The Influence of the PD-associated Gene Products PINK1 and HtrA2/Omi on Mitochondrial Integrity

Dissertation

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

der Fakultät für Biologie
und
der Medizinischen Fakultät
der Eberhard-Karls-Universität Tübingen

vorgelegt
von

Kira Margareta Holmström
aus Helsinki, Finnland

Februar 2010

Tag der mündlichen Prüfung:	14.05.2010
Dekan der Fakultät für Biologie:	Prof. Dr. H. A. Mallot
Dekan der Medizinischen Fakultät:	Prof. Dr. I. B. Autenrieth
1. Berichterstatter:	Prof. Dr. Philipp Kahle
2. Berichterstatter:	Prof. Dr. Doron Rapaport
Prüfungskommission:	Prof. Dr. Philpp Kahle
	Prof. Dr. Doron Rapaport
	Prof. Dr. Thomas Gasser
	Prof. Dr. Rejko Krüger
	PD Dr. Ulrike Naumann

I hereby declare that I have produced the work entitled: “The Influence of the PD-associated Gene Products PINK1 and HtrA2/Omi on Mitochondrial Integrity”, submitted for the award of a doctorate, on my own (without external help), have used only the sources and aids indicated and have marked passages included from other works, whether verbatim or in content, as such. I swear upon oath that these statements are true and that I have not concealed anything. I am aware that making a false declaration under oath is punishable by a term of imprisonment of up to three years or by a fine.

Tübingen, 18.02.2010

Date

Signature

Mieleni minun tekevi,
Aivoni ajattelevi,
Lähteäni laulamahan,
Saa'ani sanelemahan,
Sukuvirtta suoltamahan,
Lajivirtta laulamahan,
Sanat suussani sulavat,
Puhe'et putoelevat,
Kielelleni kerkiävät,
Hampahilleni hajoovat.

Kalevala 1:1-10

CONTENTS

1 SUMMARY

Mitochondria have been implicated in Parkinson's disease (PD) since the discovery that mitochondrial complex I inhibitors cause parkinsonian symptoms. The more recent discovery of mitochondria localised PD-associated genes has further fuelled the interest in uncovering the role of mitochondria in the progression of the disease. This study has investigated mitochondrial parameters, mainly in loss-of-function models of two of these genes, namely PINK1 and HtrA2/Omi.

PINK1 could be shown to localise to both the cytosol and mitochondria. Further investigation of mitochondrial integrity revealed a loss of mitochondrial inner membrane potential in cells lacking PINK1. A loss of mitochondrial integrity would render not just the mitochondria, but the whole cell more susceptible to cell death.

Using a mouse embryonic fibroblast cell culture model from HtrA2/Omi knock-out mice, the necessity of the protein under physiological conditions was assessed. Loss of HtrA2/Omi leads to a decrease in mitochondrial membrane potential, as well as increased ROS and ATP levels. Most interestingly, HtrA2/Omi was further found to modulate mitochondrial morphology in a protease dependent fashion. Cells lacking HtrA2/Omi show elongated mitochondria, which can be rescued by over-expression of wild-type but not a protease dead form of HtrA2/Omi. The elongated mitochondrial phenotype coincides with ultrastructural alterations in the mitochondria. These cells also display increased levels of easily extractable OPA1, a protein known to be involved in mitochondrial inner membrane fusion and the maintenance of mitochondrial cristae structures. As a final point, it could be shown that HtrA2/Omi is protective against both proteasomal and chronic oxidative stress, possibly through the regulation of the PI3K/Akt signaling pathway, as HtrA2/Omi was shown to influence Akt activation.

In brief, PINK1 and HtrA2/Omi were shown to be essential for mitochondrial integrity under physiological conditions. The loss of HtrA2/Omi further affects mitochondrial morphology, possibly through modulation of OPA1. Finally, HtrA2/Omi is protective under certain stress conditions.

2 INTRODUCTION

2.1 Parkinson's disease

What do Muhammad Ali, Michael J. Fox and Pope John Paul II have in common with about 1-2% of the aging population in the world? They all suffer or suffered from Parkinson's disease (PD), the second most common neurodegenerative disorder after Alzheimer's disease (AD) (Nutt & Wooten, 2005; Weintraub *et al.*, 2008). PD was first described by James Parkinson in 1817 in his publication "An Essay on the Shaking Palsy" (Parkinson, 1817). Originally, James Parkinson described six cases in his work, today there are millions and the prevalence of the disease is estimated to be 1-2% in people aged 65 years and over, rising with age. The incidence is slightly higher in men, with a lifetime risk of 2.0% compared to 1.3% for women (Nutt & Wooten, 2005; Weintraub *et al.*, 2008). The predicted increase in life expectancy will mean even more cases of PD in the future, causing the disease to become an even greater social and economical burden.

2.1.1 Clinical manifestation

PD is a progressive neurodegenerative disorder that entails a broad spectrum of motor and non-motor features. The usual age of onset is in the early 60s. However, up to 10% of affected are 45 years or younger (Weintraub *et al.*, 2008). The four main motor symptoms used to diagnose the disease can be grouped under the acronym TRAP: Tremor at rest (shaking and involuntary movement), Rigidity (stiffness), Akinesia (and bradykinesia, referring to the slowness in initiation and execution of movement) and Postural instability (disturbance of balance) (Jankovic, 2008; Nutt & Wooten, 2005; Weintraub *et al.*, 2008). The motor symptoms are used to define the disease but PD also entails non-motor dysfunctions that affect autonomic function, cognition and behaviour to name a few.

Today, "parkinsonism" is used as an umbrella term to encompass different parkinsonian disorders that all have a similar clinical manifestation. Idiopathic PD is the most common form of parkinsonism, responsible for up to 75% of all cases (Klein & Lohmann-Hedrich, 2007). It is sporadic in 90% of cases, with no clear aetiology, while around 10% of cases have a genetic origin (Weintraub *et al.*, 2008). PD-like symptoms can also be acquired as the result of an infection, as a side effect of medication, or due to toxins (Jankovic, 2008). Finally, other neurodegenerative

conditions such as multiple system atrophy and progressive supranuclear palsy also entail parkinsonian symptoms (Jankovic, 2008; Weintraub *et al.*, 2008).

2.1.2 Pathological features of PD

The main neuropathological hallmark of PD, and the underlying reason for the motor disturbances in the disease, is the extensive loss of dopaminergic neurons in the basal ganglia, more precisely in the substantia nigra pars compacta (SNpc), which results in depletion of dopamine in the striatum (Fig. 1). The basal ganglia are responsible for high-order motor control, which explains the clinical motor symptoms observed in patients. The clinical features, however, do not become apparent until approximately 70% of neurons in the SNpc are lost (Dauer & Przedborski, 2003; Weintraub *et al.*, 2008). As the disease progresses, other neuronal types (especially monoaminergic neurons) and other areas of the brain are also affected, giving rise to a broad spectrum of symptoms (Braak *et al.*, 2003).

Another typical feature seen in post-mortem brains of patients with PD is the presence of proteinaceous intracytoplasmic inclusions called Lewy bodies (LBs) and dystrophic Lewy neurites in the surviving neurons (Forno, 1996; Lesage & Brice, 2009). Often a definite diagnosis of PD cannot be given until after the patient is passed away, when both the loss of dopaminergic neurons in the SNpc and the LBs can be identified during autopsy. The LBs are made up of several different proteins, the main component being α-synuclein, as well as organelles such as mitochondria (Shults, 2006; Wakabayashi *et al.*, 2007). LBs are not solely found in PD, but also in other diseases such as Lewy body dementia and AD. Even aged individuals not diagnosed with PD have LBs to a lesser extent (Shults, 2006; Weintraub *et al.*, 2008).

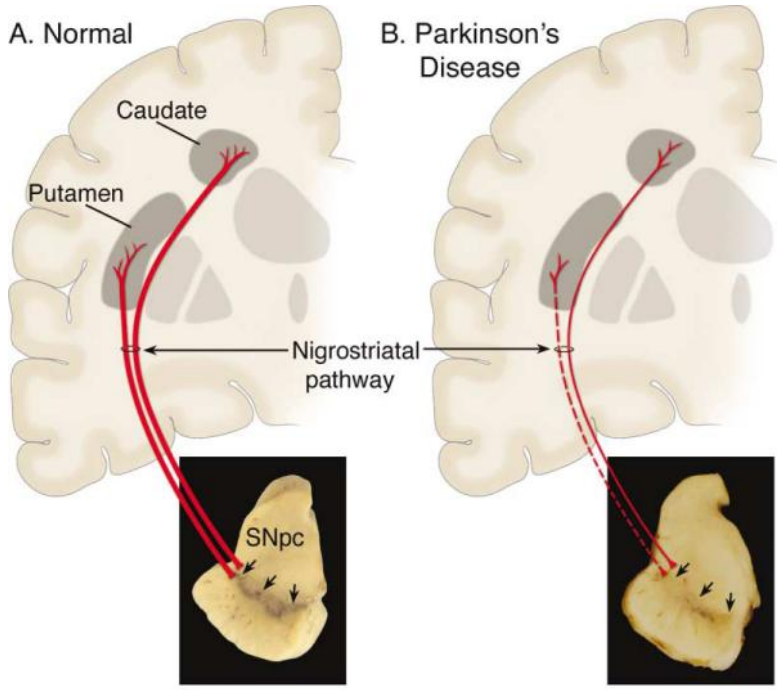

Figure 1. Neuropathology of PD.

(A) Schematic representation of the nigrostriatal pathway (in red) that projects from the SNpc to the striatum (i.e. putamen and the caudate nucleus). (B) In patients suffering from PD, the nigrostriatal pathway degenerates, especially in the branch that projects to the putamen (dashed red line), while the neurons projecting to the caudate nucleus show a milder loss (thin solid red line). In the photographs, the loss of pigmentation in the SNpc, associated with the deterioration of neuromelanin containing dopaminergic cells, can be observed in the diseased patient's brain sample. Adapted from (Dauer & Przedborski, 2003).

2.1.3 Aetiology of PD

It is generally agreed that sporadic PD is a multifactorial disorder, and that aging is the most prominent risk factor (Weintraub *et al.*, 2008). Most likely, several factors come into play for each individual, but to date, almost 200 years since identification of the disease, we do not know how these factors orchestrate the development of the disease. Both environmental factors, like exposure to pesticides and herbicides (Tanner, 1992), and genetic factors have been implicated in the pathogenesis of the disease. However, familial forms of PD are rare, amounting only to about 5-10% of all PD cases (Fitzgerald & Plun-Favreau, 2008; Lesage & Brice, 2009). So far, six genes have been proven to be associated with parkinsonism. Of these, *SNCA* (α-synuclein) and *LRRK2* (leucine-rich repeat kinase 2) cause autosomal dominant PD, while *Parkin*, *PINK1* (phosphatase and tensin (PTEN)-induced putative kinase 1), *DJ-1* and *ATP13A2* lead to recessively inherited parkinsonism (Klein & Lohmann-Hedrich, 2007; Lesage & Brice, 2009) (See Table 1). Further loci have been identified, but

their relevance to disease is not yet well established. The similarities between the familial and the sporadic forms of the disease have led researchers to speculate that similar molecular mechanisms may underlie both forms of the disease. The genes linked to PD are listed in Table 1.

PARK loci	Gene	Map position	Form of PD	Mutations	Probable function
PD-associated loci with conclusive evidence					
PARK1/ PARK4	*α-synuclein*	4q21	EOPD (AD) (sporadic)	A30P, E46K, A53T, duplications/triplications Promoter variants	Presynaptic protein, Lewy bodies
PARK2	*Parkin*	6q25-q27	EOPD (AR and sporadic)	>100 mutations, deletions, genomic partial multiplicat.	Ubiquitin E3 ligase
PARK6	*PINK1*	1p35-p36	EOPD (AR)	>40 point mutations, rare large deletions	Mitochondrial kinase
PARK7	*DJ-1*	1p36	EOPD (AR)	>10 point mutations and large deletions	Chaperone, antioxidant
PARK8	*LRRK2*	12q12	LOPD (AD and sporadic)	>40 missense variants, G2019S most common	Mixed lineage kinase
PARK9	*ATP13A2*	1p36	Kufor-Rakeb syndrome and EOPD (AR)	>5 point mutations	lysosomal P-type ATPase
PD-associated loci and genes with unknown relevance					
PARK3	*unknown*	2p13	LOPD (AD)	Not identified	unknown
PARK5	*UCHL1*	4p14	LOPD (AD)	1 mutation	Ubiquitin C-terminal hydrolase
PARK10	*unknown*	1p32	unknown	Not identified	unknown
PARK11?	*GIGYF2*	2q36-q37	LOPD (AD)	7 missense variants	Signal transduction
PARK12	*unknown*	Xq	unknown	Not identified	unknown
PARK13	*Omi/HtrA2*	2p13	unknown	3 missense variants	Mitochondrial serine protease
PARK14?	*PLA2G6*	22q13.1	EOPD (AR), dystonia-parkinsonism	2 missense mutations	Phospholipase A2
PARK15?	*FBXO7*	22q12-q13	EOPD (AR), parkinsonian-pyramidal syndrome	3 point mutations	F-box family protein

Table 1. List of PD-associated genes.

EO; early onset, LO; late onset, AD; autosomal dominant, AR; autosomal recessive (Klein & Lohmann-Hedrich, 2007; Lesage & Brice, 2009).

2.1.4 Pathogenesis of PD

After decades of research into the causes of PD, several pathological features have emerged. Abnormal protein accumulation and aggregation, faulty protein and organelle degradation, mitochondrial dysfunction and increased oxidative stress have all been implicated in the pathology of PD (Abou-Sleiman *et al.*, 2006b). There is extensive cross-talk between the different features, and they work in synergy to cause neuronal degeneration (Fig. 2). This work will focus on the mitochondrial implications in neurodegeneration and PD.

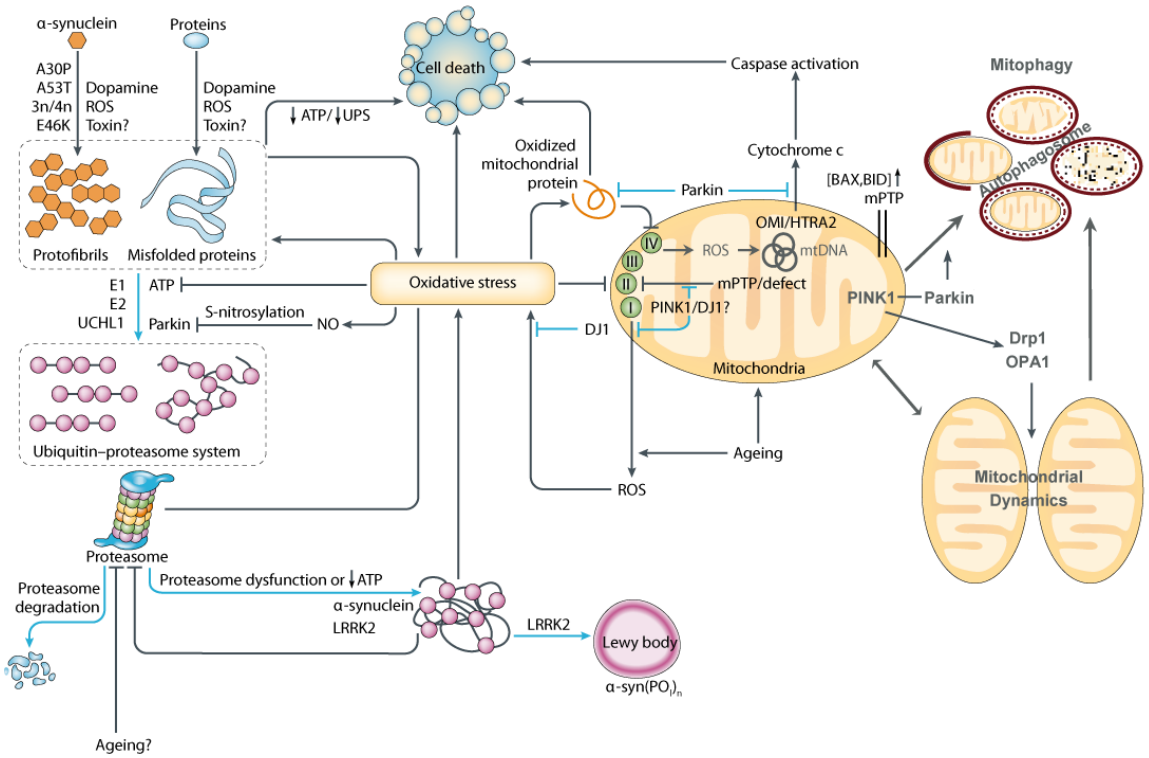

Figure 2. Pathways implicated in PD.

The main pathways indicated in the pathology of PD and their interactions are depicted here. Arrows indicate promoting interactions while blunt headed lines indicate inhibitory interactions. I; complex I, II; complex II, III; complex III, IV; complex IV, ATP; adenosine triphosphate, E1; ubiquitin-activating enzyme, E2; ubiquitin-conjugating enzyme, mPTP; mitochondrial Permeability Transition Pore, mtDNA; mitochondrial DNA, ROS; reactive oxygen species, UPS; ubiquitin proteasome system. Adapted from (Abou-Sleiman *et al.*, 2006b).

2.2 Mitochondrial functions and dysfunctions

Mitochondria are found in most eukaryotic cells. They are known as the "powerhouses of the cell", and are the main adenosine triphosphate (ATP) producers in most cells, particularly in neurons. In addition, mitochondria perform several other cellular functions ranging from metabolic reactions and calcium buffering to the control of apoptosis. According to the endosymbiotic hypothesis, mitochondria are thought to have evolved from the symbiosis between proteobacteria and their host cells (Sagan, 1967).

2.2.1 Mitochondrial structure

The mitochondrium can be divided into five subcompartments: outer mitochondrial membrane (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM), cristae and matrix (Fig. 3). The double membrane structure of mitochondria is a ruminant from the times as an independent micro organism, as is the mitochondrial DNA (mtDNA). This consists of a circular 16kb DNA molecule that encodes 37 genes, of which several hundred copies can be found in one mitochondrium (Kucej & Butow, 2007). The mtDNA is found in the matrix, the space enclosed by the IMM. This is also where most of the metabolic enzymes, involved in the tricarboxylic acid cycle, are found. Embedded in the IMM are the complexes of the respiratory chain, made up of complexes I-IV of the electron transport chain (ETC) and complex V, the ATPsynthase, which conducts ATP production. The cristae, are invaginations of the IMM, which first of all expands the surface area of the IMM, enhancing the ability to produce ATP (Zick *et al.*, 2009), as well as possibly serving a function in apoptosis control by compartmentalising the IMS protein cytochrome C (Zhang *et al.*, 2008). Finally, the IMS holds several other pro-apoptotic proteins that are released from the mitochondria upon induction of apoptosis, such as apoptosis inducing factor, Smac/DIABLO and HtrA2/Omi (Ekert & Vaux, 2005; Kroemer *et al.*, 2007). Enclosing all of this is the OMM.

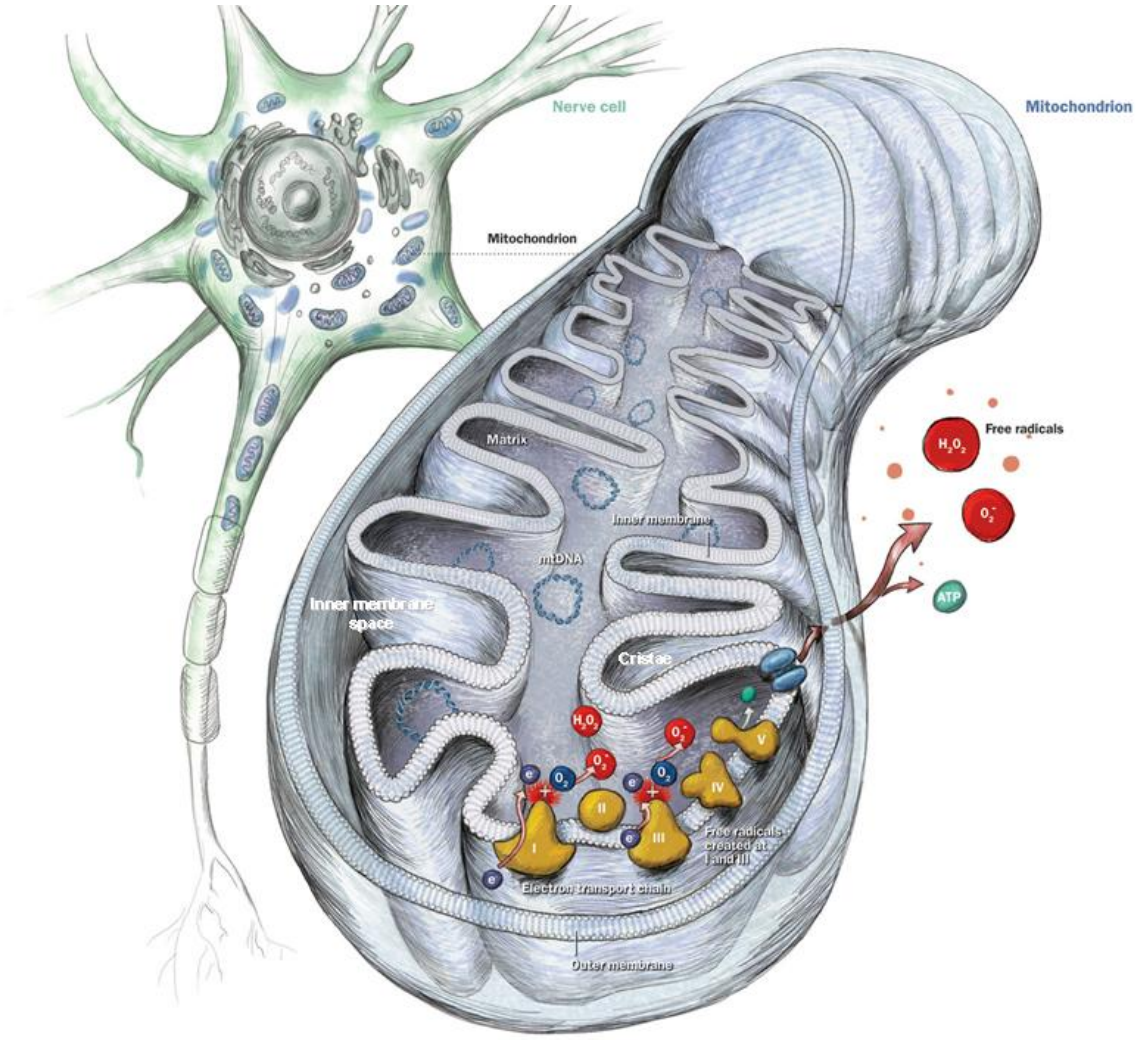

Figure 3. Schematic view of mitochondria.

The illustration shows the five mitochondrial compartments: OMM, IMS, IMM, cristae and matrix. It also depicts complexes I-V (brown) of the respiratory chain and their role in producing ATP (green) and free radicals (red) through oxidative phosphorylation. The several copies of mtDNA found in the matrix of a mitochondrium are also visible. Adapted from (Beil, 2009).

2.2.2 Mitochondrial morphology

Mitochondria are not invariant single organelles, but form a dynamic network in most cells. The connectivity of the mitochondria depends on several factors: the cell type, the stage of the cell cycle and the level of stress. The shape of the mitochondrial network comprises balancing acts of fission and fusion of the organelle. This ongoing fission and fusion is important for maintaining mitochondrial integrity, for electrical and biochemical connectivity, for mitochondrial turnover and for equal distribution of mtDNA (Berman *et al.*, 2008). Levels of cellular respiration and ATP production have been correlated with the degree of mitochondria fusion, as the loss of fusion

generally leads to a reduction in cellular respiration (Benard *et al.*, 2006; Chen *et al.*, 2005). Conversely, fragmentation of the organelle has long been associated with an early stage of apoptosis (Lee *et al.*, 2004). The morphology of the mitochondria also affects their localisation within the cell, especially in neurons where the mitochondria may need to be transported over very long distances in order to reach the synapses (Chang & Reynolds, 2006).

Mitochondrial fission and fusion

Components of the fission and fusion machineries have been identified and the main proteins involved belong to the dynamin-related family of proteins. The main player in the fission machinery is the GTPase dynamin-related protein-1 (Drp1). It is mainly localised to the cytosol but it associates in a punctae-like pattern to the OMM of the mitochondria. Some of these points later become sites of mitochondria fission (Smirnova *et al.*, 2001). In yeast, the recruitment of Dnm1 (the yeast homologue of Drp1) depends on mitochondrial fission 1 protein (Fis1), a small protein uniformly localised to the OMM of the mitochondria (Mozdy *et al.*, 2000). This has also been seen in mammalian cells (Yoon *et al.*, 2003), although knockdown of Fis1 does not affect Drp1 recruitment (Lee *et al.*, 2004). Therefore, the recruitment of Drp1 to the mitochondria is still not solved for the mammalian system.

The mitochondrial fusion process takes place in two distinct steps (OMM and IMM fusion - Fig. 4A) (Song *et al.*, 2009). The main components of the OMM fusion machinery are mitofusin-1 and -2 (Mfn1 and Mfn2), which are large, highly conserved GTPases localised to the OMM (Chan, 2006; Hoppins *et al.*, 2007) (Fig. 4A). Loss of these proteins leads to small fragmented mitochondria (Chen *et al.*, 2005). With 60% homology between Mfn1 and 2, their functions overlap. However, they have slightly different roles in healthy mammalian cells where Mfn1, for example, is more involved in tethering two adjacent mitochondria to each other to allow the OMM to fuse (Ishihara *et al.*, 2004), while Mfn2 has further fusion independent roles (de Brito & Scorrano, 2008).

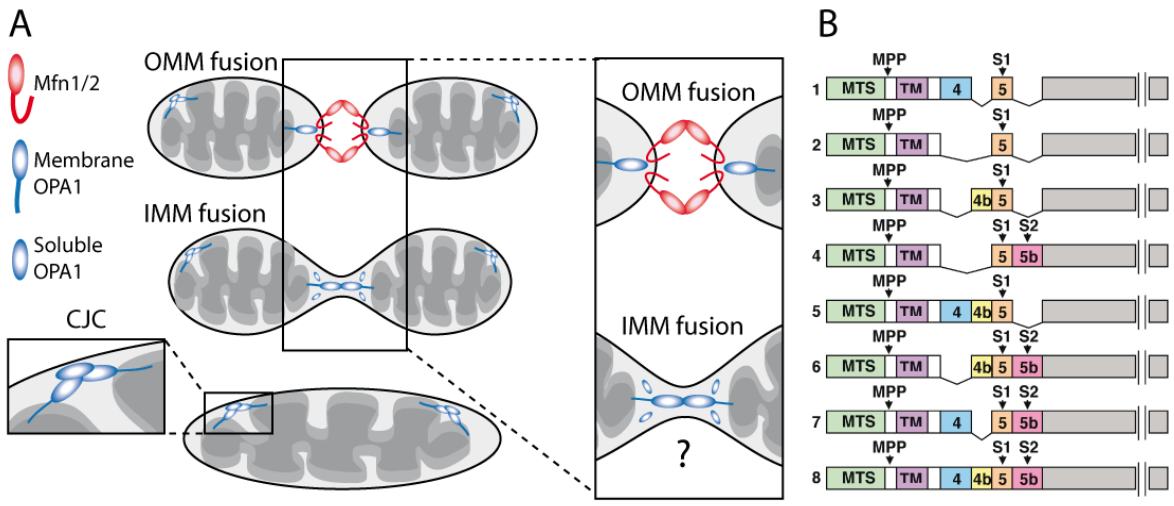

Figure 4. OPA1 and mitochondrial fusion.

(A) Schematic overview of possible mechanisms underlying mitochondrial fusion as well as a model for OPA1 engaged in the cristae junction complex. See text for more detail. (B) Schematic overview of the human OPA1 mRNA isoforms including the differentially spliced exons. Depicted are also potential protein cleavage sites (MPP, S1, S2). Adapted from (Song *et al.*, 2007). CJC; cristae junction complex, MMP; mitochondrial processing peptidase.

The key player in IMM fusion is another GTPase, optic atrophy-1 (OPA1), found in the IMS and associated with the IMM (Chan, 2006; Hoppins *et al.*, 2007) (Fig. 4A). OPA1 is a complex protein that in humans exists in eight different isoforms, which are further proteolytically processed to give rise to several protein species (Delettre *et al.*, 2001) (Fig. 4B). It is involved in IMM fusion (Song *et al.*, 2009), however little is known about the mechanisms underlying this process in mammalian cells. Most of the knowledge comes from *Saccharomyces cerevisiae*. The yeast OPA1 orthologue Mgm1, is known to exist in a membrane bound and a soluble form. The oligomerisation of the membrane bound form is known to tether and fuse the IMM (Meeusen *et al.*, 2006) (Fig. 4A). OPA1 has further been implicated in maintaining the cristae structure, by forming a complex including soluble and membrane bound forms of OPA1 at the cristae junction (Griparic *et al.*, 2004) (Fig. 4A). The exact involvement of each of the mammalian OPA1 species in either IMM fusion or cristae structure is still unclear. In addition, OPA1 and Mfn1 and 2 have been reported to interact (Guillery *et al.*, 2008), however, it is unclear whether this reflects the formation of a fusion complex, as IMM and OMM fusion are distinct steps in mammalian cells (Song *et al.*, 2009). Finally, the importance of mitochondrial fission and fusion throughout development is obvious, as knock-out of any of the large GTPases involved in mitochondrial fusion or fission leads to embryonic lethality in mice (Chan, 2006; Ishihara *et al.*, 2009).

2.2.3 Mitochondrial functions

ATP production

Although energy can be produced independently of the mitochondria, it is the most efficient producer of ATP. The first steps in ATP production, the catabolism of proteins, lipids and carbohydrates, takes place in the cytosol. This produces substrates for the citric acid cycle, followed by oxidative phosphorylation, which both take place within the mitochondria, in the matrix and across the IMM (Fig. 5). Oxidative phosphorylation involves the passing of electrons along the ETC, made up of four multimeric complexes (I-IV) and electron carriers, in the IMM. This allows the transfer of protons from the matrix into the IMS, through complexes I, III and IV, creating an electro-chemical gradient across the IMM (termed the mitochondrial membrane potential (MMP) (Rich, 2003). This gradient drives the conversion of ADP to ATP, at the ATPsynthase. Besides ATP production, the ETC is the main producer of reactive oxygen species (ROS) in the cell (Szeto, 2006).

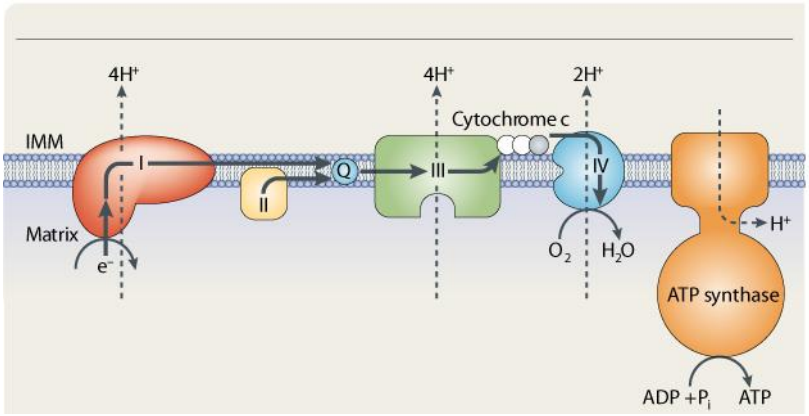

Figure 5. Oxidative phosphorylation.

Schematic overview of the electron and proton flow within the ETC, and the production of ATP by the ATPsynthase. I-IV; complexes I-IV, Q; ubiquinone, ADP; adenosine diphosphate. Adapted form (Ow *et al.*, 2008).

Apoptosis

Apoptosis, or programmed cell death, is characterised by a requirement for ATP, cell membrane blebbing, shrinkage of the cell, chromatin condensation and DNA fragmentation. It mainly takes place through two principal pathways; the extrinsic pathway, which is activated by binding of death-inducing ligands to cell surface receptors, and the intrinsic pathway, which is activated by an array of apoptotic stimuli, such as DNA damage, oxidative stress, increased calcium levels and

starvation, which act on the mitochondria (Jin & El-Deiry, 2005). Apoptosis can further be divided into at least three phases, the initiation (pre-mitochondrial events), integration (decision) and execution (degradation) phase (Kroemer *et al.*, 2007).

The fundamental event in the intrinsic pathway is OMM permeabilisation (MOMP), an event mainly controlled by the B-cell lymphoma 2 (Bcl-2) family of proteins. The Bcl-2 protein family has about 20 members that can be divided into three groups: anti-apoptotic members and two groups of pro-apoptotic proteins. The fine balance between anti- and pro-apoptotic proteins determines whether the cell will undergo apoptosis or not (Kroemer *et al.*, 2007). MOMP involves membrane permeabilisation, opening of the mitochondrial permeability transition pore and rupture of the OMM. MOMP is also driven by the pro-apoptotic Bcl-2 family members' Bcl-2 associate X protein (Bax) or Bcl-2 homologous antagonist killer (Bak) translocation to the mitochondria and their oligomerisation. Upon MOMP, either the release of molecules involved in apoptosis from the IMS upon rupture of the OMM, or the loss of mitochondrial function leads to cell death (Jin & El-Deiry, 2005).

The best known example of a pro-apoptotic factor, released from the IMS, is cytochrome C. Once in the cytosol, it binds to apoptotic peptidase activating factor 1 (Apaf-1) together with ATP, forming a structure termed the apoptosome (Bao & Shi, 2007). The apoptosome facilitates the autoprocessing of the initiator pro-caspase 9. Caspases, a group of cysteine proteases, are the main players of the integration phase and their activation is a hallmark of apoptosis (Kroemer *et al.*, 2007). Processed caspase 9 goes on to cleave and activate downstream execution caspases such as caspase 3. Further proteolytic cleavage of an array of cytosolic and nuclear targets by caspases and mitochondrial death effectors, eventually incapacitates cellular functions, and the cell dies (Jin & El-Deiry, 2005). One of the downstream targets of caspase 3 is poly[ADP-ribose] polymerase (PARP). The cleavage of PARP is often used as a marker for apoptosis (Galluzzi *et al.*, 2009). The major regulators of caspases are the inhibitor of apoptosis family of proteins (IAPs), with XIAP being the most potent one (Deveraux & Reed, 1999). For a schematic overview of the roles of cytochrome C and HtrA2/Omi in cell death see Fig. 8.

2.2.4 Implications for mitochondria in neurodegeneration

There are several factors that render the brain, and especially neurons, susceptible to damage upon mitochondrial dysfunction. One of the most prominent reasons is the high energy demand of the brain and the inability of neurons to utilise glycolysis to fulfill their high energy needs (Almeida *et al.*, 2001). This is also why classical mitochondrial encephalomyopathies, caused by mutations in mtDNA or nuclear genes involved in respiratory function, often manifest with neuronal and skeletal muscle defects (Chan, 2006). Moreover, unbalanced mitochondrial morphology has also been closely linked to neuronal survival (Chan, 2006). Mutations in the mitochondrial outer membrane fusion factor Mfn2, lead to Charcot-Marie-Tooth 2A disease, a peripheral hereditary neuropathy, while mutations in the inner mitochondrial membrane fusion factor OPA1 lead to autosomal dominant optic atrophy (Alexander *et al.*, 2000; Delettre *et al.*, 2000; Kijima *et al.*, 2005). Interestingly, so far no disease has been associated with mutations in the mitochondrial fission factor Drp1, although a newborn girl suffering from microcephaly was recorded to have a heterozygous mutation in the gene (Waterham *et al.*, 2007). This suggests that Drp1 is of such importance that mutations are strongly counter-selected. Cell culture studies have further implicated Drp1 in mitochondrial transport and synaptogenesis (Li *et al.*, 2004). The proper localisation of mitochondria at the nerve terminals is important in order to maintain the high energy demand of signal transmission and neurotransmitter release, however also for calcium homeostasis (Hollenbeck, 2005). Although the endoplasmatic reticulum (ER) is considered the foremost calcium sink in the cell, intricate bidirectional communication between the mitochondria and the ER takes place at the synapse, orchestrating signal transduction (Hollenbeck, 2005).

Mitochondrial involvement in PD

Mitochondria were first implicated in the pathogenesis of PD nearly three decades ago, when people exposed to the mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which metabolite 1-methyl-4-phenylpyridinium (MPP+) is a specific inhibitor of the respiratory complex I, suddenly developed parkinsonian symptoms (Langston *et al.*, 1983). Complex I deficiency has since been identified in autopsies of patients with sporadic PD (Schapira *et al.*, 1989). Moreover, inhibition of

complex I increases ROS levels in the cell, and markers of oxidative stress have been found up-regulated in both experimental models of PD, as well as in post-mortem PD brains (Zhou *et al.*, 2008a). Furthermore, increased levels of mitochondrial DNA deletions can be found in the SNpc compared to other dopaminergic neurons (Bender *et al.*, 2008), suggesting a role for the loss of mtDNA integrity in the pathogenic mechanisms underlying PD. Finally, more recent association of mitochondrial proteins such as PINK1 and HtrA2/Omi with PD, further highlights the possible role of dysfunctional mitochondria in the pathogenesis of PD (Valente *et al.*, 2004; Strauss *et al.*, 2005).

2.3 PINK1

2.3.1 Properties of PINK1

PINK1 maps to the 1p36 locus (PARK6) in the human genome. The *PINK1* gene contains eight exons, coding for a 581 amino acid (aa) protein (Valente *et al.*, 2004). The protein is ubiquitously expressed, with the highest levels in the heart, skeletal muscle and testis and at lower levels in the brain, placenta, liver, kidney, pancreas, prostate, ovary and small intestine (Unoki & Nakamura, 2001). It comprises an N-terminal mitochondrial targeting sequence (MTS) followed by a putative transmembrane domain (TM), a highly conserved kinase domain, resembling the Ca^{2+}/calmodulin family of kinases, and a unique C-terminal regulatory sequence that may govern its kinase activity (Mills *et al.*, 2008; Silvestri *et al.*, 2005) (Fig. 6). The full-length protein (~63kDa) is further proteolytically processed into at least two different isoforms (~55 and ~46kDa) (Mills *et al.*, 2008).

Figure 6. Schematic view of PINK1.

Depicted is a schematic overview of the domain structure of PINK1. MTS; mitochondrial targeting sequence, TM; putative transmembrane domain, reg; regulatory.

PINK1 was the first PD-associated gene with a primarily mitochondrial localisation. However, its exact sub-cellular localisation is still debated. The length of the N-terminal targeting sequence remains unclear and two different proposed lengths, 1-34 and 1-77 amino acids, have been shown to work for mitochondrial targeting of recombinant protein (Muqit *et al.*, 2006; Silvestri *et al.*, 2005). The protein, both over-expressed and endogenous, has been observed in mitochondrial fractions, and it co-localises with mitochondria and mitochondrial proteins in fluorescent stainings (Beilina *et al.*, 2005; Clark *et al.*, 2006; Gandhi *et al.*, 2006; Petit *et al.*, 2005; Valente *et al.*, 2004). However, cytosolic PINK1 can also be observed in mammalian cells (Gandhi *et al.*, 2006; Plun-Favreau *et al.*, 2007). Even once within the mitochondria, there is still controversy as to where PINK1 resides. Immunogold labeling has indicated localisation to the inner mitochondrial membrane (Muqit *et al.*, 2006;

Silvestri *et al.*, 2005). In mitochondria isolated from rat brain, both IMM and OMM localisation were detected (Gandhi *et al.*, 2006). In another study where mitochondria were isolated from PC12 cells, PINK1 showed both an IMM and IMS localisation (Pridgeon *et al.*, 2007). Recent evidence indicates that PINK1 is an OMM protein, with its kinase domain facing the cytoplasm (Zhou *et al.*, 2008b). Deletion of the putative transmembrane domain (Fig. 6) leads to further translocation into the mitochondrial matrix, suggesting that this domain is responsible for anchoring the protein in the OMM (Zhou *et al.*, 2008b).

2.3.2 The genetic association of PINK1 to PD

PINK1 was found to be associated with rare familial early-onset juvenile parkinsonism in 2004 (Valente *et al.*, 2004). The first study reported two homozygous mutations in three consanguineous PARK6 families (G309D and W437X). Today, over 40 *PINK1* mutations have been identified, of which 15 segregate in pedigrees of early onset parkinsonism, clustering mainly in the kinase domain (Fig. 6). Besides homozygous point mutations, truncations and deletions also heterozygous mutations have been reported in sporadic patients (Abou-Sleiman *et al.*, 2006a; Hiller *et al.*, 2007). The importance of these findings remains to be seen. The frequency of *PINK1* mutations ranges from 1-8% in patients (Klein & Lohmann-Hedrich, 2007). The clinical symptoms of *PINK1* patients differ to some extent from sporadic patients. Atypical psychological problems are commonly observed (Tan & Dawson, 2006). Finally, several of the other PD-related genes, such as Parkin, DJ-1 and HtrA2/Omi, have been shown to physically interact with *PINK1* (Plun-Favreau *et al.*, 2007; Xiong *et al.*, 2009) .

2.3.3 The function of PINK1

PINK1 has been shown to be protective against several cellular toxins, such as staurosporine and MPTP, in a variety of neuronal cell culture studies (Deng *et al.*, 2005; Haque *et al.*, 2008; Petit *et al.*, 2005; Wood-Kaczmar *et al.*, 2008). Interestingly, PINK1 knock-out mice do not show a neurodegenerative phenotype and only mild mitochondrial defects, including impaired mitochondrial respiration in the striatum and increased sensitivity of cortical mitochondria to exogenous stressors (Gautier *et al.*, 2008; Kitada *et al.*, 2007; Zhou *et al.*, 2007). In contrast, much

information on PINK1 has been obtained from studies with *Drosophila melongaster (D. melongaster).* In the fly, the loss of PINK1 leads to a clear degenerative phenotype, including male sterility, energy depletion, shortened life span and degeneration of flight muscles and dopaminergic neurons, due to mitochondrial dysfunction. It is accompanied by morphological changes of the organelle (Clark *et al.*, 2006; Park *et al.*, 2006b; Yang *et al.*, 2006).

PINK1 and mitochondrial dynamics

Further studies in *D. melongaster* have reported an influence of PINK1 on the mitochondrial fission and fusion machinery (Deng *et al.*, 2008; Poole *et al.*, 2008; Yang *et al.*, 2008). Genomic knock-out in *D. melongaster* has revealed a fission promoting role for PINK1, while the results from mammalian cell culture systems are more controversial (Sandebring *et al.*, 2009). We have shown that transient silencing of PINK1 with siRNAs in HeLa cells leads to fragmented mitochondria (Exner *et al.*, 2007), while transient silencing of PINK1 in COS7 cells had the opposite effect (Yang *et al.*, 2008). Further studies in mammalian neuronal cell culture systems, support the role of PINK1 in either limiting fission or promoting fusion (Dagda *et al.*, 2009; Sandebring *et al.*, 2009). The exact role of PINK1 in mitochondrial dynamics remains unclear. The mechanism underlying the effects of PINK1 on fission and fusion in mammalian cells has been reported to entail dephosphorylation of Drp1, through the calcium dependent phosphatase calcineurin (Sandebring *et al.*, 2009). However, the authors of this study argue that these effects might be secondary to the effect PINK1 has on calcium buffering, as it has been shown to be impaired in cells lacking PINK1 (Gandhi *et al.*, 2009; Marongiu *et al.*, 2009).

PINK1 and Parkin

Parkin is an E3-ubiquitin ligase, also linked to early-onset PD (Kitada *et al.*, 1998). It was first shown to genetically interact with PINK1 in *D. melongaster.* Loss of PINK1 could be compensated for by over-expressing Parkin. Furthermore, the Parkin-deficient flies show a very similar phenotype to the PINK1-deficient ones, however, they cannot be rescued by PINK1 over-expression, suggesting that PINK1 lays upstream of Parkin in a common pathway (Park *et al.*, 2006b). We could further confirm this in a mammalian system. The loss of PINK1 was shown to induce loss of MMP, fragmentation of the mitochondrial network and ultrastructural changes in the

mitochondria, and could be rescued by PINK1 and Parkin over-expression but not by PD-related mutants of either protein (Exner *et al.*, 2007). The lost MMP (Exner *et al.*, 2007; Wood-Kaczmar *et al.*, 2008), could be a secondary effect due to the impairment of the ETC (Gautier *et al.*, 2008; Gegg *et al.*, 2009). The impaired complex I and IV activity leads to a decrease in ATP production and an increase in ROS production (Gautier *et al.*, 2008; Gegg *et al.*, 2009; Wood-Kaczmar *et al.*, 2008), possibly also explaining the loss of mtDNA, reported upon loss of PINK1 (Gegg *et al.*, 2009).

PINK1/Parkin-mediated mitophagy

PINK1 seems to also be necessary for proper regulation of autophagic clearance of defective mitochondria (mitophagy) (Dagda *et al.*, 2009). In addition, Parkin has been shown to be selectively recruited to impaired mitochondria and to promote mitophagy (Narendra *et al.*, 2008). We characterised this further and showed that the translocation of Parkin is dependent on functional PINK1, on both its kinase activity and mitochondrial localisation (Geisler *et al.,* 2010). The final degradation of damaged mitochondria also involves Parkin-dependent ubiquitination of the voltage dependent anion channel 1 (VDAC1) and the autophagy adapter protein p62. PINK1 phosphorylation of Parkin at Thr175 and Thr217 has been suggested to promote translocation to the mitochondria (Kim *et al.*, 2008). However, it does not seem to be sufficient for translocation, as phospho-mimicking constructs (T175E, T217E) do not spontaneously translocate to the mitochondria, although exchanging the threonine for an alanine abrogates translocation (Narendra *et al.,* 2010).

PINK1 kinase activity

Although the main functional domain of PINK1 is the serine/threonine kinase domain, early *in vitro* kinase assays examining the autophosphorylation activity of PINK1 came to contradictory conclusions about its phosphorylation capacity (Silvestri *et al.*, 2005; Sim *et al.*, 2006). Moreover, PD-related mutations, although scattered all along the kinase domain, showed very inconsistent results for kinase activity, obscuring the importance of loss of kinase function in the pathology of PD. The identification of the tumor necrosis factor receptor-associated protein-1 (Trap1), as a putative substrate for PINK1 phosphorylation, shedded more light on the kinase activity and function of PINK1 (Pridgeon *et al.*, 2007). Trap1 is a mitochondrial intermembrane space

chaperone, of the Hsp90 family of proteins, also called mitochondrial heat-shock protein 75. Little is known about this protein except that it seems to protect against cell death induced by ROS (Hua *et al.*, 2007; Pridgeon *et al.*, 2007). Upon oxidative stress, PINK1 phosphorylates Trap1 as shown both *in vitro* and *in vivo*, an event that is necessary for some of the protective effects of PINK1 (Pridgeon *et al.*, 2007). Recently, we were also able to show that a functional kinase domain is necessary for Parkin-mediated mitophagy (Geisler *et al.,* 2010), further revealing the role of PINK1-dependent phosphorylation in PD pathogenesis.

2.3.4 PINK1 protein stability

The cytosolic Hsp90/Cdc37/p50 chaperone complex stabilises the wild type PINK1 (Lin & Kang, 2008; Moriwaki *et al.*, 2008). It appears that the Hsp90/Cdc37 complex influences the ratio of at least two of the different forms of PINK1 protein, the ratio of the premature 66kDa to the processed 55kDa form as well as the cytoplasmic localisation (Weihofen *et al.*, 2008). The authors argue that the ratio is important for PD pathogenesis as PD-related mutants (G309D) alter this ratio, as does a synthetic kinase dead mutant. This suggests that the kinase activity of PINK1 plays a role in both the maturation and function of the protein. Recent work further indicates that PINK1 undergoes constant proteolytic processing and degradation at the OMM under physiological conditions (Narendra *et al.,* 2010). Loss of MMP leads to inhibition of processing and an accumulation of PINK1 at the mitochondria, promoting Parkin translocation and mitophagy. This highlights the importance of PINK1 stability and turnover in organelle integrity. To date the mechanisms responsible for PINK1 cleavage have not been identified.

2.4 HtrA2/Omi

2.4.1 Properties of HtrA2/Omi

High temperature requirement A2 (HtrA2)/Omi was first isolated in a yeast-two hybrid screen using Mxi2, an alternatively spliced form of the stress-activated kinase p38α, as bait (Faccio *et al.*, 2000). The *(protease serine-25) PRSS25 / HtrA2/Omi* gene codes for a 458 aa protein, with great homology to bacterial HtrA family endoproteases. The HtrA family of proteases is an evolutionary highly conserved family of serine proteases, found in nearly all bacterial and eukaryotic genomes but absent in almost all archaean genomes, suggesting a common phylogenic origin with the mitochondria (Koonin & Aravind, 2002; Sagan, 1967). In humans, except for HtrA2, which is mainly localised to the IMS of the mitochondria, the other three members (HtrA1, 3 and 4) are all most likely targeted to the secretory pathway, as they all harbour a secretion signal at the N-terminus, just like the HtrA2/Omi harbours a mitochondrial targeting sequence (MTS; aa 1-31). The bacterial homologues of HtrA2/Omi are HtrA/DegP, DegQ and DegS. The *Escherchia coli* HtrA/DegP is a chaperone at low temperatures, and gains proteolytic function upon higher temperatures. DegS is known to cleave the Sigma-E factor negative regulatory protein (RseA), a transmembrane transcriptional regulator upon periplasmic stress after allosteric activation (Sohn *et al.*, 2007). The function of DegQ is much less understood – though it seems that it has redundant roles with DegS and DegQ (Vande Walle *et al.*, 2008).

HtrA2/Omi is expressed as a 49 kDa pre-peptide, targeted to the mitochondria, although small nuclear and endoplasmic reticulum (ER) pools have been reported (Gray *et al.*, 2000; Kuninaka *et al.*, 2007; Martins *et al.*, 2002). Besides the N-terminal 31 aa mitochondrial targeting sequence, the other domains of the prepeptide of HtrA2/Omi entail a putative transmembrane domain that seems to be cleaved off in the mature form of the protein, revealing an inhibitor of apoptosis protein (IAP) binding motif (IBM) (Fig. 7). C-terminally of the IBM, a conserved trimerization motif (Tyr147, Phe149, Phe256), responsible for the trimeric, pyramid-like, three-dimensional structure, necessary for active HtrA2/Omi can be found (Li *et al.*, 2002).

The protease domain is the main functional domain of the protein, however, it is regulated by a C-terminal PDZ (post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (DlgA) and zonula occludens-1 protein (zo-1)) protein interaction domain (Fig. 7). It seems that in the inactive state of the protein, the PDZ domain lies on top of the protease domain. Upon interaction with a substrate, it undergoes a conformational change, exposing the protease domain (Li *et al.*, 2002; Wilken *et al.*, 2004; Zeth, 2004). It has been reported that autoproteolytic cleavage takes place *in vitro* at Ala133 (Savopoulos *et al.*, 2000; Seong *et al.*, 2004). However, cells lacking functional HtrA2/Omi (S276C mutation, which reduces the protease function) still harbour the fully processed form of the protein (Jones *et al.*, 2003), indicating that there might be another, currently unidentified, protease involved.

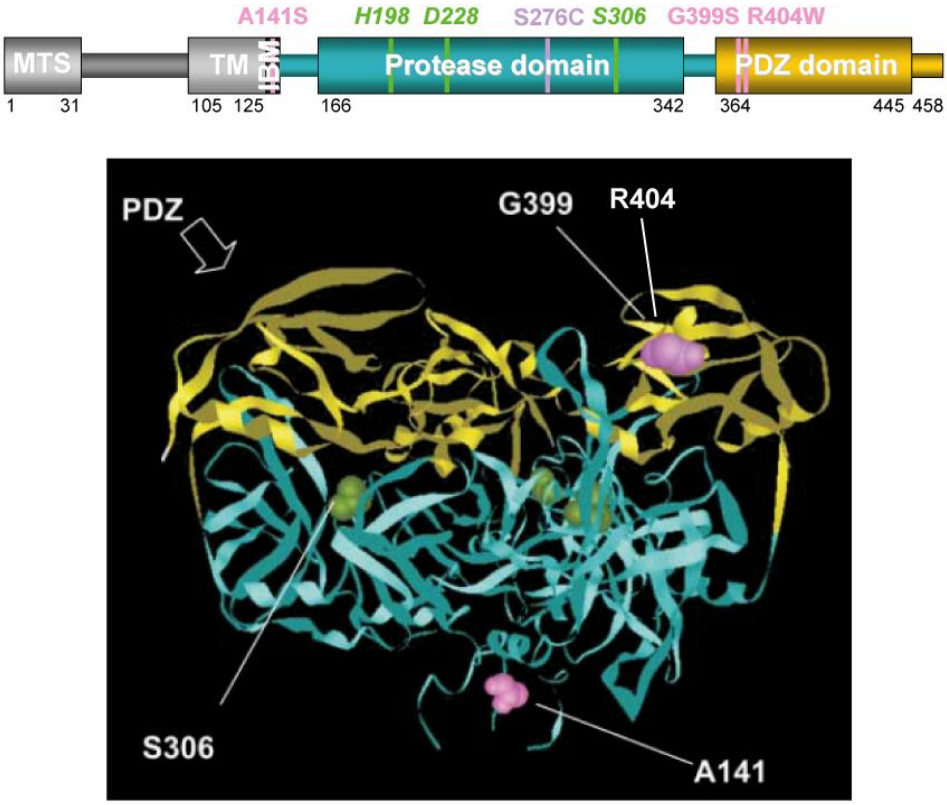

Figure 7. Schematic view of HtrA2/Omi.

Schematic overview of the domain structure of HtrA2/Omi and a three dimensional representation of the mature peptide and the PDZ domain (adapted from (Strauss *et al.*, 2005). The PD-associated mutations are depicted in pink, while the mnd2 associated mutation is in violet. In green are the residues of the catalytic triad. MTS; mitochondrial targeting sequence, TM; putative transmembrane domain, IBM; IAP-binding motif, PDZ; PSD95, DlgA, and zo-1.

2.4.2 The role of HtrA2/Omi in apoptosis

Numerous stimuli trigger apoptosis in the cell. Upon reaching the mitochondria they eventually lead to the release of proteins from the IMS, among them HtrA2/Omi (Kroemer *et al.*, 2007). The proteolytic maturation of HtrA2/Omi reveals a highly conserved IAP-binding domain (Fig. 7), homologous to the mammalian Smac/DIABLO, as well as *D. melongaster* homologs Reaper, Hind and Grim (Hegde *et al.*, 2002; Martins *et al.*, 2002; Suzuki *et al.*, 2001; van Loo *et al.*, 2002; Verhagen *et al.*, 2002). Therefore, once in the cytosol, HtrA2/Omi binds and actively cleaves IAPs in a two-step process, further activating the caspase-dependent apoptotic pathway (Srinivasula *et al.*, 2003; Yang *et al.*, 2003) (Fig. 8). In addition, experiments with the pan-caspase inhibitor ZVAD-fmk have shown that HtrA2/Omi also contributes to apoptosis in a caspase-independent manner (Kuninaka *et al.*, 2005). The knock down of HtrA2/Omi has indeed been shown to increase the resistance to apoptotic stimuli such as cisplatin, UV-radiation and anti-Fas in multiple cell lines (Cilenti *et al.*, 2005; Hegde *et al.*, 2002; Kuninaka *et al.*, 2005; Liu *et al.*, 2006; Martins *et al.*, 2002; Srinivasula *et al.*, 2003; Yang *et al.*, 2003). Its function in both caspase-dependent and -independent apoptosis seems to depend on the protease function since a specific inhibitor of HtrA2/Omi protease activity, Ucf-101, protects against HtrA2/Omi mediated cell death (Cilenti *et al.*, 2003; Cilenti *et al.*, 2004).

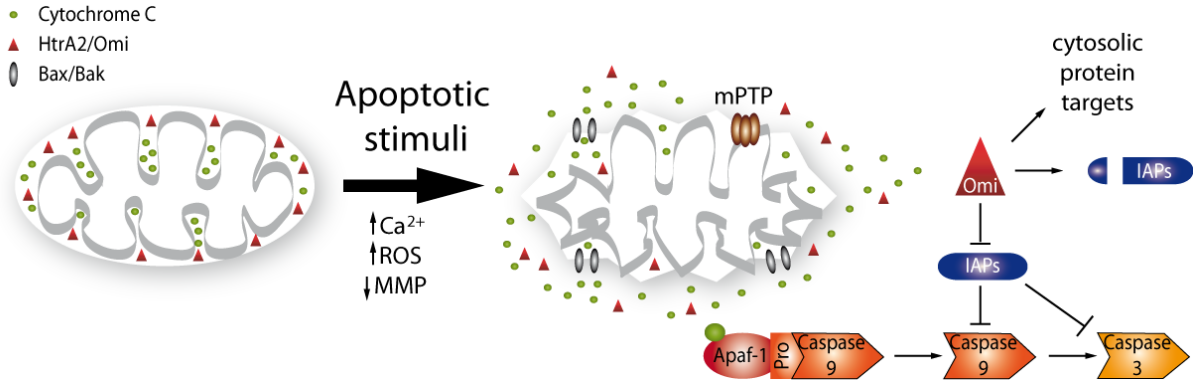

Figure 8. Roles of HtrA2/Omi in the cytosol.

HtrA2/Omi is released, together with other pro-apoptotic proteins such as cytochrome C, from the IMS into the cytosol upon induction of apoptosis by stimuli such as increased calcium (Ca^{2+}) or ROS, or decreased MMP. The induction of apoptosis leads to opening of the mitochondrial permeability transition pore (mPTP), membrane permeabilisation and rupture of the OMM. Apoptosis is also driven by Bcl-2 associate X protein (Bax) or Bcl-2 homologous antagonist killer (Bak) translocation to the mitochondria and oligomerisation, which drives OMM permeabilisation. Once in the cytosol HtrA2/Omi is known to cleave several anti-apoptotic proteins including the IAPs, to which it binds through the IBM domain, promoting apoptosis in both a caspase-dependent and -independent manner (Kroemer *et al.*, 2007). Arrows indicate promoting actions, while blunt ends indicate inhibitory actions. Apaf-1, apoptotic peptidase activating factor 1.

A few substrates beside IAPs have been shown for HtrA2/Omi, most of them associated with its pro-apoptotic function. One interesting substrate, is the tumour suppressor and mitotic regulator WARTS. It is a serine/threonine kinase that has been shown to bind the PDZ domain of HtrA2/Omi in a kinase dependent manner in the cytosol promoting HtrA2/Omi mediated cell death (Kuninaka *et al.*, 2005). However, a further publication from the same group showed that the interaction actually also includes proteolytic cleavage of WARTS and is not solely dependent on apoptotic stimuli. It seems that WARTS actually is a physiological substrate of HtrA2/Omi. They also show that the HtrA2/Omi-mediated processing of WARTS modulates the cell cycle, and HtrA2/Omi seems to be a negative regulator of cell cycle progression as silencing of the gene leads to accelerated cell proliferation, further suggesting a role for HtrA2/Omi outside apoptosis (Kuninaka *et al.*, 2007). Another kinase known to interact with HtrA2/Omi is Akt (Yang *et al.*, 2007). Phosphorylation of HtrA2/Omi by Akt1 and Akt2 on residue Ser212 attenuated its protease activity *in vivo* which leads to failure to cleave XIAP. Several other substrates for HtrA2/Omi, upon induction of apoptosis, have been identified in a large proteomics screen. The most prominent group was cytoskeletal proteins, but two

translation regulators were also identified together with other proteins involved in apoptosis. All together, 15 potential HtrA2/Omi substrates were identified, of which 10 were validated *in vitro* (Vande Walle *et al.*, 2007).

2.4.3 HtrA2/Omi in disease

HtrA2/Omi features in several different diseases, ranging from cancer and viral infection to neurodegeneration. In line with its role in apoptosis it makes sense that HtrA2/Omi is down regulated in certain cancers. For example, it has been shown that HtrA2/Omi, together with HtrA1 and HtrA3, is down regulated in endometrical cancer (Narkiewicz *et al.*, 2008). HtrA2/Omi has also been associated with the progression of certain viral infections and can be inhibited by the viral protein vMIA (mitochondrial-localised inhibitor of apoptosis) (Ma *et al.*, 2007; McCormick *et al.*, 2008). In addition, HtrA2/Omi has been implicated in AD. One study has shown that HtrA2/Omi interacts with presenilin-1, one of the proteins involved in cleaving amyloid precursor protein (APP) (Gupta *et al.*, 2004). Binding of presenilin-1 derived peptides to the PDZ domain of HtrA2/Omi up-regulates its protease activity (Gupta *et al.*, 2004). Furthermore, HtrA2/Omi was also shown to cleave APP in the mitochondria (Park *et al.*, 2006a), as well as regulating APP turnover at the ER (Huttunen *et al.*, 2007). Interestingly, HtrA2/Omi has also been implicated in another neurodegenerative disorder, namely Huntington's disease. A recent study showed that HtrA2/Omi was selectively down regulated in mutant huntingtin expressing striatal neurons, but not in cortical or cerebellar neurons. Adenoviral over-expression of HtrA2/Omi also reverted mutant htt-induced cell death in primary neurons (Inagaki *et al.*, 2008).

2.4.4 The relationship between HtrA2/Omi and PD

In 1993, a new mouse model for inherited motor neuron disease (mnd-2) was presented (Jones *et al.*, 1993). The spontaneous autosomal recessive mutation was determined to be a S276C substitution in HtrA2/Omi, which significantly reduces its protease activity. The mice showed an early onset neurodegenerative phenotype, they failed to gain weight; several of the organs were considerably smaller and the mice died before 40 days of age (Jones *et al.*, 2003). Targeted deletion of HtrA2/Omi in mice also showed a very similar phenotype with progressive loss of neurons in the

striatum and reduced body weight, in addition to lack of coordination, decreased mobility and tremor resembling the clinical manifestation of PD (Martins *et al.*, 2004). The neurodegenerative phenotype, upon loss of HtrA2/Omi, suggests an important physiological function for the protein. This is further supported by the neuron-specific over-expression in transgenic mice, which does not lead to increased cell death, highlighting the importance of its role in mitochondrial integrity rather than pro-apoptotic function in neurons (Liu *et al.*, 2007).

Further evidence supporting a role for HtrA2/Omi in PD is the identification of two polymorphisms in the *HtrA2/Omi* gene in a cohort of sporadic German patients (Strauss *et al.*, 2005). The missense mutations identified, A141S and G399S, seem to affect the enzymatic activity of the protease (Strauss *et al.*, 2005). Another study also found a new missense mutation, R404W, in a Belgian population of sporadic PD (Bogaerts *et al.*, 2008) (Fig. 7). However, these results could not be confirmed in two large independent genetic studies (Ross *et al.*, 2008; Simon-Sanchez & Singleton, 2008), where they reported the same mutations also in the control cohort, making it unclear, what role genetic alterations in HtrA2/Omi play in PD.

HtrA2/Omi and PINK1

The functional evidence speaks for an involvement of HtrA2/Omi in the pathogenesis of PD. Besides the mouse models, a direct interaction with another PD-associated gene, PINK1, also speaks for a role in the progression of the disease. A direct interaction between PINK1 and HtrA2/Omi was shown for both over-expressed and endogenous protein in three different cell lines (Plun-Favreau *et al.*, 2007). PINK1 was further shown to mediate a p38γ-dependent phosphorylation of HtrA2/Omi at Ser142. The stress-induced phosphorylation of this residue seems to increase the proteolytic activity of HtrA2/Omi, which again is protective against the parkinsonian toxins 6-OHDA and rotenone (Plun-Favreau *et al.*, 2007). Furthermore, pathological PINK1 mutations C573R and Y431H showed a loss of phosphorylation of HtrA2/Omi in postmortem brain tissue, compared to control patients, which displayed low levels of phosphorylation. Idiopathic PD patients again exhibited a clear elevation of phosphorylation at Ser142, compared to both normal neurological controls and PINK1 patients (Plun-Favreau *et al.*, 2007).

There is some disagreement in the literature, whether HtrA2/Omi is part of the PINK1/Parkin pathway. While the mouse HtrA2 shows over 95% homology to the human protein, the *D. melongaster* HtrA shows only 50% sequence homology to the human HtrA2/Omi and 68% similarity in domain structure (Yun *et al.*, 2008). The phenotypes of the PINK1- and HtrA2-deficient flies are very different: PINK1-deficient flies show an extensive mitochondrial phenotype (Yun *et al.*, 2008), while loss of HtrA2/Omi only seems to lead to mitochondrial alterations in aged flies (Tain *et al.*, 2009). Although over-expressed PINK1 and HtrA2 genetically interact in the fly, loss of both proteins leads to an accumulative effect, and none of the proteins can compensate for the others function, indicating that the two proteins might associate with two different downstream pathways. This is further supported by evidence that HtrA2 acts genetically downstream of PINK1, but parallel to Parkin (Whitworth *et al.*, 2008). Interestingly, the same group also showed that in *D. melongaster,* upstream of both PINK1 and HtrA2, is the mitochondrial protease Rhomboid-7, responsible for proteolytically processing of both PINK1 and HtrA2 (Whitworth *et al.*, 2008). The mammalian ortholog of Rhomboid-7, presenilin-associated rhomboid-like (PARL), is also known to cleave HtrA2/Omi, though in a HS1-associated protein X 1 (Hax 1) dependent manner (Chao *et al.*, 2008). However, this report was recently questioned by another publication that showed that the interaction between Hax 1 and PARL is an artefact, and which also questioned the proposed Bcl-2 family similarity of Hax-1 (Jeyaraju *et al.*, 2009). Hax 1 has already been identified as a substrate for HtrA2/Omi upon induction of apoptosis by etoposide, cisplatin and H_2O_2. This, however, seems to be an early event in the apoptotic signalling cascade after induction of cell death (Cilenti *et al.*, 2004).

2.4.5 The potential protective effects of HtrA2/Omi

The Mpv17-like (Mpv17l) is a mitochondrial IMM protein implicated in the metabolism of ROS. It has been shown to interact with the PDZ domain of HtrA2/Omi and induce the protease activity of the protein (Krick *et al.*, 2008). This action protects the mitochondria against cellular stress, reducing mitochondrial ROS and stabilising the MMP (Krick *et al.*, 2008). Interestingly, another study showed that the loss of HtrA2/Omi leads to a transcriptional upregulation of genes involved in the integrated stress response and further induction of the downstream target, transcription factor growth arrest and DNA damage/ C/EBP homology protein (GADD153/CHOP),

selectively in the brain (Moisoi *et al.*, 2009). The loss of HtrA2/Omi also leads to an accumulation of unfolded proteins in the mitochondria, defective respiration and higher levels of ROS contributing to elevated CHOP expression and eventually neuronal cell death (Moisoi *et al.*, 2009). The role of HtrA2/Omi in the protein quality control within the mitochondria is also supported by a further study that indicate that HtrA2/Omi plays a role in eliminating accumulating, misfolded proteins from the IMS of mitochondria, upon inhibition of the ubiquitin proteasome system (Radke *et al.*, 2008).

2.5 Objectives

Mitochondria have long been implicated in the pathogenesis of PD. Recent discoveries of mitochondria localised PD-associated genes has further fuelled the interest in uncovering the role of mitochondria in the progression of the disease. This study aims to unravel the physiological functions, and the impact of two PD-relevant genes, PINK1 and HtrA2/Omi, on mitochondrial integrity, mainly through the use of loss-of-function models. Loss of mitochondrial integrity, as seen by a sub-optimal function of the ETC, would render not just the mitochondria, but the whole cell more susceptible to cell death. By investigating parameters influenced by the ETC, like the mitochondrial membrane potential, ROS and ATP levels, insight is gained of the functional state of the mitochondria. Further questions would be, if the alterations in mitochondria actually affect the susceptibility of the cell to undergo apoptosis and which pathways may be involved.

Another emerging mitochondrial parameter in the field is the state of the mitochondrial network. It is becoming increasingly clear that balanced fission and fusion, and the connectivity of the mitochondrial network, are of importance for mitochondrial integrity as well as for the overall functionality of the cell. Several of the PD associated genes have been reported to influence this, but until now this has not been investigated for HtrA2/Omi.

In summary, this study seeks to investigate 1) mitochondrial integrity upon loss of either PINK1 or HtrA2/Omi, 2) how HtrA2/Omi influences the susceptibility of the cell to undergo apoptosis, 3) which pathway is involved and 4) how HtrA2/Omi influences the morphology of the mitochondrial network.

3 RESULTS

3.1 PD genes and mitochondrial function

3.1.1 A loss-of-protein cell culture model to investigate the function of PINK1

The recessive nature of PINK1-associated PD, and the reported loss of enzymatic function associated with some of the mutations, speaks for a likely loss-of-function disease mechanism. Therefore, a loss-of-protein cell culture model was created, where PINK1 was transiently silenced in human cervical cancer (HeLa) cells using an siRNA approach. HeLa cells were chosen as they express detectable levels of endogenous PINK1 and are easily transfected. They also have high amounts of mitochondria that form an elaborate, easily visualised network. Transient silencing of PINK1 using siRNA was highly efficient, as can be seen on both the mRNA level, determined by semi-quantitative reverse transcription PCR (RT-PCR) (Fig. 9A) and on protein level, as seen by Western blot analysis (Fig. 9B). Interestingly, several different protein species of PINK1 can be detected by immunoblotting, indicating proteolytic cleavage of PINK1 within the cell. Most of the PINK1 protein detected in the HeLa cells is found in a pre-cleaved form (marked with pre in Fig. 9B), suggesting that either it is not imported into the mitochondria or not fully processed within the mitochondria as it still harbours the MTS. Further processed PINK1 (marked with pro in Fig. 9B,D) most likely corresponds to PINK1 protein where the MTS has been cleaved off. The mature form, by prediction, corresponds to a protein that further lacks the putative TM domain (marked with mature in Fig. 9B,D). Finally, over-expression of wt PINK1 as well as re-transfection of silenced HeLa cells, with PINK1 constructs harbouring a silent mutation at the binding site of the siRNA, was highly efficient (Fig. 9C). This provides a good cell culture model to further investigate the influence of PINK1 on mitochondrial functions.

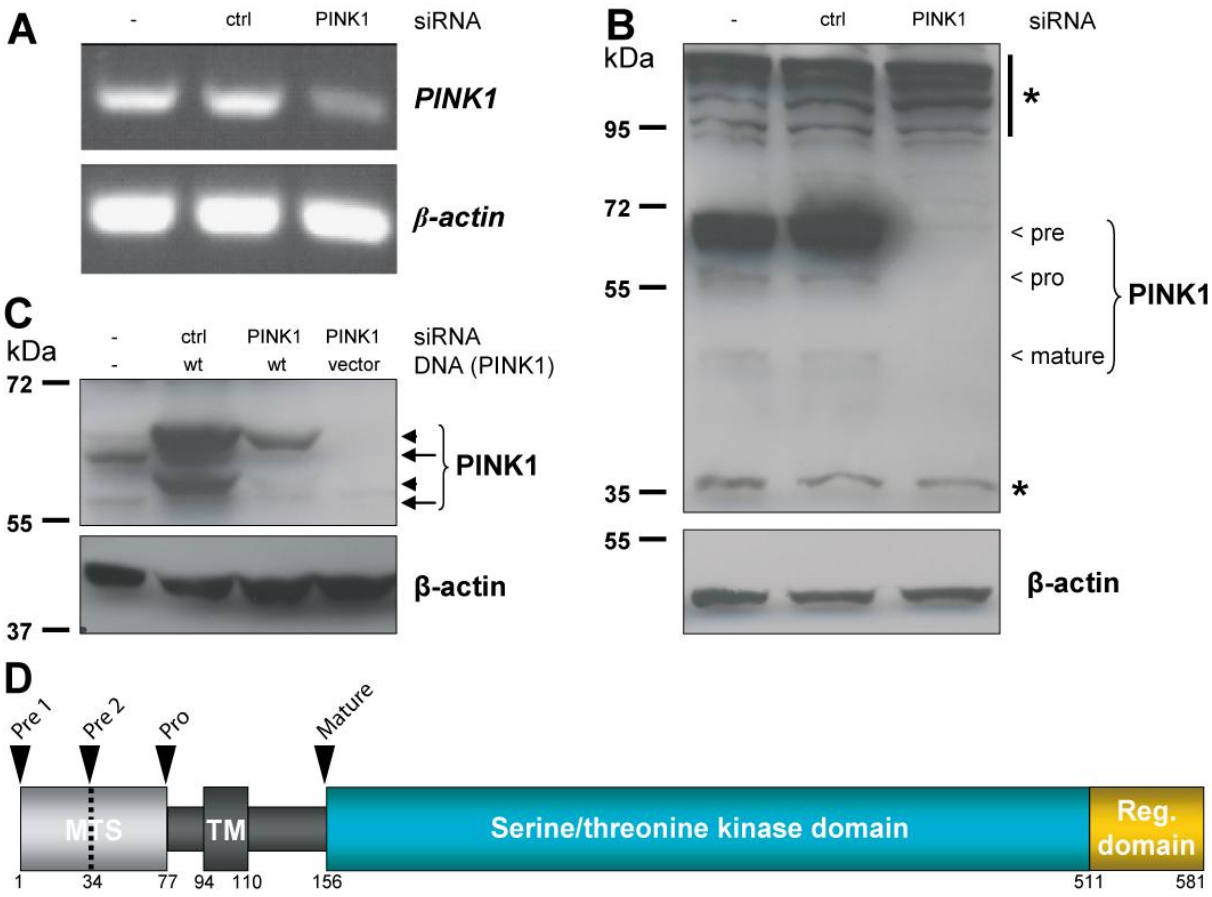

Figure 9. Transient silencing of PINK1.

HeLa cells were transiently silenced with 10nM PINK1 or control (ctrl) siRNA on three consecutive days and subjected to either RT-PCR or Western blot analysis. (A) Semi-quantitative RT-PCR analysis of efficiency of siRNA-mediated silencing of PINK1 on the mRNA level in HeLa cells. β-actin was used as a control. (B) The cells were subjected to SDS-PAGE and analysed by Western blotting. PINK1 is expressed in HeLa cells in a pre-, pro- and mature form. The antibody used (PINK1 [BC100-494] Novus Biologicals) also shows some cross-reacting bands, marked with an asterisk. β-actin was used as a loading control. (C) The silenced HeLa cells were further re-transfected with vector or wt PINK1 before subjected to SDS-PAGE and Western blot analysis. The arrowheads indicate recombinant PINK1 while the arrows show the endogenous protein. (D) A schematic overview of PINK1. Arrow heads mark the possible cleavage sites for the different bands seen by Western blot analysis (Fig. 9 and 10).

3.1.2 Sub-cellular localisation of PINK1

In order to characterise the localisation of different forms of PINK1, sub-cellular fractionation experiments in HeLa cells were performed (Fig. 10). Western blot analysis revealed both a cytosolic as well as a mitochondrial localisation of PINK1 (Fig. 10A,B). The presence of high amounts of the precursor form of PINK1, still harbouring the MTS confirms the notion of sub-optimal import of PINK1 into the mitochondria. Depending on the segregation of the bands on the Western blot and the antibody used, several forms of PINK1 could be detected. Further analysis

reveals that the pre-cleaved form actually consist of two different PINK1 peptides (pre 1 and 2) (Fig. 9D and 10B). The first pre-cleaved form most likely represents the full length form of PINK1. This PINK1 product is also not seen in the mitochondrial fraction, suggesting efficient cleavage of the MTS upon import into the mitochondria. Interestingly, the second precursor form can be found in both mitochondria and cytosol. This may be due to 1) export from the mitochondria or 2) cleavage of the pre 1 form of PINK1 in the cytosol. The two longest forms of PINK1 are the most prominent forms of the protein, both in the cytosol and overall, while within the mitochondria two further processed forms exist. The approximate molecular weight suggests that the two shorter forms (pro- and mature PINK1) represent forms of the protein where the full MTS has been cleaved off (pro) and further, where the putative transmembrane domain has been cleaved off (mature). The mature form of PINK1 is seen almost exclusively in the mitochondrial fraction (Fig. 10B).

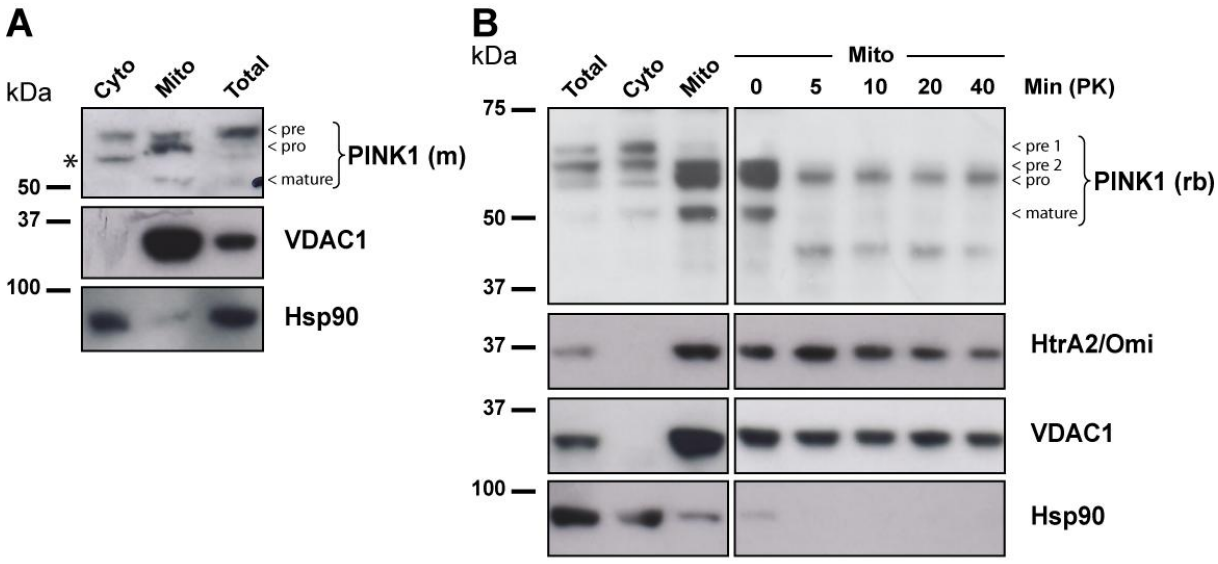

Figure 10. Sub-cellular localisation of PINK1.

HeLa cells were subjected to fractionation experiments and investigated by Western blot analysis for PINK1 localisation. (A) Blots containing a mitochondrial, cytosolic and total cell lysate fraction were probed for PINK1 (anti-mouse (m) [AM6406a]), for VDAC1 as a mitochondrial marker and for Hsp90 as a cytosolic marker. The asterisk marks a possible cross-reacting band as this is not seen with the other antibody. (B) Blots containing a total cell lysate, cytosolic and mitochondrial fraction as well as mitochondrial fractions subjected to proteinase K (PK) digestion for indicated time points were probed for PINK1 (anti-rabbit (rb) [BC100-494]), HtrA2/Omi as a marker for the IMS, VDAC1 as a marker for the OMM and Hsp90 as a marker for the cytosol.

Proteinase K digestion of isolated mitochondria suggests that the pre 2 and mature form of PINK1 might reside at the OMM, as it seems to disappear upon digestion, consistent with reports that some forms of PINK1 resides in the OMM facing the cytosol (Zhou *et al.*, 2008b). Interestingly, the disappearance of the two PINK1 forms correlates with the emergence of an even shorter form of PINK1, suggesting that a part of either the pre 2 or the mature form of PINK1 is cleaved off, while the rest of the protein remains intact.

3.1.3 Loss of PINK1 leads to reduced mitochondrial membrane potential

As PINK1 has been linked to the maintenance of mitochondrial integrity, the MMP in PINK1 silenced and control transfected HeLa cells was measured. Tetramethyl rhodamine methyl ester (TMRM), a membrane potential sensitive dye that is only taken up into active mitochondria, was used for this purpose. The TMRM signal was measured by flow cytometry and the percentage of cells above the threshold TMRM fluorescence signal was determined. There was a clear decrease in TMRM signal in cells lacking PINK1, compared to cells either left untreated or transfected with a scrambled siRNA, indicating a higher amount of defective mitochondria in PINK1 silenced cells (Fig. 11). The MMP reflects the activity of the ETC and is therefore generally considered a good marker for mitochondrial activity and integrity. Several other functions of the mitochondria are sensitive to changes in membrane potential: the activation of the permeability transition pore, protein import as well as fission and fusion of mitochondria. A decrease in membrane potential upon loss of PINK1 has since also been reported by others in neuronal cell lines (Wood-Kaczmar *et al.*, 2008).

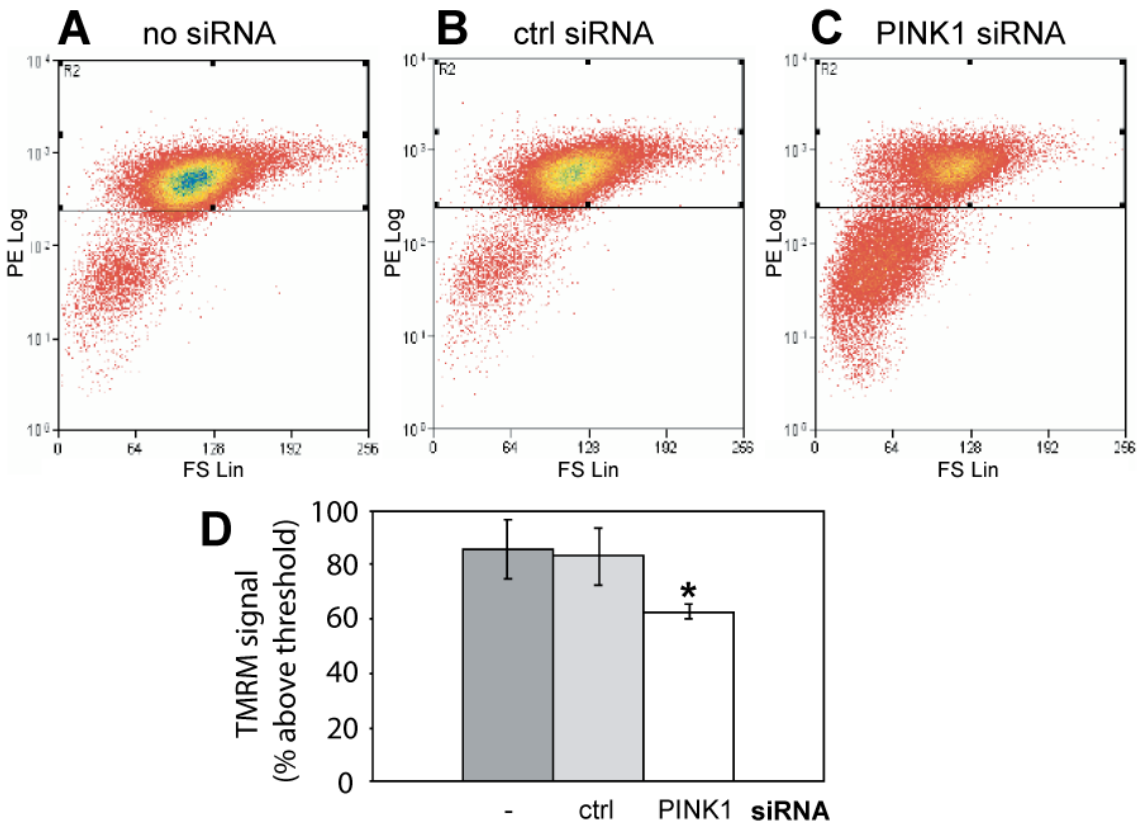

Figure 11. Loss of PINK1 affects the mitochondrial membrane potential.

HeLa cells were treated with no siRNA (A), control (ctrl) siRNA (B) or 10nM PINK1 siRNA (C) on three consecutive days before harvested and stained with 200nM TMRM for detection of MMP. Scattergraphs represent cells separated according to size (forward scatter (FSLin)) and red TMRM fluorescence (PE Log) intensity from one representative experiment. (D) Mean percentage of cells with above threshold TMRM signal (* $p<0.05$). Results represent the mean of three independent experiments. Error bars correspond to SEM.

3.1.4 A knock-out cell culture model of HtrA2/Omi

HtrA2/Omi is a nuclear encoded serine protease, which under physiological conditions localises to the IMS. Once released from the IMS, HtrA2/Omi is also known to be involved in promoting apoptosis (Vande Walle *et al.*, 2008). Recent reports have implicated HtrA2/Omi in idiopathic PD (Strauss *et al.*, 2005). This, together with the severe neurodegenerative phenotype of loss-of-function mouse models of HtrA2/Omi (Jones *et al.*, 2003; Martins *et al.*, 2004), has fuelled an interest in HtrA2/Omi, in terms of unravelling the molecular mechanisms underlying PD. The genetic link for HtrA2/Omi to PD is not as strong as for other well recognised PD-associated genes. However, the striatal neuronal loss of the HtrA2/Omi -/- mice and their parkinsonian-like motor impairment has implicated a functional link for this protein to PD. To investigate the importance of HtrA2/Omi under physiological conditions, an HtrA2/Omi -/- mouse embryonic fibroblast (MEF) cell culture model was further used in this study. HtrA2/Omi -/- and littermate control +/+ MEFs were kindly provided by Miguel Martins from Leicester (Martins *et al.*, 2004). Western blot analysis of endogenous HtrA2/Omi in both MEF cell lines shows a complete loss of the protein in the HtrA2/Omi -/- MEF cells (Fig. 12).

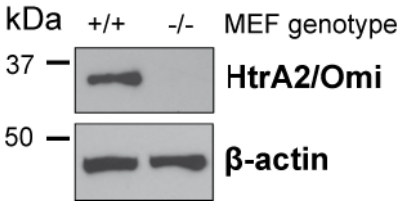

Figure 12. HtrA2/Omi +/+ and -/- MEF cells.

HtrA2/Omi +/+ and -/- MEF cells were subjected to Western blot analysis. The membranes were probed with antibodies against HtrA2/Omi and β-actin as a loading control.

3.1.5 Loss of HtrA2/Omi compromises mitochondrial integrity

Knock-out of HtrA2/Omi leads to a reduction in MMP

To observe whether the loss of HtrA2/Omi leads to a similar mitochondrial phenotype as PINK1 loss, the same parameters were investigated. To assure that the differences in MMP seen by flow cytometry are not simply due to differences in mitochondrial mass, the total mitochondrial mass was also accounted for in these experiments. Staining the cells with MitoTracker Green FM (green) labels all mitochondria, independent of MMP, while co-staining with the membrane potential sensitive MitoTracker CM-H_2XRos (red) only labels mitochondria with intact MMP. Using this dual-labeling approach on HtrA2/Omi -/- MEF cells revealed a decrease of about 20% in the MMP compared to +/+ (Fig. 13A). This reduction of MMP indicates a higher proportion of dysfunctional mitochondria in HtrA2/Omi deficient cells, consistent with previous reports (Krick *et al.*, 2008; Plun-Favreau *et al.*, 2007).

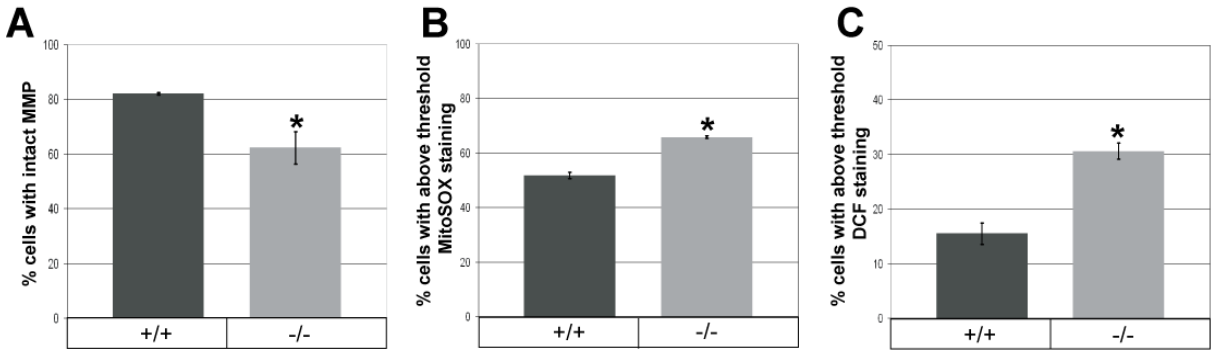

Figure 13. The mitochondrial integrity is impaired upon loss of HtrA2/Omi.

Analysis of mitochondrial membrane potential (MMP) and ROS levels in HtrA2/Omi +/+ and -/- cells. (A) MEF cells were stained with MitoTracker Green FM and MitoTracker CM-H_2XRos and subjected to FACS analysis. The ratio between these two was calculated as a readout for mitochondrial membrane potential (* $p<0.005$). (B) MEF cells were stained with MitoSOX in order to measure intramitochondrial ROS (* $p< 0.00005$). (C) MEF cells were stained with DCF to measure total ROS in the cells (* $p< 0.0005$). All experiments were performed in triplicate. Error bars represent SD. The MMP and MitoSOX measurements were performed by Nicole Kieper.

Increased oxidative stress upon loss of HtrA2/Omi

The reduction in membrane potential frequently indicates malfunction of the ETC, which, as a consequence, often leads to higher ROS levels. It was therefore investigated, whether a reduced MMP observed correlated with increased ROS. Mitochondria-specific ROS was measured using the fluorogenic dye MitoSOX Red, which is targeted to the mitochondria and readily oxidised by superoxide, but not by

other ROS- or reactive nitrogen species–generating systems. The results indicate higher ROS levels in the HtrA2/Omi -/- MEFs compared to +/+ controls (Fig. 13B). Moreover, also total cellular ROS levels, which were measured using 2',7'-dichlorohydrofluorescein diacetate (DCF), were elevated in HtrA2/Omi -/- MEF cells compared to +/+ controls (Fig. 13C).

3.1.6 Cells lacking HtrA2/Omi have more ATP

A decreased mitochondrial membrane potential and an increased ROS production usually indicate a malfunctioning ETC, which could lead to alterations in ATP levels. Interestingly, basal ATP levels in HtrA2/Omi -/- MEF cells were elevated when compared to +/+ controls (Fig. 14). In order to compensate for the faster growth of cells lacking HtrA2/Omi (personal observation and consistent with previous reports (Kuninaka *et al.*, 2007)), the ATP levels were normalised to the protein concentration. The increased ATP levels could either be a secondary effect to the elongated mitochondrial phenotype, discussed in upcoming sections, or a compensatory mechanism for the lost MMP (possibly by increased glycolysis). This would be possible as the cells are cultured under high glucose conditions (4.5g/l), which would allow them to derive most of their energy from glycolysis, further suggesting that the increased ATP levels might not reflect the functionality of the ETC.

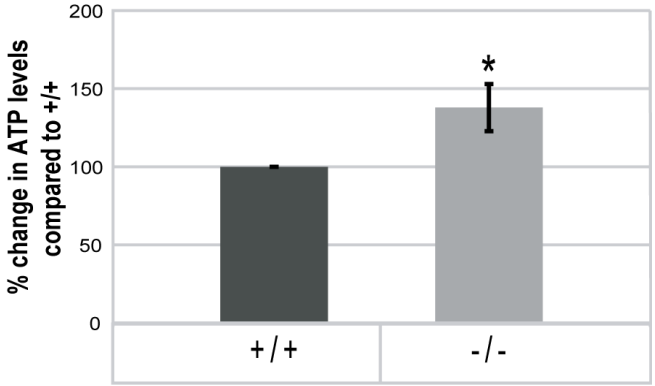

Figure 14. MEF cells lacking HtrA2/Omi have more basal ATP.

The basal ATP levels in HtrA2/Omi +/+ and -/- MEF cells were measured using the ATPlite kit (PerkinElmer) and normalised to protein levels. The results are presented as relative to +/+ where +/+ was set to 100% (* $p < 0.05$). Error bars correspond to SD.

3.2 The effects of HtrA2/Omi on mitochondrial morphology

3.2.1 HtrA2/Omi knock-out MEFs have elongated mitochondria

Interestingly, loss of both HtrA2/Omi and PINK1 leads to a decrease in MMP and increased ROS (Dagda *et al.*, 2009). Alterations in both these parameters, as well as changes in ATP levels, have been shown to coincide with changes in mitochondrial morphology (Mandemakers *et al.*, 2007). Therefore, mitochondrial morphology in HtrA2/Omi +/+ and -/- MEFs was investigated. For PINK1, a fragmentation of the mitochondrial network upon silencing of PINK1 in HeLa cells was observed (Exner *et al.*, 2007). In order to prevent fixation artefacts, a live-cell based fluorescent imaging technique was used to investigate the mitochondrial morphology. The cells were stained with MitoTracker Green FM and Z-stacks from at least seven layers of the cell where taken to be scored by an unbiased observer blinded to the genotype of the cells. Three categories of mitochondrial morphology were used for scoring the cells; middle sized (mid-sized), elongated and fragmented mitochondria (Fig. 15). The amount of cells with a fragmented mitochondrial profile was very low (4.3% for +/+ and 2.9% for -/-) and not significantly different between the cell types (Fig 15C). Interestingly, the loss of HtrA2/Omi, in contrast to the loss of PINK1, leads to a predominantly elongated mitochondrial phenotype (Fig. 15B) compared to the +/+ controls (Fig. 15A). In HtrA2/Omi -/- MEF cells, the increased amount of cells with elongated mitochondria (84.1%) was accompanied by a loss of cells with a mid-sized mitochondrial morphology profile. Elongated mitochondria have been associated with increased ATP levels, especially under mild stress conditions (Tondera *et al.*, 2009). This could possibly explain the increased ATP levels observed in HtrA2/Omi -/- MEF cells (Fig. 14).

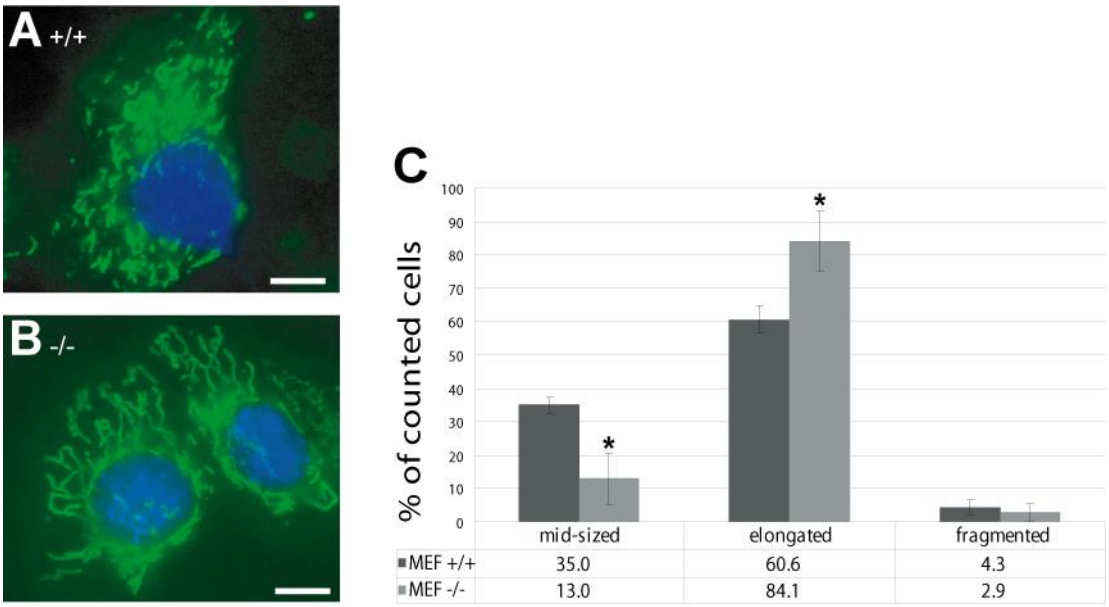

Figure 15. HtrA2/Omi influences mitochondrial morphology.

Live HtrA2/Omi +/+ (A) and -/- (B) MEF cells were stained with MitoTracker Green FM (green) and Hoechst 33342 (blue). Size bars correspond to 10µm. (C) The mitochondrial morphology was classified into three categories (elongated, mid-sized and fragmented) by an unbiased observer. The results are given as percentage of cells with mitochondria falling into each category (* $p<0.05$) Error bars represent SD. The live-cell images were obtained by Nicole Kieper and the cells were scored by Dalila Ciceri.

3.2.2 Loss of HtrA2/Omi leads to ultrastructural changes in the mitochondria

Alterations in gross mitochondrial morphology often go hand in hand with ultrastructural changes within the mitochondria. Therefore, the fine structure of the mitochondria in the HtrA2/Omi +/+ and -/- MEF cells, was further investigated by electron microscopy (EM). Investigation of ultrastructural abnormalities clearly showed pronounced morphological alterations in the HtrA2/Omi -/- cells (Fig. 16D-F) compared to the +/+ controls, which displayed mostly typical round shaped mitochondria with well formed cristae (Fig. 16A-C). In contrast to HtrA2/Omi +/+ MEFs, -/- often displayed bulged or extended mitochondria with abnormal, frequently absent, inner membrane structures and lost cristae. At many sites, in particular were the mitochondria were cut longitudinally, a local loss of cristae could be observed, whereas the other end of the mitochondrium was still normal. If the part with the lost cristae was cross sectioned, the mitochondrium seemed to be completely devoid of folded inner membrane structures. Semiquantitative analysis revealed that 44.5% of the mitochondria in HtrA2/Omi -/- MEF cells show these alterations while only 8.6%

of the mitochondria in HtrA2/Omi +/+ control MEFs have abnormal mitochondrial ultrastructure. These modifications are in agreement with the fluorescent microscopy data where an elongated mitochondrial phenotype upon loss of HtrA2/Omi was observed (Fig. 15C).

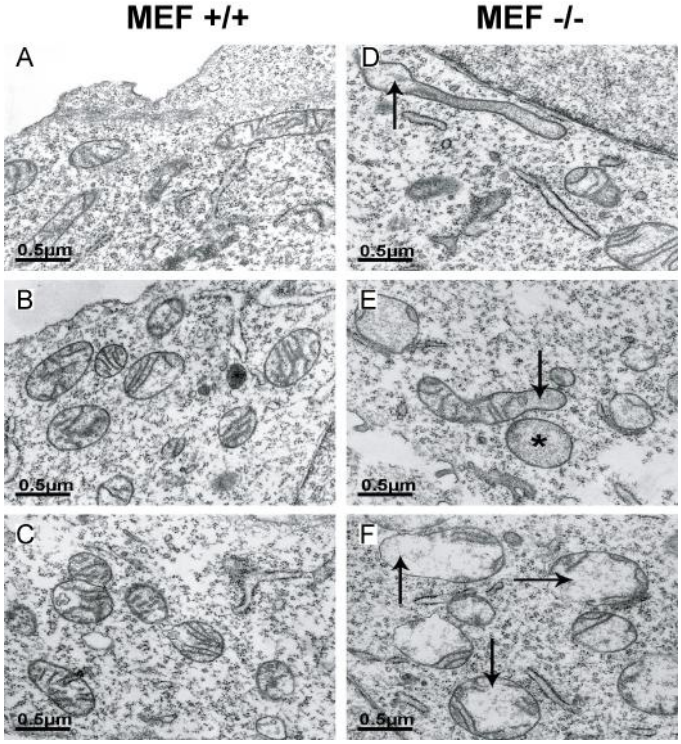

Figure 16. Ultrastructural changes in HtrA2/Omi -/- MEF cells.

(A-C) Ultrastructure of HtrA2/Omi +/+ MEFs as seen by electron microscopy. (D-F) Ultrastructure of HtrA2/Omi -/- MEFs as seen by electron microscopy. Examples of mitochondria with a local loss of cristae structures (arrow in E and F) or a complete loss of folded inner membrane structures (arrow in D and asterisk in E). The EM pictures are from Hartwig Wohlburg.

3.2.3 Transient silencing of HtrA2/Omi recapitulates the phenotype

In order to further confirm the finding in the MEF cells, and to obscure clonal artefacts, a different approach to create a loss of protein cell culture model for HtrA2/Omi was used. Transient silencing of HtrA2/Omi was achieved by siRNA transfection (Fig. 17A). Comparison of the mitochondrial morphology in HeLa cells transfected with either a control siRNA (Fig. 17B) or HtrA2/Omi specific siRNA (Fig. 17C) shows a significant difference in the mitochondrial network between the two treatments. The cells were scored by an unbiased observer into two categories; mid-sized or elongated. HeLa cells lacking HtrA2/Omi showed a significantly higher proportion of cells with elongated mitochondria compared to the control cells (Fig. 17D). Importantly, silencing of HtrA2/Omi in *Drosphila* S2R+ cells showed a similar phenotype (Kieper *et al.,* 2010). This further confirms the involvement of HtrA2/Omi in the regulation of the mitochondrial morphology and highlights that this effect is conserved across species.

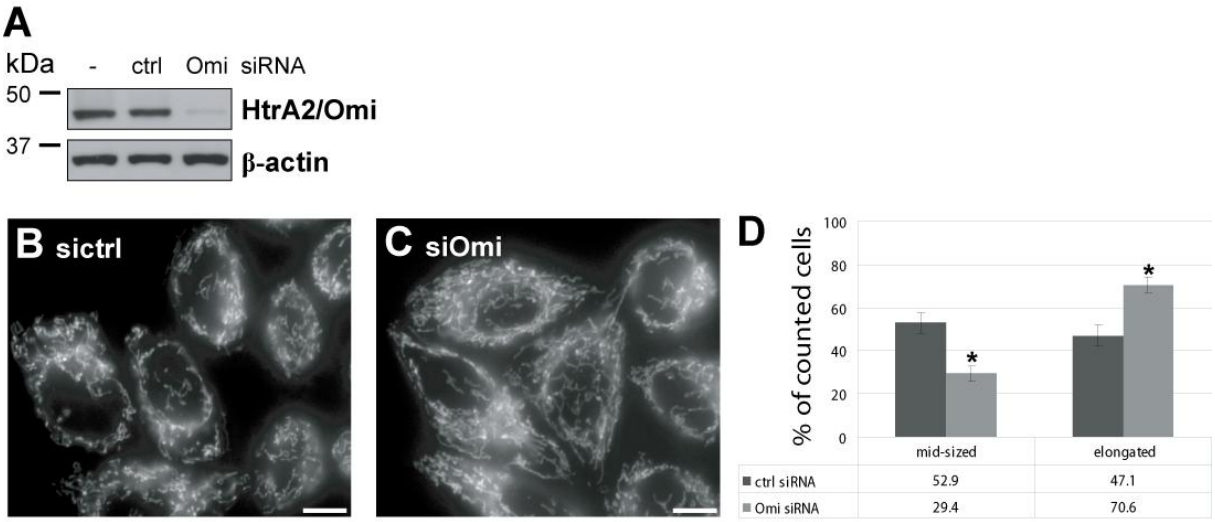

Figure 17. Transient silencing of HtrA2/Omi recapitulates the phenotype.

(A) HeLa cells were treated with a total of 15nM of either no, control (ctrl) or HtrA2/Omi (Omi) siRNA on three consecutive days and subjected to Western blot analysis for detection of silencing efficiency. β-actin was used as a loading control. The mitochondrial morphology was investigated in the control (sictrl – B) and HtrA2/Omi (siOmi – C) siRNA treated HeLa cells stained with MitoTracker Green FM. (D) The mitochondria were scored as either elongated or normal by an unbiased observer. The results are given as percentage of cells with mitochondria falling into each category (* $p<0.005$). Error bars correspond to SD. The live-cell images were scored by Nicole Kieper.

3.2.4 Mitochondrial morphology is dependent on HtrA2/Omi protease activity

As HtrA2/Omi is a serine protease, a natural question to ask is whether the observed phenotype is dependent on the protease function of HtrA2/Omi. In order to test this hypothesis MEF -/- cells stably over-expressing either an empty vector, wt or a mutant form of the protein, where the active serine 306 has been exchanged for an alanine (S306A) (Hegde *et al.*, 2002), were created. The vector line is a polyclonal pool stable, while for S306A and wt single clone lines were generated. For S306A only one clone successfully expressed the protein, while for wt, three clones were generated (Fig. 18A), of which clone 6 was used for all further experiments unless otherwise stated (Fig. 18B). The MEF cell clones expressed HtrA2/Omi at a very high level, mainly in the fully processed form (Fig. 18A). However, occasionally also the precursor form of HtrA2/Omi could be detected in the stable wt over-expressing clones (Fig. 20).

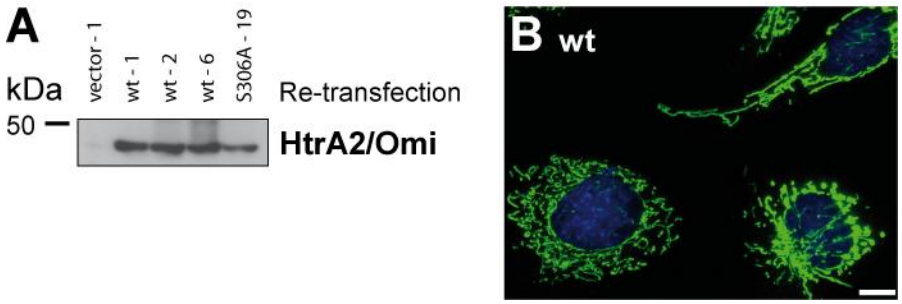

Figure 18. HtrA2/Omi -/- MEF cells stably over-expressing vector, wt or mutant protein.

(A) Western blot analysis of HtrA2/Omi -/- MEF cells stably over-expressing wt, vector or a protease dead (S306A) form of HtrA2/Omi. Shown are, besides one vector and S306A cell line, also three wt lines (1, 2, 6). All future experiments were performed with clone 6 unless otherwise stated. (B) Fixed HtrA2/Omi -/- MEF cells stably over-expressing wt HtrA2/Omi (clone 6) were stained for HtrA2/Omi (Axxora) (green) while the nucleus was counterstained with Hoechst 33342 (blue). Size bar corresponds to 10µm.

The mitochondrial morphology was investigated in the same manner as before, with live-cell imaging. HtrA2/Omi MEF cells stably over-expressing simply the empty vector did not show a change in morphology of the mitochondrial network, compared to -/- MEFs (Fig. 15 and 19). However, the HtrA2/Omi -/- MEF cells stably re-transfected with human wt HtrA2/Omi showed a significant reversal of the phenotype to less elongated mitochondria (Fig. 19B,C), indicating that HtrA2/Omi does indeed influence the mitochondrial morphology. Even though the levels of wt HtrA2/Omi in the re-transfected HtrA2/Omi -/- MEFs exceeded the endogenous wt levels (Fig. 20), they had a similar effect on morphology (Fig. 15 and 19), indicating almost a complete reversal of the phenotype upon re-transfection of HtrA2/Omi. This was additionally confirmed in two further wt over-expressing clones (clones 1 and 2, data not shown). Most interestingly, in contrast to the wt over-expressing HtrA2/Omi -/- MEF cells, over-expression of S306A did not normalise the mitochondrial morphology (Fig. 19C,D), suggesting that the protease function of HtrA2/Omi is needed for maintaining a normal mitochondrial morphology. Furthermore, Nicole Kieper could show that stable over-expression of wt HtrA2/Omi in HtrA2/Omi -/- MEF cells reduced the levels of intramitochondrial ROS, as measured by MitoSOX. Interestingly, also cells over-expressing the protease dead mutation of HtrA2/Omi showed reduced levels of intramitochondrial ROS, suggesting that this event is independent of the mitochondrial morphology phenotype (Kieper *et al.,* 2010). It also suggests that the alterations in mitochondrial morphology are not due to increased ROS production.

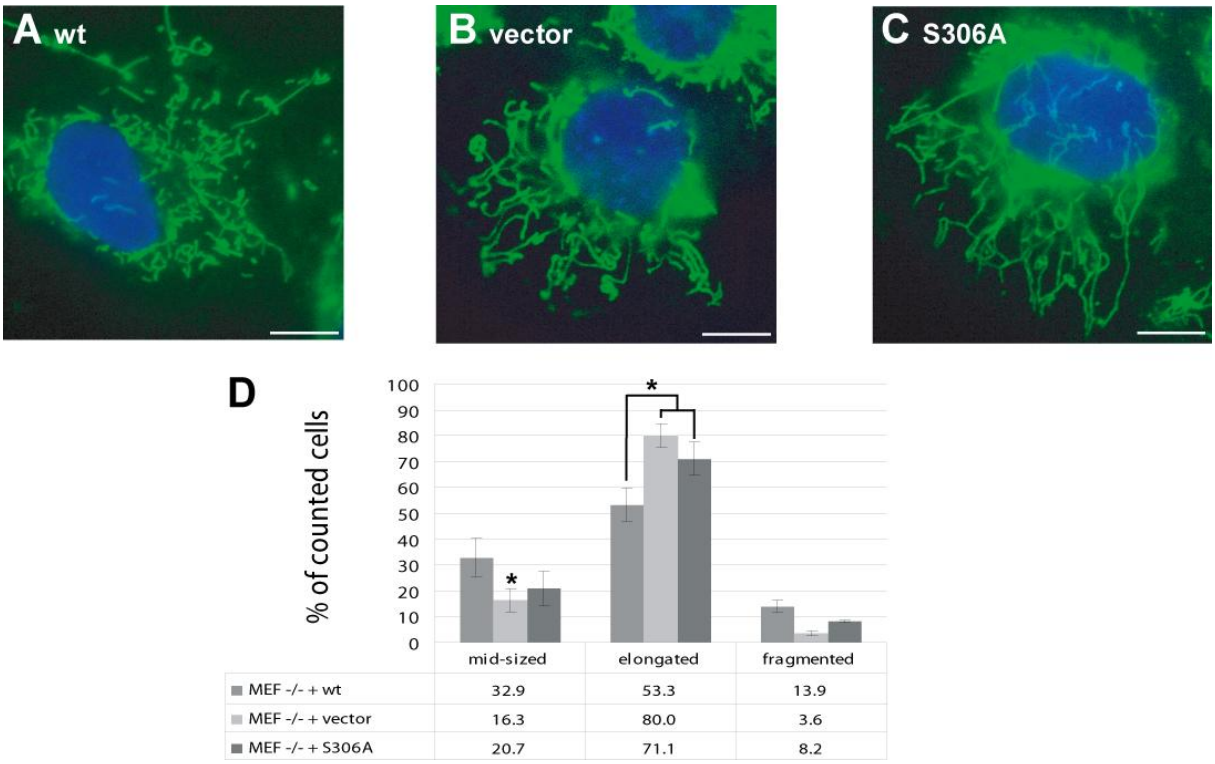

Figure 19. Re-transfection with human wt HtrA2/Omi rescues phenotype.

Live MEF -/- cells stably over-expressing human wt (A), vector (B) or S306A (C) HtrA2/Omi, stained with MitoTracker Green FM (green) and counterstained with Hoechst 33342 (blue). Size bars correspond to 10µm. (E) The mitochondrial morphology of Omi/HtrA2 -/- MEF cells stably over-expressing either human wt or S306A HtrA2/Omi or empty vector was investigated using the same procedure as for wt and -/- MEF cells (* $p<0.02$) (Fig. 15). Error bars correspond to SD. Live-cell imaging and counting was done in collaboration with Nicole Kieper.

3.2.5 Changes are not due to alterations in mitochondrial mass

In order to account for possible changes in mitochondrial mass due to the elongated mitochondria phenotype, Western blot analysis of cell lysates from HtrA2/Omi +/+ and -/- MEFs as well as HtrA2/Omi -/- MEFs stably over-expressing vector or wt HtrA2/Omi were performed. The results indicate that there are no detectable changes at the basal level of either the inner mitochondrial membrane, as seen by probing against the inner mitochondrial membrane protein adenine nucleotide transporter (ANT) (Fig. 20), or the outer mitochondrial membrane, here represented by the VDAC1. Nonetheless, it can be debated whether this method is sensitive enough to detect minor changes.

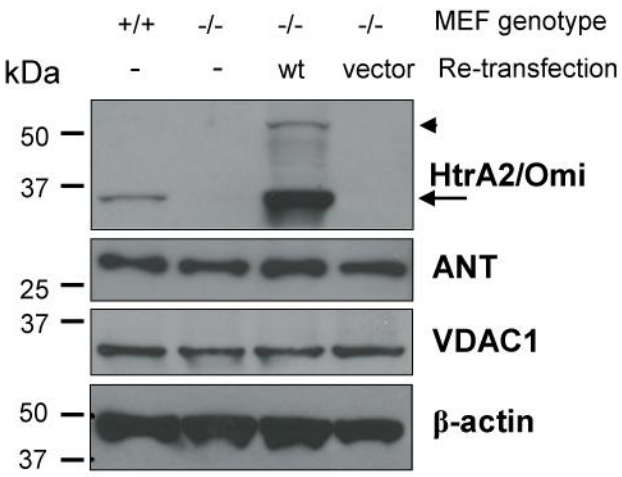

Figure 20. HtrA2/Omi does not affect mitochondrial mass.

HtrA2/Omi +/+ and -/- MEFs as well as -/- MEFs stably re-transfected with either vector or wt HtrA2/Omi were lysed with 1% Triton X-100 in TNE and subjected to Western blot analysis. The arrowhead indicates the precursor form of HtrA2/Omi while the arrow indicates the processed form. ANT was used as an IMM control, while VDAC1 is an OMM protein. β-actin was used as loading control.

3.3 HtrA2/Omi and mitochondrial fission and fusion

3.3.1 HtrA2/Omi does not influence the mitochondrial fission machinery

Mitochondrial morphology is dependent on the balance between fission and fusion and elongated mitochondrial may be a result of either increased fusion or decreased fission events. In order to monitor possible HtrA2/Omi dependent changes in mitochondrial fission and fusion, the basal protein levels of fission and fusion proteins in HtrA2/Omi +/+ and -/- MEF cells, as well as in HtrA2/Omi -/- MEFs re-transfected with either vector or wt HtrA2/Omi, were investigated. First the fission proteins Fis1 and Drp1 were investigated (Fig. 21). No decrease of either protein, upon loss of HtrA2/Omi, could be detected. On the contrary, a decrease of Fis1 levels in control MEF cells was observed. Interesting as the finding may be, this was not investigated further as this seemed not to be dependent on HtrA2/Omi protein levels.

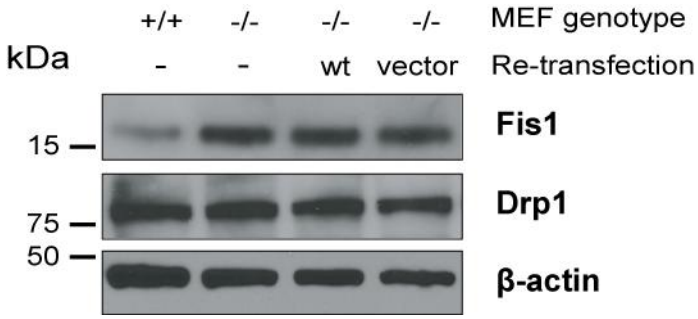

Figure 21. Mitochondrial fission factors.

HtrA2/Omi +/+ and -/- MEF cells, as well as -/- MEF cells stably expressing either wt or an empty vector were subjected to Western blot analysis. The membrane was probed with the mitochondrial fission factors Fis1 as well as Drp1, while β-actin was used as a loading control.

3.3.2 HtrA2/Omi influences the mitochondrial fusion machinery

The second step was to examine whether the basal protein levels of mitochondrial fusion factors were altered. The level of the mitochondrial outer membrane fusion protein Mfn-2 was not altered in any of the cell lines tested (Fig. 22A). On the other hand, the basal levels of OPA1, another fusion protein responsible for fusion of the inner mitochondrial membrane, were increased in the MEF cells lacking HtrA2/Omi (Fig. 22A), lysed with 1% Triton X-100 in TNE. Upon densitometric analysis, this increase in OPA1 abundance was shown to be over 1.5 fold (* $p<0.05$) for HtrA2/Omi -/- MEF cells compared to +/+ controls (Fig. 22B). For the re-transfectants the decrease upon re-transfection of HtrA2/Omi was also 1.5 fold (* $p<0.05$) (Fig. 22B).

Further analysis of the three identifiable OPA1 bands, consistently seen by Western blot analysis (marked a, b, c), indicate that the increase of OPA1 can not be attributed to a single OPA1 isoform but rather reflects total protein levels of OPA1 (Fig. 22C).

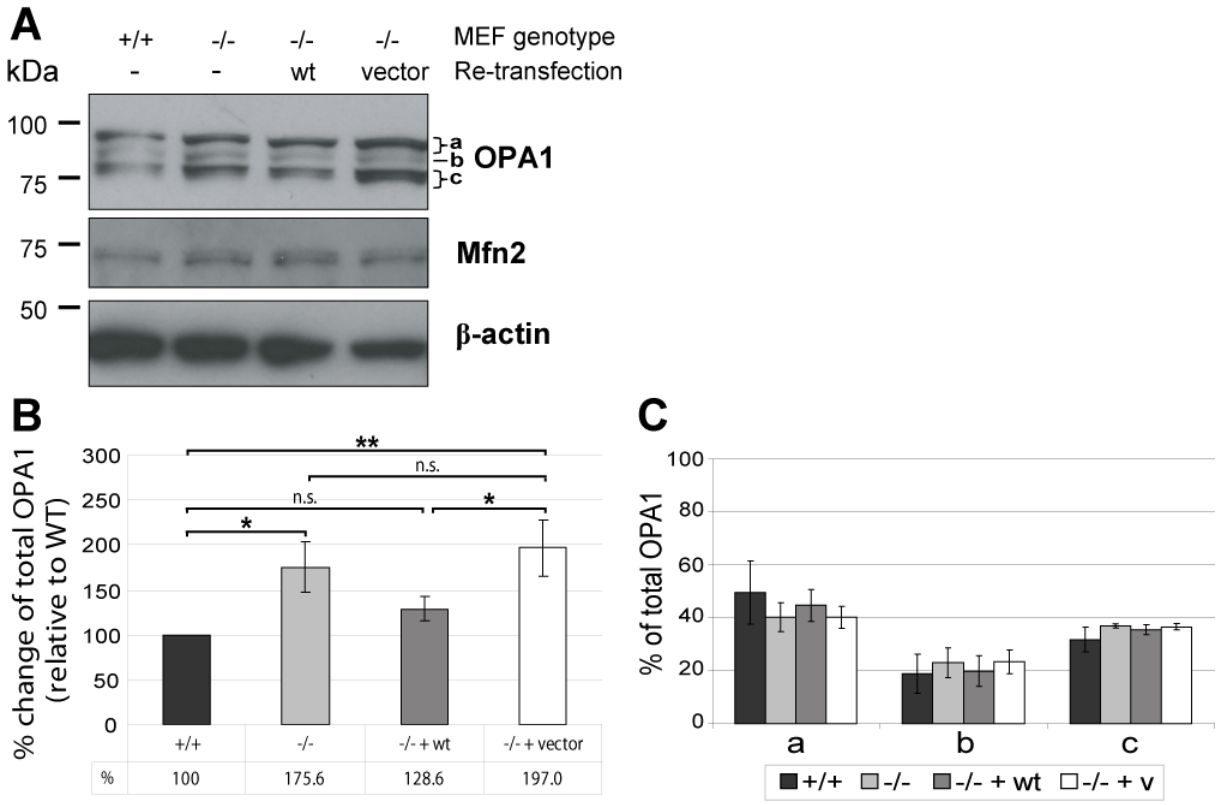

Figure 22. Effects of HtrA2/Omi on the mitochondrial fusion machinery.

HtrA2/Omi +/+ and -/- MEFs as well as -/- MEFs stably expressing vector or wt HtrA2/Omi were lysed with 1% Triton X-100 in TNE and subjected to immunoblotting. (A) Western blots were probed for the mitochondrial fusion proteins OPA1 as well as Mfn-2. β-actin was used as loading control. (B) Densitometric comparison of OPA1 Western blot signal intensities. OPA1 levels were compared to a loading control and expressed as relative to +/+. The values represent the mean of three independent experiments (n.s. = not significant, * $p<0.05$, ** $p<0.01$). (C) Densitometric comparison of the relative amount of the three different OPA1 bands identified on the Western blots (marked a, b, c) compared to total OPA1 levels. The values represent the mean of three independent experiments.

To investigate whether this effect depends on the presence of functional HtrA2/Omi protein, OPA1 levels were analysed also in HeLa cells, transiently silenced for HtrA2/Omi (Fig. 23A). These cells also display increased OPA1 levels upon lysis with 1% Triton X-100 in TNE. Interestingly, HtrA2/Omi -/- MEF cells over-expressing an empty vector or the protease dead mutant of HtrA2/Omi also reveal increased OPA1 levels upon lysis with 1% Triton X-100 in TNE, compared to wt re-transfected cells

(Fig. 23B). The close correlation between elongated mitochondria and increased OPA1 levels, suggests that these events may be related.

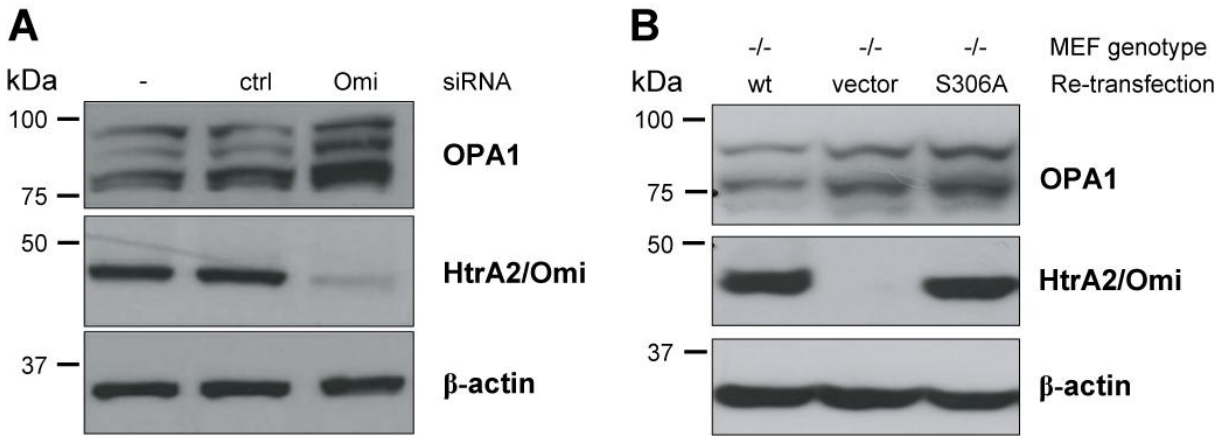

Figure 23. Effects of HtrA2/Omi on the mitochondrial fusion machinery are conserved.

(A) HeLa cells transfected with either none, 15nM control (ctrl) or 15nM HtrA2/Omi siRNA, were lysed with 1% Triton X-100 in TNE and subjected to immunoblotting. Western blots were probed for OPA1, HtrA2/Omi for silencing efficiency as well as β-actin as a loading control. (B) HtrA2/Omi -/- MEFs stably over-expressing either empty vector, wt or a protease dead form (S306A) of HtrA2/Omi were lysed with 1% Triton X-100 in TNE and subjected to immunoblotting. The Western blots were probed for OPA1 as well as HtrA2/Omi. β-actin was used as loading control.

3.3.3 Differences in OPA1 levels are due to altered protein extractability

To exclude potential transcriptional effects, OPA1 mRNA levels were investigated. Semi-quantitative RT-PCR, using primers that amplify all OPA1 isoforms, shows that there are no alterations on OPA1 mRNA levels (Fig. 24A). Additional Western blot experiments revealed different results on OPA1 protein levels upon the use of altered lysis conditions (Fig. 24B and C). HtrA2/Omi +/+ and -/- MEF cells directly lysed in SDS-PAGE sample buffer by boiling (Fig. 24B), or alternatively in RIPA buffer, which contains higher detergent concentrations than PBS or TNE with 1% Triton X-100, did not reveal any changes in OPA1 protein levels upon Western blot analysis. OPA1 levels seemed to only be altered under milder detergent lysis conditions (i.e. 1% Triton X-100) (Fig. 24C). This suggests that the difference in OPA1 levels observed upon milder extraction conditions is due to a shift in OPA1 extractability and not due to an overall increase in OPA1 protein levels.

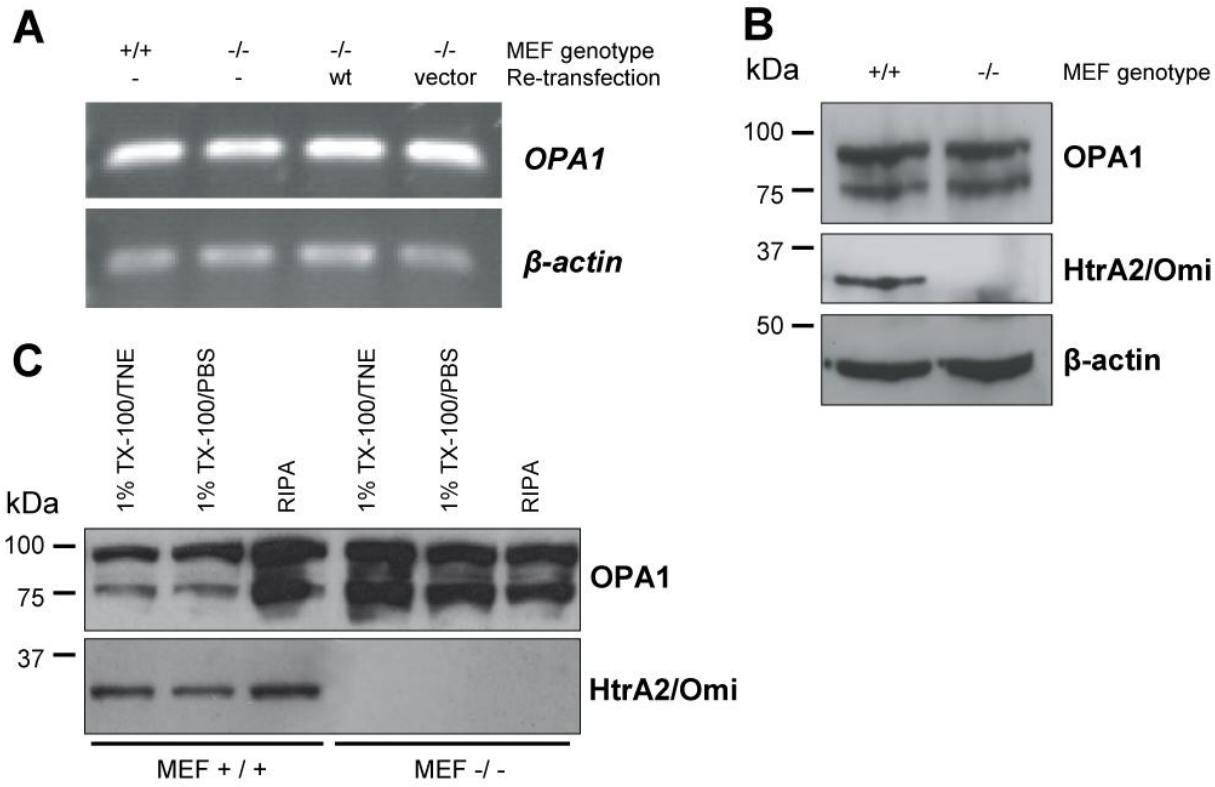

Figure 24. Differences in OPA1 levels are due to protein extractability.

(A) Semi-quantitative RT-PCR was performed on mRNA isolated from HtrA2/Omi +/+ or -/- and re-transfected MEF cells. There are no differences in the *OPA1* mRNA levels. Amplification of *β-actin* served as loading control. (B) HtrA2/Omi +/+ and -/- MEF cells were lysed directly in SDS-PAGE sample buffer, by boiling for 10 min at 95°C, before subjecting the full cell lysate to immunoblotting. The blot was probed for OPA1 and HtrA2/Omi while β-actin was used as loading control. (C) HtrA2/Omi +/+ and -/- MEF cells were lysed with either 1% Triton X-100 in PBS or TNE or with a harsher RIPA buffer and analysed by immunoblotting. Western blots were probed for OPA1 or HtrA2/Omi, as indicated.

3.3.4 OPA1 is differentially accessible depending on HtrA2/Omi

Mitochondria isolated from either HtrA2/Omi +/+ or -/- MEF cells (Fig. 25A) were subjected to proteinase K digestion for a range of time points in order to further narrow down the difference in OPA1 accessibility (Fig. 25B). The mitochondrial preparations were first probed for purity; VDAC1 was used as a marker for the mitochondrial fraction, while Hsp90 was used for the cytosolic fraction (Fig. 25A). As proteinase K digests the mitochondria from the outside in, it can help reveal a differential localisation or accessibility of OPA1 within the mitochondria. Indeed, a different kinetics of digestion for OPA1, in mitochondria isolated from HtrA2/Omi -/- MEF cells compared to +/+ controls, was observed. The OPA1 in the -/- cells was more susceptible to proteinase K digestion (Fig. 25B, C). The higher molecular weight accumulated form of OPA1 (marked with a in Fig. 25B) appears more easily

digested in -/- cells compared to lower molecular weight bands (marked with b in Fig. 25B). Densitometric analysis of the band intensities in three independent experiments confirms this, however, it also shows a slight decrease in the lower molecular weight bands in -/- cells compared to an apparent slight increase in the +/+ MEF cells (Fig. 25C). This may be due to an accumulation of the cleaved higher molecular weight forms in the lower molecular weight band. However these results need further validation. One further observation is the increase of cytosolic OPA1 in cells lacking HtrA2/Omi (Fig. 25A). Whether this is due to increased release from the IMS in cells devoid of HtrA2/Omi or simply due to loss of OMM integrity remains to be established in future experiments.

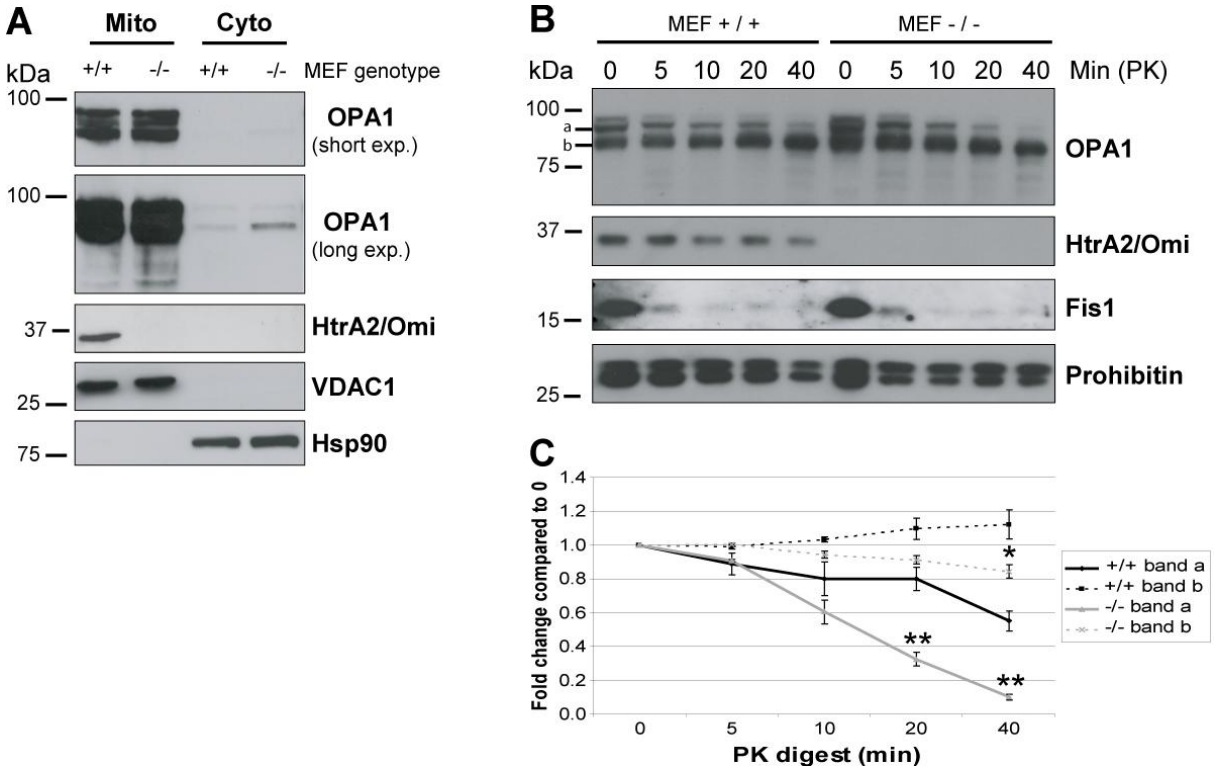

Figure 25. OPA1 is more easily digested by proteinase K in HtrA2/Omi -/- MEFs.

(A) Mitochondrial and cytosolic fractions prepared from HtrA2/Omi +/+ and -/- cells were subjected to Western blotting and analysed for localisation of both OPA1 and HtrA2/Omi. The purity of the cytosolic (Cyto) and mitochondrial (Mito) fractions was assessed by probing for Hsp90 and VDAC1 respectively. (B) Mitochondria isolated from HtrA2/Omi +/+ or -/- MEF cells were subjected to proteinase K digestion for indicated time points. Western blots were prepared and probed for OPA1 (short and long exposure) and HtrA2/Omi as indicated. The outer membrane associated protein Fis1 and the inner membrane protein prohibitin were used as controls. (C) Densitometric analysis of OPA1 intensities. The indicated OPA1 bands (marked with a and b in B) were quantified and compared to the initial amount (time point 0 min) and are represented as a fold change compared to 0 min from three independent experiments. Error bars represent SEM. For significance the HtrA2/Omi -/- time points for both band a and b were compared to the +/+ equivalent (* $p<0.01$ and ** $p<0.001$).

3.4 HtrA2/Omi and cell death

3.4.1 The loss of HtrA2/Omi renders cells more susceptible to certain types of cell death

The role of HtrA2/Omi in cell death is multifaceted. The protein can serve as both pro- and antiapoptotic, depending on the type of insult as well as the cell type and the general state of the cell. Once HtrA2/Omi is released from the mitochondria it aids in killing the cell by cleaving several different substrates. A comprehensive proteomics study identified several cytoskeletal-associated proteins as well as components of the translation machinery to be targets for HtrA2/Omi (Vande Walle *et al.*, 2008). However, within the mitochondria, HtrA2/Omi may serve to protect against damage. This is why there are several reports of both protective and promoting activities of HtrA2/Omi in cell death (Vande Walle *et al.*, 2008). In an initial attempt to investigate the protective effects of HtrA2/Omi, several stress inducing reagents such as staurosporine and H_2O_2 were explored. However, by assessment of lactatedehydrogenase (LDH) release, most of them failed to show a protective effect in this study, as seen in the case of H_2O_2 (Fig. 26A). On the contrary, it seems that loss of HtrA2/Omi is protective for the cell upon exposure to high levels of oxidative stress, most likely due to the loss of the pro-apoptotic functions of HtrA2/Omi.

Recent publications have suggested that HtrA2/Omi might be involved in protein quality control within the mitochondria. One report shows that the loss of HtrA2/Omi leads to an accumulation of misfolded proteins within the mitochondria (Moisoi *et al.*, 2009), while another report suggests that HtrA2/Omi plays a role in protecting the mitochondria against the accumulation of proteins upon proteosomal stress (Radke *et al.*, 2008). They show that the loss of HtrA2/Omi leaves the cell more prone to undergo apoptosis upon proteosomal stress, by subjecting cells to MG-132 treatment (Fig. 26B-D). MG-132 is a selective, cell-permeable inhibitor of the 26S proteasome (Lee & Goldberg, 1998). In this study it could be confirmed that HtrA2/Omi -/- MEF cells, treated with both different concentrations of MG-132 (Fig. 26B) as well as different incubation times of 1µM MG-132 (Fig. 26C), show increased susceptibility to undergo apoptosis compared to +/+ cells. Western blot analysis of caspase 3 cleavage as well as the cleavage of its substrate PARP clearly shows that cells

lacking HtrA2/Omi show earlier cleavage or alternatively at lower concentrations. Interestingly, HtrA2/Omi -/- MEF cells seem to have higher basal levels of cleaved caspase 3. Moreover, this is also seen in HeLa cells transiently transfected with siRNA against HtrA2/Omi, compared to control transfected cells (Fig. 26D).

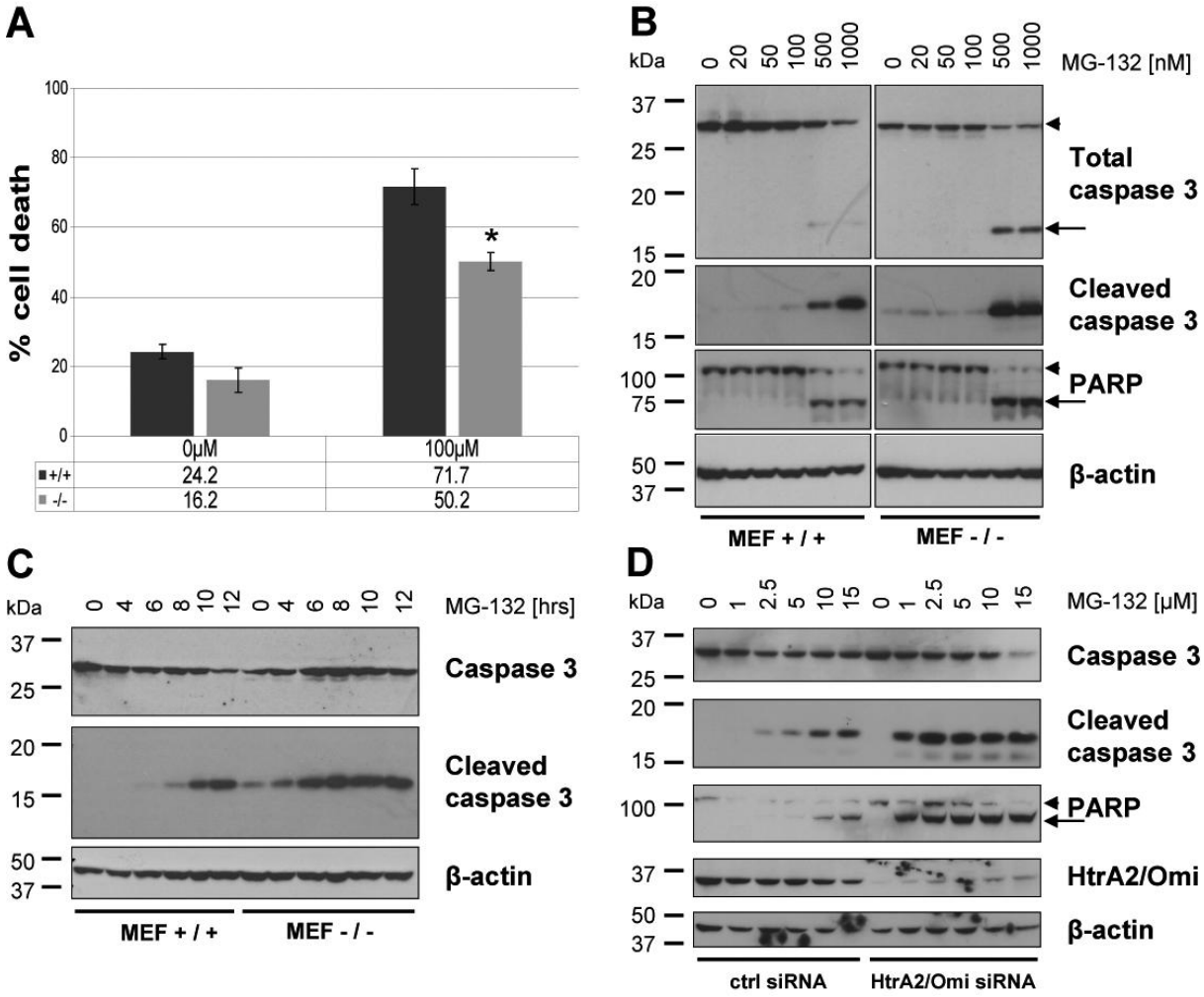

Figure 26. Cells lacking HtrA2/Omi are more susceptible to cell death upon proteasomal inhibition.

(A) HtrA2/Omi -/- and +/+ MEF cells were subjected to indicated amounts of H_2O_2 for 16 hrs after which the LDH release from the cells was measured using a CytoTox assay (Pierce), and used as a read-out for cell death. The results are the mean of three independent experiments. Error bars show SEM (* $p<0.05$). (B, C) HtrA2/Omi -/- MEF cells and +/+ controls were treated with the indicated concentrations of MG-132 for 18h (B) or with 1µM MG-132 for the indicated times (C). Lysates were prepared and Western blots sequentially probed with the indicated antibodies. Anti-caspase 3 showed breakdown of the procaspase 3 (arrowhead) to the active caspase 3 (arrow), which was confirmed by probing with a specific antibody against cleaved caspase 3. Consistently, the caspase 3 substrate PARP was cleaved from the full-length protein (arrowhead) to the 89kDa fragment (arrow). β-actin was used as loading control. (D) HeLa cells were transiently transfected with a total of 15nM either control or siRNA against HtrA2/Omi and treated with indicated concentrations of MG-132 for 18h before the cells were lysed and subjected to Western blot analysis. The membranes were probed with antibodies against both full length and cleaved caspase 3, PARP, HtrA2/Omi and β-actin as a loading control.

3.4.2 HtrA2/Omi is protective against cell death in a neuronal model

The previous result suggests a protective role for HtrA2/Omi against accumulating proteins. In order to investigate the role of HtrA2/Omi in a more PD-related model, SH-SY5Y cells stably co-expressing HtrA2/Omi and transcriptionally regulated tyrosinase were used. Tyrosinase is physiologically found mainly in skin cells (melanocytes), where it catalyses the hydroxylation of tyrosine to L-DOPA, and the downstream oxidation of L-DOPA to DOPAquinone (Hasegawa *et al.*, 2003). By ectopical over-expression of tyrosinase, which promotes the formation of dopamine (DA) and the oxidative species DOPA- and DAquinone (Fig. 27C), the model mimics the oxidative stress that takes place within the SNpc. The tyrosinase-inducible cell culture model was created by Takafumi Hasegawa and has been shown to induce increased intraneuronal dopamine and melanin, inducing chronic generation of ROS (TR8TY8; (Hasegawa *et al.*, 2003)). So far this model has also been used to show the exacerbating toxic effect of mutant α-synuclein (Furukawa *et al.*, 2006; Hasegawa *et al.*, 2006) as well as the protective effects of Parkin (Hasegawa *et al.*, 2008). HtrA2/Omi wt, as well as the three identified PD-related mutations and the synthetic protease dead mutant HtrA2/Omi, were stably co-expressed with the inducible tyrosinase in order to investigate the possible protective effects of HtrA2/Omi (Fig. 27A). The polyclonal stable pools showed similar expression levels of HtrA2/Omi for all the different variants, as well as of tyrosinase when induced (Fig. 27B). Four day induction of tyrosinase over-expression by 2µg/ml doxycycline (DOX) showed a high expression of tyrosinase and the formation of melanin, seen as pigmented granules within the cells (personal observation and consistent with (Hasegawa *et al.*, 2003)), as well as an induction of cell death as seen by the cleavage and subsequent loss of the caspase 3 substrate PARP (Fig. 27B). The cells over-expressing only vector showed increased PARP cleavage compared to cells over-expressing wt HtrA2/Omi (Fig. 27B,D and data not shown), suggesting a protective effect of wt HtrA2/Omi and a higher resistance to cell death in this model (Fig. 27B,D). Densitometric analysis of the PARP band intensities after three to four days of tyrosinase induction, in three independent experiments, confirm higher PARP levels in cells over-expressing wt HtrA2/Omi compared to empty vector controls (Fig. 27B,D). Interestingly, the protease dead mutant of HtrA2/Omi (S306A) showed significantly lower levels of full length PARP compared to wt over-expressing cells, indicating a requirement for

HtrA2/Omi protease function to protect the cells. The effects of the PD linked mutations are less clear and not significantly different from wt HtrA2/Omi (Fig. 27D).

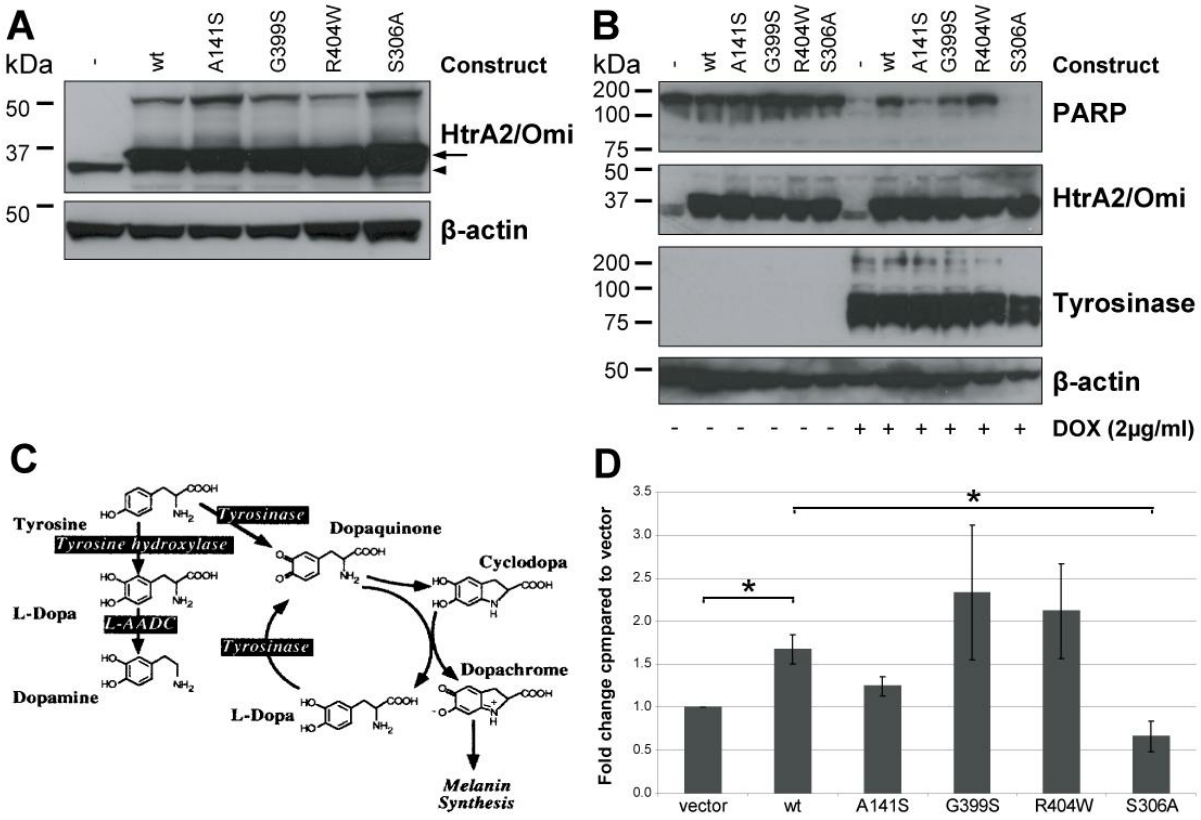

Figure 27. HtrA2/Omi is protective in a tyrosinase-induced cell death model.

(A) Lysates from neuroblastoma cells (SH-SY5Y), co-expressing inducible tyrosinase as well as vector, HtrA2/Omi wt or four mutations, were subjected to SDS-PAGE and the expression levels of HtrA2/Omi were confirmed by Western blotting. β-actin was used as a loading control. Endogenous HtrA2/Omi is indicated by an arrowhead, while the recombinant Flag-tagged HtrA2/Omi is marked by an arrow. (B) After four days with (+) or without (-) 2µg/ml DOX, the cells were lysed and subjected to Western blot analysis. The membranes were probed for PARP, as well as for HtrA2/Omi and tyrosinase expression levels. β-actin was used as a loading control. (C) Schematic overview of the roles of tyrosinase. Adapted from (Eisenhofer *et al.*, 2003). (D) Densitometric analysis of full length PARP loss in five independent experiments of TR8TY8 cells over-expressing different HtrA2/Omi constructs, induced for tyrosinase expression for three to four days with 2µg/ml DOX. Error bars represent SEM (* $p<0.005$).

3.5 The influence of HtrA2/Omi on PKB/Akt

3.5.1 Activation of Akt by H_2O_2

The PI3K/Akt pathway is one of the main pathways regulating cell survival, including cell death. It is known to be activated upon oxidative stress, and is marked by activation of Akt (also known as protein kinase B (PKB)) through phosphorylation at two different residues (Thr308 and Ser473) (Alessi *et al.*, 1996). To investigate whether HtrA2/Omi influences this signalling pathway, activation of Akt in response to oxidative stress, in cells lacking HtrA2/Omi compared to controls, was investigated. For this, H_2O_2 was used as an inducer of oxidative stress. In order to optimise the parameters used in further experiments, a range of concentrations of H_2O_2 to activate (induce phosphorylation of) Akt as well as a range of time points were tested. A time and concentration dependent increase of Akt phosphorylation at Thr308 (P-Akt) in HeLa cells, in response to acute H_2O_2 treatment, could be established (Fig. 28A). Similar kinetics was seen in wild type MEF cells (Fig. 28B). In order to further develop the parameters to be used in following experiments, the effects of glucose in the culture media were also examined. Lower levels of glucose renders the cell more dependent on oxidative phosphorylation and mitochondrial function as glycolysis cannot take place. It turned out that a lower glucose concentration or the complete absence of it leads to a higher activation of Akt as seen by higher P-Akt levels (Fig. 28C).

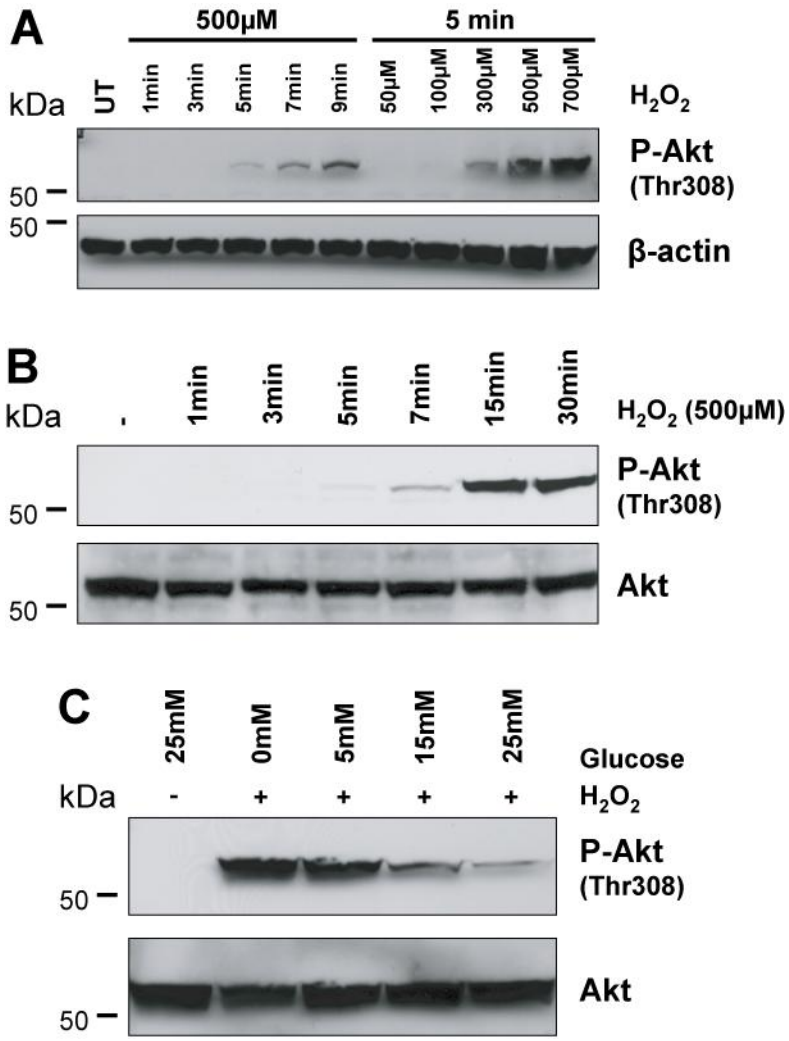

Figure 28. Phosphorylation of Akt is dependent on time and concentration of H_2O_2 as well as glucose levels.

(A) HeLa cells were treated with a range of H_2O_2 concentrations for 5 min as well as with 500µM for different times, before subjected to SDS-PAGE and immunoblotting. Western blots were probed for P-Akt induction and β-actin served as a loading control. (B) HtrA2/Omi +/+ MEF cells were subjected to 500µM H_2O_2 for the indicated time periods before subjected to SDS-PAGE and immunoblotting. The membrane was probed for P-Akt and Akt levels. (C) HtrA2/Omi -/- MEF cells were pre-treated with indicated concentrations of glucose in their growth medium 36hr prior to subjecting them to 500µM H_2O_2 treatment for 5min. The cells were then harvested and subjected to SDS-PAGE and immunoblotting. The membrane was probed for P-Akt and Akt levels.

3.5.2 MEF cells lacking HtrA2/Omi show lower levels of Akt activation upon oxidative stress

When using a range of concentrations of H_2O_2, a marked decrease in the levels of phosphorylated Akt in HtrA2/Omi -/- MEF cells compared to +/+ controls could be detected (Fig. 29A). Occasionally, a low basal activation of Akt in cells lacking Htra2/Omi could be detected under low glucose conditions, seen upon longer

exposure of the Western blots (Fig. 29A). Quantification of the marked decrease in P-Akt in MEF cells lacking HtrA2/Omi (Fig. 29B), confirmed a 50% decrease in P-Akt upon acute H_2O_2 treatment (5min, 500µM) (Fig. 29C).

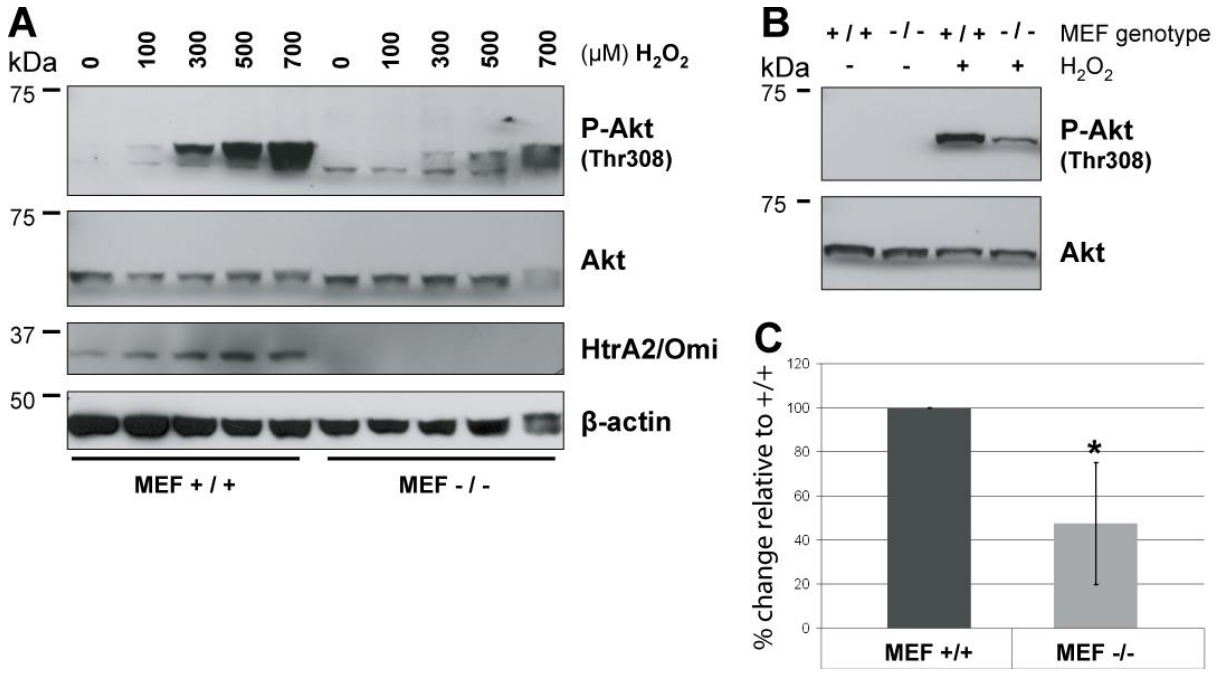

Figure 29. Cells lacking HtrA2/Omi show a weaker activation of Akt.

(A) HtrA2/Omi /+ and -/- MEF cells were treated with a range of H_2O_2 concentrations for 5min, as indicated, subjected to SDS-PAGE and immunoblotting. The membrane was probed for P-Akt, Akt and HtrA2/Omi as well as β-actin as a loading control. (B) HtrA2/Omi +/+ and -/- MEF cells were treated with 500µM H_2O_2 for 5min, before subjected to SDS-PAGE and immunoblotting. The membrane was probed for P-Akt and Akt. (C) Densitometric analysis of P-Akt Western blot signal intensity was performed. The P-Akt level were compared to a loading control and expressed as relative to +/+. The values represent the mean of four independent experiments (* $p<0.02$).

3.5.3 Lower levels of activation of Akt upon loss of HtrA2/Omi

In order to confirm that the lower levels of activation of Akt are indeed due to the lack of HtrA2/Omi protein, HtrA2/Omi -/- MEF cells stably over-expressing either vector or wt human HtrA2/Omi were used. The same acute H_2O_2 treatment to induce oxidative stress was used in these cells and they show the same kinetics of Akt phosphorylation as do HtrA2/Omi +/+ and -/- MEF cells (Fig. 30A). Cells lacking HtrA2/Omi show lower levels of Akt activation. The same is also true in HeLa cells transiently transfected with siRNA targeting HtrA2/Omi (Fig. 30B). Even though the loss of HtrA2/Omi is not as marked as in the -/- MEF cells, the decrease in P-Akt is. The lower levels of P-Akt in cells devoid of HtrA2/Omi suggest that the PI3K/Akt pathway is less active in these cells, which may render the cells more susceptible to

further cellular insults. However, one should keep in mind that the loss of HtrA2/Omi seems rather protective upon higher and prolonged levels of oxidative stress (Fig. 26A), though this is most likely due to the loss of pro-apoptotic effects of HtrA2/Omi.

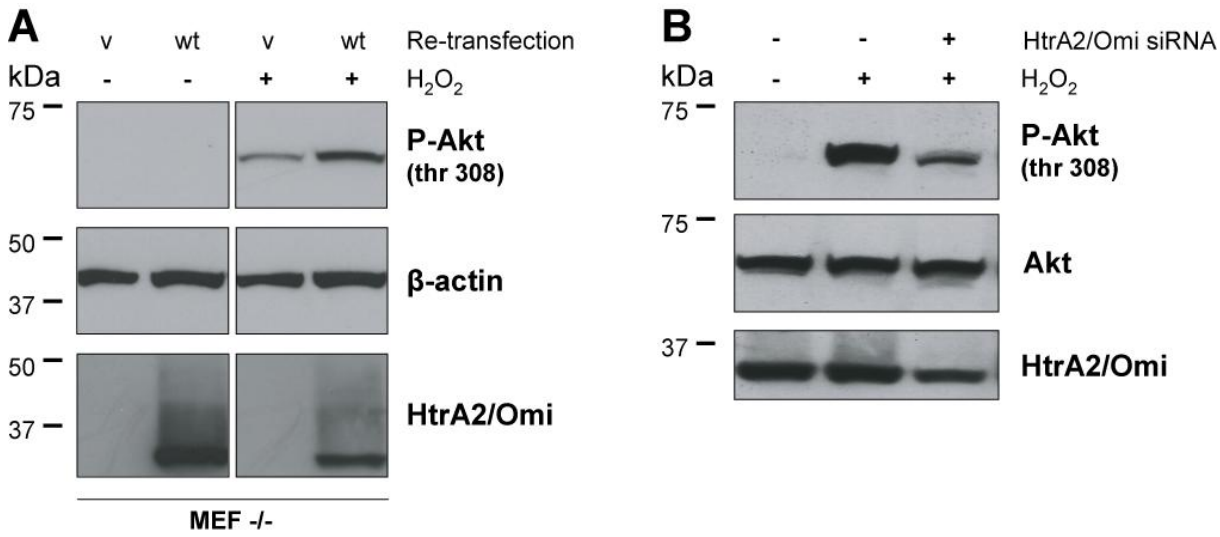

Figure 30. Phosphorylation of Akt at threonine 308 is modulated by HtrA2/Omi.

(A) HtrA2/Omi -/- MEFs stably re-transfected with either vector (v) or wt HtrA2/Omi were treated with none or 500µM H_2O_2 for 5 min and subjected to SDS-PAGE and immunoblotting. The membrane was probed for P-Akt, β-actin and HtrA2/Omi. (B) HeLa cells were transfected with either no or a total of 15nM HtrA2/Omi siRNA and subjected to 500µM H_2O_2 for 5min, as indicated. They were further subjected to SDS-PAGE and immunoblotting and the membranes were probed for P-Akt and Akt as well as HtrA2/Omi.

4 DISCUSSION

The importance of mitochondria in the pathogenesis of PD emerged with the initial discovery that the mitochondrial toxin MPTP causes parkinsonian like symptoms. Since then, mitochondrial damage has been observed in autopsies of PD patients. More recently, mitochondrial proteins have been genetically linked to PD. The identification of PINK1, associated with early onset autosomal recessive PD, and HtrA2/Omi, linked to sporadic PD, highlights the role of failing mitochondria as a possible source for the neurodegeneration in PD.

4.1 PINK1 and mitochondrial integrity

One of the main issues in PINK1 research has been the controversy over the sub-cellular localisation of PINK1. Even though PINK1 harbours a mitochondrial targeting sequence there are several reports of it being cytosolic (Gandhi *et al.*, 2006; Plun-Favreau *et al.*, 2007; Weihofen *et al.*, 2008). The main problem has been the poor quality and specificity of the available antibodies for PINK1. The antibodies used in this study also show several cross-reacting bands. However, silencing of PINK1 allowed the identification of the PINK1 specific bands. This reveals several forms of PINK1. Further cellular fractionation of HeLa cells shows both a cytosolic and mitochondrial localisation of the PINK1 protein. The full-length form, still containing the MTS, is found only in the cytosol, while further processed forms of PINK1 are found in the mitochondria as well as in the cytosol. The literature further supports the existence of several cleaved forms of PINK1, most likely due to proteolytic processing within the mitochondria but possibly also in the cytosol (Gandhi *et al.*, 2006; Weihofen *et al.*, 2008). Another option is the possible re-export of PINK1 into the cytosol, though the mechanisms underlying this possible action remain unknown. The importance of the different PINK1 cleavage products is not yet known, but it is bound to play a role in the functions of PINK1. Even though no protease responsible for PINK1 cleavage has been identified to date, other interacting proteins of PINK1 have been found to influence its distribution, activity and function. For example the Hsp90/Cdc37 molecular chaperone complex in the cytosol is known to influence the stability, localisation and processing of PINK1 (Weihofen *et al.*, 2008).

At the time of publication of the PINK1 results from this study (Exner *et al.*, 2007), little was known about the function of PINK1 in vertebrate models, especially in

regard to mitochondrial function and integrity. Since the discovery of PINK1 most of the information about the protein had been obtained using different *D. melongaster* models, either knock-out or transgenic flies. One of the aims of this study was to investigate the importance of PINK1 in a human system. In flies, PINK1 loss leads to a clear mitochondrial phenotype, namely disorganised mitochondrial morphology, energy depletion and reduced mitochondrial mass (Clark *et al.*, 2006; Park *et al.*, 2006b; Yang *et al.*, 2006). In order to address the issue in a human system, a cell culture model of PINK1 loss was created. Transient silencing by siRNA transfection was used as a fast and easy way to achieve PINK1 loss. In order to find out the necessity of PINK1 under normal physiological conditions, the MMP was assessed in these cells. As the MMP is strongly linked to the integrity of the mitochondria, a decrease in this parameter would suggest that the cells are rendered more susceptible to cellular insults. Indeed, the results showed that cells lacking PINK1 have a decrease in MMP, indicating a dysfunctional mitochondrial population. Other studies have since confirmed this finding and also linked reduced complex I activity and reduced ATP levels to the loss of PINK1 protein (Clark *et al.*, 2006; Gautier *et al.*, 2008). Besides reduced ATP production, decreased MMP has been linked to altered mitochondrial morphology, which could explain the fragmented mitochondrial phenotype found in association to this study (Exner *et al.*, 2007). A scheme of the possible outcomes of loss of PINK1 is depicted below (Fig. 31).

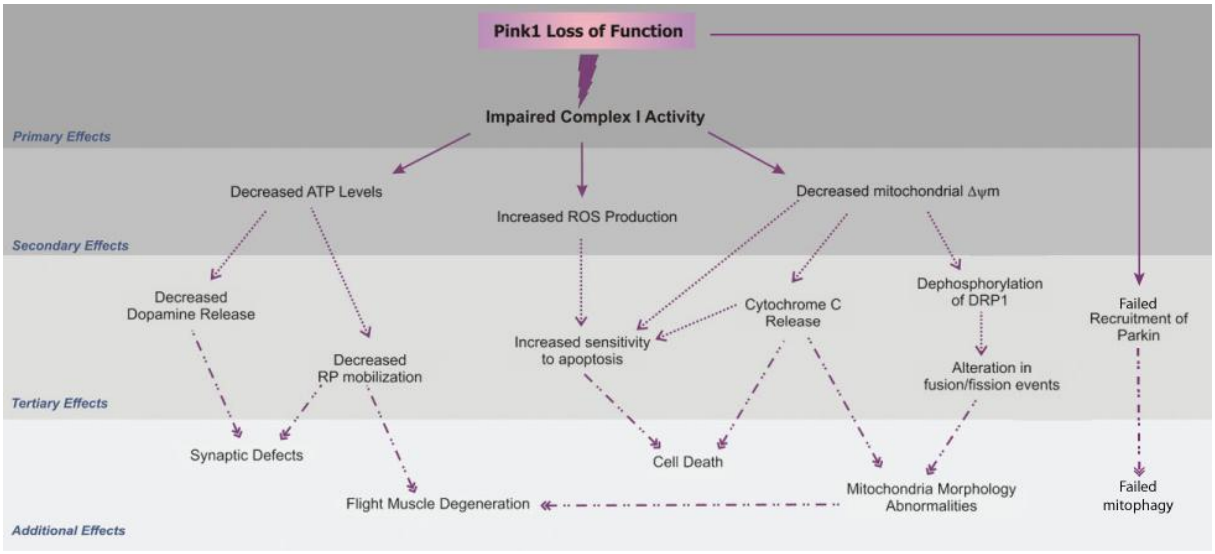

Figure 31. Schematic representation of downstream defects caused by PINK1 deficiency.

Most of the downstream effects of PINK1 deficiency, also discussed in the introduction (Section 2.3), could be explained by the impaired mitochondrial complex I activity seen in mice and cells lacking PINK1. However, new data has also implicated PINK1 in mitochondrial turnover, most likely independent of its effect on complex I. RP; reserve pool of synaptic vesicles. Adapted from (Morais *et al.*, 2009).

It is now becoming increasingly clear that PINK1 is directly involved in maintaining mitochondrial integrity and that several of the mitochondrial and cellular effects recorded, are secondary to faulty mitochondrial function, like impaired complex I activity. However, there is still no evidence for PINK1 interacting directly with complex I, therefore further physiological substrates will have to be uncovered before the exact function of PINK1 can be fully understood. Another possible role of PINK1 is as a stress and damage sensor. Recent research into the role of PINK1 in Parkin-mediated mitochondrial autophagy highlights the importance of the protein in whole organelle turnover as it is required to mediate the recruitment of Parkin to faulty mitochondria (Geisler *et al.,* 2010). The reported interactions of other PD-associated genes with PINK1, suggests that there may be a common mitochondrial signalling pathway in PD. So far, research suggests that PINK1 lays upstream of both Parkin and HtrA2/Omi, although that they are not found in the same pathway (Whitworth *et al.*, 2008).

4.2 Functions of HtrA2/Omi within the mitochondria

HtrA2/Omi is known to function as a pro-apoptotic factor, once released from the mitochondria, its role in mitochondrial protection is still unclear. The two loss-of-function mouse models of HtrA2/Omi that lead to a neurodegenerative phenotype, clearly speak for a protective or supportive role for HtrA2/Omi within the mitochondria (Jones *et al.*, 1993; Martins *et al.*, 2004). A dual function of HtrA2/Omi is intriguing. Another well documented protein with such dual function is the IMS protein cytochrome C. Under physiological conditions, cytochrome C is an essential part of the ETC, transporting electrons between complexes III and IV. Once the cell undergoes apoptosis, cytochrome C is released from the mitochondria and works as a pro-apoptotic protein by binding to Apaf-1, forming the apoptosome together with ATP and the pro-caspase 9 (Kroemer *et al.*, 2007) (Fig. 8). For HtrA2/Omi its function as a pro-apoptotic protein is relatively well documented, but its physiological role remains unknown.

4.2.1 How the loss of HtrA2/Omi influences mitochondrial integrity

In order to find out the necessity of HtrA2/Omi under normal physiological conditions, the MMP was assessed. Loss of HtrA2/Omi led to lower levels of MMP. This further correlated with an increase in both intramitochondrial as well as total cellular ROS. Both parameters indicate a sub-optimal function of the ETC. The loss of mitochondrial membrane potential and the increase in ROS production seen in this study have also been confirmed in other studies (Moisoi *et al.*, 2009). Even though the differences in MMP and ROS between HtrA2/Omi +/+ and -/- MEF cells are relatively small, they clearly indicate that the mitochondria in cells devoid of HtrA2/Omi are under higher levels of stress already under basal conditions.

For PINK1 it is known that the decreased membrane potential and increased ROS production are associated with lower ATP levels in cells lacking PINK1 (Morais *et al.*, 2009). In contrast, cells lacking HtrA2/Omi showed a substantial increase in basal ATP levels. This is inconsistent with a decreased MMP, as it would reflect a dysfunctioning ETC and therefore should lead to lower ATP levels. The loss of inner membrane structures as seen by EM also suggests a reduced capacity for oxidative

phosphorylation. So far, no other study has directly looked at ATP levels in HtrA2/Omi -/- cells. One study reports no change in brain respiratory chain complex activities (Martins *et al.*, 2004). Another report identified reduced respiration levels in brain mitochondria (Moisoi *et al.*, 2009). Interestingly, the increased ATP and ROS levels do correlate with the elongated mitochondrial phenotype observed, and it has been shown that elongated mitochondria produce more ATP (Tondera *et al.*, 2009), which again can lead to higher levels of ROS. However, the MEF cells were cultured under high glucose conditions (4.5g/l), which would allow them to derive most of their energy from glycolysis. This may have more dire consequences in neurons, as they cannot switch to glycolysis to suffice their energy demand (Almeida *et al.*, 2001). In order to identify the real influence of HtrA2/Omi on oxidative phosphorylation, the cells would have to be grown for example in galactose containing medium instead of glucose.

4.2.2 HtrA2/Omi affects the mitochondrial network

Altered mitochondrial integrity has recently been shown to further influence mitochondrial dynamics. This phenomenon has also become increasingly important in understanding the pathways underlying neurodegeneration. For neurodegenerative diseases such as Charcot-Marie-Tooth disease and autosomal dominant optic atrophy, alterations in mitochondrial morphology have been implicated in their pathology (Alexander *et al.*, 2000; Delettre *et al.*, 2000; Kijima *et al.*, 2005). In PD, it is known that PINK1 influences the mitochondrial morphology. The reports have been contradictory to whether PINK1 promotes or inhibits fission of fusion (Sandebring *et al.*, 2009), but together they highlight the importance of a balanced fission and fusion process for optimal cell survival.

For HtrA2/Omi, this study is the first to show that HtrA2/Omi influences the morphology of the mitochondrial network. Loss of HtrA2/Omi leads to elongation of the mitochondria and the presence of a more connected mitochondrial network. The elongated mitochondrial phenotype is not only clear in HtrA2/Omi -/- MEF cells, but also observed in both human (HeLa) and fly (SR2+) cells where HtrA2/Omi has been transiently silenced. This indicates that this is a much conserved effect, not just across species boarders but also when comparing transient versus permanent knock down of HtrA2/Omi, much different to the situation for PINK1.

To further confirm the specificity of the phenotype, HtrA2/Omi -/- MEF cells, stably over-expressing human wt HtrA2/Omi were created. The over-expression of wt HtrA2/Omi completely reversed the mitochondrial phenotype. Elongation was further used as a read-out for the functionality of mutants. Unfortunately, stable over-expression of the PD-linked mutations was not successful, but a clone expressing the synthetic mutation S306A was successfully used in this study. The S306A mutation is devoid of protease activity as the active serine has been exchanged for an alanine (Li *et al.*, 2002). Interestingly, the HtrA2/Omi -/- cells stably over-expressing S306A did not show a rescue of the mitochondrial morphology phenotype. This clearly suggests that the protease activity is essential for maintenance of a normal mitochondrial morphology. In contrast, another effect seen upon HtrA2/Omi knock-down, increased ROS levels, was restored also by the protease dead form of HtrA2/Omi. This suggests that the increased ROS levels are not what is driving the elongation and that there must be another mechanism than the protease activity of HtrA2/Omi that leads to the normalisation of the ROS levels.

HtrA2/Omi has long been suggested to act as a molecular chaperone, due to the homology to the bacterial chaperones Deg S, P and Q (Faccio *et al.*, 2000). However, there is very little proof of a chaperone function of mammalian HtrA2/Omi in the literature. A recent report shows that HtrA2/Omi, *in vitro*, helps delay the aggregation of the AD-related protein amyloid-β_{1-42} (Kooistra *et al.*, 2009). Most interestingly, their results indicate that the effect is partially independent of both the protease activity as well as the PDZ domain, suggesting that HtrA2/Omi also has chaperone-like functions (Kooistra *et al.*, 2009). Consistently, the results from this study also suggest that HtrA2/Omi might have a protease independent function. In addition to the ROS data, the mitochondrial morphology results also hint of such an activity. Although the S306A mutant fails to restore the mitochondrial morphology, it does slightly shorten the mitochondria, suggesting that it either still possesses some protease activity, or some other function. Although these are merely speculations, together with the ROS data, they do imply that the S306A mutant still maintains some of its functionality, possibly a chaperone-like function. Further studies would have to confirm this finding, as well as identify the chaperone acting domain of HtrA2/Omi.

4.2.3 HtrA2/Omi modulates OPA1

On the molecular level, a change in mitochondrial morphology suggests that there might be a change in the levels of any of the known mitochondrial fission or fusion proteins. Indeed, Western blot analysis of the fission factors Fis1 and Drp1 and the fusion factors Mfn2 and OPA1 revealed a selective increase in OPA1 levels under mild detergent conditions in cells lacking functional HtrA2/Omi.

OPA1 is a nuclear encoded mitochondrial protein involved in mitochondrial inner membrane fusion (Frezza *et al.*, 2006). The protein has been linked to autosomal dominant optic atrophy type 1, a neurodegenerative disorder of the axons of the optic nerve and the retinal ganglion cells, leading to progressive visual loss (Amati-Bonneau *et al.*, 2009). OPA1 is ubiquitously expressed in humans, comes in eight different isoforms (Fig. 4B) and is further proteolytically processed to give rise to a range of protein products, visualised as five bands on a Western blot. The role of the different isoforms in the process of fusion is still not clear, however, the presence of the longer forms have been linked to more fused mitochondria, while a loss of them, due to proteolytic cleavage and accumulation of shorter OPA1 protein products has been associated with shorter mitochondria (Guillery *et al.*, 2008; Song *et al.*, 2007).

OPA1 has further been suggested to be involved in formation of cristae junctions complexes (Frezza *et al.*, 2006), which are structures upholding the cristae formation and the folded organisation of the IMM. The folded nature of the IMM enhances the ATP production by expanding the surface area at which the process can take place (Zick *et al.*, 2009). It has also been shown that the F_1F_0-ATP synthase is involved in cristae formation (Soubannier & McBride, 2009; Zick *et al.*, 2009). The oligomerisation of the F_1F_0-ATP synthase, and the formation of supercomplexes seem to influence the curvature of the IMM and the formation of microdomains optimised for ATP production (Soubannier & McBride, 2009; Zick *et al.*, 2009). Besides the importance of cristae formation for ATP production, it has also been hypothesised to play a role in compartmentalising proteins such as cytochrome C. Scorrano and collagues have shown that extensive re-organisation of the IMM structures takes place upon apoptosis (Cipolat *et al.*, 2006; Frezza *et al.*, 2006). The main alteration is the opening of the cristae, which allows cytochrome C to more

easily be released from the mitochondria into the cytosol, initiating apoptosis. OPA1 has been suggested to oligomerise and form a complex, including both soluble as well as membrane bound forms of OPA1, although the exact isoforms involved in this process are not known, constricting the cristae opening trapping cytochrome C (Frezza *et al.*, 2006) (Fig. 32). This would of course influence the progression of apoptosis as cytochrome C is one of the most important pro-apoptotic proteins released form the mitochondria upon apoptosis. Interestingly, the role of OPA1 in the formation of the cristae junction complex and its role in apoptosis are thought to be independent of the role in mitochondrial fusion (Frezza *et al.*, 2006).

Further analysis of the differences in the OPA1 levels in cells devoid of HtrA2/Omi and wt MEF cells indicates that HtrA2/Omi not only affects the extractability of OPA, but also its susceptibility to proteinase K digestion. This suggests a redistribution of OPA1 within the mitochondria. The altered ultrastructure of cells lacking HtrA2/Omi supports this notion. The lack of inner membrane organisation suggest a disruption of the cristae junction complex and a redistribution of at least the soluble shorter forms of OPA1 (the ones lacking the TM domain), allowing it to be more easily extracted. The lack of a change in pattern of OPA1 forms, as seen by Western blot analysis, would imply an absence of increased proteolytic processing of the membrane bound OPA1 forms not engaged in the cristae junctions. This suggests that they remain bound to the membrane. However, the disruption of the cristae junction complex and the more accessible folding of the inner membrane render also these forms more susceptible to proteolytic processing as well as easier extraction (Fig. 32).

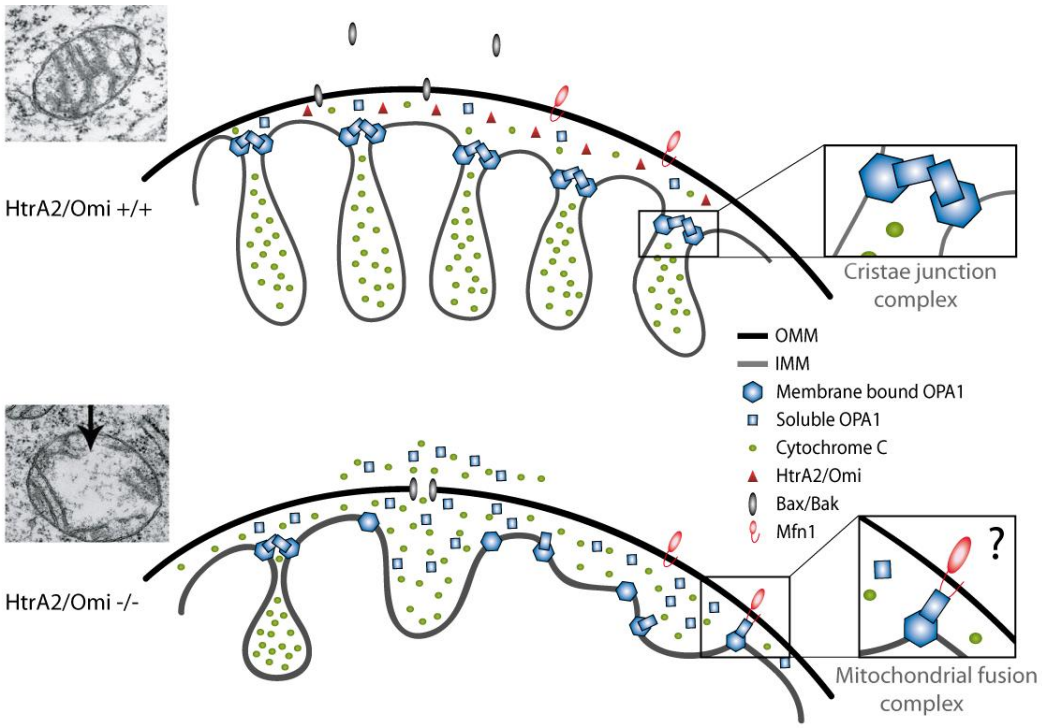

Figure 32. Schematic representation of the possible fates of OPA1 after HtrA2/Omi loss.

The possible altered mitochondrial distribution of OPA1, and the thereof following effects on mitochondrial function and morphology are depicted in the figure above. The EM pictures are taken from Fig. 16.

Whether the disruption of the cristae junction complex leads to a redistribution of OPA1 to mitochondrial fusion complexes, and this is why cells lacking HtrA2/Omi show elongated mitochondria or whether this is simply a secondary effect, is something that can only be speculated. As the exact isoforms and proteolytic products involved in either event are still unclear, it is not known whether an altered engagement of these OPA1 forms in fusion events is even possible. Further evidence is needed to confirm whether HtrA2/Omi truly influences the distribution of OPA1 from cristae junctions to fusion engaged forms. It also remains to be shown whether this effect is a primary effect on OPA1 or an indirect effect due to a loss of inner or outer membrane integrity. Nicole Kieper was able to show a physical interaction between HtrA2/Omi and endogenous OPA1 (Kieper *et al.,* 2010), however as there is no obvious influence of HtrA2/Omi on the proteolytic processing of OPA1, as seen by the absence of a change in band pattern upon Western blot analysis, it is doubtful that OPA1 is a substrate for HtrA2/Omi. The possibility of HtrA2/Omi functioning as a chaperone still remains, however as discussed before, this still

needs extensive validation and until now there is very little evidence supporting this notion.

Another interesting aspect about OPA1, in terms of neurodegeneration and PD, is its apparent link to mtDNA stability (Amati-Bonneau *et al.*, 2008). Alterations of OPA1 could influence the mitochondrial DNA mutation and deletion load. Analysis of the mitochondrial DNA from muscle tissue of patients with mutations in OPA1 showed multiple deletions of mitochondrial DNA (Amati-Bonneau *et al.*, 2008). Interestingly, higher amounts of mitochondrial DNA deletions can be found in the SNpc compared to other dopaminergic neurons (Bender *et al.*, 2008), suggesting a role for the loss of mtDNA integrity in the pathogenic mechanisms underlying PD. For HtrA2/Omi the mutation or the deletion load of mtDNA has never been investigated. Moisoi *et al.* noted that there is no change in the amount of mtDNA in brains of HtrA2/Omi -/- mice compared to littermate controls (Moisoi *et al.*, 2009), however mutations or deletions may not necessarily alter the mtDNA amount (Amati-Bonneau *et al.*, 2008). Nonetheless, it is still unclear through what mechanism OPA1 influences mtDNA stability, whether it is through maintenance of mitochondrial fusion, which seems most likely, or through some other means.

One further observation made during this study is the presence of higher levels of soluble OPA1 in the cytosol of cells lacking HtrA2/Omi, even under basal conditions. This could simply be an artifact of the mitochondrial preparation. The higher levels of soluble OPA1 in the IMS of cells lacking HtrA2/Omi may lead to higher amounts of released OPA1 during the preparation. Alternatively, it could be due to a loss of OMM integrity, leading to easier rupture of the membrane and release of IMS proteins, something that cannot completely be ruled out due to the lack of more suitable marker proteins for both the IMS and the OMM in proteinase K digest experiments. Finally, it could be due to higher induction of Bax and/or Bak and opening of a pore at the outer membrane allowing the release of soluble OPA1, possibly together with other proteins such as cytochrome C (Zhang *et al.*, 2008) (Fig. 32). Such a scenario may also explain the apparent presence of cleaved caspase 3 in HtrA2/Omi -/- MEF cells under basal conditions. OPA1 has been documented to be anti-apoptotic, though its role in the cytosol during apoptosis is unclear (Zhang *et al.*, 2008). Cytochrome C on the other hand is known to be pro-apoptotic once in the cytosol

(Kroemer *et al.*, 2007), therefore elevated level of cytochrome C, both freely available in the IMS and in the cytosol, could prime the cells to die more easily. Further experiments would have to confirm and exclude any of these hypotheses.

4.2.4 The involvement of HtrA2/Omi in cell death

The role of HtrA2/Omi in the cytosol is relatively clear and straight forward. Several cytosolic substrates have been identified, including the IAPs, and the role for HtrA2/Omi in both caspase-dependent and -independent cell death pathways is well documented. An understanding of the pro-apoptotic role of HtrA2/Omi is of therapeutic value in areas such as cancer or ischemia/reperfusion research. However, for neurodegeneration the physiological protective role of HtrA2/Omi is of importance.

A couple of reports have implicated HtrA2/Omi in the protein quality control of mitochondrial proteins (Moisoi *et al.*, 2009; Radke *et al.*, 2008). Moisoi *et al.* noted an increase accumulation of unfolded proteins in mitochondria from mice lacking HtrA2/Omi, which was even more pronounced in mitochondria isolated from the brain (Moisoi *et al.*, 2009). Radke *et al.* identified an increase of the mitochondrial IMS protein Endo G upon proteosomal inhibition, and an HtrA2/Omi dependent degradation of wt but not mutant Endo G (Radke *et al.*, 2008). In accord with these findings, a protective role for HtrA2/Omi upon proteasomal inhibition could be shown. This was true for both the HtrA2/Omi +/+ MEF cells when compared to -/- and for HeLa cells transfected with nonsense siRNA compared to those where HtrA2/Omi had been transiently silenced. The possible role of HtrA2/Omi in the protein quality control in the IMS is intriguing as very little is known about this mechanism. For the matrix and IMM, several molecular chaperones and proteases have been identified to be part of the clean up machinery in these compartments but for the IMS as well as the OMM much remains to be discovered (Tatsuta, 2009).

Besides protein aggregation and sub-optimal proteasome function, the other major cause thought to underlie PD pathology is oxidative stress. In this regard the dopaminergic cells in the SNpc are particularly susceptible to high levels of ROS due to the high propensity of dopamine and intermediates in dopamine metabolism to auto-oxidise (Hastings, 2009). The role of HtrA2/Omi in protection against oxidative

stress is less clear than upon protein aggregation. Some reports have identified a protective role for the protein upon low levels of H_2O_2 treatment (Martins *et al.*, 2004). This could however not be replicated, on the contrary, there seems to be a protective function of the loss of HtrA2/Omi upon acute H_2O_2 treatment in MEF cells.

The fickle nature of HtrA2/Omi and its possible dual role in the cell complicates the assessment of HtrA2/Omi in cell death. High levels of stress trigger permeabilisation of the IMM and a release of proteins from the IMS, including HtrA2/Omi, triggering apoptosis and highlighting the role of HtrA2/Omi as a pro-apoptotic factor. Besides the type and intensity of the toxic insult in question, the cell type also influences the role HtrA2/Omi plays in cell survival. Even though HtrA2/Omi is ubiquitously expressed, recent work identified a brain-specific up-regulation of the stress-inducible transcription factor CHOP in HtrA2/Omi -/- mice (Moisoi *et al.*, 2009). In order to investigate the role of HtrA2/Omi under oxidative stress conditions in a neuronal model, dopaminergic neuroblastoma cells (SH-SY5Y) were used. This particular model over-expresses transcriptionally regulated tyrosinase. Tyrosinase is normally found in the skin where it acts as a catalyst, promoting the formation of L-DOPA and further melanine. This resembles the situation found in the SNpc, where tyrosine hydroxylase also promotes formation of L-DOPA (Eisenhofer *et al.*, 2003). This promotes ROS production and also leads to an accumulation of melanin pigments within the cells (Hasegawa *et al.*, 2003). In order to investigate the protective functions of HtrA2/Omi in this model, wt HtrA2/Omi as well as three PD-related mutations and a synthetic protease dead mutant were stably over-expressed. Even though there seemed to be no protective effect of HtrA2/Omi upon acute oxidative stress, in a non-neuronal model, over-expression of wt HtrA2/Omi in this neuronal, more chronic model of oxidative stress did show a protective effect. This was dependent on the protease function of HtrA2/Omi as the protease dead mutant HtrA2/Omi showed no protective effect.

The mechanisms underlying the protective actions of HtrA2/Omi remain unclear. It is possible that HtrA2/Omi proteolytically cleaves oxidised proteins in the IMS, though there is no evidence for this. Oxidative stress leads to an increase of damaged proteins that need to be degraded (Breusing & Grune, 2008). Oxidation of proteins can further lead to cross-linking of them, which again can inhibit the function of the

proteasome. Especially the 26S proteasome is sensitive to oxidative stress (Breusing & Grune, 2008). Therefore, it is possible that the oxidative stress upon tyrosinase expression leads to an impairment of the proteasome, causing an increase in accumulated proteins within the mitochondria, upon which HtrA2/Omi acts.

4.2.5 The relationship between HtrA2/Omi and PKB/Akt

There are several pathways within the cell that governs its integrity, one of the main ones regulating cell viability is the phosphoinositide 3-kinase (PI3K)/Akt pathway. The serine/threonine kinase Akt has been implicated in a range of functions within the cell from cell proliferation to cell death, and it is known to be up-regulated in response to oxidative stress (Brazil *et al.*, 2002). In humans, three homologues of the protein have been found: Akt 1, 2 and 3 (Parcellier *et al.*, 2008). It has further been established that Akt is activated by sequential phosphorylation at two specific residues; Thr308 and Ser473 (Brazil *et al.*, 2002). Ser473 is phosphorylated by the rapamycin-insensitive companion of mammalian target of rapamycin (mTOR) complex 2 (mTORC2), after which Akt is phosphorylated at Thr308 by the PI3K-dependent kinase 1 (PDK1). This reaction is further enhanced by association with phosphatidylinositol (PtdIns) at the plasma membrane. Traditionally, the PI3K/Akt-pathway has been of interest in cancer research as it is dysregulated in several different cancers (Brazil *et al.*, 2002). More recently, interest for the pathway has also arisen in the field of neurodegenerative research. The cell protective function of the pathway has attracted attention as a potential therapeutic target for PD (Timmons *et al.*, 2009).

In order to examine whether HtrA2/Omi influences the PI3K/Akt pathway, H_2O_2 was used to induce activation of Akt, seen as phosphorylation at residue Thr308 (P-Akt). A clear and consistent increase in P-Akt could be seen upon both increasing concentrations and time of H_2O_2 treatment. Interestingly, the P-Akt levels were further increased by pre-treating the cells with low or no glucose, which would indicate that this phenomenon is related to mitochondrial function, or possibly to increased ROS levels in these cells. Low or no glucose conditions force the cell to rely more heavily on mitochondria respiration instead of glycolysis, also increasing the ROS production. When comparing HtrA2/Omi +/+ and -/- MEF cells in their capacity to phosphorylate Akt upon H_2O_2 treatment, the loss of HtrA2/Omi led to a

clear reduction of P-Akt. This was further verified in cells transiently treated with HtrA2/Omi siRNA as well as in HtrA2/Omi -/- MEF cells stably over-expressing human wt HtrA2/Omi. The results show that this is a conserved phenotype.

The decreased activation of Akt in cells lacking HtrA2/Omi suggests a reduced activation of the whole PI3K/Akt-pathway, rendering the cells more susceptible to undergo cell death. Akt has been reported to phosphorylate HtrA2/Omi at residue Ser212, inhibiting its protease function, however this was shown to take place in the cytosol upon release of HtrA2/Omi from the mitochondria (Yang *et al.*, 2007). The phosphorylation observed under the conditions of this study is more likely to take place before IMM permeabilisation and release of HtrA2/Omi. Although Akt is mainly cytosolic, recent reports show that there is a mitochondrial pool of the protein (Barksdale & Bijur, 2009; Bijur & Jope, 2003). Antico Arciuch *et al*. bring to light interesting new evidence that underline the role of mitochondria as a platform for signal convergence also in regards to Akt-signalling. They show that the differential phosphorylation steps of Akt upon H_2O_2 insult depend at least in part on mitochondria (Antico Arciuch *et al.*, 2009). They further show that in NIH/3T3 cells, the initial phosphorylation of Akt by mTORC2 under low (50µM) H_2O_2 conditions takes place in the cytosol. This allows the increased uptake of Akt into the mitochondria where it is further phosphorylated at Thr308 by PDK1. PDK1 and PtdIns have both been found to some extent also in the mitochondria (Antico Arciuch *et al.*, 2009; Connor *et al.*, 2005). Further results from the study indicate that the phosphorylation of Akt at Thr308 is important for nuclear translocation of Akt. This nuclear translocation was shown to promote cell proliferation. Furthermore, the results reveal that high (250µM) levels of H_2O_2 inhibit phosphorylation of Akt at Thr308, rendering the cells more susceptible to cytochrome C release and activation of apoptosis. This suggests that HtrA2/Omi could influence the mitochondrial step of Akt activation.

There are still several questions to be answered before conclusions can be made about the role of HtrA2/Omi in Akt phosphorylation. The first one is whether there are differences in basal levels of Akt in the mitochondria in cells lacking HtrA2/Omi. It would also be interesting and important to determine whether any of the Akt phosphorylation seen in this study is mitochondrial, at any point. Antico Arciuch *et al*. noticed an ablation of Thr308 phosphorylation upon high levels of H_2O_2, especially in

the mitochondria, while in this study the H_2O_2 levels used were high and there was a significant increase in P-Akt even after 30min, but in a whole cell lysate. The use of different cell lines could explain this discrepancy, or the relative low levels of mitochondrial Akt when compared to total cell levels. In this study the use of a specific antibody against Ser473 was tried, however, there seemed to be no induction upon H_2O_2 treatment (data not shown). This could be due to the insensitivity of the antibody or the loss of signal due to the treatment of the samples after induction, as a report states that the Ser473 phosphorylation is temperature sensitive and the signal can be lost after incubating the samples on ice (Oehler-Janne *et al.*, 2008). As the activation at Thr308 is a later event rendering Akt fully active, this was used for further studies. It would however be important to ensure that the lower levels of Thr308 phosphorylation are not due to lower levels of Ser473 phosphorylation. Finally, the interaction of HtrA2/Omi with any of the constituents upstream of Akt, such as DPK1, or its influence on their mitochondrial localisation would be interesting, in order to establish the role of HtrA2/Omi not just in Akt phosphorylation but within the mitochondria in general. An interesting downstream component of the Akt-pathway is the glycogen synthase kinase 3 isoform β (GSK-3β). It has been associated with a mitochondrial localisation and has been shown to influence neuronal cell death (Petit-Paitel *et al.*, 2009). Akt is known to phosphorylate GSK-3β, inhibiting its activity (Mookherjee *et al.*, 2007).

4.3 Outlook

This work was able to address the importance of both PINK1 and HtrA2/Omi under physiological conditions. The involvement of HtrA2/Omi in the modulation of both mitochondrial morphology and the PI3K/Akt-signalling pathway puts in place a few new pieces of the HtrA2/Omi puzzle. Nevertheless, a lot still remains to be discovered and needs to be addressed.

For PINK1, results from this study as well as the literature hint to roles both in maintaining mitochondrial function as well as in regulating turnover of the organelle. They further emphasise its role as a master regulator within the mitochondria, highlighting the therapeutic significance of understanding its function. The possible dual role of PINK1 in both maintaining mitochondria functions such as complex I activity, and the need for it in eliminating the organelle through autophagy, is very intriguing. It is vital to discover further substrates as well as upstream interactors or upstream activating events in order to uncover the role of PINK1 both within the mitochondria as well as in the cytosol. Possible new substrates of PINK1 could be detected using phosphoproteomics. For this the different PINK1 knock-out mouse models, or silenced human cells would come in handy. Moreover, perhaps the different processed forms of PINK1 are differentially responsible for either function? This would underline the importance of understanding the function of each form of PINK1, as well as discovering the protease(s) involved in cleaving PINK1. Creation of different length synthetic constructs of PINK1, where putative cleavage sites have been mutated could help reveal the importance of each PINK1 species. A systematic knock-down of putative, MMP sensitive, proteases either in the IMS, OMM or in the cytosol might further uncover the upstream protease responsible for PINK1 cleavage.

For HtrA2/Omi, the outcome was a successful uncovering of the influence of HtrA2/Omi on mitochondrial function under physiological conditions, in terms of decreased MMP and increased ROS as well as altered mitochondrial morphology upon loss of the protein. Nonetheless, the mechanisms underlying these events remain largely unknown. HtrA2/Omi has been proposed to mediate protein quality control in the IMS, through cleaving accumulating proteins. However, whether it has

specific protein targets or simply detects misfolded or accumulating proteins remains unclear. Some putative mitochondrial substrates are known from the literature, such as Hax1 (Cilenti *et al.*, 2004), Endo G (Radke *et al.*, 2008) and more recently the heat shock 70 kDa protein 8, pyruvate dehydrogenase E1 component beta subunit and isocitrate dehydrogenase [NAD] subunit alpha (Johnson & Kaplitt, 2009), whether there are more remains to be seen. Further, whether HtrA2/Omi interacts with these proteins under physiological conditions or only under mild stress conditions is also unclear.

One of the key issues is how HtrA2/Omi recognises its substrates. It is known that the PDZ domain is responsible for protein-protein interaction. Studies with the bacterial homologues of HtrA2/Omi, DegS and DegP, have revealed that the PDZ domain in responsible for substrate recognition (Meltzer *et al.*, 2009). Most interestingly, DegS is known to recognise envelope stress by binding to the C-termini of accumulating outer-membrane proteins with its PDZ domain (Sohn *et al.*, 2007). This initiates the cleavage of RseA, eventually facilitating enhanced transcription of genes for periplasmic chaperones, proteases and biosynthetic enzymes (Sohn *et al.*, 2007). Whether the same applies for HtrA2/Omi within the mitochondria remains to be seen. A screen of possible residues essential for interaction within the PDZ domain and site-directed mutagenesis of these residues would give tools to determine if this mode of action is conserved. The examination of recorded substrates, such as EndoG, and possible C-terminal alterations of these proteins would also help uncover the mode of action of HtrA2/Omi. Whether this can be assessed *in vivo* or would have to be done *in vitro* remains unclear.

Besides binding of substrates to the PDZ domains, there are other mechanisms that seem to influence the protease activity of HtrA2/Omi. Phosphorylation of HtrA2/Omi at Ser212 by Akt has been shown to reduce its protease activity (Yang *et al.*, 2007). PINK1 again is known to mediate phosphorylation of HtrA2/Omi at Ser142, which promotes its protease activity (Plun-Favreau *et al.*, 2007). Could phosphorylation be the possible switch within the mitochondria? In this case it would be interesting to know what triggers PINK1 mediated phosphorylation of HtrA2/Omi. For this the functions of PINK1 would have to be unravelled first. Creation of different phospho-mimicking mutants of HtrA2/Omi and their role in both protection against proteasomal

stress and mitochondrial morphology would possibly help elucidate the need of phosphorylation of HtrA2/Omi for activation.

Moreover, the apparent influence of HtrA2/Omi on OPA1 distribution and/or IMM integrity remains to be answered. The hierarchy of the effects still needs to be established, whether distorted IMM structures proceeded altered OPA1 distribution or vice versa. As of now, both the ultrastructure and the proteinase K digest experiments were performed with HtrA2/Omi +/+ and -/- MEF cells however, it would be interesting to investigate whether the morphology can be rescued by wt HtrA2/Omi and not by the protease dead mutant. Further, the actual localisation of OPA1, of all isoforms, in cells lacking HtrA2/Omi remains unknown. It remains unclear, whether the differential extractability truly reflects an altered OPA1 localisation. Further fractionation experiments, including differential centrifugation steps and percoll or sucrose gradients to isolate the different mitochondrial compartments, should help elucidate this. Carbonate extraction experiments would reveal whether the OPA1 in the HtrA2/Omi -/- cells is more soluble. To further unravel whether the OPA1, not engaged in the cristae junctions, is increasingly associated with the other mitochondrial fusion proteins Mfn 1 and 2, co-immunoprecipitation experiments with OPA1 and most importantly Mfn 1 should be performed.

Finally, the PI3K/Akt pathway is becoming increasingly important also in the field of neurodegeneration, and to further elucidate the role of HtrA2/Omi in Akt phosphorylation could prove lucrative. Of the possible downstream targets of Akt, the most promising candidate to further study is GSK-3β, which has been found to partially localise to mitochondria and is implicated in neuronal cell death (Petit-Paitel *et al.*, 2009). However, bringing to light the upstream events that influence Akt phosphorylation would probably reveal more about HtrA2/Omi function, useful also to understand its role in regards to other proteins such as OPA1. For this, the influence of HtrA2/Omi on the sub-cellular localisation of both Akt as well as the phosphorylated forms of it would be informative. The existence of possible upstream regulators of Akt, such as PDK1, within the mitochondria should also be evaluated both in wt as well as cells devoid of HtrA2/Omi. For this, fractionation experiments should be performed. Preferentially, this should also be examined in a neuronal cell

culture model, the same goes for the other HtrA2/Omi-related experiments, as studies have shown that the loss of HtrA2/Omi has differential effects in different organs (Moisoi *et al.*, 2009).

Both PINK1 and HtrA2/Omi are fascinating proteins in terms of the complexity of their functions. Especially PINK1 bares great therapeutic potential in terms of a master regulator of mitochondrial signalling pathways in PD. The possible roles of HtrA2/Omi in protein quality control within mitochondria would further reveal important new mechanistic insight about mitochondrial quality control. Therefore, further research into these two genes will improve our understanding of the mechanisms underlying not just PD but neurodegeneration in general.

5 MATERIALS AND METHODS

5.1 Chemicals and Reagents

All chemicals were purchased from Merck, Sigma, Roth, Calbiochem, Fluka or Applichem unless otherwise stated.

Reagent	Manufacturer
2',7'-dichlorohydrofluorescein diacetate (DCF)	Invitrogen
4-12% Bis-Tris NuPAGE gradient gels	Invitrogen
Agarose	Invitrogen
Agarose beads coupled to FLAG antibody	Sigma
Ampicillin	Sigma
bicinchoninic acid (BCA Protein Assay)	Pierce
BigDye Terminator v.3.1	Applied Biosystems
Bradford Reagent	Bio-Rad
Collagen	Cohesion
Complete proteinease inhibitor	Roche
Diethylpyrocarbonate (DEPC)	Roth
DMEM-F12	Biochrom AG
DNA 1 kb ladder (+6x DNA loading buffer)	Fermentas
dNTPs (dATP, dCTP, dGTP, dTTP)	Fermentas
Dulbecco's modified eagle medium (DMEM)	Biochrom AG
Ethidium bromide solution	Merck
Fetal Bovine Serum (FBS)	PAA Laboratories
FBS (tetracycline negative)	PAA Laboratories
Fluorescent mounting medium	Dako
FuGene6	Roche
GoTaq Polymerase (+ 10X buffer)	Promega
HiPerfect	Qiagen
Hoechst33342	Invitrogen
Hyperfilm ECL high performance chemiluminescence	GE Healthcare
Immobilon Western HRP Substrate	Millipore
Kanamycin	Sigma

Continued

Reagent *continued*	Manufacturer *continued*
MitoSOX	Invitrogen
MitoTracker CM-H_2XROS	Invitrogen
MitoTracker Green FM	Invitrogen
Normal goat serum	Chemicon
NuPAGE MOPS running buffer (20x)	Invitrogen
Oligonucleotides	Metabion or Sigma
Opti-MEM	Invitrogen
peqGOLD TriFast	PeqLab
Poly-D-Lysine (PDL)	Sigma
Precision Plus Protein Standard, prestained	Biorad
Protein G agarose	Millipore
PVDF membrane	Millipore
QIA prep spin MiniPrep Kit	Qiagen
QIAfilter plasmid Maxi/Midi Kit	Qiagen
QIAquick gel extraction Kit	Qiagen
QuickChange II Site-Directed Mutagenesis Kit	Stratagene
Restriction enzymes	Fermentas or Roche
RNeasy Mini Kit	Qiagen
Shrimp alkaline phosphatase (SAP)	Roche
T4 DNA Ligase (+10x ligase buffer)	Fermentas
TaKaRa *Ex Taq* polymerase (+ 10X buffer)	TaKaRa BIO INC.
Tetramethyl rhodamine methyl ester (TMRM)	Invitrogen
Transcriptor High Fidelity cDNA Synthesis Kit	Roche

Table 2. Table with all the reagents and kits used.

5.2 Solutions and Buffers

5.2.1 Molecular Biology

Ampicillin stock solution

100mg/ml in ddH_20. Aliquots were stored at -20°C.

Kanamycin stock solution

30mg/ml in ddH_20. Aliquots were stored at -20°C.

LB medium

1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 0.5% (w/v) NaCl were dissolved in ddH_20. The medium was sterilised by autoclaving. Ampicillin (100µg/ml) or Kanamycin (30µg/ml) was added and the solution stored at 4°C.

LB agar plates

1.2% (w/v) Agar was dissolved in the LB medium before autoclaving. After autoclaving Ampicillin (100µg/ml), Kanamycin (30µg/ml) or nothing was added to the solution, after which the solution was poured onto plates. The plates were stored at 4°C.

DYT medium

16% (w/v) Bactotryptone, 10% (w/v) Yeast extracts, 5% (w/v) NaCl was dissolved in ddH_2O. The medium was sterilised by autovclaving and stored at room temperature.

Tris-Borate-EDTA buffer (TBE)

90mM Tris, 89mM Boric acid, 20mM EDTA in ddH_20.

Miniprep buffer – I

0.9% Glucose, 10mM EDTA, 25mM Tris, 100µg/ml RNase A in ddH_20.

Miniprep buffer – II

200mM NaOH, 1% (w/v) SDS in ddH_20.

Miniprep buffer –III

3M Potassium acetate (pH 5.0), 11.5% acetic acid in ddH_20.

SOC medium

2% Trypton, 0.5% Yeast extract, 8.5mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM Glucose in ddH_20. Stored at 4°C.

5.2.2 Cell Biology

PBS (Phospahte buffered saline)

1.8mM KH_2PO_4, 10mM $NaHPO_4$, 137mM NaCl, 2.7 mM KCl in ddH_20 (pH 7.4).

Cell freezing medium

10% DMSO, 90% fetal bovine serum.

TNE - Cell lysis buffer

50mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1% Triton-X 100, 10mM NaPP, Complete Protease Inhibitor Cocktail in ddH_20. 2mM NaF and 100mM Na_3VO_4 was added if phosphorylated proteins were to be detected later.

RIPA lysis buffer

50mM Tris-HCl (pH 8.0), 150mM NaCl, 1% NP40, 0.5% Deoxycholate, 0.1% SDS, 10mM $NaPP_i$ and Complete Protease Inhibitor Cocktail in ddH_20.

1% Triton-X in PBS

1% Triton-X 100 and PBS. Complete Protease Inhibitor Cocktail was added if used for cell lysing.

Homogenisation buffer

50mM Tris (pH 7.5), 1% [v/v] NP-40, 2mM EDTA, 100mM NaCl and Complete protease inhibitor cocktail in ddH20.

Mitochondrial isolation buffer (MIB)

250mM Sucrose, 20mM HEPES, 3mM EDTA and Complete Protease Inhibitor Cocktail in ddH20 (pH 7.5).

Proteinase K stop solution

100mM EDTA, 40mM PMSF in ddH_20.

5.2.3 Protein Biochemistry

SDS-PAGE stacking gel buffer

0.5M Tris (pH 6.8) and 0.4% SDS in ddH_20.

SDS-PAGE separating gel buffer

1.5M Tris (pH8.8) and 0.4% SDS in ddH_20.

SDS-PAGE running buffer

25mM Tris, 193mM Glycin, 3.5mM SDS in ddH_20.

5X SDS-PAGE sample buffer (5X Laemmli buffer)

0.312M Tris-HCL (pH 6.8), 10% SDS, 25% β-Mercaptoethanol, 50% glycerol, 0.05% bromophenol blue in ddH_20.

Transfer buffer

25mM Tris, 192 mM Glycine in ddH_20.

TBS - Tris buffered saline

50mM Tris (pH 7.4), 150mM NaCl in ddH_20.

TBST – Tris buffered saline / 0.1% Tween 20

50mM Tris (pH 7.4), 150mM NaCl, 0.1% (v/v) Tween 20 in ddH_20.

Blocking solution

5% (w/v) non-fat milk powder in TBST.

Western Blot stripping solution

62.5 mM Tris (pH 7.6), 2% (w/v) SDS, 100mM β-Mercaptoethanol in ddH_20.

Primary antibody solution

5% (v/v) Roche Blocking Solution, 0.02% (w/v) Sodiumazide in TBS.

Secondary antibody solution

5% (w/v) non-fat milk powder in TBST and antibody.

5.2.4 Fluorescent Imaging

4% Paraformaldehyde (PFA)

4% paraformaldehyde, 2.5mM NaOH in PBS (dissolved at 56°C).

10% Normal Goat Serum (NGS)

10% normal goat serum in PBS.

Antibody solution

1% BSA in PBS.

5.3 Techniques

5.3.1 Molecular biology

Polymerase chain reaction, gel electrophoresis and DNA extraction

The original HtrA2/Omi constructs (pCMV-HtraA2/Omi-Flag [wt, A141S, G399S, R404W]) were obtained from Sabine Kautzmann. The S306A mutant was cloned using the Stratagene QuikChange II Site-Directed Mutagenesis Kit according to manufacturer's instructions.

The HtrA2/Omi constructs were subcloned into the pcDNA3.1-Zeo vector by PCR using the TaKaRa ExTaq DNA polymerase, according to manufacturer's protocol, with primers bearing the appropriate restriction sites. The PCR products were separated on a 1% agarose gel, visualised with UV, excised and purified using the QIAquick gel extraction kit (Qiagen). Primers used for cloning can be obtained upon request.

Enzymatic digestion of DNA

1µg DNA was digested with 10U restriction enzyme in a 20µl reaction volume for 2h at 37°C. The enzymes were subsequently heat inactivated according to manufacturer's instructions. Digested vectors were further dephosphorylated with 1µl SAP for 30min at 37°C. Both vectors and PCR products were purified using gel extraction after enzymatic digest (see above).

Ligation and transformation

The DNA fragments and vectors with compatible enzymatically digested ends were ligated using the T4 DNA ligase. The insert was added in five-fold compared to vector in a 20µl reaction mixture. The constructs were ligated at 15°C for at least two days. 10µl (for re-transformation 1µg DNA was used) of the reaction mixture was added to 125µl chemically competent DH5α *E. coli* and left on ice for 20min. The mixture was subjected to a two minute heat shock at 42°C after which it again was put on ice before adding 250µl room temperature LB medium (without antibiotics) and incubated for 1h at 37°C at 350rpm. The bacteria were then spread out on pre-warmed LB-plates with appropriate antibioticum and incubated at 37°C overnight.

Colony-PCR

In order to test whether the bacteria contained the correct construct, several clones were tested using the colony PCR method. Half of the clone was picked and lysed in 30µl ddH_20 while the other half was kept for further expansion. 1µl of the lysate was used to set up a PCR reaction using Taq DNA polymerase, according to manufacturer's instructions, using appropriate primers for the insert. The PCR product was separated on a 1% agarose gel and the size of the insert was verified.

Amplification, purification and validation of plasmid DNA

Positive colonies as verified using the colony PCR method were further amplified by doing a Miniprep or Midiprep.

For a Miniprep, 6ml LB containing the appropriate selective antibiotic was inoculated with a single colony. The bacteria were incubated at 37°C, shaking vigorously overnight. The next morning 2ml of the Miniprep culture was centrifuged at 6000rpm in a table top centrifuge for 10min. The pellet was resuspended in 150µl Miniprep buffer I after which 150µl Miniprep buffer II was added to lyse the bacteria by inverting the tube several times. 150µl of Miniprep buffer III was added and the tube inverted after which the suspension was centrifuged at 13,000 rpm for 10min at room temperature. The supernatant was transferred to another tube and 1ml of 100% ethanol was added before further centrifugation step at 13,000 rpm for 10min at room temperature was used to precipitate the DNA. The supernatant was removed and the pellet washed with 70% ethanol and centrifuged at 13,000 rpm for 10min at room temperature one to three times. After the last time the supernatant was removed and the pellet left to air-dry after which it was re-dissolved in 20µl ddH_20.

For a MidiPrep 100ml LB with appropriate antibiotica was inoculated with a single colony or some of the frozen bacterial glycerol stocks. The bacteria were grown at 37°C overnight, vigorously shaking. The next morning culture was centrifuged at 4000rpm for 30min at 4°C. For the purification of the DNA MidiPrep kits (Qiagen) were used according to manufacturer's instructions. The concentration of the DNA resuspended in EB-buffer was measured using the NanoDrop® ND-1000 device (Peqlab).

All cloned constructs and mutations were verified first by restriction digest of the vector and further by DNA sequencing. For sequencing the Big Dye Terminator v3.1-Kit (Applied Biosystems) was used according to manufacturer's instructions. For long-term storage of successfully cloned and amplified constructs 20% glycerol stocks were created from 600µl freshly grown bacteria mixed with 400µl of a 50% glycerol stock. The stocks were stored at -80°C. The vectors used in this study are listed in Table 3.

Production of chemically competent cells

DH5α bacteria were grown on a LB agar plate after which a colony was picked and grown in 20ml DYT medium, shaking at 37°C overnight. From the overnight culture 5ml was used to inoculate 195ml DYT and grown shaking at 200rpm at 37°C until the culture had reached an O.D.600 of 0.7. The bacteria were placed on ice for 15min before they were centrifuged for 10 min at 4000rpm at 4°C. The supernatant was discarded and the pellets gently resuspended in 80ml 100mM ice-cold sterile $CaCl_2$ after which the mixture was left on ice for 20 min. Once again the bacteria were spun down for 10 min at 4000rpm at 4°C. The supernatant and the cells resuspended in 16 ml 100mM ice-cold sterile $CaCl_2$. Then suspension was left on ice for 4h after which 2.3ml 70% ice-cold sterile glycerol was added and the bacteria were aliquoted, frozen in liquid nitrogen and stored immediately at -80°C. The transformation efficiency was tested using 1µl pUC19 DNA (Invitrogen) with a defined concentration of 10pg/µl.

Vector	Insert	Company	From
pCMV-Flag (C-terminal)	Omi/HtrA2	Clontech	Sabine Kautzmann
pcDNA3.1/Zeo+	Omi/HtrA2	Invitrogen	This study
pcDNA6-His/V5 (C-terminal)	Pink1	Invitrogen	Nicole Exner

Table 3. List of constructs used.

5.3.2 Cell culture

Conditions and subculturing of cells

All cell lines used were grown at 5% CO_2 and 37°C. The medias, including antibiotics, used for each cell line is listed in (Table 4). Trypsin/EDTA was used to detach the cells from the flask in order to count them and seed them for experiments or to split them for further culturing. The cells were routinely screened for mycoplasma contamination using the Venor GeM kit (Minerva Biolabs).

Cell line	Cell type	Organism	Medium	Origin
HeLa	cervical cancer cells	human	DMEM, 10% FBS	ATCC
MEF	mouse embryonic fibroblasts	mouse	DMEM, 10% FBS	Miguel Martins
MEF stable	mouse embryonic fibroblasts stably expressing HtrA2/Omi	mouse	DMEM, 10% FBS, 500µg/ml Zeocin	self made
SH-SY5Y	neuroblastoma cells	human	DMEM/Hams (F-12), 10% FBS	ATCC
TR8TY8/ Omi	SH-SY5Y cells stably expressing tetracyclin repressor inducible tyrosinase and HtrA2/Omi	human	DMEM/Hams (F-12), 10% FBS (tetracyclin negative), 5µg/ml Blasticidin, 300µg/ml Zeocin, 400µg/ml G418	TR8TY8 by Takafumi Hasegawa / self made

Table 4. List of cell lines used.

Freezing of cells

For long-term storage of cell lines the cells were pelleted by centrifugation and resuspended in cell freezing medium. The cells were then aliquoted and frozen with -

1°C/min in cryotubes before they were stored at -140°C. To bring cells back into culture the frozen cells were rapidly thawed and washed once with warm cell culture media before transferred into a cell culture flask.

Transient transfection

For transient transfection of cDNA into HeLa, MEF and SH-SY5Y cells FuGene6 (Roche) was used according to manufacturer's instructions. In brief, for a 6-well plate 1µg DNA was used per construct per well in a ratio of 1:4 with the transfection reagent in 100µl Optimem altogether. For a well in a 12-well plate all amounts were halved and for a well in a 24-well plate all the amounts were divided by four.

Stable transfection

For the stable re-transfection of HtrA2/Omi -/- MEFs, the cells were first transiently transfected with human HtrA2/Omi wt or mutant or the empty vector pcDNA3.1 Zeo+ (Invitrogen). The medium was changed 48h after transfection to medium containing 800µg/ml Zeocin to select for cells containing the construct. After about a week when most of the cells had died and only colonies resistant to Zeocin prevailed, the cells were transferred to 96-well plates in a dilution calculated to yield approximately 0.5 cells / well. Alternatively single colonies were picked using a cotton swab dipped in trypsin/EDTA and transferred onto a 24-well plate (Waldman, 1991). The single colonies were expanded and the expression of HtrA2/Omi was verified using Western blot and immunofluorescence imaging. Clones with similar expression levels were chosen for further experiments. After the initial selection, the cells were further cultured in medium containing 500µg/ml Zeocin.

For stable over-expression of HtrA2/Omi wt and mutants in TR8TY8 cells (SH-SY5Y cells stably expressing the tetracycline repressor and tyrosinase that is expressed upon induction with tetracycline or doxycycline (Hasegawa *et al.*, 2003)) the cells were first transiently transfected with HtrA2/Omi wt or mutant or empty vector pCMV-Flag. After 48h the medium was changed to medium additionally containing 400µg/ml G418 to select for cells expressing the plasmid. After about three weeks when most of the cells had died and the surviving colonies had expanded enough the cells were tested for expression of HtrA2/Omi wt and mutants using Western blot. As the level of the over-expressed protein was relatively high and equal between the different

mutations, no single clones were selected. The cells were further grown in medium containing blasticidin, Zeocin and G418 to continue stable expression of the desired proteins for future experiments.

Induction of tyrosinase expression

Neuroblastoma cells (SH-SY5Y) stably expressing tetracycline or doxycycline (DOX) inducible tyrosinase as well as over-expressing vector, HtrA2/Omi wt and four mutations, were induced to express to tyrosinase by incubating the cells in DMEM containing 2.75mM glucose, 2.75mM galactose and 2µg/ml DOX for indicated time periods. The over-expression of tyrosinase leads to an increase in dopamine and DOPA produced by the cells, which again leads to increased ROS and eventually cell death (Hasegawa *et al.*, 2003).

siRNA mediated gene silencing

Silencing of HtrA2/Omi and PINK1 was achieved by transient transfection of appropriate siRNA into HeLa cells using Hiperfect according to manufacturer's instructions. The amount of siRNA used for each gene in each experiment is indicated in the results section. Shortly, for silencing in a well of a 12-well plate 100.000 cells were seeded out the night before. For the transfection 200µl Optimem, 6µl Hiperfect was mixed with appropriate amount of siRNA. The transfection was performed three times on three consecutive days always changing media 6h after transfection. If the silenced cells were further transfected with DNA this followed 6h after the last siRNA transfection. For silencing in a 6-well, the amounts were doubled and for silencing in a 24-well the amounts halved. The siRNAs used are listed in Table 5.

siRNA	Target	Company	Product name	Catalog No.
Ctrl	none	Qiagen	AllStars Neg. Ctrl.	1027280
HtrA2/Omi	HtrA2/Omi	Qiagen	Hs_HTRA2_2_HP	SI03065496
Pink1	Pink1	Qiagen	Hs_PINK1_4_HP	SI00287931

Table 5. List of siRNAs used.

RNA isolation, reverse transcription and semi-quantitative RT-PCR

Total mRNA was isolated from MEF or HeLa cells using peqGOLD TriFast reagent (Peqlab) following manufacturer's instructions. The RNA concentration was measured using the NanoDrop® ND-1000 device (Peqlab) and 600ng total RNA was reverse transcribed (RT) using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) and anchored oligo-dT Primer to exclude non-message RNA from reverse transcription. Two µl of RT reaction was subsequently used as template for transcript amplification in a 25 µl reaction. The cycle number was chosen in the linear range of amplification for each transcript. Amplified PCR products were resolved by 1% agarose gel ectrophoresis and stained with an ethidium bromide solution (Carl Roth) to be detected under UV-light. Primers used to amplify *OPA1* were (designed to amplify all isoforms) (sense 5'-ACTCAGTTTATGTTCACCAC-3', antisense 5'-AGAGCACACAATATTCAAAC-3') and for *β-actin* (sense 5'-GGGTCAGAAGGACTCCTACG-3', antisense 5'- GGTCTCAAACATGATCTGGG-3').

5.3.3 Protein Biochemistry

Harvesting and lysing of cells

All cells were harvested by scraping and further washed three times with ice cold PBS. Unless otherwise stated the cells were lysed with cell lysis buffer for 30 min on ice. After this, the cell debris was pelleted at 18.000rpm for 15 min at 4°C, the protein content of the total cell lysate (supernatant) was determined using the BCA kit (Pierce) according to manufacturer's instructions.

Mitochondrial isolation

HtrA2/Omi +/+ or -/- MEFs were grown to 100% confluence on two 15cm plates, harvested by scraping, washed twice with ice cold PBS and re-suspended in 1ml mitochondrial isolation buffer (MIB). All further steps were performed on ice. The cells were disrupted by 50 passes with a glass douncer before they were spun down at 830 x g for 10 min at 4°C. The supernatant was retained while the pellet was re-suspended in MIB and disrupted as before and again spun down at 830 x g for 10 min at 4°C. The combined supernatants were further centrifuged at 16.800 x g for 10 min at 4°C. The supernatant was kept as the cytosolic fraction while the mitochondria enriched pellet was washed twice further with MIB before finally re-suspended in

100µl MIB. The protein concentration of both fractions was determined using the Bradford assay (Bio-Rad) according to manufacturer's instructions.

Proteinase K digestion

For proteinase K digest, 25µg mitochondria were subjected to 25ng proteinase K (Merck) digestion at 37°C for 0, 5, 10, 20, 40 min. The reaction was stopped with 1µl stop solution (100mM EDTA, 40mM phenylmethyl-sulphonyl fluoride) and kept on ice before the mitochondria were spun down at 16 000g for 10 min and resuspended in 2X SDS-PAGE sample buffer. The samples were boiled at 95°C for 5min before subjected to SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting

Samples of cell lysate or isolated mitochondria were prepared in Laemmli sample buffer to be separated on 1.5 mm polyacrylamide gels ranging from 6 to 12.5%. The gels consisted of two parts; a stacking gel on top of a separating gel, the composition can be seen in Table 6. To separate the proteins in the samples according to molecular weight they were ran through the gels first for 30 min at a constant voltage of 110V and for the remaining of the time at a constant voltage of 140V. Alternatively the samples were separated on pre-cast bis/tris gradient gels (Invitrogen). The transfer of the separated proteins onto PVDF membranes was done using wet blotting (100V for two hours at 4°C or 20V overnight, at 4°C). The membranes were further blocked (5% milk in TBST), washed (TBST) and probed with antibodies according to standard protocols (antibodies used are listed in Table 7). The primary antibodies were diluted in primary antibody solution and the secondary in 5% milk in TBST. The immunoreactive signals were detected by chemiluminescence (Immobilion Western HRP substrate). For further antibody probing the membranes were first stripped by incubation in stripping buffer in a shaking water bath (56°C) for 30 min, after which they were washed several times in TBST before they were blocked (5% milk in TBST). For densitometric analysis of Western blot band intensities the ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, USA, http://rsb.info.nih.gov/ij/, 1997-2007) was used.

Separating gel	6%	7.5%	10%	12.5%
Separating gel buffer	2.75ml	2.75ml	2.75ml	2.75ml
40% Acrylamide 19:1	1.7ml	2.1ml	2.8ml	3.4ml
ddH_2O	6.5ml	6.1ml	5.4ml	4.7ml
10% APS	0.110ml	0.110ml	0.110ml	0.110ml
TEMED	0.011ml	0.011ml	0.011ml	0.011ml

Stacking gel	4%
Stacking gel buffer	0.69ml
40% Acrylamide 19:1	0.31ml
ddH_2O	1.7ml
10% APS	0.028ml
TEMED	0.003ml

Table 6. The composition of SDS-PAGE gels.

Type	Target	Source	Dilution	Manufacturer	Prod. No.
Primary	Adenine nucleotide translocase (ANT)	m	WB - 1:1000	Calbiochem	AP 1034
	Akt	rb	WB - 1:1000	Cell Signaling	#9272
	Caspase-3	rb	WB - 1:2000	Santa Cruz	SC-7148
	Cleaved Caspase-3	rb	WB - 1:1000	Cell Signaling	#9661
	Drp1	rb	WB - 1:1000	Novus	NB110-55237
	Fis1	rb	WB - 1:2000	Alexis Biochemicals	ALX-210-907
	Heat-shock 90kDa (Hsp90)	rb	WB - 1:30000	Stressgen	SPA-846
	HtrA2/Omi	rb	WB - 1:4000 IF - 1:2000	Alexis Biochemicals	ALX-210-906
	Mitofusin 2	rb	WB - 1:1000	Sigma	M6444
	Optic atrophy 1 (OPA1)	m	WB - 1:4000	BD Transduction	612606
	poly[ADP-ribose] polymerase (PARP)	rb	WB - 1:2000	Cell Signaling	#9542
	phospho-Akt (Ser 473)	rb	WB - 1:1000	Cell Signaling	#4051
	phospho-Akt (Thr 308)	rb	WB - 1:1000	Cell Signaling	#4056
	Pink1	rb	WB - 1:1000	Novus	BC100-494
	Pink1	m	WB - 1:1000	Abgent	AM6406a
	Prohibitin	m	WB - 1:500	Thermo Scientific	#MS-261
	Tyrosinase (Clone T311)	m	WB - 1:5000	Lab Vision	#MS-800
	Voltage-dependent anion channel 1 (VDAC1)	m	WB - 1:5000	Calbiochem	529536
	β-actin (clone AC-15)	m	WB - 1:5000	Sigma	A 5441
Secondary	anti-mouse	g	WB - 1:10000	Jackson ImmunoResearch	76154
	anti-rabbit	g	WB - 1:10000	Jackson ImmunoResearch	70380
	anti-rabbit (Alexa 488)	g	IF - 1:2000	Invitrogen	A-11034

Table 7. List of antibodies used.

WB; Western Blotting, IF; immunofluorescence, m; mouse, rb; rabbit, g; goat.

LDH release assay

For detection of cell death by means of LDH release from the cells, 10000 MEF cells per well were seeded out in a 96-well plate in a volume of 200µl, 12 h before treatment. Each treatment was done at least in triplicate each time. The indicated amounts of H_2O_2 were added to the cells with a final volume of 200µl in a low glucose

containing DMEM (1g/l) for 16h. One hour before the end of the treatment the control wells, treated identically to the treatment wells, were treated with 20µl 9% Triton X-100 to completely lyse the cells and get a maximum value for the LDH release. At the end of the treatment the LDH release was measured with the CytoTox 96® kit (Promega) according to manufacturer's instructions. For analysis, the read-out for the treatment wells was compared to the control wells (100% cell death) in order to establish a relative percentage of LDH release. The relative LDH release was considered the relative percentage of cell death.

5.3.4 Fluorescent Imaging

Immunofluorescence

Glass coverslips were placed in 24-well plates and coated by covering the coverslips with Poly-D-lysine for 10min after which they were washed twice with ddH_2O before they were sterilised by UV-radiation for 1hr. The coverslips were further coated with Collagen I by covering them for 30min with a 25µg/ml solution of collagen after which they were washed twice with PBS before they were used or stored at 4°C until needed. The cells were seeded onto coverslips at least one day before they were fixed and further processed. Before fixation cells were washed once with room temperature PBS and fixed in 37°C warm 4% PFA for 20min at room temperature. The coverlips were then washed twice with PBS before they were treated with 1% Triton X-100 in PBS for 5min at RT. After washing the coverslips twice with PBS the cells were blocked in 10% normal goat serum for 1hr at RT. Before incubating the cells with the primary antibody for 1hr, they were washed twice with PBS. The cells were washed twice with PBS before the secondary antibody was added for 1hr at RT. Before mounting the coverslips onto glass slides they were washed twice more with PBS and the nucleus was stained with Hoechst 33342 (1:5000 in PBS) for 10min. Once mounted using mounting media (DakoCytomation) they were allowed to dry at least overnight. Fluorescent images were taken using a AxioImager microscope (Carl Zeiss) equipped with an Apotome system.

Live cell imaging

The cells were stained 10min at 37°C with 125nM MitoTracker Green FM and Hoechst 33342 (both Invitrogen) in DMEM medium. The cells were then washed once with DMEM and covered with DMEM. For each cell line pictures were taken

from live cells using a Cell Observer (Carl Zeiss) at 37°C and 5% CO_2. Each cell was classified into one of the following groups: elongated, middle-sized/normal or fragmented using the same criteria for each picture. At least 100 cells were scored per experiment and each experiment was done in triplicate. The scoring was done by an unbiased observer blinded to the genotype or treatment of the cells, namely Dalila Ciceri and Nicole Kieper.

ATP measurement

For ATP measurements, 15.000 MEF cells per well were seeded out in a 96-well plate in a volume of 100µl 24h before measuring. The measurement was done using ATPlite kit (PerkinElmer), according to manufacturer's instructions. In brief, 50µl Mammalian lysis solution was added to each well and the plate shaken for 5min at 700rpm. Next, 50µl of the reconstituted substrate was added to each well and the plate was again shaken for 5 min at 700rpm, after which the plate was adapted to the dark for at least 10 min. After the luminescence was measured, the protein concentration was measured using the BCA kit (Pierce) according to manufacturer's instructions. The luminescence was normalised to the protein concentration for analysis.

Fluorescence-activated cell sorting (FACS)

For all flow cytometry measurements the cells were harvested using phosphate-buffered saline (PBS) with 2mM EDTA and washed once with PBS after which they were stained with appropriate dyes and measured using a CyAn flow cytometer (DakoCytomation).

ROS

Whole cell

To measure levels of cellular ROS, the cells were stained with 10µM 2',7'-dichlorohydrofluorescein diacetate (DCF; Invitrogen) in PBS for 30min at 37°C. The cells were then washed twice with PBS, re-suspended in PBS and kept on ice for the measuring procedure. For each sample ~20,000 cells were measured using the 488nm argon laser and emission through the FITC filter (530nm).

Intramitochondrial

To measure the levels of mitochondrial superoxide, the cells were stained with 5µM MitoSOX Red (Invitrogen) in PBS for 15min at 37°C. The cells were then washed once with PBS and re-suspended in PBS. For each sample ~70,000 cells were measured using the 488 argon laser and emission through the PE filter (575nm).

Mitochondrial Membrane Potential

TMRM

To measure the mitochondrial membrane potential, the cells were stained with 200nM tetramethyl rhodamine methyl ester (TMRM) (Invitrogen) in PBS for 15min at room temperature. The cells were then washed once with PBS and re-suspended in PBS. For each sample ~50,000 cells were measured using the 488nm argon laser and emission through the PE filter (575nm).

MitoTracker Green FM / MitoTracker Red CM-H_2XRos

To measure the mitochondrial membrane potential, the cells were stained with 100nM MitoTracker Green FM and MitoTracker CM-H_2XRos (Invitrogen) in PBS for 15min at 37°C. The cells were then washed once with PBS and resuspended in PBS. For each sample ~70,000 cells were measured using the 488 argon laser and emission through the PE Texas Red filter (613nm) and the FITC filter (530nm).

For all flow cytometry measurements the percentage shift in the intensity of fluorescence activated cell sorter staining was determined with the Summit version 4.2 software (DakoCytomation).

5.3.5 Statistical Analysis

Experimental results for mitochondrial morphology were analysed for statistical significance using the Student's t-test implemented in Microsoft Excel Software. For statistical significance of the densitometrically analysed Western blot experiments a Student's t-test or a one-way ANOVA and a Studen Newman-Keuls correction were performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego California USA, www.graphpad.com.)

6 REFERENCES

Abou-Sleiman PM, Muqit MM, McDonald NQ, Yang YX, Gandhi S, Healy DG, Harvey K, Harvey RJ, Deas E, Bhatia K, Quinn N, Lees A, Latchman DS, Wood NW (2006a) A heterozygous effect for PINK1 mutations in Parkinson's disease? *Annals of neurology* **60**(4)**:** 414-419

Abou-Sleiman PM, Muqit MM, Wood NW (2006b) Expanding insights of mitochondrial dysfunction in Parkinson's disease. *Nature reviews* **7**(3)**:** 207-219

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *The EMBO journal* **15**(23)**:** 6541-6551

Alexander C, Votruba M, Pesch UE, Thiselton DL, Mayer S, Moore A, Rodriguez M, Kellner U, Leo-Kottler B, Auburger G, Bhattacharya SS, Wissinger B (2000) OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. *Nature genetics* **26**(2)**:** 211-215

Almeida A, Almeida J, Bolanos JP, Moncada S (2001) Different responses of astrocytes and neurons to nitric oxide: the role of glycolytically generated ATP in astrocyte protection. *Proceedings of the National Academy of Sciences of the United States of America* **98**(26)**:** 15294-15299

Amati-Bonneau P, Milea D, Bonneau D, Chevrollier A, Ferre M, Guillet V, Gueguen N, Loiseau D, de Crescenzo MA, Verny C, Procaccio V, Lenaers G, Reynier P (2009) OPA1-associated disorders: phenotypes and pathophysiology. *The international journal of biochemistry & cell biology* **41**(10)**:** 1855-1865

Amati-Bonneau P, Valentino ML, Reynier P, Gallardo ME, Bornstein B, Boissiere A, Campos Y, Rivera H, de la Aleja JG, Carroccia R, Iommarini L, Labauge P, Figarella-Branger D, Marcorelles P, Furby A, Beauvais K, Letournel F, Liguori R, La Morgia C, Montagna P, Liguori M, Zanna C, Rugolo M, Cossarizza A, Wissinger B, Verny C, Schwarzenbacher R, Martin MA, Arenas J, Ayuso C, Garesse R, Lenaers G, Bonneau D, Carelli V (2008) OPA1 mutations induce mitochondrial DNA instability and optic atrophy 'plus' phenotypes. *Brain* **131**(Pt 2)**:** 338-351

Antico Arciuch VG, Galli S, Franco MC, Lam PY, Cadenas E, Carreras MC, Poderoso JJ (2009) Akt1 intramitochondrial cycling is a crucial step in the redox modulation of cell cycle progression. *PloS one* **4**(10)**:** e7523

Bao Q, Shi Y (2007) Apoptosome: a platform for the activation of initiator caspases. *Cell death and differentiation* **14**(1)**:** 56-65

Barksdale KA, Bijur GN (2009) The basal flux of Akt in the mitochondria is mediated by heat shock protein 90. *Journal of neurochemistry* **108**(5)**:** 1289-1299

Beil L (2009) Mitochondria gone bad. *Science News* **175:** 20

Beilina A, Van Der Brug M, Ahmad R, Kesavapany S, Miller DW, Petsko GA, Cookson MR (2005) Mutations in PTEN-induced putative kinase 1 associated with recessive parkinsonism have differential effects on protein stability. *Proceedings of the National Academy of Sciences of the United States of America* **102**(16)**:** 5703-5708

Benard G, Faustin B, Passerieux E, Galinier A, Rocher C, Bellance N, Delage JP, Casteilla L, Letellier T, Rossignol R (2006) Physiological diversity of mitochondrial oxidative phosphorylation. *Am J Physiol Cell Physiol* **291**(6)**:** C1172-1182

Bender A, Schwarzkopf RM, McMillan A, Krishnan KJ, Rieder G, Neumann M, Elstner M, Turnbull DM, Klopstock T (2008) Dopaminergic midbrain neurons are the prime target for mitochondrial DNA deletions. *Journal of neurology* **255**(8)**:** 1231-1235

Berman SB, Pineda FJ, Hardwick JM (2008) Mitochondrial fission and fusion dynamics: the long and short of it. *Cell death and differentiation* **15**(7)**:** 1147-1152

Bijur GN, Jope RS (2003) Rapid accumulation of Akt in mitochondria following phosphatidylinositol 3-kinase activation. *Journal of neurochemistry* **87**(6)**:** 1427-1435

Bogaerts V, Nuytemans K, Reumers J, Pals P, Engelborghs S, Pickut B, Corsmit E, Peeters K, Schymkowitz J, De Deyn PP, Cras P, Rousseau F, Theuns J, Van Broeckhoven C (2008) Genetic variability in the mitochondrial serine protease HTRA2 contributes to risk for Parkinson disease. *Human mutation* **29**(6)**:** 832-840

Braak H, Rub U, Gai WP, Del Tredici K (2003) Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *J Neural Transm* **110**(5)**:** 517-536

Brazil DP, Park J, Hemmings BA (2002) PKB binding proteins. Getting in on the Akt. *Cell* **111**(3)**:** 293-303

Breusing N, Grune T (2008) Regulation of proteasome-mediated protein degradation during oxidative stress and aging. *Biol Chem* **389**(3)**:** 203-209

Chan DC (2006) Mitochondrial fusion and fission in mammals. *Annual review of cell and developmental biology* **22:** 79-99

Chang DT, Reynolds IJ (2006) Mitochondrial trafficking and morphology in healthy and injured neurons. *Progress in neurobiology* **80**(5)**:** 241-268

Chao JR, Parganas E, Boyd K, Hong CY, Opferman JT, Ihle JN (2008) Hax1-mediated processing of HtrA2 by Parl allows survival of lymphocytes and neurons. *Nature* **452**(7183)**:** 98-102

Chen H, Chomyn A, Chan DC (2005) Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *The Journal of biological chemistry* **280**(28)**:** 26185-26192

Cilenti L, Kyriazis GA, Soundarapandian MM, Stratico V, Yerkes A, Park KM, Sheridan AM, Alnemri ES, Bonventre JV, Zervos AS (2005) Omi/HtrA2 protease mediates cisplatin-induced cell death in renal cells. *Am J Physiol Renal Physiol* **288**(2)**:** F371-379

Cilenti L, Lee Y, Hess S, Srinivasula S, Park KM, Junqueira D, Davis H, Bonventre JV, Alnemri ES, Zervos AS (2003) Characterization of a novel and specific inhibitor for the pro-apoptotic protease Omi/HtrA2. *The Journal of biological chemistry* **278**(13)**:** 11489-11494

Cilenti L, Soundarapandian MM, Kyriazis GA, Stratico V, Singh S, Gupta S, Bonventre JV, Alnemri ES, Zervos AS (2004) Regulation of HAX-1 anti-apoptotic protein by Omi/HtrA2 protease during cell death. *The Journal of biological chemistry* **279**(48)**:** 50295-50301

Cipolat S, Rudka T, Hartmann D, Costa V, Serneels L, Craessaerts K, Metzger K, Frezza C, Annaert W, D'Adamio L, Derks C, Dejaegere T, Pellegrini L, D'Hooge R, Scorrano L, De Strooper B (2006) Mitochondrial rhomboid PARL regulates cytochrome C release during apoptosis via OPA1-dependent cristae remodeling. *Cell* **126**(1)**:** 163-175

Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, Yoo SJ, Hay BA, Guo M (2006) Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* **441**(7097)**:** 1162-1166

Connor KM, Subbaram S, Regan KJ, Nelson KK, Mazurkiewicz JE, Bartholomew PJ, Aplin AE, Tai YT, Aguirre-Ghiso J, Flores SC, Melendez JA (2005) Mitochondrial H2O2 regulates the angiogenic phenotype via PTEN oxidation. *The Journal of biological chemistry* **280**(17)**:** 16916-16924

Dagda RK, Cherra SJ, 3rd, Kulich SM, Tandon A, Park D, Chu CT (2009) Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *The Journal of biological chemistry* **284**(20)**:** 13843-13855

Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. *Neuron* **39**(6)**:** 889-909

de Brito OM, Scorrano L (2008) Mitofusin 2: a mitochondria-shaping protein with signaling roles beyond fusion. *Antioxidants & redox signaling* **10**(3)**:** 621-633

Delettre C, Griffoin JM, Kaplan J, Dollfus H, Lorenz B, Faivre L, Lenaers G, Belenguer P, Hamel CP (2001) Mutation spectrum and splicing variants in the OPA1 gene. *Human genetics* **109**(6)**:** 584-591

Delettre C, Lenaers G, Griffoin JM, Gigarel N, Lorenzo C, Belenguer P, Pelloquin L, Grosgeorge J, Turc-Carel C, Perret E, Astarie-Dequeker C, Lasquellec L, Arnaud B, Ducommun B, Kaplan J, Hamel CP (2000) Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. *Nature genetics* **26**(2)**:** 207-210

Deng H, Dodson MW, Huang H, Guo M (2008) The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* **105**(38)**:** 14503-14508

Deng H, Jankovic J, Guo Y, Xie W, Le W (2005) Small interfering RNA targeting the PINK1 induces apoptosis in dopaminergic cells SH-SY5Y. *Biochemical and biophysical research communications* **337**(4)**:** 1133-1138

Deveraux QL, Reed JC (1999) IAP family proteins--suppressors of apoptosis. *Genes & development* **13**(3)**:** 239-252

Eisenhofer G, Tian H, Holmes C, Matsunaga J, Roffler-Tarlov S, Hearing VJ (2003) Tyrosinase: a developmentally specific major determinant of peripheral dopamine. *FASEB J* **17**(10)**:** 1248-1255

Ekert PG, Vaux DL (2005) The mitochondrial death squad: hardened killers or innocent bystanders? *Current opinion in cell biology* **17**(6)**:** 626-630

Exner N, Treske B, Paquet D, Holmstrom K, Schiesling C, Gispert S, Carballo-Carbajal I, Berg D, Hoepken HH, Gasser T, Kruger R, Winklhofer KF, Vogel F, Reichert AS, Auburger G, Kahle PJ, Schmid B, Haass C (2007) Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. *J Neurosci* **27**(45)**:** 12413-12418

Faccio L, Fusco C, Chen A, Martinotti S, Bonventre JV, Zervos AS (2000) Characterization of a novel human serine protease that has extensive homology to bacterial heat shock endoprotease HtrA and is regulated by kidney ischemia. *The Journal of biological chemistry* **275**(4)**:** 2581-2588

Fitzgerald JC, Plun-Favreau H (2008) Emerging pathways in genetic Parkinson's disease: autosomal-recessive genes in Parkinson's disease--a common pathway? *The FEBS journal* **275**(23)**:** 5758-5766

Forno LS (1996) Neuropathology of Parkinson's disease. *Journal of neuropathology and experimental neurology* **55**(3)**:** 259-272

Frezza C, Cipolat S, Martins de Brito O, Micaroni M, Beznoussenko GV, Rudka T, Bartoli D, Polishuck RS, Danial NN, De Strooper B, Scorrano L (2006) OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* **126**(1)**:** 177-189

Furukawa K, Matsuzaki-Kobayashi M, Hasegawa T, Kikuchi A, Sugeno N, Itoyama Y, Wang Y, Yao PJ, Bushlin I, Takeda A (2006) Plasma membrane ion permeability induced by mutant alpha-synuclein contributes to the degeneration of neural cells. *Journal of neurochemistry* **97**(4)**:** 1071-1077

Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, Bazan NG, Blagosklonny MV, Blomgren K, Borner C, Bredesen DE, Brenner C, Castedo M, Cidlowski JA, Ciechanover A, Cohen GM, De Laurenzi V, De Maria R, Deshmukh M, Dynlacht BD, El-Deiry WS, Flavell RA, Fulda S, Garrido C, Golstein P, Gougeon ML, Green DR, Gronemeyer H, Hajnoczky G, Hardwick JM, Hengartner MO, Ichijo H, Jaattela M, Kepp O, Kimchi A, Klionsky DJ, Knight RA, Kornbluth S, Kumar S, Levine B, Lipton SA, Lugli E, Madeo F, Malomi W, Marine JC, Martin SJ, Medema JP, Mehlen P, Melino G, Moll UM, Morselli E, Nagata S, Nicholson DW, Nicotera P, Nunez G, Oren M, Penninger J, Pervaiz S, Peter ME, Piacentini M, Prehn JH, Puthalakath H, Rabinovich GA, Rizzuto R, Rodrigues CM, Rubinsztein DC, Rudel T, Scorrano L, Simon HU, Steller H, Tschopp J, Tsujimoto Y, Vandenabeele P, Vitale I, Vousden KH, Youle RJ, Yuan J, Zhivotovsky B, Kroemer G (2009) Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. *Cell death and differentiation* **16**(8)**:** 1093-1107

Gandhi S, Muqit MM, Stanyer L, Healy DG, Abou-Sleiman PM, Hargreaves I, Heales S, Ganguly M, Parsons L, Lees AJ, Latchman DS, Holton JL, Wood NW, Revesz T (2006) PINK1 protein in normal human brain and Parkinson's disease. *Brain* **129**(Pt 7)**:** 1720-1731

Gandhi S, Wood-Kaczmar A, Yao Z, Plun-Favreau H, Deas E, Klupsch K, Downward J, Latchman DS, Tabrizi SJ, Wood NW, Duchen MR, Abramov AY (2009) PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. *Molecular cell* **33**(5)**:** 627-638

Gautier CA, Kitada T, Shen J (2008) Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America* **105**(32)**:** 11364-11369

Gegg ME, Cooper JM, Schapira AH, Taanman JW (2009) Silencing of PINK1 expression affects mitochondrial DNA and oxidative phosphorylation in dopaminergic cells. *PloS one* **4**(3)**:** e4756

Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, Springer W (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nature cell biology* **12**(2)**:** 119-131

Gray CW, Ward RV, Karran E, Turconi S, Rowles A, Viglienghi D, Southan C, Barton A, Fantom KG, West A, Savopoulos J, Hassan NJ, Clinkenbeard H, Hanning C, Amegadzie B, Davis JB, Dingwall C, Livi GP, Creasy CL (2000) Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response. *European journal of biochemistry / FEBS* **267**(18)**:** 5699-5710

Griparic L, van der Wel NN, Orozco IJ, Peters PJ, van der Bliek AM (2004) Loss of the intermembrane space protein Mgm1/OPA1 induces swelling and localized constrictions along the lengths of mitochondria. *The Journal of biological chemistry* **279**(18)**:** 18792-18798

Guillery O, Malka F, Landes T, Guillou E, Blackstone C, Lombes A, Belenguer P, Arnoult D, Rojo M (2008) Metalloprotease-mediated OPA1 processing is modulated by the mitochondrial membrane potential. *Biol Cell* **100**(5)**:** 315-325

Gupta S, Singh R, Datta P, Zhang Z, Orr C, Lu Z, Dubois G, Zervos AS, Meisler MH, Srinivasula SM, Fernandes-Alnemri T, Alnemri ES (2004) The C-terminal tail of presenilin regulates Omi/HtrA2 protease activity. *The Journal of biological chemistry* **279**(44)**:** 45844-45854

Haque ME, Thomas KJ, D'Souza C, Callaghan S, Kitada T, Slack RS, Fraser P, Cookson MR, Tandon A, Park DS (2008) Cytoplasmic Pink1 activity protects neurons from dopaminergic neurotoxin MPTP. *Proceedings of the National Academy of Sciences of the United States of America* **105**(5)**:** 1716-1721

Hasegawa T, Matsuzaki-Kobayashi M, Takeda A, Sugeno N, Kikuchi A, Furukawa K, Perry G, Smith MA, Itoyama Y (2006) Alpha-synuclein facilitates the toxicity of oxidized catechol metabolites: implications for selective neurodegeneration in Parkinson's disease. *FEBS letters* **580**(8)**:** 2147-2152

Hasegawa T, Matsuzaki M, Takeda A, Kikuchi A, Furukawa K, Shibahara S, Itoyama Y (2003) Increased dopamine and its metabolites in SH-SY5Y neuroblastoma cells that express tyrosinase. *Journal of neurochemistry* **87**(2)**:** 470-475

Hasegawa T, Treis A, Patenge N, Fiesel FC, Springer W, Kahle PJ (2008) Parkin protects against tyrosinase-mediated dopamine neurotoxicity by suppressing stress-activated protein kinase pathways. *Journal of neurochemistry* **105**(5)**:** 1700-1715

Hastings TG (2009) The role of dopamine oxidation in mitochondrial dysfunction: implications for Parkinson's disease. *Journal of bioenergetics and biomembranes* **41**(6)**:** 469-472

Hegde R, Srinivasula SM, Zhang Z, Wassell R, Mukattash R, Cilenti L, DuBois G, Lazebnik Y, Zervos AS, Fernandes-Alnemri T, Alnemri ES (2002) Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *The Journal of biological chemistry* **277**(1)**:** 432-438

Hiller A, Hagenah JM, Djarmati A, Hedrich K, Reetz K, Schneider-Gold C, Kress W, Munchau A, Klein C (2007) Phenotypic spectrum of PINK1-associated parkinsonism in 15 mutation carriers from 1 family. *Mov Disord* **22**(1)**:** 145-147

Hollenbeck PJ (2005) Mitochondria and neurotransmission: evacuating the synapse. *Neuron* **47**(3)**:** 331-333

Hoppins S, Lackner L, Nunnari J (2007) The machines that divide and fuse mitochondria. *Annual review of biochemistry* **76:** 751-780

Hua G, Zhang Q, Fan Z (2007) Heat shock protein 75 (TRAP1) antagonizes reactive oxygen species generation and protects cells from granzyme M-mediated apoptosis. *The Journal of biological chemistry* **282**(28)**:** 20553-20560

Huttunen HJ, Guenette SY, Peach C, Greco C, Xia W, Kim DY, Barren C, Tanzi RE, Kovacs DM (2007) HtrA2 regulates beta-amyloid precursor protein (APP) metabolism through endoplasmic reticulum-associated degradation. *The Journal of biological chemistry* **282**(38)**:** 28285-28295

Inagaki R, Tagawa K, Qi ML, Enokido Y, Ito H, Tamura T, Shimizu S, Oyanagi K, Arai N, Kanazawa I, Wanker EE, Okazawa H (2008) Omi / HtrA2 is relevant to the selective vulnerability of striatal neurons in Huntington's disease. *The European journal of neuroscience* **28**(1)**:** 30-40

Ishihara N, Eura Y, Mihara K (2004) Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. *Journal of cell science* **117**(Pt 26)**:** 6535-6546

Ishihara N, Nomura M, Jofuku A, Kato H, Suzuki SO, Masuda K, Otera H, Nakanishi Y, Nonaka I, Goto Y, Taguchi N, Morinaga H, Maeda M, Takayanagi R, Yokota S, Mihara K (2009) Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. *Nature cell biology* **11**(8)**:** 958-966

Jankovic J (2008) Parkinson's disease: clinical features and diagnosis. *Journal of neurology, neurosurgery, and psychiatry* **79**(4)**:** 368-376

Jeyaraju DV, Cisbani G, De Brito OM, Koonin EV, Pellegrini L (2009) Hax1 lacks BH modules and is peripherally associated to heavy membranes: implications for Omi/HtrA2 and PARL activity in the regulation of mitochondrial stress and apoptosis. *Cell death and differentiation*

Jin Z, El-Deiry WS (2005) Overview of cell death signaling pathways. *Cancer biology & therapy* **4**(2)**:** 139-163

Johnson F, Kaplitt MG (2009) Novel mitochondrial substrates of omi indicate a new regulatory role in neurodegenerative disorders. *PloS one* **4**(9)**:** e7100

Jones JM, Albin RL, Feldman EL, Simin K, Schuster TG, Dunnick WA, Collins JT, Chrisp CE, Taylor BA, Meisler MH (1993) mnd2: a new mouse model of inherited motor neuron disease. *Genomics* **16**(3)**:** 669-677

Jones JM, Datta P, Srinivasula SM, Ji W, Gupta S, Zhang Z, Davies E, Hajnoczky G, Saunders TL, Van Keuren ML, Fernandes-Alnemri T, Meisler MH, Alnemri ES (2003) Loss of Omi mitochondrial protease activity causes the neuromuscular disorder of mnd2 mutant mice. *Nature* **425**(6959)**:** 721-727

Kieper N, Holmstrom KM, Ciceri D, Fiesel FC, Wolburg H, Ziviani E, Whitworth AJ, Martins LM, Kahle PJ, Kruger R (2010) Modulation of mitochondrial function and morphology by interaction of Omi/HtrA2 with the mitochondrial fusion factor OPA1. *Experimental cell research*

Kijima K, Numakura C, Izumino H, Umetsu K, Nezu A, Shiiki T, Ogawa M, Ishizaki Y, Kitamura T, Shozawa Y, Hayasaka K (2005) Mitochondrial GTPase mitofusin 2 mutation in Charcot-Marie-Tooth neuropathy type 2A. *Human genetics* **116**(1-2)**:** 23-27

Kim Y, Park J, Kim S, Song S, Kwon SK, Lee SH, Kitada T, Kim JM, Chung J (2008) PINK1 controls mitochondrial localization of Parkin through direct phosphorylation. *Biochemical and biophysical research communications* **377**(3)**:** 975-980

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392**(6676)**:** 605-608

Kitada T, Pisani A, Porter DR, Yamaguchi H, Tscherter A, Martella G, Bonsi P, Zhang C, Pothos EN, Shen J (2007) Impaired dopamine release and synaptic plasticity in the striatum of PINK1-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **104**(27)**:** 11441-11446

Klein C, Lohmann-Hedrich K (2007) Impact of recent genetic findings in Parkinson's disease. *Current opinion in neurology* **20**(4)**:** 453-464

Kooistra J, Milojevic J, Melacini G, Ortega J (2009) A new function of human HtrA2 as an amyloid-beta oligomerization inhibitor. *J Alzheimers Dis* **17**(2)**:** 281-294

Koonin EV, Aravind L (2002) Origin and evolution of eukaryotic apoptosis: the bacterial connection. *Cell death and differentiation* **9**(4)**:** 394-404

Krick S, Shi S, Ju W, Faul C, Tsai SY, Mundel P, Bottinger EP (2008) Mpv17l protects against mitochondrial oxidative stress and apoptosis by activation of Omi/HtrA2 protease. *Proceedings of the National Academy of Sciences of the United States of America* **105**(37)**:** 14106-14111

Kroemer G, Galluzzi L, Brenner C (2007) Mitochondrial membrane permeabilization in cell death. *Physiological reviews* **87**(1)**:** 99-163

Kucej M, Butow RA (2007) Evolutionary tinkering with mitochondrial nucleoids. *Trends in cell biology* **17**(12)**:** 586-592

Kuninaka S, Iida SI, Hara T, Nomura M, Naoe H, Morisaki T, Nitta M, Arima Y, Mimori T, Yonehara S, Saya H (2007) Serine protease Omi/HtrA2 targets WARTS kinase to control cell proliferation. *Oncogene* **26**(17)**:** 2395-2406

Kuninaka S, Nomura M, Hirota T, Iida S, Hara T, Honda S, Kunitoku N, Sasayama T, Arima Y, Marumoto T, Koja K, Yonehara S, Saya H (2005) The tumor suppressor WARTS activates the Omi / HtrA2-dependent pathway of cell death. *Oncogene* **24**(34)**:** 5287-5298

Langston JW, Ballard P, Tetrud JW, Irwin I (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science (New York, NY* **219**(4587)**:** 979-980

Lee DH, Goldberg AL (1998) Proteasome inhibitors: valuable new tools for cell biologists. *Trends in cell biology* **8**(10)**:** 397-403

Lee YJ, Jeong SY, Karbowski M, Smith CL, Youle RJ (2004) Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. *Molecular biology of the cell* **15**(11)**:** 5001-5011

Lesage S, Brice A (2009) Parkinson's disease: from monogenic forms to genetic susceptibility factors. *Human molecular genetics* **18**(R1)**:** R48-59

Li W, Srinivasula SM, Chai J, Li P, Wu JW, Zhang Z, Alnemri ES, Shi Y (2002) Structural insights into the pro-apoptotic function of mitochondrial serine protease HtrA2/Omi. *Nature structural biology* **9**(6)**:** 436-441

Li Z, Okamoto K, Hayashi Y, Sheng M (2004) The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. *Cell* **119**(6)**:** 873-887

Lin W, Kang UJ (2008) Characterization of PINK1 processing, stability, and subcellular localization. *Journal of neurochemistry* **106**(1)**:** 464-474

Liu MJ, Liu ML, Shen YF, Kim JM, Lee BH, Lee YS, Hong ST (2007) Transgenic mice with neuron-specific overexpression of HtrA2/Omi suggest a neuroprotective role for HtrA2/Omi. *Biochemical and biophysical research communications* **362**(2)**:** 295-300

Liu Z, Li H, Derouet M, Berezkin A, Sasazuki T, Shirasawa S, Rosen K (2006) Oncogenic Ras inhibits anoikis of intestinal epithelial cells by preventing the release of a mitochondrial pro-apoptotic protein Omi/HtrA2 into the cytoplasm. *The Journal of biological chemistry* **281**(21)**:** 14738-14747

Ma X, Kalakonda S, Srinivasula SM, Reddy SP, Platanias LC, Kalvakolanu DV (2007) GRIM-19 associates with the serine protease HtrA2 for promoting cell death. *Oncogene* **26**(33)**:** 4842-4849

Mandemakers W, Morais VA, De Strooper B (2007) A cell biological perspective on mitochondrial dysfunction in Parkinson disease and other neurodegenerative diseases. *Journal of cell science* **120**(Pt 10)**:** 1707-1716

Marongiu R, Spencer B, Crews L, Adame A, Patrick C, Trejo M, Dallapiccola B, Valente EM, Masliah E (2009) Mutant Pink1 induces mitochondrial dysfunction in a neuronal cell model of Parkinson's disease by disturbing calcium flux. *Journal of neurochemistry* **108**(6)**:** 1561-1574

Martins LM, Iaccarino I, Tenev T, Gschmeissner S, Totty NF, Lemoine NR, Savopoulos J, Gray CW, Creasy CL, Dingwall C, Downward J (2002) The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. *The Journal of biological chemistry* **277**(1)**:** 439-444

Martins LM, Morrison A, Klupsch K, Fedele V, Moisoi N, Teismann P, Abuin A, Grau E, Geppert M, Livi GP, Creasy CL, Martin A, Hargreaves I, Heales SJ, Okada H, Brandner S, Schulz JB, Mak T, Downward J (2004) Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. *Molecular and cellular biology* **24**(22)**:** 9848-9862

McCormick AL, Roback L, Mocarski ES (2008) HtrA2/Omi terminates cytomegalovirus infection and is controlled by the viral mitochondrial inhibitor of apoptosis (vMIA). *PLoS pathogens* **4**(5)**:** e1000063

Meeusen S, DeVay R, Block J, Cassidy-Stone A, Wayson S, McCaffery JM, Nunnari J (2006) Mitochondrial inner-membrane fusion and crista maintenance requires the dynamin-related GTPase Mgm1. *Cell* **127**(2)**:** 383-395

Meltzer M, Hasenbein S, Mamant N, Merdanovic M, Poepsel S, Hauske P, Kaiser M, Huber R, Krojer T, Clausen T, Ehrmann M (2009) Structure, function and regulation of the conserved serine proteases DegP and DegS of Escherichia coli. *Res Microbiol* **160**(9)**:** 660-666

Mills RD, Sim CH, Mok SS, Mulhern TD, Culvenor JG, Cheng HC (2008) Biochemical aspects of the neuroprotective mechanism of PTEN-induced kinase-1 (PINK1). *Journal of neurochemistry* **105**(1)**:** 18-33

Moisoi N, Klupsch K, Fedele V, East P, Sharma S, Renton A, Plun-Favreau H, Edwards RE, Teismann P, Esposti MD, Morrison AD, Wood NW, Downward J, Martins LM (2009) Mitochondrial dysfunction triggered by loss of HtrA2 results in the activation of a brain-specific transcriptional stress response. *Cell death and differentiation* **16**(3)**:** 449-464

Mookherjee P, Quintanilla R, Roh MS, Zmijewska AA, Jope RS, Johnson GV (2007) Mitochondrial-targeted active Akt protects SH-SY5Y neuroblastoma cells from staurosporine-induced apoptotic cell death. *J Cell Biochem* **102**(1)**:** 196-210

Morais VA, Verstreken P, Roethig A, Smet J, Snellinx A, Vanbrabant M, Haddad D, Frezza C, Mandemakers W, Vogt-Weisenhorn D, Van Coster R, Wurst W, Scorrano L, De Strooper B (2009) Parkinson's disease mutations in PINK1 result in decreased Complex I activity and deficient synaptic function. *EMBO Mol Med* **1**(2)**:** 99-111

Moriwaki Y, Kim YJ, Ido Y, Misawa H, Kawashima K, Endo S, Takahashi R (2008) L347P PINK1 mutant that fails to bind to Hsp90/Cdc37 chaperones is rapidly degraded in a proteasome-dependent manner. *Neuroscience research* **61**(1)**:** 43-48

Mozdy AD, McCaffery JM, Shaw JM (2000) Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. *The Journal of cell biology* **151**(2)**:** 367-380

Muqit MM, Abou-Sleiman PM, Saurin AT, Harvey K, Gandhi S, Deas E, Eaton S, Payne Smith MD, Venner K, Matilla A, Healy DG, Gilks WP, Lees AJ, Holton J, Revesz T, Parker PJ, Harvey RJ, Wood NW, Latchman DS (2006) Altered cleavage and localization of PINK1 to aggresomes in the presence of proteasomal stress. *Journal of neurochemistry* **98**(1)**:** 156-169

Narendra D, Tanaka A, Suen DF, Youle RJ (2008) Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *The Journal of cell biology* **183**(5)**:** 795-803

Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, Cookson MR, Youle RJ (2010) PINK1 Is Selectively Stabilized on Impaired Mitochondria to Activate Parkin. *PLoS biology* **8**(1)**:** e1000298

Narkiewicz J, Klasa-Mazurkiewicz D, Zurawa-Janicka D, Skorko-Glonek J, Emerich J, Lipinska B (2008) Changes in mRNA and protein levels of human HtrA1, HtrA2 and HtrA3 in ovarian cancer. *Clinical biochemistry* **41**(7-8)**:** 561-569

Nutt JG, Wooten GF (2005) Clinical practice. Diagnosis and initial management of Parkinson's disease. *The New England journal of medicine* **353**(10)**:** 1021-1027

Oehler-Janne C, von Bueren AO, Vuong V, Hollenstein A, Grotzer MA, Pruschy M (2008) Temperature sensitivity of phospho-Ser(473)-PKB/AKT. *Biochemical and biophysical research communications* **375**(3)**:** 399-404

Ow YP, Green DR, Hao Z, Mak TW (2008) Cytochrome C: functions beyond respiration. *Nat Rev Mol Cell Biol* **9**(7)**:** 532-542

Parcellier A, Tintignac LA, Zhuravleva E, Hemmings BA (2008) PKB and the mitochondria: AKTing on apoptosis. *Cellular signalling* **20**(1)**:** 21-30

Park HJ, Kim SS, Seong YM, Kim KH, Goo HG, Yoon EJ, Min do S, Kang S, Rhim H (2006a) Beta-amyloid precursor protein is a direct cleavage target of HtrA2 serine protease. Implications for the physiological function of HtrA2 in the mitochondria. *The Journal of biological chemistry* **281**(45)**:** 34277-34287

Park J, Lee SB, Lee S, Kim Y, Song S, Kim S, Bae E, Kim J, Shong M, Kim JM, Chung J (2006b) Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature* **441**(7097)**:** 1157-1161

Parkinson J. (1817) An Essay on the Shaking Palsy. Sherwood, Neely and Jones, London.

Petit-Paitel A, Brau F, Cazareth J, Chabry J (2009) Involvment of cytosolic and mitochondrial GSK-3beta in mitochondrial dysfunction and neuronal cell death of MPTP/MPP-treated neurons. *PloS one* **4**(5)**:** e5491

Petit A, Kawarai T, Paitel E, Sanjo N, Maj M, Scheid M, Chen F, Gu Y, Hasegawa H, Salehi-Rad S, Wang L, Rogaeva E, Fraser P, Robinson B, St George-Hyslop P, Tandon A (2005) Wild-type PINK1 prevents basal and induced neuronal apoptosis, a protective effect abrogated by Parkinson disease-related mutations. *The Journal of biological chemistry* **280**(40)**:** 34025-34032

Plun-Favreau H, Klupsch K, Moisoi N, Gandhi S, Kjaer S, Frith D, Harvey K, Deas E, Harvey RJ, McDonald N, Wood NW, Martins LM, Downward J (2007) The mitochondrial protease HtrA2 is regulated by Parkinson's disease-associated kinase PINK1. *Nature cell biology* **9**(11)**:** 1243-1252

Poole AC, Thomas RE, Andrews LA, McBride HM, Whitworth AJ, Pallanck LJ (2008) The PINK1/Parkin pathway regulates mitochondrial morphology. *Proceedings of the National Academy of Sciences of the United States of America* **105**(5)**:** 1638-1643

Pridgeon JW, Olzmann JA, Chin LS, Li L (2007) PINK1 protects against oxidative stress by phosphorylating mitochondrial chaperone TRAP1. *PLoS biology* **5**(7)**:** e172

Radke S, Chander H, Schafer P, Meiss G, Kruger R, Schulz JB, Germain D (2008) Mitochondrial protein quality control by the proteasome involves ubiquitination and the protease Omi. *The Journal of biological chemistry* **283**(19)**:** 12681-12685

Rich PR (2003) The molecular machinery of Keilin's respiratory chain. *Biochemical Society transactions* **31**(Pt 6)**:** 1095-1105

Ross OA, Soto AI, Vilarino-Guell C, Heckman MG, Diehl NN, Hulihan MM, Aasly JO, Sando S, Gibson JM, Lynch T, Krygowska-Wajs A, Opala G, Barcikowska M, Czyzewski K, Uitti RJ, Wszolek ZK, Farrer MJ (2008) Genetic variation of Omi/HtrA2 and Parkinson's disease. *Parkinsonism & related disorders* **14**(7)**:** 539-543

Sagan L (1967) On the origin of mitosing cells. *Journal of theoretical biology* **14**(3)**:** 255-274

Sandebring A, Thomas KJ, Beilina A, van der Brug M, Cleland MM, Ahmad R, Miller DW, Zambrano I, Cowburn RF, Behbahani H, Cedazo-Minguez A, Cookson MR (2009) Mitochondrial alterations in PINK1 deficient cells are influenced by calcineurin-dependent dephosphorylation of dynamin-related protein 1. *PloS one* **4**(5)**:** e5701

Savopoulos JW, Carter PS, Turconi S, Pettman GR, Karran EH, Gray CW, Ward RV, Jenkins O, Creasy CL (2000) Expression, purification, and functional analysis of the human serine protease HtrA2. *Protein expression and purification* **19**(2)**:** 227-234

Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD (1989) Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* **1**(8649)**:** 1269

Seong YM, Choi JY, Park HJ, Kim KJ, Ahn SG, Seong GH, Kim IK, Kang S, Rhim H (2004) Autocatalytic processing of HtrA2/Omi is essential for induction of caspase-dependent cell death through antagonizing XIAP. *The Journal of biological chemistry* **279**(36)**:** 37588-37596

Shults CW (2006) Lewy bodies. *Proceedings of the National Academy of Sciences of the United States of America* **103**(6)**:** 1661-1668

Silvestri L, Caputo V, Bellacchio E, Atorino L, Dallapiccola B, Valente EM, Casari G (2005) Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. *Human molecular genetics* **14**(22)**:** 3477-3492

Sim CH, Lio DS, Mok SS, Masters CL, Hill AF, Culvenor JG, Cheng HC (2006) C-terminal truncation and Parkinson's disease-associated mutations down-regulate the protein serine/threonine kinase activity of PTEN-induced kinase-1. *Human molecular genetics* **15**(21)**:** 3251-3262

Simon-Sanchez J, Singleton AB (2008) Sequencing analysis of OMI/HTRA2 shows previously reported pathogenic mutations in neurologically normal controls. *Human molecular genetics* **17**(13)**:** 1988-1993

Smirnova E, Griparic L, Shurland DL, van der Bliek AM (2001) Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Molecular biology of the cell* **12**(8)**:** 2245-2256

Sohn J, Grant RA, Sauer RT (2007) Allosteric activation of DegS, a stress sensor PDZ protease. *Cell* **131**(3)**:** 572-583

Song Z, Chen H, Fiket M, Alexander C, Chan DC (2007) OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. *The Journal of cell biology* **178**(5)**:** 749-755

Song Z, Ghochani M, McCaffery JM, Frey TG, Chan DC (2009) Mitofusins and OPA1 Mediate Sequential Steps in Mitochondrial Membrane Fusion. *Molecular biology of the cell*

Soubannier V, McBride HM (2009) Positioning mitochondrial plasticity within cellular signaling cascades. *Biochimica et biophysica acta* **1793**(1)**:** 154-170

Srinivasula SM, Gupta S, Datta P, Zhang Z, Hegde R, Cheong N, Fernandes-Alnemri T, Alnemri ES (2003) Inhibitor of apoptosis proteins are substrates for the mitochondrial serine protease Omi/HtrA2. *The Journal of biological chemistry* **278**(34)**:** 31469-31472

Strauss KM, Martins LM, Plun-Favreau H, Marx FP, Kautzmann S, Berg D, Gasser T, Wszolek Z, Muller T, Bornemann A, Wolburg H, Downward J, Riess O, Schulz JB, Kruger R (2005) Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. *Human molecular genetics* **14**(15)**:** 2099-2111

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R (2001) A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Molecular cell* **8**(3)**:** 613-621

Szeto HH (2006) Mitochondria-targeted peptide antioxidants: novel neuroprotective agents. *The AAPS journal* **8**(3)**:** E521-531

Tain LS, Chowdhury RB, Tao RN, Plun-Favreau H, Moisoi N, Martins LM, Downward J, Whitworth AJ, Tapon N (2009) Drosophila HtrA2 is dispensable for apoptosis but acts downstream of PINK1 independently from Parkin. *Cell death and differentiation*

Tan JM, Dawson TM (2006) Parkin blushed by PINK1. *Neuron* **50**(4)**:** 527-529

Tanner CM (1992) Epidemiology of Parkinson's disease. *Neurologic clinics* **10**(2)**:** 317-329

Tatsuta T (2009) Protein quality control in mitochondria. *J Biochem* **146**(4)**:** 455-461

Timmons S, Coakley MF, Moloney AM, C ON (2009) Akt signal transduction dysfunction in Parkinson's disease. *Neuroscience letters* **467**(1)**:** 30-35

Tondera D, Grandemange S, Jourdain A, Karbowski M, Mattenberger Y, Herzig S, Da Cruz S, Clerc P, Raschke I, Merkwirth C, Ehses S, Krause F, Chan DC, Alexander C, Bauer C, Youle R, Langer T, Martinou JC (2009) SLP-2 is required for stress-induced mitochondrial hyperfusion. *The EMBO journal* **28**(11)**:** 1589-1600

Unoki M, Nakamura Y (2001) Growth-suppressive effects of BPOZ and EGR2, two genes involved in the PTEN signaling pathway. *Oncogene* **20**(33)**:** 4457-4465

Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, Wood NW (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science (New York, NY* **304**(5674)**:** 1158-1160

van Loo G, van Gurp M, Depuydt B, Srinivasula SM, Rodriguez I, Alnemri ES, Gevaert K, Vandekerckhove J, Declercq W, Vandenabeele P (2002) The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. *Cell death and differentiation* **9**(1)**:** 20-26

Vande Walle L, Lamkanfi M, Vandenabeele P (2008) The mitochondrial serine protease HtrA2/Omi: an overview. *Cell death and differentiation* **15**(3)**:** 453-460

Vande Walle L, Van Damme P, Lamkanfi M, Saelens X, Vandekerckhove J, Gevaert K, Vandenabeele P (2007) Proteome-wide Identification of HtrA2/Omi Substrates. *Journal of proteome research* **6**(3)**:** 1006-1015

Verhagen AM, Silke J, Ekert PG, Pakusch M, Kaufmann H, Connolly LM, Day CL, Tikoo A, Burke R, Wrobel C, Moritz RL, Simpson RJ, Vaux DL (2002) HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *The Journal of biological chemistry* **277**(1)**:** 445-454

Wakabayashi K, Tanji K, Mori F, Takahashi H (2007) The Lewy body in Parkinson's disease: molecules implicated in the formation and degradation of alpha-synuclein aggregates. *Neuropathology* **27**(5)**:** 494-506

Waldman AS (1991) A simple method for retrieving colonies of cultured mammalian cells from tissue culture flasks. *BioTechniques* **10**(3)**:** 318-319

Waterham HR, Koster J, van Roermund CW, Mooyer PA, Wanders RJ, Leonard JV (2007) A lethal defect of mitochondrial and peroxisomal fission. *The New England journal of medicine* **356**(17)**:** 1736-1741

Weihofen A, Ostaszewski B, Minami Y, Selkoe DJ (2008) Pink1 Parkinson mutations, the Cdc37/Hsp90 chaperones and Parkin all influence the maturation or subcellular distribution of Pink1. *Human molecular genetics* **17**(4)**:** 602-616

Weintraub D, Comella CL, Horn S (2008) Parkinson's disease--Part 1: Pathophysiology, symptoms, burden, diagnosis, and assessment. *The American journal of managed care* **14**(2 Suppl)**:** S40-48

Whitworth AJ, Lee JR, Ho VM, Flick R, Chowdhury R, McQuibban GA (2008) Rhomboid-7 and HtrA2/Omi act in a common pathway with the Parkinson's disease factors Pink1 and Parkin. *Disease models & mechanisms* **1**(2-3)**:** 168-174; discussion 173

Wilken C, Kitzing K, Kurzbauer R, Ehrmann M, Clausen T (2004) Crystal structure of the DegS stress sensor: How a PDZ domain recognizes misfolded protein and activates a protease. *Cell* **117**(4)**:** 483-494

Wood-Kaczmar A, Gandhi S, Yao Z, Abramov AY, Miljan EA, Keen G, Stanyer L, Hargreaves I, Klupsch K, Deas E, Downward J, Mansfield L, Jat P, Taylor J, Heales S, Duchen MR, Latchman D, Tabrizi SJ, Wood NW (2008) PINK1 is necessary for long term survival and mitochondrial function in human dopaminergic neurons. *PloS one* **3**(6)**:** e2455

Xiong H, Wang D, Chen L, Choo YS, Ma H, Tang C, Xia K, Jiang W, Ronai Z, Zhuang X, Zhang Z (2009) Parkin, PINK1, and DJ-1 form a ubiquitin E3 ligase complex promoting unfolded protein degradation. *The Journal of clinical investigation* **119**(3)**:** 650-660

Yang L, Sun M, Sun XM, Cheng GZ, Nicosia SV, Cheng JQ (2007) Akt attenuation of the serine protease activity of HtrA2/Omi through phosphorylation of serine 212. *The Journal of biological chemistry* **282**(15)**:** 10981-10987

Yang QH, Church-Hajduk R, Ren J, Newton ML, Du C (2003) Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. *Genes & development* **17**(12)**:** 1487-1496

Yang Y, Gehrke S, Imai Y, Huang Z, Ouyang Y, Wang JW, Yang L, Beal MF, Vogel H, Lu B (2006) Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. *Proceedings of the National Academy of Sciences of the United States of America* **103**(28)**:** 10793-10798

Yang Y, Ouyang Y, Yang L, Beal MF, McQuibban A, Vogel H, Lu B (2008) Pink1 regulates mitochondrial dynamics through interaction with the fission/fusion machinery. *Proceedings of the National Academy of Sciences of the United States of America* **105**(19)**:** 7070-7075

Yoon Y, Krueger EW, Oswald BJ, McNiven MA (2003) The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. *Molecular and cellular biology* **23**(15)**:** 5409-5420

Yun J, Cao JH, Dodson MW, Clark IE, Kapahi P, Chowdhury RB, Guo M (2008) Loss-of-function analysis suggests that Omi/HtrA2 is not an essential component of the PINK1/PARKIN pathway in vivo. *J Neurosci* **28**(53)**:** 14500-14510

Zeth K (2004) Structural analysis of DegS, a stress sensor of the bacterial periplasm. *FEBS letters* **569**(1-3)**:** 351-358

Zhang D, Lu C, Whiteman M, Chance B, Armstrong JS (2008) The mitochondrial permeability transition regulates cytochrome C release for apoptosis during endoplasmic reticulum stress by remodeling the cristae junction. *The Journal of biological chemistry* **283**(6)**:** 3476-3486

Zhou C, Huang Y, Przedborski S (2008a) Oxidative stress in Parkinson's disease: a mechanism of pathogenic and therapeutic significance. *Annals of the New York Academy of Sciences* **1147:** 93-104

Zhou C, Huang Y, Shao Y, May J, Prou D, Perier C, Dauer W, Schon EA, Przedborski S (2008b) The kinase domain of mitochondrial PINK1 faces the cytoplasm. *Proceedings of the National Academy of Sciences of the United States of America* **105**(33)**:** 12022-12027

Zhou H, Falkenburger BH, Schulz JB, Tieu K, Xu Z, Xia XG (2007) Silencing of the Pink1 gene expression by conditional RNAi does not induce dopaminergic neuron death in mice. *International journal of biological sciences* **3**(4)**:** 242-250

Zick M, Rabl R, Reichert AS (2009) Cristae formation-linking ultrastructure and function of mitochondria. *Biochimica et biophysica acta* **1793**(1)**:** 5-19

°C	degree celcius
6-OHDA	6 –hydroxydopamine
aa	amino acid
AD	Alzheimer's disease
ADP	adenosine diphosphate
ANT	adenine nucleotide translocator
Apaf-1	apoptotic peptidase activating factor-1
APP	amyloid precursor protein
APS	ammonium persulfate
ATP	Adenine triphosphate
BCA	Bicinchoninic Acid
bp	base pair
BSA	bovine serum albumine
Ca^{2+}	calcium
CJC	cristae junction complex
ctrl	control
D. Melongaster	*Drosophila melanogaster*
DA	dopamine
DMEM	Dulbecco's Minimal Essential Medium
DNA	deoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
DOX	doxycycline
Drp1	dynamin-related protein-1
E. coli	*Escherichia coli*
EM	electron microscopy
ER	endoplasmic reticulum
ETC	electron transport chain
Fis1	mitochondrial fission 1 protein
g	gravitation constant
g	gram
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
Hsp	heat shock protein
HtrA2	high temperature requirement A2

IAP	inhibitor of apoptosis protein
IBM	IAP binding motif
IMM	inner mitochondrial membrane
IMS	inter membrane space
IP	immunoprecipitation
kb	kilobase
kDa	kilodalton
l	liter
LB medium	lysogeny broth medium
LBs	Lewy Bodies
LDH	lactate dehydrogenase
m	milli
M	molar
Mfn1	mitofusin-1
Mfn2	mitofusin-2
min	minute
mitophagy	autophagic clearance of mitochondria
MMP	mitochondrial membrane potential
MMP	mitochondrial processing peptidase
mnd-2	motor neuron disease-2
MOMP	mitochondrial outer membrane permeabilization
MPP	matrix processing peptidase
MPP+	1-methyl-4-phenylpyridinium
mPTP	mitochondrial permeability transition pore
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
MTS	mitochondrial targeting sequence
n	nano
OMM	outer mitochondrial membrane
OPA1	optic atrophy-1
PAGE	polyacrylamide gel electrophoresis
P-Akt	Akt phosphorylated at Thr308
PARL	presinilin-associated rhomoboid-like
PARP	poly[ADP-ribose] polymerase

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PDZ	post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1)
PINK1	phosphatase and tensin-induced putative kinase-1
PK	proteinase K
PT	permeability transition
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
SNCA	α-synuclein
SNpc	substantia nigra pars compacta
TEMED	N, N, N', N'-tetramethylethylenediamine
TM	transmembrane
TM	transmembrane
TMRM	tetramethyl rhodamine methyl ester
TNE	Tris-NaCl-EDTA
Trap1	tumor necrosis receptor-associated protein-1
U	units
UPS	ubiquitin proteasome system
UV	ultra violet
VDAC1	voltage dependent anion channel 1
wt	wild type
µ	micro

Acknowledgements

First of all I would like to thank my supervisor Prof. Philipp Kahle for giving me the opportunity to join his lab. His expertise and help, as well as his patience, have been invaluable to both my personal development and to the success of this project.
I am also grateful to Prof. Doron Rapaport, for his scientific input, for reviewing my thesis and for keeping up the initiative of the MitoClub.
As part of my Advisory Board committee I would further like to thank Prof. Thomas Gasser.
I would also like to thank Prof. Rejko Krüger, for input and support during the joint study.

A very special thanks goes to Nicole Kieper, for all her ideas and hard work, as well as moral support during the joint project and all the way until the end.
I would also like to thank Fabienne Fiesel for her help throughout the years, for her excellent ideas and encouragement.
To Sven Geisler, Heinrich Schell, Angela Treis, Elena Cornejo-Castro and everyone else, both current and former members of the lab, a huge thank you for all the help and for making the lab a fun place to come to. And for sharing the highs and lows of the past few years.

I am also indebted to my new family here in Germany, to the Stuttgart Rugby Club. Crouch, touch, hold…engage!

Till alla hemma i Finland och resten av familjen runt världen. Tack för att ni gett mig vingar att flyga och någonstans att landa.

Finally, I have two words for my dearest Andy Love: „Did science“!