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Impact of Parkinson disease-related mutations in PINK1 on Parkin and mitochondrial fusion/fission factors in human-derived cells

Dissertation
for fulfillment of requirements
for the doctoral degree
of the University of Lübeck

From the Section of Natural Sciences

Submitted by

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from Belgrade

Lübeck, 2011

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Tag der mündlichen Prüfung: 14.12.2011

Zum Druck genehmigt. Lübeck, den 14.12.2012

This thesis is dedicated to my family.

Ova teza je posvećena mojoj porodici.

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1 INTRODUCTION

1.1 Parkinson disease

Parkinson disease (PD) is a progressive neurodegenerative disorder of the central nervous system that impairs motor skills, cognitive processes, and other functions. The primary symptoms of the disease are motor-related, including tremor, rigidity, bradykinesia, and postural instability. The pathological hallmarks of PD are a loss of dopaminergic neurons of the midbrain, specifically the *substantia nigra* and an accumulation of α-synuclein, protein-forming inclusions in the brain, called Lewy bodies. More than 1.6 % of the population over the age of 65 year is affected by PD (de Rijk *et al.*, 1997).

The etiology of PD is still unknown. However, it was recognized that the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) is selectively toxic to the neurons in the *substantia nigra* resulting in signs and symptoms similar to those observed in PD. MPTP is a mitochondrial toxin whose metabolite 1-methyl-4-phenyl pyridinium (MPP^+) accumulates in mitochondria and inhibits complex I activity (Vyas *et al.*, 1986). These findings have prompted environmental theories of disease causation and were followed by several reports of loss of electron transport chain (ETC) activity in multiple tissues from individuals with PD. This biochemical defect has been seen in platelets (Parker *et al.*, 1989; Krige *et al.*, 1992; Benecke *et al.*, 1993; Haas *et al.*, 1995), lymphocytes (Yoshino *et al.*, 1992), brain (Schapira *et al.*, 1989), muscle (Shoffner *et al.*, 1991), and fibroblasts (Mytilineou *et al.*, 1994). Immunoblot studies of brain tissues from individuals affected with PD have demonstrated disruption of NADH dehydrogenase (complex I) subunits (Mizuno *et al.*, 1989). These findings strongly implied a connection between mitochondrial dysfunction and the pathogenesis of PD.

1.2 Familial forms of PD

The identification of causative genes for rare familial forms of PD that share clinical and neuropathological features with the more common, idiopathic form has greatly accelerated approaches to dissecting the molecular basis of PD. Indeed, in distinct populations, mutations in one single gene were found to cause the disease in up to 40% of patients formerly described as 'idiopathic' cases (Schiesling *et al.*, 2008). To date, 16 genetic loci (*PARK* loci) have been linked to or associated with PD. Within these loci, six genes, i.e. *α-synuclein, Parkin, PINK1, DJ-1, LRRK2, and HtrA2* have been unambiguously identified and reported by several groups

to carry mutations that segregate in affected family members. A list of the PD genes and gene loci and their mode of inheritance is given in Table 1.

Table 1. *PD genes and gene loci*

Locus	Mode of inheritance	Gene	Position
PARK1/PARK4	AD	*α-synuclein*	4q21-q23
PARK2	**AR**	***Parkin***	**6q25-q27**
PARK3	AD	Unknown	2p13
PARK5	AD	*Ubiquitin carboxy-terminal-hydrolase-L1(UCHL1)*	4p14
PARK6	**AR**	***PTEN-induced-putative kinase (PINK1)***	**1p36-p35**
PARK7	AR	*DJ-1*	1p36
PARK8	AD	*Leucine-rich repeat kinase 2 (LRRK2)*	12p11-q13
PARK9	AR	*ATPase type 13A2 (ATP13A2)*	1p36
PARK10	Unknown	Unknown	1p32
PARK11	AD	*Grb10-Interacting GYF Protein-2(GIGYF2)(TNRC15)*	2q36-q37
PARK12	X-linked	Unknown	Xq21-q25
PARK13	AD	*HtrA2/Omi*	2p12
PARK14	AR	*Phospholipase A2, group VI(PLA2G6)*	22q13.1
PARK15	AR	*F-box protein 7(FBX07)*	22q12-q13
PARK16	Unknown	Unknown	1q32

AD- autosomal dominant; AR- autosomal recessive Source: *Human Mol Genet*, 16:R183-94, 2007

Studies using *in-vivo* animal models and *in-vitro* cell culture have linked mutations in *α-synuclein*, *DJ-1*, *Parkin*, and *PINK1* to impairments of mitochondrial structure and function and oxidative stress response, reinforcing the idea of a general involvement of mitochondrial dysfunction and oxidative stress in PD pathogenesis (Hsu *et al.*, 2000; Palacino *et al.*, 2004; Shen *et al.*, 2004). Among these, recessively inherited *PINK1* (*PTEN-induced putative kinase 1*) (Valente *et al.,* 2004) and *Parkin* (Kitada *et al.,* 1998) mutations are known causes of a clinical syndrome closely resembling idiopathic PD with the exception of an overall earlier age of onset and slower disease progression.

1.3 PINK1 and Parkin in PD

The study of recessive forms of familial PD, such as those resulting from mutations in PINK1 and Parkin, may reveal disease mechanisms important to the development of disease in these families as well as those suffering from sporadic PD.

The *PINK1* gene encodes a 581-aa polypeptide with a predicted N-terminal mitochondrial targeting signal (MTS), a transmembrane domain (TM), and a serine/threonine kinase domain similar to that in the Ca^{2+}/calmodulin kinase family (Unoki *et al.,* 2001; Deas *et al.,* 2009). The predicted PINK1 structure is schematically presented in Figure 1. PINK1 is synthesized as a full-length form (~66 kDa) which is proteolytically processed upon entry into mitochondria to its cleaved ~55 kDa form. The physiological relevance of cleavage and the exact cleavage site, as well as the submitochondrial localization of both forms currently remain under debate (Weihofen *et al.,* 2008). Mitochondrial localization of PINK1 and its autophosphorylation and phosphorylation activities were shown in a series of experiments and could be impaired by PD-causing mutations (Valente *et al.*, 2004; Beilina *et al.*, 2005; Silvestri *et al.*, 2005; Sim *et al.*, 2006). According to the most recently proposed model, PINK1 is localized on the outer mitochondrial membrane, where it is cleaved in a voltage-dependent manner (Narendra *et al.*, 2010). Inhibition of cleavage and accumulation of full-length PINK1 can be induced by loss of the mitochondrial membrane potential (Narendra *et al.*, 2010).

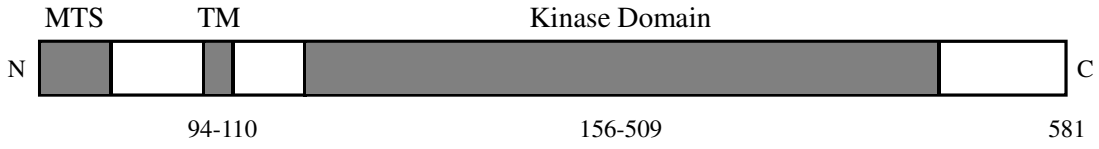

Figure 1. *Schematic representation of the PINK1 structure*. MTS- mitochondrial target sequence, TM- transmembrane domain

Studies in animal models showed a selective loss of dopaminergic cells in *PINK1* knock-out flies (Park *et al.*, 2006; Yang *et al.*, 2006). While wild-type PINK1 protects cells against mitochondrial toxins (Wood-Kaczmar *et al.,* 2008) and apoptosis induced by proteasomal stress (Klinkenberg *et al.*, 2010), stable loss or knockdown of PINK1 in mammalian cellular models and mice leads to a number of mitochondria-related abnormalities. Mitochondria in these cells or tissues exhibit ETC dysfunction, diminished mitochondrial membrane potential, increased reactive oxygen species production, mitochondrial fragmentation, and calcium dysregulation, among other abnormalities (Exner *et al.*, 2007; Dagda *et al.*, 2009; Gandhi *et al.*, 2009; Sandebring *et al.*, 2009). For instance, complex I and the putative Na^{+}/Ca^{2+} transporter seem to be dysfunctional in cultured cells following PINK1 knockdown (Gandhi *et al.*, 2009), whereas complex I and II appear to be dysfunctional in the striatum of mice

lacking PINK1 (Gautier *et al.*, 2008). It has been shown that PINK1 genetically interacts with the mitochondrial fission/fusion machinery and modulates mitochondrial dynamics. In *Drosophila* and mammalian cells, overexpression of PINK1 promotes mitochondrial fission (Yang *et al.*, 2008).

Two candidate substrates for direct or downstream phosphorylation by PINK1 in mitochondria have been found to date: PINK1 phosphorylates the mitochondrial chaperone TNF receptor-associated protein 1 (TRAP1) (Pridgeon *et al.*, 2007) and was shown to facilitate phosphorylation of HtrA2. Phosphorylation of HtrA2 contributes to an increased resistance of cells to mitochondrial stress (Plun-Favreau *et al.*, 2007). Other reported PINK1 interaction partners are Heat shock protein 90 (Hsp90) and Cdc37 protein chaperones. These chaperones likely facilitate transport of proteins to the mitochondria before transport across the mitochondrial membranes (Weihofen *et al.*, 2008). PINK1 was found to be a part of a mitochondrial multiprotein complex that contains the atypical GTPase Miro and the adaptor protein Milton, proteins involved in the trafficking of mitochondria along microtubules. This postulates a role for PINK1 in mitochondrial trafficking (Weihofen *et al.*, 2009). In addition, PINK1 and Parkin may form a complex in conjunction with DJ-1 to promote degradation of un-/misfolded proteins (Xiong *et al.,* 2009).

Parkin encodes a 465-aa protein with a modular structure containing an N-terminal ubiquitin-like domain and a C-terminal RING-IBR-RING domain. Recently, a third RING domain was identified in Parkin and, as it is located upstream of RING1, was named RING0 (Hristova *et al.*, 2009). Similar to other RING finger proteins, Parkin functions as an E3 ubiquitin ligase in the ubiquitin proteasome system (UPS), where it ubiquitinates a number of substrates (Moore, 2006). E3 ligases catalyze the addition of ubiquitin molecules to lysine residues of damaged target proteins, and the presence of a polyubiquitin chain provides a signal for the removal and degradation of these proteins by the proteolytic complex, the 26S proteasome (Pickart, 2001). Previous studies yielded conflicting conclusions on Parkin's subcellular localization, finding the protein in the cytosol or associated with the ER or mitochondria (Shimura *et al.*, 1999; Darios *et al.*, 2003). According to numerous recent findings, Parkin is predominately localized in the cytosol and can be translocated to damaged mitochondria in PINK1-dependent manner (Kim *et al.*, 2008; Geisler *et al.*, 2010; Narendra *et al.*, 2010). Parkin has been implicated in several other cellular functions including a key role in maintaining mitochondrial function and integrity (Abou-Sleiman *et al.,* 2006), mitochondrial DNA repair (Rothfuss *et al.,* 2009), as well as involvement in mitochondrial quality control and mitophagy (Narendra *et al.*, 2009; Geisler *et al.*, 2010; Narendra *et al.*, 2010; Vives-Bauza *et*

al., 2010). Mutations in *Parkin* are the most common known cause of early-onset PD, accounting for up to 77% of the cases with an age of onset <30 years (Lucking *et al.,* 2000). The vast majority of mutations in Parkin is considered to produce a loss of function and tend to differentially disrupt ubiquitin ligase activity, expression, intrinsic solubility, cellular localization or interaction with ubiquitin-conjugating enzymes (Shimura *et al.,* 2000; Zhang *et al.,* 2000) and substrates (Wang *et al.,* 2005; Hampe *et al.,* 2006; Matsuda *et al.,* 2006).

Recent studies have linked Parkin and PINK1 in a pathway critical for the maintenance of mitochondrial integrity and function. Loss of either protein in *Drosophila* results in a similar phenotype, with mitochondrial damage preceding muscle degeneration, as well as disrupted spermatogenesis and death of dopaminergic neurons. Interestingly, overexpression of Parkin can partially compensate for PINK1 loss, but PINK1 overexpression cannot compensate for Parkin loss, suggesting that PINK1 functions upstream of Parkin in a common pathway (Whitworth *et al.*, 2005; Clark *et al.*, 2006; Park *et al.*, 2006; Yang *et al.*, 2006). Additionally, null mice for either Parkin or PINK1 exhibit increased oxidative damage and decreased mitochondrial function in the striatium (which receives projections from dopaminergic neurons) (Palacino *et al.*, 2004; Gautier *et al.*, 2008); and primary cells from patients with loss-of function mutations in Parkin or PINK1 have similar abnormalities (Muftuoglu *et al.*, 2004; Exner *et al.*, 2007; Mortiboys *et al.*, 2008). More recently, involvement of both Parkin and PINK1 has also been demonstrated in mitophagy with mitochondrial translocation of Parkin depending on PINK1 (Geisler *et al.*, 2010; Narendra *et al.*, 2010; Vives-Bauza *et al.*, 2010). These findings collectively demonstrate that both PINK1 and Parkin are intimately involved in an evolutionarily conserved pathway to prevent mitochondrial dysfunction.

1.4 PINK1/Parkin pathway and mitochondrial dynamics

Furthermore, both PINK1 and Parkin have been linked to mitochondrial fission. In *Drosophila* and mammalian cells, overexpression of PINK1 promotes mitochondrial fission (Yang *et al.*, 2008). Mitochondria undergo dynamic changes in morphology through fusion and fission. Fusion is likely to protect function by providing a chance for mitochondria to mix their contents, thus enabling protein complementation, mtDNA repair and equal distribution of metabolites. Fission most likely acts to facilitate equal segregation of mitochondria into daughter cells during cell division and to enhance distribution of mitochondria along cytoskeletal tracks. In addition, fission may help to isolate damaged segments of mitochondria and thus promote their autophagy (Twig *et al.*, 2008). Although these processes have been

extensively studied in yeast, only recently have molecules regulating mitochondrial dynamics been identified in mammals. These include the homologous GTPases Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2), which mediate fusion of the mitochondrial outer membrane, as well as Optic atrophy 1 (Opa1), a GTPase required for fusion of the inner membrane. Mitochondrial fission, conversely, requires Dynamin-related protein 1 (Drp1), which is also a GTPase (Okamoto *et al.*, 2005; Detmer *et al.*, 2007; Hoppins *et al.*, 2007). In previous work, it has been shown that genetic manipulations that promote mitochondrial fragmentation, including increased drp1 gene dosage and decreased opa1 or mfn gene dosage, dramatically suppress the pink1 and parkin mutant phenotypes in Drosophila (Deng *et al.*, 2008; Poole *et al.*, 2008; Yang *et al.*, 2008; Park *et al.*, 2009).

Mfn1 and Mfn2, which are the human orthologues of the *Drosophila* mfn, are essential for mitochondrial fusion (Chen *et al.*, 2003; Eura *et al.*, 2003). These proteins are large GTPases showing 81% homology to each other and similar topologies. Both reside on the outer membrane with the N-terminal GTPase and a predicted coiled coil protruding into the cytosol (Rojo *et al.*, 2002; Santel *et al.*, 2003). A transmembrane region forms a U-shaped membrane anchor, ending in a cytosolic, C-terminal coiled coil. Cells lacking both Mfn1 and Mfn2 have completely fragmented mitochondria and show no detectable mitochondrial fusion activity. Mitochondrial fusion is important not only for maintenance of mitochondrial morphology, but also for cell growth, mitochondrial membrane potential, and respiration.

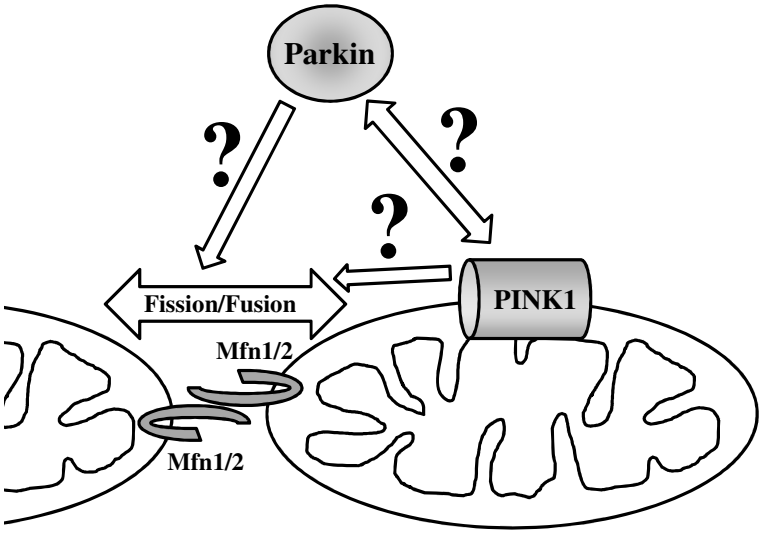

Figure 2. *The relationship between the two PD genes PINK1 and Parkin and their role in mitochondrial dynamics were mainly unknown at the start of this thesis.* Mfn 1/2- Mitofusin 1 and Mitofusin 2

1.5 Finding a novel PINK1 protein interacting partner

To date, several protein-interactors of PINK1 have been discovered (for details see 1.3). However, to gain better insight into the pathology of PD, identification of novel protein–protein interactions as well as the characterization of pathways they are involved in is needed. Traditionally, potential interacting proteins have been identified by genetic methods (yeast two–hybrid screens) with subsequent verification of the interaction by co-immunoprecipitation. While this method has been successful for the detection of a variety of interacting proteins, it is of limited utility when integral membrane proteins or transcription factors are investigated (Stagljar *et al.*, 1998). An alternative method is the tandem affinity purification (TAP) method that can be used for precipitation of protein complexes. The TAP method combines purification of a protein complex of interest using affinity purification tags with subsequent identification of unknown protein complex components by mass spectrometry. The key feature of this technology is the use of two different affinity purification tags that are fused to at least one known component of the protein complex of interest by genetic methods. Performing two consecutive purification steps using affinity purification tags that have gentle washing and elution conditions allows for isolation without disrupting the targeted complex. Since PINK1 is a putative mitochondrial membrane protein, this method provides a means to search for new PINK1-interacting partners in human-derived cells.

1.6 Studying primary cells in neurologic diseases

Studying neurons to elucidate the disease mechanisms of PD seems most logical. However, obtaining neurons from patients is highly limited and therefore not feasible for most applications. PINK1 (as well as Parkin) is ubiquitously expressed, and therefore mutated forms can also be studied in other primary cells.

Human skin fibroblasts are a common source of primary cells for studying disease mechanisms. Their advantages in comparison to neurons are: (i) they are easily obtained; (ii) fibroblast cell banks exist worldwide; (iii) they are simple to culture, to store and to recover; (iv) many cells can be obtained from small samples and multiple experiments can be performed on cells from the same patient; (v) they provide models when no or only poor animal models exist; and (vi) inherited mutations in DNA or RNA can be studied or the DNA can be modified to study how changes in genes alter their function (for further details see reference # Connolly, 1998). However, there are also disadvantages of fibroblasts as a cellular

model: (i) they are inconsistent in their response between cells from different passages or vary in growth between batches of cells; (ii) factors present *in vivo* might be missing; and (iii) fibroblasts are not fully representative of the diseased tissue and/or central nervous system dysfunction (Connolly, 1998). Still, fibroblasts have provided important insights into mechanisms underlying neurologic diseases such as DYT1 dystonia (Goodchild *et al.,* 2004), Alzheimer disease (Gibson *et al.,* 2002), amyotropic lateral sclerosis (Aguirre *et al.,* 1998) and Huntington disease (Seo *et al.,* 2004). For PD, currently only a small number of studies in human cell models exist. So far, fibroblasts of eight different PINK1 mutant individuals have been examined in two independent studies (Hoepken *et al.,* 2007; Grünewald *et al.,* 2009). For Parkin, only few papers summarizing data from fibroblast cultures of five patients with homozygous or compound heterozygous mutations have been published to date (for details see passage above and reference # Mortiboys *et al.,* 2008).

1.7 Hypotheses

The general aim of the present thesis was to explore the effect of PD-causing mutations in PINK1 on protein levels and subcellular localization of Parkin and involvement of the PINK1/Parkin pathway in the regulation of mitochondrial dynamics in fibroblasts from PD patients and controls. Specifically, the following hypotheses were addressed:

Project 1:
- PINK1 regulates subcellular localization of Parkin and its mRNA and protein levels.
- PD-causing mutations in PINK1 impair mitochondrial translocation of Parkin.

Project 2:
- PINK1 and Parkin regulate protein levels of mitochondrial fusion factors Mfn1 and Mfn2.
- Parkin-mediated PINK1-dependent ubiquitination of Mfn1 and Mfn2 is impaired by PD-causing mutations in both PINK1 and Parkin.

Project 3:
- Newly discovered interacting partners of PINK1 contribute to a better understanding of the PD pathology.

2 PATIENTS, MATERIAL AND METHODS

2.1 Patients

In the present study, primary dermal fibroblasts from Parkinson disease (PD) patients were investigated at the molecular level. The diagnosis of PD was established by movement disorders specialists based on published criteria (for parkinsonism see Gibb *et al.*, 1988; clinical features of M-D are reviewed in Klein, 2003). All patients and control individuals gave informed consent and the study was approved by the local Ethics Committee (Ethic approval 05-030).

2.1.1 PD patients with mutations in PINK1

The investigations included four PD patients carrying two different PD-causing homozygous mutations in *PINK1*. Three of them were siblings harboring a c.1366C>T (p.Q456X) nonsense mutation (one male, two female; mean age: 70.0 years [±4.8]; mean age at onset: 50.0 years [±9.3]). A detailed clinical report about these patients has been published when the family was first identified (Hedrich *et al.,* 2006).

In addition, one patient (female, age: 70 years, age at onset: 31 years) with a homozygous missense mutation c.509T>G (p.V170G) was studied. Clinical details of this patient are published elsewhere (Moro *et al.,* 2008).

The localization of the PINK1 mutations and their effect on the length of the protein are schematically shown in Fig. 3.

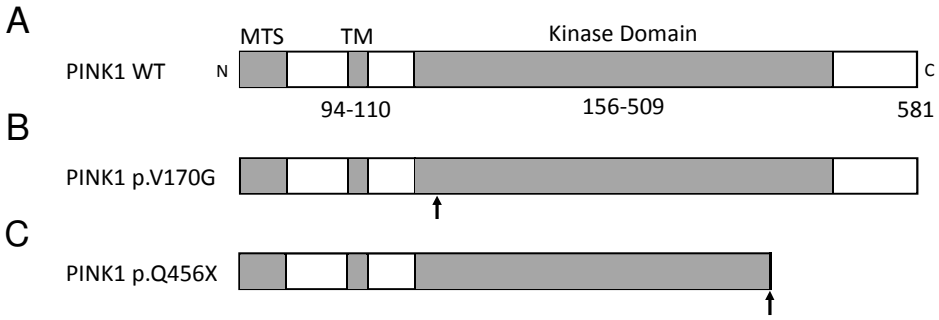

Figure 3. *Topology of the wild-type PINK1 and location of PINK1 mutations studied.* (A) Schematic representation of the PINK1 structure with, (B and C) positions of the two PINK1 mutations studied that are both located in the kinase domain of the protein. (C) The nonsense mutation results in protein truncation. MTS-mitochondrial target sequence; TM-transmembrane domain

2.1.2 PD patients with mutations in Parkin

Three PD patients carrying two different PD-causing mutations in the *Parkin* gene were investigated. Two of them carried either a homozygous c.1072delT (p.V324fsX434) mutation (male; age: 62 years, age at onset: 43 years) or a homozygous delEx7 (p.R245fsX253) mutation (male; age: 49 years, age at onset: 34 years). The third patient was a compound-heterozygous *Parkin* mutation carrier (c.1072delT+ delEx7) (female; age: 79 years, age at onset: 64 years).

2.2 Healthy controls and cell lines

Four age-matched, healthy controls, without mutations in *PINK1* or *Parkin* (three female, one male; two family members and two unrelated controls) were included in this study.
In addition, commercially available human embryonic kidney (HEK) cells were used.

2.3 Material

2.3.1 Chemicals

Acetic acid (CH_3COOH)	Merck
Accutase	PAA Laboratories
Agarose	Biozym
Ampicillin, salt	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Bromphenol blue	Sigma-Aldrich
Calcium chloride ($CaCl_2$)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Desoxyribonucleotides (dNTPs)	Amersham Biosciences
Dithiothreitol (DTT; $C_4H_{10}O_2S_2$)	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM)	PAA Laboratories
Epoxomicin	Sigma
Ethanol (C_2H_5OH)	J.T. Baker
Ethidium bromide ($C_{21}H_{20}BrN_3$)	Sigma-Aldrich
Ethylenediaminetertraacetic acid (EDTA)	Merck
Fetal bovine serum (FBS)	PAA Laboratories
Glycerol ($C_3H_5(OH)_3$)	Sigma-Aldrich
HEPES	Sigma

Hydrochloric acid (HCl)	Merck
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich
2-Mercaptoethanol (C_2H_6OS)	Sigma-Aldrich
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich
Methanol (CH_3OH)	J.T. Baker
MG132 (Z-Leu-Leu-Leu-al)	Sigma
Milk powder	Roth
MitoTracker Green FM	Molecular Probes
Nonidet P-40 (NP-40)	USB
NuPAGE antioxidant	Invitrogen
NuPAGE LDS sample buffer (4x)	Invitrogen
NuPAGE MOPS SDS running buffer (20x)	Invitrogen
NuPAGE transfer buffer (20x)	Invitrogen
Paraformaldehyde (PFA)	Sigma-Aldrich
Penicillin/Streptomycin (P/S)	PAA Laboratories
Phosphate buffered saline (10xPBS)	PAA Laboratories
PhosSTOP phosphatase inhibitor cocktail tablets	Roche
2-Propanol (Isopropanol)	Sigma-Aldrich
Protease inhibitor cocktail tablets	Roche
Protein G-agarose	Roche
Precision plus protein all blue standards	Biorad
Proteinase K	Sigma-Aldrich
Sodium chloride (NaCl)	Merck
Sodium deoxycholate (DOC)	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	Fluka
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich
Sucrose ($C_{12}H_{22}O_{11}$)	Sigma-Aldrich
Taq DNA polymerase and buffer	Qbiogene
5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1)	Molecular Probes
Tris ($C_4H_{11}NO_3$)	Roth
Triton X-100	Sigma-Aldrich
Tween-20	Biorad
Valinomycin ($C_{54}H_{90}N_6O_{18}$)	Sigma-Aldrich

2.3.2 Solutions

1xPBS	100ml 10xPBS, 900ml sterile dH_2O
10x Tris-buffered saline (TBS)	9% NaCl, 250 mM Urea, pH 7.5
1x Tris-buffered saline tween-20 (TBST)	1xTBS, 0.1% Tween 20
Homogenization buffer:	10mM Tris, 1mM EDTA, 250mM sucrose, pH 7.4
Radioimmunoprecipitation assay (RIPA) buffer	25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% DOC, 0.1% SDS
Immunoprecipitation lysis buffer	150mM NaCl, 50 mM Tris, 1% NP-40, 0.1% SDS
Immunoprecipitation washing buffer	150mM NaCl, 50 mM Tris, 1% NP-40
1x LDS sample buffer	1x NuPAGE LDS Sample Buffer (4x), 50mM DTT
2x Hepes-buffered saline (HBS)	280 mM NaCl, 50mM HEPES, 1.5mM Na_2HPO_4, pH 7.0

2.3.3 Kits

EndoFree Plasmid Maxi Kit	Qiagen
InterPlay® Mammalian TAP System	Agilent Technologies
LightCycler® FastStart DNA Master SYBR® Green	RocheDiagnostics
Nucleofector™ Technology	Lonza
One Shot® TOP10 Electrocomp™ *E. coli*	Invitrogen
PAXgene Blood RNA Kit	Qiagen
Protein D_c Assay Kit	Biorad
RNeasy Mini Kit	Qiagen
SilverQuest™ Silver Staining Kit	Invitrogen
SuperScript™ First-Strand Synthesis System	Invitrogen
SuperSignal West Pico Chemiluminescent Substrate	Pierce
Zenon® Immunolabeling Reagent	Invitrogen

2.3.4 Antibodies

Anti-β-actin (1:1x10^6)	Sigma
Anti-FLAG M2 (1:10,000)	Sigma
Anti-GRP75 (1:1x10^6)	Abcam
Anti-Hsp60 (1:1,000)	Cell Signaling
Anti-Mitofusin 1 (1:1,000)	Abcam
Anti-Mitofusin 2 (1:1,000)	Abcam
Anti-MTCO2, subunit of complex IV (1:2,000)	Mitosciences
Anti-Neomycin Phosphotransferase 2 (1:1,000)	Abcam
Anti-Parkin (1:1,000)	Cell Signaling
Anti-β-tubulin (1:50,000)	Sigma-Aldrich
Anti-Ubiquitin (1:1,000)	BostonBiochem
Anti-V5 tag (1:2,000)	Invitrogen
Anti-VDAC1 (1:10,000)	Abcam
Anti-mouse, horseradish peroxidase (HRP) -conjugated (1:10,000)	SantaCruz
Anti-rabbit, horseradish peroxidase (HRP) -conjugated (1:10,000)	SantaCruz

2.3.5 Oligonucleotides

Oligonucleotides (primers)	MWG Biotech

2.3.6 Equipment

Axiovert 200 M Inverted Microscope	Carl Zeiss
Centrifuge	Eppendorf, Neolab, Sorvall
Countess® Automated Cell Counter	Invitrogen
Electroporator 2510	Eppendorf
Homogenizer	Eurostar IKA Werke
Nucleofector Device	Lonza
Synergy HT Plate Reader	BioTek
Thermocycler - LightCycler	RocheDiagnostics
XCell SureLock Electrophoresis System	Invitrogen

XCell SureLock Blot Module	Invitrogen
ZOOM® Dual Power Supply	Invitrogen

2.3.7 Software

AxioVision	Carl Zeiss
Gen5 Data Analysis Software	BioTek
Image Processing Program	TotalLab

2.4 Methods

This section describes methods and techniques used to generate the experimental data. Routinely used techniques were outlined only in brief.

2.4.1 Real-time PCR: mRNA expression studies

For gene expression studies total RNA was isolated from fibroblast cell cultures using the RNeasy Mini Kit according to the manufacturer's protocol.

Whole RNA was then converted to complementary DNA (cDNA) by reverse transcription using the SuperScript First-Strand Synthesis system. Oligo(dTs) were used as a primers according to the manufacturer's protocol. This cDNA was later used for gene expression studies and cloning.

On the basis of the generated cDNA, quantitative real-time polymerase chain reaction (PCR) (qPCR) was performed. qPCR amplification mixtures (10µl) contained 2µl cDNA, 4µl LightCycler FastStart DNA Master SYBR Green I and 200nM forward and reverse primer. Reactions were performed in duplicate and run on a Roche LightCycler System.

To compare *PINK1* or *Parkin* mRNA levels between different samples, mRNA expression levels of the housekeeping gene *YWHAZ* (*tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide*) served as a reference.

Table 1. *List of primers used in the PINK1 and Parkin mRNA expression studies.*

Gene	Exon	Sequence
PINK1	Ex5 F	5'-ATC GCG CAC AGA GAC CTG A-3'
	Ex6 R	5'-AGC AGC CAA AAT CTG CGA TC-3'
Parkin	Ex4 F	5'-GGT AGA TCA ATC TAC AAC AGC TTT TAT G-3'
	Ex5 R	5'-TGC ACT AGT CCC AGG GCA-3'
YWHAZ	Ex3 F	5'-GTA GGT CAT CTT GGA GGG TCG T-3'
	Ex5 R	5'-GGT ATG CTT GTT GTG ACT GAT CG-3'

F-forward primer; R-reverse primer

2.4.2 Mammalian expression vectors containing *PINK1* and *Parkin*

To generate vectors expressing Parkin or PINK1 in fibroblasts and cell lines the following was performed:

1. Design of the open reading frame-cDNAs of *Parkin* and *PINK1*
2. Ligation of cDNAs in expression vectors
3. Amplification in bacteria and purification of plasmid DNA

2.4.2.1 Design of an open reading frame-cDNAs of Parkin

To design a 1,395-bp long open reading frame cDNA (ORF-cDNA) of *Parkin,* a PCR technique was used. Total RNA from healthy control was extracted and converted to cDNA as described in 2.4.1. The forward primer was specific for the region shortly before the start codon and the reverse primer for the region shortly after the stop codon. Whole *Parkin* ORF-cDNA was synthesized as a single PCR product. Each primer contains a restriction site suitable for subsequent cloning of the resulting DNA fragment in an expression vector.

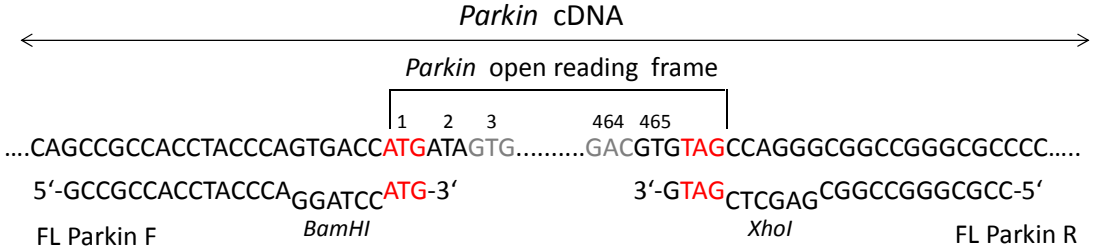

Figure 4. *Generating full-length Parkin ORF-cDNA*. Full-length *Parkin* ORF-cDNA was designed using specifically designed primers. *BamHI* and *XhoI* restriction sites were introduced and used for subsequent cloning of the resulting cDNA into an expression vector.

2.4.2.2 Design of full-length and truncated PINK1 cDNAs

Design of a 1,743-bp long ORF-cDNA of *PINK1* was performed as described in 2.4.2.1.

To generate N-terminally truncated forms of PINK1 (PINK1d93 and PINK1d110), specifically designed primers were used. Each primer introduces a start codon and a restriction site suitable for subsequent cloning of the resulting DNA fragment into an expression vector.

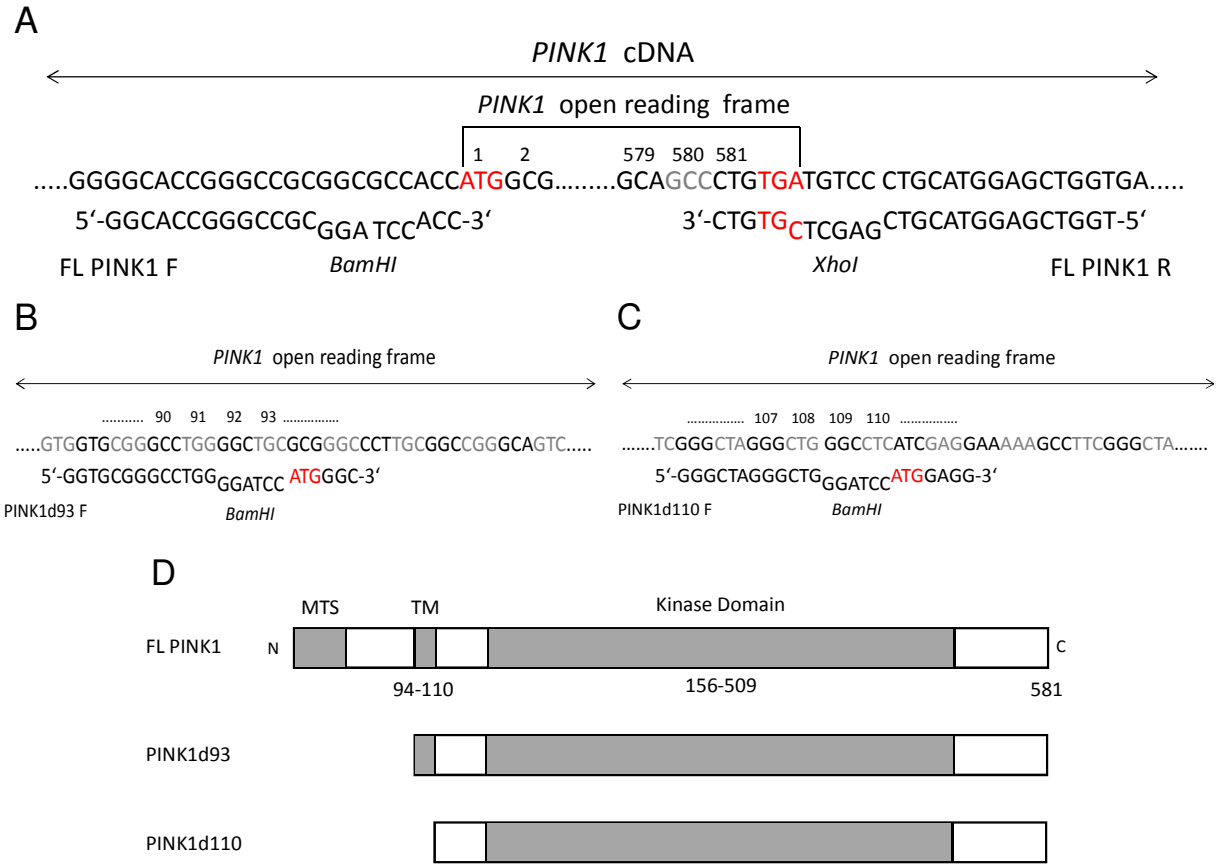

Figure 5. *Full-length- and truncated forms of PINK1.* (A) Full-length- and N-terminally truncated forms of *PINK1* ORF-cDNAs lacking (B) 93 or (C) 110 amino acids were generated by PCR using specifically designed primers. Restriction sites *BamHI* and *XhoI* were introducd and used for subsequent cloning of the resulting cDNAs into an expression vector. (D) Schematic representation of the resulting full-length (FL PINK1) and truncated forms (PINK1d93 and PINK1d110) of the PINK1 protein.

Table 2. *List of primers used to generate full-length* PINK1 and Parkin and truncated forms of PINK1.

cDNA	Primer name	Sequence
Full-length Parkin ORF-cDNA	FL Parkin F	5'-GCC GCC ACC TAC CCA GGA TCC ATG-3'
	FL Parkin R	5'-GGC GCC CGG CCG CTC GAG CTA C-3'
Full-length PINK1 ORF-cDNA	FL PINK1 F	5'-GGC ACC GGG CCG CGG ATC CAC C-3'
	FL PINK1 R	5'-ACC AGC TCC ATG CAG CTC GAG CAC AG-3'
Truncated PINK1 cDNAs	PINK1d93 F	5'-GGT GCG GGC CTG GGG ATC CAT GGG C-3'
	PINK1d110 F	5'-GGG CTA GGG CTG GGA TCC ATG GAG G-3'

ORF-open reading frame; FL-full-length; F-forward primer; R-reverse primer

2.4.2.3 Ligation of cDNAs in expression vectors

For Parkin transgenic expression we used a modified pcDNA3 mammalian expressing vector. This vector contains a FLAG tag upstream of a cloning site resulting in expression of N-terminally FLAG-tagged protein of interest.

For transgenic expression of full-length and N-terminally truncated forms of PINK1 we used a pcDNA3.1/V5-His mammalian expressing vector. This vector contains V5 and 5xHis tags downstream of a cloning site resulting in expression of the C-terminally V5-His tagged protein of interest.

To open both vectors and to generate "sticky ends" on the designed cDNAs, restriction endonucleases *BamHI* and *XhoI* were used according to the manufacturer's protocol.

Ligation of vectors and "inserts" was performed using T4 DNA ligase according to the manufacturer's protocol.

To generate a vector expressing PINK1 with the SBP and CBP affinity tags adjacent to the 3´ end of the gene, previously designed full-length *PINK1* cDNA (see section 2.4.2.2) was used. The *PINK1* insert was ligated into the pCTAP vector provided with the InterPlay® Mammalian TAP kit according to the manufacturer's protocol.

2.4.2.4 Amplification in bacteria and purification of the plasmid DNA

In order to obtain enough of the purified vector for the purpose of transgenic expression of Parkin and PINK1 in mammalian cells, One Shot® TOP10 Electrocomp™ *E. coli* were used. Bacteria were transformed with plasmid DNA using an Electroporator according to the manufacturer's protocol. After transformation bacteria were selected on LB-agar plates

containing ampicillin as a selective antibiotic. Positive colonies were grown in LB medium with ampicillin for 16h and plasmid DNA was extracted from bacteria using the EndoFree Plasmid Maxi kit.

2.4.3 Tissue culture

2.4.3.1 Cell cultures

Fibroblasts and HEK cells were grown in T-175 flasks and cultured in Dulbecco's Modified Eagle Medium (DMEM) plus 10% FBS (vol/vol), 4.5g/l glucose and 10ml/l penicillin/streptomycin in an incubator (95% air/5% CO_2) at 37 ℃ (media changed every 3-7 days).

2.4.3.2 Passage of cells

Cells were passaged when 80% confluent. Growth media was removed, the cells washed with 1xPBS, and incubated at 37°C with 2ml of accutase for 5min. Cells were then collected and pelleted by centrifugation at 1000rpm for 5min. Fibroblasts were resuspended in culturing medium composed as above. All experiments were performed with cells not older then passage number 10.

2.4.3.3 Treatment of cells

To induce a loss of mitochondrial membrane potential, 1µM valinomycin was used. This potassium ionophore induces loss of mitochondrial membrane potential by releasing K^+ ions through membranes down the electrochemical gradient.

To study the effect of oxidative stress, cell cultures were treated with the superoxide generator hydrogen peroxide (H_2O_2, 70-100µM).

For inhibition of the ubiquitin proteasome system, 10µM MG132 and 10 µM Epoxomicin were used.

2.4.3.4 Transfection of fibroblasts

To introduce expression vectors or a small interfering RNA (siRNA) into the fibroblasts, an electroporation method - Nucleofector™ Technology - was used. In brief, cells were

harvested, counted, and 10^6 cells were used for each transfection. Cells were then diluted in 100µl of the Nucleofactor solution and mixed with 5µg of the vector DNA or with 4µl of the 100µM siRNA. Transfection was performed in electroporation cuvettes according to manufacturer's protocol.

2.4.3.5 Transfection of cell lines

To introduce expression vectors into HEK cells, the $CaPO_4$ method was employed.
Cells were plated in a 150mm dish on the day before the transfection. To prepare the transfection mix, two tubes were needed. In a tube labeled with "A", 62µl of $CaCl_2$ and 30ug of DNA were mixed, and sterile dH_2O was added up to a volume of 500µl. In a tube labeled with "B", 500µl of 2xHBS was added. While vortexing tube B, the contents of tube A was added dropwise, followed by incubation at room temperature for 45min. The transfection mix was added to the cells dropwise.

2.4.4 Mitochondrial preparation

Mitochondria were isolated from fibroblasts as previously described previously (Almeida *et al.*, 1997). Cells were removed from the flasks by incubating the cells with accutase for 5min. The cells were pelleted by centrifugation at 1000rpm for 5min. (The pellet can be frozen at -80°C at this point.) To proceed, cells were resuspended in 1ml ice-cold homogenization buffer. Cells were then centrifuged at 4000 x g for 5min and the supernatant was discarded. The pellet was again resuspended in 4ml ice-cold homogenisation buffer. Cells were optimally homogenized on ice by 20 strokes of a tight fitting glass-teflon homogenizer revolving at 1000rpm. Cell homogenates were centrifuged at 1500 x g for 10min at 4 ºC, the supernatant transferred in a new tube and centrifuged once more at 1500 x g (10min, 4ºC). The pellet was discarded, and the supernatant centrifuged at 11,500 x g for 12min at 4ºC. The obtained mitochondria pellet was resuspended in 50µl of RIPA buffer or frozen at –80ºC until required.

2.4.5 Measurement of protein concentration

Sample protein concentration was determined by use of the Dc protein assay kit according to the manufacturer's protocol. Absorbance was measured on a plate reader at 750nm. Sample protein concentration was calculated from the BSA standard calibration curve (0-3000µg/ml).

2.4.6 Mitochondrial membrane potential

To analyse the mitochondrial membrane potential, the sensitive fluorescent probe JC-1 was used. This lipophilic cation changes its emitted light reversibly from 530nm to 590nm, as the mitochondrial membrane potential increases. For this, $5x10^5$cells/well were plated in a 12-well plate 24h before the experiment. Cells were treated with 1µg/ml JC-1 for 15min at 37°C. As a blank, one well per sample remained untreated. A plate reader device was used to measure fluorescence at 590nm. The mitochondrial membrane potential was expressed relative to the protein concentration per well and subtracted for the blank.

2.4.7 Western blotting

The Western blotting procedure involved sample preparation, SDS polyacrylamide gel electrophoresis (SDS-PAGE), protein transfer to a membrane and antibody detection of target proteins on the resulting blot.

2.4.7.1 Sample preparation

To extract proteins, cellular or mitochondrial pellets were resuspended in the appropriate amount of RIPA buffer. After incubation on ice for 30 min, the samples were centrifuged at 16,000xg for 20 min. The protein concentration per sample was determined according to the protocol given in 1.3.5. Before loading, 10µg of each protein sample were mixed with NuPAGE LDS loading buffer in a ratio of 4:1 (vol/vol) and boiled for 5min at 95°C.

2.4.7.2 SDS-PAGE

For protein separation, NuPAGE Novex 4-12% Bis-Tris mini gels were used.
Sample mixtures were loaded on the bottom of each well. A molecular weight standard (Precision plus protein all blue) was run on the same gel. The electrophoresis was performed in 1x MOPS SDS running buffer for about 90 min at 150V.

2.4.7.3 Protein transfer

After electrophoresis proteins were transferred from a gel to a nitrocellulose membrane using a XCell SureLock Blot Module for “wet” transfer. Sponges and filter papers were prewet in 1x NuPAGE transfer buffer before assembling the transfer unit.

The Western blot "sandwich" was built according to manufacturer's protocol. The transfer was run at 32V for 1h in 1x NuPAGE transfer buffer supplemented with 10% methanol.

2.4.7.4 Antibody staining of Western blots

The membrane was blocked in 1% Milk/TBST for 1h. The primary antibody was added into 1% Milk/TBST at an appropriate concentration (section 1.3.4) and incubated with the membrane over night at 4°C. This was followed by three washing steps in 1xTBST for 5min each. The blot was then incubated for 1h at room temperature with a HRP-conjugated secondary antibody in 1% Milk/TBST (dilution 1:10,000). This was followed by another three washing steps. The bands were detected by enhanced chemiluminescence.

2.4.8 Immunocytochemistry

To determine the subcellular localization of the protein of interest, the Zenon immunolabeling method was used. This technique is similar to "classical" immunostaining but instead of the whole secondary antibody, it uses only its Fc-specific anti-IgG Fab fragment. Fibroblasts were seeded onto glass coverslips (0,7cm^2) placed in 12-well plates. The cells were then incubated for approximately 24h. Cells were washed three times with 1ml of 1xPBS. Fibroblasts were fixed by adding 1ml pre-chilled 4% PFA per well and incubated at room temperature for 15min. PFA was then removed, cells washed briefly with 1ml 1xPBS and permeabilized with 0.1% NP-40 for 10min. After that the cells were incubated with 1% BSA for 1h to prevent non-specific binding of antibodies. In the meantime, complexes of the primary antibody and the appropriate Zenon immunolabelling reagent were generated according to manufacturer's protocol. Cells were incubated with complexes over night at 4^{0}C. Finally, the coverslips were mounted on glass microscope slides (76 x 26mm).

2.4.9 Immunoprecipitation

In order to confirm its ubiquitination, Mfn2 was precipitated out of the mitochondrial fraction using an anti-Mfn2 antibody. For that, the mitochondrial pellets of one T-175 flask were resuspended in 500µl of lysis buffer. After 30min of incubation, the homogenates were centrifuged at 13,000xg for 10min. Supernatants were transferred into a new tube and incubated with 3µg an anti-Mfn2 antibody overnight on a rotary shaker. On the next day, 50µl of washed Protein G-agarose beads were added to each sample and incubated for 2h on a

rotary shaker. Beads were pelleted by centrifugation at 10,000xg for 1min followed by incubation in 500µl of washing buffer on a rotary shaker for 5min. Pelleting and washing of the beads were repeated 3 times.

After the last centrifugation, the washing buffer was discarded and the beads were dissolved in 2x LDS sample buffer followed by boiling for 5min. Beads were pelleted at 10,000xg for 1min. Supernatants containing precipitated Mfn2 were resolved using SDS-PAGE.

2.4.10 Tandem affinity purification

The tandem affinity purification (TAP) method, followed by mass spectrometry identification of unknown protein complex components, was used to identify novel interacting partners of PINK1. Performing two consecutive purification steps using affinity purification tags that have gentle washing and elution conditions allows for isolation without disruption of the targeted complex. For this, 10^8 HEK cells were transiently transfected with TAP-tagged PINK1 using the $CaPO_4$ method. Twenty-four hours after the transfection, cells were harvested and proteins were extracted using a mild lysis buffer provided together with the kit. In brief, cells were dissolved in the lysis buffer and homogenized in a motor-driven glass-teflon homogenizer at 900 rpm (20 strokes). After 20 min of incubation on ice, lysates were "cleared" by centrifugation at 16,000xg for 15 min. Purification of protein complexes was performed according to the manufacturer's protocol. After that, proteins were resolved by SDS-PAGE and protein bands were detected using a mass spectroscopy compatible SilverQuest™ Silver Staining kit according to the manufacturer's protocol. Bands were cut out and sent for mass spectrometry analysis to the Taplin Mass Spectrometry Facility (Harvard Medical School; Boston, MA).

3 RESULTS

The results part of this thesis consists of three parts. The first part is related to the molecular interaction between PINK1 and Parkin. In this section the effect of mutations in PINK1 on the subcellular localization and protein levels of Parkin is presented (3.1). The second part describes the Parkin-mediated, PINK1-dependent ubiquitination of mitochondrial proteins, i.e. Mfn1 and Mfn2 (3.2). The third part deals with the identification of interaction partners of the PINK1 protein (3.3).

3.1 Effect of mutant and wild type PINK1 on subcellular localization and protein levels of Parkin

Four primary human dermal fibroblast cultures carrying two different PD-causing homozygous *PINK1* mutations, c.1366C>T (p.Q456X; nonsense mutation; n=3) and c.509T>G (p.V170G; missense mutation; n=1) and fibroblast cultures from four healthy controls without *PINK1* mutations were analyzed under basal and stress conditions. To test specificity of an anti-Parkin antibody, fibroblasts carrying different homozygous and heterozygous *Parkin* mutations, c.1072delT (p.V324fsX434; n=1) or delEx7 (p.R245fsX253; n=1) were used.

For experiments under stress conditions, valinomycin was used as mitochondrial stressor and hydrogen peroxide (H_2O_2) was used as a general stressor.

3.1.1 Validation of the antibodies and siRNAs used in the present study

To validate the specificity of an anti-PINK1 antibody and the knockdown efficiency of two different *PINK1* siRNAs, wild-type PINK1 (PINK1-V5) was overexpressed in the presence of scrambled siRNA or *PINK1* siRNA in fibroblasts from PINK1 nonsense mutation carriers. Western blotting showed that the anti-PINK1 antibody specifically recognizes the full-length and the cleaved form of PINK1. Furthermore, it was confirmed that both *PINK1* siRNAs efficiently knockdown expression of *PINK1* (Fig. 6A).

In order to validate the sensitivity and specificity of the anti-Parkin antibody that is directed against the C-terminus of the Parkin protein, fibroblasts from controls, from homozygous and heterozygous Parkin mutation carriers were analyzed using a Western blotting technique. These mutations (del exon 7 and c.1072delT) generate a truncated protein, which cannot be detected by antibody used in the present study. Parkin signal was found only in controls and

heterozygous Parkin mutants but not in homozygous Parkin mutants, confirming specificity of the antibody (Fig. 6B).

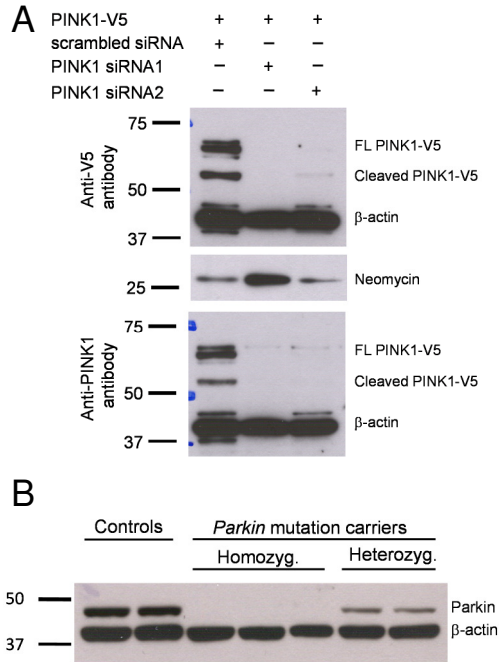

Figure 6. *Confirmation of antibody specificities and validation of knock-down efficiency of PINK1 siRNAs.* (**A**) Whole cell lysate of fibroblasts of a homozygous PINK1 nonsense mutation carrier transfected with PINK1-V5 in conjunction with either scrambled siRNA or two different PINK1 siRNAs. A Western blot probed with an antibody against the V5 tag (upper panel) showed bands of the same size as a blot probed with an antibody against PINK1 (lower panel), confirming specificity of the PINK1 antibody. Protein levels of overexpressed full-length PINK1 (FL PINK1) and cleaved ~55-kD PINK1 are abolished (siRNA1) or decreased (siRNA2) specifically by both *PINK1* siRNAs. Transfection efficiency was determined using an antibody against the Neomycin cassette (middle panel). (**B**) Western blot using whole cell lysates from fibroblasts from controls and individuals carrying homozygous or heterozygous Parkin mutations. Protein levels of Parkin were estimated using an antibody able to recognize only Parkin with an intact C-terminus of the protein. In fibroblasts from heterozygous mutation carriers, protein levels were approximately 50% of those detected in controls, and no Parkin protein was detectable in fibroblasts of homozygous Parkin mutation carriers lacking the C-terminal part of Parkin.

3.1.2 Effect of wild type and mutant PINK1 on protein levels of Parkin

To test whether PINK1 regulates Parkin levels, proteins were extracted from both PINK1 mutants and controls under basal conditions and upon valinomycin-induced stress. Under basal conditions, protein levels of Parkin were comparable between the group of mutants and controls as shown by Western blotting (Fig. 7A, upper panel). In contrast, under stress conditions using valinomycin, a loss of Parkin signal was detected in cells from healthy individuals but not in those from PINK1 mutation carriers (Fig. 7A, lower panel).

Furthermore, H_2O_2 (Fig. 7B) had the same effect on detectable Parkin levels as observed under valinomycin-induced stress.

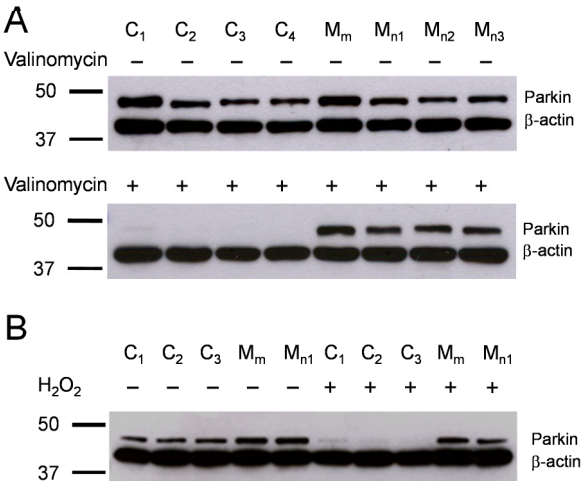

Figure 7. *Treatment with valinomycin or hydrogen peroxide promotes loss of detectable endogenous Parkin in fibroblasts from controls but not from PINK1 mutation carriers.* (**A**) Western blot analysis of whole-cell lysate from non-treated cells (upper panel) and from cells treated with valinomycin (lower panel) from four controls and four mutants. (**B**) H_2O_2 induces a decrease in the detectable levels of endogenous Parkin in cells from healthy controls but not in cells from PINK1 mutation carriers. In non-treated fibroblasts, the level of endogenous Parkin is comparable between controls and mutants. C- healthy control; M_m- *PINK1* missense mutation carrier (c.509T>G; p.V170G); M_n- *PINK1* nonsense mutation carriers (c.1366C>T; p.Q456X)

To test whether these findings on the protein level were due to changes in mRNA expression, mRNA levels of *PINK1* and *Parkin* were measured before and after valinomycin treatment in mutants and controls by real-time PCR using the house-keeping gene *YWHAZ* for normalization. As mentioned above, the *PINK1* nonsense mutation leads to a premature stop codon exerting a major effect on the *PINK1* mRNA level (80-90% reduction compared to the *PINK1* mRNA levels in controls; Fig. 8A) most likely via nonsense-mediated mRNA decay (NMD) (Grunewald *et al.*, 2007).

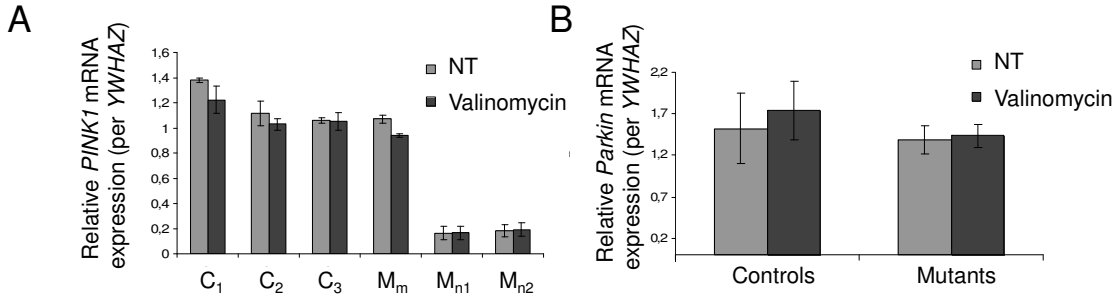

Figure 8. *Relative PINK1 and Parkin mRNA expression is not altered by valinomycin treatment in both controls and PINK1 mutants.* Fibrobasts from PINK1 mutation carriers and from controls were treated with valinomycin for 12 h. Total RNA was then extracted from stressed and non-stressed cells and quantified by real-time PCR. (A) Relative *PINK1* mRNA expression levels remain unchanged upon valinomycin treatment. Expression levels of the nonsense mutation carriers are reduced to 10-20% compared to those of controls and the missense mutation carrier due to nonsense-mediated mRNA decay in the former. (B) Relative *Parkin* mRNA expression is not significantly different between controls and mutants before or after treatment. Values represent means ± SD. C- healthy control; M_m- *PINK1* missense mutation carrier (c.509T>G; p.V170G); M_n- *PINK1* nonsense mutation carriers (c.1366C>T; p.Q456X)

In contrast, *PINK1* expression levels were comparable between the *PINK1* missense mutant and healthy controls (Fig. 8A). Importantly, no differences in *Parkin* mRNA levels were detected between PINK1 mutants and controls before or after valinomycin-induced stress (Fig. 8B), thereby excluding a possible effect of gene expression on the observed differences in protein levels.

The observed loss of Parkin signal in controls after mitochondrial stress indicates that wild-type PINK1 is involved in this process. In order to confirm this hypothesis, experimental knock-down of PINK1 in controls and a rescue experiment in PINK1 mutants were performed (Fig. 9). First, the effect of PINK1 knock-down on levels of endogenous Parkin was tested in control cells (Fig. 9B). Upon valinomycin treatment, controls transfected with *PINK1* siRNA had higher Parkin levels than controls transfected with scrambled siRNA. The same effect was observed even under basal conditions, suggesting that PINK1 is indeed involved in the reduction of detectable Parkin levels. In mutant cells, wild-type PINK1 was expressed using a mammalian expression vector containing PINK1-V5. Under basal conditions, Parkin levels were comparable between mutants transfected with PINK1 or with empty vector. As expected, valinomycin treatment induced a decrease of Parkin signal in mutants transfected with a vector containing PINK1-V5, but not in those transfected with an empty vector. Of note, an increase in the intensity of the full-length form of transgenically expressed and of endogenous PINK1 were detected, respectively (Fig. 9A).

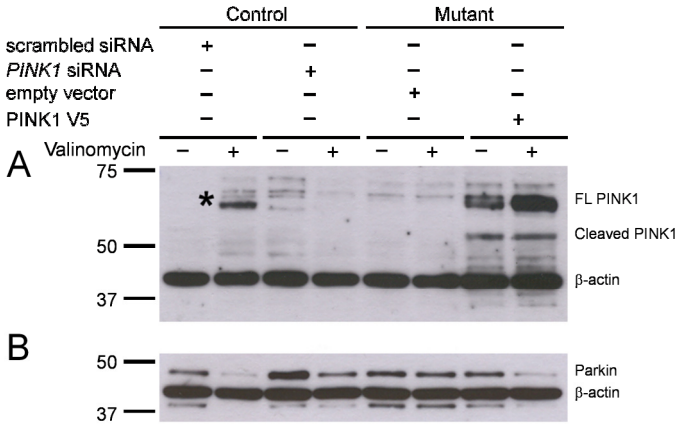

Figure 9. *PINK1 is necessary for valinomycin-induced reduction of endogenous Parkin.* Control cells transfected with *PINK1* siRNA and nonsense mutant cells transfected with a vector expressing wild-type PINK1 tagged with V5 at the C-terminus (PINK1-V5) were stressed with valinomycin for 12 h. After valinomycin treatment, proteins were extracted and analyzed by Western blotting using antibodies against (**A**) PINK1 and (**B**) Parkin. β-actin was used as loading control. In control cells, knock-down of *PINK1* prevented the reduction of Parkin signal compared to controls transfected with scrambled siRNA both under basal conditions and under valinomycin treatment. In mutant cells, expression of PINK1 promotes loss of Parkin signal upon valinomycin-induced stress. FL PINK1- full-length form of PINK1; *- endogenous full-length form of PINK1

3.1.3 Effect of wild type and mutant PINK1 on subcellular localization of Parkin

Subcellular localization of Parkin was determined using immunocytochemistry. For this, N-terminally tagged wild-type Parkin (FLAG-Parkin) was overexpressed in human dermal fibroblasts. Subcellular localization of FLAG-Parkin in control cells and in PINK1 nonsense mutant cells was determined using an anti-FLAG antibody. Mitochondria were stained using an antibody against mitochondrial protein GRP 75 (Fig. 10A and B). Under basal conditions, Parkin was localized in the cytosol in both controls and PINK1 mutants and mitochondria were organized in an intact network (Fig. 10, left lane). Treatment with valinomycin (Fig. 10, middle lane) or H_2O_2 (Fig. 10, right lane) induced loss of the mitochondrial network and its fragmentation into multiple, smaller organelles in both controls and mutants. In addition, both stressors resulted in mitochondrial translocation of Parkin in controls (Fig. 10A) but not in PINK1 mutants (Fig. 10B), suggesting that PINK1 is necessary for the stress-induced mitochondrial translocation of Parkin.

3.1.4 Inhibition of the UPS prevents stress-induced loss of Parkin

Parkin can promote self-ubiquitination and degradation through the UPS (Imai *et al.,* 2000; Shimura *et al.,* 2000; Zhang *et al.,* 2000). To explore whether the UPS is involved in the PINK1-mediated stress-induced loss of Parkin two inhibitors of the UPS were used: MG132 and Epoxomicin. First, the effect of MG132 on Parkin levels was followed over time in cells treated with valinomycin (Fig. 11). When treated with valinomycin only, a time-dependent decrease in Parkin signal intensity was observed only in control cells. In mutant cells, Parkin levels were not affected by this treatment (Fig. 11A). By contrast, in an experiment combining valinomycin and MG132 treatment, no change in intensity of Parkin signal was observed in either controls or mutants (Fig. 11B). Since MG132 has a more general function as protease inhibitor, i.e. MG132 is also able to inhibit the activity of mitochondrial proteases (Granot *et al.,* 2007), epoxomicin, a more selective inhibitor of the UPS (Meng *et al.,* 1999) was used. For this, control cells were treated with epoxomicin in the presence of valinomycin for 12 h followed by Western blotting (Fig. 11C). Valinomycin-induced degradation of Parkin was prevented by epoxomycin, as it was by MG132, suggesting that the UPS is indeed involved in the stress-induced reduction of Parkin levels.

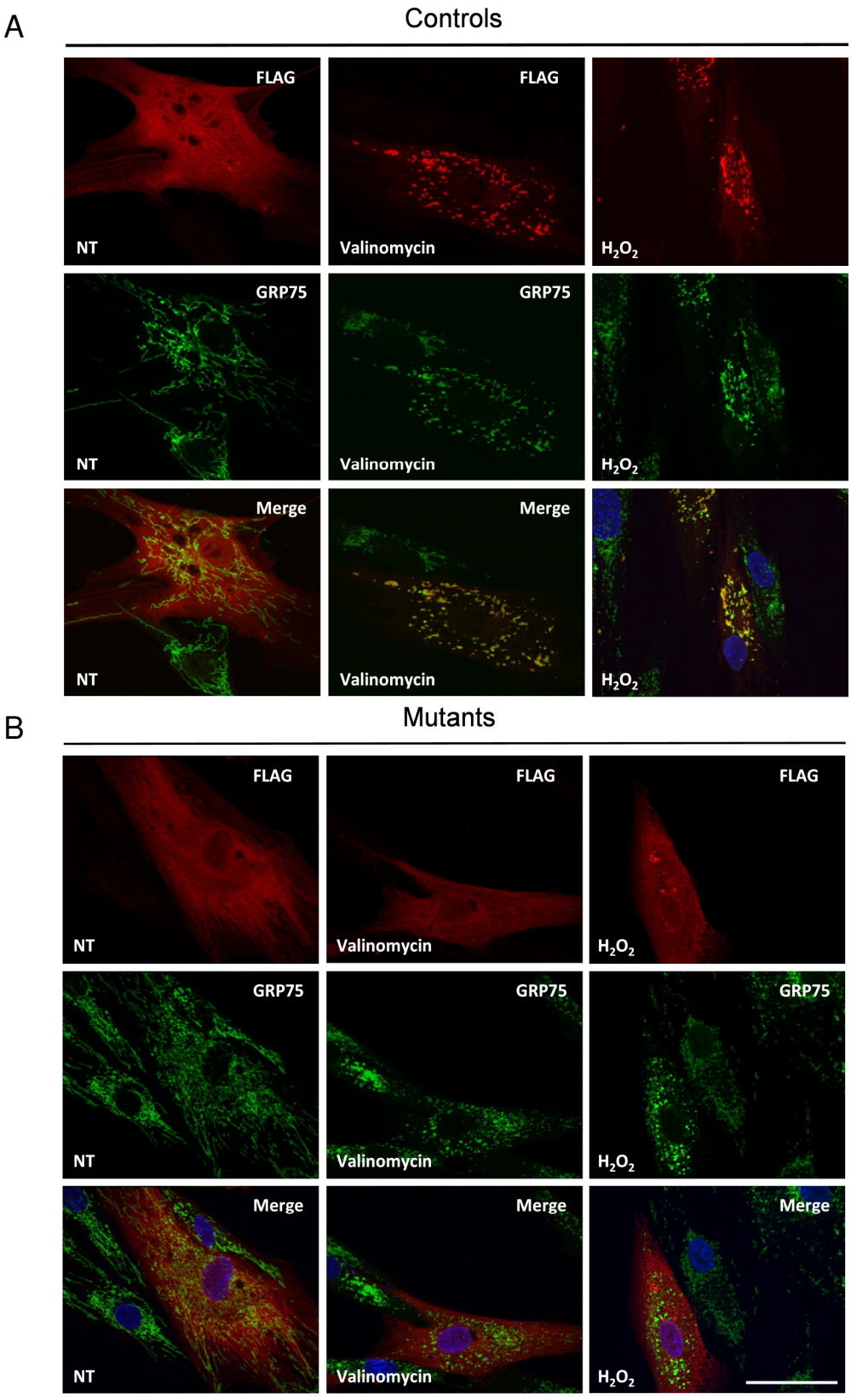

Figure 10. *Valinomycin- and hydrogen peroxide (H_2O_2)-induced stress result in mitochondrial localization of Parkin in cells from healthy controls but not in cells from PINK1 nonsense mutation carriers.* Fibroblasts transfected with FLAG-Parkin were either untreated (left lane), treated with valinomycin (middle lane) or with H_2O_2 (right lane) for 12 h. After treatment cells were fixed and immunostained with antibodies against the FLAG-tag (red) and a mitochondrial marker GRP75 (green). Upon stress, FLAG-Parkin colocalizes with mitochondria in fibroblasts originating from (**A**) controls but not in fibroblasts from (**B**) PINK1 mutation carriers. Scale bar, 50µm, NT- non-treated

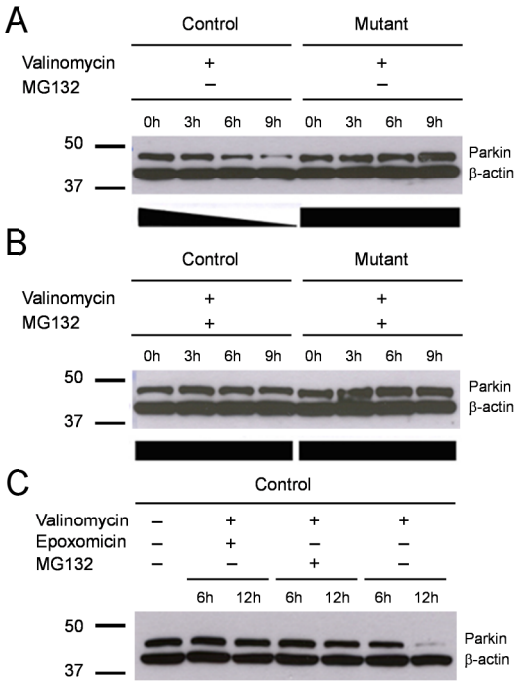

Figure 11. *Inhibitors of the UPS protect valinomycin-induced degradation of Parkin*. Fibroblasts from a healthy control and from a PINK1 nonsense mutation carrier were treated with (**A**) valinomycin or (**B**) with valinomycin plus MG132. (**C**) In addition, control cells were treated with valinomycin together with epoxomicin or with MG132 for 12 h. Proteins were extracted at different time points and analyzed by Western blotting. Only in the control cells but not in mutants, there was valinomycin-induced decrease in Parkin signal (Fig. 11A). This reduction could be prevented by MG132 treatment (Fig. 11B). The same effect was observed when using epoxomicin, a potent and selective inhibitor of the ubiquitin proteasome system (Fig. 11C).

In light of the immunocytochemistry findings showing stress-induced mitochondrial translocation of overexpressed Parkin, it now became important to clarify in which cellular compartment (mitochondrial or cytosolic) endogenous Parkin accumulates after treatment with MG132. For this, both controls and mutants were treated with MG132 alone, valinomycin alone or MG132 plus valinomycin in combination. After 12 h of treatment cells were harvested and extracted mitochondrial and cytosolic fractions by differential centrifugation. Purity of both fractions was verified by Western blotting using antibodies against β-tubulin and β-actin and antibodies against heat shock 60kDa protein (Hsp60) and voltage-dependent anion channel 1 (VDAC1) (Fig. 12). As expected, in control cells treated with valinomycin alone, the presence of Parkin was detected in both the mitochondrial and the cytosolic fraction. Compared to the previous experiments, Parkin signal was detected even after 12 h of valinomycin treatment. This can be explained by the fact that, although the same amount of proteins was loaded as in Fig. 7A, in this case, proteins from concentrated cytosolic and mitochondrial fractions were used. However, when combining valinomycin with MG132 treatment, the reduction in Parkin signal could be prevented not only in the cytosolic but also in the mitochondrial fraction (Fig. 12A). In mutant cells, Parkin was localized exclusively in the cytosolic fraction, independent of the treatment conditions (Fig. 12B). Treatment with MG132 alone had no effect on protein levels or subcellular localization of Parkin in either controls or mutants when compared to non-treated cells (Fig. 12C and D). These data collectively demonstrate that PINK1 is involved both in the stress-induced decrease and in the mitochondrial translocation of Parkin.

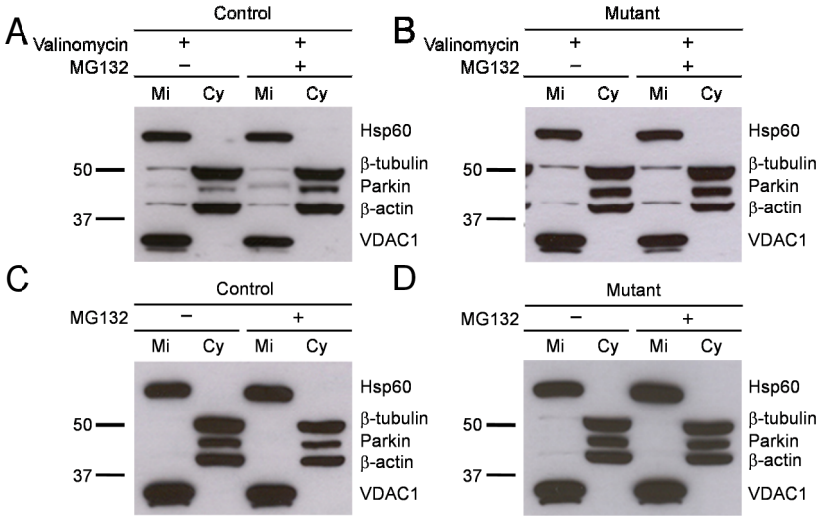

Figure 12. *MG132 prevents valinomycin-induced decrease of Parkin in the cytosolic fraction and promotes accumulation of Parkin in the mitochondrial fraction in cells from healthy controls.* Fibroblasts from controls and from PINK1 nonsense mutation carriers (Mutant) were treated with valinomycin alone, with valinomycin plus MG132 or with MG132 alone for 12 h. Non-treated cells were also analyzed. The cells were harvested to prepare mitochondrial (Mi) and cytosolic (Cy) fractions and analyzed by Western blotting. (A) In control cells, upon valinomycin treatment, the presence of Parkin was detected in mitochondrial and cytosolic fractions. Furthermore, combining valinomycin with MG132, an increase in Parkin levels was observed in both the mitochondrial and the cytosolic fraction compared to control cells treated with valinomycin only. (B) In mutants, neither treatment with valinomycin alone or in combination with MG132 induced mitochondrial translocation or loss of Parkin signal. (C and D) Treatment with MG132 alone had no effect on Parkin levels and subcellular localization compared with non-treated cells. Purity of the cellular fractionation was verified using antibodies against β-tubulin and β-actin and antibodies against Hsp60 and VDAC1. Mi- mitochondrial fraction; Cy- cytosolic fraction

3.1.5 Mitochondrial localization of PINK1 is necessary for valinomycin-induced reduction in detectable Parkin and its mitochondrial translocation

The next step was testing the hypothesis that the stress-induced reduction in Parkin levels and mitochondrial translocation of Parkin are dependent upon the mitochondrial localization of PINK1. To prevent mitochondrial targeting of PINK1, vectors expressing N-terminally truncated PINK1 were designed. The MTS has unambiguously been localized within the N-terminal part of human PINK1, however, its exact length and composition are still under debate (Zhou *et al.,* 2008). While the first 93 amino acids of PINK1 are required to target GFP to mitochondria, recent findings showed that only upon deletion within the first 110 amino acids (Haque *et al.*, 2008; Takatori *et al.*, 2008; Narendra *et al.*, 2010), PINK1 cannot be targeted to mitochondria. Based on these findings, truncated forms of PINK1 lacking its first 93 (PINK1d93) or 110 (PINK1d110) amino acids were designed. To determine their subcellular localization, HEK cells were transiently transfected with vectors containing full-length PINK1 (FL PINK1), PINK1d93 or PINK1d110. The cells were harvested and

mitochondrial and cytosolic fractions were extracted. As shown in Fig. 13A, full-length PINK1 and PINK1d93 were mainly localized in the mitochondrial fraction. PINK1d110, on the other hand, was predominately present in the cytosolic fraction (Fig. 13A). Notably, the presence of the full-length (~66 kDa) form of PINK1 was detected in the cytosolic fraction, which can be explained by overloading of the mitochondrial translocase system (Weihofen *et al.,* 2008). The major cleaved (~55 kDa) form of PINK1 was detected in the mitochondrial but also in the cytosolic fraction (Fig. 13A). There are two possible explanations for this phenomenon: i) after processing, cleaved PINK1 is translocated from mitochondria to the cytosol, as recently proposed (Narendra *et al.*, 2010) ; ii) cleaved PINK1 is generated in the cytosol by a process that is alternative to mitochondrial processing (Takatori *et al.,* 2008; Weihofen *et al.,* 2008). In accordance with previous publications (Weihofen *et al.,* 2008; Zhou *et al.,* 2008), our data suggest that processing of full-length PINK1 into its cleaved ~55 kDa form occurs around residue 110 (Fig. 13A). These findings confirm that the N-terminal end of PINK1 contains the MTS but is not restricted to the first 93 amino acids but more likely to the first 110 amino acids. Importantly, removal of the first 110 amino acids should not affect the kinase activity of PINK1, as shown by several studies, where amino acids 112-581 alone were enough to perform autophosphorylation (Beilina *et al.,* 2005; Silvestri *et al.,* 2005), as well as phosphorylation of several other proteins (Sim *et al.,* 2006) *in vitro.*

Next, mutant cells were transfected with vectors containing FL PINK1, PINK1d93 or PINK1d110. In mutant cells transfected with full-length PINK1 or with PINK1d93, a valinomycin-induced decrease in Parkin signal was detected by Western blotting. In contrast, the cytosolicly localized form of PINK1 (PINK1d110) failed to induce a decrease in signal of endogenous Parkin upon valinomycin treatment (Fig. 13B).

Finally, the effect of these PINK1 forms on the subcellular localization of Parkin was explored. For this, cells from a PINK1 nonsense mutation carrier were co-transfected with wild-type Parkin (FLAG-Parkin) together with FL PINK1, PINK1d93 or PINK1d110. Co-transfection of FLAG-Parkin with an empty vector served as a negative control. After transfection, the cells were divided into two groups. One group of cells was treated with valinomycin for 12 h and the other one remained non-treated. In non-treated cells, Parkin was diffusely localized in the cytosol. Valinomycin treatment non-selectively induced loss of the mitochondrial network and its fragmentation into multiple, smaller organelles independent of the vector used for transfection. In accordance with the data observed on Western blotting, only the cytosolically localized form of PINK1 (PINK1d110) failed to induce mitochondrial translocation of Parkin upon valinomycin-induced stress (Fig. 13C and D).

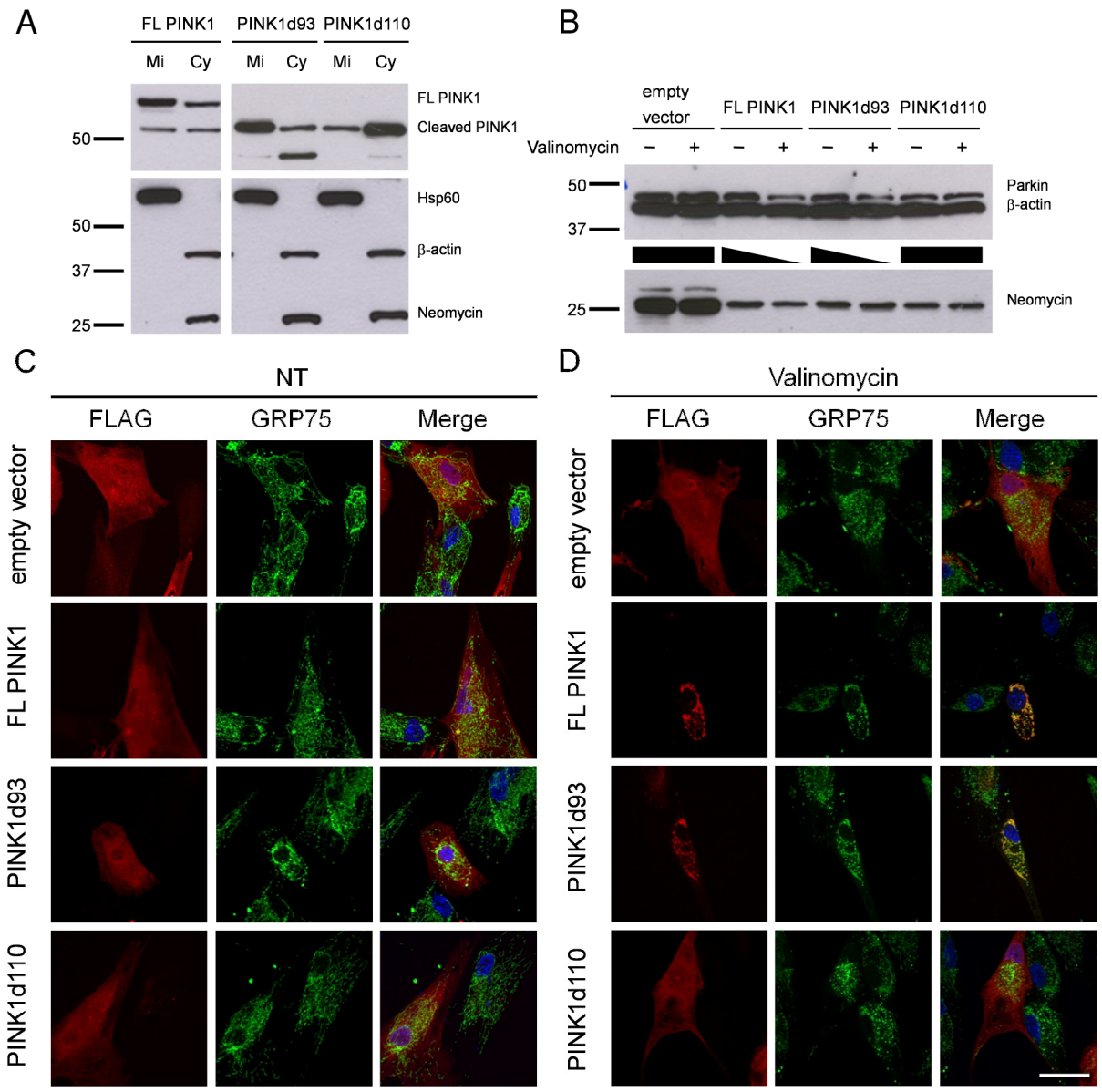

Figure 13. *Mitochondrial localization of PINK1 is necessary for valinomycin-induced decrease in detectable Parkin and its mitochondrial translocation.* (**A**) HEK cells were transfected with vectors expressing FL PINK1, PINK1d93 or PINK1d110. Mitochondrial (Mi) and cytosolic (Cy) fractions were analyzed by Western blotting. While FL PINK1 and PINK1d93 were predominately localized in mitochondria, PINK1d110 was mainly localized in the cytosol. (**B**) Fibroblasts from a PINK1 nonsense mutation carrier were transfected with empty vector and vectors expressing FL PINK1, PINK1d93 or PINK1d110 (upper panel). After transfection, cells were treated with valinomycin for an additional 12 h. Whole cell lysate was analyzed by Western blotting. In cells expressing the mitochondrially localized forms of PINK1 (FL PINK1 and PINK1d93), treatment with valinomycin induced a decrease in Parkin signal (Fig. 13B upper panel). (C and D) Immunostaining of fibroblasts from a PINK1 nonsense mutation carrier co-expressing Parkin (FLAG-tagged) together with empty vector, FL PINK1, PINK1d93 or PINK1d110. (**C**) In non-treated cells, co-transfection of Parkin with any of the PINK1 constructs had no effect on the subcellular localization of Parkin. (**D**) In contrast, in valinomycin-treated cells, expression of FL PINK1 and PINK1d93 induced mitochondrial translocation of Parkin. Only the cytosolically localized form of PINK1 (PINK1d110) failed to induce mitochondrial translocation of Parkin upon valinomycin-induced stress. (Scale bar, 50 μm)**.** FL PINK1- full-length PINK1; PINK1d93- PINK1 construct lacking the first 93 amino acids; PINK1d110- PINK1 construct lacking the first 110 amino acids; NT- non-treated

3.1.6 Relations between mitochondrial accumulation of full-length PINK1, the stress induced loss of endogenous Parkin and the mitochondrial membrane potential.

Recently it was published that Parkin is recruited to depolarized mitochondria (Narendra *et al.*, 2010) . In this study (section 3.1.2) was shown that a reduction in detectable Parkin levels (Fig. 7A and B) and its mitochondrial translocation (Fig. 10) can be induced upon either valinomycin or H_2O_2 treatment. Therefore, both of these stressors were analyzed for their effect on the mitochondrial membrane potential. For this, the mitochondrial membrane potential of fibroblasts from both PINK1 mutation carriers and controls upon H_2O_2 or valinomycin treatment was measured using the same concentrations and within the same time frame like in the remainder of the experiments in this study. While treatment with valinomycin caused an immediate loss of the mitochondrial membrane potential, H_2O_2 did not cause a drop in mitochondrial membrane potential (Fig. 14A). To additionally confirm a differential effect of H_2O_2 and valinomycin on the mitochondrial membrane potential, protein levels and processing of the glucose-regulated protein 75 (GRP75) were analyzed. GRP75 is a mitochondrial matrix chaperone, synthesized as a 679-amino acid pre-protein, which contains a 51-residue N-terminal MTS sequence. After the mitochondrial membrane potential-dependent import into mitochondria, it is cleaved into the mature protein which is ~5.5 kDa shorter than the pre-protein (Mizzen *et al.,* 1989; Dahlseid *et al.,* 1994; Szabadkai *et al.,* 2006). For this, cells from a healthy control, from a PINK1 nonsense and from a PINK1 missense mutation carrier were treated with H_2O_2 and valinomycin followed by subcellular fractionation. Accumulation of the full-length form of GRP75 was detected only upon valinomycin but not upon H_2O_2 treatment. This finding was independent of the type of PINK1 mutation (nonsense vs. missense), confirming our previous findings (Fig. 14A). Additionally, levels of endogenous PINK1 were tested in the same experiment. While both stressors induced the previously shown loss of detectable Parkin in controls (Fig. 14B, left upper panel) but not in mutants (Fig. 14B, left middle and lower panels), accumulation of FL PINK1 was found only in the mitochondrial fraction from controls treated with valinomycin. In contrast, no PINK1 was detected in non-treated controls or in controls treated with H_2O_2 (Fig. 14B, right upper panel). As expected, presence of any PINK1 was not detected in either fraction of the cells of the nonsense mutation carrier (Fig. 14B, right middle panel) due to nonsense-mediated decay. Likewise, in the missense mutation carrier, PINK1 was absent in both the mitochondrial and the cytosolic fraction, although the PINK1 expression level was the same in the missense mutation carrier as in controls (Fig. 14B, right lower panel).

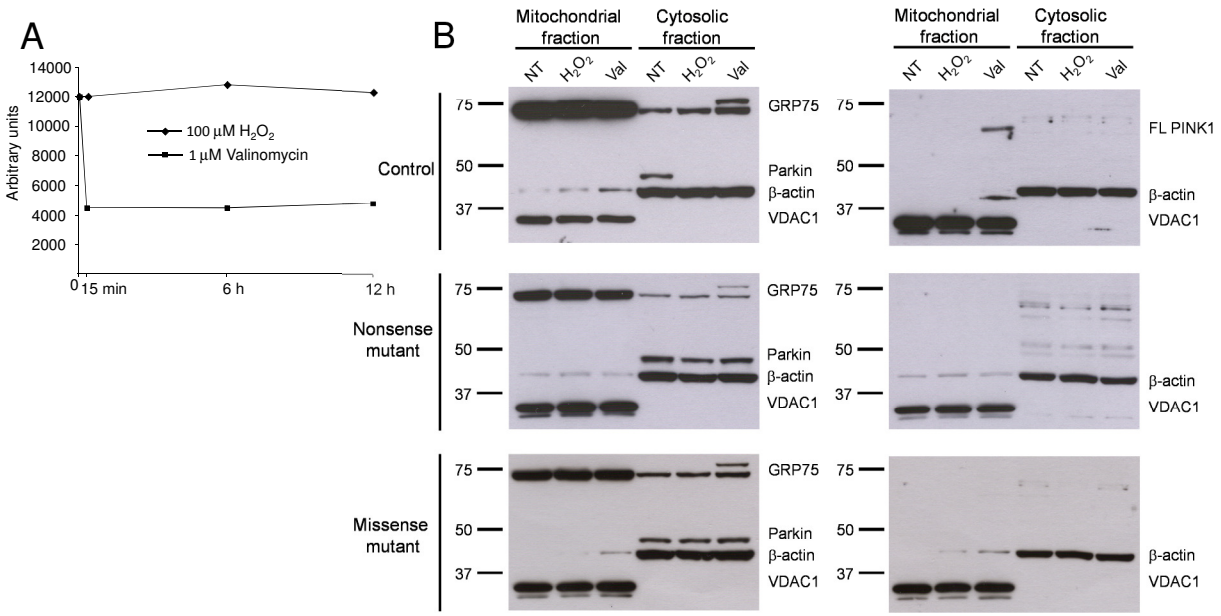

Figure 14. *Mitochondrial accumulation of full-length PINK1 is not necessary for the stress-induced reduction in the level of Parkin and is independent of the mitochondrial membrane potential.* (**A**) Fibroblasts of controls and mutants were treated with H_2O_2 or valinomycin for 12 hours. The level of the mitochondrial membrane potential was measured at different time points using JC-1 and normalized for number of cells. Valinomycin but not H_2O_2 caused an immediate loss of mitochondrial membrane potential, which remained at the same level throughout the entire experiment. (**B**) Fibroblasts from a control (upper panels), a nonsense mutation carrier (middle panels), and a missense mutation carrier (lower panels) were treated with H_2O_2 or valinomycin for 12 hours. Both the mitochondrial and the cytosolic fraction were analyzed by Western blotting. In controls loss of Parkin signal was detected in cells treated with either H_2O_2 or valinomycin and accumulation of the full-length form of GRP75 in the cytosolic fraction only in cells treated with valinomycin (upper left panel). In the mitochondrial fraction of control cells, accumulation of full-length PINK1 was found only in cells treated with valinomycin (upper right panel). In the cells from both the nonsense and the missense mutation carrier, neither of the treatments resulted in loss of Parkin signal, whereas accumulation of full-length GRP75 was present in cells treated with valinomycin but not in those treated with H_2O_2 (left middle and left lower panels). In contrast to controls, no accumulation of PINK1 was found in the mitochondrial or cytosolic fraction of cells from both the nonsense and the missense mutation carrier (right middle and right lower panels). FL PINK1- full- length PINK1

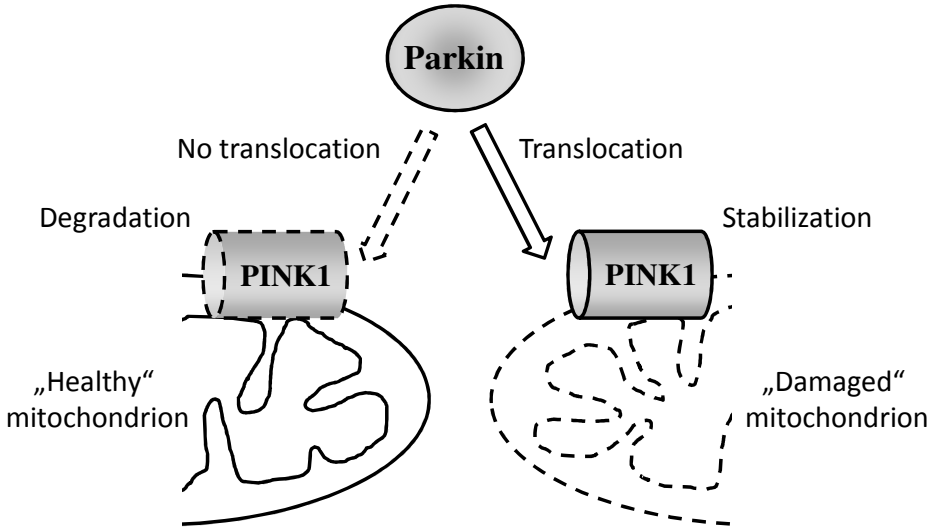

Figure 15. *PINK1 is necessary for the stress-induced mitochondrial translocation of Parkin.* The proteolytic processing of PINK1 maintains low levels of PINK1 on healthy mitochondria, but allows for the rapid accumulation of PINK1 on damaged mitochondria. In addition, accumulation of PINK1 on the outer mitochondrial membrane is sufficient for the mitochondrial recruitment of Parkin.

3.2 Parkin-mediated PINK1-dependent ubiquitination of mitochondrial fusion proteins: Mfn1 and Mfn2

In this section two primary human dermal fibroblast cultures carrying two different PD-causing homozygous *PINK1* mutations, c.1366C>T (p.Q456X; nonsense mutation; n=1) or c.509T>G (p.V170G; missense mutation; n=1), two primary human dermal fibroblast cultures carrying two different PD-causing homozygous *Parkin* mutations, c.1072delT (p.V324fsX434; n=1) or delEx7 (p.R245fsX253; n=1) and fibroblast cultures from two healthy controls without *PINK1* or *Parkin* mutations were analyzed under basal and stress conditions.

For experiments under stress conditions, valinomycin was used as mitochondrial stressor and hydrogen peroxide (H_2O_2) was used as a general stressor.

3.2.1 Validation of the antibodies against Mfn1 and Mfn2 used in present study

To test whether the used anti-Mfn1 and anti-Mfn2 antibody specifically bind Mfn1 or Mfn2, a knock-down experiment with siRNA against *Mfn1* or *Mfn2* was performed (Fig. 16). For this, fibroblasts were transfected with scrambled siRNA, *Mfn1* siRNA, *Mfn2* siRNA or with both *Mfn1* and *Mfn2* siRNAs. After two days of incubation proteins were extracted and analyzed by Western Blotting. Blots were probed with an antibody against Mfn1 or against Mfn2. This experiment showed a drop in Mfn1 levels only when siRNA against *Mfn1* was employed (Fig. 16A) and a drop in Mfn2 levels only when siRNA against *Mfn2* was employed (Fig. 16B), confirming the specificity of the antibody.

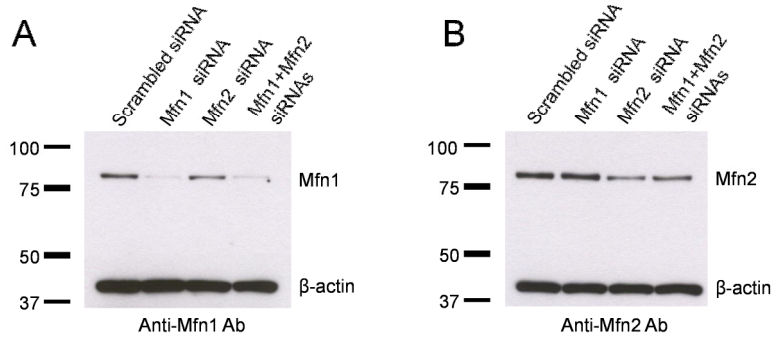

Figure 16. *Validation of the antibodies against Mitofusin 1 and Mitofusin 2 used in present study*. Fibroblasts were transfected with scrambled siRNA, *Mfn1* siRNA, *Mfn2* siRNA or a combination of *Mfn1* and *Mfn2* siRNA for 40h. Western blot analysis was performed with (A) an antibody against Mfn1 or (B) an antibody against Mfn2. The Mfn1 levels decreased only when *Mfn1* siRNA was employed and Mfn2 levels decreased only when *Mfn2* siRNA was employed, confirming the specificity of both the anti-Mfn1 and anti-Mfn2 antibodies used in present study. Mfn1 – Mitofusin 1; Mfn2 – Mitofusin 2

3.2.2 Effect of mutations in PINK1 and Parkin on Mfn1 and Mfn2

To examine the effect of mutations in PINK1 and Parkin on the endogenous levels of Mfn1 and Mfn2 fibroblasts from controls, PINK1 mutation carriers and Parkin mutation carriers were analyzed under basal conditions and after exposure to valinomycin for 12h by means of Western blotting. When compared to non-stressed cells, protein levels of both Mfn1 (Fig. 17A) and Mfn2 (Fig. 17B) were reduced only in controls but not in PINK1 or Parkin mutants. Furthermore, Mfn2 had an additional band on the Western blot, which was about 8kDa larger in size than the non-modified form, consistent with possible monoubiquitination of the protein. When analyzing whole cell lysate using an antibody against Mfn1, no additional band was detected, most probably due to a lower quality of the available anti-Mfn1 antibodies. Protein levels of β-actin and of the mitochondrial marker VDAC1 were comparable in all samples under basal and stress conditions (Fig. 17).

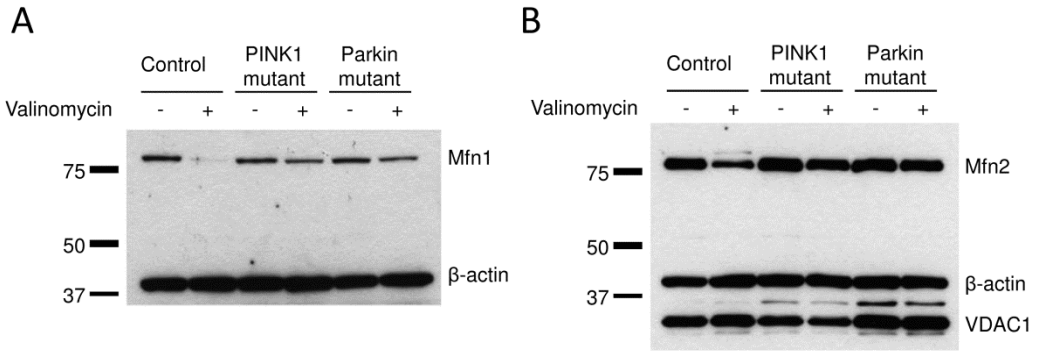

Figure 17. *Protein levels of Mfn1 and Mfn2 after valinomycin treatment.* Fibroblasts from a healthy control, a homozygous PINK1 mutant and a homozygous Parkin mutant were cultured under basal conditions or treated with 1μM valinomycin for 12h. The protein levels of Mfn1 (A) and Mfn2 (B) were investigated by means of Western blotting. Valinomycin exposure caused a drop in Mfn1 and Mfn2 levels in controls, but not in PINK1- or Parkin-mutant cells. β-actin and VDAC1 served as a loading controls.

3.2.3 Mfn1 and Mfn2 are ubiquitinated upon valinomycin treatment through the PINK/Parkin pathway and were detected in the mitochondrial fraction

The next aim was to verify whether the additional band on the Mfn2 blot (Fig. 17B), which is present only in controls after valinomycin-induced stress, is indeed explained by ubiquitination of the protein. For this, immunoprecipitation was performed using an antibody against Mfn2. Whole cell lysates from non-treated and valinomycin-treated (1μM, for 6h) controls were employed. The resulting immunoprecipitates were analyzed by Western blotting with an antibody against ubiquitin (Fig. 18, left panel) or with an antibody against Mfn2 (Fig. 18, right panel). On both blots, bands of the size of mono- and polyubiquitinated or multiple

monoubiquitinated Mfn2 were only detected in valinomycin-treated but not in non-treated controls. Taken together, these findings supported our previous results and underline that Mfn2 is ubiquitinated via the PINK1/Parkin pathway.

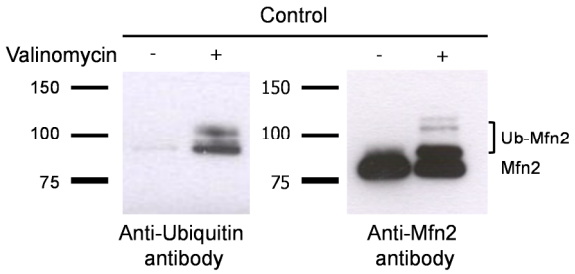

Figure 18. *Ubiquitination of Mfn2 upon valinomycin treatment.* Fibroblasts from a healthy control were treated with 1µM valinomycin for 6h. Whole cell lysates from non-treated and valinomycin treated controls were immunoprecipitated using an antibody against Mfn2. Immunoprecipitates were analyzed by Western blotting using an antibody against ubiquitin (left panel). Subsequently, the membrane was washed and reprobed with an antibody against Mfn2 (right panel). Ubiquitinated forms of Mfn2 (mono- and polyubiquitinated) are present only in valinomycin treated controls. Mfn2 – Mitofusin 2; Ub-Mfn2 – ubiquitinated Mitofusin 2

To determine the subcellular localization of ubiquitinated Mfn1 and Mfn2 in control fibroblasts, cells were incubated with 1µM valinomycin for 6h and mitochondrial and cytosolic protein fractions separated. Western blot analysis revealed that Mfn1 and Mfn2 and their ubiquitinated forms are exclusively localized in the mitochondrial fraction (Fig. 19).

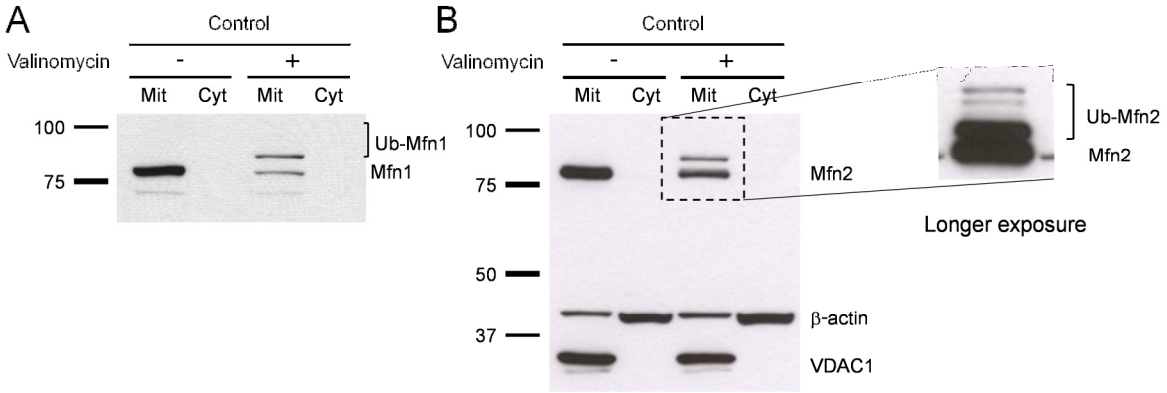

Figure 19. *Mitochondrial localization of ubiquitinated Mfn1 and Mfn2 after valinomycin treatment.* Control fibroblasts were cultured under basal conditions or treated with 1µM valinomycin for 6h. Cells were harvested and mitochondrial and cytosolic fractions were analyzed by Western blotting. The subcellular localization of Mfn2 was determined. Quality of cellular fractionation was confirmed using antibodies against VDAC1 and β-actin. The ubiquitinated forms of (A) Mfn1 and (B) Mfn2, which were observed only after valinomycin stress, are exclusively found in the mitochondrial fraction. A longer exposure of the blot showed several Mfn2 bands with higher molecular weight, indicative of Mfn2 polyubiquitination (enlarged cutout). Cyt – cytosolic fraction; Mit – mitochondrial fraction; Mfn1 – Mitofusin 1; Mfn2 – Mitofusin 2; VDAC1 – voltage-dependent anion channel 1

3.2.4 Rescue of Mfn2 ubiquitination in PINK1 and Parkin mutants

To test whether lack of ubiquitination of Mfn-2 in the mutants can be rescued, fibroblasts from a control, PINK1- and Parkin- mutant were transfected with an empty vector, a vector containing *PINK1-V5* or a vector containing *FLAG-Parkin*. Twenty-four hours after transfection, these cells were cultured under basal conditions or treated with 1µM valinomycin for an additional 12h. Whole cell lysates were analyzed by Western blotting. Using antibodies against V5 and FLAG, bands of the size of tagged full-length and cleaved PINK1 or Parkin were detected, confirming successful transfection (Fig. 20, upper panel). Furthermore, using an antibody against Mfn2, ubiquitinated Mfn2 was detected in control cells under stress conditions (Fig. 20A). In PINK1 mutant cells, ubiquitination of Mfn2 under stress was rescued through expression of PINK1-V5 but not through expression of FLAG-Parkin (Fig. 20B). By contrast, in Parkin-mutant fibroblasts, ubiquitinated Mfn2 was only detected after transfection with FLAG-Parkin (Fig. 20C).

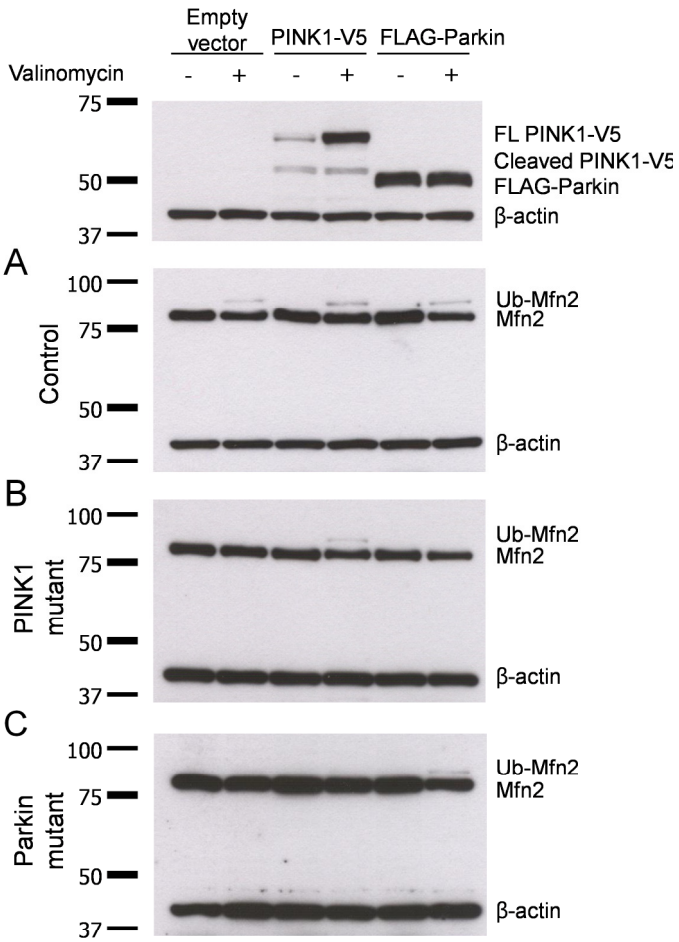

Figure 20. *Rescue of Mfn2 ubiquitination.* Fibroblasts of a control, a *PINK1* and a *Parkin* mutant were transfected with an empty vector, a vector containing *PINK1-V5* or a vector containing *FLAG-Parkin*. Twenty hours after transfection, cells were cultured under basal conditions or treated with 1µM valinomycin for an additional 6h. Western blot analysis using antibodies against V5 and FLAG showed bands of the size of full-length/cleaved PINK1 and Parkin, respectively, in treated and non-treated control cells after transfection, confirming the success of the experiment (upper panel). (A) In controls, ubiquitinated Mfn2 was detected in all three experiments after valinomycin treatment. (B) In *PINK1* mutant cells, ubiquitination of Mfn2 under stress was rescued through overexpression of PINK1-V5 but not through overexpression of FLAG-Parkin. (C) In *Parkin* mutant fibroblasts ubiquitinated Mfn2 was only detected after transfection with *FLAG-Parkin*. FL – full-length; Mfn2 – Mitofusin 2; Ub-Mfn2 – ubiquitinated Mitofusin 2

3.2.5 PINK1/Parkin-mediated ubiquitination of Mfn1 and Mfn2 and the UPS

To explore whether the ubiquitinated forms of Mfn2 are degraded by the UPS, control (Fig. 21A), PINK1- (Fig. 21B) and Parkin-mutant (Fig. 21C) fibroblasts were treated with 1µM valinomycin alone (Fig. 21, left panel) or in combination with 10µM epoxomicin (Fig.21, right panel) and proteins extracted at different time points for Western blot analysis. In control cells, valinomycin treatment initiated the ubiquitination of Mfn2 within 1h of incubation (Fig. 21A, left panel). Mfn2 ubiquitination was prevented by simultaneous exposure to epoxomicin (Fig. 21A, right panel). In PINK1 and Parkin mutants the protein levels of non-modified Mfn2 remained unchanged in all samples over time when treated with valinomycin (Fig. 21B and C, left panel) or with valinomycin plus epoxomicin (Fig. 21B and C, right panel). To further test whether epoxomicin alone has an impact on the protein levels of Mfn2, control cells were treated with 10µM epoxomicin but observed no change in Mfn2 levels during 9h of incubation (Fig. 21D).

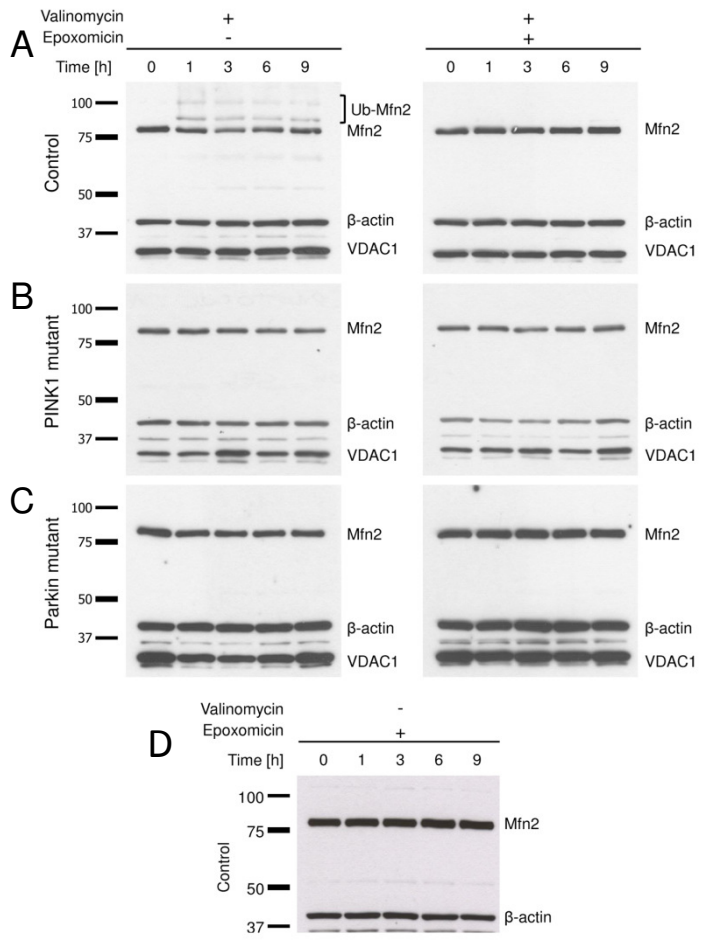

Figure 21. *Ubiquitination of Mfn2 is prevented by exposure to epoxomicin*. Fibroblasts from (A) a healthy control, (B) a homozygous *PINK1* mutant and (C) a homozygous *Parkin* mutant were treated with 1µM valinomycin alone (left panel) or with 1µM valinomycin plus 10µM epoxomicin (right panel). Proteins were extracted at different time points and analyzed by Western blotting. In control cells, valinomycin treatment initiated the ubiquitination of Mfn2 after 1h of incubation. This effect was prevented by simultaneous exposure to epoxomicin. (D) Exposure to epoxomicin alone did not affect the expression of Mfn2 over time. The mitochondrial marker VDAC1 and the cytosolic marker β-actin served as loading controls. Mfn2 – Mitofusin 2; Ub-Mfn2 – ubiquitinated Mitofusin 2; VDAC1 – voltage-dependent anion channel 1

By contrast, simultaneous treatment with valinomycin and the lysosomal inhibitor bafilomycin did not prevent Mfn2 ubiquitination in control cells (Fig. 22).

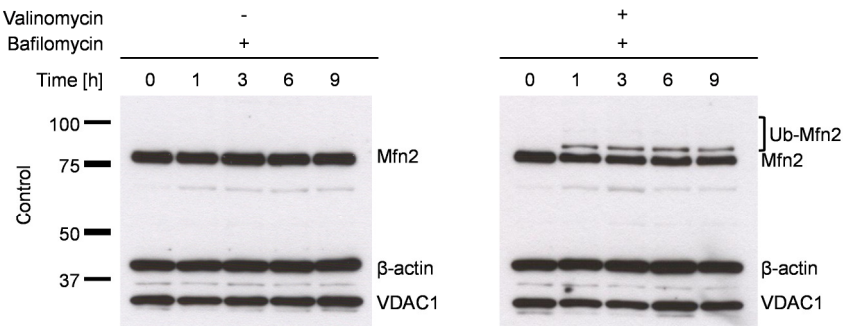

Figure 22. *Exposure to bafilomycin has no impact on Mfn2 ubiquitination.* Fibroblasts from a healthy control were treated with 10nM bafilomycin alone (left panel) or with 10nM bafilomycin plus 1µM valinomycin (right panel). Proteins were extracted at different time points and analyzed by Western blotting. Bafilomycin had no effect on the Mfn2 levels and no ubiquitination was detected. When bafilomycin and valinomycin were combined, Mfn2 ubiquitination was initiated by valinomycin and not influenced by bafilomycin. In both experiments, the mitochondrial marker VDAC1 was unchanged. β-actin served as a loading control. Mfn2 – Mitofusin 2; Ub-Mfn2 – ubiquitinated Mitofusin 2; VDAC1 – voltage-dependent anion channel 1

To exclude that proteasomal inhibition influences the effect of the potassium ionophore valinomycin, the mitochondrial membrane potential in control fibroblasts was monitored during 9h of treatment with valinomycin alone or in combination with epoxomicin. Both culturing conditions caused a similar drop in membrane potential (Fig. 23).

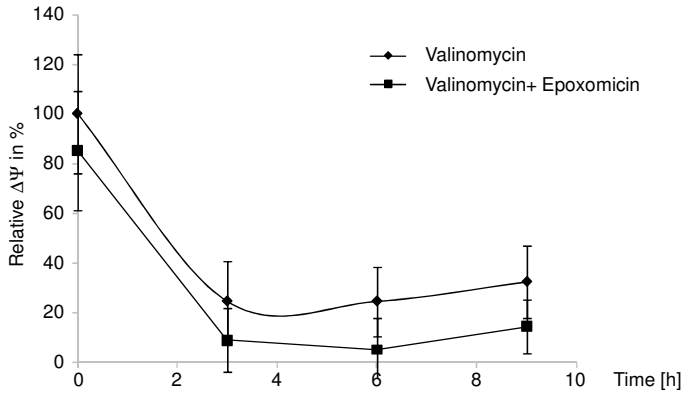

Figure 23. *Treatment with valinomycin alone or in combination with epoxomicin causes a drop in mitochondrial membrane potential.* Control fibroblasts were incubated with either 1µM valinomycin alone or with 1µM valinomycin plus 10µM epoxomicin. The membrane potential was measured at different time points and corrected for protein concentration. Exposure to the proteasome inhibitor epoxomicin did not influence the membrane potential over time. In the graph mean values +/- standard deviation of three independent experiments are given. ΔΨ – mitochondrial membrane potential

To show that inhibition of the UPS is not only preventing ubiquitination of Mfn1 and Mfn2 (Mitofusins) (as shown in 3.2.5) but is actually causing deubiquitination of already ubiquitinated Mitofusins, control fibroblasts were treated with valinomycin to induce ubiquitination. After 6h MG132 or DMSO (dissolvent for MG132) were added and cells were harvested at different time points. Western blot analysis revealed that upon 6h of UPS inhibition, levels of non-modified Mitofusins were almost at the same level as before treatment (Fig. 24). This additionally confirmed that the UPS is involved in the processing of ubiquitinated Mfn1 and Mfn2.

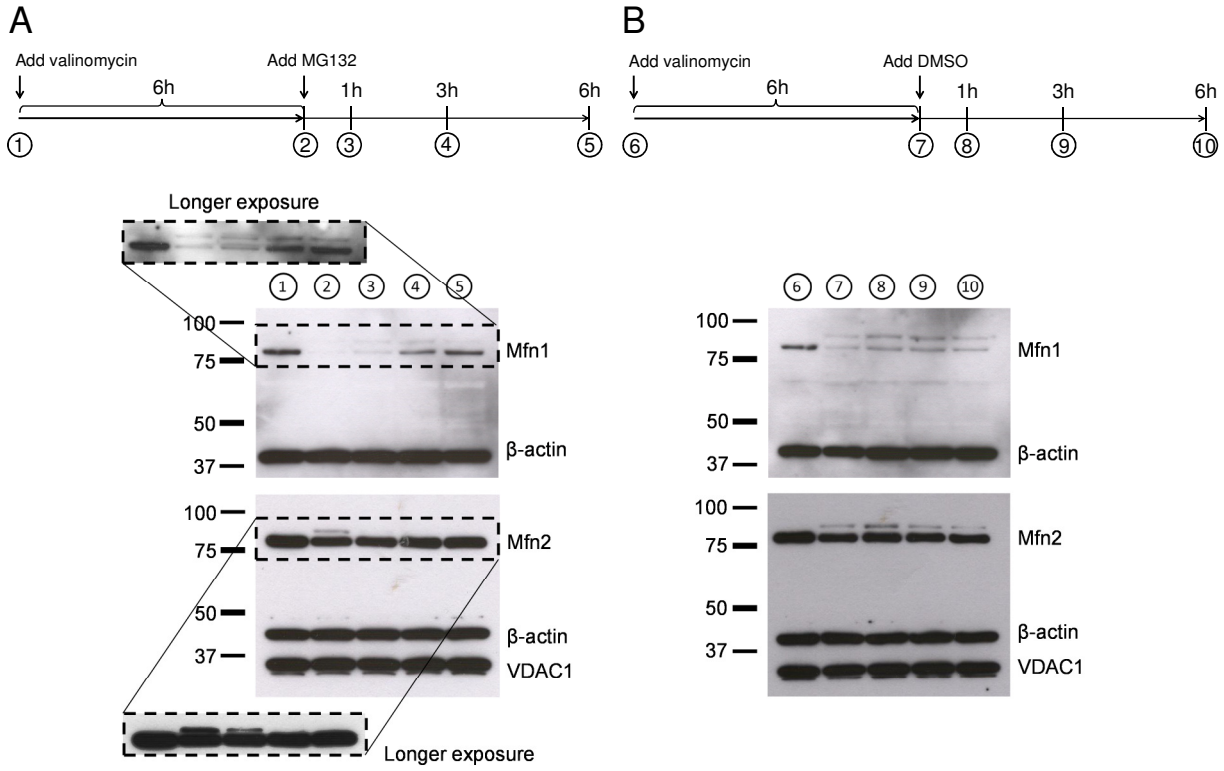

Figure 24. *MG132 promotes deubiquitination of Mfn1 and Mfn2.* Fibroblasts from a healthy control were treated with 1µM valinomycin. After 6h, MG132 (final concentration 10µM) was added to the cells. Proteins were extracted at different time points and analyzed by Western blotting. Exposure to (A) an inhibitor of the UPS, i.e.MG132 but not to (B) DMSO alone, induced deubiquitination of both Mfn1 and Mfn2. Mfn1 – Mitofusin 1; Mfn2 – Mitofusin 2; VDAC1 – voltage-dependent anion channel 1

3.2.6 Exposure to H_2O_2 causes ubiquitination of Mfn2

In the previous section (3.2) was shown that exposure to H_2O_2 induced mitochondrial translocation of Parkin. Therefore, it was investigated in control fibroblasts whether exposure to the superoxide generator H_2O_2 also results in ubiquitination of Mfn2. For this, cells were incubated with 100µM H_2O_2 for 12h and compared to cells stressed with 1µM valinomycin

for 6h and non-treated cells. Mitochondrial and cytosolic fractions of these cells were analyzed by Western blotting using antibodies against Mfn2, Parkin, VDAC1 and β-actin. The predominant presence of VDAC1 in the mitochondrial and of β-actin in the cytosolic fraction indicated good quality of the fractionation (Fig. 25A). Densitometric analysis revealed a significant drop in protein levels of non-modified Mfn2 in the mitochondrial fraction after valinomycin but also after H_2O_2 treatment (Fig. 25A and B). Under both stress conditions, high-molecular-weight bands of Mfn2 were detected, indicative of Mfn2 ubiquitination. As already demonstrated in section 3.1 both treatments caused a significant drop in protein levels of Parkin in the cytosolic fraction (Fig. 25A and C). Longer exposure of the Western blots revealed mitochondrial translocation of endogenous Parkin after both treatments (Fig. 25A, right panel).

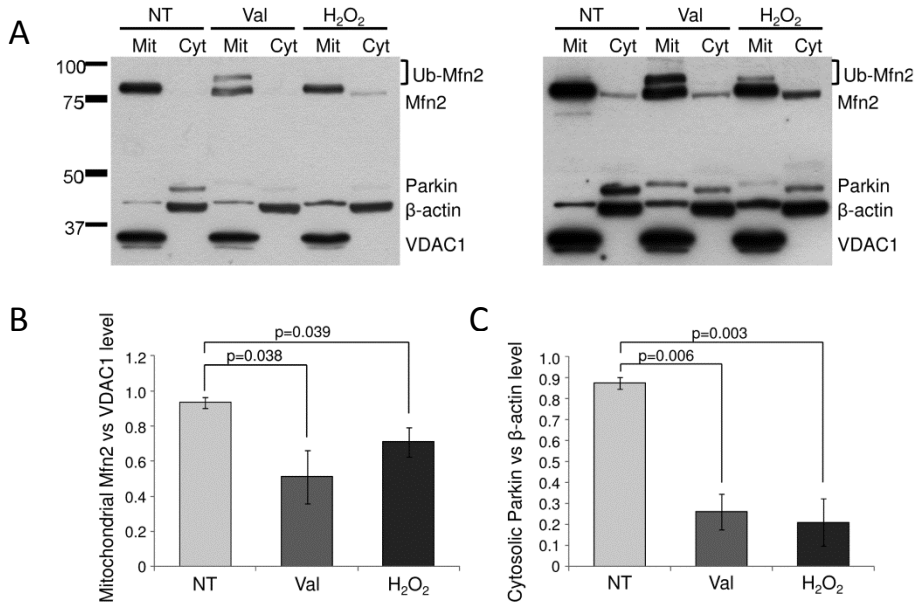

Figure 25. *Exposure to H_2O_2 caused ubiquitination of Mfn2*. Control fibroblasts were cultured under basal conditions, treated with 1µM valinomycin for 6h or with 100µM H_2O_2 for 12h. Cells were harvested and mitochondrial and cytosolic fractions were analyzed by Western blotting. (A) The subcellular localization of Mfn2 and Parkin was determined. Quality of cellular fractionation was confirmed using antibodies against VDAC1 and β-actin. (B) Densitometric analysis of the Western blot results revealed a significant drop in protein levels of non-modified Mfn2 (normalized against VDAC1 expression level) in the mitochondrial fraction after valinomycin or H_2O_2 treatment. (C) Furthermore, both treatments caused a significant drop in protein levels of Parkin (normalized against β-actin expression) in the cytosolic fraction. (A, right panel) A longer exposure of the Western blot showed mitochondrial translocation of Parkin after both treatments. For quantification of protein levels, blots of three independent experiments were evaluated. In the graphs mean intensities +/- standard deviation are given. Cyt – cytosolic fraction; H_2O_2 – hydrogen peroxide; Mit – mitochondrial fraction; Mfn2-Mitofusin 2; Ub-Mfn2 – ubiquitinated Mitofusin 2; Val – valinomycin; VDAC1 – voltage-dependent anion channel 1

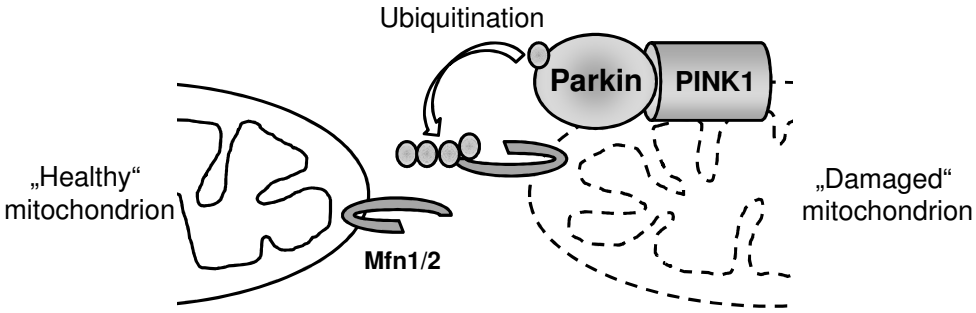

Figure 26. *The stress-induced PINK1-dependent Parkin-mediated ubiquitination of Mfn1 and Mfn2.* Damaged mitochondria can be distinguished from functional mitochondria, for instance, by a difference in mitochondrial membrane potential. Dysfunctional mitochondria with low membrane potential are detected by PINK1, which recruits Parkin. Next, Parkin ubiquitinates Mfn1/2 which localize to the outer mitochondrial membrane. Mfn1/2- Mitofusin 1 and/ or Mitofusin 2

3.3 PINK1-interacting proteins: Proteomic analysis of overexpressed PINK1

In this section, the tandem affinity purification (TAP) method was employed to isolate proteins that are directly associated with PINK1. For this, human kidney embryonic (HEK) cells overexpressing PINK1 were employed. Using this approach, the aim was to better characterize the PINK1/Parkin mitophagy pathway.

3.3.1 New PINK1 interactors

The TAP approach was used to identify proteins that form complexes with PINK1. For this, 10^8 HEK cells were transiently transfected with a mammalian vector expressing a "TAP tagged" PINK1. The "TAP tagged" PINK1 is a wild-type PINK, C-terminally tagged with both a calmoduline-binding peptide and a streptavidine-binding peptide which are necessary for subsequent affinity purification. Cells were harvested after 24h and protein extracted according the manufacturer's protocol. The resulting TAP eluate was resolved by SDS PAGE. To visualize protein bands on the PAGE gel, a mass spectroscopy-compatible silver staining was used. Seven bands (Fig. 27, a-g) were excised from the gel and respective interacting proteins identified by mass spectroscopy (MS) (Table 3).

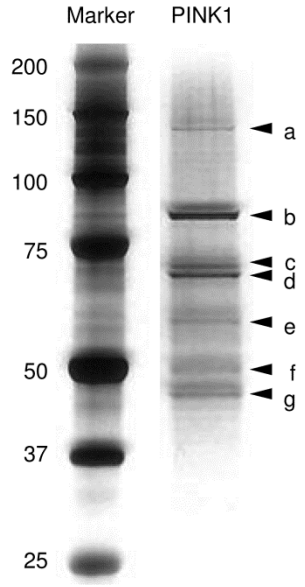

Figure 27. *SDS-PAGE result after TAP with overexpressed* PINK1. A TAP approach was performed with HEK cells overexpressing *PINK1*. Purified proteins were resolved by SDS-PAGE and visualized on the gel by silver staining. Protein bands which were excised from the gel are marked by an arrow head (a-g). A molecular weight marker was used to estimate the size of the detected protein bands.

Table 3. *Potential interactors of PINK1*

Swiss-Prot accession no.	Gene	Protein name	Subcellular localization	No. of unique peptides	Band on PAGE gel	Previous report of interaction
P42704	*LRPPRC*	Leucine-rich PPR motif-containing protein	Mitochondrion	2	a	None
P07900	*Hsp90A*	Heat shock 90 kDa protein alpha	Cytoplasm	27	b	Weihofen *et al.*, 2008
P08238	*Hsp90B*	Heat shock 90 kDa protein beta	Cytoplasm	18	b	Weihofen *et al.*, 2008
P38646	*GRP75*	75 kDa glucose-reguated protein/ Mortalin	Mitochondrion	6	c	None
P11021	*GRP78*	78 kDa glucose-reguated protein	Endoplasmic reticulum	15	c	None
P08107	*HSPA1*	Heat shock 70 kDa protein 1	Cytoplasm, organelles	10	c	None
P34931	*HSPA1L*	Heat shock 70 kDa protein 1-like	Cytoplasm, organelles	8	c	None
P54652	*HSPA2*	Heat shock 70 kDa protein 2	Cytoplasm, organelles	4	c	None
P11142	*HSPA8*	Heat shock 70 kDa protein 8	Cytoplasm	7	c	None
P10809	*Hsp60*	Heat shock 60 kDa protein	Mitochondrion	7	d	None
Q9BQE3	*TUBA1C*	Tubulin alpha-1C chain	Microtubule	2	f	None
Q13748	*TUBA3C*	Tubulin alpha-3C/D chain	Microtubule	3	f	None
Q16543	*Cdc37*	Hsp90 co-chaperone Cdc37	Cytoplasm	10	g	Weihofen *et al.*, 2008
P49411	*EFTu*	Elongation factor Tu	Mitochondrion	3	g	None

Source: *Rakovic et al., Parkinsons Dis, 2011; 2011: 153979*

A total of 14 proteins were detected which met the requirement of having two or more peptides matched to it by the database-searching program. Out of those, seven are predominantly found in the cytoplasm (Heat shock 90 kDa proteins alpha and beta, Heat shock 70 kDa proteins 1, 2, 8 and 1-like and Hsp90 co-chaperone Cdc37), two are components of microtubuli (Tubulin alpha-1C chain and Tubulin alpha-3C/D chain), one is associated with the endoplasmatic reticulum (78 kDa glucose-regulated protein) and four are mitochondrially localized (GRP75, Hsp60, leucine-rich PPR-motif containing (LRPPRC) protein and elongation factor thermo unstable (EFTu)) (for details on subcellular localization see: http://expasy.org/sprot/).

Given that PINK1 was reported to be associated with mitochondria (Narendra *et al.,* 2010; Rakovic *et al.,* 2010), a primary focus was on the mitochondrial proteins GRP75, Hsp60, LRPPRC and EFTu in the ensuing experiments.

3.3.2 Cellular abundance of GRP75, Hsp60, LRPPRC and EFTu

First, the quality of the non-commercially available LRPPRC antibody was determined by means of a knock-down approach. This experiment showed a drop in LRPPRC levels when siRNA against *LRPPRC* was employed but not when scrambled siRNA was used, confirming the specificity of the antibody (Fig. 28). LRPPRC protein levels were also not affected by a *PINK1* knock-down.

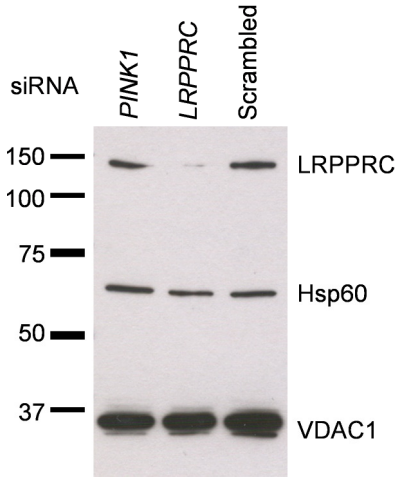

Figure 28. *Specificity of the anti-LRPPRC antibody.* Fibroblasts were incubated with *PINK1* siRNA, *LRPPRC* siRNA or scrambled siRNA for 24 h. Whole cell lysates were analyzed by Western blotting with an antibody against LRPPRC. LRPPRC levels decreased only when *LRPPRC* siRNA was employed, confirming the specificity of the anti-LRPPRC antibody used in the present study. The mitochondrial markers Hsp60 and VDAC1 served as loading controls. Hsp60 – Heat shock 60kDa protein; LRPPRC – Leucine-rich PPR motif-containing protein; VDAC1 – Voltage-dependent anion channel 1

Next, the abundance of GRP75, Hsp60, LRPPRC and EFTu was investigated in mitochondrial fractions from control and *PINK1*-mutant fibroblasts. This experiment revealed comparable levels of GRP75, Hsp60 and LRPPRC in both groups (Fig. 29A and B). The abundance of EFTu was variable in all investigated samples showing no clear trend when comparing mutants and controls (Fig. 29A). Since LRPPRC regulates expression levels of the subunit I of the mitochondrial cytochrome c oxidase (MT-CO1), its protein levels were also analyzed (Fig. 29A). GRP75 is a mitochondrial matrix chaperone, synthesized as a 679-amino acid full-length protein (MTS-GRP75), which contains a 51-residue N-terminal mitochondrial targeting sequence (MTS). After the membrane potential-dependent import into mitochondria, it is cleaved into the mature protein (GRP75) which is ~5.5 kDa shorter than the pre-protein (Mizzen *et al.,* 1989; Dahlseid *et al.,* 1994; Szabadkai *et al.,* 2006). When treating cells with the mitochondrial membrane potential inhibitor valinomycin, the MTS-GRP75 was detected in the cytosolic fractions from both mutants and controls. In the both groups, the levels of MTS-GRP75 as well as of GRP75 were comparable (Fig. 29B right half). The presence of the GRP75 in the cytosolic fraction was likely due to contamination with the mitochondrial fraction, although previous studies have shown that GRP75 can also be cytosolically localized (Ran *et al.,* 2000).

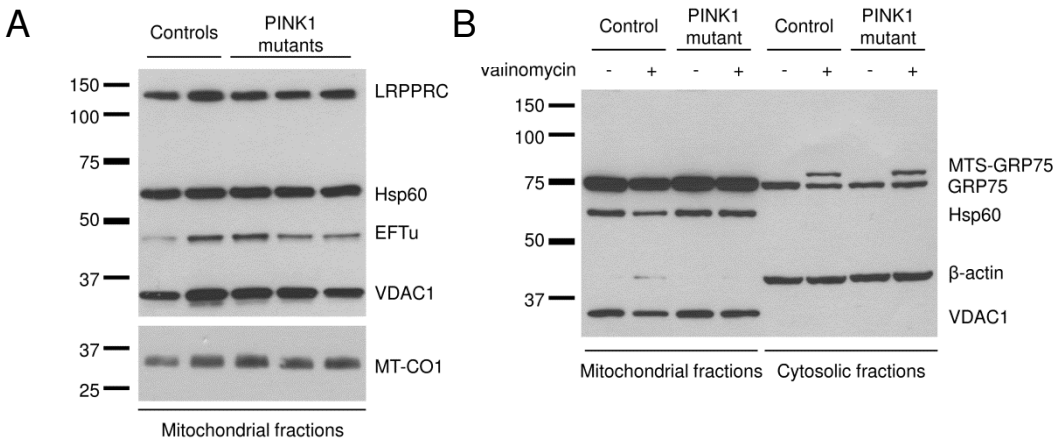

Figure 29. *Cellular abundance of potential mitochondrial PINK1-interacting proteins.* Mitochondrial and cytosolic fractions from fibroblasts were analyzed by Western blotting using antibodies against Hsp60, LRPPRC, EFTu, MT-CO1 and GRP75. (A) The mitochondrial localization of LRPPRC and EFTu was confirmed and no differences in their cellular abundance were detected when comparing *PINK1* mutants and controls. Furthermore, the level of MT-CO1 was not altered in *PINK1* mutants. (B) In the mitochondrial fractions, the abundance of (processed) GRP75 was comparable in *PINK1* mutants and controls under basal and valinomycin stress conditions (1 μM for 24 h). In the cytosol, an additional band representative of accumulation of non-processed MTS-GRP75 was detected when cells were treated with the mitochondrial membrane potential inhibitor valinomycin. Due to a possible contamination of the cytosolic fraction with mitochondria and/or partially cytosolic localization of GRP75, also the processed form of the protein is apparent in this fraction. The mitochondrial marker VDAC1 and the cytosolic marker β-actin served as loading controls. GRP75 – 75 kDa glucose-regulated protein; Hsp60 – Heat shock 60kDa protein; LRPPRC – Leucine-rich PPR motif-containing protein; MT-CO1 – Mitochondrially encoded cytochrome c oxidase I; MTS-GRP75 – full-length GRP75 (with mitochondrial targeting sequence); EFTu – Elongation factor Tu; VDAC1 – voltage-dependent anion channel 1

4 DISCUSSION

In this study human dermal fibroblasts from PD patients carrying mutations in the PINK1 and Parkin gene were used to further elucidate the relation between PINK1 and Parkin and their role in the pathogenesis of PD. Since PD is a disease where the environment plays an important etiologic role, cells were analyzed upon oxidative stress caused by H_2O_2 and upon mitochondrial stress caused by the potassium ionophore valinomycin as well as under basal conditions. The results of the projects performed as part of the present thesis are discussed as follows: first, the effect of mutations in PINK1 on protein levels and subcellular localization of Parkin (4.1); second, the effect of the PINK1/Parkin pathway on mitochondrial fusion factors (4.2); and third, findings from the proteomic analysis of overexpressed PINK1 (4.3).

4.1 Effect of mutations in PINK1 on protein levels and subcellular localization of Parkin

It has been first identified in 2006 that Parkin interacts with PINK1 (Clark *et al.,* 2006; Park *et al.,* 2006; Yang *et al.,* 2006), another protein involved in autosomal recessive early-onset parkinsonism. In *PINK1* loss-of-function *Drosophila* mutants, degeneration of flight muscles and dopaminergic neurons is observed in combination with defects in mitochondrial morphology and increased sensitivity to oxidative stress (Clark *et al.,* 2006; Park *et al.,* 2006; Yang *et al.,* 2006). This phenotype overlaps with the morphological changes seen earlier in *Parkin* mutants (Greene *et al.,* 2003; Pesah *et al.,* 2004). Transgenic expression of Parkin markedly ameliorates all PINK1 loss-of-function phenotypes, but not vice versa, suggesting that Parkin functions downstream of PINK1 (Clark *et al.,* 2006; Park *et al.,* 2006; Yang *et al.,* 2006). However, the details and functional consequences of this interaction are mainly unknown.

When protein levels of endogenous Parkin were investigated by means of Western blotting, no difference was detected between controls and PINK1 mutants under basal conditions. In contrast, upon stress, loss of endogenous Parkin was observed in controls but not in PINK1 mutants. Expression analysis showed no alteration in *Parkin* mRNA levels between controls and PINK1 mutants that could explain the observed differences in protein levels. The stress-induced loss of endogenous Parkin could be prevented by inhibitors of the UPS. This is in keeping with previous findings showing that PINK1 is required for proteasomal degradation of overexpressed Parkin (Xiong *et al.,* 2009). An alternative explanation of the loss of detectable Parkin could be a PINK1-mediated decrease in Parkin solubility, as suggested in

studies using overexpressed proteins (Shiba *et al.,* 2009). However, when endogenously expressed protein was analyzed upon stress, no Parkin was detected either in the insoluble or soluble fraction (Rakovic *et al.*, 2010). Thus, these seemingly conflicting results can be explained by different experimental conditions (endogenous vs. overexpressed protein). Data obtained on the endogenous level support the hypothesis of PINK1-mediated degradation of Parkin and underline the necessity to interpret results generated in artificial systems with caution.

In accordance with previous studies (Geisler *et al.*, 2010; Narendra *et al.*, 2010; Vives-Bauza *et al.*, 2010), immunostaining of control- and PINK1 mutant fibroblasts overexpressing Parkin revealed that valinomycin and H_2O_2 induce mitochondrial translocation of Parkin. This translocation is dependent upon PINK1 and only takes place in healthy controls but not in affected *PINK1* mutation carriers. Furthermore, accumulation of Parkin in the mitochondrial fraction upon valinomycin-induced stress was demonstrated on the endogenous level. Using cellular fractionation followed by Western blotting, the presence of endogenous Parkin was detected in the mitochondrial fraction of controls but not of PINK1 mutants confirming immunostaining results. Studying the potential role of the UPS in this process, it was shown that mitochondrial accumulation of Parkin is even more pronounced when inhibiting the UPS, whereby stress-induced degradation of Parkin was prevented. This increase in mitochondrial accumulation of Parkin is most likely a direct consequence of its accumulation in the cytosol due to UPS inhibition. Indeed, an accumulation of Parkin occurred in both the cytosolic and the mitochondrial fraction. These effects were only present in cells from control individuals but not in those derived from carriers of pathogenic *PINK1* mutations. Taken together, wild-type PINK1 is necessary for two different processes, i.e. the stress-induced mitochondrial translocation of Parkin and regulation of Parkin levels via the UPS.

It has previously been shown that Parkin is physically associated with mtDNA, most likely through an indirect interaction with TFAM, and thus localized within mitochondria (Rothfuss *et al.*, 2009). In contrast to these data, there is a growing number of studies showing that Parkin is translocated onto damaged mitochondria and promotes their autophagy. This translocation is dependent on PINK1 and can be impaired by PD-causing mutations in PINK1 (Geisler *et al.*, 2010; Matsuda *et al.*, 2010; Narendra *et al.*, 2010; Vives-Bauza *et al.*, 2010). More recently, these findings have been confirmed in dopaminergic neurons that were differentiated from induced pluripotent stem (iPS) carrying PD-causing mutations in PINK1 (Seibler *et al.*, 2011). The physiological relevance of the stress-induced PINK1-depenendent loss of Parkin through the UPS currently remains elusive. One possible explanation would be

that loss of Parkin presents a protective mechanism under conditions of high levels of stress. Indeed, it has been shown in cells overexpressing Parkin that the stress-induced mitophagy is a very fast process and leads to a complete removal of all mitochondria. In contrast, in cells expressing endogenous levels of Parkin, a complete clearance of mitochondria was not observed. At this point, the possible interplay of these two processes and the underlying mechanism(s) remain to be elucidated.

Another important finding of this thesis is that it is specifically the mitochondrial localization of PINK1 that is needed for the stress-induced changes of Parkin, as demonstrated in a series of experiments using different truncated forms of PINK1. PINK1 is a mitochondrially localized protein kinase and, as most of mitochondrial proteins, contain a MTS at its N terminus. The exact length of the MTS and the cleavage site upon delivery to mitochondria is still under debate. However, it has been demonstrated that upon deletion of amino acids 1-111 or 1-108, PINK1 could no longer be imported into the mitochondria (Haque *et al.*, 2008; Takatori *et al.*, 2008) and its cleavage site is most likely between amino acids 91 and 101 (Zhou *et al.*, 2008). Results obtained in this thesis confirmed previous reports and additionally narrowed the region of PINK1 cleavage. Since the truncated form of PINK1 lacking the first 93 amino acids was mainly localized in the mitochondrial fraction, the truncated form of PINK1 lacking the first 110 amino acids failed to be targeted to mitochondria. This shows that the region between amino acids 93 and 110 is necessary for mitochondrial targeting of PINK1 and most likely contains a cleavage site. Only recently, two independent studies designated amino acid A107 as the exact site of cleavage (Jin *et al.*, 2010; Deas *et al.*, 2011). More importantly, PINK1d93 (present thesis) was sufficient to restore the PINK1 wild-type phenotype, i.e. the stress-induced mitochondrial translocation and loss of detectable Parkin. In contrast, PINK1d110 did not have this effect. These data demonstrated that the amino acid residues between position 93 and 110 are necessary for mitochondrial targeting of PINK1 and its normal function.

In experiments with the potassium ionophore valinomycin, it has been shown that endogenous and overexpressed full-length PINK1 accumulate upon valinomycin-induced collapse of mitochondrial membrane potential. Recently, a two-step model has been proposed for the processing of PINK1: first, full-length PINK1 is cleaved into the 52-kDa short form in a voltage-dependent, proteasome-independent manner, and second, the short form of PINK1 is rapidly degraded by the proteasome. The voltage-dependent processing of PINK1 maintains low levels of PINK1 on healthy polarized mitochondria, but allows for the rapid accumulation of PINK1 on depolarized mitochondria. In addition, accumulation of PINK1 on the outer

mitochondrial membrane is necessary for the mitochondrial recruitment of Parkin (Narendra *et al.*, 2010).

In contrast, an accumulation of PINK1 was not observed in experiments with H_2O_2, which similarly to valinomycin caused the stress-induced loss of endogenous Parkin and its mitochondrial translocation. A series of experiments in the present thesis revealed that H_2O_2 had no effect on the mitochondrial membrane potential. First, direct measurement of the mitochondrial membrane potential using the fluorescent voltage-sensitive dye JC-1 showed normal levels of mitochondrial membrane potential in cells treated with H_2O_2. In addition, an accumulation of the premature, non-processed form of the mitochondrial matrix protein GRP75 was detected only upon valinomycin treatment but not upon treatment with H_2O_2. GRP75, as well as PINK1, are synthesized as a precursor protein which is cleaved into its shorter, mature form after import into mitochondria in a mitochondrial membrane potential-dependent manner (Rohe *et al.,* 2004; Silvestri *et al.,* 2005). While loss of endogenous Parkin and its mitochondrial translocation could be induced with both H_2O_2 and valinomycin, an accumulation of PINK1 was observed only upon valinomycin treatment. Taken together, these experiments revealed that accumulation of PINK1 is sufficient but not necessary for the stress-induced recruitment of Parkin to mitochondria or the PINK1-mediated loss of Parkin.

Little is known about the mechanism(s) how PINK1 might induce Parkin recruitment. The simplest explanation may be that, as PINK1 accumulates, Parkin may be recruited to mitochondria through a direct interaction with the accumulated PINK1. In support of this model, PINK1 appears to directly bind Parkin at least in some contexts (Xiong *et al.*, 2009). Alternatively, PINK1 may need to phosphorylate Parkin, a substrate of Parkin, or an adaptor between PINK1 and Parkin, and thereby increase Parkin's affinity for a substrate or receptor on mitochondria. Consistent with a role for phosphorylation in the activation of Parkin, it has been shown that a kinase-deficient version of PINK1 fails to rescue Parkin recruitment to mitochondria in PINK1 null cells (Narendra *et al.*, 2010). However, a finding suggesting that phosphorylation of Parkin by PINK1 is sufficient to induce Parkin recruitment to mitochondria (Kim *et al.*, 2008), failed to be reproducible (Narendra *et al.*, 2010). In addition, if direct phosphorylation is sufficient to induce Parkin recruitment to mitochondria, it seems difficult to explain how Parkin can be targeted to a particular subset of mitochondria, as appears to occur in cells with bioenergetically diverse populations of mitochondria (Narendra *et al.*, 2008).

The significance of the present thesis in this context is twofold: First, it was confirmed at the endogenous level that i) PINK1 regulates the stress-induced decrease in detectable Parkin

levels (Shiba *et al.,* 2009; Um *et al.,* 2009), ii) mediates the stress-induced mitochondrial translocation of Parkin (Geisler *et al.*, 2010; Narendra *et al.*, 2010; Vives-Bauza *et al.*, 2010), iii) and that endogenous PINK1 accumulates upon depolarization of mitochondria (Geisler *et al.*, 2010; Narendra *et al.*, 2010). Second, this study provides novel evidence that mitochondrial accumulation of full-length PINK1 is not necessary for the stress-induced loss of Parkin signal and its mitochondrial translocation and is independent of the mitochondrial membrane potential.

Moreover, two different types of human pathogenic PINK1 mutations, i.e. a nonsense and a missense mutation, were studied and the same phenotype was observed. The nonsense mutation is known to cause NMD (Grunewald *et al.*, 2007), whereas little is known about the consequences of the homozygous missense mutation. While mRNA expression of the missense mutation was comparable to that of controls, no accumulation of the full-length mutated protein was detected after valinomycin treatment, suggesting that protein stability or mitochondrial targeting was impaired. Since this missense mutation affects the kinase domain and not the mitochondrial targeting signal sequence, impaired protein stability would be a more sustainable explanation.

In addition to confirming and expanding on previous findings on the PINK1/Parkin pathway, this thesis revealed certain discrepancies between the behavior of endogenous vs. overexpressed protein. This highlights the need for more experiments to be performed at the endogenous level and for employing different stressors to explore their differential effects on the functional relationship of PINK1 and Parkin. More generally speaking, this study shows the necessity to introduce an environmental factor, i.e. stress, to visualize the differences in the interaction of PINK1 and Parkin in mutants vs. controls and thus provide experimental evidence for the generally held notion of PD as a condition with a combined genetic and environmental etiology. While our data support the proposition that additional stressors may be important in PD pathogenesis, such stressors do not necessarily have to be exogenous in terms of environment. In fact, endogenous stressors, such as free radicals produced over an individual's lifetime, may be equally important as contributing factors in the development of PD.

4.2 Effect of PINK1/Parkin pathway on mitochondrial fusion factors

Maintenance of mitochondrial homeostasis appears to be an important function of the PINK1/Parkin pathway in multiple model systems and is likely a key factor in mediating

neurodegeneration. Previous studies have begun to shed light on the potential mechanism by which this pathway maintains a healthy mitochondrial population. Regulated mitochondrial fission and fusion events are thought to contribute to a quality control mechanism to help "sort out" terminally damaged mitochondria for degradation (Tatsuta *et al.*, 2008; Twig *et al.*, 2008). Importantly, PINK1 and Parkin have previously been shown to genetically interact with components of the mitochondrial fission/fusion machinery and to affect mitochondrial morphology (Deng *et al.*, 2008; Poole *et al.*, 2008; Yang *et al.*, 2008; Park *et al.*, 2009); however, the molecular mechanisms are not known. Emerging evidence indicates that PINK1 is required to recruit Parkin to damaged or dysfunctional mitochondria, whereupon it promotes mitophagy (Geisler *et al.*, 2010; Narendra *et al.*, 2010; Vives-Bauza *et al.*, 2010; Springer *et al.*, 2011).

In previous work it has been shown that genetic manipulations promoting mitochondrial fragmentation, including increased drp1 gene dosage and decreased opa1 or mfn gene dosage, dramatically suppress the PINK1 and Parkin mutant phenotypes in *Drosophila* (Deng *et al.*, 2008; Poole *et al.*, 2008; Yang *et al.*, 2008; Park *et al.*, 2009). These findings suggest that the PINK1/Parkin pathway acts to promote mitochondrial fission or inhibit mitochondrial fusion, either of which would culminate in mitochondrial fragmentation.

In this thesis (Rakovic *et al.*, 2011), protein levels of both Mfn1 and Mfn2 were analyzed by means of Western blotting in human dermal fibroblasts form healthy controls and PD patients carrying mutations in PINK1 or Parkin. Consistent with studies in *Drosophila* (Poole *et al.*, 2010; Ziviani *et al.*, 2010), protein levels of engodenous Mitofusins were significantly lower in controls than in PINK1 or Parkin mutants upon valinomycin-induced stress. In controls, both Mfn1 and Mfn2 had additional bands of higher molecular weight. These band shifts are consistent with monoubiquitination (8.5 kDa). In addition, H_2O_2–induced stress resulted in a decreased Mfn2 signal in the controls but not in the mutants. However, the effect after H_2O_2 incubation was less pronounced than the effect upon valinomycin treatment. By means of immunoprecipitation, the additional anti-Mfn2 reactive bands indeed proved to represent ubiquitinated forms of the protein. These findings are in line with recent publications reporting that Mitofusins are ubiquitinated in response to mitochondrial stress in wild-type *Drosophila* and SH-SY5Y cells. This modification was, however, impaired in treated *parkin* or *pink1* knockdown cells (Gegg *et al.*, 2010; Poole *et al.*, 2010; Ziviani *et al.*, 2010). Furthermore, the ubiquitination of Mfn2 was rescued when PINK1 or Parkin was re-expressed in PINK1- or Parkin-mutant cells. In *Drosophila*, transgenic expression of parkin in pink1 loss-of-function mutants markedly ameliorated all mitochondrial phenotypes, but not vice

versa, leading to the conclusion that parkin functions downstream of pink1 (Clark *et al.,* 2006; Park *et al.,* 2006; Yang *et al.,* 2006). However, an ubiquitinated form of Mfn2 was not detected when PINK1 mutant cells were transfected with *FLAG-Parkin*. Given the weak signal of ubiquitinated Mfn2 after PINK1 transfection in the Parkin-mutant cells, a possible explanation for this discrepancy might be that the used antibody is not sensitive enough to detect the likely even lower levels of ubiquitinated Mfn2 in the PINK1-mutant fibroblasts. When the subcellular localization of non-modified and ubiquitinated Mfn2 in control fibroblasts and SH-SY5Y cells were determined, all Mfn2 forms were exclusively found in the mitochondrial fraction. Ubiquitination of Mitofusins is fast process. It occurred already within one hour of treatment with valinomycin (Rakovic *et al.*, 2011).

Longer exposures indicated further high-molecular-weight bands for Mfn2, which suggests that Mfn2 undergoes multiple ubiquitination modifications upon mitophagy induction. In contrast, when analyzing Mfn1 after longer exposure, only the monoubiquitinated form was detected. Monoubiquitination is a predominant covalent modification of Mitofusins. However, multiple ubiquitination is a subject of vivid debate. In recent studies, the number of proposed ubiquitin adducts ranges from one to four (Gegg *et al.*, 2010; Poole *et al.*, 2010; Ziviani *et al.*, 2010; Rakovic *et al.*, 2011). The type of linkage and number of ubiquitin moieties added to a particular cargo determines whether the substrate is destined for degradation by the proteasome or autophagy (Kirkin *et al.*, 2009; Chin *et al.*, 2010). Although it remains to be shown that Parkin directly mediates this ubiquitination, there is evidence that Parkin can mediate monoubiquitination (Fallon *et al.*, 2006; Matsuda *et al.*, 2006; Moore *et al.*, 2008) and Lys27 (Geisler *et al.*, 2010) and Lys63 linkages (Lim *et al.*, 2006; Olzmann *et al.*, 2007). These modes of ubiquitination are not typically linked to proteasome degradation. Typically, polyubiquitination linked via Lys48 of ubiquitin targets the protein to the proteasome, whereas monoubiquitination or oligomeric Lys63-linked ubiquitin chains target proteins to lysosomes. There is also growing evidence that ubiquitination of proteins may also affect protein function, similar to other post-translational modifications such as phosphorylation (Yonashiro *et al.*, 2006; Germain, 2008). Therefore, the type of ubiquitination and the function of the mitochondrial proteins most likely determine the role they play in mediating mitophagy. Further work is required to determine whether the post-translational modification of Mfn1 and Mfn2 is due to polyubiquitination or multiple monoubiquitination events. The ubiquitination of Mitofusins may act as tags to identify damaged mitochondria (Whitworth *et al.*, 2009; Ziviani *et al.*, 2010) and recruit the ubiquitin-binding autophagic receptors p62 and Histone deacetylase 6 (HDAC6) (Lee *et al.*, 2010;

Vives-Bauza *et al.*, 2010), and subsequently autophagosome components such as LC3-II (Kirkin *et al.*, 2009).

To study involvement of UPS in processing of ubiquitinated forms of Mitofusins, cells were analyzed in the presence of inhibitors of UPS. Treatment with valinomycin, upon inhibition of the UPS with epoxomicin or MG132, neither increased nor preserved the levels of ubiquitinated Mfn2 over time. Ubiquitination of Mfn2 in control cells was apparently absent using valinomycin in combination with a proteasomal inhibitor. However, this was not explained by interference of epoxomicin with the effect of the mitochondrial uncoupler valinomycin. According to the literature, proteins targeted for degradation can only enter the UPS after their ubiquitin chain has been removed. This deubiquitination is performed by the 19S regulatory complex of the UPS (Yao *et al.,* 2002; Kaiser *et al.,* 2005). Epoxomicin is a potent inhibitor of the 20S proteasome subunit, where protein degradation takes place, but does not influence the deubiquitinase activity of the 19S particle (Fig. 30) (Verma *et al.,* 2005).

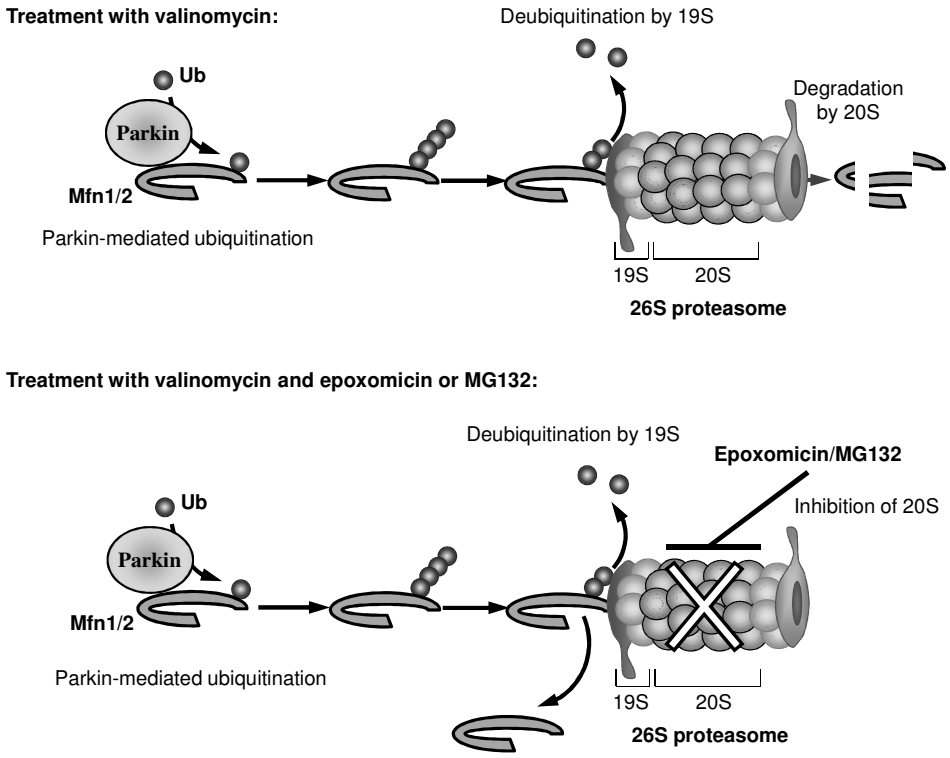

Figure 30. *Possible scenario of Mitofusins deubiquitination and degradation at the UPS*. Under valinomycin stress, Mitofusins are ubiquitinated by Parkin. Poly-ubiquitinated Mitofusins are subsequently recognized by the intrinsic ubiquitin-binding activity of the 19S particle of the 26S proteasome. At the 19S regulatory complex the ubiquitin chain is disassembled, and the substrate is unfolded before it can enter the cavity of the 20S subunit where proteolysis takes place. Simultaneous treatment with epoxomicin (or MG132) inhibits the degradation function of the 20S core particle but does not influence the deubiquitylase activity of the 19S subunit. Consequently, poly-ubiquitinated Mitofusins are deubiquitinated at the proteasome but cannot be degraded. Mfn1/2 – Mitofusins; Ub – ubiquitin

Consequently, data from the present study suggest that, in the presence of epoxomicin or MG132, ubiquitinated Mitofusins are solely deubiquitinated but not degraded by the UPS leading to constant levels of non-modified Mitofusins in the stressed control cells. Under stress conditions without UPS inhibition, however, the turnover of ubiquitinated Mitofusins in the cytosol is probably occurring too rapidly to be detected in a fractionation experiment. In line with this hypothesis, inhibition of lysosomal degradation did not prevent Mfn2 ubiquitination in control fibroblasts.

A previous study showed that the degradation of the yeast Mitofusins homologue Fzo1 is also dependent on the UPS (Cohen *et al.,* 2008). Taken together, these results suggest that Mitofusins are ubiquitinated in a PINK1/Parkin pathway-dependent manner and ubiquitinated Mitofusins are at least partially processed through the UPS. This was additionally confirmed recently in a study showing that Parkin-dependent ubiquitination of Mitofusins leads to their degradation in a proteasome- and p97-dependent manner (Tanaka *et al.*, 2010).

In a recent publication, the mitochondrial membrane potential was identified as an important cellular parameter to differentiate between functional and dysfunctional mitochondria. Following fission, the refusion of daughter mitochondria requires a membrane potential beyond a certain threshold (Twig *et al.,* 2008). It is tempting to speculate that Parkin-mediated ubiquitination and subsequent degradation of Mitofusins prevents this refusion. Such isolated dysfunctional mitochondria likely undergo mitophagy and require both functional Parkin and PINK1. This notion is supported by colocalization of PINK1 and partially also of Parkin with microtubule-associated protein 1 light chain 3 (LC3), a marker of autophagosomes (Kawajiri *et al.,* 2010), and by abrogation of Parkin-induced mitophagy upon treatment with bafilomycin, a lysosomal inhibitor (Narendra *et al.,* 2008). For a schematic representation of the putative Parkin/PINK1 mitophagy pathway, see Figure 31.

According to current knowledge, mitophagy is one of the most important mechanisms by which mitochondria are recycled (Chen *et al.,* 2009). Therefore, impairment of mitophagy due to PINK1 or Parkin mutations presumably leads to accumulation of dysfunctional mitochondria in the cell. This scenario may be an explanation for mitochondrial phenotypes, such as respiratory chain dysfunction (Mortiboys *et al.,* 2008; Flinn *et al.,* 2009; Gegg *et al.,* 2009) and elevated mitochondrial DNA mutational load (Piccoli *et al.,* 2008; Rothfuss *et al.,* 2010; Suen *et al.,* 2010) which have been observed in PINK1 or Parkin knockout models as well as PD patients with mutations in either gene (Fig. 31). In accordance with this model, two studies in HeLa cells provided evidence that overexpression of Parkin leads to a significant loss of mitochondria (Narendra *et al.,* 2008; Kawajiri *et al.,* 2010). However, when

the expression of various mitochondrial markers were compared in PINK1-and Parkin-mutant as well as control fibroblasts, no changes indicative of differences in mitochondrial mass were identified. A possible explanation for this discrepancy is that mitophagy is highly selective in an endogenous model and thus does not result in a readily observable reduction in mitochondrial mass (Kanki *et al.,* 2008; Chen *et al.,* 2009).

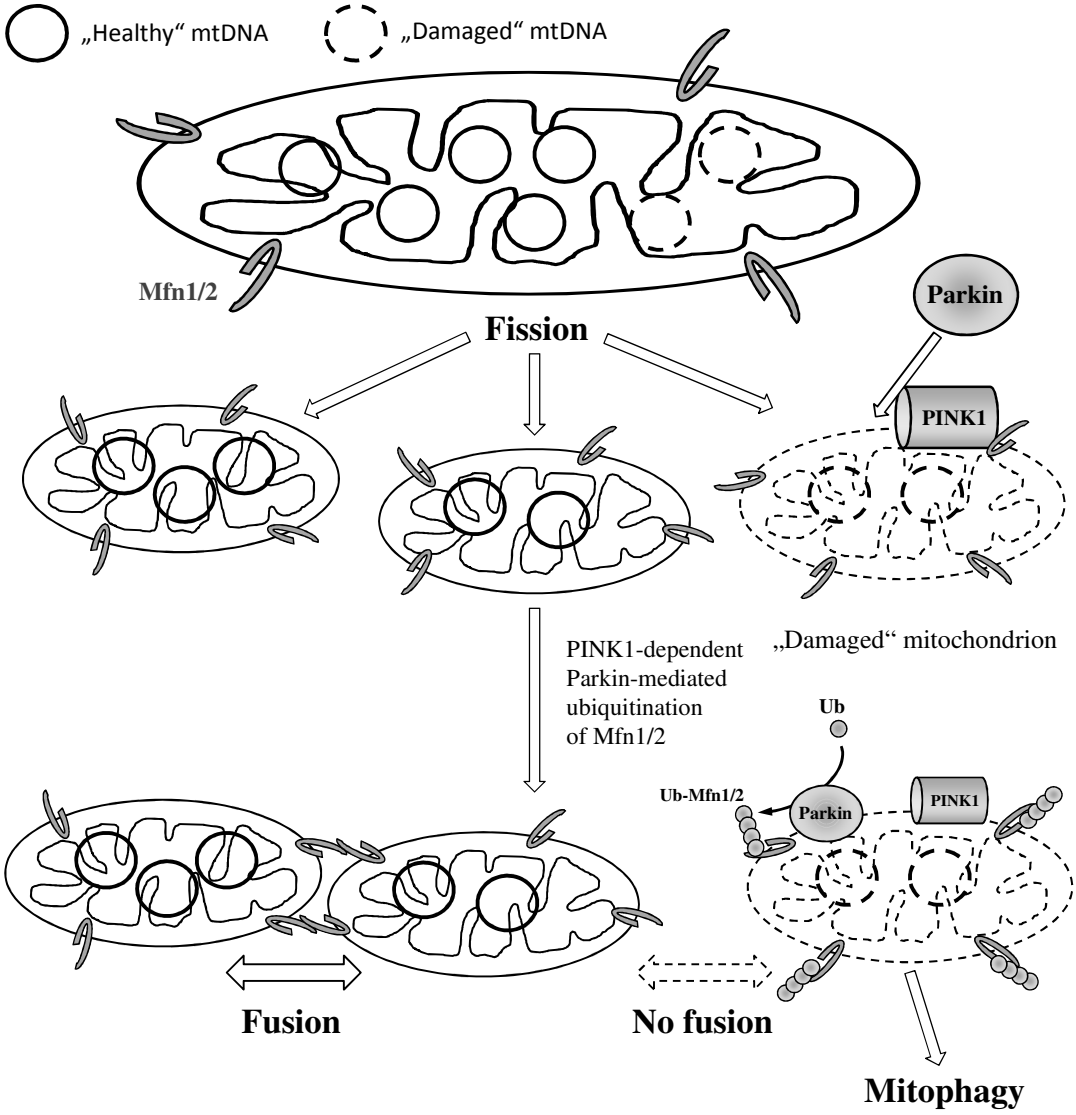

Figure 31. *Scheme summarizing PINK1's and Parkin's putative function in mitophagy*. By means of fission, mitochondria are randomly divided. Damaged mitochondria can be distinguished from functional mitochondria, for instance, by a difference in membrane potential. Dysfunctional mitochondria with low membrane potential are detected by PINK1, which recruits Parkin. Next, Parkin ubiquitinates Mitofusins which localize to the outer mitochondrial membrane. Subsequently, ubiquitinated Mitofusins are degraded by the ubiquitin proteasome system, preventing fusion of dysfunctional with functional mitochondria. By this, dysfunctional mitochondria are selected out from the general pool of mitochondria and subsequently undergo mitophagy. mtDNA-mitochondrial DNA; Mfn1/2 – Mitofusins; PINK1 – PTEN-induced putative kinase 1; Ub-Mfn1/2 – ubiquitinated Mitofusins

Confirming results from arthropode studies and expanding on previous findings, the present thesis provides novel evidence i) that endogenous mutations in PINK1 and Parkin impair ubiquitination of Mitofusins in human fibroblasts and ii) that the UPS is involved in the degradation of Mitofusins under mitochondrial stress conditions.

Although the cause of sporadic Parkinson disease is likely complex, several lines of evidence link mitochondrial dysfunction to its pathogenesis. Mitochondria within the substantia nigra (SN), a midbrain region that is preferentially affected in Parkinson disease, have a higher somatic mitochondrial DNA (mtDNA) mutation rate than all other regions of the brain examined (Soong *et al.*, 1992). Increased mitochondrial damage in the SN, particularly to mtDNA, has been associated with sporadic PD (Bender *et al.*, 2006; Kraytsberg *et al.*, 2006; Schapira, 2008), and mitochondrial dysfunction is sufficient to cause parkinsonism in patients with rare multiple mtDNA deletion syndromes and in animal models with decreased mtDNA expression (Luoma *et al.*, 2004; Baloh *et al.*, 2007; Ekstrand *et al.*, 2007).

Loss of PINK1 and Parkin affects some cell populations, like substantia nigral neurons, more than others, even though PINK1 and Parkin appear to be more widely expressed. Why some tissues are more vulnerable to loss of PINK1/Parkin than others is unclear, but it may relate to the degree of damage mitochondria sustain within that tissue (e.g., mitochondria in the SN are subject to greater oxidative stress than those in other neural tissues (Soong *et al.*, 1992); the existence of redundant mitophagy pathways (e.g., mammalian tissues may contain pathways orthologous to those recently identified in yeast (Kanki *et al.*, 2009; Okamoto *et al.*, 2009); the ability of the tissue to mitigate the damage by other means (a tissue composed of mitotic cells may be able to manage mitochondrial damage through cellular turnover rather than mitochondrial turnover); and mitochondrial demand within a particular tissue (neurons have high, local metabolic demands, and dopaminergic neurons are subject to especially high calcium fluxes that need to be buffered by mitochondria (Chan *et al.*, 2007). Some or all of these factors may contribute to the special reliance of SN neurons on PINK1 and Parkin.

PINK1 and Parkin are a significant cause of autosomal recessive parkinsonism and have been genetically linked to a pathway that protects against progressive mitochondrial damage and dysfunction. We have found that PINK1 levels and subsequently Parkin recruitment to mitochondria are dramatically regulated by the bioenergetic state of individual mitochondria, and that this unique regulation may allow PINK1 and Parkin to promote the selective and efficient turnover of mitochondria that have become damaged. Loss of PINK1 or Parkin function due to pathogenic mutations can disrupt this mitochondrial turnover pathway which may lead to the accumulation of dysfunctional mitochondria in vulnerable tissues—with a

resultant increase in oxidative stress, depression of metabolism, and, eventually, accelerated cell death, which has been observed in *Drosophila* and, to a lesser extent, in mouse models of the disease (Palacino *et al.*, 2004; Whitworth *et al.*, 2005; Park *et al.*, 2006; Yang *et al.*, 2006). Together, these findings provide a biochemical explanation for the genetic epistasis between PINK1 and Parkin observed in Drosophila, and support a novel, testable model of how loss of PINK1 and Parkin function may lead to autosomal recessive parkinsonism.

Mitochondrial dysfunction and changes in mitochondrial morphology have long been linked to the disease mechanisms underlying PD (Langston *et al.,* 1983; Schapira *et al.,* 1989; Palacino *et al.,* 2004; Clark *et al.,* 2006; Park *et al.,* 2006; Gegg *et al.,* 2009). However, only recently, several studies demonstrated that the various observed mitochondrial phenotypes can be ascribed to one common molecular cause: Apparently, a deficit in mitophagy leads to accumulation of dysfunctional mitochondria in the cell (Matsuda *et al.,* 2010; Narendra *et al.,* 2010; Rakovic *et al.,* 2010; Vives-Bauza *et al.,* 2010). The PD-associated proteins PINK1 and Parkin seem to play a central role in the initiation of mitophagy (Gegg *et al.*, 2010; Poole *et al.*, 2010; Ziviani *et al.*, 2010; Gegg *et al.*, 2011). In addition, recently it has been shown that another PD-associated protein DJ-1 may act to maintain mitochondrial function during oxidative stress and thereby alter mitochondrial dynamics and mitophagy in parallel to PINK1 and Parkin (McCoy *et al.*, 2011; Thomas *et al.*, 2011).Within this thesis, human dermal fibroblasts with homozygous PINK1 and Parkin mutations were not only established as a suitable model system to investigate the PINK1/Parkin pathway (Rakovic *et al.,* 2010) but they were also used to characterize effects of the PINK1/Parkin pathway on mitochondrial fusion and fission proteins on the endogenous level.

4.3 Proteomic analysis of overexpressed PINK1

Most biological processes involve the action and regulation of multiprotein complexes. A key goal in most areas of cell biology, therefore, is the characterization of the protein components of multiprotein complexes through reliable identification of specific protein interaction partners. This study has used a global unbiased proteomics approach in combination with the TAP method to identify proteins and pathways that are affected by expression of PINK1 and those that are present in PINK-associated complexes.

To date, out of 14 proteins identified in the present thesis as a potential interacting partners of PINK1, only the interaction between the Hsp90/Cdc37 chaperone system and PINK1 has been described so far (Weihofen *et al.,* 2008; Valente *et al.,* 2009). Cdc37 is a molecular co-

chaperone that functions with Hsp90 to promote folding of kinases (Caplan *et al.,* 2007). With respect to PINK1, the chaperone system was found to influence the protein's subcellular distribution. The authors of this study proposed that the Hsp90/Cdc37/PINK1 complex is destined for a translocation that leads to PINK1 processing, whereas in the absence of Hsp90 in the complex, PINK1 might be attached to mitochondria as full-length precursor (Weihofen *et al.,* 2008). More recently, an interaction between PINK1 and Hsp90 was confirmed. According to the authors, the Hsp90-PINK1 interaction increases mitochondrial PINK1 levels and is required for the dual subcellular distribution of PINK1 (Lin *et al.*, 2010).

Although Western blot results from this thesis are not supporting a direct link between PINK1 and any of the detected mitochondrial proteins, their molecular functions render them interesting targets in the context of PD: GRP75 serves as a major mitochondrial molecular chaperone and plays a key role in the import and partitioning of nuclear-encoded proteins within the two mitochondrial membranes and the matrix (Craig *et al.,* 1987; Voos *et al.,* 2002; D'Silva *et al.,* 2004). Furthermore, GRP75 seems to function in the management of oxidative stress via the PD-associated protein DJ-1. Mutations in DJ-1 were found to weaken the protein's interaction with GRP75 (Li *et al.,* 2005; Jin *et al.,* 2007). GRP75 has also been described as an anti-apoptotic agent. By binding of the transcription regulator p53, GRP75 prevents the formation of the pro-apoptotic p53/Bcl-xL/Bcl-2 complex (Park *et al.,* 2005; Deocaris *et al.,* 2008). Furthermore, putative mutations in *GRP75* were suggested to contribute to the risk of developing PD (De Mena *et al.,* 2009) and a decrease in GRP75 expression was detected in PD patient brains compared to controls (Jin *et al.,* 2006). Hsp60 is a mitochondrial chaperone responsible for the transport of nuclear-encoded proteins via the mitochondrial membranes and their refolding in the matrix (Cheng *et al.,* 1989; Koll *et al.,* 1992) and has been linked to the pathogenesis of Alzheimer disease. Apparently, Hsp60 provides protection against intracellular β-amyloid stress through maintenance of mitochondrial respiratory complex IV activity (Veereshwarayya *et al.,* 2006). Complex IV deficiency in turn, has been implicated in PD (Benecke *et al.*, 1993; Schapira, 1994) opening the possibility for a role of Hsp60 in the pathogenesis of the disease. EFTu is part of the translational apparatus of mitochondria. During protein biosynthesis, it mediates the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes (Ling *et al.,* 1997). Additional described functions of EFTu comprise recognition and translocation of co-translationally damaged proteins to the proteasome (Chuang *et al.,* 2005), rearrangement of cytoskeletal components (Shiina *et al.,* 1994; Gross *et al.,* 2005), and regulation of cell survival (Tong *et al.,* 2005). Mutations in the *EFTu* gene cause combined oxidative

phosphorylation deficiency type 4 due to decreased mitochondrial protein synthesis (Valente *et al.,* 2007). Interestingly, however, there is also a report connecting EFTu and PD, where EFTu was found to co-immunoprecipitate with leucin-rich repeat kinase 2 which is encoded by the PARK8 gene *LRRK2*. Co-incubation with recombinant EFTu reduced the kinase activity of LRRK2, whereas the GTPase activity remained unchanged (Gillardon, 2009).

Among the identified PINK1 interactors, LRPPRC is the only protein which is unequivocally linked to a neurodegenerative disorder. Mutations in *LRPPRC* are the cause of the French-Canadian type of Leigh syndrome (LSFC). LSFC patients suffer from progressive focal necrotizing lesions of the brainstem, basal ganglia, and cerebellum, accompanied by capillary proliferation. Besides metabolic acidosis, clinical features include generalized developmental delay, cerebellar signs, and a striking paucity of facial and limb movement, as well as hypomimia (Cooper *et al.,* 2006). LRPPRC has been linked to cytochome C oxidase deficiency. Mutations in the gene lower *MT-CO1* and *MT-CO3* mRNA levels and, in turn, impair complex IV assembly (Mootha *et al.,* 2003; Xu *et al.,* 2004). Recent functional studies further strengthen the link between LRPPRC and mitochondrial RNA metabolism (Sasarman *et al.,* 2010; Sondheimer *et al.,* 2010). The presence of parkinsonian signs in LSFC patients was a rationale to compare LRPPRC protein levels between controls and PD patients carrying mutations in PINK1. Furthermore, LRPPRC was identified as a component of the PGC-1α complex which itself is also linked to energy homeostasis in the cell (Cooper *et al.,* 2006). Like in the case of Hsp60, LRPPRC's impact on the respiratory chain offers a potential connection with PD. It should be noted that GRP75, Hsp60 and LRPPRC have been identified by proteomic analysis as potential interactors of Parkin earlier (Davison *et al.*, 2009). Recently it has been reported that Parkin regulates cellular levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Shin *et al.*, 2011) strengthening the relation between PINK1, Parkin, PGC-1α, and LRPPRC and their role in pathogenesis of PD.

In the present thesis, TAP technology was employed for the first time to identify PINK1-associated proteins. These experiments resulted in a list of 14 putative PINK1 binding partners, confirming two reported interactions (Hsp90 and Cdc37), but also introducing four novel mitochondrially localized proteins (GRP75, Hsp60, LRPPRC or EFTu) as potential components of the PINK1/Parkin mitophagy pathway. Although preliminary results from protein expression do not strengthen a link between the PINK1/Parkin pathway and any of these interactors, it cannot be excluded that their connection with the pathway may be more complex. Additional protein function studies, for instance under mitochondrial stress conditions, will be needed to fully characterize this potential link.

4.4 Conclusions

Project 1:

PINK1 regulates subcellular localization of Parkin and its mRNA and protein levels.

Gene expression analysis confirmed that the nonsense mutation c.1366C>T (p.Q456X) leads to almost complete degradation of the *PINK1* mRNA caused by NMD (cp. Fig.8A, p.25). Therefore, c.1366T exerts its pathogenic effect most likely as a functional null allele.
Using fibroblasts containing a homozygous c.1366T mutation it has been clearly shown that PINK1 is necessary for the valinomycin- and H_2O_2-induced mitochondrial translocation of Parkin (cp.Fig.10, p. 28). By means of Western blotting and immunostaining it was demonstrated that in cells lacking PINK1 endogenous and overexpressed Parkin fail to be translocated to mitochondria upon stress (cp.Fig.12, p. 30).
In addition, PINK1 is necessary for the stress-induced loss of endogenous Parkin (cp. Fig.7, p. 25). Upon valinomycin- and H_2O_2-induced stress, loss of endogenous Parkin has been detected only in the presence of PINK1. In cells lacking PINK1, the stress-induced loss of Parkin could be restored by exogenously expressed wild type PINK1 (cp. Fig.9, p. 26).
Furthermore, it has been shown by means of qPCR that *Parkin mRNA* levels were comparable between controls and cells lacking of PINK1 (cp. Fig.8B, p. 25) suggesting that PINK1 does not regulate *Parkin mRNA* levels.

PD-causing mutations in PINK1 impair mitochondrial translocation of Parkin.

Fibroblasts carrying a homozygous missense PD mutation c.509T>G (p.V170G) in PINK1 had an identical phenotype regarding the subcellular localization and protein levels of Parkin as PINK1 “null” cells. Therefore, pathogenic PD mutations in PINK1and not only loss of PINK1 impair the stress-induced mitochondrial translocation of Parkin and the stress-induced loss of endogenous Parkin. Taken together, wild-type PINK1 is necessary for two different, parallel processes, i.e. the stress-induced mitochondrial translocation of Parkin and regulation of Parkin levels via the UPS.

Project 2:

PINK1 and Parkin regulate protein levels of mitochondrial fusion factors Mfn1 and Mfn2.

In this thesis it has been shown that PINK1 and Parkin regulate protein levels of Mfn1 and Mfn2 through their ubiquitination and most likely degradation via the UPS. This is a three-

step process: i) PINK1-dependent mitochondrial translocation of Parkin, ii) Parkin-mediated ubiquitination of Mfn1 and Mfn2, and iii) degradation of ubiquitinated Mfn1 and Mfn2 via the UPS.

Parkin-mediated PINK1-dependent ubiquitination of Mfn1 and Mfn2 is impaired by PD-causing mutations in both PINK1 and Parkin.

Pathogenic mutations in PINK1 (p.Q456X and p.V170G) and Parkin (p.V324fsX434 and p.R245fsX253) impair the stress-induced ubiquitination of Mfn1 and Mfn2 (cp. Fig.17, p. 37). In Project 1 it was shown that PD mutations in PINK1 impair mitochondrial translocation of Parkin and therefore no ubiquitination of Mfn1 and Mfn2 was detected. In Parkin mutants, absence of functional Parkin is most likely the reason for impairment of Mfn1 and Mfn2 ubiquitination. The ubiquitination of Mfn1 and Mfn2 in PINK1 and Parkin mutants was restored by exogenous expression of wild type PINK1 and Parkin, respectively (cp. Fig.20, p. 39).

Project 3:

Newly discovered interacting partners of PINK1 contribute to a better understanding of the PD pathology.

Several proteins identified in this study as potential interactors of PINK1 are involved in maintenance of mitochondrial homeostasis. Identification of LRPPRC as a potential fuctional interactor could explain one of the most consistent findings in idiopathic and familial PD: dysfunction of the electron transport chain.

5 PERSPECTIVES

This thesis addressed the molecular characterization of the PINK1/Parkin pathway. Together with recent reports, this study has led to a model in which PINK1, a mitochondrially localized serine/threonine kinase, accumulates selectively in depolarized mitochondria, where it recruits Parkin, a cytosolic E3 ubiquitin ligase. Additional studies about the mechanism(s) how PINK1 induces Parkin recruitment, and further characterization of this pathway will furthermore improve our understanding of the pathophysiology of PD.

When comparing the phenotype of homozygous *PINK1* mutation carriers and healthy controls under basal conditions regarding the subcellular localization of Parkin and its protein levels, no difference was detected and only appeared after applying valinomycin or H_2O_2. Additional studies, considering the influence of environmental factors on the development of the disease, will be necessary to further clarify the pathophysiology of PD.

When translocated to mitochondria, Parkin promotes the ubiquitination of the mitochondrial fusion-promoting factors Mfn1 and Mfn2. Ubiquitin-mediated inactivation (or degradation via UPS) of Mitofusins on damaged mitochondria after a fission event might serve to produce fusion-incompetent mitochondria that are prevented from reentering the undamaged mitochondrial network. To further characterize this effect, mitochondria of healthy individuals could be analyzed upon valinomycin-induced stress for other ubiquitinated proteins. Furthermore, comparing these results with findings obtained from PINK1 mutants might lead to the identification of novel specific members of this pathway.

The final step of this multi-step process is the destruction of depolarized mitochondria through an autophagic mechanism. However, the mechanism of mitochondrial autophagy (mitophagy) is mainly unknown. Further investigations should include another PD protein, i.e. the lysosomaly localized ATP13A2. Since lysosomal degradation is the final step in autophagy, a potential lysosomal impairment due to mutations in ATP13A2 would include the third PD protein in the common pathway together with PINK1 and Parkin.

Proteomic analysis of overexpressed PINK1 using the TAP method identified 14 proteins, four of which are mitochondrially localized proteins (GRP75, Hsp60, LRPPRC or EFTu). Since PINK1 is a mitochondrially localized protein kinase, applying the TAP method on the concentrated mitochondrial fraction but not on the whole cell lysate, might improve the chances to identify less abundant PINK1 protein interactors and to reduce the background. In addition, usage of protein-protein crosslinkers might be of help to identify transient and less stable protein complexes. An alternative method would be a proteomic analysis of

phosphorylated proteins in fibroblasts from healthy individuals and in fibroblasts from PINK1 mutation carriers.

Taken together, the final goal of future studies would be finding of (a) common pathway(s) that include(s) all or at least most of the genes linked to PD. This pathway(s) would than reveal one or more potential therapeutical targets for causally treating PD.

6 SUMMARY

Recessively inherited Parkinson disease (PD), a clinical syndrome closely resembling idiopathic PD, can be caused by mutations in the *PINK1 (PTEN-induced putative kinase 1)* and *Parkin* gene. The clinical features associated with Parkin and PINK1 mutations are indistinguishable from one another; likewise, loss of *Drosophila* parkin shows phenotypes similar to loss of pink1 function. In agreement with these observations, Parkin and PINK1 have been shown to function, at least in part, in the same pathway with PINK1 acting upstream of Parkin. In addition, null mice for either Parkin or PINK1 exhibit increased oxidative damage and decreased mitochondrial function in the striatium and primary cells from patients with loss-of function mutations in Parkin or PINK1 have similar abnormalities.

The human dermal fibroblasts from healthy individuals and from PD patients carrying the homozygous mutations in PINK1 (c.509T>G; p.V170G or c.1366C>T; p.Q456X) or Parkin (c.1072delT; p.V324fsX434 or delEx7; p.R245fsX253) were used to study the relation between PINK1 and Parkin and their role in process of mitochondrial fusion/fission. In addition the human embryonic kidney (HEK) cells were used for the proteomic analysis of overexpressed PINK1. The cells were analysed under basal conditions and upon valinomycin- or hydrogen peroxide (H_2O_2) - induced stress by means of Western blotting, Immunocytochemistry, Immunoprecipitation, Tandem affinity purification (TAP) and subcellular fractionation.

In Project 1 two specific hypotheses were addressed: (i) *PINK1 regulates subcellular localization of Parkin and its mRNA and protein levels,* (ii) *PD-causing mutations in PINK1 impair mitochondrial translocation of Parkin.* The effect of pathogenic mutations in PINK1 on protein levels of Parkin and its subcellular localisation was analysed in controls and PINK1 mutants under basal conditions and upon stress. Western blot analysis showed the stress-induced loss of endogenous Parkin only in controls but not in PINK1 mutants. The observed loss of Parkin could be prevented by inhibitors of the ubiquitin proteasome system (UPS). Immunostaining and subcellular fractionation showed a mitochondrial translocation of Parkin upon stress only in controls but not in PINK1 mutants. Taken together, the stress-induced proteasomal degradation of Parkin and mitochondrial translocation of Parkin are two processes that take place simultaneously. Furthermore, functional, wild type PINK1 is necessary for both of them. Additional studies are needed to further characterize the PINK1-dependent mitochondrial recruitment of Parkin and its proteasomal degradation.

Regarding the role of PINK1/Parkin pathway on mitochondrial dynamic, the following hypotheses were formulated in Project 2: (i) *PINK1 and Parkin regulate protein levels of*

mitochondrial fusion factors Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2). (ii) *Parkin-mediated PINK1-dependent ubiquitination of Mfn1 and Mfn2 is impaired by PD-causing mutations in both PINK1 and Parkin.* To examine the protein levels of mitochondrial fusion factors Mfn1 and Mfn2 in fibroblasts from controls, PINK1 mutation carriers and Parkin mutation carriers were analyzed under basal conditions and upon stress by means of Western blotting. When compared to non-stressed cells, protein levels of both Mfn1 and Mfn2 were reduced only in controls but not in PINK1 or Parkin mutants. Furthermore, an additional band on the Western blot, which was about 8kDa larger in size than the non-modified form, consistent with possible monoubiquitination of the protein was detected only in controls. Immunoprecipitation followed by Western blotting with an antibody against ubiquitin showed that Mfn2 is ubiquitinated via the PINK1/Parkin pathway and is impaired by PD-causing mutations PINK1 and Parkin. Observed ubiquitination was prevented by UPS inhibition suggesting that UPS is involved in processing of Mfn1 and Mfn2. Taken together this suggest that the stress-induced, PINK1-dependent, Parkin-mediated ubiquitination of Mfn1 and Mfn2 present initial step in process of mitophagy. Furthermore this is impaired by pathogenic mutations in PINK1 or Parkin.

The stable loss or knockdown of PINK1 in mammalian cellular models and mice leads to a number of mitochondria-related abnormalities. However, the mechanism how PINK1 regulates mitochondrial functions is still unknown. Regarding the molecular role of PINK1, the following hypotheses were formulated in Project 3: *Newly discovered interacting partners of PINK1 contribute to a better understanding of the PD pathology.* To identify proteins that form complexes with PINK1, HEK cells transiently transfected with a mammalian vector expressing a “TAP tagged” PINK1 were used. After affinity purification, resulting PINK1 complexes were characterising by mass spectroscopy (MS). Out of 14 proteins identified by this method, four were mitochondrially localized (GRP75, Hsp60, LRPPRC and EFTu). Since PINK1 is a mitochondrial protein kinase these proteins were of the particular interest. However, when their protein levels were compared between control fibroblasts and those of PINK1- or Parkin-mution carriers no difference was found. Among the identified PINK1 interactors, LRPPRC is the only protein which is unequivocally linked to a neurodegenerative disorder. Mutations in this gene lower MT-CO1 and MT-CO3 mRNA levels and, in turn, impair complex IV assembly. Additional studies regarding phosphorylation level of LRPPRC are needed.

The natural extension of this thesis would be the search for (a) common pathway(s) that include(s) all or at least most of the genes linked to PD. This pathway(s) would than reveal one or more potential therapeutical targets for causally treating PD.

7 ZUSAMMENFASSUNG

Das rezessiv vererbte Morbus Parkinson (MP) ähnelt klinisch dem idiopathischen MP und kann durch Mutationen in den Genen *PINK1 (PTEN-induced putative kinase 1)* und *Parkin* verursacht werden. Die klinischen Symptome des *Parkin*- und *PINK1*-assoziierten MP lassen sich ebenfalls nicht voneinander unterscheiden; darüber hinaus zeigten die Drosophila-Modelle mit einem Funktionsverlust des Parkin- bzw. PINK1-Proteins vergleichbare Phänotypen. In Übereinstimmung mit diesen Beobachtungen konnte gezeigt werden, dass die Funktion von Parkin und PINK1 z. T. in einem gemeinsamen Signalweg liegt, wobei Parkin in Abhängigkeit von PINK1 agiert. In Parkin bzw. PINK1 *knockout*-Mäusen wurde zudem eine verringerte Funktion der Mitochondrien und eine erhöhte oxidative Schädigung im Striatum festgestellt, Auffälligkeiten, die auch bei primären Zellen von Patienten mit *loss-of-function* Mutationen beobachtet wurden.

Humane Fibroblasten von gesunden Individuen und MP-Patienten mit homozygoten *PINK1*-Mutationen (c.509T>G; p.V170G oder c.1366C>T; p.Q456X) oder Parkin (c.1072delT; p.V324fsX434 oder delEx7; p.R245fsX253) wurden verwendet, um die Beziehung zwischen PINK1 und Parkin sowie deren Rolle im mitochondrialen *Fusion/Fission*-Prozess zu untersuchen. Zusätzlich wurden *human embryonic kidney* (HEK)-Zellen zur Proteom-Analyse bei überexprimiertem PINK1 verwendet. Die Zellen wurden unter basalen Bedingungen und nach Zugabe von Valinomycin oder Wasserstoffperoxid (H2O2) mittels Westernblot, Immunfärbung, Immunpräzipitation, *Tandem affinity purification* (TAP) und subzellulärer Fraktionierung analysiert.

Für das erste Projekt wurden im Rahmen der vorliegenden Arbeit zwei Hypothesen formuliert: (i) *PINK1 reguliert die subzelluläre Lokalisation von Parkin sowie dessen mRNA- und Proteinlevel,* (ii) *Mutationen im PINK1-Gen stören die mitochondriale Translokation von Parkin*. Der Effekt von pathogenen *PINK1*-Mutationen auf Proteinlevel und subzelluläre Lokalisation von Parkin wurde in Kontrollen und Zellen mit mutiertem PINK1 unter basalen Bedingungen und nach Zugabe der o.g. Stressoren analysiert. Mittels Westernblot konnte ein durch Stress verursachter Verlust endogenen Parkin-Proteins nur bei den Kontrollzellen beobachtet werden, nicht aber bei den Zellen mit einer PINK1-Mutation. Durch Hemmung des Ubiquitin-Proteasom-Systems (UPS) konnte dieser Verlust bei den Kontrollen verhindert werden. Mithilfe von Immunofärbung und subzellulärer Fraktionierung wurde zudem eine Stress-abhängige mitochondriale Translokation von Parkin in den Kontrollzellen gezeigt. Es kann daraus geschlussfolgert werden, dass der proteasomale Abbau und die Translokation von Parkin simultan unter Stressbedingungen ablaufen. Zusätzliche Studien sind notwendig, um

diese beiden von der Funktion des PINK1-Proteins abhängigen Prozesse weiter zu charakterisieren.

In Bezug auf die Rolle des PINK1/Parkin-Signalweges bei den dynamischen Prozessen von Mitochondrien wurden für das zweite Projekt der vorliegenden Dissertation folgende Hypothesen formuliert: (i) *PINK1 und Parkin regulieren die Proteinlevel der mitochondrialen Fusionsfaktoren* Mitofusin 1 (*Mfn1*) *und* Mitofusin 2 *(Mfn2).* (ii) *Die in Anhängigkeit von PINK1 gesteuerte Ubiquitinierung von Mfn1 bzw. Mfn2 durch Parkin wird durch Mutationen in einem der beiden Gene gestört.* Um die Proteinlevel von Mfn1 und Mfn2 in Fibroblasten von Kontrollen, *PINK1*- und *Parkin*-Mutationsträgern zu untersuchen, wurden die Zellen unter basalen Bedingungen und nach Zugabe von Stressfaktoren mittels Westernblot analysiert. Im Vergleich zu den basalen Bedingungen waren die Proteinlevel von Mfn1 und Mfn2 nur bei den Kontrollfibroblasten reduziert. Darüber hinaus wurde bei den Kontrollen eine zusätzliche Bande identifiziert, die ein um ca. 8kDa größeres Molekulargewicht hatte als „normales“ Mfn und damit eine Monoubiquitinierung des Proteins bedeuten könnte. Durch Immunpräzipitation und anschließenden Westernblot mit einem Ubiquitin-Antikörper wurde gezeigt, dass Mfn2 über den PINK1/Parkin-Signalweg ubiquitiniert wird, und dass dieser Prozess durch eine Mutation in einem der beiden Gene gestört ist. Eine Inhibierung des UPS hatte wiederum zur Folge, dass die beobachtete Ubiquitinierung ausblieb, woraus man auf einen Einfluss des UPS bei der Prozessierung von Mfn1 und Mfn2 schließen kann. Die in Anhängigkeit von PINK1 gesteuerte Ubiquitinierung von Mfn1 bzw. Mfn2 durch Parkin stellt damit einen initialen Schritt beim Prozess der Mitophagie dar, ein Prozess, der durch pathogene Mutationen in PINK1 und Parkin gestört wird.

Der Verlust von PINK1 in Zellen von Säugetieren und Mausmodellen führte zu einer Reihe von Auffälligkeiten in den Mitochondrien. Durch welchen Mechanismus PINK1 die Funktion der Mitochondrien reguliert ist jedoch unbekannt. In Bezug auf die molekulare Rolle von PINK1 wurden folgende Hypothese im Rahmen des dritten Projekts dieser Arbeit formuliert: *Neu entdeckte Interaktionspartner von PINK1 tragen zu einem besseren Verständnis der Pathologie des MP bei*. Um Proteine zu identifizieren, die einen Komplex mit PINK1 bilden, wurden HEK-Zellen mit einem Vektor transient transfiziert, der TAP-tagged PINK1 exprimiert. Nach Affinitätsaufreinigung wurden entstandene PINK1-Komplexe mittels Massenspektroskopie (MS) charakterisiert. Von den 14 Proteinen, die dadurch identifiziert werden konnten, waren vier im Mitochondrium lokalisiert (GRP75, Hsp60, LRPPRC und EFTu), was angesichts der mitochondrialen Proteinkinase PINK1 von besonderem Interesse war. Ein Unterschied in Bezug auf die Proteinlevel zwischen Kontrollen und den Fibroblasten

mit einer *Parkin*- oder *PINK1*-Mutation, wurde allerdings nicht gefunden. Innerhalb der Gruppe der identifizierten Interaktionspartner ist LRPPRC das einzige Protein, das eindeutig mit einer neurodegenerativen Erkrankung verbunden ist. Mutationen in diesem Gen verringern die mRNA-Level von MT-CO1 bzw. MT-CO3 und stören dadurch die Zusammenlagerung von Komplex IV. Zusätzliche Studien bezüglich der Phosphorylierung von LRPPRC sind erforderlich.

Eine sinnvolle Fortsetzung der vorliegenden Arbeit wäre die Suche nach einem gemeinsamen Signalweg, in welchem alle oder zumindest die meisten der Gene vorkommen, die mit monogenetischem MP assoziiert wurden. Dieser Signalweg könnte ein oder mehrere therapeutische Ziele aufdecken, die wichtig für die ursächliche Behandlung von MP sind.

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9 APPENDIX

9.1 List of abbreviations

AD	Autosomal dominat
AR	Autosomal recessive
ATP	Adenosine triphosphate
ATP13A2	ATPase type 13A2
Bcl	B-cell lymphoma
BSA	Bovine serum albumine
CBP	Calmodulin binding peptide
cDNA	Copy (complementary) DNA
Cy	Cytosolic
del	Deletion
DJ-1	Amyotrophic lateral sclerosis-parkinsonism/dementia complex 2
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Drp1	Dynamin-related protein 1
DYT1	Dystonia type 1
$\Delta\Psi$	Mitochondrial membrane potential
ER	Endoplasmatic reticulum
ETC	Electron transport chain
Ex	Exon
Fab	Fragment antigen binding
Fc	Fragment, crystallizable region

FBX07	F-box protein 7
Fis1	Fission 1 (mitochondrial outer membrane) homolog (S. cerevisiae)
FL	Full-length
fs	Frameshift
GIGYF2	Grb10-Interacting GYF Protein-2 (TNRC15)
GRP75	Glucose- regulated protein 75
GRP78	Glucose- regulated protein 78
GTP	Guanosine triphosphate
HDAC6	Histone deacetylase 6
HEK	Human embryonic kidney
His	Histidine
HRP	Horseradish peroxidase
Hsp60	Heat shock 60kDa protein
Hsp90	Heat shock protein 90
IBR	In between ring
IgG	Immunoglobulin G
LB	Lysogeny broth
LC3	Microtubule-associated protein 1 light chain 3
LRPPRC	Leucine-rich PPR-motif containing
LRRK2	Leucine-rich repeat kinase 2
LSFC	French-Canadian type of Leigh syndrome
Mi	Mitochondrial
Mfn1	Mitofusin 1

Mfn2	Mitofusin 2
MG132	Z-Leu-Leu-Leu-CHO
MPP^+	Methyl-4-phenyl pyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin
mRNA	Messenger ribonucleic acid
MS	Mass spectroscopy
MT-CO1	Subunit I of the mitochondrial cytochrome c oxidase
MT-CO3	Subunit III of the mitochondrial cytochrome c oxidase
mtDNA	Mitochondrial Deoxyribonucleic acid
MTS	Mitochondrial targeting signal
Mut	Mutant
NADH	Nicotinamidadenindinukleotid
NMD	Nonsense-mediated mRNA decay
NT	Non-treated
Omi/HtrA2	HtrA serine peptidase 2
Opa1	Optic atrophy 1
ORF	Open reading frame
PCR	Polymerase chain reaction
PD	Parkinson disease
PFA	Paraformaldehyde
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PINK1	PTEN-induced-putative kinase
PLA2G6	Phospholipase A2, group VI

qPCR	Quantitative real-time polymerase chain reaction
RING	Really Interesting New Gene
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
SBP	Streptavidin binding peptide
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
SN	Substantia nigra
SYBR	Synergy Brands, inc.
TAP	Tandem affinity purification
TBST	Tris-Buffered Saline and Tween 20
TFAM	Transcription factor A, mitochondrial
TM	Transmembrane domain
TNF	Tumor necrosis factor
TRAP1	TNF receptor-associated protein 1
EFTu	Elongation factor thermo unstable
Ub	Ubiquitin
UPS	Ubiquitin proteasome system
VDAC1	Voltage-dependent anion channel 1
WT	Wild-type
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polipeptide

9.2 Curriculum vitae

BIOGRAPHICAL INFORMATION

Date of Birth: 10/06/1976

Place of Birth: Belgrade, Serbia

Marital Status: single

EDUCATION

10/ 1995- 02/ 2006	Student at the Faculty of Biology, University of Belgrade.
02/ 2006	Graduated in Molecular Biology and Physiology (University Diploma), University of Belgrade, Faculty of Biology, Average grade: 8.83/10.
09/ 2006- 11/ 2007	PhD student at the Faculty of Biology, University of Belgrade.
11/ 2007- Present	PhD student at the University of Luebeck.

SCIENTIFIC TRAINING

01/ 2005 – 02/ 2006	Department of Radiobiology and Molecular Genetics, Institute of Nuclear Sciences, Belgrade, Serbia. Diploma thesis, mark: 10/10.
02/ 2006- 02/2007	Department of Radiobiology and Molecular Genetics, Institute of Nuclear Sciences, Belgrade, Serbia.Engaged in projects related to the molecular genetics of cardiovascular diseases: detection of polymorphisms in genes related to cardiovascular diseases (LPL, CTLA4, ACE, ApoE, MMP-9, IL-6, MCP-1), engaged in projects related to renal diseases - CAKUT (AT1, AT2, AT1R).
02/2007- 11/ 2007	Visiting researcher at Department of Neurology, University of Lübeck, Lübeck, Germany.Engaged in projects related to the molecular genetics and functional studies of various movement disorders (TorsinA, PINK1).

11/ 2007- 05/ 2011	University of Lübeck, Department of Neurology/Neurogenetics (Laboratory of Professor C. Klein), Lübeck. PhD studies (current position). Molecular characterization of PINK1/Parkin pathway in PD.

FELLOWSHIP AND GRANT SUPPORT

02/ 2006- 02/ 2007	Engaged on a project "Genetic epidemiology and pharmacogenomics of vascular disease" (PI: Dragan Alavantić, PhD) supported by grant #145023 from Serbian Ministry of Science and Technology.
02/2007- 07/ 2007	Scholarship from Department of Neurology, University of Lübeck, Lübeck, Germany.
08/ 2007- 10/ 2007	Deutscher Akademischer Austauch Dienst (DAAD) Foundation's Kontaktstipendium.

LANGUAGE SKILLS

English	Excellent knowledge
German	Good knowledge
Russian	Basic knowledge

9.3 Publications

ORIGINAL ARTICLES

1. Osmanovic, A., A. Dendorfer, A. Erogullari, N. Uflacker, D. Braunholz, **A. Rakovic**, et al. Truncating mutations in THAP1 define the nuclear localization signal. Mov Disord 2011; [Epub ahead of print]. (IF: 4.014)
2. **Rakovic, A.**, A. Grünewald, L. Voges, S. Hofmann, S. Orolicki, K. Lohmann, et al. PINK1-Interacting Proteins: Proteomic Analysis of Overexpressed PINK1. Parkinsons Dis 2011; 2011: 153979. (no IF)
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Up to now eleven original articles were published, among these four are first author papers. The publications have a cumulative impact factor (IF) of 65.8 and an average of 6.6.

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ORAL PRESENTATIONS

1. **Rakovic, A**., A. Grünewald, P. Seibler, A. Ramirez, S. Orolicki, K. Lohmann, C. Klein. Effect of endogenous mutant and wild-type PINK1 on Parkin in fibroblasts from Parkinson disease patients. DGN Neurowoche 2010.

9.4 Acknowledgements

The work presented in this thesis was performed in the Section of Clinical and Molecular Neurogenetics under the supervision of Professor Christine Klein at the University of Lübeck, Lübeck, Germany.

I am indebted to my supervisor Professor Christine Klein for her excellent supervision, advice, and support throughout my time of research and while writing this thesis.

I thank all the members of the Section of Clinical and Molecular Neurogenetics for all their help over the past four years and for making it an enjoyable time.

I would like to thank Professor Enno Hartmann, the Head of the Institute of Biology, for providing an anti-PINK1 antibody.

Finally, I thank Anne Weißbach for all support and love.

9.5 Declaration

Ich versichere, dass ich die Dissertation ohne fremde Hilfe angefertigt und keine anderen als die angegebenen Hilfsmittel verwendet habe.
Weder vor noch gleichzeitig habe ich andernorts einen Zulassungsantrag gestellt oder diese Dissertation vorgelegt.
Ich habe mich bisher noch keinem Promotionsverfahren unterzogen.

Aleksandar Raković Lübeck, 17.05.2011