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Molecular characterization of Parkin-associated parkinsonism in a human cellular model

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AD	–	Autosomal dominat
AR	–	Autosomal recessive
ATP	–	Adenosine triphosphate
ATP13A2	–	ATPase type 13A2
CI, CII, CIII,CIV, CV	–	Complex I, complex II, complex III, complex IV, complex V
CCCP	–	Carbonyl cyanide *m*-chlorophenyl hydrazone
cDNA	–	Copy (complementary) DNA
CI	–	Confidence interval
Con	–	Control
CoQ1	–	Ubiquinone
$CoQH_2$	–	Ubiquinol
COX	–	Cytochrome oxidase
COX II	–	Cytochrome oxidase subunit II
CS	–	Citrate synthase
$\Delta\psi m$	–	Mitochondrial membrane potential
DMSO	–	Dimethyl sulfoxide
DNA	–	Deoxyribonucleic acid
Drp1	–	Dynamin-related protein 1
DTT	–	Dithiothreitol
EPS	–	Extrapyramidal system
$FADH_2$	–	Flavin adenine dinucleotide (hydroquinone form)
FBXO	–	F-box protein 7
Fis1	–	Fission factor 1 (mitochondrial outer membrane) homolog (S. cerevisiae)
FL	–	Fluorescence emission
GAK	–	Cyclin G-associated kinase
GBA	–	Glucosidase, beta, acid; Glucocerebrosidase
GD	–	Gaucher disease
GIGYF2	–	GRB10-Interacting GYF protein 2
GPi/e	–	Internal/external globus pallidus
GWAS	–	genome-wide association study

HLA-DRA	–	Major histocompatibility complex, class II, DR alpha
IBR	–	In-between-RING domain
IQR	–	Interquartile range
KCN	–	Potassium cyanide
L-Dopa	–	Levo-dopa
LRRK2	–	Leucine-rich repeat kinase 2
LRPPRC	–	Leucin-rich pentatricopeptide repeat cassette
LSFC	–	Leigh syndrome, French-Canadian type
MAPT	–	Microtubule-Associated Protein Tau
Mfn1/2	–	Mitofusin 1/2
$MgCl_2$	–	Magnesium dichloride
MGP	–	Monogenic parkinsonism
Min	–	Minutes
MPTP	–	1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine
mRNA	–	Messenger ribonucleic acid
mtDNA	–	Mitochondrial DNA
Mut	–	Mutant
NADH	–	Nicotinamide adenine dinucleotide
NBIA	–	Neurodegeneration with brain iron accumulation
Omi/HtrA2	–	HtrA serine peptidase 2
OPA1	–	Optic atrophy 1
OR	–	Odds ratio
OXPHOS	–	Oxidative phophorylation
PAGE	–	Polyacrylamide gel electrophoresis
PACRG	–	Parkin co-regulated gene
PCR	–	Polymerase chain reaction
PD	–	Parkinson’s disease
PINK1	–	PTEN-induced putative kinase 1
PLA2G6	–	Phospholipase A2, group VI
PS	–	Parkinson-Syndrome
qPCR	–	Quantitative PCR
RC	–	Respiratory chain
RING	–	Really interesting gene
ROS	–	Reactive oxygen species

RT	–	Room temperature
S	–	Seconds
SF	–	Susceptibility factor
SN	–	Substantia nigra
SNCA	–	Alpha-synuclein
SNP	–	Single nucleotide polymorphism
STD	–	Standard deviation
SYBR	–	Synergy Brands, Inc.
TAP	–	Tandem affinity purification
TFAM	–	Mitochondrial transcription factor A
UBL	–	Ubiquitin-like
UCH-L1	–	Ubiquitin C-terminal hydrolase-L1
UPDRS	–	Unified Parkinson's Disease Rating Scale
UPS	–	Ubiquitin proteasomal system
X	–	X-chromosomal

1 INTRODUCTION

The present thesis investigates the movement disorder Parkinson's disease (PD) from two different angles. First, the function of the disease-linked protein Parkin was studied in fibroblasts from patients and controls. Second, a mutational screening of *LRPPRC*, a gene potentially linked to PD, was performed.

The clinical features of PD in general (1.1) and specific phenotypic aspects of *Parkin*-associated PD (1.1.1) will be described. The current state of knowledge of the *Parkin* gene and its encoded protein (1.1.2) will also be given. The following section (1.2.1) introduces the system of oxidative phosphorylation and its attributes. Subsequently, findings from the literature linking mitochondrial function and PD are summarized (1.2.2). Next, the common pathway of Parkin and PINK1 and its role in the pathogenesis of recessive PD is outlined (1.2.3). In chapter 1.2.4 the putative interaction partners of Parkin are listed and their relevance is explained. Then, the link between the gene *LRPPRC* and PD is elucidated (1.2.5). In the last part of this introduction, the choice of the applicated cell model is explained (1.2.6).

1.1 Parkinsonism and Parkinson's disease

The first detailed description of the disease was provided in 1817 in James Parkinson's (1755–1824) publication "An essay on the shaking palsy" (Parkinson 2002). For years his achievements were not rewarded with any scientific attention until the French neurologist Jean-Martin Charcot (1825–1893) redefined the syndrome characterized by the triad of symptoms, bradykinesia, rigidity and rest tremor and named it after its first descriptor "maladie de Parkinson" (PD). Leroux and Lhirondel, two of Charcot's students at the Hôpital de la Salpêtrière in Paris, were the first who suggested a heritable component in the etiology of PD, "a true cause of paralysis agitans, and may be the only true cause, is heredity" (Leroux 1880). Since this article, numerous reports dealing with hereditary "parkinsonism" were published and led to the important breakthrough in research on genetic parkinsonism. The first discovery of a single gene mutation causing parkinsonism in a family dates back to 1997.

The term parkinsonism is used for the classification of all movement disorders with parkinsonian features independent of their etiology (Table 1). The designation "parkinsonism" encompasses the combination of the symptoms bradykinesia, rigidity, rest

tremor and postural instability with a therapeutic response to levo-dopa (L-dopa) and the development of motor complications. PD is the most common form of "parkinsonism" (approx. 75% of the cases) and belongs to the group of primary parkinsonism (Klein *et al.* 2007b). Parkinsonism is integrated into the class of akinetic rigid syndromes. Loss of dopamine in the corpus striatum is the primary defect in parkinsonism. Although the neurodegenerative progression cannot be stopped, symptomatic treatments are available. Dopamine is one of three major neurotransmitters known as catecholamines (Löffler *et al.* 2003). In the central nervous system catecholamines modulate point-to-point neurotransmission and affect complex processes including emotion, mood and attentiveness (Damrauer *et al.* 2005). In the periphery, they are the major neurotransmitter of sympathetic postganglionic neurons and are secreted by the adrenal glands in response to stress (Damrauer *et al.* 2005).

Table 1 *Classification of parkinsonian disorders.*

Primary parkinsonism	Secondary parkinsonism	Parkinson-plus syndromes	Hereditary diseases
• Idiopathic Parkinson's disease • Familial Parkinsonian syndromes • Dopa-responsive dystonia	• Vascular • Traumatic • Postencephalic • Paraneoplastic • Psychogenic • Metabolic • Infectious • Toxic • Drug-related • Hydrocephalus	• CBGD • LBD • FTDP17 • MSA • PSP	• Wilson's disease • SCA • Chorea Huntington • Gaucher disease • IBGC • NBIA disorders

CBGD, Cortical-basal ganglionic degeneration; LBD, Lewy body dementia; FTDP17, Frontotemporal dementia and parkinsonism linked to chromosome 17; MSA, Multiple system atrophy; PSP, Progressive Supranuclear Palsy; SCA, Spinocerebellar ataxia; IBGC, Idiopathic basal ganglia calcification; NBIA, Neurodegeneration with brain iron accumulation. Classification modified after Kompoliti and Goetz (2005).

The loss of dopamine in the corpus striatum is caused by reduced activity of dopaminergic cells in the pars compacta of the substantia nigra (SN) (see Figure 1) (Hornykiewicz 1962; Fearnley *et al.* 1991). This lack of dopamine in the nigrostriatal system results in a hyperactivity of inhibiting cholinergic interneurons (Crossman 2000). The SN is part of the extrapyramidal system (EPS) which has been implicated in a number of important motor functions, especially the initiation of motor responses and maintenance of posture (Crossman 2000). The EPS and the pyramidal system, which received its name because all fibers run through the medullary pyramid, are the main structures concerned with

regulation of motor activity (Ebadi 2005). The major structures of the EPS involve the putamen, globus pallidus, substantia nigra, and the subthalamic nuclei (corpus Luysii). The network is closely connected to the cerebral cortex, thalamus, brainstem nuclei and cerebellum (Martin 2003; Ebadi 2005).

The basal ganglia are a group of interconnected subcortical nuclei. They are composed of one input structure, the striatum which encompasses three subnuclei - caudate nucleus, putamen and nucleus accumbens - and three output structures, the pars reticulata of the SN, the ventral pallidum and the internal segment of the globus pallidus (GPi) (Martin 2003). The excitatory signals to the basal ganglia originate from the cerebral cortex. This afferent information is processed in the basal ganglia and transmitted as inhibitory efferents to the brainstem, partially to the motor thalamus and from there back to the cortex (Figure 1) (Martin 2003). The decreased inhibition of the striatum leads to motor inhibition whereby the clinical picture of parkinsonism emerges (Herrero et al. 2002).

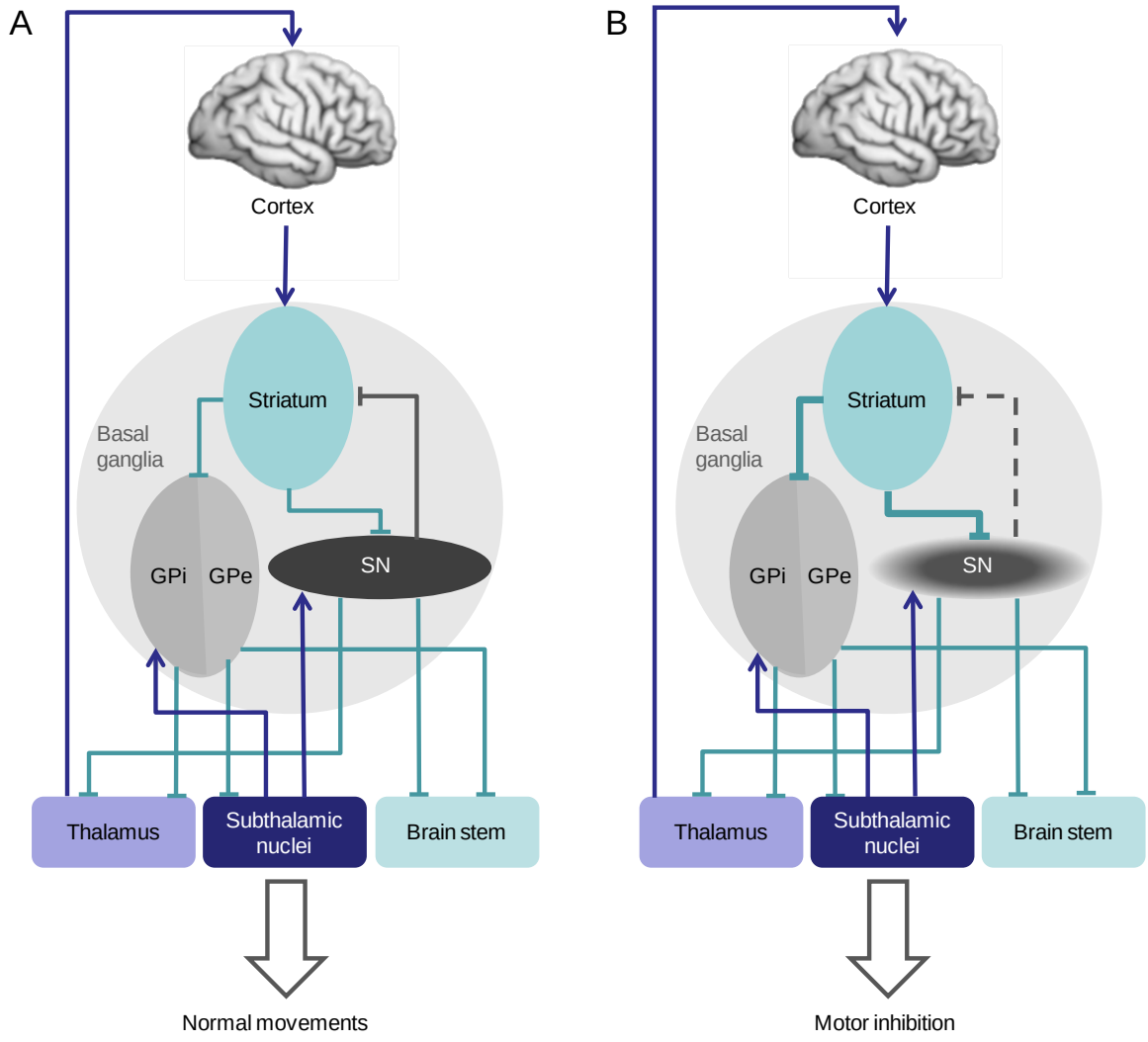

Figure 1 ***(previous page)*** *Neuronal tracts for motor control.* (A) Signaling under healthy conditions. (B) Alterations of signaling in Parkinson's disease (PD). Decreased inhibition of the striatum by the substantia nigra (SN) leads to motor inhibition. Arrows stand for activating signals, crossbars indicate inhibitory signals. Bold lines highlight increasing signal intensity, discontinuous lines emphasize a reduction of the signal intensity. Different colors of the arrows indicate different neurotransmitters (green - GABAergic projection; blue – glutamatergic projection; grey – dopaminergic projection). GPi/e – internal/external globus pallidus. For reasons of clarity the connections of the cortex to the output structures (thalamus, subthalamic nuclei and brain stem) are not illustrated in this overview. Schema modified from the literature (Todd *et al.* 1998; Herrero *et al.* 2002; Conn *et al.* 2005).

In 1997, the overall age-adjusted prevalence (per 100 population) for people of the age of 65 years or older in Europe was 2.3 for parkinsonism and 1.6 for PD. The prevalence rose gradually with age (de Rijk *et al.* 1997). Due to an aging population in industrialized countries and a longer survival of patients with parkinsonism, the prevalence will rise over the next years with a projected doubling of the prevalence between the years 2005 and 2030 (Dorsey *et al.* 2007).

Only about 25% of patients will have a positive family history, consistent with a monogenetic cause of parkinsonism. For some monogenetic forms of parkinsonism an early age of onset increases the probability that the patient will be a mutation carrier (Klein *et al.* 2006). In case of clinically classic parkinsonism with a known genetic origin, the condition is often denoted as, for example, *Parkin*-linked PD.

The etiology of parkinsonism, and especially hereditary parkinsonism, is varied (Warner et al. 2003). However, an increasing proportion of patients (albeit still a minority) have an identifiable gene mutation (Klein et al. 2006). To date, 18 genetic loci (PARK1-18), and two loci which are not yet assigned, have been identified as causes for rare forms of parkinsonism or susceptibility factors for PD (Table 2). These loci include seven autosomal dominant (PARK1 (=4), 3, 5, 8, 11, 17, 18), seven autosomal recessive (PARK2, 6, 7, 9, 10, 14, 15), one X-linked (PARK12) and two forms with still unclear mode of transmission (PARK10, 13) (see Table 2, Klein *et al.* 2007a; Bekris *et al.* 2010; Houlden *et al.* 2012). Common variants in three genes (*SNCA*, *LRRK2* and *Microtubule-Associated Protein Tau* (*MAPT*)) and loss-of-function mutations in the gene *glucerebrosidase* (*GBA*) are well-known risk factors which contribute to the development of a classical PD phenotype (Houlden *et al.* 2012). Recent genome-wide association studies (GWAS) revealed one new susceptibility locus (PARK16) which still awaits further confirmation (Bekris *et al.* 2010). The designation system for PD using the PARK

acronyms can lead to problems due to misinterpretation. Originally these acronyms were genetic locus symbols and introduced to specify chromosomal regions that had been linked to familial PD with a yet unknown gene (Marras *et al.* 2012). In consequence of the growing knowledge and the progress in research, this list was gradually amended by new insights from different perspectives. This led to a consecutively numbered system, combining causative genes and risk factor genes in the same list, and including erroneously assigned loci, duplicated loci, missing symbols, missing loci, unconfirmed loci, and discordance between phenotype and list assignment (Marras *et al.* 2012). For this reason, a substantial revision of the classification of inherited forms of parkinsonism is required (Kumar *et al.* 2012).

For the present thesis the PARK2 gene *Parkin* is of particular interest. Details about *Parkin*-associated PD are summarized in the following chapter.

Table 2 *Genetic causes of parkinsonism.*

Acronym	Mode of inheritance	Locus	Gene	Initial linkage study	Validation status	Type of parkinsonism
PARK1/ PARK4	AD	4q21	*SNCA*	Polymeropoulos *et al.* 1996 / Farrer et al. 1999	Well-validated gene for MGP and variations in the gene as well-validated SF	EOPD, susceptibility gene for PD
PARK2	AR	6q25.2-q27	*Parkin*	Matsumine *et al.* 1997	Well-validated gene for MGP	Juvenile, EOPD, even LOPD described
PARK3	AD	2p13	Unknown	Gasser *et al.* 1998	Putative locus for MGP	LOPD
PARK5	AD	4p14	*UCH-L1*	Leroy *et al.* 1998	Putative gene for MGP	LOPD
PARK6	AR	1p36-p35	*PINK1*	Valente *et al.* 2001	Well-validated gene for MGP	EOPD
PARK7	AR	1p36	*DJ-1*	Van Duijn *et al.* 2001	Well-validated gene for MGP	EOPD
PARK8	AD (incomplete penetrance)	12q12	*LRRK2*	Funayama *et al.* 2002	Well-validated gene for MGP and variation in the gene as well-validated SF	EOPD, susceptibility gene for PD
PARK9	AR	1p36	*ATP13A2*	Hampshire *et al.* 2001	Well-validated gene for MGP	Kufor-Rakeb syndrome
PARK10	AR	1p32	Unknown	Hicks *et al.* 2002	Putative locus for MGP	LOPD

Continuation Table 2						
PARK11	AD (incomplete penetrance)	2q36-q37	*GIGYF2*	Pankratz *et al.* 2003b	Putative gene for MGP	LOPD
PARK12	X-linked	Xq21-q25	Unknown	Pankratz *et al.* 2003a	Putative locus for MGP	Not clear
PARK13	Unknown	2p12	*Omi/HtrA 2*	Strauss *et al.* 2005	Putative gene for MGP	Not clear
PARK14	AR	22q13.1	*PLA2G6*	Paisan-Ruiz *et al.* 2009	Putative gene for MGP	Adult onset dystonia-parkinsonism
PARK15	AR	22q12-q13	*FBXO7*	Shojaee *et al.* 2008	Putative gene for MGP	Early-onset parkinsonian-pyramidal syndrome
PARK16	SF	1q32	Unknown	Satake *et al.* 2009	Locus as putative SF	Susceptibility locus for PD
PARK17	AD	16q11.2	*VPS35*	Zimprich *et al.* 2011/ Vilarino-Güell *et al.*	Well-validated gene for MGP	LOPD
PARK18	AD	3q26-q28	*EIF4G1*	Chartier-Harlin *et al.* 2011	Putative gene for MGP	LOPD
Not assigned	AD	3q22.1	*DNAJC13*	Vilarino-Güell *et al. 2012*	Putative gene for MGP	LOPD

Note: AD – autosomal dominant, AR – autosomal recessive, *ATP13A2 – ATPase type 13A2*, *DNAJC13 – DnaJ homolog, subfamily C, member 13*, *EIF4G1 – eukaryotic translation initiation factor 4-gamma*, EOPD – early-onset Parkinson's disease, *FBXO7 – F-box only protein 7*, *GIGYF2 – GRB10-Interacting GYF protein 2*, LOPD – late-onset Parkinson's disease, *LRRK2 – Leucine-rich repeat kinase 2*, MGP – monogenic parkinsonism, *Omi/HtrA2 – HtrA serine peptidase 2*, PD – Parkinson's disease, *PINK1 – PTEN-induced putative kinase 1*, *PLA2G6 – Phospholipases A2, group VI*, *SNCA – alpha-synuclein*, SF – susceptibility factor, *VPS35 – vacuolar protein sorting 35*, *UCH-L1 – ubiquitin C-terminal hydrolase-L1*, X – X-chromosomal; Table modified after (Houlden *et al.* 2012) and the PD Gene Online Database (Lill CM 2011).

1.1.1 Phenotype of *Parkin*-associated parkinsonism

The first description of *Parkin*-associated PD was a publication from Japan in 1998 (Kitada *et al.* 1998). The affected woman who was found to carry a mutation in the *Parkin* gene was born to consanguineous parents. The onset of the symptoms of parkinsonism was in her teens and the disease slowly progressed over years. At age 43 years, the patient

clinically presented with all four cardinal signs of PD and additionally had hyperreflexia, mild foot dystonia and diurnal fluctuation of symptoms. The response to L-Dopa treatment was excellent, but complicated by L-Dopa-induced dyskinesias (Kitada et al. 1998). In this first study, additional patients were identified with *Parkin* mutations who showed a phenotype mimicking idiopathic PD, mostly with a juvenile (<20 years) age of onset.

After this first report, hundreds of reports of patients from all over the world with mutations in *Parkin* were published. The ethnic background, when reported, was mostly Caucasian (80%), followed by Asian (14%), Latin-American (5%), and other (1%) (Kasten et al. 2010). While the clinical phenotype was in line with the first description, age of onset tended to be later for other ethnicities (average age of onset: 30-45 years). Rarely, patients with an age of onset in the sixties or early seventies have been reported (Pramstaller et al. 2005). Altogether, 18% of the published cases with a *Parkin* mutation and a documented age of onset had a juvenile onset (<20 years). Seventy percent of cases had disease manifestation between 20 and 40 years of age, and 12% at 41 years or older (Kasten et al. 2010). In a study of 956 patients with early-onset PD (defined as age at onset younger than 51 years), 6.7% had *Parkin* mutations, of which 3.9% were heterozygous, 0.6% were homozygous, and 2.2% appeared to be compound heterozygous (Marder *et al.* 2010). Furthermore, the lower the age of onset, the higher the proportion of patients carrying at least one *Parkin* mutation (Marder *et al.* 2010).

Neuropathological studies of brain specimens from patients with proven *Parkin* mutations revealed variable findings. No Lewy body inclusions were found in patients with juvenile-onset (Hayashi et al. 2000). In contrast, other studies in patients with a later age of onset revealed Lewy bodies (Farrer et al. 2001; Pramstaller et al. 2005).

'Parkin disease', as a distinct clinical entity, is characterized by a rather benign disease course including slower disease progression, an excellent response to treatment, and a rare occurrence of additional motor and non-motor signs (Lohmann *et al.* 2003; Klein *et al.* 2009; Lohmann *et al.* 2009). In recent systematic studies, patients with *Parkin* mutations did not show increased frequencies of cognitive impairment, nor of specific behavioral or psychiatric symptoms (Lohmann *et al.* 2009; Kasten *et al.* 2010), which previously were proposed as 'red flags' for *Parkin*-associated parkinsonism (Khan et al. 2003). In a systematic literature review, rates of cognitive and psychiatric symptoms in *Parkin* mutation carriers (depression (31%), anxiety (26%), hallucinations (7%), and dementia (5%)) appeared to be comparable to or even lower than those accounted for idiopathic PD patients (Kasten et al. 2010). When assessing olfaction and color discrimination in *Parkin*

mutation carriers, these were less impaired than idiopathic PD patients and *LRRK2* mutation carriers (Kertelge *et al.* 2010).

1.1.2 Parkin – the gene and the protein

The first gene locus for autosomal recessive early-onset parkinsonism was mapped to the q arm of chromosome 6 (6q25.2-q27) in 1997 with the aid of linkage analysis (Matsumine et al. 1997). By means of positional cloning the disease-causing gene was localized. A cDNA clone of 2,960bp with a 1,395-bp open reading frame was identified and the gene was named *Parkin* (Kitada et al. 1998). *Parkin* is situated adjacent to the *Parkin co-regulated gene* (*PACRG*). It contains 12 exons with large intronic regions in between. The size is about 1.3Mb (Kahle et al. 2004) and it is therefore one of the largest human genes.

The *Parkin* gene encodes a 465-amino-acid protein with modular structure. Parkin contains an N-terminal ubiquitin-like (UBL) domain and a C-terminal RING (really interesting new gene) domain. The RING domain is comprised of three RING finger motifs (RING0, RING1 and RING2) (Figure 2). RING0 and RING1 are separated by a residue of 26 amino acids, whereas RING1 and RING2 are connected by a unique Parkin sequence of approximately 160 residues without any identifiable domain structure (in-between-RING domain (IBR)) (Beasley *et al.* 2007; Hristova *et al.* 2009).

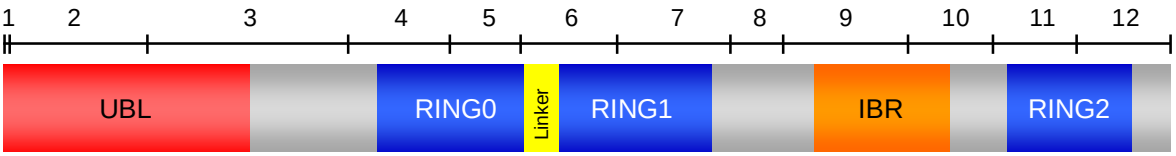

Figure 2 *Domain structure of Parkin.* The *Parkin* gene consists of 12 exons which are spliced and translated into a 465-amino-acid protein. The domains for which the exons encode are marked and their relation to each other is indicated. Functionally important domains of the protein comprise an amino-terminal ubiquitin-like (UBL) domain and three really interesting new gene finger motifs (RING0-2). RING0 and RING1 are connected by a 26-residue linker region, while an in-between RING domain (IBR) is localized between the other RING domains (1 and 2).

Parkin can be found in the endoplasmic reticulum, the Golgi apparatus and synaptic vesicles (Shimura et al. 1999; Stichel et al. 2000; Kubo et al. 2001). Notably, it has also been recognized to be associated with the outer mitochondrial membrane (Kuroda et al. 2006). Parkin is a E3 ubiquitin protein ligase (Shimura *et al.* 2000). E3 ligases are part of

an enzymatic holocomplex that adds ubiquitin chains to proteins to initialize protein degradation by the 26S proteasome (Hochstrasser 1996). Most of mutations in *Parkin* are expected to cause a loss of function by either disrupting the cellular distribution pattern, ubiquitin ligase activity, altering expression, intrinsic solubility, or interaction with substrates (Wang *et al.* 2005; Hampe *et al.* 2006a; Matsuda *et al.* 2006) and ubiquitin-conjugating enzymes (Shimura et al. 2000; Zhang et al. 2000) (see 1.2.4).

Besides its key role in PD, *Parkin* is also known as a tumor suppressor gene. Loss of heterozygosity of the third common fragile site *FRA6E* that contains the *Parkin* gene (Smith et al. 1998) was demonstrated in different types of cancers. This latter function of *Parkin* will not be further discussed in this thesis.

1.2 Mitochondria and parkinsonism

Mitochondria are the main source of energy for the eukaryotic cell under aerobic conditions. Their core function of ATP production by coordinated proton and electron transport between different compartments is maintained by the oxidative phosphorylation (OXPHOS) system, which comprises the respiratory chain (RC).

In recent years, mitochondria became a central piece in the puzzle of neurodegeneration. Mitochondrial dysfunction seems to play a role in the etiology of several neurological disorders such as PD, Alzheimer's disease, and Huntington's disease (Schapira *et al.* 1990; Gu *et al.* 1996; Bonilla *et al.* 1999).

1.2.1 The oxidative phosphorylation (OXPHOS) system

The OXPHOS system consists of approximately 85 polypeptides grouped together into four enzyme complexes, one complex facilitating adenosine triphosphate (ATP) generation, and two different electron carriers, coenzyme Q (CoQ) and cytochrome c (Smeitink *et al.* 2001; Schapira 2006) (see Figure 3). It is embedded in a lipid bilayer of the inner mitochondrial membrane, which separates the mitochondrial matrix and the intermembrane space from each other (Hatefi 1985; Saraste 1999). The passage of electrons and protons releases energy which leads to an electrochemical gradient. This gradient is the force that drives the fifth OXPHOS complex (ATP synthase, an F_1F_o-ATPase) to generate ATP from ADP and inorganic phosphate (Hatefi 1985; Saraste 1999).

The RC (CI-CIV) facilitates the transfer of electrons from nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) to oxygen, which is reduced to water at complex IV (CIV) (Michel et al. 1998). The reduction of oxygen is coupled to the synthesis of ATP (oxidative phosphorylation) by the ATP synthase (complex V) (Mitchell 1961). Complexes I–IV (CI-IV) contain bound redox centers (e.g., iron-sulphur complexes, FAD), which transfer electrons sequentially from one to another via increasing reduction potentials (Michel et al. 1998; Zhang et al. 1998). Ubiquinol ($CoQH_2$) transfers two electrons from both CI and CII to CIII, while cytochrome c transfers one electron from CIII to CIV (Crofts et al. 1999; Tormo et al. 2000). The free energy generated by the transfer of electrons is conserved by pumping of protons from the mitochondrial matrix into the intermembrane space by CI, CIII, and CIV (Mitchell 1961; Michel et al. 1998; Crofts et al. 1999). This process results in an electrochemical gradient across the inner membrane, the mitochondrial membrane potential ($\Delta\psi m$) of 150mV (Scheffler 2001). This proton motive force is dissipated by the membrane domain of the ATP synthase leading to the phosphorylation of ADP (Mitchell 1961). Several specific inhibitors of each complex are available which are used in experimental settings to measure solely the activity of a certain complex (Brandt 2003) (Figure 3).

Compared to other cell organelles, mitochondria have a unique system of genetic control, protein translation and trafficking. First, mitochondria have their own mitochondrial DNA (mtDNA) in the form of a closed circular molecule of 16,569bp length. This mtDNA molecule is present in 10^3–10^4 identical copies per cell, with the exception of sperm and mature oocytes, in which mtDNA copy numbers are ~10^2 and ~10^5, respectively (Giles *et al.* 1980; Grossmann *et al.* 1997). The number of mtDNA molecules per mitochondrion is estimated around two to ten copies (Smeitink *et al.* 2001). Second, the main differences of mutations in mitochondrially-encoded genes to nuclear ones are a different mode of inheritance and a phenomenon named heteroplasmy. Sperm mtDNA is eliminated after fertilization of oocytes, thus resulting in an exclusively maternal transmission of mutations that are present in maternal germ cells (Chinnery *et al.* 1999). Heteroplasmy implies the co-existence of wild-type and mutant mtDNA to a different extent within a cell, which can vary between different tissues in the body. In case of inheritance of heteroplasmic mtDNA point mutations, the level of heteroplasmy can shift randomly (Larsson *et al.* 1992; Chinnery *et al.* 2001; Sekiguchi *et al.* 2003). Additionally, these latter studies indicate that a certain threshold of heteroplasmy is required to cause a disease. Furthermore, the genetic code used for translation of mitochondrial genes is simplified (use of 22 tRNAs instead of

31 predicted by Crick's wobble hypothesis) and deviates from those used for nuclear genes (Barrell *et al.* 1979; Smeitink *et al.* 2001).

Mitochondrial proteins are encoded by a dual system of nuclear DNA (nDNA) and mtDNA. Just twenty-two tRNAs, two rRNAs and 13 polypeptides of the OXPHOS system (seven subunits of CI, apocytochrome b (CIII), cytochrome oxidase (CIV) subunits I, II and III, and the ATPase subunits 6 and 8) are mitochondrially encoded (see Figure 3) (Yatscoff *et al.* 1978; Chomyn *et al.* 1985; Scarpulla 2008). Thus, the majority of subunits of the OXPHOS system, the assembly factors of the complexes, the proteins of mitochondrial gene expression regulation, and the factors for mtDNA replication, maintenance and transcription (e.g. mitochondrial transcription factor A (TFAM) see 1.2.2) are encoded by nuclear genes (Scarpulla 2008).

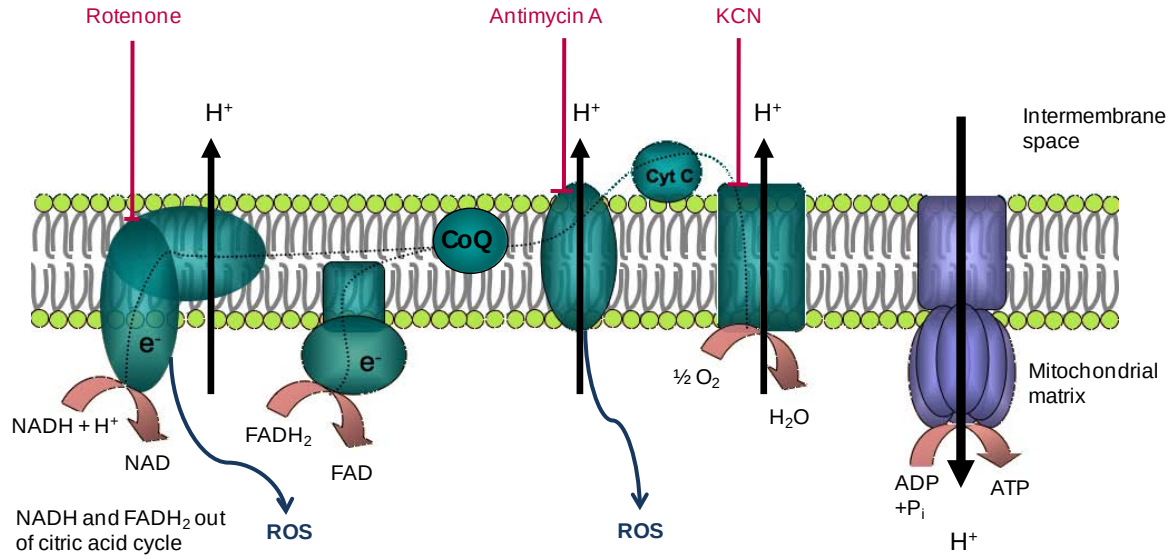

Figure 3 *The oxidative phosphorylation system with sites of ATP generation and reactive oxygen species (ROS) production.* Complexes I–IV (CI-CIV) of the electron transport chain and the ATP (adenosine-5'-triphosphate) synthase (complex V) are located in the inner membrane of the mitochondria. Electrons (e^-) enter this chain at CI and CII. Soluble NADH (nicotinamide adenine dinucleotide), a product out of the citric acid cycle, delivers two electrons to the respiratory chain (RC) via CI. At CII two electrons are channeled in via bound FAD (flavin adenine dinucleotide). At this site, different substrates (succinate as a product of the citric acid cycle, decomposition products of fatty acids, and glycerol 3-phosphate from glycolysis) can be used to transfer two electrons to coenzyme Q (CoQ). Cytochrome *c* (Cyt C) transfers those electrons, one at a time, from CIII to CIV. On acceptance of the electron, CIV converts protons (H^+) and oxygen (O_2) to water (H_2O). Except for CII, all complexes are pumping protons (H^+) out of the matrix into the intermembrane space. The resulting electrochemical gradient across the inner mitochondrial membrane is used by CV to

convert ADP (adenosine-5'-diphosphate) and inorganic phosphate (P_i) into ATP. Reactive oxygen species (ROS) produced by this process are indicated. RC complex inhibitors and their site of inhibition are shown in red: rotenone = CI inhibitor; antimycin A = CIII inhibitor, and potassium cyanide (KCN) = CIV inhibitor. The number of subunits of each complex and the type of coding DNA are given below. MtDNA = mitochondrial DNA; nDNA = nuclear DNA. Figure adapted from Bellance *et al.* (Bellance *et al.* 2009).

1.2.2 Initial links to mitochondrial dysfunction

Mitochondrial dysfunction was implicated in the pathogenesis of PD by the identification of a side product of improper synthetic opioid production, 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), as the cause of parkinsonian features in drug abusers (Langston et al. 1983). After crossing of the blood-brain barrier, MPTP is converted by glial monoamine oxidase B (MAO-B) into the 1-methyl-4-phenylpyridinium ion (MPP+). MPP+ is a potent inhibitor of complex I of the RC and a substrate for a dopamine transporter (Solute carrier family 6, member 3 (SLC6A3)). Consequently, MPP+ accumulates in dopaminergic neurons where its toxicity leads to neuronal death by CI inhibition (Nicklas et al. 1985).

Further evidence supporting the link between mitochondrial function and PD was provided by the finding of a decrease in the activity of the respiratory CI detected post-mortem in the substantia nigra of patients with idiopathic PD (Schapira et al. 1989). A recent publication strengthened the link between the exposure to CI inhibitors or oxidative stressors and the risk of development of PD. This study demonstrated a higher incidence of PD in a group of patients exposed to pesticides such as the mitochondrial CI inhibitor rotenone and the oxidative stressor paraquat (Tanner et al. 2011). In addition, rats fed with CI inhibitor rotenone developed parkinsonian features including neuronal degeneration and the formation of SNCA-rich inclusion bodies (Betarbet et al. 2000).

The first connection between Parkin and mitochondria was established based on the observation of a ‘phenotype of mitochondrial dysfunction’ in Parkin loss-of-function mice (Palacino et al. 2004). These animals carried an exon 3 deletion in *Parkin* which resulted in decreased expression of certain proteins relevant to mitochondrial function and control of oxidative stress, comprising several subunits of complexes I and IV. Their mitochondrial respiratory capacity was decreased and there was evidence for increased oxidative damage (Palacino et al. 2004). Similar results were reported for other mouse or zebrafish models (Goldberg *et al.* 2003; Itier *et al.* 2003; Perez *et al.* 2005; Flinn *et al.* 2009).

The relevance of the findings from animal studies was strengthened by investigations of mitochondrial function in human samples. Assessment of mitochondrial function in both

leukocytes and fibroblasts of patients with either homozygous or compound heterozygous *Parkin* mutations indicated dysfunction of the OXPHOS system (Muftuoglu et al. 2004; Mortiboys et al. 2008).

Besides its involvement in RC function, Parkin has also been shown to interact with mtDNA. It was demonstrated that Parkin enhances transcription and replication of mtDNA and induces mitochondrial proliferation (Kuroda et al. 2006). Potentially this process is facilitated by the interaction partner mitochondrial transcription factor A (TFAM), a regulation protein that coats and binds mtDNA for control of mtDNA transcription (Kuroda et al. 2006). A recent study confirmed in murine and human brain tissue that Parkin is associated with mtDNA (Rothfuss et al. 2009). Overexpression of *Parkin* enhances replication, transcription and repair of mtDNA, while loss-of-function of Parkin disturbs mtDNA metabolism and protection of mitochondrial genomic integrity from oxidative stress (Rothfuss et al. 2009).

1.2.3 The common pathway of Parkin and PINK1

In 2006, a new clue to the molecular basis of parkinsonism was discovered. Three studies in *Drosophila* provided evidence of a direct interaction between parkin and pink1 (Clark *et al.* 2006; Park *et al.* 2006; Yang *et al.* 2006). Pink1 is another protein causing autosomal recessive early-onset parkinsonism when mutated. In flies with *pink1* loss-of-function mutations, degeneration of flight muscles and dopaminergic neurons was demonstrated in addition to abnormal mitochondrial morphology and increased susceptibility to oxidative stress (Clark *et al.* 2006; Park *et al.* 2006; Yang *et al.* 2006). These flies exhibited features that overlap with the altered mitochondrial morphology seen in *parkin* mutants earlier. The knockdown of parkin in *Drosophila* caused swollen mitochondria accompanied by a severe disruption and disintegration of the mitochondrial cristae of the indirect flight muscles (Greene et al. 2003; Pesah et al. 2004). Transgenic expression of *parkin* rescued the pink1 loss-of-function phenotype, but not vice versa (Clark *et al.* 2006; Park *et al.* 2006; Yang *et al.* 2006). These data indicated that parkin acts in a pathway downstream of pink1 (Clark *et al.* 2006; Park *et al.* 2006; Yang *et al.* 2006). Subsequently, PINK1 was found to initiate the mitochondrial translocation of Parkin in mammals and in *Drosophila* (Kim *et al.* 2008; Sha *et al.* 2009).

Lately, research on the interaction of pink1 and parkin in *Drosophila* suggested the promotion of mitochondrial fission via this interplay (Deng *et al.* 2008; Poole *et al.* 2008; Lutz *et al.* 2009). This hypothesis was confirmed by defects indicative of impaired

mitochondrial fission and tissue integrity after silencing of either gene (Deng *et al.* 2008; Poole *et al.* 2008; Lutz *et al.* 2009). Loss of function of the dynamin-related protein 1 (drp1), a protein involved in mitochondrial fission, augmented the manifestation of the phenotypes of pink1 or parkin deficiency in *Drosophila* (Deng *et al.* 2008; Poole *et al.* 2008; Lutz *et al.* 2009). In accordance with this finding, overexpression of drp1 or knockdown of the mitochondrial fusion-promoting components optic atrophy 1 (opa1) and mitofusin (mfn) attenuated the phenotype of *pink1* and *parkin* mutants (Deng et al. 2008; Poole et al. 2008; Lutz et al. 2009).

Investigations in fibroblast cells from PD patients also addressed the effect of mutant *Parkin* on the mitochondrial integrity. Mitochondrial imaging showed enhanced branching in *Parkin* mutants when compared to controls, in line with the conclusions from the studies in flies (Mortiboys et al. 2008).

1.2.4 Putative interaction partners of Parkin

Considering this substantial role of Parkin in cell metabolism, several substrates and interactors are required to implement and regulate these functions. To date, a long list of potential interactors is known, but there is no all-encompassing pathway which explains all interactions of Parkin. A detailed overview of Parkin substrates and the consequences of their interaction are summarized in Table 3. Notably, the majority of the listed interactors still await confirmation.

Several of these putative interactors are involved in Parkin's function as an E3 ubiquitin protein ligase. In the cell, E3 ubiquitin ligases are a component of the ubiquitin proteasomal system (UPS), a major cellular pathway involved in promoting removal of damaged or misfolded proteins. E3 ligases catalyze the addition of ubiquitin molecules to lysine residues of damaged target proteins. The presence of a polyubiquitin chain provides a signal for the removal and degradation of these proteins by a proteolytic complex, the 26S proteasome (Pickart 2001). Independent of proteasomal degradation, ubiquitination of proteins commonly regulates a number of important cellular processes including post-replicative DNA repair, transcriptional and translational regulation, endocytosis, protein trafficking, and lysosomal degradation (Pickart 2001; Pickart et al. 2004).

Substrates of Parkin have been identified to function in the PINK1/Parkin pathway (Table 3, highlighted in blue). Notably, the protein products of genes causing monogenic PD show an interaction with Parkin. For the autosomal recessive forms of genetic PD (*PINK1*, *Parkin*, *DJ-1*), an underlying common pathway begins to unravel. For proteins of the

autosomal dominant forms of genetic parkinsonism, which also show an interaction with Parkin, there seems to be an involvement in protein accumulation and the formation of Lewy bodies (Chung *et al.* 2001; Smith *et al.* 2005). Other interaction partners include the protein products of mutant variants of the *glucocerebrosidase* (*GBA*) gene, responsible for Gaucher disease (GD). Concurrence of parkinsonism was observed in GD patients and mutations in *GBA* were found in patients with sporadic PD (Aharon-Peretz et al. 2004; Gan-Or et al. 2008; Sidransky et al. 2009).

Another intriguing finding is the direct interaction of two proteins which in mutant forms are responsible for two different neurodegenerative diseases with overlapping phenotype, Parkin-associated parkinsonism and spinocerebellar ataxia type 3. There is evidence of a direct binding of Parkin and ataxin-3 through two binding sites and the binding is enhanced by Parkin self-ubiquitination (Durcan et al. 2011).

Table 3 *Putative interactors of Parkin.*

Interactor	Type and consequence of interaction with Parkin	References
Ubch7 (E2 conjugating enzyme)	Interaction to initiate polyubiquitin chain assembly: targeting of proteins for proteasomal degradation	Shimura et al., 2000
Ubch8 (E2 conjugating enzyme)	Interaction to initiate polyubiquitin chain assembly: targeting of proteins for proteasomal degradation	Zhang et al., 2000
CDCrel-1	Polyubiquitination and degradation initiation of the synaptic vesicle-associated protein	Zhang et al., 2000
Synphilin-1	Linking of SNCA and Parkin via SNCA interactor synphilin-1 in a common pathogenic mechanism: molecular basis for ubiquitination of Lewy body proteins	Chung et al., 2001
Pael-R	Initiation of polyubiquitination and degradation of insoluble Pael-R	Imai et al., 2001
α-SP22	22-kD glycosylated form of SNCA as Parkin substrate, loss of Parkin function causes pathologic accumulation of α-SP22	Shimura et al., 2001
CASK	Complex formation: postsynaptic targeting or scaffolding of Parkin; no substrate for Parkin-mediated ubiquitination	Fallon et al., 2002
CHIP, HSP70, Pael-R	Complex formation, CHIP regulates Parkin E3 activity	Imai et al., 2002
CDCrel-2a	Polyubiquitination and degradation initiation	Choi et al., 2003
p38	Polyubiquitination to initiate degradation of p38, a key component of mammalian aminoacyl-tRNA complex	Corti et al., 2003
SYT11	Polyubiquitination to enhance SYT11 turnover: possible function of Parkin in regulation of the synaptic vesicle pool	Huynh et al., 2003
α-/β-Tubulin	Polyubiquitination: accelerated degradation of α-/β-tubulins	Ren et al., 2003
FBXW7, Cullin-1	Association in ubiquitin ligase complex: targeting of Parkin E3 activity to cyclin E, a regulator of neuronal apoptosis	Staropoli et al., 2003
Poly(Q)-expanded huntingtin	Complex formation with HSP70 and proteasome: example for recruitment of misfolded proteins to Parkin by HSP70	Tsai et al., 2003
Bag5, HSP70	Complex formation: Bag5 negatively regulates HSP70/Parkin function that sensitizes dopaminergic neurons to injury-induced death	Kalia et al., 2004
PSMA7	Interactor but no substrate for Parkin-dependent ubiquitination	Dachsel et al., 2005

Continuation Table 3		
DJ-1 (mutant forms)	Association with L166P and M26I mutant *DJ-1*: promotion of protein stability of these mutants	Moore et al., 2005
LRRK2	Formation of aggregates and enhanced ubiquitination of such aggregates due to coexpression of *LRRK2* and *Parkin*	Smith et al., 2005
SIM2	Polyubiquitination and degradation initiation	Okui et al., 2005
Eps15, Egfr	Binding of Eps15 to regulate interaction between Eps15 and ubiquinated Egfr: delay of Egfr internalization and degradation, and promotion of PI3K-AKT signaling	Fallon et al., 2006
FBP1	Polyubiquitination and degradation initiation	Ko et al., 2006
RanBP2	Polyubiquitination to initiate RanBP2 degradation: Controlling of intracellular levels of sumoylated HDAC4	Um et al., 2006
SUMO-1	Interaction: Increased nuclear transport and self-ubiquitination of Parkin	Um et al., 2006
PINK1	Direct phosphorylation by PINK1: promotion of mitochondrial translocation of Parkin; regulation of mitochondrial fission	Clark et al., 2006 Park et al., 2006 Kim et al., 2008
Limk1	Enhancing of ubiquitination of Lim kinase 1 leading to reduction of cofilin phosphorylation	Lim et al., 2007
HSP70	Interaction to mediate (degradation-independent) multiple mono-ubiquitination of HSP70	Moore et al., 2008
Nrdp1	Ubiquitination of Parkin by Nrdp1: negative regulation of Parkin level	Yu et al., 2008
PINK1, DJ-1	Association in ubiquitin ligase complex: targeting of proteins for proteasomal degradation	Xiong et al., 2009
Endophilin-A	The ubiquitin-like domain binds to the SH3-domain of endophilin-A, which plays a role in synaptical transmission	Trempe et al., 2009
PDCD2-1	Its ubiquitination is promoted by Parkin and programmed cell death 2 isoform 1 was found in the substantia nigra of autosomal recessive and sporadic PD patients	Fukae et al., 2009
LRPPRC	Pulled down with tandem affinity purification, mitochondrial localization	Davison et al., 2009
Mfn	Promotes ubiquitination of profusion factor Mfn to label damaged mitochondria for degradation by autophagy	Ziviani et al., 2010
Mutant *GBA*	Degredates mutant glucocerebrosidase by K48 polyubiquitination of mutant variants and promotes its accumulation in aggresome-like structures	Ron et al., 2010
26 S proteasome	N-terminal ubiquitin like domain enhances interaction between 19S protesomal subunits, activates 26S proteasome	Um et al., 2010
c-Abl	c-Abl phosphorylates tyrosine 143 of Parkin, inhibiting Parkin's ubiquitin E3 ligase activity and protective function	Ko et al., 2010
Mutant ataxin-3	The mutant form of ataxin-3 regulates Parkin ubiqitination; direct interaction with multiple domain involvement; enhanced by Parkin self-ubiquitination; promotes the clearance of Parkin via the autophagy pathway	Durcan et al., 2011

Figure legend Table 3. Note: The proteins highlighted in blue are components of the above described common pathway of Parkin and PINK1 (1.2.3). Abbreviations: α-SP22 – glycosylated SNCA derivative; Bag5 – Bcl2-associated athanogene 5; CASK – calcium/calmodulin-dependent serine protein kinase; CDCrel – cell division control-related protein; CHIP – carboxy terminus of Hsc70-interacting protein; Egfr – epidermal growth factor receptor; Eps15 – Egfr pathway substrate 15; FBP1 – far up stream element binding protein-1; FBXW7– F-box and WD repeat domain containing 7; GBA – Glucocerebrosidase; HDAC4 –

histone deacetylase 4; HSP70 – heat shock protein 70; Limk1 – Lim kinase 1; LRPPRC – Leucin-rich pentatricopeptide repeat cassette; LRRK2 – leucine-rich repeat kinase 2; Mfn – Mitofusin; Nrdp1 – neuregulin receptor degradation protein-1; p38 – subunit of the aminoacyl-tRNA synthetase complex; PDCD2-1– Programmed cell death 2 isoform 1; Peal-R – Peal receptor; PINK1 – PTEN-induced putative kinase 1; PSMA7 – proteasome subunit, alpha type, 7; RanBP2 – Ran-binding protein 2; SIM2 – single-minded 2; SUMO-1 – small ubiquitin-like modifier 1; SYT11 – synaptotagmin-11; Ubch – ubiquitin-conjugating enzyme.

Additionally, a proteomic approach revealed Leucine-rich pentatricopeptide repeat cassette (LRPPRC) as a potential interactor of Parkin (Davison et al. 2009). A tandem affinity purification (TAP) was utilized to isolate proteins that are associated with PINK1 (Rakovic *et al.* 2011b). In this approach 14 proteins were detected as potential interaction partners of PINK1 - among them the above-mentioned LRPPRC. Since LRPPRC was the only protein unequivocally linked to a neurodegenerative disorder and because of its mitochondrial localization (further explained in the following chapter), the coding gene was selected for mutational screening. Genetic analysis comprising all 38 exons and flanking intronic regions of *LRPPRC* was performed in 46 patients with atypical PD.

1.2.5 A potential role of LRPPRC in PD

Leigh syndrome, French-Canadian type (LSFC, Online Mendelian Inheritance in Man No. 220111) is a form of cytochrome oxidase (COX, equal to CIV) deficiency. It is a subacute, necrotic encephalomyelopathy which manifests typically before the age of 12 months. There are two forms of COX deficiency; one characterized by prominent muscular weakness and the other one by a neurodegenerative encephalopathy of the brainstem, cerebellum, and basal ganglia. LSFC belongs to the latter type. It is an autosomal recessive form of congenital lactic acidosis, presenting with developmental delay, hypotonia and a striking paucity of facial and limb movement. Fatal acidotic crises can occur and microvesicular hepatic steatosis and facial dysmorphism appear in almost all patients. Older, ambulatory patients show signs of truncal ataxia with a hesitant, wide-based gait and intention tremor (Cooper et al. 2006).

This Leigh Syndrome variant was found in the geographically isolated region of Quebec and occurs in 1/2,178 live births (Morin et al. 1993). Simultaneously to the initial description of the syndrome, COX deficiency was biochemically substantiated. (Merante et al. 1993).

In a genome-wide association study, a locus for LSFC was mapped to chromosome 2p16-21 with a significant linkage disequilibrium ($P<10^{-5}$) and a common ancestral haplotype was identified in all patients (Lee et al. 2001). In 2003, Mootha *et al.* detected the homozygous c.1119C>T mutation in the *LRPPRC* gene in 21 out of 22 screened LSFC patients (Mootha et al. 2003). The last patient was compound heterozygous for c.1119C>T and a truncating 8bp deletion in exon 35. *LRPPRC* has 38 exons, a length of approximately 100kb and a transcript length of 6335bps. LRPPRC is a 1394-amino-acids protein and exhibits the characteristics of a mitochondrial protein, possessing a cleavable leader sequence and a series of pentatricopeptide tandem repeats. In LSFC patients, lower levels of *COX I* and *COX III* messenger ribonucleic acid (mRNA) were shown suggesting an mRNA-stabilizing function of LRPPRC (Xu et al. 2004).

There are several reasons to suggest that LRPPRC may be involved in the pathogenesis of PD, including: (i) mitochondrial dysfunction has been implicated in the pathogenesis of PD, (ii) parkinsonian signs can be present in LSFC patients, and (iii) LRPPRC may have a mRNA-stabilizing function in the mitochondrion.

1.2.6 Studying primary cells in neurologic diseases

Studying neurons to elucidate the disease mechanisms of PD seems most logical. However, the opportunites to obtain neurons from patients are usually very limited. Parkin and PINK1 are 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 Connolly *et al,*. 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 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 2002), amyotropic lateral sclerosis (Aguirre *et al.* 1998) and Huntington disease (Seo *et al.* 2004). For PD, only a small number of studies in human cell models exist. To extend these studies in endogenous models and to subsequently allow for a better understanding of the PD pathogenesis, in this thesis, functional experiments were performed in PD patient fibroblasts with *Parkin* mutations.

1.3 Hypotheses

Project A:

- *Parkin plays a role in respiratory chain function.*
- *Loss of Parkin function leads to oxidative stress in the cell.*
- *Parkin impacts on the morphology of the mitochondrial network.*
- *Mitochondrial DNA replication and repair is influenced by mutations in Parkin.*

Project B:

- *PD patients carry mutations in* LRPPRC *which are not present in controls.*

2 PATIENTS, MATERIAL AND METHODS

2.1 Patients

In the present study, specimens of PD patients were investigated at the genetic and the protein level. The functional consequences of *Parkin* mutations were studied in primary human cells. The diagnosis of PD was made by movement disorders specialists according to the UK Parkinson's Disease Society Brain Bank criteria (Gibb et al. 1988). All patients and control individuals gave informed consent and the study was approved by the Ethics Committee of the University of Lübeck.

2.1.1 Patients included in the mitochondrial study

In the mitochondrial study all available fibroblast cultures were obtained from patients with ascertained mutations in *Parkin*. The patients were examined at the movement disorder clinic of the neurological outpatient clinic of the University of Lübeck. Among these patients were members of an Italian family with mutations in *Parkin* - one member with a homozygous c.1072delT mutation, one with a homozygous deletion of exon 7, and one compound-heterozygous case (c.1072delT/c.delEx7). Furthermore, two healthy relatives without mutations were included. In addition, three unrelated PD patients with compound-heterozygous *Parkin* mutations (c.delEx3-4/c.duplEx7-12; c.delEx4/c.924C>T and c.delEx1/c.924C>T) and three independent age-matched controls were included. These individuals were of German, Persian or Chilean origin. The six mutation-positive cases had a mean age±STD of 56.2±13.3 years (two females) at the time of assessment. The five age-matched mutation-negative controls had a mean age±STD of 51.8±11.5 years (two females). For details on genotype and phenotype of the mentioned individuals see Table 4.

Table 4 *Characterization of the genotype and clinical phenotype of the investigated individuals.*

	Code	Sex	Age (yr)	Mutation	Zygosity	First sign	Age of onset (yr)	Cardinal PD signs				H&Y scale	PD therapy		Psychiatric signs
								RT	B	R	PI		Response	Treatment complications	
Patients	B11*	M	79	delEx7/ c.1072delT	compound-heterozygous	Tremor	64	+	+	+	+	III	L-Dopa	–	n.a.
	B125*	M	62	c.1072delT	homozygous	n.a.	43	+	+	+	n.a.	n.a.	n.a.	n.a.	n.a.
	B300*	F	49	delEx7	homozygous	n.a.	34	+	+	+	n.a.	n.a.	n.a.	n.a.	n.a.
	L3035	M	49	delEx3-4/ duplEx7-12	compound-heterozygous	Tremor, depression	31	+	+	+	–	II	L-Dopa, dopamine-agonist	Dyskinesia, motor fluctuations, wearing-off, freezing	Depression
	L3048	M	57	delEx4/ c.924C>T	compound-heterozygous	Dystonia	15	+	+	+	+	III	L-Dopa	Dyskinesia, motor fluctuations , wearing-off, freezing	–
	L3244	F	41	delEx1/ c.924C>T	compound-heterozygous	Bradykinesia	37	+	+	+	–	II	Dopamine agonist, MAO-B inhibitor, anticholinergic drugs	Motor fluctuations, nausea, sleep disturbances, orthostasis	–
∅±STD			*56.2±13.3*				*37.3±16.1*								
Controls	802.1	F	60	None	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	902.1	F	68	None	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	B963*	M	44	None	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	B964*	M	44	None	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	L3293	M	43	None	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
∅±STD			*51.8±11.5*												

*Note: RT - rest tremor; B - bradykinesia; R - rigidity; PI - postural instability; H &Y - Hoehn and Yahr; + - present; – - absent; n.a. – not available, n/a – not applicable. Sex: F – female, M – male. The individuals marked with a *are part of the Italian family described in 2.1.1.*

2.1.2 Patients included in the *LRPPRC* mutation screening

Based on the interaction between LRPPRC and Parkin and common clinical features described in chapters 1.2.4 and 1.2.5, we hypothesized that mutations in *LRPPRC* may be the cause of PD. For sequencing of *LRPPRC*, 46 patients fulfulling the following criteria were selected for the present study: atypical parkinsonism and/or rapid progression (at least Hoehn and Yahr stage 3≤ five years disease duration) and/or dementia (Montreal Cognitive Assessment ≤24, Mini-Mental state examination ≤ 24) and/or depression (Beck's Depression Inventory ≥18, history of depression). After obtaining informed consent, blood was collected from all patients for DNA extraction according to a published protocol (Miller et al. 1988).

2.2 Material

Materials used in the two sections of the study are listed below with the manufacture's name.

2.2.1 Chemicals

Accutase	PAA Laboratories
Acetyl-CoA (trilithium)	Sigma-Aldrich
Agarose	Biozym
Antimycin A ($C_{28}H_{40}N_2O_9$)	Sigma-Aldrich
Ascorbic acid ($C_6H_8O_6$)	Sigma-Aldrich
Betaine	Sigma-Aldrich
Bis-tris propane ($C_{11}H_{26}N_2O_6$)	Invitrogen
Boric acid (H_3BO_3)	Merck
Bovine serum albumin (BSA)	Sigma-Aldrich
Bromphenol blue	Sigma-Aldrich
Cytochrome c (horse heart)	RocheDiagnostics
Cycloheximide ($C_{15}H_{23}NO_4$)	Alomone labs
Cytofluor mounting medium	Applied biosystems
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Dipotassium hydrogen phosphate (K_2HPO_4)	BDH

5, 5'-Dithiobis-(2-nitrobenzoic acid) (DTNB)	Sigma-Aldrich
Desoxyribonucleotides (dNTPs)	Amersham Biosciences
Dithiothreitol (DTT; $C_4H_{10}O_2S_2$)	Sigma-Aldrich
Dulbeccos Modified Eagle Medium (DMEM)	PAA Laboratories
Ethanol (C_2H_5OH)	J.T. Baker
Ethidium bromide ($C_{21}H_{20}BrN_3$)	Sigma-Aldrich
Ethylenediaminetertraacetic acid (EDTA)	Merck
Foetal Bovine Serum (FBS)	PAA Laboratories GmbH
Formamide ($HCONH_2$)	Fluka
Hydrochloric acid (HCl)	Merck
Leupeptin	Sigma-Aldrich
Low-melting point agarose	BRL
2-Mercaptoethanol (C_2H_6OS)	Sigma-Aldrich
Magnesium Chloride ($MgCl_2$)	Sigma-Aldrich
Methanol (CH_3OH)	J.T. Baker
Monopotassium phosphate (KH_2PO_4)	BDH
NADH	RocheDiagnostics
NuPAGE® MOPS SDS Running buffer	Invitrogen
NuPAGE® LDS Sample buffer (4x)	Invitrogen
NuPAGE® Transfer buffer (20x)	Invitrogen
OptimMEM® medium	Invitrogen
Oxaloacetic acid ($C_4H_4O_5$)	Sigma-Aldrich
Paraformaldehyde	Sigma-Aldrich
Paraquat (Methylviologen hydrate; $C_{12}H_{14}C_{l2}N_2$)	Sigma-Aldrich
Penicillin/Streptomycin (P/S)	PAA Laboratories
Pepstatin A ($C_{34}H_{63}N_5O_9$)	Sigma-Aldrich
Phenylmethylsulphonyl fluoride (PMSF; $C_7H_7FO_2S$)	Sigma-Aldrich
Phosphate buffered saline (PBS)	PAA Laboratories
PIPES	Sigma-Aldrich
Potassium bicarbonate ($KHCO_3$)	Sigma-Aldrich
Potassium chloride (KCl)	Merck
Potassium cyanide (KCN)	BDH
Potassium ferricyanide ($K_3[Fe(CN)_6]$)	Sigma-Aldrich
Proteinase K	Sigma-Aldrich

Rotenone ($C_{23}H_{22}O_6$)	Sigma-Aldrich
Sodium acetate	Sigma-Aldrich
Sodium borohydride ($NaBH_4$)	BDH
Sodium chloride (NaCl)	Merck
Sodium dodecyl sulphate (SDS)	Fluka
Sodium hydrogen carbonate ($NaHCO_3$)	Merck
Sodium succinate ($Na_2C_4H_{16}O_{10}$)	BDH
Sucrose ($C_{12}H_{22}O_{11}$)	BDH
Taq-DNA-Polymerase	Qbiogene
5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC1)	Moleculare Probes
Tris ($C_4H_{11}NO_3$)	BDH
Tris-Borate-EDTA buffer (TBE) 10x	Sigma-Aldrich
Triton-X100	Sigma-Aldrich
Tween	Merck
Ubiquinone (CoQ1)	EISAI
Valinomycin ($C_{54}H_{90}N_6O_{18}$)	Sigma-Aldrich

2.2.2 Solutions

Cell suspension buffer:	150mmol/l KCl, 25mmol/l Tris-HCl; pH 7.6, 2mmol/l EDTA; pH 7.4, 10mmol/l KPO_4; pH 7.4, 0.1mmol/l $MgCl_2$ and 0.1% [w/v] BSA
Homogenization buffer:	10mM Tris, 1mM EDTA, 250mM sucrose, 1mM PMSF, 1µg/ml pepstatin A, 1µg/ml leupeptin, pH 7.4
Isolation medium:	320mM sucrose, 10mM Tris, 1mM EDTA, pH 7.4
Leidener solution:	155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA, pH 8.0
Loading dye:	47.5ml formamide, 2.0ml 0.5M EDTA, 0.01g bromphenol blue, pH 8.0
Lysis buffer:	10mM Tris, 400mM NaCl, 0.2mM EDTA, pH 8.0
Phosphate buffer (10mM):	10mM KH_2PO_4, 10mM K_2HPO_4, pH 7.0
Phosphate buffer (20mM):	20mM KH_2PO_4, 20mM K_2HPO_4, 8mM $MgCl_2$, pH 7.2

Phosphate buffer (100mM):	100mM KH_2PO_4, 100mM K_2HPO_4, pH 7.4
Quenching buffer:	100mmol/l Tris-HCl, 4mmol/l EDTA pH 7.75
RIPA buffer:	25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS
Substrate buffer:	10mmol/l malate, 10mmol/l pyruvate, 1mmol/l ADP, 40µg/ml digitonin and 0.15mmol/l adenosine pentaphosphate
Tris-Buffered-Saline (TBS) 10x:	25mM Tris, 150mM NaCl, 2mM KCl, pH 7.4
Transfer buffer:	1x NuPAGE® Transfer buffer, 10% Methanol

2.2.3 Kits and ready-to-use materials

ATP Bioluminescence Assay Kit HS II	RocheDiagnostics
BCA Protein Assay Kit	Pierce
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
DNA extraction Kit	Qiagen
Genetic Analyzer Capillary Array, 50cm	Applied Biosystems
POP-7 Polymer	Applied Biosystems
Sequencing buffer (5x)	Applied Biosystems
Synergy Brands, inc.(SYBR) Green I Mix	RocheDiagnostics
NuPAGE® Novex 4-12% Bis-Tris gel 1.0mm, 10 wells	Invitrogen
Zenon immunolabelling kit	Molecular Probes

2.2.4 Antibodies

Rabbit anti-GRP75 (1:10^6)	Abcam
Rabbit anti-SOD2 (1:250)	Santa Cruz Biotechnology
Rabbit anti-mtTFA (1:2000)	Abcam
Rabbit anti-Fis1 (1:2000)	Alexis Biochemicals
Anti-Dinitrophenol (DNP)	Millipore, Billerica, MA
Anti-Parkin (1:1000)	Cell Signaling Technology
Anti-Mitofusin 2 (1:1000)	Abcam
Anti-HSP60 (1:2000)	Cell Signaling
Anti-β-actin (1:10^6)	Sigma-Aldrich

Goat anti-mouse, HRP-conjugated (1:10,000)	Santa Cruz
Goat anti-rabbit, HRP-conjugated (1:10,000)	Santa Cruz

2.2.5 Oligonucleotides

Molecular weight standard (100bp)	Invitrogen
Oligonucleotide (primer)	Biometra; MWG Biotech
Oligonucleotide, labelled	MWG Biotech

2.2.6 Equipment

Centrifuge	Eppendorf, Neolab, Sorvall
Filter papers	Whatman
Genetic Analyzer 3130 and 3130xl	Applied Biosystems
Homogeniser	Eurostar IKA Werke
Luminometer	Berthold, Detection Systems, Pforzheim
Microscope, Axiovert 40	Zeiss
Mini-transblot unit	Invitrogen
Mini-gel system	Invitrogen
PD_{10} column	Pharmacia Biotech
PVDF HyperbondP membrane	Amersham Biosciences
Scale	Satorius
Synergy HT plate reader	BIOTEK
Thermocycler - LightCycler	RocheDiagnostics
- Mastercycler	Eppendorf
- 7300 Real-Time PCR System	Applied Biosystems

2.2.7 Software

Axiovision	Zeiss
Gen5 Data Analysis	BIOTEK
SeqScape	Applied Biosystems
TotalLab Quant	Nonlinear Dynamics Ltd

2.3 Methods

2.3.1 Tissue culture

2.3.1.1 Fibroblast culture

Fibroblasts were plated in 100mm dishes and T-175 flasks and cultured in Dulbecco's Modified Eagle Medium (DMEM) plus 10% foetal bovine serum albumin (vol/vol), 10mg/l sodium pyruvate, 4.5g/l glucose and 10ml/l penicillin/streptomycin in an incubator (95% air/5% CO_2) at 37ºC.

2.3.1.2 Passaging of fibroblasts

Fibroblasts were passaged at 80% confluence. Cell media was removed, the cells were washed with phosphate buffered saline (PBS), and incubated at 37°C with 2ml Accutase for each 175cm^2 flask for 10min. Detachment of cells was assessed using microscopy and the fibroblasts were collected with 5-7ml of culturing medium. The fibroblasts were pelleted by centrifugation at 1000rpm for 5min. Fibroblasts were resuspended in culturing medium composed as above.

2.3.1.3 Treatment with toxins

To study consequences of oxidative stress, several fibroblast cultures of patients and controls were treated with the superoxide generator paraquat (0.5mM or 2mM for 24h). For the experiments described in chapter 2.3.11.3, fibroblasts were treated with 200µM H_2O_2 for 30min.

2.3.1.4 Determination of cellular growth rates

When a confluence of 90% was reached, cells were harvested, dissolved in PBS and a small proportion of this solution was mixed with Trypan blue. The viability was determined by counting in a cell counting chamber “Neubauer improved”. Stained cells were considered as dead and non-stained as alive.

2.3.2 Protein determination

Sample protein concentration was determined by use of the BCA protein assay kit according to the manufacturer's protocol. Absorbance was measured on a plate reader at 562nm. Sample protein concentration was calculated from a BCA standard calibration curve (0-1500µg/ml).

2.3.3 ATP synthesis rate

Cellular ATP synthesis rates were determined according to a published protocol (Shepherd et al. 2006). In brief, the amount of protein was determined using the BCA protein assay kit as described in chapter 2.3.2. Fibroblasts were harvested and dissolved in a cell suspension buffer to obtain a protein concentration of 1mg/ml protein. ATP synthesis was initiated by the addition of 250µl of cell suspension to 750µl of substrate buffer. Cells were incubated at 37°C for 10min. At 0 and 10min, 50µl aliquots of the reaction mixture were withdrawn, quenched in 450µl of boiling 100mmol/l Tris-HCl, 4mmol/l EDTA (pH 7.75) for 2min and further diluted 1/10 in the quenching buffer. The ATP concentration was measured using a luminometer with the ATP Bioluminescence Assay Kit HS II following manufacturer's instructions.

2.3.4 Cellular ATP concentration

ATP levels were determined with the ATP Bioluminescence Assay Kit HS II. Prior to the measurement with a luminometer, samples were corrected for cell number (for details see 2.3.1.4). The experiment was performed with 18750 cells per ml PBS. The standard curve ranged from 4.15×10^{-13} to 8.3×10^{-9}mol ATP.

2.3.5 Mitochondrial membrane potential

$\Delta\psi m$ was analyzed with the sensitive fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). This lipophilic cation changes reversibly the wavelength of its emitted light from 530nm to 590nm as $\Delta\psi m$ increases. About 3.5×10^4 cells were plated per 12-well. One well per culture was treated with 1µg/ml JC-1 in OptiMEM medium for 15min at 37°C. To detect basal fluorescence emission, an additional well per culture was treated with the ionophore valinomycin in the presence of JC-1. As blank, one well per sample remained untreated and contained medium only.

Fluorescence detection at 590nm was performed with a plate reader. Subsequently, cells were lysed by adding 200µl NaOH 100mM in each well and stored over night at 4°C. The next day, protein concentration was determined as described (2.3.2). $\Delta\psi m$ was expressed relative to the protein concentration per well and calculated as follows:

$$\Delta\psi m = \frac{FL_{Target}/[Protein]_{Target} - FL_{Blank}/[Protein]_{Blank}}{FL_{Valinomycin}/[Protein]_{Valinomycin} - FL_{Blank}/[Protein]_{Blank}}$$

FL_x - Fluorescence emission of a certain well

[x] - Protein concentration of cells of a certain well

2.3.6 Mitochondrial preparation

Mitochondria were isolated from fibroblasts as previously described (Almeida *et al.* 1997), with minor modifications as follows. To gain a sufficient amount of mitochondrial preparation for all mitochondrial assays three flasks with a surface of 175cm^2 per flask were used. Cells were harvested as mentioned above (2.3.1.2.). Next, the obtained cell pellet was re-suspended in 1-2ml ice-cold homogenization buffer. Cells were then centrifuged at 4000g for 5min and the supernatant was discarded to remove any remaining culturing medium. The pellet was resuspended in 2ml ice-cold homogenization buffer. Cells were optimally homogenized on ice by 50 strokes of a tight fitting glass-teflon homogenizer revolving at 1000rpm. To achieve a high proportion of broken cells, as indicated by isolated nuclei, the homogenate was examined under the microscope. If the ratio of nuclei to unbroken cells was under 80% the homogenization step was repeated with additional strokes until the required ratio of nuclei to whole cells was reached. 20µl of the homogenate were retained for citrate synthase (CS) activity measurements. Cell homogenates were centrifuged at 1500g for 10min at 4ºC, the supernatant was transferred into a fresh cold tube and the pellet was discarded. The supernatant was centrifuged once more at 1500g (10min, 4ºC) and the pellet discarded. In a last step, the supernatant was centrifuged at 13000rpm for 10min at 4ºC. The obtained pellet of mitochondria was frozen at -80°C over night. The next day, it was resuspended in 50µl homogenization buffer and aliquots of 2.5µl to 4.5µl were made. These were frozen in liquid nitrogen and stored at -80ºC.

2.3.7 Mitochondrial enzyme assays

The activities of complexes I to IV and of CS were determined in mitochondrial preparations by means of a spectrophotometrical approach. The activites of the complexes were expressed relative to CS activity.

2.3.7.1 Citrate synthase (CS) assay

The enzyme CS exists in nearly all living cells and stands as a pace-making enzyme in the first step of the Citric Acid Cycle. CS is localized within eukaryotic cells in the mitochondrial matrix, but is encoded by nuclear DNA rather than mitochondrial (Wiegand et al. 1986). It is therefore used as a cellular marker for mitochondrial integrity and content. CS activity was determined as previously described (Coore *et al.* 1971) using a spectrophotometrical plate reader. Mitochondrial membranes were broken by three cycles of freeze-thawing in liquid nitrogen. The mitochondrial preparations (10-20µg protein) were mixed with 100mM Tris (pH 8.0), 0.1mM acetyl coenzyme A, 0.1% (wt/vol) Triton-X100, and 0.1mM DTNB in a 96-well plate (total volume 200µl). The pathlength was calculated by the software Gen5 using the default value of 0.18 for the absorbance of water at 1cm pathlength and the following formula:

$$\text{Pathlength calculation : pathlength in cm} = \frac{(A_{977nm}-A_{900nm})_{\text{well}}}{(A_{977nm}-A_{900nm})_{\text{1.0cm water}}}$$

A= Absorbance

The background activity was registered for one minute. The reaction was started by the addition of 0.1mM oxaloacetate, and activity was measured at 412nm for 7min at 30ºC (DTNB extinction coefficient $=13.6x10^{3}M^{-1}cm^{-1}$). Samples were run against a reference well that contained distilled water instead of sample and all substrates except oxaloacetate.

2.3.7.2 Complex I assay (NADH dehydrogenase)

CI activity was determined spectrophotometrically as previously described (Ragan et al. 1987) using a plate reader. Prior to three cycles of freeze-thawing the sample (10-20µg protein) was mixed with 160µl phosphate buffer (pH 7.2, 25mM) containing 10mM $MgCl_2$. To this mixture, 2.5mg/ml BSA, 0.15mM NADH, and 1mM KCN were added in a 96-well plate. Background activity was registered for 1min and the reaction was started by

the addition of 0.25mM CoQ1. Enzyme activity was measured at 30ºC by following the decrease of absorption due to oxidation of NADH to NAD^+ at 340nm for 7min (NADH extinction coefficient =$6.81x10^3M^{-1}cm^{-1}$; total volume =200µl). After this time elapsed, 10µM rotenone was added, and rotenone insensitive NADH oxidation was measured for 7min. All results were corrected for the calculated pathlength. CI activity was calculated by subtracting the rotenone insensitive NADH oxidation rate from the NADH oxidation rate (units =nmol/min/mg protein). Notably, all wells were run against a reference well that contained distilled water instead of sample and all substrates except CoQ1.

2.3.7.3 Ferricyanide reductase assay

To further specify the results from the CI activity assay, a NADH ferricyanide reductase assay was performed. This assay allows the determination of the content of functional CI instead of its physiologic activity (Degli Esposti 2001). In this experiment a direct measurement of the CI activity was performed by using ferricyanide as a potent non-natural electron acceptor. By means of a spectrophotometer, the reduction of ferricyanide to ferrocyanide was followed corresponding to the consumption of NADH.

The probe (10-20µg protein; freeze-thawed three times in liquid nitrogen) was mixed with 20mM phosphate buffer (pH 7.0) and 1mM ferricyanide in a 96-well plate with a total volume of 200µl. After registration of the basal absorbance at 30°C, 600µM NADH was added to start the reaction. The reduction of ferricyanide was followed by tracing the decline in absorbance at 420nm. For the calculations, the extinction coefficient of $1x10^3M^{-1}cm^{-1}$ for ferricyanide was applied. In every run the measurements were compared to a reference well not containing any mitochondrial preparation. All results were corrected for the calculated pathlength and expressed in nmol/min/mg protein.

2.3.7.4 Complex II+III assay (succinate cytochrome reductase)

CII+III activity was determined spectrophotometrically as previously described (Takemori *et al.* 1964) using a plate reader. The sample (10-20µg protein; freeze-thawed three times in liquid nitrogen) was mixed with 100mM phosphate buffer (pH 7.4), 0.3mM EDTA, 1mM KCN, and 0.1mM oxidized cytochrome c (from horse heart) in a 96-well plate. The reaction was started by addition of 20mM succinate and the enzyme activity measured at 30ºC by following the reduction of cytochrome c at 550nm (cytochrome c extinction coefficient =$19.2x10^3M^{-1}cm^{-1}$; total volume =200µl). After 7min, 20µM antimycin A was

added, and the antimycin A insensitive rate of cytochrome c reduction was followed for a further 7min. All results were corrected for the calculated pathlength. CII+III activity was calculated by subtracting the antimycin A insensitive cytochrome c reduction rate from the total cytochrome c reduction rate (units =nmol/min/mg protein). All wells were run against a reference well that contained water instead of sample and all the substrates except succinate and antimycin A.

2.3.7.5 Complex IV assay (cytochrome c oxidase)

For the determination of the enzyme kinetic of CIV reduced cytochrome c is required as a substrate.

2.3.7.5.1 Reduction of oxidized cytochrome c

Oxidized cytochrome c (1.6mM) was dissolved in 10mM phosphate buffer, pH 7.0 and DTT (3.4mM) was added to reduce cytochrome c. The solution was stirred at 4°C for at least 15min. The reduced cytochrome c was passed through a PD_{10} gel filtration column (which was equilibrated before-hand by washing with 30ml of 10mM phosphate buffer, pH 7.0) to remove DTT from the reduced cytochrome c. The degree of reduction was determined by measuring the reduced cytochrome c solution against dissolved fully oxidized cytochrome c in a spectrometer at 550nm. This was performed by mixing 10µl reduced cytochrome c with 190µl H_2O in both a sample and reference well. Ferricyanide (1mM) was added to the sample well to oxidize the reduced cytochrome c. Next, the difference in absorbance between sample and reference well was noted (cytochrome c extinction coefficient $=19.2x10^3M^{-1}cm^{-1}$; total volume =1ml; path length =1cm). The concentration of reduced cytochrome c was calculated according to the Lambert-Beer law.

2.3.7.5.2 Measurement of complex IV activity

CIV activity was determined spectrophotometrically as described (Wharton *et al.* 1967) with a plate reader. In a sample and reference well, 10mM phosphate buffer (pH 7.0) and 50µM reduced cytochrome c were mixed. To the reference well, 1mM ferricyanide was added to oxidize the cytochrome c, yielding a corrected absorbance of approximately 1.0 at 550nm in the sample well prior to addition of the sample. The sample (10-20µg protein; freeze-thawed three times in liquid nitrogen) was then added to the sample well and the oxidation of cytochrome c at 550nm was measured for 10min at 30ºC (cytochrome c

extinction coefficient =19.2x$10^3$$M^{-1}$$cm^{-1}$; total volume =200µl). The values for the reference well were subtracted before calculating the activities. CIV activity is expressed by the constant k per min per mg protein. The constant k was determined by noting the highest positive absorbance following sample addition (t=0min), and the absorbance every minute afterwards for 5min. The k constant was calculated by: (ln($A_{550t=0}$/$A_{550t=n}$)/number of min)/protein concentration. The rate constant for each sample was taken as the mean of k at 1, 2, 3, 4 and 5min.

2.3.8 Western blotting

The Western blotting procedure involves sample preparation, polyacrylamide gel electrophoresis (PAGE), protein transfer and antibody detection of target proteins on the resulting blot. Western blots were carried out for whole cell homogenates and mitochondrial preparations.

2.3.8.1 Sample preparation

When mitochondrial fractions were analyzed by Western blotting, one confluent T175 flask of fibroblasts was used. For the analysis of whole cell homogenates, fibroblasts of one 6-well were harvested (2.3.1.2). Cells were pelleted at 8000rpm and the supernatant was discarded. The pellets of whole cells as well as the mitochondrial pellets were resuspended in 30-50µl of RIPA buffer with proteinase inhibitors and incubated on ice for 30min. The mixture was centrifuged at 4°C and 13000rpm for 20min. The supernatant was processed further and the pellet discarded. The protein concentration per sample was determined according to the protocol given in 2.3.2.

2.3.8.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for proteins

A mini-gel system was used for protein separation. Precast gels were utilized. These NuPAGE Noves 4-12% Bis-Tris gels (1.0mm, 10 wells) are based on a Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gel, with a separating gel that operates at pH 7.0. NuPAGE Lithium dodecyl sulphate (LDS) sample buffer in ratio 1:4 (vol/vol) and DTT in ratio 1:10 (vol/vol) were added to the samples and heated up to 95°C for 5min. Eight µg of protein per sample were loaded. A molecular weight standard was run on the same gel. The electrophoresis was performed in 1x MOPS running buffer for 90min at 150V.

2.3.8.3 Protein transfer

A piece of PVDF membrane was cut and plunged into transfer buffer. Sponges and filter papers were pre-wet in 1x transfer buffer before assembling of the mini-transblot unit. Loading wells were cut off the PAGE gel and the gel was rinsed with transfer buffer. The Western blot "sandwich" was built in the following order:

1. Sponges
2. Filter paper
3. PAGE gel
4. PVDF membrane
5. Filter paper
6. Sponges

The transfer was run at 32V for 70min in transfer buffer at room temperature (RT).

2.3.8.4 Antibody staining of Western blots

Blots were blocked in 1-4% (wt/vol) degreased milk in 1x TBS with 0.1% (vol/vol) Tween 20 for 1h at RT. The amount of milk powder used depended on the degree of blocking required for the specific antibody. The primary antibody was added into milk in 1x TBS with 0.1% (vol/vol) Tween 20 in an appropriate concentration (according to manufacturer's protocol) and the blot was incubated overnight at 4°C. This was followed by three washing steps in 1x TBS with 0.1% (vol/vol) Tween 20 for 5min each. Next, the blot was incubated with a horseradish-peroxidase (HRP)-conjugated secondary antibody in 1% (wt/vol) milk in 1x TBS with 0.1% (vol/vol) Tween 20 in a dilution of 1:10000 (vol/vol) for 1h at RT. This was followed by another three washing steps. The bands were detected by enhanced chemiluminescence. Equal loading was assessed by use of an antibody against β-actin for whole cell homogenates and HSP 60 for mitochondrial preparations. Band density was measured using the image processing program TotalLab Quant.

2.3.9 Analysis of mitochondrial network

2.3.9.1 Immunocytochemistry

Fibroblasts were harvested as described above (2.3.1.2), and $1.5x10^4$ cells in 0.5ml of culturing medium (2.3.1.1) were seeded onto glass coverslips ($5.3cm^2$) placed in 6-well

plates. The cells were then incubated for approximately 24h. The medium was removed and the cells were washed with PBS. In the following, the cells were fixed on the cover slips for 15min in 4% paraformaldehyde at RT. After washing in PBS, the cells were permeabilized in 0.1% Tergitol-type NP40 (nonyl phenoxypolyethoxyethanol) for 10min at RT. This was followed by another washing step with PBS. Following this, nonspecific binding was blocked by 1% bovine serum albumin (BSA) in PBS for 30min at RT. In a next step, the cells were incubated with rabbit polyclonal anti-GRP75 in combination with a Zenon immunolabelling complex at 4°C overnight. Subsequently, the cover slips were washed with PBS and mounted on glass microscope slides (76x26mm) with DAPI (4',6-diamidino-2-phenylindole**)**.

2.3.9.2 Fluorescence microscopy

Fluorescence was detected using an Axiovert 40 microscope with an excitation wavelength of 395nm and an emission wavelength of 509nm for the zenon immune labeling complex. For DAPI the excitation maximum of 358nm and an emission maximum at 461nm were used.

Cells were picked randomly and brought into focus. The vertical range of the cell was determined. Using the ApoTome slider module, pictures of six-ten stacks were taken of each cell. All separate stacks and an integrated picture of the stacks were saved for further image analysis with the AxioVision software. This procedure was accomplished for five different cells of each cellular sample.

2.3.9.3 Image analysis

Image analysis was carried out with the image processing program ImageJ 1.42. First, stacks were integrated to one picture. The image was converted into a binary image and the threshold was set. Mitochondrial morphology characteristics, such as area and outline of the mitochondria, were measured. To determine the degree of branching, the outline-to-area ratio and the form factor were calculated. The form factor was defined as $\frac{Pm^2}{4\pi Am}$, where Pm is the length of the mitochondrial outline and Am is the area of the mitochondrion (Mortiboys *et al.* 2008). The form factor allows quantifying the degree of branching of the mitochondrial network. Images of at least five randomly selected cells per individual were analyzed under basal conditions and after paraquat treatment.

2.3.10 Extraction of nucleic acids

For all experiments of this thesis, DNA was gained from human blood or fibroblast cell cultures.

2.3.10.1 DNA extraction from whole blood

Genomic DNA was prepared from leucocytes of peripheral blood by means of a salting out method (Miller et al. 1988). In brief, cells were lysed by adding of a hypertonic solution. Proteins were digested by proteinase K and DNA was isolated by precipitation. With this method an amount of 100 to 1000µg genomic DNA was obtained.

2.3.10.2 DNA extraction from fibroblasts

For DNA extraction from fibroblasts, the Nucleon I kit was used. Cell pellets were processed according to manufacturer's protocol.

2.3.11 Polymerase chain reaction

The polymerase chain reaction (PCR) is an enzymatic method to amplify DNA *in vitro* (Saiki et al. 1985). The specificity of the amplification is achieved by use of two chemically synthesized primers which bind complementary to the target sequence. Denaturation of double-stranded DNA into single-stranded DNA is achieved by heating to 95°C. Subsequently, the temperature is reduced to facilitate annealing of the primers. A temperature stable polymerase synthesizes the DNA region between the primers. The reaction is divided in three repeating steps: denaturing, annealing, extension. Exponential amplification of the target sequence provides high DNA concentrations for further analysis.

2.3.11.1 Standard PCR

A PCR under standard conditions was run as follows.

Reaction mixture:

Substance	**Stock concentration**	**Volume**	**Final concentration**
dH_2O		ad 20.0µl	
Buffer	10x	2.0µl	1x
dNTPs	1mM	4.0µl	0.2mM

primer forward	10µM	0.8µl	0.4µM
primer reverse	10µM	0.8µl	0.4µM
Taq polymerase	5U/µl	0.1µl	0.025U/µl
DNA	~ 10ng/µl	4.0µl	~ 2ng/µl

Cycling conditions:

		95°C	3min	initial denaturation
		95°C	30s	denaturation
35x		55°C	30s	annealing
		72°C	30-60s	extension
		72°C	10min	final extension
		4°C		storing temperature

The annealing temperature depends on the structure of the primers used. The extension time is adjusted to the expected product size. Additives like betaine and dimethyl sulfoxide (DMSO) were used to improve the PCR conditions in case of unspecific or insufficient amplification. Betaine enhances the specificity of the PCR by reducing the base pair composition dependence of DNA melting in GC-rich regions. DMSO inhibits secondary structure formation in the template DNA or primers.

PCR was performed for all 38 coding exons of *LRPPRC* (primer sequences and PCR conditions are shown in Supplementary Table 1) and for the whole mitochondrial genome (primer sequences and PCR conditions in Supplementary Table 2). To gain PCR products with practicable length for sequencing, the mitochondrial genome was divided into 28 segments according to an established protocol (Taylor et al. 2001).

2.3.11.2 Sequencing reaction

To purify 5µl of PCR product 2µl of Exo-Sap were added. This mixture of the enzymes (exonuclease I, a phosphatase and a buffer) was added to remove excess primers and dNTPs from the probe. The sample mix was incubated on a thermocycler for 15min at 37°C and for another 15min on 80°C for enzyme inactivation.

The sequencing technique used is based on the concept of the chain termination method of Sanger *et al.* (Sanger 1977) and was modified as described by Prober *et al.* (Prober et al. 1987). The sequencing reaction requires different cycles of temperatures similar to the PCR reaction. During the phase of nucleotide extension marked 2’3’-dideoxynucleotides were randomly included generating fragments of each possible length. Then, fragments

were separated by means of electrophoresis in the sequence of their length, while the tag was detected by a laser.

To prepare the samples for electrophoresis the dye terminator sequencing was performed with the ABI system using a BigDye® Terminator v3.1 Cycle Sequencing Kit. The sequencing reaction was performed on a thermocycler with the following program: (1. 1:00 at 96°C, 2. 0:10min at 96°C, 3. 0:05min at 60°C°/55°C (depending on annealing temperature of the primer), 4. 1:00min at 60°C, 25 cycles of the steps 2.-4., 5. cooling down to 4°C). Following substances are used for the sequencing reaction:

Substance	**Stock**	**Volume**	**Final concentration**
Purified PCR product	5-10ng	2µl	0,5-1ng/µl
Primer forward or reverse	10µM	0.5µl	0.5µM
Terminator mix 3.1.		0.5µl	
Sequencing buffer	5x	1.5µl	0.75x
dH_2O		Ad 10µl	

To obtain pure DNA for electrophoresis a precipitation step was performed. This was accomplished using 3M sodium acetate and 100% ethanol followed by a washing step with 70% ethanol. The precipitated DNA was dissolved in highly deionized formamide (HiDi Formamide). The fragments were separated by means of capillary electrophoresis (this procedure is described later in chapter 2.3.12.2).

2.3.11.3 Quantitative PCR

Quantitative PCR (qPCR) is a technique which allows following the amplification of DNA in real-time and, thus, measuring of DNA concentrations. The fluorescent dye SYBR Green which specifically binds double-stranded DNA was used on a LightCycler to determine the mtDNA level in lymphocytes, fibroblasts and stressed fibroblasts (200µM H_2O_2 for 30min and a recovery period of 2h).

The concentration of the mitochondrial-encoded *cytochrome oxidase subunit II* (*COX II*) gene (forward primer: 5'-AGT ACT CCC GAT TGA AGC CCC-3'; reverse primer: 5'-GGA CGA TGG GCA TGA AAC TGT-3') was calculated relative to the concentration of the nuclear single copy gene *β-globin* (forward primer: 5'-ACA CAA CTG TGT TCA CTA GC-3'; reverse primer: 5'-CAA CTT CAT CCA CGT TCA CC -3').

Both amplifications were performed in separate reactions with a total volume of 10µl each using the following reagents: 2µl SYBR Green I Mix, 0.5–1.0µM of each primer and 1–

15ng of DNA. PCR conditions were as follows: 95°C for 10min, 95°C for 5s, 60°C for 10s, 72°C for 15s (38 cycles); measurement of fluorescence in each cycle was at 80°C.

During the log-linear phase, amplification can be described as:

$N=N_0(1+E_{const})^n$

N - Number of amplified molecules

N_0 - Initial number of molecules

E - Amplification efficiency

n - Number of cycles

Since amplification efficiency during the log-linear phase is constant, the initial concentration of the sample was calculated based on the above formula, using a standard curve. This standard curve was generated using human genomic DNA in concentrations of 20, 4 and 0.8ng/µl, respectively. All standards were amplified in duplicate and a regression curve was calculated. Concentrations outside the standard range were disregarded. All samples were measured in duplicate and results were accepted only within a range of <10% of the standard deviation of the two simultaneously measured samples. After each PCR, a melting curve between 40 and 80°C was recorded to analyze the purity of the amplification product. The mtDNA level was determined in at least two independent runs per cellular sample.

2.3.12 Gel electrophoresis

2.3.12.1 Agarose gel electrophoresis

Agarose powder was mixed with electrophoresis buffer in the required ratio and heated in a microwave until completely melted. Ethidium bromide was added to the gel (final concentration 0.5µg/ml) at this point to enable visualization of DNA post electrophoresis. After cooling the solution to approximately 60°C, it was poured into a casting tray containing a sample comb and allowed to solidify at RT.

Formamide dye was added in ratio 1:1 (vol/vol) to the samples before loading. With each set of samples a molecular weight standard (100bp ladder) was loaded. The electrophoresis was performed at 120V for 30-60min in 1x TBE buffer. After running, the gel was exposed to UV light to confirm amplification of the product with expected length and to exclude any amplification in the negative control.

2.3.12.2 Capillary sequencer

During capillary electrophoresis, the products of the sequencing reaction (2.3.11.2) were injected electro-kinetically into capillaries filled with polymer. High voltage was applied to move the negatively charged DNA fragments through the polymer in the capillaries toward the positive electrode. Shortly before reaching the positive electrode, the fluorescent-labeled DNA fragments, separated by size up to one nucleotide difference in length, moved through the path of a laser beam. The laser beam excites the florescent-labels and the emitted signal is detected by an optical system. The emission wavelength of the used florescence dyes is nucleotide-specific which allows simultaneous detection of all four DNA bases after a single capillary injection.

All genes and DNA fragments in this study were sequenced on a Genetic Analyzer 3130xl by Applied Biosystems. This device was equipped with arrays of 16 capillaries with a length of 50cm. The 3130 POP-7 polymer was used as a separation matrix.

2.3.13 Computational sequence analysis

To compare sequencing results with published reference sequences for either *LRPPRC* or the mitochondrial genome the sequence analysis program SeqScape was used. Reference sequences were found in the database “Ensembl” (http://www.ensembl.org/, with Transcript ID: ENST00000260665) for *LRPPRC* and in the “Mitomap” (http://www.mitomap.org) for the mitochondrial genome.

Detected nucleotide changes were compared with single nucleotide polymorphisms (SNPs) and pathogenic variants reported in the above-mentioned databases. Additionally, the mutational burden per individual was calculated as the ratio of sequence variations observed vs. total sites possible within the coding region of the mitochondrial genome (n=15,452).

2.3.14 Statistical analysis

For statistical analysis, fibroblast samples were separated into two groups - patients and controls. In all experiments, the median and interquartile range and the minimum and maximum were calculated for each group. For reasons of comparability and a better perceivability, the median of the control group was set to 100% and all other measured values were expressed as a percent of the control median.

The Mann-Whitney U test was applied for comparisons between mutants and controls. In the case of the ATP concentration and synthesis data, a Mann-Whitney U test was performed to compare the patient with the average control values set to 100% in each run. For evaluation of the impact of stress on cells, the Wilcoxon matched-pairs signed-ranks test was used to determine differences before and after treatment. The significance level was set at 0.05.

3 RESULTS

This thesis comprises two projects which were concerned with the molecular mechanisms underlying of PD. Firstly, the impact of mutations in the recessive PD-causing gene *Parkin* on mitochondrial function and morphology was investigated. To study mitochondrial parameters on the endogenous level, fibroblasts from mutation-positive PD-patients were used.

In the second project, the *LRPPRC* gene (encoding a potential interactor of the PD-associated proteins Parkin and PINK1) was screened for mutations in a PD patient sample.

3.1 Mitochondrial function and morphology in patients with *Parkin* mutations

This project concentrated on the link between PD and mitochondria. Experiments were performed in fibroblast samples gained from related and unrelated *Parkin* mutation carriers and a number of related and unrelated control individuals. Initially, the effects of these mutations on the ATP synthesis were investigated (3.1.1). Chapter 3.1.2 is concerned with the activity of complexes I to IV (CI-CIV). Next, $\Delta\psi m$ was quantified in the *Parkin* mutants and controls (3.1.3). Furthermore, oxidative stress levels (3.1.4) and the effects of stress on the mtDNA (3.1.5) were examined in all available fibroblast samples. Then, the mitochondrial content was determined (3.1.6). Finally, the degree of mitochondrial branching (3.1.7) and the growth rates of the cells (3.1.8) were compared in *Parkin* mutants and controls.

3.1.1 ATP synthesis rate and cellular ATP concentration

ATP production is the main function of mitochondria and ATP itself is essential for cell metabolism. ATP synthesis rates and levels were determined as a general parameter of OXPHOS system function. These experiments showed significantly lower ATP synthesis rates in patients (median [interquartile range; IQR]: 69% [58%, 75%], n=6) compared to controls (100%, n=4) with a p-value of 0.002 (Figure 4A). The measurement of cellular ATP levels revealed a reduction to 39% (median [IQR]: 39% [23%, 55%], n=6) of the control level in the patient cells (Figure 4B). This difference reached significance with a p-value of 0.029.

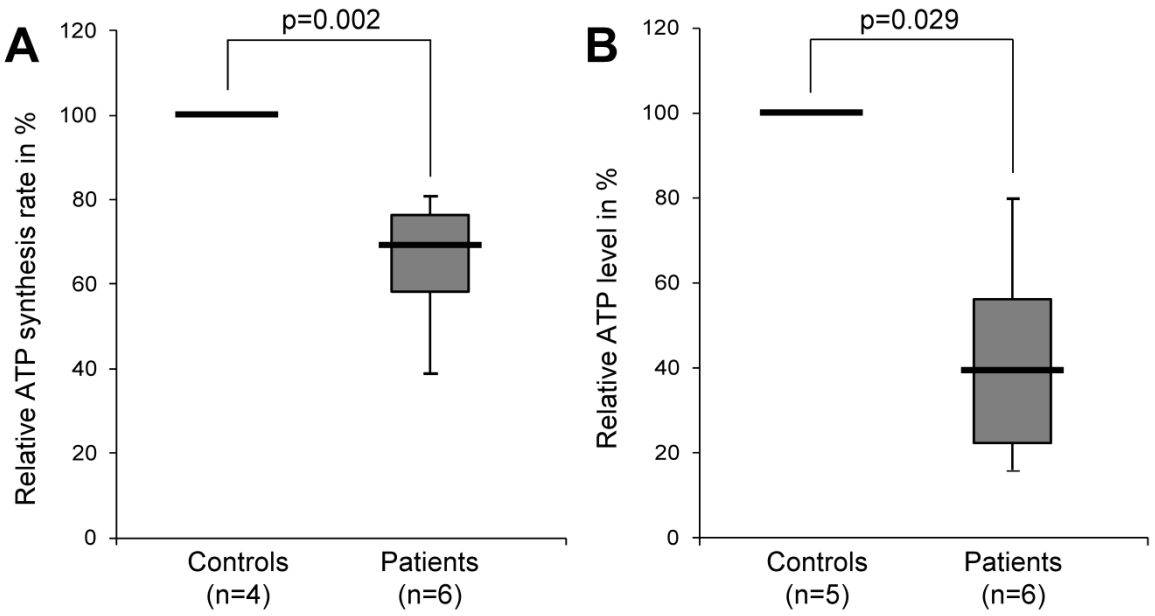

Figure 4 *Basal ATP synthesis rate (A) and basal cellular ATP level (B).* (A) The assay demonstrated a significant reduction in ATP production in the *Parkin*-mutant patients (median [interquartile range; IQR]: 69% [58%, 75%]) compared to controls (set to 100%). (B) Quantifying the overall cellular ATP concentration showed significantly lower levels in mutants (median [IQR]: 39% [23%, 55%]) than in controls (set to 100%). The median, the IQR, the minimum and the maximum value of six (A) or four (B) independent experimental runs were plotted. In each experimental run the average ATP level in the controls was set to 100%. n= number of individuals.

3.1.2 Respiratory chain enzyme activities

Next, to specify whether the lower ATP synthesis rates and cellular ATP levels in the patient samples were due to a dysfunction of RC enzymes, kinetic assays were performed in mitochondrial preparations.

These assays showed no significant differences in CI activity in patient fibroblasts (median [IQR]: 72% [66%, 87%], n=6) compared to controls (median [IQR]: 100% [80%, 102%], n=4) although a non-significant trend (p=0.1) towards lower activities for patient cells was observed (Figure 5A). Furthermore, an NADH ferricyanide reductase assay was performed, which allowed to determine the content of functional CI (Degli Esposti 2001). This assay also showed similar levels in mutants (median [IQR]: 87% [71%, 101%], n=6) and controls (median [IQR]: 100% [88%, 107%], n=5) (Figure 5B).

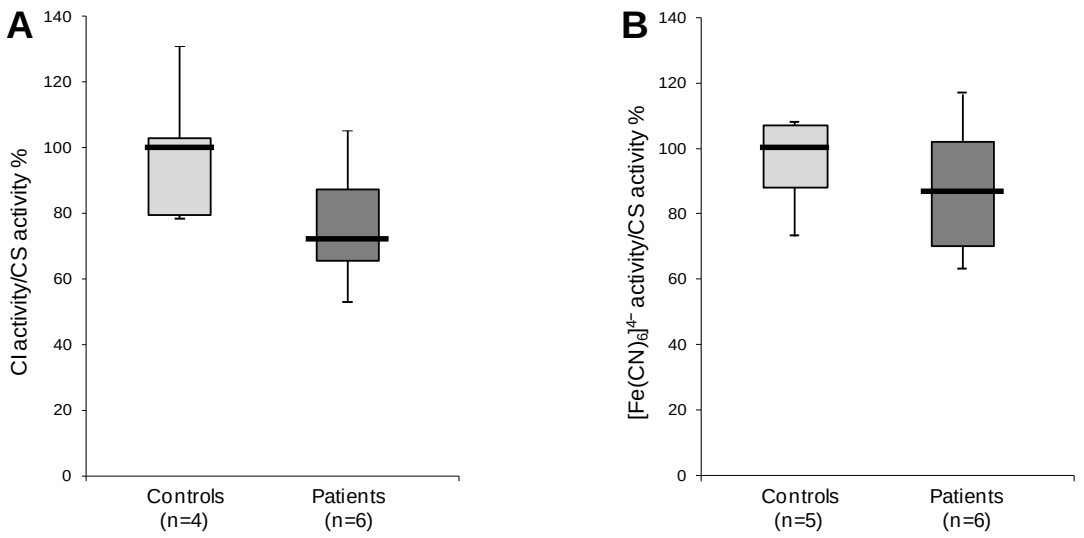

Figure 5 *Complex I (CI) activity (A) and content of functional CI (NADH ferricyanide reductase activity)(B).* (A) Fibroblasts of patients (median [IQR]: 72% [66%, 87%]) showed a non-significant trend towards lower CI activities compared to control cells (median [IQR]: 100% [80%, 102%]). (B) The NADH ferricyanide ($[Fe(CN)_6]^{4-}$) reductase assay revealed comparable enzyme activities in mutants (median [IQR]: 87% [71%, 101%]) and controls (median [IQR]: 100% [88%, 107%]). The median, the interquartile range (IQR), the minimum and the maximum value of at least three independent experimental runs per individual are plotted. The median of the controls was set to 100%. n – number of individuals, CS – citrate synthase.

The activities of complexes II+III (patients: median [IQR]: 134% [85%, 180%], n=6; controls: median [IQR]: 100% [92%, 112%], n=4) (see Figure 6A) and CIV (patients: median [IQR]: 95% [82%, 108%], n=6; controls: median [IQR] 100% [75%, 112%], n=4) (see Figure 6B) were comparable in *Parkin*-mutant fibroblasts and controls.

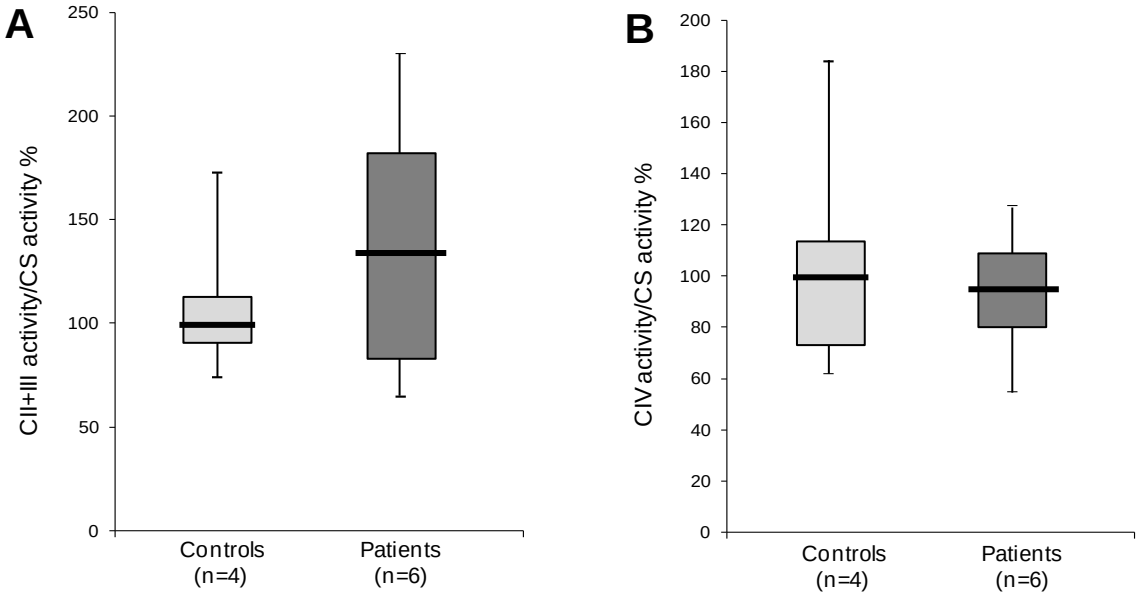

Figure 6 ***(previous page)*** *Activities of complex II +III (CII+III) (A) and complex IV (CIV) (B).* (A) The combined assays for CII and III showed comparable activities for patient (median [IQR]: 134% [85%, 180%]) and control cells (median [IQR]: 100% [92%, 112%]). (B) The measurement of the CIV activity showed similar activities for patients (median [IQR]: 95% [82%, 108%]) and controls (median [IQR]: 100% [75%, 112%]). The median, the interquartile range (IQR), the minimum and the maximum value of at least three individual runs per individual are plotted. The median of the controls was set to 100%. n- number of individuals, CS – citrate synthase.

3.1.2.1 Proof of quality of mitochondrial preparations

To verify the quality of the mitochondrial preparations, Western blot analysis was performed. HSP60 was used as a mitochondrial marker and β-actin served as cytosolic marker. In homogenates, HSP60 as well as β-actin expression was detected. By contrast, in the mitochondrial fractions, only a strong HSP60 band was observed indicative of a successful subcellular fractionation and the enrichment of mitochondria by means of differential centrifugation (Figure 7).

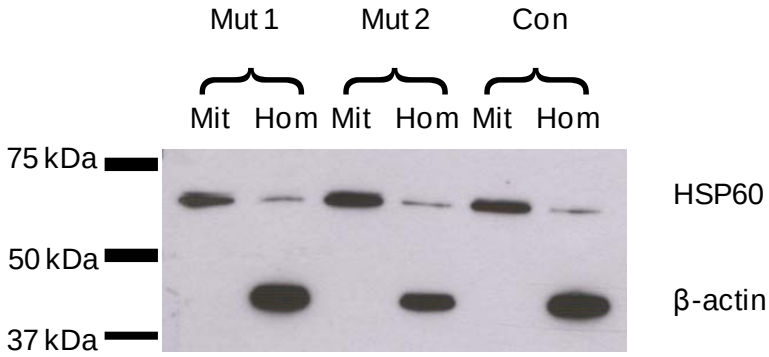

Figure 7 *Western blotting of mitochondrial preparations and homogenates.* Protein expression of the mitochondrial marker HSP60 and the cytosolic marker β-actin in mitochondrial preparations (Mit) and cell homogenates (Hom) of two *Parkin* mutants (Mut 1 and Mut 1) and one control (Con). High concentrations of mitochondria without cytosolic contamination were achieved by differential centrifugation. The precise size standard is indicated on the left, kDa – kilodalton.

3.1.3 Mitochondrial membrane potential

Δψm plays a central role in the mitochondrial biology. It provides the force that drives the influx of protons into the mitochondria. Δψm is generated through oxidation-reduction reactions catalyzed by the RC enzymes. Thus, a low Δψm indicates dysfunction of the complexes I to V (Abou-Sleiman et al. 2006).

In this thesis, the influence of mutant *Parkin* on Δψm was investigated by means of a cytofluorimetric assay (see also 2.3.5). Under normal culturing conditions, the test showed no difference between the investigated groups (controls: median [IQR]: 100% [94%, 115%]; patients: median [IQR]:113% [93%, 128%]) (Figure 8, open bars).

There is increasing evidence that free-radical damage and oxidative stress play a role in the pathogenesis of PD. Thus, the functional consequences of cellular treatment with the free radical generator paraquat were determined. Under paraquat-induced stress, Δψm in cells from patients (median [IQR]: 84% [81%, 103%]; controls under stress conditions: median [IQR]: 102% [100%, 118%]) decreased significantly from 113% to 84% of the basal control level (p=0.028) (Figure 8, hatched bars).

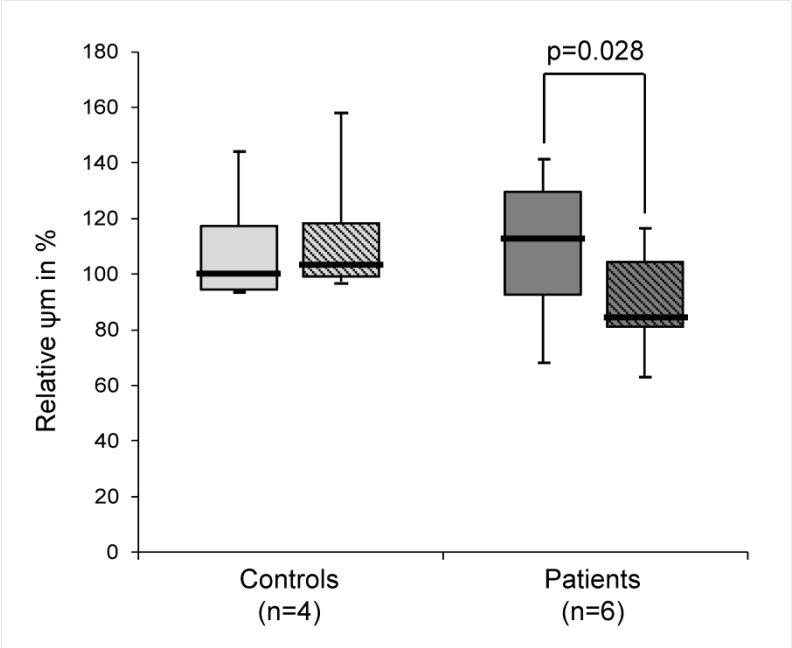

Figure 8 *Membrane potential (Δψm) under basal and stressed conditions.* In control (median [IQR]: 100% [94%, 115%]) and patient fibroblasts (median [IQR]:113% [93%, 128%]), Δψm was comparable when measured under basal conditions (open bars). When the cells were treated with paraquat (hatched bars), no relevant changes in Δψm were detected in the controls (median [IQR]: 102% [100%, 118%]). In the *Parkin* mutants, a significant reduction in membrane potential was observed (median [IQR]: 84% [81%, 103%]). The median, the interquartile range (IQR), the minimum and the maximum value of six (A) or four (B) independent experimental runs are plotted. The median of the controls under basal conditions was set to 100%. n – number of individuals.

3.1.4 Markers of oxidative stress

In order to determine basal levels of oxidative stress in the fibroblasts, an OxyBlot was applied. Densitometric quantification of this technique demonstrated significantly

(p=0.038) higher levels of oxidized proteins in the *Parkin*-mutant samples (median [IQR]: 123% [113%, 136%]) than in controls (median [IQR]: 100% [97%, 105%]) (Figure 9A, open bars). Under paraquat-induced stress, the difference in oxidation between mutants and controls increased markedly. Due to increased variability of the individual results after paraquat treatment, this result was not significant (Figure 9A, hatched bars). The findings from densitometric analyses in single individuals were supported by an OxyBlot performed with pooled control and pooled patient samples under basal and stress conditions (Figure 9B).

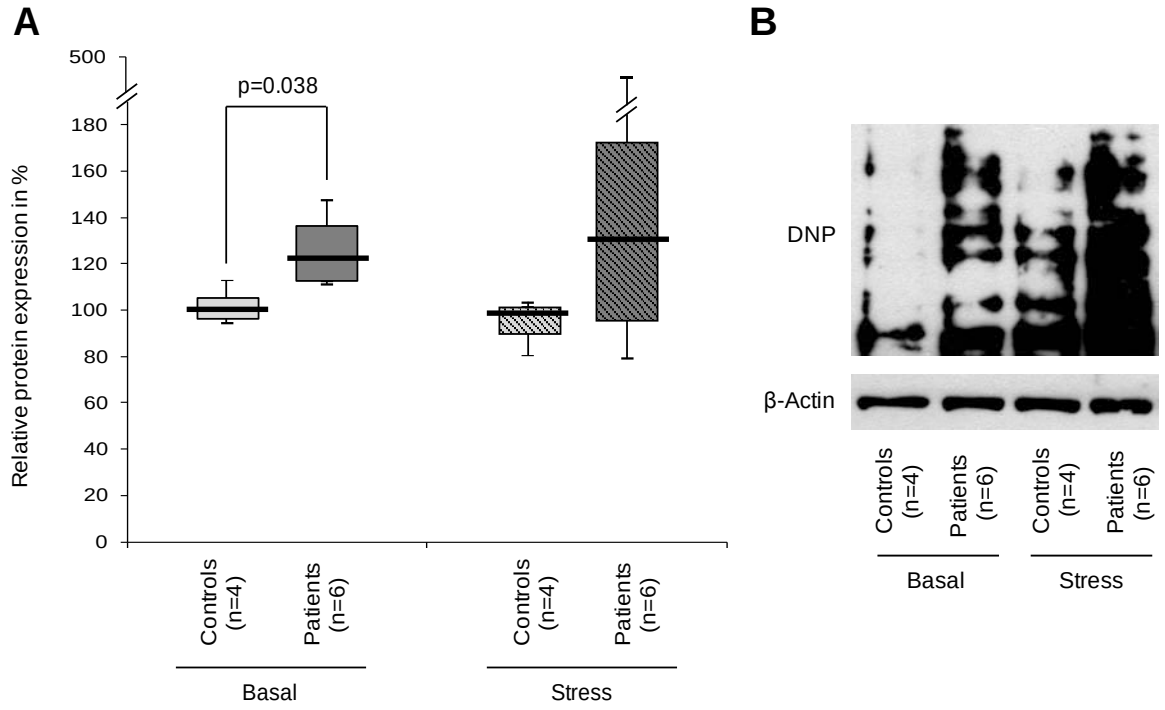

Figure 9 *Protein oxidation under basal and stress conditions.* Oxidation of proteins in *Parkin*-mutant fibroblasts and controls was determined by means of an OxyBlot. (A) When quantifying the protein oxidation in each individual using an antibody against DNP, the *Parkin* mutants (median [IQR]: 123% [113%, 136%]) showed significantly higher levels of oxidation than the controls (median [IQR]: 100% [97%, 105%], open bars). After treatment of cells with paraquat (hatched bars), the difference in oxidation between mutants (median [IQR]: 131% [96%, 172%]) and controls (median [IQR]: 100% [90%, 102%]) increased, but was no longer significant. Expression ratios of oxidized proteins vs. the cytosolic marker β-actin were calculated. The median, the IQR, the minimum and the maximum value of the investigated groups are given. (B) OxyBlot of pooled protein samples before and after paraquat treatment showing the same trend as identified by individual measurements. Equal protein loading was verified with an antibody against β-actin. n – number of individuals.

3.1.5 Mitochondrial DNA

A recent publication linked the function of Parkin to the mitochondrial genome. Accordingly, it protects the mitochondrial genome integrity from the consequences of oxidative stress and supports mtDNA repair (Rothfuss et al., 2009).

Consequently, the basal mtDNA levels in lymphocytes and fibroblast samples were measured by means of real-time PCR. The ratio of mtDNA to nDNA was calculated and the control median was set to 100% (for details see 2.3.11.3). In the blood samples the quantification showed no differences between mutants and controls (see Figure 10A).

Similarly, equal mtDNA concentrations were detected in the fibroblasts of both groups (see Figure 10B, open bars). To mimic conditions of oxidative stress, fibroblasts were treated with H_2O_2 (2.3.1.3) according to a protocol published earlier (Rothfuss et al., 2009). This treatment resulted in a significant (p=0.031) reduction of the mtDNA/nDNA ratio in patient cells (basal: median [IQR]: 80% [79%, 91%], stressed: median [IQR]: 55% [47%, %]), but showed no effect in the control group (basal: median [IQR]: 100% [53%, 160%]; stressed: median [IQR]: 114% [50%, 181%]) (Figure 10B).

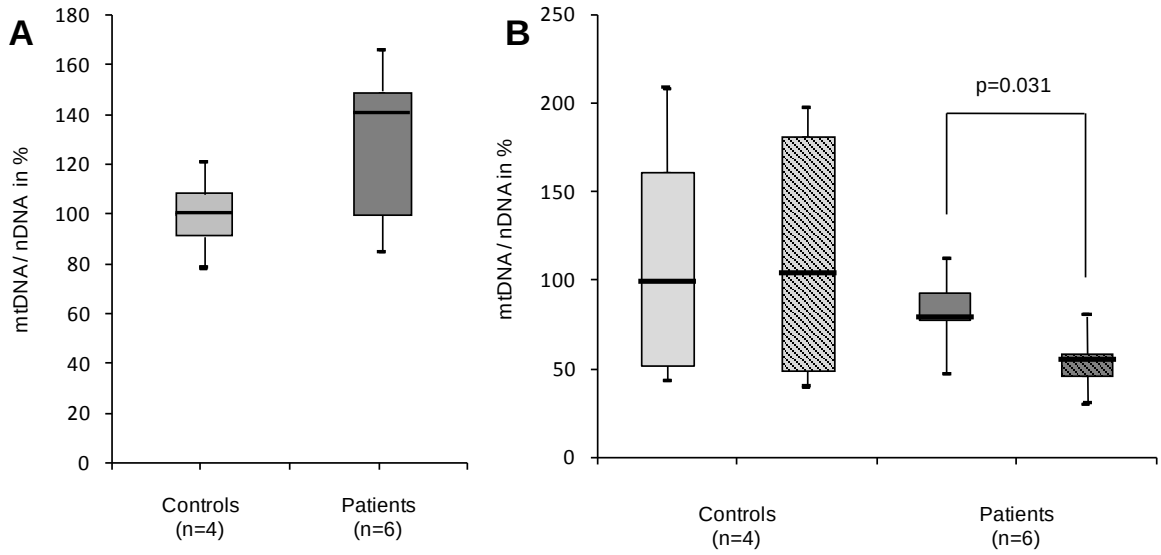

Figure 10 *Ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) in lymphocytes (A) and in fibroblasts under basal and stressed conditions (B).* (A) In lymphocytes, the mtDNA to nDNA ratio was higher in patients (median [IQR]: 141% [148%, 101%] n=6) than controls (median [IQR]: 100% [107%, 97%] n=4), but due to larger inter-individual variability this difference did not reach significance (B) In fibroblasts no difference between the mtDNA to nDNA ratio in patients (median [IQR]: 80% [79%, 91%], n=6) and controls (100% [53%, 160%], n=4) was found. Treatment with the oxidative stressor H_2O_2 for 30min prior to DNA extraction had no effect on the mtDNA-to-nDNA ratio in controls (median [IQR]: 114% [50%, 181%], n=4). In patient cells, treatment with H_2O_2 led to a significant decrease (p=0.031) in the mtDNA-to-nDNA ratio (basal: median [IQR]: 80% [79%, 91%]; stressed: median [IQR]: 55% [47%, 57%],

n=6). The median, the IQR, the minimum and the maximum value of the investigated groups are plotted in the diagrams. n – number of individuals.

To investigate the impact of *Parkin* mutations on the mutation frequency in the mitochondrial genome, the complete mtDNA was sequenced in the *Parkin*-mutant samples. In these patients (mean age 56.2±13.3 years), the mutational burden varied markedly (median [IQR]: $4.53x10^{-4}$ [$3.24x10^{-4}$, $7.28x10^{-4}$]; Figure 11), with the oldest case (B11; 79 years) carrying the highest number of sequence variations per 15,452 possible sites ($1.68x10^{-3}$). As a control, data from a study investigating 18 elderly (mean age: 77.9±4.4 years) was used. In these individuals the average mutational burden amounted to $1.6x10^{-3}$ (Coon et al. 2006). When excluding B11, all investigated PD patients of the present study were below the published control rate.

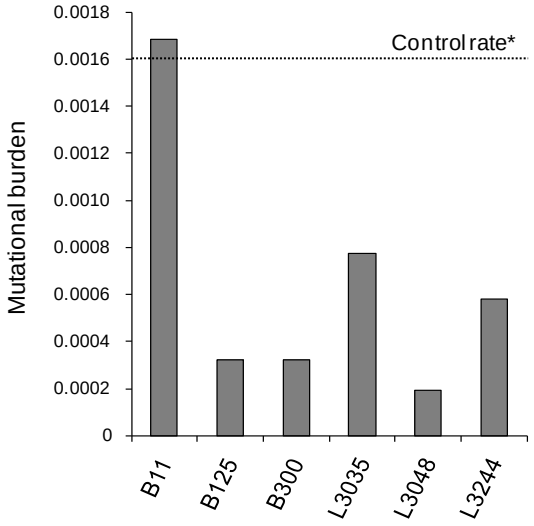

Figure 11 *Mutational burden per individual.* Mitochondrial genome mutation frequency. The mutational burden in all mutants, except for the oldest case B11 (age: 79 years), was well below the mutation frequency in a published control group (mean age: 77.9 years) (Coon et al. 2006).

3.1.6 Mitochondrial content

The differences in the activities of complexes I-IV may be caused by variations in the amount of mitochondria in the samples. Consequently, CS activity (see 2.3.7.1) as marker of mitochondrial integrity and content was measured in homogenates.

The measurement of CS activity in homogenates revealed a significantly (p=0.004) higher CS activity for patients (median [IQR]: 183% [125%, 232%]) compared to controls (median [IQR]: 100% [43%, 101%]) (see Figure 12).

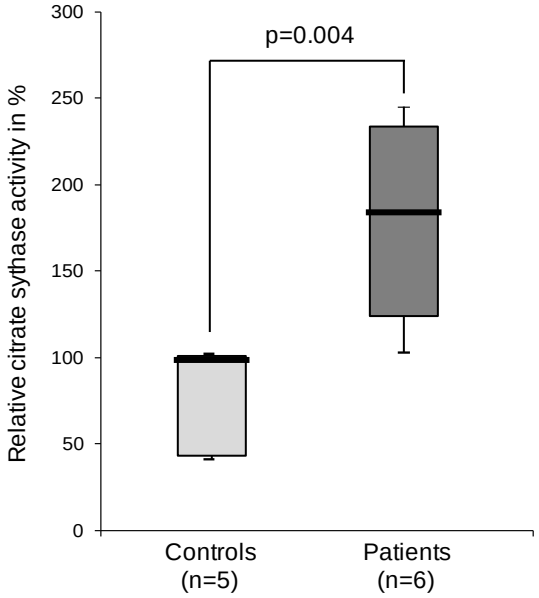

Figure 12 *CS activity in cell lysates. Parkin* mutants (median [IQR]: 183% [125%, 232%]) showed significantly higher CS activities than controls (median [IQR]: 100% [43%, 101%]), indicative of increased mitochondrial mass per cell in the former. CS activity in cell lysates was normalized for protein concentration. The median, the interquartile range (IQR), the minimum and the maximum value of the investigated groups are shown. n – number of individuals.

3.1.7 Mitochondrial network

Next, the mitochondria of the fibroblast samples were tested for morphological changes since impaired mitochondrial fission (Deng et al. 2008; Mortiboys et al. 2008; Poole et al. 2008) is a well-established finding in *Parkin* null mutants. The mitochondrial network was studied by use of immunocytochemistry (2.3.9.1). To compare the degree of branching of the mitochondrial network in *Parkin* mutants and controls, the form factor was determined. This morphological assessment demonstrated no differences between mutant and control individuals under basal conditions (Figure 13A and Figure 14, open bars). After treatment with paraquat, the degree of branching decreased by 34% within the controls and by 46% within the *Parkin*-mutant samples (Figure 13B and Figure 14, hatched bars). This drop was only significant (p=0.028) in the latter group.

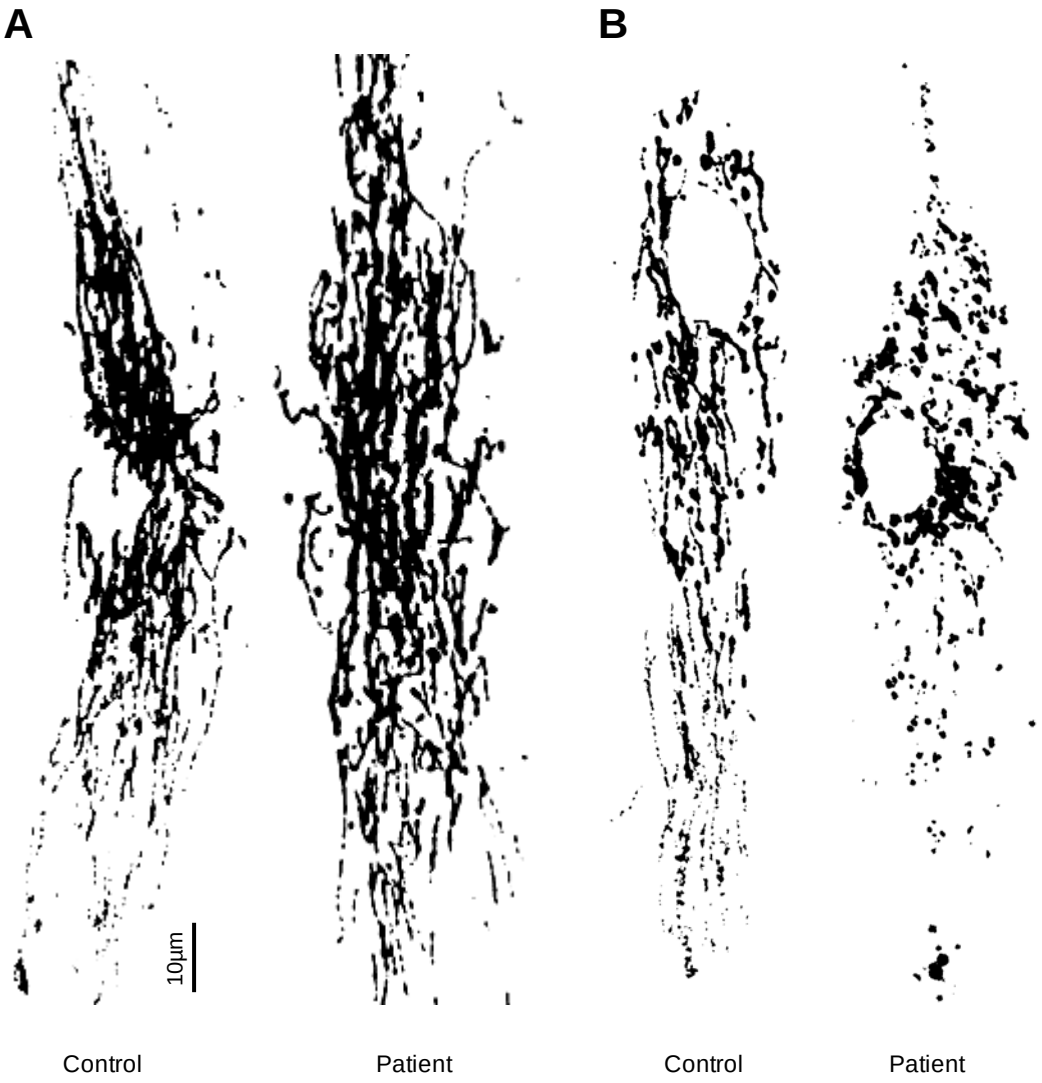

Figure 13 *Mitochondrial network in primary fibroblasts.* (A) Images of the mitochondrial network in control and patient fibroblasts demonstrating similar degrees of branching under basal culturing conditions. (B) After treatment with paraquat, the network was less branched in patients and controls. The images are bulked, a scale is given next to the first image.

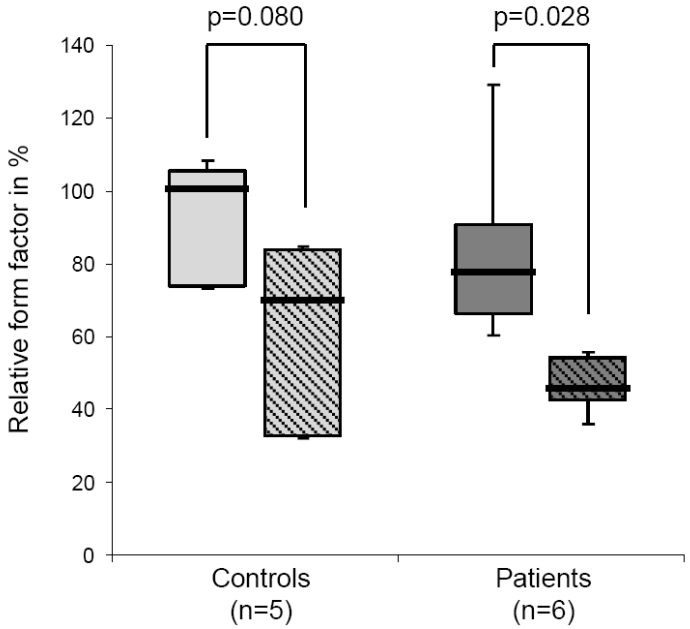

Figure 14 ***(previous page)*** *Quantification of the degree of mitochondrial branching.* The degree of mitochondrial branching (form factor) was comparable in patients (median [IQR]: 78% [66%, 90%]) and controls (median [IQR]: 100% [73%, 105%]) under standard cell culturing conditions. When treated with paraquat (hatched boxes), the form factor decreased significantly (p=0.028) in the mutant samples (median [IQR]: 46% [43%, 54%]). By contrast, the drop seen in controls (median [IQR]: 70% [32%, 84%]) was not significant.

As proteins involved in fusion and fission of mitochondria were shown in fly models to function in the PINK1/Parkin pathway (Deng et al. 2008; Poole et al. 2008; Lutz et al. 2009), Western blotting was performed with antibodies against the fusion/fission factors, Fission1 (Fis1) and Mfn2. To further investigate the impact of oxidative stress on the protein expression of fusion and fission factors, cells were treated with paraquat. In all individuals equal amounts of Fis1 and Mfn2 were observed under basal as well as oxidative stress conditions.

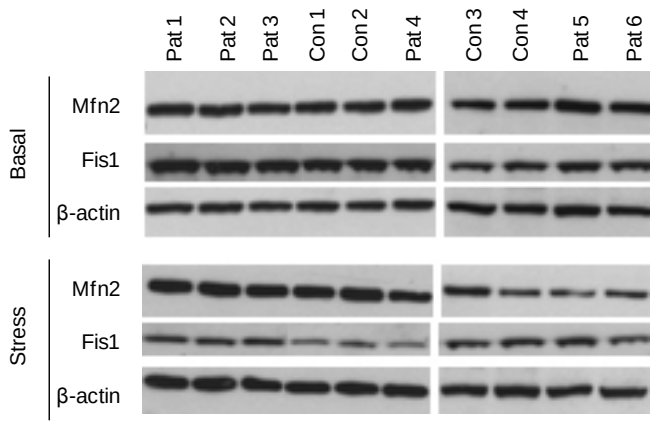

Figure 15 *Western blotting against Mfn2 and Fis1.* Western blotting was performed for six *Parkin* mutants (Pat1-Pat6) and four controls (Con1-Con4). Protein expression levels of Mfn 2 and Fis 1 were similar in all individuals. After treatment with paraquat for 24h, a marked inter-individual variety was found, but no relevant differences between mutants and controls were detected. The expression of β-actin served as loading control.

3.1.8 Cellular growth rates

To evaluate the effect of the detected mitochondrial dysfunction on cell viability, cellular growth rates in four wild-type and three *Parkin*-mutant fibroblast cultures were determined. Although a high interindividual variability was noted, this approach revealed no differences between the groups (controls: median [IQR]: 100% [54%, 156%]; *Parkin* mutants: median [IQR]: 107% [93%, 169%]).

3.2 Mutational screen of *LRPPRC*

3.2.1 Screening results

Due to the results of our own work group and the results published by Davison *et al.* 2009 (see 1.2.4, 1.2.5), it was decided to sequence the 38 exons and flanking intronic regions of *LRPPRC* in 46 patients with parkinsonism and other defined atypical features (see 2.1.2). This mutational screen revealed 24 substitutions, four of which were novel (Table 5). All of the detected changes were single nucleotide polymorphisms (SNP). Four synonymous variations were detected in the coding region (c.246G>A [p.Q82Q], c.1068A>G [p.Q356Q], c.2481A>G [p.P827P], c.4023T>C [p.Y1341Y]). Seventeen changes were found in introns, one in the 5'UTR and two in the 3'UTR. The frequencies of most substitutions in our sample were similar to those reported on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/) for studies based on populations of European origin, such as pilot.1.CEU, HapMap-CEU and AoD_Caucasian. In case of the SNPs c.-45G>A, IVS13+28T>C and IVS30+97T>C, the incidences in our group of PD patients were markedly higher than that reported in the database. For the variant c.-45G>A, an allelic frequency of 7.6% was found in our population in comparison to 1.4% in the database. The change IVS13+28T>C was observed with an allelic frequency of 9.5% in the studied population in comparison to 1.5% in reported European populations. The IVS30+97T>C substitution was present in 8.7% of patients screened here compared to 3.7% of patients in other studies.

Table 5 *Allele frequencies of sequence variations identified in LRPPRC.*

Gene position	DNA variation	NCBI No.	AF PD	AF DB	Database*
5' UTR	c.-45G>A	rs11124961	7.6%	1.4%	pilot.1.CEU
Exon 2	c.246G>A (p.Q82Q)	rs6741066	66.7%	65.5%	HapMap-CEU
Intron 3	IVS3-132C>G	rs6721144	6.8%	13.3%	HapMap-CEU
Intron 6	IVS6-70T>C	rs17031786	15.1%	13.8%	HapMap-CEU
Exon 9	c.1068A>G (p.Q356Q)	rs4953042	16.3%	19.2%	HapMap-CEU
Intron 9	IVS9+30A>G	rs7593842	15.2%	12.7%	HapMap-CEU
Intron 13	IVS13+28T>C	rs62135104	9.5%	1.5%	pilot.1.CEU
Intron 15	IVS15+11C>G	rs58811869	7.8%	13.9%	pilot.1.CEU
Intron 17	IVS17-28T>G	rs72877186	16.2%	15.3%	pilot.1.CEU
Intron 20	IVS20-40A>C	rs7594526	42.4%	47.5%	HapMap-CEU
Intron 22	IVS22+27T>G	rs28394191	43.5%	40.3%	pilot.1.CEU
Exon 23	c.2481A>G (p.P827P)	rs115993634	1.1%	none	None

Continuation Table 5

Intron 27	IVS27+26C>T	rs4952694	51.1%	53.0%	AoD_Caucasian
Intron 27	IVS27-38A>G	None	2.2%	none	None
Intron 28	IVS28+21C>A	rs7568481	43.5%	47.4%	HapMap-CEU
Intron 30	IVS30+97T>C	rs17424482	8.7%	3.7%	HapMap-CEU
Intron 32	IVS32-3C>T	rs35113761	6.5%	none	None
Intron 35	IVS35+14C>T	rs3795859	15.2%	15.0%	HapMap-CEU
Intron 35	IVS35+15C>T	rs76850904	8.7%	none	None
Intron 36	IVS36-42G>C	None	1.1%	none	None
Exon 37	c.4023T>C (p.Y1341Y)	None	1.1%	none	None
Intron 37	IVS37+37G>A	rs2955280	51.1%	53.4%	HapMap-CEU
3’UTR	*399G>A	None	2.3%	none	None
3’UTR	*556A>T	rs1136998	7.6%	8.3%	HapMap-CEU

Note: AF – allelic frequency, DB – database, * – Only studies based on European populations included.

4 DISCUSSION

The outcomes of the two projects performed in this thesis are discussed in the following order: first, the results from the mitochondrial function and morphology studies in *Parkin*-mutant fibroblasts (4.1) and, second, the findings from the mutational screening of *LRPPRC* in PD patients with defined atypical clinical features (4.2).

4.1 Mitochondrial function and morphology in *Parkin*-mutant fibroblasts

Several studies investigating Parkin in the mitochondrial context have been published to date. These data were, however, mainly obtained in recombinant cellular models, animal models or small samples of patient cells. Here, the mitochondrial phenotype in fibroblasts of six PD patients with different *Parkin* mutations was compared to that of five age-matched controls.

4.1.1 Impaired respiratory chain function in Parkin mutants

There is strong evidence that a deficit in the OXPHOS system function is involved in the pathogenesis of PD (Schapira et al. 1989; Schapira 2008). In a study on *Parkin*-mutant fibroblasts, Mortiboys *et al.* reported a decrease in CI-linked ATP production and overall ATP production. The same phenomenon was detected after *Parkin* knockdown by small interfering RNA (siRNA) (Mortiboys et al. 2008). In another study on a pair of siblings with mutations in *Parkin,* mitochondrial respiratory function was determined by measurement of oxygen consumption. The rates for oxygen consumption in patient fibroblasts were significantly lower for both the basal endogenous and the DNP-uncoupled respiration rates (Pacelli *et al.* 2011). Consistent with these data, in this study significantly reduced ATP synthesis rates and decreased cellular ATP concentrations in patient cells with *Parkin* mutations were detected in comparison to controls. Nevertheless, the reductions observed here were not as severe as in the first study mentioned above. Considering that the data were obtained in two different experimental settings, the two data sets should be compared with caution, as this difference may be explained by variable genotypes in both studies. All investigated patients had homozygous or compound heterozygous *Parkin* mutations, but within different domains of the protein. Similar differences depending on the site of the mutation within the gene were seen earlier when solubility, tendency to form aggregates, protein-protein interactions and ubiquitination

capacity were studied (Hampe *et al.* 2006b). Interestingly, the study population of Mortiboys *et al.* presented with a more severe clinical phenotype (lower age of onset: 19.2±3.7 vs. 37.3±16.1) than our patients. Similar results were obtained in fibroblasts carrying mutations in other genes causing parkinsonism. ATP production rates in patients carrying the G2019S mutation in *LRRK2* were significantly reduced with a more pronounced impairment of CIV-linked ATP production (Mortiboys *et al.* 2010). In *PINK1*-mutant fibroblasts, cellular ATP levels were significantly lower than in controls (Grünewald *et al.* 2009). Decreased ATP synthesis rates and increased oxygen consumption rates were reported for fibroblasts of patients with *ATP13A2* mutations (Grünewald *et al.* 2012).

In terms of activities of the RC complexes, the afore-mentioned study by Mortiboys *et al.* on *Parkin*-mutant fibroblasts demonstrated a reduction of CI activity in the patient group by 45% (Mortiboys *et al.* 2008). A similar alteration of CI activity was detected in two Italian siblings (Pacelli *et al.* 2011) and in leukocytes of patients with *Parkin* mutations (Muftuoglu *et al.* 2004). However, when comparing these studies, one must take into account that the measurements in fibroblasts from the Italian siblings were not carried out in purified mitochondria, but in homogenates. Additionally, in the Italian study a reduction in CIV activity in *Parkin*-mutant fibroblasts was detected (Pacelli *et al.* 2011). In contrast to these studies, here, no significant difference in CI activity was detected comparing patient samples and controls. Furthermore, the activities of CII to CIV were not significantly altered in the *Parkin*-mutant cells.

In fibroblasts of patients with mutations in other parkinsonism causing genes, normal RC activities were found for *PINK1* nonsense mutants (Grünewald *et al.* 2009) and decreased ATP production linked to CI, CII and CIV in *LRRK2*-mutant fibroblasts (Mortiboys *et al.* 2010). Another study on fibroblasts carrying *PINK1* mutations demonstrated a reduction in CI activity (Hoepken *et al.* 2007). Interestingly, a study of fibroblasts from patients with idiopathic PD showed no abnormalities in RC enzyme activities, but a specific reduction of CV activity. However, this study was restricted to assays of cell homogenates (del Hoyo *et al.* 2010). In another study on leukocytes of idiopathic PD patients a reduced CIV activity was detected in combination with a reduction in CI activity (Muftuoglu *et al.* 2004). An explanation for the divergence of the ATP and RC activity data collected in this study might be oxidation of CoQ1 or cytochrome c due to increased oxidative stress in the patients' cells. However, deficient comparability of different mutations and experimental set-ups may also impact on the results (Medja *et al.* 2009).

Further experiments will be needed to clarify the role of respiratory chain dysfunction in *Parkin*-associated parkinsonism.

4.1.2 Decreased Δψm in *Parkin*-mutant fibroblasts under stress conditions

Next, Δψm was investigated as a central factor of mitochondrial integrity (Narendra et al. 2008). In a study from 2008, theΔ ψm was found to be decreased in *Parkin*-mutant fibroblasts under basal conditions and culturing in glucose depletion medium supplemented with galactose exacerbated this disturbance (Mortiboys *et al.* 2008). Here, the *Parkin* mutants showed no impairment of Δψm under basal culturing conditions.

Familial parkinsonian syndromes typically show a gradual progression over a period of time. Therefore, treatment with toxins causing oxidative stress was performed to accentuate alterations in a tissue with a high mitotic activity. In contrast to control cells, exposure to high levels of reactive oxygen species (ROS) caused a significant decline of Δψm in patient cells. Again, the different mutational background of the investigated individuals might account for this variation between studies.

Investigations in fibroblasts of PD patients with mutations in other PD genes revealed a decreased membrane potential in patients with the G2019S mutation in *LRRK2* (Mortiboys *et al.* 2010) and no alterations in *PINK1* mutation carriers (Grünewald *et al.* 2009). Additionally, in fibroblasts of patients carrying mutations in *ATP13A2*, a decreased mitochondrial membrane potential was demonstrated (Grünewald *et al.* 2012).

4.1.3 Elevated levels of oxidative stress in *Parkin* mutants

Oxidative stress is a key element implicated in the pathophysiology of PD, as recently supported by studies on human skin fibroblasts from patients with monogenic parkinsonism (Mortiboys *et al.* 2008; Grünewald *et al.* 2009; Pacelli *et al.* 2011). Our results demonstrate increased oxidative stress levels in *Parkin*-mutant fibroblasts under basal conditions. This difference between mutants and controls became more pronounced when the cells were exposed to paraquat.

In PINK1 deficient fibroblasts, protein carbonyls were found to be elevated, but the increase did not reach significance (Hoepken *et al.* 2007). Another study reported an enhanced production of hydrogen peroxide and oxygen radicals in fibroblasts of a patient with a homozygous nonsense mutation in *PINK1* (Piccoli *et al.* 2008). A deficiency of

PINK1 has been reported to cause mitochondrial accumulation of calcium in mammalian neurons, resulting in a mitochondrial calcium overload which then stimulates the production of ROS via NADPH oxidase (Gandhi et al. 2009). The findings of this study are consistent with these previous reports suggesting a role for Parkin as a neuroprotectant.

4.1.4 Mitochondrial DNA levels in *Parkin*-mutant fibroblast decrease after H_2O_2 treatment

Somatic mtDNA point mutations and deletions were shown to accumulate progressively with age (Linnane *et al.* 1989; Corral-Debrinski *et al.* 1992; Michikawa *et al.* 1999) and the mtDNA mutational load appears to correlate with the level of mitochondrial dysfunction within the cell (Petruzzella *et al.* 1994).

Interestingly, the E3 ligase Parkin was reported to protect mtDNA from oxidative damage and to stimulate mtDNA repair (Rothfuss et al. 2009). In the respective publication, mtDNA damage and susceptibility to oxidative stress were analyzed in fibroblasts from one patient with a compound heterozygous mutation in *Parkin.* Here, cells from six *Parkin*-mutant patients were examined and no relevant changes in mtDNA levels were determined under basal conditions. In contrast, after exposure to oxidative stress, a decline in mtDNA levels was detected in the *Parkin*-mutant fibroblasts. Another study on mtDNA levels in fibroblasts of patients with mutations in *ATP13A2* demonstrated increased mtDNA levels under basal culturing conditions (Grünewald *et al.* 2012).

These findings are supported by a recent publication emphasizing the mtDNA protective role of Parkin in mitochondrial metabolism. The authors showed that long-term overexpression of Parkin eliminates mitochondria with DNA carrying deleterious *cytochrome c oxidase I* mutations in heteroplasmic cybrid-cells, resulting in an increased amount of wild-type mtDNA and restoration of cytochrome c oxidase activity (Suen *et al.* 2010).

Interestingly, an accumulation of somatic mtDNA deletions has previously also been shown in the substantia nigra of idiopathic PD patients. Similar to the mitochondrial dysfunction detected in the current study, high levels of accumulated mutations were associated with respiratory chain deficiency (Bender *et al.* 2006; Kraytsberg *et al.* 2006).

In this thesis, the sequence of the mitochondrial genome was additionally analyzed for single nucleotide changes and small deletions in blood samples from all examined patients carrying mutations in *Parkin.* The mtDNA mutational burden was then compared to

normal values as reported in an age-matched control population (Coon et al. 2006). The quantified mutational burden was comparable to the published control rate. Interestingly, in fibroblasts from PD patients with mutations in *ATP13A2*, a higher frequency of mtDNA lesions was found (Grünewald *et al.* 2012).

The absence of changes in mtDNA in lymphocytes can be explained by high energy demands due to rapid proliferation of blood stem cells leading to strong mitochondrial selection and "disappearance" of somatic mutations. For future experiments concerning mtDNA integrity, tissue with less mitochondrial selection, such as hair follicles would be preferable. Another approach might be treatment of fibroblasts with ROS prior to DNA isolation to mimic accumulative effects during aging in a short-term setting.

4.1.5 CS activity is markedly higher in *Parkin*-mutant fibroblasts

For the determination of mitochondrial content for patient and control cells, the activity of CS, the pacemaker enzyme of the citric acid cycle, was measured. For patient cells, an about 2-fold higher CS activity was demonstrated, indicative of increased mitochondrial mass in the cells. Likewise, an increased mitochondrial mass was detected in a study on fibroblasts carrying a mutation in *ATP13A2* (Grünewald *et al.* 2012). In an earlier study on *PINK1*-mutant fibroblasts the activities of CS and of the malate dehydrogenase fell within the control range (Grünewald *et al.* 2009). This result may reflect a compensatory mechanism in the cell to provide sufficient energy despite inefficient RC function.

4.1.6 Morphology of the mitochondrial network in *Parkin*-mutant cells

Recently, in *Drosophila*, parkin has been shown to act downstream of pink1 in a common pathway which appears to regulate mitochondrial morphology (Exner et al. 2007; Deng et al. 2008; Poole et al. 2008). Two studies in human cells also demonstrated an impact of mutations in *Parkin* (Mortiboys et al. 2008) and *PINK1* (Grünewald et al. 2009) on the shape of the mitochondrial network. *Parkin*-mutant cells were found to be more prone to enter fusion as reflected by a significant increase in mitochondrial branching in the patient group (Mortiboys et al. 2008). In another recent publication, *Parkin*-mutant fibroblasts showed a more fragmented mitochondrial network compared to controls (Pacelli *et al.* 2011). In contrast, in the present study no significant differences were detected in the degree of branching between *Parkin* mutants and controls under basal conditions.

In neurons of *DJ-1* knockout mice as well as in lymphoblasts from patients with *DJ-1*-linked parkinsonism, increased fragmentation of the mitochondrial network was observed (Irrcher *et al.* 2010). Interestingly, upon overexpression of SNCA in cultured cells and in *Caenorhabditis elegans*, SNCA binds to mitochondria and leads to mitochondrial fragmentation which can be rescued by coexpression of PINK1, Parkin and DJ-1 (Kamp *et al.* 2010). In summary, it can be stated that the experimental results regarding mitochondrial morphology vary within and between different organisms.

In the study by Mortiboys *et al.* on *Parkin*-mutant fibroblasts (Mortiboys et al. 2008), a higher degree of branching was seen and cells were additionally exposed to rotenone, an inhibitor of the respiratory chain CI, prior to morphology analysis. This treatment induced mitochondrial fragmentation in *Parkin*-mutant and control cells to a comparable extent. In the current study, no significant differences in branching between mutants and controls were detected after exposure to paraquat. Similarly, in fibroblasts from *PINK1* mutants, no morphologic abnormalities were detected under basal conditions (Hoepken *et al.* 2007). Other authors observed no alterations of the mitochondrial network (Piccoli *et al.* 2008) or decreased branching (Grünewald *et al.* 2009) in *PINK1* mutants. Furthermore, decreased mitochondrial branching was also observed in *DJ1*-mutant fibroblasts (Krebiehl *et al.* 2010). In contrast, increased branching of the mitochondrial network was detected for *LRRK2*-mutant fibroblasts (Mortiboys *et al.* 2010).

For prospective studies one should bear in mind that continuous observation and documentation of changes in morphology after administration of toxins etc. are preferable to snapshots at a single time point.

Research in *Drosophila* connected the mitochondrial fission and fusion factors drp1, opa1 and mfn with pink and parkin. For example, loss of function of drp1 augmented the manifestation of the phenotypes of pink1 or parkin deficiency in *Drosophila* (Deng *et al.* 2008; Poole *et al.* 2008; Lutz *et al.* 2009). Additionally, knockdown of opa1 and mfn attenuated the phenotype of *pink1* and *parkin* mutants (Deng et al. 2008; Poole et al. 2008; Lutz et al. 2009) (this context was explained in detail in chapter 1.2.3). In the present study, the expression of Fis1 and Mfn under basal and paraquat-induced stress were investigated in a human cell model, and no change in the expression of these fusion and fission factors was found.

4.1.7 Mutations in *Parkin* do not impair growth rates of fibroblasts

In PD, cell death develops in neurons of the substantia nigra over many years. However, comparable growth rates in fibroblasts from PD patients and controls detected here imply that cell viability is not affected by mutations in *Parkin* in non-neuronal tissue. Likewise, in other studies cell viability remained unaltered (Mortiboys *et al.* 2008; Piccoli *et al.* 2008). Therefore, the neuronal cell death is probably due to neuron-specific effects and accumulation of influencing factors in post-mitotic tissue over decades.

4.1.8 Integration of data into the proposed PINK1/Parkin mitophagy pathway

Recently, the process of selective clearance of damaged mitochondria has been connected with the PINK1/Parkin pathway. Several studies in mammalian cells showed a selective recruitment of Parkin to dysfunctional mitochondria as a response to low mitochondrial $\Delta\psi m$. This recruitment induces the selective engulfment of impaired mitochondria by autophagosomes and thus their elimination (Narendra et al. 2008; Narendra et al. 2009). The translocation of Parkin was subsequently found to be initiated by the phosphorylation of this protein (Kim et al. 2008; Sha et al. 2009) by PINK1 which is stabilized at depolarized mitochondria (Dagda *et al.* 2009; Matsuda *et al.* 2010; Narendra *et al.* 2010). Accumulation of PINK1 liberates the latent enzymatic activity of the E3 ubiquitin ligase Parkin (Matsuda *et al.* 2010). This theory about the mitophagy pathway is further supported by the detection of reduced chaperone-mediated autophagy markers in substantia nigra pars compacta and amygdala of PD brains (Alvarez-Erviti *et al.* 2010). In wild-type *Drosophila*, pink1-dependent translocation of parkin to the mitochondrion led to subsequent ubiquitination of mfn by parkin. By contrast, no influence of pink1 and parkin on the fission/fusion-promoting factors drp1 and opa1 was found (Poole *et al.* 2010; Ziviani *et al.* 2010). Furthermore, in human dopaminergic SH-SY5Y cells and primary human fibroblasts, the disruption of $\Delta\psi m$ was followed by ubiquitination of Mfn1 and 2 in a PINK1 and/or Parkin dependent manner (Gegg *et al.* 2010; Rakovic *et al.* 2011a). Mfn ubiquitination by Parkin may either generally mark damaged mitochondria (Gegg et al. 2010) or initiate the degradation of Mfn1/2 by the ubiquitin-proteasome system preventing fusion of dysfunctional with functional mitochondria (Rakovic *et al.* 2011a).

In light of this pathway, we can interpret our results as manifestations of the inability of mutant Parkin to perform its physiological function (see Figure 16). Mitochondria have been reported to show age-related mitochondrial decay including decline of $\Delta\psi m$ and

increased genetic heterogeneity and ROS (Hagen *et al.* 1997; Parihar *et al.* 2007). If Mfn1/2 ubiquitination and subsequent mitophagy is impaired in *Parkin* mutants under stress, it is conceivable that mitochondria with disturbed OXPHOS function are no longer separated and eliminated from the general pool but dominate cellular (dys)function. This effect is in keeping with decreased ATP synthesis rates and ATP cellular concentration, elevated oxidized protein levels, increased mitochondrial mass and the observed stress-induced decrease of $\Delta\psi m$ in the patient fibroblasts investigated here. Furthermore, one would expect that due to impaired Mfn1/2 deactivation/degradation, mitochondria should be less fragmented in *Parkin*-mutants than in control cells under stress conditions. Since mitochondrial fusion and fission are transient events, dynamic techniques to quantify the degree of mitochondrial branching would be preferable to the method established so far (Mortiboys et al. 2008). Methodological restrictions together with great inter-individual variations in branching especially after exposure to mitochondrial stressors render it impossible to detect subtle morphological differences between mutants and controls.

The absence of ubiquitinated forms of Mfn under stress conditions in the present study can be explained by usage of a different cellular toxin. Ubiquitinated forms of Mfn were shown when a direct disruption of $\Delta\psi m$ with ionophores like valinomycin or carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was performed. In contrast, here, a rather general stressor, i.e. the superoxide generator, was used. The stress induced by paraquat may have been too unspecific to cause the effect observed in other studies.

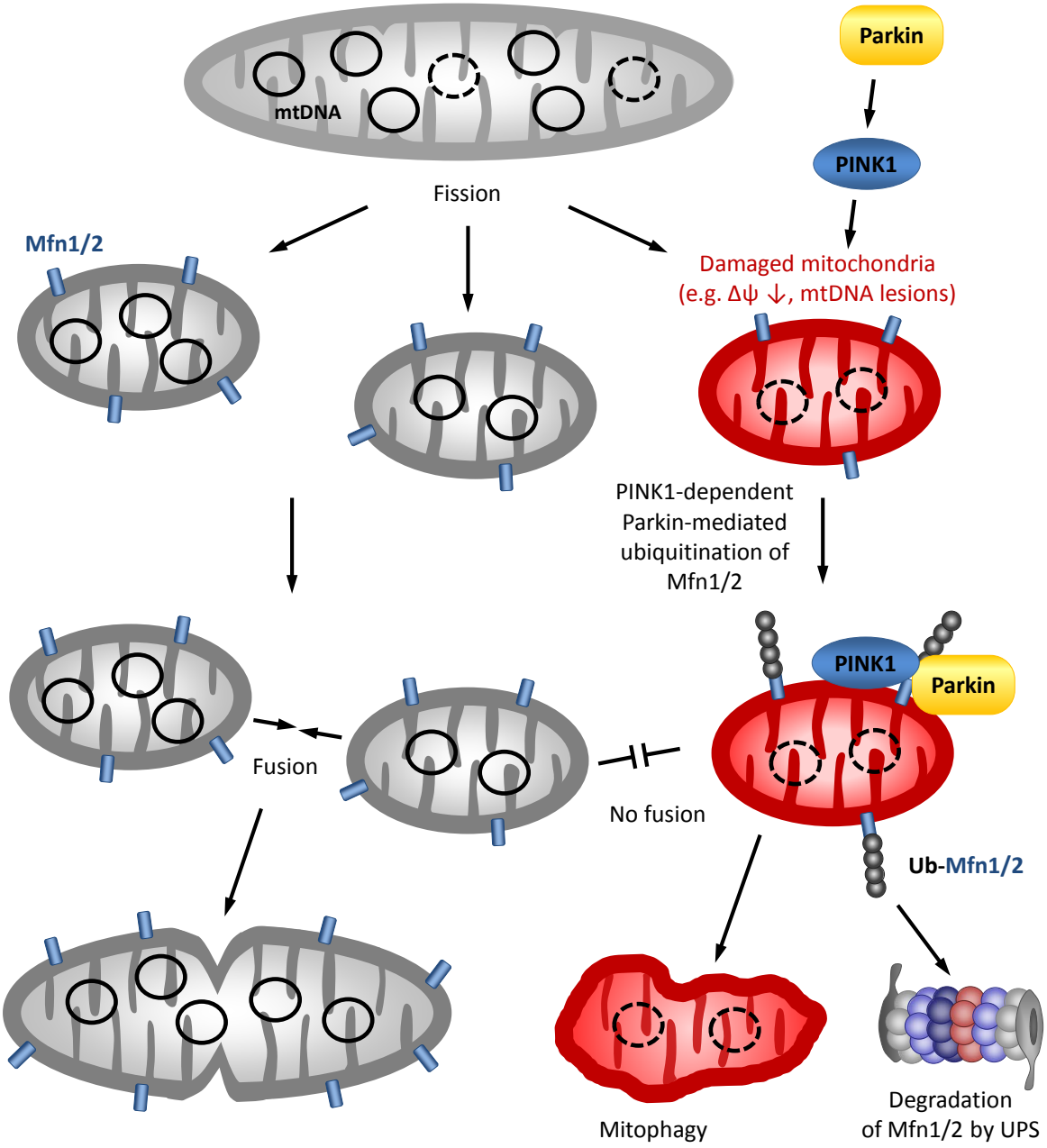

Figure 16 *The recent concept of the putative PINK1/Parkin mitophagy pathway.* Mitochondria with impaired function (e.g. loss of Δψm, mtDNA damage) are identified by PINK1 which recruits the E3 ubiquitin ligase Parkin. At the mitochondria, Parkin ubiquitinates Mfn1/2 (Ub-MFN1/2) proteins which are subsequently degraded by the ubiquitin proteasome system (UPS). By this mechanism, damaged mitochondria are prevented from fusion with functional ones and eventually undergo autophagy. Functional mitochondria are pictured in gray. Damaged mitochondria are indicated in red. (Scheme modified from Rakovic et al. (Rakovic *et al.* 2011a)).

4.2 Mutational screen of *LRPPRC* in PD patients

Mutational screening revealed no pathogenic mutations in *LRPPRC*. However, 24 SNPs were detected and four of them were novel. The frequencies of most substitutions in our sample were similar to those reported in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/) for studies based on populations of European origin, such as pilot.1.CEU, HapMap-CEU, and AoD Caucasian. Interestingly, frequencies of SNPs c.-45G>A, IVS13 + 28T>C and IVS30 + 97T>C, were markedly higher than those reported in the databases. The significance of this finding needs to be investigated in a larger sample. The screening techniques used here allow, however, only for the identification of qualitative sequence changes. Therefore, although no small deletions or insertions have been found in the *LRPPRC* gene, gene dosage variations cannot be fully excluded.

Mutational screening was performed based on the results of tandem affinity purifications by two research groups, which revealed a potential direct interaction of LRPPRC with both Parkin and PINK1 (Davison *et al.* 2009; Rakovic *et al.* 2011b) (see chapter 1.2.4 and 1.2.5). This connection was strengthened by recent insights into the molecular pathogenesis of LSFC. Accordingly, there may be another link between Parkin and LRPPRC. In 2011, Shin *et al.* reported that Parkin Interacting Substrate (PARIS (ZNF746)) is ubiquitinated by Parkin and represses the expression of the transcriptional co-activator peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1-α (PGC-1α) (Shin *et al.* 2011). PGC-1α was earlier shown to be a regulator of mitochondrial biogenesis (Wu *et al.* 1999; Scarpulla 2002b; Scarpulla 2002a). Additionally, PGC-1α polymorphisms were associated with age of onset and risk of developing PD (Clark *et al.* 2011). Interestingly, LRPPRC was found to be part of the PGC-1α holocomplex and to interact physically with PGC-1α (Cooper *et al.* 2006). These findings emphasize the connection between the two clinically distinct diseases on a molecular basis. Further research on both the genetic and the proteomic level is required to clarify the potential link between LRPPRC and the PINK1/Parkin pathway.

4.3 Conclusions

Project A:

Hypothesis: *Mutant Parkin plays a role in respiratory chain (RC) function.*

Conclusion: To test this hypothesis, several parameters of mitochondrial function were compared in *Parkin*-mutant and control fibroblasts. Mutant Parkin led to lower ATP synthesis and ATP levels in human fibroblasts (3.1.1). Furthermore, decreased $\Delta\psi m$ was detected in *Parkin*-mutant fibroblasts under conditions of oxidative stress (3.1.3). By contrast, normal respiratory chain enzyme activities (CI-IV) were measured in the mutants (3.1.2). These findings point towards no direct involvement of Parkin in oxidative phosphorylation but imply an indirect connection between Parkin and mitochondrial function, i.e. via the PINK1/Parkin mitophagy pathway (4.1.8).

Hypothesis: *Loss of Parkin function leads to oxidative stress in the cell.*

Conclusion: To investigate the impact of Parkin on oxidative stress levels, the OxyBlot technique was employed. This approach showed higher levels of oxidized proteins in *Parkin*-mutant fibroblasts than controls. After treatment with the superoxide generator paraquat, the difference between mutants and controls increased further (3.1.4). These results provide additional evidence supporting a neuroprotective role for Parkin.

Hypothesis: *Parkin impacts on the morphology of the mitochondrial network.*

Conclusion: In this study, cellular mitochondrial content was determined by quantifying CS activity. This kinetic assay revealed an increase in mitochondrial mass in the *Parkin*-mutant cells (3.1.6). However, the interconnectivity of the mitochondrial network and the expression of the fission and fusion factors Mfn2 and Fis1 were comparable between *Parkin*-mutant and control fibroblasts (3.1.7). The results of this thesis indicate a link between Parkin and the mitochondrial mass. The increased mitochondrial content in the *Parkin* mutants is likely caused by impaired mitophagy in these cells. The discrepancy between recent reports about the ubiquitination of Mfn2 by

Parkin and our findings may be due to different stressors used. Paraquat as a general superoxide generator may not act specifically enough against the mitochondrial membrane potential to induce Mfn ubiquitination. Furthermore, changes in mitochondrial network structure may be too subtle to be identified with the imaging techniques employed here.

Hypothesis: *Mitochondrial DNA replication and repair is influenced by mutations in Parkin.*

Conclusion: To investigate the integrity of the mitochondrial genome, mtDNA concentration in the cells was quantified by real-time PCR. When cells were exposed to H_2O_2, lower mtDNA levels were detected in mutant fibroblasts than in controls (3.1.5), indicating a protective effect of wild-type Parkin on the mitochondrial genome. However, the lack of functional Parkin in mutant lymphocytes did not lead to an accumulation of somatic point mutations in the mtDNA (3.1.5). An explanation for this result may be that due to strong mitochondrial selection pressure in blood stem cells, somatic mutations "disappear". For future experiments, tissue with less mitochondrial selection, such as hair follicles, is, therefore, preferable.

Project B:

Hypothesis: *PD patients carry mutations in LRPPRC which are not present in controls.*

Conclusion: To address this hypothesis, PD patients with an atypical phenotype were screened for mutations in *LRPPRC*. This screen identified no pathogenic mutations which could influence protein function in our selected patient cohort (3.2.1). Consequently, the link between LRPPRC and PD remains questionable. However, taking the recent literature on interactions between LRPPRC and PD-associated proteins into account, it may still be of interest to study the molecular function of LRPPRC in the context of PD.

5 PERSPECTIVES

The results of this thesis underline the importance of a human fibroblast model, which can be used to provide important insights into the mechanisms underlying genetic parkinsonism and sporadic PD. The validity of the results could be confirmed in a future study using a rescue experiment, e.g. overexpression of Parkin in *Parkin*-mutant fibroblasts should reverse the observed mitochondrial phenotypes.

Regarding stress tests, paraquat may be replaced by more specific stressors, i.e. valinomycin or CCCP, which target the $\Delta\psi m$. The effects of disrupted $\Delta\psi m$ on the expression of mitochondrial fission and fusion factors may also be a useful subject for a subsequent study.

Furthermore, the expression of mitophagy markers, such as microtubule-associated protein1 light chain 3 (LC3) and p62, should be investigated in *Parkin*-mutant fibroblasts and controls under basal and stress conditions. These experiments may further elucidate the mitophagy pathway and confirm impaired mitophagy as a cause for the mitochondrial phenotype detected in this study.

In order to examine the role of *Parkin* mutations in a biologically relevant setting, dopaminergic neurons can be generated from *Parkin*-mutant fibroblasts and controls using the induced pluripotent stem cells (iPS) technology (Seibler et al. 2011).

For further studies regarding the relationship between LRPPRC and Parkin, the expression and subcellular localization of LRPPRC could be investigated in fibroblasts, or possibly neurons, with Parkin deficiency and controls.

For identification of new interaction partners of Parkin, the tandem affinity purification approach could also be applied to neurons. Using this approach, the protein profiles of neuronal and connective tissue can be compared in control and *Parkin*-mutant cells.

A better understanding of the underlying PINK1/Parkin mitophagy pathway may lead to the identification of potential therapeutic intervention targets. An intervention in the pathway at the position of both the triggering oxidative stress and the subsequently impaired mitophagy is a conceivable future target to prevent PD development. Mitochondria provide for this purpose different targets, e.g. substrates, carriers, antioxidants and regulation of transcription (Schapira 2012).

6 SUMMARY

This thesis investigates the molecular background of the movement disorder Parkinson's disease (PD) from two different angles: the impact of mutations in the PD-associated gene *Parkin* on mitochondrial function and morphology in a human fibroblast model and the occurrence of mutations in the coding gene of the potential Parkin interactor leucine-rich pentatricopeptide repeat cassette (LRPPRC) in a cohort of PD patients with an unusual clinical phenotype.

Mutations in Parkin cause autosomal recessive parkinsonism. Parkin is a mitochondrially localized E3 ubiquitin ligase and its involvement in mitochondrial function and dynamics has been reported in recombinant cellular models, animal models or small samples of patient cells. The following hypotheses were addressed in this project: *(i) Mutant Parkin plays a role in respiratory chain function. (ii) Loss of Parkin function leads to oxidative stress in the cell. (iii) Parkin impacts on the morphology of the mitochondrial network. (iv) Mitochondrial DNA (mtDNA) replication and repair are influenced by mutations in Parkin.* To this end, fibroblasts were obtained from six patients with two mutations in *Parkin* (c.1072delT/c.1072delT, delEx7/delEx7, c.1072delT/delEx7, delEx3-4/duplEx7-12, delEx4/c.924C.T and delEx1/c.924C.T) and five age-matched controls. Fibroblasts were cultured under basal or paraquat-induced oxidative stress conditions. A decreased production and overall concentration of ATP were observed, in parallel to increased mitochondrial mass in *Parkin*-mutant fibroblasts. After an oxidative insult, the membrane potential decreased in patient cells but not in controls. Furthermore, higher levels of oxidized proteins in the mutants were determined under basal as well as stress conditions. Branching of the mitochondrial network was comparable in both groups and decreased to a similar extent when stressed. MtDNA levels in *Parkin*-mutant fibroblasts decreased under stress conditions. By contrast, this treatment did not lead to an accumulation of somatic mutations. These results indicate that *Parkin* mutations cause abnormal mitochondrial function in fibroblasts possibly due to impairment of the PINK1/Parkin mitophagy pathway.

Parkin was shown to interact with the protein LRPPRC, which in mutated form causes the mitochondrial disease Leigh syndrome of French Canadian type, an infantile, neurodegenerative disease with parkinsonian features. Based on this interaction the following hypothesis was tested: *(v) PD patients carry mutations in LRPPRC which are not present in controls.* When screening a cohort of patients (n=46) with parkinsonism

with atypical features no pathogenic mutations in *LRPPRC* were found. However, 20 known and four novel single nucleotide polymorphisms were detected. Despite this result, the role of LRPPRC in parkinsonism still remains to be clarified. Especially, as the protein has also been shown to interact with an additional protein involved in autosomal recessive parkinsonism, further molecular investigations may be of interest.

In conclusion (i) Parkin has no direct effect on oxidative phosphorylation but influences mitochondrial function, i.e. via the PINK1/Parkin mitophagy pathway. (ii) Higher levels of oxidized proteins and a higher susceptibility to stressors in *Parkin* mutant cells corroborate the idea of a neuroprotective role of Parkin. (iii) Parkin modifies the mitochondrial content presumably via the influence of the PINK1/Parkin mitophagy pathway. (iv) A protective effect of Parkin on mtDNA has not been detected in lymphocytes. This may be due to high mitochondrial selection pressure in this tissue. (v) In this selected patient cohort a mutational screen did not identify mutations in *LRPPRC*, but a link between LRPPRC and genetic parkinsonism remains possible.

Additional insight into the molecular function of the PINK1/Parkin mitophagy pathway will help to develop new therapeutic approaches addressing PD. Human cellular models and especially the reprogramming of these cells into induced pluripotent stem cells will likely leed to great advances in PD research in the future.

7 ZUSAMMENFASSUNG

Diese Dissertation beschäftigt sich mit den molekularen Grundlagen der Bewegungsstörung Parkinson-Syndrom (PS) aus zwei verschiedenen Blickwinkeln: Zum einen untersucht sie die Bedeutung von Mutationen im *Parkin*-Gen für mitochondriale Funktion und Morphologie in einem humanen Fibroblastenmodell. Zum anderen untersucht sie das kodierende Gen des mutmaßlichen Parkin Interaktionspartners LRPPRC in einer Kohorte von Patienten mit einem PS auf Mutationen.

Mutationen in Parkin verursachen ein autosomal rezessiv vererbtes PS. Parkin ist eine mitochondrial lokalisierte E3-Ubiquitinligase, deren Beteiligung an mitochondrialer Funktion und Dynamik bisher in verschiedenen rekombinanten Zellmodellen, Tiermodellen und kleineren Patientenstichproben belegt wurde. Folgende Hypothesen wurden in diesem Projekt untersucht: *(i) Mutiertes Parkin spielt eine Rolle für die Funktion der Atmungskette. (ii) Der Funktionsverlust von Parkin führt zu oxidativem Stress in der Zelle. (iii) Parkin hat Auswirkungen auf die Morphologie des mitochondrialen Netzwerkes. (iv) Replikation und Reparatur der mitochondrialen DNA (mtDNA) werden von Mutationen in Parkin beeinflusst.* Zu diesem Zweck wurden Fibroblasten von sechs Patienten mit zwei Mutationen im *Parkin*-Gen (c.1072delT/c.1072delT, delEx7/delEx7, c.1072delT/delEx7, delEx3-4/duplEx7-12, delEx4/c.924C.T und delEx1/c.924C.T), sowie von fünf gleichaltrigen Kontrollen gewonnen. Die Fibroblasten wurden unter basalen Bedingungen und unter oxidativem Stress, ausgelöst durch Paraquat, kultiviert. Die Ergebnisse zeigten eine verminderte Produktion und Konzentration von ATP sowie eine erhöhte mitochondriale Masse in Fibroblasten mit Mutationen in *Parkin*. Nach oxidativem Stress sank das Membranpotential der Patientenzellen ab, während es in den Zellen der Kontrollen konstant blieb. Es wurde ein hoher Anteil an oxidierten Proteinen sowohl unter basalen als auch unter gestressten Bedingungen in Zellen von Mutationsträgern festgestellt. Der Grad der Verzweigung des mitochondrialen Netzwerkes war zwischen Mutanten und Kontrollen vergleichbar und nahm unter Stress in gleichem Maß ab. Die mtDNA-Konzentration nahm in Fibroblasten mit *Parkin*-Mutation unter oxidativem Stress ab. Die Paraquatexposition führte jedoch nicht zur Akkumulation von somatischen Mutationen in Lymphozyten der Patienten. Diese Ergebnisse zeigen, dass *Parkin*-Mutationen eine mitochondriale Dysfunktion in Fibroblasten verursachen. Der mitochondriale Phänotyp lässt sich möglicherweise auf eine Störung der vor kurzem entdeckten PINK1/Parkin-Mitophagie-Kaskade zurückführen.

Parkin wurde kürzlich als potentieller Interaktionspartner von LRPPRC identifiziert. Das Protein verursacht in seiner mutierten Form eine mitochondriale Erkrankung, das so genannte Leigh-Syndrom vom französisch-kanadischen Typ. Dabei handelt es sich um eine frühkindliche, neurodegenerative Erkrankung mit Parkinson-ähnlichen Symptomen. Basierend auf diesem wissenschaftlichen Hintergrund wurde die folgende Hypothese untersucht: *PS Patienten tragen Mutationen im LRPPRC-Gen, welche nicht in Kontrollpersonen gefunden werden.* In einem Mutationsscreening einer Kohorte von Patienten (n=46), die an einem PS mit ungewöhnlichen zusätzlichen Symptomen leiden, wurden keine pathogenen Mutationen gefunden. Stattdessen wurden 20 bekannte und vier neue Single Nucleotide Polymorphismen detektiert. Trotz dieses Ergebnisses verbleibt LRPPRC in der Pathogenese des genetischen PS beachtenswert. Insbesondere, da das Protein zusätzlich mit einem anderen Protein interagiert, das auch ein autosomal rezessives PS verursachen kann. Weitere molekulare Untersuchungen zur Rolle von LRPPRC bei PS könnten von Interesse sein.

Zusammenfassend (i) hat Parkin keine direkten Auswirkungen auf die oxidative Phosphorylierung, beeinflusst aber die mitochondriale Funktion, z.B. über die PINK1/Parkin-Mitophagie-Kaskade. (ii) Ein höherer Anteil an oxidierten Proteinen und ein erhöhte Empfindlichkeit gegenüber Stressoren von Zellen mit *Parkin*-Mutation bestärken die Annahme der neuroprotektiven Funktion Parkins. (iii) Parkin verändert den mitochondrialen Gehalt vermutlich über die Beeinflussung der PINK1/Parkin-Mitophagie-Kaskade. (iv) Parkin hat vermutlich einen schützenden Effekt auf die mtDNA, jedoch lässt sich dieser in Lymphozyten, wahrscheinlich wegen des hohen mitochondrialen Selektionsdrucks in diesem Gewebe, nicht nachweisen. (v) In dieser selektierten Patientenkohorte konnte durch das Mutationsscreening keine Mutation in *LRPPRC* identifiziert werden, jedoch bleibt eine Verbindung zwischen LRPPRC und dem genetischen PS möglich.

Mehr Erkenntnisse über die molekulare Funktion der PINK1/Parkin-Mitophagie-Kaskade werden hilfreich sein um neue therapeutische Ansätze für das PS zu entwickeln. Humane Zellmodelle und im Speziellen deren Reprogrammierung in induzierte pluripotente Stammzellen werden die PS-Forschung in den nächsten Jahren vermutlich deutlich vorantreiben.

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9 APPENDIX

9.1 Supplementary material

Supplementary Table 1 *PCR conditions and primers for LRPPRC sequencing.*

Primer Name / Region	Sequence 5‘→3‘	Annealing Temperature (°C)	Cycles	PCR Additives	Length of PCR Fragment (bp)
Ex 1F	GGAGACGGAAGACGGAAAA	53	35	5% Betaine	431
Ex 1R	GCAGCTTGCCTGGAGAAAG				
Ex 2F	GAGCTTTGCAGCATAAACTG	58	35	5% Betaine	712
Ex 2R	GTAGAAATGGGGTTTTGCC				
Ex 3F	ACACTGTACATTAGGATAGGA	53	35	5% Betaine	439
Ex 3R	ATGTCACTATACCTCACCAA				
Ex 4F	ATGCTTAGCTTGCTCTGTGTGA	53	35	5% Betaine	570
Ex 5R	CTGAAACAATTTCCTCCATTAGG				
Ex 6F	AGGGGATCAAAGGGATTAG	53	35	5% Betaine	300
Ex 6R	AGCATCTCAGCCATCTATCAC				
Ex 7F	GTTTAGTATCTCGGTGCTGGT	53	35	5% Betaine	401
Ex 8R	TTCCATCATGTAAGAATAGC				
Ex 9F	TCTTCAGTATTGGAACATTGG	53	35	5% Betaine	800
Ex 11R	CATTACAAATCGGTAAAAGGC				
Ex 12F	AGGGGGATGAAGAAAGGAG	53	35	5% Betaine	485
Ex 12R	CTGGGCAATGAGAGAGAAAC				
Ex 13F	CAGGAAAACAGGATCTTTGG	63	35	5% Betaine	235
Ex 13R	GCATGCCCTCAATAGTAACTG				
Ex 14F	CTGACTCTGATTCTGCTG	53	35	5% Betaine	637
Ex 14R	GTCTGTAACTCCAACTTGC				
Ex 15F	GAATATTTGAGGTGGGTGAGTG	53	35	5% Betaine	271
Ex 15R	CCTATGGTATTGGCTTGTAACG				
Ex 16F	CTTGTTTGCTTCTCTTGCTGC	53	35	5% Betaine	322
Ex 16R	AAAAAGCCTCCCAACCCTG				
Ex 17F	GCTCCCAAACCACATAATCT	53	35	5% Betaine	693
Ex 18R	GCTTCGTTTTAATGGCTGTC				
Ex 19F	CGTGTGTTTTCTAATTCTGGGC	61	35	5% Betaine	734
Ex 20R	CATGCCATTCAAGGTGTAGC				
Ex 21F	ATATCAAGGGGCAGAGTC	53	35	5% Betaine	343
Ex 21R	CTATCAAGGTCTGTAGAGCAG				
Ex 22F	AGTTCAGGTCTTCTGATGAGC	53	35	5% Betaine	253
Ex 22R	CATGTTATTCCAGTGGCAAG				

Continuation Supplementary Table 1

Ex 23F Ex 23R	GCTGGGCTTGAAATGCTG AAGCCACAGGACCACAGG	61	35	5% DMSO	478
Ex 24F Ex 24R	CCCCATTTTAGCTTTGTGGT GCCTTCTGCTGGTTTCTCTTA	53	35	5% Betaine	363
Ex 25F Ex 25R	CCCTTATCCTCACTAGTTCTCT CAACCAGACTGCCTTTTC	53	35	5% Betaine	499
Ex 26F Ex 26R	ATTCAGCCGAAGCCTTACCT TCTCTCGAAGTCCCCAAATC	53	35	5% Betaine	213
Ex 27F Ex 27R	AGTTCAACTGCCCTTTTGG AAACTGCCTTCTGACAATCC	53	35	5% Betaine	313
Ex 28F Ex 29R	CACTTTTGGTTCCTTTCCCT GCTTACACCCCACACTGCTA	53	35	5% Betaine	584
Ex 30F Ex 30R	GGGTTTAGGTCTTTGTTTC TGTACTGCACTGTATATGGC	53	35	5% Betaine	427
Ex 31F Ex 31R	CCAACTTTGTCATCTGTCA ACGTAAAGGTCAAGCATTG	53	35	5% Betaine	326
Ex 32F Ex 32R	GCATTAAAGTCAGCACAGGTCAT CAGCAACTAGCATCAACATTTCC	53	35	5% Betaine	396
Ex 33F Ex 34R	ATGTGATTCAGGGTTTCAG ACCACACGCCTATGTCTAG	53	35	5% Betaine	641
Ex 35F Ex 35R	TGGCATAGCTTGCACTTGT AGCTAACAGACGACAAAACTGAC	53	35	5% Betaine	422
Ex 36F Ex 36R	ATACAAGGGACAATAGTGC CTTTGAGCTGAAGACTAAGAC	53	35	None	346
Ex 37F Ex 37R	CTGAGTGGCTGTTTTGGTG GCTCTTCACAGAGATCTAATCCTC	53	35	None	340
Ex 38aF Ex 38aR	ATCACCTCTTGCTGGTATCTC TTGGGTCTCTGCTTCTCAC	53	35	5% Betaine	638
Ex 38bF Ex 38bR	TTAGTCTGCTGTTTCTCTG AACTCTCCTGACTACCTATC	53	35	None	593

Supplementary Table 2 *PCR conditions and primers for mitochondrial genome sequencing.*

Primer Name / Region	Nucleotide position in mitochondrial genome, sequence of the primer	Annealing Temperature (°C)	Cycles	PCR Additives	Length of PCR Fragment (bp)
Mt_1F	516-534 CACACACACCGCTGCTAAC	58	35	5% DMSO	675
Mt_1R	1190-1172 GATATGAAGCACCGCCAGG				
Mt_2F	1138-1156 GAACACTACGAGCCACAGC	55	35	5% DMSO	664

Continuation Supplementary Table 2

Mt_2R	1801-1782 TCATCTTTCCCTTGCGGTAC				
Mt_3F	1756-1776 AATTGAAACCTGGCGCAATAG	55	35	5% Betaine	689
Mt_3R	2444-2426 TGAGCATGCCTGTGTTGGG				
Mt_4F	2395-2415 ACCAACAAGTCATTATTACCC	56	35	5% Betaine	680
Mt_4R	3074-3054 TGAACTCAGATCACGTAGGAC				
Mt_5F	2995- 3013 GGATCAGGACATCCCGATG	58	35	5% DMSO	651
Mt_5R	3645-3627 AACGGCTAGGCTAGAGGTG				
Mt_6F	3536-3553 TAGCTCTCACCATCGCTC	58	35	None	704
Mt_6R	4239-4219 GATTGTAATGGGTATGGAGAC				
Mt_7F	4184-4202 TCCTACCACTCACCCTAGC	58	35	5% DMSO	686
Mt_7R	4869-4852 GTCATGTGAGAAGAAGCA				
Mt_8F	4832-4849 CACCCCTCTGACATCCGG	58	58	None	739
Mt_8R	5570-5551 AGTATTGCAACTTACTGAGG				
Mt_9F	5526-5545 AATACAGACCAAGAGCCTTC	58	35	5% DMSO	663
Mt_9R	6188-6171 GGGAAACGCCATATCGGG				
Mt_10F	6115-6134 TACCCATCATAATCGGAGGC	58	35	5% DMSO	667
Mt_10R	6781-6761 AATATATGGTGTGCTCACACG				
Mt_11F	6730-6750 CTATGATATCAATTGGCTTCC	58	35	5% DMSO	669
Mt_11R	7398-7379 GGCATCCATATAGTCACTCC				
Mt_12F	7349-7369 CCTAATAGTAGAAGAACCCTC	55	35	5% Betaine	661
Mt_12R	8009-7990 CTCGATTGTCAACGTCAAGG				
Mt_13F	7960-7979 ATTATTCCTAGAACCAGGCG	58	35	5% DMSO	682

Continuation Supplementary Table 2

Mt_13R	8641-8621 TGATGAGATATTTGGAGGTGG				
Mt_14F	8563-8581 ACAATCCTAGGCCTACCCG	58	35	5% DMSO	669
Mt_14R	9231-9212 GATAGGCATGTGATTGGTGG				
Mt_15F	9181-9198 AGCCTCTACCTGCACGAC	58	35	5% DMSO	687
Mt_15R	9867-9848 GGATGAAGCAGATAGTGAGG				
Mt_16F	9821-9841 ACTTCACGTCATTATTGGCTC	58	35	None	696
Mt_16R	10515-10497 GTGAGATGGTAAATGCTAG				
Mt_17F	10394-10414 CTGAACCGAATTGGTATATAG	58	35	None	639
Mt_17R	11032-11013 TCGTGATAGTGGTTCACTGG				
Mt_18F	10985-11004 ACAATCATGGCAAGCCAACG	55	35	None	724
Mt_18R	11708-11689 TTATGAGAATGACTGCGCCG				
Mt_19F	11633-11651 AGCCACATAGCCCTCGTAG	58	35	5% DMSO	729
Mt_19R	12361-12341 TGGTTATAGTAGTGTGCATGG				
Mt_20F	12284-12302 CTATCCATTGGTCTTAGGC	58	35	5% DMSO	722
Mt_20R	13005-12987 TTTGCCTGCTGCTGCTAGG				
Mt_21F	12951-12969 CGCTAATCCAAGCCTCACC	58	35	5% DMSO	664
Mt_21R	13614-13595 TATTCGAGTGCTATAGGCGC				
Mt_22F	13568-13587 TTACTCTCATCGCTACCTCC	53	35	5% Betaine	709
Mt_22R	14276-14258 GGTTGATTCGGGAGGATCC				
Mt_23F	14227-14246 CCCATAATCATACAAAGCCC	58	35	None	702
Mt_23R	14928-14911 GTTGAGGCGTCTGGTGAG				
Mt_24F	14732-14752 ACTACAAGAACACCAATGACC	58	35	5% DMSO	688

Continuation Supplementary Table 2

Mt_24R	15419-15400 TGTAGTAAGGGTGGAAGGTG				
Mt_25F	15372-15391 TAGGAATCACCTCCCATTCC	58	35	5% DMSO	696
Mt_25R	16067-16048 GTCAATACTTGGGTGGTACC				
Dloop_1F	15879-15897 AATGGGCCTGTCCTTGTAG	58	35	5% DMSO	667
Dloop_1R	16545-16526 AACGTGTGGGCTATTTAGGC				
Dloop_2F	16495-16514 CGACATCTGGTTCCTACTTC	58	35	5% DMSO	446
Dloop_2R	389-370 CTGGTTAGGCTGGTGTTAGG				
Dloop_3F	315-332 CGCTTCTGGCCACAGCAC	58	35	5% DMSO	489
Dloop_3R	803-786 GGTGTGGCTAGGCTAAGC				

9.2 Curriculum vitae

9.3 Publikationsliste

Artikel:

L. Voges*, A. Grünewald*, A. Rakovic ,M. Kasten ,H. Vandebona ,C. Hemmelmann, K. Lohmann, S. Orolicki, A. Ramirez, A. H. Schapira, P. P. Pramstaller, C. M. Sue, C. Klein (2010). "Mutant Parkin impairs mitochondrial function and morphology in human fibroblasts." PLoS One **5**(9): e12962.
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Rakovic, A., A. Grünewald, **L. Voges**, S. Hofmann, S. Orolicki, K. Lohmann, and C. Klein (2011) "PINK1-interacting proteins: Proteomic analysis of overexpressed PINK1", Parkinson's Disease 2011 Mar 16;2011:153979

Poster:

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9.4 Danksagung

9.5 Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit ohne fremde Hilfe angefertigt habe und keine anderen als die in der Arbeit genannten personellen, technischen und sachlichen Hilfen oder Hilfsmittel benutzt habe.

Ich versichere, dass ich nicht vorher oder gleichzeitig andernorts einen Zulassungsantrag gestellt oder die Dissertation vorgelegt habe und dass ich mich bisher noch keinem Promotionsverfahren unterzogen habe.

Ich bestätige, dass die in dieser Arbeit durchgeführten Untersuchungen durch die Ethikkommission der Universität zu Lübeck (Aktenzeichen 05-030 vom 18.03.2005, Aktenzeichen 05-199 vom 02.12.2005 und Aktenzeichen 04-155 vom 02.03.2006) genehmigt wurden.

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