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Mechanistic Insights into the Function and Dysfunction of Parkin, an E3 ubiquitin ligase associated with Parkinson's Disease

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Erklärung

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Non quia difficilia sunt, non audemus, sed quia non audemus, difficilia sunt.

Nicht weil die Dinge schwierig sind, wagen wir sie nicht, sondern weil wir sie nicht wagen, sind sie schwierig.

Seneca, *epistulae morales*

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1 Introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is the most common movement disorder and the second most frequent neurodegenerative disease after Alzheimer's disease (AD). With increased life expectancy and an aging society, the incidence of PD is expected to dramatically increase within the next decades. Current therapy is mainly based on a dopamine replacement strategy, commonly using the dopamine precursor levodopa. However, long-term treatment is associated with motor complications, and to date there is no cure for the disease. It is thus important to search for new therapeutic options and identify novel targets for disease modification to help PD patients improve their daily lives. In order to achieve this it is necessary to provide a better understanding of the molecular pathomechanisms underlying the disease.

1.1.1 Etiology

PD is a complex disease with contribution of both environmental and genetic factors. Although most cases are sporadic with no family history, monogenetically inherited forms of PD are present in some families. In addition, large genome-wide association studies (GWAS) have identified several genetic risk factors. Aging is the major risk factor for sporadic PD. Normal aging is associated with a loss of about 1 % of total neurons in the *substantia nigra pars compacta* (SNc) per decade, while during PD pathogenesis neuronal cell death is accelerated to approximately 10 % loss of neurons within ten years.

Besides aging, environmental factors are considered important for the development of the disease. First evidence was presented in 1983, when Langston and coworkers reported a series of young drug addicts developing PD upon accidental exposure to MPTP, a toxic byproduct of the clandestine synthesis of a pethidine analogue (Langston et al., 1983). Furthermore, pesticides such as paraquat or rotenone and heavy metal exposure, in particular manganese, have been identified as environmental risk factors that contribute to the disease. Conversely, some factors seem to decrease the risk for PD. Epidemiological studies have linked cigarette smoking, caffeine intake and potentially the use of anti-inflammatory drugs to a significantly decreased risk to develop PD (Powers et al., 2008).

Although most PD cases are sporadic with no obvious family history, about 10-20 % of patients display a genetic inheritance pattern with at least one affected first degree relative. To date, mutations in six genes have been unequivocally connected to familiar forms of the disease. In addition, genetic risk factors associated with PD have been found in large GWAS. The genetic contribution to PD will be discussed in section 1.3.

In conclusion, genetic predisposition and environmental factors seem to contribute to PD pathogenesis (see Figure 1).

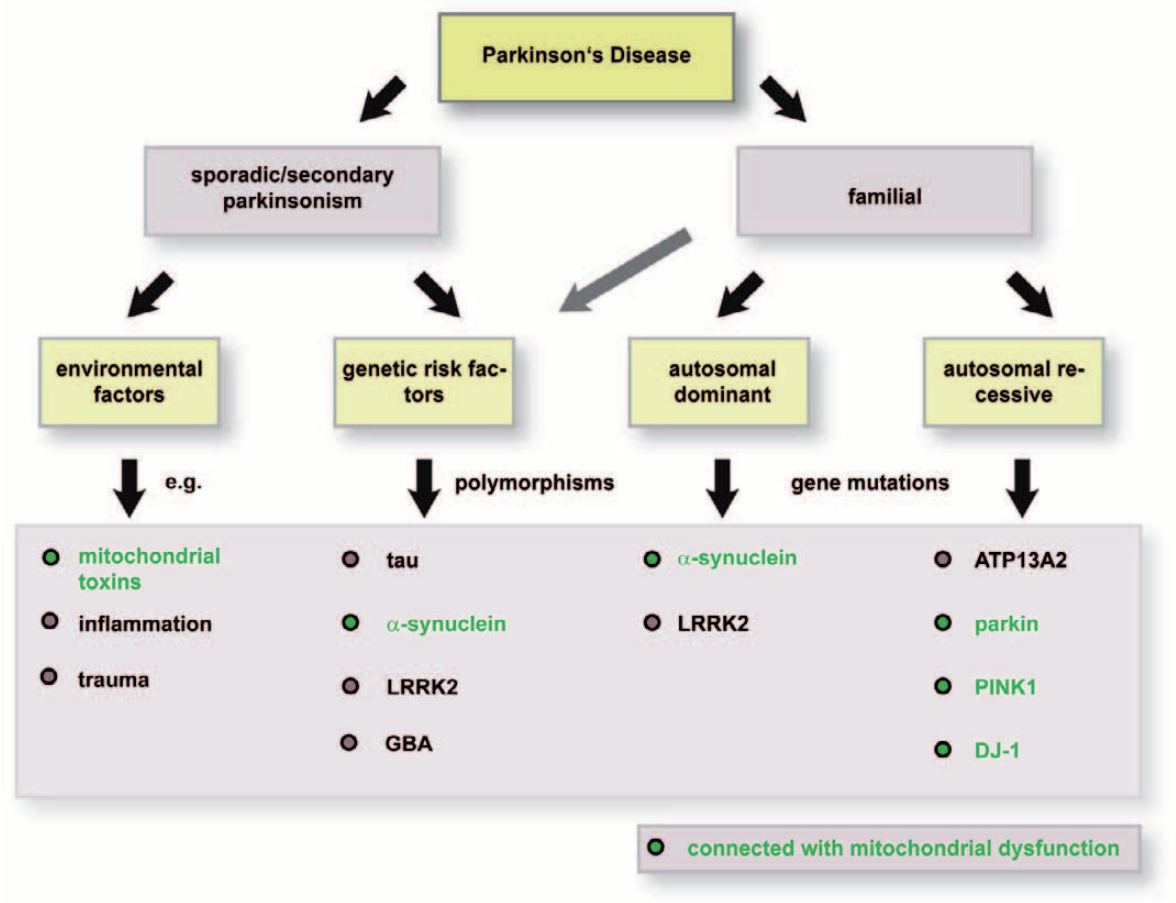


Figure 1: Etiology of PD. Most PD cases are sporadic with no obvious family history. Familiar forms of the disease are caused by genetic mutations with either autosomal dominant or recessive inheritance pattern. Several genetic risk factors are associated with PD. Environmental factors such as the exposure to toxins are also connected to an increased risk of PD development. Adopted from (Pilsl and Winklhofer, 2011).

1.1.2 Pathology

PD is characterized by a degeneration of neurons in the *substantia nigra pars compacta* (SNc) and the occurrence of Lewy Bodies (LB) and Lewy Neurites (LN). However, pathology is not restricted to the SNc, but involves areas such as cortex, amygdala, *locus coeruleus*, the *nucleus basalis* of Meynert, and the peripheral autonomic nervous system. LBs are proteinaceous cytoplasmic inclusions containing α-synuclein, chaperones, ubiquitin, neurofilament, and several other proteins. They are about 10 µM in diameter with a dense core and a clear halo (Figure 2).

Surprisingly, first PD symptoms occur when about 80 % of dopaminergic neurons in the SNc have perished. It is largely unclear what causes the neuronal cell death and why dopaminergic neurons in the SNc are particularly susceptible to demise. Scenarios explaining possible mechanisms of neurodegeneration involve mitochondrial dysfunction, oxidative stress, excitotoxicity, apoptosis and inflammation, and ubiquitin-proteasomal-system (UPS) dysfunction accompanied by deposition of abnormal protein species.

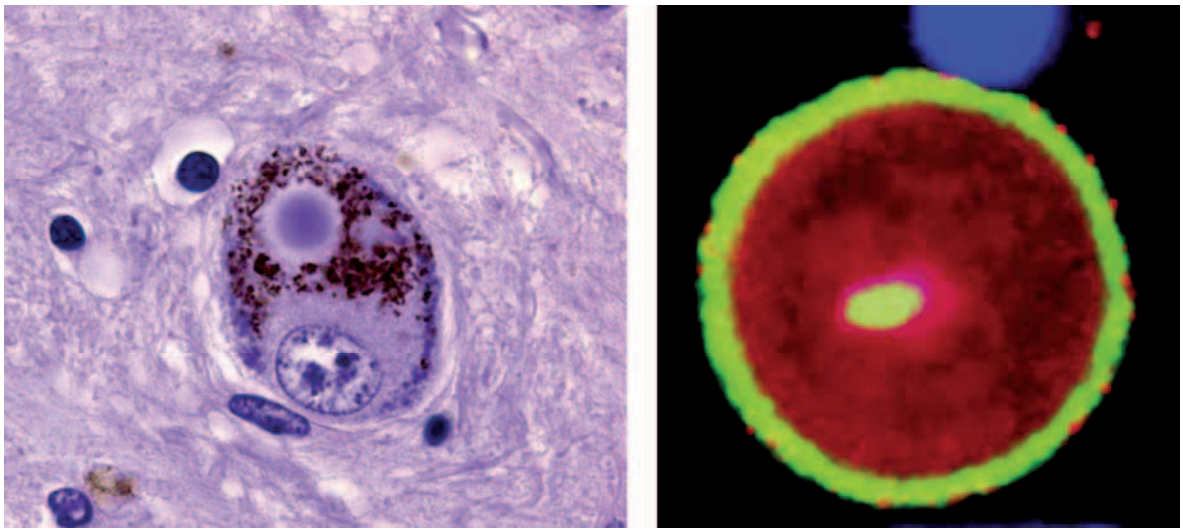


Figure 2: Morphological appearance of Lewy bodies. Left: Hematoxylin and eosin staining of a midbrain section from a PD patient. The intracellular Lewy body displays a typical dense core and a clear halo. The dark granules contain neuromelanin, a typical component of dopaminergic neurons in the SNc (source:http://neuropathology web.org/chapter9/images9). Right: Lewy body in a dopaminergic neuron of a PD patient stained for α-synuclein (yellow) and ubiquitin (red). Source: (Olanow et al., 2009).

1.1.3 Symptoms and Diagnosis

The cardinal symptoms of PD are motor symptoms: resting tremor, rigidity, bradykinesia, and postural instability. Besides these motor complications a variety of other symptoms can occur such as autonomic dysfunction (blood pressure and temperature dysregulation, sensory deficits, sleep disturbances) and neuropsychiatric complaints (depression, cognitive impairment, dementia) in a large subset of patients.

Onset of symptoms is typically between the 5th and 6th decade of life, although some monogenic forms of the disease display an earlier manifestation. To date, clinical diagnosis is largely based on exclusion with a positive response to levodopa as a major criterion. A definite diagnosis of PD can only be made upon *post mortem* autopsy.

1.1.4 Current treatment options

Current PD therapy is mainly based on dopamine replacement strategies with levodopa as the gold standard. Levodopa is usually administered in combination with peripheral decarboxylase inhibitors to increase drug availability in the central nervous system. Monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT) inhibitors slow down the metabolism of dopamine. An additional focus of therapeutic intervention is the treatment of non-motor symptoms. Several ongoing studies investigate the effect of neuroprotectants on disease progression, such as co-enzyme Q10 and vitamine E (reviewed in (Olanow et al., 2009)).

Unfortunately, most patients develop dyskinesias following several years of treatment. These involuntary movements are difficult to control and significantly impair the quality of life. Surgical approaches such as deep brain stimulation is used in patients where medical

interventions fail. Furthermore, intensive research efforts are undertaken using transplantation approaches including induced pluripotent stem cells, embryonic stem cells and tissue grafts (reviewed in (Dyson and Barker, 2011)).

1.2 PD and mitochondria

1.2.1 Importance of mitochondria for cellular homeostasis

Mitochondria – the “cellular powerhouses” – are found in almost all eukaryotic cells. They serve a wide spectrum of important functions; most notably they are involved in energy production in form of ATP. Furthermore, mitochondria are part of other metabolic pathways such as β-oxidation of fatty acids. Mitochondria control calcium homeostasis and apoptosis induction. Proper mitochondrial function is essential for cellular homeostasis, especially in cells with high energy demand such as liver, muscle, and neuronal cells. Thus, different mitochondrial quality control mechanisms are employed at different levels: mitochondrial biogenesis, the synthesis of new mitochondria, control of mitochondrial morphology via modulation of mitochondrial dynamics, and degradation of defective mitochondria through mitophagy.

In the first place, mitochondrial biogenesis is adjusted to cellular needs. Synthesis of additional mitochondria is triggered by a variety of different environmental stimuli or during cellular stress conditions including temperature, energy deprivation, and availability of nutrients and growth factors. Mitochondrial biogenesis is controlled by the interplay of nuclear and mitochondrial factors. The majority of mitochondrial proteins are transcribed from the nuclear genome, while the circular mitochondrial genome encodes parts of the electron transport chain and mitochondrial RNAs. The main regulator of mitochondrial biogenesis appears to be the peroxisome proliferator-activated receptor gamma 1 alpha (PGC-1α), which activates the mitochondrial transcription factor A (TFAM) and nuclear respiratory factors 1 and 2 (NRF1/2). As transcription factors, NRFs are directly involved in the transcription of nuclear-encoded mitochondrial genes while TFAM is a major regulator of mitochondrial gene transcription.

Mitochondrial quality is also ensured and controlled through changes in mitochondrial dynamics. The mitochondrial fusion and fission machinery is highly conserved in eukaryotic cells. In mammals, mitochondrial fusion is regulated by at least three membrane-bound GTPases: the mitofusins 1 and 2 (Mfn1/2) regulate fusion of the outer mitochondrial membrane (OMM), while optic atrophy 1 (Opa1) mediates inner mitochondrial membrane (IMM) fusion. Mitochondrial fission is accomplished by translocation of the cytosolic dynamin-related protein 1 (Drp1) to future scission sites at mitochondria (Figure 3). Mitochondria are quite dynamic organelles that constantly fuse and divide. Disturbances in mitochondrial dynamics can be devastating, in particular for neurons. As a matter of fact, mutations in key genes regulating mitochondrial dynamics can result in neurodegenerative diseases in humans: Opa1 was determined to be mutated in dominant optic atrophy, the most frequent hereditary optic neuropathy (Alexander et al., 2000, Delettre et al., 2000).

Charcot-Marie-Tooth disease type 2A, a peripheral demyelinating neuropathy, is caused by mutations in the MFN2 gene (Zuchner et al., 2004). In agreement with a protective function of mitochondrial dynamics, a decline in respiratory activities has been observed in fusion-deficient fibroblasts from mice lacking Opa1 or mitofusins (Chen et al., 2005, Chen et al., 2007).

Interestingly, it has been demonstrated that dysfunctional mitochondria can be recovered by means of mitochondrial dynamics: single mitochondria with mutations in mitochondrial DNA (mtDNA) are able to fuse with the mitochondrial network to obtain intact mtDNA from neighboring mitochondria (Twig et al., 2008a, Twig et al., 2008b), preventing the accumulation of mtDNA mutations. A balance in mitochondrial dynamics is particularly important for neurons (reviewed in (Pilsl and Winklhofer, 2011)): 1) Mitochondria require long-distance transport along microtubules from the location of synthesis in the soma to sites of high energy demand such as the synapse. 2) Neurons are postmitotic cells, thus an efficient quality control is crucial for cell survival and 3) Nerve cells are characterized by high rates of metabolic activity, thus they require effective supply of energy provided by the mitochondrial respiratory chain (reviewed in (Gusdon and Chu, 2011)).

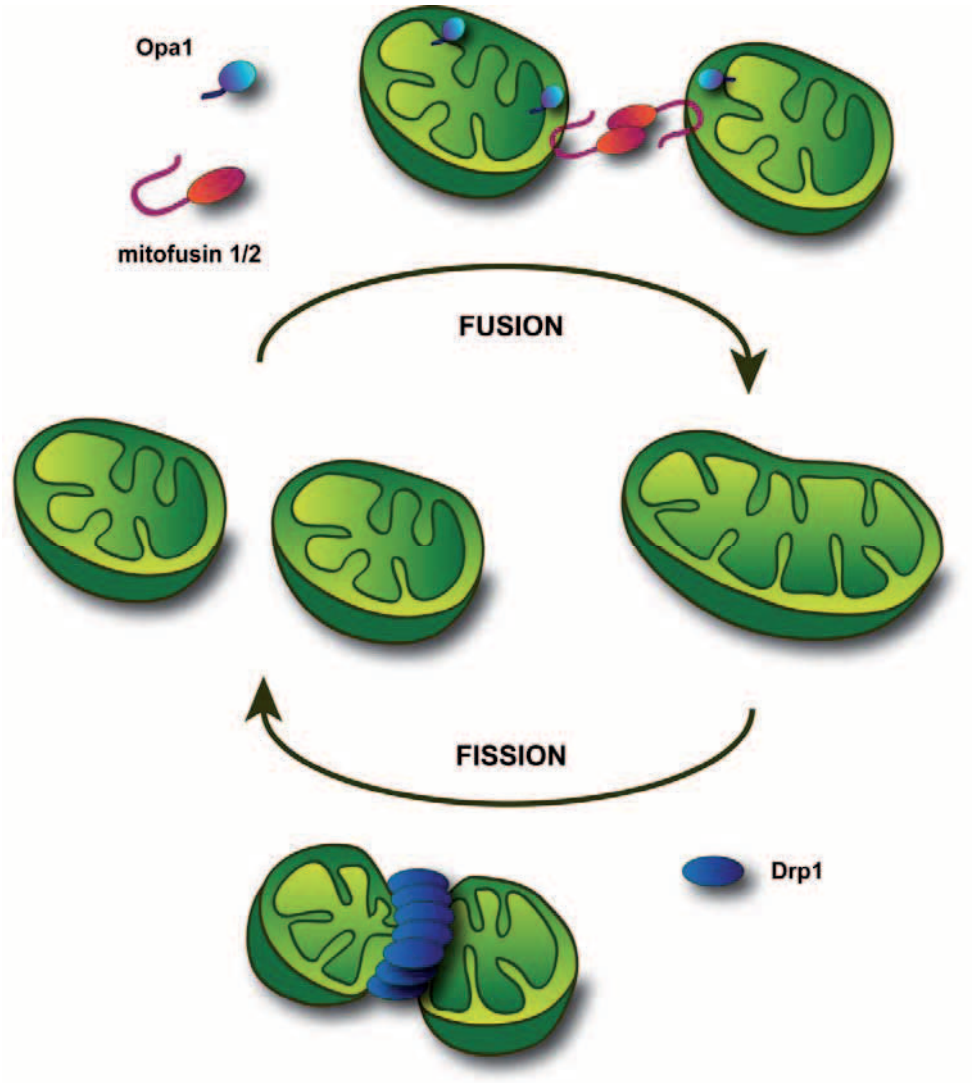


Figure 3: Mitochondrial dynamics. Mitochondrial morphology is controlled by mitochondrial fusion and fission processes. Mitochondrial outer membrane fusion is mediated by mitofusins 1 and 2, while inner membrane fusion is controlled by Opa1. During fission of mitochondria, Drp1 translocates from the cytosol to scission sites where it multimerizes and forms ring-shaped structures constricting mitochondria.

In addition, the degradation of dysfunctional or superfluous mitochondria is an essential part of mitochondrial quality control. Mitochondria are the major source of reactive oxygen species (ROS) which can be detrimental to cells. Furthermore, the intrinsic pathway of apoptosis can be induced by the release of cytochrome c from mitochondria. Removal of mitochondria is achieved via autophagic degradation of the organelles, a process termed mitophagy (discussed in detail in section 1.5.2). Mitophagy can be induced under different cellular conditions: analogous to non-selective, bulk-degradation macroautophagy, mitophagy can be triggered by starvation (reviewed in (Inoue and Klionsky, 2010)). In contrast, selective removal of mitochondria is induced in the course of erythrocyte maturation, and to prevent damage to cells when mitochondria are senescent or defective (see also section 1.5.2 and (Mortensen et al., 2010a, Mortensen et al., 2010b)).

Due to the important role of mitochondria for the cell, a tight regulation of mitochondrial biogenesis, dynamics and degradation is essential and the different quality mechanisms have to be balanced well in order to keep cellular homeostasis (Figure 4).

1.2.2 PD and mitochondrial dysfunction

As a consequence of the past decades of PD research it becomes increasingly evident that mitochondrial dysfunction plays a major role in disease pathogenesis. A first connection between PD and mitochondrial dysfunction emerged from the accidental exposure of drug addicts to the meperidine analogue 1-methyl-4-phenyl-1,2,3,6-tetrahydrodropyridine (MPTP) which induces a parkinsonian syndrome (Langston et al., 1983). MPTP is converted to the metabolite 1-methyl-4-phenylpyridinium (MPP^+) in glia cells and selectively taken up by dopaminergic neurons via the dopamine transporter where it inhibits complex I of the mitochondrial respiratory chain, ultimately resulting in the degeneration of dopaminergic neurons. In agreement with these observations, a complex I deficit has been identified in post mortem brain samples in the SNc of PD patients (Parker et al., 2008, Janetzky et al., 1994, Mizuno et al., 1989). Moreover, complex I deficiency is not restricted to SNc, but can be found in a variety of different tissues, including platelets, skeletal muscle and glia cells, although this is not a consistent observation among different studies (reviewed in (Reichmann and Janetzky, 2000)).

In brains of PD patients, an increase in reactive oxygen species (ROS) has been observed. ROS is mainly produced by mitochondrial defects or leakage of protons in the mitochondrial respiratory chain. This results in a decrease in oxidative phosphorylation and an increase in the generation of reactive oxygen and nitrogen species (ROS and RNS). The *substantia nigra* is a site of free radical damage to mtDNA, and this damage is enhanced in PD patients (reviewed in (Schapira, 2008)). Not only MPP^+ but also other environmental toxins interfering with mitochondrial function are implicated in PD pathogenesis. Rotenone, a broad-spectrum insecticide and piscicide, acts by inhibiting complex I of the electron transport chain. Similar to MPP^+, rotenone is used in rodents and primates to induce a parkinsonian phenotype and model PD in animals (reviewed in (Bove et al., 2005)). The herbicide paraquat catalyzes the formation of superoxide free radicals, a major source of ROS, and triggers degeneration of dopaminergic neurons. The exposure of humans to paraquat or rotenone is associated with increased incidence of PD in case-control studies (reviewed in (Tanner et al., 2011)).

Besides environmental factors genetic predisposition can affect mitochondrial function and subsequent PD pathogenesis. As an example, mutations in the mitochondrial polymerase POLG1 cause ophtalmoplegia and parkinsonism (Hudson et al., 2007). The interest in mitochondrial alterations linked to PD tremendously increased when it became evident that some PD-associated genes have a direct or indirect impact on mitochondrial integrity. A subset of gene products connected to familial forms of PD localize to mitochondria or influence aspects of mitochondrial quality control. The influence of PD-associated genes on mitochondria will be discussed in detail in the following sections (see section 1.3).

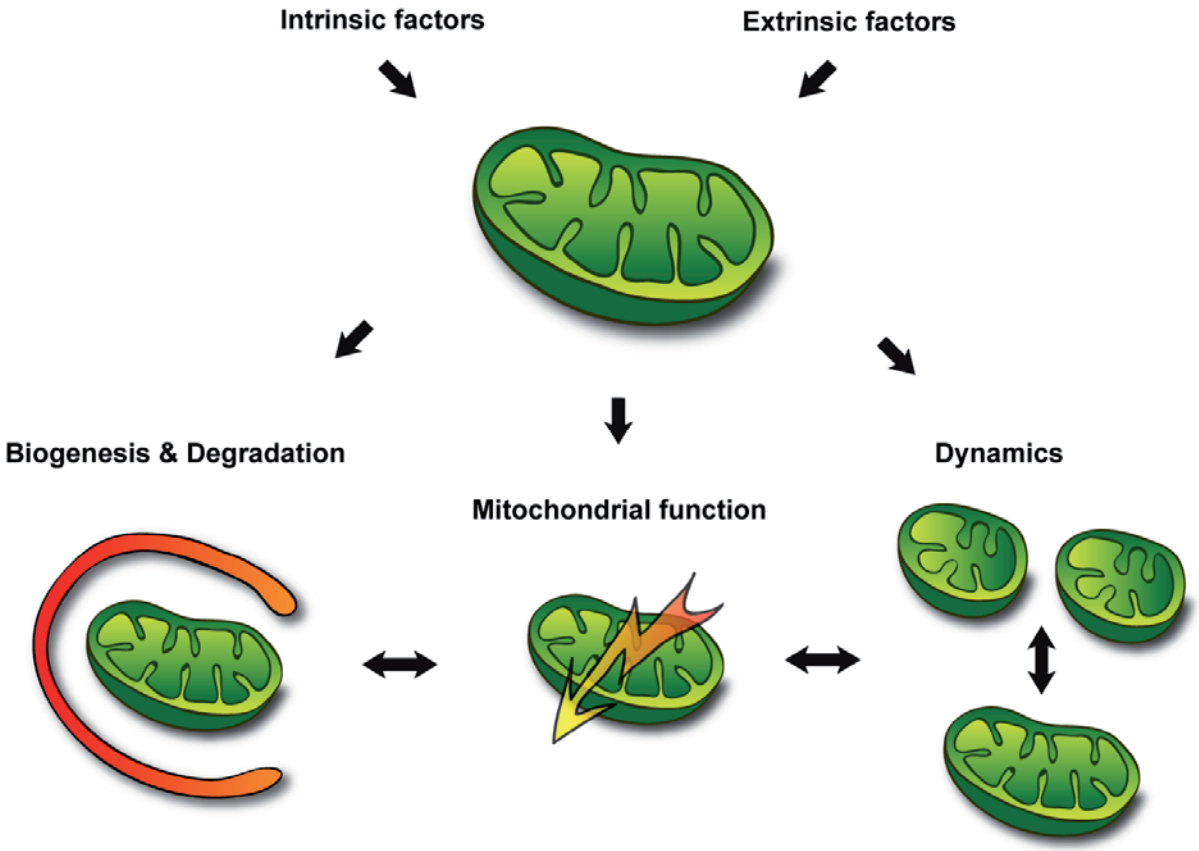


Figure 4: Mitochondrial quality control mechanisms. Both intrinsic and extrinsic factors can influence mitochondrial homeostasis. This can manifest in different ways such as altered mitochondrial biogenesis and repair, bioenergetics and/or dynamics.

1.3 Genetics of PD

Many lines of evidence indicate that sporadic or idiopathic PD is caused by the interplay of environmental factors and genetic predisposition. Genetic studies have provided valuable insights into the pathological mechanisms underlying PD. In fact, roughly 10 % of PD cases are monogenetically inherited.

Our understanding of the cellular mechanisms underlying PD pathogenesis emerged from the identification of mutations in the gene encoding α-synuclein and the demonstration that α-synuclein is the major constituent of Lewy bodies (Polymeropoulos et al., 1997, Spillantini et al., 1997). Although the exact cellular function of α-synuclein has not been elucidated, the protein has been implicated in binding to lipid membranes and possibly acting as a molecular chaperone in the formation of SNARE complexes (Kamp et al., 2010, Chandra et al., 2005, Burre et al., 2010).

Thereafter, mutations in other genes have been assigned to familial PD. Meanwhile, at least 16 loci (designated as PARK1 to PARK16, see table 1) have been associated with inherited forms of PD, six of which have been unequivocally linked to PD pathogenesis. Mutations in α-synuclein and leucine-rich repeat kinase 2 (LRRK2) display an autosomal-dominant inheritance pattern. Of note, recent GWAS have implicated genetic variability at these two

loci, α-synuclein and LRRK2, as significant risk factors for developing sporadic PD (Consortium" et al., 2011, Pankratz et al., 2009, Pankratz et al., 2012, Lill et al., 2012). Contrarily, mutations in parkin, PINK1 (PTEN-induced kinase 1), DJ-1 and ATP13A2 are autosomal-recessively inherited. Interestingly, the symptoms of patients with mutations in PD genes are clinically indistinguishable from those suffering from sporadic PD, suggesting possible common pathomechanisms. Moreover, there is mounting evidence that parkin and PINK1 function in a mutual cellular pathway, and this will be discussed in detail in the following sections (see sections 1.4 and 1.5).

Table 1: Summary of genes and loci associated with PD.

Genetic locus	Gene product	Inheritance	Gene function
PARK1/4	α-synuclein	AD, RF	vesicle dynamics, lipid binding, synaptic transmission
PARK2	parkin	AR	E3 ubiquitin ligase
PARK3	unknown	AD	unknown
PARK5	UCH-L1	AD	enzyme involved in ubiquitination and deubiquitination
PARK6	PINK1	AR	mitochondrial kinase
PARK7	DJ-1	AR	redox-sensitive protein
PARK8	LRKK2	AD, RF	serine/threonine kinase, GTPase
PARK9	ATP13A2	AR	lysosomal ATPase
PARK10	unknown	RF	unknown
PARK11	GIGYF2	AD	possibly involved in tyrosine kinase receptor signaling
PARK12	unknown	RF	unknown
PARK13	HtrA2/OMI	AD	mitochondrial serine protease
PARK14	PLA2G6	AR	lipid metabolism
PARK15	FBXO7	AR	SFC ubiquitin ligase
PARK16	unknown	RF	unknown
Not assigned	GBA	GD	lysosomal enzyme

Abbreviations:

AD: autosomal dominant; AR: autosomal recessive; RF: risk factor for sporadic PD; GD: Gaucher's Disease

FBXO7: F-box only protein 7; GBA: glucocerobrosidase A; GIGYF2: GRB10 interacting GYF protein 2; HtrA2/OMI: high temperature requirement protein A2; LRRK2: leucine-rich repeat kinase 2; PINK1: PTEN-induced kinase 1; PLA2G6: phospholipase A2, group VI; SFC: Skp1-Cullin-F-box protein; UCH-L1: ubiquitin carboxyl-terminal esterase L1

1.4 PINK1

PINK1 (PTEN-induced kinase 1) is a ubiquitously expressed mitochondrial serine-threonine kinase comprising 581 amino acids. It contains an N-terminal mitochondrial targeting signal (MTS), a transmembrane sequence (TM) and a C-terminal kinase domain (Figure 5). Mutations in PINK1 have been connected to autosomal-recessive parkinsonism (Valente et al., 2004). Approximately 50 different mutations in PINK1 have been associated with the

disease, including missense and truncation mutations as well as large genomic rearrangements and deletions (see the Parkinson Disease Mutation Database PDmutDB found at http://www.molgen.ua.ac.be/PDmutDB; also reviewed in (Nuytemans et al., 2010)). Furthermore, heterozygous PINK1 mutations might be a risk factor for sporadic PD (Abou-Sleiman et al., 2006). Similarly to parkin, the PINK1 protein is highly conserved among multicellular species. Valuable insights into the function of PINK1 resulted from the analysis of animal models. *Drosophila* PINK1 mutants display reduced life span, male sterility and apoptotic flight muscle degeneration (Clark et al., 2006, Park et al., 2006, Yang et al., 2006a). In contrast, ablation of PINK1 in mice does not result in an overt phenotype. Aged PINK1 knockout mice display only minor deficits such as a mild decrease in total dopamine levels and an impaired synaptic plasticity in the striatum (Gispert et al., 2009, Kitada et al., 2007) and minor gait alterations and olfactory dysfunctions (Glasl et al., 2012). However, no dopaminergic cell death has been observed in the *substantia nigra* or the striatum of mice lacking PINK1 (Kitada et al., 2009, Kitada et al., 2007, Gispert et al., 2009, Zhou et al., 2007).

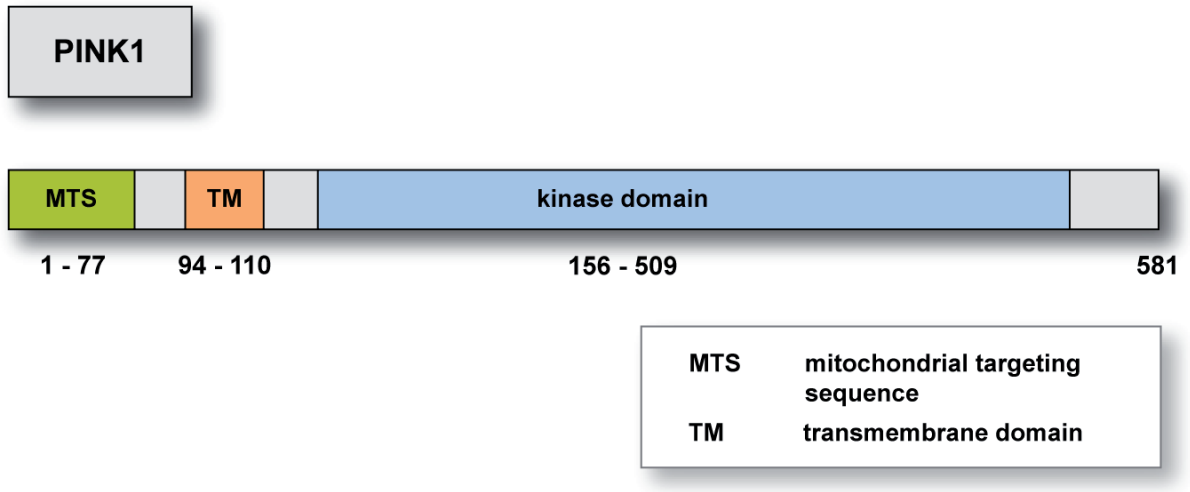


Figure 5: Modular structure of PINK1. The PINK1 protein contains an N-terminal mitochondrial targeting sequence (MTS), a transmembrane domain (TM) and a C-terminal kinase domain. Numbers below indicate the domain borders (amino acid positions).

The subcellular localization of PINK1 is still under extensive debate. Although PINK1 contains a mitochondrial targeting signal and a transmembrane sequence (Silvestri et al., 2005), several groups have identified a proportion of the protein in the cytosol (Weihofen et al., 2008, Takatori et al., 2008, Beilina et al., 2005, Haque et al., 2008, Lin and Kang, 2008, Lin and Kang, 2010). A recent report presents evidence that the PINK1 protein resides in the outer mitochondrial membrane with its kinase domain facing the cytoplasm (Zhou et al., 2008). Interestingly, cytosolic PINK1 lacking the mitochondrial import sequence and the transmembrane domain displays neuroprotective activity *in vivo*, suggesting that cytosolic PINK1 may have a function in neuronal survival (Haque et al., 2008). Analysis of PINK1

subcellular localization is difficult owed to the lack of an appropriate antibody detecting endogenous PINK1. Notably, PINK1 levels are low under basal conditions. Recent evidence indicates that PINK1 protein is kept at low levels through instant shedding of the protein during or shortly after mitochondrial import, most likely via cleavage mediated by the protease presenilin-associated rhomboid-like (PARL) or its homologue rhomboid-7 in *Drosophila* (Deas et al., 2011, Jin et al., 2010, Meissner et al., 2011, Shi et al., 2011, Whitworth et al., 2008). However, when the mitochondrial membrane potential is lost, PINK1 processing is inhibited and full-length PINK1 is stabilized at the OMM (Narendra et al., 2010b, Beilina et al., 2005, Lin and Kang, 2010, Jin et al., 2010, Deas et al., 2011). Interestingly, stablization of PINK1 at mitochondria seems crucial for the recruitment of parkin to mitochondria during mitophagy (see section 1.5.2).

Different substrates have been suggested to be phosphorylated by PINK1. The first identified substrate of PINK1 is TNF-receptor associated receptor 1 (TRAP1), a mitochondrial chaperone (Pridgeon et al., 2007). Phosphorylation of TRAP1 by PINK1 is able to confer the protective activity of PINK1 against oxidative stress by inhibiting the release of cytochrome c from mitochondria. Another PINK1 substrate identified is high temperature regulation A2 (HtrA2/OMI). Upon stimulation of the p38 stress kinase pathway, HtrA2/OMI is phosphorylated at a conserved serine residue in a PINK1-dependent manner (Plun-Favreau et al., 2007). Recently, PINK1 has also been found in a multiprotein complex together with Miro and Milton (Weihofen et al., 2009). The atypical GTPase Miro and the adaptor protein Milton are two proteins at the outer mitochondrial membrane that link kinesin heavy chains to mitochondria for anterograde axonal transport along microtubules. In accordance with this are recently published observations that PINK1 regulates Miro, possibly via phosphorylation, resulting in decreased levels of Miro and inhibiting axonal mitochondrial transport (Liu et al., 2012, Wang et al., 2011). An interaction of PINK1 and parkin has been observed in some studies, and possibly PINK1 might directly phosphorylate parkin under certain conditions (Kim et al., 2008, Shiba et al., 2009, Um et al., 2009). Notably, an epistatic interaction of parkin and PINK1 has been observed in *Drosophila*, where parkin is sufficient to rescue the PINK1 loss-of-function phenotype (see references (Clark et al., 2006, Park et al., 2006, Yang et al., 2006a) and section 1.6).

Similarly to parkin, the PINK1 protein has been implicated in cellular stress resistance (see section 1.5.1). Overexpression of PINK1 protects cells from cell death induced by staurosporine, a general kinase inhibitor (Petit et al., 2005). As mentioned before, the stress-protective effect of PINK1 can be mediated by direct phosphorylation of its substrate TRAP1, and mutations in the kinase domain of PINK1 abolish its cytoprotective activity (Pridgeon et al., 2007). In contrast, downregulation of PINK1 enhances apoptosis in the neuroblastoma cell line SH-SY5Y under cellular stress conditions (Deng et al., 2005). In line with this, *Drosophila* mutants lacking PINK1 exhibit widespread apoptosis, most prominently in indirect flight muscles. In the worm *Caenorhabditis elegans* loss of Pink-1 leads to an increased sensitivity to paraquat treatment (Samann et al., 2009). Accordingly, skin fibroblasts from PD patients harboring PINK1 mutations display increased vulnerability to proteasomal stress (Klinkenberg et al., 2010). Hence, PINK1 might be essential for

maintaining neuronal homeostasis, thus preventing neuronal degeneration. However, it remains to be determined if the protective activity of PINK1 depends on parkin and *vice versa*.

1.5 Parkin

Mutations in the parkin gene (PARK2 locus) are responsible for the majority of autosomal recessive PD cases. More than one hundred pathogenic parkin mutations have been identified, ranging from missense and nonsense mutations to large genomic deletions (see the Parkinson Disease Mutation Database PDmutDB http://www.molgen.ua.ac.be/PDmutDB; also reviewed in (Nuytemans et al., 2010)). The parkin gene comprises 12 exons, encoding for the 465 amino acid cytosolic parkin protein. The parkin protein contains an N-terminal ubiquitin-like domain (UBL), an atypical RING 0 domain involved in zinc binding and a C-terminal RING-between-RING (RBR) domain (Figure 6). Similarly to other RING finger proteins, parkin functions as an E3 ubiquitin ligase, mediating substrate specificity for the ubiquitination of substrate proteins. During the past decade a plethora of parkin substrates have been proposed, including parkin-associated endothelin receptor-like receptor (Pael-R), synphilin-1, glycosylated α-synuclein, cyclin E, synaptotagmin XI, CASK, the p38/JTV-1 subunit of the multi-tRNA synthetase complex, the PD-associated PINK1 protein and the parkin-interacting substrate (PARIS) (Zhang et al., 2000, Chung et al., 2001, Imai et al., 2001, Shimura et al., 2001, Choi et al., 2003, Corti et al., 2003, Huynh et al., 2003, Ren et al., 2003, Staropoli et al., 2003, Fallon et al., 2006a, Shin et al., 2011). Although initial studies pointed towards a role of parkin in proteasomal degradation by the attachment of lysine-48 (K48) linked polyubiquitin chains, there is *in vitro* evidence that it can also mediate the addition of lysine-63 polyubiquitin (K63) chains (Sha et al., 2010, Olzmann et al., 2007, Lim et al., 2005, Henn et al., 2007) or monoubiquitin (Fallon et al., 2006b, Hampe et al., 2006, Joch et al., 2007, Liu et al., 2008, Matsuda et al., 2006, Moore et al., 2008, Trempe et al., 2009).

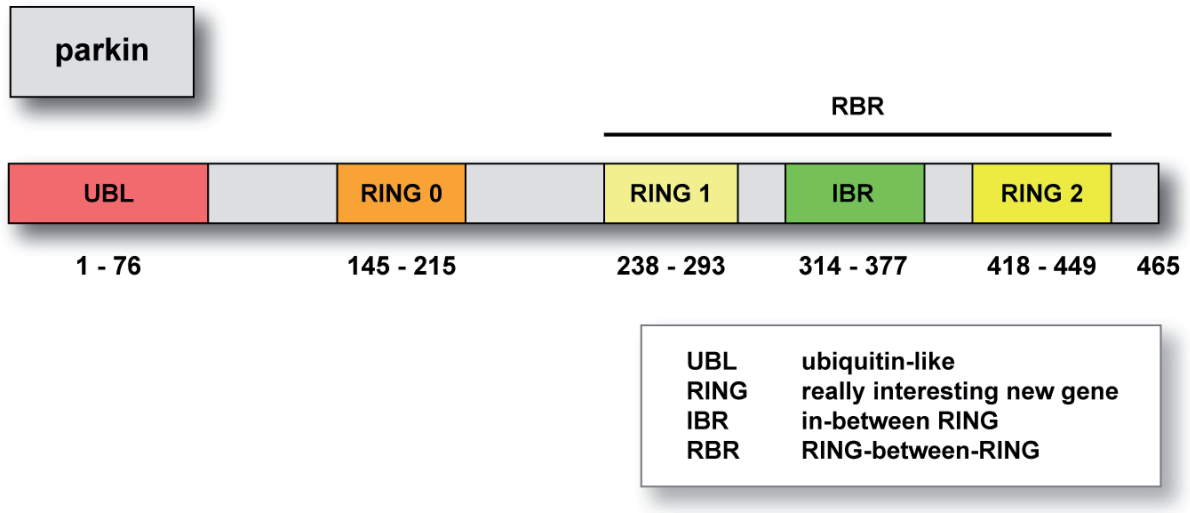


Figure 6: Modular structure of parkin. The parkin protein contains an N-terminal ubiquitin-like domain (UBL) and a C-terminal RBR (RING-between RING) domain. The RBR domain consists of two RING domains and an in-between RING domain (IBR) and mediates the E3 ubiquitin ligase activity of parkin. An additional atypical RING domain has been identified (RING 0). Amino acid positions of the individual domains are given below.

Insights into the *in vivo* function of parkin originated from the analysis of different animal models. *Drosophila* parkin null mutants display a strongly reduced lifespan, male sterility and locomotion abnormalities. Apoptotic flight muscle degeneration is observed, most likely resulting from mitochondrial alterations and functional deficits. In contrast to PD patients, no degeneration of dopaminergic neurons is detected in parkin-deficient flies (Clark et al., 2006, Greene et al., 2003, Park et al., 2006, Yang et al., 2006a). Overexpression of wildtype parkin in flies does not result in phenotypical alterations, by contrast, overexpression of the pathogenic parkin mutants Q311X or R275W causes neuronal and muscular degeneration, implying a possible toxic gain-of-function mechanism of pathogenic parkin mutants (Sang et al., 2007, Wang et al., 2007). In contrast to the severe phenotype observed in *Drosophila*, targeted deletion of parkin in rodents does not result in a severe phenotype. Parkin-deficient mice exhibit only minor changes in dopamine release and deficits in spatial learning while motor skills are not impaired (Goldberg et al., 2003, Von Coelln et al., 2004, Sato et al., 2006, Zhu et al., 2007b).

Many different functions have been attributed to parkin, the most consistent and favored being parkin-dependent cytoprotection and parkin-mediated mitophagy that are presented in the following chapters.

1.5.1 Parkin protects cells against a broad range of toxic insults

Parkin is a stress-responsive and stress-protective protein. It is upregulated upon various stress paradigms (Fett et al., 2010, Bouman et al., 2011, Imai et al., 2000, Yang et al., 2006b). Accordingly, parkin confers protectivity against different cellular stressors, thus preventing apoptotic cell death. Parkin is able to protect cells *in vitro* and in animal models

from toxicity induced by dopamine (Hasegawa et al., 2008), ceramide (Darios et al., 2003), kainate (Henn et al., 2007), rotenone (Henn et al., 2007) and various other stress conditions (Petrucelli et al., 2002, Staropoli et al., 2003, Higashi et al., 2004, Jiang et al., 2004). In contrast, downregulation of parkin enhances stress-induced cell death (MacCormac et al., 2004, Yang et al., 2007, Henn et al., 2007). In line with this, *Drosophila* parkin null mutants exhibit enhanced sensitivity to oxidative stress (Pesah et al., 2004). Additionally, parkin has been shown to maintain mtDNA integrity and supports mtDNA repair (Rothfuss et al., 2009). Another study reported an impact of parkin on mitochondria: in neuroblastoma cells, expression of wildtype parkin attenuated reactive oxygen species (ROS) production while the mutant protein enhanced ROS formation and apoptosis in these cells (Kuroda et al., 2006).

How can parkin protect cells from stress-induced apoptosis? Different pathways have been suggested to confer parkin-dependent cytoprotectivity. The impact of parkin on several signaling pathways has been demonstrated. In particular, parkin negatively regulates the c-Jun N-terminal kinase (JNK) pathway (Cha et al., 2005, Jiang et al., 2004, Liu et al., 2008). Another study presents evidence that parkin delays endocytosis and degradation of the epidermal growth factor receptor (EGFR) by ubiquitination of Eps15, thus promoting prosurvival EGF signaling through the PI3K/AKT pathway (Fallon et al., 2006b). Parkin might also contribute to the stress-induced activation of the nuclear factor kappa B (NF-κB) signaling pathway via ubiquitination of NEMO (NF-κB essential modifier) and TRAF2 (TNF receptor-associated factor 2) (Henn et al., 2007). Moreover, parkin could directly act on mitochondria as it is able to reduce the release of cytochrome c from mitochondria during cellular stress conditions (Berger et al., 2009). Recently, it has been demonstrated that parkin functions in mitophagy, the selective degradation of mitochondria via autophagy ((Narendra et al., 2008); see also next section 1.5.2). However, it is not clear if mitophagy can fully explain the protective capacity of parkin or if these functions are mediated via separate pathways.

1.5.2 Parkin mediates the removal of damaged mitochondria via mitophagy

Parkin is an E3 ubiquitin ligase, mediating the specificity of substrate protein ubiquitination. Although several substrates for parkin have been proposed, the validity of these remains elusive *in vivo*. Although it has been recognized for quite a while that parkin exhibits a broad-spectrum cytoprotective activity, the exact cellular function of parkin still remains obscure. A breakthrough in our understanding of parkin function was derived from a series of elegant cell biological studies: the identification that parkin mediates the selective removal of dysfunctional mitochondria has strengthened the link between PD pathogenesis and mitochondrial dysfunction (Narendra et al., 2008). The group of Richard Youle observed that parkin is targeted to mitochondria when the mitochondrial membrane potential is dissipated by the uncoupling reagent carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Strikingly, upon prolonged exposure to CCCP, mitochondria are cleared from parkin-expressing cells within 24 h as determined by immunostaining against several mitochondrial marker proteins. The degradation is specific for mitochondria, as peroxisomal abundance and distribution are

not changed. Mitophagy is dependent on autophagy: first, inhibition of lysosomal degradation by chemical inhibitors blocks the removal of defective mitochondria from the cells. In agreement with this, dysfunctional mitochondria are not degraded in autophagy-deficient fibroblasts (Narendra et al., 2008, Chan et al., 2011).

The initial study on mitophagy did not answer the question how parkin might be able to sense dysfunctional mitochondria for their selective degradation. Prompted by genetic interaction of parkin and PINK1 in *Drosophila* (Clark et al., 2006, Park et al., 2006, Yang et al., 2006a) and indications of an interaction of the two proteins in mammalian cells (Kim et al., 2008, Sha et al., 2010, Exner et al., 2007, Shiba et al., 2009, Um et al., 2009), the effect of PINK1 on parkin-dependent mitophagy was assessed. Several research groups demonstrated that PINK1 recruits parkin to dysfunctional mitochondria (Geisler et al., 2010a, Jin et al., 2010, Lazarou et al., 2012, Matsuda et al., 2010, Meissner et al., 2011, Narendra et al., 2010b, Shi et al., 2011, Vives-Bauza et al., 2010). Upon uncoupling of the mitochondrial membrane potential, full-length PINK1 is stabilized at mitochondria. This is most likely due to inhibited mitochondrial import of PINK1 through the TIM/TOM complex and impaired cleavage by the presenilin-associated rhomboid-like (PARL), a voltage-dependent mitochondrial protease (see (Lazarou et al., 2012) and references above).

Since the initial discovery of parkin-mediated mitophagy a lot of emphasis has been taken to dissect the molecular mechanisms of this pathway. A variety of other factors have been implicated in parkin-mediated mitophagy, most notably mitochondrial proteins such as voltage-dependent anion channel 1 (VDAC1) and the mitofusins 1 and 2, as well as proteins involved in autophagy such as p62, LC-3, Nix, HDAC6 and Ambra1 (Narendra et al., 2010a, Geisler et al., 2010a, Chan et al., 2011, Ding et al., 2010, Lee et al., 2010, Okatsu et al., 2010, Ziviani et al., 2010, Van Humbeeck et al., 2011). However, not all players and steps in mitophagy have been elucidated. Most notably, the *in vivo* relevance of parkin and PINK1 function in mitophagy is not yet clear, thus it remains to be determined if mitophagy plays a role in PD pathogenesis.

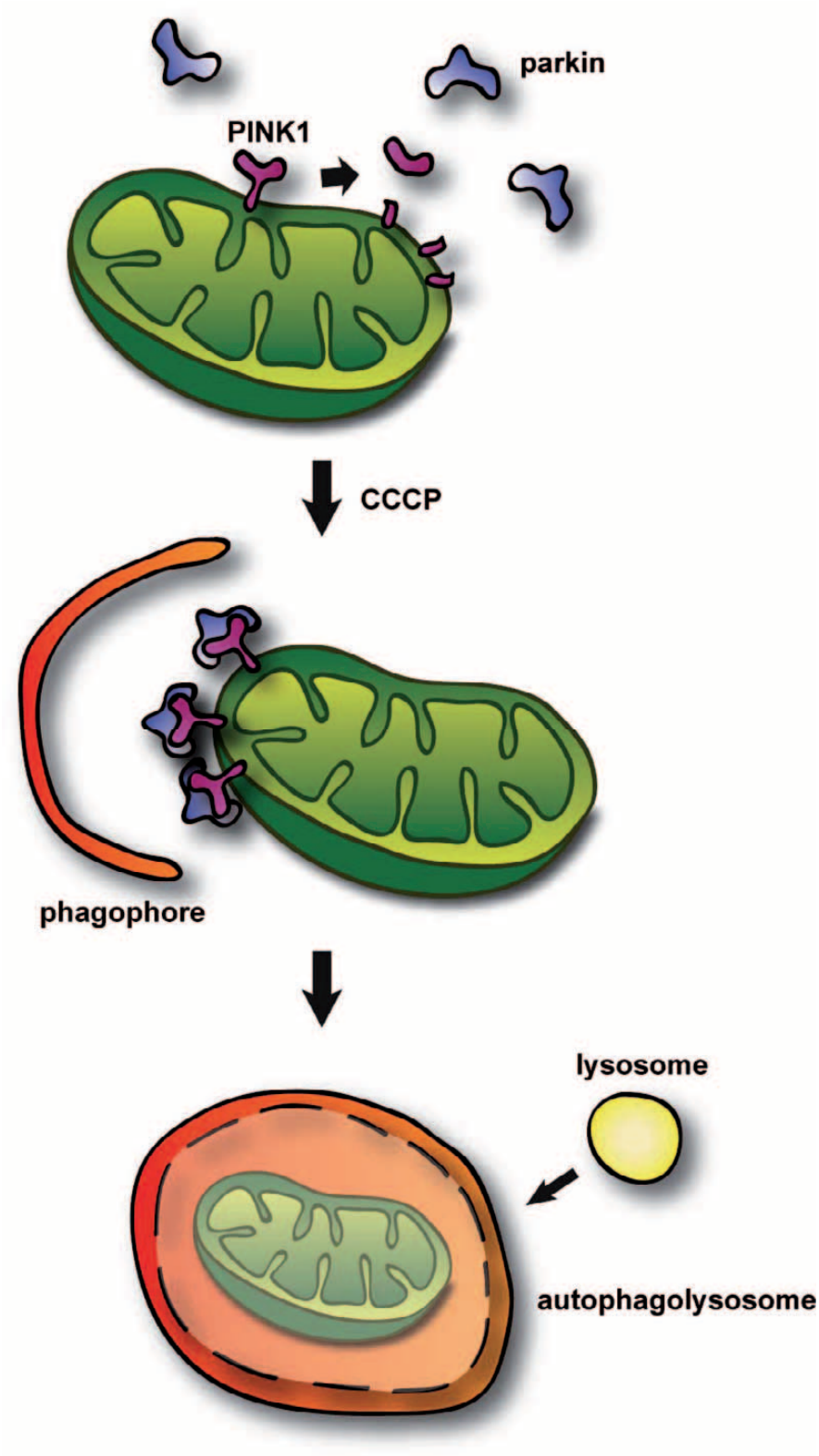


Figure 7: Sequential steps of parkin-induced mitophagy. On healthy mitochondria, steady-state levels of PINK1 are kept low due to rapid shedding, and parkin is mainly found in the cytosol. Upon dissipation of the mitochondrial membrane potential, for instance by treatment with the uncoupling agent CCCP, proteolytic processing of PINK1 is abolished, resulting in accumulation of full-length PINK1 at the outer mitochondrial membrane OMM). PINK1 stabilization is essential to recruit parkin to uncoupled mitochondria, possibly via a direct or indirect interaction between PINK1 and parkin. Parkin-induced ubiquitination of OMM proteins allows remodeling of the mitochondrial surface, facilitating the recruitment of adaptor proteins that link damaged mitochondria to the autophagic machinery. Dysfunctional mitochondria are engulfed in autophagosomes and can be degraded after fusion with lysosomes. Figure adapted from (Pilsl and Winklhofer, 2011).

1.6 Molecular interaction of parkin and PINK1

The most compelling link between the PD genes PINK1 and parkin emerged from studies in *Drosophila*. Flies deficient for parkin or PINK1 display similar phenotypes, including abnormal mitochondrial morphology, male sterility and apoptotic flight muscle degeneration (Clark et al., 2006, Park et al., 2006, Yang et al., 2006a, Greene et al., 2003). Surprisingly, overexpression of parkin in PINK1 mutant flies was able to restore the PINK1 mutant phenotype, while PINK1 could not compensate for a parkin loss-of-function. These findings suggest that PINK1 and parkin function in a common pathway with parkin acting downstream of PINK1. In cellular models, silencing of parkin or PINK1 by RNA interference causes similar mitochondrial alterations (Lutz et al., 2009, Exner et al., 2007, Sandebring et al., 2009). Subsequent analysis in mammalian cells found that parkin was able to rescue mitochondrial fragmentation induced by the loss of PINK1 (Exner et al., 2007).

Supporting evidence for a common parkin-PINK1 pathway originated from the work of several groups reporting a direct physical interaction of the two proteins (Kim et al., 2008, Sha et al., 2010, Shiba et al., 2009, Um et al., 2009, Moore, 2006). Most studies rely on protein overexpression, however, in an approach to analyze the interaction of parkin and PINK1 *in vivo*, endogenous parkin and PINK1 was co-immunoprecipitated in lysates from the striatum and *substantia nigra* of rats (Um et al., 2009) or human brain (Sha et al., 2010). However, the direct interaction of the two proteins is not a common finding. It is possible that parkin and PINK1 only interact upon particular cellular stress conditions or that the proteins function in the same pathway, but their interaction is not direct (Vives-Bauza et al., 2010, Lazarou et al., 2012).

As pointed out before, parkin and PINK1 are both essential for mitophagic degradation of mitochondria. Furthermore, parkin and PINK1 share the ability to protect cells from stress-induced aptosis. However, it remains to be determined if 1) the protective activities of parkin and PINK1 depend on each other and 2) if mitophagy is essential for the cytoprotection mediated by parkin and PINK1.

1.7 Cellular quality control mechanisms

Protein homeostasis is essential for proper cellular function. Thus, different strategies are exploited to keep a proper balance in cellular components. Consequently, already the expression of proteins is tightly regulated, with control mechanisms at the level of transcription and translation. To achieve a proper tertiary structure, the proteins are then folded with the help of chaperones. Misfolded proteins that fail to adopt a native conformation are recognized and instantly degraded to prevent potential detrimental effects for the cell. Two major pathways are responsible for decomposition of cellular components: the ubiquitin-proteasomal pathway (UPS) and autophagy. While the UPS is mainly involved in the degradation of short-lived and small proteins, the autophagy pathway has been implicated in degradation of large proteins, cellular protein aggregates and even whole organelles.

Although both pathways work independently and in parallel to each other, they are closely linked and are able to functionally compensate each another.

1.7.1 Ubiquitin-proteasomal system

The ubiquitin-proteasomal system (UPS) is an essential cellular pathway for protein degradation. Degradation of a protein via the UPS involves the covalent attachment of ubiquitin chains followed by recognition of the ubiquitinated protein by the proteasome and subsequent proteolysis (Figure 8). Ubiquitination of substrate proteins is mediated by a three step mechanism: first, an E1 ubiquitin activation enzyme covalently binds an ubiquitin moiety in an ATP-dependent manner. Second, the ubiquitin moiety is transferred to an E2 ubiquitin conjugation enzyme. In the last step, an E3 ubiquitin ligase controls for specificity of substrate ubiquitination. Several rounds of this ubiquitination steps follow to prolong the ubiquitin chain and result in polyubiquitination of the substrate protein. Several different modes of ubiquitination are used, depending on the lysine residue used to connect the different ubiquitin moieties. In particular, linkage via lysine-48 (K48) is a classical signal for proteasomal degradation. Substrates ubiquitinated through K48 are recognized by the proteasome, unfolded and eventually degraded, while ubiquitin is cleaved off and recycled.

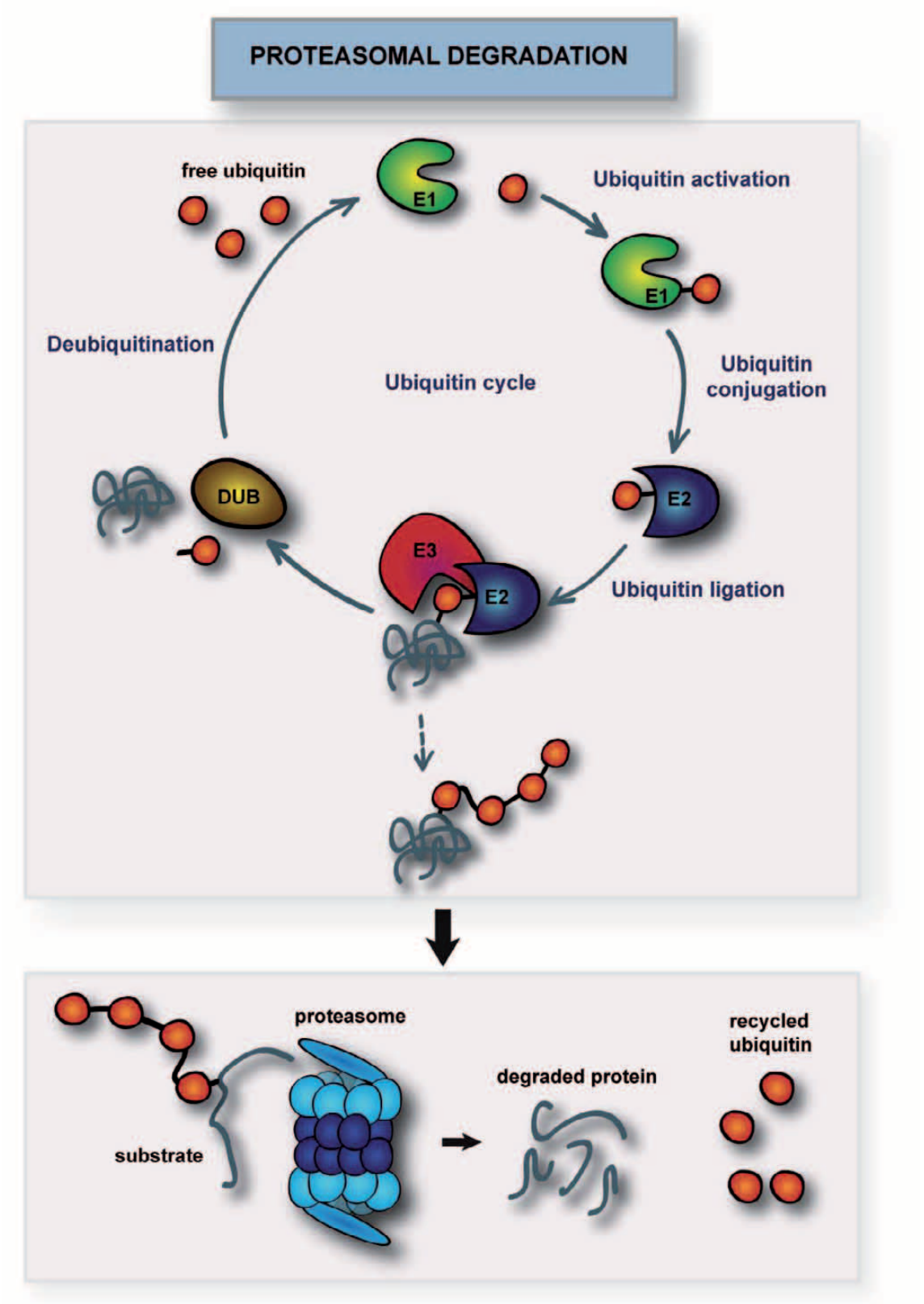


Figure 8: Ubiquitin cycle and proteasomal degradation. Substrate ubiquitination is achieved by three sequential steps: an E1 ubiquitin activating enzyme covalently binds ubiquitin in an ATP-dependent manner. Ubiquitin is then transferred to an E2-conjugating enzyme and subsequently attached to the substrate via an E3 ubiquitin ligase. The E3 ligase mediates substrate specificity. Ubiquitin can be detached from the substrate through a deubiquitinase (DUB). Alternatively, following several rounds of ubiquitination, the substrate can be targeted for proteasomal degradation. This involves unfolding of the substrate protein, uptake into the proteasome, and cleavage therein. Ubiquitin is detached from the substrate and recycled.

1.7.2 Autophagosomal-lysosomal system

Autophagy has long been classified as a bulk degradation process. Only recently it became clear that besides starvation- or stress-induced autophagy, several modes of selective autophagy are employed by cells. This includes the selective degradation of pathogens (xenophagy), endoplasmic reticulum (reticulophagy), peroxisomes (pexophagy) and mitochondria (mitophagy). Autophagy comprises the formation of a phagophore, a double-membrane structure, which engulfs the organelle or cargo determined to be degraded and forms the autophagosome. Subsequently, the autophagosome fuses with a lysosome, following the degradation of the vesicular content by acidic lysosomal proteases (Figure 9).

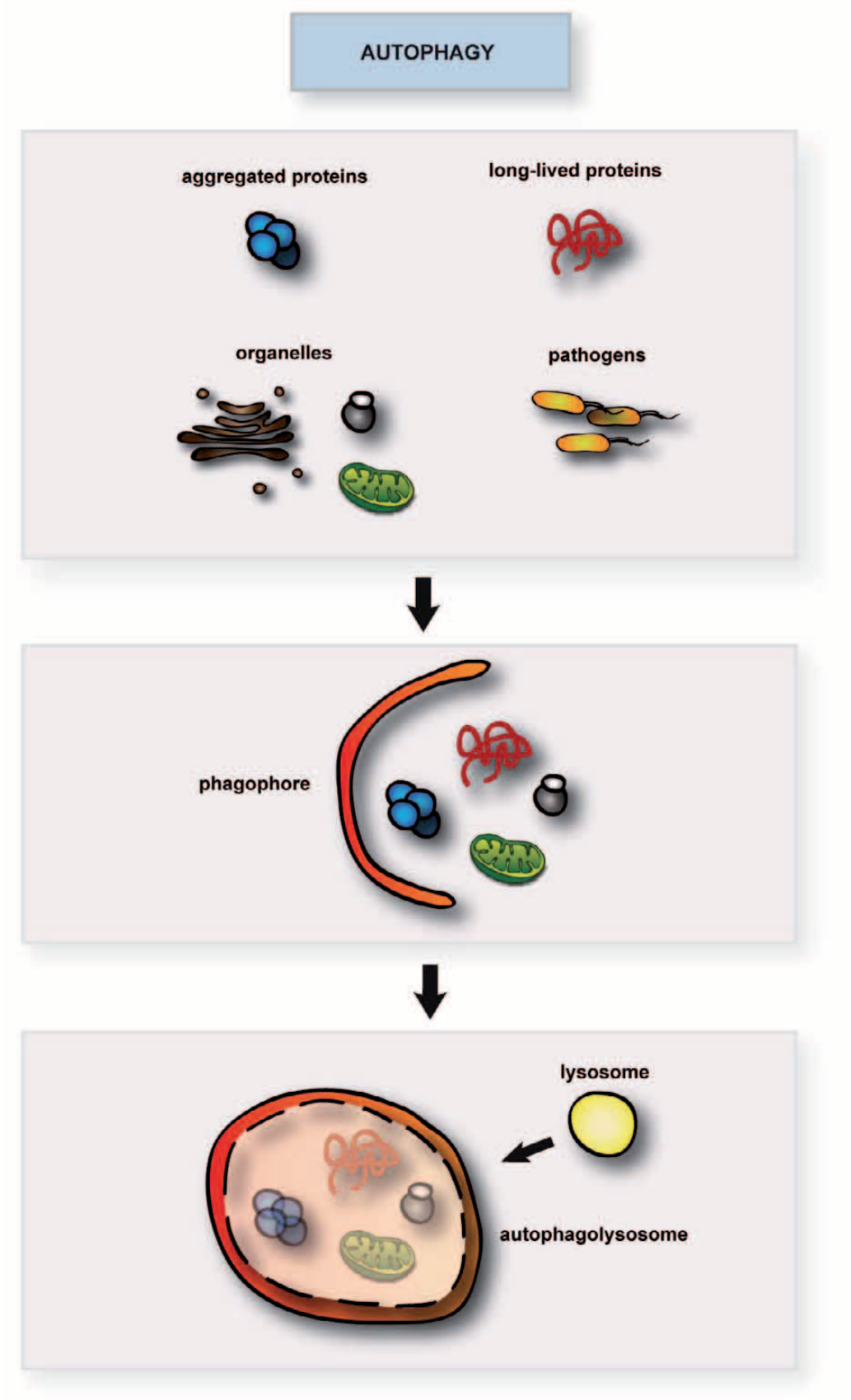


Figure 9: Autophagosomal-lysosomal pathway. During autophagy, a phagophore is formed which engulfs cytoplasmic content determined for degradation. Following formation of the autophagosome, the vesicle fuses with a lysosome and the intravesicular content is degraded by lysosomal proteases.

Interestingly, the substrate recognized for degradation by selective autophagy is tagged by ubiquitin molecules. Similarly to the UPS, polyubiquitination serves as a versatile degradation signal for selective autophagy. Substrates such as dysfunctional or surplus organelles are labeled by ubiquitin. The polyubiquitin signal can subsequently be recognized by autophagy adaptor proteins. Autophagy adaptors mediate simultaneous binding of ubiquitinated cargo and components of the autophagic machinery. Several adaptor proteins have been identified in yeast and in mammalian cells (reviewed in (Behrends and Fulda, 2012)). All of them are characterized by an ubiquitin-binding domain, and mammalian examples are neighbor of BRCA1 gene 1 (Nbr1), optineurin (OPTN), NIP3-like protein X (NIX) and p62 (also known as sequestosome-1 or SQSTM1). The p62 protein contains an ubiquitin-associated domain (UBA) involved in ubiquitin binding and a LC3-interacting region (LIR) for interaction with autophagosomes (Figure 10).

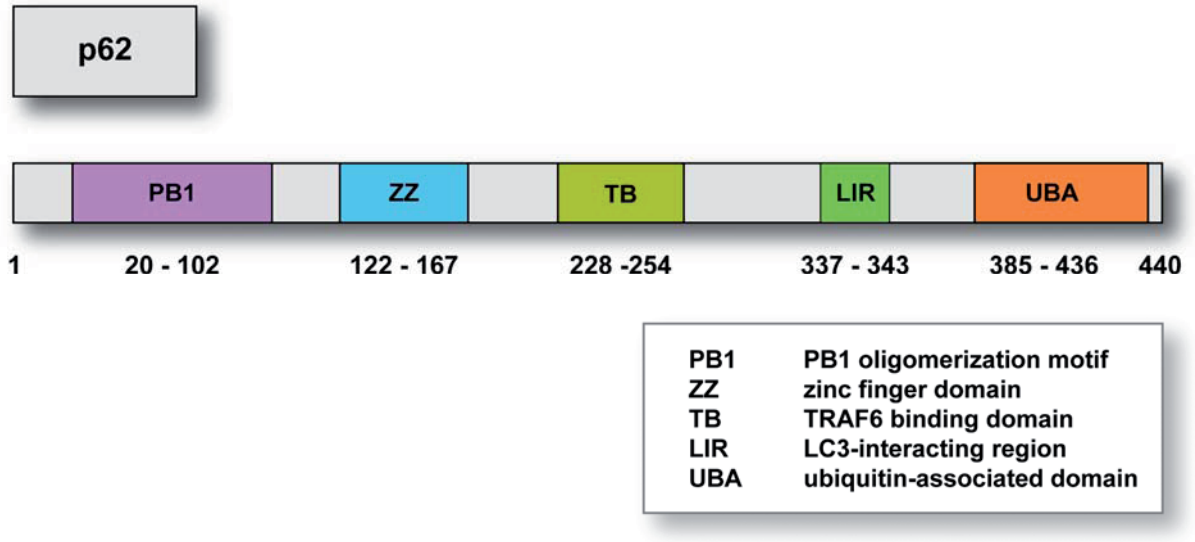


Figure 10: Modular structure of p62. The p62 protein contains an N-terminal PB1 domain important for p62 auto-oligomerization. The C-terminal ubiquitin-associated domain is involved in polyubiquitin binding. In addition, p62 has been shown to interact with TRAF6 (via the TRAF6-binding domain (TB)), which is implicated in NF-κB signaling. The ZZ domain is a zinc finger domain, and the LC-3 interacting domain (LIR) mediates the binding of p62 to autophagosomes via LC3 during selective autophagy.

It has been demonstrated that p62 plays a role in the degradation of protein aggregates. Aggregates of mutant huntingtin are encompassed by p62 and degraded by autophagy in a p62-dependent manner (Tung et al., 2010). In addition there is a possible role for p62 in mitophagy (Ding et al., 2010, Geisler et al., 2010a, Lee et al., 2010), although the experimental results obtained from different groups are ambiguous (Narendra et al., 2010a, Okatsu et al., 2010). Results from animal models unveil the critical role of selective autophagy for cellular homeostasis: mice deficient for p62 display mature-onset obesity, insulin resistance, and neurodegeneration (Rodriguez et al., 2006, Ramesh Babu et al., 2008). Additionally, mutations in p62 have been implicated in Paget's disease, a chronic disorder of the adult characterized by abnormal bone formation (Hocking et al., 2002, Laurin

et al., 2002). Of note, p62 is also used as a marker to stain for protein aggregates in a variety of proteinopathies, including neurodegeneration conditions, further implying a crucial role of selective autophagy in the pathogenesis of protein aggregation diseases (Zatloukal et al., 2002, Kuusisto et al., 2008, Kuusisto et al., 2001).

2 Aims of the thesis

The PD-associated E3 ubiquitin ligase parkin is involved in different cellular processes (Figure 11). Many lines of evidence indicate that parkin displays broad neuroprotective activity, although the underlying mechanisms are largely unknown. A landmark publication by the group of Richard Youle identified parkin as a mediator of mitophagy, the degradation of dysfunctional mitochondria via autophagy (Narendra et al., 2008). Although several factors contributing to mitophagy have been identified recently, there are still a lot of open questions. Analysis of parkin function in mitophagy is of great importance as mitochondrial dysfunction is thought to play a key role in PD pathology. In this context it remains to be determined if PD-associated mutations in parkin can affect mitophagy and how their function is compromised when parkin is mutated. Do pathogenic parkin mutations display a loss-of-physiological function in mitophagy? Or is it a toxic gain-of-function mechanism that is relevant for the disease? Parkin is an aggregation-prone protein, and several parkin mutants tend to misfold and form cellular aggregates. The goal of the second part of this thesis was to analyze how aggregates of mutant parkin can be degraded and to characterize the factors that contribute to the removal of misfolded parkin conformers.

The results are subdivided into two topics pursuing the following goals:

1. To get insights into the physiological function of parkin and its role in the degradation of dysfunctional mitochondria via mitophagy.

2. To investigate the degradation mechanism of pathogenic misfolded parkin mutants.

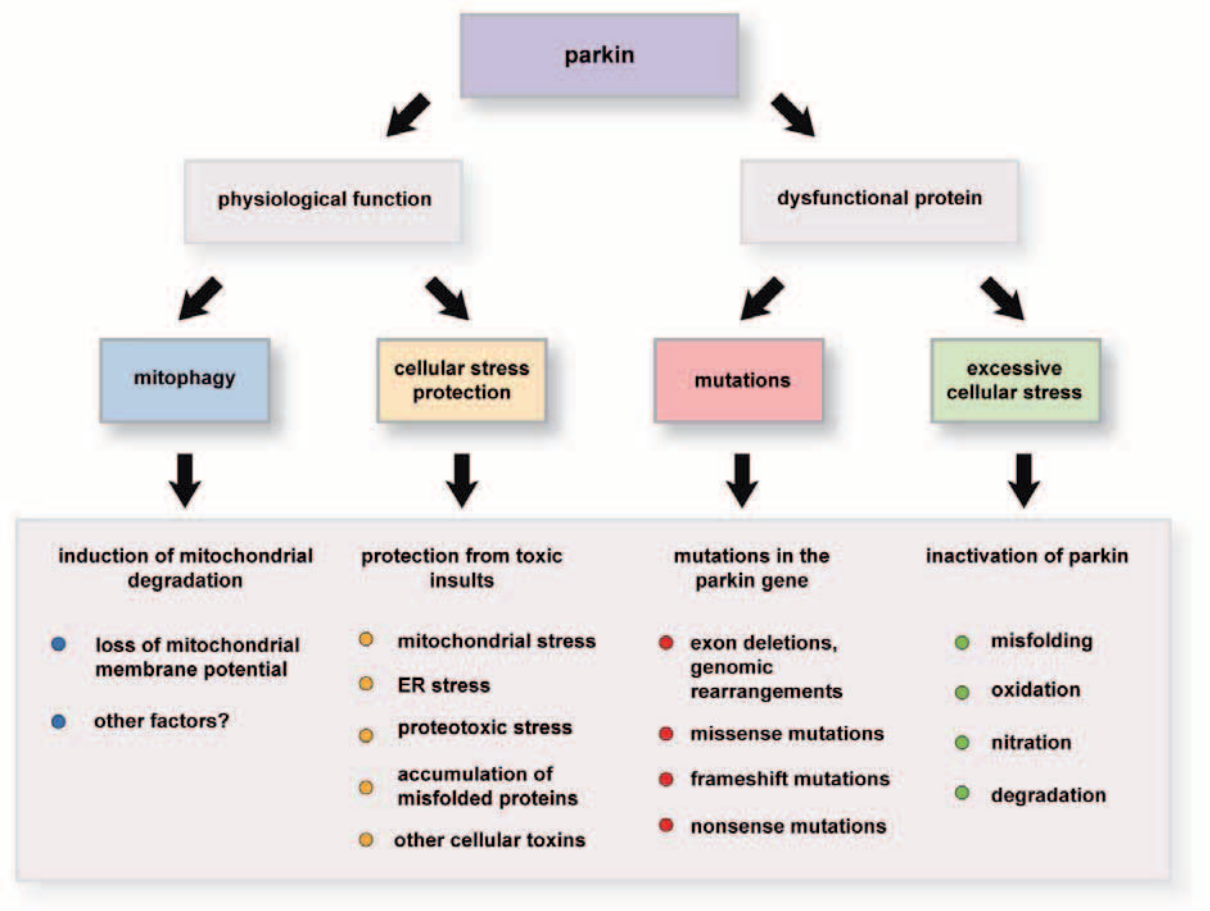


Figure 11: Physiological function and pathological dysfunction of parkin. Parkin is implicated in the degradation of dysfunctional mitochondria via mitophagy and mediates cellular stress-protection from apoptotic stimuli. Parkin function can be compromised by pathogenic mutation or upon exposure to severe cellular stress.

3 Results

3.1 Characterization of parkin-mediated mitophagy

3.1.1 Depolarization of mitochondrial membrane potential induces mitochondrial fragmentation independently of parkin

Mitochondria are the cellular powerplants, supplying cells with energy in form of adenosine triphosphate (ATP). Maintenance of mitochondrial membrane potential is essential for proper mitochondrial function. When mitochondrial membrane potential is lost, dysfunctional mitochondria have to be removed by selective autophagy, a process termed mitophagy. The mitochondrial membrane potential can be experimentally dissipated by the use of uncoupling agents, such as the protonophore CCCP. When cells are treated with CCCP, a rapid depolarization of mitochondrial membrane potential is triggered, resulting in the fragmentation of the tubular mitochondrial network (Figure 12). This fragmentation in response to uncoupling of mitochondrial membrane potential is observed in a variety of different cells. Of note, it is independent of parkin as it occurs in HeLa cells that lack endogenous parkin.

3.1.2 Parkin is recruited to dysfunctional mitochondria and initiates their degradation

Previous studies have shown that parkin is able to translocate to mitochondria that have lost their membrane potential and can initiate their degradation via mitophagy (Narendra et al., 2008). In order to reproduce these findings, HeLa cells were used as they are devoid of endogenous parkin (see (Narendra et al., 2008) and own unpublished observations). HeLa cells stably expressing human wildtype parkin were obtained by lentiviral transduction. After treatment of the cells with the uncoupling reagent CCCP for 1 or 24 h, the cells were fixed and subjected to immunofluorescence or Western blotting for mitochondrial marker proteins. Parkin was found in the cytosol under basal conditions. Following 1 h of CCCP treatment, parkin translocated to mitochondria as assessed by single cell analysis (Figure 12). While all control cells retained their mitochondrial staining at 24 hours of CCCP treatment, about two third of parkin-expressing cells completely lost mitochondrial staining (Figure 13). Analysis of whole cell lysates by Western blotting revealed a strong overall decrease in mitochondrial mass in cells parkin-expressing, while there was only a slight decrease in control cells lacking parkin (Figure 13). Protein levels of TOM20, a marker protein located in the outer mitochondrial membrane, slightly decreased upon 3 h of CCCP treatment in cells expressing parkin and even further decreased upon prolonged exposure to CCCP. Similar results were obtained with TIM44, a protein associated with the inner mitochondrial membrane. TIM44

protein levels decreased upon uncoupling of mitochondrial membrane potential only in parkin-expressing HeLa cells, but not in control cells lacking parkin protein. Protein levels of the endoplasmic reticulum (calnexin) and the cytoplasm (GAPDH) did not change in response to mitochondrial uncoupling and parkin expression (Figure 13).

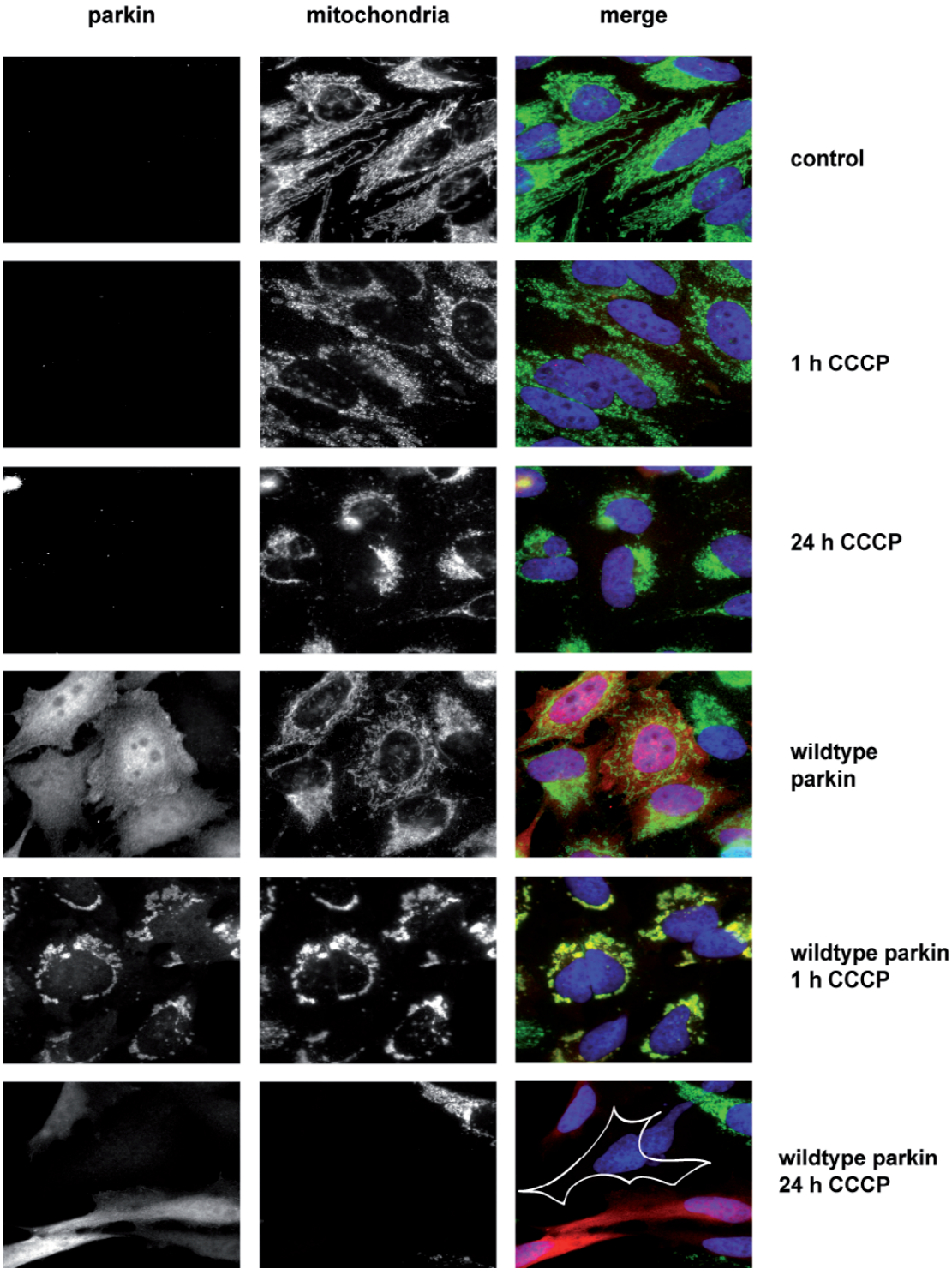


Figure 12: Parkin translocates to dysfunctional mitochondria and initiates their degradation. HeLa cells stably expressing human wildtype parkin and non-transduced control cells were treated with 10 µM of CCCP for the indicated time periods. Cells were subjected to indirect immunofluorescence with the parkin-specific antibody

PRK8 (red) and the mitochondria-specific antibody TOM20 (green). DAPI was used for nuclear counterstaining. In the last panel, one cell with complete loss of mitochondrial staining is highlighted by a white line to visualize cell borders. Note that parkin is sometimes degraded in conjunction with mitochondria, leading to a decrease in parkin staining.

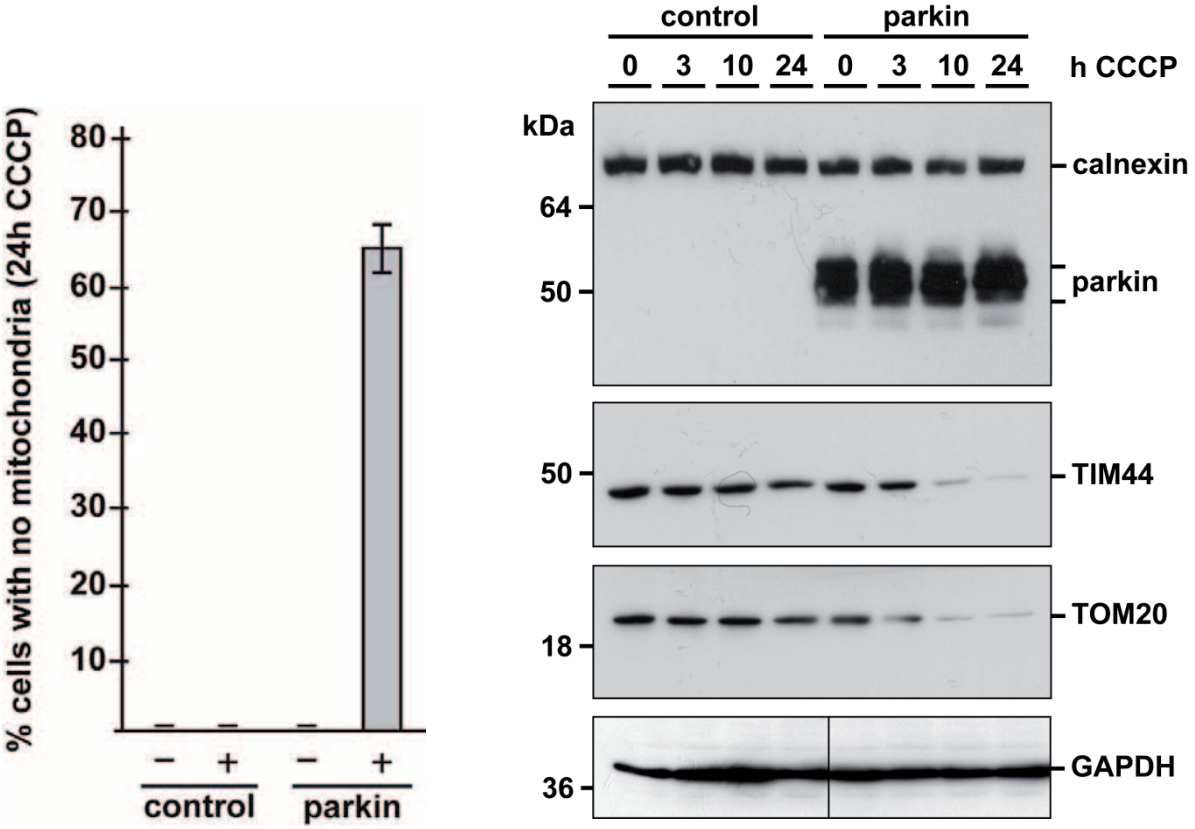


Figure 13: Parkin mediates the removal of dysfunctional mitochondria. Left panel: quantification of HeLa cells with no mitochondrial staining at 24 h of CCCP (10 µM) treatment. At least 500 cells were manually counted in three independent experiments (control: non-transduced HeLa cells, parkin: HeLa cells stably expressing wildtype parkin; - : no treatment, + 24 h treatment with 10 µM CCCP). Note that in the absence of parkin or CCCP, mitochondria are not degraded. Right panel: immunoblot analysis of HeLa cells stably expressing wildtype parkin and untransduced control cells. Cells were treated with 10 µM of CCCP for the indicated times and 10 µg of total protein were analyzed for the expression levels of mitochondrial proteins. Parkin expression was assessed in parallel and the endoplasmic reticulum marker protein calnexin and the cytosolic GAPDH served as loading controls.

3.1.3 Uncoupling of mitochondrial membrane potential but not other cellular stressors mediate parkin recruitment to mitochondria and mitophagy

Parkin has been shown to protect cells from a variety of toxic insults. Apoptosis can be prevented by parkin when a broad range of cellular stressors is applied. It is largely unknown how parkin mediates its protective activity. To determine if the induction of mitophagy can explain the anti-apoptotic function of parkin, a set of different cellular toxins was tested and parkin recruitment to mitochondria and subsequent mitophagy was assessed. SH-SY5Y neuroblastoma cells were chosen as they biochemically resemble dopaminergic neurons, thus expressing a subset of neuronal receptors and being responsive to treatment with various neurotoxins. Stable cell lines overexpressing human wildtype parkin were obtained

through lentiviral transduction. The protonophore CCCP, which induces mitochondrial uncoupling, was used as a positive control. Similar to CCCP, valinomycin (a potassium ionophore) treatment resulted in mitochondrial fragmentation followed by a strong and robust recruitment of parkin to mitochondria (Figure 14). Oligomycin, an inhibitor of the mitochondrial ATP synthase, induced parkin translocation to mitochondria only in a small subset of cells (less than 1 % of parkin-expressing cells). However, at 24 h of oligomycin treatment, no cells without mitochondria were detected, thus oligomycin treatment did not induce parkin-mediated mitophagy. Other stressors tested did not induce parkin translocation to mitochondria, neither after 3 h nor after 24 h of treatment (Figure 14 and Figure 15). Consequentially, mitochondria were not removed from the cells by mitophagy. Application of iron ions (5 mM of iron(III) citrate ($Fe3^{+}$) or 5 mM of iron(II) chloride (data not shown)) resulted in a marginal hyperelongation of mitochondria, but did not effectuate parkin translocation or mitophagy (Figure 14Figure 15). Similarly, two PD-associated toxins, 1-methyl-4-phenylpyridinium (MPP^{+}; 1 mM) and paraquat (1 mM) did not have an effect on parkin subcellular distribution and mitochondrial degradation. MPP^{+} is an inhibitor of complex I of the mitochondrial respiratory chain, while paraquat results in increased oxidative stress and free radical production. The excitotoxin glutamate (1 mM) and the sarcoplasmic/endoplasmic reticulum calcium ATPase-inhibitor (SERCA) thapsigargin (1 mM) both cause cellular toxicity by increasing intracellular calcium concentrations. Treatment of parkin-expressing SH-SY5Y cells with these chemicals did not result in parkin relocation to mitochondria and did not induce mitophagy. In conclusion, parkin translocation to mitochondria is only elicited by ionophores that directly depolarize mitochondrial membrane potential. In contrast, cellular stressors that directly or indirectly affect mitochondrial function do not alter the cytosolic distribution of parkin protein and do not elicit mitophagy.

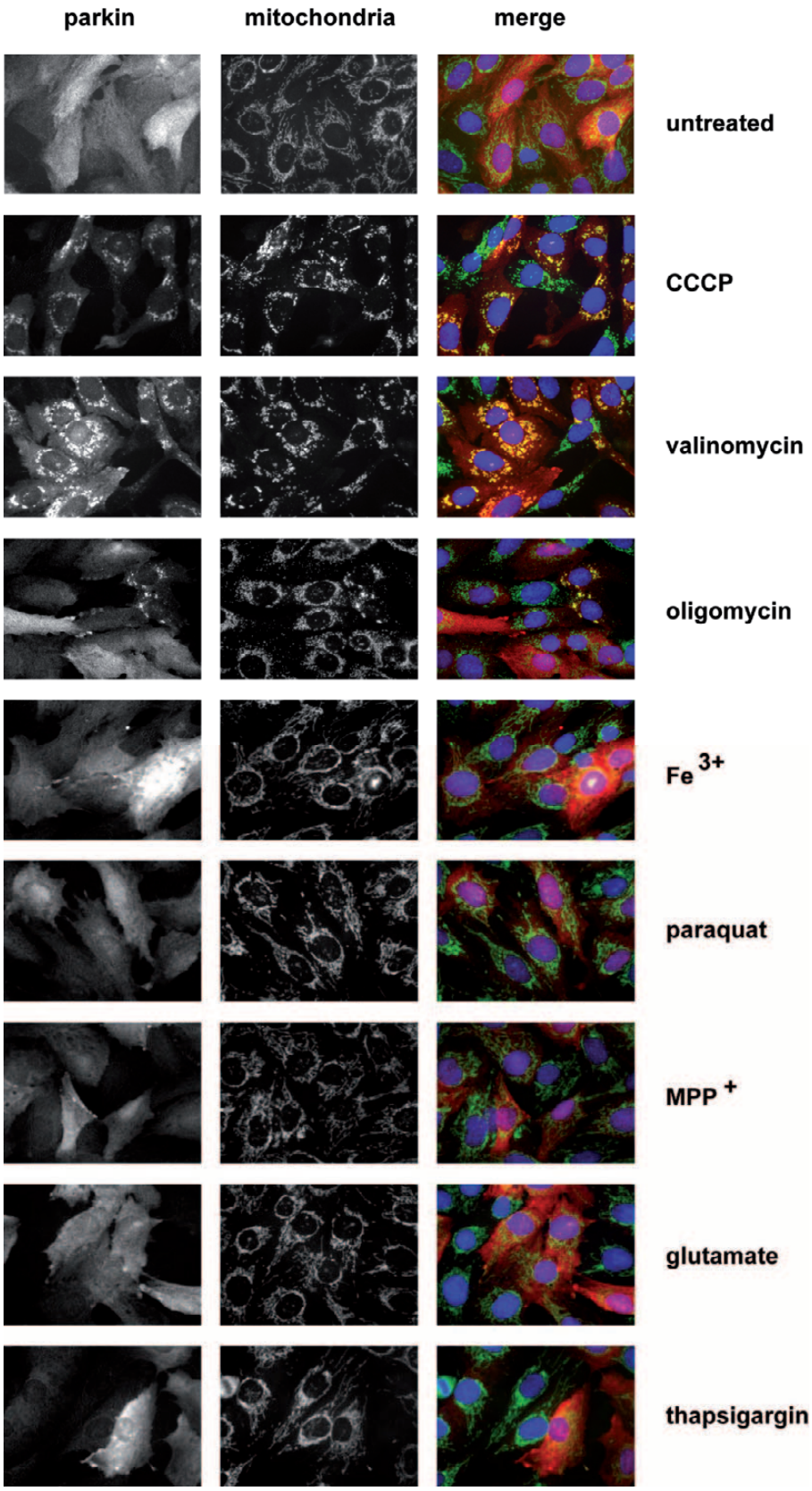


Figure 14: Uncoupling of mitochondrial membrane potential induces parkin translocation from the cytosol to mitochondria. SH-SY5Y neuroblastoma cells stably expressing wildtype parkin were treated with the indicated chemicals for 3 h. Cells were subsequently stained with the parkin-specific antibody PRK8 (red) and the anti-TOM20 antibody (green) to visualize mitochondria.

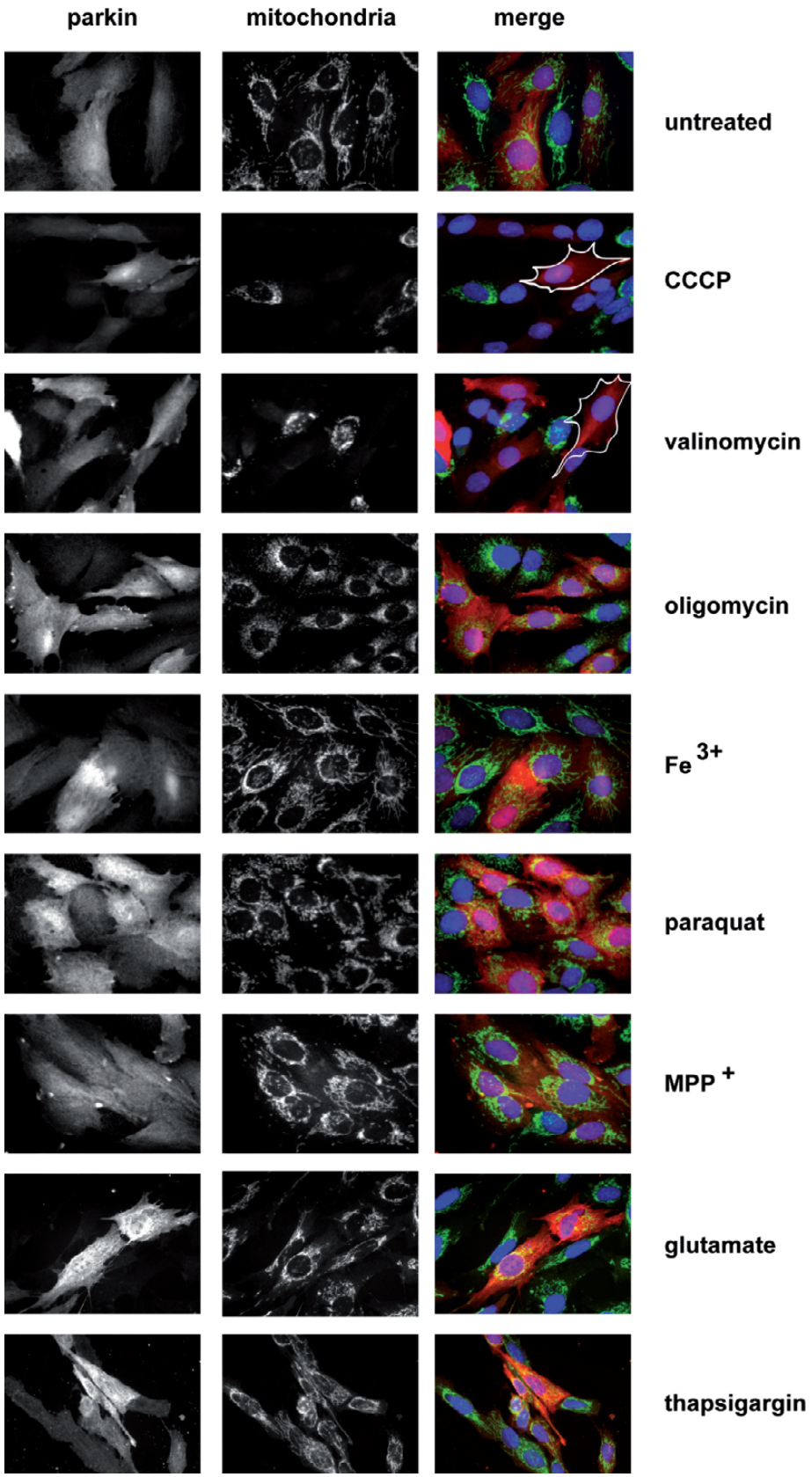


Figure 15: Uncoupling of mitochondrial membrane potential induces parkin-mediated mitophagy. SH-SY5Y cells stably expressing wildtype parkin were treated with the indicated chemicals for 24 h. Cells were stained with the parkin-specific antibody PRK8 (red) and the anti-TOM20 antibody (green) to visualize mitochondria. Representative single cells with no mitochondrial staining were surrounded by a white line to depict mitophagy.

3.1.4 Pathogenic parkin mutants are compromised in mitochondrial translocation or mitophagy completion

Several mutations in the parkin gene have been linked to autosomal recessive early-onset PD. The disease-causing mutations are distributed all over the parkin gene, but cluster in the functional domains of the protein. Different pathogenic parkin mutations have been identified, ranging from single point mutations to truncating mutations and large genomic rearrangements. Therefore it was important to analyze the effect of parkin mutations on its ability to translocate to mitochondria and initiate mitophagy. To this aim, pathogenic parkin mutants were transiently expressed in HeLa cells and parkin recruitment and mitophagy were assessed at 1 and 24 h of CCCP treatment. In contrast to wildtype parkin which is cytosolic under basal conditions, several parkin mutants showed a spotted, aggregate-like staining (Figure 16). In particular, the cysteine point mutants C212Y and C289G and the truncation mutants W453X and E409X displayed cytosolic aggregates that did not colocalize with mitochondria. In addition, these mutants lacked the ability to translocate to mitochondria when the mitochondrial membrane potential was uncoupled. Contrarily, the G340D pathogenic parkin mutant showed a cytosolic staining pattern analogous to wildtype parkin. However, when cells expressing the G430D parkin mutant were treated with CCCP, the protein did not redistribute to mitochondria. Even upon prolonged exposure to CCCP, G430D parkin remained cytosolic and did not translocate to the fragmented, dysfunctional mitochondria. The analysis of different point mutations located in the ubiquitin-like domain (UBL) of the parkin protein yielded similar results for all mutants analyzed: first, the mutant protein was soluble under basal conditions with no appearance of aggregates. Second, upon treatment of the cells with CCCP, all parkin UBL mutants displayed translocation to mitochondria, although to different extents. Upon treatment with CCCP for 24 h, mitophagy was initiated by means of all parkin UBL mutants (Figure 17). These results suggest that the parkin UBL domain might be dispensable for parkin mitochondrial recruitment and mitophagy. Furthermore, some parkin mutants might be inactivated through their propensity to misfold and aggregate formation (Figure 16).

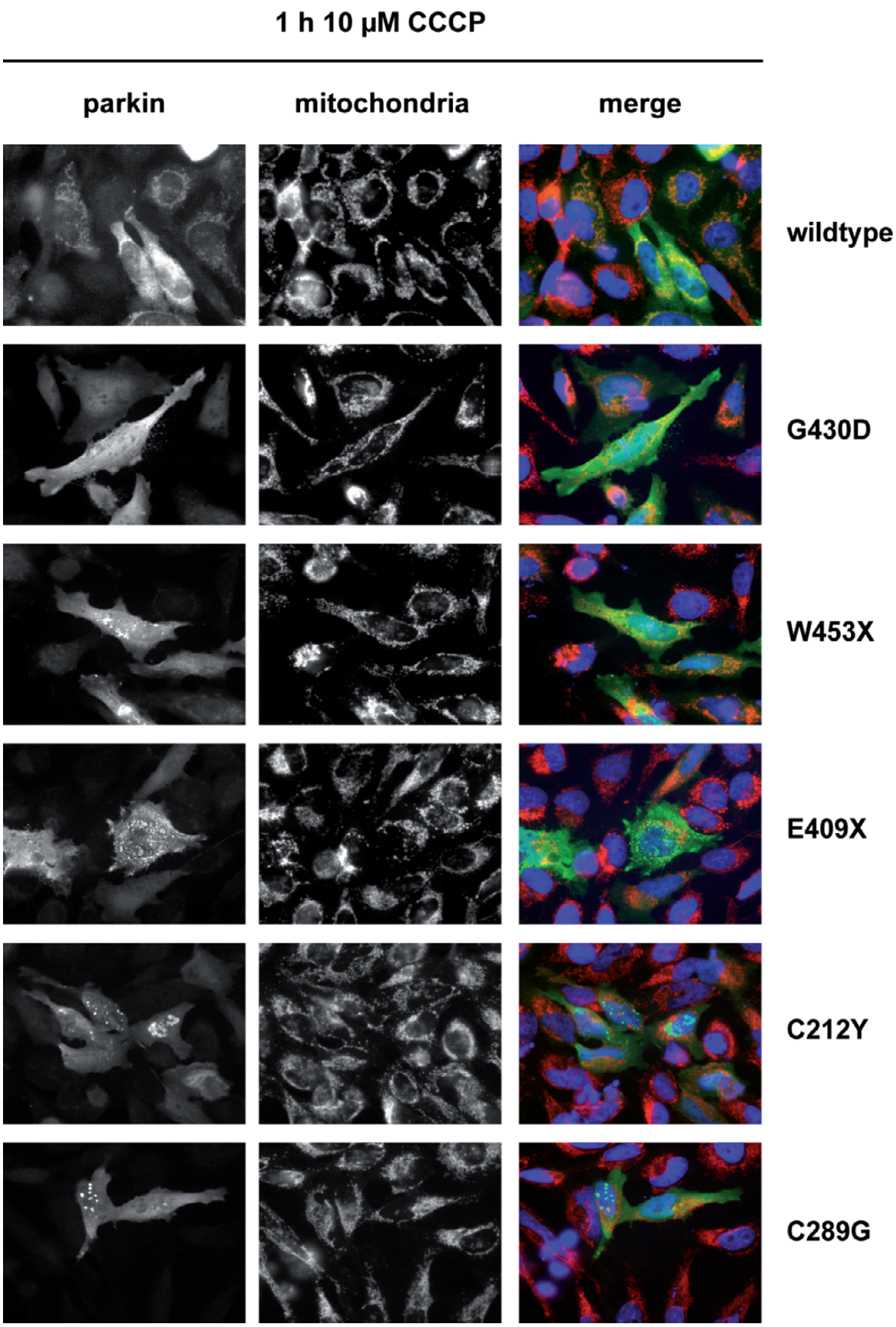


Figure 16: Pathogenic parkin mutants are compromised in mitochondrial translocation or mitophagy. HeLa cells were transiently transfected with the indicated parkin mutants and treated with 10 µM CCCP for 1 h. Parkin was visualized using the PRK8 antibody (green) and mitochondria with the anti-TOM20 antibody (red).

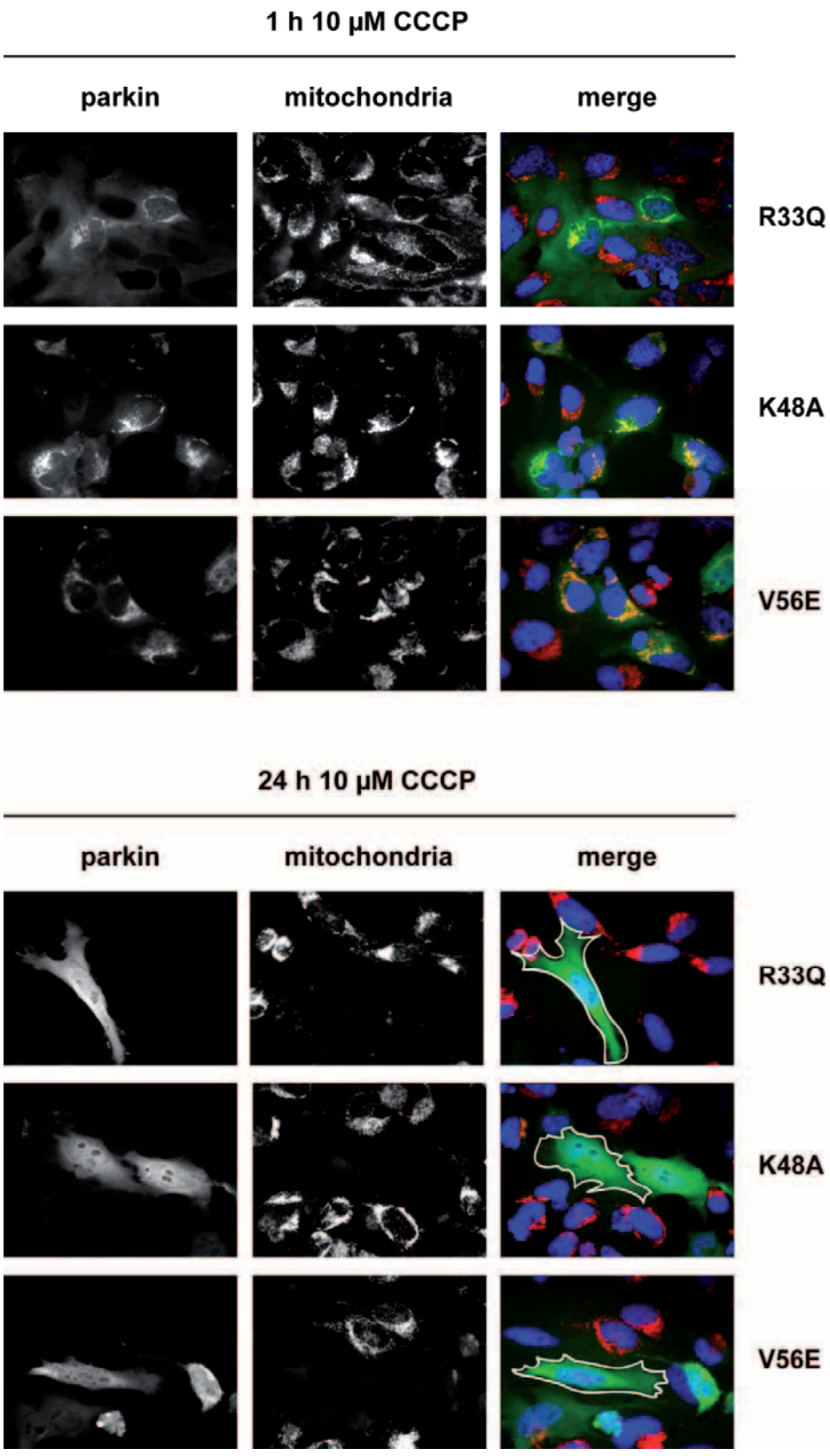


Figure 17: Pathogenic mutations in the UBL domain of parkin do not interfere with mitophagy. HeLa cells were transfected with the indicated parkin mutants (all located in the UBL domain of parkin (amino acids 1 to 76)) and treated with 10 µM of CCCP for 1 h. Cells were immunostained with the parkin-specific antibody PRK8 (green) and the mitochondria-specific TOM20 (red) antibody. A white line surrounds single cells devoid of mitochondrial staining.

3.1.5 Deletion of parkin functional domains results in parkin insolubility and loss of mitochondrial translocation and mitophagy

Parkin is an E3 ubiquitin ligase composed of an N-terminal UBL domain and a C-terminal RBR domain. The RBR domain consists of two RING domains separated by an IBR domain. To test which domains are required for parkin function in mitophagy, individual domain deletion mutants were transiently expressed in HeLa cells and mitochondrial recruitment of parkin and mitophagy were evaluated upon uncoupling of the mitochondrial membrane potential. In untreated cells, wildtype parkin was homogenously distributed throughout the cytosol (Figure 18). The ΔUBL mutant lacking the first 76 N-terminal amino acids also appeared cytosolic under basal conditions. In contrast, all other parkin domain deletion mutants showed a punctuate staining pattern in the cytosol, suggesting that proper parkin folding is impaired by the deletion of C-terminal domains. Next, CCCP was added for 1 h to assess the recruitment of the parkin mutants to uncoupled mitochondria. Wildtype parkin as well as the ΔUBL mutant colocalized with mitochondria, and upon 24 h of CCCP exposure, mitochondria were degraded under these conditions. Contrarily, deletion of any other domain impaired mitochondrial translocation of parkin and compromised mitophagy, resulting in no clearance of mitochondria via mitophagy. In concordance with the analysis of pathogenic parkin mutants (Figure 17), the UBL domain of parkin seems to be dispensable for mitophagy, while all other domains seem essential for parkin folding and function in mitochondrial removal.

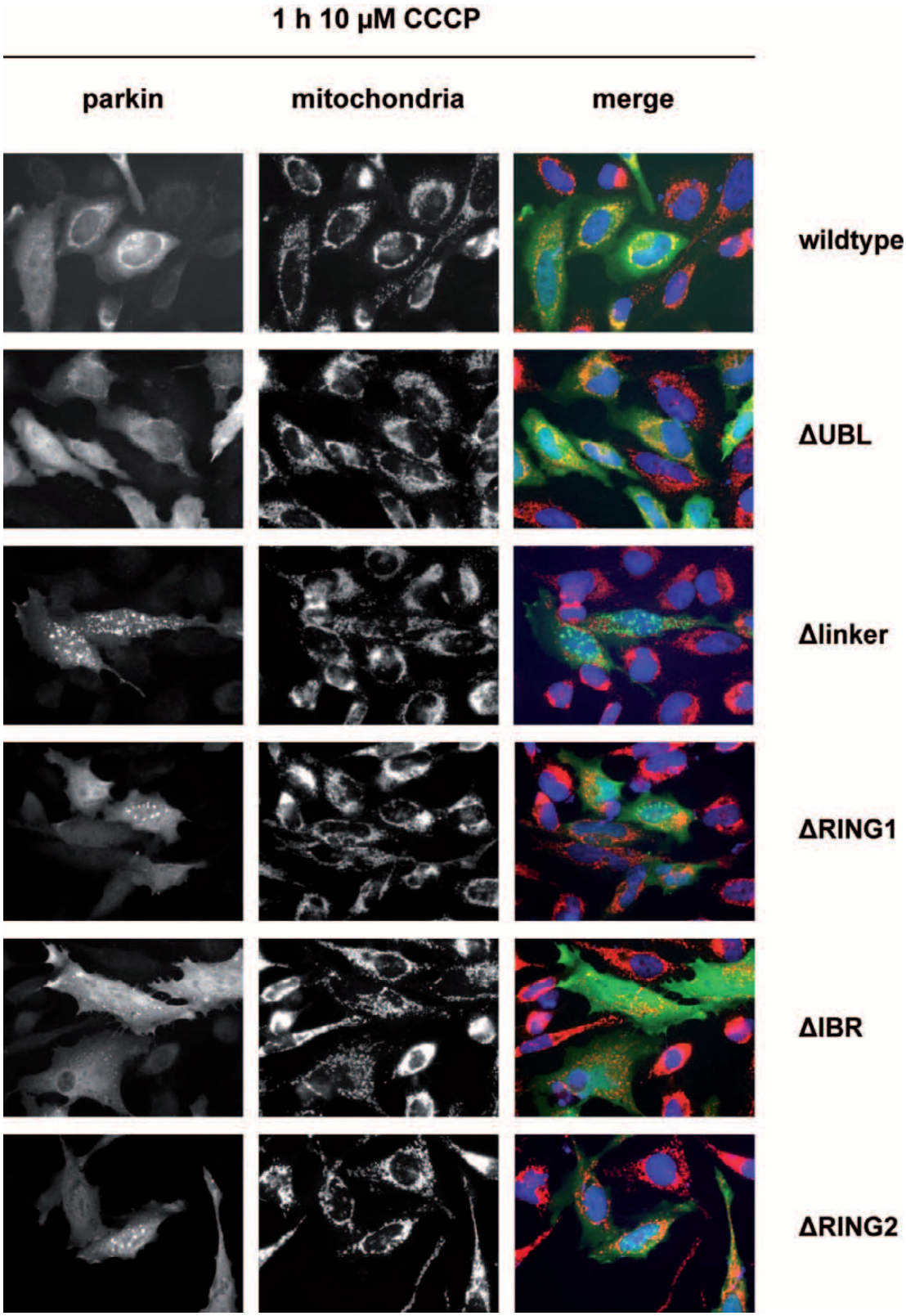


Figure 18: The UBL domain of parkin is dispensable for mitochondrial recruitment of parkin, all other domains are essential. HeLa cells were transiently transfected with the indicated parkin species: ΔUBL (deletion of amino acids 1-76), Δlinker (Δ77-237), ΔRING1 (Δ238-293), ΔIBR (Δ314-377), ΔRING2 (Δ418-449). CCCP was added for 1 h and cells were analyzed by indirect immunofluorescence for parkin subcellular distribution and colocalization of parkin and mitochondria. The PRK8 parkin-specific antibody (green) and an anti-TOM20 mitochondria-specific antibody (red) were used for immunofluorescence stainings.

3.1.6 Parkin-dependent ubiquitination of depolarized mitochondria

Ubiquitination serves as a signal for various cellular signaling pathways, including targeting of substrates for degradation via the proteasome or through selective autophagy. Parkin is an E3 ubiquitin ligase, mediating substrate specificity of ubiquitination. Thus, ubiquitination of mitochondrial proteins could trigger parkin-mediated mitophagy. To test this hypothesis, HeLa cells stably expressing wildtype parkin were treated with CCCP for 1 h and analyzed for colocalization of parkin and polyubiquitin or mitochondria and polyubiquitin. Polyubiquitin signal, as analyzed by an antibody recognizing chains that consist of three or more ubiquitin moieties (polyubiquitin-antibody clone FK1), revealed staining throughout the cells with highest intensity in the cytoplasm (Figure 19). Remarkably, general staining intensity was greatly enhanced when cells were treated with CCCP for 2 h. In addition, the polyubiquitin signal strongly colocalized with both depolarized mitochondria and parkin. However, an enhanced polyubiquitin signal was only observed in cells expressing parkin, suggesting that parkin is either directly or indirectly involved in the assembly of polyubiquitin chains at mitochondria.

A single ubiquitin moiety contains seven lysine residues that can be employed for the assembly of polyubiquitin chains. Dependent on which lysine residue is used to connect single moieties, different signaling pathways or responses can be triggered. In particular, linkage of lysine-48 (K48 ubiquitin) is a signal for proteasomal degradation, while linkage via lysine-63 (K63 ubiquitin) is involved in selective autophagy. Specific antibodies have been developed that are able to distinguish K48- and K63-linked polyubiquitin. In order to address the questions which polyubiquitin linkage is found in the parkin-mediated mitophagy pathway, these antibodies were used in indirect immunofluorescence. Interestingly, both K48- and K63-linked polyubiquitin chains were found to colocalize with mitochondria when membrane potential was dissipated (Figure 20). The analysis of parkin revealed that both K48- and K63-specific ubiquitin signal was observed to colocalize with parkin and increased intensity of both markers was determined when mitochondrial membrane potential was lost. Interestingly, polyubiquitin staining was increased only in cells expressing parkin, but remained at baseline in the absence of parkin (Figure 20 and data not shown). Consequently, the results presented above point towards a role of parkin in the ubiquitination of mitochondrial substrate/s, either directly or via a so far unknown indirect pathway.

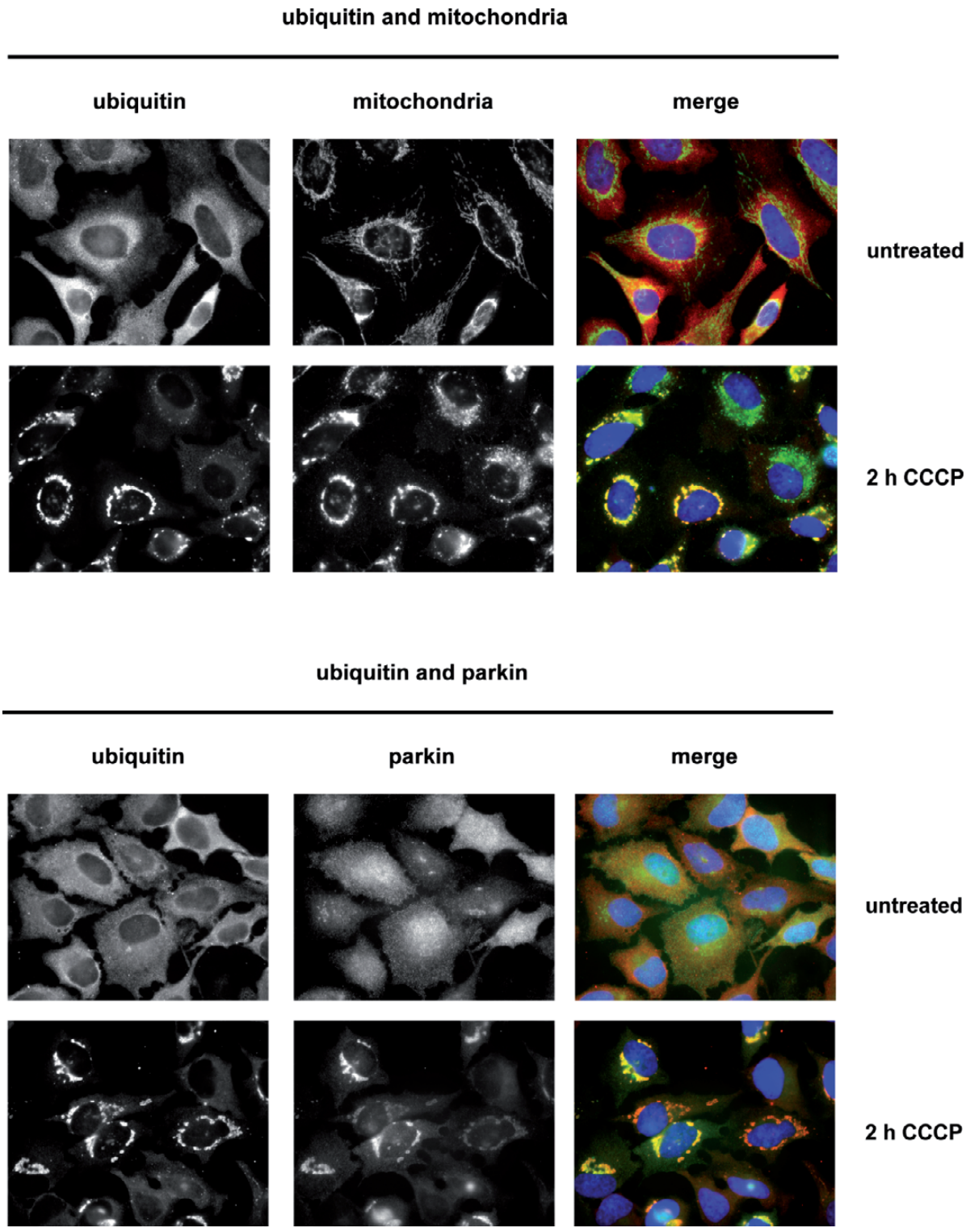
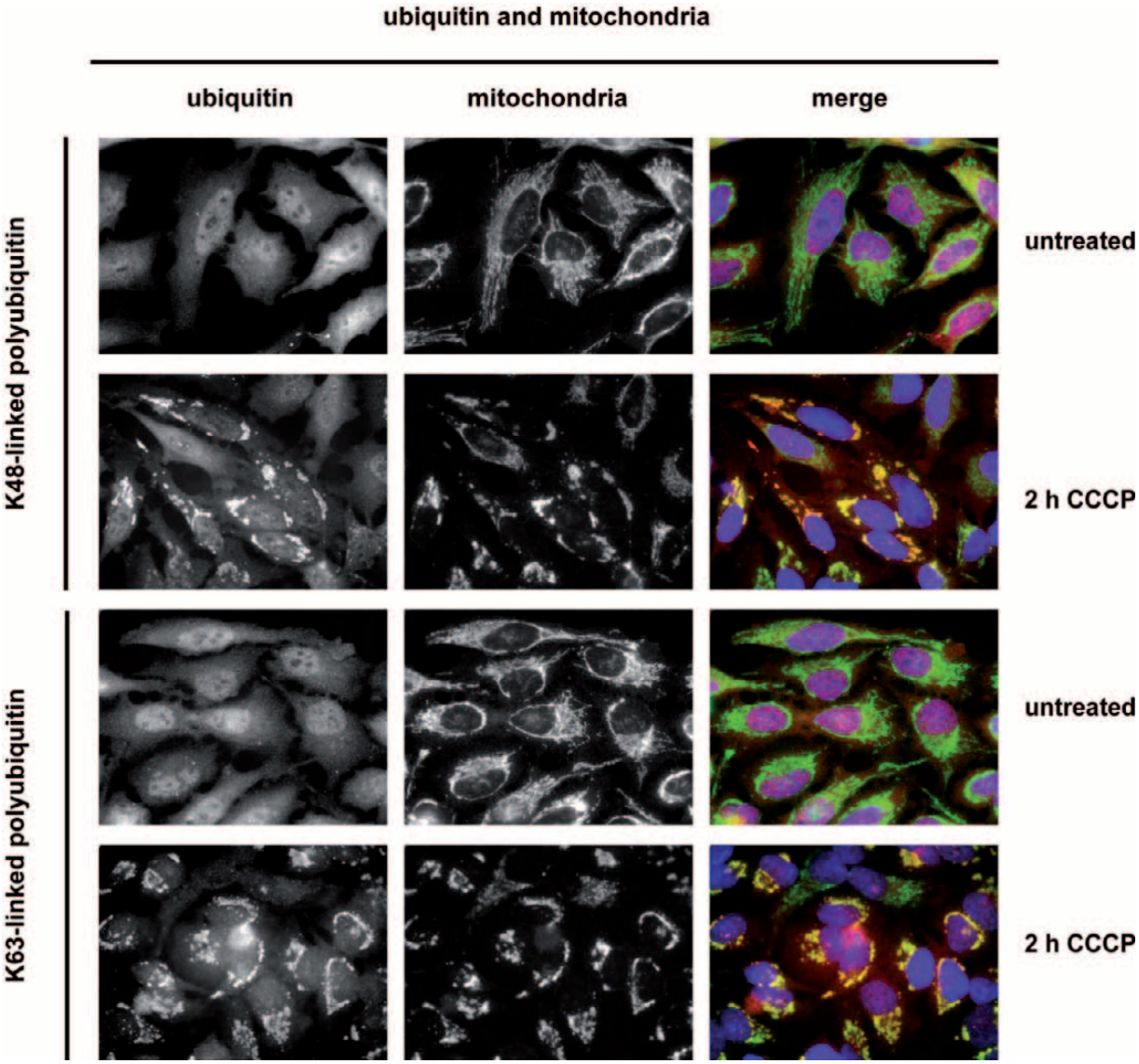


Figure 19: Increased polyubiquitin reactivity at mitochondria in the presence of parkin upon uncoupling of mitochondrial membrane potential. HeLa cells stably expressing wildtype parkin were treated with CCCP for 2 h. Upper panel: Immunofluorescence staining of total polyubiquitin (clone FK1; red) and mitochondria (TOM20; green). Note that polyubiquitin signal at mitochondria is enhanced upon CCCP treatment. Lower panel: Immunofluorescence staining of total polyubiquitin (clone FK1; red) and parkin (PRK8; green). Notably, ubiquitin staining is selectively enhanced in cells expressing parkin (see lower right picture).


ubiquitin and mitochondria
ubiquitin
mitochondria
merge
K48-linked polyubiquitin
untreated
2 h CCCP
K63-linked polyubiquitin
untreated
2 h CCCP

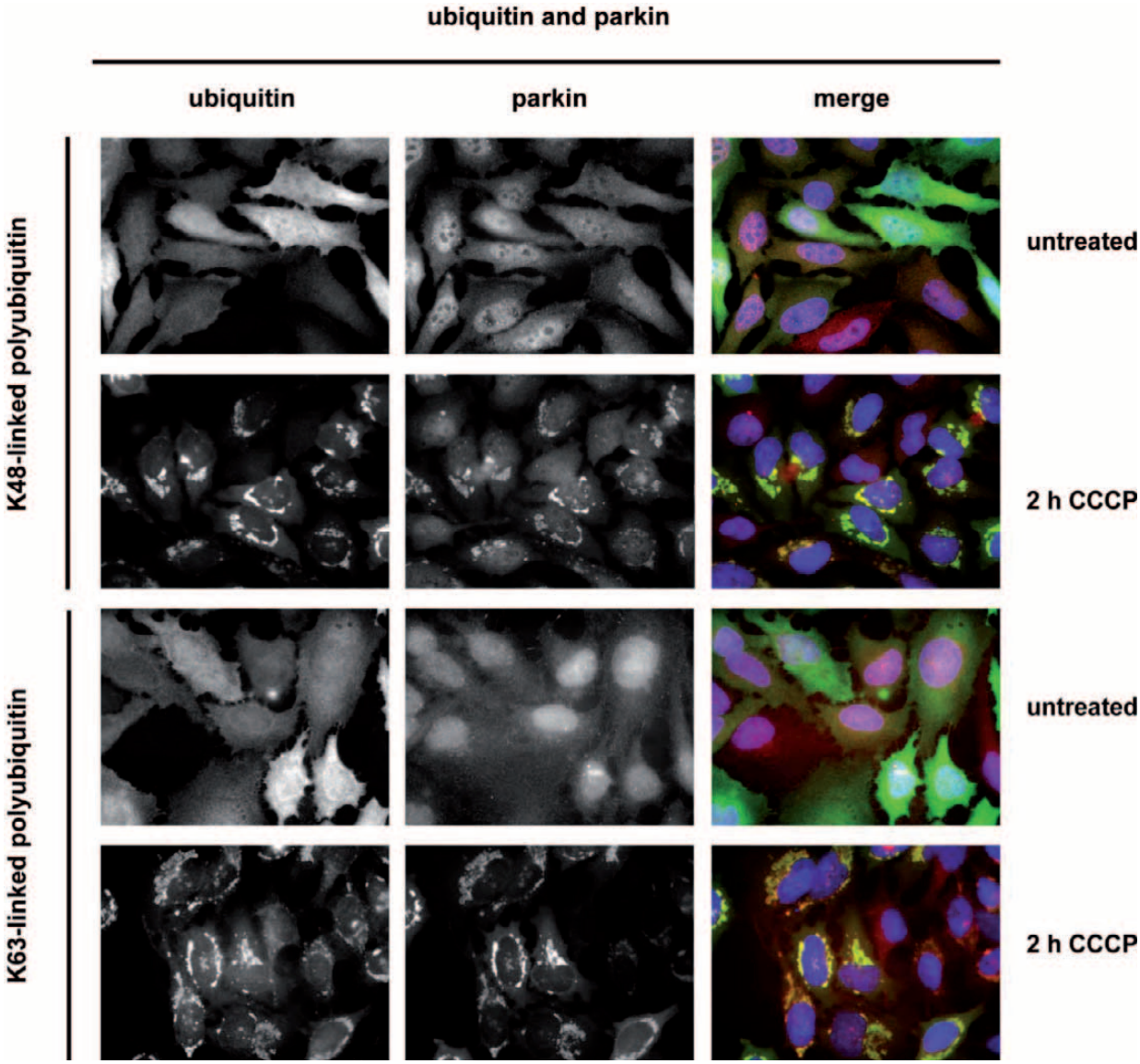


Figure 20: K48- and K63-linked polyubiquitin staining at mitochondria upon loss of mitochondrial membrane potential. HeLa cells stably expressing wildtype parkin were treated with CCCP for 2 h. Upper panel (previous page): Immunofluorescence staining of polyubiquitin (K48-linked ubiquitin (Apu2; red; rows 1 and 2) and K63-linked ubiquitin (Apu3; green; rows 3 and 4)) and mitochondria (TOM20; green). Note that polyubiquitin signal at mitochondria is enhanced upon CCCP treatment. Lower panel: Immunofluorescence staining polyubiquitin (K48-linked ubiquitin (Apu2; green; rows 1 and 2) and K63-linked ubiquitin (Apu3; green; rows 3 and 4)) and parkin (PRK8; red). Notably, ubiquitin staining is selectively enhanced in cells expressing parkin and colocalizes with parkin.

3.1.7 Both autophagy and proteasomal degradation are essential for mitophagy

The previous results pointed out, that both K48- and K63-linked polyubiquitin chains are involved in labeling dysfunctional mitochondria for degradation via parkin-dependent mitophagy. As K48-linked polyubiquitin is a *bona fide* signal for proteasomal degradation, but not autophagy, it is important to investigate the contribution of the UPS and the autophagy pathway on mitophagy. In a first approach, embryonic fibroblasts from mice lacking ATG5

(ATG5 KO MEF) and corresponding wildtype mice were chosen to see if functional autophagy is required for parkin-mediated mitochondrial removal. ATG5 is a core component of the autophagic pathway and essential for autophagosome formation. Fibroblasts from ATG5 KO mice lost their ability to implement autophagy. To see if mitophagy was impaired in these cells, ATG5 KO MEFs and corresponding wildtype fibroblasts were stably transduced with wildtype parkin by lentiviral transduction. Cells were treated with 10 µM of CCCP for 1 h to investigate parkin recruitment to mitochondria and for 24 h to quantify mitophagy. Following 1 h of mitochondrial uncoupling, parkin robustly redistributed from the cytosol to mitochondria both in wildtype and ATG5-deficient cells (Figure 21). Similar results were obtained when HSP60, a mitochondrial matrix protein, was used to stain mitochondria instead of TOM20. However, when cells were analyzed for mitophagy after 24 h of CCCP treatment, the results varied depending on which mitochondrial marker protein was used. In wildtype control MEFs expressing parkin, mitochondrial staining was lost in about half of the cell population, with no significant difference between the different mitochondrial stainings. In contrast, while mitophagy appeared to be induced when TOM20 was used to stain mitochondria, no mitophagy was observed when HSP60 was utilized. TOM20 is an outer mitochondrial membrane protein, thus it is possible that outer mitochondrial membrane proteins are degraded independently of autophagy, explaining the contradicting results obtained with the different mitochondrial markers.

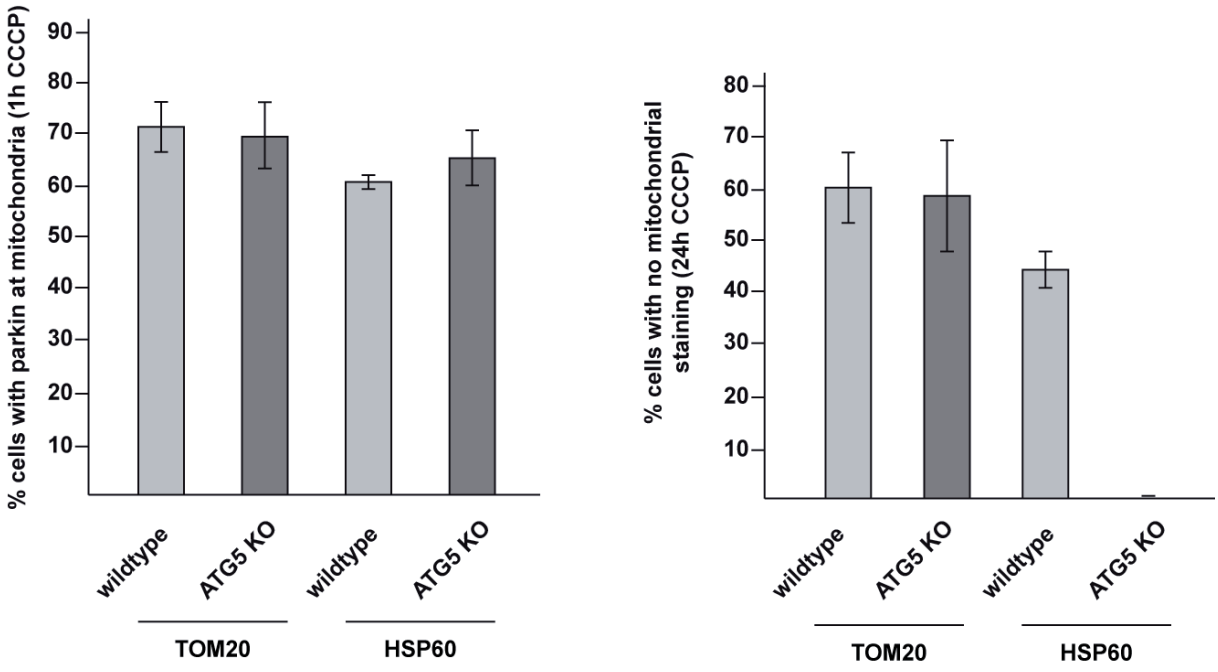


Figure 21: Mitophagy, but not mitochondrial recruitment of parkin is compromised in autophagy-deficient cells. Wildtype and ATG5 KO MEF cells were stably transduced to express human wildtype parkin. Cells were treated with 10 µM of CCCP for 1 h (left graph) or 24 h (right graph), respectively. Cells were analyzed for colocalization of parkin with mitochondria in immunofluorescence using the mitochondrial marker proteins TOM20 or HSP60. At 24 h of CCCP treatment, cells devoid of mitochondria were counted and the percentage of cells with no mitochondrial staining was quantified.

In order to further investigate the role of proteasomal degradation on mitophagy, chemical inhibitors were used to block proteasomal or lysosomal function: epoxomycin is a selective and covalent inhibitor of the proteasome; bafilomycin A1 is a specific inhibitor of vacuolar-type H^+-ATPase, thereby interfering with lysosomal acidification. Both inhibitors were applied to HeLa cells stably expressing wildtype parkin, either separately or in combination with CCCP for 1 h and 24 h, respectively. Upon dissipation of mitochondrial membrane potential, parkin was recruited to dysfunctional mitochondria within 1 h. Parkin redistribution was not altered when proteasomal or lysosomal function were blocked (data not shown). In addition, although bafilomycin A1 treatment resulted in a slight fragmentation of mitochondria, it did not induce parkin translocation to mitochondria. Epoxomycin treatment did not affect mitochondrial morphology, however cell morphology changed and cells elongated upon addition of the proteasomal inhibitor. Following treatment with the inhibitors for 24 h, parkin-dependent mitophagy was assessed (Figure 22). The percentage of cells with no mitochondrial staining was determined in three independent experiments (Figure 23). In the absence of CCCP, all cells retained their mitochondrial staining. In line with the previous results, blockage of lysosomal function with bafilomycin A1 resulted in an almost complete loss of mitophagic activity in the presence of CCCP. Interestingly, inhibition of the proteasome with epoxomycin during CCCP treatment also significantly decreased the percentage of cells with no mitochondrial staining. In summary, both intact lysosomal and proteasomal function appear to be crucial to complete parkin-dependent mitochondrial degradation.

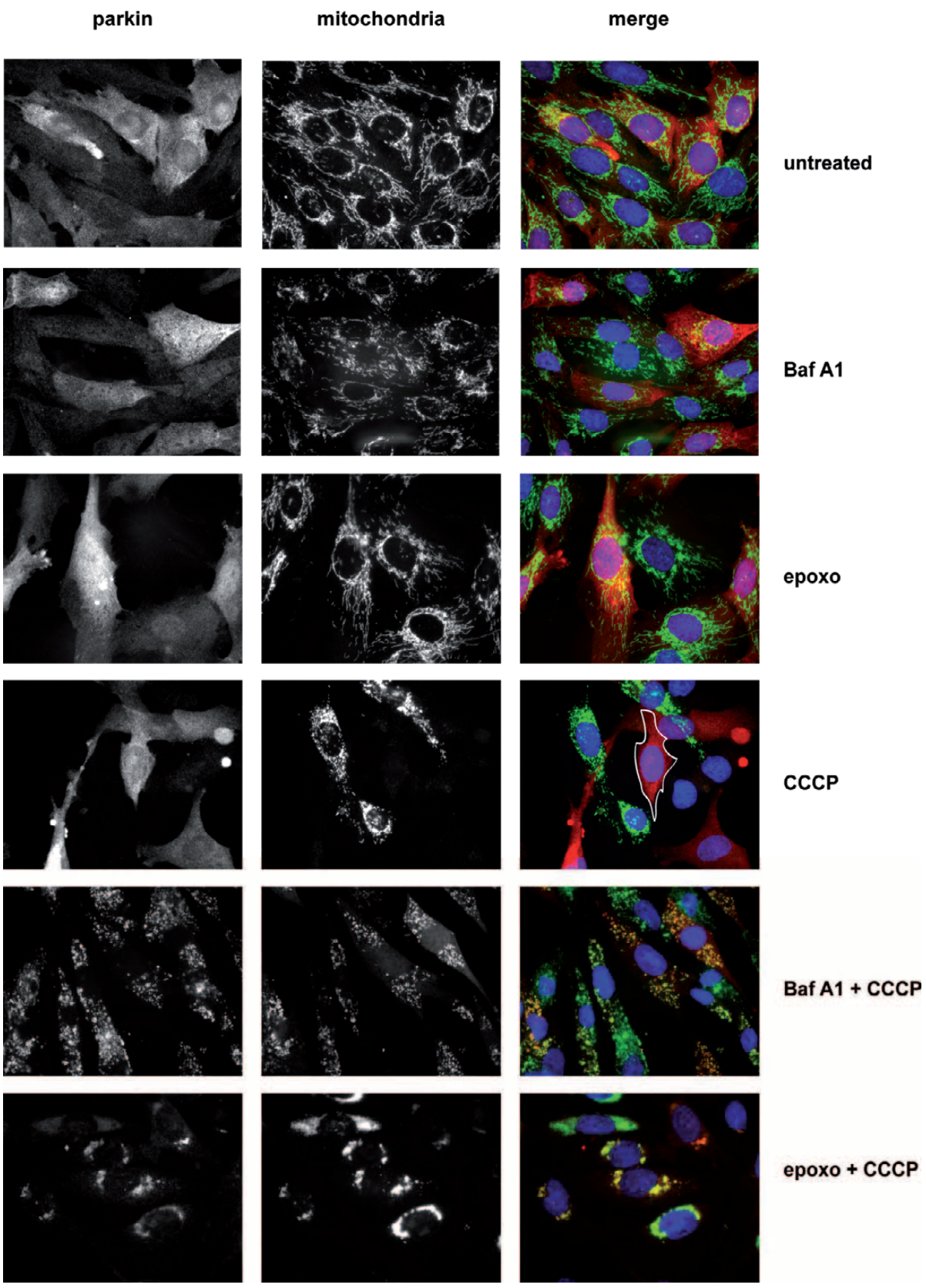


Figure 22: Mitophagy is compromised by both lysosomal and proteasomal inhibition. HeLa cells stably expressing wildtype parkin were treated with bafilomycin A1 (bafA1, 30 nM), epoxomycin (epoxo, 10 nM) or CCCP (10 µM) alone, or bafilomycin A1 and epoxomycin were applied in combination with CCCP. After 24 h of treatment with the indicated chemicals, cells were fixed and stained for parkin (PRK8; red) and mitochondria (TOM20; green). Note that bafilomycin A1 treatment results in a fragmentation of mitochondria but does not induce mitophagy.

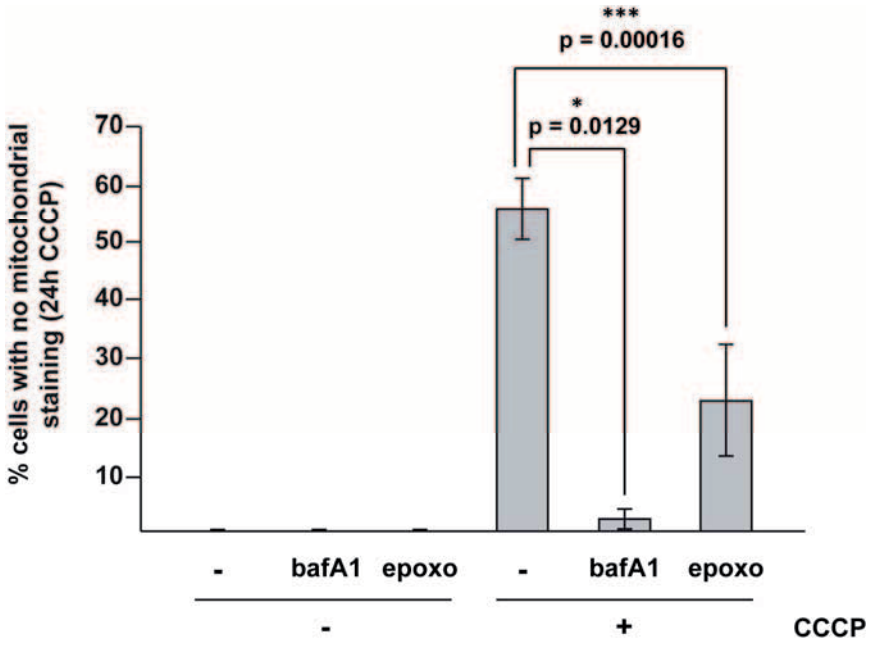


Figure 23: Inhibition of proteasomal and lysosomal function impairs mitophagy. HeLa cells stably expressing human wildtype parkin were simultaneously treated for 24 h with the indicated inhibitors (CCCP: 10 µM CCCP, epoxo: 10 nM epoxomycin, bafA1: 30 nM bafilomycin A1). Cells were stained with parkin-specific antibody PRK8 and TOM20 antibody to stain mitochondria. The number of cells with no mitochondria was counted and the percentage of mitophagy quantified.

3.1.8 PINK1 is essential for parkin-mediated mitophagy

Experimental evidence from genetic linkage analysis in *Drosophila melanogaster* suggests that the PD-associated protein PINK1 functions in a common pathway with parkin, with PINK1 acting upstream of parkin (Clark et al., 2006, Park et al., 2006, Yang et al., 2006a). Furthermore, *in vitro* studies even suggest that parkin and PINK1 might interact directly (Kim et al., 2008, Shiba et al., 2009, Um et al., 2009, Moore, 2006). In order to analyze the importance of PINK1 on parkin-mediated mitophagy, fibroblasts from mice lacking PINK1 were used. Both PINK1 KO and corresponding wildtype MEFs were transduced with a lentivirus for parkin overexpression. Of note, cells deficient for PINK1 displayed an altered mitochondrial morphology, with shortened and fragmented mitochondria. Parkin overexpression by lentiviral transduction did not alter mitochondrial morphology in these cells, and parkin was not recruited to mitochondria in the absence of mitochondrial uncoupling. To assess parkin recruitment and mitophagy in these cells, an immunostaining for parkin and the mitochondrial marker TOM20 was conducted after 1 h and 24 h of CCCP treatment. As PINK1 is a serine-threonine kinase with a mitochondrial targeting sequence (MTS), PINK1 might be involved in the recruitment of parkin to mitochondria. Indeed, while parkin readily translocated to mitochondria in wildtype fibroblasts, the protein remained cytosolic and was not recruited to mitochondria (data not shown). Even more remarkably, at 24 h of CCCP treatment, parkin was still completely cytosolic in fibroblasts lacking PINK1

and accordingly mitochondria were not degraded (Figure 24). In contrast, mitochondria were sequestered in wildtype MEFs in more than 50 % of cells expressing parkin (Figure 24 and Figure 25). The results implicate that PINK1 is essential for targeting parkin to dysfunctional mitochondria.

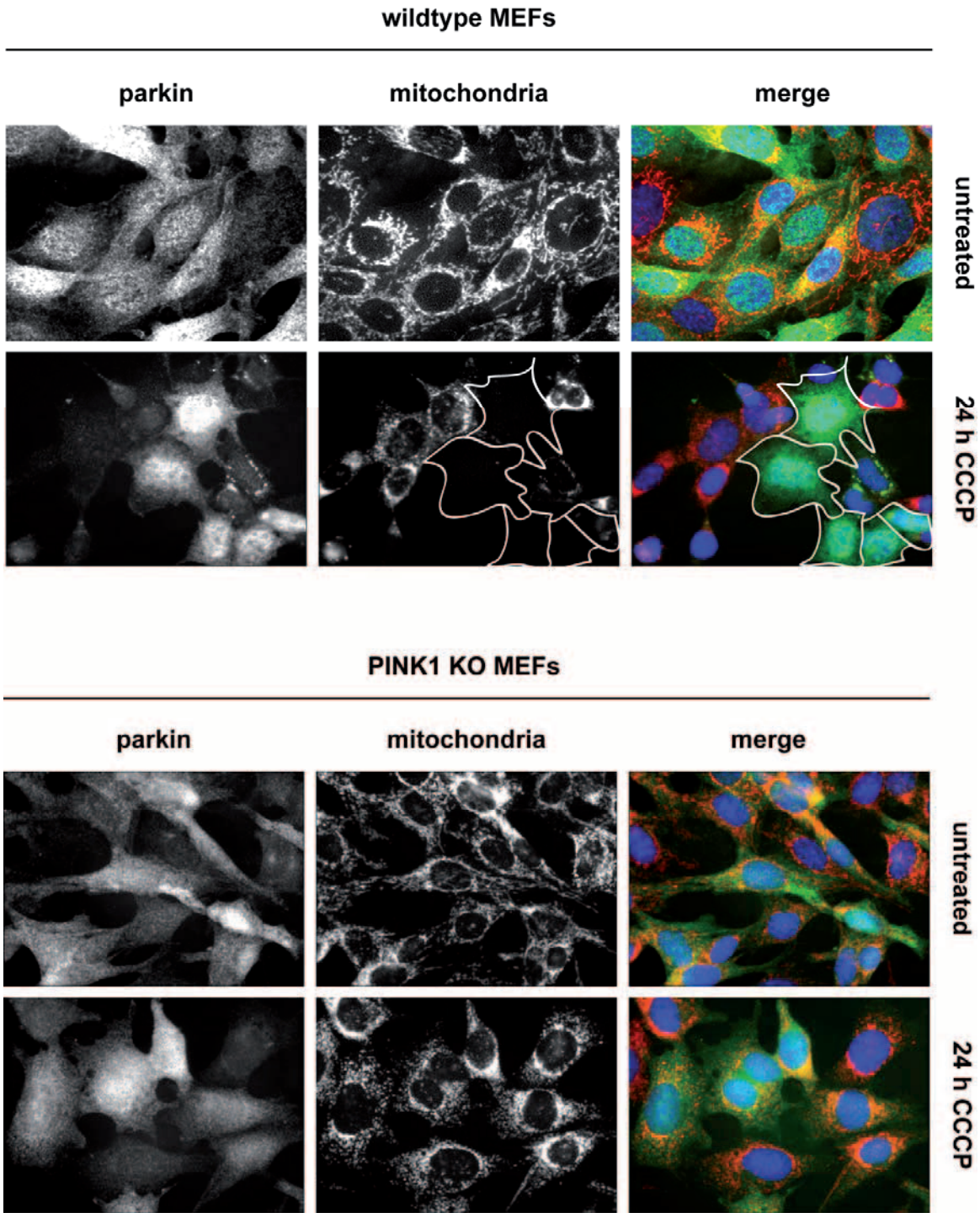


Figure 24: PINK1 is essential for parkin-mediated mitophagy. Wildtype and PINK1 KO MEFs stably overexpressing human wildtype parkin were treated with 10 µM CCCP for 24 h. Parkin (PRK8; green) and mitochondria (TOM20 polyclonal antibody; red) were stained. Exemplified cells with no mitochondrial staining are

surrounded by a white line for better visualization. Picture published in Pilsl & Winklhofer, Acta Neuropathol., 2011 (Pilsl and Winklhofer, 2011).

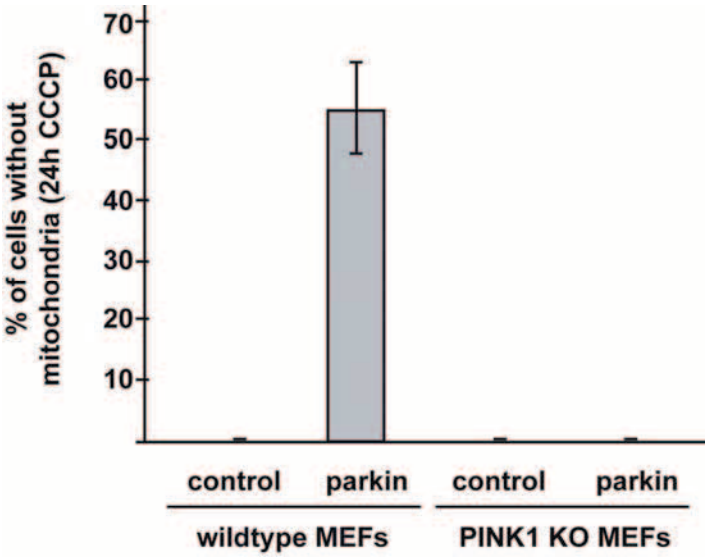


Figure 25: PINK1 is essential for parkin-dependent degradation of dysfunctional mitochondria. Wildtype and PINK1 KO mouse fibroblasts stably expressing human wildtype parkin or untransduced cells (control) were treated with 10 µM CCCP for 24 h. Cells were immunostained for parkin (PRK8) and mitochondria (TOM20) and the percentage of cells with no mitochondrial staining was quantified. Results were obtained from three independent experiments with at least 500 cells counted per condition.

3.1.9 Overexpression of parkin and PINK1 is sufficient to induce mitophagy in the absence of mitochondrial depolarization

Analysis of mitophagy in PINK1-deficient cells pointed towards an essential role of PINK1 in parkin-dependent mitophagy. Previous studies from other groups suggest that parkin and PINK1 might directly interact with each other. It is possible that the mitochondrial protein PINK1 can target parkin to dysfunctional mitochondria to initiate their degradation. To further investigate the role of PINK1 in mitophagy, HeLa cells were transiently transfected with PINK1-V5 and pcDNA3.1 empty vector as a control or together with parkin. Cells were allowed to grow for 24 h after transfection, then an immunostaining for PINK1 (detected via the C-terminal V5 tag) or parkin (PRK8) and mitochondria (TOM20) was done. Overexpression of PINK1 alone resulted in part of the protein in the cytosol and another part colocalizing with mitochondria (Figure 26). This is in line with other studies demonstrating that PINK1 is partially imported into mitochondria, but can also be found in the cytosol (Weihofen et al., 2008, Takatori et al., 2008, Beilina et al., 2005, Haque et al., 2008, Lin and Kang, 2008, Lin and Kang, 2010). Mitochondrial morphology was not affected by PINK1 overexpression, retaining a tubular network. Surprisingly, PINK1 and parkin coexpression frequently resulted in the formation of large mitochondrial clusters that colocalized with both parkin and PINK1 (data not shown). In addition, a subset of cells displayed no mitochondrial staining upon coexpression of parkin and PINK1, suggesting that both proteins can initiate

mitochondrial degradation even in the absence of mitochondrial uncoupling (Figure 26). Notably, when cells overexpressing PINK1 alone were treated with CCCP, mitochondria fragmented but were not degraded, pointing out that PINK1 itself is not sufficient to induce mitophagy in the absence of parkin. To determine if mitochondrial targeting of PINK1 is crucial for parkin-induced mitophagy, a PINK1 mutant with a deletion of the first 110 N-terminal amino acids (PINK1Δ110) was analyzed (provided by Kathrin Lutz). This mutant lacks the N-terminal mitochondrial targeting sequence (MTS) and the transmembrane domain (TM). Indeed, the mutant was expressed and displayed a cytosolic staining pattern with no colocalization of the PINK1 mutant and mitochondria (Figure 26). When wildtype parkin was coexpressed with PINK1Δ110, it remained cytosolic and was not recruited to mitochondria. Furthermore, simultaneous expression of parkin and PINK1Δ110 was not able to induce the degradation of mitochondria via autophagy. In summary, PINK1 is responsible for parkin recruitment to mitochondria and mitochondrial targeting of PINK1 is crucial for mitophagy. Overexpression of PINK1 and parkin is able to induce mitophagy, even when the mitochondrial membrane potential is intact.

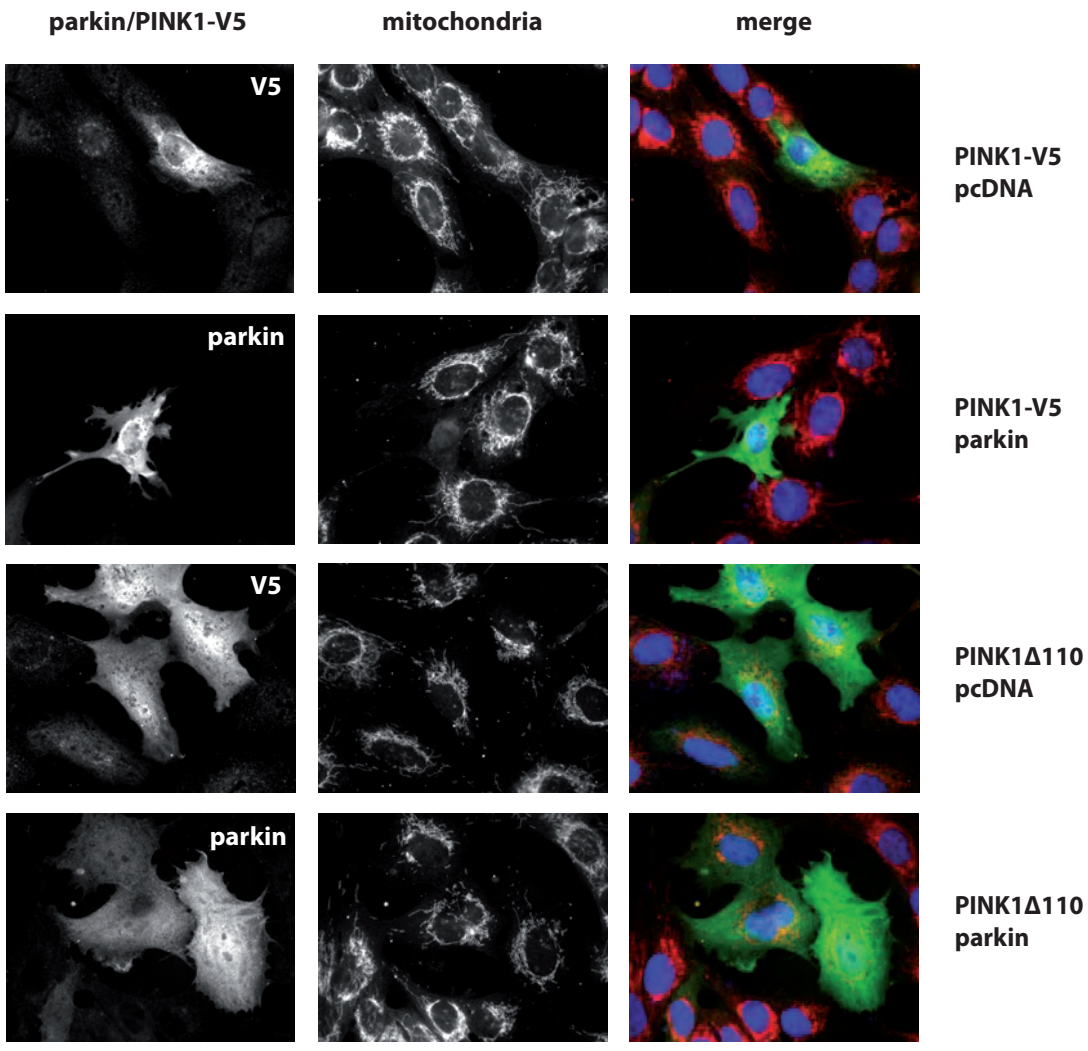


Figure 26: PINK1 expression is sufficient to induce parkin-mediated mitophagy. HeLa cells were transiently transfected with 0.5 µg of wildtype PINK1 or a mutant lacking 110 N-terminal amino acids (PINK1Δ110); either 0.5

µg of pcDNA empty vector control or parkin was cotransfected. Cells were stained with TOM20 to visualize mitochondria (red) and either parkin (PRK8; green; rows 2 and 4) or PINK1 (using a V5 monoclonal epitope antibody; green; rows 1 and 3).

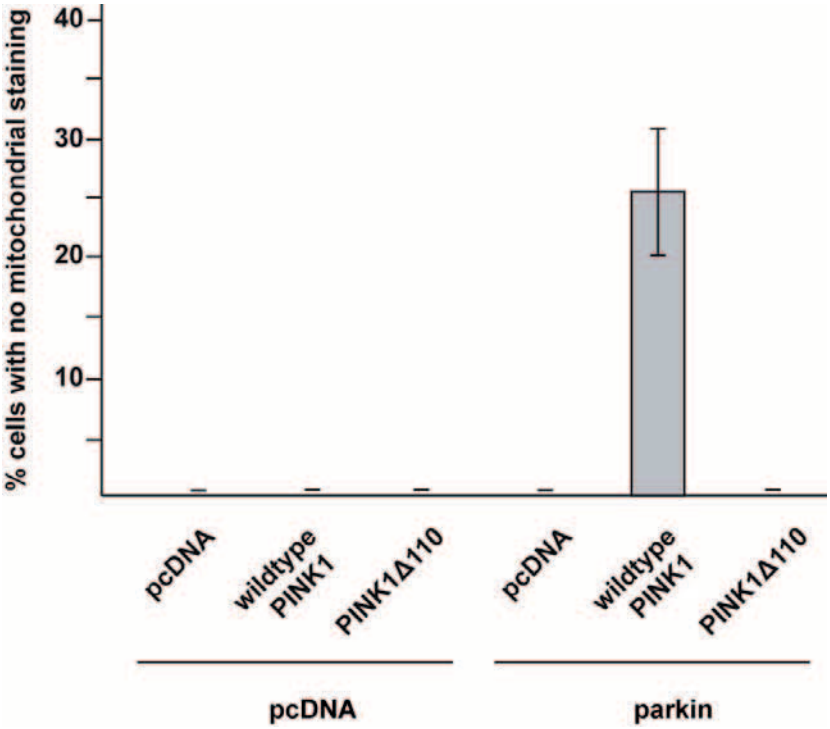


Figure 27: Coexpression of parkin and PINK1 is sufficient to induce mitophagy. HeLa cells were transiently transfected with the indicated vectors (0.5 µg each). After 24 h, the cells were fixed and analyzed by indirect immunofluorescence using the TOM20 antibody to visualize mitochondria. Transfected cells were analyzed for the presence of mitochondrial staining and the percentage of cells devoid of mitochondria was calculated. The bar graphs represent the results from three independent experiments with a minimum of 300 cells counted for each condition.

3.1.10 Overexpression of PINK1 induces insolubility of parkin

Previous analysis of the impact of PINK1 on parkin-mediated mitophagy revealed that coexpression of parkin and mitochondrially localized PINK1 is sufficient to induce mitophagy. However, the mechanism by which PINK1 influences parkin function is not clear. In cells where mitochondria were cleared from the cell by concomitant expression of parkin and PINK1, parkin appeared to be clustered rather than homogenously distributed throughout the cytosol (cf. Figure 26). Notably, parkin is an aggregation-prone protein and is rendered insoluble following a variety of cellular insults (see section 1.5). Parkin distribution upon coexpression of PINK1 reminded of aggregated parkin, thus the effect of PINK1 on parkin solubility was analyzed in a detergent-solubility assay. A mixture of the nonionic detergent Triton X-100 and the ionic detergent sodium desoxycholate (both 0.5 % (v/v) in PBS) was exploited for the analysis of parkin solubility. To experimentally address this, human embryonic kidney (HEK) 293T cells were used. HEK cells are particularly suited for the detergent-solubility assay as they are easily transfectable by liposome-mediated transfection reagents and show high transfection efficiencies. When parkin was expressed in HEK cells,

the protein was almost completely detected in the detergent-soluble (S; soluble) fraction (Figure 28). Similar results were obtained when the PD-associated protein DJ-1 was coexpressed: parkin was soluble in the presence of DJ-1. DJ-1 was chosen as a control as it displays a comparable subcellular distribution as PINK1 under certain cellular conditions, contains a C-terminal V5-tag and is expressed from the pcDNA6 vector, exactly the same as for PINK1. Surprisingly, the solubility of parkin dramatically changed in the presence of overexpressed wildtype PINK1. Under these conditions, parkin protein shifted to the detergent-insoluble fraction (P; pellet). In addition, PINK1 expression stabilized parkin protein levels; total protein levels of parkin were elevated in the presence of PINK1. Remarkably, treatment of cells expressing parkin with CCCP for 6 h also induced a partial shift of parkin to the detergent-insoluble fraction (Figure 27). Therefore, a conformational shift of parkin, reflected in altered detergent-solubility of the protein, could be a possible trigger for subsequent mitophagy.

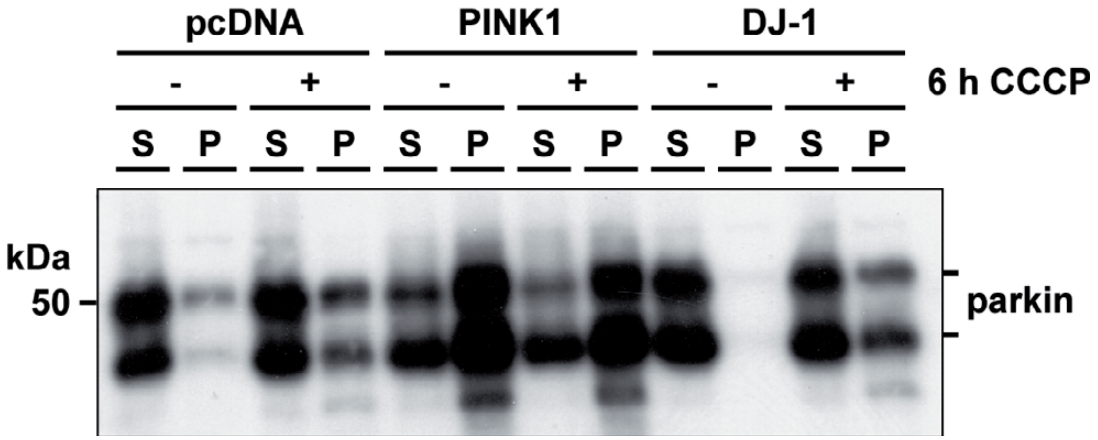


Figure 28: Overexpression of PINK1 results in parkin insolubility. HEK cells were transiently transfected with 0.5 µg of wildtype parkin and 0.5 µg of the indicated plasmids (pcDNA: empty vector control; PINK1: pcDNA6-PINK1-V5; DJ-1: pcDNA6-DJ-1-V5). Cells were either left untreated or 10 µM CCCP was added for 6 h. The cells were subjected to detergent-solubility assay (0.5 % Triton X-100 and 0.5 % desoxycholic acid in PBS) and equal amounts of detergent-soluble (S) and –insoluble fraction (P) were analyzed via Western blotting for parkin (PRK8 antibody).

3.1.11 Mitochondrial depolarization induces insolubility of parkin

The previous results showed that PINK1 is able to induce biochemical alterations of the parkin protein. Overexpression of PINK1 resulted in a shift of parkin from the detergent-soluble to the insoluble fraction (see Figure 28). Furthermore, the experiment provided evidence that CCCP treatment does have an effect on parkin solubility. To confirm these results, the solubility of parkin was assessed at different time points of CCCP treatment. Equal amounts of detergent-soluble and –insoluble fraction (0.5 % (v/v) Triton X-100 and sodium desoxycholate in PBS) were analyzed via Western blotting for parkin. Depending on the duration of CCCP treatment, parkin gradually shifted from the soluble into the insoluble subcellular fraction (Figure 29). After 16 h of CCCP application, parkin was

rendered completely insoluble, and this effect persisted to 24 h of mitochondrial uncoupling. Of note, at this time point, total levels of parkin protein were decreased. This is in accordance with investigations of parkin-dependent mitophagy in immunofluorescence, where parkin is often found to be degraded concomitantly with mitochondria. Thus, under conditions where CCCP is able to induce parkin translocation to mitochondria and mitophagy, a conformational shift of parkin is observed.

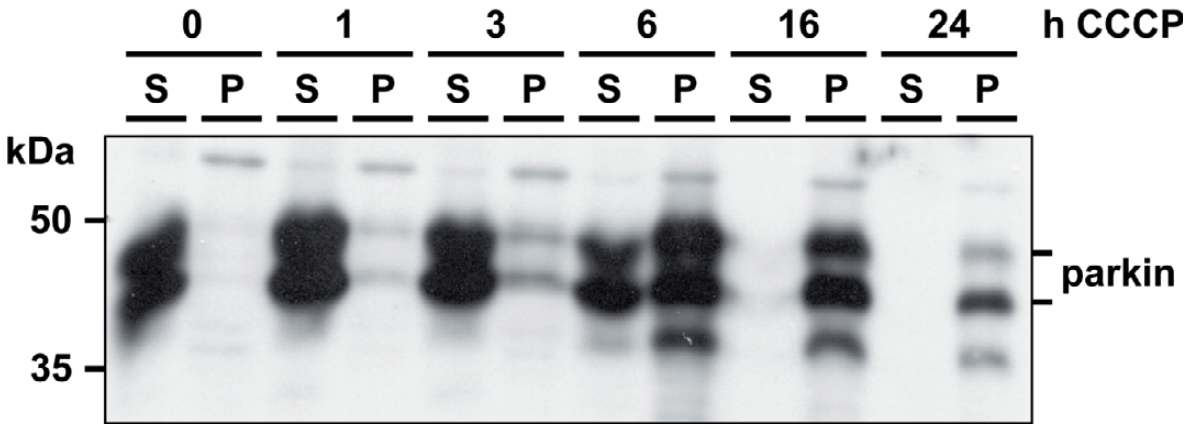


Figure 29: Uncoupling of mitochondria membrane potential alters parkin solubility. HEK cells were transiently transfected with human wildtype parkin. Following different time periods of treatment of cells with 10 µM of CCCP as indicated, the solubility of parkin was analyzed. Therefore, cells were lysed in 0.5 % (v/v) Triton X-100 and sodium desoxycholate in PBS and separated by centrifugation. Equal amounts of detergent-soluble and –insoluble fraction were analyzed by immunoblotting for parkin (PRK8).

3.1.12 In the absence of PINK1 CCCP does not render parkin insoluble

The experimental data obtained from the above analysis pointed towards a role of parkin conformation on mitophagy. Overexpression of PINK1 or treatment of cells with CCCP resulted in a change of biochemical properties of parkin protein, with parkin being rendered insoluble in detergent. Importantly, these are conditions in which parkin can initiate mitochondrial degradation via mitophagy.

In order to investigate whether endogenous PINK1 has an effect on parkin solubility upon exposure to CCCP, parkin solubility in the absence and presence of CCCP was assessed in PINK1 KO MEFs. For this purpose, fibroblasts from PINK1 KO mice and wildtype littermates were lentivirally transduced to stably overexpress human wildtype parkin. For each genotype, fibroblasts from two different animals were used. GFP transduced MEFs served as a control. Cells were treated with CCCP for 24 h and subsequently subjected to detergent-solubility assay using 0.5 % Triton X-100 and 0.5 % desoxycholic acid (v/v). While GFP was consistently found selectively in the detergent-soluble fraction, CCCP was able to induce a shift of parkin to the detergent-insoluble fraction in wildtype fibroblasts (Figure 30). In contrast, no shift of parkin was observed in MEF cells lacking PINK1. Parallel experimental assessment of mitophagy in these cell reproduced previous findings (see Figure 24) that

PINK1 is crucial for mitophagy and mitochondria with lost membrane potential are only degraded in wildtype, but not in PINK1-deficient cells. Based on these findings it becomes evident that PINK1 is essential for recruitment of parkin to dysfunctional mitochondria and their subsequent removal by mitophagy. In the absence of PINK1, parkin does not change its biochemical properties upon exposure to CCCP and does not shift to the detergent-soluble fraction.

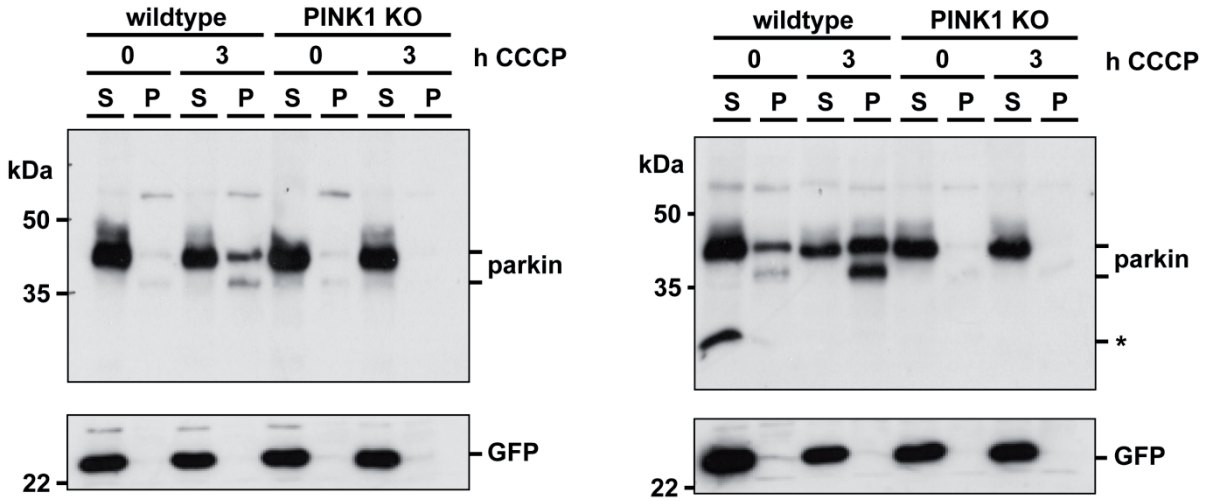


Figure 30: CCCP does not alter parkin solubility in the absence of PINK1. Wildtype and PINK1 KO MEFs were stably transduced with human wildtype parkin or GFP as a control. Cells were treated for 3 h with 10 µM CCCP, harvested and subjected to detergent solubility assay. Fibroblast cells lines were obtained from two different mice and analyzed for parkin solubility (independent MEF lines from independent mouse embryos were obtained, left panel: representative immunoblot for wildtype line 1 and PINK1 KO line 1; right panel: same representative experiment for line 2). Note that CCCP treatment results in a partial shift of parkin to the detergent-insoluble fraction in wildtype, but not in PINK1 KO fibroblasts. GFP, overexpressed as a control protein, stays completely soluble under these experimental conditions.

3.1.13 Pathogenic PINK1 mutants have differential effects on parkin solubility and mitophagy

Autosomal recessive mutations in parkin or PINK1 cause PD. The previous results revealed that several pathogenic parkin mutants are prone to misfolding and are impaired in mitochondrial translocation and mitophagy. To gain insights into the role of pathogenic PINK1 mutants in alteration of parkin solubility, the detergent-solubility assay was applied in the presence of PINK1 mutants. When DJ-1 was coexpressed with parkin as a control, parkin protein was detected in the detergent-soluble fraction. Expression of wildtype PINK1 resulted in a partial shift of parkin to the pellet fraction (Figure 31). Similar results were obtained with a PINK1 mutant lacking a part of the N-terminal bipartite mitochondrial targeting signal but retaining the transmembrane (TM) domain (PINK1Δ78; deletion of amino acids 1 to 78). In immunofluorescence, this PINK1 mutant is largely cytosolic but a subset of the protein is able to colocalize with mitochondria, a picture similar to the expression of the wildtype PINK1 protein. In contrast, the pathogenic mutant W437X, a truncated PINK1

species, did not alter parkin solubility when overexpressed in HEK cells. Similarly, the pathogenic point mutants G309P, L347P and Q126P did not result in a shift of parkin to the detergent-insoluble fraction. In conclusion, the PINK1 mutants analyzed are defective in changing the biochemical properties of parkin in respect to detergent solubility of the protein.

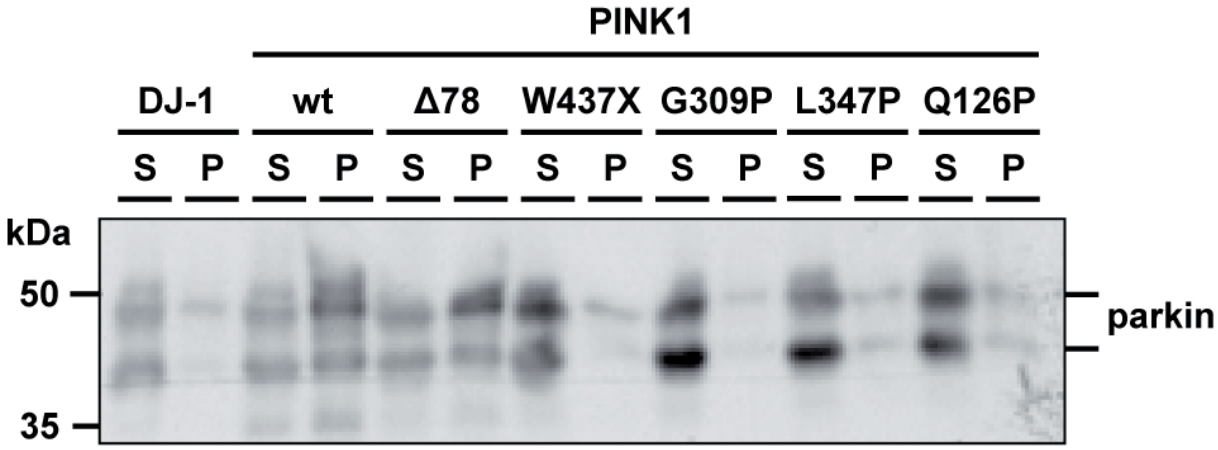


Figure 31: Pathogenic PINK1 mutants do not affect parkin solubility. HEK cells were transiently transfected with 0.3 µg of wildtype parkin and 0.3 µg of the indicated expression constructs. DJ-1 was used as a control. Cells were lysed in Triton X-100 and desoxycholic acid (0.5 % (v/v) in PBS) and separated into detergent-soluble and -insoluble fraction by centrifugation. Equal volumes of S and P fraction were analyzed by Western blot analysis for parkin using the PRK8 antibody.

3.1.14 Mitochondrially targeted parkin induces mitophagy upon dissipation of mitochondrial membrane potential

As outlined by the experiments above, parkin is recruited to depolarized mitochondria and initiates their degradation via mitophagy. Moreover, PINK1 is essential for redistribution of parkin to mitochondria, and mitochondrial targeting of PINK1 is crucial for this process. In the absence of PINK1, parkin is not targeted to mitochondria and mitochondrial removal is not induced. In addition, overexpression of PINK1 recruits parkin to mitochondria and initiates mitophagy even in the absence of CCCP treatment.

To answer the question whether recruitment of parkin is sufficient to induce mitophagy, an approach was chosen to directly target parkin to the outer mitochondrial membrane (OMM): an artificial vector construct was cloned directly anchoring parkin to the OMM and termed mitoparkin. To this aim the mitochondrial targeting sequence and the transmembrane domain (TM) of human TOM20 were N-terminally fused to the coding sequence of human wildtype parkin. A short linker sequence (6 amino acids) was introduced between the TM domain of TOM20 and parkin to allow proper parkin folding and avoid steric hindrance (Figure 32). Expression of this construct targets parkin to the outer mitochondrial membrane surface with parkin being exposed to the cytosol.

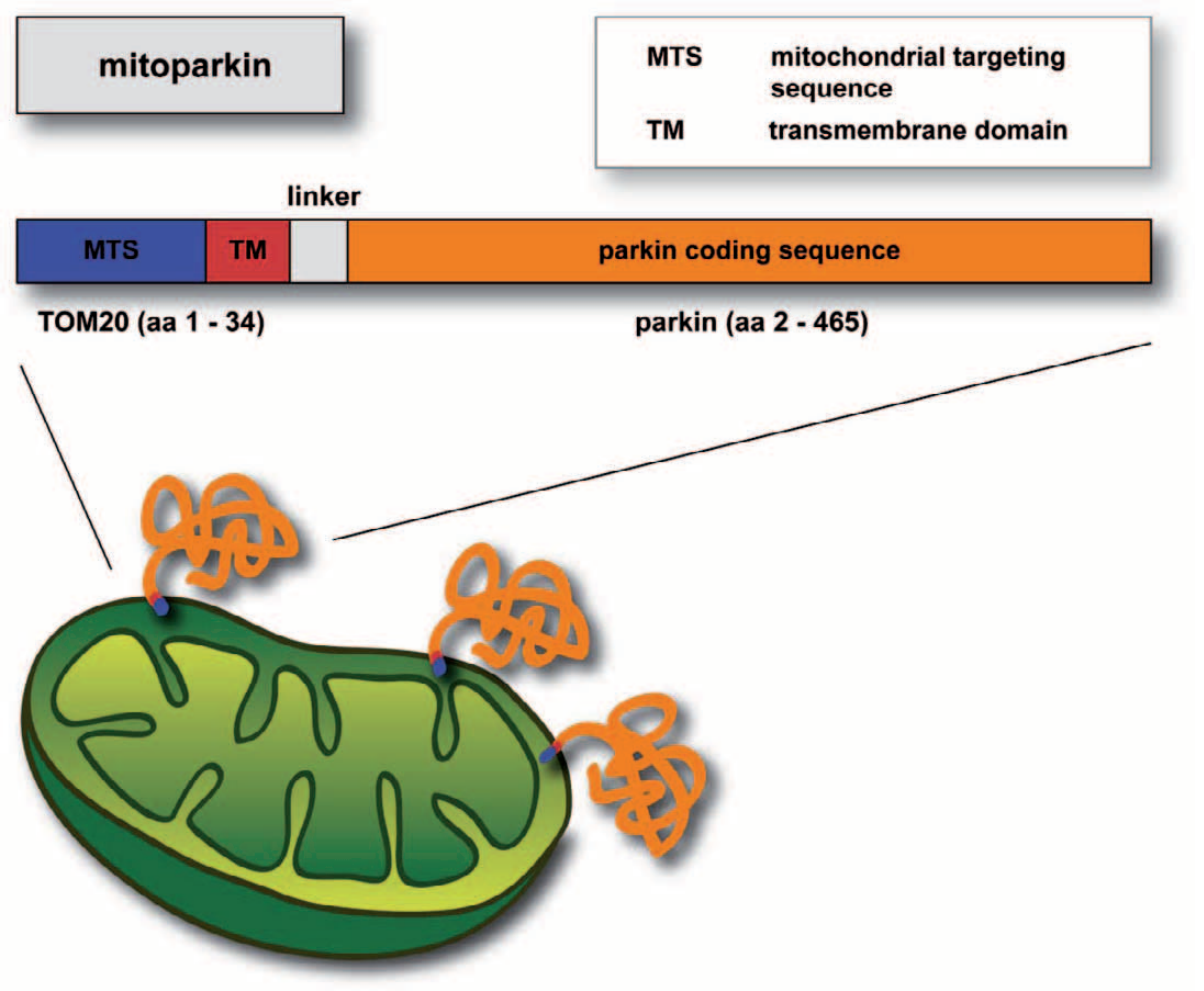


Figure 32: Schematic representation of mitoparkin. The artificial fusion protein mitoparkin consists of the first 40 amino acids of TOM20 (including the MTS and TM domain) followed by a short peptide linker of 6 amino acids and the full coding sequence of human wildtype parkin (not drawn to scale). The start codon of parkin was removed to avoid expression of untagged wildtype parkin. Mitoparkin is inserted to the outer mitochondrial membrane, with parkin facing the cytosol. aa: amino acid.

When HeLa cells were transiently transfected with mitoparkin, the artificial fusion protein was nicely expressed as determined by immunoblot analysis (Figure 33). Furthermore, it was targeted to mitochondria and did not influence mitochondrial morphology when expressed at low levels (Figure 34). Notably, expression of mitoparkin did not induce mitophagy *per se*, suggesting that targeting of parkin to functional mitochondria is not sufficient for parkin-mediated mitophagy. In addition, parkin is an aggregation-prone protein, and anchoring of the protein to mitochondria could interfere with parkin folding and function. To determine if the artificial fusion protein mitoparkin was functional, CCCP was added to mitoparkin expressing HeLa cells to depolarize mitochondrial membrane potential. Indeed, mitoparkin was fully functional and removed dysfunctional mitochondria to the same extent than untargeted wildtype parkin (Figure 34).

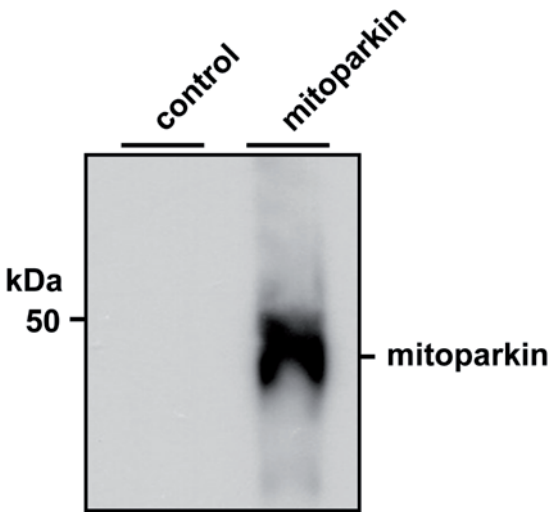


Figure 33: Mitoparkin protein is expressed. HeLa cells were transiently transfected with 0.5 µg of mitoparkin. After 24 h, cells were harvested and total lysates were subjected to immunoblot analysis using the parkin-specific antibody PRK8.

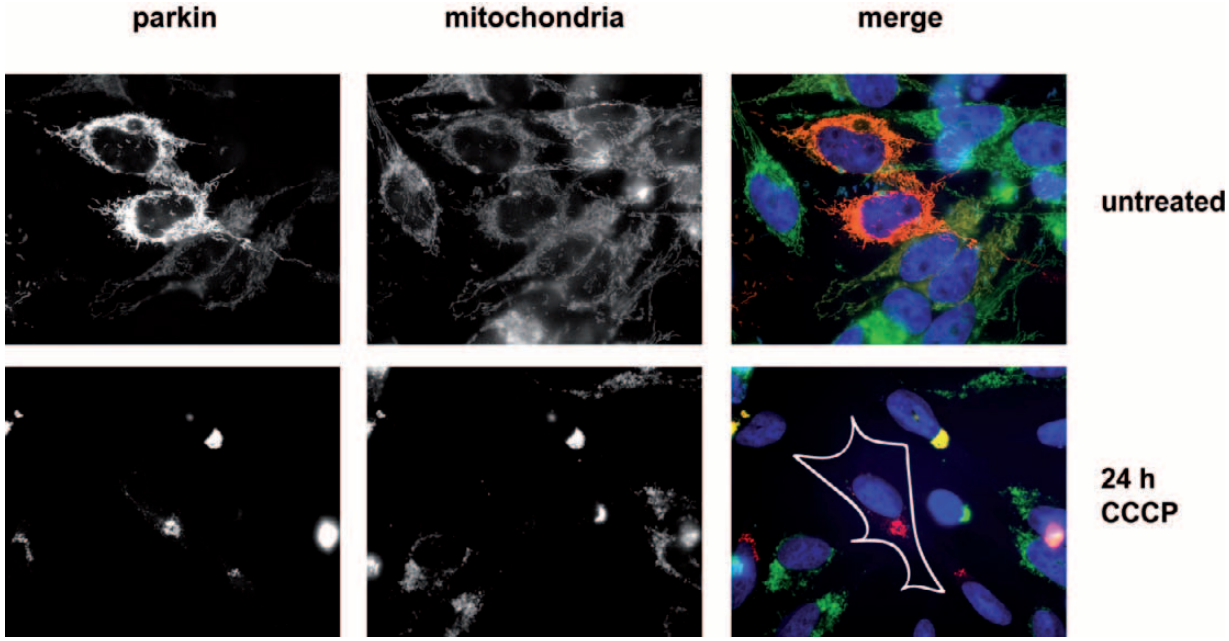


Figure 34: Mitoparkin localizes to mitochondria. HeLa cells were transiently transfected with mitoparkin and analyzed for colocalization of mitoparkin (parkin-specific PRK8 antibody; red) and mitochondria (TOM20 polyclonal antibody; green). In order to check for the functionality of mitoparkin, cells were treated with 10 µM of CCCP for 24 h. As an example, a single cell with no mitochondrial staining is highlighted by a white line, indicating the cell borders. Note that the TOM20 antibody does not cross-react with mitoparkin as the antibody was generated against a C-terminal fragment of TOM20, which is not present in mitoparkin.

3.1.15 Mitochondrial targeting of parkin is not sufficient to induce mitophagy in the absence of PINK1

The analysis of mitoparkin, an artificial fusion protein anchoring parkin to the mitochondrial OMM, has shown that mitochondrial targeting of parkin is not sufficient to induce mitophagy in the absence of mitochondrial uncoupling. PINK1 has been shown to recruit parkin to dysfunctional mitochondria and parkin is not targeted to mitochondria when PINK1 is lacking. In order to investigate if PINK1 has additional functions in mitophagy apart from parkin mitochondrial recruitment, mitoparkin was subcloned into a lentiviral expression vector to obtain stable cell lines by lentiviral transduction. In concordance with the results from HeLa cells, mitoparkin expression did not alter mitochondrial morphology and did not induce the degradation of mitochondria (Figure 35). As already outlined above, mitochondria are shorter and partially fragmented in PINK1-deficient cells compared to wildtype fibroblasts or HeLa cells, however, this effect is independent of parkin or mitoparkin expression.

Surprisingly, when the mitochondrial membrane potential was dissipated by CCCP treatment, mitophagy was not induced. After 24 h of CCCP treatment, all cells retained their mitochondrial staining as assessed by immunostaining for the mitochondrial outer membrane protein TOM20. These results suggest that PINK1 is not only necessary for the recruitment of parkin to dysfunctional mitochondria, but might have other functions apart from parkin recruitment in the mitophagy process.

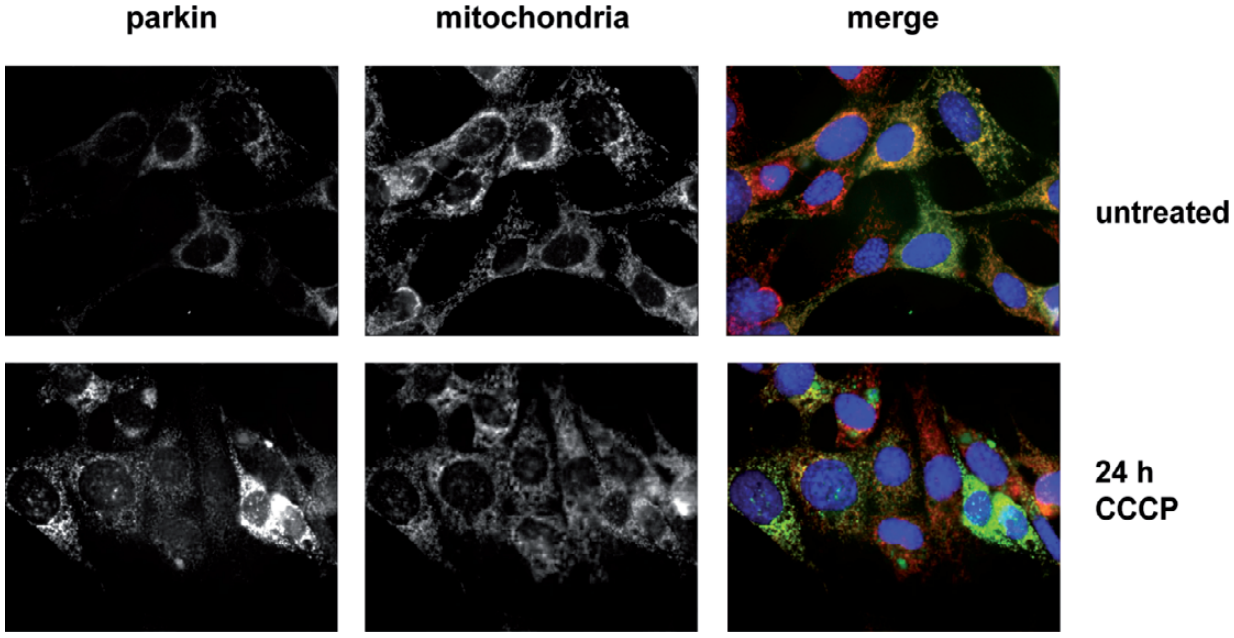


Figure 35: Mitoparkin is not sufficient to induce mitophagy in PINK1-deficient fibroblasts. PINK1 KO MEFs were lentivirally transduced to stably express mitoparkin. The cells were left untreated or 20 µM of CCCP were added for 24 h to depolarize mitochondrial membrane potential. Cells were stained with antibodies for mitoparkin (PRK8; green) and mitochondria (TOM20; red) and analyzed via indirect immunofluorescence.

3.2 Pathogenic parkin species are degraded via the proteasome in a p62-dependent manner

Lewy bodies (LB), cytoplasmic proteinaceous inclusions, are a characteristic hallmark of PD. Mutations in the parkin gene cause familiar autosomal-recessive PD with early-onset. Interestingly, parkin has been identified as a component of LBs (Schlossmacher et al., 2002), and *in vitro* experiments suggest that some mutations in the parkin coding sequence induce parkin misfolding and aggregation. However, it is not clear if and how parkin aggregates can be removed from the cell.

3.2.1 Pathogenic parkin mutants are prone to misfolding and form cytosolic aggregates

Wildtype parkin typically displays a homogenous, mainly cytosolic, cellular distribution. In contrast, it has been described that pathogenic parkin mutants are prone to misfolding and aggregate formation (Winklhofer et al., 2003). In line with this notion, several parkin mutants also display distinct biochemical properties such as reduced solubility in ionic and non-ionic detergents and differential sedimentation properties during centrifugation (Winklhofer et al., 2003, Schlehe et al., 2008). However, it is not clear how aggregated parkin protein is degraded. To determine how misfolded parkin is removed from cells, mutants were chosen that are particularly prone to misfolding: two point mutants with a single amino acid exchange (C212Y and C289G) and two truncated parkin mutants, E309X and W453X (Depicted in Figure 36). The later mutant is specifically interesting as it reflects a deletion of only 13 C-terminal amino acids without affecting the C-terminal RING domain of the parkin protein which is essential for its E3 ligase activity.

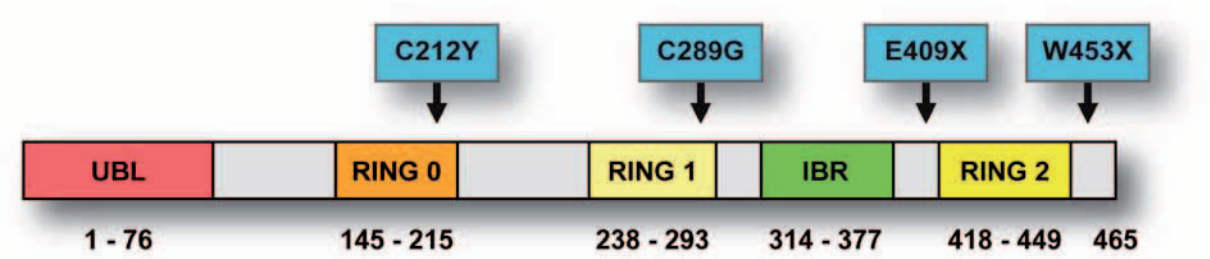


Figure 36: Modular structure of parkin protein and position of pathogenic mutations. The individual domains are colored. Numbers below designate amino acid positions of the respective domain. UBL: ubiquitin-like, RING: really interesting new genes, IBR: in-between RING.

HeLa cells were chosen for the analysis of parkin aggregate formation. HeLa cells do not express parkin protein, thus effects of endogenous wildtype parkin can be excluded. When transiently expressed in HeLa cells, wildtype parkin showed a clear homogenous distribution in the cytosol. In contrast, expression of pathogenic parkin mutants resulted in marked spotted staining due to cytosolic aggregate formation (Figure 37). Indeed, expression of

human wildtype parkin in HeLa cells never resulted in parkin aggregate formation as determined by single cell analysis via indirect immunofluorescence. In contrast, all pathogenic parkin mutants analyzed exhibited aggregate formation in a subset of cells (Figure 37). Moreover, when HeLa cells were transfected with the indicated parkin mutants and analyzed for detergent-solubility, all parkin mutants were partially found in the detergent-insoluble fraction while wildtype parkin was exclusively present in the soluble fraction (Figure 38).

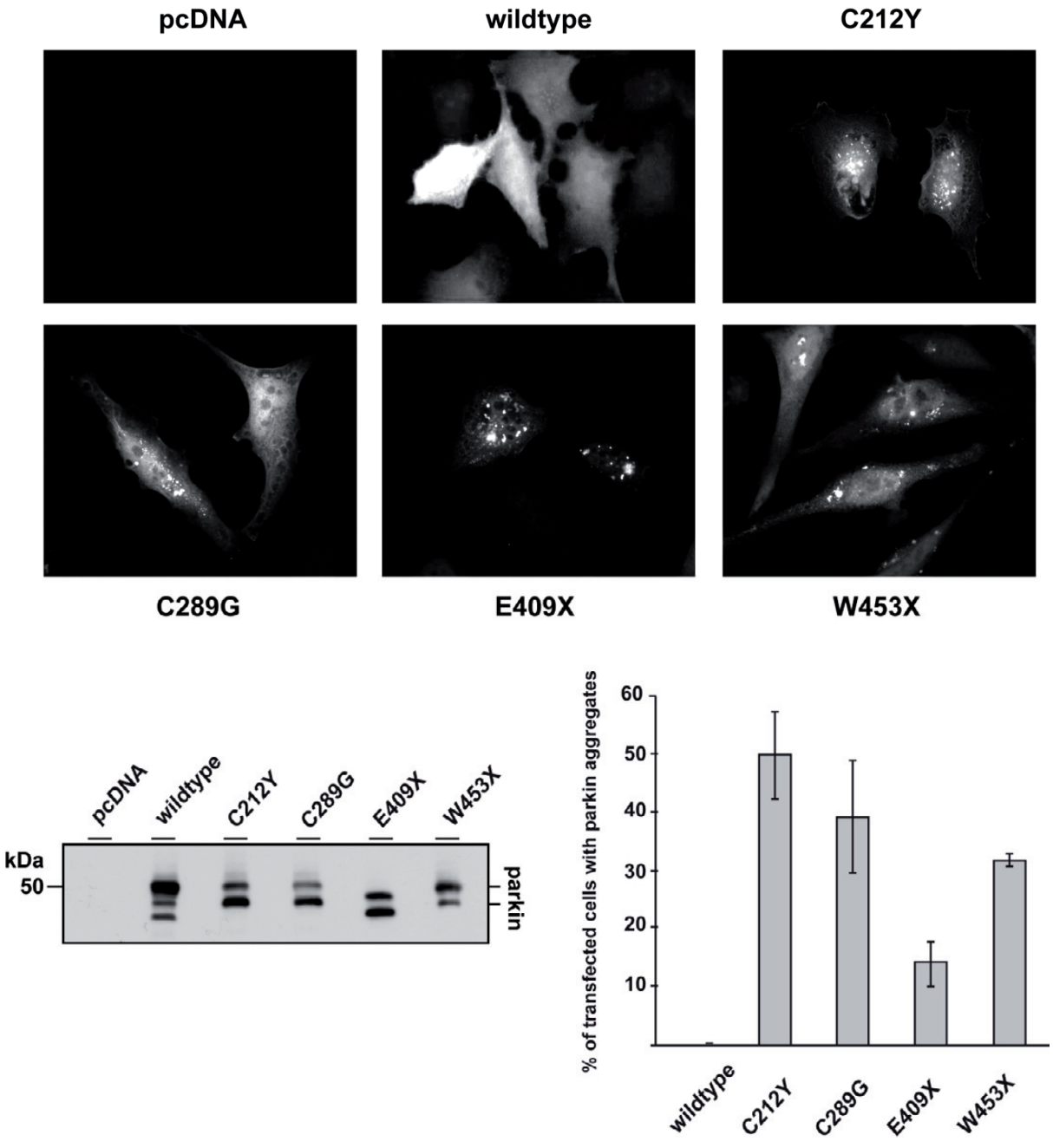


Figure 37: Pathogenic parkin mutants form cytosolic aggregates. HeLa cells were transiently transfected with the indicated parkin species. Upper pictures: Representative immunofluorescence pictures stained with the parkin-specific antibody PRK8. Lower left: Western blot analysis of parkin mutants to determine equal expression levels of the mutants (PRK8 antibody). Lower right: Single cell analysis was performed to determine the

percentage of parkin-expressing cells with parkin aggregate formation. At least 300 cells were counted in three independent experiments and the percentage of cells with parkin aggregate formation was calculated.

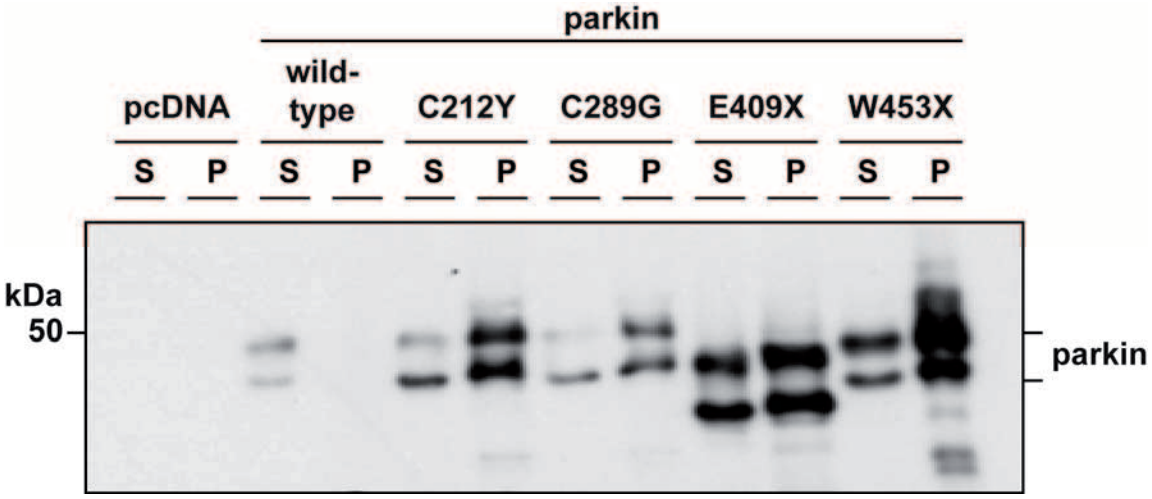


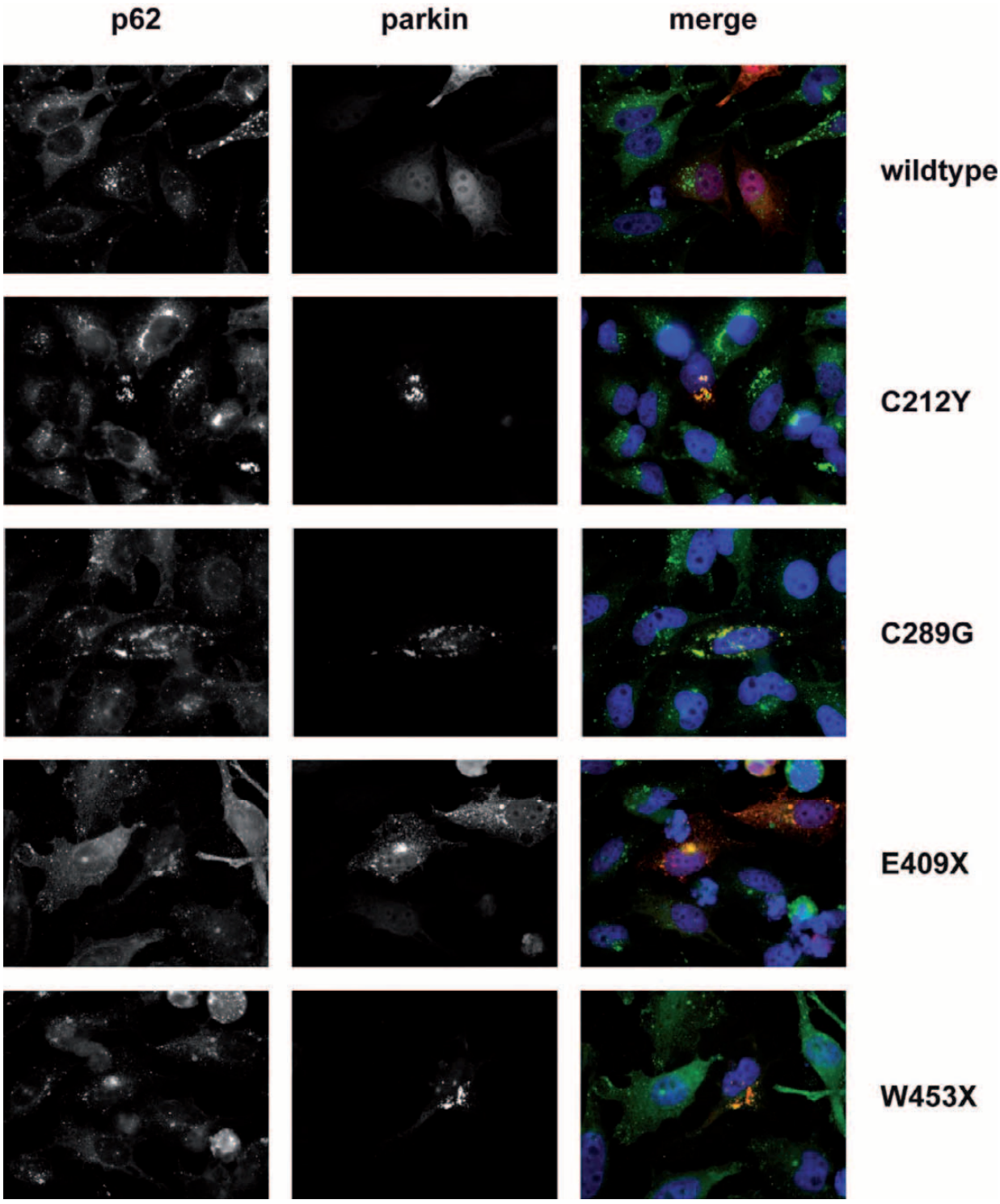
Figure 38: Pathogenic parkin mutants show reduced solubility in detergents. HeLa cells were transiently transfected with the indicated parkin species. Cells were lysed in 0.5 % Triton X-100 and 0.5 % desoxycholic acid (v/v) in PBS and separated into detergent-soluble (S) and –insoluble (P) fraction. Equal volumes of S and P fraction were subjected to immunoblot analysis using the parkin-specific antibody PRK8.

3.2.2 P62 colocalizes with mutant parkin aggregates

As a consequence of their large size, protein aggregates are often subject to autophagosomal degradation. P62 has been described as an adapter protein that mediates the interaction of protein aggregates and autophagosomes, resulting in the clearance of the aggregates through the lysosomal pathway. In agreement with this idea, p62 is found in close proximity to a number of proteinaceous deposits in different proteinopathies, such as Alzheimer's disease or Amyotrophic Lateral Sclerosis (ALS). Lewy bodies, the protein aggregates in PD, are composed of different proteins, and parkin has been identified as a constituent of the pathogenic inclusions. To experimentally address the question if p62 is found at aggregates of mutant parkin, an immunofluorescence experiment to detect parkin and p62 was performed in HeLa cells. The cells were transiently transfected with human wildtype parkin or the respective pathogenic mutants. Indeed, endogenous p62 was found to colocalize with the majority of parkin aggregates (Figure 39). In cells transfected with empty vector only, p62 showed a typical expression pattern with cytosolic staining, vesicular staining (resembling autophagosomes) and occasionally nuclear staining. The subcellular distribution or abundance of p62 did not change in the presence of wildtype parkin. Remarkably, when aggregates of pathogenic parkin mutants were present in the cell, p62 was sequestered to these cytoplasmic aggregates and displayed colocalization with parkin while the rest of the cell was frequently completely devoid of p62 staining (Figure 39).

To verify the findings obtained in HeLa cells in other cell types, the neuroblastoma cell line SH-SY5Y was analyzed. The cells were transiently transfected with pcDNA empty control vector, wildtype parkin or selected pathogenic parkin mutants. The analysis included

determination of 1) the percentage of cells with parkin aggregates, 2) the percentage of cellular parkin aggregates colocalizing with endogenous p62 and 3) the solubility of parkin mutants via the detergent-solubility assay (Figure 40 and Figure 41). The analysis of SH-SY5Y cells yielded results comparable to the HeLa cells, suggesting that p62 colocalizes with misfolded parkin independently of the cell type.



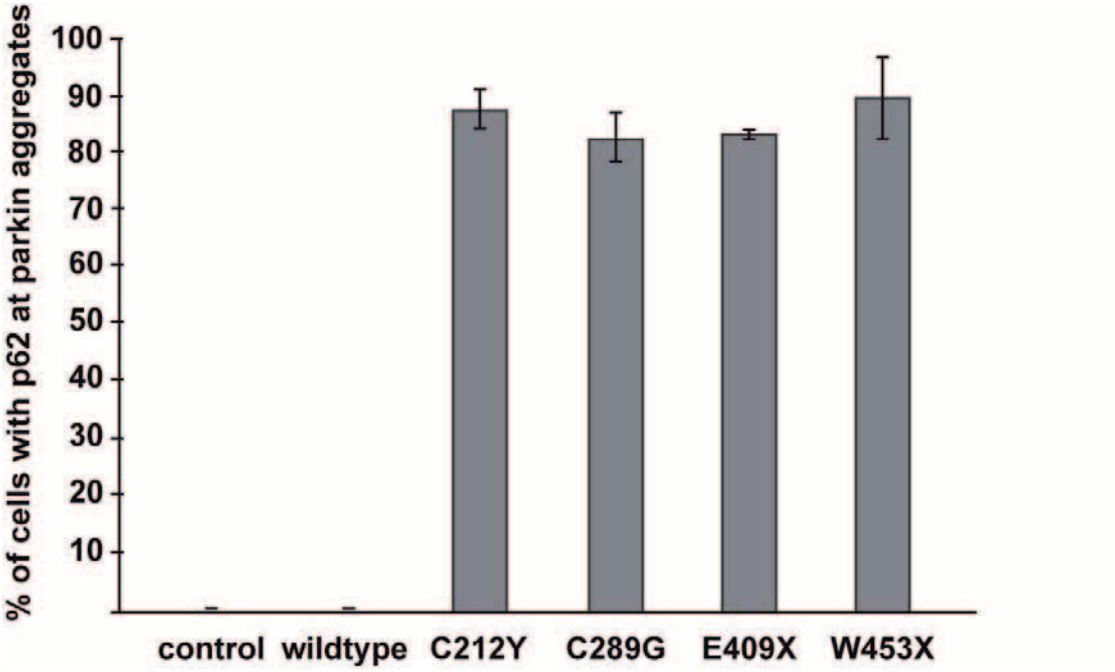


Figure 39: Pathogenic parkin mutants colocalize with endogenous p62. Upper panels (previous page): HeLa cells were transfected with pcDNA control vector, wildtype parkin or the indicated parkin mutants. Cells were stained with PRK8 parkin-specific antibody and p62 polyclonal antibody to stain endogenous p62. In the merge channel parkin is depicted in red and p62 in green. Lower panel: quantification of cells with endogenous p62 colocalizing with parkin aggregates.

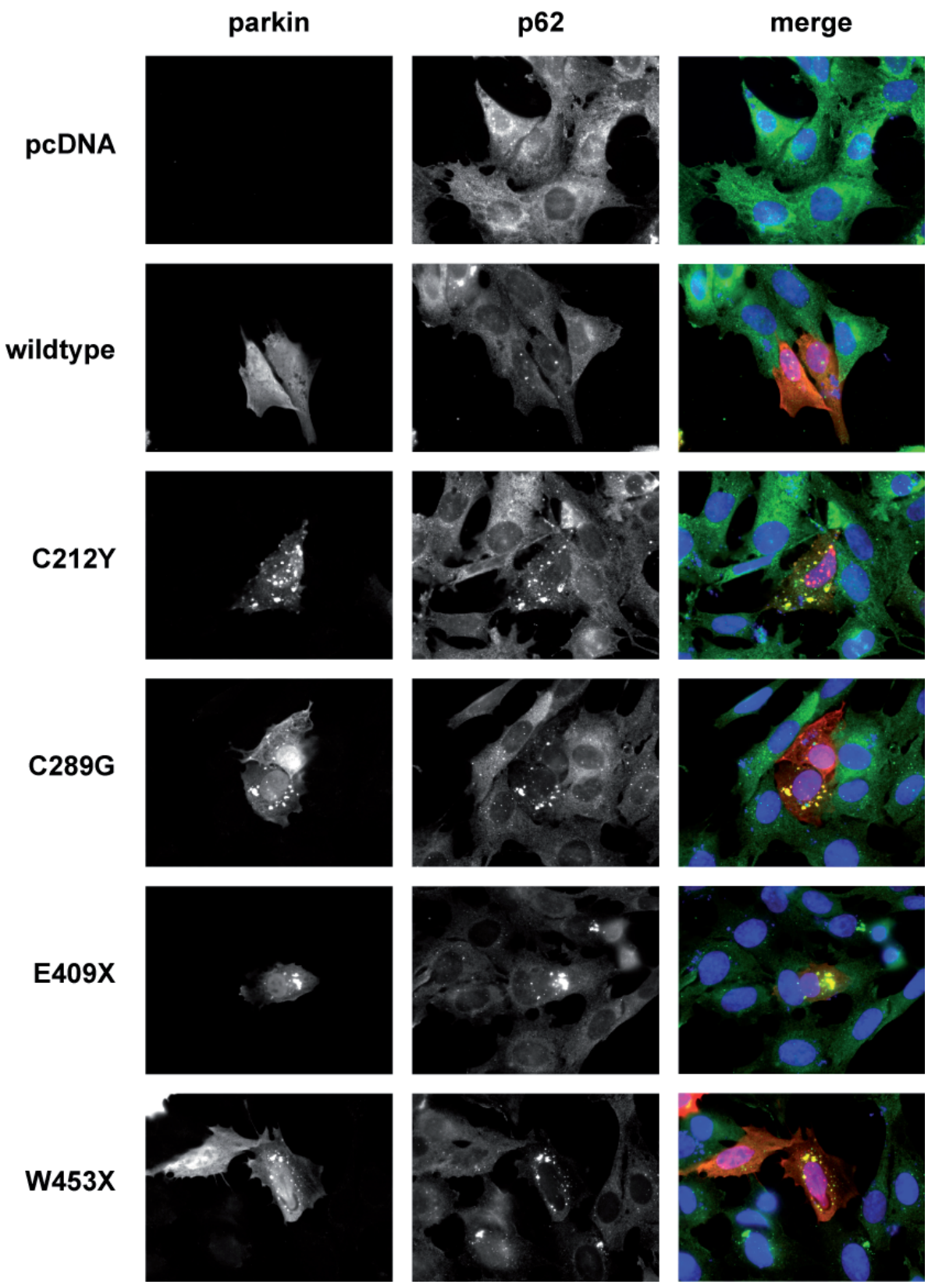
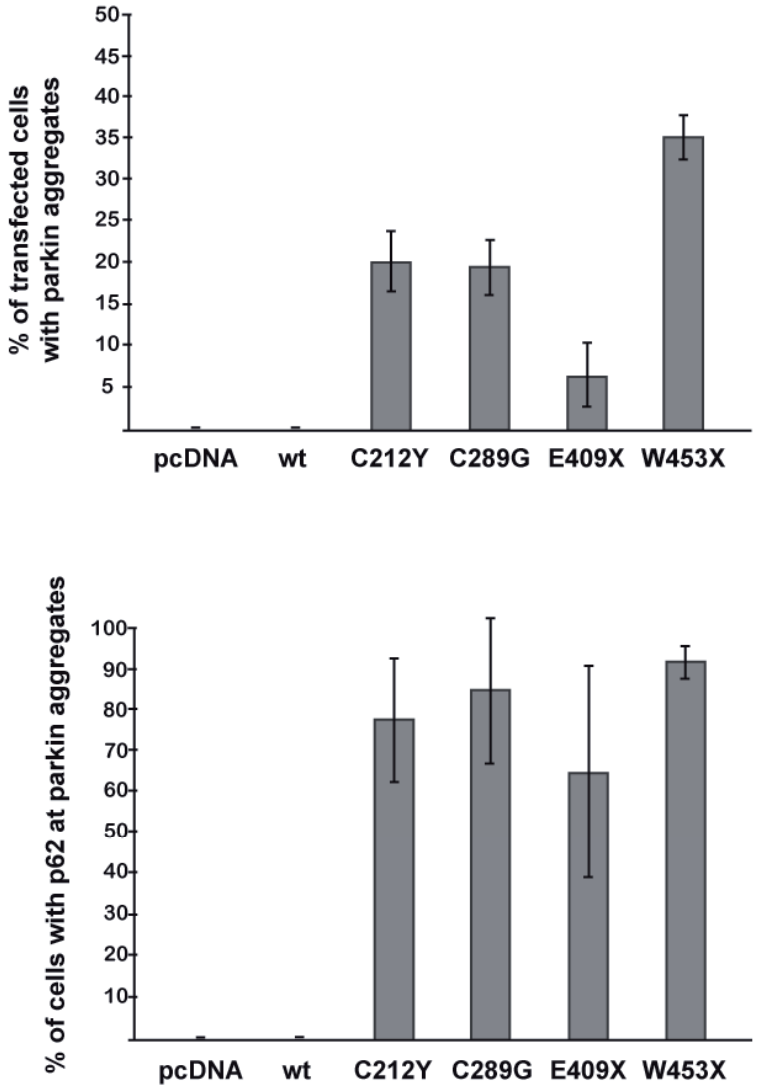


Figure 40: Pathogenic parkin mutants form cytosolic aggregates in SH-SY5Y cells and colocalize with p62. SH-SY5Y neuroblastoma cells were transiently transfected with pcDNA empty control vector, wildtype parkin or the indicated parkin mutants. Cells were stained with PRK8 parkin-specific antibody (red) and p62 polyclonal antibody (green) to stain endogenous p62.



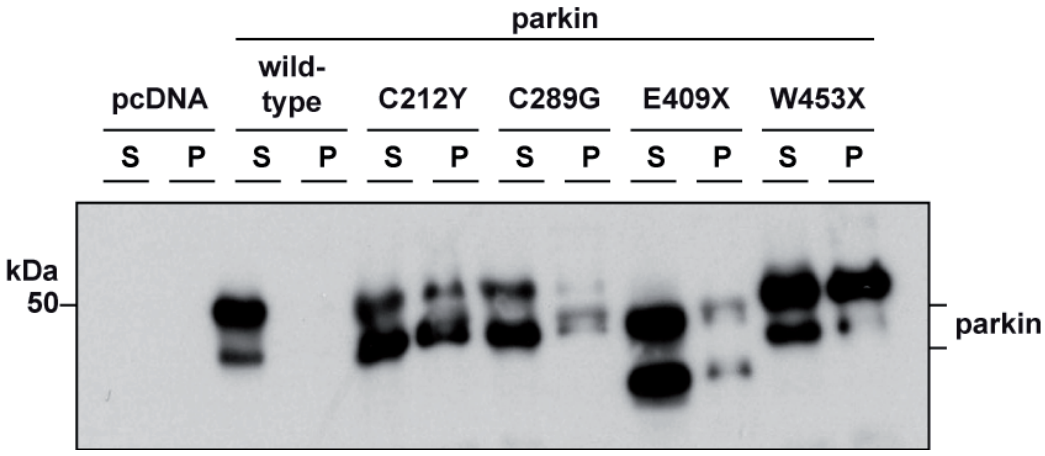


Figure 41: Pathogenic parkin mutants form aggregates in SH-SY5Y neuroblastoma cells and colocalize with p62. SH-SY5Y cells were transiently transfected with the indicated parkin mutants. The percentage of cells with parkin aggregates and the percentage of cells containing aggregates colocalizing with endogenous p62 staining was determined. Lower panel: solubility of parkin mutants in Triton X-100 and desoxycholic acid (0.5 % (v/v) each in PBS).

3.2.3 P62 levels influence the abundance of parkin aggregates

P62 has been found to colocalize with the majority of mutant parkin aggregates. In order to determine a possible influence of p62 on parkin aggregate formation, fibroblasts from wildtype and p62-deficient mice were used. Consistent with the results obtained in the human cell lines HeLa and SH-SY5Y, wildtype parkin was completely cytosolic and homogenously distributed when overexpressed in fibroblasts derived from p62 knockout (KO) animals or wildtype littermates. Contrarily, parkin mutants formed cytosolic aggregates in wildtype and p62 KO mouse embryonic fibroblasts (MEFs), which is in line with the previous findings. Exemplarily, the aggregation of the pathogenic parkin mutant W453X was assessed. The parkin stop mutant W453X exhibited spotted aggregates in a subset of cells in both wildtype and p62-deficient MEFs (Figure 42). Surprisingly, aggregates were present in a significantly higher percentage of parkin expressing p62-deficient cells. While only about 30 % of cells positive for parkin showed aggregate-like spots within the cytoplasm of wildtype MEFs, more than 50 % of cells lacking p62 presented cellular W453X parkin aggregates (Figure 43). Similar results were obtained with the parkin mutants C212Y, C289G and E309X (Figure 44). The expression of all of these parkin mutants resulted in enhanced aggregate formation in the absence of p62. To determine if the increase in parkin aggregates was due to the deficiency in p62, p62 was introduced into W453X parkin-expressing cells by transient transfection. Indeed, overexpression of wildtype human p62 in the KO MEFs significantly reduced the number of cells containing mutant parkin aggregates (Figure 42). Notably, overexpression of p62 also reduced the number of aggregates in wildtype MEFs. In contrast, expression and cytoplasmic distribution of wildtype parkin was not affected by different levels of p62. The data thus show that loss of p62 results in enhanced formation of parkin aggregates while p62 overexpression reduces the number of mutant parkin aggregates.

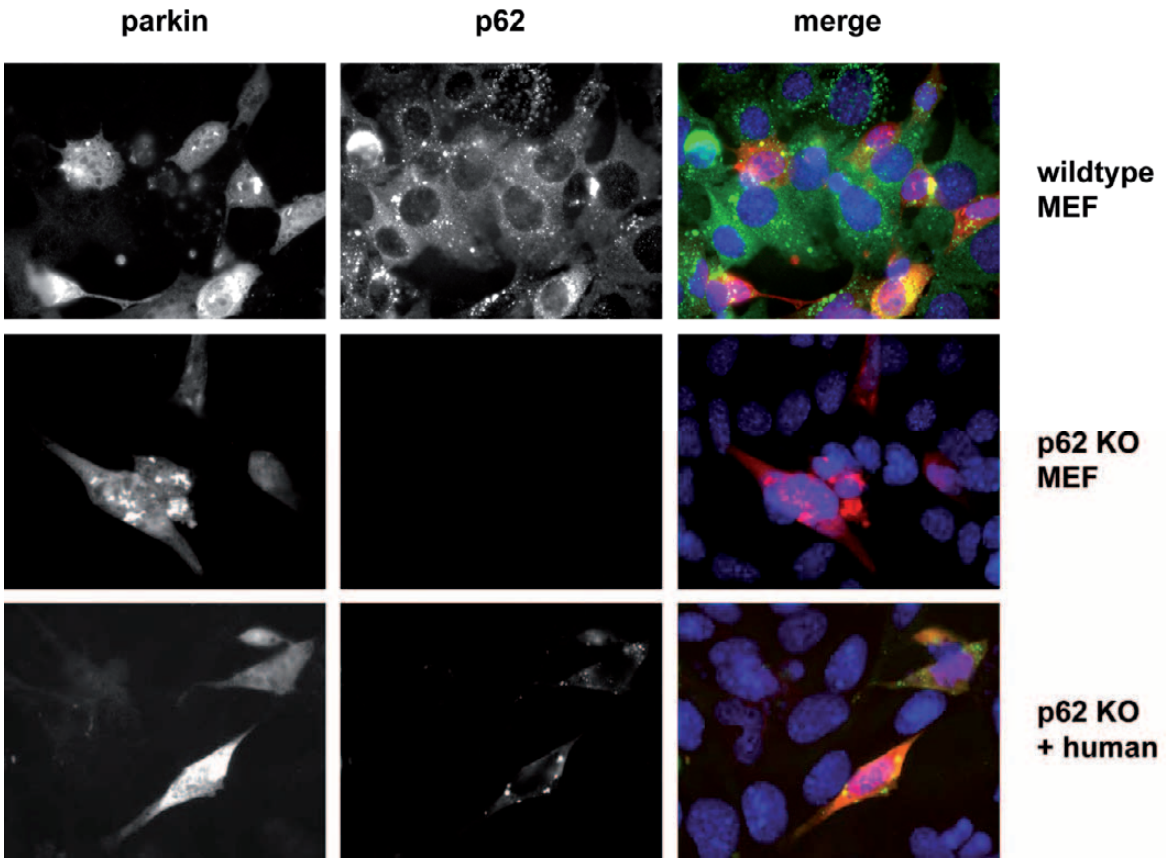


Figure 42: The parkin mutant W453X forms aggregates in wildtype and p62-deficient fibroblasts. Wildtype and p62 KO MEFs were transiently transfected with the parkin mutant W453X and stained for p62 (polyclonal p62 antibody; green) and parkin (PRK8; red channel). In the last panel, cells were cotransfected with human wildtype p62. Note that overexpression of p62 reduces parkin aggregate formation.

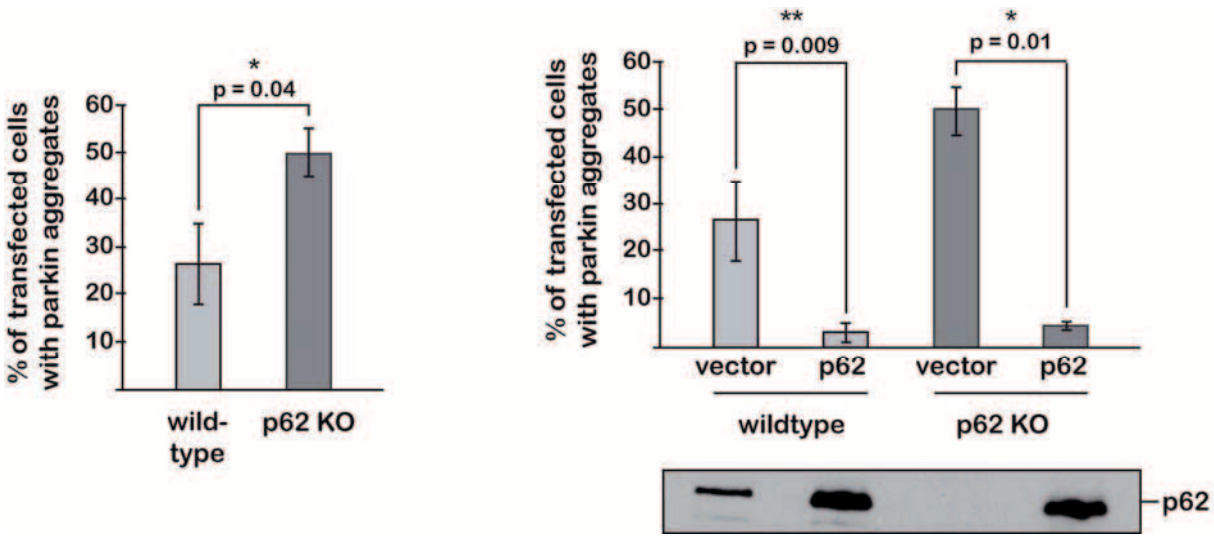


Figure 43: Aggregates of W453X mutant parkin are more abundant in fibroblasts from p62-deficient mice. Wildtype and p62 KO MEFs were transiently transfected with the parkin mutant W453X and stained for p62 (polyclonal p62 antibody) and parkin (PRK8). Left: in p62 KO MEFs, the abundance of parkin-expressing cells containing aggregates is significantly higher. Right: coexpression of p62 reduces parkin aggregate formation both in wildtype and p62 KO cells. Lower right: representative Western blot analysis (p62 polyclonal antibody) showing p62 expression levels for the individual conditions. Thanks to Veronika Hampl for experimental help.

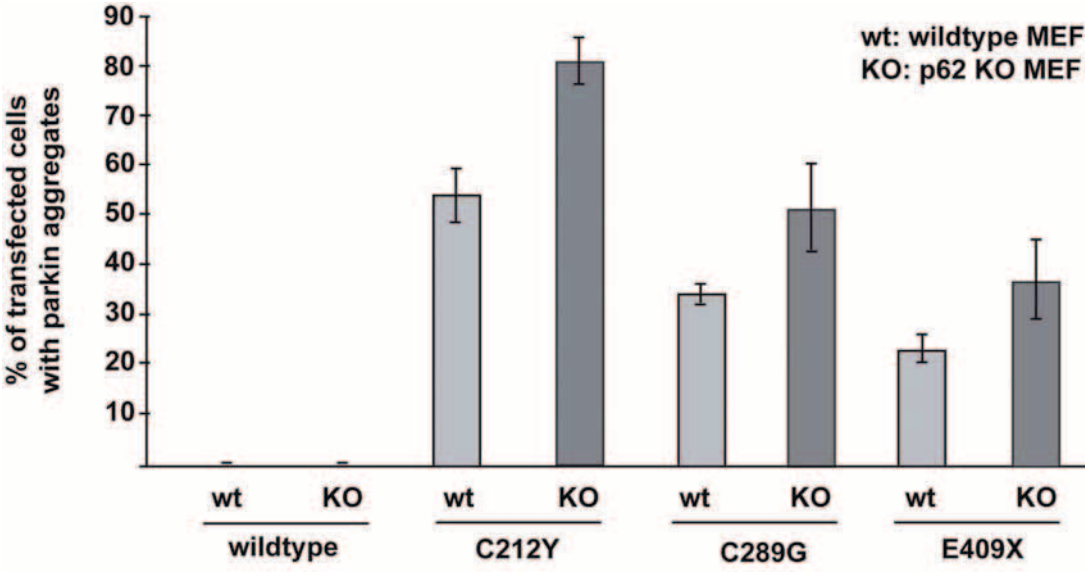


Figure 44: Aggregates of parkin mutants are more abundant in p62-deficient cells. Wildtype and p62 KO MEFs were transfected with wildtype parkin as a control or the indicated parkin mutants. Cells were analyzed for the percentage of transfected cells containing parkin aggregates via single cell analysis in indirect immunofluorescence experiments. A minimum of 500 cells per condition was assessed in three independent experiments.

3.2.4 Proteasomal inhibition enhances mutant parkin aggregate formation

The previous experimental data provided support for the notion that p62 was involved in the degradation of pathogenic parkin mutant aggregates. In order to determine if the ubiquitin-proteasomal system (UPS) or the autophagy pathway was used for removal of parkin aggregates, chemical inhibitors were used. HeLa cells were transiently transfected to express the parkin mutant W453X, and then treated with the proteasomal inhibitor epoxomycin or the lysosomal inhibitor bafilomycin A1 for 10 h. Treatment of cells with epoxomycin significantly increased the number of cells with parkin aggregates (Figure 45). In contrast, when cells were incubated with bafilomycin A1, no rise in the number of cells with parkin aggregates was observed. Bafilomycin A1 is a specific inhibitor of vacuolar proton ATPases, resulting in inhibition of fusion of autophagosomes with lysosomes. Of note, none of the chemical inhibitors induced the formation of aggregates of wildtype parkin under these conditions. Moreover, similar results were also obtained for the missense mutants C212Y and C289G and the truncated E309X mutant of parkin. The effect of enhanced parkin aggregate formation on proteasomal inhibition was not specific for HeLa cells but observed in other cell lines such as the dopaminergic neuroblastoma cell line SH-SY5Y and embryonic fibroblasts derived from wildtype mice (Figure 46). In contrast, when fibroblasts deficient for p62 were treated with proteasomal or lysosomal inhibitors, no significant change in the number of cells with parkin aggregates was found (Figure 46). Of note, reintroduction of exogenous human p62 was able to reduce formation of mutant parkin aggregates both in wildtype and p62-deficient fibroblasts. Interestingly, the reduced aggregate formation was

completely prevented when proteasomal function was blocked, suggesting that p62 is able to target aggregated mutant parkin for proteasomal degradation.

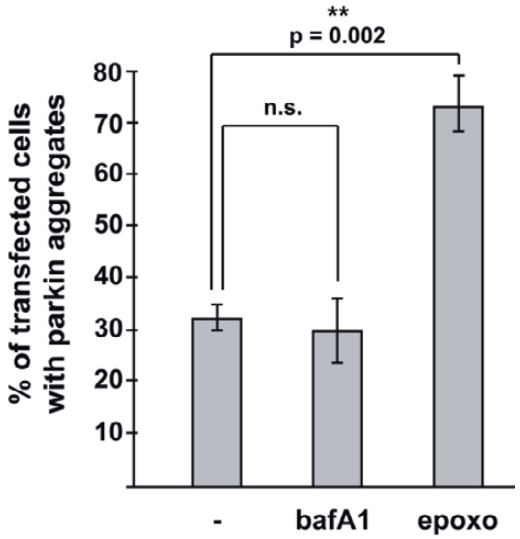


Figure 45: Proteasomal inhibition enhances parkin W453X aggregate formation in HeLa cells. HeLa cells were transiently transfected with the pathogenic parkin mutant W453X. Expression of this mutant in HeLa cells resulted in parkin aggregate formation in a subset of cells as assessed by indirect immunofluorescence. Pretreatment of cells with the lysosomal inhibitor bafilomycin A1 (30 nM; 10 h) did not change the percentage of cells with parkin aggregates. Epoxomycin treatment (100 nM; 10 h) significantly elevated parkin aggregate formation.

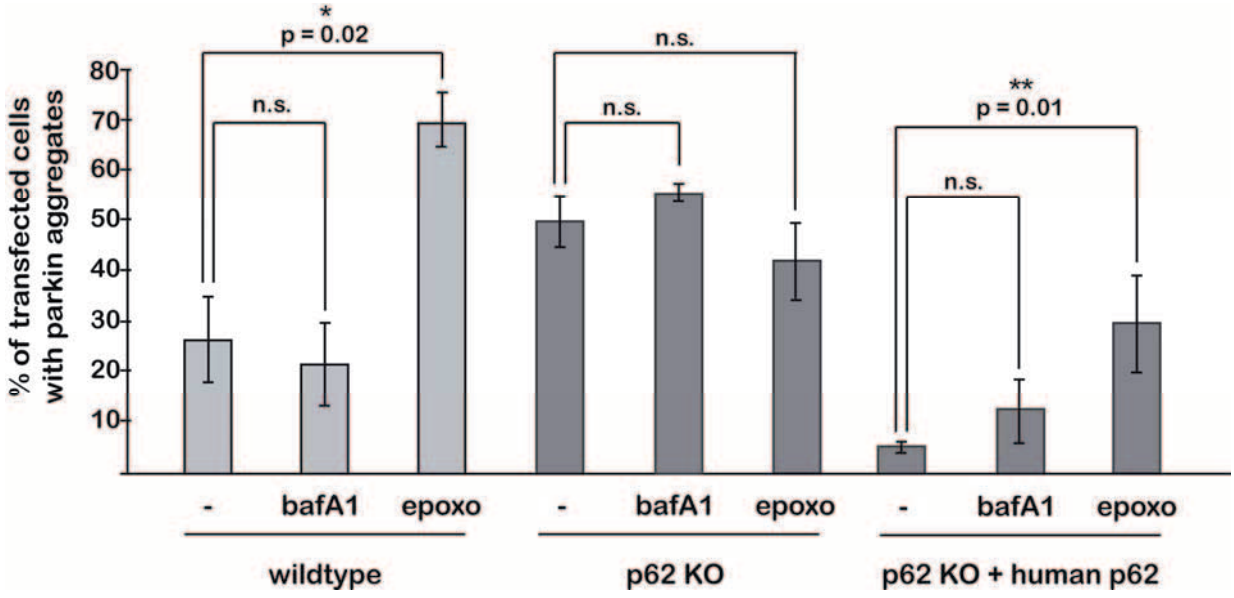


Figure 46: Expression levels of p62 and proteasomal inhibition both influence parkin aggregate formation. Wildtype and p62 KO MEFs were transfected with parkin W453X. For rescue experiments wildtype human p62 was cotransfected. Cells were treated with 30 nM of bafilomycin A1 or 100 nM of epoxomycin for 10 h and subjected to single cell analysis to determine the percentage of parkin aggregate formation.

3.2.5 The autophagosomal-lysosomal pathway is dispensable for degradation of mutant parkin aggregates

Proteasomal, but not lysosomal inhibition by chemical agents resulted in an increase of cells with parkin aggregates. The results suggested that autophagy was dispensable for the degradation of mutant parkin aggregates. To investigate the role of autophagy in parkin aggregate degradation in more detail, fibroblasts from ATG5 KO mice were used. Fibroblasts from these mice are deficient for autophagy protein 5 (ATG5), an essential component of the core autophagic machinery crucial for autophagosome formation. MEFs from ATG5 KO animals are thus defective in accomplishing autophagy (Kuma et al., 2004, Hara et al., 2006). The percentage of transfected cells with W453X parkin aggregates under basal conditions as well as upon blockage of the proteasome or lysosomes with epoxomycin or bafilomycin A1 was determined. Aggregate formation of the W453X mutant parkin protein in cells deficient for ATG5 was comparable to wildtype cells, with epoxomycin significantly increasing the number of aggregates in both cell lines (Figure 47). In agreement with all previous experiments, aggregates were never observed when wildtype parkin was expressed as a control. Further evidence for autophagy being dispensable for degradation of parkin aggregates was obtained by the analysis of autophagy markers in immunofluorescence: aggregates of mutant parkin did not colocalize with lysosomal markers such as LAMP1 (Figure 48). By contrast, a robust co-staining of parkin aggregates with polyubiquitin was observed. Making use of linkage-specific ubiquitin antibodies, a marked colocalization of parkin aggregates and K48-linked polyubiquitin was found. Contrarily, no labeling of the aggregates with a K63 polyubiquitin-specific antibody was observed (Figure 48). These findings substantiate the idea that parkin aggregates are degraded by the proteasome rather than autophagy, as polyubiquitin linked trough the lysine residue K48 is a classical recognition signal for proteasomal degradation. Taken together, the results point out that functional autophagy is not involved in the removal of mutant parkin aggregates.

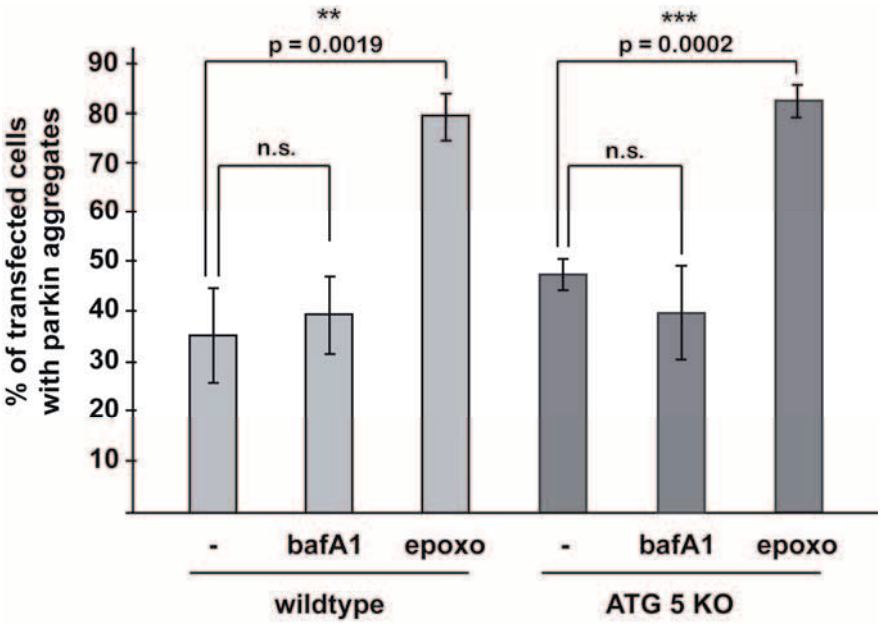


Figure 47: Proteasomal inhibition enhances aggregate formation of mutant parkin in autophagy-deficient cells. Wildtype and ATG5 KO MEFs were transiently transfected with the parkin stop mutant W453X and treated with bafilomycin A1 (30 nM; 10 h) or epoxomycin (100 nM; 10 h). The cells were stained with the parkin-specific antibody PRK8 and cells containing aggregates were determined by indirect immunofluorescence. The analysis was done by counting at least 500 cells per condition in three independent experiments.

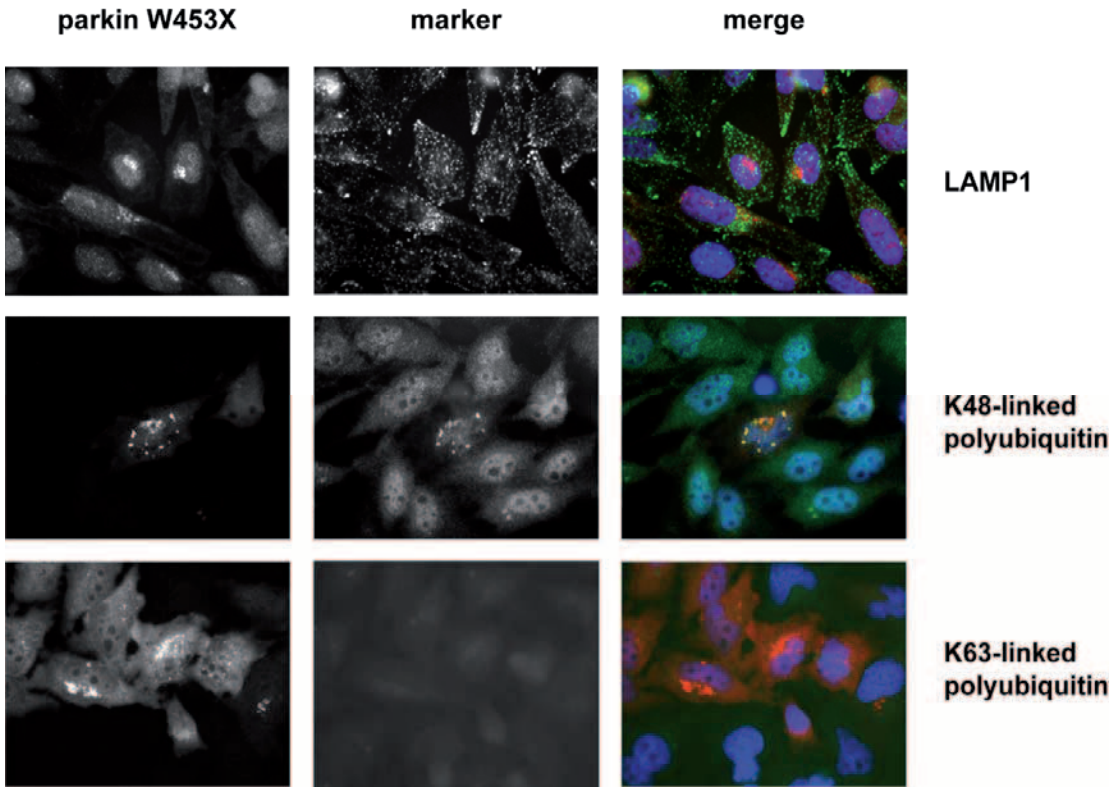


Figure 48: Aggregated W453X parkin does not colocalize with lysosomes and preferentially colocalizes with K48-linked polyubiquitin. HeLa cells were transiently transfected with the parkin mutant W453X and stained with the parkin-specific antibody PRK8 (red) and the indicated antibodies (green channel). LAMP1 is a

lysosomal transmembrane protein, staining lysosomal vesicles. Polyubiquitin antibodies specific for lysine-48 (K48) and lysine-63 (K63) polyubiquitin chains were used in the two lower panels.

3.2.6 A cell culture model to monitor the degradation of pathogenic parkin mutants

The results presented so far have revealed the importance of p62 and proteasomal degradation in the removal of parkin aggregates. In order to corroborate these findings, stable HeLa cell lines were generated to monitor the effect of proteasomal inhibition on parkin aggregate formation. Using lentiviral transduction, HeLa cells overexpressing either wildtype parkin or the stop mutant W453X were established. Interestingly, steady-state levels of wildtype parkin were much higher than the levels of the W453X stop mutant. This phenomenon could be due to rapid degradation of the mutant protein. Indeed, previous results from our group suggest that instant degradation of some parkin mutants that cannot adopt a native conformation precedes aggregation (Schlehe et al., 2008). Remarkably, wildtype parkin is quite stable when overexpressed in cells and the steady-state levels of the protein cannot be substantially increased with proteasomal or lysosomal inhibitors. In contrast, when cells stably expressing the parkin W453X mutant were treated with epoxomycin, there was a significant increase in parkin protein levels (Figure 49). Bafilomycin A1 instead did not alter the levels of W453X parkin. Indirect immunofluorescence was additionally used to monitor mutant parkin aggregation on single cell level. Consistent with the observations from immunoblot experiments, there was only weak W453X parkin signal and thus hardly any parkin aggregates detectable under basal conditions (Figure 50). However, when cells had been treated with a proteasomal inhibitor prior to analysis, there was an increase in parkin staining intensity and a significant rise in the number of cells with parkin aggregates. Cells that displayed parkin aggregation showed a marked colocalization of p62 with the aggregated parkin protein (Figure 50). Consistent with the previous findings these results show that parkin is rapidly degraded in HeLa cells stably expressing low levels of the parkin mutant W453X. However, when proteasomal function is impaired, the mutant accumulates and forms p62-positive aggregates reminiscent of the experiments with transient transfection of parkin mutants (see Figure 42, Figure 40 and Figure 50).

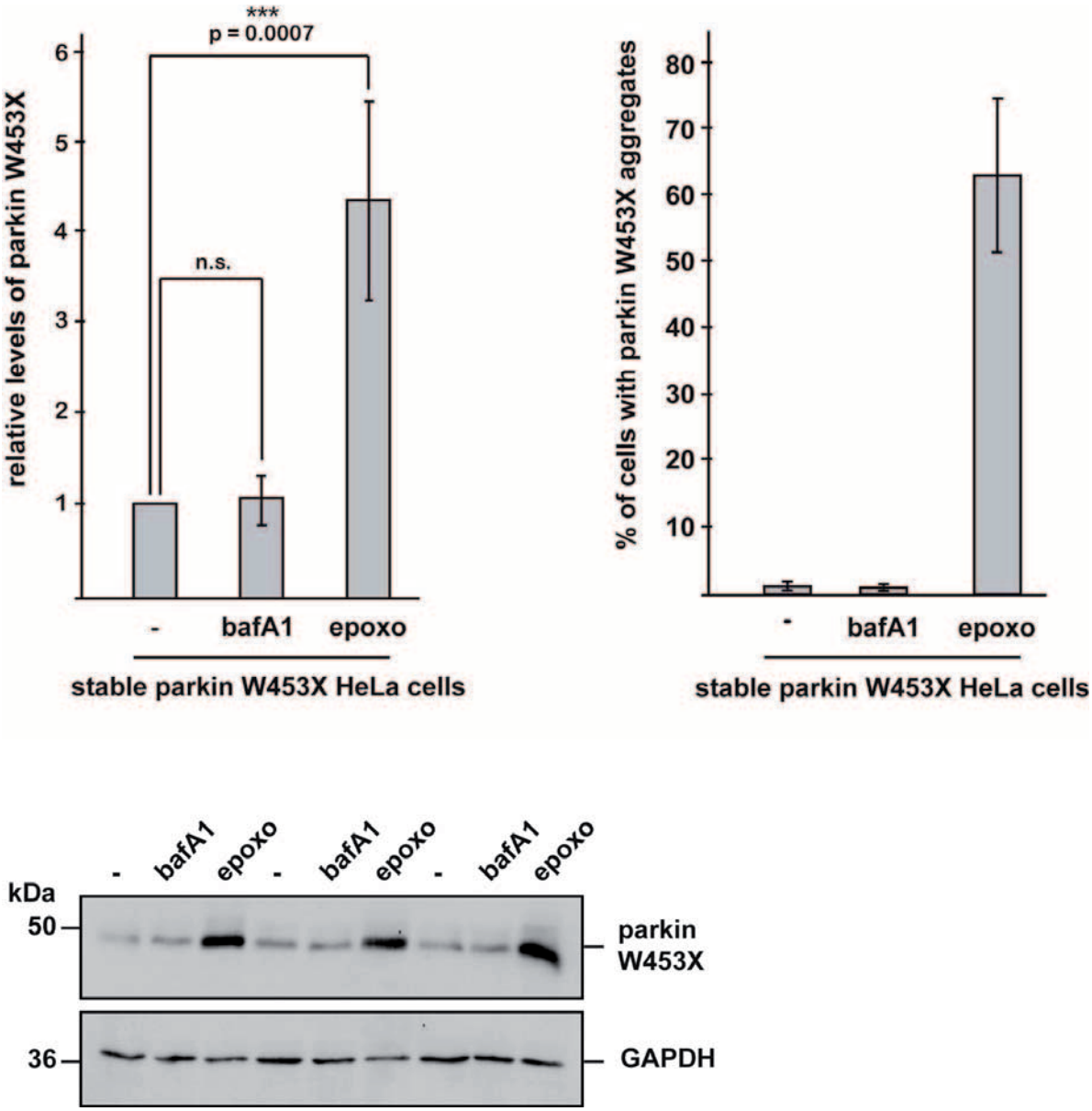


Figure 49: Proteasomal inhibition enhances parkin aggregate formation in a stable cell culture model of mutant parkin expression. HeLa cells were lentivirally transduced to express the parkin mutant W453X. Left: Western blot analysis of parkin levels in these cells following treatment with bafilomycin A1 (30 nM, 10 h) or epoxomycin (100 nM, 10 h). 20 µg of protein were separated by SDS-PAGE and immunoblotted with the parkin-specific antibody PRK8. GAPDH levels were assessed to determine equal loading. The relative levels of parkin to GAPDH were calculated from three independent experiments (upper left panel). Right: analysis of parkin aggregate formation in HeLa cells stably expressing parkin W453X via single cell analysis by indirect immunofluorescence (treatment with bafilomycin A1 (30 nM, 10 h) or epoxomycin (100 nM, 10 h)).

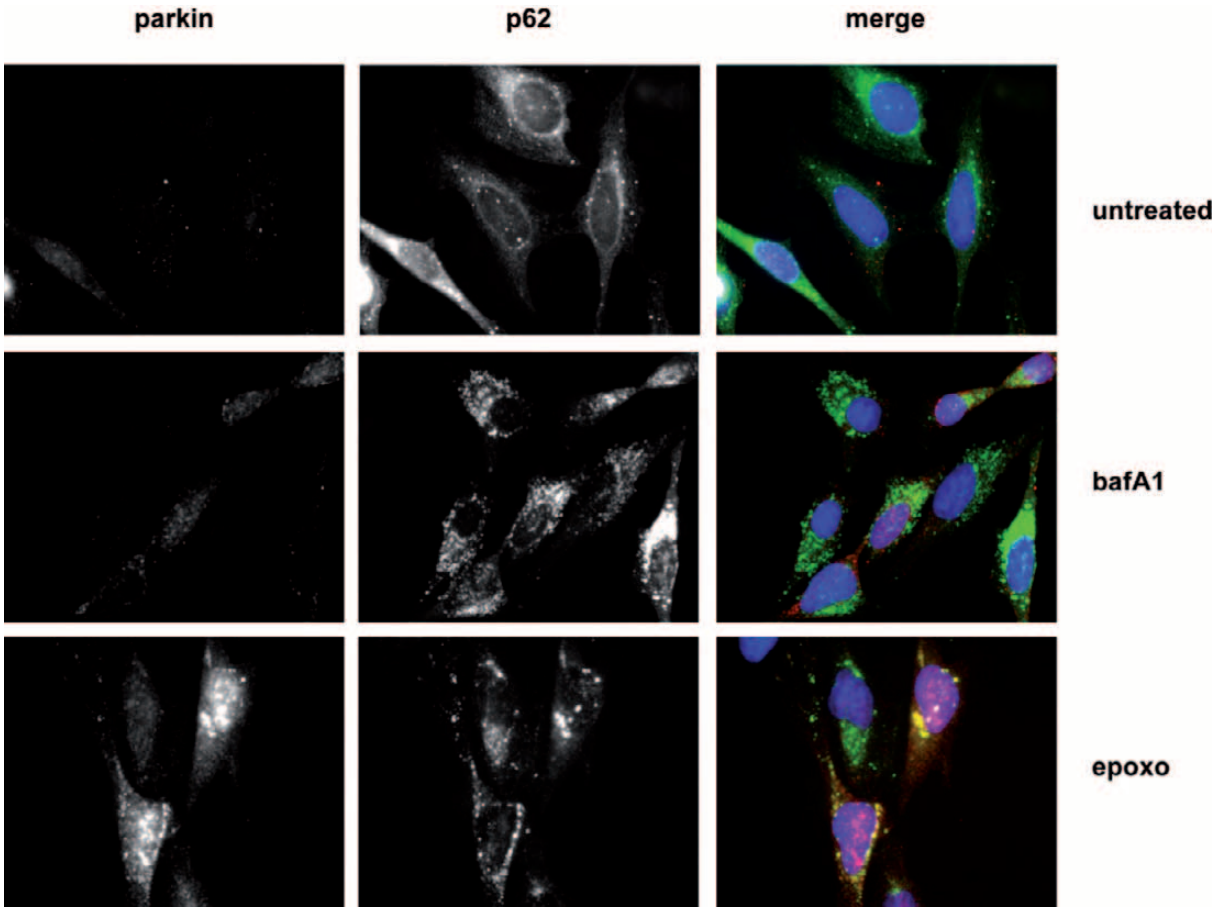


Figure 50: p62 colocalizes with parkin aggregates in stable parkin W453X HeLa cells upon proteasomal inhibition. HeLa cells stably overexpressing the parkin mutant W453X were treated with bafilomycin A1 (30 nM) or epoxomycin (100 nM) for 10 h. Cells were stained with the parkin-specific antibody PRK8 (red channel) and endogenous p62 (p62 polyclonal antibody; green). Parkin aggregates appear upon proteasomal inhibition, and a widespread colocalization with p62 can be observed. Note that p62-positive vesicles accumulate both through proteasomal and lysosomal inhibition.

In a next step an RNAi approach was used to examine the effect of p62 on degradation of mutant parkin protein in a human-derived cell line. The previous results obtained with fibroblasts from p62 KO mice pointed towards an essential role of p62 in proteasomal degradation of pathogenic parkin aggregates. Small interfering RNA (siRNA) duplexes targeting p62 were used to downregulate steady-state levels of p62 and monitor the effect on parkin degradation. Cells were transfected with a pool of scrambled control siRNA or siRNA specifically targeting p62 and analyzed 24 h after transfection. Transient knockdown of p62 in HeLa cells stably expressing the parkin mutant W453X indeed increased the levels of parkin protein (Figure 51). However, the effect of p62 knockdown was not as prominent as observed with proteasomal inhibition by epoxomycin was applied. This might be due to the fact that the knockdown was incomplete (5 to 20 % of residual p62 protein present) as assessed by Western blot analysis (Figure 51).

In conclusion, the experimental data obtained from the stable W453X HeLa cells support the idea that in contrast to wildtype parkin, the parkin stop mutant W453X is degraded by the proteasome in a p62-dependent manner.

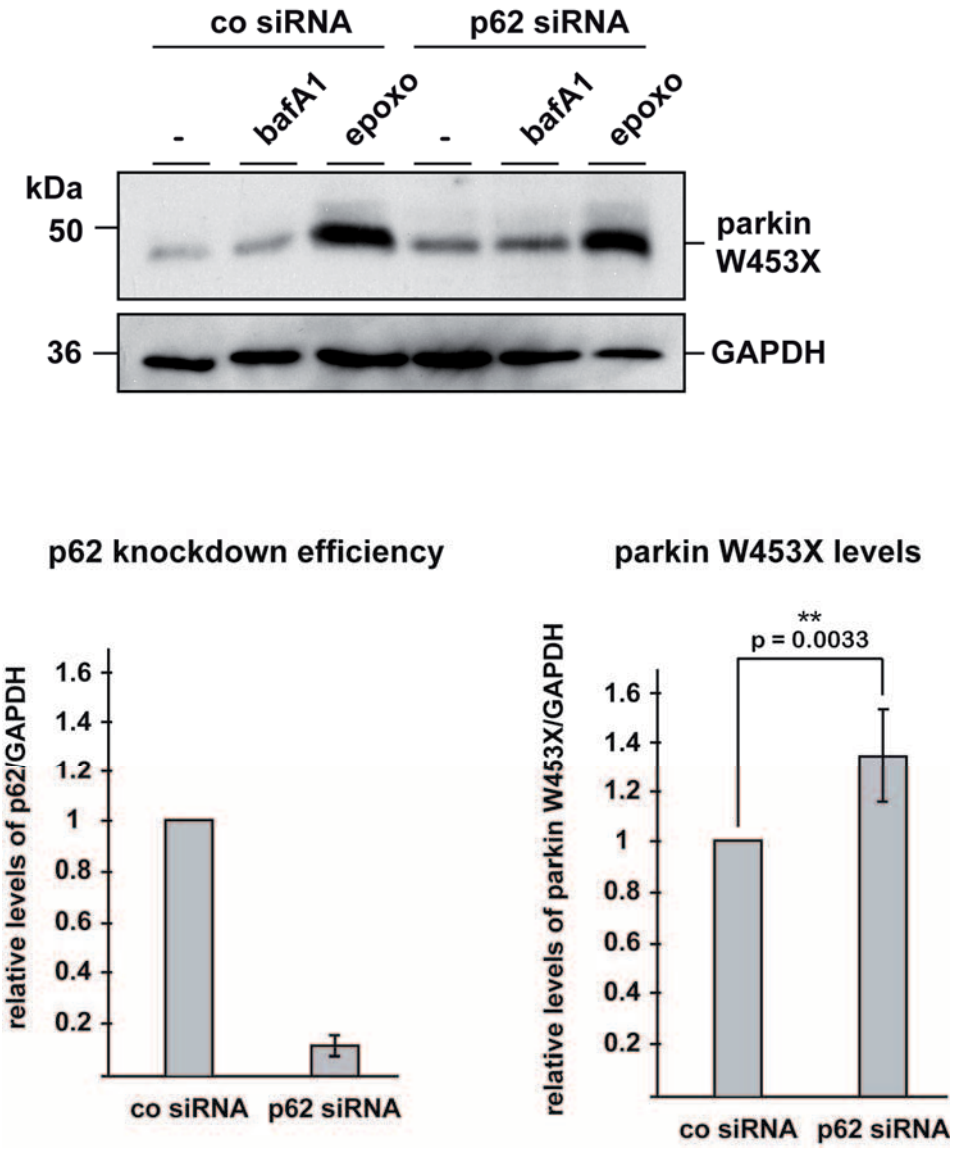


Figure 51: The parkin mutant W453X accumulates upon siRNA-mediated knockdown of p62. HeLa cells stably expressing the parkin mutant W453X were transfected with control (co siRNA) or p62-specific siRNA, respectively. Where indicated, cells were additionally treated with 30 nM of bafilomycin A1 or 100 nM of epoxomycin 10 h prior to analysis. 24 h after siRNA transfection, cells were harvested and equal amounts of total protein (20 µg for parkin and 5 µg for p62 and GAPDH immunoblots) were subjected to Western blot analysis. Upper panel: representative immunoblot showing parkin W453X levels in control and p62 siRNA conditions. GAPDH was used as a loading control. Lower left graph: quantification of p62 knockdown efficiency from five independent experiments. Lower right: quantification of parkin W453X protein levels relative to the housekeeping gene GAPDH, based on five independent experiments.

3.2.7 Analysis of mutant parkin W453X in a transgenic mouse model of parkin aggregation

The previous experiments on the removal of mutant parkin in different cellular models suggested that parkin aggregates are indeed proteasomally degraded in a p62-dependent manner. To extend our knowledge about degradation of mutant parkin in an *in vivo* model, we chose to analyze primary cortical neurons from a transgenic mouse model. The

transgenic mouse model was established in our group and overexpresses the parkin stop mutant W453X under control of the prion promoter, resulting in expression of the parkin transgene ubiquitously in the brain. The mouse line was generated, established and maintained by other members of our research group (Margit Miesbauer, Angelika Rambold, Anita Schlierf and Maria Funke). Primary cortical neurons were prepared from these mice and analyzed for parkin expression and solubility under basal conditions and following treatment with the proteasomal inhibitor epoxomycin. Spotted aggregates of parkin protein were found in a subset of neurons using indirect immunofluorescence (Figure 52). Interestingly, p62 staining was found to colocalize with these parkin aggregates. Consistent with the results from the cell lines analyzed previously, the mutant parkin protein was almost exclusively present in the pellet fraction when the detergent-solubility assay was applied (Figure 53). In addition, epoxomycin treatment substantially increased the level of mutant parkin protein. Thus, there is additional evidence from primary mouse neurons that mutant parkin aggregates can be found in the brain of these mice *in vivo* and that they are degraded by the proteasome.

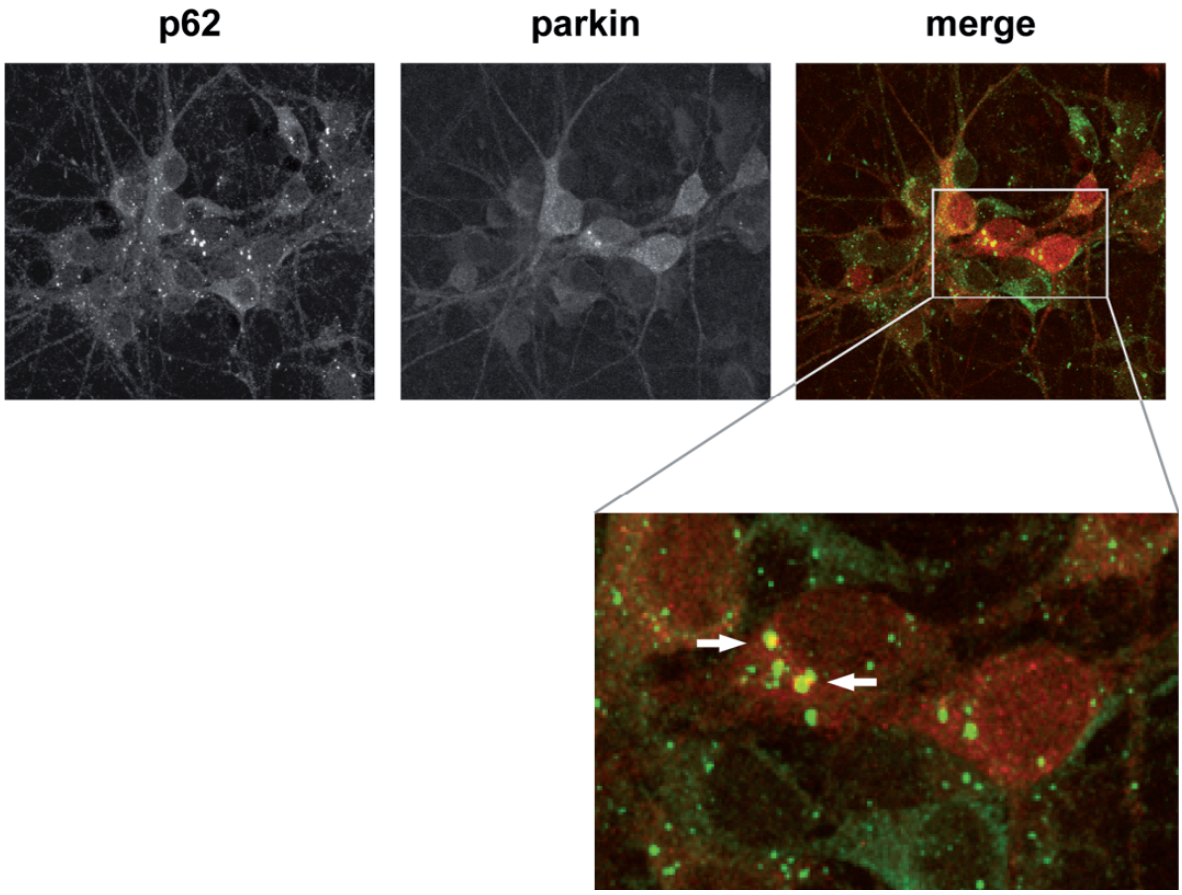


Figure 52: Parkin aggregates in primary neurons from W453X parkin-transgenic mice colocalize with p62. Primary cortical neurons from parkin W453X-transgenic mouse embryos were stained using the parkin-specific antibody PRK8 (red) and the polyclonal p62 antibody (green). Images were acquired using confocal microscopy. Thanks to Maria Funke for preparation of the primary neurons and to Kathrin Lutz for help with microscope settings and picture acquirement. The arrows in the enlargement point to parkin aggregates that colocalize with endogenous p62.

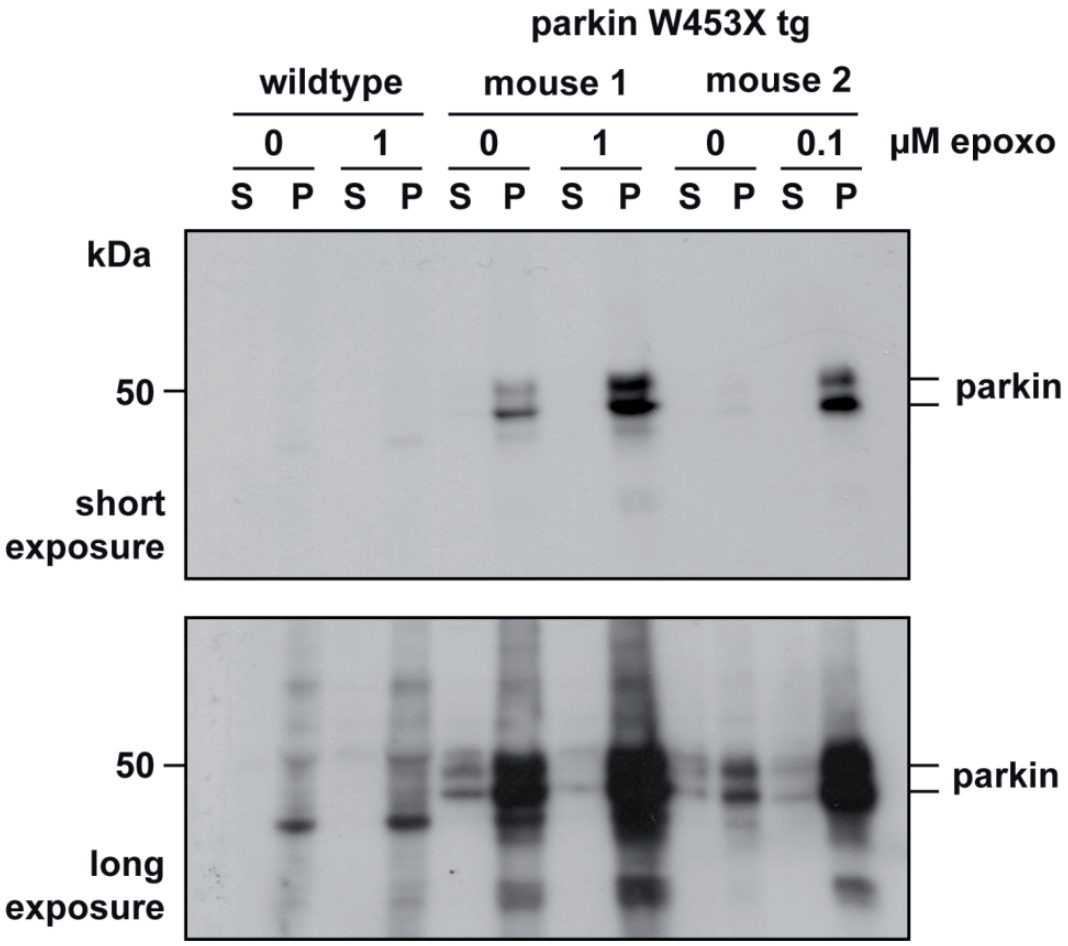


Figure 53: Mutant parkin W453X accumulates in primary neurons upon proteasomal inhibition. Primary cortical neurons were obtained from parkin W453X-transgenic mouse embryos and wildtype littermates. The neurons were exposed to 0.1 µM and 1 µM of epoxomycin for 16 h and analyzed by the detergent-solubility assay. Protein samples of neurons from two different parkin-transgenic animals (mouse 1, mouse 2) are shown in the representative immunoblot. Thanks to Alexandra Deinlein and Maria Funke for experimental assistance.

4 Discussion

4.1 Parkin and mitophagy

Mutations in the parkin gene are responsible for the majority of early-onset Parkinson's disease (PD) cases. Familiar PD caused by parkin mutations is autosomal recessively inherited, suggesting that a loss of parkin function could account for the disease. The parkin gene encodes an E3 ubiquitin ligase mediating the ubiquitination of substrate proteins. Different putative parkin substrates have been identified *in vitro*, including the parkin-associated endothelin receptor-like receptor (Pael-R), synphilin-1, glycosylated α-synuclein, cyclin E, synaptotagmin XI, CASK, the p38/JTV-1 subunit of the multi-tRNA synthetase complex, and the parkin-interacting substrate (PARIS) (Zhang et al., 2000, Chung et al., 2001, Imai et al., 2001, Shimura et al., 2001, Choi et al., 2003, Corti et al., 2003, Huynh et al., 2003, Ren et al., 2003, Staropoli et al., 2003, Fallon et al., 2006a, Shin et al., 2011). So far, the contribution of these substrates to PD and their disease relevance *in vivo* remain largely speculative. Although parkin displays a broad cytoprotective activity, the exact cellular function of parkin has long been veiled. Only recently, the finding that parkin is able to mediate the degradation of dysfunctional mitochondria via autophagy has invigorated research on the role of parkin and other PD genes on mitochondrial function (Narendra et al., 2008). Many lines of evidence indicate that mitochondrial dysfunction is a major contributor to the development of PD (reviewed in (Schapira and Gegg, 2011, Pilsl and Winklhofer, 2011). However, it remains to be clarified if mitochondrial dysfunction is a primary cause of PD directly eliciting pathogenic events or if it is only a bystander occurring late in disease pathogenesis.

In order to maintain the cellular homeostasis, dysfunctional mitochondria have to be removed to prevent damage to the cell and the induction of apoptosis. Mitochondria are remarkably dynamic organelles that constantly fuse and divide. When the mitochondrial membrane potential is lost, mitochondria undergo a rapid remodeling process, apparent through instant fragmentation of the tubular mitochondrial network. Within only a few minutes of cell treatment with the protonophore CCCP, the mitochondrial network disintegrates into small, spherical single mitochondria. This mitochondrial fragmentation is not specific for CCCP treatment but is a general response to many potentially harmful stimuli. In particular, mitochondrial fragmentation into smaller organelles is thought to be a prerequisite for subsequent degradation in lysosomes, a process called mitophagy (Twig and Shirihai, 2011, Twig et al., 2008b, Twig et al., 2008a). Given that HeLa cells do not express endogenous parkin, it becomes evident that parkin is not required for the fragmentation of tubular mitochondria following the exposure to CCCP. Nevertheless, exogenous parkin readily translocates to mitochondria that have lost their membrane potential and initiates their

degradation via mitophagy. Parkin-mediated mitophagy is not cell type specific, as it can be elicited in many cell types by the combination of parkin overexpression and subsequent dissipation of mitochondrial membrane potential. Not only overexpressed parkin, but also endogenous parkin can initiate mitophagy, as shown by recent studies in the neuroblastoma cell line SH-SY5Y (Van Laar et al., 2011, Lee et al., 2010). The mouse embryonic fibroblasts investigated in this thesis do not express endogenous parkin protein, thus, overexpression of wildtype parkin via lentiviral transduction was employed. Independently of the cell line analyzed herein, mitochondria were never depleted in the absence of parkin, revealing that parkin is essential for CCCP-induced mitophagy. The observed slight reduction in mitochondrial marker proteins upon prolonged exposure to CCCP in the absence of parkin most likely does not reflect degradation via mitophagy but is rather a consequence of impaired *de novo* biosynthesis of mitochondrial proteins or whole mitochondria. Parkin did not localize to other cellular compartments such as endoplasmic reticulum (ER) or lysosomes upon CCCP treatment, therefore the removal of mitochondria was specific for these organelles. In addition, levels of the ER marker protein calnexin and the cytoplasmic enzyme GAPDH did not change in response to CCCP or dependent on parkin protein levels, suggesting that mitochondria are selectively degraded.

Previous studies from different groups provided detailed evidence that parkin is a cytoprotective protein. It confers an anti-apoptotic effect to cells in various toxic paradigms. As an example, parkin is able to protect cells from dopamine-induced apoptosis and from excitotoxicity conveyed by kainate (Staropoli et al., 2003, Jiang et al., 2004). In addition, parkin displays neuroprotective activity *in vivo*: we were able to show that human parkin reduces the number of TUNEL-positive neurons in the spinal cord of zebrafish upon exposure to acute proteotoxic stress induced by heat-shock (Fett et al., 2010). However, the connection of parkin-mediated cytoprotection and parkin-dependent mitophagy had not been experimentally addressed this far. In principle, it is feasible that parkin exhibits its cytoprotective potential through the degradation of dysfunctional mitochondria. Removal of mitochondria that have lost their membrane potential could prevent the release of pro-apoptotic factors from these defective organelles. As an example, cytochrome c release from mitochondria is an important step in apoptosis induction, and a recent study was able to show that parkin is capable of preventing the release of cytochrome c (Berger et al., 2009). We wanted to address the connection of parkin-induced cytoprotection and mitophagy. To examine whether parkin-mediated mitophagy is sufficient to explain the cytoprotective capacity of parkin, different cellular toxins were investigated for their potential to induce mitophagy. Toxins with differential modes of action were chosen to examine a broad spectrum of cellular stress conditions. Chemicals that have a direct impact on mitochondrial membrane potential (such as the ionophores CCCP and valinomycin) effectively trigger parkin recruitment to dysfunctional mitochondria and initiate mitophagy. Importantly, parkin displays anti-apoptotic properties in response to CCCP treatment using identical experimental conditions (Bouman et al., 2011). However, cytotoxins that indirectly impair mitochondrial membrane potential did not result in a recruitment of parkin to mitochondria: the PD-associated toxins 1-methyl-4-phenylpyridinium (MPP^+) or paraquat did not trigger parkin translocation to mitochondria or mitophagy although they inhibit mitochondrial

oxidative phosphorylation and increase the amount of intracellular reactive oxygen species (ROS). Previous studies implicated that parkin is cytoprotective in cells exposed to MPP^+ or paraquat (Wang et al., 2005a), another indication that mitochondrial degradation via mitophagy is independent of parkin-mediated cytoprotection. In addition, iron ions did not induce mitophagy. Elevated brain iron levels have been described as a risk factor for PD development; PD prevalence is higher in people exposed to iron, e.g. among welders and farmers (reviewed in (Sian-Hulsmann et al., 2011). Parkin-dependent mitophagy, however, is not stimulated by iron ions. Thapsigargin, a selective inhibitor of the SERCA-ATPase, results in the elevation of cytosolic calcium levels. Treatment of cells with thapsigargin promotes acute mitochondrial hyperelongation as a compensatory cellular stress response. However, upon prolonged exposure to ER stress induced by thapsigargin, the mitochondrial network fragments. Parkin is both able to prevent thapsigargin-induced mitochondrial fragmentation and subsequent cell death (Bouman et al., 2011). Nonetheless, we were able to demonstrate that mitochondrial degradation is not triggered by thapsigargin, suggesting independent mechanisms of action. In favor of this hypothesis are unpublished findings from our group that PINK1 is dispensable for parkin-mediated cytoprotection (Kathrin Lutz, unpublished data). In PINK1-deficient mouse embryonic fibroblasts, parkin is able to prevent stress-induced apoptosis. In contrast, the findings presented here demonstrate that no mitophagy is induced in the absence of parkin. In summary, parkin is a stress-protective protein, conferring anti-apoptotic properties to cells exposed to a wide spectrum of toxins. Nevertheless, the mechanism of parkin action is not completely understood. The notion that mitophagy is not trigged by cellular toxins indirectly affecting the mitochondrial membrane potential leads to the conclusion that parkin-mediated mitophagy is not the common denominator of parkin cytoprotective activity under various conditions of cellular stress.

The E3 ubiquitin ligase parkin contains an N-terminal ubiquitin-like (UBL) domain and a C-terminal RBR (RING-between RING-RING) domain essential for ligase activity. The experimental results presented in this study provide evidence that the UBL domain of parkin is dispensable for parkin recruitment to mitochondria and subsequent mitophagy. The ΔUBL parkin mutant effectively translocated to mitochondria upon exposure to CCCP and triggered degradation of dysfunctional mitochondria to the same extent as wildtype parkin. Interestingly, a parkin species lacking the UBL domain is found *in vivo*, translated from an alternative start codon at amino acid 80 (Henn et al., 2005). This smaller parkin protein is detected in cell culture and in samples of human brain, assuming a function for this protein in humans. In contrast, deletion of any other domain of parkin resulted in partial parkin insolubility and cytosolic aggregate formation. These domain deletion mutants all displayed a loss-of-function phenotype in mitophagy initiation. Although this implicates an essential role of the C-terminal RBR domain and thus a functional E3 ligase activity, no definite conclusion can be made from the interpretation of the domain deletion mutant experiments: the parkin protein is prone to misfolding and aggregate formation (Henn et al., 2005, Winklhofer et al., 2003, Hampe et al., 2006, Wang et al., 2005a, LaVoie et al., 2005, LaVoie et al., 2007). Yet small modifications of the parkin coding sequence or the addition of an epitope tag can interfere with parkin folding and inactivate parkin function. Deletion of whole domains might therefore prevent proper parkin folding leading to a dysfunctional protein. In fact, the ΔUBL

parkin is the only deletion mutant that is fully soluble under basal conditions comparable to the wildtype protein (unpublished results from our group).

It has been demonstrated that the E3 ligase activity of parkin is crucial for the cytoprotective properties of the protein. Most pathogenic parkin mutants are impaired in the ubiquitination of substrates or in autoubiquitination and do not protect cells from apoptosis induced by 2-mercaptoethanol or tunicamycin (Imai et al., 2000). Besides the importance of the E3 ubiquitin ligase activity for parkin-mediated cytoprotection, it seems reasonable that the function of parkin as an ubiquitin ligase plays a crucial role in mitophagy as well. Following translocation of parkin to dysfunctional mitochondria, a robust ubiquitination is found at mitochondria. However, the exact substrates of ubiquitination remain to be determined. Different hypothetic scenarios are thinkable: 1) Parkin directly ubiquitinates one or several mitochondrial substrates at the outer mitochondrial membrane (OMM). This is in line with recently published data proposing mitofusin 1 and 2 (Mfn 1/2) as substrates for parkin-mediated ubiquitination and degradation (Rakovic et al., 2011, Gegg et al., 2010, Gegg and Schapira, 2011, Tanaka et al., 2010, Ziviani et al., 2010, Poole et al., 2010). Data of another study further supports this hypothesis. Using the stable isotope labeling by amino acids in cell culture (SILAC) technology the authors of this study found a variety of OMM proteins ubiquitinated by parkin and degraded following the exposure to CCCP (Chan et al., 2011). 2) Parkin function might also indirectly influence mitochondrial ubiquitination. It is possible that parkin ubiquitinates a specific, so far unidentified, substrate which in turn triggers a signaling cascade resulting in mitochondrial ubiquitination. 3) *In vitro* studies demonstrated that parkin reveals autoubiquitination activity (Imai et al., 2000, Zhang et al., 2000, Tanaka et al., 2010, Shimura et al., 2000). Conceivably, parkin autoubiquitination represents a degradation signal which in turn recruits the mitophagic machinery to mitochondria. 4) In addition, it is feasible that parkin might be ubiquitinated by another ubiquitin ligase. Ubiquitination of parkin might change functional properties of the parkin protein, resulting in parkin translocation to mitochondria.

Results from the analysis of pathogenic parkin mutants provide further evidence for the importance of parkin E3 ligase activity in mitophagy. During this study it was observed that point mutations in the UBL domain did not affect the mitophagic activity of parkin. However, this can be a result of the presence of a smaller parkin species lacking the UBL which is fully capable of mitophagy initiation (see 3.1.5). Different studies have yielded contradictory results on the capability of parkin UBL mutants on mitophagy: our findings are consistent with reports from Geisler and coworkers demonstrating that the two pathogenic point mutants of parkin, P37L and R42P, are fully functional in initiating mitophagy (Geisler et al., 2010a). In contrast, the R42P mutant was found to partially impair the mitophagic activity of parkin in two different recent studies (Lee et al., 2010, Matsuda et al., 2010). Thus, more elaborate examinations need to unravel the role of the UBL domain in mitophagy. A possibly interesting clue may be derived from a recent *in vitro* analysis of the parkin UBL domain. The experimental data from this group suggest that the UBL domain of parkin functions to inhibit its autoubiquitination. Moreover, pathogenic parkin mutations within the UBL disrupt this autoinhibition, resulting in a constitutively active molecule (Chaugule et al., 2011). In contrast

to the UBL parkin mutants that were functional in our mitophagy assay, several point mutants present in the RBR domain severely compromised parkin function. The pathogenic mutant G430D (point mutation in the RING 2 domain of parkin) was soluble under basal conditions similar to the wildtype protein but did not colocalize with mitochondria upon depolarization of the mitochondrial membrane potential. This suggests that the mutant protein is impaired in mitochondrial recruitment. Other pathogenic parkin mutants analyzed displayed cytosolic aggregate formation and lost the ability to translocate to mitochondria as a consequence. *In vitro* studies demonstrated that most pathogenic parkin mutants are impaired in E3 ubiquitin ligase activity (Shimura et al., 2000, Matsuda et al., 2006), which can be an alternative explanation for loss of mitophagic activity. Interestingly, the analysis of the pathogenic parkin mutant W453X revealed that this mutant is defective in mitochondrial recruitment upon CCCP treatment. The W453X mutant lacks only 13 C-terminal amino acids without affecting the integrity of the RBR domain. It has been demonstrated that the E3 ligase activity of this mutant is impaired in an *in vitro* assay using a recombinant maltose-binding protein (MBP) fused to parkin as a pseudosubstrate (Matsuda et al., 2006).

The ubiquitination pattern at dysfunctional mitochondria mediated by parkin is characterized by both K48- and K63-linked polyubiquitin chains. Linkage of polyubiquitin moieties through the lysine residue K48 is a typical trigger for proteasomal degradation. K63-linked polyubiquitin chains have been implicated in various cellular signaling processes and in particular selective autophagy such as mitophagy. The findings are in accordance with the notion that both proteasomal and lysosomal inhibition impair mitophagy but do not affect parkin translocation to mitochondria. The results indicate that the two cellular degradation systems ubiquitin-proteasomal system (UPS) and autophagy are crucial for successful completion of mitophagy. Recently, other research groups have published their experimental data on the pattern of parkin-mediated ubiquitination in mitophagy: a study by Geisler and coworkers indicates that voltage-dependent anion channel 1 (VDAC1) is tagged by K27- and K63-linked polyubiquitin via parkin (Geisler et al., 2010a), although the relevance of VDAC1 ubiquitination for mitophagy is controversially discussed (Narendra et al., 2010a, Chan et al., 2011). Investigation of parkin-mediated ubiquitination via the SILAC technology unraveled the presence of K48-linked polyubiquitin chains (Chan et al., 2011). However, a lot of debate is still ongoing concerning the substrate/s of parkin and the pattern of ubiquitination. Nonetheless profound evidence shows that both UPS and autophagy are involved in mitophagy. As an example, the group of Richard Youle presents evidence for an involvement of p97 and the proteasome in mitophagy (Tanaka et al., 2010). The AAA+ ATPase p97 is a cytosolic chaperone that has been found to translocate to dysfunctional mitochondria in the presence of parkin. P97 is thought to chaperone OMM proteins for proteasomal degradation. Additional evidence for UPS function in mitophagy was obtained by the notion that the ubiquitin mutant K48R, which is defective in the assembly of K48-linked polyubiquitin chains, impairs parkin-dependent mitophagy (Chan et al., 2011).

One very interesting observation results from the analysis of ATG5 KO MEFs, which are incapable of macroautophagy (see section 3.1.7). ATG5 is an essential component of the autophagic machinery (Kuma et al., 2004). When ATG5-deficient cells were tested for the

ability to degrade the OMM protein TOM20 in response to CCCP, no difference was observed compared to wildtype control cells, suggesting that autophagy is dispensable for mitochondrial degradation. However, when the mitochondrial matrix protein HSP60 was analyzed instead, no degradation of this protein was observed in cells lacking ATG5. In line with the observation that both proteasomal function and autophagy are required for mitochondrial degradation the findings indicate a differential degradation mechanism of OMM and matrix proteins. In addition, the proteasomal degradation of OMM proteins seems to be independent and upstream of lysosomal degradation.

The current experimental data support the following working model: Upon dissipation of the mitochondrial membrane potential, parkin is recruited to dysfunctional mitochondria. Parkin subsequently either directly or indirectly ubiquitinates and induces the proteasomal degradation of OMM proteins. This remodeling of the OMM surface is then supposed to be a trigger for the subsequent engulfment of the mitochondria into autophagosomes and degradation in lysosomes, although these steps are not completely understood. So how is parkin recruited to impaired mitochondria? The analysis of PINK1-deficient mouse fibroblasts established PINK1 as a key player in parkin-mediated mitophagy (see section 3.1.8). Indeed, several groups agree with the idea that PINK1 is responsible for recruitment of parkin to mitochondria (Geisler et al., 2010a, Geisler et al., 2010b, Jin et al., 2010, Kawajiri et al., 2010, Matsuda et al., 2010, Narendra et al., 2010b, Vives-Bauza et al., 2010, Kim et al., 2008). PINK1 is readily cleaved and degraded under basal conditions. However, when mitochondrial membrane potential is lost, PINK1 accumulates at dysfunctional mitochondria which in turn triggers parkin translocation. Although it is common sense that PINK1 is responsible for parkin recruitment to mitochondria, the exact mechanism still remains elusive. Some evidence points towards a potential direct interaction of parkin and PINK1 (Shiba et al., 2009); additional *in vitro* data suggest that the two proteins may also modify each other: PINK1 could phosphorylate parkin, or parkin could ubiquitinate PINK1 (Sha et al., 2010, Kim et al., 2008, Shiba et al., 2009, Um et al., 2009). PINK1 levels appear to be limiting in mitophagy. On the one hand, no mitophagy occurs in PINK1-deficient cells (see section 3.1.8) and is perturbed upon transient siRNA-mediated knockdown of PINK1 (Geisler et al., 2010b, Geisler et al., 2010a, Vives-Bauza et al., 2010). On the other hand, overexpression of PINK1 is sufficient to induce parkin-mediated mitophagy independent of mitochondrial membrane potential (see section 3.1.9). Furthermore, mitochondrial targeting of PINK1 is crucial for mitophagy. A PINK1 mutant lacking the C-terminal mitochondrial targeting sequence (MTS) did not induce parkin translocation to mitochondria with dissipated membrane potential and was deficient in initiating mitophagy (see section 3.1.9 and unpublished data from our group). These findings are accordant with the results from Narendra and coworkers, demonstrating that an artificial PINK1 mutant anchored to the OMM via the MTS and TM domain of Opa-3 induces mitophagy to a greater extent than wildtype PINK1 (Narendra et al., 2010b).

Interestingly, the data obtained in this study suggest parkin folding to be essential for mitophagy induction. The parkin protein is soluble under basal conditions. Surprisingly, parkin shifted into the detergent-insoluble fraction when cells were treated with the

protonophore CCCP. This biochemical alteration of parkin could reflect a structural change of the protein accompanied by a functional modification of the protein. Not only CCCP, but also PINK1 levels influence parkin solubility: overexpression of PINK1 affects parkin solubility. In the absence of PINK1, CCCP can no longer trigger parkin insolubility. Hence, the differential solubility of parkin seems to be crucial for mitophagy, although the underlying mechanism is unclear. It is possible that parkin adopts a misfolded conformation at mitochondria which in turn is a stimulus that tags dysfunctional mitochondria making them accessible for subsequent degradation. Additionally, it is feasible that parkin function as an E3 ubiquitin ligase is relieved only upon translocation to mitochondria and interaction with PINK1. As an example, the interaction of PINK1 could sterically modify parkin in a way that E3 ligase activity is triggered. This is in agreement with a recent finding, that parkin might be inactive when present in the cytosol and exhibit its E3 ligase activity only upon translocation to mitochondria (Matsuda et al., 2010).

One of the first steps in mitophagy is the recruitment of parkin to dysfunctional mitochondria. Our analysis of mitoparkin, an artificial chimeric protein anchoring parkin to the OMM, revealed that mitochondrial recruitment of parkin is not sufficient to induce mitophagy (see section 3.1.14). Nevertheless, mitoparkin is functional: when mitochondrial membrane potential is lost, mitoparkin triggers mitochondrial removal. The functionality of mitoparkin was shown in a parkin-deficient background, for instance in HeLa cells that lack endogenous parkin. This implicates that another stimulus besides mitochondrial targeting of parkin might be necessary to induce mitophagy. Parkin folding is eligible to play a role. Unfortunately, mitoparkin folding cannot be assessed following CCCP treatment; mitoparkin is already insoluble in detergents under basal conditions. Most likely this is due to the addition of the TOM20 TM domain which is highly hydrophobic and might interfere with the solubility of the chimeric protein in detergents. Of note, with the detergent conditions used in these experiments, endogenous TOM20 protein was partially insoluble as well (own, unpublished data). Surprisingly, mitoparkin was not able to induce mitophagy in PINK1-deficient cells, neither under basal conditions nor when the mitochondrial membrane potential was abolished. These results point towards an alternative role of PINK1 in parkin-dependent mitophagy aside from parkin recruitment to mitochondria, the nature of it having to be solved. Hypothetically, PINK1 kinase activity could be necessary for activation of parkin E3 ligase activity; it has been proposed that PINK1 can mediate phosphorylation of parkin (Kim et al., 2008, Sha et al., 2010), although other groups do not find supporting evidence for this scenario (Vives-Bauza et al., 2010). In addition, recent findings suggest that the E3 ligase activity of parkin is only released upon translocation of parkin to mitochondria (Matsuda et al., 2010, Lazarou et al., 2012) and this could in principle be mediated through the action of PINK1.

Different pathogenic mutations in parkin or PINK1 interfere with the degradation of dysfunctional mitochondria via mitophagy (see section 3.1.4 and (Geisler et al., 2010b, Geisler et al., 2010a)). Various pathogenic parkin mutants are prone to misfolding and form cytoplasmic aggregates that disable parkin translocation to mitochondria. In addition, E3 ligase activity is possibly impaired as discussed before. However, treatment of cells with

CCCP or overexpression of PINK1 induces alterations in the biochemical properties of parkin, leading to parkin insolubility in detergents (see sections 3.1.12 and 3.1.13). In addition, pathogenic PINK1 mutants are defective in altering parkin solubility and in inducing parkin-mediated mitophagy (see section 3.1.13). Reduced solubility of parkin in detergents has been observed previously, although in a different context, and is a biochemical read-out for parkin misfolding (see section 3.2.1 and (Winklhofer et al., 2003, Henn et al., 2005, Schlehe et al., 2008)). Thus, it could be speculated that a conformational change of parkin at mitochondria, possibly even misfolding of parkin, could trigger the removal of dysfunctional mitochondria. Nevertheless, the nature of this conformational change has to be addressed in more detail. Structural studies on parkin are hampered by the fact that parkin is prone to misfolding and could not be crystallized so far. The only available structural data of parkin is the three-dimensional folding of the parkin UBL domain which resembles ubiquitin both in its primary amino acid sequence (61 % of identical amino acids between ubiquitin and the parkin UBL domain) and its three-dimensional structure (Sakata et al., 2003, Tashiro et al., 2003, Tomoo et al., 2008).

Mitophagy is commonly quantified in indirect immunofluorescence using parkin overexpressing cells that are treated with CCCP and stained for a mitochondrial marker protein. This assay however, has certain drawbacks, so the results have to be interpreted carefully. First, the uncoupling agent CCCP is a chemical drug inducing an acute and drastic depolarization of mitochondrial membrane potential. This situation is quite remote from *in vivo* conditions appearing in PD patients, where a slow and moderate decrease in mitochondrial membrane potential may occur over several decades during disease development and progression. Besides CCCP, only reagents that directly uncouple mitochondrial membrane potential have been successfully tested in the *in vitro* mitophagy assay. Of note, drugs that directly cause a parkinsonian phenotype in rodents, monkeys, and humans such as rotenone, paraquat or MPP^+, do not induce parkin recruitment or mitophagy in cell culture experiments (see section 3.1.3). Second, the majority of studies investigating mitophagy rely on single cell analysis of cells stained with the mitochondrial marker protein TOM20. The outer mitochondrial membrane (OMM) protein TOM20 has been suggested to be degraded via an UPS-dependent mechanism, and might not be a reliable marker to analyze downstream autophagy-dependent mechanisms (Chan et al., 2011). The results from this study corroborate this idea: mitochondrial staining subsided in ATG5-deficient fibroblasts upon CCCP treatment when TOM20 staining was analyzed; contrarily, mitophagy was found to be abolished when the mitochondrial matrix protein HSP60 was analyzed instead (see section 3.1.7). Another drawback of the mitophagy studies is the use of protein overexpression to analyze parkin translocation to mitochondria and subsequent mitochondrial degradation. HeLa cells and most established mouse embryonic fibroblast cell lines do not express endogenous parkin; the neuroblastoma cell line SH-SY5Y expresses parkin, but only at very low levels when undifferentiated. The potential of endogenous parkin to induce mitophagy has been demonstrated in SH-SY5Y cells, although the effect is weaker and displays a slower kinetic (Lee et al., 2010, Van Humbeeck et al., 2011, Van Laar et al., 2011). Nevertheless, the use of HeLa cells is especially valuable for the analysis of parkin mutants as endogenous wildtype parkin could possibly distort the experimental results (see

sections 3.1.4 and 3.1.5). When mitophagy is analyzed in cell culture, pathogenic parkin mutants display an impairment of parkin translocation to dysfunctional mitochondria and subsequent mitophagy. It will now be important to analyze the impact of these mutations for the patients. As an example, fibroblasts from PD patients could be investigated in the context of parkin translocation to mitochondria and mitophagy efficiency upon uncoupling of the mitochondrial membrane potential. Not only PD patients with mutations in parkin or PINK1 might be reasonable to examine, but also fibroblasts from sporadic PD patients or patients with mutations in other PD genes to see whether parkin-mediated mitophagy is affected. One recent study addressed the impact of PINK1 mutations on parkin function and *vice versa* in fibroblasts derived from PD patients (Rakovic et al., 2010). Interestingly, translocation of parkin to dysfunctional mitochondria is abrogated in dopaminergic neurons differentiated from induced pluripotent stem cells (iPS cells) derived from PD patients carrying PINK1 mutations (Seibler et al., 2011).

Although quite a number of studies have investigated the role of parkin in mitophagy, the *in vivo* relevance of the findings is mostly obscure. Neurons have an especially high energy demand and rely on functional mitochondria. The initial studies of parkin-mediated mitophagy were all conducted in established tumor cell lines, thus it is of special interest to check the importance of this pathway for neurons. Tumor cells produce energy mainly through glycolysis and do not depend on oxidative phosphorylation. In contrast, ATP generation in nerve cells requires efficient energy production through oxidative phosphorylation and consequently, a functional mitochondrial network. Furthermore, mitochondrial quality control mechanisms are required especially in post-mitotic cells such as neurons (reviewed in (Detmer and Chan, 2007)). Therefore, sensing and subsequent degradation of dysfunctional mitochondria via the mitophagy pathway would be a feasible mechanism in neuronal quality control. Surprisingly, a study in primary rat cortical neurons and mixed striatal/midbrain neuronal cultures failed to observe parkin recruitment to mitochondria following CCCP treatment (Van Laar et al., 2011). However, in line with other reports, parkin translocation was observed when HeLa cells were examined. Notably, mitochondrial recruitment of parkin depended on the bioenergetical status of cells – when HeLa cells were forced to produce their energy through oxidative phosphorylation instead of glycolysis, parkin translocation to mitochondria was completely abolished (Van Laar et al., 2011). Furthermore, Larsson and coworkers recently reported on a mouse model enabling to study mitochondrial translocation of parkin *in vivo* (Ekstrand et al., 2007): A conditional knockout of the mitochondrial transcriptional factor A (TFAM) in dopaminergic neurons of mice results in mitochondrial DNA depletion and severe dysfunction of the respiratory chain (Sterky et al., 2011). Dopaminergic neurons in these mice display enlarged, rounded mitochondria in the perinuclear region. Nonetheless, parkin did not translocate to these dysfunctional organelles *in vivo*. Besides, additional deletion of parkin in dopaminergic neurons lacking TFAM did not exacerbate the observed pathology in these mice. Another study showed that parkin mediates protection of dopaminergic neurons *in vivo* against chronic MPTP toxicity, but does not influence Tom20 levels (Yasuda et al., 2011). In addition, exogenous parkin was only scarcely located at mitochondria in this mouse model, another indication that the cytoprotective function of parkin is probably independent of mitophagy. Thus, the so far

obtained results do not support an essential role of parkin-mediated degradation of mitochondria *in vivo*. However, one very recent report suggests that parkin might be able to translocate to mitochondria in primary neurons, although the kinetics of parkin recruitment to mitochondria is much slower than in tumor cell lines (Cai et al., 2012). In summary, the role of parkin-dependent mitophagy for neuronal homeostasis is still heavily debated and requires further experimental investigation. Interestingly, parkin is ubiquitously expressed and mitochondrial pathology is not restricted to dopaminergic neurons in *Drosophila* parkin mutants (Clark et al., 2006, Greene et al., 2003) and PD patients (reviewed in (Schapira, 2008, Schapira and Gegg, 2011), suggesting that parkin serves important cellular functions outside of the central nervous system. Further research on this topic will be necessary to shed light on the impact of the mitophagy pathway in nerve cells and peripheral tissue and the role of parkin in this context.

4.2 Degradation of pathogenic parkin mutants

Proteinopathies refer to a class of protein misfolding diseases characterized by abnormal protein aggregation in different organs and tissues of the body. Proteinopathies include a wide range of diseases such as type 2 diabetes, Alzheimer's disease and PD. Lewy bodies, the intracellular protein aggregates found in PD patients, mainly consist of α-synuclein, thus PD is categorized as a "synucleinopathy". Besides α-synuclein, a variety of other proteins can be found in Lewy Bodies, including ubiquitin, neurofilament, heat-shock proteins, and also parkin is found in these intracellular inclusions (Schlossmacher et al., 2002). Protein aggregates are a pathological hallmark of various neurodegenerative diseases; aggregates of β-amyloid (Aβ) and the microtubule-associated protein tau are present in Alzheimer's disease, TAR DNA-binding protein 43 (TDP-43) protein aggregates are found in frontotemporal lobar degeneration (FTLD) and amyloidotrophic lateral sclerosis (ALS), mutant huntingtin accumulates in Huntington's disease and conversion of the prion protein (PrP) from PrP^{c} to PrP^{Sc} causes transmissible spongiform encephalopathies (also known as prion diseases). Protein aggregates can confer a toxic gain-of-function, but also induce a loss-of-function when proteins essential for cellular functions are sequestered in these aggregates. To date, it is not definitely clarified if protein aggregates are beneficial or detrimental for cell survival (reviewed in (Winklhofer et al., 2008)). Although the protein aggregates in different diseases consist of distinct proteinaceous components, they share some common features: extensive ubiquitin staining is often observed at protein inclusions; pathological aggregates usually consist of fibers containing misfolded protein with a β-sheet conformation, termed amyloid; mutations in aggregation-prone proteins can directly cause the disease as it is often the case in inherited forms of the different diseases, however sporadic forms can occur. In each of these cases, the inclusions are composed of a protein that is tightly connected with the cause of the illness. Another common feature of intracellular inclusions is the presence of p62 at these protein aggregates. The ubiquitin-binding protein

p62 frequently colocalizes with protein aggregates and is used as a marker protein for the staining of aggregates in several neurodegenerative diseases (Zatloukal et al., 2002, Kuusisto et al., 2008, Kuusisto et al., 2001). For instance, p62 colocalizes with mutant huntingtin aggregates in Huntington's disease (Bjorkoy et al., 2005) and in *in vitro* and *in vivo* models of expanded polyglutamine huntingtin (Nagaoka et al., 2004). In ALS patients, p62 immunoreactivity is found at pathological inclusions in anterior horn cells of the spinal cord (Mizuno et al., 2006). Multiple system atrophy (MSA) is pathologically characterized by the formation of cytoplasmic inclusions in oligodendrocytes containing α-synuclein, and p62 staining can be detected in these inclusions (Chiba et al., 2011). In addition, a robust colocalization of p62 and Lewy bodies is observed in the *substantia nigra* of PD patients (Kuusisto et al., 2003). Nevertheless, it remains to be determined what the physiological function of p62 at these various protein inclusions is.

Pathogenic mutations in the parkin gene can result in misfolding of the parkin protein and subsequent aggregate formation. Biochemically, protein aggregation can be experimentally determined by the formation of aggregates visualized by indirect immunofluorescence or the analysis of protein solubility in different detergents such as a combination of ionic or non-ionic detergents, urea or sodium dodecylsulfate (SDS). Data from several groups and our group determined reduced detergent-solubility and scattered aggregate formation of several pathogenic parkin mutants (Winklhofer et al., 2003, Henn et al., 2005, Sriram et al., 2005, Wang et al., 2005a, Wang et al., 2005b). However, there is limited understanding how cells deal with these aggregates and by which mechanism(s) they are degraded. The experimental data within this thesis demonstrate the formation of intracellular aggregates of mutant parkin while wildtype parkin is homogenously distributed throughout the cytosol (see section 3.2.1). However, wildtype parkin is prone to misfolding induced by severe cellular stress conditions. Exposure of cells to toxins such as dopamine, MMP^+, and peroxide can lead to misfolding and aggregate formation of wildtype parkin (Meng et al., 2011, LaVoie et al., 2007, LaVoie et al., 2005, Winklhofer et al., 2003, Um et al., 2010, Wong et al., 2007). Even proteasomal inhibitors have been found to induce aggregation of wildtype parkin (Ardley et al., 2003, Junn et al., 2002, Muqit et al., 2004); however, aggregate formation of wildtype parkin was never observed when proteasomal or lysosomal inhibitors were applied in the experiments presented in this study. This is probably due to the low concentrations of inhibitors used in our experimental setups. The results provided within this thesis do not include the analysis of wildtype parkin degradation when parkin misfolding is induced by cellular toxins. Although it has been demonstrated that wildtype parkin aggregates upon severe cellular stress and is subsequently degraded by autophagy (LaVoie et al., 2005), it was not investigated before whether the same pathway is employed for the removal of mutant parkin species. Two major cellular degradation systems are feasible to mediate the removal of parkin aggregates: the ubiquitin-proteasomal system (UPS) and the autophagosomal-lysosomal system (autophagy). The UPS system preferentially degrades small and short-lived proteins. In contrast, autophagy is used for the removal of stable proteins, whole organelles and various protein aggregates. Due to the large size of protein aggregates it was reasonable to speculate that autophagy plays a role in the degradation of mutant parkin aggregates. Indeed, when the subcellular localization of the autophagy

adaptor protein p62 was analyzed, a robust colocalization of mutant parkin aggregates and p62 staining was detected (see section 3.2.2). Using indirect immunofluorescence, p62 was often sequestered to the aggregated material, with the rest of the cytoplasm being frequently completely devoid of p62 immunoreactivity.

The p62 protein contains a C-terminal ubiquitin-associated domain (UBA) which enables binding to ubiquitinated substrate proteins and an LC3 interacting region (LIR domain) which mediates binding to autophagosomes (Pankiv et al., 2007). Although p62 has been used as a bona fide marker protein to monitor autophagy, the results obtained in our study provide a link to proteasomal degradation of parkin mutants. The experimental evidence provided herein points towards a role for the protein in targeting of substrates for proteasomal degradation. Although sparse, some findings from other research groups also point towards a role of p62 in proteasomal degradation of substrate proteins. For instance, it has been proposed that p62 regulates tau levels by targeting the tau protein for proteasomal degradation (Babu et al., 2005, Ramesh Babu et al., 2008, Seibenhener et al., 2004). There is evidence that p62 can directly bind to the proteasomal subunit Rpt1 (Geetha et al., 2008), one of the six ATPases of 19S regulatory particle of the 26S proteasome. It seems conceivable that p62 is capable of mediating protein degradation both via the UPS and autophagy. Nevertheless, it remains to be demonstrated how the decision between proteasomal degradation and autophagy is made in the cellular context. What exactly determines whether aggregated proteins are degraded by macroautophagy or whether they are subject to proteasomal degradation? Are other proteins involved in decision-making? Does it depend on the function of additional factors? One such factor could be neighbor of BRCA1 gene 1 (Nbr1), another adapter protein that shares structural homology with p62 and has been found to colocalize to Lewy bodies and other pathophysiological aggregates similar to p62 (Odagiri et al., 2012). Or do protein modifications such as ubiquitination, phosphorylation or sumoylation play a decisive role? All of these questions will have to be addressed in the future to provide a better understanding of the mechanism why protein aggregates are differentially degraded depending on the protein composition of aggregates, the type of disease and the tissue affected.

Autophagy has been found dispensable for the degradation of mutant parkin aggregates in our study (see section 3.2.5). First, inhibition of lysosomal acidification by bafilomycin A1 did not affect the amount of cellular aggregates. Second, the abundance of parkin aggregates did not differ from wildtype cells when fibroblasts deficient of ATG5 were examined. ATG5 is an essential component of the autophagic machinery. However, these results have to be interpreted carefully: recently, the identification of a novel autophagy pathway functioning independently of ATG5 has promoted the idea that low levels of autophagy can still occur even in the absence of ATG5 protein (Nishida et al., 2009). Nevertheless, ATG5-deficient cells display major impairments in the execution of general autophagy, and the crucial involvement of ATG5 has been demonstrated both *in vivo* and in cell culture experiments (Hara et al., 2006, Kuma et al., 2004). Additional evidence for the notion that autophagy is not required for removal of parkin aggregates originates from the analysis of autophagy marker proteins in indirect immunofluorescence (see section 3.2.5). No colocalization of

parkin aggregates with lysosomal proteins was observed. In addition, aggregates were positive for immunoreactivity of K48-linked polyubiquitin, but not K63-linked polyubiquitin, suggesting proteasomal degradation rather than removal via autophagy. Nonetheless, a collaboration of the UPS and autophagy under certain circumstances is feasible. Reports in literature support the idea that substrates degraded by the UPS under normal conditions are subjected to lysosomal degradation in case of impaired proteasomal function (reviewed in (Zheng et al., 2009)).

How are misfolded parkin mutants recognized by p62? Two possible scenarios are thinkable: First, p62 is able to recognize polyubiquitinated proteins via its UBA domain (Wooten et al., 2005, Long et al., 2008). Mutant parkin is heavily ubiquitinated (unpublished data from our group), thus it is possible that p62 binds polyubiquitin chains that are covalently attached to the mutant parkin protein. However, the nature of the linkage of ubiquitin chains at parkin is not completely solved. The results from the immunofluorescence experiments suggest that lysine K48, but not lysine K63 is exploited for building the polyubiquitin chains found at parkin aggregates. In addition, differently conjugated polyubiquitin could also be present, such as K27-linked ubiquitin, or possibly monoubiquitination as well. In principle, one ubiquitin moiety contains seven lysine residues, all of which can be employed for the assembly of polyubiquitin chains. Additionally, only recently linear ubiquitination has been identified as an alternative mode of polyubiquitination, linking the N- and the C-termini of ubiquitin residues to form a head-to-tail linear polyubiquitin chain (Kirisako et al., 2006). Thus, a plethora of putative modes of ubiquitin linkages is hypothetically possible. Besides this, other ubiquitinated components aside of parkin might be present in aggregates which could give rise to the ubiquitin signal. Furthermore, it is not clear whether p62 can bind all types of ubiquitinated proteins with similar affinity or if the linkage pattern of the polyubiquitin chains impacts the p62 interaction. Alternatively, p62 could directly interact with parkin through a classical UBA/UBL interaction. The UBA domain of p62 is capable of binding to UBL domains, such as the UBL domain found in the parkin protein, although the interaction of the two proteins still requires experimental proof. The UBL of parkin shares a high sequence homology with ubiquitin and structurally resembles ubiquitin, thus it is possible that p62 is able to bind parkin directly through the UBL domain.

The establishment of stable cell lines overexpressing the parkin mutant W453X provided additional insights into the degradation of the pathogenic parkin protein. Cells were generated by lentiviral transduction, resulting in overexpression of the protein at lower levels than achieved by transient liposome-mediated transfection. The parkin mutant W453X was rapidly degraded in the stably transduced HeLa cells, resulting in low steady-state levels of the pathogenic protein. This observation implies a loss-of-function mechanism of pathology rather than a toxic gain-of-function of the mutant protein. The low expression of the mutant protein in stable cells might better reflect the situation occurring in PD patients. This is supported by the observation that the W453X parkin mutant does not confer toxicity in cell culture and in transgenic mice under basal conditions (own observations and unpublished results from our group). However, it is possible that in the course of PD, mutations in the parkin gene are not sufficient to trigger pathology. PD is a disorder where environmental

factors can play a crucial role in disease development. Thus, a detrimental interplay of genetic predisposition and environmental toxins might result in the degeneration of neurons in the *substantia nigra*. This is in agreement with experimental data from our group demonstrating that the parkin W453X mutant is not toxic to cells under basal conditions but exhibits toxicity when proteasomal function is impaired (unpublished results). Of note, a reduction of proteasomal activity has been found in patients suffering from PD (reviewed in (Cook et al., 2012)). In addition, proteasomal inhibition causes striatal degeneration in rodents (Zhu et al., 2007a, Zeng et al., 2006, Sun et al., 2006, McNaught et al., 2002b, McNaught et al., 2002a), although some doubts have been raised concerning the selective toxicity of proteasomal inhibitors on nigro-striatal neurons (Mathur et al., 2007).

The abundance of cellular p62 protein affects the quantity of parkin aggregation. Levels of mutant parkin can be stabilized both by proteasomal inhibitors or transient downregulation of p62 through RNA interference in cells stably expressing W453X parkin (see section 3.2.6). In addition, enhanced formation of mutant parkin aggregates in cells deficient for p62 is observed. Along with this, overexpression of p62 is able to reduce the percentage of cells with parkin aggregates both in fibroblasts derived from p62 knockout mice or wildtype littermates (see section 3.2.3). The results point towards an essential role of p62 in the degradation of misfolded parkin protein. However, it cannot be ruled out that other factors aside of p62 aid in the degradation of parkin mutants. In addition, the *in vivo* relevance of the findings from this study remains to be clarified. Some conclusions can be drawn from the analysis of W453X transgenic mice: p62 colocalized with parkin aggregates in *ex vivo* cultures of primary cortical neurons of these transgenic mice. In addition, upon treatment of the neurons with a proteasomal inhibitor, protein levels of W453X parkin were elevated, suggesting that mutant parkin protein is indeed removed by proteasomal degradation *in vivo*.

The results obtained in this study provide a novel link between proteasomal degradation of pathogenic parkin mutants and the adapter protein p62. Understanding the mechanism by which aggregates of mutant parkin are removed from cells might aid the development of strategies to overcome the cellular protein overload and eventually prevent the detrimental cascade ultimately provoking neuronal demise. It might furthermore be interesting to investigate if p62 is able to mediate the degradation of other aggregation-prone proteins that are present in various neurodegenerative diseases. The ultimate goal would be the development of therapeutic strategies to halt or even prevent the occurrence of proteinopathies.

5 Material

For all experiments, standard laboratory equipment was employed. All chemicals were purchased from Merck, Sigma, Roth or Fluka with analytical grade p. A. (*pro analysi*) if not specified differently.

5.1 Drugs and inhibitors

chemical (abbreviation)	chemical (full name)	drug target/ application	concentration
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone	protonophore	10 – 20 µM
baf A1	bafilomycin A1	lysosomal H^+ATPase inhibitor	30 nM
epoxo	epoxomycin	proteasomal inhibitor	10 – 100 nM
TG	thapsigargin	inhibitor of SERCA ATPase	10 µM
valinomycin	valinomycin	potassium ionophore	2 µM
oligomycin	oligomycin	inhibitor of mitochondrial ATP synthase	10 µM
Fe^{2+}	iron(II) chloride	induction of oxidative stress	5 mM
Fe^{3+}	iron(III) citrate	iron toxicity	5 mM
paraquat	*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride	oxidative stress; production of superoxide free radical	1 mM
MPP^+	1-methyl-4-phenylpyridinium chloride	inhibitor of mitochondrial respiratory chain complex I	1 mM
glutamate	glutamic acid	excitotoxicity	1 mM

5.2 Enzymes

Enzyme	Provider
Restriction enzymes	New England BioLabs, Fermentas, Roche Applied Science
Taq DNA polymerase	Roche Applied Science
Pfu DNA polymerase	Stratagene
Pwo DNA polymerase	Roche Applied Science
rapid shrimp alkaline phosphatase (SAP)	Roche Applied Science
T4 DNA ligase	Fermentas
Klenow fragment	Fermentas, Thermo Scientific

5.3 Primers and Oligonucleotides

Primers were synthesized by Thermo Fisher Scientific, HPLC purified and lyophilized. Stock solutions of 100 µM (100 pmol/µL) were prepared and stored at -20 °C.

5.4 Buffers

Running buffer for SDS-PAGE

25 mM Tris/HCl, pH 6.8

190 mM glycine

0.1 % (w/v) SDS

TBE (running buffer for agarose gel electrophoresis)

0.9 M Tris base, pH 8.3

0.9 M boric acid

20 mM EDTA

TBS

25 mM Tris/HCl, pH 7.2

150 mM NaCl

TBST

0.1 % (v/v) Tween-20 in TBS

PBS

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4 x 2 H_2O

2 mM KH_2PO_4

PBS for immunofluorescence

PBS supplemented with calcium and magnesium ($CaCl_2$ x $2H_2O$ 0.132 g/L; $MgCl_2$ x $6H_2O$ 0.1 g/L) (PAA Laboratories)

PBST

0.1 % (v/v) Tween-20 in PBS

Upper tris buffer for SDS-PAGE

0.5 M Tris/HCl pH 6.8,

0.4 % (w/v) SDS

Lower tris buffer for SDS-PAGE

1.5 M Tris/HCl, pH 8.8,

0.4 % (w/v) SDS

Blotting buffer

20 mM Tris-Base

150 mM glycine

0.01 % (w/v) SDS

20 % (v/v) methanol

Cell lysis buffer

0.1 % to 1 % Triton X-100 in PBS

Detergent-solubility assay buffer

0.5 % (v/v) Triton X-100

0.5 % (w/v) desoxycholic acid

in PBS

Laemmli sample buffer (LSB)

60 mM Tris, pH 6.8

1 % (w/v) SDS

10 % (v/v) glycerol

0.01 % (w/v) bromphenol blue

1 % 2-mercaptoethanol

Transformation buffer I (for chemically competent bacteria)

30 mM potassium acetate (CH_3CO_2K)

100 mM RbCl

10 mM $CaCl_2$

50 mM $MnCl_2$

15 % (w/v) glycerol

pH 5.8, sterilized by filtration

Transformation buffer II (for chemically competent bacteria)

10 mM MOPS (3-(*N*-morpholino)propanesulfonic acid)

75 mM $CaCl_2$

10 mM RbCl

15 % (w/v) glycerol

pH 6.5, sterilized by filtration

6x DNA Sample Buffer

0.25 % (w/v) bromphenol blue

0.25 % (w/v) xylene cyanol

30 % (v/v) glycerol

5.5 Mammalian cell lines and primary cortical neurons

Name	Origin	Specification	Culture conditions
HeLa	human cervical adenosarcoma	DSMZ # ACC 57	DMEM, 10 % fetal calf serum (FCS)
HEK293T	human embryonic kidney	DSMZ # ACC 305	DMEM, 10 % fetal calf serum (FCS)
SH-SY5Y	human neuroblastoma	DSMZ # ACC 209	DMEM GlutaMAX, 15 % fetal calf serum (FCS), non-essential amino acids
MEF	mouse embryonic fibroblasts	isolated from wildtype, ATG5 KO, PINK1 KO, p62 KO mice, all immortalized	DMEM, 10 % fetal calf serum (FCS)
primary cortical neurons	parkin W453X transgenic mice and wildtype littermates, E14.5 – E15.5	provided by Maria Funke	Neurobasal medium, B-27 serum-free supplement, NGF and bFGF (10 ng/mL)

5.6 Cell culture reagents and media

5.6.1 Reagents and media for mammalian cell culture

Reagent	Supplier
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen
DMEM/F12	Invitrogen
OptiMEM	Invitrogen
fetal calf serum (FCS)	Invitrogen
trypsin solution	Invitrogen
Lipofectamine2000	Invitrogen
Lipofectamine reagent	Invitrogen
PLUS reagent	Invitrogen

non-essential amino acids	Invitrogen
RNAiMAX	Invitrogen
siGENOME smart pool	Dharmacon

5.6.2 Media for bacteria

LB liquid medium

1 % NaCl

1 % bacto tryptone

0.5 % yeast extract

LB agar

1 % NaCl

1 % bacto tryptone

0.5 % yeast extract

1.5 % bacto agar

5.7 Antibodies

5.7.1 Primary antibodies

Antibody	Dilution	Supplier
HSP 60 (goat polyclonal) (N-20): sc-1052	1:2.000	Santa Cruz Biotechnology
Ubiquitin FK-1 (total polyubiquitin; mouse monoclonal)	1:500	Biomol
Ubiquitin Lys63-specific clone Apu3 (rabbit monoclonal)	1:500	Millipore
Ubiquitin Lys48-specific clone Apu2 (rabbit monoclonal)	1:500	Millipore
total ubiquitin (N-19): sc-6085 (goat polyclonal)	1:1.000	Santa Cruz Biotechnology
parkin PRK8 (mouse monoclonal)	1:1.000 (IF), 1:2.000 (WB)	Santa Cruz Biotechnology
parkin (rabbit polyclonal) #2132	1:500 (IF), 1:1.000	Cell Signaling

	(WB)	Technology
TOM20 (FL-145): sc-11415 (rabbit polyclonal)	1:2.000 (IF), 1:5.000 (WB)	Santa Cruz Biotechnology
TOM20 (mouse monoclonal), #612278	1.2.000	BD Transduction Laboratories
p62/SQSTM1 (rabbit polyclonal)	1:1.000 (IF), 1:3.000 (WB)	MBL (Medical & Biological Laboratories; Japan)
p62/SQSTM1 (mouse monoclonal)	1:1.000 (IF), 1:5.000 (WB)	BD Biosciences
cytochrome c (mouse monoclonal), #556432	1:500 (IF)	BD Biosciences
cytochrome c (mouse monoclonal), #556433	1:500 (WB)	BD Biosciences
LC3 clone 5F10 (mouse monoclonal)	1:500	nanoTools
active caspase-3 (rabbit polyclonal)	1:1.000	Promega
GAPDH (mouse monoclonal)	1:10.000	Ambion
calnexin (rabbit polyclonal)	1:20.000	Thermo Fisher Scientific, Stressgen
GFP (mouse monoclonal)	1:1.000	Clonetech
V5 epitope antibody (mouse monoclonal)	1.1.000 (IF), 1:2.000 (WB)	Invitrogen
LAMP1 H4A3 (mouse monoclonal)	1:2.000	Abcam

5.7.2 Secondary antibodies

Antibody (all from Invitrogen)	**Dilution**	**Application**
donkey-anti-mouse (HRP-conjugated)	1:12.000	WB
donkey-anti-rabbit (HRP-conjugated)	1:12.000	WB
goat-anti-mouse (ALEXA 488-conjugated)	1:1.000	IF
goat-anti-mouse (ALEXA 555-conjugated)	1:1.000	IF
goat-anti-rabbit (ALEXA 488-conjugated)	1:1.000	IF
goat-anti-rabbit (ALEXA 555-conjugated)	1:1.000	IF
donkey-anti-goat (ALEXA 546-conjugated)	1:1.000	IF

5.8 Plasmids

5.8.1 Commercial vectors

Name	Supplier
pcDNA3.1-Zeo(+)	Invitrogen
pcDNA3.1-Zeo(-)	Invitrogen
pcDNA6-V5/HIS-A	Invitrogen
pEYFP	Clontech
pCMV-HA	Clontech

5.8.2 Mammalian expression vectors

Expressed cDNA	Vector backbone	Reference/provider
human wildtype parkin	pcDNA3.1-Zeo(+)	Winklhofer *et al*., 2003
parkin W453X	pcDNA3.1-Zeo(+)	Winklhofer *et al*., 2003
parkin C212Y	pcDNA3.1-Zeo(+)	Dr. Lena Bouman
parkin C289G	pcDNA3.1-Zeo(+)	Dr. Lena Bouman
parkin G430D	pcDNA3.1-Zeo(+)	Dr. Iris Henn
parkin E409X	pcDNA3.1-Zeo(+)	Dr. Iris Henn
parkin ΔUBL	pcDNA3.1-Zeo(+)	Dr. Iris Henn
parkin Δlinker, ΔRING1, ΔIBR, ΔRING2	pcDNA3.1-Zeo(+)	Dr. Julia Schlehe
human p62	pcDNA3.1-Zeo(+)	Anna Pilsl and Benjamin Schwenk
mitoparkin	pcDNA3.1-Zeo(+)	Anna Pilsl and Veronika Hampl
mitoGFP (coding sequence of parkin replaced by GFP in the mitoparkin plasmid)	pcDNA3.1-Zeo(+)	Anna Pilsl

PINK1-V5	pcDNA6-V5/HIS-A	Dr. Nicole Exner
PINK1Δ78	pcDNA6-V5/HIS-A	Dr. Nicole Exner
PINK1Δ110	pcDNA6-V5/HIS-A	Kathrin Lutz
PINK1 W437X, G309P, L347P, Q126P	pcDNA6-V5/HIS-A	Dr. Nicole Exner
DJ-1-V5	pcDNA6-V5/HIS-A	Irrcher *et al*., 2010

5.8.3 Lentiviral expression vectors

Name of vector	**Reference/provider**
psPAX2	Addgene #12260, Peer Hendrik Kuhn
pcDNA3.1 (-)-VSV-G	Peer Hendrik Kuhn
FU-ΔZeo (empty vector)	Peer Hendrik Kuhn
FU-ΔZeo-parkin wildtype	Peer Hendrik Kuhn and Anna Pilsl
FU-ΔZeo-parkin W453X	Peer Hendrik Kuhn and Anna Pilsl
FU-ΔZeo-GFP	Peer Hendrik Kuhn

6 Methods

6.1 Nucleic acid techniques

6.1.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed to specifically amplify DNA, to screen bacterial clones for plasmids via colony PCR and to introduce mutations into plasmids. Primers used are specified in the material section. *Pwo* polymerase from *Pyrococcus woesei* was used for cloning applications owing to its proofreading activity.

PCR reactions were performed in a final volume of 50 µL containing the following components: 100 ng of template DNA, 10 pmol each of forward and reverse primer, 2 U of *Pwo* polymerase, and 100 µmol of dNTPs in 1 x PCR reaction buffer.

The PCR amplification was performed in three subsequent steps: first, the template DNA was denatured (94 °C; 20 s), followed by annealing of the primers to the DNA (55 °C to 72 °C; depending on their melting temperature). In a third step, elongation of the DNA was done at 72 ° C for 1 min per kilobase. Generally, 30 cycles of amplification were performed.

For mutagenesis, primer pairs containing the desired mutation and a flanking region were designed. Mutagenesis was either performed using site-directed mutagenesis (Stratagene) or a two step PCR approach.

6.1.2 Restriction digest

Restriction enzymes were used according to supplier's instructions. Double digestion was performed in 1 x TANGO buffer or NEB buffer 4 if feasible. Typically, 1 U of restriction enzyme was used for 1 µg of DNA in a total volume of 20 µL. Restriction digests were incubated for at least 1 h.

6.1.3 Agarose gel electrophoresis

DNA solutions were mixed with 6 x DNA loading buffer and analyzed via agarose gel electrophoresis (0.8 to 2 % agarose in TBE buffer, depending on DNA size analyzed) using GeneRuler 1 kbp as a standard. DNA was visualized with ethidium bromide and documented under UV light.

6.1.4 Purification of DNA from agarose and clean-up of PCR reactions

DNA fragments from agarose gels were excised with a clean scalpel and transferred to a fresh plastic tube. Purification of these fragments or clean-up of PCR reactions was performed using the NucleoSpin Extract kit (Macherey-Nagel) according to the manual.

6.1.5 Dephosphorylation of linearized vectors

In order to prevent religation of the vector backbone (especially when restriction digests were performed with a single enzyme), the terminal phosphates of the open vector were removed using rapid shrimp alkaline phosphatase (SAP). A typical dephosphorylation reaction was performed in 20 µL of 1 x phosphatase buffer with 2 U of SAP for 1 h at 37 °C.

6.1.6 Ligation of DNA fragments

DNA fragments were ligated using T4 DNA ligase. For the insertion of a PCR fragment into a vector, the DNA was incubated in a total volume of 20 µL of 1 x ligase buffer. Typically, vector and insert were used in a ratio of roughly 1:3. The ligation reaction was incubated at room temperature for 2 h or at 16 °C overnight.

6.1.7 Preparation of competent bacteria

Chemically competent *Escherichia coli* (E.coli) DH5α were prepared via the rubidium chloride method. 200 mL of LB medium were inoculated with 3 mL of a overnight E.coli culture and grown to an optical density of OD_{600} = 0.6. Cells were chilled on ice for 10 min, afterwards centrifuged at 800 g for 10 min at 4 °C. The cell pellet was resuspended in 100 mL of transformation buffer I and incubated on ice for 20 min. Following additional centrifugation (800 g; 10 min; 4 °C), the cells were resuspended in 10 mL of transformation buffer II and chilled on ice for another 60 min. Aliquots of 100 µL were transferred into sterile 1.5 mL plastic tubes, immediately frozen in liquid nitrogen and stored at -80 °C until use.

6.1.8 Transformation of bacteria and screening

For transformation, the internalization of DNA plasmids by bacteria, one aliquot of chemically competent bacteria was thawed on ice for a minimum of 10 min. The ligation mixture was added and incubated on ice for additional 10 min. Heat-shock was applied (42 °C, 30 sec), followed by chilling of the suspension on ice for 5 min. Afterwards, 0.5 mL of LB medium was added to the bacteria and the bacterial suspension was incubated for 30 min at 37 °C with agitation. 100 µL of the cell suspension were plated on agar plates containing the appropriate selection antibiotic and incubated at 37 °C until single colonies appeared.

Single bacterial colonies were inoculated into liquid LB medium and allowed to grow overnight. Screening for positive clones was either done by purification of DNA and analytical digest or by colony PCR screening.

6.1.9 DNA Sequencing

DNA sequencing reactions were performed by GATC Biotech using Sanger sequencing on the Applied Biosystems 3730xl DNA Analyzer. The obtained sequences were analyzed using CLC Main Workbench 6.1 or Vector NTI 10.1.

6.1.10 Preparative and analytical scale plasmid DNA preparation

For isolation and purification of plasmid DNA, E. coli were grown in LB medium (3 mL for analytical, 150 mL for preparative extraction) and harvested by centrifugation. DNA was isolated using the NucleoSpin Plasmid (Macherey-Nagel) or the Quiagen Midi kit according to the provided protocol. DNA concentration and purity were determined by photometric measurement using the NanoDrop photospectrometer (PeqLab).

6.2 Cell culture methods

6.2.1 Culture and maintenance of mammalian cell lines

Cell lines were cultured as monolayers in 10 mL of the appropriate medium (see material section) in 75 cm^2 tissue culture flasks. Cells were maintained at 37 °C in a humified atmosphere containing 5 % CO_2.

At regular intervals cells were passaged by trypsinization. For propagation, the cells were washed with sterile PBS and 1 mL of trypsin solution was added until the cells detached from the surface. The cells were resuspended in fresh medium and an appropriate amount of cell suspension (dilution typically ranging from 1:6 to 1:50) was added to a fresh tissue culture flask containing 10 mL of medium.

For experiments, cells were seeded in 3.5 cm or 2.0 cm cell culture dishes at a confluency of 30 to 50 %, usually the day before treatment or transfection.

For cryoconservation and long term storage, the cells were trypsinized and resuspended in fresh medium to inactivate the trypsin. Cells were pelleted by centrifugation (1.000 g, 3 min, 4 °C) and resuspended in medium supplemented with 10 % DMSO. Cell suspensions were aliquoted in 2 mL screw cap tubes and placed in an isopropanol cryofreezing container which allows freezing of the cells with a cooling rate of 1 °C/min. The tubes were subsequently stored in liquid nitrogen.

6.2.2 Preparation and cultivation of primary cortical neurons

Murine primary cortical neurons were provided by Maria Funke or Elisa Motori. Cortical neurons were cultured from mutant W453X parkin transgenic mouse embryos or non-transgenic littermates at E14.5–E15.5 days of gestation and individually processed. Cortices were dissected and dissociated with 20 U/mL of papain. Genotypes were determined by PCR of peripheral tissue. Neurons from each embryo were plated individually into dishes (1.5 million cells/3.5 cm well) coated with poly-D-lysine (100 mg/ml) in Neurobasal medium (Invitrogen) supplemented with B-27 serum-free supplement, NGF and bFGF (10 ng/mL). At three to four days *in vitro*, cortical neurons were treated with 0.1 µM or 1 µM epoxomycin for 16 h and further processed for immunofluorescence or detergent solubility assay.

6.2.3 Liposome-mediated transient transfection

Cells were plated 24 h prior to transfection. At 60 to 70 % confluency, cells were transfected using Lipofectamine2000 or Lipofectamine PLUS reagents according to manufacturer's instructions. The transfection mix was prepared in OptiMEM and added to the cells for 3 h. Thereafter, the medium was replaced by fresh medium and cells were incubated for at least 20 h prior to drug treatment or cell analysis.

6.2.4 Establishment of stable cell lines via lentiviral transduction

Cell lines stably overexpressing wildtype parkin or the pathogenic mutant W453X were generated by lentiviral transduction. To this end, the coding sequence of wildtype human parkin was subcloned into the Fu-ΔZeo vector (Kuhn et al., 2010, Lutz et al., 2012) using the restriction enzymes BamHI and NotI. An empty control vector and a vector containing the coding sequence of green fluorescent protein (Fu-ΔZeo-GFP) were cloned and provided by Peer Hendrik Kuhn. Expression of all constructs is under control of the ubiquitin C promoter.

Lentiviral particles were generated by transient cotransfection of HEK293T cells with the plasmids psPAX2 (0.5 µg), pCDNA3.1 (-)-VSV-G (0.5 µg) and FU-ΔZeo (0.75 µg), using Lipofectamine2000 in OptiMEM. The medium was replaced by fresh DMEM supplemented with 10 % FCS one day after transfection. Overnight conditioned medium was filtered through 0.45 mm sterile filters and directly added to the target cells. Cells were analyzed for transgene expression by immunofluorescence and Western blotting one week after lentiviral transduction.

6.2.5 Transient silencing of target genes using siRNA

Knockdown of target genes by RNA interference (RNAi) results in a transient and primarily gene-specific reduction in gene expression. To this end, 25 nM of small interfering RNA (stealth siRNA, Invitrogen or siGENOME SMART pool, Dharmacon) were transiently transfected using Lipofectamine RNAiMax. Knockdown efficiency for p62 protein levels was determined by immunoblotting of 10 µg of total protein, with GAPDH as a loading control. Cells were analyzed 24 h after transfection of the siRNA.

6.2.6 Cell harvest and lysis

Monolayers of cells were washed with PBS and detached from the dish surface using a cell scraper or 1 mL of trypsin solution. Afterwards, the cells were transferred to plastic tubes and pelleted by centrifugation (6.000 g, 5 min, 4 °C). Supernatant was aspired and the cell pellet was either lysed directly thereafter or stored at -20 °C until further analysis.

Whole cell lysated were prepared by direct immersion and homogenizations in Laemmli sample buffer containing 10 % (v/v) 2-mercaptoethanol (LSB). Post-nuclear supernatant was obtained by lysing cells in an appropriate volume of lysis buffer (typically 0.1 to 1 % Triton X-100 in PBS supplemented with complete protease inhibitor cocktail) on ice for 5 min. Following centrifugation (13.000 g, 1 min, 4 °C) the supernatant was transferred to a fresh

tube and supplemented with LSB. If applicable, total protein concentration was measured prior to addition of LSB using the colorimetric bicinchoninic acid assay (BCA).

6.3 Protein biochemistry

6.3.1 Measurement of total protein content

For determination of total protein concentration in cell lysates prior to immunoblot analysis, the colorimetric bicinchoninic acid (BCA) method (Pierce) was used according to manufacturer's instructions. Serial dilutions of bovine serum albumin (BSA; 0.1 to 2 µg/µL) were used as a standard. Absorbance was measured at λ=562 nm. Typically, 5 to 10 µg of total protein were analyzed by immunoblot analysis.

6.3.2 SDS-PAGE and immunoblot analysis

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), protein samples prepared in LSB were separated on discontinuous polyacrylamide gels (with acrylamide concentration adjusted to separation range, 6 to 15 %; stacking gels 4 %). After gel electrophoresis, proteins were transferred onto polyvinylidenfluoride (PVDF) membranes (wet blotting; 400 mA 1 h). The membranes were saturated in blocking buffer, thereafter the primary antibody was applied (diluted in blocking buffer, 4 °C, overnight). Following extensive washing in TBST, the secondary antibody (1:12.000 dilution in TBST) was added for 1 h at room temperature. Afterwards, the membranes were washed in TBST (4 times, 10 mL per membrane, 10 min). The luminescent signal was detected using ECL Western blotting detection reagents (GE Healthcare) and FUJI medical X-ray films.

6.3.3 Detergent-solubility assay

Cells were pelleted by centrifugation, then resuspended in lysis buffer (0.5 % (v/v) Triton X-100, 0.5 % (w/v) desoxycholic acid, complete protease inhibitor cocktail in PBS) and incubated on ice for 5 min. Following centrifugation (13.000 g, 20 min, 4 °C), supernatants were transferred to new vessels and supplemented with Laemmli sample buffer (LSB). The remaining pellet was washed with one volume of lysis buffer, centrifuged and supplemented with an appropriate volume of LSB. Pellet fractions were homogenized by several passages through a 23 gauge syringe. All samples were boiled at 95 °C for 10 min and equal volumes of both fractions were subjected to immunoblot analysis.

6.4 Microscopy

6.4.1 Indirect immunofluorescence

Cells were grown on 15 mm glass coverslips. Prior to fixation, cells were washed with PBS supplemented with calcium and magnesium (PAA Laboratories). Cells were fixed in 3.7 %

(w/v) aqueous formaldehyde solution and permeabilized with ice-cold methanol. After incubation in blocking buffer (5 % (v/v) horse serum and 0.1 % Tween-20 in PBS) the primary antibody was added overnight (dilution dependent on antibody, range 1:200 to 1:2.000 in PBS) at 4 °C. Following intensive washing with PBST (0.1 % Triton X-100 in PBS), ALEXA-conjugated secondary antibodies were added for 2 h at room temperature (1:1.000 in PBS). After several washes with PBST, cells were embedded in Mowiol mounting medium supplemented with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei.

6.4.2 Mitophagy assay

For the analysis of mitophagy HeLa or MEF cells stably overexpressing wildtype human parkin were used. For the analysis of parkin mutant, transient liposome-mediated transfection was used. Cells were seeded on glass cover slips, treated with 10 µM CCCP for 1 or 24 h and fixed in 3.7 % formaldehyde in PBS. Cells were stained using the PRK8 anti-parkin antibodies and TOM20 or HSP60 antibodies and ALEXA-conjugated secondary antibodies. The percentage of cells devoid of mitochondria was counted in three independent experiments with at least 500 cells being analyzed for each condition.

6.4.3 Activated caspase-3 assay

In order to quantify apoptotic cells with activated caspase-3 a single cell assay was applied. For this approach, an indirect immunofluorescence was performed with an antibody specifically recognizing activated cleaved caspase-3. The percentage of cells positive for activated caspase-3 was assessed in three independent experiments with at least 300 cells analyzed for each condition.

6.5 Statistical analysis

Data were expressed as mean +/- standard deviation (SD). Statistical analysis among groups was carried out using the one-dimensional analysis of variance (ANOVA). P-values are * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6.6 Software and databases

Microsoft Office Excel 2007 was used for calculations and statistical analyses. For the administration of plasmids, for strategic planning of clonings and the analysis of DNA sequencing reactions the programs VectorNTI 10.1 and CLC Main Workbench 6.1 were used. Research publications were obtained from the online database NCBI PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and managed with EndNote X4. DNA and protein sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/nuccore and http://www.ncbi.nlm.nih.gov/protein) and ENSEMBL (http://www.ensembl.org). Sequence homology was determined via the basic local alignment search tool (BLAST). Microscopic pictures were processed using the Zeiss Axiovision Software 4.7 and Adobe Photoshop and Illustrator CS4 were used for the assembly of figures.

7 References

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9 Publications

Parts of the results of this thesis have been submitted for publication or have already been published in peer-reviewed journals:

Parkin, PINK1 and mitochondrial integrity: Emerging concepts of mitochondrial dysfunction in Parkinson's Disease. Pilsl A and Winklhofer KF. Acta Neuropathol. 2012 Feb;123(2):173-88. Review Article.

Parkin is protective against proteotoxic stress in a transgenic zebrafish model. Fett ME*, **Pilsl A***, Paquet D, van Bebber F, Haass C, Tatzelt J, Schmid B, Winklhofer KF. PLoS One. 2010 Jul 30;5(7):e11783. *authors contributed equally

Aberrant folding of pathogenic Parkin mutants: aggregation versus degradation. Schlehe JS, Lutz AK, **Pilsl A**, Lämmermann K, Grgur K, Henn IH, Tatzelt J, Winklhofer KF. J Biol Chem. 2008 May 16;283(20):13771-9.

10 Summary

Parkinson's disease (PD) is the most common movement disorder and the second most frequent neurodegenerative disease after Alzheimer's disease. Mutations in the parkin gene account for the majority of autosomal recessive PD. Parkin functions as an E3 ubiquitin ligase mediating the specificity of substrate ubiquitination. Various substrates have been proposed to be ubiquitinated by parkin, however, the *in vivo* relevance of them is still under debate. Several different functions have been attributed to parkin, the most consistently found being parkin-dependent mitophagy in parkin-mediated cytoprotection.

The goal of the first part of this thesis was to gain deeper insights into the physiological function of parkin in mitophagy. It was shown that parkin triggers the degradation of dysfunctional mitochondria, but only when the membrane potential is uncoupled by ionophores. Other cellular stressors interfering with mitochondrial function did not induce parkin translocation to mitochondria and their subsequent degradation, suggesting that mitophagy is independent of parkin-dependent cellular stress protection. Most pathogenic parkin mutations impaired the removal of damaged mitochondria, and only mutations in or deletion of the N-terminal ubiquitin-like domain did not affect the mitophagic activity of parkin. In line with the notion that parkin functions as an E3 ubiquitin ligase, robust ubiquitination at mitochondria was observed when parkin translocated to mitochondria with disrupted membrane potential. It was demonstrated that parkin-mediated mitophagy crucially depends on both autophagy and the ubiquitin-proteasomal system. Furthermore, PINK1, a mitochondrial serine/threonine kinase associated with PD, is crucially involved in mitophagy. In fibroblasts devoid of PINK1, parkin was not targeted to dysfunctional mitochondria. Using an artificial chimeric protein anchoring parkin to the outer mitochondrial membrane, it was demonstrated that targeting of parkin to mitochondria is not sufficient to induce mitophagy. Interestingly, mitochondrially targeted parkin did not induce the degradation of dysfunctional mitochondria in the absence of PINK1, suggesting that PINK1 carries out other functions independent of recruitment of parkin. Indeed, PINK1 was able to alter the solubility of parkin in detergents, supporting the notion that structural features of parkin are crucial for its action in mitochondrial degradation. Notably, pathogenic mutants of PINK1 were impaired in their ability to render parkin insoluble. In addition, treatment with CCCP also induced a shift in parkin conformation to a similar extent as PINK1 expression, but this shift was abolished in PINK1-deficient cells.

The second part of this thesis focused on identifying the degradation mechanisms of pathogenic parkin mutants. In agreement with previous results it was demonstrated that some pathogenic missense and truncation mutants misfold and aggregate when overexpressed in cells. Reminiscent of protein aggregates in various proteinopathies, the adapter protein p62 robustly colocalized with aggregates of mutant parkin. Cellular levels of p62 influenced the abundance of parkin aggregates: an increase in the percentage of cells

with parkin aggregates was observed in fibroblasts deficient of p62. In addition, overexpression of p62 was sufficient to significantly reduce the number of cells containing parkin aggregates. In agreement with this, mutant parkin protein accumulated upon transient siRNA-mediated downregulation of p62 in cell lines stably overexpressing the pathogenic parkin mutant W453X. Analysis of primary cortical neurons from parkin W453X mutant transgenic mice further revealed aggregates of parkin colocalizing with p62 and protein levels of the mutant parkin were increased following proteasomal inhibition. Although p62 has been suggested to function as an adapter molecule for selective autophagy, inhibition of autophagy did not have an impact on the abundance of aggregates of mutant parkin. In contrast, inhibition of proteasomal function significantly increased the number of cells with parkin mutant aggregates, suggesting that pathogenic parkin mutants which are prone to misfolding are degraded by the proteasome in a p62-dependent manner.