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Folding, function and subcellular localization of parkin

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Erklärung

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Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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Summary

Idiopathic Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimers disease. The specific molecular events that provoke neurodegeneration in PD are still unknown, which is an impediment to the development of neuroprotective drugs. Only recently, genes linked to hereditary forms of PD have been identified. Idiopathic and hereditary variants of PD share important pathological features, most notably the demise of dopaminergic neurons in the substantia nigra. Functional characterization of PD-associated gene products might help to understand the molecular mechanisms underlying the pathogenesis and maybe, in the future, to find preventive and curative treatments for PD. Among the mutated genes is the parkin gene (*PARK2*), encoding a E3 ubiquitin ligase. Mutations in the parkin gene are responsible for the majority of autosomal recessive parkinsonism.

Previous work of our group revealed that misfolding and aggregation of parkin is a major mechanism of parkin inactivation, accounting for the loss-of-function phenotype of various pathogenic parkin mutants, including C-terminal deletion mutants and some missense mutants [1,2]. Remarkably, also wildtype parkin is prone to misfolding under certain cellular conditions, suggesting a more general role of parkin in the pathogenesis of PD. One aim of this thesis was to study the folding characteristics of parkin. To this end, I cloned several parkin mutants and analyzed them in cell-culture based assays to determine their folding properties. Folding analysis of these mutants revealed that pathogenic mutations can lead to aberrant parkin conformers with two distinct phenotypes. One class of mutations destabilized the native conformation of parkin, leading to its proteasomal degradation immediately after synthesis. Another class of mutants first adopted a detergent-soluble conformation, similarly to wildtype parkin. However, within hours these mutants formed relatively stable detergent-insoluble aggregates. A comparative analysis of HHARI, an E3 ubiquitin ligase with a similar modular signature, revealed that folding of parkin is specifically dependent on the integrity of the C-terminal domain, but not on the presence of a putative PDZ binding motif at the extreme C-terminus. This study provided new insight into the propensity of parkin to misfold and suggested that pathogenic mutations can induce the formation of non-native conformers at distinct steps in the folding pathway of parkin.

Another focus of this thesis was the functional characterization of parkin. We and others observed that parkin protects neurons against diverse cellular insults in different model systems, indicating that it may play a role in maintaining neuronal integrity. To address the

underlying mechanism, we analyzed the effect of parkin on different signaling pathways. Our results revealed that parkin has a permissive effect on NFκB signaling by ubiquitylating two components of the signaling cascade in a non-degradative manner. Notably, parkin lost its neuroprotective capacity in the presence of a dominant negative inhibitor of NFκB. In addition, we could show that parkin expression is significantly up-regulated in neurons under stress conditions, indicating that parkin is a stress-responsive protein.

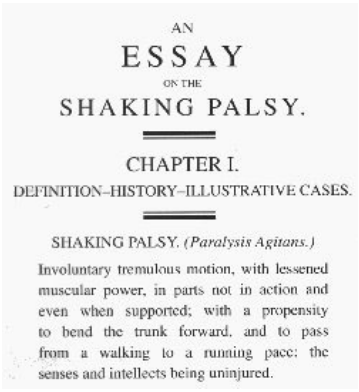
Introduction

Parkinson's Disease

Sporadic or idiopathic Parkinson's Disease (PD) is the most common movement disorder and the second most common aging-related neurodegenerative disease after Alzheimer's Disease (AD). More than 4 million people suffer from PD worldwide. The cardinal symptoms of PD can be relieved for several years after onset, but there is still no cure for the disease. The specific molecular events that provoke neurodegeneration in PD are still unknown, which is an impediment to the development of neuroprotective drugs. Functional characterization of mutated gene products might help to understand the molecular mechanisms underlying the pathogenesis and maybe, in the future, to find preventive and curative treatments for PD.

History

The clinical symptoms of PD were first described in 1817 by the English physician and pharmacist James Parkinson (1755-1824) in his monograph "Essay on the Shaking Palsy". He characterized the disease as an "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellects being uninjured." In this definition, he summarizes some of the cardinal symptoms of the disease: resting tremor, akinesia, postural instability and gait problems.



AN

ESSAY

ON THE

SHAKING PALSY.

CHAPTER I.

DEFINITION–HISTORY–ILLUSTRATIVE CASES.

SHAKING PALSY. *(Paralysis Agitans.)*

Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellects being uninjured.

Figure 1. First documentation of Parkinson's disease, written by James Parkinson, 1817. The first documentation of a patient showing the cardinal symptoms of PD " An Essay on the Shaking Palsy". (Source: http://www.pdmdcenter.com/articles/HopkinsWeb/index.html)

The disease was named after Parkinson by Jean-Martin Charcot (1825-1893) 67 years after the initial publication. Charcot also expanded the list of symptoms by including the mask face, rigidity and akathesia. More than a century passed before the pathology of PD was being documented, specifically, the demise of neurons in the substantia nigra (SN). In 1958, Arvid Carlsson found dopamine (DA) as a neurotransmitter in the mammalian brain, and in this line, Ehringer and Hornykiewicz discovered that a lack of dopamine and neuron loss in the SN causes PD (1960). Since the 1960s the DA precursor levo-dopa (L-3,4-dihydroxyphenylalanine) has been used as medication to treat PD. Until today, PD medication can relieve symptoms, but none are able to halt or retard dopaminergic neuron degeneration.
Like other neurodegenerative diseases, PD occurs sporadically, or very rarely, in heritable forms. A breakthrough came more than a decade ago, when the A53T mutation in the SCNA gene, which encodes for α-synuclein, was the first to be found as a cause for heritable PD [3]. Since then, more genetic factors have been determined, and today there are 12 loci identified which are associated to heritable forms of PD (PDGene database). Finding out more about the molecular mechanisms of heritable disease might lead to the discovery of ways to treat and prevent PD.

Clinical characteristics, symptoms and treatment

The average age of onset for PD is 55 years, with an increasing prevalence with age: 1-2% of more than 60 years old individuals develop PD, and more than 4% of the population by the age of 85 [4]. Some monogenic forms show an earlier manifestation. PD is a slowly progressing disease, with the first symptoms occurring when at least 60% of the SNpc dopaminergic neurons are dead and dopamine release is reduced by about 80%. The loss of DA in a PD affected brain can be imaged by positron emission tomography scans, as depicted in Figure 2.

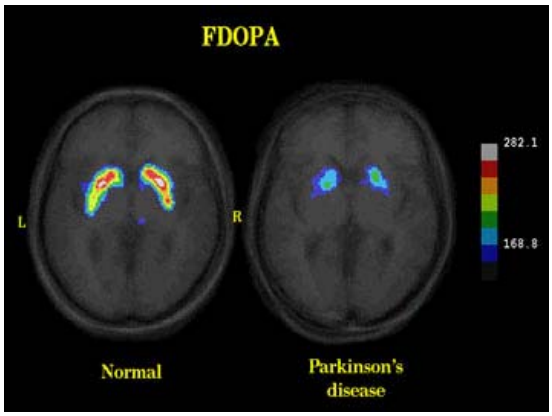


Figure 2: Comparison of normal brain and Parkinson's brain positron emission tomography (PET) scans. Left: Healthy control brain has a normal uptake of ^{18}F-Dopa into the striatum. Right: Brain of a PD patient shows a reduced uptake of ^{18}F-Dopa. Alan Dagher, Montreal Neurological Institute, Mc Gill University.

The major clinical symptoms encompass rigidity, bradykinesia, hypokinesia, akinesia, hypomimia, hypophonia, drooling, micrographia, decreased stride length and freezing. Also, abnormalities of affect and cognition can be part of the disease. Patients may show a loss of initiative, anhedonia, slowed cognitive processes, depression, and, especially in older patients, also dementia.

Treatment strategies are mainly aimed at compensating the lack of dopamine. To this end, the DA precursor L-Dopa is administered to patients, usually in combination with Carbidopa. It helps to increase the dose of L-Dopa that reaches the brain by inhibiting DA Decarboxylase (DDC), which is present in the periphery and breaks down DA. Other strategies involve the inhibition of dopamine catabolism: inhibition of Monoamine Oxidase B (MAO-B; converts DA to DOPAC (3,4-Dihydroxyphenyl acetic acid) keeps concentrations of DA high and is used to treat mild symptoms. It also prolongs the L-DOPA effect. Catechol-O-methyl-transferase (COMT) inhibitors (Entacapone) are given together with L-DOPA when severe symptoms occur. COMT reduces DA levels by methylating DA to 3-Methoxytyramine. COMT also acts in the periphery, resulting in too small amounts of L-Dopa reaching the brain. In some cases, tremor is treated with anticholinergics, albeit rarely, due to side effects. Patients that cannot be treated conventionally can receive deep brain stimulation, a surgical strategy where a microelectrode is introduced into specific regions within the basal ganglia.

All available treatment strategies can alleviate the symptoms of the disease, but the neuronal degeneration cannot be stopped or slowed down.

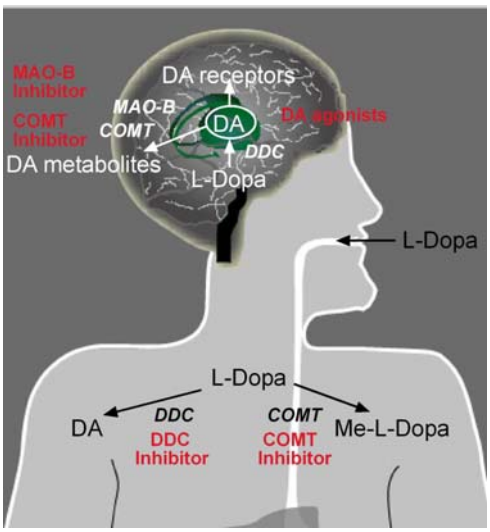


Figure 3: Treatment strategies of PD. Red: Site of drug action. Inverse: Key enzymes of DA metabolism. Black letters: Periphery. White letters: Brain. Adapted from www.learningcommons.umn.edu/neuro/mod6/ldopa.html.

Neuropathological characteristics

A pathological hallmark of PD is the degeneration of dopaminergic neurons in the SN and the loss of their nigrostriatal projections to the putamen, which account for the motor symptoms of PD. These dopaminergic neurons produce the pigment neuromelanin. Anatomically, a depigmentation of the SN in post mortem PD patient brain tissue can be observed due to the loss of these cells. Apart from DAergic neurons, neuronal death also occurs in noradrenergic, cholinergic and serotonergic systems of the brain as well as in the cerebral cortex, olfactory bulb and autonomic nervous system [5].

Histopathological features of sporadic PD and some familial PD forms encompass Lewy bodies (LBs) and Lewy neurites (LN). Lewy neurites are dystrophic neurites that are present in surviving neurons. Lewy bodies are eosinophilic, cytoplasmic, intraneuronal inclusions that contain a variety of proteins, including α-synuclein, ubiquitin, heat shock proteins, neurofilaments and parkin. Figure 4, left panel, shows an immunohistochemical α-synuclein staining of a Lewy body. It has a dense core surrounded by a halo and a size of about 15 µm. The right panel shows a ubiquitin staining, which is more diffuse in the center of the Lewy body.

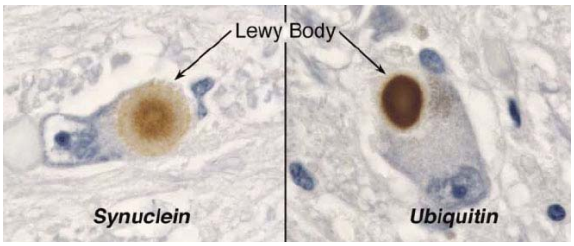


Figure 4: Immunohistochemical stainings of intra-neuronal inclusions (Lewy bodies). Left: α-synuclein staining; right: Ubiquitin staining. From Dauer and Przedborski, Neuron 2003 [5].

Lewy bodies are not only observed in PD, but also in multiple system atrophy (MSA) dementia with Lewy body disease (DLB), also called diffuse Lewy body disease (DLBD) [6], and in other neurodegenerative diseases. Such neurodegenerative diseases are generally referred to as synucleinopathies. Lewy bodies are frequently observed in surviving DA neurons of the SN. Braak *et al.* proposed, based on anatomical investigations, that the pathological process of sporadic PD starts from the lower brain stem and spreads to the midbrain, limbic brain and cerebral cortex, and that the movement disorder of PD appears at a late stage when the nigro-striatal DA neurons become involved [7]. In relation to this wide distribution of Lewy bodies in sporadic PD, various non-DA symptoms, e.g. REM sleep behaviour disorder (RBD) [8], olfaction disturbance, cardiac sympathetic denervation or constipation [9], have been noticed as early signs of sporadic PD before the appearance of parkinsonism.

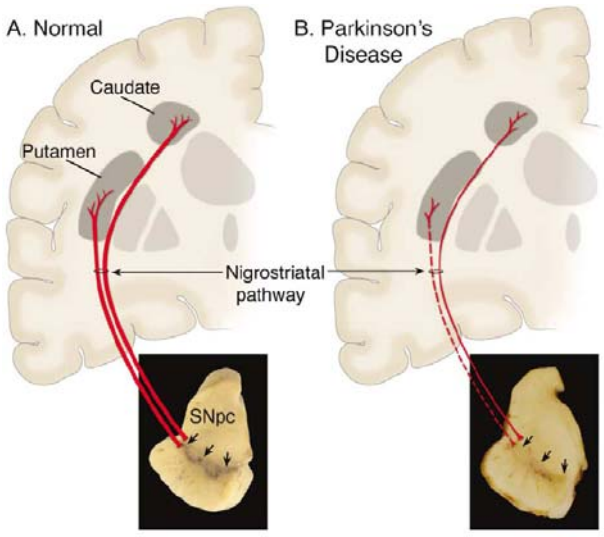


Figure 5: Neuropathology of PD. Schematic representation of A) normal nigrostriatal pathway between SNpc and putamen/caudate nucleus (solid red line) and B) degenerate neuronal projection of a PD patient (dashed red line). From Dauer and Przedborski, Neuron 2003[5].

Etiology

Cases of parkinsonism can be etiologically classified: sporadic or idiopathic PD (80%); inherited or familial PD (10%), and secondary or symptomatic parkinsonian syndromes (10%). The latter 10% refer to an inhomogenous group of disorders with multiple possible causes. Examples are toxin- or drug-induced parkinsonism, tumors, traumata, ischemia, metabolic dysfunctions or inflammation. Additionally, atypical parkinsonism can occur in the context of other neurodegenerative diseases, such as MSA, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) or dementia with lewy bodies (DLB). In the 80% of sporadic PD cases, no clear etiology can be found. They are thought to be caused by an interplay of both individual genetic predisposition and environmental influences [10]. The environmental hypothesis claims that the exposure to a dopaminergic toxin can induce PD by chronic exposure or inititation of a self-perpetuating cascade. Examples in support of this hypothesis are the inhibitors of the mitochondrial respiratory chain Complex-1 MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [11] and rotenone [12]. Another hypothesis proposes that an endogenous toxin causes onset of PD. Normal metabolism could be disturbed due to inherited differences or environmental toxins, which might lead to toxic substances, e.g originating from normal DA metabolites which generate reactive oxygen species (ROS) [13].
The remaining 10% of familial PD cases show a Mendelian pattern of inheritance. An overview on PD etiology is given in Figure 6.

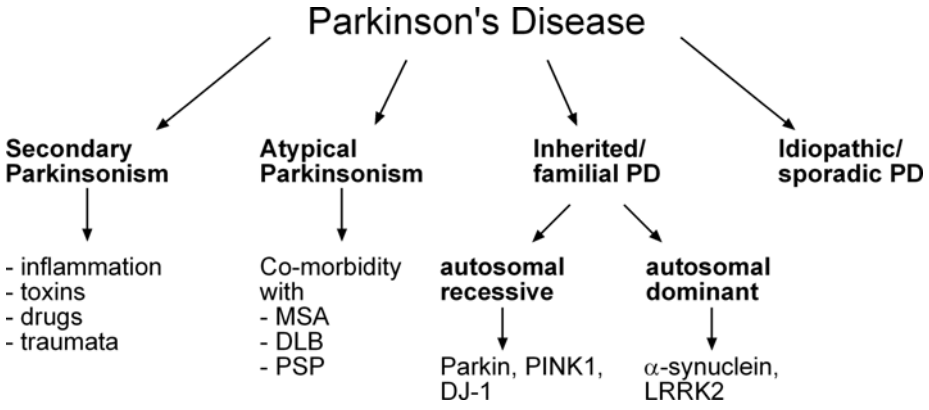


Figure 6: Etiology of Parkinon's Disease. MSA: multiple systems atropy. DLB: dementia with lewy bodies. PSP: progressive supranuclear palsy.

Familial forms of PD and their genetics

The discovery of the genes that cause familial forms of PD accelerated the progress in research on Parkinson's Disease. Since 1997, 13 loci could be identified, and for 8 of them, the corresponding genes are known (Table 1). The insight into genes associated with the disease can help to establish animal and cell culture models to discover the pathophysiological mechanisms underlying PD. Moreover, several pathways could be indentified that are implicated in the neurodegeneration of the nigrostriatal system (see below) [14].

Gene locus	Chromosome	Gene product	Inheritance pattern	Putative function
PARK1/4	4q21-q23 / 4p15 (duplications/triplications)	α-synuclein	AD	Vesicle trafficking/ synaptic plasticity
PARK 2	6q25.2-27	Parkin	AR	E3 ubiquitin ligase
PARK 3	2p13	?	AD	
PARK 5	4Q14	UCH-L1	AD?	Ubiquitin hydrolase?
PARK 6	1p35-36	PINK1	AR	Mitochondrial kinase
PARK 7	1p36	DJ-1	AR	Cytosolic redox-sensitive protein
PARK 8	12p11.2-q13.1	LRRK2	AD	MAPKK kinase
PARK 9	1p36	ATP13A2	AR	Lysosomal H^+-ATPase
PARK 10	1p32	RNF11?	SUS	
PARK 11	2q34	?	AD	
PARK 12	Xq21-q25	?	SUS	
PARK 13	2p12	Htra2/Omi	SUS	Mitochondrial protease

Table 1: Gene loci involved in monogenic PD cases. AR: autosomal recessive. AD: autosomal dominant. SUS: susceptibility locus for idiopathic PD. UCH-L1: Ubiquitin carboxyl-terminal esterase L1. PINK1: Phosphatase and Tensin (PTEN)-induced kinase 1. LRRK2: leucine-rich repeat kinase 2. RNF11: RING-finger protein 11. Htra2/Omi: High temperature regulation A serine peptidase2/Omi.

Dominant genes

α-synuclein

The first gene to be indentified in the context of PD was the one coding for α-synuclein (SCNA). In 1997, Polymeropoulos and colleagues discovered the dominant A53T mutation in a small number of Italian-greek families [3]. Shortly after that, Spillantini and co-workers could show that α-synuclein is a major component of Lewy bodies. The synucleins form a family of proteins consisting of α-, β- and γ-synuclein, and are prominently expressed in the central nervous system. α-synuclein is natively unfolded, has an N-terminal domain which forms an α-helix in association with membranes, an unfolded acid C-terminal domain and a hydrophobic NAC-domain (non-amyloid component of plaques). This NAC-domain has a tendency to aggregate [15]. In a solution, a monomeric unfolded structure of a-synuclein is in equilibrium with a folded form of α-synuclein associated to vesicles [16]. The α-helical N-terminus and the NAC domain binds to vesicles, whereas the C-terminus remains unfolded. Thus, α-synuclein might physiologically be involved in vesicular transport and synaptic transmission in the brain [17]. In contrast to its family members, α-synuclein has been shown to be an aggregation-prone protein [18]. The propensity to form oligomeric assemblies is especially increased in all the 4 α-synuclein mutants that are associated with PD: A53T, E46K, A30P and genomic multiplications of the wildtype. Increased gene dosage presumably fosters aggregation of α-synuclein, which is a concentration- as well as nucleation-dependent process [19]. Additionally, an increased phosphorylation at serine 129 in the C-terminal domain of wildtype α-synuclein can lead to increased aggregation and formation of fibrils. This posttranslational modification has been detected in brain tissue from PD patients [20].

It is highly controversial if the species that are toxic for the cell are the oligomers, the protofibrils or the fibrils, which form later during the aggregation process. The protofibrils can form ring-like structures that could act as pores and thus disturb vesicular membranes [21], whereas the formation of Lewy bodies containing fibrillar α-synuclein is strongly associated with PD.

LRRK2

The leucine-rich repeat kinase 2 (LRRK2), which is also named Dardarin, has been discovered in the context of PD in 2004 [22,23]. The gene consists of 51 exons that code for an extremely large protein of about 250 kDa. It has several functional domains: an N-terminal ankyrin domain, a leucine rich repeat, a ROC (Ras in complex proteins) domain, a COR (C-terminal of ROC) domain, a MAPKKK (Mitogen activated protein kinase kinase kinase) domain and a WD 40 domain [24].

LRRK 2 mutations are the most frequent cause of late onset familial autosomal dominant PD. Among the 19 different pathogenic LRRK2 mutations that are known, the most abundant mutation is the G2019S within the kinase domain. Mutations in the kinase domain seem to increase kinase activity of LRRK2 [24,25]. The physiological function of LRRK2 and its role in PD is unclear. Patients with LRRK2 mutations show typical symptoms of idiopathic PD, which occur usually at the age between 50 and 60 years. The pathological characteristics of the patients are rather inhomogenous, e.g. in regards of Lewy body formation or tau pathology [26].

Recessive genes

Parkin

Kitada and colleagues identified the first recessive PD gene *parkin* in 1998 [27]. They found mutations in parkin leading to familial autosomal recessive juvenile PD (AR-JP). Until today, a wide spectrum of parkin mutations have been described. Parkin plays a prominent role among PD associated genes, because the majority of familial early onset cases are due to a mutation in the parkin gene. A more detailed summary on parkin is given below.

PINK1

The ubiquitously expressed PINK1 transcript encodes a protein containing a serine/threonine kinase domain, similar to Ca^{2+} Calmodulin (CaM) kinases. It has an N-terminal mitochondrial targeting sequence and is mainly localized in mitochondria [28]. The kinase activity has been shown autocatalytically [29], and for the mitochondrial molecular chaperone TRAP1 as substrate, which is also called Hsp 75 [30]. PINK1 can protect cultured neuronal cell lines from apoptosis when they are subjected to proteasomal inhibitors or oxidative stress [30,31]. Only recently, data from *Drosophila* and cell culture was published that implied PINK1 to play a major role in the maintenance of mitochondrial integrity and dynamics [32,33,34,35].

Two mutations in the PINK1 (PTEN-induced kinase 1) gene have been identified in 2004 in three consanguineous families suffering from PD [28]. Since then, more than 20 pathogenic mutations were described, which are the second most frequent cause for autosomal recessive PD. PINK1 associated cases show a broad phenotypic spectrum, spanning from an early manifestation with atypical symptoms to late manifestation with the typical clinical PD symptoms. To date, no neuropathological data is available.

DJ1

DJ-1 mutations were first identified as a cause for autosomal-recessive PD in families from the Netherlands and Italy [36]. One mutation was a homozygous deletion of exon 1-5, the other a L166P missense mutation [37].

The DJ-1 gene codes for a ubiquitously expressed 189 amino acids protein and was assumed to have a function as a tumor suppressor gene [38]. Several studies showed that DJ-1 appears to function as a dimer [39,40,41]. A three-dimensional structure determined by X-ray cristallography suggested that the pathogenic L166P mutation accounts for the destabilisation of the dimer interface [41]. The same mutant has been shown to be rapidly degraded after ectopic expression [42]. These findings, rapid turnover and structural changes, might be significant for the disease pathogenesis [43]. DJ-1 has been implicated in several cellular functions. Structural homologies to the *E.coli* chaperone Hsp 31 give rise to the speculation that DJ-1 has a chaperone like function [44,45]. It has been reported to modulate transcriptional processes by interacting with the androgen receptor modulator PIASxα, as determined by yeast two hybrid screen and cell cultures studies [46]. The most commonly held view is that DJ-1 is a redox-sensitive protein with cytoprotective potential towards oxidative stress. This has been shown in DJ-1 knock out mice, which displayed an increased sensitivity of striatal dopaminergic neurons after treatement with the parkinsonism-inducing drug MPTP [47].

Pathogenesis/ assumed cellular mechanisms of PD

Until today, the molecular causes for PD remain obscure. The findings about the recessive genes and toxin-induced PD models imply a mechanistic coherence between mitochondrial dysfunction/oxidative stress and the ubiquitin-proteasome system (UPS) [5]. Below, both aspects are described in detail.

Mitochondrial dysfunction and oxidative stress

The function of neuronal mitochondria is significantly impaired in PD [48]. Specifically, complex-I and -III of the respiratory chain show altered activity. Complex-I normally transfers two electrons via coenzyme Q to complex-III. From there, electrons go on to cytochrome c and to complex-IV. Sometimes, electrons are transferred to molecular oxygen instead of cytochrome c, which results in the formation of highly reactive radical oxygen species (ROS) that are noxious for cells. ROS include superoxide anions ($^{\cdot}O_2^-$), hydroxyl radicals ($OH^{\cdot}$), peroxyl radicals of lipid ($LOO^{\cdot}$), alkoxyl radicals of lipids ($LO^{\cdot}$), stable molecular oxidants like hydrogen peroxide (H_2O_2), ozone (O_3), hypochloric acid (OCl^-) and metastable states like singlet oxygen ($^1O_2$). These reactive species can be inactivated by a network of antioxidative systems like glutathione (GSH) and glutathione peroxidases, catalases and superoxide dismutases (SOD). The formation of ROS and their elimination by antioxidative systems are in a very delicate equilibrium. A disturbance of this equilibrium can have devastating effects on the cell [49].

Post-mortem brain tissue of PD patients showed an increased content of oxidized lipids, proteins and DNA [48] in the SN, as well as a reduced content of antioxidative GSH and an increase of ROS. These findings gave rise to the hypothesis that oxidative polymerisation of DA to neuromelanine subjects the SN to increased oxidative stress. This oxidative stress seems at least to contribute to cell death of dopaminergic neurons in this region. Whether this is the cause or the consequence of pathogenic processes is unclear. Additionally, the SN of PD patients show typically a reduced activity of complex-I. Reduced complex-I activity can lead to an energy deficit of the cell, as described above.

Another reason for increased oxidative stress in dopaminergic neurons is the DA metabolism. Auto-oxidation of DA leads to toxic quinones and semi-quinones, which can damage proteins by reacting with their cysteine residues. When DA is enzymatically metabolized by the monoaminoxidase B (MAO-B) and Catechol-O-methyl-transferase (COMT) (Figure 7), reactive hydrogen peroxide (H_2O_2) can form next to the metabolites 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT).

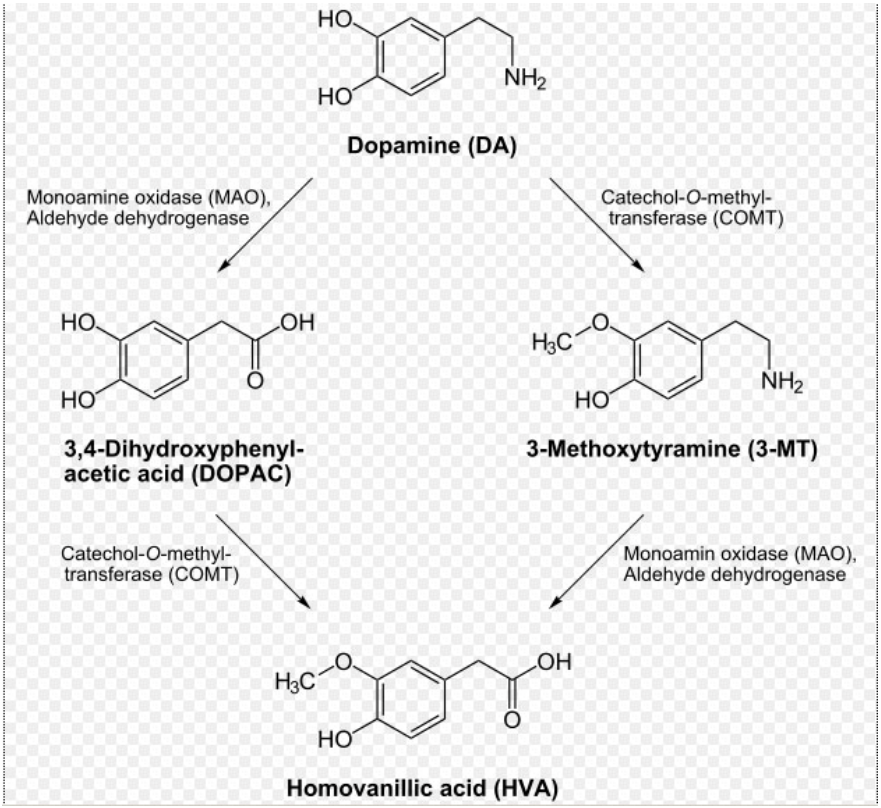


Figure 7: Enzymatic degradation of dopamine (http://en.wikipedia.org/wiki/Dopamine).

Animal models of sporadic PD support the hypothesis that mitochondrial dysfunction precedes cell death. In mice and primates, the mitochondrial complex-I inhibitor MPTP induces a specific loss of dopaminergic neurons and a PD-like pathology [5,50,51].

The poisons rotenone, an insecticide and fish poison, and paraquat, an insecticide, which are both used in PD animal models, also inhibit complex I of the respiratory chain and thus lead to oxidative stress for the cell.

Protein aggregation and dysfunction of the ubiquitin proteasome system

Abnormally aggregated proteins are characteristic for all neurodegenerative diseases. Recent studies imply that not the end products of the aggregation process are toxic, but the oligomeric intermediates (e.g. α-synuclein, Aβ, polyQ-huntingtin). Protein aggregates also occur in PD as Lewy bodies (see above). The implication of Lewy bodies is mainly unclear, however, there are hints that Lewy bodies per se do not have a toxic potential and presumably act in a protective way [52].

The ubiquitin proteasome system (UPS) is essential for the non-lysosomal degradation of short-lived, mislocalized, misfolded, mutated or damaged proteins and thus plays a crucial

role for the survival of the cell. The degradation of proteins via the UPS is a regulated multi-step process that is catalyzed by specific enzymes [52].

The ubiquitin-activating enzyme (E1) catalyzes in an ATP-dependent manner a thioester bond between the C-terminus of ubiquitin, a 76 amino acid residue protein, and the reactive cysteine of E1. The activated ubiquitin is transferred to a cysteine of the ubiquitin conjugating enzyme (E2). The ubiquitylation of the substrate protein occurs via an E2/E3 (E3: ubiquitin ligating enzyme) complex by forming an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of a substrate lysine (K). Specificity of the ubiquitylation process is provided by the functional interaction between E2/E3 complex and the substrate. In the case of poly-ubiquitylation, additional ubiquitin units are being bound to the initial ubiquitin via iso-peptidic bonds between the C-terminal glycine of each ubiquitin molecule and a specific lysine residue of the previous ubiquitin. Sometimes this is mediated in conjunction with an additional multichain assembly factor (E4), to ensure efficient substrate multi-ubiquitylation. The linkage via K63 is involved in various other processes of the cell, such as endocytosis, DNA-repair or signal transduction. For degradation by the proteasome, the polyubiquitin chain is linked via K48 [53].

The 26S proteasome is a large protease complex consisting of the barrel-shaped 20S proteolytic core and two 19S regulatory caps, one on each side of the 20S core openings (Figure 8). The catalytically active 20S core has three distinct proteolytic activities: chymotrypsin-like, trypsin-like and post-glutamyl peptidyl hydrolytic-like. The degradational products are peptides of 4-9 amino acids, which are further degraded by cellular peptidases to amino acids that are then reused for protein synthesis. The 19S subunits play important roles in substrate recognition, the initial steps of substrate proteolysis, unfolding and translocation of the substrate proteins to the proteolytic 20S core. Also, deubiquitylation prior to the degradation of the substrate is mediated via the 19S subunits[54]. The released ubiquitin chains are further processed to monomers by the ubiquitin carboxy terminal hydrolases, such as UCHL-1 [55].

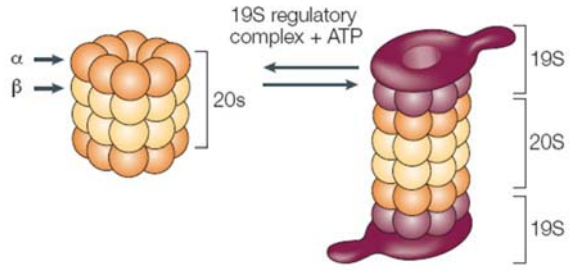


Figure 8: The proteasome. Scheme of the proteasome multi-enzyme complex, composed of the 20S complex, which comprises α- and β-subunits, and two 19S regulatory complexes. Together with ATP, these form the 26S proteasome [56].

Implications for the UPS to play a role in the pathogenesis of PD are given by the fact that several monogenic forms of PD involve mutations in genes that might play a role in the UPS: Parkin has been described to be an E3 ubiquitin ligase [57,58,59]. UCHL-1, a deubiquitylating enzyme, has also been implicated in familial PD [60]. For α-synuclein it has been reported that its overexpression inhibits proteasome function in brains of transgenic mice [61,62,63].

Also in sporadic cases, indication for a role for the UPS in PD pathogenesis is given. Components of the UPS might be sequestered into aggregates, as the proteasomal subunit levels and activity are reduced in SN of PD brains compared to healthy controls [64]. Another report showed that aggregated proteins can directly inhibit the proteasome [65].

Further, lessons from toxin-induced PD models indicate a role for the UPS in PD pathogenesis. Rotenone impairs the proteasome *in vitro* [66,67], and MPTP infusion in mice impair the proteasome function [68].

Presumably, changes in the UPS involve age-related increases in oxidative stress, mitochondrial impairment and thus energy depletion, which leads to impairment of the ATP-dependent proteasomal function [69]. Further, the heat shock proteins Hsp70 and Hsp40 function in an ATP-dependent way. Mitochondrial impairment induced by oxidative stress thus leads to energy deficiency of the cell, which inactivates the main cellular defence systems against protein misfolding, the proteasome and heat shock proteins. Taken together, there is a tight relationship between oxidative species and protein metabolism. Therefore, both has to be taken into account for neurodegeneration in PD.

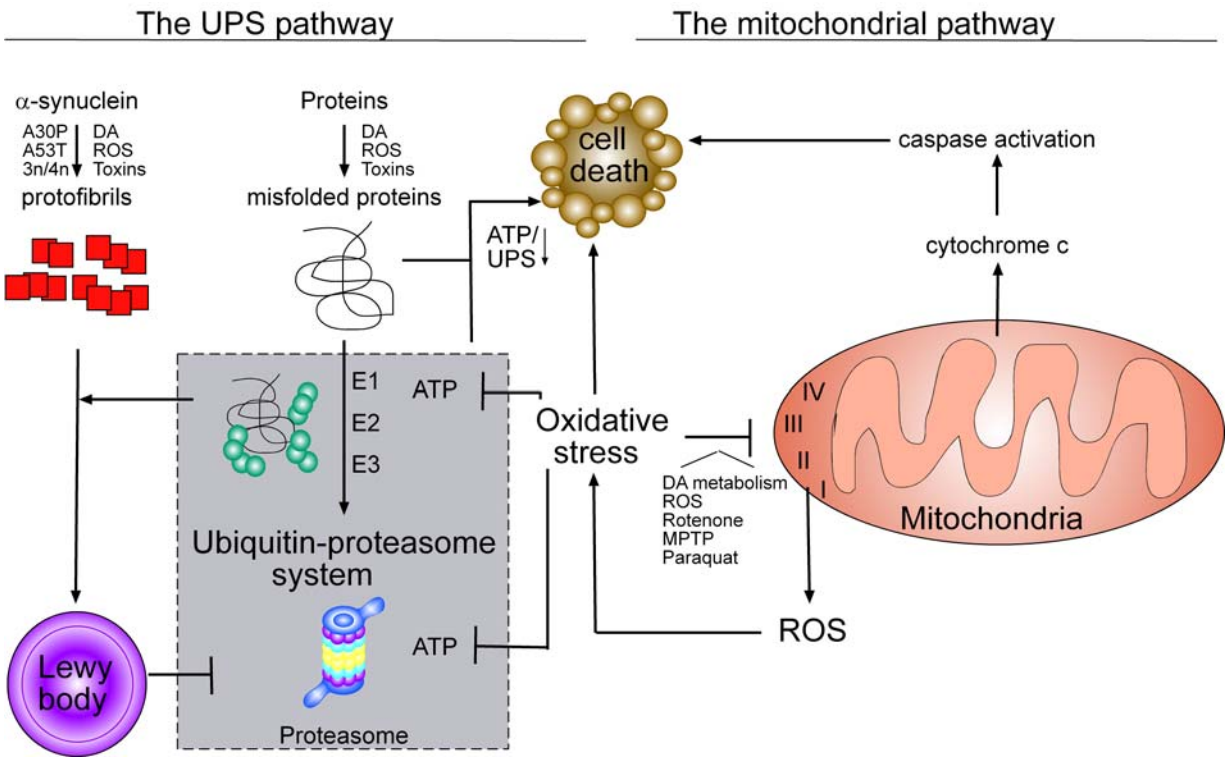


Figure 9: Overview on pathways to parkinsonism. α-synuclein, protein misfolding and aggregation form one main pathway of cell toxicity (left). Accumulation of misfolded proteins and failure of clearance by the UPS lead to the formation of fibrils and Lewy bodies. Another important pathway is the mitochondrial pathway (right). Impaired oxidative phosphorylation and Complex-1 deficiency lead to ROS and energy deficit of the cell. Loss of membrane potential of the mitochondrial membrane leads to cytochrome c release, caspase activation and cell death. Dysfunction of both pathways lead to oxidative stress, which causes further dysfunction of these pathways, leading to feedback and feedforward mechanisms and ultimately to cell death. Adapted from Abou-Sleiman et al., 2006 [70].

Parkin-associated Parkinson's Disease

Mutations in the parkin gene are responsible for an autosomal recessive form of PD [27]. The majority of the cases have homozygous mutations, several cases show monoallelic mutations, and rarely, compound heterozygote mutations have been detected in patients. The clinical symptoms are generally undistinguishable from sporadic PD and are characterized by a good response to levodopa.

So far only few parkin-associated PD cases could be neuropathologically examined. They have homozygous deletions in the PARK2 gene and show a selective loss of dopaminergic neurons in the SN and the locus coeruleus [71,72,73,74]. Initially, it has been described that patients with parkin mutations had no Lewy body pathology, but recently parkin-associated cases with Lewy bodies were discovered. The neuropathological changes seem to be - like in LRRK2 patients - multifaceted and possibly depend on the age of the patient or the type of the mutation. More autopsies are needed for a clearcut conclusion [14,75].

Molecular genetics and cell biology of parkin

With its size of 1.3 Mb, the parkin gene is one of the largest in the human genome. It is localized on chromosome 6q25.2-q27 and consists of 12 exons which code for a protein of 465 amino acids and an approximate molecular mass of 52 kDa [27].

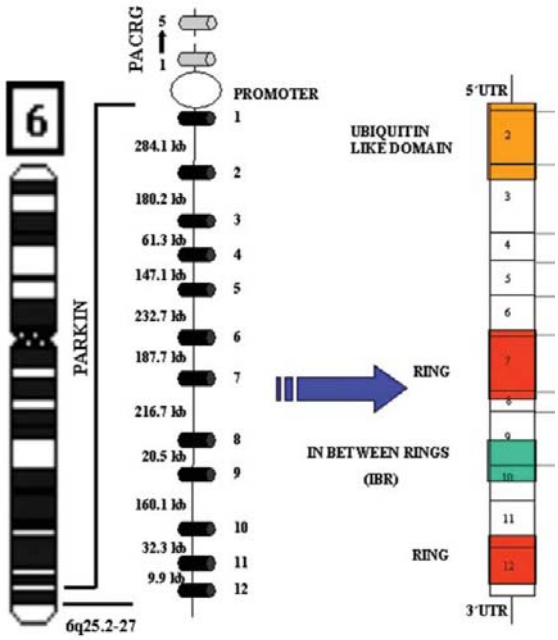


Figure 10: Genomic structure and model of protein domain structure of parkin. Left: Chromosome location of *PARK2*. Middle: Schematic representation of exon/intron structure (not to scale). Right: Scheme of the protein and its domains. Exons coding for each region are indicated by numbers. From Mata *et al.*, [76].

Parkin is highly conserved during evolution. It is not only present in vertebrates such as human, rat or mouse, but also in invertebrates like *C. elegans* and *D. melanogaster*. A comparison of amino acid sequences reveals a high homology between species (Figure 11) [77]. For example, the mouse parkin orthologue has 82% homology to human parkin. Interestingly, especially the functional domains are highly conserved.

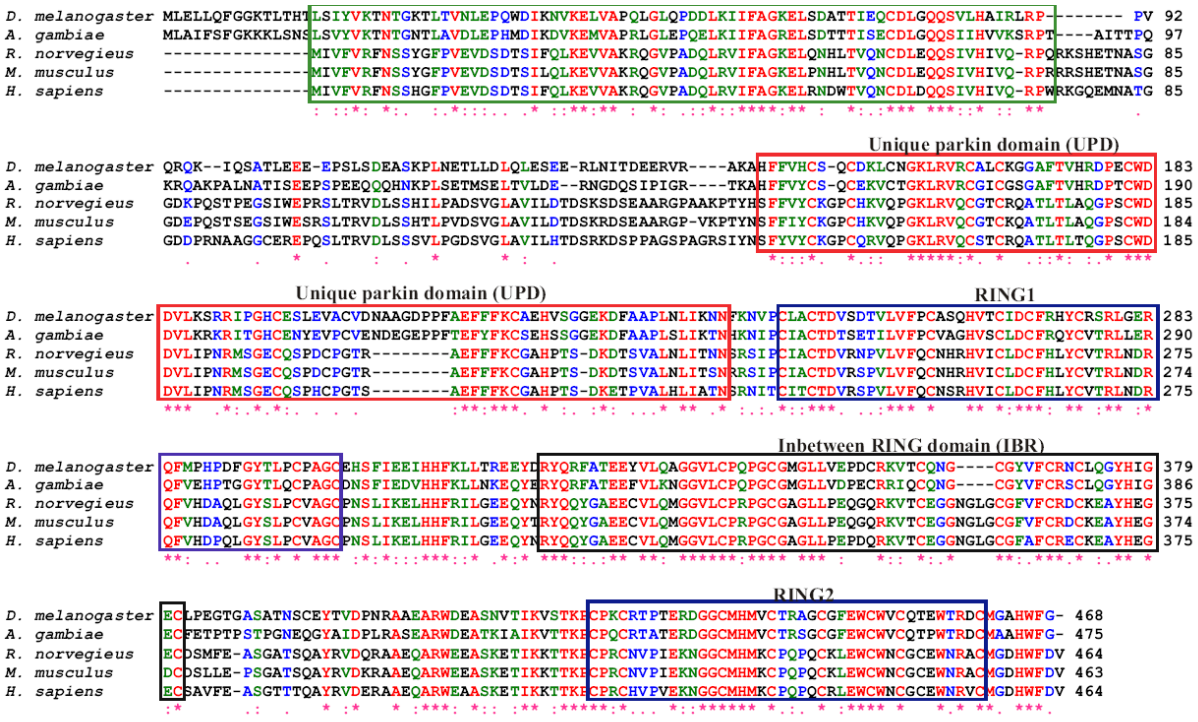


Figure 11: Parkin proteins are highly conserved during evolution. ClustalW alignment of parkin sequences of *D. melanogaster, A. gambiae, R. norvegicus, M. musculus*, and *H. sapiens*. Parkin domains are highlighted with boxes.* and red letters indicate identical amino acids; : and green letters conserved amino acids; . and blue letters semi-conserved amino acids. From Haywood & Staveley, 2004 [77].

Parkin is ubiquitously expressed, with high expression levels in brain, heart, testis and skeletal muscle [27]. It is differentially expressed in the brain regions, but curiously only weakly in the SN [27]. In a cell, parkin mainly localizes to the cytoplasm [78]. Furthermore, associations of parkin with the trans Golgi network [79], actin and tubulin filaments [80,81] and synaptic vesicles [79,82] were described.

An analysis of the primary structure of parkin reveals several domains: An N-terminal ubiquitin-like (UBL) domain, and a C-terminal RBR (RING-between-RINGS) domain, consisting of two RING (really interesting new gene) motifs and an in between RING (IBR) domain.

The N-terminal ubiquitin-like domain of parkin comprises amino acids 1-76 and is homologous to human ubiquitin to a degree of 62% [27]. The N-terminal domain has been

described to play a role in the control of parkin expression [83], involvement in substrate recognition [58] and interaction with the Rpn10 subunit of the 26S proteasome [84].

The C-terminal RBR box of parkin presumably mediates its function as an E3 ubiquitin ligase by interacting with E2 conjugating enzymes and substrates. It makes parkin a member of the RING-type E3 ligase family. RING domains have cysteine and histidine residues for zinc binding. In contrast to classical DNA-binding zinc fingers, RING domains provide an interface for protein protein interactions. This family is widespread among eukaryotes and is implicated in various functions, such as cell lineage determination, oncogenesis, and embryogensis [85]. RING domains can be assembled in various ways, thus constituting different subfamilies of RING-type E3 ligases, among them the RBR family. More than 400 RBR domain containing proteins have been identified in various genomes. The closest relative of parkin in human is the Human Homologue of Ariadne (HHARI). The structure of HHARI RING2 has been resolved by NMR, and a molecular model of the parkin RING2 has been generated. This analysis revealed that the parkin and HHARI RING2 domains have a unique topology compared to other RING domains, since they only bind one zinc atom instead of two, and also use a different hydrophobic network than classical RINGs [86].

Parkin-mediated proteasomal degradation

Consistent with its function as an E3 ubiquitin ligase, parkin has been shown to interact with several E2 ubiquitin conjugating enzymes (table 2). In addition to the human E2s UbcH7 and UbcH8, the ER-associated E2s Ubc6 and Ubc7 were identified as binding partners. It has been postulated that the E2/E3 complex catalyzes K48-linked ubiquitylation and thus targets substrate proteins to proteasomal degradation [57,58,59,87,88]

E2	**Model system**	**Citation**
UbcH7, UbcH8	Cell culture (overexpression)	Shimura *et al.,* 2000 Zhang *et al.,* 2000 Imai *et al.,* 2000
UbcH7	Human brain	Shimura *et al.,* 2001
Ubc6, Ubc7	Cell culture (overexpression)	Imai *et al.,* 2001
Ubc13/Uev1a	*In vitro* (recombinant proteins)	Doss-Pepe *et al.,* 2005 Matsuda *et al.,* 2006

Table 2: Parkin associated E2 ubiquitin conjugating enzymes. Ubc: ubiquitin conjugating enzyme E2, Uev1a: Ubiquitin conjugating enzyme E2 variant 1a.

Substrates for parkin were identified by yeast-two-hybrid screens or co-immunoprecipitations (Table 3). The putative substrate proteins fulfil various functions in the cell: proteins with a vesicular and synaptic function such as CDCrel-1 (*cell division control-related protein*) [59] CDCrel-2a [89], synaptotagmin [80], O-glycosylated α-synuclein (αSp22) [88], synphilin-1 [90] and the dopamine transporter (DAT) [91]; control proteins of the cell cycle like cyclin E [92], of protein synthesis like the amino acyl tRNA subunit p38/JTV-1 [93,94]; proteins of the cytoskeleton like α/β tubulin [81]; nuclear export proteins like RanBP2 [95], and proteins of signal transduction like Pael-R (*parkin associated endothelin-like receptor*) [87], and Eps-15, an EGF receptor adaptor protein [96]. The relevance and authenticity of most of the substrates has so far not been proven consistently. Neuropathologic examination of parkin-associated PD patient brains could show a slight accumulation of non-ubiquitylated αSp22, Pael-R, cyclin E, CDCrel-1, CDCrel-2a, FBP1 and p38/JTV-1 in some brains, but in parkin knock out mice, only for FBP1 and p38/JTV-1 an accumulation in the brain could be shown [87,89,92,94,97].

Putative parkin substrates	**Possible function**
	Vesicular and synaptic function
CDCrel-1	Zhang *et al.,* 2000
CDCrel-2a	Choi *et al.,* 2003
Synaptotagmin XI	Huynh *et al.,* 2003
αSp22	Shimura *et al.,* 2001
Synphilin-1	Chung *et al.,* 2001
DAT	Jiang *et al.,* 2004
	Cell cycle
Cyclin E	Staropoli *et al.,* 2003
	Protein biosynthesis
p38/JTV-1	Corti *et al.,* 2003
	Ko *et al.,* 2005
FBP1	Ko *et al.,* 2006
	Cellular signal transduction
Pael-R	Imai *et al.,* 2001
Eps-15	Fallon *et al.,* 2006
	Nuclear export
RanBP2	Um *et al.,* 2006

Table 3: Putative parkin substrates and their function. CDCrel: cell division control-related protein, αSp22: O-glycosylated α-synuclein, DAT: dopamine transporter, FBP1: far upstream sequence element-binding protein 1, Pael-R: Parkin associated endothelin-like receptor, RanBP2: Ran-binding protein 2.

Parkin-mediated regulatory ubiquitylation

Only recently, the E2 heterodimer UbcH13/Uev1a was shown to interact with parkin *in vitro* [98,99]. Earlier studies already describe that UbcH13/Uev1a catalyze ubiquitin linkage via K63 [100]. More recent *in vivo* studies showed parkin to catalyze ubiquitylation via K63 next to the conventional K48 ubiquitylation [101]. A parkin mediated multi-monoubiquitylation via K63 could be observed for p38/JTV1 and Hsp70 [102,103]. The physiological role of parkin-mediated ubiquitylation is mainly unclear.

Putative substrates for a parkin mediated regulative ubiquitylation	**Possible function**
Eps15	EGFR-Endocytosis Fallon *et al.,* 2006
Synphilin-1 (poly-Ub)	Synaptic function Chung *et al.,* 2005
p38/JTV-1 (multi-Ub)	Biosynthesis Hampe *et al.,* 2006
Hsp70 (multi-Ub)	Chaperone Moore *et al.,* 2005

Table 4: Putative parkin substrates for a parkin-mediated regulatory ubiquitylation and their possible function. Eps15: *epidermal growth factor receptor pathway substrate 15*, Hsp70: *Heat shock protein 70*, poly-Ub: polyubiquitylation, multi-Ub: monoubiquitylation at several K residues.

Conclusively, parkin can mediate polyubiquitylation via K48 and K63 as well as a multi-monoubiquitylation. Thus, parkin could act as a multi-functional E3 ligase. Based on these findings, one could speculate that an accumulation of toxic substrates, or the loss of a regulatory ubiquitylation, could be the cause for parkin-associated PD.

Other parkin-interacting proteins

Next to the already mentioned E2 enzymes and putative parkin substrates, several other parkin-interacting proteins could be identified.

Parkin has been described to be part of a functional larger ligase complex, the Skp1-Cullin-F-box (SCF) complex [92]. Imai *et al*. reported a complex of parkin and the chaperones CHIP (*carboxyl terminus of the Hsc70-interacting protein*) and Hsp70 [104]. The interaction with the scaffold protein CASK (Ca2+-calmodulin-dependent serine protein kinase) suggests parkin as a component of a complex which colocalizes with postsynaptic membranes and lipid rafts in the brain [82]. In this line, another study from the same group showed parkin to monoubiquitylate PICK1, a scaffold protein that regulates the activity of Acid Sensing Ion Channels (ASIC), which contribute to excitotoxicity in neurons [105].

Putative parkin interacting proteins	**Possible function**	**Citation**
Actin	Cytoskeleton	Huynh *et al*., 2000
CASK PICK	Postsynaptic PDZ Scaffold protein	Fallon *et al*., 2002
Cullin-1	Multiprotein ligase	Staropoli *et al*., 2003
γ-tubulin	Centrosome	Zhao *et al*., 2003
Rpn-10 α4	Proteasomal subunit	Daechsel *et al*., 2005 Sakata *et al*., 2003
14-3-3η	Signal regulation	Sato *et al*., 2006
BAG5	Co-chaperone	Kalia *et al*., 2004
CHIP	Chaperone	Imai *et al*., 2002
LRRK2	Kinase	Smith *et al*., 2005
DJ-1 mutants	Redox protein?	Moore *et al*., 2005
PINK1	Mitochondrial kinase	Moore *et al*., 2006

Table 5: Potential parkin-interacting proteins and their function. CASK: Ca2+ calmodulin dependent serine protein kinase, PDZ: Postsynaptic density-95, disc large, zona occludens, BAG5: Bcl-2 associated anthanogene 5, CHIP: Carboxyl terminus of the Hsc70 interacting potein, LRRK2: Leucin rich repeat kinase 2, PINK1: PTEN-induced kinase1.

Parkin has a neuroprotective potential

In several cell culture systems and animal models, a broad neuroprotective spectrum of parkin could be observed. Parkin protects cultured cells from apoptosis induced by kainic acid [92], proteasomal inhibition [106,107], ceramide [108], manganese [109], dopamine [91] and overexpression of parkin substrates or other proteins like α-synuclein [107], Pael-R [87], p38/JTV-1, expanded polyQ ataxin 3 fragment [110] and ataxin-2 [111]. In *Drosophila*, an

overexpression of parkin could rescue dopaminergic neurons from cell death induced by α-synuclein and Pael-R overexpression. Moreover, viral overexpression of parkin inhibits dopaminergic neuron degeneration induced by α-synuclein or tau in rats and saves skeletal muscles of mice from mitochondrial toxins [112,113,114].

Parkin mediates neuroprotection from	**Model system**	**Citation**
Proteasomal inhibition	Primary murine neurons (viral overexpression) Cell culture (SH-SY5Y)	Petrucelli *et al*., 2002 Muquit *et al*., 2004
Ceramide induced cell death	Cell culture (PC12)	Darios *et al*., 2003
Kainate induced excitotoxicity	Primary murine neurons	Staropoli *et al*., 2003
Manganese induced toxicity	Cell culture (SH-SY5Y)	Higashi *et al*., 2004
Dopamine induced apoptosis	Cell culture (SH-SY5Y)	Jiang *et al*., 2004
Mitochondrial toxins: MPP+, Rotenone	Parkin k.o. mice Cell culture (NT-2 and SK-N-MC) Primary muscle cells	Casarejos *et al*., 2006 Hyun *et al*., 2005 Rosen *et al*., 2006
Toxicity induced by overexpression of parkin substrates or other proteins: Pael-R P38/JTV-1 α-synuclein (mutant) tau (mutant) Elongated polyQ ataxin 3 fragment Ataxin2 Aβ	 Cell culture (SH-SY5Y) *Drosophila* Cell culture (SH-SY5Y, SK-N-MC) Mouse primary neurons Rats *Drosophila* Transgenic mice (Parkin$^{-/-}$ /tau^{VLW}) rat Cell culture (N18) Cell culture (PC12) Primary muscle cells	 Imai *et al*., 2001 Yang *et al*., 2003 Ko *et al*., 2005, Corti *et al*., 2003 Petrucelli *et al*., 2002 LoBianco *et al*., 2004 Yang *et al*., 2003 Mendenez *et al*., 2006 Klein *et al*., 2006 Tsai *et al*., 2003 Huynh *et al*., 2007 Rosen *et al*., 2006

Table 6: Neuroprotective potential of parkin against various stressors. Tau^{VWL}: Tau with a triple FTDP-17 mutation (G272V, P301L, R406W), FTDP-17: Frontotemporal dementia with parkinsonism-17.

These observations give rise to the assumption that parkin plays a central role for neuronal integrity under cellular stress conditions. Due to the dopamine metabolism, dopaminergic neurons are exposed to high oxidative stress. The mechanism that could clarify the neuroprotective potential is not resolved yet.

Parkin mutations

Large homozygous deletions were described for the first time in a Japanese population [27]. Follow-up studies revealed multiplications, small deletions/insertions and a variety of point mutations in different ethnic groups. Until today, more than 100 pathogenic parkin mutations were described in PD patients. Figure 12 shows missense and nonsense mutations schematically.

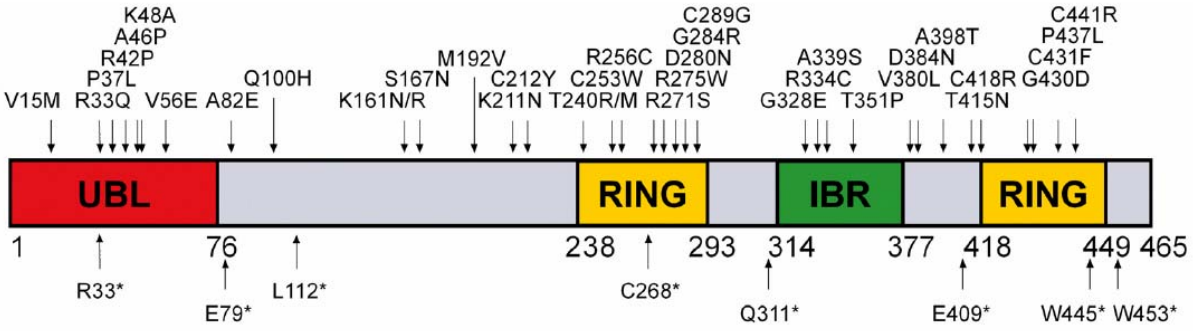


Figure 12: Point mutations in parkin. Schematic representation of parkin and its functional domains. Missense and nonsense mutations are indicated by arrows. Stop mutations are marked with an asterisk. UBL: ubiquitin-like; RING: really interesting new gene; IBR: in-between RINGs. Kindly provided by I. Henn.

Although mutations occur almost everywhere in the coding region of the parkin gene, an accumulation of mutations in the functional domain is obvious. The localization of mutations and identification and characterization of amino acids that are essential for parkin function can give important insights into the role of parkin in PD pathogenesis. Interaction analyses revealed that mutations in the RBR region can inhibit interaction with E2 ubiquitin conjugation enzymes and/or substrate binding [57,59,88,90].

Parkin-deficient animal models

To uncover the molecular pathogenesis of parkin-linked PD and to clarify the physiological role of parkin *in vivo*, several groups established parkin deficient animal models. Below, the most important results from mice and *Drosophila* are summarized.

Parkin knockout mice

Parkin knockout (k.o.) mice strains targeting several exons of murine parkin were established: (i) deletion of exon 2, which corresponds to the UBL domain [115,116]; (ii) deletion of exon 3, which is the most common deletion in AR-JP [117,118]; (iii) deletion of exon 7, which corresponds to RING1 of the RBR domain [119]. All deletions lead to complete loss of the parkin protein.

The published k.o. mice have no significant phenotype which recapitulate the symptoms of the human disease, namely a motor phenotype and the demise of dopaminergic neurons of the SN. The strains containing deletions targeted against exon 2 have been described to have no phenotype. The other strains have various phenotypes which are not very pronounced. Behavioral changes, such as reduced explorative behavior, indicate disturbances of the nigrostriatal pathway, but neuropathologically, no loss of dopaminergic neurons or nigrostriatal degeneration occurred. Small changes were observed in DA metabolism and dopaminergic neurotransmission, as well as deficits in mitochondrial respiration. Steady-state levels of some proposed parkin substrates, CDCrel-1, synphilin1, and α-synuclein, were not altered in parkin -/- mice with deletion of exon 3, which raises the question about the authenticity of these substrates. Interestingly, compared to wildtype mice, the dopaminergic neurons of k.o. mice are much more sensitive to oxidative stress induced by rotenone [120] .

In conclusion, deletion of parkin in mice did not lead to a phenotype that recapitulates the situation of AR-JP in humans, but leads to only slight changes in DA metabolism and DA receptor expression.

Drosophila model

A parkin deficient *Drosophila* strain has been established in 2003 by Greene *et al.* [121] by targeted deletion of the highly conserved *Drosophila* parkin orthologue. Like in mice, no loss of dopaminergic neurons was observed, which is the hallmark of AR-PD in humans. However, a null phenotype was characterized that might indicate some functional aspects of parkin: male infertility, a deficit to fly and jump, and flight muscle degeneration point to a

problem of energy-demanding tissues and a possible involvement of mitochondria, which has indeed later been shown by Clark et al. and Park *et al.* in 2006 [32,33]. During analyzing PINK1 deficient flies, they found a strong and similar phenotype of the mitochondrial ultrastructure in PINK1- and parkin-deficient flies, as indicated by the gross phenotype. Interestingly, a genetic interaction indicated that both proteins seem to act in the same pathway, with PINK1 upstream of parkin: the PINK phenotype was rescued by overexpression of parkin, but not vice versa.

An impact on mitochondrial dynamics by PINK1 and parkin has been shown with similar genetic interaction experiments by several groups [35,122]. They showed that key regulatory proteins of mitochondrial fission and fusion could aggravate or alleviate the phenotypes induced by PINK1 and parkin.

Results

Determinants of parkin folding

Parkin has been shown to be an aggregation-prone protein. Particularly, it is sensitive to oxidative stress, as shown in an earlier study of our group. H_2O_2 treatment of cultured cells overexpressing wildtype (wt) parkin leads to massive misfolding of the protein. Also, a heat shock can induce parkin misfolding [2].

Other groups could show that upon application of dopamine to cultured cells, parkin becomes functionally inactivated and is found in detergent-insoluble aggregates in brains from PD patients [123,124]. Previous studies of our group also demonstrated that there is an array of pathogenic point mutations and deletion mutations, e.g. the W453X, E409X, and Q311X, that lead to misfolding of the protein. Misfolding of parkin has therefore been postulated to be an important molecular mechanism for the pathogenesis of parkin associated early onset familial PD [1,2].

Analysis of parkin deletion mutants

A unique feature of parkin is its modular structure, which is schematically represented in Figure 13A. It contains an N-terminal ubiquitin-like domain (UBL) and a C-terminal RBR domain [27,125]. Providing insight into the functional role of the single domains could help to understand the molecular pathogenic mechanisms of parkin associated PD, as well as basic functional aspects of parkin.

Domain deletions

Since proper folding of parkin is rather sensitive [1,2], the first issue to investigate was the importance of the domains of parkin for its native folding. To this end, domain deletion mutants of parkin were cloned to analyze the functional relevance of the parkin domains. Each of the cloned mutants lacks one functional domain, as indicated in Figure 13B. To compare wt parkin with the domain deletion mutants in regards of their folding behaviour, HEK293T cells and SH-SY5Y cells were transiently transfected with wt or mutant parkin constructs. The two cell lines were used due to their particular features: HEK293T cells are more appropriate for Western blot analysis because their transfection efficiency is much higher compared to SH-SY5Y, and therefore detection of the protein by Western blot is facilitated. SH-SY5Y cells

have a morphology that disposes them for immunofluorescence studies, as they are much larger than HEK293T cells and also have a more favorable cytosol-to-nucleus ratio.

Transfected HEK293T cells were used for the detergent solubility assay [126]: cells were lysed in detergent buffer containing 0.1% Triton-X 100 and separated into detergent soluble (S) and insoluble (P) fraction by centrifugation at 16,000x g for 20 min at 4°C. The lysis conditions were chosen on the basis of previous work in our group that showed that wt parkin is soluble under these conditions, whereas a pathogenic deletion mutant, W453X, is not. Equal amounts of the soluble and insoluble fraction were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blot. Two parkin species can be detected: a higher molecular weight band of around 52 kDa corresponds to the full length protein; the lower molecular weight band of around 42 kDa appears due to an internal translational start site at position 80 [1]. Therefore, the smaller parkin species lacks the UBL domain.

To analyze the folding characteristics of wt parkin and the domain deletion mutants, Western blot analysis of wt and mutant parkin was carried out. This analysis revealed that wildtype parkin and the ΔUBL mutant (aa 1-76 deleted) were predominantly present in the soluble fraction, whereas all the other mutants, ΔLinker, ΔRING1, ΔIBR and ΔRING2, shift to the 0.1% Triton X-100 insoluble fraction (Figure 13B). Immunofluorescence analysis, which was performed with the transfected SH-SY5Y cells, confirmed these results by showing homogenous staining when wildtype parkin and the ΔUBL mutant was transfected. In contrast, a scattered punctate parkin-positive staining was visible in cells transfected with the other mutants, indicating aggregated parkin. These experiments were also performed in different cell lines to show that parkin folding and misfolding is not dependent on a specific cell type (data not shown).

Thus, the UBL is the only domain which can be deleted without interfering with proper folding of parkin, whereas all the other domains are essential for this process. This also implicates that the ΔUBL is the only domain deletion mutant that can be used for functional assays of parkin, since the detergent insolubility of the other domain deletion mutants reflects non-native conformational alterations, thus, these mutants are probably not functional.

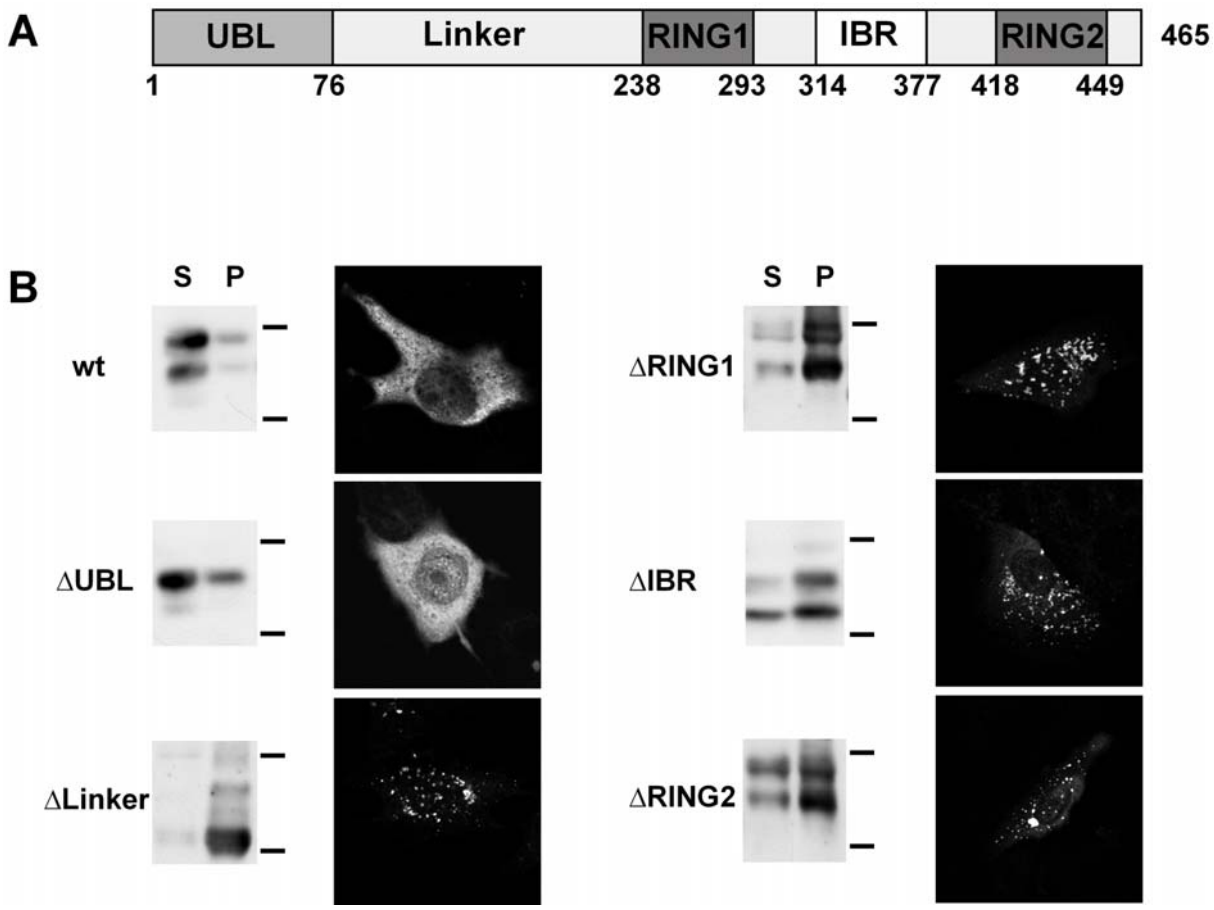


Figure 13. Domains of parkin and their role in folding. A. Parkin has a modular structure of conserved domains. UBL – ubiquitin like, IBR – in between RINGs. B. HEK293T cells and SH-SY5Y cells were transfected with wt or mutant parkin. Transfected HEK293T cells were harvested, lysed in 0.1% Triton X-100 in PBS, separated in detergent soluble (S) and insoluble (P) fraction by centrifugation, and analyzed by Western blot. Transfected SH-SY5Y cells were PFA-fixed, permeabilized and stained with hP1, a rabbit polyclonal antibody (pAb) raised against parkin.

The domain deletion analysis revealed that the ΔUBL mutation was the only one that was tolerated in regards of folding. Therefore, an interesting question was how many amino acids in addition to the UBL can be deleted without disturbing the correct folding of parkin. To this end, constructs were generated which lack larger parts of the N-terminal domain: Δ1-100 (amino acids (aa)1-100 deleted) which spans the UBL and a small part of the linker region, Δ1-144 (aa 1-144 deleted) which is deleted until the middle of the linker region, and Δ1-238 (aa 1-238 deleted) which deletes the UBL and the complete linker region until the beginning of RING1. HEK293T cells were transfected with these constructs and analyzed by detergent solubility assay (Figure 14). The Western blot analysis showed that almost half of the linker region can be deleted (Δ1-144) without interfering with parkin folding. Only when the RING-IBR-RING part is left, as for the Δ1-238 mutant, detergent solubility was impaired.

These results indicate that N-terminal deletion is tolerated over a large stretch of the protein, whereas the more C-terminal domain is highly sensitive. This is in line with the fact that the deletion of more than 3 C-terminal amino acids is sufficient to interfere with parkin folding [2].

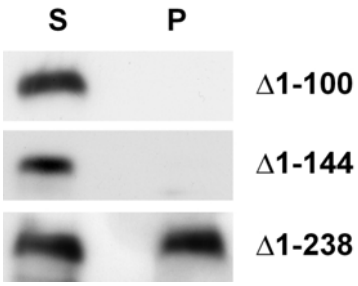


Figure 14: The role of the N-terminal domain in parkin folding. HEK293T-cells have been transfected with the indicated N-terminal deletion constructs of parkin and analyzed with the detergent solubility assay as described in Figure 13.

Deletion of 3 C-terminal amino acids

Since it was known that the deletion of 2 amino acids did not interfere with parkin folding, but the deletion of only 4 amino acids (W462X) had devastating effects on parkin folding [2], a mutant lacking three amino acids was cloned, so that the highly conserved phenylalanine (see below, Figure 16) was replaced by a stop codon. As shown by transient transfection of HEK293T cells followed by a detergent solubility assay in Figure 15 (upper panels), and immunofluorescence of transfected SH-SY5Y cells in the lower panels, the deletion of the 3 terminal amino acids had a severe effect on parkin folding. In the course of the study, this data has also been reproduced by Joch *et al*. in 2007 [105].

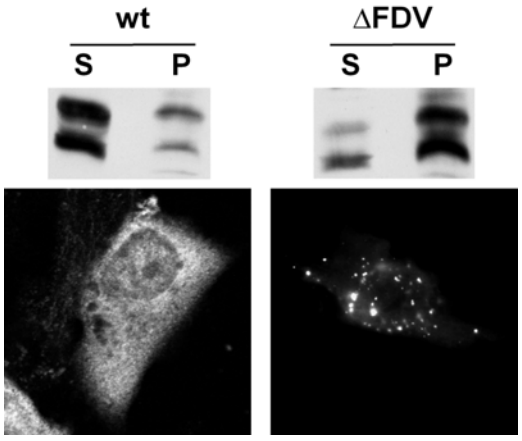


Figure 15: The last 3 amino acids are crucial for parkin folding. HEK293T and SH-SY5Y cells were transfected with wt parkin or a mutant lacking the terminal 3 amino acids. Detergent solubility analysis was carried out as described before, and is supported by immunofluorescence which was carried out with the anti-parkin pAb hP1 and anti rabbit Alexa 555 antibody.

Impact of the putative PDZ-binding motif on parkin folding

Interestingly, an alignment of the protein sequences of the extreme C-terminus after the RING2 domain of several species revealed a high degree of conservation during evolution, but a putative PDZ binding motif comprising the three C-terminal amino acids is not conserved (Figure 16) [82].

PDZ-binding domains are small C-terminal peptide motifs which can bind to PDZ domains of interaction partners. PDZ domains are modular protein interaction domains of 80-90 amino acids in length involved in transport, localization and assembly of multiprotein signalling complexes at cellular membranes. The most prominent example are postsynaptic densities in neuronal membranes, where protein scaffolds emerge due to PDZ-dependent protein-protein interactions. PDZ domains can also interact with phosphoinositides or dimerize, multiplying their functional diversity [127]. They are named after the three proteins in which these sequence motifs were originally identified: **P**SD-95 (postsynaptic density protein 95), **d**iscs large, **z**ona occludens 1. PDZ domains have a conserved peptide binding groove that interacts with short peptide sequences at the extreme C-terminus of other proteins, the PDZ-binding motifs. Based on their target sequence specificity, they have been categorized into three classes. Class I PDZ domains bind to (S/T)X(V/I/L), class II PDZ domains bind Φ-X-Φ (Φ is a hydrophobic residue) and class III PDZ domains bind (D/E)X(V/L). The last three amino acids of parkin at the C-terminus (FDV) have been suggested to function as a PDZ-binding motif corresponding to class II, mediating binding to the PDZ domain-containing proteins CASK and PICK1 [82,105].

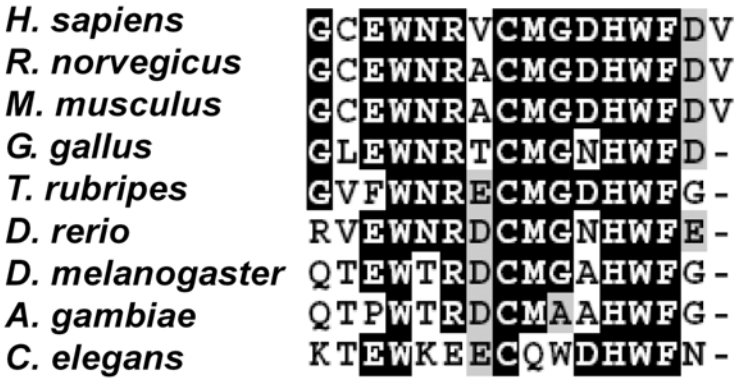


Figure 16: The C-terminus of parkin is highly conserved throughout evolution. C-termini of different species, mammalian, avian, fish, insect and nematode, have been aligned with the ClustalW algorithm. Identical amino acids are black boxed, similar amino acids are grey boxed.

Based on the alignment in Figure 16, several mutants were designed in order to find out about the role of the PDZ binding motif for parkin folding and function.

Mutational analysis of the putative PDZ-binding motif

Other functional domains of parkin, such as the UBL and the RBR domain, are highly conserved between species. The FDV motif is only present in mammals, but not in non-mammalian species (*Gallus gallus, Fugu rubripes, Danio rerio*) or non-vertebrates (*Drosophila melanogaster, Anopheles gambiae,* and *Caenorhabditis elegans,* Figure 16). To analyze the role of the putative PDZ-binding motif in parkin folding, two mutants were cloned that lack a functional PDZ-binding motif: (i) the C-terminal domain of human parkin was replaced by the *D. melanogaster* sequence of parkin (parkin-*D.m.*-CT; aa 468-482) according to the alignment in Figure 16; (ii) the terminal valine at position 465 was replaced by glutamic acid (V465E), a mutation that has previously reported to disrupt PDZ-dependent interactions because the requirements of the Φ-X-Φ class II PDZ-binding motif is no longer fulfilled [82,128]. A detergent solubility assay has been carried out and analyzed by Western blot as described before. The upper panels of Figure 17 show that the PDZ mutant forms of parkin adopted a stable fold similar to wildtype parkin. This is supported by immunofluorescence staining of the overexpressed mutants, showing a homogenous staining comparable to wildtype parkin. Thus, the integrity of the PDZ binding motif seems not to be essential for parkin folding.

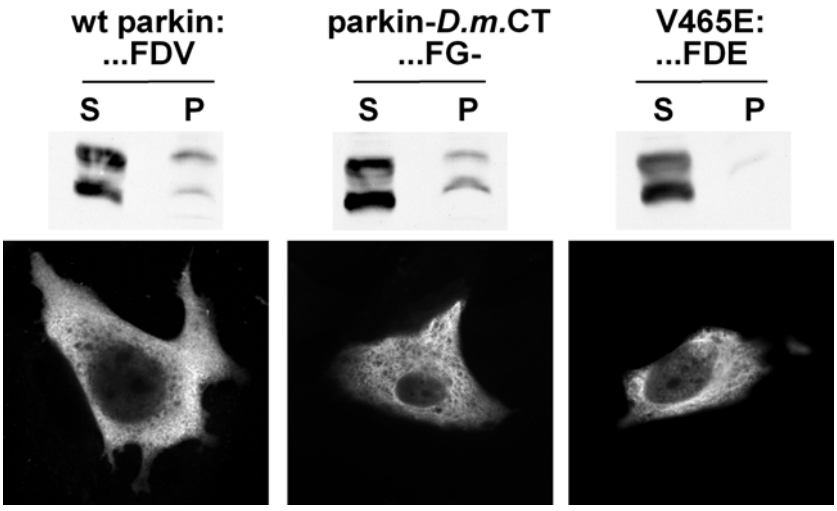


Figure 17: The integrity of the putative PDZ binding motif is dispensable for the formation of detergent-soluble parkin. HEK293T and SH-SY5Y cells were transfected with wt parkin or PDZ-defective mutants. Detergent solubility analysis was carried out as described before, and is supported by indirect immunofluorescence using the anti-parkin pAb hP1.

To analyze which amino acid of the putative PDZ binding motif is crucial for parkin folding, we replaced each of the last three amino acids with alanine (V465A, D464A, F463A). Detergent solubility analysis accompanied by immunofluorescence revealed that the exchange of the last two amino acids in the mutants V465A and D464A had no effect on parkin folding, but when the phenylalanine 463 is mutated to an alanine, parkin shifted to the insoluble fraction (Figure 18). Thus, the phenylalanine 463 is crucial for parkin folding, which is in line with the conservation of this amino acid in all species (Figure 16).

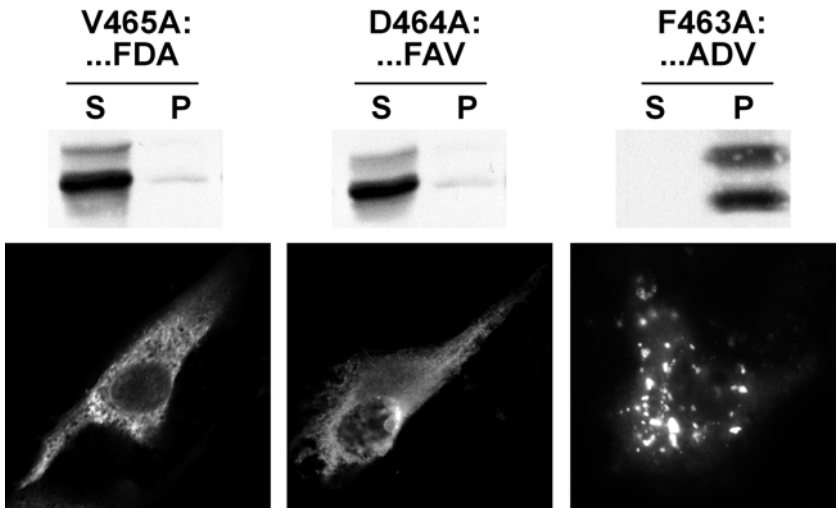


Figure 18: F463 is essential for correct parkin folding. HEK293T and SH-SY5Y cells were transfected with wt parkin or mutants where the last three amino acids were sequentially replaced by alanin. Detergent solubility analysis has been carried out as described before, and is supported by indirect immunofluorescence using the anti-parkin pAb hP1.

The folding analysis of the putative PDZ-binding motif of parkin revealed that its integrity is not essential for parkin folding. The question then occurred whether the putative PDZ-binding motif of parkin might be essential for other established properties of parkin: (i) membrane association of parkin [1], (ii) neuroprotective potential of parkin, and (iii) ubiquitylation activity of parkin [129].

(i) Influence of C-terminal mutations on membrane association of parkin

As described above, PDZ interactions are crucial for proteins to form scaffolds at membranes, where e.g. receptors interact with adaptor or signalling proteins. Since parkin can be found in association with membranes [79,82,130], an interesting question was whether the putative PDZ-binding domain is involved in membrane targeting of parkin. To this end, we applied a membrane flotation experiment on wt parkin and the PDZ mutants V465E and parkin-*D.m.*-

CT. Homogenates of transiently transfected HEK293T cells were overlayered by several steps of an iodixanol density gradient and ultracentrifuged for 3 hours, so that membranes could float up in the gradient. Fractions were collected from top to bottom of the ultracentrifugation tube and analyzed by Western blot (Figure 19). The purity of the fractions was confirmed by probing against GAPDH as a cytosolic marker and transferrin receptor (TFR) as a marker for the fraction containing cellular membranes. Parkin occured mainly in cytosolic fractions (fraction 7 and 8), but a portion of the protein also floated up with the cellular membranes (fraction 3 or 4). Similar experiments were performed previously in our group by using N2a cells and a renografin gradient [1], supporting the results described here.

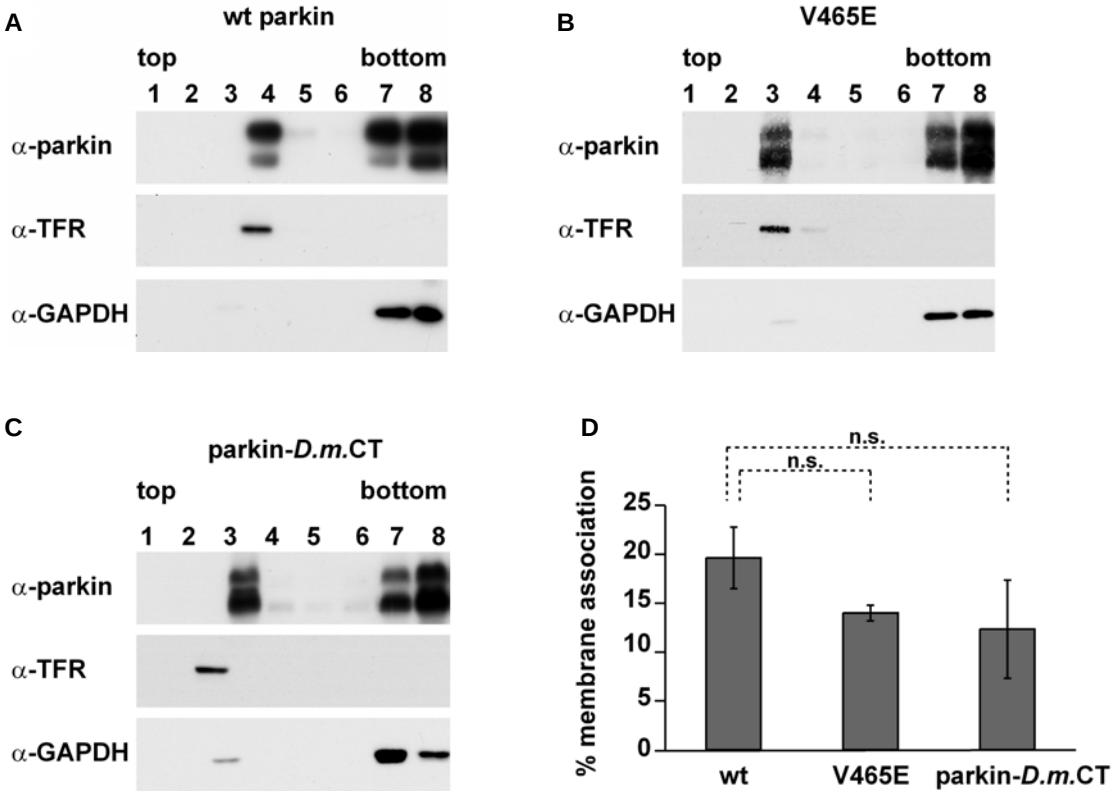


Figure 19: The putative PDZ binding domain is not involved in membrane targeting of parkin. A-C: HEK293T cells were transiently transfected with wt or PDZ mutant parkin. Total cell homogenates were subjected to density gradient centrifugation. Eight fractions were collected from top to bottom of the centrifugation tube, and aliquots of those were analyzed by Western blotting against parkin, transferrin receptor (TFR) or GAPDH. **D:** Quantification of signal intensity of Western blots of three independent membrane flotation experiments with the constructs indicated. Membrane-associated fraction versus total content of the overexpressed parkin construct has been determined. Error bars indicate +/- S.E.; *n.s.*, not significant.

Quantification of the membrane-associated fraction versus total content of overexpressed wildtype or mutant parkin revealed that there is no significant difference between wildtype and PDZ mutant parkin. Thus, the integrity of the C-terminal putative PDZ-binding motif seems not to be essential for membrane targeting of parkin.

(ii) Impact of the putative PDZ-binding motif on the neuroprotective potential of parkin

In several cell culture systems and animal models, a broad neuroprotective activity of parkin has been shown (see introduction). Therefore, to further characterize the PDZ mutants in terms of functionality, they were subjected to the neuroprotection assay established in our lab [129]. With this assay, it has been shown that wt parkin shows cytoprotective activity when cultured cells are treated with kainate or a variety of other toxins. Kainate is a compound which activates ionotrophic glutamate receptors and induces apoptosis by excitotoxicity.
Mock-, parkin- or mutant parkin transfected SH-SY5Y cells were incubated in the presence or absence of 500 µM kainate for 3 h. Apoptotic cells were identified by indirect immunofluorescence using an antibody specific for activated caspase-3. The results of three independent experiments show that the integrity of the putative C-terminal PDZ-binding domain is not essential for the neuroprotective activity of parkin (Figure 20).

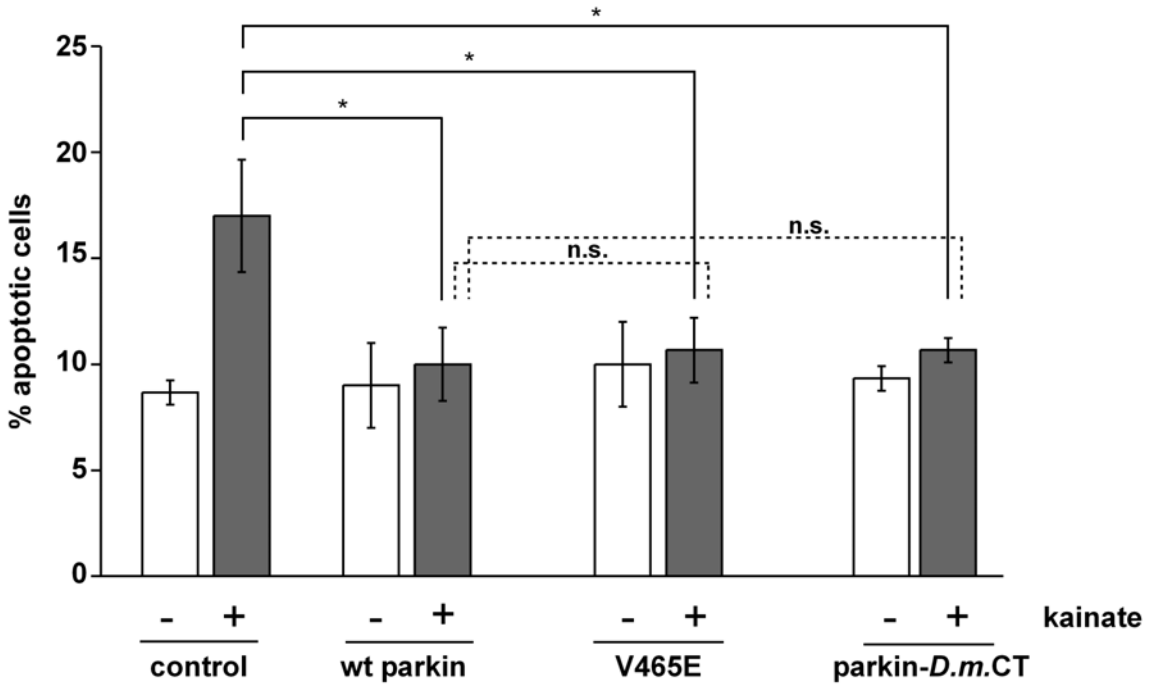


Figure 20: Neuroprotective activity of parkin is independent of the putative PDZ binding motif. SH-SY5Y cells were transiently cotransfected with wt or mutant parkin and YFP to visualize transfected cells. 24 h after transfection, cells were incubated with 500 µM kainate for 3 h at 37°C, PFA-fixed, permeabilized and analyzed by indirect immunofluorescence using an antibody against active casapase 3. Shown is the percentage of apoptotic cells among the transfected cells. Error bars indicate +/- S.E. *$p < 0.05$ (ANOVA).

(iii) Impact of the putative PDZ-binding motif on the ubiquitylation activity of parkin

Consistent with its function as an E3 ubiquitin ligase, parkin has been shown to ubiquitylate various substrates (see introduction). To address the question whether the putative PDZ binding domain has an impact on parkin function, the ubiquitylation patterns of overexpressed parkin and the PDZ mutants were investigated. Firstly, an autoubiquitylation assay of parkin, which is a surrogate marker of its E3 ligase activity, was carried out, and secondly, the ubiquitylation of IKKγ upon overexpression of parkin was assessed. For the first approach, wt or mutant parkin and HA-tagged ubiquitin were co-expressed in HEK293T cells and immunoprecipitated under denaturing conditions with hP1 anti parkin antibody. Prior to immuoprecipitation, a denaturation step was performed to ensure that the HA-ubiquitin signal stems only from the immunoprecipitated parkin and no other parkin-binding proteins, for example a parkin substrate like IKKγ or Traf2, which could coimmunoprecipitate under 0.1% Triton X-100 lysis conditions (see below, Figure 39; [129]). The Western blot analysis of the ubiquitylation assay was carried out using HRP-conjugated HA-antibody to avoid cross-reaction with IgG heavy and light chains and is shown in the upper panel of Figure 21. To monitor differences in expression levels of the transfected parkin constructs, the input (lower panel) was analyzed by Western blotting. No significant differences in the amount of ubiquitylated parkin could be detected, which indicates that the PDZ-binding motif has no impact on auto-ubiquitylation of parkin.

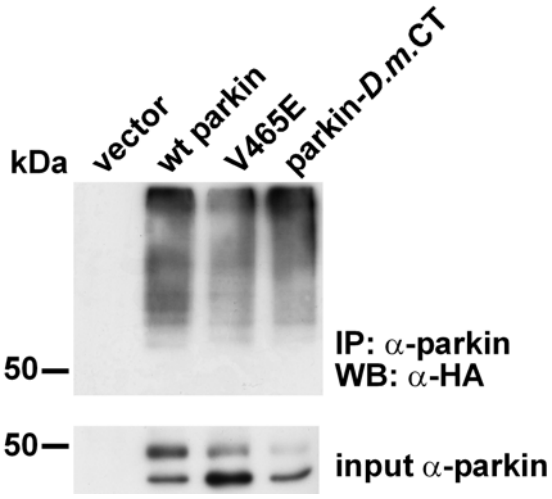


Figure 21: Ubiquitylation of parkin is intact upon PDZ-binding motif disruption. HEK293T cells were transiently transfected with HA-ubiquitin and wt parkin or the mutants indicated. Overexpressed parkin was immunoprecipitated under denaturing conditions and analyzed by Western blot against HA-ubiquitin. An input sample was probed with hP1 antibody to compare expression levels of the parkin constructs.

For the second approach, we took advantage of a recent finding by our group. We identified interaction of the NFκB modulator IKKγ and parkin, as well as increased ubiquitylation of IKKγ upon overexpression of parkin (see below, Figure 39/40; [129]). To test the activity of wildtype and mutant parkin to enhance ubiquitylation of IKKγ, HEK293T cells were cotransfected with wildtype or mutant parkin and flag-tagged IKKγ and HA-tagged ubiquitin. Immunoprecipitation after denaturation of the lysate was carried out as described above, but with a FLAG-antibody to precipitate IKKγ. Western blot against HA-ubiquitin (Figure 22) revealed no signicicant difference in ubiquitylation of IKKγ after overexpression of wt or PDZ mutant parkin. The difference in signal intensities between parkin-*D.m.*-CT and wildtype can be explained with differences in expression levels of the parkin constructs. Thus, the disruption of the putative PDZ binding motif does not interfere with the capacity of parkin to promote ubiquitylation of IKKγ.

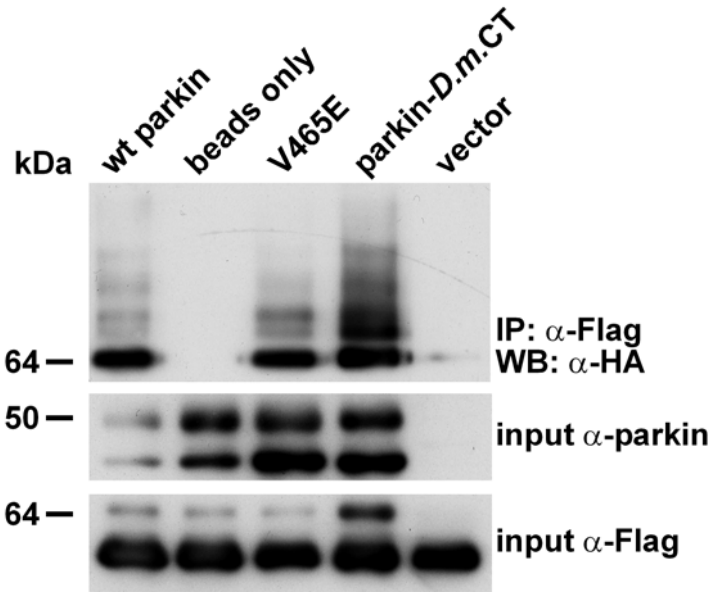


Figure 22: Ubiquitylation of IKKγ is preserved upon disruption of the PDZ-binding motif. HEK293T cells were transiently cotransfected with wt or mutant parkin, IKKγ-flag and HA-ubiquitin. Immunoprecipitation with FLAG-M2 coupled agarose has been performed under denaturing conditions. Input samples have been probed against parkin and FLAG in order to detect differences in expression levels. In the uppermost panel, a ubiquitin smear becomes visible at higher molecular weight. In the lowermost panel, the lower band corresponds to IKKγ, and the upper band to monoubiquitylated IKKγ.

Conclusively, the putative PDZ binding domain had no impact on the native folding pathway of parkin; two major functions of parkin, neuroprotective activity and ubiquitylation activity, were not impaired after disruption of the putative PDZ binding motif; parkin membrane association was not dependent on the integrity of the PDZ binding motif. Thus, the putative PDZ binding motif has no influence on important properties of parkin.

A role in translational regulation for the parkin C-terminus?

A common observation made for all the C-terminal missense mutants described in the paragraphs above is that compared to wt parkin, the ratio of the 52 kDa band, which corresponds to full-length (fl) parkin, to the 42 kDa band of the smaller parkin species is changed as soon as the amino acid sequence is altered, irrespective of proper folding. Due to that, we hypothesized that the human wt C-terminal sequence might play a role in the stability of the protein. One explanation would be that the C-terminal missense mutants are degraded by the proteasome. To test this, wt and C-terminal mutants were overexpressed in HEK293T cells with or without proteasomal inhibition. In Figure 23A, a comparison of wt and one C-terminal mutant which is not impaired in folding, the parkin-*D.m.*-CT, under conditions of proteasomal inhibition is shown. The Western blot analysis showed that proteasomal inhibition did not change the shift in ratio of fl and ΔN parkin displayed by wt and C-terminal mutant parkin. So, the phenomenon of shift in ratio might be due to a different mechanism.

To test whether the answer for the shift in ratio was lying in the mRNA sequence, a mutant was cloned that contained silent mutations in the last three codons. The amino acid sequence remained wildtype but the mRNA was changed in a similar manner like in the other C-terminal point mutations. This mutant was named wt silent. A first detergent solubility assay of wt and wt silent revealed that the ratio of larger and smaller translational product was shifted (Figure 23B). Thus, the construct containing wildtype amino acid sequence but altered mRNA behaved similar to the C-terminal missense mutants.

This result indicates an effect on the mRNA sequence on the translation of parkin. Further investigation is needed here, e.g. confirming mRNA stability on Northern blot, or analyzing the formation of secondary structure formation of the mRNA and the role of several open reading frames of wt parkin.

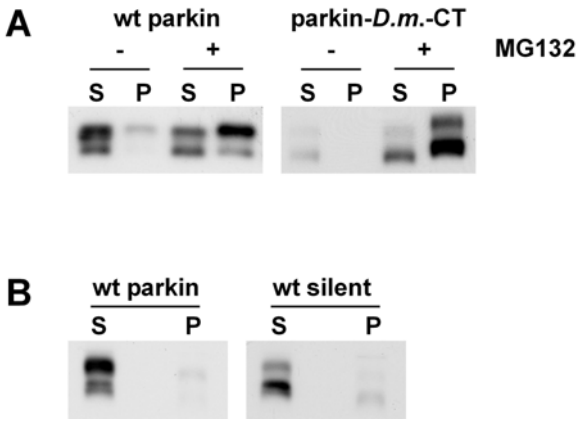


Figure 23: C-terminal mutations influence the ratio of smaller and larger parkin species. HEK293T cells were transfected with wt parkin or mutants. Detergent solubility analysis was carried out as described before. A. Wt parkin and parkin-*D.m.*-CT with and without MG132 treatment (5 µM overnight). B. Solubility profile of wt and wt silent.

Comparative analysis of parkin and HHARI

Parkin is a member of the RBR family, a gene family that is characterized by two RING finger domains separated by an IBR domain (RBR motif; Figure 24). Many RBR proteins function as E3 ubiquitin ligases [131]. The closest relative of parkin in humans is the Human Homologue of Ariadne (HHARI). Ariadne family proteins have first been characterized in *Drosophila,* and have been shown to interact with ubiquitin-conjugating proteins (E2) [132]. Also, HHARI has been shown to have an E3-ligase activity, [133] and to regulate the transcription factor single-minded 2 (SIM2) [134]. The structure of its RING2 domain has been resolved by NMR. A molecular model of parkin RING2 has been proposed based on the structure of HHARI RING2, taking advantage of the high degree of homology between these two regions [86].

In Figure 24, a schematic representation of parkin and HHARI is depicted. Homologous regions in the C-terminal halfs of the proteins are indicated by similar shading.

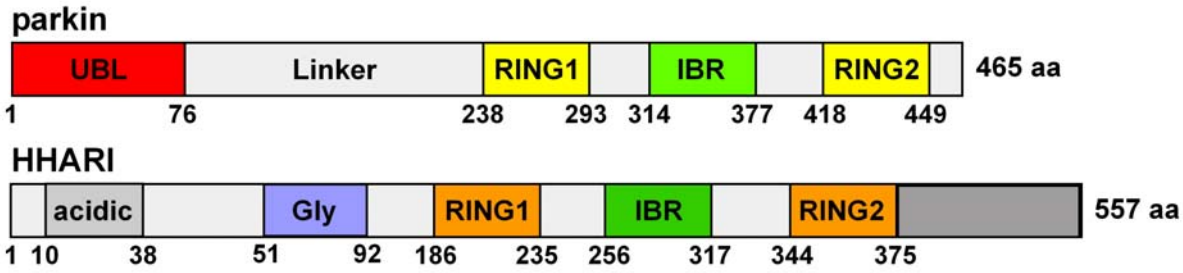


Figure 24: Modular structure of parkin and HHARI. Parkin and HHARI have a similar modular domain structure. The complete RBR domain of parkin and HHARI is highly homologous to each other and determines them as members of a subfamily of RING-finger containing proteins.

Sensitivity to oxidative stress

The RING domains are highly conserved in parkin and HHARI, and are rich in cysteine [86]. To test whether the sensitivity to oxidative stress [2] is specific to parkin, or whether this is an intrinsic feature of the RING domain-containing proteins, a comparative analysis of wildtype parkin and HHARI was conducted. HA-tagged HHARI-overexpressing cells were subjected to oxidative stress by treatment with increasing concentrations of H_2O_2. As a control, YFP-expressing cells were treated in the same way. The detergent solubility assay showed that in the case of parkin and HHARI, a major part of the protein shifts to the detergent-insoluble fraction and thus misfolds under oxidative stress, whereas for YFP, the distribution in the two fractions remains the same under all conditions tested (Figure 25).

Thus, the sensitivity to oxidative stress seems to reside in the RING domains, probably due to their high content in cysteines. During the course of the study, this has also been confirmed by LaVoie et al. in 2007 [135].

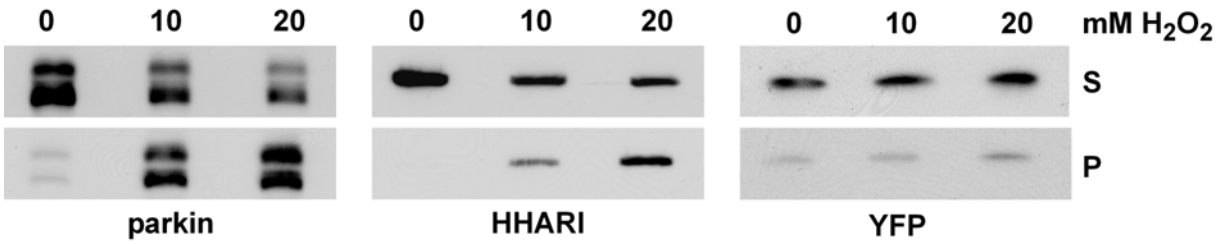


Figure 25: Parkin and HHARI behave similarly under oxidative stress. Transiently transfected HEK293T cells were treated with H_2O_2 at the indicated concentrations. After 30 min, cells were harvested, lysed in 0.1% Triton-X 100 in PBS and analyzed by Western blot using hP1 pAb, anti-HA and anti-GFP antibodies.

C-terminal truncations of parkin and HHARI

Alignment of the protein sequences of parkin and HHARI revealed that they not only have a high homology at the RING domains, but that the C-terminal part of parkin distal to RING2 has some homology to the HHARI sequence shortly after its RING2 domain. Since parkin folding is very sensitive to C-terminal truncation [2], it was an interesting question if HHARI behaves in a similar manner. To investigate this, a C-terminally truncated mutant of HHARI has been cloned which corresponds to the pathogenic parkin W453X mutant according to the alignment. Both wildtype and C-terminally truncated proteins were analyzed by the detergent solubility assay. Different lysis conditions were used, (i) 0.1% Triton-X 100, a non-ionic detergent, in PBS; (ii) 0.1% Triton-X 100/ 0.5% sodium deoxycholate (DOC) in PBS. DOC is a strong ionic detergent, which is more capable to solubilize protein aggregates than Triton-X 100. The upper panels of Figure 26 show the results of 0.1 % Triton-X 100 treatment. Wt parkin, wt HHARI and HHARI P378X were mainly present in the soluble fraction, and only parkin W453X was entirely in the detergent insoluble fraction. In the lower panels, the results are similar, however, the small portion of HHARI and wt parkin which was visible under the milder detergent conditions in the pellet fraction was completely removed when DOC was present in the lysis buffer. This experiment shows clearly that HHARI is not as sensitive to C-terminal truncation as parkin. Thus, the folding sensitivity to C-terminal truncation is a unique feature of parkin.

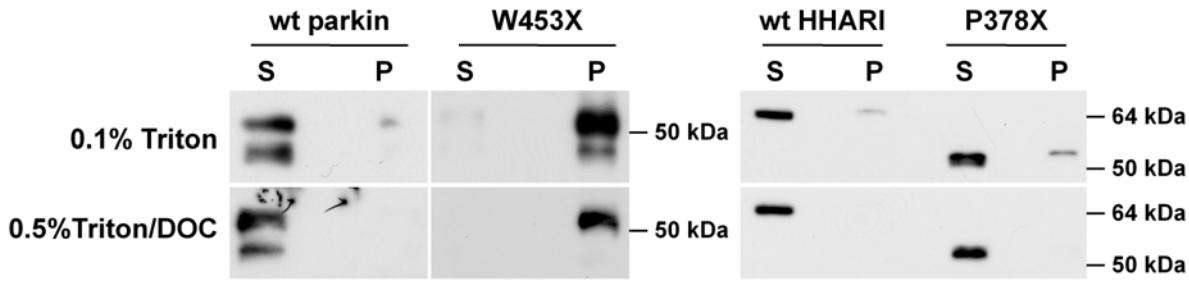


Figure 26: Parkin is more sensitive to C-terminal truncation than HHARI. HEK 293T cells were transfected with wildtype and truncated forms of parkin and HHARI and lysed with non-ionic and/or ionic detergent. For detection of the proteins on Western blot the hP1 antibody was used for the parkin constructs, and HRP conjugated HA antibody was used for HA-tagged HHARI constructs.

Can the C-terminal domain of HHARI replace that of parkin?

To better understand the role of the C-terminal domain in parkin folding, we asked whether the C-terminal domain distal to RING 2 of HHARI could replace that of parkin. To this end, three chimeric parkin-HHARI constructs were generated according to alignment of the protein sequences. The first one, 449/376, comprises the parkin sequence from start until the end of its RING2. There, it is fused to the RING2 distal part of the HHARI sequence according to Moynihan *et al.*, who proposed in 1999 that the HHARI RING2 domain spans to amino acid 375 (Figure 27, left)[133].

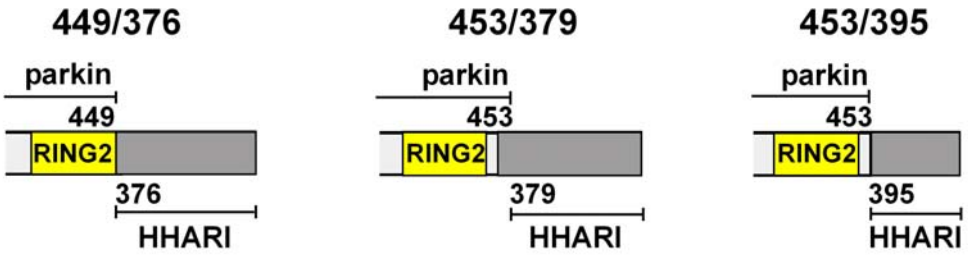


Figure 27: Chimeric constructs of parkin and HHARI. The fusion constructs of parkin and HHARI were designed according to alignment of protein sequences and reports from the literature. 449/376: parkin aa 1-449 (end of RING2) and HHARI aa 376-557. 453/379: parkin aa 1-453 (pathogenic W453X mutant) and HHARI aa 379-557. 453/395: parkin aa 1-453 and HHARI aa 395-557.

Surprisingly, after the first transient transfection and following Western blot analysis, no signal was detectable (data not shown). The next step was to check if the protein was translated at all. Therefore, an in vitro translation with 35-S labelled methionine/cysteine and rabbit reticulocyte lysate was performed. The autoradiograph in Figure 28 shows that mRNA of the chimeric construct is synthesized and translated to protein *in vitro*.

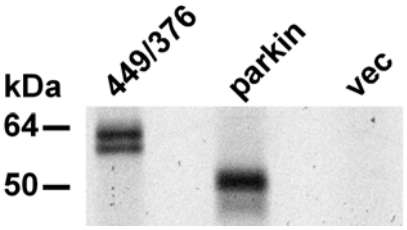


Figure 28: In vitro translation of 449/376. The vectors containing the 449/376 fusion protein, wt parkin or empty vector have been in vitro translated with the TNT T7 Quick coupled Transcription/Translation System (Promega) in the presence of 35S-labelled methionine and cytosine mix. The translational products have been analyzed by SDS-PAGE and exposed to film. Specific bands of the predicted sizes show that both proteins are translated in vitro.

The reason for the empty blot in transient transfection was therefore not due to impaired translation, as Figure 28 suggests. Another possibility was that the fusion protein disappears quickly because of enhanced proteasomal degradation. So, a metabolic labelling experiment of the transfected cells with 35S-methionine/cysteine was carried out. Figure 29 shows the autoradiograph of the 449/376 mutant in the left panel and as a control wt parkin in the right panel. Pulse and chase times where 1 h, and have been performed with and without the proteasomal inhibitor MG 132. Interestingly, a very weak signal can be detected from the 449/376 fusion protein without inhibition of the proteaseome during pulse time, while after 1 h of chase, the signal disappeared. In the presence of MG 132, a much stronger signal was appearing in pulse and chase. In contrast, wildtype parkin was present in both cases in a well detectable manner. This experiment showed that the 449/376 chimeric protein is rapidly degraded by the proteasome after its synthesis.

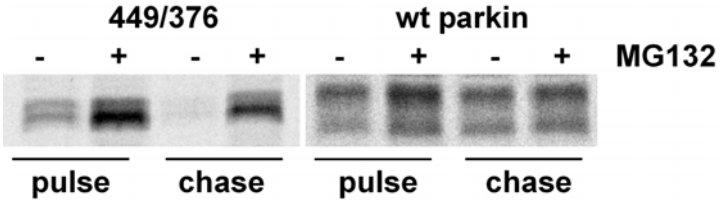


Figure 29: Metabolic labelling of 449/376 fusion protein and wt parkin. HEK293T cells transiently expressing the 449/376 fusion mutant or wt parkin were metabolically labelled with 35S methionine/cysteine for 1 h (pulse) and chased for 1 h in the absence or presence of the proteasomal inhibitor MG 132. The cell lysates were subjected to immunoprecipitation with hP1 antibody and analyzed by SDS-PAGE.

The predictions for the RING2 domain of HHARI are variable: as mentioned before, Moynihan *et al*. propose that RING2 stretches from amino acid 344 to 375[133]; Ardley *et al*. postulate amino acid 325 to 382 [136]; Capili *et al*., who resolved the structure of the HHARI RING 2, define it from amino acid 326 to 395 [86]. The SwissProt database notes 344-389 (http://expasy.org/sprot/). One possibility for the rapid degradation of the chimeric parkin-

HHARI 449/375 protein could be that a severe instability is induced due to interrupting the structure of the RING2 domains. To exclude this, we cloned two other fusion proteins with the parkin sequence spanning aa 1-453 (where the pathogenic mutant has a stop codon instead of a tryptophane), and a sequence more C-terminal than in the previous mutant of HHARI is fused to it (453/379 and 453/395, see Figure 27, middle and right). HEK293T cells were transfected with these constructs, and since the first mutant 453/376 was unstable, MG 132 was added for 16 h. The result of the detergent solubility analysis of their cell lysates is shown in Figure 30, where all three constructs are present only under conditions of proteasomal inhibition, with the majority in the detergent-insoluble fraction (left panel). The membrane has been reprobed with an anti-actin antibody (lower panels) as a control for protein loading.

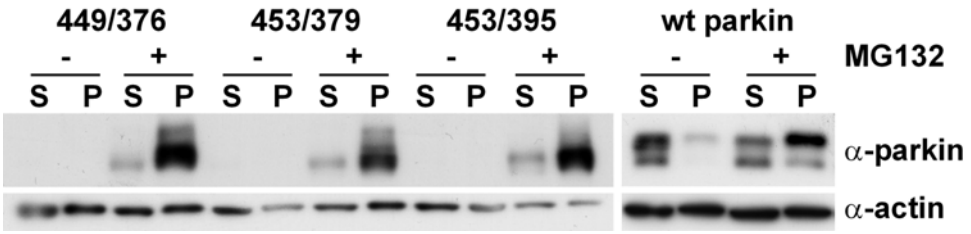


Figure 30: Detergent solubility assay and proteasomal inhibition of chimeric parkin-HHARI mutants. The three parkin-HHARI fusion constructs 449/376, 453/379 and 453/395 and wt parkin were transiently overexpressed in HEK293T cells, incubated with the proteasomal inhibitor MG 132 (5 µM) where indicated for 16 h and analyzed by the detergent solubility assay. As a control for protein loading, the membrane was probed against actin.

Thus, the C-terminal domain of HHARI could not compensate for the folding defect observed for the C-terminal deletion mutant of parkin. The replacement of the C-terminal domain of parkin next to RING2 by that of HHARI not only induced the formation of detergent-insoluble parkin, but in addition led to a destabilization of the chimeric protein.

Previous work by our group demonstrated that fusion of the terminal 3 amino acids, which are crucial for parkin folding, to the pathogenic W453X mutant, could not render the resulting W453FDV mutant detergent-soluble, emphazising that the presence of the terminal 3 amino acids was not sufficient to restore parkin folding [2]. Therefore, the parkin C-terminus was further analyzed to test the possibility that the 10 amino acids C-terminal to RING2 might play a sterical role in bridging a gap between the RING2 domain and the last 3 amino acids which are crucial for parkin folding. The parkin sequence of 10 amino acids between RING2 and the terminal 3 amino acids were replaced by the myc (EQKLISEEDL) sequence. A scheme of the mutant, named hP-mycFDV, is shown in Figure 31. Detergent solubility analysis of wt versus W453X, a C-terminally truncated pathogenic mutant of parkin, and the hP-

mycFDV mutant, revealed that this mutation leads to detergent insolubility, similar to the W453X mutant. Thus, the wildtype sequence seems to play an essential role for parkin folding, since it could not be replaced by myc without interfering with folding of parkin .

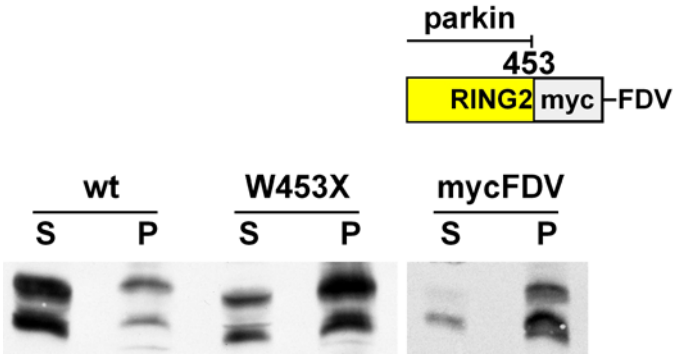


Figure 31: Partial replacement of the C-terminal domain by the myc sequence. HEK293T cells were transfected with wildtype and mutant forms of parkin as indicated, and lysed with 0.1% Triton-X 100 in PBS. Supernatant and pellet fractions were separated by centrifugation and analyzed by Western blot. For detection of the proteins, the hP1 pAb was used.

Two consequences of parkin misfolding

The instability of the parkin-HHARI fusion proteins reminds of a similar phenotype that has been observed for pathogenic mutants of parkin. The point mutants R42P and K48A in the UBL domain are rapidly degraded by the proteasome, as previously shown by our group [1]. Not investigated was the folding behaviour of these pathogenic mutants, and since the parkin-HHARI chimeras appeared mostly in the pellet fraction (Figure 30), it might be that the instability of the pathogenic mutants could be another manifestation of parkin misfolding. To test this, the R42P mutant, which has the stronger phenotype of the two pathogenic mutations, was expressed in the absence and presence of the proteasomal inhibitor MG132 and subjected to the detergent solubility assay. The respective lanes in the Western blot in Figure 32 show that the 52 kDa species of the R42P mutant is not visible without proteasomal inhibition. The 42 kDa band of parkin is visible because the R42P mutation resides in the UBL, which is not present in the smaller parkin species. Under conditions of proteasomal inhibition, the 52 kDa species mainly appears in the detergent insoluble fraction. An example for the behaviour of wildtype parkin under the same conditions is given in Figure 30. There, much more of the high molecular weight band is present in the soluble fraction when the proteasome is inhibited. This experiment indicates that the R42P mutant is rapidly degraded by the proteasome because it is not able to adopt a correctly folded conformation, similarly to the parkin-HHARI fusion proteins.

To check whether aggregation or increased proteasomal degradation might be dominant, the R42P-W453X mutant was cloned, which contains prerequisites for both processes. On the one hand, the destabilizing R42P point mutation is present, which leads to proteasomal degradation. On the other hand, it contains the W453X mutation, which leads to C-terminal truncation and aggregation of the protein. To facilitate the interpretation of the results, the mutant was cloned in the M80L background of parkin, which disrupts the internal translation initiation site and occurs in non-human species. The R42P-W453X double mutant could only be detected when the proteasome was inhibited with MG132, and in an almost exclusively detergent-insoluble conformation (Figure 32). The results of the detergent solubility assay were confirmed by immunofluorescence studies, shown in Figure 32B. A homogenous cytosolic staining was observed in the case of wt parkin, whereas the mutants, which are degraded more rapidly, show a weaker staining, which in the case of the R42P mutant might arise from the smaller parkin species that carries no mutation. The misfolded W453X mutant seemed to form more stable aggregates which are not degraded quickly by the proteasome but accumulate in the cytosol.

This indicates that the rapid proteasomal degradation of parkin is dominant over the formation of rather stable aggregates, which would be visible without proteasomal inhibiton.

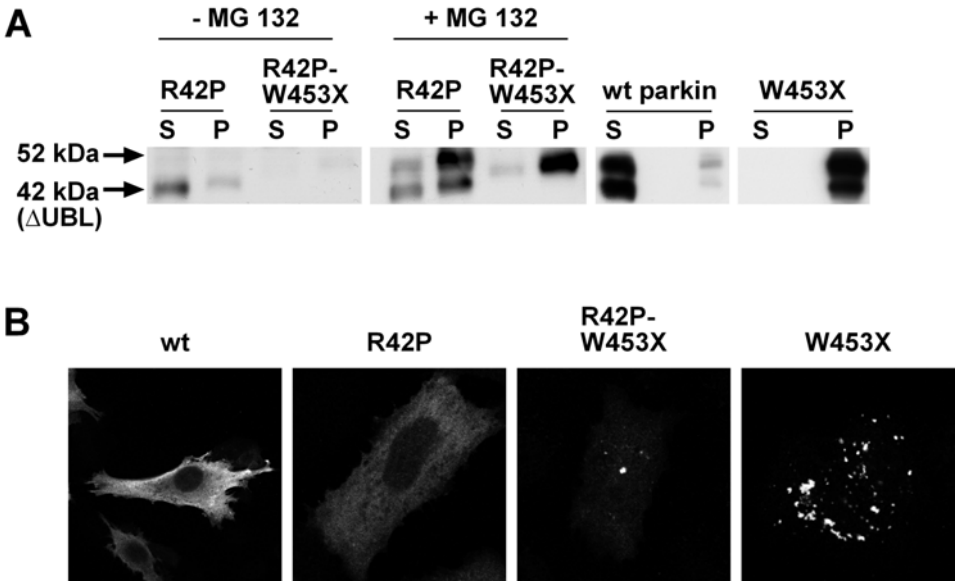


Figure 32: Proteasomal degradation of parkin is dominant over formation of aggregates. A. HEK293T cells were transiently transfected with mutant or wt parkin and treated with the proteasomal inhibitor MG132 (5 µM, 16 h). Parkin present in the soluble and insoluble fraction was analyzed as described before. B. SH-SY5Y cells transiently expressing wt parkin or the mutants indicated were analyzed by indirect immunofluorescence using the hP1 pAb. The confocal pictures were taken under constant conditions of laser intensity and detector gain and offset.

Subcellular localization of parkin

Knowledge about the localization of a protein can give insight into its physiological and pathophysiological function. Numerous proteins change their localization when cellular conditions change, e.g. Bax, a member of the Bcl-2 family, is located in the cytosol and at the endoplasmatic reticulum in a monomeric form in a healthy cell. Early during apoptosis, it translocates to mitochondria to increase the mitochondrial outer membrane permeability [137,138].

Since parkin has a neuroprotective capacity, as reported by several groups (see introduction) and shown earlier in this work, it might undergo similar translocations when exerting its neuroprotective function under conditions of cellular stress. So far, the subcellular localization of parkin has remained controversial. As an E3 ligase, its most obvious localization is the cytosol (http://expasy.org/sprot/), but there are also reports that it is found at mitochondria in PC12 cells [108], synaptic vesicles of rat brain [79,82], microtubules [81], the nucleus and the ER membranes [130].

Due to the controversial reports in literature, we analyzed the subcellular localization of parkin under basal and cellular stress conditions.

Detection of endogenous parkin

Western blot and immunofluorescence

Prerequisite to study the subcellular localization of a protein is the availability of an antibody that can detect a protein on endogenous levels. The monoclonal antibodies PRK 28 and PRK 8, which are suited for this, were provided by Virginia Lee [139]. Only recently, PRK8 became available commercially.

Another limiting factor for the detection of parkin is the fact that it is expressed at very low levels. Interestingly, in the case of SH-SY5Y cells, the levels of endogenous parkin change when the cells are passaged many times. Early passages of them contained considerable amounts of parkin, but after 30 to 40 passages, they seem to lose it (Figure 33, SH-SY5Y-e = early passage, SH-SY5Y-l = late passage).

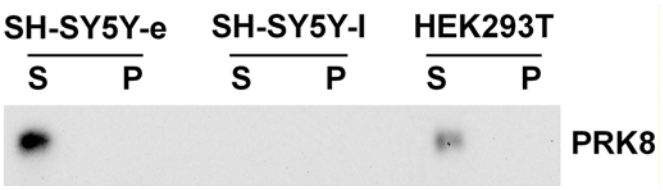


Figure 33: Endogenous levels of parkin in different cell lines. SH-SY5Y cells of an early passage (SH-SY5Y-e), of a late passage (SH-SY5Y-l) and HEK293T cells were lysed in 0.1% Triton-X 100 in PBS. Pellet and supernatant were separated by centrifugation. Equal amounts of protein were analyzed SDS-PAGE and Western blot using the monoclonal PRK8 antibody, anti-mouse HRP conjugated IgG and the Millipore ECL system.

Immunofluorescence studies have been carried out with SH-SY5Y cells to analyze the subcellular localization of parkin, as mentioned above. Upon overexpression of parkin in SH-SY5Y cells, an homogenous cytosolic and sometimes also nuclear staining, is visible by indirect immunofluorescence. By the use of confocal microscopy, it became visible that the staining of overexpressed parkin was slightly inhomogenous and structured (Figure 13B).
Unfortunately, none of the antibodies available was suited for the detection of endogenous parkin in immunofluorescence (data not shown), even under different fixation and permeabilisation methods. So, all localization experiments were conducted upon overexpression.

Association of parkin with membranes

Several reports have shown parkin to be associated with membranes [1,79,82,130]. This was recapitulated in this study during the analysis of the role of the putative PDZ-binding domain at the C-terminus of parkin for its membrane targeting. Since these data are based on overexpression of wt and mutant parkin constructs, an interesting question was if also endogenous parkin in our cell culture system was associated to membranes. To this end, SH-SY5Y homogenates were used because they express considerably more endogenous parkin than HEK293T cells (Figure 33), and subjected to a membrane flotation experiment described before (Figure 19). Figure 34 shows that next to the signals in the cytosolic fractions 7 and 8, a strong signal could be detected in fraction 3, which is the membrane-enriched fraction, as verified by antibodies against TFR and GAPDH.
Thus, endogenous parkin protein is associated to membranes.

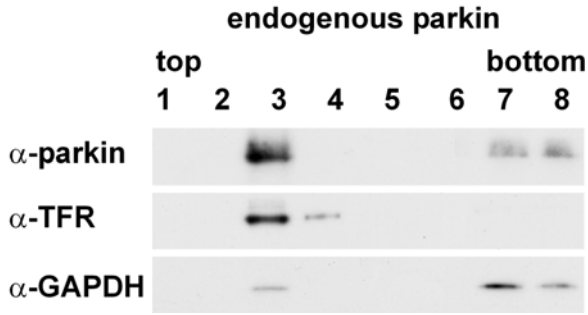


Figure 34: Endogenous parkin is found in a membrane-enriched fraction. SH-SY5Y cells were homogenized and subjected to density gradient centrifugation and Western blotting as described under Figure 19.

Finally, mouse brain was tested for membrane association, but a different fractionation protocol than for cultured cells was used. Mouse brains of wildtype mice were homogenized in hypotonic buffer, frozen and thawn, treated with Brij 35, which helps to stabilize functional membrane protein complexes, and then differentially centrifuged to obtain a cytosolic and a membrane fraction. In Figure 35, the upper panel reveals that a small portion of endogenous parkin from mouse brain was present in the microsomal fraction, whereas the majority resided in the cytosolic fraction. The fractions were identified and tested for purity by reprobing with a TFR antibody and an anti-lactate-dehydrogenase (LDH) antibody.

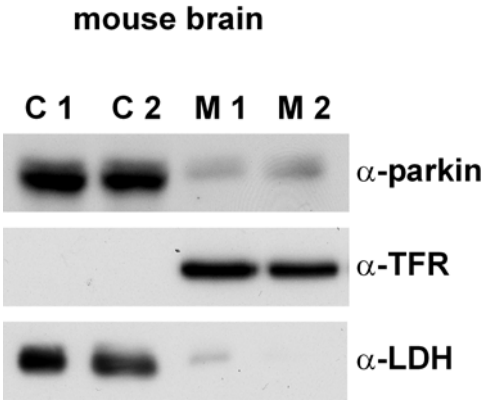


Figure 35: Endogenous parkin in mouse brain. 2 months old mouse brain was dounce homogenized and frozen and thawn. Cytosolic (C) and microsomal (M) fraction were obtained by differential centrifugation. To confirm the purity of the fractions, TFR and Lactate Dehydrogenase (LDH) antibodies were used.

Thus, the association of a certain fraction of endogenous parkin to membranes could be confirmed using mouse brain tissue, and strengthens the possibility that parkin has a functional role close to cellular membranes.

Mitochondrial association

The membrane fractionation experiments in the paragraph before showed a crude membrane fraction which contained all kinds of membranes, including mitochondria (data not shown). Mitochondria might play a major role in PD pathogenesis [140]. There are several reports that link parkin to mitochondrial integrity [108,114,121]. A localization experiment would give first insight into the question whether parkin plays a role for mitochondria and is a player in the mitochondrial pathogenesis for PD. So, a highly pure mitochondrial fraction has been obtained by differential centrifugation and high salt wash of parkin transfected HEK293T cell homogenates. In addition to wt parkin, the ΔUBL mutant and the M80T mutant (fl parkin), which lacks the internal start site, were investigated for their capacity to copurify with mitochondria. A Western blot analysis of cytosolic and mitochondrial fractions is depicted in Figure 36. To confirm the purity of the fractions, actin β was used as a cytosolic control protein, and the mitochondrial matrix protein TIM44 was used to identify intact mitochondria. In case of a damage of mitochondria during the preparation procedure, TIM 44 would diffuse into the cytosolic fraction. The Western blot analysis showed that a considerable amount of parkin is present in the mitochondrial fraction. For the parkin blot, half of the mitochondrial fraction has been loaded on the gel, and 1/30 of the cytosolic fraction in order to not get a too strong signal.

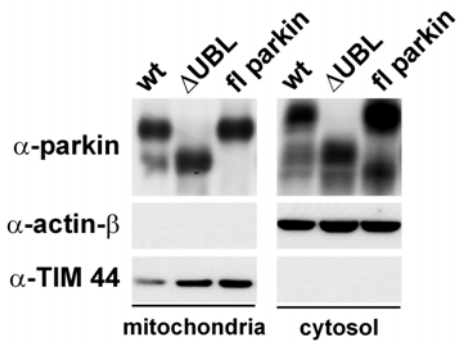


Figure 36: Parkin is found in a mitochondria endriched fraction. Transiently transfected HEK293T cells were homogenized, differentially centrifuged, subjected to a high salt wash, and analyzed by Western blot. 1/30 of the cytosolic fraction was used for SDS-PAGE, whereas the mitochondrial fraction was split in two halfs to probe against parkin or actin as a cytosolic marker and TIM44 as a mitochondrial marker.

Thus, a portion of overexpressed parkin is present in a mitochondrial fraction of HEK293T cells.

The reports concerning parkin and mitochondria are quite diverse. One of them even shows that parkin is present inside the mitochondrial matrix [141], which is hard to understand since there is no N-terminal sequence that targets parkin to mitochondria. To localize the position of

parkin at mitochondria, a protease protection experiment with purified mitochondria from parkin transfected cells has been performed. Isolated mitochondria were treated with increasing concentrations of trypsin. Figure 37 shows the result of this experiment. Parkin was degraded already by small amounts of trypsin, as the signal decreased when 5 µg/ml of trypsin is added (upper panel). TIM 23, a protein that is integral to the inner mitochondrial membrane, needed a larger concentration of trypsin, namely 20 µg/ml, to be affected by this protease (lower panel).

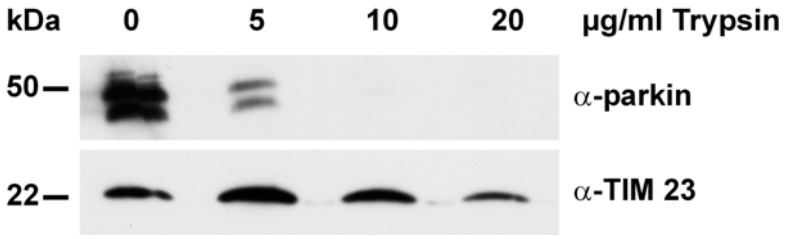


Figure 37: Limited trypsin digest of mitochondrially associated parkin. Mitochondrial fractions were obtained by differential centrifugation of parkin-transfected HEK293T cells. The isolated mitochondria were incubated with the indicated concentrations of trypsin for 10 min on ice. To stop the reaction, trypsin inhibitor was added.

These data gave rise to the assumption that parkin is associated to the outer mitochondrial membrane.

Further support for the hypothesis that parkin is associated to the outer mitochondrial membrane and not localized inside mitochondria is given in Figure 38. Parkin and a version of GFP that contains a mitochondrial target sequence were coexpressed in SH-SY5Y cells. An immunofluorescence analysis revealed that they effectively do not colocalize, as no visible yellow signal can be spotted in the overlay. In contrast, the mito-GFP signal seems to be particularly strong at subcellular sites where weak or no parkin staining is visible.

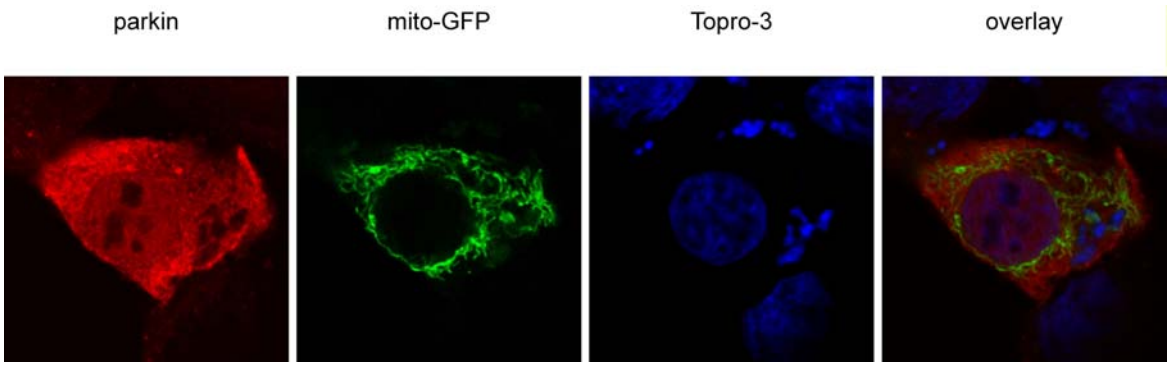


Figure 38: Parkin does not colocalize with mito-GFP. SH-SY5Y cells were cotransfected with wt parkin and mito-GFP. After PFA-Fixation and permeabilisation with 0.2% Triton-X 100 parkin has been stained with hP1 antiserum and Alexa 555, and nuclei are made visible with Topro-3. The green fluorescence stems directly from mito-GFP.

Taken together, these data indicate that a small portion of parkin is associated with the outer mitochondrial membrane.

Co-localization with IKKγ and Traf2

During the course of this study, our group found out that parkin can activate the NFkB pathway in an anti-apoptotic manner [129]. In the course of this study we could show that parkin can interact and ubiquitylate the substrates IKKγ and Traf2, which are important regulatory proteins in the NFκB pathway. The physical interaction of two proteins can be confirmed by co-immunoprecipitation (co-IP).

After the establishment of suitable conditions for parkin immunoprecipitation, a co-IP experiment with IKKγ and Traf2 revealed an interaction of these cytosolic proteins with parkin. This experiment has been performed by Iris Henn and the results are shown in Figure 39. SH-SY5Y cell lysates were used for immunoprecipitation with hP1 pAB crosslinked to protein A agarose (PAA) beads, and membranes were blotted against IKKγ and Traf2. The specific signals indicate that parkin can interact with IKKγ and Traf2 in the cytosol, and provide evidence that parkin plays a role in the NFκB pathway.

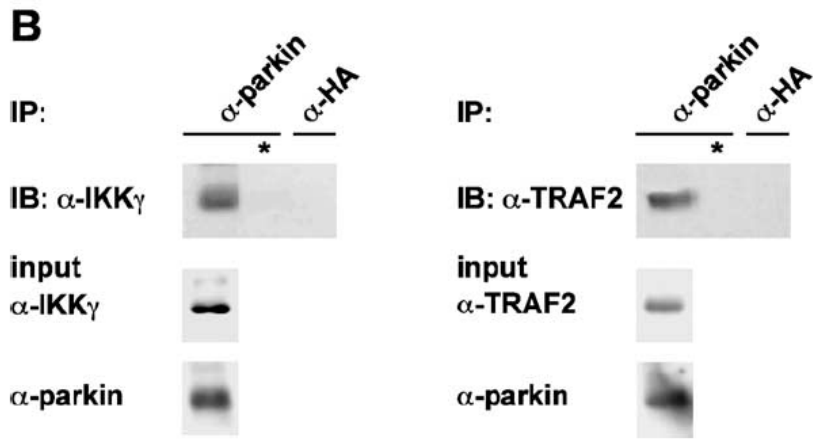


Figure 39: Co-immunoprecipitation of endogenous parkin and IKKγ or Traf2. SH-SY5Y cell lysates were incubated with hP1 crosslinked to PAA. Immunoprecipitates were dissolved in 2x Laemmli buffer and analyzed by Western blot with antibodies directed against IKK γ and Traf2 (upper panels). The lower panels show input controls of the respective proteins. * indicates buffer instead of cell lysate. The crosslinked polyclonal HA antibody has been used to prove specificity of the co-IP. The experiment has been performed by I. Henn.

To support the co-IP experiments and to strengthen the finding that these proteins interact with each other, the subcellular localization of parkin, IKKγ and Traf 2 was determined by indirect immunofluorescence. To that end, parkin and each of the two other proteins were co-

expressed in SH-SY5Y, fluorescently labelled by specific antibodies and analyzed by confocal microscopy. To avoid cross-talk of fluorescence, the pictures for each channel have been taken sequentially after adjusting them with samples that were stained with only one antibody. The individual proteins showed a homogenous distribution in the cytosol (Figure 40). The overlay pictures in the rightmost panel show that parkin colocalized with IKKγ and Traf2, since the red and green signals merge to yellow. This is specified by a quantification of the signal intensities along a line, which is shown in the lowest panel. Interestingly, upon co-expression, a more perinuclear staining of the overexpressed proteins was visible, which is consistent with an activation of the NFκB-pathway and its translocation to the nucleus.

These observations support that parkin specifically interacts with Traf2 and IKKγ and thus might be implicated in the NFκB-signalling pathway.

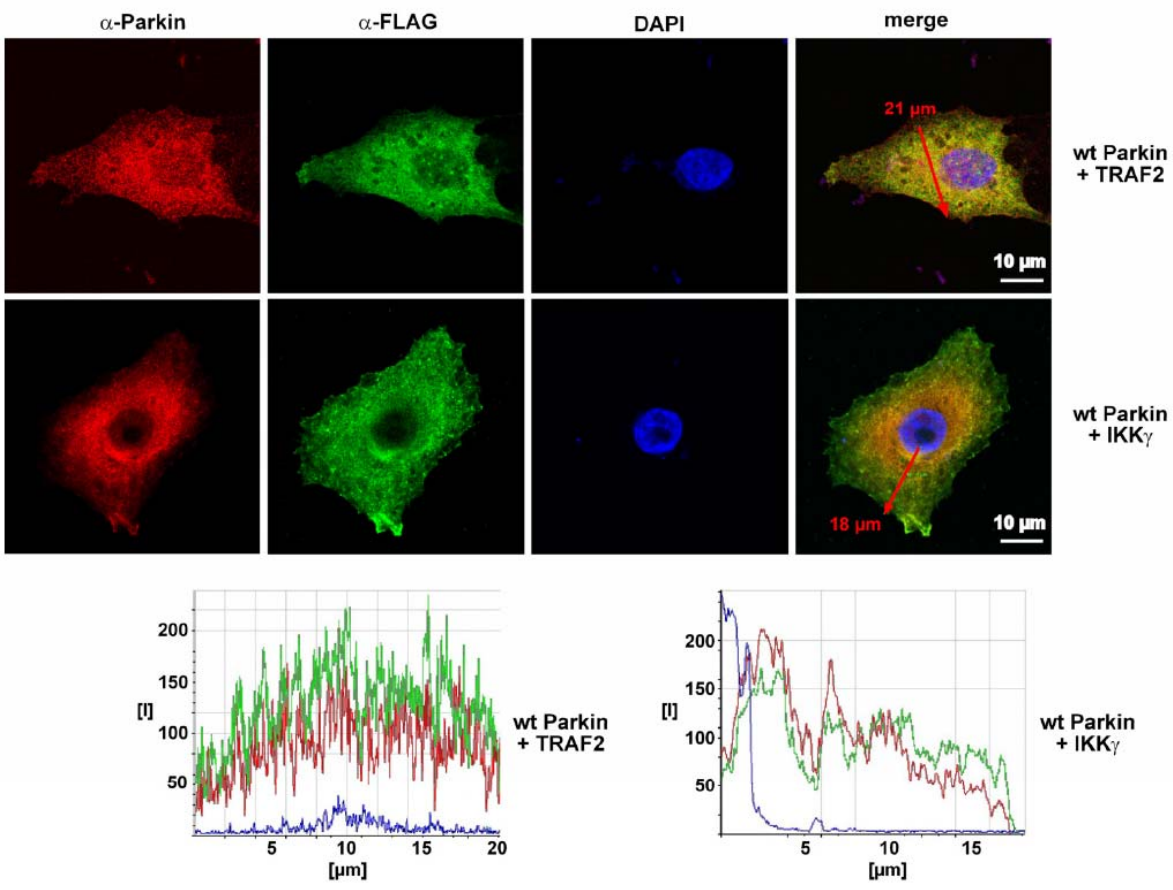


Figure 40: Costaining of parkin and IKKγ or Traf2. SH-SY5Y cells were transiently transfected with parkin and flag-tagged IKKγ or Traf2. 24h post transfection, cells were fixed with PFA, permeabilized and incubated with rabbit hP1 and mouse anti-FLAG antibodies followed by an incubation with the respective fluorescently labelled secondary antibodies. The stained cells were analyzed by confocal microscopy. Signal intensity plots were obtained with the Leica software.

Summary results

Misfolding and aggregation is a major pathway of parkin inactivation. In the course of this study, the native folding pathway of parkin has been investigated, and its impact on the subcellular localization and function of parkin. Several new insights were provided into the following issues:

- The putative PDZ-binding domain has no impact in terms of folding, localization and neuroprotective function of parkin.
- The N-terminal UBL is the only domain that can be deleted without significantly interfering with parkin folding.
- The C-terminus of parkin plays a specific role in parkin folding and cannot be replaced by that of a highly homologous RING-type E3 ubiquitin ligase (HHARI).
- Two phenotypes of parkin misfolding can occur, leading to either aggregation or destabilization.

These observations gave rise to two new mechanistic hypotheses: pathogenic mutations might induce the formation of different misfolded conformers, or they might affect parkin folding at different stages of the folding pathway. The fact that misfolding of parkin can occur in two phenotypes, namely aggregation or destabilization, is an interesting new feature which needs further mechanistic analysis.

Discussion

The specific molecular events that provoke neurodegeneration in Parkinson's Disease are mostly unknown. Recently, several genes linked to hereditary forms of PD have been identified. Idiopathic and hereditary variants of PD share important pathological features, most notably the selective demise of dopaminergic neurons in the substantia nigra. The functional characterization of PD-linked gene products is likely to facilitate our understanding of the molecular mechanisms underlying the pathogenesis. Loss-of-function mutations in the parkin gene (*PARK2*) are responsible for the majority of autosomal recessive parkinsonism. The parkin gene encodes a stress-responsive E3 ubiquitin ligase with a wide neuroprotective capacity. Previous work of our group could show that parkin has a propensity to misfold. A detailed investigation and characterization of the folding pathway, of the subcellular localization, and the neuroprotective activity of parkin were the aims of this work.

Subcellular localization of parkin

As variable as the substrates and functions of parkin that have been reported until today (see introduction), as various are the subcellular localizations of parkin: although the general agreement in the field is that as an E3 ligase, the localization is mainly cytosolic, there are reports that describe parkin to be associated to synaptic vesicles [78,79], at [108] and, although no corresponding signal sequence has been described for parkin, even inside [141] of mitochondria, in the nucleus [95], and associated to the cytosceleton [81,142]. One possibility for this diversity is that parkin might function at all these subcellular sites in different cellular contexts and different cell types. Various cell lines or primary cells were used for these studies, which complicates a direct comparison of these studies. Another explanation might lie in the experimental setup, as for all these reports, different "homemade" antibodies were used that in some cases have not unequiqocally been shown to be specific for parkin and might cross-react with other proteins. Therefore, they may give misleading data concerning the subcellular localization of parkin, and also reproducing the data is difficult due to a lack of availability of these antibodies. In the course of this study, highly specific antibodies also directed against endogenous parkin became available (PRK8 and PRK28) [139]. Therefore it was essential to define the subcellular localization of endogenous versus wt overexpressed and mutant parkin in the cell culture system used for this work.

Differential centrifugation of cell homogenates revealed a colocalisation of overexpressed parkin in a mitochondrial fraction, though the majority of the protein localized to the cytosol

of the cells used in our study. We also tested mitochondrial association, which was loosely, as a limited trypsin digest quickly degraded mitochondrially associated parkin, leading to the conclusion that parkin seems to be associated to the outer mitochondrial membrane. We could not detect endogenous parkin in the mitochondrial fraction (data not shown), which might indicate that only a small amount of endogenous parkin is associated to mitochondria under steady-state conditions, which is below the detection limit of the methods used herein. Ongoing work of our group showed that parkin plays a role in maintaining mitochondrial morphology, confirming a functional relevance of the mitochondrial localization of parkin. Also other groups showed data supporting a mitochondrial localization of parkin [108,141], leading to the notion that at least a subset of parkin molecules may play a functional role at mitochondria.

We also found parkin to be present in large amounts in a crude membrane fraction obtained by density gradient centrifugation. This membrane fraction also contained mitochondrial membranes (data not shown). The majority of overexpressed parkin was again cytosolic. A density gradient can be easily "contaminated" by overloading with cell homogenate. To exclude such a contamination, the blots have been reprobed against an endogenous cytosolic protein. Unfortunately, it was not possible to confirm the biochemical data by immunofluorescence, as endogenous parkin could not be detected with any of the available antibodies, and overexpressed parkin showed a strong cytosolic staining that did not allow a clear discrimination of other subcellular structures to which parkin is associated. The detailed mechanistic relevance of mitochondrial and membrane associated parkin remains to be determined, although some publications and work by our group allow speculations: Fallon *et al.* published in 2006 that parkin can modulate EGF receptor endocytosis and trafficking by interaction with and ubiquitylation of Eps15, a EGF receptor adaptor protein [96]. This implicates a role for parkin in neuronal survival by indirectly modulating EGF recepotor signalling close to membranes. Ongoing work of our group shows that parkin influences mitochondrial morphology, suggesting a functional role for parkin close to mitochondria. This is supported by recent reports from *Drosophila* parkin, which was also shown to influence mitochondrial morphology of the flight muscle and spermatids [122].

For cytosolic parkin, a functional role has been elucidated by our group during this work: Parkin has been shown to have a wide neuroprotective activity [92,107,120]. To address the mechanistic background of this function in our cell culture model, transfected cells were exposed to moderate stress. These conditions were chosen because higher levels of stress can induce misfolding of wt parkin [2]. Parkin has been shown to protect against Complex-1 inhibition by rotenone, and also against excitotoxicity induced by kainate [92,120]. Our

analysis of several stress response pathways by luciferase reporter assays revealed that parkin can modulate the NFkB pathway by interacting with and increasing ubiquitylation of IKKγ and Traf2. The co-immunoprecipitation and ubiquitylation data was complemented in this work by immunofluoresence analysis that showed a high degree of colocalization of parkin and IKKγ or Traf2, especially in the perinuclear region. Up to now it is not clear whether parkin increases the ubiquitylation of IKKγ and Traf2 directly or indirectly. Interestingly, an *in vitro* interaction of parkin with the E2 heterodimer Ubc13/Uev1a was described, which is the essential E2 complex for Traf2 and Traf6 NFκB activation. It remains to be elucidated if Ubc13/Uev1a is a possible interaction partner of parkin, and if it catalyzes the regulative ubiquitylation of the NFκB pathway by parkin.

Determinants of parkin folding

Protein misfolding is a common pathological denominator for many neurodegenerative diseases, such as AD, ALS, Huntington's disease and PD. In PD, misfolding can have several consequences: a gain of toxic function, as in the case of α-synuclein by gene multiplications, a loss of function, as shown for parkin, or a combination of both, which seems to apply for α-synuclein. Misfolding of parkin due to pathogenic mutations or cellular stress has been established as a major mechanism for parkin inactivation, underlining a possible pathological role of parkin in sporadic PD [2]. One aim of this work was to determine the native folding pathway of parkin.

In this study, parkin folding was analyzed with a previously described cell culture model: Overexpression of the constructs in HEK293T cells was followed by lysis in detergent buffer, separation of detergent-soluble and insoluble fractions by centrifugation, and Western blot analysis of the fractions. Complementary immunofluorescence experiments with SH-SY5Y cells were performed, in which discrete intracellular parkin-positive aggregates were visible, indicative of parkin misfolding, or a homogenous cytosolic staining when parkin was present in the detergent-soluble fraction. This cell culture model is well established in the field to analyze protein folding *in vivo* [1,143]. In previous work of our group, complementary experiments showed a difference of wt and mutant parkin regarding sedimentation in a sucrose gradient and resistance to a limited proteolytic digestion [2].

Different cell lines have been used for this study because SH-SY5Y cells are well suited for immunofluorescence experiments due to their high cytoplasm-to-nucleus ratio and their relatively large size. However, the transfection efficiency in SH-SY5Y cells is low, which has no impact on a single cell based analysis like immunofluorescence, but impedes Western blot

analysis, since the amount of parkin protein is not sufficient to obtain a signal that is easy to interprete (data not shown). In contrast to SH-SY5Y, HEK293T cells are small and have an unfavorable cytoplasm-to-nucleus ratio regarding immunofluorescence, whereas their high transfection efficiency makes them well suited for Western blot analysis. *In vitro* studies with recombinant parkin isolated from *E.coli* are hampered by the existence of 35 cysteine residues in the RBR domain, which favors misfolding of recombinant wildtype parkin when overexpressed in *E.coli*.

In this work, the intrinsic determinants of parkin folding and misfolding were addressed by analyzing the role of specific domains on parkin folding. Therefore, several domain deletion mutants were cloned and analyzed in the cell culture model discussed above. This approach revealed that only the N-terminal ubiquitin-like domain can be deleted without interfering with the folding of parkin, whereas deletion of any other domain lead to accumulation of misfolded parkin in the detergent-insoluble fraction, determined by Western blot and formation of parkin aggregates determined by indirect immunofluorescence.

Of note, such deletion constructs of parkin have widely been used in previous studies to map interactions with putative substrates or other interacting proteins. However, interaction studies with non-natively folded proteins can not be interpreted conclusively, as authentic interactions may be lost by misfolding of the protein and not by the specific loss of the domain analyzed. On the other hand, unspecific interactions can occur when hydrophobic patches of the protein are exposed due to misfolding.

Our finding that deletion of the N-terminal UBL is the only one that was tolerated in regards of folding led to the question of how many amino acids in addition to the UBL could be deleted without disturbing the correct folding of parkin. Mutants containing larger N-terminal deletions were cloned, specifically, Δ1-100, Δ1-144, Δ1-238, and analyzed by the detergent solubility assay. The analysis revealed that the former deletions are tolerated in regards of folding, whereas the latter one, which basically leads to the overexpression of the RBR domain, has a clear tendency to misfold. The results led to the conclusion that the C-terminal part of the linker domain is important for the folding stabilization of the RBR domain.

In the course of this study, also other groups reported domain deletions, which recapitulated some of the results reported here [79,83]. Kubo *et al*. reported an exon1-5 deletion of parkin found in patients [79], which results in a similar deletion to the Δ1-238 analyzed in this work, and also found the protein to misfold. These findings indicate that the adjacent parts close to the RBR domain are important to stabilize the folding of this complex domain.

The results of the domain deletion analysis are in line with the previously reported sensitivity of parkin to misfolding due to diverse insults. In addition to the modifications described in

this work, namely the targeted deletion of RING1, IBR, RING2 and the linker domain, also oxidative stress, covalent modification of cysteines by dopamine and C-terminal truncation lead to misfolding [2,123,124]. These modifications take place at the RBR domain, or at the C-terminus which is crucial for parkin folding, respectively (see below). Interestingly, the deletion of the RBR domain in patients leads to an earlier onset of disease [144].

Parkin has been proposed to contain a putative PDZ binding domain, which by definition resides in the last 3 amino acids of the C-terminus [82,105]. The putative PDZ-binding motif of parkin can only be found in mammalian species, compatible with an additional function evolved in mammals. Based on the previously reported membrane association of parkin [1,79,130] and the striking role of these terminal three amino acids in regards of folding, one aim was to investigate a possible functional role of this domain in membrane targeting of parkin. As PDZ-binding domains mediate PDZ interactions in protein scaffolds close to membranes, we hypothesized that the putative PDZ-binding domain might target parkin to interaction partners close to membranes. Given the wide range of substrates of parkin, the differential subcellular localization of parkin could provide a mechanism that confers substrate selectivity.
To address this question, two modifications have been introduced into parkin: replacing the last 13 C-terminal amino acids of human parkin by the sequence of Drosophila parkin (parkin-D.m.), which lacks a PDZ-binding motif, and exchanging the terminal valine to glutamate (V465E). Both modifications result in a disruption of the putative PDZ binding motif, because the sequence prerequisite Φ-X-Φ are no longer fulfilled [128]. The latter mutation has been shown by Fallon et al. to have altered PDZ binding abilities in a pull-down experiment [82]. We analyzed the folding behavior of both mutants, and showed that they fold like wt parkin as assessed by detergent solubility behaviour and immunofluorescence. However, we could not detect differences between wt parkin and the PDZ deficient mutants in regards of membrane association, neuroprotective activity and ubiquitylation activity of parkin. However, it cannot be excluded that under certain cirumstances, for example in response to a specific stimulus or cellular signalling, a transient PDZ dependent interaction of parkin with PDZ domain containing proteins can occur, which serves different functions than neuroprotective activity, ubiquitylation activity and membrane association of parkin.

The next question we addressed was whether the folding sensitivity of parkin is unique for this protein, or if a protein of a similar modular structure behaves the in a similar way. Therefore, a comparative analysis of parkin with its closest relative of the RBR family,

Human Homologue of Ariadne (HHARI), has been performed. Both proteins have a C-terminal RBR domain, which is the region of highest homology, and both have been shown to have an E3-ligase activity.

Firstly, we compared parkin and HHARI in regards of folding sensitivity upon oxidative stress. Previous work by our group reported misfolding of parkin after treatment with hydrogen peroxide [2]. To compare parkin and HHARI, both proteins were overexpressed in HEK293T cells and treated with increasing concentrations of hydrogen peroxide. Detergent solubility analysis revealed that both proteins respond in a similar manner to oxidative stress, namely, a large portion of the protein shifts to the insoluble fraction, indicative of misfolding of the protein. The propensity to misfold of wt parkin and HHARI can be explained by their homology in the RBR domain, since a high content in cysteines is integral to this domain. During the course of the study, other groups published similar results, confirming our data [135,145].

Secondly, parkin and HHARI were compared regarding the sensitivity to C-terminal truncation. Previous work by our group reported a sensitivity of parkin to C-terminal truncation. Notably, the deletion of 3 C-terminal amino acids is sufficient to cause parkin misfolding, explaining the loss-of-function phenotype of pathogenic C-terminal deletion mutants [2]. To compare parkin and HHARI, a C-terminally truncated HHARI mutant was cloned similarly to the pathogenic W453X parkin, according to an alignment of protein sequences. Detergent solubility analysis of the overexpressed proteins revealed that truncated HHARI remains in the soluble fraction, indicating proper folding of C-terminally truncated HHARI. Thus, the propensity to misfold upon C-terminal truncations was specific for parkin. We then replaced the C-terminal portion of parkin by that of HHARI, to test whether the tolerance to C-terminal truncations can be transferred to parkin. Surprisingly, different strategies to generate such a chimeric parkin-HHARI protein resulted in the formation of misfolded, unstable conformers. The half-life of the fusion protein was dramatically shorter than that of wildtype parkin.

In conclusion, the RBR domain of both proteins shows a high degree of homology, possibly the same fold and a sensitivity to oxidative stress, but the role of the C-terminus of parkin in regards of folding is unique.

These results confirm the observation by Capili *et al*. [86]. They observed in an NMR study that hydrophobic residues C-terminal to RING2 in HHARI show nuclear Overhauser enhancements to other regions within RING2, therefore most probably stabilizing its fold. Due to the high homology of sequence of RING2 and the adjacent C-terminal region of parkin and HHARI, the same could be true for parkin.

The instability of the parkin-HHARI fusion proteins were similar to a phenomenon that has been observed before by our group [1]: Pathogenic point mutations at positions 42 and 48 within the UBL domain induce a destabilization and rapid degradation by the proteasome. The appearance of the parkin-HHARI fusion proteins mostly in the insoluble fraction upon proteasomal inhibition led us to uncover the reason for the instability of the pathogenic R42P mutant, which is the most unstable UBL mutant. Our results indicate that the indeed rapid proteasomal degradation of this mutant is due to the formation of a non-native conformer, which appeared in the detergent-insoluble fraction after proteasomal inhibition.
This finding is in line with a recent study on the folding and structure of the UBL domain of parkin: Safadi and Shaw [146] showed by NMR spectroscopy the complete unfolding of the UBL induced by the R42P mutation.
The results of the folding analysis demonstrated that conformational alterations of parkin induced by pathogenic mutations can induce either aggregation of parkin, or a destabilization of parkin. Even though these results are based on overexpression of pathogenic parkin mutants in cell culture, and aggregate formation not necessarily occurs in patients, there are consistent biochemical differences between wildtype parkin and mutant parkin. These differences are not dependent on expression levels or the cell type that was used, and explain the loss-of-function phenotype of the pathogenic parkin mutants.

Our new observations in regards of parkin misfolding contribute to better understanding of the loss-of-function mechanism of pathogenic parkin mutants. Characteristic for parkin misfolding is its sensitivity to oxidative stress, which is most probably due to its high content in cysteines, which is supported by our finding that HHARI, the closest relative in human to parkin, shares the same features. In contrast, folding sensitivity towards C-terminal deletion is a feature only specific for parkin.
At the first glance, a paradoxical situation arises from these data: why is a protein which protects cells from stress-induced cell death inacitvated by stress-induced misfolding? Considering all aspects we know about parkin, this specific feature makes sense in a physiological context and helps to understand why dopaminergic neurons might be particularly vulnerable to an inactivation of parkin. We and others provided evidence that parkin can deal with mild and moderate stress conditions, while stress-induced misfolding of parkin occurs under high level stress. When cellular stress exceeds a critical threshold, resulting in an irreversible damage, it is not favorable to execute an anti-apoptotic program, which would interfere with the elimination of damaged cells. Hence, an inactivation of pro-survival proteins under such a condition is useful from an organismal viewpoint.

Dopaminergic neurons in the substantia nigra have to cope with a variety of stress conditions, in particular oxidative stress resulting from the metabolism of dopamine and excitotoxicity. Thus, they require effective stress response systems. The fact that parkin is specifically sensitive to dopamine-mediated inactivation and to oxidative stress might explain why they are at high risk. In addition, stress-induced up-regulation of parkin gene expression, which has been demonstrated in response to transient stress conditions (9,41,42), may be impaired by constant stress at higher levels, and could be compromized in aging dopaminergic neurons, leading to sporadic PD.

Two phenotypes of parkin misfolding could be observed from our experiments: on the one hand aggregation, on the other hand degradation. Pathogenic mutations might induce the formation of different parkin conformers, or they might affect parkin folding at distinct stages of the folding pathway. This highlights the importance to analyze the parkin folding pathway in more detail and to identify chaperones that are essential for parkin folding and stablization, and thus to provide further mechanistical insight into the folding pathway of parkin.

Methods

DNA techniques

Polymerase chain reaction (PCR) and site-directed mutagenesis

DNA fragments were amplified by PCR [147] using thermostable DNA-polymerase and primers as listed below (see primer lists). To clone parkin mutants, the cDNA of wt parkin in pcDNA 3.1 (Zeo+) [2] containing a polymorphism at S223P was used. To substitute single amino acids or to delete entire domains of parkin, a two step PCR strategy was used: the first PCRs were performed using forward and reverse primers containing the desired mutations, and the respective flanking primers at the 5' and 3' end of the cDNA. The PCR products were isolated and purified. Aliquots of the PCR products were used as templates for the second PCR, together with the outermost primer pairs.
HHARI cDNA was amplified from the RZPD clone IRATp970D0877D and subcloned into pCMV-HA vector, using primer pairs and PCR conditions as described below. Deletion and substitution of domains was performed in a similar manner as for parkin mutants using the respective primer pairs.

Reaction mixture for PCR:

H_2O dd	38,5 µl
forward primer 10µM	1 µl
reverse primer 10µM	1 µl
plasmid (1 µg/µl)	1 µl
Pfu-Buffer 10x with $MgSO_4$	5 µl
dNTPs 10 mM	2,5 µl
Pfu-Polymerase (2,5U/µl)	1 µl
final volume	50 µl

Table 7: Reaction mixture for PCR.

Parkin PCR program

To amplifiy parkin cDNA, the following program has been used:

temperature	time	cycle
95°C	5 min	1x
95°C	50 sec	
52°C	50 sec	30x
72°C	2 min	
72°C	5 min	1x
10°C	∞	

Table 8: PCR program for parkin amplification.

HHARI PCR program

To amplifiy HHARI cDNA, the annealing temperature had to be increased stepwise to obtain a PCR product.

temperature	time	Cycles
95°C	5 min	1x
95°C	1 min	
45/47/49/52°C	1 min	5/5/5/15x
72°C	1 min	
72°C	10 min	1x
10°C	∞	

Table 9: PCR program for HHARI amplification.

Agarose gel electrophoresis

To separate linearized DNA fragments from supercoiled DNA or to analyze PCR products, 1-2% (w/v) agarose gels in 1x TBE buffer and 0,2 µg/ml Ethidium Bromide were used depending on the expected size of the fragment. A 1 kb size marker was used to define the size of the fragment. 6x loading dye was added to the DNA samples, and gels were run at 80 V.

Isolation and purification of DNA fragments from agarose gels

DNA fragments were cut out of the agarose gel on a UV screen and purified with the Nucleo Spin Extract kit (Macherey-Nagel) according to the manufacturer's instructions.

Enzymatic modification of DNA fragments

Purified DNA fragments were digested with 10U restriction enzyme (see below) and respective reaction buffer according to the manufacturer's instructions either overnight for digestion close to the end of DNA fragments or 1 h at 37°C to digest circular DNA. DNA fragments were purified as described above.

Alkaline phosphatase treatment

To avoid self-ligation, the linearized vectors were dephosphorylated with shrimp alkaline phosphatase (SAP) before ligation. SAP and SAP reaction buffer was added to the digested vector according to the manufacturers instructions. The mixture was incubated at 37°C for 10 min and heat inactivated at 65°C for 10 min.

Ligation of cDNA fragments into vector DNA

To ligate the digested and purified DNA fragment into a respectively linearized plasmid, 100-200 ng of the plasmid was mixed with 1-2 µg DNA fragment, T4 ligase buffer and T4 ligase in a final volume of 20 µl. The mixture was incubated for 3 h at room temperature and heat inactivated for 10 min at 65°C. 7 µl were used for transformation of competent bacteria of the *E. coli* strain DH5α (see below).

Preparation of competent *E.coli*

An overnight culture of DH5α in 3 ml LB medium was used to inoculate 200 ml LB. Bacteria were grown to a density of 0,2 at 600 nm (OD_{600} = 0,2) at 37 °C. Cells were chilled on ice for 10 min and centrifuged at 5000 g for 10 min. The pellet was resuspended in 100 ml of transformation buffer, incubated for 20 min on ice, centrifuged as before and resuspended in 10 ml transformation buffer. 200 µl aliquots were stored at -80°C.

Transformation of competent *E.coli*

Competent DH5α were thawed on ice, mixed gently with DNA and incubated for 30 min on ice. After a heat shock of 42°C for 90 sec, cells were incubated on ice for 5 min. 400 µl LB was added and bacteria were incubated for 1 h at 37°C with shaking. The mixture was shortly centrifuged, the pellet resuspended in 100-200 µl of LB and then plated on LB-agar-plates containing the respective antibiotics to select positive clones. LB-agar plates were incubated at 37°C over night, single clones were analyzed as described below.

Preparation of plasmid DNA from *E.coli*

Single clones were used to inoculate LB-medium for small-scale DNA preparation (Macherey-Nagel), which was performed according to the manufacturers instructions. To identify positive clones, the DNA was digested with the same restriction enzymes that were used to get sticky ends before ligation and analyzed by agarose gel electrophoresis. Large-scale DNA-preparation (Maxi Macherey-Nagel) was used to obtain higher amounts of DNA according to the manufacturers instructions. DNA amounts and purity were determined by measuring absorbance at 260/280 nm.

Sequencing

All cDNA constructs were confirmed by sequencing by GATC Biotech AG (Konstanz, Germany).

Cell culture

Cell lines

Cell line	Organism / cell type	Culture medium
SH-SY5Y	Human neuroblastoma; DSMZ-Nr. ACC 209	DMEM/Ham's F12, 15% FCS, 1% non-essential amino acids, P/S
N2a	Murine neuroblastoma; ATCC-Nr. CCL 131	MEM, 10% FCS, P/S
HEK 293T	Human embryonic kidney; ATCC-Nr. CRL-1573	DMEM, 10% FCS, P/S

Cells were cultivated in 25 or 75 cm2 tissue culture flasks. Upon confluency, cells were trypsinised and passaged 1:4 or 1:8 in new flasks. For transfection, cells were counted and plated in the desired confluency in cell culture dishes.

Transient transfection

Cells were plated 24 h before transfection; HEK and N2a cells were plated $1x10^6$ on a 35 mm dish, SHSY5Y were plated $7x10^5$ for Western blot or $3x10^5$ on coverslips for indirect immunofluorescence. For transient transfection, DNA was mixed with Lipofectamine and Plus (Invitrogen) in Optimem according to the manufacturers instructions. Transfection mixture and cells were incubated for 24 h before processing for the indicated experiment.

Protein analysis

Detergent solubility assay

Transfected cells were harvested and lysed in detergent buffer (0,1% Triton X-100 or 0,5% Triton X-100/sodium desoxycholate (DOC) in PBS). After centrifugation at 16,000 x g for 20 min at 4°C, supernatant and pellet fraction were separated. The pellet fraction was washed with lysis buffer and resuspended in Laemmli sample buffer in a volume equal to the supernatant. To compare the relative distribution of the protein of interest, equal amounts of detergent-soluble and -insoluble fractions were analyzed by Western blot [143].

Western blot Analysis

Proteins were analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using a polyvinylidenfluorid (PVDF) membrane (Millipore). The PVDF-membranes were blocked with blocking solution containing 5% non-fat dry milk in TBS containing 0,1%Tween-20 (TBS-T) for 1 h at room temperature and then incubated with the primary antibody in blocking solution for 16 h at 4°C. After extensive washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibody. Following washing with TBS-T, the antigen was detected with the enhanced chemoluminescence (ECL) detection system (Amersham Biosciences). HRP-conjugated epitope antibodies were incubated for 16 h at 4°C, washed extensively and detected with the ECL system.

Metabolic labelling of cellular proteins

Cells were starved for 30 min in methionine-free Dulbeccos modified Eagles medium (Invitrogen) and then labelled with 300 µCi/mL Pro-mix L-[^{35}S] *in vitro* cell label mix (Amersham Biosciences) in methionine-free DMEM (pulse) for 1 h. When indicated, the proteasomal inhibitor MG123 (Calbiochem) was present during labelling and chase periods. For the chase, labelling medium was removed, cells were washed twice and then incubated in complete DMEM for 1h. Radiolabelled cells were lysed in detergent buffer and fractionated into detergent-soluble and –insoluble fractions as described above. The supernatants were precleared with protein A-Sepharose (Pierce) for 30 min, the primary antibody hP1 was added and the samples were incubated at 4°C for 16h. The antigen-antibody complexes were captured by the addition of immobilized protein A and then washed three times with detergent buffer. Proteins present in the immunoprecipitates were released from the protein A-

Sepharose by the addition of Laemmli sample buffer containing 1% SDS and heating at 95°C for 5 min. Immunoprecipitates were analyzed by SDS-PAGE. Gels were impregnated with Amplify (Amersham Biosciences), dried and exposed to film.

Crosslinking of hP1 to a protein A agarose (PAA) matrix

To covalently crosslink the hP1 antibody to the PAA beads, the PAA slurry was washed twice with PBS. 5 µl hP1 per 50 µl slurry was rotated for 1 h at room temperature. After thorough washes with PBS to remove unbound antibody and serum, the crosslinking reaction was prepared by adding disuccinimidyl suberate in PBS in a final concentration of 3 mg/ml and rotation for 1 h at RT. The incubation was followed by 4 washes with TBS and 4 washes with acid glycine buffer to remove non-covalently bound antibody, and two more washes with TBS to readjust the pH. Beads were either used directly for immunoprecipitation or stored at 4°C after addition of 0.02% of NaN_3.

Co-immunoprecipitation (Co-IP)

Lysates of untransfected SH-SY5Y were prepared in 0,1% Triton-X 100 in PBS containing protease inhibitor. The samples were incubated with crosslinked hP1 under rotation at 4°C overnight. Prior to immunoblotting, the beads were washed with PBS and boiled in 2x Laemmli sample buffer in order to release the precipitated proteins. For detection, the monoclonal IKKγ and Traf2 antibodies were used.

Ubiquitylation assay

Parkin or parkin mutants, HA-ubiquitin and when indicated IKKγ–flag were cotransfected in HEK293T cells. One day after transfection, protein lysates were prepared in denaturing lysis buffer and incubated at 95°C for 5 min. Protein extracts were diluted 1:10 with non-denaturing lysis buffer. Immunoprecipitation of parkin was performed with hP1 pAb followed by an incubation with protein A beads (Pierce); immunoprecipitation of IKKγ-flag was performed with flag-M2 agarose (Sigma). Immunoprecipitated proteins and input samples were analyzed by Western blotting using the antibodies indicated in the respective figures.

Immunocytochemistry and fluorescence microscopy

SH-SY5Y were grown on glass coverslips, transfected, fixed 16 h after transfection in 3% paraformaldehyde/sucrose in PBS for 10 minutes at room temperature, and permeabilized with 0.2% Triton X-100 in PBS. Fixed cells were incubated with primary antibody (diluted in 1% BSA and 10% goat serum) for 1 h at room temperature. After washes with PBS, the coverslips were incubated with fluorescently labelled secondary antibodies. Nuclei were stained with DAPI (Invitrogen). Finally, cells were embedded in Mowiol mounting medium (Calbiochem). Images were obtained on a Zeiss LSM 510 or Leica confocal microscope.

Subcellular fractionation:

Preparation of cellular membranes

Transfected cells were harvested, incubated in hypoosmotic buffer and dounce homogenized. After a low speed spin the homogenate was mixed with 60% iodixanol (Optiprep, Axis Shield) to obtain a final iodixanol concentration of 40 %. The mixture was overlayered in a SW55 tube with 2,5 ml 28% iodixanol diluted with TNE and 1ml TNE on top. After ultracentrifugation in a MLS 50 swing-out rotor (Beckman) at 165,000 g, fractions were collected from top to bottom. Aliquots of these fractions were analyzed by Western blot.

Preparation of Mitochondria

Mitochondrial fractions were obtained by differential centrifugation. Transfected cells were incubated in resuspension buffer, pelleted by low speed centrifugation, dounce homogenized in a 1:1 mixture of resuspension buffer and MS-buffer and low speed centrifugated. After an additional 600 g centrifugation, the supernatant was centrifuged at 12 000 g to obtain a mitochondrial pellet. The supernatant (cytosol and microsomes) was TCA-precipitated. The isolated mitochondria were washed with M1 buffer containing 250 mM NaCl to minimize cytosolic contamination, pelleted at 12,000 g and analyzed by Western blot in comparison to the cytosolic fraction.

Preparation of a membrane fraction from mouse brain

Mouse brains were isolated from 2 months old mice, homogenized with 10 strokes in a glass potter in hypotonic buffer. The tissue was further processed by a step of freeze-thaw using liquid nitrogen. After addition of 1% Brij 35 (Pierce), homogenates were low-spin centrifuged to yield a post-nuclear supernatant (PNS). Glycerol was added to the PNS to a final concentration of 5%. An ultracentrifugation step of 130,000 x g for 60 min at 4°C resulted in

a cytosolic fraction and a membrane fraction. The membrane fraction was resuspended in hypotonic buffer containing 1% Triton-X 100 and ultracentrifuged for another 30 min. This supernatant (membrane fraction) and cytosolic fraction was analysed by Western blotting.

Apoptosis assay

SH-SY5Y cells were grown on glass coverslips. 24 h after transfection, cells were incubated with kainate (500 μM) for 3 h. The cells were then fixed with 3% PFA for 20 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and blocked with 1% BSA in PBS for 1 h at room temperature. Fixed cells were incubated with anti-active caspase-3 antibody overnight at 4°C, washed, and incubated with Alexa 555-conjugated secondary antibody for 1 h at room temperature. After extensive washing, cells were mounted onto glass slides and examined by fluorescence microscopy using a Zeiss Axioscope 2 plus microscope. To detect cells undergoing apoptosis, the number of activated caspase-3-positive cells out of at least 300 transfected cells was determined. Quantifications were based on at least three independent experiments.

Statistical analysis

Data were expressed as means ± SE. All transfections were performed in triplicates and repeated at least three times. Statistical analysis among groups was performed using ANOVA. P-values are as follows: *$P < 0.05$, **$P < 0.005$ and *** $P< 0.0005$.

Material

Material for DNA techniques

LB-Medium

1% Tryptone, 0,5% NaCl, 0,5 % yeast extract in dH_20, pH 7,0; autoclaved; Ampicillin 100 µg/ml or Kanamycin 30 µg/ml

LB-Agar

1,5% Agar in LB-Medium, autoclaved; Ampicillin 100 µg/ml or Kanamycin 30 µg/ml

TBE

9mM Tris-borate, 2mM EDTA in dH_20

Transformation buffer

50 mM $CaCl_2$, 15% glycerol, 10 mM PIPES, pH 6.6

1 kb marker	Gibco Invitrogen
Agarose	Serva
Ethidium Bromide	Sigma
dNTPs	Sigma
Pfu-polymerase and 10x buffer	Promega
Restriction enzymes and buffers	NEB/Fermentas
Shrimp Alkaline Phosphatase and buffer	Roche
T4 ligase and buffer	Roche

Primer list

Parkin	5’ 3’
Hind III forward (fw)	CCCAAGCTTATGATAGTGTTTGTCAGGTTC
Not I reverse (r)	ATAAGAATGCGGCCGCCTACACGTCGAACCAGTGGTCCCC
dUBL fw	CCCAAGCTTGGTCAAGAAATGAATGC
dLinker fw	AGACCGTGGAGAAAAATTACGTGCACAGAC
dLinker r	TCTGTGCACGTAATTTTTCTCCACG
dRING1 fw	CAAATAGTCGGAACATCACTCCCAACTCCTTGATTAAAG
dRING1 r	CCTTAATCAAGGAGTTGGGAGTGATGTTCCGACTATTTG
dIBR fw	CTGGGAGAAGAGCAGTACAACAGTGCCGTATTTGAAGCCTC
dIBR r	GAGGCTTCAAATACGGCACTGTTGTACTGCTCTTCTCCCAG

dRING2 fw	CAAGAAAACCACCAAGCCCGGCTGCGAGTGGAACCGC
dRING 2 r	GCGGTTCCACTCGCAGCCGGGCTTGGTGGTTTTCTT
d1-100 fw	CCCAAGCTTATGAGCTTGACTCGGGTGGAC
d1-144 fw	CCCAAGCTTATGCAGCTTTTATGTGTATTG
d1-238 fw	GATTACGCGATTACGTGCACAGACGTC
d1-238 r	GACGTCTGTGCACGTAATCGCGTAATC
d450-457 r	ATAAGAATGCGGCCGCCTACACGTCGAACCAGTGGTCCCCCATA CAGTTCCAGCACCACTCGAGCC
dFDV r	ATAAGAATGCGGCCGCCTACCAGTGGTCCCCCATGCAGAC
FDV-AAA r	ATAAGAATGCGGCCGCCTAGCAGCAGCACCAGTGGTCCCCCATG CAGAC
W453mycFDV r	ATAAGAATGCGGCCGCCTACACGTCGAACAGATCCTCTTCTGA
Parkin-D.m. r	ATAAGAATGCGGCCGCCTAGGCCGAACCAGTGGGCTCCCATGCA GTCGCGTGTCCACTCAGTCTGACAGTTCCAGCACCACTCGAGCCT GCACTGG
V465E r	ATAAGAATGCGGCCGCCTACTCGTCGAACCAGTGGTCCCC
V465A r	ATAAGAATGCGGCCGCCTATGCGTCGAACCAGTGGTCCCCCATG CAGAC
D464A r	ATAAGAATGCGGCCGCCTACACTGCGAACCAGTGGTCCCCCATG CAGAC
F463A r	ATAAGAATGCGGCCGCCTACACGTCTGCCCAGTGGTCCCCCATGC AGAC
K48E fw	GTGATTTTCGCAGGGGAGGAGCTGAGGAATGAC
K48E r	GTCATTCCTCAGCTCCTCCCCTGCGAAAATCAC
K76E fw	AGACCGTGGAGAGAGGGTCAAGAAATGAATGC
K76E r	GCATTCATTTCTTGACCCTCTCTCCACGGTCT
P2C3_silent1	ATAAGAATGCGGCCGCTCATACATCAAACCAGTGGTCCCC

HHARI	5’ 3’
HHARI fw	GCGGCTGAATTCGGGACTCGGACGAGGGCTAC
HHARI r	GGAGGCGGCCGCTCAGTCCTCAATGTACTCCCACAG
P378X r	GGAGGCGGCCGCTCAAAGACACACCCAGCAAAAC
PH449/376 fw	GGTGCTGGAACTGTCTTGGCCCATGGGAAC
PH449/376 r	GTTCCCATGGGCCAAGACAGTTCCAGCACC
PH453/379 fw	GGAACTGTGGCTGCGAGTGGGAACCACATGGATCTG
PH453/379 r	GGCAGATCCATGTGGTTCCCACTCGCAGCCACAGTTCC
PH453/395 fw	GGCTGCGAGTGGGATGCAAAGGCAGCAAGAG

PH453/395 r	TGCCTTTGCATCCCACTCGCAGCCACAG

Plasmids

pcDNA3.1/Zeo(+)	Invitrogen, Karlsruhe
pEYFP, pEGFP	Clontech, Mountain View, CA, USA
HA-Ubiquitin	Krappmann, D. *et al.* 1996[148]
Traf2-flag	Krappmann, D. *et al.* 2000 [149]
IKKγ-flag,	Tegethoff, S. *et al.* 2003[150]
Mito-GFP	Invitrogen, Karlsruhe
pCMV-HA	Clontech, Mountain View, CA, USA

Equipment

Agarose Gelelectrophoresis chambers	Hoefer
Bacterial culture shaker Scientific 4518	ThermoQuest, Egelsbach
Gel documentation System	MWG Biotech, Ebersberg
PCR machine T3 Thermocycler	Biometra GmbH, Göttingen
Benchtop microcentrifuge	Eppendorf
Incubator	Heraeus
Microwave	Bosch
Thermomixer	Eppendorf
Gel extraction kit	Macherey-Nagel
DNA preparation kit mini/midi	Macherey-Nagel

Material and equipment for cell culture

Dulbecco's Modified Eagle's Medium (DMEM)	Gibco Invitrogen
DMEM/Ham's F12	Cambrex
Fetal Calf Serum	Gibco Invitrogen
H_2O_2 (30%)	Sigma
Lipofectamine/Plus	Invitrogen
Minimal Essential Medium (MEM)	Gibco Invitrogen
Non essential amino acids	Gibco Invitrogen
Optimem	Gibco Invitrogen
Penicillin/Streptavidin (P/S)	Invitrogen
PBS	Gibco Invitrogen
Promix	[^{35}S] Methionine/Cysteine; Amersham
Pulse-medium	Starvation Medium, P/S, Promix
Starvation medium	DMEM without Methionine/Cysteine; Gibco Invitrogen
Trypsin-EDTA	Invitrogen
Incubator	Heraeus, Hanau
Plastic pipets, sterile	Sarstedt
Cell culture dishes and flasks	Nunc

Material and equipment for protein biochemistry

PBS

3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4

TBS

25 mM Tris/HCl, 150 mM NaCl, pH 7.2

Glycine buffer

100 mM glycine, pH 2.7

Denaturing lysis buffer

50 mM Tris/HCl pH 7.4, 5 mM EDTA, 1% SDS, 15 U/ml DNAse, protease inhibitor

Non-denaturing lysis buffer

50 mM Tris/HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton-X 100, protease inhibitor

Hypotonic buffer

10 mM Tris/HCl pH 8, 1 mM $MgCl_2$

TNE

50 mM Tris/HCl pH 7.4, 150 mM NaCl

Resuspension buffer

10 mM Tris/HCl pH 7.4, 10 mM NaCl, 1,5 mM $CaCl_2$

MS buffer

420 mM Mannitol, 140 mM Saccharose, 10 mM Tris/HCl pH7.4, 5 mM EDTA

M1 buffer

600 mM Saccharose, 50 mM Tris/HCl pH 7.4, 10 mM EDTA pH8

Hypotonic buffer (mouse brain)

20 mM citrate, 1 mM EDTA, protease inhibitor

Laemmli sample buffer 4x

240 mM Tris/HCl pH 6.8, 4% SDS, 40 % glycerol, 2% bromophenol blue, 4% β-mercapto-ethanol

Disuccinimidy suberate (DSS)	Molecular Biosciences
ECL Amersham	Amersham
Immobilon Western ECL	Millipore
Goat serum	Sigma-Aldrich
Kainic acid	Calbiochem
MG132	Merck
Na-Desoxycholate	Sigma
Optiprep	Axis Shield
PBS +/+ Mg^{2+}/Ca^{2+}	Invitrogen
PFA	Sigma
PVDF-membrane	Millipore
Protein A Sepharose	Pierce
Triton-x100	USB
Super RX film (chemiluminescence)	Fuji
Biomax MR film (autoradiography)	Kodak
Film developer X-Omat	Kodak
SDS-PAGE chamber	BioRad
Blotting Chamber	BioRad
Gel dryer SGD 300	Savant

Whatman paper	Schleicher & Schüll
Cell homogenisator 1 ml	B.Braun
96 well plates for proein assay	Nunc
Amplify	Amersham
Ultracentrifuge Optima TLX	Beckman Coulter

Antibody list

Detected protein	name	species	company
Actin		Mouse	Sigma
Calreticulin		Mouse	Calbiochem
GAPDH		Mouse	Ambion
GFP		mouse	clontech
Hsp-60		Mouse	Stressgen
HA-epitope	3F10-HRP	Mouse	Roche
Parkin	#4230	rabbit	Cell signaling
	hP-1	rabbit	Winklhofer *et al*. [2]
	PRK 8	Mouse	Prof. V. Lee
	PRK 28	Mouse	Prof. V. Lee
TIM23		mouse	BD Biosciences
TIM44		Mouse	BD Biosciences
Transferrin Receptor		Mouse	Zymed
α-tubulin		mouse	Sigma

Appendix

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Publications

Schlehe J.S., Lutz A.K., Pilsl A., Lämmermann K., Grgur K., Henn I.H., Tatzelt J., Winklhofer K.F.
Aberrant folding of pathogenic parkin mutants: aggregation versus degradation
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Winklhofer K.F., **Schlehe J.S.**, Bouman L., Henn I.H., Tatzelt J
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The E3 ubiquitin ligase parkin mediates neuroprotection through activation of IKK/NF-kappa B signaling
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Henn I.H., **Schlehe J.S.**, Bouman L., Tatzelt J., Winklhofer K.F.
Mechanisms of parkin inactivation by pathogenic mutations: insights into the folding and degradation pathway of parkin
"Neuroscience" 2005, Washington DC, USA

Conferences attended

Molecular chaperones and Stress response
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Poster: "Aberrant folding of pathogenic parkin mutants: aggregation versus degradation"

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Ringberg Castle, Rottach-Egern, Germany, March 5-8 2008

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Munich, Germany, Dec. 7, 2007
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Grant to attend the Lindau Nobel Laureates Symposium, June 2003