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A Role of Parkin in Stress Response Pathways

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1 Summary

Parkinson's Disease (PD) is a neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the *substantia nigra pars compacta*. The etiology of sporadic PD remains poorly understood, however the recent identification of causative gene mutations responsible for monogenic familial variants of PD has provided significant new insights into the molecular mechanisms underlying the neuronal degeneration. So far, six genes have been associated with monogenic familial variants of PD, such as α-synuclein and LRRK2 for autosomal dominant PD and parkin, PINK1, DJ-1 and ATP13A2 for autosomal recessive PD. The parkin gene (*PARK2*) seems to play a prominent role, accounting for the majority of autosomal recessive PD. The modular structure of parkin suggests that it acts as a RING-type E3 ubiquitin ligase.

Endoplasmic reticulum (ER) stress has been implicated in the pathogenesis of PD. In this study I show that parkin plays an important role in the cellular ER stress response. Under ER stress parkin is significantly up-regulated on the mRNA and protein level due to the binding of the unfolded protein response (UPR)-specific transcription factor, ATF4, to a cis-acting element within the parkin promoter region. Interestingly, another transcription factor, c-Jun, can bind to the same regulatory region, but acts as a transcriptional repressor of parkin gene expression. Moreover, increased expression of parkin protects cells against ER stress-induced cell death, while cells lacking endogenous parkin are highly vulnerable to an imbalance in ER homeostasis. Interestingly, we could show that the protective activity of parkin is independent of the proteasome, indicating that parkin mediates non-degradative ubiquitylation. Moreover, we could provide evidence that the protective activity of parkin is associated with the regulation of cell survival/death pathways, like the JNK or the NF-κB pathway. The latter one is activated by parkin-mediated non-degradative ubiquitylation. In addition, parkin is able to suppress mitochondrial dysfunction and damage induced by ER stress, indicating an important role for parkin in the interorganellar crosstalk between the ER and mitochondria to promote cell survival under stress.

In the second part of my thesis I studied the transcriptional regulation of PD-associated genes under stress conditions playing a role in the pathogenesis of PD, such as mitochondrial stress, ER stress and oxidative stress. This study revealed that parkin is transcriptionally up-regulated in response to all stress conditions tested, while the other genes only were responsive to ER stress, emphasising the significance of ER stress in the pathogenesis of PD.

2 Introduction

2.1 PARKISON'S DISEASE

Idiopathic Parkinson's Disease (PD) is the most common movement disorder and the second most common neurodegenerative disorder after Alzheimer's disease (AD). In 2005, the number of people suffering from PD worldwide was estimated between 4,1 and 4,6 million with the tendency to be doubled in 2030 (Dorsey et al., 2007). The disease is characterized by the progressive loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (*SNpc*), and the subsequent lack of dopamine in the *corpus striatum*, responsible for the major PD motor symptoms. The symptoms of the disease can be ameliorated by medication, but medication cannot cure the disease. To develop more effective treatments, it is necessary to shed light on the complex and virtually unexplored molecular basis of the disease. Research on genetic cases of PD can increase the understanding of the molecular mechanism behind the symptoms of PD and may serve as a future basis for the development of drugs which could delay, or even halt the disease's progression.

2.1.1 History

In 1817, the clinical symptoms of Parkinson's disease (PD) were first described by the British physician James Parkinson in his script: "An Essay on the Shaking Palsy" (Fig. 1).

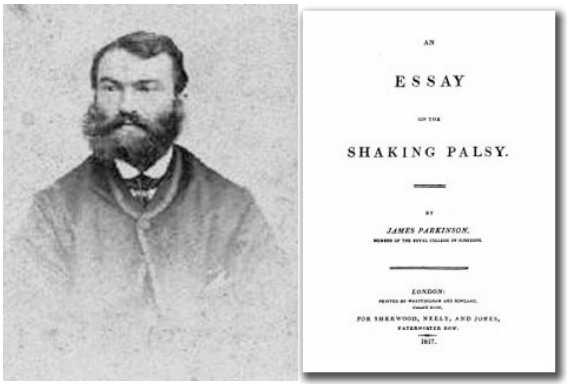


Fig. 1: First description of Parkinson's Disease written by James Parkinson, 1817. On the left: James Parkinson (1755-1824) (source: http://www.parkinsonsinjury.info); on the right: title page of his monograph "An Essay on the Shaking Palsy" from 1817 (source: www.pdmdcenter.com).

He systematically characterized the medical history of six individuals and concludes his work with following disease symptoms: “Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellect being uninjured”. With this he had already described the cardinal symptoms of Parkinson’s disease: tremor, akinesis and postural instability. Rigor, another cardinal symptom of PD was described 67 years later, by the French neurologist Jean-Martin Charcot. Charcot also recognized the disease as a uniform assembly of symptoms and named it after the man who had first documented it, James Parkinson. The discovery of pathological changes in the brain took place more than a century later. The starting point of this was the work of the Swedish scientist Arvid Carlsson, who discovered dopamine as a neurotransmitter in the mammalian brain. Neurons in the *SNpc* were then found to be lost in PD patients resulting in the consecutive lack of dopamine in the *corpus striatum*, which is believed to induce the classical clinical motor deficits seen in PD patients (Ehringer and Hornykiewicz, 1960; Sourkes and Poirier, 1965). It was this discovery that lead to the first effective medical treatment of the disease. In 1967 the drug levodopa (L-3,4-dihydroxyphenylalanine, L-DOPA), the precursor of dopamine, entered clinical practice and the first large study reporting improvements in patients with PD resulting from treatment with L-DOPA was published in 1968. Although the discovery of L-DOPA revolutionized the treatment of PD, and it certainly diminishes PD symptoms neither this, nor any other medication available at present is able to halt or retard dopaminergic neuron degeneration.

2.1.2 Neuropathological characteristics

The neuropathological hallmark of PD is the widely selective degeneration of dopaminergic neurons in the *SN* and the loss of projections to the *corpus striatum putamen*. The striatal dopaminergic nerve terminals are suggested to be the primary target of the degenerative process and the neuronal cell death is the following consequence (Bernheimer et al., 1973). Dopaminergic neurons contain conspicuous amounts of the dark-brown pigment neuromelanin (Marsden, 1983), a catabolism product of dopamine. The loss of these neurons results in depigmentation of the *SN*, which can be observed in the *post mortem* brains of PD patients (Fig. 2).

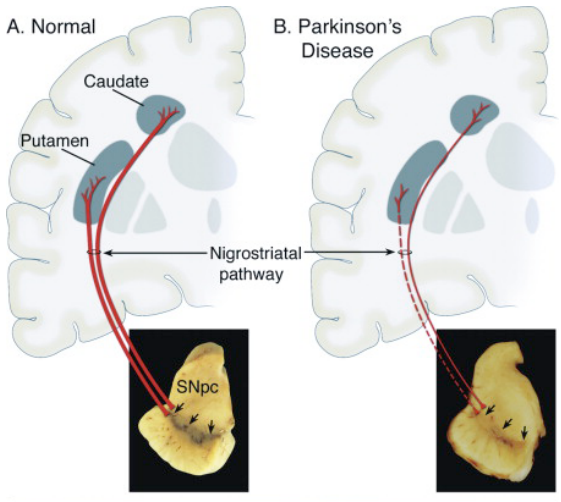


Fig. 2: Schematic representation of the nigrostriatal projections. (A) Normal nigrostriatal pathway between the *SNpc* and the *putamen/caudate nucleus* (solid red line). The photograph demonstrates the normal pigmentation of the *SNpc* due to the production of neuromelanin within the dopaminergic neurons. **(B)** Degenerated nigrostriatal pathway in PD. The loss of dopaminergic neurons that project to the *putamen* is more severe (dashed red line) than the loss of dopaminergic neurons that project to the *caudate* (thin red solid line). The photograph demonstrates depigmentation of the *SNpc* due to the marked loss of dopaminergic neurons. From (Dauer and Przedborski, 2003).

To understand the possible therapeutic treatments of PD, it is useful to pay attention to the neuropathological changes in the basal ganglia circuits. The loss of nigrostriatal projections in the striatum produces an imbalance within both the direct pathway (striatum to the *Globus pallidus internus, GPi*) as well as the indirect pathway (striatum to the *GPi* via *GP externus* and *Subthalamic nucleus*), leading to a reduced activation of the direct pathway and a reduced inactivation of the indirect pathway. The balance between these circuits allows smooth coordinated movement behaviour. The net-effect of the non-equilibrium present in PD is an increased inhibitory output activity of the *GPi* neurons to the thalamus, resulting in reduced motor cortex activity and clinically in a reduced regulation of movements.

Apart from the degeneration of dopaminergic neurons in the *SN*, neuronal cell death occurs also in the noradrenergic (*locus coeruleus*), serotonergic (*raphe nucleus*), and cholinergic (*nucleus basalis of Meynert*, *dorsal motor nucleus of vagus*) systems of the brain as well as in the cerebral cortex, olfactory bulb and autonomic nervous systems (Dauer and Przedborski, 2003; Forno, 1996).

Another pathological feature of sporadic and some familial PD forms (detailed explanation in chapter “Familial forms of PD and their genetics”) is the occurrence of intraneuronal, cytoplasmatic, eosinophilic inclusions, so called Lewy bodies, and dystrophic neuritis, so called Lewy neurites, present in the remaining intact nigral

neurons (Dickson et al., 1991). Lewy bodies are composed of numerous proteins, for example neurofilaments, ubiquitin, α-synuclein, molecular chaperones and parkin (Forno, 1996; Spillantini et al., 1998; Spillantini et al., 1997). Immunohistochemical staining reveals an organized structure with a dense round core surrounded by a clear halo with a diameter of about 15 μm (Fig. 3) (Pappolla, 1986). Nevertheless, Lewy bodies are not specific for PD and also other diseases exhibit Lewy-body pathology such as dementia with Lewy bodies (DLB) or multiple system atrophy (MSA). These diseases are collectively termed "α-synucleinopathies" (Duda et al., 2000). The role of Lewy bodies and Lewy neurites in the pathogenesis of PD is widely controversial.

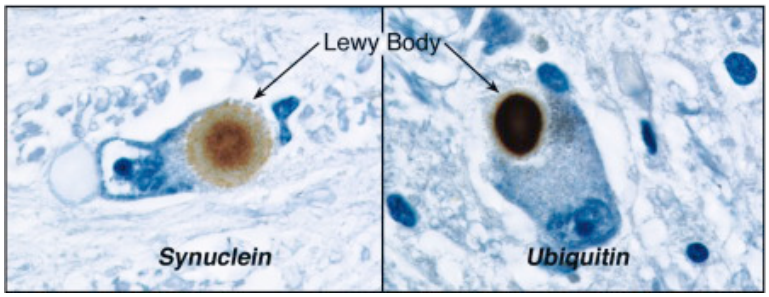


Fig. 3: Immunohistochemical staining of Lewy bodies in a *SNpc* dopaminergic neuron. Left panel: Immunostaining against α-synuclein; right panel: Immunostaining against ubiquitin. From (Dauer and Przedborski, 2003).

2.1.3 Symptoms and therapeutic approaches

Although the age of onset of the cardinal symptoms of PD does vary across patients and can occur prior to the age of 40 in early-onset PD, most cases generally occur in patients over 55 years. Prevalence of PD increases from approximately 1%-2% of those over age 60 to about 4% of those over age 80 (de Lau and Breteler, 2006). The disease prevalence rises markedly with age and will expand due to the expected increased average life-expectancy of the population, giving PD more and more relevance in an aging society.

The major clinical PD symptoms are resting tremor, muscle rigidity, slowness or absence of voluntary movements without paralysis (bradykinesia, akinesia), postural instability, paucity of normal facial expression (hypomimia), decreased voice volume (hypophonia), drooling (failure to swallow without thinking about it), decreased size and speed of handwriting (micrographia), decreased stride length and freezing (inability to begin a voluntary movement) (Hughes et al., 1991; Lang, 1998; Lang and Lozano, 1998). Olfactory and autonomic nervous system dysfunction (Herting et al., 2008), adverse effects in the visual system (Bodis-Wollner, 1990), psychological symptoms like depression, slowness of cognitive processes and also dementia typically develop

as the disease progresses and often become a major cause of disability (Aarsland et al., 1996; Cummings, 1992). The non-nigral lesions are considered to account for the main cognitive aspects of PD. A growing number of clinical results speak for an etiologically heterogenic syndrome rather than a homogenous disease. When the first cardinal symptoms of the disease appear, 50-60% of DA neurons in *SNpc* have already been lost and the dopamine release in the striatum has been reduced by more than 80%, indicating that the brain is able to compensate deficits in the nigro-striatal dopaminergic system for quite a long time.

For diagnosis of the disease the brain of PD patients can be examined by imaging methods such as by a positron emission tomography (PET) scan. During such a scan the uptake of [^{18}F]-Fluoro-L-Dopa by the presynaptic dopaminergic terminals is monitored. The uptake differs between a normal individual and an individual suffering from PD (Fig. 4).

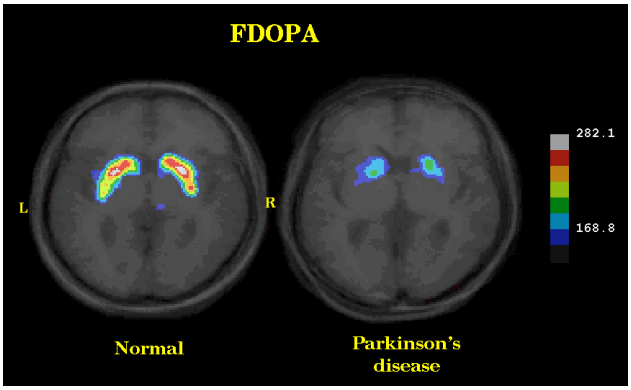


Fig. 4: Comparison of PET (positron emission tomography) scans from a normal individual with one from a PD patient, demonstrating the massive dopaminergic neuron loss that occurs in PD. Left: Control brain of a healthy individual has a normal uptake of ^{18}F-DOPA into the striatum. Right: Brain of a PD patient shows a reduced uptake of ^{18}F-DOPA. From G. Leger and A. Dagher, Montreal Neurological Institute, Mc Gill University.

The therapeutic approaches mainly focus on compensating the lack of dopamine and the consequent imbalance of neurotransmitters. The basis of therapy is the prescription of L-DOPA, the precursor of dopamine, as dopamine is not able to pass the blood-brain barrier. L-DOPA is taken up into the central nervous system and is converted into dopamine in dopaminergic neurons by the DA (dopamine) decarboxylase. In addition to L-DOPA, an inhibitor of peripheral DA decarboxylase, e.g. carbidopa is administered, which increases the L-DOPA concentrations that reach the brain, decreases the dosage, which is needed and inhibits the premature degradation of L-DOPA (Fig. 5). Further, dopaminergic agonists can be used to directly stimulate dopamine receptors. Another possibility of treatment is the inhibition of the dopamine

catabolism: The monoamine oxidase B (MAO-B) inhibitor prevents the conversion of dopamine to DOPAC (3,4-Dihydroxyphenyl acetic acid). It is mostly used to treat mild symptoms of PD. The catechol-O-methyl-transferase (COMT) inhibitor reduces the dopamine methylation to 3-methoxytyramine. MAO-B and COMT inhibitors can also be prescribed in combination with L-DOPA, to prolong the action of dopamine. Anticholinergics are only given in some tremor cases, as they cause severe side effects. Medical surgery by introducing microelectrodes in specific regions of the basal ganglia (deep brain stimulation) is mainly carried out in patients who cannot be treated conventionally. All available treatments are merely ameliorating the symptoms of PD, however they are not able to modify the disease process.

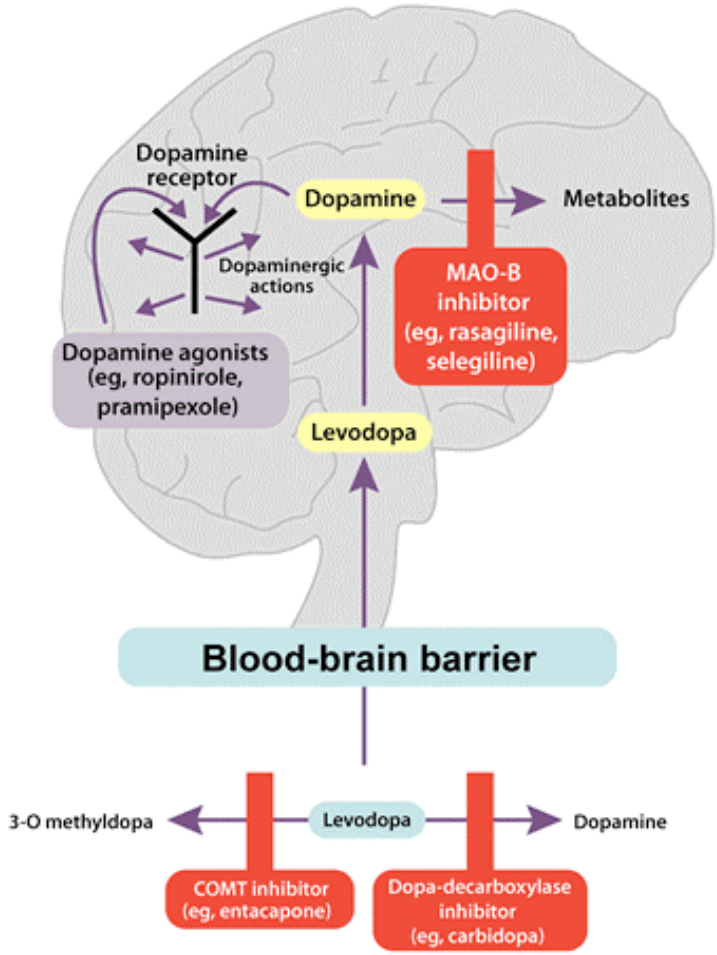


Fig. 5: Current pharmacologic therapies for PD and their potential sites of action on the central nervous system. Red: site of drug action. COMT: Catechol-O-methyl-transferase; MAO-B: Monoamine oxidase-B. (Adapted from parkinsonsdiseasecme.com/cme-modules/neuroprotection-parkinsons-disease/introduction.html.)

2.1.4 Etiology

No familiar accumulation can be observed in about 80% of the PD cases. As the reason for the loss of dopaminergic neurons is broadly unclear, they are referred to as idiopathic or sporadic PD cases. About 10% of the cases are familiar forms of the disease with autosomal dominant or recessive inheritance. The remainder (10% of cases) are associated with the symptomatic Parkinson syndrome also known as secondary Parkinson syndrome. This inhomogenous group of disorders can have multiple possible causes, for example drug- or toxin-induced parkinsonism, inflammation, metabolic dysfunctions, tumours, ischemia or traumata (Dauer and Przedborski, 2003). Further, atypical parkinsonism can also occur in other neurodegenerative diseases such as dementia with Lewy bodies (DLB), progressive supranuclear palsy (PSP), MSA and corticobasal degeneration (CBD) (Fig. 6).

For the majority of individuals with sporadic PD the cause of the disease remains unclear. Aging seems to be the major risk factor. The difference in the age of onset may relate to the combination of risk factors underlying an individual's particular disease, a combination of environmental influences and genetic predisposition is suggested. An example for an environmental toxin induced destruction of dopaminergic neurons is the accidental intoxication of young drug addicts with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), resulting in the development of a syndrome nearly identical to PD (Langston et al., 1983). Human epidemiological studies revealed an elevated risk of developing PD symptoms for people who live in rural environments and have exposure to herbicides and pesticides (Tanner, 1992). Examples of herbicides and pesticides, which are under suspicion of inducing PD, are paraquat and rotenone, both shown to inhibit like MPTP complex I of the mitochondrial respiratory chain. Also endogenous toxins are suspected to be responsible for PD neurodegeneration. Inherited differences or environmental toxins might distort the normal metabolism, creating toxic substances; e.g. the normal dopamine metabolism, which generates reactive oxygen species (ROS) (Cohen, 1984). Thirteen different loci are described for familial forms of PD, showing all a mendelian pattern of inheritance.

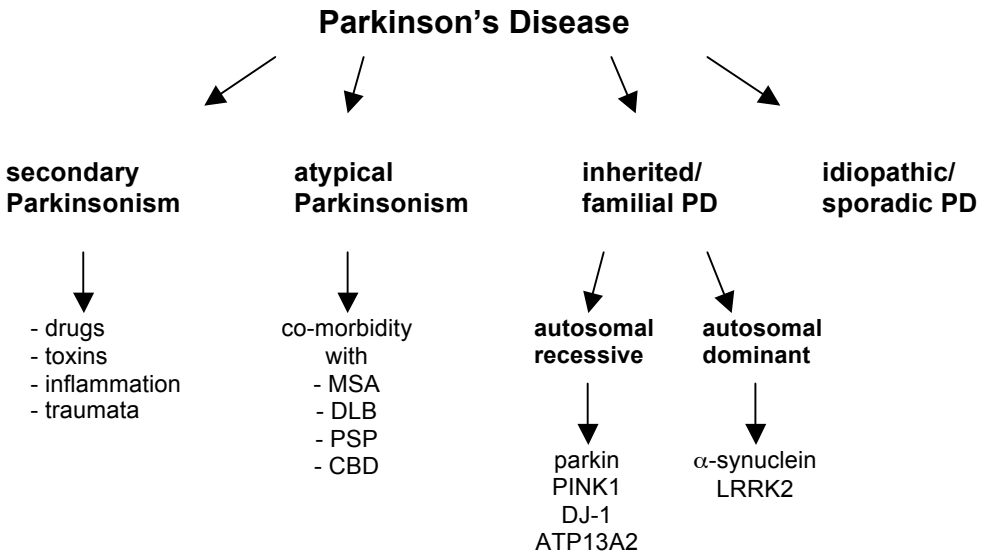


Fig. 6: Etiology of Parkinson's Disease. MSA: multiple systems atropy; DLB: dementia with Lewy bodies; PSP: progressive supranuclear palsy.

2.2 FAMILIAL FORMS OF PD AND THEIR GENETICS

The identification of genes responsible for the rare familial forms of PD dramatically improved our understanding of the molecular mechanisms underlying the pathogenesis of PD. Since 1997, linkage data has identified at least 13 loci associated with PD. Currently the genes are known for 8 of them (Table 1). Their discovery opened new and exciting research opportunities to track the molecular pathways involved in the disease pathophysiology and provided new opportunities to generate cell culture or animal models for the disease. The typical and extremely consistent phenotype of both sporadic and hereditary PD suggests a common molecular mechanism for the disease (Thomas and Beal, 2007). Consequently, new insights into the pathomechanism of idiopathic PD will also facilitate a better understanding of sporadic PD and will help to develop novel therapies for this common and so far incurable neurodegenerative disorder. In the following some PD-associated genes will be described in detail.

gene locus	map position	gene product	Inheritance pattern	putative function
A. Loci and genes implicated in PD with conclusive evidence from genome-wide linkage screens				
PARK1/4	4q21-q23/4p15 (duplication/triplications)	α-synuclein (SCNA)	AD	vesicle trafficking / synaptic plasticity
PARK2	6q25-q27	parkin	AR	E3 ubiquitin ligase
PARK6	1p36-p35	PINK1	AR	mitochondrial kinase
PARK7	1p36	DJ-1	AR	cytosolic redox-sensitive protein
PARK8	12p11-q13	LRRK2	AD	MAPKK kinase
PARK9	1p36	ATP13A2	AR	lysosomal ATPase
B. Further loci identified in genome-wide linkage screens				
PARK3	2p13	unknown (SPR?)	AD	
PARK10	1p32	unknown	unclear	
PARK11	2q36-q37	unknown (GIGYF2?)	unclear	
PARK12	Xq	unknown	unclear	
C. Genes proposed to be implicated in PD etiology in studies adapting a candidate-gene approach				
PARK5	4p14	UCHL1	AD?	ubiquitin carboxy-terminal hydrolase
PARK13	2p12	HtrA2/omi	unclear	mitochondrial protease

Table 1: Catalogue of genes and loci for parkinsonism. AR: autosomal recessive; AD: autosomal dominant; PINK1: phosphatase and tensin (PTEN)-induced kinase 1; LRRK2: leucine-rich repeat kinase 2; SPR: sepiapterin reductase; GIGYF2: GBR10 interacting GYF protein-2; HtrA2/omi: high temperature requiring protein A 2/omi (Bonifati, 2007; Gasser, 2009).

2.2.1 Autosomal-dominant genes

2.2.1.1 α-synuclein (PARK1 and PARK4)

α-Synuclein (SCNA) was the first gene to be described in the context of familial PD (Polymeropoulos et al., 1997). An autosomal dominant mutation (A53T) was found in the α-synuclein gene of a small number of Greek/Italian families. Up to the present two more dominant mutations have been identified: A30P and E46K (Kruger et al., 1998; Zarranz et al., 2004). Subsequently, a genomic duplication or triplication at the α-synuclein locus, resulting in an increase of the gene dosage was also reported to cause autosomal dominant PD (Farrer et al., 2004; Singleton et al., 2003). The natively unfolded relatively small (140 aa) α-synuclein protein contains three regions: First, an N-terminal region taking up a α-helical confirmation after binding to membranes, and characterized by repetitive imperfect repeats (Davidson et al., 1998; Eliezer et al., 2001), second, a central hydrophobic NAC-domain (non-amyloid component of plaques) responsible for the aggregation potential of α-synuclein (Bodles and Irvine, 2004; Giasson et al., 2001), and third an acidic C-terminal region, containing several phosphorylation sides and being able to give the protein a chaperone like activity (Okochi et al., 2000; Park et al., 2002). Normally, α-synuclein is an abundant soluble neuronal cytoplasmic protein, predominantly localized to pre-synaptic terminals in close association with synaptic vesicles (Maroteaux et al., 1988). The function of α-synuclein is not well understood, but its localization to synaptic membranes implicates a role in the regulation of vesicle dynamics and neuronal plasticity (Abeliovich et al., 2000; Sidhu et al., 2004). Interestingly, α-synuclein is known to be a major constituent of Lewy bodies (LBs), therefore providing the most obvious link between sporadic and familial PD (Spillantini et al., 1997). *In vitro* the α-synuclein protein tends to aggregate in a concentration-dependent manner to form fibrils similar to those observed in LBs (Conway et al., 2000; Serpell et al., 2000). Moreover, for the pathogenic mutations an increased propensity to self-assembly and fibrillization was described (Greenbaum et al., 2005). Further, the genomic multiplication of the α-synuclein loci, and the resulting enhanced expression of α-synuclein, fosters insoluble α-synuclein aggregation (Miller et al., 2004). In addition, α-synuclein protofibrils were shown *in vitro* to build up ring-like structures able to permeabilize vesicles by acting like pores (Volles and Lansbury, 2003). These findings point to the ongoing debate, whether the oligomeric intermediates, the protofibrils, or the fibrils (formed later in the aggregation process) are the toxic species for the cell. Studies of PD patient-derived brain tissue demonstrated an increased phosphorylation of α-synuclein at serine 129 in the C-terminal domain in LBs (Anderson et al., 2006; Okochi et al., 2000). However, this posttranslational

modification was recently shown to reduce the α-synuclein induced toxicity (Gorbatyuk et al., 2008), further demonstrating the controversy in the field.

2.2.1.2 LRRK2 (PARK8)

Leucine-rich repeat kinase 2 (LRRK2), also named Dardarin, was recently discovered to play a role in the context of PD (Funayama et al., 2002; Paisan-Ruiz et al., 2005; Zimprich et al., 2004). The *LRRK2* gene is a very large protein with a molecular mass of 285 kDa. It consists of multiple domains: an N-terminal ankyrin domain, a leucin-rich repeat, a GTPase/ROC (ras of complex proteins) domain, a COR (C-terminal of ROC domain), a kinase domain and a WD40 domain (Taylor et al., 2006). *LRRK2* mutations are the most common cause of familial PD, typically associated with late onset. Until now there are about twenty known mutations, with the G2019S located in the kinase domain being the most frequent one. Mutations in the kinase domain and also in other domains appear to increase the kinase activity, which suggests a toxic gain of function mechanism (Brice, 2005; West et al., 2007). However, mutations in the *LRRK2* gene give rise to diverse, widespread neuropathological features and the physiological function of LRRK2 is still unclear.

2.2.2 Autosomal-recessive genes

2.2.2.1 Parkin (PARK2)

Parkin was the first identified recessive gene involved in PD. In 1998, Kitada and colleagues described mutations in the *parkin* gene as the cause of recessive PD with juvenile onset in a Japanese family (Kitada et al., 1998). A broad spectrum of parkin mutations have since been described, accounting for the majority of autosomal recessive Parkinsonism and pointing to a prominent role among the other PD-associated genes. For a detailed description of parkin see section “Parkin-associated Parkinson’s Disease”.

2.2.2.2 PINK1 (PARK6)

In 2004, PTEN (phosphatase and tensin)-induced kinase 1 (PINK1) mutations were found to be associated with *PARK6* autosomal recessive PD in a large Sicilian family (Valente et al., 2004). PINK1 is a 581 amino acid protein and contains a mitochondrial-targeting motif at the N-terminus, and there is evidence that PINK1 is targeted to mitochondria (Beilina et al., 2005; Silvestri et al., 2005; Valente et al., 2004). In addition, PINK1 contains a conserved serine/threonine kinase domain. The kinase activity was shown by autophosphorylation *in vitro* (Beilina et al., 2005; Silvestri

et al., 2005), and additionally for two putative substrates, TRAP1 (TNFR-associated protein 1; Hsp75), a mitochondrial localized chaperone (Pridgeon et al., 2007) and HtrA2/omi *in vivo* (Plun-Favreau et al., 2007). Increased PINK1 expression in neurons protects from oxidative and proteasomal stress and staurosporine induced apoptosis (Haque et al., 2008; Petit et al., 2005; Valente et al., 2004), whereas loss of PINK1 function increases the vulnerability of the cell for stress (Deng et al., 2005a; Wood-Kaczmar et al., 2008). Recently, published data from *Drosophila* and cell culture demonstrate a major function of PINK1 in maintaining mitochondrial integrity and dynamics (Clark et al., 2006; Exner et al., 2007; Gandhi et al., 2009; Gegg et al., 2009; Marongiu et al., 2009; Morais et al., 2009; Park et al., 2006; Poole et al., 2008). Evidence exists that parkin is functionally linked to PINK1 and that they act in a common cellular pathway with PINK1 upstream of parkin (Clark et al., 2006; Exner et al., 2007; Lutz et al., 2009; Yang et al., 2006a).

To date, approximately 20 pathogenic mutations, mainly missense and nonsense mutations, but also whole gene deletions have been described (Marongiu et al., 2007). *PINK1* mutations are the second most frequent cause for autosomal recessive, early onset parkinsonism (Bonifati, 2007). Most pathogenic mutations occur in the kinase domain of PINK1, and were shown to affect its activity, essential for the neuroprotective potential of PINK1. Yet no neuropathological data from patients with PINK1 mutations have been reported.

2.2.2.3 DJ-1 (PARK7)

Mutations in the *DJ-1* gene are the least common among the known cases of early onset autosomal recessive parkinsonism (Bonifati et al., 2003). The first mutations identified were a deletion mutant (exon 1-5) in a Dutch family and a missense mutation (L166P) in an Italian family, indicating that the loss of DJ-1 function can cause parkinsonism (Bonifati et al., 2004). The *DJ-1* gene encodes a ubiquitously expressed 189 amino acids protein found to be localized in the cytoplasma and nucleus as well as within mitochondria (Junn et al., 2009; Zhang et al., 2005). It was first identified as an oncogene (Nagakubo et al., 1997), and diverse cellular functions have been attributed to it. The most commonly held view might implicate a role in the response to oxidative stress. In several cell culture and animal models DJ-1 was shown to be protective against oxidative stress-induced cell death (Canet-Aviles et al., 2004; Kim et al., 2005; Yokota et al., 2003). The mechanism behind this protective effect is still unclear. DJ-1 could have intrinsic chaperone-like activity as structural studies demonstrated similarities with the bacterial chaperone Hsp13 (Lee et al., 2003; Shendelman et al., 2004) and/or serve as an antioxidant and/or a stress sensor for

oxidative stress, since it can be oxidized at the cysteine residue C106 (Canet-Aviles et al., 2004; Mitsumoto et al., 2001). Notably, this residue was demonstrated to be essential for the protective activity of DJ-1 (Taira et al., 2004; Waak et al., 2009; Yokota et al., 2003) Additionally, DJ-1 could have an impact on stress-signalling pathways: The pro-apoptotic ASK (apoptotic signal-regulating kinase1)-pathway was inhibited and the anti-apoptotic Akt-pathway was shown to be activated by DJ-1 (Gorner et al., 2007; Junn et al., 2005; Yang et al., 2005). Of note, DJ-1 does not seem to be part of the PINK1/parkin-pathway as it was not able to compensate for mitochondrial alterations in parkin or PINK1 deficient cells (Exner et al., 2007; Yang et al., 2008).

Some evidence exists that the inactivation of DJ-1 can also lead to Parkinsonism. Extensive oxidation was shown to damage DJ-1 (Zhou et al., 2006), supported by the finding of oxidatively damaged DJ-1 in brains of sporadic PD patients (Choi et al., 2006). Further, the pathogenic L166P mutant impairs the functional dimer formation of DJ-1, leading to a very unstable protein, which is rapidly degraded by the proteasome (Miller et al., 2003; Olzmann et al., 2004).

2.2.2.4 HtrA2/omi (PARK13)

Mutations in the *high temperature requirement protein A2 (HtrA2/omi)* encoding gene were implicated with PD etiology. In PD patients a heterozygous mutation (G399S) and a polymorphismn (A141S) of *HtrA2/omi* were proposed to be responsible for PD pathology (Bogaerts et al., 2008; Strauss et al., 2005). However, the genetic proof of pathogenicity is still lacking as the linkage evidence from large families is missing (Bonifati, 2007; Gasser, 2009). However, loss-of-function mice show neurodegeneration with a parkinsonian phenotype (Jones et al., 2003; Martins et al., 2004).

HtrA2/omi is a mitochondrially located serine protease and is assumed to play an important role in apoptosis. Upon apoptotic stimuli it is released from the mitochondrial intermembrane space into the cytosol where it interacts with IAPs (inhibitor of apoptosis proteins), thus inducing caspase activity, and also mediating caspase- independent death through its own protease activity. However, its functional role may be context-dependent as HtrA2/omi mutations result in a defective activation of their protease activity, which leads to mitochondrial dysfunction and increased susceptibility to apoptosis (Hegde et al., 2002; Martins et al., 2002). On the contrary, phosphomimetic HtrA2/omi mutants showed an increase in protease activity and enhanced the protection capacity of the cell for rotenone -or 6-OHDA-induced stress, presuming a neuroprotective function for HtrA2/omi. Furthermore, HtrA2/omi was shown to interact with PINK1, which phosphorylates HtrA2/omi on serine 142 in

dependency of p38 (Plun-Favreau et al., 2007). It seems therefore likely that HtrA2/omi functions in a similar manner to that of its bacterial homologues DegP and DegS, which are involved in transducing stress response signals and protection against cell stress (Clausen et al., 2002; Schlieker et al., 2004).

2.3 PATHOGENESIS OF PD AND ITS ASSOCIATED CELLULAR PROCESSES

The molecular mechanisms responsible for the degeneration of dopaminergic neurons remain very difficult to pinpoint. However, the findings from familiar forms of PD coupled with those from toxin-induced PD models implies a multifactorial cascade of deleterious factors for the pathogenesis of PD. In this cascade mitochondrial dysfunction, accumulation of oxidative stress, protein aggregation, impairment of the ubiquitin-proteasome system (UPS), increased ER stress, and activation of stress kinase signalling pathways are involved. These aspects are described in detail below.

2.3.1 Mitochondrial dysfunction and oxidative stress

Mitochondrial dysfunction has long been implicated in PD pathogenesis. The primary function of mitochondria is the generation of cellular energy in the form of ATP by oxidative phosphorylation. In addition, they play a role in metabolism of e.g. amino acids and lipids, as well as calcium homeostasis, signalling pathways and apoptosis.
The mitochondrial electron transport chain is composed of five multi-subunit complexes. Electrons are fed from NADH (derived from the Krebs cycle) to complex-I in the electron transport chain and transported via ubiquinone (coenzyme Q) to complex-III ($FADH_2$ directly transfers its electrons to ubiquinone). From there the electrons are transferred to cytochrome C and passed to complex-IV. The transport of electrons down the respiratory chain produces energy, which is used to pump protons across the inner mitochondrial membrane by the complex-I, -III and -IV, thus creating a proton and electrochemical gradient. This gradient forms the basis for the mitochondrial membrane potential and is used by complex-V to drive ATP synthesis by reducing oxygen to water. However, 2% of electrons passing through the electron transport chain, mostly at complex-I and complex-III, react with molecular oxygen to superoxide anion (O_2^-) (Beal, 2003), which is the precursor of most other reactive oxygen species (ROS) (Turrens, 2003), such as the highly reactive hydroxyl radicals (·OH) or the stable molecular oxidant hydrogen peroxide (H_2O_2). These reactive species can be inactivated by a network of antioxidative systems, consisting of glutathione (GSH), and glutathione peroxidases, catalases and superoxide dismutases (SOD). A very delicate

equilibrium exists between the formation and the elimination of ROS and a disturbance can have disastrous effects for the cell. Increased formation of ROS or the defective removal can lead to oxidative damage of proteins, DNA and lipids (Raha and Robinson, 2000). The damage can also affect mitochondrial constituents such as the mtDNA or components of the mitochondrial electron transport chain, in particular because of their proximity to ROS production. In turn, the damage of the electron transport chain again increases oxidative stress producing a vicious cycle. As time goes on oxidative alterations accumulate, resulting in further mitochondrial dysfunction and finally to cell death, pointing to the fact that increasing age is the most consistent risk factor for sporadic PD (Wallace, 2005).

A possible reason why in particular dopaminergic neurons are affected in PD is their exposure to high oxidative stress levels due to their dopamine metabolism. Dopamine is degraded enzymatically by monoaminoxidase (MAO) or catechol-O-methyl-transferase (COMT) or non-enzymatically by autooxidation. The degradation by MAO or COMT results besides the formation of the desaminated metabolites 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT) also in reactive hydrogen peroxide (H_2O_2) (Maker et al., 1981) (Fig. 7). The autooxidation of dopamine leads to the production of toxic quinones and semi-quinones, able to damage proteins with their cysteine residues (Stokes et al., 1999). Further, also the production of neuromelanine by oxidative polymerisation of dopamine leads to the release of free radical species.

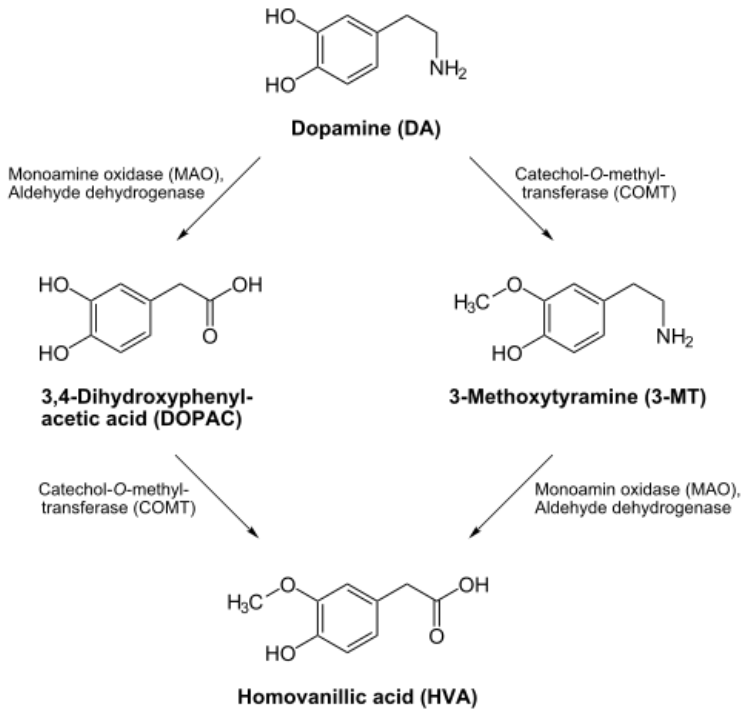


Fig. 7: Enzymatic degradation of dopamine. (source: http://en.wikipedia.org/wiki/dopamine)

Several studies support the hypothesis that mitochondrial dysfunction, increased ROS production and oxidative stress at least contribute to dopaminergic cell death in the *SN*. Concretely, post-mortem brain analysis of PD patients showed a strong reduction of complex-I activity in the *SNpc* (Schapira et al., 1990). Low activity of complex-I leads to an energy deficit for the cell, while oxidative stress is increased (Chan et al., 1991). Furthermore, the *SN* of patients showed an increase in oxidative modifications of lipids, proteins and DNA (Dexter et al., 1989; Giasson et al., 2000; Jenner, 2003; Zhang et al., 1999), as well as reduced amounts of the antioxidative GSH (Sian et al., 1994) and increased ROS. Animal models support the thesis of a dysfunction of mitochondria in PD. Thus the treatment of mice or primates with mitochondrial complex-I inhibitor MPTP results in a specific loss of dopaminergic neurons, and PD-like pathology (Bloem et al., 1990; Dauer and Przedborski, 2003). Additional, also the fish poison rotenone and the herbicide paraquat, were shown in animal models to reduce complex-I activity (Betarbet et al., 2000; Brooks et al., 1999; McCormack et al., 2002; Sherer et al., 2003). Conclusively, mitochondrial dysfunction and oxidative stress play a major role in the pathogenesis of PD, whether they are the cause or the consequence is still unclear.

It is notable that ROS are not always harmful or toxic for the cell and increasing evidence shows that they are also important as signalling molecules for the regulation of diverse cellular pathways such as the JNK (c-Jun N-terminal-kinase) pathway and the NF-κB (nuclear factor kappa enhancer binding protein) pathway (Shen and Liu, 2006; Yoon et al., 2002).

2.3.2 Mitochondria-induced apoptosis

Besides ATP production, mitochondria are important regulators of Ca^{2+} signalling. Mitochondria respond actively and sensitively to a local increase of Ca^{2+} concentration by a transient and massive uptake of the ion into the organelle (Rizzuto et al., 1999). This uptake can affect various cellular processes ranging from the induction of mitochondrial ATP production, regulation of subcellular processes by Ca^{2+}-dependent enzymes, control of mitochondrial movement but also the induction of apoptosis. Specially, for the latter case mitochondria play an integral role. The trigger for apoptosis can be mitochondrial complex inhibition, increased ROS production or misfolded proteins for example (Bredesen et al., 2006). Apoptosis is executed via two main pathways. Firstly, the death receptor (or extrinsic) pathway, which is initiated by the activation of cell-surface death receptors, for example Fas, and secondly, the mitochondrial (or intrinsic) pathway characterized by the release of mitochondrial pro-apoptotic factors. The permeabilization of the outer mitochondrial membrane plays a

key role in this process. This effect can occur via two mechanisms: the first one involves the opening of the mitochondrial permeability transition pore and the second one is dependent on the inactivation or activation of Bcl-2 (B-cell leukaemia/lymphoma 2) family members, being anti- (like Bcl-2 and Bcl-x_L) or pro-apoptotic (like Bid (BH3-interacting domain death agonist), Bax (Bcl-2 associated X protein) and Bak (Bcl-2 antagonist/killer), respectively. Upon permeabilization of the mitochondrial membrane, several intermembrane space proteins are released, such as cytochrome c, apoptosis inducing factor (AIF), endonuclease G, HtrA2/omi and second mitochondria-derived activator of caspases (Smac). Cytochrome c release leads to the formation of the protein complex known as the apoptosome, together with apoptosis protease-activating factor 1 (Apaf1) and procaspase 9 (pro cysteine-aspartic protease 9). The formation of the complex results in the activation of caspase 9, which then triggers the activation of caspase 3 and other caspases in an amplification cascade, ultimately causing cell death. Inhibitors of apoptosis (IAPs) are able to halt activated caspases, however, they are deactivated by Smacs and HtrA2/omi. The two proteins AIF and endonuclease G translocate to the nucleus and induce chromatin condensation and DNA fragmentation independent of caspase activation (Fig. 8).

The JNK pathway is also implicated in the execution of apoptosis in response to different stress stimuli by catalyzing the phosphorylation of anti- and pro-apoptotic Bcl-2 family members (Davis, 2000; Schroeter et al., 2003) (see Fig. 11).

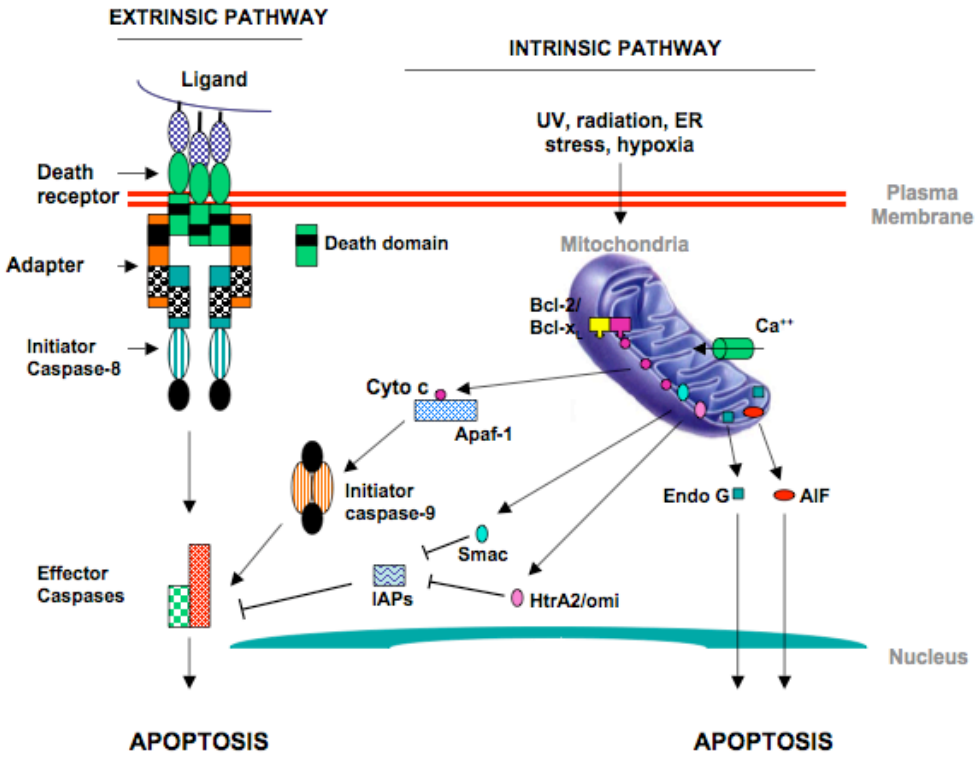


Fig. 8: Extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway is mediated by cell-surface death receptors. Following interaction with death receptor ligand, the cytoplasmic death domains of death receptors undergo trimerization and recruit a set of adaptor proteins and initiator caspases (caspase-8 and -10), forming a death-inducing signalling complex (DISC). Initiator caspases are activated by homodimerization and when released from the DISC into the cytoplasm they cleave effector pro-caspases to generate active effector caspases (caspase-3, -6, -7), which cleave a large number of cytoplasmic and nuclear substrates to induce morphological and biochemical features of apoptosis. The intrinsic pathway is dependent on the mitochondrial permeability transition pore and inactivation or activation of anti- or proapoptotic Bcl2 family members (e.g. Bcl2 (B-cell leukaemia/lymphoma) or Bcl2-X_L). Upon induction of apoptosis the outer mitochondrial membrane is permeabilized and several intermembrane space proteins are released. Cytochrom c (cyto c), Apaf1 (apoptosis protease factor 1), and pro-caspase 9 form a complex, which leads to the activation of caspase 9 and to the generation of active effector caspases. IAPs (inhibitor of apoptosis) inhibit activated caspases, however they are deactivated by Smac (second mitochondria-derived activator of transcription) and HtrA2/omi (high temperature requiring enzyme A2). AIF (apoptosis inducing factor) and Endo G (endonuclease G) translocate to the nucleus and induce chromatin condensation and DNA fragmentation independent of caspase activation. Ca^{2+} plays a major role for the induction of apoptosis. Modified from (Gupta et al., 2006).

2.3.3 Protein aggregation and dysfunction of the ubiquitin proteasome system

Protein aggregates are characteristic for many age-related neurodegenerative diseases. It is uncertain whether the presence of these aggregates per se, or a loss of normal protein function due to the sequestering is associated with their toxicity. However, new studies suggest that it is not the aggregates themselves that are toxic, but the oligomeric intermediates (e.g. α-synuclein, Aβ, poly-Q-huntingtin), associated with them. Moreover, aggregates may also occur as a result of attempts by neurons to protect themselves from toxic proteins, indicating a protective potential for the cell, rather than a toxic one (Ciechanover and Brundin, 2003). In PD protein aggregates occur as cytosolic Lewy-bodies (see also the section “Neuropathological characteristics” of PD).

One reason for an accumulation of proteins to aggregates could be their impaired degradation. Normally, short-lived, mislocalized, misfolded, mutated or damaged proteins are degraded by the ubiquitin-proteasome system (UPS) (Sherman and Goldberg, 2001). This degradational process is a highly complex and tightly regulated multistep mechanism, catalyzed by several specific enzymes (Ciechanover and Brundin, 2003). In a first step the protein to be degraded is tagged covalently by a ubiquitin chain to be recognized by the proteasome. This process is catalysed by three enzymatic steps: (1) formation of an ATP-dependent thioester bond between the C-terminus of ubiquitin and a reactive cysteine of the activating enzyme E1; (2) transfer of the activated ubiquitin to a cysteine residue of a ubiquitin-carrier enzyme E2; and (3) attachment of ubiquitin to a target protein through an isopeptide bond between a C-terminal glycine of ubiquitin and a specific ε-amino group of a lysine residue within the

substrate in the presence of an E3 ubiquitin-protein ligase. Target specificity is dictated by the functional interaction between the E2/E3 complex and its substrate (Fig. 9).

Three E3 ubiquitin ligase families have been identified: RING (Really Interesting New Gene), U-box E3s and HECT (homologous to the E6-AP C-terminus) E3s. The first two facilitate conjugation by acting as bridging factors, whereas the latter directly catalyzes the final attachment of ubiquitin to the substrate, by first forming a thiol ester intermediate with ubiquitin. In multiple rounds additional ubiquitin moieties may then be attached to the first ubiquitin, leading to the generation of polyubiquitin chains. The 76 amino acid residue protein ubiquitin contains seven lysine residues that in principle can all engage in the formation of polyubiquitin chains. Typically, ubiquitin linkage via Lys48 (K48) targets a protein for proteasomal degradation (at least four ubiquitin molecules must be attached) (Hershko and Ciechanover, 1998; Hershko et al., 2000), whereas the linkage via K63 is implicated in various regulatory processes, such as endocytosis, autophagy, protein trafficking, DNA-repair or signal transduction (Hicke, 2001; Pickart, 2001; Pickart and Fushman, 2004). Additionally, a substrate can be monoubiquitylated at one or several lysine residues, also leading to the regulation of different cellular processes including endocytosis, membrane trafficking, histone regulation and DNA repair.

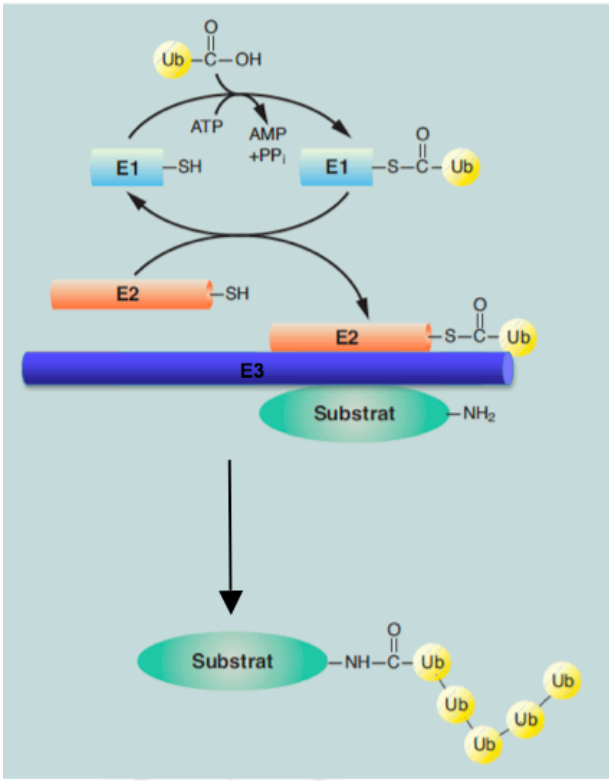


Fig. 9: Ubiquitylation of proteins. 1. Formation of an ATP-dependent thioester bond between the C-terminus of ubiquitin and a reactive cysteine of E1 (ubiquitin-activating enzyme), 2. Transfer of the ubiquitin to a cysteine residue of E2 (ubiquitin-conjugating enzyme), 3. Formation of an isopeptide bond between a C-terminal glycine of ubiquitin and the ε-amino group of a lysine residue within the substrate in the presence of E3 (ubiquitin ligase). Ub: ubiquitin. Modified from (Winklhofer, 2006).

The 26S proteasome is a large multiprotein complex, consisting of two subcomplexes, one barrel-like 20S catalytic core, and two 19S regulatory cap structures binding on each side of the 20S core openings. The 20S core contains four stacked rings creating a central pore. The inner two rings consist of β-subunits comprising of proteolytic activity located at the interior surface of the pore to degrade the entered polypeptides. The outer two rings are built up of α-subunits, which gate the polypeptides for the entry into the pore. The 19S complex recognizes the polyubiquitin chains of the substrates, regulates their unfolding and deubiquitylation, and thereby initiates their degradation. The degradational products are small peptides (4-9 amino acids), which are further hydrolyzed by cellular peptidases to their amino acids (Baumeister et al., 1998). The ubiquitin chains are decomposed by ubiquitin carboxy terminal hydrolases (like UCH-L1) into ubiquitin monomers (Healy et al., 2004).

It is speculated that an impairment of the UPS is implicated in the pathogenesis of PD. In fact, several familiar forms of PD are thought to be associated with the UPS: On the one hand parkin and UCH-L1 are components of the system, which interfere with the processes that normally recognize or process misfolded proteins. On the other hand modified or/and mutated α-synuclein and DJ-1 are targets of the UPS, which induce abnormal and possibly toxic protein conformations, able to disrupt proteasomal function (Chen et al., 2006; Miller et al., 2003; Petrucelli et al., 2002; Shimura et al., 2001; Stefanis et al., 2001; Tanaka et al., 2001). Also in the sporadic form of PD, indication for a role for the UPS in the pathogenesis of the disease is given. A reduced level and activity of a proteasomal subunit was observed in the *SNpc* of PD patients, suggesting that components of the UPS may be sequestered into aggregates (McNaught et al., 2003; McNaught et al., 2002). On the other hand protein aggregates were shown to directly block the UPS (Bence et al., 2001). In addition, experiments with toxin-induced animal models also showed an involvement of the UPS. MPTP infusion in mice was shown to interfere with proteasomal function (Fornai et al., 2005) and an *in vitro* model showed that rotenone impaired the proteasome (Betarbet et al., 2006; Wang et al., 2006). Further, treatment of rats with a proteasomal inhibitor resulted in a progressive loss of dopaminergic neurons, α-synuclein-positive aggregates and development of PD-like symptoms, however subsequent studies failed to reproduce this effect (McNaught and Olanow, 2006; McNaught et al., 2004).

Presumably, the ability of the cell to cope with oxidative stress decreases with aging. Oxidative stress leads to decreased mitochondrial activity, resulting in energy deficiency for the ATP-dependent proteasomal degradation. In addition, the function of the heat shock proteins Hsp70 and Hsp40 is ATP-dependent. Oxidised proteins accumulate, provoking a vicious cycle by increasing ROS production and damage of

the mitochondria. The fact that the capability of the cell to handle misfolded proteins also decreases with age enhances the toxic result (Abou-Sleiman et al., 2006; Sullivan et al., 2004).

2.3.4 Endoplasmic Reticulum (ER) stress

A second reason for increased accumulation of aggregated proteins in PD patient brain could be a dysfunction of the folding machinery. Unfolded proteins are characterized by a high number of hydrophobic surface patches, making them prone to interaction and aggregation with other unfolded proteins in the crowded environment of the cell (Stevens and Argon, 1999). To circumvent this interaction and to ensure correct folding of the proteins, molecular chaperones promote productive protein folding by preferentially interacting with hydrophobic surface patches on unfolded proteins. In the Endoplasmic Reticulum (ER) protein folding is even more complex, as post-translational modifications of nascent polypeptide chains e.g. N-linked glycosylation and formation of disulfide bonds, additionally take place. Besides the capability of the cell to extract properly folded proteins from the ER it is also essential for a functional protein folding machinery that slowly folding or incompletly folding polypeptide chains are also extracted and targeted for proteolytic degradation, a process called ER-associated protein degradation (ERAD) (Ellgaard and Helenius, 2003). A balance between the influx of newly synthezised, completely unfolded polypeptide chains into the ER, and the sum of the effluxes of correctly folded proteins to the Golgi complex and unfolded proteins targeted for degradation, is necessary to ensure homeostasis of protein folding in the ER. Various different insults lead to the disruption of this sensitive homeostasis such as glucose/energy deprivation, ischemia, redox changes, viral infections, perturbations in calcium homeostasis, elevated secretory protein synthesis, altered glycosylation, and mutations that impair correct protein folding (Kaufman et al., 2002). Alteration in ER homeostasis consequently disrupts protein folding and leads to the accumulation of unfolded proteins and protein aggregates, resulting in ER stress. As a consequence, the cell has evolved various protective strategies, collectively termed the unfolded protein response (UPR). This coordinated adaptive response regulates the expression of numerous genes, which lead to a reduction in the protein load into the ER and up-regulates the protein folding and degradation pathways of the cell. However, if these mechanisms fail to restore normal ER homeostasis, signalling switches from pro-survival to pro-apoptotic.

The UPR is mediated through three ER transmembrane receptors: double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), activating

transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). In unstressed cells, all three ER stress receptors remain inactive through their binding to the ER chaperone BiP (binding immunoglobulin protein; also named GRP78 [glucose related protein 78]). Upon accumulation of unfolded proteins in the ER, BiP dissociates from the three receptors resulting in their activation. The activation occurs sequentially, with PERK being the first, rapidly followed by ATF6, with IRE1 being activated last.

When BiP binding to PERK is disrupted, PERK homodimerizes and phosphorylates itself on its threonine kinase domain, thereby generating an active kinase. Activated PERK phosphorylates the eukaryotic initiation factor 2α (eIF2α) at Ser51, which inhibits the guanine exchange factor eIF2B that normally recycles eIF2α to its active, GTP-bound from. Lower levels of active eIF2α result in lower levels of translation initiation, globally reducing new protein synthesis, thereby decreasing the load of nascent proteins arriving at the ER. However, this attenuation of translation is not absolute; genes carrying certain regulatory sequences in their 5'-untranslated regions are only translated under these conditions, among them activating transcription factor 4 (ATF4) (Lu et al., 2004). ATF4 translocates to the nucleus and induces the transcription of genes regulating amino acid metabolism, redox reactions, stress response and protein secretion (Harding et al., 2003). However, pro-apoptotic genes are also induced, such as the transcription factor C/EBP homologous protein (CHOP). Notably, also several other signalling pathways unrelated to ER stress are triggered by amino-acid starvation, double-stranded RNA accumulation or haem depletion converge on eIF2α phosphorylation and activate a common set of target genes.

Dissociation of BiP from ATF6 leads to ATF6's translocation to the Golgi apparatus, where it is proteolytically cleaved by site-1 and site-2 proteases, resulting in an active transcription factor (Ye et al., 2000). ATF6 mainly regulates the expression of ER chaperones and XBP-1 (x-box binding protein-1).

After the dissociation of BiP, IRE1, like PERK, homodimerizes and autophosphorylates itself. Activated IRE1 cleaves the mRNA of XBP-1 with its endoribonuclease domain to remove a 26 nucleotide intron, generating a translational frameshift. The frameshift in the XBP-1 mRNA leads to the translation of an active stable transcription factor. XBP-1 has several targets, including ER chaperones, proteins involved in protein degradation and $p58^{IPK}$, which provides a negative feed back loop to PERK (Fig. 10).

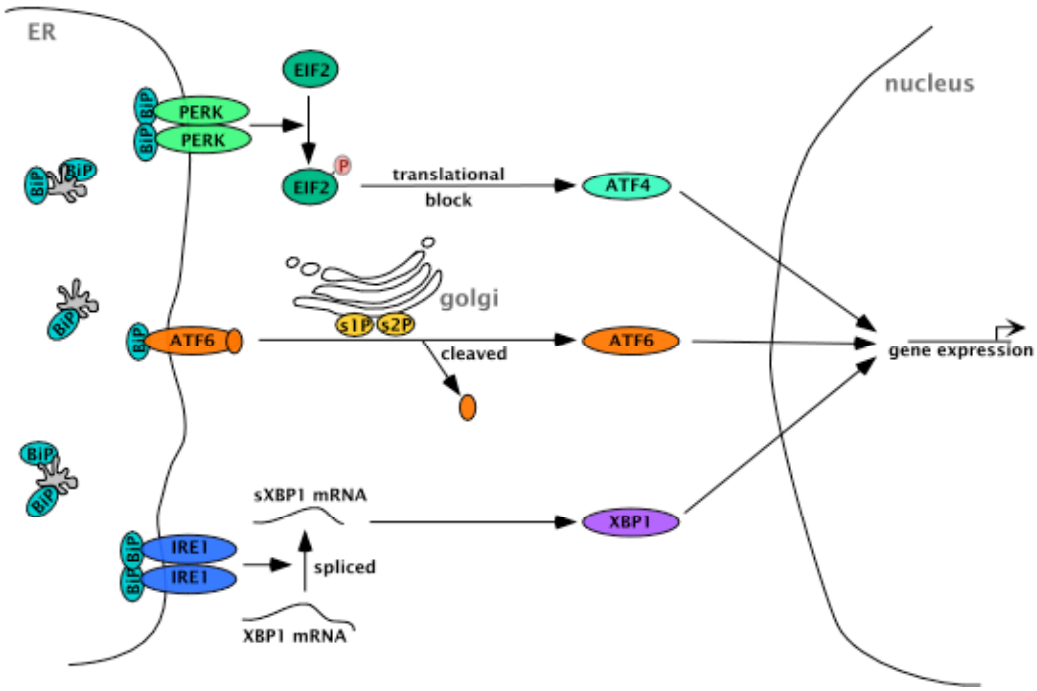


Fig. 10: The unfolded protein response. Upon aggregation of unfolded proteins, BiP (binding immunoglobulin protein) translocates from the three ER stress receptors, PERK (PKR-like ER kinase), ATF6 (activating transcription factor 6), IRE1 (inositol requiring enzyme 1) allowing their activation. Upon translocation of BiP PERK dimerizes and autophosphorylates itself, leading to its activation. Activated PERK phosphorylats eIF2α (eucaryotic initiation factor 2), resulting in a general protein synthesis block. However, ATF4 (activating transcription factor 4) is only translated in case of phosphorylated eIF2α. Translated ATF4 transclocates to the nucleus and induces the transcription of genes for example to restore ER homeostasis. After its translocation from the ER ATF6 is activated by limited proteolysis by site1 or site2 proteases (s1P, s2P) in the Golgi apperatus. Cleaved ATF6 translocates into the nucleus and drives transcription of target genes. Upon dimerization and autophosphorylation, resulting in the activation of IRE1, IRE1 splices the XBP1 mRNA (sXBP1; x-box binding protein 1), which leads to the translation of the active transcritpition factor XBP1. XBP1 transclocates to the nucleus and controls the transcription of target genes.

In case too excessive and/or prolonged ER stress occurs, pro-apoptotic pathways are activated. Among these downstream pro-apoptotic proteins are JNK and CHOP. Both JNK, activated by the IRE1-TRAF2-ASK1 branch of the UPR (Nishitoh et al., 2002; Urano et al., 2002; Urano et al., 2000) and CHOP, transcriptionally regulated by ATF4, are able to suppress the anti-apoptotic effect of Bcl-2. CHOP blocks the expression of Bcl-2 (Matsumoto et al., 1996; McCullough et al., 2001), whereas JNK phosphorylates it. Additionally, JNK can phosphorylate the pro-apoptotic protein Bim, leading to its release from its inhibitory association with the cytoskeleton and to its activation (Lei and Davis, 2003). Altogether, these alterations lead to the activation of Bax and Bak, resulting in caspase activation and ultimately to cell death. Furthermore, caspases (caspase-12) were reported to be directly activated at the ER membrane through interaction with IRE1 and TRAF2 (Nakagawa et al., 2000; Rao et al., 2002; Szegezdi et al., 2003; Yoneda et al., 2001). However, caspase-12 is only expressed in

rodents, the human homologue carries several functional mutations. Caspase 4 has been proposed to fulfil the function of caspase-12 in humans, but this is still under debate (Fig. 11).

The potential clinical relevance of ER stress in PD was supported by the finding that several ER stress markers were found to be up-regulated in brain of sporadic PD patients by a *post mortem* analysis, such as phospho-PERK, phospho-eIF2α and caspase-4 (Hoozemans et al., 2007; Moran et al., 2007). In addition, neurotoxins used as model compounds to mimic the disease process, such as MPTP, 6-OHDA and rotenone were shown to trigger ER stress both in cell culture and *in vivo* models (Conn et al., 2004; Ghribi et al., 2003; Holtz and O'Malley, 2003; Holtz et al., 2006; Holtz et al., 2005; Ryu et al., 2005; Ryu et al., 2002; Yamamuro et al., 2006). Gene profiling revealed an up-regulation of ER chaperones and other components of the UPR such as CHOP, in addition to the phosphorylation of IRE1 and PERK (Ryu et al., 2002). One possible reason for the induction of ER stress by these mitochondrial toxins could be their effect on mitochondrial respiration, causing damaged oxidized proteins to accumulate in the cell, resulting in ER stress. Alternatively, oxidative stress can directly compromise proteasomal components or the folding machinery of the ER (Friedlander et al., 2000; Reinheckel et al., 2000). Also for the familial form of PD ER stress is implicated to play a role in the pathogenesis of the disease. Parkin as a proposed E3 ligase is suggested to ubiquitylate proteins for degradation. In case of parkin dysfunction some substrates accumulate and could cause neurotoxicity. One substrate for parkin has been described as the Pael-receptor (parkin-associated endothelin receptor-like receptor; Pael-R). The folding of the multipass G protein-coupled transmembrane protein proves a massive challenge for the cell and when overexpressed in cell culture Pael-R was reported to become unfolded and insoluble. Accumulation of Pael-R was shown to induce ER stress. Parkin suppresses Pael-R induced toxicity by ubiquitylation and promoting the degradation of the protein (Imai et al., 2001). In a Drosophila model from the same group parkin was shown to suppress Pael-R-induced dopaminergic neuronal loss (Yang et al., 2003). Experiments with overexpressed Pael-R in parkin null mice revealed conflicting data about the induction of ER stress in these mice, however an increased death of dopaminergic neurons of *SNpc* was observed (Dusonchet et al., 2009; Kitao et al., 2007; Wang et al., 2008). Furthermore, ER stress induces up-regulation of parkin mRNA per se, and cells overexpressing parkin, but not pathogenic parkin mutants, are particularly resistant to unfolded protein-induced stress (Imai et al., 2000). In addition, also DJ-1 was shown to play a role in the protection from ER stress. DJ-1-deficient cells were more vulnerable to ER stress-induced cell death than wild-type cells (Canet-Aviles et al., 2004). In

contrast, α-synuclein was reported to cause ER stress. Several studies reported that overexpression of α-synuclein mutants, but also α-synuclein wild-type induces ER stress (Cooper et al., 2006; Smith et al., 2005a; Sugeno et al., 2008). Interestingly, parkin was shown to suppress the α-synuclein-induced toxicity (Yang et al., 2003). In addition, α-synuclein was reported to block ER to Golgi vesicular trafficking in different model systems, leading to ER stress (Cooper et al., 2006).

Conclusively, the involvement of ER stress in the familial forms and sporadic forms of PD, coupled with the evidence from the neurotoxin models raises the possibility of wide spread involvement of ER stress-mediated cell death in the pathogenesis of PD.

2.3.5 Interaction of ER and mitochondria

Interestingly, the ER and mitochondria physically and functionally interact with each other to allow the exchange of metabolites and determine the efficiency of Ca^{2+} signalling and regulation of apoptotic pathways (Hayashi et al., 2009; Pizzo and Pozzan, 2007). Ca^{2+} seems to be the most important key molecule in this interaction. The ER is the principal internal store of calcium ions. The binding of IP_3 (inositol 1,4,5-trisphosphate) to the IP_3 receptor (IP_3R) or the binding of cyclic adenosine diphosphate ribose (cADPr) to the ryanodine receptor (RyR) stimulates a rapid efflux of Ca^{2+} from the ER into the cytoplasm, where the Ca^{2+} concentration is significantly lower (about 100 μM in the ER and about 100 nM in the cytosol). However, the speed of Ca^{2+} diffusion is slow, leading to the formation of high concentrated Ca^{2+} patches, so called microdomains. Strategic location of the mitochondria close to the opening Ca^{2+} channels is the key to rapid and regulated Ca^{2+} uptake by the mitochondria (Rizzuto and Pozzan, 2006). As already mentioned above, the Ca^{2+} uptake into mitochondria triggers several cellular pathways, including cell death pathways. In the context of apoptosis it should be mentioned that several proteins of the Bcl-2 family with pro-and anti-apoptotic features are located on the ER membrane, influencing among other things the Ca^{2+} concentration in the ER, again indicating that the two organelles act in concert in this key process (Kim et al., 2008a; Oakes et al., 2006) (Fig. 11)

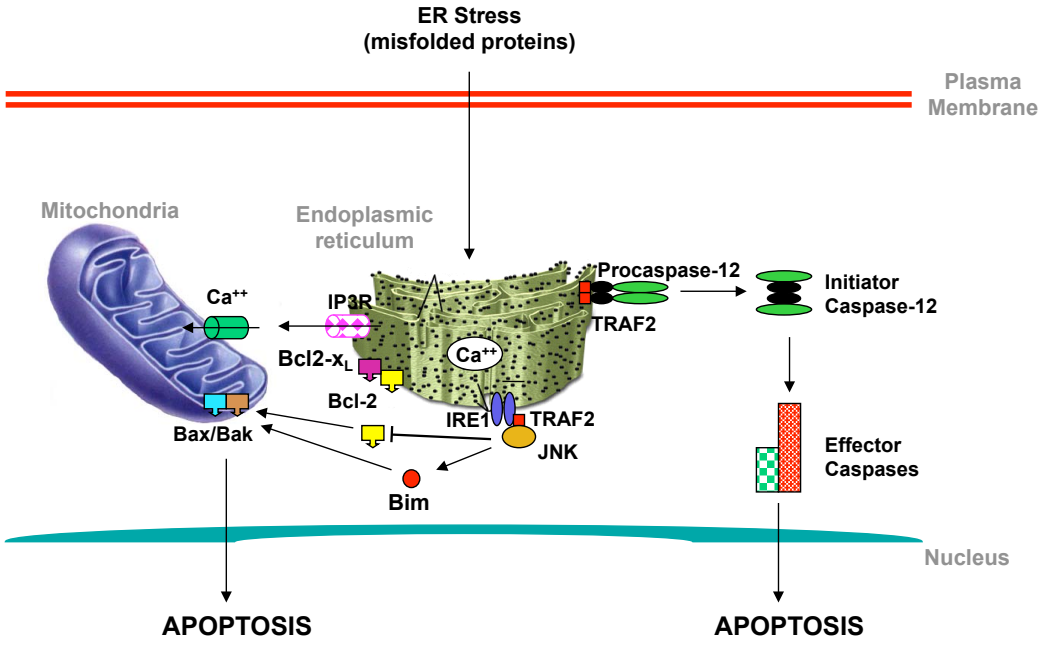


Fig. 11: ER stress triggers apoptosis. Upon prolonged ER stress caspase-12 is activated through interaction with IRE1 and TRAF2. Activated caspase-12 cleaves effector pro-caspases to active caspases. The activated IRE1/TRAF2 complex recruits ASK1 and JNK. Activated JNK phosphorylates Bcl-2, resulting in its inactivation, and Bim, leading to its release from its inhibitory association with the cytoskeleton and to its activation. Upon stimulation of the UPR CHOP is expressed, leading to the reduction of Bcl-2 expression (not illustrated here). Altogether, suppression of Bcl-2 and phosphorylation of Bim lead to the activation of the pro-apoptotic proteins Bax and Bak in the mitochondrial membrane and to the induction of the mitochondrial apoptotic pathway. ER and mitochondria are in close contact. Ca^{2+} signalling from the ER to the mitochondria through IP_3R (IP_3 receptor) or RyR (ryanodine receptor; not illustrated here) can induce mitochondrial toxicity events, including mitochondrial membrane depolarization, increased production of free oxygen radicals and release of cytochrom c, which triggers the activation of caspases. Modified from (Gupta et al., 2006).

2.3.6 Pathways implicated in neuronal survival and cell death

2.3.6.1 NF-κB pathway

The nuclear factor kappa enhancer binding protein (NF-κB) is a key transcription factor, which controls various biological processes, including immunity, differentiation and apoptosis (Baeuerle and Henkel, 1994; Barnes, 1997; Ghosh and Karin, 2002). It regulates the expression of a broad spectrum of target genes (Pahl, 1999), which is achieved by the existence of five diverse NF-κB family members: RelA (p65), RelB, c-Rel, p50 and p52 (p50 and p52 are derived from the larger precursors p105 and p100, respectively, through proteolytic processing). For functional diversity and specificity, these five NF-κB proteins form hetero- and homodimers and interact with different co-factors in a cell type- and/or promoter-specific manner (Hayden and Ghosh, 2004). All NF-κB proteins contain a highly conserved REL-homology domain that is responsible for DNA binding, dimerization, nuclear translocation and interaction

with the IκB (inhibitor of NF-κB) protein. The IκB proteins, including IκBα, β and ε, bind to NF-κB and block its nuclear import by sequestering it in the cytoplasma and thereby inhibit its transcriptional activity (Baldwin, 1996; Ghosh et al., 1998). Various stimuli can induce NF-κB translocation through distinct upstream pathways that converge at the IκB-kinase-complex (IKK), composed of two catalytic subunits IKKα and IKKβ, and a regulatory subunit NEMO (NF-κB essential modifier, also known as IKKγ). The most predominant NF-κB signalling pathway is the canonical pathway, stimulated by treatment with cytokines, like the tumour necrosis factor-α (TNF-α) or interleukin 1 (IL-1) as well as with bacterial antigens, like lipopolysaccharide (LPS) (Hayden and Ghosh, 2004). Upon activation of the IKK-complex, IKKβ mediates the phoshporylation of IκBα at two N-terminal serine residues (Ser32 and Ser36), which targets the inhibitor for ubiquitylation and subsequent proteasomal degradation. The NF-κB molecules are thereby released from their inhibitors and translocate due to their now uncovered nuclear signal sequence into the nucleus, where they are further modified (phosphorylation, acetylation) for transcriptional regulation of target genes (Delhase et al., 1999; Karin, 1999a; Karin, 1999b). Among these target genes are genes coding for c-IAPs (cellular inhibitors of apoptosis), anti-oxidative enzymes such as MnSOD (manganese superoxide dismutase) and proteins of the Bcl-2 family (Karin and Lin, 2002; Mattson et al., 2000). The regulatory subunit IKKγ/NEMO is crucial for the activation of the IKK-complex. At the beginning of the canonical pathway, activating stimuli induce the regulatory ubiquitylation (via K63) of several signal molecules, like TRAF2 (TNFR-associated factor 2) and TRAF6 (TNFR-associated factor 6). These activated upstream regulators in turn ubiquitylate IKKγ/NEMO (also via K63). This ubiquitylation mediates the interaction of the IKK-complexes with kinases, resulting in the phosphorylation of IKKβ, and its activation as IκB kinase (Chen, 2005; Haglund and Dikic, 2005; Krappmann and Scheidereit, 2005; Ravid and Hochstrasser, 2004) (Fig. 12).

The transcriptional activity of NF-κB is regulated by a negative feed back loop. NF-κB induces the expression of IκBα, which binds to deacetylated RelA and mediates nuclear export of NF-κB. Thus, the transcriptional activity is stopped and the pool of inactive NF-κB complexes in the cytosol restored (Arenzana-Seisdedos et al., 1995; Arenzana-Seisdedos et al., 1997).

NF-κB is present throughout the whole nervous system, and accumulating evidence indicates that it plays a central role in neuronal integrity and synaptic plasticity (Karin and Lin, 2002). Recent cell culture and animal models demonstrated that NF-κB is able to protect neuronal cells from apoptosis induced by oxidative stress, excitotoxicity or glucose deprivation (Kaltschmidt et al., 2005; Mattson et al., 2000).

These observations suggest a possible involvement of NF-κB in neurodegenerative diseases, in particular PD. And indeed, increased NF-κB activation was found in mesencephalic neurons from sporadic PD patients (Hunot S 1997). In addition, parkin along with proteins associated with the NF-κB pathway was observed to be up-regulated in the lateral *SN* of PD patients (Moran et al., 2007).

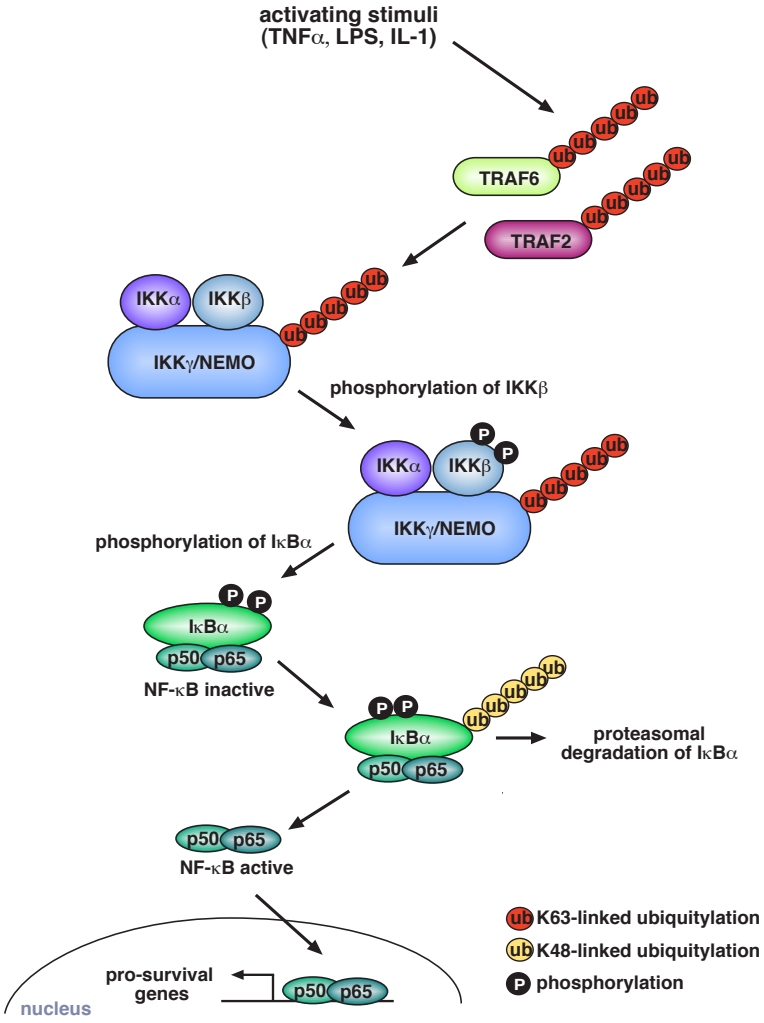


Fig. 12: Canonical NF-κB pathway. Activating stimuli like TNFα, IL-1 or LPS induce the regulatory ubiquitylation via K63 of several signal molecules like TRAF2 or TRAF6, which in turn ubiquitylate IKKγ/NEMO (also via K63). The ubiquitylation of the IKK-complex (consisting of the subunits IKKα and IKKβ and the regulatory subunit IKKγ/NEMO) mediates the interaction with kinases, resulting in the phosphorylation of IKKβ. The activated IKK-complex in turn phosphorylates IκB, leading to its ubiquitylation and subsequent proteasomal degradation. The NF-κB molecules are thereby released from their inhibitor and translocate into the nucleus, where they induce the transcription of the appropriate target genes.

2.3.6.2 JNK pathway

The NF-κB pathway has been implicated in crosstalks to other signalling pathways, and in particular to the JNK (c-Jun N-terminal kinase) pathway at various levels (Papa et al., 2004). JNK, or stress-activated protein kinase (SAPK), is an important member of the mitogen-acitvated protein kinase (MAPK) superfamily, which also includes ERK and p38 MAP kinases. The JNK family includes three isoforms: JNK1, JNK2 and JNK3. JNK1 and 2 are ubiquitously expressed, while JNK3 is primarily found in the brain, heart and testes. The JNK pathway is activated by various environmental stimuli, including UV radiation, heat shock, oxidative stress, ER stress, cell death ligand Fas-L and proinflammatory cytokines, like TNF-α or IL-1. Similar to other members of the MAPK family, JNK activation is mediated by the MAPK cascade comprising MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAPK. Activated JNK phosphorylates and activates besides the transcription factor AP1 (mainly c-Jun, JunB, and ATF-2) other transcription factors, such as p53 or c-myc (Davis, 2000; Minden and Karin, 1997) as well as other non-transcription factors, such as Bcl-2 family members. The functional specificity and diversity is achieved by phosphorylation and functional modification of these molecular targets in stimuli- and cell type-dependent manners.

A large number of studies implicated a role for the JNK pathway in the mediation of neuronal cell death in neurodegenerative diseases (Bogoyevitch et al., 2004; Peng and Andersen, 2003; Waetzig and Herdegen, 2004) and in particular in PD, as a very potent stimulus for JNK activation is oxidative stress. Thus, *post mortem* brain samples from PD patients showed an increased JNK activity (Hunot et al., 2004; Teismann et al., 2003). In mouse models JNK was activated in nigrostriatal dopaminergic neurons upon treatment with the neurotoxins paraquat and MPTP (Hunot et al., 2004; Peng et al., 2004; Saporito et al., 2000), and it was suggested that the JNK pathway is an obligatory signalling pathway utilized by these neurotoxins to selectively induce apoptosis (Peng et al., 2004). Subsequently, the use of a JNK inhibitor (CEP1347), which inhibits MLK (mixed lineage kinase; a MAPKKK) protected MPTP-treated mice against dopaminergic cell death (Maroney et al., 2001; Saporito et al., 1999). Notably, because of the promising effects in mice and primates, CEP1347 has recently been tested in a clinical trial, which unfortunately failed to detect favourable effects in PD patients. Another JNK inhibitor, which directly targets JNK (SP600125) was shown to protect dopaminergic neurons in MPTP models (Wang et al., 2004b; Xia et al., 2001). In *Drosophila* the loss of parkin was determined to result in an up-regulation of JNK, and a dominant negative form of JNK was able to rescue the phenotype of impaired dopaminergic neurons. In the same study the authors showed

that parkin overexpression inhibited JNK activation in human neuroblastoma cells (Cha et al., 2005), indicating a role for parkin as an upstream physiological inhibitor for the pro-apoptotic JNK pathway.

2.4 PARKIN-ASSOCIATED PARKINSON'S DISEASE

2.4.1 Clinical and neuropathological characteristics of parkin-associated PD

Mutations in the parkin gene account for the majority of known autosomal recessive PD cases (Kitada et al., 1998). The patients have either homozygous or compound heterozygous (different mutations in each allele) mutations of parkin. It is currently under discussion, whether heterozygous carriers bear an increased risk of developing PD and exhibit an earlier age of onset (Farrer et al., 2001; Foroud et al., 2003; Khan et al., 2003), however for some cases it is also possible that a complementary mutation at the other allele, perhaps within the promoter or an intron, has been overlooked.

The clinical phenotype is characterized by early onset parkinsonism with the vast majority of patients manifesting the disease before the age of 40 (Klein et al., 2000) and a good and prolonged response to levodopa (Lohmann E 2003). Relatively little is known about the neuropathology of molecularly confirmed parkin associated cases. A severe and selective degeneration of dopaminergic neurons and gliosis in the *SNpc*, and to a somewhat lesser degree in the locus coeruleus has been reported (Mori et al., 1998; Takahashi et al., 1994; van de Warrenburg et al., 2001). Initially, it was described that patients with parkin mutation do not harbour Lewy body pathology. In addition, in sporadic PD cases parkin was found to localize to Lewy bodies, giving rise to the hypothesis that functional active parkin is essential for Lewy body formation (Schlossmacher et al., 2002). However, several parkin-linked cases with Lewy body formation were discovered recently (Farrer et al., 2001; Pramstaller et al., 2005; Rawal et al., 2003), suggesting that the neuropathological changes are influenced by additional genetic or environmental factors and possibly depend upon the age of the patient and the type of parkin mutation. Interestingly, a lack of Lewy bodies was also reported in LRRK2 patients (Gaig et al., 2007), raising the question of whether Lewy bodies are necessary for the definition of PD or not.

2.4.2 Molecular genetics and cell biology of parkin

Parkin is with 1.3 Mb of genomic DNA one of the largest genes in the genome. The gene has 12 exons with a super-expanded intronic structure encoding a 465 amino

acid protein with an approximate molecular mass of 52 kDa (Kitada et al., 1998). The parkin locus on chromosome 6q25.2-q27 lies adjacent to the 6q telomere and within *FRA6E*, the third most common fragile site in tumor tissue (Denison et al., 2003), however a possible additional role of parkin in cancer is not yet determined. The core promoter of parkin is 204 bp long (Asakawa et al., 2001; West et al., 2001). Parkin shares it with the parkin co-regulated gene (PACRG), which is transcribed on the opposite strand in an opposite orientation (Fig. 13).

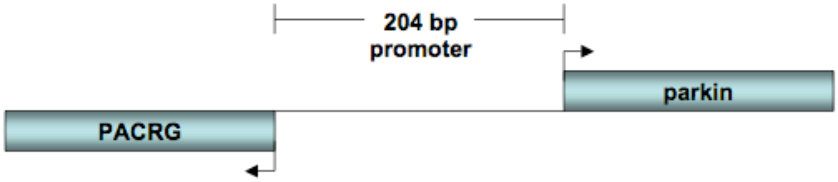


Fig. 13: Schematic model of the parkin and PACRG promoter. Parkin and PACRG share the same promoter, but are transcribed on opposite strands in opposite direction. The promoter region between the two genes is 204 bp long.

Given their physical proximity and common transcription factor binding sites, a co-regulation of both genes to some degree may occur (West et al., 2003a). For parkin a possible enhancing element between -890 and -1500 bp and between -2280 and -4500 bp was observed. A silencing element may exist between -1500 and -2280 bp (West et al., 2001). Several binding sites of transcription factors were described for the core promoter such as SP-1, AP-4, AP-1/CREB and NF-1. In addition, the promoter contains several CpG islands but no TATA box, which would be typical for a housekeeping gene (Asakawa et al., 2001; West et al., 2001). To date, no single mutation or small deletion located in the parkin promoter has been found. Only a complete deletion of the parkin/PACRG promoter resulted to early-onset Parkinsonism (Lesage et al., 2007). In addition, a single nucleotide polymorphismen (-258T/G) was reported to influence the transcriptional regulation of parkin and increases the risk factor in elderly PD patients (Sutherland et al., 2007; Tan et al., 2005; West et al., 2002).

The expression of parkin is ubiquitous with high expression levels in the brain, heart, skeletal muscle and testis. In the brain the expression levels vary between different regions, curiously in the *SNpc* parkin is only weakly expressed (Kitada et al., 1998). In the cell, parkin is mainly localized to the cytoplasma (Shimura et al., 1999), but also an association with the trans-Golgi-network (Kubo et al., 2001), actin- and tubulin-filaments (Ren et al., 2003), synaptic vesicles (Fallon et al., 2002; Kubo et al.,

2001) as well as mitochondria was described (Darios et al., 2003; Kuroda et al., 2006; Stichel et al., 2000).

Parkin consists of several structural domains: An ubiquitin-like domain (UBL) at the N-terminus and a RING box close to the C-terminus. The RING box consists of two RING (Really Interesting Gene) finger motifs, which are separated by a cysteine-rich in-between RING domain (IBR) (Fig. 14). The N-terminal UBL domain comprises the amino acids 1-76 and is to 62% homologues to human ubiquitin (Kitada et al., 1998). It is suggested that the N-terminus plays a functional role in the control of parkin expression (Finney et al., 2003), substrate recognition (Shimura et al., 2000) and interaction with the Rpn10 subunit (protein of the 19S complex) of the 26S proteasome (Sakata et al., 2003). The C-terminal RING box implicates an E3 ligase activity for parkin, which mediates the interaction between substrate and E2 ubiquitin conjugating enzyme, leading to the covalent attachment of ubiquitin to lysine residues of specific substrates (Joazeiro and Weissman, 2000).

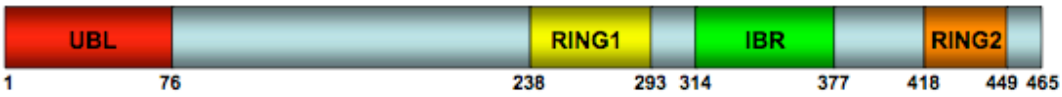


Fig. 14: Modular structure of the parkin protein. Parkin consists of 465 amino acids and has a molecular mass of 52 kDa. It comprises different structural domains: a N-terminal UBL domain and a C-terminal RING-box domain, which consists of two RING-finger motifs, separated by an IBR domain. UBL: ubiquitin-like; RING: really interesting new gene; IBR: in-between RING.

2.4.3 Parkin mutations

To date more than 100 parkin mutations have been described in PD cases of different ethnic groups worldwide. All types of mutations were identified, including missense mutations leading to amino acid exchanges, nonsense mutations resulting in premature termination of translation and exonic rearrangements (deletions, duplications and triplications) (Lucking et al., 2000). The mutations are distributed over almost the whole coding region of parkin, but hot spots are in particular the functional domains, suggesting the importance of these regions for function (Fig. 15). The localization of mutation and the identification and characterization of amino acids essential for the function of parkin can provide insights into the role of parkin for the pathogenesis of Parkinsonism. Genetic and biochemical studies demonstrated that pathogenic parkin mutations induce a loss of function. Mutations may either impair the catalytical E3 activity of parkin or its interaction with substrates, with E2 ubiquitin enzymes or with other proteins of functional complexes. Interaction analyzes revealed

that mutations in the RING box are able to interfere with the binding of E2-enzymes and/or substrates (Chung et al., 2001; Hershko et al., 2000; Imai et al., 2000; Shimura et al., 2001; Zhang et al., 2000). However, increasing evidence indicates that the majority of parkin mutations are characterized by misfolding and aggregation, leading to non-functional parkin (Cookson et al., 2003; Gu et al., 2003; Henn et al., 2005; Schlehe et al., 2008; Sriram et al., 2005; Wang et al., 2005a; Wang et al., 2005b; Winklhofer et al., 2003).

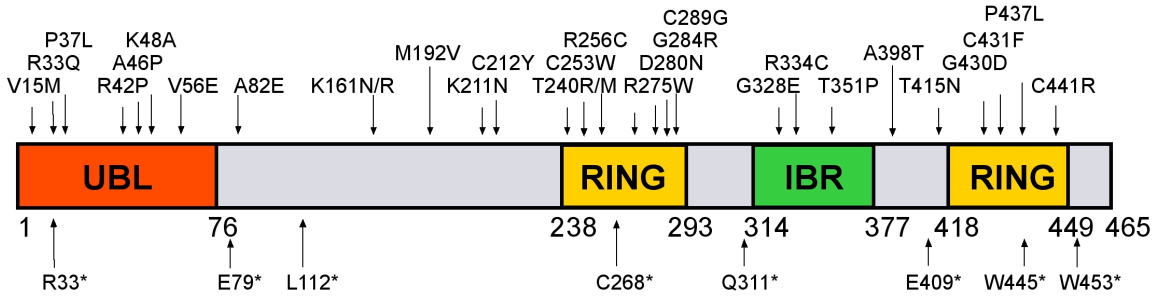


Fig. 15: Missense and nonsense mutations in the parkin gene. Schematic representation of parkin with its functional domains. Localization of pathogenic missense and nonsense mutations in the parkin gene are indicated by arrows. Stop mutations are marked with an asterisk. UBL: ubiquitin-like; RING: really interesting new gene; IBR: in-between RINGs. (Kindly provided by Iris Henn.)

2.4.4 Putative parkin substrates

A variety of putative parkin substrates have been reported by different approaches, however these proteins are highly diverse and so far have not contributed to understanding the physiological role of parkin. Due to its RING box parkin is suggested to act as an E3 ubiquitin ligase. Recent evidence indicated that ubiquitin, in addition to its traditional role in proteasomal degradation can also have a regulatory function (Winklhofer, 2007).

2.4.4.1 Parkin-mediated ubiquitylation for proteasomal degradation

Consistent with its assumed function, parkin was shown to interact with several E2 ubiquitin conjugating enzymes, including UbcH7 and UbcH8 (Imai et al., 2000; Shimura et al., 2000; Shimura et al., 2001; Zhang et al., 2000) and the ER-associated E2 enzymes Ubc6 and Ubc7 (Imai et al., 2001). It was postulated that the E2/E3 enzyme complex catalyzes the ubiquitylation via K48, and thus targets the substrates for proteasomal degradation.

The association of parkin to the proteasome system and the knowledge that pathogenic mutation lead to a loss of parkin function gave rise to the hypothesis that an accumulation of parkin substrates leads to toxicity for the cell, resulting in dopaminergic

neuronal cell death. Using Yeast-two-hybrid and pull-down approaches a long list of putative parkin substrates were identified, however these substrates show a wide functional diversity and do not fit into a common pathway: Proteins with a vesicular and synaptic function such as CDCrel-1a (cell division and control-related protein) (Zhang et al., 2000), CDCrel-2a (Choi et al., 2003), synaptotagmin XI (Huynh et al., 2003), O-glycosylated α-synuclein (αSp22) (Shimura et al., 2001), the α-synuclein-interacting protein synphillin-1 (Chung et al., 2001) and the dopamine transporter (DAT) (Jiang et al., 2004), control proteins of the cell cycle such as cyclin E (Staropoli et al., 2003), proteins of the protein synthesis, for example aminoacyl-tRNA synthetase-subunit p38/JTV-1 (Corti et al., 2003; Ko et al., 2005), transcription factors such as FBP1 (far upstream sequence element-binding protein 1) (Ko et al., 2006), nuclear export proteins like RanBP2 (Um et al., 2006), proteins involved in cell death pathways, for example PDCD2-1 (programmed cell death 2 isoform 1) (Fukae et al., 2009), and signal transduction proteins like Pael-R (parkin associated endothelin-like receptor) (Imai et al., 2001).

The relevance and authenticity of most of the mentioned substrates has still not been proven consistently. For some (αSp22, Pael-R, cyclin E, CDCrel-1, CDCrel2a, FBP1 and p38/JTV-1, PDCD2-1) a possible accumulation of their non-ubiquitylated forms could be shown in brain tissue from parkin-linked PD cases. Only for p38/JTV-1 and FBP1 also a mild accumulation in the brain of parkin-deficient mice and in sporadic PD cases could be observed (Ko et al., 2006; Ko et al., 2005), increasing the evidence for authentic substrates. However, the pathophysiological relevance for these findings remains unclear.

2.4.4.2 Parkin-mediated regulatory ubiquitylation

Recent research has also revealed a proteasome-independent ubiquitylation function for parkin. An *in vitro* approach showed the interaction of parkin with the heterodimeric E2-ubiquitin-conjugating enzyme Ubc13/Uev1a (Doss-Pepe et al., 2005; Matsuda et al., 2006). This enzyme was reported to catalyze ubiquitylation via K63 and not via the conventional K48 (McKenna et al., 2001). Furthermore, a direct K63 linked ubiquitylation by parkin was observed for synphilin-1, besides the already known K48 linkage (Lim et al., 2005). Fallon and colleagues showed that parkin is able to mono-ubiquitiylate PICK1, a scaffold protein that regulates the activity of acid sensing ion channels (ASIC), which contribute to excitotoxicity in neurons (Fallon et al., 2002). The same group also reported a regulatory mono-ubiquitylation of Eps-15, resulting in the delay of EGF receptor internalization and degradation, and the promotion of the PI3K/Akt signalling (Fallon et al., 2006). In addition, parkin can also mediate the

multiple mono-ubiquitylation of several lysine residues of p38/JTV-1 and HSP70 (Hampe et al., 2006; Moore et al., 2005a), and in vitro ubiquitylation experiments revealed the capability of parkin to mediate its own multiple mono-ubiquitylation (Matsuda et al., 2006).

Conclusively, it seems that under experimental conditions parkin can catalyze all modes of ubiquitylation (K48, K63 as well as multiple mono-ubiquitylation), however, the physiological role of parkin-mediated regulatory ubiquitylation is still largely unknown. In summary, it could be speculated that either an accumulation of toxic substrates and/or the loss of the regulatory E3 function of parkin could be the reason for dopaminergic cell death.

2.4.4.3 Other parkin interacting proteins

In addition to the E2 enzymes and the putative substrates of parkin, which have already been mentioned, several other parkin-interacting proteins have been identified. For example, parkin has been proposed to be a functional component of a larger ligase complex, the Skp1-Cullin-F-box (SCF) complex (Staropoli et al., 2003). Further, it was reported to be in a complex together with the chaperones CHIP (Carboxyl terminus of the Hsc70-interacting protein) or Hsp70 (Imai et al., 2001). The interaction between the scaffold protein CASK (Ca^{2+}-calmodulin-dependent serine protein kinase) suggests that parkin is part of a complex, which co-localizes with postsynaptic membranes and lipid rafts in the brain (Fallon et al., 2002). In addition, further interactions of parkin with following proteins were described: proteins of the cytoskeleton like α/β-tubulin (Ren et al., 2003), actin filaments (Huynh et al., 2000), γ-tubulin (Zhao et al., 2003), and HDAC6 (histone deacetylase 6) (Jiang et al., 2008), proteasomal proteins such as Rpn10 α4 (Dachsel et al., 2005; Sakata et al., 2003), other PD-associated genes like LRRK2 (Smith et al., 2005b), DJ-mutants (Moore et al., 2005b), PINK1 (Moore, 2006; Shiba et al., 2009; Um et al., 2009), and PACRG (Imai et al., 2003).

Furthermore several proteins were reported to influence the E3 ligase activity of parkin. For example PINK1 was proposed to control mitochondrial localization of parkin through direct phosphorylation (Kim et al., 2008b), also casein kinase-1 and protein kinase A and C as well as cyclin-dependent kinase 5 (Cdk5) were reported to phosphorylate parkin and thereby reduce parkin E3 activity (Avraham et al., 2007; Yamamoto et al., 2005). HtrA2/omi was described to inactivate parkin function by cleaving parkin (Park et al., 2009a), the co-chaperone BAG5 (Bcl-2 associated anthogene 5) (Kalia et al., 2004) and 14-3-3η, a chaperone-like protein (Sato et al., 2006b) were shown to interact with parkin, both leading to the down-regulation of its E3 activity.

2.4.5 Parkin-deficient animal models

Parkin is highly conserved during evolution. It exists not only in vertebrates such as human, mouse and rat, but also in invertebrates, for example *Caenorhabditis elegans*, *Drosophila melanogaster* and *Danio rerio* (zebra fish). A comparison of the amino acid sequence of parkin in vertebrates and invertebrates reveals a high homology between the different species (Haywood and Staveley, 2004) e.g. mouse or rat parkin orthologues show a 82% or 83% homology to human parkin, respectively. Interestingly, the functional domains are especially highly conserved.

Parkin knockout mice were generated by deletion of several exons in the murine parkin gene: exon 2, corresponding to the UBL domain (Perez and Palmiter, 2005; Sato et al., 2006a), exon 3, the most common deletion of parkin-linked PD (Goldberg et al., 2003; Itier et al., 2003; Palacino et al., 2004) or exon 7, corresponding to the RING1 of the RBR domain (von Coelln et al., 2004). All deletions lead to the complete loss of parkin protein.

As with other knockout mouse models of PD-associated genes, none of the published parkin knockout mice showed any significant pathological phenotype, in particular the loss of dopaminergic neurons or nigrostriatal degeneration, which is a key pathological feature of PD, was not apparent. The strains carrying the exon 2 deletion of parkin have been described to have no phenotype at all (Perez and Palmiter, 2005). The other strains show various, but not very pronounced phenotypes. Mild alterations of dopaminergic neurotransmission, and of the DA metabolism were observed in addition to subtle deficits in behaviour. The behavioural phenotype, such as reduced explorative behaviour, indicates a disturbance of the nigrostriatal pathway, but neuropathologically a loss of dopaminergic neurons or nigrostriatal degeneration was not detected (Fleming et al., 2005; Goldberg et al., 2003; Itier et al., 2003; Kitada et al., 2009a). The proteomic analysis of the ventral midbrain revealed a slight decrease in the abundance of proteins involved in mitochondrial function (in particular complex-I and -VI of the respiratory chain) and protection from oxidative stress (Palacino et al., 2004). Subsequently, dopaminergic neurons from knockout mice show a higher sensitivity to oxidative stress induced by rotenone compared to wild-type animals (Casarejos et al., 2006). Another parkin knockout model has its cause in a spontaneous deletion of a 1,17 Mb region, encompassing the complete parkin promoter, the first 5 exons of parkin, PACRG and the promoter of the quaking gene. The loss of the quaking gene leads to the dysmyelination of the central nervous system (Sidman et al., 1964). Additionally, the mice are characterized by a slight change in their DA-metabolism, which might be due to the loss of parkin, but no neurophatological feature was detected (Lockhart et al., 2004; Lorenzetti et al., 2004;

Nikulina et al., 1995). Of note, also the recently published parkin/DJ-1/PINK1 triple knockout mice did not show any nigral degeneration (Kitada et al., 2009b).

In conclusion, parkin knockout mice do not recapitulate the human phenotype of PD, they only exhibit mild defects. One explanation for this could be a compensatory mechanism of other E3 ubiquitin ligases for the loss of parkin function.

By contrast, *Drosphila melanogaster* appears more susceptible to PD-type pathology. Parkin deficient flies show a marked phenotype, which includes reduced life span, male infertility, locomotor deficits, and flight muscle degeneration. This indicates some of the functional aspects of parkin and points to a problem of energy demanding tissues and the involvement of mitochondria. Indeed, mitochondrial dysfunction was confirmed to play a role in parkin null mutants (Clark et al., 2006; Park et al., 2006) and was underlined by the discovery of swollen mitochondria and disintegrated cristae (Greene et al., 2003; Wang et al., 2007a). However, here the loss of parkin did not lead to dopaminergic neuronal degeneration either. Strikingly, the parkin null flies showed a phenotype with marked similarities to that observed in PINK1-deficient flies. In a genetic interaction study parkin was able to compensate the PINK1 loss-of function phenotype but not *vice versa*, suggesting that parkin functions downstream of PINK1 in the same molecular pathway (Clark et al., 2006; Park et al., 2006; Yang et al., 2006a).

Subsequent studies suggested a role of PINK1 and parkin in modulating mitochondrial morphology and dynamics. Key regulator proteins that decrease mitochondrial fusion and increase mitochondrial fission were able to suppress the PINK1/parkin flight muscle phenotype (Deng et al., 2008; Park et al., 2009b; Poole et al., 2008; Yang et al., 2008).

2.4.6 Parkin has a neuroprotective potential

Parkin can protect cells against a remarkably broad spectrum of toxic or stressful agents. In a variety of cell culture systems and animal models, parkin was shown to protect from apoptosis induced by mitochondrial toxins such as MPTP (Hyun et al., 2005; Paterna et al., 2007), 6-OHDA (Manfredsson et al., 2007; Vercammen et al., 2006) and rotenone (Casarejos et al., 2006; Rosen et al., 2006), kainic acid-induced excitotoxicity (Staropoli et al., 2003), endoplasmic reticulum stress (Imai et al., 2000; Takahashi and Imai, 2003), proteasomal inhibition (Muqit et al., 2004; Petrucelli et al., 2002), treatment with sphingolipide ceramide (Darios et al., 2003), dopamine (Jiang et al., 2004), H_2O_2 (Hyun et al., 2005) or manganese (Higashi et al., 2004), and overexpression of parkin substrates or other proteins, for example Pael-R (Imai et al., 2001; Yang et al., 2003), p38/JTV-1 (Corti et al., 2003; Ko et al., 2006), α-synuclein (Lo Bianco et al., 2004; Petrucelli et al., 2002; Yang et al., 2003), LRRK2 G2019S mutant

(Ng et al., 2009), mutant tau (Menendez et al., 2006), β-amyloid (Aβ) (Burns et al., 2009; Rosen et al., 2009; Rosen et al., 2006), Ataxin-2 (Huynh et al., 2007) or expanded polyQ ataxin-3-fragment (Tsai et al., 2003) (all listed in Table 2).

Obviously, parkin seems to play a central role for neuronal integrity in response to stress. Moreover, dopaminergic neurons are in particular exposed to a high oxidative stress burden, due to their dopamine metabolism. The mechanism behind this broad protective role of parkin is still not known.

There is evidence that parkin could play a general role in the pathogenesis of sporadic PD based on the fact that severe oxidative stress can induce misfolding of native parkin (Henn et al., 2005; Winklhofer et al., 2003). Indeed, misfolded parkin was found in the *substantia nigra* of sporadic PD patients (LaVoie et al., 2005). This concept is supported by the discovery that parkin is also inactivated by nitrosative stress and correspondingly, S-nitrosylated parkin was found in the brains of sporadic PD patients (Chung et al., 2004; Yao et al., 2004). In addition, dopamine was shown to covalently modify and functionally inactivate parkin (LaVoie et al., 2005). These observations suggest that the protective properties of parkin might be lost over time, contributing to sporadic PD.

In line with this central role of parkin in maintaining neuronal viability, parkin gene expression is up-regulated in various stress paradigms such as after induction of ER stress (Imai et al., 2000; Koch et al., 2009; Ledesma et al., 2002; Oda et al., 2008; Wang et al., 2007b) and treatment with rotenone (Koch et al., 2009), H_2O_2 (Tan et al., 2005), dopamine (Yang et al., 2006b) or MPP+ (Hyun et al., 2005; Yang et al., 2006b).

parkin mediates neuroprotection from	model system	citation
mitochondrial toxins: MPTP, 6-OHDA, rotenone	• parkin knock out mice • cell culture (NT-2 and SK-NMC) • primary skeletal muscle cells • mouse model (viral gene transfer) • rat model (viral gene transfer)	(Casarejos et al., 2006) (Hyun et al., 2005) (Rosen et al., 2006) (Paterna et al., 2007) (Manfredsson et al., 2007; Vercammen et al., 2006)
kainic acid induced excitotoxicity	• primary murine neurons (viral overexpression)	(Staropoli et al., 2003)
endoplasmatic reticulum stress induced cell death	• cell culture (SH-SY5Y)	(Imai et al., 2000)
proteasomal inhibition	• primary murine neurons (viral overexpression) • cell culture (SH-SY5Y)	(Petrucelli et al., 2002) (Muqit et al., 2004)
ceramide induced cell death	• cell culture (PC12)	(Darios et al., 2003)
dopamine induced apoptosis	• cell culture (SH-SY5Y)	(Jiang et al., 2004)
H_2O_2 induced oxidative stress	• cell culture (NT-2 and SK-N-MC)	(Hyun et al., 2005)
manganese induced toxicity	• cell culture (SH-SY5Y)	(Higashi et al., 2004)
Toxicity induced by overexpression of parkin substrates or other proteins:		
Pael-R	• cell culture (SH-SY5Y) • *Drosophila* model	(Imai et al., 2001) (Yang et al., 2003)
P38/JTV-1	• cell culture (SK-N-MC, SH-SY5Y)	(Corti et al., 2003; Ko et al., 2005)
α-synuclein	• mouse primary neurons • rat model (viral gene transfer) • *Drosophila* model	(Petrucelli et al., 2002) (Lo Bianco et al., 2004) (Ng et al., 2009; Yang et al., 2003)
LRRK2 G2019S mutant	• *Drosophila* model	(Ng et al., 2009)
tau (mutant)	• transgenic mice (parkin$^{-/-}$ /tau^{VLW}) • rat-model (viral gene transfer)	(Menendez et al., 2006) (Klein et al., 2006)
Aβ	• primary skeletal muscle cells • cell culture (M17; viral overexpression) • cell culture (SH-SY5Y) • primary neuronal cells	(Rosen et al., 2006) (Burns et al., 2009) (Rosen et al., 2009)
Ataxin-2	• cell culture (PC12)	(Huynh et al., 2007)
expanded polyQ ataxin 3 fragment	• cell culture (N18)	(Tsai et al., 2003)

Table 2: Neuroprotective potential of parkin against various stressors. Tau^{VLW}: Tau with a triple FTDP-mutation (G272V, P301L and R406W); FTDP-17: Frontotemporal dementia with parkinsonism-17.

3 Results

3.1 PARKIN IS A TARGET OF THE UNFOLDED PROTEIN RESPONSE AND PROTECTS CELLS FROM ER STRESS-INDUCED MITOCHONDRIAL DAMAGE

3.1.1 Parkin is up-regulated in response to ER stress

Previous studies showed a possible transcriptional up-regulation of parkin in response to ER stress (Imai et al., 2000; Ledesma et al., 2002). To reproduce and extend these studies, we incubated human neuroblastoma-derived SH-SY5Y cells with the ER Ca^{2+}-ATPase inhibitor thapsigargin or with the N-glycosylation inhibitor tunicamycin. Parkin-specific mRNA levels were analyzed by quantitative RT-PCR. Both ER stressors significantly increased the level of parkin-specific mRNA with a maximum at 12 hours after drug treatment (Fig. 16A). Notably, the increase in parkin mRNA in response to ER stress was translated into elevated parkin protein levels (Fig. 16B).

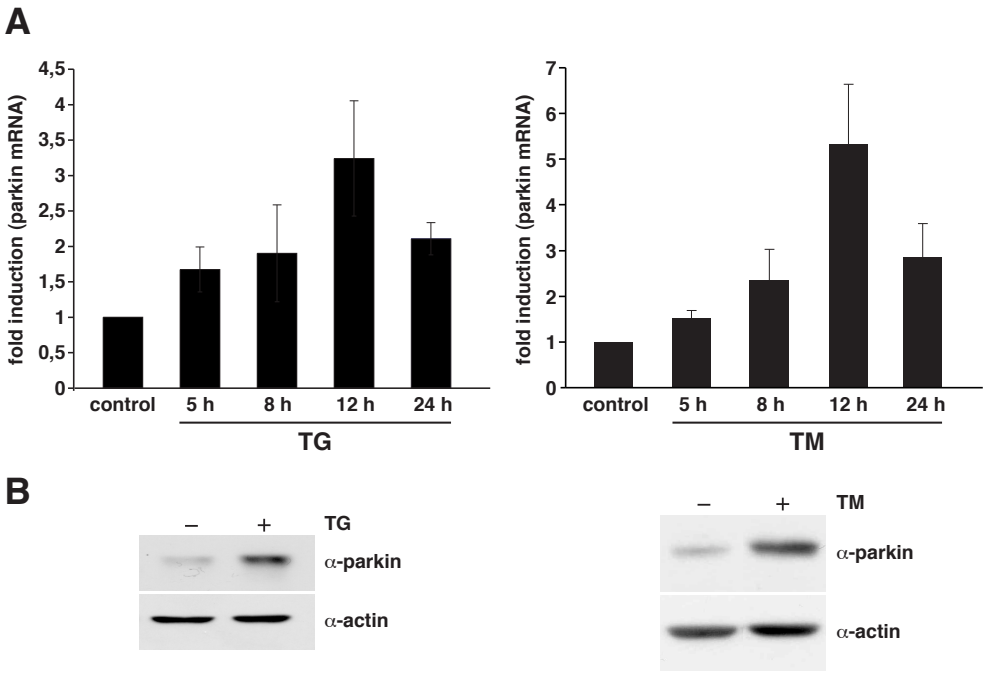


Fig. 16: Parkin gene expression is up-regulated in response to ER stress. (A) Parkin mRNA levels are increased under ER stress induced by thapsigargin or tunciamycin. SH-SY5Y cells were incubated with 1 μM thapsigargin (TG) or 2 μg/ml tunicamycin (TM) for the indicated time. Cells were harvested and total cellular RNA was isolated and subjected to quantitative RT-PCR using parkin-specific primers. The amount of RNA of each sample was normalized with respect to the endogenous housekeeping gene β-actin. Shown is the fold increase of parkin-specific mRNA compared to untreated control cells. **(B)** Expression of endogenous parkin after treatment of SH-SY5Y cells with TG or TM for 12 h was analyzed by Western blotting using the anti-parkin mAb PRK8. Loading was controlled by re-probing the blots for β-actin. The Western blot image for TM was re-arranged by excluding one line, as indicated by a white line; all samples originate from one gel.

In addition to SH-SY5Y cells, human embryonic kidney (HEK293T) cells (Fig. 17A), mouse embryonic fibroblasts (MEFs) (Fig. 17B) and primary neuronal cultures (Fig. 17C) prepared from mouse cortex also showed a robust up-regulation of parkin mRNA in response to thapsigargin or tunicamycin treatment. *(MEF cells were kindly prepared by Anita Schlierf and primary neuronal cultures were provided by Carsten Culmsee.)*

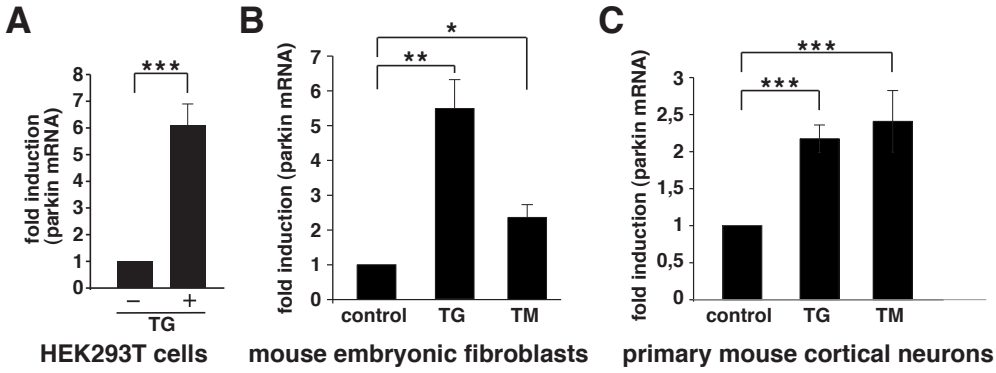


Fig. 17: Parkin mRNA is up-regulated upon ER stress in HEK293T cells, mouse embryonic fibroblasts and mouse primary cortical neurons. (A-C) HEK293T cells were incubated with 1 μM TG for 14 h (A), mouse embryonic fibroblasts (B) or primary cortical neurons derived from embryonic mouse brain (C) were incubated with 1 μM TG or 2 μg/ml TM for 12 h and then analyzed as described in Fig. 16. The amount of RNA of each sample was normalized with respect to the endogenous housekeeping gene β-actin. Shown is the fold increase of parkin-specific mRNA compared to untreated control cells. ***p<0.001, **p<0.01,*p<0.05.

3.1.2 Parkin as target of the unfolded protein response (UPR)

The previous experiments showed an up-regulation of parkin in response to ER stress. Transcription/translation of proteins after ER stress is mainly mediated by the UPR. The UPR comprises three pathways: the PERK (protein kinase-like ER kinase), the ATF6 (activating transcription factor 6) and the IRE1 (inositol requiring kinase 1) pathway. They all lead to the induction of various transcription factors, helping the cell to restore cell homeostasis. Thus, we wondered if the parkin promoter contains a cis-acting element for one of the activated transcription factors.

The promoter analysis of the human parkin gene (TFSEARCH: Searching Transcription Factor Binding Sites, http://www.rwcp.or.jp/papia; (Heinemeyer et al., 1998)) revealed a putative CREB/ATF site at position –168 to position –162. Further in-depth analysis identified this binding site as a possible binding sequence for ATF4 (activating transcription factor 4). Of note, the transcription factor ATF4 is activated by the PERK pathway. To test whether this ATF4-binding site in the parkin promoter is responsible for the up-regulation of parkin after ER stress, a reporter construct was

cloned containing the putative ATF4-binding site in triplicate in front of a sequence coding for luciferase (park-luc). As a control, a reporter construct was cloned, comprising an already confirmed ATF4-binding site from the insulin growth factor binding protein 1 (IGFBP1) promoter in triplicate in front of a luciferase gene analogously to the park-luc construct. This control construct was termed ATF4RE-luc (ATF4 response element-luc) (Fig. 18).

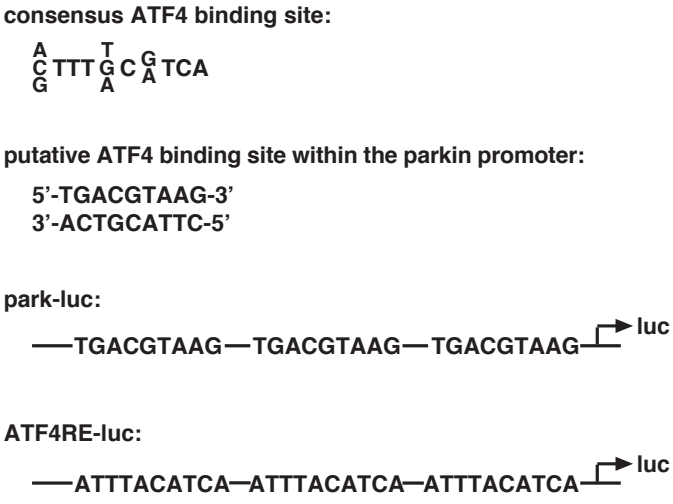


Fig. 18: Schematic representation of the consensus ATF4-binding site, the putative ATF4-binding site within the parkin promoter and the luciferase reporter constructs cloned for the analysis. The letters written one upon the other for the consensus ATF4-binding site are alternatively used. Of note, the putative binding site for ATF4 within the parkin promoter is located on the complementary strand in 5'→3' direction. The luciferase constructs are described in the following: park-luc contains the putative ATF4-binding site of the parkin promoter in triplicate in front of a luciferase gene and ATF4RE-luc contains the confirmed ATF4-binding site of the insulin growth factor binding protein 1 (IGFBP1) promoter in triplicate in front of a luciferase gene.

The park-luc construct as well as the control construct (ATF4RE) were tested under ER stress conditions. Both constructs showed to a similar extent an increase in luciferase expression after treatment with thapsigargin in comparison to the vector control, lacking a binding site for ATF4 (Fig. 19A). The experiment was performed in HEK293T as well as in SH-Y5Y cells (data not shown). In addition, the park-luc construct was also tested after treatment with tunicamycin. This treatment resulted likewise in an induction of luciferase activity (Fig. 19B).

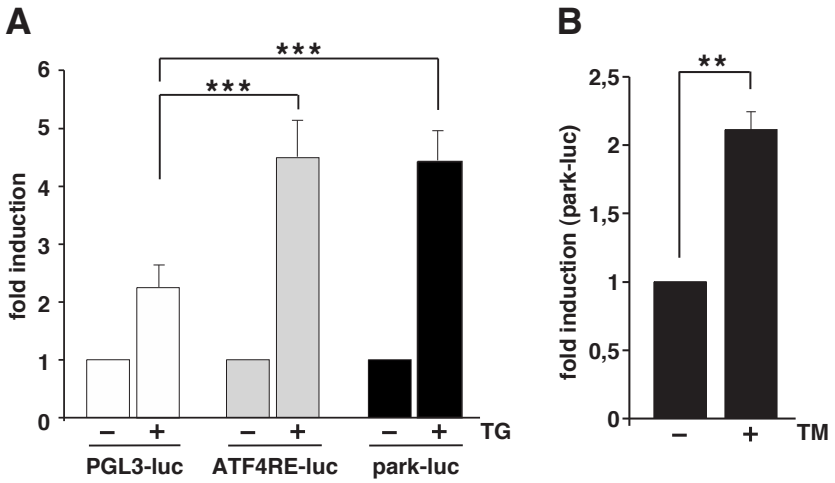


Fig. 19: The park-luc reporter construct is induced after ER stress. (A, B) HEK293T cells were transfected with either the control luciferase reporter construct PGL3-luc, the ATF4RE-luc construct containing the confirmed ATF4-binding site or the park-luc construct. 8 h after transfection cells were incubated with 1 μM TG (A) or with 2 μg/ml TM (B) and harvested after additional 14 h. Shown is the fold induction of luciferase activity in stressed cells compared to non-stressed control cells based on triplicates of at least three independent experiments. *** $p < 0.001$; ** $p < 0.01$.

To test whether ATF4 itself is responsible for the up-regulation of parkin, ATF4 was co-expressed together with the park-luc reporter construct. Indeed, overexpression of ATF4 stimulated the induction of the park-luc construct. In parallel, the control construct ATF4RE-luc was likewise induced. Additionally, the overexpression of PERK, the kinase located upstream of ATF4, showed an increase in luciferase expression of the park-luc construct as well as of the control construct. Notably, the activated transcription was higher for the park-luc construct than for the ATFRE control construct (Fig. 20). The experiment was performed in HEK293T as well as in SH-Y5Y cells (data not shown).

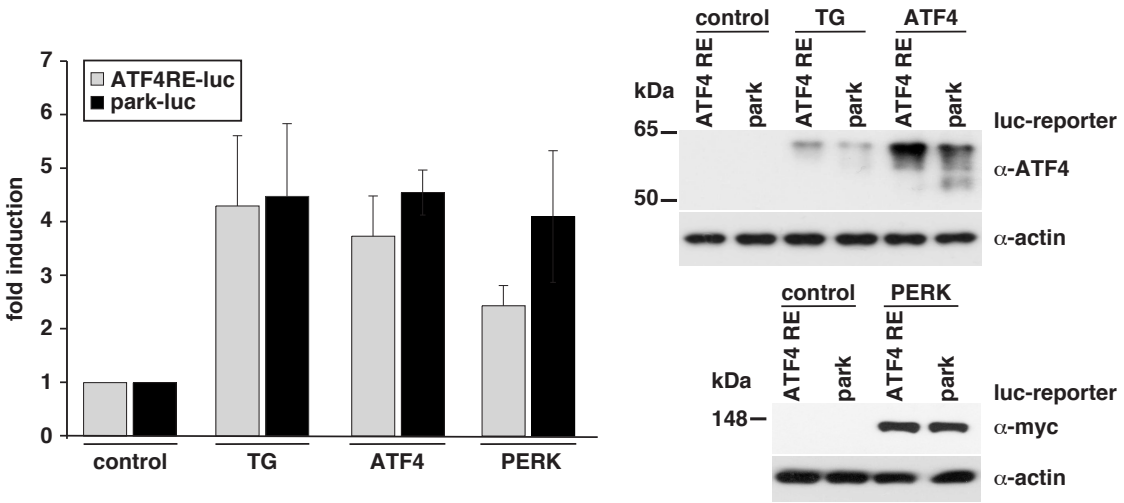


Fig. 20: Increased expression of ATF4 or upstream PERK induces transcription from the park-luc reporter construct. HEK293T cells were co-transfected with either the ATF4RE-luc reporter plasmid or the park-luc reporter plasmid and ATF4 or PERK or GFP (as a control). 8 h after transfection cells were incubated with 1 μM TG for 14 h. Shown is the fold induction of luciferase activity compared to GFP-expressing control cells based on triplicates of at least three independent experiments. Expression levels of AFT4 and PERK were analyzed by immunoblotting using the anti-ATF4 pAb C-20 or the anti-myc mAb 9E10 (right panels). Notably, TG treatment (1 μM, 14 h) induced the increased expression of endogenous ATF4. The cells were lysed in urea lysis buffer. Loading was controlled by re-probing the blots for β-actin.

In line with these findings, the transient expression of a dominant negative mutant of ATF4, ATF4ΔN, lacking the transcriptional activation domain located at the N-terminus, failed to induce the park-luc construct and consistently decreased the activation of the park-luc after ER stress (Fig. 21).

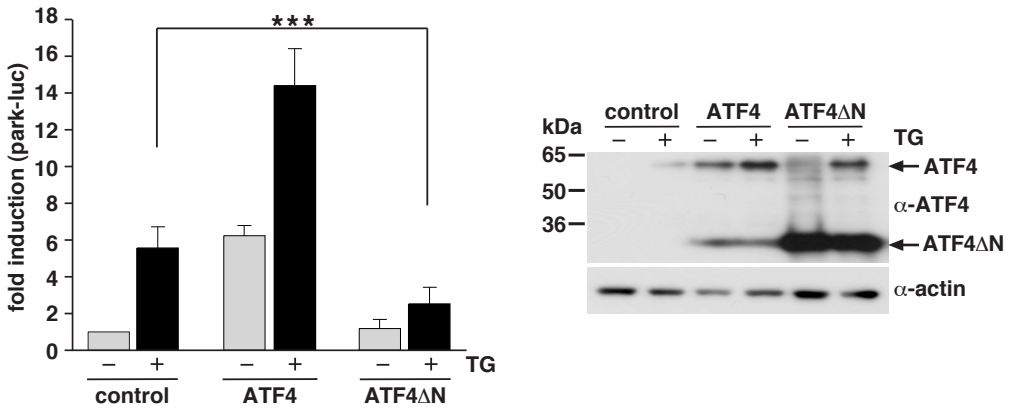


Fig. 21: A dominant negative mutant of ATF4 (ATF4ΔN) fails to activate the park-luc reporter construct. HEK293T cells were co-transfected with the park-luc reporter plasmid and ATF4 or ATF4ΔN or GFP (as a control). 8 h after transfection cells were incubated with 1 μM TG for 14 h. Shown is the fold induction of luciferase activity in comparison to GFP-expressing control cells based on triplicates of at least three independent experiments. Expression levels of ATF4 and ATF4ΔN were analyzed by immunoblotting using the anti-ATF4 pAb C-20. The cells were lysed in urea lysis buffer. Loading was controlled by re-probing the blots for β-actin (right panel). *** $p < 0.001$.

To confirm the data that ATF4 binds to the parkin promoter, an electromobility shift assay (EMSA) using the putative ATF4-binding site of the parkin promoter as a probe (park oligo) was established. As a positive control the ATF4RE oligo was used, comprising the confirmed ATF4 binding site of the IGFBP1 promoter. The park oligo was radiolabeled and incubated with nuclear extract from HEK293T cells treated with 2 μM thapsigargin for 3 hours (Fig. 22A). Upon treatment with thapsigargin, a DNA-protein complex appeared (lane 2), which could be supershifted by an anti-ATF4 antibody (lane 7), but not with a polyclonal antibody against TRAF6 (tumor necrosis factor (TNF) receptor associated factor 6; lane 8). Furthermore, this complex was

competed out by a 100-fold excess of homologous unlabeled park oligo (lane 3) or a 100-fold excess of cold ATF4RE oligo (lane 4). In contrast, the complex was not displaced by an oligonucleotide mutated at the ATF4 composite sequence of the parkin promoter (park mut; lane 5) or by an oligonucleotide that binds the octamer binding transcription factor 1 (Oct1; lane 6), suggesting specific binding of ATF4 (Fig. 22A). In addition, cells expressing ATF4 showed likewise the appearance of the same DNA-ATF4 complex under non-stress conditions (Fig. 22B). Next, we compared the park oligo to the ATF4RE oligo (Fig. 22A). The ER stress-induced ATF4RE oligo-protein complex migrated similarly to the complex observed with the park oligo (lane 10) and could be supershifted in the presence of the anti-ATF4 antibody (lane 13). The complex of DNA and protein could be competed out by a 100-fold excess of cold ATF4RE oligo (lane 11) as well as with a 100-fold excess of cold park oligo (lane 12).

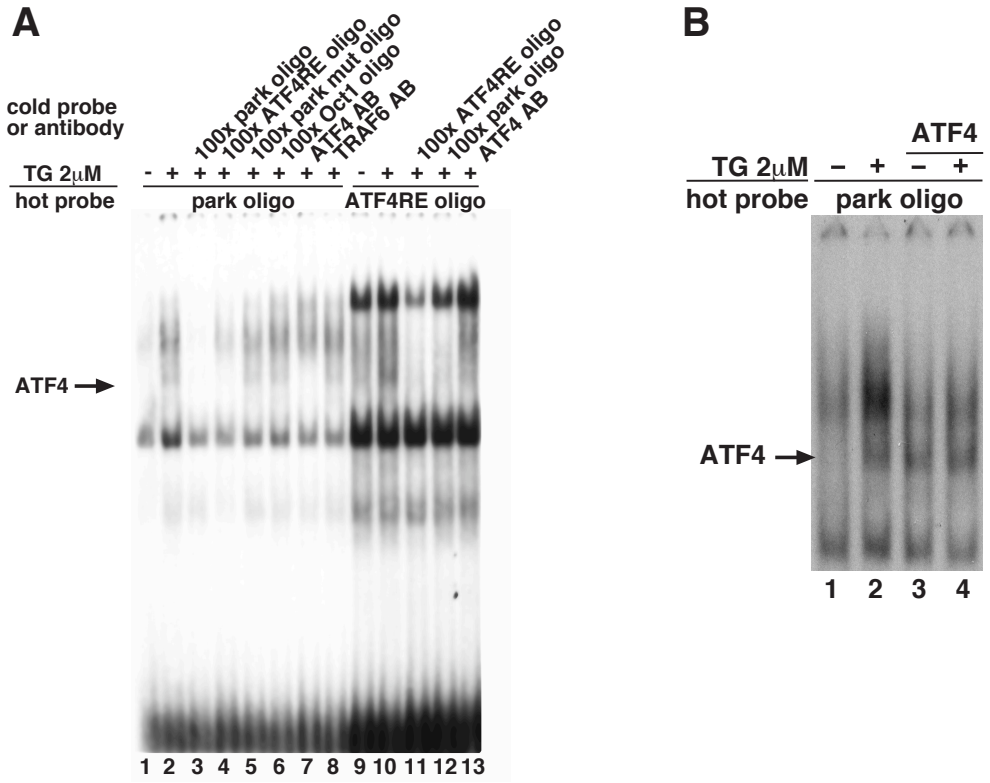


Fig. 22: ATF4 binds to the to the putative binding site within the parkin promoter. (A) ER stress induces ATF4 binding to the parkin promoter. HEK293T cells were incubated with 2 μM TG and harvested after 3 h. Nuclear extracts were prepared and tested for binding to a ^{32}P-labeled oligonucleotide comprising the putative ATF4-binding site of the parkin promoter (park oligo; lanes 1-8) by an electrophoretic mobility shift assay (EMSA). As a positive control a ^{32}P-labeled oligonucleotide comprising the confirmed ATF4-binding site of the IGFBP1 promoter (ATF4RE oligo; lanes 9-13) was used. The labelled oligonucleotides were incubated with nuclear extracts in the absence or presence of a 100-fold excess of unlabelled park oligo (lanes 3 and 12), ATF4RE oligo (lanes 4 and 11), mutated park oligo (lane 5), or Oct1 oligo (lane 6) to test for competition with the binding reaction. For supershift assays, the anti-ATF4 pAb C-20 (lanes 7 and 13) or the anti-TRAF6 pAb H-274 (lane 8) was added to the binding reaction. **(B)** Overexpressed ATF4 binds to the parkin promoter. HEK293T cells were transfected with ATF4 (lanes 3 and 4) followed by treatment with 2 μM TG for 3 h one day later (lanes 2 and 4) and EMSA was performed as described above.

To further confirm our results that ATF4 mediates the transcriptional up-regulation of parkin under ER stress *in vivo*, an ATF4 knockdown approach was established. SH-SY5Y cells were reversely transfected with siRNA against ATF4 or control siRNA. Two days later the cells were again transfected, to interfere with the up-regulation of ATF4 mRNA after induction of ER stress. As expected, the mRNA level of parkin in the control transfected cells was increased after ER stress. However, in the ATF4-deficient cells the ER stress-induced up-regulation of parkin mRNA was significantly decreased (about 69%) (Fig. 23A) The knockdown of ATF4 was verified on the mRNA as well as on the protein level (Fig. 23B). The same result was obtained with a second siRNA targeted against ATF4 (data not shown).

As a positive control the mRNA levels of the ATF4-regulated gene CHOP (C/EBP homologue protein) was examined. In the ATF4 knockdown cells the response of CHOP mRNA to ER stress was decreased to a similar extent than for the parkin mRNA, about 69%, compared to control transfected cells (Fig. 23C).

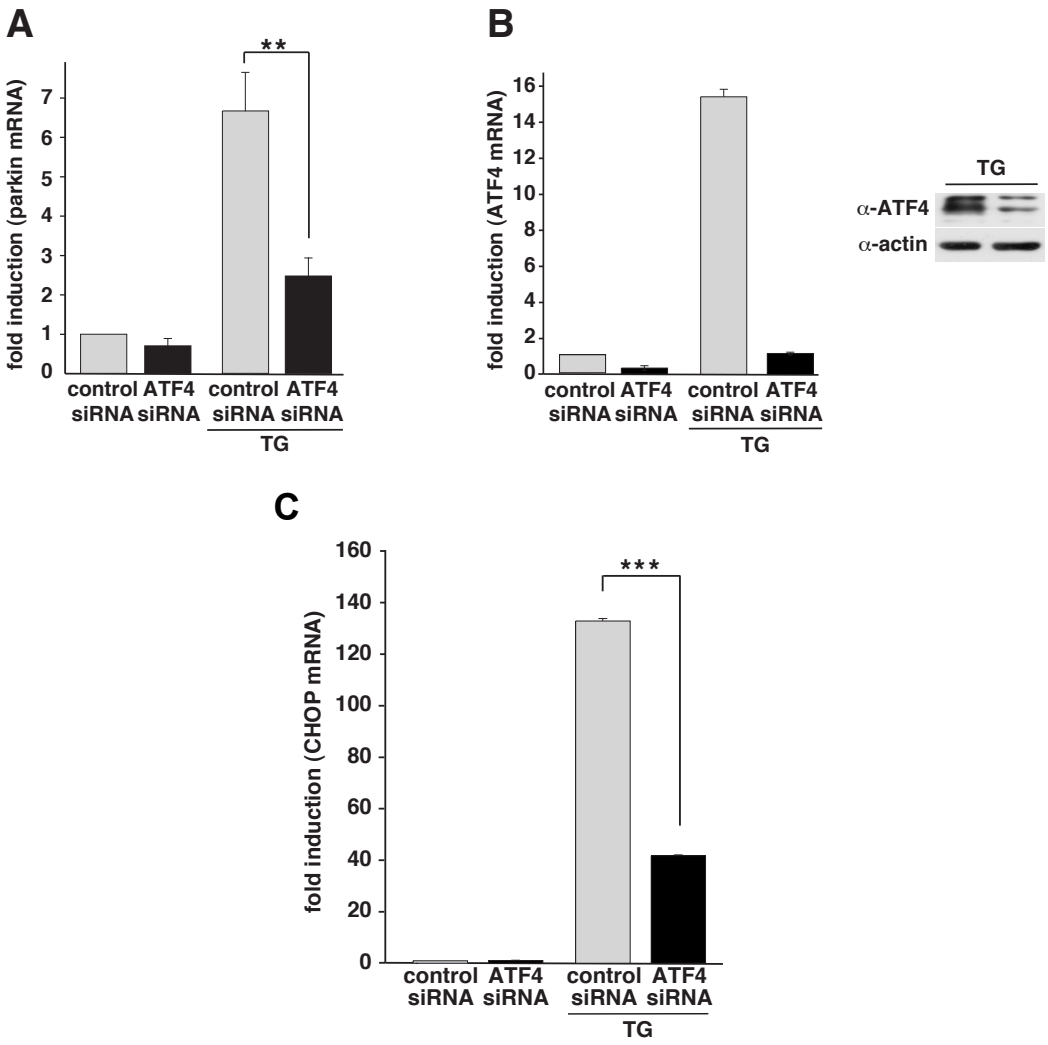


Fig. 23: ER stress-induced up-regulation of parkin is impaired in ATF4-deficient cells. (A-C) SH-SY5Y cells were reversly transfected with ATF4-specific or control siRNA duplexes. 2 days later cells were transfected again with siRNA duplexes and then incubated with 1 μM TG for 16 h. The cells were harvested and analyzed as described in Fig. 16 for quantitative RT-PCR using parkin-specific (A), ATF4-specific (B) or CHOP-specific primers (C). The amount of RNA of each sample was normalized with respect to β-actin. (A) Shown is the fold increase of parkin mRNA in response to TG treatment. (B) The efficiency of ATF4 downregulation was determined by quantitative RT-PCR and Western blotting using the anti-ATF4 pAb C-20. Cells were lysed in urea lysis buffer. Loading was controlled by re-probing the blots for β-actin. (C) Shown is the fold increase of CHOP mRNA in response to TG treatment. ** $p < 0.01$; *** $p < 0.001$.

To increase experimental evidence for an *in vivo* interaction of ATF4 with the parkin promoter during ER stress, chromatin immunoprecipitation (ChIP) assays were performed using an ATF4-specific antibody. A rabbit polyclonal antibody against chicken IgG was used as non-specific control. After isolation of cross-linked chromatin from cells incubated with or without 300 nM thapsigargin, immunoprecipitated DNA was analyzed by real time PCR. The ChIP analysis revealed binding of ATF4 to the parkin promoter after 2 and 8 hours of thapsigargin treatment (Fig. 24). *(The ChIP was done in cooperation with Jixiu Shan in the group of Michael S. Kilberg.)*

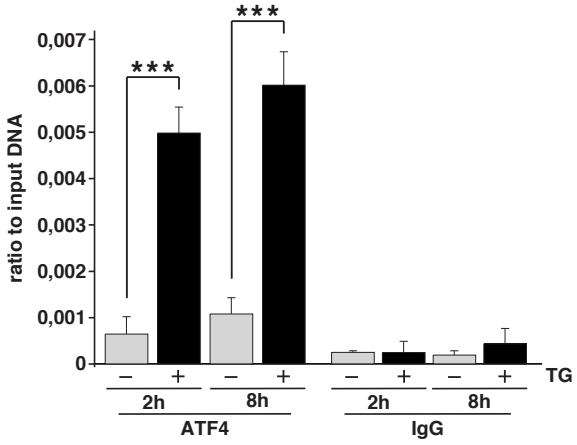


Fig. 24: ATF4 binds to the parkin promoter *in vivo*. HEK293T cells incubated with or without 300 nM TG for 2 and 8 h were used to perform a ChIP analysis using a pAb specific for ATF4 in comparison to a non-specific rabbit IgG. For the final real time PCR step, primers specific for the parkin promoter region were used. *** $p < 0.001$. *(This experiment was kindly done by Jixiu Shan.)*

Notably, also starvation of SH-SY5Y cells with L-histidinol resulted in the up-regulation of parkin protein (Fig. 25). L-histidinol is a histidine analogue which blocks

charging of the histidyl-tRNA synthetase, thus mimicing histidine deprivation. Amino acid deprivation in general leads to an increase in uncharged tRNA, which binds to and activates the eIF2α kinase GCN2 (general control non-derepressible-2). GCN2 specifically phosphorylates eIF2α, which results in the activation of ATF4 but no other UPR branch, indicating a specific regulation of parkin by ATF4.

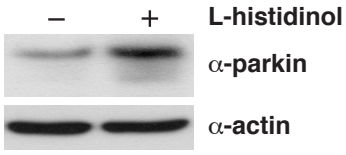


Fig. 25: Starvation leads to the up-regulation of parkin protein expression. SH-SY5Y cells were treated with 2 mM L-histidinol in cell culture medium containing 10% dialysed FCS for 16 h and expression of endogenous parkin was analyzed by Western blotting using the anti-parkin mAb PRK8. Loading was controlled by re-probing the blots for β-actin. *(With experimental help of Vincenza Palmisano.)*

3.1.3 c-Jun represses the ER stress-induced transcriptional activation of parkin

The EMSAs performed with the park oligo showed in addition to the ATF4–park complex another protein complex, with reduced electrophoretic mobility behaviour. This protein complex was also very specifically increased after ER-stress (Fig. 26A; lane 2). It has been described previously that ATF4 can form heterodimers with other ATF family members as well as with c-Jun family members. Therefore different antibodies were tested for their potential to supershift the upper band. By using a specific antibody against c-Jun a supershift for the larger protein-DNA complex but not the smaller ATF4-park-DNA complex could be observed (lane 3). Additionally, the respective band could be competed out by an excess of unlabeled park oligo (lane 5). Further experiments using the AP1 (activator protein 1) site, the common binding site of c-Jun as probe, revealed that thapsigargin was able to induce a c-Jun/AP1 complex (lane 2) which could be competed out by a 100-fold excess of cold AP1 oligo (lane 3; Fig. 26B). The same band also appeared after PMA (phorbol 12-myristate 13-acetate) treatment, a specific stimulus for JNK/c-Jun activation (lane 2; Fig. 26C). This complex could be competed out with a 100-fold excess of cold park oligo, supporting the hypothesis of c-Jun binding to the park oligo (lane 4). Cells expressing c-Jun showed likewise the appearance of the same band for the AP1 oligo (lane 2; Fig. 26D) as well as for the park oligo (lane 5). Of note, increasing amounts of c-Jun expression in HEK293T cells resulted in an increasing signal for the c-Jun/park oligo complex (Fig. 26C).

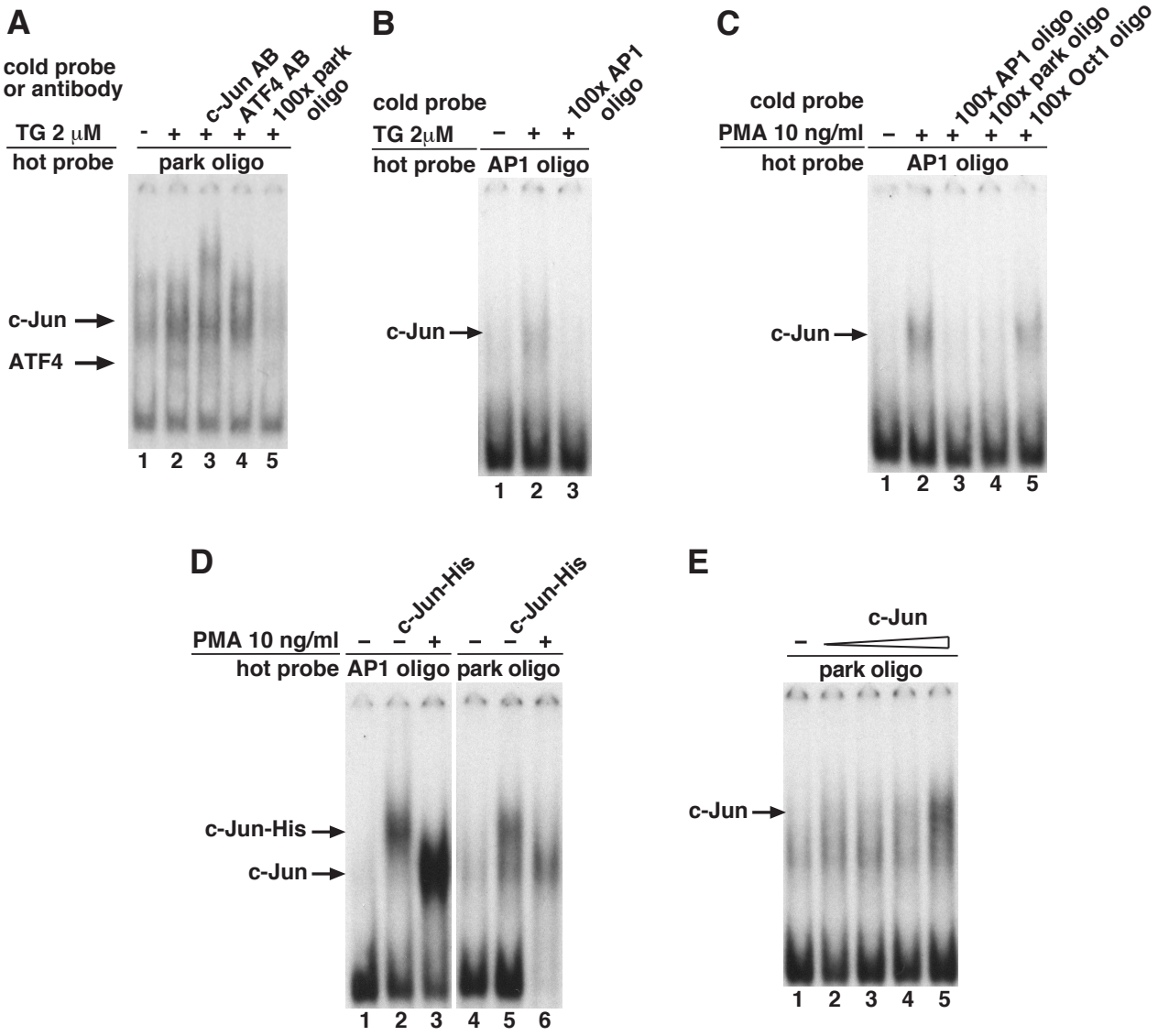


Fig. 26: c-Jun binds to the parkin promoter. (A) ER stress induces c-Jun binding to the parkin promoter. HEK293T cells were incubated with 2 μM TG and harvested after 3 h. Nuclear extracts were prepared and tested for binding to the ^{32}P-labeled oligonucleotide comprising the putative ATF4-binding site of the parkin promoter (park oligo; lanes 1-5) by an electrophoretic mobility shift assay (EMSA). The labelled oligonucleotides were incubated with nuclear extracts in the absence or presence of a 100-fold excess of unlabeled park oligo (lane 5) to compete with the binding reaction. To test for supershift activity, the anti-c-Jun pAb (N) sc-45 X (lane 3) or the anti-ATF4 pAb C-20 (lane 4) was added to the binding reaction. (*The EMSA was kindly performed by Anita Schlierf.*) **(B)** c-Jun binds to the AP1 site in response to ER stress. EMSA with TG-treated HEK293T cells (lane 2) was performed as described above. As ^{32}P-labeled oligonucleotide the common c-Jun binding site AP1 was used. To show the specificity of the bands, a 100-fold excess of unlabeled AP1 oligo (lane 3) was added. **(C)** PMA-stimulated binding of c-Jun to the AP1 site is competed by an excess of cold park oligo. HEK293T cells were incubated with 10 ng/ml PMA for 45 min (lanes 2-5) and prepared as described above. The labelled AP1 oligonucleotide was incubated with nuclear extracts with a 100-fold excess of unlabeled AP1 oligo (lane 3), park oligo (lane 4) or unspecific Oct1 (lane 5). **(D)** Overexpressed c-Jun binds to the parkin promoter. HEK293T cells were transcfected with His-tagged c-Jun (lane 2 and lane 5) or treated with 10 ng/ml PMA for 45 min (lane 3 and 6). Cells were harvested and the EMSA was performed as described above by incubation with labelled park oligo (lanes 4-6). As a control nuclear extracts were incubated with labelled AP1 oligo instead of the park oligo (lanes 1-3) The image was re-arranged by excluding one line, as indicated by a white line; all samples originate from one gel. **(E)** Increasing amounts of c-Jun expression result in a stronger binding to the parkin promoter. Nuclear extracts of HEK293T cells transfected with 0.1 μg (lane 2), 0.5 μg (lane 3), 1 μg (lane 4), 2 μg (lane 5) c-Jun DNA were incubated with labelled park oligo.

To better understand the role of c-Jun in the transcriptional regulation of parkin expression, c-Jun was co-transfected together with the park-luc construct. As expected, upon thapsigargin treatment the transcription of the park-luc construct was induced. However, in cells overexpressing c-Jun the luciferase expression was remarkably reduced under basal as well as under ER stress conditions (Fig. 27A). Moreover, the overexpression of c-Jun together with ATF4, resulted in a suppression of ATF4-mediated activation of the park-luc construct under non-stress conditions as well as under ER stress conditions, indicating a dominant negative effect of c-Jun on ATF4 (Fig. 27B).

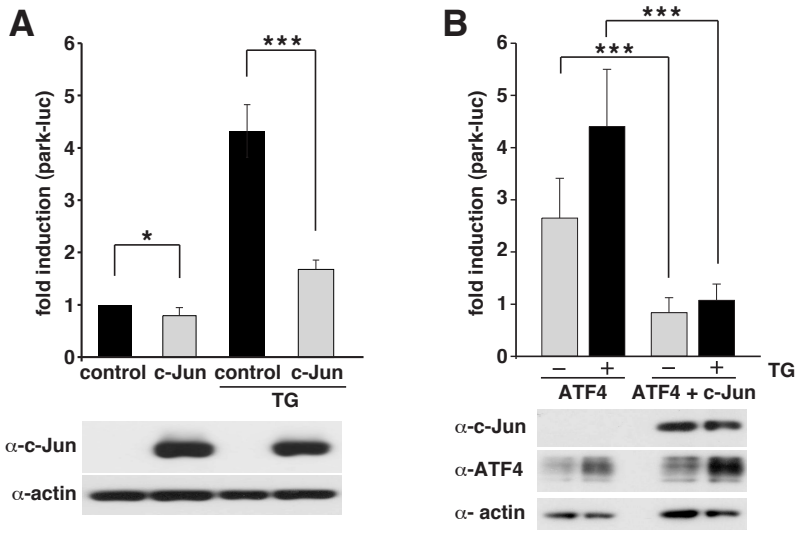


Fig. 27: c-Jun decreases transcription from the park-luc reporter after ER stress. (A) HEK293T cells were co-transfected with the park-luc reporter plasmid and c-Jun or GFP (as a control). 8 h after transfection, the cells were treated with 1 μM TG for 14 h. Shown is the fold induction of luciferase activity in c-Jun-expressing cells in comparison to GFP-expressing control cells based on triplicates of at least three independent experiments. Expression levels of c-Jun were analyzed by immunoblotting using the anti-c-Jun pAb (N) sc-45. Loading was controlled by re-probing the blots for β-actin (lower panel). **(B)** c-Jun suppresses the ATF4-mediated activation of the park-luc construct. HEK293T cells were co-transfected with the park-luc reporter plasmid and either GFP, ATF4 or ATF4 plus c-Jun. 8 h after transfection, the cells were treated with 1 μM TG for 14 h. Shown is the fold induction of luciferase activity in ATF4-expressing cells in comparison to ATF4- and c-Jun-expressing cells based on triplicates of at least three independent experiments. Expression levels of ATF4 and c-Jun were analyzed by immunoblotting using the anti-ATF4 pAb C-20 or the anti-c-Jun pAb (N) sc-45. Cells were lysed in urea-lysis buffer. Loading was controlled by re-probing the blots for β-actin (lower panel). *** $p < 0.001$.

For further *in vivo* evidence that c-Jun suppresses the up-regulation of parkin expression under ER stress conditions a c-Jun knockdown approach was established. c-Jun specific siRNA duplexes were reversely transfected into SH-SY5Y cells. One day

later the cells were again transfected with siRNA, to interfere with the up-regulation of c-Jun mRNA after the induction of ER stress. The knockdown efficiency was controlled by real-time RT PCR, revealing a reduction of c-Jun mRNA of about 87% under basal conditions and of about 69% under ER stress conditions. In parallel, the knockdown efficiency was also controlled on the protein level (Fig. 28B). Already under normal conditions c-Jun knockdown cells showed a slightly higher parkin mRNA expression than control cells. Notably, under ER stress conditions parkin mRNA was significantly stronger induced in c-Jun-deficient cells compared to control transfected cells (Fig. 28A). The same result was obtained with a second siRNA targeted against c-Jun (data not shown). As a control for specific effect of c-Jun on parkin gene expression, the mRNA levels of the pro-apoptotic protein CHOP (Fig. 28C) and the PD-associated protein PINK1 (Fig. 28D) was examined in c-Jun deficient cells. The mRNA levels of both, CHOP and PINK1 were increased after ER stress, however the knockdown of c-Jun had no significant influence on the level of induction.

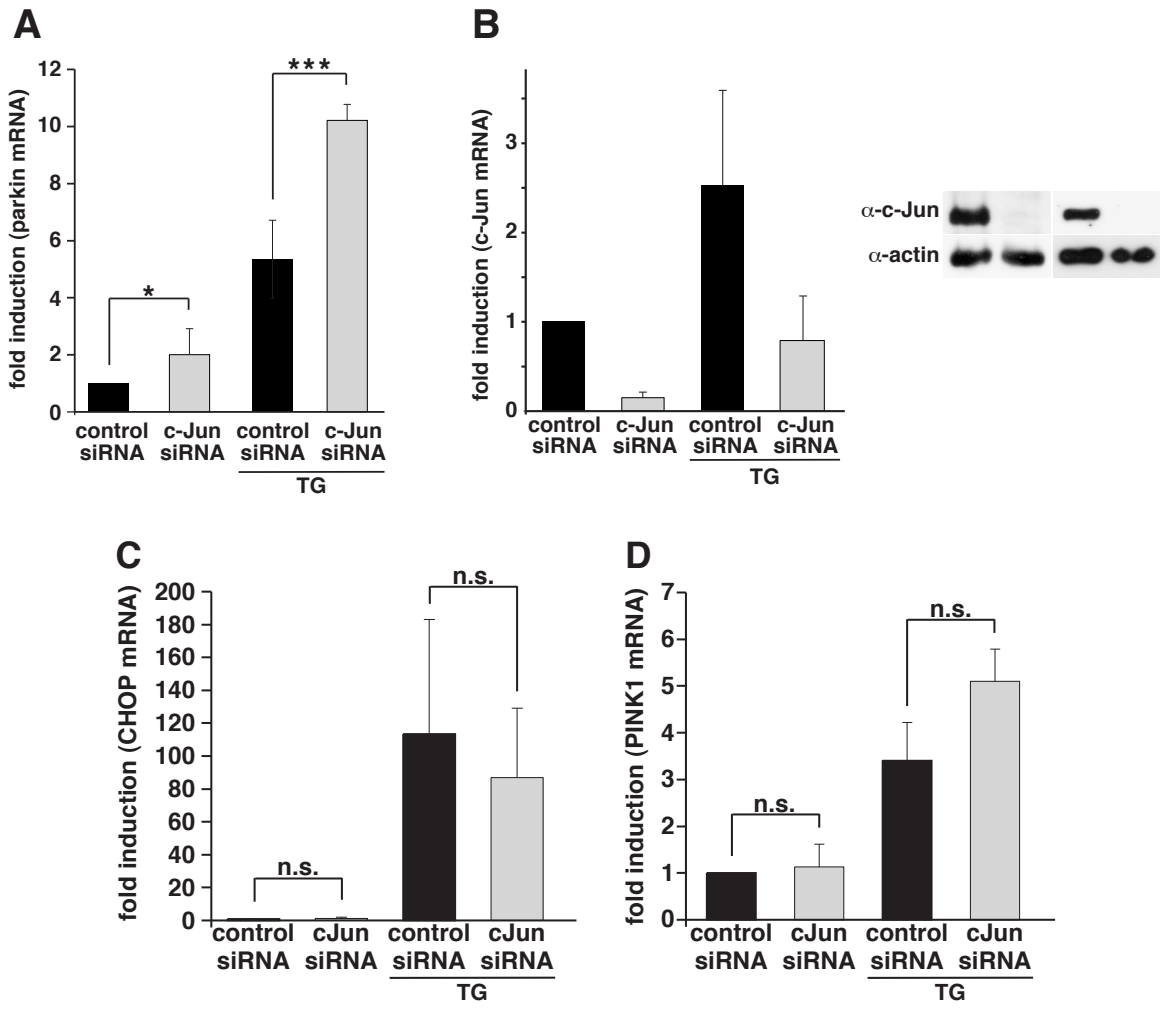


Fig. 28: ER stress-induced up-regulation of parkin is increased in c-Jun-deficient cells. (A-D) SH-SY5Y cells were reverse transfected with c-Jun-specific or control siRNA duplexes. One day later cells were transfected again with siRNA duplexes and incubated with 1 μM TG for 16 h. The cells were harvested and analyzed as described in Fig. 16 for quantitative RT-PCR using parkin-specific (A), c-Jun-specific (B), CHOP-specific (C) or PINK1-specific (D) primers. The amount of RNA of each sample was normalized with respect to β-actin. (A) Shown is the fold increase of parkin mRNA in response to TG treatment. (B) The efficiency of c-Jun downregulation was determined by quantitative RT-PCR and Western blotting using the anti-c-Jun pAb (N) sc-45. Loading was controlled by re-probing the blots for β-actin (right panel). (C, D) Shown is the fold increase of CHOP mRNA (C) and PINK1 mRNA (D) in response to TG treatment. ** $p < 0.01$; n.s. = not significant.

To get more insight into the binding behaviour of ATF4 and c-Jun on the parkin promoter, recombinant proteins were tested in an EMSA. c-Jun and ATF4 were either incubated alone or together with the park oligo for 30 min on ice. As expected, increasing amounts of protein resulted in more protein bound to the park oligo (c-Jun: lane 1-4; ATF4: lane 9-11) (Fig. 29). Remarkably, the binding properties of the two proteins were changed when first ATF4 was incubated with the park oligo and after 15 min c-Jun was added to the reaction. The bound ATF4 was able to recruit c-Jun to the parkin promoter (lane 5-8). However, *vice versa* this was not the case, c-Jun was not able to recruit ATF4 to the parkin promoter as visualized by the reduced intensity of the signal. This observation suggests that c-Jun might compete with ATF4 for the transcriptional regulation of parkin and that it might serve as a terminator for the up-regulation of parkin after ER stress. Of note, the recombinant c-Jun used for these experiments was not phosphorylated, thus it cannot be excluded that the binding of c-Jun to the park oligo is influenced by the phosphorylation status of c-Jun.

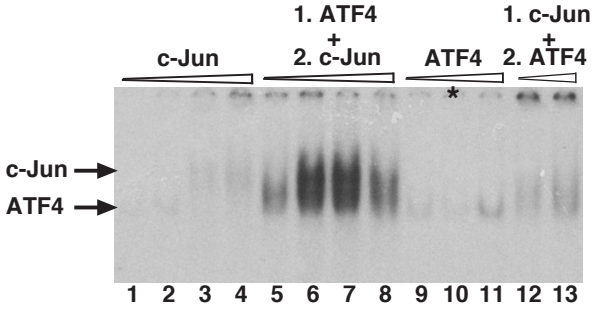


Fig. 29: ATF4 increases binding of c-Jun to the parkin promoter. Increasing amounts of recombinant ATF4 or c-Jun were incubated with ^{32}P-labeled oligonucleotide comprising the putative ATF4-binding site of the parkin promoter (park oligo) to monitor formation of DNA-protein complexes by EMSA. Lanes 1-4: 200, 300, 350 or 400 ng recombinant c-Jun was incubated with the park oligo. Lanes 5-8: 100 ng recombinant ATF4 was incubated with the park oligo for 15 min on ice followed by the addition of c-Jun (200, 300, 350 or 400 ng) for another 15 min. Lane 9-11: 100, 50 (*) or 200 ng of recombinant ATF4 was used. Lanes 12 and 13: 350 ng recombinant c-Jun was incubated with the park oligo for 15 min on ice followed by the addition of ATF4 (50 and 100 ng) for another 15 min.

c-Jun is regulated by the JNK (c-Jun N-terminal kinase) pathway. JNK phosphorylates and thereby activates c-Jun. This subfamiliy of mitogen-activated protein kinases (MAPK) consists of three isoforms JNK1, JNK2 and JNK3. JNK1 and 2 are ubiquitously expressed, while JNK3 is primarily found in the brain, heart and testes. JNK3 has been linked to cell death in several models of neurodegeneration. To test if JNK3 also has an impact on parkin expression, JNK3 was cotransfected with the park-luc construct. Indeed, overexpression of JNK3 resulted in a reduced induction of the park-luc construct both under ER stress and basal conditions (Fig. 30).

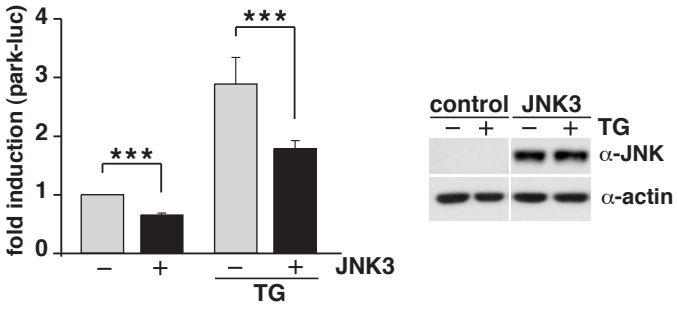


Fig. 30: JNK3 decreases transcription from the park-luc reporter. HEK293T cells were co-transfected with the park-luc reporter plasmid and JNK3 or GFP (as a control). 24 h after transfection, the cells were treated with 1 μM TG for 8 h. Shown is the fold induction of luciferase activity in JNK3-expressing cells in comparison to GFP-expressing control cells based on triplicates of at least three independent experiments. Expression levels of JNK3 were analyzed by immunoblotting using an anti-JNK pAB. Loading was controlled by re-probing the blots for β-actin. The Western Blot image was re-arranged by excluding one line, as indicated by a white line; all samples originate from one gel. (right panel). *** $p < 0.001$.

3.1.4 Parkin protects cells from ER stress-induced cell death

Based on these results, the question arises, what are the consequences for the cell upon the up-regulation of parkin? The main task of the UPR is to restore ER function. However, additionally also the expression of pro-survival genes and genes which preserve cell function is induced. To test if parkin up-regulation might protect cells from ER stress-induced toxicity, SH-SY5Y cells transiently expressing parkin or pathogenic parkin mutants were treated with thapsigargin or tunicamycin.

Please note, for these experiments we chose SH-SY5Y cells, which show a reduced endogenous parkin expression due to long passage number (Fig. 31A, B).

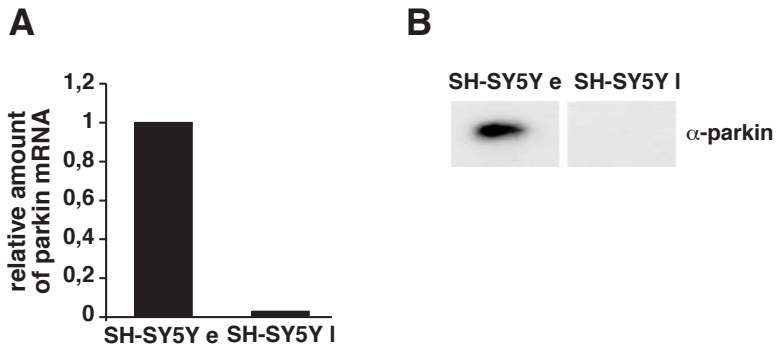


Fig. 31: The level of endogenous parkin in SH-SY5Y cells changes, when the cells are passaged over a long period of time. Early passages of SH-SY5Y cells still contain considerable amounts of parkin (SH-SY5Y e), but after 30-40 passages parkin seems to be lost (SH-SY5Y l). Of note, parkin is expressed at very low levels in SH-SY5Y cells. **(A)** Parkin mRNA levels were analyzed as described in Fig. 16 using parkin-specific primers. The amount of mRNA of each sample was normalized with respect to β-actin. Shown is the fold reduction of parkin mRNA in SH-SY5Y e cells compared to SH-SY5Y l cells. **(B)** Expression levels of parkin were analyzed by immunoblotting using the anti-parkin PRK8 mAb. The Western Blot image was re-arranged by excluding one line, as indicated by a white line; all samples originate from one gel. *(The Western Blot was kindly done by Julia Schlehe.)*

Cells undergoing apoptosis in response to ER stress were analyzed by indirect fluorescence using an antibody specific for activated caspase-3. The control cells showed a significant increase in apoptosis in response to ER stress induced by either thapsigargin or tunicamycin. However, cells overexpressing parkin were protected against ER stress-induced cell death. Notably, the parkin mutants G430D and ΔUBL, which are associated with autosomal recessive parkinsonism, failed to protect the cells from ER stress-induced apoptosis (Fig. 32).

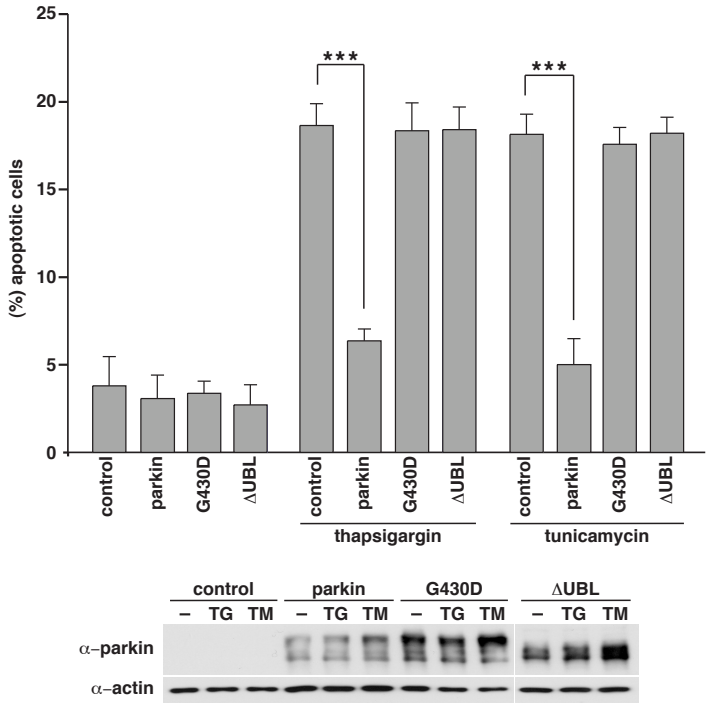


Fig. 32: Increased expression of parkin protects cells from ER stress-induced cell death. SH-SY5Y cells were cotransfected with EYFP (as a control) and wild-type parkin or the pathogenic parkin mutants G430D or ΔUBL. 24 h after transfection, cells were incubated with 10 μM TG or 5 μg/ml TM at 37°C for 8 h, fixed, permeabilized, and then the activation of caspase-3 was analyzed by indirect immunofluorescence using an anti-active caspase-3 pAb. For quantification at least three independent experiments were performed. Per experiment ≥ 300 transfected cells were counted. Shown is the percentage of apoptotic cells among transfected cells. Parkin expression levels were determined by immunoblotting using the anti-parkin PRK8 mAb. Loading was controlled by re-probing the blots for β-actin (lower panel). (*Caspase assays were kindly counted by Kathrin Lutz.*) *** $p < 0.001$.

To increase the evidence for a role of endogenous parkin in coping with ER stress, the consequences of a parkin knockdown induced by RNA interference were analyzed. First, HEK293T cells were transfected with parkin-specific siRNA duplexes, resulting in a >80% reduction of parkin mRNA and protein levels three days after transfection (Fig. 33B). Cell viability was measured with the MTT viability assay. Parkin knockdown cells showed a significant decrease in cell vitality after ER stress in comparison to control siRNA-transfected cells, indicating an increased vulnerability of cells to ER stress in the absence of parkin (Fig. 33A).

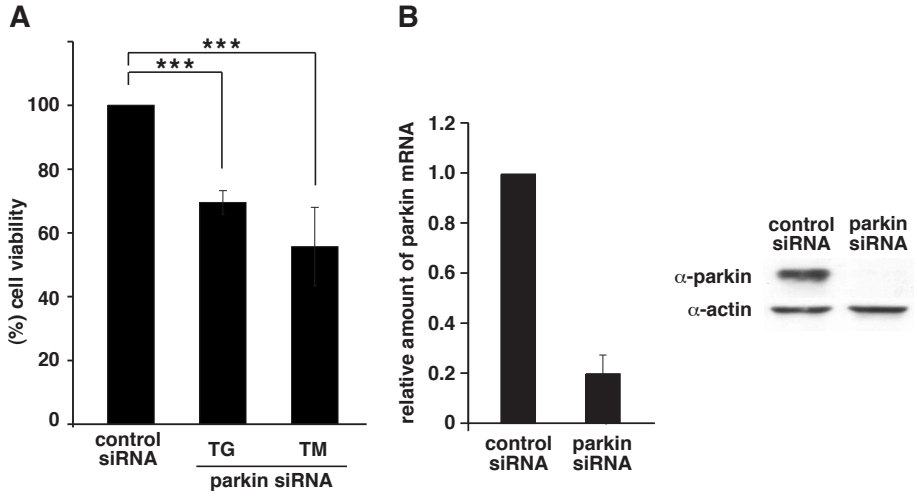


Fig. 33: Parkin deficiency impairs the viability of cells under ER stress. (A, B) HEK293T cells were transfected with parkin-specific or control siRNA duplexes. (A) Three days later the cells were stressed with TG (10 μM) or TM (2 μg/ml) for 16 h. Cellular viability was determined by the MTT assay. Shown is the relative viability of cells transfected with parkin siRNA in comparison to control siRNA-transfected cells treated with ER stressors based on triplicates of at least three independent experiments. (B) The parkin knockdown efficiency was verified by quantitative RT-PCR and Western blotting using the anti-parkin mAb PRK8. Loading was controlled by re-probing the blot for β-actin. *** $p < 0.001$.

In addition, also the parkin knockdown in SH-SY5Y resulted in an increase of apoptotic cells as determined by the activated caspase 3 assay. This effect could be rescued by the addition of a siRNA resistant parkin wild-type construct (rescue parkin). The parkin rescue construct contained three silent mutations at the same site of the DNA recognized by the specific parkin siRNA. Because of the mutations, the siRNA lost its ability to bind to the construct, which would otherwise lead to its destruction (Fig. 34).

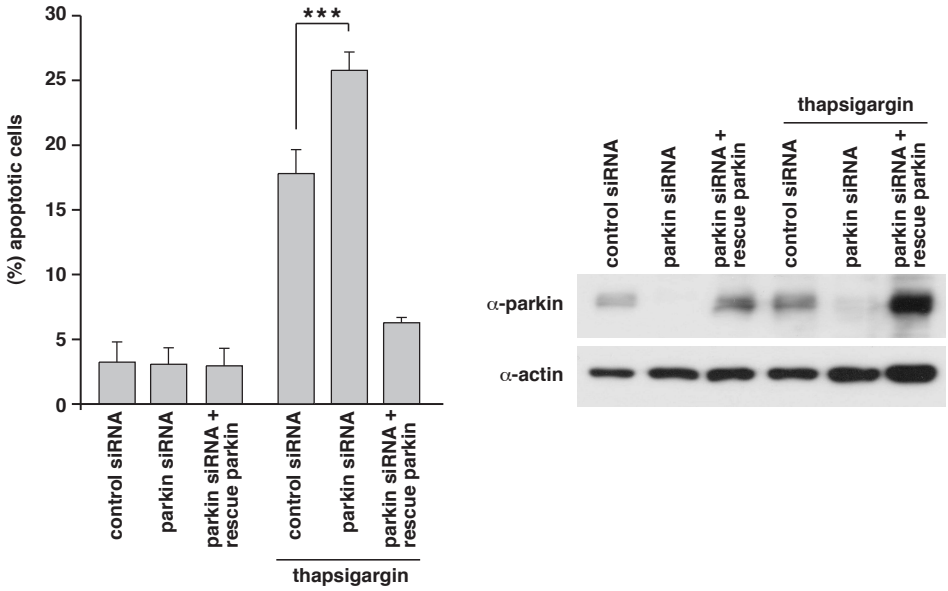


Fig. 34: The increased vulnerability of parkin-deficient cells to ER stress can be rescued by siRNA-resistant wild-type parkin. SH-SY5Y cells were transfected with parkin-specific or control siRNA duplexes and co-transfected with EYFP (as a control) or siRNA-resistant wild-type parkin (rescue parkin). Three days later the cells were stressed with TG (10 μM) for 8 h fixed, permeabilized, and then activation of caspase-3 was analyzed by indirect immunofluorescence as described in Fig. 32. Parkin expression levels were determined by immunoblotting using the anti-parkin PRK8 mAb. Loading was controlled by re-probing the blots for β-actin (right panel). (*Caspase assays were kindly counted by Kathrin Lutz.*) *** $p < 0.001$.

Finally, mouse embryonic fibroblast (MEF) cells derived from parkin knockout mice were tested for their viability after ER stress. The MEF cells were treated with thapsigargin overnight and analyzed by the MTT viability assay. Interestingly, the MEF cells derived from knockout mice were more vulnerable to ER stress than wild-type MEF cells (Fig. 35). (*MEF cells were kindly prepared by Anita Schlierf.*)

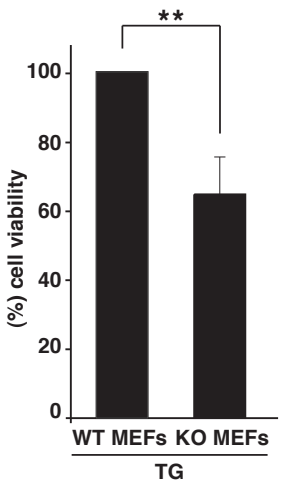


Fig. 35: Mouse embryonic fibroblasts (MEFs) derived from parkin knockout mice are more vulnerable to ER stress than wild-type MEFs. MEFs from wild-type (WT) or parkin knockout (KO) mice were stressed with TG (10 μM) for 16 h and then cellular viability was determined by the MTT assay. Shown is the relative viability of KO MEFs in comparison to WT MEFs after TG treatment. Quantification is based on five independent experiments. ** $p < 0.01$.

3.1.5 Parkin has no direct effect on ER stress

The experiments above established a protective role of parkin in response to ER stress. To gain insight into the mechanism underlying this effect, the question was addressed whether parkin might have an influence on the severity of ER stress. Therefore, a knockdown of parkin in SH-SY5Y cells was performed (Fig. 36C) and the mRNA levels of the UPR target genes BIP and CHOP in response to ER stress were examined. After thapsigargin-induced ER stress BiP mRNA was highly up-regulated (15 fold compared to untreated cells). Interestingly, the knockdown of parkin had no significant impact on the mRNA level of the chaperone BiP under basal as well as under ER stress conditions (Fig. 36A). In parallel, the mRNA of the pro-apoptotic protein CHOP was highly up-regulated after ER stress. However, also here the knockdown of parkin had no influence on the level of induction (Fig. 36B).

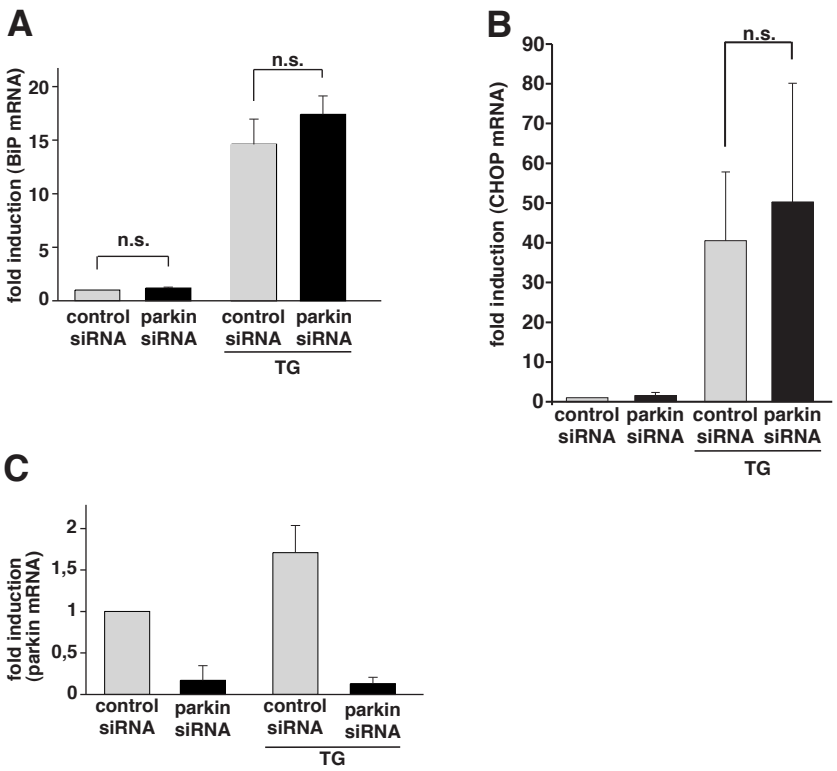


Fig. 36: The transient knockdown of parkin has no impact on the ER stress markers BiP and CHOP. (A-C) SH-SY5Y cells were transfected with parkin-specific or control siRNA duplexes. Three days later the cells were stressed with 1 μM TG for 5 h. As an indicator of ER stress BiP (A) and CHOP (B) mRNA levels were analyzed by quantitative RT-PCR as described in Fig. 16. The amount of RNA of each sample was normalized with respect to the endogenous housekeeping gene β-actin. Shown is the fold increase of BiP-specific mRNA (A) or CHOP-specific mRNA (B) compared to untreated control cells. (C) To test for the efficiency of parkin knockdown, parkin mRNA levels were quantified in parallel. n.s. = not significant.

Additionally, MEF cells derived from parkin knockout mice were examined in comparison to wild-type MEFs in their capability to induce BiP mRNA after ER stress. In line with the findings of transient parkin knockdown cells, the parkin knockout MEFs also showed no significantly altered levels of BiP mRNA after thapsigargin or tunicamycin treatment compared to control MEFs (Fig. 37). *(Experiment was kindly performed by Anita Schlierf.)*

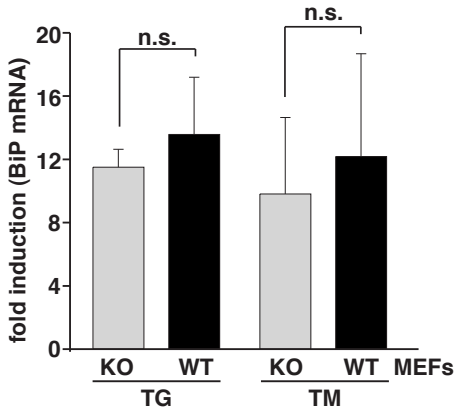


Fig. 37: The level of ER stress is not increased in mouse embryonic fibroblasts (MEFs) derived from parkin knockout mice. MEFs from wild-type (WT) or parkin knockout (KO) mice were stressed with 1 μM TG or 2 μg/ml TM for 5 h. The levels of BiP mRNA were analyzed by RT-PCR as described in Fig. 16. The amount of RNA of each sample was normalized with respect to the endogenous housekeeping gene β-actin. Shown is the fold increase of BiP-specific mRNA compared to untreated control cells. *(Experiment was kindly done by Anita Schlierf.)* n.s. = not significant.

To test whether the induced expression of parkin has an effect on the UPR, several reporter constructs were cloned. Given that transcriptional activation of UPR target genes is mediated by three signalling cascades PERK/eIF2α/ATF4, ATF6 and IRE1/XBP1, the reporter constructs contained different ER stress response elements in front of a luciferase gene, allowing to differentiate between these three pathways. Interestingly, especially for adaptation to chronic stress the selective activation of signalling through one or two of the limbs of the UPR is proposed (Rutkowski and Kaufman, 2007). To the different ER stress response elements one or two of the three known UPR transducers ATF4, ATF6 and XBP1 were able to bind (Fig. 38). The first luciferase construct contained the ER stress response element (ERSE), which is fully activated by ATF6, even in the absence of XBP1, but dependent on NF-Y (Yoshida et al., 1998). The second one contained the unfolded protein response element (UPRE), only depending on XBP1 (Wang et al., 2000). The third one, ERSE-II, allowed binding of ATF6 dependent on NF-Y as well as NF-Y-independent binding of XBP1 (Kokame et al., 2001). The last one, ATF4 response stress element (ATF4RE), contained the binding site for ATF4 from the promoter of the Insulin growth factor binding protein 1 (IGFBP1) (Marchand et al., 2006).

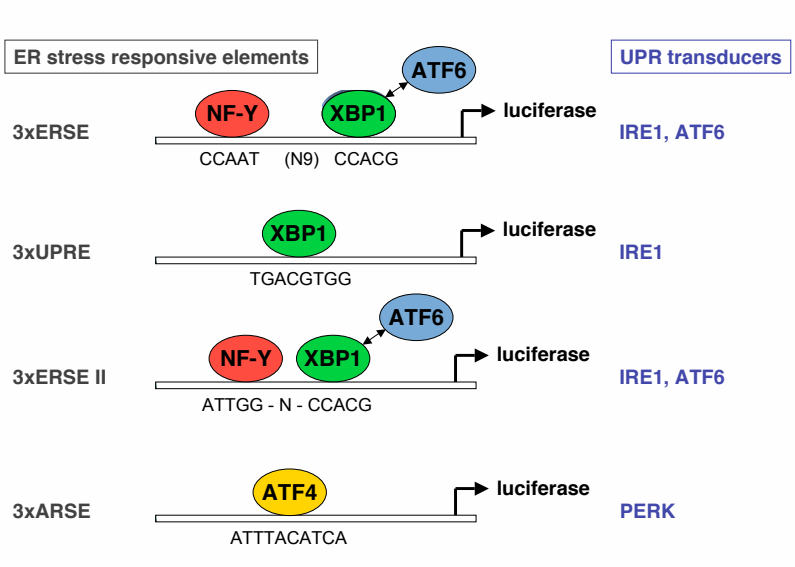


Fig. 38: ER stress luciferase reporter constructs cloned to quantify the ER stress response. Binding of ER stress-specific transcription factors to each construct and UPR transducers involved are schematically shown. ERSE: ER stress response element, UPRE: unfolded protein response element, ERSE II: ER stress response element II, ATF4RE: ATF4 response element, NF-Y: nuclear factor-Y, XBP1: X-box binding protein 1, ATF6: activating transcription factor 6, IRE1: inositol requiring enzyme 1, ATF4: activating transcription factor 4, PERK: PKR (double-stranded RNA-activated protein kinase)-like ER kinase.

HEK293T cells were transiently transfected with one of the ER stress reporter constructs and a plasmid coding for parkin or GFP as a control. To test whether parkin has an impact on ER stress, cells were additionally subjected to thapsigargin-induced ER stress (Fig. 39). Luciferase activity was clearly increased after thapsigargin treatment for all of the four reporter constructs. Interestingly, parkin did not significantly influence ER stress-dependent transcription, neither under basal conditions nor under ER stress. The same results were obtained in SH-SY5Y cells (data not shown).

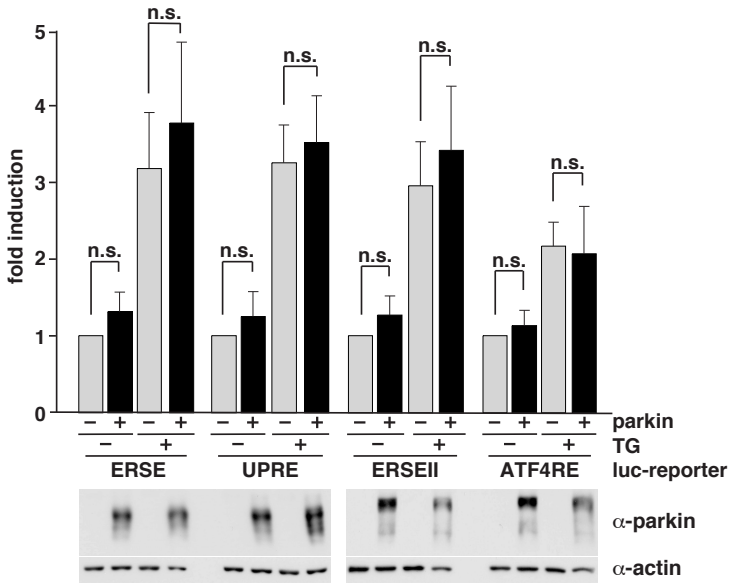


Fig. 39: Overexpression of parkin has no influence on the ER stress level determined by ER stress reporter constructs. HEK293T cells were cotransfected with the ER stress reporter plasmids and either parkin or GFP (as a control). 24 h after transfection, the cells were treated with 1 μM TG for 8 h. Shown is the fold induction of luciferase activity in parkin-expressing cells in comparison to GFP-expressing control cells. Quantification is based on triplicates of at least three independent experiments. Expression levels of parkin were analyzed by immunoblotting using the anti-parkin pAB 2132. Loading was controlled by re-probing the blots for β-actin (lower panel). n.s. = not significant.

Besides wild-type parkin also other parkin constructs were tested for their ability to influence ER stress. The UPRE reporter was co-transfected with wild-type parkin, full-length (fl) parkin devoid of the internal initiation site, the smaller parkin species lacking the N-terminal UBL domain (ΔUBL) and the loss of function mutant

W453STOP. However, all parkin constructs tested showed no difference in the induction of the luciferase expression under basal nor under ER stress conditions (Fig. 40A). For further evidence the ER stress reporter constructs were also tested in parkin deficient HEK293T cells. But also the loss of parkin did not lead to significant alterations in luciferase activity under ER stress as well as under normal conditions (Fig. 40B).

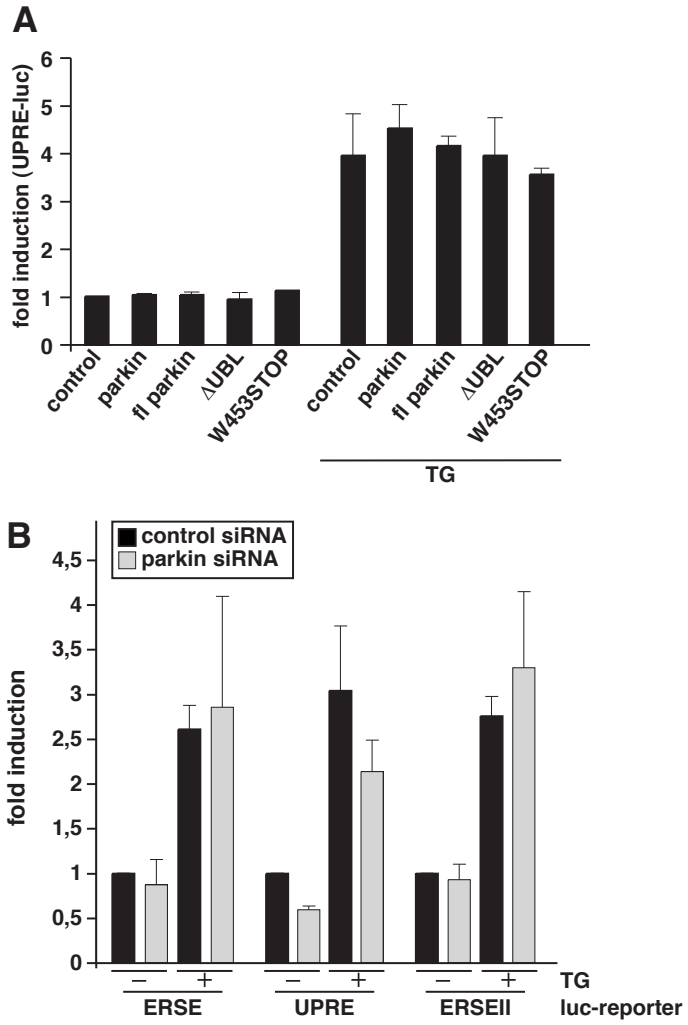


Fig. 40: Loss of parkin function has no impact on ER stress reporter constructs. (A) Parkin mutants have no influence on ER stress reporter constructs. HEK293T cells were cotransfected with the UPRE construct and either GFP, wild-type parkin, full-length (fl) parkin or the pathogenic parkin mutants ΔUBL and W453STOP. 24 h after transfection, the cells were treated with 1 μM TG for 8 h. Shown is the fold induction of luciferase activity in parkin-expressing cells in comparison to GFP-expressing control cells. Quantification is based on triplicates of at least three independent experiments. **(B)** Parkin deficiency has no impact on ER stress reporter constructs. HEK293T cells were transfected with parkin-specific or control siRNA duplexes and the ER stress reporter constructs UPRE, ERSE and ERSEII. Two days later the cells were stressed with 1 μM TG for 8 h. Shown is the fold induction of luciferase activity in parkin-knockdown cells in comparison to control cells. Quantification is based on triplicates of at least three independent experiments.

In addition, to exclude that parkin might have an influence on the induction of ER stress, which is not due to drug treatment rather than to protein expression, the Pael receptor (Pael-R) and the receptor tyrosinkinase Ret were co-expressed together with parkin and the reporter construct ERSE. The Pael-R is reported to be a substrate of parkin and when overexpressed to become unfolded and insoluble and to induce ER stress (Imai et al., 2001). Ret is a highly glycosylated receptor molecule at the cell membrane, associated with GDNF (glial cell line derived neurotrophic factor) signalling. Several splice variants exist, the longest one is 1114 bp long (Ret-long). Overexpression of Ret-long is also likely to misfold and to induce ER stress. Indeed, the overexpression of the Pael-R (Fig. 41A) and Ret-long (Fig. 41B) resulted in increased luciferase activity of ERSE-luc, indicating an induction of ER stress. However, also in this case the co-expression of parkin had no effect on the level of ER stress.

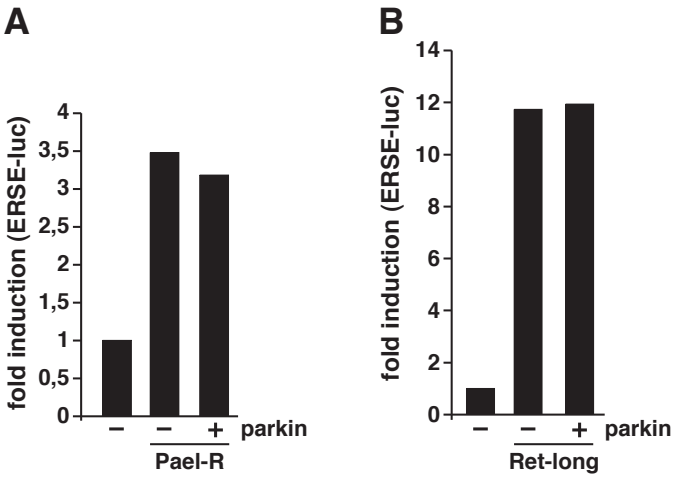


Fig. 41: Parkin has no influence on ER stress due to accumulating proteins in the ER. (A, B) HEK293T cells were cotransfected with the ERSE reporter construct and either GFP, Pael-R or Ret-long together with GFP or wild-type parkin. Shown is the fold induction of luciferase activity in Pael-expressing (A) or Ret-long-expressing (B) cells in the presence or absence of parkin in comparison to GFP-expressing control cells.

Parkin has an E3 ubiquitin ligase activity and was previously reported to be involved in the proteasomal degradation of misfolded secretory pathway proteins and thereby to protect the cell for toxic accumulation of parkin substrates, such as Pael-R. Therefore, the question was addressed whether the protective activity of parkin is dependent on the proteasome. To induce proteasomal inhibition, low parkin expressing SH-SY5Y cells were treated with a low concentration of epoxomycin. The efficiency of proteasome inhibition was controlled by analyzing the steady state level of the rapidly

turned over transcription factor p53 and the total ubiquitin load in the cell (Fig. 42B). As expected, thapsigargin treatment resulted in an increase in apoptotic cells and parkin overexpression was able to rescue this effect. Interestingly, parkin was also protective in cells treated with both thapsigargin and epoxomycin, indicating that protein degradation via the proteasome is not required for the protective function of parkin after ER stress. Of note, for this experiment we used a non-toxic concentration of epoxomycin which was sufficient to inhibit proteasomal degradation (Fig. 42A).

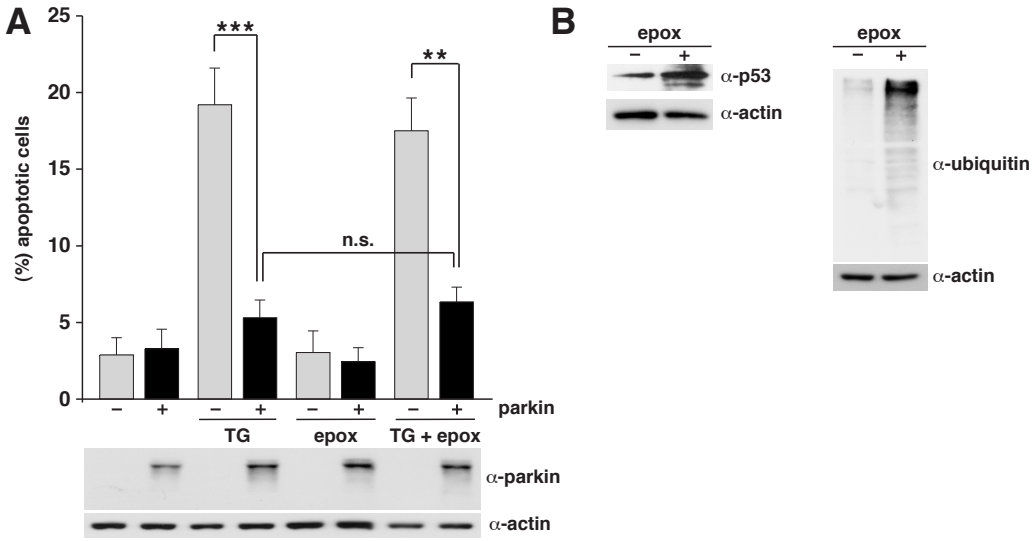


Fig. 42: The protective activity of parkin after ER stress is independent of the proteasome. (A, B) (A) SH-SY5Y cells were cotransfected with EYFP (as a control) and wild-type parkin. 24 h after transfection, cells were incubated with 10 μM TG and/or 0.1 μM epoxomycin (epox) for 8 h, fixed, permeabilized, and then activation of caspase-3 was analyzed as described in Fig. 32. Parkin expression levels were determined by immunoblotting using the anti-parkin PRK8 mAb. Loading was controlled by re-probing the blots for β-actin (lower panel). (*Caspase assays were kindly counted by Kathrin Lutz.*) (B) Efficiency of proteasomal inhibition by epoxomycin was controlled by immunoblotting for p53 (mAb) and for ubiquitin (mAb). Loading was controlled by re-probing the blots for β-actin. *** $p < 0.001$, ** $p < 0.01$, n.s. = not significant.

In addition, we tested the potential of parkin to protect cells from cell death induced by toxic concentrations of proteasomal inhibitors. After treatment with MG132 for 8 hours, apoptotic SH-SY5Y cells were visualized with anti-active caspase-3 antibody. The proteasomal block resulted in an increase of apoptotic cells, however, cells overexpressing parkin showed a reduced apoptotic cell rate (Fig. 43).

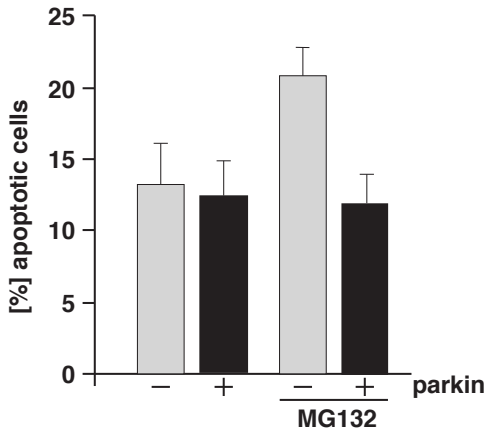


Fig. 43: Parkin is protective against cell death induced by proteasomal inhibition. SH-SY5Y cells were cotransfected with EYFP (as a control) and wild-type parkin. 24 h after transfection, cells were incubated with 5 μM MG132 for 4 h, fixed, permeabilized, and then activation of caspase-3 was analyzed as described in Fig. 32.

3.1.6 Involvement of parkin in protective signaling pathways

The experiments above showed a protective function for parkin after ER stress. In addition, previous experiments also from our group, showed that parkin is also protective after various other stress factors like rotenone or kainic acid. Our analysis demonstrated that the protective activity of parkin was not dependent on the proteasome as postulated recently. How does parkin mediate its protective effect? One hypothesis could be that parkin is influencing major anti-stress pathways. One pathway which is involved in the up-regulation of anti-apoptotic proteins is the NF-κB (nuclear factor kappa enhancer binding protein) pathway. NF-κB transcription factors regulate various biological processes, including apoptosis, differentiation, and immunity. The IκB kinase (IKK) complex, consisting of two catalytic (IKKα and IKKβ) and one regulatory (IKKγ/NEMO [NF-κB essential modifier]) subunit, regulates the degradation-dependent activation of NF-κB by lysine 48 (K48)-linked ubiquitylation of the NF-κB inhibitor IκB. The activation of the IKK complex and other upstream located regulators such as TRAF2 (tumor necrosis factor [TNF] receptor associated factor 2) and TRAF6 is dependent on the degradation independent ubiquitylation by lysine 63 (K63)-linked ubiquitin chains.

To test whether parkin could have an influence on the NF-κB pathway by ubiquitylating IKKγ and/or TRAF2 in a degradation-independent manner, first co-immunoprecipitation experiments and then ubiquitylation assays were performed. Full-length parkin and FLAG-tagged IKKγ or TRAF2 were co-expressed in HEK293T cells and the immunoprecipitation was performed with a polyclonal anti-IKKγ or TRAF2 antibody. Co-precipitated proteins were subjected to SDS-PAGE and immunoblotted

with a monoclonal anti-parkin antibody. IKKγ as well as TRAF2 co-immunoprecipitated together with parkin in a complex. The vector control and parkin transfected without IKKγ (Fig. 44A) or TRAF2 (Fig. 44B) did not show any complex formation. These findings indicate a specific interaction between IKKγ or TRAF2 and parkin in a direct or indirect way.

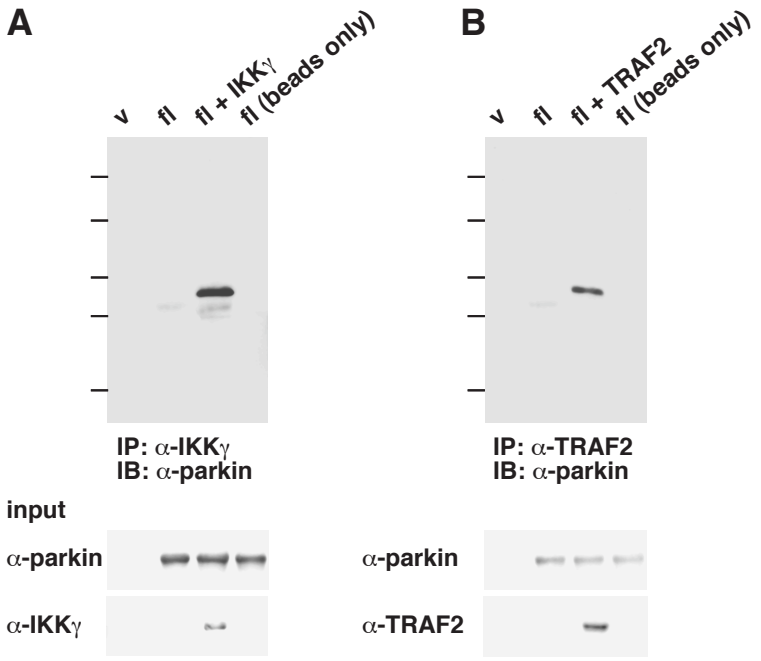


Fig. 44: Parkin is found in a complex together with IKKγ and TRAF2. (A, B) HEK293T cells were cotransfected with full-length (fl) parkin and either IKKγ (A) or TRAF2 (B). One day after transfection cells were harvested, lysed, and cleared by centrifugation. Equal protein amounts of the supernatant were incubated with a pAb against IKKγ or TRAF2 overnight at 4°C. Proteins present in the immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB) with the anti-parkin mAb PRK28. Aliquots of the supernatant were immunoblotted with antibodies against parkin (pAb hP1), IKKγ (mAb), TRAF2 (mAb), respectively (input controls: bottom panels). *(This experiment was performed by Iris Henn.)*

Subsequently, ubiquitylation assays were performed. Therefore, full-length parkin, HA-tagged ubiquitin and FLAG-tagged IKKγ or TRAF2 were co-expressed in HEK293T cells. The immunoprecipitation was done under denaturating conditions with an anti-FLAG antibody followed by Western Blot analysis using an anti-HA antibody. Notably, parkin was able to increase the ubiquitylation of IKKγ or TRAF2 compared to the vector control. Remarkably, the co-expression of the pathogenic parkin mutants ΔUBL and R42P showed a reduced ubiquitylation pattern for IKKγ (Fig. 45A) as well as for TRAF2 (Fig. 45B). In addition, also the mutant TRAF2ΔN, lacking the N-terminal RING domain, essential for its E3 ligase activity, was ubiquitylated by parkin, indicating that the increase of ubiquitylation was not due to autoubiquitylation capacity of TRAF2

itself. On the other hand, the ubiquitylation of TRAF6, located upstream of IKKγ and also regulated by ubiquitylation, was not increased after parkin co-expresssion (Fig. 45C).

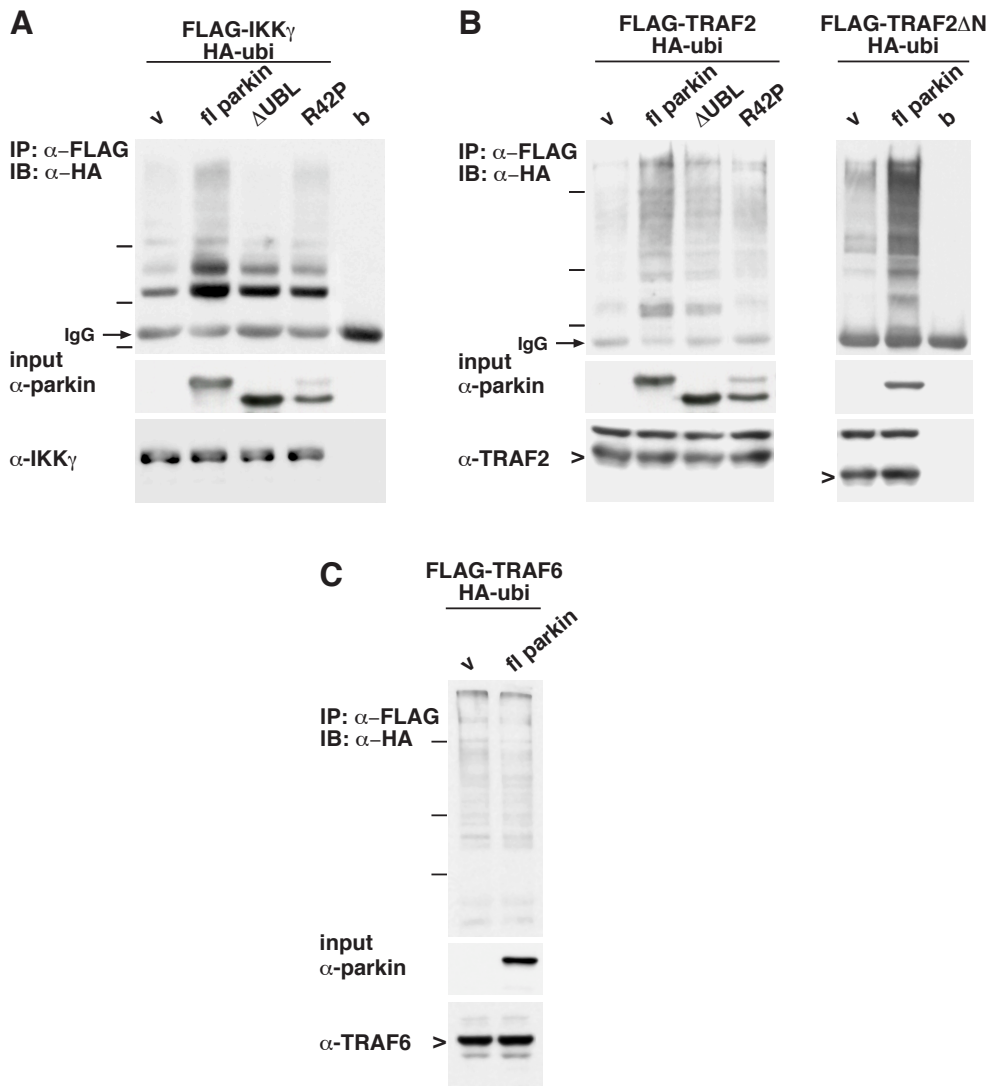


Fig. 45: Parkin promotes ubiquitylation of IKKγ and TRAF2. (A-C) Full-length (fl) parkin, ΔUBL parkin, or R42P, HA-tagged ubiquitin, and either FLAG-tagged IKKγ (A), TRAF2 (B), or TRAF6 (C) were cotransfected into HEK293T cells. At 24 h after transfection, cells were harvested, lysed and cleared by centrifugation. Equal protein amounts of the supernatant were subjected to an immunoprecipitation (IP) under denaturating conditions using an anti-FLAG mAb. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted (IB) with the anti-HA mAb (top panels). Aliquots of the supernatant before the immunoprecipitation were immunoblotted with antibodies against parkin (pAb hP1), IKKγ (mAb), TRAF2 (mAb), and TRAF6 (mAb) respectively (input controls: bottom panels). Molecular size markers are indicated as bars at the left side of the panels and represent 148, 98, and 64 kDa (A, B) or 98, 64, and 50 kDa (C). v: vector control; b: buffer control (no lysate). *(This experiment was performed by Iris Henn.)*

To determine the mode of ubiquitin linkage of parkin-mediated ubiquitylation of IKKγ, ubiquitin mutants were used. These ubiquitin mutants lack all but one lysine, K48-

only or K63-only, thereby allowing only the assembly of ubiquitin chains via lysine 48 or lysine 63, respectively. The ubiquitylation assays were performed with IKKγ as described above. The use of the ubiquitin mutant K48-only reduced the potential of parkin to ubiquitylate IKKγ. In contrast the K63-only mutant promoted ubiquitylation of IKKγ by parkin to a similar extent as wild-type ubiquitin. This finding indicates that parkin preferentially promotes ubiquitylation via K63-linked ubiquitin chains, supporting a non-proteolytic regulatory function of parkin (Fig. 46).

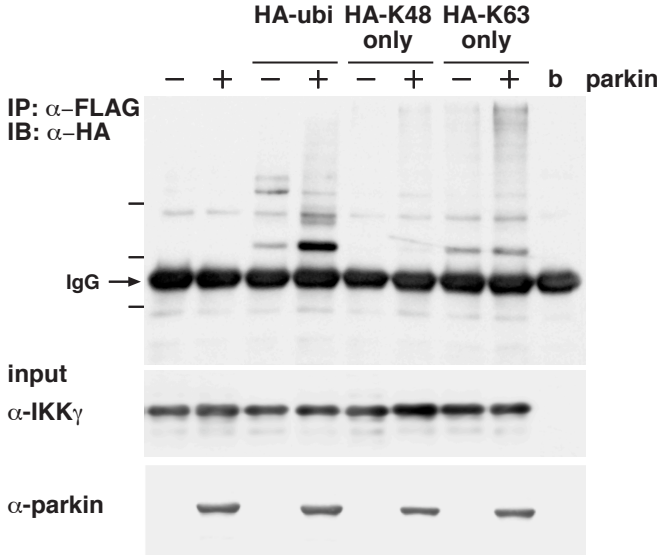


Fig. 46: Parkin preferentially promotes K63-linked polyubiquitylation of IKKγ. Full-length parkin, FLAG-tagged IKKγ, and HA-tagged wild-type ubiquitin, K48-only, or K63-only ubiquitin mutant were cotransfected into HEK293T cells. At 24 h after transfection, cells were harvested and analyzed as described in Fig. 45. b: buffer control (no lysate). *(This experiment was performed by Iris Henn.)*

For further evidence that parkin stimulates NF-κB activation, a reporter assay with a NF-κB regulated inducible promoter in front of a firefly luciferase (NF-κB-luc), in was performed parkin-deficient cells. HEK293T cells were co-transfected with the NF-κB-luc and control or parkin siRNA. After mild stimulation with PMA, an activator of the NF-κB pathway, the NF-κB dependent-expression of the luciferase construct was induced. However, the parkin-deficient cells showed a drastically reduced transcriptional activation than the control siRNA transfected cells, demonstrating that parkin is involved in the regulation of NF-κB activation after the stimulation with PMA (Fig. 47).

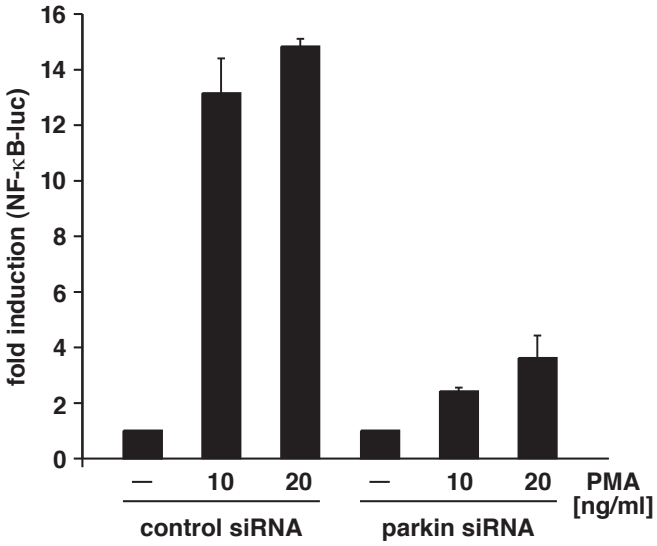


Fig. 47: Loss of parkin compromises NF-κB activation in response to stress. HEK293T cells co-transfected with parkin-specific or control siRNA duplexes and the NF-κB reporter plasmid (NF-κB-luc) were incubated 48 h after transcfection with 10 and 20 ng/ml PMA for 3 h. Cells were harvested and analyzed for luciferase activity 5 h later.

A possible role of NF-κB in protecting cells for ER stress induced cell death was supported by the finding that it is activated after ER stress (Hu et al., 2006; Pahl and Baeuerle, 1995). Therefore, an EMSA was performed, monitoring the NF-κB DNA binding affinity in response to ER stress. The induction of ER stress by thapsigargin lead to a slight increase of NF-κB binding to the NF-κB consensus sequence. Interestingly, the binding affinity of NF-κB was not influenced by parkin as demonstrated with parkin deficient cells. However, compared to other stressors the NF-κB binding capacity after ER stress was quite low, either indicating only a slight increase of NF-κB activation to ER stress or the problematic to find the right time point to look at NF-κB activation, as this is possible a quite transient event. In addition, the NF-κB response is influenced by other factors as the signal differes between untransfected and control transfected cells (Fig. 48). In conclusion, a direct link of parkin in modulating NF-κB activation after ER stress was not dectably by this approach.

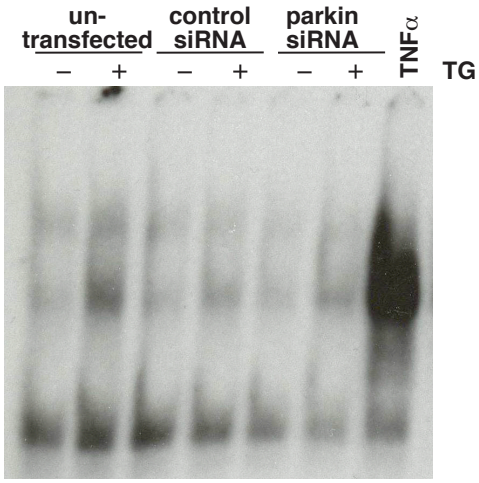


Fig. 48: Parkin has no impact on ER stress induced NF-κB activation. HEK293T cells transcfected with parkin-specific or control siRNA complexes were incubated 48 h after transfection with 5 μM TG or 25 ng/ml TNFα. Cells were harvested 2 h later and extracts were prepared and tested for binding to the ^{32}P-labeled oligonucleotide comprising the common NF-κB binding site by an electrophoretic mobility shift assay (EMSA).

Recent studies indicated a signalling crosstalk between JNK and NF-κB at various levels (Papa et al., 2004). Furthermore, JNK was shown to be activated in response to ER stress. In figure 26C c-Jun binding to the AP1 site was observed after treatment with thapsigargin, supposing an activation of upstream located c-Jun kinase (JNK) in response to ER stress. As we could show that parkin has a positive effect on the activation of the NF-κB pathway, the question arouse whether parkin would also have an impact on the JNK-pathway. To test this hypothesis wild-type parkin and the pathogenic parkin mutants ΔUBL or R42P were co-transfected with a JNK responsive reporter construct. Additionally, the cells were treated with thapsigargin to stimulate JNK activation. The JNK reporter construct was induced after ER stress, however, neither the expression of wild-type parkin nor the expression of the parkin mutants had any influence on the JNK activity under basal or under ER stress conditions (Fig. 49).

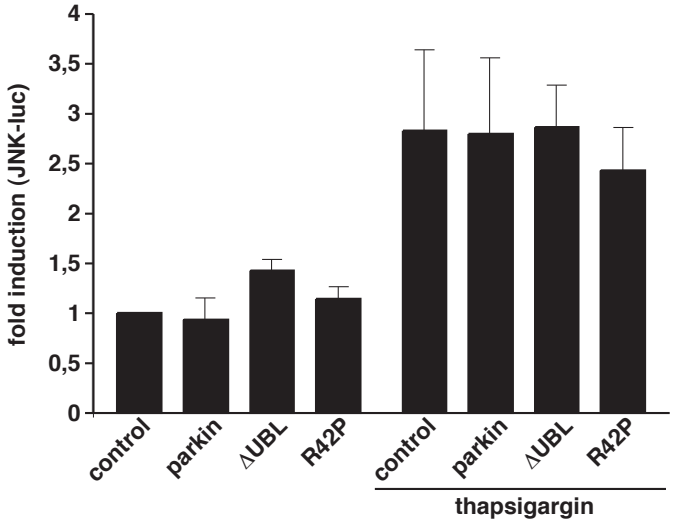


Fig. 49: Parkin has no effect on JNK reporter assay. HEK293T cells were cotransfected with the JNK reporter construct and either GFP, wild-type parkin, or the parkin mutants ΔUBL or R42P. 8h after transfection cells were treated with 1μM thapsigargin for 14h. Shown is the fold induction of luciferase activity in comparison to GFP-expressing control cells.

In a further approach, components of the JNK pathway were tested for their ability to be activated by parkin. The activation of c-Jun and JNK is regulated by phosphorylation, therefore phospho-specific antibodies were used to monitor the activation status. HEK293T cells expressing parkin were incubated under UV for 20 min and the protein lysates were analyzed by Western blotting. JNK and c-Jun were phosphorylated after UV treatment, however the presence of parkin had no influence on the strength of the phosphorylation signal (Fig. 50).

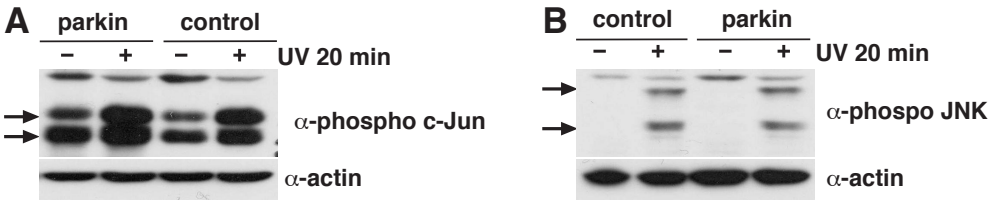


Fig. 50: Parkin has no impact on c-Jun or JNK phosphorylation. HEK293T cells were transfected with parkin or GFP as control. The next day cells were exposed to UV light for 20 min. Cells were lysed in cell lysis buffer containting phosphatase and protease inhibitors and immunoblotted using pAb against α-phospho c-Jun or α-phospho JNK. Loading was controlled by reprobing the blots for β-actin.

Interestingly, the knockdown of parkin resulted in transcriptional up-regulation of the JNK suppressing protein A20 (also called tumour necrosis factor alpha induced protein 1), especially under ER stress conditions. This observation points to a compensatory mechanism of JNK suppression in the absence of parkin (Fig 51).

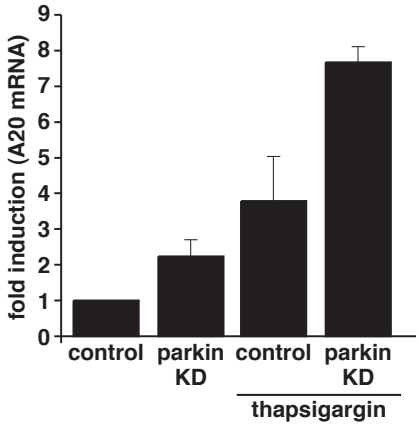


Fig. 51: A20 mRNA is up-regulated in parkin-deficient cells. SH-SY5Y cells were reversly transfected with parkin or control siRNA duplexes. Three days after transfection cells were treated with 1 μM thapsigargin and harvested 14 h later. Total cellular RNA was isolated and subjected to quantitative RT-PCR using A20-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of A20-specific mRNA in comparison to the untreated control.

Another survival mechanism of the cell that could be influenced by parkin is the autophagic-lysosomal pathway. Autophagy is an intracellular, lysosome-mediated degradation process for damaged cellular constituents, including membranes, organelles, and proteins, thereby contributing to the maintenance of cellular homeostasis (Dice, 2007). It is also an adaptive survival mechanism that is activated in response to various environmental challenges (Levine, 2005; Shintani and Klionsky, 2004). As a marker for autophagy activity, the microtuble-associated protein 1 light chain 3 (LC3) is used. During autophagy, the cytoplasmic form LC3-I (18kDa) is processed and recruited to the autophagosomes, where the smaller form LC3-II (16kDa) is generated by site-specific proteolysis and lipidation near the C-terminus. The amount of LC3-II correlates well with the number of autophagosomes, as they are transient structures, resulting in a short half-life for LC3-II. We were now interested whether parkin had an influence on autophagy after stress. As our previous experiments showed an up-regulation of parkin after ER stress and in addition, autophagy was reported to be induced after ER stress, we chose to look at LC3 levels after thapsigargin treatment. The autophagy inhibitor chloroquine (CQ) was used to analyze the autophagic flux. Chloroquine disrupts lysosomal function inhibiting the acid-dependent degradation of autophagosome contents, which results in the accumulation of autophagic vesicles that cannot be cleared. Parkin deficient HEK293T cells were incubated with thapsigargin or chloroquine and analyzed by immunoblotting for LC3. As expected, the treatment with chloroquine resulted in a strong increase of LC3-II, demonstrating an accumulation of autophagic vesicles containing the autophagosome bound LC3-II from. In parallel also the treatment with thapsigargin resulted in an increase of the LC3-II band, indicating an activation of autophagy after ER stress. However, the knockdown of parkin had only a slight influence on the ratio between LC3-II and LC3-I level and was not significantly changed under ER stress conditions (Fig. 52). Collectively, parkin does not seem to play a role for the induction of autophagy after ER stress.

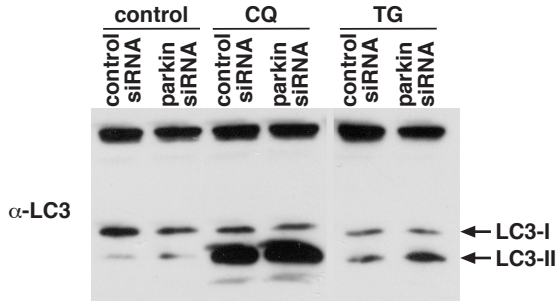


Fig. 52: Parkin has no significant impact on autophagy after ER stress. HEK293T cells were transfected with control or parkin siRNA and treated after 48 h with 50 μM chloroquine (CQ) or 1 μM TG for 15 h. Cell lysates were lysed in 0.5% Triton X-100 and analyzed by immunoblotting using a pAb against LC3. The Western blot image was re-arranged by excluding one line, as indicated by a white line; all samples originate from one gel.

3.1.7 Parkin maintains mitochondrial integrity after ER stress

In conclusion, so far our study could not show a direct involvement of parkin in the activation of pro-survival pathways such as the NF-κB-pathway and the autophagic-lysosomal-pathway or inhibition of pro-apoptotic pathways such as the JNK-pathway after ER stress to mediate its protective function. Interestingly, ER stress was shown to lead to alterations in the morphology of mitochondria (Hom et al., 2007). Additionally, loss-of-parkin was demonstrated to influence mitochondria integrity (Lutz et al., 2009). Therefore we asked the question, whether parkin could have an impact on mitochondrial integrity after ER stress. The mitochondrial morphology was imaged by fluorescence microscopy in living SH-SY5Y cells after treatment with thapsigargin or tunicamycin for 6 or 15 hours. Under normal physiological conditions the majority of cells showed a network of tubular mitochondria, only about 30% of mitochondria are truncated or fragmentated. Upon ER stress the percentage of fragmented mitochondria was significantly increased up to 70%. Longer exposure to ER stress resulted in a slight increase in the amount of fragmented mitochondria. Remarkably, increased expression of parkin could significantly reduce ER stress-induced mitochondrial fragmentation (Fig. 53A,B).

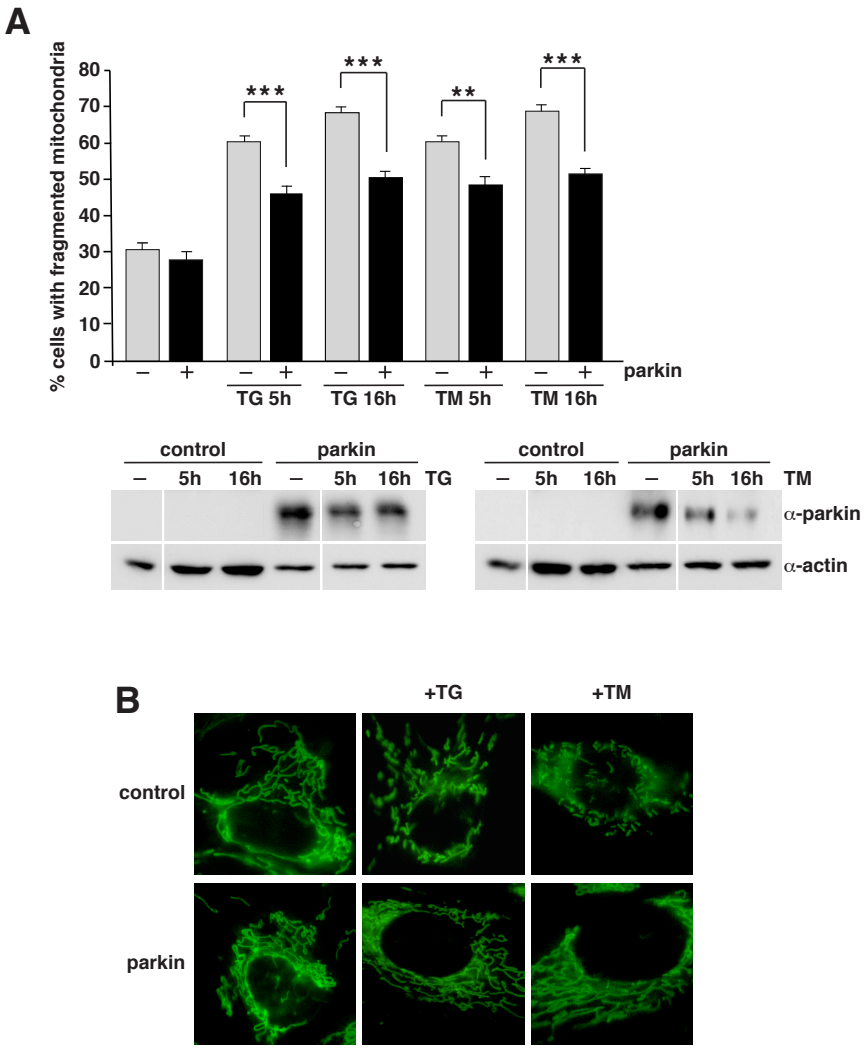


Fig. 53: Increased expression of parkin suppresses ER stress-induced mitochondrial fragmentation. (A, B) SH-SY5Y cells were transfected with parkin or mCherry (as a control). One day after transfection, the cells were treated with 1 μM TG or 2 μg/ml TM for 5 or 16 h. Mitochondria were visualized by life cell microscopy after incubating cells with the fluorescent dye $DiOC_6(3)$. (A) The mitochondrial morphology was classified as tubular, in case of an intact network of tubular mitochondria or as fragmented, when this network was disrupted and mitochondria appeared predominantly spherical or rod-like. Shown is the percentage of cells with fragmented mitochondria. Quantifications were based on three independent experiments. Per experiment ≥ 300 cells per coverslip of triplicate samples were assessed. Expression levels of parkin were analyzed by immunoblotting using the anti-parkin pAb 2132. Loading was controlled by re-probing the blots for β-actin (lower panel). (B) Examples of mitochondrial morphologies of the experiment described in (A). Treatment of cells with TG or TM cause a disruption of the tubular mitochondrial network, which can be suppressed by increased parkin expression. *(The experiment was kindly done by Kathrin Lutz.)* *** $p < 0.001$, ** $p < 0.01$.

The effect of parkin on mitochondrial morphology was demonstrated to be specific as the anti-apoptotic protein Bcl-2 failed to rescue the ER stress-induced fragmented mitochondria phenotype (Fig. 54A). ER stress also had an impact on the mitochondrial function. The cellular ATP production was drastically decreased in tunicamycin treated cells. Interestingly, the additional knockdown of parkin worsened

the mitochondrial energy production after ER stress. Obviously, parkin maintains mitochondrial integrity and, remarkably, even under ER stress it is able to protect mitochondria by preventing mitochondrial morphology alterations and changes in their bioenergetics (Fig. 54B). *(Mitochondria morphology assays and the ATP assays were kindly performed by Kathrin Lutz.)*

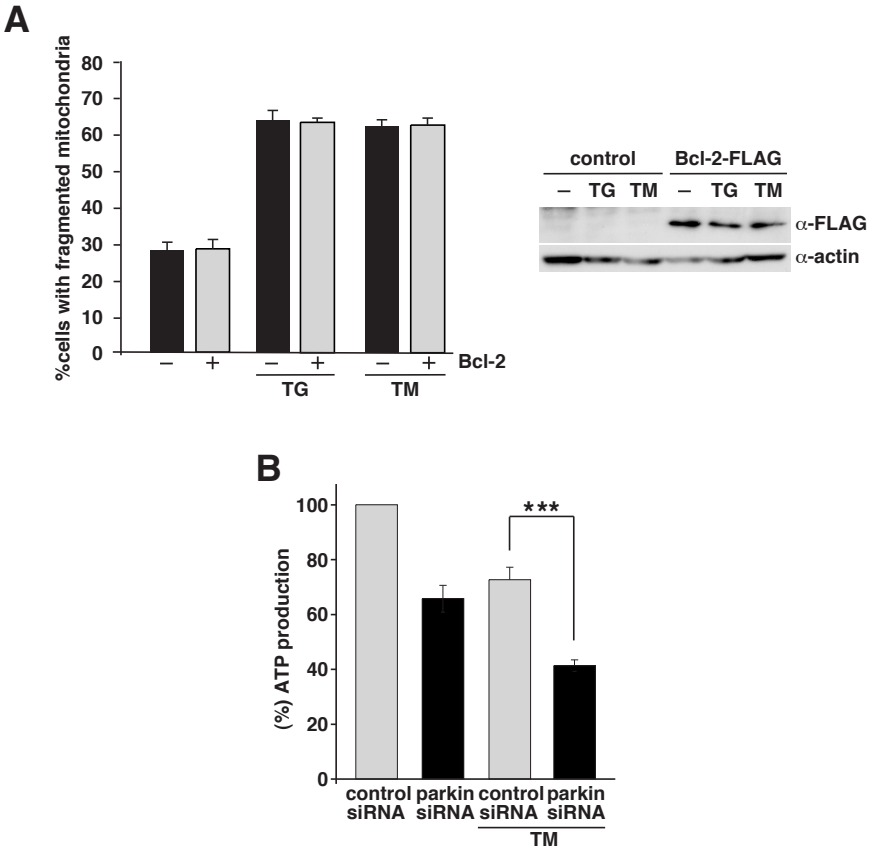


Fig. 54: The effect of parkin on mitochondrial integrity after ER stress is specific and parkin deficiency increases mitochondrial dysfunction. (A) Anti-apoptotic Bcl-2 has no effect on ER stress-induced mitochondrial fragmentation. SH-SY5Y cells were transfected with Bcl-2-FLAG. One day after transfection, the cells were treated with 1 μM TG or 2 μg/ml TM for 5 h. Mitochondria were visualized, categorized and quantified as described in Fig. 53. Expression level of Bcl-2 was analyzed by immunoblotting using the anti-Flag M2 mAb. Loading was controlled by re-probing the blots for β-actin (right panel). **(B)** Parkin deficiency increases ATP depletion in response to ER stress. SH-SY5Y cells were reversly transfected with either parkin or control siRNA duplexes. Three days after transfection the cells were treated with 2 μg/ml TM for 5 h and the steady state cellular ATP levels were measured. *(The experiment was kindly done by Kathrin Lutz.)* *** $p < 0.001$.

3.2 REGULATION OF PD-ASSOCIATED GENES IN RESPONSE TO PATHOPHYSIOLOGICAL STRESS CONDITIONS

3.2.1 Parkin gene expression is stress-regulated

Our studies showed an up-regulation of parkin in response to ER stress. We wondered now whether parkin is also regulated in response to other stressors especially *as* parkin has been shown to maintain neuronal integrity under various moderate stress conditions for example excitotoxicity, oxidative stress and proteasomal inhibition in different model systems. In a first attempt we used two stress conditions, which play a prominent pathophysiological role in PD, namely the complex-I inhibiton induced by rotenone and excitotoxicity induced by kainic acid. Experiments from our group demonstrated for both stressors a protective function of parkin (Henn et al., 2007). SH-SY5Y cells were treated with either rotenone or kainic acid and the parkin-specific mRNA levels were analyzed by quantitative real-time PCR (Fig. 55A). The real-time PCR revealed that parkin mRNA levels were significantly increased in response to mitochondrial and excitotoxic stress with a maximal increase 5-8 hours after drug treatment. In parallel, the rotenone induced increase of parkin mRNA was translated into elevated endogenous parkin protein levels (Fig. 55B).

In addition, stress induction of parkin mRNA was also observed in primary cortical neurons derived from embryonic mouse brain after treatment with glutamate for 6 hours. Glutamate is an excitotoxic stressor like kainic acid (Fig. 55C).

To test whether parkin up-regulation was due to an induction of the UPR, the park-luc construct was tested in response to kainic acid and rotenone treatment in SH-SY5Y cells. Both stressors did not result in an increase in the transcriptional response of luciferase, indicating an ATF4 independent mechanism (data not shown).

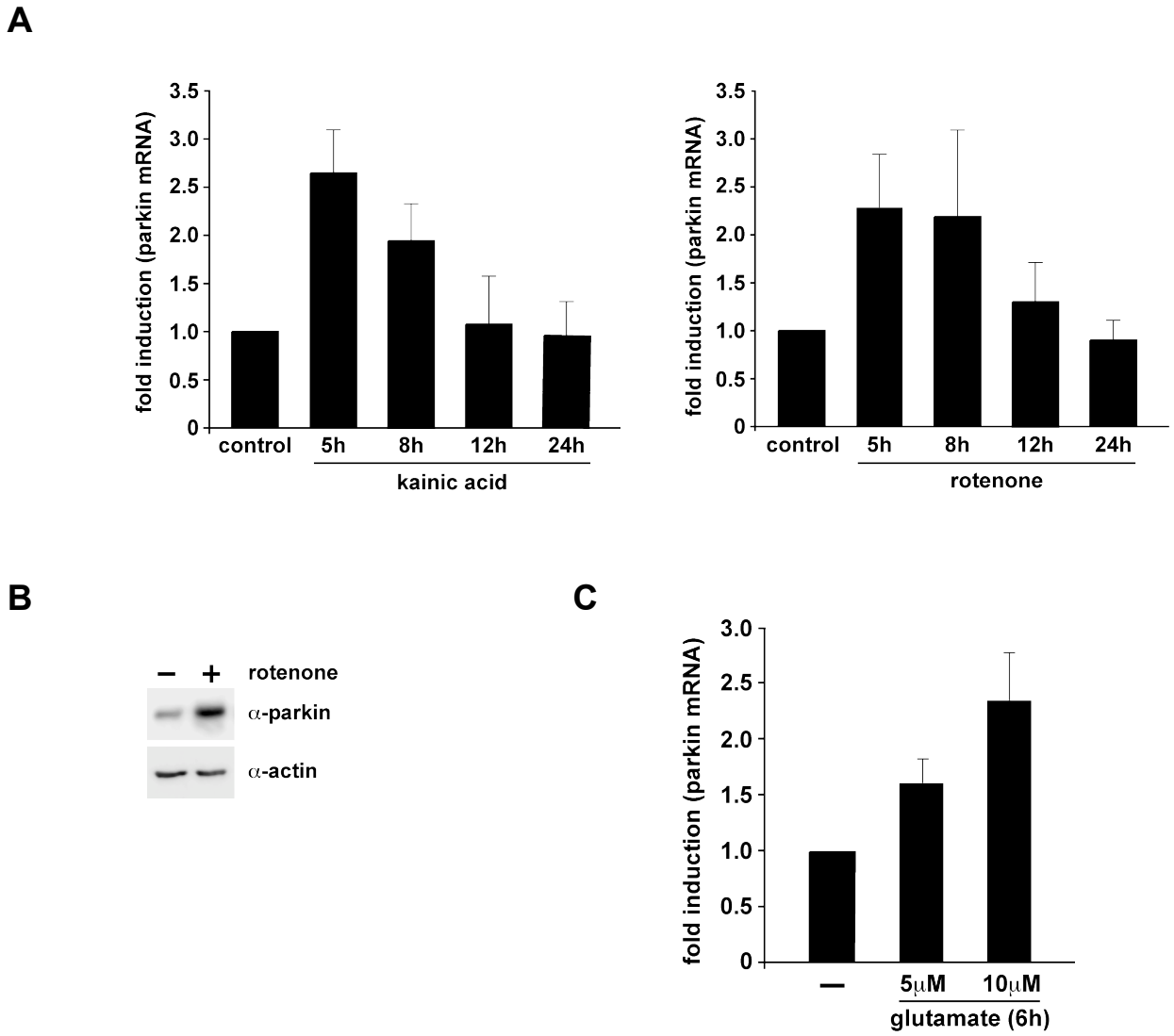


Fig. 55: Parkin is up-regulated in response to mitochondrial and excitotoxic stress. (A) Parkin mRNA is up-regulated in SH-SY5Y cells after excitotoxic and oxidative stress. SH-SY5Y cells were stressed with 50 μM kainic acid (left panel) or 1 μM rotenone (right panel) for 3 h and harvested 5, 8, 12 and 24 h after drug treatment, respectively and analyzed as described in Fig. 16 for quantitative RT-PCR using parkin-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of parkin-specific mRNA in rotenone- or kainic acid-treated cells in comparison to the untreated control. **(B)** The increase of parkin mRNA was translated into elevated endogenous parkin protein levels. SH-SY5Y cells were treated with rotenone (1 μM, 3 h) or mock-treated, and expression of endogenous parkin was analyzed by Western blotting using the PRK8 mAb. **(C)** Parkin mRNA in primary neurons is up-regulated in response to excitotoxic stress. Primary cortical neurons derived from embryonic rat brain were incubated with glutamate (5 or 10 μM) for 6 h and then analyzed as described in (A).

Next, dopamine was tested as a potential stressor and inducer of parkin mRNA expression. Dopamine is the principal neurotransmitter produced in the neurons of the *substantia nigra*. It is a highly reactive molecule that possesses a great propensity for oxidation to form multiple reactive oxygen species. High doses of dopamine were found

to covalently modify and functionally inactivate parkin (LaVoie et al., 2005), therefore a low dopamine concentration was used to stimulate the cells (80 μM). SH-SY5Y cells treated with dopamine showed more than a 2,5-fold up-regulation of their parkin mRNA after 24 hours (Fig. 56A).

The association of parkin to the proteasome system and due to the fact that it is able to protect cells from cell death induced by proteasomal inhibition (Fig. 42 and 43) provokes the question, whether parkin expression is also regulated after proteasomal inhibition. The proteasome was inhibited by the treatment with MG132. Interestingly, in contrast to the other tested stressors proteasomal stress did not lead to an up-regulation of parkin mRNA (Fig. 56B).

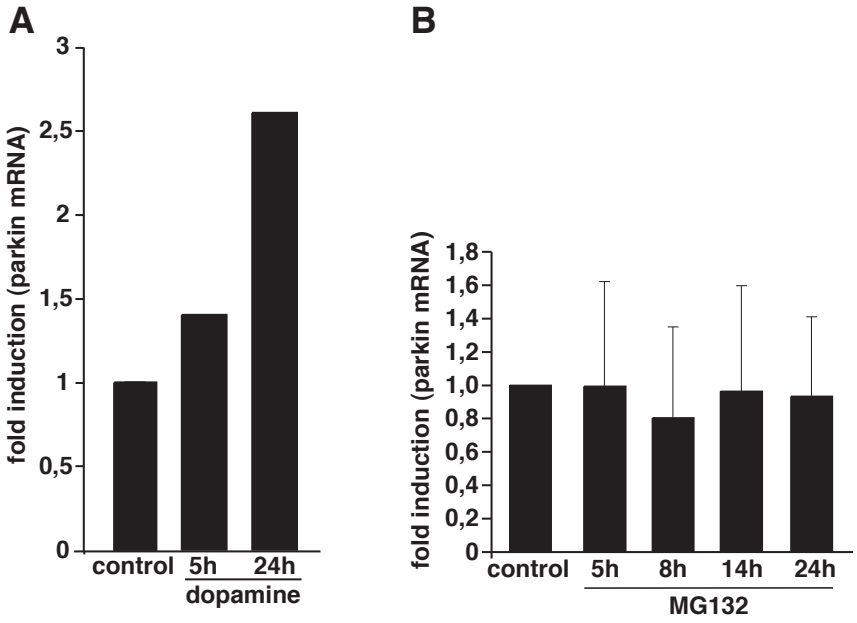


Fig. 56: Parkin mRNA is up-regulated in response to dopamine treatment, but not after protesomal inhibition. (A) Parkin mRNA expression responds to dopamine treatment. SH-SY5Y cells were treated with 80 μM dopamine for 3 h and harvested after 5 h or 24 h and analyzed as described in Fig. 16 for quantitative RT-PCR using parkin-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase of parkin mRNA in response to dopamine treatment. **(B)** Parkin mRNA is not up-regulated after proteasomal inhibition. SH-SY5Y cells were treated with 30 μM MG132 for 3 h and harvested after 5, 8, 14 or 24 h, respectively and analyzed as described in Fig. 16 for quantitative RT-PCR using parkin-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of parkin-specific mRNA in MG132-treated cells in comparison to the untreated control.

3.2.2 PACRG is up-regulated in response to ER stress

The parkin co-regulated gene (PACRG) is transcribed from the same bi-directional promoter as parkin, but on the opposite DNA strand in the opposite direction, suggesting some common regulatory binding motifs used for both genes. We were now interested whether PACRG transcription is up-regulated in response to stress treatment to the same extent as parkin transcription. As an example, PACRG expression was examined after ER stress. The treatment with thapsigargin resulted in a 2,7-fold increase of PACRG mRNA compared to the non-treated control (Fig. 57A). To exclude that parkin directly influences the expression of PACRG, PACRG mRNA expression in parkin deficient cells was examined. The reduced expression of parkin had no impact on the expression of PACRG under normal conditions (Fig. 57B) as well as under ER stress conditions (data not shown).

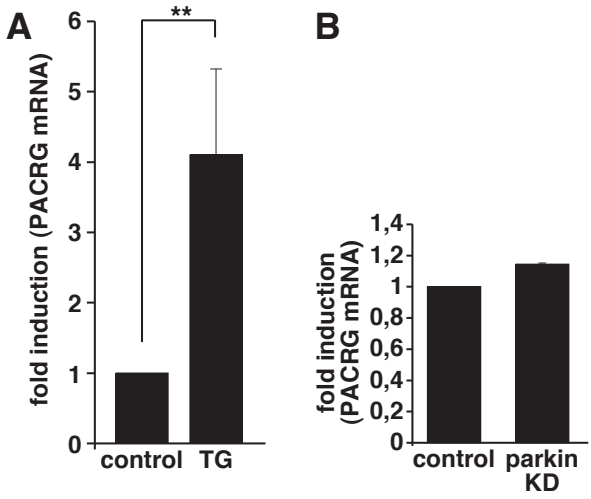
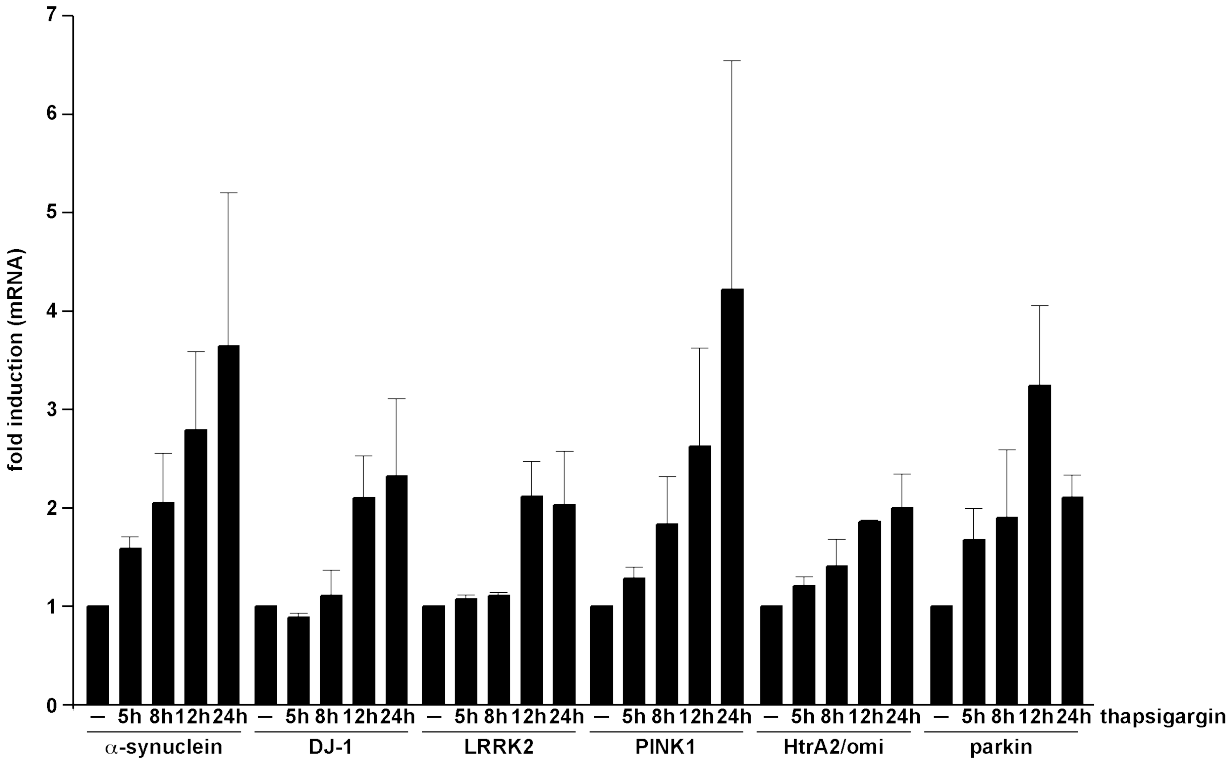


Fig. 57: PACRG is up-regulated in response to ER stress, but PACRG transcription is not dependent on parkin. (A) PACRG mRNA is up-regulated after ER stress. SH-SY5Y cells were treated with 1 μM TG for 14 h. Total cellular RNA was isolated and subjected to quantitative RT-PCR using PACRG-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of PACRG-specific mRNA in comparison to the untreated control. **(B)** Parkin deficiency has no effect on PACRG mRNA expression. SH-SY5Y cells were reversely transfected with parkin or control siRNA duplexes. Three days after transfection cells were harvested and analyzed as described in A. Shown is the fold increase of PACRG-specific mRNA in parkin-deficient cells compared to control transfected cells.

3.2.3 α-synuclein, DJ-1, PINK1, LRRK2 and HtrA2/omi are up-regulated in response to ER stress

Besides parkin other PD-associated genes are known. At least thirteen chromosomal loci have been associated with monogenic familial variants of PD, among them the genes coding for α-synuclein, DJ-1, PINK1, LRRK2 and HtrA2/omi. As parkin showed an impressive up-regulation after various stressors, the question was addressed whether these PD-associated genes are also regulated in response to stress.

In a first attempt, the expression of these PD-associated genes was tested after ER stress. ER stress was induced by treatment of the cells with either thapsigargin (upper panel) or tunicamycin (lower panel). All PD-associated genes tested showed an up-regulation of their mRNA. Remarkably, for all genes, the mRNA expression was still increased after 24 hours (Fig. 58).



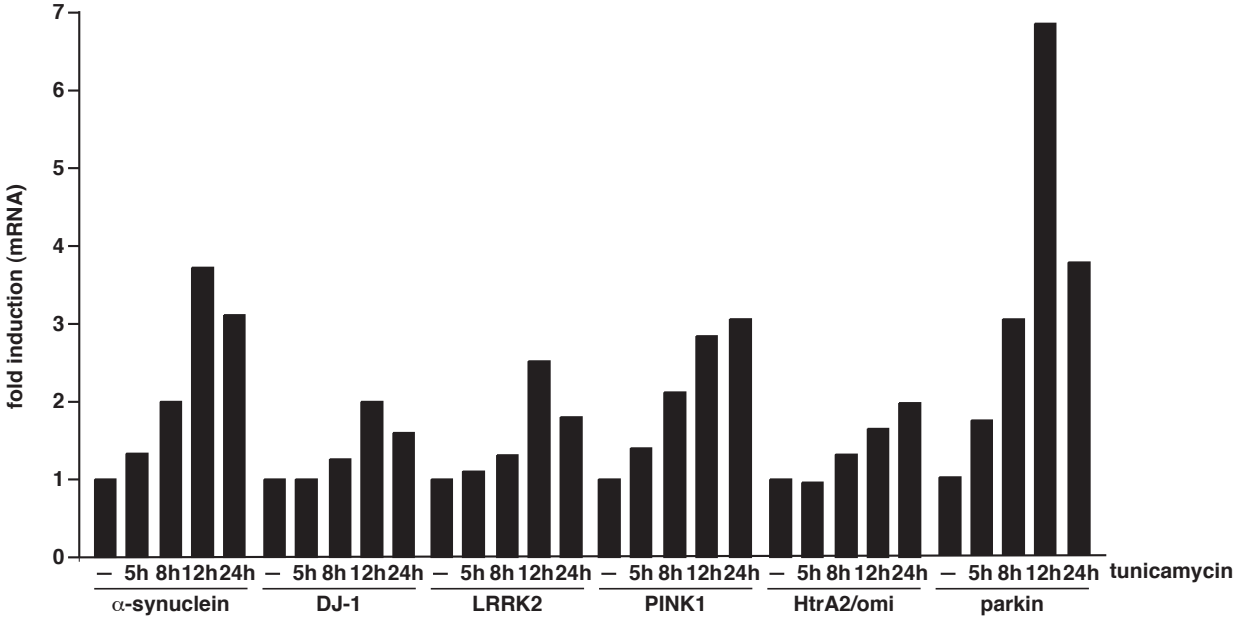


Fig. 58: α-synuclein, DJ-1, PINK1, LRRK2, and HtrA2/omi mRNA is up-regulated in response to ER stress. SH-SY5Y cells were incubated with 1 μM TG (upper panel) or 2 μg/ml TM (lower panel) for 5, 8, 12 and 24 h, respectively and analyzed as described in Fig. 16 for quantitative RT-PCR using α-synuclein, DJ-1, PINK1, LRKK2, HtrA2/omi and parkin-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of mRNA in comparison to the untreated control. In addition, parkin mRNA in response to ER stress is shown.

The up-regulation of parkin after ER stress was shown to be specifically regulated by the PERK/ATF4 branch of the UPR, therefore the mRNA of α-synuclein (Fig. 59A), DJ-1 (Fig. 59B), PINK1 (Fig. 59C) and HtrA2/omi (Fig. 59D) was examined after the knockdown of ATF4. However, the quantitative RT-PCR revealed that the knockdown of ATF4 had no influence on the up-regulation of their mRNA after ER stress. In summary, in contrast to the regulation of parkin expression after ER stress the regulation of the PD-associated genes DJ-1, HtrA2/omi, PINK1 and α-synuclein is not dependent on the PERK/ATF4 branch.

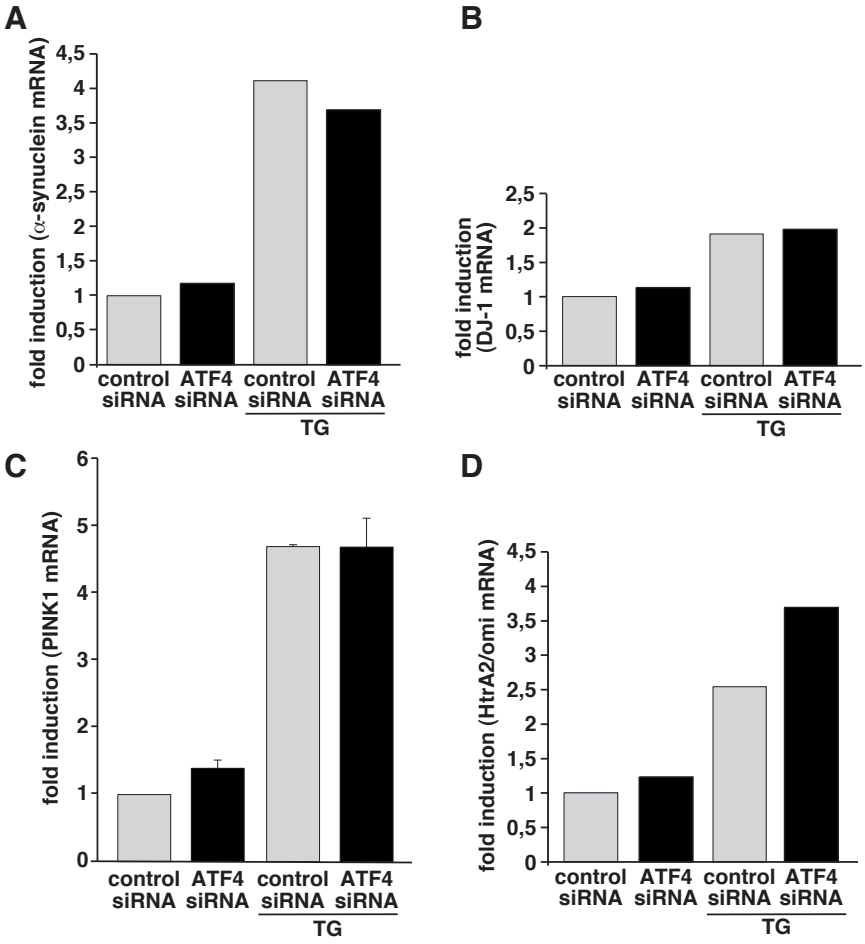


Fig. 59: α-synuclein, DJ-1, PINK1, and HtrA2/omi are not regulated by ATF4. (A-D) SH-SY5Y cells were reversely transfected with ATF4-specific or control siRNA duplexes. 2 days later cells were transfected again with siRNA duplexes and followed by incubation of 1 μM TG for 16 h. The cells were harvested and analyzed as described in Fig.16 for quantitative RT-PCR using α-synuclein, DJ-1, PINK1, or HtrA2/omi-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of mRNA in comparison to the untreated control.

3.2.4 The expression of α-synuclein, DJ-1, PINK1, LRRK2 and HtrA2/omi is not changed in response to mitochondrial stress, excitotoxicity and dopamine treatment

In a next step, SH-SY5Y cells were stressed with rotenone or kainic acid. Remarkably, in contrast to parkin all the other examined PD-associated genes, α-synuclein, DJ-1, PINK1, LRRK2 and HtrA2/omi were not induced after rotenone (Fig. 60A) or kainic acid (Fig. 60B) treatment. In addition, also the treatment with dopamine did not result in a significant up-regulation of their mRNA in contrast to parkin (Fig. 60C).

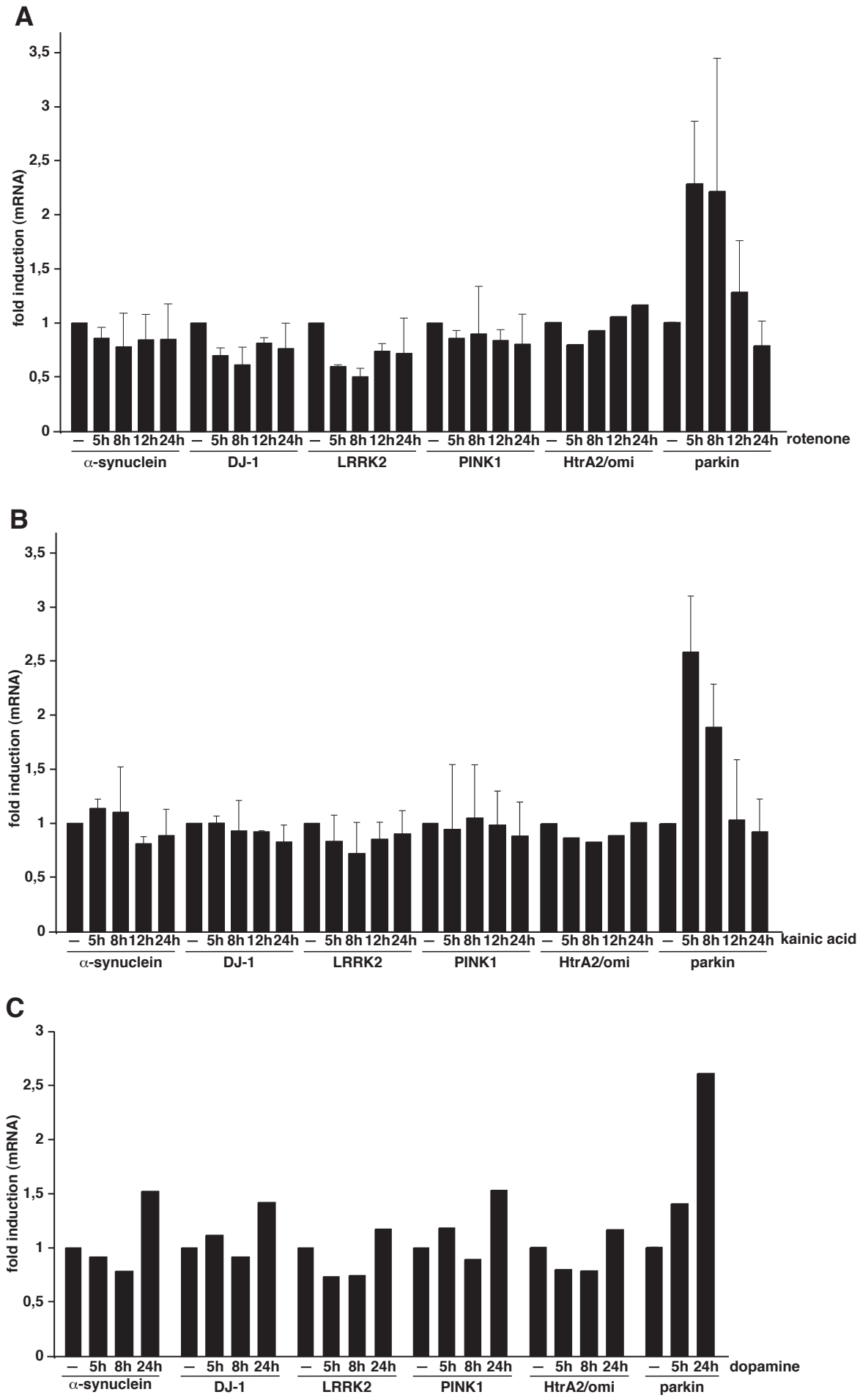

A
fold induction (mRNA)
3,5
3
2,5
2
1,5
1
0,5
0
– 5h 8h 12h 24h
α-synuclein
DJ-1
LRRK2
PINK1
HtrA2/omi
parkin
rotenone
B
fold induction (mRNA)
kainic acid
C
fold induction (mRNA)
– 5h 8h 24h
dopamine

Fig. 60: α-synuclein, DJ-1, PINK1, LRRK2, and HtrA2/omi mRNA is not up-regulated in response to mitochondrial stress, excitotoxic stress, or dopamine treatment. (A-C) SH-SY5Y cells were incubated with 1 μM rotenone (A), 50 μM kainic acid (B) or 80 μM dopamine (C) for the indicated time and analyzed as described in Fig. 16 for quantitative RT-PCR using α-synuclein, DJ-1, PINK1, LRKK2, HtrA2/omi or parkin-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of mRNA in comparison to the untreated control. In addition, parkin mRNA in response to these stressors is shown.

3.2.5 PINK1 and parkin expression are not transcriptionally linked to each other

Several studies with different models indicated a genetic link between parkin and PINK1, suggesting that parkin acts downstream of PINK1 (Clark et al., 2006; Park et al., 2006; Yang et al., 2006a). As PINK1 is up-regulated after ER stress similarly to parkin, we asked the question whether the regulation of parkin and PINK1 expression might be dependent of each other. Therefore, skin fibroblasts from patients with two different PINK1 homozygous mutations (Q126P and G309D) were examined for their parkin mRNA and protein levels. The mutations were shown to impair PINK1 function (Exner et al., 2007). From the fibroblast line carrying the mutation Q126P two different patients were analyzed with their respecitve controls. Unfortunately, the results were not consistent between the two patients. The first mutation carrier showed an increase in parkin expression on the mRNA (Fig. 61A) as well as on the protein level (Fig. 61B) compared to his age-matched control. In contrast, the second carrier did not show an increase in parkin expression on the mRNA and protein level, when compared to his age-matched control. However, comparison of this latter patient with the control fibroblasts matching to the first patient revealed an increase in parkin mRNA and protein. For the patient carrying the second PINK1 mutation, G309D, an elevated level of parkin mRNA as well as parkin protein was observed compared to the age-matched control (Fig. 61C).

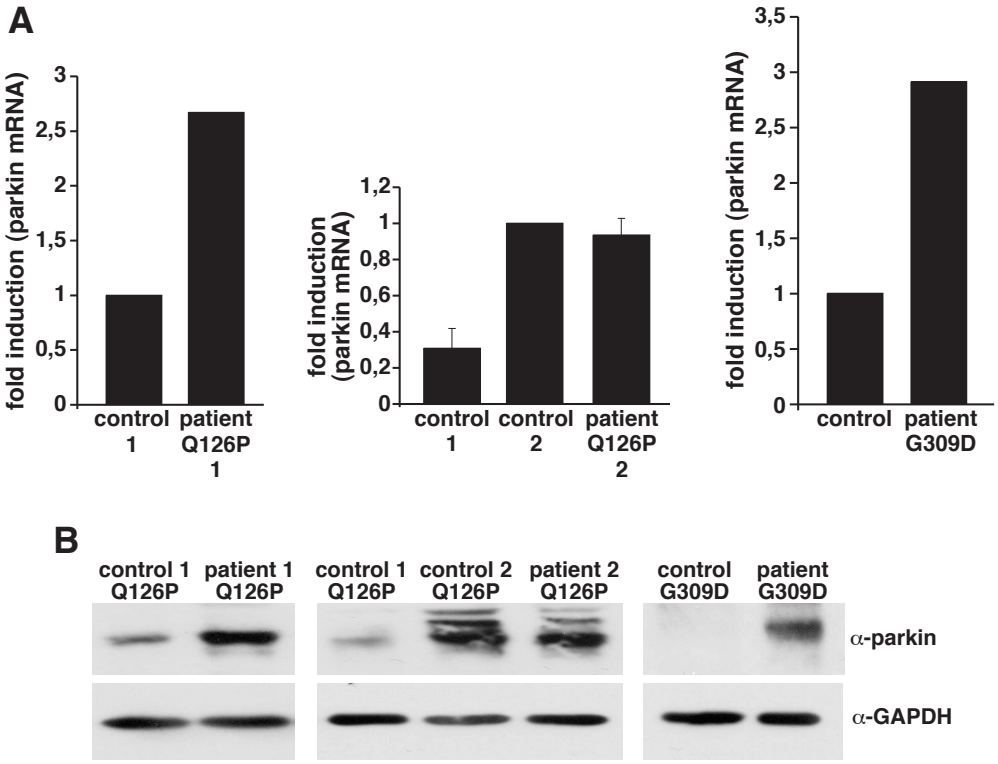


Fig. 61: Parkin expression in fibroblasts of PD patients carrying PINK1 mutations is not consistently up-regulated. (A) Different PINK1 mutation carrier differ in their parkin mRNA content. Fibroblasts of controls or PD patients carrying the PINK1 Q126P (left and middle panel) or PINK1 G309D (right panel) mutation were analyzed as described in Fig. 16 for quantitative RT-PCR using parkin-specific primers. The amount of RNA of each sample was normalized with respect to GAPDH (Glycerinaldehyde-3-phosphate-dehydrogenase). Shown is the fold increase in the amount of parkin-specific mRNA in fibroblasts derived from PINK1 mutation carriers in comparison to the age-matched control fibroblasts. Right panel: Shown is the parkin mRNA of the first PD patient carrying the Q126P (patient Q126P 1) mutation in comparison to his age-matched control (control 1). Middle panel: Shown is the parkin mRNA amount of the second Q126P mutation carrier (patient Q126P 2) in comparison to his age-matched control (control 2), and to the control 1. Left panel: Shown is the parkin-specific mRNA amount in the PD patient carrying the G309D mutant compared to his age matched control. **(B)** The parkin mRNA is translated into corresponding parkin protein levels; different PINK1 mutation carrier differ in their parkin protein amount. Expression of endogenous parkin in fibroblasts of controls or PD patients carrying the PINK1 Q126P (left and middle panel) or PINK1 G309D (right panel) mutation were analyzed by Western blotting using the anti-parkin mAb PRK8. Loading was controlled by re-probing the blots for GAPDH. Right panel: Shown is the parkin protein level of the first PD patient carrying the Q126P (patient 1 Q126P) mutation in comparison to his age-matched control (control 1 Q126P). Middle panel: Shown is the parkin protein amount of the second Q126P mutation carrier (patient 2 Q126P) in comparison to his age-matched control (control 2 Q126P), and to the control 1 Q126P. Left panel: Shown is the parkin protein amount in the PD patient carrying the G309D mutant compared to his age matched control.

To extent our study, the brain of PINK1 knockout mice, carrying a homozygous truncation after exon 1 was analyzed (Morais et al., 2009). The analysis of the parkin mRNA by quantitative RT-PCR did not show an increase compared to wild-type mice (Fig. 62A). In addition, also the transient knockdown of PINK1 in SH-SY5Y cells did not

lead to an increase of parkin mRNA or protein (Fig. 62B). In summary, these results argue for a PINK1-independent regulation of parkin. The different amounts of parkin expression in the PINK1 and control fibroblasts could be due to individual variations in parkin expression and/or to variations in the isolation of fibroblasts. *(PINK1 knockout and wild-type mice were provided by Anne Röthig; mice brains were prepared with experimental help of Anita Schlierf.)*

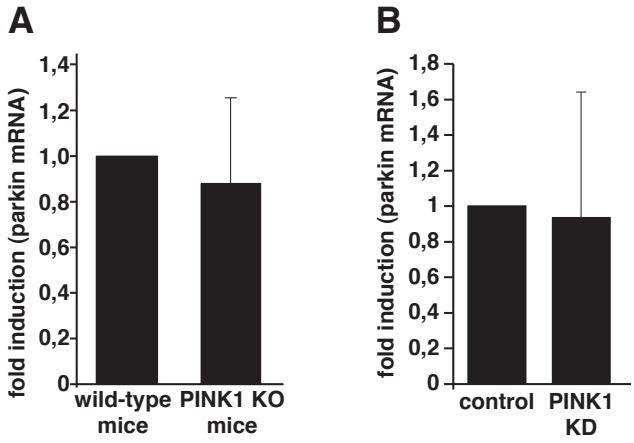


Fig. 62: PINK1 deficiency in mice and humans does not lead to an increase in parkin expression. (A) Parkin mRNA levels are not changed in PINK1 knockout mice compared to wild-type mice. Brains of PINK1 knockout mice and wild-type mice were homogenized, lysed in trizol and total cellular RNA was isolated and subjected to quantitative RT-PCR using parkin-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of parkin-specific mRNA in knock out mice in comparison to age-matched wild-type mice. **(B)** PINK1 deficiency has no impact on parkin mRNA amount. SH-SY5Y cells were reversely transfected with control or PINK1-specific siRNA duplexes. 3 days later cells were harvested and analyzed as described in Fig.16 for quantitative RT-PCR using parkin-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of parkin mRNA in PINK1-deficient cells in comparison to control transfected cells.

To test whether, parkin could have an influence on PINK1 expression, PINK1 mRNA was quantified by RT-PCR in parkin-deficient cells. The knockdown of parkin did not result in a change of PINK1 mRNA expression (Fig. 63A). Subsequently, fibroblasts, from a patient carrying a homozygote exon two deletion of the parkin gene, resulting in an undetectable parkin expression (Nakaso et al., 2006), were checked for their PINK1 mRNA content. Also in this case no increase of PINK1 mRNA could be found (Fig. 63B). *(Parkin fibroblast cells were provided by Kazuhiro Nakaso.)*

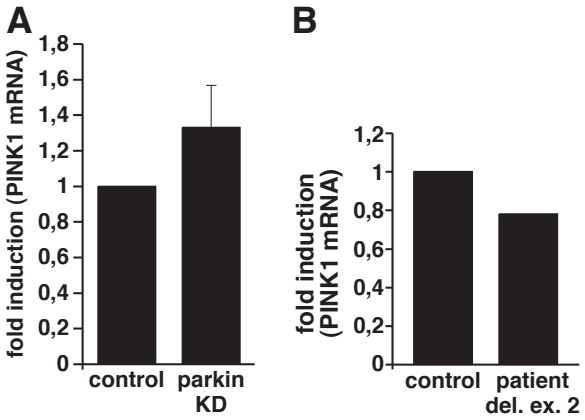


Fig. 63: Parkin deficiency has no impact on PINK1 expression. (A) Downregulation of parkin has no influence on the PINK1 mRNA amount. SH-SY5Y cells were reversely transfected with control or parkin-specific siRNA duplexes. 3 days later cells were harvested and analyzed as described in Fig.16 for quantitative RT-PCR using PINK1-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of PINK1 mRNA in parkin-deficient cells in comparison to control transfected cells. **(B**) Fibroblasts from PD patients carrying an exon 2 deletion of the parkin gene show the same PINK1 mRNA amount as age-matched control fibroblasts. Fibroblasts of controls or PD patients carrying the parkin exon 2 deletion were analyzed as described in Fig. 16 for quantitative RT-PCR using PINK1-specific primers. The amount of RNA of each sample was normalized with respect to β-actin. Shown is the fold increase in the amount of PINK1-specific mRNA in fibroblasts derived from a parkin deletion patient in comparison to the age-matched control fibroblasts.

3.2.6 Parkin point mutations lead to misfolding

Accumulating evidence indicates that misfolding of pathogenic parkin mutations is the major mechanism of inactivation. So far, mainly C-terminal deletion mutations have been tested for their capacity to misfold. In this study, pathogenic parkin point mutations were analyzed to check whether they are likewise capable for misfolding and aggregation. The parkin point mutations C212Y and C289G were recently described in PD patients (Gu et al., 2003; Morales et al., 2002). To test if these mutations are prone to misfolding, the mutations were introduced by PCR into wild-type parkin and tested for their detergent solubility and for their cellular distribution. To analyze the folding, wild-type and mutant parkin were transiently transfected into N2a cells and the cells were lysed in detergent buffer containing 0.1% Triton X-100. The cell lysates were fractionated by centrifugation into detergent-soluble (supernatant) and detergent insoluble fraction (pellet) and analyzed by immunoblotting. In contrast to wild-type parkin, the pathogenic parkin mutants were mainly present in a detergent insoluble confirmation (Fig. 64A). The indirect immunoflourescence of transiently transfected SH-SY5Y cells showed in contrast to wild-type parkin that the mutants are distributed in

aggregates scattered throughout the cytosol. These finding indicates that also parkin point mutations can lead to parkin-loss of function due to misfolding and aggregation (Fig. 64B).

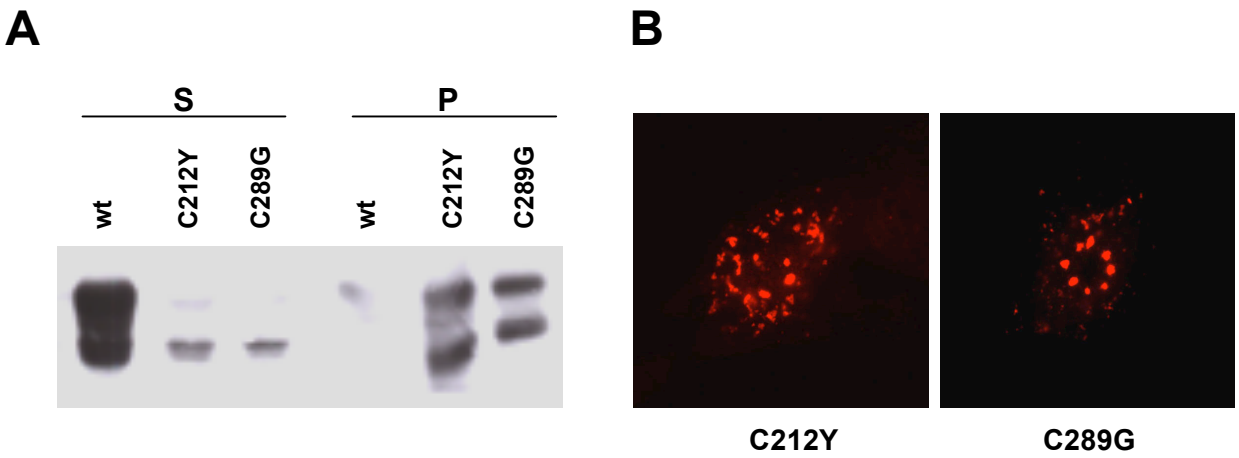


Fig. 64: Pathogenic parkin point mutations lead to misfolding. (A) The parkin point mutations C212Y and C289G lead to misfolding. N2a cells were transiently transfected with wild-type parkin (wt) or the pathogenic parkin mutants C212Y and C289G, harvested, lysed in 0.1% Triton X-100. Detergent-soluble (S) and insoluble (P) fractions were obtained by centrifugation and analyzed by Western blotting. Parkin was detected by the hP1 antiserum. **(B)** The parkin point mutations C212Y and C289G lead to aggregation of mutant parkin in the cell. SH-SY5Y cells transiently expressing wt parkin or the parkin mutants C212Y and C289G were analyzed by indirect immunofluorescence using the anti-parkin antiserum hP1.

4 Discussion

4.1 PARKIN IS A TARGET OF THE UNFOLDED PROTEIN RESPONSE AND PROTECTS CELLS FROM ER STRESS-INDUCED MITOCHONDRIAL DAMAGE

Parkinson's Disease (PD) is characterized by a preferential and progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (*SNpc*). The major question, which arises, is why are these cells particularly vulnerable, more than other cells and also than other neurons? Dopaminergic neurons are exposed to intrinsic stress, due to the enzymatic and non-enzymatic metabolism of dopamine (Maker et al., 1981). In the course of dopamine metabolism reactive oxygen species (ROS) are generated, which can damage proteins, lipids and DNA, resulting in severe damage for the cell such as restriction in mitochondrial energy generation as well as in the endoplasmatic reticulum (ER) folding capacity (Raha and Robinson, 2000). But also other intrinsic stressors like excitoxicity have an influence on mitochondrial integrity and the function of the ER (Ruiz et al., 2009; Sokka et al., 2007; Yu et al., 1999). In addition, also extrinsic stressors like the environmental toxins MPTP, rotenone or paraquat were shown to damage mitochondria leading to ROS production and ER stress (Conn et al., 2004; Ghribi et al., 2003; Holtz and O'Malley, 2003; Holtz et al., 2006; Holtz et al., 2005; Ryu et al., 2002; Yamamuro et al., 2006). If the damage of cellular processes is too severe, apoptotic pathways are activated, which result in the death of dopaminergic cells. Both mitochondria and the ER are crucial organelles in regulating these elementary pathways and therefore are playing both a major role in the pathology of PD.

Accumulating evidence over the last years demonstrates parkin as an important regulator of cell protective processes after stress (Winklhofer, 2007), which might explain why dopaminergic neurons are particularly vulnerable to a loss of parkin function. In this study we were interested to gain more insight into the role of parkin after ER stress with emphasis on its protective capacity and the possible involvement of mitochondria.

Our study revealed that parkin is a low abundant protein under normal conditions but is highly up-regulated in response to ER stress. We could show that this up-regulation is mediated by the binding of ATF4 (activating transcription factor 4) to the parkin promoter. ATF4 is a transcription factor the translation of which is induced by phosphorylated eIF2α (eucaryotic initiation factor 2α). eIF2α is specifically phosphorylated by different kinases such as the GCN2 (general control non-derepressible-2) kinase, mainly activated by amino acid starvation, the double-stranded

RNA-activated protein kinase (PKR), which participates in an anti-viral defence mechanism, the HRI (haem regulated inhibitor) kinase that is activated by haem deprivation or the PKR-like ER kinase (PERK), which is activated after ER stress (Wek et al., 2006). We showed that ER stress induced by tunicamycin or thapsigargin as well as starvation results in an increase of parkin mRNA and protein levels in several cell lines including primary mouse neurons. Furthermore, increased expression of ATF4 and also PERK can directly support the transcription from the parkin promoter. Moreover, a dominant negative mutant of ATF4 interferes with the transcriptional up-regulation of parkin after ER stress. These findings were further supported by following *in vivo* experiments: First, the knockdown of ATF4 resulted in a reduced parkin mRNA induction after ER stress. Second, a ChIP (chromatin immunoprecipitation) experiment demonstrated an increased binding of ATF4 to the parkin promoter after ER stress.

Moreover, our experiments revealed a second factor bound to the parkin promoter. We identified this transcription factor as c-Jun. However, in contrast to ATF4 this factor reduces the activation of the parkin promoter and even more also interferes with the activation potential of ATF4. In agreement with these findings, the knockdown of c-Jun results in an increase of parkin mRNA already under normal conditions, but particularly after ER stress, indicating that c-Jun is a transcriptional repressor of parkin. What might be the physiological relevance of this observation? c-Jun could terminate ATF4 mediated up-regulation of parkin. This hypothesis is supported by the finding that ATF4 can recruit c-Jun to the parkin promoter but not *vice versa*. After ER stress first cell survival pathways are activated to restore cellular homeostasis, in case of severe and prolonged ER stress pro-apoptotic pathways are induced to favour the elimination of irreversible damaged cells (Kim et al., 2006; Szegezdi et al., 2006). Several studies implicate an activation of the IRE1 (inositol requiring enzyme)/TRAF2 (TNF receptor associated factor 2)/ASK1 (apoptosis signal-regulating kinase 1) pathway after severe ER stress, resulting in the activation of JNK (c-Jun N-terminal kinase) (Nishitoh et al., 2002; Urano et al., 2002) and other pro-apoptotic factors like Bim, Bax (Bcl-2 associated x protein) and Bak (Bcl-2 antagonist/killer) (Kim et al., 2008a; Kim et al., 2006; Szegezdi et al., 2006). c-Jun activated by the JNK pathway could bind to the parkin promoter, interfere with the activation potential of ATF4 after ER stress and lead to the suppression of the protective protein parkin to push the cellular fate towards apoptosis. This hypothesis is supported by the fact that neuronal cultures from PERK deficient mice revealed an increased sensitivity to treatment with 6-hydroydopamine (6-OHDA), suggesting that neurons lacking PERK were unable to mount a proper unfolded protein response (UPR), including the up-regulation of parkin and therefore were more vulnerable to 6-OHDA induced cell death (Ryu et al., 2002). The PERK

pathway is responsible for the early UPR response, which seems to have mainly a protective potential, while a sustained ER stress later through IRE1 signalling leads to the suppression of proteins that inhibit cell death, activation of proteins that induce cell death such as JNK and CHOP (C/EBP homologous protein) and to a suppression of the protective PERK branch by $p58^{IPK}$ (Kim et al., 2008a; Kim et al., 2006; Szegezdi et al., 2006). Of note, CHOP is also regulated by the PERK pathway, however, for its full pro-apoptotic function further stimuli such as modification or increased transcriptional activity are necessary (Harding et al., 2003; Scheuner et al., 2001; Szegezdi et al., 2006; Wang et al., 1996).

The involvement of the JNK-pathway in the suppression of parkin expression is supported by the finding that JNK3, a JNK isoform primarily found in the brain, heart and testes, is also able to interfere with the transcriptional up-regulation of parkin after ER stress. The JNK pathway is implicated in neuronal cell death and may contribute to the loss of neurons in neurodegenerative diseases (Bogoyevitch et al., 2004; Peng and Andersen, 2003; Waetzig and Herdegen, 2004). Interestingly, parkin was shown to suppress JNK activity in several cellular models and in studies with *Drosophila melanogaster* (Cha et al., 2005; Hasegawa et al., 2008; Jiang et al., 2004; Liu et al., 2008; Ren et al., 2009). However, we were not able to show in preliminary experiments a direct impact of parkin on the JNK pathway as analyzed by phosphorylation studies with c-Jun and JNK as well as by a JNK-reporter assay. These negative results can be due to the transient activation of c-Jun and the timing and conditions of analysis. It is necessary to perform an in depth analysis by using different stressors in different concentrations and varying the stress conditions. In addition, compensatory mechanism could decrease the effect of parkin on JNK, as demonstrated in this study for A20, which is transcriptionally up-regulated in parkin deficient cells and is able to suppress JNK activity.

It is therefore tempting to speculate whether there is a reciprocal interaction between parkin and the JNK-pathway. Dependent on the cellular context and the severity of stress conditions, parkin could attenuate JNK signalling and thereby shift the balance towards cell survival. In case JNK3 is gaining the upper hand pro-apoptotic pathways are induced and in parallel pro-survival pathways are suppressed, for example by inhibiting the protective protein parkin.

To further support the hypothesis that parkin is a protective protein playing a role for pro-survival pathways, parkin was tested for its ability to protect cells from ER stress-induced cell death. We could clearly show that overexpression of parkin reduces ER stress-induced cell death, whereas a loss of parkin results in increased apoptosis in response to ER stress. Interestingly, the pathogenic parkin mutants fail to prevent ER

stress-induced cell death. These findings demonstrate that parkin is an important factor for the cell to switch the gear towards cell survival and that the loss of parkin and also mutations in the parkin gene can lead to higher vulnerability of the cell to ER stress. Notably, the protective function of parkin seems not to be dependent on the transcriptional regulation of anti-apoptotic genes, as the knockdown of parkin had no effect on the transcription of the anti-apoptotic proteins XIAP (x-linked inhibitor of apoptosis protein), $Bcl2_XL$ and MIHC (Inhibitor of apoptosis protein 1) (data not shown).

But still the question is open how parkin mediates this protective effect for the cell? One possibility could be a direct impact of parkin on the severity of ER stress. As parkin has an E3 ubiquitin ligase activity and was reported to ubiquitylate proteins for proteasomal degradation parkin could play a role in the ER-associated degradation (ERAD) machinery, thereby reducing the amount of misfolded proteins in the ER resulting in a reduction of ER stress. However, our studies showed that the loss of parkin, neither acute nor permanent, does cause ER stress. Furthermore, also induced parkin expression is not able to decrease the severity of ER stress. Interestingly, the protective activity of parkin is independent of the proteasome, as parkin was still protective after ER stress when the proteasome was blocked. These findings speak against a role of parkin in the ERAD machinery and stand in contrast to the believed opinion that parkin ubiquitylates its substrates for degradation and thereby reduces the toxic protein load for the cell as for example reported for the PAEL-receptor (PAEL-R) by Y. Imai and colleagues (Imai et al., 2001). Notably, another mechanism to remove misfolded or unfolded proteins leading to reduced ER stress is autophagy. However, also for this pathway we were not able to show in preliminary experiments an influence of parkin.

Which activity of parkin might then be responsible for its protective effect under ER stress? The pro-survival NF-κB (nuclear factor kappa enhancer binding protein) pathway is reported to be activated after ER stress (Deng et al., 2004; Hu et al., 2006; Kaneko et al., 2003). In the course of our study we could show that parkin activates the NF-κB pathway. Parkin is able to interact with two factors involved in the NF-κB signalling cascade, TRAF2 and IKKγ (IκB kinase γ or NEMO). Both proteins are activated by lysine 63 (K63)-linked ubiquitination. In contrast to K48-linked ubiquitin, which marks proteins for proteasomal degradation, K63-linked ubiquitin regulates the activity of proteins. Our analysis showed that parkin is able to increase K63-linked ubiquitiylation of IKKγ as well as TRAF2. Furthermore, the loss of parkin leads to a decrease of NF-κB activity after PMA (phorbol 12-myristate 13-acetate) stimulation, supporting a role for parkin in the activation of NF-κB. However, we were not able to show in preliminary experiments a direct involvement of parkin in the activation of the

NF-κB pathway after ER stress. This could be due to the difficulties to see NF-κB activation in response to ER stress in our assays. Compared to other stressors, the signal was quite weak, either indicating only a slight increase in the response of NF-κB to ER stress or difficulties in finding the right time point to look at NF-κB activation, as this is possibly a highly transient event. Due to the weak signal it was difficult to observe a difference between parkin knockdown and control cells in their activation potential. Yet, it is still possible that parkin might have an impact on modulating NF-κB activation after ER stress. Of course, our experiments need follow up studies to provide evidence or to rule out the possibility for a direct influence of parkin on this pro-survival pathway.

To increase the understanding of the mechanism, which is underlying the protective capacity of parkin, it could be necessary to pay attention to all organells involved after ER stress and not only to the ER alone in an isolated manner. The ER is closely associated with mitochondria providing the conditions for a local and privileged communication between the two organelles (Pizzo and Pozzan, 2007). Specifically, we and others could show that ER stress leads to a disruption of mitochondrial integrity. The mitochondrial morphology is changed as well as their energy production reduced. Accumulating evidence also from our group indicates an essential role of parkin in maintaining mitochondrial integrity (Lutz et al., 2009). Remarkably, our study found that parkin is indeed able to restore mitochondrial morphology and influence cellular ATP production, demonstrating that parkin is able to prevent pathophysiological consequences of ER stress on mitochondrial integrity. Obviously, this effect is not mediated by an impact of parkin on the transcription of various mitochondrial proteins, such as the mitochondrial fusion protein Drp1 (dynamin related protein 1), NAD(P)H (2,4-dienoyl CoA reductase 1; DECR1), an accessory enzyme for β-oxidation and metabolism of unsaturated fatty enoyl-CoA esters, the ctytochrome c oxidase subunit II (cox2) of the mitochondrial respiratory chain, the mitochondrial chaperone mortalin (MOT), the ATP-dependent mitochondrial matrix protease LON and the antioxidant enzyme MnSOD2 (mitochondrial superoxide dismutase 2) (data not shown).

But how is parkin able to mediate this effect? A key player in this interorganellar crosstalk seems to be Ca^{2+} (Pizzo and Pozzan, 2007; Rizzuto et al., 2009; Rizzuto and Pozzan, 2006). The ER acts as the intracellular calcium storage. Upon stimulation resident Ca^{2+} channels are opened, resulting in a Ca^{2+} efflux to the cytosol. As ER and mitochondria are in close contact to each other Ca^{2+} is ultimately taken up by the mitochondria. This direct Ca^{2+} exchange between these two organelles regulates various cellular processe, ranging from the induction of mitochondrial ATP production,

regulation of subcellular processes by Ca^{2+}-dependent enzymes, controlling of mitochondrial movement and the activation of apoptotic signals (Celsi et al., 2009). Especially for the latter scenario mitochondria seem to play an important role. In case of massive and/or a prolonged accumulation of Ca^{2+} in the mitochondria, cell death pathways are activated. It is tempting to speculate that parkin could have an effect on the prevention of Ca^{2+} overload or its consequences, thereby preventing the induction of apoptotic pathways. This hypothesis is supported by the finding that the loss of PINK1, acting possibly in the same pathway, yet upstream of parkin, leads to mitochondrial Ca^{2+} overload, which results in cell death (Gandhi et al., 2009).

Regarding the fact that cells have to adapt to increasing stress, parkin could also play a key role in circumventing the induction of cell death to the adaptation to stress. Besides the melioriation of protein folding and processing the cell needs to regulate the threshold for the execution of the apoptotic cascades to adapt to chronic stress (Lin et al., 2007; Rutkowski et al., 2003) here parkin could enter the stage for example by influencing JNK activity or the sensible calcium homeostasis. As chronic stress is also implicated to play a major role in sporadic PD and loss of parkin function is associated with early onset parkinsonism, it is tempting to speculate, whether parkin dysfunction could also have an impact on sporadic PD by loosing the adaptation potential of dopaminergic neurons to prolonged stress.

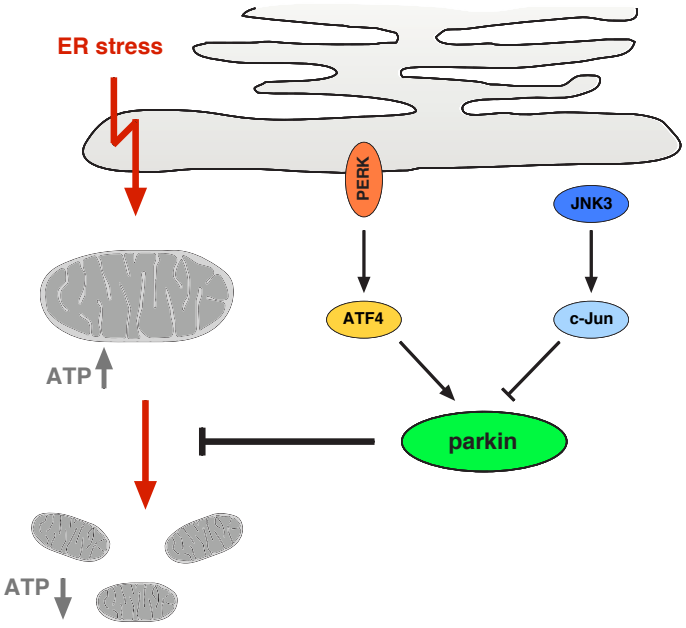


Fig. 65: Model: Parkin and ER stress. In response to ER stress parkin is transcriptionally up-regulated via the PERK/ATF4 branch of the UPR by binding of ATF4 to a specific site within the parkin promoter. Increased parkin expression alleviates the cellular consequences of ER stress by preventing ER stress-induced mitochondrial fragmentation and ATP depletion. Activation of the JNK3/c-Jun pathway leads to transcriptional repression of parkin gene expression, probably to terminate ATF4-mediated up-regulation of parkin or to inhibit pro-survival pathways under conditions of severe ER stress, when cells are irreversibly damaged.

4.2 REGULATION OF PD-ASSOCIATED GENES IN RESPONSE TO PATHOPHYSIOLOGICAL STRESS CONDITIONS

As mentioned above, dopaminergic neurons are exposed to a variety of intrinsic and extrinsic stressors. Different lines of evidence indicate exactly under those stress triggers, such as mitochondrial dysfunction, excitotoxicity, ER stress, proteasome inhibition and overexpression of tau, α-synuclein or expanded polyglutamine fragments, a remarkable potential of parkin to protect neuronal cells (Moore, 2006; Winklhofer, 2007). In line with a central role of parkin in mainting neuronal viability, the question arises, whether the cell includes also in its responding cell stress management program the induction of parkin expression? In this study different stressors were chosen, playing an important role in the pathogenesis of PD. First, we tested rotenone, a mitochondrial stressor, which can easily cross the blood brain barrier, and blocks complex-I of the mitochondrial electron transport chain. Inhibition of complex-I causes mitochondrial dysfunction associated with increased oxidative stress. Mitochondria dysfunction plays a central role in the pathogenesis of PD, starting with the early observation that the accidental toxication of young drug addicts with the complex-I inhibitor MPTP can cause parkinsonism (Langston et al., 1983). Second, excitotoxicity was induced by kainic acid. Kainic acid binds to the non-NMDA-glutamate-receptor and stimulates the opening of cation channels, leading to the influx of Ca^{2+}. Overstimulation of glutamate receptors leads to an uncontrolled influx of Ca^{2+}, resulting in excitotoxicity. As glutamate is an important neurotransmitter in the brain, changes in the glutamate concentration, the damage of the NMDA-receptor or the glutamate transporter can have severe effects on the cell, reported to accompany neurological disorders. Third, dopamine was used to induce stress. Dopamine is the principal neurotransmitter produced in the neurons of the *substantia nigra*. It is a highly reactive molecule that possesses a great propensity for oxidation to form multiple reactive oxygen species. As fourth stressor MG132 was used, which blocks the proteasome. As parkin is reported to be an E3 ligase that might ubiquitinate proteins for degradation, this experiment was of special interest for us. In addition, the ubiquitin-

proteasome system is thought to play an important role in the pathogenesis of PD, for example the formation of Lewy bodies is seen as a failure of the cell to degrade misfolded or aggregated proteins (Conway et al., 2000; Miller et al., 2004; Tanaka et al., 2004).

Mitochondrial stress, excitotoxicity as well as dopamine treatment resulted in an increase of parkin mRNA expression in a human neuroblastoma-derived cell line as well as in primary neurons. Please note that in the first part of this study we could already observe an up-regulation of parkin after ER stress in different cell lines. Remarkably, the up-regulation of parkin expression after treatment with kainic acid and rotenone was independent of ATF4 activation, as the reporter construct containing the ATF4-binding site of the parkin promoter was not induced in response to these stressors, pointing to an ER stress-independent activating pathway. These results demonstrate a wide response of parkin expression to neuronal stressors and point to a role of parkin in protecting the cell against various insults, indicating a broad protective capacity.

Interestingly, parkin expression is not significantly up-regulated after the inhibition of the proteasome. Nevertheless, parkin is able to protect the cell for proteasomal stress as shown in the first part of this work. One possible explanation could be that proteasomal inhibition is not a normally *in vivo* occurring physiological stress and therefore the cell did not develop a specific transcriptional program in response to this stressor. Excitotoxicity, ER and mitochondrial stress, however, are stressful insults, which normally occur in the cell *in vivo*, therefore transcription responds to these stressors.

In the course of our study we were also interested in the transcriptional regulation of other PD-associated genes, such as DJ-1, PINK1, α-synuclein, HtrA2/omi and LRRK2 in response to different stress triggers. Notably, our analysis showed that only parkin gene expression is regulated by mitochondrial stress and excitotoxicity. These results are quite surprising, as especially PINK1, as a mitochondrially located kinase, is strongly associated with maintaining mitochondrial integrity (Clark et al., 2006; Exner et al., 2007; Gandhi et al., 2009; Gegg et al., 2009; Marongiu et al., 2009; Morais et al., 2009; Park et al., 2006; Poole et al., 2008), and DJ-1 has been shown to protect cells against dopamine, MPP+ and oxidative stress-induced cell death (Canet-Aviles et al., 2004; Kim et al., 2005; Martinat et al., 2004; Taira et al., 2004; Yokota et al., 2003) and is believed to be a sensor for oxidative stress (Kinumi et al., 2004). Additionally, HtrA2/omi was shown to protect from rotenone-induced cell death (Martins et al., 2004). Remarkably, all PD-associated genes studied are induced after ER stress, pointing to an important role of ER stress in PD. This hypothesis is supported by

the finding that dopaminergic neurons in the *substantia nigra* of sporadic PD cases show an increased phosphorylation of PERK and eIF2α, indicating an activation of the UPR in these patients (Hoozemans et al., 2007). Futhermore, the observation suggests also a possible involvement of all or at least a part of the examined familial PD genes in common pathways, as already demonstrated for parkin and PINK1 (further discussed below). However, in contrast to parkin, DJ-1, HtrA2/omi, PINK1 and α-synuclein expression is not regulated by the PERK/ATF4 pathway, indicating a different mechanism for the transcriptional up-regulation in response to ER stress. Nevertheless, the analysis of the PINK1 promoter could not reveal a binding site for another ER stress induced transcription factor.

Several lines of evidence also from our group indicate that parkin and PINK1 function in a common genetic pathway. Parkin is able to compensate for the PINK1 loss-of-function phenotype in *Drosophila* and different cellular models but not *vice versa*, implying that parkin acts downstream of PINK1 (Clark et al., 2006; Exner et al., 2007; Lutz et al., 2009; Yang et al., 2006a). In contrast to the functional link, our study revealed that the transcriptional regulation and expression of parkin works independently of PINK1 and *vice versa*. So was the transcriptional up-regulation of parkin in fibroblasts of PD patients carrying homozygous PINK1 mutations not consitently up-regulated. Also PINK1 knockout mice and the transient knockdown of PINK1 did not lead to an increase in the parkin mRNA level. *Vice versa* in fibroblasts derived from PD patients carrying a deletion in the parkin gene and in parkin-deficient cells the level of PINK1 mRNA was not changed.

Up to date no mutations in the parkin promoter as well as in the relatively uncharacterized promoters of the other PD-associated genes were described. Only one single nucleotide polymorphismus at bp -258 was reported to influence the transcription of parkin and to increase the risk factor in elderly PD patients (Sutherland et al., 2007; Tan et al., 2005; West et al., 2001; West et al., 2002). It is possible that due to the nucleotide exchanged the binding of a nuclear protein expressed in the *substantia nigra* to this site is influenced, resulting in transcriptional alterations (West et al., 2002). Furthermore another binding protein n-myc was reported to bind to the parkin promoter and to negatively influence parkin transcription. This finding implicates parkin function in cell cycle and neuronal differentiation (West et al., 2004). The studies of Shuichi Asakawa and Andrew West as well as our analysis of the parkin promoter revealed auxiliary potential transcription factor binding sites such as SP-1, AP-4, NF-1, STATx, HSF1, HSF2, GATA-1 and of course AP1/CREB (Asakawa et al., 2001; West et al., 2001). Interestingly, the GATA-1 transcription factor was also associated with the

transcriptional regulation of α-synuclein in the *substantia nigra* (Scherzer et al., 2008). In addition, the expression of GATA-1 seems to be influenced by age (Budovskaya et al., 2008), indicating a probably role in PD.

Further screening of the first intron of parkin could not identify special transcription factor binding sites. However, the length of the promoter (up to 5 kb [core promoter: 250 bp]) and the large introns (first intron: 284 kb) makes a detection of further potential transcription regulatory elements very difficult.

The length and the yet relatively uncharacterized structure are probably the reason why still no mutation in the promoter or intron has been described to count for parkin-associated parkinsonism. Therefore it is possible that heterozygous carriers, which still develop PD, late onset but also early onset cases are reported (Farrer et al., 2001; Foroud et al., 2003; Khan et al., 2003), have an additional mutation in the non-coding region of the parkin promoter or intron. This complementary mutation could be responsible for their increased risk to develop PD (Abbas et al., 1999; Lucking et al., 2000).

Interestingly, the parkin co-regulated gene (PACRG), sharing with parkin the same promoter but transcribed on the opposite strand in an opposite orientation showed also an up-regulation of its mRNA after ER stress (Wang et al., 2007b), indicating that parkin and PACRG share some regulatory binding sites. Both proteins demonstrate overlapping but not identical tissue expression profiles (West et al., 2003b). How interleaving their regulation is has to be further investigated. PACRG and parkin were shown to interact (Imai et al., 2003). However, in our study we could not show that a reduced expression of parkin resulted in any regulatory changes for PACRG transcription. Whether PACRG contributes to the pathogenesis of PD for example by influencing the function of parkin has still to be elucidated. Immunostainings of post mortem brains of PD patients as well as of multiple system atrophy (MSA) and progressive supranuclear palsy (PSP) patients revealed PACRG in areas of neurodegeneration (Taylor et al., 2007) yet, a significant link between single nucleotide exchanges and early onset PD could not be demonstrated so far (Deng et al., 2005b).

Of all examined PD-associated genes, parkin expression seems to be the most sensitive one for a wide spectrum of different stressors. Additionally, parkin was shown to protect cells against several different stressors, supporting the assumption that parkin plays an important role for the cell to handle cellular stress and to maintain cellular integrity. This observation also seems to be of pathological relevance to sporadic PD as elevated parkin levels were found in the *substantia nigra* of sporadic

PD patients (Moran et al., 2007). Drawing the circle back to familiar PD, also here the loss of parkin function due to pathogenic mutations in the parkin gene seems to result in difficulties for the dopaminergic neurons to cope with cellular stress, as the patients suffer from early onset parkinsonism. Notably, different lines of evidence indicate that parkin can be inactivated by misfolding and aggregation of the pathogenic parkin mutations (Cookson et al., 2003; Gu et al., 2003; Henn et al., 2005; Schlehe et al., 2008; Sriram et al., 2005; Wang et al., 2005b; Winklhofer et al., 2003). Yet, even wild-type parkin is prone to misfolding especially under high-level oxidative stress, resulting in a loss of function (LaVoie et al., 2007; LaVoie et al., 2005; Wang et al., 2005a; Winklhofer et al., 2003; Wong et al., 2007). On the other hand, parkin is postulated to play a remarkably protective role under stress. How can this discrepancy be explained? This finding makes sense in a physiological context. Parkin is functional under mild and moderate stress conditions, whereas under severe stress, which irreversibly might damage neurons parkin is inactivated by misfolding. When cellular stress exceeds a critical threshold, the damage for the cell will be irreversible, reaching a point of no return, cell-survival programs will be shut down, and pro-apoptotic pathways induced, resulting in the death of the cell. As sporadic PD cases are usually late onset cases, age is considered as the major risk factor. Aging neurons are compromised in their capability to cope with stress, for example it could be that constant stress levels may impair parkin up-regulation. In addition, dopaminergic neurons are characterized by a high oxidative burden. Over the time reactive oxygen species are able to accumulate particularly in the metabolism of dopamine, which then may lead to misfolding and inactivation of parkin. This observation may explain why dopaminergic neurons are particularly vulnerable in sporadic PD.

The analysis of parkin and the other PD-associated genes gives us significant new insights into the molecular mechanisms underlying hereditary PD, but also sporadic PD. The discovery of pathways, dependent on parkin or other PD-associated gene regulation may serve as a basis for the development of drugs, which could halt or delay the disease progression.

5 Methods

5.1 CELL CULTURE METHODS

5.1.1 Cell culture

All used cells lines and primary cell cultures (fibroblasts) grew as adherent monolayers. Cells were cultivated in 25 or 75 cm^2 tissue culture flasks, maintained at 37°C with 5% CO_2. For cultivating, confluent grown cells were washed once with PBS (Phosphate Buffered Saline) followed by incubation with 0.5 g/l trypsin with EDTA (Invitrogen, GIBCO) for some minutes at room temperature. Cells were then taken up into fresh media and passaged into new culture flasks. For transfection, cells were counted and plated in the desired confluency in cell culture dishes. All cell lines and their desired medium are listed in Table 3.

Table 3: List of cell lines.

Cell line	Organism / cell type	Culture medium
HEK 293T	human embryonic kidney; ATCC-Nr. CRL-1573	DMEM (Dulbecco's Modified Eagle's Medium), 10% FCS (fetal calf serum), penicillin (50 U/ml) /streptomycin (50 μg/ml)
N2a	murine neuroblastoma; ATCC-Nr. CCL 131	MEM (Minimal Essential Medium), 10% FCS, penicillin/streptomycin
SH-SY5Y	human neuroblastoma; DSMZ-Nr. ACC 209	DMEM/Ham's F12, 15% FCS, 1% non-essential amino acids, penicillin/streptomycin
SH-SY5Y I	human neuroblastoma with reduced parkin expression	DMEM plus pyruvate, 10% FCS, 2 mM glutamate, penicillin/streptomycin

5.1.2 Preparation and cultivation of mouse embryonic fibroblasts

Primary mouse fibroblasts were isolated from parkin knockout and wild-type mice (Itier et al., 2003). Embryos at embryonic stage 12.5 days post coitum were extracted and all inner organs were removed. The remaining tissue was washed 5 times with PBS before incubation with trypsin for 20 min at 37°C. The cells were dissociated by passing several times through a pipette and spun down at 1000 rpm for 5 min. The pellet was resuspended and plated into complete DMEM media (10% FCS, Pen/Strep).

5.1.3 Preparation and cultivation of mouse and rat primary cortical neurons

Cortices were removed from the brains of embryonic wild-type day 18 rats or day 14.5 mice. Cells were dissociated by mild trypsination and trituration. The dissociated neurons were plated onto polyethyleneimine-coated dishes and grown in neurobasal medium (Invitrogen) supplemented with 5 mM HEPES, 1.2 mM glutamine, B27 supplement (20 ml/L; Invitrogen) and gentamicin (0.1 mg/ml). All experimental treatments were performed on 8 to 10 day old cultures. To induce glutamate excitotoxicity rat neurons were exposed to 5 and 10 μM glutamate in EBSS medium (6800 mg/l NaCl, 400 mg/l KCl, 264 mg/l $CaCl_2$, 200 mg/l $MgCl_2$, 2200 mg/l $NaHCO_3$ and 140 mg/l NaH_2PO_4 pH 7.2) with 10 mM glucose. After the indicated time cells were harvested for RNA extraction. To induce ER stress, mouse neurons were exposed to 1 μM thapsigargin or 2 μg/ml tunicamycin. After 12 h cells were harvested for RNA extraction as described below.

5.1.4 Transient transfection

Cells were plated 24 h before transfection; HEK 293T cells were plated 0.6 x 10^6, SH-SY5Y cells 0.3 x 10^6 and N2a 1 x 10^6. For transient transfection, DNA was mixed with Lipofectamine and Plus (Invitrogen) in Opti-MEM medium (Invitrogen) according to the manufacturers instructions. The incubation was conducted for 3-5 hours in the transfection mix and Opti-MEM, which was than replaced by normal growth medium.

5.1.5 RNA interference

For RNA interference the Stealth small interfering RNA (siRNA) from Invitrogen was used. Stealth siRNA is a chemical modified dsRNA, able to reduce the induction of cellular stress response pathways. Reverse transfection showed the highest knock down efficiency. This method works as follows: HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen). First, 5 μl Lipofectamine 2000 and likewise the appropriate amount of siRNA (indicated below) were diluted separately in 250 μl Opti-MEM medium. After 15 min incubation at room temperature siRNA and Lipofectamine dilution were combined and incubated for another 15 min at room temperature to allow complex formation. The mixture was transferred into poly-L-lysine coated 6-wells and 0.3 x 10^6 HEK 293T cells in 1.5 ml Opti-MEM were added on top. The next day 2 ml growth medium without antibiotics was added to the cells.

The transfection of SH-SY5Y was performed with 5 μl Lipofectamine RNAiMAX incubated directly with the appropriate amount of siRNA (indicated below) in 500 μl

Opti-MEM for 15 min at room temperature followed by the transfer to a 6-well dish and the addition of 0.2 x 10^6 SH-SY5Y cell–suspension in 1.5 ml Opti-MEM. The next day 2 ml growth medium without antibiotics was added to the cells.

For endogenous parkin knock down the Stealth siRNA duplex 1 (PARK2-HSS107593) was the most efficient one followed by duplex 2 (PARK2-HSS107594). For one 6-well dish 100 pmol (5 µl of 20 µM stock) of parkin siRNA was used. The highest knock down efficiency was achieved at day 4 after transfection.
For the ATF4 knock down the best siRNA was duplex 3 (ATF4-HSS141299) followed by duplex 1 (ATF4-HSS141297). For an efficient knock down 100 pmol siRNA was used. To interfere with the up-regulation of the ATF4 mRNA after induction of ER stress, the cells were again transfected 3 days after the first transfection with the same siRNA. Therefore, siRNA was incubated for 15 min with RNAiMAX, before added directly on top of the Opti-MEM washed cells. 5 h later cells were stressed and harvested 14 h later.

The best siRNA for c-Jun knock down was duplex 2 (Jun-HSS105641) and duplex 3 (Jun-HSS105642). To get acceptable knockdown efficiency 200 pmol of siRNA was used per 6-well dish. 1 day after the first transfection the cells were again transfected with the same amount of siRNA, stressed, and harvested as described for the ATF4 knock down.

As control the negative control duplex siRNA (medium GC-content) from Invitrogen was used.

5.1.6 Starvation of cells

For starvation assays cells were plated with medium containing dialysed FCS. To starve the cells L-histidinol (2 mM) was added, which blocks the charging of the amino acid histidine to its tRNA. The cells were harvested after 16 h of incubation.

5.2 NUCLEIC ACID BIOCHEMISTRY

5.2.1 Polymerase chain reaction (PCR)

DNA fragments were amplified by PCR (Saiki et al., 1988). The reaction was performed in a 50 µl reaction volume containing 100 ng template, 10 pmol of each primer (used primers are listed below), 0.25 mM dNTPs (dATP, dCTP, dGTP and dTTP), 1 x PCR reaction buffer and 2.5 units of DNA Polymerase. For amplification the following program was applied: step 1 (denaturation): 5 min at 95°C; step 2 (denaturation): 50 sec at 95°C; step 3 (primer annealing): 50 sec at 50 - 60°C

(dependent on the calculated melting temperature); step 4 (elongation): 2 min / kilo base pair of to be amplified DNA at 72°C; step 5 (final elongation): 5 min at 72°C; step 6 (end): 4°C. Steps 2 - 4 were repeated for a total of 28 - 30 cycles.

Two step PCR

To substitute single amino acids, 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 fort the second PCR, together with the outermost primer pair.

5.2.2 Agarose-gelelectrophoresis

To analyze the resulting PCR products or to separate linearized DNA fragments from supercoiled DNA, the DNA was loaded onto a 1% (w/v) agarose gel in 1x TBE buffer containing 0,1 μg/ml Ethidium Bromide. The appropriate volume of 6x DNA loading buffer was added to the DNA, and gels were run at a constant voltage (10 V/cm) until sufficient separation of the fragments. To determine the different size of the DNA fragments 1 kB or 100 Bp marker was loaded in parallel.

5.2.3 Isolation and purification of DNA fragments from agarose gels

The right sized DNA fragments, verified under the UV lamp, were cut out from the agarose gel and purified with the Nucleo Spin Extract kit (Macherey-Nagel) according to the manufacturer's instructions.

5.2.4 Restriction digest

All analytical and preparative restriction digests were performed in total volumes of 20-50 μl, using 10 U restriction enzyme and the appropriate amount of the respective 10x reaction buffer. The reaction was incubated at 37°C for several hours or over night, unless otherwise noted in the manufacturer's direction. DNA fragments were purified as described above.

5.2.5 Ligation

To insert DNA fragments into the appropriate linearized vector, fragment and vector were incubated in a 1:3 ratio in final reaction volume of 20 μl, buffered with 5x T4 ligation buffer and including 1U of T4 Ligase. The mixture was incubated either for 3

h at room temperature or overnight at 4°C, before used to transform competent bacteria.

5.2.6 Preparation of competent bacteria

For competent *Escherichia coli (E.coli)* cells, the cell membrane was destabilized by divalent cations (Sambrook et al, 1989) to make the cell compatible to take up plasmid-DNA. An overnight culture of DH5α in 3 ml *Luria Broth* (LB)-Medium without antibiotics was used to inoculate 200 ml LB. Bacteria were grown to a density of 0.3-0.6 at 600 nm (OD_{600} = 0.3 – 0.6) at 37°C. Cells were chilled on ice for 10 min and centrifugated at 5000 rpm for 10 min. The pellet was resuspended in 100 ml of transformation buffer (50 mM $CaCl_2$, 15% Glycerol, 10 mM PIPES-buffer; pH 6.6), incubated for 20 min on ice, centrifugated as before and resuspended in 10 ml transformation buffer. 100 µl aliquots were shock frozen in liquid nitrogen and stored at -80°C.

5.2.7 Transformation of competent *E.coli*

50 µl competent DH5α were thawed on ice, mixed gently with DNA and incubated for 30 min on ice. As next step the cells were heat shocked by placing the reaction tube for 90 sec into a 42°C heat block and immediately chilled on ice for 3min. Afterwards 400 µl of antibiotic free LB media was added followed by incubation at 37°C for 60min. Subsequently, in case of a ligation the transformed cells were shortly centrifugated, the pellet resuspended in 50 µl of LB and then plated on LB-agar plates containing the respective antibiotic to select positive clones. In case of a re-transformation an aliquot of 50 µl was plated directly on an agar plate. LB-agar plates were incubated at 37°C over night, single clones were analyzed as described below.

5.2.8 Plasmid DNA preparation

Single clones were used to inoculate LB-medium for small-scale DNA preparation (Macherey-Nagel), which was performed according to the manufacturers instructions. Large-scale DNA-preparation (Midi Macherey-Nagel) was used to obtain higher amounts of DNA according to the manufacturer's instructions. To obtain endotoxin free DNA samples the Endofree Plasmid Maxi Kit (Qiagen) was used according to the manufacturer's instructions. DNA amounts and purity were determined by measuring absorbance at 260/280 nm.

5.2.9 Sequencing

For sequencing purified DNA samples were analyzed by GATC Biotech AG (Konstanz, Germany).

5.2.10 Generation of DNA constructs

5.2.10.1 ER stress reporter constructs

The luciferase reporter constructs were cloned by subcloning the unfolded protein response element (UPRE), the ER stress response element (ERSE), the ER stress response element II (ERSEII), the ATF4 binding site of the IGFBP-1 promoter (ATF4RE) or the ATF4 binding element of the parkin promoter (Park) into the pGL3 vector (Promega). The oligonucleotides were synthesized containing the binding site in triplicates separated by nine random nucleotides and flanked by NheI and BglII restriction sites (oligonucleotides are listed below). The oligonucleotides (100 pmol of each) were annealed by boiling for 3 min in TE. After cooling them down they were directly inserted by ligation into the digested pGL3 vector.

5.2.10.2 Generation of the renilla luciferase construct

To generate the renilla luciferase construct, the SV40 promoter from the pGL3 vector was subcloned into the phRL-SV40 vector (Promega) by digestion with BglII and HindIII, thereby replacing the SV40 early enhancer/promoter.

5.2.10.3 Generation of hP rescue (siRNA resistant parkin)

Into human parkin wild type four silent mutations were introduced into the parkin siRNA1 (Invitrogen) target sequence (1035-1059 bp). Following base pairs were mutated: C 1038 to T, G 1044 to A, C 1053 to A and A 1059 to G. The PCR fragment of the hP rescue construct was subcloned into pcDNA3.1/Zeo(+) by using the restriction enzymes HindIII and NotI and the primer pairs listed below.

5.2.10.4 Generation of parkin mutants

To clone parkin mutants, the cDNA of human wild-type parkin in pcDNA3.1/Zeo(+) was used. The pathogenic point mutations C212Y and C289G were inserted into the wild-type parkin cDNA using the primer pairs listed below and the PCR fragment was ligated via the restriction sites HindIII and NotI into pcDNA3.1/Zeo(+).

5.2.10.5 Generation of c-Jun constructs

The human c-Jun cDNA in the pMT35 vector (Krappmann et al., 1996) was subcloned into the pcDNA3.1(-) vector by digestion with BamHI and HindIII (name: c-Jun His), into the pcDNA3.1(-) vector using the primer pairs Flag c-Jun XhoI F and Flag c-Jun HindIII listed below, where the N-terminal primer contains a Flag-Tag, and the restriction sites XhoI and HindIII (name: Flag c-Jun) and into the pcDNA3.1(-) vector using the primer pairs Kozak c-Jun EcoRI F and Flag c-Jun HindIII R listed below, where the N-terminal primer contains a Kozak sequence, and the restriction sites EcoRI and HindIII (name: Kozak c-Jun; *with experimental help of V. Palmisano*).

5.2.10.6 Generation of ATF4 constructs

The human ATF4 cDNA (Tanabe et al., 2003) was subcloned into the pCMV-HA vector using the primer pairs HA ATF4 EcoRI F and HA ATF4 NotI R listed below and the restriction sites EcoRI and NotI (name: pCMV HA-ATF4, *with experimental help of V. Palmisano*), into the pcDNA6 V5/His vector using the primer pairs ATF4 V5 BamHI F and ATF4 V5 XbaI R listed below and the restriction sites BamHI and XbaI (name: ATF4 V5/His), into the pcDNA3.1(-) vector using the primer pairs Flag ATF4 XhoI F and Flag ATF4 HindIII R listed below, where the N-terminal primer contains a Flag-Tag, and the restriction sites XhoI and HindIII (name: Flag-ATF4), into the pCMV-TAG (Flag) vector the primer pairs Flag ATF4 BamHI F and Flag ATF4 HindIII R listed below and the restriction sites BamHI and HindIII (name: pCMV-Flag ATF4) and into the pcDNA3.1(-) vector using the primer pairs Kozak ATF4 XhoI F and Flag ATF4 HindIII R listed below, where the N-terminal primer contains a Kozak sequence, and the restriction sites XhoI and HindIII (name: Kozak ATF4; *with experimental help of V. Palmisano*).

5.2.11 RNA preparation from cells

For analysis of mRNA induction under various stress conditions, SH-SY5Y cells, mouse embryonic fibroblasts or mouse/rat neurons were incubated with 1 μM thapsigargin, 2 μg/ml tunicamycin, 1 μM rotenone, 50 μM kainic acid, 30 μM MG123 or 80 μM dopamine for the indicated time. Total cellular RNA was isolated and treated with DNaseI according to manufacturer's instructions (RNaesy mini kit, QIAGEN).

5.2.12 RNA preparation from mouse brain

To prepare RNA from mouse brain, brains were pesteled in liquid nitrogen and total RNA was isolated using the RNaesy lipid tissue kit (QIAGEN) according to manufacturer's instructions.

5.2.13 cDNA synthesis

cDNA was synthesized from 1 μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad).

5.2.14 Real-time RT-PCR

Each quantitative PCR reaction (final volume 20 μl) included 0.5–2 μl cDNA solution, 10 μl PCR Master Mix and 0.5 μl of each primer. For the quantification of human parkin or PINK1 mRNA the TaqMan Gene Expression Assay (parkin: Hs00247755_m1; PINK1: Hs02330592_s1, β-actin: P/N 4326315E; GAPDH: P/N 4326317E; 18sRNA: P/N 4319413E) (Applied Biosystems) was used. For all other mRNA quantifications, PCR reactions were performed with 2x Power SYBR Green PCR Master Mix (Applied Biosystems) and 1 μM of each primer pair (listed below). Quantification was performed with 7500 Fast Real Time System (Applied Biosystems) based on triplicates per primer set. The mid-linear range was used to establish the threshold for each oligonucleotide set. Gene expression was normalized with respect to endogenous housekeeping control genes, β-actin, glyceraldehyde phosphate dehydrogenase (GAPDH) and 18sRNA, which were determined not to have significantly changed under the different conditions. Relative expression was calculated for each gene using the $\Delta\Delta C_T$ method.

5.3 PROTEIN BIOCHEMISTRY

5.3.1 Lysate preparation

For parkin detection cells were lysed in detergent buffer containing 0.1% Triton X-100 in PBS supplemented with protease inhibitor cocktail for 5 min on ice, followed by centrifugation for 10 min at 13,000 rpm.

For detection with phospho-antibodies the cells were lysed using a lysis buffer containing phosphatase inhibitors (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, phosphatase

inhibitor and protease inhibitor) likewise for 5 min on ice. To disturb nuclear envelope and DNA cells were sonicated for 5 sec 5 times and cell debris were pelleted by centrifugation (16,000 x g, 10 min). The cleared supernatants were transferred to a new tube.

For detection of ATF4, c-Jun and PERK urea lysis buffer was used to lyse the cells (6 M urea, 50 mM HEPES pH 8.0, 0.5% SDS and protease inhibitor cocktail) for 10 min on ice. To disrupt DNA the cells were needled about ten times on ice.

For electrophoretic mobility shift assays the cells were lysed in hypotonic cell lysis buffer (20 mM HEPES (pH 7.9), 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1% Triton-x 100, 20% glycerol, add before use protease inhibitor and 2 mM DTT) to obtain cytoplasmic cell extract. To obtain nuclear extracts cell pellets of step one were resuspended in hypotonic cell lysis buffer plus 380 mM NaCl.

Protein concentration was determined by BCA protein assay (Uptima).

5.3.2 Detergent solubility assay

Transfected cells were harvested and lysed in detergent buffer (0,1% Triton X-100 in PBS). After centrifugation at 16,000x 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.

5.3.3 SDS-PAGE

Proteins were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Samples were boiled for 5 min in the presence of 4x SDS-PAGE-loading buffer. Equal amounts of protein were run on 8, 10 or 12% SDS-PAGE gels, depending on the size of the protein. The electrophoresis was made using the minigel system (BIORAD). The gels were run at constant current of 25 mA per gel.

5.3.4 Western Blot

After electrophoresis, proteins were electro-transferred from the SDS-gel onto a polyvinylidenfluorid (PVDF) membrane (Millipore) using the minigel system (BIO RAD) (Towbin et al., 1979). Membranes were blocked directly in 5% nonfat dry milk or 5% BSA in TBS containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature and then probed with the primary antibody in TBS-T with 5% milk or 5% BSA for 2 h at RT or overnight at 4°C with gentle agitation. After extensive washing with TBS-T, the

membranes were incubated with HRP-conjugated secondary antibody. After washing again with TBS-T, the antigens were detected with the enhanced chemoluminescence detection system (Amersham Biosciences) or the Immobilon Western chemoluminiscent HRP substrate (Millipore).

5.3.5 Co-immunoprecipitation

HEK293T cells were co-transfected with full-length parkin and FLAG-IKKγ or FLAG-TRAF2. The next day, cells were washed twice with cold PBS, scraped off the plate, pelleted by centrifugation, and lysed in cold lysis buffer (0.1% Triton X-100 in PBS, supplemented with protease inhibitor cocktail). Cell lysates were cleared by centrifugation (16,000 x g, 20 min, 4°C), and pre-cleared with immobilized protein A for 1 h at 4°C. Equal amounts of protein of the detergent-soluble fraction were incubated overnight at 4°C with the antibody indicated. The antigen-antibody complexes were captured by the addition of protein A agarose for 90 min. Prior to immunoblotting, the beads were washed three times with lysis buffer and boiled in 2x Laemmli sample buffer in order to release the precipitated proteins.

5.3.6 Ubiquitylation assay

Parkin or parkin mutants, HA-tagged ubiquitin or the ubiquitin mutants HA-K48only-ubiquitin, HA-K63only–ubiquitin and either FLAG-IKKγ, FLAG-TRAF2, FLAG-TRAF2ΔN or FLAG-TRAF6 were co-transfected into HEK293T cells. One day after transfection, protein lysates were prepared in denaturing lysis buffer (50 mM Tris/HCl pH 7.4, 5 mM EDTA, 1% SDS, 15 U/ml DNase I, and protease inhibitor cocktail) and boiled for 5 min at 95°C. Protein extracts were diluted 1:10 with nondenaturing lysis buffer (50 mM Tris/HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail), and immunoprecipitation was performed with FLAG-M2 agarose (Sigma). Proteins present in the immunoprecipitates were analyzed by Western blotting using an anti-HA antibody.

5.3.7 Luciferase assay

Luciferase and renilla reporter plasmids (0.3 μg / 6-well) and the indicated DNA construct (0.5 - 1μg / 6-well) were cotransfected in HEK293T or SH-SY5Y cells. 8 h or 24 h later, the cells were treated with 1 μM of thapsigargin or 10 to 20 ng/ml PMA for 8 h or 14 h. Luciferase activity of cell lysates was determined luminometrically using an LB96V luminometer (Berthold Technologies) by the dual luciferase assay system (Promega) as specified by the manufacturer. The measured values were analyzed with

WinGlow Software (Berthold Technologies). Quantification was based on at least three independent experiments. Per experiment, each transfection was performed at least in dublicates.

5.3.8 Immunocytochemistry and fluorescence microscopy

SH-SY5Y cells were grown on glass coverslips. 24 h after transfection cells were fixed and permeabilized in ice cold methanol for 10 min, followed by incubation with the primary antibody (diluted 1:200 in PBS, 1% BSA) for 1 h at room temperature. After washes with PBS, the coverslips were incubated with fluorescently labelled Alexa 555-conjugated secondary antibody (diluted 1:200 in PBS, 1% BSA) for 30 min at room temperature. Finally, cells were embedded in Mowiol mounting medium (Calbiochem) supplemented with 4', 6-diamidimo-2-phenylindole (DAPI; Sigma) to stain the nuclei. Images were obtained on a Zeiss LSM 510 microscope.

5.3.9 Apoptosis and cell viability assays

Active caspase-3

SH-SY5Y cells were grown on glass coverslips. 24 h after transfection (for parkin knockdown three days later), cells were incubated with thapsigargin (10 μM), tunicamycin (5 μg/ml) and/or epoxomycin (0.1 μM) for 8 h. The cells were then fixed with 3% paraformaldehyde (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 (Carl Zeiss). 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 triplicates of at least three independent experiments.

Vybrant MTT Cell Proliferation Assay

For the cell viability test equal numbers of HEK293T cells or mouse embryonic fibroblasts were plated into 12-well plates. The HEK293T cells were reversely transfected with parkin or control siRNA. 2 days later the cells were stressed with tunicamycin (2 μg/ml) or thapsigargin (10 μM) for 16 h and the Vybrant MTT Cell Proliferation Assay was performed according to manufacturer's instructions (Invitrogen).

5.3.10 Electrophoretic mobility shift assay (EMSA)

Oligonucleotide annealing

Oligonucleotides (1.25 μg of each oligonucleotide) were annealed by boiling for 10 min in 50 μl 100 mM KCl.

Preparation of shift probe

The DNA probe was ^{32}P end-labeled with γ^{32}P-ATP (50 μCi) by using the T4 ploynucleotide kinase (Promega). To purify the labeled oligonucleotides the ProbeQuant G-50 columns were used, according to the manufacturer's direction.

EMSA

For the binding reaction 10 μg of extracts or the indicated amount of recombinant protein were incubated with binding buffer (10 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol), 2 mM DTT, 10 μg BSA, 2 μg poly(dI-dC) and 0.2 ng (20,000 cpm) of ^{32}P-labeled, double-stranded park oligonucleotide (5'-CCC CGG TGA CGT AAG ATT GC-3'), ATF4RE oligonucleotide (ATF4 binding site from the IGFBP1 promoter) (5'-AGA TTT ACA TCA TCC CCT G-3') or AP1 oligonucleotide (5'-CGC TTG ATG AGT CAG CCG GAA-3') in a final volume of 20 μl. For supershift assays 0.2 μg of the ATF4 antibody or 2 μg of the c-Jun(N)X antibody, for competition experiments 50 ng (100 x) of cold oligonucleotide was added to the binding reaction. After binding on ice for 30 min, mixtures were loaded onto nondenaturating 4% polyacrylamide gels in 0.5 x TBE (45 mM Tris borate and 1 mM EDTA). Gels were electrophoresed at 4°C for 4 h at 160V, dried, and exposed for autoradiography at -80°C.

5.3.11 Chromatin immunoprecipitation (ChIP)

HEK293T cells were seeded at 1.5 x 10^7 / 150-mm dish with complete medium and grown for 24 h. Cells were replenished with fresh medium to ensure that the cells were in the basal state, 12 h before transfer to either complete medium or medium containing 300 nM thapsigargin. Protein-DNA was cross-linked by adding formaldehyde directly to the culture medium to a final concentration of 1% and then stopped 10 min later by adding 2 M glycine to a final concentration of 0.125 M. Cross-linked chromatin was solubilised by sonication using a Sonic Dismembrator (Model 60, Fisher Scientific Co) for five bursts of 40 sec at power 10 with 2-min cooling on ice between each burst. To monitor ATF4 binding the extracts from 1 x 10^7 cells was incubated with 2 μg of rabbit polyclonal ATF4 antibody (Cocalico Biologicals). A rabbit anti-chicken IgG was used as the nonspecific antibody control. The antibody-bound complex was precipitated by protein A-Sepharose beads (Amersham Biosciences). The DNA fragments in the immunoprecipitated complex were released by reversing the

cross-linking at 65°C for 5 h and purified using a QIAquick PCR purification kit (Qiagen). Purified, immunoprecipitated DNA was analyzed by quantitative real time PCR. A 5 µl aliquot of DNA was mixed with 62.5 pmol of each PCR primer (F: 5'-GTT GCT AAG CGA CTG GTC AA-3', R: 5'-CAG CCC CCC ACC GCC GCC-3') and 12.5 µl of SYBR Green PCR master mix (Applied Biosystems) in a 25 µl total volume. The real-time PCR was performed with a DNA Engine Opticon 3 system (Bio-Rad). The reaction mixtures were incubated at 95°C for 15 min, followed by amplification at 95°C for 15 sec and 60°C for 60 sec for 35 cycles. All experiments were performed in triplicate and each sample was subjected to PCR in duplicate.

5.3.12 Fluorescent staining of mitochondria

SH-SY5Y cells were grown on 15 mm glas coverslips, and were fluorescently labeled with 0.1 µM DiOC6(3) in cell culture medium for 15 min, before replacing them for 30 min in normal medium. The coverslips with the living cells were analyzed for mitochondrial morphology by fluorescence microscopy using a Leica DMRB microscope. Cells were categorized in two classes according to their mitochondrial morphology: tubular or fragmented. Cells displaying an intact network of tubular mitochondria were classified as tubular. When this network was disrupted and mitochondria appeared predominantly spherical or rod-like they were classified as fragmented. Quantifications were based on three independent experiments. Per experiment the mitochondrial morphology of more than 300 transfected cells per coverslip of triplicate samples was assessed.

5.3.13 Measurement of cellular ATP levels

Cellular steady state ATP levels were measured using the ATP Bioluminescence Assay Kit HS II (Roche) according to the manufacturer's instructions. This kit uses the ATP dependency of the light-emitting luciferase catalyzed oxidation of luciferin for the measurement of extremely low concentrations of ATP. The quantitative determination of ATP was done in SH-SY5Y cells, reversely transfected with parkin or control siRNA. 24 hours prior to harvesting cells, the culture medium was replaced containing 3 mM glucose, 5 hours before the measurement cells were treated with 2 µg/ml tunicamycin. Cells were washed twice with PBS, scraped off the plate and lysed according to the provided protocol. ATP content of the samples was determined using an LB96V luminometer (Berthold technologies), analyzed with WinGlow Software (Berthold technologies) and normalized to total protein levels. Quantification was based on at least three independent experiments. Per experiment, each transfection was performed at least in triplicates.

5.3.14 Statistical analysis

Data were expressed as means ± SE. Statistical analysis among groups was carried out using ANOVA. P-values are as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

6 Material

6.1 TECHNICAL DEVICES AND FURTHER EQUIPMENT

Autoclave (Tuttnauer 3850 EL)	Systec
Camera (CCD Video Camera Module)	Raiser
Cell counter chamber, Neubauer	Labor Optik
Cell culture dish (60x15 mm, 100x17 mm, 24 well, 12 well)	Nunc
Cell culture flasks	Nunc
Cell homogenisator 1 ml	B.Braun
Cell scraper	Corning Inc.
Centrifuge (J-20XP) Rotors: Type JA10	Beckman
Centrifuge for Eppendorf tubes (Biofuge pico)	Heraeus, Kendro
Centrifuge for Eppendorf tubes/4°C (Biofuge pico)	Heraeus, Kendro
Centrifuge/4°C/ swing rotor (Megafuge 1.0R)	Heraeus, Kendro
Centrifuge/4°C/ swing rotor (Multifuge 3L-R)	Heraeus, Kendro
Clean bench (Hera Safe HS12)	Heraeus, Kendro
CO^2-Incubator (Hera cell)	Heraeus, Kendro
Disposable cuvette (10x4x45 mm)	Sarstedt
Electrophoresis chamber Hoefer SE600 with Power Supply EPS	Pharmacia Biotech
Electrophoresis chamber: Mini-PROTEAN 3 electrophoresis cell	Bio-Rad
Eppendorf tubes (200 µl, 1,5 ml, 2 ml)	Eppendorf
Falcon tubes (15 ml; 50 ml)	Genaxxon
Freezer -20°C	Elektrolux/Liebherr
Freezer -80°C (HFU 86-450)	Heraeus, Kendro
Fridge +4°C	Elektrolux / Siemens
Gas burner (Gas profi 1scs)	WLD-Tec
Gel dryer SGD300	Savant
Gel running chamber	PeqLab technologies
Heatblock	Liebisch
Incubator 37°C (Function line)	Heraeus
Incubator 56°C	Heraeus
LB96V luminometer	Berthold Technologies
Luminometer WinGlow Software	Berthold Technologies
Magnet stirler (IKAMAG REO)	IKA Labortechnik
MicroAmp optical 96-well plate	Applied Biosystems
Microscope (Wilovert S 10x 4/10/20)	Hund
Microscope cover glasses (∅ 15 mm)	Marienfeld
Microscope Zeiss Axioscope 2 plus	Carl Zeiss
Microscopic slide (76 x 26 mm)	Thermo scientific
Microwave	Bosch
Pasteur pipettes	VWR International
PCR Real Time System 7500 Fast	Applied Biosystems
PCR-machine Master Cycler	Eppendorf
pH-Meter (Inolab pH Level 1)	WTW
Photometer (SmartSpecTM 3000)	Bio-Rad

Pipette tips (2 µl, 20 µl, 200 µl, 1 ml)	Sarstedt
Pipette tips with sterile filter (2 µl, 20 µl, 200 µl, 1 ml)	Sarstedt
Pipettes (P2, P20, P200, P1000)	Gilson
Pipettes sterile (2 ml, 5 ml 10 ml, 25 ml)	Sarstedt
Pipett-boy (Accu-Jet)	Brand
Pipetts disposable (2 ml, 5 ml, 10 ml, 25 ml)	Sarstedt
Power supply (Power Pac 300)	Bio-Rad
Printer (p91)	Mitsubishi
Quarz cuvette (10x10x45 mm)	Hellma
Rotator (Rotator shaker genie 2)	Scientific Industries
Scale (Analytical +200 g - 0.0001 g)	Ohaus
Scale (Standard 2000 g - 0.01 g)	Ohaus
Shaker (KM2)	Edmund Buhler
Shaker 37°C (Certomat BS-1)	B. Braun Biotech International
Thermomixer 5439 compact	Eppendorf
Tumbler	Heidolph
UV-Lamp (White/Ultraviolet Transilluminator)	UVP
Vortex apparatus (Vortex Genie 2)	Scientific Industries
Water bath (Typ 1002; Typ 1003)	GFL
Water deionizing machine (Milli-Q academic)	Millipore

6.2 MATERIAL, SOLUTIONS AND BUFFER FOR CELL CULTURE

6.2.1 Material

B27 supplement	Invitrogen
DMEM plus pyruvate	Gibco Invitrogen
DMEM/Ham's F12	Lonza
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco Invitrogen
dialysed FCS (Fetal Calf Serum)	Gibco Invitrogen
Fetal Calf Serum (FCS)	Gibco Invitrogen
gentamicin	Gibco Invitrogen
glucose	Gibco Invitrogen
glutamate	Gibco Invitrogen
glutamine	Gibco Invitrogen
Lipofectamine/Plus	Invitrogen
Lipofectamine 2000	Invitrogen
Neurobasal medium	Invitrogen
non-essential amino acids	Gibco Invitrogen
Opti-MEM	Gibco Invitrogen
PBS (Phosphate buffered saline)	Gibco Invitrogen
Penicillin/Streptomycin (Pen/Strep)	Invitrogen
Poly-L-lysine hydrobromide (10 mg/ml)	Sigma
RNAiMAX	Invitrogen
Stealth small interfering RNA	Invitrogen
Trypan Blue	Invitrogen
Trypsin-EDTA	Gibco Invitrogen

Table 4: List of inhibitors and drugs

chloroquine	Sigma
dopamine	Sigma
epoxomycin	Calbiochem
glutamate	Sigma
kainic acid	Calbiochem
L-histidinol	Sigma
MG132	Merck
PMA	Sigma
rotenone	Sigma
thapsigargin	Sigma
TNFα	Biomol
tunicamycin	Fluka/Sigma

Table 5: Stealth siRNA/control (Invitrogen)

negative control duplex	medium GC-content
parkin	
duplex 1	PARK2-HSS107593
duplex 2	PARK2-HSS107594
duplex 3	PARK2-HSS107595
c-Jun	
duplex 1	Jun-HSS105640
duplex 2	Jun-HSS105641
duplex 3	Jun-HSS105642
ATF4	
duplex 1	ATF4-HSS141297
duplex 2	ATF4-HSS141298
duplex 3	ATF4-HSS141299

6.2.2 Solutions and buffer

EBSS medium

6800 mg/l NaCl, 400 mg/l KCl, 264 mg/l $CaCl_2$, 200 mg/l $MgCl_2$, 2200 mg/l $NaHCO_3$ and 140 mg/l NaH_2PO_4 pH 7.2,

6.3 MATERIAL, SOLUTIONS AND BUFFER FOR NUCLEIC ACID BIOCHEMISTRY

6.3.1 Material

Agarose	Serva
DNA preparation kit mini/midi	Macherey-Nagel
dNTPs	Sigma
EndoFree Plasmid Maxi kit	Qiagen
Ethidium Bromide	Sigma
iScript cDNA Synthesis Kit	Bio-Rad
Nucleo spin extract kit	Macherey-Nagel
Pfu-polymerase and 10x buffer	Promega
Power SybrGreen PCR Master Mix (2x)	Applied Biosystems
Pre-stained protein standard: 1kB, 100Bp	NEB
QIAquick PCR purification kit	Qiagen
QIAshredder	Qiagen

Restriction enzymes and buffers	NEB/Fermentas
RNaesy mini kit	Qiagen
RNase-free DNase set	Qiagen
T4 ligase and buffer	Roche
TaqMan Fast Real-time PCR universal Master Mix (2x)	Applied Biosystems
TaqMan Gene expression assay	Applied Biosystems
Primer/oligo	Thermo Scientific
Bacteria	
DH5α	Genotyp: *sup*E44, *Δlac*169, (Φ80*lac*ZΔM15), *hsd*R17, *rec*A1, *gyr*A96, *thi*-1, *rel*A1 Source: Hanahan, 1983

Table 6: List of DNA constructs

name	source
parkin human wild-type	(Winklhofer et al., 2003)
parkin mutant ΔUBL	(Henn et al., 2005)
parkin mutant G430D	(Henn et al., 2005)
parkin mutant C212Y	See Methods
parkin mutant C289G	See Methods
ATF4	(Tanabe et al., 2003)
pcMV HA-ATF4	See Methods
ATF4-V5/His	See Methods
Flag-ATF4	See Methods
pcMV-Flag-ATF4	See Methods
Kozak-ATF4	See Methods
ATF4ΔN	(Steinmuller and Thiel, 2003)
PERK	(Harding et al., 1999)
c-Jun His (pMT35)	(Krappmann et al., 1996)
c-Jun His	See Methods
Flag c-Jun	See Methods
Kosak-c-Jun	See Methods
JNK3	(Waetzig and Herdegen, 2003)
Bcl-2-Flag	(Rambold et al., 2006)
mCherry	(Shaner et al., 2004)
Parkin siRNA resistant (hP rescue)	See Methods
EYFP	Clontech
cGFP	Clontech
3x UPRE-luc	See Methods
3x ERSE-luc	See Methods
3x ERSEII-luc	See Methods
3x ATF4RE-luc	See Methods
3x Park-luc	See Methods
1x ERSE-luc	See Methods
Renilla	See Methods
pGL3 vector	Promega
Flag-TRAF6	(Rothe et al., 1995)
Flag-TRAF2	(Krappmann et al., 2000)

HA-ubiquitin	(Krappmann et al., 1996)
HA-K63R-ubiquitin, HA-K48R-ubiquitin	(Evans et al., 2004)
PAEL-R	(Imai et al., 2001)
Ret	(Wang et al., 2004a)
Flag-IKKγ	(Tegethoff et al., 2003)
IRE1	(Kaneko et al., 2003)
NF-κB-luc	(Krappmann et al., 2001)
pcDNA3.1/Zeo(+)	Invitrogen
pcDNA3.1/Zeo(-)	Invitrogen
Flag-TRAF2ΔN	(Rothe et al., 1995)
pCMV-HA	Clontech
phRL-SV40 vector	Promega

Table 7: List of Primers

name	sequence
1x ERSE F	5'-GAT CCT TCA CCA ATC GGC GGC CTC CAC GAC GGA-3'
1x ERSE R	5'-GAT CTC CGT CGT GGA GGC CGC CGA TTG GTG AAG-3'
1x ERSEII F	5'-GAT CCG CCG ATT GGG CCA CGT TGG GAG AA-3'
1x ERSEII R	5'-GAT CTT CTC CCA ACG TGG CCC AAT CGG CG-3'
1x UPRE F	5'-GAT CCG TGC TGA CGT GGC GAT A-3'
1x UPRE R	5'-GAT CTA TCG CCA CGT CAG CAC G-3'
ATF4RE R	5'-GAT CTC AGG GGA TGA TGT AAA TCT CAG GGG ATG ATG TAA ATC TCA GGG GAT GAT GTA AAT CTG CGG GAT CCA TGA CCG AAA TGA GC-3'
ATFRE F	5'-CTA GCA GAT TTA CAT CAT CCC CTG AGA TTT ACA TCA TCC CCT GAG ATT TAC ATC ATC CCC TGA-3'
Park-Luc F	5'-CTA GCC CCG GTG ACG TAA GAT TGC CCC GGT GAC GTA AGA TTG CCC CGG TGA CGT AAG ATT GCA-3'
Park-Luc R	5'-GAT CTG CAA TCT TAC GTC ACC GGG GCA ATC TTA CGT CAC CGG GGC AAT CTT ACG TCA CCG GG-3'
3x ERSE F	5'-CTA GCT TCA CCA ATC GGC GGC CTC CAC GAC GGT TCA CCA ATC GGC GGC CTC CAC GAC GGT TCA CCA ATC GGC GGC CTC CAC GAC GGA-3'
3x ERSE R	5'-GAT CTC CGT CGT GGA GGC CGC CGA TTG GTG AAC CGT CGT GGA GGC CGC CGA TTG GTG AAC CGT CGT GGA GGC CGC CGA TTG GTG AAG-3'
3x ERSEII F	5'-CTA GCT GCC GAT TGG GCC ACG TTG GGA GAG CCG ATT GGG CCA CGT TGG GAG AGC CGA TTG GGC CAC GTT GGG AGA A-3'
3x ERSEII R	5'-GAT CTC TCC CAA CGT GGC CCA ATC GGC TCT CCC AAC GTG GCC CAA TCG GCT CTC CCA ACG TGG CCC AAT CGG CAG-3'
3x UPRE F	5'-CTA GCT CGT GCT GAC GTG GCG ATC GTG CTG ACG TGG CGA TCG TGC TGA CGT GGC GAT A-3'
3x UPRE R	5'-GAT CTA TCG CCA CGT CAG CAC GAT CGC CAC GTC AGC ACG ATC GCC ACG TCA GCA CGA G-3'
ATF4 V5 BamHI F	5'-CGG GAT CCA TGA CCG AAA TGA GC-3'
ATF4 V5 XbaI R	5'-CGT CTA GAG GGG ACC CTT TTC TTC C-3'
Flag ATF4 BamHI F	5'-GGC GGA TCC ACC GAA ATG AGC TTC-3'

Flag ATF4 HindIII R	5'-CGA TAA GCT TCT AGG GGA CCC TTT CTT-3'
Flag ATF4 XhoI F	5'-GAC CTC GAG ATG GAT TAC AAG GAT GAC GAC GAT AAG ACC GAA ATG AGC TTC C-3'
Kozak ATF4 XhoI F	5'-GAC CTC GAG GCC ACC ATG ACC GAA ATG AGC TTC C-3'
HA ATF4 EcoRI F	5'-GGA ATT CGG ACC GAA ATG AGC TTC C-3'
HA ATF4 NotI R	5'-TAT TCT TAC GCC GGC GCT AGG GGA CCC-3'
Flag c-Jun HindIII R	5'-CGA TAA GCT TTC AGA ATG TTT GCA ACT GCT GCG T-3'
Flag c-Jun XhoI F	5'-GAC CTC GAG ATG GAT TAC AAG GAT GAC GAC GAT AAG ACT GCA AAG ATG GAA ACG-3'
Kozak c-Jun EcoRI F	5'-GGA ATT CGC CAC CAT GAC TGC AAA GAT GGA AAC-3'
hP C212Y F	5'-TTT TTC TTT AAA TAT GGA GCA CAC CCC-3'
hP C212Y R	5'-GGG GTG TGC TCC ATA TTT AAA GAA AAA-3'
hP C289G F	5'-GGC TAC TCC CTG CCT GGT GTG GCT GGC TGT CC-3'
hP C289G F	5'-GGA CAG CCA GCC ACA CCA GGC AGG GAG TAG CC-3'
hP siRNA resc. F	5'-TGC CGG AGC CTG ATC AGA GAA AAG TCA CAT GCG AGG GGG GCA ATG-3'
hP siRNA resc. R	5'-CAT TGC CCC CCT CGC ATG TGA CTT TTC TCT GAT CAG GCT CCG GCA-3'

Table 8: List of EMSA oligonucleotides

name	sequence
park oligo	5'-CCC CGG TGA CGT AAG ATT GC-3'
park mut oligo	5'-CCC CGG TGA GCG CAC ATT GC-3'
ATF4RE oligo (IGFBP1)	5'-AGA TTT ACA TCA TCC CCT G-3'
NF-κB oligo	5'-AGT TGA GGG TTT CCC AGG C-3'
Oct1 oligo	5'-TGT CGS ATG CAA ATC ACT AGA A-3'
AP1 oligo	5'-CGC TTG ATG AGT CAG CCG GAA-3'

Table 9: List of Real time PCR Primers

M=mouse; h=human; r=rat; F=forward; R=reverse

name	sequence
hA20 F	5'-TCC TCC TCT CCT ACC AAG CA-3'
hA20 R	5'-CAC GCG ACT TGT GTG TCT TT-3'
hactin-β F	5'-TGG ACT TCG AGC AAG AGA TG-3'
hactin-β R	5'-AGG AAG GAA GGC TGG AAG AG-3'
hATF4 F	5'-CCC TTC ACC TTC TTA CAA CCT C-3'
hATF4 R	5'-GTC TGG CTT CCT ATC TCC TTC A-3'
hBcl2A1 F	5'-TCT CAG CAC ATT GCC TCA AC-3'
hBcl2A1 R	5'-GTC CTG AGC CAG CCT GTA A-3'
hBcl2L1 F	5'-CCT CTC CCG ACC TGT GAT A-3'
hBcl2L1 R	5'-ACT GAG TCT CGT CTC TGG TTA GTG-3'
hBiP F	5'-GCT CGA CTC GAA TTC CAA AG-3'
hBiP R	5'-GAT CAC CAG AGA GCA CAC CA-3'
hc-Jun F	5'-CGC CTG ATA ATC CAG TCC A-3'
hc-Jun R	5'-CCT GCT CAT CTG TCA CGT TC-3'
hChop F	5'-CCC TTG GTC TTC CTC TCT T-3'
hChop R	5'-CCT CTC TGG CTT GGC TGA CT-3'
hcox2 F	5'-TCG ATGCTG TGG AGC TGT AT-3'
hcox2 R	5'-GCT TCC AGT AGG CAG GAG AA-3'

hDJ-1 F:	5'-CTG GCT AAA GGA GCA GAG GA-3'
hDJ-1 R:	5'-ATG ACC ACA TCA CGG CTA CA-3'
hDrp1 F	5'-ACA GGC AAC TGG AGA GGA AT-3'
hDrp1 R	5'-GCA ACA GGA ACT GGC ACA T-3'
hFTHC1 (Ferritin heavy polypeptide) F	5'-TGG AGC TCT ACG CCT CCT AC-3'
hFTHC1 R	5'-AGC ATG TTC CCT CTC CTC AT-3'
hFTMT (ferritin mitochondrial) F	5'-GAA CAG GAC GAC TGG GAA AG-3'
hFTMT R	5'-CCA GCA ACG ACT GGT TCA-3'
hGadd45b1 F	5'-ACC CAT GAA CTC CCA GTT TG-3'
hGadd45b1 F	5'-CTT CCC ATC TCG CTC TCA GT-3'
hGAPDH F	5'-ATG GGT GTG AAC CAT GAG AA-3'
hGAPDH R	5'-GTG CTA AGC AGT TGG TGG TG-3')
hHsp72 F	5'-CAA GAT CAC CAT CAC CAA CG-3'
hHsp72 R	5'-TCG TCC TCC GCT TTG TAC TT-3'
hIRF1 F (interferon regulatory factor 1)	5'-AGC TCA GCT GTG CGA GTG TA-3'
hIRF1 R	5'-CCT CTT GGC CTT GCT CTT A-3'
hLon P1 F	5'-CCT TAT GTC GGC GTC TTT CT-3'
hLon P1 R	5'-CCC CGT GTG GTA GAT TTC AT-3'
hLRRK2 F:	5'-CCT TCT TGG CTT GGT CCT T-3'
hLRRK2 R:	5'-AGG CTG TTC CTT CTT CCA CA-3'
hMIHC F (inhibitor of apoptosis protein 1)	5'-GAG GAG ACA GTC CTA CTG AAA AGC-3'
hMIHC R	5'-GGA AAA GTA GGC TGA GAG GTA GC-3'
hmot2 / mito. Hsp70 F	5'-GAA GGA CTA TCG CTC CAT GC-3'
hmot2 / mito. Hsp70 R	5'-GCC ACC CAC AAG AAT CAC TT-3'
hmyc F (v-myc myelocytomatosis viral oncogene homolog)	5'-GAA CAG CTA CGG AAC TCT TGT G-3'
hmyc R	5'-AGT CTC AAG ACT CAG CCA AGG T-3'
hNAD(P)H F	5'-AAA GGA CCC TTC CGG AGT AA-3'
hNAD(P)H R	5'-GTG GAT CCC TTG CAG AGA GT-3'
hNFKBIA F	5'-ATG CTC AGG AGC CCT GTA AT-3'
hNFKBIA R	5'-GGT GAG CTG GTA GGG AGA ATA G-3'
hp53 F	5'-GGA AGA GAA TCT CCG CAA GA-3'
hp53 R	5'-GGA GAG GAG CTG GTG TTG TT-3'
hPACRG F	5'-AAC AAA TGC CCA GAC AAG ATG-3'
hPACRG R	5'-TGT GAA ACC CTC AGA AAC CA-3'
hParkin F	5'-CGA CCC TCA ACT TGG CTA CT-3'
hParkin R	5'-GAC ACA CTC CTC TGC ACC ATA C-3'
hPINK1 F:	5'-CCA ACA GGC TCA CAG AGA AG-3'
hPINK1 R:	5'-AGC GTT TCA CAC TCC AGG TT-3'
hSOD2 F (superoxide dismutase 2)	5'-AAG GAA CGG GGA CAC TTA CA-3'
hSOD2 R	5'-ATC CCC AGC AGT GGA ATA AG-3'
hTau F	5'-CCA AAG CCA AGA CAG ACC A-3'
hTau R	5'-TGG AGG AGA CAT TGC TGA GA-3'
hTH (Thyrosin Hydroxylase) F	5'-GCA GTT CTC GCA GGA CAT T-3'
hTH R	5'-CTG CTT ACA CAG CCC GAA-3'
hXIAP F	5'-ATG CTC ACC TAA CCC CAA GA-3'
hXIAP R	5'-AGG AAA GTG TCG CCT GTG TT-3'
hα-syn F:	5'-AAC CAA GGA GGG AGT GGT G-3'
hα-syn R:	5'-TGT CTT CTG GGC TAC TGC TG-3'

LUC F	5'-AAA CGC TGG GCG TTA ATC-3'
LUC R	5'-GTC TTC GTC CCA GTA AGC TAT G-3'
m/ractin-β R	5'-GGT CTT TAC GGA TGT CAA CG-3'
mactin-β F	5'-AGC CTT CCT TCT TGG GTA TG-3'
mBiP F	5'-GCC TCA TCG GAC GCA CTT-3'
mBiP R	5'-GGG GCA AAT GTC TTG GTT-3'
mGAPDH F	5'-CAT CAA GAA GGT GGT GAA GC-3'
mGAPDH R	5'-AGG TGG AAG AGT GGG AGT TG-3'
mparkin F	5'-AAA CCG GAT GAG TGG TGA GT-3'
mparkin R	5'-AGC TAC CGA CGT GTC CTT GT-3'
ractin-β F	5'-CTC TCT TCC AGC CTT CCT TC-3'
rGAPDH F	5'-ACT CTA CCC ACG GCA AGT TC-3'
rGAPDH R	5'-TAC TCA GCA CAG CAT CAC C-3'
rparkin F	5'-AGA CAA GCA ACC CTC ACC TT-3'
rparkin R	5'-TGG CAC TCT CCA CTC ATC C-3'

Table 10: TaqMan gene expression assay (Applied biosystems)

hparkin	Hs00247755_m1
hPINK1	Hs02330592_s1
hβ-actin	P/N 4326315E
hGAPDH	P/N 4326317E
h18sRNA	P/N 4319413E

6.3.2 Solutions and buffer

LB-Medium

1% Tryptone, 0.5% NaCl, 0.5 % yeast extract in dH_2O 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

9 mM Tris-borate, 2 mM EDTA in dH_2O

Transformation buffer

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

6x DNA sample buffer

0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol

TE

10 mM Tris-Cl, pH 8,0, 1 mM EDTA, pH 8,0

6.4 MATERIAL, SOLUTIONS AND BUFFER FOR PROTEIN BIOCHEMISTRY

6.4.1 Material

4',6'-Diamidino-2-phenylindole (DAPI)	Sigma
Amplify	Amersham
Biomax MR film (autoradiography)	Kodak
Bovine serum Albumine (BSA; 2mg/ml)	New England Biolabs
Complete protease inhibitor (PI)	Roche
Dual luciferase assay system	Promega
ECL Amersham	Amersham
Goat serum	Sigma-Aldrich

Immobilon Western ECL	Millipore
Mito-tracker green $DIOC_6(3)$	Invitrogen
Mowiol 4-88	Calbiochem
Na-Deoxycholate	Sigma
PBS +/+ Mg^{2+}/Ca^{2+}	Invitrogen
PFA	Sigma
Protein A Sepharose	Pierce
PVDF-membrane	Millipore
recombinant full-length ATF4	Abnova
recombinant full-length c-Jun	Sigma
See BluePlus2	Invitrogen
Super RX film (chemiluminescence)	Fuji
Vybrant MTT cell proliferation Assay kit	Molecular Probes
Whatman paper	Schleicher & Schüll

Table 11: List of Antibodies

name	source
PRK8, monoclonal	Millipore
PRK28, monoclonal	(Pawlyk et al., 2003)
Parkin #2132, polyclonal	Cell Signaling Technology
hP1, polyclonal	(Winklhofer et al., 2003)
Actin, monoclonal	Sigma
CREB2 (ATF4) C-20, polyclonal	Santa Cruz Biotechnology
Myc 9E10, monoclonal	Sigma
TRAF6 H274, polyclonal	Santa Cruz Biotechnology
c-Jun (N) sc45, polyclonal	Santa Cruz Biotechnology
c-Jun (N) sc45X, polyclonal (EMSA)	Santa Cruz Biotechnology
SAPK/JNK, polyclonal	Cell Signaling Technology
p53, monoclonal	Calbiochem/Merck
Ubiquitin P4D1, polyclonal	Cell Signaling Technology
IKKγ (B-3), monoclonal	Santa Cruz Biotechnology
IKKγ (FL-419), polyclonal	Santa Cruz Biotechnology
TRAF2 (C-20), polyclonal	Santa Cruz Biotechnology
TRAF2 (33a1293), monoclonal	Santa Cruz Biotechnology
Phospho-c-Jun (Ser63) II, polyclonal	Cell Signaling Technology
Phospho-c-Jun (Ser73), polyclonal	Upstate
Phospho-JNK (Thr183/Tyr185), polyclonal	Cell Signaling Technology
LC3, monoclonal	nanoTools
GAPDH, monoclonal	Ambion
Active-caspase-3, polyclonal	Promega
Flag M2, monoclonal	Sigma
Alexa 555-conjugated goat anti rabbit	Sigma
Horseradish peroxidase-conjugated anti mouse IgG	Promega
Horseradish peroxidase-conjugated anti rabbit IgG	Promega

6.4.2 Solutions and buffer

PBS
3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl pH 7.4
TBS
25 mM Tris / HCl, 150 mM NaCl pH 7.2
TBS-T
TBS containing 0.1% Tween-20
Denaturing lysis buffer
50 mM Tris / HCl pH 7.4, 5 mM EDTA, 1% SDS, 15 U/ml DNAse, protease inhibitor cocktail
Non-denaturing lysis buffer
50 mM Tris/HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitor cocktail
Detergent buffer
0.1% Triton X-100 in PBS, protease inhibitor
Phosphatase inhibitor lysis buffer
20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluorid (PMSF)
Urea lysis buffer
6 M urea, 50 mM HEPES pH 8.0, 0.5% SDS and protease inhibitor cocktail
Laemmli sample buffer 4x
240 mM Tris / HCl pH 6.8, 4% SDS, 40 % glycerol, 2% bromophenol blue, 4% β-mercaptho-ethanol
APS
10% ammoniumperoxodisulfate in PBS
Lower Tris Buffer for SDS-PAGE
0.5 M Tris/HCl pH 6.8, 0.4% SDS
Upper Tris Buffer for SDS-PAGE
1.5M Tris/HCl pH 8.8, 0.4% SDS
Running Buffer for SDS-PAGE
25 mM Tris/HCl, 190 mM glycine, 0.1% SDS
Blotting buffer
25 mM Tris, 20 mM glycine
Blocking buffer
5% nonfat dry milk or 5% BSA in TBS-T
Stripping buffer
70 mM Tris pH 6.7, 2% SDS add 350 μl β-ME to 50 ml buffer before use
Hypotonic cell lysis buffer
20 mM HEPES (pH 7.9), 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1% Triton-x 100, 20% glycerol, add before use protease inhibitor, 2 mM DTT
EMSA binding buffer
10 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol
Blocking buffer (caspase assay)
5% goat serum, 0.1% Tween 20 in PBS
Mowiol mounting medium
3.26 M glycerine, 2.72 M mowiol 4-88, 0.12 M Tris pH8.5 in UPW, add 0.5 μl DAPI/ml Mowiol before use

7 Appendix

7.1 REFERENCES

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8 Publications

Bouman L., Schlierf A., Lutz A.K., Shan J., Palmisano V., Kilberg M.S., Tatzelt J. and Winklhofer K.F.
Parkin is a target of the unfolded protein response and protects cells from ER stress-induced mitochondrial damage. *Submitted.*

Lutz A.K., Exner N., Fett M.E., Schlehe J.S., Kloos K., Lämmermann K., Brunner B., Kurz-Drexler A., Vogel F., Reichert A.S., **Bouman L.**, Vogt-Weisenhorn D., Wurst W., Tatzelt J., Haass C., Winklhofer K.F.
Loss of parkin or PINK1 function increases Drp1-dependent mitochondrial fragmentation. *J Biol Chem. 2009 Aug 21;284(34):22938-51.*

Henn I.H., **Bouman L**., Schlehe J.S., Schlierf A., Schramm J.E., Nakaso K., Culmsee C., Berninger B., Krappmann D., Tatzelt J., Winklhofer K.F.
Parkin mediates neuroprotection through activation of IKK/NF-κB signaling. *J. Neurosci. 2007 Feb 21;27(8):1868-78.*

Bouman L., Sanceau J., Rouillard D., Bauvois B.;
Gamma-Glutamyl transpeptidase expression in Ewing's sarcoma cells: up-regulation by interferon. *Biochem J. 2002 Jun 15; 364(Pt 3):719-24*.

9 Curriculum Vitae

PERSONAL DETAILS

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Date of birth	January 18, 1979
Birthplace	Heidelberg, Germany

EDUCATION

April 2005 – present	**PhD** under K. F. Winklhofer, MD PhD Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE), Ludwig-Maximilians-University Munich, Adolf-Butenandt-Institute, Neurobiochemistry, Chair: Prof. C. Haass April 2005 - Dec. 2005: Max-Planck-Institute for Biochemistry, Departement of Cellular Biochemistry, Munich, Chair: Prof. F. U. Hartl Titel of PhD thesis: A Role of Parkin in Sress Response Pathways
Nov. 2003 – July 2004	**Diploma thesis** in the laboratory of Prof. J. Darnell, Rockefeller University, New York, USA Supervisor: PhD M. Henrikson, Rockefeller University and Prof. A. Nordheim, University Tübingen Title of Diploma thesis: Novel Characteristics in STAT Function: Insights into the Inactivation-/Activation Cycle of STAT1 and the Synergistic Interaction of STAT3 with GR
Oct. 2001 – Dec. 2004	**Graduate studies** Eberhard-Karls-University Tübingen, Faculty of Biology Main subject: Cell Biology/Immunology Side subjects: Genetics, Human Physiology, Virology
Oct. 2000 – Sept. 2001	**Socrates/Erasmus exchange** Université de Paris-Sud 11, Faculté des Sciences, Département de Biologie
Oct. 1998 – Sept. 2000	**Undergraduate studies** Philipps-University Marburg, Faculty of Biology
1989-1998	**School** Gymnasium des Englischen Instituts Heidelberg

PRESENTATIONS AND ATTENDECES TO CONFERENCES

Eibsee Meeting on Molecular Mechanisms of Neurodegeneration
Eibsee, Grainau/Garmisch-Patenkirchen, Germany, Nov. 11 - 14, 2009
Poster: Opposing Effects of Cytosolic and ER Stress Pathways on the Transcriptional Regulation of Parkin

Alzheimer's and Parkinson's Diseases International Conference (AD/PD 2009)
Prague, Czech Republic, March 11 - 15, 2009
Presentation: Opposing Effects of Cytosolic and ER Stress Pathways on the Transcriptional Regulation of Parkin

Eibsee Meeting on Molecular Mechanisms of Neurodegeneration
Eibsee, Grainau/Garmisch-Patenkirchen, Germany, Oct. 29 - Nov. 1, 2008
Poster: Opposing Effects of Cytosolic and ER Stress Pathways on the Transcriptional Regulation of Parkin

Molecular Chaperones and Stress Response
Cold Spring Harbor Laboratories, USA, April 30 – May 4, 2008
Poster: Parkin Integrates Cytosolic and ER Stress Pathways

Ringberg Symposium: Molecular Mechanisms on Prion Diseases and Parkinson's Disease
Ringberg Castle, Rottach Egern, Germany, March 5 - 8, 2008

Interact PhD Symposium
Munich, Germany, Dec. 7, 2007
Poster: Role of Parkin in Stress Response Pathways

Eibsee Meeting on Molecular Mechanisms of Neurodegeneration
Eibsee, Grainau/Garmisch-Patenkirchen, Germany, Nov. 28 – Dec. 1, 2007
Poster: Role of Parkin in Stress Response Pathways

43. Herbsttreffen auf Schloss Hochhausen am Neckar
Hochhausen am Neckar, Germany, Oct. 18 – 20, 2007

Alzheimer's and Parkinson's Diseases International Conference (AD/PD 2007)
Salzburg, Austria, March 14 - 18, 2007

Eibsee Meeting on Molecular Mechanisms of Neurodegeneration
Eibsee, Grainau/Garmisch-Patenkirchen, Germany, April 17 - 20, 2005