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Effects of Parkin and mutant Huntingtin on the NF-κB signaling pathway

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Erklärung

Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Frau Prof. Dr. Konstanze F. Winklhofer betreut.

Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

München, _____

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(Maria Patra)

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“Experience is what you get,

when you didn’t get what you wanted.”

(Randy Pausch)

“SCIENTIIS ET ARTIBUS”

(dedicated to the sciences and arts)

Acknowledgement

„No one can whistle a symphony (alone); it takes a whole orchestra to play it."
(Halford E. Luccock)

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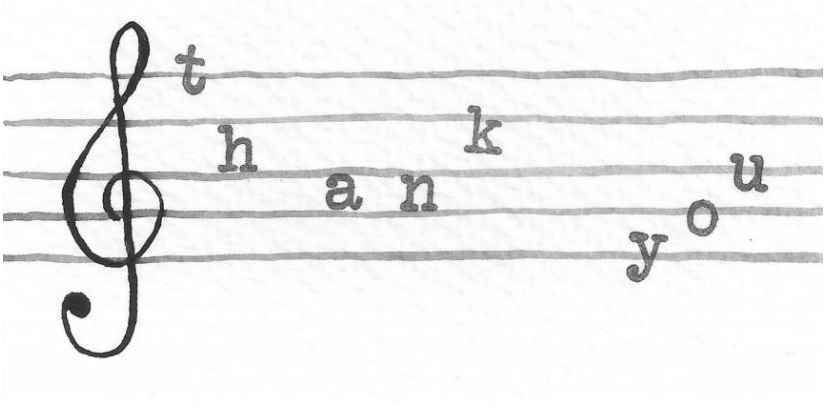

Meine Laborzeit erinnert mich an ein Lied der Toten Hosen
und hat mich zu folgenden Zeilen inspiriert:

10 kleine Forschergeister

Zehn kleine Forschergeister forschten im Labor,
der eine ging nach Tübingen, da waren's nur noch neun.

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Einer für alle, alle für einen,
wenn einer fort ist, wer wird denn gleich weinen?
Einmal trifft's jeden, ärger dich nicht,
so geht's im Leben, du oder ich.

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Einer für alle, alle für einen,
wenn einer fort ist, wer wird denn gleich weinen?
Einmal trifft's jeden, ärger dich nicht,
so geht's im Leben, du oder ich.

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die eine mit dem Ruderboot, die and’re mit der Geig’n.

Einer für alle, alle für einen,
wenn einer fort ist, wer wird denn gleich weinen?
Einmal trifft's jeden, ärger dich nicht,
so geht's im Leben, du oder ich.
Einmal muss jeder gehen
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davon wird die Welt nicht untergehn -
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I. Abstract

Recently, we showed that the E3 ubiquitin ligase Parkin, which is associated with Parkinson's Disease (PD), influences NF-κB signaling via increased activation of the linear ubiquitin assembly complex (LUBAC). Huntington's Disease (HD) shares pathologic features such as mitochondrial alterations and augmented neuronal cell death with PD. Therefore, we were interested in the question if similar signaling pathways might be affected in HD and PD. To address this issue, we examined whether and how Huntingtin aggregates might alter the pro-survival NF-κB pathway.

Using HD cell culture models we discovered that Huntingtin aggregates sequester and trap components of the LUBAC, thereby blocking one of the first steps of the TNF-α-induced NF-κB pathway. As a consequence, nuclear translocation of the transcription factor NF-κB is decreased leading to reduced expression of NF-κB responsive target genes. OPA1, a key regulator of mitochondrial fusion, belongs to the group of NF-κB responsive target genes, which can explain why increased mitochondrial fragmentation is found in HD. Due to the impairment of the pro-survival NF-κB pathway, two other TNF-α-induced pro-apoptotic pathways that are usually suppressed by a subset of NF-κB targets, are up-regulated in cells with Huntingtin aggregates: the JNK signaling pathway and the caspase cascade. This switch from pro-survival NF-κB signaling to programmed cell death caused by an expansion of the polyQ stretch in Huntingtin can explain the increased vulnerability to stress-induced cell death. Interestingly, overexpression of Parkin as well as HOIP, which is the catalytic subunit of the LUBAC, can restore defective NF-κB signaling and thereby rescue from mitochondrial fragmentation and increased JNK activation induced by polyQ aggregates. Moreover, knockdown of the two proteins Parkin or HOIP results in increased toxicity of Huntingtin aggregates.

Having demonstrated that the NF-κB pathway is not only affected in PD, but also in HD, it is tempting to speculate that also other neurodegenerative diseases might share an impairment in this common pathway.

II. Zusammenfassung

Vor kurzem konnten wir zeigen, dass die E3 Ubiquitin-Ligase Parkin, die mit der Parkinson-Krankheit (Parkinson's Disease, PD) in Verbindung steht, den NF-κB-Signalweg durch eine erhöhte Aktivierung des linearen Ubiquitin-Assemblierungskomplexes (Linear Ubiquitin Assembly Complex, LUBAC) beeinflusst. Die Huntington-Krankheit (Huntington's Disease, HD) weist gemeinsame pathologische Eigenschaften wie mitochondriale Veränderungen und vermehrten neuronalen Zelltod mit der Parkinson-Krankheit auf. Deshalb waren wir an der Frage interessiert, ob ähnliche Signalwege bei HD und PD betroffen sind. Um diese Hypothese zu addressieren, untersuchten wir, ob und wie Huntingtin-Aggregate den überlebensfördernden NF-κB-Signalweg beeinflussen können.

Wir entdeckten mittels HD Zellkulturmodellen, dass Huntingtin-Aggregate Komponenten des LUBAC binden und abfangen, wodurch sie einen der ersten Schritte des TNF-α-induzierten NF-κB-Wegs blockieren. Folglich tritt eine verminderte nukleäre Translokation des Transkriptionsfaktors NF-κB auf, die zu reduzierter Expression von NF-κB-responsiven Zielgenen führt. OPA1, ein wichtiger Regulator von mitochondrialer Fusion, gehört zur Gruppe der NF-κB-responsiven Zielgene, was die erhöhte mitochondriale Fragmentierung bei HD erklären kann. Wegen der Beeinträchtigung des überlebensfördernden NF-κB-Signalwegs sind zwei andere TNF-α-induzierte apoptosefördernde Signalwege, die normalerweise durch einen kleinen Teil der NF-κB Zielgene unterdrückt werden, in Zellen mit Huntingtin-Aggregaten hochreguliert: der JNK-Signalweg und die Caspase-Kaskade.

Dieses Umschalten von überlebensfördernder NF-κB Wirkung hin zu programmiertem Zelltod aufgrund des expandierten polyQ-Strangs in Huntingtin kann die gesteigerte Stress-induzierte Zelltod-Rate erklären. Interessanterweise kann Überexpression von Parkin oder HOIP, der katalytischen Untereinheit des LUBAC, eine gestörte NF-κB-Aktivierung wiederherstellen und dadurch vor mitochondrialer Fragmentierung und erhöhter JNK-Aktivierung schützen, die durch polyQ-Aggregate induziert werden. Zudem führt eine verminderte Expression von Parkin oder HOIP zu einer erhöhten Toxizität der Huntingtin-Aggregate.

Nachdem gezeigt wurde, dass der NF-κB Signalweg nicht nur bei PD, sondern auch bei HD betroffen ist, könnte man mutmaßen, dass sogar noch weitere neurodegenerative Erkrankungen eine Beeinträchtung dieses Stoffwechselwegs gemeinsam haben könnten.

1. Introduction

1.1 Parkin

1.1.1 Parkinson's Disease

a) History

Parkinson's Disease (PD) is the most common movement disorder and after Alzheimer's Disease the second most common neurodegenerative disease [Winklhofer and Haass, 2010]. It is estimated that PD affects four to six million people worldwide and 1.2 million people in Europe [http://www.epda.eu.com]. The disease was first documented by the English physician James Parkinson, who described the symptomes of idiopathic PD in his famous "Essay on the Shaking Palsy" in 1817. Another notable contribution to the understanding of the disease was made by the French neurologist Jean-Martin Charcot with his studies differentiating between rigidity, weakness and bradykinesia. He also pleaded for renaming the disease in honor of James Parkinson [Lees 2007]. In 1912, Friedrich Heinrich (Frederic) Lewy discovered microscopic particles in affected PD brains, which were later named "Lewy bodies" [Holdorff 2002]. It took almost a whole century, until α-Synuclein was identified as the main component of the Lewy bodies in 1997 [Spillantini 1997, Schulz-Schaeffer 2010].

Fig. 1: (left) James Parkinson (1755 - 1824) and (right) Frederic Lewy (1885 - 1950).
[http://www.biography.com/people/james-parkinson-21226395; http://lewybody.org/science]

b) Etiology

So far, little is known about the etiology of Parkinson's Disease. A number of environmental factors have been associated with an increased risk of sporadic PD including insecticides, pesticides, such as rotenone or paraquat, and herbicides, such as Agent Orange [de Lau and Breteler 2006]. Besides that, the most important risk factor for the development and progression of PD seems to be aging. With increasing age, the prevalence of PD increases as

well and this age-specific prevalence is remarkably similar in the majority of countries in Europe [Hindle 2010]. The mean age of onset is typically around 60 years, although in 5 to 10% of cases, classified as young onset, patients are affected between the age of 20 and 50 [Samii 2004]. The process of aging is thought to be a stochastic combination of predictable and random effects that lead to the accumulation of unrepaired cellular damage, weakened cellular repair and compensatory mechanisms. Aging is associated with mitochondrial dysfunction, increased free radical production and oxidative stress, which may lead to genomic instability and DNA mutations, with shortening of telomeres promoting reduced survival [Kirkwood 2003, Migliore and Coppedè 2009]. Another characteristic feature is the age-related decline in proteasomal activity leading to a lack in the degradation of damaged or ubiquitinated proteins and an increase in abnormal deposition of cellular brain proteins [Tai and Schuman 2008]. All of these age-related changes are relevant to the etiology and pathogenesis of PD, although it is not clear yet, if PD reflects a failure of the normal cellular compensatory mechanisms in vulnerable brain regions, and if this vulnerability is increased by aging [Hindle 2010].

Furthermore, several genetic mutations could be identified, which are responsible for monogenic familial PD forms (up to 10% of all PD cases). Genetic studies revealed mutations in 13 genes, which are mostly associated with rare forms of PD with early onset. However, only six of these genes have conclusively been linked to PD so far (Table 1).

gene	gene product	inheritance	putative function
PARK1/4	α-Synuclein (SNCA)	AD	vesicle trafficking/ synaptic plasticity
PARK2	Parkin	AR	E3 ubiquitin ligase
PARK6	PINK1	AR	mitochondrial kinase
PARK7	DJ-1	AR	cytosolic redox-sensitive protein
PARK8	LRRK2	AD	MAPKK kinase
PARK9	ATP13A2	AR	lysosomal ATPase

Table 1: Only genes with compelling evidence for association with PD are listed.
AD = autosomal dominant, AR = autosomal recessive

c) Genetics

Through the identification of genes responsible for the rare familial forms of PD, our understanding of the molecular mechanisms underlying the pathogenesis of PD was tremendously improved.

PARK1/PARK4 (α-Synuclein)

In 1997, the first discovered risk gene for familial PD was PARK1/PARK4 [Polymeropoulos 1997]. The corresponding protein is localized in the cytoplasm of neurons, where it is closely associated with synaptic vesicles [Maroteaux 1988]. As mentioned in the previous chapter, α-Synuclein is a major component of Lewy bodies, which provides an interesting link between sporadic and familial PD. Pathogenic mutations in this protein lead to increased self-assembly and fibrillization [Greenbaum 2005]. Moreover, genomic multiplication of the α-Synuclein locus enhances insoluble α-Synuclein aggregation. An ongoing debate discusses which species of the protein implicates the highest toxicity for the cell - oligomers, protofibrils or fibrils.

PARK2 (Parkin)

Parkin was the first recessive gene which was found to be connected to PD. Mutations in this E3 ubiquitin ligase were described in 1998 by Kitada and co-workers, who identified it as the cause of recessive PD with juvenile onset in a Japanese family [Kitada 1998]. Mutations in PARK2 account for the majority of autosomal recessive Parkinsonism. Interestingly, patients with Parkin mutations tend to develop symptoms at a much younger age, such as below 30 years [Mata 2004]. A more detailed description of Parkin will be given in chapter 1.1.2.

PARK6 (PINK1)

PTEN-induced putative kinase 1 (PINK1) is a ubiquitously expressed protein, which is highly conserved between species. It contains an N-terminal mitochondrial targeting sequence, a transmembrane domain and a C-terminal serine-threonine kinase domain. Mutations in the PINK1 gene are the second most common cause of autosomal recessive Parkinsonism. Pathogenic PINK1 mutations cluster in the kinase domain leading to an effect on either kinase activity or protein stability supporting a loss-of-function mechanism.

In contrast to *Drosophila* PINK1 mutants showing reduced life span, male sterility and apoptotic flight muscle degeneration, PINK1 knockout (KO) mice do not display an overt phenotype. Impaired mitochondrial respiration in the striatum of PINK1 KO mice and decreased complex I activity were observed in PINK1-deficient mice and flies pointing to a bioenergetic deficit when PINK1 is missing [Pilsl and Winklhofer 2011].

First evidence for a genetic interaction of PINK1 and Parkin came from studies in fruit flies, which indicated that Parkin-deficient flies show a similar phenotype as PINK1-deficient flies.

Importantly, the PINK1 mutant phenotype could be restored by overexpression of Parkin, but not *vice versa*. These observations suggest a function of PINK1 and Parkin in the same pathway with Parkin acting dowstream of PINK1.

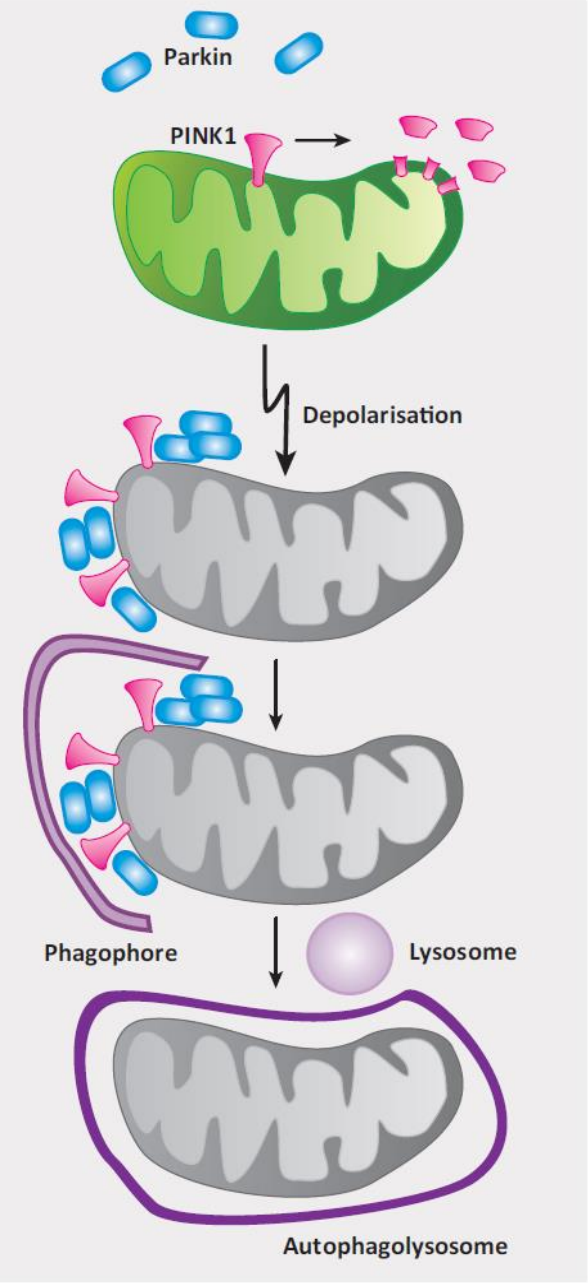


Fig. 2: Concept of mitophagy. Mitochondrial depolarization leads to stabilization of PINK1 on the surface of mitochondria. PINK1 then recruits and activates Parkin, which in turn fosters ubiquitination and remodeling of the outer mitochondrial membrane to attract the autophagic machinery. These damaged mitochondria become engulfed by autophagosomes fusing with lysosomes resulting in formation of autophagolysosomes [Winklhofer 2014].

Additional evidence for such a linear pathway was provided by cell-culture studies demonstrating that Parkin promotes autophagic degradation of depolarized mitochondria in a

PINK1-dependent manner. When HeLa cells overexpressing Parkin are treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP), mitochondrial depolarization leads to stabilization of PINK1 at the outer mitochondrial membrane. Then, PINK1 recruits and activates Parkin at the outer mitochondrial membrane resulting in Parkin-dependent ubiquitination and remodeling of the outer mitochondrial membrane, which attracts the autophagic machinery. Next, autophagosomes engulf damaged mitochondria and finally fuse with lysosomes to form autophagolysosomes. This process is termed mitophagy (Fig. 2). At present, it is a great challenge to transfer this artificial setting with harsh treatment of cells to more physiological conditions in order to examine the relevance of the mitophagy concept to the observed phenotypes from PINK1- or Parkin-deficient animal models and, what is even more important, to pathogenic mechanisms in patients [Winklhofer 2014].

PARK7 (DJ-1)

In 2003, Bonifati et al. discovered a large deletion and missense mutation in the DJ-1 gene in Italian and Dutch PD patients, leading to the identification of the DJ-1 gene as a causative gene for familial PD with recessive inheritance [Bonifati 2003]. Compared to Parkin and PINK1, the number of mutations in the DJ-1 gene is relatively small. Diverse functions including transcriptional regulation, antioxidative stress reaction as well as chaperone, protease, and mitochondrial regulation have been described for DJ-1. Its activity is regulated by the oxidative status, especially that of cysteine 106 [Ariga 2013].

PARK8 (LRKK2)

Discovered in 2004, Leucine-rich repeat kinase 2 (LRRK2)/Dardarin encodes a protein consisting 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 and a WD40 domain [Taylor 2006]. In 2008, LRKK2 was also linked to Crohn's disease by genome-wide association studies (GWAS) [Barrett 2008].

Among the six clearly PD-associated gene loci, only α-Synuclein and LRRK2 mutations cause autosomal dominant forms of the disease. So far, LRRK2 has been found to be the most frequent cause of late-onset PD. The mutations are found in 5-6 % of patients with familial PD and have also been implicated in sporadic PD [Ho 2014].

Currently, LRRK2 is considered to be a potential therapeutic target for the treatment of PD. Several pathogenic mutations were found and the majority of them is located in the dual catalytic domains of LRRK2. The most common mutation G2019S results in increased kinase activity of LRRK2 and therefore, much effort is put into the development of potent and specific inhibitors of LRRK2 kinase activity [Dzamko 2013].

PARK9 (ATP13A2)

Mutations in the ATP13A2 (PARK9) gene lead to the Kufor-Rakeb syndrome (KRS), a severe early-onset autosomal recessive form of PD with dementia [Ramirez 2006]. ATP13A2 is a lysosomal transmembrane protein belonging to the P5-type ATPase family and seems to be a critical regulator of lysosomal functions. As overexpression of ATP13A2 suppresses α-Synuclein toxicity, two genetic risk factors of PD are connected, highlighting the central role of ATP13A2 in PD. Loss of ATP13A2 function was also linked to neuronal ceroid lipofuscinosis (NCL), a lysosomal storage disorder, implicating an impairment of the lysosomal pathway in KRS and PD [van Veen 2014].

d) Symptoms

The primary symptoms of Parkinson's Disease are all related to voluntary and involuntary motor function. Moreover, they usually start on one side of the body. Being mild at first, the symptoms will progress over time. Studies have shown that by the time when primary symptoms appear, individuals with PD have already lost 60 to 80% of the dopaminergic neurons. The four primary motor symptoms include tremor, rigidity, bradykinesia or hypokinesia and postural instability (Fig. 3), while additional non-motor symptoms like sleep abnormalities, depression and cognitive impairment occur [Lang and Lonzano 1998, Pilsl and Winklhofer 2011].

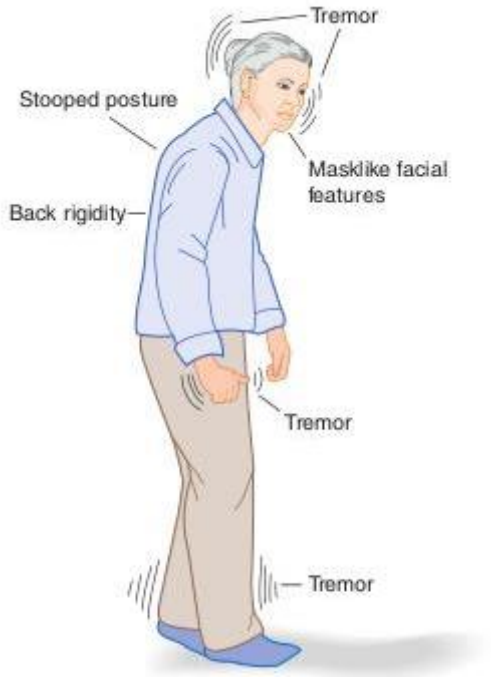


Fig. 3: Primary motor symptoms of Parkinson's Disease:
Tremor (involuntary, rhythmic shaking of a limb, head, or entire body) – The most recognised symptom of Parkinson's Disease, tremor often starts with an occasional tremor in one finger that eventually spreads to the whole arm. The tremor may affect only one part or side of the body, especially in the early stages of the disease. Not everyone with Parkinson's Disease has tremor.
Rigidity (stiffness or inflexibility of the limbs or joints) – The muscle rigidity experienced with Parkinson's Disease often begins in the legs and neck. Rigidity affects most people. The muscles become tense and contracted, and some people may feel pain or stiffness.
Bradykinesia or akinesia (slowness of movement or absence of movement) – Bradykinesia is one of the classic symptoms of Parkinson's Disease. After a number of years, people suffering from PD may experience akinesia, or "freezing," and not be able to move at all.
Postural Instability (impaired balance and coordination) – A person with postural instability may have a stooped position, with head bowed and shoulders drooped.

[https://bootheels33.wordpress.com/2013/04/03/when-p-was-for-parkinsons-disease; http://www.medtronic.co.uk/your-health/parkinsons-disease]

e) Diagnosis

Making an accurate diagnosis of PD is difficult, as there is no standard diagnostic test available. Because PD symptoms occur due to decreased dopamine levels, one possible test is to administer the drug Levodopa (L-Dopa/L-3,4-dihydroxyphenylalanine) to the patient and monitor the response to the treatment. Significant improvement with this medication often confirms the diagnosis of PD.

However, there are additional imaging techniques to analyze brain activity, which can be applied to support diagnostics. At present, either PET (Positron Emission Tomography) or DaT (Dopamine Transporter) scans help to distinguish between the different diseases linked to Parkinsonian symptoms. First, the patient receives an injection of an imaging agent, which can be visualized by a special detector. Then, the scan measures either glucose metabolism or dopamine transporter activity in the brain. If the measured activity is reduced in the patient compared to a control brain and can be elevated by a drug like L-Dopa, the diagnosis of PD is likely (Fig. 4) [www.parkinson.org].

Moreover, there is evidence from numerous studies that impairment of olfaction is a characteristic and early feature of PD. Deficits in the sense of smell may precede clinical motor symptoms by years and can be used to assess the risk for developing PD in otherwise asymptomatic individuals. It was observed that over 95% of patients with PD displayed

significant olfactory loss. Thus, olfactory dysfunction should be considered as a reliable diagnostic marker of the disease [Haehner 2009].

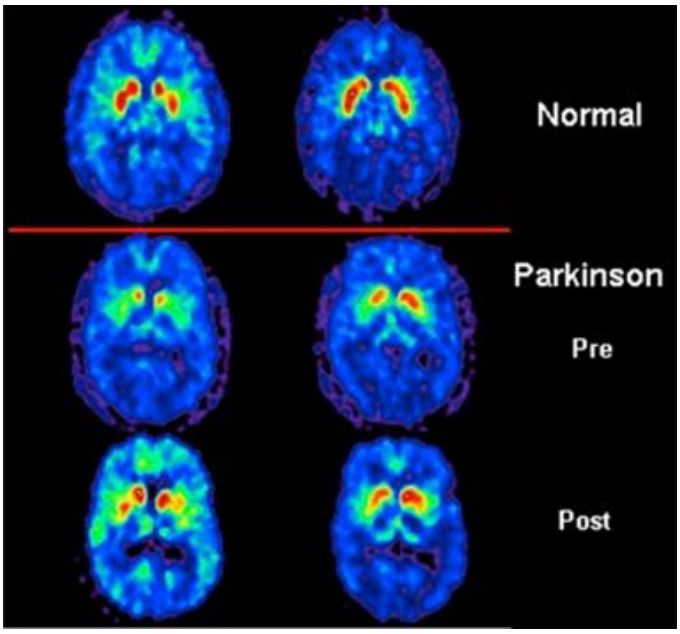


Fig. 4: PET scans from a healthy and a PD-affected individual. Top panel: normal scan, middle panel: abnormalities in the putamen (red uptake in the figure) in a patient with Parkinson's Disease, lower panel: a return to an almost normal scan following the introduction of levodopa. [parkinson.org].

f) Therapy

Currently, no treatment is available to cure Parkinson's Disease. However, there are drugs to treat both motor and non-motor symptoms associated with PD. Most of the therapeutic approaches focus on the compensation of the dopaminergic deficit, thereby alleviating the cardinal symptoms of the disease. Soon after the discovery of the nigral dopamine loss occurring in PD, treatment with the dopamine precursor Levodopa revolutionized the therapy. For the last five decades, Levodopa has been seen as the gold-standard to treat the motor symptoms. In addition to L-Dopa, an inhibitor of the peripheral Dopamine decarboxylase (DDC), like Carbidopa, is administered (Fig. 5). This blocks peripheral conversion of L-Dopa and allows a high amount of applied L-Dopa to reach and pass the blood-brain barrier. Within the central nervous system, L-Dopa is then converted into dopamine in neurons. Besides this precursor molecule, dopaminergic agonists can be utilized to stimulate dopamine receptors or the dopamine catabolism can be blocked by monoamine oxidase B (MAO-B) inhibitors. MAO-B inhibitors intervene at the conversion of dopamine to 3,4-Dihydroxyphenylacetic acid (DOPAC) and are mostly used to treat mild symptoms of PD. Another possibility is to use catechol-O-methyl-transferase (COMT) inhibitors which reduce the methylation of L-Dopa and dopamine and thereby increase the bioavailability of these substances. One critical

aspect regarding the application of the mentioned drugs is the appearance of severe non-motor side effects like psychiatric disorders and fatigue. Severe side effects also occur when anticholinergic drugs are administered for some tremor cases.

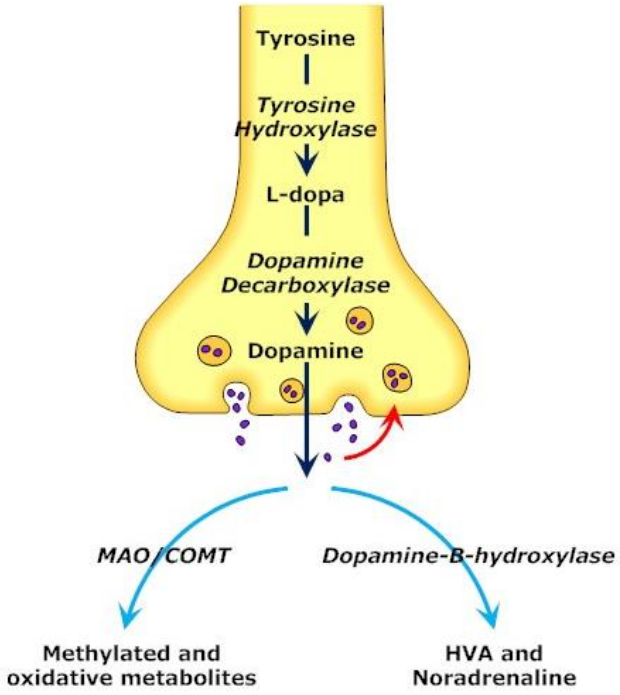


Fig. 5: Dopamine metabolism.
The neurotransmitter Dopamine originates from the amino acid tyrosine and the precursor L-Dopa. Dopamine is either processed by the enzymes MAO/COMT via oxidation/methylation or by the Dopamine-B-hydroxylase. [http://www.smartdraw.com/examples/view/neurology+of+dopamine+metabolism]

For treatment of non-motor symptoms, Clozapine is the most efficient antipsychotic agent in PD patients, but due to the possible development of agranulocytosis, Quetiapine is the first-line antipsychotic drug being used.

Besides medication, another option for some PD patients is deep brain stimulation (DBS). Medical surgery and introduction of microelectrodes in specific regions of the basal ganglia can only be offered to few patients who fulfill several criteria [Smith 2012].

Unfortunately, the goal to provide neuroprotective interventions, which would be able to modify the progression of Parkinson's Disease, has not been met over the last decades, despite potentially encouraging results with compounds, such as Rasagiline [Rascol 2011].

g) Neuropathology

One characteristic feature of PD is the preferential loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNc) projecting to the striatum. The hormone and neurotransmitter dopamine, which belongs to the family of catecholamines, is involved in the

regulation of voluntary movements. As a consequence, high dopamine levels lead to high levels of motor activity, while low levels of dopamine lead to slowed movement.

Cell death of dopaminergic neurons, which convert dopamine to a dark-pigmented product called Neuromelanin, leads to depigmentation of the *substantia nigra*. This phenomenon can be investigated in *post mortem* brains of PD patients. PD symptoms occur when approximately 80% of striatal dopamine are depleted [Winklhofer 2007].

It should be noted that the neurodegenerative process is not limited to dopaminergic neurons. In addition, noradrenergic, serotonergic and cholinergic systems, the cerebral cortex, brain stem, spinal cord and the peripheral autonomic nervous system are also affected [Pilsl and Winklhofer 2011].

A further pathologic hallmark of PD is the presence of Lewy bodies and Lewy neurites containing aggregated α-Synuclein as a main component [Spillantini 1997]. On the one hand, Lewy bodies could implicate a protective function via sequestration of toxic misfolded protein species. On the other hand, they could provide a reservoir for toxic protein species. Importantly, Lewy bodies are not present in all Parkinsonian syndromes, leading to the conclusion that PD comprises diverse disease forms [Pilsl and Winklhofer 2011].

1.1.2 Features of the E3 ubiquitin ligase Parkin

a) Modular structure of Parkin

The Parkin gene encodes a protein consisting of 465 amino acids and a molecular weight of 52 kDa [Kitada 1998]. The gene shares a bidirectional promoter with the Parkin-coregulated gene (PACRG), which is transcribed on the opposite strand in the opposite direction [Lockhart 2004]. Similarly to PINK1, Parkin is ubiquitously expressed and high expression levels were found in the brain, heart, skeletal muscle and testis. Surprisingly, Parkin shows only low expression in the *substantia nigra pars compacta* [Kitada 1998]. Within cells, Parkin is localized in the cytoplasm [Shimura 1999].

The modular structure of Parkin contains several structural elements: a ubiqutin-like (UBL) domain at the N-terminus and a RING-between-RING (RBR) domain at the C-terminus (Fig. 6). This RBR domain consists of two different RING (Really Interesting New Gene) domains - RING1 and RING2 - and an IBR (in-between RING) domain. An additional, atypical RING domain - RING0 - was identified lying N-terminal to the RBR domain. As Parkin contains an RBR motif, it belongs to the RBR class of E3 ubiquitin ligases [Wenzel and Klevit 2012].

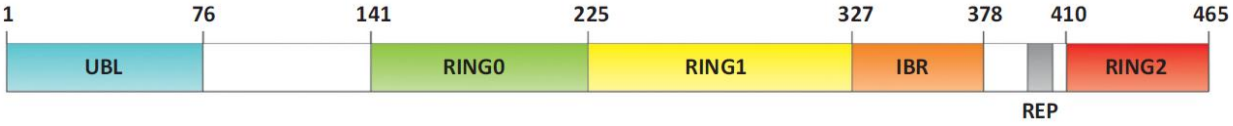


Fig. 6: Domain architecture of Parkin. UBL: ubiquitin-like, RING: really interesting new gene, IBR: in-between RING, REP: repressor element of Parkin. Numbers indicate amino acid residues [Winklhofer 2014].

Altogether, the structure of Parkin is formed in a relatively compact manner with two occluded regions: the active site cysteine in RING2 (C431) and the E2-binding site in RING1 are not accessible, indicating an autoinhibited state (Fig. 7). The phemomenon of autoinhibition was also reported for other E3 ubiquitin ligases, such as HHARI (Human Homolog of Drosophila Ariadne-1), HOIP (HOIL-1-interacting protein) and HOIL-1L (haem-oxidized IRP2 ubiquitin ligase 1), which points to autoinhibition as a general feature of RBR ligases. However, the inhibitory mechanisms differ between these RBR ligases [Winklhofer 2014].

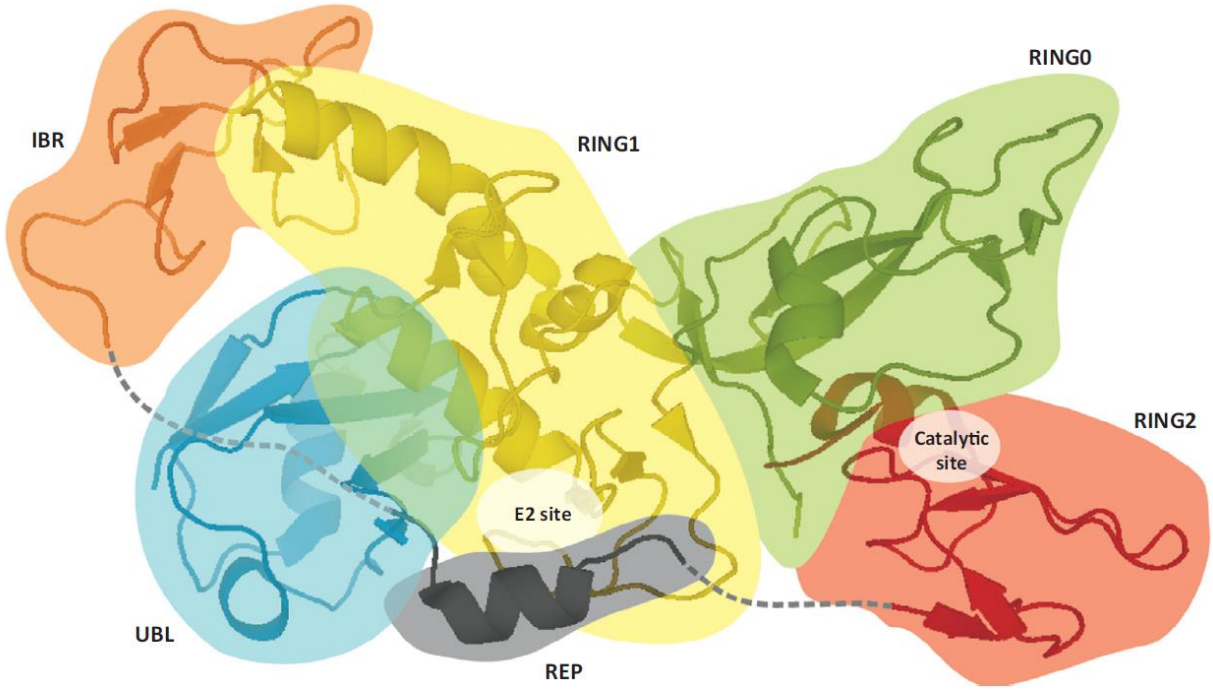


Fig. 7: Schematic structure of Parkin. The compact structure of Parkin represents an autoinhibited state with an inaccessible E2-binding site in RING1 and occluded C431 in RING2 [Winklhofer 2014].

b) RBR E3 ligase mechanism

Ubiquitination is carried out by a trio of enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase. In humans, the number of E3 ligases with over 600 greatly exceeds the number of E2 enzymes with less than 40, which underscores their role in substrate selection [Wenzel and Klevit 2012].

E3 ligases are divided into three groups: RING, HECT (homologous with E6-associated protein C-terminus) and RBR (RING-between-RING) ligases. Parkin belongs to the RBR class of E3 ubiquitin ligases (Fig. 8), which consists of 14 complex multidomain enzymes [Smit and Sixma 2014]. Other well-known candidates of RBR E3 ubiquitin ligases are HOIP and HOIL-1L, which are components of the multiprotein complex LUBAC [Spratt 2014].

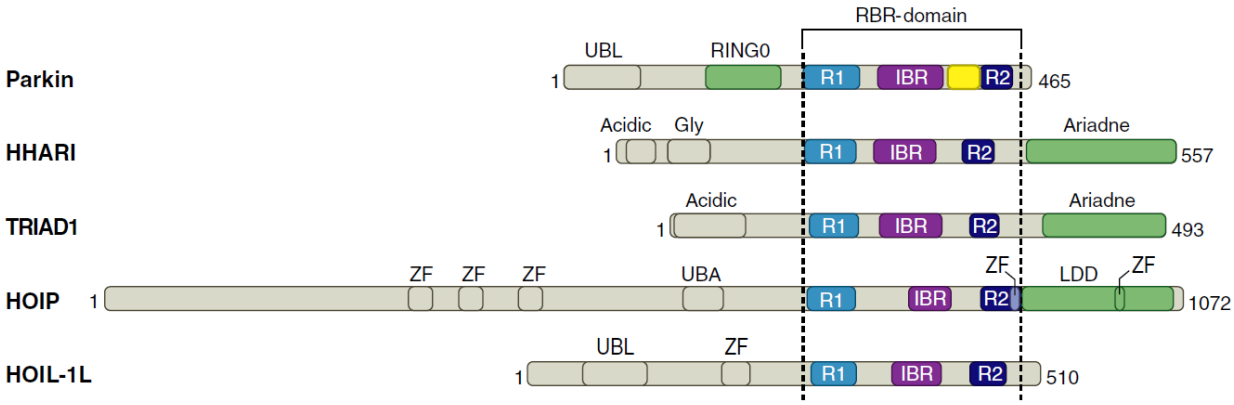


Fig. 8: Domain organisation of the RBR E3 ligases Parkin, HHARI, TRIAD1, HOIP and HOIL-1L. UBL: ubiquitin-like, acidic, Gly: glycine-rich, ZF: zinc finger, UBA: ubiquitin-associated, R1: Ring 1, IBR : in-between RING, R2: Ring 2. Only HHARI, TRIAD1 and HOIP contain an Ariadne or linear ubiquitin determining domain (LDD) [Smit 2014].

The RBR E3 ubiquitin ligases use a hybrid mechanism combining features of RING and HECT ligases to transfer ubiquitin to substrate proteins (Fig. 9). RING ligases are known to facilitate the direct transfer of ubiquitin from a ubiquitin-charged E2 to the substrate, whereas HECT ligases form a thioester intermediate with ubiquitin via a catalytic cysteine residue. In a following step, the ubiquitin moiety is transferred to the lysine residue of a target protein by generation of an isopeptide bond. The RING/HECT hybrid mechanism of RBR E3 ligases comprises the binding of an E2 ubiquitin-conjugating enzyme via RING1 as seen in RING ligases and the transfer of the ubiquitin from E2 to a catalytic cysteine residue in RING2 forming a transient thioester intermediate like described for HECT ligases [Wenzel and Klevit 2012, Winklhofer 2014].

This hybrid mechanism of RBR ligases was first identified in HHARI [Wenzel 2011]. In contrast to Parkin and HOIP, HHARI contains an additional Ariadne domain at its C-terminus, which blocks access to the catalytic cysteine residue in the RBR domain and is thereby responsible for an intramolecular autoinhibition mechanism.

Showing structural and functional similarities to Parkin, HHARI is suggested as a possible candidate to compensate for the loss of Parkin function in other neurons than dopaminergic

ones. Furthermore, endogenous HHARI was found in Lewy bodies of both PD and diffuse Lewy body disorder patients [Parelkar 2012].

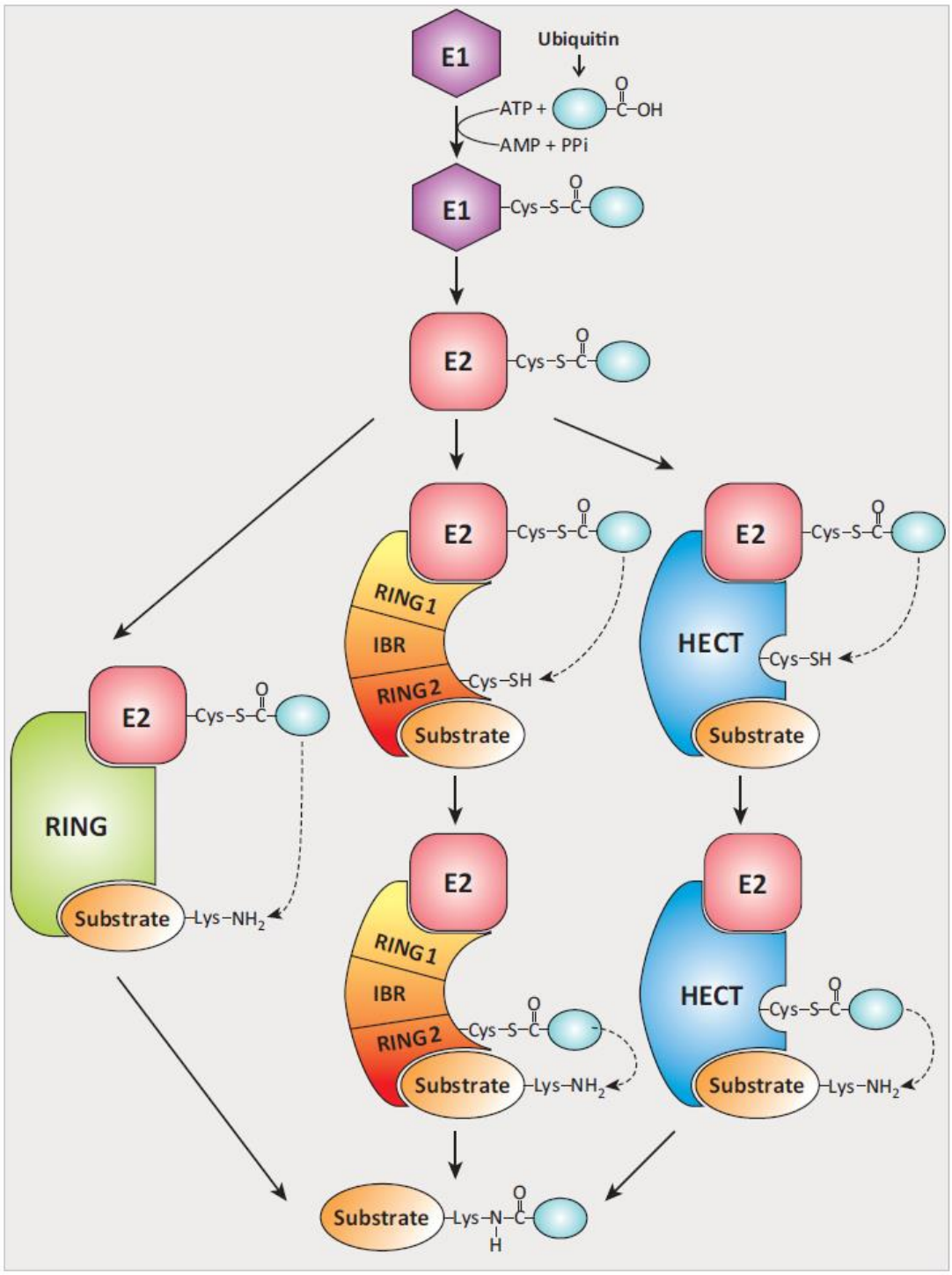


Fig. 9: Mechanism of ubiquitin transfer mediated by RING, HECT or RBR ligases. Three enzymes cooperate to accomplish ubiquitination: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase. RBR ligases combine features of the RING and HECT ligases by binding of an E2 ubiquitin-conjugating enzyme via RING1 like seen in RING ligases and the transfer of the ubiquitin from E2 to a catalytic cysteine residue in RING2 forming a transient thioester intermediate like reported for HECT ligases. [Winklhofer 2014]

c) Pathogenic mutations

So far, more than 120 different pathogenic Parkin mutations have been described, which account for most cases of autosomal-recessive Parkinsonism. Various types of mutations were identified: missense mutations, nonsense mutations and exonic rearrangements like duplications or deletions [www.molgen.ua.ac.be/PDmutDB].

Although the mutations are distributed over almost the whole gene, clusters of mutations can be found in the functional domains pointing to the significance of these regions. A loss-of-function mechanism was suggested for pathogenic Parkin mutations by genetic and biochemical studies, whereby the mutations are thought to impact either intramolecular or interactions with E2, substrate or adaptor proteins. Parkin folding and stability or catalytic activity could also be affected by the mutations [Winklhofer 2014].

d) Parkin-deficient animal models

Parkin-deficient mice do not develop nigrostriatal neurodegeneration, but evidence for mitochondrial alterations was described in several reports. Parkin knock-out (KO) mice show decreased levels of proteins which are involved in mitochondrial function or oxidative stress response in the ventral midbrain [Dawson and Dawson 2010, Palacino 2004]. Parkin KO mice do not recapitulate the human phenotype of PD, which could be explained by compensatory mechanisms through other E3 ubiquitin ligases.

In contrast, Parkin- as well as PINK1-deficient flies show a clear phenotype with reduced life span, male infertility, locomotor deficits and flight muscle degeneration [Pilsl and Winklhofer 2011]. Intriguingly, genetic interaction studies revealed that Parkin was able to rescue the PINK1 loss-of-function phenotype but not *vice versa*. This observation suggests a position of PINK1 upstream of Parkin within the same pathway [Clark 2006, Park 2006, Yang 2006, Exner 2007].

e) Function and putative substrates of Parkin

With its characteristic RBR domain, Parkin is assigned to the RBR E3 ubiquitin ligases and supposed to play a crucial role in ubiquitination. Ubiquitination is a post-translational modification that determines the fate of many proteins in the cell, as this process is involved in cell-cycle progression, transcriptional regulation, DNA repair, signal transduction and protein turnover by the proteasome [Wenzel and Klevit 2012, Spratt 2014].

Ubiquitin molecules are linked via their C-terminal glycine residue to the ε-amino group of a lysine residue belonging to the target protein or another ubiquitin molecule. Different modes of ubiquitination exist to target the proteins for different purposes. Monoubiquitination (attachment of a single ubiquitin molecule), multi- (attachment of several single ubiquitin molecules) and polyubiquitination (attachment of ubiquitin chains) can be discriminated (Fig. 10) [Sadowski 2012].

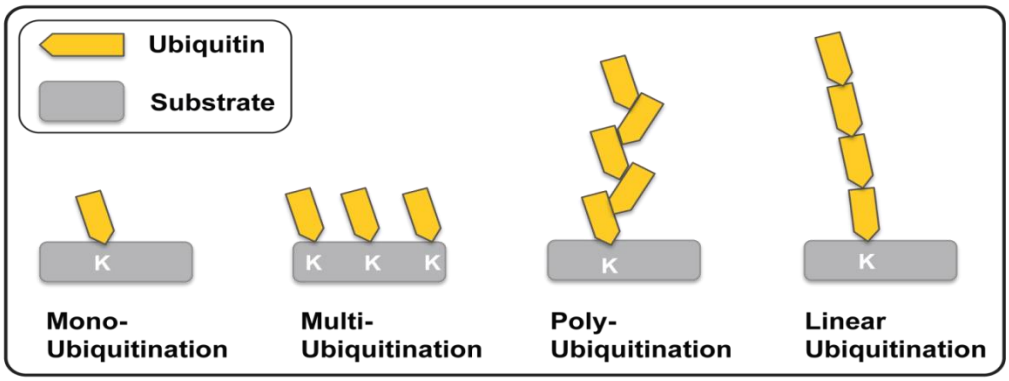


Fig. 10: Proteins may be ubiquitinated in diverse ways: mono-, mulit-, poly- and linear ubiquitination as well as combinations thereof. [http://sysimg.ifrec.osaka-u.ac.jp/udb/about.html]

Furthermore, ubiquitin has seven lysine residues, opening the possibility of forming seven different linkage types, namely K6, K11, K27, K29, K33, K48 and K63. Besides, a donor ubiquitin can also be attached to an acceptor ubiquitin via the amino terminal methionine (M1) resulting in the formation of M1- or linear linkages, making a total of eight different inter-ubiquitin linkage types [Zinngrebe 2014]. Each linkage type fulfills a different physiological function (Fig. 11): K48-linkage targets proteins for proteasomal degradation, while K63-linkage is required for cell signaling in DNA damage response, endocytosis and kinase activation and preferentially involved in the lysosomal pathway. The roles of the other atypical linkag types are not yet fully understood. K11-linkage, for instance, was connected to proteasomal degradation. Linear ubiquitination is catalyzed by a complex called linear ubiquitin chain assembly complex (LUBAC) and associated with NF-κB signaling [Sadowski 2012, Zinngrebe 2014].

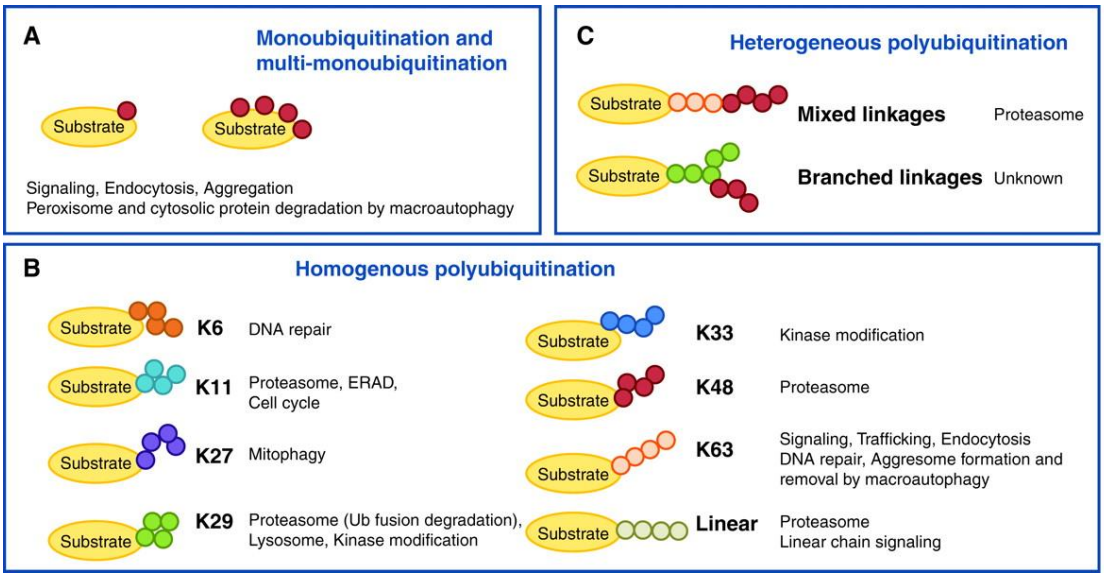


Fig. 11: Different modes of ubiquitination lead to different substrate fates.
[http://cshperspectives.cshlp.org/content/2/12/a006734/F5.expansion.html]

Although Parkin is known to be an E3 ubiquitin ligase, the intriguing question of key substrates is still unanswered. The long list of putative substrates includes α-Synuclein, Pael-R and CDCrel-1, all of which accumulate in patients with heritable Parkinson's Disease. They point to a role for Parkin in the clearance of misfolded or aggregated proteins. But none of them could so far serve as an explanation for the selective death of dopaminergic neurons. Mitofusin-1 and -2, being regulators of mitochondrial dynamics, are two other reported substrates for ubiquitination by Parkin [Wenzel and Klevit 2012].

In 2011, PARIS (Parkin-interacting substrate) was identified as a new Parkin substrate. It was reported that Parkin regulates the expression levels of PARIS through the ubiquitin-proteasome system. PARIS in turn binds to the promoter of the mitochondrial regulator PGC-1α (peroxisome proliferator-activated receptor gamma-coactivator 1-alpha) to inhibit its expression. Consequently, degradation of PARIS by Parkin leads to increased gene expression of PGC-1α and mitochondrial biogenesis, whereas loss of Parkin leads to accumulation of PARIS and thereby repressed PGC-1α expression [Shin 2011, Winklhofer 2014].

f) Parkin as tumor suppressor gene

Studies from PD resesarch demonstrate an antiapoptotic effect of Parkin under stress conditions [Henn 2007, Bouman 2011]. In contrast, cancer studies suggest a role of Parkin as a tumor suppressor gene (TSG) [Cesari 2003]. During the last few years, Parkin has been

linked to cancer, as mutations in this gene were found in several different cancer types. One study underpinning the role of Parkin as a TSG demonstrated that Parkin mutations in cancer cells decrease its E3 ubiquitin ligase activity. The authors argued that the ability of Parkin to ubiquitinate and degrade Cyclin E was compromised in cancer cells leading to mitotic instability of the cells [Veeriah 2010].

A general link between neurodegenerative diseases and cancer was assumed because some of the genes involved in neurodegenerative disorders play a central role in cell cycle control, DNA repair and kinase signaling. Furthermore, genetic studies have shown a reduced incidence of some cancer types, such as lung cancer, but an increased incidence of other cancer types, such as breast and skin cancer, in people suffering from Parkinson's Disease [Plun-Favreau 2010, Olsen 2005]. As NF-κB and the signaling pathways that are involved in its activation are also important for tumor development, the NF-κB network might represent a an additional link between neurodegeneration and cancer [Karin 2002]. Furthermore, the cytokine TNF-α, which can induce activation of the NF-κB pathway, is known to be a multi-functional regulator of diverse pathways, which can induce pro-apoptotic as well as anti-apoptotic cell responses [Park and Bowers 2010].

g) Neuroprotective potential of Parkin

The neuroprotective activity of Parkin has been observed in several cellular and animal models, which proved that Parkin protects from cell death in stress paradigms like mitochondrial, ER (endoplasmatic reticulum) or proteotoxic stress. Parkin is transcriptionally upregulated when cells are exposed to various stress conditions, indicating its neuroprotective function. In addition, Parkin deficiency leads to an increased vulnerability under stress conditions [Henn 2007, Bouman 2010, Müller-Rischart 2013].

In principal, there are at least three hypotheses how Parkin could mediate its neuroprotective activity: I) promoting the removal of damaged mitochondria via mitophagy; II) increasing proteasomal degradation of toxic substrates; and III) modulating nondegradative ubiquitin signaling within cell death or cell viability pathways. These alternatives require either the interaction of Parkin and PINK1 to induce autophagic clearance of depolarized mitochondria, ubiquitination and proteasomal degradation of Parkin substrates or the influence of a general prosurvival pathway like NF-κB and a proapoptotic pathway like caspase signaling [Winklhofer 2014].

1.1.3 Parkin, PINK1 and mitochondria

Mitochondria are extremely dynamic organelles whose morphology is determined by fusion and fission events. Mitochondrial fusion is controled by Mitofusin 1 and 2 (Mfn1 and 2) located in the outer mitochondrial membrane and OPA1 (Optic atrophy 1) located in the inner mitochondrial membrane. OPA1 is regulated by alternative splicing and proteolysis, which produce several long and short OPA1 isoforms. The cytosolic protein Drp1 (Dynamin-related protein 1) and the mitochondrial-anchored protein Fis1 (Mitochondrial fission 1) are GTPases and regulate fission events.

Well-balanced mitochondrial dynamics lead to tubular mitochondrial network, whereas increased fission produces small rod-like or spherical mitochondria and increased fusion generates elongated, strongly interconnected mitochondria. An imbalance of the fusion and fission machinery implicates severe effects on mitochondrial bioenergetics, transport and clearance [Pilsl and Winklhofer 2011].

In the last decade, PD-associated genes have been linked to effects on mitochondrial dynamics. Transient silencing of PINK1 as well as Parkin expression in cell culture models results in increased mitochondrial fragmentation and decreased mitochondrial ATP production. This phenotype can be rescued by overexpression of the fusion proteins Mfn2 or OPA1. Moreover, Parkin and PINK1 protect cells from mitochondrial fragmentation caused by overexpression of the fission factor Drp1 [Lutz 2009]. These findings provide a link of the PD-associated genes Parkin and PINK1 with factors controling mitochondrial fusion and fission events.

Parkin and PINK1 also have a different effect on mitochondria by assisting in their autophagic clearance. Upon treatment of cells with CCCP and disruption of the mitochondrial membrane potential, Parkin and PINK1 collaborate to attract the autophagosomal machinery for clearance of damaged mitochondria (mitophagy). However, the role of mitophagy has to be addressed in future *in vivo* models to prove its relevance for disease progression.

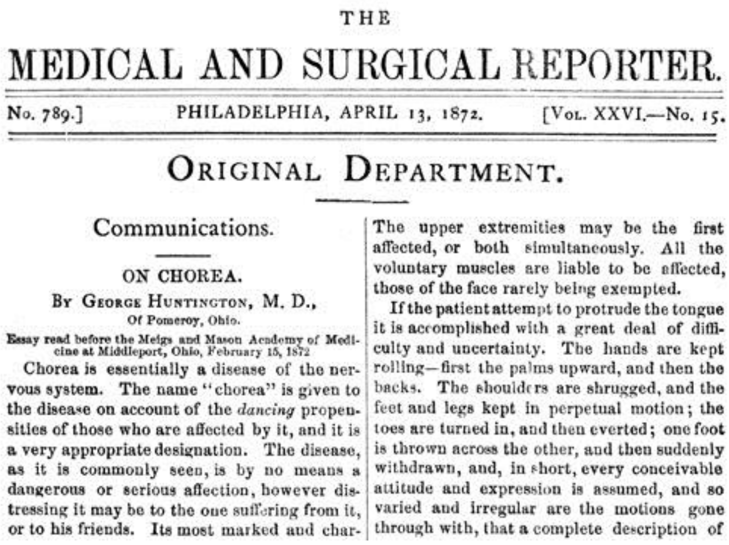
The detailed mechanism how Parkin executes its protective effects on mitochondria is still not fully understood. A convincing concept is the context- and tissue-specificity of Parkin function: Under mild stress conditions, Parkin could prevent mitochondrial fragmentation. When the stress level exceeds a certain limit and severe damage of mitochondria has taken place, Parkin could promote the elimination of these defective organelles via mitophagy [Winklhofer 2014].

1.2 Huntingtin

1.2.1 Huntington's Disease

a) History

Huntington's Disease (HD) is a rare neurodegenerative disease with a prevalence of 5-7 in 100,000 in Europe [Huntington Study Group 2011]. It is the most common genetic cause of abnormal involuntary writhing movements called chorea. The term "chorea" is derived from the Greek word for "dance", as the movements of the feet or hands are comparable to dancing movements. George Huntington was the first one to provide a thorough description of this disease in 1872 (Fig. 12). He examined the combined medical history of several generations of a family exhibiting similar symptoms and realized that their conditions must be linked [Huntington 1872]. In 1993, the disease-associated gene Huntingtin was identified [The Huntington's Disease Collaborative Research Group, 1993].


THE
MEDICAL AND SURGICAL REPORTER.
No. 789.] PHILADELPHIA, APRIL 13, 1872. [Vol. XXVI.—No. 15.

ORIGINAL DEPARTMENT.

Communications.

ON CHOREA.

By George Huntington, M. D.,
Of Pomeroy, Ohio.

Essay read before the Meigs and Mason Academy of Medicine at Middleport, Ohio, February 15, 1872

Chorea is essentially a disease of the nervous system. The name "chorea" is given to the disease on account of the *dancing* propensities of those who are affected by it, and it is a very appropriate designation. The disease, as it is commonly seen, is by no means a dangerous or serious affection, however distressing it may be to the one suffering from it, or to his friends. Its most marked and char-

The upper extremities may be the first affected, or both simultaneously. All the voluntary muscles are liable to be affected, those of the face rarely being exempted.

If the patient attempt to protrude the tongue it is accomplished with a great deal of difficulty and uncertainty. The hands are kept rolling—first the palms upward, and then the backs. The shoulders are shrugged, and the feet and legs kept in perpetual motion; the toes are turned in, and then everted; one foot is thrown across the other, and then suddenly withdrawn, and, in short, every conceivable attitude and expression is assumed, and so varied and irregular are the motions gone through with, that a complete description of

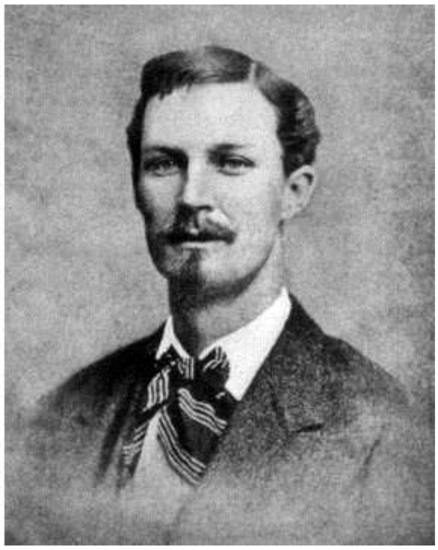


Fig. 12: George Huntington (1850 - 1916) and the first description of Huntington's Disease „On Chorea".
[http://en.wikipedia.org/wiki/File:On_Chorea_with_photo.jpg]

b) Etiology

HD is caused by a single genetic factor: an autosomal dominant mutation in the Huntingtin gene. An abnormal expansion of a CAG triplet repeat (>36) in this gene leads to the formation of a mutant protein with an expanded polyglutamine (polyQ) tract (Fig. 13) [Rubinsztein 1996]. Due to this elongation of the polyQ stretch, the mutant protein is prone for aggregation. Interestingly, there is an inverse correlation between the length of the polyQ tract

and the age of onset for HD symptoms, while the length of the polyQ stretch directly correlates with the severity of the symptoms [Andrew 1993]. In contrast to other neurodegenerative diseases, polyQ diseases are always inherited and do not occur sporadically.

Fig. 13: Comparison of normal and mutant Huntingtin protein. Individuals expressing a normal Huntingtin protein with a repeat length of 10-36 (left) are not affected by Huntington's disease, whereas individuals expressing a mutated form of the protein with repeat lengths larger than 36 (right) are affected. [https://embryology.med.unsw.edu.au/embryology/index.php?title=File:Healthy_Huntingtin_protein_and_Huntingtin_gene_mutated_by_Huntington%27s_Disease.jpg]

c) Symptoms

The first symptoms of HD are uncontrollable movements (choreic movements). When the disorder progresses, more severe physical symptoms like rigidity, writhing motions or abnormal posturing appear. Non-motor symptoms such as cognitive impairment, memory deficits and depression accompany the progression of the disease.

d) Diagnosis

Medical diagnosis of the onset of HD is often made by following the appearance of physical symptoms specific to the disease. Futhermore, genetic testing can also be used to confirm a physical diagnosis if there is no family history of HD. The genetic test for HD consists of a blood test determining the numbers of CAG repeats in each of the Huntingtin alleles.

e) Therapy

Similar to PD, there is no cure for HD, but there are treatments available to reduce the severity of some of its symptoms. In 2008, Tetrabenazine was approved in the US for treatment of chorea in Huntington's disease [Hayden 2009]. Additionally, rigidity can be treated with antiparkinsonian drugs.

f) Neuropathology

In parallel to PD, HD is also characterized by the loss of specific neurons. However, the most affected region in HD is the striatum (with 50-60% loss in the *Nucleus caudatus* and the

Putamen in advanced disease, Fig. 14). Besides, cerebellar atrophy is most frequently reported in cases with a juvenile onset of the disease [Rubinsztein and Carmichael 2003]. The loss of striatal neurons leads to decreased levels of the neurotransmitter GABA (γ-aminobutyric acid), which serves as an inhibitory signal and influences Dopamine levels. A drop in GABA levels causes an imbalance in the regulation of movements.

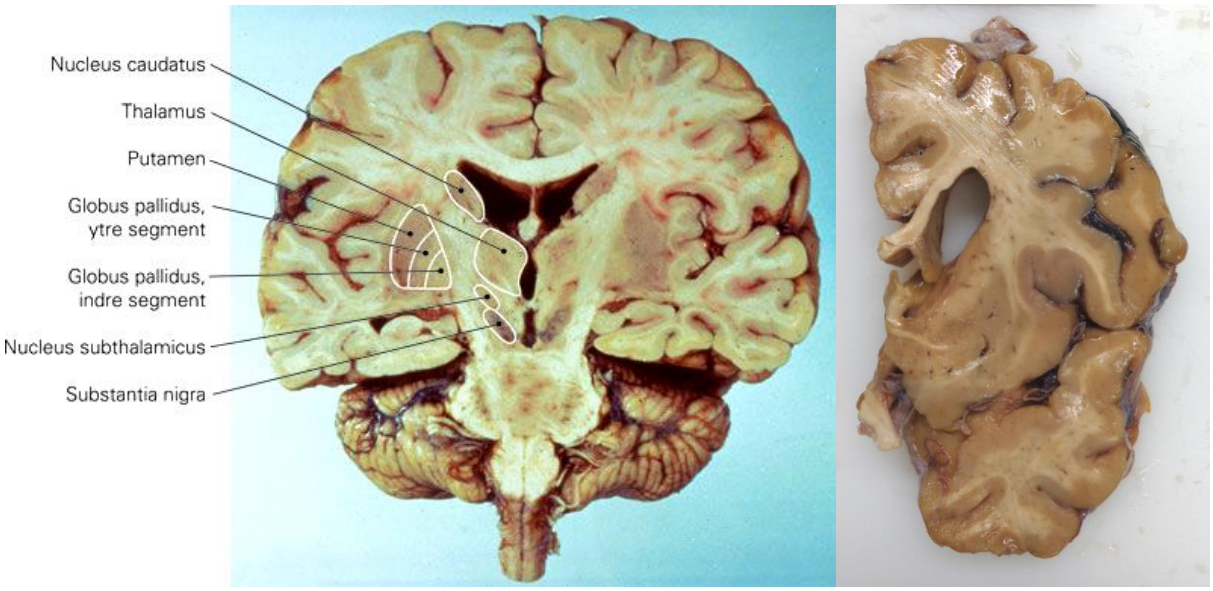


Fig. 14: Huntington's Disease is characterized by a specific loss of neurons in the striatum (*Nucleus caudatus* and *Putamen*). The left panel shows a normal brain with clearly visible *Nucleus caudatus* and *Putamen*, whereas the right panel shows one hemisphere of a HD patient brain with mild atrophy of the *Nucleus caudatus*. [http://tidsskriftet.no/article/1730507; Thomas Arzberger]

An additional pathological feature of HD is the presence of protein aggregates. In *post mortem* brains, neuronal intranuclear inclusions (NIIs) are found, which contain an N-terminal fragment of Huntingtin [DiFiglia 1997]. Besides, extranuclear aggregates like neuropil aggregates and cytoplasmic aggregates were also observed [Gutekunst 1999, Hackam 1999].

1.2.2 Features of Huntingtin

a) Modular structure of Huntingtin

The Huntingtin (Htt) gene contains 67 Exons with a polyQ region located in Exon1 [Ambrose 1994]. In healthy individuals, the polyQ region consists of up to 36 repeats. Additionally, Huntingtin features a high number of HEAT repeats (Huntington, Elongation Factor 3, PR65/A, TOR) and a putative nuclear export signal (NES) near the C-terminus (Fig. 15). The

multiple cleavage sites for caspases and other proteases lead to the formation of several N-terminal fragments [Ross and Tabrizi 2011].

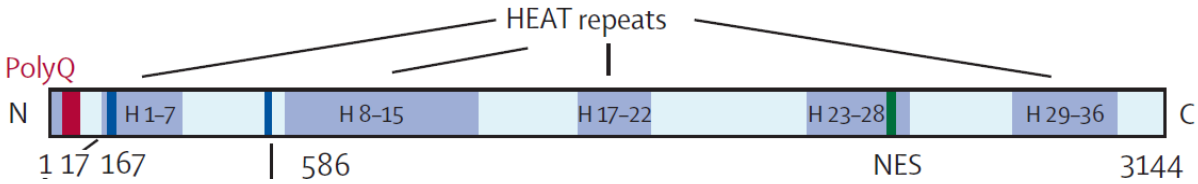


Fig. 15: Domain structure of Huntingtin. Human Huntingtin predominantly consists of HEAT repeats. A polyglutamine stretch (polyQ) is located at the N-terminus and a nuclear export signal (NES) in proximity to the C-terminus. Furthermore, proteolytic cleavage by caspases and other proteases forms N-terminal fragments [Ross and Tabrizi 2011].

b) Huntingtin-deficient and -overexpressing animal models

As Huntingtin KO mice die already at the embryonic stage before day 8.5 [Duyao 1995], this points to a putative role of Htt in embryonic development. Besides that, mice with less than 50% of wild-type (WT) Huntingtin display defects in neurogenesis and formation of the cortex and striatum [White 1997].

The best characterized mouse model for HD is the R6/2 mouse line, which expresses an N-terminal fragment of Huntingtin with an extended polyQ stretch and shows motoric impairment and reduction of brain size and weight after two months. After 12 months, these transgenic mice develop first signs of neurodegeneration [Mangiarini 1996, Turmaine 2000]. Both intranuclear inclusions as well as neuropil aggregates were observed in this mouse line [Davies 1997, Li 1999].

Moreover, HD mouse models expressing the full-length Htt gene carried by either a yeast or a bacterial artificial chromosome (YAC or BAC) exist. However, these full-length transgenic models show relatively normal survival rates and a gradual development of the disease over many months in contrast to N-terminal transgenic models with shortended life spans and early development of the disease.

Another group of HD models consists of knock-in (KI) models, which are generated by homologous recombination using mouse embryonic stem cells. Similar to HD patients, these KI mice are heterozygous for one wild-type Huntingtin allele and one CAG-expanded allele. In summary, the KI models display milder initial behavioral abnormalities than transgenic lines and normal life spans [CHDI Foundation, Jackson Laboratory 2014].

c) Putative function of wild-type and malfunction of mutant Huntingtin

To date, the cellular function of Huntingtin is still unclear. Whether neuronal degeneration in HD is due to the loss of normal Htt function or due to a toxic gain of function, or both, is not fully understood [Roze 2011].

Wild-type Htt is a ubiquitously expressed protein present in most cells of the organism [Sharp 1995]. It is mostly located in the cytoplasm and was reported to shuttle into the nucleus.

Multiple interaction partners like HAP1 (Huntingtin-associated protein 1), HIP-1 (Huntingtin-interacting protein 1) or PGC-1α were described for Huntingtin [Li 1995, Kalchman 1997, Ross and Tabrizi 2011]. Several transcription factors such as CREB (cAMP response element-binding protein) were also reported as binding partners of Htt. The Htt protein is considered to be a scaffold protein, which orchestrates intracellular trafficking, signaling pathways and transcriptional activity being requirements for neuronal homeostasis [Roze 2011].

Due to the extended polyQ stretch, mutant Huntingtin shows increased formation of β-sheets and aggregates. One possibility is that these aggregates sequester either wild-type Htt or interaction partners and thereby influence the fate of neuronal cells. Another important aspect of Huntingtin aggregates is their effect on the ubiquitin-proteasome system (UPS). On the one hand, UPS components were found in polyQ aggregates of HD transgenic mice and HD *post mortem* brains suggesting a sequestration of these components. On the other hand, polyQ containing aggregates cannot be cleared by the UPS machinery and may block the proteasome resulting in insufficient degradation of other proteins [Davies 2007, Roze 2011].

d) Neuroprotective potential of Huntingtin

Interestingly, Huntingtin exhibits a neuroprotective potential like Parkin. Rigamonti and co-workers showed that WT Htt protects CNS cells from a variety of apoptotic stimuli [Rigamonti 2000]. *In vivo* experiments with overexpression of WT Htt confirmed a significant protection against NMDAR (N-methyl-D-aspartate receptor)-mediated apoptotic neurodegeneration. Therefore, Huntingtin may regulate the balance between neuronal survival and death following acute stress conditions and the levels of Huntingtin may modulate neuronal sensitivity to excitotoxic neurodegeneration [Leavitt 2006].

A couple of mechanisms were proposed how this neuroprotective potential might be carried out. First, WT Htt was reported to interfere with the activity of the apoptosome and thereby inhibiting neuronal cell death. Cells overexpressing WT Htt showed continuous cytochrome c

release, but no processing of Procaspase-9 upon exposure to apoptotic stimuli [Rigamonti 2001].

Second, the production of the neurotrophic factor BDNF (brain derived neurotrophic factor) was found to be regulated by WT, but not mutant Huntingtin. BDNF is involved in synaptic glutamate release and excitotoxicity [Zuccato 2001, Zuccato 2003]. Moreover, WT Htt promotes the axonal transport of BDNF from cortical neurons and the release of BDNF into the striatum [Gauthier 2004].

Third, data focusing on the proapoptotic molecule HIP-1 suggest that sequestration of HIP-1 underlies the antiapoptotic effect of Htt. Normally, HIP-1 is associated with Huntingtin, whereas an expansion of the polyglutamine stretch in Huntingtin leads to reduced binding of HIP-1. Free HIP-1 can then bind to the protein HIPPI (Hip-1 protein interactor) and together they form a heterodimer. In the following step, this heterodimer is thought to recruit Procaspase-8 and thereby induce apoptosis, which might explain the death of neuronal cells in HD [Gervais 2002].

1.2.3 Huntingtin and mitochondria

Mitochondrial dysfunction and energy deficits have been found in multiple analyses of *post mortem* HD patient brains as well as mouse and cell culture models. These analyses comprise altered mitochondrial morphology (Fig. 16), Ca^{2+} buffering capacity, enzymatic activity (complex II/III/IV), membrane potential, ATP levels and transport processes [Damiano 2010, Reddy and Shirendeb U.P. 2012, Costa and Scorrano 2012].

Mitochondria play an essential role during apoptosis, as these organelles sense cell damage and release cytochrome c accompanied by mitochondrial fragmentation and christae remodeling [Danial and Korsmeyer 2004, Frank 2001]. Furthermore, neurons rely mainly on ATP production of mitochondria, as they cannot switch to glycolysis, which makes them extremely susceptible to mitochondrial dysfunction. Due to their extensive physiological functions including a fluctuating plasma membrane potential, release and uptake of neurotransmitters as well as trafficking of organelles, neurons also exhibit especially high-energy demands [Mitchell 1999, Pickrell 2011]. A large number of neurodegenerative disorders share similar mitochondrial alterations [Bossy-Wetzel 2003], implying the importance of mitochondrial integrity to the development of HD.

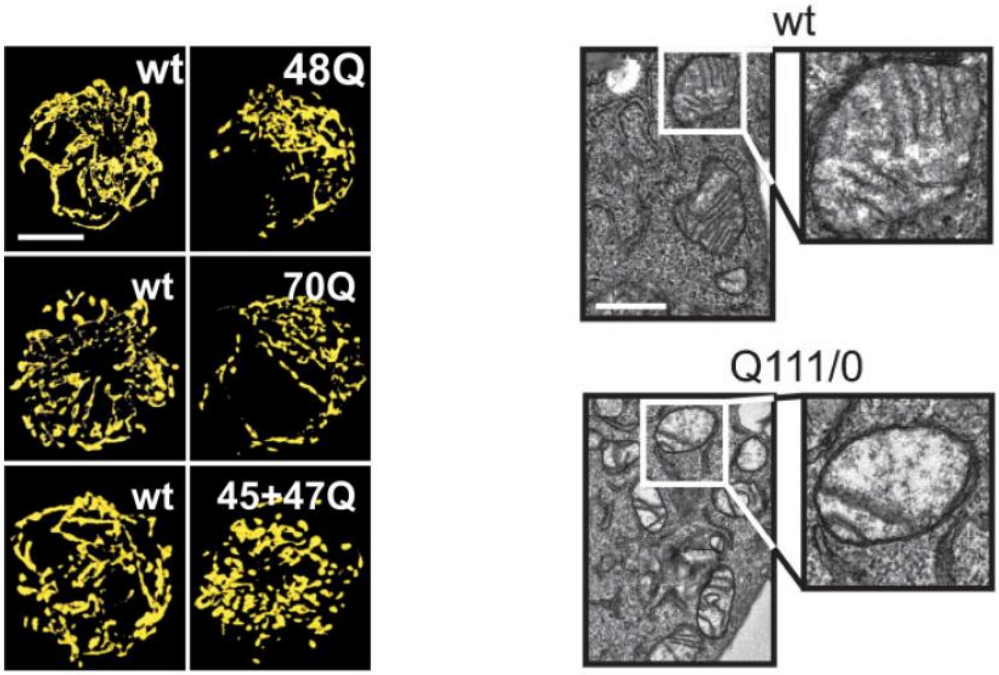


Fig. 16: HD lymphoblasts and striatal precursors show mitochondrial fragmentation (left) and cristae derangement (right). Left: Lymphoblasts from HD patients carrying extendended polyQ tracts of 48, 70 or 45+47 reveal fragmentation and clustering of mitochondria compared to wt controls. Right: Electron micrographs of Q111 striatal neurons from Htt knockin mice show altered mitochondrial cristae structure compared to wt neurons. [Costa 2010]

Specific interactions of mitochondrial proteins or regulators with Htt have been described in the last decade (Fig. 17). Several investigations imply a link between transcriptional dysregulation and defects in energy metabolism demonstrated in patient's brain tissue and HD transgenic mice. PGC-1α is a master regulator of mitochondrial biogenesis and oxidative phosphorylation. First evidence for a role of PGC-1α in the pathogenesis of HD came from observations of PGC-1α null mice revealing selective striatal lesions. Moreover, PGC-1α levels and activity are reduced in different models of HD. In neuronal cells, a direct link between CREB phosphorylation and transcriptional regulation of the PGC-1α promoter was established. For instance, stimulation of extracellular NMDA receptors in HD neuronal cells with impaired CREB/ PGC-1α signaling increases cell vulnerability [Lin 2004, Cui 2006, Okamoto 2009]. The transcriptional coactivator PGC-1α displays an interesting overlap between HD and PD, as both Htt as well as Parkin have been connected to this mitochondrial regulator in the past years.

In addition to PGC-1α, Drp1 was identified as an interactor of mutant Htt. This interaction between the fission-promoting factor and the polyQ protein stimulates the GTPase activity of Drp1. A dominant-negative Drp1 mutant with reduced GTPase activity rescues mitochondrial

fragmentation, transport defects and neuronal cell death caused by mutant Htt [Costa 2010, Song 2011].

Recently, the group around Robert Friedlander discovered an interaction between mutant Htt and TIM23 (translocase of the inner membrane, subunit 23). Mitochondrial protein import was impaired by mutant Htt in a presymptomatic HD stage and the import defect and neuronal cell death could be rescued by overexpression of TIM23 complex subunits [Yano 2014].

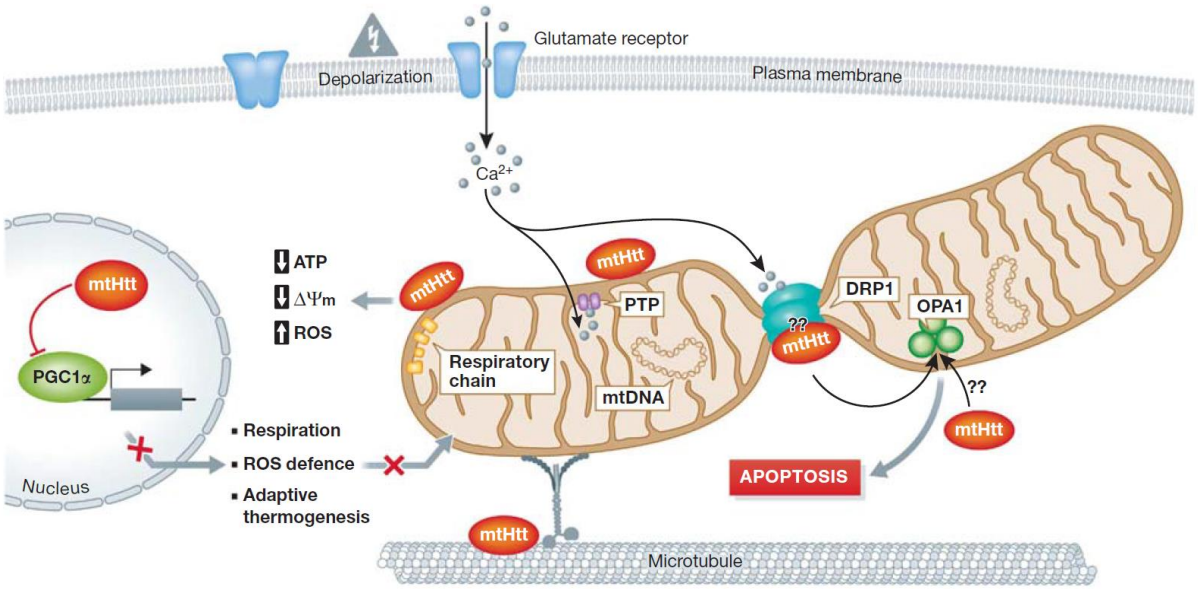


Fig. 17: Functional alterations in HD mitochondria. The cartoon depicts the functional alterations occurring at the level of mitochondrial function, opening of the permeability transition pore (PTP) and morphology in the context of HD. Mutated Huntingtin can act at the transcriptional level through inhibiton of PGC-1α, directly at the mitochondrial level, by increasing the opening probability of the PTP and affecting mitochondrial respiratory chain. Moreover, mutant Htt can also influence DRP1 causing fragmentation and remodeling of the mitochondrial cristae. [Costa and Scorrano 2012]

1.2.4 Huntingtin and Parkin

With Parkin being involved in ubiquitination and degradation of substrate proteins and Htt showing high resistance to degradation processes, the question arised, if Parkin deficiency would deteriorate the HD phenotype in mouse models. To address this question in a first attempt, Parkin null mice were crossed with an HD mouse model (R6/1) and the results indicated a slightly more severe HD phenotype, when Parkin expression was partially suppressed [Rubio 2009]. It had already been observed before that Parkin is able to facilitate the elimination of expanded polyglutamine proteins. Experiments with a polyQ Ataxin-3 fragment suggested a role for Parkin in the preservation of proteasomal function [Tsai 2003]. These findings hint at a possible protective role of Parkin in HD.

1.3 TNF-α stimulated pathways

In 1984, Aggarwal and coworkers isolated and identified the two cytotoxic factors TNF-α and β (TNF: tumor necrosis factor) in macrophages and lymphocytes. Until now, 19 members of the TNF superfamily and 29 TNF receptors (TNF-R) have been identified. The TNF superfamily members participate in inflammation, apoptosis, proliferation, invasion, angiogenesis, metastasis and morphogenesis with apparent roles in different diseases. Furthermore, these molecules represent attractive drug targets and TNF blockers are used for treatment of, amongst others, rheumatoid arthritis and osteoporosis.

The disproportionate number of TNF ligands and receptors implies that some of the ligands interact with more than one receptor. For instance, TNF-α was reported to bind to two different receptors: TNF-R1 and TNF-R2. While TNF-R1 containing an intracellular death domain (DD) is expressed universally, the expression of TNF-R2 is only found in cells of the immune system, endothelial cells and nerve cells [Aggarwal 2003, Aggarwal2012].

Being a type-II transmembrane protein, TNF-α is processed by proteolytic cleavage through TACE (TNF-α cleaving enzyme) resulting in a soluble form of TNF-α. Both exogenous signals including exposure to bacterial or viral proteins like LPS (lipopolysaccharide) as well as cell-intrinsic stimuli lead to increased TNF-α expression. The cytokine TNF-α is a multi-functional regulator of diverse pathways, which can induce pro-apoptotic as well as anti-apoptotic cell responses [Park and Bowers 2010]. In the following chapters, the three major underlying pathways of these opposing activites will be illuminated in more detail: NF-κB, JNK (c-Jun N-terminal kinase) and caspase signaling.

1.3.1 NF-κB pathways

Since the identification of the transcription factor NF-κB (nuclear factor κB) in 1986, intensive research revealed NF-κB as the central orchestrator of inflammation and immune responses by maintenance of prosurvival gene expression [Sen and Baltimore 1986]. To celebrate the tremendous progress made in 25 years for understanding the role and regulation of NF-κB, Nature Immunology published a special edition with focus on NF-κB in 2011 (Fig. 18).

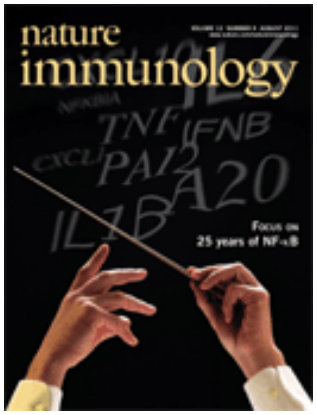


Fig. 18: Special edition of Nature Immunology featuring a series of specially commissioned review articles to mark the 25th anniversary of the discovery of NF-κB. [http://www.nature.com/ni/journal/v12/n8/covers/index.html]

The NF-κB family consists of five members: RelA (p65), RelB and c-Rel as well as the precursor proteins NF-κB1 (p105) and NF-κB2 (p100) being processed into p50 and p52, respectively. The different NF-κB transcription factors form homo- or heterodimers, which bind to NF-κB binding sites in gene promoters and enhancers to either induce or suppress transcription. A common feature of all NF-κB proteins is their Rel homology domain (RHD) located at the N-terminus for DNA binding, interaction with inhibitory proteins and dimerization [Oeckinghaus 2011, Hayden and Ghosh 2004].

As a wide range of stimuli mediated by receptors like TNF-R, TLR(Toll-like receptor) or IL-1R (interleukin 1 receptor) can induce NF-κB activation and dysregulation of this transcription factor is not only linked to inflammatory, autoimmune and metabolic diseases, but also cancer, NF-κB activity has to be tightly regulated by multiple elements. Therefore, one regulatory mechanism includes that NF-κB dimers are present in an inactive state in the cytoplasm by binding to IκB (inhibitor of κB) proteins [Hayden and Ghosh, 2012].

a) Canonical and noncanonical NF-κB signaling

In general, there exist two main NF-κB-activating pathways in cells (Fig. 19). First, the more prominent canonical or classical pathway, which is activated via cytokine receptors like TNF-R or IL-1R and pattern-recognition receptors like TLR, and dependent on IKKβ (IκB kinase) and the regulatory subunit NEMO (NF-κB essential modifier/IKKγ). This pathway mainly results in phosphorylation and degradation of IκB-α followed by nuclear translocation of p65-containing dimers and activation of target gene transcription. Second, the noncanonical or alternative pathway, which is induced by specific TNF family members like CD40R (CD40 receptor) or LTβR (lymphotoxin-β receptor). The noncanonical pathway depends on activation of IKKα and phosphorylation of p100 being associated with RelB. Processing of p100 by the proteasome generates a heterodimeric complex of p52 and RelB translocating to the nucleus. Next, down-regulation of NF-κB activation is achieved by a well-characterized feedback loop: newly synthesized IκB-α protein binds to nuclear NF-κB, which is exported to the cytosol. In both pathways, the proteasome plays an essential role. However, within the classical pathway it is responsible for degradation of IκB-α and in the alternative pathway for constitutive processing of p100 [Oeckinghaus 2011, Hayden and Ghosh 2012].

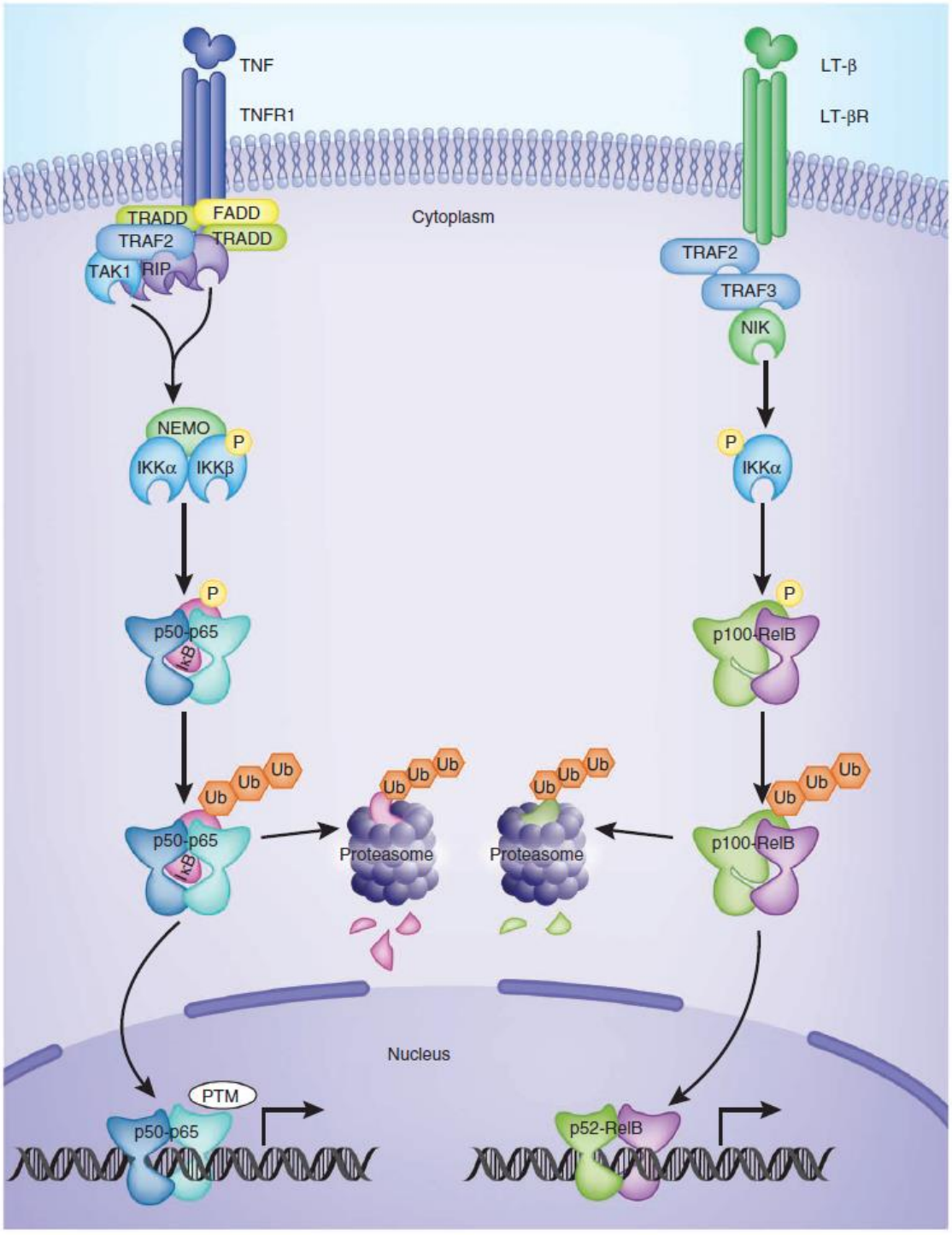


Fig. 19: The canonical and noncanonical pathway for activation of NF-κB.
The canonical pathway (left) is induced by most physiological NF-κB stimuli such as TNF-R1 signaling. Stimulation of TNF-R1 leads to binding of TRADD (Tumor necrosis factor receptor type 1-associated death domain), FADD (FAS-associated death domain) and TRAF2 (TNF receptor-associated factor 2). TRAF2 associates with RIP1 for IKK activation. In the following, IκBα is phosphorylated in an IKKβ- and NEMO-dependent manner, which results in nuclear translocation of mostly p65-containing heterodimers and initiation of gene transcription. In contrast, the noncanonical pathway (right), induced by certain TNF family cytokines, such as CD40L and lymphotoxin-β (LT-β), involves IKKα-mediated phosphorylation of p100 associated with RelB, which leads to partial processing of p100 and the generation of transcriptionally active p52-RelB complexes. IKKα activation and phosphorylation of p100 depend on NIK, which is regulated by TRAF3, TRAF2 and additional ubiquitin ligases. [Oeckinghaus 2011]

Besides targeting of proteins for proteasomal degradation, ubiquitination also fulfills several tasks within the NF-κB signaling pathways. On the one hand, phosphorylation of IkB-α by the IKK complex is followed by rapid K48-linked ubiquitination and targeting for 26S proteasome mediated degradation. On the other hand, a more untypical mode of ubiquitiation - linear ubiquitination - was shown to be involved in activation of NEMO and the IKK complex. Additionally, ubiquitination can be reversed by a large family of proteins termed deubiquitination enzymes (DUBs). These DUBs represent an emerging focus in current research [Liu and Chen 2011, Harhaj and Dixit 2012]. Some other still remaining questions concerning the variety of NF-κB family members include what the contribution of specific NF-κB dimers to the physiological outcome is or which genes are regulated by which dimers.

b) Parkin and NF-κB signaling

In 2007, our research group linked the E3 ubiquitin ligase Parkin for the first time to increased NF-κB signaling. Blocking NF-κB activation by an IκB-α repressor or inactivation of IKKβ was shown to impair the neuroprotective activity of Parkin. Moreover, analysis of Parkin mutants revealed decreased stimulation of NF-κB-dependent transcription. Thus, this work connected the PD-associated protein Parkin with the NF-κB pathway playing a key role in inflammation and immune responses [Henn 2007].

A few years later, we were able to deliver a more detailed insight into the role of Parkin in NF-κB activation. We demonstrated that Parkin is recruited to the LUBAC under stress conditions, which leads to enhanced linear ubiquitination of NEMO and increased canonical NF-κB signaling (Fig. 20). Furthermore, the regulator of mitochondrial dynamics OPA1 could be identified as a new target gene, whose transcription is activated by the transcription factor NF-κB. These findings were confirmed in Parkin-deficient models to verify the physiological relevance of Parkin's antiapoptotic and neuroprotective function [Müller-Rischart 2013].

Before knowing the molecular mechanism how Parkin influences NF-κB signaling, this pathway was already discussed as a therapeutic strategy for PD, which is characterized by chronic inflammation. To test the relevance of drugs targeting the NF-κB pathway, specific inhibitors for IKKβ or IKKγ were used, which showed that degeneration of dopamine producing neurons was reduced by these inhibitors in several models of PD. In short, targeting the NF-κB pathway might serve as a useful approach for future PD treatment [Flood 2011]. However, it has to be considered that this fundamental transcription factor targets a

large number of genes and physiological responses, which might lead to unwanted side effects.

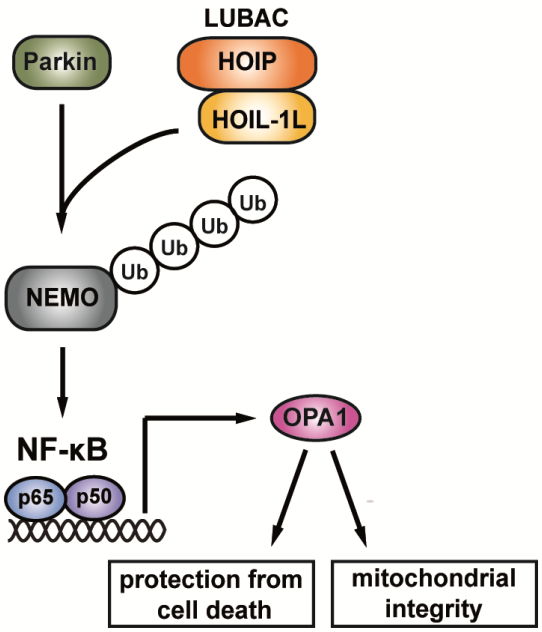


Fig. 20: Parkin confers stress protection via NEMO, NF-κB and OPA1. Under cellular stress conditions, Parkin can increase the activity of LUBAC to mediate linear ubiquitination of NEMO, which in turn activates the IkB kinase complex. This results in activation of NF-κB, which regulates transcription of NF-κB -responsive genes. OPA1 as an NF-kB target links Parkin, linear ubiquitination, and NF-κB signaling to mitochondrial integrity. [Müller-Rischart 2013]

c) Huntingtin and NF-κB signaling

In 2010, two publications reported different effects of mutant Htt on the transcription factor NF-κB. In the first paper, the Finnish researchers around Korhonen found that extended polyQ repeats induced ER stress, which triggers the unfolded protein response (UPR) via the inositol-requiring enzyme-1 (IRE1). Furthermore, decreased p65 levels together with increased JNK signaling, cell death and calpain activation were observed in cells expressing mutant Huntingtin. Calpain was suggested to promote p65 degradation [Reijonen 2010]. In summary, the findings demonstrated a link between mutant Huntingtin and decreased NF-κB signaling, although the underlying mechanism and the contribution of calpain were not illustrated in a satisfying profoundness.

The second publication focused on the function of wild-type Huntingtin in neuronal cells. Beside its neuroprotective role, the function of wild-type Htt is still poorly understood, as already mentioned before. Using biochemical and imaging approaches, wild-type Htt was found to foster the transport of activated NF-κB from dendritic spines into the nucleus, where the transcription factor activates gene transcription. Next, the group of Kennedy analyzed if

the transport of NF-κB was impaired by polyQ expansions, which was the case. To recapitulate the results, an inhibitory effect of mutant Huntingtin on NF-κB signaling was demonstrated [Marcora and Kennedy 2010].

Recently, a contradicting publication argued that expression of mutant Htt influences the NF-κB pathway by an interaction with IKKγ. This interaction led to enhanced degradation of IκB and enhanced nuclear translocation of p65, which was supported by transcriptional upregulation of intracellular immune factors. Application of glucan-encapsulated small interfering RNA particles to lower endogenous Huntingtin levels in human HD macrophages decreased cytokine production and induced transcriptional alterations [Träger 2014]. Similar findings were reported on analyses of HD cell culture models and R6/2 HD mice: expression of mutant Htt exon1 resulted in activation of the NF-κB pathway via direct interaction with IKKγ [Khoshnan 2004, Hsiao 2013].

These publications on Htt and NF-κB provided first evidence suggesting a connection of HD and this major transcription factor of the immune response, although they do not agree in the manner how mutant Htt is affecting the NF-κB pathway (increase or decrease). The activation status of NF-κB might be one possible explanation why neurons are especially vulnerable to the presence of mutant Htt in comparison to non-neuronal cells: NF-κB is mostly kept in an inactive state in non-neuronal cells, whereas it is constitutively activated in neuronal cells by basal glutamergic transmission [Meffert 2003].

d) Other neurodegenerative diseases and NF-κB signaling

In addition to HD and PD, also other neurodegenerative diseases were connected to NF-κB dysfunction. Researchers concentrating on amyotrophic lateral sclerosis (ALS), which is characterized by TDP-43 (TAR DNA-binding protein 43) inclusions, identified TDP-43 as a co-activator of the NF-κB subunit p65. Using an inhibitor of NF-κB activity, ALS disease symptoms could be deminished in TDP-43 transgenic mice. Moreover, enhanced NF-κB activation in familial and sporadic ALS patients was confirmed [Swarup 2011]. A subsequent work discovered that only NF-κB inhibition in microglia, but not in astrocytes was able to rescue motor neurons from cell death and extend survival of ALS mice [Frakes 2014].

Moreover, Progranulin (PGRN) being involved in frontotemporal lobar degeneration (FTLD) was found to attenuate NF-κB signaling and thereby execute its protective role [Hwang 2013, Zhao 2013].

Very recently, a mechanistic explanation was published deciphering the possible role of NF-κB in Alzheimer's Disease (AD). Besides the identification of the complement protein C3 as a target of the transcription factor NF-κB, exposure to Aβ (Amyloid beta) was shown to activate NF-κB and increase the astroglial release of C3. As brain tissues from AD patients and APP (Amyloid precursor protein) transgenic mice also exhibit high C3 levels, the application of C3 receptor agonists might be therapeutically beneficial [Lian 2015].
The growing number of neurodegenerative diseases linked to NF-κB signaling implies that this pathway could display a unifying feature of the different facets of neurodegeneration.

1.3.2 JNK pathway

JNK signaling also termed SAPK (stress-activated protein kinase) pathway is another pathway, which is stimulated by cytokines like TNF-α. JNKs are master protein kinases that regulate many physiological processes including inflammatory responses, morphogenesis, cell proliferation, differentiation, survival and death (Fig. 21) [Bubici and Papa 2014]. These various cellular responses can even be antagonistic demonstrated by data showing that JNK activation can on the one hand lead to apoptosis and on the other hand promote proliferation. Important factors to determine the direction of the response comprise cell type, context of other regulatory factors as well as intensity and duration of the signal. For instance, transient JNK activation was supposed to promote cell survival, while prolonged JNK activation is thought to mediate apoptosis [Leppä and Bohmann 1999, Wicovsky 2007, Davies and Tournier 2012].
So far, three different JNKs have been identified: JNK1, 2 and 3. JNK1 and 2 are ubiquitously expressed, whereas JNK3 was mainly found in brain, testis and heart tissue. JNK belongs to the MAPK (mitogen-activated protein kinase) family with the two other members ERK (extracellular-signal-regulated kinases) and p38. The activation of JNK is achieved by dual phosphorylation executed by MKKs (MAPK kinase/MAPKK), which in turn are also activated by phosphorylation through MAPKKKs. MKK4 and MKK7 were discovered as activators of JNK, which were suggested to work synergistically to phosphorylate JNK [Davies and Tournier 2012].

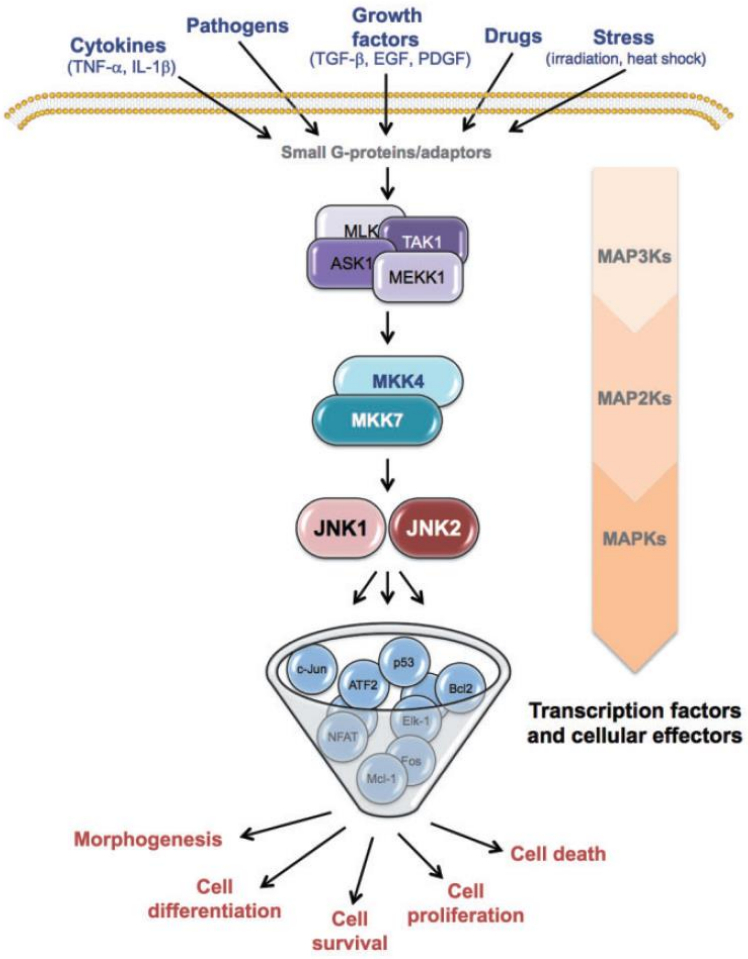


Fig. 21: Schematic representation of JNK signaling. The JNK signaling cascade consists of a three-component module: upstream, MAP3Ks couple signals from the cell surface to intracellular protein effectors. MAP3Ks phosphorylate and activate components of the MAP2K module, such as MKK4 and MKK7. These two kinases in turn phosphorylate and stimulate the activity of different JNK isoforms belonging to the MAPK module.Upon its activation, each JNK protein itself can phosphorylate specific substrates to target different cellular responses. [Bubici 2014]

A major target of the JNK signaling pathway is the activation of the transcription factor AP-1 (Activator protein-1). The dimeric complex AP-1 consists of subunits from the Jun, Fos or ATF families and its activation is achieved by phosphorylation. JNK obtained its name due to its ability of phosphorylating c-Jun at two sites within its N-terminal transactivation domain [Liu and Lin 2005]. C-Jun can form homo- or heterodimers with Fos or ATF proteins [Vogt 2001]. Upon activation by phosphorylation, JNK translocates from the cytosol to the nucleus in order to phosphorylate and activate the nuclear protein c-Jun (Fig. 22). Phosphorylation enables c-Jun to bind to DNA and initiate target gene transcription [Nadruz 2004].

In addition to c-Jun, JNK can phosphorylate transcription factors like c-Fos or ATF. All of these transcription factors regulate the expression of several stress-responsive genes. Furthermore, JNK is not only able to mediate its effects by influencing gene transcription, but

also by direct phosphorylation of pro- and anti-apoptotic proteins like Bim or Bcl-2 [Weston and Davis 2002].

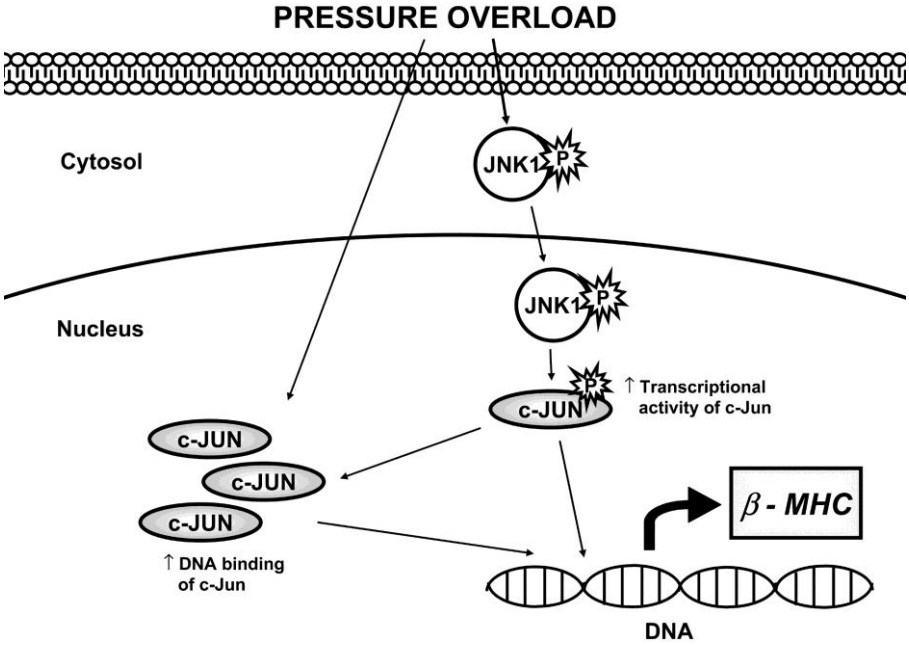


Fig. 22: Schematic model of acute JNK and c-Jun activation induced by various stimuli including TNF-α or pressure overload in the heart. Upon activation of JNK, phosphorylated JNK translocates to the nucleus and activates c-Jun by phosphorylation. Activated c-Jun contributes to the dimeric transcription factor AP-1 and results in gene transcription. [Nadruz 2004]

Increased levels of JNK activation in brain homogenates of patients with different tauopathies provide evidence that the JNK pathway plays a critical role in the pathogenesis of various neurological disorders [Cui 2007].

It becomes also more and more apparent that persistent activation of JNKs is involved in cancer development and progression. Therefore, JNKs represent attractive targets for therapeutic intervention. However, a deeper understanding of the molecular mechanisms determining a pro- or anti-apoptotic outcome and the crosstalk with other pathways has to be gained, before new drugs can be developed into successful therapies [Bubici and Papa 2014, Davies and Tournier 2012].

1.3.3 Caspase pathways

a) Apoptosis and caspases

The TNF receptor belongs to the family of death receptors (DR) and can also initiate an apoptotic pathway besides NF-κB and JNK signaling. Apoptosis or programmed cell death (PCD) consists of a well-regulated process including activation of an enzyme cascade

resulting in cell death. PCD can occur as apoptosis (type I), autophagy (type II) or necrosis (type III) [Ouyang 2012].

Apoptosis includes two major pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. These two pathways, mediated by different death stimuli, were found to influence each other and to converge on the same execution reaction [Elmore 2007]. The central components of the apoptotic response are conserved enzymes termed caspases (cysteine proteases cleaving after an aspartate residue), which irreversibly commit a cell to die. Two categories of caspases are involved in the apoptotic process: initiator caspases including Caspase-2, -8, -9 and -10 versus effector caspases including Caspases-3, -6 and -7. To protect the cell from constant apoptotic signaling, caspases are present as catalytically inactive zymogens and have to undergo proteolytic activation when apoptosis is initiated [Riedl and Shi 2004].

b) Extrinsic and intrinsic apoptotic signaling

The intrinsic apoptotic pathway is triggered by stress-inducing stimuli like DNA damage or oncogene activation. Stress-induced apoptosis results in formation of pores in the outer mitochondrial membrane, which is followed by the release of proteins such as cytochrome c, SMAC (second mitochondria-derived activator of caspase)/ DIABLO (direct inhibitor of apoptosis-binding protein with low pI) from the intermembrane space of mitochondria into the cytoplasm (Fig. 23). Mitochondrial cytochrome c release is regulated by anti-apoptotic (Bcl-2, Bcl-xL) and pro-apoptotic (Bax, Bak, tBid) Bcl-2 family members and activates Apaf1 (apoptotic protease-activating factor 1) in the cytoplasm allowing formation of the apoptosome, which mediates activation of the initiator Caspase-9. Finally, activation of Caspase-9 triggers a caspase cascade.

Within the extrinsic pathway, death receptors like TNF-R or Fas containing an extracellular region and a cytoplasmic death domain (DD) bind to homotrimeric ligands like TNF-α or FasL. This leads to binding of FADD and Procaspase-8 forming the death-inducing signaling complex (DISC). This complex drives the auto-catalytic activation of Procaspase-8. Upon activation of Caspase-8, the execution phase of apoptosis is triggered [Riedl and Shi 2004].

The caspase cascade or execution phase, which unifies both the intrinsic and extrinsic pathway, consists of the activation of effector caspases (mainly Caspase-3 and -7), which cleave important cellular substrates like PARP (Poly ADP-ribose polymerase) resulting in biochemical and morphological changes characteristic for the apoptotic phenotype.

The protein Bid (BH3 interacting-domain death agonist), which is converted by Caspase-8 to tBid (truncated Bid), additionally connects the extrinsic to the intrinsic pathway. Interaction of tBid with Bax, a Bcl-2 family protein, results in cytochrome c release [Elmore 2007, MacFarlane and Williams 2004].

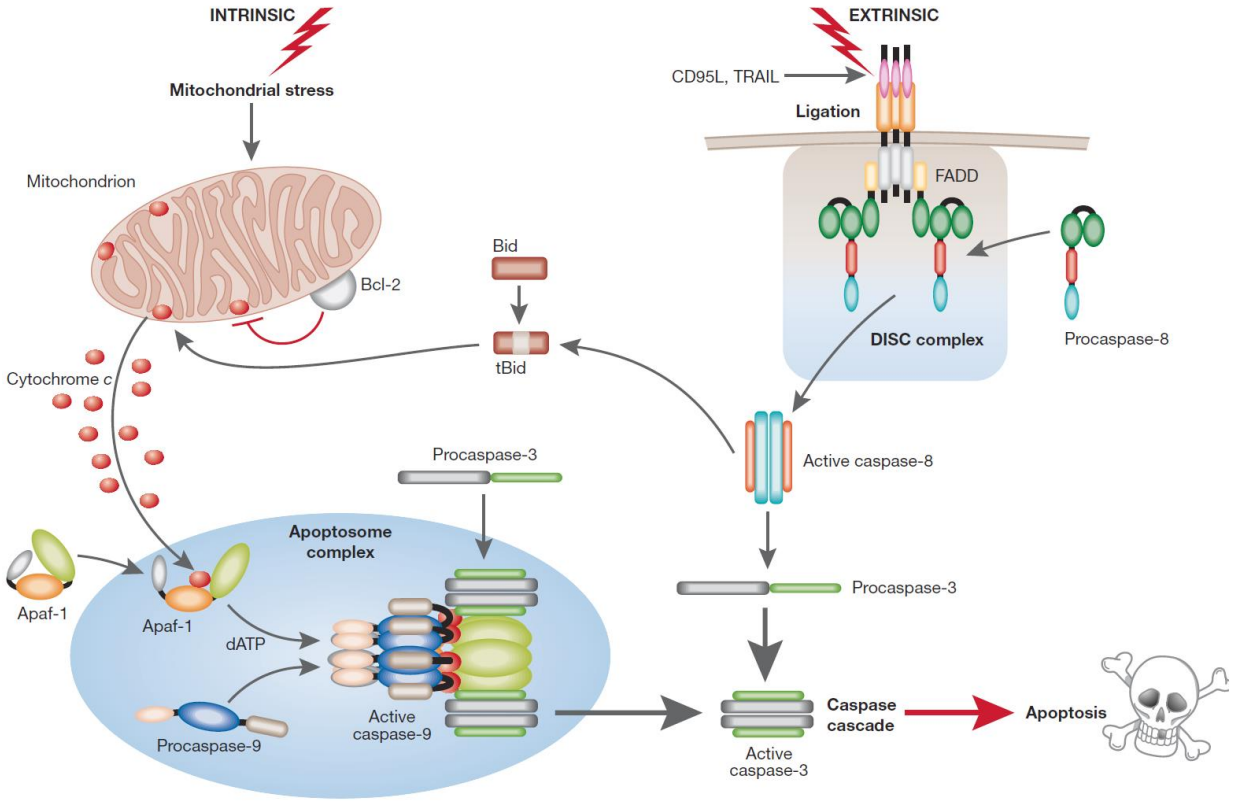


Fig. 23: The intrinsic and extrinsic pathway. The intrinsic pathway (left) is activated by stress-inducing stimuli resulting in mitochondrial release of proteins, such as cytochrome c. Released cytochrome c binds to apoptotic protease-activating factor 1 (Apaf1), which promotes formation of the apoptosome complex and activation of Caspase-9. The extrinsic pathway (right) is triggered by activation of death receptors such as the TNF receptor and results in rapid activation of Caspase-8. Both the intrinsic and extrinsic pathway share the last part, where the activated initiator Caspases-8 or -9 activate the effector Caspases-3, -6 and -7. These effector caspases are responsible for apoptosis of the cell. [MacFarlane and Williams 2004]

One important regulatory mechanism of programmed cell death is the presence of IAPs (inhibitors of apoptosis proteins). XIAP, c-IAP, Livin and Survivin are some members, which belong to this protein family. So far, the best characterized one is XIAP (X-chromosome-linked inhibitor of apoptosis), which binds Caspase-3 and -7, thereby inhibiting their activation and preventing apoptosis [Scott 2005]. For instance, binding of DIABLO, which is released from mitochondria upon induction of apoptosis, blocks XIAP activity. How the different IAPs inhibit apoptosis mechanistically at the molecular level is to date not completely understood.

1.3.4 Crosstalk between NF-κB, JNK and caspase pathways

For an organism, the balancing act between cell survival and cell death is a key function. The elimination of dysfunctional cells like cancer cells is crucial for surviving, but too excessive cell death results in diseases, such as neurodegenerative diseases [Papa 2004]. One mechanism to regulate this decision is the crosstalk between the NF-κB, the JNK and the caspase signaling pathways.

An example to illustrate the crosstalk between prosurvival NF-κB and proapoptotic caspase signaling can be given by RIP1 (receptor-interacting protein 1). RIPs are kinases important for sensing cellular stress and controlling cell death. RIP1 features a death domain, which mediates binding to other DD-containing receptors like the TNF receptor. It was observed that NF-κB activation by RIP1 can on the one hand lead to induction of antiapoptotic genes, whereas RIP1 overexpression can on the other hand lead to apoptosis. These seemingly opposing effects can be elegantly explained by formation of two distinct TNF-induced signaling complexes (Fig. 24): Complex I, including TRADD, RIP1 and TRAF2 promotes rapid NF-κB activation and gene expression of antiapoptotic factors, whereas complex II, composed of TRADD, RIP1, TRAF2 and additionally FADD as well as caspase-8 and -10, has a proapoptotic effect. In contrast to the membrane-bound complex I, complex II is located in the cytosol. Upon TNF-α stimulation, complex I is formed at the TNF receptor and triggers NF-κB activation. Within one hour, the components of complex I (TRADD, RIP1, TRAF2) have already been released from the receptor and bind to FADD and caspases in the cytosol for activation of apoptosis. This highlights the transformation of complex I to complex II as a central point in the decision of whether to live or to die. It remains unclear, how this dissociation of the complex I factors is regulated. Complex II migth only be able to trigger apoptosis when NF-κB is defective or inhibited [Oeckinghaus 2011].

Within the last decade, the Yuan lab discovered a novel regulated necrotic cell death pathway termed necroptosis, which also requires RIP1 and was added to the already known pathways via complex I and II [Degterev 2008, Degterev 2005]. Hence, complex II can exist in two forms: complex IIa (former complex II) and complex IIb (the newly discovered necroptotic complex). Complex IIb is characterized by the two additional members RIP3 and MLKL (mixed lineage kinase domain-like). Furthermore, necroptosis is suppressed by Caspase-8/FADD-mediated apoptosis [Zhou and Yuan 2014].

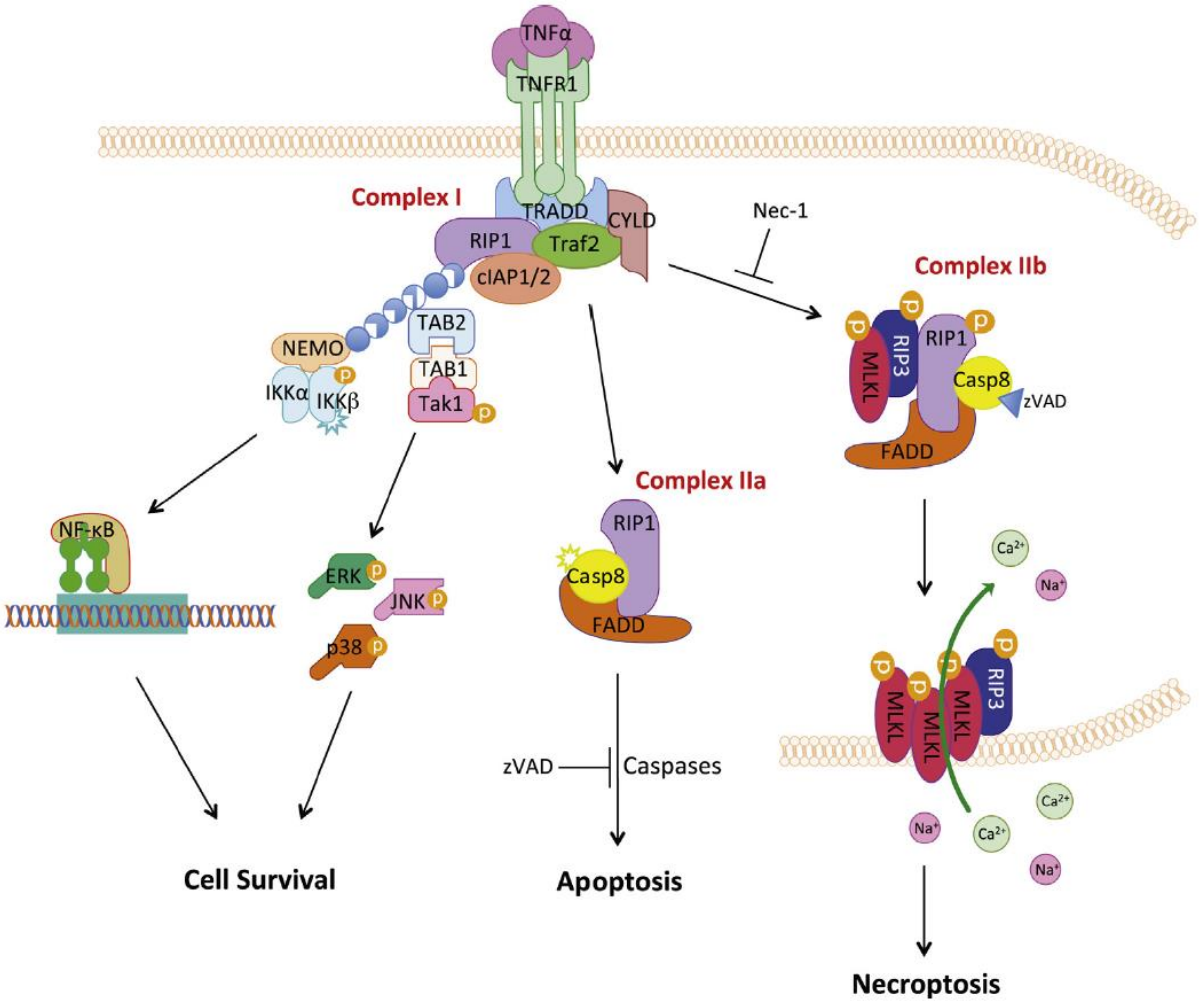


Fig. 24: TNF-R1-mediated pathways of cell survival, apoptosis and necroptosis. Stimulation of the TNF receptor leads to the formation of complex I consisting of TRADD, TRAF2, RIP1, and cIAP1 located at the cellular membrane. Polyubiquitination of RIP1 by cIAP1, fosters the recruitment of the IKK complex activating the NF-κB survival pathway. In the absence of cIAP1 or cFLIP, a switch takes place and RIP1, FADD and Caspase-8 form the cytosolic complex IIa to activate the caspase cascade and induce apoptosis. When Caspase-8 activity is inhibited, RIP1 interacts with RIP3 and MLKL to form complex IIb which is involved in mediation of necroptosis. RIP3 and MLKL are phosphorylated in complex IIb and translocate to the plasma membrane, where the complex mediates membrane permeabilization. [Zhou and Yuan 2014]

Recently, a connection of TNF signaling and the LUBAC was described. Several groups demonstrated that deficiency in one of the LUBAC components HOIP or Sharpin results in increased TNF-R1-mediated cell death. As HOIP is the catalytically important component of the LUBAC, HOIP deficiency leads to aberrant complex-IIa formation upon TNF stimulation mediating apoptosis instead of survival. These findings confirm the importance of the LUBAC to prevent cells from TNF-induced cell death by maintenance of complex I action [Peltzer 2014, Kumari 2014, Rickard 2014].

An additional interplay occurs between the NF-κB and JNK pathway. NF-κB provides its survival signals via inhibiton of JNK. Experiments in NF-κB-deficient cells showed an

impaired downregulation of JNK signaling [Papa 2009]. Moreover, analysis of these NF-κB-deficient cells treated with JNK inhibitors or silenced for MKK7 expression revealed a rescue from TNF-α induced cell death, which points to a proapoptotic role of JNK. Another paradigm for the crosstalk of NF-κB and JNK signaling has been discussed during the inflammatory response. There, TNF-α activates NF-κB signaling and JNK signaling can be suppressed by either upregulation of Gadd45β (Growth arrest DNA damage-inducible gene 45 β), XIAP or other factors, which hinder JNK activation. Gadd45β binds to the JNK kinase MKK7 and thereby blocks its activity (Fig. 25) [Papa 2004].

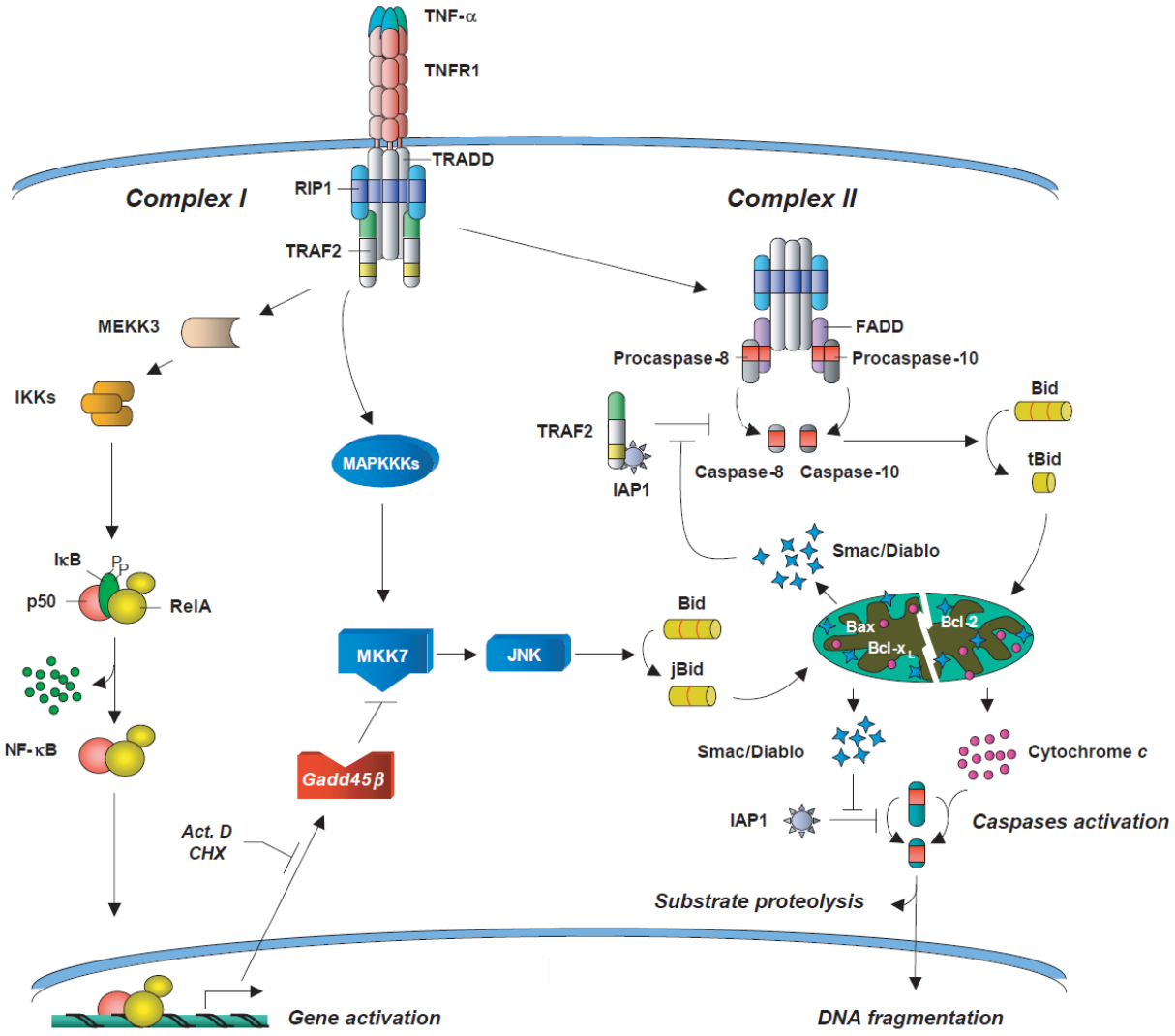


Fig. 25: Crosstalk between TNF-R-induced pathways. Formation of complex I leads to NF-kB activation, Gadd45b induction, JNK inhibition and cell survival (left). Alternatively, formation of complex II leads to Caspase-8/10-mediated cleavage of Bid, which then induces cytochrome c release from mitochondria and cell death (right). The scheme also depicts JNK activation, which promotes programmed cell death by triggering release of Smac/Diablo into the cytosol, inhibiting the TRAF2-IAP1 complex and consequently activating Caspase-8. Moreover, the Gadd45b-MKK7 interaction linking the JNK and NF-kB pathways is shown. [Papa 2004]

Interestingly, a JNK substrate, which fosters TNF-α induced apoptosis, was missing for a long time. In 2006, it was reported that JNK activation by TNF-α leads to reduced levels of an NF-κB-induced antiapoptotic protein termed c-FLIP inhibiting Caspase-8. The underlying mechanism involves phosphorylation and activation of the E3 ligase Itch by JNK; Itch in turn ubiquitinates c-FLIP to promote its degradation via the proteasome [Chang 2006]. In conclusion, several players participating in the crosstalk of NF-κB and JNK signaling could be identified so far underpinning the importance of this counterregulation.

2. Aim of the thesis

Recently, our group demonstrated that the PD-associated protein Parkin can influence the NF-κB pathway through increased activation of the LUBAC [Müller-Rischart 2013]. In the past few years, mutant Huntingtin and HD were also linked to NF-κB signaling by a few publications [Reijonen 2010, Marcora 2010, Hsiao 2013, Träger 2014]. However, these reports were inconsistent and argued either for decreased or increased activation of the NF-κB pathway caused by mutant Huntingtin. In addition, a common convincing molecular mechanism how mutant Huntingtin leads to the monitored effects on this TNF-α mediated pathway was missing. Furthermore, it is still unclear, how such an interference in NF-κB signaling or its consequences can serve as an explanation for the observed mitochondrial alterations and the death of neurons in HD.

To minimize or clear these ambiguities, I focused on the following questions within the project:

- Confirm and clarify effect of Huntingtin on NF-κB signaling (up- or downregulation)
- Identify step of the NF-κB pathway, which is affected by mutant Huntingtin
- Investigate if other TNF-mediated pathways (JNK and caspase signaling) are also influenced by the presence of polyQ aggregates
- Analyze if dysregulation of pathways can explain impact of mutant Huntingtin on mitochondrial function and apoptotis
- Investigate if Parkin or HOIP can rescue from altered TNF signaling caused by mutant Huntingtin

3. Results

NF-κB is a key transcription factor that regulates expression of genes involved in cell survival, differentiation and proliferation [Hayden 2012]. After having discovered a connection between the PD-associated protein Parkin and the NF-κB signaling pathway [Müller-Rischart 2013], we were interested in identifying a possible link between the HD-associated protein Huntingtin and the transcription factor NF-κB.

For Parkin, we had observed that it can increase the activity of the LUBAC in response to cellular stress (Fig. 26). The LUBAC mediates linear ubiquitination of NEMO resulting in activation of the IKK complex. The IKK complex in turn activates NF-κB by phosphorylation and subsequent degradation of the inhibitory protein IκB-α, so that the NF-κB heterodimer (p50-p65) can translocate to the nucleus and regulate transcription of NF-κB responsive genes like OPA1.

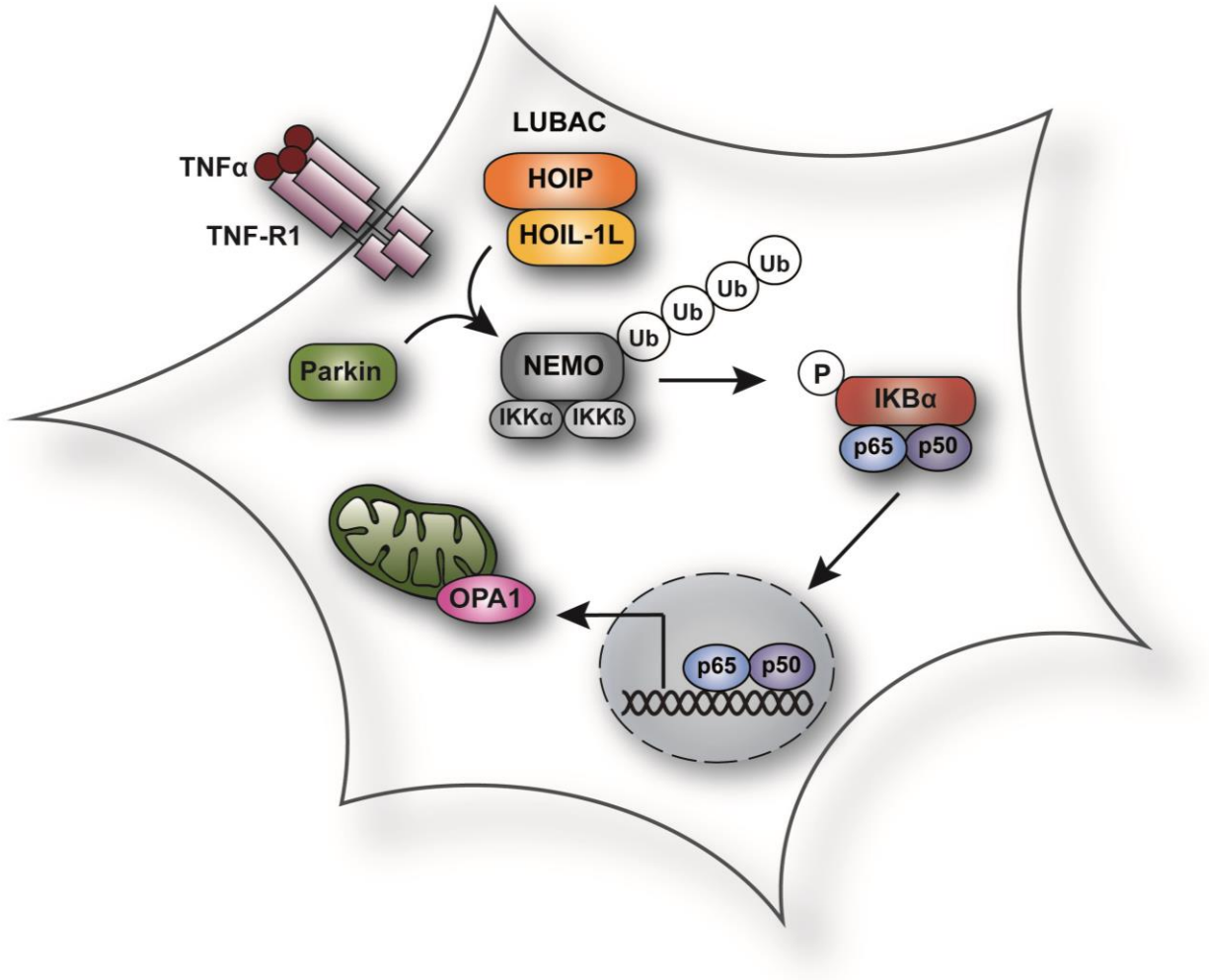


Fig. 26: Parkin exerts its stress protective function via activation of LUBAC, NEMO, NF-κB and OPA1. [Graphical abstract, Müller-Rischart 2013].

In the following, the knockdown of Parkin in human neuroblastoma SH-SY5Y cells serves as an example to demonstrate the effect of Parkin expression levels on NF-κB activation. To monitor NF-κB activation, we focused on one of the last steps of the NF-κB signaling pathway – the translocation of the activated NF-κB-heterodimer from the cytosol to the nucleus. For analysis via indirect immunofluorescence, we concentrated on the subunit p65. Upon stimulation with TNF-α, p65 is normally imported into the nucleus in control cells, whereas p65 is still mainly localized in the cytosol of Parkin-deficient cells (Fig. 27). This finding supports the functional relevance of endogenous Parkin within the NF-κB pathway.

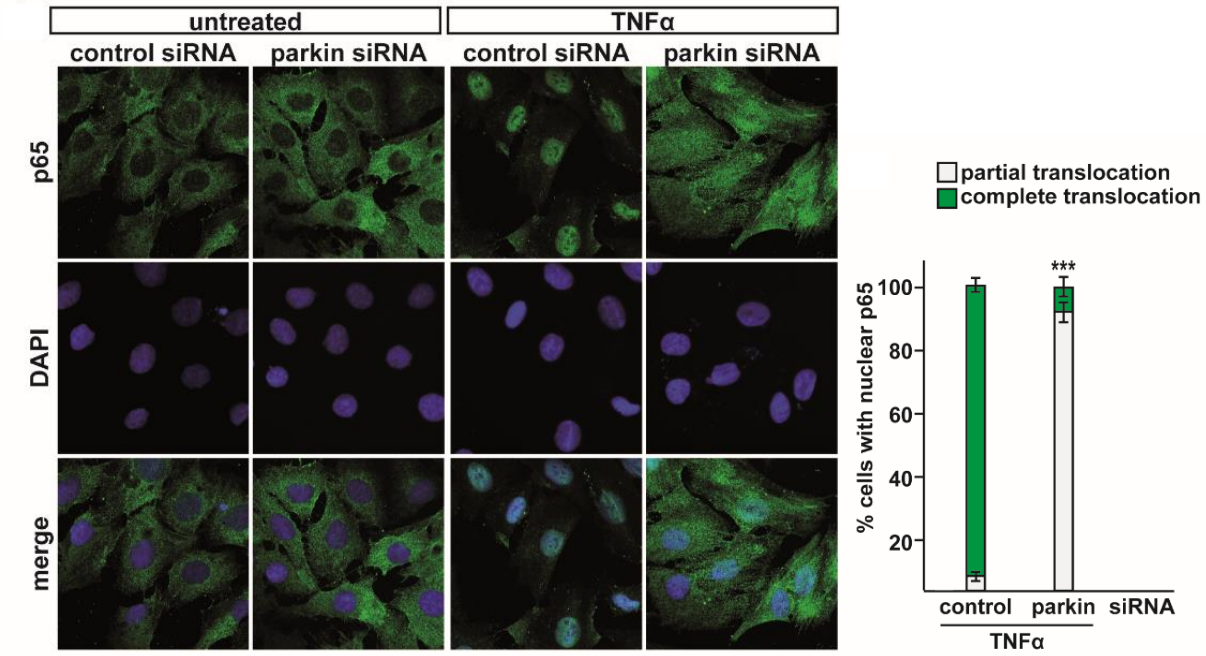


Fig. 27: Nuclear translocation of p65 is decreased in SH-SY5Y cells silenced for Parkin expression.
Left: p65 translocation was analyzed by indirect immunofluorescence of cells transfected with control or Parkin siRNA and treated with TNF-α (15 min, 20 ng/ml). Right: Quantifications are based on four independent experiments performed in duplicate (n ≥ 2,500). Data represent the mean ± SEM. *p ≤0.05; **p ≤0.01; ***p ≤0.001. [Müller-Rischart 2013]

3.1 NF-κB signaling is impaired by mutant Huntingtin

3.1.1 Nuclear translocation of p65 is impaired in cells with polyQ aggregates

Having seen that nuclear translocation of p65 is a convenient tool for monitoring NF-κB activation, we also applied it to study a possible effect of Huntingtin aggregates on the NF-κB signaling pathway. SH-SY5Y cells were transfected with myc-tagged Htt-Q20 and -Q96 for transient expression of wild-type and mutant Huntingtin. After treatment with TNF-α to induce NF-κB activation, the cells were fixed and stained against p65.

For quantification, the following three categories were defined: full p65 translocation (p65 is mostly localized in the nucleus and hardly detected in the cytosol), partial p65 translocation

(staining intensity for p65 in the nucleus is equal to the cytosol), no p65 translocation (p65 is mainly located in the cytosol and hardly detected in the nucleus). Full p65 translocation was observed in 100% of the cells expressing Q20, while only 7 % of the cells expressing Q96 showed full translocation (Fig. 28). Approximately 50 % of transfected cells with aggregates showed no p65 translocation at all.

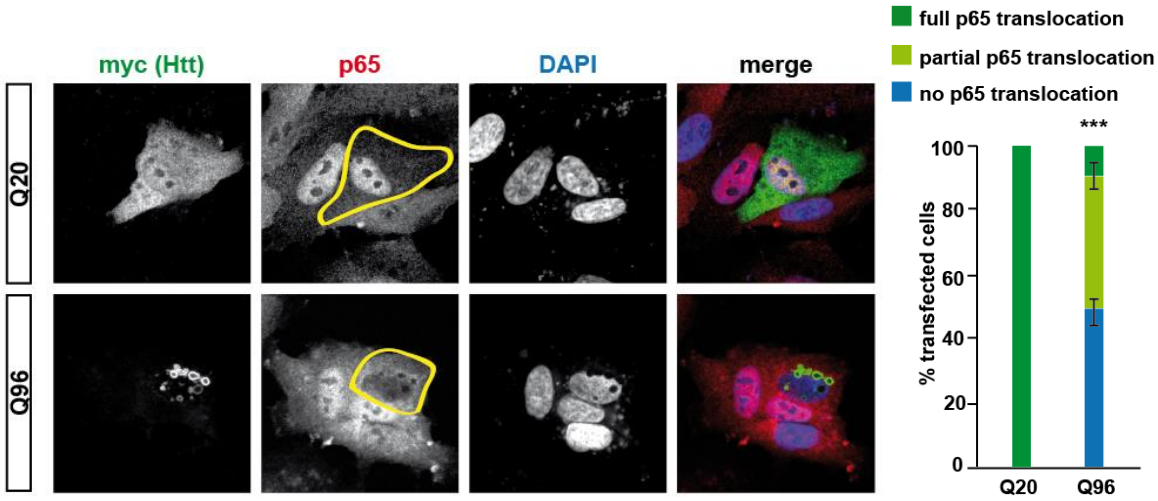


Fig. 28: Nuclear translocation of p65 is decreased in SH-SY5Y cells expressing polyQ aggregates. Left: p65 translocation was analyzed by indirect immunofluorescence of cells transfected with control (Q20) or mutant (Q96) myc-tagged Huntingtin treated with TNF-α (15 min, 20 ng/ml). Right: Quantifications are based on three independent experiments performed in triplicates (n ≥ 1,000). Data represent the mean ± SEM. Statistical analysis was first performed separately for each subgroup and the total p-value was determined by the mean value of the single statistical results. *p ≤0.05; **p ≤0.01; ***p ≤0.001.

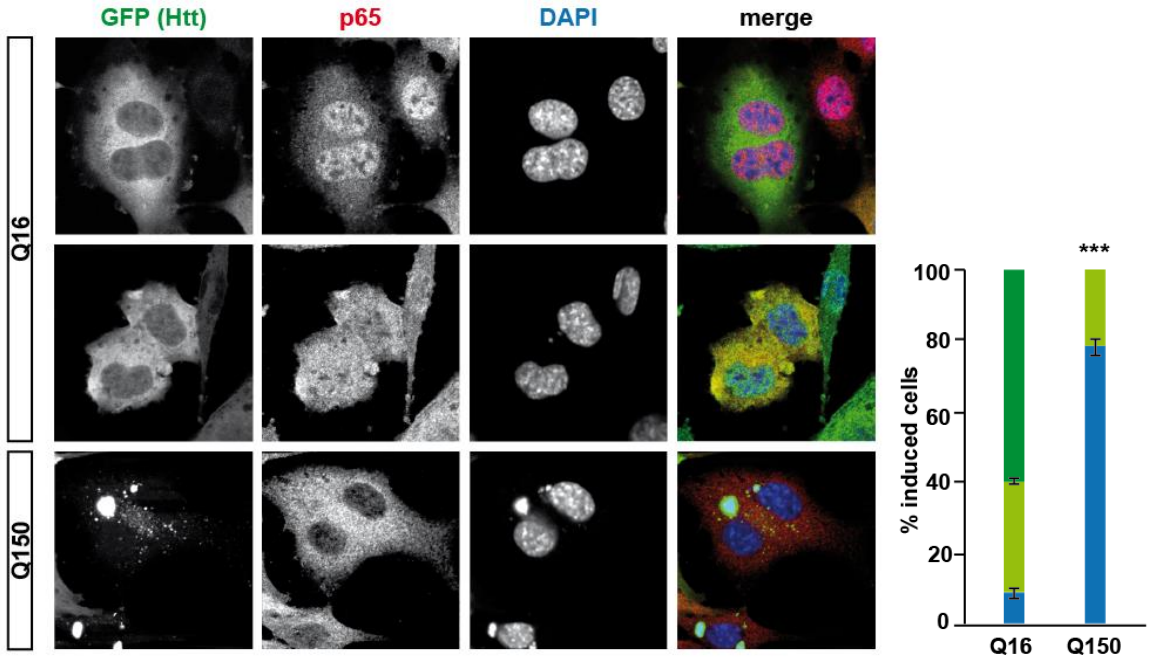


Fig. 29: Nuclear translocation of p65 is decreased in inducible N2a cells expressing polyQ aggregates. Left: p65 translocation was analyzed in induced N2a-cells expressing polyQ aggregates upon TNF-α treatment (30 min, 20 ng/ml) and induction with Muristerone A for 72 h. Induced cells express GFP. Right: Quantifications are based on three independent experiments performed in triplicates (n ≥ 1,000). Data represent the mean ± SEM. *p ≤0.05; **p ≤0.01; ***p ≤0.001.

Similarly, inducible murine neuroblastoma N2a cells were analyzed (Fig. 29). Upon induction with Muristerone A, these inducible cell lines express a fusion protein consisting of N-terminal Huntingtin fragments with different polyQ stretches (Q16, Q150) and EGFP as a reporter protein. 59 % of the Q16-expressing cells showed full translocation in comparison to none of the Q150-expressing cells. Overall, the lowered p65 translocation in the N2a cells confirmed the previous finding in SY-SY5Y cells that NF-κB activation is impaired in cells displaying cytosolic Huntingtin aggregates.

3.1.2 Phosphorylation and degradation of IκB-α is reduced in cells with polyQ aggregates

It was not clear from the obtained data, whether polyQ aggregates directly block NF-κB translocation or an impairment of the NF-κB pathway occurs already at an earlier step upstream of the translocation. Therefore, we next investigated the phosphorylation and degradation of IκB-α located upstream of NF-κB translocation. Within the NF-κB pathway, binding of TNF-α to its receptor leads to activation of the IKK complex, which is followed by phosphorylation and degradation of the inhibitory protein IκB-α. Thereby, the transcription factor NF-κB is liberated and can translocate to the nucleus to activate gene transcription.

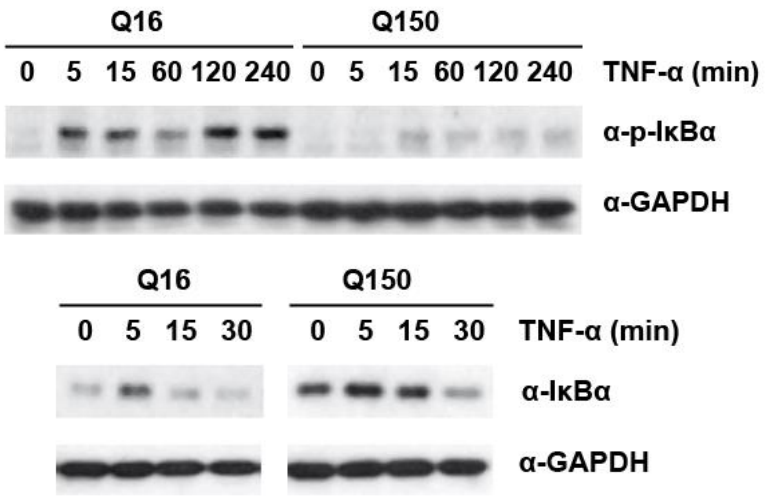


Fig. 30: N2a-Q150 cells show reduced phosphorylation (upper panel) and impaired degradation of IκB-α (lower panel) in comparison to control N2a-Q16 cells upon TNF-α treatment. Cells were treated with TNF-α for 0-240 min (upper panel) and 0-30 min (lower panel). p-IκB-α, IκB-α and GAPDH as control were analyzed by western blotting.

Analysis of phospho- IκB-α and IκB-α protein levels after treatment of induced N2a cells with TNF-α revealed that both steps – phosphorylation and degradation of IκB-α – were significantly impaired in cells expressing Q150 (Fig. 30). Furthermore, it should be noted that

the IκB-α protein seems to be expressed at higher levels even in untreated cells, which points to the possibility that the defect in NF-κB signaling caused by polyQ aggregates occurs already upstream of the IκB-α degradation.

3.2 Impaired NF-κB signaling occurs due to sequestration of LUBAC E3 ligases

3.2.1 LUBAC components show increased aggregation upon co-expression of polyQ aggregates

The higher basal IκB-α levels in N2a-Q150 cells supported the assumption that the impairment in NF-κB signaling occurs at an early step of the pathway. Knowing from recent studies that the PD-associated protein Parkin intervenes at one of the top steps of NF-κB signaling to increase activity of the LUBAC and thereby NF-κB activation, we were wondering if the Huntingtin aggregates might also interfere with LUBAC activation [Müller-Rischart 2013]. One possibility would be that polyQ aggregates sequester and trap components of the LUBAC. To test this hypothesis, we first performed filter trap assays to analyze protein aggregation. For filter trap assays, the cell lysate is soaked through a cellulose acetate membrane (pore size 0.2 µm), which cannot be passed by large protein aggregates. The aggregates are trapped at the membrane and can be detected via Western Blotting. In our assays, HEK293T cells were transfected with myc-tagged Htt-Q20, -Q96 or in combination with either Parkin, HOIP or HHARI-HA as control.

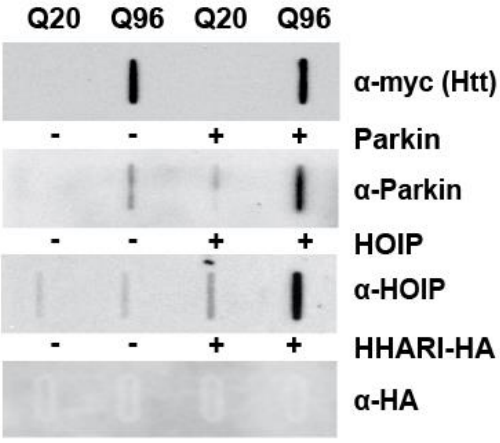


Fig. 31: Aggregation of Parkin and HOIP but not HHARI is increased when Htt-Q96 is co-expressed. Filter trap assays of HEK293T cells transfected with Htt-Q20 or Q96 and Parkin, HOIP or HHARI-HA.

Analysis of the different Htt constructs produced only detectable aggregates, when Htt-Q96 was expressed and not -Q20 (Fig. 31). For Parkin (both endogenous as well as overexpressed), aggregation was significantly increased in the presence of Q96 and a strong

increase of HOIP aggregation was also detected when Q96 was co-expressed (only seen for overexpressed HOIP). HHARI was analyzed as control protein, as it is an RBR E3 ubiquitin ligase similar to Parkin and HOIP that was so far not associated with the LUBAC. Aggregation of HHARI was not altered by Q96 co-expression.

To confirm the co-aggregation of mutant Huntingtin with other LUBAC components, HOIL-1L and SHARPIN were also examined. These filter trap assays showed similar findings as an increased signal for HOIL-1 L or SHARPIN was only detected when Q96 was co-expressed (Fig. 32).

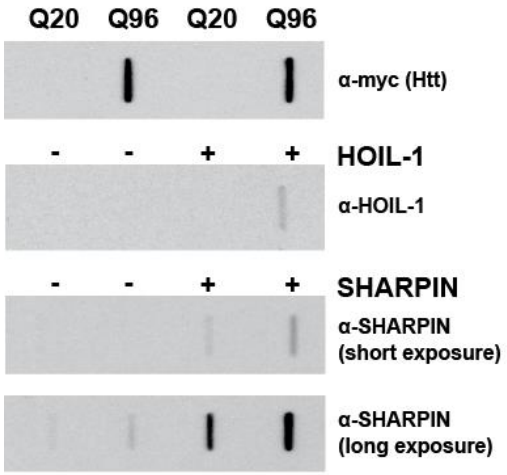


Fig. 32: Aggregation of HOIL-1L and SHARPIN is increased when Htt-Q96 is co-expressed. Filter trap assays of HEK293T cells transfected with Htt-Q20 or Q96 and HOIL-1l or SHARPIN.

3.2.2 LUBAC components co-localize with polyQ aggregates

For further clarification of the implied sequestration caused by the presence of polyQ aggregates, confocal microscopy was performed. In SH-SY5Y cells overexpressing mutant Htt, Parkin and HOIP were clearly enriched at the region of the aggregates (Fig. 33). In contrast, the RBR E3 ubiquitin ligase HHARI was equally distributed within the cytosol. Similarly to HOIP and Parkin, HOIL-1L was co-localizing with polyQ aggregates, whereas co-localization between SHARPIN and the aggregates was weaker and did not result in cytosolic depletion. These findings confirmed the data from the filter trap assays and the hypothesis that mutant Huntingtin sequesters LUBAC components. As a consequence, the LUBAC E3 ligases might not be able anymore to fulfill their functions including promotion of NF-κB signaling.

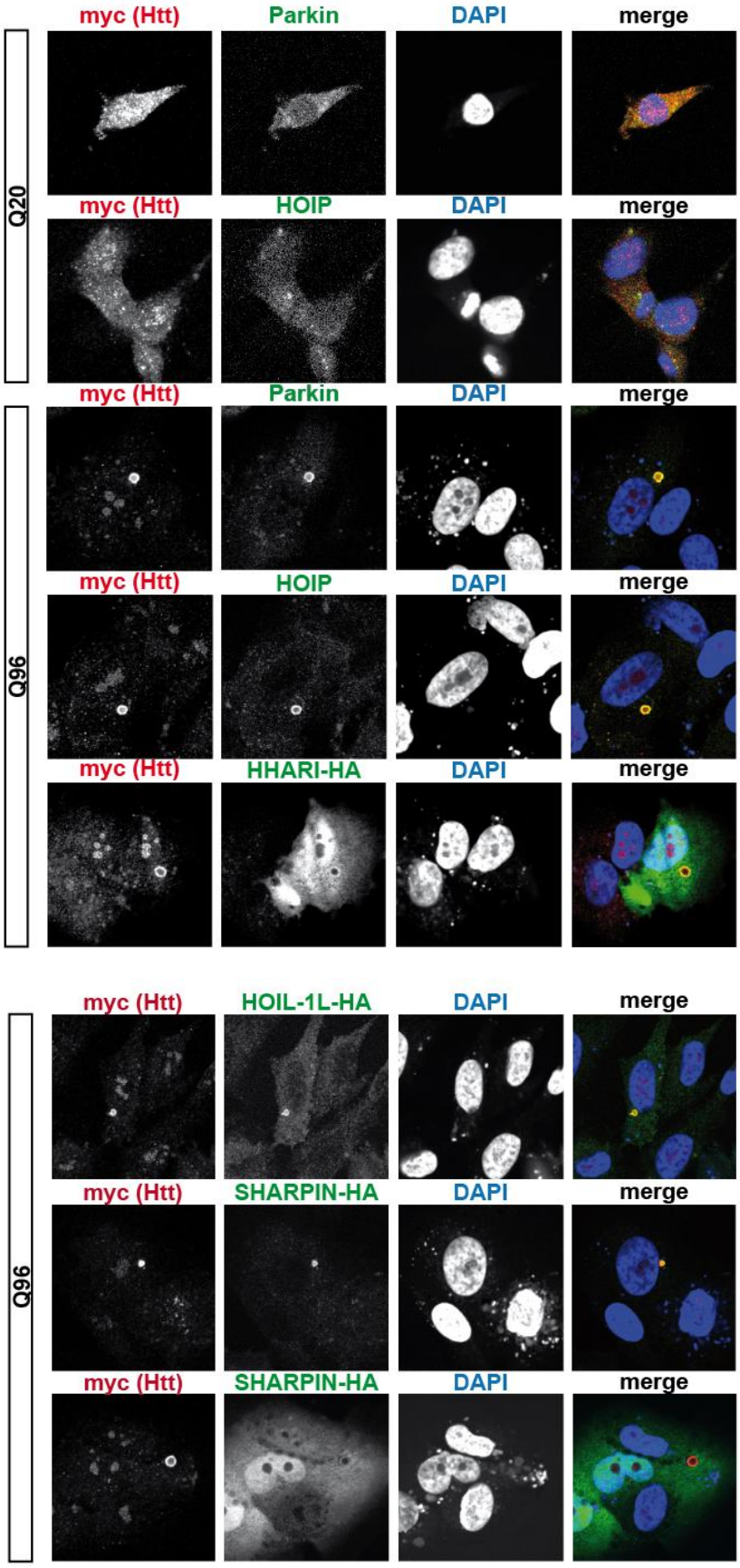


Fig. 33: Parkin, HOIP, HOIL-1L and SHARPIN co-localize with polyQ aggregates, while HHARI is still distributed equally within the cytosol. SH-SY5Y cells were co-transfected with the indicated constructs and analyzed via indirect immunofluorescence.

3.3 Wild-type Parkin or HOIP can rescue from impaired NF-κB signaling

3.3.1 Impaired p65 translocation in SH-SY5Y cells can be rescued by wild-type Parkin or HOIP

If sequestration of Parkin and other LUBAC components by Huntingtin aggregates is the reason for impaired NF-κB signaling, overexpression of these proteins should result in a rescue of the defective pathway. To test this hypothesis, p65 translocation was used as a first read-out. SH-SY5Y cells were transfected with Htt-Q96 in combination with wild-type or mutant Parkin or HOIP. Co-expression of both wild-type Parkin and HOIP significantly decreased the number of cells impaired in p65 translocation (Fig. 34). Besides this, neither the catalytically inactive mutants of Parkin (C431F) or HOIP (C885A, C885S) nor the pathogenic loss-of-function Parkin mutants (ΔUBL, G430D) could revert the impairment in p65 translocation.

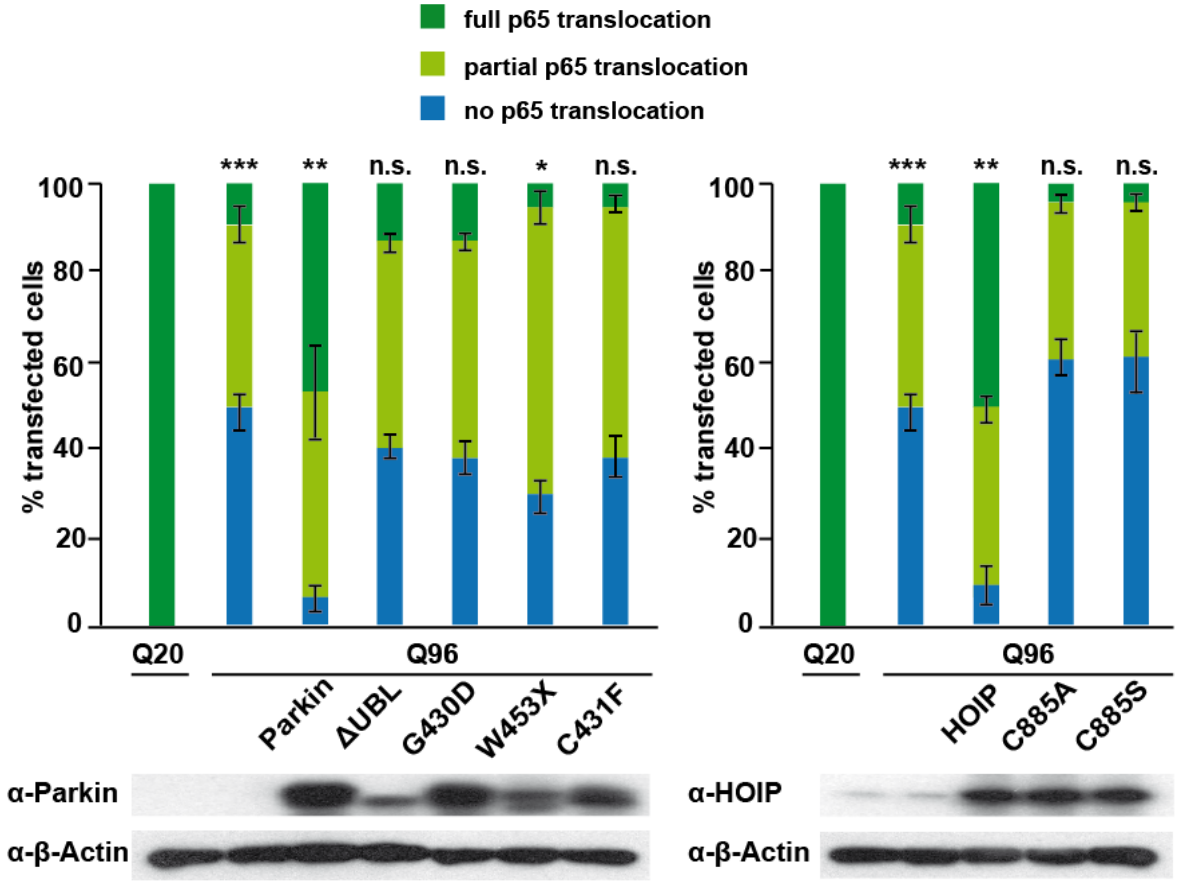
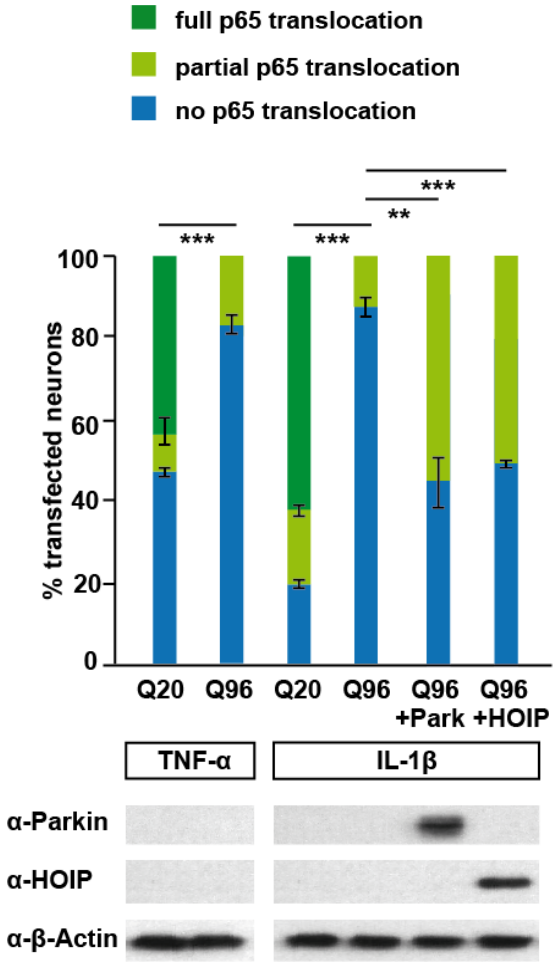


Fig. 34: Impaired p65 translocation in SH-SY5Y cells expressing mutant Huntingtin can be rescued by overexpression of wild-type but not mutant Parkin or HOIP TNF-α treatment was conducted for 15 min with a concentration of 20 ng/ml. Data represent the mean ± SEM of at least three independent experiments, each performed in triplicate. n ≥ 1,000 induced or transfected cells were assessed per condition. Statistical analysis was first performed separately for each subgroup and the total p-value was determined by the mean value of the single statistical results. *p ≤0.05; **p ≤0.01; ***p ≤0.001.

3.3.2 Impaired p65 translocation in neurons can be rescued by wild-type Parkin or HOIP

Moreover, the confirmation of this observation in a more disease-relevant model was of interest. Therefore, nuclear p65 translocation was additionally analyzed in primary hippocampal neurons, which were transfected with Htt-Q20 or -Q96 in combination with wild-type Parkin or HOIP. Both TNF-α as well as IL-1β, another NF-κB activator, were used as stimulators for p65 translocation. As the translocation efficiency of p65 was slightly higher with IL-1β, this stimulus was applied in the rescue experiments with co-expression of Parkin or HOIP (Fig. 35). While approximately 84 % of the Q96-expressing neurons showed no p65 translocation, co-expression of Parkin or HOIP reduced this number to 44 and 50 %, respectively. However, this rescue was only partial as no cells with full p65 translocation could be discovered like in cells expressing the control construct Htt-Q20. Taken together, co-expression of wild-type Parkin or HOIP was able to rescue p65 translocation in immortalized cells as well as in primary neurons.



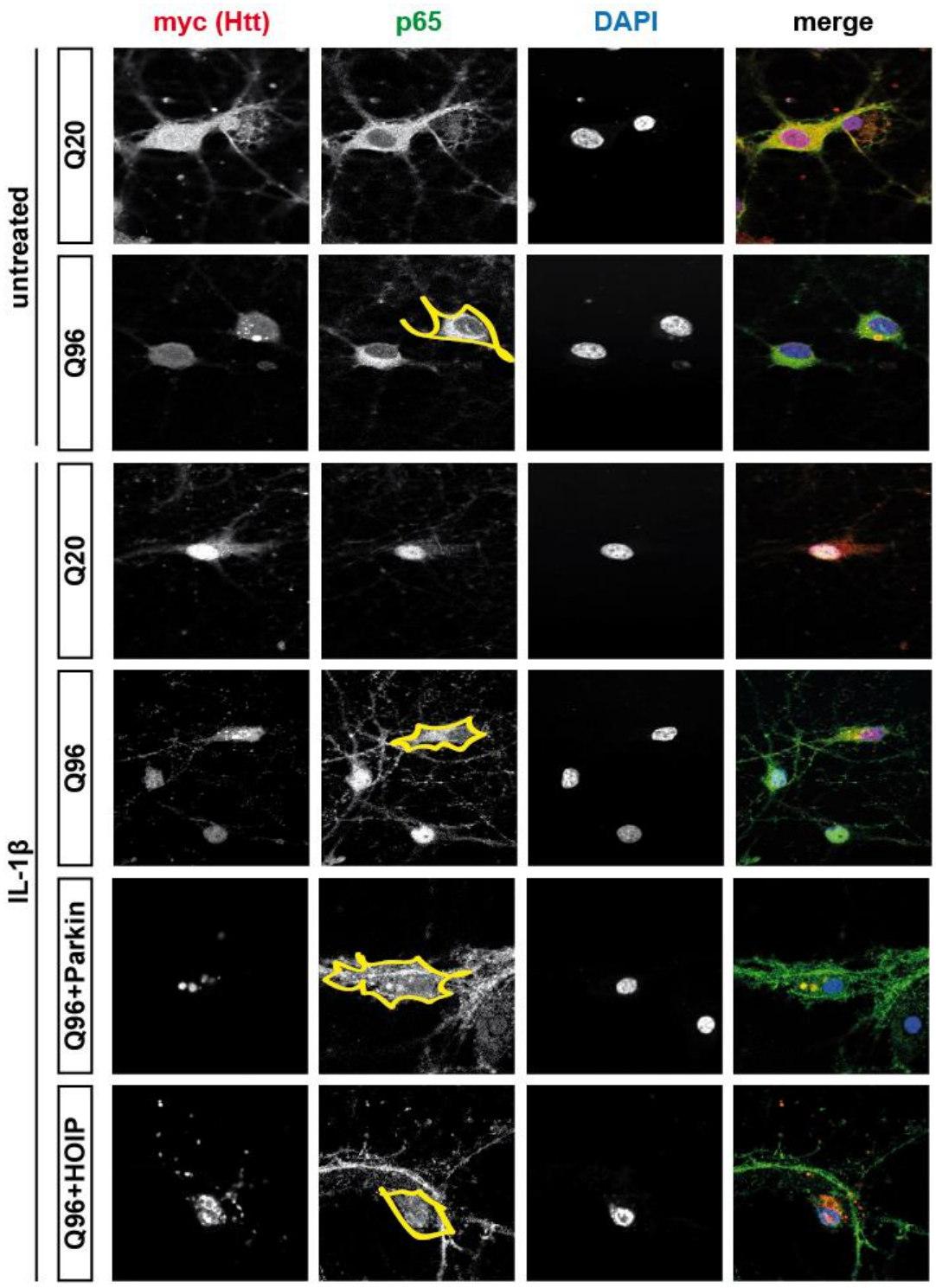


Fig. 35: Nuclear translocation of p65 is decreased in hippocampal neurons expressing polyQ aggregates, which can be increased by co-expression of wild-type Parkin or HOIP.
Neurons were transfected with Q20 or Q96 together with Parkin or HOIP and treated with TNF-α (60 min, 20 ng/ml) or IL-1β (30 min, 10 ng/ml) before fixation. Data represent the mean ± SEM of at least three independent experiments, each performed in triplicate. n ≥ 100 cells were assessed per condition. Statistical analysis was first performed separately for each subgroup and the total p-value was determined by the mean value of the single statistical results. *p ≤0.05; **p ≤0.01; ***p ≤0.001.

3.3.3 Impaired NF-κB activity in inducible N2a cells can be rescued by wild-type Parkin or HOIP

NF-κB luciferase reporter assays with the inducible N2a cells (Q16, Q150) and stimulation with TNF-α provided further evidence for the rescue properties of Parkin or HOIP regarding defective NF-κB signaling due to the presence of mutant Huntingtin (Fig. 36). TNF-α treatment induced an almost 3-fold increase of NF-κB activity in Q16 control cells. In Q150 N2a cells expressing polyQ aggregates, this increase was dramatically reduced to 1.3-fold. However, transient expression of wild-type Parkin or HOIP increased NF-κB-dependent transcription to approximately 3-fold, which was comparable to control cells. The two catalytically inactive mutants Parkin C431F and HOIP C885S did not significantly increase the levels of induction in Q150 cells. In conclusion, several experiments have so far supported the potential of wild-type Parkin or HOIP to overcome the NF-κB impairment caused by polyQ aggregates.

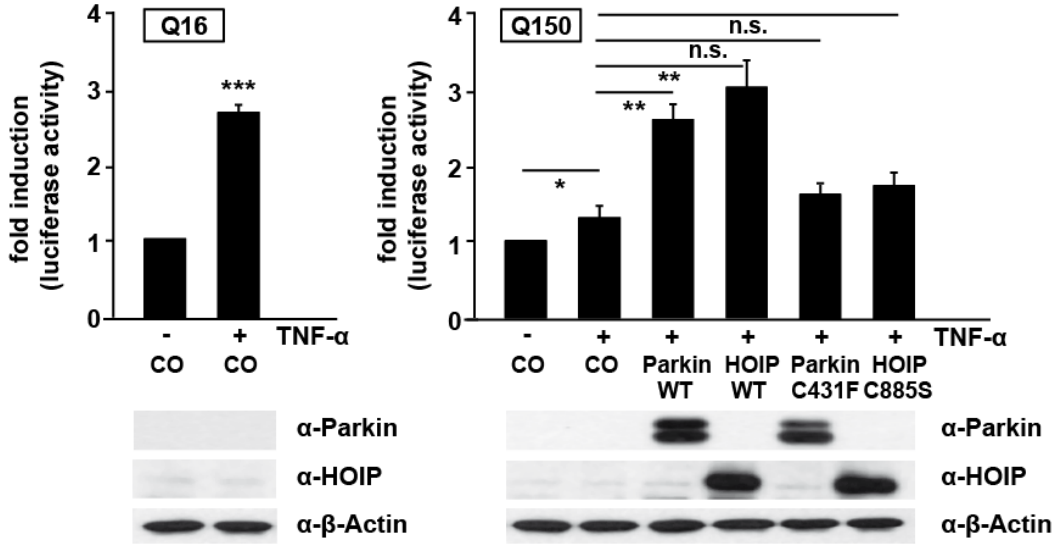


Fig. 36: Wild-type Parkin or HOIP can restore impaired NF-κB activity. Induced N2a cells (Q16 and Q150) were transfected with an NF-κB luciferase reporter and control, Parkin or HOIP construct. After TNF-α treatment for 16 h (10 ng/ml) luciferase activity in cell lysates was determined. Data represent the mean ± SEM of at least three independent experiments, each performed in triplicate. *p ≤0.05; **p ≤0.01; ***p ≤0.001.

3.4 Mutant Huntingtin causes mitochondrial alterations

3.4.1 Mutant Huntingtin provokes increased mitochondrial fragmentation in inducible N2a cells

As mitochondrial alterations have already been described in several models of polyQ diseases [Scorrano 2012], we next investigated mitochondrial morphology upon overexpression of Htt

aggregates. As we could not observe any severe effects of transient overexpression of Htt-Q96 on the mitochondrial fragmentation in SH-SY5Y cells (data not shown), we moved on to the inducible N2a cells, which are stably overexpressing Htt with different polyQ lengths. Indeed, we found that Htt-Q16 control cells showed less than 5 % of cells with fragmented mitochondria in comparison to 56 % of the Htt-Q150 cells (Fig. 37).

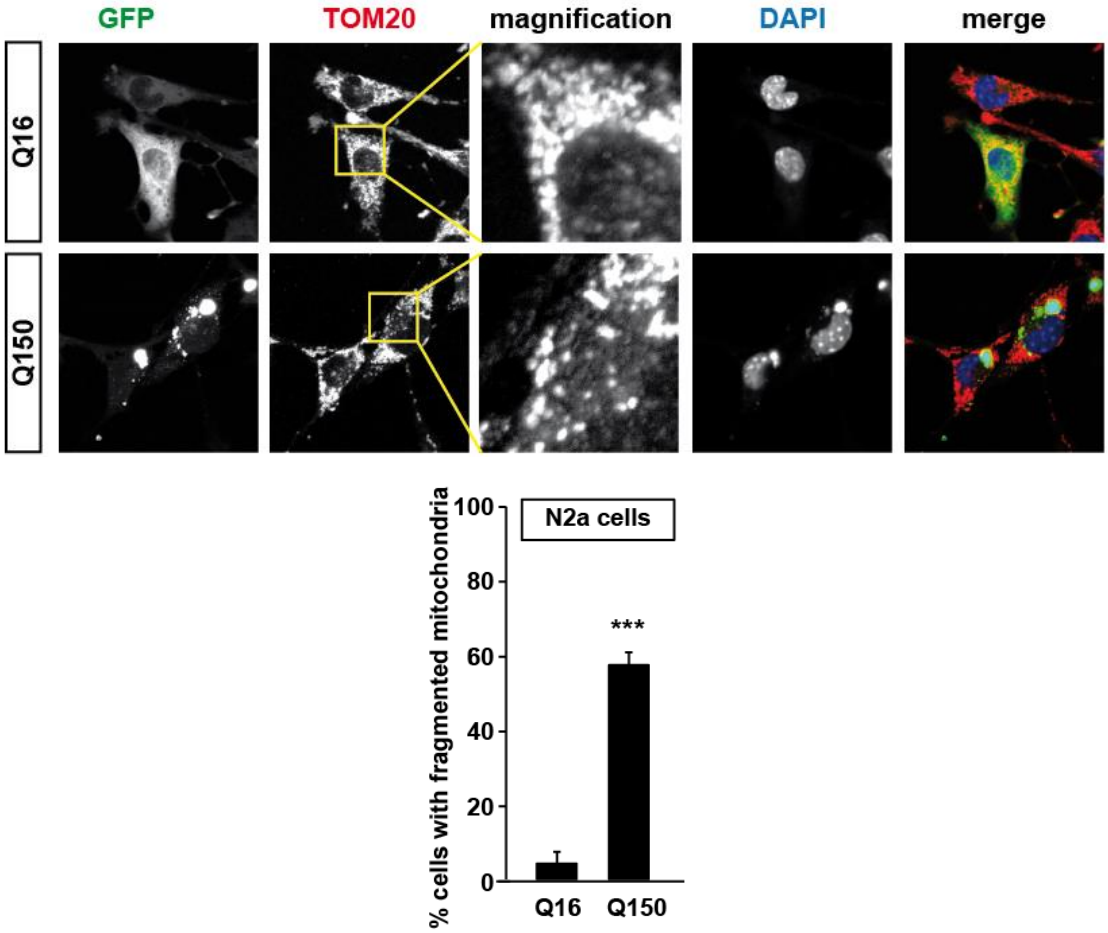


Fig. 37: Induced Q150-N2a cells show increased mitochondrial fragmentation. Upper panel: The cells were stained with TOM20 to visualize mitochondria. Cells displaying an intact network of mitochondria were classified as non-fragmented, while cells with a disrupted mitochondrial network were classified as fragmented. Lower panel: Quantifications are based on three independent experiments performed in triplicates (n ≥ 1,000). Data represent the mean ± SEM. *p ≤0.05; **p ≤0.01; ***p ≤0.001.

3.4.2 Increased mitochondrial fragmentation in neurons can be rescued by Parkin or HOIP

A similar increase in mitochondrial fragmentation was seen in primary hippocampal neurons (from 12 % in Q20- to 62 % in Q96-expressing neurons). Here, we tested if overexpression of wild-type Parkin or HOIP influences the mitochondrial phenotype in Q96-expressing cells. Strikingly, mitochondrial fragmentation observed upon Q96-overexpression was in fact significantly alleviated in neurons co-expressing either Parkin or HOIP (Fig. 38).

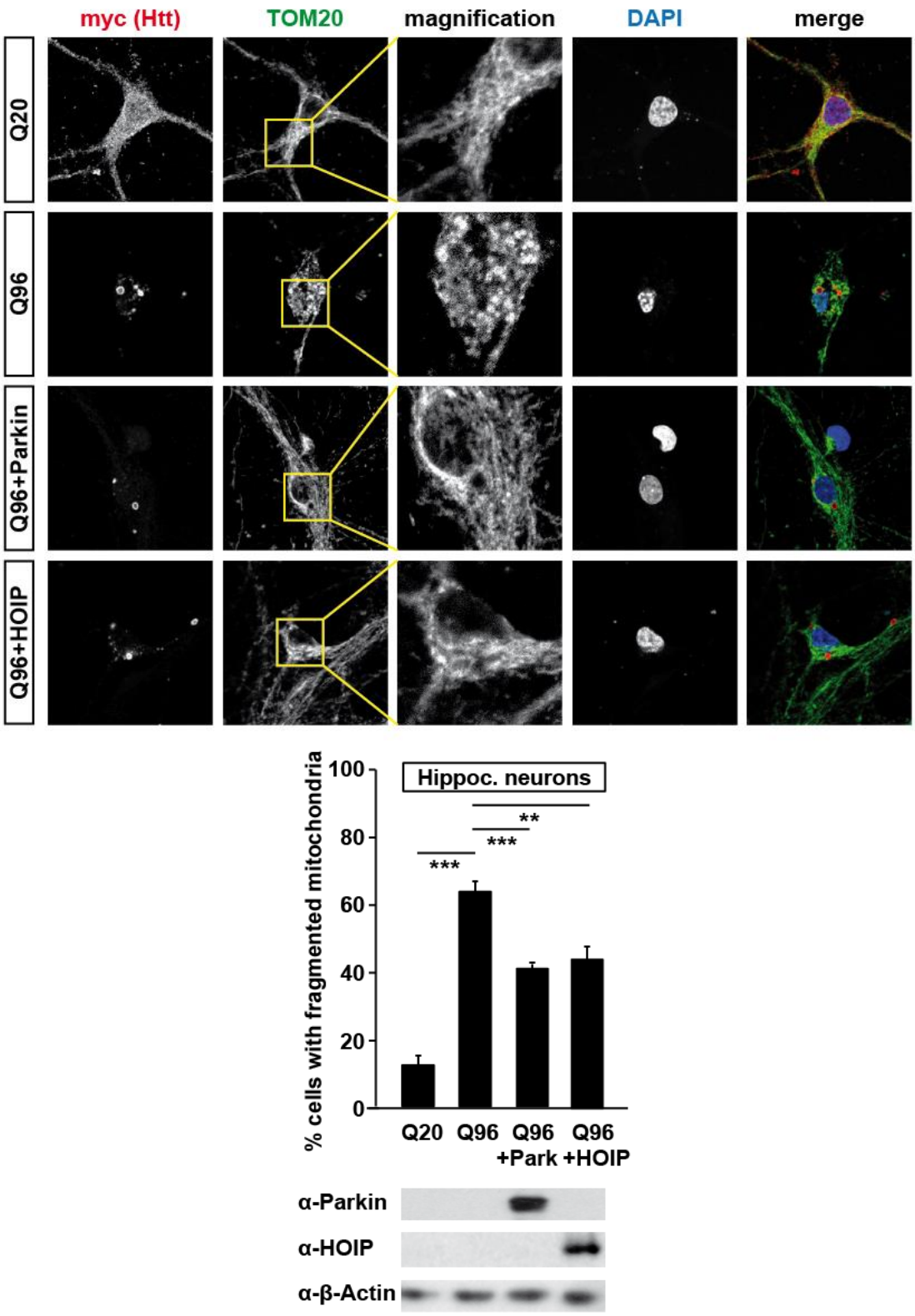


Fig. 38: Hippocampal neurons expressing polyQ aggregates show an impaired mitochondrial network. A tubular mitochondrial network can be restored by co-expression of Parkin or HOIP. The neurons were transfected with Q20 or Q96 together with wild-type Parkin or HOIP. Cells displaying an intact network of tubular mitochondria were classified as non-fragmented. When this network was disrupted and mitochondria appeared predominantly spherical or rod-like the cells were classified as fragmented. Quantifications are based on three independent experiments performed in triplicates ($n \geq 100$). Data represent the mean ± SEM. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

3.4.3 Cells expressing mutant Huntingtin lack OPA1 upregulation

In our recent work focusing on Parkin and its effect on NF-κB signaling, we identified OPA1 as an NF-κB target gene [Müller-Rischart 2013]. OPA1 is a mitochondrial GTPase and an essential regulator of structural and functional mitochondrial integrity. For this reason, we were asking the question if NF-κB-mediated expression of OPA1 is altered in cells expressing mutant Huntingtin, as we have already observed changes in mitochondrial morphology in these cells. To answer this question, we analyzed expression levels of mitochondrial proteins in induced N2a cells after stimulation with TNF-α.

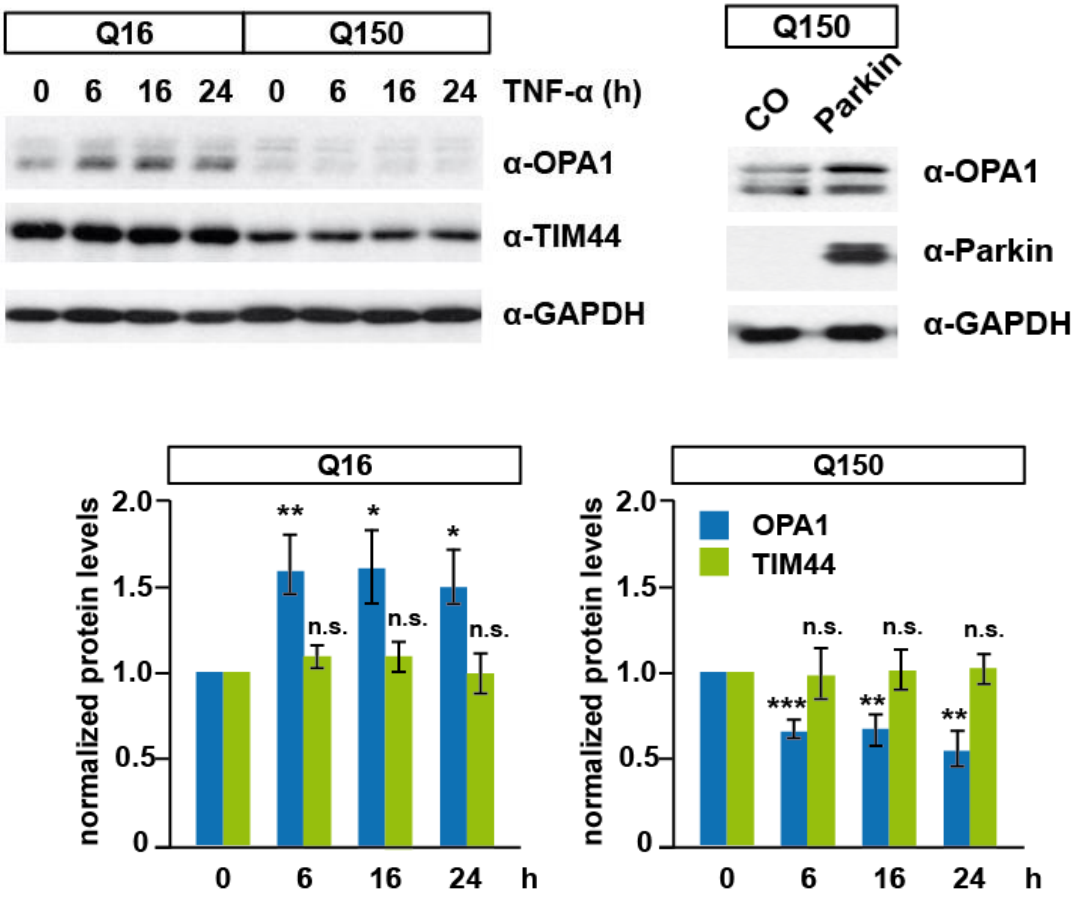


Fig. 39: Defective OPA1 protein upregulation is observed in Q150 cells upon TNF-α treatment (upper panel, left). OPA1 upregulation can be reconstituted by viral overexpression of wild-type Parkin in Q150 cells (upper panel, right). Other mitochondrial marker proteins such as TIM44 are also slightly decreased in Q150 cells. Upon TNF-α treatment (10 ng/ml) for the indicated hours, expression levels of mitochondrial proteins and the loading control GAPDH were analyzed by western blotting. Lower panel: Quantification of mitochondrial protein levels in Q16 and Q150 cells upon TNF-α treatment. Protein levels are normalized to GAPDH and quantification is based on three independent experiments. Quantification of protein levels upon viral overexpression of Parkin was not possible due to insufficient repetitions of the experiment. Data represent the mean ± SEM. *p ≤0.05; **p ≤0.01; ***p ≤0.001.

Similar to previous observations in other cell lines such as SH-SY5Y or MEF cells [Müller-Rischart 2013], OPA1 was upregulated in control Htt-Q16 N2a cells upon treatment with TNF-α, while another mitochondrial protein, TIM44, was not changed significantly in its expression pattern (Fig. 39). Interestingly, in cells expressing mutant Htt, this upregulation of OPA1 was completely abolished and even a slight decrease of OPA1 protein levels was observed. Furthermore, the expression level of the other mitochondrial marker protein was also decreased in these cells expressing aggregates suggesting a decrease in mitochondrial biogenesis in polyQ-expressing cells, as has been reported previously [Scorrano EMBO 2012].

Through viral transduction and overexpression of wild-type Parkin, OPA1 protein levels could be elevated in Q150 cells, which were treated with TNF-α (Fig. 39, upper panel, right).

3.5 PolyQ aggregates induce increased JNK and caspase signaling

3.5.1 Phosphorylation of c-Jun is increased in hippocampal neurons expressing polyQ aggregates

TNF-α is a proinflammatory cytokine signaling both cell survival as well as cell death (Wajant 2003). The biological outcome of TNFα treatment is determined by the balance between NF-κB and Jun kinase (JNK) signaling: NF-κB promotes survival, whereas JNK can enhance cell death (Chang 2006). Typically, activation of NF-κB results in inhibition of Caspase-8 and suppresses pro-apoptotic signaling via JNK. As our data show that TNF-α induced pro-survival NF-κB activation is blocked in cells expressing mutant Huntingtin, the balance might be shifted towards pro-apoptotic signaling in these cells. To test this hypothesis, we focused on the phosphorylation of c-Jun, which was identified as the essential substrate of JNK signaling to regulate stress-induced apoptosis [Behrens 1999].

Primary hippocampal neurons were transfected with polyQ constructs and nuclear phosphorylated c-Jun was analyzed by indirect immunofluorescence. Surprisingly, we found a significant increase in c-Jun phosphorylation in Q96-expressing neurons (67 % compared to 8 % in Q20-expressing neurons) even without TNFα-stimulation (Fig. 40). Beyond that, co-expression of wild-type Parkin or HOIP was able to reduce the number of phospho-c-Jun-positive cells significantly to 29 % and 13 % when Huntingtin aggregates were present.

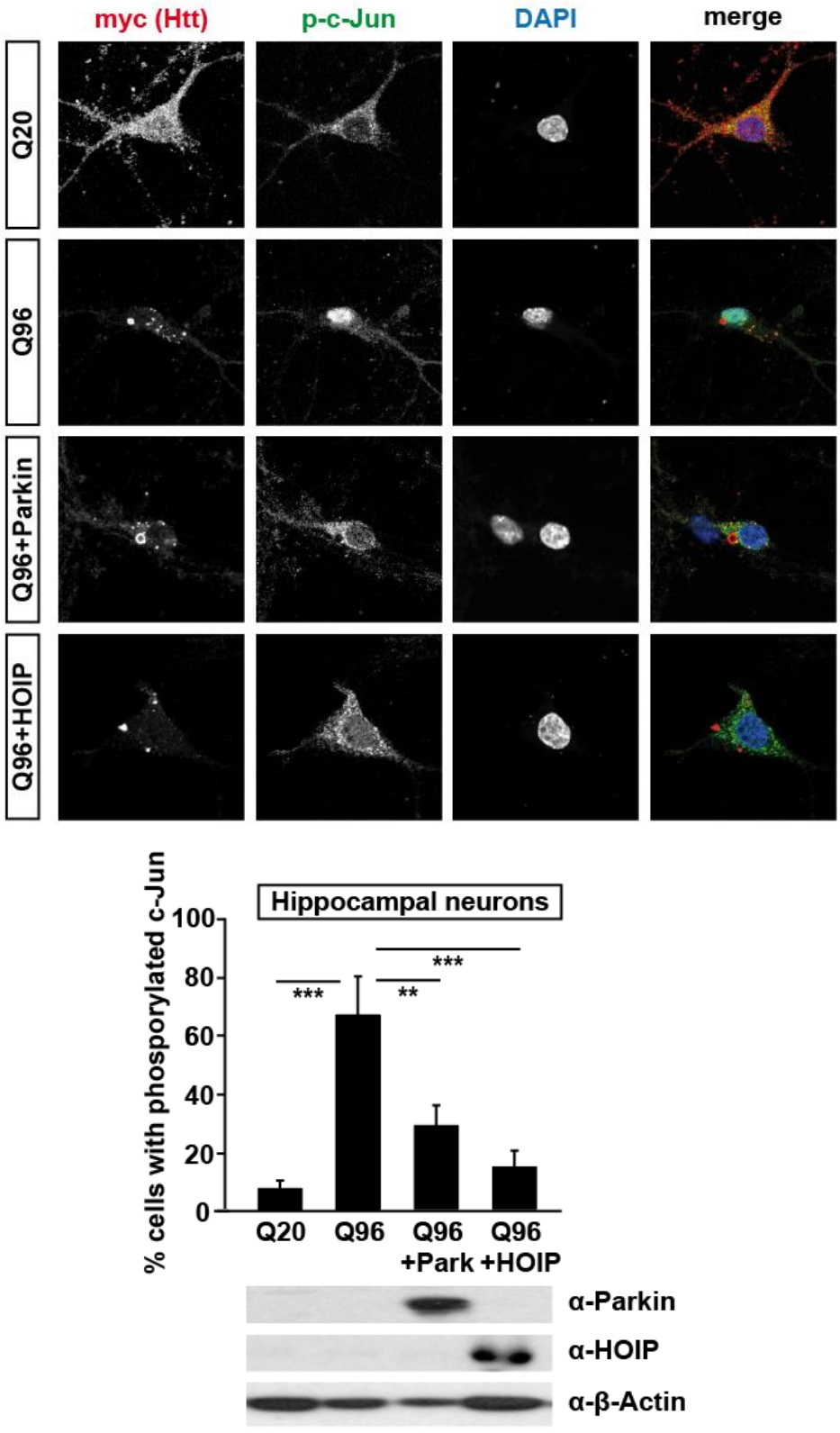
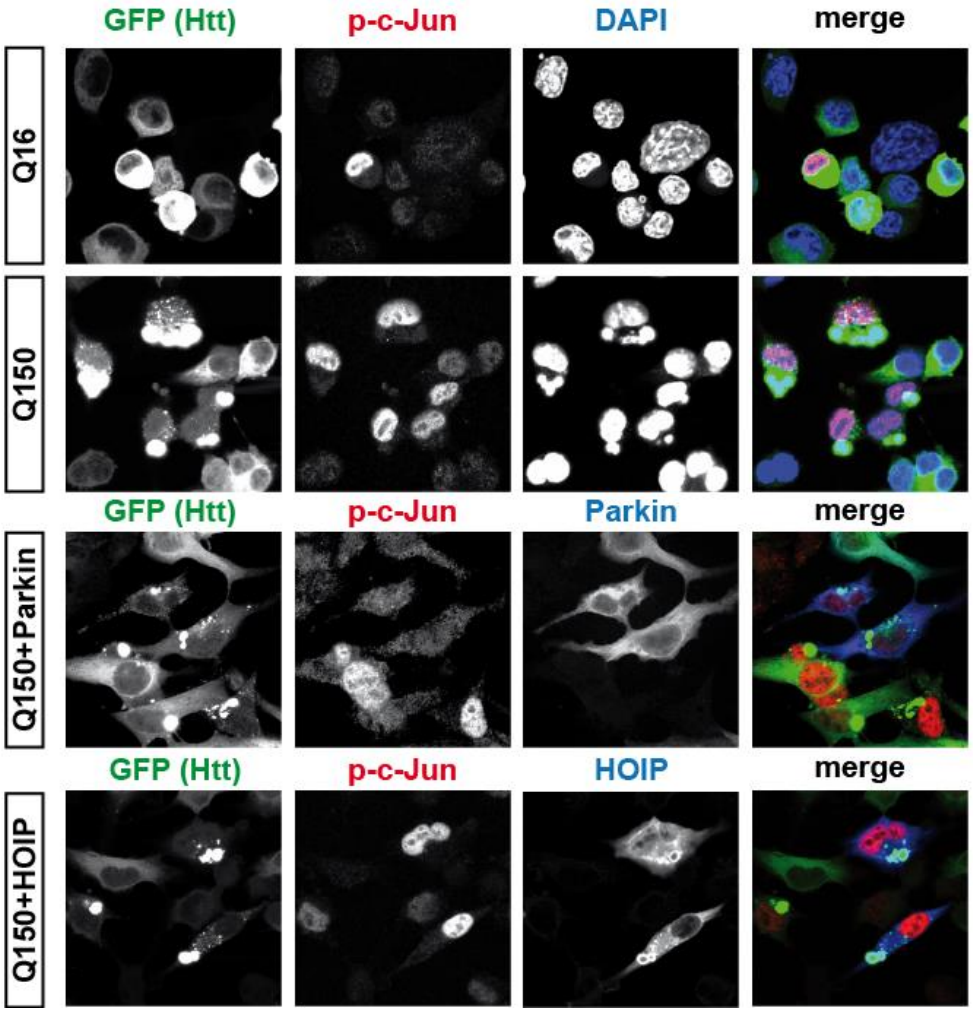


Fig. 40: Phosphorylation of c-Jun is increased in hippocampal neurons expressing Q96 aggregates. Upper panel: Overexpression of Htt-Q96 results in increased phosphorylation of c-Jun, while co-expression of Parkin or HOIP decreases the number of phospho-c-Jun positive cells. Cells displaying strong nuclear p-cJun staining were classified as phosphorylation-positive, while cells with no or weak staining in the nucleus were classified as phosphorylation-negative. Lower panel: Quantifications are based on three independent experiments performed in triplicates ($n \geq 100$). Data represent the mean ± SEM. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

3.5.2 Parkin reduces increased phosphorylation of c-Jun in N2a cells with polyQ aggregates

When the analog experiment was performed in inducible N2a cells (Fig. 41), similar numbers were obtained for cells showing phosphorylated c-Jun in control Htt-Q16 cells (17 %) versus mutant Htt-Q150 cells (72 %).



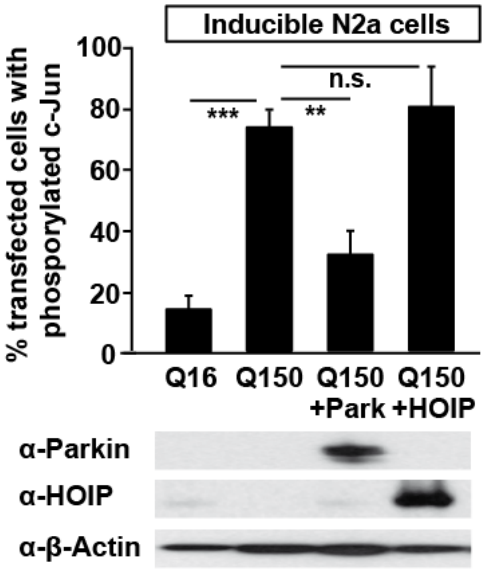


Fig. 41: In induced N2a cells with Q150 aggregates phosphorylation of c-Jun is increased. Overexpression of Parkin decreases the number of phospho-c-Jun positive cells. Quantifications are based on three independent experiments performed in triplicates (n ≥ 300). Data represent the mean ± SEM. *p ≤0.05; **p ≤0.01; ***p ≤0.001.

It should be noted that overexpression of Parkin or HOIP in these induced cells had to be verified by additional immunofluorescence staining of the transfected cells and visualizing them in a third channel (Fig. 41, lower panels, blue). Unexpectedly, only overexpression of Parkin could lower the level of c-Jun-phosphorylation. The failure of the HOIP rescue in this case might be explained by the quantitative sequestration of most HOIP molecules through the aggregates at this expression level. As seen in the immunofluorescence images, overexpressed Parkin shows mostly equal distribution within the cytosol, while HOIP seems to be enriched around the aggregates. Overall, the DNA amount used for transfection is supposed to determine if there is still free Parkin or HOIP available, which is not sequestered by mutant Huntingtin and can still activate NF-κB and block JNK signaling.

3.5.3 JNK and caspase signaling are elevated in N2a cells expressing polyQ aggregates

In addition to indirect immunofluorescence analysis of phosphorylated c-Jun, we examined expression levels of different markers for JNK and caspase activation by western blotting to prove the increased apoptotic signaling in cells overexpressing Htt aggregates (Fig. 42).

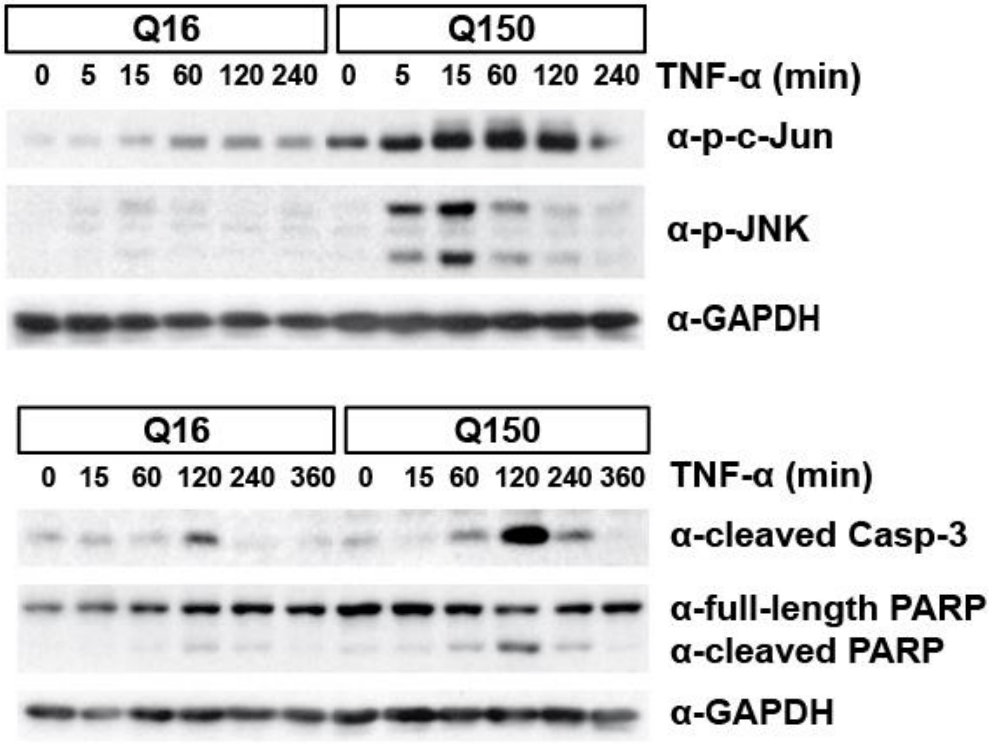


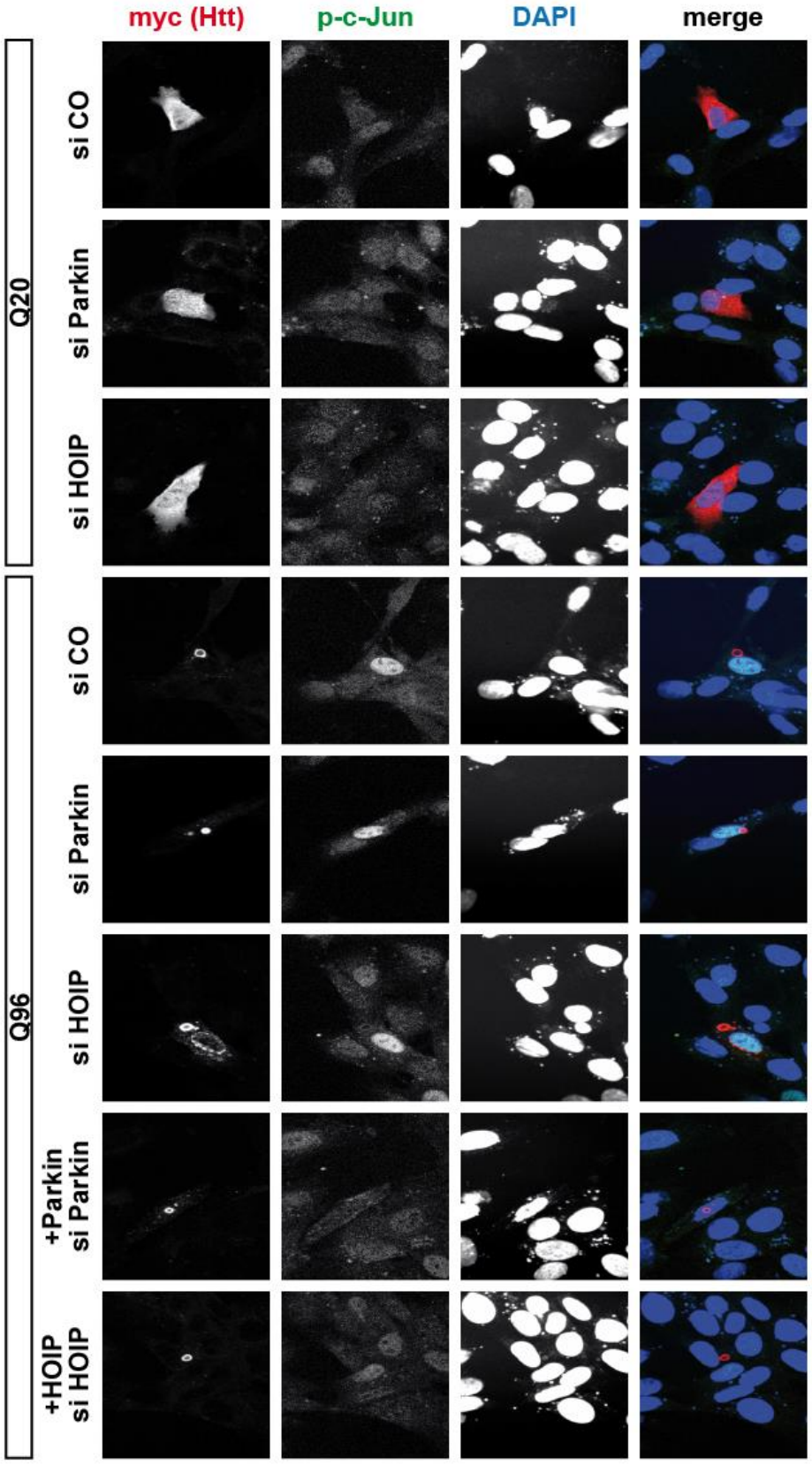
Fig. 42: Induced Q150-N2a cells show elevated levels of JNK and caspase activation upon TNF-α treatment. For JNK activation, phosphorylated c-Jun and phosphorylated JNK were analyzed; for caspase activation, cleaved Caspase-3 and cleaved PARP were investigated.

After the phosporylation of JNK, p-JNK translocates to the nucleus and phosporylates c-Jun which then serves as transcription factor. Both, p-JNK and p-c-Jun were found to be increased in Q150-N2a cells upon TNF-α treatment. Besides this, elevated caspase activation in cells expressing mutant Huntingtin could be demonstrated by increased levels of cleaved Caspase-3 and cleaved PARP.

3.6 Toxicity of polyQ aggregates is amplified in Parkin- or HOIP-deficient cells

As overexpression of Parkin or HOIP was able to reverse the impairment induced by polyQ aggregates on NF-κB signaling (e.g. nuclear translocation of p65), we wanted to test if silencing of Parkin or HOIP in cells expressing mutant Huntingtin would lead to a further increase in polyQ toxicity. SH-SY5Y cells expressing Htt-Q96 showed a marked increase in phosphorylation of c-Jun, which was used as an indicator for JNK signaling, and additional silencing of Parkin or HOIP expression in these cells by RNA interference led to an additional increase in c-Jun phosphorylation (Fig. 43). When Parkin or HOIP was reintroduced in the silenced cells, the level of phosphorylated c-Jun could be significantly lowered.

In conclusion, these data confirm that both endogenous Parkin as well as HOIP reduce polyQ-mediated toxicity, while silencing of these two E3 ubiquitin ligases leads to intensified JNK signaling in cells with Huntingtin aggregates.



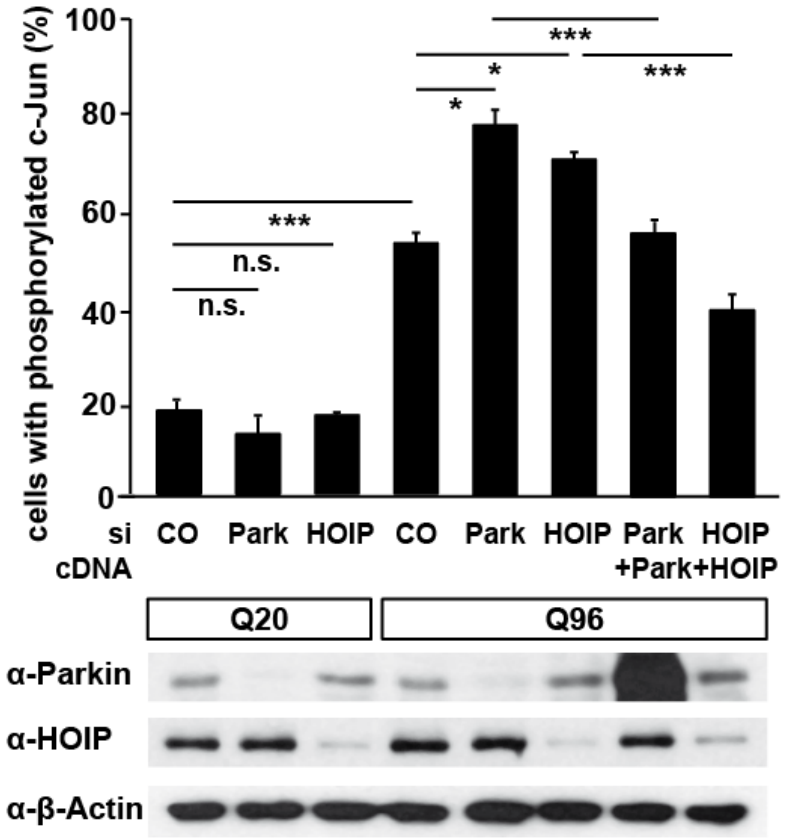


Fig. 43: Phosphorylation of c-Jun is increased in SH-SY5Y cells expressing Htt-Q96 aggregates. Moreover, knockdown of Parkin or HOIP leads to an additional increase in the number of phospho-c-Jun positive cells, whereas reintroduction of Parkin or HOIP decreases the number of phospho-c-Jun positive cells. 24 h after knockdown, cells were transfected with the indicated constructs and fixed 72 h after transfection. Data represent the mean ± SEM of at least three independent experiments, each performed in triplicate. n ≥ 300 transfected cells were assessed per condition. *p ≤0.05; **p ≤0.01; ***p ≤0.001.

3.7 Sequestration of LUBAC components in brain sections from a HD patient

3.7.1 Huntingtin antibody 1HU-4C8 detects cytosolic Huntingtin aggregates

To validate if our data from immortalized and primary cells pointing to a sequestration of LUBAC components by Htt aggregates, can also be seen in human patient brains, we collaborated with the two neuropathologists Dr. Thomas Arzberger and Dr. Kohji Mori.

First, we had to establish the staining of Huntingtin aggregates in brain sections. In the beginning, the well-established antibody mEM48 was used for detection. However, as this antibody stains only nuclear aggregates (Fig. 44) and our focus lies on cytosolic ones, another antibody had to be selected. Surprisingly, the main focus of HD research is concentrated on nuclear aggregates although wild-type Htt is known to be localized in the cytosol and it is rarely studied how wild-type cytosolic Htt can develop into a nuclear aggregate.

After intensive search in the long list of available Huntingtin antibodies, 1HU-4C8 was identified as a potential candidate for staining of cytosolic aggregates in paraffin-embedded sections, as this antibody was raised against an immunogen containing a bigger C-terminal part than the mEM48 antibody (Table 2).

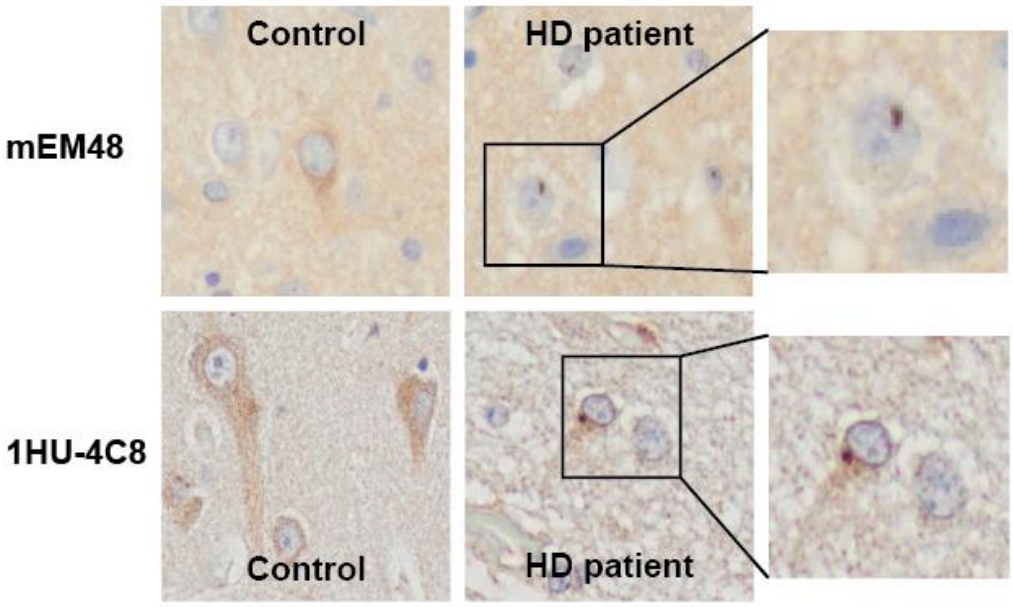


Fig. 44: DAB (Diaminobenzidine) staining of Huntingtin aggregates in control (left) and HD patient brains (right) with the antibodies mEM48 (above) and 1HU-4C8 (below). While the mEM48 antibody detects mostly nuclear aggregates, the 1HU-4C8 antibody shows mainly cytosolic aggregates on HD patient tissue. Both antibodies show cytoplasmic staining in the control tissue.

mEM48	GST fusion protein from the **first 256 amino acids** from human Htt with the deletion of the polyglutamine tract
1HU-4C8	Htt fragment from **amino acids 181 to 810** as a fusion protein

Table 2: Immunogen species of Huntingtin antibodies mEM48 and 1HU-4C8. (www.emdmillipore.com)

DAB staining with 1HU-4C8 showed normal cytosolic distribution of wild-type Htt in control cases and indeed enabled us to detect cytosolic aggregates in HD patient brain tissue (Fig. 44). These rare cytosolic aggregates found in HD brain were mostly located in close proximity to the nucleus and an additional thin ring structure could sometimes be observed around the nucleus. Interestingly, we detected cytosolic aggregates with this antibody only in brain sections from patient 1, while staining of patient 2 brain did not result in any positive signal for cytosolic Huntingtin aggregates. As patient 1 was characterized with pronounced atrophy of the *Nucleus caudatus* (late stage) and patient 2 showed almost no atrophy of the

Nucleus caudatus (early stage), this was a surprising finding (Table 3). Initially, we had assumed that cytosolic aggregates might appear with higher frequency only in earlier and not advanced HD cases. This question will be adressed with further experiments on different HD cases representing different stages of the disease and different brain regions including the striatum.

HD patient 1	male, 57 years, pronounced atrophy of the *Nucleus caudatus* (late stage)
HD patient 2	male, 74 years, little atrophy of the *Nucleus caudatus* (early stage)

Table 3: Clinical data of HD patients 1 and 2.

3.7.2 Cytosolic Huntingtin aggregates co-localize with LUBAC element HOIP

Next, another important question was addressed: Do cytosolic aggregates in HD patient brains co-localize with LUBAC components? For answering this crucial question, immuno-histochemistry staining with antibodies against Parkin and HOIP was established first (Fig. 45).

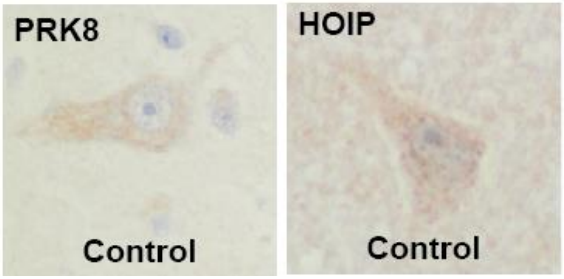


Fig. 45: DAB staining of PRK8/ Parkin (left) and HOIP (right) antibody in control brains.

Co-staining of Htt and HOIP revealing a co-localization of these two proteins supports the notion that LUBAC components are sequestered by Huntingtin aggregates in HD patient brain (Fig. 46).

Unfortunately, a convincing co-staining of Parkin (PRK8) and the 1HU-4C8 could not be performed as the antibodies 1HU-4C8 and PRK8 are derived from the same species (mouse) and therefore did not yield a suitable signal in co-staining experiments.

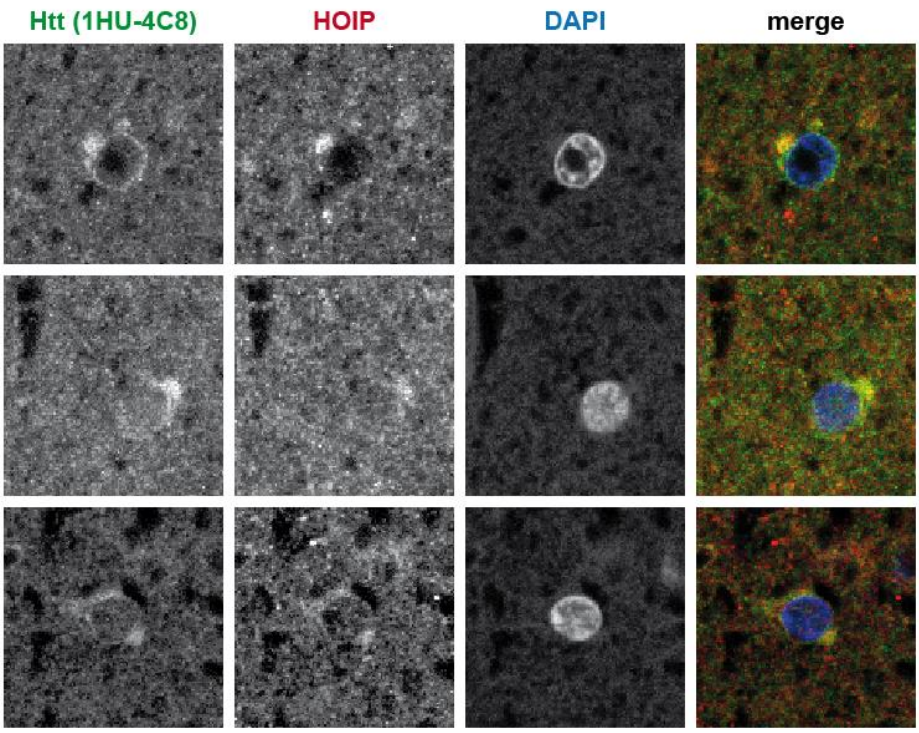


Fig. 46: Immunofluorescence staining of Huntingtin aggregates and HOIP in HD patient brain indicates co-localization.

Nuclear Htt aggregates are reported to be positive for the ubiquitin-binding scaffold protein p62, which colocalizes with ubiquitinated protein aggregates. Therefore, we also tested if the cytosolic aggregates we detected with the 1HU-4C8 antibody, co-localize with this degradation marker. Indeed, we found that the cytosolic Htt aggregates observed in HD patient brain were positive for p62 (Fig. 47).

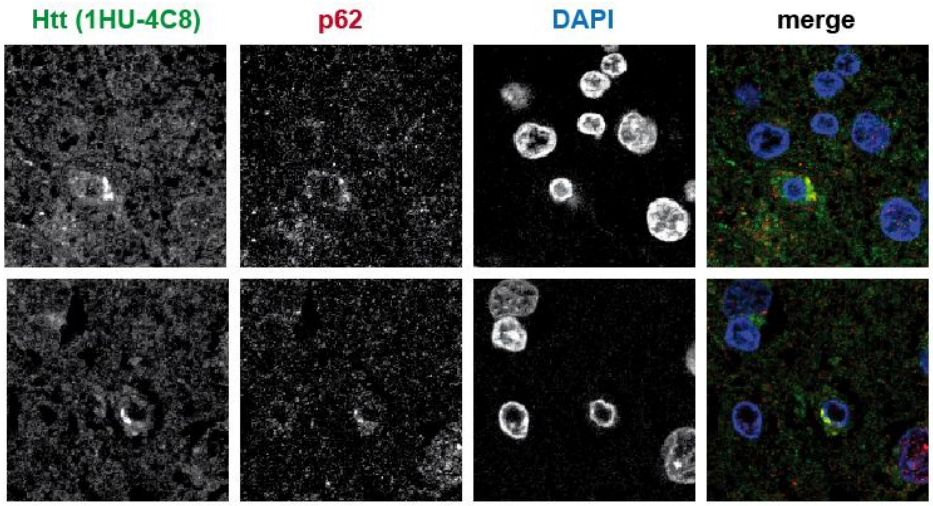


Fig. 47: Immunofluorescence staining of Huntingtin aggregates and p62 in HD patient brain shows co-localization.

To summarize, the obtained results give insight into the impact of mutant Htt on the NF-κB signaling pathway. Cytosolic polyQ aggregates sequester and trap several LUBAC components involved in the activation of the IKK complex. Without activation of the IKK complex, phosphorylation and degradation of the inhibitor protein IκB-α are blocked. Further downstream, nuclear translocation of p65 and activation of gene transcription are also lowered leading to defective OPA1 upregulation and increased mitochondrial fragmentation. TNF-α stimulation causes either a pro-survival response via NF-κB or a pro-apoptotic response via long-lasting JNK signaling and the caspase cascade. As the cell-protective pathway is blocked by the Huntingtin aggregates, destructive JNK and caspase signaling are activated instead (Fig. 48). Importantly, overexpression of wild-type E3 ubiquitin ligases Parkin and HOIP can at least partially restore impaired NF-κB signaling and protect cells from programmed cell death, as demonstrated by several approaches. This switch from NF-κB-mediated survival to apoptosis signaling might be of special relevance to neurons and thereby explain the increased vulnerability of this cell population to an impairment in NF-κB signaling by polyQ aggregates.

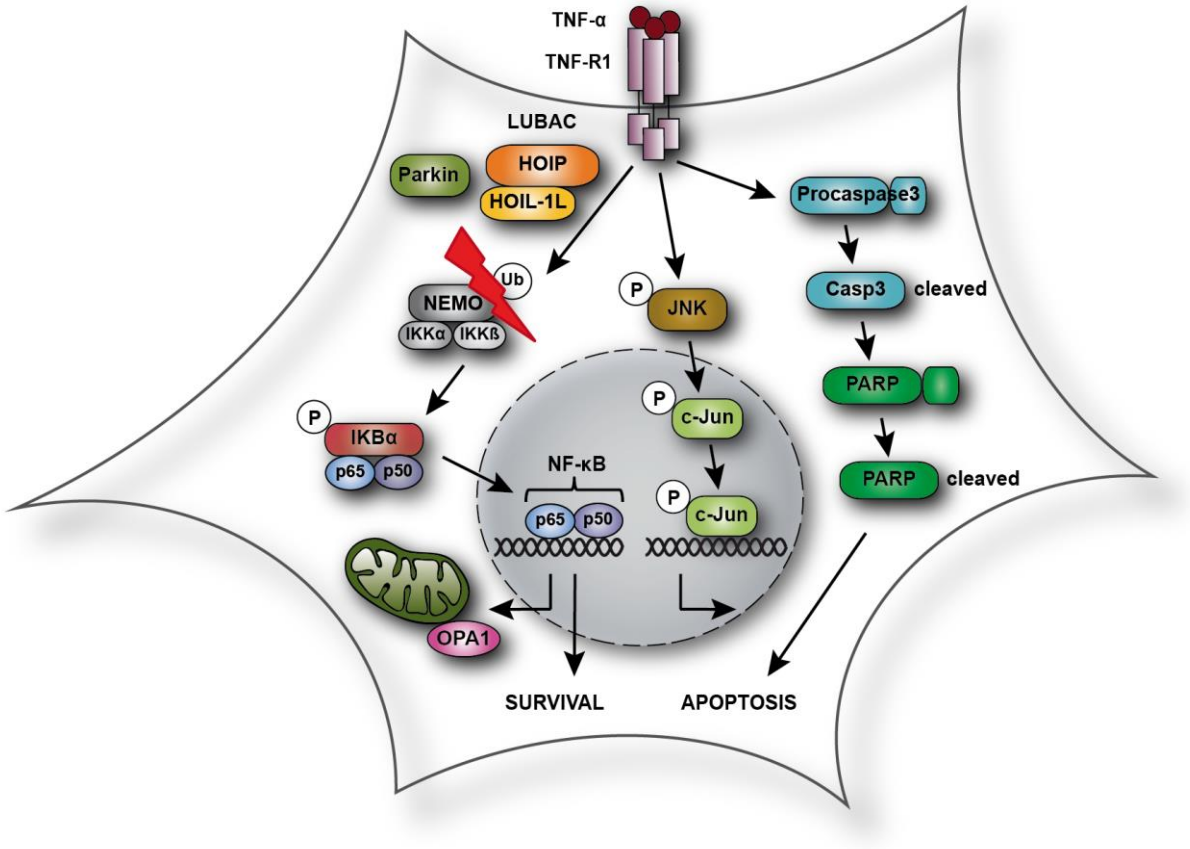


Fig. 48: When the pro-survival NF-κB pathway is blocked by sequestration of LUBAC components through Huntingtin aggregates, JNK and complex IIa-mediated apoptotic signaling are activated instead and promote programmed cell death [modified from graphical abstract, Müller-Rischart 2013].

4. Discussion

4.1 Role of LUBAC in prevention of programmed cell death

Expansion of the polyQ stretch in the Huntingtin gene leads to severe impairments in mitochondrial integrity and neuronal survival. In this work, an interplay of cytosolic Huntingtin aggregates with components of the LUBAC was identified resulting in a switch from pro-survival NF-κB signaling to pro-apoptotic JNK and caspase signaling within a cell. Previously, the E3 ubiquitin ligase Parkin was shown to increase LUBAC activation, whereas HOIP was found to be the catalytic subunit of the LUBAC [Müller-Rischart 2013, Smit 2012]. Therefore, we addressed the question, if Parkin and HOIP can rescue from defective NF-κB signaling in cells expressing Huntingtin aggregates. Our experiments demonstrate that overexpression of either Parkin or HOIP was able to alleviate the impact of mutant Huntingtin not only on NF-κB activation measured by p65 translocation or luciferase reporter assays but also on mitochondrial morphology and on JNK activation. But it is still unclear, how this rescue effect of Parkin or HOIP on impaired NF-κB signaling caused by Huntingtin aggregates can be explained on a mechanistic level. One possible explanation would be that these components of the LUBAC might be able to revert aggregate formation and could be involved in dissolving of polyQ aggregates. However, we could not confirm this hypothesis by any experimental evidence, as the number and size of Htt aggregates was not altered by co-expression of Parkin or HOIP - at least not at the time points and in the cell types we looked at.

The more plausible explanation is that overexpression of these mediators of the NF-κB pathway restores cytosolic protein levels, which can perpetuate the signaling and lead to a prosurvival response. Most of the endogenously available Parkin and HOIP is probably sequestered by mutant Huntingtin aggregates and therefore not sufficient unbound protein is available anymore for downstream signaling to activate NF-κB and protect the cell from apoptosis. This idea is supported by the observation that experiments with low levels of overexpressed HOIP, for instance, show mainly a ring-like staining around the aggregates and cytosolic depletion for HOIP when co-expressed with Htt-Q150. Expression of higher amounts of HOIP results in a mostly equal cytosolic distribution of the LUBAC component and only a slightly increased staining intensity around the aggregates compared to the cytosol.

During the last few years, the LUBAC and its different components have increasingly gained importance, as their contribution to the NF-κB pathway was shown to be crucial. Despite the

remaining uncertainty regarding the exact complex composition and the detailed role of Parkin or Sharpin, it is beyond all question that this complex plays a major part in cell protection. Therefore, its functional efficiency and capability are essential for inhibition of programmed cell death. Very recently, three publications focusing on HOIP and Sharpin reported that HOIP- or Sharpin-deficient cells show increased TNF-R1 mediated cell death. Furthermore, it was demonstrated that Sharpin can prevent TNF-R1 mediated cell death [Kumari 2014, Peltzer 2014, Rickard 2014]. Taken together, these findings underline the significance of the LUBAC in the context of complex I mediated survival signals versus complex II mediated death signals, as the LUBAC maintains complex I mediated signaling to prevent switching to the cell death program via complex IIa.

4.2 Huntingtin aggregates

Huntingtin aggregates were found to be present in brains of R6/2 mice as well as human HD brains and, in addition, showed positive for ubiquitin staining [Davies 1997, DiFiglia 1997]. Both, the length of the polyQ stretch and the length and amount of Huntingtin fragments determine the aggregation process of mutant Huntingtin. It was demonstrated by biochemical analyses that nuclear and cytosolic aggregates are made of different components: While nuclear aggregates consist mainly of N-terminal Huntingtin fragments, extranuclear inclusions are composed of full-length and fragmented Huntingtin. A model termed the toxic fragment hypothesis, argues that toxic Huntingtin fragments are liberated by proteolytic cleavage. These fragments contain the expanded polyQ stretch and their accumulation might promote activation of caspases and programmed cell death. Experiments on cleavage processes and existence of N-terminal Huntingtin fragments evidenced that both normal and mutant Huntingtin undergo cleavages. However, it was shown that mutant Huntingtin is more susceptible to proteolysis [Zuccato 2010].

It is still an issue of intensive debate, which of the species is the most toxic form within these aggregation pathways. Are small monomers or oligomers more toxic than large fibers or aggregates themselves or is it the other way around? And, is toxicity of the aggregates dependent on the cellular compartment? Previously, it was assumed that nuclear Huntingtin aggregates are particularly toxic and that they sequester and block transcription factors and regulators in the nucleus [Yang 2002]. However, current views favor the hypothesis that Huntingtin inclusions are a protective mechanism of cells by sequestering toxic factors [Bennett 2007]. Preliminary data from our group on the translocation of p65 in cells

expressing mutant Htt showed that impaired NF-κB activation was already seen at time points, when Htt was not yet in an aggregated state. However, the impairment was not as pronounced as in cells where the aggregates had already formed. These findings point to toxic features of both soluble and insoluble cytosolic mutant Huntingtin species, which could be prevented by inclusion of these species together with their removal and transport into the nucleus. In this work, we focused on cytosolic aggregates as wild-type Huntingtin is known to be localized in the cytoplasm and our aim was to investigate early cellular changes occurring due to aggregate formation. At present, mainly nuclear Huntingtin aggregates are examined, because of their numerous appearance in *post mortem* HD brains. Although it is a great challenge to find less abundant cytosolic aggregates in advanced stages of the disease, it will be essential to tackle this problem for a better understanding of HD. In the future, it would therefore be important to concentrate also on early cytosolic aggregation steps within Huntingtin research and clarify the identity of the pathogenic species as well as the mechanism of how cytosolic soluble Huntingtin can turn into a nuclear insoluble aggregate.

4.3 Selectivity of neurodegeneration

Our observations demonstrate that the presence of mutant Huntingtin results in a clear switch from prosurvival signaling via NF-κB to proapoptotic cellular signaling, which explains the caspase-induced death of affected cells. However, this finding of increased cell death upon exposure to polyQ aggregates cannot explain why a selective degeneration of a particular neuronal population occurs. It is known that affected brain regions in Alzheimer's Disease are involved in memory, while affected brain regions in PD and HD are responsible for motor function. Furthermore, degeneration in PD is mainly seen in dopaminergic neurons of the *substantia nigra*, whereas degeneration in HD hits mostly striatal GABAergic neurons.

Several possibilities are discussed how specific neuronal loss could be explained. Proteostasis is one of the major factors suggested to play a role in this context. As neurons are postmitotic cells, they require consistent maintenance of their protein quality and rely on a functional proteostasis machinery [Margulis and Finkbeiner 2014, Saxena and Caroni 2011]. Formerly, Huntingtin aggregates were thought to block the proteasome in HD [Bence 2001]. This was emended by findings implying that mutant Huntingtin indirectly affects the proteasome by impairment of the chaperone system leading to increased load of misfolded proteins that finally overburden the ubiquitin proteasome system (UPS) [Hipp 2012]. Lower activity of the UPS in the striatum was recently reported, suggesting the necessity of functional capability of

the striatal UPS [Tsvetkov 2013]. Another argument supporting this hypothesis is based on the regional selectivity of inclusion body (IB) formation. IBs are only detected in particular brain regions although mutant Huntingtin is ubiquitously expressed [Margulis and Finkbeiner 2014].

The activation status of NF-κB might be another feasible explanation why neurons are especially vulnerable to the presence of mutant Huntingtin in comparison to non-neuronal cells: In non-neuronal cells, NF-κB is mostly kept in an inactive state, while being constitutively activated by basal glutamatergic transmission in neuronal cells [Meffert 2003]. This permanent activation of the NF-κB pathway, which is blocked by Huntingtin aggregates according to our results, would followingly be converted into permanent apoptotic signaling finally leading to programmed cell death.

In 2009, the protein Rhes (Ras homolog enriched in striatum), which is selectively enriched in the striatum, was identified as an interaction partner of mutant Huntingtin. It was reported that Rhes induces sumoylation of mutant Huntingtin and thereby causing cytotoxicity. For this reason, Rhes and its interaction with Huntingtin was used as further possible explanation for the specific striatal neurodegeneration seen in HD [Subramaniam 2009]. Though, *in vivo* data confirming this interaction are still missing.

Despite all attempts of explanation, the selective vulnerability of specific brain regions remains one of the most intruiging mysteries in neurodegenerative disease research and a better understanding of the underlying mechanisms is necessary for the development of targeted therapeutic interventions [Jackson 2014].

4.4 Cellular defects caused by mutant Huntingtin

Apparently, the cellular effects of mutant Huntingtin are complex and comprise impaired gene transcription, cytoplasmic sequestration of important factors, insufficient protein folding, impairment of the UPS as well as increased cell death and mitochondrial dysfunction (Fig. 49). Mutant Huntingtin could either inhibit transcription factors by aggregate formation in the nucleus or sequester transcription factors in the cytoplasm and block their translocation into the nucleus. For instance, CBP (CREB binding protein) and TBP (TATA box binding protein) were detected in intranuclear polyQ aggregates. Furthermore, chaperones, ubiquitin and proteasome subunits accumulate in Huntingtin aggregates, which points to an involvement of the UPS machinery [Landles and Bates 2004, Costa and Scorrano 2012].

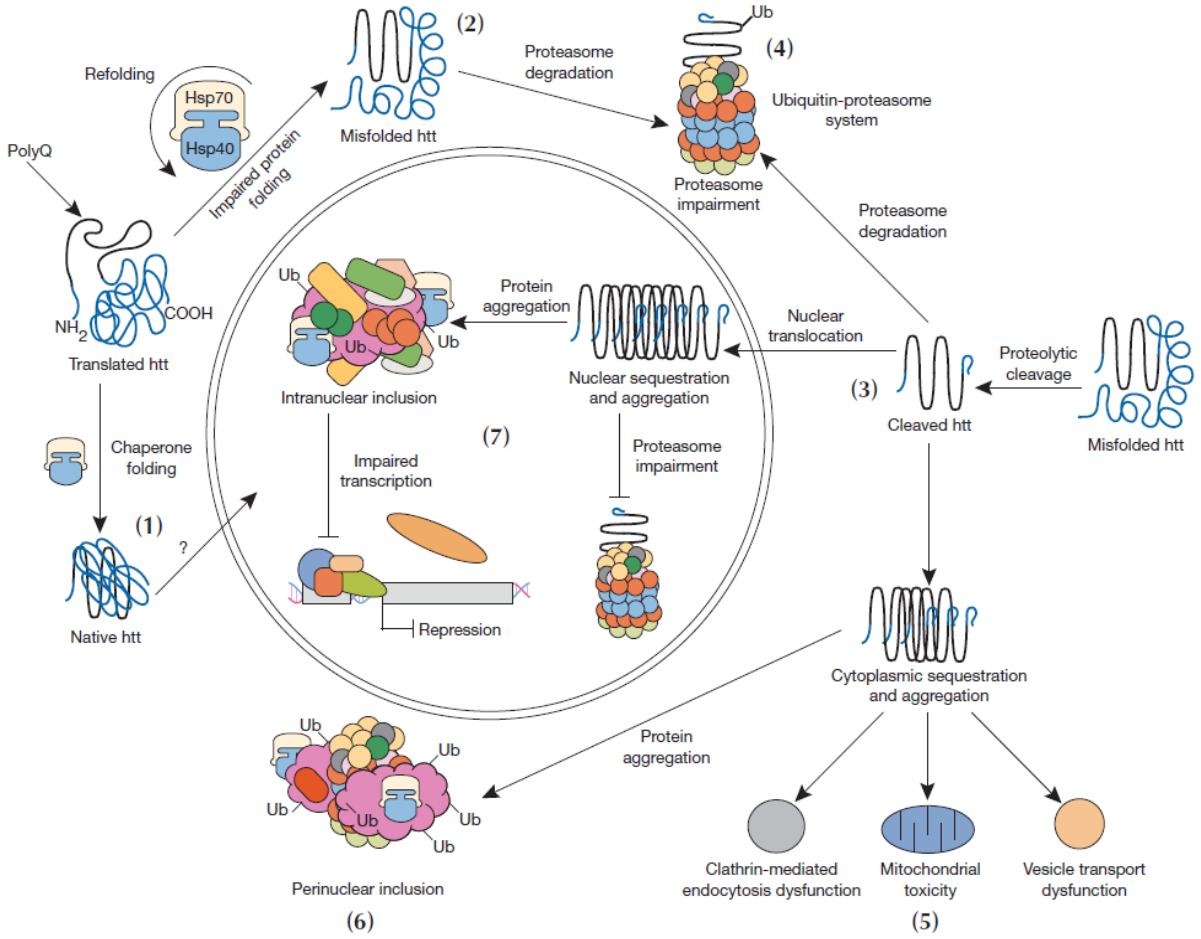


Fig. 49: Model for cellular pathogenesis in HD. (1, 2, 4) An HD mutation causes abnormal folding of Huntingtin, which should be corrected by chaperones or cleared by proteasomal degradation. (3) Alternatively, mutant Huntingtin might also undergo proteolytic cleavage producing N-terminal fragments. In the cytoplasm, mutant Huntingtin might impair the proteasomal function directly or indirectly, which leads to accumulation of misfolded proteins. (5) Aggregation and sequestration of other proteins in the cytoplasm could lead to mitochondrial damage. (6) To protect itself, the cell presumably incorporates toxic proteins into cytoplasmic perinuclear aggregates. (7) An additional protection mechanism is assumed to comprehend the translocation of mutant Huntingtin into the nucleus, where nuclear inclusions may block gene transcription. [Landles and Bates 2004]

Our data especially emphasize both mitochondrial fragmentation and dysregulation as well as increased cell death signaling caused by the presence of Htt aggregates. Decreased p65 translocation, reduced OPA1 protein levels and increased levels of JNK signaling (phosphorylated c-Jun and JNK) and the caspase pathway (Caspase-3 and PARP) in cells containing polyQ aggregates are clear indicators for a switch from NF-κB-mediated signaling to complex IIa-induced apoptosis. How the other observed cellular defects such as hindered protein degradation by the proteasome can be integrated into the dysregulation of these pathways, has to be adressed in future projects. In addition, it should be investigated and distinguished what the primary and what the secondary effects of aggregated Huntingtin are.

4.5 Balanced crosstalk of pathways

In general, an essential function of a cell is the decision about living or dying. Therefore, the underlying signaling pathways have to be well-regulated and adjusted to the respective circumstances. While the prosurvival pathway via the transcription factor NF-κB should predominantly be active in cells, the proapoptotic pathways including JNK and caspase signaling should be repressed to ensure a proper immune response to inflammatory stimuli. Only when cells are irreparably damaged, the program should be switched from the pro-survival to the pro-apoptotic channel through suppression of canonical NF-κB signal transduction and activation of the JNK and the caspase cascade.

As described before, mutant Htt was found to influence the NF-κB pathway directly by sequestration of LUBAC components and thereby to affect the JNK and the caspase pathways indirectly, as suppression of NF-κB signaling is accompanied by elevated cell death signaling. Our findings focusing on Huntingtin and HD, taken together with earlier reports on other diseases such as liver diseases indicate that the balance between activation and repression of the investigated pathways as well as the functional crosstalk between these pathways is crucial for the cellular fate.

Few mediators and mechanisms of the crosstalk could be discovered so far: On the one hand, NF-κB activation blunts JNK activation by inducing JNK inhibitory proteins such as XIAP and controling the levels of reactive oxygen species (ROS). [Papa 2009, Wullaert 2006]. On the other hand, Siva was found to interact with XIAP thereby shifting the balance from NF-κB-mediated prosurvival signaling to proapoptotic JNK and Caspase-3 signaling [Resch 2009]. These mediators between the pathways might be interesting targets for therapeutic interventions.

One of the main future challenges will be the identification of further balance regulating mechanisms within this pathway network. In particular, targeting and tackling of these regulators to overcome deficient signaling caused by multiple factors including protein aggregates like in HD will be important. In the end, the interplay of NF-κB, JNK and caspase signaling determines the cellular response to TNF-α stimulation (Fig. 50).

The most fascinating current question is whether the imbalanced pathway network observed in HD models might also be involved in other neurodegenerative diseases and serve as a general concept to explain the mitochondrial alterations as well as effects on viability seen in most neurodegenerative diseases. For Parkin and PD, the link to the LUBAC and NF-κB signaling was already demonstrated. This indicates the importance of common pathways for

the development of at least HD and PD and tempts one to speculate that other cytosolic aggregates occurring in different diseases could also sequester and trap components of the NF-κB pathway such as the LUBAC. Thereby, the increased vulnerability of neurons under stress conditions might be explained, as the aggregates induce a switch from an anti- to a pro-apoptotic cellular response.

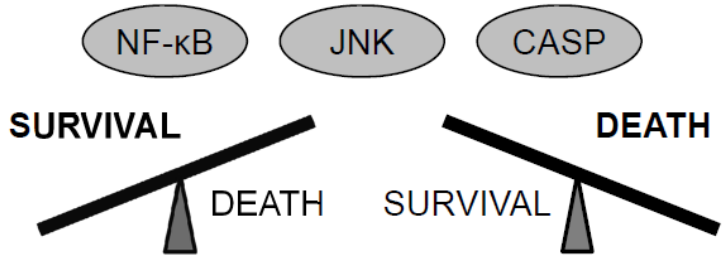


Fig. 50: Sensitive balance between pro-survival and pro-apoptotic signaling. Activation of the NF-κB pathway and suppression of JNK and caspase signaling lead to a pro-survival outcome, while inhibition of NF-κB signaling connected with increased activation of the JNK and caspase pathway promotes programmed cell death [modified from Resch 2009].

5. Methods

5.1 Cell culture

5.1.1 Cultivation

Both immortal as well as primary cell lines were cultivated as adherent monolayers in either 25 cm^2 or 75 cm^2 tissue culture flasks at 37°C and 5% CO_2. Every 3 – 4 days, the cells were splitted by washing once with PBS (Phosphate Buffered Saline), trypsination at 37°C, inhibition of trypsin by fresh FCS-containing medium and transfer of a cell aliquot into a new flask with fresh medium for further cultivation. All cell lines, the appropriate media and splitting ratios are listed in the following table (table 3).

Cell line	type	medium	splitting ratio
SH-SY5Y	Human neuroblastoma	DMEM F-12 + 15% FCS + 1% NEAA (+1% PS)	1:6 – 1:12
HEK 293T	Human embryonic kidney	DMEM GlutaMax + 10% FCS (+1% PS)	1:10 – 1:20
N2a Htt-Q16/ N2a Htt-Q150	Murine neuroblastoma	DMEM + 10% FCS + 2 mM L-Glutamine + 0.1 mg/ml G418 + 0.1 mg/ml Zeocin (+1% PS)	1:5 – 1:20

Table 4: Cell lines and cultivation details.

5.1.2 Plating

For transfections or treatments, cells were counted and plated in the desired confluency in different cell culture dish types (table 4).

Cell line	experiment	dish	density
SH-SY5Y	WB	6-well plate	3-5 x 10^5
	IF	6-well plate	1-1,75 x 10^5
HEK 293T	WB	6-well plate	1 x 10^6
HEK 293T	LUC	12-well plate	4 x 10^5
HEK 293T	Filter trap	12-well plate	1,5 x 10^5
HEK 293T	Lin Ubi assay	10 cm culture dish	2 x 10^6
N2a	IF	12-well plate	1 x 10^5
N2a	WB	6-well	5 x 10^5

Table 5: Plating details of cell lines for different experiments. WB = Western Blot, IF = immunofluorescence, LUC = Luciferase assay, Lin Ubi Assay = Linear ubiquitination assay.

5.1.3 Preparation and cultivation of mouse or rat primary neurons

Cortices of mouse embryos (E14 - 15) were isolated and treated with Papain for 15 – 20 minutes (min) at 37°C to digest the tissue. After removing the Papain solution, cells were dissociated by mild trituration with a 2 ml pipette and plated onto Poly-L-Lysine-coated dishes. For immunofluorescene experiments, 4 x 10^5 cells were seeded on coverslips in 12-well-plates (DMEM + 10% FCS + 1% PS). The medium was switched to neuronal medium (Neurobasal + 5 mM HEPES + 0.5 mM L-Glutamine + 2% B27 supplement + 1% PS) after 3 to 4 hours. One week after preparation, fresh medium was added to the cultured neuronal cells.

Further rat cortical and hippocampal neurons were kindly provided by Dieter Edbauer's group. These neurons were prepared at stage E19 and cultivated in Neuronal Basal Medium supplemented with SM1 Neuronal Supplement, L-Glutamine and PS.

5.1.4 Transient transfection

First, transient transfection for overexpression of genes was mainly performed 24 h after plating or 24 to 48 hours after a knockdown. DNA (0.2 - 1 µg per 6-well) was mixed with Plus reagent in Opti-MEM, while Lipofectamine reagent was added to Opti-MEM in a separate tube (for details see table 5). The separate mixes were incubated for 15 min at room temperature (RT), merged and incubated for another 15 min at RT. Before the transfection mix was dripped onto the cells, the medium was switched to Opti-MEM. After 3 to 5 hours of incubation, normal growth medium was added. Cells were harvested 24 to 72 hours after transfection for analysis.

Cell line	ratio Plus: Lipo
SH-SY5Y	8:6
HEK 293T, N2a	6:8

Table 6: Ratio of transfection reagents for different cell lines. Specifications are valid for cells plated in 6-well-format. Lipo = Lipofectamine.

Second, transfection of small interfering RNA (siRNA) was used to silence the expression of specific genes. These transfections were performed as reverse transfections together with the plating of the cells. For this purpose, siRNA for the specific gene was incubated together with 5 µl of the transfection reagent RNAimax and Opti-MEM in 6-well plates for15 min at RT

(for details see table 6). A negative control duplex siRNA with medium GC-content served as control. In the meantime, cells were trypsinated and counted, before they were plated in an appropriate density in the 6-well plates containing the siRNA transfection mix. After 5 to 24 hours, normal growth medium was added to the cells.

Gene	volume	siRNA
Parkin	5 µl (stock solution 20 µM)	si1 or si2
HOIP	6 ul (stock solutions 20 µM)	si1+2+3 (1:1:1)

Table 7: Optimized siRNA composition for specific genes. Specifications are valid for SY-SY5Y and HEK 293T cells.

Third, transfection of neurons was mostly performed on day 12 post preparation and the cells were harvested on day 3 or 4 post transfection. Neuronal experiments were mainly conducted in 12-well plates with neurons seeded on glass coverslips for immunofluorescence analyses. For the transfection, 2 separate mixes (1.8 µg DNA in Neurobasal and 3.3 µl Lipofectamine 2000 in Neurobasal per 12-well) were made separately, mixed and incubated at RT for 30 min. Meanwhile, a new 12-well plate was prepared with incubation medium (Neurobasal + PS + Glutamine), the neurons on coverslips were washed with warm Neurobasal medium and transfered to the new 12-well plate for transfection. The old plate was kept warm in the incubator. Then, transfection followed by dripping the transfection mix onto the cells and incubation at 37°C for 45 min. Afterwards, the coverslips were washed twice with Neurobasal medium and transfered back into the original plate, where they stayed for 72 to 96 hours until their harvest.

5.1.5 Lentiviral transduction

N2a cells were transduced with lentiviruses for stable overexpression of either wild-type Parkin or a control vector as lentiviral transduction allows higher transfection efficiencies compared to transient transfection of plasmid DNA with Lipofectamine and Plus. Generation of the lentiviral expression vector Fu-ΔZeo-ParkinWT and purification of the lentiviruses was kindly performed by Anna Pilsl [Müller-Rischart 2013].

5.1.6 Induction of stable N2a cell lines

The ecdysone-inducible mammalian expression system from Invitrogen was used for stable expression of a Huntingtin fragment (90 amino acids from exon1 of a HD patient) combined

with different polyQ lengths and the reporter protein GFP (green fluorescent protein). We tested the N2a cell lines expressing a polyQ stretch of 16 or 150. Different ecdysone analogs can be used to induce this expression system, among them the herbal steroids Muristerone A and Ponasterone A. For induction, we used 1 µM Muristerone A and for differentiation of the N2a cells, 2.5 - 5 mM dbcAMP (Dibutyryl cyclic adenosine monophosphate, (N6,2'-O-dibutyryl)-adenosine-3',5'-mono-phosphate) were applied in differentiation and induction medium (DMEM + 1%FCS + 2mM L-Glutamine + 0.1 mg/ml G418 + 0.1 mg/ml Zeocin +1% PS). The cells were mainly treated for 72 to 96 hours, as sufficient aggregate production was observed at these time points in the case of N2a Htt-Q150 cells.

5.1.7 Treatment of cells

To stimulate TNF receptor-mediated signaling pathways, the cytokines TNF-α or IL-1β were utilized. TNF-α was applied at a concentration of 10 - 20 ng/ml, while IL-1β was applied at a concentration of 10 ng/ml.

5.2 Protein biochemistry

5.2.1 Cell harvest and lysate preparation

For harvesting, cells were washed twice with PBS on ice and scraped off the plates with a cell scraper. Next, they were transfered to an Eppendorf tube and centrifuged for 5 min at 5000 rpm and 4°C. The cell pellet was either stored at -20°/ -80°C or subsequently lysed.

Lysis conditions depended on the carried out experiment, but a detergent buffer containing 0.5 - 1 % Triton X-100 in PBS (+ protease inhibitor cocktail) was mainly used for cell lysis. The lysate was incubated for 5 min on ice. For some experiments such as a Parkin knockdown, the complete lysate was used and needled, while for most other experiments an additional centrifugation step of 15 min at 13000 rpm and 4°C followed to pellet the cell debris. The supernatant was then transfered to a new tube and only this soluble fraction was used for analysis.

To prepare samples for Western Blot analysis, protein concentration of the cell lysates was measured by BCA protein assay, Laemmli sample buffer (+β Mercaptoethanol) was added to the lysate and the samples were incubated for 10 min at 95°C. Then, the samples were either analyzed after the boiling step or stored at -20°C until further usage.

5.2.2 SDS PAGE

Sodium dodecylsulphate polyacrylamide gelelectrophoresis (SDS-PAGE) under denaturing conditions was used for separation of proteins according to their molecular weight. Equal amounts of protein (5 - 20 ug per sample) were loaded onto 10 - 15% separation gels depending on the size of the considered proteins. The electrophoresis gels ran on the minigel system from BIORAD at a constant current of 25 mA per gel for 90 to 120 min.

5.2.3 Western Blot

After the electrophoresis step, the separated proteins were transfered from the gel onto a PVDF (polyvinylidene difluoride) membrane by applicaton of the same minigel system from BIORAD and 400 mA for 1 hour. To block unspecific binding, the membranes were incubated in 5% milk in TBS-T at RT after the protein blotting, before the membrane was transfered to a tube containing the respective primary antibody in milk solution. Incubation at 4°C overnight or for 1 hour at RT followed. Next, three washing steps with TBS-T were performed and the second antibody, which was conjugated to the enzyme HRP (horseradish peroxidase), was added to the membrane. Finally, three washing steps and detection of the antigen via an enhanced chemoluminescence (ECL) reaction and exposure to an X-ray film was conducted.

5.2.4 Filter trap assay

To analyze protein aggregates, filter trap assays were performed. As aggregates are bigger in size and cannot pass through a membrane with a pore size of 0.2 µm, they are retained when the cell lysate is soaked through the membrane, while non-aggregated proteins can easily slip through the membrane. Afterwards, the retained aggregates can be detected via antibodies and chemoluminescence similar to the last part of Western Blotting.

For this assay, a different lysis buffer was used, which additionally contained DNase (lysis buffer: 1% Triton X-100 + 15 mM $MgCl_2$ + 0.2 mg/ml DNase in PBS). Cells were lysed and ultracentrifuged at 55 000 rpm and 4°C for 30 min. Next, the pellet was resuspended in SDS-buffer (2% SDS in100 mM Tris pH 7) and incubated for 1hour at RT. When the incubation was finished and the pellet dissolved, the slot-blot equipment containing the activated cellulose acetate membrane was assembled. The samples were diluted 1:5 in three steps, loaded in four lanes (undiluted, 1:5, 1:25, 1:125) into the slots and soaked through the membrane by application of a vacuum. After three washing steps with PBS, the membrane

was removed from the slot blot chamber and blocked with milk solution. The antibody detection worked as described in the previous chapter (see "Western Blot").

5.2.5 Linear ubiquitination assay

The linear ubiquination assay served to detect proteins, which were pulled down by recombinant Strep-tagged UBAN domain from the cell lysate and visualized with a ubiquitin antibody. As the UBAN domain, which is part of the IKK subunit NEMO, binds to linear ubiquitin chains, it can be used to pull down proteins with linear ubiquitin chains.

Transfections of large quantities for linear ubiquitination assays were performed with calcium phosphate, because this method is more cost-saving than transfection with Lipofectamine and Plus reagent. For this purpose, DNA was mixed with $CaCl_2$ and HeBs buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4) and incubated for 15 min at RT. In the following step, the transfection mix was dripped onto the cells in 10 cm dishes. After 16 h of incubation, fresh medium was added to the cells.

Upon harvest, lysis of the cells included resuspension in a denaturing buffer (1% SDS in PBS), incubation for 10 min at 95°C and needling for sufficient dissolving. Then, the lysate was diluted 1:10 with 1% Triton X-100 in PBS and centrifuged for 15 min at 4600 rpm and 4°C. The supernatant was transfered to a new tube for subsequent co-immunoprecipitation (Co-IP) and a small aliquot was taken as input control.

For the Co-IP, recombinant UBAN protein was added to the cleared lysate and the samples were incubated with mild swinging overnight at 4°C. On the following day, Strep Tactin Sepharose beads were used to bind the Strep-tag attached to the UBAN domain. After 3 to 4 hours of incubation at mild swinging and 4°C, a centrifugation step for 5 min at 1500 rpm and 4°C pelleted the beads together with the bound complexes of the UBAN domain and linear ubiquitinated proteins. Several washing steps of the beads followed. Finally, addition of Laemmli sample buffer (+β Mercaptoethanol) and an incubation step for 10 min at 95°C led to the dissociation of the bound protein complexes from the beads. Input controls and supernatants of the pulldown samples were loaded onto SDS gels and Western Blot analysis was conducted. For the detection of ubiquitinated proteins, a nitrocellulose membrane was used and boiled after the blotting step for 5 min, before the first antibody was added.

This linear ubiquitination assay was performed both with HEK cells transiently expressing polyQ aggregates as well as inducible Htt-expressing N2a cells, but all experiments revealed indefinite results regarding a possible effect of Htt aggregates on linear ubiquitination levels.

5.2.6 Luciferase reporter assay

Luciferase reporter assays are based on the expression of the reporter gene luciferase, which is combined with promoter sequences or transcription factor binding sites to be considered. In our case, several NF-κB binding sites were put in front of the luciferase gene. Upon binding of NF-κB to the respective responsive elements, transcription of the reporter gene is activated and luciferase protein is produced. The resulting protein converts the substrate luciferin into oxyluciferin accompanied with the production of light, which can be measured by a luminometer.

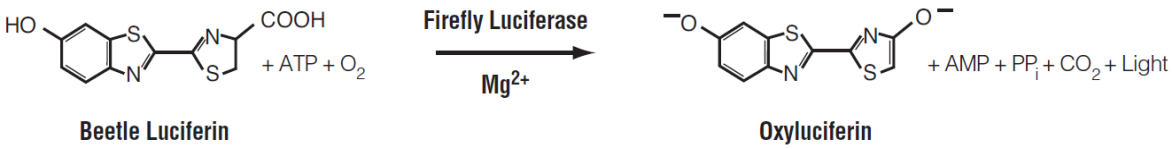


Fig. 51: Bioluminescent reaction catalyzed by firefly luciferase. [Promega]

Cells were co-transfected with the NF-κB reporter construct and other plasmids to be analyzed. Transfected cells were harvested and resuspended in reporter lysis buffer (provided by the Luciferase Assay System, Promega). After incubation for 5 min on ice and vortexing, the samples were centrifuged for 15 min at 13000 rpm and 4°C. The supernatant was used for luminometric measurements and the obtained raw values were normalized to protein concentration.

5.2.7 ATP assay

To determine the ATP content of cells, the same principle as described for luciferase reporter assays was applied: the luciferase reaction produces light, which can be quantified.

24 hours prior to the cell harvest, the culture medium was replaced with low Glucose medium (3 mM Glucose) to induce a metabolic shift from glycolysis to mitochondrial respiration for ATP production. Furthermore, control cells were treated with 10 µM CCCP three hours before the harvest. For the measurement, cells were scraped off the plates, resuspended carefully in PBS and lysed in the cell lysis buffer of the ATP Bioluminescence Assay Kit HS II (Roche). After 5 min of incubation at RT, the lysates were centrifuged for 1 min at 10000 rpm and 4°C. The supernatant was used for luminometric measurement and the obtained raw values were normalized to protein concentration.

ATP assays were performed with inducible Htt-expressing N2a cells. However, the obtained results did not yield a clear effect of Huntingtin aggregates on ATP levels.

5.2.8 Immunofluorescence of cultured cells

Immunofluorescence stainings of cultured cells can be used for different assays such as nuclear translocation of p65, assessment of mitochondrial morphology via staining of mitochondrial proteins such as TOM20, phosphorylation of c-Jun or activation of Caspase-3.
The cells were washed with PBS and fixed with 3.7% Formaldehyde in PBS for 15 min at RT. Then, 0.1% Triton X-100 in PBS was applied for 10 min to permeabilize the cell membranes. For blocking of unspecific binding, the coverslips were transfered to a humid chamber and covered with blocking buffer (5% horse serum, 0.1% Tween20 in PBS). There, the coverslips were incubated for 2 hours at RT. In the following, the first antibody was added to the samples and incubation of 1 hour at RT was sufficient, before several washing steps with PBS and PBS-T were performed. Further incubation with the secondary antibody was conducted for 1 hour at RT and the coverslips were finally mounted with DAPI-containing mounting medium onto slides.
Quantification of cells and capturing of the images was done either at the fluorescence microscope Axio Imager A2 or the confocal microscope LSM 710.

5.2.9 Immunofluorescence of human sections

Immunofluorescence as well as immunohistochemistry experiments with paraffin-embeded sections were kindly performed by Dr. Kohji Mori. All patient materials were generously provided by Dr. Thomas Arzberger from the Neurobiobank Munich, Ludwig-Maximilians-University (LMU) Munich and were collected and distributed according to the guidelines of the local ethical committee.
First, the sections were deparaffinized with xylene and ethanol treatment and briefly washed with deionized water. After antigen retrieval through microwaving in 100 mM citrate buffer pH 6.0 and blocking with 2% FBS, the primary antibody was added and incubation at 4°C overnight followed. Washing with 0.02 % Brij35 or 0.05% Tween20 in PBS and incubation with the secondary Alexa-conjugated antibody were the next steps. For staining of the nucleus, either TO-PRO-3 or DAPI were used. Confocal images were taken with the confocal microscopes LSM510 or LSM710.

5.2.10 Immunohistochemistry of human sections

Similar to immunofluorescence experiments with patient sections, the sections were first deparaffinized with xylene and ethanol treatment and briefly washed with deionized water.

Upon antigen retrieval through microwaving in 100 mM citrate buffer pH 6.0 and blocking of endogenous peroxidase with 5% H_2O_2 in methanol, sections were put in PBS with 0.02% Brij35. Blocking with 2% FBS in PBS for 5 min followed and the respective primary antibody was applied for 1.5 h at RT or overnight at 4°C. Next, the secondary antibody was applied for 1 h. After rinsing with 0.02 % Brij35 in PBS, antibody binding was detected and enhanced by DCS Super Vision 2 HRP-Polymer-Kit or NovoLink DS Polymer Detection Systems using the chromogen DAB. Counterstaining for cellular structures was performed with haematoxylin. Microscopic images were obtained with a BX50 microscope and Cell-D software (Olympus).

5.2.11 Statistics

Data represent the mean ± SEM (standard error of the mean). The student's t-test or ANOVA were applied to calculate p-values for statistical analysis. P-values were assigned as listed in the following: *p ≤0.05; **p ≤0.01; ***p ≤0.001.

6. Material

6.1 Cell culture

6.1.1 Material

a) Cell lines

Cell line	Type	Origin
SH-SY5Y	Human neuroblastoma	DMSZ, ACC-209
HEK 293T	Human embryonic kidney	ATCC, CRL-1573
N2a Htt-Q16/ N2a Htt-Q150	Murine neuroblastoma	Ulrich Hartl

b) Media

Medium	Company
DMEM	Biochrom
DMEM GlutaMax	Invitrogen
DMEM F12	Invitrogen
HBSS	Invitrogen
Neurobasal	Invitrogen
Neuronal basal medium	Stemcell technologies
Opti-MEM	Invitrogen

c) Supplements

Supplement	Company
B27 supplement	Invitrogen
FCS (fetal calf serum)	Invitrogen
G418/ Geneticin	Invitrogen
Glucose	Invitrogen
L-Glutamine	Invitrogen
NEAA (non-essential amino acids)	Invitrogen
PS (Penicillin/ Streptomycin)	Invitrogen
SM1 Neuronal supplement	Stemcell technologies
Zeocin	Invitrogen

d) Transfection reagents

Tranfection reagent	Company
Lipofectamine	Invitrogen
Plus	Invitrogen
Lipofectamine 2000	Invitrogen
RNAiMAX	Invitrogen

e) siRNAs

Target	Product number	Company
Parkin	HSS107593 (si1), HSS107594 (si2)	Invitrogen
HOIP	HSS123836 (si1), HSS123837 (si2), HSS182838 (si3)	Invitrogen
Negative control	45-2001	Invitrogen

f) Plasmids

Plasmid	Features	Origin
GFP	pcDNA3.1	Anna Pilsl
HOIP wt	pcDNA3.1	Kathrin Müller-Rischart
HA-HOIP	pcDNA3.1	Kathrin Müller-Rischart
HOIP C885A	pcDNA3.1	Maria Patra
HOIP C885S	pcDNA3.1	Maria Patra
HOIL-1L	pcDNA3.1	Kathrin Müller-Rischart
HOIL-1L-HA	pcDNA3.1	Kathrin Müller-Rischart
HA-HHARI	pCMV	Julia Schlehe
Myc-Htt-Q20	pcDNA3.1	Ulrich Hartl
Myc-Htt-Q96	pcDNA3.1	Ulrich Hartl
NF-κB LUC	pGL3-LUC	Daniel Krappmann
LUC	pGL3-LUC	Promega
Parkin wt	pcDNA3.1	Winklhofer et al, 2003
Parkin ΔUBL	pcDNA3.1	Julia Schlehe

Parkin C431F	pcDNA	Richard Youle
Parkin G430D	pcDNA3.1	Iris Henn
Parkin W453X	pcDNA3.1	Iris Henn
Sharpin-HA	pcDNA3	Anna Pilsl

g) Chemicals and enzymes

Reagents	**Company**
CCCP	Sigma Aldrich
dbcAMP	Sigma Aldrich
IL-1β	R&D Systems
Muristerone A	Sigma Aldrich
Papain	Sigma Aldrich
Poly-L-Lysine	Sigma Aldrich
TNF-α	Biomol
Trypsin-EDTA	Sigma Aldrich

h) Equipment

Product	**Company**
Cell counter chamber	Neubauer Labor Optik
Cell culture dishes	Nunc
Cell culture flasks	Nunc
Cell scraper	Corning Inc.
Centrifuge Multifuge 3 S-R	Heraeus
Centrifuge for Eppendorf tubes	Eppendorf
Incubator	Heraeus

6.1.2 Solutions and buffers

PBS (Phosphate buffered saline):

3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl pH 7.4

6.2 Protein biochemistry

6.2.1 Material

a) Antibodies

Primary antibody	Company	Product nr.	Applic.	Dilution	Species
Active Caspase 3	Cell Signaling	9664L	IF WB	1:250 1:1000	rabbit
β-Actin	Sigma Aldrich	A5316	WB	1:5000	mouse
GAPDH	Life Technolog.	AM4300	WB	1:4000	mouse
GFP	Roche	11814460001	WB		mouse
HA 1.1	Covance	MMS-101P	IF WB	1:100 1:2000	mouse
HHARI/ ARIH1 (B-2)	Santa Cruz Biot.	Sc-390763	IHC	1:50	mouse
HOIL-1L (E2)	Santa Cruz Biot.	Sc-365523	WB	1:1000	mouse
HOIP	Acris	AP16062PU-N	IF	1:500	goat
HOIP	Sigma Aldrich	Sab2102031	IF WB IHC	1:500 1:1000 1:50-200	rabbit
Hsp60	Santa Cruz Biot.	Sc-1052	WB	1:2000	goat
Hsp75/ Trap1	Santa Cruz Biot.	Sc-135944	WB	1:2000	mouse
Huntingtin mEM48	Millipore	MAB5374	IHC	1:20-100	mouse
Huntingtin 1HU-4C8	Millipore	MAB2166	IHC	1:50-200	mouse
IκBα	Cell Signaling	9242	WB	1:1000	rabbit
IκBα	Cell Signaling	4814	WB	1:1000	mouse
Myc (9E10)	Santa Cruz Biot.	Sc-40	IF WB	1:500 1:1000	mouse
Myc (A14)	Santa Cruz Biot.	Sc-789	IF	1:500	rabbit
NF-κB p65 (C-20)	Santa Cruz Biot.	Sc-372	IF WB	1:500 1:1000	rabbit, goat
OPA1	BD Biosciences	612607	WB	1:1000	mouse
PRK8 (Parkin)	Santa Cruz Biot.	Sc-32282	IF WB IHC	1:1000 1:2000 1:10-20	mouse

PARP	Cell Signaling	9542S	WB	1:1000	rabbit
Phospho-IκBα	Cell Signaling	9246	WB	1:1000	mouse
Phospho-JNK (E11)	Cell Signaling	4668	WB	1:1000	rabbit
Phospho-c-Jun	Cell Signaling	3270S	IF WB	1:1000 1:1000	rabbit
P62	MBL	PM045	IHC	1:10000	rabbit
Sharpin	Cell Signaling	4444S	WB	1:1000	rabbit
TIM44	BD Biosciences	612583	WB	1:4000	mouse
TOM20	Santa Cruz Biot.	Sc-11415	IF WB	1:1000 1:2000	rabbit
Tubulin	Sigma Aldrich	T5168	WB	1:5000	mouse
Ubiquitin (P4D1)	Santa Cruz Biot.	3270S	WB	1:1000	mouse

Secondary antibody	**Company**	**Product nr.**	**Applic.**	**Dilution**
mouse-HRP	Promega	W4021	WB	1:10000
rabbit-HRP	Promega	W4011	WB	1:10000
goat-HRP	Santa Cruz Biot.	Sc-2020	WB	1:10000
mouse-Alexa488	Invitrogen	A-11029	IF	1:500
mouse-Alexa555	Invitrogen	A-21424	IF	1:500
mouse-Alexa647	Invitrogen	A-21236	IF	1:500
rabbit-Alexa488	Invitrogen	A-21245	IF	1:500
rabbit-Alexa555	Invitrogen	A-11034	IF	1:500
rabbit-Alexa647	Invitrogen	A-21429	IF	1:500

b) Beads

Beads	**Company**
Strep-Tactin Sepharose	IBA

c) Kits

Kit	**Company**
ATP Bioluminescence Assay Kit HS II	Roche
Luciferase Assay System	Promega

d) Chemicals and enzymes

Reagents	Company
Amersham ECL Western Blotting Detection Reagent	GE Healthcare
BSA (Bovine serum albumine)	New England Biolabs
DAPI (4',6'-Diamidino-2-phenylindole)	Sigma Aldrich
DNAse	Sigma Aldrich
Horse serum	Sigma Aldrich
Mowiol 4-88	Millipore
PFA (Paraformaldehyde)	Sigma Aldrich
PI (complete protease inhibitor)	Roche

e) Equipment

Product	Company
Cellulose acetate membrane, 0.2 µm	GE Healthcare
Centrifuge Multifuge 3 S-R	Heraeus
Centrifuge for Eppendorf tubes	Eppendorf
Freezer -20°C	Elektrolux, Liebherr
Freezer -80°C	Heraeus, Kendro
Fridge 4°C	Elektrolux, Siemens
Gel chamber Mini Trans-Blot Cell	Bio-Rad
Thermomixer comfort	Eppendorf
Luminometer LB96V	Berthold Technologies
Microscope Axio Imager A2	Zeiss
Microscope Confocal LSM710	Zeiss
Microscope cover glasses	Marienfeld
Microscope slide	Thermo Scientific
Milli-Q academic	Millipore
Nitrocellulose membrane, 0.2 µm	Invitrogen
pH-Meter	WTW
Power supply	Bio-Rad
PVDF membrane	Millipore
See Blue Plus2 (pre-stained protein standard)	Invitrogen

Slot Blot PR 648	Hoefer Scientific Instruments
Super RX (medical x-ray film)	FUJIFILM
Thermomixer compact	Eppendorf
Ultracentrifuge Optima MAX-XP	Beckman Coulter
Whatman paper	Schleicher & Schüll

f) Software

Sotware	Company
Adobe Illustrator CS5	Adobe
Adobe Photoshop CS5	Adobe
Axiovision 4.7	Zeiss
Microsoft Excel 2010	Microsoft
Multi-Gauge V3.0	FUJIFILM
WinGlow	Berthold Technologies
ZEN 2011	Zeiss

6.2.2 Solutions and buffers

a) General buffers

PBS: 3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl pH 7.4

b) Lysis buffers

Detergent buffer: 0.5 - 1% Triton X-100 in PBS, protease inhibitor

c) SDS gels and Western Blot buffers

PBS-T: PBS containing 0.1% Tween-20

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

TBS-T: TBS containing 0.1% Tween-20

Laemmli sample buffer (4x):

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

4% β-mercaptoethanol

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% milk powder in TBS-T

d) Immunofluorescence buffers

Fixing solution:	3.7% Formaldehyde in PBS
Permeabilization buffer:	0.1-0.2% Triton X-100 in PBS
Blocking solution:	5% horse serum, 0.1% Tween20 in PBS (or 2.5% BSA in PBS)
Mounting medium:	3.26 M glycerine, 2.72 M Mowiol 4-88, 0.12 M Tris pH8.5 in H_2O

e) Filter Trap buffers

Lysis buffer:	1% Triton X-100, 15 mM $MgCl_2$, 0.2 mg/ml DNAse in PBS
SDS buffer:	2% SDS in 100 mM Tris pH 7

f) Ubiquitination assay buffers

HeBs buffer (2x):	50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4
$CaCl_2$ solution:	2.5 M $CaCl_2$
Denaturing lysis buffer:	1% SDS in PBS
Dilution buffer:	1% Triton X-100 in PBS

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8. Publications

Mutant Huntingtin traps LUBAC – switching from the prosurvival NF-κB pathway to apoptotic signaling

Patra M. et al.

(manuscript in preparation)

Cytoplasmic protein aggregates interfere with nucleo-cytoplasmic protein and RNA transport.

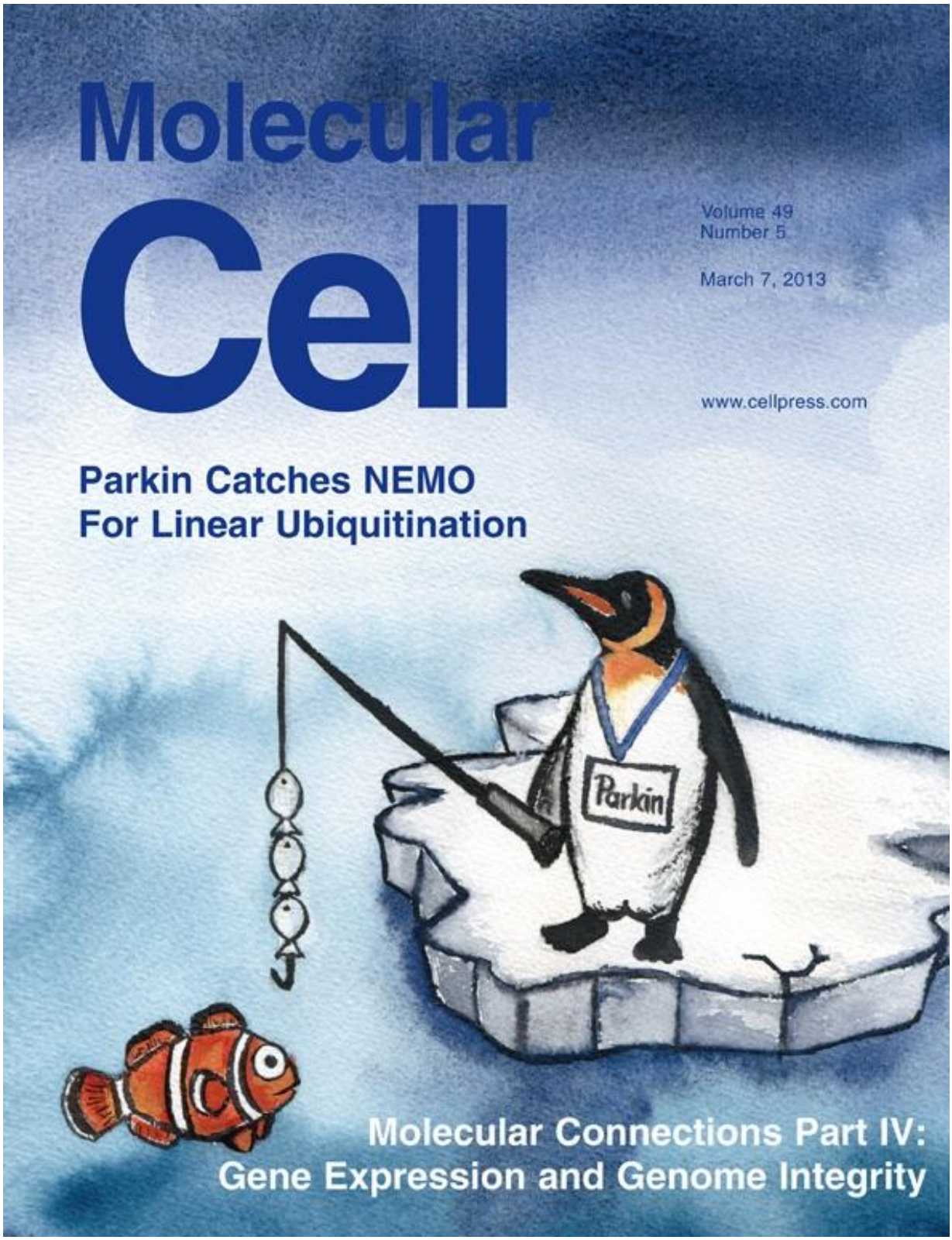
Wörner A.C., Hornburg D., Feng L.R., Patra M., Tatzelt J., Winklhofer K.F., Mann M., Hartl F.U., Hipp M.S.

(manuscript in preparation)

The E3 ligase parkin maintains mitochondrial integrity by increasing linear ubiquitination of NEMO.

Müller-Rischart A.K., Pilsl A., Beaudette P., Patra M., Hadian K., Funke M., Peis R., Deinlein A., Schweimer C., Kuhn P.H., Lichtenthaler S.F., Motori E., Hrelia S., Wurst W., Trümbach D., Langer T., Krappmann D., Dittmar G., Tatzelt J., Winklhofer K.F..

Mol Cell. 2013. 49(5):908-21.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