Parkin interacts with the 26S proteasome via Rpn13, a novel ubiquitin receptor

Miguel Angel Aguileta

Department of Neurology and Neurosurgery

McGill University, Montreal

A thesis submitted in partial fulfillment of the requirements of a Master's Degree

Abstract

Mutations in the Parkin gene cause a juvenile-onset for of Parkinson's disease (PD) that is transmitted in an autosomal recessive way. Studies have shown that mutations in parkin account for almost 50% of all juvenile-onset forms of PD, making parkin one of the most studied genes as a causative factor for PD.

Ubiquitin (Ub), a small 76 amino acid protein, can be covalently attached to other proteins. This post-translational modification is used as a signal for degradation in the ubiquitin-proteasome pathway (UPP). Ubiquitination requires the concerted activity of 3 basic enzymes: Ub-activaying enzyme (E1), Ub-conjugating enzyme (E2) and Ub-ligase (E3).

Studies have shown that parkin functions as a RING type Ub ligase, thus targeting potentially toxic proteins for degradation in the UPP. Parkin's E3 ligase activity is linked to its C-terminal RING1-IBR-RING2 domain. However, the function of its N-terminal ubiquitin-like domain (Ubl) remains unclear. Here, we present evidence suggesting that parkin's Ubl domain mediates an interaction between parkin and the proteasome, specifically Rpn13, a novel ubiquitin receptor.

Résumé

Des mutations dans le gène parkin causent une forme juvénile de la maladie de Parkinson qui est transmise de façon autosomique récessive. Des études ont démontré que ces mutations comptent pour près de 50% de toutes les formes juvéniles d'apparition de la maladie de Parkinson, rendant ainsi le gène parkin un candidat important comme facteur causal de cette maladie.

Ubiquitine (Ub) est une protéine de 76 acides aminés qui interragie de façon covalente à d'autres protéines. Cette modification post-traductionnelle est utilisée comme un signal pour la dégradation des protéines ciblées par le complexe protéolytique du protéasome. L'ubiquitination requiert l'activité concertée des enzymes E1, E2 et E3-ligases.

Des études ont démontré que la protéine parkin peut agir comme une ligase ubiquitine de type “RING”, ciblant ainsi les protéines potentiellement toxiques pour la dégradation protéasomale. Le motif “RING1-IBR-RING2” situé dans le domaine C-terminal contribue à la fonction ligase de parkin. Cependant, la fonction du domaine “ubiquitin like (Ubl)” situé au terminus aminé n’est pas encore claire. Ici, nous présentons des preuves suggérant que le domaine Ubl de la parkin agie en tant que médiateur pour l’interaction entre parkin et le protéasome, en particulier Rpn13, un nouveau récepteur d’ubiquitine.

Table of Contents

Acknowledgements

First of all, I would like to take this opportunity to thank my supervisor, Dr. Ted Fon. His endless patience and advice kept me motivated trough all this process. All the members from my committee; Dr. Phil Barker, Dr. Simon Wing and my mentor Dr. Edith Hamel, for their advice and help for this project

To all the members from my lab, I had a lot of fun working side by side with all of them and having their support during the roughest times, with a special mention to our lab manager, Maria Kontogiannea.

Our collaborating groups, Dr. Jean-Francois Trempe and Dr. Kalle Gehring from McGill University, Dr. Koraljka Husnjak and Dr. Ivan Dikic from Frankfurt, Germany.

All my friends from the BTRC and MNI, they have made a huge impact on my spare time. Extensive discussions and designing new ways to inhibit PKMζ kept me distracted when things were not smooth.

Most of all, I have to thank my family. Who have supported me throughout my studies. They always had faith in me and I could not have done it without their help.

Thanks to all of you… E.S.S., M.S.S., G.F., M.M., S.O., R.M., M.H., P.M., T.D., T.D., G.M., A.G, G.T., A.D., R.T., P.M. and K.M.

List of figures

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, and is only preceded by Alzheimer's disease. It has a prevalence of about 2-4% among the aging population between 65-85 years (de Rijk et al., 1995). It presents characteristic clinical abnormalities such as bradykinesia, resting tremor, muscular rigidity and postural instability (de Rijk et al., 1997). The cause of these symptoms has been mainly attributed to the selective loss of dopaminergic neurons in the substantia nigra; however, noradrenergic and serotoninergic systems are affected as well (Gibb, 1992; Braak, 2004). As with most neurodegenerative disorders, PD presents characteristic protein aggregates, known as Lewy bodies (LBs). These intracytoplasmic inclusion bodies are ubiquitin (Ub)-positive, and also contain lipids, neurofilaments, α-synuclein, synphilin-1 and some ubiquitin-protesomal pathway (UPP)-related enzymes. Although, to date, this is still considered as the pathological hallmark for PD, some forms of Parkinsonism do not present LBs (Dekker et al., 2003).

Identified in 1998, PARK2, encodes a protein called Parkin, now proven to be a causative gene in autosomal-recessive juvenile Parkinsonism (AR-JP), a form of PD characterized by an early onset (20 years of age on average) (Kitada et al., 1998; Shimizu et al., 2000). Parkin has been shown to function as a RING-type E3 ubiquitin ligase within the ubiquitin-proteasome pathway (UPP), the main cellular system involved in protein degradation (Shimura et al., 2000).

Ubiquitin is a highly conserved 8.6 KDa protein and it is used as a degradation signal in the UPP. Proteins are targeted for degradation by the 26S proteasome by the covalent attachment of Ub, in the form of a ploy-Ub tag, using the concerted action of 3 enzymes: Ub-activating enzyme (E1),

Ub-conjugating enzyme (E2) and a Ub-ligase enzyme (E3). The E3 ligases form the largest and most diverse family in this pathway and appear to be involved in substrate recognition; therefore, conferring specificity to ubiquitination.

Parkin's C-terminus region is composed of two RING-finger motifs (RING1 and RING2), flanking an In-Between RING (IBR). This Cysteine-rich region is known to be required for its E3 ligase activity. Recently, studies realized by Hristova and colleagues identified 2 new Cysteine-rich motifs spanning amino acids 145-237. Since these motifs are characteristic of RING domains, they have decided to include a third RING domain in parkin, now known as RING0.

Parkin's N-terminal ubiquitin-like domain (Ubl) remains poorly understood (Kitada et al., 1998; Hristova, 2008). Studies examining other Ubl-domain containing proteins, such as hHR23B and hPlic2, have shown that this domain is important to mediate interactions with the 26S proteasome; furthermore, these proteins have been suggested to function as "shuttles", delivering ubiquitinated substrates to the proteasome (Hiyama et al., 1999. Kleijnen et al., 2000).

Given that parkin is an E3 ubiquitin ligase, and that it has a Ubl domain on its N-terminus. Michael Haber (a former laboratory member (2004)) tested and confirmed that parkin is able to interact with the 26S proteasome, and that this interaction is abrogated or severally impaired upon deletion of the Ubl domain or using an AR-JP mutant form of parkin.

The specific aims of this project were to: 1) determine the specific subunit within the proteasome that parkin is interacting with; 2) determine the specific amino-acid residues mediating this interaction, as well as the affinity; and 3) to elucidate the function of this interaction.

Elucidating these 3 aims will increase our understanding of how parkin functions in the UPP. Also, it will provide us with insights about Parkinson's related mutations, specifically, within the Ubl domain of parkin.

Literature review

History of Parkinson's disease

First described in the *Ayurveda* (Sanskrit: *ayur*, life; *veda*, science), Paralysis agitans or Parkinson's disease (PD) has its first treatise completed at around 1000 B.C. The Ayurveda describes PD as a disorder, manifesting symptoms such as: rigidity, akinesia, tremors, depression and somnolence "loss of mind". It was treated with seeds from Mucuna pruriens, a plant in the Leguminosae family. Interestingly, the mature seeds contain about 3-6% of L-3,4-dihydroxiphenylalanine (L-DOPA), traces of 5-hydroxytriptamine (serotonin), nicotine, DMT-n-oxide, among other compounds. It is worth mentioning at this point that, L-DOPA is the current treatment for PD; although, it alleviates some of the symptoms, it doesn't treat the causing factors and DA neurons will die regardless of the treatment. During the 1930s the active compound L-DOPA coming from these seeds was isolated; however, this finding had no impact at that time, since the involvement of dopamine in PD had not yet been discovered (Damodaran and Ramaswamy; 1937).

What we commonly know as PD is mainly due to the studies realized by James Parkinson's. He describes and documents the symptoms in *An Essay on Shaking Palsy (1817)*. Latter on, the name, Parkinson's disease was coined by Jean-Martin Charcot (Parkinsons., 1817 (2002)). The biochemical changes underlying PD were first characterized by Arvid Carlson. He describes DA as a neurotransmitter and not only as a precursor for norepinephrine. He also noticed that mice treated with reserpine (decreases DA), were responsive to L-DOPA, which led other doctors to

begin treating PD patients with L-DOPA. His studies made such a huge impact in the field, that he was awarded with the Nobel Prize in physiology or medicine in 2000, along with Eric Kandel and Paul Greengard.

For long, PD has been attributed to environmental factors, and it was considered as a typical non-genetic disorder. However, the fact that PD has been present since ancient times, suggests that environmental factors may not be the only underlying cause for PD.

Environment

The use of animal models for any given disease provides useful insights into the pathology. PD is not an exception, and there are two toxin based models that mimic some crucial aspects of this disorder such as preferential loss of catecholaminergic neurons (leading to a parkinsonian-like motor dysfunction), olfactory loss and to a lesser degree, formation of intracellular inclusion bodies.

The first model relies on the cerebral administration of 6-hydroxy-dopamine (6-OHDA), which is an analog of DA. 6-OHDA rapidly and selectively destroys dopaminergic and noradrenergic neurons in the central nervous system (CNS). This toxin is taken up by dopamine and noradrenaline membrane transporters and accumulates in the cell cytosol. Cell death is caused by the formation of reactive oxygen species (ROS) and mitochondrial respiratory chain deficiency (Blum et al., 2001).

The second model comes from studies realized during the 80s, in which, certain groups of drug addicts in California were developing sudden PD-like syndrome (Langston et al., 1983). The

cause for these symptoms is attributed to heroin contaminated with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Consumption of this compound was found to induce Parkinsonism within 7-14 days.

MPTP is a lipophilic molecule which readily crosses the blood-brain barrier. In non-dopaminergic cells, monoamine oxidase B (MAO-B), converts MPTP into 1-methyl-4-phenilpiridinium (MPDP) which is quickly oxidized into 1-methyl-4-pyridinium (MPP^+) (Przedborski et al., 2003). The active metabolite MPP^+ is taken up by the dopamine transporter (DAT), and acts as an inhibitor of complex 1 in the mitochondria (Shen et al., 1985 and Nicklas et al., 1978). Inhibition of complex 1 in mitochondria generates ROS. Therefore, besides impairing the production of ATP, it exacerbates the damage in cells through a mechanism similar to 6-OHDA. (Choi et al., 1999). It is known that the brain is especially susceptible to damage caused by ROS because of its constant requirement of oxygen consumption, and it contains higher amounts of membrane lipids with polyunsaturated fatty acyl chains, making them prone to oxidation (Paik et al., 2003).

In addition to MPTP, rotenone, a commonly used herbicide, has been shown to cause selective degeneration of substantia nigra neurons in rats that are receiving systemic administration of this compound. Although it is known that rotenone is a powerful mitochondrial complex 1 inhibitor, unlike MPTP, it also induces the formation of cytoplasmic inclusions (lewy bodies), a hallmark of PD and other neurological disorders (Betarbet et al., 2000).

Genetics

Today, the underlying pathology of PD is well known, but our understanding in its etiology is limited. Long considered a purely environmental disorder, the majority of the PD cases are still classified as idiopathic. Nonetheless, over the past two decades our view of PD as a sporadic disorder has changed. Genetic risk factors are standing out as the major causative factors, possibly in combination with environmental circumstances.

There are some forms of PD that can be inherited as autosomal-dominant (PARK1 and PARK4/α-Synuclein; PARK5/UCHL1; PARK8/LRRK2; or autosomal-recessive (PARK2/Parkin; PARK6/PINK1; PARK7/DJ-1; PARK9/ATP13A2) forms. Although several families have been identified with Mendelian patterns of inheritance, it was not until 1997, with the discovery of the first PD gene (α-synuclein), that there was a clear genetic basis for the disease (Lesage et al., 2009 and Polymeropolus et al., 1997).

Autosomal dominant PARK genes

α-synuclein (PARK 1 and PARK 4)

In 1996 a pioneering genetic finding was made by Polymeropoulus and colleagues. They found a genetic linkage in an Italian-American family with an autosomal dominant form of PD. This finding lead to the discovery of the first PD linked gene, SNCA (α-synuclein) (Polymeropolus et al., 1997).

The locus PARK1 is located on chromosome 4q21, in this region; SNCA encodes a neuronal protein of 140 amino acids named α-synuclein. To date, three point mutations have been characterized. The first mutation, being A53T, was found in Italian and Greek families (Polymeropolus et al., 1997). Another mutation, A30P substitution was later found in German kindred (Kruger et al., 1998), and E46K found in a Spanish family (Zarranz et al., 2004), as well as whole SNCA gene multiplications (PARK4) (Singleton et al., 2003; Ross et al., 2008).

Mutations in α-synuclein are rare, but fully penetrant, and it is consistently found to be a major fibrillar component of LBs (Spillantini et al., 1997). Furthermore, the previously mentioned mutations have been shown to produce conformational abnormalities, making α-synuclein prone to self-aggregation and deposition (Lee et al., 2002).

α-Synuclein is an abundant presynaptic phosphoprotein that is normally "unstructured", it has been suggested to be involved in cellular trafficking within the endoplasmic reticulum/Golgi network, since overexpression of α-synuclein in Drosophila and yeast disrupts cargo trafficking (Cooper et al., 2006 and Gitler et al., 2008).

The function of Synucleins remain poorly understood, although highly expressed in the brain, α-synuclein KO mice are both viable and fertile, do not exhibit abnormal brain structure, and have a normal compliment of dopaminergic cell bodies, fibers and synapses (Dawson and Dawson. 2003).

As mentioned earlier, multiplications of the whole SNCA gene have been found in cases of early-onset PD with dementia and LBs. As a result, these patients have increased levels of α-synuclein. Interestingly, the differences in the severity of clinical phenotype between patients were found to be dependent on the number of copies of SNCA (3 or 4). Being the more severe

cases those who had 4 copies instead of 3. This findings, suggests that abnormal α-synuclein expression is a risk factor for PD (Ibanez et al., 2004 and Singleton et al., 2004).

A number of α-synuclein transgenic mice have been developed, using a variety of promoters to overexpress WT α-synuclein, or mutant forms of it. None of these models have accurately modeled PD, in that there is no progressive loss of DA neurons; however, there are several functional abnormalities in the nigrostriatal system, some of which are DA or L-DOPA responsive (Chesselet. 2008 and Chesselet et al., 2008).

Ubiquitin C-terminal Hydroxylase (UCH-L1) (PARK5)

Mutations in ubiquitin carboxyl-terminal esterase L1 (UCH-L1) located at the PARK5 locus have been suggested to cause autosomal dominant PD. Linkage to chromosome 4p14 has been established, however the finding has been questioned since it has only been found in rare cases from a single PD family. An I93M mutation in UCH-L1 was found in a Parkinson's disease German family (Leroy et al., 1998) and this finding has latter been followed by the discovery of a more common variant S18Y (Lincoln et al., 1999), which is associated with a decreased risk for PD (Satoh and Kuroda, 2001).

UCH-L1, a member of the ubiquitin C-terminal hydrolase family, hydrolyses C-terminal ubiquitins to generate ubiquitin monomers that can be recycled and reused to tag other proteins for degradation. The mutation I93M has been shown to diminish the protein's enzymatic activity. Although immunoflurescence studies have shown that LBs stain positive for UCH-L1, at present, no neuropathological data is available to determine whether this mutation affects LB pathology.

A second enzymatic activity for UCH-L1 has been reported that is dependent on its dimerization. Liu et al., found that UCH-L1 also possesses a ubiquitin ligase function, which is "opposed" to its hydrolase role. Furthermore, they found that the polymorphic variant S18Y, associated with a lower risk for PD, has reduced ligase while maintaining normal hydrolase activity. These results suggest that the balance of both its activities may contribute to the pathogenesis of PD, but how either is regulated remains unknown (Liu et al., 2002).

Leucine-rich repeat kinase 2 (LRRK2) (PARK8)

Located on chromosome 12p11.2-q13.1 the locus PARK8 contains the gene for leucine-rich repeat kinase 2 (LRRK2) (Paisan-Ruiz et al., 2004). A large number of genetic variants (mainly missense mutations) have been found within this 51 exon long protein kinase, some of them, PD related mutations.

Since the discovery of LRRK2 mutations in Basque Parkinson's families, the gene and its variable sites have been studied extensively. The G2019S mutation, accounts for up to 2-6% of all familial cases and 1-2% of the "sporadic" cases. However, in certain populations, like Ashkenazi Jews and North African Arabs mutation frequencies have been reported of up to 30-40% (Lesage et al., 2006 and 2008). Another common LRRK2 variant, G2385R polymorphism, has been found to be present in 10% of Asian PD cases but only 4% in age matched controls; although, considered as a big risk factor among Asians, this polymorphism is rare or absent in most other populations (Tan et al., 2008).

PD patients carrying mutations in LRRK2 exhibit a typical late onset form of the disease that is usually responsive to L-DOPA. Surprisingly, these patients show variations in the pathology, between and even within families, for example presence or absence in LBs.

The LRRK2 protein consists of several domains including ARM (Armadillo), ANK (Ankyrin repeat), LRR (Leucine-rich repeat), Roc (Ras of Complex. GTPase domain), COR (C-terminal of Roc), MAPKKK (mitogen activated protein kinase kinase kinase) and WD40 repeats (Lesage and Brice 2009). LRRK2 function is yet poorly understood; however, it has been implicated in apoptosis, regulation of neuronal survival, maintenance of neurites and protein-protein interactions. It is localized to membranous and vesicular structures such as mitochondria, lysosomes and endosomes (Biskup et al., 2006). Unlike other PD linked genes, LRRK2 is markedly, but not exclusively, expressed in the striatal dopamine area (Galter et al., 2006), further arguing to an important role for PD.

In cellular models, overexpression of disease-causing mutations of LRRK2 are toxic, and the toxicity is kinase and GTP-binding dependent (Smith et al., 2006. West et al., 2007); although, overexpressing LRRK2 in Drosophila or C. elegans leads to age-dependent loss of DA neurons (Liu et al., 2008), mammalian models using this system have not been able to fully develop a PD-like syndrome. Nonetheless, abnormalities in the nigrostriatal pathway have been observed, and motor deficits that are L-DOPA responsive (Lin et al., 2009).

Recently, using Drosophila models a genetic interaction between parkin and Thor was found in which mutation in Thor markedly decrease parkin and Pink1 mutant viability. Importantly, overexpression of 4E-BP was able to suppress parkin and Pink1 KO phenotypes. It is known that 4E-BP activity is regulated by its level of phosphorylation, and that this event is achieved via the

TOR signaling pathway. Interestingly, 4E-BP has been found to be a substrate of pathogenic LRKK2 mutants, leading to its hyper-phosphorylation and consequently to its inhibition (Tain et al., 2009).

Autosomal recessive PARK genes

DJ-1 (PARK7)

In 2001, having narrowed a 5.6-Mb region in chromosome 1 trough fine mapping single nucleotide polymorphisms (SNP) and using a systematic screening with reverse-transcriptase-polymerase chain reaction (RT-PCR) for this region, Bonifati et al., identified a gene for autosomal recessive early-onset Parkinsonism. Located on chromosome 1p36 at the PARK7 locus, the gene DJ-1 was identified from two genetically isolated communities in the Netherlands and Italy. In the Dutch family, a homologous deletion encompassing exons 1-5 was found, and showed complete co-segregation. The Italian family, on the other hand, had complete co-segregation of a single T→C transition at position 497. This missense mutation resulted in the substitution of a highly conserved leucine at position 166 for a proline (Bonifati et al., 2003).

The clinical phenotype in both the Dutch and Italian families is characterized by an early onset (30s), but with a slow disease progression. Consistent with patterns observed in PD, functional brain imaging studies in patients of the Dutch family, showed severe abnormalities in the

dopamine transporter system, revealing presynaptic dysfunctions in the nigrostriatal pathway (Bonifati et al., 2003).

DJ-1 is a member of the Thi/Pfpl family of molecular chaperones (Moore et al., 2003), and mutations on DJ-1 play a small but important role in early-onset Parkinsonism. Penetrance appears to be complete in individuals carrying two disease-causing mutations (Heutnik et al., 2006). Although, ubiquitously expressed through all the body, DJ-1 has its greatest expression in subcortical regions including the caudate nucleus, thalamus and substantia nigra (Bonifati et al., 2003).

In cells, DJ-1 is found in the cytosol, mitochondrial matrix and intermembrane space (Zhang et al., 2005). It has been suggested that DJ-1 acts as a sensor of oxidative stress since it shifts its isoelectric point (pI) to a more acid form following oxidative stress (Mitsumoto and Nagakawa., 2001), this change in pI seems to cause a re-localization of DJ-1 from the cytosol to the mitochondria (Canet-Aviles et al., 2004); however, this property of DJ-1 and its relation to PD has yet to be determined.

PTEN induced putative kinase 1 (PINK1) (PARK6)

PD due to PTEN induced putative kinase 1 (PINK1) mutations is inherited in an autosomal recessive fashion, and it manifests with an early-onset (<50 years). Localized on chromosome 1p35-36 at the PARK6 locus, PINK1 mutations are thought to lead to a loss of function (Valente et al., 2004).

Structurally, PINK1 contains a conserved serine/threonine kinase domain with an N-terminus mitochondrial targeting motif. In cells, PINK1 is localized to the mitochondrial outer membrane space, with its kinase domain facing the cytosol (Silvestri et al., 2005 and Zhou et al., 2008), thus, PINK1 substrates should reside in the cytosol.

Drosophila lacking PINK1 exhibit an inability to fly and slower climbing speed, along with these behavioral deficits, mitochondrial degeneration is present in flight muscles. It is worth mentioning, that this phenotype is almost identical to Drosophila parkin KO; however, PINK1 mutants or KO phenotype can be rescued or strongly improved by overexpressing parkin. Strongly suggesting, that PINK1 and parkin function in a common pathway, and positioning parkin downstream of PINK1 (Clark et al., 2006 and Park et al., 2006).

Unfortunately, for PD-associated recessive genes, there is no mammalian model that shows any striking phenotype. Perhaps there is a redundancy in these systems, or the life span of these animals is too short to display any PD like phenotype.

ATP13A2 (PARK9)

Located on the PARK9 locus on chromosome 1p36, mutations on the ATP13A2 have been linked to an autosomal recessive parkinsonism in families with Kufor-Rakeb syndrome (Najim al-Din et al., 1994 and Hampshire et al., 2001). The patients presents a juvenile-onset atypical parkinsonism, accompanied by pyramidal cell degeneration and cognitive dysfunctions, usually not seen in PD; however, some genetic variants in ATP13A2 have recently being associated with a more typical early-onset PD in families from Brazil and Italy (Di Fonzo. et al., 2007).

Parkin (PARK2)

Identified in 1998, the locus PARK2 harbors the gene PRKN. This gene is located on chromosome 6q25.2-q27 and it encodes a protein called parkin (Kitada et al., 1998; Shimizu et al., 2000).

The parkin gene, one of the largest in the genome, spans more than 500Kbp and its 12 exons encode for a 465 amino acid protein (Kahle et al., 2000). To date, >70 PD-linked mutations have been identified (Dekker et al., 2003, Kitada et al., 1998, Shimura et al., 2000 and Zhang et al., 2000), and it is believed that parkin loss of function is the underlying cause for PD.

Mutations in parkin constitute the most common cause of early-onset parkinsonism, and it accounts for almost 50% of AR-JP. Patients carrying mutations in this gene, exhibit clinical symptoms resembling sporadic PD, marked reduction of dopamine uptake in the putamen and caudate nucleus and selective neurodegeneration of the nigrostriatal tract (Westerlund et al., 2010).

The C-terminal region of parkin is composed of a class II PDZ-binding motif (Fallon et al., 2002) and three RING-finger motifs (termed RING0, RING1 and RING2) flanking an In-between RING (IBR) it is RING1 and RING2 cys-rich motifs. The N-terminal 76 amino acids of parkin have 31% identity and 62% similarity to ubiquitin, thus, it is called ubiquitin like domain (Ubl).

Parkin's far C-terminus PDZ-binding motif, which is selectively truncated in a PD-associated mutation, binds to the class II PDZ protein CASK, the mammalian homolog of *Caenorhabditis elegans* Lin-2 (Fallon et al., 2002). They also found that parkin is part of a large multimeric

complex at the postsynaptic density (PSD), possibly through its interaction with CASK. This might suggest a role for parkin in regulating synaptic transmission (Fallon et al., 2002).

N-terminal to the PDZ-binding motif is the RING-IBR-RING domain. To date, several proteins containing a RING finger motif have been identified, and it is well accepted that RING fingers mediate protein-protein interactions, particularly in the ubiquitin-dependent proteasomal degradation pathway (UPP). Among the proteins bearing a RING finger domain, the ariadne E3 ubiquitin ligase, which interacts with the E2 conjugating enzyme UbcH7, are the most similar to Parkin's C-terminal. In 2000, it was proven that parkin acts as an E3 ubiquitin ligase; furthermore, it was shown that the full region RING1-IBR-RING2 is both necessary and sufficient for binding to the E2 ubiquitin-conjugating enzyme UbcH7. Also, AR-JP-linked mutations in Parkin's C-terminal, show a loss of ubiquitin protein ligase activity (Shimura et al., 2000).

As an E3 ubiquitin ligase, parkin's function is to tag unfolded proteins with ubiquitin to undergo degradation in the ubiquitin-proteasome pathway, or, as observed in our laboratory, to monoubiquitinate its substrates (Fallon et al. 2006; Joch et al. 2007). Monoubiquination of substrates leads to trafficking, as is the case for Eps15, or to regulate their functions as seen for PICK1. These findings implicate parkin in roles other than degradation. For example, our laboratory has shown that epidermal growth factor receptor (EGFR) endocytosis and degradation is accelerated in parkin deficient cell lines and parkin KO animals.

Among parkin's substrates, the most cited are: p38, CDCrel-1, α-synuclein, Pael-R, Cyclin E and Synphilin. The degradation of these putative substrates has been followed upon parkin overexpression in cell lines; Although, its degradation seemed a consequence of parkin's

activity, most of them do not accumulate in parkin KO animal models (Ko et al., 2005), and their role in PD pathogenesis is not known.

As previously mentioned, PD hallmark is the presence of ubiquitin-positive protein aggregates, known as Lewy bodies (LBs). Interestingly, most PD cases associated with parkin mutations presents no such abnormalities.

Among the PD-linked genes, parkin and UCHL-1 activities can be linked with the UPP, whose function is key in almost every cellular process.

Ubiquitin proteasome pathway UPP

In eukaryotic cells, the selective degradation of short-lived proteins, unfolded proteins and factors whose activity has to be terminated, is carried out by the ubiquitin-proteasome pathway (UPP).

Ubiquitin (Ub), a highly conserved 8.6 KDa protein, is used as the degradation signal. Proteins are targeted for degradation by the 26S proteasome by the covalent attachment of Ub, in the form of a poly-Ub tag, through the concerted action of 3 basic enzymes as described in figure 1.

An E1 or activating enzyme, "activates" ubiquitin in a reaction that requires ATP. It consists of the formation of ubiquitin adenylate and the release of pyrophosphate (PP_i) followed by the binding of ubiquitin to a cystein residue in the E1 that involves the formation of a thiolester bond. The next step is the transfer of this ubiquitin to a cystein of an E2 enzyme or ubiquitin-carrier, both processes are mediated through a thiolester bond. Although, in some cases, the E2

enzyme can transfer the activated ubiquitin to a target protein, an E3 enzyme is often required to confer substrate recognition; thus, achieving high specificity.

There are two main kinds of E3s and they are classified according to their catalytic domains. The HECT type (homologous to E6-AP C-terminus), is an E3 that transiently binds ubiquitin from the E2 (using the same type of bond and also using a Cystein). After being charged with ubiquitin, this HECT type E3 ubiquitinates its substrates using an amide bond in an isopeptide linkage. The second type of E3s, are the RING type (Really Interesting New Gene), parkin belongs to this type of E3s. For this type of ubiquitination, a substrate and an E2 are simultaneously bound by the RING type E3, and ubiquitin is transferred directly from the E2 into the substrate, without the need to transfer ubiquitin into the E3 (Hershko et al., 1998).

Ubiquitin-dependent degradation pathway (UPP)

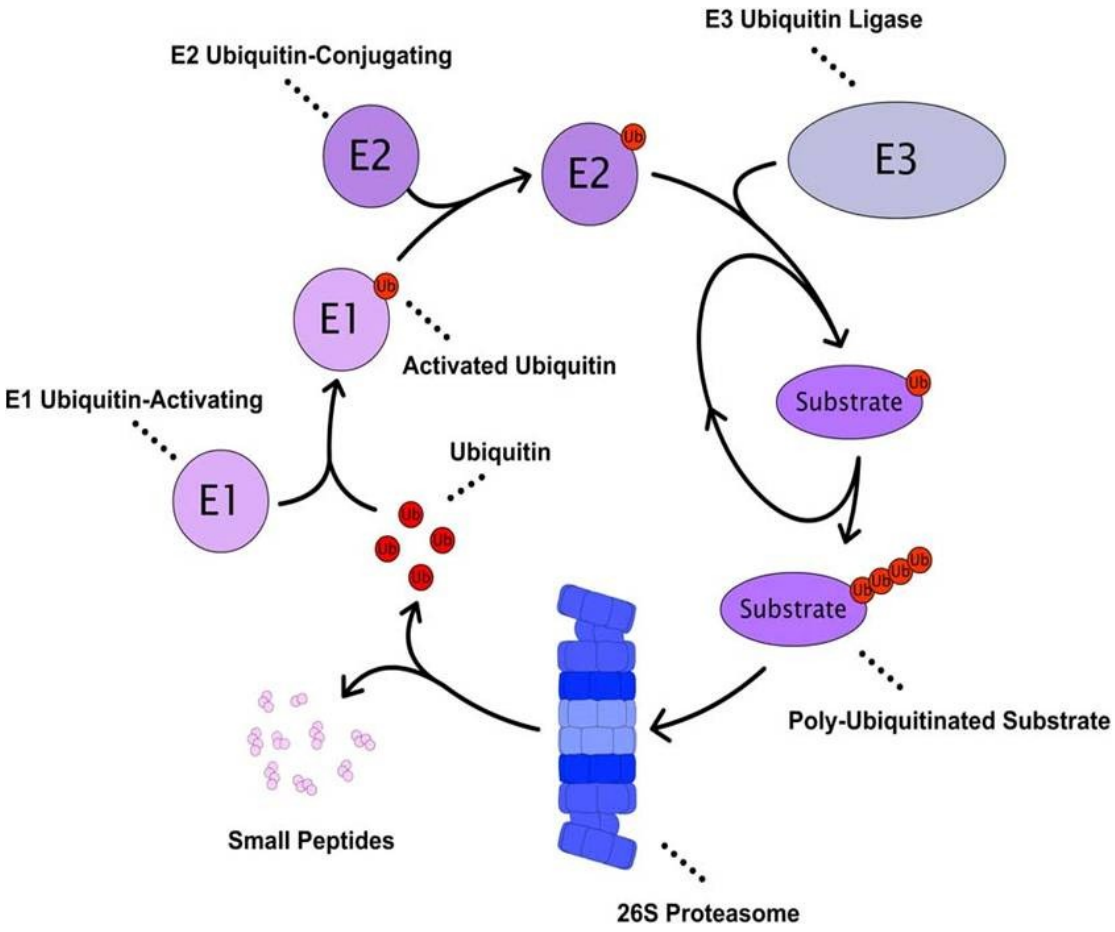

Figure 1. Substrates are targeted for degradation by the 26S proteasome by the covalent attachment of ubiquitin (Ub) in the form of a poly-Ub tag. The concerted activity of 3 basic enzymes is depicted. An E1 activates Ub and transfers it to an E2. Together with the E3 ligase, activated ubiquitin is transferred to a substrate. Through multiple iterations of the cycle, a poly-Ub tag is formed and recognized by the 26S proteasome. Eventually the substrate is degraded into small peptides and Ub is recycled.

The 26S proteasome

Once a substrate has been tagged with ubiquitin, its canonical fate will be degradation. For this purpose, eukaryotic cells have developed a highly conserved structure, the proteasome (Hwan-Ching et al., 2008).

Depicted in figure 2, the 26S proteasome consists of two major complexes (Tanaka. 2009): a central 20S protease core that can be found as single or double capped by a 19S regulatory complex. The 20S complex is composed of two copies of seven α and β subunits, that form two juxtaposed rings with the β subunits facing the center of the "cylinder" these subunits harbors the proteases activities found in the proteasome. There are three β-type subunits in each inner ring that contain active threonine residues at their N-terminus and show N-terminal nucleophile hydrolase activity, implying that the proteasome is a threonine protease that does not fall into the known seryl, thiol, carboxyl and metalloprotease families. It is well known that the 20S proteasome can in fact degrade unfolded proteins in the absence of the 19S regulatory particle, ATP and or ubiquitin; however, in order to achieve specificity, the 20S core requires at least one 19S regulatory particle.

The 19S complex, recognizes poly-ubiquitinated substrates, removes their ubiquitin chains, unfolds the substrates, opens the α-ring on the 20S, and transfers the unfolded substrates into the 20S core for degradation.

The 19S regulatory particle comprises about 20 different subunits that are classified in two subgroups: regulatory particle of AAA-ATPase (Rpt) family and regulatory particle of the non-ATPase (Rpn) family. Furthermore, the 19S particle is also subdivided into a lid subcomplex and a base subcomplex.

The lid complex is composed of at least nine non-ATPase subunits: Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn11, Rpn12 and Rpn15. Its main function is to deubiquitinate the captured substrates in order to prevent ubiquitin from being degraded. Rpn11 is one of the best described subunits in the lid that performs this task; it cleaves the polyubiquitin chain at a proximal site, and leaves this "big" ubiquitin chain to be further disassembled down to its monomeric components by other deubiquitinating enzymes (DUBs). In addition to Rpn11, there are two other DUBs associated with the base complex, Usp14 (Ubp6 in yeast) and Uch37, the main difference with these DUBs is that they cleave ubiquitin from substrates at the distal ends. These DUBs are bound to the 19S base via Rpn1 and Rpn13, respectively (Tanaka, K., 2009).

The base complex is composed of six homologous AAA-ATPase subunits (Rpt1-6) and four non-ATPase subunits (Rpn1, Rpn2, Rpn10 and Rpn13). The base complex has three major roles: to capture substrates via recognition of ubiquitin, to promote substrate unfolding and to open the α-ring to thread the substrates into the 20S core. The opening of the α-ring, is not fully understood, but is mainly attributed to Rpn2, and Rpn1 to a lesser extent (Rosenzweig et al. 2008). The presence of ATP is a requirement and it is for this reason that some Rpt subunits have been proposed to have an important role, such as Rpt2, Rpt3 and Rpt5 (Saeki et al. 2007 and Rabl et al 2008). The opening of the 20S core subunit and the unfolding of the substrates is closely related, the six ATPases in the base subcomplex is known to have a reverse chaperon-like activity, which, by means of hydrolyzing ATP, unfolds and threads the substrate into the core of the proteasome (Braun et al. 1999).

The specificity of the substrates degraded in the proteasome, relies on their proper identification by the 19S particle. In order to achieve this function, the proteasome has two main ubiquitin receptors. Rpn10 is part of the base in the 19S particle, it achieves the recognition of

ubiquitinated substrates by interacting with ubiquitin via a C-terminal Ubiquitin-interacting motif (UIM). This is an α-helix motif that binds ubiquitin with high specificity. The binding of ubiquitin to the UIM utilizes a hydrophobic patch in ubiquitin composed of L8, L71, V70, I44, G47 and A46 amino acid residues. These residues are also important for the binding of ubiquitin to the other receptor, Rpn13. This 19S proteasome subunit has been recently identified as an Ub-receptor by our collaborating group in Frankfurt, Germany, leaded by Dr. Ivan Dikic (Schreiner et al. 2008 and Husnjak et al. 2008). Unlike Rpn10, Rpn13 binds ubiquitin via a pleckstrin-homology domain receptor for ubiquitin (Pru). This domain is composed of a series of β-strands and has a bigger surface to interact with ubiquitin as compared with the UIM domain, although as previously mentioned, both domains interact with ubiquitin using the same hydrophobic patch within it.

Ubiquitin-like domain proteins

Besides many missense mutations within the RING-IBR-RING domain, only deleting and truncating mutations were described for the N-terminus of parkin. In 2001, however, Terreni et al., identified a novel homozygous amino acid substitution, R42P (Terreni et al., 2001). The N-terminus of parkin (aa1-76) it is known as the ubiquitin-like domain (Ubl), the reason for this name, relies simply in the fact that it resembles ubiquitin. In fact, it is 31% identical and 61% similar to ubiquitin.

Although the function of Parkin's Ubl remains unclear, many other Ubl containing proteins have been well characterized.

The 26S proteasome

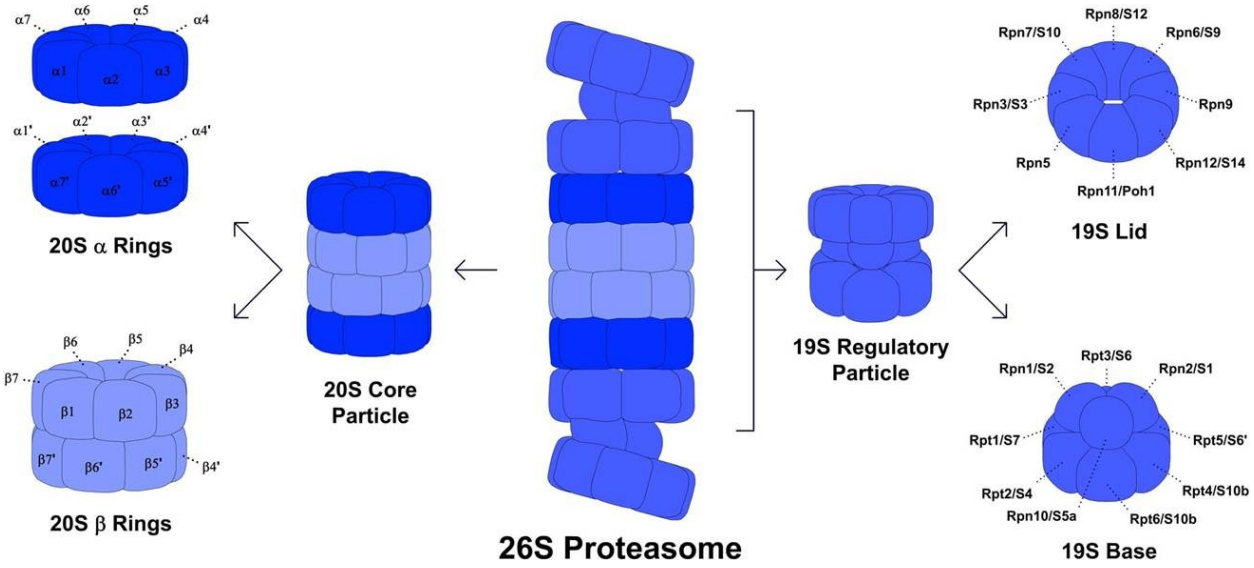

Figure 2. The 26S proteasome: composed of 20S core particle, made up by seven copies of alpha and beta subunits that form juxtaposed rings. It can be found as single or double capped by the 19S regulatory particle, which is further subdivided into lid and base.

Unlike ubiquitin or other post-translational modifiers (PTM) such as Nedd8, SUMO1, Ufm1, ISG15 etc… that can be covalently attached to client proteins, Ubl domain containing proteins do not serve as PTM. Instead, compelling evidence suggests that this domain is important in mediating interactions with the 19S regulatory particle in the proteasome. Some of these interactions are depicted in figure 3.

Ubl-Uba-domain proteins (Extrinsic Ub receptors)

Among the Ubl domain containing proteins, those harboring a ubiquitin-associated (Uba) domain have been the most characterized. Uba domains are implicated in ubiquitin binding. Yeast proteins RAD23 and DSK2 as well as their human homologs, hHR23A/B and hPlic-2 respectively, contain Uba domains downstream of their Ubl domains. In addition to binding ubiquitin on their Uba domains, they also are able to directly interact with the proteasome using their Ubl domains (Hiyama et al., 1999. and Kleijnen et al., 2000). This particular characteristic to, on the one hand bind ubiquitin chains via their Uba domains, and on the other hand, bind the proteasome via their Ubl domains, suggest that these proteins are involved in shuttling substrates destined for degradation to the proteasome (Figure 3).

Evidence for the potential role in substrate recognition for these Ubl-Uba domain proteins came first from a yeast genetics study. Although yeast null mutants in Rpn10 and Rad23 or Dsk2 are viable, likely due to the contribution of the other Ub receptor Rpn13, a cross between Rad 23/Dsk2 null with Rpn10 null mutant, results in either lethal or a very sick phenotype (Rao and Sastry 2002). Furthermore, Elsasser et al., found that Rpn10 and Rad23 serve as alternative ubiquitin receptors for the proteasome. Specifically, they found that Rad23 and Dsk2 dock

ubiquitin conjugates to the proteasome directly, confirming the hypothesis that these proteins are involved in shuttling substrates to the proteasome (Elsasser et al., 2004).

In mammals, hHR23A/B and hPlic-2 have been shown to bind the 19S regulatory subunit S5a (yeast homolog of Rpn10). Among all the subunits in the proteasome, S5a has been the most studied. It was the first subunit identified to mediate the recognition of ubiquitinated substrates, specifically proven to recognize poly-Ub chains with high affinity. Latter, Young et al., identified two independent poly-Ub binding sites in the C-terminal region of S5a, originally termed PUbS1 and PUbS2, but commonly known as ubiquitin-interacting motifs (UIM). These sites are separated by about 50 residues and are approximately 30 amino acids in length. UIMs consists of 5 hydrophobic residues forming an alternating pattern of large and small side-chains (an α-helix), which is essential for poly-Ub recognition (Young et al., 1998).

Both hHR23A/B and hPlic-2 have been shown to interact specifically with the second UIM domain of S5a (Hiyama et al., 1999. and Kliejnen et al., 2000). Furthermore, the solution structure of the Ubl-UIM interaction has been characterized and confirmed (Walters et al., 2002).

Structurally, the Ubl domains of hHR23A/B and hPlic-2 are quite similar to ubiquitin and between themselves. hHR23A/B and hPlic-2 have a well conserved hydrophobic surface involving similar amino acid residues: A77, I75, I80, V101 and I102 in hPlic-2, that corresponds to A51, I49, I54, M75 and V76 in hHR23A/B. This hydrophobic region is involved in binding with S5a (Walters et al., and Figure 4). It should be noted, however, that parkin contains atypical polar residues within the corresponding hydrophobic region in both hHR23A/B and hPlic-2 involved in proteasome binding, shown in figure 4. Moreover, the Ubl domain of parkin more closely resembles ubiquitin, which only interacts weakly with S5a as a monomer.

Ubl domain containing proteins and extrinsic Ub receptors

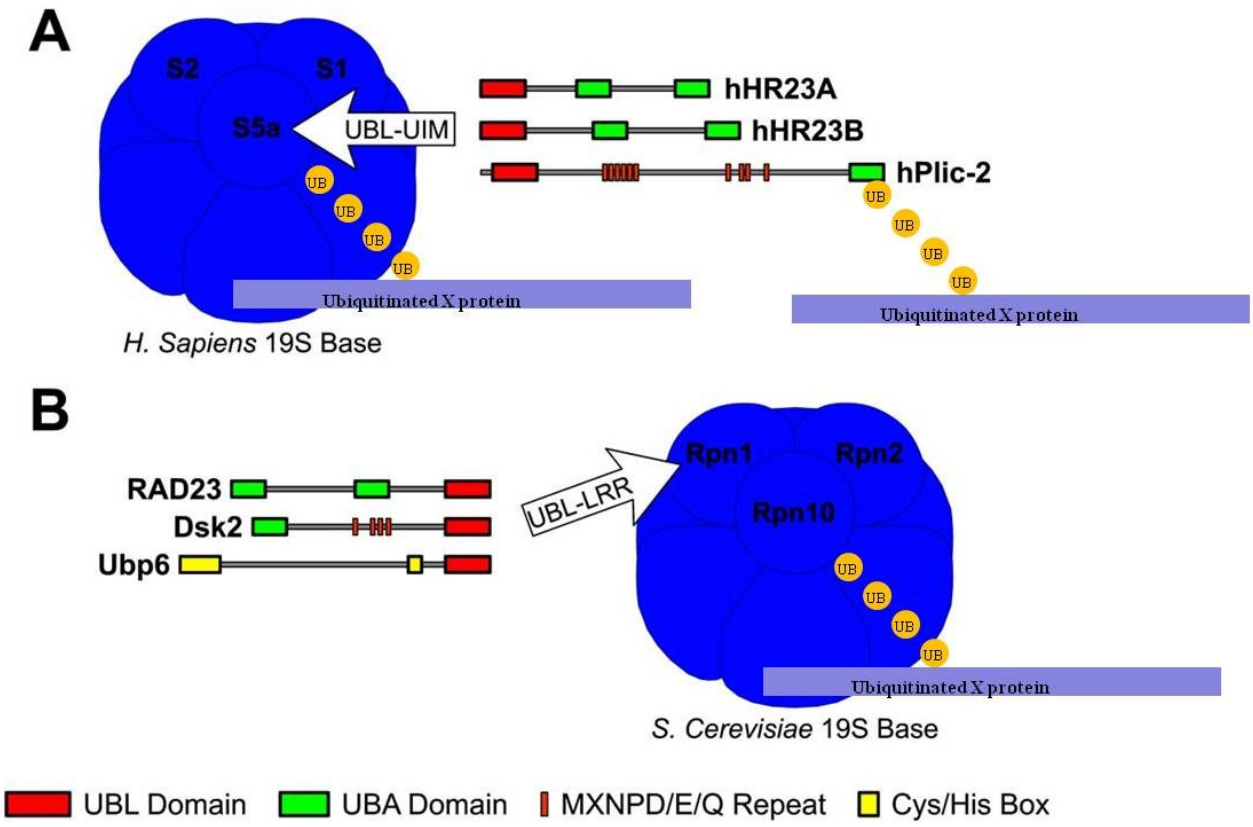

Figure 3. (A) In H. sapiens, Ubl domain proteins hHR23A/B and hPlic-2 have been shown to interact directly to the second ubiquitin-interacting motif (UIM) of 19S base subunit S5a. **(B).** In S. cerevisiae, however, Ubl domain proteins RAD23, Dsk2 and Ubp6 have all been shown to interact directly to a leucine-repeat-like (LRR) region of 19S base Rpn1.

Ubl domains of hHR23A, hPlic-2, parkin and ubiquitin

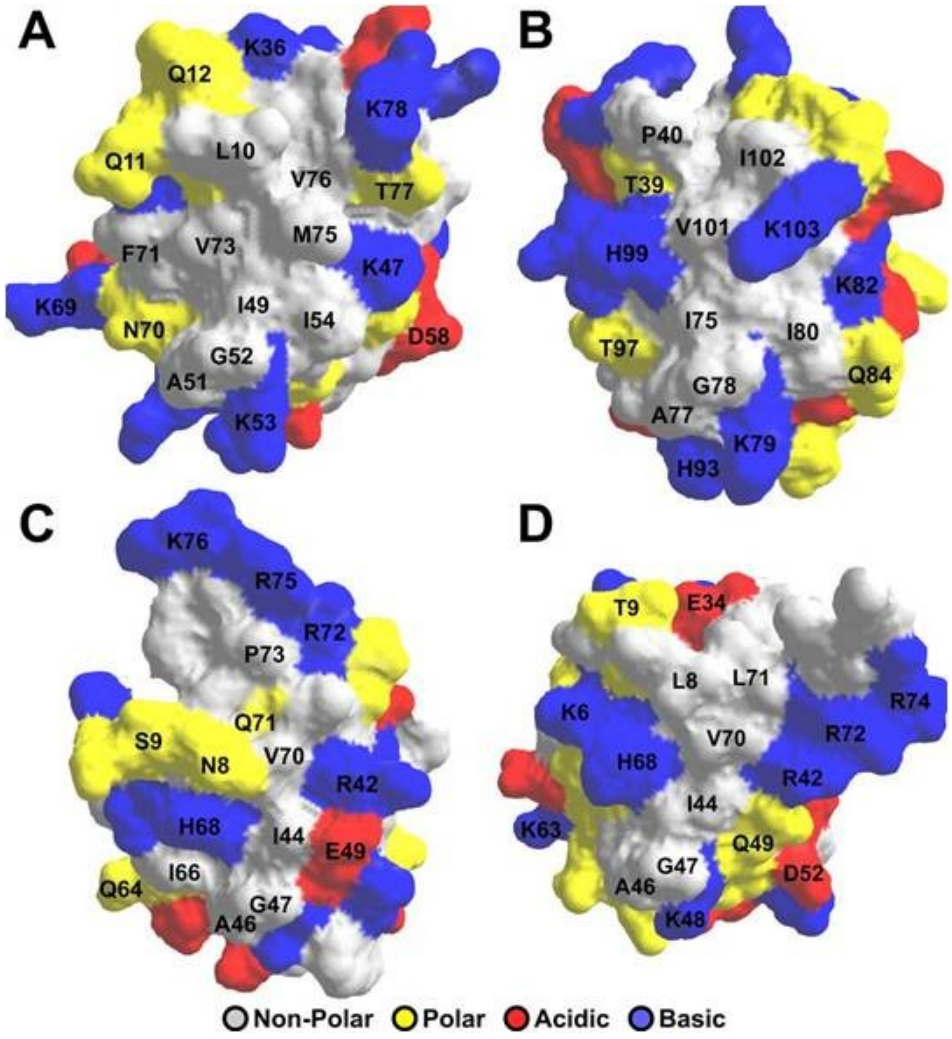

Figure 4. Comparison of the molecular surface of human (**A**) hHR23A, (**B**) hPlic-2, (**C**) parkin and (**D**) ubiquitin (RCSB Protein Data Bank structures 1P98, 1J8C, 1IYF and 1D3Z respectively). The structures are oriented to compare the putative S5a-binding regions. Both hHR23A and hPlic-2 form a hydrophobic, non-polar binding patch (residues L10, A51, G52, I49, I54, M75 and V76 in hHR23A and P40, A77, G78, I75, I80, V101 and I102 in hPlic-2). The corresponding region in parkin includes atypical polar residues N8 and Q71, also an acidic glutamic residue E49 situated in a position typically occupied by a non-polar residue (I54 and I80 in hHR23A and hPlic-2 respectively). Overall parkin more closely resembles ubiquitin. The panel was generated using DeepView/Swiss-pdbViewer.

Although all higher eukaryote S5a sequences contain at least two UIM domains, or even three as is the case for *Drosophila*. *S. cerevisiae* has only one UIM. Furthermore, yeast Rpn10's UIM domain is more similar to UIM1 of its mammalian homolog S5a, and thus lacks the UIM required for binding Ubl domain containing proteins. Elsasser et al., showed that Dsk2 and Rad23 overcome this problem by binding a leucine-repeat-like (LRR-like) region in the 19S base subunit Rpn1, thus, still functioning as shuttles for client ubiquitinated proteins (Elsasser et al. 2002. and figure 4).

Ubl domain proteins as chaperone co-factors

In mammalian cells, all the isoforms of Bag1 (Bag1L, Bag1M and Bag1S) contain a Ubl domain. Bag1 displays anti-apoptotic activity when interacting with Bcl2, Raf and some other hormone receptors. In the C-terminus region of these proteins, there is a Bcl2-associated athanogene (BAG) domain, known to mediate interactions with molecular chaperones (Townsend et al., 2003). Also, it has been shown that Bag1 modulates the chaperone activity of Hsc70 and Hsp70 in the cytosol and in the nucleus (Luders et al., 2000). Upon binding to Hsp70, Bag1 stimulates the release of its substrate, and potentially functions to direct unfolded proteins for degradation. This idea has gain strength because of two findings: Bag 1 cooperates with CHIP, an E3 ubiquitin ligase, to promote the degradation of the glucocorticoid hormone receptor (Demand et al., 2001) and also, Bag1 reduces and promotes the degradation of mutant huntingtin (mtH), possibly down-regulating seven in absentia homolog 1, an E3 ligase whose function has been shown to promote the nuclear import of mtH (Sroka et al., 2009).

Just like the Ubl-Uba proteins discussed, Bag1 has been shown to bind the 26S proteasome via its Ubl domain, however, it remains unclear whether this interaction is mediated through S5a UIM domains. Furthermore, Bag1 was found to get ubiquitinated by CHIP, although canonically, ubiquitination is a signal for degradation, this is not the case for Bag1. Bag1 gets ubiquitinated using a not so well understood type of Ub linkage (K27), surprisingly this type of ubiquitination strengthens the interaction between Bag1's Ubl and the proteasome (Alberti et al., 2002).

To date, Bag-1 is the only Ubl domain protein whose association with the proteasome has been shown to be regulated by ubiquitination. However, both ubiquitinated and non-ubiquitinated forms of Bag1 interact with the proteasome.

Ubl domain proteins as de-ubiquitinating enzymes

In yeast, Ubp6 as well as the human homolog Usp14 harbors a Ubl domain and they are de-ubiquitinating enzymes (DUBs). As previously mentioned, ubiquitination is a reversible process, in fact, is a requirement prior to undergo proteosomal degradation. In the proteasome, the 19S subunit Rpn11 is the most well characterized DUB, and its activity is a rate-limiting step in the UPP. Besides Rpn11 that cleaves ubiquitin chains in a proximal site, there are other DUBs, such as Ubp6 whose activity is needed for the cleavage of ubiquitin chains at the distal sites.

Ubp6 has been demonstrated to bind directly to the 26S proteasome. As is the case with the aforementioned yeast Ubl-Uba domain proteins, Ubp6 binds directly to the 19S proteasome subunit Rpn1. Interestingly, Ubp6 bound to the proteasome has an increase in its DUB activity of about 300 fold (Leggett et al., 2002). Furthermore, yeast strains lacking Ubp6 exhibit an

accelerated ubiquitin turnover. As previously mentioned, DUB activity highly contributes to the recycling of Ub.

E3 ligase interactions with the proteasome

Besides Ubl-domain containing proteins, some E3 ligases have also been shown to interact with the 26S proteasome. One of them KIAA10, a member of the HECT family of E3 ligases, interacts with purified proteasomes via a direct interaction with the 19S regulatory subunit S2 (You and Pickart, 2001). KIAA10's ortholog in yeast; Hul5, is an E3 ligase whose activity seems to oppose that of Ubp6 by adding ubiquitin chains into substrates destined for degradation. The purpose for this activity is not yet fully understood, but it seems, Hul5 modifies the type of linkages in ubiquitin onto these substrates. Because of this opposing activity, Hul5 has recently been recognized as an E4, making it a new type of enzyme in the UPP (Crossas et al., 2006). Other E3s shown to bind the proteasome include Ubr1p and Ufd4p (Xie and Varshavsky, 2000). Specifically, Ubr1p was shown to bind to 19S regulatory subunits Rpn2, Rpt1 and Rpt6. Ufd4p was shown to bind Rpt4 and Rpt6 in yeast (Xie and Varshavsky, 2000 and Xie and Varshavsky, 2002). Furthermore, they showed that Ufd4p lacking the proteasome-binding region, while still able to catalize ubiquitination, its substrates are impaired in degradation (Xie and Varshavsky, 2002). In this model, Ufd4p catalyzes ubiquitination of its substrates, but also delivers them directly into the 26S proteasome through its direct interaction with the 19S particle.

Materials and Methods

DNA Constructs

The entire parkin coding region was PCR-amplified from a PC12 cDNA library (gift of Dr. Steven Morris, MNI) and subcloned into EcoRI and NotI sites of pcDNA 3.1 (Invitrogen). Glutathione-S-transferase (GST)-Parkin, GST-Parkin^{UBL}, and GST-Parkin$^{\Delta UBL}$ were prepared by PCR amplification and by subcloning the corresponding fragments into pGEX-5X-1 (Amersham Biosciences). GST-Parkin$^{UBL\text{-}I44A}$ was prepared using site-directed mutagenesis kit (Stratagene). All Rpn13 constructs were provided by our collaborator, Ivan Dikic (Franfurt, Germany). Myc-Rpn13 WT miRNA^{R} (resistant to KD) were produced using site-directed mutagenesis kit (Stratagene).

Expression of fusion proteins

GST-tagged Parkin^{FL}, Parkin^{UBL}, Parkin$^{UBL\text{-}I44A}$, Parkin$^{\Delta UBL}$, hPlic2^{UBL}

Parkin^{FL}, Parkin^{UBL}, Parkin$^{UBL\text{-}I44A}$, Parkin$^{\Delta UBL}$, and hPlic2^{UBL} GST-tagged fusion proteins were transformed into BL21 Rosetta^{TM} chemically competent *E. coli* (Novagen). Fresh colonies were inoculated into 10 ml of LB broth with appropriate antibiotic and incubated overnight at 37°C with shaking (250 rpm). 500 ml of LB broth with appropriate antibiotic was then inoculated with 5 ml of overnight culture and grown at 37°C with shaking (250 rpm) for approximately 2 hours until grown to mid-log phase (OD_{600} = 0.6). 1M IPTG was then added to a final concentration of 0.04M to induce expression and incubated at room temperature (~25°C) with shaking (250 rpm) for 3 hours. Cells were harvested by centrifugation at 5000 rpm for 10 min, resuspended in 15ml of phosphate-buffered saline (PBS) and protease cocktail (0.02 mg/ml PMSF, 0.1 mg/ml

benzamidine, 0.5 µg/ml aprotonin, 0.5 µg/ml leupeptin) and lysed by sonication. 10% Triton X-100 (Fisher Biotech) was then added to a final concentration of 1% and incubated for 30 minutes at 4°C with rotation before centrifugation at 12 000 rpm for 15 minutes. The soluble extract was stored at -20°C in 10% glycerol (Fisher Biotech).

Purification of GST-tagged fusion proteins

GST-tagged proteins were purified by incubating 5-10 ml (depending on the level of expression) with 150 µl of glutathione-sepharose beads (50% v/v in cold PBS) for 2 hours at 4°C on a rotator. Beads were then washed 3 times with 1 ml of cold PBS and resuspended in a final volume of 500 µl in PBS with protease inhibitor cocktail.

Purification of HIS-tagged fusion proteins

His-tagged $Rpn13^{Fl}$, $Rpn13^{\Delta Pru}$ and $Rpn13^{Pru}$

HIS-tagged proteins were expressed following the same conditions as GST-tagged proteins, but they were purified by incubating 5-10 ml (depending on the level of expression) with 500 µl of 50% Ni-NTA slurry (Qiagen) for 4 hours at 4°C on a rotator. Beads were then washed 3 times with 1 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, adjusted to pH 8.0 with NaOH) and eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 200 mM imidazole, and protease inhibitor cocktail, adjusted to pH 8.0 with NaOH) to a final volume of 500 µl.

SDS-PAGE

Samples were prepared by SDS-PAGE with either a 2-gel mini-gel apparatus (BIO-RAD) or a large 4-gel vertical slab apparatus (Hoefer Pharmacia Biotech). A typical 10% gel was prepared in two parts. First, the resolving gel by combining 2.5 ml of 1.5M Tris-HCl (pH 8.8), 100 µl of 10% SDS, 5.0 ml of bis-acrylamide, 50 µl of 10% ammonium persulfate, 10 µl of TEMED, and water to 10 ml. Second, the stacking gel by combining 1.75 ml of 0.5M Tris-HCl (pH 6.8), 10% SDS, 1.65 ml bis-acrylamide, 50 µl of 10% ammonium persulfate, 10 µl TEMED and 3 ml of water.

Samples to be separated were first prepared in sample buffer to a final concentration of 0.05M Tris-HCl (pH 6.8), 6.7% glycerol, 1.3% SDS, 100 mM DTT, and bromophenol blue and then boiled for 10 minutes prior to loading on a gel. Large gels were run for approximately 3 hours at 280 V and either stained with Coomassie blue to visualize total protein, or transferred to nitrocellulose membrane for immunoblotting (Western Blot).

Western Blot

Following SDS-PAGE, large gels were transferred onto nitrocellulose membranes using blotting cassette (Hoefer) between 2 sponges and 5 whatman paper squares. Gels were transferred for 3 hours at 0.85 A. Ponceau Red was used to verify protein transfer and rinsed away with 0.1% Tween in phosphate-buffered saline (PBS-T). Membranes were blocked with 5% skim milk in PBS-T for a minimum of 1 hour at room temperature. Primary antibody incubation was overnight at 4°C followed by a three 10 minute wash in PBS-T. Secondary antibodies, conjugated to HRP, were prepared in 5% skim milk in PBS-T and incubated at room temperature

for 1 hour followed by three 10 wash in PBS-T. Antibody-protein complexes were visualized using Western Lighting Chemiluminescence (Perkin Elmer).

Antibodies

The antibodies used for the immunoblots were acquired and used as follows:

α-Rpt1 (Biomol cat no. pw8825) the dilution used was 1:5K. α-His (NEB cat no.2366) dilution 1:10K. α-Myc (Santa Cruz cat no. SC40) dilution 1:20K. α-Rpn2 (Biomol cat no. pw9270) dilution 1:5K. α-Rpn13 (Biomol cat no. pw9910) dilution 1:5K. α-actin (Chemicon catno. MAB1501) dilution 1:35K. α-parkin (Santa Cruz cat no. sc32282) dilution 1:10K. α-p38 (kindly provided by Dr. Olga Corti. Paris, France) dilution 1:5K. α-CDCrel (Chemicon catno. MAB 5358) dilution 1:5K. α-ubiquitin (Covance cat no. MMS-258R) dilution 1:20K. α-Eps15 (BDtransduction cat no. 610806) dilution 1:10K. α-PICK1 (Affinity cat no. PA1-073) dilution 1:5K.

Cell culture and transfection

HEK 293T cells, derived from human kidney, were maintained in Dulbecco's modified eagle medium supplemented with 10% v/v fetal bovine serum (heat-inactivated), 1% v/v penicillin/streptavidin, and 4mM L-Glutamine. Cells were kept into petri dishes as required for experimentation at 37°C and 5% CO_2.

Myc-Rpn13 WT, F76R and miRNA^R transfection

Cells dishes, 70-80% confluent, were transiently transfected using standard lipofectamine reagent protocol (Invitrogen)

Cell lysate preparation

Cell culture dishes were washed 3 times in cold PBS and harvested into microcentrifuge tubes (1.5ml) in Lysis hypotonic buffer (25mM Tris HCl pH 8.0, 5mM $MgCl_2$, 0.5mM EDTA and protease inhibitors cocktail). Cells were then sonicated for 15 seconds using wave amplitude of 20 and incubated at 4°C for 30 minutes in constant agitation. Lysates were centrifuged at 12,000g for 10 minutes at 4°C. The final supernatant was then assayed for protein content using BCA protein assay kit (Pierce).

GST fusion affinity chromatography (GST-pulldown)

Bacterially-expressed and purified GST-fusion proteins were incubated with appropriate lysate (1mg) or purified protein in stated binding buffer (10% v/v Glycerol, 50mM Tris HCl pH 7.4, 10mM $MgCl_2$, 1mM DTT, 25mM NaCl and protease cocktail inhibitors) for 4 hours at 4°C with rotation, unless specified otherwise. The beads and bound complexes were then washed 3 times in 1ml of binding buffer. Samples were finally resuspended in SDS-PAGE sample buffer.

Lentivirus based shRNAmiR constructs

First, the oligonucleotides encoding the shRNA were designed using the software BLOCK-iT from invitrogen. Oligos were then resuspended to a final concentration of 1ug/ul. 2.5 ul from each oligo were combined (sense with antisense) and annealed using annealing buffer (20mM

Tris HCl pH 7.8, 100mM NaCl and 0.2mM EDTA) and heating to 95°C with a slow cool down to about 40°C.

Oligo 356 sense.	TGCTGTTGAACTTCAGCACGTAGACCGTTTTGGCCACTGACTGACGGTCTACGCTGAAGTTCAA
Oligo 356 antisense.	CCTGTTGAACTTCAGCGTAGACCGTCAGTCAGTGGCCAAAACGGTCTACGTGCTGAAGTTCAAC
Oligo 1048 sense	TGCTGTATTCTGGATCTCATCCGCGGGTTTTGGCCACTGACTGACCCGCGGATGATCCAGAATA
Oligo 1048 antisense	CCTGTATTCTGGATCATCCGCGGGTCAGTCAGTGGCCAAAACCCGCGGATGAGATCCAGAATAC

Phosphorylation of the annealed oligos was done following the specifications from T4 polynucleotide kinase (Invitrogen). These oligos were cloned into a previously digested (BsaI) vector pcDNA6.2/GW-EmGFP.miR (Invitrogen) using standard cloning procedures and following the specifications for T4 DNA ligase (Invitrogen).

Positive constructs were selected according to their restriction pattern using PvuII, and sequenced to confirm that there were not point mutations and recombination. Using these constructs in transient transfection of HEK 293T cells allowed us to determine to some extent the efficiency at which our gene of interest was knocked down (Rpn13).

Lentivirus production

To get our construct into the final vector for viral production, we PCR amplified the expression cassette using the following primers:

miRGFP BglII sense	GCGCAGATCTACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGC
miRXFP XhoI antisense	GCGCCTCGAGTGCGGCCGGATCTGGGCCATTTGTTCCATGTGAGTGC

and standard PCR specifications for platinum *pfx* (Invitrogen). The PCR product was then digested accordingly (BglII and XhoI) and cloned into a previously digested (BamHI and SalI) pRRLsinPPTeGFP vector (kindly provided by Dr. Brigitte Ritter). Positive clones were selected according to the restriction pattern generated by EcoRI and XbaI, and sequenced to discard point mutations.

For the production of viral particles, we used The Miami System (Addgene), which is an HIV-based system composed of Expression vector (our previous construct) pRRLsinPPTeGFP-(356 or 1048), and three packaging mix; pRSV-Rev, pMD2.g and pMDLg/pRRE. The viral particles were produced in regular HEK 293T cells following the standard procedure for the Miami System and following the security regulations from Standard Operating Procedure (SOP) written by Dr. Phillip Barker (McGill) (appended).

Nuclear magnetic resonance (NMR)

The Rpn13 PRU (1-130) and 15N-labeled parkin Ubl (1-76) were purified by size-exclusion chromatography in NMR buffer (30mM sodium phosphate pH 7.0, 200mM NaCl, 0.5mM DTT, 0.1mM EDTA). Proteins were concentrated using ultrafiltration devices (MWCO 3K) and 5% D2O was added. 1H-15N HSQC spectra were recorded at a temperature of 303K using proteins at the following concentrations (in uM):

[15N-UBL]	[PRU]
800	0
410	180
270	240
207	260

Weighted-average chemical shift perturbations were measured as the difference between the first and last spectra using the following formula:

SQRT (ΔH^2 + (ΔN/5)^2).

Surface plasmon resonance (SPR)

SPR was performed using a Ni-NTA chip on a BIAcore T100 instrument (GE Healthcare) at 25C, using the SPR buffer (10mM HEPES, 150mM NaCl, 0.05% P20, pH 7.4). 100mM NiSO4 was injected to activate the surface, 125nM His6-PRU was injected to 1000 RU. Then, concentrations series of parkin Ubl (0.05-25uM) or ubiquitin (0.2-50uM) were injected for one minute. Affinities were determined by fitting the steady state equilibrium responses (50-60sec post-injection) as a function of concentration using a simple 1:1 binding isotherm.

Previous results from our laboratory

Given that Ubl domains are known to bind the proteasome, that parkin has a Ubl domain in its N-terminus and that it functions as an E3 ligase, Michael Haber, a former lab member, began to test whether parkin could interact with the proteasome. In his M.Sc thesis in 2004, he showed that parkin could interact with the proteasome. In collaboration with Dr. Suzanne Elsasser and Dr. Daniel Finley, Mike Haber tested whether the Ubl domain of parkin was able to interact directly with purified bovine proteasomes (figure 5). They performed a series of non-denaturing gel-mobility shift assays with GST, GST-parkin^{Ubl} and GST-RAD23^{Ubl} combined with purified bovine proteasomes. As expected, a reduction in proteasome mobility was observed for RAD23^{Ubl} (It is a well known Ubl-Uba protein that interacts with the proteasome), but also, the same effect was observed for parkin^{Ubl}. This results, strongly suggested that the Ubl domain of parkin is able to interact with the proteasome. Furthermore, in order to test whether the purified proteasomes were active, and presumably containing all its subunits, the artificial proteasome substrate suc-LLVY-AMC was added. This artificial substrate fluoresces upon its cleavage by the chimotrypsin activity of the β-subunits within the 20S core.

After identifying a direct interaction between parkin and the 26S proteasome, Mike Haber tested whether a mutation in the parkin Ubl domain (R42P), which is a cause of Parkinson's disease, has an effect on proteasome binding. To test this possibility, a series of *in vitro* binding assays were carried out. HEK293 cell lysates (as a source of proteasomes) were incubated with different GST constructs (figure 6) and probed for a known 19S subunit, S7 (Rpt1 in yeast). His prediction was correct, and he showed that this mutation causes a reduced interaction between parkin and the proteasome.

Parkin's Ubl domain interact with the 26S proteasome

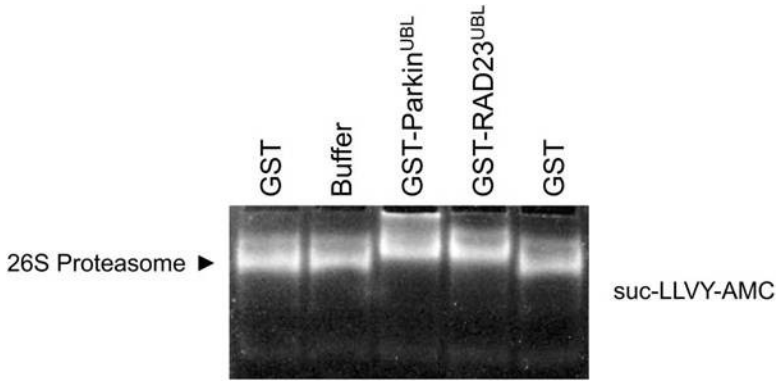

Figure 5. The Ubl domain of parkin interacts directly with the 26S proteasome. Gel mobility-shift assay of the interaction between purified bovine 26S proteasome and both parkin and RAD23 Ubl domains. The mobility of the proteasome following non-denaturing PAGE was visualized with fluorogenic substrate suc-LLVY-AMC. Binding of both parkin and RAD23 Ubl domains decreases the mobility of the proteasome (In collaboration with Dr. Suzanne Elsasser).

Parkin's AR-JP causing mutation disrupts the interaction with the proteasome

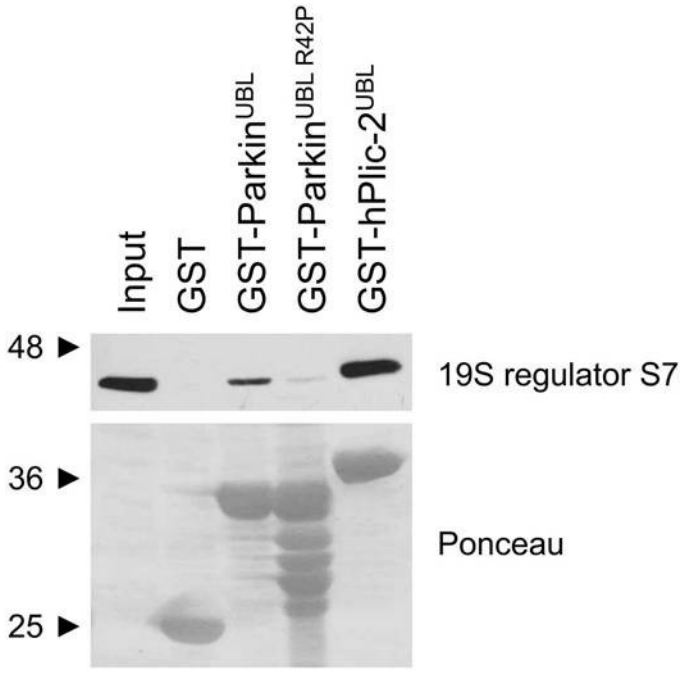

Figure 6. Parkin AR-JP-linked R42P mutation inhibits binding to the proteasome. Glutathione-Sepharose-immobilized GST, GST-Parkin^{Ubl}, GST-Parkin$^{Ubl\text{-}R42P}$ and GST-hPlic-2^{Ubl} was incubated with HEK293 cell lysates, analized by SDS-PAGE and immunoblotted with anti-19S regulator S7.

At the time, there was no knowledge about Rpn13 as an ubiquitin receptor, hence, the most likely candidate to mediate the interaction parkin-proteasome was S5a (Rpn10 in yeast). In order to identify a direct interaction between parkin and Rpn10, a series of *in vitro* experiments were performed exactly as in figure 5, but this time, they used yeast purified proteasomes with or without Rpn10 (mutant yeast strain). They found comparable binding between parkin and the proteasome (figure 7), regardless of the presence or absence of Rpn10. This data, strongly suggested that parkin interacts with the proteasome via a different subunit.

Rpn10 does not mediate the interaction parkin-proteasome

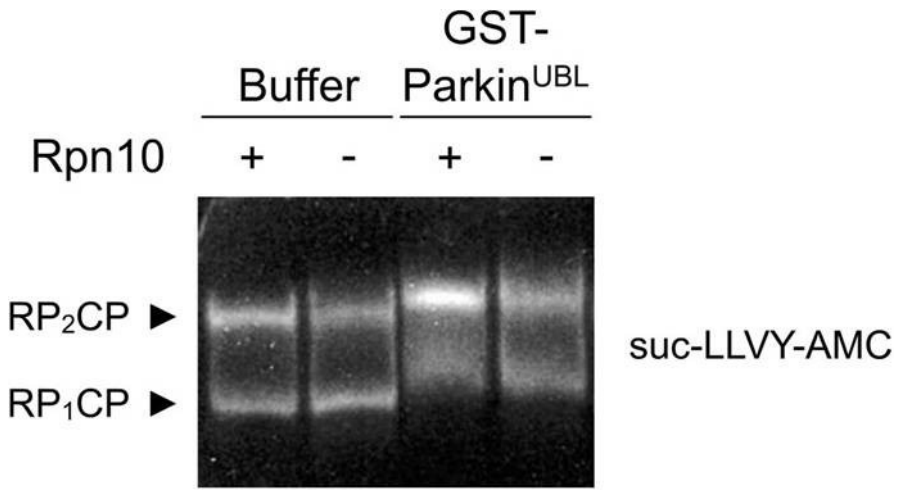

Figure 7. The Ubl domain of parkin interacts directly with *S. cerevisiae* proteasomes with or whitout Rpn10. Gel mobility-shift assay of the interaction between the Ubl domain of parkin and the proteasome purified from wild type or Rpn10Δ mutant *S. cerevisiae*. The mobility of the proteasome following non-denaturing PAGE was visualized with fluorogenic substrate suc-LLVY-AMC. Binding of the Ubl domain of parkin decreases the mobility of the proteasomes with or without Rpn10 present. RP_1CP and RP_2CP refers to singly or doubly 19S capped 20S proteasomes respectively (In collaboration with Dr. Suzane Elsasser).

Results

Parkin's Ubl domain interacts with purified Rpn13

Previous work in our laboratory conducted by Michael Haber in 2004, showed unequivocally that parkin's Ubl domain interacts with the 26S proteasome. Unfortunately, he could not identify the subunit within the 26S proteasome responsible for this interaction. His work however, showed among other things that S5a/Rpn10 is not the subunit mediating this interaction (figures 5-7).

In 2008, Dr. Ivan Dikic's group in Frankfurt, Germany, described a new subunit within the 19S particle, Rpn13. Their work proved that Rpn13 is an Ub receptor that mediates this interaction using a novel Ub recognizing domain, called Pru domain (pleckstrin-homology domain receptor for ubiquitin). This domain, not only serves to recognize Ub, but also interacts with Ubl-Uba domains containing proteins (Husnjak et al., 2008; Schreiner et al., 2008). During this work, they found in a series of yeast two-hybrid assays (Y2H) that parkin interacts with Rpn13 (unpublished). This finding lead to a collaboration between Dr. Dikic's laboratory and ours.

To determine whether the Ubl domain of parkin can interact with Rpn13, we performed affinity chromatography with bacterially expressed and purified GST-fusion proteins: GST, GST-Ub, GST-parkin^{Ubl}, GST-4X-Ub and GST-hPlic-2^{Ubl}. These constructs were incubated with bacterially expressed and purified His-fusion proteins: His-Rpn13^{FL}, His-Rpn13$^{\Delta Pru}$ and His-Rpn13^{Pru} (binding buffer: Tris-HCl 50mM pH 7.4, NaCl 50mM, DTT 1mM, EDTA 0.1mM, Tx-100 1% and protease cocktail inhibitors) (Figure 8).

Under these conditions, and probing for the Histidine tag, we were able to determine that parkin's Ubl domain is able to directly bind both full-length Rpn13 and its isolated Pru domain. This interaction is completely abrogated when Rpn13's Pru domain is absent.

As a positive control, we included GST-Ub, GST-4xUb and GST-hPlic2^{Ubl}. Although Rpn13 is an ubiquitin receptor, it was not able to bind GST-Ub in these assays; the most likely explanation might involve the relative low affinity for mono-Ub compared to poly-Ub or Ubl domains, as described for another Ub receptor, Rpn10/S5a (Walters et al., 2002). Indeed, even though the Ubl domains of Ubl-Uba proteins highly resemble ubiquitin; there are slight differences in the hydrophobic patch known to interact with Ub-receptors (figure 4). In 2002, in a series of nuclear magnetic resonance (NMR) experiments, Kylie Walters et al., proved that S5a has a lower affinity for mono-Ub than for the Ubl domains of hPlic-2 or hHR23A. Thus, if we consider a similar mechanism for Rpn13, in which preference is given for poly-Ub chains, our results remain well in line with previous data.

Parkin-Rpn13 interaction is mediated through Ubl-Pru domains

Having identified direct binding between parkin's Ubl and Rpn13, we next sought to map the domain or domains in both, Rpn13 and parkin responsible for this interaction. In order to do this, we used different His-tagged Rpn13 constructs combined with different GST-Parkin constructs in an affinity chromatography assay (GST-pulldown) (figure 9 (b)). When immunoblotting for His, it became clear that the Ubl domain of parkin as well as the Pru domain of Rpn13 were a requirement for this interaction to take place. Interestingly, this interaction seems to be strengthened when both, parkin and Rpn 13 were combined in their full length forms, or, when using only the Ubl domain of parkin and the isolated Pru domain of Rpn13. At the time that this experiment took place, it was not known that Rpn13 requires to bind the 19S base proteasomal subunit Rpn2 in order to expose its Pru domain (Chen et al., 2010), since these experiments are lacking Rpn2, we speculated that when both proteins are in its full-length form, the C-terminus part of both proteins prevents the Pru domain to fold back into its "hidden" state, thus an increase in binding is observed when both proteins are in their full-length forms (further discussed on page 69).

In parallel, Dr. Koraljka Husnjak from Dr. Ivan Dikic's group, further corroborated our results using yeast two hybrid assay (Y2H) in which, she used the Ubl domain of parkin as prey, and different constructs of Rpn13 as bait: hRpn13 aa1-407 ($Rpn13^{Fl}$), hRpn13 aa1-150 ($Rpn13^{Pru}$) and hRpn13 aa151-407 ($Rpn13^{\Delta Pru}$) (figure 9 (A)).

The Ubl domain of parkin directly binds Rpn13

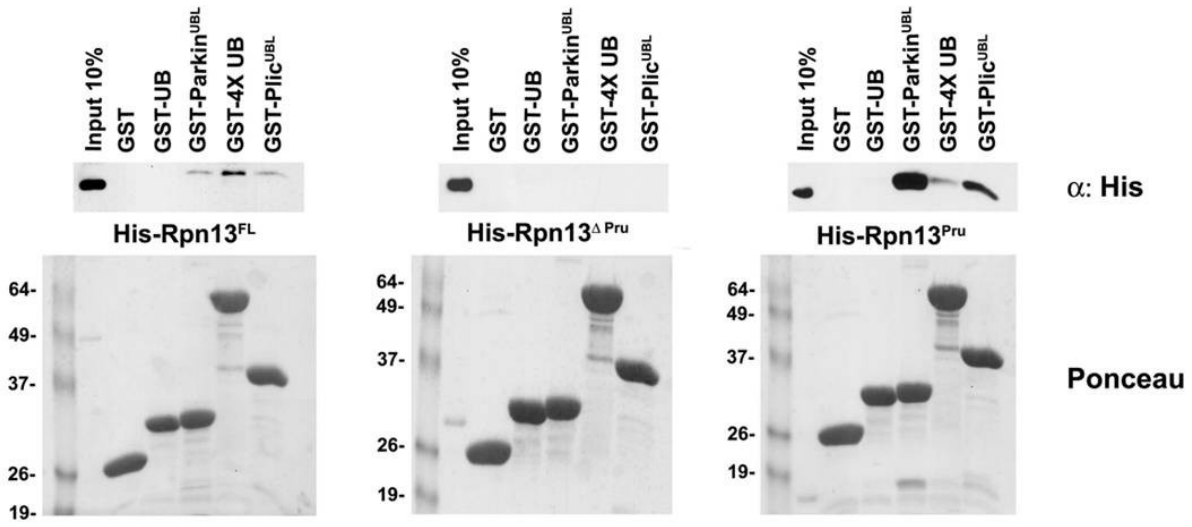

Figure 8. The Ubl domain of parkin directly interacts with Rpn13. His-tagged Rpn13 constructs: His-Rpn13^{FL} (Rpn13 full length), His Rpn13$^{\Delta Pru}$ (Rpn13 lacking its Pru domain) and His-Rpn13^{Pru} (Only the Pru domain of Rpn13) were incubated with different GST fusion proteins: GST-Ub (ubiquitin), GST-Parkin^{Ubl} (Ubl domain of parkin), GST-4xUb (4 ubiquitins linked N-terminus to C-terminus) and GST-Plic^{Ubl} (the Ubl domain of hPlic-2). Affinity blotting for His showed, the Ubl domain of parkin has high affinity for the Pru domain of Rpn13, and a moderate affinity for Rpn13 in its full-length form. This interaction is completely abrogated when Rpn13 is missing its Pru domain. Ponceau staining is shown to prove that similar amounts were used for the different GST-fusion proteins.

Point mutations within the Ubl domain of parkin or the Pru domain of Rpn13 disrupt parkin-Rpn13 interaction *in vitro*

The interaction between Rpn13's Pru domain and Ub has been previously characterized and described by our collaborating group (Scheirner et al. 2008). This interaction takes place using a series of loops within the Pru domain of Rpn13 and a very important hydrophobic interaction mediated by I44 in Ub and F76 in Rpn13. Since parkin's Ubl domain and ubiquitin share homology in the hydrophobic patch that is know for ubiquitin to interact with its receptors (compare figure 4 (C) and (D)), and I44 is well conserved between Ub and the Ubl domain of parkin, we hypothesized that should be a critical residue mediating this interaction. To test whether this holds true in a more "physiological way" we transfected HEK 293T cells with: pcDNA 3.1 (empty), myc-Rpn13 (wild type) and myc-Rpn13 F76R (point mutation). Lysates from these transfected cells were incubated with bacterially expressed and purified GST-fusion proteins: GST, GST-parkin^{Ubl} and GST-Parkin$^{Ubl\text{-}I44A}$. When probing for myc, we saw that only GST-Ubl (wild type) was able to pull-down myc-Rpn13 (wild type), (figure 10 middle panel); furthermore, this interaction is impaired when using either I44A mutation in parkin's Ubl or when using cell lysates coming from myc-Rpn13 F76R mutant (figure 10).

The interaction between parkin and Rpn13 is mediated by both, the Ubl domain of parkin and the Pru domain of Rpn13

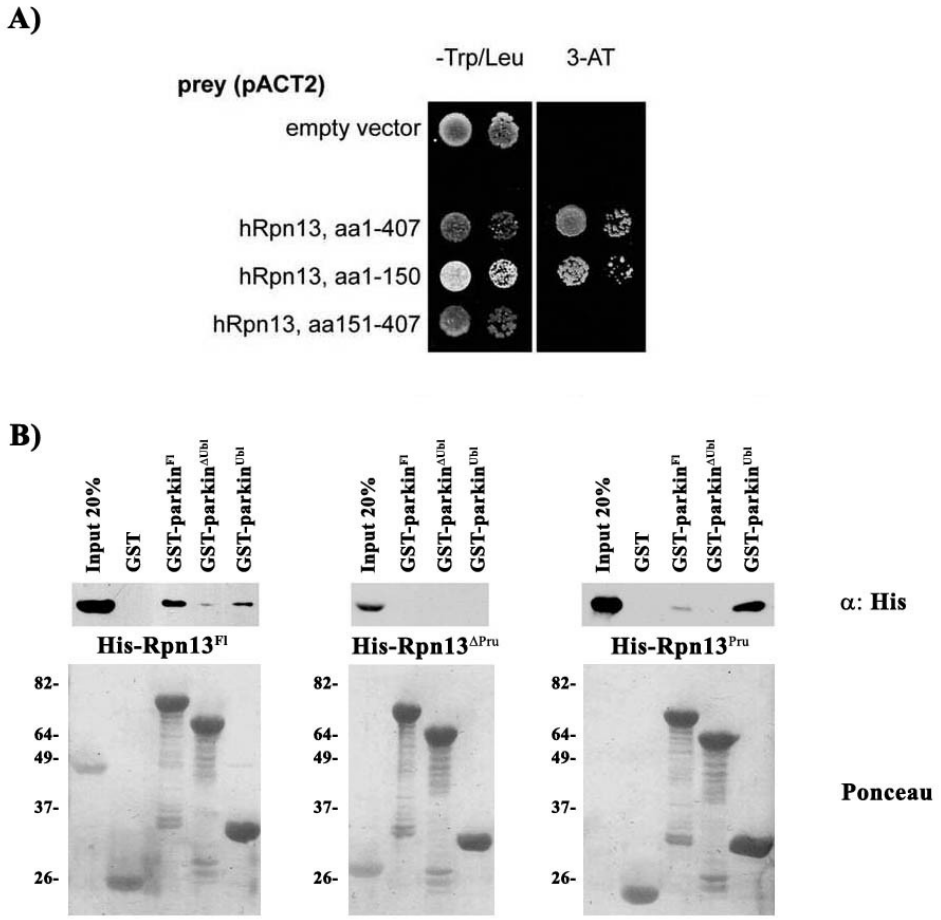

Figure 9. (A) Yeast two hybrid assay using parkin's Ubl domain as a prey and different Rpn13 constructs as bait: hRpn13 aa 1-407 (full-length), hRpn13 aa 151-407 (ΔPru) and hRpn13 aa 1-150 (Pru). Done by Koraljka Husnjak. Frankfurt, Germany. (**B**) His-Rpn13 constructs were incubated with different GST-Parkin constructs. Immunoblot for His shows that Parkin-Rpn13 interaction is mediated trough Parkin's Ubl and Rpn13's Pru domain. Ponceau staining is shown to prove that similar amounts were used for the different GST-fusion proteins.

Point mutations within the Ubl domain of parkin or the Pru domain of Rpn13 disrupt parkin-Rpn13 interaction

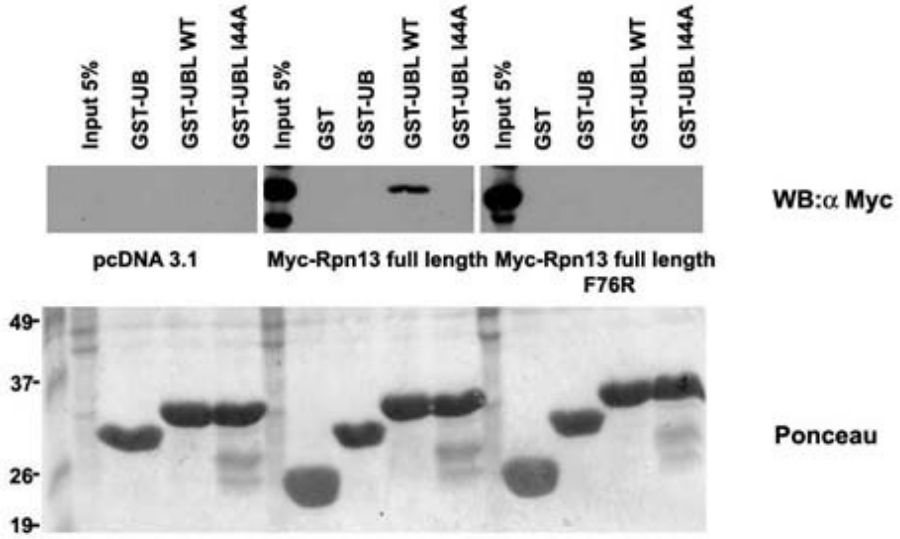

Figure 10. HEK 293T cells were transfected for 48h with either the empty vector (pcDNA3.1), Myc-Rpn13 full-length WT and Myc-Rpn13 full-length F76R. Lysates coming from these cells were used for affinity chromatography (GST-pulldown) using GST, GST-Ub, GST-Parkin^{Ubl} (GST-UBL) and GST-Parkin$^{Ubl\text{-}I44A}$ (GST-UBL I44A). When probing for Myc, we observed that only GST-Parkin^{Ubl} WT was able to bind Myc-Rpn13 WT (middle panel). Ponceau staining is shown to prove that similar amounts were used for the different GST-fusion proteins.

A designed mutation I44A within the Ubl domain of parkin, disrupts its ability to bind the 26S proteasome

Having tested that I44 is a critical residue within parkin's Ubl domain to bind Rpn13, we next decided to prove if this point mutation would render the Ubl domain of parkin unable to bind the 26S proteasome (figure 11 (B)).

In order to confirm our prediction, we incubated lysates coming from HEK 293T cells (as a source of proteasomes) with different GST-fusion proteins: GST, GST-Parkin^{Ubl}, GST-Parkin$^{Ubl\text{-}I44A}$ and GST-hPlic2^{Ubl}. When immunoblotting for different 19S particle subunits, Rpn2 and Rpt1 (arrows in figure 11 (A)), we saw a dramatic binding reduction in parkin's Ubl harboring the I44A substitution; compare the Ubl WT with Ubl I44A.

The reasoning behind these experiments is preceded by Mike Haber's experiments (figure 5). Knowing that parkin's Ubl domain is able to interact with the proteasome, we speculated that, under the right conditions (binding buffer) it should be able to pull-down the entire 26S proteasome, or at least the 19S regulatory particle (figure 11 (A)). Thus, instead of probing for Rpn13, we probe for other subunits such as Rpn2 and Rpt1 (figure 11 (B)). As a positive control we've included GST-hPlic-2^{Ubl} which is known to bind S5a/Rpn10.

Although, as previously tested, I44A substitution within the Ubl domain of parkin disrupts the binding with Rpn13 and presumably the binding with the 26S proteasome, our results in figure 11 can not completely exclude the possibility for this mutation to disrupt the interaction with other subunits within the 19S particle. Hence, further experiments need to be performed.

I44A substitution within the Ubl domain of parkin, disrupts its interaction with the 26S proteasome

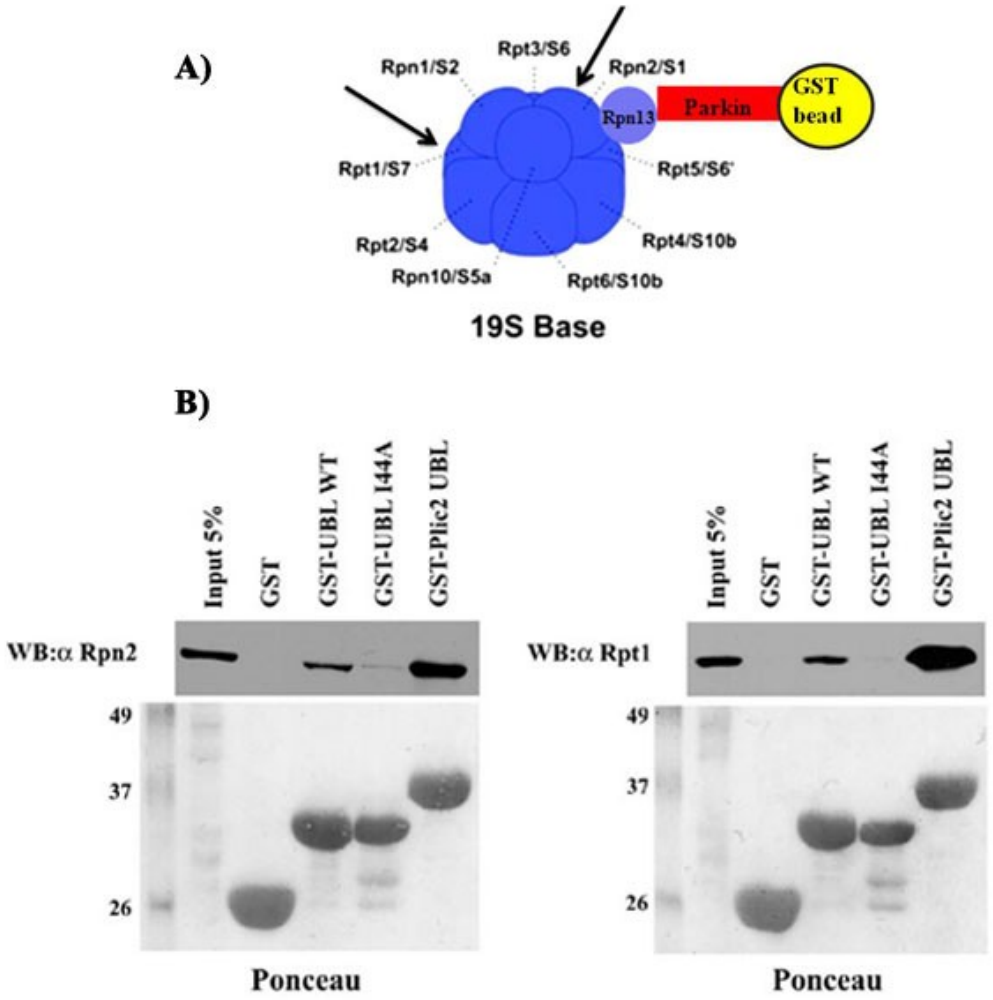

Figure 11. A) Scheme showing the reasoning behind the experiment in (B), arrows mark the relative position of the subunits in 19S that were probed. B) Glutathione-sepharose-immobilized GST, GST-Parkin$^{Ubl\text{-}WT}$ (GST-UBL WT), GST-Parkin$^{Ubl\text{-}I44A}$ (GST-UBL I44A) and GST-hPlic-2^{Ubl} (GST-plic2 UBL) were incubated with lysates from HEK 293T cells as a source of proteasomes. Bound proteins were immunoblotted for 19S Rpn2 subunit and 19S Rpt1 subunit after SDS-PAGE.

Parkin's Ubl domain does not interact with Rpn10/S5a, Rpn2/S1 or Rpn1/S2

Since the beginning of this decade there has been evidence that parkin interacts with the 26S proteasome; studies suggest, the 19S particle subunit S5a/Rpn10 (Sakata et al., 2003; Safadi et al., 2010). Although, these studies have proved an interaction between parkin and Rpn10, the experimental procedures are mainly based in a series of NMR experiments. Due to the sensitivity of this instrument in which the chemical shifts on amino acid residues coming from two purified proteins are measured, an interaction between the Ubl domain of parkin and the UIM domains of Rpn10 would be expected. However, the lack of functional experiments renders these conclusions weaker.

Other subunits within the 19S particle, namely S1/Rpn2 and S2/Rpn1 have also been implicated in binding parkin, at least theoretically (Elsasser et al., 2002; Saeki et al., 2002), and even the 20S core particle subunit α4 has been implicated in mediating parkin-proteasome interaction (Dächsel et al., 2005).

Due to the controversies regarding the subunit responsible for parkin-proteasome interaction, our collaborating group, specifically Dr. Koraljka Husnjak decided to test for parkin's Ubl interaction with other subunits in the 19S regulatory particle. For this purpose, a series of Y2H assays were carried out (figure 12).

Using the Ubl domain of parkin as prey, and different constructs of S5a/Rpn10, S1/Rpn2 and S2/Rpn1 as bait, they were able to determine that only the Pru domain of Rpn13 is able to sustain the growth of yeast when challenged in conditional media (Figure 12 second row). Strongly suggesting that, even if there is an interaction between parkin's Ubl and Rpn10, this interaction is not as strong as the one with Rpn13.

Y2H assay to challenge other subunits in the 19S regulatory particle that could be mediating parkin-proteasome interaction

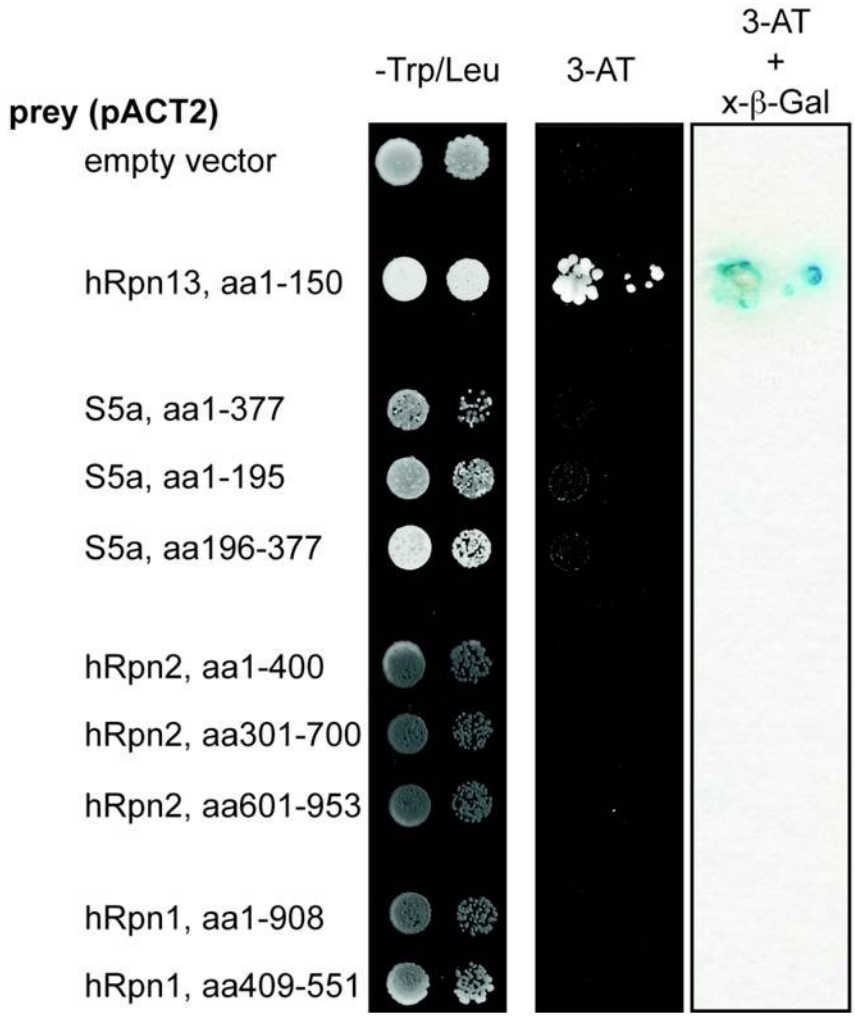

Figure 12. Y2H assay using parkin's Ubl domain as prey and different 19S particle subunits as bait: hRpn13 aa 1-150 (Rpn13's Pru domain); S5a aa 1-377 (hRpn10 full-length), S5a aa 1-195 (hRpn10 UIM 1), S5a aa 196-377 (Rpn10 UIM 2); hRpn2 aa 1-400 (First LRR-like motif), hRpn2 aa 301-700 (Fisrt LRR-like motif, KEKE motif and second LRRR-like motif), hRpn2 aa 601-953 (Second LRR-like motif); hRpn1 aa 1-908 (S2/Rpn1 full-length: LRR-like and KEKE motifs), hRpn1 aa 409-551 (Just LRR-like motif). LRR-like stands for Leucine-rich-repeat like motif. KEKE motif is composed of alternating positive and negative residues. Both motifs have been shown to mediate protein-protein interactions.

Knocking down Rpn13 abolishes parkin-proteasome interaction

After having established that other subunits such as S5a/Rpn10, S2/Rpn1 and S1/Rpn2 within the 19S regulatory particle were not mediating the parkin-proteasome interaction (figure 12). We've decided to fully test our model, in which Rpn13 mediates this interaction. For this purpose, three shRNAmiR lentivirus based constructs were designed to knock down (KD) Rpn13 in cells. shRNAmiR-control: produces a miRNA that has no mRNA target, although, as for all the constructs, infection of the cells is achieved; shRNAmiR-356: produces an miRNA that targets the mRNA of Rpn13 starting at nucleotide 356; shRNAmiR-1048: produces an miRNA that targets the mRNA of Rpn13 starting at nucleotide 1048.

When infecting HEK 293T cells with the 3 constructs previously mentioned, it became clear that construct shRNAmiR-356 was able to KD Rpn13 with high efficiency, although construct shRNAmiR-1048 was able to KD Rpn13 to some extent, it was not very efficient. Finally, our control construct had no apparent consequence; either on Rpn13 or the general phenotype of the cells (figure 13, bottom right).

Following the same principle as in figure 11 and using lysates coming from these previously infected cells, Glutathione-sepharose-immobilized GST, GST-Parkin^{Ubl} and GST-hPlic-2^{Ubl}, were incubated overnight at 4°C. After running an SDS-PAGE and probing for Rpt1 or Rpn2 we were able to determine that Rpn13 is a requirement for the Ubl domain of parkin to pull-down the proteasome (Figure 13 middle panel). As a control, the Ubl domain of hPlic-2 was used in these assays. As expected, and because hPlic-2 binds Rpn10, there was no reduction in hPlic-2^{Ubl}-proteasome interaction, regardless of the presence or absence of Rpn13.

Lentivirus based KD of Rpn13 in HEK 293T cells

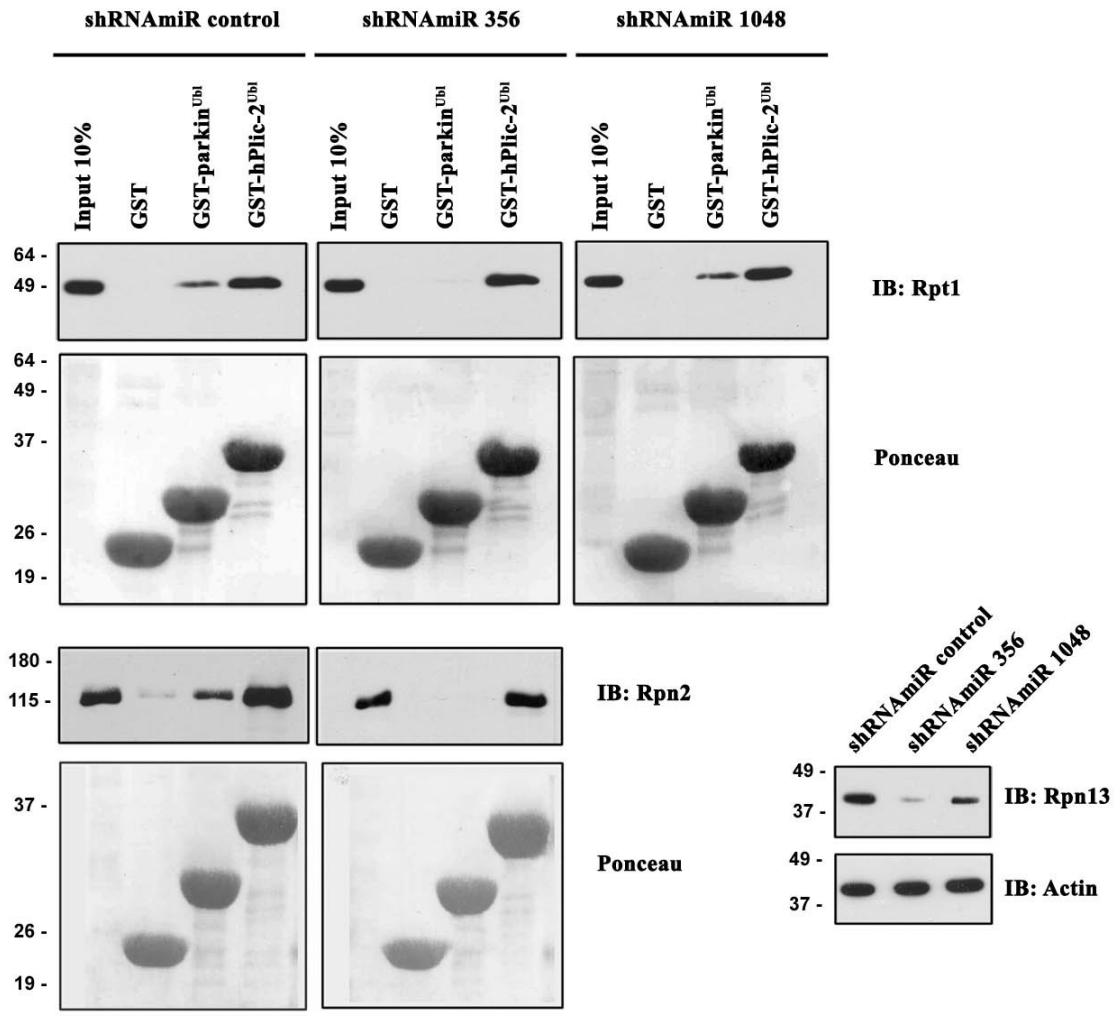

Figure 13. Proteasome pull-down using Glutathione-sepharose-immobilized GST, GST-Parkin^{Ubl} and GST-hPlic-2^{Ubl}, combined with lysates from HEK 293T cells previously infected with a lentivirus control (shRNAmiR-control), a lentivirus 356 (shRNAmiR-356) or lentivirus 1048 (shRNAmiR-1048). Pull-down efficiency was tested by immunoblotting for 19S regulatory complex subunits Rpt1 and Rpn2. Knock down of Rpn13 was assessed by immunoblotting for Rpn13 and actin (bottom right panel).

Transfection of Myc-Rpn13 rescues parkin-proteasome interaction

To further prove that the loss of binding observed between the parkin Ubl domain and the proteasome was caused by silencing Rpn13 and not a general effect due to the infection. The rescue of this phenotype should be possible by introducing exogenous Rpn13. For this reason, we decided to transfect a myc-tagged murine construct of Rpn13 and check for a rescue of the interaction between the Ubl and the 26S proteasome (figure 14).

Because murine Rpn13 and its human homolog are strikingly similar in both DNA coding sequence and amino acid composition, a series of point mutations were generated in order to prevent our myc-construct from getting knocked down by the miRNA upon infection. Because our lentivirus based KD is designed to target a zone (20 nucleotides) starting at position 356 in the mRNA of Rpn13, these point mutation were performed around that particular position. It is worth mentioning, that the point mutations we've done in this construct, changes the mRNA on its nucleotide composition, but not in its coding potential (the protein will remain the same in its amino acid composition).

To determine that knocking down Rpn13 can be rescued with our myc-Rpn13-shRNAmiR-resistant (myc-resistant-c) construct, we performed GST-pulldowns with GST, GST-Parkin^{Ubl} and GST-hPlic-2^{Ubl} incubated with lysates coming from cells previously infected with shRNAmiR-356. These infected cells were either transfected with myc-resistant-c or mock transfected for 48h before the lysis. When probing for the 19S subunit Rpt1, it became evident that our transfected cells (myc-resistant-c) were rescued. Parkin's Ubl domain was able to pulldown the proteasomes from these cells (figure 14 compare panel A) with B)). The extent of

Rpn13 KD is shown in figure 14 panel C), as well as the level of expression of our myc-resistant-c.

Taken together, our results suggest that Rpn13 is crucial for the parkin-proteasome interaction, and the fact that we were able to rescue the KD phenotype (proteasome pull-down) strongly implicates a level of specificity for this interaction.

Nuclear magnetic resonance (NMR) shows chemical shifts on the hydrophobic patch of parkin's Ubl domain upon binding Rpn13's Pru domain

After establishing that the Ubl domain of parkin as well as the Pru domain of Rpn13 is mediating the interaction parkin-proteasome, we've decided to map the amino acid residues that are critical for this interaction.

In collaboration with Dr. Jean-Francois Trempe and Dr. Kahle Gehring (McGill University) a series of NMR measurements were recorded and chemical shift perturbations were measured (figure 15).

The Rpn13 PRU (1-130) and 15N-labeled parkin Ubl (1-76) were purified by size-exclusion chromatography in NMR buffer. Proteins were concentrated using ultrafiltration devices (MWCO 3K) and 5% D2O was added. 1H-15N HSQC spectra were recorded at a temperature of 303K using proteins at the following concentrations (in uM):

[15N-UBL]	[PRU]
800	0
410	180
270	240
207	260

Weighted-average chemical shift perturbations were measured as the difference between the first and last spectra using the following formula:

SQRT (ΔH^2 + (ΔN/5)^2).

The NMR results were consistent with our previous experimental data. It shows that parkin's Ubl domain is indeed mediating its interaction with the Pru domain of Rpn13 using the hydrophobic patch shown previously in figure 4; Furthermore, it corroborates the importance of the well conserved residue I44 between ubiquitin and parkin's Ubl domain.

The largest chemical shift changes were mapped onto the surface of parkin's Ubl. Most residues are localized primarily on the five-strand β-sheet face of the Ubl with little perturbation of the long α-helix positioned on the back side of the domain (Figure 15, highlighted in blue on the structure within the graph).

Interestingly, our NMR results showed the exact same pattern of chemical shifts perturbations previously described in (Safadi et al., 2010); although, their results were acquired using the UIM domains of Rpn10. However, our data shows a higher chemical shifts when using the Pru domain of Rpn13.

KD rescue, transfecting Myc-Rpn13 shRNAmiR resistant

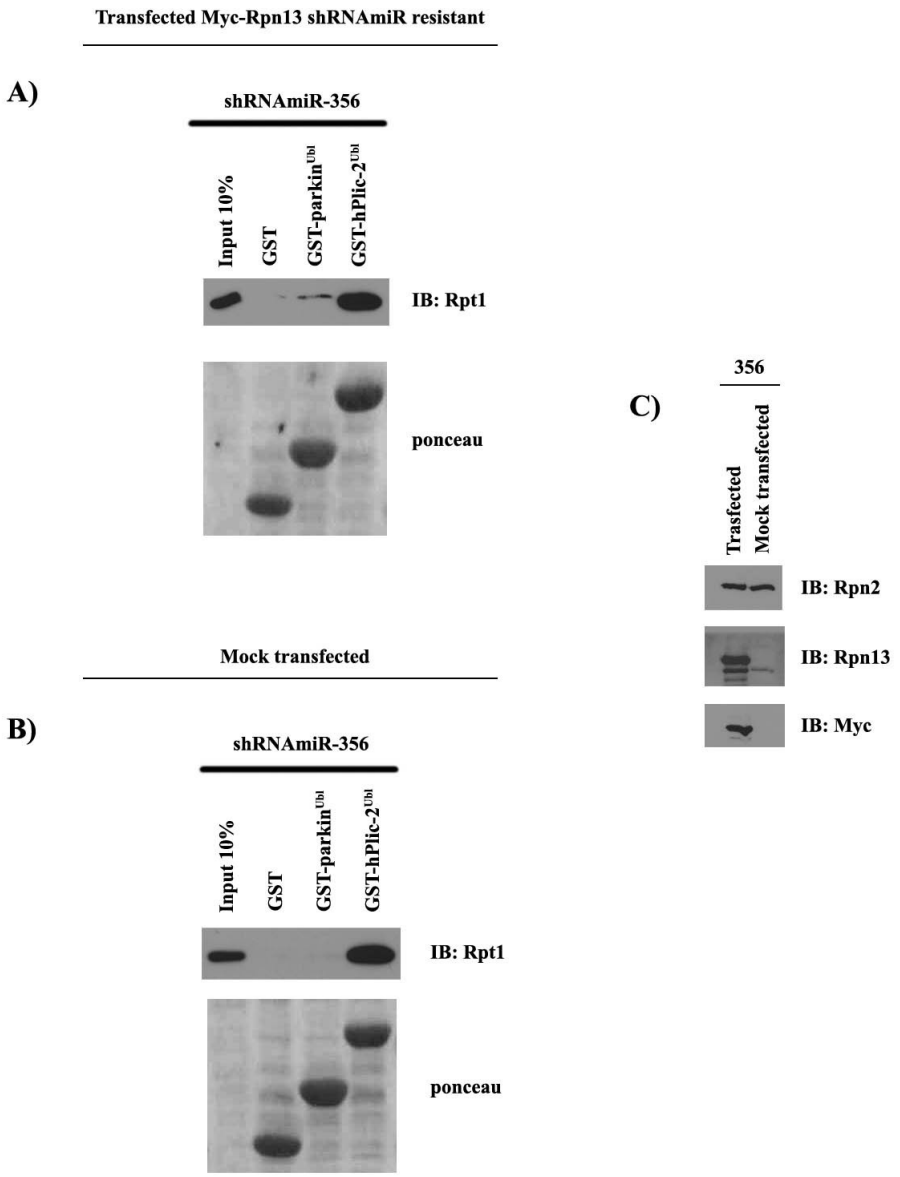

Figure 14. Proteasome pull-down using Glutathione-sepharose-immobilized GST, GST-Parkin^{Ubl} and GST-hPlic-2^{Ubl}, combined with lysates from HEK 293T cells previously infected with shRNAmiR-356. These cells were transfected with myc-Rpn13-shRNAmiR-resistant or mock transfected A) and B). Pull-down efficiency was tested by immunoblotting for 19S regulatory complex subunit Rpt1. C) Shows the extent of expression of myc-Rpn13 shRNAmiR-resistant, the KD of Rpn13 and the levels of Rpn2 (as loading control).

NMR showing the residues within the Ubl domain of parkin being shifted upon binding the Pru domain of Rpn13

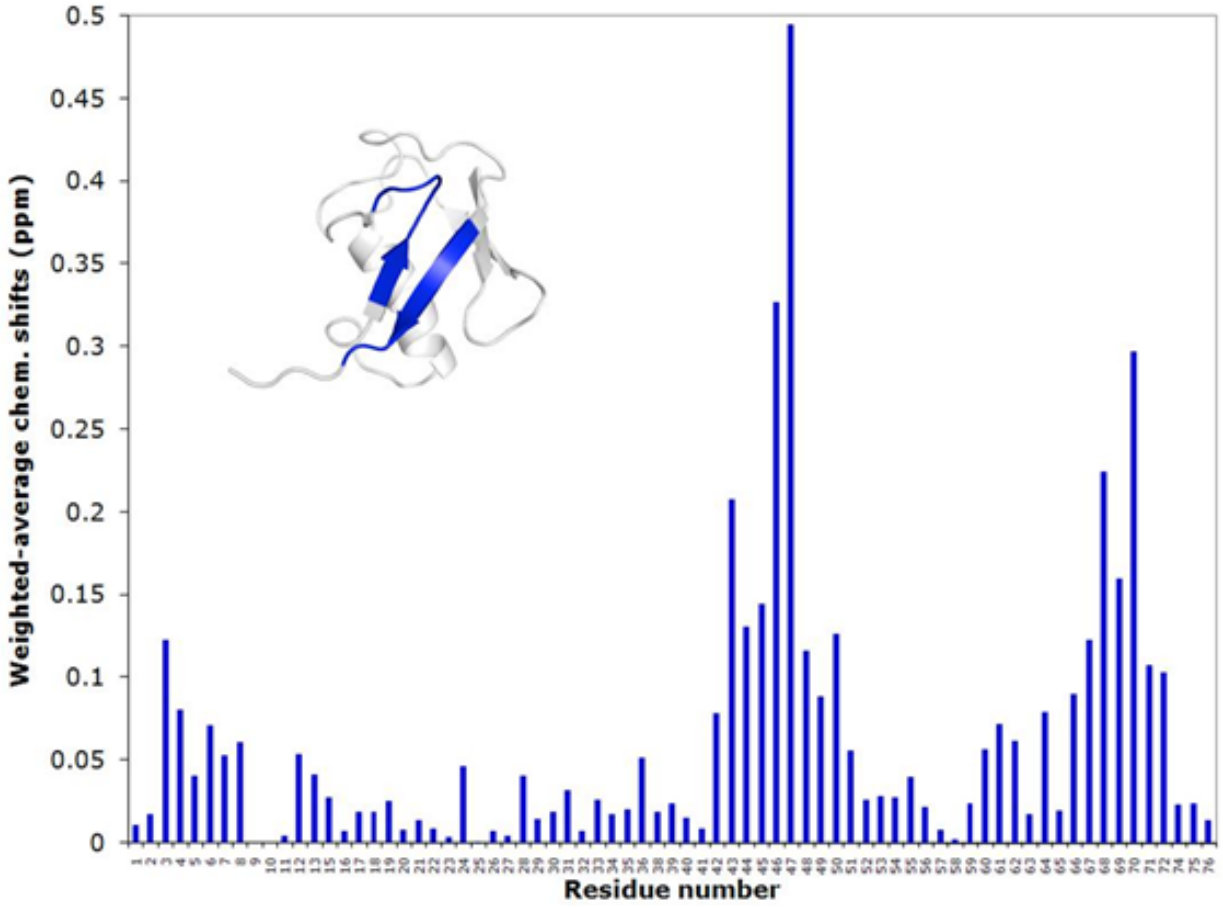

Figure 15. NMR showing the weighted average chemical shifts perturbations in Parkin's Ubl domain upon binding the Pru domain of Rpn13. shift perturbations were measured as the difference between the first and last spectra using the following formula:
SQRT (ΔH^2 + (ΔN/5)^2). The structure of the Ubl domain of parkin is shown within the graph, highlighted is the hydrophobic patch predicted to interact with the Pru domain of Rpn13.
In collaboration with Dr. Jean-Francois Trempe and Dr. Kahle Gehring (McGill University).

Rpn13's Pru domain has higher affinity for parkin's Ubl domain than mono-ubiquitin

Due to the high degree of similarity between our NMR results and a previously reported interaction parkin-Rpn10 (Safadi et al., 2010), we decided to measure the affinity between parkin-Rpn13 as well as the dissociation constant (K_d).

In collaboration with Dr. Jean-Francois Trempe and Dr. Kahle Gehring (McGill University) a series of surface plasmon resonance (SPR) measurements were carried out using a Ni-NTA chip on a BIAcore T100 instrument (GE Healthcare) at 25°C.

To activate the NI-NTA chip, 100mM $NiSO_4$ was injected followed by 125nM 6His-Pru to a 1000 response units (RU). Then, concentrations series of parkin Ubl (0.05-25uM) or ubiquitin (0.2-50uM) were injected for one minute. Affinities were determined by fitting the steady state equilibrium responses (50-60sec post-injection) as a function of concentration using a simple 1:1 binding isotherm.

Interestingly, our SPR results showed a higher K_d between ubiquitin and Pru domain (40-90uM) than Ubl-Pru (3uM); furthermore, the shape of the slope after 60 seconds (the dissociation signal) strongly suggests a higher affinity between Pru-Ubl than Pru-Ub (Figure 16). This effect is well in line with previous results in which Ubl-Uba containing proteins display higher affinity for Ub receptors than mono-ubiquitin (Walters et al., 2002).

We also noticed that the calculated K_d between parkin's Ubl and Rpn13's Pru domain is around 3 orders of magnitude lower than the one reported for parkin's Ubl and Rpn10's UIMs (217±51uM) (Safadi et al., 2010); once again, arguing that parkin-proteasome interaction is achieved via Rpn13.

SPR: Higher affinity and lower K_d for parkin's Ubl than ubiquitin

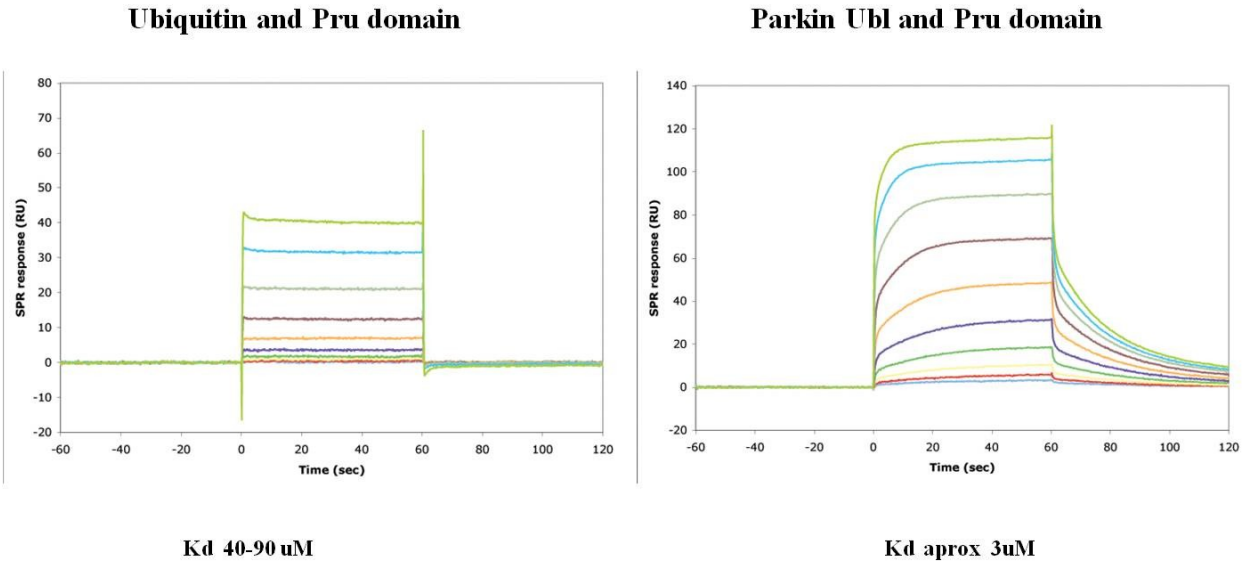

Figure 16. SPR was performed using a Ni-NTA chip on a BIAcore T100 instrument (GE Healthcare) at 25C, using the SPR buffer (10mM HEPES, 150mM NaCl, 0.05% P20, pH 7.4). 100mM NiSO4 was injected to activate the surface, 125nM His6-PRU was injected to 1000 RU. Then, concentrations series of parkin Ubl (0.05-25uM) or ubiquitin (0.2-50uM) were injected for one minute. Affinities were determined by fitting the steady state equilibrium responses (50-60sec post-injection) as a function of concentration using a simple 1:1 binding isotherm. In collaboration with Dr. Jean-Francois Trempe and Dr. Kahle Gehring (McGill University).

Knocking down Rpn13 has no effect on the steady-state levels of "parkin substrates"

Having thoroughly tested that there is an interaction between parkin and the proteasome, we sought to investigate about the function for this interaction.

Because parkin is an E3 ubiquitin ligase and it has a Ubl domain, we thought that its interaction with the proteasome could be mediating substrate delivery, as is the purpose for the shuttles previously described (hPlic-2 and hHR23A/B). If this is the case, parkin will not only be delivering substrates, but ubiquitinating them on their way to the 26S proteasome. In order to test for this possibility, we decided to check for the levels of some parkin substrates.

Using lysates from HEK 293T cells in which Rpn13 had previously been knocked down, we probed for parkin substrates whose degradation has been related to parkin function, such as CDCrel-1 and p38 (Zhang et al., 2000 and Corti et al., 2003); substrates whose mono-ubiquitination is parkin dependent such as Eps15 and PICK1 (Fallon et al., 2006 and Joch et al., 2007) and finally, the overall levels of protein-Ub conjugates and the levels of parkin itself (figure 17). Interestingly, we didn't see any effect on these substrates, but even more surprising was the fact that there were no differences in the overall ubiquitination. However, very recently, 3 independent groups have found that knocking down Rpn13 did not affect the general short-lived protein degradation. Thus, differences in overall ubiquitination might not be expected, consistent with our own findings (Hamazaki et al 2006, Jorgensen et al., 2006 and Yao et al., 2006) (figure 17).

In contrast, it appears that parkin itself becomes up-regulated upon Rpn13 KD (compare shRNAmiR-control with shRNAmiR-356 figure 17); however more experiments are required to confirm this effect.

Parkin substrates upon Rpn13 KD

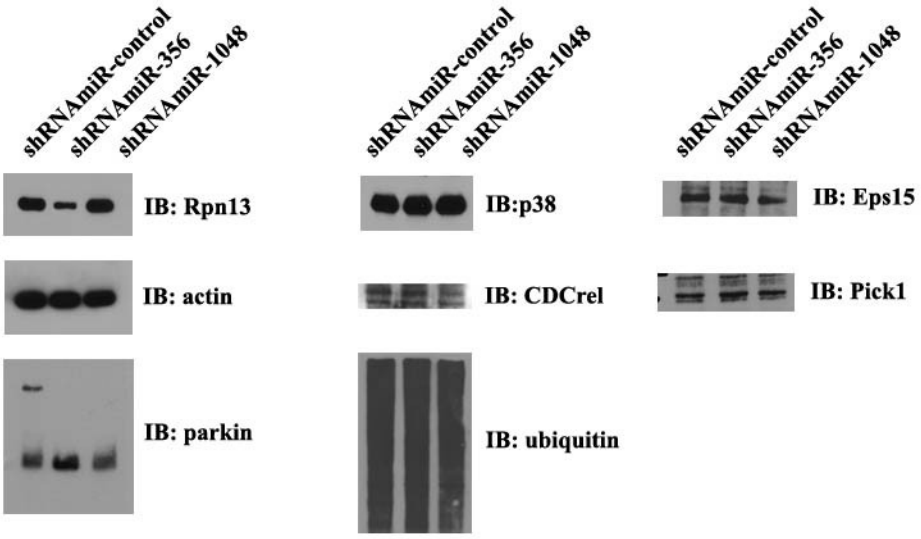

Figure 17. HEK 293T cells were infected with our 3 lentivirus based constructs. 100ug were run on SDS-PAGE and probe for different parkin substrates, ubiquitin and parkin itself, a consistent up-regulation of parkin was observed (bottom left panel). Immunoblots for Rpn13 and actin are shown to depict the loading and the extent of KD respectively.

Rpn13 KD and parkin

Since we noticed a slight effect on parkin once Rpn13 is knocked down, we decided to increase the MOI of our lentivirus based constructs, and try to examine this effect in more detail. All our previous experiments using shRNAmiR-lentivirus-based constructs were done using an MOI of 2. Because of their efficiency and the results obtained, we saw no reason to go higher and maybe create secondary effects due to the infection.

HEK 293T cells were infected with our 3 shRNAmiR constructs using an MOI of 4. After 7 days post-infection, the cells were lysed and 300ug were loaded for SDS-PAGE. When probing for parkin, Rpn13 and actin, two things were evident. Rpn13 KD was very efficient and parkin levels seemed to become highly up-regulated (52KDa band) (figure 18). Interestingly, our parkin antibody revealed a band of about 82KDa on our control cells. This band disappeared when using both our KD constructs (356 and 1048); furthermore, this time our construct 1048 was able to reduce Rpn13 levels to some extent and was able to cause a slight increase on parkin levels.

Since 2000, it has been know that parkin is able to ubiquitinate itself, this activity is mediated trough its RING1 IBR RING2 domain (Shimura et al., 2000). With the experiments presented so far in this manuscript, we can not determine if the 82KDa band shown in figure 18 is the ubiquitinated form of parkin (4 ubiquitins) or just an unspecific band recognized with this antibody.

Rpn13 KD has consequences on endogenous parkin

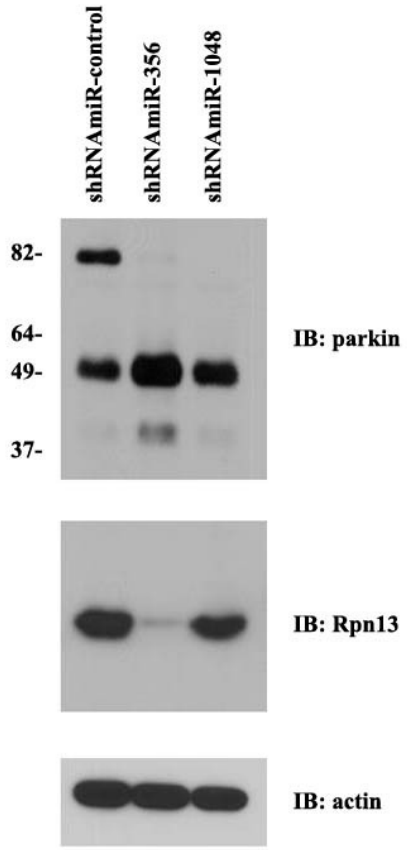

Figure 18. HEK 293T cells were infected for 7 days using an MOI of 4 for each of the shRNAmiR constructs. 300µg were loaded and ran on an SDS-PAGE. Immunoblots are shown for parkin, Rpn13 and actin. Parkin gets up-regulated upon Rpn13 KD.

Discussion

In this work, we characterized a direct interaction between parkin and Rpn13. This interaction is mediated via the Ubl domain of parkin, which has high resemblance to ubiquitin and the Pru domain of Rpn13, recently proven to be a ubiquitin receptor. Although, the Ubl domain of parkin mediates other interactions, including those with the UIM domains of Eps15 and the SH3 domain of endophilin-A (Fallon et al., 2006., Trempe et al., 2009), here we described yet another interaction with the Pru domain of Rpn13. Importantly, we do not believe that parkin is exclusively binding the proteasome or the substrates we have previously described, but, that it has the ability to mediate different kinds of interactions according to the cellular requirements.

Here we have shown that the Ubl domain of parkin is not interacting with any other proteasome subunit, at least the ones that have been proposed. Also, we were able to determine the K_d for this interaction, which is lower than the previously reported interaction with Rpn10. Although our results have not shown a function for this interaction, we have shown a high degree of specificity that causes an up-regulation in parkin when knocking down Rpn13. Our current efforts are focused in proving a specific function for parkin-proteasome interaction, as well as definitely excluding Rpn10/S5A as the subunit mediating this interaction, by means of KD coupled with proteasome pull-down.

The UPP is known to be the major degradation pathway for intracellular proteins, many of which are of particular interest in the context of neurodegenerative disease. These include parkin and α-synuclein, previously discussed, and other proteasome substrates whose accumulation might have potentially toxic effects such as oxidized or unfolded proteins. The 26S proteasome which is at the center of the pathway, has thus been widely studied. First thought as a discrete complex,

more and more proteasome–interacting proteins (PIP) are being discovered with a diverse range of functions such as chaperones, DUBs, E3s and more recently E4s, to name a few.

In 2000, Shimura et al., proved that parkin is an E3 ligase, whose activity is mediated trough its C-terminal RING1-IBR-RING2 domains (Shimura et al., 2000). Ever since, a wide variety of parkin-protein interactions have been described, and some substrates have been assigned to parkin. Importantly, the N-terminal Ubl domain of parkin has been shown to mediate some of these interactions, of particular interest are the interactions between parkin-Eps15 (Fallon, et al., 2006) and parkin-endophilin-A (Trempe, et al., 2009), which implicates parkin in receptor trafficking and synaptic transmission respectively. Furthermore, although a wide number of parkin's substrates have been proposed, and their degradation has been attributed to parkin's activity, most of them have controversies due that they do not accumulate in parkin KO mice (Ko et al., 2005). It is worthy to mention that our lab has only found substrates that are mono-ubiquitinated by parkin (Fallon et al.,2006; Joch et al., 2007 and Trempe et al., 2009), perhaps suggesting a different role other than degradation for its E3 ligase activity.

Just like many others Ubl containing proteins, we've found that parkin also interacts with the 26S proteasome. This interaction is mediated via its Ubl domain, using a contiguous surface containing residues Phe^{13}, Arg^{42}-Ile^{44}, Ala^{46}, Lys^{48}, Glu^{49}, Arg^{51}, Leu^{61}, Ile^{66}-Val^{67}, and Gln71 among others. Importantly, when testing a direct interaction between parkin and Rpn13 (figures 8 and 9), differences were evident when both proteins were in their full-length form or just the domains involved in this interaction. This effect can easily be explained by recent data (Chen et

al., 2010) showing that Rpn13's Pru domain remains hidden, up until it interacts with the 19S base subunit Rpn2, in our case, once it interacts with parkin's Ubl domain. It is likely that once this interaction takes place, the C-terminal region of both proteins will prevent the Pru domain to fold back into its non exposed form, thus an increase of this interaction is seen when both proteins are in its full-length form.

Our SPR data showed that parkin just like the Ubl-Uba shuttles has a higher affinity for Ub receptors than they do for mono-Ub. Considering the amount of ubiquitinated proteins in the cell cytosol, this higher affinity does not mean that parkin will be competing for these proteins, but that it might serve to deliver a substrate or substrates that otherwise wouldn't display such a high affinity for the proteasome. Evidence for such a model has been published (Xie and Varshavsky 2002), in which Ufd4p (an E3 ligase) is responsible for both, the formation of substrate-linked poly-ubiquitin chain, and its delivery to the proteasome. Furthermore, there are some AR-JP causing mutations in parkin such as R33Q, R42P, K48A and V56E that disrupt the Ubl domain but they do not impair parkin's E3 ligase activity (data from our lab and others, Sriram et al., 2005). Also, using the crystal structure of murine parkin's Ubl, Tomoo et al., showed in a series of simulations how the aforementioned mutants should be impaired in binding the proteasome (Tomoo et al., 2008).

Unfortunately, our data (figure 17) did not prove that parkin is functioning as an E3 ligase-Shuttle, but then again, most parkin substrates have controversies. To fully test this model, a reliable parkin substrate whose degradation is linked to parkin activity is a requirement, and to date there is not such known substrate. It is important to mention, that our data is not excluding

the aforementioned model, but, our current knowledge about parkin substrates are making it difficult to prove.

Another possible function for parkin while binding Rpn13 might come from what we know about the other proteasome Ub receptor, Rpn10. Among the proteasome interacting proteins (PIPs), the previously mentioned Ubl containing DUB, Ubp6, binds the Ub receptor Rpn10; furthermore, its DUB activity is opposed by the E3 ("E4") enzyme Hul5 (Crosas et al., 2006). In this model, Crosas et al., suggests that poorly ubiquitinated substrates or those who have been ubiquitinated using unusual Ub linkages, namely K6, K11, K27, K29 and K33 are "trimmed" by Ubp6 and ubiquitinated again using the conventional K48 type of linkage by Hul5. This model is strengthened by the fact that there is already a described DUB that binds Rpn13, Uch37 (Hamazaki et al., 2006 and Yao et al., 2006). It would be interesting if parkin's E3 ligase activity is also opposed by Uch37.

Finally, and currently being tested is whether parkin's E3 ligase activity is enhanced or diminished upon its binding to Rpn13. As previously discussed, Ubp6 activity is highly increased when binding Rpn10; furthermore, ubiquitinated Ubp6 with the unusual K27 linkage increases its affinity for Rpn10 (Alberti et al., 2002). It is a well known fact that parkin self-ubiquitinates, and even more interesting is that we have found that it mainly uses a poorly understood K6 type of linkage (unpublished data). It should be a "straight forward" model to test.

Here, we have presented strong evidence implying that parkin binds the 26S proteasome, specifically through its Ubl domain and via Rpn13's Pru domain. We also have shown (Michael Haber 2004; figure 6) that an AR-JP causing mutation, R42P disrupts this interaction, suggesting an important role for parkin-proteasome interaction. Also, it is important to stress that we were able to see an up-regulation of parkin when KD Rpn13 (figure 18), once again arguing for the relevance for this interaction. Future experiments are now focused on determining the exact role for parkin when bound to the proteasome, and we are confident that a good publication should emerge from this work.

Bibliography

1. Alberti, S., Demand, J., Esser, C., Emmerich, N., Schild, H. and Hohfeld, J. (2002). Ubiquitylation of BAG-1 suggests a novel regulatory mechanism during the sorting of chaperone substrates to the proteasome. J. Biol. Chem. *277*, 45920-45927.
2. Betarbet, R., Shere, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V., and Greenamyre, J.T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat.Neurosc. *2*, 434-439.
3. Biskup, S., Moore, D.J., Celsi, F., Higashi, S., West, A.B., Andrabi, S.A., Kurkinen, K., Yu, S.W., Savitt, J.M., Waldvogel, H.J., Faull, R.L., Emson, P.C., Torp, R., Ottersen, O.P., Dawson, T.M. and Dawson, V.L. (2006). Localization of LRRK2 to membranous and vesicular structures in mammalian brain. Ann. Neurol. *60*, 557-569.

4. Blum, D., Torch, S., Lambeng, N., Nissou, M., Benabid, A.L., Sadoul, R., and Verna, J.M. (2001). Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. Prog. Neurobiol. *65*, 135-172.

5. Bonifati, V., Rizzu, P., van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C., Squitieri, F., Ibanez, P., Joose, M., van Dongen, J.W., Vanacore, N., van Switen, J.C., Brice, A., Meco, G., van Duijn, C.M., Oostra, B.A. and Heutink, P. (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science. *299*, 256-259.

6. Braak, H., Ghebremedhin, E., Rub, U., Bratzke, H. and Del, T.K. (2004). Stages in the development of Parkinson's disease-related pathology. Cell. Tissue. Res. *318*, 121-134.

7. Braun, B.C., Glickman, M., Kraft, R., Dahlman, B., Kloetzel, P.M., Finley, D. and Shmidt, M. (1999). The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat. Cell. Biol. *1*, 221-226.

8. Canet-Aviles, R.M., Wilson, M.A., Miller, D.W., Ahmad, R., McLendon, C., Bandyopadhyay, S., Baptista, M.J., Ringe, D., Petsko, G.A. and Cookson, M.R. (2004). The Parkinson's disease protein DJ-1 is neuroprotective due to cystein-sulfinic acid-driven mitochondrial localization. Proc. Natl. Acad. Sci. U.S.A. *101*, 9103-9108.

9. Chen, X., Lee, B.H. and Walters, K.J. (2010). Structure of preoteasome ubiquitin receptor hRpn13 and its activation by the scaffolding protein hRpn2. Mol. Cell. *38*, 404-4015.

10. Chesselet, M.F. (2008). In vivo alpha-synuclein overexpression in rodents: a useful model of Parkinson's disease? Exp. Neurol. *209*, 22-27.

11. Chesselet, M.F., Fleming, S., Mortazavi, F. and Meurers, B. (2008). Strengths and limitations of genetic mouse models of Parkinson's disease. Parkinsonism. Relat. Disord. *14*, (Suppl 2), S84-S87.

12. Choi, W.S., Yoon, S.Y., Oh, T.H., Choi, E.J., O'Malley, K.L., and Oh, Y.J. (1999). Two distinct mechanisms are involved in 6-hydroxidopamine and MPP+ induced dopaminergic neuronal cell death: role of caspases, ROS and JNK. J.Neurosci.Res. *57*, 86-94.

13. Clark, I.E., Dodson, M.W., Jiang, C., Cao, J.H., Huh, J.R., Seol, J.H., Yoo, S.J., Hay, B.A. and Guo, M. (2006). Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature. *441*, 1162-1166.

14. Cooper, A.A., Gitler, A.D., Cashikar, A., Haynes, C.M., Hill, K.J., Bhullar, B., Liu, K., Xu, K., Strathearn, K.E. and Liu, F. (2006). Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. Science. *313*, 324-328.

15. Corti, O., Hampe, C., Koutnikova, H., Darios, F., Jacquier, S., Prigent, A., Robinson, J.C., Pradier, L., Ruberg, M., Mirande, M., Hirsch, E., Rooney, T., Fournier, A. and Brice, A. (2003). The p38 subunit of the aminoacyl-tRNA synthetase complex is a parkin substrate: linking protein biosynthesis and neurodegeneration. Hum. Mol. Genet. *12*, 1427-1437.

16. Crosas, B., Hanna, J., Kirkpatrick, D.S., Zhang, D.P., Tone, Y., Hathaway, N.A., Buecker, C., Leggett, D.S., Schmidt, M., King, R.W., Gygi, S.P. and Finley, D. (2006). Ubiquitin chains are remodeled at the proteasome by opposing ubiquitin ligase and deubiquitinating activities. Cell. *127,* 1401-1413.

17. Dawson, T.M. and Dawson, V.L. (2003). Rare genetic mutations shed light on the pathogenesis of Parkinson's disease. J. Clin. Invest. *111*, 145-151

18. Dekker, M.C., Bonifanti, V., and van Duijn, C.M. (2003). Parkinson's disease: piecing together a genetic jigsaw. Brain *126*, 1722-1733.

19. de Rijk, M.C., Breteler, M.M., Graveland, G.A., Ott, A., Grobbee, D.E., van der Meche, F.G., and Hofman, A. (1995). Prevalence of Parkinson's disease in the elderly: the Rotterdam Study. Neurology *45*, 2143-2146.

20. de Rijk, M.C., Tzourio, C., Breteler, M.M., Dartigues, J.F., Amaducci, L., Lopez-Pousa, S., Manubens-Bertran, J.M., Alperovitch, A., and Rocca, W.A. (1997). Prevalence of parkinsonism and Parkinson's disease in Europe: The EUROPARKINSON Collaborative Study. European Community Concerted Action on the Epidemiology of Parkinson's disease. J.Neurol. Neurosurg. Psychiatry *62*, 10-15.

21. Damodaran, M., and Ramaswamy, R. (1937). Isolation of L-3,4-dihydroxyphenilalanine from the seeds of Mucuna pruriens. Biochem.J. *31*, 2149-2152.

22. Dächsel, J.C., Lücking, C.B., Deeg, S., Schultz, E., Lalowski, M., Casademunt, E., Corti, O., Hampe, C., Patenge, N., Vaupel, K., Yamamoto, A., Dichgans, M., Brice, A., Wanker, E.E., Kahle, P.J. and Gasser, T. (2005). Parkin interacts with the proteasome subunit alpha4. FEBS. Lett. *18*, 3913-3919.

23. Dekker, M.C., Bonifati, V. and van Duijn, C.M. (2003). Parkinson's disease: piecing together a genetic jigsaw. Brain. *126*, 1722-1733.

24. Demand, J., Albertis, S., Patterson, C. and Hohfeld, J. (2001). Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. Curr. Biol. *11*, 1569-1577.

25. Di Fonzo, A., Chien, H.F., Socal, M., Giraudo, S., Tassorelli, C., Iliceto, G., Fabbrini, G., Marconi, R., Fincati, E., Abbruzzese, G., Marini, P., Squitieri, F., Horstink, M.W., Montagna, P., Libera, A.D., Stocchi, F., Goldwurm, S., Ferreira, J.J., Meco, G., Martignoni, E., Lopiano, L., Jardim, L.B., Oostra, B.A., Barbosa, E.R. and Bonifati, V. (2007). ATP13A2 missense mutations in juvenile parkinsonism and young onset Parkinson's disease. Neurology. *68,* 1557-1562.

26. Elsasser, S., Chandler-Militello, D., Muller, B., Hanna, J. and Finley, D. (2004). Rad23 and Rpn10 serve as an alternative ubiquitin receptors for the proteasome. J. Biol. Chem. *279*, 26817-26822.

27. Elsasser, S., Gali, R.R., Schwickart, M., Larsen, C.N., Leggett, D.S., Muller, B., Feng, M.T., Tubing, F., Dittmar, G.A. and Finley, D. (2002). Proteasome subunit Rpn1 binds ubiquitin-like protein domains. Nat. Cell. Biol. *4*, 725-730.

28. Fallon, L., Belanger, C.M., Corera, A.T., Kontogiannea, M., Reagan-Klapisz, E., Moreau, F., Voortman, J., Haber, M., Rouleau, G., Thorainsdottir, T., Brice, A., van Bergen En Henegowen, P.M. and Fon, E.A. (2006) A regulated interaction with the UIM protein Eps15 implicates parkin in EGF receptor trafficking and PI(3)K-Akt signaling. Nat.Cell.Biol. *8*, 334-342.

29. Fallon, L., Moreau, F., Croft, B.G., Labib, N., Gu, W.J. and Fon, E.A. (2002). Parkin and CASK/LIN-2 associate via a PDZ-mediated interaction and are co-localized in lipid rafts and postsynaptic densities in brain. J. Biol. Chem. *277*, 486-491.

30. Galter, D., Westerlund, M., Carmine, A., Lindqvist, E., Sydow, O. and Olson, L. (2006). Ann. Neurol. *59*, 714-719.

31. Gibb, W.R. (1992). Neuropathology of Parkinson's disease and related syndromes. Neurol. Clin. *10*, 361-376.

32. Gitler, A.D., Bevis, B.J., Shorter, J., Strathearn, K.E., Hamamichi, S., Su, L.J., Caldwell, K.A., Caldwell, G.A., Rochet, J.C., McCafferey, J.M., Barlowe, C. and Lindquist, S. (2008). The Parkinson's disease protein alpha-synuclein disrupts cellular Rab homeostasis. Proc. Natl. Acad. Sci. U.S.A. *105*, 145-150.

33. Hamazaki, J., Iemura, S., Natsume, T., Yashiroda, H., Tanaka, K. and Murata, S. (2006) A novel proteasome interacting protein recruits the deubiquitinating enzyme Uch37 to 26S proteasomes. EMBO. J. *25*, 4524-4536.

34. Hampshire, D.J., Roberts, E., Crow, Y., Bond, J., Mubaifin, A., Wriekat, A.L. Al-Din, A. and Woods, C.G. (2001). Kufor-Rakeb syndrome, pallid-pyramidal degeneration with supranuclear upgaze paresis and dementia, maps to 1p36. J.Med. Genet. *38,* 680-682.

35. Hershko Avram and Ciechanover Aaron. (1998). The ubiquitin system. Annu.Rev.Biochem. *67*, 425-79.

36. Heutnik, P. (2006). PINK-1 and DJ-1-new genes for autosomal recessive Parkinson's disease. J. Neural. Transm. Suppl. 215-219.

37. Hristova, V.A., Beasley, S.A., Rylett, R.J. and Shaw, G.S. (2008). Identification of a novel Zn^{2+} - binding domain in the autosomal recessive juvenile Parkinson-related E3 ligase parkin. J. Biol. Chem. *284*, 14978-14986.

38. Hiyama, H., Yokoi, M., Masutani, C., Sugasawa, K., Maekawa, T., Tanaka, K., Hoeijmakers, J.H. and Hanaoka, F. (1999). Interaction of hHR23 with S5a. The ubiquitin-like domain of hHR23 mediates interaction with S5a subunit of 26S proteasome. J. Biol. Chem. *274*, 28019-28025.

39. Husnajak, K., Elsasser, S., Zhang, N., Chen, X., Randles, L., Shi, Y., Hofman, K., Walters, K.J., Finley, D. and Dikic, I. (2008). Proteasome subunit Rpn13 is a novel ubiquitin receptor. Nature *453*, 481-488.

40. Hwan-Ching, T. and Schuman, E.M. (2008). Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. Nat Rev Neur *9*, 826-838.

41. Ibanez, P., Bonnet, A.M., Debarges, B., Lohman, E., Tison, F., Pollak, P., Agid, Y., Durr, A. and Brice, A. (2004). Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. Lancet. *364*, 1169-1171.

42. Joch, M., Ase, A.R., Chen, C.X., MacDonald, P.A., Kontogiannea, M., Corera, A.T., Brice, A., Seguela, P. and Fon, E.A. (2007). Parkin-mediated monoubiquitination of the PDZ protein PICK1 regulates the activity of acid sensing ion channels. Mol.Biol.Cell. *18*, 3105-3118.

43. Jørgensen, J.P., Lauridsen, A.M., Kristensen, P., Dissing, K., Johnsen, A.H., Hendil, K.B. and Hartmann-Petersen, R. (2006). Adrm1, a putative cell adhesion regulating protein, is a novel proteasome-associated factor. J. Mol. Biol. *360*, 1043-1052.

44. Kahle, P.J., Laimer, U. and Haass, C. (2000). Does failure of parkin-mediated ubiquitination cause juvenile parkinsonism? Trends. Biochem. Sci. *25*, 524-527.

45. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura,Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature, *392*, 605-608.

46. Kleijnen, M.F., Shih, A.H., Zhou, P., Kumar, S., Soccio, R.E., Kedersha, N.L., Gill, G. and Howley, P.M. (2000). The hPLIC proteins may provide a link between the ubiquitination machinery and the proteasome. Mol. Cell. *7*, 1143-1152.

47. Ko, H.S., von Coelln, R., Sriram, S.R., Kim, S.W., Chung, K.K., Pletnikova, O., Troncoso, J., Johnson, B., Saffary, R., Goh, E.L., Song, H., Park, B.J., Kim, M.J., Kim, S., Dawson, V.L., and Dawson, T.M. (2005). Accumulation of the authentic parkin substrate aminoacyl-tRNA synthetase cofactor, p38/JTV1, leads to catecholaminergic cell death. J. Neurosci. *35*, 7968-7978.

48. Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L. and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet. *18*, 106-108.

49. Langston, J.W., Ballard, P., Tetrud, J.W. and Irwin, I. (1983). Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. Science *219*, 979-980.

50. Lee, M.K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A.S., Dawson, T.M., Copeland, N.G., Jenkins, N.A. and Price, D.L. (2002) Human alpha-synuclein-harboring familial Parkinson's disease-linke A53T mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. Proc. Natl. Acad. Sci. U.S.A. *99*, 8968-8973.

51. Leggett, D.S., Hanna, J., Borodovsky, A., Crosas, B., Schmidt, M., Baker, R.T., Walz, T., Ploegh, H. and Finley, D. (2002). Multiple associated proteins regulate proteasome structure and function. Mol. Cell. *10*, 495-507.

52. Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Wilkinson, K.D. and Polymeropoulos, M.H. (1998). The ubiquitin pathway in Parkinson's disease. Nature. *395*, 451-452.

53. Lesage, S., Belarbi, S., Troiano, A., Condroyer, C., Hecham, N., Pollak, P., Lohman, E., Benhassine, T., Ysmail-Dahlouk, F., Durr, A., Tazir, M. and Brice, A. (2008). Is the common LRRK2 G2019S mutation related to dyskinesias in North African Parkinson's disease?. Neurology. *71*, 1550-1552.

54. Lesage, S., and Brice, A. (2009). Parkinson's disease: from monogenic forms to genetic susceptibility factors. Hum.Mol.Genetics. *18*, R48-R59.

55. Lesage, S., Durr, A., Tazir, M., Lohman, E., Leutenegger, A.L., Janin, S., Pollak, P. and Brice, A. (2006). LRRK2 G2019S as a cause of Parkinson's disease in North African Arab. N. Engl. J. Med. *354*, 422-423.

56. Lin, X., Parisiadou, L., Gu, X.L., Wang, L., Shim, H., Sun, L., Xie, C., Long, C.X., Yang, W.J., Ding, J., Chen, Z.Z., Gallant, P.E., Tao-Cheng, J.H., Rodow, G., Troncoso, J.C., Liu, Z., Li, Z. and Cai, H. (2009). Leucine-rich repeat kinase 2 regulates the progression of neuropathology induced by Parkinson's-disease-related mutant alpha-synuclein. Neuron. *64*, 807-827.

57. Lincoln, S., Vaughan, J., Wood, N., Baker, M., Adamson, J., Gwinn-Hardy, K., Lynch, T., Hardy, J. and Farrer, M. (1999). Low frequency of pathogenic mutations in the ubiquitin carboxy-terminal hydrolase gene in familial Parkinson's disease. Neuroreport. *10*, 427-429.

58. Liu, Y., Fallon, L., Lashuel, H.A., Liu, Z. and Lansbury, P.T. Jr. (2002). The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. Cell. *111*, 209-218.

59. Liu, Z., Wang, X., Yu, Y., Li, X., Wang, T., Jiang, H., Ren, Q., Jiao, Y., Sawa, A. and Moran, T. (2008). A Drosophila model for LRRK2-linked parkinsonism. Proc. Natl. Acad. Sci. U.S.A. *105*, 2693-2698.

60. Luders, J., Demand, J. and Hohfeld, J., (2000). The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. J. Biol. Chem. *275*, 4613-4617.

61. Mitzumoto, A. and Nagakawa, Y. (2001). DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free. Radic. Res. *35*, 885-893.

62. Moore, D.J., Zhang, L., Dawson, T.M. and Dawson, V.L. (2003). A missense mutation (L166P) in DJ-1, linked to familial Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization. J. Neurochem. *87*, 1558-1567.

63. Najim al-Din, A.S., Wriekat, A., Mubaidin, A., Dasouki, M. and Hiari, M. (1994). Pallidopyramidal degeneration, supranuclear upgaze paresis and dementia: Kufor-Rakeb syndrome. Acta. Neurol. Scand. *89*, 347-352.

64. Nicklas, W.J., Youngster, S.K., Kindt, M.V. and Heikkila, R.E. (1987). MPTP, MPP+ and mitochondrial function. Life Sci. *40*, 721-729.

65. Paik, S.R., Lee, D., Cho, H.J., Lee ,E.N., and Chang, C.S. (2003) Oxidized glutathione stimulated the amyloid formation of alpha-synuclein.FEBS Lett. *535*, 63-67.

66. Paisan-Ruiz, C., Jain, S., Evans, E.W., Gilks, W.P., Simon, J., van der, B.M., Lopez de, M.A., Aparicio, S., Gil, A.M., Khan, N., Johnson, J., Martinez, J.R., Nicholl, D., Carrera, I.M., Pena, A.S., de, S.R., Lees, A., Marti-Masso, J.F., Perez-Tur, J., Wood, N.W. and Singleton, A.B. (2004). Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron. *44*, 595-600.

67. Park, J., Lee, S.B., Lee, S., Kim, Y., Song, S., Kim, S., Bae, E., Kim, J., Shong, M., Kim, J.M. and Chung, J. (2006). Mitochondrial disfunction in Drosophila PINK1 mutants is complemented by parkin. Nature. *441*, 1157-1161.

68. Parkinsons, J. (2002). **Classic articles**. J Neuropsychiatry Clin Neurosci 14:223-236.

69. Polymeropolus, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. *276*, 2045-2047.

70. Przedborski, S. and Vila, M. (2003). The 1-methyl-4-phenil-1,2,3,6-tetrahydropyridine mouse model: a tool to explore the pathogenesis of Parkinson's disease. Ann. N. Y. Acad. Sci. *991*, 189-198.

71. Rabl, J., Smith, D.M., Yu, Y., Chang, S.C., Goldberg, A.L. and Cheng, Y. (2008). Mechanisms of gate opening in the 20S proteasome by the proteasomal ATPases. Moll. Cell. *30*, 360-368.

72. Rao, H. and Sastry, A. (2002). Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. J. Biol. Chem. *277*, 11691-11695.

73. Rosenzweig, R., Osmulski, P.A., Gaczynska, M. and Glickman, M.H. (2008). The central unit within the 19S regulatory particle of the proteasome. Nat. Struct. Mol. Biol. *15*, 573-580.

74. Ross, O.A., Braithwaite, A.T., Skipper, L.M., Kachergus, J., Hulihan, M.M., Middleton, F.A., Nishioka, K., Fuchs, J., Gasser, T., Maraganore, D.M., Adler, C.H., Larvor, L., Chartier-Harlin, M.C., Nilsson, C., Langston, J.W., Gwinn, K., Hattori, N. and Farrer, M.J. (2008). Genomic investigation of alpha-synuclein multiplication and parkinsonism. Ann. Neurol. *63*, 743-750.

75. Saeki, Y., Sone, T., Toh-e, A. and Yokosawa, H. (2002). Identification of ubiquitin like protein-binding subunits of the 26S proteasome. Biochem. Biophys. Res. Commun. 296, 813-819.

76. Saeki, Y. and Tanaka, K. (2007). Unlocking the proteasome door. Mol. Cell. *27*, 865-867.

77. Safadi, S.S. and Shaw, G.S. (2010). Differential interaction of the E3ligase parkin with the proteasomal subunit S5a and the endocytic protein Eps15. J. Biol. Chem. *285*, 1424-1434.

78. Sakata, E., Yamaguchi, Y., Kurimoto, E., Kikuchi, J., Yokoyama, S., Yamada, S., Kawahara, H., Yokosawa, H., Hattori, N., Mizuno, Y., Tanaka, K. and Kato, K. (2003). Parkin binds the Rpn10 subunit of 26S proteasome through its ubiquitin-like domain. EMBO. *3*, 301-306.

79. Satoh, J.I. and Kuroda, Y. (2001). A polymorphic variation of serine to tyrosine at codon 18 in the ubiquitin C-terminal hydrolase-L1 gene is associated with a reduced risk of Parkinson's disease in a Japanese population. J. Neurol. Sci. *189*, 113-117.

80. Scheirner, P., Chen, X., Husnjak, K., Randles, L., Zhang, N., Elasasser, S., Finley, D., Dikic, I., Walters, K.J. and Groll, M. (2008). Ubiquitin docking at the proteasome through a novel pleckstrin-homology domain interaction. Nature *453*, 548-552.

81. Shen, R.S., Abell, C.W., Gessner, W., and Brossi, A. (1985). Serotonergic conversion of MPTP and dopaminergic accumulation of MPP+. FEBS lett. *189*, 225-230.

82. Shimizu, N., Asakawa, S., Minoshima, S., Kitada, T., Hattori, N., Matsumine, H., Yokochi, M., Yamamura, Y., and Mizuno, Y. (2000). PARKIN as a pathogenic gene for autosomal recessive juvenile parkinsonism. J.Neural Transm. Suppl 19-30.

83. Shimura, H., Hattori, N., Kubo, S.,Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K.., Chiba, T., and Tanaka, K. (2000). Familial Parkinson's disease gene product, parkin, is a ubiquitin-protein ligase. Nat. Genet. *25*, 302-305.

84. Silvestri, L., Caputo, V., Bellachio, E., Atorino, L., Dallapicola, B., Valente, E.M. and Casari, G. (2005). Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. Hum. Mol. Genet. *14*, 3477-3492.

85. Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M.R., Muenter, M., Baptista, M., Miller, D., Blancato, J., Hardy, J. and Gwinn-Hardy, K. (2003). Alpha-synuclein locus triplication causes Parkinson's disease. Science. *302*, 841.

86. Smith, W.W., Pei, Z., Jiang, H., Dawson, V.L., Dawson, T.M. and Ross, C.A. (2006). Kinase activity of mutant LRRK2 mediates neuronal toxicity. Nat. Neurosci. *9*, 1231-1233.

87. Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R. and Goedert, M. (1997). Alpha-synuclein in Lewi bodies. Nature. *388*, 839-840.

88. Sriram, S.R., Li, X., Ko, H.S., Chung, K.K., Wong, E., Lim, K.L., Dawson, V.L. and Dawson, T.M. Familial-associated mutations differentially disrupt the solubility, localization, binding and ubiquitination properties of parkin. Hum. Mol. Genet. *14*, 2571-2586.

89. Sroka, K., Voigt, A., Deeg, S., Reed, J.C., Schulz, J.B., Bahr, M. and Kermer, P. (2009). BAG1 modulates huntingtin toxicity, aggregation, degradation, and subcellular distribution. J. Neurochem. *111*, 801-807.

90. Tain, L.S., Mortiboys, H., Tao, R.N., Ziviani, E., Bendmann, O. and Whitworth, A.J. (2009). Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. Nat. Neurosci. *12*, 1129-1135.

91. Tan, E.K., Lee, J., Lim, H.Q., Yuen, Y. and Zhao, Y. (2008). Essential tremor and the common LRRK2 G2385R variant. Parkinsonism Relat. Disord. *14*, 569-571.

92. Tanaka, K. (2009). The proteasome: Overview of structure and functions. Proc. Jpn. Acad. *85*, 12-36.

93. Terreni, L., Calabrese, E., Calella, A.M., Forloni, G. and Mariani, C. (2001). New mutation (R42P) of the parkin gene in the ubiquitin like domain associated with parkinsonism. Neurology. *56*, 463-466.

94. Tomoo, K., Mukai, Y., In, Y., Miyagawa, H., Kitamura, K., Yamano, A., Shindo, H. and Ishida, T. (2008). Crystal structure and molecular dynamics simulation of ubiquitin-like domain of murine parkin. Biochim. Biophys. Acta. *1784*, 1059-1067.

95. Townsend, P.A., Cutress, R.I., Sharp, A., Brimmell, M. and Packhman, G. (2003). BAG-1: a multifunctional regulator of cell growth and survival. Biochim. Biophys. Acta. *1603*, 83-98.

96. Trempe, J.F., Chen, C.X., Grenier, K., Camacho, E.M., Koslov, G., McPherson, P.S., Gehring, K. and Fon, E.A. (2009). SH3 domains from a subset of BAR proteins define a Ubl-binding domain and implicates parkin in synaptic ubiquitination. Mol. Cell. *36*, 1034-1047.

97. Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., Albanese, A., Nussbaum, R., Gonzalez-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W.P., Latchman, D.S., Harvey, R.J., Dallapiccola, B.,

Auburger, G. and Wood, N.W. (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science. *304*, 1158-1160.

98. Walters, K.J., Kleijnen, M.F., Goh, A.M., Wagner, G. and Howley, P.M. (2002). Structural studies of the interaction between ubiquitin family proteins and proteasome subunit S5a. Biochemistry. *41*, 1767-1777.

99. West, A.B., Moore, D.J., Choi, C., Andrabi, S.A., Li, X., Dikeman, D., Biskup, S., Zhang, Z., Lim, K.L., Dawson, V.L. and Dawson, T.M. (2007). Parkinson's disease-associated mutations in LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity. Hum. Mol. Genet. *16*, 223-232.

100. Westerlund, M., Hoffer, B. and Olson, L. (2010). Parkinson's disease: Exit toxins, enter genetics. Progr. Neurobiol. *90*, 146-156.

101. Xie, Y. and Varshavsky, A. (2000). Physical association of ubiquitin ligases and the 26S proteasome. Proc. Natl. Acad. Sci. U.S.A. *97*, 2497-2502.

102. Xie, Y. and Varshavsky, A. (2002). UFD4 lacking the proteasome-binding region catalyses ubiquitination but is impaired in proteolysis. Nat. Cell. Biol. 4, 1003-1007.

103. Yao, T., Song, L., Xu, W., DeMartino, G.N., Florens, L., Swanson, S.K., Washburn, M.P., Conaway, R.C., Conaway, J.W. and Cohen, R.E. (2006). Proteasome recruitment and activation of the Uch37 deubiquitinating enzyme by Adrm1. Nat. Cell. Biol. *8*, 994-1002.

104. You, J. and Pickart, C.M. (2001). A HECT domain E3 enzyme assembles novel poyubiquitin chains. J. Biol. Chem. 276, 19871-19878.

105. Young, P., Deveraux, Q., Beal, R.E., Pickart, C.M. and Rechsteiner, M. (1998). Characterization of two polyubiquitin binding sites in the 26S protease subunit 5a. J. Biol. Chem. *273*, 5461-5467.

106. Zarranz, J.J., Alegre, J., Gomez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B., Llorens, V., Gomez, T.E., del, S.T., Munoz, D.G. and de Yebenes, J.G. (2004). The new mutation E46K, of alpha-synuclein causes Parkinson's and Lewy body dementia. Ann. Neurol. *55*, 164-173.

107. Zhang, L., Shimoji, M., Thomas, B., Moore, D.J., Yu, S.W., Marupudi, N.I., Torp, R., Torgner, I.A., Ottersen, O.P., Dawson, T.M. and Dawson, V.L. (2005). Mitochondrial localization of the Parkinson's disease related protein DJ-1: implications for pathogenesis. Hum. Mol. Genet. *14*, 2063-2073.

108. Zhang, Y., Gao, J., Chung, K.K., Huang, H., Dawson, V.L., and Dawson, T.M. (2000). Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc.Natl.Acad.Sci. U.S.A. *97*, 13354-13359

109. Zhou, C., Huang, Y., Shao, T., May, J., Prou, D., Perier, C., Dauer, W., Schon, E.A. and Przedborski, S. (2008). The kinase domain of mitochondrial PINK1 faces the cytoplasm. Proc. Natl. Acad. Sci. U.S.A. *105*, 12022-12027.