



2012



## DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA  
UNIVERSIDADE DE COIMBRA

Explore the role of parkin in endoplasmic reticulum stress and ER-associated protein degradation:  
cellular models for juvenile PD.

Miguel Fernando Alves Ferreira

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Explore the role of parkin in endoplasmic reticulum stress and ER-associated protein degradation:

cellular models for juvenile PD.

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Isabel da Conceição Moreira Pereira Alonso (Universidade do Porto) e do Professor Doutor Carlos Jorge Bandeira Duarte (Universidade de Coimbra)

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## Abbreviations

$\chi^2$	Chi-square
a.u.	Arbitrary units
ACTB	$\beta$ -actin gene
ANOVA	Analysis of variance
ARJP	Autosomal Recessive-Juvenile Parkinson's disease
ASK1	Apoptosis signal-regulating kinase 1
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BiP	Immunoglobulin heavy chain-binding Protein
CHP	Co-chaperone carboxyl terminus of Hsp70-interacting protein
DMEM	Dulbecco's Modified Eagle Medium
DN	Dominant Negative
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
DNA	Deoxyribonucleic acid
DUB	Deubiquitylating enzymes
eIF2	Eukaryotic initiation factor 2
EOPD	Early onset PD
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated protein degradation
ERQC	ER quality control
ERSE	ER-stress response element
FBS	Fetal bovine serum
h	Hour
IBR	In between RING fingers
IRE1	Inositol requiring kinase 1
JNK	c-Jun NH <sub>2</sub> -terminal kinase
LB	Luria Bertani
LB	Lewy bodies
LRRK2	Leucine-rich repeat kinase 2
min	Minute
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mRNA	Messenger ribonucleic acid
NMD	Nonsense-mediated decay
Pael-R	Parl receptor
PBS-T	Phosphate buffered saline-0.1% Tween20
PD	Parkinson's disease
pDNA	Plasmid DNA
PERK	Protein kinase-like endoplasmic reticulum kinase
PINK1	PTEN-induced putative kinase 1
PQ	Paraquat
PTC	Premature termination codons
PVDF	Polyvinylidene Difluoride
RBR	RING-between-RING
RING	Really interesting new gene
RNA	Ribonucleic acid
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SNc	Substantia nigra pars compacta
SP	Site protease
TRAF2	TNF receptor-associated factor 2
Ubl	Ubiquitin-like domain
UCHL1	Ubiquitin carboxyl-terminal esterase L1
UIM	Ubiquitin interacting motifs
UPD	Unique parkin domain
UPR	Unfolded protein response
UPRE	Unfolded protein response element
UPS	Ubiquitin proteasome system
XBP	X-box binding protein

## Abstract

Parkinson disease (PD) is the second most common neurodegenerative disorder and is mainly characterized by motor dysfunction, as the result of selective loss of dopaminergic neurons. Autosomal Recessive-Juvenile Parkinson's disease (AR-JP) is a rare genetic form, showing symptoms resembling idiopathic PD, although being characterized by a young age-at-onset (usually under 40). This disorder is caused by mutations in the *PARK2* gene which encodes parkin, an E3-ubiquitin ligase.

The unfolded protein response (UPR), leading to the activation of three protective cellular pathways, is required for the cells to cope with stressful conditions and results in the upregulation of endoplasmic reticulum (ER) stress genes. Recent observations support the existence of escorting machinery, which connects the ubiquitinated substrates at the ER-membrane with the proteasome, in a process known as ER-associated degradation (ERAD). Also, it has been shown that misfolded proteins and aggregates are able to induce ER stress, probably by suppressing proteasome function, an essential component of the ERAD machinery.

Our aim was to clarify the role of different cellular response mechanisms in the formation and clearance of parkin aggregates, including the endoplasmic reticulum stress response and ERAD. To accomplish this we studied nine mutations covering all parkin domains. First, we evaluated the role of ERAD in wild-type and mutant parkin clearance, by potentiating or suppressing ERAD in the different cellular models. Then, we explored the effect of the different parkin mutants on ER stress by assessing the levels of UPR-related proteins in these cellular models.

Our data show that VCP may have a role in the clearance of parkin aggregates and that VCP overexpression may result in an increase in parkin expression levels. Moreover, our data do not support the activation of an UPR in the presence of the different parkin mutants. Also, we observed a striking increase in the expression of ER molecular chaperones in cells co-expressing parkin and WT or DN VCP.

In conclusion, our results suggest that mutations in *PARK2* lead to parkin misfolding, contributing to the formation of aggregates that co-localize with VCP. With this work we enlarge the amount of data relating parkin with the ER and in particular with ERAD, raising the possibility that modulation of VCP and ERAD activity might have potential therapeutic significance for AR-PD.

Keywords: Parkinson disease; *PARK2*; Parkin; ER-Stress; ERAD

## Sumário

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum e é caracterizada principalmente por disfunção motora, como resultado da perda selectiva de neurónios dopaminérgicos. A doença de Parkinson Juvenil de transmissão Autossómica Recessiva (ARJP) é uma forma genética rara, apresentando sintomas semelhantes à DP idiopática, embora seja caracterizada por uma idade de início jovem (normalmente antes dos 40). Esta doença é causada por mutações no gene *PARK2* que codifica a parkina, uma E3-ubiquitina ligase.

A resposta a proteínas *unfolded* (UPR) leva à activação de três vias celulares protectoras, necessárias para a célula lidar com as situações de *stress* e resulta no aumento da expressão de genes de *stress* do Retículo Endoplasmático (RE). Dados recentes reforçam a existência de uma maquinaria de suporte, que liga os substratos ubiquitinados da membrana do RE ao proteossoma, num processo conhecido como degradação de proteínas associadas ao RE (ERAD). Além disso, foi demonstrado que as proteínas *misfolded* e os agregados são capazes de induzir uma resposta de stress do RE, provavelmente devido à supressão da função do proteossoma, um componente essencial da maquinaria da ERAD.

O nosso objectivo era esclarecer o papel de diferentes mecanismos de resposta celular na formação e na limpeza de agregados de parkina, incluindo a resposta de *stress* do RE e a ERAD. Para isso, estudamos nove mutações que cobrem todos os domínios da proteína parkina. Primeiro, foi avaliado o papel da ERAD na limpeza da parkina normal e mutada, através da potenciação ou supressão da ERAD nos diferentes modelos celulares. Em seguida, foram explorados os efeitos de diferentes mutantes da parkina no stress do RE, de modo a avaliar os níveis de proteínas relacionadas com a UPR nestes modelos celulares.

Os nossos dados mostram que a VCP pode ter um papel na limpeza de agregados de parkina e que a sua sobreexpressão pode resultar no aumento dos níveis de expressão da parkina. Por outro lado, os nossos dados não suportam a activação da UPR na presença dos diferentes mutantes de parkina. Além disso, observou-se também um aumento na expressão das chaperones moleculares do RE nas células que co-expressam a parkina e VCP WT ou DN.

Em conclusão, os nossos resultados sugerem que mutações no gene *PARK2* podem levar ao *misfolding* da parkina, contribuindo para a formação de agregados, que co-



localizam com a VCP. Com este trabalho, conseguimos obter mais dados que relacionam a parkina com o RE e em particular com a ERAD, reforçando a hipótese de que a modulação da VCP e da actividade da ERAD pode ter um potencial terapêutico significativo na ARJP.

Palavras-chave: Doença de Parkinson, PARK2; Parkin; Stress do ER; ERAD

## **1. Introduction**

Neurodegenerative diseases have a high worldwide prevalence, being defined as hereditary and sporadic conditions characterized by progressive and irreversible degeneration of specific groups of neurons, the cell that processes and transmits information, responsible for the functions of the nervous system. When this happens, depending on the disease, it causes substantial disability with gradual loss of motor functions, physiological and / or cognitive capacities creating a burden on individuals, families, communities and societies all over the world. Neurodegenerative disorders are currently the major cause of morbidity, disability and mortality, as the result of an increased life span, and are a fundamental issue in both medical care and research for the 21<sup>st</sup> century. Therefore, new efforts have focused on identifying crucial changes on genetic, epigenetic, or environmental factors that hamper normal neuronal function.

Protein misfolding and aggregation occurs due to a chronic imbalance in the generation and clearance of misfolded proteins, potentiated by alterations in primary structure caused by mutations, RNA modification, environmental insults or oxidative damage. These accumulations of misfolded proteins could play a role in the dysfunction and neuronal death that characterizes several common neurodegenerative disorders, such as Parkinson's disease (PD), one of the protein misfolding diseases.

## **1.1. Parkinson Disease**

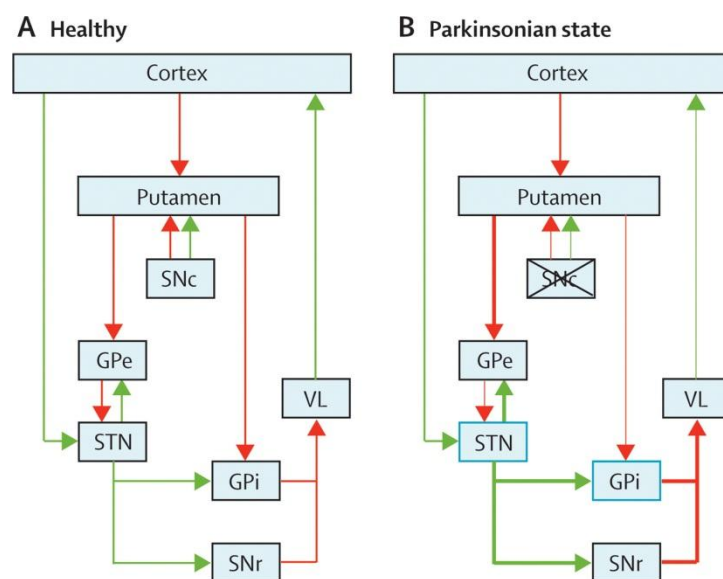
Parkinson disease, first described by James Parkinson in 1817, is the second most common neurodegenerative disease and the most frequent movement disorder. Approximately 1% of the population above 65 years suffers from this slowly progressive neurodegenerative disease with no known cure and its prevalence increases to approximately 5% above 85 years[1]. In Portugal, in 1994 a cross-sectional study was performed in which PD prevalence was estimated to be 130/100,000[2]. The mean age of diagnosis of PD is in the seventh decade of life but the disease can be diagnosed at any age, and it is estimated that 3% of the cases are initially recognized in individuals younger than 50 years old [3, 4]. The mean life expectancy following diagnosis is approximately 15 years, although affected individuals can frequently survive two decades or longer [5, 6].

PD is clinically characterized by four cardinal signs: resting tremor, rigidity (increased muscular tone), postural instability and bradykinesia (slowed movements) [7]. Nevertheless, non-motor symptoms are also present, including constipation, urinary symptoms, sleep disturbances, and olfactory impairment, which are now believed to presage the clinical recognition of bradykinesia, tremor or gait impairment by many years [3]. However, the cardinal motor symptoms apparently appear at a late stage of the disease when 60 to 70% of the substantia nigra dopaminergic cells are already dead, resulting in a concomitant 80% depletion in striatal dopamine [8, 9].

### **1.1.1. Pathophysiological mechanisms**

PD pathology results from the highly specific progressive and selective loss of dopaminergic neurons in the pars compacta of the substantia nigra (SNc) and from the

dysfunction of the basal ganglia which is accompanied by a dramatic reduction of striatal dopamine levels. In normal conditions (figure 1), dopamine from the SNc facilitates putaminal neurons in the direct pathway (putamen- GPi) and inhibits those in the indirect pathway (putamen-GPe-STN-GPi/SNr). Activation of the direct pathway leads to reduced neuronal firing in the GPi/SNr and movement facilitation, while activation of the indirect pathway suppresses movements. In Parkinson's disease (B), dopamine deficit leads to an increased activity of the indirect circuit by STN hyperactivity (key feature) and hypoactivity in the direct circuit, resulting in an increase of the inhibitory output from the Gpi to the VL and in the reduction of the activation of cortical and brainstem motor regions. Thus, this mechanism is repressing the initiation of movements and leading to the main motor features (tremor and rigidity) [9].



**Figure 1.** Summary of the classic pathophysiological model of the basal ganglia in healthy (A), parkinsonian (B) states. (adapted by Rodriguez-Oroz MC, 2009). Green arrows indicate excitatory activity and red arrows indicate inhibitory activity. GPe=globus pallidus pars externa. GPi=globus pallidus pars interna. SNc=substantia nigra pars compacta. SNr=substantia nigra pars reticulata. STN=subthalamic nucleus. VL=ventrolateral nucleus.

The most popular treatment for PD is through medication that increases the level of dopamine in the brain (e.g. L-dopa), enhancing abnormal putaminal dopaminergic activation which will lead to hypoactivity in the indirect circuit and hyperactivity in the direct circuit, resulting in reduced inhibitory output activity from the basal ganglia [6, 9]. Dopamine supplementation in the early stages of the disease can suppress the symptoms but, with PD progression, dopamine supplementation becomes ineffective and exacerbates motor symptoms [10].

Why and how DA neurons are preferentially lost in PD is not clear. A potential clue to the vulnerability of these neurons is the opening of L-type calcium channels during autonomous pacemaking, resulting in sustained calcium entry into the cytoplasm of SNc DA neurons, increasing mitochondrial oxidative stress and susceptibility to toxins known to induce PD in animal models [11]. Hence, oxidation of cytosolic DA (and its metabolites) leads to the production of damaging free radicals resulting in neuronal loss. Also, dopaminergic neurons have been shown to exhibit distinct physiology intrinsically associated with elevated reactive oxygen species production, which has been indicated as determinant for substantia nigra degeneration under oxidative stress conditions. Although there are diverse causes for PD, the disease pathogenic mechanism converges in a subset of pathways namely mitochondrial impairment, oxidative stress and impaired protein handling. Nevertheless, the role for this type of cellular stress in normal aging and PD is still to be fully understood [12, 13].

### **1.1.2. Histopathology**

PD is characterized by the intracytoplasmic accumulation of amyloide-like inclusions, known as Lewy bodies (LB), in the spared dopaminergic cells. PD pathology is not restricted to the DA system, progressively involving noradrenergic and serotonergic

neurons within the locus coeruleus, dorsal motor nucleus of the vagus, nucleus basalis of Meynertm, the olfactory systems and nerve cells of peripheral autonomic ganglia [14-16].

### **1.1.3. Environment and Genetics**

In the recent years, it has become clear that genetic factors are implicated in the etiopathogenesis of PD although environmental factors are also major determinants and cannot be discarded [17]. Environmental causes are generally associated with toxins and/or free radicals, in part due to pesticide exposure (specifically, the pesticides rotenone and Paraquat (PQ) and the fungicide Maneb) and intravenous drugs contaminated with methyl-phenyl-tetrahydropyridine (MPTP) [18-20]. Some meta-analysis studies show that both cigarette smoking and coffee consumption are associated with reduced PD susceptibility but the biological mechanisms underlying this potentially protective effect are still poorly understood [21]. Nevertheless, a causal role for these environmental factors in the etiology of PD has yet to be definitively established, although it is known that exposure to these agents lead to an array of consequences such as lipid peroxidation, oxidative DNA damage and mitochondrial dysfunction [22, 23].

Most of PD cases occur sporadically and the specific etiology of the disease remains unknown. In the last few years, however, the discovery of genes linked to rare familial forms of the disease, supports a substantial genetic component (Table1) [24].

**Table 1** – The major genetic forms of PD.

<b>Locus</b>	<b>Chr.</b>	<b>Gene/Protein</b>	<b>Function</b>	<b>Heredit.</b>	<b>Phenotype/ AO</b>
<b>PARK1</b>	4q21	<i>SNCA</i> /α-Synuclein	Synaptic protein	AD	PD, ~46y
<b>PARK4</b>			Vesicle traffic		Lewy bodies
<b>PARK5</b>	4p14	<i>UCHL1</i>	Ubiquitin hydrolase/ligase	AD	PD ~50y
<b>PARK2</b>	6q25	<i>PARK2</i> /Parkin	Ubiquitin E3 ligase	AR	Parkinsonism, Juvenil < 40y
<b>PARK6</b>	1p36	<i>PINK1</i>	Mitochondrial kinase	AR	Parkinsonism, 30-50y
<b>PARK7</b>	1p36	<i>DJ-1</i> /DJ-1	Oxidative stress defence	AR	Parkinsonism, 20-40y
<b>PARK8</b>	12p12	<i>LRRK2</i> /LRRK2	Kinase 2	AD	Late onset

The genes encoding alpha-synuclein (*SNCA*), ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) (*UCHL1*), leucine-rich repeat kinase 2 (*LRRK2*) are responsible for autosomal dominant PD, whereas mutations in the genes encoding Parkin, PTEN (phosphatase and tensin homologue deleted on chromosome 10)-induced putative kinase 1 (PINK1) and DJ-1 cause autosomal recessive parkinsonism with early onset [25-27].

## 1.2. Juvenile Parkinson Disease

First identified in Japan by Yamamura et al. in 1973, Autosomal Recessive-Juvenile Parkinson's disease (ARJP), is the most frequent type of familial PD and is characterized by a young age at onset (usually under 40). In general, the pathogenic parkin mutations are inherited in a recessive manner in patients and ARJP has been mostly linked to homozygous or compound heterozygous mutations. Nevertheless, single heterozygous mutations in parkin have also been found but their role in disease causality is controversial [28]. The symptoms of ARJP resemble those of idiopathic PD besides prominent foot dystonia, hyperactive tendon reflexes, diurnal fluctuation, sleep benefit (with an excellent response to levodopa) and frequent and early occurrence of L-dopa-induced dyskinesias.[29] ARJP diverges from idiopathic PD because patients do not appear to develop non-motor manifestations of PD (e.g., loss of olfaction) and because Lewy bodies, the diagnostic hallmark of PD, are usually not present. Nevertheless, LB pathology has infrequently been described in patients with parkin mutations, suggesting that parkin may play a more direct role in the typical LB parkinsonism [30-33].

The gene *PARK2* is responsible for ARJP in some patients and, is located on chromosome 6 (6q25.2-q27), spanning more than 500kb and comprising 12 exons [34]. Mutations in *PARK2* range from point mutations to complex rearrangements, including deletions and/or duplications of one or more exons [35]. Mutations in *PARK2* are the most common cause of early onset PD (EOPD), being responsible for approximately 50% of all ARJP, and of 77% of sporadic cases with disease onset before the age of 20 [28, 36].



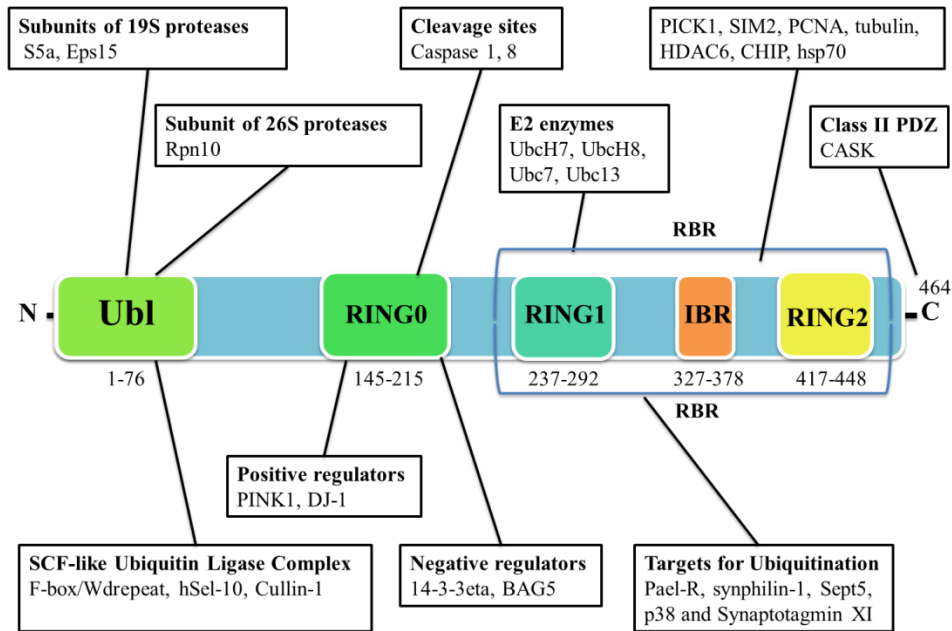
The parkin protein is derived from a 4.5 kb transcript, has 465 amino acids and a molecular weight of 52 kDa. Parkin has remained essentially unchanged throughout evolution, with orthologs in many different organisms, including *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, *Rattus norvegicus*, and other species [37-40]. Parkin is predominantly a cytosolic protein but also co-localizes to cellular vesicles, the Golgi complex, endoplasmic reticulum, and the mitochondrial outer membrane [25]. Although expressed in many human tissues this protein is abundant in the brain, including the substantia nigra, as well as in heart and skeletal muscle, testis, stomach, adrenal gland, thyroid, and spinal cord [41]. Parkin has structural and functional homology to E3 ubiquitin ligases that specifically recognizes its substrate protein, promoting its ubiquitination and subsequent degradation by the proteasome [42, 43]. Furthermore, parkin is involved in mitochondrial maintenance and might induce subsequent autophagy of dysfunctional mitochondria [44-46].

### **1.2.1. Parkin Domain Structure and Function**

Parkin is a multidomain E3 ubiquitin ligase with an ubiquitin-like domain at its NH<sub>2</sub>-terminus followed by a RING0 domain (a unique parkin-specific domain) and a RBR (RING-between-RING) motif at its COOH-terminus (Figure 2).

The N-terminus ubiquitin-like domain (Ubl) interacts with ubiquitin interacting motifs (UIMs), found in the 19S proteasome regulatory subunit S5a and Eps15, positioning substrate proteins for degradation by the ubiquitin proteasome system (UPS) [47]. Even though not directly involved in E2 interactions, the Ubl domain has been shown to be essential for the ligase activity of parkin because deletion or mutation of this domain results in impaired E3 ligase activity [48]. The U1b domain also binds the Rpn10 subunit of 26S proteasomes, which is likely to recognize proteins that are poly-

ubiquitinated by parkin [49]. Additionally, this domain may work as a part of the SCF-like (Skp1-Cullin-F-box protein) ubiquitin ligase complex where parkin interacts with F-box/WDrepeat protein hSel-10 and Cullin-1[50].



**Figure 2.** Domain structure of parkin and its interaction partners. (adapted by Suzuki, 2006).

Hristova et al, in 2009, identified the RING0 domain within the UPD (unique parkin domain), previously thought to have no recognizable domain structure. RING0, a novel  $Zn^{2+}$ -binding domain, is located in the central ~150 residues of parkin separating the Ubl from the RBR region [51]. This region contains cleavage sites for the pro-apoptotic caspase 1 and 8 and is positively regulated by PINK1 and DJ-1, proteins that when mutated are responsible for familial PD [52, 53]. Mutations in this segment of parkin have been shown to lead to dysfunction of parkin E3 ligase activity [54, 55]. 14-3-3eta, one of the 14-3-3 family members, interacts directly with the RING0 region as BAG5 and inhibits parkin E3 ubiquitin protein ligase activity [54].

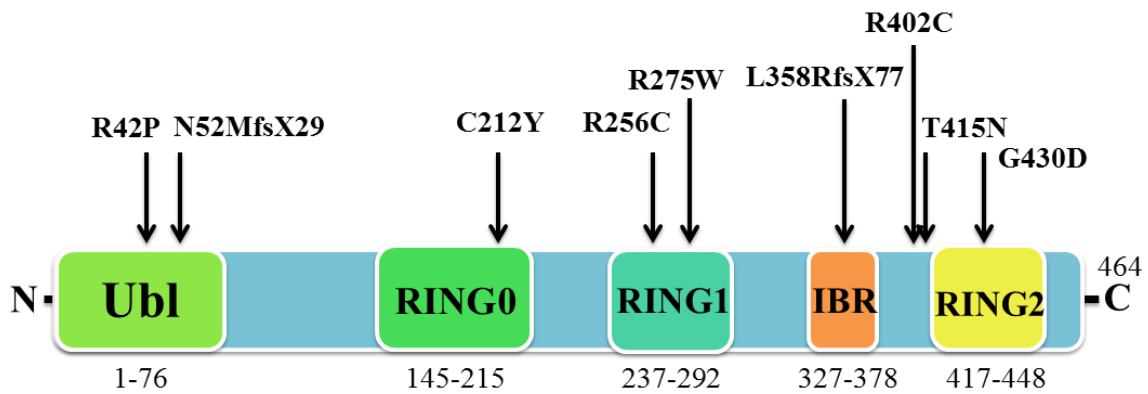
The parkin C-terminus consists of three consecutive domains, each of approximately 50 amino acids: two really interesting new gene (RING1 and RING2) fingers flanking a cysteine-rich domain, known as the in between RING fingers (IBR) region [56]. These domains appear to be important for binding and ubiquitination of ligase substrates (E2 enzymes) [57, 58]. Parkin interacts with the E2 ubiquitin-conjugating enzymes UbcH7, UbcH8, Ubc7, and Ubc13 and controls parkin-mediated ubiquitination of a variety of substrates such as Pael-R, synphilin-1, Sept5, p38 and synaptotagmin XI among others, through its C-terminal RING finger domains [59-61].

Some proteins interact with RBR domain but are not necessarily targets for ubiquitination, like PICK1, SIM2, PCNA, tubulin, HDAC6, CHIP and hsp70 [62-67].

At the extreme C-terminus of parkin, is present one specific short peptide sequence (amino acids FDV), functioning as a class II PDZ (PSD-95/discs large/ZO-1) binding motif that binds the PDZ protein, CASK [68].

### **1.2.2. Parkin Mutations**

In 1998, a linkage study discovered, for the first time, mutations of *PARK2* in Japanese ARJP families [41]. A large spectrum of parkin gene mutations has been identified, including missense and nonsense mutations, exon deletions and duplications and frameshifts, distributed in all the different domains along the protein. The clinical manifestations are influenced by the number of mutations, their location and if the mutations found were present in heterozygosity [56]. (Figure 3)



**Figure 3.** Representation of the mutations found in parkin, linked to familial PD. (adapted by Hristova et al, 2009)

Mutations that result in a truncated N-terminal, which includes the ubiquitin-like domain (UBL), as well as the pathogenic parkin mutant R42P, may induce a conformational change, decreasing the stability of parkin and leading to rapid degradation by the proteasome of these mutants [48]. The mutation C212Y, in RING0 domain, compromise zinc-coordinating residues by affecting zinc binding to parkin in order to maintain its three-dimensional structure, which causes near complete unfolding of the protein. Mutations in this domain could affect parkin solubility and/or ubiquitination [51]. Wang et al (2005) reported that C212Y mutation produces an insoluble form of parkin, leading to the formation of aggresome-like inclusions in SH-SY5Y cells. Until now, ubiquitination studies have not been reported on the RING0 C212Y mutants [51]. The R256C and R275W RING finger 1 mutations, have been shown previously to retain activity towards at least one of its putative substrates, synphilin-1. These mutations which reduce Parkin solubility were found in cellular inclusions which may contribute to the pathogenic mechanism of these specific mutations [69]. In the C-terminus IBR-R2, T415N is considered “ligase dead” due to its inability to self and substrate ubiquitination; additionally, this mutant has been shown to impair self-degradation [70]. Also, this mutation shows highly reduced abilities to bind

synphilin-1 and induces the formation of large mitochondrial aggregates in the perinuclear region [71]. Kahsn et al, tested whether the mutation G430D affected the caspase activity; however cleavage was not significantly affected [52]. Other studies evaluated the location of parkin based on its behavior in Triton X-soluble and Triton X-insoluble fraction although the mutation G430D showed a similar intracellular localization to wild-type parkin [70]. The effects in the structure and consequently in the function of parkin of the mutations T415N and L358RfsX77, located in the interdomain IBR-RING2 and in the IBR domain, have not yet been clarified. Therefore, gathered data show that parkin mutations result in a broad range of effects such as in the ubiquitination-degradation process, parkin-protein interaction, intracellular localization and enzymatic function.

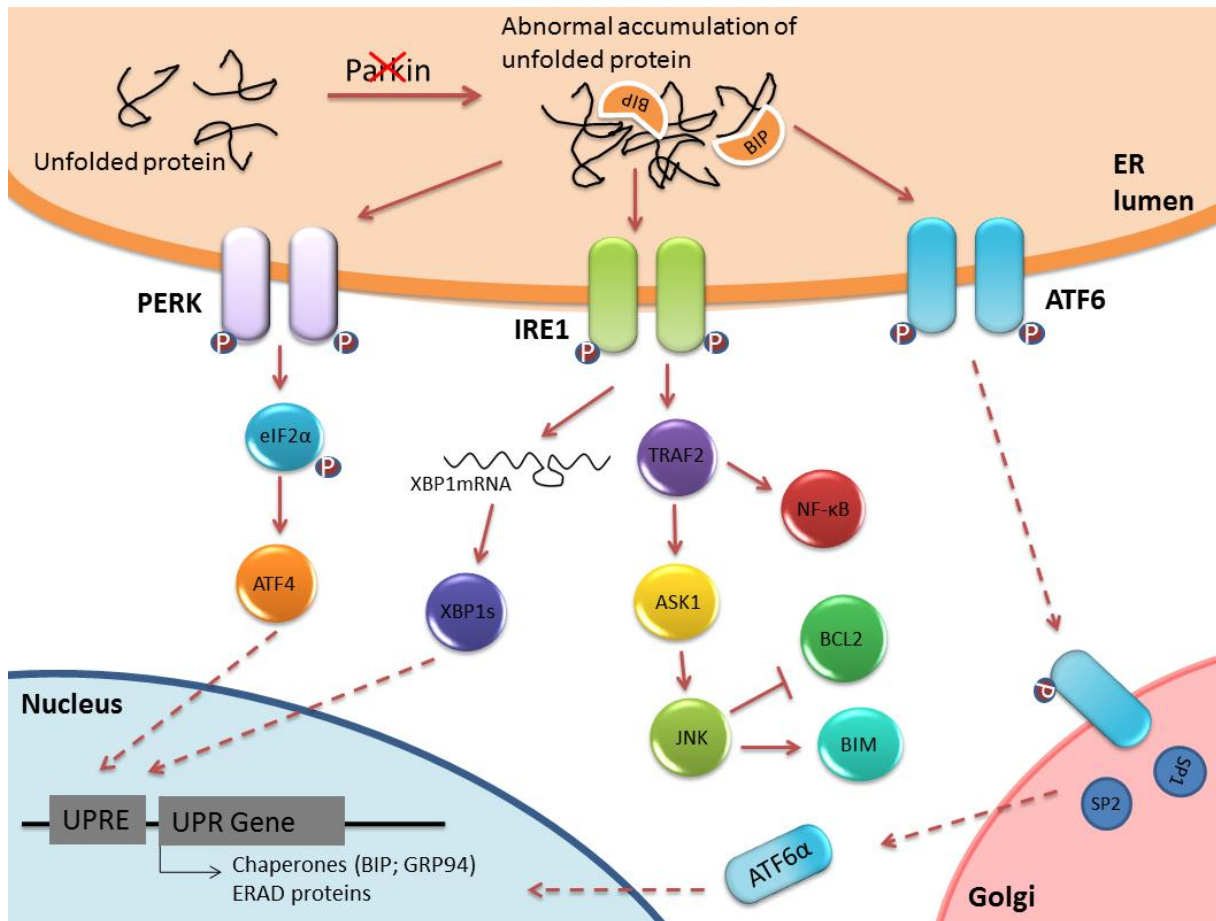
### **1.3. Endoplasmic Reticulum**

Recent studies have investigated the role of the endoplasmic reticulum (ER) in protein degradation and in neurodegeneration. Due to this fact, the physiological and pathophysiological function of parkin in ER stress is discussed.

The ER is a eukaryotic organelle responsible for the maintenance of calcium homeostasis, lipid biogenesis, synthesis and maturation of cell surface and secretory proteins. Among the many functions of ER it is noteworthy the important function in protein quality control through the central role in folding and processing newly synthesized secretory proteins. ER quality control (ERQC) is a surveillance mechanism that allows that only properly folded proteins exit the ER, on their way to other intracellular organelles and the cell surface [72]. Cellular stress, chemical environment, toxic compounds and fundamentally genetic mutations are some of the factors that can compromise folding efficiency. Thus, the ER is the entry gate for the vast majority of newly synthesized proteins; these proteins are transported into the ER lumen where chaperones and folding sensors often assist and alert the cell to the presence of misfolded proteins, triggering the folding process. Once folded, the protein is transported by vesicular trafficking to the Golgi apparatus for further processing. Disruption of these functions is a severe source of cellular stress [73].

### 1.3.1 ER-Stress

Sustained accumulation of unfolded or misfolded proteins results in the failure of the ER to cope with the excess of protein load, which is termed ER stress. These proteins form aggregates (which are hallmarks of degenerative disorders) that can elicit an ER stress response, the unfolded protein response (UPR). This signaling pathway is characterized by the induction of chaperones, degradation of misfolded proteins and attenuation of protein translation [73]. It is thought that the UPR is an evolutionarily conserved response that is triggered to adapt to the changing environment, and reestablish normal ER function.



**Figure 4.** Representation of the three transmembrane proteins in response of an unfolded proteins.

In mammalian ER there are three ER transmembrane proteins that operate as stress sensors: double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring kinase 1 (IRE1) (Figure 4) [74]. These proximal sensors are regulated by chaperones such as binding immunoglobulin protein (BIP). In normal unstressed conditions BIP maintains the UPR sensor proteins in an inactive state, by bounding to them and thus prevent their activation and downstream signaling. Under conditions associated with ER dysfunction, BIP binds the unfolded protein promoting its refolding and preventing its transport to the cis-Golgi.

PERK (PKR-like ER kinase) is a Ser/Thr protein kinase, that upon removal of BIP leads to PERK homodimerization and auto-phosphorylation resulting in its activation, and subsequent phosphorylation, on Ser51, of the translation eukaryotic initiation factor 2 (eIF2 $\alpha$ ). Phosphorylation of eIF2 $\alpha$  will promote a selective translation of activating transcription factor 4 (ATF4) mRNA, thereby inhibiting protein biosynthesis to reduce the workload on the ER.

ATF6 is a type II ER transmembrane protein encoding a bZIP transcriptional factor on its cytosolic domain that when released from BIP control is translocated to the Golgi compartment where it is cleaved by SP1 (site-1 protease) and SP2 (site-2 protease). The cleaved ATF6 $\alpha$  migrates to the cell nucleus where, in the presence of the transcription factor NF-Y, recognizes ER-stress response element (ERSE) minimal motif (CCAAT(N9)CCACG), (e.g. in the XBP1), and by binding to unfolded protein response element (UPRE) induces the expression of genes encoding ER resident molecular chaperone proteins, such as BIP, GRP4, PDI.

Finally, after dissociation of BIP, IRE-1 is activated, leading to dimerization, to activate its kinase and RNase activities. IRE-1 cuts out a sequence of 26 bases from the mRNA



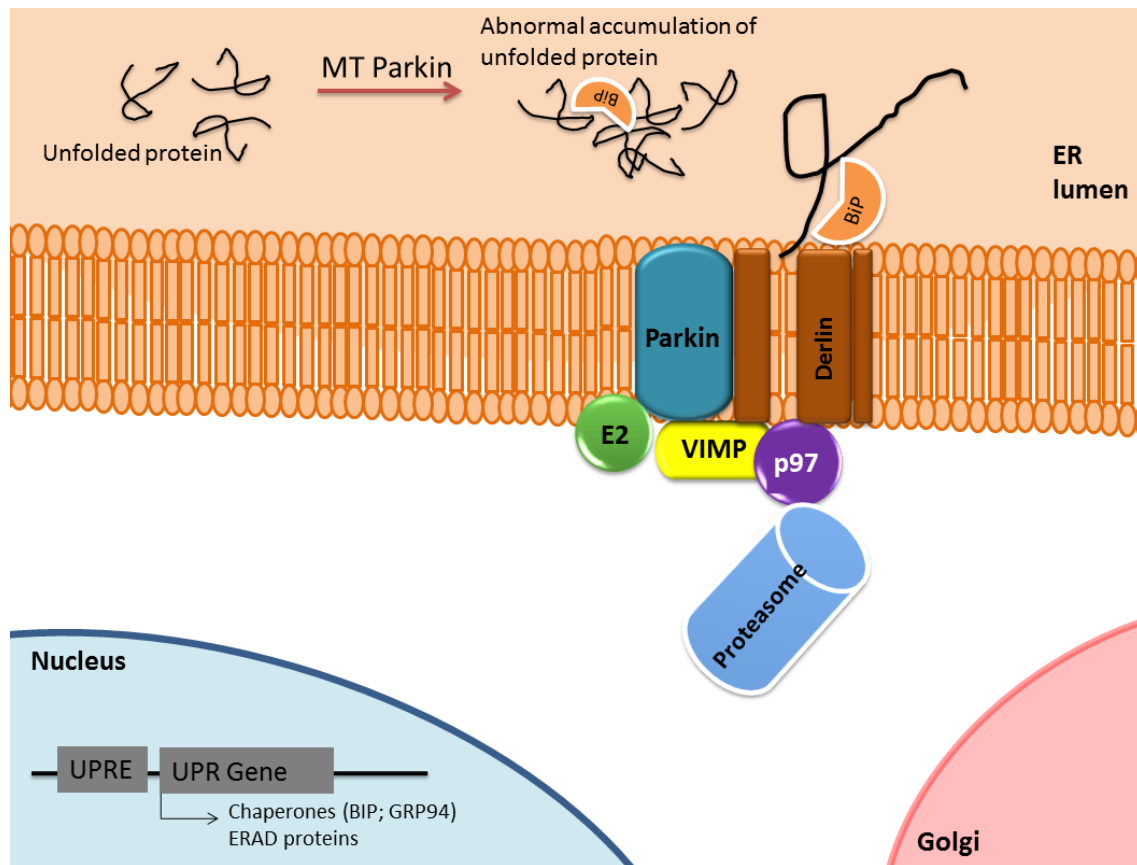
encoding X-box binding protein (XBP) 1, generating a 54kDA protein (XBP1s) with considerably higher stability that translocates to the nucleus and functions as a specific transcription factor that binds to UPRE or ERSE sequences in many UPR target genes. When oligomerized, IRE1 also binds TNF receptor-associated factor 2 (TRAF2) leading to the activation of protein kinases like apoptosis signal-regulating kinase 1 (ASK1), which by its turn activates c-Jun NH<sub>2</sub>-terminal kinase (JNK) and kinases linked to NF- $\kappa$ B [75, 76]. Moreover, JNK then inhibits anti-apoptotic BCL-2 and activates pro-apoptotic BIM. An excessive and/or prolonged stress leads to the activation of an ER-mediated apoptotic pathway and in the consequent cell death [77].

These three protective cellular responses, required for the cells to cope with stressful conditions, allow for adjustment of the endoplasmic reticulum associated protein degradation (ERAD)-related genes involved in ER expansion, folding, protein maturation, as well as export and degradation of misfolded proteins.

### **1.3.2. ERAD**

In general, protein aggregates do not accumulate in unstressed, healthy neurons, in part due to the existence of the ERQC, which include the endoplasmic reticulum associated protein degradation (ERAD). The ERAD components and the molecular chaperones that increase the folding capacity of the ER and the clearance of accumulating misfolded proteins are upregulated by UPR [78]. To ensure efficient dispatching of the improperly folded proteins, this complex ubiquitin-proteasome-mediated process involves a multi-protein complex on the ER membrane, and interacts with proteasome in the cytosol. ERAD was found in yeast, however, the ERAD ubiquitination machinery in both yeast and mammals comprises the same mechanism: the protein conducting channel, the ER-resident transmembrane E3s and their cognate E2s, the complex that

intermediate the retrotranslocation from the ER lumen back into cytoplasm and the 26S proteasome which degrades the misfolded proteins (figure 5) [78].



**Figure 5.** Representation of the ERAD machinery in mammals.

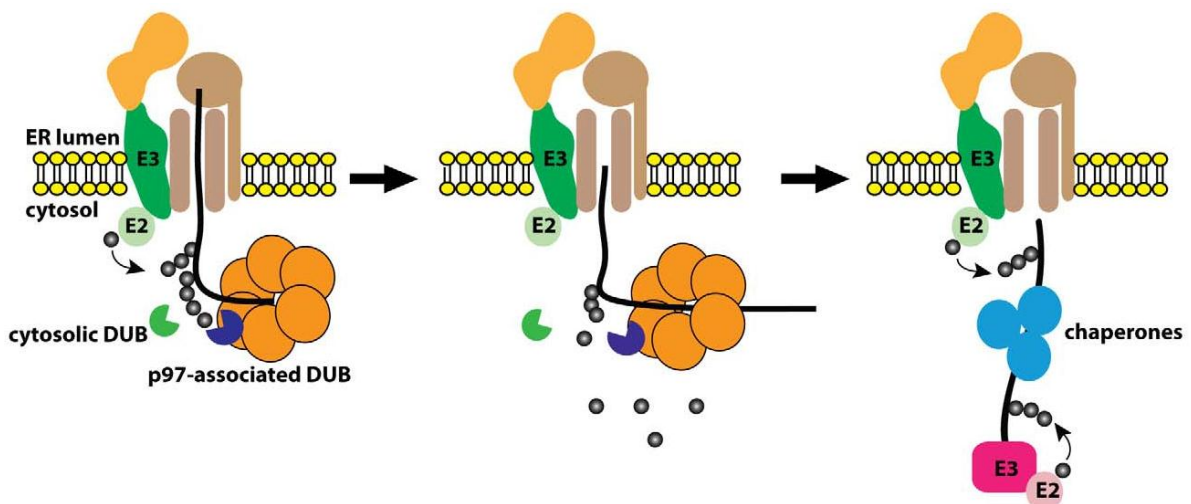
The ERAD process can be divided in 3 stages, which are going to be described in more detail, based on the mammalian proteins and processes:

### Recognition of misfolded proteins in the endoplasmic reticulum

The recognition of misfolded or mutated proteins depends on the chaperones (BIP), lectin-like chaperones (OS-9, XTP3-B) and others proteins (SEL1L, ERdj, EDEM) that interact with ERAD transmembrane complex, contributing to the maintenance of protein folding efficiency and help in the extraction of misfolded proteins by conducting channels [78, 79].

### Retro-translocation into the cytosol

In general, proteins are co-translationally inserted into the ER in order to be evaluated by ERQC. After this, they have to be transported from the ER lumen back into cytoplasm because the ubiquitin-proteasome system (UPS) is localized in this cellular compartment. This process is referred as “retro-translocation”, which occurs through conducting channels like Sec61 and a multi-spanning membrane protein, Derlin-1 [80]. Further, this retro-translocation requires a driving force that determines the direction of transport. Over 500 different E3 (such as HRD1, Parkin) and E2 (UBC6 and UBC7) conjugating proteins work together with ubiquitin to perform the highly regulated ubiquitination process, an essential driving force for ER substrate export [81]. Derlin-1, interacts with VIMP, forming a membrane protein complex that serves as receptor for p97. The AAA-ATPase p97, which is recruited by VIMP, facilitates the translocation of misfolded ER-proteins into the cytosol, by an ATP-driven process [82]. A recent study has provided a new model for transport the ubiquitinated proteins out of the ER.



**Figure 6.** Model based on results in Ernst et al. (adapted by Yien Tsai et al, 2011)

Ernst et al, showed, based in their results, that it is necessary a deubiquitination for protein dislocation from the ER [83]. So, the p97 complex (hexamer of the AAA-ATPase p97 and accessory proteins such as Ufd1 and Npl4) recognizes polyubiquitin

chains on the proteins as it moves through the protein-conducting channel. But, the protein dislocation might require trimming off the polyubiquitin chain to let the substrate enter the central channel of the p97 complex during retro-translocation, so, there are deubiquitinating enzymes (DUBs) associated with the p97 complex or potentially free in the cytosol that trim off the polyubiquitin chain on the substrate, allowing it to be threaded into the narrow channel of the p97 complex [84].

#### Ubiquitin-dependent degradation by the proteasome

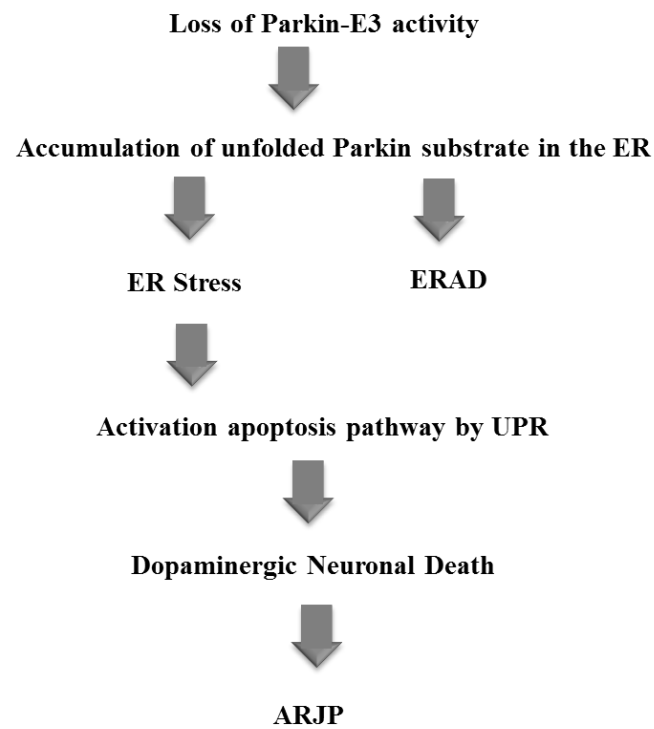
For the 19S capping complex of the 26S proteasome recognize and degrade proteins, these must be ubiquitinated. Therefore, E3 and their cognate E2 ligases, will again conjugate and bind the ubiquitin molecules to the dislocated substrate for targeting to the proteasome. Hereafter, the proteins are conducted into the central chamber of the 20S core region that contains the degradation machinery (proteolytically active sites) [84, 85].

### **1.3.3. Parkin and ER Stress/ERAD**

It has been shown that misfolded proteins and aggregates, even when involving cytosolic proteins, are able to induce ER stress, probably by suppressing proteasome function, an essential component of the ERAD machinery. Additionally, Imai et al. (2000) reported that overexpression of parkin specifically prevent cell death resulting from ER stress [43].

One of the putative substrates of parkin is Pael-R, which has been associated with inherited PD, because there is a possibility that accumulation of unfolded Pael-R in the ER might lead to the death of dopaminergic neurons as a result of unfolded protein stress (Figure 7) [86]. Pael receptor (Pael-R) is a G protein-coupled orphan receptor, that is abundantly expressed in dopaminergic neurons in the substantia nigra and that is

detected in an insoluble form in the brain of ARJP patients. Defects in parkin lead to the accumulation of Pael-R, due to the direct involvement of parkin in ERAD. Parkin binds to the ER-associated E2s involved in ERAD (Ubc6 and Ubc7) and ubiquitinate Pael-R to target it for proteasomal degradation [56].



**Figure 7.** Putative mechanism of parkin mutants in the etiology of ARJP. (adapted by Takahashi et al, 2003)

## 2. Objectives

Our main aim was to clarify the role of different cellular response mechanisms in the formation and clearance of parkin aggregates, including the endoplasmic reticulum stress and endoplasmic reticulum (ER)-associated protein degradation.

To accomplish this, we studied nine mutations, located in different parkin domains, including the N52MfsX29 (c.155delA) and R275W (c.823C>T), the most frequent mutations in Portuguese patients with juvenile Parkinson disease (our unpublished results).

Our specific objectives were:

- 1) To evaluate the role of ERAD in wild-type and mutant parkin clearance, by potentiating or suppressing ERAD in the different cellular models.
- 2) To explore the effect different parkin mutants on ER stress by assessing the levels of UPR-related proteins in these cellular models.

### **3. Material and methods**

#### **3.1. Expression constructs**

A pEGFP-C1 vector with the cDNA for the human parkin was kindly provided by Dr. Sumihiro Kawajiri from Juntendo University School of Medicine, Tokyo, Japan. A pCMV6-myc vector with the cDNA for the human VCP was purchased from *TrueORF Gold, OriGene*. A pCMV6-myc empty vector was generated by excising VCP with *MluI* and *AsiSI* restriction sites.

#### **3.2. Site-directed mutagenesis**

Nine parkin mutants (Table 2) were generated with the *QuikChange*® Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. Briefly, and for each intended mutation, 1,5 µg of plasmid DNA were added to a reaction mix containing 125 ng of each primer (forward and reverse, see table 3), 10x reaction buffer, 50 ng (10 mM) of a dNTP mix, 250 U of *PfuTurbo* DNA polymerase and ddH<sub>2</sub>O up to a final volume of 50 µL. In order to amplify the mutant plasmids, all samples were denatured at 95°C for 30 s, followed by 18 cycles comprising a cycle of denaturation at 95°C for 1 min, annealing at 65°C for 1 min, and then an extension at 68°C for 25 min. Following the reaction, the product was incubated with *DpnI*, at 37°C for 2 hours, in order to degrade the methylated non-mutated DNA (template). The reaction product was transformed into competent cells, XL-1 Blue *Escherichia coli* using heat shock method: 50 µL of Library Efficiency XL-1 Blue Competent Cells (Invitrogen) were thawed on ice, mixed gently with 2 µL of plasmid DNA (pDNA) and incubated for 30 minutes on ice. After a heat shock for 45 seconds at 42°C, cells were incubated on ice for 2 minutes. The culture was re-suspended in 0.5 mL of SOC medium, incubated for 1 hour at 37°C with vigorous shaking. Cells were centrifuged for

5 minutes at 3000 rpm and the pellet re-suspended in the remaining medium. Transformed bacteria were plated on Luria Bertani (LB) agar plates, containing 50 µg/mL kanamycin for positive clone selection, and incubated at 37°C overnight.

**Table 2.** Parkin mutants generated by site-directed mutagenesis.

<b>Mutation</b>		<b>Domain</b>
<b>Protein</b>	<b>cDNA</b>	
p.R42P	c.125G>C	Ubl
p.N52MfsX29	c.del155delA	Ubl
p.C212Y	c.635G>A	RING0
p.T240M	c.719C>T	RING1
p.R275W	c.823C>T	RING1
p.L358RfsX77	c.1072_1073delCT	IBR
p.R402C	c.1204C>T	IBR-RING2
p.T415N	c.1244C>A	IBR-RING2
p.G430D	c.1289G>A	RING2

A VCP<sup>E305Q/E578Q</sup> double mutant was generated from the pCMV6-VCP<sup>wt</sup>-Myc construct also by site-directed mutagenesis with specific primers (table 3) and following the same procedure.

Single isolated colonies were inoculated in liquid media with selection and incubated overnight, at 37°C, for plasmid DNA isolation.



**Table 3.** Primers for site-directed mutagenesis. Fw- Forward; Rv-Reverse

<b>Parkin Mutation (cDNA/protein)</b>	<b>Direction</b>	<b>Primer Sequence</b>
c.125G>C / p.R42P	Fw Rv	5'-CGGCTGACCAGTTGCCTGTGATTTTCGCAGG-3' 5'-CCTGCGAAAATCACAGGCAACTGGTCAGCCG-3'
c.del155delA / p.N52MfsX29	Fw Rv	5'-GGGAAGGAGCTGAGGATGACTGGACTGTGC-3' 5'-GCACAGTCCAGTCATCCTCAGTCTCCTTCCC-3'
c.635G>A / p.C272Y	Fw Rv	5'-GGACTAGTGCAGAATTTTTCTTTAAATATGGAGCACACCCCA-3' 5'-TGGGGTGTGCTCCATATTTAAAGAAAAATTCTGCACTAGTCC-3'
c.719C>T / p.T240M	Fw Rv	5'-AGTCGGAACATCACTTGCATTATGTGCACAGACGT-3' 5'-ACGTCTGTGCACATAATGCAAGTGATGTTCCGACT-3'
c.823C>T / p.R275W	Fw Rv	5'-CTGTGTGACAAGACTCAATGATTGGCAGTTTGTTCACG-3' 5'-CGTGAACAACTGCCAATCATTGAGTCTTGTGCACACAG-3'
c.1072_1073delCT / p.L358RfsX77	Fw Rv	5'-CGAAGGGGGCAATGGCAGGGCTGTGG-3' 5'-CCACAGCCCTGCCATTGCCCCCTTCG-3'
c.1204C>T / p.R402C	Fw Rv	5'-CGCCGAGCAGGCTTGTGGGAAGCAGC-3' 5'-GCTGCTTCCCAACAAGCCTGCTCGGCG-3'
c.1244C>A / p.T415N	Fw Rv	5'-ACCATCAAGAAAACCAACAAGCCCTGTCCCCG-3' 5'-CGGGGACAGGGCTTGTGGTTTTCTTGATGGT-3'
c.1289G>A / p.G430D	Fw Rv	5'-CAGTGGAAAAAATGGAGACTGCATGCACATGAAGTG-3' 5'-CACTTCATGTGCATGCAGTCTCCATTTTTTCCACTG-3'
<b>VCP Mutation (cDNA/protein)</b>	<b>Direction</b>	<b>Primer Sequence</b>
c.913G>C / p.E305Q	Fw Rv	5'-CTCCTGCCATCATCTTCATTGATCAGCTAGATGCCA-3' 5'-TGGCATCTAGCTGATCAATGAAGATGATGGCAGGAG-3'
c.1732G>C / p.E578Q	Fw Rv	5'-CCCTGTGTGCTATTCTTTGATCAGCTGGATTCTGA-3' 5'-TCGAATCCAGCTGATCAAAGAATAGCACACAGGG-3'

### 3.3. Plasmid DNA isolation

Plasmid DNA was extracted using the Qiaprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. In brief, the liquid cultures were centrifuged for 10 minutes at 3000 rpm and the supernatant was discarded. The pelleted bacteria were then re-suspended in buffer P1, containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA and 100 µg/mL of RNase A. Cells were lysed in 200 mM NaOH and 1% SDS (Buffer P2). After centrifuging cell extracts at 13000 rpm for 10 minutes at room temperature, the supernatant was applied to the QIAprep spin columns, which were then centrifuged for

1 minute. The columns were afterwards washed with PB and PE buffers, with 1 minute centrifugation after each wash. Any remaining buffer was removed from the columns by one extra 1 minute centrifugation. The spin columns were then incubated with EB (10 mM Tris-Cl, pH8.5) and the plasmid DNA recovered with a final 1 minute centrifugation. Plasmid DNA concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific).

### **3.4. Direct sequencing**

All mutations and the entire inserts were confirmed by direct sequencing with the primers presented in table 4. Sequencing reactions were performed using 2  $\mu$ L of Big Dye Terminator Cycle Sequencing v1.1 Ready Reaction (Applied Biosystems), 0.5  $\mu$ L of primers (forward or reverse), 1  $\mu$ L of purified DNA fragment and 6.5  $\mu$ L of ddH<sub>2</sub>O. The cycling conditions used were: an initial denaturing at 95°C for 5 minutes, followed by 35 cycles of denaturation for 10 seconds at 96°C, annealing for 5 seconds at 50°C, and an extension for 4 minutes at 50°C. Then, the samples were purified using DyeEx 96 well plates (QIAGEN) according to manufacturer's instructions, and were loaded in an ABI-PRISM 3130 XL genetic analyzer (Applied Biosystems).

**Table 4.** Primers designed for cDNA sequencing.

<b>Parkin –pEGFP</b>	
<b>Primer ID</b>	<b>Primer Sequence</b>
PARK2_F1	5'-CTGGATCAGCAGAGCATTGTTTCAC-3'
PARK2_F2	5'-TCCAAACCGGATGAGTGGTGAATG-3'
PARK2_F3	5'-AGTATGGTGCAGAGGAGTGTGT-3'
PARK2_R1	5'-GTCGCCTCCAGTTGCATTCATTTC-3'

<b>pCMV6-VCP-Myc</b>	
<b>Primer ID</b>	<b>Primer Sequence</b>
VCP_F1	5'-CAGATCCTAGCCCTTATTGC-3'
VCP_F2	5'-GTCCTTGAATGAAGTAGGGT-3'
VCP_F3	5'-GGTAGATATTGGAATTCCTG-3'
VCP_F4	5'-GGGGAGTCTGAGGCCAATGT-3'
VCP_R1	5'-ATCAGCCATGGAGGTAGAAG-3'

### 3.5. Cell Culture and transfection

The human neuroblastoma cell line SH-SY5Y (from ATCC) was grown in DMEM (Dulbecco's Modified Eagle Medium) with GlutaMAX (Gibco by life technologies), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 1% of antibiotic/antimycotic solution (Invitrogen) at 37°C in 5% CO<sub>2</sub>, 95% humidified air atmosphere. At 80% of confluence, SH-SY5Y cells were detached by trypsinization with a trypsin-EDTA (0.25% Trypsin and 1 mM EDTA, Invitrogen) for 5 minutes at 37°C. The medium containing cells was centrifuged at 500g for 4 minutes and then the pellet was re-suspended in 6 ml of fresh medium. Cells were counted in a hemocytometer, and seed at  $5 \times 10^5$  cells per well for 12-well plates or  $39,5 \times 10^3$  cells per well for 96-wells plates.

SH-SY5Y cells were co-transfected 24h after plating with parkin and wild-type VCP; parkin and mutant VCP; and parkin withpCMV6-myc empty vector. The co-transfections were performed using a 3:1 ratio between FuGENE HD (Roche) and the plasmid DNA, 75 µL of the resulting complex mixture were used for each transfection.

### **3.6. RNA isolation**

Total RNA was isolated from cells at 24h, 48h and 72h post-transfection using the TRIzol Reagent (Invitrogen), according to the manufacturer's recommendations. Briefly, cells were lysed in 500  $\mu$ L of TRIzol and incubated for 5 minutes at room temperature, after which 100  $\mu$ L of chloroform were added and mixed with each sample. After 3 minutes of incubation, samples were centrifuged at 12000g, for 15 minutes and at 4°C, for phase separation. The supernatant was removed, and the RNA was precipitated with 250  $\mu$ L of isopropyl alcohol and pelleted, after 10 minutes of incubation at room temperatures, by centrifugation at 12000g for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet was washed with 500  $\mu$ L of 75% ethanol. Samples were centrifuged again at 7500g for 5 minutes and at 4°C, and the RNA pellet was air dried and re-suspended in DEPC-treated ddH<sub>2</sub>O. Finally, the samples were incubated at 50°C for 10 minutes to allow RNA solubilization and stored at -80°C.

Total RNA quantification was performed using the NanoDrop 2000 spectrophotometer (Thermo Scientific).

### **3.7. Reverse transcriptase and real-time PCR**

After RNA isolation, reverse transcription was performed with Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to manufacturer's instructions, using 1 $\mu$ g of total RNA of each sample and the standard oligo(dT)<sub>20</sub> primer.

To measure the *PARK2*, *VCP* and the housekeeping gene *ACTB* expression in the transfected cells, analysis was conducted using quantitative Real-time PCR. Real-time PCR was performed using a iQ Real-time PCR detection system (Bio-Rad) with a mix

of 10  $\mu\text{L}$  of iQ SYBR Green Supermix (Bio-Rad), 0.25  $\mu\text{L}$  of the 10  $\mu\text{M}$  primer (forward and reverse) (table 5), 1  $\mu\text{L}$  of cDNA at 0.1  $\mu\text{g}/\mu\text{L}$  (10-fold dilution of the cDNA synthesis reaction) and 8.5  $\mu\text{L}$  of ddH<sub>2</sub>O. After an initial denaturing cycle at 95°C for 3 minutes, amplification was performed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and an extension at 72°C for 30 seconds. Two replicas of each sample and for each gene were done and the average Ct was determined. Expression levels of *PARK2* and *VCP* were normalized to the housekeeping gene *ACTB*.

**Table 5.** Primers used for Real Time PCR.

Gene	Direction	Primer Sequence	Amplicon size (bp)	T <sub>annealing</sub>
Parkin	Fw	5'-CAGCCTCCAAAGAAACCATCAAG-3'	149	57°C
	Rv	5'-CAGCCTCCAAAGAAACCATCAAG-3'		
VCP	Fw	5'-GGTAGAGGTGCCACAGGTAAC-3'	162	57°C
	Rv	5'-CAGCCAGGAGGTCCATAGAAC-3'		
ACTB	Fw	5'-GCACTCTTCCAGCCTTCCTTC-3'	176	57°C
	Rv	5'-GTGATCTCCTTCTGCATCCTGTC-3'		

### 3.8. Protein extracts and western blotting

SH-SY5Y cells expressing the target proteins were lysed 24h, 48h and 72h after transfection using a cell-lysis buffer (RIPA buffer; Sigma-Aldrich) supplemented with a cComplete Protease Inhibitor Cocktail (Roche). The cell extracts were sonicated and protein concentration was determined using a colorimetric assay (DC Protein Assay, Bio-Rad) in microplates according to the manufacturer's instructions.

Protein equivalent samples were denatured for 10 minutes at 100°C in an equal volume of 2x Laemmli buffer (with 0.7%  $\beta$ -mercaptoethanol). Protein were separated on 8% and 10% denaturing polyacrylamide gels and then transferred to Polyvinylidene Difluoride (PVDF) membranes. The membranes were blocked in a 3% non-fat powder

milk PBS-T solution, for 1 hour at 4°C and then incubated overnight at 4°C with the primary antibodies: anti-KDEL antibody detecting GRP78/BiP and GRP94 (mouse monoclonal, 1:500, Abcam), anti-Parkin (rabbit monoclonal, 1:1000, Novus Biologicals), anti-VCP (rabbit polyclonal, 1:1000, Cell Signaling) and anti-β-actin (mouse monoclonal, 1:10000, Santa Cruz) diluted in 3% milk PBS-T solution. After three washes with PBS-T, the membranes were incubated with the secondary antibodies (diluted in 3% milk PBS-T solution): anti-mouse (1:5000, Santa Cruz) or anti-rabbit (1:10000, Calbiochem) for 1 hour or 1 hour and 40 minutes, respectively, at 4°C. After three washes with PBS-T, chemiluminescent detection was performed by FemtoMax Chemiluminescent Western blot Kit (Rockland) and bands were visualized by autoradiography with Hyperfilm ECL (GE Healthcare). Quantification of band intensity was performed using GS-800 calibrated imaging densitometer (Bio-Rad).

### **3.9. Immunofluorescence assays**

Cells were seeded in 12-well plates with a coverslips previously coated with 0.2mg/ml collagen type I (Stemcell), after an acid wash. Cells were plated and allowed to adhere for 48h before transfection. After 24h, 48h and 72h post-transfection, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, washed in PBS and permeabilized with 0.5% Triton X-100 solution for 15 minutes. After washed in PBS, cells were blocked for 1 hour at room temperature using 10% FBS. Cells were incubated overnight at 4°C with the primary antibody anti-myc (1:1800, Roche). After three washes with PBS, cells were incubated at room temperature for 1 hour with the secondary antibody, Alexa Fluor-568 (1:800, Invitrogen). Nuclei were stained with Hoechst 33342 (1:10000, Invitrogen) at room temperature for 5 minutes. After washed, the coverslips were mounted in ProLong Gold antifade reagent (Invitrogen), allowed to

dry and sealed with nail polish. Images were acquired using a Carl Zeiss Axio Imager Z1 microscope with a coupled device camera, using 10X or 63X (oil) objectives.

### **3.10. Quantification of intracellular parkin aggregates**

The quantification of parkin aggregates was performed using the ImageJ software. The 12 bit images were converted to 8 bit and a ten-pixel background was subtracted to the GFP channel. The number of particles per cell was analyzed by counting the area of every particle with  $0.01 \mu\text{m}^2$  and higher, which allowed the exclusion of single pixels. At least 20 cells were analyzed per condition.

### **3.11. Statistical analysis**

Number of cells with and without aggregates was compared between wild-type and mutant cells using the  $\chi^2$  test.

Aggregate quantification data are expressed as mean  $\pm$  SEM. Comparison of the number and area of parkin aggregates was done using one-way ANOVA with a Bonferroni post-hoc test for multiple comparisons. This test was applied to the three tested conditions (cells expressing only parkin, cells expressing parkin and wild-type VCP and cells expressing parkin and mutant VCP). Comparisons between the different testing conditions, to assess the effect of wild-type and mutant VCP on parkin aggregation, was performed by the non-parametric Kruskal-Wallis test. Differences were considered to be significant when  $p < 0.05$ . Statistical analysis was performed using PASW Statistics 18.

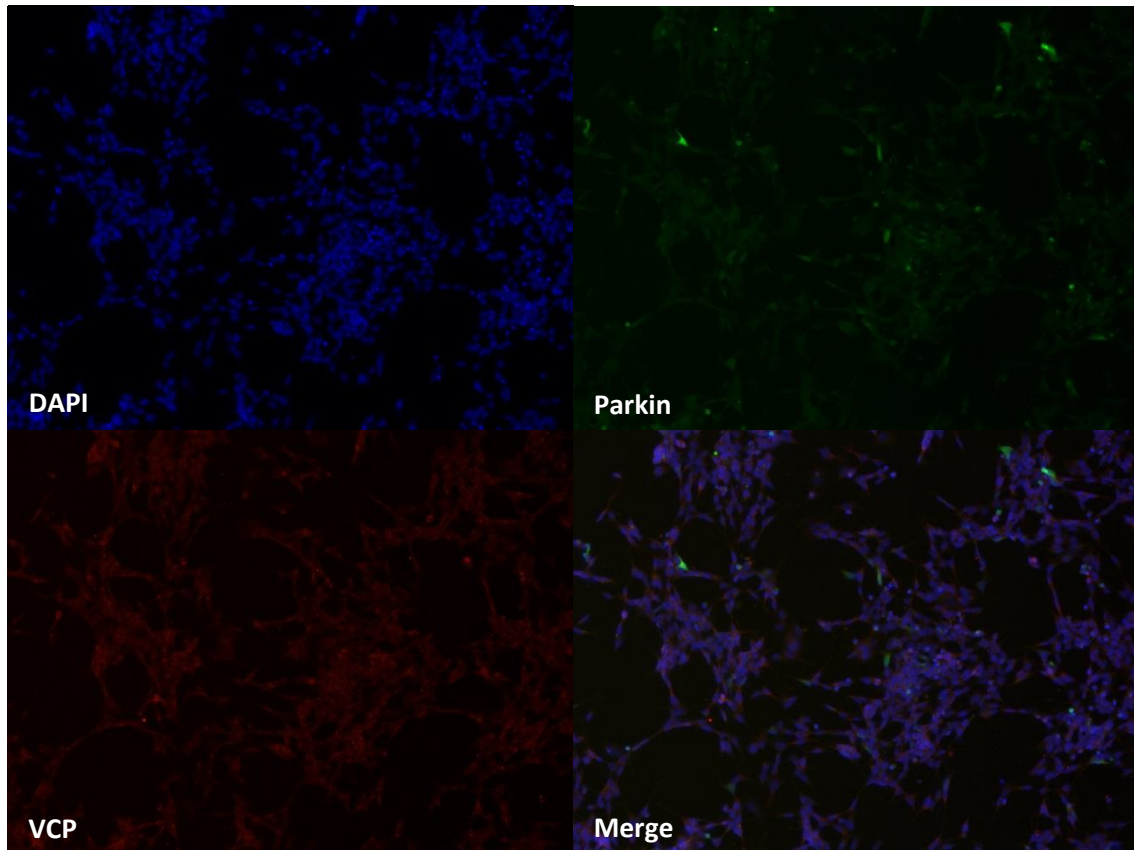
## **4. Results**

During this project we have explored the effect of VCP, a protein involved in the ERAD machinery, in the clearance of parkin aggregates. We selected mutations located in different parkin domains in order to have a broader insight into the impact of ERAD on the diverse mutations. Our data show that VCP may have a role in the clearance of parkin aggregates and that VCP overexpression may result in an upregulation of parkin expression levels. Moreover, our data do not support the involvement of an UPR in the presence of the different parkin mutants, at least after 24 hours of expression. Nevertheless, the number of experiments needs to be enlarged and other time-points explored. Also, we have observed a striking increase in the expression of ER chaperones when co-expressing parkin and WT or DN VCP that we will further explore in order to get additional insight into the role of ER stress in parkin-associated pathogenesis.

### **4.1. Co-transfection efficiency in SH-SY5Y cells**

Although neuroblastoma cells have been shown to be a difficult cell line to transfect we have optimized our transfection protocol and obtained high transfection efficiencies for parkin and VCP co-transfection. We have applied a 3:1 ratio between the transfection reagent and the plasmid DNA that resulted in a good balance between transfection efficiency and cytotoxicity. Under these conditions we were able to estimate transfection efficiencies between 80% and 90% in the SH-SY5Y cultures (Figure 8).





**Figure 8. Co-transfection efficiency.** SH-SY5Y cells transfected with pEGFP-parkin and pCMV6-VCP using FuGENE HD (Roche). Images obtained with a 10X objective on a Zeiss Axio Imager Z1, 48h after transfection.

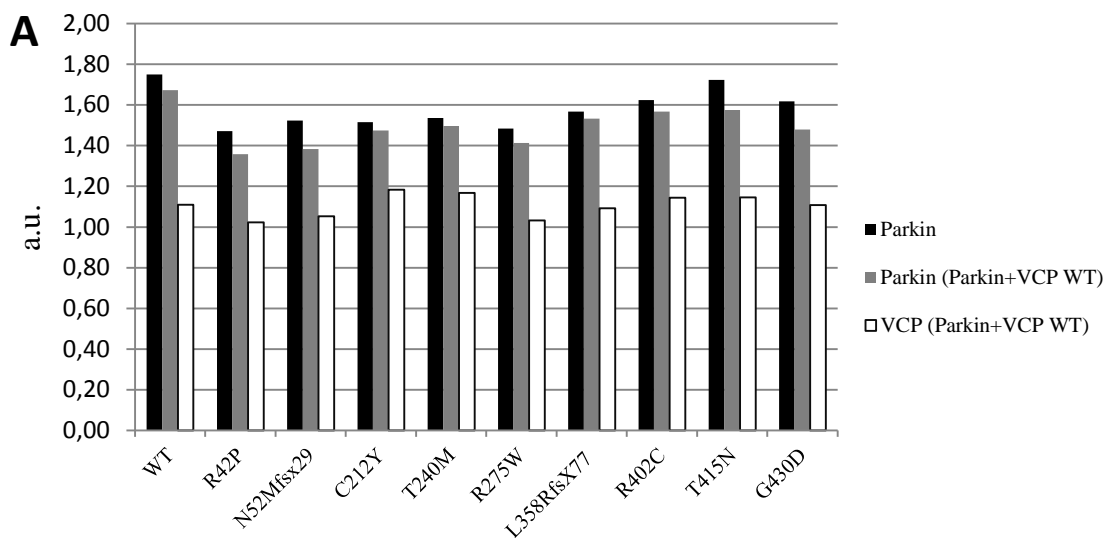
#### **4.2. Influence of VCP in parkin aggregates clearance**

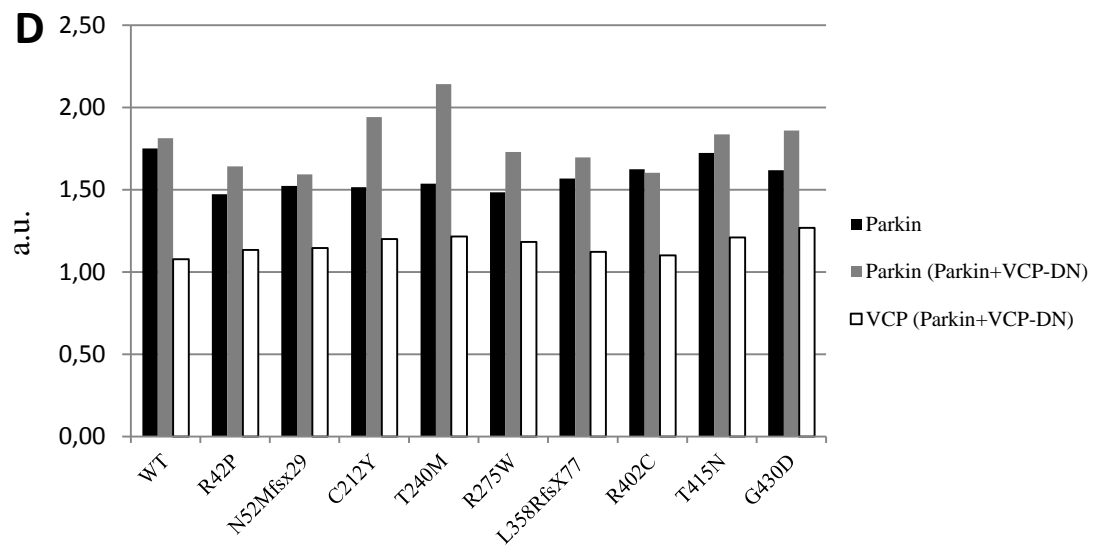
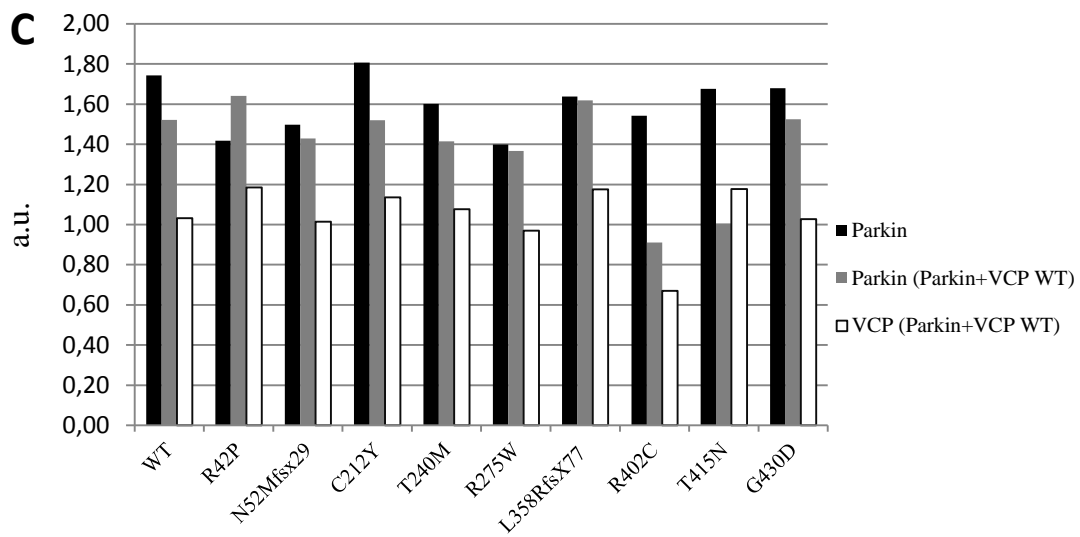
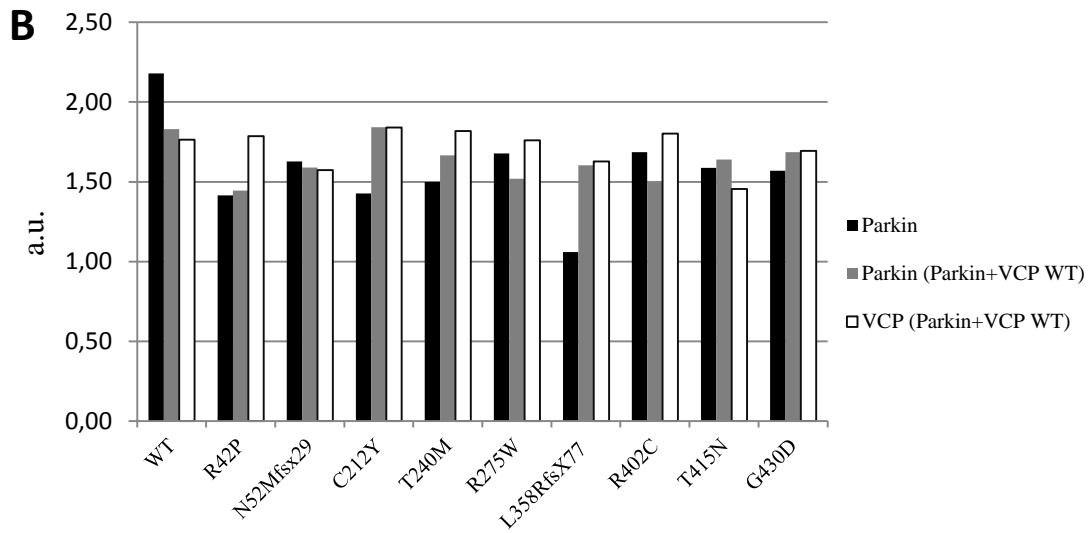
VCP is a component of the ERAD machinery, which contributes to the elimination of misfolded proteins by the proteasome. Previous data from our lab showed that some parkin mutants result in aggregate formation, when overexpressed in neuroblastoma cell lines. In order to explore the role of the ERAD machinery in aggregate clearance we co-transfected cells with parkin – wild-type (WT) and nine different mutants – and VCP, either WT or double-negative (DN), and assessed aggregate formation.

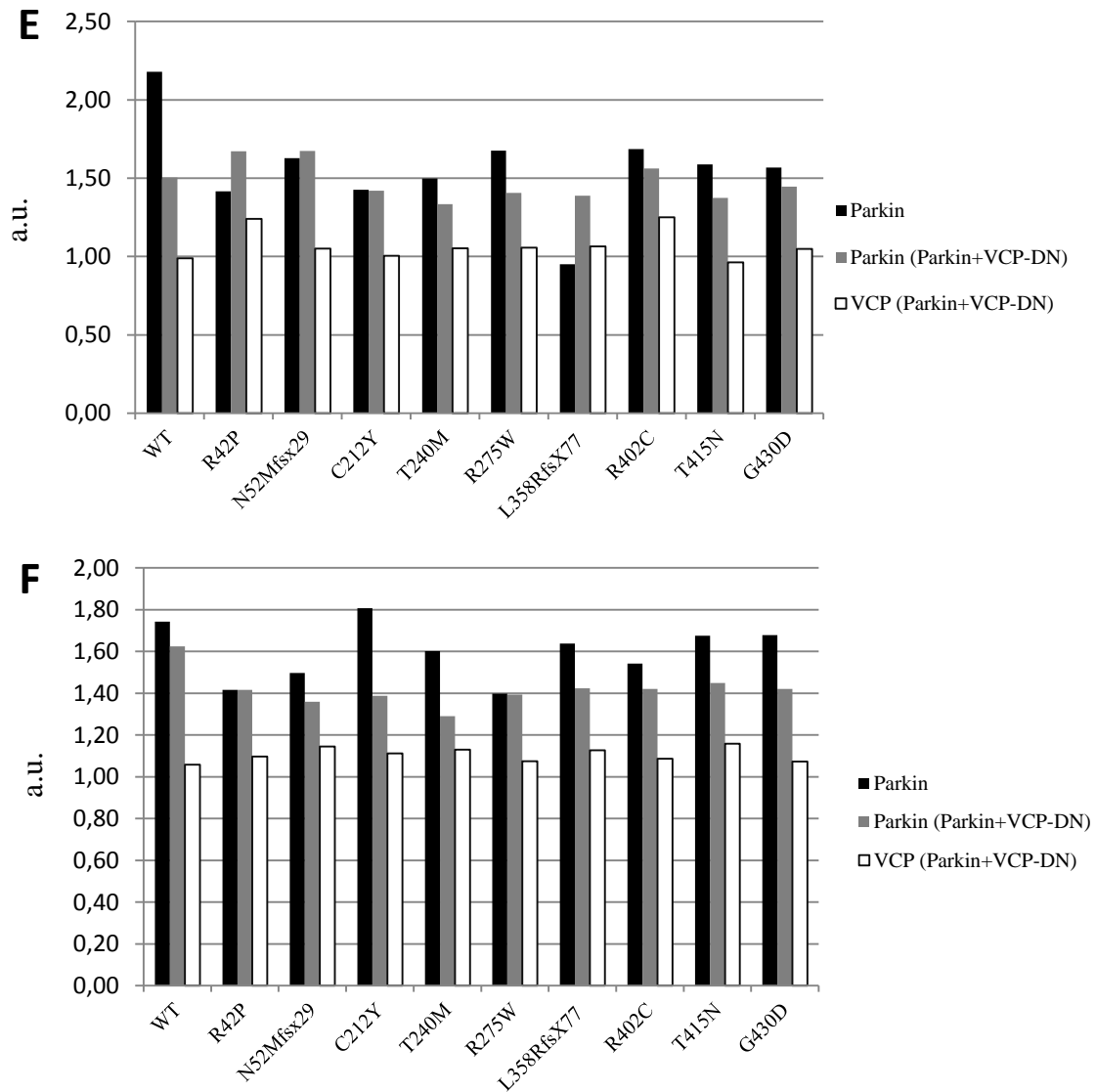
##### **4.2.1. Parkin and VCP expression levels**

In order to assess co-transfection efficiency *PARK2*, *VCP* WT and *VCP* DN mRNA expression levels were quantified through real-time PCR and their expression levels

were normalized towards *ACTB*. Quantitative analysis results from two replicas, of each sample and for each gene from one experiment, at 24, 48 and 72 hours after transfection are shown in Figure 9. Overall our data suggests that the relative expression of normal and all *PARK2* mutants do not differ at each time point. Thus, we can infer that transfection efficiency of the different construct does not present significant variation allowing us to proceed with mutants' characterization and comparison. In co-transfection of *PARK2* with WT or DN *VCP*, although we have slightly less *VCP* expression when compared to *PARK2*, the expression does not differ between the different mutants, and thus, allows us to proceed with mutants' characterization and comparison. These results still need to be replicated in additional experiments in order to perform the statistical analysis to confirm the comparable transfection efficiency.

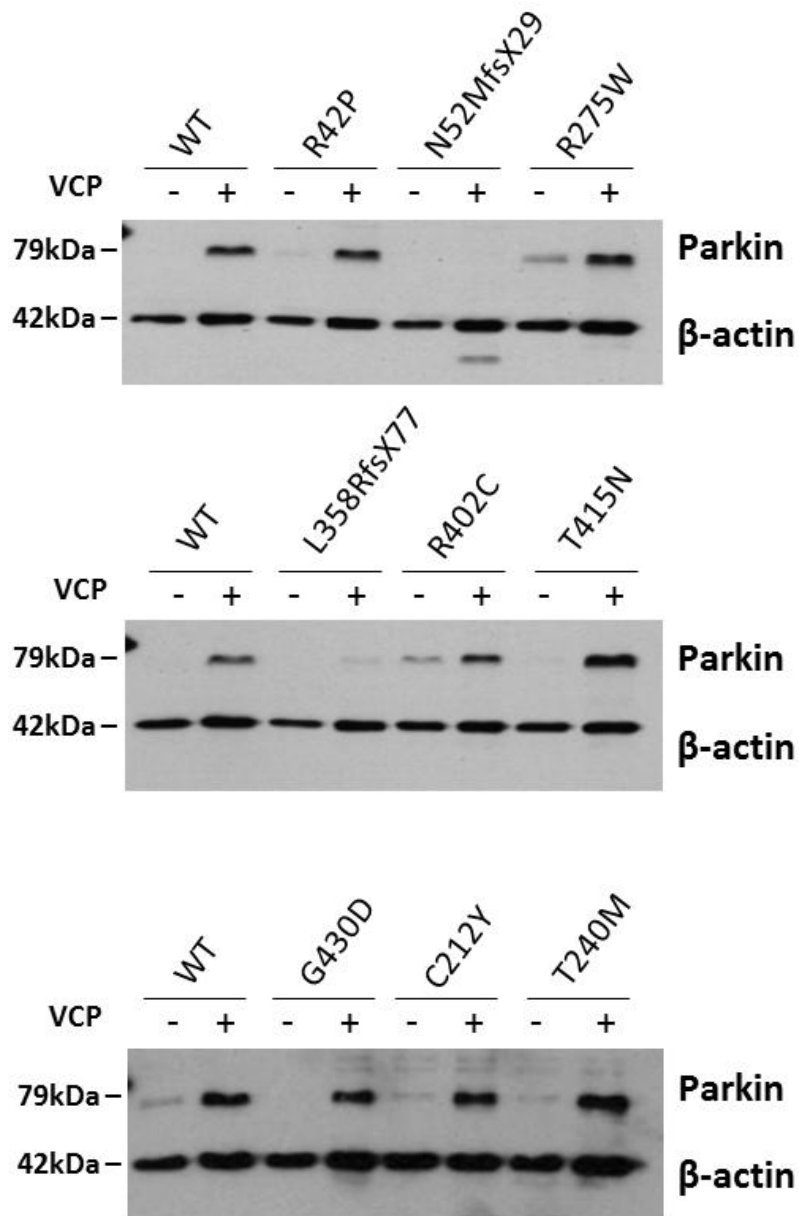






**Figure 9.** Quantitative analysis of parkin and VCP mRNA levels at 24 h (A; D), 48 h (B; E), and 72 h (C; F) after co-transfection with VCP WT or DN.

Quantitative analysis of parkin expression levels by western-blot, in SH-SY5Y cells has been a challenging task, with often-inconsistent results, that are consequently not shown here. Nevertheless, immunoblots of protein extracts collected 24h after transfection are shown in figure 10 as an example.

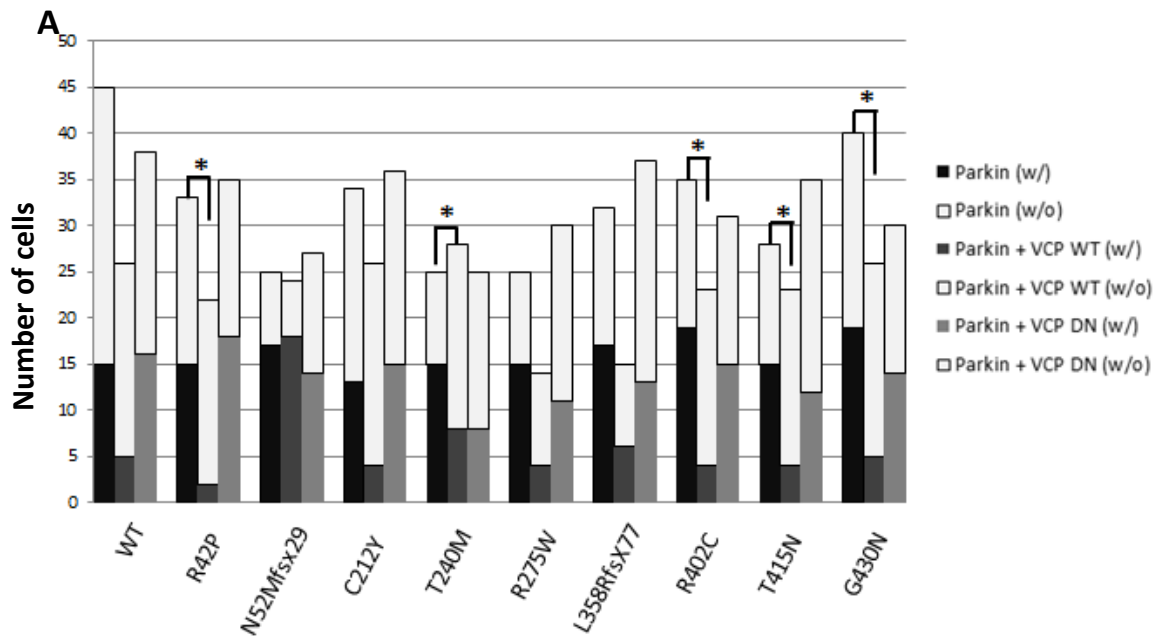


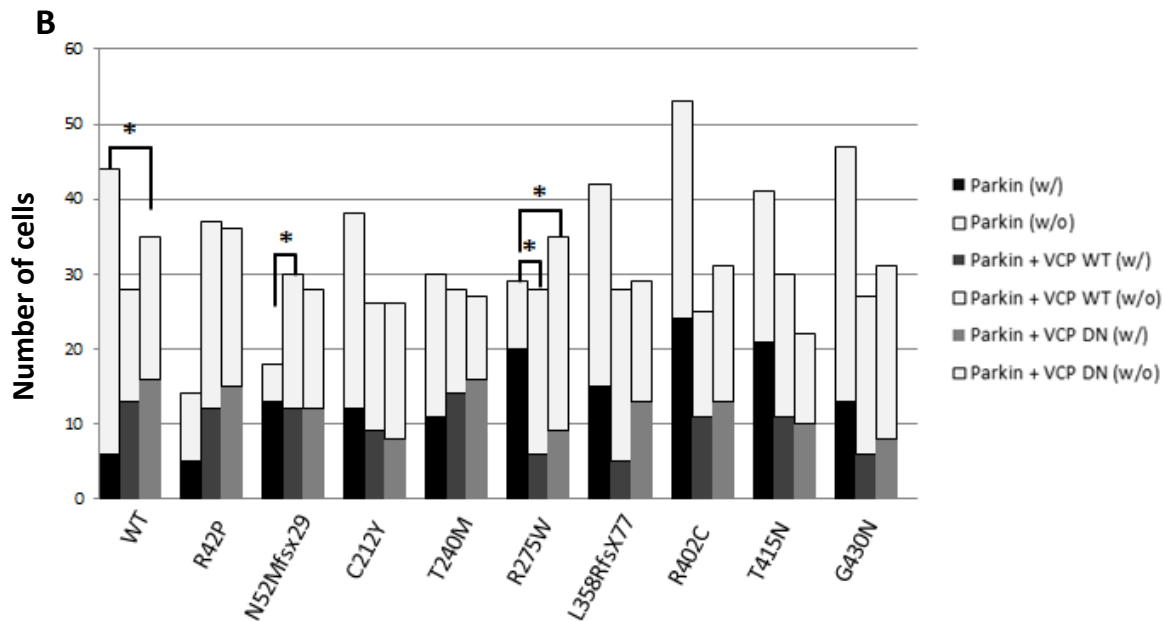
**Figure10.** Immunoblot of wild-type and mutant parkin protein levels in SH-SY5Y, 24 hours after transfection in cells expressing only parkin and co-transfected with WT VCP, using an anti-parkin antibody.

The analysis of the immunoblots suggests increased parkin protein levels in the cells co-transfected with parkin (WT and mutant) and WT VCP. Nevertheless, there is still the need to reproduce these results and to exclude an effect of the overexpression of WT VCP in  $\beta$ -actin protein levels that was used as loading control.

#### 4.2.2. Wild-type and mutant parkin aggregation: modulation by VCP

In order to explore the role of VCP in aggregate formation of mutant parkin, fluorescent imaging was carried out. Data shown in figure 11 reflects the results for one of the experiments, where the total numbers of cells analyzed are depicted, as well as the relative number of cells with or without aggregates, at 24 and 48 hours post transfection. In our model system, at a given time point only some of the mutants showed statistically significant differences from the wild-type form. Moreover, our analysis suggests that VCP is involved in the clearance of these aggregates.

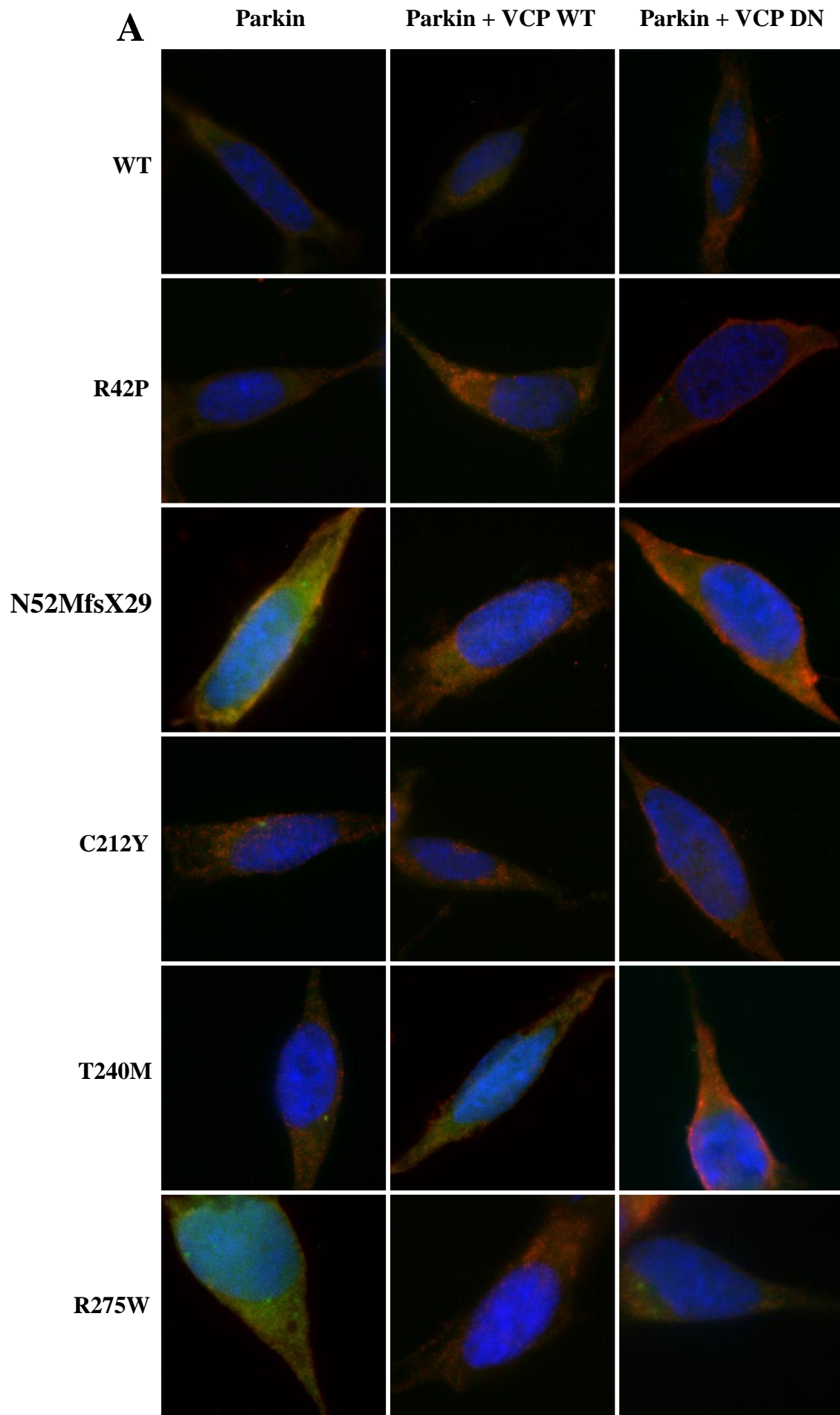




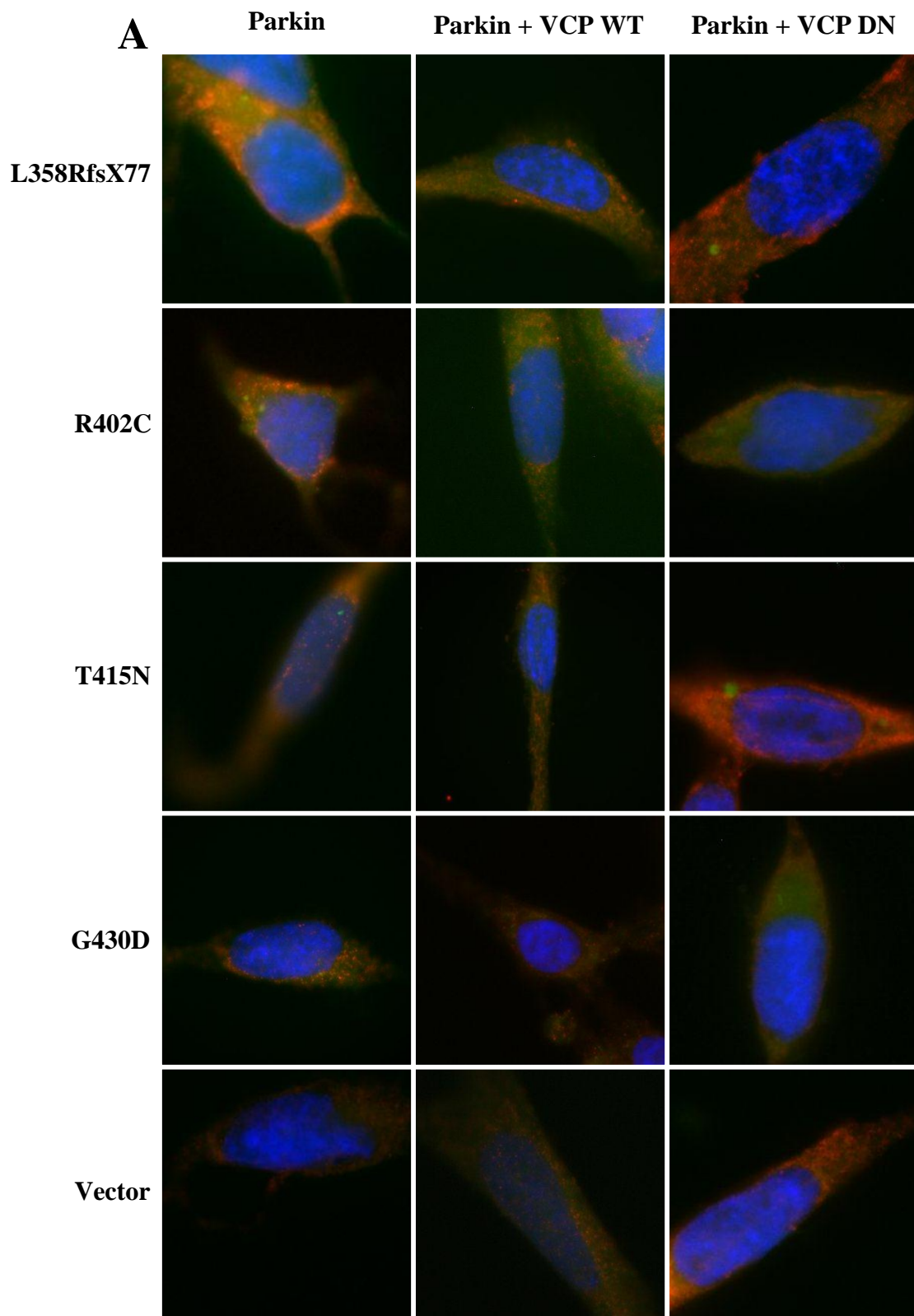
**Figure 11.** Quantitative analysis of aggregate formation of wild-type and mutant parkin in SH-SY5Y when co-transfected with WT or DN VCP, at 24 h (A) and 48 h (B) after transfection. Representation of one experiment is shown. \* $p < 0.05$ . (w/- cells with aggregates; w/o- cells without aggregates)

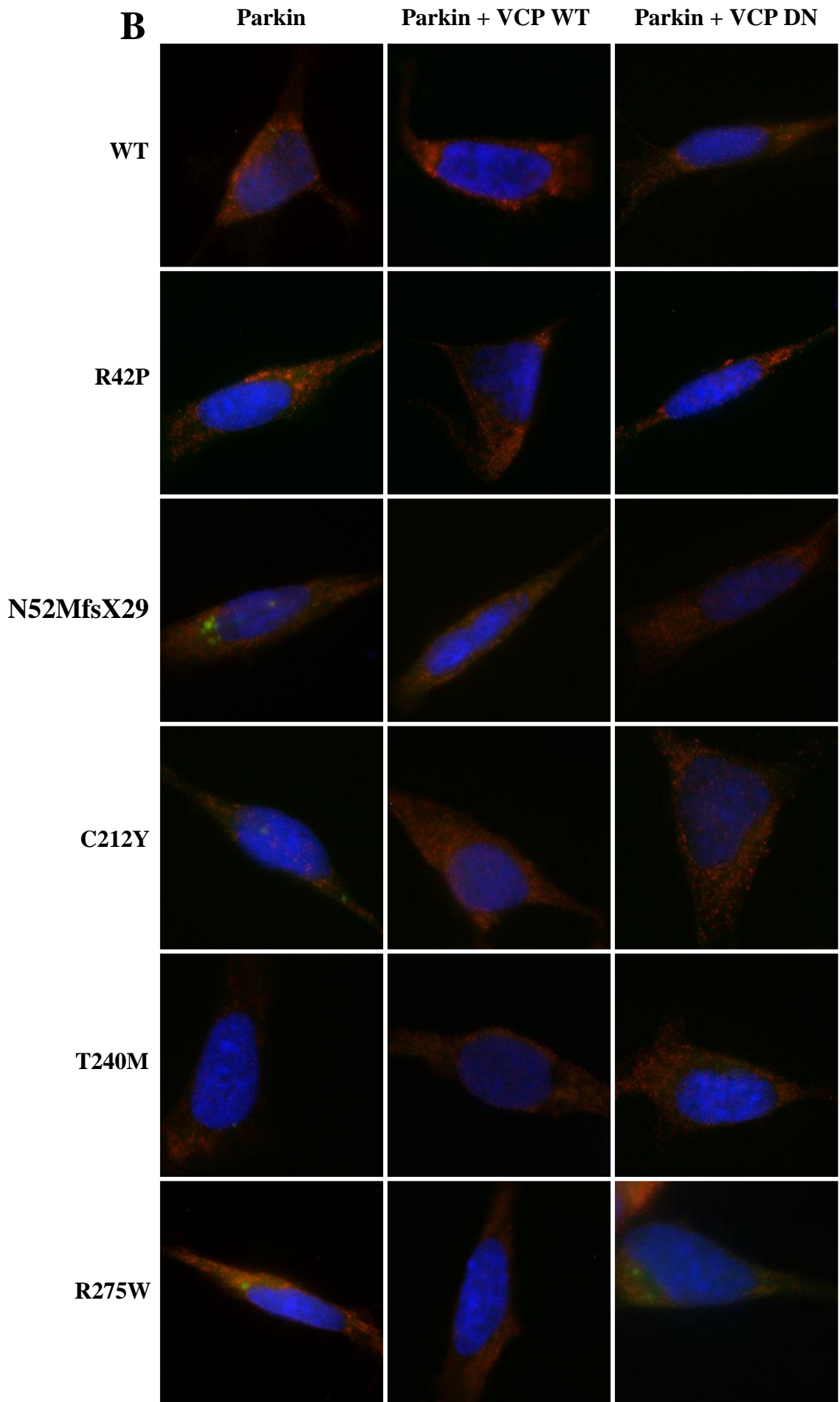
At 24 hours after transfection we have found a significant effect of wild-type VCP on aggregate clearance in five different parkin mutants: R42P ( $p = 0.004$ ), T240M ( $p = 0.021$ ), T402C ( $p = 0.005$ ), T415N ( $p = 0.008$ ) and G430D ( $p = 0.020$ ), showed by a reduction in the number of cells with aggregates. At 48 hours after transfection our results indicate a significant effect of wild-type VCP in the number of cells with aggregates for N52Mfsx29 ( $p = 0.031$ ) and R275W ( $p < 0.001$ ).

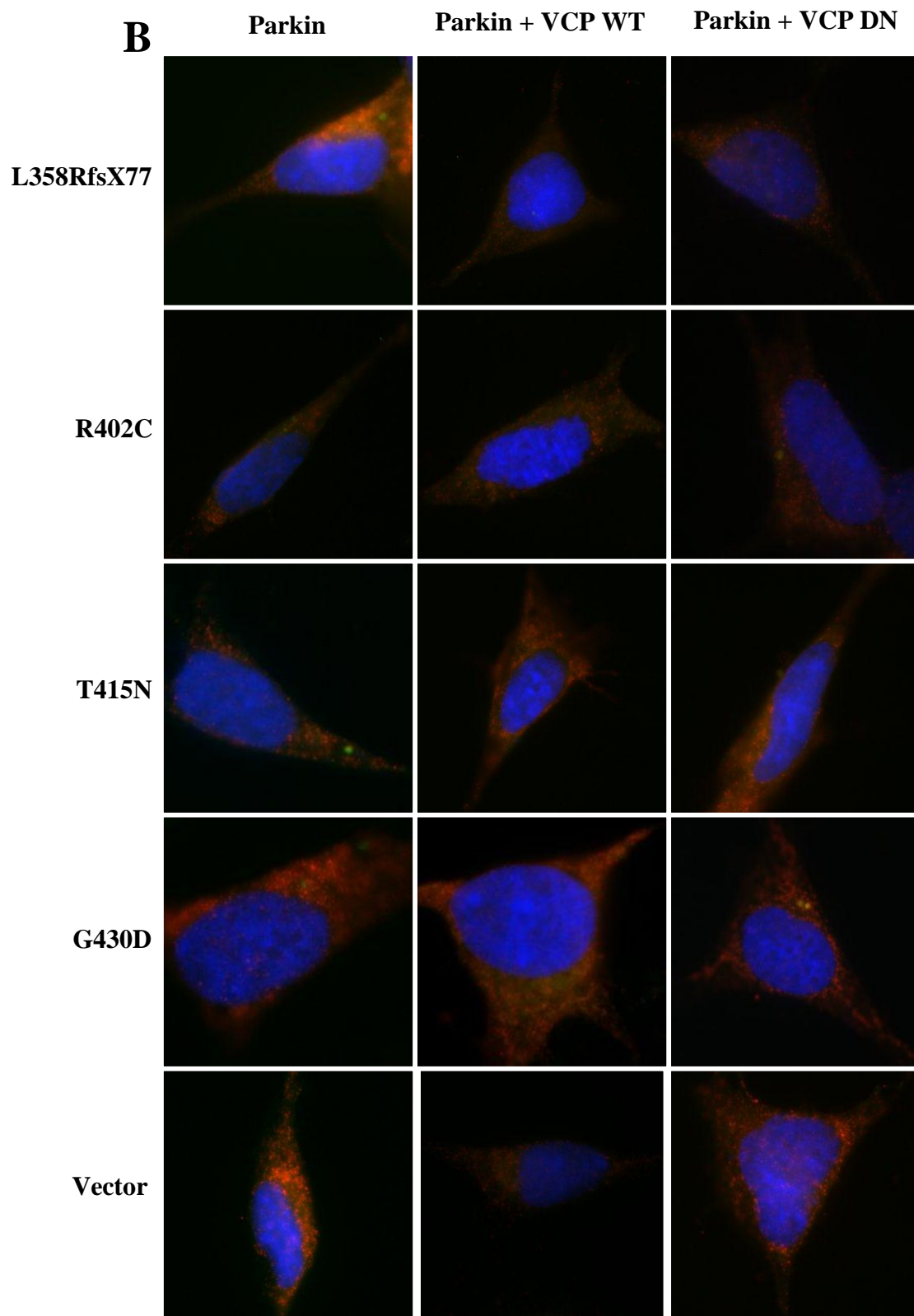
Regarding, the effect of the double-negative VCP in parkin mutants, our analysis does not correspond to the expected results that would be an increase in the number of cells with aggregates, which was not observed either at 24 or 48 hours after transfection. However, at 48 hours the overexpression of the dominant negative VCP appears to significantly increase the number of cells with aggregates for wild-type parkin ( $p = 0.002$ ). On the other hand, the effect of this dominant negative VCP on parkin R275W results in a significant reduction of the number of cells with aggregates ( $p = 0.001$ ).





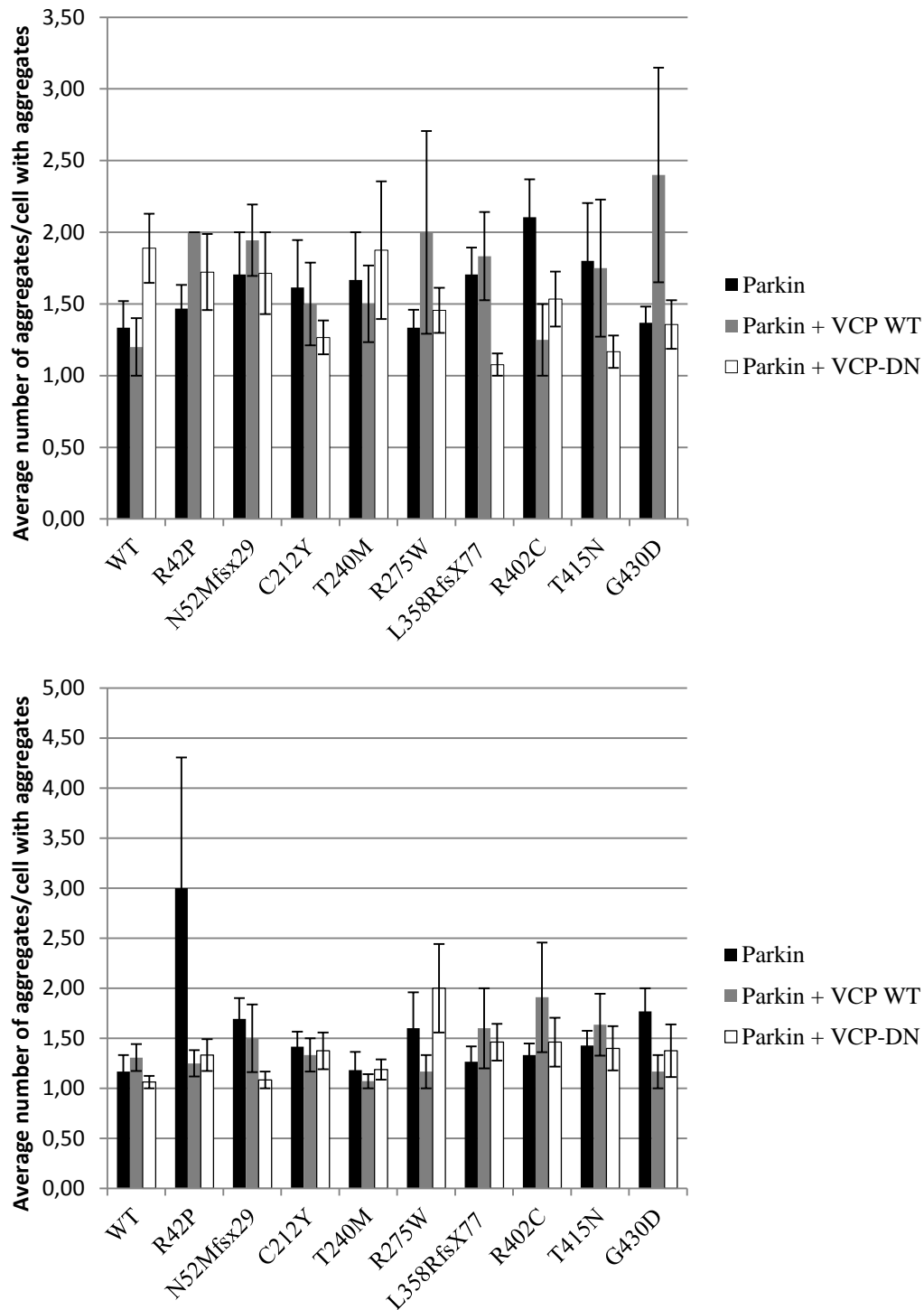






**Figure 12.** Representative images of SH-SY5Y, 24 hours (A) or 48 hours (B) after transfection in cells expressing only Parkin (first column) or co-transfected with Parkin and WT VCP (second column) or Parkin and DN VCP (third column). The cell nucleus is counterstained with Hoechst 3342.

Regarding the mean number of aggregates per cell showing parkin aggregation we did not observe any statistically significant differences at 24h and 48h (figure 13).

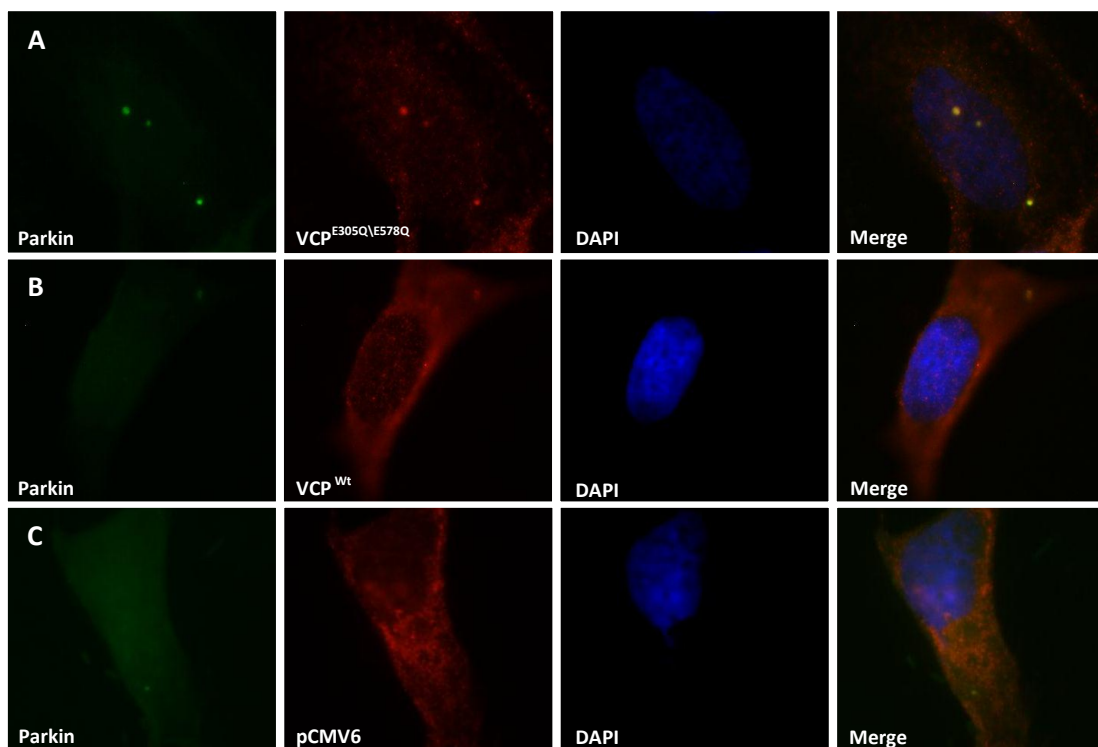


**Figure 13.** Mean number of aggregates per cell with parkin aggregates at 24 h (A) and 48 h (B) after co-transfection. Results are presented as mean  $\pm$  SEM. No statistically significant differences were found.

Regarding the cellular distribution of aggregates, no particular distribution in any intracellular location was observed. Representative images of parkin aggregation are displayed in figure 12, randomly selected from each condition at a given time point.

#### 4.3. VCP co-localizes with parkin aggregates

Concerning the cellular location of parkin and VCP in the cells showing aggregates our data suggests that parkin aggregates co-localize with WT and DM VCP. In particular, for the R275W mutant aggregates formed in neuroblastoma cells (Figure 14).

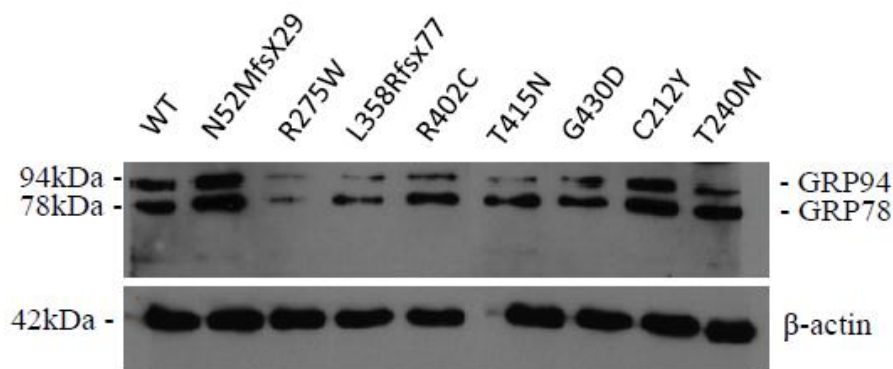


**Figure 14. VCP co-localizes with parkin aggregates** (A) Co-localization of parkin aggregates (green) and DN VCP (red) in SH-SY5Y cells. (B) Co-localization of parkin aggregates (green) and WT VCP (red) in SH-SY5Y cells. (C) Parkin aggregates (green) in cells co-transfected with pCMV6-myc empty vector (red). Co-localization is shown in the merge pictures (fourth column). Images were obtained 48 hours post-transfection (63x).

#### 4.4. Expression of GRP78 and GRP94 in SH-SY5Y expressing parkin and VCP

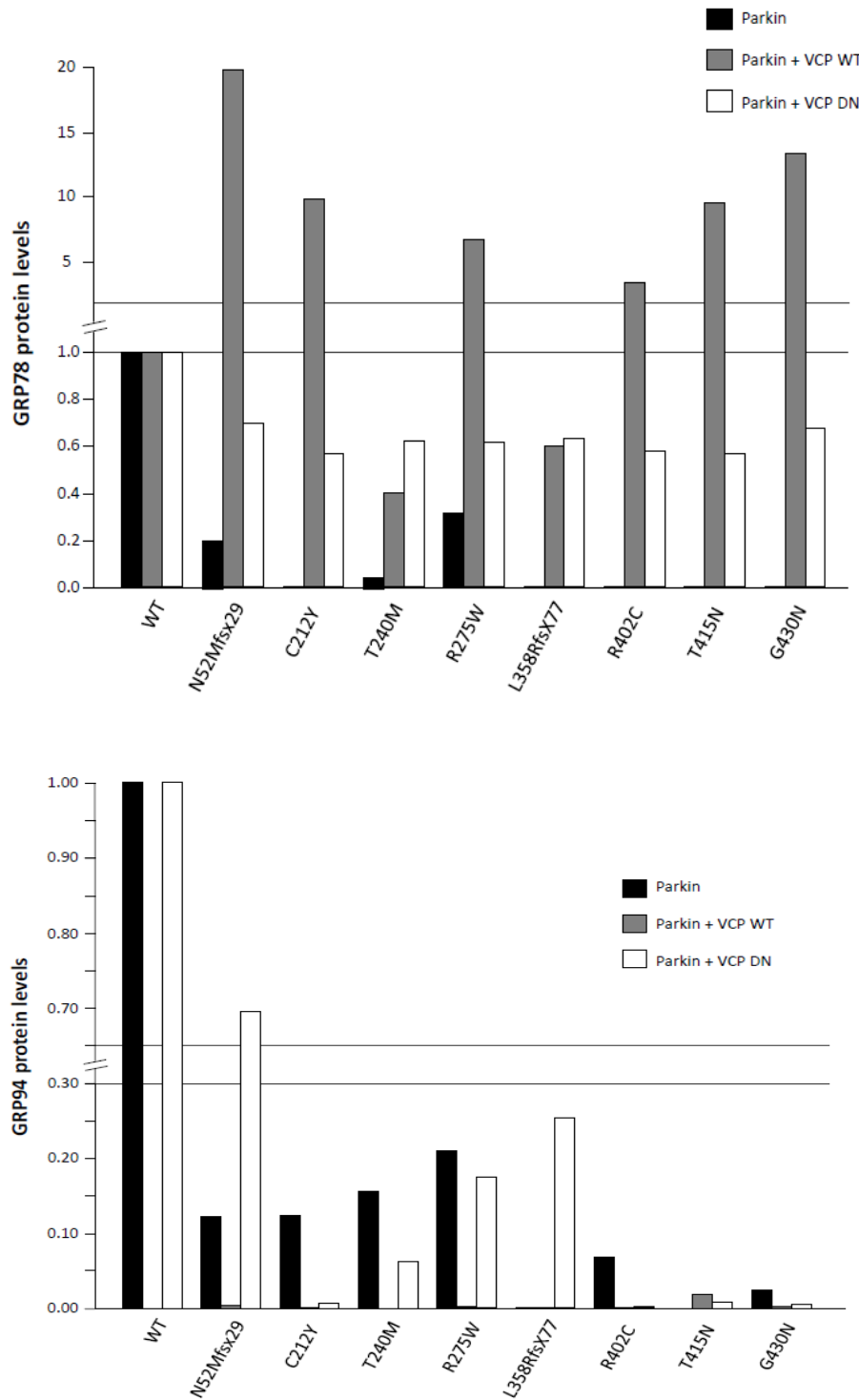
Sustained accumulation of misfolded proteins within the ER may activate an ER stress response known as UPR, which results in three protective cellular responses, leading to the upregulation of ER stress genes, including binding immunoglobulin protein (GRP78/BIP).

To explore the possibility of ER stress activation by parkin mutants, and the effect of WT and DN VCP in this stress response we started to evaluate ER stress activation by assessing GRP78/BIP and GRP94 expression levels through western-blot in extracts of cells transfected only with parkin and co-transfected with parkin and WT or DN VCP (figure 15).



**Figure 15.** An example of immunoblot of GRP78 and GRP94 protein levels in SH-SY5Y, 48 hours after co-transfection with DN VCP, using an anti-KDEL antibody.

Data obtained from one experiment at 24 hours after transfection are shown for GRP78/BIP and GRP94 (Figure 16).



**Figure 16.** Quantitative analysis by western-blot of GRP78 (A) and GRP94 (B) protein levels in SH-SY5Y cells expressing parkin or parkin and WT or DN VCP, at 24 hours after transfection, using anti-KDEL antibody.

Our results show a tendency towards an increase in GRP78/BIP expression in cells expressing parkin mutants in the presence of WT VCP. Even in cells expressing parkin and the DN VCP, GRP78/BIP levels although decreased when compared to the cells expressing parkin and WT VCP, are higher than the levels observed in cells transfected only with Parkin (Figure 16A).

Regarding GRP94 expression, the levels of this protein are increased in cells expressing only parkin when compared with cells co-expressing parkin and WT VCP for all the mutants, except for the mutant T415N. Conversely, in cells co-expressing parkin and the DN VCP, GRP94 shows higher protein levels for both frameshift mutations (N52MfsX29 and L358RfsX77) (Figure 16B).



## 5. Discussion

Although the aetiology and pathogenesis in PD are still poorly understood, central players causing rare genetic forms of PD should prove useful to understand idiopathic PD. Among those is a juvenile, autosomal recessive, form of PD (*PARK2* gene), caused by mutations in parkin, an E3-ubiquitin ligase. In this study we selected nine mutations covering all protein domains, including the most frequent mutations found in Portuguese patients (our group's unpublished results).

Parkinson's disease is a proteinopathy or protein conformational disorder that is characterized by the accumulation of misfolded proteins into aggregates that adversely affect neuronal function and result in cell death. Aggregate formation in AR-PD is controversial, but recent studies showed that missense mutations resulting in retention of ubiquitin ligase activity lead to parkin aggregation [69]. In this context we aimed to clarify the role of different cellular response mechanisms in the formation and clearance of parkin aggregates, namely the endoplasmic reticulum stress and endoplasmic reticulum (ER)-associated protein degradation.

In this study, we used a neuroblastoma cell line, SH-SY5Y, to take advantage of their neuronal-like nature and of the properties shared with dopaminergic neurons, namely, the intracellular substrates for dopamine synthesis, metabolism and transportation. This neuronal cell line is one of the most widely used for study the mechanisms involved in PD [87]. Although previous work from our group has shown that three of the parkin mutants, N52MfsX29, R275W and L358RfsX77, are highly prone for aggregation (unpublished results), in the present project we did not observed this severe phenotype. One possible explanation is the fact that in this study we are using co-transfection and thus less parkin construct in each experiment. This is corroborated by the fact that in the

present work we have more cells with low intensity of GFP expression than when transfecting only parkin constructs, that most probably will need increased expression times to develop a comparable phenotype, a question we will further explore.

### **5.1. ER-associated protein degradation is involved in parkin clearance**

Recent studies confirm the involvement of VCP in the clearance aggregates in different neurodegenerative disorders. Namely, VCP was found to mediate and reduce ataxin-3 aggregation in a dose-dependent manner and to reduce the toxicity of this polyQ expansion responsible for Machado-Joseph disease [88]. Also, VCP was found to modulate TDP-43 neuronal inclusions in frontotemporal lobar degeneration [89].

VCP is an AAA-ATPase that facilitates the translocation of misfolded ER-proteins into the cytosol and thus is an essential component of the ERAD machinery [90]. Interestingly, parkin is an ubiquitin ligase (E3) directly involved in ERAD that ubiquitinates specific substrate proteins playing a protective role by tagging misfolded proteins [42].

To explore the involvement of ERAD in parkin-associated PD we co-expressed WT or DN VCP together with WT or different parkin mutants and assessed aggregate formation in these cellular models.

Here, we show that parkin is probably a new substrate for VCP, which will promote its retrotranslocation and delivery to the proteasome to be degraded. Also, VCP may have a role in the clearance of misfolded mutated parkin.

Our results show that at 24 hours after transfection the expression of WT VCP leads to a generalized reduction in the number of cells with parkin aggregates for all the studied

mutants except the N52MfsX29 mutation. This mutant shows a particular behavior, as this reduction on the number of cells with aggregates is not observed even at 48 hours after transfection. The number of cells with aggregates for this mutant does not differ much in the three different conditions (parkin, parkin co-transfected with WT VCP and parkin co-transfected with DN VCP). This mutation causes the deletion of the entire RBR domain and thus the resulting protein consists of only the UBL domain. We may hypothesize that somehow this small protein may be interpreted as an ubiquitin molecule and thus not be recognized by VCP for translocation.

Another hypothesis may be the involvement of a nonsense mediated mRNA decay (NMD) mechanism in the modulation of this frameshift, as we see reduced levels of this mutant by western-blot. NMD is an evolutionarily conserved mRNA surveillance pathway that preferentially destroys mRNAs harboring premature translation termination codons (PTCs), as it occurs in the N52MfsX29 mutation, to protect the cells from potentially harmful effects [91].

Although we still need to enlarge the amount of data and to quantify aggregation at 72 hours after transfection we have evidence for the involvement of ERAD in wild-type and mutant parkin clearance. Also, our results suggest that perhaps the ERAD machinery needs more time to be able to clear the parkin with frameshift mutations, and consequently result in a reduction on the number of cells with aggregates.

Previous data from our group, shows that two mutants N52MfsX29 (located in the ubiquitin-like domain) and R275W (located in the RING1 domain) are highly prone to aggregation, resulting an increased number of cells with aggregates, as well as an increased number of aggregates in cells with aggregates when compared with WT parkin. In contrast, our data are not consistent regarding the aggregation-proneness of these two mutants, maybe due to the lower levels of parkin expression under co-

transfection conditions. Interestingly, our results indicate that these are the only two mutants, that when co-transfected with WT VCP show a significant decrease in the number of cells with aggregate only at 48 hours, corroborating the need to increase expression time in order to have comparable results.

Additionally, we co-transfected SH-SY5Y cells with WT and mutant parkin with a DN VCP. Dominant negative (DN) VCP consists of mutations in both the D1- and D2-domains which abolishes ATPase activity and functions as dominant negative when expressed exogenously, and this mutant has been extensively used to interrogate VCP function [92]. Contrary to what we would expect, our results did not show evidence of a major increase in the number of cells with parkin aggregates when we co-transfected DN VCP, when compared to the cells transfected only with parkin. However, Taeko et al, showed similar results with experiments with expanded polyQ aggregates, in which VCP had been knockdown through RNA interference. Other studies with severe VCP KD or expression of a DN VCP have been reported to diminish aggresome formation [93, 94]. In order to clarify this issue in parkin-associated aggregation we need to assess aggregation at 72 hours after transfection with the DN VCP and maybe assess parkin aggregation in cells with silenced VCP instead of the overexpression of a DN.

Several studies have reported a co-localization of VCP with ubiquitin-positive inclusions in patients with Alzheimer's and Parkinson's disease [95]. Moreover, VCP also co-localized with nuclear inclusions in neurons of Huntington's and Machado–Joseph's disease [96].

In this study, we have also found VCP labeling co-localized with parkin aggregates, which may suggest that these proteins do share the same biological pathway, and that they may interact. However, additional co-localization experiments and immunoprecipitation assays are needed to confirm this hypothesis. In particular it will

be very interesting to explore the co-localization and interaction of VCP with the different parkin mutants.

## **5.2. Parkin mutants do not induce an ER stress response**

The accumulation of unfolded proteins can induce ER stress and activate an unfolded protein response (UPR), which results in three protective cellular pathways, required for the cells to cope with stressful conditions; nevertheless, too severe and prolonged ER stress results in the induction of apoptosis.

In general, protein aggregates do not accumulate in unstressed, healthy neurons, in part due to the existence of cell “quality control machineries”, which include molecular chaperones. Chaperones are believed to provide a defence mechanism against the toxicity of misfolded proteins, by preventing inappropriate interactions, within and between polypeptides, and promoting protein refolding.

It has been shown that misfolded proteins and aggregates, even when involving cytosolic proteins, are able to induce an UPR, resulting in the upregulation of ER stress genes, such as *BIP/GRP78* or *GRP94* or even *PARK2*.

We explored the possibility of an UPR activation by assessing the expression of ER chaperones protein levels, namely GRP78 and GRP94, through western-blot in extracts from cells expressing only parkin or co-expressing parkin and WT or DN VCP.

The results obtained until now do not support the activation of an UPR in the presence of mutant parkin, although we still need to assess GRP78 and GRP94 levels after prolonged parkin expression. Interestingly, we observed an increase in the expression of the ER chaperone GRP78 in cells expressing parkin and WT VCP when compared with cells expressing only parkin, 24 hours after transfection. These results are consistent

with an increase of parkin protein levels in the cells co-transfected with WT VCP. Yuzuru et al, demonstrated that Parkin is up-regulated in response to unfolded protein stress and suppresses unfolded protein stress-induced cell death, suggesting that the physiological role of Parkin involves dealing with unfolded protein stress responses [43]. Also, recently it has been shown that stress-induced upregulation of parkin is mediated by ATF4 a transcription factor of the unfolded protein response (UPR) [97]. In contrast, West et al, evaluated endogenous parkin in the SH-SY5Y cell line at the promoter, RNA, and protein levels in response to unfolded protein stress induced by tunicamycin and no significant changes were detected in parkin levels, thus suggesting that parkin is not regulated by the unfolded protein response in human neuroblastoma cells [98].

In addition, we observed an increase of GRP78 and even GRP94 levels in SH-SY5Y cells expressing parkin and the DN VCP. This probably relates to the increased burden on the cell, as it has to deal with the additional expression of the dominant negative VCP. We can even hypothesize that in this condition the cell might have to react earlier and may trigger an ER-stress response earlier than 24 hours.

In an ER-stress response situation, GRP78 is the first ER molecular chaperone that was shown to bind assembled immunoglobulin and prevent its transport from the ER lumen [99]. Afterwards, it was demonstrated that prolonged unfolded mutants trigger another molecular chaperone, GRP94, leading to the designation of this signaling pathway as the UPR [100].

The role of aggregates in pathology is still controversial and it is also not clear whether the different evidences of ER stress activation in PD is largely neuroprotective or whether it directly contributes to the disease process.

## 6. Conclusion

In the present study we explored the effect of parkin and VCP on ER function, in order to get additional insights into ER stress in Parkinson disease, and to better understand the crosstalk between ER and UPS, through ERAD.

Our results show that VCP overexpression results in a decrease in the number of cells with parkin aggregates and thus supports the hypothesis that parkin is a VCP substrate and the role of ERAD in parkin-associated PD. Also, we found an up-regulation of parkin in response to ER stress, when dopaminergic neuroblastoma cells were co-expressing WT VCP. Also, we observed a striking increase in the expression of ER molecular chaperones in cells co-expressing parkin and WT or DN VCP.

In conclusion, our results suggest that mutations in *PARK2* lead to parkin misfolding, contributing to the formation of aggregates that co-localize VCP. Also, we do not have evidence of the activation of an UPR in the presence of mutant parkin. With this work we enlarge the amount of data relating parkin with the endoplasmic reticulum and in particular with ERAD, raising the possibility that modulation of VCP and ERAD activity might have potential therapeutic significance for AR-PD.

## 7. Future Perspectives

Taking into account the obtained results it will be most important to assess aggregate formation in all conditions at 72 hours after transfection. Also, some of these results still need to be further explored and other time-points assessed in order to perform the statistical analysis and confirm the present data. In particular for the ER stress response, we can also assess expression levels at the mRNA level through real-time PCR.

To better understand the neuroprotective or cytotoxic effect of the three pathways activated in the UPR, specific inhibitors will be used for each, as well as the expression levels of proteins downstream GRP78 and GRP94.

Other important points to clarify are the total ubiquitination level in the different experimental conditions as well as proteasome activity level to better understand the involvement of parkin aggregates in UPS-ERAD crosstalk.

Co-localization of VCP and parkin needs to be carefully explored paying special attention to the impact of the different mutants in these proteins' subcellular location.

To clarify the impact of VCP functional impairment in aggregate formation and in ER stress this will be addressed by silencing VCP by small interference RNA.

Finally, it will be important to evaluate the effect of VCP expression in cell viability, using SH-SY5Y cells co-transfected with parkin and WT or DN VCP, or under VCP silencing.



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## **9. Communications**

### **9.1. Posters in conferences**

M. Alves-Ferreira, S. Morais, J. Sequeiros, I. Alonso. Explore the impact of parkin mutations in ER-associated protein degradation: cellular models for juvenile PD. 62nd American Society of Human Genetics Annual Meeting; San Francisco, California, November 6-10, 2012. (accepted)

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