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Discovering mitochondrial alterations in Parkinson's disease: the role of mitophagy impairment

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Table of contents

Abbreviations

AMBRA1, activating molecule in BECN1-regulated autophagy protein 1; AP, affinity purification; BNIP3L, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; Clp, ATP-dependent Clp protease proteolytic subunit; COMT, catechol-O-methyl transferase; COX5β, cytochrome *c* oxidase subunit 5β; CREB, cyclic AMP-responsive element-binding protein; CS, citrate synthase; DA, dopamine; DAT, dopamine transporter; DDSA, dodecenylsuccinic anhydride; DMEM, high glucose Dulbecco's modified Eagle's medium; DMP, dimethylphthalate; DMSO, dimethylsulfoxide; DRP1, dynamin related protein 1; ETC, electron transport chain; FA, formic acid; FBS, fetal bovine serum; Fis1, mitochondrial fission 1 protein; FUNDC1, FUN14 domain-containing protein 1; GCase, β-glucocerebrosidase; GSK3β, glycogen synthase kinase-3 beta; HSP, heavy strand promoter; HtrA2/OMI, Serine protease HTRA2; IAA, iodoacetamide; IMM, inner mitochondrial membrane; IMS, intermembrane space; LB, Lewy bodies; LETM1, Mitochondrial proton/calcium exchanger protein; Lon, Lon protease homolog; LRRK2, leucine-rich repeat serine/threonine-protein kinase 2; LSP, light strand promoter; MAO-B, monoamine oxidase B; Mff, mitochondrial fission factor; MFNs, mitofusins; MiD49, mitochondrial dynamics protein MID49; MiD51, mitochondrial dynamics protein MID51; Miro, mitochondrial Rho GTPase 1; MPP, mitochondrial processing peptidase; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mtDNA, mitochondrial DNA; NOS, reactive nitrogen species; NRFs, nuclear respiratory factors; OMA1, metalloendopeptidase OMA1; OMM, outer mitochondrial membrane; OPA1, dynamin-like 120 kDa protein; p62/SQSTM1, sequestosome-1; PARIS, zinc finger protein 746; Parkin, E3 ubiquitin-protein ligase parkin; PARL, presenilins-associated rhomboid-like protein; PD, Parkinson's disease; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PINK1, Serine/threonine-protein kinase PINK1; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; POLG, DNA polymerase γ; POLRMT, DNA-directed RNA polymerase; ROS, reactive oxygen species; SNpc, Substantia nigra pars compacta; TBST, tris-buffered saline with 0.05% tween 20; TCEP, tris(2-carboxyethyl)phosphine; Tfam, transcription factor A; TFB, dimethyladenosine transferase 1; TIM, translocase of inner mitochondrial membrane; TOM, translocase of outer mitochondrial membrane; UPS, ubiquitin-proteasome system; VDACs, voltage-dependent anion channels; VMAT2, synaptic vesicular amine transporter 2; YME1L, ATP-dependent zinc metalloprotease YME1L1; α-syn, α-synuclein.

Abstract

Parkinson's disease (PD) is one of the most prevalent neurodegenerative disorders, characterized by the loss of dopaminergic neurons in the Substantia nigra pars compacta. Although the cause of PD is currently unknown, strong evidences indicate that a complex interplay between several factors including genetic susceptibility, environmental factors, abnormal protein handling and oxidative stress could be involved. Many of the molecular pathways implicated in PD etiology converge on mitochondria, resulting in their dysfunction, which could impact on neuronal survival.

In this intricate pathogenetic landscape, altered dopamine homeostasis seems to be an important mechanism involved in the neurodegeneration process of PD. In this context, the SH-SY5Y neuroblastoma cell line may be used to mimic the improper handling of this neurotransmitter by the administration of exogenous dopamine in the culture medium. It is also well known that complex I deficiency is associated to PD pathogenesis. For this reason, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its metabolite 1-methyl-4-phenylpyridinium (MPP^+) are widely used to reproduce parkinsonian symptoms and mechanisms in animal and cellular models, respectively.

Given the importance of mitochondrial dysfunctions in PD, we decided to investigate the missed removal of damaged mitochondria due to mitophagy impairment as the possible trigger of pathogenesis. To this purpose, we characterized specific mitochondrial alterations in Substantia nigra specimens from PD patients, comparing them to what observed by inducing mitochondrial impairment in human neuroblastoma SH-SY5Y cells using dopamine or MPP^+. In particular, mitochondrial alterations were investigated from a molecular point of view by analyzing the mtDNA content, the proteins involved in fusion (i.e. OPA1 and MFN1) and fission (i.e. DRP1) processes, the proteins involved in mitochondrial functions (i.e. VDAC1, VDAC2 and COX5β) and in mitophagy pathway (i.e. PINK1). As a reference model of mitophagy induction, treatment with the IMM uncoupler CCCP was used in all experimental designs as a positive control.

As a result, we found that mitophagy impairment is a common feature of both Substantia nigra of PD patients and PD cellular models. Indeed, we never observed PINK1 accumulation, a sign of triggered mitophagy. However, the analysis of mitochondrial molecular markers (i.e. VDAC1, VDAC2, COX5β and mtDNA) showed a discordant behavior between the cellular model of altered dopamine homeostasis and that of complex I inhibition. In MPP^+-treated cells, we simultaneously observed the decrease of COX5β, OPA1 and mtDNA, the increase of VDAC1 and VDAC2, and no effect on MFN1 level. This molecular picture was clarified by TEM

images, showing empty organelles, with disrupted cristae. The treatment with dopamine causes a different mitochondrial alteration. Indeed, COX5β and MFN1 did not change, whereas OPA1, VDAC1 and VDAC2 were decreased and the mtDNA content was increased. TEM images evidenced the presence of damaged mitochondria, although presenting different features if compared to those in MPP^+-treated cells, in line with a different molecular behavior. In PD patients, the molecular landscape was identical to the one of dopamine-treated cells. TEM images gave us the suggestion that also electron dense deposits are present in mitochondria. Therefore, the acute inhibition of complex I only partially reproduces the mitochondrial alterations related to PD pathogenesis.

Since mitophagy impairment seems to play a central role in the development of this pathology, the second part of this thesis focused on mitochondrial alterations that occur in skin fibroblasts obtained from *PARK2*-mutated patients. First, we characterized the mitochondrial network morphology in order to understand if the loss of function of Parkin can have a negative impact on the mitochondrial dynamics. As a result, we found that mutations in the *PARK2* gene do not cause any significant morphological alterations in the mitochondrial network shape.

In order to better decipher the molecular pathways altered in the *PARK2* pathology, we used the global proteomics and interactomics approaches to highlight the difference of protein expression levels and of protein-protein interactions between *PARK2*-mutated patients and control subjects. The proteomics analysis revealed an impairment of proteins involved in several mitochondrial functions. In particular, we found that the expression of NADH dehydrogenase iron-sulfur protein 3 and ATP synthase subunit g, two proteins involved in the respiratory chain, is reduced in PARK2-mutated patients. This result is in line with the lower complex I activity and the lower ATP production found in skin fibroblasts cells of these patients. Moreover, the altered expression of persulfide dioxygenase ETHE1 and Lon protease revealed a general impairment of the mitochondrial quality control system. Eventually, the increased expression of LETM1, a protein involved in the maintenance of mitochondrial tubular networks and in the maintenance of the mitochondrial cristae organization, justifies the absence of mitochondrial network alterations found with our morphological analysis.

So far, the size-exclusion chromatography (SEC) coupled with LC-MS/MS analysis was not made for the fractions obtained from the three control subjects, thus making impossible the comparison of the interactome of the two groups analyzed. Nevertheless, our preliminary results conducted on the interactome of the *PARK2*-mutated patients revealed the activation of one apoptotic pathway and the presence of a protein-protein interaction network involved in the positive regulation of mitochondrial network morphology.

Overall, this project contributes to a complete definition of the *PARK2*-related molecular signature, that will be crucial for providing new insights into disease mechanisms and identifying new therapeutic targets for this pathology.

1. Introduction

1.1. Parkinson's disease

Parkinson's disease (PD) is one of the most common neurodegenerative disorders, with increasing prevalence in the aging population. Indeed, this pathology affects around 14 per 100000 people in the total population, and 160 per 100000 people aged 65 years or older (Ascherio and Schwarzschild, 2016).

The most important pathological hallmark that characterized PD is the progressive loss of dopaminergic neurons of the substantia nigra pars compacta (SNpc), which produces the classic neuropathological finding of SNpc depigmentation. This particular neurodegenerative process leads to the onset of typical motor symptoms (i.e. rigidity, tremor at rest, bradykinesia and postural instability) (Figure 1) (Fahn, 2003; Jankovic, 2008).

Despite PD is predominantly characterized as a movement disorder, its clinical spectrum also includes non-motor symptoms, which emerge during the disease progression. They encompass depression, anxiety, sleepy disturbances, constipation, bladder and other autonomic disturbances, sensory symptoms (pain, numbness, tingling and burning in the affected limbs) (Fahn, 2003; Gallagher et al., 2010).

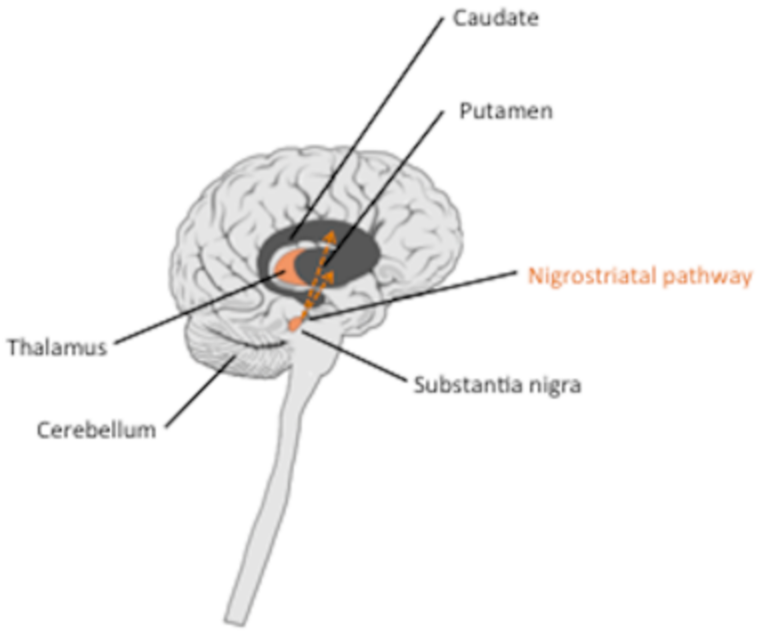


Figure 1. Schematic representation of the nigrostriatal pathway (in orange). It is composed of dopaminergic neurons whose cell bodies are located in the SNpc. These neurons project (orange arrows) to the basal ganglia and synapse in the striatum (i.e., putamen and caudate nucleus). In Parkinson's disease there is a marked loss of dopaminergic neurons that project to the putamen and a much more modest loss of those that project to the caudate. The loss of these neurons affects the signaling in the basal ganglial circuit leading to decreased output from cortex and brainstem, thus causing the onset of the typical motor symptoms.

Another important pathological feature of PD is the appearance of intracellular proteinaceous inclusions, termed Lewy bodies (LB), in the dopaminergic neurons (Shults, 2006). LB are mainly composed by aggregated proteins, among which the most abundant is the α-synuclein (α-

syn), a protein that has been linked to familial PD (Polymeropoulos et al., 1996; Polymeropoulos et al., 1997; Vekrellis et al., 2011).

Since the motor symptoms appear only when the putamenal dopamine (DA) is depleted by 80% and the 60% of SNpc dopaminergic neurons have already been loss, the only possible treatment of PD is currently symptomatic and aimed at restoring DA levels in the striatum (Dauer and Przedborski, 2003). Indeed, pharmacological treatment with L-DOPA (a precursor of DA that can pass the blood-brain-barrier which is metabolized to DA in the brain) and parallel administration of COMT- (catechol-O-methyl transferase) and MAO-B- (monoamine oxidase B) inhibitors achieve this purpose (Poewe et al., 2017).

For these reasons, the discovery of molecular mechanisms that are involved in the initial step of the disease and of biomarkers that allow the earlier diagnosis has become a priority in the field.

1.1.1. Etiology of Parkinson's disease

PD is a multifactorial disorder whose etiology is not completely understood. The discovery of several responsible genes (e.g. *PARK1*, *PARK2*, *PARK6*, *PARK7*, *PARK8*, *PARK13*) that cause early-onset parkinsonism highlighted the importance of mitochondrial impairment in PD pathogenesis (Saiki et al., 2012; Burchell et al., 2013). Moreover, it has long been known that the exposition to specific environmental mitochondrial toxins (e.g. paraquat, rotenone, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)) represents an high risk factor for developing PD (Subramaniam and Chesselet, 2013). For these reasons, it is believed that sporadic form of PD is caused by a complex interplay between genetic susceptibility and environmental factors (Figure 2) (Schapira and Tolosa, 2010; Schapira and Jenner, 2011).

In spite of the involvement of several biological mechanisms (e.g. increased production of reactive oxygen species (ROS), autophagy and UPS impairment and neuronal protein aggregation), many of the molecular pathways involved in PD pathogenesis converge on mitochondria, resulting in their dysfunction, which could in turn induce the neuronal cell death.

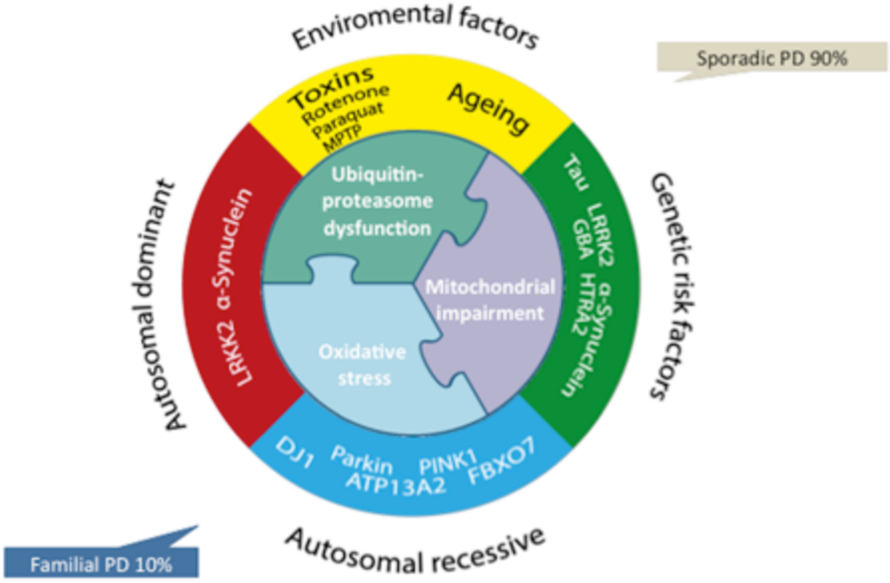


Figure 2. Schematic representation of PD etiology. The complex interplay between genetic susceptibility and environmental factors plays a key role in the development of sporadic PD. Both familial and sporadic forms of the disease are united by common pathogenetic mechanisms (i.e. oxidative stress, mitochondrial impairment and ubiquitin-proteasome dysfunctions).

1.1.1.1. Genetic forms of Parkinson's disease

Approximately the 5 – 10% of PD patients suffer from a familial form of this pathology. Till now, 26 PD risk loci have been identified through genome-wide association studies (Lill, 2016). Given the high number of discovered mutated genes, only the most important ones will be described below.

SNCA (PARK1)

SNCA (*PARK1*) was the first gene identified to be associated with familial PD (Polymeropoulos et al., 1996; Polymeropoulos et al., 1997). To date, three different missense mutations (A53T, A30P and E46K) and duplication or triplication of this gene have been associated with the onset of autosomal dominant forms of PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Singleton et al., 2003; Zarranz et al., 2004).

SNCA gene encodes for α-synuclein (α-syn), a presynaptic protein that plays several roles in neuronal cells (e.g. regulation of cellular trafficking and endocytosis and interaction with synaptic vescicles) (Vekrellis et al., 2011; Benskey et al., 2016).

α-Syn is prone to form fibrillar aggregates, which are the major components of LB (Vekrellis et al., 2011), thus underlining the strong link between the familial and the sporadic forms of this pathology. The aggregation of α-syn causes several cellular damage that can contribute to the development of PD, such as the disruption of lysosomal and proteasomal functions (Chu et al., 2009; Wang and Mao, 2014; Xilouri et al., 2016). Moreover, it has been demonstrated that

overexpression of mutant or wild type form of α-syn leads to the association of this protein to the mitochondrial membrane, thus causing the impairment of mitochondrial functions (e.g. inhibition of complex I and high ROS production) and the activation of the apoptotic process due to the release of cytochrome c (Parihar et al., 2008; Vekrellis et al., 2011; Reeve et al., 2015).
Mutations in the *SNCA* gene affect also the mitochondrial shape. In these cases, mitochondrial fragmentation is determined by the decreased association between mitochondria and endoplasmic reticulum and by the protease cleavage of OPA1 into its pro-fission form (Guardia-Laguarta et al., 2014).

LRRK2 (PARK8)

PARK8 gene encodes a large multidomain protein, named leucine-rich repeat serine/threonine-protein kinase 2 (LRRK2), with GTP-regulated serine/threonine kinase activity (Martin et al., 2014). Mutations in *PARK8* are associated with the onset of autosomal dominant forms of PD of late onset (Zimprich et al., 2004; Di Fonzo et al., 2005). However, mutations in this gene do not account only for rare familial forms of the disease, but they are also associated with the 1% of sporadic cases (Gilks et al., 2005; Lesage and Brice, 2009).
To date, it has been demonstrated that seven sequence variants (N1437H, R1441H, R1441C, R1441G, Y1699C, I2020T and G2019S) cause the development of PD (Klein and Westenberger, 2012). Most of the LRRK2 mutations are located in the GTPase or in the kinase domain, thus causing an increased kinase activity (Cookson, 2010; Martin et al., 2014).
LRRK2 plays a key role in the maintenance of the mitochondrial functions and morphology. Indeed, this protein is involved in the translocation of DRP1 from the cytosol to the mitochondria, thus allowing the fission of the mitochondrial network (Wang et al., 2012). Indeed, it has been demonstrated that G2019S mutation determines the formation of a more elongated and interconnected mitochondrial network and the impairment of mitochondrial functions (i.e. lower ATP production and lower mitochondrial membrane potential) (Mortiboys et al., 2010). Moreover, inhibition of LRRK2 activity causes mitochondrial fission and increased ROS production (Saez-Atienzar et al., 2014).

Parkin (PARK2)

PARK2 gene encodes for Parkin, a protein with E3-ubiquitin ligase activity. Several mutations in this gene have been linked to the onset of autosomal recessive juvenile Parkinsonism (Kitada et al., 1998; Shimura et al., 2000). Most of the point mutations cause alterations in the cellular localization or in the protein solubility. Instead, insertions and deletions of this gene determine a loss-of-function of Parkin (Sun et al., 2006).

Parkin plays a role in protein and organelle degradation via the proteasome. For this reason, mutations in *PARK2* gene cause the accumulation of its substrates, which may have toxic effect on DA neurons (Zhang et al., 2000; Giasson and Lee, 2001). Parkin is also involved in the regulation of mitochondrial dynamics and turn over (Geisler et al., 2010). Eventually, it has been proposed an indirect role for Parkin in the regulation of mitochondrial biogenesis (Shin et al., 2011).

PINK1 (PARK6)

PARK6 gene encodes for PINK1, a serine/threonine kinase protein that acts upstream to Parkin in the regulation of mitochondrial disposal (mitophagy) (Kim et al., 2007; Narendra et al., 2010). Mutations in this gene cause the onset of autosomal recessive form of PD. However, mutations in *PARK6* gene were also found in patients suffering from sporadic PD (Valente et al., 2004a): for this reason, it has been proposed that heterozygous mutations in PINK1 might be a risk factor for PD (Valente et al., 2004b). Most of the mutations reside in the kinase domain of PINK1, thus determining a loss of function of this protein (Sim et al., 2006).

PINK1 is involved in the mitochondrial quality control: indeed, following mitochondrial depolarization, PINK1 accumulates on the outer mitochondrial membrane, thus recruiting Parkin (Kim et al., 2007; Narendra et al., 2010). The PINK1/Parkin pathway plays also an important role in mitochondrial dynamics and functions. Indeed, when mitochondria are damaged, PINK1 phosphorylates Miro to induce a parkin and proteasomal-dependent degradation of this protein, thus inhibiting mitochondrial motility (Wang et al., 2011a). Moreover, the activation of this mitophagy pathway causes the ubiquitination and the consequent degradation of mitochondrial fusion proteins (e.g. mitofusins) (Gegg et al., 2010; Chen and Dorn, 2013; Wang et al., 2011b). Eventually, it has been demonstrated that PINK1 deficiency causes lower complex I activity and increased sensitivity to apoptotic stress (Morais et al., 2009).

HtrA2/OMI (PARK13)

PARK13 gene encodes for HtrA2/OMI, a mitochondrial serine protease. Mutations in *PARK13* gene are associated with familial dominant form of PD (Strauss et al., 2005). The loss of function of HtrA2/OMI causes mitochondrial dysfunction and mutation of the mtDNA (Goo et al., 2013). Eventually, it has been suggested that PINK1 acts upstream of HtrA2/OMI, thus facilitating its phosphorylation and the resulting cellular resistance to mitochondrial stress (Plun-Favreau et al., 2007).

GBA

GBA gene encodes for β-glucocerebrosidase (Gcase), a lysosomal enzyme with an important role in glycolipid metabolism. Mutations in this gene have been found to increase the risk of developing sporadic PD and are found in 8%-14% of autopsy-proven diagnoses of PD (Velayati at al., 2010).

Some *GBA* mutations result in misfolded protein, which might induce lysosomal insufficiency and autophagic pathways impairment, thus causing neurodegeneration (Bekris et al., 2010). Moreover, it has been demonstrated that reduction in Gcase determines an increase in α-syn and conversely, overexpression of α-syn reduces Gcase levels (Manning-Boğ et al., 2009; Schapira, 2015).

1.1.1.2. Environmental factors

MPTP

The discovery that the administration of MPTP toxin causes parkinsonism was done in the late 1970s (Davis et al., 1979; Langston et al., 1983). Indeed, subjects exposed to MPTP developed PD due to the degeneration of dopaminergic neurons. However, these patients did not show LB formation (Davis et al., 1979; Langston et al., 1999).

After this discovery, MPTP toxin became of common use in research in several *in vitro* and *in vivo* models in order to recapitulate the sporadic PD pathology.

The toxicity of the MPTP molecule is due to its ability to cross the blood-brain-barrier. Once MPTP enter in the glial cells, it is metabolized in MPP^+ by the monoamine oxidase enzyme. Then, MPP^+ accumulates in the mitochondria of the dopaminergic neurons thanks to the dopamine transporter (DAT) (Langston et al., 1984; Markey et al., 1984; Nicklas et al., 1985; Ramsay et al., 1986).

In vitro and *in vivo* studies demonstrated that MPTP treatment cause several cellular alterations (e.g. dopamine signaling, ubiquitin-proteasome system, calcium signaling, apoptosis and mitochondrial dysfunctions) (Zhang et al., 2010; Burte et al., 2011). In particular, at mitochondrial level, this toxin inhibits the activity of the complex I of the electron-transport-chain, thus leading to lower ATP production, increased ROS generation and neuronal cell death (Dauer and Przedborski, 2003; Perier et al., 2005; Perier et al., 2007). MPTP exposure causes also the reduction of several mitochondrial gene expressions (Hoang et al., 2009; Piao et al., 2012).

Recently it has been demonstrated that MPP^+ exposure causes a mitophagy impairment due to the degradation of BNIP3L protein, the decreased protein ubiquitination and the p62 inactivation

(Gao et al., 2015; Navarro-Yepes et al., 2016).

Rotenone

Rotenone is a lipophilic molecule that is able to cross the blood-brain barrier and biological membranes without using a specific receptor or transporter. Once in neuronal cells, rotenone is able to inhibit the complex I of the mitochondrial electron-transport-chain, thus causing high ROS generation, lower ATP production and apoptotic cell death (Johnson and Bobrovskaya, 2015). The use of rotenone also causes proteolityic stress due to the inhibition of the proteasome activity (Chou et al., 2010).

Chronic treatment with rotenone causes the selective death of dopaminergic neurons and the formation of LB-like cytoplasmic inclusions *in vivo* (Betarbet et al., 2000; Sherer et al., 2002b; Sherer et al., 2003). Moreover, rotenone related symptoms better resembles typical parkinsonian motor features (Tanner et al., 2011). For these reasons, this toxin is widely used in research to reproduce PD. Lastly, it has been demonstrated that people exposed to this toxin show an increased risk in developing PD (Tanner et al., 2011).

Paraquat

Paraquat is another compound commonly used in research to reproduce some clinical and pathological features of PD. Unlike the two mitochondrial toxins described above, paraquat is not able to cross the blood-brain barrier. However, it enters the brain through the neutral amino acid transporter (Shimizu et al., 2001; McCormack and Di Monte, 2003). Once in the cells, paraquat accumulates in the mitochondria, where it convert free radicals to superoxide and other ROS, thus increasing the cellular oxidative stress (Jones and Vale, 2000; Yumino et al., 2002). Moreover, paraquat interacts with glutamate, thus leading to excitotoxicity and NOS generation (Shimizu et al., 2003).

Paraquat treatment causes the selective loss of dopaminergic neurons, with consequent decreasing in motor functions (Brooks et al., 1999; McCormack et al., 2002; Ossowska et al., 2005). Administration of paraquat also determines the over-expression and aggregation of α-syn, thus leading to the formation of LB-like structures (Manning-Bog et al., 2002). Eventually, the exposure to this herbicide represents a risk factor for the development of PD in human (Tanner et al., 2011; Wu et al., 2012; Nandipati and Litvan, 2016).

1.1.1.3. Sporadic Parkinson's disease

The 90% of PD cases is not determined by a genetic cause (Ascherio and Schwarzschild, 2016). The most significant features of sporadic PD pathogenesis are mitochondrial dysfunctions,

increased production of ROS and neuronal protein aggregation (Subramaniam and Chesselet, 2013). Moreover, the ubiquitin-proteasome-system and autophagy-lysosomal pathway are impaired in PD, thus leading to the worsening of the molecular features cited above (Lim and Tan, 2007; Moors et al., 2016; Migdalska-Richards and Schapira, 2016).

The reduction of complex I activity is one of the main hallmark of sporadic PD: indeed, mitochondrial complex I deficiency are reported not only in the SNpc of PD patients (Schapira et al., 1990) but also in other cells and tissues, e.g. fibroblasts (Mytilineou et al., 1994), skeletal muscle (Blin et al., 1994) and lymphocytes (Haas et al., 1995). Moreover, as described above, the exposure to toxins that cause the inhibition of complex I represent a crucial risk factor for developing PD.

Oxidative stress is another typical feature of PD pathogenesis (Jenner, 2003). Normally the production and the detoxification of ROS are well balanced (Wang and Hai, 2016). However, when mitochondrial complexes of electron-transport-chain are inhibited, the production of free radical is increased, thus allowing ROS to activate specific signaling pathway, eventually inducing cell death (Fiskum et al., 2003; Jenner and Olanow, 2006). This has been confirmed by the presence of oxidized proteins and of high level of mtDNA deletions in substantia nigra of PD patients (Jenner, 2003; Bender et al., 2006).

Neurons of the SNpc are more sensitive to ROS production due to the presence of dopamine, which is able to produce reactive quinones at neutral pH. This process leads to the formation of endogenous toxins, which cause mitochondrial dysfunction and oxidative damage (Muñoz et al., 2012).

1.1.2. Dopamine metabolism and oxidative stress

Despite mitochondrial toxins are widely used in research, the parkinsonism induced by these compounds results from an acute toxic insult and differs from the slow and progressive disease process that characterize sporadic PD.

Notwithstanding the initial triggers of the pathogenetic mechanisms is still unknown, altered dopamine homeostasis might be a key factor in the early steps of PD pathogenesis (Alberio et al., 2012; Bisaglia et al., 2013; Herrera et al., 2017).

Dopamine is synthesized from tyrosine by tyrosine hydroxylase and aromatic amino acid decarboxylase. After this, dopamine does not accumulate in the cytosol but is immediately stored in monoaminergic synaptic vesicles thanks to the vesicular monominergic transporter-2 (VMAT-2) (Hastings, 2009; Segura-Aguilar et al., 2014). However, when dopamine is not properly handled and accumulates in the cytosol, dopamine is able to oxidize to o-quinones that is able to form adducts with several proteins (e.g. Parkin, tyrosine hydroxylase, mitochondrial complex I,

III and V and superoxide dismutase 2) (Hastings, 2009; Segura-Aguilar et al., 2014) (Figure 3). Moreover, dopamine quinones can be oxidized to aminochrome, which is able to induce and stabilize the formation of neurotoxic protofibrils of α-syn (Conway et al., 2001), to cause dysfunction of proteasome system (Zhou and Lim, 2009) and to prevent the fusion of autophagy vacuoles with lysosomes (Paris et al., 2010) (Figure 3).

It has been demonstrated that α-syn play an important role in dopamine homeostasis and that protofibrils of α-syn can modify the permeability of vesicles by forming pores, thus causing the leakage of dopamine from synaptic vesicles to the cytoplasm (Lotharius and Brundin, 2002; Mosharov et al., 2006; Hastings, 2009) (Figure 3).

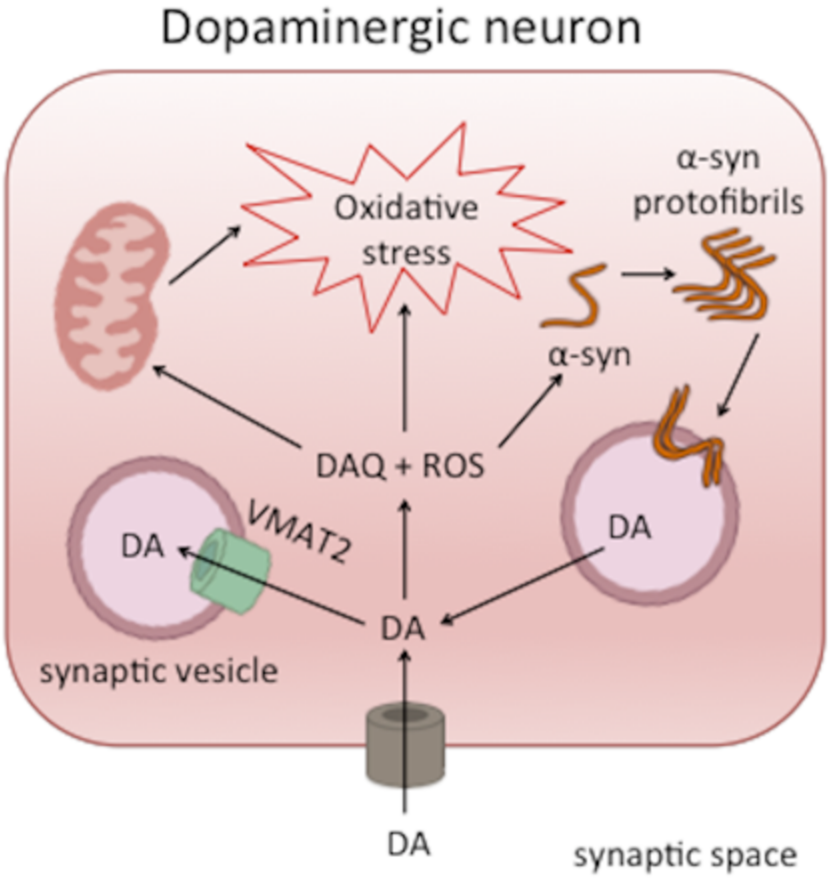


Figure 3. Schematic representation of altered dopamine homeostasis. When dopamine (DA) is not properly stored in the synaptic vesicle, it accumulates in the cytosol, thus forming quinones (DAQ) and increasing the ROS production. DAQ form adducts with several mitochondrial proteins and stabilize the formation of α-syn neurotoxic protofibrils. In this way, altered dopamine homeostasis cause cellular oxidative stress and apoptotic cell death.

On this basis, several efforts have been made to develop cell models for dopamine toxicity. Several investigators increased the level of α-synuclein by transient or conditional expression, observing extensive protein aggregation and increased susceptibility to dopamine toxicity (Maguire-Zeiss et al., 2005). Moreover, altered dopamine homeostasis in mice with reduced VMAT2 expression was sufficient to cause dopamine-mediated toxicity and progressive loss of

dopaminergic neurons (Caudle et al., 2007).

For these reasons, it seems plausible that dopamine oxidation plays an important role in the degeneration of dopaminergic neurons by inducing mitochondrial dysfunctions, neuroinflammation and oxidative stress (Herrera et al., 2017).

1.2. Cellular models of Parkinson's disease

1.2.1. SH-SY5Y

SH-SY5Y is a human neuroblastoma cell line widely used to study PD. Indeed, the majority of genes associated with PD molecular pathways are intact in SH-SY5Y genome. Moreover, the somatic mutations that characterize this cell line do not alter PD-related pathways (Krishna et al., 2014).

SH-SY5Y cells possess a complete dopaminergic system. In particular, this cellular model couples the good activity of the dopamine transporter (DAT) with the low activity of VMAT-2, thus impairing the dopamine storage into vesicles (Mena et al., 1989; Alberio et al., 2012). Thanks to this molecular characteristic, the cytoplasmic dopamine concentration can be raised by the administration of exogenous dopamine in the culture medium, thus reproducing the impairment of dopamine homeostasis (Gómez-Santos et al., 2003; Van Laar et al., 2009; Alberio et al., 2012).

Eventually, it is possible to induce the differentiation of SH-SY5Y cells using specific molecules (e.g. retinoic acid). In this way, SH-SY5Y cells lose their neuroblast-like morphology and become more similar to primary neurons (Kovalevich and Langford, 2013).

1.2.2. Primary skin fibroblasts cell lines

Primary skin fibroblast cells are widely used in research to investigate PD mechanisms. This cellular model represent an easily accessible source of proliferating cells that share the same genetic complexity of neurons, mirror the polygenic risk factors and reflect cumulative cell damage at the age of the patient (Connolly, 1998; Auburger et al., 2012). Several studies carried out on fibroblasts obtained from skin biopsies from PD patients have reported that fibroblasts show some deficits associated with PD pathogenesis that are typical of neuronal tissue (McNeill et al., 2014; Lippolis et al., 2015; Kilpatrick et al., 2016). For example, fibroblasts derived by *PARK2*-mutated patients can be used successfully to study the mitochondrial impairment that characterizes these subjects. Indeed, fibroblast's mitochondria show lower membrane potential, lower complex I activity and lower ATP cellular levels (Zanellati et al., 2015). Also fibroblasts from LRRK2 G2019S mutated PD patients are characterized by mitochondrial dysfunctions (Mortiboys et al., 2010).

Thus fibroblasts may be considered easily accessible patients-derived cells to be used in discovery studies. Moreover, it is possible to transform primary skin fibroblasts in iPSC-derived dopaminergic neurons, thus giving a way to study the patient's tissue directly affected by the disease (Beevers et al., 2013).

1.3. Mitochondria

Mitochondria are double membranes-enclosed cytoplasmic organelles. The structure of mitochondria can be divided in four components: outer mitochondrial membrane (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM) and mitochondrial matrix. The inner membrane is highly folded, forming tubular or lamellar structures called cristae where the complexes of the respiratory are embedded.

Mitochondria are intimately involved in cellular homeostasis. First of all, these organelles are mainly known because they provide energy in form of ATP to the cell. Moreover, they play a key role in the biosynthesis of several macromolecules, such as nucleotides, lipids, heme, and iron-sulfur clusters. Another important property of mitochondria is their capacity of calcium buffering which helps to regulate the intracellular Ca^{2+} homeostasis (Vakifahmetoglu-Norberg et al., 2017).

As mitochondria are essential for a plethora of cellular processes, regulation and maintenance of mitochondrial function is very important for the cell.

1.3.1. Mitochondrial dynamics

Mitochondria are very dynamic organelles that forms complex network. This mitochondrial network changes continuously in response to the activation of a specific signaling pathway or the presence of a particular metabolic stimulus. Two different processes (i.e. fusion and fission) are involved in the mitochondrial network dynamics.

1.3.1.1. Mitochondrial fusion

Mitochondrial fusion allows the exchange of components between mitochondria, thus enabling the maintenance of their functional state (Chen and Chan, 2006). This process is made possible by three different proteins (i.e. MFN1, MFN2 and OPA1) (Figure 4).

Mitofusin 1 (MFN1) and mitofusin 2 (MFN2) are integral outer mitochondrial membrane proteins with GTPase activity that are responsible for the fusion of the outer mitochondrial membranes (Chen et al., 2003; Dimmer and Scorrano, 2006). The first step of fusion is mediated by their respectively heptad-repeat domains that are able to form a dimeric antiparallel coiled-coil structure, which can be homotypic (MFN1-MFN1 or MFN2-MFN2) or heterotypic (MFN1-

MFN2) (Chen et al., 2003; Koshiba et al., 2004). Because of the higher GTPase activity of MFN1 protein, mitochondria that own only MFN1 protein show a better tethering efficiency than mitochondria with only MFN2 protein (Ishihara et al., 2004). For this reason, MFN1 is able to partially rescue the defects caused by MFN2 mutations (Detmer and Chan, 2007). However, it has been demonstrated that MFN2 protein plays other crucial functions in the cell beyond mitochondrial fusion. Indeed, it is involved in the tethering between mitochondria and endoplasmic reticulum and in the maintenance of the Ca^{2+} signaling between these organelles (de Brito and Scorrano, 2008). Moreover, MFN2 play also a role in the mitochondrial motility, thanks to its connection with the Miro/Milton transport complex (Misko et al., 2010). It seems also that this protein exerts a protective function against neurodegeneration (Chen et al., 2007) and in dopaminergic neurons (Lee et al., 2012; Pham et al., 2012). The turnover of MFN1 and MFN2 proteins depends on the recruitment of the AAA-ATPase p97 that permits the degradation of the ubiquitinated mitofusins through the proteasome (Tanaka et al., 2010; Pallanck, 2010). It has been demonstrated that deletion of MFN1 or MFN2 causes mitochondrial fragmentation and poor mitochondrial function, although low levels of mitochondrial fusion remain (Chen et al., 2005; Chen and Chan, 2005; Perier and Vila, 2012).

Dynamin-like 120 kDa protein (OPA1) is an integral GTPase protein that is responsible for the fusion of the inner mitochondrial membranes (Chan, 2006a). This protein is imported in the intermembrane space, where it is processed by several proteases, such as PARL, YME1L and OMA1 (Cipolat et al., 2006; Ishihara et al., 2006; Hoppins et al., 2007; MacVicar and Langer, 2016), thus leading to the formation of both long (L-OPA1) and short (S-OPA1) protein forms. In physiological conditions, a correct balance between L-OPA1 and S-OPA1 is maintained in order to preserve the mitochondrial network morphology (Ishihara et al, 2006; Song et al, 2009). However, mitochondrial depolarization causes the activation of OMA1 protease, which determines the accumulation of the short form of OPA1 (Head et al., 2009). This process leads to the inhibition of the inner mitochondrial membranes fusion, thus causing mitochondrial fragmentation (Twig et al, 2008; Song et al, 2009). OPA1 is also involved in the protection against apoptotic cell death. Indeed, this protein plays a key role in the maintenance of cristae structure and junctions, thus preventing the release of cytochrome c from mitochondria (Lee et al., 2004; Zhang and Chan, 2007; Ramonet et al., 2013). Moreover, OPA1 knock down leads to Bax translocation, release of cytochrome c and caspase activation (Scorrano et al., 2002; Olichon et al., 2003).

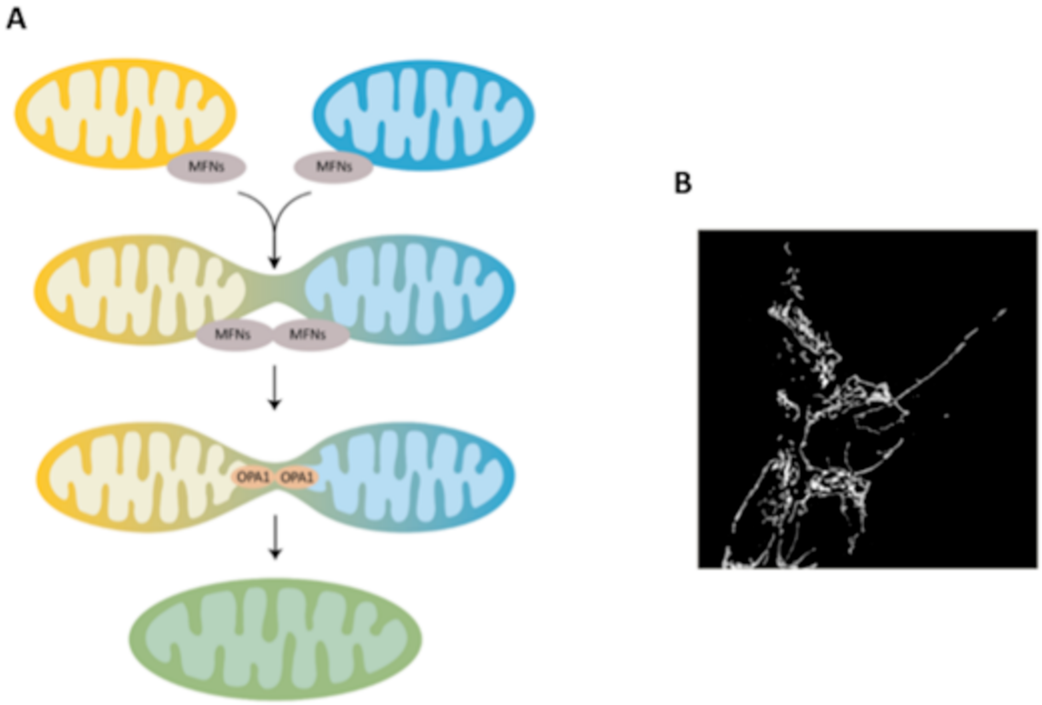


Figure 4. A: Schematic representation of mitochondrial fusion process. Both MFN1 and MFN2 are responsible for the fusion of the outer mitochondrial membranes, while OPA1 plays a key role in the fusion of the inner mitochondrial membranes. B: Representative immunofluorescence image of mitochondrial networks in SH-SY5Y cells with filamentous shape.

1.3.1.2. Mitochondrial fission

Mitochondrial fission is important to allow the proper cellular distribution of these organelles and the degradation of damaged mitochondria through the mitophagy process (Otera and Mihara, 2011). This process is made possible by the GTPase activity of the cytosolic protein DRP1. Indeed, once this GTPase protein localized on mitochondria, DRP1 oligomerizes into ring-like structures and constrict mitochondria thanks to the GTP hydrolysis (Ingerman et al., 2005).

The recruitment of DRP1 to the outer mitochondrial membranes depends on specific post-translational modifications. For example, fission process is allowed when calcineurin dephosporilates DRP1 protein, while it is prevented when PKA phosphorylates the conserved residue Ser-637 of DRP1. Also sumoylation and ubiquitination processes have an impact on fission machinery (Otera and Mihara, 2011).

The recruitment of DRP1 on the outer mitochondrial membrane requires also the presence of specific mitochondrial receptors (Parone et al., 2008). The most characterized receptor that interacts with DRP1 is Fis1 (Mozdy et al., 2000; Legesse- Miller et al., 2003; Yoon et al., 2003; Karren et al., 2005) (Figure 5). This protein consists of several domains, but the fisrt alpha-helix of its N-terminal is the most important for its oligomerization and its fission activity (Jofuku et al., 2005). There are also other mitochondrial membrane proteins that interact with DRP1, thus causing the fission process. For example, Mff proteins can recruit DRP1 independently of Fis1

and its overexpression determines mitochondrial fragmentation (Otera et al., 2010). Eventually, MiD49 and MiD51 are involved in the fission process through their capacity to recruit DRP1 on the outer mitochondrial membrane (Palmer et al., 2011).

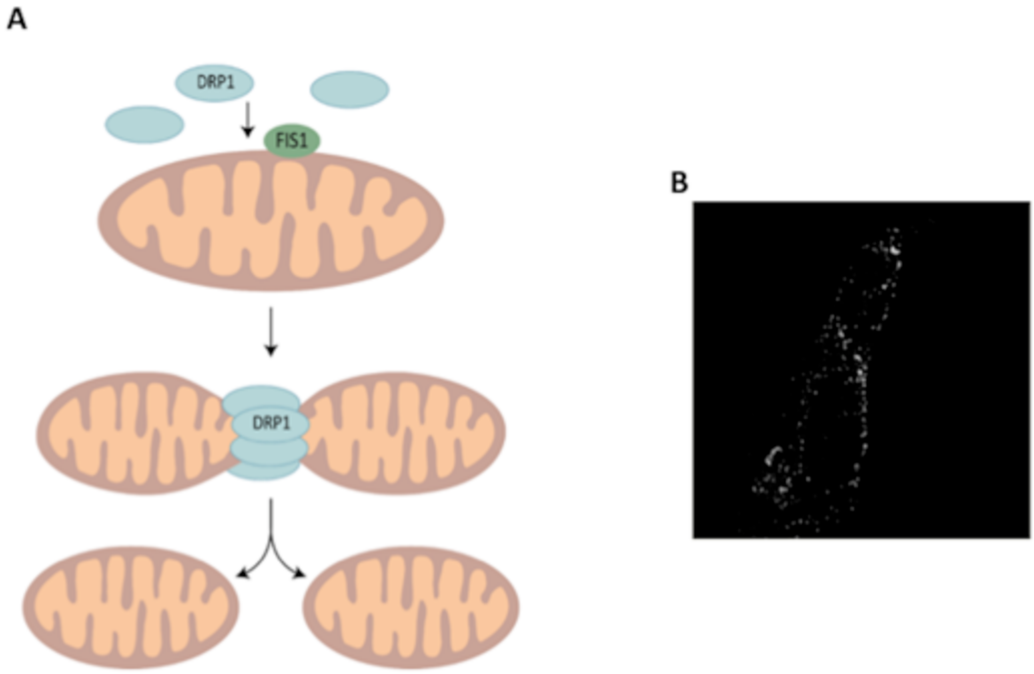


Figure 5. A: Schematic representation of mitochondrial fission process. Fis1 is a mitochondrial membrane receptor involved in the recruitment of DRP1 protein. Once DRP1 localized and oligomerized on the outer mitochondrial membrane, it constricts mitochondria thanks to the hydrolysis of GTP molecules. B: Representative immunofluorescence image of mitochondrial networks in SH-SY5Y cells with fragmented shape.

1.3.1.3. Mitochondrial dynamics in Parkinson's disease

Several studies demonstrated that mutations in the genes associated with the familial forms of the disease impaired the dynamics of the mitochondrial network. PINK1 and Parkin proteins, for example, act in a common pathway that modulates the mitochondrial shape (Exner et al., 2007; Yang et al., 2008; Poole et al., 2008). In particular, it has been demonstrated that the overexpression PINK1 act in a pro-fission manner, thus determining the formation of a more fragmented mitochondrial network. On the contrary, knockdown of PINK1 cause the formation of a more elongated and interconnected mitochondrial network (Yu et al., 2011). Moreover, a study on fibroblasts obtained from *PARK2*-mutated patients has shown that loss-of-function of Parkin protein cause the appearance of "chain-like" networks (Zanellati et al., 2015)

Several studies also demonstrated the interaction between LRRK2 and DRP1. Indeed, LRRK2 phophorilates DRP1 and cause the translocation of DRP1 from the cytosol to the mitochondria, thus causing the formation of mitochondrial network with fragmented shape (Niu et al., 2012; Wang et al., 2012a). Moreover, also α-syn protein has an impact on mitochondrial morphology: A30P and A53T mutations increase the cleavage of OPA1 protein (Guardia-Laguarta et al.,

2014). Eventually, a fragmented mitochondrial shape was also observed in cells transfected with DJ1 mutated proteins (Wang et al., 2012b).

1.3.2. Mitochondrial homeostasis

The life cycle of mitochondria includes both the division of pre-existing organelles (mitochondrial biogenesis) and the degradation of older or damaged organelles (mitophagy). The balance between these two processes is indispensable to optimize the mitochondrial functions. In this complex contest, the correct balance between fusion and fission process is also indispensable to regulate the expansion of the mitochondrial network or to segregate the damaged organelles that will be eliminated through the mitophagy process. Moreover, mitochondria contain their own proteolytic system that is able to degrade misfolded or unfolded proteins inside mitochondrial compartments. Also the proteasome system is involved in the mitochondrial homeostasis: indeed, it is involved in the elimination of damaged outer mitochondrial proteins and proteins that fail to be imported (Palikaras and Tavernarakis, 2014).

1.3.2.1. Mitochondrial biogenesis

Several factors participate in the transcription of the mtDNA: a single RNA polymerase (POLRMT), two stimulatory transcription factors (Tfam, TFB) and a termination factor (MTERF1) (Bonawitz et al., 2006; Scarpulla, 2008). The transcription of mtDNA occurs bi-directionally from two different promoters, known as LSP and HSP, which are inside the D-loop regulatory region (Neupert, 2016).

Several nuclear-encoded proteins play a key role in the mitochondrial biogenesis process. Tfam, for example, is able to stimulate mtDNA transcription through specific promoter recognition (Fisher and Clayton, 1988; Neupert, 2016). Moreover, NRF1 and NRF2 are two nuclear transcription factors that positively regulate the expression of some key genes (e.g. Tfam and TFB) involved in the mitochondrial biogenesis process through their ability to bind several promoters, thus enabling their transcription (Virbasius and Scarpulla, 1994; Gleyzer et al., 2005). NRF1 and NRF2 are also involved in the positive regulation of the transcription of other genes that encode for mitochondrial proteins (e.g. cytochrome c, TOMM20 and several COX subunits) (Scarpulla, 1999; Takahashi et al., 2002; Ongwijitwat and Wong-Riley, 2005; Ongwijitwat et al., 2006; Blesa et al., 2007; Scarpulla, 2011).

PGC-1α is an important transcriptional coactivator that plays a key role in the mitochondrial biogenesis process. Indeed, PGC-1α is able to bind both NRF1 and NRF2 proteins, thus activating the transcription of their target genes (e.g. Tfam, TFB1M and TFB2M) and positively influencing the mitochondrial functions (Wu et al., 1999; Gleyzer et al., 2005). Two different

signaling pathways are able to induce the transcription of PGC-1α. For example, when cAMP cellular levels are elevated, PKA phosphorylates CREB, which, in turn, induces the expression of PGC-1α (Herzig et al., 2001; Puigserver and Spiegelman, 2003). Also the c-GMP signaling pathway, which is activated following an increased of nitric oxide cellular levels, is able to induce the transcription of PGC-1α, thus inducing an increase in the mitochondrial biogenesis process (Nisoli et al., 2003; Nisoli et al., 2004).

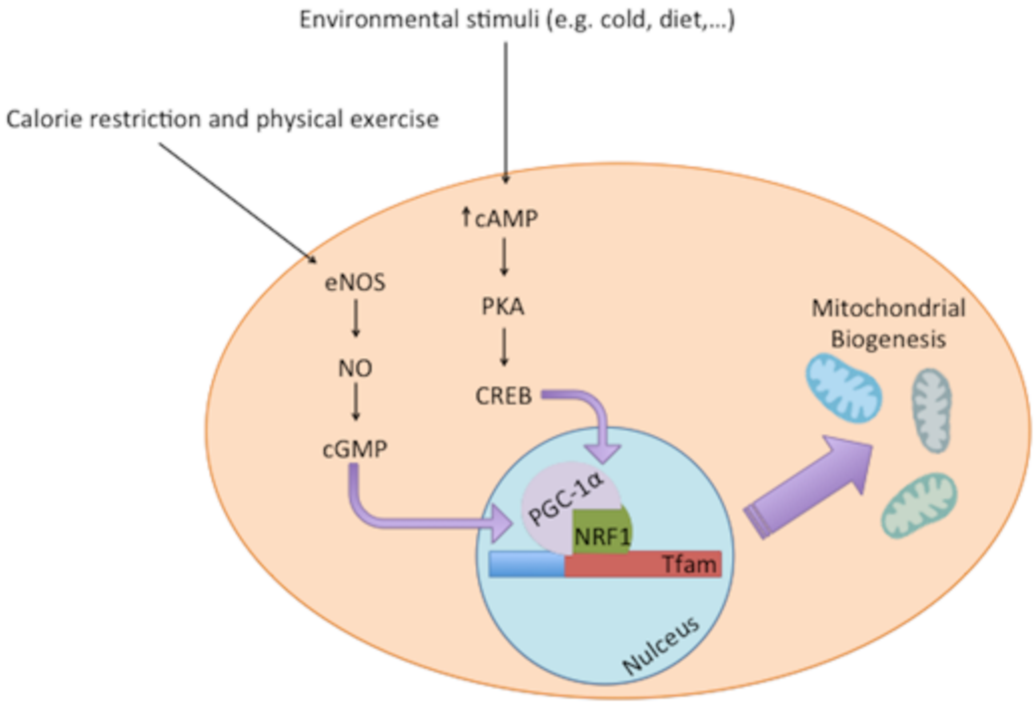


Figure 6. Schematic representation of mitochondrial biogenesis process. cGMP and cAMP signaling pathway increase the expression of PGC-1α, which in turn positively regulate the transcription of Tfam. Eventually, Tfam stimulates the mtDNA transcription through specific promoter recognition.

Strong evidences suggest that the impairment of mitochondrial biogenesis process is tightly linked to both sporadic and genetic forms of PD. mtDNA deletions, which are caused by high levels of ROS occurring in dopaminergic neurons, are an hallmark of sporadic PD (Dias et al., 2013). Moreover, it has been demonstrated that the conditional disruption of Tfam determines the appearance of levodopa-responsive motor deficits, coupled with lower mtDNA expression and respiratory chain impairment (Ekstrand et al., 2007). Moreover, mutations in the gene that encode for the mitochondrial protein POLG, which is responsible for the synthesis and the proofreading of mtDNA, are associated with the development of a more complex syndrome, characterized by levodopa-responsive parkinsonism (Luoma et al., 2004; Davidzon et al., 2006). Taken together these results suggest that the impairment in the mtDNA synthesis may be a leading event in the PD pathogenesis.

Recently it has been proposed the involvement of the PINK1/Parkin-mediated mitophagy pathway in the activation of mitochondrial biogenesis process. Indeed, the activation of NRF2, TFEB and PGC-1α that occur following the activation of the mitophagy pathway, allow the activation of mitochondrial biogenesis process and the transcription of genes with an antioxidant response element (Ivankovicet al., 2016). Moreover, it has been demonstrated that the overexpression of Parkin protein induces an increased mtDNA transcription and replication through Tfam activity (Kuroda et al., 2006). The strong involvement of Parkin in the mitochondrial biogenesis process was further confirmed by the discovery that *PARK2*-mutated patients showed lower mRNA levels of Tfam, NRF1 and NRF2 (Pacelli et al., 2011). Furthermore, sporadic PD human brains and parkin knockout mice show an increased expression of PARIS protein, which is the major repressor of PGC-1α. This molecular event causes the impairment in the mitochondrial biogenesis process (Shin et al., 2011).

Eventually, it has been proposed that the mitochondrial protease OMI is involved in the regulation of mitochondrial biogenesis process. Indeed the loss of OMI protease activity causes the downregulation of PGC-1α and the increased expression of GSK3β kinase, which is responsible for the PGC-1α degradation (Xu et al., 2014).

1.3.2.2. Mitophagy

Mitophagy is an important quality control process that allows the recognition and the consequent lysosomal degradation of the damaged mitochondria (Youle and Narendra, 2011). The missed mitochondrial disposal causes the accumulation of dysfunctional mitochondria (Tal et al., 2009; Egan et al., 2011) that can lead to cell death (Green and Kroemer, 2004) or inflammation activation pathway (Green et al., 2011) and participate in disease pathogenesis (Wallace, 1999; Chan, 2006b; Itoh et al., 2013).

The most characterized mitophagy mechanism is the PINK1/Parkin pathway. Under basal conditions, the precursor of PINK1 is synthesized in the cytosol and is imported into the OMM via translocase of outer mitochondrial membrane complex (TOM). Full-length PINK1 is further transferred into the inner membrane of mitochondria through the translocase of inner mitochondrial membrane (TIM) complex in a membrane potential dependent manner. Here, PINK1 is processed by the mitochondrial processing peptidase (MPP), that cleaves the MTS sequence, resulting in a ~60-kDa PINK1 form. This MPP-cleaved form of PINK1, which now spans the IMM, is then further cleaved by the rhomboid protease of the IMM, presenilin associated rhomboid-like protease (PARL), to give rise to the 52-KDa mature form. PINK1 is then degraded through the proteasome. This pathway keeps endogenous PINK1 levels very low

in polarized mitochondria to prevent mitophagy of healthy mitochondria. (Jin et al., 2010; Matsuda et al., 2010; Eiyama and Okamoto, 2015) (Figure 7).

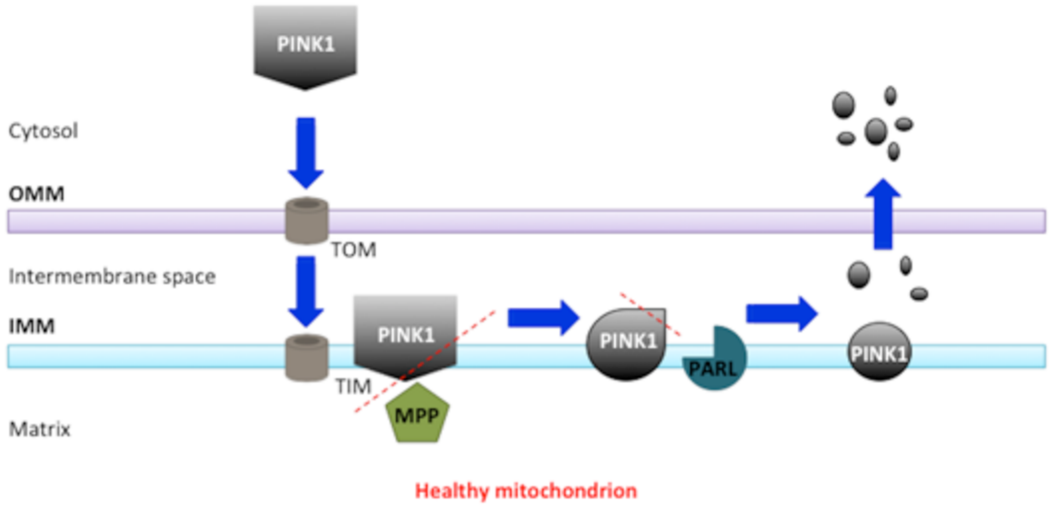


Figure 7. Schematic representation of PINK1 degradation in healthy mitochondria. In healthy mitochondria, the precursor of PINK1 (64 kDa) is imported into the OMM via TOM and further transferred into IMM through TIM complex in a membrane potential dependent manner. Here, 64-kDa PINK1 is processed by the MPP, that remove the MTS sequence. This form of PINK1 is then cleaved by the rhomboid protease of the IMM, PARL, to give rise to the 52-kDa mature form and subsequently degraded by mitochondrial peptidases and by the proteasome.

On the contrary, when mitochondria are damaged and lose their membrane potential, the import of PINK1 and its proteasomal degradation are inhibited, thus causing the accumulation of this protein onto the outer mitochondrial membrane. This process leads to the mitochondrial recruitment of the E3-ubiquitin ligase Parkin, where it catalyzes the covalent attachment of ubiquitin moieties onto specific mitochondrial proteins (e.g. MFNs, VDACs, Miro, and TOMM70) (Narendra et al., 2010a; Sarraf et al., 2013; Pickrell and Youle, 2015) (Figure 8). The recruitment of Parkin to mitochondria depends on the phosphorylation and consequent activation of this protein by PINK1 (Shiba-Fukushima et al., 2012; Ordureau et al., 2014), on VDAC proteins (Geisler et al., 2010; Sun et al., 2012) and on MFN2 (Chen and Dorn, 2013).

Once the OMM proteins are ubiquitylated, several autophagy receptors are recruited to damage mitochondria in order to bind the ubiquitin-tagged OMM proteins. In particular, p62/SQSTM1 is an autophagy receptor involved in the mitochondrial clustering during mitophagy and it is required for mitochondrial disposal (Narendra et al., 2010b; Okatsu et al., 2010; Geisler et al., 2010) (Figure 8). Moreover, parkin protein is able to bind AMBRA1, an autophagy-promoting protein that determines the local formation of autophagosomes, thus causing the PINK1/parkin-mediated mitophagy (Fimia et al., 2007; Van Humbeeck et al., 2011; Strappazzon et al., 2011).

The impairment of PINK1/Parkin mitophagy axis could lead to accumulation of dysfunctional mitochondria that may contribute to dopaminergic cell death due to the increased production of

ROS and the enhanced release of mitochondrial apoptogenic factors (Vila and Przedborski 2003; Fernández-Moriano et al., 2015).

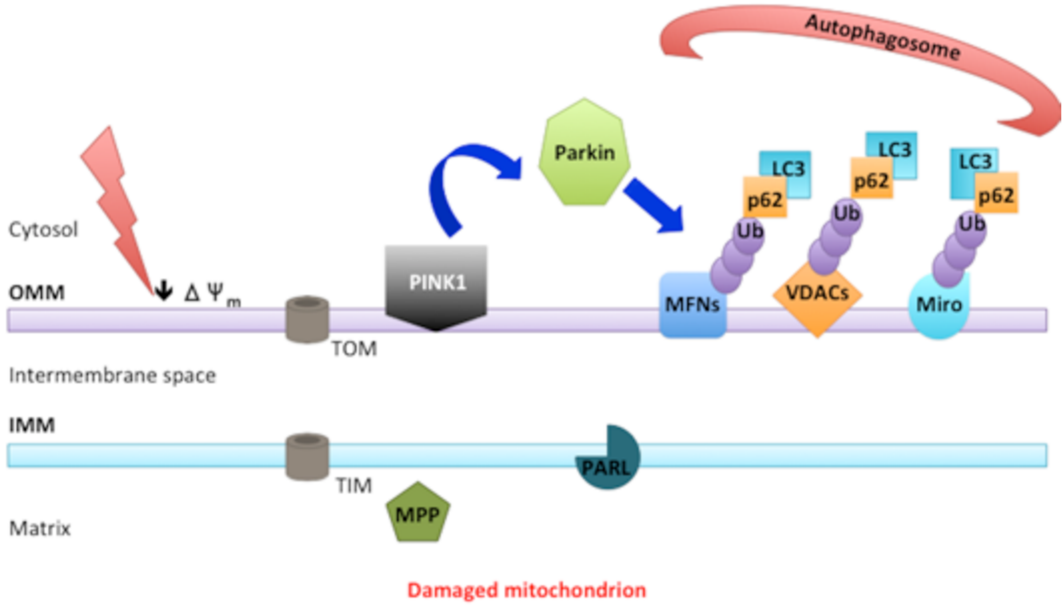


Figure 8. Schematic representation of PINK1/Parkin mitophagy pathway. When mitochondria are depolarized, PINK1 accumulates onto the OMM, thus recruiting Parkin. Parkin catalyzes the covalent attachment of ubiquitin moieties onto specific mitochondrial proteins. This process leads to the selective engulfment of damaged organelles by the autophagosome.

One of the most common risk factors for developing PD are the presence of mutations in the *GBA* gene, which encode for GCase, a protein involved in the autophagy - lysosome pathway. Indeed, loss of function of GCase leads to the accumulation of damaged macromolecules and dysfunctional organelles (Gegg and Schapira, 2016).

In addition to the PINK1/Parkin/ubiquitin axis, mitophagy can occur independent of Parkin. Several autophagy receptor proteins have been shown to localize on mitochondria and interact with LC3 to recruit autophagosomes to damaged mitochondria including Parkin-independent mediators such as Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), NIX (also called BNIP3L), and Fun14 Domain containing 1 (FUNDC1). BNIP3 and NIX activate autophagy by binding to Bcl-2, which dissociates the complex of Bcl-2 and Beclin-1, a protein necessary for initiation of autophagosome formation (Bellot et al., 2009). However, BNIP3 can also work in conjunction with Parkin, by recruiting DRP1 and Parkin to the mitochondria to promote fission and mitophagy (Lee et al, 2011).

1.3.2.3. Intraorganellar quality control system

The first line of defense operates within mitochondria. Indeed, several chaperones and proteases are involved in the mitochondrial quality control by promoting the folding of newly imported proteins, by protecting mitochondrial proteins against heat stress and by degrading irreversibly damaged polypeptides.

In particular, mitochondrial proteases are able to recognize specifically misfolded proteins and degrade them to peptides, which are subsequently exported from the organelle or further degraded to amino acids by the action of oligopeptidases (Rugarli and Langer, 2012).

The mitochondrial matrix contains two ATP-dependent proteases: the first is Lon, a serine protease that is able to degrade the denatured and oxidized proteins; the second ATP-dependent protease is Clp, which is involved in the degradation of damaged proteins in the mitochondrial matrix space (Hamon et al., 2015).

Since the mitochondrial inner membrane contains the respiratory chain and several other proteins, there are multiple possible target proteins for oxidative and nitrosative stress and other protein damage. The quality control of the inner mitochondrial membrane is mediated by two membrane-embedded metalloprotease complexes, called AAA proteases, which are involved in the processing and in the degradation of proteins localized in the matrix, the inner mitochondrial membrane or the inner mitochondrial space. Indeed, the i-AAA protease faces towards intermembrane space, while the m-AAA protease exposes the catalytic domain to the matrix side of the inner membrane (Koppen and Langer, 2007; Hamon et al., 2015).

Eventually, mitochondrial quality control includes also chaperones. The important role of chaperone proteins (e.g. HSP60, HSP70 and HtrA2/OMI) is due to the continuous import of the vast majority of mitochondrial proteins and to the predisposition to oxidation of misfolded proteins (Dukan et al., 2000; Wiedemann et al., 2004; Vande Walle et al., 2008).

1.3.3. Mitochondrial proteome

Mitochondria have their own genetic system that is characterized by several bacteria-like features, such as a compact circular DNA genome (mtDNA) and a simple transcription system that produces multigenic RNA transcripts. The mitochondrial genome has 37 genes, among which only 13 encode proteins, which are all essential subunits of the respiratory chain. The remaining genes encode for 22 tRNAs and 2 rRNAs, which are required for protein translation within the mitochondrial matrix (Scarpulla et al., 2012). The majority of mitochondrial proteins are encoded by nuclear genes, synthesized in the cytosol and eventually imported into the organelle. Mitochondria have several multimeric translocation machines that allow the import of

mitochondrial proteins from the cytosol to the appropriate mitochondrial subcompartment (e.g. TOM and TIM) (Kang et al., 2017).

Nowadays, the estimate number of strictly mitochondrial proteins in human cells is 1100–1900 (Fasano et al., 2016; Palmfeldt and Bross, 2017). In addition, there are a big variety of proteins that are more dynamically present and that are able to interact with the core of mitochondrial proteins. Therefore, the estimate number of these proteins that do not belong to the strictly concept of mitochondrial proteome is 6592. These proteins can deeply influence the mitochondrial biology (Fasano et al., 2016) (Figure 9). For this reason, the mitochondrial proteome is dynamic and it is adjusted depending on the cellular requirements (Rafelski, 2013; Hoppins, 2014; Yin and Cadenas, 2015). Moreover, a recent study demonstrated that three different mitochondria isolation methods (i.e. differential centrifugation, sucrose gradient and a commercial kit based on surfactants) coupled with mass spectrometry analysis allow the understanding of the mitochondrial proteome. The best mitochondrial enrichment protocol must be chosen depending on the experimental design and on the cell line used (Alberio et al., 2017).

The mitochondrial proteome is deeply influenced by PD. Several proteomics studies conducted on both toxin and genetic-based models of PD confirmed once againe the importance the mitochondrial proteome in PD pathology (Pienaar et al., 2010). Gel-based and gel-free proteomics approaches can be successfully used in order to highlight the alterations of the mitochondrial proteome in PD. The big quantity of data originating from these proteomics approaches can be then manage and integrate using some bioinformatics tools, such as PANTHER, STRING and Cytoscape. Only in this way, it will be possible to infer and understand the biological meaning of the data.

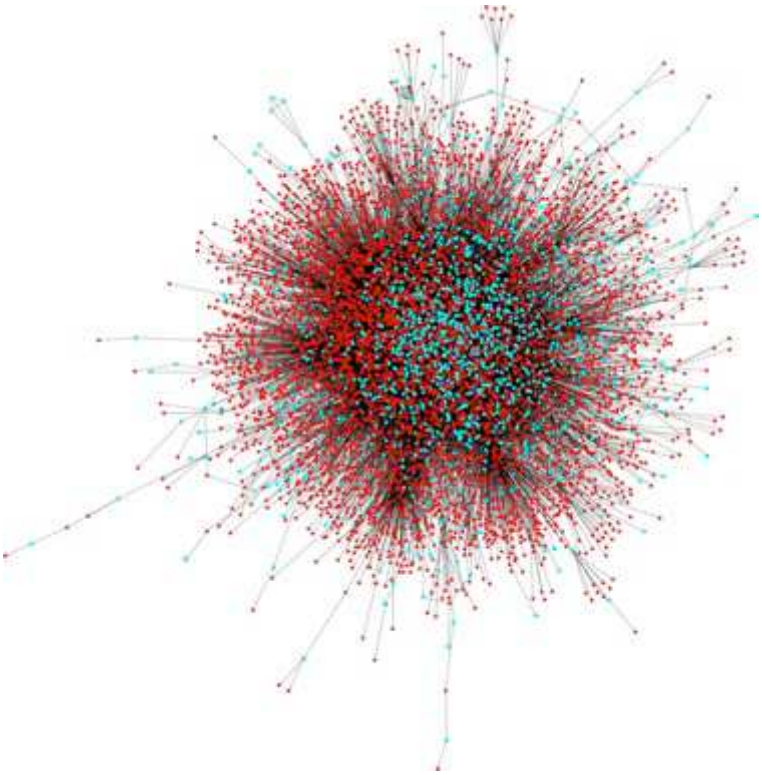


Figure 9. The functional mitochondrial human proteome. Cyan nodes represent proteins encoded by the mitochondrial genome or translocated to the mitochondrion. Red nodes represent interactors of the cyan nodes.

1.4. Proteomics

Since PD is a multifactorial disorder characterized by a complex and not completely understood etiology, the research in this field is shifting in the use of –omics techniques (particularly transcriptomics, proteomics and metabolomics) in order to elucidate the pathogenetic processes of the disease.

Proteomics is a discipline that studies the structure and function of proteins by a variety of methods. The term proteome was first coined by Wilkins with the meaning of the entire PROTein complement expressed by a genOME, or by a cell or a tissue type at a given time (Wilkins et al., 1996). Proteomics is a global and an unbiased approach that allows the investigations of proteins that are not a priori expected to be linked to any condition, without the restrictions imposed by more traditional methods, such as antibody-based targeting of candidate proteins (Tyers and Mann, 2003; Villoslada et al., 2009; Alberio and Fasano, 2011). The list of proteins thus generated can be integrated with data mining methods, thus revealing the involvement of specific biochemical pathways that were hidden by the complexity of data themselves (Alberio et al., 2010; Alberio and Fasano, 2011). This in turn allows the discovery of novel molecular mechanisms and eventually new therapeutic strategies.

Nowadays, there are two different classes of proteomics techniques:

1) Gel-based proteomic approaches, which include separation of proteins by gel electrophoresis followed by mass spectrometry (MS) analysis;
2) Gel-free proteomics, which includes various isotopic labeling strategies for quantitative proteomics analysis as well as label-free approaches.

1.4.1. Gel-based proteomics

This approach requires as first step the separation of proteins by a simple SDS-PAGE or by two-dimensional electrophoresis (2DE). In particular, 2DE methodology allows the separation of proteins by their isoelectric point (isoelectric focusing or IEF) and then by their molecular weight. During the IEF, proteins migrate through a thin gel-strip embedded with immobilized pH gradients (IPG). Migration terminates when the protein reaches the point where the net charge is neutral (i.e., the isoelectric point, or pI). Proteins are then separated in the second dimension using a common SDS-PAGE, which allows proteins to separate according to their molecular weight. After separating proteins through 2DE electrophoresis approach, the gel thus obtained are stained with specific chemical stains (e.g. Coomassie blue) or fluorescent protein dyes (e.g. SYPRO stains and ruthenium-based dye) in order to visualize and to detect the differences in protein staining intensities between samples (Pienaar et al. 2008). However, the limit of this

technique is the great experimental variation obtained generating a distinct 2DE image for each gel. In order to decrease this variation, it was developed the differential in-gel electrophoresis (DIGE) technology, which is based on labelling proteins with fluorescent Cy2, Cy3, and Cy5 dyes. All of these dyes have identical Mr and pI but differ in excitation and emission wavelengths. Therefore, up to three different protein mixtures can be labelled individually but separated and analyzed on a single gel (Ünlü et al., 1997, Lilley and Friedman, 2004).

In order to process the information from the 2DE-gel, translation of gel information into digital data is required. Depending on the staining method, this is carried out using an appropriate imaging system, such as laser-based detectors, CCD cameras or flatbed scanners. Translation of complex data into appropriate biological information is carried out using computerized data analysis provided by software packages developed for this scope. After this analysis, proteins are identified using mass spectrometry (MS). In this way it is possible to measure the molecular mass of a charged particle by calculating its mass-to-charge (m/z) ratio.

To sum up, 2DE gel-based proteomics allow to visualize the proteins distribution and the resolution of multiple proteoforms (i.e. post-translational modifications) of the same protein. Therefore, 2DE is able to resolve thousands of spots simultaneously and represents the only technique that can be routinely applied for parallel quantitative profiling of large sets of complex protein mixtures such as whole-cell lysates (Görg et al., 2004; Albrecht et al., 2010). Nevertheless, the less represented proteins are lost due to the reduced dynamic range of the detection technique. Moreover, proteins of extreme hydrophobicity and those of extreme basic or acidic pI are not considered in the majority of published studies (Albrecht et al., 2010).

1.4.2. Gel-free proteomics

Due to the intrinsic limitations of gel-based proteomics approaches, nowadays the most promising techniques used to study the cellular proteome are based on gel-free proteomics (Abdallah et al., 2012).

The term “shotgun proteomics” refers to the application of bottom-up proteomics analysis to protein mixtures, thereby indirectly measuring proteins by analyzing the peptides generated from proteolysis of intact proteins using proteolytic enzymes (e.g. trypsin). Peptides in the mixture thus obtained are separated by liquid chromatography (LC), which is then coupled to tandem mass spectrometry (MS/MS) for further analysis (McDonald and Yates, 2002).

In shotgun proteomics, peptides are identified by comparing tandem mass spectra of fragmented peptides with deposited theoretical tandem mass spectra available in protein databases. The identified proteins are classified and scored depending on their peptides, since they may refer to a single protein or to more than one protein (Zhang et al, 2013). The advance represented by this

technique made it possible to avoid some of the limitations of gel-based proteomics, including the requirement for a large amount of material, limited dynamic range, low-throughput analysis, difficulties in the identification of acidic, basic, hydrophobic, very small or very large proteins and bias toward abundant proteins (Walther and Mann, 2010).

Nowadays, two different approaches are used to quantify the proteome: the differential isotopic labeling and the label-free quantification. These two approaches have different advantages, isotope- labeling methods measure protein abundance with higher accuracy, whereas label-free approaches have a greater dynamic range and achieve higher levels of proteome coverage.

1.4.2.1. Differential isotope labeling

The isotope labeling can be applied either *in vitro* (labeling of isolated proteins and peptides) or *in vivo* (incorporation of isotope-labeled amino acids through metabolic labeling, which is also called stable isotope labeling with amino acids in cell culture (SILAC)). This modern LC-MS/MS technique allows to overcome the two major problems of the classical proteomics quantification methods (i.e., 2DE gels coupled with dyes). Indeed, the applicability of 2DE approach is limited to abundant and soluble proteins. Moreover this technique does not reveal the identity of the underlying protein. However, it must keep in mind that mass spectrometry is not inherently quantitative because proteolytic peptides exhibit a wide range of physicochemical properties, such as size, charge, and hydrophobicity. For these reasons, it is necessary to compare each individual peptide between experiments in order to give an accurate quantification (Bantscheff et al., 2007).

One major quantitative approach is built on the theory of stable isotope dilution (Ong and Mann, 2005). This theory states that the relative signal intensity obtained in a mass spectrometer of two analytes that are chemically identical but have different stable isotope composition, represents the relative abundance of the two analytes in the sample. Therefore, protein abundance in two or more different biological samples can be analyzed in a LC–MS/MS experiment on the basis of observable mass shifts caused by differential isotope labeling (Ong and Mann, 2005).

Isotope labels can be introduced as an internal standard into amino acids, metabolically, chemically, or enzymatically. The first possibility to introduce a stable isotope signature into proteins came from the metabolic labeling during cell growth and division, i.e., stable isotope labeling by amino acids in cell culture (SILAC). The main advantage of this strategy is that the differentially treated samples can be combined at the level of intact cells. This excludes all sources of quantification error introduced by biochemical and mass spectrometric procedures as these will affect both protein populations in the same way. However, the main application of metabolic labeling in higher eukaryotes to date is SILAC in immortalized cell lines. Indeed, the

cost and time required for creating and maintaining this approach in *in vivo* models is often incommensurate with the value of the information provided (Bantscheff et al., 2007).

Post-biosynthetic labeling of proteins and peptides is performed by chemical or enzymatic derivatization in vitro. Enzymatic labeling can be performed either during proteolytic digestion or, more commonly, after proteolysis in a second incubation step with the protease. This process leads to the incorporation of different isotope into C-termini of peptides, thus causing a mass shift. Because peptides are enzymatically labeled, artifacts (i.e., side reactions) common to chemical labeling can be avoided. A practical disadvantage is that full labeling is rarely achieved and that different peptides incorporate the label at different rates, thus complicating the analysis of data (Bantscheff et al., 2007).

1.4.2.2. Label-free quantification

Label-free quantitative MS methods are based either on spectral counting or on peptide precursor ion intensities that are obtained using the first mass spectrometer (MS1) of a tandem mass spectrometer (Colinge et al., 2005). This method is based on the assumption that the rate at which a peptide precursor ion is selected for fragmentation in a mass spectrometer is correlated to its abundance. For relative protein quantification, the spectral counts are then averaged into a protein abundance index. Therefore, label-free quantification is based on the accurate mass and time tag approach (Strittmatter et al., 2003) and builds on the alignment of high-mass accuracy spectra that are obtained from the analysis of two different sample conditions by separate LC–MS/MS experiments. Peptides are identified across different LC runs based on their specific retention time coordinates and precise mass to charge (m/z) values, which in principle allows the quantification of all of the peptides detected from a biological sample that are within the sensitivity range of a MS analyzer, independent of MS/MS acquisition (Bantscheff et al., 2007).

Label-free approaches are less accurate compared to the isotope labeling methods. Indeed, all the systematic and non-systematic variations between experiments are reflected in the obtained data. Consequently, it is important to maintain the number of experimental steps to a minimum and to control reproducibility at each step. Nonetheless, label-free quantification has many advantages. Indeed, the time-consuming steps of introducing a label into proteins or peptides can be omitted and there are no costs for labeling reagents. For these reasons, it is possible to conduct and to compare several numbers of experiments. This is certainly an advantage over stable isotope labeling techniques that are typically limited to 2–8 experiments that can be directly compared. Moreover, unlike for most stable isotope labeling techniques, the mass spectral complexity is not increased in label-free approaches, thus increasing the number of detected peptides/proteins in an experiment. Eventually, there is evidence that label-free methods provide higher dynamic range

of quantification than stable isotope labeling and therefore may be advantageous when large and global protein changes between experiments are observed (Bantscheff et al., 2007).

1.5. Interactomics

The proteomics approach unveils only qualitative and quantitative changes of the cellular proteome and gives back a static view of what is occurring in biological samples. For this reason, research is increasingly shifting towards the understanding of the cellular interactome, in order to get a more dynamic and complete picture of the molecular pathways that are altered in a specific pathology.

Nowadays, two different main techniques are used to study the interactome: affinity purification (AP) and biochemical fractionation, both coupled with MS. Indeed, AP and biochemical fractionation are used for the detection of stable protein complexes (Havugimana et al., 2012).

1.5.1. Affinity purification

AP coupled with MS analysis is one of the most successful methods for identifying stable protein interactions and for characterizing the protein function in various model organisms (Polanowska et al., 2004; Hu et al., 2009; Guruharsha et al., 2011; Babu et al., 2012).

This approach consists in the fusion of a protein-coding sequence with an epitope tag compatible with protein purification. For example, the tandem affinity purification (TAP)-tag (which contains two IgG binding domains of Protein A and the calmodulin binding peptide (CBP) that flank a tobacco etch virus (TEV) cleavage site) has been used successfully for the characterization of protein-protein interactions in yeast (Krogan et al., 2006; Babu et al., 2012; Mak and Moffat, 2012). However, it has been demonstrated that the yield of purified proteins obtained with this technique is low in mammalian cells (Bürckstümmer et al., 2006; Mak and Moffat, 2012). In comparison with TAP-tag technique, the dual protein G-streptavidin binding peptide (GS)-TAP tag in mammalian cells can increase the protein-complex efficiency. Indeed, the GS-TAP tag proposed by Bürckstümmer and colleagues offers the opportunity to perform single-step purification by streptavidin followed by specific elution with biotin or straight boiling. Therefore, using the same cell lysate, the researcher has the choice of obtaining different levels of yield and purity depending on the nature of the protein complex and the type of question being asked (Bürckstümmer et al., 2006). However, this approach does not yield sufficient affinity purified protein from the tagged baits required for MS analysis (Mak and Moffat, 2012).

Another common strategy is the use of commercial antibodies that bind the epitope tag of the target protein (immunoprecipitation). However, the success of this approach depends on the

availability of antibodies with high specificity for a single human protein (Mak and Moffat, 2012).

For these reasons, the best affinity purification method used to study the interactome in mammalian cells is the versatile affinity (VA)-tag constructed in-frame with a Gateway cassette consisting of 3 × Flag, 6 × hexahistidine (His), and 2× Streptactin (Strep) epitopes. Indeed, the VA-tag can be integrated in-frame with the target bait protein through viral or non-viral based transfection approaches. The lentiviral-based procedure has the advantage to transduce these viruses into a broad range of host cells and to have a high efficiency of transfection. Therefore, following chromosomal integration in a specific cell line, this technique provides a high-level and stable expression of the specific heterologous gene (Karra and Dahm, 2010). Moreover, the VA-tag allows to monitor the subcellular localization of the tagged baits, thus being able to check if the affinity tagging or overexpressing target proteins affects their proper localization. The VA-tag gives also the opportunity to select the bait purification schemes (single or multi-step purifications) for the optimization of weak or stable PPIs, or for controlling sample purity. Eventually, another advantage of the VA-tag is that the bait protein can be eluted using non-denaturing conditions, which may be required for downstream applications (e.g., enzyme assays or maintaining PPIs for a second round of purifications) (Mak and Moffat, 2012). However, when the protein is expressed from the exogenous promoter, the resulting protein over-production can alter the physiological state of the protein, thus resulting in false positive identification of protein-protein interactions. Moreover, the lentiviral-based procedure determines the formation of low numbers of stable clones (Karra and Dahm, 2010).

The non-viral based tagging approach is based on the use of specific chemical reagent-mediated transfection (i.e. liposome and non-liposome). This approach allows high transient and stable transfection rates and reduces the toxicity effects of the lentiviral-based procedure (Karra and Dahm, 2010). However, the non-viral based tagging approach is effective in dividing cells, but not in post-mitotic cells, such as mature neurons (Washbourne and McAllister, 2002; Karra and Dahm, 2010).

Eventually, the further use of chemical cross-linking allows to capture interactions between flexible regions of solubilized proteins by covalently linking the functional groups of amino acid side chains. This technique provides high-confidence protein interaction data with very little chemical background noise (Miteva et al., 2013).

1.5.2. Biochemical fractionation

The intrinsic limitations of affinity purification methods (e.g. the availability of antibodies with high specificity for a single human protein (immunoprecipitation technique) and the low

transfection efficiency in post-mitotic cells (non-viral based tagging approach)), can be overcome using a recent integrative global proteomic approach, which allows the identification of disease interaction networks from human cells or tissues (Havugimana et al., 2012). The biochemical fractionation approach determines the separation of protein complexes by their size (Size-Exclusion chromatography, SEC) or by their charge (Ion-Exchange chromatography, IEX), using high performance liquid chromatography system (HPLC).

The SEC technique allows the separation of proteins under native conditions, thus preserving the biological interactions and activity of the macromolecule (Hong et al., 2012). Recently, it has been proposed to combine the protein correlation profiling–stable isotope labeling by amino acids in cell culture approach (PCP–SILAC) with high-performance liquid chromatography using SEC in order to determine the composition of the human interactome as well as its change over time (Kristensen et al., 2012). PCP-SILAC was initially used to assign the protein localization to specific organelles proteins thanks to a sucrose gradient coupled with mass spectrometry. However, proteins are poorly resolved using this method. For this reason, PCP-SILAC was coupled with SEC technique, which is the best method for resolving protein complexes. So, SEC-PCP-SILAC not only allows the determination of an interactome, but this approach is also able to highlight the heterogeneity of complexes within the cell. Indeed, the conventional interactome approaches cannot detect the distribution of a protein among different complexes. Therefore, this technique can reveal the biological outcomes of the interactome, which is normally hidden by the other interactomics approach (Kristensen et al., 2012).

The IEX approach allows the separation of protein complexes by the Coulombic interactions between charged groups of the analyte and the charged stationary phase. In order to elute the proteins, it is necessary to add salts into the mobile phase. At the beginning, the mobile phase is characterized by a higher concentration of salts in order to elute the more strongly bound (more highly charged) proteins. Over time, the concentration of salts slow down, thus forming a gradient mobile phase, in order to elute the less charged proteins (Di Palma, et al., 2012). Principally, there are two main forms of IEX; cation exchange (CX) and anion exchange (AX). In CX the stationary phase has an anionic functional group that enables binding of molecules with cationic groups. On the contrary, in AX the stationary phase contains positively charged residues that undergo Coulombic interactions with anionic sites of the analytes (Di Palma, et al., 2012).

Eventually, the use of biochemical fractionation techniques coupled with LC-MS/MS approach allows the sensitive detection of protein complexes in cell lines or tissues. Moreover, following protein identification in each fraction, a post-processing scoring step (i.e. the employment of

three different scoring scheme: Pearson correlation coefficient (pcc), weighted cross correlation (wcc), and co-apex score) is necessary to predict protein-protein interactions (Havugimana et al., 2012).

2. Aim of the project

PD is a multifactorial disorder whose etiology is not completely understood. Strong evidences suggest that a complex interplay between genetic susceptibility and environmental factors are involved in the development of the sporadic form of the disease. In this intricate pathological landscape, in which several molecular mechanisms are implicated, mitochondrial dysfunctions and mitophagy impairment seems to play a key role in PD pathogenesis (Schapira and Jenner, 2011). For this reason, the aim of this research project was to investigate the mitochondrial alterations caused by the impaired clearance of these organelles and to understand how mitophagy alteration can impact the development of this pathology.

The first part of the project is focused on the characterization of mitochondrial state and dynamics in two different cellular models of PD (i.e. dopamine or MPP^+-treated SH-SY5Y human neuroblastoma cells) and in autoptic samples from Substantia nigra of PD patients. Altered dopamine homeostasis seems to be an important cellular pathogenetic mechanism involved in neurodegeneration in PD. In this context, it is possible to use the SH-SY5Y neuroblastoma cell line in order to mimic the improper handling of this neurotransmitter by administration of exogenous dopamine in the culture medium (Alberio et al., 2012). Moreover, MPP^+ is a commonly used toxin in PD research due to its ability to induce parkinsonism in *in vivo* studies through the inhibition of complex I (Zhang et al., 2010). As a reference model of mitophagy induction, treatment with the IMM uncoupler CCCP was used in all experimental designs as a positive control. The mitochondrial alterations were investigated from a molecular point of view by analyzing the mtDNA content, the proteins involved in fusion (i.e. OPA1 and MFN1) and fission (i.e. DRP1) processes, the proteins involved in mitochondrial functions (i.e. VDAC1, VDAC2 and COX5β) and in mitophagy pathway (i.e. PINK1). Furthermore, a morphological analysis of mitochondrial structure was carried out using transmission electron microscope.

The second part of the project investigates the mitochondrial proteome and interactome alterations that characterize *PARK2*-mutated PD patients. To this purpose, primary skin fibroblasts of three *PARK2*-mutated patients and three control subjects were used to obtain mitochondria-enriched fractions. These samples were subjected to quantitative shotgun proteomics analysis and to biochemical fractionation coupled with MS. A systems biology approach was used in order to identify the molecular pathway altered by mutations in *PARK2* gene. Eventually, the impact of Parkin loss-of-function on the mitochondrial network morphology was evaluated.

3. Materials and methods

3.1. Cell cultures and treatments

3.1.1. SH-SY5Y

The human neuroblastoma SH-SY5Y cells (ECACC, Cat No. 94030304; Lot No. 11C016) were maintained at 37°C under humidified conditions and 5% CO_2 in high glucose dulbecco's modified eagle's medium (DMEM) (Euroclone) supplemented with 10% fetal bovine serum (FBS) (Euroclone), 100 U/mL penicillin (Euroclone), 100 g/mL streptomycin (Euroclone) and 2 mM L-glutamine (Euroclone).

Cells were seeded at a density of 6×10^6 per T75 flask for 24 hours before treatments. Cells were treated with 250 μM dopamine (Sigma-Aldrich) or with 2.5 mM MPP^+ (Sigma-Aldrich) in DMEM. For both treatments, 700 U/ml catalase (Sigma-Aldrich) was added to DMEM in order to eliminate the extra-cellular H_2O_2. Eventually, SH-SY5Y cells were treated with 20 μM CCCP (Sigma-Aldrich) or an equal volume of vehicle, the dimethyl sulfoxide (DMSO) (Sigma-Aldrich). All treatments lasted for 24 hours.

At the end of the treatments, cells were detached from T75 flask with trypsin-EDTA (Euroclone) and centrifuged at 310 g at 4°C for 10 minutes. Cellular pellets were maintained at -80°C for subsequent analysis.

3.1.2. Human primary skin fibroblasts cell lines

Human primary skin fibroblasts cell lines (Table 1) were obtained from the Telethon bio-bank. A written consent was obtained for all the 3 *PARK2*-mutated patients and the 3 control subjects (Table 1). Genetic and clinical information were taken for each subject.

Human primary skin fibroblasts were maintained at 37°C under humidified conditions and 5% CO_2 in DMEM (Euroclone) supplemented with 10% FBS (Euroclone), 100 U/mL penicillin (Euroclone), 100 g/mL streptomycin (Euroclone) and 2 mM L-glutamine (Euroclone).

Cells were seeded at a density of $5x10^5$ per T25 flask in order to reach the 80% of confluence. The culture medium was changed every two days. The cells used in this research were between passages 8 and 12.

Once the right amount of cells were reached ($20x10^6$), skin fibroblasts cells were detached with Accutase (Euroclone) and centrifuged at 310 g at 4°C for 10 minutes. The cellular pellet was then used to obtain the mitochondrial-enriched fractions by a commercial kit based on surfactants in order to perform our proteomic and interactomic studies (Sigma-Aldrich).

Patients code	Sex	PD onset (years)	Age at skin sampling (years)	*PARK2* mutation
S1	F	61	74	Del_1/p.R275W
S4	F	14	18	Dupl_2/Del_3-4-5
S5	M	40	52	p.Q34Rfs*X5 homozygous
S6	F	-	27	CTRL subject
S7	F	-	27	CTRL subject
S8	M	-	33	CTRL subject

Table 1. Summary of *PARK2*-mutated patients and control subjects characteristics. M: male; F: female.

3.2. Human Substantia nigra

Substantia nigra from six sporadic PD patients and six age-matched controls (Table 2) were obtained from the Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam. A written consent for a brain autopsy was obtained from all donors. Eventually, clinical information for each subject was obtained from the NBB.

Autopsy number	Sample type	Sex	Age	Braak Stage (Tau/amyloid)	Braak Stage LB	Pmd (hr:min)	Diagnosis
07/055	Frozen	M	61	1 O	-	07:35	PD
09/007	Frozen	M	62	1 O	0	07:20	NDC
07/121	Paraffin/Frozen	M	67	1 O	6	04:55	PD
99/249	Frozen	M	78	0 O	-	04:20	NDC
06/191	Frozen	M	87	1 B	5	03:40	PD
09/001	Paraffin/Frozen	M	88	2 A	-	04:43	NDC
09/146	Frozen	F	59	1 A	4	09:35	PD
01/016	Frozen	F	64	0 B	-	08:35	NDC
09/254	Frozen	F	88	-	6	04:15	PD
99/232	Frozen	F	88	3 B	-	05:40	NDC
92/080	Paraffin	M	75	0 B	5	04:15	PD
94/325	Paraffin	F	51	-	-	07:40	NDC

Table 2. Summary of sporadic PD patients and control subjects characteristics. M: male; F: female; Braak stage (Tau/amyloid): this is a scale for Alzheimer's pathology (50, 51); Braak stage LB: this is a scale for Parkinson's disease (52); Pmd: postmortem delay; NDC: non-demented control; PD: Parkinson's disease.

3.3. Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1× protease inhibitor cocktail (Sigma-Aldrich)) and sonicated on ice (0.5 cycles, 10 pulses, three times). Cellular lysates were then centrifuged at 15000 g for 30 minutes at 4°C in order to collect the supernatants in new eppendorf tubes.

Human Substantia nigra were lysed in 500 µl of a specific tissue lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton, 2 mM EDTA, 1 mM DTT, 1x phosphatase inhibitors (Roche), 1× PMSF, 1× protease inhibitor cocktail (Sigma-Aldrich)). The tissues were manually lysed using a Potter homogenizer and then sonicated on ice (0.5 cycles, 15 pulses, twice). After this procedure, the lysates were centrifuged at 18000×g for 15 minutes at 4°C in order to collect the supernatants in new eppendorf tubes.

Equal amounts of proteins, previously quantified using the BCA method (Thermo Fisher Scientific), were incubated in Laemmli loading buffer, resolved in 10% or 16% SDS-gels and transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore) at 1.0 mA/cm2, 1.5 h (TE77pwr, Hoefer). Membranes were then saturated for 2 hours at room temperature in tris-buffered saline with 0.05% tween 20 (TBST) containing 5% dried skimmed milk. Eventually, blots were probed with antibodies against: OPA1 (HPA036927, 1:250; Sigma-Aldrich), MFN1 (sc-50330, 1:1000; Santa Cruz Biotechnology), VDAC1 (ab15895, 1:1000; Abcam), VDAC2 (HPA043475, 1:250; Sigma-Aldrich), COX5β (#C4498, 1:1000; Sigma-Aldrich), PINK1 (#6946, 1:1000; Cell Signaling) histone H3 (H0164, 1:2500; Sigma-Aldrich), CS (AMAb91006,

1:1000; Sigma-Aldrich) or β-actin (GTX23280, 1:8000; GeneTex) in 5% milk-TBST. Blots were incubated with proper peroxidase-conjugated secondary antibodies: anti-rabbit (#AP132P, 1:1500; Millipore Corporation) and anti-mouse (#12-349, 1:3000; Millipore Corporation) in 5% milk-TBST. Peroxidase signals were visualized by chemiluminescence using ECL substrate (Millipore). Images (16 bit grayscale) were acquired with G:BOXChemi XT4 (Syngene) system and analyzed using ImageJ software (https://imagej.nih.gov/ij/) (Schneider and Rasband, 2012).
In order to conduct a correct densitometric analysis, the human Substantia nigra lysates were loaded in two gels that were prepared and transferred to PVDF simultaneously. In this way it was possible to normalize the signal of each protein to that of the sample CTRL 99/249, loaded in both gels, and then to that of β-actin for loading correction.
Data were analyzed by two-tailed, unpaired Student's t-test. $P < 0.05$ was considered significant.

3.4. Immunofluorescence stain and imaging

SH-SY5Y cells were seeded at a density of 5×10^4 per well onto 18 mm glass coverslips in 12 multiwell plates and treated with dopamine, MPP^+ or CCCP, as described above. Human primary skin fibroblasts cells were instead seeded at a density of $5x10^3$ per well.
Cells were washed with PBS and fixed with paraformaldehyde (4% w/v) for 15 min, permeabilized with Triton X-100 solution (Triton X-100 0.2% in PBS) for 5 min and blocked with 5% FBS in PBS for 2 h at RT. Coverslips were incubated overnight at 4°C with primary antibody against DRP1 (sc-32898, 1:100; Santa Cruz Biotechnology) and/or ATP Synthase β (A9728, 1:400; Sigma-Aldrich) in 5% FBS diluted in PBS. Then cells were incubated with the proper Alexa Fluor 488 anti-rabbit and 647 anti-mouse secondary antibodies (1:1000; Thermo Fisher Scientific) in 5% FBS in PBS. Coverslips were mounted with ProLong Gold Antifade mountant (Thermo Fisher Scientific) and imaged using a laser-scanning confocal microscope (Leica TCS SP5) through a 63×/1.40 NA oil-immersion objective (HCX PL APO lambda blue). Z-stacks with 0.2 µm step size were acquired with sequential excitation at 1024 x 1024 pixels resolution and 1.5× or 2× magnification, 2 frames average. Eventually, all image processing and analysis were performed using ImageJ software.

3.4.1. Immunofluorescence quantification and co-localization analysis

In order to quantify the amount of DRP1 in the mitochondrial surface, the six most representative planes of ATP synthase β z-stack signals were chosen and then selected. After this process, the mitochondrial areas thus selected were transposed on the corresponding planes of DRP1 signal. In this way it was possible to measure its intensity normalized on mitochondrial area.

Moreover, the degree of co-localization of ATP synthase β and DRP1 proteins was also evaluated using the JaCoP plugin (Bolte and Cordelières, 2006) that measures the Manders' Overlap Coefficient, which expresses the amount of DRP1 signal overlapping to ATP synthase β (Manders et al., 1992).

Data were analyzed by two-tailed, unpaired Student's t-test. $P < 0.05$ was considered significant.

3.4.2. Mitochondrial network morphology analysis

For the analysis of the mitochondrial network morphology, z-stacks of human primary skin fibroblasts cells labeled with ATP Synthase β antibody were used. Six fovs randomly taken from each coverlips were analyzed.

Before image manipulation, spatial calibration was performed, according to the magnification used during image acquisition. A hybrid filter, unsharp mask, was applied to all z-stack slices. This filter sharpens and enhances edges by the subtraction of a weighed copy of a smoothed version of the image from the original image. Then, an automatic threshold (Huang algorithm) was applied to generate a binary image. 1-bit images were further processed to remove aberrantly detected objects and to improve the mask's representation of the experimental data. Small objects (<0.35 μm2, 3×3 pixels) that violate the Nyquist limit of resolution were eliminated by Remove Outliers function. Following post-processing, the final masks were stored and used for subsequent analysis and measure of mitochondrial network.

A panel of several spatial and shape-descriptor parameter was evaluated among all z-planes using the Analyze Particles function:

-*Area*: the area of the particle is calculated as the sum of the areas of each individual pixel within the borders of the object and it is expressed in calibrated square units (μm2);

-*Perimeter*: the total length of the object boundary;

-*Major and minor axis*: the length of the primary and the secondary axis of the best fitting ellipse for each particle;

-*Angle*: angle between the primary axis and a line parallel to the x-axis of the image;

-*Aspect ratio*: ratio between major and minor axis of the best fitting ellipse. It takes into account of the elongation degree of the particle;

-*Circularity*: measurement of both form and roughness of an object. It is defined as the degree to which an object is similar to a circle, basing on the smoothness of its perimeter;

-*Roundness*: a measure of the particle shape relative to a perfect circle built on the major axis of the particle;

-*Solidity*: the overall concavity of a particle and is defined as the area of the particle divided by the area of the convex hull of the particle.

3.4.3. Statistical analysis of network morphology data

The distribution density of all parameters was calculated for each fov and compared to the distribution density of all controls (i.e., particles from six fovs from each control subject) by the non-parametric Kolmogorov-Smirnov test to determine the distance between the empirical distribution functions of the samples. Significant ($p<10^{-3}$) distances were analyzed by the non-parametric Kruskal-Wallis test followed by the post-hoc Dunn's test for multiple comparisons. All data analysis and statistics procedures were written using the R environment for statistical computing (http://www.r- project.org/).

3.5. Light and transmission electron microscopy

A section of paraffin-embedded human tissues from two sporadic PD patients and from two age-matched controls were stained with hematoxilyn and eosin and analyzed at light microscope in order to identify the best area to study at the transmission electron microscope (TEM). After this selection process, small fragments of brain tissues were removed from paraffin blocks with a blade and put into chloroform for 30 minutes to remove paraffin. Human Substantia nigra were then rehydrated and post-fixed with 2% Karnovsky fixative (2% paraformaldehyde diluted in cacodylate buffer 0.05 M pH 7.3 and 2% glutaraldehyde) for 2 h at 4°C. After several washes in cacodylate buffer, human tissues were put in 1% osmium tetroxide for 1 h at RT and then dehydrated and embedded in a specific resin made by a mixture of araldyte, epon 812, dodecenylsuccinic anhydride (DDSA) and dimethylphthalate (DMP). The polymerization of the resin was carried out at 60°C for 48 hours.

A similar process was performed also for SH-SY5Y cells. After treatments (i.e. dopamine, MPP^+ and CCCP), neuroblastoma cells were detached from T75 flasks using trypsin-EDTA and then pelleted. After a PBS washing, cellular pellets were fixed for 2 h at 4°C with 2% Karnovsky fixative, washed in cacodylate buffer, post-fixed with 1% osmium tetroxide, dehydrated and embedded as previously described.

Eventually, both resin-embedded Substantia nigra tissues and SH-SY5Y have been cut in order to obtain ultrathin sections that were stained with 5% uranyl acetate and Reynold's lead citrate. Ultrathin sections were then observed with a Morgagni Philips/FEI transmission electron microscope.

3.6. mtDNA quantification

For the evaluation of mtDNA, total DNA was extracted from SH-SY5Y treated with dopamine, MPP^+ and CCCP using the QIAamp DNA extraction kit (Qiagen).

Cellular pellets were lysed in a specific lysis buffer made by 150 µl PBS, 15 µl proteinase K, 4 µl RNAsi A stock solution and 150 µl Buffer AL and then incubated at 56°C for 10 minutes. After this process, 200 µl of ethanol (96-100%, Sigma-Aldrich) were added to each sample. This final mixture was applied to QIAamp Mini spin column in order to centrifuge the samples at 6000 g for 1 minute at RT. After several washes with Buffer AW1 and Buffer AW2, total DNA was eluted using 100 µl Buffer AE and quantified by UvVis Optizen Pop 810 Nano Bio spectrophotometer (Mecasys).

The mtDNA amount was measured by quantitative Real-Time PCR (DNA Engine Opticon 2; MJ Research, USA). To this purpose, specific primers have been used:

-mtDNA FW: 5'-CATCTGGTTCCTACTTCAGGG-3';

-mtDNA RV: 5'-TGAGTGGTTAATAGGGTGATAGA-3';

-TK2 FW: 5'-TCCTGCAGATGCCACTTTGA-3';

-TK2 RV: 5'-CCCCAAGTCTGAAGAAAACG-3'.

The PCR amplification profile was as follows: one cycle of 95°C for 3 minutes and 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds (annealing step), followed by the melting curve (55-95°C).

The quantitative Real-Time PCR was performed using a 96 well plate. The reaction mixture was prepared using the GoTaq® Probe 2-Step RT-qPCR System (Promega) and was made by 12.5 µl GoTaq® Probe qPCR Master Mix, 5.5 ul of Nuclease-Free Water, 1 ul Forward Primer 10 uM, 1 ul Reverse Primer 10 uM and 5 ul of DNA 5 ng/ul per well.

For each sample a technical triplicate was performed. Moreover, four different biological replicates were analized. The mean of mtDNA Ct was normalized to that of the genomic DNA housekeeping (TK2). The relative level of mtDNA expression was expressed as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ is the difference between the ΔCt of the treated samples and the ΔCt of the control. Eventually, due to the high variability of mtDNA quantity between each biological replicate, the values of the control samples have been always set to 1 and the amount of mtDNA of treated cells was expressed as relative to that value. Data were analyzed by two-tailed, unpaired Student's t-test. $P < 0.05$ was considered significant.

3.7. Mitochondrial enriched fractions

Human primary skin fibroblasts cells were detached with trypsin-EDTA and collected by centrifugation (310 g, 4° C, 10 min). Mitochondrial enriched fractions were obtained using the MITOISO2 kit (Sigma-Aldrich). After two washes in ice cold PBS, cellular pellets were resuspended in 1 ml of Lysis buffer and incubated on ice for 10 minutes. At the end of this first lysis process, 2 ml of Extraction buffer were added to the lysates in order to centrifuge the samples at 600 g for 10 minutes at 4°C. The supernatant thus obtained was discarded and the cellular pellets were subjected to a second lysis with 400 µl of Lysis buffer and incubated on ice for 10 minutes. After adding two volumes of Extraction buffer, lysates were centrifuged to eliminate cell debris (600×g, 4° C, 10 min) and the supernatants were centrifuged to isolate the mitochondrial-enriched fraction (11000×g, 4° C, 10 min). The resulting mitochondrial pellets were washed in Extraction buffer 1x and stored at -80°C for further investigations. The efficiency of the isolation procedure was tested by Western blotting quantification of histone H3 (nuclear marker, H0164, 1:2500; Sigma-Aldrich), VDAC1 (marker of outer mitochondrial membrane, ab15895, 1:1000; Abcam) and CS (marker of mitochondrial matrix, AMAb91006, 1:1000; Sigma-Aldrich).

3.8. Quantitative Shotgun proteomics

Mitochondrial-enriched fractions obtained from human primary skin fibroblasts cells were lysed in 0.1% RapiGest SF Surfactants (Waters) diluted in 50 mM $(NH_4)_2CO_3$, pH 8.0, according to the manufacturer instructions, and the protein amount was determined with the Bradford method using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad). Tryptic digestion was performed in RapidGest SF using a final protease:protein ratio of 1:50 (w/w) of sequence-grade trypsin (Promega). Prior to proteolysis, mitochondrial fractions were subjected to reduction with 10 mM TCEP (30 minutes at 55°C) and alkylation with 20 mM IAA (30 minutes at RT). Peptide digestion with trypsin was instead conducted at 37 °C overnight. The reaction was stopped by acidification with 0.1% formic acid (FA) at 37°C for 30 minutes. To get rid of the acid-labile surfactant RapidGest SF, samples were dried in a vacuum centrifuge at 16200 g for 30 minutes at 60°C. Samples were then diluted with an aqueous solution of 0.1% FA, 3% CH_3CN (at a final peptide concentration of 0.4 µg/µL). 150 fmol/µL of MassPrep Yeast Enolase digestion standard (Waters), prepared by digesting Yeast Enolase (UniProtKB/Swiss-Prot AC: P00924) with sequencing grade trypsin, were added to each sample as internal standard.

0.25 ug of each digested samples were then loaded on a 5 µm Symmetry C18 trapping column 180 µm × 20 mm (Waters) and separated by a 170 minutes reversed phase gradient at 250 nl/min

(3–40% CH3CN over 145 min) on a nano ACQUITY UPLC System (Waters), using a 1.7 µm BEH 130 C18 Nano Ease 75 µm × 25 cm nano scale LC column (Waters). Separated peptides will be mass analyzed by High Definition Synapt G2-Si Mass spectrometer directly coupled to the chromatographic system.

3.8.1. Shotgun label free proteomics analysis

Differential protein expression was evaluated with a data-independent acquisition (DIA) of shotgun proteomics analysis by high definition Expression mode (HDMSe). Continuum LC-MS data from four technical replicate experiments for each sample was processed for qualitative and quantitative analysis using the software ProteinLynx Global Server v. 3.0.2. (PLGS, Waters). The qualitative identification of proteins was obtained by searching in human database (i.e. UniProt KB/Swiss-Prot Protein Knowledgebase restricted to Homo Sapiens Taxonomy) to which sequence from Saccharomyces cerevisiae Enolase will be appended. Data was filtered in order to have a fold difference larger than 30% among protein hits (corresponding to a ratio of + or - 1.3) (Pieroni et al., 2015; Piras et al., 2015). The search parameters included: automatic tolerance for precursor ions and for product ions, minimum of 3 fragment ions matched per peptide, minimum of 7 fragment ions matched per protein, minimum of 2 peptides matched per protein, 1 missed cleavage, carbamidomethylation of cysteine as fixed modification and oxidation of methionine as variable modification, false positive rate fixed at 4% and 150 fmol of the Yeast Enolase internal standard set as calibration protein concentration.

3.8.2. Statistical analysis of shotgun proteomics data

EMRT clusters tables (i.e., the list of peptide Exact Masses paired to their Retention Times) and Protein tables were generated upon normalization with the endogenous protein Human ATP Synthase subunit 5 β mitochondrial (ATP 5β, UniProtKB/Swiss-Prot AC: P06576). ATP 5β was chosen since, in the qualitative analysis, it was observed being identified within the first 15 most abundant protein (i.e., with a high PLGS score) in all the replicates analyzed.

Protein quantitative data was then analyzed by the univariate non-parametric Wilcoxon test. In order to avoid type-II (beta) errors in the subsequent bioinformatics analysis, no correction for multiple testing will be considered (Alberio et al., 2014a).

3.9. Size-Exclusion Chromatography

Mitochondrial-enriched fractions obtained from human primary skin fibroblasts cells were subjected to lysis in preparation for size-exclusion chromatography (HPLC). Mitochondrial pellets underwent approximately 10 minutes of homogenization on ice using a glass pestle grinder and mortar tube (Corning Life Sciences). The lysis buffer consisted of 20 mM HEPES pH 7.4, 5% glycerol, 0.015 % Triton X-100, 1x phosphatase inhibitors (Sigma-Aldrich), 1x protease inhibitors (Sigma-Aldrich). The suspension was centrifuged for 3 minutes at 10000 g at RT. Supernatant was then collected and protein concentration was determined using the Bradford assay (Bio-Rad).

HPLC fractionations were performed using an Agilent 1100 semi-preparative HPLC equipped with binary pump system (Agilent Technologies, ON, Canada). Protein elution was monitored by absorption at 280 nm. For the size-exclusion chromatography (SEC), a total of 50 µg protein were applied to a 300 × 7.8 mm BioSep4000 Column (Phenomenex). Elution was performed with 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.015% Triton X-100 and 1%-Glycerol. The protein fractions of 0.1 mL were collected after the void volume at a flow rate of 0.5 mL·min−1, thus obtaining 96 fractions for each sample.

Tryptic digestion of each fraction was performed in 2M Urea after reduction (5 mM TCEP 45 minutes at RT) and alchilation (15 mM IAA 1 h at RT). Peptide digestion will be conducted using a final protease:protein ratio of 1:50 (w/w) of Trypsin/Lys-C Mic Mass Spec Grade (Promega) at 37°C overnight. After this process, desalting was performed using Top-Tip Reversed phase (C18) chromatography (Glygen Corp.) and two different solutions, the binding solution (0.1% FA in ultrapure water) and the elution solution (70% acetonitrile in 0.1% FA). At the end, samples were put in the speedvac (16200 g overnight at 60°C).

Samples were then diluted with an aqueous solution of 1% FA and loaded in a micro-column packed with 10 cm of 3 µm Luna-C18 resin. This micro-column was interfased to a nano electrospray ion source that was placed in line with the LTQ Orbitrap Velos Tandem Mass Spectrometer. Nano flow binary HPLC pump was used to deliver a stable tip flow rate of 300 nl/min during the peptide separation. The qualitative identification of proteins was obtained by searching in UniProt KB/Swiss-Prot Protein Knowledgebase restricted to Homo Sapiens Taxonomy.

3.10. Systems biology analysis of the interactome

The use of SEC approach has allowed to obtain for each fraction a list of identified proteins with similar co-elution profiles. The protein list of the same fraction obtained from the three *PARK2*-mutated patients was used to generate protein-protein interaction networks using cy3sbml Cytoscape application (http://apps.cytoscape.org/apps/cy3sbml) and IntAct database as reference. The nodes obtained for each network were used to perform an over-representation analysis using Gene Ontology Consortium database (http://www.geneontology.org) in order to highlight the cellular component of each protein and to reveal the biological process in which the protein network is involved.

4. Results

4.1. CCCP treatment triggered the PINK1/parkin mitophagy process

4.1.1. CCCP treatment caused mitochondrial fission

In order to verify if the fusion process was inhibited following CCCP treatment in SH-SY5Y cell line, the abundance of two different proteins involved in mitochondrial fusion, i.e. mitofusin 1 (MFN1) and optic atrophy 1 (OPA1), were measured by Western blot analysis. MFN1 protein level was reduced by one half after CCCP treatment in SH-SY5Y cells (Figure 10 A and B). As shown in Figure 1 A, the treatment with this protonophore also caused the appearance of the ubiquitinated form of MFN1 at higher molecular weight. Regarding the OPA1 protein, treatment of SH-SY5Y cells with CCCP caused the complete disappearance of its long form (OPA1-L) and the increased expression of its pro-fission short form (OPA1-S) (Figure 10 C and D).

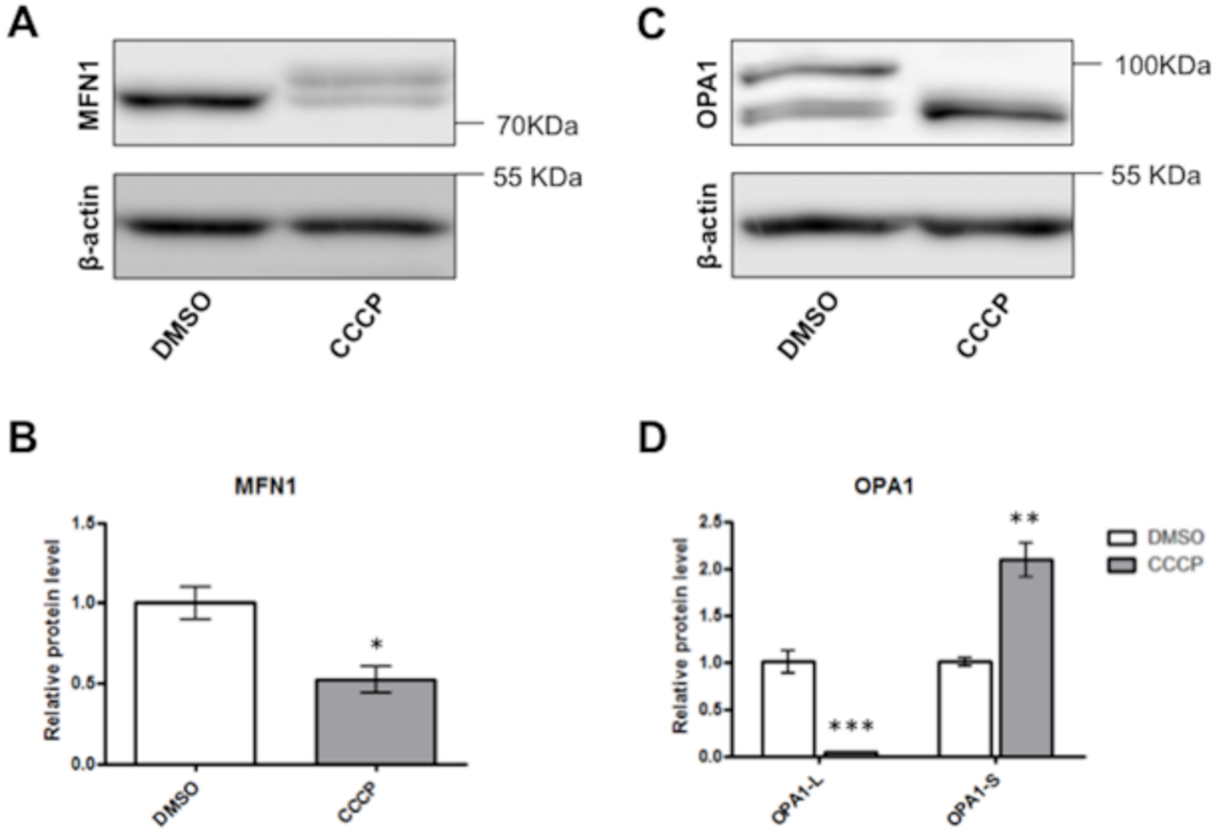


Figure 10. Mitochondrial fusion process is prevented in CCCP-treated SH-SY5Y cells. A: Representative Western blot image of MFN1 protein in SH-SY5Y after CCCP treatment. B: Relative protein level is expressed as mean ± SEM (N = 5). C: Representative Western blot image of OPA1 protein following CCCP treatment. D: Relative protein level is expressed as mean ± SEM (N = 5). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

To better explore the activation of the fission process, we decided to investigate the recruitment of dynamin-related protein 1 (DRP1) to mitochondria. To this end, both quantification and co-

localization analyses between ATP synthase β, a mitochondrial marker, and DRP1 proteins were performed. As shown in figure 11, DRP1 is recruited to mitochondria following CCCP treatment, indeed we obtained a positive co-localization result using both the quantification of the DRP1 signal intensity on the mitochondrial area and the Manders' overlap coefficient.

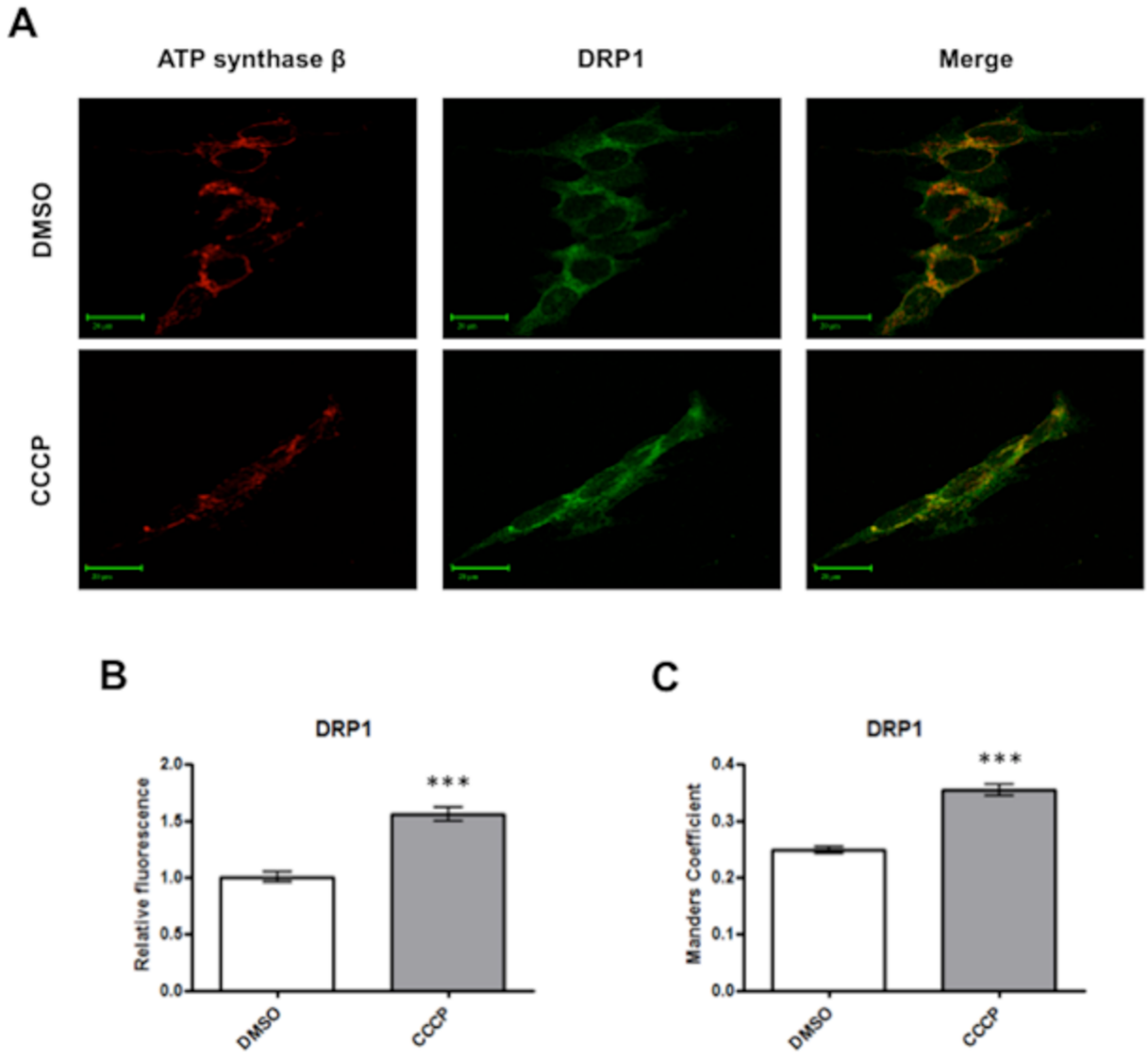


Figure 11. DRP1 protein is recruited to mitochondria after CCCP treatment in SH-SY5Y cell line. A: Representative immunofluorescence images of ATP synthase β (used as mitochondrial marker), DRP1 and their overlap (scale bar: 20 μm). B: DRP1 signal quantification in the mitochondrial area. C: Correlation between immunofluorescence probes using the Manders' coefficient. Data are expressed as mean ± SEM (N = 6). *** $p < 0.001$.

4.1.2. Mitophagy process was activated in CCCP-treated SH-SY5Y cells.

In order to investigate if the mitophagy process was activated after CCCP treatment, we decided to evaluate the expression level of three different mitochondrial proteins, i.e. voltage-dependent

anion channel 1 (VDAC1), voltage-dependent anion 2 (VDAC2) and cytochrome c oxidase 5β (COX5β). The expression levels of all these proteins were reduced by one half following the treatment with this protonophore (Figure 12 A-E). Also the mtDNA level was clearly decreased in SH-SY5Y cells treated with CCCP (Figure 12 F). As shown in figure 12 G, PINK1 protein was clearly visible after CCCP treatment, while it was completely undetectable in its own control, thus suggesting that exposure to this protonophore caused the activation of PINK1/Parkin mitophagy pathway.

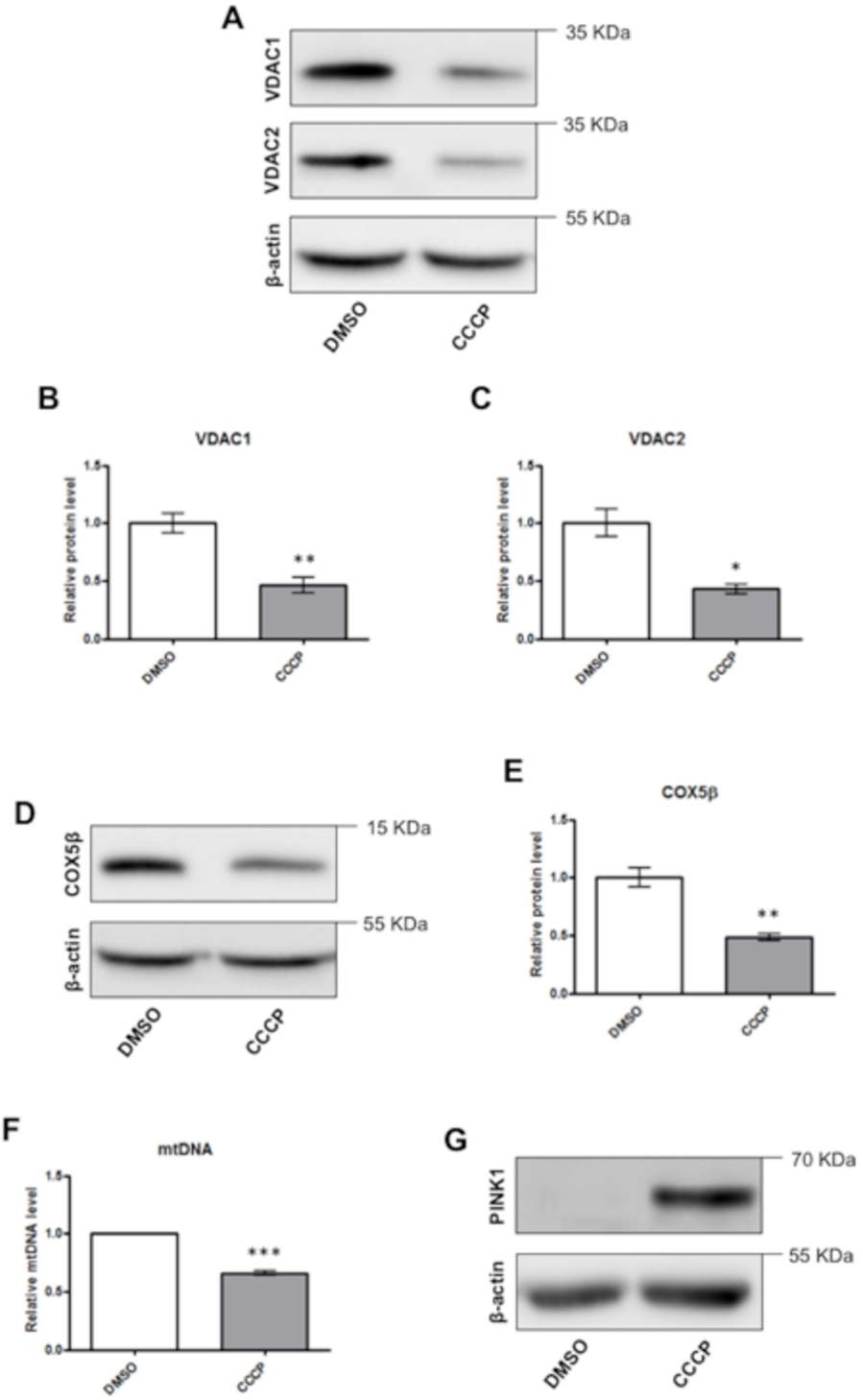


Figure 12. Pink1/Parkin mitophagy process is activated in CCCP-treated cells. A: Representative Western blot images of VDAC1 and VDAC2 proteins in CCCP-treated SH-SY5Y cells. B and C: Relative protein levels are expressed as mean ± SEM (N = 5). D: Representative Western blot image of COX5β protein in CCCP-treated SH-SY5Y cells. E: Relative protein level is expressed as mean ± SEM (N = 5). F: Relative mtDNA level is expressed as mean ± SEM (N = 4). G: Representative Western blot images of PINK1 protein in SH-SY5Y cells after CCCP treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.1.3. A visual proof of mitophagy activation in CCCP-treated cells

To better demonstrate the activation of the mitophagy process following CCCP treatment in SH-SY5Y cells, we decided to use the transmission electron microscopy technique in order to visualize the specific cellular damages caused by this protonophore and the selective engulfment of mitochondria by autophagosomes. CCCP treatment caused the dilatation of endoplasmic reticulum and the appearance of several vacuoles (Figure 13 A). Moreover, CCCP treatment determined the appearance of severely damaged and swollen mitochondria, and their fusion with autophagic vacuoles (Figure 13 B).

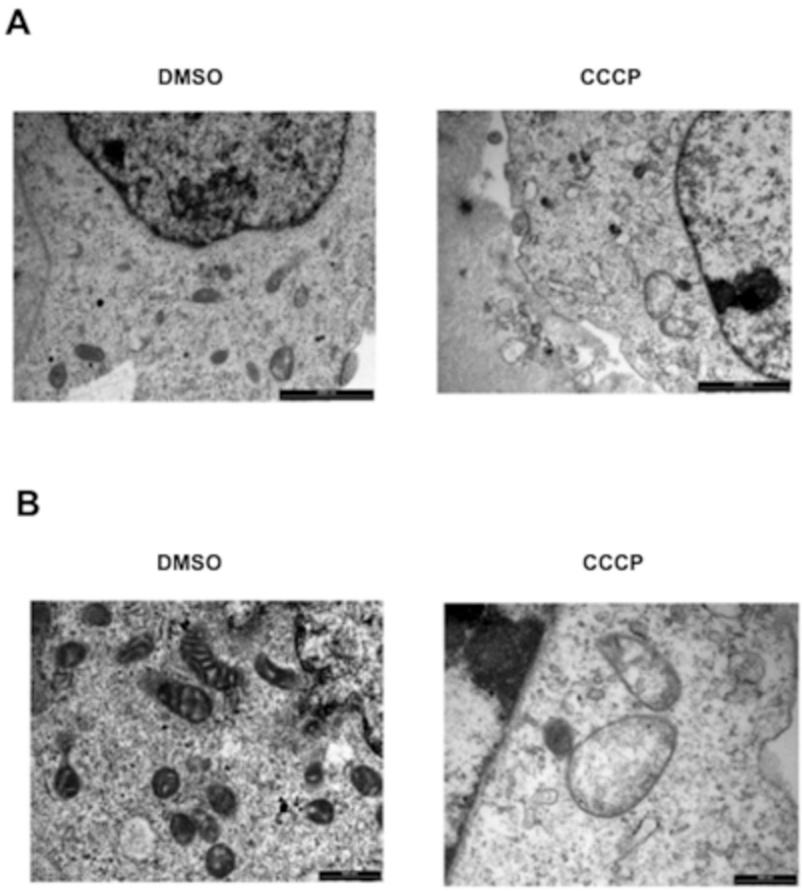


Figure 13. CCCP treatment cause several cellular and mitochondrial damage. A: Representative TEM images of SH-SY5Y cells after CCCP treatment (A: scale bar: 2000 nm; B: scale bar: 500 nm).

4.2. Mitochondrial dynamics and mitophagy process were altered in two different cellular models of PD

4.2.1. Fusion process was impaired in dopamine and MPP^+-treated cells

In order to verify if dopamine and MPP^+ molecules caused an alteration of the mitochondrial fusion machinery, we evaluated the expression levels of both MFN1 and OPA1 proteins in SH-SY5Y cell line. MFN1 protein level was not altered by these two different treatments (Figure 14 A and B). However, the expression of both long and short forms of OPA1 protein was drastically reduced in dopamine and MPP^+-treated cells. In particular, these treatments caused a decrease in the OPA1-L levels of about 50%, while dopamine treatment affected in a deeper way the expression of OPA1-S form if compared with those caused by the MPP^+ treatment (Figure 14 C and D).

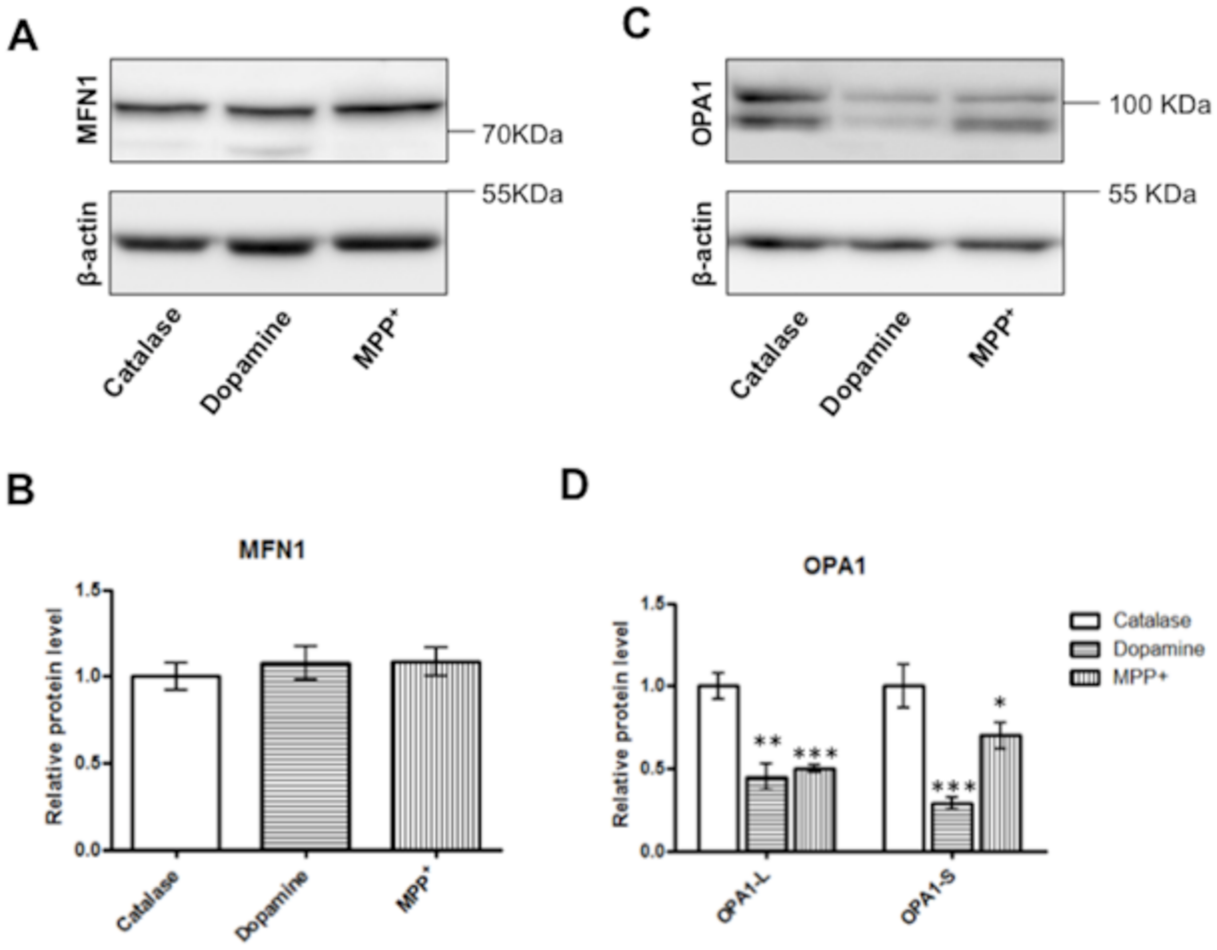


Figure 14. Fusion of the inner mitochondrial membranes is altered by both dopamine and MPP+ treatments. A: Representative Western blot image of MFN1 protein in dopamine and MPP^+-treated SH-SY5Y cells. B: Relative protein level is expressed as mean ± SEM (N = 5). C: Representative Western blot image of OPA1 protein in dopamine and MPP^+-treated SH-SY5Y cells. D: Relative protein level is expressed as mean ± SEM (N = 5). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.2.2. DRP1 was not recruited to mitochondria following both dopamine and MPP^+ treatments

To verify if the fission process was activated in dopamine and MPP^+-treated cells, we assessed the cellular localization of DRP1 protein. As shown in figure 15, these treatments did not cause the recruitment of DRP1 to mitochondria, using both the quantification of the DRP1 signal intensity on the mitochondrial area (Figure 15 B) and the Manders' overlap coefficient (Figure 15 C).

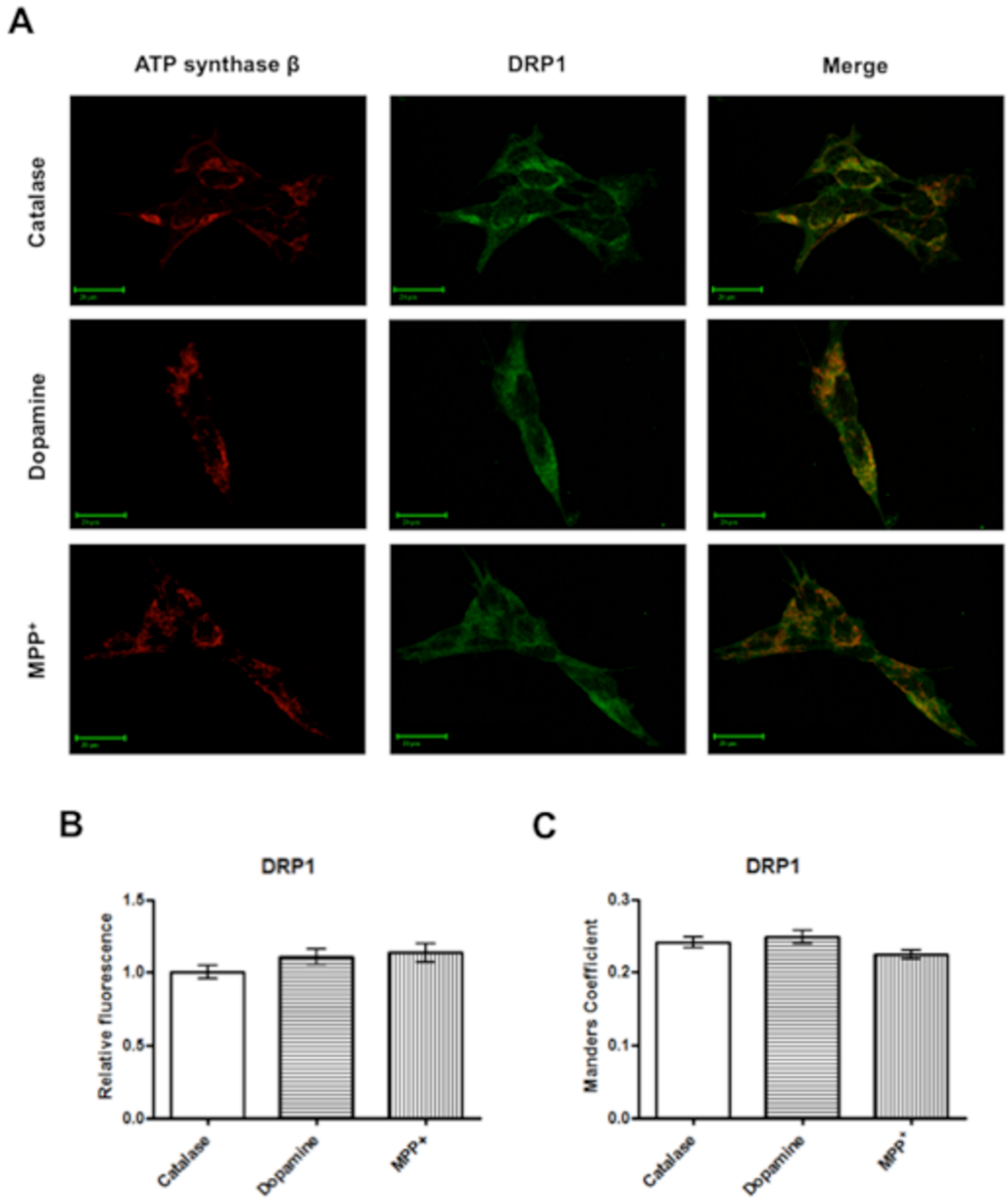


Figure 15. DRP1 protein is not recruited to mitochondria after dopamine and MPP^+ treatment in SH-SY5Y cell line. A: Representative immunofluorescence images of ATP synthase β, DRP1 and their overlap (scale bar: 20 µm). B: DRP1 signal quantification in the mitochondrial area. C: Correlation between immunofluorescence probes using the Manders' coefficient. Data are expressed as mean ± SEM (N = 6).

4.2.3. Dopamine and MPP^+ treatment caused two different types of mitochondrial alterations

To better investigate the mitochondrial alterations caused by these two different cellular treatments, we decided to evaluate the expression of VDAC1, VDAC2 and COX5β proteins. Dopamine treatment caused the reduction of VDAC1 of approximately 60% and a more prominent abatement of VDAC2 protein level. On the other hand, MPP^+ treatment induced an increase of approximately 50% of both anion channels in SH-SY5Y cells (Figure 16 A-C).

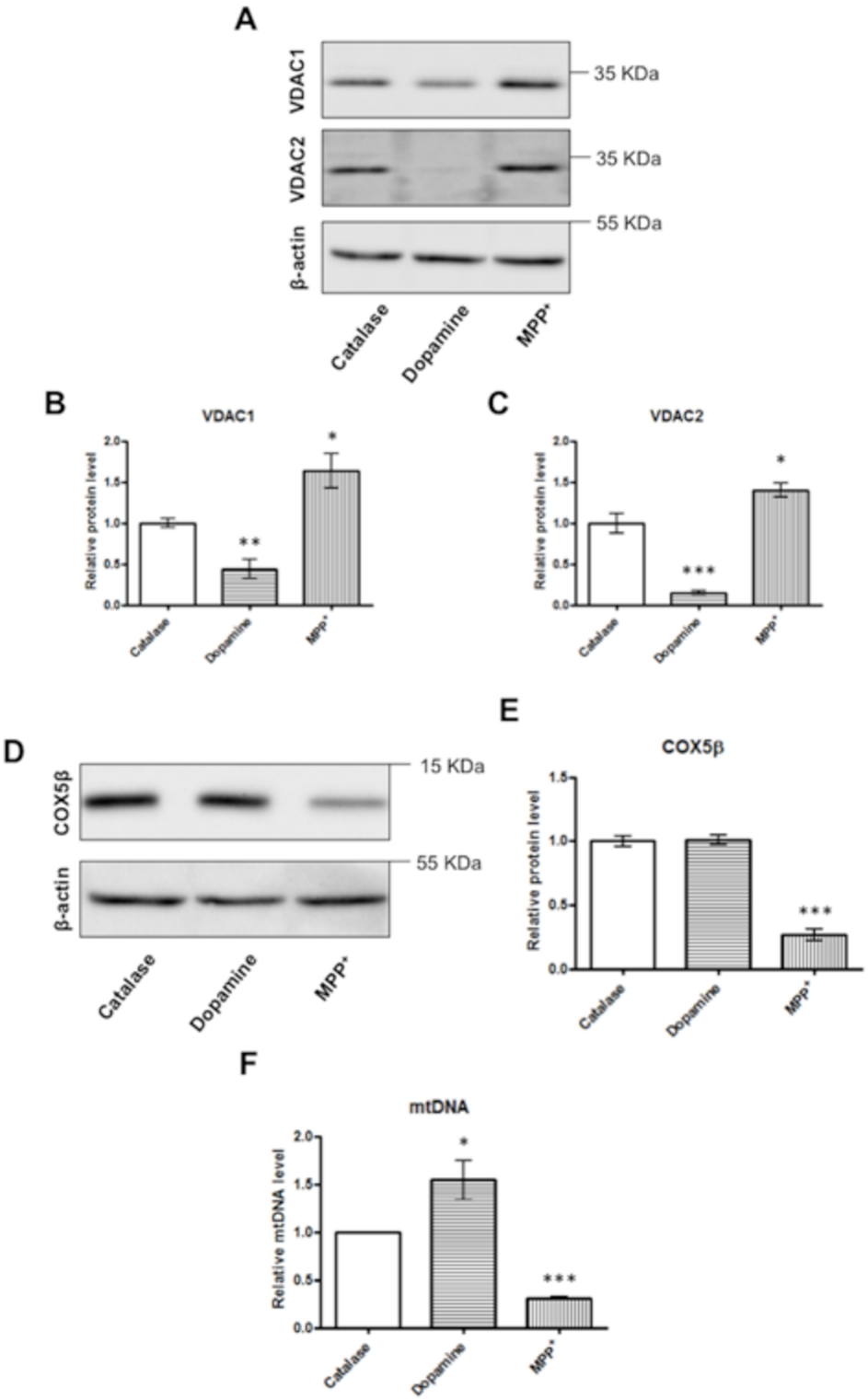


Figure 16. Dopamine and MPP+ treatments cause different mitochondrial alterations. A: Representative Western blot images of VDAC1 and VDAC2 proteins in dopamine and MPP^+-treated SH-SY5Y cells. B and C: Relative protein levels are expressed as mean ± SEM (N = 5). D: Representative Western blot image of COX5β protein in dopmanie and MPP^+-treated SH-SY5Y cells. E: Relative protein level is expressed as mean ± SEM (N = 5). F: Relative mtDNA level is expressed as mean ± SEM (N = 4). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

On the contrary, COX5β protein level was not altered after dopamine treatment, while the use of MPP^+ toxin caused a considerable reduction of this mitochondrial protein (Figure 16 D and E). Also the mtDNA level showed an opposite behavior in dopamine and MPP^+-treated cells. Indeed, dopamine treatment determined an increase of approximately one half of mtDNA level, while MPP^+ treatment caused its decrease of about 70% (Figure 16 F).

4.2.4. Mitophagy process was not activated following both dopamine and MPP^+ treatments in SH-SY5Y cell line

In order to verify if the mitophagy process was impaired by dopamine and MPP^+ treatments, we evaluated the PINK1 protein level. As shown in figure 17, PINK1 protein was not detectable in SH-SY5Y cells treated with dopamine or MPP^+, while CCCP treatment, used as mitophagy positive control, determined the accumulation of this protein, as demonstrated above.

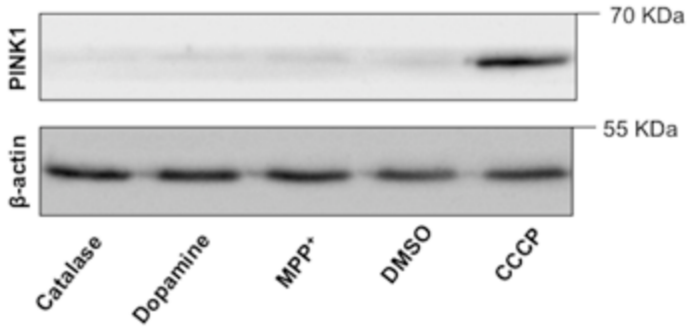


Figure 17. Dopamine and MPP^+ treatments do not determined PINK1 accumulation. Representative Western blot image of PINK1 protein in dopamine, MPP^+ and CCCP-treated SH-SY5Y cells.

4.2.5. Morphological analysis explained the different molecular behaviors of dopamine and MPP^+-treated cells

To get a more detailed vision of the cellular and mitochondrial damages caused by dopamine and MPP^+ molecules, we used the transmission electron microscopy technique to perform the morphological analysis. Cells treated with both MPP^+ and dopamine showed abundant rough endoplasmic reticulum and Golgi cysternae, nuclei with dispersed chromatin and nucleoli (Figure 18A). However, at higher magnification, in SH-SY5Y cells treated with MPP^+, mitochondria appeared emptied, due to a marked disruption of cristae, while dopamine treatment determined the appearance of dense mitochondria, showing fusion of cristae and often containing markedly electron dense deposits (Figure 18B).

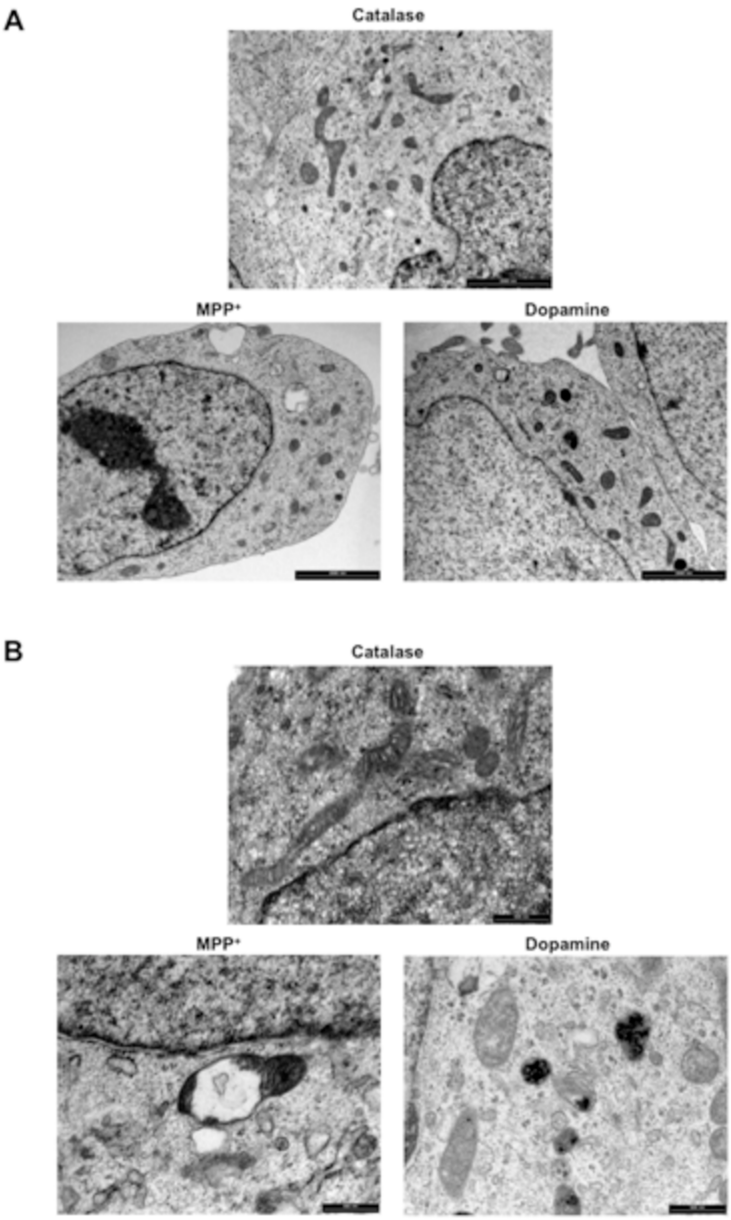


Figure 18. MPP^+ and dopamine treatments cause different morphological changes at mitochondrial level. Representative transmission electron microscopy images of control and treated cells. A: scale bar: 2000 nm; B: scale bar: 500 nm.

4.3. Mitochondrial dynamics and mitophagy were altered in Substantia nigra of sporadic PD patients

4.3.1. Substantia nigra of sporadic PD patients showed a mitochondrial fusion impairment similar to that of dopamine or MPP^+-treated cells

We decided to assess the impairment of the fusion machinery also in Subtantia nigra of PD patients. Also in this case, both MFN1 and OPA1 proteins were considered to characterize the alterations that occur in this mitochondrial process. No difference was observed in MFN1 protein level between sporadic PD patients and control subjects (Figure 19 A and B). Regarding OPA1 protein, both its long and short forms were reduced in Substantia nigra of PD patients (Figure 19 C and D), thus resembling the results obtained for dopamine and MPP^+- treated SH-SY5Y cells.

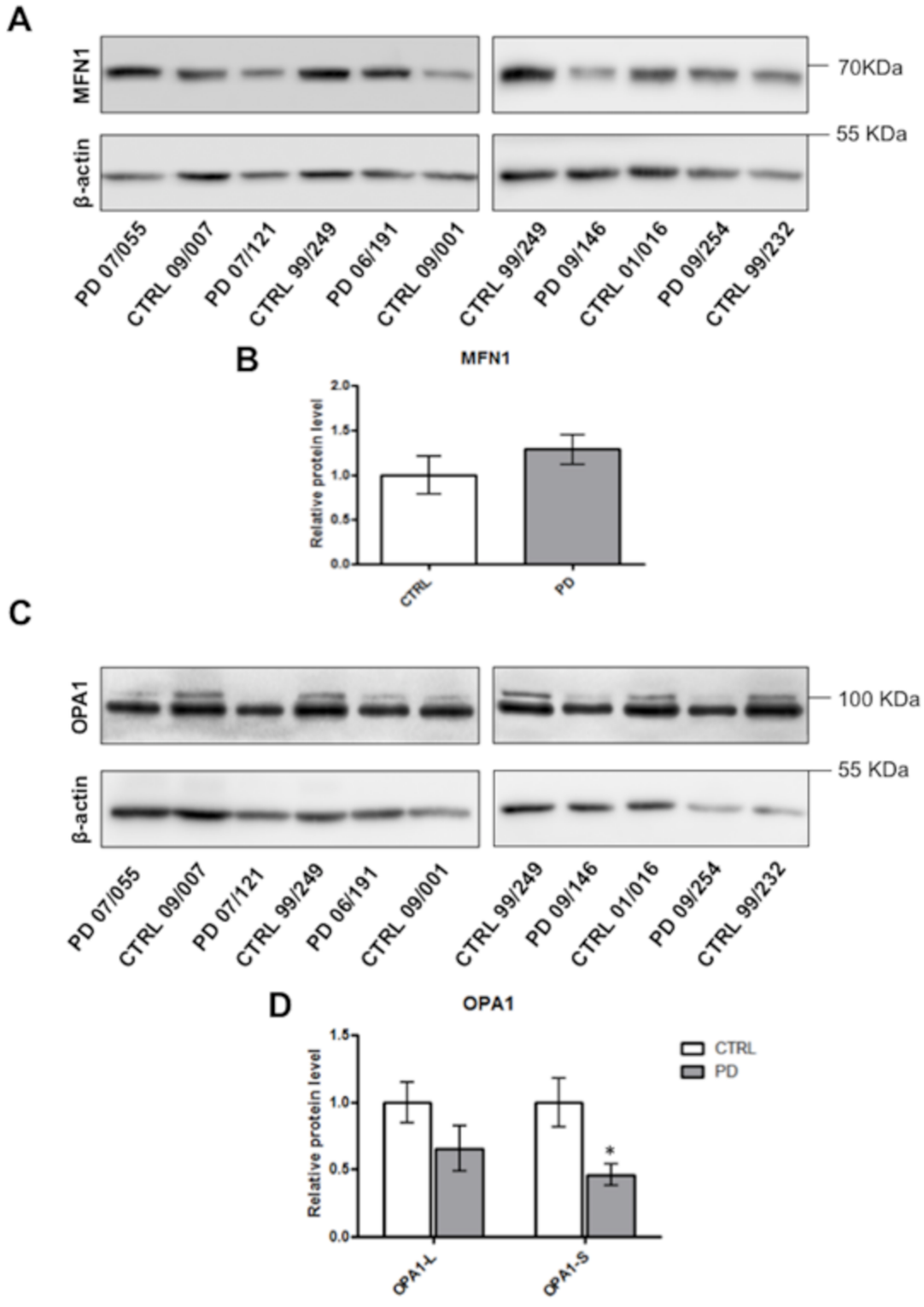


Figure 19. Fusion of the inner mitochondrial membranes is altered in Substantia nigra of sporadic PD patients. A: Representative Western blot image of MFN1 protein in control subjects and PD patients. B: Relative protein level is expressed as mean ± SEM (n = 5 CTRL subjects, n = 5 PD patients). C: Representative Western blot image of OPA1 protein in control subjects and PD patients. D: Relative protein level is expressed as mean ± SEM (n = 5 CTRL subjects, n = 5 PD patients). * $p < 0.05$.

4.3.2. The mitochondrial damage that occur in Substantia nigra of PD patients was better recapitulated by dopamine-treated cells

To better define the mitochondrial alterations that characterize sporadic PD patients, we analyzed VDACs, COX5β and PINK1 proteins levels. As shown in figure 20, both VDAC1 and VDAC2 proteins were reduced by one half in Substantia nigra of PD patients.

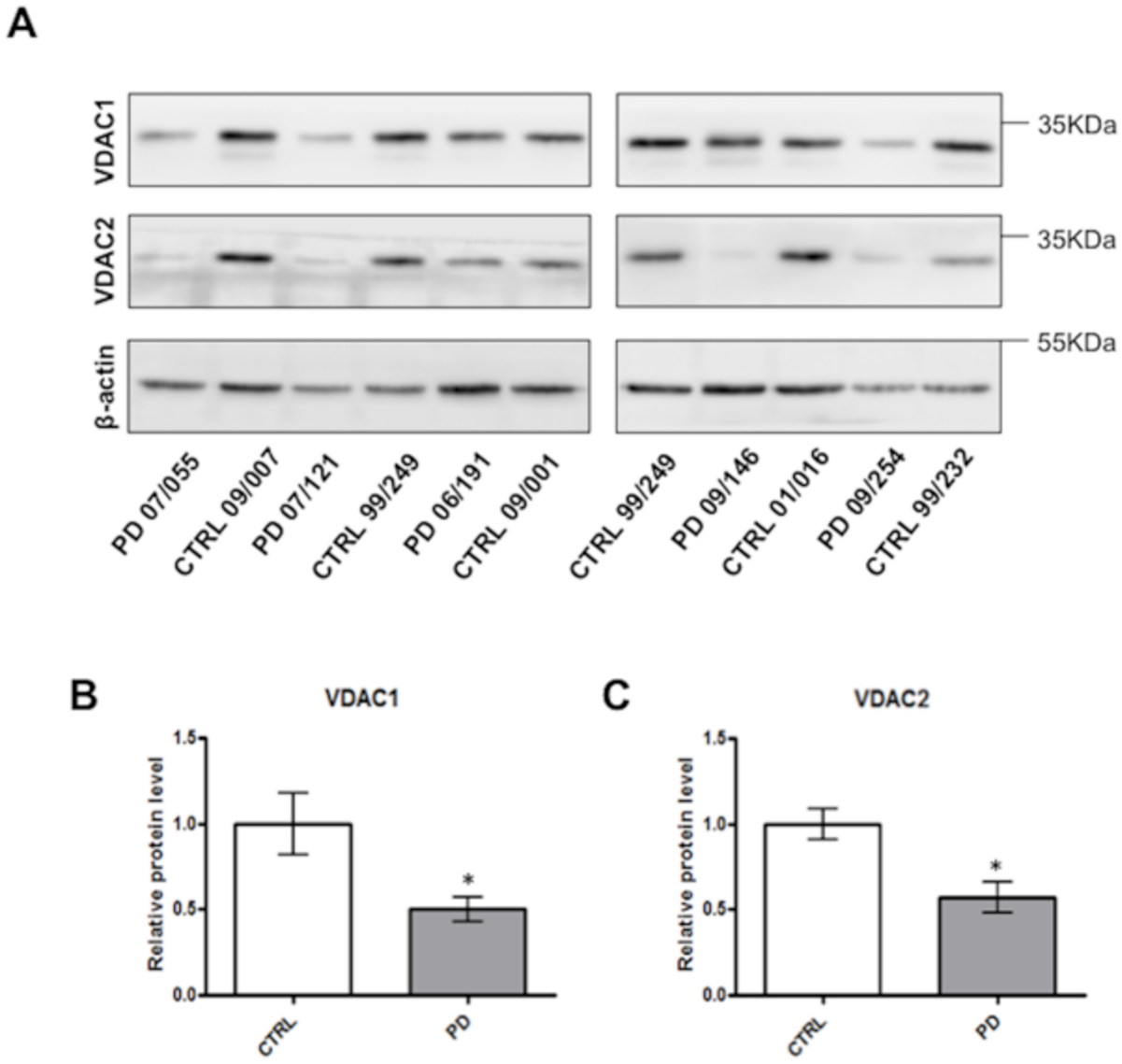


Figure 20. VDACs protein levels are reduced in Substantia Nigra of PD patients. A: Representative Western blot images of VDAC1 and VDAC2 proteins in human samples. B and C: Relative protein levels are expressed as mean ± SEM (n = 5 CTRL subjects, n = 5 PD patients). * $p < 0.05$.

On the other hand, no difference was observed in COX5β protein level when the Substantia nigra of sporadic PD patients was compared to that of control subjects (Figure 21). Moreover, no PINK1 accumulation was observed in Substantia nigra from PD patients (data not shown).

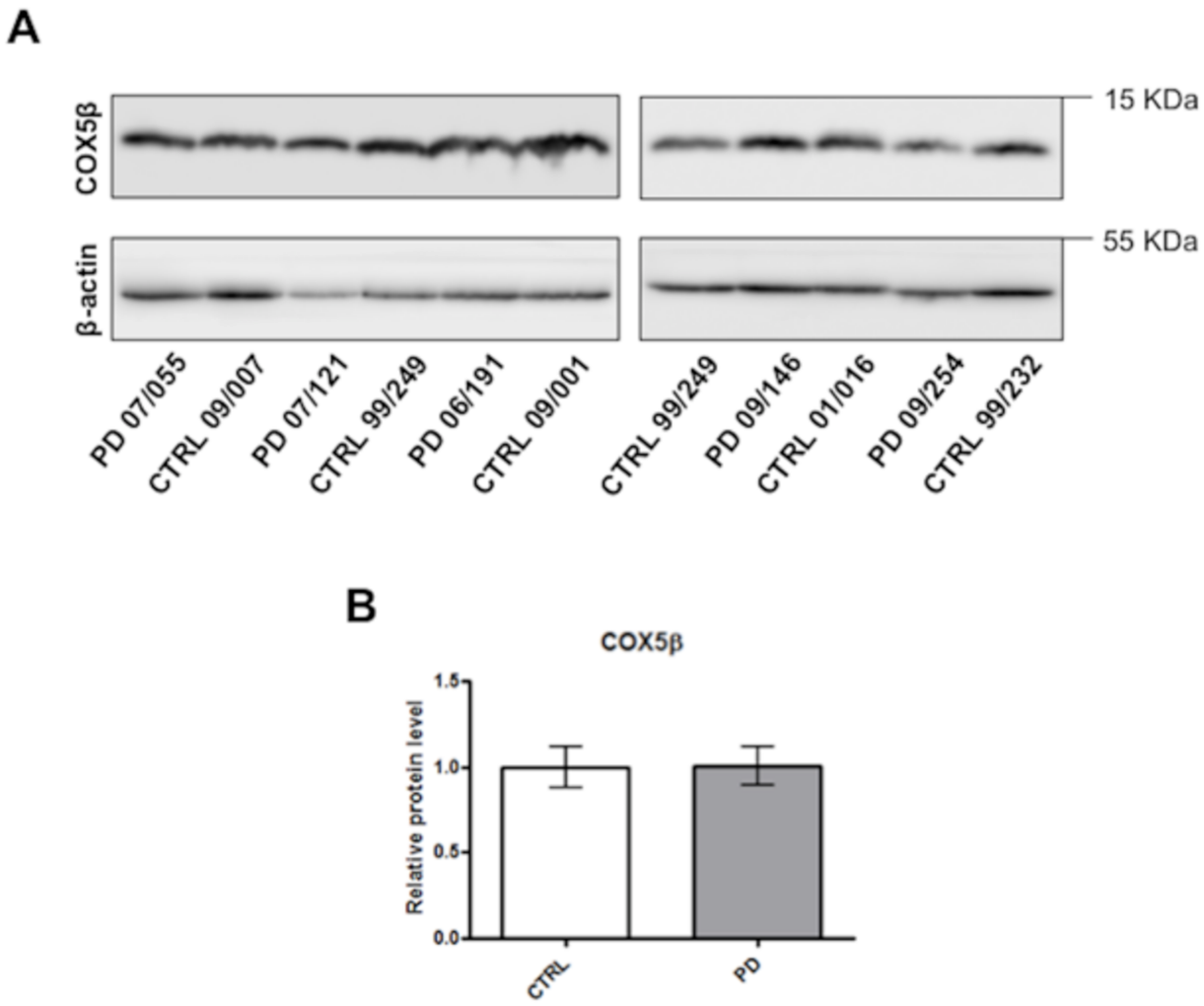


Figure 21. COX5β protein is not altered in Substantia Nigra of PD patients. A: Representative Western blot images of COX5β protein in human samples. B: Relative protein levels are expressed as mean ± SEM (n = 5 CTRL subjects, n = 5 PD patients).

These results are in line with those obtained in SH-SY5Y cells treated with dopamine, thus suggesting that the scenario observed in Substantia nigra PD specimens is better explained by the alteration of dopamine homeostasis rather than the inhibition of complex I caused by MPP^+ toxin.

4.3.3. Mitochondrial morphology was altered in sporadic PD patients

To get a clearer and a more detailed vision of the mitochondrial alterations that occur in sporadic PD patients, the morphological analysis was carried out by light and transmission electron microscopy. First, a section of paraffin-embedded Substantia nigra tissues from two sporadic PD patients and from two age-matched controls were stained with hematoxylin and eosin and observed with a light microscope, in order to have a generic tissue characterization and to identify the best area to study at TEM (Figure 22 A). This first morphological analysis showed

that the Substantia nigra of control subjects had more neurons and abundant cytoplasmic pigments. Instead, Substantia nigra of PD patients had fewer neurons, characterized by a smaller size, by the presence of fewer pigments and of Lewy bodies, as expected (Figure 22 A).

TEM analysis demonstrated that PD neurons showed irregular nuclei and just few melanin bodies, if compared to control neurons (Figure 22 B). Moreover, mitochondria of sporadic PD patients appeared more swollen and irregular and were also characterized by a cristae derangement and by the presence of some small electron dense deposits (Figure 22 C and Supplementary Figure 1).

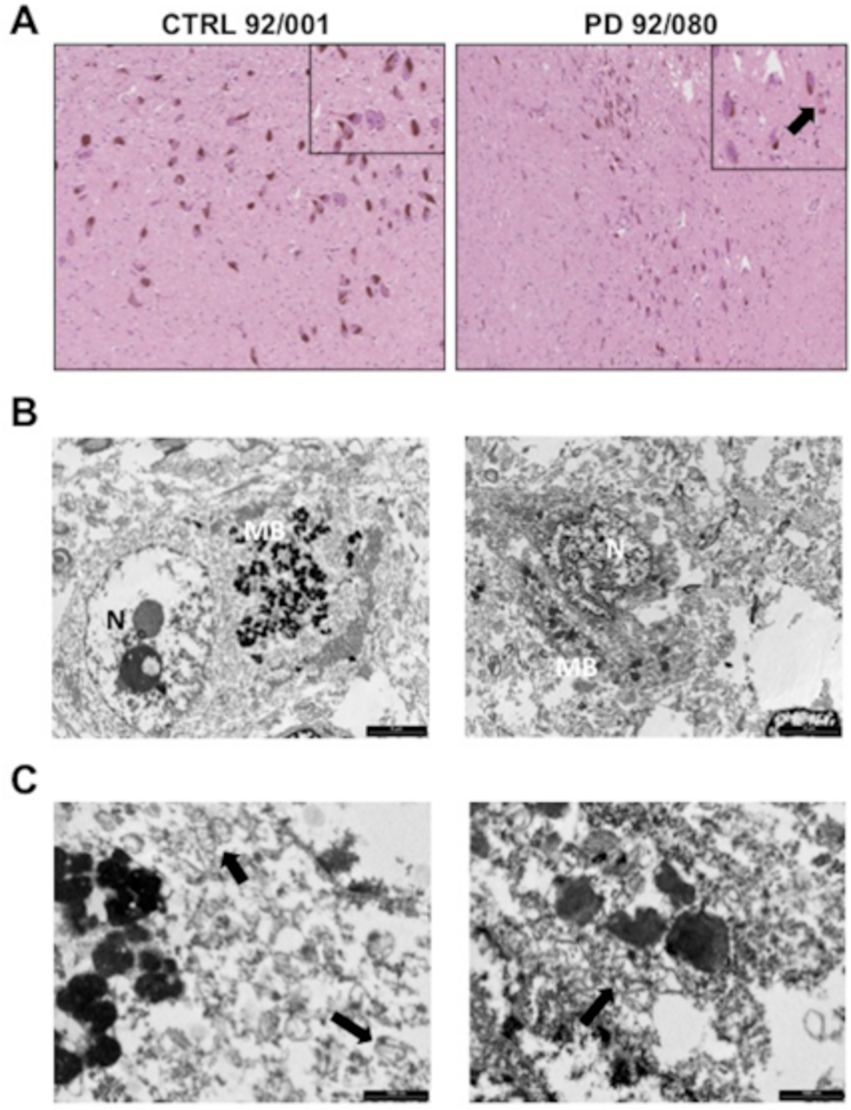


Figure 22. Morphological analysis of one control subject and one sporadic PD patient. A: Representative light microscopy images of Substantia nigra of one control subject and one PD patient stained with hematoxylin and eosin (magnification: 40X and inset 200X). A Lewy body is visible in the cytoplasm of a PD neuron (arrow). B: Representative transmission electron microscopy images of Substantia nigra neurons of one control subject and one PD patient (scale bar: 5 μm). N: nuclei; MB: melanin bodies. C: Representative transmission electron microscopy images of the same Substantia nigra neurons taken at higher magnification (scale bar: 1 μm). Arrows indicate mitochondria of both control subject and PD patient.

4.4. Focus on *PARK2*-mutated PD patients

4.4.1. Mitochondrial network was not altered in *PARK2*-mutated PD patients

In order to verify if the loss of parkin protein had an impact on the morphology of the mitochondrial network, primary skin fibroblast cells from 3 *PARK2*-mutated patients and 3 control subjects were labeled with ATP synthase β antibody (Figure 23). In this way, it was possible to acquire z-stacks of these cells through confocal microscopy.

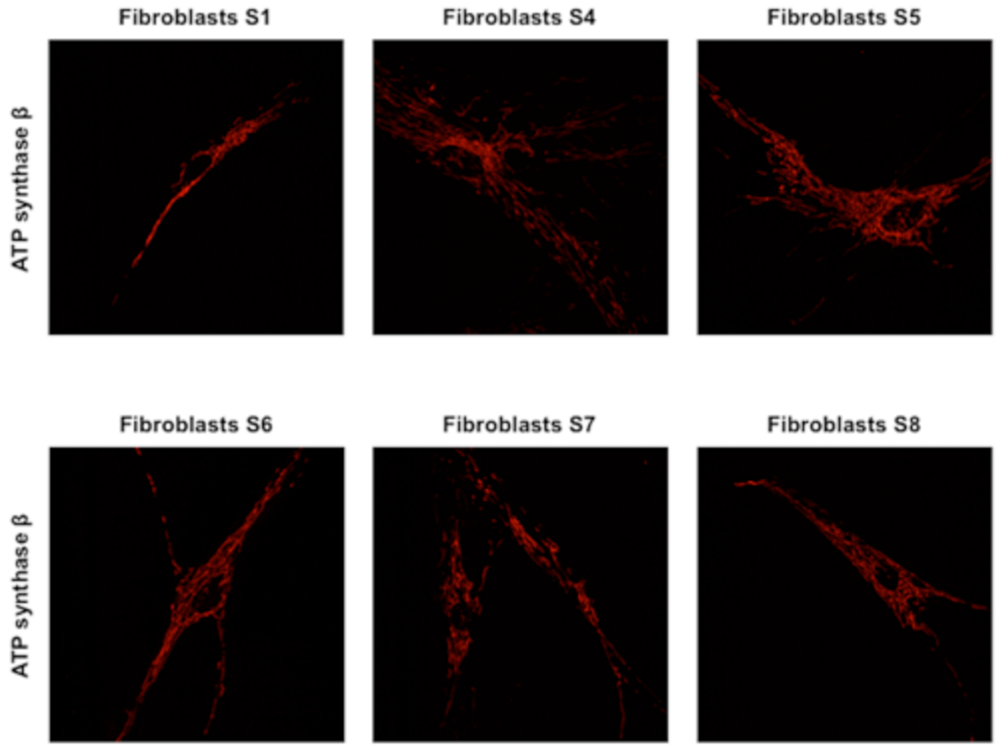


Figure 23. Representative immunofluorescence images of primary skin fibroblasts cells from 3 *PARK2*-mutated patients (Fibroblasts S1, S4 and S5) and 3 control subjects (Fibroblasts S6, S7 and S8) labeled with ATP synthase β antibody.

As shown in Figure 23, fibroblast cells from both *PARK2*-mutated patients and control subjects showed a filamentous mitochondrial network, with mitochondria distributed all over the soma.

To confirm the results of this first qualitative analysis, several morphological parameters were evaluated in order to quantitatively define the morphology of these mitochondrial networks.

Among all parameters (Supplementary Figure 2), the distribution density of circularity, roundness and solidity were taken into account (Figure 24). Indeed, as previously demonstrated, these parameters better describe the mitochondrial network dynamics alterations (Bondi et al., 2016).

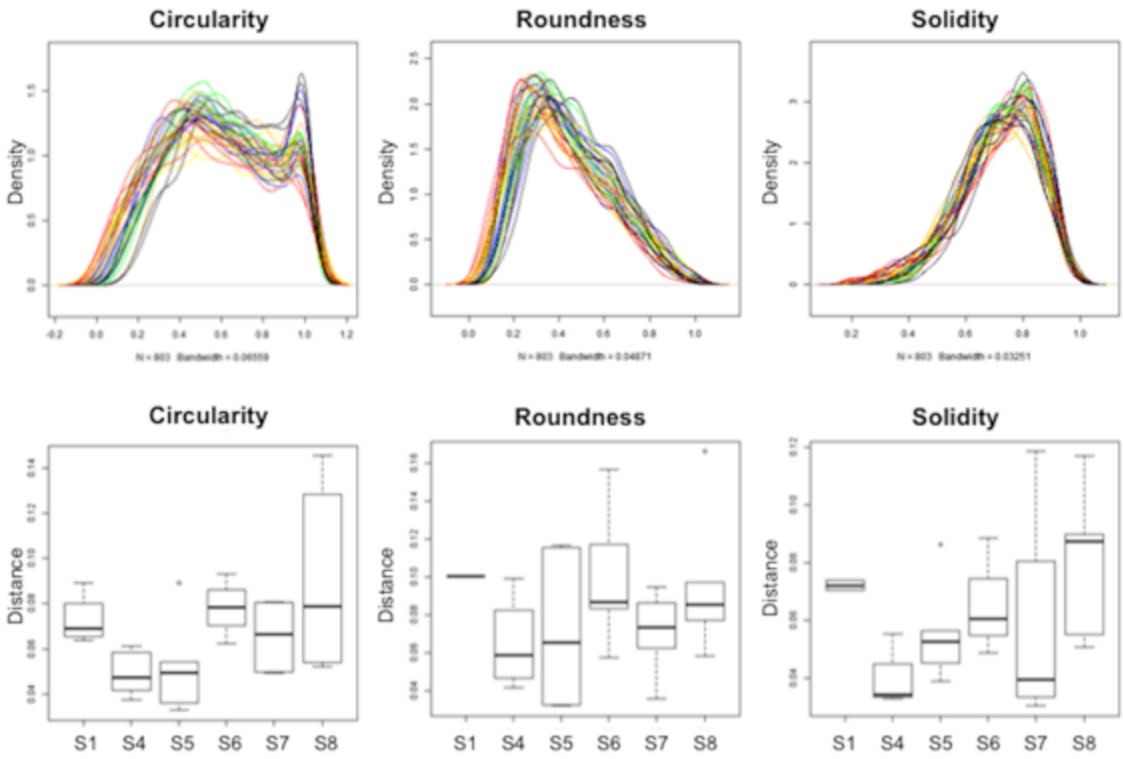


Figure 24. Analysis of mitochondrial network morphology. Distribution density (for all particles analyzed in six fovs from each subject) and Kolmogorov-Smirnov distances of circularity, roundness and solidity. *PARK2*-mutated patients (Fibroblasts S1, S4 and S5); control subjects (Fibroblasts S6, S7 and S8). Yellow: S1; Green: S4; Blue: S5; Red: S6; Orange: S7; Black: S8.

No significant difference was observed for these three parameters between the distribution density of each *PARK2*-mutated subject and the distribution densities of all controls. This preliminary result suggested that the loss of parkin protein did not affect the morphology of the mitochondrial network.

4.4.2. Mitochondrial fraction enrichment

In order to analyze the mitochondrial proteome and interactome alterations that occur in *PARK2*-mutated patients, primary skin fibroblast cells were used to isolate mitochondria from both control subjects and *PARK2*-mutated patients with a commercial kit based on surfactants. The effectiveness of the mitochondrial enrichment procedure was evaluated by Western blotting. Figure 25 shows that both mitochondrial markers citrate synthase (CS) and voltage-dependent anion channel 1 (VDAC1) were noticeably enriched in the mitochondrial fraction and almost undetectable in the nuclear fraction. Conversely, the nuclear marker histone H3 was clearly detectable only in the nuclear fraction.

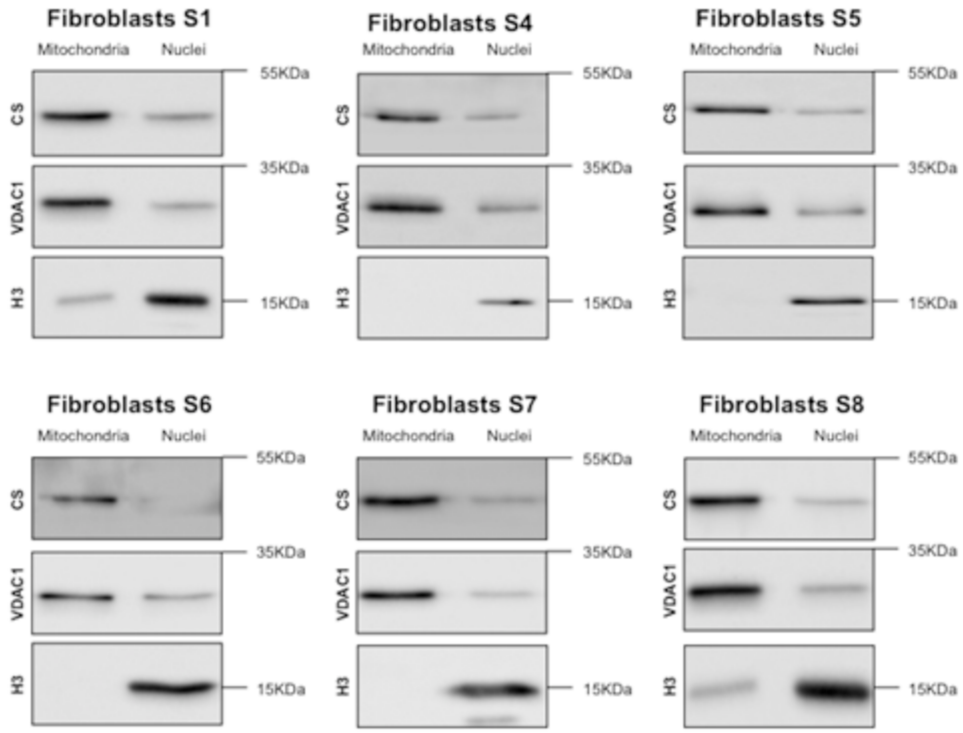


Figure 25. Mitochondrial markers are noticeably enriched in the mitochondrial pellet, while histone H3 is clearly visible only in the nuclear fraction. Representative Western blot images of CS, VDAC1 and H3 proteins. *PARK2*-mutated patients (Fibroblasts S1, S4 and S5); control subjects (Fibroblasts S6, S7 and S8).

Mitochondrial pellets obtained from primary skin fibroblast cells were then subjected to quantitative shotgun proteomics or size-exclusion chromatography.

4.4.3. Mitochondrial proteome alterations of *PARK2*-mutated patients

In order to highlight the proteome alterations that occur in the mitochondrial-enriched fractions obtained from *PARK2*-mutated patients, we decided to use a quantitative shotgun proteomics approach. This global and unbiased technique allows us to identify around 300 proteins in each mitochondrial sample. However the number of proteins that significantly change between the control subjects and the *PARK2*-mutated patients are very low due to the small size of the two groups. To fill this gap, we are recruiting other subjects in order to find a greater number of proteins that are affected by the loss of function of Parkin.

Nevertheless, our preliminary results suggest that these patients are characterized by an impairment of the mitochondrial respiratory chain, the mitochondrial quality control and the maintenance of the mitochondrial network.

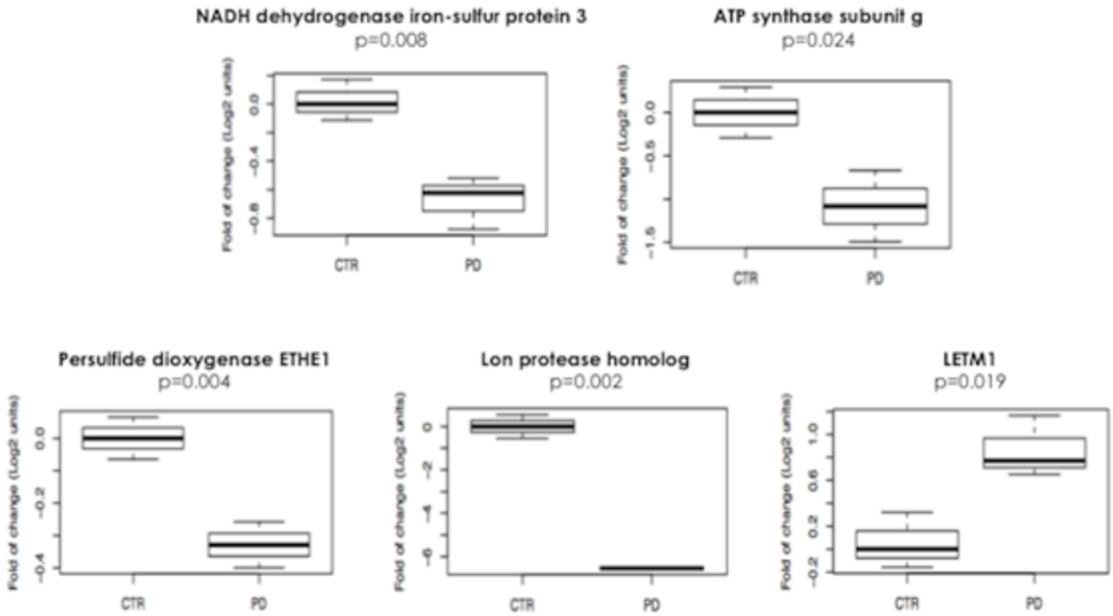


Figure 26. Quantitative shotgun proteomics data obtained from mitochondrial-enriched fractions of three control subjects and of three *PARK2*-mutated patients. These preliminary data suggest that the loss of function of Parkin determine an impairment of the mitochondrial respiratory chain, the mitochondrial quality control and the maintenance of the mitochondrial network.

Figure 26 shows 5 proteins that quantitatively or qualitatively change between the two groups. In particular, the expression of two proteins of the respiratory chain (i.e. NADH dehydrogenase iron-sulfur protein 3 and ATP synthase subunit g) is reduced in PARK2-mutated patients. Likewise, the expression of persulfide dioxygenase ETHE1, a protein involved in the mitochondrial quality control, is decreased in these PD subjects. Moreover, Lon protease, another important mitochondrial protein involved in the quality control mechanism, qualitatively changes between the two groups. Eventually, the expression of LETM1 is increased in *PARK2* mutated patients. This protein plays a central role in the maintenance of the mitochondrial network morphology.

4.4.4. Preliminary interactomics results

So far, LC-MS/MS analysis was not made for the fractions obtained from the three control subjects. For this reason, it was carried out only a preliminary interactome analysis of the fractions obtained from the three *PARK2*-mutated patients. This chapter shows the procedure used to analyze the protein-protein interactions and the result obtained considering only the proteins present in the same fraction of all the three *PARK2*-mutated patients.

Figure 27 shows a protein network obtained from fraction 58 using IntAct database. In this fraction, 122 proteins could interact and form macromolecular complexes involved in a specific biological process. Among these nodes, only 50 proteins are strictly mitochondrial (green nodes) according to Gene Ontology Consortium database.

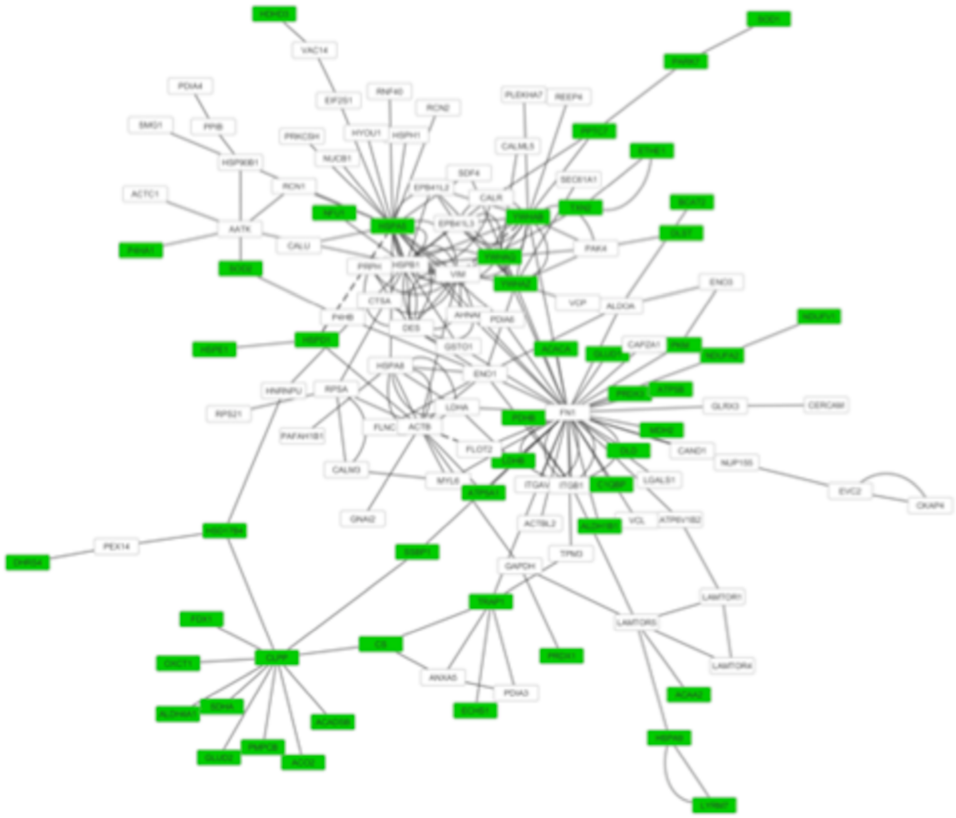


Figure 27. Protein-protein interaction network obtained from fraction 58 of the three *PARK2*-mutated patients. Among these 122 proteins, only 50 proteins (green nodes) are strictly mitochondrial. The other 72 white nodes are proteins that could interact with at least one of the 50 mitochondrial proteins.

This first network was used to identify the protein complexes involved in a specific biological process. As example, Figure 28 show two smaller protein networks obtained using Gene Ontology Consortium database. One of these networks is involved in the redox process (Figure 28 A), while the second is involved in the positive regulation of apoptotic signaling pathway and of mitochondrial membrane permeability (Figure 28 B).

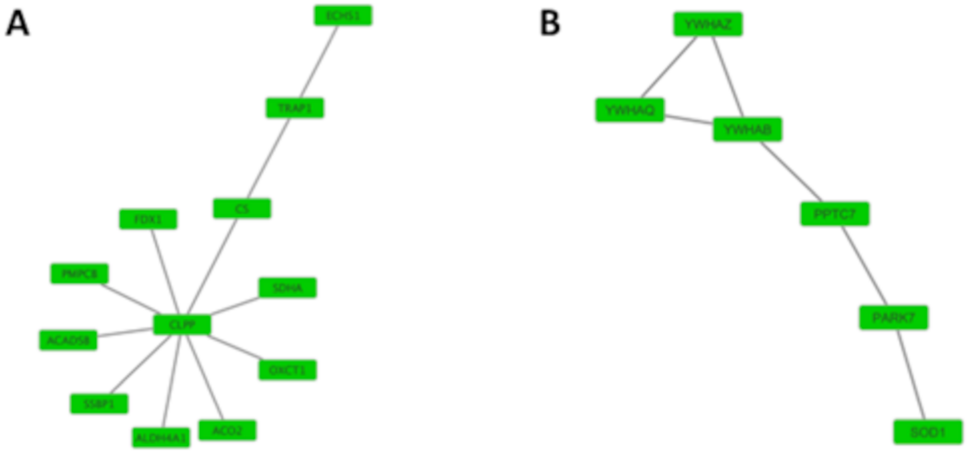


Figure 28. Example of protein networks obtained from fraction 58 that are involved in a specific biological process. A: Protein network involved in the redox process. B: Protein network involved in the positive regulation of apoptotic signaling pathway and of mitochondrial membrane permeability.

The protein network in Figure 28 B is characterized by the presence of DJ1, a protein that is encode by the gene *PARK7* and whose mutations cause familial forms of PD.

Eventually, figure 29 shows a protein network obtained from fraction 51 using IntAct database. In this fraction, 72 proteins could interact and form macromolecular complexes involved in a specific biological process. Among these nodes, only 19 proteins are strictly mitochondrial (green nodes) according to Gene Ontology Consortium database.

Figure 29. Protein-protein interaction network obtained from fraction 51 of the three *PARK2*-mutated patients. Among these 72 proteins, only 19 proteins (green nodes) are strictly mitochondrial. The other 53 white nodes are proteins that could interact with at least one of the 19 mitochondrial proteins.

This network then was used to identify the protein complexes involved in a specific biological process. As example, Figure 30 shows one smaller protein networks obtained using Gene Ontology Consortium database. This network, characterized by the presence of LETM1, is

involved in the positive regulation of mitochondrial membrane organization, cristae formation, and maintenance of mitochondrial tubular organization.

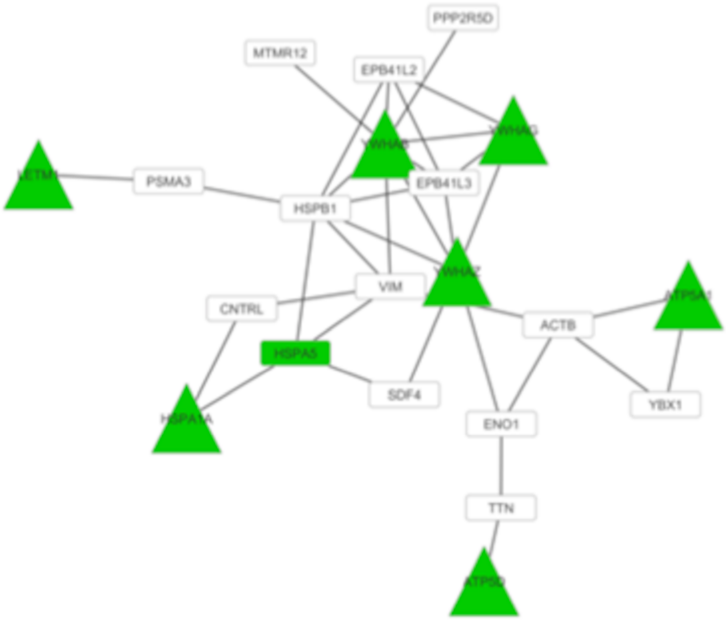


Figure 30. Example of protein network obtained from fraction 51 that are involved in a specific biological process. This protein network is involved in in the positive regulation of mitochondrial membrane organization, cristae formation, and maintenance of mitochondrial tubular organization.

5. Discussion

5.1. Impairment of mitochondrial dynamics and mitophagy process in PD

Although the cause of PD is currently unknown, strong evidences suggest that a complex interplay between environmental factors and genetic susceptibility is involved in the development of the sporadic form of the disease (Schapira and Tolosa, 2010; Schapira and Jenner, 2011). In this intricate pathological landscape, many of the molecular mechanisms implicated in PD pathogenesis converge on mitochondria, resulting in their dysfunction (Schapira and Jenner, 2011; Subramaniam and Chesselet, 2013; Dupuis, 2014). Moreover, the missed mitochondrial disposal causes the accumulation of these damaged organelles (Tal et al., 2009; Egan et al., 2011) that can lead to cell death (Green and Kroemer, 2004), and participate in disease pathogenesis (Wallace, 1999; Chan, 2006b; Itoh et al., 2013). Mitochondrial complex I dysfunction has been indicated as a key player in the development of PD (Schapira et al., 1990; Papa and De Rasmo, 2013). For this reason, MPTP toxin and its metabolite MPP^+ are widely used in research to reproduce parkinsonian symptoms and mechanisms in animal and cellular models, respectively (Dauer and Przedborski, 2003; Perier et al., 2005; Perier et al., 2007; Zhang et al., 2010; Burte et al., 2011). Nevertheless, the parkinsonism induced by this compound results from an acute toxic insult and differs from the slow and progressive disease process that characterize sporadic PD. In this context, altered dopamine homeostasis might be a key factor in the early steps of PD pathogenesis (Alberio et al., 2012; Bisaglia et al., 2013; Herrera et al., 2017). Indeed, the oxidation of dopamine at neutral pH causes the formation of endogenous toxins, which contribute to mitochondrial dysfunction (e.g., inhibition of complex I) and oxidative damage (Hastings, 2009; Segura-Aguilar et al., 2014).

Given the importance of mitochondrial dysfunctions in PD, we decided to investigate the permanence of depolarized mitochondria due to mitophagy impairment as the possible trigger of the pathogenesis. To this purpose, we characterized the mitochondrial alterations in Substantia nigra specimens from PD patients, comparing them to what observed by inducing mitochondrial impairment in human neuroblastoma SH-SY5Y cells using dopamine or MPP^+. As reference, we have always kept the SH-SY5Y cells treated with CCCP, used as mitophagy positive control.

First, we focused our attention on mitochondrial fusion and fission defects. Several studies demonstrated that fusion process is inhibited upon mitochondrial depolarization and mitophagy induction. Indeed, MFN1 is ubiquitinated and consequently degraded by the proteasome, thus preventing the fusion of the outer mitochondrial membranes (Gegg et al., 2010; Karbowski and Youle, 2011; Rakovic et al., 2011; Bondi et al., 2016). Moreover, the induction of the mitophagy process determines the activation of the zinc metalloprotease OMA1, which in turn causes the accumulation of the short form of OPA1, thus inhibiting the fusion of the inner mitochondrial

membranes (Head et al., 2009). These results were perfectly reproduced by CCCP treatment in SH-SY5Y cells. However, SH-SY5Y cells treated with dopamine or MPP^+ and Substantia nigra of PD patients did not show significant alterations in MFN1 protein levels, thus suggesting that fusion of the OMM is not blocked. Moreover, both long and short forms of OPA1 dramatically decreased after dopamine or MPP^+ treatment in SH-SY5Y cells. In PD patients, only OPA1-S was significantly reduced, although the long isoform of OPA1 also showed the same tendency. These results suggest an impairment of the IMM fusion in a cellular model of impaired dopamine homeostasis, in a cellular model of complex I inhibition, and in Substantia nigra specimens from PD patients. This molecular event has been linked to the loss of mitochondrial membrane integrity and in turn to the release of cytochrome *c* and apoptotic cell death (Olichon et al., 2003; Ramonet et al., 2013).

Given the important role of mitochondrial fission in the mitophagy process (Otera and Mihara, 2011), we investigated the behavior of DRP1 in SH-SY5Y cells treated with CCCP, dopamine or MPP^+. As a result, we found that DRP1 co-localized with mitochondria only after CCCP treatment. Therefore, either the perturbation of dopamine homeostasis or the inhibition of complex I caused the impairment of the fission machinery. Unfortunately, we could not verify this molecular event in the Substantia nigra tissues of sporadic PD patients since it is not possible to perform the co-localization analysis on whole neurons.

In order to better describe the mitochondrial alterations occurring in the two cellular models of PD and in Substantia nigra specimens from PD patients, we decided to measure the levels of VDACs. Actually, VDACs are involved in several mitochondrial processes, such as energetic metabolism, formation of the permeability transition pore, regulation of calcium homeostasis, and mitochondria-mediated apoptosis (Shoshan-Barmatz et al., 2008; Naghdi and Hajnóczky, 2016). Furthermore, it has been suggested that VDACs play an important role in recruiting Parkin to damaged mitochondria in order to eliminate them through mitophagy (Geisler et al., 2010; Narendra et al., 2010b; Sun et al., 2012). Next, we decided to analyze the levels of COX5β protein and the mtDNA content, two mitochondrial markers normally used to have an estimate of the number of these organelles. Eventually, we measured the accumulation of PINK1 protein in order to verify the impairment of the mitophagy process. As expected, all the mitochondrial proteins analyzed (i.e. VDAC1, VDAC2 and COX5β) were reduced by one-half following the activation of PINK1/Parkin pathway. The mtDNA content also showed the same behavior after CCCP treatment. Moreover, the transmission electron microscope images showed the presence of many vacuoles and mitochondria fused with phagosomes, thus confirming the activation of the mitophagy process.

Completely different results were instead observed in SH-SY5Y cells treated with dopamine or MPP^+ and in human tissues of sporadic PD patients. Indeed, VDAC1 and VDAC2 protein levels were drastically reduced in dopamine-treated SH-SY5Y cells and in subjects with PD, probably because of degradation by mitochondrial proteases, as already suggested (Alberio et al., 2014a; Di Pierro et al., 2016). On the contrary, the inhibition of complex I caused an increased expression of both VDACs. This molecular event is probably caused by the formation of the permeability transition pore and by the induction of the apoptotic process (Shoshan-Barmatz et al., 2008). Moreover, treatment with dopamine did not cause any alteration in COX5β protein levels. The same result was obtained in human tissues of sporadic PD patients. Interestingly, COX5β levels were decreased following MPP^+ treatment. The mtDNA analysis showed an opposite behavior for dopamine and MPP^+ treatment. Indeed, the mtDNA copy number was increased following dopamine administration. This is probably caused by oxidation of dopamine in the cytosol, which in turn determines the formation of nitric oxide (Antunes et al., 2005), a molecule that has been positively linked to mtDNA transcription (Nisoli et al., 2003; Nisoli et al., 2004). On the contrary, the mtDNA copy number was drastically reduced following the inhibition of complex I. However, the reduced COX5β protein levels and mtDNA content observed in MPP^+-treated cells were not correlated with the accumulation of PINK1, which causes the activation of the mitophagy pathway and the degradation of damaged mitochondria (Narendra et al., 2010a; Sarraf et al., 2013; Pickrell and Youle, 2015). Unfortunately, we could not perform the mtDNA analysis on Substantia nigra of PD patients and control subjects due to the little amount of tissues, which were all used to extract proteins and perform the Western blot analysis.

In order to better describe the mitochondrial alterations that occur in our models, we decided to perform a morphological analysis of mitochondria using transmission electron microscopy. In MPP^+-treated SH-SY5Y cells, we observed swelling and emptying of mitochondria, which accounts for the drastic decrease of the IMM COX5β and OPA1 proteins. On the other hand, VDACs, which are in the OMM, are increased. Moreover, no appearance of vacuoles and mitochondria engulfed by the autophagosomes were observed after MPP^+ treatment. This result justifies the lack of PINK1 accumulation and the consequent mitophagy impairment that occur in our cellular model of complex I inhibition.

On the other hand, treatment with dopamine determined the fusion of cristae, in line with the reduced levels of OPA1, responsible of cristae maintenance (Ramonet et al., 2013). Moreover, some electron dense deposits are visible inside mitochondria, representing melanized organelles, due to the entry of dopamine and the following action of dopamine-derived reactive species

leading to their covalent addition to mitochondrial proteins (Brenner-Lavie et al., 2008; Van Laar et al., 2009). Interestingly, also in neurons of PD subjects, mitochondria contained deranged cristae and some small electron dense deposits.

To sum up, these results suggest that mitophagy impairment is a common feature of both Substantia nigra of PD patients and PD cellular models. Indeed, we never observed PINK1 accumulation, a sign of triggered mitophagy. However, the analysis of mitochondrial molecular markers (i.e. VDAC1, VDAC2, COX5β and mtDNA) showed a discordant behavior between the cellular model of altered dopamine homeostasis and that of complex I inhibition. In MPP^+-treated cells, we simultaneously observed the decrease of COX5β, OPA1 and mtDNA, the increase of VDAC1 and VDAC2, and no effect on MFN1 level. This molecular picture was clarified by TEM images, showing empty organelles, with disrupted cristae. The treatment with dopamine causes a different mitochondrial alteration. Indeed, COX5β and MFN1 did not change, whereas OPA1, VDAC1 and VDAC2 were decreased and the mtDNA content was increased. TEM images evidenced the presence of damaged mitochondria, although presenting different features if compared to those in MPP^+-treated cells, in line with a different molecular behavior. In PD patients, the molecular landscape was identical to the one of dopamine-treated cells. TEM images gave us the suggestion that also electron dense deposits are present in mitochondria.

In conclusion, the acute inhibition of complex I only partially reproduces the mitochondrial alterations related to PD pathogenesis. Indeed, even if some alterations are similar (i.e. fusion impairment), the MPP^+ toxin causes a peculiar mitochondrial damage, which cannot explain the complexity of mitochondrial impairment occurring in Substantia nigra of PD patients. On the other hand, the altered dopamine homeostasis model better recapitulates the molecular changes that occur in the human autoptic samples. Indeed, dopamine-treated SH-SY5Y cells and substantia nigra of PD patients were both characterized by the marked reduction in levels of both VDACs proteins without induction of mitophagy, the degradation of both OPA1 forms, and the presence of residual granules within mitochondria. These results suggest a fundamental role of impaired dopamine homeostasis and of the altered mitochondrial disposal in PD pathogenesis.

5.2. Proteomics and interactomics characterization of *PARK2*-mutated patients

Several pathogenetic mechanisms are involved in the development of PD, such as mitochondrial impairment, oxidative and nitrative stress, and autophagy dysfunction. Consequently, the identification of specific molecular pathways altered by the disease is necessary to understand its etiology and to find new therapeutic targets for this pathology. In this intricate molecular landscape, mitophagy impairment seems to play a central role in the development of this pathology. Indeed, the improper mitochondrial disposal generates higher levels of ROS, a lower ATP production and the activation of the apoptotic pathway (Pickrell and Youle, 2015). The most characterized mitophagy mechanism is the PINK1/Parkin pathway, which is able to tag the damaged mitochondria (Narendra et al., 2010a), thus causing their selective lysosomal degradation (Fimia et al., 2007). For these reasons, mutations in the *PARK2* gene, which encodes for the Parkin protein, are responsible for the accumulation of damaged mitochondria.

In such a multifaceted scenario, it is particularly important to identify experimental models that simplify the study of the different networks of proteins/genes involved. Several studies have been recently relying on skin fibroblasts, as a substrate to investigate PD mechanisms. Indeed, skin fibroblasts represent an easily accessible source of proliferating cells that share the same genetic complexity of neurons, mirror the polygenic risk factors and reflect cumulative cell damage at the age of the patient (Connolly, 1998, Auburger et al., 2012). Moreover, several studies carried out on fibroblasts obtained from skin biopsies from PD patients have reported that fibroblasts show some deficits associated with PD pathogenesis that are typical of neuronal tissue (McNeill et al., 2014; Lippolis et al., 2015; Kilpatrick et al., 2016).

Nowadays, the molecular mechanisms underlying mitophagy impairment are not yet completely understood. For these reasons, we decided to investigate the mitochondrial alterations that occur in skin fibroblasts obtained from *PARK2*-mutated patients. First, we characterized the mitochondrial network morphology in order to understand if the loss of function of Parkin can have a negative impact on the mitochondrial dynamics. To this purpose, we performed an immunofluorescence assay on skin fibroblasts of three control subjects and three *PARK2*-mutated patients in order to stain the mitochondrial network with the ATP Synthase β antibody. We then measured the distribution densities of several morphological parameters, a method previously used by our group in order to characterize the mitochondrial network in SH-SY5Y cell lines treated with different toxins (Bondi et al., 2016). Surprisingly, the measurement of the distribution densities of these morphological parameters (e.g. solidity, roundness and circularity) demonstrated that mutations in the *PARK2* gene do not cause any significant morphological

alterations in the mitochondrial network. This is probably due to the mitophagy impairment that characterizes these patients. Indeed, fission and mitophagy processes are strictly connected (Otera and Mihara, 2011).

In order to decipher the molecular pathways altered in the *PARK2* pathology, we used a global and unbiased proteomics approach to highlight the difference of protein expression levels between *PARK2*-mutated patients and control subjects. Quantitative shotgun proteomics analysis was performed on the mitochondrial-enriched fractions (obtained using a commercial kit based on surfactants) of these subjects. Before conducting the proteomics analysis on these samples, we decided to check the quality of the mitochondrial isolation procedure by Western Blot. As a result, we found that VDAC1 and CS, two mitochondrial proteins, were detected only in the mitochondrial-enriched fractions, while the Histone H3, a nuclear protein, was clearly detected only in the nuclear fractions.

The subsequent quantitative shotgun proteomics performed on the mitochondrial-enriched fractions revealed only the alterations of a low number of proteins between the two groups. This issue could be solved by increasing the number of subjects recruited for this research. So far, we found that the expression of two proteins involved in the respiratory chain (i.e. NADH dehydrogenase iron-sulfur protein 3 and ATP synthase subunit g) was reduced in *PARK2*-mutated patients. A previously research conducted on skin fibroblasts of *PARK2*-mutated patients demonstrated that mitochondria show lower membrane potential, lower complex I activity and lower ATP cellular levels (Zanellati et al., 2015). Our proteomics result is in line with these data. Indeed, the decreased expression of NADH dehydrogenase iron-sulfur protein 3, a subunit of the complex I, can explain the lower complex I activity found in these subjects. Moreover, the reduced expression of ATP synthase subunit g, a subunit of the ATP synthase, clarifies the lower ATP levels found in these cells. Our preliminary proteomics results also showed that the mitochondrial quality control system was impaired in *PARK2*-mutated subjects. Indeed, persulfide dioxygenase ETHE1, a protein that plays an important role in the metabolic homeostasis in mitochondria by metabolizing hydrogen sulfide and preventing the accumulation of supraphysiological H_2S levels, which have cellular toxic effects due to the ability of this molecule to inhibit the cytochrome c oxidase (Tiranti et al., 2009), is reduced in these patients. Moreover, Lon protein, an ATP-dependent serine protease that mediates the selective degradation of misfolded, unassembled or oxidatively damaged polypeptides in the mitochondrial matrix and that have also a chaperone function in the assembly of inner membrane protein complexes (Bota and Davies, 2002; Pinti et al., 2016), was not expressed in *PARK2*-mutated patients. Eventually, *PARK2*-mutated patients show an increased expression of LETM1.

This protein is crucial for the maintenance of mitochondrial tubular networks and for the maintenance of the mitochondrial cristae organization (Tamai et al., 2008). This result can justify the lack of mitochondrial morphological alterations obtained with the analysis of the mitochondrial network shape in *PARK2*-mutated patients. Given the mitophagy impairment of these subjects, the increased expression of LETM1 and the maintenance of the tubular shape of the mitochondrial networks may represent a compensation mechanism implemented by the cells.

Since the shotgun proteomics technique gives only a static view of the alterations that occur in *PARK2*-mutated patients, we decided to use also an interactomic approach to better deciphering the molecular landscape affected by mutations in the *PARK2* gene. Indeed, thanks to the biochemical fractionations coupled with mass spectrometry analysis and to the systems biology approach, it is possible to reveal the protein-protein interactions affected by the Parkin loss and to discover the molecular pathway altered in this pathology.

So far, the size-exclusion chromatography (SEC) coupled with LC-MS/MS analysis was not made for the fractions obtained from the three control subjects. For this reason, a preliminary interactomics analysis was carried out on the fractions obtained from the three *PARK2*-mutated patients. As a preliminary result, we found that a protein network of the fraction 58 is involved in the positive regulation of apoptotic signaling pathway and of mitochondrial membrane permeability. Since the mitochondrial functions of these patients are impaired (e.g. lower membrane potential, lower complex I activity and lower ATP cellular levels) (Zanellati et al., 2015), it is probable that some apoptotic pathways are activated in these cells. Eventually, we found a protein-protein interaction network in the fraction 51 that was characterized by the presence of LETM1, a protein that is up-regulated in *PARK2*-mutated patients. This protein network is positively involved in the mitochondrial membrane organization, cristae formation, and mitochondrial network organization. Once again, this result is in line with our morphological data, which demonstrate that mutations in *PARK2* gene do not affect the mitochondrial tubular network shape.

Since the interactomic data obtained with SEC are very complex (indeed, we found a total of around 3600 proteins in all the 84 fractions of the 3 *PARK2*-mutated mitochondrial samples), we are now preparing other mitochondrial-enriched fractions of both control and *PARK2*-mutated subjects in order to perform also the ion-exchange chromatography (IEC). Using the results obtained with both biochemical fractionations techniques it will be possible to remove the false positive and to reveal the false negative protein-protein interactions data. Moreover, a more complex bioinformatics analysis will be conducted on these samples in order to decipher the interactome alterations that characterize the *PARK2*-mutated patients. Indeed, to identify

individual protein pairs with similar co-elution profiles, three complementary scoring schemes will be employed: 1) Pearson correlation coefficient (pcc); 2) weighted cross correlation (wcc); and 3) co-apex score. For each protein pair, 2 pcc scores, 2 wcc scores, and 2 co-apex scores will be generated, corresponding to each of the separation techniques. These scores will be integrated with functional association evidence predicted by genomic context for human using a machine-learning classifier to construct a network of proteins linked by probabilistic interaction scores.

5.3. Concluding remarks

This research project has contributed to pave the road for a better understanding of the molecular factors involved in *PARK2* pathogenesis, unravelling also possible biochemical pathways altered in the sporadic form of the disease. Indeed, it is known that in sporadic cases the genetic/epigenetic background and the environment lead over time to mitochondrial impairment and to the accumulation of damaged organelles. On the contrary, the loss of function of Parkin that occurs in *PARK2*-mutated patients and the consequent mitophagy alteration lead directly to the specific neurodegeneration of the Substantia nigra pars compacta. For these reasons, we aimed at clarifying the role of mitophagy impairment in PD pathogenesis. To reach this goal, the use of proteomics integrative approach is necessary. Moreover, the interactomics results that we will obtain soon could give a dynamic vision of the macromolecular complexes altered by the disease. Overall, the definition of a *PARK2*-related molecular signature will be crucial for providing new insights into disease mechanisms and identifying new therapeutic targets for this pathology.

6. References

Abdallah C, Dumas-Gaudot E, Renaut J, Sergeant K. (2012) Gel-based and gel-free quantitative proteomics approaches at a glance. Int J Plant Genomics. 2012:494572.

Alberio T, Bossi AM, Milli A, Parma E, Gariboldi MB, Tosi G, Lopiano L, Fasano M. (2010) Proteomic analysis of dopamine and α-synuclein interplay in a cellular model of Parkinson's disease pathogenesis. FEBS J. 277:4909-4919.

Alberio T, Fasano M. (2011) Proteomics in Parkinson's disease: An unbiased approach towards peripheral biomarkers and new therapies. J Biotechnol. 156:325-337.

Alberio T, Lopiano L, Fasano M. (2012) Cellular models to investigate biochemical pathways in Parkinson's disease. FEBS J. 279:1146-1155.

Alberio T, Bondi H, Colombo F, Alloggio I, Pieroni L, Urbani A, Fasano M. (2014) Mitochondrial proteomics investigation of a cellular model of impaired dopamine homeostasis, an early step in Parkinson's disease pathogenesis. Mol. BioSyst. 10:1332-1344.

Alberio T, Pieroni L, Ronci M, Banfi C, Bongarzone I, Bottoni P, Brioschi M, Caterino M, Chinello C, Cormio A, Cozzolino F, Cunsolo V, Fontana S, Garavaglia B, Giusti L, Greco V, Lucacchini A, Maffioli E, Magni F, Monteleone F, Monti M, Monti V, Musicco C, Petrosillo G, Porcelli V, Saletti R, Scatena R, Soggiu A, Tedeschi G, Zilocchi M, Roncada P, Urbani A, Fasano M. (2017) Toward the Standardization of Mitochondrial Proteomics: The Italian Mitochondrial Human Proteome Project Initiative. J Proteome Res. doi: 10.1021/acs.jproteome.7b00350.

Albrecht D, Kniemeyer O, Brakhage AA, Guthke R. (2010) Missing values in gel-based proteomics. Proteomics. 10:1202-1211.

Antunes F, Nunes C, Laranjinha J, Cadenas E. (2005) Redox interactions of nitric oxide with dopamine and its derivatives. Toxicology. 208:207-212.

Aroso M, Ferreira R, Freitas A, Vitorino R, Gomez-Lazaro M. (2016) New insights on the mitochondrial proteome plasticity in Parkinson's disease. Proteomics Clin Appl. 10:416-429.

Ascherio A, Schwarzschild MA. (2016) The epidemiology of Parkinson's disease: risk factors and prevention. Lancet Neurol. 15:1257-1272.

Auburger G, Klinkenberg M, Drost J, Marcus K, Morales-Gordo B, Kunz WS, Brandt U, Broccoli V, Reichmann H, Gispert S, Jendrach M. (2012) Primary skin fibroblasts as a model of Parkinson's disease. Mol Neurobiol. 46:20-27.

Babu M, Vlasblom J, Pu S, Guo X, Graham C, Bean BD, Burston HE, Vizeacoumar FJ, Snider J, Phanse S, Fong V, Tam YY, Davey M, Hnatshak O, Bajaj N, Chandran S, Punna T, Christopolous C, Wong V, Yu A, Zhong G, Li J, Stagljar I, Conibear E, Wodak SJ, Emili A, Greenblatt JF. (2012) Interaction landscape of membrane-protein complexes in Saccharomyces cerevisiae. Nature. 489:585-589.

Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. (2007) Quantitative mass spectrometry in proteomics: a critical review. Anal Bioanal Chem. 389:1017-1031.

Beevers JE, Caffrey TM, Wade-Martins R. (2013) Induced pluripotent stem cell (iPSC)-derived dopaminergic models of Parkinson's disease. Biochem Soc Trans. 41:1503-1508.

Bekris LM, Mata IF, Zabetian, CP. (2010). The genetics of Parkinson disease. Journal of geriatric psychiatry and neurology. 23:228-242.

Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouysségur J, Mazure NM. (2009) Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol. 29:2570-2581.

Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, Jaros E, Hersheson JS, Betts J, Klopstock T, Taylor RW, Turnbull DM. (2006) High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet. 38:515-517.

Benskey MJ, Perez RG, Manfredsson FP. (2016) The contribution of alpha synuclein to neuronal survival and function - Implications for Parkinson's disease. J Neurochem. 137:331–359.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci. 3:1301-1306.

Bisaglia M, Greggio E, Beltramini M, Bubacco L. (2013) Dysfunction of dopamine homeostasis: clues in the hunt for novel Parkinson's disease therapies. FASEB J. 27:2101-2110. doi:10.1096/fj.12-226852.

Blesa JR, Prieto-Ruiz JA, Hernandez JM, Hernandez-Yago J. (2007) NRF-2 transcription factor is required for human TOMM20 gene expression. Gene. 391:198-208.

Blin O, Desnuelle C, Rascol O, Borg M, Peyro Saint Paul H, Azulay JP, Billé F, Figarella D, Coulom F, Pellissier JF, et al. (1994) Mitochondrial respiratory failure in skeletal muscle from patients with Parkinson's disease and multiple system atrophy. J Neurol Sci. 125:95-101.

Bolte S, Cordelières FP. (2006) A guided tour into subcellular colocalization analysis in light microscopy. J Microsc 224:213-232.

Bonawitz ND, Clayton DA, Shadel GS. (2006) Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. Mol Cell. 24:813-825.

Bondi H, Zilocchi M, Mare MG, D'Agostino G, Giovannardi S, Ambrosio S, Fasano M, Alberio T. (2016) Dopamine induces mitochondrial depolarization without activating PINK1-mediated mitophagy. J Neurochem. 136:1219-1231.

Bota DA, Davies KJ. (2002) Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. Nat Cell Biol. 4:674-680.

Brenner-Lavie H, Klein E, Zuk, R., Gazawi, H., Ljubuncic, P., and Ben-Shachar, D. (2008). Dopamine modulates mitochondrial function in viable SH-SY5Y cells possibly via its interaction with complex I: relevance to dopamine pathology in schizophrenia. *Biochim. Biophys. Acta.* 1777, 173-185.

Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HJ. (1999) Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. Brain Res. 823:1-10.

Burchell VS, Nelson DE, Sanchez-Martinez A, Delgado-Camprubi M, Ivatt RM, Pogson JH, Randle SJ, Wray S, Lewis PA, Houlden H, Abramov AY, Hardy J, Wood NW, Whitworth AJ, Laman H, Plun-Favreau H. (2013) The Parkinson's disease-linked proteins Fbxo7 and Parkin interact to mediate mitophagy. Nat Neurosci 16:1257-1265.

Bürckstümmer T, Bennett KL, Preradovic A, Schütze G, Hantschel O, Superti-Furga G, Bauch A. (2006) An efficient tandem affinity purification procedure for interaction proteomics in mammalian cells. Nat Methods. 3:1013-1019.

Burte F, De Girolamo LA, Hargreaves AJ, Billett EE. (2011) Alterations in the mitochondrial proteome of neuroblastoma cells in response to complex 1 inhibition. Journal of Proteome Research. 10:1974–1986.

Caudle WM, Richardson JR, Wang MZ, Taylor TN, Guillot TS, McCormack AL, Colebrooke RE, Di Monte DA, Emson PC, Miller GW. (2007) Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. J Neurosci. 27:8138-8148.

Chan DC. (2006a) Dissecting mitochondrial fusion. Dev Cell. 11:592-594.

Chan DC. (2006b) Mitochondria: dynamic organelles in disease, aging, and development. Cell. 125:1241–1252.

Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. (2003) Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J Cell Biol. 160:189-200.

Chen H, Chomyn A, Chan DC. (2005) Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J Biol Chem. 280:26185-26192.

Chen H, Chan DC. (2006) Critical dependence of neurons on mitochondrial dynamics. Curr Opin Cell Biol. 18:453-459.

Chen H, McCaffery JM, Chan DC. (2007) Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell. 130:548-562.

Chen Y, Dorn 2nd GW. (2013) PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science. 340:471–475.

Chou AP, Li S, Fitzmaurice AG, Bronstein JM. (2010) Mechanisms of rotenone-induced proteasome inhibition. Neurotoxicology. 31:367-372.

Chu Y, Dodiya H, Aebischer P, Olanow CW, Kordower JH. (2009) Alterations in lysosomal and proteasomal markers in Parkinson's disease: relationship to alpha-synuclein inclusions. Neurobiol Dis. 35:385-398.

Cipolat S, Rudka T, Hartmann D, Costa V, Serneels L, Craessaerts K, Metzger K, Frezza C, Annaert W, D'Adamio L, Derks C, Dejaegere T, Pellegrini L, D'Hooge R, Scorrano L, De Strooper B. (2006) Mitochondrial rhomboid PARL regulates cytochrome crelease during apoptosis via OPA1-dependent cristae remodeling. Cell. 126:163-175.

Colinge J, Chiappe D, Lagache S, Moniatte M, Bougueleret L. (2005) Differential proteomics via probabilistic peptide identification scores. Anal Chem. 77:596-606.

Connolly GP. (1998) Fibroblast models of neurological disorders: fluorescence measurement studies. Trends Pharmacol Sci. 19:171-177.

Conway KA, Rochet JC, Bieganski RM, Lansbury PT Jr. (2001) Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. Science. 2294:1346-1349.

Cookson MR. (2010) The role of leucine-rich repeat kinase 2 (LRRK2) in Parkinson's disease. Nat Rev Neurosci. 11:791-797.

Dauer W, Przedborski S. (2003) Parkinson's disease: mechanisms and models. Neuron. 39:889-909.

Davidzon G, Greene P, Mancuso M, Klos KJ, Ahlskog JE, Hirano M, DiMauro S. (2006) Early-onset familial parkinsonism due to POLG mutations. Ann Neurol. 59:859-862.

Davis GC, Williams AC, Markey SP, Ebert MH, Caine ED, Reichert CM, Kopin IJ. (1979) Chronic Parkinsonism secondary to intravenous injection of meperidine analogues. Psychiatry Research. 1:249–254.

de Brito OM, Scorrano L. (2008) Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature. 456:605-610.

Detmer SA, Chan DC. (2007) Complementation between mouse Mfn1 and Mfn2 protects mitochondrial fusion defects caused by CMT2A disease mutations. J Cell Biol. 176:405-414.

Di Fonzo A, Rohé CF, Ferreira J, Chien HF, Vacca L, Stocchi F, Guedes L, Fabrizio E, Manfredi M, Vanacore N, Goldwurm S, Breedveld G, Sampaio C, Meco G, Barbosa E, Oostra BA, Bonifati V; Italian Parkinson Genetics Network. (2005) A frequent LRRK2 gene mutation associated with autosomal dominant Parkinson's disease. Lancet. 365:412-415.

Di Palma S, Hennrich ML, Heck AJ, Mohammed S. (2012) Recent advances in peptide separation by multidimensional liquid chromatography for proteome analysis. J Proteomics. 75:3791-3813.

Di Pierro A, Bondi H, Monti C, Pieroni L, Cilio E, Urbani A, Alberio T, Fasano M, Ronci M. (2016) Experimental setup for the identification of mitochondrial protease substrates by shotgun and top-down proteomics. EuPA Open Proteomics. 11:1-3.

Dias V, Junn E, Mouradian MM. (2013) The role of oxidative stress in Parkinson's disease. J Parkinsons Dis. 3:461-491.

Dimmer KS, Scorrano L. (2006) (De)constructing mitochondria: what for? Physiology (Bethesda). 21:233-241.

Dukan S, Farewell A, Ballesteros M, Taddei F, Radman M, Nyström T. (2000) Protein oxidation in response to increased transcriptional or translational errors. Proc Natl Acad Sci U S A. 97:5746-5749.

Dupuis L. (2014) Mitochondrial quality control in neurodegenerative diseases. Biochimie. 100:177-183.

Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, Asara JM, Fitzpatrick J, Dillin A, Viollet B, Kundu M, Hansen M, Shaw RJ. (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science. 331:456–461.

Eiyama A, Okamoto K. PINK1/Parkin-mediated mitophagy in mammalian cells. (2015) Curr Opin Cell Biol. 33:95-101.

Ekstrand MI, Terzioglu M, Galter D, Zhu S, Hofstetter C, Lindqvist E, Thams S, Bergstrand A, Hansson FS, Trifunovic A, Hoffer B, Cullheim S, Mohammed AH, Olson L, Larsson NG. (2007) Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. Proc Natl Acad Sci U S A. 104:1325-1330.

Exner N, Treske B, Paquet D, Holmström K, Schiesling C, Gispert S, Carballo-Carbajal I, Berg D, Hoepken HH, Gasser T, Krüger R, Winklhofer KF, Vogel F, Reichert AS, Auburger G, Kahle PJ, Schmid B, Haass C. (2007) Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. J Neurosci. 27:12413-12418.

Fahn S. (2003) Description of Parkinson's disease as a clinical syndrome. Ann N Y Acad Sci. 991:1-14.

Fasano M, Alberio T, Babu M, Lundberg E, Urbani A. (2016) Towards a functional definition of the mitochondrial human proteome. EuPA Open Proteomics.10:24-27.

Fernández-Moriano C, González-Burgos E, Gómez-Serranillos MP. (2015) Mitochondria-Targeted Protective Compounds in Parkinson's and Alzheimer's Diseases. Oxid Med Cell Longev. 2015:408927.

Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, Corazzari M, Fuoco C, Ucar A, Schwartz P, Gruss P, Piacentini M, Chowdhury K, Cecconi F. (2007) Ambra1 regulates autophagy and development of the nervous system. Nature. 447:1121–1125.

Fisher RP, Clayton DA. (1988) Purification and characterization of human mitochondrial transcription factor 1. Molecular and Cellular Biology. 8:3496–3509.

Fiskum G, Starkov A, Polster BM, Chinopoulos C. (2003) Mitochondrial mechanisms of neural cell death and neuroprotective interventions in Parkinson's disease. Annals of the New York Academy of Science. 99:111-119.

Gallagher DA, Lees AJ, Schrag A. (2010) What are the most important nonmotor symptoms in patients with Parkinson's disease and are we missing them? Mov Disord. 25:2493-2500.

Gao F, Chen D, Si J, Hu Q, Qin Z, Fang M, Wang G. (2015) The mitochondrial protein BNIP3L is the substrate of PARK2 and mediates mitophagy in PINK1/PARK2 pathway. Hum Mol Genet. 24:2528-2538.

Gegg ME, Cooper JM, Chau KY, Rojo M, Schapira AH, Taanman JW. (2010) Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. Hum Mol Genet. 19:4861–4870.

Gegg ME, Schapira AH. (2016) Mitochondrial dysfunction associated with glucocerebrosidase deficiency. Neurobiol Dis. 90:43-50.

Geisler S, Holmström KM, Treis A, Skujat D, Weber SS, Fiesel FC, Kahle PJ, Springer W. (2010) The PINK1/Parkin-mediated mitophagy is compromised by PD-associated mutations. Autophagy. 6:871-878.

Giasson BI, Lee VM. (2001) Parkin and the molecular pathways of Parkinson's disease. Neuron. 31:885–888.

Gilks WP, Abou-Sleiman PM, Gandhi S, Jain S, Singleton A, Lees AJ, Shaw K, Bhatia KP, Bonifati V, Quinn NP, Lynch J, Healy DG, Holton JL, Revesz T, Wood NW. (2005) A common LRRK2 mutation in idiopathic Parkinson's disease. Lancet. 365:415-416.

Gleyzer N, Vercauteren K, Scarpulla RC. (2005) Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. Mol Cell Biol. 25:1354–1366.

Gómez-Santos C, Ferrer I, Santidrián AF, Barrachina M, Gil J, Ambrosio S. (2003) Dopamine induces autophagic cell death and alpha-synuclein increase in human neuroblastoma SH-SY5Y cells. J Neurosci Res. 73:341-350.

Goo HG, Jung MK, Han SS, Rhim H, Kang S. (2013) HtrA2/Omi deficiency causes damage and mutation of mitochondrial DNA. Biochim Biophys Acta. 1833:1866–1875.

Görg A, Weiss W, Dunn MJ. (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics. 4:3665-3685.

Green DR, Galluzzi L, Kroemer G. (2011) Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. Science. 333:1109–1112.

Green DR, Kroemer G. (2004) The pathophysiology of mitochondrial cell death. Science. 305:626–629.

Guardia-Laguarta C, Area-Gomez E, Rüb C, Liu Y, Magrané J, Becker D, Voos W, Schon EA, Przedborski S. (2014) α-Synuclein is localized to mitochondria-associated ER membranes. J Neurosci. 34:249-259.

Guruharsha KG, Rual JF, Zhai B, Mintseris J, Vaidya P, Vaidya N, Beekman C, Wong C, Rhee DY, Cenaj O, McKillip E, Shah S, Stapleton M, Wan KH, Yu C, Parsa B, Carlson JW, Chen X, Kapadia B, VijayRaghavan K, Gygi SP, Celniker SE, Obar RA, Artavanis-Tsakonas S. (2011) A protein complex network of Drosophila melanogaster. Cell. 147:690-703.

Haas RH, Nasirian F, Nakano K, Ward D, Pay M, Hill R, Shults CW. (1995) Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. Ann Neurol. 37:714-722.

Hamon MP, Bulteau AL, Friguet B. (2015) Mitochondrial proteases and protein quality control in ageing and longevity. Ageing Res Rev. 23:56-66.

Hastings TG. (2009) The role of dopamine oxidation in mitochondrial dysfunction: implications for Parkinson's disease. J Bioenerg Biomembr. 41:469-472.

Havugimana PC, Hart GT, Nepusz T, Yang H, Turinsky AL, Li Z, Wang PI, Boutz DR, Fong V, Phanse S, Babu M, Craig SA, Hu P, Wan C, Vlasblom J, Dar VU, Bezginov A, Clark GW, Wu GC, Wodak SJ, Tillier ER, Paccanaro A, Marcotte EM, Emili A. (2012) A census of human soluble protein complexes. Cell.150:1068-1081.

Head B, Griparic L, Amiri M, Gandre-Babbe S, van der Bliek AM. (2009) Inducible proteolytic inactivation of OPA1 mediated by the OMA1 protease in mammalian cells. J Cell Biol. 187:959-966.

Herrera A, Muñoz P, Steinbusch HWM, Segura-Aguilar J. (2017) Are Dopamine Oxidation Metabolites Involved in the Loss of Dopaminergic Neurons in the Nigrostriatal System in Parkinson's Disease? ACS Chem Neurosci. 8:702-711.

Herzig S, Long FX, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, Montminy M. (2001) CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature. 413:179-183.

Hoang T, Choi DK, Nagai M, Wu DC, Nagata T, Prou D, Wilson GL, Vila M, Jackson-Lewis V, Dawson VL, Dawson TM, Chesselet MF, Przedborski S. (2009) Neuronal NOS and cyclooxygenase-2 contribute to DNA damage in a mouse model of Parkinson disease. Free Radical Biology & Medicine. 47:1049–1056.

Hong P, Koza S, Bouvier ES. (2012) Size-Exclusion Chromatography for the Analysis of Protein Biotherapeutics and their Aggregates. J Liq Chromatogr Relat Technol. 35:2923-2950.

Hoppins S, Lackner L, Nunnari J. (2007) The machines that divide and fuse mitochondria. Annu Rev Biochem. 76:751-780.

Hoppins S. (2014) The regulation of mitochondrial dynamics. Curr. Opin. Cell Biol. 29:46−52.

Hu P, Janga SC, Babu M, Díaz-Mejía JJ, Butland G, Yang W, Pogoutse O, Guo X, Phanse S, Wong P, Chandran S, Christopoulos C, Nazarians-Armavil A, Nasseri NK, Musso G, Ali M, Nazemof N, Eroukova V, Golshani A, Paccanaro A, Greenblatt JF, Moreno-Hagelsieb G, Emili A. (2009) Global functional atlas of Escherichia coli encompassing previously uncharacterized proteins. PLoS Biol. 7:e96.

Ingerman E, Perkins EM, Marino M, Mears JA, McCaffery JM, Hinshaw JE, Nunnari J. (2005) Dnm1 forms spirals that are structurally tailored to fit mitochondria. J Cell Biol. 170:1021-1027.

Ishihara N, Eura Y, Mihara K. (2004) Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. J Cell Sci. 117:6535-6546.

Ishihara N, Fujita Y, Oka T, Mihara K. (2006) Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. EMBO J. 25:2966-2977.

Itoh K, Nakamura K, Iijima M, Sesaki H. (2013) Mitochondrial dynamics in neurodegeneration. Trends Cell Biol. 23:64–71.

Ivankovic D, Chau KY, Schapira AH, Gegg ME. (2016) Mitochondrial and lysosomal biogenesis are activated following PINK1/parkin-mediated mitophagy. J Neurochem. 2016 Jan;136(2):388-402.

Jankovic J. (2008) Parkinson's disease: clinical features and diagnosis. Journal of Neurology, Neurosurgery & Psychiatry, 79:368-376.

Jenner P. (2003) Oxidative stress in Parkinson's disease. Ann Neurol. 53 Suppl 3:S26-36.

Jenner P, Olanow CW. (2006) The pathogenesis of cell death in Parkinson's disease. Neurology. 66:S24-36.

Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ. (2010) Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. J Cell Biol. 191:933–942.

Jofuku A, Ishihara N, Mihara K. (2005) Analysis of functional domains of rat mitochondrial Fis1, the mitochondrial fission-stimulating protein. Biochem Biophys Res Commun. 333:650-659.

Johnson ME, Bobrovskaya L. (2015) An update on the rotenone models of Parkinson's disease: their ability to reproduce the features of clinical disease and model gene-environment interactions. Neurotoxicology. 46:101-116.

Jones GM, Vale JA. (2000) Mechanisms of toxicity, clinical features, and management of diquat poisoning: a review. J Toxicol Clin Toxicol. 38:123-128.

Kang Y, Fielden LF, Stojanovski D. (2017) Mitochondrial protein transport in health and disease. Semin Cell Dev Biol.. S1084-9521:30021-30026.

Karbowski M, Youle RJ. (2011) Regulating mitochondrial outer membrane proteins by ubiquitination and proteasomal degradation. Curr Opin Cell Biol. 23:476-482.

Karra D, Dahm R. (2010) Transfection techniques for neuronal cells. J Neurosci. 30:6171–6177.

Karren MA, Coonrod EM, Anderson TK, Shaw JM. (2005) The role of Fis1p-Mdv1p interactions in mitochondrial fission complex assembly. J Cell Biol. 171:291-301.

Kilpatrick BS, Magalhaes J, Beavan MS, McNeill A, Gegg ME, Cleeter MW, Bloor-Young D, Churchill GC, Duchen MR, Schapira AH, Patel S. (2016) Endoplasmic reticulum and lysosomal Ca^{2+} stores are remodelled in GBA1-linked Parkinson disease patient fibroblasts. Cell Calcium. 59:12-20.

Kim I, Rodriguez-Enriquez S, Lemasters, JJ. (2007) Selective degradation of mitochondria by mitophagy. Archives of biochemistry and biophysics, 462:245-253.

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

Klein C, Westenberger A. (2012) Genetics of Parkinson's disease. Cold Spring Harb Perspect Med. 2:a008888.

Koppen M, Langer T. (2007) Protein degradation within mitochondria: versatile activities of AAA proteases and other peptidases. Crit Rev Biochem Mol Biol. 42:221-242.

Koshiba T, Detmer SA, Kaiser JT, Chen H, McCaffery JM, Chan DC. (2004) Structural basis of mitochondrial tethering by mitofusin complexes. Science. 305:858-862.

Kovalevich J, Langford D. (2013) Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. Methods Mol Biol. 1078:9-21.

Krishna A, Biryukov M, Trefois C, Antony PM, Hussong R, Lin J, Heinäniemi M, Glusman G, Köglsberger S, Boyd O, van den Berg BH, Linke D, Huang D, Wang K, Hood L, Tholey A, Schneider R, Galas DJ, Balling R, May P. (2014) Systems genomics evaluation of the SH-SY5Y neuroblastoma cell line as a model for Parkinson's disease. BMC Genomics. 15:1154.

Kristensen AR, Gsponer J, Foster LJ. (2012) A high-throughput approach for measuring temporal changes in the interactome. Nat Methods. 9:907-909.

Krogan NJ, Cagney G, Yu H, Zhong G, Guo X, Ignatchenko A, Li J, Pu S, Datta N, Tikuisis AP, Punna T, Peregrín-Alvarez JM, Shales M, Zhang X, Davey M, Robinson MD, Paccanaro A, Bray JE, Sheung A, Beattie B, Richards DP, Canadien V, Lalev A, Mena F, Wong P, Starostine A, Canete MM, Vlasblom J, Wu S, Orsi C, Collins SR, Chandran S, Haw R, Rilstone JJ, Gandi K, Thompson NJ, Musso G, St Onge P, Ghanny S, Lam MH, Butland G, Altaf-Ul AM, Kanaya S, Shilatifard A, O'Shea E, Weissman JS, Ingles CJ, Hughes TR, Parkinson J, Gerstein M, Wodak SJ, Emili A, Greenblatt JF. (2006) Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. Nature. 440:637-643.

Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O. (1998) Ala30Pro mutation in the gene encoding alphasynuclein in Parkinson's disease. Nature genetics. 18:106–108.

Kuroda Y, Mitsui T, Kunishige M, Shono M, Akaike M, Azuma H, Matsumoto T. (2006) Parkin enhances mitochondrial biogenesis in proliferating cells. Hum Mol Genet. 15:883-895.

Langston JW, Ballard PA Jr. (1983) Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N Engl J Med. 309:310.

Langston JW, Irwin I, Langston EB, Forno LS. (1984) 1-Methyl-4-phenylpyr- idinium ion (MPP+): identification of a metabolite of MPTP, a toxin selective to the substantia nigra. Neuroscience Letters. 48:87–92.

Langston JW, Forno LS, Tetrud J, Reeves AG, Kaplan JA, Karluk D. (1999) Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann Neurol. 46:598-605.

Lee S, Sterky FH, Mourier A, Terzioglu M, Cullheim S, Olson L, Larsson NG. (2012) Mitofusin 2 is necessary for striatal axonal projections of midbrain dopamine neurons. Hum Mol Genet. 21:4827-4835.

Lee Y, Lee HY, Hanna RA, Gustafsson ÅB. (2011) Mitochondrial autophagy by Bnip3 involves Drp1-mediated mitochondrial fission and recruitment of Parkin in cardiac myocytes. Am J Physiol Heart Circ Physiol. 301:H1924-1931.

Lee YJ, Jeong SY, Karbowski M, Smith CL, Youle RJ. (2004) Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol Biol Cell. 15:5001-5011.

Legesse-Miller A, Massol RH, Kirchhausen T. (2003) Constriction and Dnm1p recruitment are distinct processes in mitochondrial fission. Mol Biol Cell. 14:1953-1963.

Lesage S, Brice A. (2009) Parkinson's disease: from monogenic forms to genetic susceptibility factors. Hum Mol Genet. 18:R48-59.

Lill CM. (2016) Genetics of Parkinson's disease. Mol Cell Probes. 30:386-396. doi: 10.1016/j.mcp.2016.11.001.

Lilley KS, Friedman DB. (2004) All about DIGE: quantification technology for differential-display 2D-gel proteomics. Expert Rev Proteomics. 1:401-409.

Lim KL, Tan JM. (2007) Role of the ubiquitin proteasome system in Parkinson's disease. BMC Biochem. 8 Suppl 1:S13.

Lippolis R, Siciliano RA, Pacelli C, Ferretta A, Mazzeo MF, Scacco S, Papa F, Gaballo A, Dell'Aquila C, De Mari M, Papa S, Cocco T. (2015) Altered protein expression pattern in skin fibroblasts from parkin-mutant early-onset Parkinson's disease patients. Biochim Biophys Acta. 1852:1960-1970.

Lotharius J, Brundin P. (2002) Impaired dopamine storage resulting from alpha-synuclein mutations may contribute to the pathogenesis of Parkinson's disease. Hum Mol Genet. 11:2395-2407.

Luoma P, Melberg A, Rinne JO, Kaukonen JA, Nupponen NN, Chalmers RM, Oldfors A, Rautakorpi I, Peltonen L, Majamaa K, Somer H, Suomalainen A. (2004) Parkinsonism, premature menopause, and mitochondrial DNA polymerase gamma mutations: clinical and molecular genetic study. Lancet. 364:875-882.

MacVicar T, Langer T. (2016) OPA1 processing in cell death and disease - the long and short of it. J Cell Sci. 129:2297-2306.

Maguire-Zeiss KA, Short DW, Federoff HJ. (2005) Synuclein, dopamine and oxidative stress: co-conspirators in Parkinson's disease? Brain Res Mol Brain Res.134:18-23.

Mak AB, Moffat J. (2012) A versatile lentiviral expression system to identify mammalian protein-protein interactions. Methods. 57:409-416.

Manders EM, Stap J, Brakenhoff GJ, van Driel R, Aten JA. (1992) Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. J Cell Sci 103:857-862.

Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA. (2002) The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. J Biol Chem. 277:1641-1644.

Manning-Bog AB, Schüle B, Langston JW. (2009) Alpha-synuclein-glucocerebrosidase interactions in pharmacological Gaucher models: a biological link between Gaucher disease and parkinsonism. Neurotoxicology. 30:1127-1132.

Markey SP, Johannessen JN, Chiueh CC, Burns RS, Herkenham MA. (1984) Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. Nature. 311:464–467.

Martin I, Kim JW, Dawson VL, Dawson TM. (2014) LRRK2 pathobiology in Parkinson's disease. J Neurochem. 131:554-565.

Matsuda N, Sato S, Shiba K, Okatsu K, Saisho K, Gautier CA, Sou YS, Saiki S, Kawajiri S, Sato F, Kimura M, Komatsu M, Hattori N, Tanaka K. (2010) PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J Cell Biol. 189:211–221.

McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, Di Monte DA. (2002) Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. Neurobiol Dis. 10:119-127.

McCormack AL, Di Monte DA. (2003) Effects of L-dopa and other amino acids against paraquat-induced nigrostriatal degeneration. J Neurochem. 85:82-86.

McDonald WH, Yates JR 3rd. (2002) Shotgun proteomics and biomarker discovery. Dis Markers. 18:99-105.

McNeill A, Magalhaes J, Shen C, Chau KY, Hughes D, Mehta A, Foltynie T, Cooper JM, Abramov AY, Gegg M, Schapira AH. (2014) Ambroxol improves lysosomal biochemistry in glucocerebrosidase mutation-linked Parkinson disease cells. Brain. 137:1481-1495.

Mena MA, de Yebenes JG, Dwork A, Fahn S, Latov N, Herbert J, Flaster E, Slonim D. (1989) Biochemical properties of monoamine-rich human neuroblastoma cells. Brain research, 486:286-296.

Migdalska-Richards A, Schapira AH. (2016) The relationship between glucocerebrosidase mutations and Parkinson disease. J Neurochem. 139 Suppl 1:77-90.

Misko A, Jiang S, Wegorzewska I, Milbrandt J, Baloh RH. (2010) Mitofusin 2 is necessary for transport of axonal mitochondria and interacts with the Miro/Milton complex. J Neurosci. 30:4232-4240.

Miteva YV, Budayeva HG, Cristea IM. (2013) Proteomics-based methods for discovery, quantification, and validation of protein-protein interactions. Anal Chem. 85:749-768.

Moors T, Paciotti S, Chiasserini D, Calabresi P, Parnetti L, Beccari T, van de Berg WD. (2016) Lysosomal Dysfunction and α-Synuclein Aggregation in Parkinson's Disease: Diagnostic Links. Mov Disord. 31:791-801.

Morais VA, Verstreken P, Roethig A, Smet J, Snellinx A, Vanbrabant M, Haddad D, Frezza C, Mandemakers W, Vogt-Weisenhorn D, Van Coster R, Wurst W, Scorrano L, De Strooper B. (2009) Parkinson's disease mutations in PINK1 result in decreased Complex I activity and deficient synaptic function. EMBO Mol Med. 1:99-111.

Mortiboys H, Johansen KK, Aasly JO, Bandmann O. (2010) Mitochondrial impairment in patients with Parkinson disease with the G2019S mutation in LRRK2. Neurology. 75:2017-2020.

Mosharov EV, Staal RG, Bové J, Prou D, Hananiya A, Markov D, Poulsen N, Larsen KE, Moore CM, Troyer MD, Edwards RH, Przedborski S, Sulzer D. (2006) Alpha-synuclein overexpression increases cytosolic catecholamine concentration. J Neurosci. 26:9304-9311.

Mozdy AD, McCaffery JM, Shaw JM. (2000) Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. J Cell Biol. 151:367-380.

Muñoz P, Huenchuguala S, Paris I, Segura-Aguilar J. (2012) Dopamine oxidation and autophagy. Parkinsons Dis. 2012:920953.

Mytilineou C, Werner P, Molinari S, Di Rocco A, Cohen G, Yahr MD. (1994) Impaired oxidative decarboxylation of pyruvate in fibroblasts from patients with Parkinson's disease. J Neural Transm Park Dis Dement Sect. 8:223-228.

Naghdi S, Hajnóczky G. (2016) VDAC2-specific cellular functions and the underlying structure. Biochim. Biophys. Acta. 1863:2503-2514.

Nandipati S, Litvan I. (2016) Environmental Exposures and Parkinson's Disease. Int J Environ Res Public Health. 13. pii: E881.

Narendra D, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, Cookson MR, Youle RJ. (2010a) PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 8:e1000298.

Narendra D, Kane LA, Hauser DN, Fearnley IM, Youle RJ. (2010b) p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. Autophagy. 6:1090–1106.

Navarro-Yepes J, Anandhan A, Bradley E, Bohovych I, Yarabe B, de Jong A, Ovaa H, Zhou Y, Khalimonchuk O, Quintanilla-Vega B, Franco R. (2016) Inhibition of Protein Ubiquitination by Paraquat and 1-Methyl-4-Phenylpyridinium Impairs Ubiquitin-Dependent Protein Degradation Pathways. Mol Neurobiol. 53:5229-5251.

Neupert W. (2016) Mitochondrial Gene Expression: A Playground of Evolutionary Tinkering. Annu Rev Biochem. 85:65-76.

Nicklas WJ, Vyas I, Heikkila RE. (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neu- rotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sciences. 36:2503– 2508.

Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C, Bracale R, Valerio A, Francolini M, Moncada S, Carruba MO. (2003) Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science. 299:896-899.

Nisoli E, Falcone S, Tonello C, Cozzi V, Palomba L, Fiorani M, Pisconti A, Brunelli S, Cardile A, Francolini M, Cantoni O, Carruba MO, Moncada S, Clementi E. (2004) Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. Proc Natl Acad Sci USA. 101:16507-16512.

Niu J, Yu M, Wang C, Xu Z. (2012) Leucine-rich repeat kinase 2 disturbs mitochondrial dynamics via Dynamin-like protein. J Neurochem. 122:650-658.

Okatsu K, Saisho K, Shimanuki M, Nakada K, Shitara H, Sou YS, Kimura M, Sato S, Hattori N, Komatsu M, Tanaka K, Matsuda N. (2010) p62/SQSTM1 cooperates with Parkin for perinuclear clustering of depolarized mitochondria. Genes Cells. 15:887–900.

Olichon A, Baricault L, Gas N, Guillou E, Valette A, Belenguer P, Lenaers G. (2003) Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. J Biol Chem. 278:7743-7746.

Ong SE, Mann M. (2005) Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol. 1:252-262.

Ongwijitwat S, Wong-Riley MT. (2005) Is nuclear respiratory factor 2 a master transcriptional coordinator for all ten nuclear-encoded cy- tochrome c oxidase subunits in neurons? Gene. 360: 65-77.

Ongwijitwat S, Liang HL, Graboyes EM, Wong-Riley MT. (2006) Nu- clear respiratory factor 2 senses changing cellular energy demands and its silencing down-regulates cytochrome oxidase and other target gene mRNAs. Gene. 374:39-49.

Ordureau A, Sarraf SA, Duda DM, Heo JM, Jedrychowski MP, Sviderskiy VO, Olszewski JL, Koerber JT, Xie T, Beausoleil SA, Wells JA, Gygi SP, Schulman BA, Harper JW. (2014) Quantitative proteomics reveal a feedforward mechanism for mitochondrial PARKIN translocation and ubiquitin chain synthesis. Mol Cell. 56:360–375.

Ossowska K, Wardas J, Smiałowska M, Kuter K, Lenda T, Wierońska JM, Zieba B, Nowak P, Dabrowska J, Bortel A, Kwieciński A, Wolfarth S. (2005) A slowly developing dysfunction of dopaminergic nigrostriatal neurons induced by long-term paraquat administration in rats: an animal model of preclinical stages of Parkinson's disease? Eur J Neurosci. 22:1294-1304.

Otera H, Wang C, Cleland MM, Setoguchi K, Yokota S, Youle RJ, Mihara K. (2010) Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J Cell Biol. 191:1141-1158.

Otera H, Mihara K. (2011) Molecular mechanisms and physiologic functions of mitochondrial dynamics. J Biochem. 149:241-251.

Pacelli C, De Rasmo D, Signorile A, Grattagliano I, di Tullio G, D'Orazio A, Nico B, Comi GP, Ronchi D, Ferranini E, Pirolo D, Seibel P, Schubert S, Gaballo A, Villani G, Cocco T. (2011) Mitochondrial defect and PGC-1α dysfunction in parkin-associated familial Parkinson's disease. Biochim Biophys Acta. 1812:1041-1053.

Palikaras K, Tavernarakis N. (2014) Mitochondrial homeostasis: the interplay between mitophagy and mitochondrial biogenesis. Exp Gerontol. 56:182-188.

Pallanck LJ. (2010) Culling sick mitochondria from the herd. J Cell Biol. 191:1225-1227.

Palmer CS, Osellame LD, Laine D, Koutsopoulos OS, Frazier AE, Ryan MT. (2011) MiD49 and MiD51, new components of the mitochondrial fission machinery. EMBO Rep. 12:565-573.

Palmfeldt J, Bross P. (2017) Proteomics of human mitochondria. Mitochondrion. 33:2-14.

Papa S, De Rasmo D. (2013) Complex I deficiencies in neurological disorders. Trends. Mol. Med. 19:61-69.

Parihar MS, Parihar A, Fujita M, Hashimoto M, Ghafourifar P. (2008) Mitochondrial association of alpha-synuclein causes oxidative stress. Cell Mol Life Sci. 65:1272-1284.

Paris I, Perez-Pastene C, Cardenas S, Iturriaga-Vasquez P, Muñoz P, Couve E, Caviedes P, Segura-Aguilar J. (2010) Aminochrome induces disruption of actin, alpha-, and beta-tubulin cytoskeleton networks in substantia-nigra-derived cell line. Neurotox Res. 18:82-92.

Parone PA, Da Cruz S, Tondera D, Mattenberger Y, James DI, Maechler P, Barja F, Martinou JC. (2008) Preventing mitochondrial fission impairs mitochondrial function and leads to loss of mitochondrial DNA. PLoS One. 3:e3257.

Perier C, Tieu K, Guegan C, Caspersen C, Jackson-Lewis V, Carelli V, Martinuzzi A, Hirano M, Przedborski S, Vila M. (2005) Complex I deficiency primes Bax- dependent neuronal apoptosis through mitochondrial oxidative damage. Proceedings of the National Academy of Sciences of the United States of America. 102:19126–19131.

Perier C, Bove J, Wu DC, Dehay B, Choi DK, Jackson-Lewis V, Rathke-Hartlieb S, Bouillet P, Strasser A, Schulz JB, Przedborski S, Vila M. (2007) Two molecular pathways initiate mitochondria-dependent dopaminergic neurode- generation in experimental Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America. 104:8161–8166.

Perier C, Vila M. (2012) Mitochondrial biology and Parkinson's disease. Cold Spring Harb Perspect Med. 2:a009332.

Pham AH, Meng S, Chu QN, Chan DC. (2012) Loss of Mfn2 results in progressive, retrograde degeneration of dopaminergic neurons in the nigrostriatal circuit. Hum Mol Genet. 21:4817-4826.

Piao Y, Kim HG, Oh MS, Pak YK. (2012) Overexpression of TFAM, NRF-1 and myr-AKT protects the MPP(+)-induced mitochondrial dysfunctions in neuronal cells. Biochimica et Biophysica Acta. 1820:577–585.

Pickrell AM, Youle RJ. (2015) The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. Neuron. 2015;85:257–273.

Pienaar IS, Daniels WM, Götz J. (2008) Neuroproteomics as a promising tool in Parkinson's disease research. J Neural Transm (Vienna). 115:1413-1430.

Pienaar IS, Dexter DT, Burkhard PR. (2010) Mitochondrial proteomics as a selective tool for unraveling Parkinson's disease pathogenesis. Expert Rev Proteomics. 7:205-226.

Pieroni L, Levi Mortera S, Greco V, Sirolli V, Ronci M, Felaco P, Fucci G, De Fulviis S, Massoud R, Condò S, Capria A, Di Daniele N, Bernardini S, Urbani A, Bonomini M. (2015) Biocompatibility assessment of haemodialysis membrane materials by proteomic investigations. Mol Biosyst.11:1633-1643.

Pinti M, Gibellini L, Nasi M, De Biasi S, Bortolotti CA, Iannone A, Cossarizza A. (2016) Emerging role of Lon protease as a master regulator of mitochondrial functions. Biochim Biophys Acta. 1857:1300-1306.

Piras C, Soggiu A, Greco V, Martino PA, Del Chierico F, Putignani L, Urbani A, Nally JE, Bonizzi L, Roncada P. (2015) Mechanisms of antibiotic resistance to enrofloxacin in uropathogenic Escherichia coli in dog. J Proteomics. 127:365-376.

Plun-Favreau H, Klupsch K, Moisoi N, Gandhi S, Kjaer S, Frith D, Harvey K, Deas E, Harvey RJ, McDonald N, Wood NW, Martins LM, Downward J. (2007) The mitochondrial protease HtrA2 is regulated by Parkinson's disease-associated kinase PINK1. Nat Cell Biol. 9:1243-1252.

Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkmann J, Schrag AE, Lang AE. (2017) Parkinson disease. Nat Rev Dis Primers. 3:17013.

Polanowska J, Martin JS, Fisher R, Scopa T, Rae I, Boulton SJ. (2004) Tandem immunoaffinity purification of protein complexes from Caenorhabditis elegans. Biotechniques. 36:778-780.

Polymeropoulos MH, Higgins JJ, Golbe LI, Johnson WG, Ide SE, Di Iorio G, Sanges G, Stenroos ES, Pho LT, Schaffer AA, Lazzarini AM, Nussbaum RL, Duvoisin RC. (1996) Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. Science. 274:1197-1199.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. 276:2045-2047.

Poole AC, Thomas RE, Andrews LA, McBride HM, Whitworth AJ, Pallanck LJ. (2008) The PINK1/Parkin pathway regulates mitochondrial morphology. Proc Natl Acad Sci U S A. 105:1638-1643.

Puigserver P, Spiegelman BM. (2003) Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. Endocr Rev. 24:78-90.

Rafelski SM. (2013) Mitochondrial network morphology: building an integrative, geometrical view. BMC Biol. 11:71.

Rakovic A, Grünewald A, Kottwitz J, Brüggemann N, Pramstaller PP, Lohmann K, Klein C. (2011) Mutations in PINK1 and Parkin impair ubiquitination of Mitofusins in human fibroblasts. PLoS One. 6:e16746.

Ramonet D, Perier C, Recasens A, Dehay B, Bové J, Costa V, Scorrano L, Vila M. (2013) Optic atrophy 1 mediates mitochondria remodeling and dopaminergic neurodegeneration linked to complex I deficiency. Cell Death Differ. 20:77-85.

Ramsay RR, Salach JI, Dadgar J, Singer TP. (1986) Inhibition of mitochondrial NADH dehydrogenase by pyridine derivatives and its possible relation to experimental and idiopathic parkinsonism. Biochemical and Biophysical Research Communications. 135:269–275.

Reeve AK, Ludtmann MH, Angelova PR, Simcox EM, Horrocks MH, Klenerman D, Gandhi S, Turnbull DM, Abramov AY. (2015) Aggregated α-synuclein and complex I deficiency: exploration of their relationship in differentiated neurons. Cell Death Dis. 6:e1820.

Rugarli EI, Langer T. (2012) Mitochondrial quality control: a matter of life and death for neurons. EMBO J. 31:1336-1349.

Saez-Atienzar S, Bonet-Ponce L, Blesa JR, Romero FJ, Murphy MP, Jordan J, Galindo MF. (2014) The LRRK2 inhibitor GSK2578215A induces protective autophagy in SH-SY5Y cells: involvement of Drp-1-mediated mitochondrial fission and mitochondrial-derived ROS signaling. Cell Death Dis. 5:e1368.

Saiki S, Sato S, Hattori N. (2012) Molecular pathogenesis of Parkinson's disease: update. J Neurol Neurosurg Psychiatry 83:430-436.

Sarraf SA, Raman M, Guarani-Pereira V, Sowa ME, Huttlin EL, Gygi SP, Harper JW. (2013) Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. Nature. 496:372–376.

Scarpulla RC. (1999) Nuclear transcription factors in cytochrome c and cytochrome oxidase expression. Frontiers of Cellular Bioenergetics. 553-591.

Scarpulla RC. (2008) Transcriptional paradigms in Mammalian mitochondrial biogenesis and function. Physiol Rev. 88:611–638.

Scarpulla RC. (2012) Nucleus-encoded regulators of mitochondrial function: integration of respiratory chain expression, nutrient sensing and metabolic stress. Biochim Biophys Acta. 1819:1088-1097.

Scarpulla RC, Vega RB, Kelly DP. (2012) Transcriptional integration of mitochondrial biogenesis. Trends Endocrinol Metab. 23:459-466.

Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. (1990) Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem. 54:823-827.

Schapira AH, Tolosa E. (2010) Molecular and clinical prodrome of Parkinson disease: implications for treatment. Nature Reviews Neurology, 6:309-317.

Schapira AH, Jenner P. (2011) Etiology and pathogenesis of Parkinson's disease. Mov Disord. 26:1049-1055.

Schapira AH. (2015) Glucocerebrosidase and Parkinson disease: Recent advances. Mol Cell Neurosci. 66:37-42.

Schneider CA, Rasband WS, Eliceiri KW. (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671-675.

Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA, Korsmeyer SJ. (2002) A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell. 2:55-67.

Segura-Aguilar J, Paris I, Muñoz P, Ferrari E, Zecca L, Zucca FA. (2014) Protective and toxic roles of dopamine in Parkinson's disease. J Neurochem. 129:898-915.

Sherer TB, Betarbet R, Stout AK, Lund S, Baptista M, Panov AV, Cookson MR, Greenamyre JT. (2002) An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. J Neurosci. 22:7006-7015.

Sherer TB, Kim JH, Betarbet R, Greenamyre JT. (2003) Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. Exp Neurol. 179:9-16.

Shiba-Fukushima K, Imai Y, Yoshida S, Ishihama Y, Kanao T, Sato S, Hattori N. (2012) PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy. Sci Rep. 2:1002.

Shimizu K, Ohtaki K, Matsubara K, Aoyama K, Uezono T, Saito O, Suno M, Ogawa K, Hayase N, Kimura K, Shiono H. (2001) Carrier-mediated processes in blood—brain barrier penetration and neural uptake of paraquat. Brain Res. 906:135-142.

Shimizu K, Matsubara K, Ohtaki K, Fujimaru S, Saito O, Shiono H. (2003) Paraquat induces long-lasting dopamine overflow through the excitotoxic pathway in the striatum of freely moving rats. Brain Res. 976:243-252.

Shimura H, Hattori N, Kubo Si, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K, Suzuki T. (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat Genet. 25:302-305.

Shin JH, Ko HS, Kang H, Lee Y, Lee YI, Pletinkova O, Troconso JC, Dawson VL, Dawson TM. (2011) PARIS (ZNF746) repression of PGC-1α contributes to neurodegeneration in Parkinson's disease. Cell. 144:689-702.

Shoshan-Barmatz V, Keinan N, Zaid H. (2008) Uncovering the role of VDAC in the regulation of cell life and death. J. Bioenerg. Biomembr. 40:183-191.

Shults CW. (2006) Lewy bodies. Proc Natl Acad Sci U S A. 103:1661-1668.

Sim CH, Lio DS, Mok SS, Masters CL, Hill AF, Culvenor JG, Cheng HC. C-terminal truncation and Parkinson's disease-associated mutations down-regulate the protein serine/threonine kinase activity of PTEN-induced kinase-1. Hum Mol Genet. 365:412-415.

Singleton AB, 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 MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K. (2003) Alpha-Synuclein locus triplication causes Parkinson's disease. Science. 302:841.

Song Z, Ghochani M, McCaffery JM, Frey TG, Chan DC. (2009) Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. Mol Biol Cell. 20:3525-3532.

Strappazzon F, Vietri-Rudan M, Campello S, Nazio F, Florenzano F, Fimia GM, Piacentini M, Levine B, Cecconi F. (2011) Mitochondrial BCL-2 inhibits AMBRA1-induced autophagy. EMBO J. 30:1195–1208.

Strauss KM, Martins LM, Plun-Favreau H, Marx FP, Kautzmann S, Berg D, Gasser T, Wszolek Z, Müller T, Bornemann A, Wolburg H, Downward J, Riess O, Schulz JB, Krüger R. (2005) Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. Hum Mol Genet. 14:2099-2111.

Strittmatter EF, Ferguson PL, Tang K, Smith RD. (2003) Proteome analyses using accurate mass and elution time peptide tags with capillary LC time-of-flight mass spectrometry. J Am Soc Mass Spectrom. 14:980-991.

Subramaniam SR, Chesselet MF. (2013) Mitochondrial dysfunction and oxidative stress in Parkinson's disease. Prog Neurobiol. 106-107:17-32.

Sun M, Latourelle JC, Wooten GF, Lew MF, Klein C, Shill HA, Golbe LI, Mark MH, Racette BA, Perlmutter JS, Parsian A, Guttman M, Nicholson G, Xu G, Wilk JB, Saint-Hilaire MH, DeStefano AL, Prakash R, Williamson S, Suchowersky O, Labelle N, Growdon JH, Singer C, Watts RL, Goldwurm S, Pezzoli G, Baker KB, Pramstaller PP, Burn DJ, Chinnery PF, Sherman S, Vieregge P, Litvan I, Gillis T, MacDonald ME, Myers RH, Gusella JF. (2006) Influence of heterozygosity for parkin mutation on onset age in familial Parkinson disease: the GenePD study. Arch Neurol. 63:826-832.

Sun Y, Vashisht AA, Tchieu J, Wohlschlegel JA, Dreier L. (2012) Voltage-dependent anion channels (VDACs) recruit Parkin to defective mitochondria to promote mitochondrial autophagy. J Biol Chem. 287:40652-40660.

Takahashi Y, Kako K, Arai H, Oishi T, Inada Y, Tkehara A, Fukamizu A, Munekata E. (2002) Characterization and identification of promoter elements in the mouse COX17 gene. Biochim Biophys Acta. 1574:359–364.

Tal MC, Sasai M, Lee HK, Yordy B, Shadel GS, Iwasaki A. (2009) Absence of autophagy results in reactive oxygen species-dependent amplification of RLR signaling. Proc Natl Acad Sci USA. 106:2770–2775.

Tamai S, Iida H, Yokota S, Sayano T, Kiguchiya S, Ishihara N, Hayashi J, Mihara K, Oka T. (2008) Characterization of the mitochondrial protein LETM1, which maintains the mitochondrial tubular shapes and interacts with the AAA-ATPase BCS1L. J Cell Sci. 121:2588-2600.

Tanaka A, Cleland MM, Xu S, Narendra DP, Suen DF, Karbowski M, Youle RJ. (2010) Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. J Cell Biol. 191:1367-1380.

Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, Marras C, Bhudhikanok GS, Kasten M, Chade AR, Comyns K, Richards MB, Meng C, Priestley B, Fernandez HH, Cambi F, Umbach DM, Blair A, Sandler DP, Langston JW. (2011) Rotenone, paraquat, and Parkinson's disease. Environ Health Perspect. 119:866-872.

Tiranti V, Viscomi C, Hildebrandt T, Di Meo I, Mineri R, Tiveron C, Levitt MD, Prelle A, Fagiolari G, Rimoldi M, Zeviani M. (2009) Loss of ETHE1, a mitochondrial dioxygenase, causes fatal sulfide toxicity in ethylmalonic encephalopathy. Nat Med. 15:200-205.

Twig G, Hyde B, Shirihai OS. (2008) Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. Biochim Biophys Acta. 1777:1092-1097.

Tyers M, Mann M. (2003) From genomics to proteomics. Nature. 422:193-197.

Unlü M, Morgan ME, Minden JS. (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis. 18:2071-2077.

Vakifahmetoglu-Norberg H, Ouchida AT, Norberg E. (2017) The role of mitochondria in metabolism and cell death. Biochem Biophys Res Commun. 482:426-431.

Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, González-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, Wood NW. (2004a) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science. 304:1158-1160.

Valente EM, Salvi S, Ialongo T, Marongiu R, Elia AE, Caputo V, Romito L, Albanese A, Dallapiccola B, Bentivoglio AR. (2004b) PINK1 mutations are associated with sporadic early-onset parkinsonism. Ann Neurol. 56:336-341.

Van Humbeeck C, Cornelissen T, Hofkens H, Mandemakers W, Gevaert K, De Strooper B, Vandenberghe W. (2011) Parkin interacts with Ambra1 to induce mitophagy. J Neurosci. 31:10249–10261.

Van Laar VS, Mishizen AJ, Cascio M, Hastings TG. (2009) Proteomic identification of dopamine-conjugated proteins from isolated rat brain mitochondria and SH-SY5Y cells. Neurobiol Dis. 34:487-500.

Vande Walle L, Lamkanfi M, Vandenabeele P. (2008) The mitochondrial serine protease HtrA2/Omi: an overview. Cell Death Differ. 15:453-460.

Vekrellis K, Xilouri M, Emmanouilidou E, Rideout HJ, Stefanis L. (2011) Pathological roles of α-synuclein in neurological disorders. Lancet Neurol. 10:1015-1025.

Velayati A, Yu WH, Sidransky E. (2010) The role of glucocerebrosidase mutations in Parkinson disease and Lewy body disorders. Current neurology and neuroscience reports. 10:190-198.

Vila M, Przedborski S. (2003) Targeting programmed cell death in neurodegenerative diseases. Nat Rev Neurosci. 4:365-375.

Villoslada P, Steinman L, Baranzini SE. (2009) Systems biology and its application to the understanding of neurological diseases. Ann Neurol. 65:124-139.

Virbasius JV, Scarpulla RC. (1994) Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. Proc Natl Acad Sci U S A. 91:1309-1313.

Wallace DC. (1999) Mitochondrial diseases in man and mouse. Science. 283:1482–1488.

Walther TC, Mann M. (2010) Mass spectrometry-based proteomics in cell biology. J Cell Biol. 190:491-500.

Wang G, Mao Z. (2014) Chaperone-mediated autophagy: roles in neurodegeneration. Transl Neurodegener. 3:20.

Wang H, Song P, Du L, Tian W, Yue W, Liu M, Li D, Wang B, Zhu Y, Cao C, Zhou J, Chen Q. (2011) Parkin ubiquitinates Drp1 for proteasome-dependent degradation: implication of dysregulated mitochondrial dynamics in Parkinson disease. J Biol Chem. 286:11649-11658.

Wang X, Winter D, Ashrafi G, Schlehe J, Wong YL, Selkoe D, Rice S, Steen J, LaVoie MJ, Schwarz TL. (2011) PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell. 147:893–906.

Wang X, Yan MH, Fujioka H, Liu J, Wilson-Delfosse A, Chen SG, Perry G, Casadesus G, Zhu X. (2012a) LRRK2 regulates mitochondrial dynamics and function through direct interaction with DLP1. Hum Mol Genet. 21:1931-1944.

Wang X, Petrie TG, Liu Y, Liu J, Fujioka H, Zhu X. (2012b) Parkinson's disease-associated DJ-1 mutations impair mitochondrial dynamics and cause mitochondrial dysfunction. J Neurochem. 121:830-839.

Wang X, Hai C. (2016) Novel insights into redox system and the mechanism of redox regulation. Mol Biol Rep. 43:607-628.

Washbourne P, McAllister AK. (2002) Techniques for gene transfer into neurons. Curr Opin Neurobiol. 12:566-573.

Wiedemann N, Frazier AE, Pfanner N. (2004) The protein import machinery of mitochondria. J Biol Chem. 279:14473-14476.

Wilkins MR, Sanchez JC, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF, Williams KL. (1996) Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Eng Rev. 13:19-50.

Wu B, Song B, Tian S, Huo S, Cui C, Guo Y, Liu H. (2012) Central nervous system damage due to acute paraquat poisoning: a neuroimaging study with 3.0 T MRI. Neurotoxicology. 33:1330-1337.

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell. 98:115-124.

Xilouri M, Brekk OR, Stefanis L. (2016) Autophagy and Alpha-Synuclein: Relevance to Parkinson's Disease and Related Synucleopathies. Mov Disord. 31:178–192.

Xu R, Hu Q, Ma Q, Liu C, Wang G. (2014) The protease Omi regulates mitochondrial biogenesis through the GSK3β/PGC-1α pathway. Cell Death Dis. 5:e1373.

Yang Y, Ouyang Y, Yang L, Beal MF, McQuibban A, Vogel H, Lu B. (2008) Pink1 regulates mitochondrial dynamics through interaction with the fission/fusion machinery. Proc Natl Acad Sci U S A. 105:7070-7075.

Yin F, Cadenas E. (2015) Mitochondria: the cellular hub of the dynamic coordinated network. Antioxid. Redox Signaling. 22:961−964.

Yoon Y, Krueger EW, Oswald BJ, McNiven MA. (2003) The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. Mol. Cell. Biol. 23:5409–5420.

Youle RJ, Narendra DP. (2011) Mechanisms of mitophagy. Nat Rev Mol Cell Biol. 12:9–14.

Yu W, Sun Y, Guo S, Lu B. (2011) The PINK1/Parkin pathway regulates mitochondrial dynamics and function in mammalian hippocampal and dopaminergic neurons. Hum Mol Genet. 20:3227-3240.

Yumino K, Kawakami I, Tamura M, Hayashi T, Nakamura M. (2002) Paraquat- and diquat-induced oxygen radical generation and lipid peroxidation in rat brain microsomes. J Biochem. 131:565-570.

Zanellati MC, Monti V, Barzaghi C, Reale C, Nardocci N, Albanese A, Valente EM, Ghezzi D, Garavaglia B. (2015) Mitochondrial dysfunction in Parkinson disease: evidence in mutant PARK2 fibroblasts. Front Genet. 6:78.

Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atares B, Llorens V, Gomez Tortosa E, del Ser T, Munoz DG, de Yebenes JG. (2004) The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Annals of neurology. 55:164–173.

Zhang A, Sun H, Wu G, Sun W, Yuan Y, Wang X. (2013) Proteomics analysis of hepatoprotective effects for scoparone using MALDI-TOF/TOF mass spectrometry with bioinformatics. OMICS. 17:224-229.

Zhang X, Zhou JY, Chin MH, Schepmoes AA, Petyuk VA, Weitz KK, Petritis BO, Monroe ME, Camp DG, Wood SA, Melega WP, Bigelow DJ, Smith DJ, Qian WJ, Smith RD. (2010) Region-specific protein abundance

changes in the brain of MPTP-induced Parkinson's disease mouse model. Journal of Proteome Research. 9:1496–1509.

Zhang Y, Gao J, Chung KK, Huang H, Dawson VL, Dawson TM. (2000) Parkin functions as an E2-dependent ubiquitin–protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proceedings of the National Academy of Sciences. 97:13354-13359.

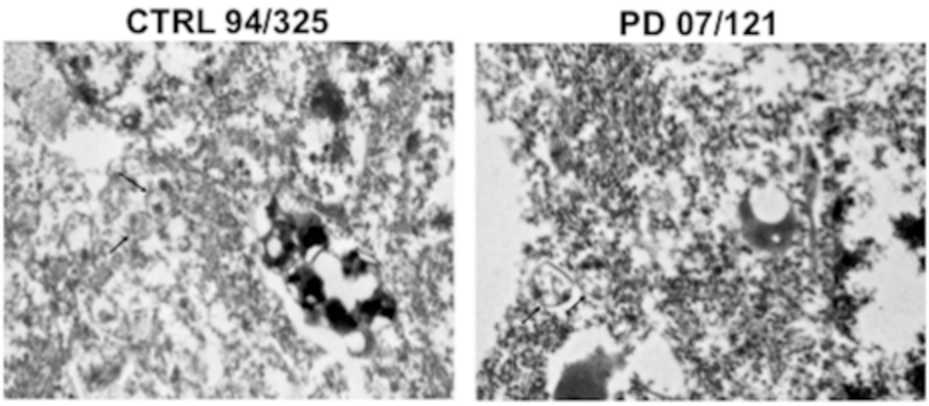
Zhang Y, Chan DC. (2007) New insights into mitochondrial fusion. FEBS Lett. 581:2168-2173.

Zhou ZD, Lim TM. (2009) Dopamine (DA) induced irreversible proteasome inhibition via DA derived quinones. Free Radic Res. 43:417-430.

Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S, Kachergus J, Hulihan M, Uitti RJ, Calne DB, Stoessl AJ, Pfeiffer RF, Patenge N, Carbajal IC, Vieregge P, Asmus F, Müller-Myhsok B, Dickson DW, Meitinger T, Strom TM, Wszolek ZK, Gasser T. (2004) Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron. 44:601-607.

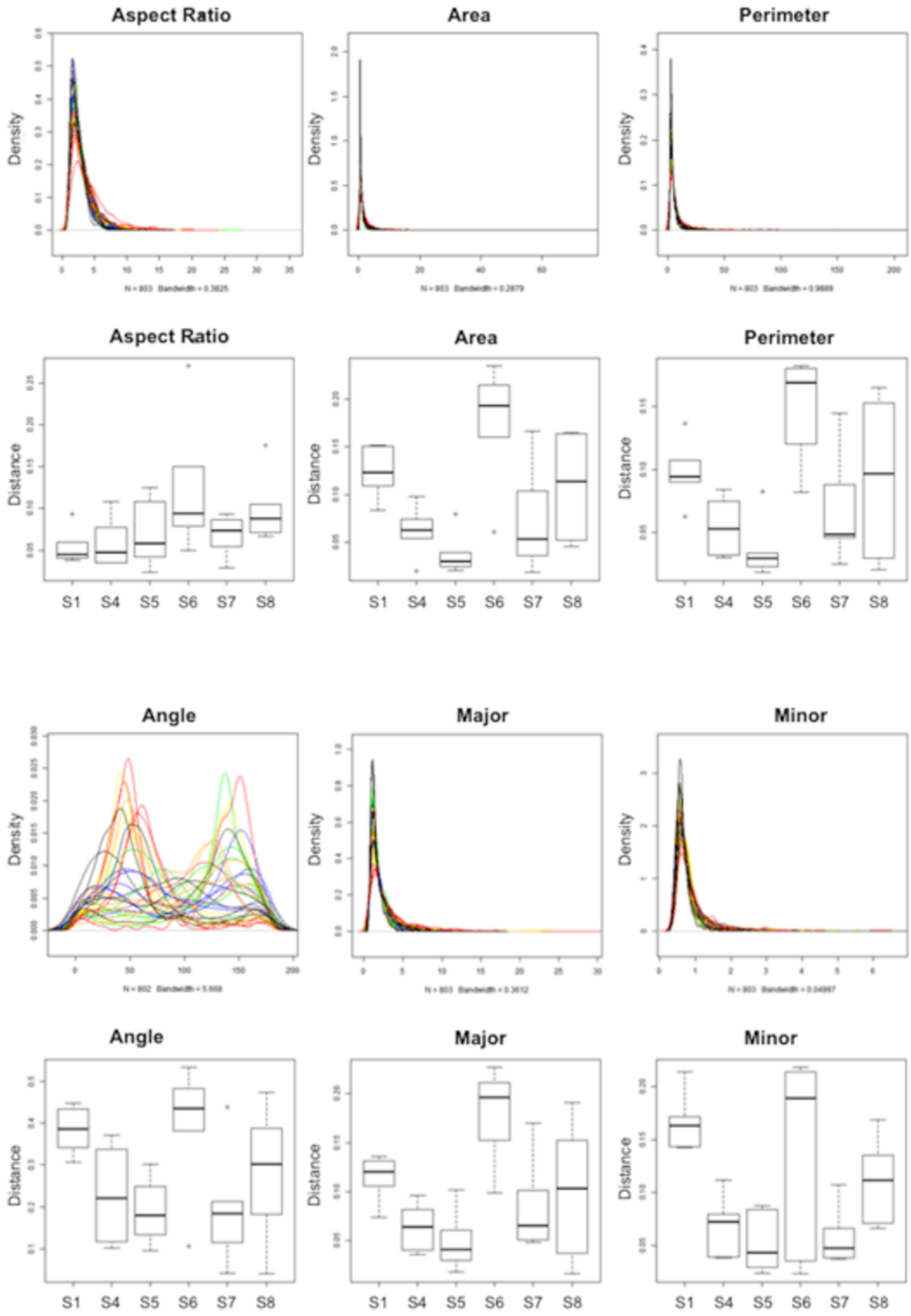
7. Supplementary figures

Supplementary Figure 1



Supplementary Figure 1. Morphological analysis of one control subject and one sporadic PD patient. Representative transmission electron microscopy images of Substantia nigra neurons taken at high magnification Arrows indicate mitochondria of both control subject and PD patient.

Supplementary Figure 2



Supplementary Figure 2. Analysis of mitochondrial network morphology. Distribution density (for all particles analyzed in six fovs from each subject) and Kolmogorov-Smirnov distances of aspect ratio, area, perimeter, angle, major axis and minor axis. PARK2-mutated patients (Fibroblasts S1, S4 and S5); control subjects (Fibroblasts S6, S7 and S8). Yellow: S1; Green: S4; Blue: S5; Red: S6; Orange: S7; Black: S8.

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9. Publications, Poster and Awards

- ✓ Bondi H, **Zilocchi M**, Mare MG, D'Agostino G, Giovannardi S, Ambrosio S, Fasano M, Alberio T. Dopamine induces mitochondrial depolarization without activating PINK1-mediated mitophagy. J Neurochem. **2016**. doi: 10.1111/jnc.13506.
- ✓ Alberio T, Pieroni L, Ronci M, Banfi C, Bongarzone I, Bottoni P, Brioschi M, Caterino M, Chinello C, Cormio A, Cozzolino F, Cunsolo V, Fontana S, Garavaglia B, Giusti L, Greco V, Lucacchini A, Maffioli E, Magni F, Monteleone F, Monti M, Monti V, Musicco C, Petrosillo G, Porcelli V, Saletti R, Scatena R, Soggiu A, Tedeschi G, **Zilocchi M**, Roncada P, Urbani A, Fasano M. Toward the Standardization of Mitochondrial Proteomics: The Italian Mitochondrial Human Proteome Project Initiative. J Proteome Res. **2017**. doi: 10.1021/acs.jproteome.7b00350.
- ✓ Article submitted and under review: "Mitophagy and mitochondrial dynamics are impaired in Parkinson's disease human samples and cellular models". **Zilocchi M**, Finzi G, Sessa F, Fasano M, Alberio T.
- ✓ Article submitted and under review: "Mitochondrial proteins in the development of Parkinson's disease". **Zilocchi M**, Fasano M, Alberio T.
- ✓ IX Annual Congress - EuPA 23-28th June 2015. **Poster**: "Identification of FBXO7 interactors: the role of UBL domain in Parkinson's disease". **Zilocchi M**, Alberio T, Mandemakers W, Peroni D, Pieroni L, Urbani A, Bonifati V, Fasano M.
- ✓ XI Annual Conference - ItPA 16-19th May 2016. **Poster**: "Mitochondrial proteomics in cellular models of Parkinson's disease". **Zilocchi M**, Pieroni L, Ronci M, Alberio T, Fasano M. **Award: Best poster competition, third position.**
- ✓ **Award:** Fellowship "**PREMI DI STUDIO GIOVANI MERITEVOLI**" 2016 Fondazione ItPA Onlus to go to Regina (Canada) for 2 months in the lab. of Prof. Mohan Babu.
- ✓ XII Annual Conference – ItPA 12-15th June 2017. **Selected oral presentation**: "Proteomic and interactomic characterization of PARK2-mutated Parkinson's disease patients". **Zilocchi M**, Colugnat I, Pieroni L, Garavaglia B, Minic Z, Fasano M, Babu M, Alberio T.
- ✓ XVI Annual Conference – HUPO 17-21th September 2017. **Poster**: "Discovering proteome alterations in PARK2-mutated Parkinson's disease patients". **Zilocchi M**, Colugnat I, Pieroni L, Garavaglia B, Alberio T, Fasano M.