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Elucidating the functional interplay between Parkinson’s disease-related proteins and the mitochondrion

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“Ne abbiamo viste tante di cose io e te, eppure abbiamo ancora la voglia di guardare...”

- Mercante di liquori italiano del XIV secolo -

“We have seen so many things, me and you, but we still have the desire to observe...”

- Italian liquor tradesman of the XIV century -

τούτο γάρ ἐστὶν ὁ χρόνος, ἀριθμὸς κινήσεως κατὰ τὸ πρότερον καὶ ὕστερον
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To Aristotle, for conceiving Time as circular entity

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Abbreviations

ATGs: Autophagy-Related Proteins

Ca²⁺: calcium

CCCP: carbonyl cyanide 3-chlorophenylhydrazone; protophore (mitochondrial depolarizer)

DA: dopamine

DRP1: Dynamin-Related Protein 1; protein involved in mitochondrial fission

$\Delta\Psi$: mitochondrial membrane potential

FIS1: Fission Protein 1, protein involved in mitochondrial fission

FRET: Fluorescence Resonance Energy Transfer

IMM: Inner Mitochondrial Membrane

IMS: Intermembrane Space

HSD17B10: type 10 17hydroxysteroid dehydrogenase; mitochondrial matrix enzyme

Kb/Mb: 1.000/1.000.000 base pair

kDa: 1.000 Daltons

MFF: Mitochondrial Fission Factor, protein involved in mitochondrial fission

MFN 1/2: Mitofusin 1/2, proteins involved in mitochondrial fusion

MOMP: mitochondrial outer membrane permeabilization

MPP: Mitochondrial Processing Peptidase; mitochondrial matrix protease

mtDNA: mitochondrial DNA

MTS: Mitochondrial targeting sequence

OMM: Outer Mitochondrial Membrane

PINK1: P-TEN induced kinase 1

OPA1: Optic Atrophy 1, protein involved in mitochondrial fusion

PD: Parkinson's Disease

ROS: Reactive Oxygen Species

SNPc: Substantia Nigra Pars Compacta

TOM: Translocase of Outer Membrane; complex devoted to mitochondrial import throughout the OMM

TOMM subunits: TOMM5, 6, 7, 20, 22, 40, 70

TIM: Translocase of Inner Membrane; complex devoted to mitochondrial import throughout the IMM

TIMM subunits: TIMM9, 10, 12, 17, 18, 21, 22, 23, 50, 54

UPS: Ubiquitin-Proteasome System

VDAC1/2/3: Voltage-Dependent Anion Channel 1/2/3 located on the OMM

Chapter I. Introduction

I.1. Parkinson's disease

I.1.1 Physiopathological and genetic bases of Parkinson's disease

A. Overview on Parkinson's Disease.

Clinical signs and neuropathology

Parkinson's disease (PD) is a severe and progressive neurodegenerative disorder affecting nearly 2% of the world population above 60 years of age and 4-5% above 80, with a mean age of onset at 70 years [1]. The history of this syndrome is old: already known in ancient India and Egypt and observed by Leonardo da Vinci in the 16th century [2], it was first described in 1817 by James Parkinson in his "Essay on the shaking palsy" and then defined in greater detail by the French neurologist Jean-Martin Charcot in 1877 [3]. At least three world-leading dictators of the 20th century suffered from this syndrome: Adolf Hitler, Francisco Franco and Mao Tse-Tung. Nowadays, the incidence rate of the disease is 8-18 new cases over 100.000 subjects each year in developed countries, with a higher prevalence in the male population than in the female one. From the clinical point of view, PD is diagnosed when a classic tetrad of motor symptoms is present: uncontrollable resting tremor, postural imbalance, muscles rigidity, slowness of movement (bradykinesia) [4]. Nevertheless, this pathology is far more heterogeneous and to date, different subtypes of PD can be defined according to age at onset, progression of the disease and symptom manifestation; several degrees of cognitive decline and dementia are also ascribed [5][6]. Noteworthy, evidence indicates that a plethora of non-motor features, such as cardiac, urinary and olfactory dysfunctions, pain, sleep disturbance and depression, precede motor disability and are thus of importance for early diagnosis of PD [7][8].

The neuropathological hallmark of PD is the massive loss of dopaminergic neurons in *the substantia nigra pars compacta* (SNPc). The SNPc is a brain region belonging to the basal ganglia loop, in charge of evaluating environmental *stimuli* and further signalling to the striatum to promote or inhibit voluntary movements; this function requires the release of the neurotransmitter dopamine (DA) from dopaminergic neurons to striatal neurons through a vesicular transport pathway [9]. Another distinctive feature of PD is the presence of cytosolic proteinaceous inclusions termed Lewy Bodies (LBs) and composed of the PD-related protein α -synuclein, ubiquitin, neurofilaments and subcellular organelles, as mitochondria [10][11]. Concerning PD progression, six stages of the disease can be described according to diffusion of LBs in different regions of the brain [12]. Remarkably, as LBs spread and reach the SNPc, the four distinctive symptoms of the disease manifest; at present no direct correlation can be made between LB appearance and dopaminergic cell death, raising the question whether these aggregates are harmful to the nerve cell or rather innocuous.

In addition to dopaminergic cell death, non-dopaminergic neuronal populations in other brain regions are also partially lost, as serotonergic cells in raphe nuclei, noradrenergic neurons in the locus coeruleus, cholinergic cells in the pedunculopontine tegmental nucleus and in the *nucleus basalis* of Meynert, and hypocretin cells in the hypothalamus [13]. The partial loss of these neuronal subtypes (it attains up to 50% in late-stage PD) is of extreme importance to the progression of the disease, as it is responsible for the appearance of non-motor symptoms in the early phase of the syndrome. As pure motor features in PD patients appear only when 80% of dopaminergic neurons attained cell death [4], huge compensatory mechanisms are thought to be activated in the first phase of the disease to counteract the degeneration of the above brain regions at this stage.

Etiology of PD: mechanisms behind the vulnerability of dopaminergic neurons

During the last decades of research, a single or unifying cause for the onset of PD has not emerged yet, although several possible pathogenic mechanisms have been proposed so far. Given that dopaminergic cells appear to be the most susceptible neuronal subtype in PD, a first appealing hypothesis linked DA metabolism, oxidative stress and the death of neurons in the SNpc [14]. DA is neurotransmitter, a protein involved in the transmission of chemical signals from a neuron to a target cell through a synapse. DA is a highly reactive molecule synthesized in the cytoplasm of the nerve cell: rapidly auto-oxidized under its free form at physiological pH, it leads to the formation of dopamine-quinone species, superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals as a byproduct. When these compounds come in contact with proteins, lipids and nucleic acids in the cytosol, neurotoxic reactive species are produced. Alternatively, the cytosolic fraction of DA not readily used as a neurotransmitter is converted into a non-toxic compound through deamination by the mitochondrial enzyme monoamine oxidase (MAO); again, to fulfil this reaction H_2O_2 and hydroxyl radicals are produced [15]. Noteworthy, these reactions are all catalyzed by iron, which is found in higher concentrations in the SNpc than in other brain regions [16]. Altogether, to supply the neuron with dopamine for synaptic transmission and to protect the cell from the uncontrolled production of radicals, DA is readily sequestered into synaptic vesicles, which function as storage sites at low pH to impede DA auto-oxidation. Conversely, reduced sequestration of DA into synaptic vesicles and thus increased DA amounts in the cytosol could constitute a vulnerability factor for dopaminergic cell loss in PD. However, this dopamine toxicity hypothesis is not supported by the observation that administration of L-DOPA (a precursor of DA) as an alternative source of free DA does not enhance oxidative stress in PD patients [17]. Indeed, the administration of L-DOPA attenuates PD motor symptoms in the early phase of the disease, called "honeymoon", indicating that these features in PD depend on a reduced availability of DA. However, during long-term treatment, L-DOPA gradually becomes less efficient and motor features worsen again, and side-effects as dyskinesia and dystonia appear. As the mode of action of L-DOPA cannot be controlled after its administration, these side-effects are believed to be due to a toxic action of L-DOPA on the remaining dopaminergic neurons: additional oxidative stress is potentially produced through the formation of auto-

oxidation products of L-DOPA, increased DA availability and turnover [18]. A second possibility for the selective cell death of dopaminergic neurons compared to other catecholaminergic cells involves the composition in proteins of this neuronal cell type. Dopaminergic neurons are particularly rich in $\text{Ca}_v1.3 \text{ Ca}^{2+}$ channels, a selective ion channel subtype located on the plasma membrane, which allows the entry of Ca^{2+} from the extracellular space to the cytoplasm [19]. Whereas the majority of neurons use Na^+ and Na^+ -channels to regulate their pacemaking activity, dopaminergic neurons rely on Ca^{2+} [20]. Furthermore, compared to other regions of the brain where these channels are rare and open episodically in synaptic transmission events, in dopaminergic cells $\text{Ca}_v1.3 \text{ Ca}^{2+}$ channels are almost permanently active, enabling high concentrations of Ca^{2+} to enter the cytosol. Accordingly, deregulation of Ca^{2+} homeostasis in this particular subset of neurons has been proposed as a risk factor in PD [21].

The mitochondrial hypothesis

Environmental agents and toxins have long been considered as predisposing factors for PD onset. When viral agents causing the spread of the influenza pandemic after the First World War were identified, they were suspected to be the one of the principal causes for PD, as a subset of affected individuals developed postencephalitic parkinsonism (PEP), a syndrome presenting the symptomatic tetrad of PD, although without LB pathology [22]. Secondly, in 1980s it was observed that the neurotoxic compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), found as a contaminant of a synthetic opiate and acting as a heroine analogue, could induce symptoms highly resembling the ones observed in PD cases [23]. In the attempt to elucidate the mode of function of MPTP, it was discovered that its active metabolite 1-methyl-4-phenylpyridinium (MPP^+) inhibits the MAO enzyme. This potentially leads to an increase in the concentration of DA in the cytoplasm of nerve cells and in an enhancement of the production of H_2O_2 and hydroxyl radicals, resulting in an overall raise of DA-dependent oxidative stress [24]. Therefore, administration of MPTP to non-human mammals and rodents was widely employed for the creation of PD-related models [25]. As expected, degeneration of dopaminergic neurons in the SNpc was observed in these models after intoxication with MPTP, given that MPP^+ and dopamine rely on the same molecular transporter, the dopamine transporter (DAT), to enter this neuronal subtype [26–28]. Interestingly, no LBs were detected in brain samples from patients examined in the first study reporting the effects of MPTP in humans [23]; accordingly, acute administration of MPTP in primates and rodents does not induce the formation of LBs, although chronic treatment produces proteinaceous inclusions, positive for α -synuclein [29].

Broad-spectrum pesticides as rotenone were observed to trigger dopaminergic cell loss, α -synuclein- and ubiquitin-positive LBs, and a behavioural phenotype including bradykinesia and rigidity in rodents. Nevertheless, this compound does not enter via the DAT transporter and neuronal cell loss is not restricted to dopaminergic cells only [30,31]. The herbicide paraquat, widely used in agriculture, can also trigger a parkinsonian-like syndrome in animal models with modest nigrostriatal cell loss, although at present there is no

compelling evidence that exposure to paraquat causes PD in humans [32]. Remarkably, as DA, MPP⁺ and paraquat share common chemical features, it has been hypothesized that they act through a common molecular mechanism and enter *via* the DAT transporter [33], although this theory was confirmed only partially [34]. Accordingly with previous reports, this study indeed confirms that DA, MPTP and rotenone are lipophilic and they easily cross the blood-brain barrier using the DAT transporter, but it uncovers that the positively-charged paraquat molecule requires a neutral amino-acid transporter to enter the brain. Importantly, this paper corroborated the finding of the inhibitory activity of MPTP and rotenone towards the first complex of the mitochondrial respiratory chain (complex I), although paraquat was demonstrated not to share the same properties. The consequences of this blockade are severe: reduced ATP synthesis, Reactive Oxygen Species (ROS) production and oxidative stress, inhibition of proteasomal activity and a concomitant generation of proteolytic stress by accumulation of unfolded protein precursors, followed by cell death by apoptosis [35]. This mechanism of action nurtured the hypothesis that “mitochondrial dysfunction” may be central to the pathophysiology of PD. These observations were supported by the discovery that complex I alterations are present in the SNpc, in the frontal cortex and in peripheral tissues as platelets and skeletal muscle of sporadic PD patients [36–38], and complex III deficiencies in lymphocytes and platelets were also retrieved in patients with idiopathic PD [39]. Multiple somatic mitochondrial DNA (mtDNA) mutations and deletions challenging the mitochondrial respiratory chain accumulate in dopaminergic neurons of patients with late-stage idiopathic PD but also in aged-matched control cases [40], whereas a recent study provides the first body of evidence for an increase in the overall somatic mtDNA point mutations rate in patients with sporadic PD at an early stage [41]. Furthermore, it has to be noted that Ca²⁺ levels in the cytoplasm are tightly controlled by mitochondria, as Ca_v1.3 Ca²⁺ channels need a massive mitochondrial ATP production to pump Ca²⁺ ions inside the cell. In the attempt of providing additional elements linking mitochondria and PD, it has been proposed that maintenance of Ca²⁺ gradients would greatly enhance ATP production to support the pacemaking activity of dopaminergic neurons; such a situation may further enhance ROS production, oxidative stress and in the long term mtDNA mutations [21]. Taken together, mitochondrial stress in PD has emerged as a primary risk factor which may weaken dopaminergic neurons. The complex plethora of mitochondrial dysfunctions in PD is now the target of intensive research both at the molecular and at the therapeutic level, corroborated by the discovery that several genes linked to autosomal recessive PD code for proteins partially localized at the mitochondrion (c.f. Par 1.3.1).

In conclusion, it seems unlikely that a unique cause for PD exists. A large consensus has now been reached on the importance of mitochondrial dysfunctions. However, “multiple hits”, possibly combining toxic stress, other environmental risk factors and genetic susceptibility are today considered to be most often responsible for the disease [9][32]. Therefore, developing new tools to predict the interactions between these elements will most likely constitute the direction of basic and clinical research in the upcoming years.

Research in this direction will probably shed light on the mechanisms underlying variability in PD onset and symptoms and possibly allow for anticipation of disease diagnosis, offering new hope for the development of efficient pharmacological treatments to cure this multifaceted pathology.

Involvement of the ubiquitin-proteasome system in PD

Sporadic PD is a disorder characterized by the accumulation of misfolded proteins and the presence of proteinaceous LBs. Therefore it is conceivable that this disease is linked to protein handling systems, which become defective. Indeed, it has been reported that the structure and the function of the proteasome are decreased in the SNpc and in lymphocytes of PD patients with idiopathic PD, compared to aged-matched controls (for insights on the structure and function of the proteasome and on ubiquitylation c.f. Paragraph I.1.2A) [42,43]. In contrast, an increased proteasomal activity was observed in brain regions that do not degenerate during the course of this disease, including frontal cortex, striatum and cerebellum, suggesting that these areas provide compensatory responses potentially protecting against neurodegeneration [42,44,45]. Activators of the hydrolytic activity of the proteasome also show changes in abundance: in PD patients, several subunits of the activator protein PA700 were increased in non-affected brain regions as the frontal cortex and the striatum compared to controls. Concomitantly, the levels of the protein PA28 decreased in the SNpc of subjects carrying the disease [42]. These observations contribute to the hypothesis that in PD paradigms there could be a loss of proteasomal activity, leading to the formation of cytosolic inclusions as LBs. Accordingly, it has been proposed that increased proteasomal abundance in brain regions in which there is no marked DA loss could not be sufficient to mount a satisfactory compensatory response and thus overcome PD [42]. Remarkably, it has been observed that the administration of proteasomal inhibitors in rodents constitutes a model for PD, and although these data couldn't be fully reproduced, this model shows marked DA loss and the presence of proteinaceous aggregates highly similar to LBs [46,47]. A possible link for PD onset and proteasomal dysfunction has been corroborated by the extensive body of evidence reporting the involvement of PD-related proteins in the regulation of the UPS. Firstly two of these proteins, Parkin and UCHL1, act in the proteasome-targeted ubiquitylation cascade as a E3 ubiquitin-protein ligase and as a deubiquitinase (c.f. Paragraph I.1.2A). Secondly, Parkin physically interacts with the proteasome subunit Rpn10/S5a, which modulates the recognition of ubiquitylated substrates by the proteasome activator P700 [48]. Loss-of-function mutations on Parkin gene could potentially impair the ubiquitylation of key substrates of the protein, leading to the impaired degradation of proteasomal activity; nevertheless, this concept is yet to be fully proven.

Indeed, two major issues concerning the involvement of UPS dysfunctions in PD remain to be solved. First of all, the relationship between familial PD and UPS dysfunctions has not been fully established. In addition, it is not clear whether alterations of the UPS in idiopathic PD cases are a primary factor PD onset, or they rather arise as a consequence to a general disease-prone condition. Future research is mandatory to shed light on this potentially pivotal component of PD pathogenesis.

Conclusive remarks: how might we develop PD? A puzzled scenario

A possible pathway of SNpc degeneration leading to PD is visually represented in the figure below.

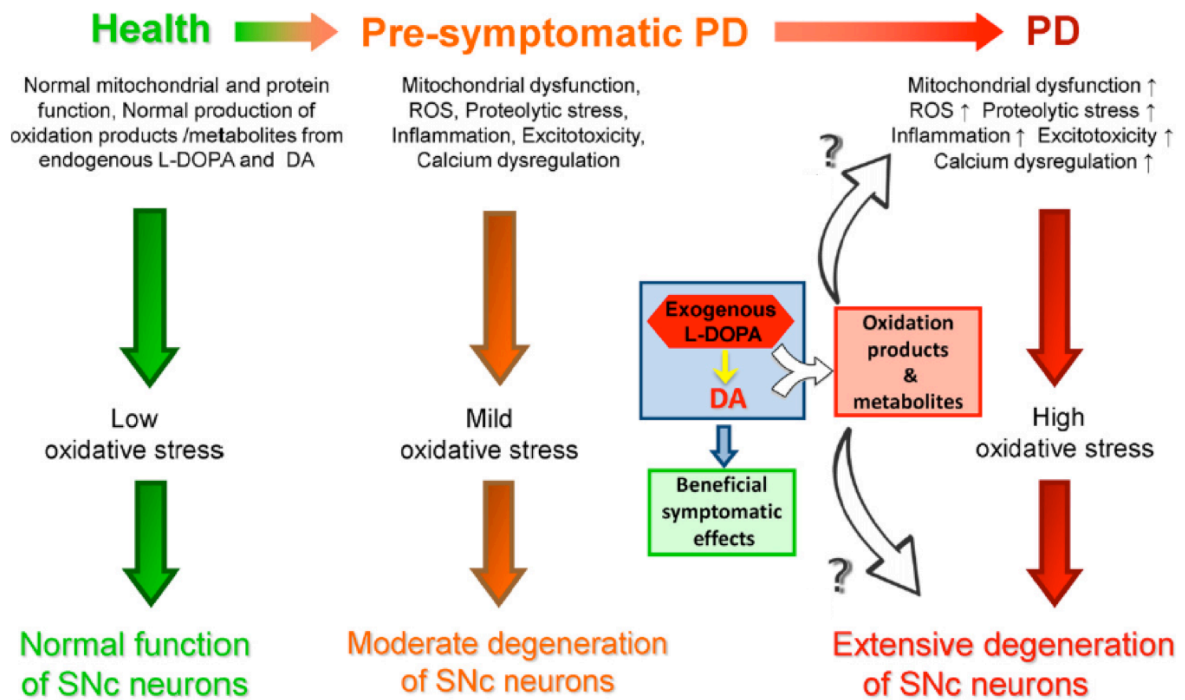


Figure 1. Schematic representation of the putative evolution of PD pathogenesis. Healthy DA neurons in the SNpc (green) suddenly start to show impaired features, as increased oxidative stress, mitochondrial dysfunctions, Ca^{2+} dyshomeostasis and proteolytic stress; due to these events, a moderate percentage of these neurons undergo cell death (orange). Treatment of patients with an exogenous source of L-DOPA initially promotes synthesis of DA and ameliorates motor symptoms, although prolonged administration of this compound might increase the concentration of oxidative stress byproducts and enhance mitochondrial/proteasomal impairments and alterations in Ca^{2+} abundance (red). This situation could potentially lead to a worsening of nigrostriatal cell loss and to PD. Adapted from [18].

B. One or several Parkinson's diseases? Sporadic versus monogenetic forms.

Until 1997, PD was classified as a typical non-genetic disorder. The diverging hypotheses on its etiology and the lack of concordance in studies concerning monozygotic twins privileged the idea of a disease caused mainly by environmental factors [49–51]. Nowadays, although the majority of PD patients show an idiopathic form of the disease, mutations in several genes with typical Mendelian inheritance have been found to be responsible for familial forms that constitute 10% of the cases. These genes played an essential role in the discovery of molecular pathways and biological processes altered in sporadic PD and suspected to involve the Ubiquitin-Proteasome System, lysosomal and mitochondrial dysfunctions, protein aggregation [4]. Genes were identified by taking advantage of three different approaches: linkage analyses, next-generation sequencing and Genome-Wide Association Studies (GWAS). With the first method, geneticists calculate the probability that a selected genomic region (locus) and/or specific genetic markers segregate with the disease;

loci linked to PD historically carried the PARK name and an identification number, until the mutated gene was found and given a specific name. Historically, this technique was the method employed the most to detect common polymorphism of PD-candidate genes in large families of patients, with a Mendelian transmission pattern. More recent techniques as Genome-Wide Association Scan (GWAS) and next-generation sequencing approaches (whole-genome and exome sequencing) aim at identifying factors present in the affected population but not in the age-, region-, ethnicity- and sex-matched controls. These factors are not disease-causing genes, but rather elements potentially increasing the risk to develop the disease. These approach based on the computational alignment of short sequences (between 70 and 125 bases) covering a selected genomic region in its entirety, and search for nucleotide differences from the human genome reference sequence in the attempt of associating these variants with a specific disease, of identifying potentially pathogenic repetitive elements and copy number changes [52]. Specific polymorphic variants, some of which bore by known PD-associated genes and thought to be risk factors to develop PD were identified; nevertheless, the results obtained with these approaches have not always been confirmed and our knowledge of the factors conferring risk to develop PD is unfortunately still partial [53].

The first evidence that PD has a genetic component was the discovery of mutations in the SNCA gene lead to PD [10]. It codes for α -synuclein, a protein successively found to be the major fibrillar constituent of LBs, therefore becoming a pivotal the neuropathological hallmark of sporadic and familial PD [54]. To date, 16 loci and 11 genes, both confirmed or putative ones, have been associated with Mendelian forms of PD, with autosomal dominant or recessive inheritance. Remarkably, there is a growing body of evidence showing a role for some of these PD-linked genes in idiopathic PD as well, in particular concerning the existence of polymorphic variants on *SNCA* and *LRRK2*, which are emerging as risk factors thanks to GWAS analyses [55–57]. Mutations on *PARK2* were also found in “isolated cases” of PD, indicating patients developing PD and unambiguously devoid of a Mendelian transmission pattern for the disease in their families [58] (Table 1). These data support the hypothesis that the “two syndromes” might be closely interlinked and might even represent two faces of the same disease, and highlight the essential contribution of genetics to decipher the molecular causes leading to PD onset.

PARK Loci	Gene	Map Position	Inheritance	Disease Onset	Mutations	Susceptibility Variants
<i>PD-associated loci and genes with conclusive evidence</i>						
PARK1/PARK4	SNCA	4q21	Dominant; rarely sporadic	Early onset	A30P, E46K, A53T genomic duplications/triplications	Promotor Rep1, 5' and 3' variants increase risk for PD
PARK8	LRRK2	12q12	Dominant; sporadic	Late onset	>80 Missense variants, >7 of them pathogenic, including the common G2019S	G2385R, R1628P increase risk for PD in Asian populations
PARK2	parkin	6q25-q27	Recessive; sporadic	Juvenile; early onset	Approximately 170 mutations (point mutations, exonic rearrangements)	Promoter polymorphisms increase risk for PD; heterozygous mutations may increase risk for late-onset PD
PARK6	PINK1	1p35-p36	Recessive	Early onset	Approximately 50 point mutations, rare large deletions	Heterozygous mutations may increase risk for late-onset PD
PARK7	DJ-1	1p36	Recessive	Early onset	Approximately 15 point mutations and large deletions	Heterozygous mutations may increase risk for late-onset PD
PARK9	ATP13A2	1p36	Recessive	Juvenile KRS, early-onset PD	>5 Point mutations	Heterozygous variants increase risk for PD
<i>PD-associated loci and genes with unknown relevance</i>						
PARK3	Unknown	2p13	Dominant	Late onset	Not identified	SPR variants may increase risk for PD
PARK5	UCHL1	4p14	Dominant	Late onset	One mutation in a single PD sibling pair	S18Y variant decreases risk for PD
PARK10	Unknown	1p32	Unclear	Late onset	Not identified	Unknown
PARK11	GIGYF2	2q36-q37	Dominant	Late onset	7 Missense variants	None
PARK12	Unknown	Xq21-q25	Unclear	Late onset	Not identified	Unknown
PARK13	Omi/HTRA2	2p13	Unclear	Late onset	2 Missense variants	Regulatory variants may contribute to risk for PD
PARK16	Unknown	1q32	Unclear	Unclear	Not identified	Polymorphic SNPs
<i>Loci and genes associated with atypical parkinsonism</i>						
PARK14	PLA2G6	22q12-q13	Recessive	Juvenile levodopa-responsive dystonia-parkinsonism	2 Missense mutations	Not investigated
PARK15	FBXO7	22q12-q13	Recessive	Early-onset parkinsonian-pyramidal syndrome	3 Point mutations	Not investigated
<i>PD-associated genes proposed by candidate gene approach</i>						
Not assigned	SCA2	12q24.1	Dominant for SCA2	Unclear	Low-range interrupted CAG expansions in SCA2	Not investigated
Not assigned	GBA	1q21	Recessive for GD	Unclear		Heterozygous GD-associated mutations increase risk for PD
<p>PD, Parkinson's disease; GD, Gaucher's disease; SCA2, spinocerebellar ataxia type 2; KRS, Kufor Rakeb syndrome; SNCA, α-synuclein; PINK1, PTEN-induced kinase 1; LRRK2, leucine-rich repeat kinase 2; SPR, sepiapterin reductase; UCHL1, ubiquitin C-terminal hydrolase 1; GIGYF2, GRB10-interacting GYF protein 2; PLA2G6, group VI phospholipase A₂; GBA, β-glucocerebrosidase; SNP, single polymorphism nucleotide.</p>						

Table 1. Summary of PD-associated loci and genes, chromosomal position, mode of transmission and age at onset, disease-causing mutations and susceptibility variants. From [4].

C. PD-related genes with autosomal dominant mode of inheritance

I. SNCA

Structurally, the SNCA gene is 117 kb long and comprises six exons. Linkage analyses essentially identified two types of PD-causing mutations, as single nucleotide substitutions and whole-locus rearrangements. Five rare pathogenic missense mutations leading to single amino acid substitutions in the α -synuclein protein sequence have been described: A30P, E46K, H50N, G51D, A53T [54,58–63]. These substitutions cause a parkinsonian syndrome with a wide phenotypic spectrum, varying from late to early onset, multiple degrees of dementia and motor symptoms; interestingly, the clinical phenotype induced by the A30P mutation is strikingly

similar to idiopathic PD, with late age at onset, LB pathology, presence of the classic tetrad of motor symptoms and cognitive decline [64].

Additional *SNCA* copies, ranging from two to 20 but usually present as duplicates or triplicates, are a more common cause of autosomal dominant PD: they represent nearly 2% of familial parkinsonism overall, and are also rarely found in PD cases with a sporadic origin [4]. *SNCA* multiplications induce a parkinsonian syndrome with a plethora of symptoms, including a predominantly early age at onset, LBs and different degrees of dementia; remarkably, the severity of the disease appears to be directly correlated with the *SNCA* copy number [65–67]. This observation has been corroborated by the discovery that a polymorphic increase in the dinucleotide repeat REP1, located in the promoter region of *SNCA*, enhances *SNCA* transcription and *SNCA*-induced toxicity in vitro and in vivo [68–71].

II. *LRRK2*

With its 51 exons, the large Leucine-rich repeat kinase 2 (*LRRK2*) covers a region of 144 Kb positioned close to the centromere of chromosome 12 [72,73]. Patients carrying mutations on this gene present clinical and neuropathological features similar to sporadic PD: age of onset at 60 years, tremor (the discovery of this gene in the basque population granted the protein *LRRK2* the alternative name of “Dardarin”, the basque word for tremor), presence of LB and neuronal loss in the SNpc, although penetrance was shown to differ according to age and population [74–76]. The majority of *LRRK2* mutations identified so far are single missense nucleotide substitutions, consistent with a gain-of-function feature. These mutations represent nearly 10% of all cases of familial PD with an autosomal dominant mode of transmission, and are also found in nearly 4% of patients affected by idiopathic PD [77]. Therefore, to date, mutations on *LRRK2* seem to be the most frequent cause of the disease, nevertheless, among the 80 variants known only seven are considered as pathogenic and their relevance is corroborated by the observation that they all affect key functional domains of the *LRRK2* protein. The PD-related variant most commonly retrieved in worldwide population is the c.6055G>A mutation, causing the G2019S substitution at the protein level. In addition, mutations on c.4321 and c.4322 can cause the substitution of the arginine residue at position 1441 by a cysteine (the most common variant), a glycine or a histidine; other variants as the Y1699C, the I2020T and the N1437H are found less frequently and in restricted populations only [4]. It has to be noted that, unlike for *SNCA*, homozygous *LRRK2* mutations do not worsen the symptoms of the disease or anticipate the age at onset compared to heterozygous mutations.

III. Other genes lacking conclusive relevance for PD: *UCHL1*, *HTRA2*, *GIGYF2*, *EIF4G1*

Besides the identification of risk factors for PD on *SNCA* and *LRRK2*, classical linkage analyses, GWAS and next-generation sequence approaches often failed to provide robust evidence for the implication of other loci and genes in familial autosomal-dominant PD [78]. However, thanks to its specific neuronal localisation and its presence in LBs, *Ubiquitin C-Terminal Hydrolase 1 (UCHL1)* was believed to be a good candidate gene for PD [79]. Two missense mutations on *UCHL1* have initially been associated with PD: the I93M and the S18Y. The

I93M substitution, which was found in one single family and without a pure dominant segregation, was demonstrated to reduce the enzymatic activity of the protein by half in vitro. Nevertheless, this mutation was never retrieved in any additional PD-affected family [80]; accordingly, the role of the S18Y polymorphic variant, identified as a protective factor against sporadic PD in population studies, is still highly debated [4,81]. Similarly, mutations in *HTRA2*, e.g. G399S and A141S, were identified but never reproduced, although in vivo and in vitro studies supported a physiopathological role for this mitochondrial serine-threonine protease [82–84]. Another gene, *GRB10-interacting GYF protein 2 (GIGYF2)*, has recently been linked to PD after GWAS analyses [85], although none of the missense mutations identified in the examined populations were unequivocally defined as pathogenic [4]. At last, there is controversial evidence for an implication of *Eukaryotic Translation Initiation Factor 4 gamma 1 (EIF4G1)* in PD. A dominantly inherited R1205H mutation has been associated with late-onset PD with LB pathology, but two recent studies generated controversial pieces of data, suggesting that the pathological relevance of this variant cannot be ascertained yet [86–88].

D. PD-related genes with autosomal recessive mode of inheritance

I. PARK2/PARKIN

PARK2 is a large gene of 1.35 Mb comprising 12 exons; mutations in *PARK2* account for nearly 20% of familial autosomal recessive PD cases with onset between 40 and 50 years of age at diagnosis, a percentage raising to 80% for patients with juvenile parkinsonism (< 20 years of age at onset) [89,90]. The phenotype of the disease induced by mutations in this gene presents some specific features which distinguish it from idiopathic PD: early age at onset, dystonia at onset, hyperreflexia, slow disease progression, possible cerebellar impairment and development of dyskinesia due to L-DOPA treatment [91,92]. In addition, as for *SNCA* and *LRRK2*, *PARK2* mutations have been found in nearly 15% of PD cases with a sporadic origin [93]. Concerning the neuropathological traits of *PARK2*-associated PD, there is a severe loss of the dopaminergic neurons in the SNpc and a modest one in the locus coeruleus. LBs are absent from carriers of the mutation at the homozygous state, although LB-like inclusions were observed in few patients carrying two different disease-associated alleles at the same locus, termed compound heterozygotes [94,95]. To date, more than 170 mutations on *PARK2* have been identified, including single nucleotide missense or nonsense mutations, deletions, insertions and exon rearrangements throughout the entire gene sequence. Given that *PARK2* codes for the E3 ubiquitin-protein ligase Parkin, these mutations often lead to heavily truncated dysfunctional proteins lacking selective domains regulating the enzymatic activity [58]. However, it is less clear how missense mutations lead to loss of protein function, particularly if they do not affect the catalytic domain of the protein, although they have been reported to decrease the solubility of the protein and lead to the formation of aggregates in cell models [96,97].

II. *PINK1*

PINK1 is a gene of 38 Kb and 8 exons, and mutations in its sequence account for 4% to 15% of autosomal recessive PD cases in the Caucasian and Asian populations [58]. The clinical phenotype resembles that associated with *PARK2* mutations, presenting key features such as early disease onset, dystonia, hyperreflexia and early L-DOPA-induced dyskinesia, but also peculiar characteristics as psychiatric disturbances, slower disease progression and generally mild symptoms [4]. Concerning the neuropathology of this subtype of PD, the only study describing *PINK1*-related parkinsonism at autopsy presented neuronal loss in the SNpc and presence of LBs [98]. Mutations on *PINK1* sequence can be of several types: point mutations, frameshifts and small or whole gene deletions, resulting in the destabilization of the conformation of the protein or an impairment of its enzymatic activity [99].

III. Genes less frequently mutated in autosomal recessive PD: *DJ-1*, *ATP13A2*, *GBA*.

DJ-1 is a gene constituted by 51.2 Kb and 7 exons; when mutated, it causes a parkinsonian syndrome similar to that induced by *PARK2* or *PINK1* mutations, including early onset and slow disease progression [4]. Incomplete penetrance has been observed in conditions of compound heterozygosity with *PINK1* [100]. Patients presenting *DJ-1* mutations are rare (about 1% of cases with autosomal recessive PD) and it is therefore difficult to provide a full neuropathological characterization. The mutations identified thus far include large deletions spanning one or more exons, frameshifts and missense mutations. Particular attention has been given to the protein carrying the L166P substitution, which was first identified in two families from Italy and the Netherlands and which seems to impair the conformation of the protein, targeting it to the Ubiquitin-Proteasome System (UPS) for degradation [101–103].

The lysosomal *type 5 P-type ATPase (ATP13A2)* gives rise, when mutated, to an atypical form of juvenile parkinsonism (< 20 years) with autosomal recessive transmission, called Kufor-Rakeb syndrome and sharing common features with PD: pyramidal signs, akinesia (postural freezing), dementia and brain atrophy [104,105]. However, these symptoms can vary within a broad spectrum whenever homozygous or compound heterozygous mutations in the *ATP13A2* gene are present: the first ones presents features highly resembling the parkinsonian ones, including rigidity, dyskinesia and responsiveness to L-dopa administration [106]; the latter show a broader phenotype with dementia, pyramidal signs and less responsiveness to L-dopa [107]. In addition, the pathogenic role of the variants F182L and G504R, recently identified in the homozygous state, requires further investigation [105,108].

Mutations in one or both *GBA* alleles, which in the homozygous or in the compound heterozygous state lead to a lysosomal storage disorder termed Gaucher's disease, have been identified as the most common genetic risk factor for the development of PD with early onset and LB pathology [98]. The interconnection between PD and Gaucher's disease was discovered with the observation that patients with

Gaucher's disease present typical parkinsonian features from the neuropathological point of view, including α -synuclein immunopositive LB and dopaminergic neuronal loss; on the other hand, PD patients at autopsy show higher frequencies of mutations in *GBA* compared to controls [109,110]. Successive screenings of PD patients for mutations on *GBA* normally causing Gaucher's disease, revealed that heterozygous *GBA* mutations increased the risk to develop PD in specific populations as the Ashkenazi Jewish and the French one [111,112]; nevertheless, the pathological effects of *GBA* mutations and the molecular mechanisms by which these variants could constitute a risk factor for PD have not been fully elucidated yet [113]. *GBA* codes for the Glucocerebrosidase, a lysosomal enzyme catalyzing the hydrolysis of the plasma membrane glycolipid glucocerebroside to glucose and ceramide. Intuitively, loss-of-function of the enzymatic activity of glucocerebrosidase might cause lysosomal insufficiency, leading to autophagic dysfunctions and accumulation of toxic and/or unfolded proteins in the cytoplasm [114].

1.1.2 PD-related proteins: focus on enzymatic function and subcellular localisation.

Among the proteins implicated in autosomal dominant or recessive PD, some common elements can be detected. Although for some proteins as α -synuclein the function remains uncertain, for other proteins as LRRK2, PINK1 and Parkin, insights on specific structural features or protein domains helped scientist to decipher their biological activity. In general, PD-related proteins can be grouped in two classes: kinases and proteins implicated in degradation pathways as the UPS and the autophagy systems, with a growing body of evidence indicating that dysfunctions in protein phosphorylation and/or in the clearance of aberrant proteins or organelles could play an key role in the pathogenesis of PD. In parallel, several lines of evidence corroborate the central role played by mitochondria and mitochondrial dysfunctions in the physiopathology of this disease, after the discovery that the PD-related protein PINK1 has a mitochondrial localization, and that Parkin, DJ-1 and LRRK2 are also partially found at the organelle and play a role in oxidative stress responses. Nevertheless, present literature is sometimes contradictory and future studies will be required to decipher the entirety of molecular mechanism regulated by PD-related proteins at the mitochondrion.

A. PD-related proteins with kinase or kinase-related activity: LRRK2, PINK1 and DJ-1

Kinases are among the most abundant families of proteins in the human proteome (nearly 500), and their biological role consists in catalyzing the modification of target proteins by adding a phosphate group obtained from the hydrolysis of an ATP molecule: this is a process termed phosphorylation, which allows the modification of roughly 30% of all proteins within a cell (Figure 2).

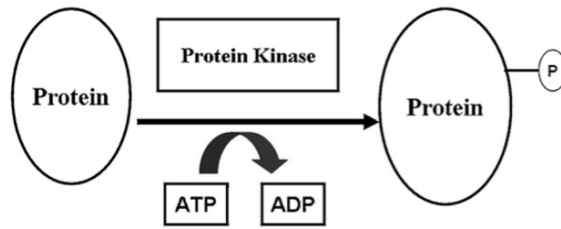


Figure 2. Representation of a general phosphorylation reaction, where the protein kinase shifts the target protein from a basal to a phosphorylated state. From [115].

Kinases regulate a multitude of biological processes within the cell, including gene transcription, cell cycle progression, signal transduction, cell proliferation/survival and death by apoptosis; they function as molecular switches, turning the target protein from a basal, inactive state to an active form after phosphorylation [116]. Two major classes of kinases are known: serine/threonine and tyrosine kinases, according to their biological property of phosphorylating OH groups of serine and threonine residues or tyrosine side-chains.

LRRK2 has a multidomain composition sharing structural features compatible with a protein involved in signal transduction, although its precise biological activity has not been fully characterized. The kinase domain is located in proximity of the C-terminus and shows sequence homology with serine/threonine kinases, but also with Receptos-Interacting Protein Kinases (RIPKs) and with Mitogen-Activated Protein Kinase Kinase Kinases (MAPKKKs), which supports the idea that LRRK2 primarily acts as an intracellular signal propagator *via* its kinase activity [117]. Furthermore, the simultaneous presence of a ROC (Ras in Complex) and a COR (C-ter of ROC) domain indicate that the protein is a member of the ROCO GTPase family (Figure 3). Three additional domains are involved in protein-protein interactions: ANK (Ankyrin-like repeat), LRR (Leucine-Rich Repeat) and WD40.

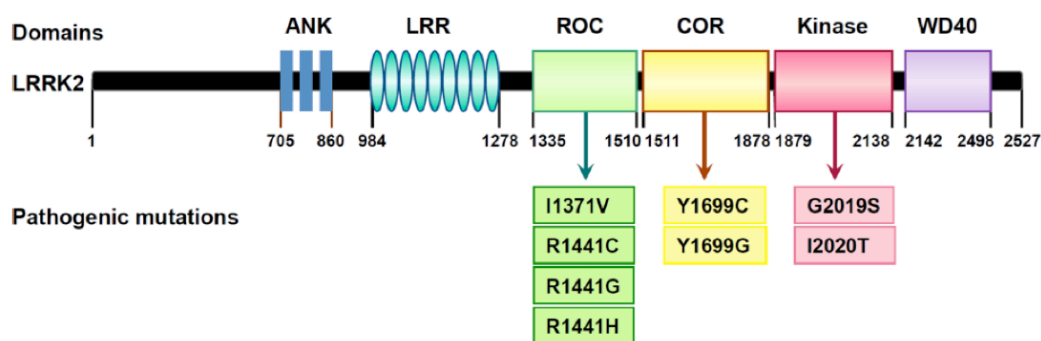


Figure 3. Organization of key functional domains along the sequence of the LRRK2 protein; PD-causing variants affecting the ROC, COR and kinase domains are indicated in the coloured boxes below each domain . From [118].

Thus, several scenarios concerning the enzymatic activity of LRRK2 can be evoked: (i) the protein works as a conventional kinase, therefore activating or inhibiting its substrates by phosphorylation; (ii) the phosphorylation activity of LRRK2 is controlled by its GTPase activity, although it has been observed that LRRK2 undergoes auto-phosphorylation on the ROC and COR domains [119–121]; and (iii) LRRK2 mediates

protein-protein interactions, as the ANK, LRR and WD40 domains are reminiscent of structural features of scaffolding proteins belonging to the MAPK family, where they regulate the subcellular localization and the enzymatic activity of the protein within they are found [118]. In principle, these three roles potentially played by LRRK2 within the cell are not mutually exclusive; nevertheless, they seem to indicate that the protein is involved in several biological pathways focused on neuronal survival. Overproduction of LRRK2 in *in vitro* models causes neuronal cell death; in this paradigm, apoptosis is further increased by PD-causing pathogenic mutations, which are localised throughout the different domains of the protein. This phenomenon is particularly significant for the G2019S mutation, which increases the kinase activity of the protein, providing a functional link between the most frequent LRRK2 mutation and possible mechanisms of neurodegeneration in PD [122,123]. Accordingly, these screenings in *Drosophila melanogaster* and in *Caenorabditis elegans* confirmed that decreasing the kinase activity of LRRK2 by taking advantage of pharmacological inhibitors partially prevents neurodegeneration. Unfortunately, these studies did not provide evidence for cell signalling pathways in which the protein is implicated. Nevertheless, preliminary reports indicate that LRRK2, but not pathogenic variants of the protein, could play a role in the MAPK signalling cascade, by changing the protein abundance of the Extracellular Signaling-Related Kinase (ERK) 1 and 2 [123,124]. The Tumor Necrosis Factor α /Fas Ligand (TNF α /FasL) pathway is also a potential target of LRRK2 *in vitro*: Fas-associated protein with Death Domain (FADD) was found to physically interact with overproduced LRRK2, therefore linking the LRRK2-induced neurotoxic effect to the extrinsic apoptotic pathway. In this paradigm, cell death by apoptosis was abolished by a dominant-negative FADD or a kinase-dead mutant of LRRK2, whereas it was enhanced by four pathogenic LRRK2 mutations [125]. Finally, there is evidence that LRRK2 interacts with the Disheveled (DVL) proteins 1, 2 and 3 and the Glycogen Synthase-kinase 3 (GSK-3), members of the Wnt signalling pathway; pathogenic *LRRK2* mutations impair or abolish these interactions, supporting their relevance to the pathogenesis of PD [126,127].

PINK1 is a serine/threonine kinase, which was first identified in a transcriptomic profile of tumor-inducing cells as a protein activated by the tumor suppressor gene Phosphatase and Tensin Homolog (PTEN), and thus named PTEN-Induced Kinase1 [128,129]. The domain architecture of PINK1 reveals that the N-terminal portion of the protein contains a Mitochondrial Targeting Sequence (MTS), whereas the kinase domain is located in the core region, which also regulates binding to substrates (Figure 4) [130,131].

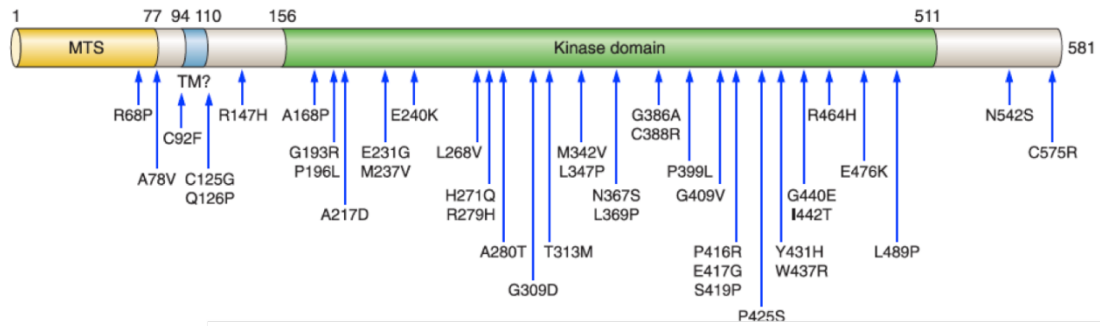


Figure 4. Organization of key functional domains of the PINK1 protein; MTS: Mitochondrial Targeting Sequence, TM: putative Transmembrane domain. PD-causing mutations span the three domains and are indicated below each of them. Adapted from [118].

To date, there is evidence that PINK1 phosphorylates several mitochondrial proteins [132]. The first mitochondrial substrate to be identified was the Tumor Necrosis Factor Receptor-Associated Protein 1 (TRAP1), a molecular chaperone involved in the oxidative stress response [133]. In this study, the PINK1-dependent phosphorylation of TRAP1 *in vitro* and *in vivo* allowed to overcome cell death by blocking cytochrome *c* release, a situation associated with protection against the proapoptotic stimuli activated in conditions of oxidative stress. A second study showed that the PD-causing mitochondrial protein Omi/HTRA2 is modified by phosphorylation in a PINK1-dependent manner; this post-translational modification was found to be reduced in brain samples from PD patients carrying *PINK1* mutations [134]. In a recent report, phosphorylation by PINK1 in *Drosophila* was shown to activate the cytosolic kinases Mechanistic Target of Rapamycin 2 (mTORC2) and its downstream effector Tricorned (Trc), which then translocates to mitochondria when phosphorylated [135]. As these kinases modulate cell growth and motility through their interaction with cytoskeletal elements [136], it has been hypothesized that the interaction of mTORC and Trc with PINK1 is essential for a correct distribution of mitochondria through the cytoskeleton. In parallel, as mutations on PINK1 were shown to affect mitochondrial complex I activity [137,138] and given that PINK1 does not bind directly to mTORC2, Wu et al. further demonstrated that a dysfunctional complex I mimics the PINK1 loss-of-function phenotype, and observed that in conditions where complex I is impaired, mTORC is enzymatically inactive. The authors also demonstrated that PINK1 physically interacts with the subunits I and III of complex I, providing supplemental evidence for a role of PINK1 in maintaining the activity of this complex and thus regulating mitochondrial quality control.

In addition, PINK1 was shown to modulate the protective effects of the anti-apoptotic AKT pathway, by mediating Insulin Growth Factor 1 (IGF-1)-dependent signalling in the cytoplasm [139]. In this study, IGF-1 phosphorylation of AKT and the AKT-dependent neuroprotective effects were found to be impaired in a *PINK1* knockout mouse model, suggesting that alteration of this molecular cascade by *PINK1* deficiency could increase the susceptibility of dopaminergic neurons to apoptotic cell death. Supporting evidence has been provided by another recent study, showing that overproduction of PINK1 regulates the AKT pathway through the activation of the Phosphoinositol-3 kinase (PI3K), thereby protecting against ceramide-induced apoptosis

[140]. Intriguingly, the PD-related protein Parkin was also shown to exert a protective effect in this cell death paradigm (c.f. Paragraph 1.1.2B) [141].

DJ-1 is a protein belonging to the family of the ThiJ/PfpI-like molecular chaperones, and is activated in response to oxidative stress events (Figure 5). The precise role of the ThiJ domain is still unclear: it is structurally related to the type 1 glutamine amidotransferase domain (GAT1 domain), which catalyzes the transfer of a molecule of H₂O to a molecule of glutamine to release ammonia, a substrate widely used in biosynthetic reactions [142]. Nevertheless, it appears that the ThiJ domain has different enzymatic activities, including RNA-protein interactions and proteolysis, although its primary function remains unknown. DJ-1 is structurally related to the *Escherichia coli* Hsp31, a molecular chaperone activated by heat shock and which also has a functional sub-domain (a catalytic triad) with protease activity [143]. Accordingly, the yeast homolog of DJ-1, YDR533C, was found to carry a domain with protease activity, although it seems unlikely that this domain exists on DJ-1 [144]. In a study based on the use of embryonic stem cells, it has been proposed that DJ-1 acts as a molecular chaperone in oxidized environments and it can prevent the accumulation of aggregation-prone proteins as α -synuclein [145]. Furthermore, the protein was identified as a positive regulator of the PI3K/AKT pathway in a *Drosophila melanogaster* model: in this context, inactivation of DJ-1 led to defective phosphorylation of key members of this pathway, resulting in enhanced production of ROS [146]. A recent study reported that mice cortical neurons devoid of DJ-1 show reduced levels of phosphorylated AKT in response to oxidative stress induced by H₂O₂ or MPTP treatment *in vitro* and *in vivo*, reinforcing the neuroprotective role of this protein in parkinsonian-like models [147].



Figure 5. Representation of DJ-1; PD-causing mutations are indicated with blue arrows. From [118].

In addition, overproduction of DJ-1 was reported to regulate the MAPK pathway *in vitro* and *in vivo* by activating ERK1/2 downstream signalling events in response to oxidative stress, such as the transcriptional upregulation of Superoxide Dismutase 1 (SOD1), which catalyzes the conversion of oxygen superoxide (O₂⁻) into O₂ and H₂O [148,149]. In other studies, DJ-1 was proposed to exert its antioxidative function by activating the nuclear transcription factor Nurr1, which is required for the differentiation and survival of dopaminergic neurons in the SNpc. Nurr1 modulates the transcriptional activation of the gene encoding tyrosine hydroxylase (TH), which catalyzes the rate-limiting step in the dopamine biosynthesis pathway. Interestingly, the S125C Nurr1 variant, recently identified in a sporadic PD patient, abolished this neuroprotective function of DJ-1 [150,151].

B. PD-related proteins implicated in protein clearance pathways: Parkin, α -synuclein, ATP132A, UCHL1

It is well recognized that the synthesis and the degradation of proteins are mechanisms strictly balanced in healthy cells; alteration of this homeostatic equilibrium triggers the abnormal accumulation of aggregation-prone proteins, a process believed to lead to the formation of inclusions in several neurodegenerative disorders, including PD. Importantly, it is still unclear whether these proteinaceous aggregates, composed by insoluble misfolded proteins are neurotoxic (as the presence of these agglomerates in the cytoplasm of nerve cells might alter cell shape or trafficking cascades), or whether these aggregates are a repository for potentially harmful proteins and are thus protective for the cell. Another important issue to solve is whether the intracellular accumulation of proteins is the outcome of increased protein synthesis or of defective degradation. Therefore, in the field of PD the last decades of research concentrated on the mechanisms of protein accumulation in LBs and the key role played by PD-related proteins in quality control mechanisms, demonstrating that these aspects are closely intertwined, and further corroborating the contribution of genetics to our understanding of the pathogenesis of this disease.

There are two major protein degradation routes which may, at some stage, become dysfunctional in PD: the Ubiquitin-Proteasome system (UPS) and the autophagy/lysosomal pathway [152]. The UPS degrades proteins with short half-lives, involved in signal transduction, cell death, cell cycle progression and cell differentiation. Additional targets of the UPS are mutated, misfolded proteins and/or proteins damaged by cytotoxic events, such as oxidative stress [153,154]. Recognition of dysfunctional proteins that need to undergo proteolysis by the UPS starts with their ubiquitylation, which is the covalent post-translational linkage to several molecules of ubiquitin, a small polypeptide of 76 residues. Ubiquitin can be attached to target proteins as a single entity on one or more sites (a process termed mono- or multi-monoubiquitylation) or as a series of residues forming a chain on target proteins (polyubiquitylation); in a polyubiquitin chain, molecules can be linked through one of the seven lysine residues present in the ubiquitin sequence: lysine 6, 11, 27, 29, 33, 48 and 63 [155]. Ubiquitin monomers connected through one type of residue are referred as homotypic chains and are usually linear, whereas chains containing mixed linkages are called heterotypic and they may be found in a branched conformation. Chains with amide (connected through their ϵ -amino groups) linkages between the C-terminus of one ubiquitin residue and the lysine in position 48 (K48) of the next one are the most common ubiquitin chains; when at least four residues of ubiquitin are bound together, they have been demonstrated to bind to the proteasome *in vitro*, constituting a target signal for degradation [156]. Conversely, polyubiquitin chains involving lysine 63 (K63) have been shown to promote non-proteolytic functions, as modulation of intracellular trafficking, endocytosis, topography, DNA damage repair, ribosomal biogenesis and cellular signalling. A cascade of three proteins is involved in the attachment of ubiquitin residues to target proteins: E1 and E2-conjugating enzymes, and E3 ubiquitin ligases (Figure 6). A single E1 enzyme triggers the

formation of a thiol ester bond between the C-terminus of a ubiquitin residue and a E1 cysteine residue (step A). Successively, ubiquitin is transferred by one of the dozens of E2 enzymes from the E1 to the target protein, previously recognized by one of the hundreds of E3 ubiquitin ligase (steps B-D) [152]. Ubiquitin ligases can be differentiated according to the properties of their catalytic residues required for the E3 function: HECT, RING and U-box ligases. Homologous to E6-associated protein C-terminus (HECT) have a catalytic cysteine residue directly binding incoming ubiquitin from a cognate E2 enzyme, creating a covalent intermediate. Really Interesting New Gene (RING) and U-box ligases instead do not form this covalent intermediate, but rather function as scaffold proteins, bringing the target in close proximity to the E2 enzyme [155].

The proteasome is a large protease complex, made of a core cylindrical structure called the 20S subunit with trypsin-, chymotrypsin-like and post-glutamyl-peptide hydrolytic activities; two regulatory subunits, called 19S, are located at each end of the 20S cylinder (Figure 6). The function of the 19S subunit located in the lid is to recognize and bind the proteins bearing a K48 polyubiquitin chain only, therefore not to destroy indiscriminately other cytosolic proteins. The 19S subunit located at the base instead, possesses six ATPase subunits with an intrinsic chaperone-like activity, to help unfolded substrates enter the 20S core. Together, the two 19S subunits and the 20S one constitute the 26S proteasome.

Importantly, ubiquitylation is a dynamic process: while the substrate protein is degraded in small polypeptides within the 20S subunit in an ATP-dependent reaction, ubiquitin chains are disassembled thanks to the hydrolase enzymatic activity of de-ubiquitylating enzymes (DUB). These enzymes are also in charge of removing ubiquitin residues from proteins and residual peptides associated to the proteasome [157].

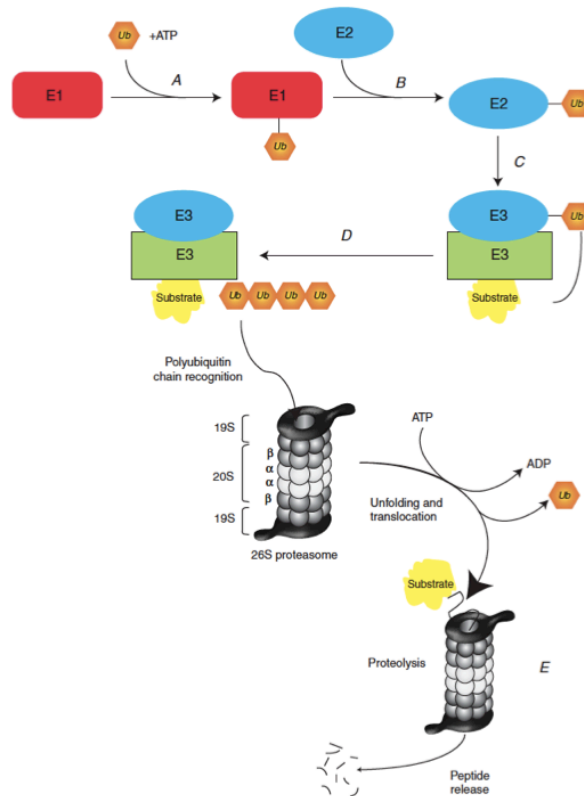


Figure 6. The ubiquitylation cascade leads to the proteasome-dependent degradation of target proteins: the E1-, E2-, E3-driven reactions are represented in steps A-D. The 26S proteasome recognises the ubiquitylated protein through the 19S lid, which is subsequently cleaved within the 20S subunit and released (step E). From [152].

The autophagy/lysosomal pathway is a series of events activated by the cell in parallel or alternatively to the UPS, mainly to eliminate excessive or defective subcellular organelles as mitochondria or peroxisomes, proteins with long half-lives (e.g. integral membrane proteins) or abnormal protein aggregates. Autophagy is generally triggered in nutrient deprivation, stress or infection conditions, but it has also been associated to physiological conditions such as development, and cell differentiation [152]. This pathway consists in encapsulating the substrates targeted for degradation in double-membrane vesicular structures, the autophagosomes. Autophagosomes subsequently fuse with organelles rich in acidic hydrolases, the lysosomes, to enzymatically destroy the content of these vesicles: this is a “bulk” autophagy mechanism, also known as macroautophagy. Another type of autophagy, termed chaperone-mediated autophagy, takes advantage of cytosolic chaperones to carry specific targets to the lysosomal membrane: the selectivity of this pathway consists in a lysosomal receptor, LAMP2A, which will recognize the chaperone and internalize its cargo into the lysosomal lumen, a phase followed by unfolding and cleavage of the selected protein [158] (Figure 7).

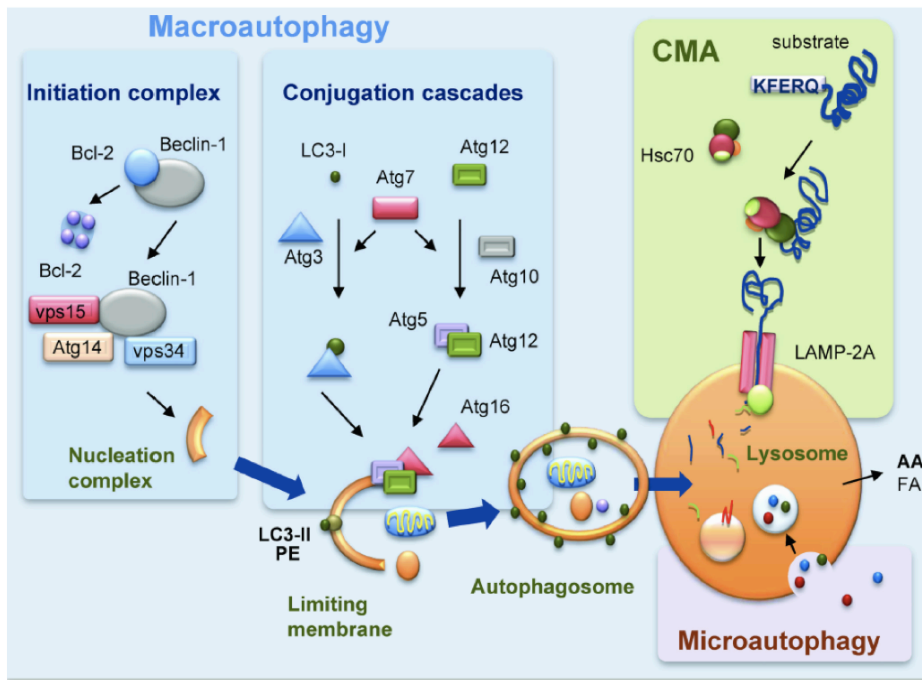


Figure 7. Schematic representation of protein degradation through macroautophagy, microautophagy and chaperone-mediated autophagy. For macroautophagy, an initiation complex is formed after association of Beclin-1 and Bcl-2 with the “pre-autophagic” machinery (Vps34, 35 and Atg14), so the formation of the phagophore can start. Lipidation of LC3-I into LC3-II and the activation of several autophagy-related proteins (ATGs) in cascade complete the formation of the phagophore/autophagosome. This structure has a physiological pH, therefore protein degradation occurs only when the autophagosome fuses with the lysosome in which the acidic hydrolases eliminate unfolded proteins and/or damaged organelles. Microautophagy is used to internalize small cytosolic cargoes through the invagination of the lysosomal membrane. Chaperone-mediated autophagy is activated when a chaperone (here Hsc70) recognizes the KFERQ pentapeptide motif of a cytosolic protein and carries it to the lysosomal receptor LAMP-2A in an unfolded form; this protein will then be internalized and degraded within the lysosome. From [158].

Parkin is an E3 ubiquitin-protein ligase initially linked to the degradation of selected substrates by the UPS [159–161]. It is a 465-amino acid protein organised in five domains: an N-terminal domain homologous to ubiquitin (UBL), three Really Interesting New Gene domains (RING 0, 1 and 2) and an In-Between Ring domain (IBR) (Figure 8).

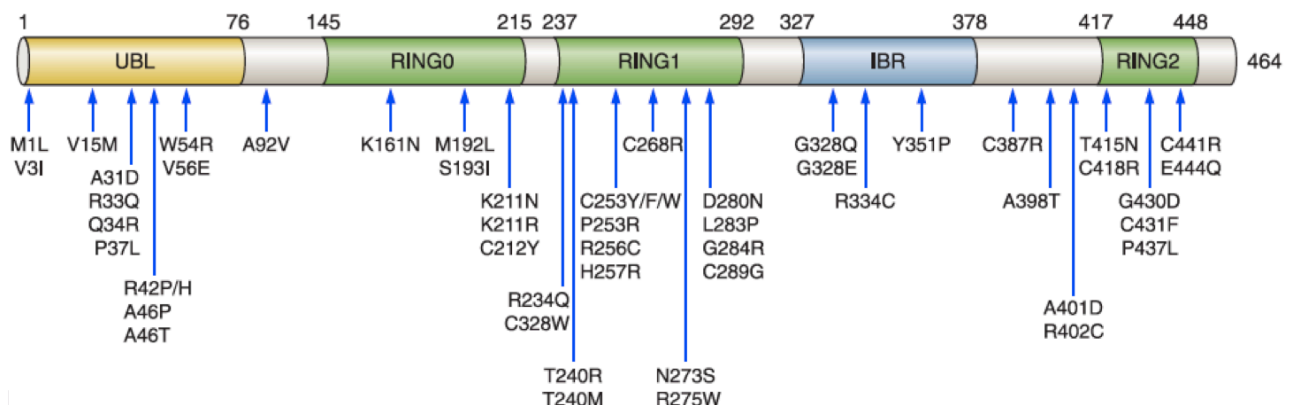


Figure 8. Organization of the functional domains of Parkin. At the N-terminus: the Ubiquitin-like domain (UBL). Three Really Interesting New Gene domains cover the core of the structure (RING 0, 1 and 2), and an In-between Ring domain is found between the RING1 and RING2 domain. PD-causing missense mutations are indicated by the blue arrow. From [4].

Because of the autosomal recessive mode of inheritance of *PARK2* mutations and the severity of a great number of them, since the discovery of the enzymatic activity of the protein and together with the finding that LBs are rarely found in PD cases carrying *PARK2* mutations, the most straightforward hypothesis was that defective clearance of specific targets of Parkin could be responsible for *PARK2*-linked PD and that it is necessary for LB formation, although this role is nowadays controversial [159]. Accordingly, among the constantly increasing number of substrates of Parkin, several appear to be aggregation-prone proteins accumulating in *PARK2*-knockout mouse models or in PD patients with *PARK2* mutations, as the Parkin Endothelin Receptor-like Receptor (Pael-R), a transmembrane protein belonging to a molecular supercomplex of Parkin interactors together with the chaperone Hsp70 and the Carboxyl Terminus of Hsp70-interacting protein (CHIP) enhancing the E3 ubiquitin-ligase function of Parkin, the α -synuclein interactor synphilin-1, and the ubiquitin-conjugating E2 enzymes UbcH7 and UbcH8, which appear to mediate the auto-ubiquitylation function of Parkin [162–165]. Moreover, Parkin was found to interact with PINK1 and DJ-1 in a 200-kDa supercomplex which controls the proteasome-mediated degradation of Parkin itself and of synphilin-1 [166]. In support to the “proteasomal hypothesis”, Parkin was also found to interact physically with subunits of the 26S proteasome [167,168]; concomitantly, abnormalities in the composition of proteasomal subunits or in the proteasomal activity have been detected in the SNpc of patients with sporadic PD, further supporting the possibility that the alteration of this degradation machinery is an important feature in the pathogenesis of this disease [169]. Importantly, Parkin cooperates with PINK1 for the elimination of dysfunctional mitochondria by autophagy (c.f Paragraph I.3.1C), thus providing outstanding evidence for multiple roles played by this protein and particularly at the crossroad of the two major quality control mechanisms within the cell [170,171]. Finally, a growing body of evidence demonstrate the involvement of Parkin in the regulation of cell death. The first *in vitro* study concerning the role played by Parkin in apoptosis described that overproduction of this protein in cell models prevents against ceramide-induced cell death [141]. This function was shown to be dependent on the E3 ligase activity of Parkin and it was abrogated by PD-inducing *PARK2* mutations. Subsequent *in vitro* reports demonstrated that the overproduction of Parkin is protective in apoptotic paradigms induced by DA or 6-hydroxyDA, and that silencing of the endogenous protein is sufficient to induce cell death [172,173]. These observations have been supported by *in vivo* data obtained in *Drosophila melanogaster* models. *PARK2* knock-out flies show reduced lifespan compared to control ones, and locomotor deficits triggered by the apoptotic death of the indirect flight muscles [174]. In addition, it has been proposed that Parkin exerts its protective effect on specific paradigms of programmed cell death, as the Endoplasmic Reticulum-Unfolded Protein Response (UPR^{ER}). The UPR^{ER} provides a mechanisms by which the ER expands its folding capacity when unfolded proteins start to accumulate in the ER lumen; the apoptotic cascade is activated when an unresolved chronic accumulation of these unfolded proteins takes place [175]. Parkin was shown *in vitro* and *in vivo* to ubiquitinate and to promote the UPS-mediated degradation of the aggregation-prone substrate Pael-

R, a protein able to activate the UPR^{ER}, due to its high difficulty in folding and thus triggering cell death [163,176].

α -synuclein is a small 140 amino-acid protein, characterized by seven imperfect repeats (KTKEGV) at the N-terminus, forming an amphipathic α -helix domain and allowing the protein to associate with lipid rafts; an amyloidogenic domain in the core region (NAC), which confers the protein the capacity to aggregate and to form polymers of fibrils; and an acidic C-terminus (Figure 9) [4].

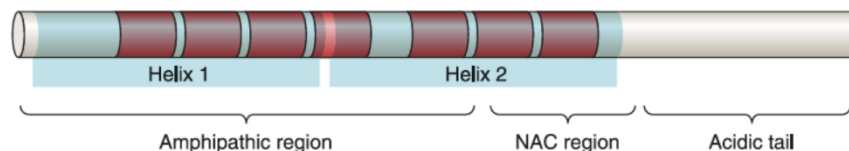


Figure 9. Organization of the functional domains of α -synuclein. In red: seven imperfect repeats (KTKEGV); the blue rectangles indicate the regions of the protein forming two amphipathic α -helices. From [4].

As it has been postulated that α -synuclein accumulates into LBs because of its defective degradation in pathological conditions, understanding the molecular mechanisms of its elimination from the cell would provide helpful insights on how LBs are formed. It has been reported that α -synuclein can be degraded by the proteasome [177,178]; however, the discovery in 2004 that the protein carries a characteristic pentapeptide sequence (KFERQ) recognized by the cytosolic chaperone Hsc70 and responsible for its autophagic elimination *via* the CMA system, opened an exciting new field of research and corroborated the hypothesis that both the UPS and the autophagic degradation pathways regulate the clearance of this protein [179]. In *in vitro* and *in vivo* models, α -synuclein interacts physically with the Lysosome-Associated Protein 2A (LAMP-2A), which appears to be necessary for the translocation of the protein in the lysosomal lumen; α -synuclein variants carrying PD-causing substitutions have increased affinity for the LAMP-2A receptor to which they stick, resulting in a drastic reduction of the activity of the CMA system [180]. Furthermore, a comparable reduction of the CMA activity was described when α -synuclein was exposed to DA, a condition previously shown to trigger the formation of α -synuclein-DA adducts *in vitro* and potentially constituting a detrimental situation for PD pathogenesis [181]. Therefore, Martinez-Vicente et al. proposed that high concentrations of cytosolic α -synuclein in PD could potentially lead to a blockade of the CMA pathway and further accumulation of α -synuclein in the cytoplasm of dopaminergic neurons, potentially constituting the cause for cell death in the SNPc.

ATP13A2 is a neuronal specific lysosomal ATPase, playing a key role in lysosomal acidification. As discussed above, the involvement of this protein in PD pathogenesis is still debated, nevertheless, recent data unexpectedly linked ATP13A2 to α -synuclein [182]. Dopaminergic cell loss induced by the overproduction of α -synuclein both *in vitro* and *in vivo* could be rescued by increasing amounts of ATP13A2, whereas silencing of this latter triggered α -synuclein accumulation and misfolding. Furthermore, PD-correlated mutations or

deletions in *ATP13A2* in *in vitro* paradigms led to permeabilization of the lysosomal membrane and alkalinization of the lysosomal lumen, causing impaired autophagosomal clearance and decreased rate of α -synuclein degradation, followed by the formation of α -synuclein adducts in the cytosol [183,184].

In 1989, Wilkinson et al. discovered that the protein product of UCHL1 gene codes for a neuron-specific deubiquitylating enzyme with a hydrolase activity, originally called PGP 9.5 and today known as UCHL1 [185]. The possibility that this protein could be dysfunctional in ubiquitin-rich protein aggregates as LBs raised a great interest, and a subsequent study from the same team provided evidence for UCHL1-positive LBs in immunohistochemical samples obtained from patients with sporadic PD [186]. In addition, recent data demonstrated that the UCHL1 protein possesses a non-canonical ubiquitin-ligase activity that can polyubiquitylate α -synuclein *in vitro*: as this polyubiquitylation appears to be K63-linked and not K48-linked, it has been suggested that the modified α -synuclein could not be recognized and degraded by the proteasome, resulting in an accumulation of the protein within the cell [187]. How this phenomenon is linked to PD pathology is totally unclear; a possibility is that the degradation and aggregation of α -synuclein are closely intertwined, and tightly controlled by the balanced action of the two enzymatic activities of UCHL1. Remarkably, the PD-linked S18Y putatively protective polymorphism reduced α -synuclein aggregation, leading to the hypothesis that this mutation might inhibit the ubiquitin-ligase activity in favor of the ubiquitin-hydrolase one. Conversely, overproduction of the PD-linked variants in cell models, which is known to abolish the hydrolase activity of the protein, was shown to increase the abundance of α -synuclein. Recent evidence proposed an involvement of UCHL1 in the autophagy/lysosome pathway by showing that the protein interacts with the key components of the CMA machinery, the lysosomal receptor LAMP-2A and the chaperones Hsc70 and Hsp90, required to transport through the cytoplasm the proteins to be degraded by the CMA pathway [188]. The PD-linked I93M variant enhances all these interactions, possibly leading to the overwhelming of the LAMP-2A receptor by α -synuclein and resulting in an impaired degradation of this protein through the autophagic pathway.

I.1.3. Conclusive remarks

Although formal evidence still needs to be provided, it appears that a functional link between kinases and proteins involved in multiple degradation pathways exists, as symbolized by the serine/threonine kinase PINK1 playing a direct role in the regulation of the autophagy/lysosomal clearance system. DJ-1 has indirectly been linked to these quality control mechanism, thanks to its chaperone activity preventing unfolded α -synuclein accumulating in the cytosol and ensuring the phosphorylation of members of the PI3K/AKT pathway [189,190]. Other kinases as the Extracellular signal regulated protein kinases 1/2 (ERK1/2), c-Jun N-

terminal kinases (JNK) and Mitogen-Activated Protein Kinase (MAPK) have been shown to modulate the catalytic activity of LRRK2 and are suspected to play a role in the elimination of mitochondria by autophagy (Reviewed in [134]). Phosphorylation reactions are normally extremely rapid, and the employment of these post-translational modifications might be a primary mechanism in PD for the elimination of unfolded or defective cellular components, potentially constituting a global response initiated by the cell to overcome damage before it becomes too harmful or it induces cell death.

Accordingly, The deregulation of the catalytic activity of PD-related kinases appears to increase oxygen stress, leading to mitochondrial dysfunction and protein aggregation, which strongly contribute to the pathogenesis of PD. The goal of the next years of research will be to develop innovative therapeutic strategies to potentially modulate the catalytic activity of these kinases; at the same time, the fine tuning of proteasomal, autophagy and apoptotic degradation pathways towards selective substrates could help preventing the relentless dopaminergic cell death in PD.

I.2. Structure and function of mammalian mitochondria

I.2.1 Generalities on mitochondria

Mitochondria are unique organelles within the mammalian cell, as already illustrated by their origin. As for chloroplasts in the plant cell, the so-called “endosymbiotic hypothesis” suggests that mitochondria were originally α -proteobacteria which entered the ancestral eukaryotic cell nearly two thousand million years ago and fused with their host, becoming an endosymbiotic entity [191]. This theory is supported by the evidence that both chloroplasts and mitochondria possess their own genetic system: during evolution, their integration within the host cell was probably done at the expenses of the “guest” genetic material, which was transferred and included almost entirely in the host nucleus. This process ended up with only 13 genes left in mitochondrial DNA (mtDNA) molecules nowadays, coding for core subunits of the OXPHOS machinery. Nearly 100 genes were left in the chloroplast DNA (cpDNA), encoding four RNA polymerase subunits, four ribosomal rRNAs, around 20 ribosomal proteins, 30 transfer RNA (tRNA) molecules, 30 proteins involved in photosynthesis and 11 subunits of a complex mediating the transport of electrons [192].

Together with the nucleus, mitochondria are the only organelles present in the cytoplasm to be enveloped by a double-membrane system (Figure 10) [193].

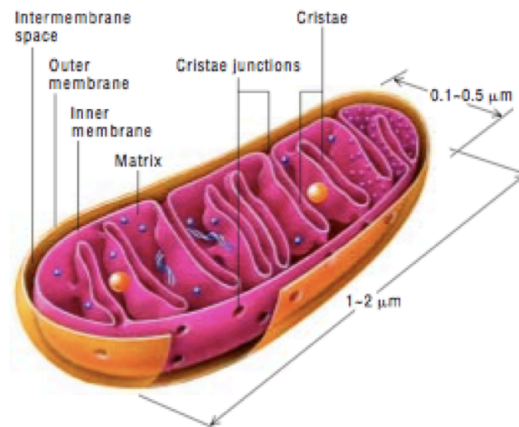


Figure 10. Schematic representation of a eukaryotic mitochondrion; the main compartments and the dimensions of the organelle are indicated. The blue ribbons symbolize mtDNA, the blue spheres mitochondrial ribosomes, the orange spheres the ATP synthase complex. Adapted from [192].

Each membrane is structurally and functionally distinct: the smooth external membrane, defining the perimeter of the organelle and known as Outer Mitochondrial Membrane (OMM), is extremely rich in sphingolipids (mainly phosphatidylcholine and phosphatidylethanolamine), which make this compartment highly permeable to ions and small molecules [194]; conversely, the Inner Mitochondrial Membrane (IMM) presents several invaginations known as *cristae* and is enriched in cardiolipin (diphosphatidyl glycerol), which contributes in determining its permeability properties and membrane potential. The passage of protons across the IMM is

tightly regulated to generate a proton gradient, with the mitochondrial lumen charged negatively. Two aqueous subcompartments are physically separated by the two above-mentioned membranes: the Intermembrane Space (IMS), located between the OMM and the IMM, and the matrix, the innermost portion of the organelle where multiple copies of mtDNA molecules are found.

The primary function of mitochondria is the production of the major intracellular energy carrier, Adenosine Triphosphate (ATP), via the oxidative phosphorylation system (OXPHOS) located in the *cristae* of the IMM [195]. To obtain ATP, required for the majority of intracellular reactions, the chemical bond energy produced by the oxidation of carbohydrates and fats is processed in the matrix through the reduction of the enzymatic cofactors NAD^+ and FAD into NADH and FADH_2 , in a series of reactions known as Krebs cycle. The reducing potential of NADH and FADH_2 is then, in brief, converted into an electrochemical proton gradient across the IMM by the first four complexes of the OXPHOS chain: the NADH dehydrogenase-ubiquinone reductase (complex I), the succinate dehydrogenase (complex II), the ubiquinol-cytochrome *c* reductase (complex III) and the cytochrome *c* oxidase (complex IV). The protons pushed into the IMS through these complexes are then re-imported into the matrix by the ATP synthase (complex V) using a retrograde transport against the proton gradient of the IMM to catalyze the formation of a third high energy phosphate bond on an Adenosine Diphosphate (ADP) molecule, converting it into ATP (Figure 11).

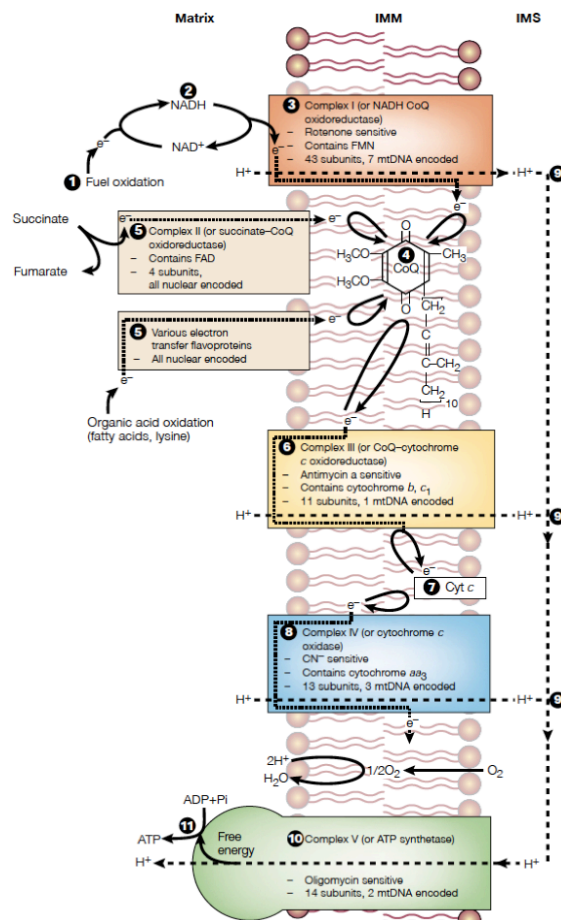


Figure 11. Schematic representation of the oxidative phosphorylation chain. The five OXPHOS complexes (I to V) are embedded in the lipid bilayer of the IMM; electrons (e^-) from the oxidation of carbohydrates (step 1 and dotted lines) are transferred from NADH (step 2) to complex I (step 3) and transported to the Coenzyme Q (CoQ, step 4), along with additional electrons from alternative carbon sources to complex II (step 5). Electrons are then shuttled through complex III (step 6) and cytochrome *c* (Cyt *c*, step 7) to the electron acceptor complex IV (step 8) to catalyze the formation of a molecule of water. For each of the steps 3, 6 and 7 a proton molecule is formed and is shuttled into the IMS (step 9), thus generating the proton gradient required by complex V to produce a molecule of ATP (step 11). For each OXPHOS complex, the number of subunits, the subunits encoded by mtDNA and the pharmacological inhibitors are indicated. Adapted from [196].

In conclusion, thanks to their small but highly specialized DNA, these multifunctional organelles control the bioenergetic status of eukaryotic cells; however, they also fulfil diverse roles in the maintenance of cellular homeostasis, ranging from the production of ROS to the regulation of intracellular Ca^{2+} levels and the synthesis and metabolism of essential cellular components, such as Fe-S clusters, heme, lipids and nucleotides [197]. In conclusion, mitochondria also participate to selective cell death programs in response to specific intracellular stimuli as Endoplasmic Reticulum (ER) stress or DNA damage [198].

A. Crosstalk between mitochondria and the cytosol: mitochondrial biogenesis and dynamics

Nowadays, the concept of “mitochondrial biogenesis” is quite elusive, raising several still open questions. The first one is whether the generation of new mitochondria is regulated intrinsically by the organelles (fully autonomous) or independently, thus completely relying on nuclear and/or cytosolic factors (nonautonomous),

rather than a process “in between” (partially autonomous) [199]. In addition, evidence from the literature demonstrating that mitochondria are part of a dynamic network which constantly undergoes fusion or fission events, turned the simplistic idea of a general “replacement of mitochondria” in a more complex and defined question: is there any biosynthetic program for the selective replacement of damaged proteins, lipids and mtDNA? In alternative, is there an isolation of defective mitochondria through fission and a complete substitution of organelles? To date, the most convincing model corresponds to a partially autonomous biogenesis program, according to which existing and/or damaged mitochondria undergo fission events to divide or restructure their architecture prior to the incorporation of newly synthesized components [200].

As mentioned before, nearly 99% of mitochondrial proteins is encoded from the nuclear genome and only few rely on mtDNA, indicating that transcriptional activation in both compartments is necessary for the production of functional organelles [201]. Despite our knowledge of the “bigenomic origin” of mitochondria, the mechanisms coordinating the nuclear and mitochondrial programs of biogenesis and the molecular components involved in these phases are still poorly understood. The mitochondrial transcription factor A (TFAM) appears to be one of the key actors in this process: transcriptionally activated in the cytoplasm by the nuclear transcription factor, Nuclear Respiratory Factor 1 (Nrf-1), it is required for the initiation of mtDNA replication and the regulation of mtDNA copy number [202]. Importantly, TFAM is necessary for the expression of mitochondrially-encoded OXPHOS genes, in conjunction with its activator Nrf-1, which promotes the expression of the *COXIV* gene. In a retrograde perspective, little is known about the factors acting upstream of Nrf-1 activation: activation of its expression is believed to be controlled by the Peroxisome Proliferator-Activated Receptor (PPAR)- γ Coactivator 1- α (PGC-1 α), another nuclear transcription factor, thus reinforcing the requirement for the nuclear genome for mitochondrial function [203]. A cell- and context-specific network of nuclear transcription factors is likely to be present for the regulation of these early events, although our current knowledge of these issues is poor and requires corroboration by further studies. Importantly, other parameters as mitochondrial fusion and fission events and their regulation must be integrated to the complex scenario of organelle turnover.

A growing body of evidence indicates that mitochondria are highly organized in a dynamic network, capable of shifting from a tubular, elongated state to a fragmented configuration [204]. These two phases can be considered as an equilibrium adjusting to specific cellular demands or signalling cascades, to specific metabolic demands in different regions of the cell (e.g. in neurons, fission events allowing movement of organelles along the axon have been described, [205]) or to enable the elimination of defective mitochondria by detaching these organelles from the mitochondrial network. This is achieved through the proteasomal degradation of the OMM protein Miro and its cytoskeletal receptor Milton, two key proteins driving the transport of mitochondria along microtubules and filaments of actin [206–208]. Although the regulation of mitochondrial dynamics is a matter of intense investigation, several molecular mechanisms have been identified [204].

Mitochondrial fission is regulated by the massive recruitment of the cytosolic GTPase Dynamin-Related Protein 1 (DRP1) on its recently discovered OMM receptor Mitochondrial Fission Factor (Mff), and in close proximity with the Mitochondrial Fission 1 (Fis1) protein [209]. DRP1 and Fis1 form an oligomeric ring-shaped structure around the portion of membrane to be separated from the mitochondrial network, in conjunction with the Ganglioside-induced Differentiation Associated Protein 1 (GDAP1), a mitochondrial fission factor integral to the OMM (Figure 12).

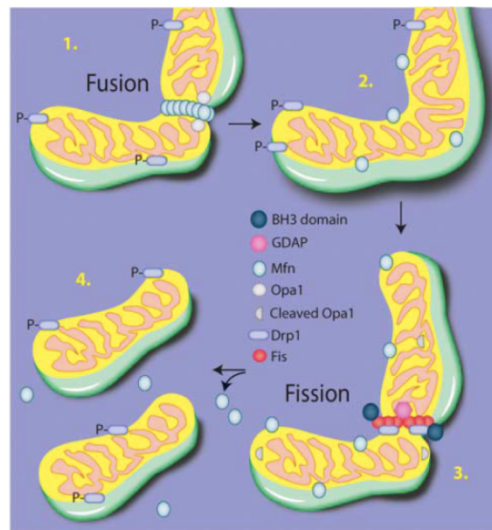


Figure 12. Schematic representation of mitochondrial fusion and fission events. Fusion of the OMMs of two adjacent organelles relies on Mfn 1 and 2, whereas Opa1 is required to fuse IMMs from different organelles (Steps 1 and 2). Conversely, fission requires several steps and actors, including dephosphorylation of the mitochondrially-recruited pool of Drp1; the molecular receptor of Drp1, Mff, the mitochondrial fission factors Fis1 and GDAP and possibly members of the Bcl-2 family carrying a BH-3 domain as Bax or Bak, to pinch off the targeted mitochondrial region (Steps 3 and 4). From [210].

Conversely, mitochondrial fusion events occur in a two-step program: first, OMM of adjacent organelles are tethered together by the dimerization of Mitofusins 1 and 2 (Mfn1/2), while cardiolipin is hydrolyzed by the mitochondrial Phospholipase D to facilitate the curvature of the two OMMs prior to fusion [211]; the second phase is controlled by the dynamin-like GTPase Optic Atrophy 1 (Opa1) and prohibitins, which mediate fusion of the two IMMs by forming a fusion pore, proposed to allow chemical and electrical continuity between the two adjacent matrices [212–214].

It is conceivable that, as the dynamics balance shifts towards mitochondrial fission or fusion in response to, two signalling cascades could possibly be activated: a global, nuclear-initiated (anterograde) cascade, and a local, mitochondrial (retrograde) cascade. As far as the first cascade is concerned, a broad activation of nuclear transcription factors, such as PGC1- α , PPAR and Nrf-1 could enhance the transcription of mitochondrial genes coding for OXPHOS subunits and mtDNA replication, as mentioned before. The increase in the global pool of mitochondrial transcripts and the consequent adaptation of the dynamics of the organelle could in turn directly affect mitochondrial functionality, activating a retrograde signalling pathway

from mitochondria back to the nucleus, to block mitochondrial biogenesis or enhance organelle clearance [210,215]. Future studies will be required to shed light on the impact of these pathways on mitochondrial and cellular homeostasis.

B. Crosstalk between mitochondria and the cytosol: mitochondrial protein import

Despite the central role played by mitochondria in the production of energy, the 13 genes carried by the mtDNA and encoding core subunits of the OXPHOS machinery are not essential for cell viability, at least in organisms capable of fermentation as the budding yeast and cultured cells. Conversely, what appears to be strictly required are complexes regulating the import of pre-mitochondrial proteins, hence proteins encoded by the nuclear genome with a mitochondrial subcompartment as final destination [216]. Mitochondrial preproteins, including those involved in import reactions, have specific sequence characteristics determining the pathway they will take to enter the organelle. A first import pathway is the so-called “Presequence pathway”, used by the majority of pre-mitochondrial proteins that possess a cleavable MTS at the N-terminus; the MTS is usually 15-100 residues long and is capable of folding into amphipatic α -helices due to the presence of positively-charged aminoacids. The positive net charge of the MTS is necessary for the precursor protein to cross the IMM, due to the presence of a negative mitochondrial membrane potential ($\Delta\Psi$) on the matrix side (Figure 12). Proteins sharing these features are mainly targeted to the mitochondrial matrix, where the MTS is cleaved by the dimeric Mitochondrial Processing Peptidase (MPP) protein. After cleavage of the N-terminal MTS, a second, hydrophobic MTS located after the matrix-targeting signal localizes the pre-mitochondrial protein in the IMM or promotes its release in the IMS [217]. The characteristics of this second MTS determine the submitochondrial compartment of destination of the preprotein: if it is uncleavable, the preprotein will be released into the lipid phase of the IMM, whereas if the preprotein is targeted to the IMS it is cut off by IMM proteases, such as the Presenilin-Associated Rhomboid-Like protease (PARL).

A large family of mitochondrial preproteins are not synthesized with a presequence, but carry an internal MTS located in the core structure of the protein, at its C-terminus (tail anchor), or at the N-terminus (signal anchor sequence) (Figure 13); this class of proteins are mainly targeted to the lipid portion of the OMM and IMM, but proteins reaching the IMS and/or the matrix can also be ascribed to this group [201]. In addition, these sequences can be found in one or multiple copies, suggesting that they do not follow a unique insertion pathway. The majority of pre-mitochondrial proteins targeted to the OMM have a α -helical three-dimensional conformation and they may carry an N-terminal, C-terminal or internal MTS (Figure 12). However, a subset of OMM-targeted proteins sharing a β -barrel conformation carry a C-terminal MTS only, which is formed by the last β strand of the preprotein and is recognised by the Sorting and Assembly Machinery (SAM) [218]. Metabolite carriers, as the ADP/ATP carrier or the phosphate carrier, show six MTS distributed along the protein sequence, each containing multiple 10-residue-elements targeting the protein to the IMM [219]. Finally,

a few proteins with a “presequence-like” domain preceded by a hydrophobic region were shown to be targeted to the IMM (Figure 13) [216]. These proteins belong to the “Carrier pathway” and are transported to the IMM with the help of cytosolic chaperones: after transcription from the nuclear genome and translation in the cytoplasm, these precursors are shuttled throughout the cytosol by members of the Hsp70 or Hsp90 families, which bind the preprotein in an unfolded state and prevent its aggregation [220].

Targeting and sorting signals of mitochondrial precursor proteins			
A Presequences and variations		Imported via	Destination of proteins
Presequence Amphipathic α helix Typically cleaved after import		TOM TIM23 PAM	Matrix
Presequence + uncleaved hydrophobic anchor (sorting signal)		TOM TIM23 (PAM)	Inner membrane
Presequence + cleaved hydrophobic sorting signal (bipartite presequence)		TOM TIM23 (PAM)	Intermembrane space
B Noncleavable signals of hydrophobic proteins			
β signal		TOM SAM	Outer membrane (β -barrel)
Signal-anchor sequence		Mim1	
C tail anchor		?	Outer membrane (α -helical)
Internal signal		(TOM) (SAM)	
Multiple internal signals		TOM Tim9-Tim10 TIM22	Inner membrane (metabolite carrier)
Presequence-like internal signal (after hydrophobic region)		TOM TIM23	Inner membrane

Figure 13. General classification of pre-mitochondrial proteins according to their MTS properties, the mitochondrial complexes through which they are imported, and their final suborganellar localization. Adapted from [201].

Regardless of the import pathway followed to reach the organelle, the entry gate for mitochondrial precursor proteins is the Translocase of Outer Membrane (TOM), a multisubunit complex composed by a dimeric channel, TOMM40, and several receptors: TOMM20, TOMM22 and TOMM70. Minor TOM subunits, TOMM5, TOMM6 and TOMM7, coordinate the three-dimensional architecture and stability of the complex (Figure 13). TOMM20 is the initial recognition site for proteins belonging to the “Presequence pathway”, while TOMM70 docks the proteins with internal MTS, mainly with a metabolite carrier activity [221]. These two receptors transfer the mitochondrial precursor to the General Receptor TOMM22, which provides structural support to the whole TOM complex and a functional connection between the organelle and the cell, as it faces both the cytosol and the IMS; from here, the proteins reach the import channel TOMM40. Once the import through TOM is achieved, the dichotomy of precursors belonging to the “Presequence pathway” or to the “Carrier pathway” is further illustrated by the different molecular interactors they encounter in the IMM and the mitochondrial matrix. A complex located on the IMM and known as the Translocase of Inner Membrane (TIM) regulates the import of preproteins with a charged N-terminal MTS into the IMS, the mitochondrial matrix or the IMM bilayer, by structurally and functionally cooperating with the TOM machinery. The TIM complex

shares structural similarities with its homolog on the OMM. It is formed by a tetrameric channel, TIMM23, and two major receptor subunits: TIMM50, facing the IMS and required for the delivery of mitochondrial precursors to this compartment; and TIMM17, necessary for the lateral release of preproteins into the IMM. A fourth subunit, TIMM21, physically connects the TOM and TIM import machineries when mitochondrial precursors are brought into contact with the TOM complex in the early stages of the import process. TIMM21 also facilitates the integration of nuclearly-encoded OXPHOS subunits into the mitochondrial respiratory chain. In addition, in targeting mitochondrial preproteins to the matrix, the TIMM23 machinery interacts with the Presequence Associated Motor (PAM), constituted by several subunits with chaperone-like enzymatic activity (the major one being the Mitochondrial Heat Shock Protein 70, mtHsp70) involved in the ATP-dependent refolding of the preprotein in its final conformation (Figure 14).

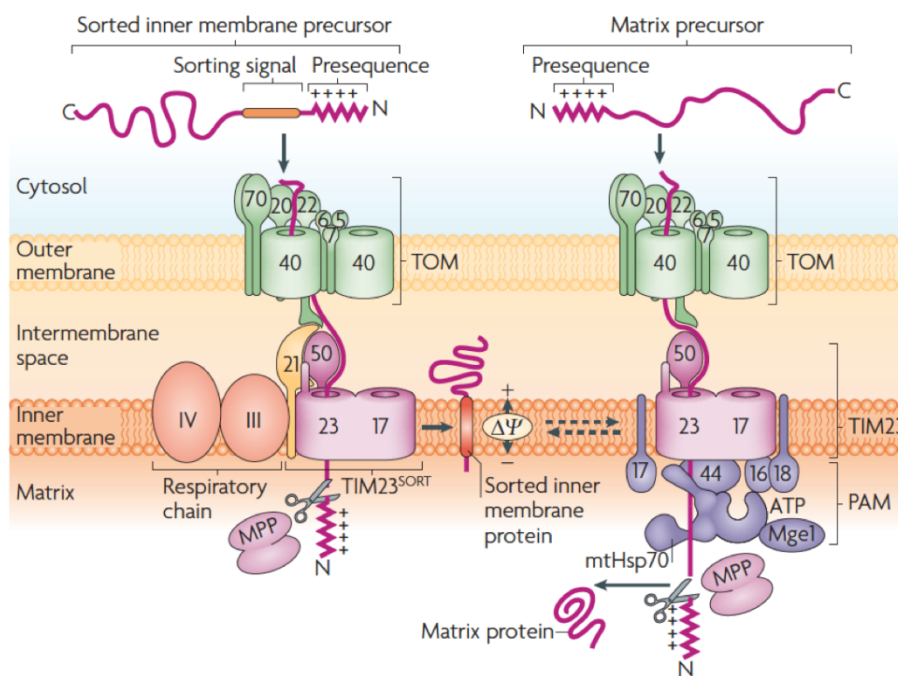


Figure 14. Schematic representation of the import pathway followed by mitochondrial preproteins targeted to the IMM (left panel) or to the matrix (right panel). Numbers inside the TOM, TIMM23 and PAM complexes represent the subunit composition of these machineries. Archetypal features of the imported preprotein (purple) are given: “+” indicates a positively-charged domain at the N-terminal portion of the precursor, the IMM sorting signal is represented in orange (left panel only). The TIMM23 complex (The TIMM17 and 23 subunits in particular) recognizes this signal and drives the lateral release of the preprotein in the IMM (TIMM23^{SORT}). Scissors represent the proteolytic cleavage of the N-terminal MTS by the MPP protease. Adapted from [221].

For mitochondrial preproteins belonging to the “Carrier pathway” and acting as metabolite carriers, the import journey is different [216]. To fulfil translocation across the OMM the TOM complex works in conjunction with the hexameric dimer TIMM9/TIMM10, which acts as a chaperone in the IMS to shuttle precursor proteins to the IMM. A TIM complex is present at this stage, although with a subunit composition different from that of TIMM23, termed TIMM22: a small TIM protein, TIMM12, joins the chaperone dimer TIMM9/10 and promotes binding of the precursor protein to the IMM receptor TIMM54, which exposes a large domain to the IMS.

TIMM54 thus acts as the binding site of the pre-protein on the IMM before its delivery to the TIMM22 dimeric channel, which in turn internalizes the protein in a $\Delta\Psi$ -dependent manner. A third subunit, TIMM18, is probably required for the correct assembly of the TIMM22 complex [222]. The protein is then released into the lipid bilayer of the IMM, where it is folded into its final three-dimensional conformation by an as yet unknown mechanism (Figure 15).

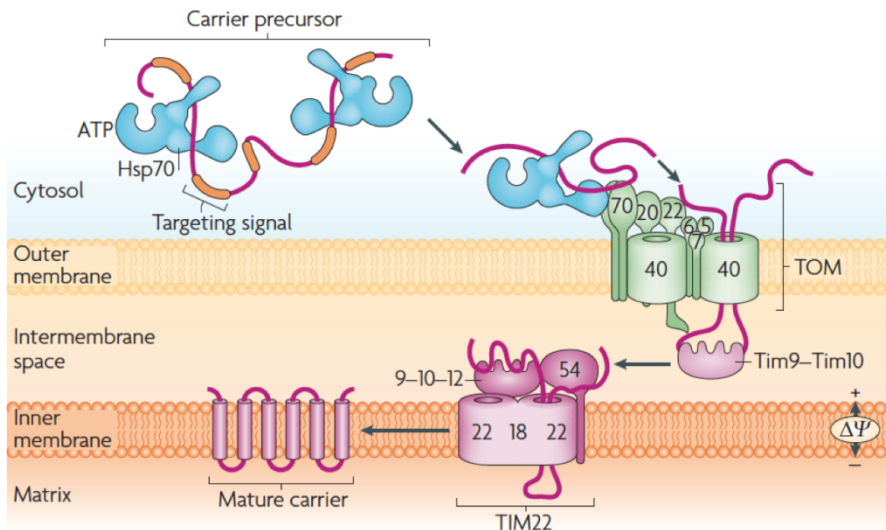


Figure 15. Schematic representation of the import pathway followed by metabolite carrier preproteins, imported through the “Carrier pathway”. Numbers inside or close to the TOM and TIMM22 complexes represent the subunit composition of these machineries. Internal MTS in the mitochondrial preprotein are symbolized in orange. Cytosolic chaperones (here, Hsp70) shuttling the protein to the TOM complex in an ATP-dependent manner are represented in light blue. Adapted from [221].

Conversely, OMM integral proteins are imported through the TOM machinery in cooperation with small TIM dimers (9/10 and 8/13), acting as chaperones to maintain the precursor into an unfolded state. The cooperation between the two major subunits of the SAM complex, the channel SAMM50 and the receptor Sam35 mediate the physical integration of the protein with α -helical or β -barrel features into the OMM [201].

In vitro reconstitutions infer that import reactions are quite fast: once the mitochondrial precursor reaches the TOM complex, it is imported in an unfolded state, so as to partially span the TOM and the TIM machineries. As translocation across the two machineries is completed, the precursor rapidly reaches the IMM or the matrix, where it is retained for cleavage of the presequence and refolding to avoid its accumulation and aggregation; alternatively, it is exported to reach its final submitochondrial localization [201]. In quantitative terms, only a fraction of the protein is usually imported and the remainder is released back into the cytosol, where it is rapidly degraded by the UPS or the autophagy-dependent degradation systems.

C. Crosstalk between mitochondria and the cytosol: mitophagy

Given that mitochondria are at dynamic equilibrium, capable of generating and replacing selective components, we should consider that biogenesis events must be counterbalanced by selective degradation programs. First identified in yeast, particular paradigms of mitochondrial degradation, termed mitophagy, have been discovered by a growing number of studies in mammalian cells, where they lead to extraction of organelles from the mitochondrial network by fission events followed by their elimination [210,223,224]. Under stress-free conditions, several types of mitophagy are known, indicating that this cascade of events can definitely be considered the flipside of organelle biogenesis. First, maturation of erythrocytes derived from red blood cell precursors, involves a series of events including enucleation and elimination of the majority of cellular components, including mitochondria, which are cleared by autophagy [225,226]. Second, whenever an asymmetric repartition of mitochondria takes place in daughter cells during mitosis, it leads to loss of $\Delta\Psi$ in a proportion of the organelles, followed by their destruction by autophagy [227]. Third, sublethal stress conditions, as photoirradiation, blockade of mitochondrial biogenesis or dissipation of electrochemical proton gradient of the IMM by pharmacological means, can also trigger the elimination of the damaged organelles [170,228,229]. Unfortunately, the current literature underlines the lack of a functional or mechanistic link between these three situations (cell differentiation, cell cycle and stress) suspected to share several features of their mitochondrial degradation programs.

As mentioned before, mitophagy is a targeted autophagic-like system that relies on cytosolic actors regulating macroautophagy: these proteins tether autophagic membranes to trigger engulfment of whole mitochondria into autophagosomes, which subsequently fuse with lysosomes to promote the degradation of their content. These molecular players are early autophagosomal adaptors, such as LC3 and p62, as well as several cytosolic factors, as for example Beclin1, GABARAP and the Autophagy machinery of proteins termed ATGs, which in yeast and in mammalian cells coordinate autophagosome formation and fusion with the lysosome [230]. Some intrinsic mitochondrial factors are also needed for mitochondrial clear, e.g. the Bcl-2 family member Nix or its homolog Bnip3; these BH3 domain-containing OMM proteins (Bcl-2 Homology domain), appear to be required for the elimination of mitochondria from red blood cells, both in physiological and in particular stress conditions, such as hypoxia. As they contain an LC3-interacting motif, they have been proposed to be the mitochondrial receptors for the components of the autophagy machinery, although further studies are needed to confirm these observations [231,232]. Noteworthy, the molecular signals triggering the cascade of events leading to the elimination of mitochondria remain largely unknown.

In recent years, mitochondrial removal by autophagy has been the subject of intense investigation in PD-related models, and is today suspected to play a key role in the pathogenesis of autosomal recessive PD forms. The discovery that PD-causing genes of autosomal-recessive inheritance as *PINK1* and *PARK2* code for proteins constitutively or partially localized to the mitochondrion, and that these proteins are involved in the

mitophagy pathway further raised the interest of the scientific community in the study of mitochondrial dysfunction in PD. According to current models, PINK1 translocates to mitochondria thanks to its N-terminal MTS; in healthy conditions it is imported and rapidly degraded by a still unknown mechanism involving the mitochondrial proteases MPP and PARL and the proteasome machinery [233,234]. When mitochondrial protein import is blocked by the down-regulation of the mitochondrial protease MPP, or when $\Delta\Psi$ is dissipated by pharmacological means, PINK1 is stabilized on the OMM, where it recruits Parkin to trigger mitophagy, often monitored with the disappearance of the whole mitochondrial network from cultured cells [189][234]. Although this discovery opened the way for an exciting new field of research, i.e. the investigation of mitophagy in *in vivo* paradigms of PD, its supposed protective effect under physiological conditions and possible dysfunctions thereof in PD await confirmation.

1.2.2 Mitochondria and the regulation of cell death by apoptosis

Cell death is a pivotal event in vertebrates, and a process critical for development, organization of tissues and homeostasis of the organism. In mammals, the molecular mechanism used to eliminate superfluous, ectopic or damaged cells is, in the majority of the cases, the activation of a programmed cell death program called apoptosis. It consists of a tightly regulated molecular cascade leading to drastic morphological changes, including chromatin condensation, nuclear rupture and cell shrinkage; defective or impaired apoptosis are common traits of a plethora of pathologies, i.e. oncogenesis, infectious diseases and septic shocks [235].

As illustrated by the last decades of research, mitochondria play a key role in apoptosis. The first molecular event of this cell death program is the Mitochondrial OMM Permeabilization (MOMP), during which mitochondrial proteins normally found in the IMS, such as cytochrome *c* and apoptotic factors, are released in the cytosol after the formation of size-indeterminate openings in the OMM. This appears to be mediated by members of the Bcl-2 family, a large group of cytosolic proteins with structural and functional similarities (the presence of selected domains known as BH1, 2 and 3), which activate or inhibit cell death and play a role in the selective removal of mitochondria by autophagy. Bcl-2 proteins can be classified into three groups: direct proapoptotic members, such as Bax, which shuttles from the cytosol to the mitochondrion to trigger the formation of pores on the OMM, thus leading to MOMP; prosurvival (antiapoptotic) members of this family, including Bcl-2 and closely related proteins like Bcl-XL, Bcl-W and Mcl-1, which inhibit cell death by interacting physically with proapoptotic members and antagonizing their function; BH3-only proteins, sharing a BH3 motif, such as Bim and Bid, which directly activate the proapoptotic factors Bax or Bak, or antagonize the function of the antiapoptotic Bcl-2 family members, as in the case of Noxa, Bim or Bik. In general, BH3-only proteins are inactive in the cytosol under normal conditions, whereas the presence of proapoptotic stimuli activates the expression of the corresponding genes or post-translational modifications of the proteins. This leads to the mitochondrial translocation of Bax and the conformational change of Bax and Bak on the OMM from inactive to

active states through oligomerization [236]. However, it is yet unclear how this phenomenon is mechanistically causative of MOMP, although it has been speculated that oligomerization of Bax and Bak leads to the formation of a large pore on the OMM, responsible for the release of IMS resident factors [237]. In concomitance with MOMP, a profound re-organization of the OMM and IMM structure takes place. First, massive mitochondrial fragmentation events are promoted, with components of the mitochondrial fusion and fission machinery, Drp1 and Mfn2, co-localizing with Bax at fission sites [238]. Accordingly, remodelling of the IMM and the opening of mitochondrial *cristae* has been described, possibly due to a defective oligomerization of Opa1 monomers. The Bax- and Bak-dependent disruption of the physical interaction between Opa1 and Bnip-3 constitutes a second hypothesis for the physical collapse of this mitochondrial subcompartment during apoptosis [239–241]. Intriguingly, MOMP appears to be both the first step of the apoptotic cascade and the point of no return of this cell death program, as it leads to the irreversible activation of a series of cytosolic caspases and, ultimately, to apoptosis. Once the OMM permeabilized, cytochrome *c* released from the IMS binds to the cytosolic protein Apaf-1 and activates the formation of a heptameric caspase-9 complex, known as the apoptosome. In turn, this complex proteolytically cleaves the executioner caspases-3 and -7, which translocate to the nucleus as transcription factors and activate cell death; concomitantly, these caspases can alter the integrity of the plasma membrane and induce the externalization of the apoptotic marker phosphatidylserine. Furthermore, SMAC/Diablo and Omi/HtrA2 are released from mitochondria into the cytoplasm, where they inhibit the activity of anti-apoptotic proteins, such as XIAP, which prevent activation of caspase-9 and cleavage of caspase-3 in normal conditions [242].

Intriguingly, a growing body of evidence demonstrates that IMM can actively participate in apoptotic cell death; pores termed the Permeability Transition Pore (PTP) can form in this compartment to allow the passage of water and molecules up to 1.5 kDa [243]. Opening of the PTP leads to $\Delta\Psi$ dissipation as ions reach the osmotic equilibrium across the membrane; in addition, H₂O enters the mitochondrial matrix. This events triggers the swelling of these organelles and accordingly to the extent of this phenomenon, MOMP can be induced. Although the molecular composition of this pore has been debated for years, recent evidence demonstrated that it is formed by dimers of the complex V of the mitochondrial respiratory chain [244,245].

1.2.3. Conclusive remarks

Mitochondria are perfectly integrated in the eukaryotic cell and share with their “host” different degrees of crosstalk. The two compartments share a high degree of co-regulation and act in complementary ways to respond to extracellular stimuli and stress factors. Furthermore, it is uttermost fascinating that the principal mechanisms employed by the cell to dispose of exceeding or damaged proteins and cellular components, or to trigger cell death indicate that this organelle is a key player for the efficient outcome of these degradation

processes. This is the reason why, concerning the physiopathology of PD, mitochondria are an excellent target to decipher the molecular events that could potentially lead to the death of dopaminergic neurons in the SNPC and a growing body of evidence indicates that they play a role in the early response of the cell to PD-inducing stress. It has to be considered that over the past 10 years, a huge matter of debate was whether apoptosis causes or contributes to dopaminergic cell death detected in idiopathic PD. A formal demonstration for the role played by apoptosis in PD is still lacking, given the late onset of sporadic PD (possibly because the neuronal death rate at a given time is slow) and a potentially rapid removal of dead nerve cells [246]. In addition, SNPC post-mortem samples usually derive from patients where the disease is advanced and, as a consequence, most dopaminergic neurons have undergone apoptosis already. Nevertheless, elements hinting at an involvement of mitochondrion-dependent apoptosis in sporadic PD are the activation of Bax and of caspase-3 [247–249]. Accordingly, MPTP-treated mice show cytochrome *c* release, activation of Bax and of caspase-3/9, and apoptotic cell death were described [250]. Although MPP⁺ and rotenone block mitochondrial complex I, this blockade is not the molecular cause activating apoptosis *per se*. Alteration of OXPHOS has been hypothesized to trigger a cascade of intra-mitochondrial deleterious events, including an increased ROS production, inducing peroxydation of cardiolipin and of other IMM compounds. This is thought to modify the binding of cytochrome *c* to the IMM, massively releasing it into the IMS in a soluble form, and in the cytosol after MOMP [250,251]. Furthermore, as mentioned in the previous section (Paragraph I.1.2), PD-related proteins entirely or partially localized at the mitochondrion, as Parkin and PINK1, have been linked to apoptotic cell death, corroborating the existence of a link between mitochondria, apoptosis and the pathogenesis of PD.

To date, it is clear that the components of the mitochondrion-dependent apoptotic cascade appear as promising molecular targets for blocking or slowing down dopaminergic cell death in PD. Nevertheless, it must be considered that this cascade requires to be fully elucidated before launching novel therapeutic strategies, which, by consequence, need to be addressed specifically in affected neurons and not in other cell populations.

I.3. Mitochondrial dysfunctions in familial Parkinson's disease

PD-related proteins localize to the mitochondrion

As mitochondrial dysfunctions in sporadic PD patients may represent a molecular hallmark of the disease, deciphering the roles played by PD-related proteins at the mitochondrion could shed light on commonalities between idiopathic and monogenic PD. With the exception of PINK1, which carries an N-terminal MTS, none of the PD-related proteins is primarily localized to the mitochondrion. Notwithstanding an extensive, albeit contradictory series of reports attributes to the majority of these proteins partial association with this organelle and the ability to modulate mitochondrial homeostasis, as illustrated in the following section.

The features of the N-terminal MTS of PINK1 and the presence of a transmembrane domain indicate that the protein could be imported into the organelle as a mitochondrial precursor of 66 kDa localizing to the IMM *cristae*, facing the IMS [130,252]. However, additional studies reported the association of PINK1 with the OMM, both in cell lines and in extracts from human or rodent brains [131,133,253]. The incongruencies concerning PINK1 localization have been extended by the fact that under basal conditions, the endogenous protein is only barely detectable by commercially available antibodies. This indicates that PINK1 is expressed at very low levels in physiological conditions; blockade of its mitochondrial import appears to be necessary to visualize the protein and study its sub-mitochondrial localization. Following $\Delta\Psi$ loss, the C-terminus of the protein protrudes from the OMM and faces the cytosol, whereas its N-terminus is first cleaved in the mitochondrial matrix by the MPP protease, and is then subjected to a second cleavage in the IMM by the PARL protease, turning into a full mitochondrial protein of 52 kDa [233,254] Taking advantage of *in vitro* import assays, a recent study suggested that both full-length and 52 kDa PINK1 localize to the OMM facing the cytosol [255] (For the issues concerning PINK1 import and degradation, see the paragraph I.3.1C "Insights on the lifecycle of PINK1").

Parkin is a cytosolic protein, and it was described to associate to various intracellular organelles, including the nucleus, the endoplasmic reticulum and mitochondria [141,256]. Although a fraction of the protein constitutively localizes to the OMM [141], Parkin is lacking a formal MTS. Remarkably, the protein was found to massively shuttle to this submitochondrial compartment in specific experimental paradigms, particularly after $\Delta\Psi$ dissipation triggered by the protonophore CCCP or upon paraquat-induced oxidative stress [170,257]. Intriguingly, a recent report showed that Parkin translocates to the organelle following blockade of mitochondrial protein import by siRNA-mediated gene silencing of the β subunit of the mitochondrial protease MPP, a condition not associated with $\Delta\Psi$ loss [234]. In this context, PINK1 was found to accumulate in the lipid phase of the OMM, to recruit Parkin and prime defective mitochondria for mitophagy; however, the nature of the molecular signal prompting the mitochondrial translocation of Parkin remains highly debated. Furthermore, compelling evidence demonstrated that this translocation depends on the PINK1-dependent

phosphorylation of Parkin, although the precise residue required for this shuttling remains controversial [258–260].

To date, the mitochondrial localization of DJ-1 has been poorly investigated. As for Parkin, DJ-1 is mostly a cytosolic protein devoid of a MTS, although in cell models and in the mouse brain, a proportion of it has been retrieved in enriched mitochondrial fractions by biochemical methods, or co-localizing with mitochondrial markers by immunocytochemistry and confocal analyses [261]. Miller and colleagues proposed that the DJ-1 is recruited to the OMM in conditions of oxidative stress [261]; however, another study did not confirm this finding, and reported that the protein is found in the IMS and in the mitochondrial matrix [262].

Proteins related to autosomal dominant PD have also been described to be associated with mitochondria. A first study analyzing the import of α -synuclein inside the organelle by mitochondrial import assays reported that the protein was found to be associated with the IMM [263]. Its intramitochondrial translocation was mediated by its charged N-terminus, occurred via the import channel TOMM40 and required an active $\Delta\Psi$ and an active ATP pool. On the contrary, according to a second report using purified mitochondria from mice brain and taking advantage of FRET-based reporters and of biochemical approaches, the import of this protein is rather instantaneous and irrespective of the functional state of the organelle [264].

At last, LRRK2 was also found to associate at the OMM in mammalian cell models [265]. However, a contradictory study in mouse brain tissues reported association of LRRK2 either with the OMM or with purified mitochondria devoid of the OMM, termed mitoplasts [266]. Again, co-localization of the protein with mitochondrial markers was found to be weak in primary neuronal cultures and in post-mortem samples of SNPC obtained from PD patients [267].

I.3.1. The PINK1/Parkin Pathway is centred on mitochondrial homeostasis.

A. Modulation of mitochondrial morphology

In 2003, a study from our laboratory suggested that Parkin could regulate mitochondrial shape, as observed in a *in vitro* paradigm of neuronally-differentiated rat pheochromocytoma cells (PC12) overproducing Parkin: electron microscopy approaches revealed that these cells show a consistent reduction in mitochondrial area compared to control cells [141]. An extended series of studies in *Drosophila melanogaster* models provided outstanding support to this initial observation. Analyses by electron microscopy of *PARK2* null mutant flies revealed a complex mitochondrial phenotype in the indirect flight muscles, defined in general terms as “mitochondrial pathology” and characterized by swollen organelles, few remaining *cristae* and loss of electron-dense material [174,268]. These defects were accompanied by the degeneration of the indirect flight muscles by apoptosis, abnormal wing posture “downturned” wing phenotype), the inability of the flies to fly or jump, defects in spermiogenesis leading to male infertility, and reduced lifespan [174,269]. Successive reports

highlighted the presence of a strikingly similar phenotype in *PINK1* deficient mutants, providing the first line of evidence for the existence of a close relationship between the two genes [270–273]. Additional elements in support of this hypothesis were provided by the surprising finding that overproduction of Parkin in *PINK1* mutant flies recovers a normal phenotype, whereas the reverse does not [270–273]. These observations indicated that *PARK2* and *PINK1* belong to a common linear pathway, with *PARK2* acting downstream of *PINK1*; since then, this pathway is known in the literature as the PINK1/Parkin pathway. In agreement with the existence of this pathway, the phenotype of double null *PARK2/PINK1* mutant flies was no more severe than that observed in each mutant alone [270]. Parallel studies in dopaminergic neurons from *Drosophila melanogaster* revealed neuronal atrophy and loss [174,271,272,274,275]; these findings could only be partially confirmed, as death of dopaminergic nerve cells was not observed in concomitant reports [268,270]. In addition, modeling *PINK1* or *PARK2* deficiency in mammalian cell lines led to similar mitochondrial alterations, including enhanced mitochondrial fragmentation, *cristae* abnormalities, $\Delta\Psi$ loss and defective ATP production [276–279].

Later studies reported that loss of function of a third PD-related gene in *Drosophila melanogaster*, *DJ-1*, leads to a phenotype strikingly similar to that of *PARK2* or *PINK1* null flies. Conversely, overproduction of human or *Drosophila* DJ-1 rescued the wing abnormalities and muscle degeneration observed in *PINK1* mutant flies, although no effect was observed in *PARK2*-deficient strains, suggesting that DJ-1 also acts downstream of *PINK1*, though possibly in a pathway parallel to that of Parkin [280]. Two other putative components of the PINK1/Parkin pathway were identified in the visual system of *Drosophila*: *Omi/HTRA2*, a second gene acting as a downstream effector of *PINK1* but in parallel to *PARK2*, and the IMM protease PARL ortholog, *Rhomboid-7*, suggested to intervene upstream of *PINK1* and *PARK2* [281]. However, the interaction between *PINK1* and *Omi/HTRA2* is controversial since it was not confirmed by a second study [282]; moreover the phenotype induced in *Drosophila* by loss of *Omi/HTRA2* function or introduction of PD-linked mutations was mild and did not involve muscle degeneration, loss of dopaminergic neurons or behavioural defects.

Altogether, these studies clearly demonstrated that *PARK2* and *PINK1* interact genetically, but until 2010 evidence for physical association of the two proteins was missing. *In vitro* studies taking advantage of the co-production of Parkin and PINK1 in cell models revealed interaction between the proteins by co-immunoprecipitation [171], and FRET-based experiments confirmed the existence of this interaction [283]; the association of the endogenous proteins was further ascertained in protein extracts from the rat striatum and SNPc or the human brain [284,285].

B. Regulation of mitochondrial dynamics

As described previously (c.f. Paragraph 1.2.1B), the actors regulating mitochondrial fusion and fission belong to a molecular pathway evolutionary conserved from yeast to mammals; however, our knowledge concerning the functions of its components and their impact on essential cellular processes as organelle biogenesis, quality control pathways and regulation of cell death is still fragmentary. Mitochondrial homeostasis relies on a correct balance between fusion and fission events, and the result of this equilibrium in mammalian cells is usually a tubular network of organelles. Spherical, rod-like mitochondria are the outcome of increased mitochondrial fission, and a highly interconnected network with elongated mitochondria is generated when organelle fusion is prevalent. Mitochondrial fusion and fission processes depend on the activity of key proteins involved in mitochondrial dynamics (DRP1, Fis1, Mff for mitochondrial fission, Mfn1/2 and Opa1 for mitochondrial fusion, c.f. Paragraph 1.2.1B), which is regulated by post-translational modifications, including phosphorylation, ubiquitylation, proteolytic cleavage and SUMOylation; regulation at the transcriptional level has also been described [286].

*Evidence from *Drosophila melanogaster* models*

In 2008, three parallel genetic interaction studies in *Drosophila melanogaster* indicated for the first time that the PINK1/Parkin pathway is implicated in the modulation of mitochondrial fusion and fission [273,287,288]. These reports demonstrated rescue of the phenotypes in *PINK1* null flies by an additional copy of *Drp1*, and discovered that *Drp1* loss-of-function was lethal on these genetic backgrounds [273,287]. Similarly, the *PINK1* and *PARK2* null background phenotypes were ameliorated by RNAi-mediated knockdown or loss-of-function mutations in genes encoding the *Drosophila* profusion proteins, *Opa1* or *Mfn*. Therefore, artificial enhancement of mitochondrial fission was proposed to mimic the activity of the PINK1/Parkin pathway in this model organism (Figure 16, left part). Interestingly, although the morphological defects of mitochondria in *PINK1* mutants were recovered by the downregulation of *Mfn* or *Opa1* homologs [273,287,288], electron micrographs revealed that organelle network had a “hyperfissioned” appearance, possibly due to overactivated fission events. Altogether, data from this *in vivo* model suggest that the PINK/Parkin pathway promotes mitochondrial fission or inhibits mitochondrial fusion.

Evidence from mammalian cells

Observations in cultured mammalian cells and in primary neurons indicated initially that the regulation of mitochondrial dynamics by the PINK1/Parkin pathway may differ from *Drosophila*. A series of studies showed that silencing of *PARK2* or *PINK1* promotes mitochondrial fission, accompanied by dissipation of $\Delta\Psi$ and defects in ATP production, or mitochondrial fusion: the outcome can differ according to cell type and experimental context [276–279,289–293]. In contrast to what observed in flies, the mitochondrial phenotypes

in mammalian cells were rescued by overproducing the fusion-inducing proteins Mfn2 and Opa1, or by decreasing organelle fission, most often with a dominant-negative form of Drp1 [277,279,290,294] (Figure 16). Therefore, the PINK1/Parkin pathway in mammalian cells appears to stimulate fusion, rather than fission. Interestingly, mitochondrial fragmentation induced by silencing of *PARK2* or *PINK1* did not occur in cells depleted for Drp1, suggesting that Drp1 is indeed a key player in the modulation of mitochondrial dynamics depending on the PINK1/Parkin pathway even in mammalian cells [279,294]. Remarkably, mitochondria in cell lines stably silenced for *PARK2* or *PINK1* and in fibroblasts from PD patients bearing pathogenic mutations on both genes did not display increased fission [257,293,295]. These indicate that additional compensatory mechanisms may maintain the mitochondrial dynamic balance in case of constitutive absence of PINK1 or Parkin. Nevertheless, *PARK2* or *PINK1* mutant fibroblasts obtained from PD patients were more sensitive to rotenone or paraquat-induced oxidative stress, not observed in control cells [257,293,295]. These studies indicated that *PARK2* mutant fibroblasts show a lowered $\Delta\Psi$ and a decreased production of ATP in basal conditions compared to controls. Results in fibroblasts from PD patients suggest that the PINK1/Parkin pathway increases mitochondrial length and branching, activating a protective response for the cell in the attempt to overcome oxidative stress [257]. An additional consideration needs to be done to explain the differences between *Drosophila melanogaster* models and mammalian cells: although the actors of the mitochondrial dynamics balance are conserved in these models, the molecular mechanisms regulating the fusion and fission balance, and the putative compensatory mechanisms to overcome Parkin/PINK1 deficiency may simply be different.

In support of this hypothesis, Lutz et al. discovered that the silencing of *PARK2* or *PINK1* in *Drosophila* S2 cells leads first to mitochondrial fission, then rapidly to mitochondrial fusion [294]. Organelle fusion could potentially be detrimental in the long term, as cells with high energy demands as neurons might not be able to rely on organelle fragmentation to maintain the mitochondrial turnover rate: fission-dependent events, as for example the activation of mitophagy and the engulfment of organelles by autophagosomes could be severely impaired in conditions where mitochondrial fusion is favoured. Support to this possibility is given by the observation that stimulating organelle fragmentation by an extra *Drp1* allele rescues the mitochondrial phenotype in *PINK1/PARK2* null mutant flies, possibly because of enhanced organelle clearance, as previously suggested [273].

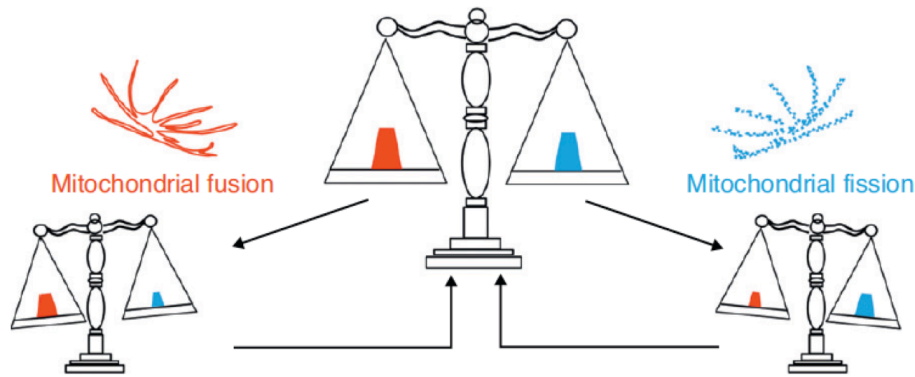


Figure 16. PINK1/Parkin pathway controls the balance between mitochondrial fusion and fission. As this pathway is altered (e.g. by down-regulation of one of the two proteins or by PD-related mutations), the outcomes on the two paradigms are different according to the model organism analyzed. In flies, the PINK1/Parkin pathway seems to trigger mitochondrial fission, so its alteration pushes the dynamic balance towards fusion. Conversely, in mammals the pathway stimulates mitochondrial fusion or fission according to cell type and experimental context; its deregulation induces the equilibrium to shift towards mitochondrial fragmentation or organelle hyperfusion. Adapted from [296].

Finally, recent data also provided evidence for direct interactions between Parkin and proteins of the mitochondrial fusion/fission machinery. In *in vitro* models, Parkin was shown to interact with and ubiquitlyate Drp1 via K48 polyubiquitin chains, addressing the protein to the UPS for degradation [291]. Unfortunately, to date it is not known whether Parkin ubiquitylates the fraction of Drp1 actively promoting organelle fission on the mitochondrion, rather than a quiescent pool localized in the cytosol and enzymatically. The protein Fis1 is also ubiquitylated by Parkin, although *via* monoubiquitylation and not leading to proteasomal degradation; strikingly, however, overproduction of Parkin and ubiquitin led to Fis1 degradation via the proteasome, and proteasomal inhibition was demonstrated to stabilize Fis1 [290]. Therefore, Parkin-dependent monoubiquitylation may be an early step triggering recognition of Fis1 by a still unknown E3 ligase targeting it to the proteasome for degradation. Further studies will be required to determine the identity of this E3 ligase and its direct or indirect relation to Parkin. Finally, Parkin was shown to promote the K48-mediated polyubiquitylation of Mfn proteins both in mammalian cells and in *Drosophila* prompting them for proteasomal degradation [297–299]. Consequently, this step enhances mitochondrial fission, a prerequisite for initiation of mitophagy; in addition, enhanced mitochondrial fission could possibly inhibit the fusion of defective mitochondria with intact organelles, thus facilitating the isolation of impaired organelles to be degraded by mitophagy [298].

C. Regulation of mitochondrial quality control

The PINK1/Parkin pathway and its interactors co-operate to clear dysfunctional mitochondria

In 2008, Narendra et al. published an outstanding observation: Parkin massively relocalizes at the mitochondrion, selectively accumulating on damaged organelles which aggregate in the perinuclear region when $\Delta\Psi$ is pharmacologically dissipated by the use of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or

paraquat [170][300]; upon prolonged exposure to depolarizing agents, Parkin targets the elimination of defective mitochondria *via* the autophagy/lysosomal pathway in a process termed mitophagy (c.f. Paragraph 1.2.1D). This study paved the way for an extensive series of reports, as several groups focused on the research of key molecular interactors of Parkin in this clearance paradigm. In light of the genetic interaction studies in *Drosophila* models, indicating a functional link between *PARK2* and *PINK1* and maintenance of mitochondrial morphology and dynamics, several independent groups investigated on the role played by PINK1 in Parkin-dependent mitophagy [171,301–304]. These reports indicated that PINK1 is indispensable for the mitochondrial translocation of Parkin and for the clearance of defective organelles after $\Delta\Psi$ loss: siRNA-mediated gene silencing of PINK1 in mammalian cell lines and PINK1 deficiency in fibroblasts from knockout mice completely abrogates mitophagy [171,301]. Accordingly, PD-associated PINK1 variants or an artificial kinase-dead protein impaired the mitochondrial recruitment of Parkin and mitophagy following CCCP treatment [301,303,305]. In the same paradigm, PD-related Parkin variants affected differentially the mitochondrial recruitment of Parkin and mitophagy, although these aspects are not univocally linked to the E3 ligase activity of the protein [171,283,303,306,307]. Interestingly, co-production of PINK1 and Parkin without an additional depolarizing stimulus was sufficient to trigger the mitochondrial relocation of Parkin and mitophagy [258,304]; moreover, additional studies indicated that the mitochondrial recruitment of Parkin requires the PINK1-dependent phosphorylation of Parkin, as mentioned before [259,260,305]. Therefore, PINK1 appears to behave as the receptor of Parkin at the OMM; further research is required to understand the mechanisms by which Parkin reaches the OMM and whether other stimuli and/or proteins, are needed in addition to phosphorylation by PINK1 to promote this process.

Is there any intrinsic mitochondrial protein other than PINK1 acting as the receptor of Parkin on the OMM and is this protein a molecular switch in mitophagy? To date, a conclusive answer to these questions is still lacking, although several mitochondrial and cytosolic factors were shown to be part of this mitochondrial elimination program (Figure 17). The silencing of the autophagy adaptor p62/SQSTM1, which recognizes ubiquitylated substrates and interacts with the autophagosomal marker LC3, was shown to impair the elimination of defective organelles by Parkin [306–308]. Although p62/SQSTM1 was demonstrated to be required for mitochondrial clustering at the perinuclear region, its role in mitophagy is debated, with two studies indicating it is dispensable [300,309]. Another Parkin substrate benefiting from considerable attention is the Voltage Dependent Anion Channel 1 (VDAC1). VDAC1 was shown to be ubiquitylated by Parkin after $\Delta\Psi$ loss and to be necessary for effective mitophagy [306]; again, these findings were contradicted by a subsequent report, indicating that VDAC1 was not mandatory for this process [300]. As mentioned before, the Parkin-dependent ubiquitylation of Mfn1/2 has been identified as a key event in CCCP-induced mitophagy, suggesting it constitutes a possible hallmark for recognition of defective organelles, thus enhancing the rate of mitochondrial removal [298]. Nevertheless, a recent study showed that PINK1-phosphorylated Mfn2 is the

receptor of Parkin on the OMM of cardiac myocytes: it appears to be necessary for PINK1/Parkin-mediated removal of mitochondria and its knockout leads to cardiac myopathies [310]. K48 polyubiquitin chains and the AAA+ ATPase p97, previously involved in the extraction from the ER membrane and the degradation of membrane-spanning ER proteins through the UPS, were shown to drive the proteasomal degradation of both Mfns [311]. Once more, this post-translational modification and these proteins appear not to be needed for Parkin-dependent mitochondrial clearance, as *Mfn1/2*-depleted cells correctly activate this program; conversely, a dominant-negative p97 variant abrogated organelle clearance [297–299]. Altogether, it appears that Parkin triggers the elimination of integral OMM proteins, although they seem not to play any role in mitophagy.

Support for this evidence was given by two studies reporting widespread ubiquitylation and proteasomal degradation of OMM proteins following the CCCP-induced recruitment of Parkin at the mitochondrion [312,313]. Subunits of the TOM machinery, VDAC1 and the related channels VDAC2 and 3, and Mfn1 and 2 are among these proteins. Since then, the cytosolic proteins hexokinase 1 and 2 - which catalyze the first step of the glycolytic reaction by converting glucose in glucose 6-phosphate during a transient interaction with the OMM were also discovered to directly interact with Parkin after the induction of mitophagy [314,315,316]. Finally, a recent outstanding paper reported the complete ubiquitylome of Parkin in mammalian cell lines after CCCP treatment [316]. This study confirmed many interactors of the PINK1/Parkin pathway in the the clearance of mitochondria, including VDAC1/2/3, Mfn1/2, the TOMM70 subunit of TOM, previously found to be ubiquitylated by Parkin after $\Delta\Psi$ loss [317], hexokinase 1/2 and subunits of the proteasome. Future studies will have to dissect the role played by PINK1 in the Parkin-dependent ubiquitylation of OMM proteins during mitophagic events.

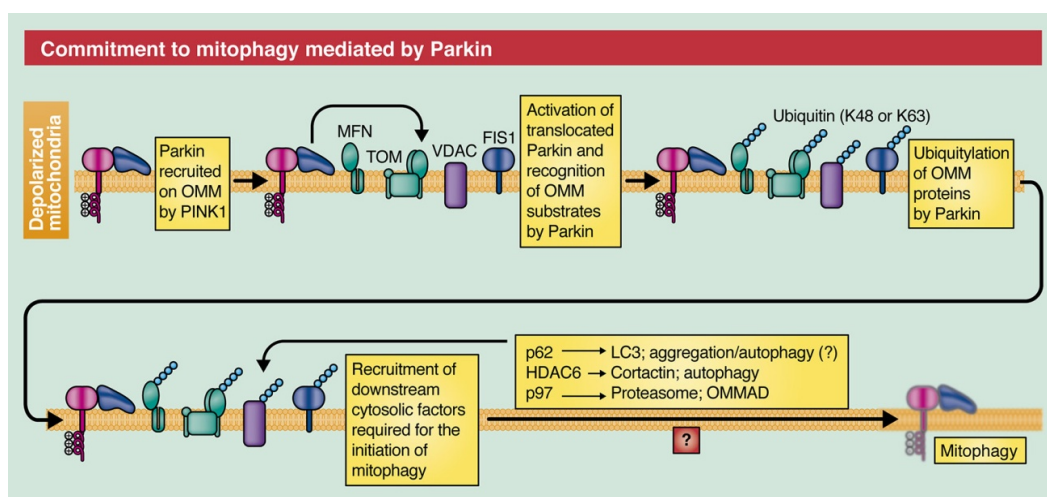


Figure 17. Schematic representation of PINK1/Parkin-dependent selection of damaged mitochondria and their priming for autophagy. Some of the proteins belonging to this degradation program are indicated. OMMAD: OMM-associated degradation. From [318].

In addition, as the kinase domain of PINK1 is believed to face the cytosol [130,255,319], it has been proposed that PINK1 interacts with and recruits Parkin to the mitochondrion through phosphorylation. Indeed, PINK1-dependent phosphorylation of Parkin on the Thr175 and Thr217 residues (located in the RING0 domain) was shown to mediate Parkin recruitment to the mitochondrion [258]; another report indicated that the auto-phosphorylation of PINK1 on Ser228 and Ser402, is sufficient to trigger the translocation of Parkin at the organelle [259], while a third study showed that the PINK1-dependent phosphorylation of Parkin on Ser65 activates the E3 ligase activity of the protein after CCCP treatment [260]. Another study reported that phosphorylation by PINK1 stimulates the E3 ligase activity of Parkin towards the generation of K63 polyubiquitin chains, although the phosphorylation residue found to be responsible for this effect was not determined [285]. Despite this body of evidence, further studies are required to determine whether the interactors of Parkin on the OMM are also phosphorylated by PINK1 in paradigms of mitochondrial clearance.

Insights in the lifecycle of PINK1

What is the peculiarity in the structure/function of PINK1 that makes it absolutely required for mitophagy? Is PINK1 the molecular sensor of defective mitochondria and, if so, how does it accomplish this function? Answers to these questions are of key importance to dissect the molecular changes driven by this protein at the mitochondrion during Parkin-dependent mitophagy. Although PINK1 has an N-terminal MTS (c.f. Paragraph I.1.2A), its import appears to be unconventional. In functional organelles, PINK1 is processed by at least four proteases: the IMM proteases PARL and mAAA and the matrix proteases MPP and ClpXP [233,320]. This generates a rapidly degraded protein sensitive to proteasomal inhibition, suggesting that it shuttles back to the cytosol or that it remains partly accessible to the cytoplasm once imported [255]; these observations raise the questions of the putative function of the protein processed within the IMM and the mechanisms of its potential “export” into the cytoplasm.

In cells treated with CCCP, $\Delta\Psi$ collapse triggers the stabilization and the accumulation of full-length PINK1 at the OMM, with the C-terminus facing the cytosol [130,255,319], prior to Parkin recruitment and the induction of mitophagy. Interestingly, these phenomena were reproduced by the siRNA-mediated downregulation of MPP, a condition in which mitochondrial protein import is blocked without apparent $\Delta\Psi$ loss [320]. This observation introduces an additional level of complexity: is the dissipation of $\Delta\Psi$ or mitochondrial import blockade the signal inducing the accumulation of PINK1 at the OMM? Unfortunately, this question remains still unresolved, as our own observations provided evidence that a direct blockade of the mitochondrial import complex TOM by siRNA-mediated gene silencing is correlated with $\Delta\Psi$ loss [Bertolin et al, in press]. Furthermore, by crosslinking and blue-native gel electrophoresis, a recent report showed that $\Delta\Psi$ collapse leads to the stable association of PINK1 with the TOM machinery within a 300 kDa complex; Parkin was not

found as part of this complex [321]. PINK1 was demonstrated to interact specifically with the TOMM20 receptor, thus suggesting that the protein is imported through the “Presequence pathway”, and that it could remain stuck in the TOM complex until $\Delta\Psi$ is restored or mitochondria enter autophagy. A parallel study also retrieved PINK1 within a 300 kDa complex by affinity purification approaches, although in this case western blot analysis did not reveal the presence of any of the TOM subunits [255]. In addition, a recent study taking advantage of cell-free systems demonstrated that TOMM70, but not TOMM20 or TOMM40 are necessary for the import of PINK1 inside the organelle [322]. Surprisingly, no MPP-induced cleavage of the N-terminal MTS of PINK1 could be observed in this study, which suggests that PINK1 is imported through a non-canonical pathway relying on the receptor TOMM70 only. These observations further complicate the yet hazy scenario of PINK1 lifecycle, including its import and degradation under physiological conditions, its stabilization on the OMM and the molecular events controlling its activity under mitochondrial stress conditions; nevertheless, it is becoming increasingly clear that PINK1 could potentially “sense” mitochondrial dysfunction translated into an impaired mitochondrial protein import and a loss in $\Delta\Psi$, so as to selectively accumulate on damaged organelles [320][Bertolin et al, in press]

Mitophagy requires the interplay between the UPS and autophagy-dependent degradation

As explained previously (c.f. Paragraph I.2.1D), mitophagy is a complex process achieved through a cascade of sequential events. This program relies, in the end, on the fusion between autophagosomes and lysosomes, and pharmacological treatments blocking this step, as for example bafilomycin A1 (which dissipates the acidic pH of lysosomes) or 3-methyladenine (which inhibits the formation of autophagosomes), were shown to abolish PINK1/Parkin-dependent mitophagy [170,323]; accordingly, silencing of the essential components of the autophagic machinery Atg5 and Atg7 [170,303,309] or of the ubiquitin-binding deacetylase HDAC6 [307] also compromises PINK1/Parkin-mediated organelle clearance. Nevertheless, as mentioned the previous paragraph, new breakthroughs indicated that the UPS is also involved in mitochondrial elimination [298,312,313,316,323]. This raised the hypothesis that two differential phases are required to achieve mitophagy: the first one relies on the proteolytic activity of the proteasome, leading to the degradation of OMM proteins ubiquitylated by by Parkin-dependent; in the second phase the mitochondrial remnants are possibly eliminated through the recruitment of components of the autophagic machinery by Parkin, including its interactor Ambra1, which regulates Beclin1/Atg6 and activates class III PI3K, essential for autophagosome nucleation (c.f. paragraph I.1.2B) [324][323]. However, some data need to be reconciled: how could organelles with a double membrane become accessible to the proteasome, although they have been found encapsulated by autophagosomes [313]? As the overproduction of the K48L variant of ubiquitin unable to generate K48 polyubiquitin chains appears to impair Parkin-dependent mitochondrial degradation [303,307], what is the role of alternative polyubiquitin linkages, such as K63 and K27, found for example to modify VDAC1 in the

presence of Parkin following $\Delta\Psi$ loss [306,312]? Again, the view that mitochondrial degradation is executed by two independent phases seems simplistic; future data are required to unravel the molecular actors allowing the switch between putative phase I (UPS-dependent) and phase II (autophagy-dependent), and to clarify the molecular interplay between these degradation pathways.

I.3.2. Conclusive remarks

As exposed in this last chapter, the PINK1/Parkin pathway directly regulates a plethora of mitochondrial functions, including mitochondrial morphology, dynamics and degradation. In addition, this protein pair appears to be implicated in the regulation of other mitochondrial functions, potentially linked with the possibly primary role of these proteins in mitochondrial quality control. For instance, Parkin and PINK1 may modulate the transport of mitochondria through cytoskeletal elements, thus being part of the trafficking machinery for the shuttling of defective organelles to the lysosomes for degradation [208]. This function may be exerted by the physical interaction of PINK1 with the OMM GTPase Miro and the kinesin-adaptor protein Milton, potentially anchoring mitochondria to cytoskeletal “engines” for transport [289,325]. However, recent evidence rather suggests that PINK1-dependent phosphorylation of Miro, which appears to be a prerequisite for Parkin to target the protein to the proteasome for degradation, arrests mitochondrial trafficking, sequestering the damaged organelles in a (putative) “quarantine” prior to mitophagy [208]. In addition, the absence of Parkin in rodents and in samples from PD patients was shown to enhance the accumulation of Parkin Interacting Substrate (PARIS), a KRAB and zinc-finger protein normally targeted to the proteasome by Parkin-dependent ubiquitylation [326]; PARIS was demonstrated to be a transcriptional repressor of the nuclear transcription factor PGC-1 α , one among the master gene regulators of mitochondrial biogenesis as illustrated in Paragraph I.2.1B. Therefore, Parkin appears to positively regulate mitochondrial biogenesis, such that its absence may not only compromise the degradation of damaged organelles but also the renewal of mitochondrial components.

In conclusion, our current knowledge of the activities of the PINK1/Parkin pathway may only be the tip of the iceberg: we gained a considerable number of “snapshots” concerning possible mitochondrial functions controlled by this protein pair. Nevertheless, the molecular mechanisms connecting these functions and the overall impact of this pathway in terms of number of substrates, totality of catalyzed reactions and their dynamics in time and space remain largely unknown. Additional studies are therefore required to gain insight into these aspects for a more precise picture of the overall consequences of dysfunction of the PINK1/Parkin pathway and its relevance for autosomal recessive parkinsonism and to PD.

Chapter II. Preface and objectives

I initiated my PhD project in Alexis Brice's group in October 2009, with the aim of studying the molecular mechanisms underlying the regulation of mitochondrial homeostasis by Parkin in the team led by Olga Corti. This group had already shown in 2003 that a fraction of Parkin in the cell is associated with the OMM in physiological conditions [141]. In a differential proteomic analysis of protein extracts from striatum and cortex of *PARK2*-deficient and wild-type mice at 12 and 24 months of age [327], the team had identified the mitochondrial matrix enzyme 17 β -hydroxysteroid dehydrogenase 10 (HSD17B10) as a protein moderately, but reproducibly upregulated (30%) in the absence of *PARK2*. This multifunctional enzyme had been previously reported to protect against dopaminergic neuron degeneration caused by MPTP intoxication in mice, and to be downregulated in the substantia nigra in *post-mortem* samples of cases with sporadic PD [328]. Complementary analyses in our laboratory had shown that, although HSD17B10 was increased in abundance in total protein extracts from *PARK2*-knockout mice, its levels were significantly lowered in mitochondrion-enriched fractions from these mice compared to wild-type controls. In addition, the results of protein interaction and ubiquitylation assays suggested that HSD17B10 is a direct substrate of the E3 ubiquitin-protein ligase activity of Parkin. Intriguingly, when overproduced in cells, HSD17B10 had been shown to recruit Parkin to the mitochondrion, suggesting interaction between the proteins at the OMM, prior to the intramitochondrial translocation of HSD17B10. While this work was in progress, Richard Youle and colleagues at NIH made a ground-breaking discovery: they reported that treatment of cells in culture with the protonophore CCCP, which dissipates the mitochondrial transmembrane potential required for various processes relevant to proper mitochondrial function including mitochondrial protein import, leads to recruitment of Parkin to dysfunctional mitochondria, thereby initiating their degradation by autophagy [170]. At that time, the role of PINK1 in this process remained to be defined.

Based on these observations and context, I began my PhD work with the following main objectives:

1. To investigate the role of mitochondrial import blockade in the mitochondrial recruitment of Parkin;
2. To explore the role of the Translocase of Outer Membrane as a scaffold mediating protein-protein interactions involving Parkin, HSD17B10 and PINK1 at the OMM;
3. To evaluate the role of Parkin (and PINK1) in the mitochondrial import of HSD17B10, and possibly in that of PINK1 itself;
4. To address the role of HSD17B10 and the TOM machinery in the PINK1/Parkin-dependent mitochondrial elimination program;

Finally, since mitochondrial degradation is coupled with mitochondrial fission to promote separation of dysfunctional mitochondria from the healthy network, to gain insight into the mechanisms underlying the coordination between these processes, in a final objective, we sought:

5. To determine whether the mitochondrial recruitment of Drp1 in cells subjected to CCCP treatment is coordinated with that of Parkin on the OMM.

Chapter III. Materials and methods

This section is comprised in the bodies of Article 1, Article 2 and Article 3.

An insight concerning FRET microscopy is presented below.

FRET microscopy

Förster's Resonance Energy Transfer (FRET) microscopy relies on the non-radiative transfer of energy from one fluorescent molecule (the "donor") to a second fluorescent molecule (the "acceptor"): if both molecules are located in close proximity (< 10 nm), the photon emitted by the donor after laser excitation will be transferred to the acceptor, which will emit a photon at its specific wavelength [329]. A widely employed method to explore interaction between two putative molecular partners is the so-called acceptor photobleaching FRET (acPbFRET) technique, whereby the presence of FRET between a given donor/acceptor pair is revealed as an increase in the donor fluorescence after destruction of the acceptor by laser-mediated photobleaching (Figure 1). During this procedure, the elimination of the acceptor fluorophore "releases" the energy of the donor, which instead of being transferred to its partner, is emitted as a fluorescence characteristic of the donor.

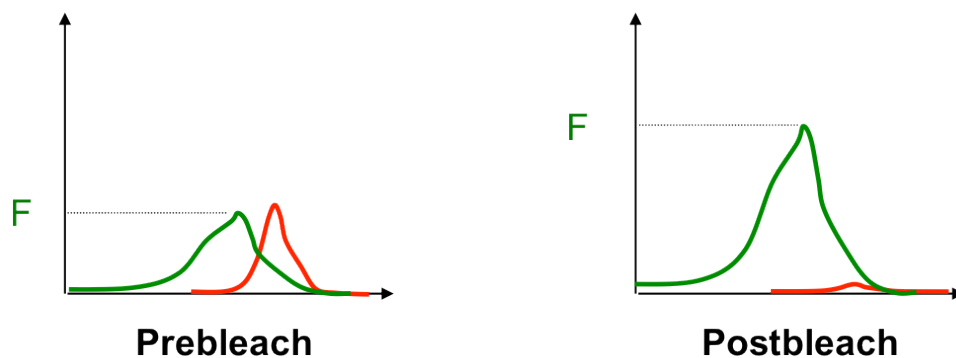


Figure 1. Representation of the increase in donor (green) fluorescence after acceptor (red) photobleaching, indicative of FRET. F = fluorescence intensity.

In our studies, we investigated interactions between ectopic and exclusively endogenous proteins, by staining candidate donor/acceptor pairs with the corresponding primary antibodies and species-specific secondary antibodies conjugated to appropriate fluorophores with partially overlapping emission/excitation spectra (Figure 2): Alexa488 for the donor and Alexa 568 for the acceptor.

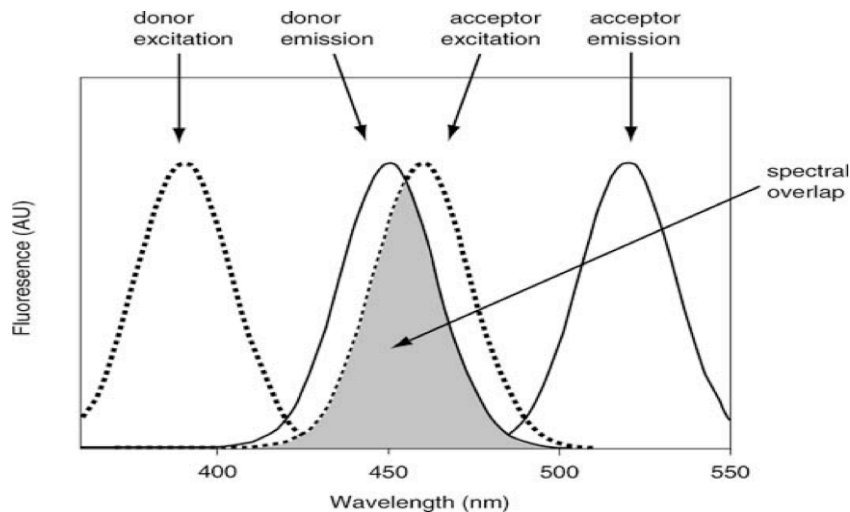


Figure 2. Illustration of the superposition (grey area) between the donor emission and acceptor excitation spectra, required to detect FRET. AU: arbitrary units.

We then acquired confocal images of the fluorescence of the donor before and after photobleaching of the acceptor in 10-20 cells per condition, with the Olympus FV-1000 confocal microscope. FRET efficiencies (% FRET) were calculated pixel by pixel within manually selected regions of interest (ROI, with in general 1 ROI/cell), according to the following equation [329]:

$$\% \text{ FRET} = (I_D \text{ postbleach} - I_D \text{ prebleach}) / I_D \text{ postbleach} \quad (I: \text{ fluorescence intensity; D: donor})$$

Mean values per ROI (10-20 per condition) were used to generate FRET efficiencies per donor/acceptor pair reported on the graphs. Each of these values was also accompanied by an estimate of the proportion of cells displaying FRET.

As an alternative approach, Fluorescence Lifetime Imaging Microscopy (FLIM) was used to corroborate FRET results. This technique relies on the measurement of the lifetime (τ) of the excited state of a fluorescent donor; due to the FRET effect, τ is shorter for a donor molecule in proximity of an acceptor molecule than for a donor molecule alone (Figure 3) [330].

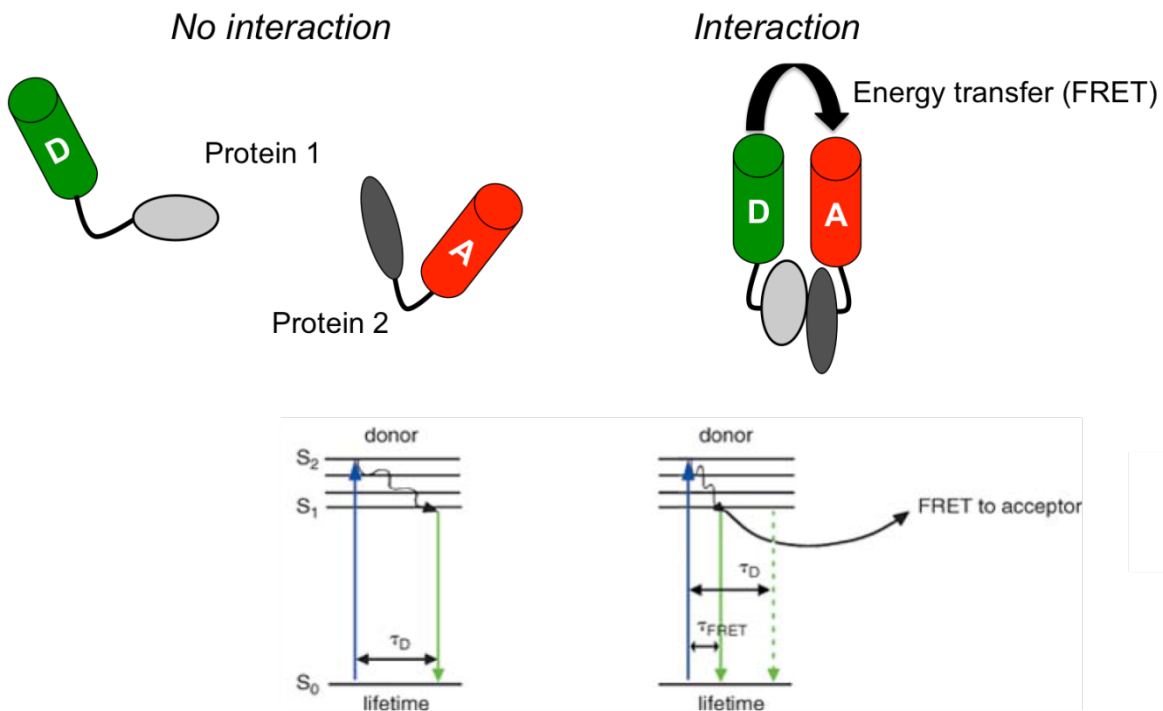


Figure 3. Representation of FRET measured by FLIM. Left: the donor and acceptor are at a distance not compatible with FRET; the lifetime measured is characteristic of the donor molecule only (τ_D). Right: the donor is in close proximity to an acceptor molecule; its initial lifetime (τ_D) is shortened due to energy transfer to the acceptor and becomes τ_{FRET} . S1 and S2 indicate the energy levels reached by a donor molecule following its excitation by a laser; S0 indicates the basal state.

In our experiments, we acquired images of the donor in the presence or absence of the acceptor in 10-20 cells per condition, with a Nikon TE-2000 inverted microscope and a custom-built system based on a commercial module (Lifa, Lambert instruments). Pixel-by-pixel lifetime (τ) values in a manually-selected ROI (1 ROI/cell) were calculated with the Lifa Software; mean values from each of 10-20 ROIs were used calculate the mean lifetime values per donor/acceptor pair reported in the graph.

Chapter IV. Results

This section contains the bodies of Article 1 (Accepted, in press), Article 2 (Submitted) and Article 3 (Submitted).

For convenience, the articles were inserted in order of acceptance and of authorship.

Article 1: **The TOMM machinery is a molecular switch in PINK1 and PARK2/PARKIN-dependent mitochondrial clearance (*Autophagy*, 9(11) in press).**

In 2010 a wave of studies confirmed and extended the initial discovery of Richard Youle's team that the pharmacological dissipation of the mitochondrial membrane potential ($\Delta\Psi$) by the protonophore CCCP triggers the recruitment of PINK1 and Parkin to the OMM, ultimately leading to mitophagy [283,303,306]; these studies also showed that the mitochondrial translocation of Parkin occurs in a PINK1-dependent manner.

By reasoning that blockade of mitochondrial protein import is a concomitant event in CCCP-induced $\Delta\Psi$ collapse, we hypothesized that this is the molecular signal triggering the recruitment of Parkin in proximity of PINK1, accumulating on the OMM. Using Förster resonance energy transfer imaging, we here showed in several models of mitochondrial protein import impairment that Parkin is recruited in proximity of PINK1 at the TOM machinery, the general entry gate for the vast majority of mitochondrial proteins encoded by the nuclear genome. These molecular proximities were impaired by PD-causing *PARK2* mutations, suggesting they are of physiopathological relevance to PD.

Since subunits of the TOM complex are among the first proteins to be degraded during the early proteasome-dependent phases of Parkin-dependent mitochondrial clearance, we sought to determine whether this event is alone sufficient to trigger mitochondrial degradation. We provided evidence that mimicking the degradation of the channel subunit (TOMM40) or the central receptor (TOMM22) of the TOM complex by RNA interference was sufficient to trigger the clearance of OMM and IMS proteins in cells treated with CCCP in the absence of exogenous Parkin or endogenous PINK1. However, under these conditions exogenous Parkin was required to promote the degradation of whole organelles by autophagy, whereas PINK1 was dispensable for this step. Finally, by using a semi-quantitative method to evaluate mitochondrial loss, we demonstrated that depletion of TOMM40 triggers Parkin-dependent mitochondrial degradation even in the absence of CCCP.

Altogether, the results presented in this manuscript suggest that the TOM complex acts as a molecular switch in the PINK1/Parkin pathway, coupling loss of mitochondrial protein import efficiency with the initiation of mitochondrial degradation. We propose that dysfunction of this quality control mechanism may play a key role in the physiopathology of autosomal recessive PD.

(This is a peer-reviewed copy of the manuscript in press)

ARTICLE 1

The TOMM machinery is a molecular switch in PINK1-PARK2/PARKIN-dependent mitochondrial clearance

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Abbreviations: ACTA1, actin, alpha 1, skeletal muscle; AMBRA1, autophagy/beclin-1 regulator 1; ATG5/7, autophagy-related 5/7; ATP5A1, ATP synthase subunit alpha; CCCP, carbonyl cyanide m-chlorophenylhydrazone ; CYCS; cytochrome c; Dox, doxycycline; dsRed, red fluorescent protein from *Discosoma*; GFP, green fluorescent protein; GST, glutathione-S-transferase; HSD17B10, hydroxysteroid (17-beta) dehydrogenase 10; LC3, microtubule-associated protein 1 light chain 3 alpha; LONP1, lon peptidase 1, mitochondrial; MFN1/2, mitofusin 1/2; OXPHOS, oxidative phosphorylation system; PARK2, parkinson protein 2, parkin; PARL, presenilin associated, rhomboid-like; PINK1, phosphotensin-induced kinase 1; PMPCB, peptidase (mitochondrial processing) beta; TOM, translocase of outer mitochondrial membrane; TOMM20/22/40/70A, translocase of outer mitochondrial membrane 20/22/40/70A homolog; VDAC1, voltage-dependent anion channel 1.

Loss-of-function mutations in *PARK2/PARKIN* and *PINK1* cause early-onset autosomal recessive Parkinson disease (PD). The cytosolic E3 ubiquitin-protein ligase *PARK2* cooperates with the mitochondrial kinase *PINK1* to maintain mitochondrial quality. A loss of mitochondrial transmembrane potential ($\Delta\psi$) leads to the *PINK1*-dependent recruitment of *PARK2* to the outer mitochondrial membrane (OMM), followed by the ubiquitination and proteasome-dependent degradation of OMM proteins, and by the autophagy-dependent clearance of mitochondrial remnants. We show here that blockade of mitochondrial protein import triggers the recruitment of *PARK2*, by *PINK1*, to the TOM machinery. PD-causing *PARK2* mutations weakened or disrupted the molecular interaction between *PARK2* and specific TOMM subunits: the surface receptor, TOMM70A, and the channel protein, TOMM40. The downregulation of TOMM40 or its associated core subunit, TOMM22, was sufficient to trigger OMM protein clearance in the absence of *PINK1* or *PARK2*. However, *PARK2* was required to promote the degradation of whole organelles by autophagy. Furthermore, the overproduction of TOMM22 or TOMM40 reversed mitochondrial clearance promoted by *PINK1* and *PARK2* after $\Delta\psi$ loss. These results indicate that the TOM machinery is a key molecular switch in the mitochondrial clearance program controlled by the *PINK1-PARK2* pathway. Loss of functional coupling between

mitochondrial protein import and the neuroprotective degradation of dysfunctional mitochondria may therefore be a primary pathogenic mechanism in autosomal recessive PD.

Keywords: Parkinson disease, mitochondria, outer mitochondrial membrane, mitophagy, PARK2, PINK1, TOM machinery.

Introduction

Parkinson disease (PD) is a frequent neurodegenerative disease and the most common movement disorder. It is in most cases sporadic, but nearly 10% of the cases are caused by mutations in genes with autosomal dominant or recessive inheritance. At least seven of these genes have been identified; the *PARK2* and *PINK1* genes, encoding the cytosolic E3 ubiquitin-protein ligase, PARK2, and the mitochondrial kinase, PINK1, account for clinically similar early-onset autosomal recessive forms of PD.¹

There is growing evidence that mitochondrial dysfunction plays a central role in the pathogenesis of autosomal recessive PD. Genetic studies in *Drosophila* demonstrate that *PARK2* and *PINK1* belong to a common pathway implicated in the maintenance of mitochondrial morphology and dynamics.²⁻⁷ In 2008, it has been shown in mammalian cells that dissipation of $\Delta\Psi$ leads to recruitment of PARK2 to mitochondria, followed by their elimination by autophagy.⁸ Translocation of PARK2 to mitochondria depends on the accumulation of PINK1 on the outer mitochondrial membrane (OMM), and PD-causing gene mutations compromise these events.⁹⁻¹³ Ubiquitination by PARK2 and proteasomal degradation of the OMM mitochondrial fission proteins MFN1/2 are early events triggered by $\Delta\Psi$ collapse;¹⁴ this study provided the first indication that the proteasome is also required for effective mitochondrial clearance. This machinery is critical for the PARK2-dependent proteolysis of a series of OMM proteins, a process associated with OMM rupture that precedes the degradation of dysfunctional organelles by autophagy.^{15,16}

Protein import into mitochondria relies on $\Delta\Psi$ and depends on the translocase of the OMM (TOM), a multiprotein complex responsible for importing the majority of mitochondrial proteins, which are encoded by the nuclear genome.¹⁷ PINK1 is imported into mitochondria and following $\Delta\Psi$ loss, it forms a high molecular weight complex with the TOM machinery.¹⁸ Here we investigate the role of the TOM complex in the recruitment of PARK2 and explore its implication in the different phases of the mitochondrial elimination program controlled by the PINK1-PARK2 pathway.

Results

Mitochondrial import blockade recruits PARK2 to the OMM. We investigated whether the recruitment of PARK2 to the OMM following $\Delta\psi$ collapse was due to the disruption of mitochondrial import. We addressed this issue in three models of mitochondrial import impairment (**Fig. 1 and Fig. S1**): COS7 cells treated with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), widely used to study PINK1-PARK2-mediated mitochondrial clearance; HeLa cells modified for stable, doxycycline (Dox)-inducible depletion of the mitochondrial matrix LONP1 protease (HeLa-T-REx-shLONP1), a multifunctional enzyme essential for mitochondrial biogenesis;^{19,20} and HEK293T cells transfected with modest amounts of an siRNA targeting the channel-forming TOMM40 subunit of the TOM machinery. Mitochondrial protein import efficiency was investigated by studying the cleavage of the N-terminal mitochondrial targeting signal (MTS) of a modified GFP protein (mitoGFP) by the mitochondrial processing peptidase (MPP), a process which is coupled to the import of preproteins (**Fig. 1A**).²¹⁻²³ Abundance of the cleaved, intramitochondrial form of mitoGFP was significantly lower in cells treated with CCCP or depleted of LONP1 than in control cells, demonstrating the impairment of mitochondrial protein import. As the effect was less clear in cells with lower TOMM40 levels, due to the moderate downregulation of its expression, we studied the abundance of mitoGFP in mitochondrion-enriched fractions treated with trypsin to eliminate exposed OMM proteins, including non-imported mitochondrial precursors (**Fig. 1A**). Under these conditions, we observed a decrease in mitoGFP levels proportional to the concentration of TOMM40 siRNA, confirming the import defect.

We then studied the distribution of endogenous PARK2 in HEK293T cells and exogenous PARK2 in COS7 cells, in which the endogenous protein is undetectable with the available antibodies, and in HeLa cells, which lack it. PARK2 was recruited to mitochondria clustered around the nucleus in all three models (**Fig. 1A and B**). As expected, CCCP treatment led to $\Delta\Psi$ loss, indicated by a strong decrease in the intensity of staining with the potentiometric mitochondrial dye tetramethylrhodamine methyl ester (TMRM) (**Fig. 1C**). Mitochondria were also moderately depolarized in cells lacking LONP1 (+Dox) or containing low levels of TOMM40.

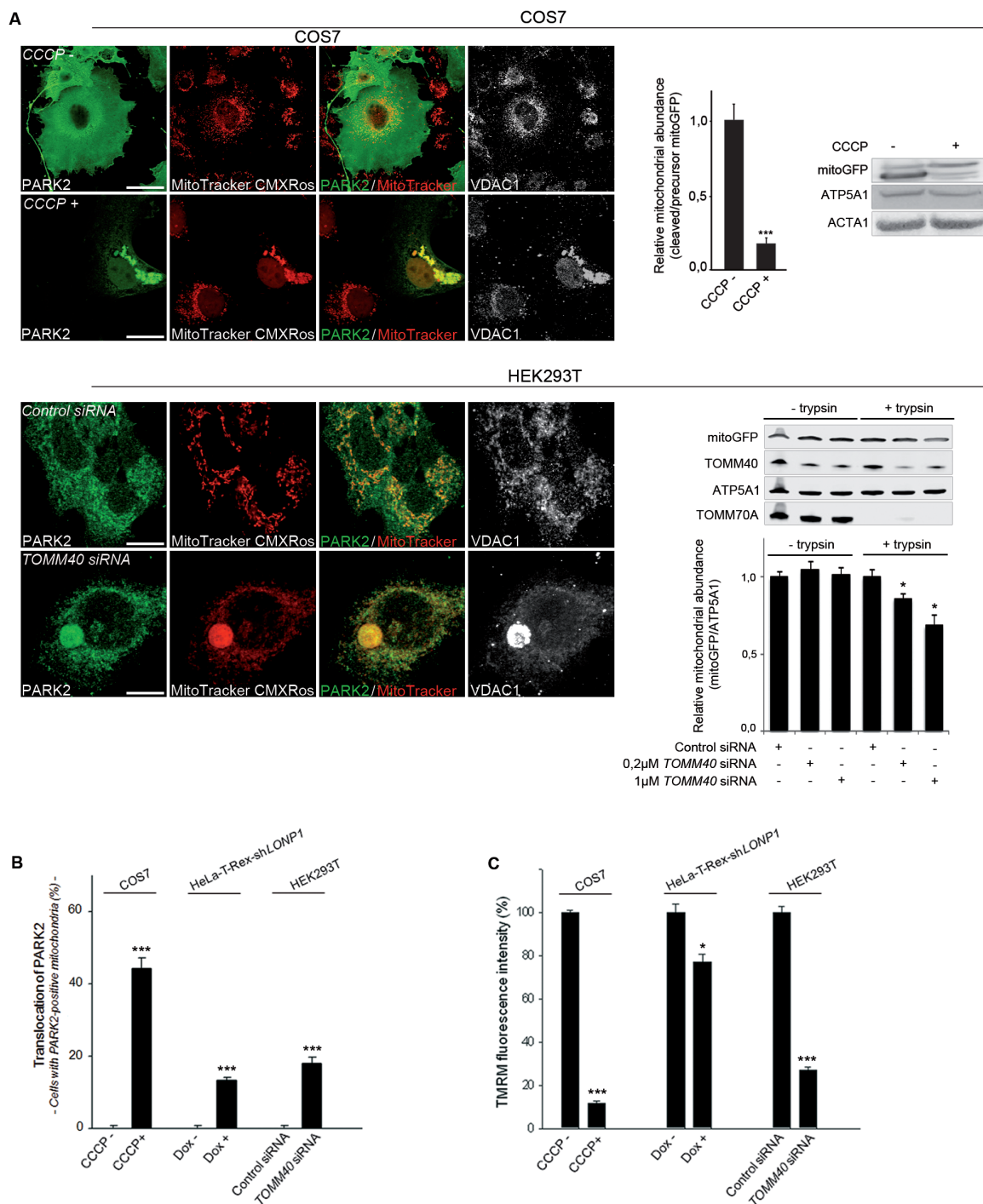


Figure 1. Impaired mitochondrial import triggers PARK2 recruitment to mitochondria. **(A)** Left panels: mitochondrial redistribution of PARK2 in models of defective mitochondrial protein import. Mitochondria were visualized with MitoTracker CMXRos and anti-VDAC1 staining. Treatments are indicated in italics. Right panels: mitochondrial import impairment of mitoGFP (western blots) and quantification of the abundance of the imported mitoGFP protein in total lysates from CCCP treated cells, or in mitochondrion-enriched fractions subjected to trypsin digestion in cells with lower TOMM40 levels ($n = 3$ independent experiments). Loading controls: ATP synthase subunit alpha (ATP5A1), ACTA1/actin (actin, alpha 1, skeletal muscle). Note the disappearance of the TOMM70A immunoreactive band in trypsin-digested mitochondrial fractions. **(B)** Proportion of cells with colocalization of PARK2 and VDAC1 immunostainings in the various models. **(C)** Fluorescence intensity of TMRM in live cells. $n = 3$ independent wells from one of at least three independent experiments. * $p < 0.05$, *** $p < 0.001$ versus the corresponding control condition. Scale bar, 10 μm .

PARK2 is recruited by PINK1 to the TOM machinery. We hypothesized that PARK2 would be recruited to the OMM close to the TOM machinery when mitochondrial import is impaired. The TOM complex has a highly stable functional core unit consisting of the channel protein TOMM40 and the central receptor TOMM22, which holds the translocating preproteins and is essential for cell viability.^{17,24-26} Several small subunits and two loosely bound receptors for mitochondrial preproteins (TOMM20 and TOMM70A) are associated with the central core. To investigate the interaction between PARK2 and TOM machinery subunits, we used Förster resonance energy transfer (FRET) microscopy. This technique, due to its great sensitivity, is particularly well suited to the detection of proximity between two proteins compatible with their interaction *in cellulo*.²⁷ We chose a FRET approach based on the detection of quenched donor fluorescence after photobleaching of the acceptor, with endogenous donor-acceptor protein pairs labeled with secondary antibodies conjugated to appropriate fluorophores (**Fig. 2**). FRET between pairs occurs only if the two fluorophores are separated by distances compatible with single protein molecules (≤ 10 nm).²⁷ In HEK293T cells treated with CCCP or with *TOMM40* siRNA, FRET was detected between endogenous PARK2 and TOMM70A, and to a lesser extent between PARK2 and TOMM40 (**Fig. 2A and B**); surprisingly, FRET was not detected between PARK2 and VDAC1, previously reported to be ubiquitinated by PARK2 after CCCP treatment.¹¹ These interactions were also observed with exogenous PARK2 in COS7 cells treated with CCCP and in HeLa-T-REx-sh*LONP1* cells depleted of *LONP1*, and for the TOMM70A-PARK2 pair, in control cells (**Fig. S2A and B**). FRET was never observed in cells immunostained with secondary antibodies only (**Fig. S2C**). To confirm these observations with a biochemical approach, we used the GST pull-down technique to test the interaction of PARK2 with subunits of the TOM machinery in COS7 cells and HEK293T cells (not shown). TOMM70A bound specifically to GST-PARK2 but not to a GST control protein incubated with lysates from the two cell types (**Fig. S2D**). However, we did not detect a direct interaction between GST-PARK2 and TOMM40 or VDAC1.

We then investigated whether PINK1 is required for the recruitment of PARK2 to the TOM machinery. In HEK293T cells treated with *TOMM40* siRNA or CCCP, FRET was detected between endogenous PINK1 and each of the four TOMM subunits examined, and between endogenous PINK1 and PARK2 (**Fig. 2A and B**). In

cells silenced for *PINK1* or *PARK2*, no molecular interactions involving *PARK2* were detected at the TOM machinery (Fig. 2B). We then confirmed the above-described protein-protein interactions in mitochondrion-enriched fractions isolated from HEK293T cells. As expected, CCCP treatment resulted in stabilization of the long form of *PINK1* at the mitochondrion and recruitment of *PARK2* (Fig. S3A). Furthermore, we detected FRET between *PARK2* and all components of the TOM machinery tested and between *PARK2* and *PINK1*, but not *VDAC1* (Fig. S3B). Again, the TOMM70A-*PARK2* interaction appeared to be the strongest, as it was also detected in mitochondria from untreated cells.

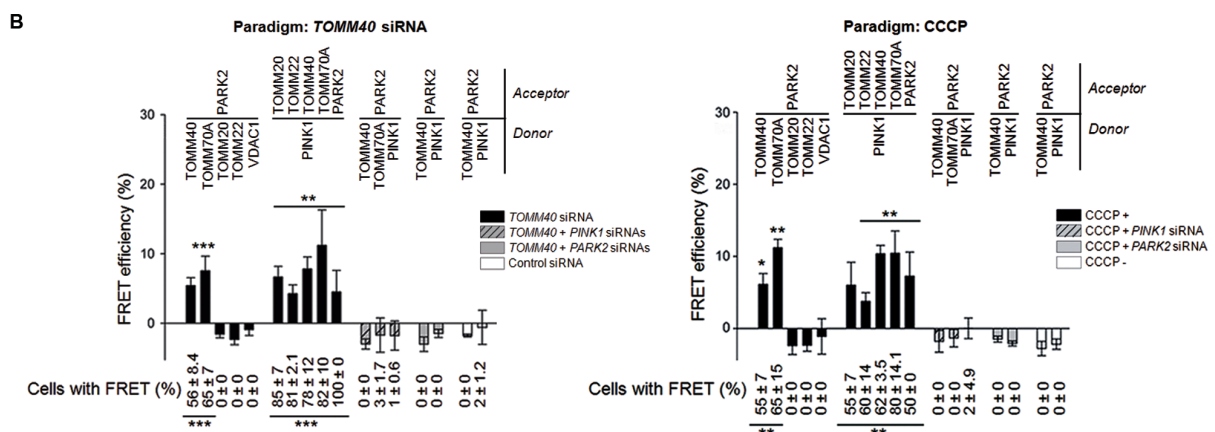
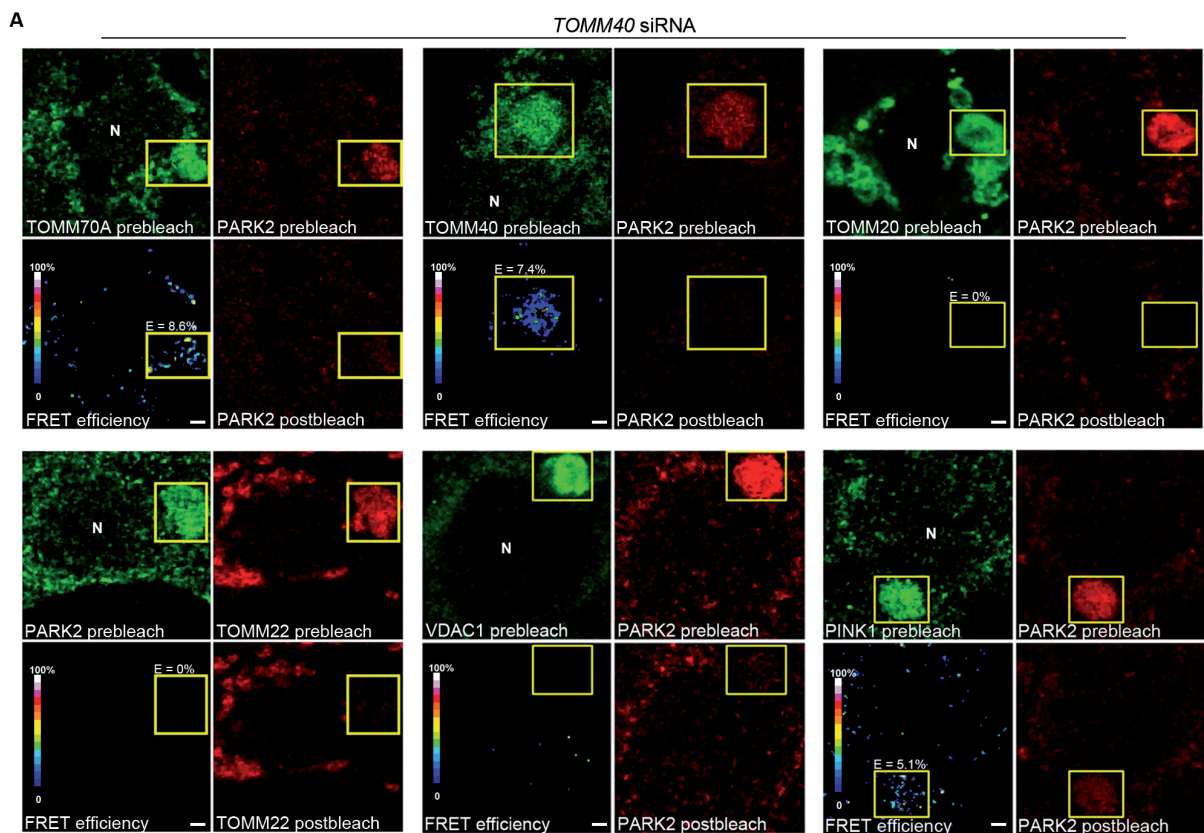


Figure 2. PARK2 interacts with the TOM machinery in a PINK1-dependent manner. **(A)** Representative FRET images for the indicated donor (green)-acceptor (red) pairs in HEK293T cells with low TOMM40 levels. Pseudocolor scale: FRET efficiencies reflecting individual pixel donor fluorescence changes after acceptor photobleaching. E: mean FRET efficiency within the region of interest (ROI, yellow frame). N: nucleus. **(B)** Quantitative FRET analysis and percentage of cells with FRET for all donor-acceptor pairs analyzed in HEK293T cells transfected with siRNA against *TOMM40* or treated with CCCP. $n = 10$ to 35 cells from two independent experiments. Comparisons were made with VDAC1-PARK2 for donor-PARK2 pairs, PINK1-PARK2 in the corresponding “Control siRNA” or “CCCP-” conditions for PINK1-acceptor pairs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar, 3 μm .

PD-causing mutations disrupt the interaction between PARK2 and the TOM complex. To evaluate the relevance of the identified interactions, we then explored the effects of PD-causing missense *PARK2* mutations on PARK2 recruitment to the TOM machinery. FRET efficiencies between TOMM70A and each of nine *PARK2* variants overproduced in COS7 cells were lower than those between TOMM70A and normal *PARK2* after CCCP treatment. Furthermore, all the mutations tested abolished energy transfer from TOMM40 to *PARK2* (**Fig. 3A and B**), suggesting that they alter the orientation of *PARK2* within the complex, thereby modifying its behaviour in FRET experiments.²⁷ We confirmed these findings by using fluorescence lifetime imaging microscopy (FLIM) as an alternative technique to analyze the impact of selected *PARK2* mutations with modest (K161N) or large (K211N, R256C) effects on the interaction of the protein with TOMM70A (**Fig. 3B**).²⁷ This approach is based on the shortening of the fluorescence lifetime of a donor in close proximity of a fluorescence acceptor, due to the FRET effect. Consistent with FRET measurements, the presence of fluorescently labeled *PARK2* or its K161N variant, but not the K211N and R256C variants, significantly decreased the mean lifetime of TOMM70A fluorescence; normal *PARK2* had a stronger effect than the K161N variant.

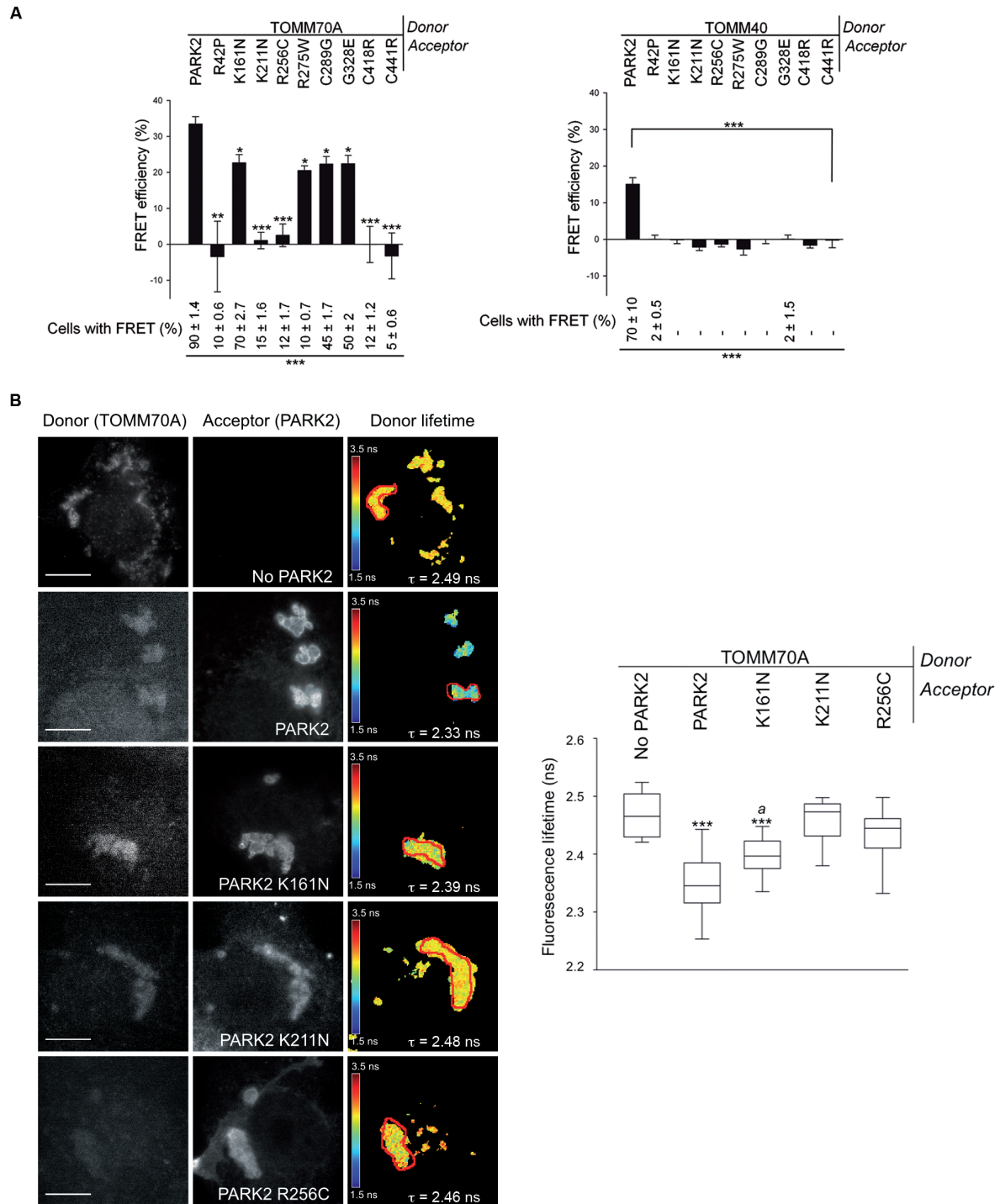
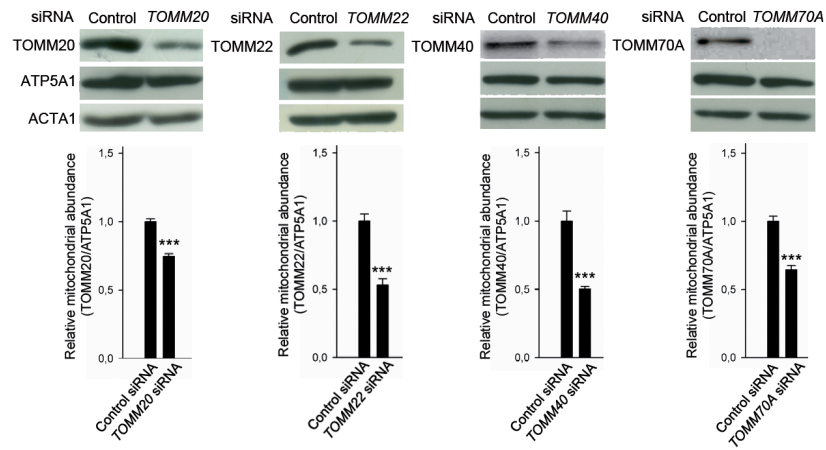
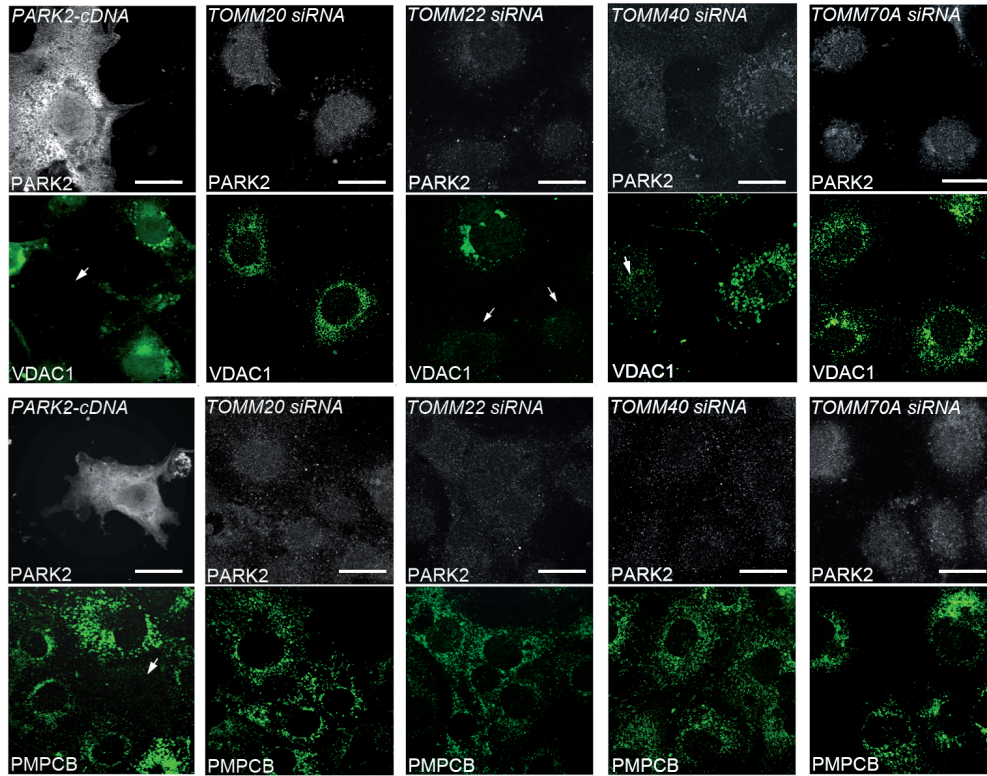


Figure 3. PD-causing *PARK2* mutations disrupt the molecular interaction between *PARK2* and the TOM machinery. **(A)** FRET efficiencies and percentages of cells displaying FRET for the indicated donor (endogenous TOMM subunits)-acceptor (exogenous *PARK2* variants) pairs in COS7 cells treated with CCCP. $n = 10$ to 20 cells from one experiment representative of two (TOMM40) or three (TOMM70A). **(B)** FLIM analysis of TOMM70A (donor) in the presence of *PARK2* (acceptor) variants in COS7 cells treated with CCCP. Pseudocolor scale, τ : individual and mean lifetimes within the ROI (red frame). No *PARK2*: immunostaining procedure excluded fluorophore-conjugated secondary antibody for *PARK2*. $n = 20$ ROIs per condition from three independent experiments. Comparisons were made with normal *PARK2* in **(A)** or with TOMM70A-No *PARK2* (*) or TOMM70A-*PARK2* (a) in **(B)**. *, a , $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar, 10 μm .

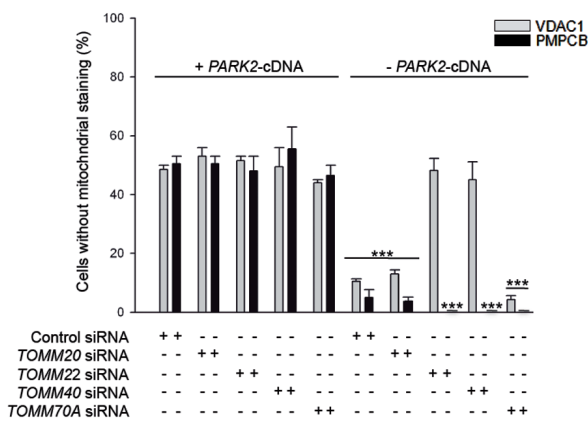
Depletion of subunits of the TOM core complex primes mitochondria for OMM clearance. TOM machinery subunits are among the first proteins to be degraded in a PARK2-dependent manner during OMM proteolysis and rupture.^{15,16} On the basis of the preceding observations, we hypothesized that the degradation of TOMM subunits might be a key event in mitochondrial degradation promoted by PINK1 and PARK2. We therefore analyzed the consequences of moderate depletion of specific TOMM subunits on the clearance of mitochondrial markers in COS7 cells treated with CCCP (**Fig. 4**). As expected, PARK2 promoted degradation of the dysfunctional mitochondrial network, as illustrated by the disappearance of OMM (VDAC1) and mitochondrial matrix markers (β subunit of MPP, PMPCB/MPPB) in cells in which it was overproduced (**Fig. 4A and B**).⁸ The depletion of TOMM subunits did not further enhance the mitochondrial loss triggered by PARK2 (**Fig. 4B**). However, the partial silencing of *TOMM22* or *TOMM40* alone, but not of *TOMM20* or *TOMM70A*, mimicked the effect of PARK2 on VDAC1 and the intermembrane space protein CYCS/cytochrome *c*, leading to their loss (**Fig. 4A and B, and Fig. S4A**). Decreases in *TOMM22* or *TOMM40* levels did not result in the loss of PMPCB in the absence of exogenous PARK2, although staining for this marker was systematically fragmented and fainter than in control cells, indicating that PARK2 is indispensable for complete mitochondrial degradation (**Fig. 4A and B, and Fig. S4B**).

To control for the specificity of the observed effects, we designed “rescue” experiments by overexpressing cDNAs resistant to siRNA. As expected, reintroduction of *TOMM40* or *TOMM22* in the corresponding paradigms of downregulation significantly reduced the loss of VDAC1 triggered by CCCP (**Fig. 4C and Fig. S4C**). Importantly, overproduction of *TOMM40* or *TOMM22*, but not *TOMM70A*, significantly reduced the loss of VDAC1 and PMPCB triggered by PARK2 following CCCP treatment. Altogether, these findings indicate that degradation of the core structure of the TOM machinery is a crucial step in the mitochondrial clearance program, downstream of mitochondrial recruitment of PARK2 by PINK1.

A



B



C

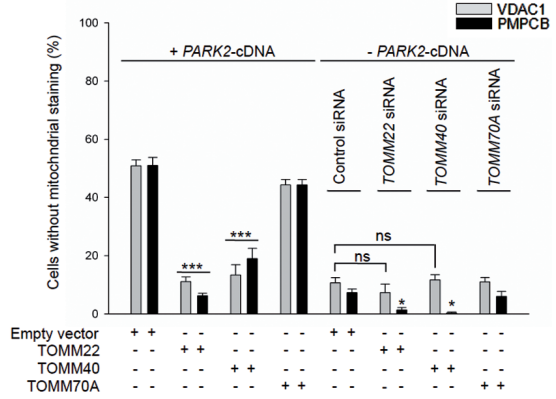


Figure 4. Depletion of TOM core complex subunits initiates the mitochondrial clearance program mediated by the PINK1-PARK2 pathway. **(A)** Fate of VDAC1 or PMPCB after CCCP treatment in COS7 cells overproducing PARK2 or with low levels of the indicated TOMM subunits. Arrows: loss of mitochondrial markers. Western blot analyses and corresponding quantifications: efficacy of downregulation for each TOMM subunit in total cell extracts. $n = 3$ independent wells from one representative experiment. ATP5A1, ACTA1: loading controls. **(B)** Quantification of the proportion of cells in which the indicated mitochondrial marker proteins were absent, in the experiment shown in **(A)** or **(C)** from COS7 cells with exogenous PARK2 or treated with the indicated siRNAs, after overproduction of the indicated TOMM subunits and CCCP treatment. $n = 3$ independent experiments. * $P < 0.05$, *** $P < 0.001$ versus **(A)** “Control siRNA”; **(B)** the corresponding condition with exogenous PARK2; or **(C)** “Empty vector” within the “+ PARK2” or “- PARK2” condition. ns: not significant. Scale bar, 10 μm .

PARK2, but not PINK1, is indispensable for the degradation of whole mitochondria. We then asked whether PINK1 plays a role beyond recruitment of PARK2 to mitochondria and the degradation of subunits of the TOM machinery in mitochondrial clearance. We, therefore, investigated whether the requirement for PINK1 in mitochondrial degradation could be bypassed by silencing *TOMM40* (**Fig. 5**). The concomitant downregulation of *TOMM40* and *PINK1* did not prevent OMM clearance, as demonstrated by the loss of VDAC1 and CYCS staining in similar proportions of cells in the presence and absence of PINK1 (**Fig. 5A and B, and Fig. S5A**). Of note, PINK1 was also dispensable for the degradation of whole organelles, which, in this model, was only accomplished in the presence of exogenous PARK2, as illustrated by clearance of the matrix marker PMPCB. As expected, PINK1 was indispensable for mitochondrial clearance induced by PARK2 in cells not treated with *TOMM40* siRNA exposed to CCCP (**Fig. 5A and B**).⁹⁻¹³

As previously mentioned, complete disappearance of PMPCB staining was never observed in cells with lower TOMM40 levels in the absence of exogenous PARK2. However, the staining was always less widespread than in control cells after CCCP treatment, possibly reflecting mitochondrial clearance promoted by endogenous PARK2. We, therefore, measured the area occupied by mitochondrial staining in cells in different conditions (**Fig. 5C and D**). This is a more quantitative method for estimating mitochondrial loss than the simple scoring of cells with or without labeling, a method commonly used by researchers in the field.^{8-10,14,15,18,28-31} With this method, CCCP treatment decreased PMPCB staining in cells overproducing PARK2 by nearly 80%, validating the approach and demonstrating that it is more sensitive than cell scoring. This decrease was completely reversed when *PINK1* was downregulated. Remarkably, depletion of TOMM40

resulted in a nearly 50% decrease in the area covered by PMPCB staining after CCCP treatment in the absence of exogenous PARK2. As expected from our above-mentioned results, the absence of PINK1 did not prevent loss of PMPCB staining under these conditions. In addition, we observed a nearly 20% reduction in PMPCB staining in the absence of CCCP in cells depleted of TOMM40, which was again unaffected by the downregulation of *PINK1*, but completely reversed by knockdown of endogenous *PARK2* (Fig. 5E). Altogether, these findings support the idea that the degradation of TOMM subunits is an event intrinsic to the PINK1-PARK2 pathway, situated temporally downstream of the PINK1-dependent recruitment of PARK2 to the OMM and upstream of the PARK2-dependent degradation of whole organelles.

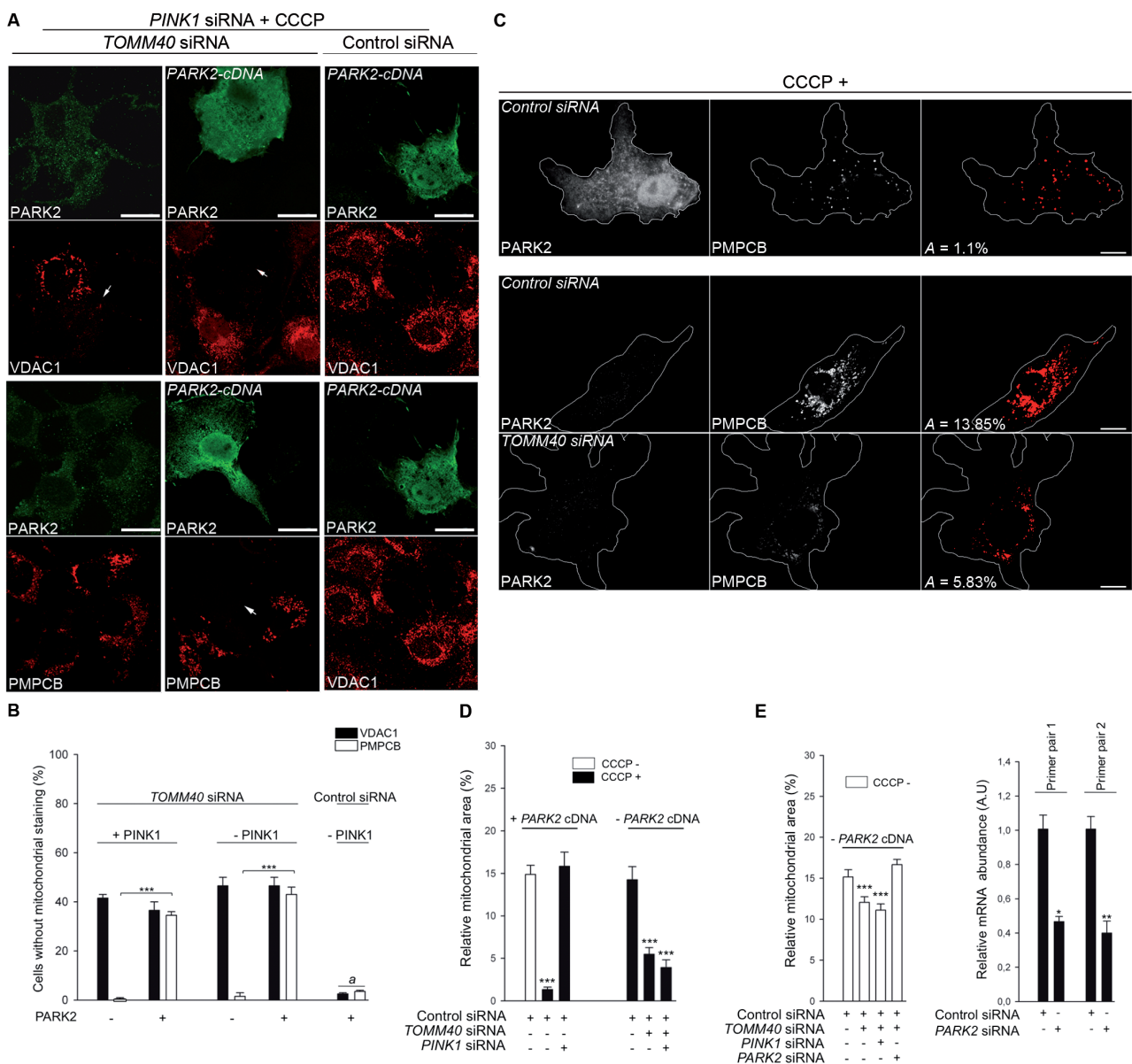


Figure 5. Depletion of TOM core complex subunits bypasses the requirement for PINK1, but not PARK2, in mitochondrial clearance. **(A)** Immunostaining and **(B)** corresponding quantifications illustrating the effect of PINK1 depletion on the fate of VDAC1 and PMPCB in COS7 cells with low TOMM40 levels, with or without exogenous PARK2, or in cells with exogenous PARK2 only. Arrows: loss of mitochondrial markers. $n = 3$ independent wells from one experiment representative of five. **(C)** Images illustrating the relative loss of PMPCB immunostaining in representative conditions. The right panels show PMPCB staining after application of a threshold mask, with A corresponding to mitochondrial area relative to total cell area (%). **(D and E, left graph)** Quantification of the cell area covered by PMPCB (relative mitochondrial area), estimated as in **(C)**, in the indicated conditions. $n = 3$ independent wells from one experiment representative of three. **(E, right graph)** Effect of siRNA-mediated silencing of endogenous *PARK2*, estimated by real-time PCR with two different primer pairs on mRNA extracted from COS7 cells and retrotranscribed into cDNA. $n = 3$ independent wells from one representative experiment out of two. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the corresponding control condition: “+ PARK2” in **(B)**,” Control siRNA” **(D, E)**. ^a $p < 0.001$ versus the corresponding “*TOMM40* siRNA” condition in **(B)**. Scale bar, 10 μm .

To confirm that TOM is a primary target of PINK1 and PARK2 in OMM clearance, and exclude non-specific triggering of the process by depletion of any protein involved in maintenance of mitochondrial import, we investigated mitochondrial loss following downregulation of *PMPCB*, encoding the catalytic subunit of the mitochondrial matrix protease MPP (**Fig. 6 and Fig. S5B**). MPP processes mitochondrial preproteins by cleaving their MTS; it is thought to be essential for mitochondrial import, including that of PINK1.^{22,23,32} Contrary to downregulation of *TOMM40* or *TOMM22*, moderate downregulation of *PMPCB* did not result in a massive loss of mitochondrial markers after CCCP treatment: no more than 15 to 20% of the cells lacked VDAC1 or the matrix enzyme HSD17B10 in the absence of exogenous PARK2. Moreover, loss of these markers was further limited by the simultaneous silencing of *PINK1* in cells with lower *PMPCB* levels, demonstrating complete dependence on the PINK1-PARK2 pathway. Again, the downregulation of *TOMM40* was sufficient to trigger PARK2-dependent elimination of mitochondrial markers in the absence of PINK1 in this model (**Fig. 6, graph**). In addition, the effects of *PMPCB* silencing, *TOMM40* downregulation and PARK2 overproduction were not additive, demonstrating that mitochondrial degradation in this context occurs within the PINK1-PARK2 pathway.

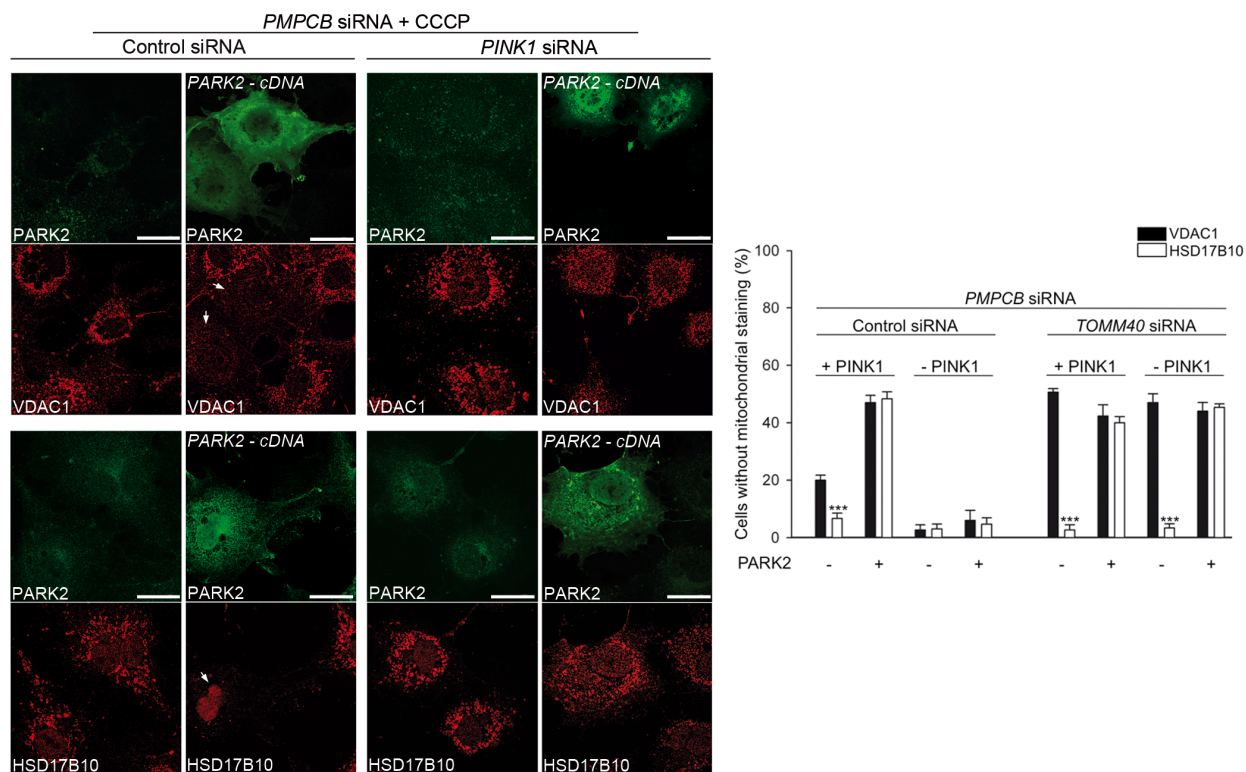


Figure 6. *PMPCB* downregulation does not prime mitochondria for OMM clearance. Effect of *PMPCB* downregulation on the clearance of VDAC1 and HSD17B10 (matrix marker) in COS7 cells with or without PINK1 or exogenous PARKIN. $n = 3$ independent wells from one experiment representative of three. *** $p < 0.001$ versus the corresponding “+ PARK2 condition”. Scale bar, 10 μm .

Downregulation of *TOMM40* triggers PARK2-dependent mitophagy. To formally demonstrate that the loss of mitochondrial markers observed following downregulation of *TOMM40* is indeed caused by autophagy, we quantified the relative proportion of LC3-positive vesicles associated with mitochondria in cells overproducing the fluorescent autophagosome marker LC3-dsRed (**Fig. 7A**). As expected, in cells treated with CCCP, overproduction of PARK2 resulted in a nearly 100% increase in the number of LC3-positive vesicles covering mitochondria compared to control cells not treated with CCCP or not overproducing PARK2. In cells with low *TOMM40* levels, this increase was 200% in the presence of CCCP and already 50% in its absence, supporting the involvement of autophagy in the clearance of mitochondrial markers previously observed in these paradigms (**Fig. 5B**).

To strengthen these findings, we tested the involvement of specific degradation pathways by treating the cells with (i) the proteasomal inhibitor epoxomicin, (ii) 3-methyladenine, to block autophagosome formation,

and (iii) the lysosome inhibitor bafilomycin A₁ (**Fig. S6**). All three treatments prevented PINK1 and PARK2-dependent clearance of mitochondrial markers, demonstrating the cooperation among these pathways in this process already reported.^{8,14-16} In addition, these treatments prevented the loss of VDAC1 and CYCS in cells with low TOMM40 levels.

We then explored the role of key components of the autophagy pathway, ATG5, ATG7 and AMBRA1, by siRNA-mediated gene silencing (**Fig. 7B**). As previously shown, scoring the proportion of cells without mitochondrial markers demonstrated that the three proteins are essential for PARK2-mediated mitophagy, as illustrated by the loss of the matrix marker PMPCB.^{8,9,16,30} In contrast, the clearance of the OMM marker VDAC1 did not depend on these proteins in cells with exogenous PARK2 or with low TOMM40 levels after CCCP treatment, in agreement with the role of the proteasome in the digestion of OMM proteins (**Fig. S6**).^{15,16} To demonstrate that the reduction in PMPCB staining (**Fig. 5C**) is also due to mitophagy, we further evaluated the effect of the downregulation of *ATG5*, *ATG7* and *AMBRA1* in cells with low TOMM40 levels, by quantifying the relative area occupied by PMPCB staining in this model (**Fig. 7C**). Again, loss of this staining was prevented in all cases, demonstrating the involvement of autophagy; this provides further evidence that the mechanism underlying mitochondrial degradation triggered by downregulation of *TOMM40* are the same as those underlying PARK2-dependent mitophagy.

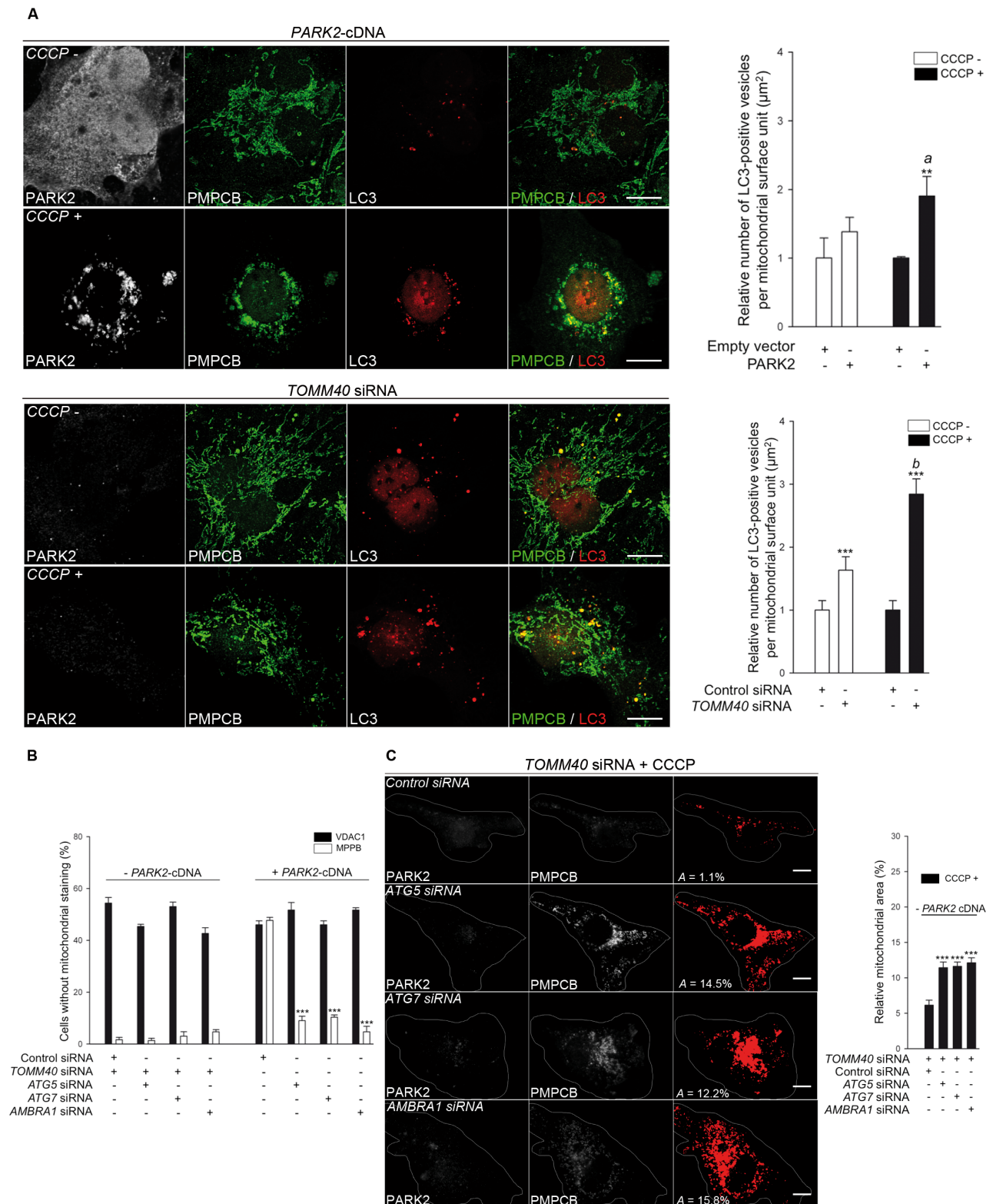


Figure 7. *TOMM40* downregulation triggers *PARK2*-dependent mitophagy. **(A)** Representative images illustrating LC3-positive vesicles associated with PMPCB staining in COS7 cells, in the indicated conditions. Right graphs: quantification of the number of LC3-positive vesicles contained for PMPCB relative to the total number of LC3 vesicles in cells, as explained in the Materials and Methods section. The control conditions were arbitrarily set at 1. $n = 20$ cells from three independent experiments. **(B)** Quantification of the proportion of cells without VDAC1 or PMPCB staining after overproduction of *PARK2* or downregulation of *TOMM40*, and silencing of *ATG5*, *ATG7* or *AMBRA1*, as indicated. Cells were treated with CCCP in all conditions. $n = 3$

independent wells from one experiment representative of three. (C) Images showing the relative loss of PMPCB immunostaining and (graph) quantification of the relative mitochondrial area covered by PMPCB staining, estimated in the indicated conditions as explained in the legend of **Figure 5C**. A: relative mitochondrial area (%). $n = 30$ cells from one experiment representative of three. $**p < 0.01$, $***p < 0.001$ versus (A) the corresponding control condition within “CCCP +” or “CCCP -”; (B, C) the corresponding “Control siRNA” condition. $^ap < 0.01$, $^bp < 0.001$ versus the corresponding “CCCP -” condition. Scale bar, 10 μm .

Discussion

We provide evidence that PINK1 and PARK2 constitute a molecular quality control sensor-effector system, coupling mitochondrial import efficiency with the clearance of dysfunctional mitochondria (**Fig. 8**). We show that PARK2 translocates to the OMM in two models in which protein import is impaired, associated with a less dramatic mitochondrial depolarization than that caused by CCCP. These observations are consistent with the recent demonstration that depletion of PMPCB is sufficient to stabilize PINK1 and recruit PARK2 to mitochondria in the absence of $\Delta\Psi$ loss, and suggest that impaired mitochondrial import through the TOM machinery is the primary signal for PINK1 and PARK2-mediated mitochondrial elimination.³² In support of this hypothesis, Lazarou *et al.* have discovered, using biochemical approaches, that PINK1 interacts with TOMM20 in the TOM machinery following CCCP treatment.¹⁸ PARK2 was not identified in this complex, but, as the authors of the study stated, this does not exclude association; it rather reflects experimental conditions in which transient or labile interactions are not preserved. By using FRET-based microscopy, we confirm here that PINK1 accumulates at the TOM machinery when mitochondrial import is blocked. In addition, we demonstrate that PARK2 is brought into proximity to this complex, highlighting the power of FRET imaging techniques for detecting localized, endogenous protein-protein interactions.

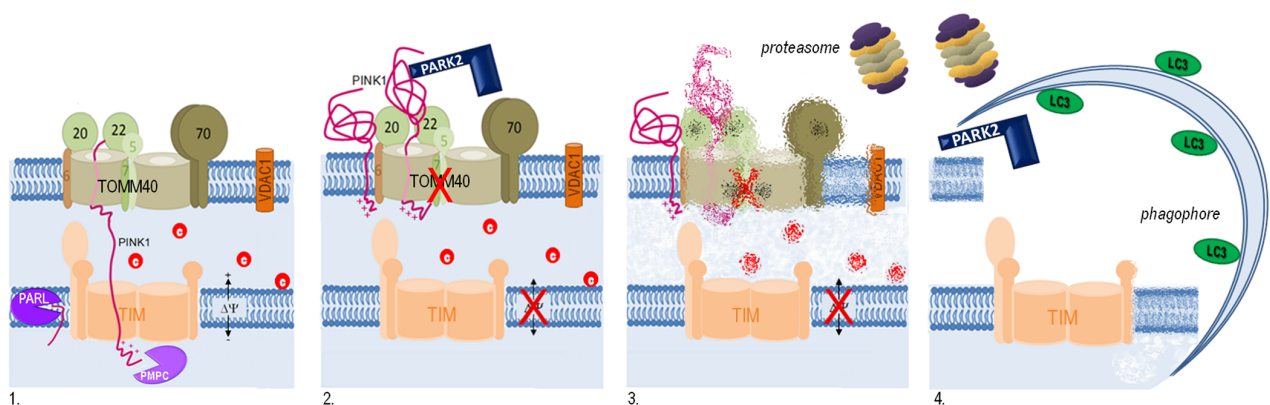


Figure 8. Model illustrating putative steps in the mitochondrial clearance program mediated by the PINK1-PARK2 pathway. PINK1 is imported into healthy mitochondria through the TOM and TIM complexes, and processed by MPP and the presenilin-associated rhomboid-like protease PARL (1).⁴³ Loss of mitochondrial import efficiency, associated or not with $\Delta\psi$ collapse, leads to accumulation of PINK1 at the OMM, recruitment of PARK2 in proximity of the TOM complex (2), destabilization of the machinery, possibly caused by proteasomal degradation of key subunits, OMM rupture and proteolysis (3) and elimination of whole mitochondria by the proteasome and lysosome-dependent pathways (4). PINK1 and PARK2 are both required to trigger step 3., whereas only PARK2 is essential for step 4.

TOMM70A may be the preferential target of PARK2 in the complex, since FRET levels were consistently higher for the TOMM70A-PARK2 pair than for the other pairs tested, and TOMM70A but no other TOM subunit was retrieved by GST pull-down. Accordingly, two independent mass spectrometry studies have recently identified the TOMM70A receptor as one of the proteins binding to and ubiquitinated by PARK2 following CCCP treatment.^{33,34} Remarkably, the nine PD-causing missense *PARK2* mutations analyzed significantly reduced fluorescence energy transfer between TOMM70A and PARK2, indicating that this interaction is of physiopathological relevance. In addition, these mutations abolished FRET between TOMM40 and PARK2. This further supports the hypothesis that TOMM70A is the physical target of PARK2, and that weakening of this primary association modifies the relative configuration of the partners of the complex, precluding FRET from TOMM40 to PARK2. Indeed, although the presence of energy transfer in FRET experiments is unlikely to be an artifact, lack of FRET does not necessarily indicate an absence of molecular proximity; it may also reflect non-permissive orientations of the donor and acceptor.¹⁸ Such a situation may explain why we did not detect FRET between VDAC1 and PARK2, which based on previous studies should interact.^{11,31,33,34}

Our findings provide evidence that the TOM machinery is a switch in the mitochondrial clearance program regulated by the PINK1-PARK2 pathway. They suggest that proteolytic destabilization of the TOM core complex follows upon the recruitment of PARK2 and acts as the molecular trigger that initiates the degradation of proteins of the OMM and intermembrane space. The degradation of TOMM70A, which precedes that of other TOMM subunits according to previous studies,^{15,16} may permit the subsequent degradation of the core structure, with the intervention of PINK1 and PARK2. Consistent with this hypothesis,

Sarraf *et al.*, using anti-HA affinity purification-mass spectrometry to identify proteins associated with HA-tagged PARK2, have found TOMM70A after 1h of CCCP treatment and TOMM22 after 8h.³⁴ Future studies are required to define the specific mechanisms underlying the degradation of the different subunits of the TOM complex and their spatiotemporal regulation.

Intriguingly, Lazarou *et al.* excluded that the TOM complex is a key regulator of mitochondrial clearance.¹⁸ They showed that an exogenous PINK1 protein lacking the N-terminal MTS and targeted to the OMM by an artificial mitochondrial anchor was able to recruit PARK2 and initiate organelle degradation, despite the absence of stable associations with the TOM complex. However, stable association might not be essential for PINK1 and PARK2 to degrade the machinery, and previous data do not rule out the possibility that ectopically targeted mitochondrial PINK1 is located close to the TOM machinery. It remains to be determined how PINK1 targeted to peroxisomes can recruit PARK2 and activate pexophagy in the absence of a TOM complex. However, since the roles of ubiquitin signaling in pexophagy and mitophagy are different, intrinsically distinct programs might be involved.^{29,35}

The investigation of the role of key elements of the autophagy machinery in our study confirmed that OMM proteolysis triggered by PINK1 and PARK2 relies on the ubiquitin-proteasome pathway; in addition, it demonstrated that PARK2 cooperates subsequently with ATG5, ATG7 and AMBRA1 in the absence of PINK1 to promote the degradation of mitochondrial remnants. As PINK1 is dispensable for this second phase, physical association of PARK2 with mitochondria may not be necessary at this stage, or PARK2 may bind other mitochondrial proteins. VDACs are excellent candidates, since they have been identified as major partners of PARK2 in cells treated with CCCP and may play a role in mitophagy.^{11,31,33,34} These channels are abundant OMM proteins and may thus still be available to interact with PARK2 once the initial phase of mitochondrial degradation is launched. The degradation of the entire OMM may indeed not be necessary for entry into the second phase, since mitochondria with a largely intact OMM are found inside autophagosomes in cells treated with CCCP.¹⁶ The requirement for both the proteasome and autophagy machinery for efficient mitochondrial clearance also supports the view that the two phases of the mitochondrial degradation program are closely interlinked; rupture of the OMM caused by the degradation of critical OMM components may act as

a signal for massive recruitment of the ubiquitin-proteasome and autophagy-dependent pathways. Permeabilization and rupture of the OMM are key events in mitochondrion-dependent apoptosis,^{36,37} but no apoptotic features are observed after the triggering of mitochondrial degradation by the PINK1-PARK2 pathway,¹⁶ consistent with the existence of reciprocal regulations between mitochondrial clearance and cell death programs.³⁸

In summary, our findings suggest that mitochondrial quality control by the PINK1-PARK2 pathway becomes essential when protein flux through the TOMM machinery is impaired. *TOMM40* influences the age at onset of Alzheimer disease and changes in protein import efficiency have been reported in neurodegenerative conditions in humans and animal models, including *pink1*-deficient mice.³⁹⁻⁴¹ The loss of functional coupling between mitochondrial import and the neuroprotective degradation of dysfunctional OMM components or mitochondria may therefore be a primary pathogenic mechanism in autosomal recessive PD caused by *PARK2* or *PINK1* mutations.

Materials and Methods

Mammalian expression vectors, siRNAs, cell culture and transfection. The vectors used were: pcDNA3-HA encoding human *PARK2* or *PARK2* variants,⁴² *TOMM70A* and siRNA-resistant *TOMM22* and *TOMM40*; pCB6 encoding an HA-tagged GFP fused to the N-terminal MTS of COX8A (kindly provided by M. Rojo), and pDsRed2-LC3 (kindly provided by G. Kroemer). The *TOMM70A* cDNA was cloned by PCR from a human cDNA bank obtained from HeLa cells (F. Lacroute) and inserted into pcDNA3-V5-His-TOPO TA (Invitrogen, K4800-01). siRNAs-resistant cDNAs for human *TOMM22* and *TOMM40* were produced by Genosphere Biotechnologies (catalog numbers: no catalog number available; synthesis according to our indications) and cloned into the HindIII/BamHI sites of pcDNA3-HA (A. Fournier). The siRNA-resistant cDNA for *TOMM40* was designed by targeting the corresponding siRNA-binding site (validated siRNA, Qiagen, SI00301966) with the following sequence: 5'-aacaattggtagctaccgta-3', carrying four silent mutations in the original cDNA sequence starting at position +768 from the ATG codon. As the binding site of the siRNA for *TOMM22* (validated siRNA, Qiagen, SI00125650) was located in the 3' UTR, the corresponding coding sequence was by itself resistant. Other siRNAs used were: validated *TOMM20* (SI00301959), *TOMM70A* (SI00301973), *ATG5* (SI02655310), *ATG7* (SI02655373) and *AMBRA1* (SI00397306) siRNAs (Qiagen); *PARK2* (HSS107593) and *PINK1* (HSS127945) stealth siRNAs (Invitrogen); *PMPCB* siRNA (Dharmacon, L-004747-00-0005); AllStars negative control siRNA (Qiagen, 1027281). HeLa-*tet-on-shLONP1* cells were generated by annealing a 68-base oligonucleotide starting at position 476 of the *LONP1* coding sequence (5'-gatctccgttcgtctcgcccagcctttcaagagaaaggctggcgagacgaacttttgaaagcctt-3') to its complementary sequence and inserting the resulting sequence into pENTR/H1/T0⁺ (Block-it, Invitrogen, K4920-00). HeLa T-Rex cells (Invitrogen R71407) were transfected, by the calcium phosphate method, with the siRNA construct and stable clones were selected in Dulbecco's modified Eagle's medium (DMEM; Gibco, 41966-052) supplemented with 10% fetal bovine serum (Gibco, 10270-106), 5 µg/ml blasticidin (Invitrogen R210-01), and 200 µg/ml zeocin (Invitrogen, R250-01). Production of the LONP1 protease was controlled by adding Dox to the culture medium (2 µg/ml, Sigma, D-9891). COS7 cells and HEK293T cells were grown in DMEM supplemented with 10% fetal

bovine serum, 1% L-glutamine (Invitrogen, 25030024) and 1% penicillin-streptomycin (Invitrogen, 15140-122). Cells were plated, at 80% confluence, on glass coverslips (Thermo Scientific, CB00140RA1)) in 24-well cell plates for immunocytochemistry, in six-well plates for western blot analyses of total cell lysates, or on 15 cm Petri dishes for subcellular fractionation. Transfections were performed by the calcium phosphate method (HeLa cells) or with Lipofectamine 2000 (Invitrogen, 11668019), according to the manufacturer's instructions. Moderate downregulation of TOMM subunits was achieved with the corresponding siRNAs (0.2 μ M). Where indicated, the cells were incubated with dimethylsulfoxide (DMSO, Sigma, D5879), 100 nM bafilomycin A₁ (Sigma, B1793) for 6 h (LC3-positive vesicles) or 48 h (clearance of mitochondrial markers), 10 mM 3-MA (Sigma, M9281) and 50 nM epoxomycin (Sigma, E3652) for 48 h, or with 10 μ M CCCP (Sigma, C2759) for 6 h (mitochondrial translocation of PARK2, FRET and FLIM analyses, quantification of LC3-positive vesicles) or 48 h (clearance of mitochondrial markers).

Quantitative Real-Time RT-PCR. To demonstrate the efficiency of the siRNA-mediated silencing of endogenous *PARK2* in COS7 cells, total RNA was isolated from cells transfected with control or *PARK2* siRNA using the RNeasy plus Mini Kit (Qiagen, 74134). RNA from each sample (1 μ g) was reverse-transcribed into cDNA. Real-time PCR was performed with the LightCycler® 480 System (Roche Applied Science, Germany) and LightCycler® 480 DNA SYBR Green I Master kit (Roche Applied Science, 04707516001). Results were analyzed using LightCycler 480 sw 1.5 quantification Software (Roche Applied Science). *RPL13* was used as the reference gene for normalization. Primers used for *PARK2* were as follows: forward 1, 5'-cgcaacaaatagtcggaac-3'; reverse 1, 5'-gagggtcgtgaacaaactg-3'; forward 2, 5'-gacctcaacttgctactc-3'; reverse 2, 5'-acgcttctttacattcccg-3'.

Subcellular fractionation, trypsin digestion assay, GST pull-down and western blot analyses.

Total protein fractions were obtained from cells lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM DTT supplemented with phosphatase inhibitors (0.2 mM Na₃VO₄, 4 mg/ml NaF, 5.4 mg/ml β -glycerophosphate) and protease inhibitors (Complete Cocktail, 11836145001, Roche), after centrifugation at 12,500 g for 20 min at 4°C. Alternatively, cells were lysed in 20

mM HEPES (pH 7.0), 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.5 mM DTT, with phosphatase and protease inhibitors, and passed through a 22G needle. Mitochondrion-enriched fractions were obtained by differential centrifugation, from cells homogenized with a Dounce homogenizer (80 manual strokes), as previously described,³² or with a mitochondrion isolation kit used according to the manufacturer's instructions (human, 130-094-532 and separation columns, 130-042-401, MACS, Miltenyi Biotech). Mitochondrion-enriched fractions obtained by differential centrifugation were digested with 1 µg of trypsin (Sigma, T1426) per mg of protein for 20 min at 37°C, where indicated. GST pull-down was performed as previously described.⁴² Protein concentrations were determined with the BCA (Pierce, 23225) or Bradford (Bio-Rad, 500-0006) protein assays. Samples were boiled in protein sample buffer, resolved by SDS-PAGE, transferred onto a nitrocellulose membrane (Protran, GE 10401196, Whatman) and analyzed by western blotting with selected primary and secondary antibodies (**Table 1**). Membranes were incubated with enhanced chemiluminescence substrate (Pierce, 34076) and chemiluminescence and fluorescence signals were revealed on a film (ECL, Amersham Hyperfilm, 28906837), or captured with a KODAK Image Station 4000 MM (Carestream Health, U.S.A.), with the MF-ChemiBIS Gel (DNR Bio-imaging systems, Israel) or Odyssey Imaging (Li-COR, U.S.A.) systems and quantified with ImageJ software (NIH).

Immunocytochemistry and confocal microscopy. Cells were fixed in 4% paraformaldehyde (PFA, Sigma, 252549-500 ml) and stained with standard procedures. Mitochondrion-enriched fractions obtained by differential centrifugation were fixed in 4% PFA, washed in phosphate-buffered saline (PBS), incubated overnight in a suspension of primary antibodies in PBS supplemented with 2% normal goat serum (NGS-PBS; Gibco, 16210-072) and then with secondary antibodies diluted in NGS-PBS for 45 min. The mitochondrial pellet was obtained by centrifugation at 660 g for 5 min, rinsed in PBS, resuspended and mounted in ProLong Gold Antifade reagent (Molecular Probes, P36930). The antibodies used for staining are indicated in **Table 2**. Mitochondrial dyes were obtained from Invitrogen and used at a concentration of 10 nM (TMRM, T-668) and 500 nM (MitoTracker DeepRed, M22426/CMXRos, M7512) according to the manufacturer's instructions. TMRM intensities were quantified with ImageJ software, on images of at least 200 live cells per condition cells taken with an inverted Zeiss Axiovert 200 videomicroscope (Zeiss, Germany) and a 20 X oil immersion

objective. Mitochondrial translocation of PARK2 or the lack of specific mitochondrial markers were quantified with a Zeiss Axioplan 2 microscope (Zeiss, Germany) driven by ExploraNova FluoUp Imaging software (4.2.2, Explora Nova, France) and a 100 X oil immersion objective (N.A. 1.40), with 60 to 90 cells scored per well. The relative mitochondrial area was calculated with ImageJ software on images from at least 30 cells per experiment, acquired as above; it was expressed as the ratio between the area covered by the PMPCB stain, selected with an automatic threshold mask, and the total cell area. Colocalization images were taken with an Olympus FV-1000 confocal microscope (Olympus, Japan) driven by Olympus FV-1000 Software, or with a Leica SP5 confocal microscope (Leica, Germany) (63X oil immersion objective, N.A 1.40) driven by Leica Imaging Software. For FRET analysis, a ROI was photobleached with a 543 nm helium/neon laser for 20 ms. Images of donor and acceptor fluorescence were acquired before and after photobleaching with a 60 X (N.A. 1.35) oil immersion objective. Images of the donor after the photobleaching procedure were corrected with ImageJ software for the general decrease in fluorescence intensity due to the acquisition process and quantified as described.²⁷ To quantify the number of LC3 vesicles per mitochondrial surface unit (μm^2), Z-stacks were acquired from 20 cells per condition and processed with ImageJ software. After applying median and background elimination filters, a manually-determined threshold mask was created for PMPCB and LC3-specific fluorescent stains. The particles counted were then selected by pixel size (from 0.1 to infinity) and by circularity (from 0.00 to 1.00), before applying watershed segmentation. The relative number of vesicles per mitochondrial surface unit was calculated as the ratio of the number of PMPCB-positive LC3 vesicles relative to the total number of LC3 vesicles per cell in cells treated with bafilomycin A₁, normalized to the same ratio in untreated cells. FLIM measurements were carried out in the frequency domain, by phase modulation on a custom-built system based on a commercial module (Lifa, Lambert Instruments) attached to a Nikon TE-2000 inverted microscope (Nikon, Japan) with a 100 X (N.A. 1.49) TIRF oil immersion objective (CFI Apochromat), a Coolsnap HQ CCD camera (Photometrics), a 473 nm modulated laser diode (Omicron) and a TIRF module for wide-field laser illumination. Fluorescence emission was selected with a band-pass filter (500 to 550 nm). Cells were analyzed with Li-FLIM software (Lambert Instruments).

Statistical analyses. Two-way ANOVA and the Holm-Sidak method were used to analyze the effects of siRNAs and PARK2 overproduction on mitochondrial marker loss (**Fig. 4B**); the effect of donor and treatment on FRET efficiencies for given donor-PARK2 pairs (**Fig. 2C, and S2A**) or the effects of CCCP and PARK2 overproduction or *TOMM40* downregulation on the relative number of LC3-positive vesicles (**Fig. 7A**). One-way ANOVA and the Holm-Sidak method were used to analyze the effects of: *TOMM40* siRNA in the trypsin digestion assay (**Fig. 1A**); donors or acceptors on FRET efficiencies for given donor-acceptor pairs (**Fig. 2B, Fig. S2B and S3B**); *PARK2* mutations on FRET efficiencies and FLIM values (**Fig. 3A and B**); pharmacological treatments on the loss of mitochondrial markers after *PARK2* overproduction or *TOMM40* downregulation (**Fig. S6**); the overproduction of siRNA-resistant TOMM subunits on clearance of mitochondrial markers within the “+ *PARK2*” or “– *PARK2* conditions” (**Fig. 4C**); *ATG5*, *ATG7* or *AMBRA1* gene silencing strategies on the clearance of mitochondrial markers and on relative mitochondrial surface after *PARK2* overproduction or *TOMM40* downregulation (**Fig. 5D and E, and Fig. 7B and C**). ANOVA on ranks and Tukey’s test were used to compare the effects of *PINK1* downregulation on mitochondrial marker loss (**Fig. 5A and 6, and Fig. S5A**). The Mann-Whitney U test was used to compare TMRM intensities between conditions within each cell type (**Fig 1C**). Student’s *t* test was used to analyze the effect of CCCP (**Fig. 1A**) or Dox treatment (**Fig. S1A**) on the relative mitochondrial abundance of mitoGFP; *PARK2* recruitment to mitochondria in each cell type (**Fig 1B**); the effect of CCCP treatment on FRET efficiencies for donor-acceptor pairs in cells stained with secondary antibodies (**Fig. S2C**); the effect of *PINK1* downregulation on loss of mitochondrial markers in cells with or without *TOMM40* siRNA (**Fig. 5B**); the efficacy of the siRNA-mediated downregulation of *PARK2* by Real Time RT-PCR (**Fig. 5C**), or of genes encoding TOMM subunits by western blot (**Fig. 4A**). Parametric statistical tests were used unless the data were not normally distributed.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing financial interests.

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Table 1. Antibodies used for western blotting

Primary antibodies				
Type	Company	Catalog #	Host	Dilution
PARK2, clone Park8	Millipore	MAB5512	mouse	1:2000
Respiratory chain complexes (Total OXPHOS)	Mitosciences	458099	mouse	1:1000
GFP	Abcam	Ab290	rabbit	1:4000
VDAC1	Abcam	Ab15895	rabbit	1:1000
TOMM70A	Abcam	Ab106193	mouse	1:5000
TOMM40 clone H7	Santa Cruz	Sc-365466	mouse	1:500
TOMM20	Santa Cruz	Sc-11415	rabbit	1:500
TOMM22	Abcam	Ab10436	mouse	1:500
LONP1 (G. Isaya, Mayo Clinic Foundation Rochester, MN)	-		rabbit	1:1000
ACTA1	Sigma	A2066	rabbit	1:2000
MPPB	Proteintech	16064-1-AP	rabbit	1:4000
Secondary antibodies				
Type	Company		Host	Dilution
CY3-conjugated anti-mouse/rabbit	Sigma	C2181/C2306	sheep	1:1000
Alexa Fluor 488-conjugated anti-mouse/rabbit	Invitrogen	A11070/A11029	goat	1:1000
IRDye 800CW-conjugated anti-mouse/rabbit	Li-Cor	926-32211/926-32210	goat	1:10000
IRDye 680RD-conjugated anti-mouse/rabbit	Li-Cor	926-68071/926-68070	goat	1:10000
horseradish peroxidase-conjugated anti-mouse/rabbit	Jackson ImmunoResearch	115-035-146/111-035-144	goat	1:50000

Table 2. Antibodies used for immunocytochemical staining

Primary antibodies				
Type	Company	Catalog #	Host	Dilution
PARK2, clone Park8	Millipore	MAB5512	mouse	1:1000
TOMM20	Abcam	Ab56783	mouse	1:1000
TOMM20	Santa Cruz	Sc-11415	rabbit	1:500
TOMM22	Abcam	Ab10436	mouse	1:5000
TOMM22	Atlas antibodies	HPA003037	rabbit	1:1000
TOMM22	Santa Cruz	Sc-14894	goat	1:200
CYCS, clone 7H8.2C12	BD Pharmingen	556432	mouse	1:1000
HSD17B10	Abcam	Ab10260	mouse	1:2000
PARKIN	Millipore	MAB5112	rabbit	1:7000
TOMM70A	Sigma	HPA014589	rabbit	1:200
TOMM70A	Abcam	Ab106193	mouse	1:2000
TOMM40	Santa Cruz	Sc-11414	rabbit	1:100
TOMM40 clone D2	Santa Cruz	Sc-365467	mouse	1:200
TOMM40	Santa Cruz	Sc-11022	goat	1:100
MPPB	Proteintech	16064-1-AP	rabbit	1:400
VDAC1	Abcam	Ab15895	rabbit	1:200
LONP1 (G. Isaya, Mayo Clinic Foundation Rochester, MN)	-		rabbit	1:3000
PINK1	Novus biologicals	BC100-494	rabbit	1:2500
ATG5	Novus biologicals	NB110-53818	rabbit	1:500
ATG7	Abcam	Ab53255	rabbit	1:500
AMBRA1	Novus biologicals	FLJ20294	rabbit	1:250
Secondary antibodies				
Type	Company		Host	Dilution
Alexa Fluor 488 anti-mouse/rabbit	Invitrogen	A11070/A11029	goat	1:2000; 1:10000*
Alexa Fluor 568 anti-mouse/rabbit	Invitrogen	A11036/A11031	goat	1:10000*; 1:30000*
Alexa Fluor 633 anti-mouse/rabbit	Invitrogen	A21050/A21070	goat	1:500
CY3-conjugated anti-mouse/rabbit	Sigma	C2181/C2306	sheep	1:2000

*Dilution used for FRET and FLIM studies

Supplemental material

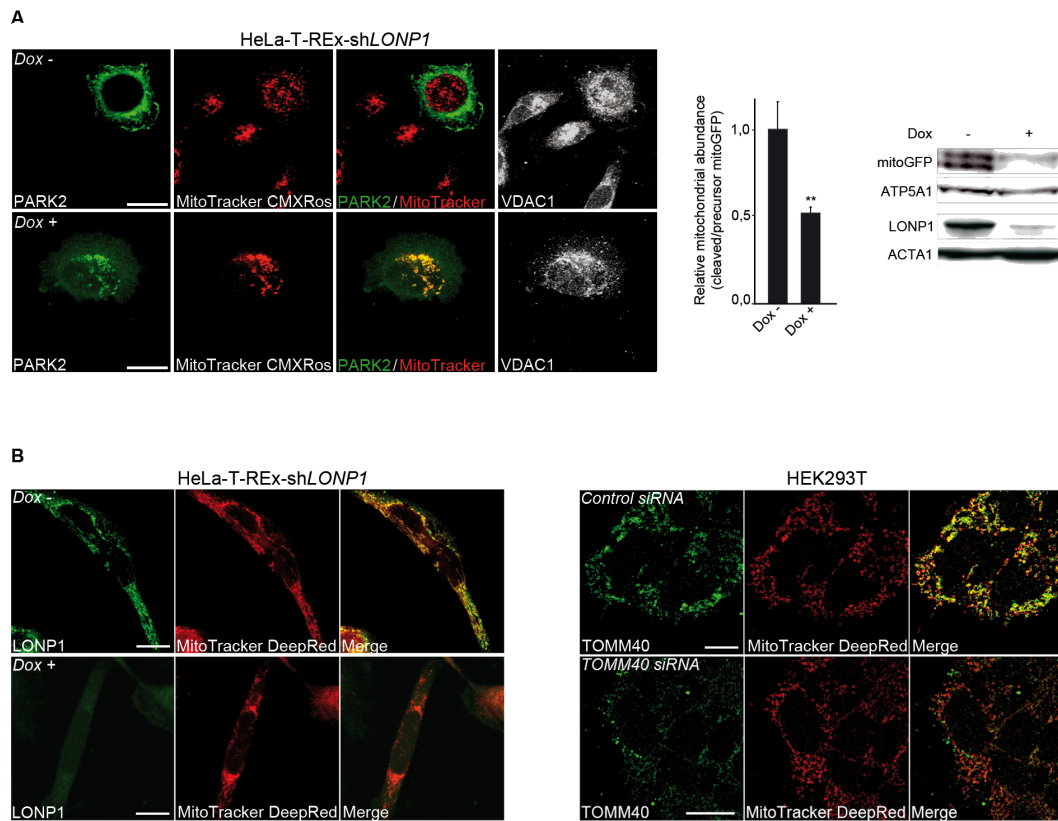


Figure S1. Mitochondrial import blockade recruits PARK2 to mitochondria. **(A)** Mitochondrial redistribution of PARK2 (left panels) in LONP1-depleted HeLa cells and quantification of the abundance of the imported mitoGFP protein in total cell lysates (western blot and graph). ($n = 3$ independent experiments). Mitochondria (left panels) were visualized with MitoTracker CMXRos and anti-VDAC1 staining. Loading controls in western blots: ATP5A1, ACTA1. **(B)** Representative immunofluorescent images illustrating the efficiency of the gene silencing strategy for *LONP1* in HeLa-T-Rex-shLONP1 cells treated with Dox, and for TOMM40 in HEK293T cells. Mitochondria were labeled with MitoTracker DeepRed. Scale bar, 10 μm . ** $p < 0.01$.

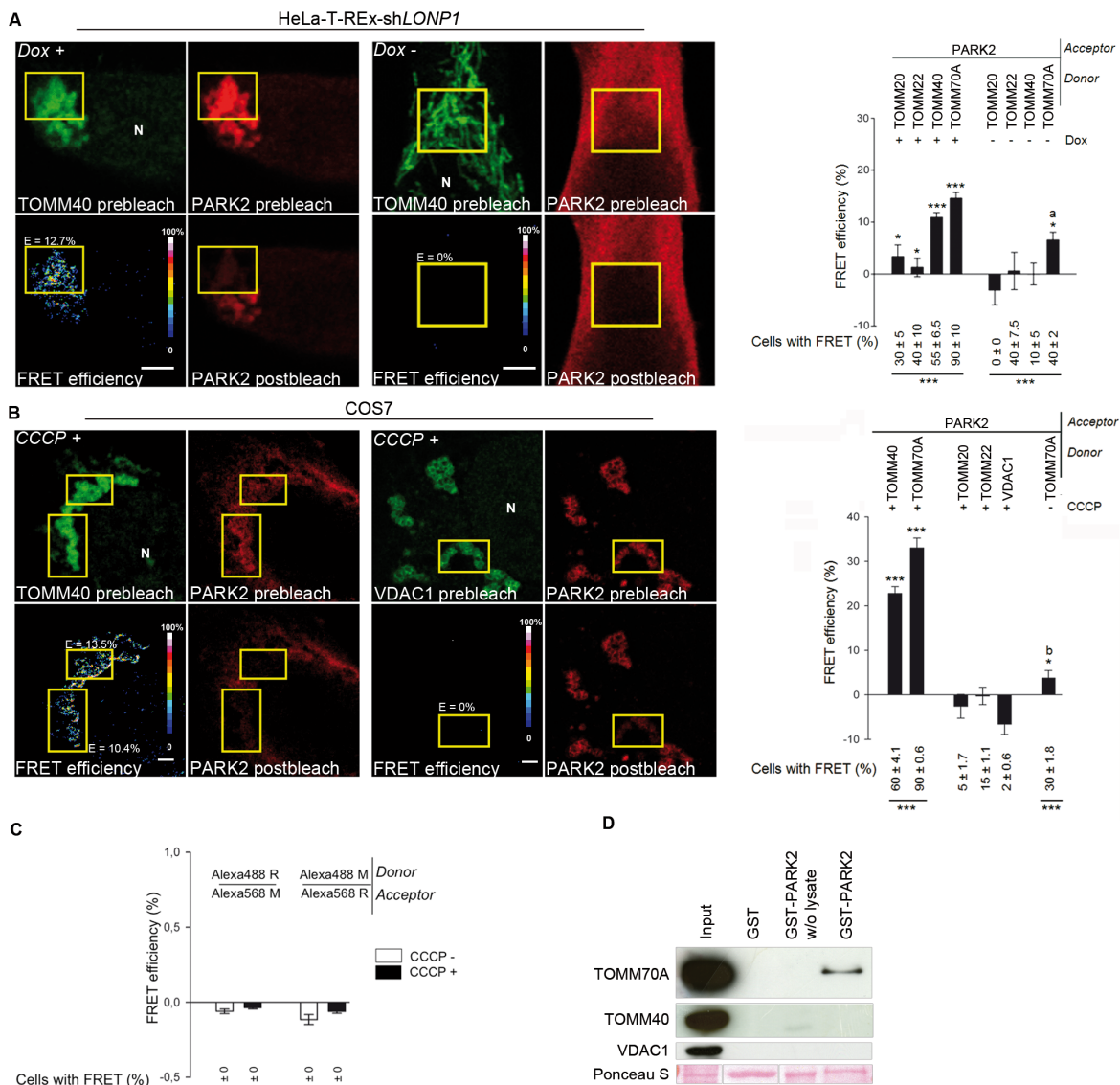


Figure S2. PARK2 interacts with the TOM machinery in various models of mitochondrial import blockade. Representative FRET images and quantitative analysis of FRET efficiencies for the indicated donor-acceptor pairs in **(A)** HeLa-T-Rex-shLONP1 cells with and without Dox treatment or **(B)** COS7 cells with and without CCCP treatment. Comparisons were made with the TOMM40-PARK2 pair in the “Dox -” condition (*) or versus the corresponding “Dox +” condition (a) in **(A)**, and with the VDAC1-PARK2 donor-acceptor pair (*) or the corresponding “CCCP +” condition (b) in **(B)**. Pseudocolor scale: pixel-by-pixel FRET efficiencies. E: mean FRET efficiency within the ROI. N: nucleus. $n = 10$ to 20 cells per condition from one experiment representative of three. *, a, b $p < 0.05$, *** $p < 0.001$. **(C)** FRET analysis of COS7 cells overproducing PARK2, treated or untreated with CCCP and immunostained with secondary antibodies only. M: anti-mouse IgG; R:

anti-rabbit IgG. $n = 10$ cells from one representative experiment out of three. (D) Specific pull-down of TOMM70A but not TOMM40 or VDAC1 by a recombinant GST-tagged PARK2 protein incubated with total lysates from COS7 cells. Ponceau S illustrates the recombinant GST and GST-PARK2 proteins in the pull-down assay (5 μ g). Input corresponds to an aliquot of the lysate (40 μ g) prior to pull-down. w/o: pull-down without cell lysate. Results from one representative experiment out of three. Scale bar, 10 μ m.

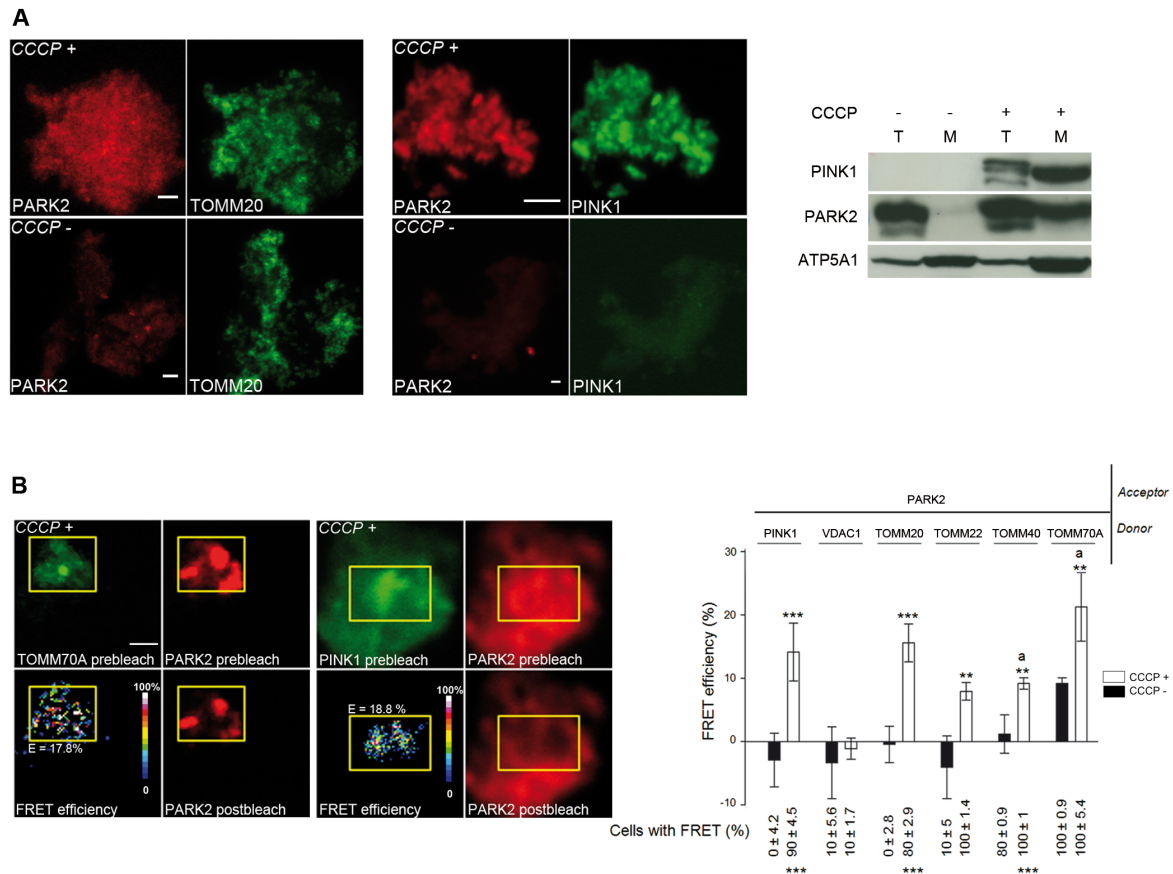


Figure S3. PARK2 interacts with the TOM machinery in mitochondrion-enriched fractions. **(A)** Representative images and western blots showing mitochondrial accumulation of endogenous PINK1 and PARK2 in mitochondrion-enriched fractions from HEK293T cells treated with CCCP. (T): total protein extracts; (M): mitochondrion-enriched fraction. Western blotting for ATP5A1 confirms mitochondrial enrichment in M. **(B)** Representative FRET images, quantitative analysis of FRET efficiencies and percentage of ROIs with FRET in enriched mitochondrial fractions from HEK293T cells. $n = 5$ to 10 ROIs per condition from one experiment representative of two. Comparisons were made with VDAC1-PARK2 in “CCCP+” conditions (*) or corresponding “CCCP-” condition (a). **, $p < 0.01$, *** $p < 0.001$. Scale bar, 3 μm .

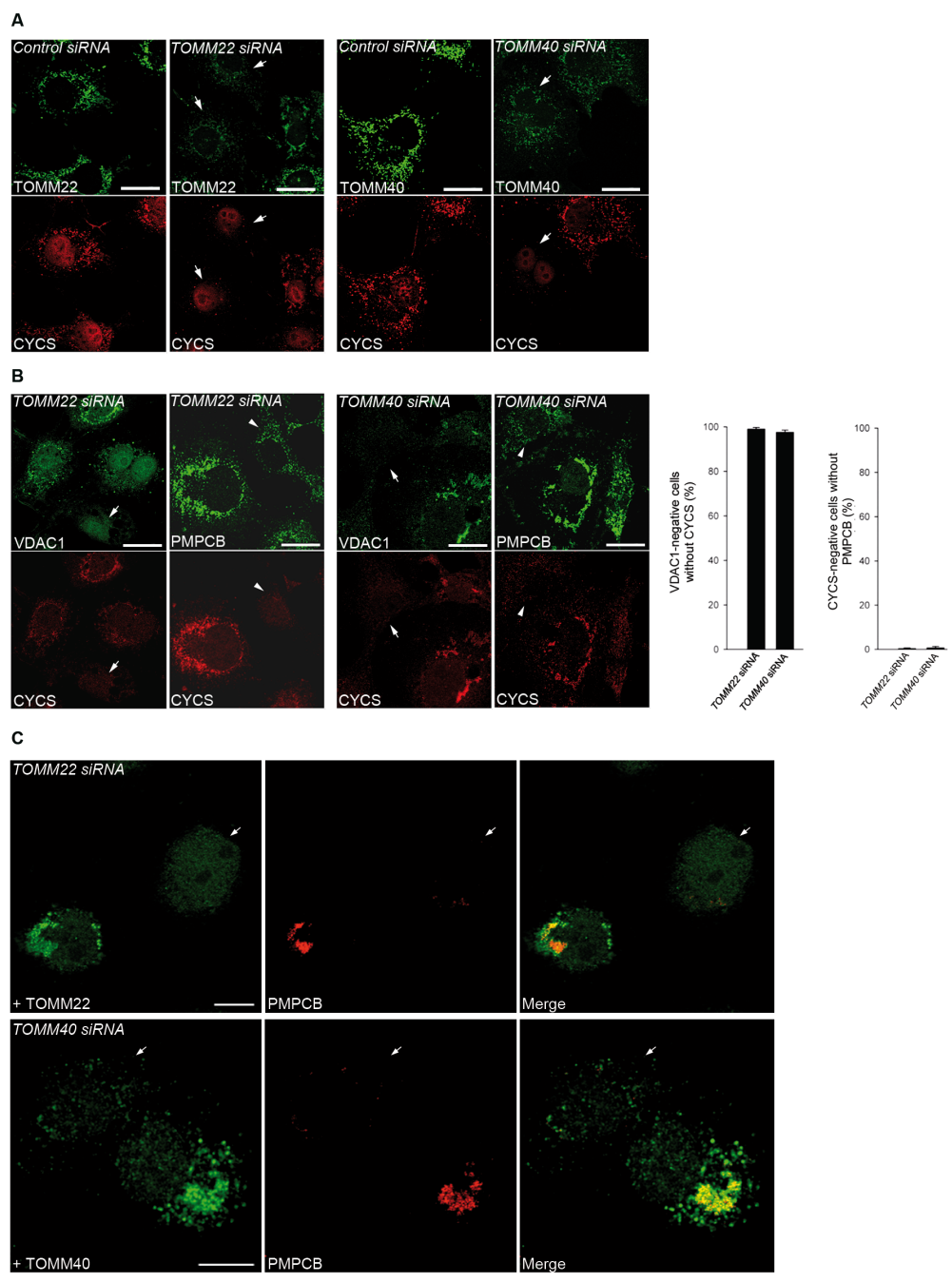


Figure S4. Silencing of *TOMM40* or *TOMM22* initiates mitochondrial clearance. **(A)** Fate of CYCS in cells with low *TOMM22* or *TOMM40* levels. **(B)** Representative images and corresponding quantifications illustrating the concomitant loss of CYCS and VDAC1 (arrows), but not PMPCB (arrowheads), in cells treated with the indicated siRNAs. $n = 3$ to 5 independent wells from one experiment representative of three. **(C)** Representative images illustrating the presence of mitochondria immunostained for PMPCB after CCCP treatment in cells silenced for *TOMM22* or *TOMM40* and transfected to express the corresponding siRNA-

resistant cDNAs. Arrows: PARK2-positive cells not overproducing the corresponding TOM subunit and showing loss of PMPCB staining. Scale bar, 10 μ m.

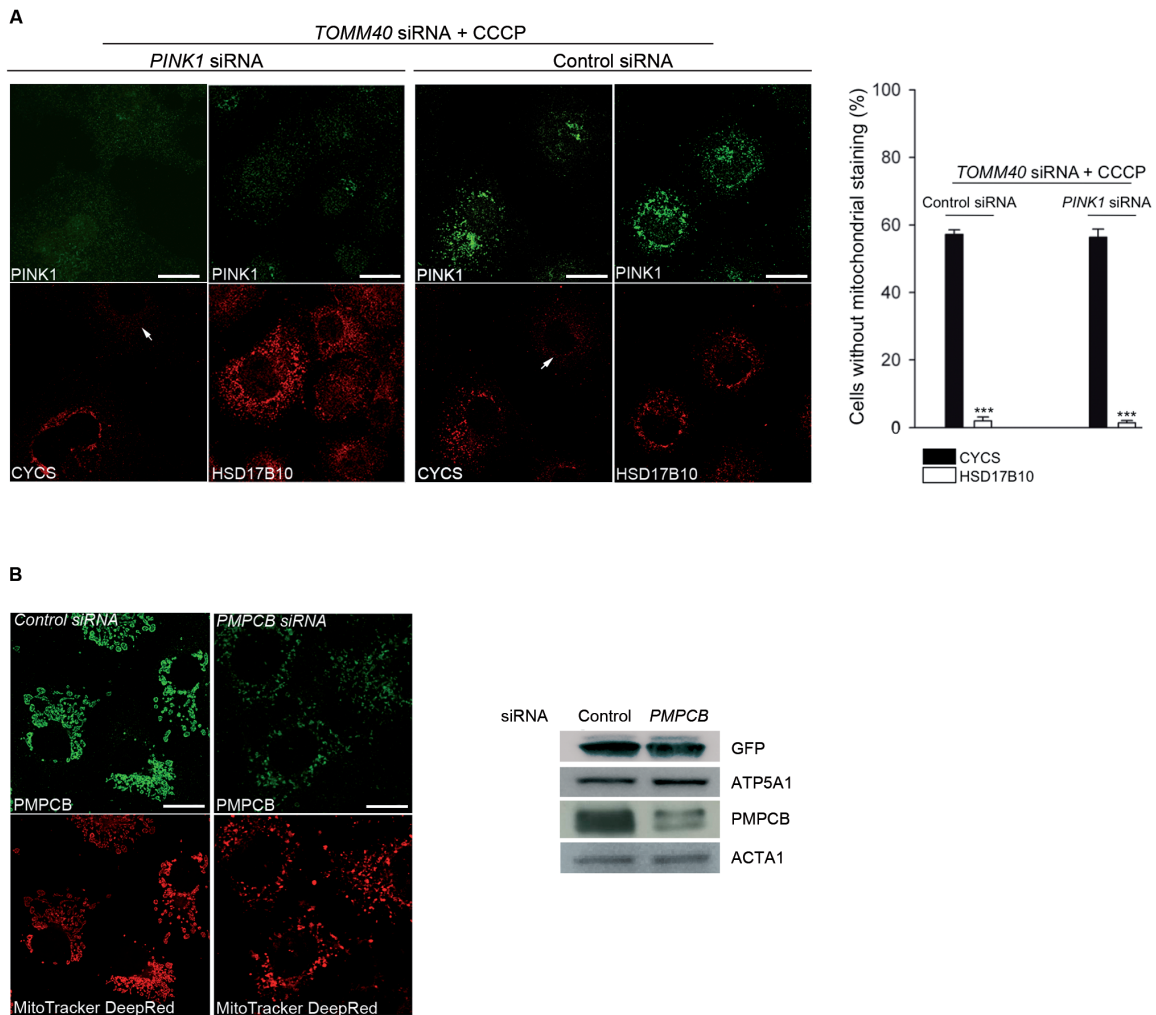
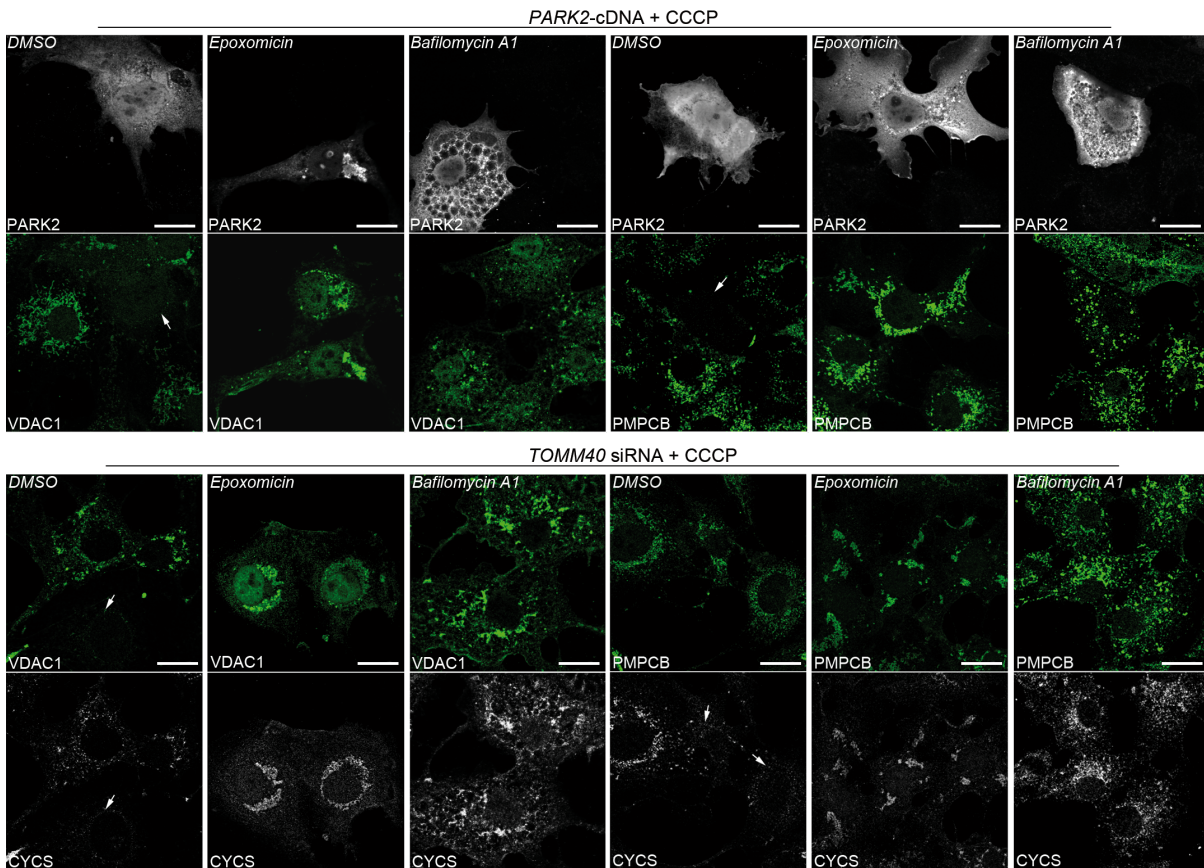


Figure S5. Depletion of subunits of the TOM machinery leads to CYCS clearance in cells silenced for *PINK1*. (A) Representative images illustrating the fate of the mitochondrial markers CYCS or HSD17B10 (matrix marker) in COS7 cells with low TOMM40 levels and with or without *PINK1* silencing, and corresponding quantitative analysis. Arrows: loss of CYCS. $n = 3$ to 5 independent wells from one experiment representative of three. $***p < 0.001$ versus the “CYCS” condition. (B) Representative images illustrating the efficacy of *PMPCB* gene downregulation in COS7 cells. Mitochondria were labeled with MitoTracker DeepRed. Western blot analyses: mitochondrial import impairment of mitoGFP in total extracts from COS7 cells with lower *PMPCB* levels. Loading controls: ATP5A1, ACTA1. Scale bar, 10 μm .

A



B

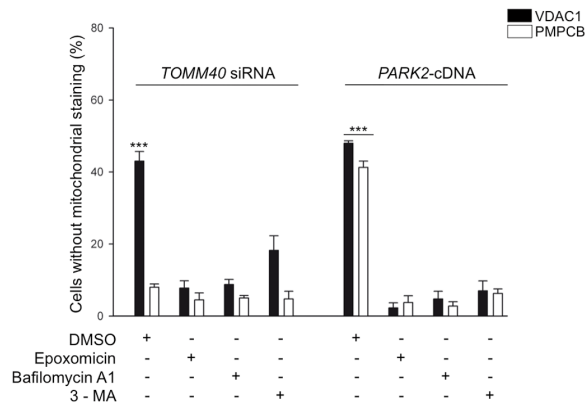


Figure S6. The proteasome and lysosome are required for OMM proteolysis and whole-organelle clearance. **(A)** Representative images illustrating the fate of mitochondrial markers in COS7 cells overproducing PARK2 or with low TOMM40 levels after CCCP treatment and the addition of the vehicle DMSO, the proteasome inhibitor epoxomicin or the lysosome inhibitor bafilomycin A₁, to the culture medium. Arrows: loss of mitochondrial markers. **(B)** Percentage of cells without VDAC1 or PMPCB immunostaining in cells transfected and treated as in **(A)** or with the inhibitor of autophagosome formation, 3-methyladenine. *n* = 3 independent

wells from one experiment representative of four. ***p < 0.001 versus all other conditions in each paradigm.

Scale bar, 10 μ m.

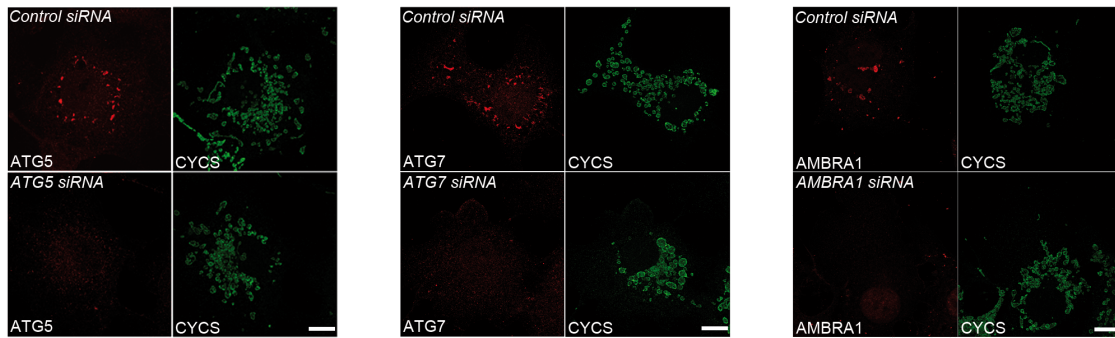


Figure S7. siRNA-mediated silencing of *TOMM40* initiates PARK2-dependent mitophagy. Representative images illustrating the efficacy of the downregulation strategies in COS7 cells transfected with the indicated siRNAs and stained for CYCS and the corresponding autophagy marker. Scale bar, 10 μ m.

Article 2: **Parkin regulates the mitochondrial abundance of the protective Parkinson's disease-related enzyme 17 Beta-Hydroxysteroid Dehydrogenase type 10 (Submitted to *Cell Death and Differentiation*).**

In this manuscript, we describe the identification of the mitochondrial matrix enzyme 17 β -hydroxysteroid dehydrogenase type 10 (HSD17B10), as a novel substrate of Parkin. HSD17B10 is a multifunctional protein involved in isoleucine metabolism, in steroidogenesis and is a functional component of the mitochondrial RNase P [333]. In addition, HSD17B10 is required for the maintenance of mitochondrial integrity, and loss of this protein leads to apoptotic cell death *in vivo* [334]. Mutations in *HSD17B10* are associated with rare X chromosome-linked inborn errors of metabolism (Reviewed in [333]). The protein is suspected to play a role in at least two of the most frequent neurodegenerative disorders, Alzheimer's disease, where it modulates the toxicity of the A β peptide [335,336], and PD, where it protects against MPTP-induced intoxication and where it was found to be downregulated in post-mortem samples of sporadic PD patients [337].

Accordingly with these observations, we here illustrated that HSD17B10 is downregulated in PD patients carrying *PARK2* mutations, leading to the hypothesis that Parkin positively regulates the levels of HSD17B10. By using FRET microscopy, we demonstrated that Parkin, PINK1 and HSD17B10 physically interact at the TOM machinery following mitochondrial import blockade, and showed that several missense PD-causing *PARK2* mutations severely reduced FRET between Parkin and HSD17B10 under these conditions. In addition, by analyzing mitochondrial protein levels of HSD17B10, neosynthesized in transfected cells concomitantly depleted for or overproducing Parkin, we showed that Parkin directly plays a role in maintaining mitochondrial HSD17B10 abundance, possibly with the intervention of PINK1.

In exploring whether HSD17B10 plays a role in PINK1/Parkin-dependent mitochondrial clearance, we discovered that the down-regulation of HSD17B10 by RNA interference does not impair mitophagy triggered by CCCP, but rather enhances this process. Conversely, we observed that the overproduction of HSD17B10 protects against CCCP-induced mitochondrial dysfunction, and suggest that it may do so by inducing a drastic mitochondrial elongation. By taking advantage of enzymatically inactive variants of HSD17B10, we demonstrated that the protective function of HSD17B10 does not depend on the catalytic activity of the protein.

Based on these observations, we propose that Parkin and PINK1 contribute to maintenance of the mitochondrial levels of HSD17B10, an enzyme essential for proper mitochondrial function, possibly by modulating its import through the TOM complex. This may be an additional mechanism by which Parkin and PINK1 cooperate in preserving mitochondrial homeostasis; its impairment may result in reduced levels of mitochondrial HSD17B10, thereby contributing to neuronal vulnerability in autosomal recessive PD.

Article 3: **Functional interplay between Parkin and Drp1 in mitochondrial fission and clearance (Submitted to *Biochimica et Biophysica Acta, Molecular Cell Research*).**

As illustrated in the introduction section, the PD-related proteins Parkin and PINK1 regulate a number of processes relevant to maintenance of mitochondrial physiology. What are the underlying mechanisms of this regulation and whether the proteins always act concertedly in the context of the PINK1/Parkin pathway is currently a matter of intense investigation.

Under conditions of mitophagy induction, Parkin and PINK1 have been shown to interact functionally with molecular actors involved in mitochondrial trafficking (the Miro-Milton complex, [208,289,339]), mitochondrial dynamics (Mfn1 and 2, [298,310]) and autophagy (Ambra 1, [323]). Unravelling the spatio-temporal basis of these multiple interactions would also shed light on possible novel crosstalk levels between Parkin and PINK1.

In this manuscript, we first analyzed the reciprocal roles of Parkin and PINK1 in Drp1-dependent mitochondrial fission. We observed that Parkin enhances mitochondrial fragmentation by mechanisms at least partially independent of the presence of PINK1. In addition, we demonstrated that pharmacological inhibition or antagonization of the Calcium/Calmodulin/Calcineurin signaling pathway, which controls the phosphorylation of Drp1 on serine 637, abolished the pro-fission effect of Parkin, suggesting that Parkin modulates this process.

We then explored the relation between Drp1-dependent mitochondrial fission and initiation of organelle clearance, and the role of PINK1 and Parkin therein. By taking advantage of PD-related *PARK2* mutations with a differential impact on these two processes, we demonstrated that Parkin-dependent mitochondrial fragmentation was not required to achieve the elimination of defective organelles through autophagy in cells treated with CCCP. However, FRET microscopy revealed that Drp1 was co-recruited with Parkin on mitochondrial fission sites following $\Delta\Psi$ loss where PINK1 also accumulated, indicating that mitochondrial fission and mitophagy are spatially co-ordinated.

This work provided novel insights into the regulation of mitochondrial dynamics by PINK1 and Parkin in the context of mitophagy induction, two processes appearing to be spatially coordinated. The alteration of this functional interplay could potentially be a previously unsuspected element in the physiopathology of autosomal recessive PD. Future work is required to determine the temporal regulation of the molecular events triggering the recruitment of Drp1 and Parkin to the OMM.

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Chapter V. Discussion

Based on our objectives and on data from recent literature, the results obtained during my PhD allowed us to gain novel insights on the role of Parkin and PINK1 in mitochondrial quality control.

First, we provided evidence that blockade of mitochondrial protein import is sufficient to trigger the stabilization of PINK1 on the OMM and the recruitment of Parkin, suggesting that the mitochondrial degradation program may be directly coupled with loss of mitochondrial import efficiency. We confirmed these data in indirect and direct models of mitochondrial import impairment triggered by: the protonophore CCCP; silencing of the mitochondrial protease LONP1, involved in organelle biogenesis and in the response to the accumulation of unfolded proteins in the matrix; the mild downregulation of the import channel TOMM40. Given that the degree of mitochondrial depolarization was variable in these models, $\Delta\Psi$ collapse does not appear to be mandatory for the accumulation of PINK1 on the OMM and the translocation of Parkin to the mitochondrion, but may rather be a secondary phenomenon. A very recent study from Youle's team demonstrated that the activation of the UPR^{mt} induces the stabilization of PINK1 and PINK1/Parkin-dependent mitophagy [340]: the authors confirmed previous findings from our group, showing that PINK1 and Parkin accumulate on mitochondria without an apparent $\Delta\Psi$ loss both in this alternative model of mitochondrial protein import impairment and after the downregulation of the LONP1 protease. Mitophagy appeared to be activated in the attempt to eliminate organelles with an overloaded matrix. Our results are also in agreement with recent data provided by Greene and co-workers, indicating that blockade of mitochondrial import following down-regulation of the matrix protease MPP β , a condition not associated with $\Delta\Psi$ loss, also leads to the recruitment of PINK1 and Parkin to the OMM [234]. It is not excluded that the massive translocation of Parkin to the mitochondrion triggered by the overproduction of the mitochondrial enzyme HSD17B10 in the absence of obvious mitochondrial depolarization is also reflecting the consequence of the overwhelming of the TOM machinery; however, further studies are required to establish whether local mitochondrial import alterations indeed occur in this model.

Second, FRET analyses revealed that Parkin, PINK1 and HSD17B10 interact with the TOM machinery both on functional mitochondria and on mitochondria entering the mitochondrial degradation program. These findings strongly support the possibility that the TOM machinery is the primary target of Parkin on the OMM, constituting a molecular platform for the recruitment of HSD17B10 and potentially other mitochondrial proteins. Consistent with another study from Youle's team published while our work was in progress [341], we also detected PINK1 in proximity of this complex.

Third, we also provided evidence for an involvement of Parkin in maintenance of mitochondrial levels of the protective enzyme HSD17B10, although the underlying mechanisms remain to be explored; preliminary studies suggest that PINK1 may cooperate with Parkin in this function. Intriguingly, the overproduction of

Parkin also enhanced the mitochondrial levels of PINK1 (Article 2, supplementary results). These observations hint at a new interplay between Parkin and PINK1 in the regulation of mitochondrial protein import *via* their interaction with TOM. Further studies are required to explore in detail this hypothesis and determine whether the import of mitochondrial proteins other than HSD10B10 and PINK1 is also subjected to this type of regulation. Nevertheless, these observations hint at an additional regulatory loop involving this quality control pair in maintenance of mitochondrial homeostasis.

Fourth, we discovered that the core subunits of the TOM machinery act as a molecular switch promoting mitochondrial clearance following the accumulation of Parkin and PINK1 at the TOM complex; in contrast HSD17B10 is unlikely to play a direct role in this process. Together with our previous observations, these findings support the possibility that Parkin and PINK1 orchestrate different mitochondrial quality control functions by acting on the same molecular scaffold.

Of note, although some TOM subunits are targeted to the proteasome for degradation by Parkin-dependent ubiquitylation [312,313,316,317], others may not. Therefore, future studies will need to address the mechanisms involved in the degradation of the core of the TOM machinery for a conclusive scenario of how this event triggers mitochondrial elimination. Finally, we showed that in the early phases of PINK1/Parkin-dependent mitophagy, Drp1 is corecruited with Parkin on the OMM in direct proximity of PINK1. These data provide a first line of evidence that mitochondrial fission and clearance are spatially coordinated, although future studies are required to determine the time scale of this functional interaction and whether it occurs in proximity of the TOM machinery to regulate the crosstalk between organelle fission and mitophagy.

The work summarized here raised a number of questions that require further discussion.

A. Can dynamic protein-protein interactions be fully explored by FRET microscopy?

Sometimes it is the only key to open the door: FRET microscopy versus biochemical approaches

FRET microscopy is a powerful technique to detect protein-protein interactions *in cellulo* and *in organello* [342]. In our case, this approach allowed the identification of the physical proximities between Parkin, PINK1, HSD17B10 and subunits of the TOM complex. To detect FRET, we chose to immunolabel endogenous proteins with fluorophore-conjugated secondary antibodies serving as donor and acceptor of energy. Since subunits of the TOM machinery are very abundant proteins, we hypothesized that their overproduction could severely impair mitochondrial morphology and dynamics, possibly affecting their ability to transfer energy with putative partners in FRET experiments. The approach we set up bypasses the use of fluorescent fusion proteins with donor or acceptor properties, but it needs to be performed on fixed cells. Because of this technical aspect, we could not determine the stoichiometry and the dynamics of the interactions examined, but rather the presence or absence of a given proximity in a given experimental

paradigm. Nevertheless, this technique turned out to be a robust and reproducible approach for the analysis of protein-protein interactions, as demonstrated by several controls used to corroborate our findings. First of all, the interactions we detected were conditional, as they were observed only when the efficiency of mitochondrial protein import was reduced by different means (i.e. CCCP treatment, moderate downregulation of TOMM40, overproduction of HSD17B10, etc.) and Parkin was recruited to the OMM, in cells showing in general large mitochondrial aggregates. Only the strongest interactions, e.g. that between Parkin and TOMM70, were detected even in basal conditions, suggesting that they normally follow rapid kinetics and that the proportion of molecules performing FRET at a given time in isolated mitochondria goes beyond the sensitivity threshold of the technique. Secondly, the interactions involving Parkin, PINK1 and HSD17B10 at the TOM complex were found in several cell models, with exclusively endogenous as well as overproduced proteins; remarkably, the interaction between PINK1, Parkin and subunits of the TOM machinery were also found in mitochondrion-enriched fractions from HEK293T cells, revealing the suitability of FRET microscopy for the detection of endogenous protein-protein interactions *in organello*. In this paradigm, Parkin interacted with all the subunits of the TOM machinery compared to studies *in cellulo*, indicating that detection of FRET indeed depends on the proportion of molecules interacting at a given time. Last, it should be considered that lack of FRET does not univocally mean absence of physical interaction, as it may also indicate an unfavourable 3D conformation of the fluorophores. We suspected that this could be the case for the analyzed PD-related Parkin variants, which severely impaired the ability of Parkin to interact with TOMM40, but at least in part preserved the molecular proximity with TOMM70 or HSD17B10. Accordingly, lack of FRET may also hint at modified or simply different interaction kinetics between specific partners. For instance, Parkin G328E did not interact with TOMM70 or with Tom 40, as shown in Fig. 3a of Article 1, although it retained the ability to trigger mitophagy after CCCP-induced $\Delta\Psi$ loss (data not shown and [306]). Therefore, the disadvantage of FRET microscopy on fixed cells is that it does not provide access to the dynamic dimension of the protein-protein interactions analyzed.

This technique turned out to be particularly advantageous compared to biochemical approaches: the interaction between Parkin and TOMM70, which showed the highest FRET efficiency in our experiments, was the only one retrieved by GST pull-down. Accordingly, Lazarou and co-workers failed to observe Parkin in a 300 kDa complex constituted by PINK1 and the TOM machinery and isolated by immunoprecipitation techniques [341]. As the authors themselves suggested, these interactions are likely to be dynamic and difficult to detect; FRET microscopy thus appears as a suitable solution to investigate molecular proximities at the cell level.

Finally, in an attempt to confirm the existence of a tripartite protein complex involving PINK1 and Parkin at the TOM complex, we set up a protocol based on the use of three chromophores to detect energy transfer. Although preliminary data are extremely encouraging and did confirm FRET between Parkin, PINK1

and the TOMM40 subunit (Article 1, supplementary results), future studies are required to dissect the dynamics of the formation of this complex.

In conclusion, our studies show that FRET performed on fixed samples is indeed a powerful technique to investigate molecular proximities involving endogenous proteins. However, this technique probably only detects the tip of the iceberg, as it indicates the presence or absence of an interaction at a given time point. Therefore, studies in live cells are absolutely required to deepen our understanding of the mechanisms by which Parkin, PINK1 and HSD17B10 act on the TOM complex in time and space. In addition, a future challenge will be to detect these protein-protein interactions in physiological conditions, and to understand how they are affected by the absence of *PARK2* or *PINK1*.

B. The degradation of TOM subunits acts as a molecular switch in PINK1/Parkin-dependent mitophagy; what are the mechanisms behind?

Protein-protein interactions open the way to TOM degradation.

Several studies reported that a plethora of OMM proteins, including TOMM22 and TOMM70, are targeted to the proteasome for degradation in a Parkin-dependent manner [312,313,316,317], and that this constitutes the first phase of the mitophagy cascade. By revealing the existence of a complex involving Parkin and PINK1 at the TOM machinery, our FRET analyses led us to suspect that the degradation of specific TOM subunits might be a key event in this cascade. We thus discovered that mimicking the degradation of the TOMM22 and TOMM40 subunits of TOM by RNA interference initiates mitochondrial degradation. In our hands, FRET analyses revealed that Parkin preferentially interacts with the TOMM70 subunit and at least four independent studies provided evidence that this protein is rapidly ubiquitylated by Parkin and degraded after induction of mitophagy [312,313,316,317]. The most straightforward hypothesis would postulate a direct role for this subunit in the initiation of PINK1/Parkin-mediated mitochondrial elimination; however we clearly demonstrated that mimicking its degradation by RNA interference does not have any substantial effect in this degradation process. This observation suggests either that the role of TOMM70 is not pivotal to this end, or that degradation of this subunit is functionally coupled with a second event dependent on PINK1 and/or Parkin, which rapidly destabilizes the core structure of the TOM complex. Future studies are required to dissect the mechanisms underlying the degradation of specific TOM subunits during mitophagy, and their spatiotemporal regulation.

Remarkably, TOMM40 was not identified among the early targets of Parkin-dependent degradation [312,313,316], suggesting that it does not follow a proteasome-dependent elimination pathway. Given that TOMM40 is an integral protein and only a limited portion is exposed to the cytoplasm, it is conceivable that its extraction from the OMM requires the activation of particular molecular mechanisms. As previously shown for

Mfn1/2, it may rely on the intervention of p97 to physically remove it from the lipid bilayer of this submitochondrial compartment [298]; however the activity of this chaperone has always been associated with proteasomal elimination of substrates and the degradation of TOMM40 seems insensitive to proteasomal inhibitors, such as lactacystin [313].

Alternatively, other yet unexplored mechanisms of degradation could potentially involve Mitochondria-Derived Vesicles (MDVs), a recently identified system of vesicles containing mitochondrial proteins targeted either to peroxisomes, or to the lysosomes for degradation [343,344]. Interestingly, the TOMM20 and the small Tom7 subunits were found as cargoes within these vesicles. Although this hypothesis is fascinating, to date evidence is lacking for a molecular engine driving the formation of these vesicles; nevertheless, the role of PINK1 and Parkin in this process is currently under investigation; such a role would not be surprising, given that PINK1 and Parkin have already been reported to regulate the activity of the two main degradation systems in the context of their mitochondrial quality control function.

Thirdly, it is not excluded that proteins playing a role in mitochondrial fission could selectively isolate small portions of the OMM containing TOMM40 channels. We provided evidence that Drp1-dependent mitochondrial fission and organelle clearance are spatially co-ordinated [Article 3]. It is possible that Drp1, and possibly the pro-fission factors Mff and/or Fis1, tear apart OMM fragments containing TOMM40 after $\Delta\Psi$ collapse, but not the proteasome-degraded receptors TOMM22 or TOMM70. This may represent an efficient way to temporarily stock integral, potentially difficult to degrade, OMM proteins, in the attempt to recreate functional TOM machineries re-fusing with the healthy network after the re-establishment of mitochondrial protein import. Aggregated or unfolded proteins persisting in the TOMM40 channel could possibly constitute a regulatory loop to induce the definitive degradation of these mitochondrial remnants. In light of these hypotheses, it would be interesting to determine whether fission-promoting proteins directly interact with TOM subunits, as well as to explore whether the fusion proteins Mfn1/2 are ubiquitylated and degraded with the intervention of Parkin and PINK1 in proximity of these putative fission sites, prior to mitophagy. This would deepen our understanding of the spatio-temporal interplay between mitochondrial dynamics and quality control, and help define the precise “cascade” of events driven by the PINK1/Parkin pathway.

Lastly, a differential mechanism triggering the differential elimination of TOM subunits may involve distinctive post-translational modifications, which also needs to be investigated. The regulation of TOM subunits by phosphorylation has recently been described in *Saccharomyces cerevisiae*, with the identification of cytosolic kinases controlling the assembly and the translocase activity of the TOM machinery [345]. Although the existence of this post-translational regulation has not yet been described in mammalian cells, analysis of the role of PINK1 in the phosphorylation of the subunits of the TOM complex may uncover a new mechanism controlling the turnover of the import machinery. Consistent with this possibility, recent evidence in budding yeast indicates that a great proportion of ubiquitylation sites are used in conjunction with

phosphorylation to modulate protein degradation via the proteasome, although it is not excluded that a common phosphorylation/ubiquitylation signature could be recognized by other elimination systems as well [346]. Therefore, PINK1-mediated phosphorylation of TOM subunits in conjunction with Parkin-dependent ubiquitylation may constitute a molecular hallmark for a differential elimination of this complex. This could reveal unexpected interplays between the catalytic activities of Parkin and PINK1 for the degradation of selective OMM proteins, possibly causing OMM rupture and mitophagy. Alternatively, we could envisage that Parkin could promote the production of different types of ubiquitylation chains after CCCP treatment, according to the TOM subunit to be degraded. As mentioned previously (c.f. Paragraph 1.3.1C), alternatively to K48 chains [312], Parkin has been shown to trigger the formation of K63 chains, already linked to lysosomal degradation [306] and of K27 ones [306].

C. Is there room left for a role of other mitochondrial proteins in PINK1/Parkin-dependent mitochondrial degradation?

Conclusive evidence for the role of VDACs, hexokinases and Mfn1/2 in mitophagy is still lacking.

As already mentioned in the Introduction section of this manuscript, several studies reported on the role of specific OMM proteins in mitophagy, although with controversial findings. VDAC1/3 initially appeared to be preferential substrates of Parkin in this process [298,306,316]; in Geisler's study mitophagy was impaired in the absence of VDAC1, but Narendra and colleagues reported that mitophagy proceeds in cells deficient for VDAC1 and VDAC3. We were unable to confirm the interaction between Parkin and VDAC1 by FRET analyses or by GST pull-down; again, absence of FRET does not exclude physical proximity between the two proteins, although our results were extremely reproducible, so that we chose to use the Parkin/VDAC1 interaction as a negative control in most of our FRET experiments. Other OMM proteins have controversial roles in mitophagy: Mfn1/2 were among the first substrates found to be ubiquitylated by Parkin in cells treated with CCCP, but they were reported to be dispensable organelle clearance in mammalian cell lines; their early degradation in the mitophagy program was rather proposed to be essential to inhibit fusion of damaged organelles with the healthy network [298]. Surprisingly, recent data revealed that Mfn2, but not Mfn1 is strictly necessary for Parkin to complete mitophagy in primary cardiac myocytes, where Mfn2 apparently behaves as the receptor of Parkin on the OMM [310]. Although it is conceivable that the PINK1/Parkin pathway works differently according to cell type, additional data are required to establish the exact role of Mfn1/2 in this experimental context, as in this last study no quantification of mitochondrial clearance was provided.

Lastly, recent evidence revealed that hexokinases 1/2 are ubiquitylated by Parkin rapidly after CCCP treatment [315,317]. Interestingly, it was shown that the two hexokinases [315] or all three VDAC channels [347] need to be silenced simultaneously to block mitophagy, indicating molecular redundancy between these proteins. Our observations identifying the TOM machinery as a docking site for PINK1 and Parkin on the

OMM, and a switch in mitochondrial degradation, do not exclude the contribution of other mitochondrial proteins in this program. However the functional relationships between these proteins and the temporal scale of their intervention downstream of the TOM-dependent initiation of the program remain to be determined. Interestingly, the analysis of the Parkin ubiquitylome after CCCP treatment indicated that HSD17B10 is among the interactors of Parkin, together with other members of the Short-Chain dehydrogenase/reductase superfamily (SDR) [316,348]. Our results suggest that this protein does not play a role in mitophagy *per se*, but rather counteracts this process by potentially exerting a protective function on mitochondria. It would be interesting to determine whether the members of the SDR family interacting with Parkin share some kind of redundant action in mitochondrial degradation, as previously observed for VDACs and Hexokinases [315,347], or whether their simultaneous knockdown alters the efficiency of organelle clearance.

In conclusion, future studies are required to explore the effect of the absence of VDACs, hexokinases 1/2, Mfn1/2 and possibly members of the SDR family, in conjunction with lowered levels of TOM, on the ability of Parkin to trigger mitophagy.

D. How does the degradation of the TOM complex intrinsically cause mitophagy?

OMM disruption is strikingly similar to MOMP.

Our findings raise the question as to the mechanisms by which the degradation or destabilization of the TOM machinery following recruitment of Parkin initiates mitochondrial degradation. According to our observations, it is conceivable that the elimination of TOM subunits alters the composition and the integrity of the OMM, by physically opening a “pore” which brings IMS proteins, such as cytochrome *c*, in contact with the cytosol. This hypothesis is supported by the ultrastructural analyses of Yoshii and co-workers, who reported that the initial proteasome-dependent step in mitochondrial degradation triggered by CCCP is associated with rupture of the OMM [313]. It remains to be clarified whether this event is related to MOMP, an event characteristic of mitochondrion-dependent apoptosis (c.f. Paragraph 1.2.2); Yoshii et al. reported that upon induction of Parkin-dependent mitophagy, cytochrome *c* was degraded by the proteasome and by autophagy after OMM rupture, thus implying that this protein may be in contact with pro-apoptotic factors in the cytosol; interestingly, OMM rupture was not accompanied by signs of apoptosis. Our own preliminary studies provided evidence that the pharmacological inhibition of the PTP by cyclosporin A partially prevents Parkin-dependent mitophagy induced by CCCP, and completely abolishes OMM rupture triggered by the siRNA-mediated down-regulation of TOMM40 (Article 1, supplementary results). Based on these elements, it can be inferred that Parkin-induced OMM rupture in the early phases of mitophagy indeed shares some common features with MOMP, and that apoptosis could be activated together with mitophagy. Is there any molecular mechanism blocking apoptosis in favour of organelle clearance under these conditions? Intriguingly, a recent study

provided a first insight into the mechanisms by which cells undergoing mitophagy may escape apoptosis by showing that the anti-apoptotic protein Bcl-2 and its interactor FKBP38, required for mitochondrial localization of Bcl-2, relocate from the OMM to the ER [349]; this event appears to act as a last “exit strategy” for the cell, in an attempt to avoid unwanted apoptosis under conditions of mitophagy activation. Unravelling how the ER participates in Parkin-dependent mitophagy remains a challenge for future research and it is already a topic of interest in our team.

E. From OMM rupture to the elimination of mitochondrial remnants: what is the downstream signal to complete mitophagy?

Our data demonstrate that downregulation of TOMM40 or TOMM22 triggers OMM proteolysis at a time-point at which IMM and matrix proteins are only partially cleared. As mentioned earlier in this manuscript, this observation corroborates previous studies suggesting the existence of two phases in mitochondrial degradation, the first one involving the UPS to proteolyze the OMM, the second driving the elimination of mitochondrial remnants by the autophagy system [312,313]. This has been inferred partly on the basis of the analysis of electron micrographs of Parkin-positive cells after long-term CCCP treatment, illustrating organelles with an impaired ultrastructure and surrounded by one mitochondrial membrane only [313]. However, the scenario presenting two different (and potentially separated) phases for mitochondrial elimination appears to be too dichotomic to be true. We and others demonstrated that pharmacological blockade of the proteasome or the autophagy pathway were both able to abolish OMM rupture and organelle clearance [170,298,312,313][Article 1], strongly suggesting that the two phases are instead closely intertwined. This might explain why even organelles with an intact OMM were found surrounded by autophagosomes [313], and why selected OMM proteins, such as TOMM40, do not appear to be targeted to the proteasome for degradation.

We also demonstrated that Parkin, but not PINK1, is necessary to complete mitophagy following OMM rupture/proteolysis. It is conceivable that Parkin interacts with other proteins of the IMM that might play a similar role to that of TOM to signal for degradation of the IMM and mitochondrial matrix. A possible mechanism for the degradation of mitochondrial remnants could involve the interaction of Parkin with import-dedicated complexes on the IMM, such as TIMM. We already started to investigate this hypothesis, by exploring whether silencing selective TIM subunits triggers mitochondrial clearance regardless of the presence of Parkin. Alternatively, components of the respiratory chain may be crucial to the second phase of mitophagy; indeed, recent data in *Drosophila* revealed that several subunits of the five OXPHOS complexes undergo a more rapid, Parkin-dependent turnover *in vivo* compared to other mitochondrial proteins [350]. The authors suggested that this event happens distinctively from mitophagy; however, it could be hypothesized that, when this turnover mechanism becomes secondary following loss of mitochondrial integrity, the degradation of a

specific OXPHOS subunit may represent the molecular trigger driving the elimination of mitochondrial remnants. Altogether, the search for potential preferential partners of Parkin on the IMM is open and future work is required to complete the puzzle of the PINK1/Parkin-dependent mitophagy cascade.

Finally, it would be interesting to determine whether the ER plays a role in this phase. The origin of the membranes constituting the phagophore is currently highly debated, and it is unclear whether they originate from the ER, as for regular autophagy [351], from mitochondria themselves [352], or from ER-mitochondria contact sites, as recently suggested [353]. Future studies are therefore necessary to shed light on this particular aspect, and whether PINK1 and Parkin could regulate the formation of autophagosomes.

F. A novel role for the PINK1/Parkin pathway in the regulation of mitochondrial import?

The puzzle of the mitochondrial quality control functions of PINK1 and Parkin still misses key pieces

Our data suggest that Parkin is involved in maintenance of the mitochondrial abundance of HSD17B10. One possible mechanism may be the direct regulation of the import of HSD17B10 through the TOM machinery; in addition, Parkin may regulate the mitochondrial import of PINK1 in physiological conditions, i.e. in absence of ongoing mitophagy (Article 2 and corresponding supplementary results). This hints at a new mechanism but which Parkin maintains mitochondrial homeostasis by selectively promoting the import of proteins with a protective function. However, our data do not formally demonstrate that Parkin actively regulates mitochondrial protein import and additional experiments are required to explore this possibility. *In vitro* import assays would be straightforward, although basic protocols would not take into account the requirement for the catalytic activities of Parkin and PINK1 and for other cytosolic factors potentially involved in the process. Therefore, these protocols should be optimized and coupled with ubiquitylation and phosphorylation assays, as well as addition of cytosolic fractions in future research. Direct analysis of the mitochondrial import of HSD17B10 is further complicated by the fact that the N-terminal MTS of the protein is not cleaved by MPP, which complicates the quantification of the proportion of the protein inside the organelle, compared to the non-imported cytosolic protein. To discriminate between the two forms of HSD17B10, we created a synthetic HSD17B10 construct in frame with the cleavable MTS of COXVIII; interestingly, we were unable to overproduce this protein, suggesting that this artificial MTS does not warrant a functional import for HSD17B10, or that it might interfere with the canonical import route of the protein. This may result in the production of non-importable precursor, which may be rapidly degraded by the cell. Strategies to develop import-sensitive probes to study *in cellulo* the effect of Parkin and PINK1 on the mitochondrial import of selected substrates of the TOMM20 and TOMM70-dependent import pathway are currently investigated in the team in close interaction with a private company.

Interestingly, the analysis of the Parkin ubiquitylome after CCCP treatment indicated that HSD17B10 is among the interactors of Parkin, together with other members of the Short-Chain dehydrogenase/reductase superfamily (SDR) [316,348]. Our results suggest that this protein does not play a role in mitophagy *per se*, but rather counteracts this process by potentially exerting a protective function on mitochondria. It would be interesting to determine whether the members of the SDR family interacting with Parkin share some kind of redundant action in mitochondrial degradation, as previously observed for VDACs and Hexokinases [315,347], or whether their simultaneous knockdown alters the efficiency of organelle clearance.

G. Is there any role for PINK1/Parkin-dependent mitophagy in models relevant to PD?

Evidence of mitophagy in neurons and in vivo remains elusive.

The majority of the studies concerning the activity of the PINK1/Parkin pathway in the regulation of mitochondrial clearance employed immortalized cell lines mainly relying on glycolysis for ATP production, while neurons depend on lactate and on oxidative phosphorylation [354]. Therefore, it is crucial to confirm the existence of the PINK1/Parkin-dependent control of mitophagy, and to explore the consequences of *PARK2* or *PINK1* deficiency in this more relevant cell type. At present, data from the literature appear to be quite contradictory in this regard. Van Laar et al. observed that Parkin is not recruited to mitochondria following CCCP treatment in rat cortical neurons and in mixed striatal/midbrain neurons, in contrast to established cell lines where Parkin was efficiently translocating to the organelle [355]. Comparatively, in HeLa cells forced to depend on oxidative phosphorylation, no mitochondrial translocation of Parkin was observed. Accordingly, induced-pluripotent stem cells (iPS)-derived dopaminergic neurons grown on galactose did not display Parkin translocation and PINK1-dependent mitophagy after prolonged mitochondrial depolarization, indicating that cells with higher energy demands do not easily get rid of all of their mitochondria, and that in whole organisms in which mitochondria rely on oxidative phosphorylation, complete disappearance of the mitochondrial network would be unlikely to occur [356]. Nevertheless, this does not exclude that mitophagy occurs, although to a reduced scale compared to the loss of the entire mitochondrial network. To verify this hypothesis, the findings discussed above should be re-examined after the analysis of mitochondrial clearance with more sensitive, perhaps image-based techniques to quantitatively measure mitochondrial loss, rather than the simple scoring of cells devoid of mitochondrial markers.

In addition, Rakovic et al provided evidence that endogenous Parkin was insufficient to promote mitophagy in iPS-derived dopaminergic neurons and in fibroblasts from PD patients with PINK1 mutations, again suggesting that the loss of the entire mitochondrial network is an unlikely event in conditions more relevant to the disease. By taking advantage of more sensitive quantification approaches, it would be

interesting to explore whether Parkin induces small-scale mitophagy [357]. Conversely, Cai et al. demonstrated that Parkin co-localized with mitochondrial markers when overproduced in mouse primary cortical neurons treated with CCCP and grown on glucose [358]. These cells could undergo mitophagy on selective groups of organelles, although with slower kinetics compared to those observed in cell lines. Interestingly, Joselin et al showed that the culture media for primary neurons is determinant, since the presence of apoptotic inhibitors and anti-oxidants such as B-27 appears to counteract the action of chemical uncouplers as CCCP or valinomycin [359,360], potentially explaining the differences concerning the recruitment of Parkin to mitochondria and the efficiency of mitophagy in the studies employing neurons [360].

Is there any conclusive evidence for the existence of PINK1/Parkin-dependent mitophagy in *in vivo* models? Few pioneering studies investigated the role of this pathway in disease-relevant models. In a pioneer study, the presence of mitophagy was assessed in a mouse model obtained by crossing *PARK2* knockout mice with MitoPark mice [361]. MitoPark mice present progressive degeneration of dopaminergic neurons and impaired locomotion, due to the accumulation of mtDNA deletions and OXPHOS defects caused by the selective deletion of the gene encoding TFAM in dopaminergic neurons (c.f. paragraph I.2.1A) [362,363]. In this model, clustering of dysfunctional mitochondria was observed, but overproduced Parkin did not co-localize with these structures. In addition, the absence of *PARK2* did not increase the average number of mitochondrial aggregates in MitoPark mice, indicating that Parkin does not trigger the elimination of dysfunctional mitochondria in this context [361], or that an upstream signal lacking in MitoPark mice is required to trigger mitochondrial recruitment of Parkin and induction of mitophagy. Again, a more sensitive quantification of the loss of mitochondrial markers was not performed, and the issue concerning the existence of small-scale mitophagy in this paradigm remains unresolved. This observation raises the question of the specificity of the paradigms currently used in the literature to investigate Parkin-dependent mitophagy. The $\Delta\Psi$ collapse induced artificially by CCCP or by valinomycin, is a condition most likely never occurring in physiological conditions, and if it does, it would probably not affect the entire mitochondrial network of the cell, but only portions thereof. Therefore, if the PINK1/Parkin pathway is indeed activated by a mitochondrial protein import blockade, as we and others propose [234][Article 1], modelling TOM-dependent import dysfunction may represent an alternative solution to investigate the activities of these proteins in *in vivo* paradigms.

The PINK1/Parkin pathway has been extensively explored in *Drosophila melanogaster*. Rana and co-workers showed that the overexpression of *PARK2* in adult flies leads to increased mitochondrial fission and lowered abundance of the fly ortholog of Mfn1/2, dMfn [364]. This corroborates the role of Parkin in promoting mitochondrial fission in flies, a role potentially activated prior to mitochondrial quality control mechanisms; however, the presence of mitophagy was not explored in this model. Mitochondrial clearance was investigated in two recent studies: as mentioned earlier in this discussion, Vincow and colleagues showed that *PARK2*-null flies present a slower turnover of mitochondrial proteins, compared to wild-type ones. The authors drove this

conclusion after comparison of the mean half-life of mitochondrial proteins and non-mitochondrial ones, which is a direct and quantitative parameter to measure the clearance of mitochondrial proteins by Parkin [350]. Second, Chen and co-workers illustrated that the absence of Parkin causes cardiomyopathy and respiratory defects in flies, and provided indirect evidence that these dysfunctions occurred because of an impaired Parkin-dependent ubiquitylation of Mfn2 [310].

It is clear that investigating the relevance of the PINK1/Parkin pathway for mitochondrial quality control *in vivo* requires further analyses; novel molecular or pharmacological strategies and more adequate methodologies need to be developed, to study the relevance of this pathway to neuronal physiology and the consequences of its dysfunction in PD.

Chapter VI. Conclusions and perspectives

Mendelian inheritance accounts for nearly 10% of the overall PD cases. In the past fifteen years, the discovery of genes responsible for these familial forms and the investigation of the functions of their protein products, such as Parkin and PINK1, brought great hope not only to deepen our knowledge of the pathogenic pathways involved in each of these specific genetic diseases, but also to find early neuropathological markers in common with idiopathic PD. This would potentially allow anticipated diagnosis and thus, a better efficacy of pharmacological treatments.

A great body of evidence in the last decade of research demonstrated the key role of that the PINK1/Parkin pathway in maintenance of mitochondrial homeostasis; an ever-increasing number of partners have been found to interact with Parkin and PINK1 on this organelle. Despite these observations, formal evidence for a unifying mechanism of action of this pathway is lacking, and our knowledge of the spatio-temporal orchestration of the different processes reported to be regulated by the PINK1/Parkin pathway is fragmentary.

The results obtained during my PhD internship opened new perspectives on the mode of action of the PINK1/Parkin pathway. One of these is the analysis of the implication of Parkin and PINK1 in the modulation of the mitochondrial protein process via the TOM machinery, as a new mechanism by which these proteins preserve mitochondrial physiology. We propose that this function could be achieved by direct interaction of Parkin and PINK1 with the TOM complex. The role of this pair in the regulation of TOM-dependent mitochondrial import, and the potential dysfunction of this process in sporadic and genetic forms of PD is currently under investigation in the context of a recently established partnership between our team and a pharmaceutical laboratory. We also provided evidence that the TOM complex is a key target and major player of the mitochondrial degradation program mediated by the PINK1/Parkin pathway. Functional coupling between the efficiency of the mitochondrial import process through the TOM machinery and different levels of mitochondrial quality control by as single sensor/effector pair appears as an intuitive and “economic” solution to ensure the preservation of an organelle of crucial relevance to cell survival. In this respect, it would be interesting to investigate the potentially broader role of the TOM machinery as a putative platform for the recruitment of different actors of the processes reported to be regulated by PINK1 and Parkin, including mitochondrial dynamics and trafficking, autophagy and apoptosis. The possibility that Drp1-mediated fission takes place at sites where PINK1 and Parkin initiate mitochondrial clearance, as suggested by our results, supports this view.

In conclusion, our study identified the TOM machinery and the mitochondrial import process as a new target in PD, and opened the way for identification of pharmacological agents modulating this process and evaluation of their therapeutic potential in autosomal-recessive PD.

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Summary

Elucidating the functional interplay between Parkinson's disease-related proteins and the mitochondrion

Parkinson's disease (PD) is a common neurodegenerative disorder of unknown etiology, affecting nearly 5% of the world population over the age of 80. Nearly 10% of PD cases are familial forms with Mendelian inheritance pattern. Mitochondrial dysfunction has long been suspected to play a role in the physiopathology of sporadic PD. This possibility has been recently corroborated by major discoveries in the field of autosomal recessive PD. Parkin and PINK1, the products of two genes associated with these forms, participate in a common molecular pathway focused on maintenance of mitochondrial quality, with roles in mitochondrial transport, dynamics, biogenesis and clearance.

The aim of this work was to elucidate some of the molecular mechanisms underlying the regulation of mitochondrial homeostasis by Parkin and PINK1. We used a combination of approaches in molecular and cell biology, biochemistry and confocal microscopy to identify and characterize molecular interactors of Parkin and PINK1 on the outer mitochondrial membrane (OMM).

In the first part of my project, we discovered that Parkin and PINK1 associate on dysfunctional mitochondria in proximity of the translocase of the OMM (TOM), a complex devoted to the mitochondrial import of the vast majority of the mitochondrial proteins. We provided evidence that these associations play a key role in activation of the mitochondrial degradation program mediated by the PINK1/Parkin pathway. We also observed that the dynamin-related GTPase Drp1, involved in mitochondrial fission is recruited to defective mitochondria in proximity of Parkin and PINK1, suggesting that mitochondrial fission occurs at sites where mitochondrial clearance is initiated.

In the second part of my project, we characterized the functional interaction between Parkin and the multifunctional neuroprotective mitochondrial matrix enzyme 17 β -hydroxysteroid dehydrogenase type 10 (HSD17B10), previously found by the team to be altered in abundance in Parkin-deficient mice. We demonstrated that HSD17B10 exerts a mitochondrion-protective function independent of its enzymatic activity. In addition, we provided evidence that Parkin directly interacts with HSD17B10 at the TOM machinery and that it positively regulates its mitochondrial levels, possibly through the regulation of its mitochondrial import.

Altogether, these results provide novel insights into the molecular mechanisms by which Parkin and PINK1 control mitochondrial quality, and deepen our understanding of the role of these proteins in the physiopathology of autosomal recessive PD.

Keywords: Parkinson's Disease, Parkin, PINK1, mitochondria, mitochondrial quality control, TOM complex, Drp1, HSD17B10

Résumé

Etude de l'interaction fonctionnelle entre les protéines impliquées dans la maladie de Parkinson et la mitochondrie

La maladie de Parkinson (MP) est une affection neurodégénérative fréquente d'étiologie inconnue, touchant environ 5% de la population mondiale après 80 ans. Environ 10% des cas correspondent à des formes familiales à transmission mendélienne. Pendant longtemps, un dysfonctionnement mitochondrial a été soupçonné jouer un rôle dans la physiopathologie de la MP. Cette possibilité a été récemment corroborée par des découvertes majeures réalisées dans le cadre des formes autosomiques récessives. Parkine et PINK1, les produits de deux gènes associés à ces formes familiales, participent au sein d'une même voie moléculaire au contrôle de la qualité mitochondriale, par la régulation du transport, de la dynamique, de la biogenèse et de la clairance de ces organites.

L'objectif de ce travail a été d'élucider certains des mécanismes moléculaires sous-jacents à la régulation de l'homéostasie mitochondriale par Parkine et PINK1. Nous avons utilisé un ensemble d'approches de biologie moléculaire et cellulaire, de biochimie et de microscopie confocale, afin d'identifier et de caractériser des interacteurs moléculaires de Parkine et PINK1 à la membrane mitochondriale externe (MME).

Dans la première partie de ce travail, nous avons découvert que la Parkine et PINK1 s'associent sur la MME de mitochondries dysfonctionnelles à proximité de la translocase de la MME (TOM), un complexe dédié à l'import de la grande majorité des protéines mitochondriales. Nous avons montré que ces interactions protéiques jouent un rôle clé dans l'activation du programme de dégradation mitochondriale régulé par la voie PINK1/Parkine. Nous avons également observé que la GTPase de type dynamine Drp1, impliquée dans la fission mitochondriale, est recrutée au niveau de mitochondries endommagées à proximité de Parkine et PINK1 ; ainsi, les processus de fission et de dégradation mitochondriales pourraient être spatialement coordonnés.

Dans la deuxième partie de ce projet, nous avons caractérisé l'interaction fonctionnelle entre la Parkine et l'enzyme neuroprotectrice multifonctionnelle de la matrice mitochondriale, 17 β -hydroxystéroïde déshydrogénase de type 10 (HSD17B10), dont les taux s'étaient révélés être diminués chez la souris déficiente en Parkine. Nous avons mis en évidence un effet protecteur d'HSD17B10 vis-à-vis de la mitochondrie qui était indépendant de son activité catalytique. Nous avons de plus montré que la Parkine interagit directement avec HSD17B10 à proximité de la machinerie TOM et qu'elle régule positivement l'abondance mitochondriale de cette protéine ; cela suggère qu'elle pourrait promouvoir son import.

Dans l'ensemble, ces résultats approfondissent notre connaissance des mécanismes moléculaires mis en jeu par la Parkine et PINK1 dans le contrôle de la qualité mitochondriale, élargissant ainsi notre compréhension de leur rôle dans la physiopathologie des formes autosomiques récessive de MP.

Mots-clé: Maladie de Parkinson, Parkine, PINK1, mitochondrie, contrôle de la qualité mitochondriale, machinerie TOM, Drp1, HSD17B10.