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PARKINSON'S DISEASE: IDENTIFICATION OF NOVEL SUBSTRATES FOR THE MITOCHONDRIAL KINASE PINK1

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular com Especialização em Neurobiologia orientada pela Professora Doutora Vanessa Alexandra Morais e pelo Professor Doutor Carlos Bandeira Duarte e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Outubro de 2020

Departamento de Ciências da Vida

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The work presented in this thesis was performed at the Mitochondrial Biology and Neurodegeneration Laboratory at the Instituto de Medicina Molecular João Lobo Antunes of the Lisbon School of Medicine (Faculdade de Medicina da Universidade de Lisboa – FMUL), under the supervision of Dr. Vanessa Morais (IMM, FMUL) and Dr. Carlos Duarte (DCV, UC).

This work was financed by FCT – Fundação para a Ciência e a Tecnologia.

Acknowledgements

I would like to thank to the people that have in some way helped me or supported me during this year.

First of all, I would like to start by thanking my supervisor, Dr. Vanessa Morais, for accepting me in her lab to do my dissertation. She always supported me from the beginning and tried her best to make me comfortable with my questions and concerns. In this atypical year, her calm and encouragement were very important to keep me focused on my work, and during all the ups and downs that happened along the way. To all my lab colleagues, for always being by my side in the busiest of the bench workdays, and for always having a kind word to say and a smile in any situation. Their constant humour and pleasure at work always made me enjoy going to the lab every day. A special thanks to Elvira and Filipa, for all the help during this year and for putting me at ease with my questions, being it science-related or not. Their support was very important and without it this year would not have been the same.

I also want to thank the important people in my life that in one way or the other contributed to this work. I want to start by thanking my mother, because without her this thesis would not have been possible. From very early on, she always made an effort towards my education and always supported my dreams and choices in life. She always knows the right thing to say in any situation, and her constant support is a driving force in my life. Thank you for believing in me. To my father, for always taking care of me and supporting me. To my brother, whose silliness and nonsense can cheer me up at any time. To Marcos, for being by my side constantly and for all his help during this year. From the start, he was always encouraging, and his presence gave me strength to continue. Thank you for being my safe harbour during the troublest times. To all my family, to which I will always be thankful for, for making me who I am today. To my friends, for sharing this incredible journey with me.

Science has no expiration date, that is one thing I learned from this year. Despite all the troubles that might happen along the way, as long as you work hard and give yourself to it, you are one step closer to making a difference.

Abstract

Parkinson's disease (PD) is a multifactorial progressive movement disorder, characterized by the degeneration of dopaminergic neurons from substantia *nigra* and by intracellular accumulation of protein aggregates termed Lewy bodies. While the exact etiology of this disease remains to be clarified, mitochondrial dysfunctions have been strongly implicated in its pathophysiology. Mitochondria are acknowledged as the "powerhouse of the cell" but also perform other critical cellular functions, making them extremely important for cell survival. Many mitochondrial genes have been implicated in PD, and among them mutations in the PTEN-induced putative kinase 1 (PINK1), a nuclear encoded mitochondrial targeted kinase, were already identified in patients with early-onset recessive PD. PINK1 plays a dual function in mitochondria, depending on its polarization state. When mitochondria are damaged, PINK1 interacts with Parkin, a cytosolic E3 ubiquitin ligase, to signal defective organelles for degradation. Conversely, under basal conditions, PINK1 is internalized and phosphorylates different proteins, namely the Complex I subunit NDUFA10, hence regulating the overall energetic balance of mitochondria. Currently, many PINK1 substrates have been identified; however, the mechanism underlying the PINK1 substrate selection remains to be understood. Moreover, it seems that none of these substrates can fully restore all PINK1 associated phenotypes. Therefore, the identification of novel PINK1 substrates will help to elucidate the mechanisms associated with PINK1 substrate selection and consequent maintenance of a healthy pool of mitochondria in neurons.

To achieve this, a 2D-DIGE electrophoresis was performed using isolated mitochondria from wildtype and PINK1 null cells. Differential protein spots were further analysed by LC/MS and a phosphoproteome was generated. After bioinformatic analysis, a list of top substrate candidates for PINK1 was obtained. In order to validate these candidate proteins as bonafide PINK1 substrates, immunoblot analysis and a cell-based phosphorylation assay were performed. From the phosphoproteomics screen analysis, five top candidate substrates were selected based on an *in-silico* protein analysis and literature revision. The choice of these five hits was based in the increasing evidences linking PINK1 to alternative mitochondrial pathways, namely lipid metabolism and electron shuttling to the mitochondrial respiratory chain. Western blot analysis revealed distinct expression profiles in WT and PINK1 KO cells, with most proteins having a decreased expression in the PINK1 null samples. Phos-Tag SDS PAGE analysis showed different phosphorylation patterns for some of the proteins, suggesting an interplay with PINK1.

The different protein expression profiles and altered phosphorylation status observed in PINK1 null cells highlight the link between PINK1 and those candidates. Although we did not perform the validation of the substrates or assessed their function in the PINK1-mediated pathways, these assays will help to uncover the molecular mechanisms linking these proteins with PINK1, additionally revealing the cross-talk between mitochondrial homeostasis and PINK1 function.
Our findings have identified possible novel PINK1 substrates and provided new insights on the intrinsic mechanisms that regulate mitochondrial function, particularly in the context of PD.

Although preliminary, they are a first step towards the validation of new PINK1 substrates that will ultimately contribute to the current knowledge of the PINK1-biology and development of novel therapeutic strategies for the treatment of human degenerative disorders.

Keywords: Parkinson's Disease, mitochondria, PINK1, substrate phosphorylation

Resumo

A doença de Parkinson (DP) é uma doença multifactorial progressiva associada ao movimento, que é caracterizada pela degeneração dos neurónios dopaminérgicos da *substantia nigra* e pela acumulação intracelular de agregados proteicos designados corpos de Lewy. Apesar da sua etiologia não ser totalmente conhecida, defeitos nas mitocôndrias já foram identificados como possíveis causas da doença. As mitocôndrias são geralmente conhecidas pela sua função na produção de energia, mas possuem outras funções importantes nas células, tornando-as essenciais para a sua sobrevivência. Diversos genes mitocondriais têm sido implicados nesta doença, e entre eles, mutações no gene da PINK1, uma cinase Ser/Thr que quando expressa no citosol é direcionada para a mitocôndria, já foram identificadas em pacientes com formas familiares recessivas da DP. A PINK1 apresenta diferentes funções neste organelo, dependendo do seu potencial membranar. Quando as mitocôndrias estão danificadas, a PINK1 interage com a Parkin (uma ligase de ubiquitina citosólica) para sinalizar estes organelos para degradação. Em condições basais, a PINK1 é internalizada neste organelo, onde medeia a fosforilação de diversas proteínas, incluindo a da subunidade NDUFA10 do complexo I da cadeia respiratória, regulando a produção de energia. Apesar de terem sido identificados vários substratos para a PINK1, não são ainda conhecidos os mecanismos associados à seleção desses substratos pela enzima. Para além disto, nenhum destes substratos parece conseguir restaurar todos os fenótipos associados a uma deficiência na PINK1. Assim, a identificação de novos substratos de fosforilação da PINK1 poderá elucidar os mecanismos associados a esta seleção e elucidar como é que uma população saudável de mitocôndrias é mantida nos neurónios.

De forma a clarificar estas questões, foram analisadas mitocôndrias isoladas de células wildtype e PINK1 knockout por eletroforese bidimensional, o que possibilitou a identificação de proteínas expressas de forma diferencial, as quais foram posteriormente analisadas por CL-EM. Após análise bioinformática, foi gerada uma lista de candidatos. Para validar estas proteínas como verdadeiros substratos da PINK1, foi efetuada uma análise dos respetivos níveis de expressão e do seu padrão de fosforilação. A partir da análise fosfoproteómica, e com base em revisão da literatura e estudo *in silico*, cinco candidatos foram selecionados. A escolha destes candidatos foi baseada nas evidências crescentes que relacionam a PINK1 com o metabolismo dos lípidos e o transporte de eletrões para a cadeia respiratória mitocondrial. A análise dos níveis de expressão das proteínas por western blot revelou diferenças nos seus níveis entre células wildtype e PINK1 knockout. Para além disso, foram observadas alterações nos padrões de fosforilação de algumas destas proteínas através da análise por eletroforese *Phos-Tag*.

As alterações nos padrões de expressão e estado de fosforilação das proteínas que foram observados na ausência da PINK1 sugerem uma interação entre estes substratos e a cinase. Apesar de não termos validado estes substratos ou avaliado a importância da sua função nas vias mediadas pela PINK1, estes ensaios revelar-se-ão úteis não só para elucidar os mecanismos

moleculares que relacionam estas proteínas com a cinase, mas também para clarificar a interação entre a homeostasia mitocondrial e a função da PINK1.

Os nossos resultados permitiram a identificação de possíveis novos substratos da PINK1 e forneceram pistas para explicar os mecanismos intrínsecos da regulação da função mitocondrial. Estes resultados preliminares são um passo importante para validar estas proteínas como verdadeiros substratos da PINK1, contribuindo para o conhecimento atual da biologia desta cinase e para o desenvolvimento de novas terapias para o tratamento de doenças neurodegenerativas humanas.

Palavras-chave: Doença de Parkinson, mitocôndria, PINK1, fosforilação de substratos

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Abbreviation List

ACAA2	3-ketoacyl-CoA thiolase
ACAD9	Acyl-CoA dehydrogenase member 9
Acetyl-Coa	Acetyl-Coenzyme A
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
ATP	Adenosine triphosphate
Bax	Bcl-2 Associated X protein
Bak	Bcl-2 homologous antagonist
Bcl-2	B-cell lymphoma 2 proteins
Ca^{2+}	Calcium ion
Cyt C	Cytochrome C
CO_2	Carbon dioxide
Complex I	NADPH-ubiquinone oxidoreductase
Complex II	Succinate dehydrogenase-ubiquinone oxidoreductase
Complex III	Ubiquinone-cytochrome c oxidoreductase
Complex IV	Cytochrome c oxidase
Complex V	ATP synthase
COMT	Catechol-O-methyl transferase
DHTKD1	2-oxoglutarate dehydrogenase E1
DIGE	Differential In-Gel Electrophoresis
DNA	Deoxyribonucleic acid
DJ-1	Daisuke-Junko-1
Drp1	Dynamin-Related Protein 1
ETC	Electron transport chain
ETF	Electron transfer flavoprotein
ETFA	Electron transfer flavoprotein subunit alfa
ER	Endoplasmatic reticulum
FAD	Flavin adenine dinucleotide
FADH2	Reduced flavin adenine dinucleotide

GABA	γ-aminobutyric acid
GTP	Guanosine-5'-triphosphate
HD	Huntington's Disease
HtrA2/Omi	High temperature requirement protein A2/Omi
IMM	Inner mitochondrial membrane
iPS	Induced pluripotent stem cells
KI	Kinase inactive
KO	Knock-out
L-DOPA	Levodopa
LC3	Microtubule-associated protein 1A/1B-light chain 3
LC/ MS	Liquid Chromatography/ Mass Spectrometry
LPP	Lambda Protein Phosphatase
LRRK2	Leucine-rich repeat kinase 2
mFAO	Mitochondrial fatty acid oxidation
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MAO-B	Monoamine oxidative B
MCIA	Mitochondrial Complex I assembly complex
MCU	Mitochondrial calcium uniporter
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MIP	Mitochondrial intermediate peptidase
Miro1	Mitochondrial Rho-GTPase 1
MOMP	Mitochondrial outer membrane permeabilization
MPP	Mitochondrial processing peptidase
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
MTS	Mitochondrial targeting sequence
Na^+	Sodium ion
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide

NCLX	Na^+/Ca^{2+} exchanger
NDUFA10	NADH:Ubiquinone oxidoreductase subunit A10
OGDHC	Oxoglutarate dehydrogenase complex
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
p38	Mitogen-activated protein kinase (MAPK)
p62	Sequestosome-1
PARL	Presenilin-associated rhomboid-like protein
PD	Parkinson's disease
PINK1	PTEN-induced putative kinase 1
PTEN	Peptide phosphatase and tensin homolog
Q	Ubiquinone coenzime
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SARDH	Sarcosine dehydrogenase
SDS-PAGE	Sulphate Polyacrylamide Gel Electrophoresis
SN	Substantia nigra
SNCA	α-synuclein
SNpc	Substantia nigra *pars compacta*
TBK1	Tank Binding Kinase 1
TCA	Tricarboxylic acid cycle
TNF	Tumour necrosis factor
TOM	Translocase of the outer membrane
TRAP1	TNF receptor associated protein 1
Ub	Ubiquitin
VDAC	Voltage dependent anion channel
WT	Wild-type
ΔΨm	Mitochondrial membrane potential

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Chapter 1
INTRODUCTION AND PROJECT AIM

1.1. Into Parkinson's Disease

Since its first clinical characterization by James Parkinson in 1817, the knowledge around Parkinson's disease (PD) has grown throughout the years, with many evidences contributing to the perception on this disorder, including its hallmarks and pathological mechanisms (Goetz, 2011).

PD is described as a progressive degenerative movement disorder affecting the nervous system, that is characterized by a wide variety of motor symptoms, such as bradykinesia, resting tremor, muscle rigidity, impaired posture and imbalance (Poewe et al., 2017). Although these symptoms are the most classical manifestations of PD, the majority of patients with the disorder also display non-motor symptoms comprising several different functions, including sleep alterations, autonomic dysfunction, cognitive impairment (that may include dementia, decreased frontal executive functions and memory deficits), sensory symptoms and pain (Stacy, 2011). It has been reported in some cases that these non-motor features can precede the onset of the classical symptoms by years, but they usually became more prevalent over the progression of the disease, considerably contributing to the overall disease picture.

The characteristic motor deficits observed in patients with PD are caused by a specific degeneration of the dopaminergic neurons from the substantia nigra *pars compacta* (SNpc) (Lang & Lozano, 1998a; Polito, Greco, & Seripa, 2016) **(Figure 1)**. However, the precise mechanism that explains the loss of these dopaminergic neurons remains to be clarified. The specific neurodegeneration in the SN neurons affects the thalamo-cortico-basal ganglia circuits, particularly hampering the dopaminergic transmission and decreasing the levels of this neurotransmitter in the dorsal striatum, which in turn diminishes the control over voluntary actions. The observed effects result from an increased inhibition of the thalamocortical and brainstem projections to the cortex by the γ-aminobutyric acid (GABA) output neurons, caused by a decreased D1-mediated direct pathway activity (which normally promotes movement through thalamus disinhibition) and an over-activation of the D2-mediated indirect pathway from basal ganglia, that inhibits the thalamus and suppresses movement (Mcgregor & Nelson, 2019). Even though macroscopic brain atrophy is not commonly observed in patients with this disorder, the specific neuronal loss is progressive and related with the diseases' stage. While in early stages of the disease the degeneration of dopaminergic neurons appears to specifically

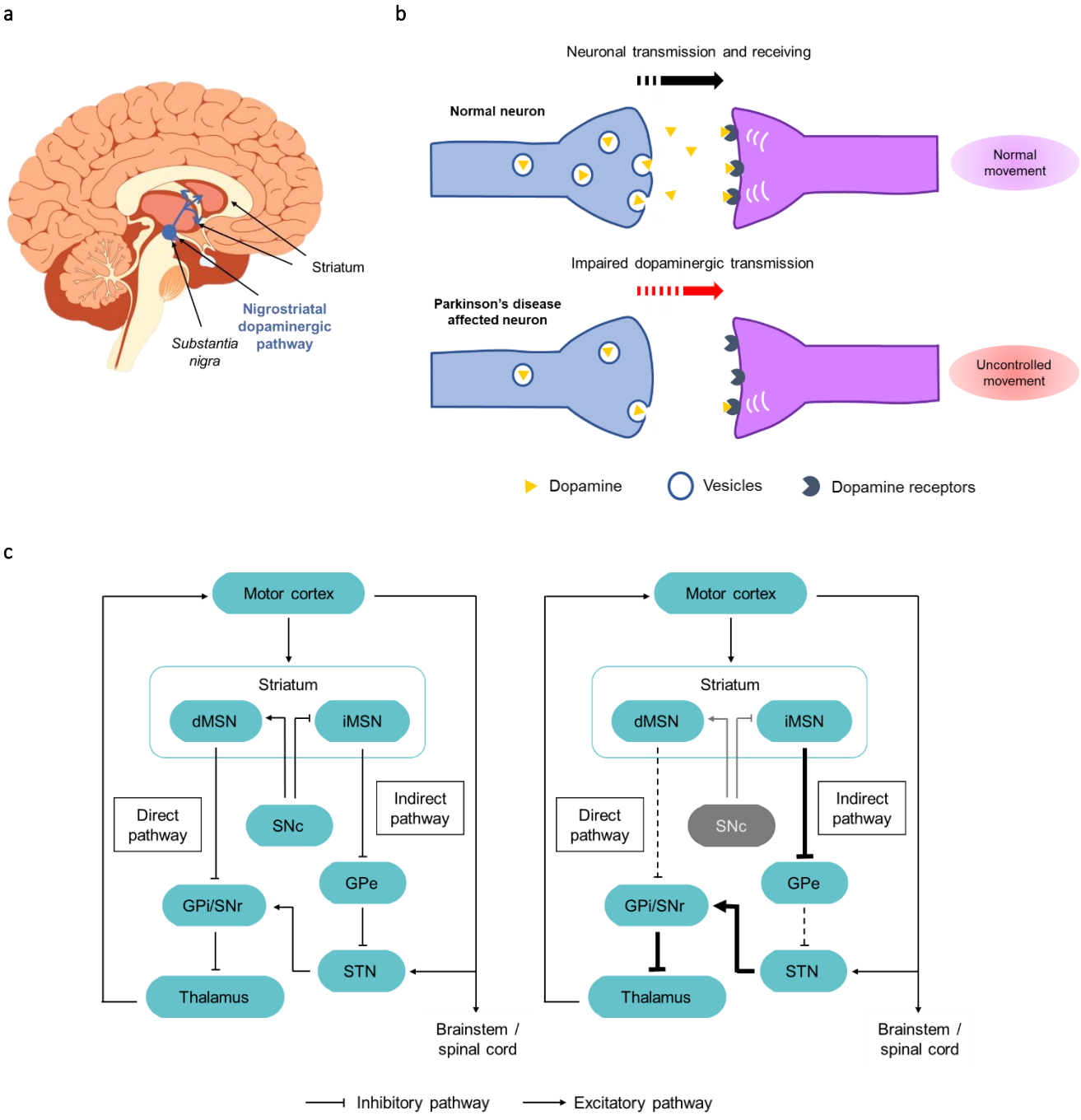

Figure 1: The dopaminergic neurotransmission is affected in Parkinson's disease.

(a) Dopaminergic neurons projecting from the substantia nigra to the striatum, particularly to the putamen, are a target for degeneration in Parkinson's disease. (b) This specific neurodegeneration of dopaminergic cells results in the release of low levels of the neurotransmitter in the striatum, impairing the thalamo-cortico-basal ganglia circuits that control and coordinate movement, and producing the characteristic motor symptoms of the disease. (c) In healthy conditions (left), the dopaminergic neurons from SN are projected to the striatum, where they simultaneously activate the direct pathway medium spiny neurons (dMSNs), while inhibiting the indirect pathway MSNs (iMSNs). The consequent effect of this dopaminergic signalling is the supression of the globus pallidus pars interna (GPi) output, which reduces the inhibitions on the thalamus and cortex, overall promoting movement. In PD (right), the dopaminergic neurons loss causes an imbalanced activity between the two pathways in the striatum, resulting in decreased activation of the direct pathway and increased activation of the indirect pathway. This imbalanced activity causes the over inhibition of the thalamus and cortex, ultimately impacting the body movement (adapted from Mcgregor & Nelson, 2019).

target the ventrolateral SN without affecting other brain regions and circuits (Damier, Hirsch, Agid, & Graybiel, 1999), by the end-stage of PD the neuronal loss becomes widespread along several brain regions. These findings support the idea that the dopaminergic neurons loss consists in an early event of the disease, possibly starting before the onset of the symptoms (Dijkstra et al., 2014).

Molecularly, PD is characterized by the intracellular deposition and accumulation of misfolded protein aggregates, termed Lewy bodies (Lang & Lozano, 1998b). These molecular structures constitute the hallmark lesions of the disease and have been appointed as one of the main mechanisms leading to the degeneration of dopaminergic neurons from the SN, since their accumulation and propagation can compromise neuronal function (Poewe *et al.*, 2017). Among the different proteins that made up these structures, misfolded α-synuclein (SNCA) aggregates were the first to be described and are recognized as one of its principal components (Braak et al., 2003). Moreover, there is evidence suggesting that SNCA may self-propagate throughout the nervous system and induce neuroinflammation, thus favouring the progression of PD (Recasens & Dehay, 2014) (Figure 2).

Being the second most common neurodegenerative disorder (only behind Alzheimer's' disease (AD)), PD is associated with a high socioeconomic and emotional burden to the patient and family members. Currently, there are different treatments available for PD, that delay the disease progression and/or ameliorate some of the diseases' symptoms; however, the current therapeutics are not completely efficient, only partially treating the diseases' manifestations (Maiti et al., 2017). Moreover, in some cases, the treatments are not sufficient and there is the need to perform coadjuvant therapies. The most commonly used treatments include dopaminergic drugs, such as Levodopa (L-DOPA) (Barbeau, 1969). Since dopamine is unable to cross the blood brain barrier, dopaminergic precursors are used to restore the dopamine levels in the striatum. The application of dopamine precursors is very effective in the reduction of the most predominant motor symptoms, but they are not able to prevent the degeneration of dopaminergic neurons from the SN (Foster & Hoffer, 2004). Other drugs such as monoamine oxidase B inhibitors (MAO-B inhibitors), catechol-O-methyl transferase (COMT) inhibitors and dopamine agonists, can also be used to prevent the rapid enzymatic breakdown of dopamine in the synapses and increase the available levels of the neurotransmitter in the brains of PD patients (Brooks, 2000; Krishna et al., 2014). In addition to these drug treatments, many other

types of therapeutics have been developed and are currently available for PD patients, such as non-dopaminergic drugs (Fox, 2013), immunotherapy (Masliah et al., 2005) and surgical procedures, namely deep brain stimulation (Groiss et al., 2009). These treatments can partially overcome the faults of dopaminergic drugs, efficiently diminishing the motor symptoms and targeting other pathological manifestations of the disease. Moreover, one of the most promising therapeutics for the disease is the transplantation of neuronal stem cells into brains of PD patients (Goodarzi et al., 2015).

Although different efforts have been made to develop new and better treatments for PD, at the moment there is no available cure for the disease. Furthermore, the existing treatments are only moderately efficacious or can only be applied to a small percentage of patients. Given that these therapies cannot totally restore the degenerated dopaminergic neurons, nor reverse or halt the disease progression, it is crucial to develop novel and more adequate treatments that can efficiently ameliorate the symptoms of the disease and prevent its rapid development.

Typically, PD can be related with two different forms, according to the time of onset of the disease and to the cause of the pathogenic manifestations. Generally, most PD patients exhibit a sporadic form of the disease, of unknown cause and late time of onset of the classical symptoms. In these cases, the time of onset of the motor symptoms is beyond 45 or 50 years old, and even though they are majorly idiopatic, different risk factors seem to contribute to the diseases' establishment and development. Among them, aging is the most predominant risk factor, since it can affect normal cellular functions (Reeve et al., 2014), including the oxidative stress, inflammation, mitochondrial function and autophagy. The hampering of all these molecular and cellular mechanisms is consistent with the ones observed in other well-known pathologies and has been strongly associated with a predisposition for the degeneration of the dopaminergic neurons from the SN, as well as with the development of PD (Pang et al., 2019). Exposure to specific environmental toxins present in pesticides and herbicides, bacterial and viral infections and illicit drugs are also known to contribute to the development of sporadic PD and can be partially mimicked in different experimental animal models. However, none of the environmental toxins that have been isolated for the development of animal models of PD seems to totally represent the different pathogenic aspects commonly observed in patients, lacking the classical motor symptoms or failing to recapitulate the pathogenic mechanisms of dopaminergic

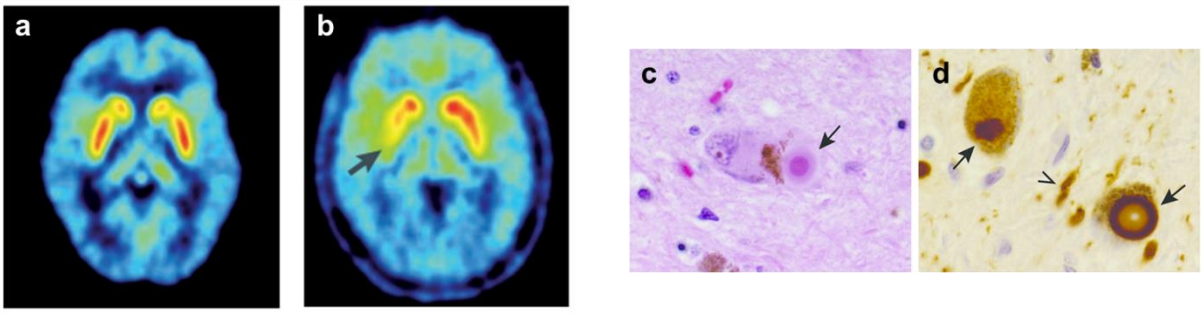

Figure 2: Brain imaging (PET scan) of dopaminergic projections to the striatum, assessed by 18fluorodopa (a, b), and Lewy body pathology in substantia nigra neurons affected with Parkinson's disease (c, d).

(a) PET scan from a healthy (control) subject, with high uptake of ^{18}fluorodopa in the striatum. (b) PET scan from a Parkinson's disease patient, showing a low striatal uptake of the radiotracer (Redgrave et al., 2010). (c) Standard hematoxylin and eosin staining of a eosinophilic and spherical Lewy body inclusion. (d) Lewy body inclusions with an immunohistochemical α-synuclein staining and an immunopositive α-synuclein neurite (Burdick & Leverenz, 2013).

neuronal loss (Bové et al., 2005). Lastly, a genetic predisposition has also been linked to sporadic PD (Calne & Langston, 1983), with several genes being identified as contributors for the development of the pathology.

Even though the sporadic cases of PD are more common, a smaller percentage of patients displays a familial form of the disease, which has a genetic-based phenotype. This form of the disease is commonly defined as a young-onset PD, since motor symptoms become prominent before 45 years of age. To date, several different genetic mutations have been linked to early-onset dominant and recessive forms of PD, and are thought to be directly associated with its pathophysiology (Singleton et al., 2013). One of the first genes to provide insights into the molecular genetics of PD was the α-synuclein (SNCA) gene. SNCA is a small presynaptic neuronal protein, specifically localized to the axon terminals, which has a role in neurotransmitter release and neuronal plasticity (Murphy, Rueter, Trojanowski, & Lee, 2000; Bendor, Logan, & Edwards, 2013). This protein has been genetically and neuropathologically associated to PD. The link between this gene and the disorder was first described in small unrelated families from Italy and Greece, which presented an autosomal dominant inheritance for the PD phenotype (Polymeropoulos et al., 1997). Later on, it was demonstrated that SNCA constitutes the principal component of Lewy bodies (Spillantini et al., 1998). Even though several mutations in the gene encoding for SNCA have been related with familial variants of PD, genetic studies have also linked mutations in this gene to sporadic forms (Stefanis, 2012).

Pathogenic alterations in the gene coding for the Leucine-rich repeat kinase 2 (LRRK2) have also been associated with both familiar and sporadic PD (J.-Q. Li et al., 2014). LRRK2 is a large widely expressed protein containing two important enzymatic domains, that are critical for its function: a kinase domain, that catalyzes phosphorylation, and a GTPase domain, which is involved in the hydrolysis of GTP (Rideout & Stefanis, 2014). Furthermore, the structure of this protein contains several protein-protein interaction regions, suggesting a function as a scaffolding protein that participates in the formation of signaling complexes. Even though the exact physiological functions of LRRK2 remained unclear during several years, studies with LRRK2 knock-out (KO) animal models and PD-associated mutants have identified different important cellular functions for this protein, such as vesicle trafficking (Piccoli et al., 2011), cytoskeletal upkeep (Gillardon, 2009; Kawakami et al., 2012), autophagic protein elimination and neurite outgrowth (Plowey et al., 2008).

Since these discoveries, other different genes and proteins involved in distinct cellular functions have been associated with PD. Particularly, loss-of-function mutations in genes such as Parkin, PTEN-induced putative kinase 1 (PINK1) and Daisuke-Junko-1 (DJ-1) have been identified as putative causes of some rare forms of early-onset PD (Vincenzo Bonifati, 2012). Alterations in the Parkin gene account for the most frequent cause of autosomal recessive PD, and a wide variety of pathogenic mutations have already been identified. Mutations in the PINK1 and DJ-1 genes are less common, but there is evidence suggesting they still play an important role in the development of the disorder in these cases.

DJ-1 is a ubiquitously expressed, small and highly conserved protein, that is encoded by the PARK7 gene. Although different physiological roles for this protein have already been determined in cells, including protein folding and protein cleavage (Olzmann et al., 2004; Shendelman et al., 2004), it is commonly accepted that DJ-1 has a neuroprotective role against oxidative stress (Batelli et al., 2015; R. H. Kim et al., 2005; Ottolini et al., 2013; Taira et al., 2004). Evidences have also linked this protein with mitochondria, since a DJ-1 loss-of-function was shown to alter the organelles polarization state and increased mitochondria fragmentation (Thomas et al., 2011). Different pathological variants of the PARK7 gene have been discovered and associated with both DJ-1 protein loss-of-function and parkinsonism. Comparative studies identified differential expression of the PARK7 gene in post-mortem PD and control brain regions, with decreased DJ-1 mRNA and protein levels in PD brains versus controls (Kumaran et

al., 2009). This study also revealed modifications of the oxidative state of the protein in patients brains, since PD samples presented higher amounts of acidic isoforms of the protein in specific brain regions, suggesting that an impairment of the neuroprotective role of DJ-1 could contribute to the pathogenesis of the disease.

Parkin is a cytosolic E3 ubiquitin ligase protein, encoded by the PARK2 gene, whose main role in cells is related with the maintenance of the quality control and turnover of mitochondria. Functioning as an ubiquitin ligase, Parkin ubiquitinates various cytosolic and non-cytosolic proteins for degradation by the ubiquitin proteasome system (Kahle & Haass, 2004; Shimura et al., 2000). Through the signaling of specific mitochondrial membrane proteins, Parkin tags defective organelles for elimination through selective autophagy. Studies using loss-of-function Parkin models have demonstrated the importance of the protein in the maintenance of mitochondria homeostasis: Parkin-mutant *Drosophila* flies display not only fewer organelles, but also swollen mitochondria with significant cristae disintegration (Greene et al., 2003; Pesah et al., 2004); Parkin KO mice did not show any morphological alterations in mitochondria but exhibited decreased mitochondrial respiratory function and increased tissue damaged induced by reactive oxygen species (ROS) (Palacino et al., 2004). A number of PARK2 genetic mutations has been linked with different forms of early onset autosomal recessive PD (Lucking et al., 2000; Periquet et al., 2003), although alterations in this proteins gene have also been suggested to play a role in sporadic forms of the disease (Kay et al., 2010). The activity of Parkin is modulated, among others, by phosphorylation by PINK1, that by being of great importance for this thesis, will be later discussed.

1.2. Link between Parkinson's Disease and mitochondria

Currently, PD is broadly recognized as a multifactorial disease with both genetic and environmental contributions (Cannon & Greenamyre, 2011; Klein & Westenberger, 2012). Furthermore, different molecular and biochemical mechanisms, that were already identified and linked with PD and its pathophysiology, have been pointed out as possible causes for the development of the disease.

Many evidences (Orth & Schapira, 2001; Reddy, 2009) have suggested the importance of mitochondria in neuronal damage and disease. Particularly, mitochondrial dysfunction appears

to have a role in the establishment and development of both sporadic and familial PD (Hauser & Hastings, 2013; Park, Davis, & Sue, 2018).

For the sporadic PD, different endogenous and exogenous inhibitors of the mitochondrial function have been identified. One of the first evidences pointing out to the association between toxic agents, mitochondria and high risk of developing PD was obtained through exposure to 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) present in illicit drugs (Ballard et al., 1985), and by studies on the pathogenic mechanisms of this compound. After crossing the blood brain barrier, MPTP enters glial cells and is metabolized into the toxic metabolite MPP+. MPP+ is then integrated in dopaminergic neurons from the SN through dopamine transporters and selectively targets the Complex I (NADPH-ubiquinone oxidoreductase) of mitochondria, inhibiting its activity and rupturing the oxidative phosphorylation (OXPHOS). This specific disruption of the mitochondrial electron transport chain (ETC) broadly affects the organelles functions, impairing adenosine triphosphate (ATP) production and increasing the oxidative stress, which results in neuronal damage and ultimately causes cell death and parkinsonism (Tipton & Singer, 1993; Vyas et al., 1986). Given the characteristics of the drug, MPTP is commonly used to obtain severe and persisting parkinsonian phenotypes in different animal models for the study of the molecular mechanisms of PD (Jackson-Lewis & Przedborski, 2007).

Different environmental toxic compounds present in pesticides and herbicides, such as paraquat and rotenone, were also identified and linked to parkinsonism (Tanner et al., 2011), with evidences suggesting that rural populations exposed to some agricultural products had an increased risk for developing sporadic PD (Pezzoli & Cereda, 2013). Studies on the molecular mechanisms of these toxic compounds demonstrated a specific targeting of the mitochondria in cells, with inhibition of the mitochondrial respiratory chain (particularly of Complex I) and increased production of ROS, resulting in activation of different apoptotic pathways (Castello et al., 2007; N. Li et al., 2003). It is also possible to produce animal models of the disease from the exposure to these different toxic agents (Bové et al., 2005).

Several clinical studies, mostly related with the discovery of gene mutations in inherited forms of PD, also demonstrated that 3-5% of sporadic cases of the disease have a genetic base (Klein & Westenberger, 2012), leading to the assumption that some mutations (namely in genes related with mitochondria) may increase the susceptibility for developing sporadic PD (Gandhi & Plun-Favreau, 2017). In addition, it is already known that aging plays an important role in

mitochondrial dysfunction, given the great amount of evidences linking it with loss of mitochondrial DNA (mtDNA) integrity, accumulation of mtDNA mutations, increased ROS production and respiratory deficiency (Bender et al., 2006; Kraytsberg et al., 2006). Analysis of *postmortem* brains from aged subjects and idiopatic PD patients showed an increased amount of mtDNA mutations in neurons (Bender et al., 2006). Furthermore, these high amounts of mtDNA alterations were linked to a declined enzymatic activity of Complex IV (Cytochrome c Oxidase) of mitochondria, as well as to an increase in the production of ROS.

Further evidences suggested that in some early-onset familial forms of PD, several of the already identified genes that caused a parkinsonian phenotype were linked with the mitochondria, since different pathogenic mutations were associated to both disease and mitochondrial dysfunction (Lill, 2016; Park *et al.*, 2018), as it was previously discussed. Such genes appear to be related with different important functions of the organelle, such as calcium (Ca^{2+}) buffering, energy production/metabolism and cell death, but also with its dynamics, biogenesis and trafficking in cells (Schapira, 2007; Park *et al*., 2018). Among the different genes that have been identified as causes of PD, mutations in the PINK1 gene have been greatly studied given their relevance to the mitochondrial functions and to its contribution to PD (Valente et al., 2004).

All these findings suggest that mitochondria are a target organelle in PD, with mitochondrial alterations being recognized as a main pathogenic event resulting in the different manifestations of the disease. Given all the evidences that point out to the role of mitochondria in the mechanisms leading to cellular dysfunction and neuronal loss, the functions that these organelles play in cells, and in particular, in neurons, must be critical for the normal cellular activities.

1.3. Importance of mitochondria in cells

The importance of mitochondria within cells is well-known, given its role in the regulation of cell homeostasis and survival. Besides participating in the bioenergetics and metabolism of cells (Mitchell, 1961), mitochondria also have a part in other critical cell functions, namely in the redox reaction regulation and production of oxidative stress molecules (Lee et al., 1964), ion homeostasis (Azzone & Massari, 1973) and apoptosis (Kroemer et al., 2007). Moreover, the mitochondrial function is intrinsically regulated by different quality control pathways, that assure

the normal activities of the organelle. Therefore, the mechanisms that are responsible for the regulation of the intrinsic mitochondrial activity command cell survival and fate.

Even though mammalian cells rely on mitochondria for their overall activity, brain cells are particularly dependent on these organelles' activity for their function and survival. Being a high energy demanding organ, the brain requires great amounts of energy to fuel the intracellular energy-dependent mechanisms and maintain all its critical functions namely brain plasticity and synaptic activity. The overall mitochondrial functions seem to be particularly important for neurons, given all their characteristic morphological and molecular features. Neurons are long and highly differentiated cells, whose function greatly relies on the energetic and oxidative metabolism to perform critical tasks such as synaptic transmission and neuronal communication, but also to perform other functions, namely to maintain the neuronal plasticity and establish membrane excitability (Schon & Manfredi, 2003; Kann & Kovács, 2007). Being very unique cells, neurons are segmented into different parts (dendrites, soma, axon and pre-synapse), each one with different energetic demands. Because of this difference, there needs to exist a constant adaptation of the energy fueling as well as a dynamic interconnection between cell and mitochondria, to assure all neuronal activities, which can only be achieved by the maintenance of a healthy pool of mitochondria within the cells.

Mitochondria are very dynamic intracellular membrane enclosed organelles, that play complex functions in the majority of eukaryotic cells (Perier & Vila, 2012). Structurally, mitochondria are organized in four different compartments, which are crucial for the organelles morphology and function: the outer mitochondrial membrane (OMM), the inner mitochondrial membrane (IMM), the intermembrane space and the mitochondrial matrix (located inside the IMM). The IMM is very well folded and forms protrusions called mitochondrial cristae into the matrix, which largely increase the surface area of the inner membrane. Furthermore, is in these invaginations of the IMM that the ETC of the mitochondria is located. Aside from the nucleus, mitochondria are the only organelles in mammalian cells that carry DNA (mtDNA), as well as their own RNA and protein synthesis machinery. Inside each mitochondrial matrix, there are manifold copies of the small circular mitochondrial genome, encoding for 13 mitochondrial proteins. Since all of these proteins are a part of the OXPHOS system, responsible for the energetic metabolism of cells, the proteins that are required for the maintenance of a functional organelle are encoded in the nuclear DNA. Typically, after being synthesized in the cytosol, these

proteins are imported to the organelle and targeted to one of the mitochondrial compartments through a mitochondrial targeting sequence present in their gene sequence.

Overall, mitochondria are recognized for their crucial function in the energetic metabolism of cells. To obtain the necessary energy to execute all their functions, cells rely on multiple mechanisms, such as the tricarboxylic acid (TCA) cycle (an important pathway in which the acetyl-CoAs' acetyl group is oxidized to CO_2, and the electron transporting enzymes NAD^+ and FAD are reduced), pyruvate oxidation and in the metabolism of different fuel molecules. Among these, glucose is the preferred energy source, but other molecules can also be used to fuel cells, namely amino acids and fatty acids. Such pathways are important since they give rise to different molecules that are necessary for the generation of ATP as an energy source. To perform this function, mitochondria rely on the ETC, which integrates the OXPHOS system, present in the IMM (Figure 3). The OXPHOS chain comprises five enzymatic complexes with multiple subunits: Complex I, Complex II (succinate dehydrogenase-ubiquinone oxidoreductase), Complex III (ubiquinone-cytochrome c oxidoreductase), Complex IV and the enzyme ATP synthase (also denominated Complex V). Aside from these complexes, the electron carriers ubiquinone (or coenzyme Q) and cytochrome c are necessary for the OXPHOS system to work. To produce ATP, electrons obtained from energetic substrates such as NADH and FADH2 are transported along the different enzymatic complexes of the ETC and transferred to an oxygen molecule, resulting in the generation of water. Simultaneously, hydrogen protons are pumped from the matrix to the intermembrane space, across the IMM, by Complexes I, III and IV, which generates an electrochemical gradient (also designated mitochondrial membrane potential ($\Delta\Psi m$)) through a series of oxidation-reduction reactions. The influx of these protons again to the mitochondrial matrix via the ATP synthase generates ATP, which can then be exported to the cytosol and used by the cell as energy (Perier & Vila, 2012). The normal mitochondrial respiration also produces different ROS, that if accumulated can compromise the basal cellular activities and promote excitotoxicity or cell damage, resulting in cell death.

In addition to these functions, mitochondria are responsible for the regulation of the intracellular Ca^{2+} homeostasis (Vandecasteele et al., 2001). Ca^{2+} homeostasis is of particular importance in neurons since this ion serves as a major regulator of enzymes (such as kinases, phosphatases and proteases), ion channels and transcription factors.

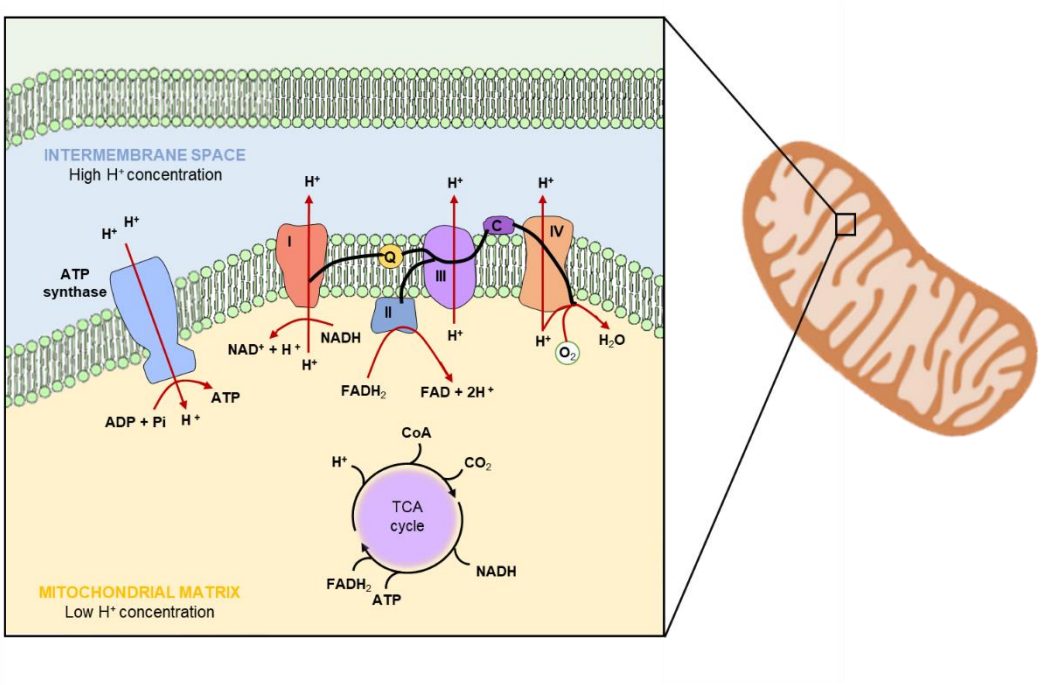

Figure 3: Electron transport chain (ETC) and tricarboxylic acid cycle (TCA).

The production of ATP by mitochondria is performed by the oxidative phosphorylation system (ETC) present in the inner membrane of the organelle. This system is formed by five enzymatic complexes (complexes I to V) and require the electron carriers ubiquinone (Q) and cytochrome c (C). The production of energy results from a series of redox reactions, in which electrons are transferred by the enzymatic complexes from different donors (such as NADH and $FADH_2$) to electron acceptors. Simultaneously, protons are pumped across the inner mitochondrial membrane from the matrix, which produces an electrochemical proton gradient (H^+) that is used by the ATP synthase (Complex V) to generate ATP. The TCA supplies the ETC with the electron donors NADH and $FADH_2$ and is important in the production of other intermediates and overall energetic metabolism of cells.

Additionally, Ca^{2+} functions as an intracellular messenger for several different mechanisms, including exocytosis, vesicle trafficking and metabolism, and participates in specific cellular pathways such as membrane excitability and apoptosis (Carafoli et al., 2001), all of these essential for the neuronal activity. To regulate Ca^{2+} homeostasis, mitochondria transiently contact with the endoplasmatic reticulum (ER) of cells (which constitutes the main intracellular Ca^{2+} storage deposit), allowing for the transfer of the ion to mitochondria. After being released from the ER, Ca^{2+} crosses the OMM through the voltage dependent anion channel (VDAC) and then reaches the matrix through the mitochondrial calcium uniporter (MCU) present in the IMM (Baughman et al., 2011; Stefani et al., 2012), therefore regulating its intracellular levels. However, there is evidence also suggesting that the Ca^{2+} imported to the mitochondrial matrix regulates the inner organelles metabolism and other activities, such as cell death (Duchen, 2000).

Mitochondria also participate in the regulation of cell death through several different molecular mechanisms (Tait & Green, 2013), that can be activated through diverse stimuli, such as DNA damage or radiation. Studies performed in mammalian animal models demonstrated that mitochondria can regulate caspase activation and consequently cell death, through a pathway termed mitochondrial outer membrane permeabilization (MOMP) (Lindsten et al., 2000). This specific mitochondrial permeabilization results in the release of multiple intermembrane space proteins that activate the proteases, thereby resulting in cell death. Other intervenients of the mitochondrial induced-apoptosis are the B-cell lymphoma 2 (Bcl-2) family of proteins, whose interaction between members, particularly of the BH3-only proteins with other Bcl-2 peptides, promotes cell death by the immediate activation of Bax and Bak proteins (that directly mediate this process) or by inhibition of various anti-apoptotic proteins (Shamas-din et al., 2011). Furthermore, there is evidence demonstrating that the permeabilization of the OMM is regulated by this Bcl-2 family of proteins (Youle & Strasser, 2008). Other apoptotic mechanism mediated by mitochondria is related with the release of the cytochrome c to the cytosol, in response to a loss of integrity of the OMM (Green, 2000), which activates the caspase cascade and induces apoptosis.

Being highly dynamic organelles, mitochondria can undergo different processes that overall ensure an appropriate mitochondrial activity. Theses mechanisms (also termed mitochondrial quality control pathways) are necessary for the mitochondria to actively move in cells, to fuse and divide, and to be recycled at the proper moments. A balance between these processes is crucial for maintaining a normal mitochondrial function and the loss of the organelles dynamics can not only compromise the general cell activity, but also cause neuronal damage, ultimately contributing to disease states.

Fusion and fission events regulate the general mitochondrial morphology, controlling features such as length, size, shape and also the amount of organelles in cells (Chan, 2006). Mitochondria typically form ramified tubular networks in cells, and in normal conditions, the cycles of fusion and fission events are equilibrated; however, under both experimental and disease conditions, alterations can occur that affect the organelles morphology: while cells with an increased fusion-to-fission ratio have fewer, more elongated and interlinked mitochondria, a low ratio results in higher amount of organelles, with a round structure and fragmented (Bleazard et al., 1999; H. Chen et al., 2003). Moreover, different molecular intervenients regulate

mitochondrial division, such as the GTPase Dynamin-Related Protein 1 (Drp1) (Ugarte-Uribe et al., 2014), and Mitofusins 1 and 2 (Mfn1 and Mfn2).

Mitochondria can also be transported throughout the cell and have specific subcellular distributions. In mammalian cells, the overall organelles trafficking is dependent on the actin and microtubule cytoskeleton; however, in neurons, the mitochondrial motility seems to be very well regulated to attend to the exquisite cellular morphology of these cells (R L Morris & Hollenbeck, 1995). Mitochondrial transport in axons is dependent on cytoskeletal motor proteins (kinesin-1 and dynein), that coordinate the bidirectional movement of the organelles along the microtubules and mediate its distribution in the cell. Different studies suggest that mitochondria are recruited to high energy demand cellular domains, that require local ATP production for intracellular activities, such as neuronal growth and synaptic communication (Chang et al., 2006; Robert L Morris & Hollenbeck, 1993). Moreover, the ΔΨm can help in the distribution of mitochondria throughout the cell, since organelles with a high membrane potential tend to migrate towards the axon terminal (in anterograde mode), while mitochondria with a lower membrane potential are transported in a retrograde manner to the cell soma (Miller & Sheetz, 2004). Studies in *Drosophila melanogaster* have also demonstrated that the mitochondrial Rho-GTPase 1 (Miro1) and the Milton motor-adaptor protein are important for the axonal transport of mitochondria, since mutations in the fly gene' variant exhibit an abnormal distribution of organelles in cells, with an accumulation of mitochondria in the neuronal soma and a decreased translocation to axons and dendrites (Guo et al., 2005), as well as an abnormal synaptic activity (Stowers et al., 2002). All these evidences suggest that the migration patterns of mitochondria within cells are very well regulated, possibly by specific molecular signaling pathways, and that these dictate the subcellular location of the organelles and its turnover according to the cellular requirements.

Other important process responsible for the turnover of mitochondria in the cells is the selective removal of dysfunctional organelles through autophagosome-mediated autophagy, or mitophagy (I. Kim et al., 2009). In this pathway, defective mitochondria are targeted and sequestered by double-membrane-enclosed structures (autophagosomes), which later fuse with lysosomes and are enzymatically degraded. Mitophagy is commonly triggered by functional modifications in the organelle, such as alterations in the ΔΨm or permeabilization of the OMM, and the activation of this autophagic pathway probably acts to narrow potential noxious effects

in cells derived from the accumulation of defective organelles, namely enhanced production of ROS or increased release of pro-apoptotic factors (Tait & Green, 2010).

Since mitochondria are so important for the regulation and maintenance of cell homeostasis, an alteration of any of the organelles functions would have catastrophic consequences that could affect the cellular fate. One of the primary effects of mitochondrial disruption in neurons would be an impairment of the general ATP production at the OXPHOS chain, causing an overall bioenergetic failure in the cell, that could be insufficiently compensated by the glycolytic ATP synthesis (Ankarcrona et al., 1995). Apart from compromising the normal ATP production, mitochondrial dysfunction could also impair other "downstream" cellular functions, with a severe outcome in neurons (Schon & Manfredi, 2003; Park *et al.*, 2018). An alteration in the OXPHOS chain would lead to a major production of ROS, which could build up in cells and exert different toxic effects, ultimately causing neuronal damage and degeneration. Mitochondrial dysfunction could also compromise the quality-control pathways that maintain a healthy pool of mitochondria in cells, which would further contribute to the accumulation of damaged organelles and further enhance this effect. Moreover, given that neurons are highly excitable cells, a disturbance of the mitochondrial function would also cause an inadequate Ca^{2+} sequestration that would not only impact the normal ion cycle and therefore disrupt the Ca^{2+}-dependent signaling pathways and enzymatic functions, but also result in an intracellular Ca^{2+} overload that could be harmful to the cell (Wolf et al., 2001), overall impacting the neuronal activity. Globally, an impairment in the mitochondrial function would result in an overall imbalance between the healthy and damaged organelle populations within cells, negatively impacting the molecular and cellular pathways that contribute to the regulation and maintenance of the neuronal functions. Since neurons deeply rely on mitochondria to thrive and establish neuronal networks, an alteration of the mitochondrial processes would result in cell degeneration and apoptosis, ultimately reducing neuronal communication and causing brain damage. Among the many evidences pointing out to the neuronal reliance over mitochondrial function is the underlying mitochondrial dysfunction etiology of several neurodegenerative disorders, such as PD, AD, Huntington's Disease (HD), Amyotrophic Lateral Sclerosis (ALS), among others (Johri & Beal, 2012). Aside from this, multiple *in vitro* and *in vivo* studies linking mutations in several proteins involved in mitochondrial function and morphology, have

demonstrated the importance of quality and functional mitochondria in neuronal maintenance and survival (V. Bonifati et al., 2003; Cherra III et al., 2013; R. K. Dagda et al., 2011).

Given all the evidences that clearly highlight the importance of mitochondrial functions in neurons, as well as the link between this organelles' impairment and neurodegeneration, it is critical to study the molecular pathways that regulate the activities of this organelle in cells, particularly in neurons. Thus, a better understanding of the processes involved in the maintenance of a healthy pool of mitochondria in cells can potentially lead to improved treatments of several brain diseases.

1.4. Role of PINK1 in mitochondria and Parkinson's Disease

The first evidence linking PINK1 to PD came from the identification of a novel *locus* for an autosomal recessive early-onset form of parkinsonism (Valente et al., 2001). This new gene, located in the short arm of chromosome 1, was detected in a large Italian family whose members had a specific PARK6 locus mutation and possessed clinical symptoms identical to those observed in sporadic PD patients (Valente, Brancati, Caputo, et al., 2002; Valente, Brancati, Ferraris, et al., 2002). Later on it was discovered that the PARK6 gene comprised 8 exons and encoded the 581-residue peptide phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) (Valente et al., 2004), whose name derived from its first description as a ubiquitous protein with suppressed expression due to increased PTEN signaling in cells from cancerous tumors (Unoki & Nakamura, 2001).

PINK1 is a serine/threonine kinase protein that structurally comprises multiple protein domains, including an N-terminal mitochondrial sequence, a transmembrane helix, a serine/threonine kinase domain and a C-terminal region (Trempe & Fon, 2013) (Figure 4). The presence of a mitochondrial targeting sequence at the N terminus of the protein suggested a translocation of this kinase to the mitochondria after expression in the cytosol, which was also compatible with the subcellular location of the protein in the organelle. Moreover, this specific localization supported previous studies on the possible role of PINK1 in the mitochondrial maintenance, as well as the importance of mitochondrial dysfunction for the development of PD (Valente et al., 2004).

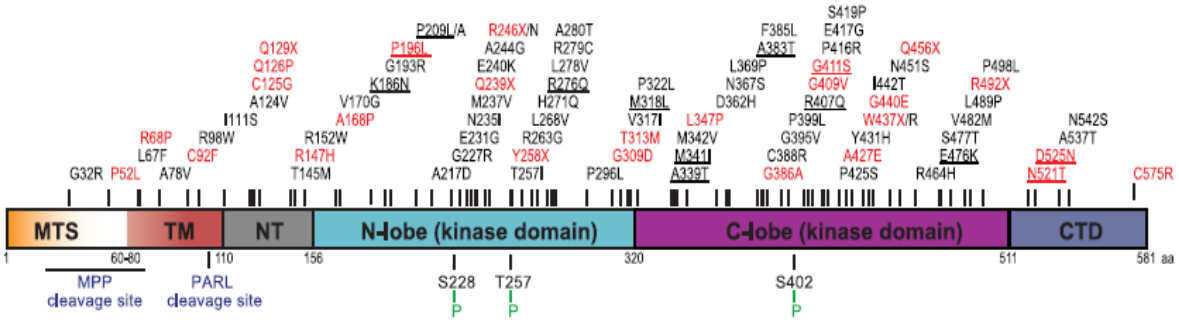

Figure 4: PTEN-induced putative kinase 1 (PINK1) gene structure and mutations linked to autosomal recessive familial forms of parkinsonism.

The PINK1 gene is comprised of different domains: mitochondrial targeting sequence (MTS; in orange), transmembrane region (TM; in red), N-terminal regulatory domain (NT; in gray), N-lobe and C-lobe of the kinase domain (cyan and purple, respectively), and the C-terminal region (CTD; blue). Various PD-associated mutations are shown above the respective domain where they are localized. Represented in black are different gene variants whose impairment has not been linked with a pathogenic PINK1 loss-of-function, while the red mutants have been established experimentally as pathogenic mutations (Truban et al., 2017).

The functions that PINK1 plays in cells, particularly in mitochondria, are dependent on its kinase activity. A downregulation of PINK1 seems to affect several important functions of the organelle, with different mutations in the PINK1 gene (specially in its kinase domain) being identified and linked with early-onset inheritable forms of PD (Ibánez et al., 2006). Furthermore, the importance of this kinase in the maintenance of the organelles homeostasis has been highlighted by several studies on different *in vivo* and *in vitro* models. It has been shown that a PINK1 knockdown impairs the function of the mitochondrial Na^{+}/Ca^{2+} exchanger (NCLX), resulting in a decreased Ca^{2+} removal from the cell (Kostic et al., 2015), and that in HeLa cells transfected with RNA interference to downregulate PINK1, mitochondria had an altered membrane potential and defective morphology (Exner et al., 2007).

Different evidences also point out that PINK1 loss-of-function can impair the respiratory chain. In a study using PINK1 knockout mouse embryonic fibroblasts, the authors showed an overall decrease in the enzymatic activity of the four complexes of the oxidative chain, with a greater impact in both complexes I and III (Amo et al., 2014). Another study determined that both PINK1 knockout cells and induced pluripotent stem (iPS) cells derived from PD patients with PINK1 mutations had a decreased activity of enzymatic Complex I, and that a loss of phosphorylation of a specific residue in the Complex I subunit NDUFA10 could be at the base of this decreased activity in PINK1 knockout mice (Morais et al., 2014). Apart from this, PINK1 also

seems to have a role in the regulation of important features of mitochondrial biology. Different *Drosophila* and mammalian cell models have documented that loss of PINK1 severely impacts different aspects of the mitochondrial function and dynamics, including trafficking in cells (Das Banerjee et al., 2017), modulation of fusion/fission events (Y. Yang et al., 2008) and mitochondrial cristae architecture (Ruben K. Dagda et al., 2009; Exner et al., 2007).

Indeed, PINK1 is associated with the phosphorylation of several substrates, and so, it regulates different molecular pathways and mitochondrial processes (Aerts, Strooper, et al., 2015). To achieve all these important functions, the kinases' activity needs to be very well regulated by multiple mechanisms, that dictate its localization, transport and turnover in cells (Figure 5). Interestingly, the function that PINK1 plays within cells and in mitochondria appears to be dependent on the mitochondrial polarization state, but the mechanisms leading to the activation of this kinase remain to be clarified. When mitochondria are depolarized (in other words, when mitochondria are damaged or unhealthy), PINK1 acts as a mediator of mitophagy, removing defective organelles from the cells through a selective autophagic pathway (Pickrell & Youle, 2015). This enzyme acts together with Parkin, a cytosolic E3 ubiquitin ligase, that has also been associated to some familial forms of parkinsonism (Geisler et al., 2010). Studies have demonstrated that both PINK1 and Parkin participate in the same mitophagic pathway, with Parkin acting downstream of PINK1, since an overexpression of Parkin is able to rescue the phenotype induced by a PINK1 downregulation in *Drosophila* models (Y. Yang et al., 2006). Furthermore, other studies also performed in *Drosophila* models with either Parkin or PINK1 mutants demonstrated that the loss-of-function of these proteins resulted in a typical parkinsonian phenotype and suggested that mitochondrial dysfunction was the primary cause of this alteration, since ultrastructural analysis of the flight muscles revealed an abnormal organelle morphology (Greene *et al.*, 2003; J. Park *et al.*, 2006). When the ΔΨm is decreased, PINK1 rapidly accumulates in the OMM and immobilizes the organelle by phosphorylation of the protein transporter Miro (Wang et al., 2011). Parkin is then activated by PINK1 through phosphorylation and recruited to the depolarized mitochondria, where it ubiquitinates specific OMM proteins for signaling, such as the guanosine triphosphatase Mfn1 and Mfn2 (Gegg et al., 2010). Mfn2 has also been demonstrated to be phosphorylated by PINK1 and to act as a receptor for Parkin, mediating its recruitment (Y. Chen & Dorn, 2013). The autophagosomal protein microtubule-associated protein light chain 3 (LC3) then binds together with the polyubiquitin-

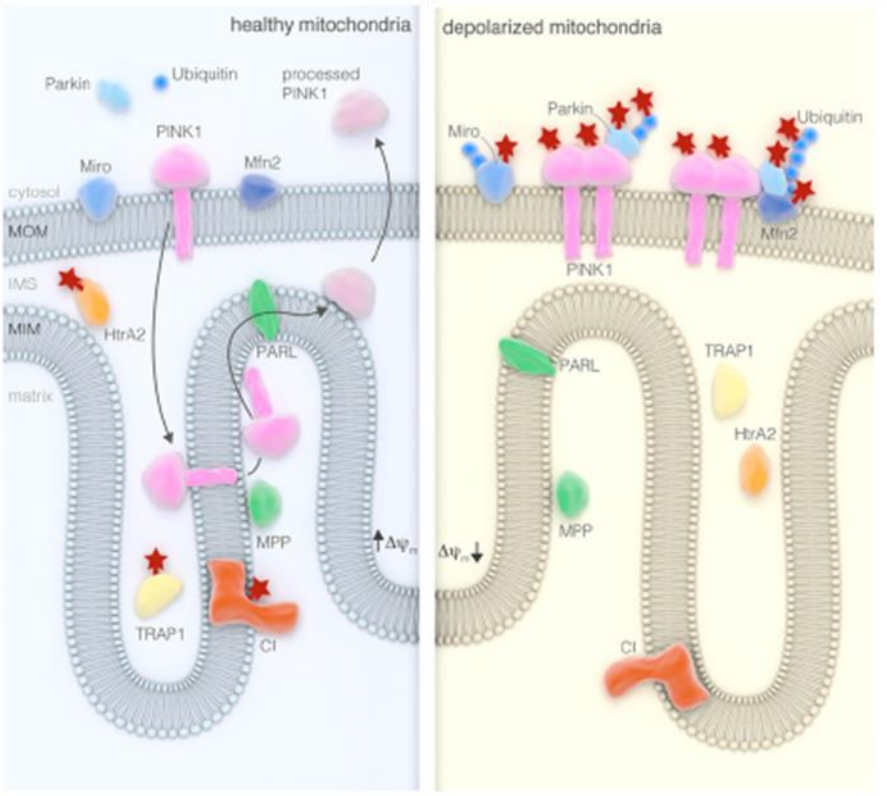

Figure 5: PINK1 substrates and function in healthy and depolarized mitochondria.

When the mitochondrial membrane is polarized, PINK1 is imported to the organelle and phosphorylates different substrates such as TRAP1, HtrA2 (Omi) and the Complex I subunit NDUFA10 (left panel). Moreover, PINK1 can undergo several proteolytical cleavages by the MPP and PARL proteases, and the processed kinase is re-translocated to the cytosol, where it can be degraded by the proteasome or it can coordinate other cellular functions. When the membrane potential is low (right panel), PINK1 is rapidly accumulated in the mitochondrial outer membrane (MOM), and phosphorylates different substrates such as Miro, Mfn2 and Parkin, signaling the defective organelle for degradation (Aerts, Strooper, et al., 2015).

binding protein p62, resulting in the degradation of the dysfunctional organelle, via engulfment by autophagosomes and later fusion with lysosomes (J.-S. Park et al., 2018) **(Figure 6)**. More recently, the PINK1-Parkin mediated autophagy pathway was suggested to influence the cell cycle progression through the activation and sequestration of the Tank Binding Kinase 1 (TBK1) during mitophagy, supporting an interplay between the mitochondrial quality control pathways and the maintenance of cellular homeostasis (Sarraf et al., 2019).

Activation of Parkin by PINK1 is typically mediated by its' kinase domain, that directly phosphorylates the S65 residue of the ubiquitin ligase leading to its translocation to mitochondria. However, this process can also be achieved by trans-activation through coupling to Parkin following phosphorylation of ubiquitin at the residue S65 (Koyano et al., 2014).

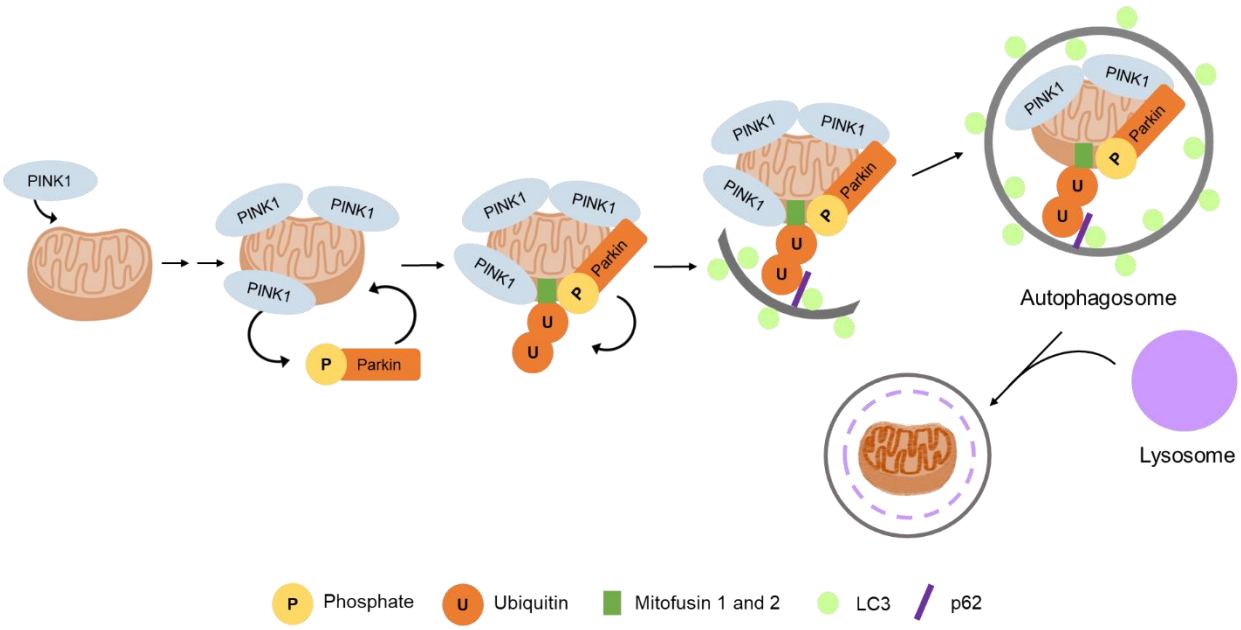

Figure 6: PINK1 mediates the degradation of dysfunctional mitochondria through selective autophagosomal-mediated autophagy (mitophagy).

When the mitochondrial membrane is depolarized, PINK1 is recruited and stabilized in the outer mitochondrial membrane, where it activates Parkin through phosphorylation. Parkin then ubiquitinates several mitochondrial membrane proteins, such as Mfn1 and Mfn2, signaling the organelle for the interaction between the microtubule-associated protein light chain 3 (LC3) and the ubiquitin-binding protein p62, leading to the engulfment of the defective mitochondria by autophagosomes and hydrolytic degradation by fusion with lysosomes.

Evidences further demonstrate that the PINK1 activation is dependent on its own autophosphorylation, after accumulation and dimerization on the OMM (Okatsu et al., 2013).

PINK1 also participates in the maintenance of the mitochondria under basal conditions. When the mitochondria membrane is polarized, PINK1 is directed to the organelle through its mitochondrial targeting sequence (Silvestri et al., 2005), and imported to mitochondria, where it mediates the phosphorylation of different downstream substrates **(Figure 5)**. The mitochondrial chaperone tumour necrosis factor (TNF) receptor-associated protein 1 (TRAP1) was the first to be described as a target of PINK1, and studies performed in *Drosophila* PINK1 null mutants demonstrated that an upregulation of TRAP1 could rescue the mitochondrial impairments caused by the kinase' loss-of-function (Costa et al., 2013). Moreover, in Parkin mutant flies, TRAP1 was only partially able to restore the mitochondrial functions, whereas in TRAP1 mutants an overexpression of Parkin completely rescued the induced phenotype, suggesting that the chaperone functions in parallel with Parkin but downstream of PINK1. Other downstream substrates of PINK1 include the mitochondrial serine protease Omi, which is

phosphorylated in a PINK1 manner after activation of the p38 pathway and contributes to the protection of cells to mitochondrial stress (Plun-Favreau et al., 2007), and the Complex I subunit NDUFA10 (Pogson et al., 2014), whose phosphorylation regulates the enzymatic activity of Complex I, consequently modulating ATP production. Studies in *Drosophila* PINK1 mutants revealed lower ATP levels compared with controls, with these levels being rescued by re-introducing the PINK1 transgene, demonstrating that an alteration of this kinases activity does affect mitochondrial function (Clark et al., 2006).

Furthermore, growing evidences have linked PINK1 with other mitochondrial functions, including lipid and amino acid metabolism, which is in line with some of the pathophysiological alterations already observed in PD and neurodegeneration (Lim et al., 2012; Tyurina et al., 2015), and further confirms the participation of PINK1 in several parallel pathways in cells.

After import to the mitochondria, the levels of PINK1 can also be regulated by a series of cleavages performed by the matrix protease mitochondrial processing peptidase (MPP) and by the presenilin-associated rhomboid-like protein (PARL), located in the IMM (Meissner et al., 2011; Sekine & Youle, 2018). This high turnover regulates the abundance of endogenous PINK1 in the cell, as well as its cellular localization and possible activation (Lin & Kang, 2008). The PINK1 processing gives rise to a 52 kDa form of the protein, that is then re-translocated to the cytosol where it can either be degraded by the proteasome (Yamano & Youle, 2013) or it can coordinate other cellular functions, such as cell growth (through nuclear transport and regulation of the cellular cycle) (Flanagan et al., 2014), or suppress mitophagy, preventing the PINK1/Parkin pathway.

All these evidences clearly highlight the importance of PINK1 in the maintenance of mitochondrial homeostasis. Different factors, particularly the $\Delta\Psi m$, seem to mediate this kinase activity through the regulation of the PINK1 trafficking in cells, the substrate recognition process and the substrates phosphorylation, allowing for the enzyme to change between molecular pathways and to play distinct functions in cells. Furthermore, given the link between PINK1 and mitochondrial health, the signaling mechanisms that are regulated by this kinase are responsible for the overall mitochondria status, contributing to the preservation of a healthy pool of mitochondria in cells.

1.5. Identification of novel PINK1 substrates to understand mitochondria role in the disease

The importance of PINK1 in the mitochondrial regulation and cellular activity is notorious. PINK1 is a critical element of the mitochondrial quality control pathway that selectively degrades defective organelles from cells, whose accumulation could compromise the normal cellular functions and induce cell death. Moreover, PINK1 also participates in other important pathways under basal conditions, such as Ca^{2+} buffering, regulation of the oxidative phosphorylation and ATP production, and maintenance of the global mitochondrial dynamics and biology.

The way PINK1 can achieve all these different functions in cells is by the phosphorylation of multiple mitochondrial and cytosolic substrates, which allow for the regulation of many important pathways related with the general mitochondrial homeostasis. In fact, PINK1 is able to detect the conditions of the cellular environment, namely mitochondrial dysfunction, and play different biological functions accordingly, in both healthy and diseased status. However, the mechanisms by which PINK1 senses the mitochondrial homeostasis and decides which substrate to preferentially modify, and consequently which function to execute, remain to be clarified. Moreover, how this enzyme identifies the defective mitochondria and promotes their segregation from the healthy pool of organelles in cells is still poorly understood. Finally, even though the current investigation has already discovered and isolated multiple PINK1 substrates, none of these substrates is able to fully restore the morphological and physiological phenotypes related with a cellular PINK1 deficiency. Therefore, the identification of additional and novel PINK1 targets is critical to further elucidate the catalytic activities of this kinase and to better understand its role on the maintenance of a healthy pool of mitochondria in cells, particularly in neurons.

Although multiple mutations on the PINK1 gene have been identified and linked to both mitochondrial dysfunction and autosomal recessive parkinsonism, the consequences of such pathogenic alterations on the substrate recognition process and catalytic activity of PINK1 in cells still need clarification. So ultimately, through the identification of new PINK1 substrates and by understanding the intrinsic mechanisms preferentially used by mitochondria in neurons to maintain their general health, new therapeutic targets for the treatment of human degenerative diseases related with mitochondrial dysfunction, particularly PD, may be identified.

1.6. Aims of the project

Considering the important role of PINK1 in cells, under both basal and pathogenic conditions, as well as the lack of bona-fide PINK1 phosphorylation substrates able to fully rescue the PINK1 associated phenotypes, we aim with this work to identify novel PINK1 substrates and provide new insights on the intrinsic mechanisms that regulate the mitochondrial function in neurons, specifically in the context of PD. This will not only elucidate on the molecular and cellular pathways underlying PINK1 substrate selection in cells, but also help to understand how a healthy pool of mitochondria is sustained in neurons. Ultimately, this project will feed into the development of new therapeutic strategies for the treatment of human degenerative disorders.

Therefore, the main goals of this project are:

1) To identify new candidate substrates for PINK1 through a phosphoproteomics screen and literature review;
2) To validate the candidate PINK1 substrates through an *in vitro* kinase assay;
3) To evaluate the importance of the bona-fide PINK1 substrates in the kinase function in mitochondria, namely Parkin recruitment and mitochondrial clearance, Complex I enzymatic activity and regulation of ATP content in cells;
4) To understand the role of the novel PINK1 substrates in PD, in the presence of human PINK1 clinical mutants.

Chapter 2
MATERIALS AND METHODS

2.1. Phosphoproteomics and bioinformatics analysis

Briefly, lysates from mitochondrial enriched fractions of PINK1 wildtype (WT) and PINK1 knock-out (KO) mouse liver were obtained and separately labelled with different fluorescent cyanine dyes (Cy3 and Cy5, respectively). These samples were then mixed in equal proportions, loaded on the same PAGE gel, and a 2D-Differential In-Gel Electrophoresis (DIGE) was performed, to compare the differences in the proteomic profiles of functionally distinct samples. Proteins were initially separated in a first-dimension gel according to their isoelectric focusing (IEF) point. Secondly, an orthogonal separation through sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) was performed, where proteins are separated according to their molecular weight (second dimension). Since post-translational modifications can contribute to the proteins isoelectric point by altering the proteins' migration in an electrical charged field, shifts in the proteins' mobility could correspond to phosphorylation alterations in our samples.

A comparison between the obtained fluorescent spot pattern between PINK1 WT and KO samples was performed using a 2D-DIGE software, where differences in signal intensity were quantified. Significant spots were then extracted from the acrylamide gel, enzymatically digested (trypsin digestion) and sent for Liquid chromatography followed by Mass Spectrometry (LC/MS). From the MS data, a list of peptides was obtained and identified proteins were ranked according to their peptide coverage (at least 85% coverage of the total protein for it to be a significant valid hit).

2.2. Plasmids

Plasmids used were acquired from Origene Technologies: Acad9 (NM_172678) Mouse Tagged ORF Clone (#MR209542); Acaa2 (NM_177470) Mouse Tagged ORF Clone (#MR206222); Dhtkd1 (NM_001081131) Mouse Tagged ORF Clone (#MR223852); Etfa (NM_145615) Mouse Tagged ORF Clone (#MR204913) and Sardh (NM_138665) Mouse Tagged ORF Clone (#MR211181). All the plasmids for the candidate substrates of PINK1 contain the cDNA of the full-length form of the respective proteins, as well as a C-terminal Myc-DDK tag.

These plasmids were amplified and further purified to obtain high quality purified plasmid DNA. Briefly, for transformation of each plasmid, a 50ul aliquot of competent DH5α cells (ThermoFisher Scientific #18265017) was thawed on ice, and 10ng of plasmid DNA was added

followed by a 30 minutes incubation on ice. Next, a heat shock was performed at 42°C for 45 seconds, followed by a 2 minutes incubation on ice. After this, 300µL of pre-warmed SOC broth medium (Nzytech #MB28001) was added and the cells were incubated at 37°C for 1 hour at 300rpm. Since all the plasmids carry the antibiotic resistance gene to kanamycin, LB agar (Sigma-Aldrich #L2897) plates with 25ug/mL kanamycin (Nzytech #MB02001) were used. The transformed bacteria were plated on the agar plates and incubated overnight at 37°C. A colony from each plate was picked and grown in pre-inoculum of 3mL of LB broth medium (Sigma-Aldrich #L3022) with 25ug/mL kanamycin in a shaker at 37°C and 250 rpm for approximately 6 hours, after which the bacteria were inoculated in 100mL of LB broth medium and incubated on a shaker at 37°C overnight.

DNA extraction was performed as indicated in the manufacturer's protocol provided by the QIAGEN® Plasmid Purification Kit Handbook (Midi-prep, #12145). Briefly, the collected overnight bacterial cultures were centrifuged at 200xg for 15 minutes and pellets were resuspended in Resuspension Buffer (50mM Tris-HCL pH 8.0; 10mM EDTA; 100µg/mL RNaseA), then lyzed with Lysis Buffer (200mM NaOH; 1% SDS), and lastly neutralized with Neutralization Buffer (3.0M potassium acetate pH 5.5). Plasmidic DNA was purified and washed in a QIAGEN-Tip 100 column (that was fist equilibrated using Equilibration Buffer) and eluted in Elution Buffer (1.25M NaCl; 50mM Tris-HCl pH 8.5; 15% isopropanol). After this, DNA is precipitated with isopropanol and washed in ethanol 70%. Plasmid DNA was then resuspended in Nuclease free water (Sigma-Aldrich #W4502). DNA concentration was measured at a wavelength of 280nm and purity was determined based on the ration between 260/280/320nm. Measurements were performed on a UV-Vis spectrophotometer NanoDrop™2000 (ThermoFisher).

2.3. Cell Culture and Cell Lines

Henrietta Lacks (HeLa) cells, COS-1 (immortalized CV-1 derived cells from monkey kidney tissue) cells and Mouse Embryonic Fibroblasts (MEF) were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12 medium; ThermoFisher #11039047) enriched with 10% fetal bovine serum (FBS) (ThermoFisher Scientific #10270098). All cell lines were preferably handled with approximately 80% confluency.

The HeLa-CrispR/Cas9-PINK1 cell line (henceforth described as HeLa PINK1 KO cell line) was previously described in Aerts, Craessaerts, et al., 2015. These cells were produced using

clustered regularly interspaced short palindromic repeats/Cas technology. A target sequence was picked from the first exon spanning the start codon of PINK1 and cloned in pX330-U6-Chimeric-BB-CBh-hSpCas9 from Addgene. This plasmid was then transfected in HeLa WT cells and the PINK1 expression was analysed through Western Blot. Clones in which PINK1 expression was absent were selected and subjected to MiSeq Next Generation sequencing analysis (Illumina) for the PINK1 gene sequence and the top five off-target regions in the HeLa genome for the clustered regularly interspaced short palindromic repeats guide RNA.

The PINK1$^{-/-}$ MEF cell line was previously described in Morais et al., 2009. Briefly, the truncated PINK1 mutants were obtained first by the generation of the targeting vector construct 129/Ola by genomic DNA polymerase chain reaction (PCR) amplification. A deletion of the exons 2 and 3 of the PINK1 gene was accomplished by Cre-Lox-mediated recombination, resulting in a frameshift and creating a truncation of the PINK1 protein after exon 1. The linearized vector was then electroporated into TBV2 embryonic stem cells, and targeted neomycin resistant clones were expanded and injected into blastocysts from C578L/6 mice. Blastocysts were transferred into pseudopregnant mothers and the obtained chimeras were bred with C578L/6 WT mice. After screening of the offspring, PINK1 conditional KO mice were bred with Cre Deleter animals with C578L/6 background to originate PINK1 complete KO mice. After a complete PINK1 KO mice model was generated, fibroblasts from mutant animals were obtained by dissection of Embryonic day 13 embryos. After removal of the heads and red organs, the remaining bodies were chopped in cell culture dishes containing DMEM with 50% fetal calf serum and 1% penicillin/streptomycin. The cultures were afterwards expanded and when they attained consistent growth, they were immortalized.

Wild-type HeLa cells (ATCC®) were also used.

2.4. Cell platting, transfection and preparation of cell lysates

HeLa cells (from both genotypes) were platted in 6-well multi-well plates and transfected at approximately 60-70% confluency. These cells were transfected using the FuGENE 6 transfection reagent (Promega #E2692), according to the manufacturer's instructions. Briefly, 2.5µg of plasmid DNA (described in section 2.2) was used for 7.5µL of transfection reagent (1:3 ratio). At 48 hours post transfection the cells were harvested. Untransfected HeLa cells and MEF cells

(from both genotypes) were also platted on 6-well plates and were harvested and lysed at approximately 80% confluency.

To harvest and lyse the cells, these were first washed with Dulbecco's phosphate-buffered saline (DPBS, ThermoFisher Scientific #14190169) and then lysed in either lysis buffer (50mM Tris-HCl pH 7.4; 150mM NaCl; 5mM EDTA; 1% Triton-X100) or RIPA buffer (50mM Tris-HCl pH 8.0; 1mM EDTA, 1% Triton X-100; 0,5% sodium deoxycholate; 0,1% SDS; 150mM NaCl) for western blot. Protease inhibitors (100:1) from Complete EDTA-free protease inhibitor cocktail (Roche #4693132001) were added to the lysis buffers. For the Lambda protein phosphatase (LPP) treatment and Phos-Tag SDS-PAGE (section 2.7), cells were lysed either with lysis buffer or with RIPA buffer, supplemented with protease inhibitors and/or phosphatase inhibitors Cocktail 2 and 3 (Sigma-Aldrich #P5726 and #P0044). Cells platted in multi-well plates were scrapped on ice and collected to a 1.5mL eppendorf tube, where homogenization by pipetting up-and-down was performed. After 1 hour incubation on ice, the cell lysates were centrifuged for 10 minutes at 4°C at 14,000xg. Supernatants were collected and stored at -20°C.

2.5. Protein Quantification

Protein concentration was determined using either the Pierce™ BCA® Protein Assay (Thermo Scientific #23225) or the Bio-Rad® Bradford Protein Assay (#5000006 and #5000007), according to manufacturer's instructions. The choice of the protein quantification method used was determined by the compatibility of the lysis buffer with the protein detection method. Cell lysates obtained using a Triton X-100 based lysis buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA, 1% Triton X-100) were quantified with the Bradford Protein Assay. This assay is based on a colour shift of the Coomassie Blue G-250 dye from red to blue that occurs upon protein binding and protonation. This shift is spectroscopically measured at an absorbance wavelength of 595nm. The amount of the blue anionic form of the dye is directly proportional to the quantity of protein in the sample. For the RIPA prepared lysates (that contain SDS) protein concentration was determined by the BCA Protein Assay. This method is based on the protein-induced reduction of the cupric ion (Cu^{2+}) to Cu^{+} (proportional to the amount of protein in sample) and on the colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid (BCA) chelation. The BCA/copper complex originates a purple coloured reaction product that

displays a linear absorbance at 562nm. For both methods, a protein standard curve using known concentration of BSA was used to determine protein concentration.

2.6. Western Blotting

Briefly, samples were treated with NuPAGE Lithium dodecyl sulphate (LDS) Sample Buffer (SB) (106mM Tris-HCl, 141mM Tris Base, 2% LDS, 10% glycerol, 0.51mM EDTA, 0.22mM SERVA Blue G250, 0.175mM Phenol Red; pH 8.5) (Invitrogen #NP0008) with 10% β-mercaptoethanol (Bio-Rad #1610710) (4x) and incubated for 10 minutes at 70°C. 30µg of total protein lysate were loaded per lane and separated on PreCast NuPAGE or Bolt gels from Invitrogen. The gel type and percentage was chosen according to each protein's molecular weight. The gels used were: NuPAGE 3-8% Tris-Acetate (#EA03752) for SARDH; NuPAGE 4-12% Bis-Tris (#NP0322) for ACAD9; NuPAGE 10% Bis-Tris (#NP0302) for ACAA2 and ETFA; and Bolt 8% Bis-Tris (#NW00085) for DHTKD1. To assess protein molecular weight, a molecular weight protein standard ladder was used (Bio-Rad #1610374). For the electrophoresis, samples were run at constant voltage, initially at 90V (until samples entered the stacking gel) and then at 150V (samples in the resolution gels) for approximately 1 hour in a Tris-MOPS based Running buffer (50mM MOPS, 50mM Tris Base, 0.1% SDS, 1mM EDTA; pH 7.7, from NuPAGE or Bolt) or a Tris-Acetate Running Buffer (50mM Tricine, 50mM Tris Base, 0.1% SDS; pH 8.24) (Invitrogen #NP0001, #B0001 and #LA0041). Gels were electrotransfered to 0.20nm nitrocellulose membranes (GE Healthcare #10600001) at 30V for 1 hour in 1x Transfer Buffer (Transfer Buffer (25mM Bicine, 25mM Bis-Tris (free base), 1mM EDTA; pH 7.2) (Invitrogen #NP00061), 20% methanol). Transfer efficiency was evaluated by briefly incubating membranes with the reversible protein-binding dye Ponceau-S (0.1% Ponceau S (w/v) and 5.0% acetic Acid (w/v)) (Sigma-Aldrich). Membranes were blocked with 5% non-fat milk in TBS-T (20mM Tris-HCl, pH 7.5; 150mM NaCl with 0.1% Tween-20) for 1 hour, followed by incubation with the respective primary antibodies overnight at 4°C. Antibodies and dilutions used were: rabbit anti-Acad9 (ProteinTech #15770-1-AP) (1/500); mouse anti-Acaa2 (Origene #TA506155) (1/500); rabbit anti-Dhtkd1 (ThermoFisher Scientific #PA5-24208) (1/250); mouse anti-Etfa (Abcam #ab110316) (1/500); rabbit anti-Sardh (Aviva Systems #ARP42344_T100) (1/500); mouse anti-α-tubulin (Abcam #ab7291) (1/5000), mouse anti-β-actin (Sigma-Aldrich #A5441) (1/20000), mouse anti-flag (Sigma-Aldrich #F3165) (1/5000) and mouse anti-c-myc 9E10 clone (Sigma-Aldrich #M4439) (1/1000). Membranes were washed three times for 5

minutes in TBS-T. Membranes were incubated with the corresponding secondary antibody: Goat anti-mouse or Goat anti-rabbit IgG conjugated to the Horseradish peroxidase (HRP) at a dilution of 1/10000 (Bio-Rad #1706516 and #1706515) for 2 hours at room temperature. Membranes were washed three times in TBS-T and developed using standard chemiluminescence, by incubating the membranes during one minute with ECL (GE Healthcare #RPN2106). This method is based on the emission of light during the HRP-catalysed oxidation of luminol, allowing for the chemiluminescent detection of specific immobilized antigens conjugated to HRP-labelled antibodies. Membranes were developed in the Amersham 680 RGB Imaging System and quantification was performed using the ImageStudio Lite Ver 5.2 software.

2.7. Lambda protein phosphatase treatment and Zn^{2+}-Phos-Tag SDS-PAGE

The dephosphorylation reaction was performed as previously described in Aerts, Craessaerts, et al., 2015. Briefly, transfected HeLa WT and PINK1 KO cell lysates resuspended in lysis buffer supplemented with protease inhibitors were incubated for 1 hour at 30°C with or without Lambda PP (Protein MetalloPhosphatases) according to manufacturer protocol (New England Biolabs #P0753L, #B0761L and #B1761). HeLa cell extracts resuspended in lysis buffer with both protease and phosphatase inhibitors were used as control. Phosphorylation status of protein candidates was then analysed by a Phos-Tag SDS-PAGE assay. The Phos-Tag SDS-PAGE uses a dinuclear metal complex (Zn^{2+} or Mn^{2+}) co-polymerized with acrylamide to detect a protein phosphorylation pattern by producing a shift in the electrophoretic mobility of phosphorylated peptides in relation to their non-phosphorylated forms. Briefly, samples were treated with Laemmli SB (4x) (62.5mM Tris-HCl, pH 6.8; 10% glycerol; 1% LDS; 0.005% Bromophenol Blue) (Bio-Rad #1610747) with 10% β-mercaptoethanol and incubated for 10 minutes at 70°C. 30µg of total protein lysate or dephosphorylation reaction mix were loaded in each lane on SuperSep™ Phos-Tag™ Precast Gels (50µmol/l), 7.5%, 17well, 100×100×6.6 mm from FUJIFILM Wako Pure Chemical Corporation (#192-18001). Electrophoresis was performed at a constant current of 20 mA/gel in Tris-based Running buffer (25mM Tris-HCl pH 8.3, 192mM glycine, 0.1% SDS). For immunoblot analysis, gels were first washed three times during 20 minutes in Transfer buffer (25mM Tris-HCl pH 8.3, 192mM glycine, 0.1% SDS, 20% methanol) with 10mM EDTA, to remove metal ions, followed by one wash in Transfer buffer for 10 minutes. Proteins were electrotransfered to 0.45nm polyvinylidene difluoride (PVDF) membranes (GE

Healthcare #10600023) at 30V for 90 minutes. Membranes were blocked with 5% non-fat milk in TBS-T and incubated with anti-flag primary antibody (section 2.6) overnight at 4°C. Control western blots were performed for each Phos-Tag gel.

2.8. Immunoprecipitation of candidate substrates

COS-1 cells cultured in 6-well plates and transfected with the plasmids for the candidate proteins were rinsed with TBS, and harvested and lysed with lysis buffer (section 2.4.). Cell lysates were incubated with Anti-c-Myc Agarose beads (Thermo Scientific, #20168) overnight at 4°C with end-over-end rotation. The beads were pelleted and washed twice with lysis buffer with 0,1% Triton-X and once with TBS with 0,05% Tween-20. Beads were incubated with 0.5mg/mL Pierce c-Myc Peptide (Thermo Scientific, #20170) for 15 minutes at 37°C (300rpm), to obtain the first two elutes. After collecting the second elute, the proteins were boiled in LDS (4x) sample buffer at 70°C for 10 minutes to obtain the last elute. To assess the efficiency of this assay, samples were treated with LDS SB with 10% β-mercaptoethanol, boiled and resolved by SDS-PAGE electrophoresis, followed by western blot analysis (section 2.6) with anti-Flag and anti-α-tubulin or anti-β-actin antibodies for loading control.

2.9. Statistical analysis

Statistical significance was analysed using GraphPad Prism 8.4.3 software through unpaired Student's *t*-test (*, $p<0.05$; **, $p<0.01$; ns, non-significant). Data are presented as mean ± standard errors of the mean (SEM).

Chapter 3
RESULTS

3.1. PINK1 candidate substrates selection

Many studies have now demonstrated that PINK1 regulates several mitochondrial pathways through the phosphorylation of multiple proteins in cells. Even though different evidences have pointed out to this function by PINK1 (Y. Chen & Dorn, 2013; Costa et al., 2013; Morais et al., 2014; Wang et al., 2011), the molecular processes by which this phosphorylation occurs remain to be clarified, namely if PINK1 directly targets these proteins or if the regulation by this kinase is part of a more complex molecular mechanism within the cells. Furthermore, manifold proteins were already identified as PINK1 phosphorylation substrates, not only under diseased states but also under steady-state conditions, which points out to the important regulatory role this kinase plays in mitochondria and in cells. However, it seems that none of the already discovered and isolated PINK1 substrates are able to reinstate all the morphological phenotypes related with a PINK1 deficiency.

To identify additional and new PINK1 targets, a phosphoproteomics screen analysis was carried out. Lysates from mitochondrial enriched fractions of PINK1 WT and PINK1 KO mouse liver were obtained and labelled with Cy3 and Cy5 fluorescent dyes, respectively. A 2D-DIGE gel electrophoresis was performed and a spot map was obtained. Spot analysis was performed using a 2D-DIGE software and spots with a statistical difference of $p<0.05$ of WT compared to KO were further processed by MS. From this analysis, a list of proteins was obtained, and the candidates were ranked according to their peptide coverage.

From this list, and based on protein analysis and literature search, a list of candidate proteins was made, which included multiple mitochondrial and cytosolic proteins. In order to prioritize these candidate proteins, a thorough literature search was conducted to evaluate the state-of-the-art for each candidate protein, the importance of their cellular and mitochondrial functions and possible interactions with the PINK1 kinase protein (Table 1).

Apart from having a very well-known role in the regulation of specific aspects of the mitochondrial morphology and physiology, growing evidence as emerged on PINK1 acting in parallel in the coordination of other cellular and mitochondrial pathways such as the lipid and amino acid metabolism, the electron shuttling to the ETC and molecular signalling (Furlong et al., 2019; Senyilmaz et al., 2015; Stauch et al., 2016; Vos et al., 2017).

Table 1: List of significantly altered proteins in PINK1 KO mice (8-weeks old) obtained from the phosphoproteomic screening.

The candidates presented below were selected based on literature-based review, to which the cellular location of the protein and general function were considered. Proteins previously evidenced as being linked with PINK1 are identified with an asterisk (*). Statistical analysis was performed on all hits ($p < 0.05$; peptide coverage: 90%).

Uniprot	Gene	Protein name	Cellular compartment	Biological process
Q16891	Immt*	Isoform 1 of Mitochondrial inner membrane protein	Mitochondria	Mitochondrial cristae morphology maintenance and formation (Alkhaja et al., 2012; John et al., 2005; Ott et al., 2015); Protein import and biogenesis (von der Malsburg et al., 2011)
Q96HY7	Dhtkd1	Probable 2-oxoglutarate dehydrogenase E1 component DHKTD1	Mitochondria	TCA cycle and ATP production (Bunik & Degtyarev, 2008); Mitochondrial biogenesis and function (W. Xu et al., 2013); Amino acid metabolism (Araújo et al., 2013; McLain et al., 2011); Probable interaction with Complex I (Sumegi & Srere, 1984)
O94826	Tomm70a*	Mitochondrial precursor proteins import receptor	Mitochondria	Mitochondrial protein import (Edmonson et al., 2002)
P00367	Glud1	Glutamate dehydrogenase 1	Mitochondria	Glutamate synthesis and catabolism (Spanaki et al., 2017)
Q9H845	Acad9	Very-long-chain acyl-CoA dehydrogenase VLCAD homolog / Complex I assembly factor ACAD9	Mitochondria	Fatty acid metabolism (Zhang et al., 2002); Complex I assembly (Nouws et al., 2010, 2014)
P36871	Pgm1	Phosphoglucomutase-1	Cytoplasm	Glucose synthesis and catabolism (Gururaj et al., 2004)
Q13217	DNAjc3b	DnaJ homolog subfamily C member 3	Endoplasmatic reticulum (ER)	Unfolded protein response in the ER and protein folding (van Huizen et al., 2003; Zarouchlioti et al., 2018);
Q9UL12	Sardh	Sarcosine dehydrogenase	Mitochondria	Sarcosine degradation (Porter et al., 1985)
Q9NPH3	Il1Rap	Interleukin-1 (IL-1) receptor accessory protein	Plasma membrane; Cytoplasm; Extracellular domain	Inflammatory response and IL-1 family signalling pathways (Acuner Ozbabacan et al., 2014; Wesche et al., 1997)
P13804	Etfa*	Electron transfer flavoprotein subunit alpha	Mitochondria	Electron transport chain (Watmough & Frerman, 2010)
O95497	Vnn1	Pantetheinase precursor	Plasma membrane	Pantothenate (vitamin B_5) metabolism and CoA synthesis (Naquet et al., 2014; Rock et al., 2000)
Q8TD30	Gpt2	Alanine aminotransferase 2	Mitochondria	Glucose, amino acid and fatty acid metabolism (R. Z. Yang et al., 2002)
P42765	Acaa2*	3-ketoacyl-CoA thiolase	Mitochondria	Fatty acid metabolism (Kiema et al., 2014)
O95299	NDUFA10*	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10	Mitochondria	Complex I function (Loeffen et al., 1998)

Given this increasing evidence, we set out to investigate specific pathways in which PINK1 might participate in cells by selecting specific hits for further study. Therefore, from the previous list, five candidate proteins were chosen.

The acyl-CoA dehydrogenase member 9 (ACAD9) was first described as a member of the acyl-CoA dehydrogenase family of proteins (ACADs), catalysing the initial step of the mitochondrial fatty acid β-oxidation (Ensenauer et al., 2005). Given its specificity for long-chain unsaturated fatty acids, ACAD9 was primarily proposed to be a very-long-chain acyl-CoA dehydrogenase (VLCAD) enzyme homolog (Zhang et al., 2002); however, following studies demonstrated that these two enzymes seem to be involved in different physiological functions in cells (He et al., 2007; Nouws et al., 2010). Furthermore, deficiencies in this protein as well as in other ACADs have been identified as important causes of different human metabolic disorders (He et al., 2007; Spiekerkoetter et al., 2003). Apart from its function in the fatty acid β-oxidation, ACAD9 was also described as having a role in the mitochondrial complex I assembly, particularly in building the intermediate ND2-module. This function is achieved by interaction with two mitochondrial chaperones (NDUFAF1 and ECSIT), that together with ACAD9 form the mitochondrial complex I assembly complex (MCIA complex) (Nouws et al., 2010). Furthermore, given the involvement of this protein in the assembly of the first ETC complex, ACAD9 plays a role in the OXPHOS. Since it was demonstrated that alterations in the ACAD9 gene result in complex I deficiencies without affecting the long-chain fatty acids β-oxidation, it is believed that these two functions of ACAD9 are independent of one another and that the dehydrogenase catalytical activity of this enzyme is not required for the complex I biogenesis (Nouws et al., 2010, 2014). However, following studies have suggested that even though these two activities of the enzyme are not dependent of one another, they can both contribute to disease severity in the case of ACAD9 deficiencies (Schiff et al., 2014). All these evidences raise several arguments on the primary role of ACAD9 in cells, which remains to be fully understood. As PINK1 has also been associated with Complex I regulation (Morais et al., 2014), we decided that ACAD9 would be an interesting candidate protein to further investigate in the context of PINK1-biology.

The 3-ketoacyl-CoA thiolase (ACAA2 or MCKAT) enzyme is a member of the thiolase family of proteins, which can be found in different regions and organelles of the cell and that participate in various metabolic pathways such as the peroxisomal fatty acid oxidation, cholesterol synthesis in the cytosol and in different mitochondrial pathways. In the mitochondrial matrix, ACAA2

catalyses the last step of each cycle of the fatty acid β-oxidation (Kiema et al., 2014; Middleton, 1973) and seems to be specific for medium-chain to short-chain unbranched fatty acids (Mao et al., 1995; Miyazawa et al., 1981; Staack et al., 1978). Evidences also suggest that ACAA2 might function as a biosynthetic enzyme, being involved in the synthesis of acetoacetyl-CoA and in the production of ketone bodies (Kiema et al., 2014), as well as to display hydrolase activity towards several fatty acyl-CoA molecules. Apart from these functions, ACAA2 was proposed to attenuate the mitochondrial damage and apoptotic effects exhibited by the pro-apoptotic protein BNIP3, through the abolishment of the ΔΨm alterations and decrease of the overall cellular apoptosis (Cao et al., 2008). Even though all these suggestions have been made on the cellular activities of this enzyme, its physiological role remains to be clarified. While no direct connection was made between ACAA2 and the PINK1 kinase, increasing evidence has linked PINK1 with proteins involved in the lipid and amino acid metabolism (Course et al., 2018; Senyilmaz et al., 2015; Vos et al., 2017). Therefore, the study of this thiolase in the context of the PINK1-mediated pathways could help elucidate the role of this enzyme in the energetic metabolism of cells, particularly of neurons.

The 2-oxoglutarate dehydrogenase E1 (DHTKD1) is a mitochondrial matrix protein that plays a critical role in the energy production by mitochondria. This protein is a component of the multienzyme oxoglutarate dehydrogenase complex (OGDHC), whose other elements include the dihydrolipoamide succinyltransferase (E2) and the lipoamide dehydrogenase (E3) enzymes. Being the first element of the OGDHC, DHTKD1 initiates and catalyses the overall conversion of 2-oxoglutarate to succinyl-CoA and CO_2 in the TCA cycle, a highly regulated step of the global energetic metabolism of cells (Bunik & Degtyarev, 2008), therefore contributing to the general production of ATP. DHTKD1 has also been considered to participate in the amino acid metabolism (Araújo et al., 2013; Biagosch et al., 2017; McLain et al., 2011). Mutations in the DHTKD1 gene have been related with different disorders, including the neurodegenerative Charcot-Marie-Tooth type 2Q disease, and the human 2-aminoadipic and 2-oxoadipic aciduria, which have multiple associated neurological symptoms (Danhauser et al., 2012; Luan et al., 2020; W. Y. Xu et al., 2012). The evidences on the physiological and pathological roles of this enzyme imply a critical role for DHTKD1 in both mitochondrial metabolism and neurological diseases. More recently, DHTKD1 has been linked to mitochondrial biogenesis, ATP production and mitochondrial general function (Luan et al., 2020; W. Xu et al., 2013). As previously

described, PINK1 plays a panoply of functions in the mitochondria, also having a role in the regulation of those same pathways, making DHTKD1 an interesting candidate for further studies.

The electron transfer flavoprotein subunit alfa (ETFA), together with the correspondent subunit beta, comprises the electron transfer flavoprotein (ETF) complex. ETFs are soluble heterodimeric proteins located on the mitochondrial matrix that act as specific electron acceptors for several dehydrogenases of the fatty acid β-oxidation and amino acid metabolism, including many acyl-CoA dehydrogenase enzymes (such as ACAD9) and the glutaryl-CoA dehydrogenase (GCDH). These electrons are then transferred to the main mitochondrial respiratory chain through the ETF:ubiquinone oxidoreductase (ETF:Q oxidoreductase) enzyme (Toogood et al., 2007; Watmough & Frerman, 2010), contributing to the overall OXPHOS function. To achieve its function, ETF requires the flavin adenine dinucleotide (FAD) cofactor and adenosine monophosphate (AMP), which are essential elements for the function of this enzymatic complex. Recently, the protein LYR motif-containing protein 5 (LYRM5), also known as Electron transfer flavoprotein regulatory factor 1 (ETFRF1), was identified as a key regulator of the ETF, through the binding and removal of the FAD cofactor from the enzymatic complex (Floyd et al., 2016). Defects in ETF enzymes are a cause of some metabolic disorders, including the Glutaric aciduria type 2, which is characterized by the accumulation of multiple metabolites resultant of the incomplete breakdown of proteins and fats (Goodman et al., 2002; Wolfe et al., 2010).

This pathway also seems to accept electrons from dehydrogenases involved in mitochondrial one-carbon metabolism, including the sarcosine dehydrogenase (SARDH), other of our protein hits. SARDH is a mitochondrial matrix protein involved in the oxidative demethylation of sarcosine (also known as N-methylglycine), which requires the FAD cofactor and consists in the primary step of the formaldehyde and glycine synthesis from the sarcosine amino acid (Porter et al., 1985). The biological significance of this protein apart from the sarcosine degradation remains to be uncovered. Given that multiple studies have revealed a role for PINK1 in cellular pathways associated with the energetic metabolism and shuttling of electrons to the ETC from alternative routes, the study of both ETFA and SARDH proteins can contribute to the current knowledge on the PINK1 function in cells.

In sum, these five candidate proteins were selected based on their, at present, known function and on a putative connection to PINK1 biological function. Next, we used cell-based and *in vitro* assays to confirm whether these candidate proteins are bona-fide PINK1 substrates.

3.2. Analysis of the candidate PINK1 substrates expression

To assess the protein levels of our selected candidates, we performed Western Blotting experiments in two different cell lines (HeLa cells and MEF), both in WT and in PINK1 KO conditions (Aerts, Craessaerts, et al., 2015; Morais et al., 2009), using commercially available antibodies. In order to confirm the specificity of the antibodies, we also tested cell extracts from HeLa WT and PINK1 KO cells transiently transfected with plasmids expressing these candidate proteins.

For the ACAD9 protein, the immunoblot analysis revealed a two-band pattern in both HeLa and MEF cell lines, with an upper band appearing at the estimated molecular weight of 69 kDa, and a lower band at approximately 65 kDa (Figure 7). This cleavage pattern was consistent with the previously described mitochondrial processing of this dehydrogenase (Ensenauer et al., 2005; Zhang et al., 2002). The nuclear encoded 69 kDa full-length form of ACAD9 is targeted to the mitochondria due to the presence of an N-terminal mitochondrial targeting sequence (MTS). After being imported into the organelle, this protein undergoes a two-step cleavage by the mitochondrial processing peptidase (MPP), followed by the mitochondrial intermediate peptidase (MIP), which results in the sequential removal of the first 37 amino acids from the N-terminal sequence of this peptide, forming a 65 kDa cleaved form of ACAD9. Our Western blot results are in agreement with these previously reported findings as we observe both forms of the protein in our results (Figure 7a and 7b).

Immunoblot analysis revealed a decrease of both the full-length and cleaved ACAD9 proteins in the HeLa PINK1 KO cells when compared to HeLa WT cells. However, for the cleaved form of this protein, statistical analysis showed that this difference was not significant. In the MEF cell line, while the cleaved ACAD9 levels were lower in the PINK1 KO condition, the full-length form is significantly accumulated in cells where PINK1 was absent, suggesting that the presence of PINK1 is required for ACAD9 processing. In order to validate the specificity of our antibody towards endogenous ACAD9, we evaluated the multi-band pattern in HeLa cells transiently

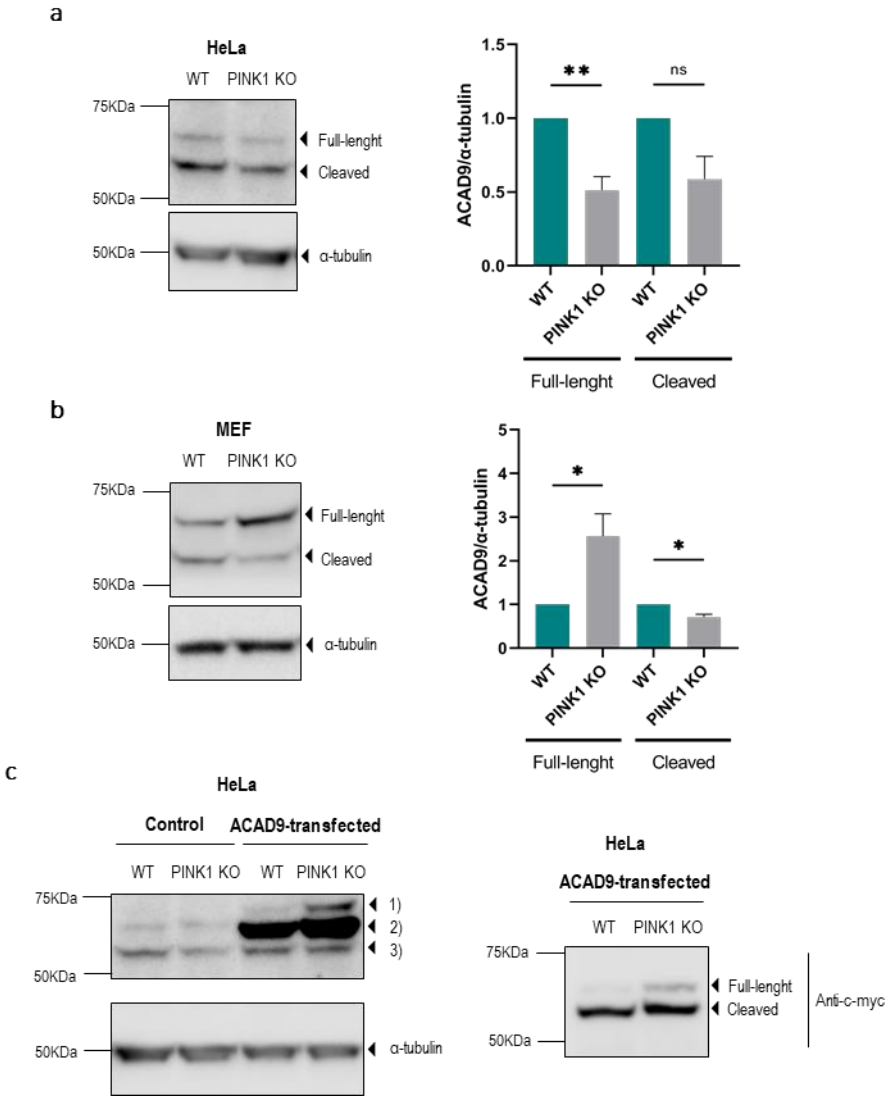

Figure 7: Western blot analysis of ACAD9 protein levels in WT and PINK KO HeLa and MEF cell lines.

(a, b) Representative immunoblot images of the endogenous ACAD9 protein in wild-type (WT) and PINK1 KO HeLa and MEF cell lines. Quantification graphs are presented for each cell line (N=3 experiments). Results were normalized to α-tubulin and for the quantification all lanes were normalized between cell lines to the correspondent WT lysates (untreated cells). (c) Immunoblotting analysis of the overexpressed ACAD9 protein in HeLa cells, and respective control (treated with the transfection reagent). Validation of the anti-ACAD9 antibody was performed using anti-c-myc. Statistical analysis was calculated by using unpaired t-test (*, $p<0.05$; **, $p<0.01$; ns, non-significant). Data are expressed in mean ± SEM (MEF cell line: full-length ACAD9: 1,566 ± 0,5129; cleaved ACAD9: -0,2915 ± 0,06847; HeLa cell line: full-length ACAD9: -0,4867 ± 0,09062; cleaved ACAD9: -0,4126 ± 0,1541). *Legend:* 1) Full-length exogenous ACAD9; 2) cleaved exogenous and full-length endogenous ACAD9; 3) cleaved endogenous ACAD9.

transfected with a mammalian plasmid expressing ACAD9 containing a C-terminal tag (Figure 7c, left panel). The exogenous protein was detected at a higher molecular weight due to the presence of the DDK and Myc tags on the C-terminal of the gene sequence (making an addition of approximately 2 kDa). For the transfected HeLa cell lysates, although we were expecting to

observe the full-length and cleaved ACAD9 forms for both the endogenously expressed and exogenous transfected protein, we observed a superimposition of the cleaved exogenous ACAD9 with the full-length endogenous protein. Despite this, both forms of the exogenous ACAD9 protein were detected in WT and PINK1 KO transfected HeLa cells using an antibody against the endogenous protein (Figure 7c, left panel) or an antibody against the C-terminal Myc tag (Figure 7c, right panel). Moreover, we were able to validate the specificity of our antibody against the endogenous form of ACAD9 as the same detected band pattern was attained for when western blot membranes were probed with the anti-myc antibody (Figure 7c, right panel).

Immunoblot analysis of the ACAA2 protein revealed a band at approximately 42 kDa, which is the estimated molecular weight of this protein (Figure 8). The results showed an increase of the ACAA2 protein levels in MEF PINK1 KO cells when compared with the MEF WT cells (Figure 8b). As for HeLa cells, no significant differences were observed between the WT and the PINK1 KO conditions (Figure 8a). We further validated these results and the specificity of our antibody towards the endogenous ACAA2 in transiently transfected HeLa cells with an ACAA2-expressing plasmid. The exogenous protein was detected at a higher molecular weight due to the tags on the C-terminal of the plasmid and similar to the endogenous protein, no differences were detected between the HeLa WT and PINK1 KO transfected cells in the immunoblots (Figure 8c).

When examining the DHTKD1 protein levels in HeLa cells, we were not able to detect the endogenously expressed protein in either normal or PINK1 KO conditions by western blot, suggesting very low basal levels of this protein in this specific cell line. For the MEF cell line, we observed bands at the expected molecular weight of this protein (approximately 103 kDa). High exposure time was required probably also indicating that this protein has low basal expression levels in these cells as well. The specificity and functionality of the antibody used against endogenous DHTKD1 was validated as this antibody in these conditions was able to detect the exogenous protein transiently transfected in HeLa WT and PINK1 KO cell lysates (Figure 9b), where increased protein levels in HeLa PINK1 null cells were observed compared to the WT condition. Nevertheless, we analysed the protein levels in WT and PINK1 KO MEF cell lines, and no significant alteration of expression levels was observed between the genotypes (Figure 9a).

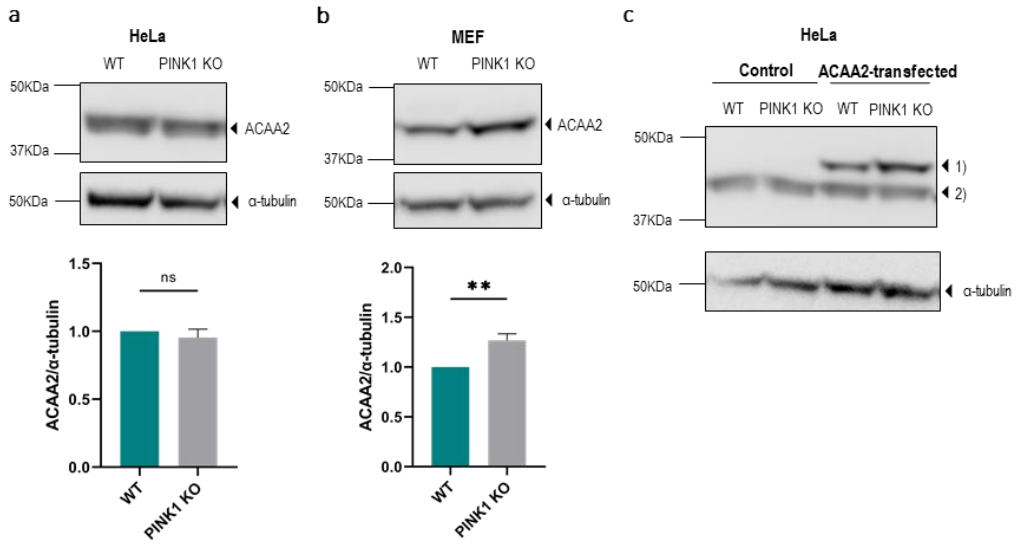

Figure 8: Western blot analysis of ACAA2 protein levels in WT and PINK KO HeLa and MEF cell lines.

(a, b) Representative immunoblot images of the endogenous ACAA2 protein in WT and PINK1 mutant HeLa and MEF cells. Quantification graphs are presented for each cell line (N=7 experiments). Results were normalized to α-tubulin and all lanes were normalized between cell lines to the correspondent WT lysates. (c) Immunoblotting analysis of the overexpressed ACAA2 protein in HeLa cells, and respective control (treated with the transfection reagent). Statistical analysis was calculated by using unpaired t-test (**, p<0.01; ns, non-significant). Data are expressed in mean ± SEM (MEF cell line: 0,2685 ± 0,06746; HeLa cell line: -0,04636 ± 0,06308). *Legend:* 1) overexpressed ACAA2; 2) endogenous ACAA2

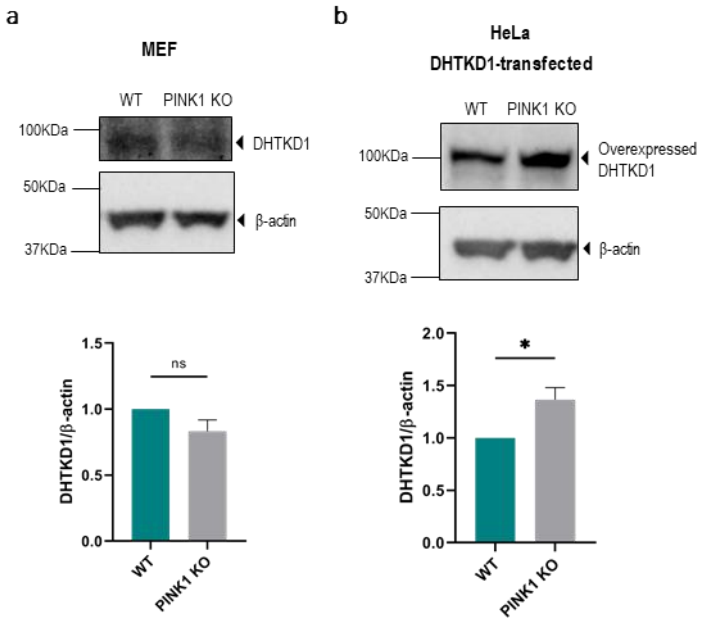

Figure 9: Western blot analysis of DHTKD1 protein levels in WT and PINK KO HeLa and MEF cell lines.

(a, b) Representative immunoblot images of the endogenous and overexpressed DHTKD1 protein in WT and PINK1 KO HeLa and MEF cells. Quantification graphs are presented for each cell line (N=3 experiments for HeLa cells and N=5 experiments for MEFs). Results were normalized to β-actin and all lanes were normalized between cell lines to the correspondent WT lysates). Statistical analysis was calculated by using unpaired t-test (*, p<0.05; ns, non-significant). Data expressed in mean ± SEM (MEF cell line: -0,1677 ± 0,08636; HeLa cell line: 0,3650 ± 0,1163).

Western blot analysis revealed a specific band at approximately 35 kDa corresponding to the ETFA protein. PINK1 null HeLa and MEF cells demonstrated increased ETFA protein levels when compared to counterpart WT cells in the immunoblots, even though statistical analysis revealed this difference to be non-significant (Figure 10a and 10b). Moreover, detection of exogenous protein levels in transiently transfected HeLa cells validated the specificity of the antibody used against endogenous ETFA protein. For the overexpressed protein, a two-band pattern was observed that was not detected for the endogenous protein, which could suggest some form of processing inside the cell (Figure 10c).

For the SARDH protein, immunoblot analysis revealed a protein band at the expected weight of approximately 101 kDa (Figure 11a and 11b). Additionally, an overall decrease of protein levels in both PINK1 KO HeLa and MEF cells was observed, that was further sustained by the exogenously expressed protein in SARDH-transfected HeLa cells (Figure 11c).

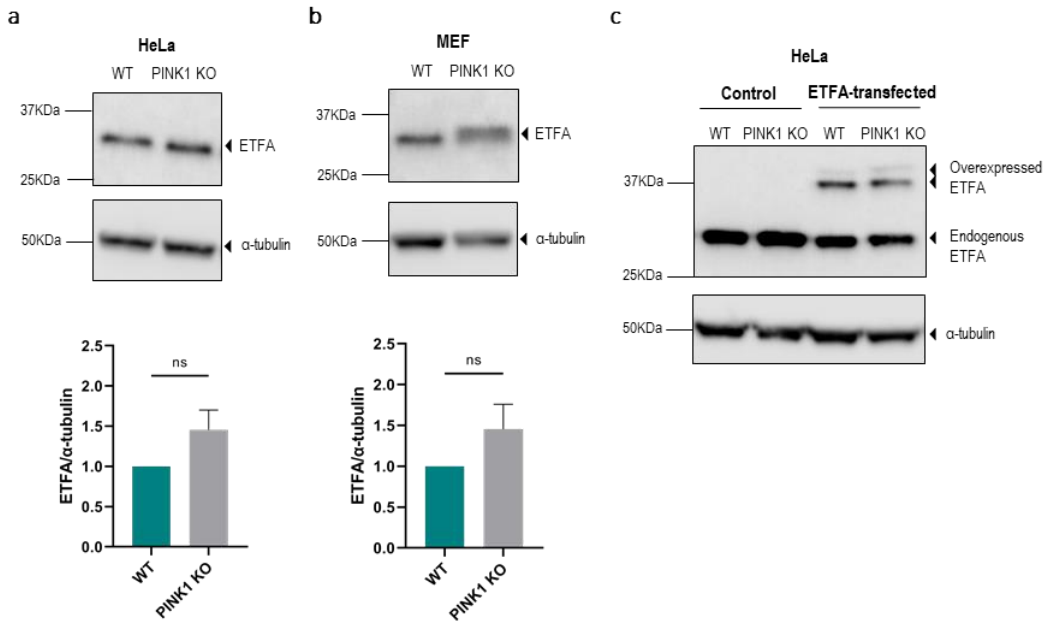

Figure 10: Western blot analysis of ETFA protein levels in WT and PINK KO HeLa and MEF cell lines.

(a, b) Representative immunoblot images of the endogenous ETFA protein levels in WT and PINK1 KO HeLa and MEF cells. Quantification graphs are presented for each cell line (N=3 experiments). Results were normalized to β-actin and to the correspondent WT lysates. (c) Immunoblotting analysis of the overexpressed ACAA2 protein in HeLa cells, and respective control (treated with the transfection reagent). Statistical analysis was calculated by using unpaired Student's t-test (ns, non-significant). Data expressed in mean ± SEM (MEF cell line: 0,4554 ± 0,3046; HeLa cell line: 0,4548 ± 0,2455).

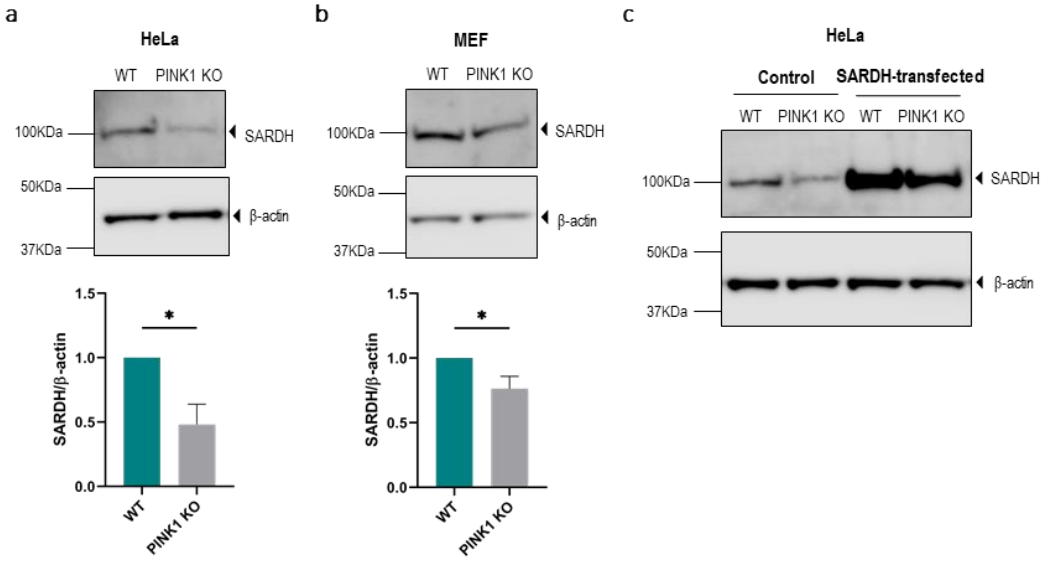

Figure 11: Western blot analysis of SARDH protein levels in WT and PINK KO HeLa and MEF cell lines.

(a, b) Representative immunoblot images for the endogenous SARDH protein levels in WT and PINK1 KO HeLa and MEF cells. Quantification graphs are presented for each cell line (N=4 experiments). Results were normalized to β-actin and within cell lines to the correspondent WT lysates (c) Immunoblot analysis of the basal SARDH protein levels in untreated HeLa cells and of the endogenous and exogenous protein in stably transfected HeLas. Statistical analysis was calculated by using unpaired Student's t-test (*, $p<0.05$). Data expressed in mean ± SEM (MEF cell line: -0,2365 ± 0,09563; HeLa cell line: -0,5184 ± 0,1582).

3.3. Assessment of substrate phosphorylation status using Phos-Tag SDS-PAGE

To assess the phosphorylation pattern of the candidate PINK1 substrates, Phos-Tag SDS-PAGE electrophoresis was performed using HeLa WT and PINK1 KO cells. For this, we transfected these cells with plasmids for ACAD9, ACAA2, DHTKD1 and SARDH. However, and most probably due to low expression levels of DHTKD1, we did not further analyse this candidate (data not shown). Also, due to lack of transfection efficiency with the ETFA plasmid, we did not further analyse this candidate either. For these two candidate substrates, we would need additional troubleshooting to identify an antibody with higher specificity for DHTKD1 and other plasmid and transfection conditions would need to be performed for ETFA.

In a first approach, transfected HeLa WT and PINK1 KO cell lysates prepared with RIPA buffer supplemented with protease and phosphatase inhibitors were analysed. The Phos-Tag blot probed with anti-Flag revealed lower phosphorylation levels for ACAD9 and ACAA2 proteins in the PINK1 KO cells compared with WT cells **(Figure 12a)**. For ACAA2, this difference in the phosphorylation of the protein was even more pronounced in the PINK1 KO conditions, suggesting an interplay between the two proteins. For the ACAD9 protein, while we were able to observe a two-band pattern in the Phos-Tag gel, we could not confirm if these corresponded to different phosphorylated forms of the same protein or if they were the full-length and cleaved forms already described. Since no western blot control was performed to assess the levels of the proteins in these samples, we could not confirm if the differences detected in both genotypes were indeed caused by an alteration in the phosphorylation of the protein or if resulted from lower protein amounts. Furthermore, since the Phos-Tag gels can detect both the phosphorylated and non-phosphorylated forms of the same protein, it was not possible to validate to which counterpart the observed bands corresponded.

Because of this, we next performed Lambda protein phosphatase (LPP) treatments on newly obtained cell lysates. HeLa WT and PINK1 KO cells transfected with the substrates plasmids were lysed with Triton-X buffer supplemented with protease inhibitors. These samples were incubated with LPP for 1 hour to induce dephosphorylation of proteins. As a control for the LPP reaction, samples were not incubated with LPP but processed in the same way as LPP-treated samples and were also loaded on the Phos-Tag gel. Moreover, cell lysates prepared with

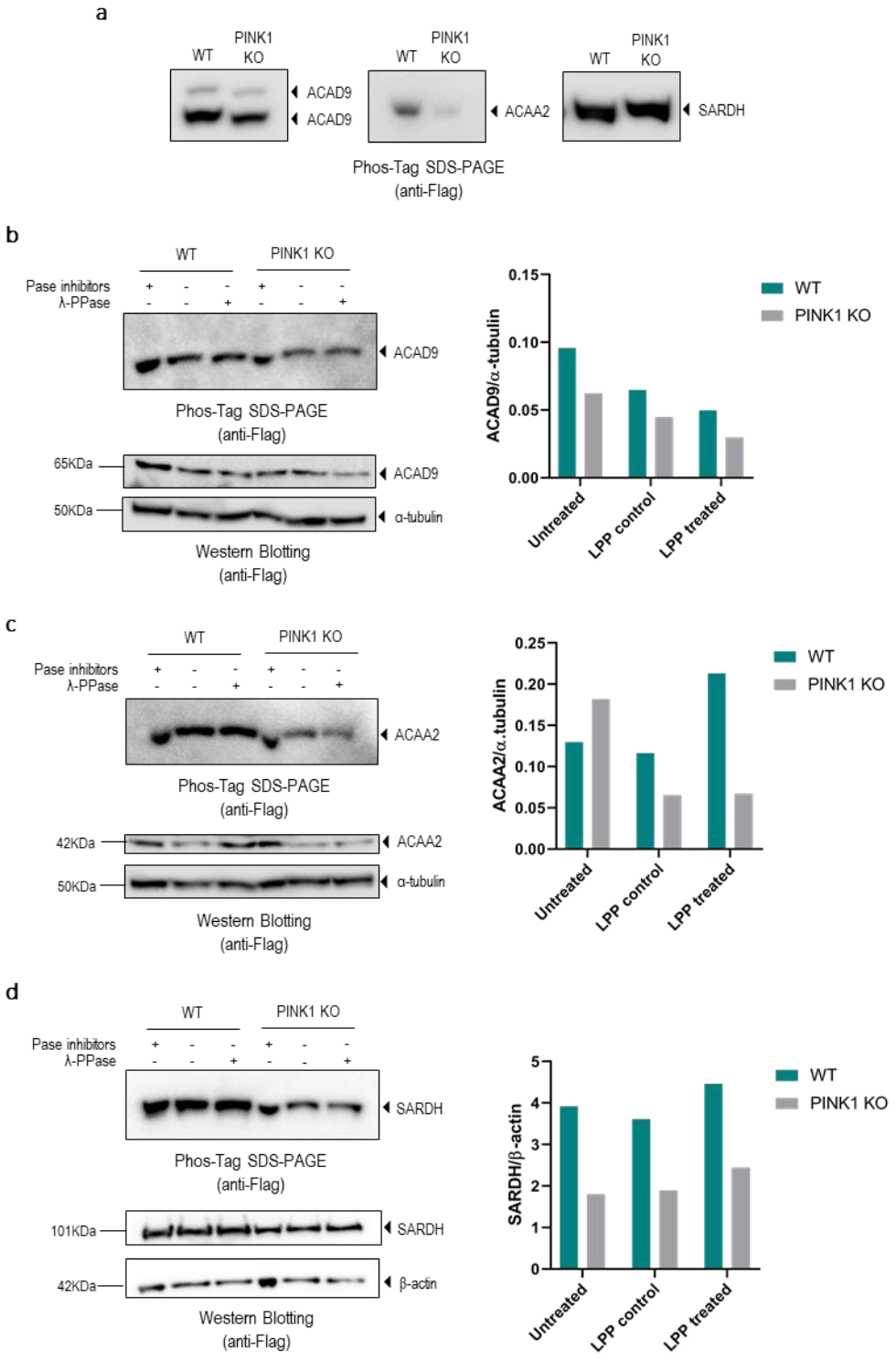

Figure 12: Difference in phosphorylation status of WT and PINK1 mutant HeLa cells overexpressing the candidate substrates by Phos-Tag SDS-PAGE assay.

(a) Phos-Tag SDS-PAGE electrophoresis followed by western blotting analysis with anti-Flag antibody on transfected HeLa cell lysates. (b, c, d) Flag-tagged ACAD9 (a), ACAA2 (b) and SARDH (d) overexpressed in HeLa cells were analysed by Phos-Tag SDS-PAGE (upper panels) and conventional SDS-PAGE (lower panels) and probed with anti-Flag. For the LPP dephosphorylation reaction, 100ug of protein were used. Graphs from the quantification of one experiment are shown for each protein. *Legend:* Pase: phosphatase; λ-PPase: protein phosphatase.

Triton-X supplemented with both protease and phosphatase inhibitors were also used in the Phos-Tag gel as a positive control. Control western blots were performed to assess the levels of the proteins in all treatment conditions for both genotypes. The Phos-Tag SDS-PAGE revealed lower phosphorylation levels of the ACAD9, ACAA2 and SARDH proteins in PINK1 KO cells in all conditions (Figure 12b, 12c and 12d). For the ACAD9 protein, while only one band was observed in both the Phos-Tag gel and in the control blot, it is possible that the full-length and cleaved forms of this protein are superimposed. The control western blots also demonstrated no significant differences in the protein levels between WT and PINK1 KO cell lysates. Even though this tendency towards decreased phosphorylation was observed when PINK1 was absent from cells, it was also noticeable that the LPP dephosphorylation did not worked in our conditions, since the bands correspondent to the LPP-treated and LPP control samples had the same intensity. To confirm if PINK1 is indeed mediating the phosphorylation of the overexpressed proteins in these conditions, a positive control for the LPP dephosphorylation reaction would need to be performed (e.g. phosphorylated-ERK enzyme), in order to assess if the phosphatase is working in our assay.

The differences observed in the overall phosphorylation status of these proteins in the WT and PINK1 KO conditions, with a tendency towards decreased phosphorylation levels when PINK1 is absent from cells, suggest that this kinase could be involved in the phosphorylation of the candidate substrates.

3.4. Immunoprecipitation of candidate substrates

To validate our proteins as putative PINK1 substrates, we aimed to perform an *in vitro* phosphorylation assay, in which the candidate substrates would be incubated with recombinant human PINK1 WT or the kinase inactive (KI) form in the presence of radiolabelled ATP to assess the incorporation of the labelled phosphate by the substrate. For this, we obtained enriched fractions of our substrates by immunoprecipitation of COS-1 cell lysates transfected with the proteins-expressing plasmids. Since these plasmids contain the Myc-tag at the C-terminal of their gene sequence, we used anti-c-Myc agarose beads to enrich for our protein of interest and then the Myc-peptide to elute these fractions. Results are shown in Figure 13.

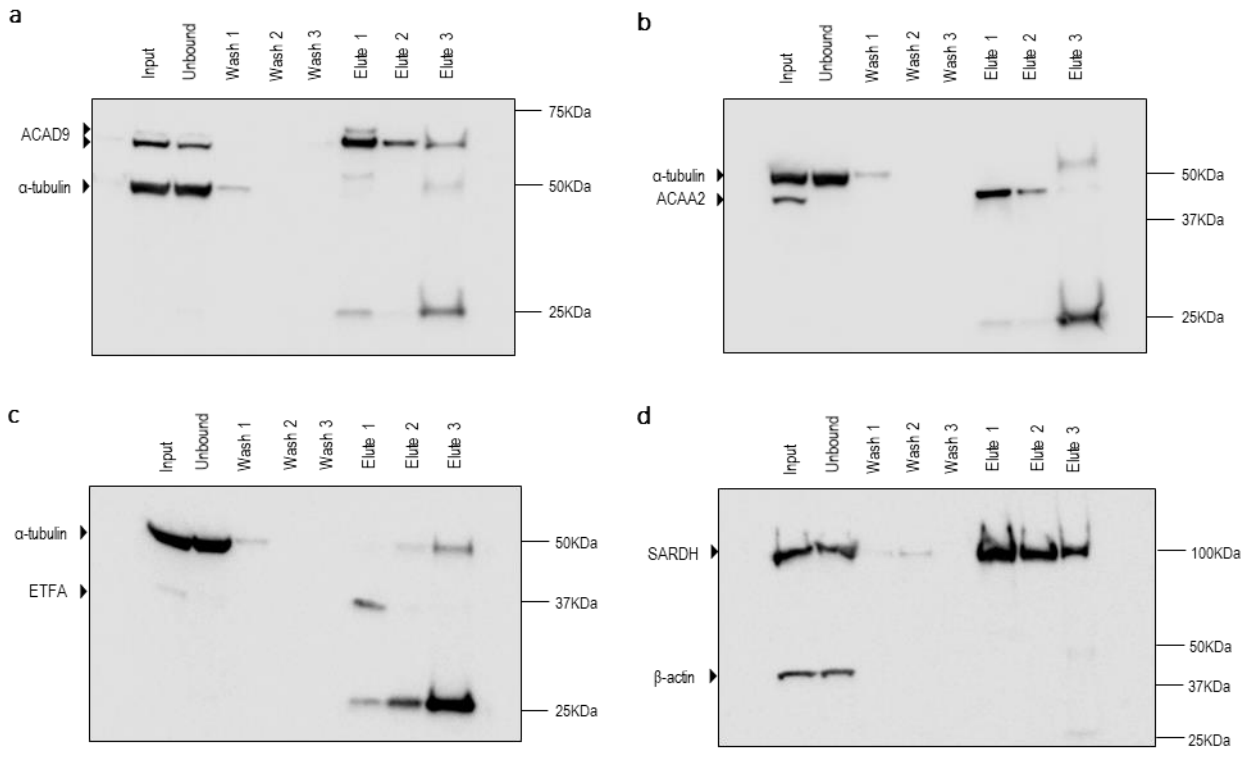

Figure 13: Immunoprecipitation of candidate substrates using anti-c-Myc beads and elution with myc peptide.

(a, b, c and d) Immunoblot analysis of the fractions obtained from the immunoprecipitation of ACAD9 (a), ACAA2 (b), ETFA (c) and SARDH (d) proteins from transfected COS-1 cell lysates.

The results of the immunoblot analysis performed with the different fractions from the immunoprecipitation (IP) assay not only confirmed the efficiency of this immunoprecipitation but also revealed high protein yields in the elute 1 when compared with the other elutes **(Figure 13)**. While this was the case for ACAD9, ACAA2 and SARDH, for the ETFA protein the immunoprecipitation was compromised by specific issues related with cell transfection, as it was revealed by lower protein levels in the input fraction. However, it was still possible to observe an enrichment of the protein in the elute 1. Probing with loading control further confirmed the specificity of the IP towards our substrates, since no α-tubulin or β-actin were detected in the elutes.

With these results, we were able to enrich for our candidate substrates, an essential first step for us to perform the *in vitro* phosphorylation assay.

Chapter 4
DISCUSSION

PD, the second most common neurodegenerative disorder worldwide, is a complex disease that mainly affects the motor system but that also comprises other non-motor symptoms (Poewe et al., 2017). With most of the patients displaying a sporadic form of the disease, only a small percentage exhibit a genetic-background form of PD. Among the genes with varying penetrance that have already been linked with familial PD, mutations in the PTEN-induced putative kinase 1 (PINK1) gene have been continuously studied (Vincenzo Bonifati, 2012). The identification of several recessive pathogenic mutations in the PINK1 gene throughout the years has strongly pointed out to a pathophysiological role of mitochondria in the development of PD. In fact, PINK1 plays different roles in mitochondria that are overall related with the maintenance of the organelles' homeostasis, including the regulation of the ETC, ATP production and trafficking in cells (Aerts, Strooper, et al., 2015; Das Banerjee et al., 2017; Exner et al., 2007). The localization, as well as all the different functions that this kinase plays in cells, seem to be dependent on the mitochondrial polarization state, with PINK1 being stabilized in the OMM and mediating the organelles clearance when mitochondria are damaged, or else being imported to the organelle to phosphorylate various substrates or undergo further processing under basal conditions. Even though clear evidences have pointed out to these multiple roles of the kinase in cells, various questions remain to be answered, including the underlying biochemical and physiological mechanisms of action of PINK1. Although different PINK1 phosphorylation substrates have already been identified, it is still uncertain how PINK1 recognizes the overall mitochondrial status in order to dictate its catalytic activity. Furthermore, none of these recognized PINK1 substrates can fully restore the phenotypes associated with a PINK1 loss-of-function. Therefore, to answer these questions, this study aimed to identify and validate novel PINK1 substrates and to assess the importance of such proteins for the overall kinase function in the mitochondria.

In order to identify novel putative PINK1 kinase substrates, a phosphoproteomics screen was performed using mitochondrial enriched fractions from WT and PINK1 KO mouse liver. A MS analysis was performed on statistically significant protein spots obtained by performing a 2D-DIGE analysis on these samples. The MS results gave rise to a list of proteins whose phosphorylation pattern was altered between these two genotypes. From this list, and based on protein analysis and literature research, a final candidates list was obtained (Table 1), from which five protein candidates were chosen for further validation in this study. The choice of these

candidates from the initial list was done after a thorough current knowledge review, with a research on the state-of-the-art for each candidate, as well as their functions in the cell (specifically on the mitochondria), and possible links with the PINK1 kinase protein.

The five chosen candidate protein substrates are mitochondrial related proteins, all nuclear-encoded and synthesized in the cytosol, that exhibit different roles in this organelle: the acyl-CoA dehydrogenase member 9 (ACAD9) and the 3-ketoacyl-CoA thiolase (ACAA2) are enzymes mainly involved in the fatty acid β-oxidation, catalysing the initial and final steps of that process, respectively. The 2-oxoglutarate dehydrogenase E1 (DHTKD1) catalyses one important step of the TCA cycle and lastly, the electron transfer flavoprotein subunit alfa (ETFA) and sarcosine dehydrogenase (SARDH) enzymes participate in a pathway that shuttles electrons to the main respiratory chain, contributing to the overall energy production.

Growing evidence has linked PINK1 with functions such as the lipid and amino acid metabolism, electron transfer to the ETC and overall maintenance of the respiratory chain and function. The metabolite stearic acid (an unsaturated fatty acid with an 18-carbon chain) was identified as a signalling molecule involved in the regulation of the mitochondrial function, through the de-stearoylation of the human transferrin receptor 1 (TFR1), in response do diet (Senyilmaz et al., 2015). A C18:0 supplementation was found to ameliorate the otherwise-altered mitochondrial activity in PINK1 and Parkin mutant flies, namely the ATP content and phenotypic defects commonly observed in these animals. More recent evidences have also linked this kinase with enzymes involved in the lipid synthesis and breakdown. The fatty acid synthase (FASN) enzyme, that catalyses the synthesis of palmitate, was shown to act as a negative regulator of PINK1, since inhibition of this enzyme was able to rescue specific PINK1-associated phenotypes in mutant flies (Vos et al., 2017). Furthermore, the downregulation of this synthase also increased the cardiolipin levels and restored the Complex I enzymatic activity in PINK1 KO mutant MEFs, correlating this lipid pathway with an already described PINK1 function. A different study based on an unbiased phosphoproteomic screening in a *Drosophila* model demonstrated that the medium-chain acyl-CoA dehydrogenase (MCAD), critical in the fatty acid metabolism, is phosphorylated in a PINK1-dependent manner, and that by mimicking phosphorylation of a specific residue of the peptide it was possible to partially restore some behaviour and morphological PINK1 mutant-flies deficiencies (Course et al., 2018). PINK1 was also shown to regulate the PI3-kinase-Akt signalling pathway, key in the modulation of cell

survival and growth, through Akt activation and Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) mobilization at the Golgi. Furthermore, PINK1 deletion resulted in Golgi fragmentation, and this phenotype was rescued simultaneously by this kinase overexpression and activation of Akt (Furlong et al., 2019). Given that these evidences point out to a link between these molecular pathways and PINK1, there seem to exist several clues on the importance of these biochemical mechanisms in the PD neuropathology, but also increasing evidences on the physiological role and participation of PINK1 in such pathways.

When assessing the candidate protein expression levels in MEF cells, decreased levels of the cleaved form of ACAD9 were observed in PINK1 null cells compared with the full-length form, which was surprising considering that the nuclear-encoded protein is normally targeted to the mitochondria and cleaved, becoming active on the fatty acid β-oxidation pathway and on the Complex I assembly. While in HeLa cells the absence of PINK1 seemed to impact both the full-length and cleaved forms of this protein, for MEF cells the increase in the full-length form of the protein in the absence of PINK1 suggests an impairment of the mitochondrial processing of ACAD9. Under basal conditions, PINK1 can participate directly or indirectly in the regulation of this processing and allow for the ACAD9 enzyme to normally function inside the mitochondria. However, when PINK1 is dysfunctional or absent from cells this may result in a cytosolic accumulation of ACAD9, therefore explaining the decrease of the cleaved form of the protein. This could be further confirmed by immunofluorescence experiments, where the cellular sub-localization of ACAD9 would be assessed in PINK1 WT and KO cells. Among the different possible explanations for this alteration are that PINK1 can somehow affect the ACAD9 import to the mitochondria through the translocase of the outer membrane (TOM), given previous evidences that PINK1 can associate with this complex, especially with the import receptor Tom20, in depolarized mitochondria (Lazarou et al., 2012). Furthermore, evidences have shown that absence of PINK1 can induce a decrease in the mitochondrial membrane potential (Morais et al., 2009), which can also impact the import of some mitochondrial targeted proteins, further supporting this theory. PINK1 could also be involved in the regulation of the mitochondrial processing of ACAD9 or have a role in this proteins stability via phosphorylation, given that this kinase is also imported to the mitochondria under basal conditions and phosphorylates several known substrates. While we could not validate ACAD9 as a putative PINK1 substrate, the observation of decreased phosphorylation levels of this protein in PINK1 KO cells overexpressing

ACAD9 through the Phos-Tag assay suggests an interplay between the two proteins in cells. One could hypothesize that PINK1 mediates the phosphorylation of the long-chain acyl-CoA dehydrogenase in the mitochondria under basal conditions, a function that could be required for activation of ACAD9 inside the organelle. An absence of phosphorylation could therefore result in an alteration of this proteins activation or stability inside the cells, overall impacting the ACAD9-mediated pathways. Although the preliminary data seems to suggest that PINK1 has indeed an impact on the mitochondrial processing of ACAD9, further work is needed to confirm this theory.

The observation of increased levels of the ACAA2 protein in PINK1 KO MEF cells was not in line with previous studies that had demonstrated through an analysis of the metabolome and mitochondrial proteome that this protein was downregulated in the brain of PINK1 KO rats (Villeneuve, Purnell, Boska, et al., 2016; Villeneuve, Purnell, Stauch, et al., 2016). A possible explanation for the upregulation of ACAA2 in our conditions were PINK1 is absent may be the fact that this enzyme is a critical component of the β-oxidation reaction, capable of metabolizing more than its specific medium-chain substrates. Even though mitochondrial fatty acid oxidation (mFAO) enzymes feature a higher specificity towards certain intermediates, each enzyme of this reaction can catalyse the conversion of different fatty-acid derivatives from a wide range of chain lengths (Houten & Wanders, 2010). Furthermore, the continuous repetition of the same cycles of reversible reactions can provoke a competition between fatty acids with different chain-lengths for the same unselective enzymes, and alternative compensation mechanisms may be evoked. While this may be the case for a rapid response by the cell to cope with the deficiencies at the fatty acid metabolism, the impact of such compensations on mitochondria and on the cell is perhaps insufficient for the long term survival, having at the end catastrophic effects on the cell. Recently, Martines and co-workers have demonstrated through a computational model of the mFAO pathway that substrate overload (specifically of palmitoyl-CoA, a sixteen-carbon fatty acid) impacts the general mitochondrial fatty acid β-oxidation via the generation of an unfavourable equilibrium of the different acyl-CoA molecules by the ACAA2 protein (Martines et al., 2017). Following palmitoyl-CoA overload, the medium-chain substrates of the ACAA2 enzyme start to built-up in the mitochondria due to a change in substrate specificity, which results in an accumulation of acyl-CoA esters from the previous reaction, in a depletion of the acyl carrier coenzyme A (CoA), and finally in a decline of the reactions flux. These alterations produce a

continuous cycle of enzymatic inhibition and further substrate overload, having an ultimate impact on the lipid oxidation metabolism over time.

Considering the alterations observed for the ACAD9 protein when the PINK1 kinase is absent from MEF cells (particularly the decrease of the cleaved form of the protein in the mitochondria), and the specificity of this enzyme towards long-chain acyl-CoA molecules, the observed increase of the ACAA2 protein levels in the same cellular conditions could suggest a mitochondrial compensation mechanism to cope with the increment of long-chain intermediates in the fatty acid oxidation reaction and to avoid a cataclysmic decline in energy and metabolite production. The apparent promiscuity of the ACAA2 protein could therefore function as an auto-propelling event that could lead to an increase in the availability of ACAA2 to metabolize all the accumulated intermediates and avoid an overall deficiency in the mFAO chain. Apart from this, the higher ACAA2 enzyme levels that are observed in PINK1 KO MEF cells could also result from a decrease in this enzymes degradation or decrease of its inhibition in cells, two processes that could be mediated by PINK1 under basal conditions and that may also be contributing to the accumulation of the protein in cells. The increase in the expression levels of the protein or decrease in its degradation could be due to an absent PINK1-mediated phosphorylation of this protein, since we observed lower levels of phosphorylation for this protein in PINK1-null cells through Phos-Tag SDS-PAGE. Although an *in vitro* kinase assay would need to be performed to validate ACAA2 as a bona-fide PINK1 substrate, the significant alterations observed by immunoblot suggest that PINK1 may be affecting this proteins activity or its stability in cells.

No significant alterations in the DHTKD1 protein levels were detected in PINK1 KO MEF cells. While the observation of the endogenous protein amounts in both HeLa and MEF cells was possibly hindered by antibody detection issues and low basal levels of this protein, the assessment of the exogenous DHTKD1 expression revealed higher protein levels in the lack of the PINK1 kinase. Even though we were able to make this observation for transfected HeLa cells, no solid conclusions could be taken from these results, since the DHTKD1 overexpression could most likely be affecting the normal intracellular pathways for this protein in these cells.

Although the subunit beta of the electron transfer flavoprotein (ETF) complex was not among the detected hits from the phosphoproteomics screening, alterations in the ETF subunit alpha (ETFA) were observed in the PINK1 KO mice. Given its enzymatic role in the oxidation of many specific mitochondrial matrix flavoprotein dehydrogenases that catalyse the oxidation of fatty

acids and some amino acids, the ETF complex is a major contributor to the OXPHOS chain, making the ETFA protein an interesting candidate of study. While our immunoblot analysis revealed a tendency towards increased protein levels in PINK1 null cells (although not statistically significant), previous studies have shown a downregulation of this electron carrier protein in PINK1-deficient rats through proteomic analysis of different brain regions (Villeneuve, Purnell, Boska, et al., 2016), as well as on isolated striatal synaptic mitochondria from PINK1 KO rats (Stauch et al., 2016). Even though our results are not in accordance with the already published data on the ETFA protein, the observed difference could be explained by the fact that the studies used different research models in addition to separate types of samples and preparations (whole cell lysates versus mitochondrial-enriched fractions).

The observed tendency towards upregulation of ETFA in our PINK1 KO conditions might be a part of a biological mechanism to compensate the overall impairment of the ETC and ATP production caused by the absence of PINK1. Given the recognized role of ETFA as an electron acceptor and shuttler to the respiratory chain through the ETF:Q oxidoreductase enzyme, the upregulation of this pathway could make up for the decreased Complex I activity that is typically detected when PINK1 is dysfunctional or absent from the cell (Morais et al., 2014). Furthermore, the alterations induced by the upregulation of the ETFA enzyme in our conditions could overall impact the OXPHOS chain and the cellular ATP content, which has also been observed as being decreased in PINK1 mutant cells (Morais et al., 2014). However, the apparent upregulation of the ETF subunit alpha alone may not be sufficient to elicit significant alterations on the activity of the Complex I or in the overall ATP levels in cells, especially in long-term responses. To clarify these questions and understand if the increased ETFA levels have indeed an impact on the overall energetic balance of the mitochondria, an evaluation of the Complex I enzymatic activity could be performed, together with an analysis of the ATP profile in MEF mutant cells.

The higher ETFA levels observed in PINK1 mutant cells may also be associated with the Complex I assembly function of the ACAD9 enzyme. As previously described, the ACAD9 protein operates as an assembly factor essential for the biogenesis of the OXPHOS chain Complex I. Pathogenic mutations in the ACAD9 gene have also been described has having negative effects over the expression of Complex I subunits and in the in-gel activity of this complex, without affecting other ETC complexes (Nouws et al., 2010). Moreover, these mutations do not seem to impact the long-chain fatty acid oxidation reaction also regulated by this enzyme. Although the

two known functions of ACAD9 can operate autonomously, it is possible that the biochemical alterations that are being induced in PINK1 KO cells are affecting both functions of the ACAD9 enzyme, even if by different pathways, through the decrease in the mitochondrial processing of this protein. Apart from inducing a decrease in the oxidation of long-chain fatty acids, the accumulation of ACAD9 outside of the mitochondria could also compromise the overall Complex I assembly, negatively impacting the OXPHOS and energy production. In the PINK1 KO cells, the higher ETFA protein levels could be a part of a compensatory mechanism to overcome an inefficient electron transfer from Complex I to ubiquinone and lower proton pumping. The increase in the ETF complex activity would therefore increase the shuttling of electrons from alternative pathways to the ETC, in an effort to maintain the ETC functional for a longer period of time. However, this effort may not be sufficient to resolve the energetic unbalance caused by the Complex I deficiencies, especially if the alternative metabolic pathways from which the ETF complex accepts electrons from (namely the fatty acid β-oxidation) are also affected by a PINK1 absence. Since we were not able to assess the phosphorylation status of ETFA through the Phos-Tag assay due to transfection issues or with the *in vitro* kinase assay, we could not determine if this protein is indeed a target of the PINK1 phosphorylation. Therefore, these experiments would need to be performed to determine the interplay of these proteins in cells.

One of the proteins that contributes to the electron pool of the ETF complex is SARDH. As it was observed by immunoblot, PINK1 KO cells have significant lower levels of this dehydrogenase, whose known function consists in the oxidative demethylation of the N-methylglycine amino acid into glycine in the mitochondrial matrix. The observation of decreased SARDH protein levels in PINK1 mutant cells further supports previous published data connecting PINK1 to the lipid and amino acid catabolism, ETC supply and general energy production (Course et al., 2018; Villeneuve, Purnell, Boska, et al., 2016; Villeneuve, Purnell, Stauch, et al., 2016). Moreover, since the reaction catalysed by SARDH is only one of the steps involved in the sarcosine degradation mechanism, itself a sub-pathway of the choline metabolism, it is possible that other enzymes that mediate the amino acid metabolism in the mitochondria are also affected by the absence of PINK1. Together, the alterations in these enzymes could elicit significant reductions in the electron shuttling to the ETC from specific mitochondrial pathways associated with both lipid and amino acid metabolism, ultimately having an impact on the overall ETC function and ATP balance in the cell. Phos-Tag gel analysis revealed that the phosphorylation levels of this protein

in PINK1 KO cells was decreased when compared to WT cells. Although only one assay was performed, the observed differences between genotypes suggest that PINK1 could contribute to the overall phosphorylation status of the protein in cells under basal conditions. The absence of PINK1 will therefore compromise this proteins function through alterations in its stability or activity, that could ultimately lead to an impairment in the SARDH-mediated pathways.

Based on our observations, we constructed a model of the substrates-mediated pathways in conditions where the PINK1 phosphorylation is compromised in cells (Figure 14). In this model, we present the alterations in the currently known molecular mechanisms in which our candidate substrates are involved in cells, based on our results from immunoblot analysis. Pathways marked in red are downregulated, while pathways in green suggest an upregulation of the proteins. The β-oxidation enzymes ACAD9 and ACAA2 are affected differently in PINK1 null MEF cells: while ACAD9 levels decrease when the kinase is absent, ACAA2 appears to be upregulated in these cells. Since the reaction catalysed by ACAA2 is downstream from the dehydrogenation of long-chain acyl-CoA molecules mediated by ACAD9, we propose that the increase of ACAA2 in our test conditions may result from the downregulation of the ACAD9 protein in this system. Since both proteins are involved in the same pathways, but one is upstream from the other, the observed increase in ACAA2 could result from an accumulation of long chain intermediates and alteration of the specificity of this protein towards their specific substrates. ACAD9 has also been described as mediating the assembly of the ND2 module of Complex I; therefore, our results could also suggest a compromised biogenesis of this ETC complex, overall impacting the OXPHOS chain and energetic balance of cells. Although statistical analysis revealed that the differences in the ETFA levels were not significant, immunoblots seem to suggest a tendency towards an upregulation of this protein in PINK1 null MEF cells. Moreover, given the known role of the ETF complex as a mediator of the electron transport to the ETC, an increase of the ETFA levels could be a part of a cellular mechanism to compensate the decreased activity of Complex I, which is known to be affected when PINK1 is dysfunctional. Furthermore, given that this complex accepts electrons from alternative energetic pathways such as from the fatty acid β-oxidation and amino acid catabolism, and we also observed significant decreases in the ACAD9 and SARDH proteins, the upregulation of ETFA could enhance the electron acceptance from these or other pathways in an effort to compensate the energetic deficits induced by the PINK1 deficiencies.

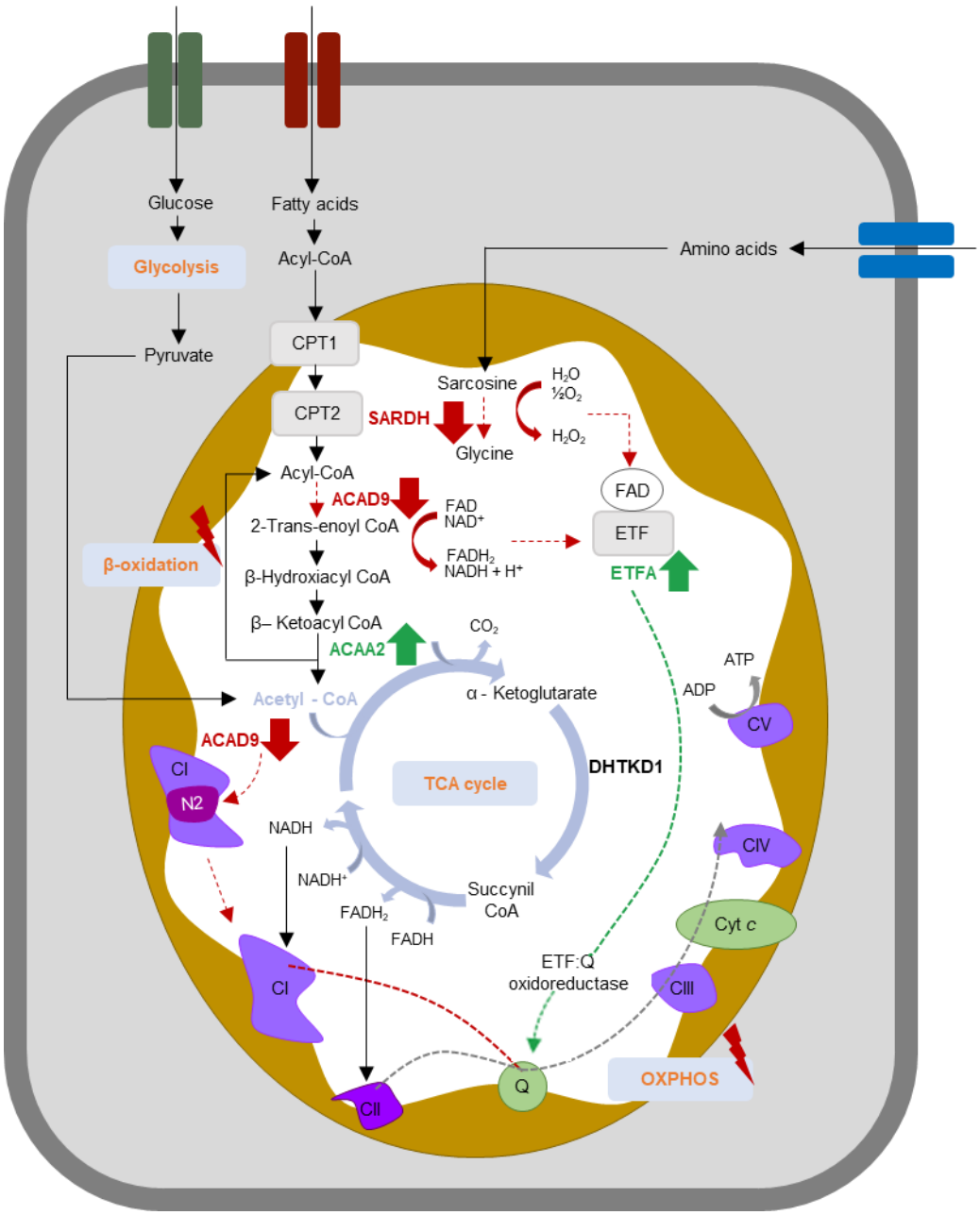

Figure 14: Overview of the cellular and mitochondrial pathways mediated by the PINK1 candidate substrates and alterations in disease.

While cells mostly rely on glucose to produce ATP and sustain their activities, alternative energetic pathways such as fatty acid and amino acid catabolism are major contributors for the cells energetic balance. In PINK1 null MEF cells, protein levels and phosphorylation patterns of specific proteins involved in these routes seem altered, including in ACAD9 and ACAA2 (two proteins involved in the β-oxidation of fatty acids), ETFA and SARDH, that overall contribute to the pool of electrons shuttled to the ETC. These alterations could affect these proteins-mediated pathways in disease states, possibly contributing to the mitochondrial defects induced by a PINK1 deficiency in cells (adapted from Tang et al., 2020).

Thus, the alterations of the phosphorylation status of our candidate proteins in PINK1 KO MEF cells could result in the observed alterations in the substrates activation or stability, strongly suggesting an interplay between these proteins and PINK1.

Overall, the differences observed in the proteins levels by immunoblot analysis suggest an interaction between our candidate substrates and PINK1. Further analysis of their phosphorylation patterns in WT and PINK1 KO cells supported that the PINK1 kinase could in fact be involved in the phosphorylation of these substrates. Additional evidence arising from the *in vitro* phosphorylation assays are needed to further validate this connection. Apart from given clues on the PINK1-biology under basal conditions and support previous evidences linking PINK1 with novel cellular and mitochondrial functions, these results could also contribute to the current knowledge of the PINK1 disease mechanisms in PD in the future.

Chapter 5
CONCLUSION AND FUTURE PERSPECTIVES

Being widely studied in the context of PD, the PINK1 enzyme has been progressively identified as a key protein in the regulation of many mitochondrial processes, greatly contributing to the general cellular maintenance and survival. While evidences have pointed out to the significance of this protein in the regulation of the mitochondrial quality control (one of PINK1 most studied functions), it has become increasingly clear that PINK1 can act in parallel in different pathways, both under steady-state and diseased conditions. Although multiple PINK1 phosphorylation substrates were already identified in the mitochondria, establishing a connection between this kinase and important aspects of the organelles morphology and physiology, none of the already identified putative substrates seems to be able to completely restore the enzymes signalling pathways in PINK1 loss-of-function contexts. In this light, the identification and validation of novel and bona-fide PINK1 phosphorylated targets in cells is of great importance to further understand this kinase signalling pathways under basal conditions, as well has to provide insights on how substrate recognition and consequent PINK1-mediated phosphorylation are relevant for the PD mechanism in neurons.

Based on a high-throughput phosphoproteomic approach, five protein hits that were altered in PINK1 KO mice were identified and selected for further study after literature review. Following this, immunoblot analysis of the candidate substrates was performed, which revealed statistically significant alterations in three of those proteins in PINK1 mutant MEF cells. To help understand if PINK1 could be mediating the phosphorylation of our candidate substrates, Phos-Tag SDS-PAGE analysis was performed for each of the proteins. We detected alterations in the phosphorylation pattern of the ACAD9, ACAA2 and SARDH proteins between WT and PINK1 KO cells, that could suggest a role for PINK1 in the activation or stability of these proteins in cells. However, given that only one experiment was performed, we cannot confirm this interaction, and more assays would need to be performed to validate our observations, with more controls for our test conditions. Additionally, these results could further help to support the validation of the candidate substrates as putative PINK1 targets that is going to be obtained with the *in vitro* kinase assay. Furthermore, the general phosphorylation of the ETFA protein still needs to be determined after overcoming the current transfection problems.

In order to validate our PINK1 candidate substrates with an *in vitro* kinase phosphorylation assay, we successfully immunoprecipitated our candidate proteins, obtaining substrate-enriched fractions to test our main hypothesis.

Although preliminary, our results are a first step towards the identification and validation of novel PINK1 substrates.

Due to different challenges that were faced during the year of this thesis, but especially because of the pandemic situation that affected the country, we were not able to complete all the tasks that had been planned for this project. While we successfully validated new antibodies for our selected substrates and evaluated their levels in two different cell lines, in the presence or absence of PINK1, we had planned to assess if the previously identified candidate substrates were true targets of the PINK1 phosphorylation by performing an *in vitro* kinase assay. This assay can be used to measure the enzymatic activity of a specific kinase towards the phosphorylation of a given substrate outside of the cell, and is based on the removal of a radioactively labelled phosphate group from a radioisotope ([γ-^{32}P]ATP) and transfer to the target substrate. Then the activity of the kinase can be measured by running an SDS-PAGE and visualized by quantification of the amount of radiolabelled substrate on a screen. For this project, we had aimed to assess if our proteins were indeed phosphorylated in a PINK1 dependent manner by incubating our proteins with human recombinant PINK1 WT or with the kinase inactive (KI) form of the enzyme, in the presence of [γ-^{32}P]ATP. Since the choice of our substrates was based on a phosphoproteomics study, we expected to confirm if indeed our proteins are phosphorylated by PINK1 in cells and if the alteration of that phosphorylation status is related with the alterations in activity or stability of the substrates that were observed by western blot. However, due to the pandemic situation the country faces, standard procedure such as training to work in the radioactive facility and the actual purchase of radioactive reagents, became a major hurdle and considerably delayed these experiments.

After validation of the candidate PINK1 phosphorylation substrates, we aimed to assess the functional relevance of the bona-fide substrates in specific PINK1-mediated pathways in the mitochondria, both under basal and diseased states. An initial approach towards this goal would be to generate phosphomimetic mutants of the PINK1 substrates and transfect them into PINK1 KO cells, in order to assess whether these proteins could restore the PINK1 functions related with the Complex I activity and selective removal of defective mitochondria from cells. If the substrates were able to restore the mitophagy defects induced by the absence of PINK1 from cells, an evaluation of the impact of these substrates on Parkin recruitment and mitochondrial

clearance would be performed. Using a cell-based assay, WT and PINK1 KO cells, expressing both Parkin and the substrates phosphomimetic mutants, would be labelled with markers for the mitochondria and Parkin. Then, after inducing mitophagy by incubating cells with an uncoupling agent (CCCP), we would assess the recruitment of the E3 ubiquitin ligase to the defective organelles through immunofluorescence techniques. Moreover, the evaluation of the mitochondrial morphology could further confirm the initiation of the mitophagy process and removal of the dysfunctional organelles from cells. Lastly, to determine if PINK1-mediated phosphorylation of these substrates is required for the recruitment of autophagic receptors, namely p62 and LC3, we would further evaluate the expression levels and cellular localization of these receptor-proteins by western blot and immunofluorescence, respectively. Overall, if we observed that the phosphomimetic substrate could recruit Parkin to the diseased-organelle in the absence of PINK1, this would suggest that the substrate in question is downstream of this kinase and that it participates in the PINK1-mediated quality control pathways that maintain a healthy pool of mitochondria in cells.

To assess the impact of the PINK1 substrates on the mitochondrial bioenergetics, the Complex I enzymatic activity would be measured using a spectrophotometric assay. The assessment of the activity of this particular ETC complex is important in the context of PD and PINK1 given that many evidences have linked PINK1 deficiencies with Complex I defects *in vitro*, but also because this particular OXPHOS complex is the only one which has been identified as significantly affected in PD patients with mutations in the PINK1 gene. This *in vitro* colorimetric assay analyses the oxidation-reduction reactions associated with the enzymatic function of this complex on the ETC, namely the oxidation of the electron transporting molecule NADH and reduction of the ubiquinone co-enzyme, after substrate administration. In the present study, we would evaluate the Complex I activity in WT and PINK1 KO cell lysates transfected with the plasmids for the putative PINK1 substrates or with their specific targeting shRNA to downregulate the proteins, in order to understand if the novel bona-fide PINK1 substrates could restore the Complex I defects induced by the absence of PINK1. To further determine the impact of the newly validated PINK1 substrates on the overall OXPHOS chain activity and energetic balance of the cell, a bioluminescence assay would be performed to evaluate the ATP content in WT and PINK1 KO cell lysates. This *in vitro* assay requires the presence of the recombinant firefly luciferase enzyme and its substrate D-luciferin, that is oxidized in an ATP-dependent manner,

resulting in the emission of light at 560 nm. Since this reaction is dependent on ATP, an increase in the ATP levels will enhance the luciferase enzyme activity, therefore emitting more light that can be measured spectrophotometrically and compared with a standard ATP curve. Since many evidences have also linked PINK1 dysfunction with lower ATP amounts in cells, this assay would further corroborate the functional importance of our substrates in the PINK1-mediated pathways.

After completing the third task, we hoped to have identified at least one putative PINK1 substrate whose function could restore, totally or partially, the PINK1 associated functions.

For the last task of this project, we aimed to determine the relevance of the validated PINK1 substrates in PD, in order to provide insights on how substrate recognition and consequent PINK1-mediated phosphorylation are relevant for the underlying disease mechanism. To achieve this, we would perform the same functional assays used in the validation of the novel PINK1 substrates but in the presence of PINK1 clinical mutants. For this, mutant cell lines could be used. Moreover, samples from PD patients with PINK1 mutations could be used to transpose the results observed in animal model to the human species.

Ultimately, the identification of novel PINK1 substrates will elucidate the mechanisms that underlie PINK1 substrate selection, and consequent maintenance of a healthy pool of mitochondria in cells, and more relevant in neurons.

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