Filipa Alexandra Barroso Gonçalves

Bachelor Degree in Human Biology

Dissecting the effect of Parkinson's Disease related PINK1 mutations on kinase activity

Dissertation to obtain the Master of Science Degree in Molecular Genetic and Biomedicine

Supervisor: Professora Doutora Vanessa Alexandra Morais

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

September 2016

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Acknowledgments

I would like first to thank my supervisor Dr.ª Vanessa Morais for the opportunity to develop this work at her lab, in addition all the support, time, patient and energy spent teaching me. Her office door was always open when I had some question. In this all year she helped me becoming not only a better scientist, but a better person. I couldn't have a better advisor and mentor.

Very important too where mine two laboratory colleagues Elvira Leites and Andreia Pereira. To them I want to thank not only the help and upright advices, but the good environment, provided since the beginning of this journey. Loving theirs job and transmitting it to me, was a good encouragement at my hardest times.

A very big thanks to the most especial persons in my life, my parents, my little one and my family, that always, not matter what, have been there for me, giving me unfailing support and affection. I want to acknowledge too the courage and strength, always given, not only at this stage, but from the very early days of my life.

To all my friends, especially José Albuquerque besides the endless patient and help, he was always there for taking care of me; Catarina Lopes, for her good mood, keeping me always smiling; Margarida Ferreira, which support was very important in the beginning of this journey; and Vânia Martins, that proves herself to be more than family.

Finally I want to express my gratitude to everyone that directly or indirectly, contributed to this work.

I hope I would made everyone as proud as I am for all the work and achieves reached.

Abstract

Parkinson's disease (PD), the second most common neurodegenerative movement disorder, affects approximately 2% of the population over 65. At present, there is only symptomatic but no causal cure for PD. Mitochondria are double membrane-bound organelles that are essential for energy production and cellular homeostasis in eukaryotic cells. Defects in this organelle are the underlying cause of several neurological disorders, namely PD. This mitochondrial connection has been furthered strengthened by the identification of mutations in the PINK1 gene that are linked to early-onset recessive PD. PINK1, a mitochondria targeted Ser/Thr kinase, regulates ATP production in healthy mitochondria by phosphorylating Complex I of the Electron Transport Chain. However, in damaged mitochondria PINK1 will phosphorylate Parkin and signal mitochondria for clearance via mitophagy. While understanding the regulation of PINK1 activity is pivotal to interpret how PINK1 executes its different functions in both healthy and damaged mitochondria it still remains unclear how PINK1 induced loss-of-function can affect the kinase activity and the overall (auto)phosphorylation status of PINK1.

To scrutinize the impact that the PINK1 clinical mutation have on PINK1 function, we systematically analysed five PD-causing clinical mutations G309D, L347P, E417G, H271Q and W437X. In order access their ability to phosphorylate the known PINK1 substrate Parkin and to (auto)phosphorylate PINK1 an in vitro phosphorylation assay was implemented. To determine their effect towards Parkin recruitment and sequential induction of mitophagy an immunofluorescence techniques was used where staining against Parkin and a mitochondria reside protein was performed.

Our results indicate that PINK1 is essential for Parkin recruitment, however the kinase activity is not required for this Parkin-mediated mitophagy pathway.

Keywords: Mitochondria, PINK1, Mitophagy, Parkinson's Disease

Resumo

A doença de Parkinson (DP), é a segunda doença neurodegenerativa mais comum, afetando aproximadamente 2% da população acima dos 65 anos. Actualmente, o único tratamento que existe é sintomático. As mitocôndrias são organelos com duas membranas, essenciais para a produção de energia e homeostase celular nas células eucarióticas. Defeitos nestes organelos estão aproximadamente 1% da população acima dos 65 anos. Actualmente, não existe nenhuma mas apenas existe um tratamento sintomático. As mitocôndrias são organelos com duas membranas, essenciais para a produção de energia e homeostase celular nas células eucarióticas. Defeitos nestes organelos estão na base de diversas doenças neurológicas, nomeadamente na DP. Esta conexão com a mitocôndria foi reforçada com a identificação de mutações no gene *PINK1* associadas à forma juvenil recessiva de DP. PINK1 é uma cinase Ser/The que regula a produção de ATP numa mitocôndria saudável, através da fosforilação do complexo I da cadeia de transporte de electrões. No entanto, em mitocôndrias danificadas, a PINK1 fosforila a Parkin sinalizando a mitocôndria para degradação, através de um processo chamado mitofagia. Desta forma, a compreensão da regulação da actividade da PINK1 é essencial para a interpretação de como esta proteína executa as suas diferentes funções, tanto na mitocôndria saudável como na danificada. Ainda existem dúvidas sobre como é que a perda de função por parte da PINK1 afecta a actividade de cinase e, consequentemente a (auto)fosfoforilação.

De forma a clarificar o impacto de mutações na função da PINK1, foram analisadas cinco mutações clínicas, G309D, L347P, E417G, H271Q e W437X, que culminam em DP. Para aceder à sua capacidade de fosforilar Parkin, já conhecido substrato da PINK1, assim como a própria autofosforilação desta, foi implementado um ensaio *in vitro*. Adicionalmente, para determinar o potencial efeito destas mutações no recrutamento da Parkin e posterior indução da mitofagia, recorreu-se a técnicas de imunofluorescência, utilizando marcação para ambas, Parkin e uma proteína mitocondrial endógena.

Os nossos resultados sugerem que PINK1 é necessária para o recrutamento da Parkin, mas a sua actividade de cinase não é imprescindível para a via de degradação mediada pela Parkin.

Palavras-Chave: Mitocôndria, PINK1, Mitofagia, Doença de Parkinson

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1. General Introduction

Parkinson's Disease

In 1817 James Parkinson describes the disorder that bears his name, publishing as *"An Essay on the Shaking Palsy*". At the time, he dubbed the disorder as "Shaking Palsy", once he observed what he thought that might be a neurological illness, consisting of resting tremor and a peculiar form of progressive motor disability, attending the signs and symptoms seen in six individual, three of whom were merely seen on London's streets (Parkinson, 2002). Later that century, these symptoms, including bradykinesia, muscular rigidity, resting tremor and postural and gait impairment, were refined by Jean-Martin Charcot (Charcot 1872).

Currently Parkinson's Disease (PD) is a known progressive multifactorial neurodegenerative disorder, characterized by the preferential loss of dopaminergic neurons in the region of the brain known as the *substantia nigra*, the disease's pathological feature. Importantly, neurodegeneration is not limited to the *substantia nigra*, the ventrolateral tier brain region which contains neurons that project to the dorsal putamen of the *striatum* is also strongly affected with disease progression, resulting in movement symptoms (Figure 1.1) (Farrer, 2006; Kalia & Lang, 2015). Clinical symptoms only appear when loss of dopaminergic neurons of the *substancia nigra pars compact* (SNpc DA) is approximately 50-70% (Orth & Schapira, 2002; Chinta & Andersen, 2008).

Neurodegeneration causes an imbalance of excitatory (acetylcholine) and inhibitory (dopamine) neurotransmitters and, it's believed that lesions in this specific area leads to the characteristic motoric symptoms of PD (reviewed in Kalia & Lang, 2015). Pathologically, a second hallmark is fibrillary α-synuclein intracellular inclusions, where the insoluble α-synuclein aggregates form inclusion bodies within the cell body (Lewy bodies) and processes (Lewy neurites) of neurons (reviewed in Capriotti & Terzakis, 2016).

PD affects approximately 2% of adults over the age of 65 and 4% of adults over the age of 80, which makes it one of the most common neurodegenerative disorders, second in prevalence to Alzheimer disease (Kalia & Lang, 2015; Capriotti & Terzakis, 2016). In an aging population where life expectancy is rising, this occurrence should rise by more than 50% by 2030 (Kalia & Lang, 2015). This raising in prevalence has been motivating the investigation around this disease as it is still unknown what triggers its initiation. However, the cause presumably is a combination of genetic and environmental factors where age is the most consistent risk factor (Capriotti & Terzakis, 2016). Despite this, little is known about the etiology of the sporadic form of the disease.

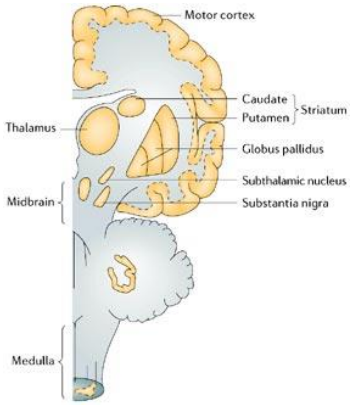

Figure 1.1 - The main brain regions affected in Parkinson disease. Represented is a lateral brain's section, with the anterior to the left. The yellow areas are affected in PD (Farrer, 2006)

Presently, there is no cure for PD, the only existing treatment is symptomatic (Samii *et al.*, 2004; Capriotti & Terzakis, 2016). The most common pharmacologic treatment used is levodopa, since it provides the greatest symptomatic help, but it losses it effectiveness due to habituation. Another therapeutic options includes surgical options, from deep-brain stimulation to restorative treatment, but negative results have dampened enthusiasm in these approaches (Samii *et al.*, 2004; Capriotti & Terzakis, 2016). There has also been some substances describe as potential neuroprotective agents, such as Vitamin E, selegiline and coenzyme Q10, but no irrefutable evidences exists (Samii *et al.*, 2004).

Mitochondria and PD

The etiopathogenesis of sporadic cases is complex, but it is believed that genetic susceptibility and environmental factors contributes to this disease progression and both influence various mitochondrial aspects, such as bioenergetics, quality control, dynamics and transport (Moon & Paek, 2015). It is well known that aberrant mitochondrial forms and functions are connected with idiopathic (or sporadic) and familial PD (Henchcliffe & Beal, 2008). However, the mechanism still remains to be clarified. Mitochondrial dysfunctions are mainly characterized by the generation of reactive oxygen species (ROS), a decrease in the electron transport chain (ETC) Complex I activity, ATP depletion and cleaved caspase-3 activation (reviewed in Moon & Paek, 2015).

There is increasing evidence that link Complex I function to PD. Complex I is the first complex of the multimeric enzymatic system of the ETC, whose overall function is the generation of ATP.

The role of mitochondria in PD became evident when it was discovered that the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) metabolite, MPP^+ (N-methyl-4-phenylpyridinium) inhibits Complex I of the ETC leading to DA loss in a way very similar to PD. After MPTP enters the cells through monoamine transporters, MPP^+ binds and inhibits NADH CoQ10 reductase, decreasing ATP synthesis and increasing generation of free radicals (Orth & Schapira, 2002; Pesah *et al.*, 2004).

Besides MPTP, rotenone also inhibits the Complex I by binding to the Complex I subunit ND-1 protein and leading to increased ROS levels. Rotenone induces a Parkinsonism syndrome in animal models and also in humans (Narendra, Tanaka, Suen, & Youle, 2008; Schapira et al., 1990). Another poisonous reagent is paraquat, whose toxicity is executed in a similar fashion as MPP^+, causing generation of free radicals and oxidative stress (Schapira *et al.*, 1990; Berry *et al.*, 2010).

It is also worth noting that the brain consumes 20% of total resting body energy, therefore with such a high demand in mitochondria driven ATP production it is not surprising that hampered mitochondria will lead to a diseased brain (Orth & Schapira, 2002; MacAskill & Kittler, 2010). Further, SNpc DA have been characterized as a highly energy demanding population of neurons, thus it should be expected an increased mitochondria biogenesis, as well as increased basal oxidative phosphorylation (Henchcliffe & Beal, 2008). This feature must be due to the metabolic sustaining of their enormous axonal arborization, demonstrated by Pacelli *et al.*, 2015. It was also shown that mitochondrial reactive oxygen species (mROS) production is higher in these neurons due to the dopamine oxidative metabolism. Further, antioxidants such as reduced glutathione, are weakly synthesized in SNpc DA (Chinta & Andersen, 2008). This bioenergetics and morphological characteristics make SNpc DA more vulnerable to mitochondrial dysfunctions.

Then, what makes Mitochondria so special?

Known as the energy powerhouse of the cell, Mitochondria are double membrane organelles that have 4 distinct sub-mitochondrial compartments: the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM), and the matrix. The compartmentalization is crucial for vital mitochondrial functions. They actively sustain a highly negative potential across their inner membrane ($\Delta\Psi$) that is maintained by four protein complexes I, II, III and IV of the mitochondrial ETC, that together with the F0/F1-ATP-synthase (Complex V), constitute the oxidative phosphorylation (OXPHOS) system. Maintenance of a highly negative potential across their inner membrane ($\Delta\Psi$) is essential for mitochondrial function and cell viability (Schapira, 2010).

Mitochondria are semi-autonomous since they contains their own DNA (mtDNA), a double stranded 16.6-kb circular molecule consisting of a heavy (H) and a light (L) chain, without any histone

coating. The mtDNA encodes 13 proteins, 2 ribosomal RNAs and 22 transfer RNAs. The mtDNA has some particularities: it is only inherited from the mother; exists approximately 8-10 mtDNA's per mitochondrion, varying in different tissues; and the 13 proteins translated are all components of the ETC. Even having their own DNA, an overwhelming number of nuclear encoded proteins are targeted to the mitochondria, such as replication, transcription, translation and repair proteins (Orth & Schapira, 2002; Palikaras & Tavernarakis, 2014). The mtDNA does not have a complete repertoire of repair mechanisms to eliminate mutated DNA sequences, making it therefore rather vulnerable to mutations that accumulate with aging (Orth & Schapira, 2002). To prevent and reduce potential mitochondrial stress generators, these organelles have a mitochondrial unfolded protein response (UPR^{mt}) system that deals with the accumulation of misfolded and toxic proteins (Moon & Paek, 2015). The UPR^{mt} is composed by controlling chaperones and transcriptional proteases (Roberts *et al.*, 2016). In addition, evidences suggest that vesicles derived from mitochondria engulf selected mitochondrial cargos and deliver them to peroxisomes for degradation. At the same time anti-oxidant pathways are activated, for example mitochondrial superoxide dismutase and glutathione (Roberts *et al.*, 2016). In addition to these processes, mitochondria are able to regulate their internal quality by two processes: one that allows degradation of OMM proteins through the ubiquitin-proteasome system; and another one that uses the autophagy-lysosome pathway for elimination of mitochondria as whole organelles, known as mitophagy (Palikaras & Tavernarakis, 2014; Scarffe *et al.*, 2014; Eiyama & Okamoto, 2015)

Mitochondria have an important role in cellular processes by supporting cellular metabolic events, as iron-sulfur cluster biogenesis, amino acid synthesis and lipid metabolism. In order to maintain cellular homeostasis, mitochondria are able to regulate the calcium influx and inhibit apoptosis. Some of these cellular reactions may lead to oxidative stress with ROS formation, as superoxide anions, hydroxyl radical and hydrogen peroxide (Orth & Schapira, 2002). Mitochondria are fueled by pyruvate and fatty acids, which are used as carbon sources for the tricarboxylic acid cycle (or Krebs cycle) in the mitochondrial matrix.

The aim of mitochondrial quality control is the maintenance of a healthy pool of mitochondria within the cell. This term is used to describe the coordination of mitochondrial dynamics, biogenesis and mitophagy (Scarffe *et al.*, 2014). Mitophagy is a specialized mitochondria autophagy, or more specifically macroautophagy, since it involves sequestration in a double membrane structure called autophagosome of mitochondria and selectively triggering them for clearance (Fig. 1.2) (Hattori *et al.*, 2014; Scarffe *et al.*, 2014).

Mitochondria can change shape, size and inner membrane organization. All these processes are regulated by fusion and fission events. In mammalian cells, mitochondrial fission depends on the GTPase dynamin-related protein (Drp1) (Büeler, 2010; Roberts *et al.*, 2016). Dpr1 translocate from

the cytosol and accumulates on OMM, where it oligomerizes into ring-like structures that will constrict the mitochondria through GTP hydrolysis, leading to the formation of new "daughter" mitochondria. This process is essential in facilitating mitochondrial transport as well as the autophagic degradation of damaged mitochondria. On the other hand, fusion depends on the action of 3 GTPases: Mitofusins 1 and 2 (Mfn1/2) that are OMM proteins that tether organelles to the membrane for OMM fusion; and Optical atrophy 1 (Opa1), an IMM protein that mediates inner membrane fusion (Büeler, 2010; Roberts *et al.*, 2016). Fusion process is crucial for maintain a functional mitochondrial population within a cell, as mitochondria do not function in isolation but rather in a complex extensive network, its morphology undergoes continuous changes in response to metabolic stimuli and signaling pathways. Fusion also allows possible exchanges of contents between mitochondria (Detmer & Chan, 2007).

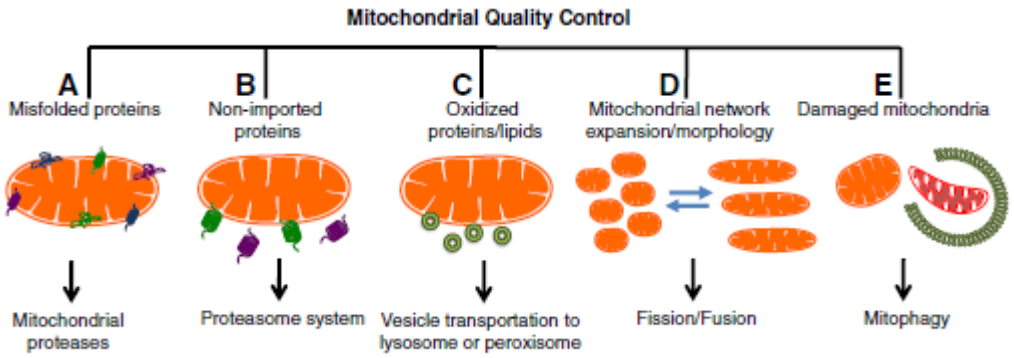

Figure 1.2 - Mitochondrial Quality Control. Mitochondria have different pathways to promote mitochondrial biogenesis and dynamics. (A) Proteolytic system; (B) Proteasome system; (C) Transportation to lysosome or peroxisome; (D) Fission/Fusion; (E) Mitophagy (Palikaras & Tavernarakis, 2014)

Genetics in PD

Nowadays, it is accepted that the involvement of mitochondria in PD is not only restricted to a decrease in ATP and increase in ROS production arising from the defective function of the respiratory chain. Defects in mitochondrial trafficking, dynamics, identification of mutations in genes involved in mitochondrial mitophagy) or defects in mitochondrial calcium buffering are emerging as mitochondrial dysfunctions related to PD (Aroso *et al.*, 2016). Thus, the regulation of these mechanisms is essential to maintain mitochondria healthy.

The past 15 years were marked by important discoveries which have led to a better understanding of the molecular pathogenesis of PD. Although 90% of cases are considered sporadic, the identification of genes responsible for familial forms of PD where a clear "mendelian" autosomal dominant (where one mutated allele is sufficient to cause the disease) or autosomal recessive pattern

(where two mutated alleles are needed to originate the disease) of inheritance is observed have been crucial for a better understanding of the disease (Gasser, 2009; Scarffe *et al.*, 2014; Kalia & Lang, 2015).

More than ten genes have been identified, and six of these genes were identified as mediating the autosomal dominant forms of PD, being the most common *SNCA* and *LRRK2* (table1.1). The gene *SNCA* encodes the α-synuclein protein, the principal constituent of Lewy bodies (Polymeropoulos *et al.*, 1996). So far, reports have identified three different missense mutations, as well as duplications and triplications (Klein & Westenberger, 2012). Three missense mutations impair the amino-terminal domain of α-synuclein causing misfolding and aggregation of the protein, a feature that is strongly correlated with PD (Klein & Westenberger, 2012; Recasens & Dehay, 2014).

The most frequent cause of autosomal dominant PD are mutations in *LRRK2* (Klein & Westenberger, 2012). This gene encodes the protein leucine-rich repeat kinase 2 LRRK2, a large multidomain enzyme, coupling kinase and GTPase activities with a number of protein/protein interaction domains (Paisán-Ruiza *et al.*, 2013).

Autosomal recessive PD occurs less frequently but occurs in early-onset of the disease. In the form the associated genes are *Parkin* (Kitada *et al.*, 1998), *PINK1* (Valente *et al.*, 2004) and *DJ-1* (Bonifati *et al.*, 2003) (table1.1), interesting all implicated within mitochondria pathways.

Table 1.1 – Most frequently genes implicated in monogenetic PD. Most frequently genes confirmed to be implicated in autosomal dominant (AD) or autosomal recessive (AR) monogenetic PD. (adapted from Spatola & Wider, 2014).

Gene	Mutations	Inheritance	Gene poduct
SNCA	A53T, A30P, H50Q, G51D, E46K, triplication, duplication	AD	α-synuclein
LRRK2	G2019S, N1437H, R1441C/G/H, Y1699C, I2020T	AD	Leucine-rich repeat kinase 2
Parkin	>100 mutations	AR	Parkin, E3 ubiquitin ligase
PINK1	>50 mutations	AR	PTEN-induced kinase 1
DJ-1	>10 mutations	AR	Daisuke Junko 1

Parkin, the second largest gene in human genome (Kitada *et al.*, 1998; Klein & Westenberger, 2012), encodes an E3 ubiquitin ligase that catalyzes the ubiquitination of a range of proteins (Sarraf *et*

al., 2013), particularly on damaged mitochondria (Narendra *et al.*, 2008). In agreement, mutations in this protein appear to lead to mitochondrial dysfunctions (Greene *et al.*, 2003). Pathologically patients with these alterations, although lacking α-synuclein aggregates, display clinical features of idiopathic (or sporadic) PD (Greene *et al.*, 2003; Pesah *et al.*, 2004).

PINK1, another autosomal recessive gene, encodes for the a serine/threonine (Ser/Thr) kinase PINK1, whose mutations are less common than Parkin (Gasser, 2009). There is a genetic link between PINK1 and Parkin as mutant models for both proteins seem to have the same phenotypes, such as flight and climbing defects in the *Drosophila* model (Greene *et al.*, 2003; Clark *et al.*, 2006; Park *et al.*, 2006). Together, PINK1 and Parkin regulate mitochondrial quality control via clearance of damaged mitochondria (Narendra *et al.*, 2010).

Mutations in the *DJ-1* gene are the least common and its function it is still not well understood (Bonifati *et al.*, 2003). The protein encoded is member of ThiJ/PfpI family and has H_2O_2 responsiveness, functioning as a sensor for oxidative stress and is an antioxidant (reviewed in Cheon, Chan, Chan, & Kim, 2012).

PINK1

Encoded by the *PARK6* gene in chromosome 1p36, alterations in this protein are the second most common cause of early onset autosome recessive PD (Hatano *et al.*, 2004; Bonifati *et al.*, 2005; Singleton *et al.*, 2013; Requejo-Aguilar & Bolaños, 2016). Phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (*PINK1*) encodes a 581 amino acid protein with an N-terminal mitochondrial targeting sequence (MTS) spanning from residues 1-34, a conservative serine/threonine kinase domain from residues 150-513 and a C-terminal non catalytic region from residues 541-581 (Valente *et al.*, 2004; Kondapalli *et al.*, 2012). A hydrophobic patch formed by 11 amino acids can also be found after the MTS. Bioinformatic analysis suggests that residues Gly-193 to Lys-507 form the ATP-binding cassette whereas residue Thr-313 is a autophosphorylation regulatory residue (Petit *et al.*, 2005).

PINK1 is ubiquitously expressed in all brain regions and in all cells types. Sporadic cases of PD or PD-related clinical mutations do not affect expression levels or localization pattern of PINK1 (Gandhi *et al.*, 2006). PINK1 has been detected in both cytosol and mitochondria (Valente *et al.*, 2004; Beilina *et al.*, 2005; Silvestri *et al.*, 2005; Lin & Kang, 2008).

Drosophila PINK1 mutants exhibit male sterility, wing postural instability with rigidity that lead to locomotion difficulties, flight muscle degeneration, mitochondrial impairment, low ATP levels and apoptosis (Clark *et al.*, 2006; Park *et al.*, 2006; Scarffe *et al.*, 2014). Studies showed that PINK1

kinase is essential for maintaining mitochondrial integrity and functions *in vivo*, as well as to dictate Parkin localization through direct phosphorylation (Kim *et al.*, 2008). PINK1 knockout mouse or human dopaminergic neurons besides showing a high sensitivity to apoptosis also have abnormalities in mitochondrial morphology, a reduced membrane potential, and an increased ROS generation (Morais *et al.*, 2009; Moon & Paek, 2015; Pacelli *et al.*, 2015)

In healthy mitochondria, PINK1 is guided into mitochondria through the mitochondrial import machinery translocase of the outer membrane (TOM) and of the inner membrane (TIM) complexes, in a mitochondrial membrane potential dependent manner (Fig.1.3). PINK1 is translocated, partially, through TOM and TIM exposing the positively charged MTS to the matrix, which is removed by mitochondrial processing peptidase (MPP), and then cleaved by the inner mitochondrial membrane protease presenilin-associated rhomboid like protease (PARL) (Nguyen *et al.*, 2016). Thus, in cells normal conditions, three forms of PINK1 protein are detected: the full-length form (63 kDa) and two cleaved forms one form at approximately 55kDa that represents an intermediate PINK1 species produced by the inner mitochondrial membrane protease presenilin-associated rhomboid like protease (PARL); and 45kDa form cleaved by MPP-mediated cleavage, between aminoacids Alanine 103 (Ala103) and Phenylalanine 104 (Phe104) (Deas *et al.*, 2011; Meissner *et al.*, 2011) and then translocated to the cytosol where it is rapidly degraded through N-end rule pathway (Greene *et al.*, 2012; Song *et al.*, 2013; Yamano & Youle, 2013; Voigt *et al.*, 2016).

Complex I of the ETC is pivotal in generating the electrochemical gradient across IMM. Compromised Complex I activity has been related with PD sporadic cases (Schapira *et al.*, 1990) and further corroborated in PINK1 null mice (Morais *et al.*, 2009). Vilain and co-workers suggested a connection between PINK1 and Complex I where PINK1 is acting with or in parallel with Complex I (Vilain *et al.*, 2012). The yeast Complex I Ndi1p rescued several phenotypes observed in *Drosophila PINK1* mutants further strengthening Complex I deficiency as the underlining cause of PINK1 related phenotypes. Later, Morais et al., 2014 showed that PINK1 regulates Complex I function by phosphorylating NdufA10, one of the 46 subunits present in Complex I.

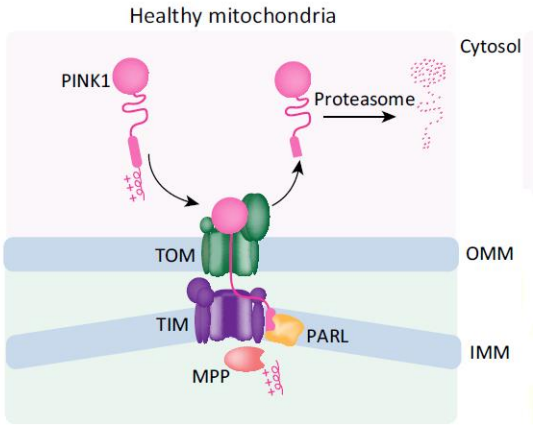

Figure 1.3 - PINK1 processing in healthy mitochondria; firstly PINK1 is imported into OMM through TOM, and then IMM over TIM, where it is processed by MPP and PARL, exposing N-end rule substrate cytosolic and promoting PINK1 degradation (adapted from Nguyen, Padman, & Lazarou, 2016).

When mitochondria are depolarized, import of PINK1 within the mitochondria is inhibited and PINK1 is stabilized on the mitochondrial outer membrane triggering the damaged mitochondria for clearance via aPINK1/Parkin mediated mitophagy pathway (Narendra *et al.*, 2010; Seirafi *et al.*, 2015). PINK1initiates mitophagy by phosphorylating Ubiquitin (Koyano *et al.*, 2014), Mitofusin 2 (Chen & Dorn, 2013) and Parkin (Clark *et al.*, 2006; Park *et al.*, 2006; Matsuda *et al.*, 2010). For this, PINK1 has to be dimerizated and autophosphorylated on residues Ser^{228} and Ser^{402} (Okatsu *et al.*, 2012; Aerts *et al.*, 2015).

Approximately 50 pathogenic PINK1 mutations were identified; being that large amount of them located within the kinase domain, suggesting that this kinase activity plays a crucial role in the PD pathogenesis (Fig.1.4) (Rogaeva *et al.*, 2004; Bonifati *et al.*, 2005; Criscuolo *et al.*, 2006; Kawajiri *et al.*, 2011). These homozygous point mutations R246X, H271Q, E417G and L347P, involving exons 3, 4, 5 and 6, and two nonsense mutations Q239X and R492X were identified in Asian families (Hatano *et al.*, 2004). The residues implicated in H271Q, E417G and L347P mutations appear to be highly preserved between PINK1 homologs (Hatano *et al.*, 2004). Two missense mutations (E420K and L489P) were described to abrogate PINK1 protective effect against cell death (Petit *et al.*, 2005). All patients shown early age onset, long disease duration and good response to L-dopa, therefore there does not exist any clinical features that will distinguish *PINK1* mutations from *Parkin* or *DJ*-1 mutations. Atypical clinical features have been observed including psychiatric disturbances, dystonia at onset and sleep benefit (Hatano *et al.*, 2004; Valente *et al.*, 2004).

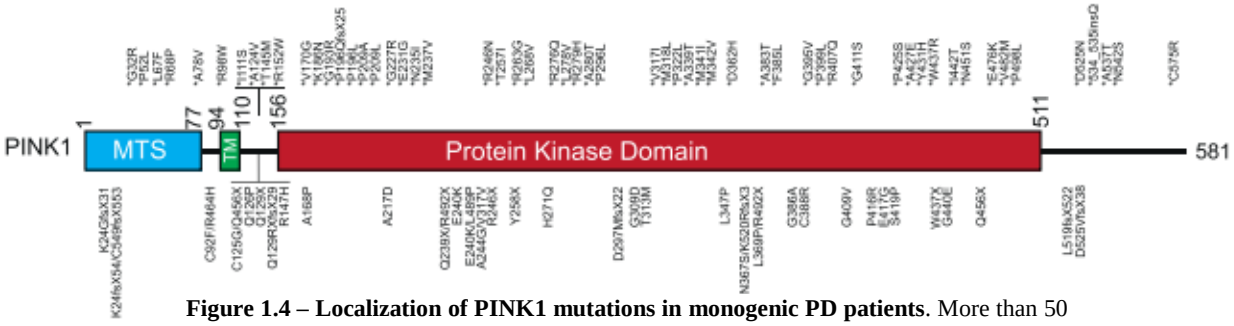

Figure 1.4 – Localization of PINK1 mutations in monogenic PD patients. More than 50 mutations have already been identifies; MTS = Mitochondrial Targetting Sequence, TM = Transmembrane Domain (Pickrell & Youle, 2015)**.**

PINK1 is known to be included in numerous pathways, and to interact with several substrates related to mitochondrial homeostasis and mitochondrial quality control (Table 1.2) (Gómez-Sánchez *et al.*, 2016). PINK1 is also responsible for blocking the activation of apoptotic signalling pathways by reducing caspase-3 activity and Cytochrome *c* translocation (Petit *et al.*, 2005). Other studies have also shown that PINK1 prevents the transport of defective mitochondria along microtubules by phosphorylating Miro (Weihofen *et al.*, 2009).

PINK1 is pivotal in promoting cell survival by interacting with a mitochondrial molecular chaperone that protects against oxidative-stress-induced apoptosis, TNF receptor-associated protein 1 (TRAP1) (Pridgeon *et al.*, 2007). The clinical PINK1 mutants G309D and L347P were unable to phosphorylate TRAP1, in contrast with W437X clinical mutations where no difference was observed when compared to WT PINK1. These results suggest that cell survival may be affected by PINK1 function, especially in the case of the G309D and L347P clinical mutants.

It was also shown that kinase activity of PINK1 is involved in the regulation of mitochondrial apoptotic pathway, as its depletion increases cells susceptibility to oxidative stress induced apoptosis (Pridgeon *et al.*, 2007). It is conceivable to assume that alterations in this domain may lead to dysfunctions in kinase activity of this protein, and indeed L347P mutations appears to lead to a protein instability (Beilina *et al.*, 2005). But studies about this possible dysfunction have been a little controversial. In some studies the in vitro phosphorylation assay were performed with hPINK1 orthologues instead, such as *Tribolium castaneum* TcPINK1, that is remarkably more active than hPINK1(Woodroof *et al.*, 2011). Recently, it was shown that different PINK1 orthologues have different substrate selectivity, so the extrapolation of obtained results back to human scenario need to be performed with caution (Aerts *et al.*, 2016).

Table 1.2 – Resume of PINK1 Substrates mentioned. Besides (auto)phosphorylates itself, PINK1 as another substrates that allows it to control pathways as mitophagy, mitochondrial motility and oxidative phosphorylation (OXPHOS). Only Miro,

TRAP1, Parkin and Ubiquitin are described as directly phosphorylated by *human* PINK1 (MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; IMS, intermembrane space).

Substrate	Localization	Function
Miro	MOM	Mitochondrial motility
Mitofusin 2	MOM	Mitophagy
NDUFA10	MIM	OXPHOS
Parkin	Cytosol/MOM	Mitophagy
TRAP1	IMS/MIM	Stress response
Ubiquitin	Cytosol/MOM	Mitophagy

Parkin

Mutations in the *PARK2* gene, also known as *Parkin*, are the most common cause of autosomal recessive PD. The gene encodes a 465-amino acid E3 ubiquitin ligase, member of the RING1-in Between-RING-RING2 (RBR) family of E3's, capable of mediating mono or poli-ubiquitination (Scarffe *et al.*, 2014; Koyano & Matsuda, 2015; Roberts *et al.*, 2016). An E3 ubiquitin-protein ligase, ubiquitinates proteins and labels them for degradation. Ubiquitination consists of a 76 amino acid polypeptide covalently conjugated to a lysine residue or N-terminal amino group of a substrate protein allowing 3 enzymes to act sequentially: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) (Seirafi *et al.*, 2015; Chin & Li, 2016).

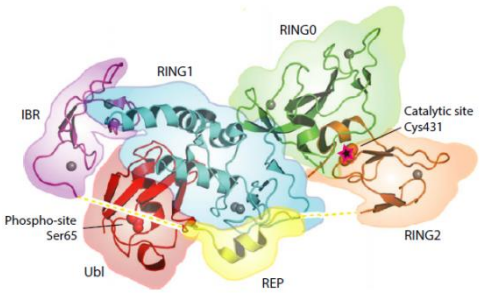

Figure 1.5 - Parkin's structure; 4 domains: ubiquitin-like (Ubl), four zinc-binding RING0, RING1, IBR (in-between RING), repressor element of Parkin (REP) and an N-terminal RING2 domain. Catalytic Site (Cys431) and Phospho-site (Ser^{65}) (Seirafi, M. *et al*. 2015).

The protein structure of Parkin is formed by the following independent domains (Fig.1.5): N-terminal ubiquitin-like (Ubl), four zinc-binding RING0, RING1, IBR (in-between RING), repressor element of Parkin (REP) and an N-terminal RING2 domain. The Ubl domain is involved in substrate recognition, binding SH3 and ubiquitin interacting motif (UIM) domains, proteasome association, and regulation of cellular Parkin levels and activity (Trempe, 2014; Koyano & Matsuda, 2015; Seirafi *et al.*, 2015). The IBR domain is attached through a flexible linker (Trempe, 2014). REP domain in line with the catalytic center, is important in regulation of Parkin activity, once it is blocking E2 binding site on RING1 (Trempe, 2014; Seirafi *et al.*, 2015).

Parkin is a cytosolic protein, not only expressed in *substantia nigra* and others brain regions, but also in many tissues, including heart, testis and skeletal muscle (Kitada *et al.*, 1998). Results obtained from the crystal structure, reveal that under basal conditions, Parkin exists as an autoinhibited form (Trempe, 2014). The catalytic active center, which accepts ubiquitin from E2 and transfers it onto substrates of Parkin is residue Cys 431 that lies in RING2 domain and beneath RING0 domain. This cysteine forms a thioester bond with ubiquitin, which is then transferred to the lysine residue of the substrate via an acyl transfer reaction (Koyano & Matsuda, 2015; Seirafi *et al.*, 2015; Wauer *et al.*, 2015). The phosphorylation site is between IBR and RING2 domains, close to REP, in the autoinhibited conformation (Seirafi *et al.*, 2015) (Fig.1.6).

Presumably, for Parkin activation it is needed some alterations that make both catalytic center and phosphorylation sites available. Reports have demonstrated that ubiquitin is necessary for Parkin activation, as it unlocks repression of the Cys 431 leading to destabilization of the Ubl domain and RBR core interactions (Koyano *et al.*, 2014; Wauer *et al.*, 2015).Structural analysis suggests a conformational flexibility around Ser^{65}, so after interaction with PINK1, the Ubl domain may undergo conformational changes, enabling PINK1 access (Kondapalli *et al.*, 2012) (Fig .1.6).

Once activated, Parkin ubiquitinates several mitochondrial proteins that are involved in numerous mitochondrial dependent processes, such as mitochondrial motility, fission and fusion (FIS1; OPA1; MIRO, Mitofusins), small molecule transport (VDACs); apoptosis (MLC1 and BAX), mitochondrial autophagy (p62); and protein translocation (TOMM70) (Sarraf *et al.*, 2013; Scarffe *et al.*, 2014; Seirafi *et al.*, 2015).

Drosophila Parkin mutants show locomotor defects namely in flight and climbing due to muscle degeneration; defects in spermatogenesis culminating in male sterility; female infertility most probably owing to functional or behavioral functions; fragmentation of mitochondrial cristae; and a reduced longevity and body size at eclosion, indicating defects of the growth and proliferating cell mechanisms (Greene *et al.*, 2003; Pesah *et al.*, 2004; Clark *et al.*, 2006; Park *et al.*, 2006).

As Parkin and PINK1 mutants have similar phenotypes one could suspect that they work in parallel genetic pathways or that one is able to regulate the other. Studies have revealed (Clark *et al.*, 2006; Park *et al.*, 2006) that when *Parkin* is overexpressed the *PINK1* null phenotype is restored. However, *PINK1* overexpression has no effect on Parkin-null phenotypes. Thus, these studies suggest that both proteins function in a common genetic pathway, with PINK1 acting upstream of Parkin. And also, PINK1 accumulation is independent on Parkin function as PINK1 accumulates in equal amounts in the absence or presence of Parkin (Narendra *et al.*, 2010).

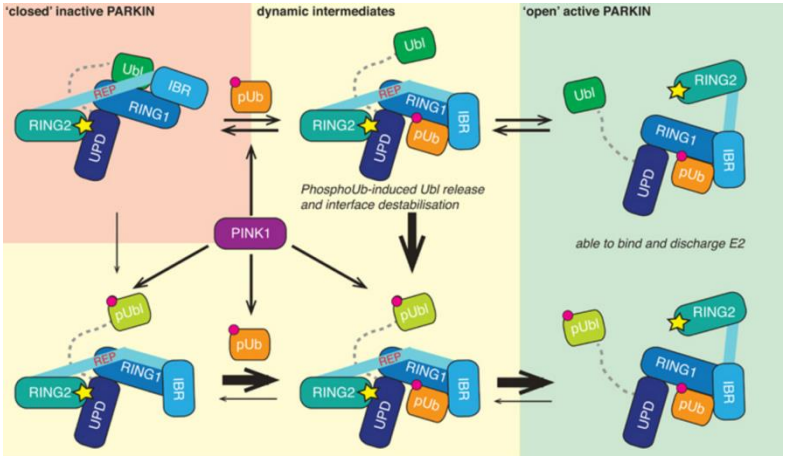

Figure 1.6 –Model of Parkin Activation. In the cytosol, Parkin exists in a "closed" conformation, with RING0, Ubl and REP, are obstructing, RING2 and E2 binding to RING1, respectively. Parkin is translocated to OMM thanks to the high affinity S^{65}-phosphorylated ubiquitin. Because of this interaction, RING1 and IBR originates a displacement of the inhibitory UBL and REP, and consequently Parkin structure to open. In the open conformation, E2 charged enzymes are able to bind to RING1 domain and expose RING2 catalytic cysteine to participate in ubiquitination; also, PINK1 is able to phosphorylate at S^{65} (Wauer, T. *et al.* 2015)

Parkin is selectively recruited to Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) treated depolarized mitochondria, and stimulates the autophagic removal of damaged mitochondria (Matsuda *et al.*, 2010; Koyano & Matsuda, 2015). Parkin mutated in the Ubl domain leads to a moderate loss in mitophagy activity, while mutations in RBR conserved cysteines results in loss of RING2, exhibits a severe mitophagy defect; when the whole Ubl domain is truncated Parkin is also inhibited (Narendra *et al.*, 2010; Wauer *et al.*, 2015).

Parkin is involved in the proteasome degradation of several substrates, prevents cytochrome *c* release and α-synuclein aggregation, its loss of function causes accumulation of potentially toxic protein aggregates eventually involved in PD, since their role in protecting mitochondria is defected (Requejo-Aguilar & Bolaños, 2016).

PINK1/Parkin pathway

When mitochondria become depolarized, PINK1 accumulates stably on the OMM, due to interaction with TOM complex, dimerizes and is autophosphorylated on residues Ser^{228} and Ser^{402} (Okatsu *et al.*, 2012; Aerts *et al.*, 2015). Then, PINK1 phosphorylates Parkin at Ser^{65}, a highly conserved residue within Ubl domain, leading to Parkin E3 ligase activity (Narendra *et al.*, 2008, 2010; Kondapalli *et al.*, 2012; Kazlauskaite *et al.*, 2014), and PINK1 also phosphorylates ubiquitin (Koyano *et al.*, 2014).

PINK1-phosphorylated ubiquitin recruits receptors for mitophagy, such as OPTN and NDP52, Parkin ubiquitination of mitochondrial substrates, generating ubiquitin chains, amplifies this signal,

and recruit ULK1, DFCP1, WIPI1 and LC3 autophagy receptors (Lazarou *et al.*, 2015; Seirafi *et al.*, 2015). OMM-bound PINK1 also mediates the recruitment of the autophagic protein p62 by Nrf2 and TFEB transcription factors (Ivankovic *et al.*, 2016).

This process represents a positive feedback model (Fig1.7), where PINK1 phosphorylates a basal level of ubiquitin and subsequently Parkin is activated, increasing the amount of conjugated ubiquitin, which is then phosphorylated by PINK1 to recruit more Parkin, that will then in turn ubiquitinate other OMM substrates (Seirafi *et al.*, 2015), namely Mfn1/2 (Gegg *et al.*, 2010) that regulates mitochondrial fusion.

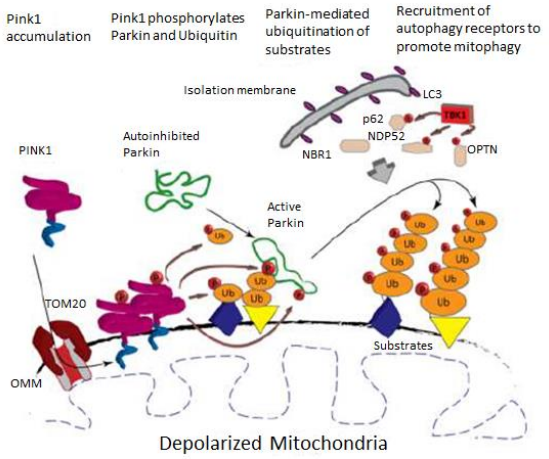

Figure 1.7 – Model of PINK1/Parkin mitophagy induction; PINK1 is barred, accumulates, phosphorylates Ubiquitin and Parkin, enhancing mitophagy signal. Then PINK1 recruits autophagic receptors to promote mitophagy (Roberts *et al.*, 2016).

In sum, this mitochondrial quality control allows taking away damaged and superfluous mitochondria and does not allow the accumulation of oxidized lipids, proteins and DNA, limiting the risk of apoptosis. The disruption of the PINK1/Parkin pathway results in an accumulation of abnormal mitochondria and overproduction of reactive oxygen species (ROS). This pathway regulates the autophagic degradation of damaged mitochondria, through ubiquitin-proteasome and autophagy pathways (Narendra *et al.*, 2008, 2010; Matsuda *et al.*, 2010).

2. Aims

Both *PINK1* and *Parkin* genes are known to be dysregulated in familiar forms of PD. PINK1 through phosphorylation of its downstream targets regulates multiple mitochondrial processes like dynamics and quality control. This pathway responsible for the specific removal of damaged mitochondria depends also on Parkin, a PINK1 substrate.

Given the importance of these encoded proteins in mitochondrial biology, it will be not surprising to find that their dysfunction is associated with damaged mitochondria.

In fact, some groups have described different levels of Parkin phosphorylation and recruitment in the presence of clinical mutant forms of PINK1. However, these results vary depending on the PINK1 specie that is used in these studies, making extrapolation of these findings into the disease context rather difficult to interpret.

In this work, we aim to understand how the kinase activity of the human form of PINK1 harboring PD clinical mutations affect Parkin recruitment and Parkin phosphorylation, as well as the (auto)phosphorylation of PINK1 itself.

3. Methods

Plasmids

The plasmids used were pcDNA 3.1 hPINK1 WT (plasmid expressing human PINK1 wild type); pcDNA 3.1 hPINK1 KI (plasmid expressing hPINK1 kinase inactive); pcDNA 3.1 hPINK1 ΔN WT (plasmid expressing a truncated form of hPINK1WT lacking the first 113 aminoacids); pcDNA 3.1 hPINK1 ΔN KI (plasmid expressing a truncated form of hPINK1 KI lacking the first 113 aminoacids). The cDNA of hPINK1 was also cloned into the pMSCV vector, a vector that has the low expressing promotor LTR, leading to near-to-endogenous PINK1 expression. The pGEX-4T-1 vector was used for the bacterial expression system. These constructs were previously described (Aerts *et al.*, 2015). The mutant Kinase Inactive form of human PINK1 consists of two point mutations of the residues K219 and D362 to Ala. These residues were predicted by computer modelling analysis as crucial residues of the catalytic pore of the kinase domain in PINK1 (Beilina *et al.*, 2005).

All materials used in these experiments are described in appendix.

Construction of Clinical PINK1 Mutants

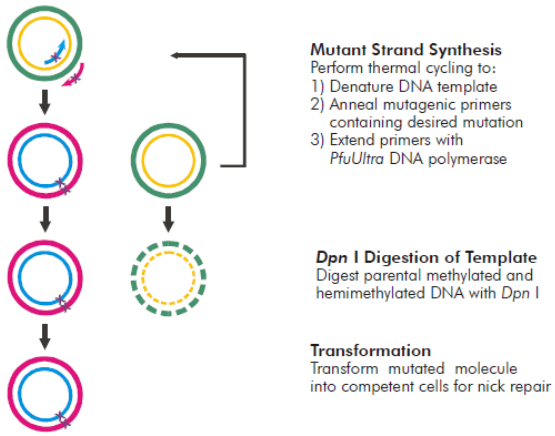

Figure 3.1 - Schematic representation of the QuikChange Site-Directed Mutagenesis protocol

Mutant PINK1 constructs were inserted into pcDNA3.1 hPINK1-WT and hPINK1-ΔN using QuikChange II XL Site-Directed Mutagenesis (Agilent technologies). This technique allows the alteration, deletion and insertion of a base pair in our coding DNA sequence. In our case we performed single point mutations that gave rise to the following amino acid change: a glycine to aspartic acid in G309D, a leucine to proline for L347P, a glutamic acid to glycine in E417G, a histidine to glutamine for H271Q; and tryptophan to a STOP codon for W437X.

The protocol consists of a three step procedure (Fig.3.1). Before moving to the first, the mutagenic oligonucleotide primers must be designed according to the desired mutation. For their design, the QuikChange Primer Design Program available online (at

www.agilent.com/genomics/qcpd) was used, where the melting temperatures, that should be ≥78°C, are calculated with the following equation: $Tm = 4x(G + C) + 2(A + T)$. In addition primers should be between 25 to 45 bases in length, with the desired mutation in the middle, a minimum GC content of 40% and end with a C or G bases. Primers obtained are described in table 3.1.

Table 3.1 - Primers used for quick change mutagenesis and sequencing; F=forward; R=reverse

Sequencing Primers	hPINK1-middle	F	a c c t c t t c c g t g c c g
		R	c g g c a c g g a a g a g g t
	pcDNA3.1-T7	F	t a a t a c g a c t c a c t a t a g g g
	pcDNA3.1-BGH	R	t a g a a g g c a c a g t c g a g g
Mutagenesis primers	Mutagenesis G309D	R	g t c c g g c c a t g g t c c a g g c c t t c a g
		F	c t g a a g g c c t g g a c c a t g g c c g g a c
	Mutagenesis L347P	R	g t c c a c g c c t t c c g g c a g c t g c a g c a g
		F	c t g c t g c a g c t g c c g g a a g g c g t g g a c
	Mutagenesis E417G	R	g c c g t g g a c a c c c c t g g g g c c a t c a
		F	t g a t g g c c c c a g g g g t g t c c a c g g c
	Mutagenesis H271Q	R	g c c g t g g a c a c c c c t g g g g c c a t c a
		F	c c a a g c a a c t a g c c c c t c a g c c c a a c a t c a t c
	Mutagenesis W437X	R	c a a g g c t g a t g c c t g a g c a g t g g g a g c
		F	g c t c c c a c t g c t c a g g c a t c a g c c t t g

First step is the Polymerase Chain Reaction (PCR) that is initiated by adding the following components into a PCR tube: reaction buffer (appropriate for the polymerase used), dNTP mix, QuikSolution reagent, milliQ water, pcDNA 3.1 hPINK1 ΔN WT, primers forward and reverse for each mutation and Pfu Turbo polymerase. To guarantee the kit's effectiveness and absence of contamination from any PCR reaction components, two control samples were used, one provided with the kit that consists in a control plasmid, and another one without the template pcDNA 3.1 hPINK1 ΔN WT, respectively. Reactions were performed with the cycling parameters mentioned in table 3.2.

Table 3.2 - Cycling parameters used for pcDNA 3.1 hPINK1 WT and ΔN mutagenesis reaction

Cycles	Temperature	Time
1	95°C	1 minute
18	95°C	50 seconds
	60°C	50 seconds
	68°C	7,5 minutes
1	68°C	7 minutes
-	4°C	∞

Secondly, after the cycle is done, the parental non mutated DNA is digested with DpnI, leaving the PCR amplified DNA intact, therefore only mutated plasmid DNA will be transformed into XL-gold ultracompetent bacteria, which offers a highest transformation efficiency and ideal for large plasmid DNA. As pcDNA 3.1 plasmids carry an antibiotic resistance genes to ampicillin, we used LB agar plates with Ampicilin (100µg/mL) resistance. Transformed bacteria were incubated for overnight at 37°C. To proceed with the DNA extraction protocol, colonies had to be picked from the plates and incubated with 3mL LB Broth Medium and 100µg/µL Ampicillin, in a shaker at 37°C and 225rpm, overnight. The protocol used for DNA extraction is according to the one described at QIAprep® Miniprep Handbook. Briefly, collected overnight bacterial cultures were spin and pellets were first, ressuspended in Ressuspension Buffer (50mM Tris-HCL pH8.0; 10mM EDTA; 100µg/mL RNaseA), secondly lyzed with Lysis Buffer (200mM NaOH; 1%SDS), and finally neutralized with Neutralization Buffer (4,2M Gu-HCL; 0,9M potassium acetate; pH4,8). Then DNA is purified in QIAprep spin column, and eluted in Buffer EB (10mM Tris-HCL; pH 8,5) added to the column center. DNA concentrations and purity were measured using UV-Vis spectrophotometers NanoDrop™2000. Based on incident and transmitted light intensity, spectrophotometer produces an optical density that correlates with Lambert–Beer law and determines the unknown concentrations.

Then the plasmids were analyzed via Sanger sequencing, where 5µg DNA and 2,5µg primer were used. All sequences obtained were analyzed using the GATC viewer program, which allows checking the chromatogram and DNA sequence. After the analysis, one colony with the desired mutation was selected and further expanded and purified using the Genopure Plasmid Midi Kit (Roche), in order to obtain a highly quality purified plasmid DNA. Briefly, the selected colony is inoculated in a bacterial culture of 50ml and incubated overnight at 37°C at 225rpm. The bacterial culture is centrifuged and pellets are ressuspended in Suspension Buffer (complemented with lyophilized enzyme RNase A), lysed and then neutralized. DNA was purified and washed, within a column, and then eluted with Elution Buffer. At that point, DNA is precipitated with isopropanol and washed in ethanol 70%. Concentrations were measured using NanoDrop™2000, as described above.

Cell Culture and cell lines

The HeLa-CrispR/Cas9-PINK1 cell line (here within referred to as HeLa PINK1 KO) were previously described (Aerts *et al.*, 2015). Briefly, these cells were generated using clustered regularly interspaced short palindromic repeats/Cas technology. A target sequence was selected from the first exon spanning the start codon of PINK1, cloned in pX330-U6-Chimeric-BB-CBh-hSpCas9 (Addgene), and the plasmid was transfected in WT HeLa cells. PINK1 expression was analyzed, via Western Blot and clones in which PINK1 expression was absent were selected. These were 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. Product generated, on all chromosomal copies, were an 84-bp deletion spanning the start codon of PINK1.

Other cell lines used were COS and HeLa WT. Cells were cultured at 37°C with 5% CO_2 in DMEM/F12 medium containing 10% fetal bovine serum (Life technologies). All cell lines were ideally manipulated with approximately 80% confluence.

Parkin expression and purification

The procedure is described in Aerts *et al.*, 2015. Briefly, BL 21 bacteria were transformed with pGEX-4T-1 expressing an N-terminal GST-tagged Ubl-domain of Parkin. Parkin expression was induced with 100μM IPTG, a reagent that induces protein expression where the gene is under the control of the *lac* operator; and cells were incubated at 37°C with 280rpm of agitation for 2hours. After a 15 minutes centrifugation, bacterial pellets were lyzed in 50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Triton X-100, 2mM EDTA, 0.1% beta-mercaptoethanol, 0.2mM PMSF and 1mM benzamidine. GST-Ubl Parkin was purified using Glutathione Sepharose™ 4B (GE Healthcare), according to manufacturer's instructions. Control samples were retained in every purification step, quality and purity was evaluated via western blot. Briefly, this technique allows proteins separations based on molecular weight, by gel electrophoresis, producing a band for each protein. The proteins in the gel are then transferred to a nitrocellulose membrane, which is then incubated with antibodies to the protein of interest, and then develop (Mahmood & Yang, 2012). For Parkin membranes it was used for primary antibody rabbit anti-GST (1/1000; Sigma) and secondary antibody GARPO (1/10000; Bio-Rad).

Parkin recruitment

The procedure was adapted from Aerts *et al.*, 2015. HeLa cells were plated in a 24-well plate, as schematized in figure 3.2, on top of 13mm coverslips and transfected at approximately 80% confluence.

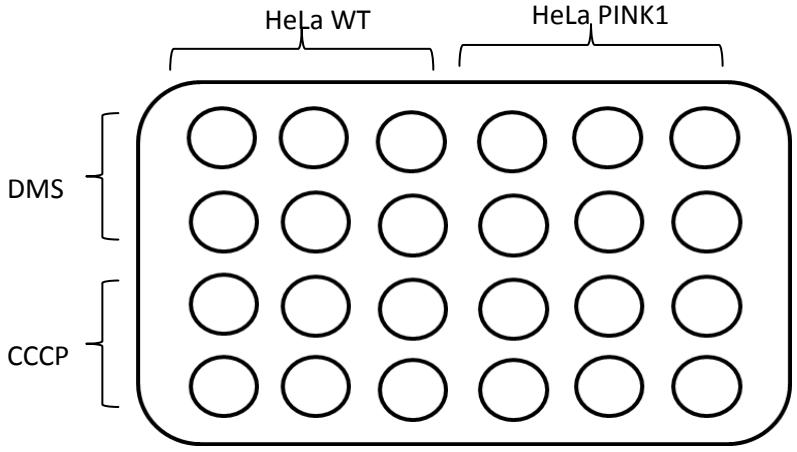

Figure 3.2 - Exemplifying scheme of HeLa cell line plating

All the cell lines were transfected using FuGENE transfection reagent, agreeing to the manufacturer's instructions. This transfection protocol uses a ratio transfection reagent to DNA of 3:1. HeLa cells were transfected with mParkin-GFP and pcDNA3.1-hPINK1 WT or corresponding PINK1 mutants as described in table 3.3, and according to the manufacturer's instructions.

Twenty-four hours post transfection, cells were treated with 10µM CCCP for 3hours or, as control, the equivalent volume of DMSO. CCCP is an uncoupling agent that is used to induce mitochondrial membrane depolarization by increasing membrane permeability to H^+). Cells were washed 3 times in $PBS^{+/+}$ (100 ml 10x $PBS^{-/-}$; 2M $CaCl_2$; 1M $MgCl_2$), fixed for 20 minutes in 4% formaldehyde in $PBS^{+/+}$, washed 3 times in $PBS^{+/+}$, permeabilized in 0,1% Triton X-100 in $PBS^{+/+}$ for 10 minutes, and washed 3times in $PBS^{+/+}$ afterwards. Cells were blocked for 1 hour in Blocking Buffer (0,2% gelatin, 2% fetal bovine serum, 2% bovine serum albumin, 0.3% Triton X-100 in $PBS^{-/-}$) and 5% goat serum (Dako). Cells were stained using the mouse Turbo-GFP antibody (1/1000; Evrogen) and sheep Cytochrome *c* antibody (1/500; Sigma) for 2 hours. Cells were further washed 3 times with $PBS^{+/+}$, and further incubated with secondary antibodies Alexa 488 donkey anti-rabbit and Alexa 568 donkey anti-sheep (Life Technologies), in a 1/500 dilution. Images were acquired on a Zeiss LSM 710 confocal microscope, using a 40x objective, and analyzed with Image J and Photoshop software's.

Table 3.3 - List of plasmids used to transfect HeLa cells

Plasmids
pCMV6 mParkin-GFP
pMSCV hPINK1 FL WT
pMSCV hPINK1 FL KI
pMSCV hPINK1 FL G309D
pMSCV hPINK1 FL L347P
pMSCV hPINK1 FL E417G
pMSCV hPINK1 FL H271Q
pMSCV hPINK1 FL W437X

Human PINK1 purification and in vitro kinase assay

The guidelines for this procedure were optimized and are described in Aerts *et al.*, 2015. COS-1 cells were transfected with the plasmids described in table 3.4, according to manufacturer's instructions, using FuGENE transfection reagent (Promega). Forty-eight hours post transfections, cells were washed and harvest using a cell scrapper and collected in PBS. After a 10 minutes centrifugation

step, cells were lyzed in Lysis buffer (25mM Tris-HCl pH 7.5, 150mM NaCl, 5mM NaF, 1mM MgCl2, 1mM MnCl2, 0.5% Igepal-NP40 (Sigma), 50mg/L DNAse (Sigma), 50mg/L RNAse (Sigma), 1mM DTT), with 20% protease inhibitor cocktail for mammalian cell and tissue extraction (Sigma), 2X complete protease inhibitor (Roche), 4X PhosSTOP tablets (Roche) and homogenized using a 22-G needle in 5 strokes. Lysates were centrifuged during 25 minutes at maximal rpm, and then incubated for 45 minutes at 4°C with FLAG-magnetic beads (Sigma). The unbound fraction was removed, and beads were washed 2 times with Lysis buffer and 3 times with kinase assay buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 10mM MgCl2, 3mM MnCl2 and 0.5mM DTT).

The kinase assay was executed immediately after the binding step where purified hPINK1-FLAG bound on the beads was incubated with 3,35µL of Parkin (2µg) , 10mM DTT and 100µM ATP containing 5µCi [γ-^{32}P] ATP. Reactions were incubated for 1hour at 22°C.

Samples were analyzed by SDS-PAGE followed by Western blotting. For this, samples were incubated for 10 minutes at 70°C with Sample Buffer (77.8mM Tris-HCl pH6.8; 44,4% (v/v) glycerol; 4,4% LDS; 0,02% bromophenol blue and 4% β-mercaptoethanol). Samples were loaded on Mini-PROTEAN 7.5% Tris-Glycine Gels, the electrophoresis separation occurred for approx. 1 hour in Running Buffer (25mM Tris-HCl pH 8.3, 190mM glycine, 0.1% SDS). After SDS-PAGE, samples were transferred onto a PVDF 0.45µm membrane in Transfer buffer (25mM Tris-HCl pH 8.3, 190mM glycine, 20% methanol) for 1 hour at 100V. After transfer, PVDF membrane was stored in an autoradiography cassette with an amplifying film. Incorporation of radiolabelled phosphor was assed via a storage phosphor screen and development on Typhoon (GE Healthcare Life Sciences). Image studio lite software was used for signal quantification.

After radiolabelled phosphor quantification, the PVDF membranes were blocked for 1hour in 5% milk in TBS-T (50 mM Tris-HCl pH 7.5; 150 mM NaCl, 0.1% Tween-20), and incubated with agitation in primary antibody mouse anti-Flag M2 (1/5000; Sigma) and rabbit anti-GST (1/5000; Sigma), overnight.

Table 3.4 - Plasmids transfected on COS-1 cell line

Plasmids
pcDNA 3.1 hPINK1 ΔN WT
pcDNA 3.1 hPINK1 ΔN KI
pcDNA 3.1 hPINK1 ΔN G309D
pcDNA 3.1 hPINK1 ΔN L347P
pcDNA 3.1 hPINK1 ΔN E417G
pcDNA 3.1 hPINK1 ΔN H271Q
pcDNA 3.1 hPINK1 ΔN W437X

Statistical analysis

Statistical significance between the different test conditions was analysed using GraphPad Prism 5.03 software, through unpaired Student's *t*-test (*: p<0,05; **; p<0,01; ***<0,001; ns: no significant). Data are shown as mean +/- standard errors of the mean (SEM), with 95% of confidence interval, in a minimum of 2 independent replicates.

4. Results and Discussion

Parkin recruitment

The mutations studied have been previously identified as altered in PD, as earlier described in Chapter 1 (Fig.4.1). Quite a few studies have reported PINK1 G309D, L347P, H271Q and W437X mutants behaviour relatively to Parkin recruitment. However there is some contradiction in these reports due to the use of different experimental conditions (such as concentration or exposure time to CCCP) and quantification methods, so facts still remain unclear in the field.

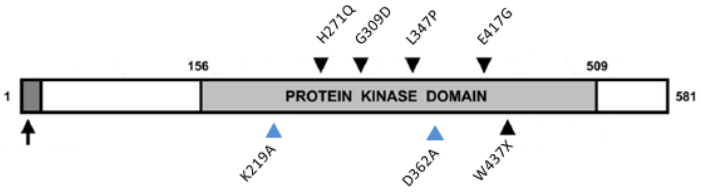

Figure 4.1 – Schematic representation of PINK1
PINK1 is a 581 amino acid protein which localizes to the mitochondria via an N-terminal mitochondrial targeting sequence (black arrow) and contains a catalytic serine/threonine kinase domain. PINK1 clinical mutations (Black arrow head) and a residue responsible for kinase activity (blue arrow head) of localized within the kinase domain of PINK1. Adapted from (Bonifati, V., *et al.* (2005)

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In order to verify the impact of hPINK1 clinical mutants on Parkin recruitment, we transfected HeLa WT and HeLa-PINK1-KO cells with PINK1 constructs and with a GFP-tagged Parkin construct. Dual staining was performed in all cell lines studied. We confirmed that in basal conditions, Parkin is predominately located in the cytosol as expected (Fig.4.2B and Fig.4.2H), and does not colocalize with mitochondria (Fig.4.2C and Fig.4.2I). Although, when HeLa WT cells were treated with CCCP, a loss of mitochondrial network is observed when staining for a mitochondrial resident protein Cytochrome *c* and a perinuclear clustering of mitochondria is observed (Fig.4.2.D), and more interestingly Parkin is recruited to the mitochondria (Fig.4.2F). This does not happen in HeLa PINK1 KO cells treated with CCCP where, in the absence PINK1, Parkin is not recruited to mitochondria (Fig.4.2L). This observation is in agreement with previous studies (Narendra *et al.*, 2010) that show that Parkin recruitment to depolarized mitochondria requires the presence of PINK1.

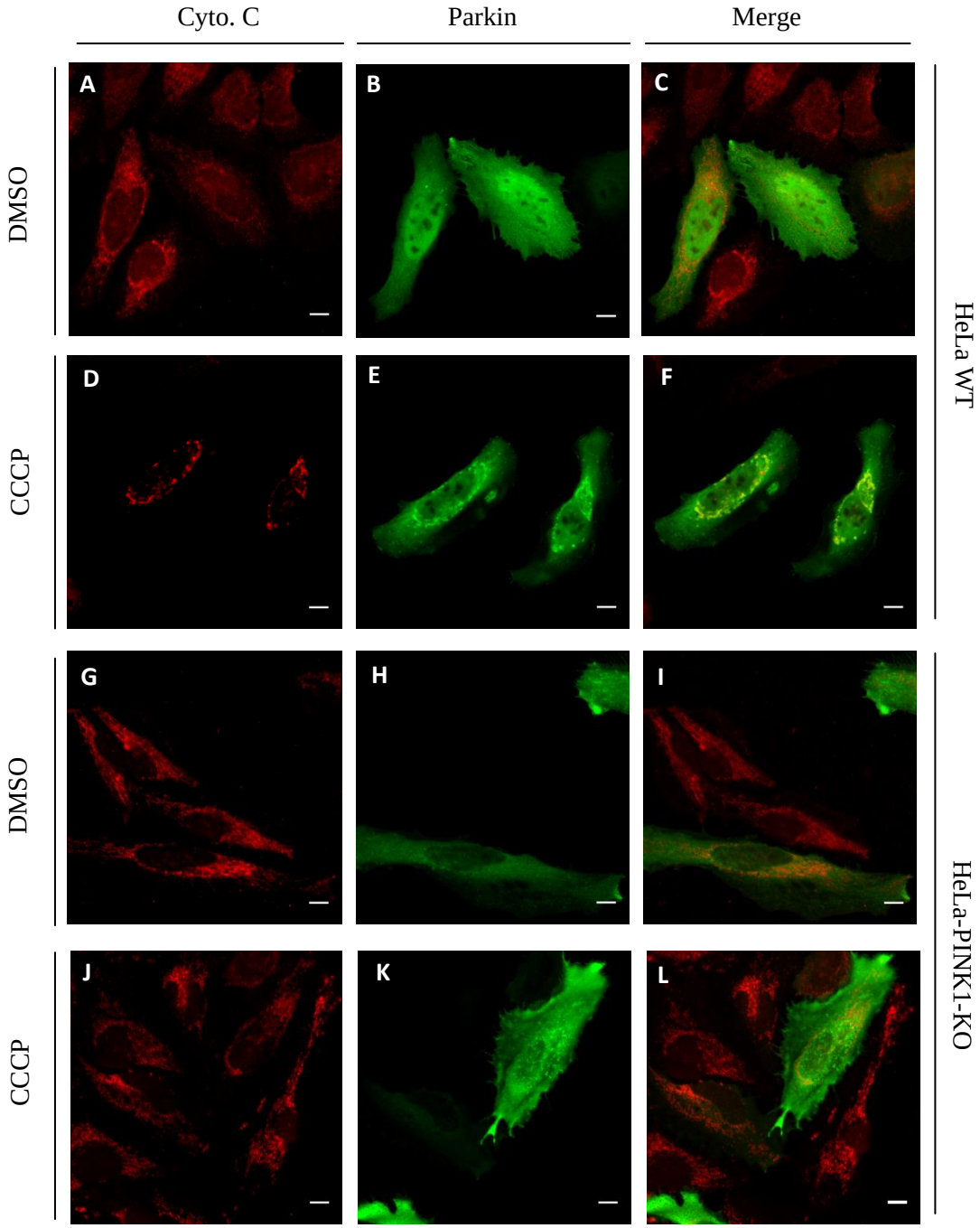

Figure 4.2 – Parkin recruitment to depolarized mitochondria in HeLa WT and HeLa-PINK1-KO. Cells were transfected with mParkin-GFP and treated with DMSO or 10μM CCCP in serum for 3h. Mitochondria were immunostained for Cyto. *C*. The images in column on the right are merged images of the middle (Parkin staining) and left-hand (Mitochondria staining) columns. Scale bar=10μM

Additionally, to further confirm that Parkin recruitment is dependent not only on the presence of the PINK1 protein but also requires an active form of PINK1, we transfected HeLa WT and HeLa-PINK1-KO cells with a kinase inactive mutant form of PINK1, the hPINK1 KI, and Parkin recruitment was evaluated. At present, there is not a known stoichiometry between PINK1 levels and Parkin recruitment (Seirafi *et al.*, 2015), therefore, in order to quantify the percentage of cells where Parkin recruitment is occurring we quantified cells that presented staining for Cytochrome *c* and Parkin simultaneously. The quantified data for cells treated with DMSO and CCCP are represented in Fig. 4.3.

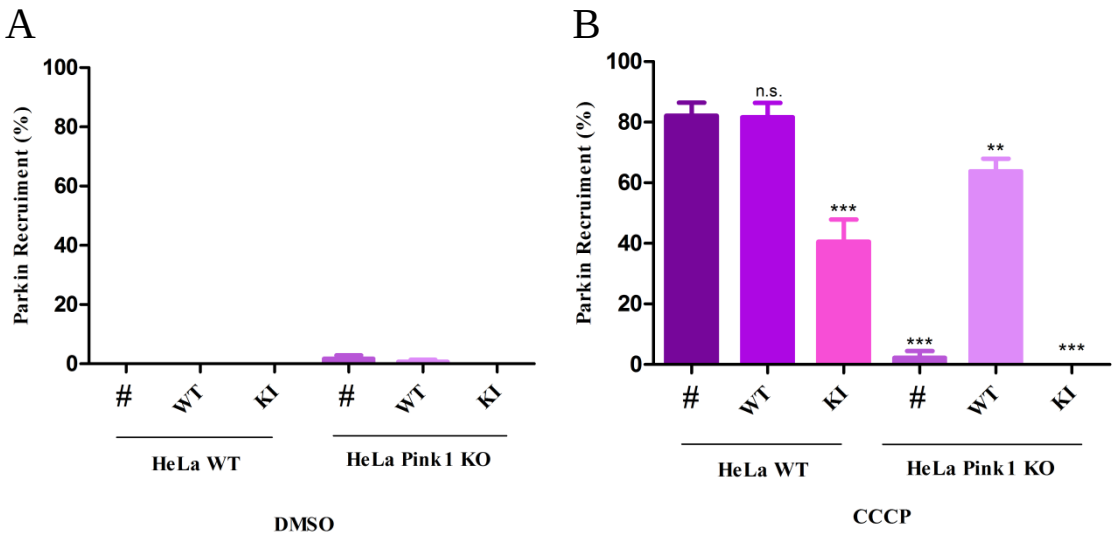

Figure 4.3 – Quantification of Parkin recruitment to mitochondria. A. Parkin recruitment in HeLa WT and Hela PINK1 KO cell lines, non-transfected, and transfected with hPINK1 WT and KI, in DMSO conditions; #=Cell line not transfected. **B.** Parkin recruitment in HeLa WT and HeLa PINK1 KO cell lines, non-transfected, and transfected with WT and KI, in CCCP conditions; #=Cell line not transfected

In DMSO conditions (Fig. 4.3A), Parkin recruitment shows no significant alterations between the different cell lines analysed. This is due to the fact that mitochondria are healthy with no defects at the level of mitochondrial membrane potential (Δψm).

When loss of Δψm induced by CCCP treatment leads to a pool of damaged mitochondria represented by a loss in mitochondrial network and the formation of fragmented mitochondria (Fig. 4.3B), there is a complete different response from Parkin which is dependent on the presence of PINK1. In HeLa WT cells transfected with PINK1 WT and treated with CCCP, we did not observe a difference between transfected and non-transfected cells. On the other hand, Parkin recruitment is significantly decreased in the presence of hPINK1-KI (Fig.4.3.B). Parkin recruitment can be recovered in Hela-PINK1-KO cells by expressing PINK1 WT (Fig.4.4F), where 63,78% of recruitment is observed when compared to hPINK1 WT; but recruitment is not restored with PINK1 KI (Fig.4.3B; Fig4.4L), indicating that PINK1 has a crucial role in Parkin recruitment to mitochondria and cooperate functionally to clear damaged mitochondria via mitophagy.

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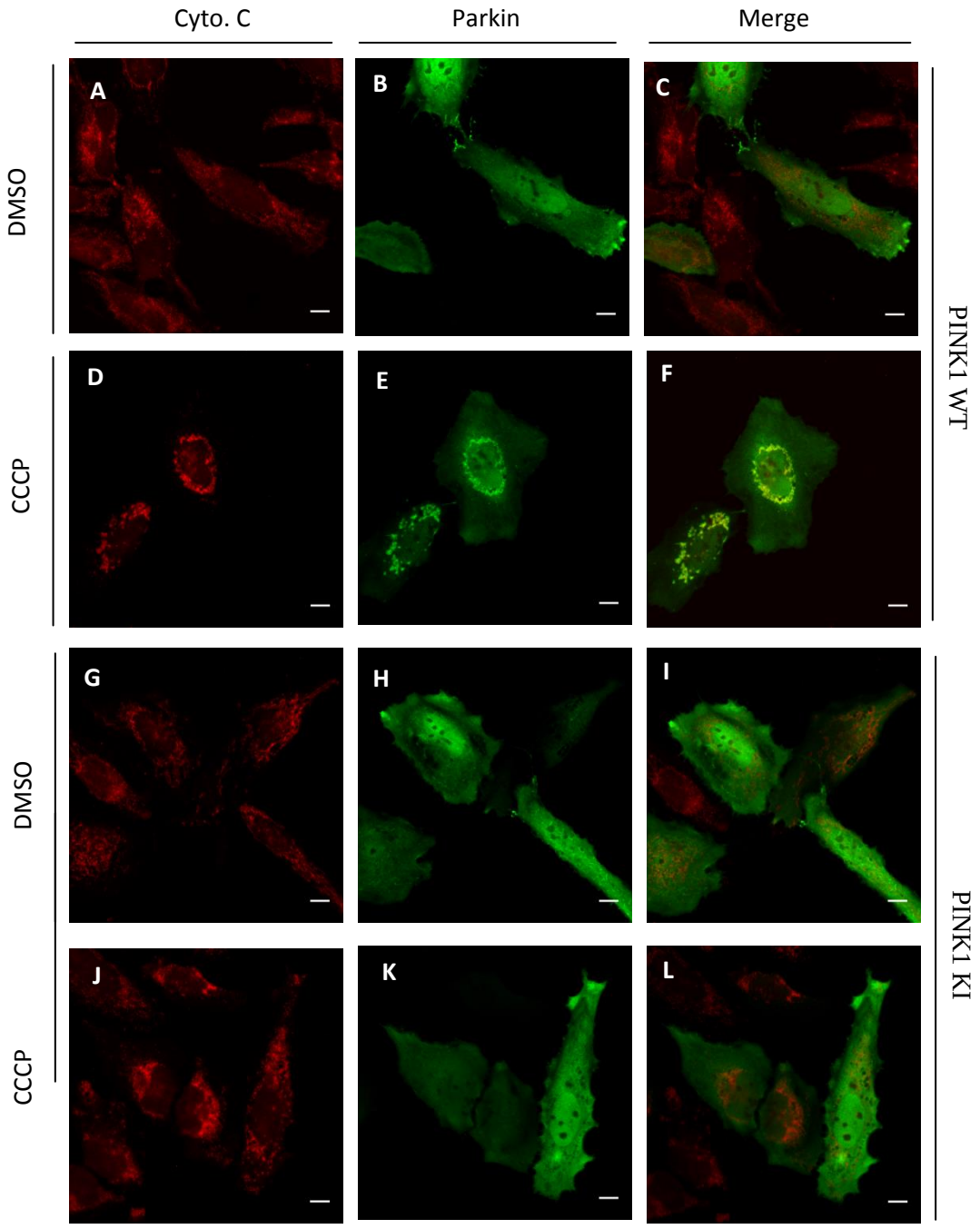

Figure 4.4 - Parkin recruitment to depolarized mitochondria in HeLa-PINK1-KO. HeLa-PINK1-KO cells were cotransfected with mParkin-GFP and pcDNA 3.1 .ΔN PINK1 WT or pcDNA 3.1 .ΔN PINK1 KI, and treated with DMSO or 10μM CCCP in serum for 3h. Mitochondria were immunostained for Cyto. *C*. The images in column on the right are merged images of the middle (Parkin staining) and left-hand (Mitochondria staining) columns. Scale bar=10μM.

In order to elucidate the impact of clinical PINK1 mutants on Parkin recruitment to mitochondria, we transfected HeLa-PINK1-KO with mParkin-GFP and hPINK1 clinical mutants, and investigated Parkin recruitment in the presence of CCCP treatment. Immunofluorescent images and consequent quantification was obtained for all studied mutants.

Interestingly, in healthy mitochondria (DMSO treatment) the PINK1 clinical mutants induced a subtle, albeit significant, increase in Parkin recruitment when compared to hPINK1 WT (Fig.4.5C; Fig.4.6C; Fig.4.6I; Fig.4.7C). However, the mutant hPINK1 L347P does not present a loss of mitochondrial network and Parkin maintains a preferential cytosolic localization (Fig.4.5L). Suggesting that overexpressing of these mutants can produce an increase in toxicity leading to mitochondria death.

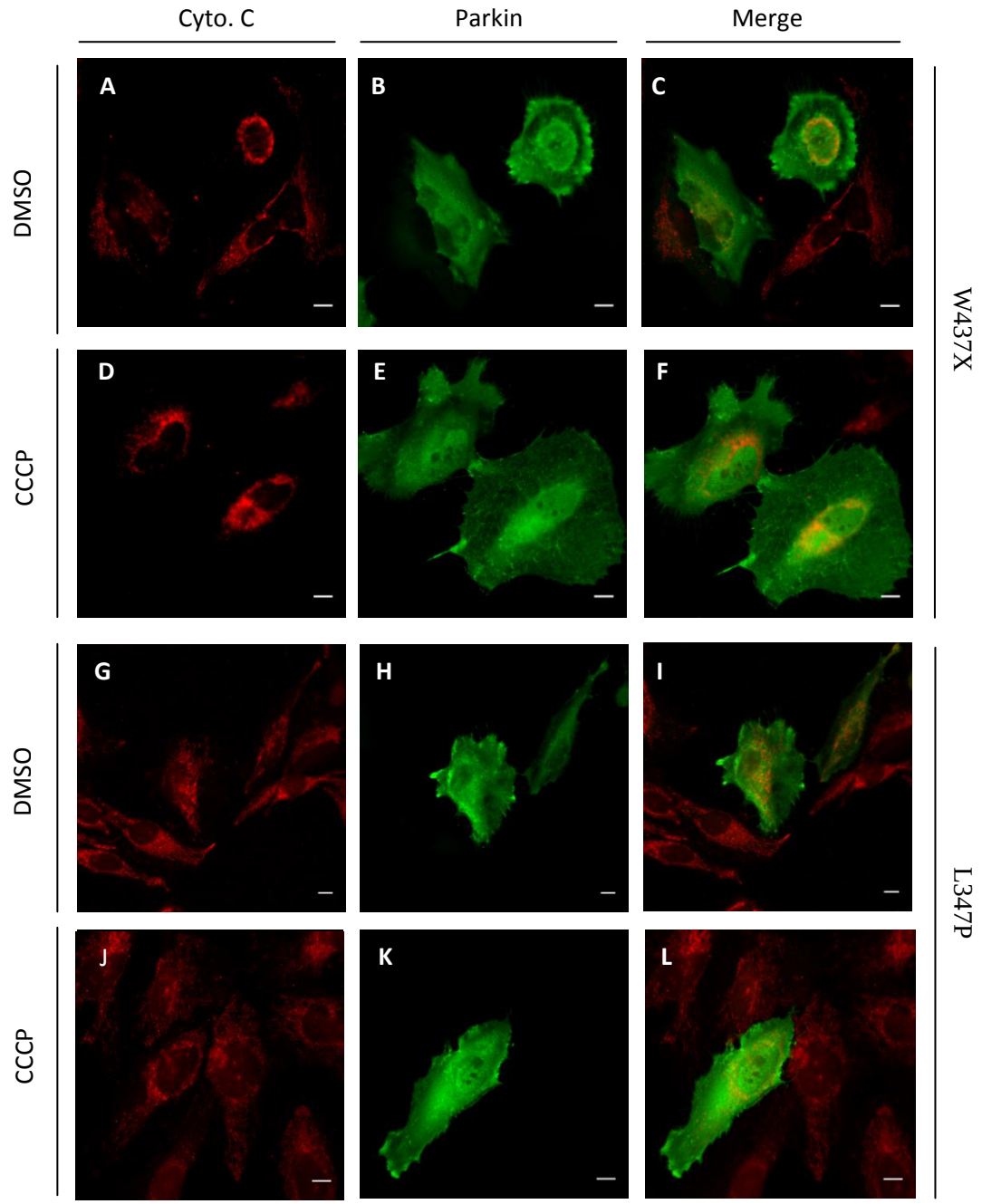

Figure 4.5 - Parkin recruitment to depolarized mitochondria in PINK1 W437X and L347P mutants. HeLa-PINK1-KO cells were cotransfected with mParkin-GFP and pcDNA 3.1 .ΔN PINK1 W437X or pcDNA 3.1 .ΔN PINK1 L347P ,and treated with DMSO or 10μM CCCP in serum for 3h. Mitochondria were immunostained for Cyto. *C*. The images in column on the right are merged images of the middle (Parkin staining) and left-hand (Mitochondria staining) columns. Scale bar=10μM.

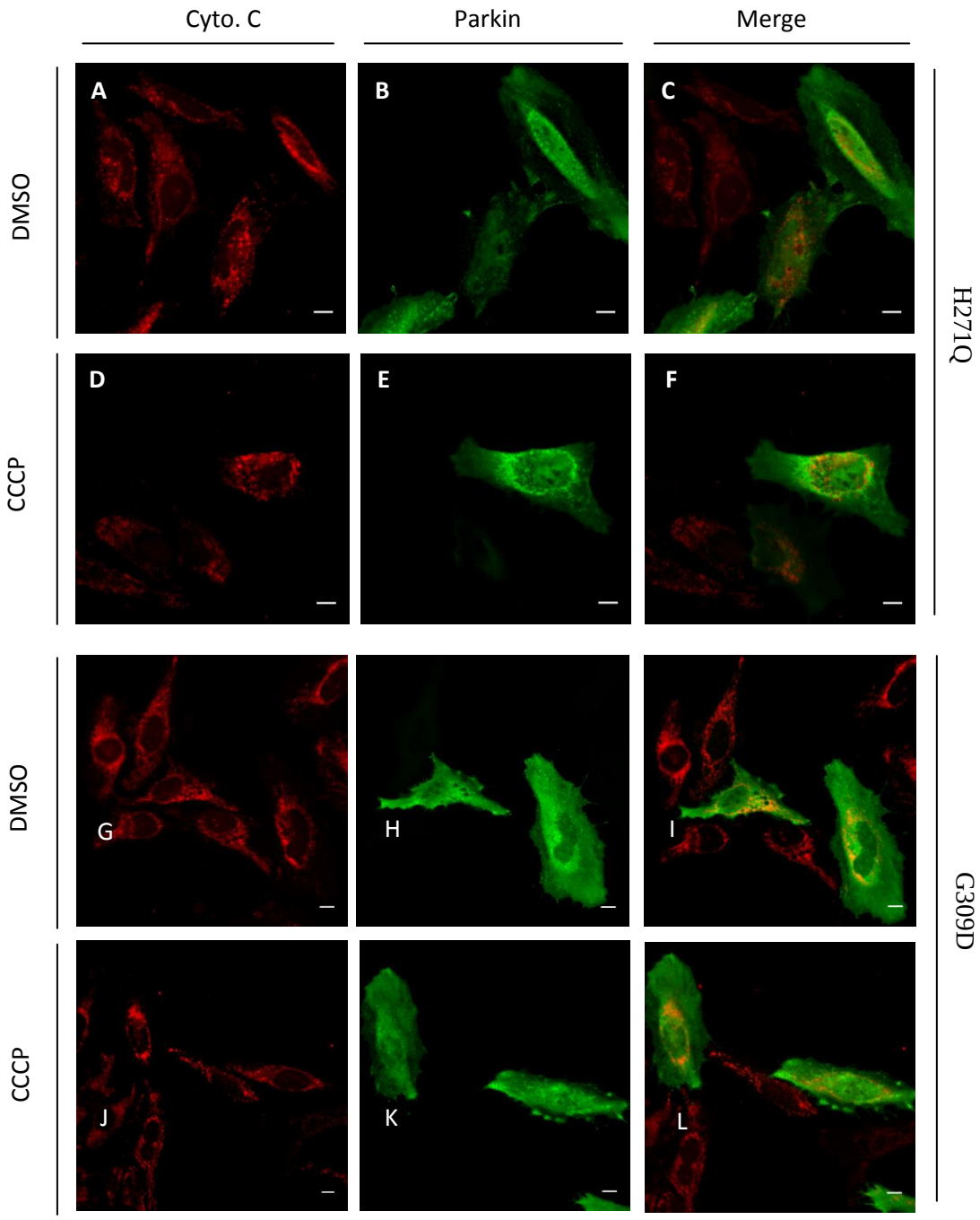

Figure 4.6 - Parkin recruitment to depolarized mitochondria in PINK1 H271Q and G309D mutants. HeLa-PINK1-KO cells were cotransfected with mParkin-GFP and pcDNA 3.1 .ΔN PINK1 H271Q or pcDNA 3.1 .ΔN PINK1 G309D, and treated with DMSO or 10μM CCCP in serum for 3h. Mitochondria were immunostained for Cyto. *C*. The images in column on the right are merged images of the middle (Parkin staining) and left-hand (Mitochondria staining) columns. Scale bar=10μM.

In order to mimic depolarized mitochondria, cells were treated with CCCP. In this case all mutants showed Parkin recruitment and presented total or partial colocalization with the mitochondrial marker Cytochrome c (Fig.4.5D-F; Fig.4.5J-L; Fig.4.6D-F; Fig.4.6J-L; Fig.4.7D-F). However, the clinical mutants are not able to restore Parkin recruitment to levels comparable with hPINK1 WT, indicating that these mutations that occur in hPINK1 lead to the expression of a hampered active form of this kinase.

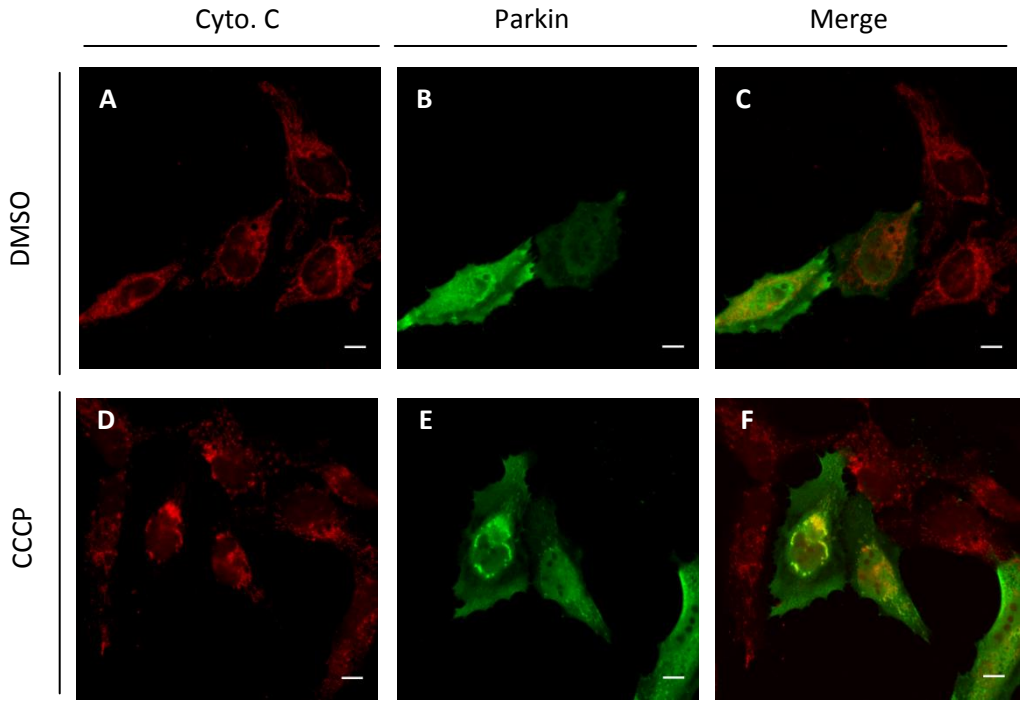

Figure 4.7 - Parkin recruitment to depolarized mitochondria in PINK1 E417G mutant. HeLa-PINK1-KO cells were cotransfected with mParkin-GFP and pcDNA 3.1 .ΔN PINK1 E417G, and treated with DMSO or 10μM CCCP in serum for 3h. Mitochondria were immunostained for Cyto. *C*. The images in column on the right are merged images of the middle (Parkin staining) and left-hand (Mitochondria staining) columns. Scale bar=10μM.

Transfected cells were scored for presence of Parkin-GFP (Figure 4.9) and further quantified for Parkin recruitment (Fig.4.8). All clinical mutants presented similar percentage of Parkin-GFP transfection, indicating that observed results within clinical mutants is not due to lower Parkin-GFP transfection efficiency. In cells treated with DMSO, PINK1 clinical mutants where able to significantly recruit Parkin when compared to non-transfected HeLa-hPINK1-KO cells, with the exception of PINK1 L347P. These results show that mitophagy can occur in healthy mitochondria, and that mitochondria turnover also occurs at basal levels. Nevertheless, this phenotype is massively increased with cells are treated with CCCP.

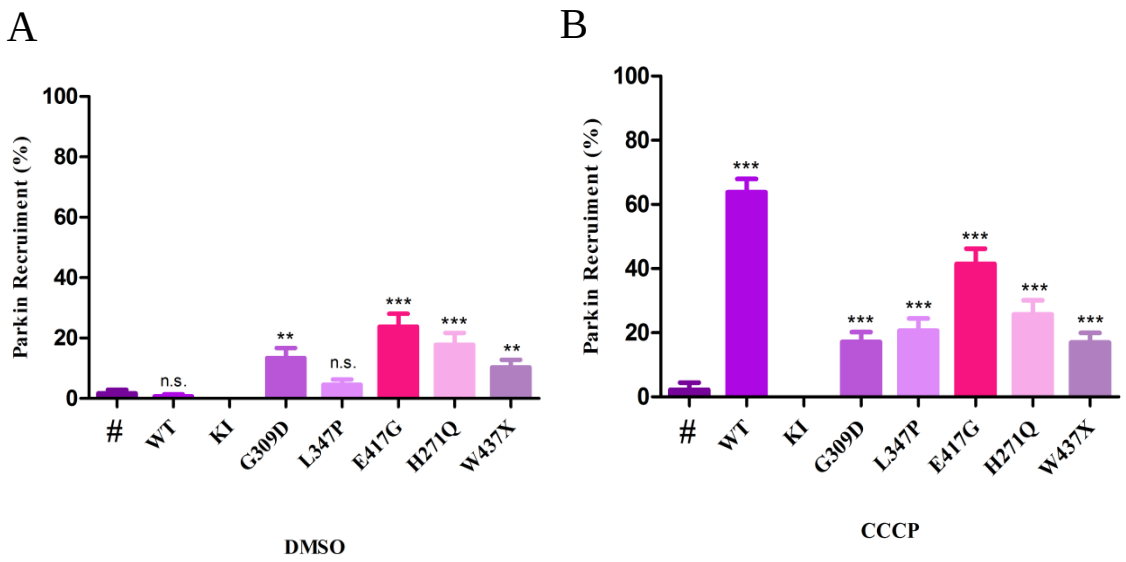

Figure 4.8 – Quantification of Parkin recruitment to mitochondria in PINK1 mutants. A. Parkin recruitment in Hela PINK1 KO cells, non-transfected, and transfected with hPINK1 WT, KI and mutants, in DMSO conditions; #: Cell line not transfected. **B.** Parkin recruitment in Hela PINK1 KO cell line, non-transfected, and transfected with hPINK1 WT, KI and mutants, in CCCP conditions; #: Cell line not transfected.

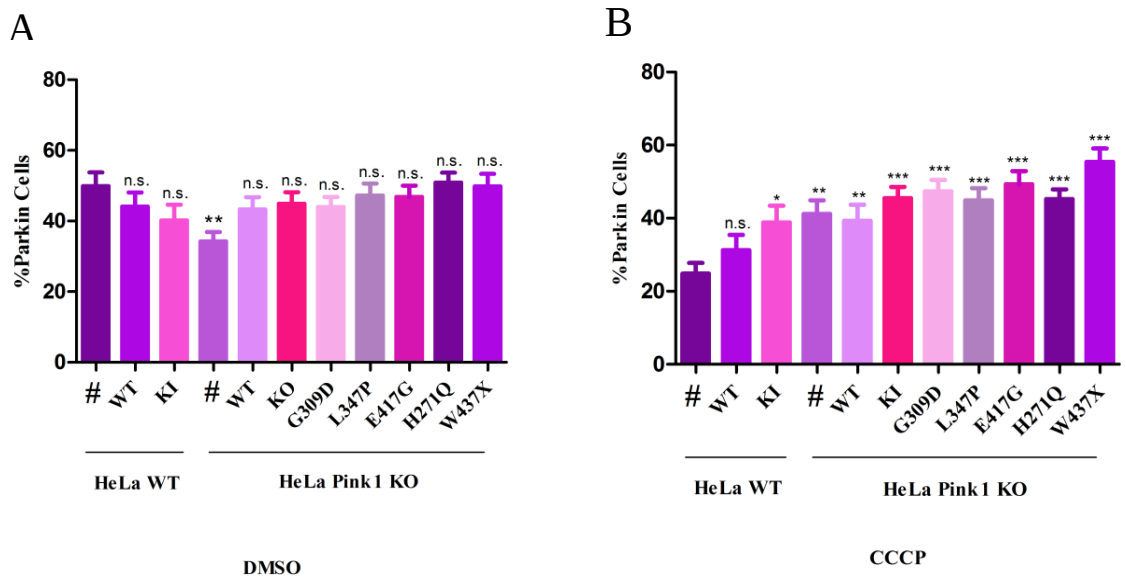

Figure 4.9 - Cells transfected with mParkin. A. Hela WT and HeLa PINK1 KO cells transfected with Parkin (%), in DMSO conditions. There is a significant difference between Hela WT non-transfected (#) and HeLa WT KO non-transfected (#). **B.** Hela WT and HeLa PINK1 KO cells transfected with Parkin (%), in CCCP conditions. There is a significant difference between Hela WT non-transfected (#) and all samples, except HeLa WT transfected with WT

Neuronal cells expressing the PINK1 W437X mutant show a significant increase in mitochondria size and loss of mitochondria cristae, as well as loss of $\Delta\psi m$ and decrease in ATP production. This mutant exacerbates the accumulation of α-synuclein by mitochondrial calcium flux dysregulation (Marongiu *et al.*, 2009). While Fallaize and co-workers have shown that PINK1 W437X abolishes the ability of PINK1 to translocate to OMM following CCCP treatment (Fallaize *et al.*, 2015), another study has shown that the expression of PINK1 W437X results in more pronounced initial mitochondrial localization, even in the absence of mitochondrial damage (Geisler *et al.*, 2010). Our results go in line with reports from Geisler and co-workers, where even without CCCP treatment we observed a residual Parkin recruitment (Fig.4.5C and Fig.4.8A). The same happens with PINK1 H271Q (Fig.4.6C), G309D (Fig.4.6I) and E417G (Fig.4.7C) clinical mutants, where a significant recruitment of Parkin occurs in Hela-hPINK1-KO non-transfected cells (Fig.4.8A).

Previous studies have shown that the PD linked mutation L347P has no effect on PINK1 sub-mitochondrial localization in either healthy or CCCP-depolarized mitochondria (Fallaize *et al.*, 2015). Narendra and co-workers have reported that PINK1 mutant L347P is unstable and fails to reconstitute Parkin recruitment to depolarized mitochondria, as well as H271Q (Narendra *et al.*, 2010). Taking into account our results, we could postulate that the kinase activity of this protein may not be required for mitochondrial depolarization-induced PINK1 OMM translocations. On the other hand, for PINK1 H271Q we observed Parkin recruitment for both DMSO and CCCP conditions. (Fig.4.6.A-F) (Fig.4.8A and Fig.4.8B).

Reports have shown that PINK1 G309D mutation does not alter the production of full length WT PINK1, but abrogates the protective function of PINK1 (Valente *et al.*, 2004). The PINK1 G309D mutant restores the mitochondrial localization and Parkin activation after CCCP treatment (Matsuda *et al.*, 2010). Also G309D, can partially reconstituted Parkin recruitment (Narendra *et al.*, 2010). Our results are in agreement with these findings.

In vitro PINK1 phosphorylation assay

Parkin expression and purification

Recombinant GST-Ubl Parkin protein was expressed using a bacterial protein expression system. Ubl-Parkin protein expression was induced using IPTG and further purified from the bacterial lysate by performing a gluthathione chromatography against the GST-tag present in the recombinant protein. In order to evaluate the efficiency of purification of Ubl-Parkin, aliquots from all relevant steps from the purification where analyzed by SDS-PAGE followed either by Coomassie staining or by Western Blot analysis: lysate, the supernatant obtained after bacterial lysis; unbound, the fraction of protein that wasn't retained at the column; and 3 elutes, the elution fraction of our protein of interest. Coomassie dye binds to proteins through ionic interactions between dye sulfonic acid groups and positive protein amine groups as well as through Van der Waals attractions. Dye that is not bound to protein diffuses out of the gel during the destain steps. Then the proteins are detected as blue bands on a clear background (Fig.4.10A).

Ubl Parkin enrichment was assessed by Western blotting where Parkin protein was probed using the rabbit anti-GST antibody, which will specifically bind to the GST-tag present in our protein of interest. As a secondary antibody, we used the goat anti-rabbit IgG (H+L) fused with horseradish peroxidase. This enzymatic reporter will interact with the luminol-based substrate producing a chemiluminescent light, which consequently is captured by Chemidoc Imager (Bio-Rad) (Fig 4.10B).

Figure 4.6 – Ubl-Parkin expression and purification. A. Coomassie staining of GST Ubl Parkin (red arrow). **B**. Membrane obtained from Western Blot of GST Parkin (red arrow).

The analysis of the Coomassie stained gel (Fig.4.10A) reveals an efficient purification of GST-Ubl-Parkin, as the major band present in the lysate that corresponds to the predicted molecular weight of GST-Ubl-Parkin (36kDa) is clearly enriched in the Elute fraction 2 and 3 (10µg Lysate and 2µg Elute 2 were loaded on the SDS-PAGE). Additionally, the absence of this band in the unbound fraction indicates an efficient depletion of our protein of interest in the binding procedure.

The protein concentration was measured using the Bradford Protein Assay (Bio-Rad). Briefly, this is method for protein quantification based on the dye Coomassie Brilliant Blue G-250 binding to proteins. The binding will induce pH changes; predominantly dye is in a protonated cationic form (red); but when dye binds to protein, it is converted to an anionic form (blue). This protein-dye interaction in a blue form is detected at the absorbance wavelength of 595nm. In table 4.1 is represented the estimated molecular weight of protein bands detected in our Coomassie stained gel, as well as the concentration of each fraction analysed (20µg).

Table 4.1 - Results for GST-Ubl Parkin Quantification;µL, were the quantity of each sample pipetted for 96-well plate; µg sample/well, is the amount of protein quantified at absorbance 595nm.M. W.=molecular weight

Samples	M.W. (KDa)	µL	µg sample/well
Lysate	35,363575	10	7,87
Elute 1	34,313031	2	3,35
Elute 2	35,900845	2	3,35
Elute 3	36,722097	2	1,57

Considering the three elutes, it was decided to proceed with Elute 2, as either in Coomassie or Western blot results this elution fraction present the highest amount of Ubl-Parkin..

hPink expression and purification

PINK1 is a delicate protein to work with, as it is highly instable, and several steps are required in order to obtain a functionally purified form of PINK1 (Hertz *et al.*, 2013; Aerts *et al.*, 2016). Diverse protein tags were tested in order to obtained a more effective purification and expression of this protein, it was concluded that the tandem affinity purification tag 3xFLAG-Streptavidin was the most efficient (Aerts *et al.*, 2016). Briefly, this expression and purification procedure requires and overall short time of manipulation as PINK1 has a short half-life of approximately five hours (Choo *et al.*, 2012); the kinase assays temperature shifts from 30°C to 22°C; and the reducing agent DTT was added, since it improves phosphorylation detection.

Transient expression of the PINK1 clinical mutants was checked by Western blotting against the FLAG tag present in these mutants (Fig.4.11). Typically, immunoblotting for PINK1 reveal not only FL PINK1 (blue arrow), but also its three processed forms: first (orange arrow) resulting from MPP cleavage (ΔMTS); secondly (red arrow), resulting from PARL cleavage (ΔN1); and ΔN2 for another process form (green arrow) (Greene *et al.*, 2012).

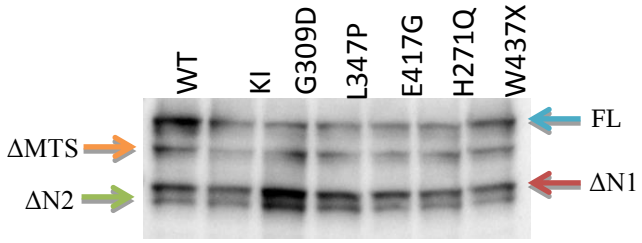

Figure 4.7 – COS Tranfection. COS Transfected with PINK1 WT, KI and 5 studied mutants, expressing different hPINK1 forms. Blue arrow represents FL PINK1; Orange arrow represents ΔMTS form; Red arrow for ΔN1 form; and Green arrow for ΔN2

Analysis of the results obtained from the in vitro phosphorylation assay clearly show that FL PINK1 phosphorylates the Ubl domain of Parkin (Fig.4.12B), however phosphorylation of PINK1 itself is not observed (Fig.4.12A).

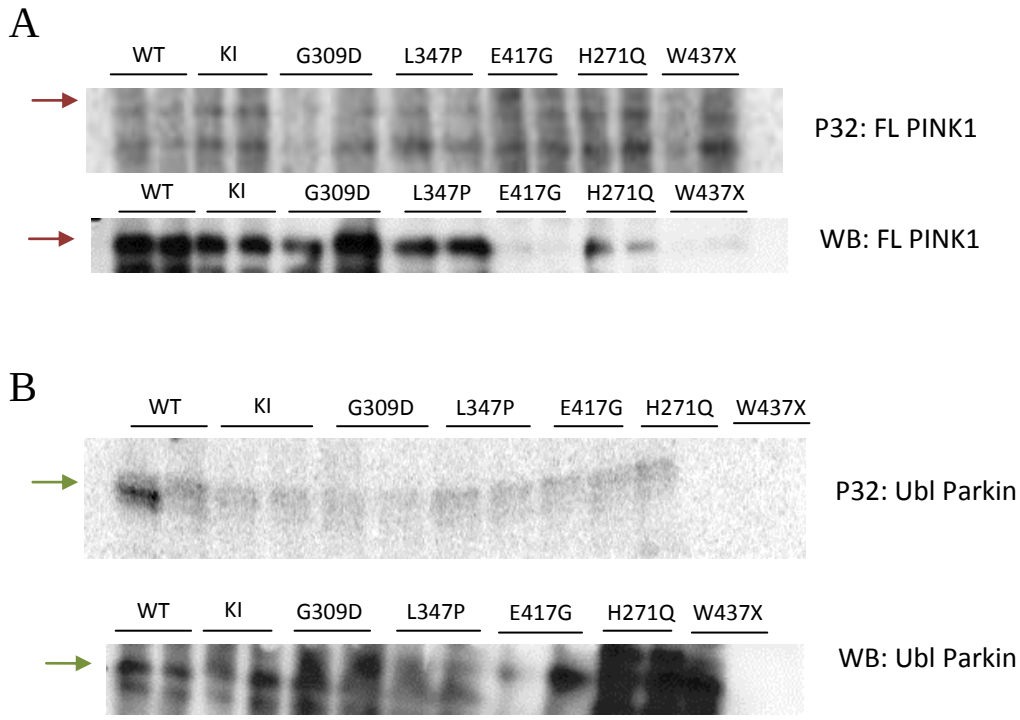

Figure 4.8 – *In vitro* assay with FL PINK1. A. Phosphorylation assay using [ϒ-^{32}P]-ATP performed with purified FL PINK1 (red arrow). Although WB shows FL PINK1 expression, the autoradiogram does not indicate FL PINK1 phosphorylation, neither in the WT, KI or any mutant. **B.** Phosphorylation assay using [ϒ-^{32}P]-ATP performed with purified FL PINK1 and Ubl Parkin. Autoradiogram shows that Ubl Parkin (green arrow) is phosphorylated by FL PINK1. (*n* = 2 independent experiments).

But when the assay was performed employing the PINK1 form lacking the N-terminal region (ΔN PINK1), both substrate phosphorylation (Fig.4.13B) and (auto)phosphorylation (Fig.4.13A) was detected. These results suggest that N-terminal of hPINK1 may play a role in PINK1 (auto)phosphorylation. Still Parkin phosphorylation appears not affected in either of the cases. Previous studies from this laboratory have reported (Aerts *et al.*, 2015) that lack of PINK1 (auto)phosphorylation by FL PINK1 is not due to a prior phosphorylation and occupancy of sites sides by non-radioactive phosphates. They conducted studies using lambda protein phosphatase (LPP) to confirm phosphorylation form of PINK1.

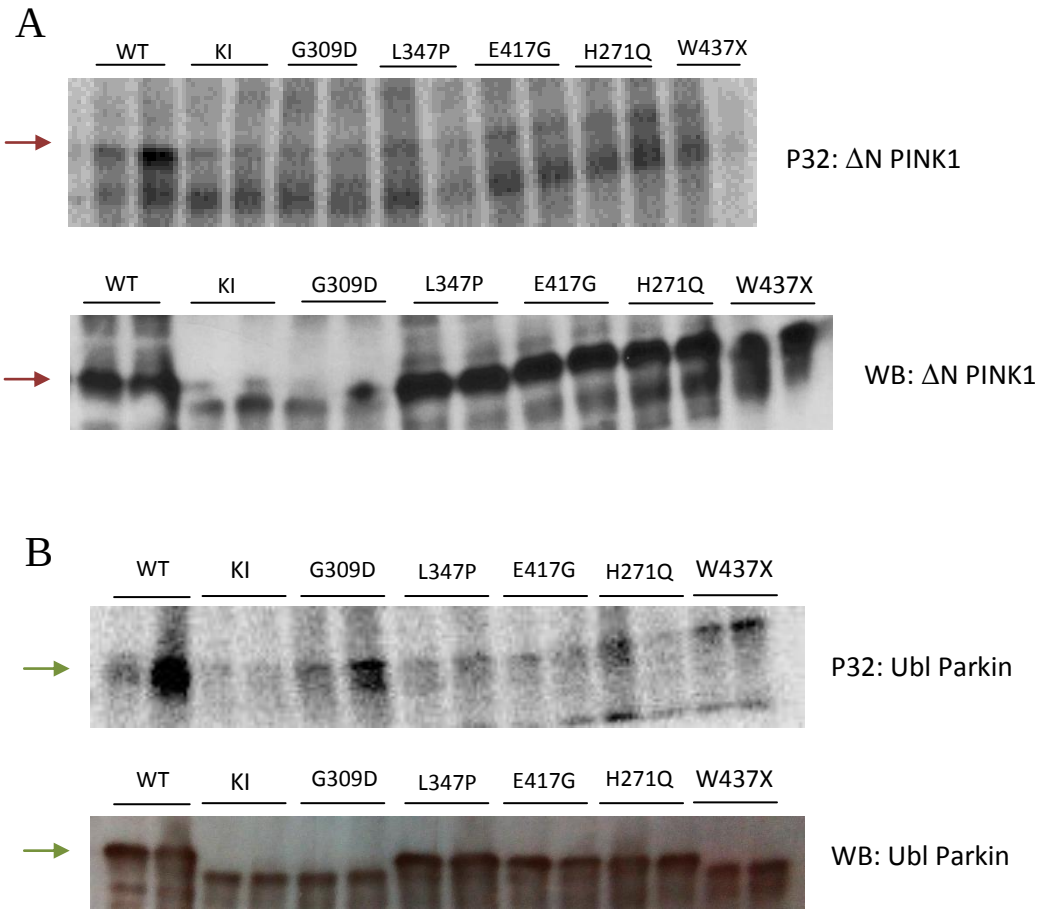

Figure 4.9 - *In vitro* assay with ΔN PINK1. A. Phosphorylation assay using [ϒ-^{32}P]-ATP performed with purified ΔN PINK1 (red arrows). PINK1 mutants seem to follow a phosphorylation level near to PINK1 KI. **B.** Phosphorylation assay using [ϒ-^{32}P]-ATP performed with purified ΔN PINK1 and Ubl Parkin. Autoradiogram shows that Ubl Parkin (green arrows) is phosphorylated by ΔN PINK1, with PINK1 G309D and PINK1 W437X presenting phosphorylation levels close to PINK1 WT (*n* = 3 independent experiments).

The protein bands obtained in the autoradiogram and in the Western blot were quantified using Image Studio Lite software, and the obtained data collected is summarized in Fig.4.14.

For PINK1 (auto)phosphorylation (Fig.4.14A), we report that all clinical mutants present a kinase activity comparable to the kinase inactive form of PINK1, with the exception of PINK1 G309D mutant where an activity of 46,93% was observed.

For phosphorylation of the substrate Parkin (Fig.4.14B), only the PINK1 W437X mutant presented a phosphorylation pattern comparable to PINK1 WT (61,67%). All the other PINK1 mutants (G309D 47,25%; L347P 38,02%; H271Q 26,66% and E417G 20,44%) presented a phosphorylation intensity of Parkin near to the levels detected for PINK1 KI. In conclusion, the PINK1 clinical mutants have a decreased ability to phosphorylate the substrate Parkin as well as to (auto)phosphorylating PINK1.

In sum, PINK1 L347P, PINK1 E417G and PINK1 H271Q show low phosphorylation levels, for both PINK1 and Parkin. Indicating that, the referred alterations, in the kinase domain affect PINK1 kinase activity. The PINK1 G309D mutant shows a defective phosphorylation towards (auto)phosphorylation of PINK1; the mean *per se* is not differing in large percentage of PINK1 KI or the other mutants, suggesting that the difference may be due to a higher experimental variation reflected by the calculated SEM value.

PINK1 W437X behaves the other way around: for PINK1 (auto)phosphorylation this mutant presents levels comparable to the ones obtained for PINK1 KI, but for Parkin phosphorylation this mutant seems not to be affected. Ideally additional experiments should be performed to confirm this tendency that we are observing in our results.

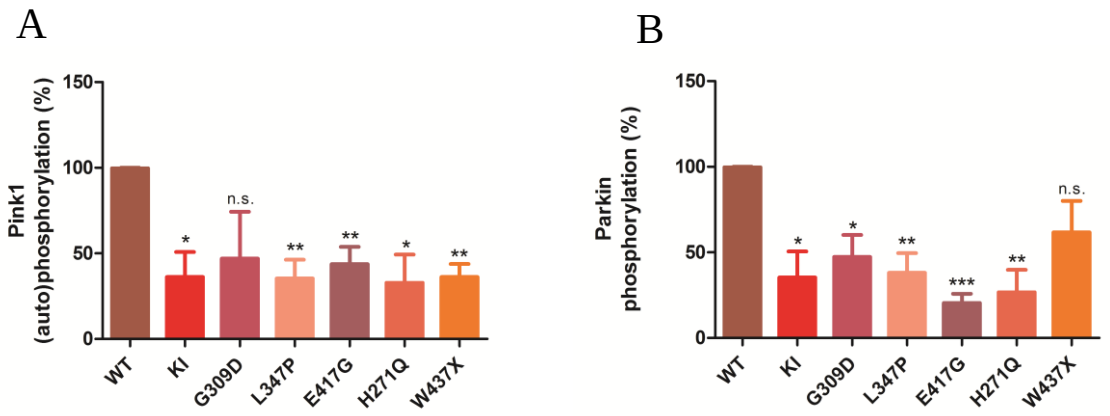

Figure 4.10 - Quantification of in vitro [ϒ-^{32}P]-ATP phosphorylation. A. Phosphorylation by purified ΔN PINK1. Besides PINK1 G309D, all other PINK1 mutants show significant difference to the WT, proximate to PINK1 KI levels. **B**. Phosphorylation by purified ΔN PINK1 with Ubl Parkin. All PINK1 mutants show phosphorylation levels next to PINK1 KI levels.

As for Parkin recruitment to mitochondria, it has previously been described that some mutations in PINK1 could dysregulate PINK1 (auto)phosphotylation.

Beilina et al. (2005) reported that residues G309 and L347 are not predicted to be within the active site of the kinase domain but in areas important for protein folding. The G309 residue is predicted to be near the ATP binding site, and residue L347 in the cyclin binding surface (cyclin-dependent protein kinases). Therefore, a G309D point mutation is unlikely to grossly destabilize the fold but could interfere electrostatically with ATP binding or hydrolysis, justifying PINK1 G309D incompetence to phosphorylate Parkin. Also the fact that PINK1 L347P has a deficient enzymatic activity is probably explained by impaired cyclin binding surface, which could lead to the phosphorylation alterations that we report. Indeed the L347P residue is known to disrupt PINK1 kinase activity (Pridgeon et al., 2007), and it was reported to be unstable, because it does not bind to the Hsp90-Dcd37 chaperone complex (Song et al., 2013).

Catalytically active mutants orthologues of PINK1 have been studied and revealed that residuesH271Q, E417G and L347P appear to virtually abolishes or ablates kinase activity (Woodroof et al., 2011).

For PINK1 W437X mutant, it has previously been reported that this mutant presents increased kinase activity towards substrates (Sim et al., 2006) and also for PINK1 (auto)phosphorylation (Silvestri et al., 2005) when compared to a kinase inactive form. But these assays were performed with different forms of full length PINK1 and with different in vitro assay settings, which may lead to discrepancy when comparing results. For example, delaying PINK1 activity measurement strongly reduces phosphorylation signal, once an unclear rapid loss of PINK1 kinase activity is observed. A lower incubation temperature improves results, and using strong reducing conditions PINK1 catalytic activity is stimulated (Aerts et al., 2016).

5. General conclusion and future work

Parkinson's disease is the second most common neurodegenerative disorder, thus clarification of its pathogenic mechanism and the development of new diagnostic approaches and effective therapeutics are eagerly awaited. In the past few years a connection between this disease and mitochondria has been strengthened. Additionally, the autophagic removal of damaged mitochondria (mitophagy) is of crucial importance in postmitotic cells such as neurons, as the lack of cell division reduces the clearance of dysfunctional damaged mitochondria, resulting in energy deficiency and increasing oxidative stress. These events will further damage mitochondria and other macromolecules, causing an ever-increasing spiral of damage that eventually leads to cell death.

We report that Parkin recruitment occurs in PINK1 mutants, however we were not able to detect a significant phosphorylation levels for the substrates PINK1 or Parkin. Thus, our results suggest that the presence of PINK1 is essential for Parkin recruitment to occur, however PINK1s' kinase activity is not required for this process. Consequently, although Parkin is recruited to the mitochondria, mitochondria present a donut-shaped morphology typically observed when mitophagy is initiated (Haddad *et al.*, 2013). As PINK1 is not able to phosphorylate Parkin and therefore mitophagy does not proceed, accumulation of these donut-shaped mitochondria occurs in the presence of our PINK1 clinical mutants. In order to further validate this hypothesis, a time-course spanning up to 72 hours should be performed for the CCCP treatment. This experiment will determine if inhibition of the PINK1/Parkin regulated mitophagy pathways occurs.

Recently (Zhang *et al.*, 2016) reported that in mammalian cells, BNIP3 inactivation promotes PINK1 proteolytic processing and promotes PINK1/Parkin regulated mitophagy, once this protein can recruit Parkin to mitochondria independently of PINK1. As BNIP3 interacts with LC3 directly promoting mitophagy, this would suggest that PINK1 activity is not obligatory for this pathway. We think that this rather recent findings may explain why the lack of PINK1 activity in these mutants is not interfering with Parkin recruitment. BNIP3 may be compensating for the PINK1 deficiency and restoring mitophagy. To address this issue, the gain- and loss-of-function of BNIP3 should be assessed within the mitophagy pathway, and also expression levels of BNIP3 should be determined in a PINK1 null background. Further, it would be interesting to investigate how BNIP3 is performing in PINK1 mutants.

Additionally, another interesting aspect that could explain our results concerning the fact that Parkin recruitment is still occurring in the presence of PINK1 clinical mutants could be that there is an increase in PGC-1α mediated mitochondria biogenesis (Henchcliffe & Beal, 2008). To tackle this

hypothesis we would initially need to determine if an increase in the PGC-1α signaling pathways is occurring in our PINK1 clinical mutants.

6. References

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7. Appendix

Material and Equipment

Mutants Construction:

Mutant Strand Synthesis Reaction

- PCR tube
- QuikChange II XL Site-Directed Mutagenesis Kit reagents:
  - 10x reaction buffer;
  - dNTP mix;
  - QuikSolution reagent;
- Double distilled water (ddH_20);
- PfuUltra HF DNA polymerase (2.5U/µL).

For the control reactions:

- pWhitescript 4.5-kb control plasmid (5ng/µL)
- Nucleotide control primer #1 [34-mer(100ng/µL)
- Nucleotide control primer #2 [34-mer(100ng/µL)

For the samples:

- pcDNA 3.1 hPINK1 WT ΔN
- Primers refered in table 2.1 "Mutagenesis Primers" section

Dpn I digestion

- Products of amplification
- 1µL DpnI restriction enzyme (10U/µL)

Transformation of XL-Gold Ultracompetent cells

- 1,5mL eppendorfs
- XL-gold ultracompetent
- β-mercaptoethanol (β-ME) mix
- Samples (DpnI treated DNA)
- Thermomixer
- SOC Medium
- Ampicillin Plate (100mg/mL)
- Beads

Cell Culture – cells maintenance

- Water bath
- DMEM (Thermo Fisher Scientific) +10%FBS (Thermo Fisher Scientific)
- DPBS (Thermo Fisher Scientific)
- Trypsin-EDTA (0,05%) (Thermo Fisher Scientific)
- Flux
- Incubator
- Micropipettes (1mL, 200µL,100µL, 20µL, 10µL and 2µL)
- Micropipette tips
- Pipettes (Thermo Fisher Scientific)
- Automatic pipettor
- T75 flasks (Nunc)
- T175 flasks (Nunc)

Cell Culture – cells plating

- 24 well plate (Corning)
- T175 flasks (Nunc)
- Water bath
- DMEM (Thermo Fisher Scientific) +10%FBS (Thermo Fisher Scientific)
- DPBS (Thermo Fisher Scientific)
- Trypsin-EDTA (0,05%) (Thermo Fisher Scientific)
- Falcons 15mL (Frilabo)
- Pipettes (Thermo Fisher Scientific)
- Pipette tips
- Eppendorfs 1,5mL
- Tweezer
- Coverslip 13mm
- Incubator

Cell Culture – cells transfection

- Water bath (37°C)
- DMEM (Thermo Fisher Scientific) +10%FBS (Thermo Fisher Scientific)
- DMEM (serum free) (Thermo Fisher Scientific)
- 1,5 mL Eppendorf
- FuGENE 6 transfection reagent
- Plasmid DNA (represented in table 1)
- Falcons 15mL
- Micropipettes
- Micropipette tips

Mini-preps

- LB Broth Medium
- Ampicillin 100μg/mL
- PS tube sterile
- Micropipette tip
- Petri Dish
- Shaker at 37°C and 225rpm, overnight.
- Overnight bacterial cultures
- 2mL and 1,5mL eppendorf
- Centrifuge
- Nanodrop
- QIAprep Spin Miniprep Kit reagents:
    - Buffer P1
    - Buffer P2
    - Buffer N3
    - QIAprep spin column
    - Buffer PE
    - Buffer EB

Sequencing

- 1,5mL eppendorf tubes
- Provided barcodes
- Primers (10μM), described at table 2.1.
- ddH_2O water

Midi-preps

- Bacterial culture
- Genopure Plasmid Midi Kit (Roche)
    - RNase A (lyophilized enzyme)
    - Suspension Buffer
    - Lysis Buffer
    - Neutralization Buffer
    - Equilibration Buffer
    - Wash Buffer
    - Elution Buffer
    - NucleoBond AX 100 Columns
    - Folded filters
    - Sealing rings
- Nanodrop
- Pipettes: 2mL, 5mL, 10mL, 25mL (Fisher Scientific)
- Pipette tips

Parkin recruitment

- Cell lines: HeLa WT and HeLa-PINK1-KO
- 24 well plate
- FuGENE transfection reagent (Promega)
- DMSO (Sigma-Aldrich)
- 0,1M CCCP (Sigma-Aldrich)
- $PBS^{+/+}$(100 ml 10x $PBS^{-/-}$; 2M $CaCl_2$; 1M $MgCl_2$)
- Formaldehyde (Electron Microscopy Sciences)
- Triton-X100 (Sigma-Aldrich)
- Coverslips 13mm
- Blocking Buffer (0,2% gelatin, 2% fetal bovine serum, 2% bovine serum albumin, 0.3% Triton X-100 in $PBS^{-/-}$)
- Goat serum (Dako)
- Primary antibodies:
    - mouse Turbo GFP (Evrogen)
    - sheep Cytochrome *c* (Sigma)
- $PBS^{-/-}$ (1370 mM Sodium chloride, 27 mM Potassium chloride, 81 mM Disodium hydrogen phosphate, 14.7 mM Potassium dihydrogen phosphate, 9.01 mM Calcium chloride, 4.92 mM Magnesium chloride)
- Secondary antibodies:
    - Alexa 488 donkey anti-rabbit
    - Alexa 568 donkey anti-sheep
- Mowiol
- Nail polish
- Microscope slide

Parkin purification

- BL21 bacteria
- IPTG (Biochemica)
- Lysis Buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Triton X-100, 2mM EDTA, 0.1% beta-mercaptoethanol, 0.2mM PMSF and 1mM benzamidine)
- Glutathione Sepharose™ 4B (GE Healthcare)
- Running Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, H_2O)
- Transfer Buffer (10x Tris/glycine)
- Electrophoresis and Trans-Blot® Cell systems:
    - Tetra Electrode Assembly;
    - Buffer Tank and Lid;
    - Gel Holder Cassette;
    - Thick Blot Filter Paper;
    - Foam Pads;
    - Trans-Blot Central Core.
- Clarity™ and Clarity Max™ Western ECL Blotting Substrates (Bio-Rad)
- Dry Milk (Nestlé)
- TBS-T (1x TBS; 0,1% Tween)
- Primary antibody:
    - α-GST (1/5000; Sigma)

- Secondary antibody:
    - GARPO (1/10000; Bio-Rad)
- Nitrocellulose membranes (GE Healthcare)
- Precision Plus Protein Dual Color Standards (Bio-Rad)
- 4x Laemmli Sample Buffer (Bio-Rad)
- Mini-Protean gels (Bio-Rad)
- 4% β-mercaptoethanol (Bio-Rad)

Human PINK1 purification and in vitro kinase assay

- Cell line: COS
- Plasmids (described in table 2)
- FuGENE transfection reagent (Promega)
- DPBS
- Lysis Buffer (25mM Tris-HCl pH 7.5, 150mM NaCl, 5mM NaF, 1mM MgCl2, 1mM MnCl2, 0.5% Igepal-NP40 (Sigma), 50mg/L DNAse (Sigma), 50mg/L RNAse (Sigma), 1mM DTT), with 20% protease inhibitor cocktail for mammalian cell and tissue extraction (Sigma), Complete protease inhibitor (Roche), PhosSTOP tablets (Roche)
- 22-G needle
- Centrifuge
- FLAG-magnetic beads (Sigma)
- Kinase assay buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 10mM MgCl2, 3mM MnCl2 and 0.5mM DTT)
- Parkin purified (obtained from Parkin purification protocol)
- ATP containing 5μCi [ϒ-^{32}P] ATP
- Thermomixer
- Mini-Protean gels (Bio-Rad)
- 4% β-mercaptoethanol (Bio-Rad)
- 4x Laemmli Sample Buffer (Bio-Rad)
- PVDF membrane (GE Healthcare Life Sciences)
- Precision Plus Protein Dual Color Standards (Bio-Rad)
- P^{32} cassette
- Typhoon
- Dry Milk (Nestlé)
- TBS-T (1x TBS; 0,1% Tween)
- Primary antibody:
    - Mouse anti-Flag M2 (1/5000; Sigma)
    - α-GST (1/5000; Sigma)
- Secondary antibody:
    - GARPO (1/10000; Bio-Rad)
    - GAMPO (1/10000; Bio-Rad)
- Running Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, H_2O)
- Transfer Buffer (10x Tris/glycine)
- Electrophoresis and Trans-Blot® Cell systems:
    - Tetra Electrode Assembly;
    - Buffer Tank and Lid;

    - Gel Holder Cassette;
    - Thick Blot Filter Paper;
    - Foam Pads;
    - Trans-Blot Central Core.
- Clarity™ and Clarity Max™ Western ECL Blotting Substrates (Bio-Rad)