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CENTRO DE INVESTIGACION

PINK1: a critical protein kinase in the molecular mechanisms involved in Cancer and Parkinson's disease

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INFORMAN:

Que la presente Tesis Doctoral, titulada "PINK1: a critical protein kinase in the molecular mechanisms involved in Cancer and Parkinson's disease", ha sido realizada bajo su dirección en el Departamento de Biología Molecular del Cáncer del Centro de Investigación Príncipe Felipe, por JUDIT JIMÉNEZ SÁINZ, licenciada en Biología por la Universidad de Valencia y en el Programa Oficial de Postgrado de Biotecnología; y que habiendo revisado el trabajo, consideran que reúne las condiciones necesarias para optar al grado de Doctor Internacional en Biología y Biotecnología.

Y para que así conste a los efectos oportunos, se expide el presente escrito.

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El mundo perdido
Arthur Conan Doyle

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Abbreviations

4E-BP	4E-binding protein
$\Delta\Psi$m	Mitochondrial membrane potential
aa	Amino acids
Acetyl Co-A	Acetyl coenzyme A
ADP	Adenosine-5'-diphosphate
AIF	Apoptosis-inducing factor
Akt/PKB	Protein kinase B
ANOVA	Analysis of variance between groups
Apaf-1	Apoptotic protease activating factor 1
ATCC	American type culture collection
ATP	Adenosine-5'-triphosphate
ATP13A2	ATPase type 13A2
A. U.	Arbitrary units
Bax	Bcl-2 associated X protein
BD matrigel	Basement membrane matrix
Beclin-1	Coiled-coil, moesin-like Bcl-2 interacting protein
BCA	Bicinchoninic acid
Bcl-2	B-cell CLL/Lymphoma 2
Bcl-xl	B-cell lymphoma-extra large
BrdU	Bromodeoxyuridine
BRPK/PINK1	Brap2-related protein kinase
BSA	Bovise serum albumin
Bub1	Budding uninhibited by benzimidazols 1
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CCD	Charge-coupled device
Cdc37	Cell division cycle 37
Cdks	Cyclin-dependent kinases
cDNA	Complementary DNA
cIAP1	Cellular inhibitor of apoptosis 1
CLIK1	CLP-36 interacting kinase
CLSM	Confocal laser scanning microscopy
cROS	Cytosolic ROS
C-terminal	Carboxilo terminal
cyt c	Cytochrome c
DA	Dopaminergic
$DiOC_6$(3)	3,30-dihexylo-xacarbocyanine iodide
Dkk-3/Reic	Dickkopf-related protein 3
Dlg7	Discs large homolog 7
DAPI	4,6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenylhydrazone
dNTPS	Deoxyribonucleic triphosphates
Dox	Doxycycline
dPINK1	*D.melanogaster* PINK1
Drp-1	Dynamin-1-like protein
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate acid
EdU	5-ethynyl-2'-deoxyuridine (BrdU analog)
EOJP	Early-onset juvenile Parkinson's disease
ERα	Estrogen receptor α
EtBr	Ethidium bromide
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FBXO7	F-box protein 7
FCCP	Carbonylcyanide-4-trifluoromethoxyphenylhydrazone
Fis1	Mitochondrial fission 1 protein
FL	Full length
FOXO3a	Forkhead box transcription factor O 3a
GABARAP	GABA(A) receptor-associated protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBA	Glucosidase β acid
GFP	Green fluorescent protein
GSH	Glutathione
GST	Glutathione S-transferase
GTPase	GTP-binding protein
H_2O_2	Hydrogen peroxide
Ham's F12	Nutrient mixture F-12 Ham medium
HBSS	HEPES-buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEt	Dihydroethidium

HPRT	hypoxanthine-guanine phosphoribosyltransferase
Hsp90	Heat shock protein 90
Htra2/omi	5-hydroxytryptamine (serotonin) receptor 2a
IB	Immunoblot
IF	Immunofluorescence
IHC	Immunohistochemistry
IKKγ	IKB kinase γ
IMM	Inner mitochondrial membrane
IMS	Intermembrane mitochondrial space
INK4	Inhibitor of transporter inner membrane
KIP	Kinase inhibitor protein
KD	Knock-down
KO	Knock-out
LB (LBA)	Lysogeny broth (LB-Agar)
LC3/ MAP1LC3	Microtubule associating protein (MAP)1 light chain 3
Let-7	MicroRNA precursor 7
LOH	Loss of heterozigosity
LRRK2	Leucine-rich repeat kinase 2
m-AAA	Matrix AAA protease
MAPKs	Mitogen-activated protein kinases
MARK2	Microtubule affinity-regulating kinase 2
MCB	Monochlorobinane
MEFs	Mouse embryonic fibroblasts
Mfn	Mitofusin
MG132	N-(benzyloxycarbonyl) leucinylleucinylleucinal Z-Leu-Leu-Leu-al
MLS	Mitochondrial localisation sequence
MPP	Mitochondrial processing protease
mPTP	Mitochondrial permeability transition pore
MPTP/MPT$^+$	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
mTOR(2)	Mammalian target of rapamycin (2)
mRNA	Messenger RNA
ncNAT	Non-coding natural antisense
NET-Gel	NET-Gelatine
NF-$\kappa\beta$	Nuclear factor-Kappa β
NIX	NIP3-like protein X
NSC	Neural stem cell
NSCLC	Non-small-cell lung cancer
N-terminal	Amino terminal
NSKs	Nervous system kinases
OMM	Outer mitochondrial membrane
Opa-1	Optic atrophy protein 1
p62	p62/SQSTM1 Sequestosome 1
PAGE	Polyacrylamide gel electroforesis
PARP	Poly (ADP-ribose) polymerase
PARL	Presenilin-associated rhomboid like
PBS	Phosphate-buffered saline
PCR	Polimerase chain reaction
PD	Parkinson's disease
PFA	Paraformaldehyde
PGAM5	Phosphoglycerate mutase family member 5
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PINK1/BRPK	PTEN-induced kinase 1
PINK1-Δ1	First PINK1 cleaved form (54kDa)
PINK1-Δ2	Second PINK1 cleaved form (45kDa)
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
Rb	Retinoblastome
RNA	Ribonucleic acid
RNAi	RNA interference
RNase A	Ribonuclease A
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SD	Standard desviation
SDHA	Succinate dehydrogenase complex, subunit A
SDS	Sodium dodecyl sulfate
Ser/Thr	Serine/Threonine
SEM	Standard error of the mean
siRNA	Small interfering RNA
siPINK1	Silenced PINK1
SNCA	α-synuclein
SNpc	Substantia nigra pars compacta
STK1	Serological thymidine kinase 1
SYBR	N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylide-2-ne)methyl]-1-phenyl-quinolin-1-ium-2-yl]-N-propyl-propane-1,3-diamine
svPINK1	Splice variant PINK1
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline

TIM	Transporter inner membrane	**T-TBS**	Tween-TBS
TOM	Transporter outer membrane	**TRAP1**	TNF receptor-associated protein 1
TK2	Nuclear thymidine kinase 2		
TM	Transmembrane domain	**UCHL1**	Ubiquitin carboxyl-terminal esterase 1
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine	**UPS**	Ubiquitin-proteasome system
TMRM	Tetramethylrhodamine methylester	**VDAC1**	Voltage-dependent anion channel 1
T-PBS	Tween-PBS	**wt**	Wild-type

Abstract

Cancer and Parkinson's disease (PD) are two disorders for which the final pathophysiological mechanism is not fully defined. Epidemiological data indicate that PD patients have a decreased risk of cancer, with the exception of malignant melanoma, skin, thyroid and breast cancers, suggesting a functional linkage between PD and cancer. In favour of this, misregulation of mitochondria homeostasis is considered an important hallmark in the pathogenesis of both diseases. Recently, several genes associated to PD, including Parkin, LRRK2, DJ-1, or PINK1, have been proposed as modulators of cancer processes. Mutations on PINK1 gene are associated with hereditary early-onset PD. PINK1 is a protein kinase whose mRNA is up-regulated in several carcinoma cell lines and in PTEN over-expressing cells. PINK1 protects cells against mitochondrial-mediated apoptosis, controlling mitochondrial homeostasis through electron transport chain, mitochondrial membrane potential, calcium homeostasis and ROS generation. Moreover, PINK1 together with Parkin, has been associated to mitochondrial dynamics and mitophagy.

To define the putative role of PINK1 in cancer and Parkinson's disease in relation with the mitochondrial homeostasis, we present the analysis of apoptosis, mitochondrial function, mitophagy and survival/proliferation processes on SH-SY5Y (human dopaminergic cells) and MCF-7 (human breast cancer cells) cells over-expressing PINK1 wild-type (wt), and missense (catalytically inactive mutation (K219M), some PD-linked mutations (A168P, G309D, L347P and W437X) and truncated (ΔC, ΔN and ΔN-C) mutations of PINK1. Moreover, we examine the mRNA expression of PINK1 and PINK1-related genes in breast cancer samples.

Our results demonstrate that PINK1 wt, but not missense or truncated mutations, controls mitochondrial function and has an anti-apoptotic role in mitochondrial-mediated apoptosis. In addition, PINK1 wt blocks tumourigenic processes such as invasion, anchorage-independent growth, proliferation and cell cycle progression.

Resumen

El cáncer y la enfermedad de Parkinson (PD) son dos enfermedades en las que el mecanismo pato-fisiológico final no está completamente definido. Datos epidemiológicos indican que los pacientes con PD poseen bajo riesgo de cáncer, con la excepción de melanoma maligno y cánceres de piel, tiroides y mama, lo que sugiere una conexión funcional entre PD y cáncer. Apoyando esta conexión, la desregulación de la homeostasis mitocondrial es una característica importante en la patogénesis de ambas enfermedades. Recientemente, varios genes asociados a PD, tales como Parkin, LRRK2, DJ-1, y PINK1, han sido propuestos como moduladores de procesos cancerígenos. Mutaciones en el gen de PINK1 están asociadas con PD hereditaria de comienzo temprano. PINK1 es una quinasa de proteínas cuyo mRNA está expresado a altos niveles en varias líneas celulares de carcinoma y en células que sobre-expresan PTEN. PINK1 protege a las células de la apoptosis mediada por la mitocondria, controlando la homeostasis mitocondrial a través de la cadena de transporte electrónico, el potencial de membrana mitocondrial, la homeostasis del calcio y la producción de ROS. Además, PINK1, junto con Parkin, ha sido asociado a procesos de dinámica mitocondrial y mitofagia.

Con objeto de definir la función de PINK1 en cáncer y la enfermedad de Parkinson, en relación con la homeostasis mitocondrial, presentamos el análisis de procesos de apoptosis, función mitocondrial, mitofagia y supervivencia/proliferación en células SH-SY5Y (células dopaminérgicas humanas) y MCF-7 (células de carcinoma mamario humano) que sobre-expresan PINK1 *wild-type* (wt) o mutaciones de cambio de aminoácido (mutación cataliticamente inactiva (K219M), mutaciones asociadas a PD (A168P, G309D, L347P y W437X) o formas truncadas (ΔC, ΔN y ΔN-C)) de PINK1. Además, examinamos la expresión del mRNA de PINK1 y de genes relacionados con PINK1 en muestras de tumores de mama.

Nuestros resultados demuestran que PINK1 wt, pero no las mutaciones de cambio de aminoácido o formas truncadas, controla la función mitocondrial y posee una función anti-apoptótica en la apoptosis mediada por la mitocondria. Además, PINK1 wt bloquea procesos tumorogénicos tales como invasión, crecimiento independiente de anclaje, proliferación y progresión del ciclo celular.

Prologue

PINK1 world between cancer and Parkinson's disease

Nowadays, cancer and Parkinson's disease are two common disorders for which the population is worried about and where the essential molecular mechanism impaired is not yet well known. Parkinson's disease, first described by James Parkinson in 1817, is the second most common neurodegenerative disease in elderly, affecting approximately 4% of the population beyond age above 65 years. The major clinical symptoms of Parkinson's disease patients include resting tremor, rigidity and bradykinesia. This disease is caused by pathological changes in the brain that implicate massive cell death with neuronal loss and degeneration in several midbrain areas (such as the substantia nigra pars compacta, the brain stem, the autonomic nervous system, some regions in the basal ganglia and the cortex) and, thereby loose of the connection to the striatal neurons, like a blocked road without connection between important cities of the country. This impairment in neuronal transmission produces significative changes in emotional and motivation aspects of behaviour and in normal movement body. One more important hallmark of Parkinson's disease is the deposition of proteinaceous inclusions, known as Lewy bodies, in the neurons and the dendrites at the substantia nigra, like potholes in the cells that are still alive [Schapira, 2006; Cookson, 2005].

Cancer, known as malignant neoplasm, is a wide group of various diseases, all involving up-regulated cell growth. The earliest written record concerning cancer was made by egiptians 3000 BC about breast cancer. Cancer is a cause of misregulated "crazy" dividing cells that do not respond to stop proliferation signals nor to inducing apoptosis/cell death stimuli.

Back in 1954 Doshay claimed cancer is rare in "paralysis agitans" [Doshay, 1954], and a growing body of several epidemiological studies suggests that people with Parkinson's disease have a decreased risk of almost all cancers. In contrast, Parkinson's disease patients have a significant increased risk of malignant melanoma, skin, breast, and thyroid cancer occurrence [Bajaj et al., 2010; Inzelberg and Jankovic, 2007]. Indeed, a PUBMED search using the keywords "cancer" and "Parkinson's disease" revealed over 2000 articles studying the link between these two diseases, for the period between 1970 and August 2012.

What may account for this association?

The general idea that Parkinson's disease provide "biologic protection" against some types of cancer recently become true because common molecular pathways and genes have shown to be essential in the progression of both diseases [Inzelberg and Jankovic, 2007]. Nevertheless, they represent two opposite forces within the cell, because Parkinson's disease is due to cell death and cancer is due to uncontrolled cell proliferation [Gao et al., 2009].

At first, cancer and Parkinson's disease were described as sporadic diseases mainly caused by some environmental exposures such as pesticides or solvents. The oxidative stress produced by toxins has been widely associated as a main cause of both diseases [Green et al., 2002].

In the last two decades, since some genes were described associated to both diseases, cancer and Parkinson's disease research has been focused on the study of the molecular mechanism modulated by these genetic factors. Expression alterations or mutations of Parkinson's disease associated-genes such as α-synuclein, Parkin, LRRK2, DJ-1 or PINK1 have been identified in a number of human cancers. Some of those genes are involved in common disease pathways like cell cycle, apoptosis, mitochondria and oxidative stress, protein aggregation and degradation within the cellular body, survival/proliferation pathways (PI3K/Akt and MAPKs pathways), etc. Indeed, recent papers have already concluded that several of these genes, such as Parkin have clear and defined functions in the balance of proliferation and cell death [Devine et al., 2011; West et al., 2005].

Who is PINK1? Possible connection between cancer and Parkinson's disease

One of the genes linked to Parkinson's disease, PINK1 (PTEN Induced Kinase 1), has been associated to cancer processes. Mutations on PINK1 gene are associated with hereditary early-onset Parkinson's disease [West et al., 2005]. PINK1 gene was discovered in 2001 as a protein kinase whose mRNA is expressed at high levels in several carcinoma cell lines and in PTEN (one of the major tumour suppressor proteins in humans) over-expressing cells [Nakajima et al., 2003; Unoki and Nakamura, 2001]. Besides, several groups pointed out PINK1 as a marker of survival prognosis in adenocarcinomas [de Reyniès et al., 2009; Fragoso et al., 2012].

PINK1 protein is associated to mitochondria (the power energy source of the cell) promoting the mitochondrial health and optimising its function [Valente et al., 2004a; Gegg et al., 2009]. PINK1 protects cells against mitochondrial-mediated apoptosis produced by several cell death inducing agents [Petit et al., 2005; Beilina et al., 2005]. Moreover, PINK1 has been associated to survival/proliferative pathways (PI3K/Akt and MAPKs pathways), which could be linked to cancer progression [Murata et al., 2011a,b].

PINK1 protein is likely to have more than one function (in apoptosis, mitochondria homeostasis, survival and proliferation processes) and could potentially play different roles in different diseases. Furthermore, PINK1 could undergo differential regulation depending on the tissue environment. The balance of the PINK1 functional read-out could lead to neurodegeneration or cell transformation, which could be important for future therapies to fight Parkinson's disease or cancer. Further studies need to be done to understand the differential role of PINK1 in cancer and Parkinson's disease.

Chapters to come

During my PhD thesis, I have worked with experts in both fields: cancer and Parkinson's disease. This thesis is my personal effort to explain the recent advances in the understanding of PINK1 functions and its relation to the pathogenesis of both diseases.

In **Chapter 1**, first we recapitulate the main evidences found for cancer and Parkinson's disease connection and introduce the findings on PINK1 identification, regulation and function. Lately, we include the literature describing the PINK1 implication in different diseases such as cancer, Parkinson's disease, diabetes and Alzheimer's disease.

In **Chapter 2**, we emphasise the main objective of this thesis: to investigate how PINK1 may control cellular homeostasis through mitochondrial quality control and its pivotal role in two relevant human diseases: cancer and Parkinson's disease. It is followed by the methods and materials (**Chapter 3**) used in our study in SH-SY5Y and MCF-7 cultured cells and in human tissues. At the end of **Chapter 3**, we introduce the scheme with all the missense and truncated mutations used in this study.

In **Chapter 4**, we present the study of PINK1 functions in SH-SY5Y cells as a Parkinson's disease model. In particular, we show the implication of wild type PINK1 in mitochondrial function through the ATP production by the electron transport chain, the maintenance of the mitochondrial membrane potential, and control of oxidative stress. Furthermore, we explore these processes with different PINK1 missense and truncated mutations, showing disruption of these functions.

In **Chapter 5**, we study the possible implications of PINK1 on important tumourigenic cancer processes such as invasion, adhesion, anchorage-independent cell growth, proliferation and cell cycle progression using MCF-7 breast cancer cells. We outline the control of these processes by PINK1 and how PINK1 mutations or truncations affect them.

To follow with the possible cancer and Parkinson's disease connection through PINK1 protein and other PINK1-related genes, we examine the expression of these genes in normal and neoplastic tissues in **Chapter 6**. Moreover, the cellular distribution of PINK1 is examined in these cultured cell models (i.e. SH-SY5Y and MCF-7) and in tissues (**Chapters 4**, **5**, and **6**).

Finally we end up with the discussion of the results and future perspectives (**Chapter 7**) as well as with the conclusions (**Chapter 8**) that come out from this thesis.

Prólogo

El mundo de PINK1 entre el cáncer y la enfermedad de Parkinson

Hoy en día el cáncer y la enfermedad de Parkinson son dos enfermedades frecuentes que preocupan a la población y donde el mecanismo molecular esencial dañado es desconocido. La enfermedad de Parkinson se describió por primera vez por James Parkinson en 1817, es la segunda enfermedad neurodegenerativa más común en edad avanzada, afectando aproximadamente a un 4% de la población por encima de los 65 años de edad. Los principales síntomas de los pacientes con la enfermedad de Parkinson incluyen temblor en posición de reposo, rigidez y bradiquinesía o inestabilidad postural. La enfermedad es causada por cambios patológicos en el cerebro que implican la muerte masiva de neuronas y la degeneración de áreas mesencefálicas (como la parte compacta de la sustancia negra, el tallo cerebral, el sistema nervioso autónomo, regiones en los ganglios basales y en la corteza) y, por ello, la perdida de conexión con las neuronas del estriado, como una carretera bloqueada sin conexión entre ciudades importantes de un país. Esta deficiencia en la transmisión neuronal produce cambios significativos en los aspectos emocionales y de motivación del comportamiento, y en el movimiento normal del cuerpo.

Una característica más importante de la enfermedad de Parkinson es la deposición de inclusiones proteicas, conocidas como cuerpos de Lewy, en las neuronas y dendritas de la sustancia negra, como baches en las células que aún están vivas [Schapira, 2006; Cookson, 2005].

El cáncer, conocido como neoplasia maligna, es un amplio grupo de enfermedades diversas, que involucran todas ellas el aumento del crecimiento celular. El registro escrito más antiguo sobre cáncer fue hecho por los egipcios en el 3000 aC, en concreto sobre el cáncer de mama. El cáncer es la causa de la división descontrolada “ loca” de las células, que no responden a las señales de parada de proliferación ni a estímulos de inducción de la apoptosis/muerte celular.

En 1954 Doshay afirmó que el cáncer es poco frecuente en “parálisis agitante” [Doshay, 1954], y un número creciente de varios estudios epidemiológicos sugieren que las personas con enfermedad de Parkinson tienen un menor riesgo de casi todos los tipos de cáncer. Por el contrario, los pacientes con enfermedad de Parkinson tienen un riesgo significativamente mayor de incidencia de melanoma maligno, y de cáncer de mama, piel, y tiroides [Bajaj et al., 2010; Inzelberg and Jankovic, 2007]. De hecho, una búsqueda en PUBMED usando las palabras clave “ cáncer” y “enfermedad de Parkinson” reveló más de 2000 artículos que estudian la relación entre estas dos enfermedades, para el período comprendido entre 1970 y agosto de 2012.

¿Qué puede explicar esta asociación?

La idea general de que la enfermedad de Parkinson puede proporcionar "protección biológica" contra algunos tipos de cáncer recientemente se ha convertido en un hecho cierto porque se ha demostrado la existencia de vías moleculares y genes comunes que son esenciales en la progresión de ambas enfermedades [Inzelberg and Jankovic, 2007]. Sin embargo, representan dos fuerzas alejadas opuestas dentro de la célula debido a que la enfermedad de Parkinson se debe a la muerte celular y el cáncer se debe a la proliferación y supervivencia celular descontrolada [Gao et al., 2009].

En un principio, el cáncer y la enfermedad de Parkinson se describieron como enfermedades esporádicas causadas principalmente por algunas exposiciones ambientales como pesticidas o disolventes. El estrés oxidativo producido por toxinas ha sido ampliamente asociado como una causa principal en ambas enfermedades [Green et al., 2002].

En las dos últimas décadas, ya que algunos genes se han descrito asociados a ambas enfermedades, la investigación en cáncer y en la enfermedad de Parkinson se ha centrado en el estudio del mecanismo molecular modulado por estos factores genéticos. Alteraciones de expresión o mutaciones de los genes asociados a la enfermedad de Parkinson tales como α-sinucleína, Parkina, LRRK2, DJ-1 o PINK1 se han identificado en un número de cánceres humanos. Algunos de estos genes están implicados en vías comunes de la enfermedad como ciclo celular, apoptosis, mitocondria y estrés oxidativo, agregación y degradación de proteínas dentro de la célula, vías de supervivencia/proliferación (vías PI3K/Akt y MAPKs), etc.

De hecho, estudios recientes ya han concluido que varios de estos genes, como Parkina tienen funciones claras y definidas en el equilibrio de la proliferación y la muerte celular [Devine et al., 2011; West et al., 2005].

¿Quién es PINK1? Posible conexión entre el cáncer y la enfermedad de Parkinson

Uno de los genes ligados a la enfermedad de Parkinson, PINK1 (PTEN Induced Kinase 1), se ha asociado a los procesos de cáncer. Las mutaciones en el gen de PINK1 están asociadas con la enfermedad de Parkinson hereditario de comienzo temprano [West et al., 2005]. El gen PINK1 fue descubierto en 2001, una quinasa de proteínas cuyo ARNm se expresa en altos niveles en varias líneas celulares de carcinoma y en células que sobre expresan PTEN (una de las principales proteínas supresoras de tumores en seres humanos) [Nakajima et al., 2003; Unoki and Nakamura, 2001]. Además, varios grupos han señalado a PINK1 como un marcador de pronóstico de supervivencia en adenocarcinomas [de Reyniès et al., 2009; Fragoso et al., 2012].

La proteína PINK1 está asociada a la mitocondria (la fuente de energía de la célula) promoviendo la salud mitocondrial y optimizando su función [Gegg et al., 2009; Valente et al., 2004a]. PINK1 protege a las células contra la apoptosis mediada por la mitocondria producida por varios agentes que inducen muerte celular [Petit et al., 2005; Beilina et al., 2005]. Por otra parte, PINK1 se ha asociado a las vías de supervivencia/proliferación (vías PI3K/Akt y MAPKs), que podrían estar relacionadas con la progresión del cáncer [Murata et al., 2011a,b].

La proteína PINK1 probablemente tenga más de una función (apoptosis, homeostasis mitocondrial, supervivencia y procesos de proliferación) y podría desempeñar diversas

funciones en diferentes enfermedades. Además, PINK1 podría regularse dependiendo del entorno tisular. El balance de las funciones de PINK1 puede inducir neurodegeneración o transformación celular, que podría ser importante para el desarrollo de futuras terapias, para combatir la enfermedad de Parkinson o el cáncer. Nuevos estudios deben hacerse para comprender el papel diferencial de PINK1 en el cáncer y la enfermedad de Parkinson.

Capítulos siguientes

Durante mi tesis doctoral, he trabajado con expertos en ambos campos: el cáncer y la enfermedad de Parkinson. Esta tesis es mi esfuerzo personal para explicar los avances recientes en la comprensión de la funciones de PINK1 y su relación con la patogénesis de ambas enfermedades.

En el **capítulo 1**, primero recapitulamos las principales evidencias encontradas para la conexión entre el cáncer y la enfermedad de Parkinson e introducimos los hallazgos en la identificación, regulación y función de PINK1. Por último, se incluye la bibliografía que describe la implicación de PINK1 en diferentes enfermedades tales como el cáncer, la enfermedad de Parkinson, la diabetes y la enfermedad de Alzheimer.

En el **capítulo 2**, hacemos hincapié en que el objetivo principal de esta tesis: la de investigar cómo PINK1 puede controlar la homeostasis celular a través del control de calidad mitocondrial y su papel fundamental en dos enfermedades importantes: el cáncer humano y la enfermedad de Parkinson. Le siguen los materiales y métodos (**capítulo 3**) utilizados en nuestro estudio en cultivos de células SH-SY5Y y MCF-7 y en tejidos humanos. Al final del **capítulo 3**, se presenta el esquema con todas las mutaciones puntuales y formas truncadas de PINK1 utilizadas en este estudio.

En el **capítulo 4**, se presenta el estudio de las funciones de PINK1 en células SH-SY5Y como modelo de la enfermedad de Parkinson. En particular, se demuestra la implicación de PINK1 de tipo salvaje en la función mitocondrial a través de la producción de ATP por la cadena de transporte de electrones, el mantenimiento del potencial de membrana mitocondrial, y el control del estrés oxidativo. Además, se exploran estos procesos con diferentes mutaciones puntuales y formas truncadas, mostrando alteración de estas funciones.

En el **capítulo 5** se estudian las posibles implicaciones de PINK1 en importantes procesos de cáncer tumorogénesis como la invasión, adherencia, anclaje independiente de crecimiento, proliferación celular y la progresión del ciclo celular utilizando células MCF-7 de cáncer de mama. Planteamos el control de estos procesos y cómo las mutaciones puntuales o formas truncadas pueden afectarlos.

Para seguir con la conexión entre el cáncer y la enfermedad de Parkinson, a través de la proteína PINK1 y de otros genes relacionados con PINK1, en el **capítulo 6** se examina la expresión de estos genes en los tejidos normales y neoplásicos. Además, la distribución celular de PINK1 se examina en estos modelos de cultivos celulares (es decir, SH-SY5Y y MCF-7) y en los tejidos (**capítulos 4**, **5**, y **6**).

Por último nos encontramos con la discusión de los resultados y perspectivas futuras (**capítulo 7**), así como con las conclusiones (**capítulo 8**) que se obtienen de esta tesis.

Chapter

1 Introduction

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1.1 Cancer and Parkinson's disease

1.1.1 Parkison's disease

Parkinson's disease (PD, MIM 168600) is the second most common neurodegenerative disorder of the elderly, affecting ∼4% of the population over the age of 65. It was first described by James Parkinson in an 1817 monograph, "Essay on the Shaking Palsy". Patients typically present resting tremor, slow movements (bradykinesia), limb stiffness (rigidity), and a shuffling gait. Many patients also suffer from autonomic, cognitive, and psychiatric disturbances.

Table 1.1: Overview of genetic loci "*PARK*" associated with a monogenic form of PD.

Locus	Gene	Chromosome position	Inheritance	Onset[1]	Pathology	Reference
PARK1	α-synuclein	4q21-q23	Dominant	Early-Late	Nigral degeneration with Lewy bodies	Polymeropoulos et al., 1997
PARK2	Parkin	6q25.5-q27	Recessive	Early	Nigral degeneration without Lewy bodies	Matsumine et al., 1998
PARK3	n.r.	2p13	Dominant	Late	Nigral degeneration with Lewy bodies. Plaques	Gasser et al., 1998
PARK4	α-synuclein	4q21-q16.3	Dominant	Early-Late	Nigral degeneration with Lewy bodies. Plaques and tangles	Farrer et al., 1999
PARK5	UCHL1	4p14	Dominant	Early	Nigral degeneration with Lewy bodies, vacuoles in neurons of the hippocampus	Leroy et al., 1998
PARK6	PINK1	1p35-36	Recessive	Early	Nigral degeneration with Lewy bodies in some cases	Valente et al., 2001
PARK7	DJ-1	1p36.33-p36.12	Recessive	Early	Nigral degeneration	Van Duijn et al., 2001
PARK8	LRRK2	12p11.23-q13.11	Dominant	Late	Nigral degeneration and/or Lewy bodies and/or neurofibrillary tangles	Funayama et al., 2002
PARK10	n.r.	1p32	Dominant	Late	n.r.	Hicks et al., 2002
PARK11	GIGYF2	2q36-q37	Dominant	Late	n.r.	Pankratz et al., 2003
PARK12	n.r.	Xq21-q25	X-linked	Late	n.r.	Pankratz et al., 2002
PARK13	Htra2/omi	2p12	Dominant	Late	n.r.	Strauss et al., 2005
PARK14	n.r.	18q11	Recessive	n.r.	n.r.	Gao et al., 2009
PARK15	FBXO7	22q12-q13	Recessive	n.r.	n.r.	Shojaee et al., 2008
PARK16	n.r.	1q32	Recessive	n.r.	n.r.	Satake et al., 2009

Based on PDgene website `http://www.pdgene.org/` [Lill et al., 2012]. [1]: Early: 20s, 30s and 40s; Late: 50s, 60s and later. n.r.: not reported.

The major symptoms of PD result from the loss of monoaminergic neuron populations in the brainstem, the most profound of which is a depletion of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). Lewy bodies are intraneuronal inclusion bodies composed of many proteins including heavily aggregated forms of a small protein, α-synuclein.

Approximately 10-15% of patients with the typical clinical picture of PD have a positive family history compatible with a Mendelian inheritance. In recent years, several new genes and environmental factors have been implicated in PD, and their impact on DA neuronal cell death is coming into focus. **Table 1.1** shows the loci associated to PD described so far [Cookson, 2005; Wood-Kaczmar et al., 2006; Lill et al., 2012].

The mutations of PD loci are present in proteins involved in protein quality control, oxidative stress, and mitochondrial function, processes that are closely interlinked. Environmental toxins used to model PD in animals similarly inhibit mitochondrial function, increase formation of free radicals and, in some cases, cause protein aggregation. Therefore, there is a strong link between mitochondrial dysfunction and PD pathology [Schapira, 2006, 2008; Kubo et al., 2006].

Mitochondria are essential organelles for aerobic metabolism present in all eukaryotic cells. Mitochondria have a number of physiological roles within the cell including the generation of ATP through the electron transport chain, regulation of apoptosis, and the storage and maintenance of intracellular calcium levels. Mitochondria are dynamic organelles and via the processes of fission and fusion are capable of eliminate dysfunctional mitochondria and remodel mitochondrial network within the cell. The mitochondria possess its own genome (mtDNA) but is also dependent on proteins codified by nuclear genome. Mitochondria are composed of three structures: the outer mitochondrial membrane (OMM), the inner mitochondrial membrane (IMM), and the matrix. The matrix contains the mtDNA and the cristae protrude into it. The electron transport chain complexes are situated within the inner mitochondrial membrane. Electrons are transferred through these complexes to the terminal electron acceptor O_2. During this transfer a number of protons are translocated across the inner mitochondrial membrane which establishes a proton gradient and the mitochondrial membrane potential ($\Delta\Psi$m). This potential is used by ATP synthase to generate ATP and is also responsible for producing reactive oxygen species (ROS) within the cell [Alberts et al., 2002b; Mathews et al., 2002].

Mitochondria are vulnerable organelles highly susceptible to damage from oxidative phosphorylation, generation of ROS and damage to mtDNA. Therefore, neurons, which are forced to high energy demands, are very susceptible to mitochondrial damage, connecting to PD disease [Reeve et al., 2008].

1.1.2 Cancer

Cancer is a disease in which cells no longer respond to the environmental signals that normally govern their behaviour in a tissue. Tumour cells grow and divide when they should not, and fail to die when they should. They loose their attachments from the tissue and spread to other tissues, a process known as metastasis. Cancer is a large and complex group of related but distinct diseases, each with unique features that depends on the biological characteristics of the cell type or tissue in which the disease originates (**Figure 1.1**).

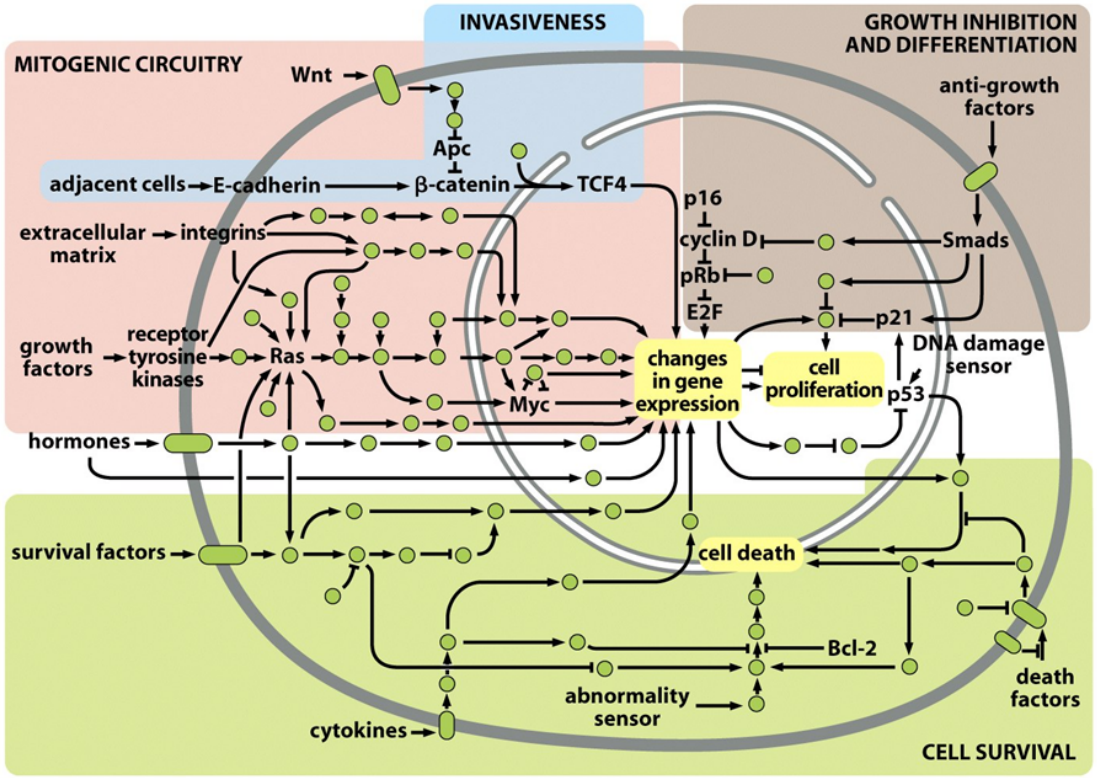

Figure 1.1: Cancer is a complex disease. The intracellular signalling circuitry and collaboration between cancer-associated genes are displayed here. The growth-promoting, mitogenic circuit (light red), the circuit governing growth-inhibitory signal (light brown), the circuit governing apoptosis (light green), and the circuit governing invasiveness and metastasis (light blue) can be affected in cancer Source: Weinberg, 2007.

Cancer of all tissues share the same antisocial patterns of proliferative and invasive behaviour, which results from the gradual loss by mutation of the mechanisms that normally limit these processes (**Figure 1.1**).

The plasma membrane is vulnerable to mechanical changes and it is required for invasive and proliferative properties [Chen, 1981; Alberts et al., 2002a; Weinberg, 2007]. Pathological levels of mechanical stress such as cancer can exacerbate these constitutive levels of cell wounding and contribute to tumour progression.

Regulation of the metazoan cell cycle is critical for processes such as proliferation, differentiation, apoptosis and tumourigenesis. A tightly controlled interplay between inhibitory, activating and "checkpoint" factors ensures (1) the unidirectional progression through G_1, S, G_2 and mitosis phases, (2) the exit from the cell cycle into the quiescent G_0 state, or (3) the re-entry into the cell cycle via G_1. A complex network of regulatory proteins governs progression through the steps of the cell cycle (**Figure 1.2**).

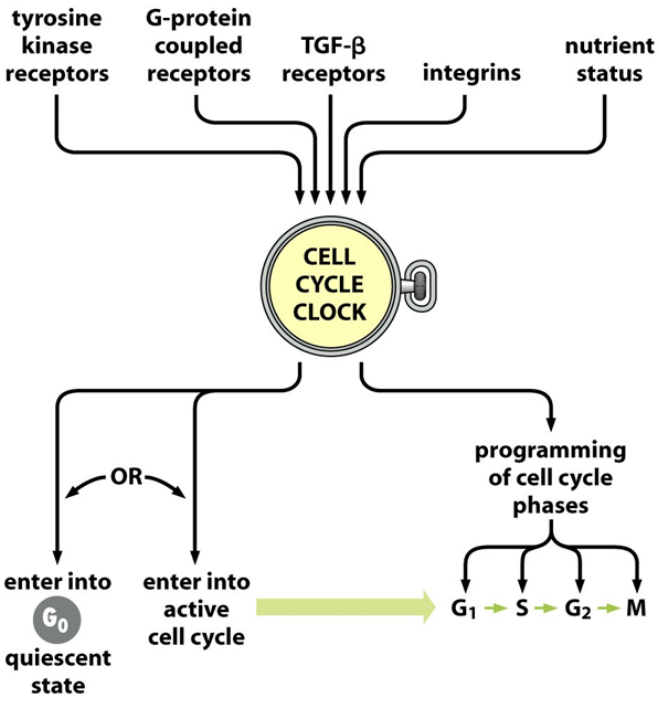

Figure 1.2: The central governor of growth and proliferation. The term "cell cycle clock" denotes a molecular circuitry operating in the cell nucleus that processes and integrates a variety of afferent (incoming) signals originating from outside and inside the cell, and decides whether or not the cell should enter into the active cell cycle or retreat into a non-proliferating state. In the event that active proliferation is decided upon, this circuitry proceeds to program the complex sequence of biochemical changes that enable the cell to double its contents and to divide into two daughters. Source: Weinberg, 2007.

Cell cycle progression is regulated by a group of cyclin-dependent kinases consisting of catalytic subunits, designated Cdks, as well as activating subunits, designated cyclins. Orderly progression through the cell cycle requires the activation and inactivation of different cyclin-Cdk complexes at appropriate times (**Figure 1.3**). Several proteins function as mitotic inhibitors, such as p27, which interacts with D-type cyclins and Cdk4 and subsequently promotes its degradation, allowing the G_1 progression; or p21, which is dependent of the p53 levels induced by DNA damage [Donovan and Slingerland, 2000]. The cyclin D1 protein increases in G_0/G_1 phase, binds Cdk4 and p27, and, after p27 binding, its degradation is activated with G_1 progression and the G_1 phase progresses. The expression of cyclin D has been associated with many forms of cancer and also plays a critical role in mammary tissue maturation [Zwijsen et al., 1998; Dufourny et al., 2000; Tashiro et al., 2007]. The cyclin A protein is involved in control of S phase and has been associated to Cdk2. In addition, cyclin A has been implicated in cell transformation [Zerfass et al., 1995]. The cyclin B1 protein is required to activate the mitosis together with maturation-promoting factor [Morgan, 2007] (**Figure 1.3**).

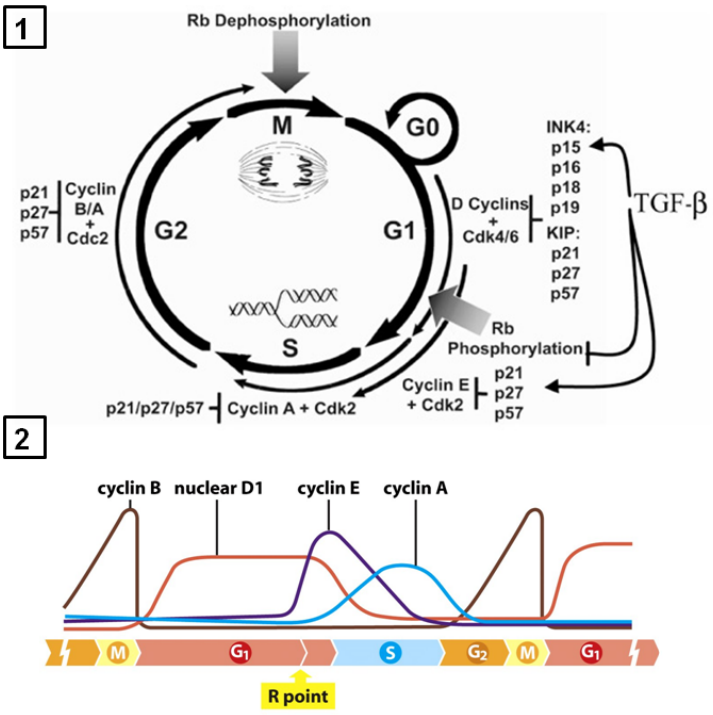

Figure 1.3: The cell cycle scheme. 1) Cell cycle progression is governed by Cdks, the activities of which are regulated by binding of cyclins, by phosphorylation and by the Cdk inhibitors (the INK4 (inhibitors of Cdk4) family: p15, p16, p18 and p19; and the kinase inhibitor protein (KIP) family: p21, p27 and p57). **2)** Fluctuation of cyclin levels during the cell cycle. These fluctuations are tightly coordinated with the schedule of advances through the various cell cycle phases. The levels of cyclin-D are highly regulated by extracellular signals and growth factors. Sources: Weinberg, 2007; Morgan, 2007.

1.1.3 Connection between cancer and Parkinson's disease

Cancer and Parkinson's disease (PD) are two common disorders for which the final pathophysiological mechanism is not yet fully known. Several epidemiological and clinical studies (**Appendix 8**) have illustrated that individuals with PD show lower occurrence of cancer than expected, with the exception of higher risk of malignant melanoma, skin, breast and thyroid cancers [Inzelberg and Jankovic, 2007].

In cancer, cell regulation mechanisms result in increased cell survival and/or proliferation. Conversely, PD is associated with increased neuronal death, caused by, or concomitant with, accumulation of proteinaceous Lewy bodies. The possibility exists that common perturbations of mechanisms involved in cell survival/death regulation could be involved in both disorders [West et al., 2005].

The exact mechanisms underlying the observed cancer-PD association are not clear, but different hypothesis have been raised for a positive or an inverse link between these diseases. Oxidative stress and environmental exposition to pesticides or solvents have been associated to both diseases [Checkoway and Nelson, 1999; Jenner and Olanow, 1996]. Also,

the administration of levodopa to PD patients (a melanin precursor) increases susceptibility to skin and melanoma cancers [Elbaz et al., 2005; Inzelberg and Israeli Korn, 2009]. On the other hand, epidemiological studies have reported an inverse relation between PD and cancer, which can be dependent on the amount of potassium and detoxifying enzymes in the body [Jansson and Jankovic, 1985; Checkoway et al., 1998]. Furthermore, the ubiquitin-proteasome system has been inversely linked to the pathogenesis of both diseases [McNaught et al., 2001; Mitchell, 2003; Lim and Tan, 2007; Devoy et al., 2005].

In addition, the genes associated with familial forms of PD (the *PARK* loci) are now somehow implicated in tumourigenesis and some of them have been found to be abnormally expressed, inactivated, or deleted in several tumour types (**Table 1.2**) [Devine et al., 2011].

The effects of *PARK* mutations on tumourigenesis are unclear and, due to the unknown functions of some of these PD-associated genes, conclusions about the nature of the mutations (gain-of-function *versus* loss-of-function) are difficult to establish. For example, α-synuclein (SNCA; *PARK1, PARK4*) expression is up-regulated in glioma cell lines, schwannomas, medulloblastomas, and breast and ovarian carcinomas. Deletions of Parkin (*PARK2*) have been identified in hepatocellular carcinomas and in breast, ovarian and non-small-cell lung cancers. Finally, ubiquitin C-terminal hydrolase (UCHL1; *PARK5*) is over-expressed in oesophageal and squamous cell carcinomas, and in pancreatic and colorectal cancers [West et al., 2005]. Moreover, loss/gain-of-function mutations in PD-linked genes may have distinct effects on tumourigenesis. Nevertheless, genes associated with familial PD have been shown to regulate cell death and/or the cell cycle, and several lines of evidence imply that malfunction of a shared biochemical pathway may lead to PD or cancer [West et al., 2005].

1.2 *PARK6*, PTEN-induced kinase 1 (PINK1)

PINK1 (MIM 608309) (PTEN-induced kinase 1) was first identified as a protein associated with cancer and PD. PINK1 isolation and identification came from two approaches: an association study in a PD family, as a new locus linked to PD, and in tumour cell lines where the tumour suppressor PTEN (Phosphatase and tensin homolog) was over-expressed ectopically [Unoki and Nakamura, 2001; Valente et al., 2001].

1.2.1 PINK1 locus/gene

PINK1 gene (Enseml ID: ENSG00000158828), known as *PARK6*, is localised in a 3.7 cM region, 2.8Mb region of chromosome 1p36-p35 (1:20959948-20978004(1) strand). The PINK1 gene contains eight exons spaning $\sim$ 1.8Kb.

1.2.2 PINK1 mRNA

PINK1 mRNA is expressed in all adult tissues from *Mus musculus* and *Homo sapiens*, with more abundant expression in the brain, heart, testis and skeletal muscle, and low levels in ovary, thymus and colon tissues (**Table 1.3**) [Unoki and Nakamura, 2001; Nakajima et al., 2003; Chiba et al., 2009; Berthier et al., 2011]. The brain distribution of PINK1 mRNA in mouse and rat is homogeneous throughout the tissue, including PD relevant structures such as SNpc, caudate-putamen, cortex and hippocampus [Taymans et al., 2006; Blackinton et al., 2007].

Table 1.2: Parkinson's disease genes related to cancer.

Gene (protein)	Alteration and putative role in: PD	Cancer	Implicated cancers
PARK1 *PARK4* (SNCA)	•Heterozygous mutations or over-expression •Synaptic dysfunction •Mitochondrial dysfunction	Unknown	Over-expressed in brain tumours with neuronal differentiation [Kawashima et al., 2000] Expressed in malignant and benign melanocytic lesions but not in normal skin [Matsuo and Kamitani, 2010] Expressed in ovarian cancer but not in normal ovarian tissue [Bruening et al., 2000]
PARK2 (Parkin)	•Homozygous mutations and deletions •E3 ubiquitin ligase targets Mfn, VDAC1 and cyclin E •Transcriptional repression of p53	E3 ubiquitin ligase targeting cyclin E	Inactivating mutations and deletions in glioblastoma, and colon and lung cancers [Veeriah et al., 2009]
PARK5 (UCHL1)	•Heterozygous mutations •Role unknown	Deubiquitylates p53	Silenced (via CpG methylation) in nasopharyngeal carcinoma [Li et al., 2010], colorectal and ovarian cancers [Okochi-Takada et al., 2006], and renal cell carcinoma [Kagara et al., 2008]
PARK6 (PINK1)	•Homo-heterozygous mutations •PI3K/Akt/mTOR pathway •Mitophagy	Unknown	Heterozygous mutations in ovarian cancer (627G>A, P209P; 1444G>A, V482M) and glioma (644C>T, P215L)*
PARK7 (DJ-1)	•Homozygous mutations •PI3K/Akt/mTOR pathway •Response to oxidative stress •Mitophagy	•PI3K/Akt/mTOR pathway •Modulation of p53	Over-expressed in NSCLC [MacKeigan et al., 2003] and prostate cancer cell lines [Hod, 2004]
PARK8 (LRRK2)	•Heterozygous mutations •PI3K/Akt/mTOR pathway •MicroRNA regulation (let-7)	•PI3K/Akt/mTOR pathway •MicroRNA regulation (let-7)	Amplification or over-expression in papillary renal cell carcinoma and thyroid cancer [Looyenga et al., 2011]
PARK9 (ATP13A2)	•Heterozygous mutations •Lysosomal dysfunctional	Unknown	Missense mutations in ovarian cancer, melanoma and glioma
GBA	•Homozygous mutations •Microglial activation •Lysosomal dysfunction •UPS dysfunction	•Decreased immune surveillance of tumours •Cytokine release	Homozygous deficiency in haematological malignancies (especially multiple myeloma) and hepatocellular carcinoma [De Fost et al., 2006]
PARK15 (FBXO7)	•Homozygous mutations •Stabilization of cyclin D-Cdk6 complexes	Inhibition of cIAP1	Over-expression in lung and colon cancer [Laman et al., 2005]

*Data source: COSMIC database `http://www.sanger.ac.uk` [Bamford et al., 2004].
See **Appendix8**. Modified from Devine et al., 2011.

In *Drosophila melanogaster*, PINK1 mRNA is expressed throughout the lifespan, with the highest levels in pupal stages and slightly reduced levels in adults, and is highly abundant in brain and in the retina [Wang et al., 2006]. In *Danio rerio*, PINK1 mRNA is distributed uniformly in larvae stage with increased intensity in muscle and in the nervous system, where many DA neurons exist [Anichtchik et al., 2008; Sallinen et al., 2010], and in *Oryzias latipes* increased expression was detected in telencephalon, diencephalon and optic tectum [Matsui et al., 2010].

1.2.3 PINK1 protein

PINK1 is a 581 amino acid protein (63 kDa) composed of a mitochondrial localisation sequence (MLS), a transmembrane domain (TM), a highly conserved Serine/Threonine (Ser/Thr) kinase domain, and a regulatory C-terminal region (**Figure 1.4**). Noticeably, PINK1 is the first kinase that exhibits a canonical mitochondrial targeting sequence with several cleavage sites defined. PINK1 full length (FL, 63 kDa) leads to the generation of at least three forms, suggesting the presence of mutiple cleavage sites: PINK1 FL, PINK1-Δ1 cleaved form upon entry into the mitochondria (54 kDa), and PINK1-Δ2 cleaved form (45 kDa). In addition, a third PINK1 protein of 60 kDa has been recently described [Sim et al., 2012; Jin et al., 2010; Greene et al., 2012]. The specific functional role and the topology of these PINK1 protein fragments are not yet well characterised (see below).

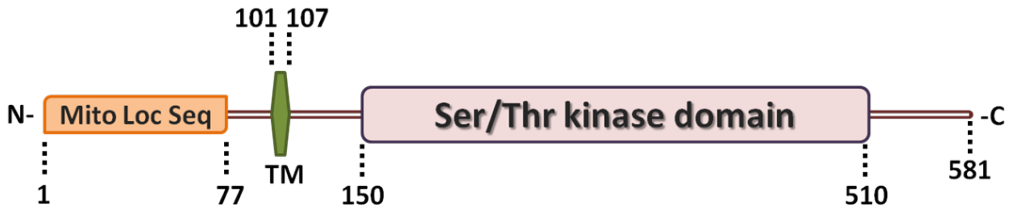

Figure 1.4: PINK1 domain organization. PINK1 is composed of a mitochondrial localisation sequence (MLS, residues 1-77), a transmembrane domain (TM, residues 101-107), a Ser/Thr kinase domain (residues 150-510), and a regulatory C-terminal domain (residues 510-581).

In humans, PINK1 protein is highly expressed in liver and epithelial tissues including adrenal gland, mammary gland, prostate, pancreas, kidney tubules, stomach, intestine and hypophysis. PINK1 protein expression is weak or absent in bladder and skin epidermis. Moreover, PINK1 protein is widely expressed in the brain, including grey matter, but not white matter. PINK1 is also expressed in cortex, striatum, and brainstem with cortical neurons and Purkinje cells [Berthier et al., 2011]. Additional studies have shown that PINK1 is expressed at low levels in white matter and in all cell types, including glia, endothelial cells, and blood vessel smooth muscle cells (**Table 1.3**) [Gandhi et al., 2006; Murakami et al., 2007].

In *Danio rerio*, PINK1 is expressed within the paraventricular regions of the adult, including the hypothalamus and the deep layers of the optic tectum, and in dopaminergic tyrosine hydroxylase positive neurons [Anichtchik et al., 2008].

Table 1.3: PINK1 mRNA and protein expression studies.

Organism	Expression			Reference
	Lifespan	**Body**	**Brain**	
mRNA expression				
D. rerio	Uniformly in larvae stage	High in muscle	High in the nervous system, mainly in grey matter	[Anichtchik et al., 2008; Sallinen et al., 2010]
O. latipes	—	—	Telencephalon, diencephalon and optic tectum	[Matsui et al., 2010]
D. melanogaster	High in pupal stages and reduced levels in adults	—	Whole brain and in the retina	[Wang et al., 2006]
M. musculus	Ubiquitously expressed	Heart, skeletal muscle and testis	—	[Unoki and Nakamura, 2001; Nakajima et al., 2003]
	—	—	Broadly with high expression in cerebral cortex, striatum, hippocampus, amygdala, thalamus, SNpc and cerebellar areas	[Taymans et al., 2006]
	—	High in liver, tongue and adrenal cortex	Strong in grey matter. High in cortex, DA and hippocampal neurons	[Blackinton et al., 2007]
	—	—	Nerve cells of cortex, hippocampus, and cerebellum	[Chiba et al., 2009]
H. sapiens	—	—	Hippocampus, cerebellum, striatum, amygdala and caudate nucleus	[Blackinton et al., 2007]
Protein expression				
D. rerio	—		Paraventricular regions of the adult, including hypothalamus, the optic tectum, and DA neurons.	[Anichtchik et al., 2008]
M. musculus	—	Testis and heart	Brain	[Berthier et al., 2011]
H. sapiens	—	Endothelial cells and smooth vessels	Grey and white matter (less). Frontal and temporal cortex, caudate, putamen and cerebellum. Glia and neurons. Lewy bodies.	[Gandhi et al., 2006]
	—	—	Increased expression in the SNpc of PD patients	[Muqit et al., 2006]
	—	—	Cortex and glial inclusions. Lewy bodies	[Murakami et al., 2007]
	—	Liver and epithelial tissues (prostate, adrenal and mammary glands)	Cortical neurons, ventricular epithelium, cerebellum (Purkinje cells)	[Berthier et al., 2011]

1.2.4 PINK1 homology

PINK1 protein is highly conserved in evolution and it is present in metazoans, from nematodes to mammals (**Figure 1.5**) [Nakajima et al., 2003; Valente et al., 2004a; Rogaeva et al., 2004; Petit et al., 2005; Marongiu et al., 2007; Cardona et al., 2011]. The whole PINK1 protein and, in particular, the kinase domain is highly conserved between PINK1 orthologues, as **Table 1.4** shows.

Table 1.4: Identity of PINK1 and domains in differents organisms.

Organism	Percentage of identity			
	Total	**Kinase domain**	**MLS**	**C-terminal tail**
H. sapiens	100	100	100	100
M. mulatta	97	96	98	99
M. musculus	81	82	80	86
M. domestica	77	81	66	75
G. gallus	64	66	41	62
D. rerio	54	63	45	38
A. gambiae	45	45	—	—
D. melanogaster	43	45	—	—
C. quinquefasciatus	41	45	—	—
A. aegypti	40	42	—	—
C. elegans	32	36	—	—

Percentage of identity compared to PINK1 *H.sapiens* amino acid sequence;
—: below 30% of identity.

1.2.5 PINK1 Ser/Thr kinase and phosphorylation

The terciary structure of protein kinases is highly conserved and contains different subdomains, according to the classification of Hanks and Hunter, 1995 [Hanks and Hunter, 1995] (**Figure 1.6**). The N-terminal part comprises the subdomains I-IV and it is involved in ATP binding and orientation. This contains the AIK motif where the lysine involved in ATP orientation is localised (subdomain II). The C-terminal part of kinase domain is composed of catalytic loop and activation loop and contains subdomains VIa-XI. The catalytic loop (subdomain VI) is characterised by HRD motif (HRDLKxxN). The activation loop is composed of the highly conserved DFG motif that orients the γ-phosphate of ATP and chelates Mg^{2+} (subdomain VII) and the APE motif which stabilises the kinase catalytic domain (subdomain VIII). The rest of the subdomains are structurally important (subdomains IV-V) and are involved in substrate recognition (subdomains IX-X).

PINK1 conserves the typical Ser/Thr kinase subdomains wiht high homology to Ca^{2+}/ calmodulin family kinases. The PINK1-related high homology kinases are STK1 (serological thymidine kinase 1) [Nakajima et al., 2003; Silvestri et al., 2005], CLIK1 (CLP-36 interacting kinase) and PKA (protein kinase A) [Sim et al., 2006]. PINK1 has been classified in the NSKs family (nervous system function and development kinase with a partially conserved putative calcium-binding site) [Cardona et al., 2011].

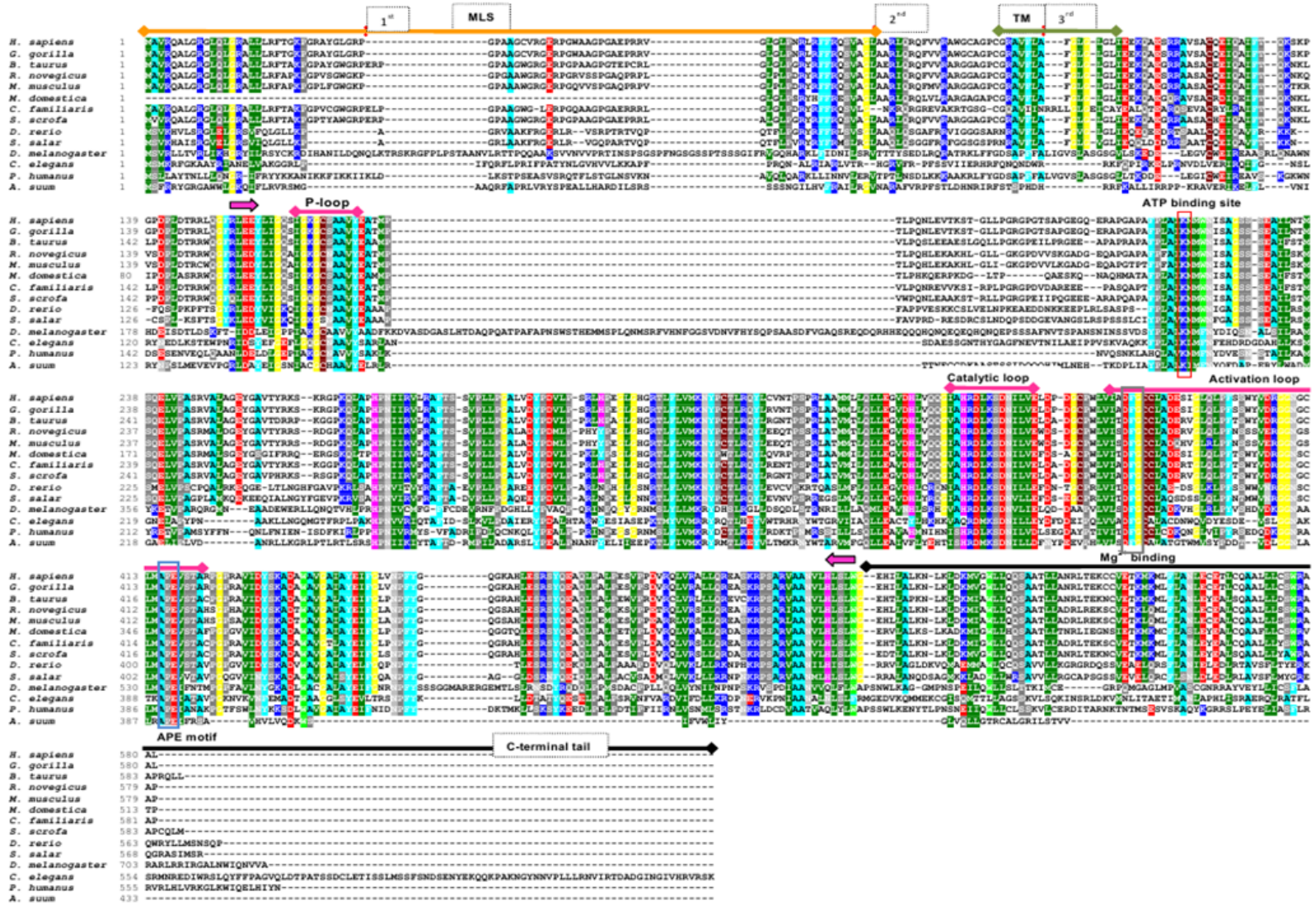

Figure 1.5: Alignment of PINK1 amino acid sequence from different organisms. MLS (orange line), TM (green line), Ser/Thr kinase domain (region between the pink arrows), C-terminal tail (black line), and the reported cleavage sites (1^{st}, 2^{nd} and 3^{rd} in red dotted line) are indicated. Within the kinase domain, the P-loop, the ATP binding site, the catalytic loop and the activation loop with Mg^{2+} binding site and APE motif are designated. PINK1 amino acid sequences were obtained from Uniprot, and ClustalX (70% threshhold for shading) was used for the alignment.

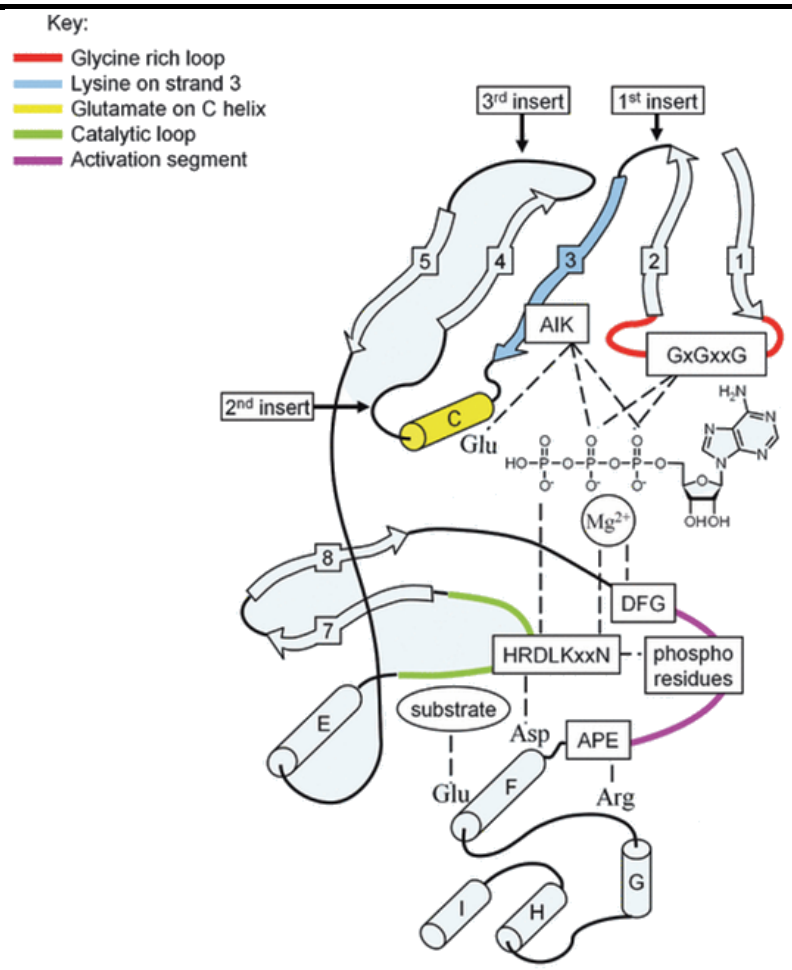

Figure 1.6: Ser/Thr kinase domain of PINK1. Schematic diagram of the Ser/Thr kinase domain, with conserved functional motifs boxed, and predicted locations of insert regions marked. Dotted lines indicate some of the interactions between specific residues, the substrate, ATP, and the cofactor Mg^{2+}. Modified from Mills et al., 2008.

The primary human sequence of PINK1 includes the three conserved Ser/Thr kinase motifs: AIK (amino acids 216-219), HRD (amino acids 236-362), and DFG (amino acids 384-386) described above (**Figure 1.7**) [Albanese et al., 2005; Beilina et al., 2005; Silvestri et al., 2005; Sim et al., 2006; Wang et al., 2006; Mills et al., 2008; Cardona et al., 2011]. A special feature of PINK1 is the presence of three insertions in the N-terminal lobe of the kinase domain which may modify the kinase activity (**Figure 1.6 and 1.7**) [Mills et al., 2008; Cardona et al., 2011; Sim et al., 2012], and can cause the decrease of the intrinsic kinase activity, as several authors have postulated [Pridgeon et al., 2007; Plun-Favreau et al., 2007; Plun-Favreau and Hardy, 2008].

Phosphorylation of artificial substrates and auto-phosphorylation of PINK1 have been described in PINK1 over-expressing cultured cells or using PINK1 protein purified from bacteria [Nakajima et al., 2003; Silvestri et al., 2005; Beilina et al., 2005; Liu et al., 2009].

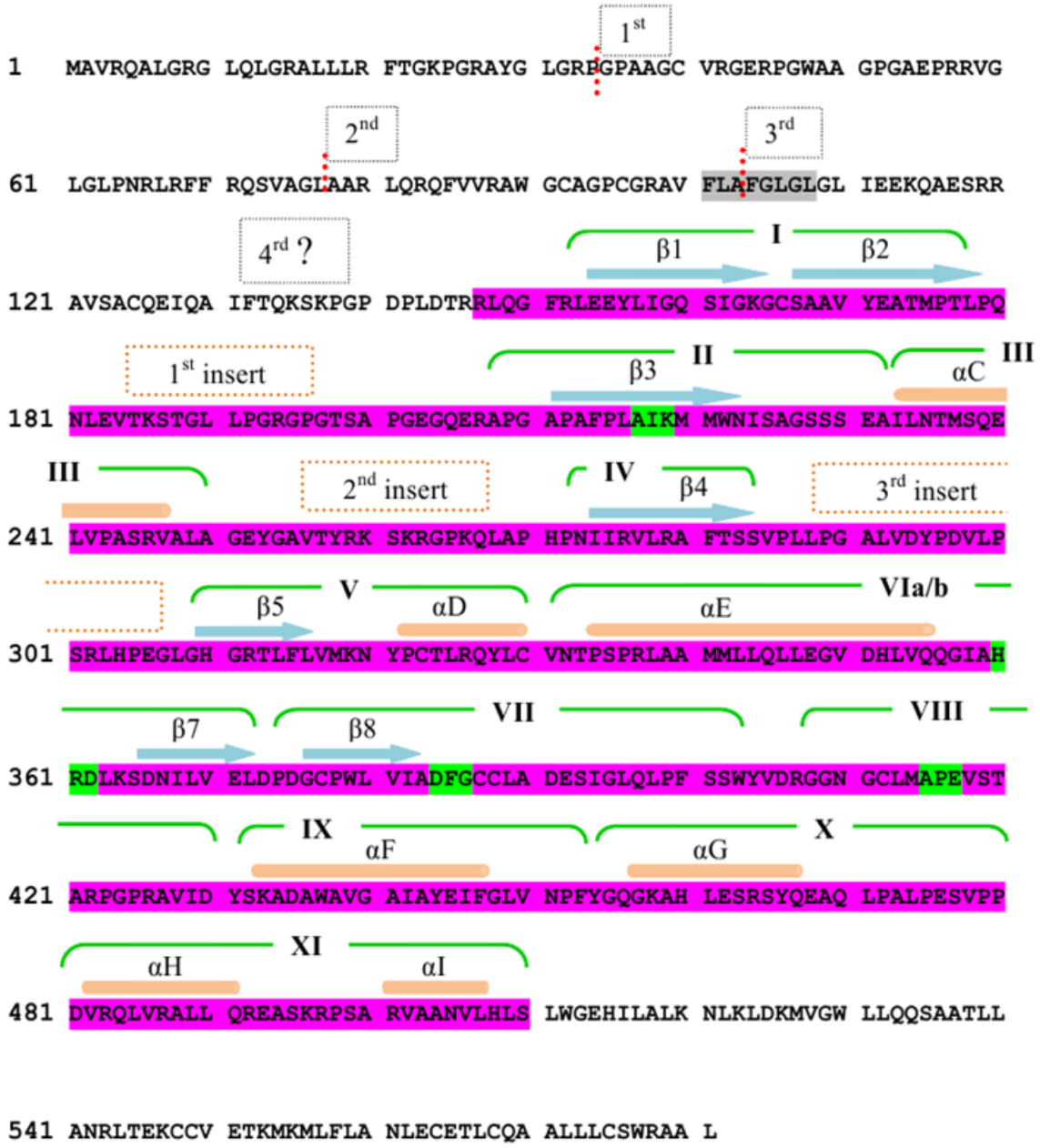

Figure 1.7: Amino acid sequence of PINK1 protein. The MLS (residues 1-77) with the cleavage sites (1^{st} and 2^{nd}), TM region (grey) with the 3^{rd} cleavage site, the kinase domain (pink) with the kinase subdomains (green brackets), and the secondary structure (β-sheet in blue and α-helix orange) are indicated. There is a putative 4^{th} cleavage site after TM. The AIK, HRD and DFG motifs within the kinase domain are shadowed in green and the three insertions indicated. The C-terminal tail is after the kinase domain. Based on Petit et al., 2005; Beilina et al., 2005.

Nevertheless, PINK1 exhibits low kinase activity, and only PINK1 insect ortologue presents significant kinase activity [Woodroof et al., 2011]. PINK1 undergoes auto-phosphorylation in multiple Ser and Thr residues. Auto-phosphorylation of Thr 257 occurs upon CCCP (carbonyl cyanide m-chloro phenyl hydrazone) treatment and increases the PINK1 phosphorylation activity over Parkin [Kondapalli et al., 2012]. Recently, the PINKtide (WIpYRR*S*PRRR), which is the peptide able to be phosphorilated by insect PINK1, and the consensus phosphorylation sequence (S/T E L/M) have been determined [Woodroof et al., 2011; Sim et al., 2012; Wang et al., 2012]. The C-terminus of PINK1 regulates its kinase activity and both positive and negative regulations have been reported [Sim et al., 2006; Silvestri et al., 2005]. Most of the PINK1 mutations analysed (K219A, G309D, L347P, D362A, D386A, G409D, E417G) are associated with reduced kinase activity [Beilina et al., 2005; Silvestri et al., 2005; Sim et al., 2006; Pridgeon et al., 2007].

The microtubule affinity-regulating kinase 2 (MARK2) has been described to phosphorylate PINK1 at Thr 313, in the third insertion region, and, subsequently, in other residues. This cooperates with MARK2 function, which regulates mitochondrial trafficking in neurons [Matenia et al., 2012].

PINK1 is involved in the phosphorylation of the mitochondrial chaperone TRAP1 (TNF receptor-associated protein 1) in hydrogen peroxide-treated PC12 cells, which can modulate mitochondrial protection [Pridgeon et al., 2007]. Cytosolic substrates such as Parkin and Miro are directly phosphorylated by PINK1. Parkin is phosphorylated at Thr 175 (in the linker region), which promotes its translocation to the mitochondria [Kim et al., 2008; Sha et al., 2010]. Upon CCCP treatment, another Parkin residue (Ser 63) is phosphorylated by insect PINK1, which is important for Parkin E3-ubiquitin ligase activation [Kondapalli et al., 2012]. Miro is directly phosphorylated by PINK1 at the Ser 156 and Thr 299 [Wang et al., 2012], which regulates mitochondrial trafficking. PINK1 modulates the phosphorylation status of the mitochondrial protein Htra2 (5-hydroxytryptamine (serotonin) receptor 2a)/omi by an indirect mechanism involving stress-activated kinase p38 [Plun-Favreau et al., 2007, 2008]. Finally, PINK1 induces phosphorylation of Rictor and thereby activates mTOR2 (mammalian target of rapamycin complex 2) [Murata et al., 2011a].

1.3 PINK1 localisation and import

The subcellular and submitochondrial locations of PINK1 remain controversial. PINK1 has been reported to be located in the inner mitochondrial membrane (IMM) [Silvestri et al., 2005; Gandhi et al., 2006; Muqit et al., 2006; Beilina et al., 2005; Pridgeon et al., 2007; Weihofen et al., 2008; Marongiu et al., 2009], in the mitochondrial intermembrane space (IMS) [Silvestri et al., 2005; Plun-Favreau et al., 2007; Pridgeon et al., 2007; Meissner et al., 2011] and in the outer mitochondrial membrane (OMM) [Zhou et al., 2008b; Narendra et al., 2010] (**Figure 1.8**).

The PINK1 positive charged MLS presents, by bioinformatic prediction, several cleavage sites which could be targeted by distinct proteases during mitochondrial import (**Figure 1.7**). The cleavage sites described include residues at 34, 76 and 103 (within the TM) positions [Silvestri et al., 2005; Deas et al., 2010; Jin et al., 2010]. Indeed, the first 34 or 93 amino acids of PINK1 suffice to target fused-GFP to the IMM via TOM/-TIM23 complexes [Silvestri et al., 2005; Muqit et al., 2006; Jin et al., 2010; Jin and Youle, 2012]. Keeping with this notion, studies using various PINK1 N-terminal deletions (at

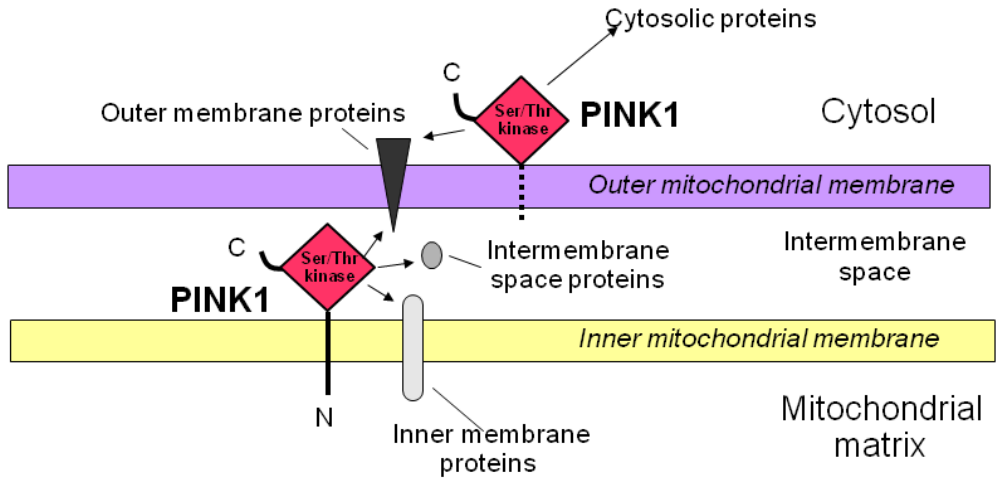

Figure 1.8: Localisation of PINK1 in the mitochondria. PINK1 localises at the IMS with the kinase domain facing this compartment and interacting with mitochondrial substrates, like TRAP1 or Htra2/omi; or at the OMM, facing the cytosol and binding to proteins such as Parkin, Rictor and Miro.

residues 35, 108, 111 and 150) concluded that the deleted PINK1 can not be imported to the mitochondria [Haque et al., 2008; Takatori et al., 2008; Wang et al., 2007; Zhou et al., 2008b]. However, PINK1 without MLS is still associated to the OMM, which could be due to binding to others protein like Miro/Milton in an MLS-independent fashion [Weihofen et al., 2009; Kane and Youle, 2012; Liu et al., 2012].

Experimental data on PD-linked PINK1 mutations show no effects on the localisation of PINK1, suggesting that the topology of the kinase alone can not explain the pathology due to mutation [Valente et al., 2004a; Silvestri et al., 2005; Beilina et al., 2005; Petit et al., 2005; Muqit et al., 2006; Pridgeon et al., 2007; Wang et al., 2007; Zhou et al., 2008b; Becker et al., 2012]. Only in the case of C-terminal truncated mutations, the localisation seems to be more mitochondrial than in PINK1 wt [Becker et al., 2012].

Recent studies have shed light on the mechanism of PINK1 mitochondria import and cleavage. PINK1 imported into the mitochondria is first cleaved by the mitochondrial processing protease (MPP), at an unknown cleavage site, to form a 60 kDa intermediate spanning the IMM [Sim et al., 2012]. Presenilin-associated rhomboid-like protein (PARL) and/or matrix-AAA protease (m-AAA), and ClnXP proteases then cleave this 60 kDa intermediate to generate a 54 kDa processed form of PINK1 attached to the IMM (**Figure 1.9**) [Whitworth et al., 2008; Deas et al., 2010; Becker et al., 2012; Jin et al., 2010; Meissner et al., 2011; Greene et al., 2012]. Findings from *D. melanogaster* and cultured cells concluded the role of Rhomboid-7 (human PARL homologue) in PINK1 cleavage at Ala residue 103, generating PINK1-Δ1, which is dependent on mitochondrial membrane potential ($\Delta\Psi$m) [Whitworth et al., 2008; Deas et al., 2010; Meissner et al., 2011]. Another important determinant on PINK1 topology is its TM sequence, that acts as a hydrophobic "stop transfer" signal of the MLS of PINK1 into the IMM. Indeed, deletion of the transmembrane domain leads to PINK1 accumulation within the matrix [Deas et al., 2010; Jin et al., 2010].

The ΔΨm dissipation upon valinomycin or CCCP treatment blocks PINK1 cleavage and import inside the mitochondria, retaining PINK1 FL at the OMM through direct binding to TOM20 where it participates in regulating mitochondrial fission/fusion processes and mitophagy [Lin and Kang, 2008; Zhou et al., 2008b; Lazarou et al., 2012].

PINK1 does not only localise to mitochondrial fractions, as cytosolic and microsomal fractions were found to contain all cleaved forms of PINK1 [Petit et al., 2005; Gandhi et al., 2006; Zhou et al., 2008b; Haque et al., 2008; Beilina et al., 2005; Weihofen et al., 2008; Narendra et al., 2010]. Moreover, PINK1 has been found in aggresomes and in Lewy bodies [Samaranch et al., 2010].

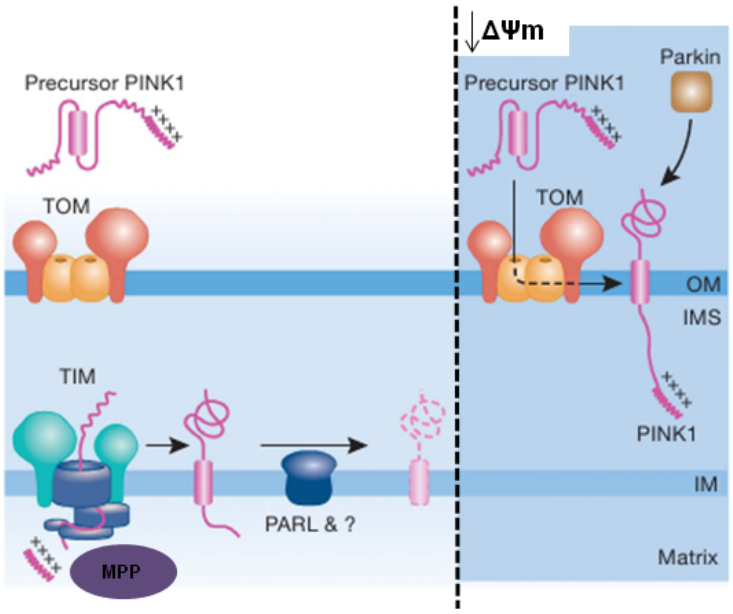

Figure 1.9: Model of PINK1 import and processing in the mitochondria. PINK1 precursor proteins are imported into the mitochondria via the TOM complex in a ΔΨm-dependent manner, accompanied by maturation, likely by the MPP. Newly imported PINK1 is degraded in the IMM by PARL. A dysfunction of mitochondria (low ΔΨm) impairs import but not mitochondrial targeting of PINK1, resulting in its accumulation at the mitochondrial surface. Modified from Rugarli and Langer, 2012.

1.4 PINK1 regulation

1.4.1 PINK1 locus regulation

PINK1 mRNA expression was originally associated to PTEN regulation and expression [Unoki and Nakamura, 2001]. Indeed, PINK1 mRNA expression correlates inversely with PI3K/Akt activation status. Activated Akt translocates out of the nucleus and inactivates transcription function of FOXO3a. FOXO3a up-regulates PINK1 mRNA expression and thus, up-regulates the expression of downstream genes involved in the protection against cellular stress [Mei et al., 2009].

PINK1 mRNA levels are up-regulated during differentiation of human NSCs into neurons and localise to soma and neurites of post-mitotic DA cells, suggesting a possible function in differentiated neurons [Wood-Kaczmar et al., 2008].

Intriguingly, it has been described in human and mouse that the PINK1 gene locus originates a cis-transcribed non-coding natural antisense (ncNAT), and a predicted novel short splice variant protein (svPINK1) which represents the C-terminal portion of PINK1 [Scheele et al., 2007; Chiba et al., 2009]. The ncNAT displays near complete sequence overlap with the svPINK1 and only partial overlap with PINK1 at the 3' end (**Figure 1.10**). The functional role and regulation of these variants are still open questions.

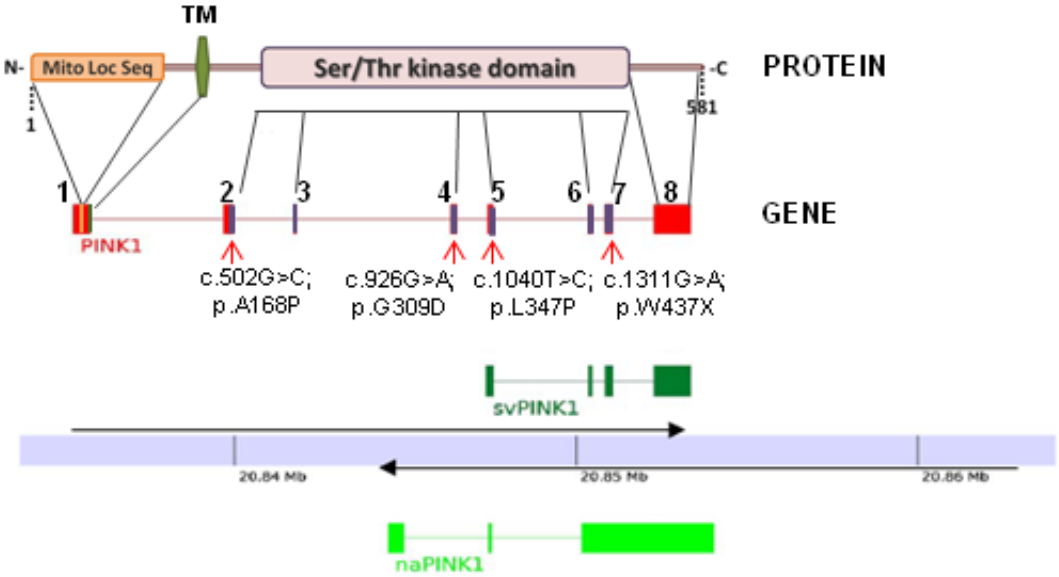

Figure 1.10: Scheme of the exon-intron organization and regulation of human PINK1 locus. Exons (red boxes) and introns (lines) are drawn to scale in PINK1 gene. The PINK1 protein corresponding to the exons is indicated: MLS (orange), TM (green) and kinase domain (purple). The PD-linked mutations used in this study are indicated with red arrows. The bottom diagram represents the chromosomal coordinates for the PINK1 gene annotated in the Ensembl database ver.67 May, 2012 (20,832,535–20,850,591, chromosome 1). Black arrows indicate direction of transcription: PINK1 and svPINK1 are transcribed from left to right, while naPINK1 is transcribed from right to left. Modified from Scheele et al., 2007.

1.4.2 PINK1 protein levels regulation

PINK1 protein is a multiple form protein which is transcribed in the nucleus as full length (FL), translated in the cytoplasm, and imported intact into the mitochondria, with subsequent processing and intra-mitochondrial or cytosolic sorting.

As we previously mentioned, it has been described that PINK1 FL (63 kDa) is dependent on PARP-cleavage under basal conditions, producing PINK1-Δ1 [Muqit et al., 2006; Lin and Kang, 2008; Weihofen et al., 2008; Petit et al., 2005; Takatori et al., 2008]. The other form, PINK1-Δ2 is from unclear origin, and it has been proposed to be generated

from ans alternative translation initiation site or by another cleavage event [Jin et al., 2010; Becker et al., 2012] . Conversion of the FL protein to these two cleavage products is rapid, occuring within 3 min after PINK1 FL protein synthesis and being dependent on the mitochondrial integrity [Lin and Kang, 2008].

PINK1 is a short-life protein of only 30 min half-life under basal conditions, and is mainly degraded via the proteasomal pathway [Muqit et al., 2006; Lin and Kang, 2008; Um et al., 2009]. The three PINK1 forms are accumulated upon proteasomal inhibition by MG132 or epoxomicin, although the PINK1-Δ1 form of 54 kDa is prominent [Tang et al., 2006; Takatori et al., 2008]. It has been described that PINK1 FL and PINK1-Δ2, but not PINK1-Δ1, are stabilised by binding to hsp90/cdc37 chaperones [Lin and Kang, 2008].

The levels of PINK1 are directly related to Parkin protein, which inhibits PINK1 ubiquitination upon binding through its RING2 domain [Um et al., 2009]. Binding to DJ-1 also stabilised PINK1 [Xiong et al., 2009].

Recent evidence indicates that PINK1 protein levels are kept low through instant shedding of the protein during or shortly after mitochondrial import. However, when the mitochondrial membrane potential is dissipated or the mitochondrial integrity is lost, PINK1 processing is inhibited and PINK1 FL is stabilised at the OMM [Jin and Youle, 2012; Narendra and Youle, 2011].

Finally, PINK1 protein is also regulated through dimers formation via the region at residues 246-509, as well as through association to the mitochondrial membranes and mitochondrial protein complexes, as a highly hydrophobic protein [Silvestri et al., 2005; Liu et al., 2009].

1.5 Animals models to study PINK1 function

The PINK1 functions described are mainly based on PINK1 relation to PD where PINK1 is inactivated.

Flies deficient for PINK1 show progressive degeneration of a subset of dopamine neurons in the brain, degeneration of flight muscle tissue, and defective sperm cell development. PINK1 deficiency leads to fragmented mitochondrial cristae and hypersensitivity to oxidative stress [Park et al., 2006; Clark et al., 2006]. This deficiency can be rescued by Parkin and the fission factors Drp1 and Fis1, suggesting a physiological role for PINK1 in dynamics and/or degradation of damaged mitochondria [Deng et al., 2008; Yang et al., 2008; Poole et al., 2008] (see **Section 1.6.3 and 1.6.4**).

By contrast, knock-out PINK1 mice have a mild phenotype and do not recapitulate the dopamine neuron loss observed in humans. Nevertheless, these mice have nigrostriatal physiological defects, suggesting a role for PINK1 in dopamine transmission [Kitada et al., 2007].

Other brain phenotypes of PINK1 knock-out (KO)/ knock-down (KD) mice include [Kitada et al., 2007; Zhou et al., 2007; Gautier et al., 2008; Gispert et al., 2009; Martella et al., 2009; Akundi et al., 2011; Gautier et al., 2012; Wang et al., 2011]:

- Elevated susceptibility to H_2O_2 or heat-shock stress, with decreased activities of the oxidative-stress vulnerable respiratory complexes as well as of aconitase activity.

- Increased calcium levels and vulnerability with subsequent excess of ROS production,

decreased glucose availability and loss of $\Delta\Psi$m, causing pathological opening of the mitochondrial permeability transition pore.

- Reduced synaptic dopamine release and plasticity in the striatum.
- Sensitization to activation of group II metabotropic glutamate receptors at corticostriatal synapses.
- Reduced viability of cortical neurons.

Finally, Billia et al. also reported in a PINK1 KD mouse model higher degree of cardiomyocyte apoptosis with greater levels of oxidative stress, suggesting that PINK1 is indispensable for normal heart function [Billia et al., 2011].

Additional PINK1 mutant models have been generated in *C. elegans*, where increased susceptibility to oxidative stress and mitochondrial morphology defects were observed [Samann et al., 2009], as well as in *D. rerio*, which exhibited a decrease in dopamine-producing cells and reduced mitochondrial activity [Sallinen et al., 2010].

These models have limitations as their phenotypes do not recapitulate well the PD phenotype observed in humans. Others models such as culture cells are needed to throw light on PINK1 functions linked to human disease.

1.6 Functions of PINK1

1.6.1 Mitochondrial-mediated apoptosis

The programmed cell death, known as apoptosis, encompasses various pathways. The mitochondrial pathway begins with the permeabilization of the OMM and the subsequent release from the IMS to the cytosol of several apoptogenic proteins such as apoptosis-inducing factor (AIF) or cytochrome c (cyt c). In the cytosol, cyt c forms a complex with apoptotic protease activating factor 1 (Apaf-1) and pro-caspase 9 (the apoptosome), which activated caspase 9 and induces the activation of downstream executioner caspases, including caspase 3 [Nijhawan et al., 2000; Green and Kroemer, 2004].

During mitochondrial apoptotic cascade, the opening of mitochondrial permeability transition pore (mPTP) is believed to cause mitochondrial membrane permeabilization and cyt c release [Kinnally and Antonsson, 2007]. Proteosome inhibitors like MG132, electron transport chain inhibitors like rotenone, and increased production of ROS, activate mitochondrial apoptotic pathways and cause the mitochondrial release of cyt c.

PINK1 was first described as a mitochondrial-mediated anti-apoptotic protein [Valente et al., 2004a]. PINK1 deficient cells are more susceptible to apoptosis after exposure to mitochondrial toxins like MPP^+, staurosporine or rotenone [Deng et al., 2005; Wang et al., 2006; Pridgeon et al., 2007; Haque et al., 2008; Wood-Kaczmar et al., 2008]. In addition, over-expression of PINK1 wt, but not PINK1 kinase inactive or PD-linked mutations, protects against cell death mediated by chemical insults such as MPTP, MG132 or staurosporine [Valente et al., 2004a; Petit et al., 2005; Wang et al., 2006; Tang et al., 2006; Pridgeon et al., 2007; Wang et al., 2007; Haque et al., 2008; Chu, 2009] (**Figure 1.11**).

PINK1 over-expression blocks the release of cyt c and the cleavage and activation of the pro-caspase 3 (**Figure 1.11**). Surprinsingly, over-expression experiments using PINK1 with N-terminal mutation (R68P) or deletion (first 111 aa) revealed protection against cell death, even in the asbsence of the MLS [Haque et al., 2008; Tan et al., 2009; Tan, 2009].

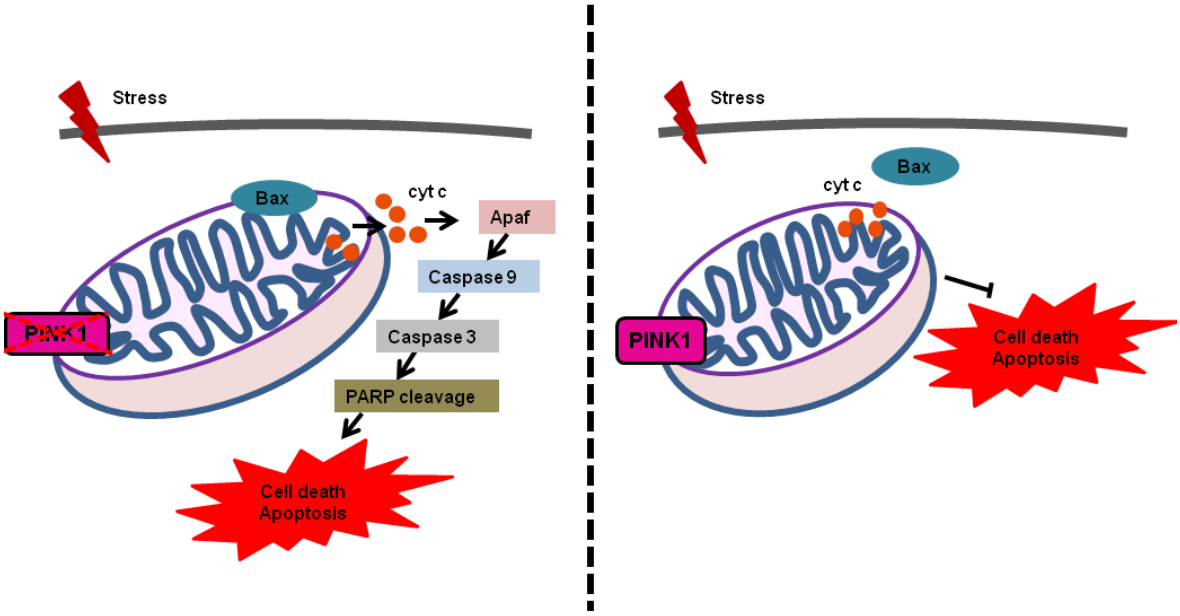

Figure 1.11: PINK1 protects against mitochondrial-mediated apoptosis. In the absence of PINK1, upon stress stimuli (staurosporine, rotenone, MPP^+, paraquat), cytosolic Bax (Bcl-2–associated X protein) is recruited to the mitochondria, followed by release of intermembrane space proteins (such as cyt c) to the cytosol and activation of caspases cascade with increased cell death. Over-expression of PINK1 wt blocks the activation of apoptosis and cell death.

However, the deletion of the first 34 aa does not prevent the mPTP opening [Wang et al., 2007].

It has been proposed that the PINK1 mediated protection in response to mitochondrial stressors is due to the interaction with substrates (**Figure 1.12**). PINK1 might provide protection against oxidative stress-induced apoptosis by the phosphorylation and the activation of the mitochondrial chaperone TRAP1 [Pridgeon et al., 2007], and by the modulation of the phosphorylation of the protease Htra2/omi [Plun-Favreau et al., 2007, 2008]. Moreover, PINK1 phosphorylates Parkin, which increases E3-ubiquitin ligase and favours the K63-linked polyubiquitination of IKKγ. Degradation of IKKγ is a critical step in the activation of NF-$\kappa\beta$, a ubiquitously expressed transcription factor of pro-survival genes [Sha et al., 2010]. Parkin activation by PINK1 enhances mitophagy, which recently has been proposed as a protective cell death pathway. It has been demonstrated that PINK1 activates phosphorylation of Rictor, a component of mTOR2, promoting cell survival mediated by Akt activation in neurons [Deas et al., 2010; Murata et al., 2011a] (**Figure 1.12**).

1.6.2 Mitochondrial function

In mitochondria, electrons travel through the electron transport chain (complexes I-IV) to generate energy by the respiration process.

Reactive oxygen species (ROS, such as oxygen ions, free radicals and peroxides) are formed as a natural byproduct of oxygen metabolism, where roughly 1-5% of the oxygen

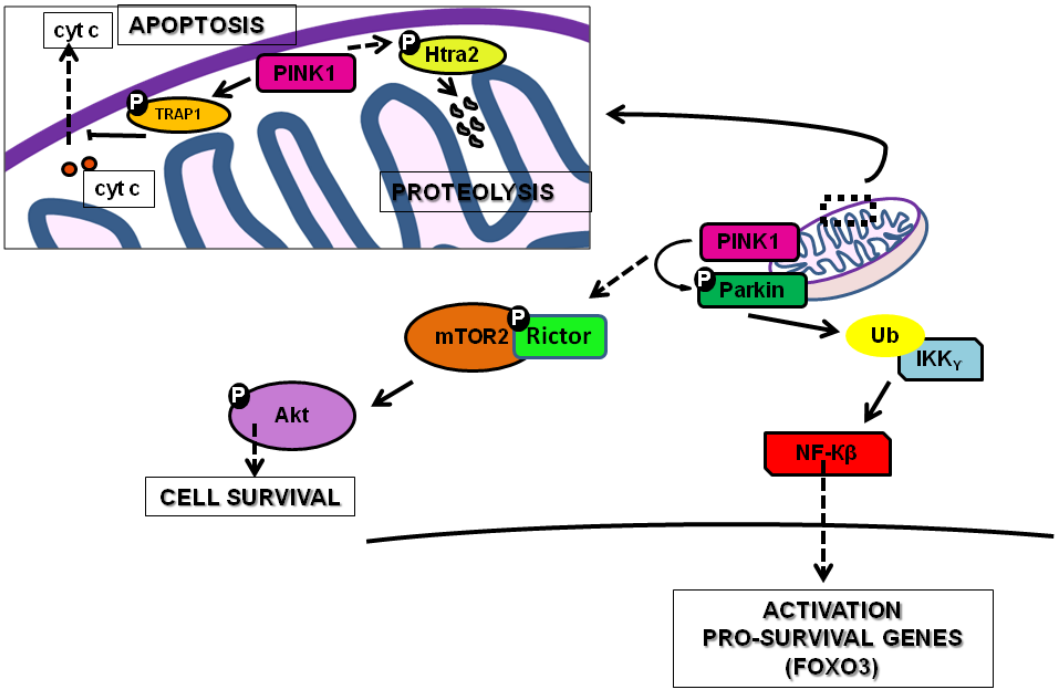

Figure 1.12: PINK1 protection is mediated by its substrates. Phosphorylation of mitochondrial PINK1 associated proteins TRAP1 and Htra2/omi can block activation of apoptosis and clearance of damaged proteins by proteolysis. Moreover, phosphorylation of cytosolic PINK1 associated proteins such as Parkin and Rictor favours the expression and activation of pro-survival effects. Arrows indicate PINK1 substrates and dotted arrows indicate PINK1-associated proteins.

consumed is converted into ROS [Westermann, 2008]. ROS can be useful to the immune system and also involved in cell signalling events, but if the levels overcome the cell's ability to neutralize and eliminate them, ROS can inflict damages on DNA, lipids and proteins [Zhou et al., 2008a]. This situation is known as oxidative stress and is caused by reduced antioxidant capacity or by the over-production of ROS.

There are many findings indicating that the mitochondria in PINK1 deficient models are vulnerable and unhealthy. Gegg et al. have shown that PINK1-silenced SH-SY5Y cells resulted in a decrease in mtDNA levels and synthesis, impairment of respiratory chain, and complex IV deficiency [Gegg et al., 2009]. Primary cultured neurons and mouse embryonic fibroblasts (MEFs) derived from PINK1 deficient mice showed loss of $\Delta\Psi$m and defects of complex I and oxygen consumption, causing pathological opening of the mPTP [Gandhi et al., 2009; Gautier et al., 2012; Amo et al., 2010; Samann et al., 2009; Wang et al., 2011]. Moreover, PINK1 deficiency increased intracellular calcium levels and vulnerability, decreased glucose availability and excess in ROS production in the mitochondria and the cytosol [Gandhi et al., 2009] (**Figure 1.13**).

Patient fibroblasts and immortalized lymphoblasts from individuals carrying a PINK1 mutation (G309D) have also been reported to display mid decrease of complex I activity, induced antioxidant defence and enhanced lipid peroxidation [Hoepken et al., 2007, 2008]. Fibroblasts from a patient with W437X mutation displayed significant decrease in the

respiratory activity and ATP, accompanied by ROS accumulation [Piccoli et al., 2008]. Other patient fibroblasts studies (V170G and Q456X mutations) have described decrease in $\Delta\Psi$m and altered redox state, but normal glutathione levels and no increase in cytosolic oxydation [Grünewald et al., 2009; Abramov et al., 2012]. PINK1 mutations over-expressed in SH-SY5Y cells showed defects in oxygen consumption [Liu et al., 2009].

Mitochondrial PKA and inhibition of mTOR pathway by rapamycin or 4E-BP over-expression can suppress the mitochondrial defects of PINK1 loss in *D. melanogaster*, maybe through activation of pro-survival genes and increased mitochondrial electron transport chain activity, but the molecular mechanism underlying this process needs to be clarified [Tain et al., 2009; Dagda et al., 2011].

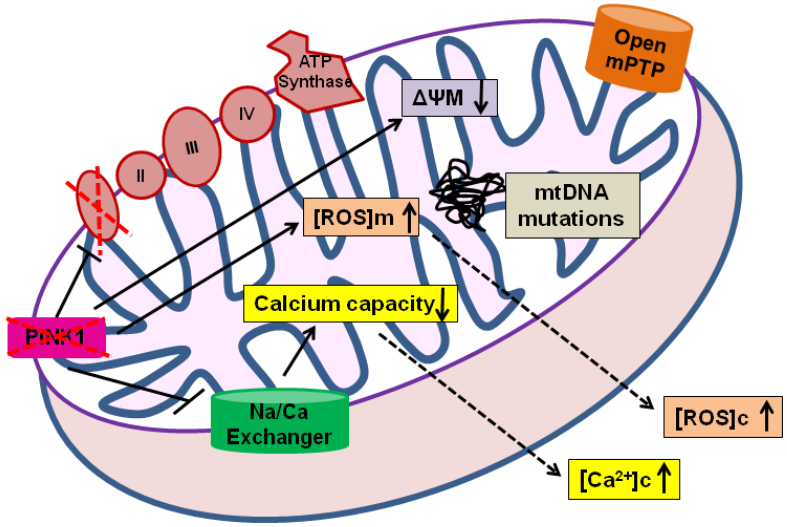

Figure 1.13: PINK1 in mitochondrial function. Loss of PINK1 impairs mitochondrial respiration, decreases complex I activity and reduces ATP production, accompanied by increased mitochondrial and cytoplasmic ROS, which produces mtDNA damage. Also, PINK1 deficiency decreases calcium capacity, blocking the Na^+/Ca^{2+} exchanger and increasing cytosolic Ca^{2+} levels, which favours the opening of mPTP. Opening of the mPTP releases pro-apoptotic factors such as cyt c from the mitochondria and induces apoptosis.

1.6.3 Mitochondrial dynamics

Mitochondrial fission and fusion are membrane-remodeling processes that control *in vivo* dynamics, distribution, and structure of the mitochondrial network. These processes respond to the cell energy status and are necessary for proper mitochondrial function. Dysfunction of mitochondrial fission/fusion has been linked to the pathogenesis of neurodegenerative [Twig et al., 2008; Westermann, 2008].

A role for PINK1 in the regulation of mitochondrial fission/fusion dynamics has recently been proposed [Deng et al., 2008; Park et al., 2006; Poole et al., 2008; Yang et al., 2008]. *D. melanogaster* PINK1 (dPINK1) RNAi, or dPINK1 mutant flies, display altered mitochondrial morphology with mitochondrial aggregates, swollen or enlarged mitochon-

dria, and the presence of a tubular mitochondrial network in high-energy demand tissues, such as flight muscle and DA neurons. This phenotype can be modified by genetic complementation of the flies with either an extra copy of the fission promoting gene Drp-1 or removal of a copy of the fusion promoting gene Opa-1 [Poole et al., 2008; Yang et al., 2008]. Overall, the combined results from flies studies suggest that dPINK1 is involved in promoting mitochondrial fission and/or inhibiting mitochondrial fusion. In support of this, Morais et al. reported similar defects in PINK1 knockout flies to those observed in Drp-1 deficient cell lines [Morais et al., 2009]. However, the PINK1/Parkin pathway directly promotes degradation of mitofusin (Mfn) which leads to mitochondrial fragmentation [Poole et al., 2008; Ziviani et al., 2010]. Recently, it has been described that PGAM5 is a PINK1 binding protein, which can modulate the defects in mitochondrial dynamics upon PINK1 loss, but without contribution to PINK1/Parkin pathway in fission/fusion [Imai et al., 2011].

Nevertheless, in mammalian cells, PINK1 is thought to function as a pro-fusion protein, since PINK1 KD, G309D or Q126P human cells showed increase in fragmented mitochondria with abnormal morphology [Exner et al., 2007; Wood-Kaczmar et al., 2008]. Contrary to fly results, Drp1 loss or dominant negative over-expression rescued the fragmented and abnormal mitochondrial, but not the ROS production associated with PINK1 loss. This suggests that mitochondrial dysfunction is the direct impairment, and fission/-fusion processes are secondary effects in PINK1 deficiency. Indeed, Sandebring et al. have proposed that accumulation of damaged mitochondria by PINK1 inactivation results in mitochondrial calcium efflux, which activates Drp1 in human cells through calcineurin-mediated dephosphorylation [Sandebring et al., 2009] In line with this, studies in PINK1 KO mice showed increased of mitochondrial size [Gautier et al., 2008] and in mammalian cells PINK1 over-expression leads to aggregation and fragmentation [Lutz et al., 2009]. Opposite to these results, it has been recently described that PINK1 and/or Parkin over-expression leads to increase fission in post-mitotic mammalian cells (hippocampal and DA neurons) [Yu et al., 2011; Liu et al., 2012].

PINK1 is associated with another aspect of mitochondrial dynamics: mitochondrial trafficking. Mitochondrial trafficking is a vital aspect of cellular homeostasis, especially in large cells such as neurons, where trafficking allows the redistribution of mitochondria to regions of high-energy demand such as synapses and areas of neuronal outgrowth. PINK1 forms a complex with Miro, an atypical GTPase of the mitochondrial outer membrane, and Milton, a cytoplasmic adaptor protein [Weihofen et al., 2009]. This protein complex is responsible for the anterograde movement of mitochondria along microtubules, which is thought to involve the attachment of mitochondria to microtubules via an interaction between Milton and the kinesin heavy chain I. This allows the transport of mitochondria to regions of high intracellular calcium (**Figure 1.14**) [Wang et al., 2012; Liu et al., 2012]. Without the formation of this complex, mitochondria are no longer transported and can not efficiently meet the energy demands of the cells. Moreover, PINK1 is activated by MARK2, an important regulator of cell polarity through phosphorylation and inactivation of several microtubule-associating proteins. The activation of PINK1 by MARK2 regulates the mitochondrial trafficking in neurons [Matenia et al., 2012]. Together, these findings indicate that PINK1 may influence both short- and long-range mitochondrial dynamics.

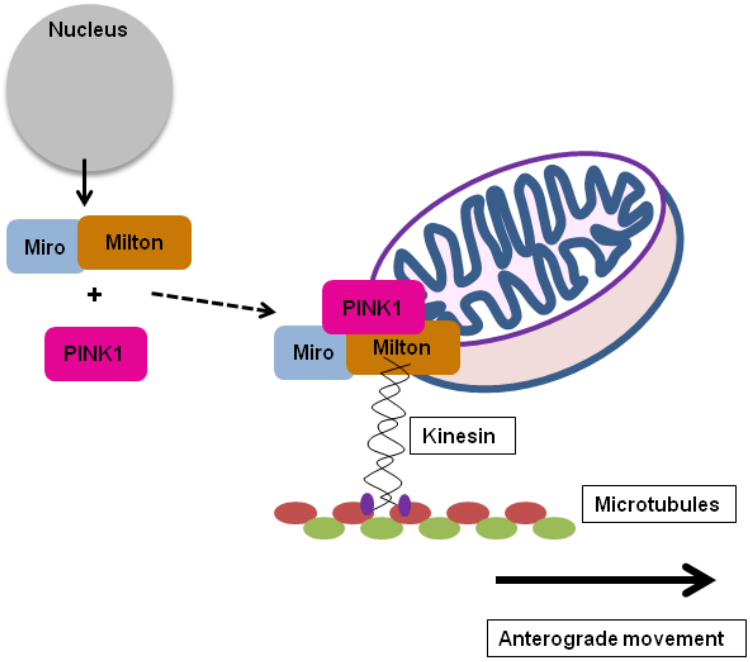

Figure 1.14: PINK1 and mitochondrial trafficking. PINK1 interacts with Miro and Milton and may play a role in anterograde mitochondrial transport within cells.

1.6.4 Mitophagy

Autophagy is a tightly regulated process which involves the degradation of cellular components. Autophagy can be divided into three types of pathways depending of the cargo: macroautophagy, microautophagy, and chaperone-mediated autophagy. Autophagy is often considered to be a nonselective pathway for the degradation of bulk cytoplasmic components. However, in some cases autophagy displays substrate specificity like the selective degradation of mitochondria, also known as mitophagy [Lynch-Day et al., 2012].

The term "mitophagy", coined by Lemasters in 2005, defines how damaged mitochondria are removed from the cell via a form of selective macroautophagy. This process has been linked to mitochondrial dynamics, whereby mitochondria are selectively fragmented depending on $\Delta\Psi$m, a marker of mitochondrial health [Lemasters, 2005; Twig et al., 2008]. Dysfunctional mitochondria undergo fragmentation, and these mitochondria, which can not be recovered through fission/fusion processes, are subsequently eliminated from the network by components of autophagy machinery, and are degraded via the lysosomal pathway.

Mitophagy can be induced in cultured cells by toxic agents such as the mitochondrial membrane uncoupling agents CCCP and valinomycin, and the ROS-producing herbicide paraquat. Prolonged treatment with these toxins has been shown to lead to a complete removal of mitochondria from the cell, a phenomenon exclusively dependent on autophagy [Ziviani et al., 2010; Narendra et al., 2008].

Although the complete mechanism mediating mitophagy is not yet fully understood, recent studies have shown that PINK1 accumulates in the outer membrane of dysfunctional

mitochondria, and recruits Parkin, which activates the clearance of damaged mitochondria by mitophagy [Narendra et al., 2010; Vives-Bauza et al., 2010a; Matsuda et al., 2010; Dagda et al., 2009; Geisler et al., 2010]. Reduced $\Delta\Psi m$ inhibits PINK1 cleavage and stabilises PINK1 FL at the OMM, triggering Parkin relocalisation from the cytosol to the mitochondria and activating mitophagy. However, the question of how PINK1/Parkin communicate each other and how the Parkin recruitment occurs is still opened. One hypothesis involves Parkin phosphorylation by PINK1 and subsequent activation of Parkin E3 ubiquitin ligase needed for mitophagy activation [Kim et al., 2008; Moore, 2006; Um et al., 2009; Shiba et al., 2009; Kondapalli et al., 2012]. In fact, the kinase activity of PINK1 is essential for Parkin recruitment and mitophagy activation [Vives-Bauza and Przedborski, 2010].

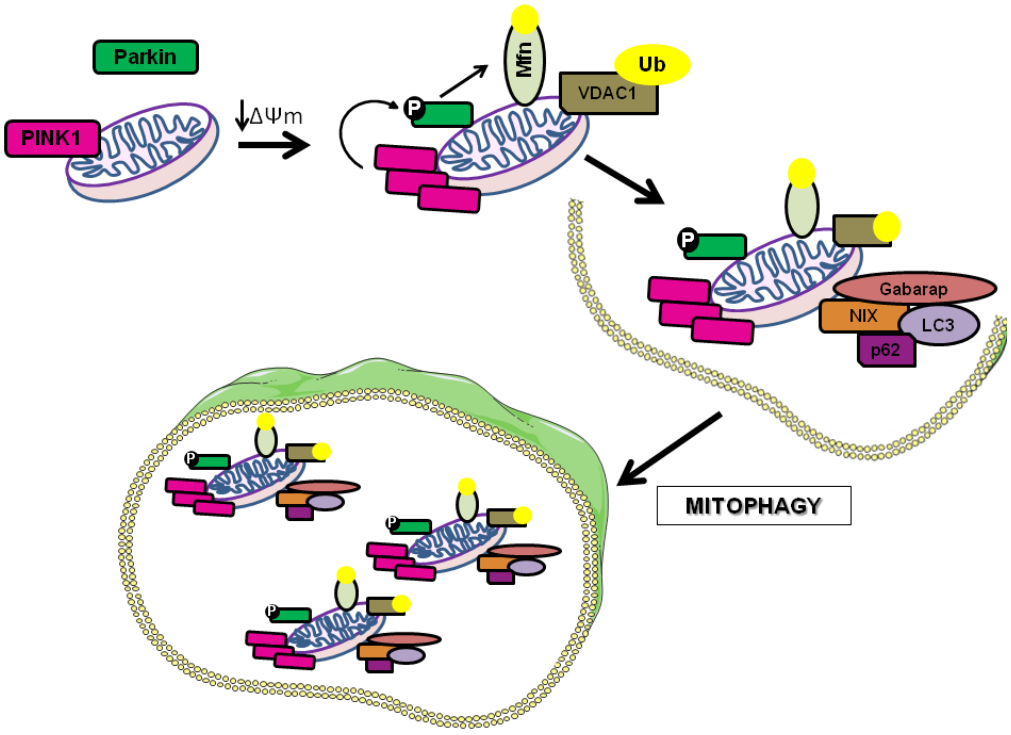

Figure 1.15: Model of PINK1 role in mitophagy. Upon mitochondrial membrane depolarization, PINK1 FL accumulates at the OMM. Parkin is recruited and phosphorylated by PINK1, which facilitates ubiquitination of VDAC1 and Mfn. Subsequently, NIX, GABARAP, p62 and LC3 are recruited for autophagosome formation and depolarized mitochondria are removed by mitophagy.

Recent studies have added further insight into the mechanism of PINK1/Parkin - mediated mitophagy by revealing downstream targets of Parkin ubiquitination. Reports in *D. melanogaster* and human cells found that the pro-fusion protein Mfn is ubiquitinated in a Parkin-dependent manner [Ziviani et al., 2010; Poole et al., 2008; Gegg et al., 2010]. In addition, a separate study using human cells reported that the voltage-dependent anion channel 1 (VDAC1), a proposed component of the mPTP, was also ubiquitinated by Parkin [Geisler et al., 2010]. The consequences of the ubiquitination may be the blockage of fusion

processes and of the release of pro-apoptotic factors, triggering the mitochondrial removal (**Figure 1.15**).

Prior to mitophagic degradation, mitochondria need to be clustered to the perinuclear area, a process occurring in PINK1 over-expressing cells upon stress conditions [Narendra et al., 2010]. In addition, under basal conditions, PINK1-silenced cells showed increased autophagosome number and autophagy with mitochondrial localised close to the perinuclear area [Chu, 2009, 2010]. Thus, in the case of PINK1 loss, protective autophagic pathways can be activated to clean up damaged mitochondria.

Finally, PINK1 FL interacts physically with Beclin-1 (Coiled-coil, moesin-like Bcl-2 interacting protein), a protein that regulates the initiation of autophagosome formation, increasing basal levels of autophagy [Michiorri et al., 2010]. This suggests that PINK1 not only plays a role in recruitment of Parkin but also in recruitment of autophagy machinery components to mitochondria.

1.7 PINK1 and human disease

1.7.1 PINK1 and PD

PINK1 (*PARK6*) represents the second most frequent cause of early-onset juvenile Parkinson's disease (EOJP) after Parkin, accounting for approximately 4-5% of autosomal recessive and 1-2% of sporadic cases. The initial report described three pedigrees with a G309D point substitution in one family and a truncation mutation W437X in two additional families [Valente et al., 2004a]. So far, more than 100 homozygous mutations (missense mutations, genomic rearrangements, truncating mutations) throughout PINK1 gene have been identified [Deas et al., 2009] (**Appendix 8**) and linked to autosomal-recessive PD in diverse populations. Many heterozygous mutations of PINK1 have been noted in late-onset PD patients and rarely in recessive families, suggesting a possible role of PINK1 mutations as a susceptibility factor (**Figure 1.16**) [Valente et al., 2004b; Bonifati et al., 2005; Abou-Sleiman et al., 2006; Ibanez et al., 2006; Tang et al., 2006; Gandhi et al., 2006; Klein and Schlossmacher, 2007].

The missense mutations of PINK1 are distributed through the different PINK1 domains. However, the majority of the missense mutations are localised in the Ser/Thr kinase domain, suggesting that loss of kinase activity plays a crucial role in the pathogenesis of PINK1-linked PD. Moreover, several nonsense mutations in the C-terminal region of the kinase domain and in the tail of PINK1 have been found in PD patients [Bonifati et al., 2005; Sim et al., 2012].

1.7.1.1 Clinical features

PINK1-related disease is clinically similar to idiopathic PD. The PINK1-associated phenotype is characterised by a wider range of ages at onset than the Parkin-associated phenotype, and responds well to dopamine replacement therapies.

The consistent features in the PINK1 phenotype include [Gispert et al., 2009; Samaranch et al., 2010]:

- Onset in most cases before age 40.
- A much more benign course despite decades of Parkinsonism.

- Symptoms primarily referable to DA substrates with well-preserved levodopa responsiveness (but with levodopa dyskinesias and motor fluctuations).
- No substantial cognitive decline (although psychiatric symptoms may occur).
- Minimal dysautonomia (confined to urinary urgency, male impotence and autonomic symptoms attributable to medications).
- Some cases of Lewy bodies presence.

Although the genotype-phenotype correlation has not been confirmed, the mean age at onset in patients with single heterozygous mutations is higher than that in patients with homozygous mutations [Kumazawa et al., 2008]. Homozygous mutations in PINK1 invariably cause PINK1-linked PD, whereas heterozygous mutations have been suggested to be a susceptibility factor for sporadic PD [Klein et al., 2007; Klein and Schlossmacher, 2007].

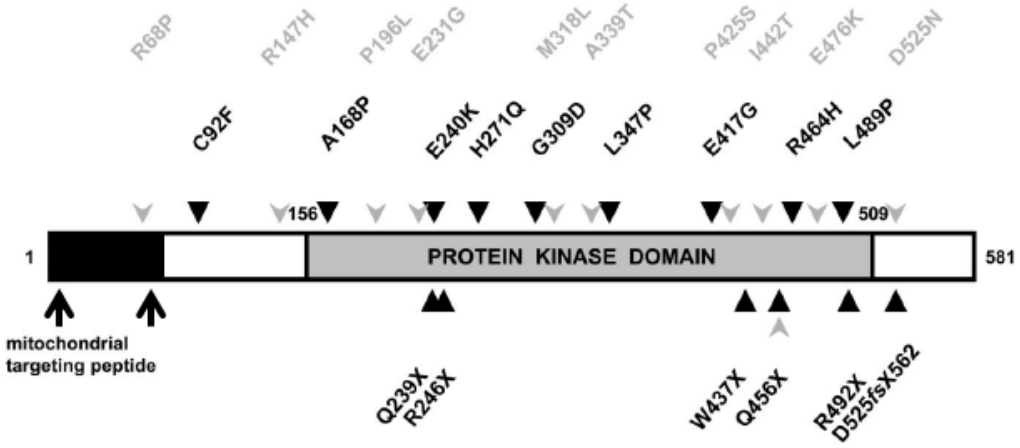

Figure 1.16: Some of the PINK1 mutations reported in PD patients. Missense and truncated mutations are depicted on top and at the bottom of the protein bar, respectively. Mutations found in homozygous or compound heterozygous states are in black. Mutations found in heterozygous state are in grey. Data from Valente et al.; Valente et al.; Hatano et al.; Healy et al.; Rogaeva et al.; Bonifati et al.. Modified from Bonifati et al., 2005.

1.7.1.2 PINK1 interaction with other PD-linked genes

As previously mentioned, PINK1 has been functionally associated to Parkin. It has been described in *D. melanogaster* and in mammalian cultured cells that Parkin over-expression rescues the phenotype of PINK1 deficiency, but not viceversa. Parkin stabilises PINK1 through direct interaction, and it was concluded that PINK1 functions upstream of Parkin in a common pathway [Park et al., 2006; Clark et al., 2006; Yun et al., 2008].

Parkin, PINK1 and DJ-1 form a ubiquitin E3 ligase complex that promotes the degradation of unfolded proteins [Xiong et al., 2009]. In *D. melanogaster*, DJ-1 can revert the consequences of PINK1 loss (except for infertility), but not the consequences of Parkin

loss. Moreover, Parkin can not rescue DJ-1 loss, suggesting that DJ-1 may not be a direct downstream effector of PINK1 [Hao et al., 2010]. In human neuroblastoma cells, PINK1 and Parkin protect against the loss of DJ-1, and, although DJ-1 does not alter PINK1 deficient mitochondrial phenotypes, DJ-1 protects against rotenone-induced damage in the absence of PINK1 [Kim and Son, 2010; Thomas et al., 2010]. These findings suggest that DJ-1 works in parallel to the PINK1/Parkin pathway to maintain mitochondrial function.

PINK1 KD causes proteasome dysfunction, accompanied by increased α-synuclein aggregation [Liu et al., 2009], and α-synuclein over-expression leads to increased mitochondrial fragmentation and dysfunction, which can be rescued by Parkin, PINK1 or DJ-1 co-expression [Kamp et al., 2010].

PINK1 has been associated to LRRK2. In *C.elegans*, the mitochondrial and axonal dysfunction caused by PINK1 deficiency can be rescued by the absence of LRRK2 [Samann et al., 2009]. In flies, over-expression of LRRK2 promotes the phenotype associated to PINK1 deficiency [Venderova et al., 2009]. Moreover, Parkin protects against LRRK2-induced neurotoxicity *in vivo* [Ng et al., 2009]. These evidences suggest that dysfunction of several PD causative gene products might contribute to pathogenesis of PD and may be linked through common pathways.

1.7.2 PINK1 association to cancer

First genomic analysis showed that PINK1 lies next to chromosome 1p31.1-34.3 region, where losses of heterozygosity (LOH) are frequently observed in endometrial cancers [Arlt et al., 1996].

The first description of PINK1 related to cancer disease was done by Unoki and Nakamura in 2001. They showed PINK1 mRNA up-regulation in cancer cells(HEC-151 and Ishikawa3-H12 endometrial cells) with exogenous PTEN over-expression but this was not linked to changes in colony-formation assay or tumour suppression. The PTEN gene is a tumour suppressor gene encoding a multifunctional phosphatase, which plays an important role in inhibiting the PI3K/Akt pathway, and PTEN mutations have been found in many human cancers. In addition, PINK1 (also named as BRPK) showed increased expression in mouse cancer cell lines with higher metastatic potential [Nakajima et al., 2003]. Also, PINK1 has been identified in a sensitized siRNA kinome and phosphatome screen as a protein essential for survival, making PINK1 important as a potential anti-cancer target [MacKeigan et al., 2003]. Finally, PINK1 has been identified in a microarray study as a strong predictor, together with Dlg7 and Bub1, of overall survival and disease-free survival in adenocarcinomas [de Reyniès et al., 2009], and the prognostic value of the combined expression of these three genes has been validated in different samples of adult adenocarcinomas [Fragoso et al., 2011, 2012].

Recently, down-regulation of PINK1 has been shown to sensitise bladder cancer cells that are resistant to adenovirus carrying Reic/dkk-3. This sensitisation was associated with increasing production of ROS, down-regulation of Bcl-xL and TRAP1 proteins, and up-regulation of Bax protein [Jin et al., 2012].

1.7.3 PINK1 and other human diseases

PINK1 has also been reported to be linked with Alzheimer disease and multiple sclerosis lesions [Wilhelmus et al., 2011]. Interestingly, PINK1 immunostaining was observed in both diseases predominantly in reactive astrocytes associated with these lesions, and in

classic senile plaques and vascular amyloid depositions, suggesting that the increase in astrocytic PINK1 protein might be an intrinsic protective mechanism to limit cellular injury [Wilhelmus et al., 2011].

PINK1 has been associated to type 2 diabetes, obesity and impairment in glucose transport. In skeletal muscle cells of type 2 diabetes patients, transcripts from the PINK1 locus were down-regulated and the gene expression correlated with diabetic status, since aerobic training increased PINK1 transcript abundance. RNA interference of PINK1 impaired basal glucose uptake of human neuronal cell lines, and PINK1 abundance correlated with plasma glucose levels [Scheele et al., 2007; Franks et al., 2008].

Chapter

2 Objectives

The main objectives of this study have been:

- To investigate how PINK1 may control cellular homeostasis through mitochondrial quality control.
- To elucidate PINK1 pivotal interplay in two relevant human diseases: cancer and Parkison's disease.

1. To investigate PINK1 role in Parkinson's disease:
    - We analyse the functional role of PINK1 in a human neuroblastoma model (SH-SY5Y cell line), in terms of mitochondrial function, homeostasis and mitophagy processes.
2. To investigate PINK1 role in cancer:
    - We explore the functional role of PINK1 in a human breast cancer model (MCF-7 cell line), in terms of growth, survival, apoptotic and transformation features.
    - We examine the level of expression of PINK1 in human breast cancer tumours and in human cancer cell lines.
3. To investigate the functional properties of PINK1 catalytically inactive, PINK1 PD-associated mutations and PINK1 truncations.

Chapter

3 Material and Methods

Chapter Outline

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3.1 Sequence alignment

PINK1 amino acids sequences from different organisms were obtained from *Uniprot Knowledgebase* database `http://www.uniprot.org/` [Consortium et al., 2011]. Alignments were done with ClustalX [Larkin et al., 2007] `http://www.ch.embnet.org/software/ClustalW.html` or Bioedit `http/www.mbio.ncsu.edu/BioEdit/biioedit/html` [Hall, 1999]. The UniprotKB codes of the sequences used were: *Homo sapiens*, Q9BXM7; *Gorilla gorilla*, G3RF52; *Bos Taurus*, A5PJP5;*Rattus norvegicus*, B5DFG1; *Mus musculus*, Q99MQ3; *Monodelphis domestica*, F7FR77; *Canis familiaris*, E2RCD1; *Sus*

scrofa, F1SU10; *Danio rerio*, B5TXD9; *Salmo salar*, B5X237; *Drosophila melanogaster*, QOKHV6; *Caenorhabditis elegans*, QO9298; *Pediculus humanus*, EOW1I1; *Ascaris suum*, F1LON5.

3.2 cDNAs, plasmids and mutagenesis

The cDNA encoding full length human PINK1 (pCMV-sport6-hPINK1) was provided from *GeneService* (Mammalian Gene Collection, IMAGE ID 5214483; GeneService, Cambridge, United Kingdom). As this cDNA contained the mutation P209A, the original cDNA clone was changed by site-directed mutagenesis to reconstitute the wild-type (wt) Pro209.

The mammalian expression plasmids to generate stable non-inducible human SH-SY5Y cell lines, pcDNA3.1-PINK1 wt and mutations, were obtained by PCR and cloning (BamHI/EcoRI) into pcDNA3.1.

The mammalian expression plasmids to generate inducible double stable Tet-On MCF-7 human cell lines, pTRE2hyg-PINK1wt and mutations, were obtained by PCR, cloning into pBluescript and subcloning (BamHI/ClaI) into pTRE2hygromicine. For all vectors, a 5' primer containing a Kozak sequence for the initiation of translation was used.

All sequences were verified by DNA sequencing. Preparative PCR contained 25 ng template, 0.2 mM dNTPs, 0.3 μM of each primer, and 0.2 μl of the termostable DNA polymerase G-C rich (Roche Diagnostics), in a total volume of 50 μl. PCR conditions included an initial denaturation step at 95^{o}C for 5 minutes (min), followed by 30 cycles of 94^{o}C denaturation for 2 min, 60^{o}C annealing for 2 min, 72^{o}C extension for 1 min and a last 72^{o}C step of final elongation for 10 min. **Table 3.1** shows the primers used for cloning and mutagenesis. T4 DNA ligase (Invitrogen) and restriction endonucleases (Roche Diagnostics and Fermentas) were used according to the manufacturer's instructions.

3.3 DNAs and bacterial methods

3.3.1 Plasmid DNA purification

Plasmid DNA was extracted from *Escherichia coli* (*E. coli*) DH5α strain (a recombination deficient strain) transformants by two methods based on alcaline lysis.

- Small-scale purification of plasmid DNA (miniprep) was carried out to identify positive transformants.
- Large-scale purification of plasmid DNA (maxiprep) was done using an alkaline lysis based maxiprep kit (Plasmid DNA Purification, Machery-Nagel).

For mini- and maxiprep, cells were grown in 3 ml and 100 ml LBA media, respectively (10 g.l^{-1} peptone, 5 g.l^{-1} yeast extract, 0.17 M NaCl, 50 μg.ml^{-1} ampicillin) 16 hours (h) on a shaker at 37^{o}C.

3.3.2 Agarose gel electrophoresis and purification of DNA fragments

Agarose gel electrophoresis (1-2%) was used to separate, identify and/or purify DNA fragments according to size. Gels, containing 0.6 μg.ml^{-1} EtBr to visualise DNA fragments, were run at 100-120 V in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA). To obtain

Table 3.1: Primers used for cloning and mutagenesis.

Primer name	Sequence
PINK1 forward (pBluescript)	AAAGGATCCCGGCCGGCCGCCACCATGGCGGTGCGAC AGGCGC
PINK1 reverse (pBluescript)	CCGGAATTCCTCGAGTCACAGGGCTGCCCTCCATG
PINK1 forward (pcDNA3.1)	GCTCTAGAGGCCGCCACCATGGCGGTGCGACAGGCGC
PINK1 reverse (pcDNA3.1)	CCCAAGCTTTCAGAGGCTAGCATAATCAGGAACATCAT ACTCGAGCAGGGCTGCCCTCCATGAGCA
P209A forward	AGCGAGCTCCGGGGGGCC
P209A reverse	GGCCCCCGGAGCTCGCT
A168P forward	GGCTGCAGTCCTGCTGTGTAT
A168P reverse	ATACACAGCAGGATCGCAGCC
K219M forward	TTGGCCATCATGATGATGTGG
K219M reverse	CCACATCATCATGATGGCCAA
G309D forward	AAGGCCTGGACCATGGCCG
G309D reverse	CGGCCATGGTCCAGGCCTT
L347P forward	TGCAGCTGCCCGGAAGGCGT
L347P reverse	ACGCCTTCCGGGCAGCTGCA
W437X (pBluescript)	CCCGAATTCAAGCTTTCAGGCATCAGCCTTGCTGTAGT
W437X (pcDNA3.1)	CCCGAATTCAAGCTTTCAGAGGCTAGCATAATCAGGAA CATCATAGGCATCAGCCTTGCTGTAGT
510 reverse (pBluescript)	CCCGAATTCCTCGAGTCAGCTTAGATGAAGCACATTTG CG
510 reverse (pcDNA3.1)	CCCAAGCTTTCAGAGGCTAGCATAATCAGGAACATCAT AGCTTAGATGAAGCACATTTGCG
150 forward (pBluescript)	AAAGGATCCCGGCCGGCCGCCACCATGGGCTTTCGGCT GGAG
150 forward (pcDNA3.1)	GCTCTAGAGGCCGCCACCATGGGCTTTCGGCTGGAG

DNA fragments for ligation, samples were run on low melting agarose gels and gel slices containing the DNA were melted and added directly to the ligation mix. For PCR restriction products, DNA samples were separated on agarose gels and DNA fragments were purified using the QIAEX II Gel Extraction Kit (Qiagen).

3.3.3 Bacterial methods

Cells from *E. coli* DH5α strain were made competent by treatment with $CaCl_2$. *E. coli* cells were transformed with the suitable plasmid by the heat shock method (30 min at 4^oC, 50 seconds at 42^oC and 2 min at 4^oC) and incubated on a shaker (1 h at 37^oC) with LB before plating. To select single transformant colonies, bacteria were plated on LBA plates containing the appropriate selection marker (LBA media with 5 $g.l^{-1}$ agar) and incubated for 16 h at 37^oC. Cultures of positive *E. coli* colonies were grown overnight in LBA media on a shaker at 37^oC.

3.4 Cell culture, treatments, stable transfections and siRNA

3.4.1 Cell culture

All parental cell lines were obtained from American Type Culture Collection (ATTC), and were grown at 37^oC in a 5% CO_2 humidified atmosphere.

The human dopaminergic neuroblastoma SH-SY5Y cell line (**Figure 3.1**) was cultured in 1:1 (v/v) DMEM (Dulbecco's Modified Eagle Medium):Ham's F12 (Nutrient mixture F-12 Ham medium) media (Gibco, Invitrogen) containing 3.6 $g.l^{-1}$ glucose and supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1 mM non-essential aminoacids, 100 $U.ml^{-1}$ penicillin and 100 $\mu g.ml^{-1}$ streptomycin. SH-SY5Y stable cells were grown in SH-SY5Y cell medium supplemented with 200 $\mu g.ml^{-1}$ G418 (Fisher BioReagents).

The human breast carcinoma MCF-7 cell line (**Figure 3.1**) was cultured in Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco, Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 $U.ml^{-1}$ penicillin and 100 $\mu g.ml^{-1}$ streptomycin. MCF-7 stable Tet-On cell line containing Tet-On plasmid was provided by R.P. Shiu [González et al., 2006; Venditti et al., 2002]. MCF-7 Tet-On double-stable cells were grown in MCF-7 cell medium, supplemented with 200 $\mu g.ml^{-1}$ G418 (Gibco, Invitrogen) and 100 $\mu g.ml^{-1}$ hygromycin (Sigma-Aldrich).

The human breast MDA-MB-231 cell line was cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 $U.ml^{-1}$ penicillin and 100 $\mu g.ml^{-1}$ streptomycin.

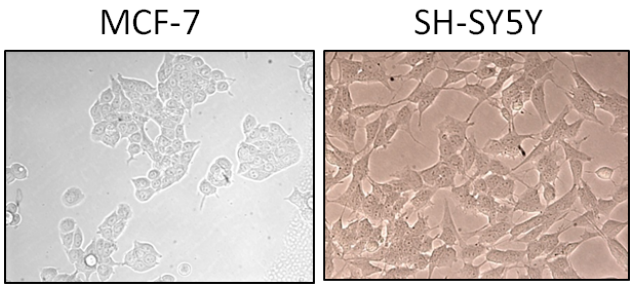

Figure 3.1: MCF-7 and SH-SY5Y cells.

3.4.2 Stable transfections

For the generation of the different PINK1 over-expressing SH-SY5Y stable cell lines, SH-SY5Y cells were stably transfected with pcDNA3.1 vector (Invitrogen) using Fugene HD (Roche Diagnostics) (**Figure 3.2**). For selection of positive transformants, the optimal drug concentration was determined at 200 $\mu g.ml^{-1}$. To select stable cells, single colonies were isolated using 3 mm cloning discs or cloning cylinders (Sigma-Aldrich), and transferred to 24-well plates. From 24-well plates, half of the cells were tested for expression of ectopic proteins, and half of the cells were transferred to 12-well plates. From 12-well

plates the clones were expanded to 6-well plates, and then to 10 cm plates. Stocks of each clone were frozen after expanding the cell cultures.

To generate double-stable cell lines, MCF-7 Tet-On cell line was transfected with the pTRE2hyg plasmid (Clontech) using FuGENE 6^{TM} (Roche Diagnostics) (**Figure 3.2**). Pilot experiments to generate the double-stable MCF-7 Tet-On cell lines, with inducible expression of the different PINK1 cDNAs, were done according to the Tet-On® Advanced Inducible Gene Expression System User Manual (Clontech) (**Figure 3.2**). For selection of double-stable transformants, the optimal drug concentration was determined at 100 μg.ml^{-1} hygromycin, and the optimal plating density of cells was determined at 1x10^6 cells/10 cm dish containing 10 ml of the appropriate selective media. To select double-stable cells, single colonies were isolated as above. To induce PINK1 expression in MCF-7 Tet-On double-stable cells, the cell lines were pre-treated with 100-500 ng.ml^{-1} doxycycline (Dox) for 24 h before processing or analysis.

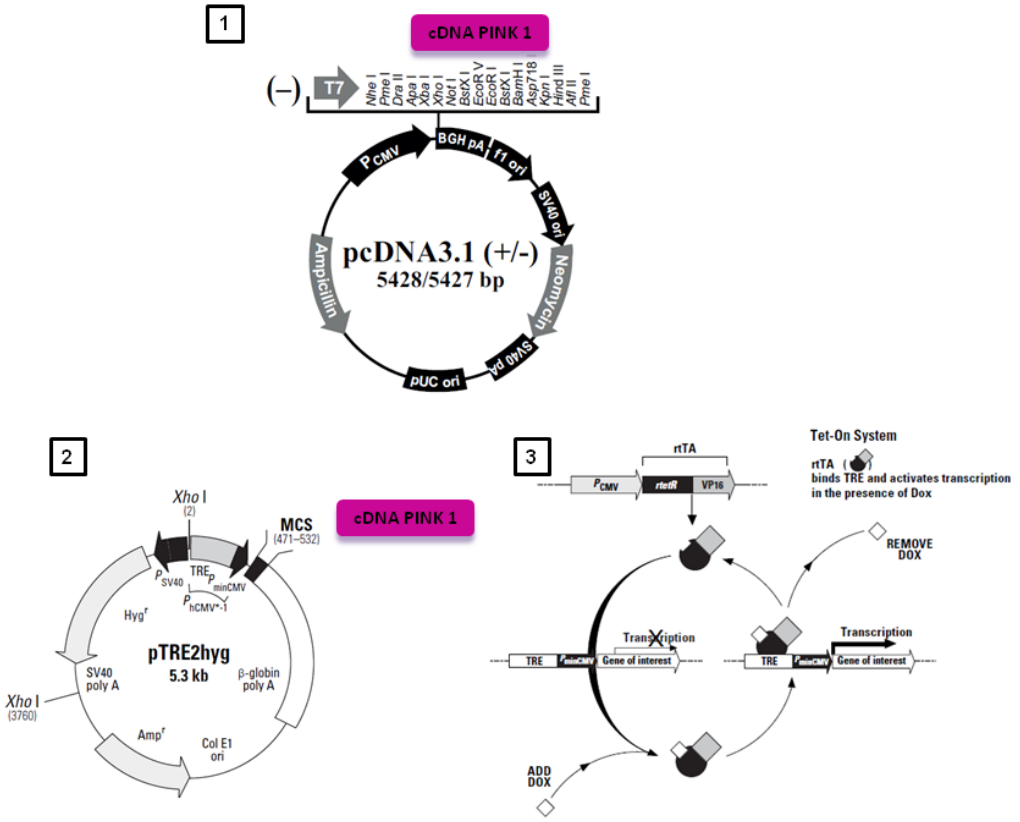

Figure 3.2: Generation of PINK1 stable over-expressing MCF-7 and SH-SY5Y cells. **1)** Scheme of pcDNA3.1(-) vector used to clone the different PINK1 cDNAs and to transfect SH-SY5Y to generate stable clones (Modified from `http://xlaevis.cpsc.ucalgary.ca/other/static/methods/vector-info/maps/pcdna3_1p-_map.jpg`). **2)** Scheme of pTRE2hyg vector used to clone the different PINK1 cDNAs and to transfect MCF-7 to generate stable clones (modified from `http://www.clas.ufl.edu/jur/200308/images/piacenti_1.gif`). **3)** Scheme of the Tet-On Advanced System: rtTA-Advanced binds TRE-Tight and fully activates transcription in the presence of low concentrations of doxycycline. Source: http://www.clontech.com.

3.4.3 siRNA

Silencing of MCF-7 and MDA-MB-231 cells was performed by transfection with Lipofectamine RNAiMAX or Lipofectamine 2000 (Invitrogen), respectively, of validated scrambled, GAPDH and PINK1 siRNAs (Ambion Applied Biosystems), following manufacturer's instructions. Silencing of SH-SY5Y cells was performed by transfection of scrambled, or PINK1 siRNAs as above, using HiPerfect Transfection (Qiagen), following manufacturer's instructions. Cells were processed 48 h after transfection.

3.4.4 Cellular treatments

The cellular treatments used are displayed in **Table 3.2**.

Table 3.2: Cellular treatments.

Treatment	Cellular effect	Use	Supplier
SH-SY5Y cells			
Oligomycin	ATP synthase inhibitor	2 μg.ml^{-1}	Sigma-Aldrich O4876
Rotenone	Inhibitor of mitochondrial electron transport	5 μM	Sigma-Aldrich R8875
CCCP (carbonyl cyanide m-chloro phenyl hydrazone)	Mitochondrial uncoupler	10μM	Sigma-Aldrich C2759
FCCP (carbonylcyanide-4-trifluoromethoxyphenylhydrazone)	Mitochondrial uncoupler	100 μM	Sigma-Aldrich C2920
MCF-7 cells			
H_2O_2 (hydrogen peroxide)	Apoptosis inductor by increasing ROS	0.15 mM	Merck 107209

3.5 Semiquantitative PCR and real-time quantitative PCR analysis

Semiquantitative PCR was performed from a human normal tissue cDNA collection (PrimeExpress II, PrimeGen, USA), using GC-Rich PCR kit (Roche). Total RNA was extracted from MCF-7 and SH-SY5Y cells using illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). Human brain, breast and thyroid total RNA were provided from Ambion Applied Biosystems. Breast cancer tissue samples were provided by Dr. Jaime Ferrer, Biobanco FIHCUV-INCLIVA, Valencia. Total RNA from 10-15 breast cancer tissue slides (10 μm each) was extracted with RNeasy Universal Plus Mini kit (Qiagen). Then, RNA was quantified by spectrometry and measured the RIN quality (Nano Drop ND1000, NanoDrop Technologies, Wilminton, Delaware USA). 1 μg total RNA was reverse transcribed using RevertAid^{TM} reverse transcriptase, oligo(dT)^{18} primers and RiboLock RNase inhibitor (all from Fermentas). The primer conditions and the cDNA amount were optimized, getting efficiency values between 1.9 to 2.1. Each qPCR reaction contained 100 ng cDNA template, 0.5 μM of each primer, and 5 μl SYBRGreenI Master (Roche) and was performed in Lightcycler 480 (Roche) in a total volume of

Table 3.3: Primers used for semiquantitave and quantitative PCR.

mRNA	Forward Primer	Reverse Primer	Amplified fragment
Semiquantitative PCR			
PINK1	AGTGATTGACTACAGCAAG GCTGAT	ATCTTGTCTAACTTCAGATT CTTCAGG	300 bp
β-actin	CCAAGGCCAACCGCGAGAA GATGAC	AGGGTACATGGTGGTGCCG CCAGAC	350 bp
qPCR of cells and normal tissues			
PINK1	Validated (Qiagen)		112 bp
HPRT	GACCAGTCAACAGGGACAT	GTGTCAATTATATCTTCCACA ATCAAG	90 bp
qPCR of breast cancer tissues			
PINK1	GCCTCATCGAGGAAAAACAGG	GTCTCGTGTCCAACGGGTC	114 bp
Parkin	TCCAAACCGGATGAGTGGTG	TTGCGATCAGGTGCAAAGCTA	128 bp
SNCA	AAGAGGGTGTTCTCTATGTAGGC	GCTCCTCCAACATTTGTCACTT	106 bp
SNCAIP	GAAGCCCCTGAATACCTTGATTT	CGTATCACATCTTCGGCACAGT	108 bp
LRRK2	AGGAAAACAGATAGAAACGCTGG	GTGCTCGGAGTACGTGAACAC	67 bp
DJ-1	GAGCTGGGATTAAGGTCACCG	GACCACATCACGGCTACACT	71 bp
UCHL1	CCTGTGGCACAATCGGACTTA	CATCTACCCGACATTGGCCTT	201 bp
GBA	CTGGCGATGTAGCTTGTGG	GAAGCGGTAATGAGTCAATCCAA	86 bp
ATP13A2	GTGCTGCGGTATTACCTCTTC	TGGGTCTCGATCCAGATATAGC	53 bp
Htra2/omi	ACCTGGTGAGTGAGACATCCT	GCTCAGAAACTTGACCAAACATC	104 bp
PTEN	TGGATTCGACTTAGACTTGACCT	TGGCGGTGTCATAATGTCTTTC	182 bp
TRAP1	AGGACGACTGTTCAGCACG	CCGGGCAACAATGTCCAAAAG	145 bp
MTP18	GTGTGCTGCCTCTCTCTATGT	GCCAGGGTCTTTGGACACC	299 bp
ER	AATGTGCCTGGCTAGAGATCC	CTGTCCAAGAGCAAGTTAGGAG	97 bp
PGR	TCAACTAGGCGAGAGGCAACT	GCCACATGGTAAGGCATAATGA	251 bp
HER2	TGACACCTAGCGGAGCGAT	GGGGGATGTGTTTTCCCTCAA	185 bp
Ki67	ACGCCTGGTTACTATCAAAAGG	CAGACCCATTTACTTGTGTTGGA	209 bp
HPRT	GAAAAGGACCCCACGAAGTGT	AGTCAAGGGCATATCCTACAACA	89 bp

10 μl. Relative quantifications were performed using the comparative method $\Delta\Delta$Ct and HPRT (Hypoxanthinephophoribosyl-transferase) as a reference gene with the mathematical method described in Pfaffl, 2001 [Pfaffl, 2001].

The set of genes analysed was selected from STRING software (`http://string-db.org/`) [Von Mering et al., 2007] and from PDgene database (`http://www.pdgene.org/`) [Lill et al., 2012]database. The sets of specific primers used to analyse expression in breast cancer samples were designed by PrimerBank (`http://pga.mgh.harvard.edu/primerbank/`) [Spandidos et al., 2010]. All the primers used are displayed in **Table 3.3**.

3.6 Immunoperoxidase staining of tissue sections

Immunohistochemistry (IHC) was performed as previously described [Torres et al., 2001], with some modifications. Briefly, home-made tumour microarrays were created from tumour tissue samples obtained by standard surgical procedures, fixed in buffered 10% formaldehyde and embedded in paraffin. 5 μm-sections were quenched for 15 min in 3% hydrogen peroxide in methanol, washed with phosphate-buffered saline (PBS), and incubated with 20% horse serum for 20 min. Then, sections were incubated with anti-

PINK1 89B mAb (culture supernatant; 10 μg.ml^{-1} approximately) for 45 min at 20^{o}C, followed by washing and incubation with the secondary streptavidin-conjugated antibody and avidin-biotin for 30 min at 20^{o}C. Samples were washed and developed using an automated processor (Autostainer, Dako; Envision Plus, Dako, Glostrup, Denmark). As a control of specificity, incubation of the samples in the presence of an excess of recombinant GST-PINK1 150-510 blocked the reactivity of the anti-PINK1 89B mAb. As negative controls, anti-PINK1 89B mAb was substituted for a non-reactive Ig from mouse ascites. Immunostaining of human normal tissues was performed on a multi-tissue control block (Biomeda Corporation); tissue sections stained included brain and breast.

3.7 Protein expression analysis

3.7.1 Cellular extracts

Whole cell protein extracts from mammalian cells were prepared as follows: cells were harvested and lysed on ice in lysis buffer A (0.25% Triton X-100 in PBS, supplemented with protease inhibitors (1 mM PMSF (phenylmethanesulfonyl fluoride), 1 μg.ml^{-1} pepstatin A, 1 μg.ml^{-1} leupeptin) and 1 nM Na_3VO_4) or lysis buffer B (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Igepal (Nonidet P-40), 2 mM Na_3VO_4, 100 mM NaF, 1 mM PMSF, 1 μg.ml^{-1} aprotinine, 20 mM $Na_4P_2O_7$, 0.25% Triton X-100) for 30 min, followed by centrifugation at 16000xg for 10 min. The supernatant (soluble fraction) was collected and stored at -20^oC before processing for immunoblot (IB).

3.7.2 Immunoblot

For immunoblot (IB), the soluble fraction (25-50 μg of protein) was resolved in 9% (PINK1 blots), 15% (p27 and LC3 blots) or 10% SDS-PAGE under reducing conditions. Protein concentrations were determined using the Bradford (Biorad) method or BCA (bicinchoninic acid, Pierce). Prestained molecular weight markers (Sigma-Aldrich) were used to estimate protein sizes. The proteins were transferred to a Hybond-P PVDF membrane (Amersham, GE Healthcare) activated with methanol. The membrane was blocked for at least 1 h prior to incubation with primary antibody (**Table 3.4**), 16 h at 4^{o}C or 2 h at 20^{o}C . Blocking and washing of the membranes were done in NET-gelatine (NET-gel) buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, 0.25% gelatine), 0.1% Tween-PBS (T-PBS), or 0.1% Tween-TBS (T-TBS) (see **Table 3.4**). The blocking was done with 5% non-fat dry milk and antibodies incubation with 3% BSA in the case of T-TBS and P-PBS conditions, except for anti-DNP with 1% BSA for blocking and antibody incubation. After membrane incubation with the appropriate horseradish peroxidase-linked secondary antibody, blots were developed with enhanced chemiluminescence reagent (Roche Diagnostics and Pierce) and exposed to X-ray film (Kodak BioMax MS). In some cases, in order to probe a second primary antibody, the membranes were stripped twice for 5 min with stripping solution (0.2 N NaOH, 1% SDS). Quantification of the immunoblots was done using the Image J (v1.44, Wayne Rasband, NIH, Bethesda, USA).

Table 3.4: Antibodies.

Antibody name	IB use	IF use	Condition	Source	Reference
Primary antibodies					
α-PINK1 Ag	1:1000	1:100	T-TBS	Mouse	Abgent
α-PINK1 108-200	1:500	1:50	NET-gel	Rabbit	Generation in our group
α-PINK1 89B culture supernatant (89B mAb)	1:1 or 1:2	1:1 or 1:2	NET-gel	Mouse	Generation in our group[Berthier et al., 2011]
α-PINK1 BC100-494	1:500	—	T-TBS	Rabbit	Novus Biologicals
α-TOM20 (FL-145)	1:2000	1:500	T-PBS	Rabbit	SC Biotechnology, Inc. Sc-11415
α-SDHA	1:1000	—	T-PBS	Rabbit	Abcam (ab 66484)
α-β-actin	1:1000	—	T-PBS	Rabbit	Sigma-Aldrich A2066
α-GAPDH (6C5)	1:10000	—	T-PBS	Mouse	Abcam (clone 6C5)
α-GAPDH	1:1000	—	NET-gel	Mouse	SC Biotechnology, Inc. Sc-32233
α-LC3	1:1000	—	T-PBS	Rabbit	Cell signaling 2775
α-p62	1:1000	—	T-PBS	Mouse	BS Transduction Lab. 610833
α-DNP	1:150	—	T-PBS	Rabbit	Millipore 90451
α-Click iT® Alexa Fluor 488EdU	Manufacturer's conditions				Invitrogen C35002
α-cyclin D1 (DCS6)	1:1000	—	NET-gel	Mouse	Cell signaling 2926
α-cyclin A (C19)	1:1000	—	NET-gel	Goat	SC Biotechnology, Inc. Sc-596G
α-cyclin B1 (GNS1)	1:1000	—	NET-gel	Mouse	SC Biotechnology, Inc. SC-245
α-p27 (N20)	1:500	—	NET-gel	Rabbit	SC Biotechnology, Inc. Sc-527
α-p27 (C19)	1:500	—	NET-gel	Rabbit	SC Biotechnology, Inc. Sc-5286
Secondary antibodies					
α-Rabbit (SH-SY5Y)	1:5000	—	—	Goat	Calbiochem DC03L
α-Rabbit (SH-SY5Y, oxyblot)	1:300	—	—	Goat	Millipore 90452
α-Mouse (SH-SY5Y)	1:5000	—	—	Goat	Calbiochem DC02L
α-Rabbit (MCF-7)	1:5000	—	—	Goat	Oncogene
α-Mouse (MCF-7)	1:5000	—	—	Goat	Promega
α-Sheep	1:20000	—	—	Donkey	Sigma-Aldrich A2066
α-Rabbit Alexa Fluor 544	—	1:100	—	Goat	Invitrogen A11001
α-Mouse Alexa Fluor 488	—	1:100	—	Goat	Invitrogen A11006

3.7.3 Immunofluorescence

For immunofluorescence (IF) analysis, the different cell lines were grown onto glass coverslips in 24-well or 6-well plates at approximate density 0.9x10^5 cells/coverslip. Then, cells were washed with PBS, fixed in 4% paraformaldehyde (PFA)-PBS for 20 min at 20^{o}C and washed three times with PBS. Staining of mitochondrial network was done with a preincubation step of 1 h with 0.5 μM Mitotracker CMXROS red (λex_{max} : 579nm λem_{max} :

597nm, Invitrogen) in living cells. PINK1 over-expressing SH-SY5Y cells kept untreated or treated with carbonyl cyanide m-chloro phenyl hydrazone (CCCP, 3 hours) were fixed and washed as above. Then, cells were treated with 10 mM Sodium Citrate pH6, 20 min, 95^{o}C, and permeabilised with methanol for 15 min at -20^oC. Cells were blocked with 5% goat serum in PBS, and incubated with antibodies against PINK1 (AM6406a, Abgent) and TOM20 (sc-11415 Santa Cruz Biotechnology) followed by goat anti-mouse and anti-rabbit secondary antibodies conjugated to the fluorophore alexa-488 and alexa-594 (alexa-488: λex_{max} : 495nm λem_{max} : 519nm; alexa-594:λex_{max} : 590nm λem_{max} : 617nm, Invitrogen), respectively. Coverslips were mounted on slides with Citifluor supplemented with 4'-6-diamidino-2-phenylindole (DAPI, λex_{max} : 350nm λem_{max} : 470nm). Images were taken using a Zeiss 710 UV-visible CLSM confocal microscope and fluorescence microscope. PINK1 over-expressing MCF-7 Tet-On cells were fixed as above, permeabilized with 0.5% Triton X-100-PBS for 5 min at 20^{o}C and blocked with 3% bovine serum albumin (BSA)-PBS 1 h at 20^{o}C. Cells were incubated for 2 h at 37^{o}C in wet-chamber with anti-PINK1 108-200 or anti-PINK1 89B mAb primary antibodies diluted in 3% BSA-PBS, followed by incubation with goat anti-mouse or anti-rabbit secondary antibodies conjugated to the fluorophore alexa-488 for 1 h. Nuclei were stained with Hoechst 33258 (λex_{max} : 352nm λem_{max} : 461nm, Sigma-Aldrich). Coverslips were mounted onto microscope slides using fluorescence mounting medium. Images were obtained with a DM2000 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DFC420 camera and analyzed by ImageJ software.

3.7.4 Cellular subfractionation and mitochondrial isolation

PINK1 over-expressing SH-SY5Y cells were harvested by trypsinisation, washed in PBS supplemented with 50 mM dithiothreitol (DTT), protease (1 mM PMSF, 1 μg.ml^{-1} pepstatin A and 1 μg.ml^{-1} leupeptin) and phosphatase (1 nM Na_3VO_4 and 1 mM NaF) inhibitors, and homogenised with a glass-teflon homogeniser in isolation medium (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH7.4 supplemented with 50 mM DTT, protease (1 mM PMSF, 1 μg.ml^{-1} pepstatin A and 1 μg.ml^{-1} leupeptin) and phosphatase (1 nM Na_3VO_4 and 1 mM NaF) inhibitors). Nuclei were removed by centrifugation (1500xg), and mitochondrial were obtained by further centrifugation at 11800xg (pellet fraction) and resuspended in isolation medium. Cytosolic fractions were concentrated with Amicon Ultra-4 10 K columns (Millipore). Antibodies against mitochondrial (TOM20 and SDHA) and cytosolic (GAPDH (6C5) and β-actin) proteins were used as markers of these compartments.

3.8 Cellular assay methods

3.8.0.1 Cell viability

SH-SY5Y cells were plated at a density of 4000 cells per well and incubated from 2 to 5 days. Cells were stained with 20 μl Cell Titer Blue Reagent (CellTiter-Blue® Cell Viability Assay (Promega)) for 4 h, and fluorescence (560/590nm) was measured. Data are presented as the average fluorescence corrected for background.

MCF-7 cells were plated at a density of 3000 cells per well and incubated from 2 to 6 days. Cells were washed with PBS and fixed with 4% PFA-PBS for 20 min. Subse-

quently, cells were stained with crystal violet solution, and after three washes, the staining was solubilized with 1% SDS. The absorbance (580nm) was measured with VICTOR3 (PerkinElmar) plate reader, using Wallac 1420 Workstation software. Data are presented as the average absorbance corrected for background.

3.8.1 Neuroblastoma cell lines

3.8.1.1 ATP synthesis

PINK1 over-expressing SH-SY5Y cells were trypsinized and washed three times with ice-cold PBS. Cells were resuspended at 2.5x10^5 cells.ml^{-1} in incubation medium (25 mM Tris, 150 mM KCl, 2 mM K^+-EDTA, 10mM K_2HPO_4, pH7.4). An aliquot of cells (2.5x10^4 cells) was mixed with an equal volume of incubation buffer containing 1 mg.ml^{-1} of BSA, 1 mM ADP and substrates (complexes I, III, IV: glutamate+malate (10 mM); complexes II, III, IV: succinate (10 mM)+rotenone (10 μM); complex IV: ascorbate (2 mM)+N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD; 50 μM)), permeabilized with digitonin (30 μg.ml^{-1}), and incubated at 37^{o}C for 30 min. The reaction was stopped with perchloric acid, and samples neutralized with 3 M K_2CO_3/0.5 M triethanolamine. Debris was removed by centrifugation and ATP measured with the ATP Bioluminesence Assay kit HSII (Roche). Data were expressed as pmoles ATP synthesized.$minute^{-1}$.$mgprotein^{-1}$. ATP synthesised in cells in the absence of substrates was substracted from the data.

3.8.1.2 Confocal imaging of mitochondrial membrane potential, reactive oxygen species and glutathione

For measurements of $\Delta\Psi$m, cells were loaded with 25 nM tetramethylrhodamine methylester (TMRM, λex_{max} : 548nm λem_{max} : 573nm, Invitrogen) in a HEPES-buffered salt solution (HBSS, 156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_2, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES pH7.35) for 30 min at 20^{o}C, and the dye was present throughout the experiment. In these experiments, TMRM is used in the redistribution mode [Duchen et al., 2003] to assess $\Delta\Psi$m and therefore a reduction in mitochondrial localised TMRM fluorescence represents $\Delta\Psi$m depolarization. Oligomycin (2 μg.ml^{-1}), rotenone (5 μM) and FCCP (100 μM, carbonylcyanide-4-trifluoromethoxyphenylhydrazone) were added after basal TMRM measurements to assess changes of $\Delta\Psi$m. Confocal images were obtained using a Zeiss 710 UV-visible CLSM microscope equipped with a META detection system and a 63x oil immersion objective. The 543nm argon laser line was used to excite TMRM and fluorescence measured using a 560nm long-pass filter.

To measure cytosolic ROS (cROS) production, 2 μM dihydroethidium (HEt, λex_{max} : 543nm λem_{max} : 560nm, Invitrogen) was present in the solution during the experiment (the absence of previous loading step limited the intracellular accumulation of oxidised products). HEt is a non-fluorescent derivative of the red fluorescent ethidium, and an increase in red fluorescence gives a measure of the rate of oxidation. The ratio between the oxidised and reduced species was considered as the measurement of ROS generation.

For GSH measurement, cells were incubated with 50 μM monochlorobinane (MCB, λex_{max} : 351nm λem_{max} : 430 − 480nm, Invitrogen) for 30 min. Non-fluorescent MCB undergoes a reaction with glutathione catalysed by glutathione-transferase, to yield a fluorescent adduct which gives a measure of GSH content [Keelan et al., 2001].

HEt and GSH fluorescence measurements were obtained on an epifluorescence inverted microscope equipped with an 20x fluorite objective. Emitted fluorescence light was reflected to a cooled CCD camera. All data presented were obtained from at least three coverslips from different cell preparations.

3.8.1.3 Oxyblot: detection of protein carbonyls

Protein carbonyl levels were measured in PINK1 over-expressing SH-SY5Y cells using the Oxyblot® protein oxidation detection kit (Millipore). Briefly, cells were lysed with 0.25% Triton X-100-PBS supplemented with 50 mM DTT and protease inhibitors (1 mM PMSF, 1 μg.ml^{-1} pepstatin A and 1 μg.ml^{-1} leupeptin). Following removal of insoluble material, protein carbonyls were derivatized to 2,4-dinitrophenylhydrazone (DNP) by reaction with 2,4-dinitrophenylhydrazine. The DNP-derivatized protein samples were then separated by SDS-PAGE followed by immunoblot (**section 3.7.2**). Carbonyls were detected by incubation with an antibody specific to DNP. Note that protein samples which were not derivatized to DNP were not detected by the DNP antibody. Equal loading was determined by reprobing the same blot with anti-GAPDH (6C5) antibody.

3.8.1.4 Mitochondrial DNA copy number

To measure mitochondrial DNA (mtDNA) copy number, total cellular DNA was isolated with E.N.Z.A genome DNA kit (Omega Bio-tek, Doraville, GA). qPCR analysis of the mtDNA copy number was performed using primers in the D-loop of mitochondria: forward 5'-CATCTGGTTCCTACTTCAGGG-3' and reverse 5'-TGAGTGGTTAATAGGGTGATAGA-3' and in the nuclear thymidine kinase 2 (TK2), exon 5: forward 5'-TCCTGCAGA TGCCACTTTGA-3' and reverse 5'-CCCCAAGTCTGAAGAAAACG-3'. Amplification of the D-loop of mitochondria was measured using the QuantiTect SYBR Green kit, and was expressed relative to the single copy nuclear gene. Relative expression was calculated using the $\Delta\Delta C_t$ method.

3.8.1.5 Mitophagy analysis and citrate synthase activity

Following treatment with 10 μM CCCP, cells were washed once with PBS and lysed (Buffer A) as in **section 3.7.1**. Supernatant was used for immunoblot analysis to check LC3 and p62 expression and to measure citrate synthase activity by following the oxidation of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent). Oxydation was measured over time in a spectrophotometer (absorbance at 412nm) at 30^{o}C in the presence of Acetyl Co-A and oxaloacetate [Coore et al., 1971; Shepherd and Garland, 1969]. Protein concentration from the same aliquot was measured using the BCA protein assay (Pierce). Enzyme activity was expressed as nmol.min^{-1}.mgprotein^{-1}.

3.8.2 Breast cancer cell lines

3.8.2.1 Apoptosis

MCF-7 inducible cell lines were plated at density 2.5x10^5 cells per well in 6-well plates 24 h before treatment with 0.15 mM hydrogen peroxide (H_2O_2) for 24 h, to induce mitochondrial-mediated apoptosis through mitochondrial release of cyt c-dependent path-

way [Brookes et al., 2002]. Cellular viability and mitochondrial membrane potential ($\Delta\Psi_m$) were measured by flow cytometry (FACS).

Cell death was determined by 5 min incubation with propidium iodide (PI; λex_{max} : $493nm$ λem_{max} : $617nm$; Sigma-Aldrich), a membrane impermeable nucleic acid dye which enters the cells upon cell death due to loss of membrane integrity.

$\Delta\Psi_m$ was measured upon 30 min incubation with 40 nM 3,30-dihexylo-xacarbocyanine iodide ($DiOC_6(3)$), λex_{max} : $484nm$ λem_{max} : $511nm$, Invitrogen). $DiOC_6$ is a membrane-permeable lipophilic cationic dye that stains mitochondria of live cells at physiologic $\Delta\Psi_m$ because of its affinity to cardiolipines [Özgen et al., 2000].

Fluorescence was quantified on a Cytomomics FC 500 flow cytometer (Beckton-Dickinson). For each sample, 10000 cells were acquired.

3.8.2.2 Adhesion assay

Cells were pretreated with Dox and plated at a density of $1x10^5$ cells per well in 96-well plates pre-coated with 1:20 diluted BD Matrigel (Basement Membrane Matrix, BD Bioscience), 10 $\mu g.ml^{-1}$ fibronectin (Sigma-Aldrich), 10 $\mu g.ml^{-1}$ colagen IV (BD Pharmigen) or 1 $\mu g.ml^{-1}$ gelatine (Merck) in PBS for at least 16 h. Before plating the cells, plates were pre-coated for 1 h with 0.1% BSA. Cells were plated with MCF-7 medium with or without Dox, containing 0.1% BSA, and were incubated 3 h for attachment. Non-attached cells were washed with PBS, and the attached cells were fixed with 4% PFA-PBS, stained with 0.1% crystal violet, and re-solubilised with 10% acetic acid. The absorbance was measured at 570nm with VICTOR3 (PerkinElmar) plate reader, and data are presented as the average absorbance corrected for background.

3.8.2.3 Invasion assay

Cells were plated at a density of $1.8x10^5$ cells per well in 24-well plates and grown with or without Dox for 48 h. Subsequently, the cell monolayers were scratched with a sterile micropipette tip and incubated for another 24 h. For each sample, the scratch boundary area was monitored during this period. The photographs were taken every 30 min during 24 h at 5x magnification with an automatised time-lapse Leica DMI6000 microscope. The analysis of the photographs was done with Image J as followed (**Figure 3.3**):

- Separation of the different positions stored in single files in one file per position using the automation "separate-time series" and saving as *.tif files.
- Open a *.tif file in Image J and process it as follows: Process → Find Edges; Process → Sharpen.
- Make threshold. Image → Adjust → Threshold; Select Black/White. Upper slider to 0 (left). Set lower slider in such a way that it is clear where the cells are.
- Second process to count particles. Process → Find Edges; Image → Look up Tables → Invert LUT
- Analyse Particles. Size: select a number between 50000-50000000. Circularity: 0.00-1.00. Show: Outlines. Flag: Summarise. Save both the summarised data file and the file with outlines. The data file will show the percentage of wound area in the

image on every time point. Initial and final photographs were analysed with Image J and invasion index (i) area were represented as:

$$i = \frac{area_{tf} - area_{t0}}{area_{t0}}$$

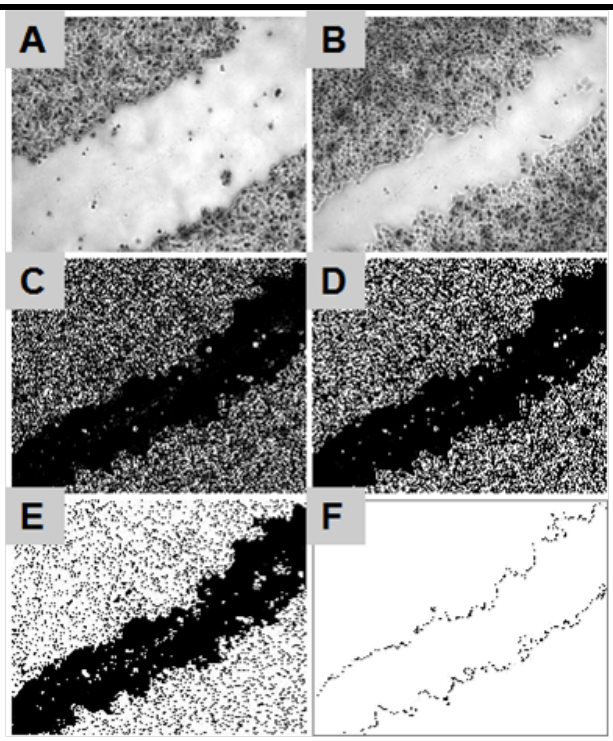

Figure 3.3: Image processing of invasion time lapse photographs. A) and **B)** Example of images taken by time lapse: initial **(A)** and final photographs **(B)**. After first image processing **(C)**, threshold selected **(D)**, and selection of Invert-LUT **(E)**, and the output outline **(F)**.

3.8.2.4 Colony-formation assay

Soft agar growth assays of PINK1 over-expressing MCF-7 Tet-On cells were carried out in 12-well plates (Modified from Koleske et al., 1995). In each well, a 0.75 ml top layer containing the cell suspension (2500 cells in 0.38% cell culture tested agar (Sigma-Aldrich), 10% FBS, 100 $U.ml^{-1}$ penicillin, 100 $\mu g.ml^{-1}$ streptomycin, 200 $\mu g.ml^{-1}$ G418, 100 $\mu g.ml^{-1}$ hygromycin B, 100 $ng.ml^{-1}$ Dox) was added to a hardened bottom layer (0.75 ml previous medium, 0.45% agar). Cells were cultured for two weeks and colonies were stained with 0.05% crystal violet (Sigma-Aldrich) and were photographed at 40x magnification. The culture was analysed in five independent experiments, and colonies larger than 100 μm in diameter were counted. Number of colonies was quantified with ImageJ software as followed (**Figure 3.4**):

- Open a *.tif file in Image J and duplicate as follows: Image → Duplicate; Image → Adjust → Brightness/Contrast → Auto → Apply.

- Obtain the background: Image → Duplicate (duplicate original image). Process → Filters → Maximum. Select Radious 10 pixeles.

- Substract the background to the image of interest: Process → Image Calculator. Image 1: image with colony to quantify; Select Substract; Image 2: image with background created previously. Create a new window; 32-bit result.

- Count and analyse the colonies: Image → Adjust → Threshold (Select the same for all the images in which all the colonies are selected) → Apply. Analyse → Analyse Particles. Options selected: Size 100-Infinity; Circularity 0.00-1.00; Show masks; Display results; Summarise.

The number of colonies counted was normalised and represented as 100% in control cells.

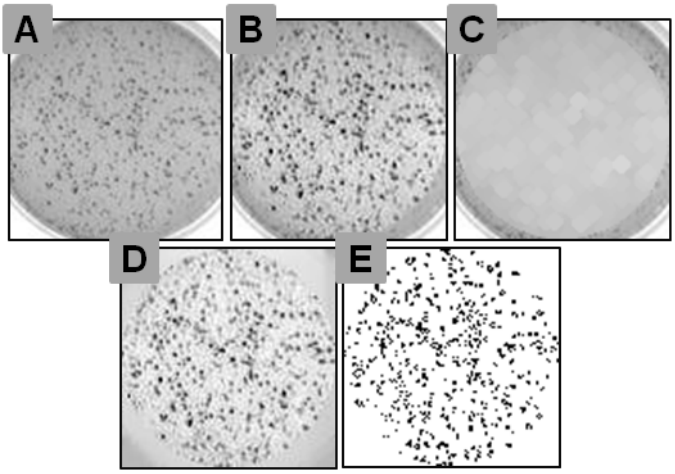

Figure 3.4: Image processing of colony formation in soft agar photographs. **A)** Example of image of colony formation in soft agar. **B)** After the first adjustment. **(C)** Background generation of the same image. **(D)** Result of the original image original after background substraction.**(E)** Last processed step with threshold adjustment.

3.8.2.5 Cell cycle and EdU proliferation

Cells were plated at a density of 2.5x10^5 cells per well in 6-well plates, grown and incubated for 24 h. Then, cells were arrested in G_0/G_1 by FBS starvation for 18 h [Kehn et al., 2007], and cells were re-entered to cell cycle by adding FBS, and were harvested at 12, 24 and 48 h. Subsequently, cells were trypsinised, washed with PBS and resuspended in 300 μl cell-cycle buffer (100 mg $Na_3C_6H_5O_7$, 100 μl Triton X-100, 5 mg PI, 10 mg RNase A). PI binds to DNA by intercalating between the bases with a stoichiometry of one dye per 45 base pairs of DNA, allowing the quantification of DNA content.

The FACS cell cycle analysis was represented as fluorescence intensity histograms of the three characteristic cell cycle populations: two Gaussian curves corresponding to G_0/G_1 (2N) and G_2/M (4N), and an inter-region representing the S phase population (**Figure 3.5**). WinMDI 2.8 software (J. Trotter, Purdue University, USA) was used to adjust the

histograms with correct mathematical models, and representation was done in Cylchred software (Cardiff University, UK).

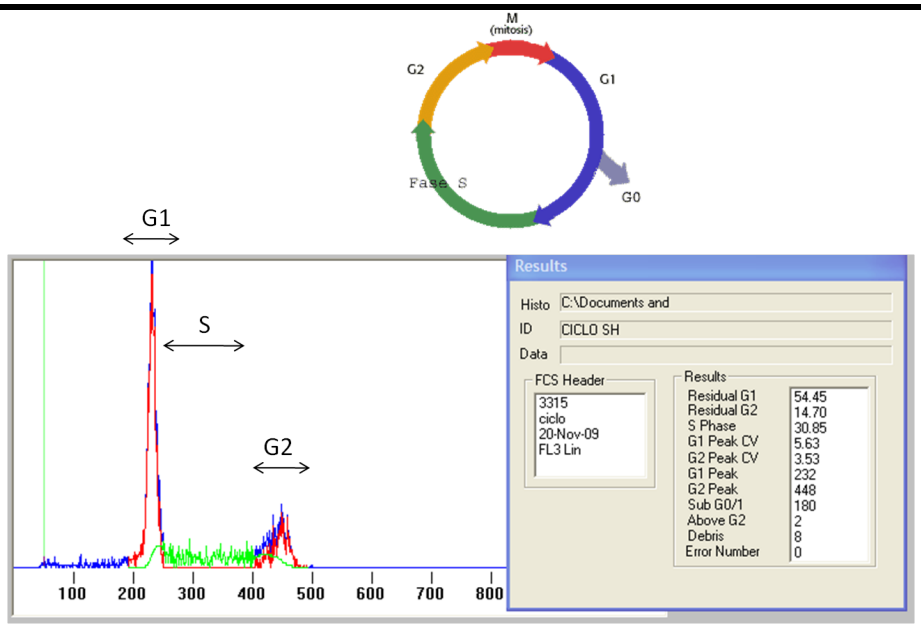

Figure 3.5: Example of cell cycle FACS graphic. Fluorescence intensity histogram with the three characteristic cell cycle phases: G_1 phase, S phase and G_2/M phase.

Sincronised cells were lysed (Buffer B) at different times and cell cycle protein expression (cyclin D1, cyclin A, cyclin B1 and inhibitor p27) was analysed by immunoblot.

For the analysis of cells in S phase (proliferating cells), cells were plated and starved as in cell cycle experiments. The EdU compound (5-ethynyl-2'-deoxyuridine, an analog to BrdU, λex_{max} : $360nm$ λem_{max} : $449nm$) was added at starvation point and kept for 18 h. Then, the Click-iT^{TM} EdU Flow Cytometry assay kit protocol was followed, which is based on the detection of EdU by antibodies after its incorporation to the DNA during S phase. Percentages of cellular proliferation events, proliferating cells in G_0/G_1 and proliferating cells in G_2/M were represented.

3.9 Statistical Analysis

Statistical analysis was performed with PRISM software (GraphPad Software, San Diego, CA) by using the Newman's Keuls multiple comparison test for one-way analysis of variance ANOVA. Student t-test was performed for mtDNA, adhesion and cell cycle/proliferation experiments. Data are expressed as the mean ± standard error of the mean (S.E.M) of separate experiments (n).

3.10 Mutations used in this study

We have functionally studied PINK1 missense and truncated mutations in the context of human neuronal (SH-SY5Y cells) and breast cancer (MCF-7 cells) models. **Table 3.5** and **Figure 3.6** summarize the PINK1 mutations analysed.

Table 3.5: PINK1 mutations used in this study.

Name	Mutations	References
PINK1 wt	—	Unoki and Nakamura, 2001; Valente et al., 2001
K219M	Catalytically inactive mutation. Lysine proposed to bind ATP.	Petit et al., 2005
A168P	Missense PD-linked mutation. Decreased kinase activity, localised inside the putative ATP binding site	Valente et al., 2004b
G309D	Missense PD-linked mutation. Decreased kinase activity, localised in the kinase subdomain V	Valente et al., 2004a
L347P	Missense PD-linked mutation. Decreased stabilisation, localised in the kinase subdomain VI	Hatano et al., 2004; Rogaeva et al., 2004
W437X	Nonsense PD-linked mutation. Increased/decreased kinase activity	Valente et al., 2004a
ΔC	PINK1 1-510 (deletion of C-terminal tail)	Silvestri et al., 2005
ΔN	PINK1 150-581 (deletion of N-terminal tail)	Takatori et al., 2008; Wang et al., 2007; Haque et al., 2008
ΔN-C	PINK1 150-510 (deletion of N- and C-terminal tails)	Sim et al., 2006; Berthier et al., 2011

All mutations have been over-expressed in SH-SY5Y and MCF-7 cells, except for K219M (only in SH-SY5Y cells) and ΔN-C (only in MCF-7 cells).

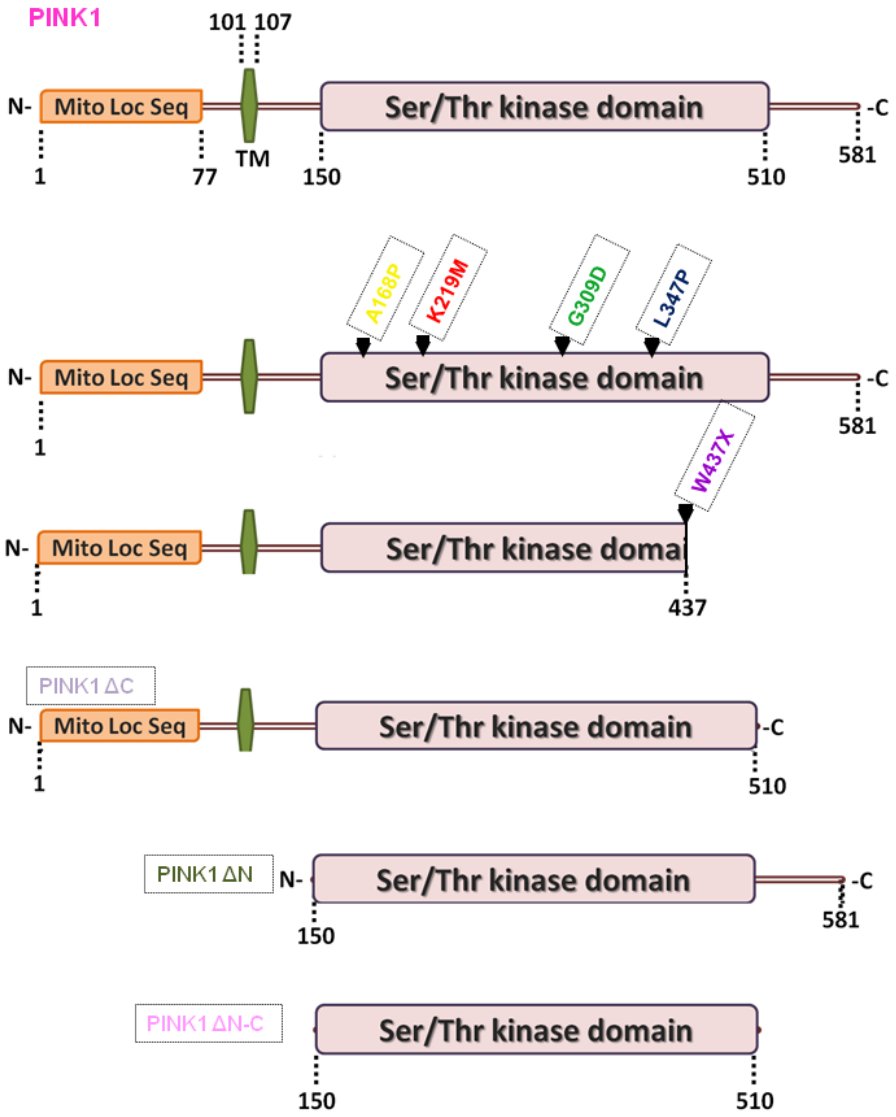

Figure 3.6: PINK1 domain organisation and schematic of the PINK1 mutations and truncations used in this study. Missense and truncated mutations are indicated with amino acid numbering. See **Table 3.5** for further details.

Chapter

4 Results I: PINK1 function in SH-SY5Y cells

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4.1 PINK1 over-expressing SH-SY5Y cells

Recent reports have determined the mitochondrial dysfunction underlying PINK1 loss of function (see **Section 1.6**). However, little is known regarding how PD-linked PINK1 mutations, as well as PINK1 subdomains, affect PINK1 function in physiological settings.

To investigate the role of PINK1 in neuronal mitochondrial function and mitophagy, as well as the functional consequences of missense or truncated mutations on the PINK1 protein, we generated SH-SY5Y human neuroblastoma cells stably over-expressing wild type, mutated or truncated PINK1 proteins. The scheme and the features of the PINK1 over-expressing cells generated are displayed in **Chapter 3, Table 3.5** and **Figure 3.6**.

4.1.1 PINK1 expression and cell viability

PINK1 mRNA expression levels in the SH-SY5Y cell lines were measured by qPCR, compared to HPRT as a reference gene. Over-expression of PINK1 mRNA in all cell lines was similar, compared to control cells (**Figure 4.1-1**). PINK1 protein was undetectable in parental SH-SY5Y cells, probably due to low endogenous PINK1 expression levels.

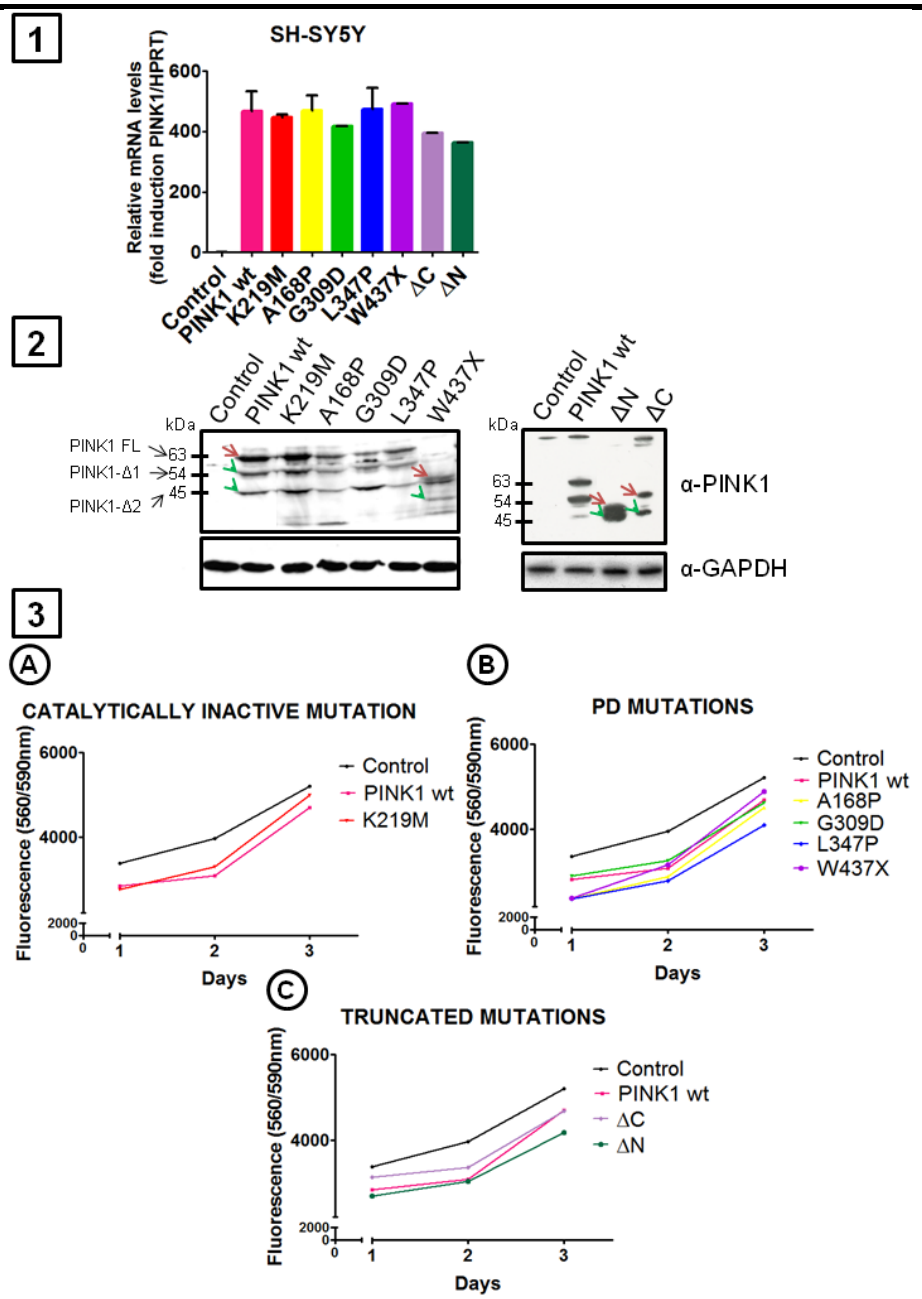


Figure 4.1: PINK1 expression levels and cell viability were similar in all PINK1 over-expressing SH-SY5Y cells generated. 1) qPCR analysis of PINK1 mRNA levels. Total RNA was subjected to reverse transcription. qPCR was performed and quantification was normalized to HPRT as a reference gene. **2)** PINK1 protein expression levels detected by immunoblot with anti-PINK1 BC100 antibody. GAPDH (6C5) was used as a loading control. Red arrows indicate the unprocessed PINK1 full length protein. Green head arrows indicate the processed PINK1 proteins. **3)** Cell viability, measured by CellTiter-Blue®, of SH-SY5Y cells over-expressing missense (**A** and **B**) and truncated (**C**) PINK1 mutations, compared to control and PINK1 wt over-expressing cells.

PINK1 protein expression levels were similar in all PINK1 over-expressing cells generated (**Figure 4.1-2**). Cell lysates were analysed with anti-PINK1 BC100 antibody which detects three PINK1 protein forms: the unprocessed form (or full length (FL), 63 kDa); and two processed forms: PINK1-Δ1 and PINK1-Δ2 (54 kDa and 45 kDa, respectively), as indicated in **Figure 4.1-2**.

The cellular viability under normal growth conditions of PINK1 over-expressing cells was analysed to rule out deleterious effects. The cellular growth of PINK1 over-expressing cells did not show differences compared to control cells (**Figure 4.1-3**).

4.1.2 PINK1 localisation

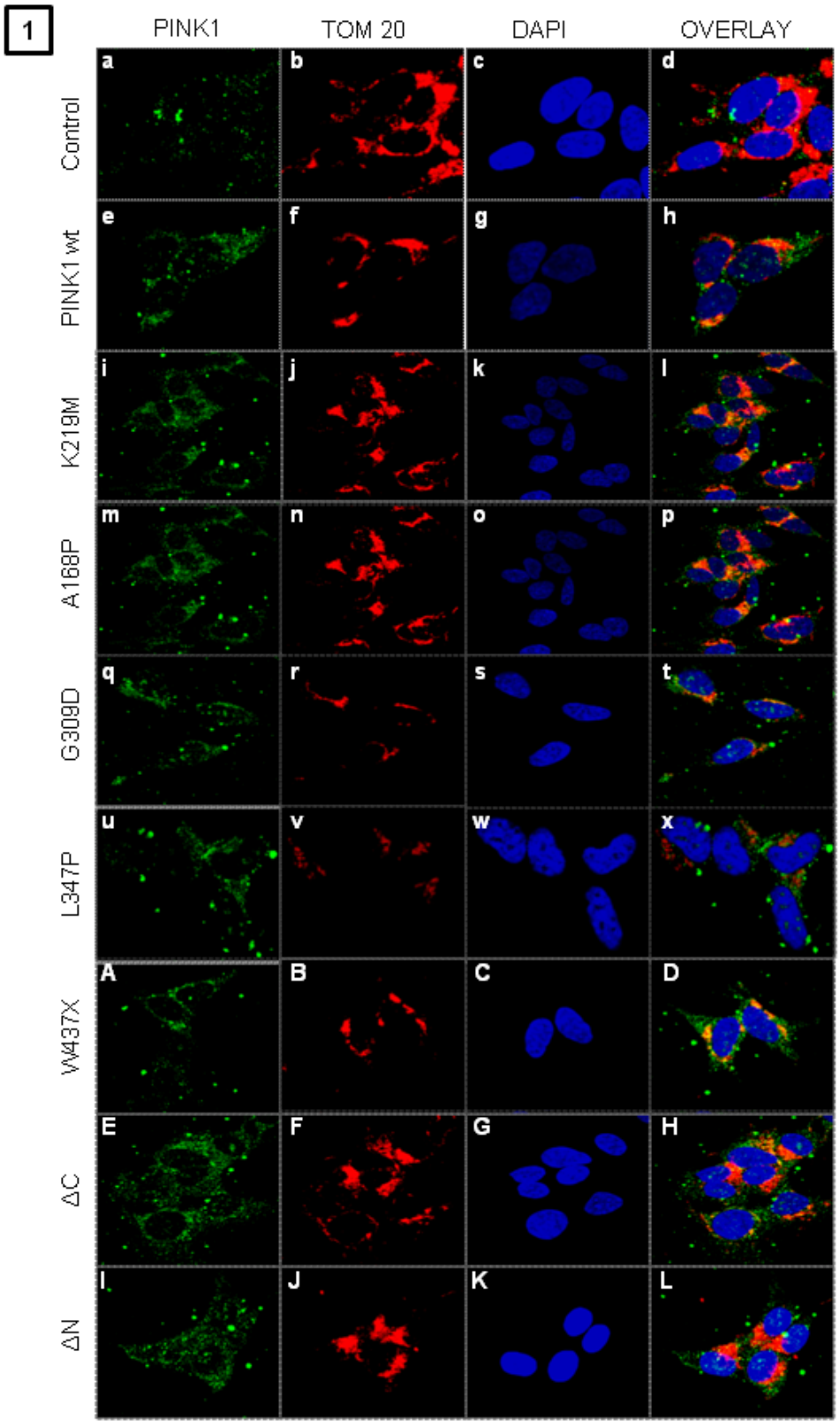
PINK1 is localised in mitochondria and cytosol compartments in cultured cells (see **Section 1.3**). Thus, we analysed PINK1 localisation in our over-expressing cells by immunofluorescence. Ectopic expression of PINK1 resulted in increased green staining compared to control cells. PINK1 wt overlapped with the staining of the outer mitochondrial membrane protein TOM20 in over-expressing SH-SY5Y cells (**Figure 4.2-1**). The PINK1 missense and C-terminal truncated mutations (PINK1 PD-linked W437X, ΔC: residues 1-510) co-localised with TOM20 as PINK1 wt, and in the case of C-terminal truncations the mitochondrial localisation was higher than PINK1. The N-terminal truncation (ΔN: residues 150-581) mostly localised to the cytosol, but still slightly associated to mitochondria compartment.

We also verified the PINK1 localisation by subcellular fractionation. PINK1 over-expressing cells were fractionated by differential centrifugation, and the mitochondrial and cytosolic fractions were analysed. As shown, PINK1 forms were present in both compartments (**Figure 4.2-2**). In PINK1 wt cells, the three PINK1 forms (PINK1 FL, Δ1 and Δ2) were detected in the cytosol fraction, but PINK1 FL was mostly in the mitochondrial fraction. PINK1 K219M and G309D over-expressing cells showed the same distribution pattern of the protein compared to PINK1 wt cells. Besides, in PINK1 missense mutation cells, PINK1 Δ1 form was detected in the mitochondrial fraction. Curiously, in the PINK1 C-terminal truncated over-expressing cells the three PINK1 forms were localised in both compartments, but with higher detection in the mitochondrial compartment. The PINK1 ΔN over-expressing cells presented two PINK1 protein forms, mainly but not exclusively, localised in the cytosolic fraction (**Figure 4.2-2**).

In summary, we demonstrate that PINK1 is localised in both compartments and that the localisation is unaffected by missense mutations, whereas truncated mutations partially affected the distribution of PINK1 protein.

4.2 Mitochondrial function

PINK1 deficiency leads to an impairment in ATP production through the mitochondrial electron transport chain, decreases mitochondrial membrane potential ($\Delta\Psi m$), increases reactive oxygen species (ROS) production, and decreases the mtDNA content (see **Section 1.6.2**). Thus, we analysed the phenotypes of the different PINK1 over-expressing SH-SY5Y cells for those mitochondrial functional parameters.


1
PINK1
TOM 20
DAPI
OVERLAY
Control
PINK1 wt
K219M
A168P
G309D
L347P
W437X
ΔC
ΔN
a
b
c
d
e
f
g
h
i
j
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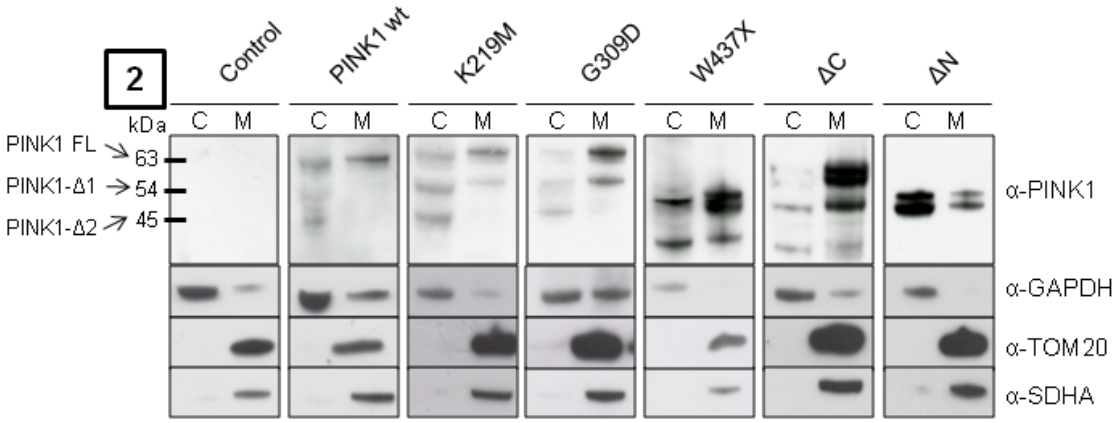

Figure 4.2: PINK1 wt, PD-linked mutations, and C-terminal truncated mutation are distributed in mitochondria and cytosolic compartments. The N-terminal truncated mutation is mostly localised in cytosol. 1) Control (a-d), PINK1 wt (e-h), K219M (i-l), A168P (m-p), G309D (q-t), L347P (u-x), W437X (A-D), ΔC (E-H), and ΔN (I-L) over-expressing SH-SY5Y cells were studied. Immunofluorescence was carried out with anti-PINK1 Ag antibody [green] (a, e, i, m, q, u, A, E, I), anti-TOM20 [red] (b, f, j, n, r, v, B, F, J), DAPI [blue] (c, g, k, o, s, w, C, G, K). In panels d, h, l, p, t, x, D, H, and L, the overlay of the three colours is shown. **2)** Subcellular fractionation of control, PINK1 wt, K219M, G309D, W437X, ΔC, and ΔN over-expressing SH-SY5Y cells. C: cytosolic fraction, M: mitochondrial fraction. GAPDH (6C5) was used as cytosolic marker, and SDHA and TOM20 as mitochondrial markers.

4.2.1 ATP production through the mitochondrial electron transport chain

We measured ATP production through the mitochondrial electron transport chain, using PINK1 over-expressing SH-SY5Y cells permeabilized with digitonin, and supplemented with the different mitochondrial electron transport chain substrates for complex I: glutamate+malate, complex II/III: succinate+rotenone or complex IV: ascorbate and TMPD (**Figure 4.3**).

Over-expression of PINK1 wt had no effect on ATP synthesis produced through complexes I, II/III, or IV, compared to control cells (**Figure 4.3**). ATP synthesis was significantly inhibited in the PINK1 K219M over-expressing cells via complexes II/III and complex IV (**Figure 4.3-1**). The cells over-expressing C-terminal truncated mutations (PINK1 W437X and ΔC) also showed an inhibition in ATP synthesis, as well as the cells over-expressing the PD-linked mutations A168P, G309D and L347P (**Figure 4.3**). The PINK1 ΔN over-expressing cells did not show impaired ATP production, but rather a slight increase in ATP production. Therefore, the PINK1 kinase activity and the C-terminal domain are important to maintain ATP production through the electron transport chain.

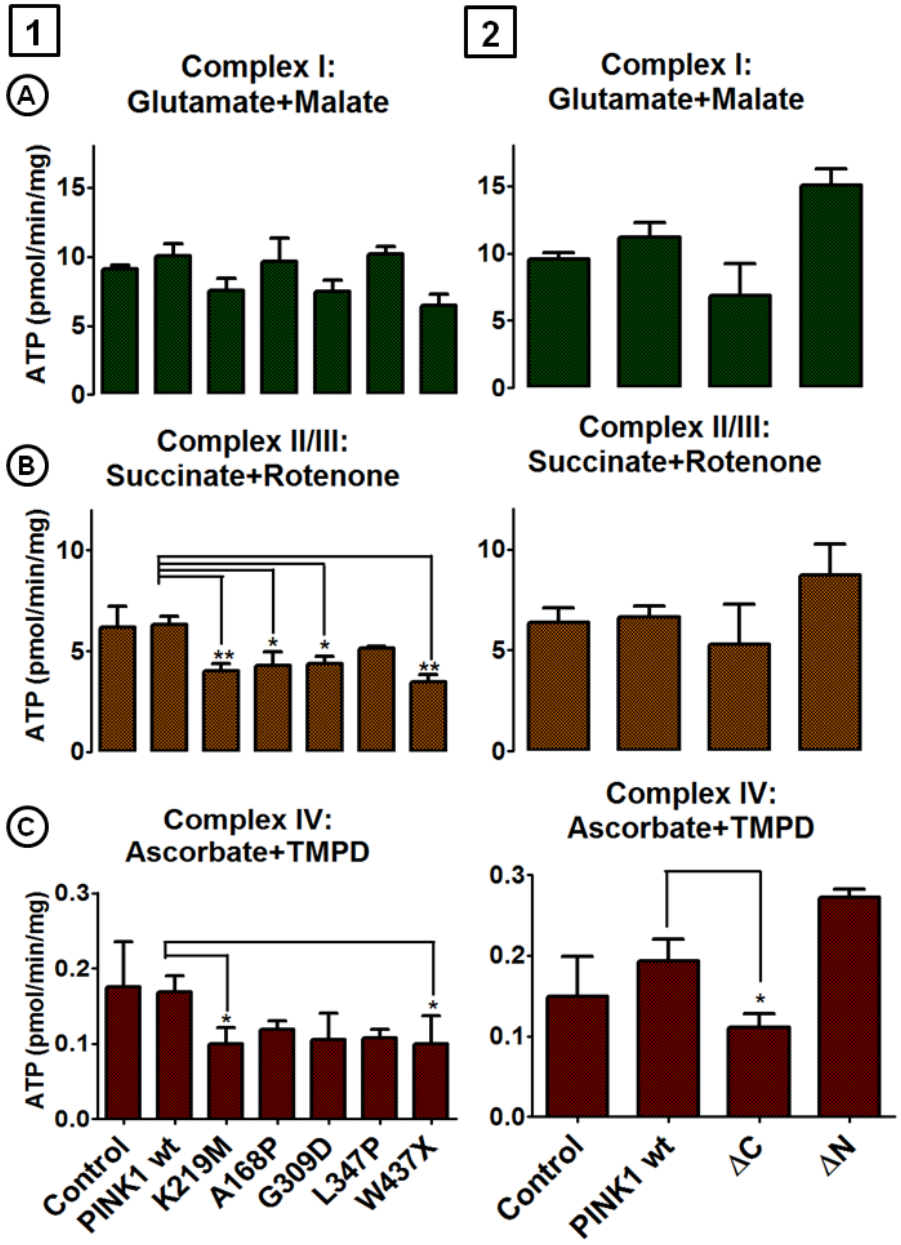

Figure 4.3: PINK1 kinase activity and C-terminal domain modulate mitochondrial ATP synthesis. PINK1 over-expressing SH-SY5Y cells were permeabilised and ATP synthesis was measured following incubation with glutamate+malate **(panels A)**, succinate+rotenone **(panels B)** or ascorbate+TMPD **(panels C)** for 30 minutes. ATP was measured by luciferase assay. Data are shown as the mean±SEM from five independent experiments. Statistical significance *p<0.05 and **p<0.01 versus control cells.

4.2.2 Mitochondrial membrane potential

The impairment of ATP synthesis mediated by PINK1 missense and C-terminal truncated mutation can be due to a decrease in $\Delta\Psi$m (see **Section 1.6.2**). Thus, we monitored the $\Delta\Psi$m in our cellular model using tetramethylrhodamine methylester (TMRM) staining. PINK1 wt over-expressing SH-SY5Y cells showed two-fold increase in basal $\Delta\Psi$m, compared to control cells (**Figure 4.4-1**). PINK1 K219M, A168P, L347P, W437X and ΔC over-expressing SH-SY5Y cells showed decreased in $\Delta\Psi$m compared to PINK1 wt over-expressing cells. In some of the mutations the decrease was below the control cells value, suggesting a dominant negative effect. We included silenced PINK1 SH-SY5Y cells to confirm the reported decrease in $\Delta\Psi$m [Gandhi et al., 2009].

PINK1 wt over-expressing cells displayed a mitochondrial network more interconnected, with mitochondria scattered through the whole cytoplasm. In the case of PINK1 K219M, A168P, L347P, W437X and ΔC over-expressing cells, the mitochondrial network is less interconnected, compared to PINK1 wt cells. Interestingly, PINK1 G309D over-expressing cells, with no changes in $\Delta\Psi$m, displayed ring-like and more fragmented mitochondrial network (**Figure 4.4-2**), compared to the rest of the mutations. PINK1 ΔN did not show significant changes in $\Delta\Psi$m nor mitochondria morphology changes in comparison to control cells (**Figure 4.4-1 and -2**).

In summary, we conclude that PINK1 controls $\Delta\Psi$m and ATP production through the electron transport chain. PINK1 catalytic activity and C-terminus are important to maintain the mitochondrial function and mitochondrial morphology.

To test the maintenance of $\Delta\Psi$m in those cells, we analysed the TMRM staining in the presence of various mitochondrial inhibitors: oligomycin (complex V inhibitor), rotenone (complex I inhibitor) and FCCP (mitochondrial uncoupling agent). Control, PINK1 wt, A168P, G309D, L347P and ΔN cells showed either no response or slight hyperpolarization upon oligomycin treatment. However, PINK1 K219M, W437X and ΔC cells showed depolarization in response to ATP synthase inhibition by oligomycin. Subsequent inhibition of complex I by rotenone, and whole mitochondrial depolarization by FCCP, caused a rapid loss of mitochondrial potential in all the PINK1 over-expressing cells analysed (**Figure 4.5**). In the case of PINK1 wt and ΔN over-expressing cells, the addition of rotenone caused less decrease in mitochondrial membrane potential compared to the rest of the cells.

Therefore, the low $\Delta\Psi$m is largely maintained by the hydrolysis of ATP by complex V, rather than by respiration, in the K219M catalytically inactive mutant and in the C-terminal truncated mutants, whereas in the rest of the cells analysed $\Delta\Psi$m is not affected by blocking complex V.

4.3 Oxidative Status

PINK1-silenced cells and fibroblasts from PD patients display an increase in oxidative stress linked to an increase in reactive oxigen species (ROS) and a decrease in antioxidant capacity (see **Section 1.6.2**). However, the exact defect of PINK1 mutations and truncations in these processes are still unclear. Thus, we determined the oxidative stress status and the sensitivity to free radical generators of PINK1 over-expressing SH-SY5Y cells.

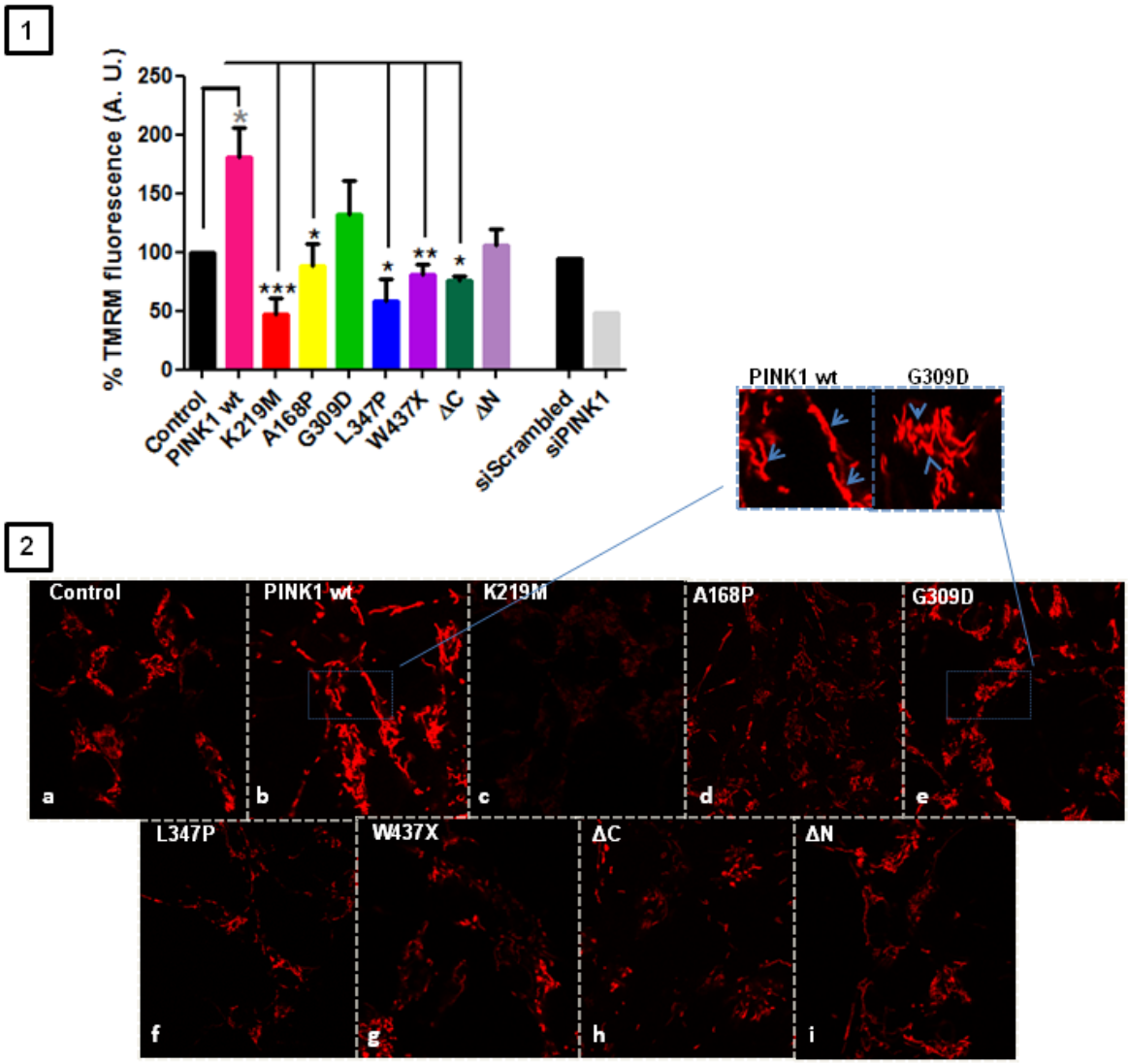

Figure 4.4: Most of PINK1 mutations over-expressing SH-SY5Y cells display mitochondrial membrane potential dissipation and less interconnected mitochondria network. The measurements of inner mitochondrial membrane potential ($\Delta\Psi$m) were made using confocal imaging of TMRM fluorescence. The signal intensity was quantified per pixel in a confocal slice after thresholding to remove background signal. **1)** Relative TMRM fluorescence intensity is expressed as the percentage of the signal from control cells in arbitrary units (A. U.). Data are the mean±SEM from five independent experiments (*$p<0.05$, **$p<0.01$ and ***$p<0.001$). PINK1 silenced cells were included as a control of the experiment. **2)** Confocal overlapped images of TMRM staining in the different SH-SY5Y cells. Note the more interconnected mitochondrial network in PINK1 wt, and the ring-like and more fragmented mitochondrial network in PINK1 G309D over-expressing SH-SY5Y cells.

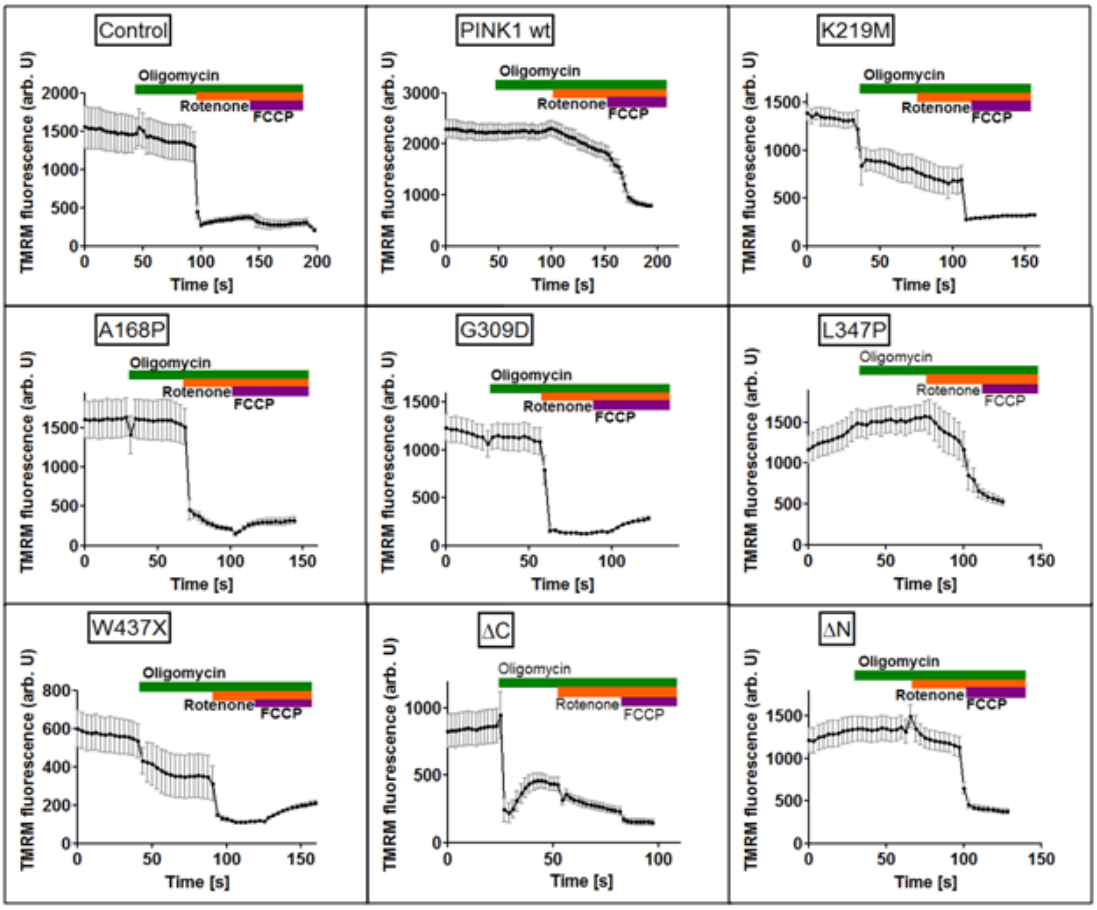

Figure 4.5: The mitochondrial membrane potential in PINK1 K219M, W437X and ΔC over-expressing SH-SY5Y cells is maintained by the hydrolysis of ATP by complex V. Kinetic changes in TMRM measurements after oligomycin, rotenone and FCCP treatments in the different PINK1 over-expressing cells.

4.3.1 Free radical species generation

To evaluate the level of oxidative stress in our model, we measured by kinetic confocal imaging the rates of intracellular cROS generation using dihydroethidium (HEt), a non-fluorescent derivative of ethidium which is oxidised to a fluorescent product by *in situ* superoxide production. Under basal conditions, in PINK1 K219M, A168P, G309D, L347P and W437X cells, the rate of cROS production was significantly higher compared to PINK1 wt over-expressing cells (**Figure 4.6-2**). Upon rotenone treatment, control cells increased cROS production as expected, as well as cells over-expressing PINK1 missense and C-terminal truncated mutations. Over-expressing PINK1 wt and ΔN cells did not display increased cROS generation rates compared to untreated conditions. Thereby, PINK1 wt and ΔN protect against generation of oxidative stress, but not PINK1 missense or truncated mutations (**Figure 4.6**).

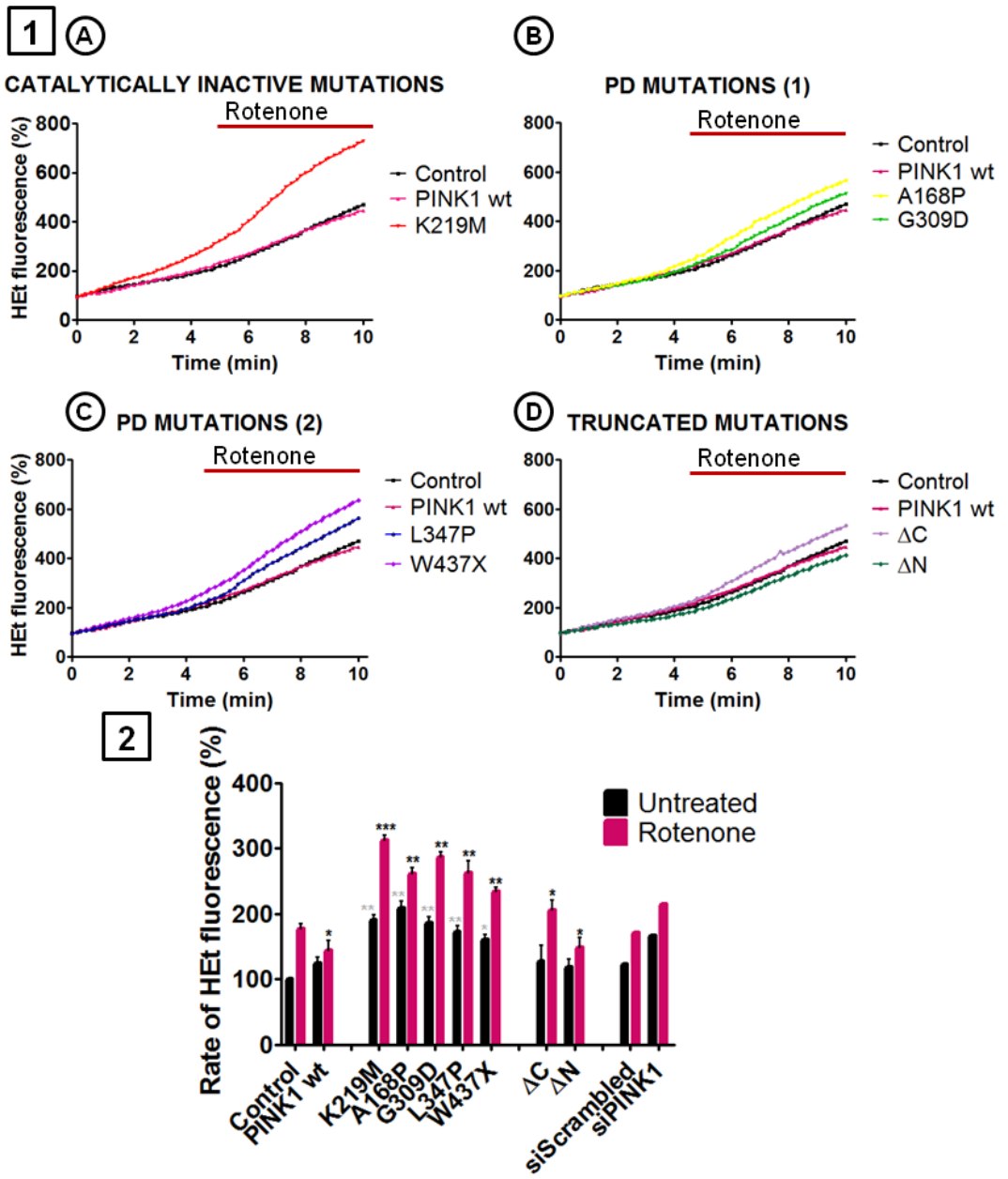

Figure 4.6: PINK1 missense and C-terminal truncated mutations increase ROS generation. The measurement of cROS production was made using imaging of HEt fluorescence. **1)** The traces represent changes of HEt fluorescence over the time before and after rotenone treatment. **2)** The mean rates of HEt fluorescence change per minute, obtained from plot in **1)** are shown. Results are expressed as percentage changes with regard to control cells. Silenced PINK1 SH-SY5Y cells were used as a positive control. Grey stars: compared to untreated control cells (*p<0.05; **p<0.01). Black stars: compared to rotenone-treated control cells (*p<0.05; **p<0.01; ***p<0.001). Data are the mean±SEM of five independent experiments.

4.3.2 Glutathione measurements

To analyse the antioxidant defense in our model, we measured the levels of the antioxidant glutathione (GSH) in basal conditions, using monochlorobimane (MCB), which forms a fluorescent adduct following an enzyme catalysed reaction with GSH. In parallel with the increase in ROS, GSH was significantly depleted in PINK1 K219M, A168P, G309D and L347P over-expressing cells, when compared with control cells or PINK1 wt over-expressing cells (**Figure 4.7-1**). The C-terminal truncated mutations displayed a trend of GSH decreased but it was no significant.

4.3.3 Oxyblot

To assess if the increase in ROS and decrease in GSH are related to increase in mitochondria oxidated species, we analysed the carbonylation of proteins by oxidizing species in isolated mitochondria of PINK1 over-expressing cells, using the Oxyblot protein oxidation detection kit. Oxyblot analysis showed increase in carbonylated proteins in SH-SY5Y cells over-expressing PINK1 missense or truncated mutations but not in cells over-expressing PINK1 wt (**Figure 4.7-2**).

4.3.4 mtDNA content

Alterations in the rates of intracellular ROS generation are associated with changes in mitochondrial abundance and mitochondrial DNA (mtDNA) copy number. PINK1 K219M, G309D and W437X cells displayed a significant decrease in their mtDNA content, as compared with PINK1 wt cells (**Figure 4.7-3**).

4.4 Mitophagy/Autophagy processes

PINK1, together with Parkin, is involved in clearance of damaged mitochondria by selective autophagy, known as mitophagy (see **Section 1.6.4**). PINK1 deficiency leads to a decrease in Parkin recruitment to the mitochondria and produces accumulation of damaged mitochondria. Next, we monitored the mitophagy/autophagy response of our PINK1 over-expressing SH-SY5Y cells, in a CCCP-induced model, and in a starving model. CCCP is a protonophore that pokes a hole in the mitochondrial membrane, making it more permeable to the protons, which causes loss of mitochondrial membrane potential.

4.4.1 PINK1 activated mitophagy/autophagy

To monitor mitophagy/autophagy activation, we measured the cleavage of the protein LC3 in control, PINK1 wt, K219M and W437X over-expressing cells (**Figure 4.8**). LC3 has two isoforms, cytosolic LC3-I (18 kDa), and its processed and lipidated form LC3-II (16 kDa), which is recruited to autophagosomes and can be used as an autophagosome formation marker [Cuervo, 2004]. Under CCCP-induced mitophagy or starvation, LC3-II levels increased compared to untreated control SH-SY5Y cells, and were further increased in PINK1 wt over-expressing cells. The levels of LC3-II in the two PD-linked mutations (K219M and W437X) over-expressing cells were lower than in control or PINK1 wt over-expressing cells (**Figure 4.8**).

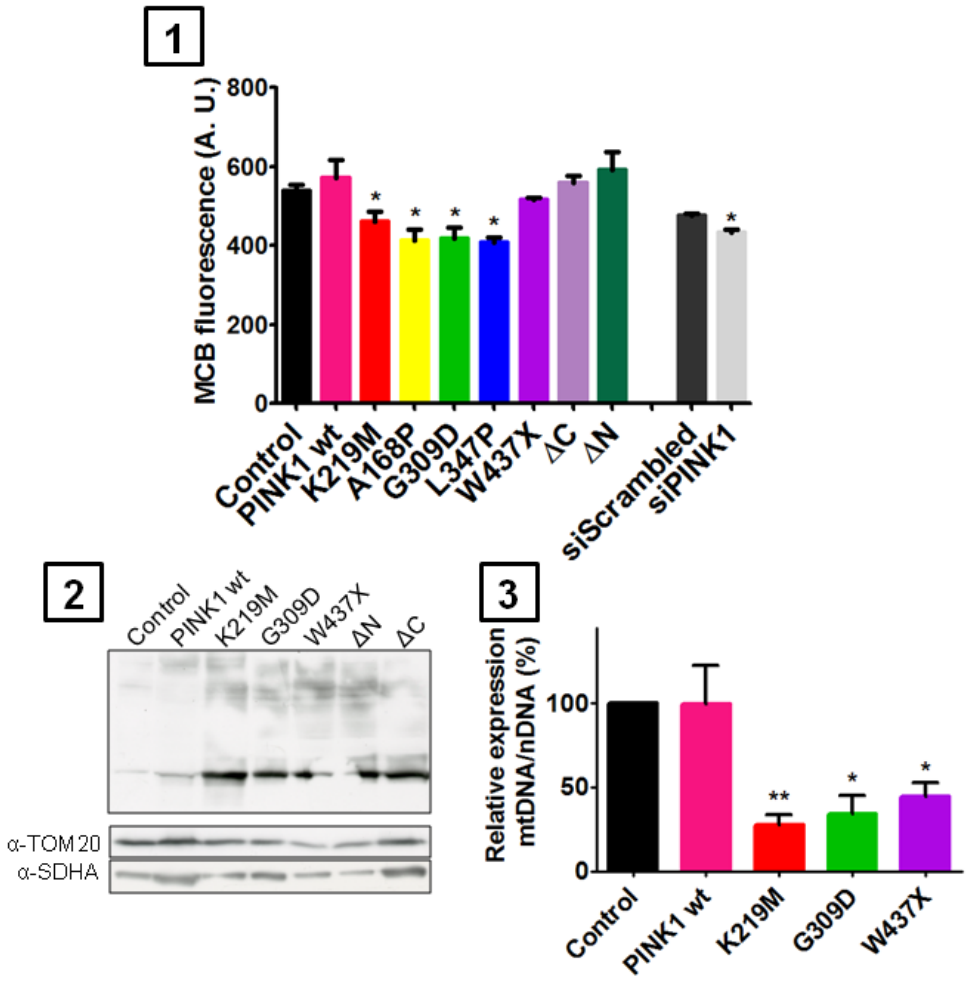

Figure 4.7: PINK1 mutations decrease GSH status and mtDNA, and increase carbonylated proteins. 1) Intracellular glutathione containing was measured by MCB. Relative intensity of MCB fluorescence is expressed in A. U. as the mean±SEM from four independent experiments. **2)** The protein carbonylation was determined by Oxyblot. Loading was assessed by re-probing the immunoblot with anti-TOM20 and anti-SDHA antibodies. **3)** Relative abundance of mtDNA, compared to nDNA, was analysed by qPCR. (Statistical significance: *p<0.05; **p<0.01, compared to control cells).

We analysed other PINK1 mutations in the CCCP-induced mitophagy model, in parallel with measurement of mitochondrial content using the citrate synthase assay and TOM20 protein (mitochondrial marker), and p62 (autophagy marker) levels. All tested mutations displayed reduced LC3-II levels, compared to control or PINK1 wt over-expressing cells (**Figure 4.9-1**). Cells over-expressing PINK1 wt, but not PINK1 mutations, displayed diminished mitochondrial content. Furthermore, PINK1 wt expression, but not K219M expression, increased the levels of p62, a protein that connects ubiquitinated proteins with LC3 for autophagic degradation [Pankiv et al., 2007] (**Figure 4.9-2,3**).

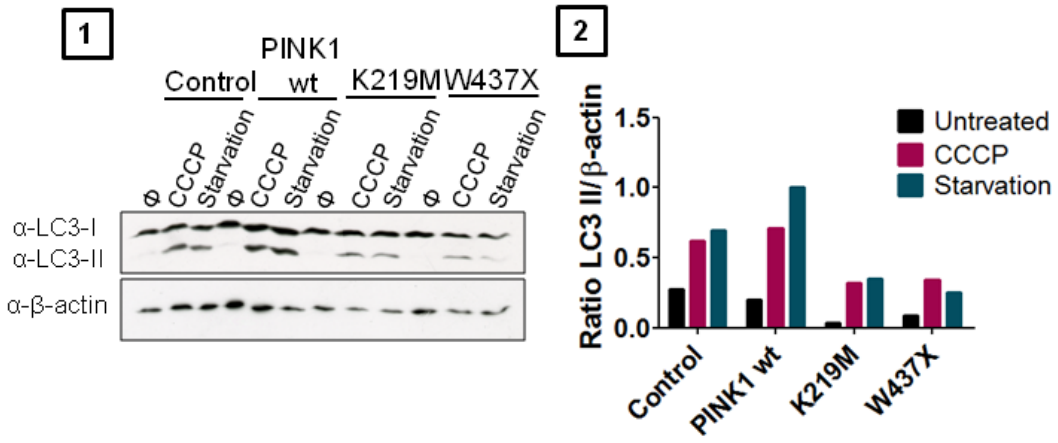

Figure 4.8: PINK1 wt activates mitophagy and autophagy processes through increasing LC3 cleavage. 1) Immunoblot analysis, using anti-LC3 antibody, of PINK1 wt, K219M and W437X SH-SY5Y cells, untreated (ϕ), treated 3 h with CCCP or incubated 4 h in free nutrient medium (starving conditions). LC3 cleavage was detected as a 16 kDa band (LC3-II). β-actin was used as a loading control. **2)** Quantification of LC3-II bands with respect to β-actin.

These results indicate that PINK1 wt, but not PINK1 mutations or truncations, increases mitophagy in SH-SY5Y cells.

4.4.2 PINK1 localisation

PINK1 mitochondrial localisation and accumulation upon CCCP treatment is necessary to activate clearance of damaged mitochondria (see **Section 1.6.4**). To investigate the possible defects on mitophagy in the PINK1 mutations and truncations over-expressing cells, we analysed by immunofluorescence and by subcellular fractionation whether PINK1 localisation is altered upon CCCP treatment. PINK1 wt cells treated with CCCP for 3 h showed clear PINK1 mitochondria localisation (co-localisation index: 0.93) (**Figure 4.10-1**), as previously reported [Narendra et al., 2010]. The PINK1 mutations K219M, A168P, G309D and L347P cells showed diminished mitochondria localisation of PINK1 protein upon 3 h of CCCP treatment, whereas in the C-terminal PINK1 W437X and ΔC and N-terminal truncated cells, PINK1 protein did not accumulate in the mitochondria after CCCP treatment. In untreated conditions, control cells showed low co-localisation index between PINK1 and TOM20 proteins, perhaps due to the detection threshold of the anti-PINK1 Ag antibody, while PINK1 over-expressing cells presented higher co-localisation index.

Subcellular fractionation after 18 h of CCCP treatment in PINK1 over-expressing SH-SY5Y cells showed high mitochondrial localisation of the PINK1 protein, even in the case of PINK1 ΔN cells (**Figure 4.10-2**). In conclusion, upon CCCP treatment, initial PINK1 localisation seems to be affected in cells over-expressing PINK1 mutations or truncations, although subfractionation analysis of long term CCCP treatment was not altered in the different PINK1 over-expressing cells analysed.

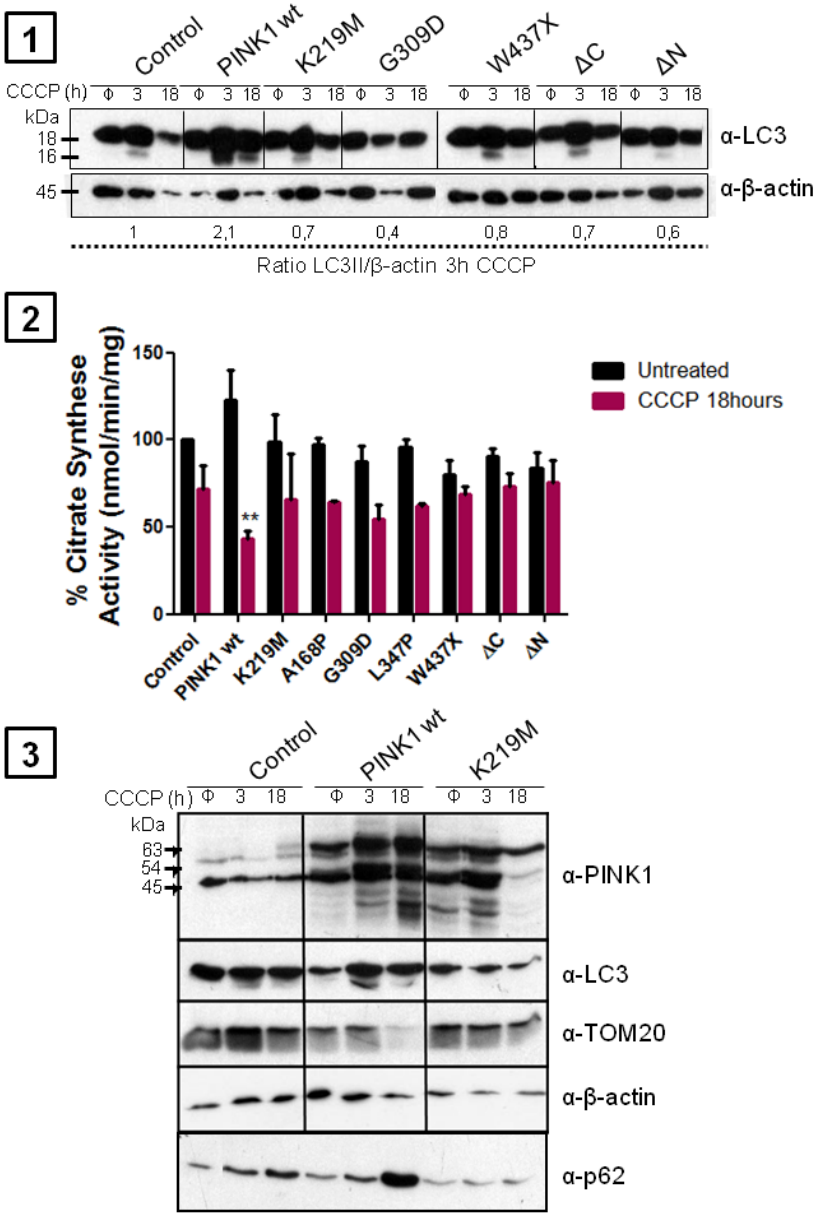

Figure 4.9: PINK1 wt, but not PINK1 mutations and truncations, increases LC3 cleavage and decreases mitochondria content. 1) Immunoblot analysis, using anti-LC3 antibody, of PINK1 over-expressing SH-SY5Y cells, untreated (ϕ) or treated for 3 h or 18 h with CCCP. β-actin was used as a loading control. Quantification of LC3-II bands with respect to β-actin is indicated as the ratio at 3 h of CCCP treatment. **2)** Citrate synthase assay, as a measurement of the mitochondria content (Statistical significance: **p<0.01 compared to control cells). **3)** Immunoblot analysis of PINK1 wt and K219M over-expressing cells, using anti-PINK1 BC100, anti-LC3 and anti-p62 antibodies (autophagy markers), and anti-TOM20 antibody (mitochondria marker) from cells untreated (ϕ) or treated for 3 h or 18 h with CCCP. β-actin was used as loading control.

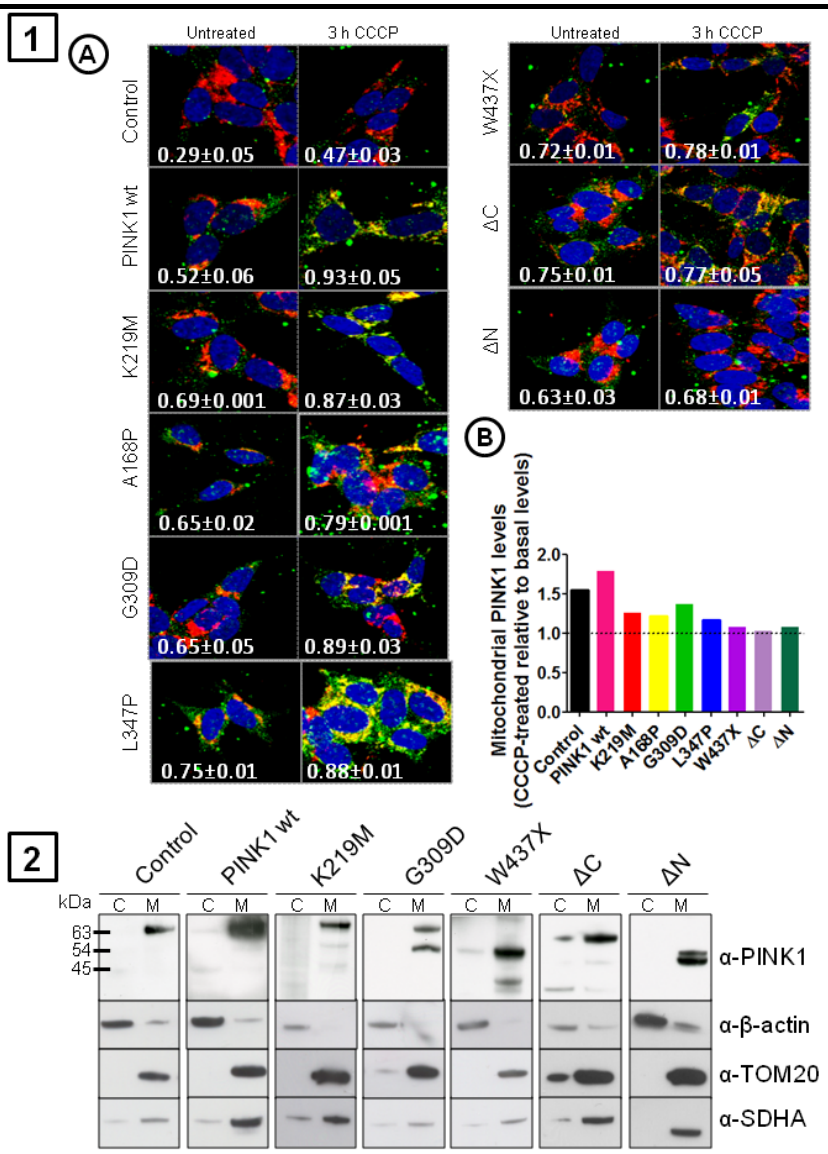

Figure 4.10: PINK1 wt cells, but not PINK1 mutations and truncations, co-localised with mitochondria after short period of CCCP treatment whereas, after long times of CCCP treatment, PINK1 co-localised to mitochondrial compartment in all PINK1 over-expressing SH-SY5Y cells. 1) A) Immunofluorescence of control, PINK1 wt, K219M, A168P, G309D, L347P, W437X, ΔC and ΔN over-expressing SH-SY5Y cells was performed in untreated and 3 h CCCP-treated conditions, and co-localisation indexes are indicated. Immunofluorescence was carried out with anti-PINK1 Ag antibody [green], anti-TOM20 antibody [red], and DAPI [blue]. The overlay of the three colours is shown in all panels. **B)** PINK1 mitochondrial levels in CCCP-treated conditions related to basal conditions in PINK1 over-expressing cells were analysed. **2)** Cellular subfractionation of control, PINK1 wt, K219M, G309D, W437X, ΔC and ΔN over-expressing cells, after 18 h of CCCP treatment. C: cytosolic fraction, M: mitochondrial fraction. β-actin was used as cytosolic marker and SDHA and TOM20 as mitochondrial markers.

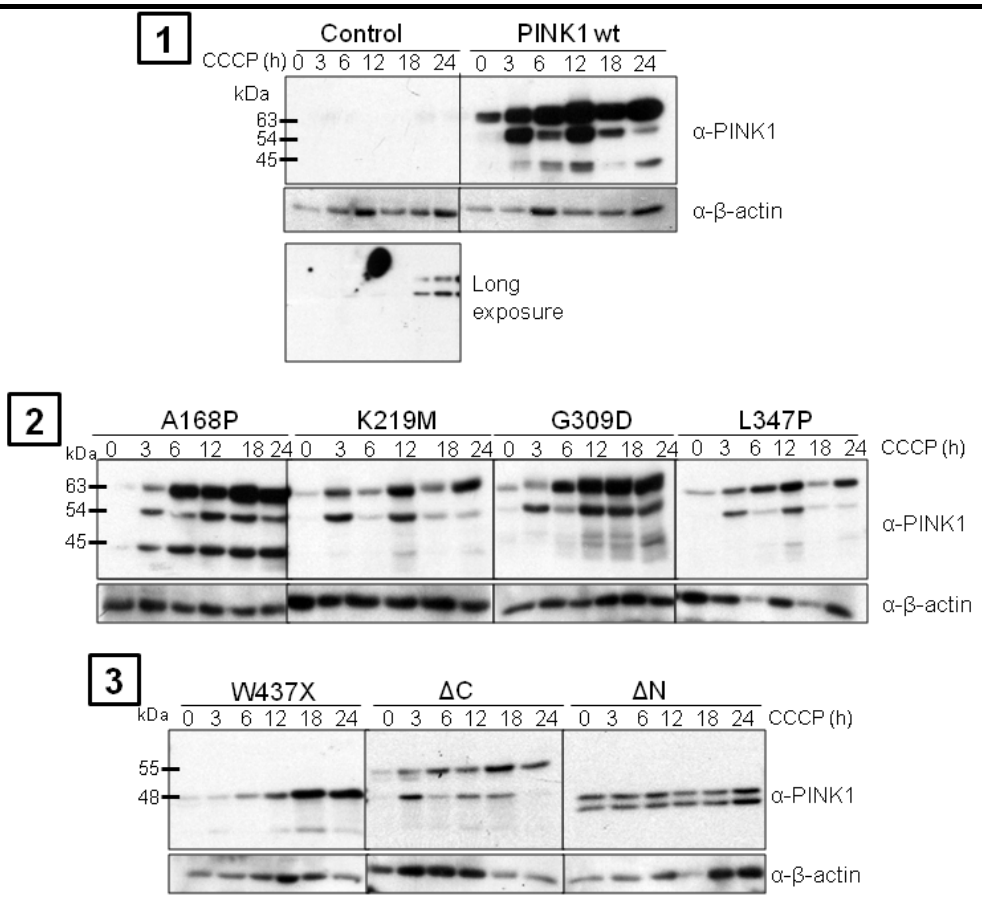

Figure 4.11: PINK1 mutations and truncations displayed defects in PINK1 protein accumulation after CCCP treatment. In PINK1 wt over-expressing cells **(1)**, PINK1 protein is accumulated at short term and over the time of CCCP treatment. Protein accumulation in PINK1 missense **(2)** and C-terminal truncation **(3)** mutations is lower than in PINK1 wt. PINK1 accumulation does not occur in PINK1 ΔN over-expressing cells. PINK1 protein expression levels were detected by immunoblot using anti-PINK1 BC100 antibody. β-actin was used as a loading control.

4.4.3 PINK1 accumulation or expression upon CCCP

It has been reported that PINK1 is accumulated upon CCCP and localised to the mitochondria [Narendra et al., 2010]. To know how the accumulation occurs in the PINK1 over-expressing cells, we performed immunoblot analysis upon a time course of CCCP treatment. Upon long term of CCCP treatment, endogenous PINK1 FL is accumulated in parental SH-SY5Y cells, suggesting a stabilization and/or new synthesis of the protein (**Figure 4.11**). In PINK1 wt over-expressing cells, the levels of PINK1 increased after 3 h of CCCP incubation and accumulated during time of CCCP treatment, suggesting stabilization of the protein. The accumulation in the cells with PINK1 mutations K219M, A168P, G309D and L347P showed a similar trend, but it was lower compared to PINK1 wt cells, whereas the cells with truncated mutations did not show a clear accumulation of PINK1 over the time with CCCP. In the case of PINK1 W437X and ΔC cells, the protein accumulation at short time was disrupted, but at long times of CCCP treatment the accumulation was observed. Remarkably, PINK1 protein levels, in PINK1 ΔN cells did not change (**Figure 4.11**) upon CCCP treatment.

In conclusion, PINK1 wt, but not mutated or truncated PINK1, is accumulated in the mitochondria upon CCCP treatment, with a concomitant increase in autophagosome formation and mitophagy, and decrease in mitochondria mass.

Chapter

5 Results II: PINK1 function in MCF-7 cells

Chapter Outline

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5.1 PINK1 inducible over-expressing MCF-7 cells

MCF-7 breast cancer cell line is an epithelial-like estrogen receptor α (ERα) positive cell line. Stable MCF-7 cells were generated to over-express, upon induction with doxycycline (Dox), PINK1 wt, and different PINK1 missense or truncated mutations (**Chapter 3, Figure 3.6 and Table 3.5**).

5.1.1 PINK1 expression and cell viability

In all MCF-7 stable cell lines, PINK1 mRNA and protein were efficiently induced upon Dox treatment (**Figure 5.1-1 and 5.1-2**). PINK1 protein expression was analysed with two different polyclonal antibodies: anti-PINK1 108-200 and anti-PINK1 BC100 (**Figure 5.1-2.A-B**). PINK1 protein levels were comparable in the different cell lines. The PINK1 full length (FL, 63 kDa), and the PINK1-Δ1 cleaved form (54 kDa) were detected in the PINK1 inducible over-expressing cells. However, the PINK1-Δ2 cleaved form (45 kDa) was weakly detected or undetectable (only with anti-PINK1 108-200 antibody) in the PINK1 over-expressing cells (**Figure 5.1-2**). Leakage expression was very low in the absence of Dox but PINK1 antibodies in MCF-7 displayed several nonspecific bands, and perhaps the sensitivity is limited (**Figure 5.1-2**). Next, cell viability was tested to discard some deleterious effects of the induced PINK1 over-expression. As shown, the induction of PINK1 over-expression in MCF-7 cells did not cause significant changes in cell viability, and all cells displayed similar growth rate profiles (**Figure 5.1-3**).

5.1.2 PINK1 localisation

We have previously described that PINK1 is localised in mitochondrial and cytosolic compartments (**Figure 4.2**.)

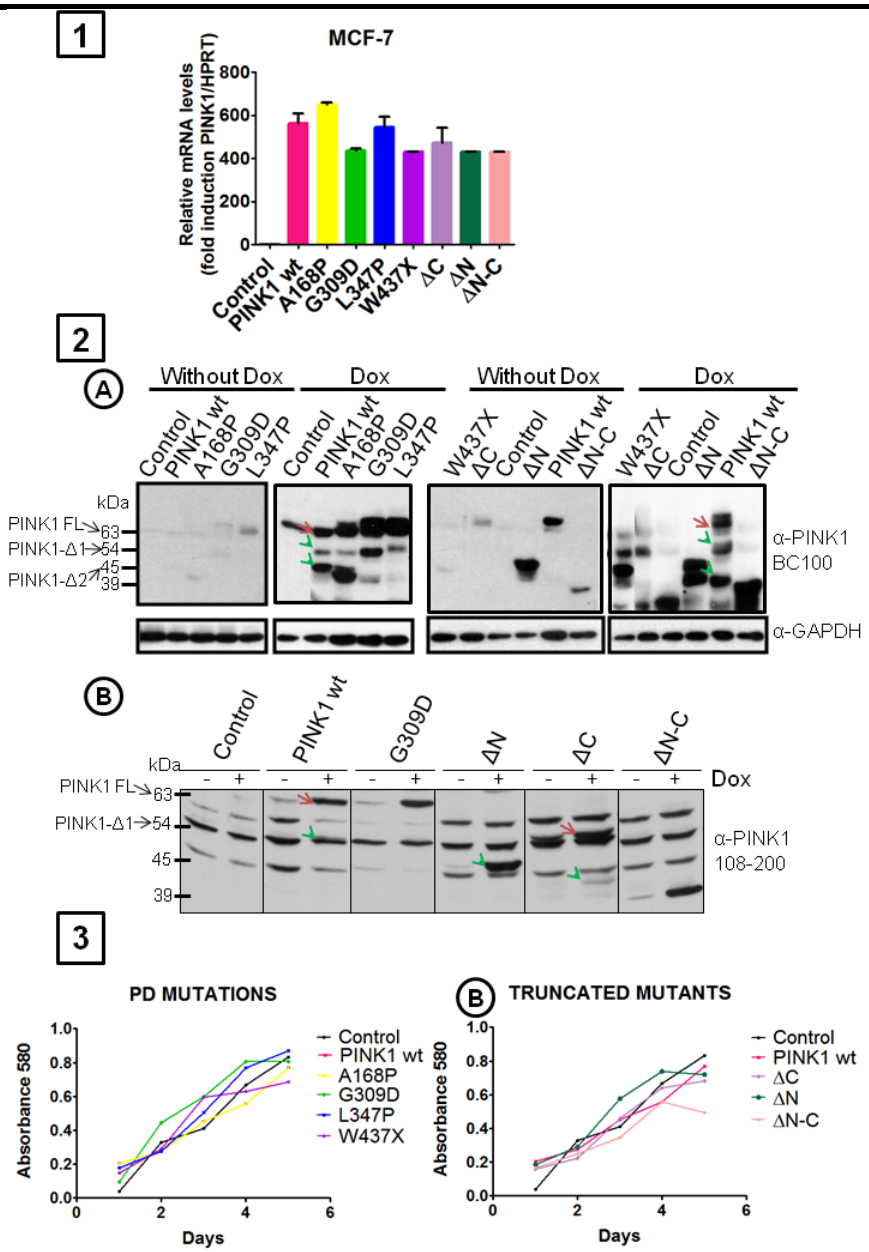

Figure 5.1: PINK1 expression levels and cell viability were similar in all PINK1 over-expressing MCF-7 cells generated. 1) qPCR analysis of PINK1 mRNA levels in the presence of Dox. Total RNA was subjected to reverse transcription. qPCR was performed and quantification was normalized to HPRT as a reference gene. **2)** PINK1 protein expression levels in the presence and absence of Dox, detected by immunoblot with anti-PINK1 polyclonal antibodies (anti-PINK1 BC100 **(A)** and a home-made anti-PINK1 108-200 **(B)**). GAPDH was used as a loading control. Red arrows indicate the full length unprocessed PINK1 protein. Green head arrows indicate the processed PINK1 proteins. **3)** Cell viability, measured by crystal violet method, of MCF-7 cells over-expressing missense **(A)** or truncated **(B)** PINK1 mutations, compared to control and PINK1 wt over-expressing cells in the presence of Dox.

To monitor PINK1 localisation in our MCF-7 inducible over-expression system, we analysed by immunofluorescence the localisation of PINK1, in comparison with the staining of the mitochondrial marker Mitotracker CMX-ROS (**Figure 5.2**). In control cells, the PINK1 signal (green) was very weak and nonspecific (**Figure 5.2 panel a**), likely due to low levels of endogenous PINK1 in MCF-7 cells (see **Figure 5.1-2**). Induction of PINK1, in PINK1 wt cells, increased the green specific staining compared to the signal of control cells, with a punctate pattern which co-localised with Mitotracker (**Figure 5.2 panel e**). The same punctate pattern and mitochondrial co-localisation of PINK1 protein were observed in the PINK1 A168P, G309D, L347P, W437X and ΔC cells (**Figure 5.2 panels i, m, q, u, A**). The PINK1 ΔN and PINK1 ΔN-C cells showed cytosolic localisation of PINK1 with a diffuse cytosolic pattern, and little or no clear mitochondria co-localisation (**Figure 5.2 panels E, I**).

The Mitotracker staining of the induced PINK1 over-expressing MCF-7 cells did not reveal differences in terms of mitochondrial content, network or shape (**Figure 5.2 Mitotracker panels**).

5.2 Cell death and apoptosis upon H_2O_2 treatment

It has been previously reported the anti-apoptotic role of PINK1 in several cell lines, but it is not known its role in breast cancer cells (see **Section 1.6.1**). Thus, we analysed the cell death response in the PINK1 over-expressing MCF-7 cells treated with hydrogen peroxide (H_2O_2) for 24 h. Upon H_2O_2 treatment, PINK1 wt cells displayed more protection to cell death than control cells, as monitored by phase contrast microscopy (**Figure 5.3**). On the other hand, PINK1 A168P, G309D, L347P and W437X cells showed less protection to H_2O_2-induced cell death, whereas the truncated forms PINK1 ΔC, ΔN and ΔN-C exhibited partial protection (**Figure 5.3**).

To study in more detail PINK1 anti-apoptotic function in MCF-7 cells, we performed FACS analysis of cell death and mitochondrial membrane potential ($\Delta\Psi$m). PI (propidium iodide) nuclear staining was used as a measurement of cell death. As shown in **Figure 5.4-1**, PINK1 A168P, G309D, L347P and W437X cells exhibited increased permeability to PI upon H_2O_2 treatment, compared to PINK1 wt cells. PINK1 wt cells showed a trend of cell death protection, although this effect was not statistically significant. The truncated forms PINK1 ΔC, ΔN and PINK1 ΔN-C cells showed no significant differences with respect to PINK1 wt.

Next, the cell-permeant probe $DiOC_6(3)$ was used to monitor the $\Delta\Psi_m$ (**Figure 5.4-2**). PINK1 wt and ΔN cells displayed significant protection against H_2O_2-induced mitochondrial depolarization. This protection was not observed in PINK1 A168P, G309D L347P, W437X, ΔC and ΔN-C over-expressing cells. All the PINK1 over-expressing cells showed, under untreated conditions, comparable percentage of cells permeable to PI and with depolarized mitochondria, suggesting that PINK1 mutations do not inducted basal cell death.

Together, these results indicate that the N-terminus of PINK1 is not required for the mitochondrial anti-apoptotic role of PINK1, whereas the intact PINK1 kinase domain and C-terminus are relevant for this PINK1 anti-apoptotic function.

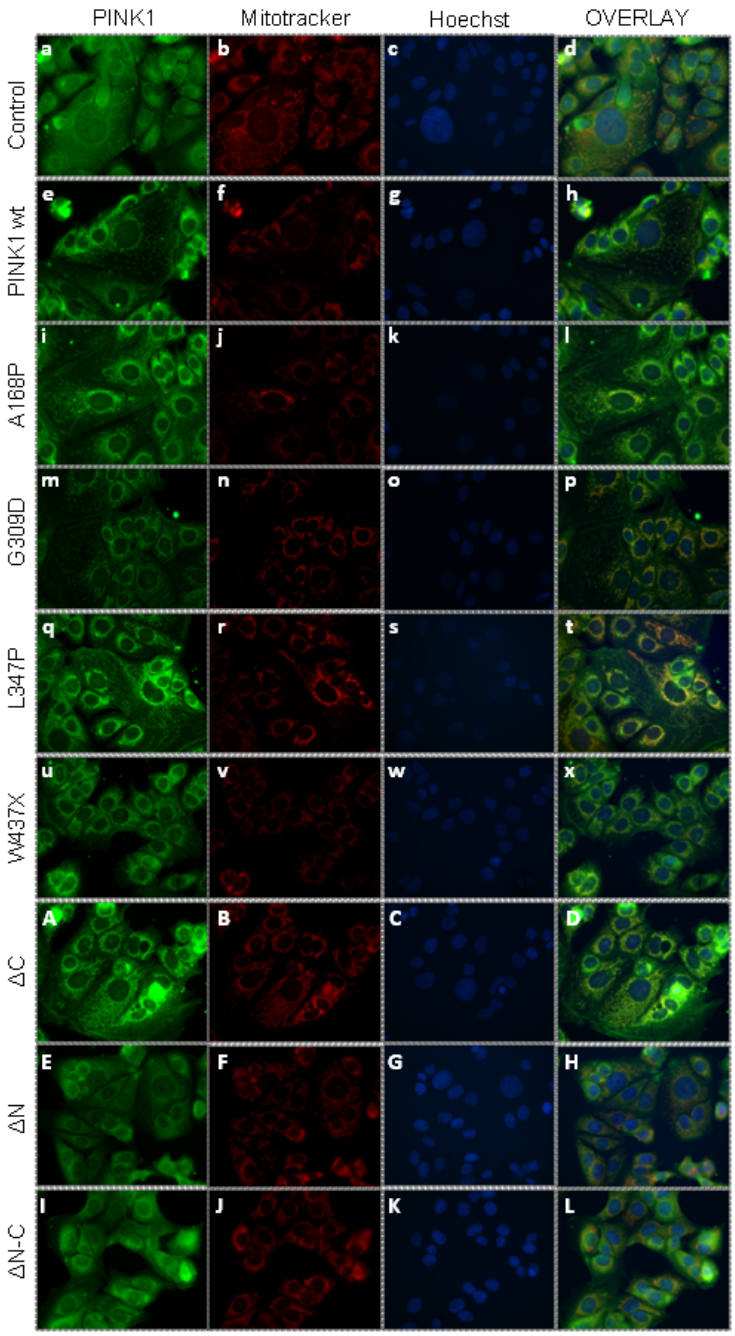

Figure 5.2: PINK1 wt, PD-linked mutations and C-terminal truncations are mostly localized at the mitochondria. The N-terminal truncations are mostly localised in cytosol. Control (a-d), PINK1 wt (e-h), A168P (i-l), G309D (m-p), L347P (q-t), W437X (u-x), ΔC (A-D), ΔN (E-H) and ΔN-C over-expressing MCF-7 cells were studied. Immunofluorescence was carried out with anti-PINK1 108-200 polyclonal antibody [green] (a, e, i, m, q, u, A, E, I), Mitotracker CMX ROS [red] (b, f, j, n, r, v, B, F, J) and Hoescht 33258 [blue] (c, g, k, o, s, w, C, G, K). In panels d, h, l, p, t, x, D, H and L, the overlay of the three colours is shown.

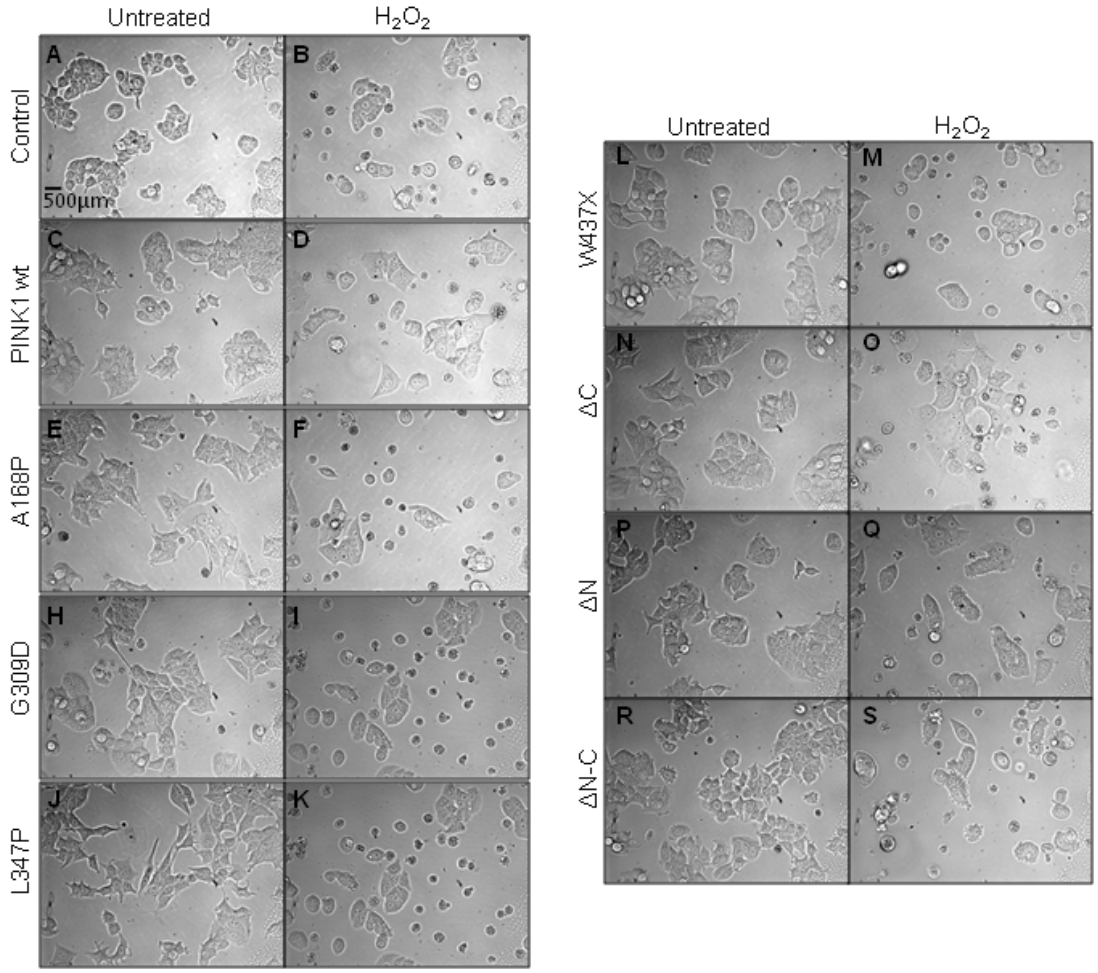

Figure 5.3: Cell death sensitivity of PINK1 over-expressing MCF-7 cells upon H_2O_2 treatment. Morphology studies of cells untreated (A, C, E, H, J, L, N, P, R) or treated with 0.15 mM H_2O_2 for 24 h (B, D, F, I, K, M, O, Q, S). H_2O_2-treated PINK1 wt over-expressing cells (D), but not the different PINK1 mutations over-expressing cells (A168P (F), G309D (I), L347P (K), and W437X (M)) are more resistant to H_2O_2-induced apoptosis. The truncated PINK1 forms (ΔC (O), ΔN (Q), and ΔN-C (S)) show a trend to resistance to apoptosis. Cells were photographed after 24 h in culture in the presence of H_2O_2 and Dox.

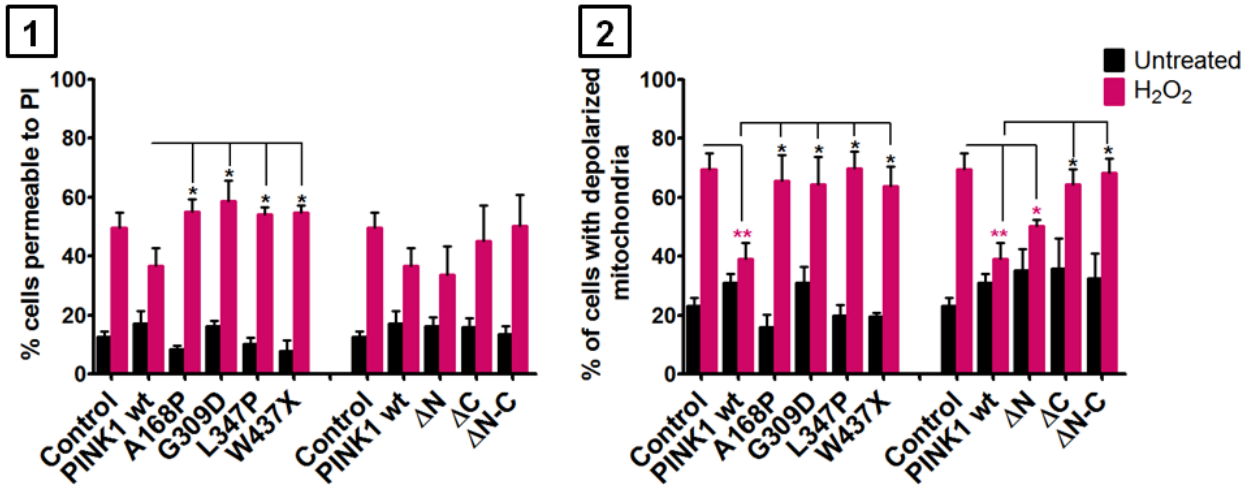

Figure 5.4: PINK1 wt and ΔN, but not the PD-linked mutations (PINK1 A168P, G309D and L347P) nor PINK1 ΔC and ΔN-C, protect against H_2O_2-induced apoptosis in PINK1 over-expressing MCF-7 cells. Apoptosis and cell death were measured by FACS analysis of cells untreated (black) or treated with 0.15 mM H_2O_2 (pink) for 24 h. Percentage of cells that present permeability to PI **(1)** and of cells with depolarized mitochondria membrane **(2)** are shown. (Statistical significance: **p<0.01 PINK1 wt and *p<0.05 PINK1 ΔN, versus control cells; and *p<0.05 PINK1 mutations and PINK1 C-terminal truncations, versus PINK1 wt.

To confirm the anti-apoptotic role of PINK1 in breast cancer cells, we silenced PINK1 in two breast cancer cell lines, MCF-7 and MB-MDA-231. Quantification of the decrease in PINK1 mRNA after transfection with validated siPINK1 (siGAPDH as a control) was performed by qPCR analysis. As shown in **Figure 5.5-1**, almost 50% reduction of PINK1 mRNA (65% reduction of GAPDH mRNA) was observed in both MCF-7 and MB-MDA-231 cells upon PINK1 silencing. The analysis of cell growth in these cells revealed no inhibitory effect, compared to control or siGAPDH cells (data not shown).

The percentage of cells permeable to PI was higher in siPINK1 MCF-7 and MB-MDA-231 cells after H_2O_2 treatment, compared to control cells (**Figure 5.5-2A**). Also, PINK1 silencing in both breast cancer cell lines increased mitochondrial depolarization upon H_2O_2 treatment (**Figure 5.5-2B**).

In conclusion, we have demonstrated that PINK1 wt and ΔN, but not other mutations or C-terminal truncations, decrease the cell death triggered by H_2O_2 in breast cancer cells.

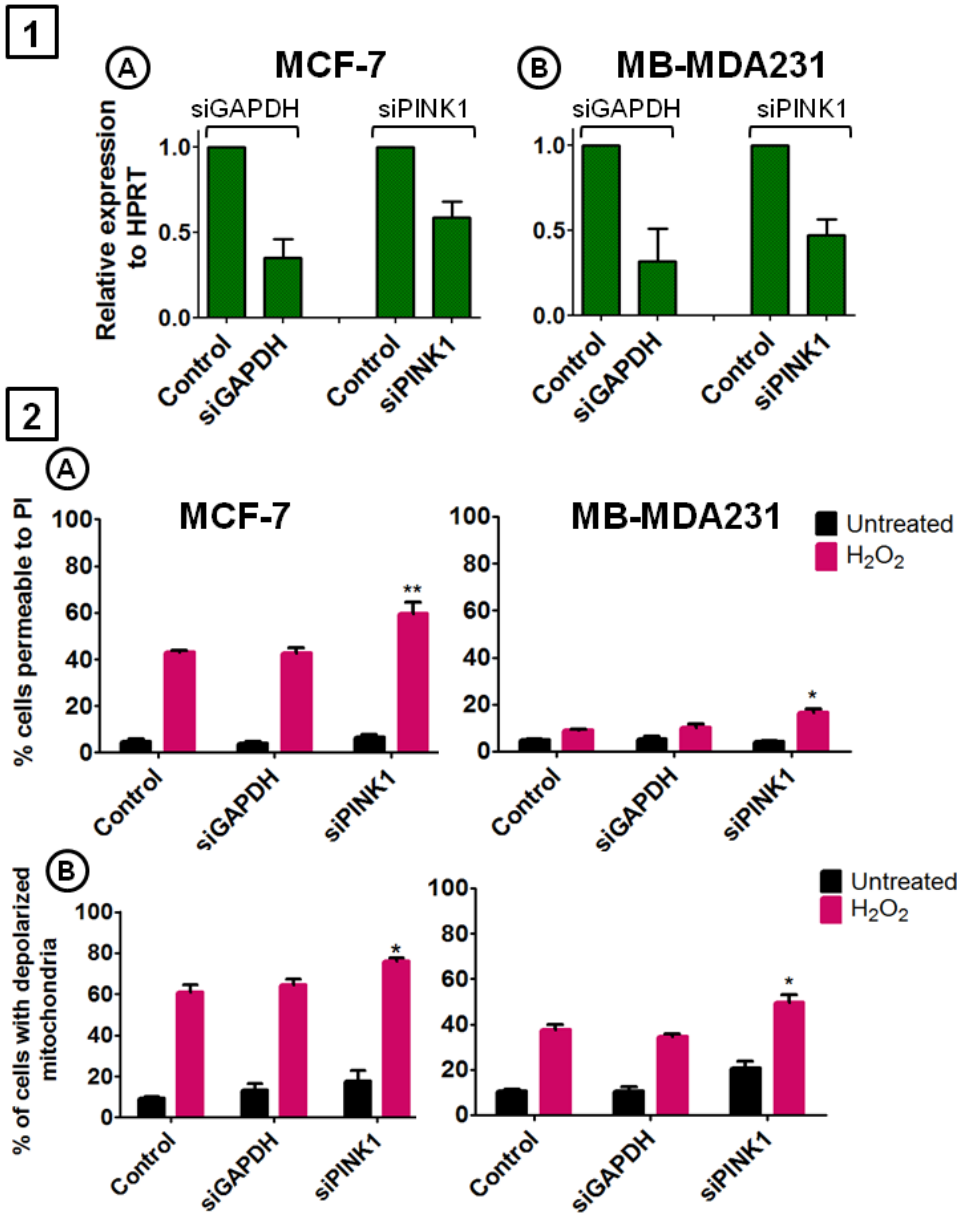

Figure 5.5: Apoptotic sensitivity of PINK1-silenced MCF-7 and MB-MDA-231 breast cancer cells. 1) qPCR analysis of PINK1-silenced MCF-7 **(A)** and MB-MDA-231 **(B)** cells. Total RNA was subjected to reverse transcription. qPCR was performed and quantification was normalized to HPRT as a reference gene. Silencing efficiency of PINK1-siRNA was approximately 50% at mRNA level. **2)** FACS analysis of cells untreated (black) or treated with 0.15 mM H_2O_2 (pink) for 24 h. Percentage of cells that present permeability to PI **(A)** and of cells with depolarized mitochondria membrane **(B)** in PINK1-silenced MCF-7 and MB-MDA-231 cells are shown. (Statistical significance: $^{**}p<0.01$ and $^{*}p<0.05$ versus control). PINK1-silenced MCF-7 cells are more sensitive than MB-MDA-231 to H_2O_2-induced apoptosis.

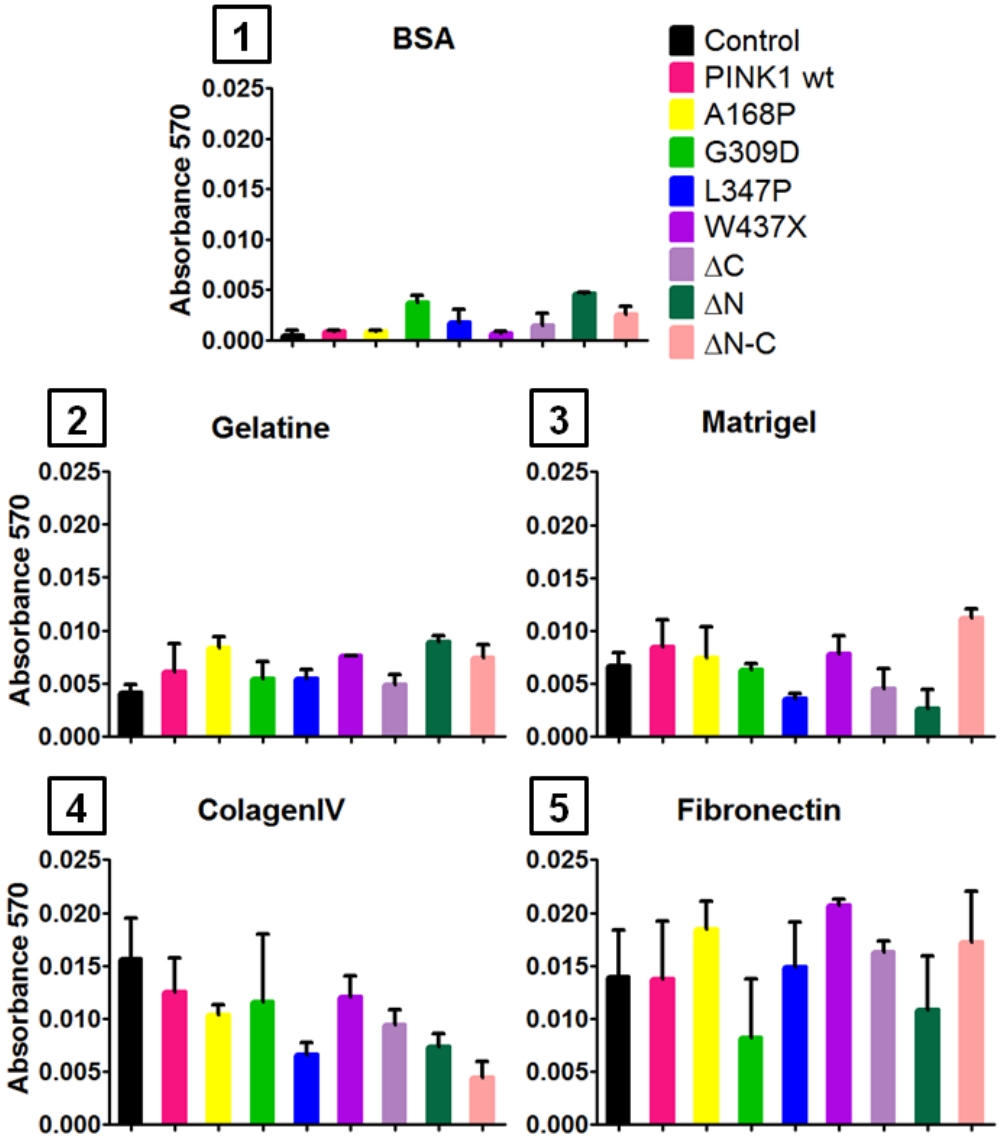

Figure 5.6: Adhesion of PINK1 over-expressing MCF-7 cells to different matrix substrates. After incubation for 24 h with Dox, cells were plated onto gelatine- **(2)**, matrigel- **(3)**, colagen IV- **(4)** or fibronectin- **(5)** coated wells and incubated for 4 h, allowing cell adhesion. Adherent cells were fixed and stained with crystal violet. BSA was used as control of adhesion **(1)**. Cell adhesion was quantified by absorbance measuring. Results represent the mean±SEM of four independent experiments.

5.3 Adhesion capacity onto different substrates

Previous analysis of PINK1 expression in human tumours suggests a role for PINK1 in cancer (**Section 1.7.2**). Thus, we examined the role of PINK1 in adhesion, tumourigenicity and proliferation properties of PINK1 over-expressing MCF-7 breast cancer cells.

To measure the adhesion capacity of PINK1 over-expressing cell lines, cells were plated onto gelatine, matrigel, fibronectin or colagen IV-coated wells, and the attachment was allowed for four hours. BSA-coated wells were used as control. As shown in **Figure 5.6** no significant differences were observed in the attachment of PINK1 over-expressing cells to the distinct substrates, in comparison with control cells. In gelatine and matrigel-coated wells, cell attachment was lower than in fibronectin and colagen IV-coated wells (**Figure 5.6**).

These results suggest that PINK1 does not affect the adhesion capacities of MCF-7 breast cancer cells.

5.4 Wound healing invasion

Next, wound healing invasion assays were performed. Confluent cells were scratched and invasion of the cell-free area was measured. PINK1 wt cells migrated less into the scratch area after 24 h of culture, compared to control cells, suggesting less invasive capacity. However, PINK1 A168P, G309D and L347P cells occupied more of the free scratch area after 24 h of growing, suggesting more invasive capacity than PINK1 wt cells. In PINK1 C-terminal (W437X and ΔC) and N-terminal (ΔN and ΔN-C) truncation cells, no differences in invasion were observed with respect to control cells. In the absence of Dox, all cell lines displayed similar invasion capacity (**Figure 5.7**).

These results suggest a negative role for PINK1 in the control of invasion processes.

5.5 Anchorage-independent growth and colony formation in soft agar

To further study the role of PINK1 in tumourigenesis, we performed anchorage-independent growth assays with the PINK1 over-expressing MCF-7 cell lines. Cells were plated in soft agar with or without Dox, and colony formation was monitored after two weeks. MCF-7 control cells formed round and numerous large colonies after 2 weeks of growth in soft agar, and were unaffected in the presence of Dox. In contrast, PINK1 wt cells (**Figure 5.8**) exhibited a reduced capacity for soft agar colony formation in the presence of Dox, with lower number of colonies and reduced colony size. The PINK1 A168P, G309D, and W437X over-expressing cells, and the PINK1 ΔC and ΔN-C over-expressing cells formed similar number of colonies but larger than control cells. The PINK1 L347P and ΔN over-expressing cells grew in soft agar similarly to PINK1 wt.

These results indicate that PINK1 attenuates anchorage-independent growth in MCF-7 cells, and suggest that PINK1 has a role in proliferation and tumour formation *in vitro*.

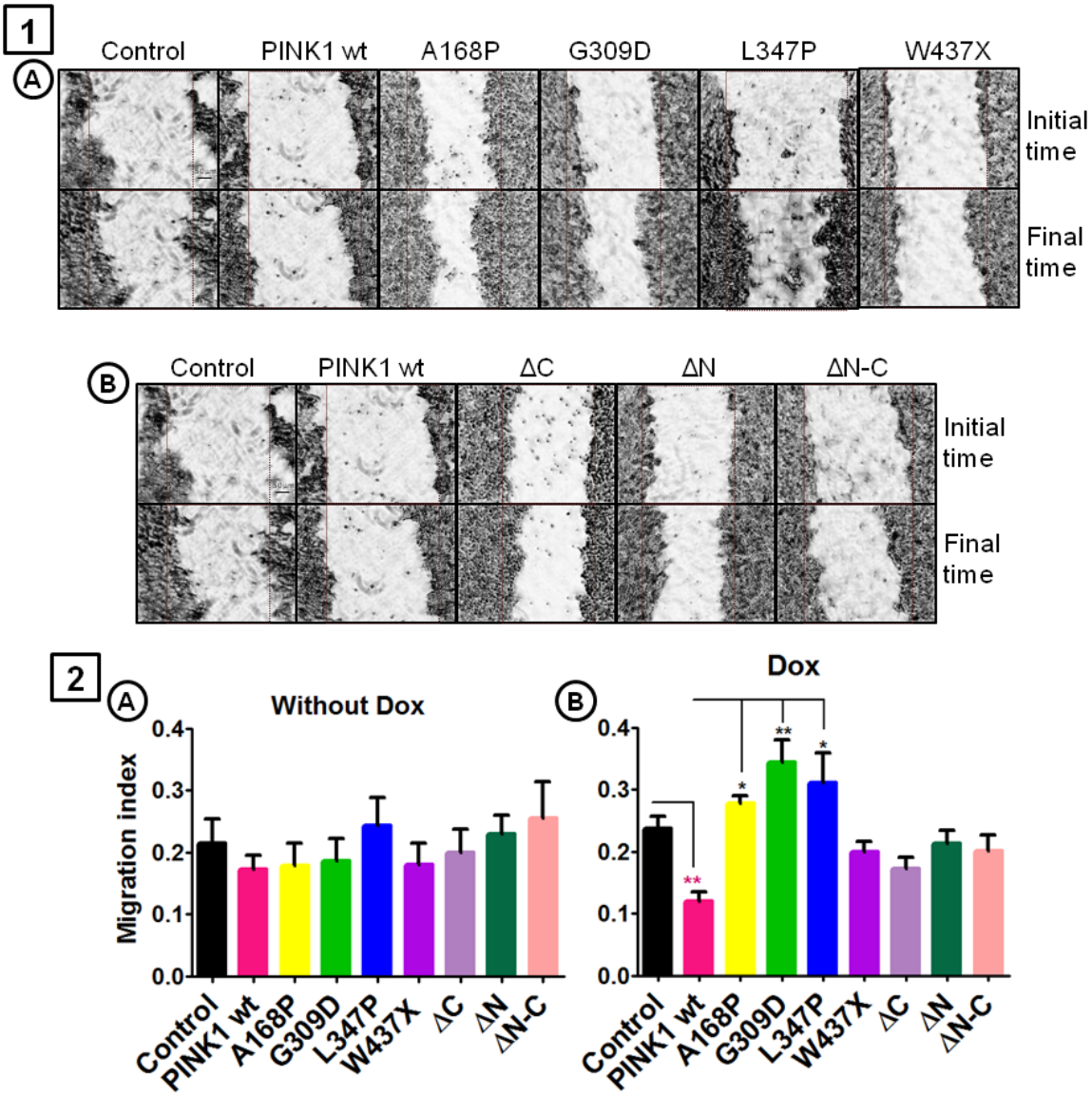

Figure 5.7: PINK1 wt, but not PINK1 missense mutations, decreases invasive capacity of PINK1 MCF-7 cells. 1) Confluent PINK1 over-expressing cells were scratched and cell invasion over the cell-free area was photographed every 30 min for 24 h. Initial and final photographs are displayed in **(A)** and **(B)**. **2)** Quantification of invasion assay was measured over the free area before and after 24 h of culture after scratching. Results represent the mean±SEM of the migration index. (Statistical significance: **(pink)p<0.01 versus control; *<p0.05, **<0.01 versus PINK1 wt)

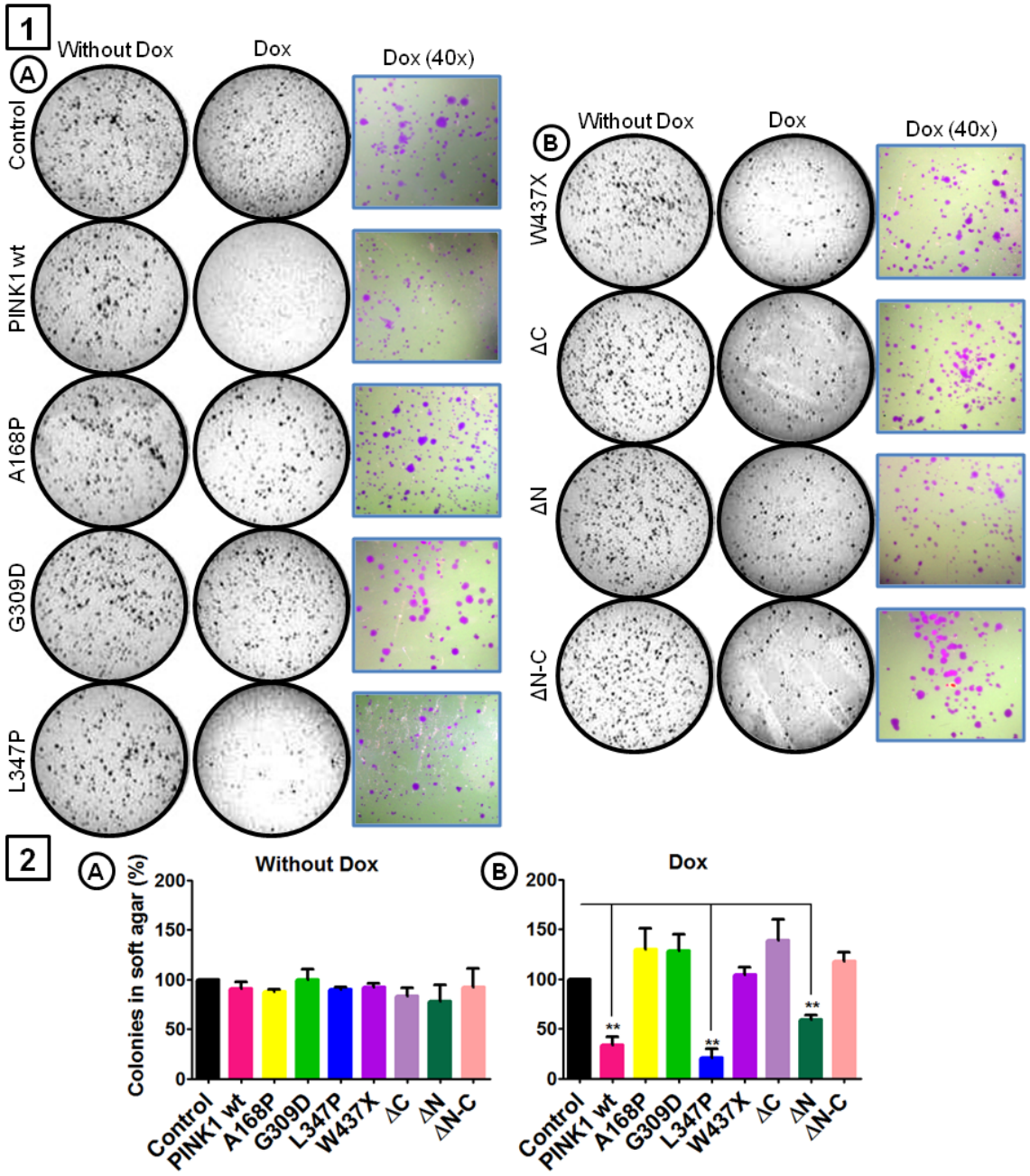

Figure 5.8: PINK1 modulates the anchorage-independent growth in soft agar of MCF-7 cells. PINK1 wt, ΔN and L347P, but not the other missense mutations or truncations, inhibit colony formation in soft agar. Cells were grown for 2 weeks in soft agar allowing colony formation. Colonies were stained with crystal violet and were photographed. **1)** Photographs of PINK1 MCF-7 cells without or with Dox, 5x and 40x. **2)** Quantification of the colonies grown in soft agar of PINK1 MCF-7 cells without or with Dox, using by Image J. (Statistical significance: *p<0.05 **p<0.01 versus control).

5.6 Cell cycle progression, study of cell cycle proteins, and proliferation.

Finally, we analysed the cell cycle and proliferation properties of PINK1 wt and G309D over-expressing MCF-7 cells.

Cells were synchronized at G_0/G_1 by 24 h growth in the absence of serum (FBS), and cell cycle re-entry and proliferation were restored by addition of serum to the medium. Samples were harvested at different times and analysed by FACS. Control, PINK1 wt and G309D over-expressing MCF-7 cells were equally arrested in G_0/G_1 upon withdrawal of serum. Interestingly, the S phase population was higher in PINK1 wt cells at 12 and 24 h than in control cells, but at 48 h the G_0/G_1 population was recovered. The cells carrying PINK1 G309D mutation showed the same profile as PINK1 wt cells at 12 h, with higher S phase population, but at longer times, the G_0/G_1 population increased faster than in control cells (**Figure 5.9**).

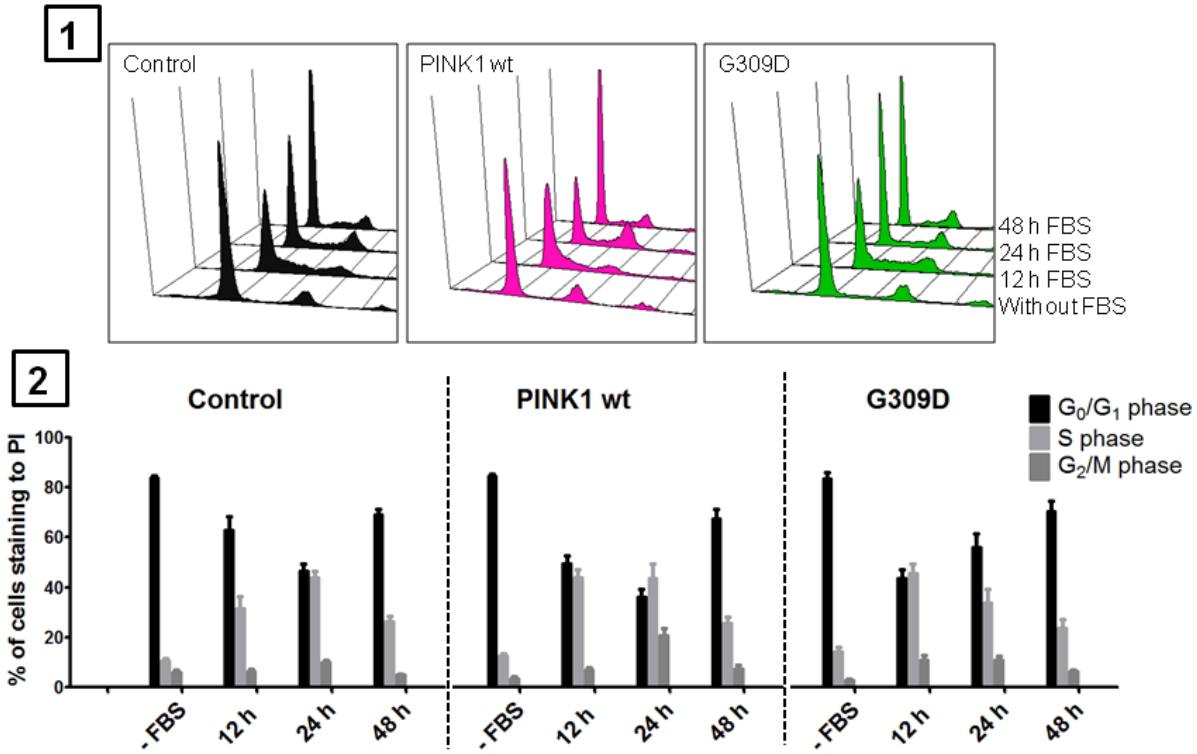

Figure 5.9: PINK1 alters the cell cycle progression of MCF-7 cells. PINK1 wt, but not G309D, blocks cell cycle in S phase and decreases the progression through the cell cycle in MCF-7 cells. Equal number of control, PINK1 wt and G309D over-expressing MCF-7 cells, were grown in the presence of Dox for 2 days, reaching 50% confluency. Then, cells were grown in FBS medium for 12 h, 24 h, or 48 h. **1)** FACS analysis of the cells was carried out by standard cell cycle protocol (fixation and PI staining). **2)** Percentage of cells in each cell cycle phase is shown in histograms.

Next, we checked by immunoblot the levels of several cell cycle proteins: cyclin D1 (G_0/G_1 phase), cyclin A (late S phase), cyclin B1 (G_2/M phase) and p27 (inhibitor and required progressor of G_0/G_1 phase). In the absence of serum, the levels of cyclins are low in all the MCF-7 cells analysed, with the exception of cyclin D1 in PINK1 G309D over-expressing cells which was highly expressed. In control, PINK1 wt, and G309D over-expressing cells, the levels of p27 were higher in the absence of serum, likely due to cell cycle arrest. In the parental MCF-7 cell line, the cells re-entered into cell cycle with increased levels of cyclin D1 (at 12 and 24 h) and subsequently, higher levels in cyclin A and B1 (at 24 and 48 h, respectively). The PINK1 wt over-expressing cells re-entered into cell cycle with lower levels of D1, A and B1, compared to control cells. In PINK1 wt cells, the kinetics of cyclin A activation is shorter than in control cells, characterised by a slight increase at 12 h (as control cells) and a decrease afterwards. PINK1 G309D over-expressing cells presented higher levels of cyclin D1 at every time point compared to control and PINK1 wt cells, and cyclin A levels were increased earlier (12 h) than in control cells, and were higher than PINK1 wt cells. This suggests a more rapid progression of cell cycle in the PD-linked missense mutation G309D over-expressing MCF-7 cells. It is important to note that p27 protein is accumulated over the time in control cells because the cells reached the G_0/G_1 restriction point, whereas in PINK1 wt over-expressing cells the levels of p27 were low at long times, suggesting a delay in the progression of the cell cycle. The p27 levels in PINK1 G309D over-expressing cells were lower than in control cells, indicating cell cycle activation at later times of serum addition (**Figure 5.10**).

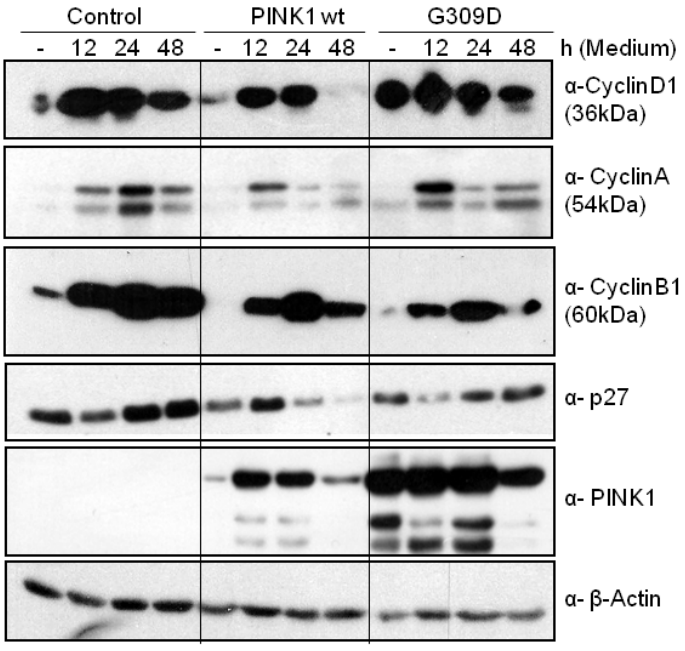

Figure 5.10: PINK1 modulates cyclin and p27 levels during the cell cycle progression of MCF-7 cells. PINK1 wt cells, but not G309D cells, show decrease cyclin D1, cyclin A and p27 levels. Protein expression analysis was made by immunoblot using anti-cyclin D1 (phase G_0/G_1 cyclin), anti-cyclin A (phase late S cyclin), and anti-cyclin B1 (phase G_2/M cyclin), anti-p27 (inhibitor of G_0/G_1 progression), or anti-PINK1 BC100 antibodies. β-actin was used as a loading control.

Finally, we measured, by EdU (5-ethynyl-2'-deoxyuridine, an analog to BrdU) assay, the proliferation rate of the PINK1 over-expressing MCF-7 cells. Cells were synchronized as above and incubated in the presence of serum and EdU for 18 h, followed by FACS analysis. PINK1 wt, but not PINK1 G309D, over-expressing MCF-7 cells, displayed 20% less of total proliferating cells. Moreover, we detected in control cells an EdU positive population in G_0/G_1 phase which corresponds to EdU incorporation (S phase, proliferating cells), progression to G_2/M phase and captured in G_0/G_1 phase (green circle). This population is smaller in the case of PINK wt over-expressing cells but not in G309D cells (**Figure 5.11**). This suggests that PINK1 wt over-expressing MCF-7 cells proliferate slower and the PD-linked missense mutation G309D over-expressing MCF-7 cells proliferate faster, than control cells.

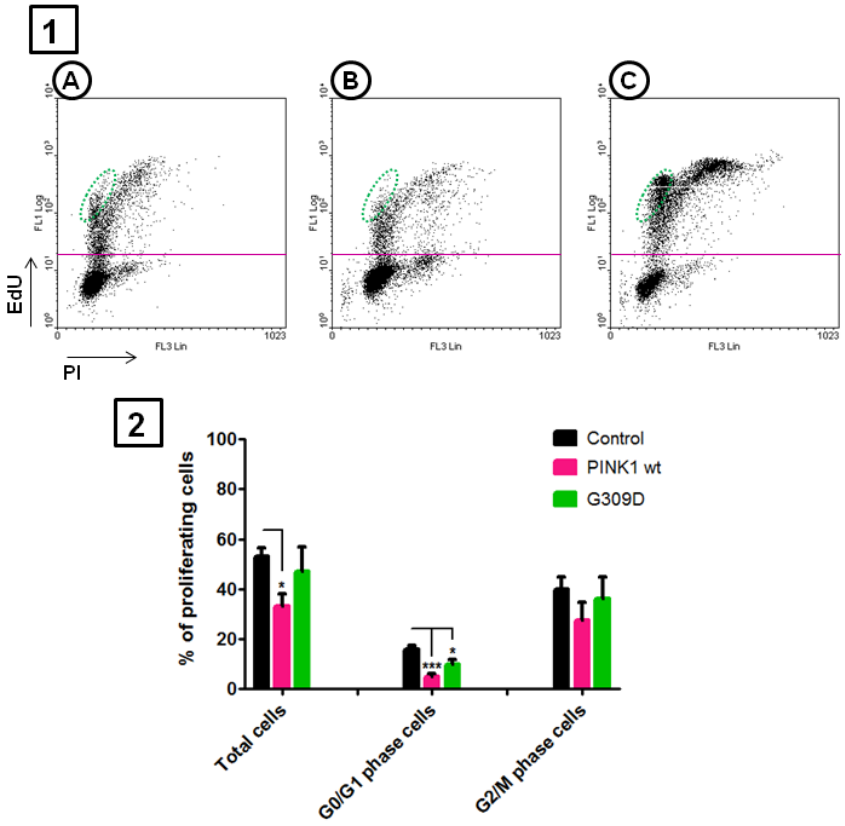

Figure 5.11: PINK1 alters proliferation of MCF-7 cells. PINK1 wt, but not G309D over-expressing cells, showed lower percentage of total proliferating cells and a decrease in G_0/G_1 phase proliferating cells. Equal number of PINK1 over-expressing MCF-7 cells were grown in the presence of Dox, and FBS was removed for 24 h to synchronize cells in G_0/G_1, followed by 18 h incubation in medium with FBS and EdU compound (5-ethynyl-2'-deoxyuridine, incorporates in S phase: proliferation measurement). **1)** FACS analysis of EdU and PI in control **(A)**, PINK1 wt **(B)** and G309D **(C)** cells was carried out. **2)** Percentage of total proliferating cells (cells above the pink line), G_0/G_1 proliferating cells (green circle), and G_2/M phase proliferating cells (the rest of proliferating cells) are shown in the histogram. (Statistical significance: ***$p<0.001$ *versus* control; *$p<0.05$ *versus* PINK1 wt).

Chapter

6 Results III: Expression of PINK1 and PINK1-related genes in normal and neoplastic tissues

Chapter Outline

6.1 PINK1 expression in normal and neoplastic tissues **83**

6.2 Parkinson's disease linked genes in normal and neoplastic breast cancer **86**

6.1 PINK1 expression in normal and neoplastic tissues

PINK1 mRNA is expressed in a wide variety of human tissues (**Figure 6.1-1**), in agreement with the northern blot analyses from mouse tissues performed by Nakajima et al. [Nakajima et al., 2003]. Analysis of PINK1 mRNA by qPCR revealed high expression in brain and breast tissues, while thyroid tissue showed low expression. We also tested the expression of PINK1 mRNA in the cell lines used in this study and found that SH-SY5Y cells displayed higher expression levels than MCF-7 cells, in acccordance with the endogenous PINK1 protein levels found in these cell lines (**Figure 6.1-2**) [Berthier et al., 2011].

PINK1 protein analysis was performed by immunoblot with the anti-PINK1 89B mAb on several human and mouse tissue extracts (**Figure 6.1-3**). In mouse brain sample, the 89B mAb recognized a 55 kDa band that co-migrated with the mitochondrial-processed form of PINK1. Human liver, a mitochondria-enriched tissue, revealed a noticeable band that migrated at the molecular weight predicted for the processed PINK1 form, whereas in human bladder a weak immunoreactive band was detected, likely representing low levels of PINK1 expression in this tissue. Finally, in mice tissue samples, the 89B mAb recognized a 55 kDa band, corresponding to processed PINK1, in testis and heart, but not in colon (data not shown). Thus, the 89B antibody recognises both human and mouse PINK1, which is in accordance with the high degree of amino acid sequence identity of PINK1 protein (82%) between these two species.

In summary, our biochemical results reveal that the 89B mAb is specific for PINK1 protein, and indicate a wide tissue distribution pattern for PINK1, not restricted to the brain.

Next, immunohistochemical analysis of PINK1 was performed with the anti-PINK1 89B mAb in a multi-tissue array containing a wide representation of human tissues. A summary of 89B immunostaining of human tissue sections is shown in Berthier et al. [Berthier et al., 2011]. In the brain, cortical neurons were highly positive for 89B immunoreactivity. Conversely, glial cells and white matter were negative. Ventricular epithelium was strongly stained by the 89B mAb. In the cerebellum, only Purkinje cells displayed staining (**Figure 6.2**). These findings are in agreement with PINK1 mRNA expression pattern in the brain reported by Blackinton et al., but show differences with that reported by Gandhi et al.

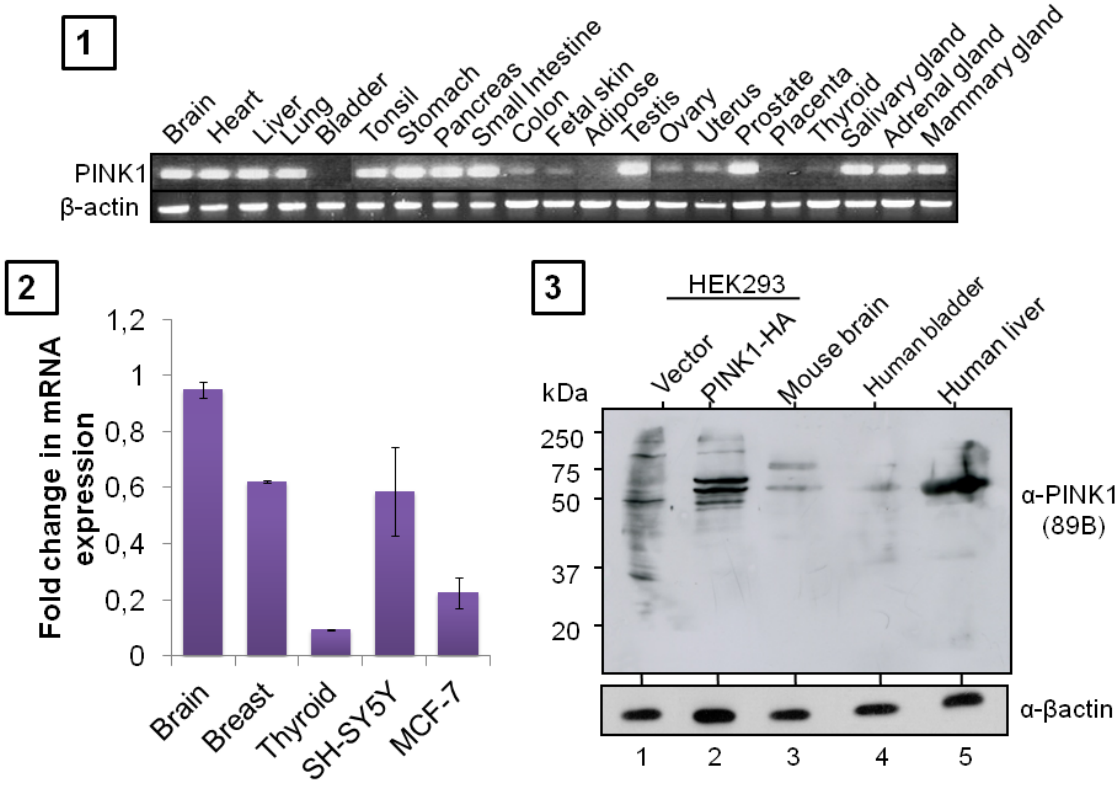

Figure 6.1: PINK1 mRNA and protein expression in human and mouse tissues. 1) PINK1 mRNA expresion is widely expressed in human tissues. cDNA samples from different normal human tissues were subjected to PCR using PINK1 and β-actin primers. Amplified bands were visualised in agarose gels. **2)** qPCR analysis of PINK1 expression from total RNA from SH-SY5Y and MCF-7 cell lines, and from brain, breast and thyroid human tissues. HPRT was used as a reference gene to quantify the relative mRNA expression by comparative $\Delta\Delta$Ct method (mean$\pm$SD from two experiments is shown). **3)** PINK1 protein expression in human and mouse tissues. Lysates from transfected HEK293 cells (50 g) (lanes 1,2) or from tissue samples (150 g) (lanes 3-5) were subjected to SDS-PAGE, followed by immunoblot with the anti-PINK1 89B mAb or with the anti-β-actin antibody, as indicated.

using anti-PINK1 polyclonal antibodies, that showed PINK1 expression in all brain cell types, including glial cells [Blackinton et al., 2007; Gandhi et al., 2009]. This difference is likely due to the different nature and epitope specificity of the anti-PINK1 antibodies used in Gandhi's and in our study. Positive 89B reactivity was detected in epithelial tissues, including adrenal gland, mammary gland, prostate, pancreas, kidney tubules, stomach, intestine, and hypophysis (**Figure 6.2**) [Berthier et al., 2011].

Interestingly, the subcellular pattern of immunostaining with the 89B was variable, depending on the tissues. In general, three distinct patterns were observed:

- Cytoplasmic granular pattern in brain neurons, Purkinje cells, stomach and liver, suggesting mitochondrial localisation.

- Cytoplasmic diffuse pattern in cardiac and striated muscles, and lymph node.
- Membrane-associated pattern: acini of the mammary gland.

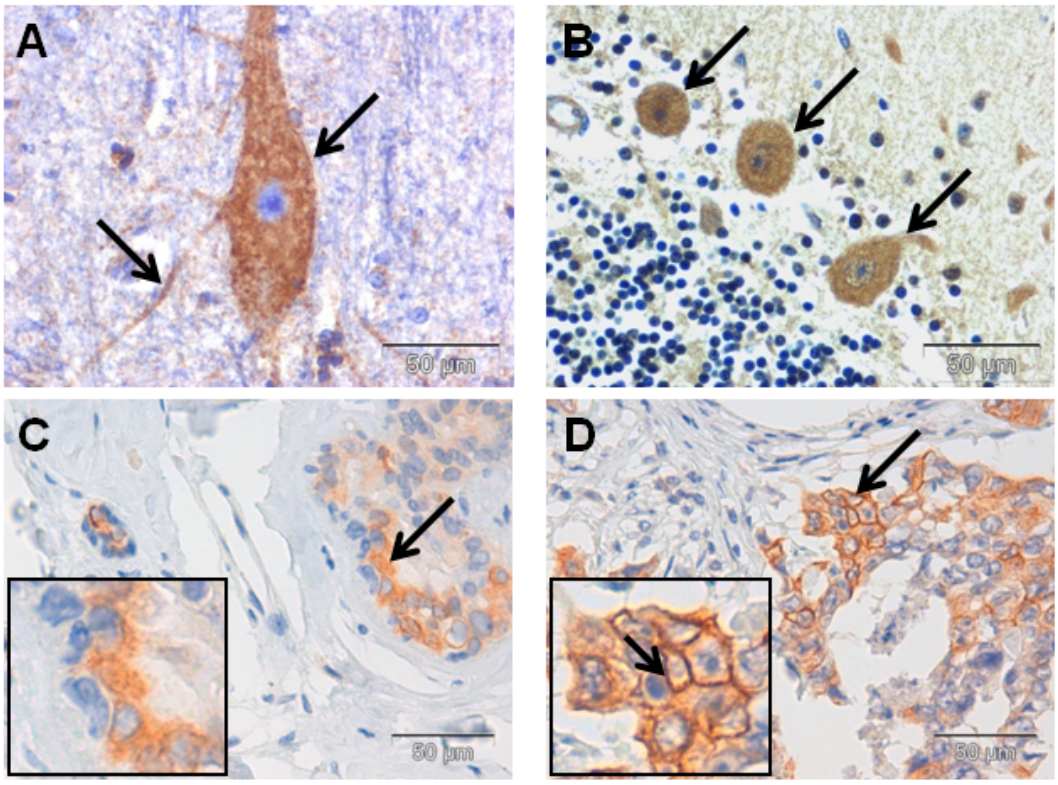

Figure 6.2: Immunoperoxidase staining with the anti-PINK1 89B mAb of human normal and neoplastic tissues. **(A)** Normal cortical brain. The arrows indicate stained neurons. **(B)** Normal cerebellum. The arrows indicate stained Purkinje cells. **(C)** Normal breast acini. The arrow indicates stained apical pole of secretory cells. **(D)** Breast carcinoma. The arrows indicate the membrane-like staining of cancer cells.

Analysis of human neoplastic tissues revealed that PINK1 expression was absent or very weak in blastomas and sarcomas but very high in carcinomas. Breast carcinomas displayed cytoplasmic diffuse staining, together with a prominent membrane staining, which differed from the granular cytoplasmic pattern and the low membrane staining observed in normal breast (**Figure 6.2**).

We further examined the expression of PINK1 mRNA in breast cancer. We analysed PINK1 mRNA expression in 43 breast cancer samples, and found that 60% of the breast tumours analysed displayed over-expression of PINK1 mRNA (**Figure 6.3**).

6.2 Parkinson's disease linked genes in normal and neoplastic breast cancer

We selected a group of genes related to PD and PINK1 (including the variants naPINK1 and svPINK1), and analysed the mRNA expression pattern in the breast tumour samples collection. We used String software to identify PINK1-related proteins (`http://string-db.org/`) [Von Mering et al., 2007], and we included in the analysis other PD genes whose expression is regulated in cancer (**Figure 1.2**). Finally, we added markers of proliferation and hormone growth factor receptors, which have been involved in breast cancer progression. A list of the selected genes is showed in **Table 6.1**.

Table 6.1: Expression of PINK1 and PINK1-related genes in breast cancer.

mRNA	PINK1-/PD-/cancer- link	Percentage of tumours (expression)*		
		Normal	Low	High
Htra2/omi	PARK13-binding partner	5	0	95
ATP13A2	PARK9	7	2	91
LRRK2	PARK8-associated partner	9	2	89
GBA	Associated protein	30	3	67
PINK1	PARK6	30	9	61
DJ1	PARK7-binding partner	40	2	58
TRAP1	Binding partner	49	2	49
MTP18	Associated protein	51	5	44
Parkin	PARK2-binding partner	53	21	26
UCHL1	Associated protein	74	12	14
SNCA	PARK1-4-associated partner	79	14	7
SNCAIP	Associated protein	39	56	5
PTEN	Associated protein	77	21	2
svPINK1	Variant	28	23	49
naPINK1	Variant	51	5	44
ER	Breast cancer related receptor	19	7	74
PGR	Breast cancer related receptor	39	28	33
HER2	Breast cancer related receptor	74	10	16
Ki67	Proliferation marker	40	9	51

*Expression is indicated in **Figure 6.3**

We detected high expression of LRRK2, DJ-1, GBA, ATP13A2, Htra2/omi, MTP18 and TRAP1 in many tumours, compared to normal breast tissue. Moreover, high percentage of tumours showed low expression of PTEN and SNCAIP. The PINK1 variants naPINK1 and svPINK1 were highly expressed in half of the tumours (**Table 6.1 and Figure 6.3**).

The high expression of PINK1 is associated with high expression of other PD-related genes in the same breast cancer samples. For example, high expression of PINK1 is associated in almost 50% of cases with high expression of LRRK2, DJ-1, GBA, ATP13A2, Htra2/omi, TRAP1 and MTP18. Indeed, in 30% of the breast cancer samples all these genes are associated, suggesting a common regulation of their expression linked to breast cancer. On the other hand, high or low expression of Parkin or PTEN were not associated to high or low expression of PINK1 (**Table 6.2**). Each of PINK1 variant is associated with PINK1 expression in 30% of the breast cancer samples, and the two variants are

associated in 40% of the samples, suggesting a positive regulation of PINK1 function and its variants in breast cancer.

In conclusion, PINK1 is up-regulated in many breast cancer tumours, and expression of PINK1 and PINK1-related genes could be linked to progression of breast cancer. Further analyses need to be done to correlate the expression of PINK1-related genes with breast cancer prognosis.

Table 6.2: Association of expression of PINK1 and PINK1-related genes in breast cancer tissues.

Associated mRNAs	**Percentage of tumours with the same expression**	
	Low	**High**
1. PINK1-GBA	2	65
2. PINK1-Htra2/omi	2	60
3. PINK1-LRRK2	2	58
4. PINK1-ATP13A2	2	58
5. PINK1-DJ-1	2	49
6. PINK1-TRAP1	2	44
7. PINK1-MPT18	5	33
8. PINK1-GBA-Htra2-LRRK2-ATP13A-DJ1-TRAP1-MPT18	2	30
9. PINK1-Parkin	5	23
10. PINK1-PTEN	2	2
11. PINK1-naPINK1	5	33
12. PINK1-svPINK1	2	30
13. naPINK1-svPINK1	5	44
14. PINK1-naPINK1-svPINK1	2	28

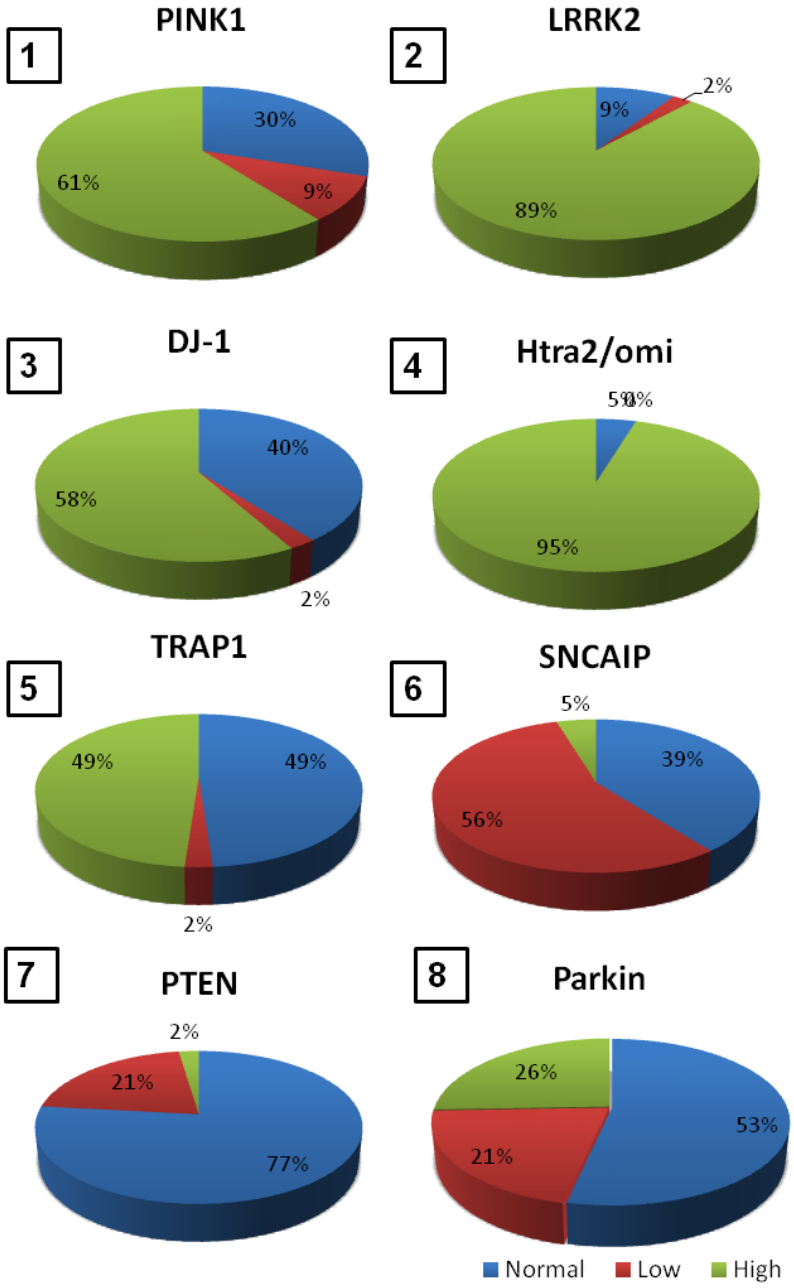

Figure 6.3: Expression of PINK1 and PD-related genes in breast cancer samples. qPCR analysis of PINK1 **(1)**, LRRK2 **(2)**, DJ-1 **(3)**, Htra2/omi **(4)**, TRAP1 **(5)**, SNCAIP **(6)**, PTEN **(7)** and Parkin **(8)** from 43 breast cancer samples, normalised to expression in normal breast and HPRT as a reference gene. Fold change value ($\Delta\Delta$Ct) was calculated and high expression was defined as $\geq$2, low expression as $\leq$0.25, and normal expression between 0.25 and 2. The percentage of each class is shown.

Summary Results

The results obtained with PINK1 wt or mutations in SH-SY5Y cells are summarized in the **Table 6.3**, according to four functions: PINK1 localisation, control of mitochondrial function, oxidative stress production and regulation of CCCP-inducted mitophagy. In the case of the mitochondrial function analysis, ATP production, $\Delta\Psi$m value and mitochondrial network is illustrated. Oxidative stress production is shown as ROS production under basal and rotenone-treated conditions, glutathione levels (MCB), carbonylation of proteins (protein damage) and mitochondrial DNA content. In the case of mitophagy function, we compile increase in LC3-II and decrease in damaged mitochondria, PINK1 protein levels and localisation in a time course of CCCP treatment. The symbols shown are with respect to control cells.

The summary of the results from PINK1 over-expressing MCF-7 cells is shown in **Table 6.4**, according to seven functions: PINK1 localisation, activation of apoptosis, adhesion, invasion, colony formation, cell cycle progression and cyclin levels, and proliferation. The activation of apoptosis via cellular permeability (PI) and mitochondrial membrane depolarization ($DiOC_6(3)$) in a H_2O_2-induced model is shown. The symbols shown are with respect to control cells.

Table 6.3: Summary of SH-SY5Y results.

	Localisation	Mitochondrial Function			Oxidative Stress					CCCP-induced mitophagy				
					ROS					Activation		Localisation		Protein levels
		ATP	ΔΨm	Network	Basal	Rot.	MCB	Protein damage	mtDNA	LC3II	Mito	3 h	18 h	
PINK1 wt	Mito/Cito	↔	↑	Connected	↔	↓	↔	↓	↔	↑	↓	Mito	Mito	↑↑
K219M	Mito/Cito	↓↓ II/III, IV	↓↓	Fragmented Oligomycin sensitive	↑	↑	↓	↑	↓	↔	↔	↓ Mito	Mito	↑
A168P	Mito/Cito	↓↓ II/III, IV	↓	Fragmented	↑	↑	↓	—	—	↔	↔	↓ Mito	Mito	↑
G309D	Mito/Cito	↓↓ II/III, IV	↔	Fragmented Round-like	↑	↑	↓	↑	↓	↔	↔	↓ Mito	Mito	↑
L347P	Mito/Cito	↓↓ II/III, IV	↓	Fragmented	↑	↑	↓	—	—	↔	↔	↓ Mito	Mito	↑
W437X	Mito/Cito	↓↓ II/III, IV	↓↓	Fragmented Oligomycin sensitivity	↑	↑	↔	↑	↓	↔	↔	↔	Mito	↔
ΔC	Mito/Cito	↓↓ II/III, IV	↓	Fragmented Oligomycin sensitive	↔	↑	↔	↑	—	↔	↔	↔	Mito	↔
ΔN	Cito	↑	↔	↔	↔	↓	↔	↑	—	↔	↔	↔	Mito	↔

↓, ↓↓: low, very low; ↔: normal; ↑, ↑↑: high, very high; (compared to SH-SY5Y control cells) —: not done.

Table 6.4: Summary of MCF-7 results.

	Localisation	Apoptosis		Adhesion	Invasion	Colony formation	Cell cycle progression	Proliferation
		PI	$DiOC_6(3)$					
PINK1 wt	Mito/Cito	↔	⇓	↔	⇓	⇓	↓: ↓ cyc D1, A and B1 ↓ p27	↓
A168P	Mito/Cito	↑	↑	↔	↑	↔ Larger colonies	—	—
G309D	Mito/Cito	↑	↑	↔	↑	↔ Larger colonies	↑: ↑ cyc D1, A and B1 ↑ p27	↑
L347P	Mito/Cito	↑	↑	↔	↑	↓	—	—
W437X	Mito/Cito	↑	↑	↔	↔	↔	—	—
ΔC	Mito/Cito	↔	↑	↔	↔	↔	—	—
ΔN	Cito	↔	↓	↔	↔	↓	—	—
ΔN-C	Cito	↔	↑	↔	↔	↔	—	—

↓, ⇓: low, very low; ↔: normal; ↑, ⇑: high, very high; (compared to MCF-7 control cells.) —: not done.

Chapter

7 Discussion

In this thesis, we have studied the role of PINK1 and several PINK1 mutations (missense: kinase inactive or PD-linked, or truncated mutations) in the pathogenesis of cancer and Parkinson's disease. We have used a human neuroblastoma cell line (SH-SY5Y) as a PD model and a human breast carcinoma cell line (MCF-7) as a cancer model.

Since its discovery, PINK1 has been associated to both diseases: it is up-regulated in high tumourigenic tumours and in cells that ectopically over-express PTEN [Unoki and Nakamura, 2001; Nakajima et al., 2003], and mutations in PINK1 gene have been linked to early onset Parkinson's disease [Valente et al., 2004a]. Nevertheless, the molecular mechanism and the functional role of PINK1 linked to these diseases are not well defined. Moreover, the association with cancer is weak and not fully clarified.

Our results indicate that PINK1 is an anti-apoptotic protein required to maintain the correct mitochondrial function and to eliminate damaged mitochondria by mitophagy, and an anti-proliferative protein which affects tumourigenic processes such as invasion, colony formation, cell cycle progression and proliferation.

In summary, the results obtained in our study support the notion that PINK1 plays important roles as a pro-survival protein against stressors and as an anti-proliferative protein in transformation processes. PINK1 requires an intact C-terminal and kinase domain to exert its protective mitochondrial function. Also, PINK1 wt, but not a PD-linked missense mutation, exerts anti-proliferative effects in breast cancer cells.

7.1 PINK1 mutations analysed and functional implications

The crystal structure of PINK1 has not yet been resolved. The analysis of the mutations chosen in our study in several 3D models [Marongiu et al., 2008; Cardona et al., 2011] suggests that all of them disrupted important parts of PINK1 kinase domain and may affect the physiological role of PINK1 diminishing its kinase activity. Most of the PINK1 mutations identified in PD patients are predicted to impair the kinase activity of the protein, suggesting that loss of PINK1 catalytic function could be one of the causative mechanism of the disease. However, *in vivo* and *in vitro* PINK1 kinase activity is very low *per se*, and it has been suggested that PINK1 may be a pseudokinase that does not phosphorylate substrates directly but facilitate the phosphorylation activity of other kinases [Pogson et al., 2011]. Further analysis on PINK1 kinase activity and PINK1 potential substrates or regulatory proteins needs to be done [Sim et al., 2012].

Bioinformatic analysis of the PD-linked PINK1 missense mutations used here (A168P, G309D and L347P) shows that Ala168, Gly309 and Leu347 are conserved between PINK1 orthologues and are within the Ser/Thr kinase domain, as the majority of the PINK1 mutations described from PD patients (**Figure 7.1, 7.2** and **Appendix 8**).

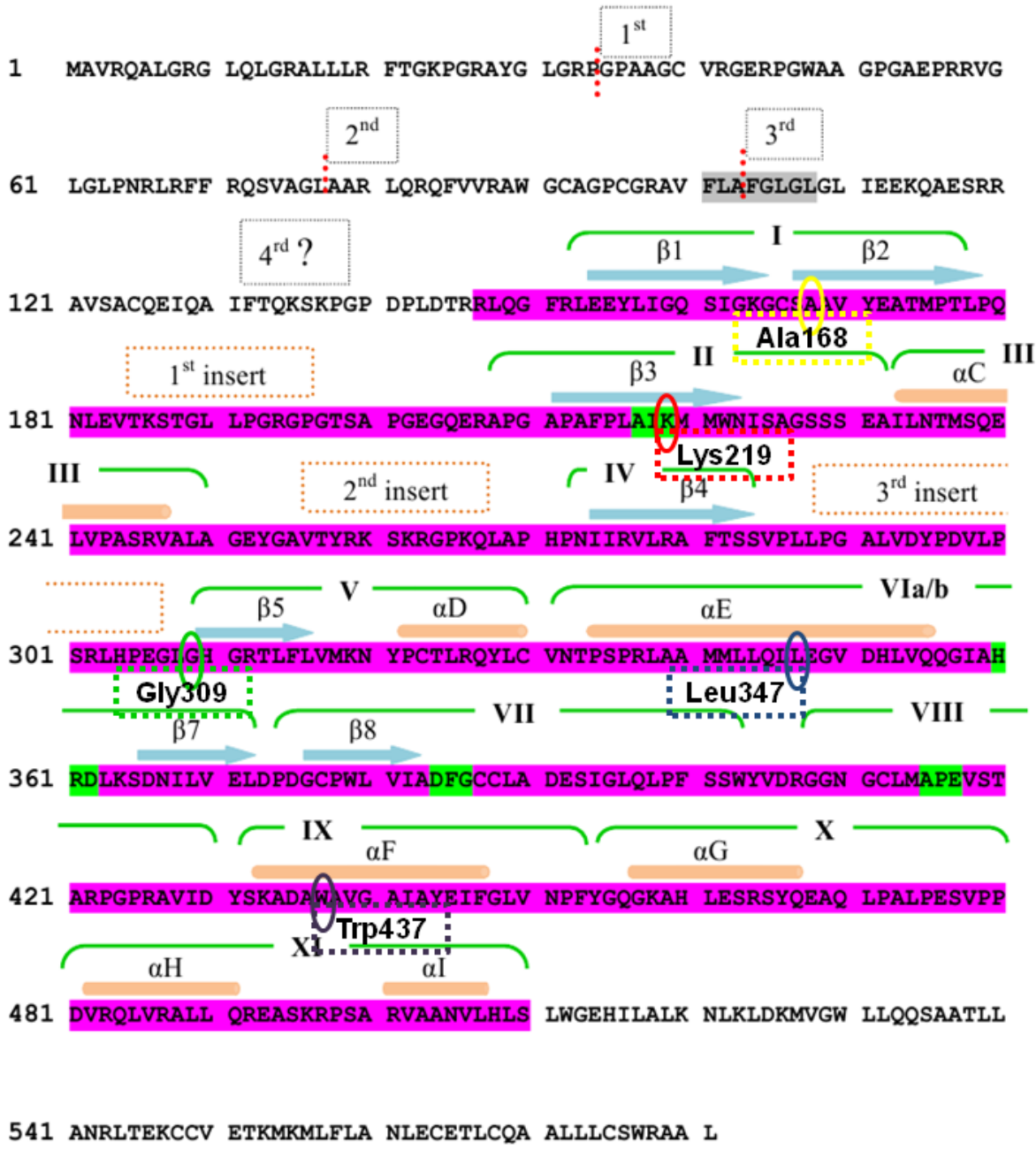

Figure 7.1: Amino acid sequence of PINK1 protein with the residues mutated in this study. Figure modified from **Chapter 1** (**Figure 1.7**). Amino acids correspondig to the mutations used are encircled.

The nonsense W437X mutation truncates the protein at position 436 and produces a stable protein of about 48 kDa, where the important motifs (AIK, HRD and DFG) for kinase activity are maintained. The protein portion missing in PINK1 W437X is predicted to have several residues in contact with the substrate which would disable the substrate access to the active site, thus increasing the propensity of the truncated protein toward non-specific protein phosphorylation [Silvestri et al., 2005]. Moreover, the subdomains IX, X, XI, and the C-terminal PINK1 region are important for folding of the protein [Beilina et al., 2005; Cardona et al., 2011].

The nonsense W437X mutation truncates the protein at position 436 and produces a stable protein of about 48 kDa, where the important motifs (AIK, HRD and DFG) for kinase activity are maintained. The nonsense mutation disrupts the conserved C-terminal part of the protein, composed of the C-terminal kinase subdomains involved in substrate recognition (subdomains IX, X and XI) and the C-terminal regulatory tail (residues 510-581). The protein portion missing in PINK1 W437X is predicted to have several residues in contact with the substrate which would disable the substrate access to the active site, thus increasing the propensity of the truncated protein toward non-specific protein phosphorylation [Silvestri et al., 2005]. Moreover, the subdomains IX, X, XI, and the C-terminal PINK1 region are important for folding of the protein [Beilina et al., 2005; Cardona et al., 2011]. The Lys219 has been proposed to bind ATP [Beilina et al., 2005; Albanese et al., 2005]. In the case of the N- and C-terminal truncated forms, important parts of the protein that may modulate the kinase activity and localisation are deleted.

Our functional results in SH-SY5Y and MCF-7 cells support the idea that different mitochondrial functions are impaired in the different PINK1 mutations [Tan, 2009; Grünewald et al., 2009; Murata et al., 2011b]. It is still unclear how these mutations affect PINK1 kinase activity under pathologic conditions, and whether other biological important functions of PINK1 are affected.

7.2 Localisation of PINK1 within the cell and in tissues

Although subcellular distribution of PINK1 is not fully clear, there is a broad agreement that at least some fraction of PINK1 protein localises to mitochondria, with its kinase domain facing the inter-membrane space or the cytoplasm [Valente et al., 2004a; Zhou et al., 2008b]. As most mitochondrial proteins, PINK1 is encoded by the nuclear genome and synthesised as a precursor polypeptide at cytosolic ribosomes. Thus, PINK1 has to be targeted to its final mitochondrial localisation in a process that is usually governed by specific signal sequences and accomplished via the protein translocation machinery present in the mitochondrial membranes [Pfanner et al., 2001; Chacinska et al., 2009]. At this regard, it has been proposed that the MLS directs PINK1 to the mitochondria via TOM20. In addition, the cleavage sites which generate PINK1-Δ1 and PINK1-Δ2 forms have been described in the MLS and TM of PINK1 protein [Greene et al., 2012].

The majority of the studies on PINK1 subcellular distribution have been done in over-expressing systems. This is because the expression of endogenous PINK1 is low and subjected to a rapid turn-over under basal conditions, and to the lack of sensitive anti-PINK1 antibodies [Lin and Kang, 2008, 2010; Narendra et al., 2010]. Nevertheless, we generated a monoclonal anti-PINK1 antibody [Berthier et al., 2011] able to recognise by immunohistochemistry endogenous human PINK1 in normal and neoplastic tissues.

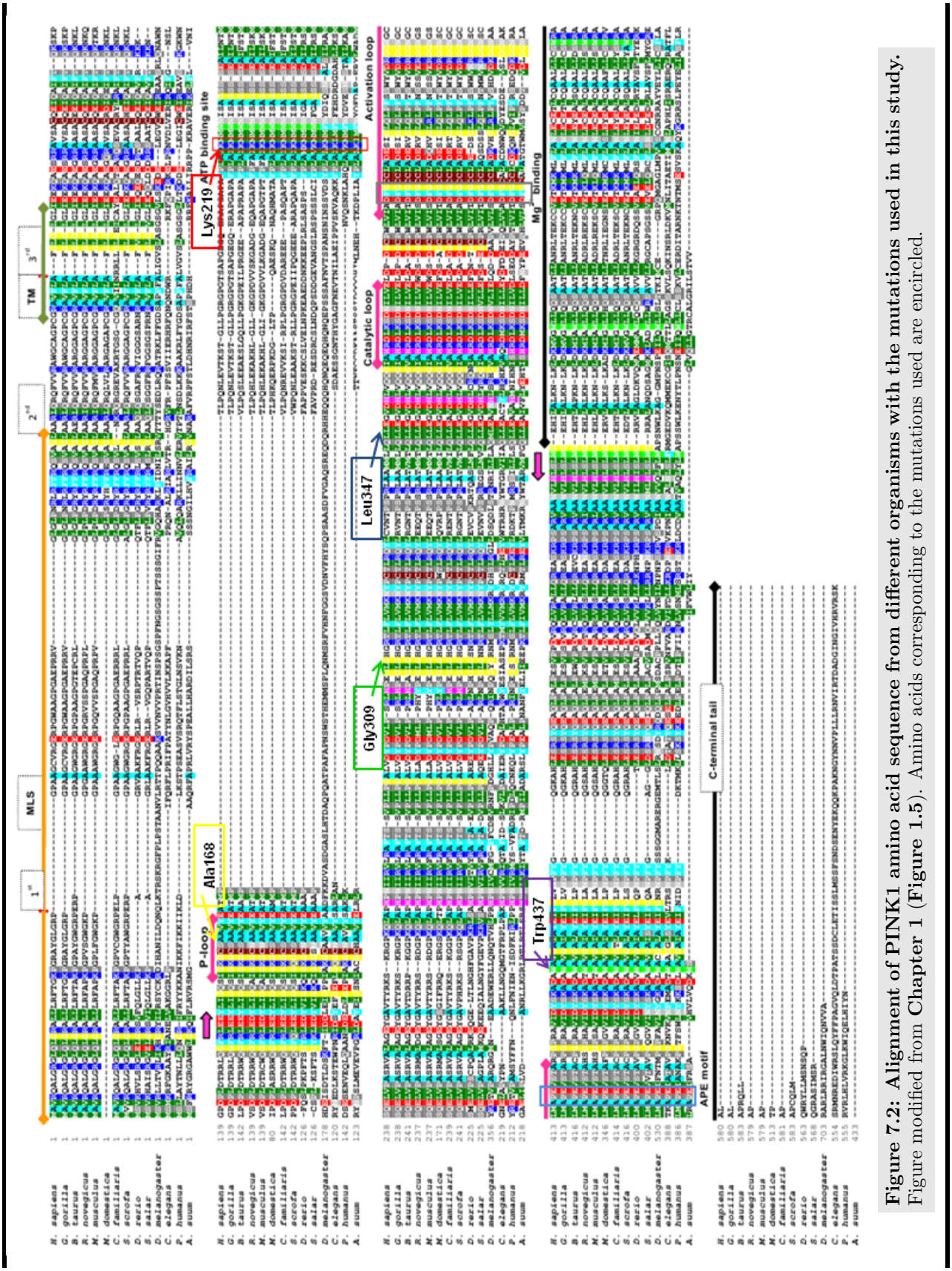

Figure 7.2: Alignment of PINK1 amino acid sequence from different organisms with the mutations used in this study. Figure modified from **Chapter 1** (**Figure 1.5**). Amino acids corresponding to the mutations used are encircled.

In the majority of the tissues analysed, PINK1 showed a cytosolic punctate pattern, characteristic of a mitocondrial protein. Together with PINK1 protein results, PINK1 mRNA expression studies revealed that PINK1 is widely expressed in normal human tissues.

In our SH-SY5Y and MCF-7 cell models, PINK1 wt co-localises with mitochondrial markers such as Mitotracker and TOM-20 (**Figure 7.3**), as other groups have described [Silvestri et al., 2005; Gandhi et al., 2006; Muqit et al., 2006; Beilina et al., 2005; Pridgeon et al., 2007; Weihofen et al., 2008; Marongiu et al., 2009]. In agreement with others subfractionation studies, PINK1 FL and PINK1-Δ1 are mostly localised in the mitochondrial fraction in our SH-SY5Y over-expressing cells. Besides, the three PINK1 protein forms (FL, Δ1 and Δ2) are also localised in the cytosolic fraction. In favour of the PINK1 partial cytosolic localisation, endogenous protein has been recently localised in the cytosol, through a mechanism involving cdc37/hsp90 and Parkin [Takatori et al., 2008; Weihofen et al., 2008]. Also, cytosolic localisation of PINK1 come from its accumulation in the cytosol under proteasome inhibition, its ubiquitination to be degraded by cytosolic proteasome [Muqit et al., 2006; Tang et al., 2006], and its presence in cytosolic aggresomes or Lewy bodies in brain samples from PD patients [Gandhi et al., 2006]. The nature and consequences of this dual localisation is an open question, and suggests the existence of different PINK1 functions, depending on its subcellular compartmentation and its interaction with substrates from mitochondria, such as TRAP1 or Htra2/omi, or from cytosol, such as Parkin, Rictor or hsp90/cdc37.

The missense mutations analysed (including catalytically inactive and PD-linked mutations) do not show any significant changes in localisation or in protein distribution either by immunofluorescence or by subcellular fractionation (**Figure 7.3**), and corroborate studies done with other PINK1 mutations [Valente et al., 2004a; Silvestri et al., 2005; Beilina et al., 2005; Petit et al., 2005; Muqit et al., 2006; Pridgeon et al., 2007; Wang et al., 2007; Zhou et al., 2008b; Becker et al., 2012]. Therefore, the mitochondrial localisation, import, processing and distribution of PINK1 in the cell do not seem to be affected by catalytically inactive or PD-linked mutations in the protein, suggesting that changes in PINK1 distribution alone do not explain PD pathology. Elucidation of how changes in PINK1 localisation may affect PINK1 physiologic functions requires further studies.

C-terminal truncations of PINK1 localised more at the mitochondria, as we describe here (**Figure 7.3**) and others have recently showed, and these mutations displayed higher kinase activity than PINK1 wt [Becker et al., 2012]. Thus, in addition to the proposed regulatory role of the C-terminal tail in PINK1 kinase activity [Silvestri et al., 2005; Sim et al., 2006], it is possible that the compartmentation of PINK1 could modulate its catalytic activity. In favour of this hypothesis, a study described that PINK1 accumulation at the OMM upon CCCP treatment enhanced Parkin phosphorylation and PINK1 autophosphorylation [Kondapalli et al., 2012].

Different deletions of the N-terminus of PINK1 are localised almost totally in the cytosolic area, but associated to the mitochondria [Weihofen et al., 2009]. In our analysis, we confirmed that PINK1 ΔN (without MLS and TM) is mainly in the cytosolic fraction but it is still associated to the mitochondria (**Figure 7.3**). We speculate that PINK1 lacking the N-terminal tail can associate to other proteins and be recruited to the mitochondria by an MLS-independent import This accounts for the control of mitochondrial anterograde transport by complex Miro/Milton, which can bind N-terminal deleted PINK1 and localises it to the mitochondria [Weihofen et al., 2009; Liu et al., 2012; Wang et al., 2012].

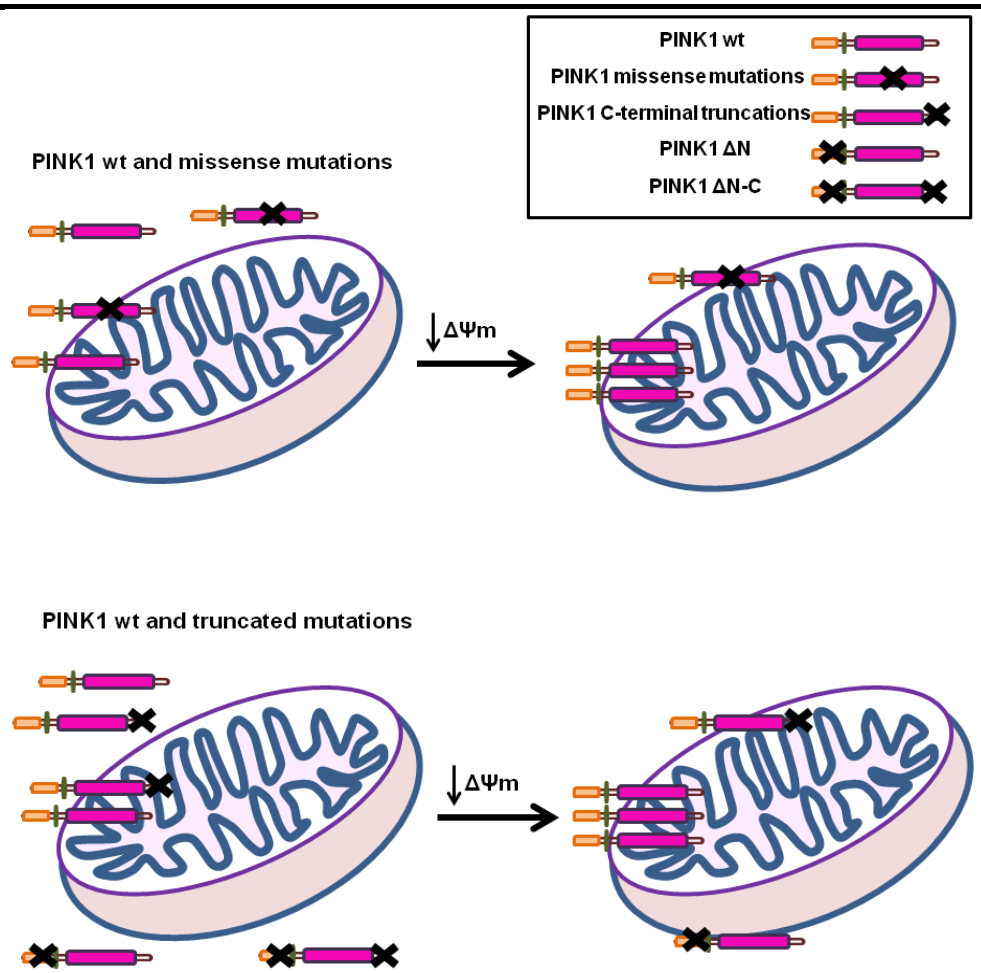

Figure 7.3: Localisation of PINK1 wt, mutations and truncations under basal and uncoupling-mitochondrial conditions. PINK1 wt and missense mutations are distributed between mitochondria and cytosol compartments under basal conditions. Dissipation of mitochondrial membrane potential leads to the mitochondrial accumulation of PINK1 wt and, at a lesser extent, missense mutations. The C-terminal truncations are distributed like PINK1 wt (but more mitochondrial) under basal conditions, while the N-terminal truncations are mostly localised in the cytosol. Both truncations localise to the mitochondria after long term dissipation of mitochondrial membrane potential, but with no increase in PINK1 protein levels.

PINK1 full length is processed in its N-terminus to generated PINK1-Δ1 [Deas et al., 2010; Becker et al., 2012; Jin et al., 2010]. Our results demostrate that the ratio and the existence of PINK1 FL and PINK1-Δ1 is unaffected in PINK1 missense and C-terminal truncated mutations. One point to highlight regarding ΔN and ΔN-C is the detection of two bands by immunoblot with the same intensity (**Figure 4.2, 5.1**, contrary to previous reports showing one band in ΔN [Lin and Kang, 2010; Matsuda et al., 2010; Becker et al., 2012]. We postulate that the second band (lower migrating band) could be a degradation product or likely, due to the same intensity, another PINK1 cleavage product. Further experiments are required to test these hypothesis.

Recently, it has been reported that PINK1 FL is stabilised at the OMM, facing the cytosol, upon depolarised mitochondria by CCCP or valinomycin treatment [Narendra et al., 2010]. At this regard, in our SH-SY5Y model, PINK1 FL localised to the mitochondria at short CCCP treatment times. The missense mutations, which showed mitochondrial location defects at short CCCP treatment times, localised to the mitochondria at long CCCP tteatment times. In addition, the deletion of N- or C-terminal parts of PINK1 impaired the mitochondrial localisation induced by CCCP at short times, but not at long times (**Figure 7.3**). It is possible that PINK1 kinase activity, and integrity of the N- and C-terminal portions, are needed to facilitate the import of PINK1 and its location in the OMM upon uncoupling mitochondrial conditions. Furthermore, PINK1 localisation at OMM at short times of mitochondrial uncoupling could be important for the downstream activating pathways, which is disrupted in the PINK1 mutations.

7.3 Role of PINK1 in mitochondrial function and mitophagy

7.3.1 PINK1 regulates mitochondrial function

Mitochondria are important mediators of generation of ATP and energy to the cell. PINK1 deficiency leads to mitochondrial dysfunction, impairment in complex I function, decrease in mtDNA, as well as enhanced oxidative stress in neuronal cell, in flies and mice, which is believed to contribute to PD pathology [Gandhi et al., 2006; Wang et al., 2006; Kitada et al., 2007; Gegg et al., 2009].

We observed that the expression of PINK1 missense mutations and C-terminal truncated mutations in neuroblastoma SH-SY5Y cells leads to a combined impairment of ATP generation, decreased mitochondrial membrane potential and mitochondrial DNA, and increase in ROS production, which is consistent with a deficit in the mitochondrial bioenergetic function. Fibroblasts bearing PINK1 missense and C-terminal truncated mutations showed decrease activity of complex I and IV [Grünewald et al., 2009; Piccoli et al., 2008; Abramov et al., 2012]. However, our results in SH-SY5Y cells indicated that impairment of ATP production was due to complexes II/III and IV, but not to complex I. This discrepancy could be due to differences in the bionergetic status of the models used. PINK1 N-terminal deletion did not affect ATP production, supporting the notion that deletion of PINK1 N-terminal tail does not affect mitochondria function.

The maintenance of $\Delta\Psi$m is mainly due to the activity of the mitochondrial electron transport chain complexes, which pump up protons through the inner mitochondria membrane generating the proton electrochemical gradient. Indeed, in the majority of the mutations analysed in this study, the decrease in ATP production is connected to a decrease in mitochondrial membrane potential (K219M, A168P, L347P, G309D, W437X and ΔC), as loss of PINK1 studies have already described. Moreover, as a result of the lowered activity of the oxidative phosphorylation complexes, the mitochondria switch from the production of ATP to its consumption by ATP synthase in order to maintain their mitochondrial membrane potential [Campanella et al., 2008]. This likely occurs in K219M and C-terminal truncated mutations, where the maintenance of $\Delta\Psi$m is mediated by hydrolysis of ATP, as PINK1-deficiency studies have previously shown [Gandhi et al., 2006; Abramov et al., 2012].

Several studies on $\Delta\Psi$m in other cell types, where different PINK1 missense PD-

linked or kinase inactive mutations were over-expressed, showed no significant changes in $\Delta\Psi$m under basal conditions, whereas we detected the opposite in SH-SY5Y cells. This discrepancy can be explained by the type of dye used, its concentration and the type of analysis done, as saturating concentrations of $\Delta\Psi$m dye and FACS analysis can hide the deleterious defects of $\Delta\Psi$m. In favour of this, we do not detect changes in $\Delta\Psi$m, analysed by FACS with $DiOC_6(3)$, in MCF-7 cells. However, in our SH-SY5Y model, we study the distribution of TMRM at low concentration and at single cell level as a more sensitive method to analyse $\Delta\Psi$m [Perry et al., 2011].

The analysis of the mitochondrial morphology revealed that in the mutations with low $\Delta\Psi$m and ATP production, the mitochondrial network was altered and mitochondria were closer to the perinuclear area. Noticeably, PINK1 G309D, which did no show changes in $\Delta\Psi$m, displayed swollen and more fragmented ring-like mitochondria network. This reinforces the observations from fibroblasts that PINK1 G309D mutation produced more drastic mitochondrial changes than other PINK1 mutations [Grünewald et al., 2009; Exner et al., 2007]. Together, these data suggest that the PINK1 deficiency firstly caused impairment in ATP and respiration, and secondly, changes in mitochondrial morphology and collapsed $\Delta\Psi$m. In accordance with this, a study using PINK1-deficient MEFs showed that the deficit of respiration produced the opening of the mPTP, and subsequently a decrease in $\Delta\Psi$m and calcium overload, that finally caused changes in the mitochondrial network [Gautier et al., 2012]. In addition, Gandhi et al. revealed that neurons with loss of PINK1 display enhanced sensitivity to the toxic effect of dopamine, with increased mPTP permeability, respiratory chain impairment, and mitochondrial calcium overload [Gandhi et al., 2012].

The amount and assembly of the respiration chain complexes in PINK1 mutations seem to be correct [Grünewald et al., 2009; Liu et al., 2009]. Therefore, another possible regulatory role of PINK1 on the oxidative phosphorylation could be the control of the appropriate phosphorylation of the mitochondrial complex proteins, which is required for the correct function of the electron transport chain [Liu et al., 2009]. At this regard, it has been recently shown that the activation of mitochondrial PKA, which phosphorylates complex I subunits, is sufficient to rescue mitochondrial dysfunction induced by PINK1 deletion [Dagda et al., 2011]. We speculate that PINK1 could be important to maintain the phosphorylation status of the mitochondrial respiratory complexes.

The impairment in ATP production and the decrease in $\Delta\Psi$m favour the generation of ROS in the cell. In our SH-SY5Y model, the oxidative stress induced by rotenone was increased in the PINK1 mutations and C-terminal truncations. Furthermore, the generation of cROS in the mutations was associated to an increase in mitochondrial carbonylated proteins and a decrease in antioxidant GSH capacity. The decrease in antioxidant capacity of the mutations can be explained by GSH conversion into GS-SG due to increase in ROS production. One mechanism explaining how PINK1 blocks the ROS production induced by stress could be the new synthesis of detoxifying enzymes, such as glutathione transferase and/or MnSOD [Hoepken et al., 2007]. Our study corroborates other reports showing lipid damage and mtDNA decrease upon PINK1 mutation or silencing conditions [Gegg et al., 2009; Hoepken et al., 2007], as well as previous studies reporting reduced $\Delta\Psi$m and elevated oxidative stress in PINK1 KD human dopaminergic neuron cultures [Wood-Kaczmar et al., 2008], and reduced mitochondrial respiration and increased sensititvity to oxidative stress in PINK1 KO mice and flies [Wang et al., 2006, 2007; Gautier et al., 2012].

Together, these observations reinforce the notion that PINK1 plays an important role in the integrity of mitochondria function and oxidative metabolism (**Figure 7.4**).

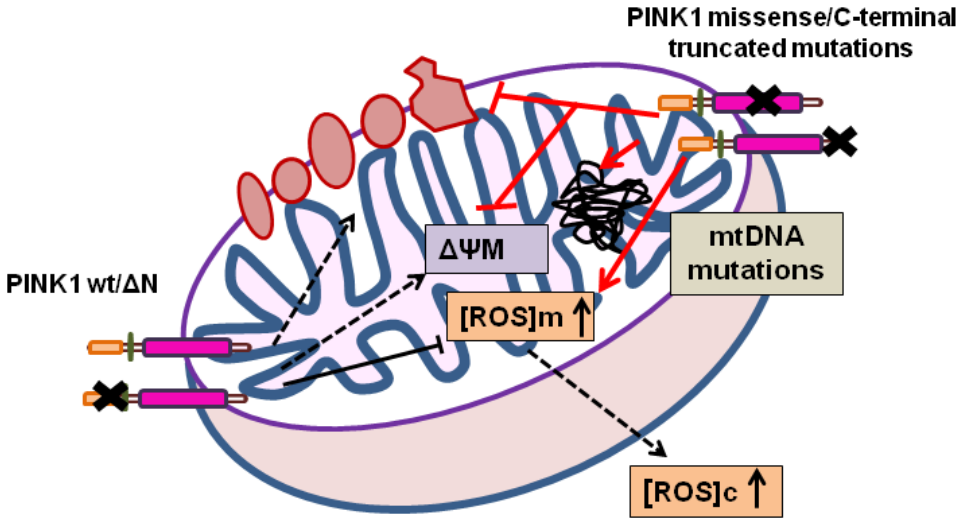

Figure 7.4: PINK1 wt and ΔN, but not PINK1 missense and C-terminal truncated mutations, control mitochondrial homeostasis. Over-expression of PINK1 missense and C-terminal truncated mutations decrease ATP production, mitochondrial membrane potential and mtDNA content, and increase ROS production, whereas over-expression of PINK1 wt and ΔN maintain the physiologic mitochondrial ATP production and mitochondrial membrane potential, and block the ROS production.

7.3.2 Mitophagy modulation by PINK1

Mitophagy activates selectively the clearance of damaged mitochondria [Twig et al., 2008; Sandoval et al., 2008; Elmore et al., 2001], and PINK1, together with Parkin, has been recently described to regulate this process [Narendra et al., 2008]. Several studies have shown that PINK1 accumulates in the outer membrane of dysfunctional mitochondria, recruits Parkin and facilitates the activation of its E3 ubiquitin ligase function. Parkin is able to ubiquitinate other substrates and promotes the triggering of mitophagy [Dagda et al., 2009; Narendra et al., 2010; Vives-Bauza et al., 2010b; Matsuda et al., 2010; Geisler et al., 2010; Koh and Chung, 2011]. Moreover, PINK1 FL binds Beclin-1, a protein involved in the main autophagy pathway [Michiorri et al., 2010].

In our study, we have found that PINK1 wt promotes autophagosome formation and clearance of damaged mitochondria in SH-SY5Y upon mitochondrial uncoupling by CCCP or starvation. Moreover, the CCCP treatment induces accumulation and association of PINK1 FL to the mitochondria, and inhibition of its processing into PINK1-Δ1 and PINK1-Δ2. Processing and mitochondrial import of PINK1 is dependent on mitochondrial membrane potential, and perhaps the dynamics of PINK1 processing and localisation could determine its function in different processes and cell compartments. This idea could

explain why PINK1 and its multiple cleavage forms are regulated and involved in different functions depending on the cellular status.

Our results show that mutated PINK1 impaired the activation of autophagosome formation and the consequent clearance of damaged mitochondria. The mitochondrial accumulation of PINK1 missense mutations was impaired only at short times of CCCP treatment, suggesting a crucial role for PINK1 in early stages of mitophagy. Rapidly after CCCP treatment, PINK1 FL wt recruits Parkin to the mitochondria and induces its phosphorylation at Ser63, activating downstream pathways of mitophagy and survival [Kondapalli et al., 2012]. In the case of the C-terminal truncations, the PINK1 mitochondrial accumulation occured at later times of CCCP treatment, and the protein levels were lower. The N-terminal truncation showed late mitochondrial accumulation and unaffected protein levels of PINK1 after CCCP treatment. At this regard, it has been documented that some PINK1 mutations or truncations do not recruit Parkin to the mitochondria, blocking mitophagy [Narendra et al., 2010; Becker et al., 2012].

The majority of the studies associating PINK1 to mitophagy have been done in an induced-mitophagy system. However, it has been described that cultured cells silenced for PINK1 can activate mitophagy under basal conditions to clear up the damaged mitochondria prior to cell death [Chu, 2009]. We and others detect that PINK1 deficiency or mutations lead to increased oxidative stress and fragmented mitochondria, which activates mitophagy [Narendra et al., 2008]. Nevertheless, our results show that missense or truncated mutations of PINK1 do not increase the autophagosome formation at basal conditions, compared to PINK1 wt. Further studies addressing the PINK1 role in autophagy need to be done.

7.4 Role of PINK1 in apoptosis, tumourigenic and proliferative processes in breast cancer

7.4.1 PINK1 as an anti-apoptotic protein in breast cancer cells

PINK1 is involved in the protection of the cell from a broad range of mitochondrial insults and apoptotic inductors, including rotenone, 1-methyl-4-phenylpyridinium (MPP^+) and staurosporine [Valente et al., 2004a; Petit et al., 2005; Muqit et al., 2006; Tang et al., 2006; Wang et al., 2007; Pridgeon et al., 2007; Chu, 2009; Tan, 2009; Kim and Son, 2010].

In our study using breast cancer cells over-expressing PINK1 wt, we show lack of dissipation of $\Delta\Psi_m$ (an early event of apoptosis) and inhibition of cell death produced by H_2O_2. PINK1 missense mutations and PINK1 deficiency in MCF-7 and MB-MDA-231 cells disrupted this anti-apoptotic role of PINK1, and cells displayed increased cell death and depolarised mitochondria, as it has been shown in other cell systems [Valente et al., 2004a; Wood-Kaczmar et al., 2008; Deng et al., 2005; Haque et al., 2012]. It is important to note that MB-MDA-231 are more resistant to H_2O_2-induced cell death and induction of mitochondrial depolarisation than MCF-7, perhaps due to the fact that MB-MDA-231 comes from of a more aggressive breast cancer tumour [Pozo-Guisado et al., 2002].

MCF-7 cells over-expressing the C-terminal truncations, but not those over-expressing the N-terminal truncation, were more sensitive to cell death, when compared with PINK1 wt over-expressing cells, suggesting an important role for the PINK1 C-terminus in apoptosis [Silvestri et al., 2005; Petit et al., 2005]. Interestingly, PINK1 ΔN cells, lacking the mitochondrial localization sequence, conserved the same anti-apoptotic function than

PINK1 wt in MCF-7 cells, which has also been observed in other cell types [Haque et al., 2008; Tan et al., 2009; Tan, 2009]. Nevertheless, Wang et al. reported that PINK1 lacking the first 35 residues did not protect against apoptosis triggered by proteasome inhibition [Wang et al., 2007].

Taking together, our results show that the blocking effect of PINK1 in mitochondrial-induced apoptosis is not cell type-specific, and it is observed in non-neuronal cells. In breast cancer and neuronal cells, the activation of the MAPKs p38, JNK and ERK1/2 by several mitochondrial stimuli is important for cell death/survival [Ruffels et al., 2004; Newhouse et al., 2004; Deng et al., 2010]. PINK1 has been related to p38 and JNK activation upon stress [Pridgeon et al., 2007; Plun-Favreau et al., 2007], and subsequently, it modulates the activation of Htra2/omi as a cytoprotective role. This makes PINK1 a potential modulator or effector of MAPKs. Also, PINK1 phosphorylates TRAP1 and Parkin upon stress conditions, and activates downstream pro-survival pathways, such as the NF-$\kappa\beta$ pathway [Sha et al., 2010]. Thus, PINK1 may exert its anti-apoptotic role acting on different pathways and molecular effectors (**Figure 7.6**).

7.4.2 PINK1 mediated invasion, colony formation, proliferation and cell cycle progression

Cancer is a complex disease where cells divide uncontrollably, missing out the cell cycle checkpoints and invading the surrounding tissue. PINK1 expression is altered in tumours but PINK1 role in tumourigenicity is unknown [Nakajima et al., 2003; Unoki and Nakamura, 2001; Berthier et al., 2011; Fragoso et al., 2011]. In our study, we have characterised for the first time the role of PINK1 in tumourigenic processes in breast cancer cells, including adhesion, invasion, colony formation, proliferation and cell cycle progression.

Our results demonstrate that PINK1 wt does not affect the adhesion capacities of MCF-7 cells to different matrix substrates, but decrease their invasion and the number and size of colonies formed in soft agar. This reduction in the number and size of colonies could indicate inhibition of the most highly proliferating cells and/or presence of more differentiated cells. At this regard, the morphology of PINK1 wt over-expressing MCF-7 cells is more spread out, differentiated-like, compared to control cells (data not shown). On the other hand, cell lines over-expressing PINK1 mutations presented higher number of colonies than control cells, suggesting an increase in tumourigenic properties with more proliferating and/or less differentiated cells, which could be due to a dominant negative effect over the endogenous PINK1 [Tan, 2009; Kawajiri et al., 2011]. This suggests a role for PINK1 in cell differentiation and blockage of cell division. In line with this possibility, it has been described that PINK1 is up-regulated and required for neuron differentiation [Wood-Kaczmar et al., 2008] [unpublished data].

The analysis of invasion also suggests that PINK1 wt negatively regulates tumourigenic properties, since cell invasion was lower in PINK1 wt over-expressing cells than in control cells. Consequently, cells over-expressing PINK1 PD-linked mutations (A168P, G309D and L347P) presented higher increase invasion rate. The PINK1 truncated mutations showed invasion values similar to control cells. PINK1 L347P over-expressing cells displayed increased invasion and decreased colony formation. This could be explained because L347P is a destabilising mutation with normal kinase activity [Beilina et al., 2005]. It is possible that the amount of PINK1 could determine differential regulation of these two processes

PINK1 wt and PINK1 G309D over-expressing SH-SY5Y cells display increased cell motility through Matrigel-coated membrane, which dependent on pro-survival Akt/mTOR2 activation [Murata et al., 2011a]. This partial discrepancy with our results could be due to the differents assays performed in the two studies or to the existence of different PINK1 functions depending on the cell type.

MCF-7 cells over-expressing PINK1 wt showed decreased proliferation, blockage in cell cycle progression, and diminished expression of cyclins. The inhibition in cell cycle progression was not due to a complete growth arrest because cells with over-expressing PINK1 wt still proliferated, although at lower levels. PINK1 could either lengthen G_0/G_1 transition time or cause arrest in late G_0/G_1 or S phases. On the other hand, PINK1 G309D over-expressing cells increased cell cycle progression and increase cyclin D and A expression. The activation of cyclin D is essential for cell cycle progression and increased levels of cyclin D have been associated to tumourigenic processes in breast cancer [Zwijsen et al., 1998; Dufourny et al., 2000; Laman et al., 2005; Kehn et al., 2007; Tashiro et al., 2007]. This points out for a putative role of PINK1 mutations in the progression of cancer. Further studies will be needed to test this hypothesis.

In other ways, PINK1 can exert its anti-proliferative role through the association to related proteins like PTEN, which up-regulates PINK1 levels, and it can modulated the PINK1 function in the cell. It has been described that PTEN prevents spreading, migration on extracellular matrix proteins and invasion, wchich leads to G_1 arrest in glioma, breast, endometrial and prostate cancer cells [Yamada and Araki, 2001; Kim et al., 2005; Kim and Mak, 2006]. The mechanism of PTEN cell cycle arrest is dependent on down-regulating PKB/Akt pathway which increase forkead transcription factors (like FOXO3a), glycogen synthase kinase and decreased levels of cyclin D [Maehama et al., 2001]. This process can be link to increase of PINK1 mRNA levels. Moreover, the function of PTEN is affected by the oncogenic role of cytoplasmic p27 protein and is dependent on the cellular compartement, nucleus versus cytosol [Andrés-Pons et al., 2011].

PTEN, apart from its tumour-suppressor role, positively regulates apoptosis, inducing cell death and inhibit mTOR inducing autophagy. Our results showed that PINK1 plays as anti-apoptotic and autophagy induction protein so PTEN and PINK1 can have some linked functions but not other. The relation between them, far from expression is unclear so further experiments need to be done.

The expression of Parkin is diminished or absent in breast and ovarian cancer [Cesari et al., 2003]. Recently, Parkin has been proposed as a tumour-suppressor protein that decreases cancer progression as well as the levels of cyclin E in MCF-7 cells by inducing the ubiquitination and subsequent degradation [Tay et al., 2010]. Therefore, PINK1 anti-proliferative or tumour-suppressor activity could be mediated through Parkin. suppression capacity.

The results reported here suggest that PINK1 functions as an anti-proliferative protein, blocking the G_0/G_1-S phases transition and modulating directly or indirectly cyclins and inhibitors of the cell cycle. It is important to keep in mind that PINK1 is a kinase (or a pseudokinase) and phosphorylation is very important in controlling cell cycle [Pogson et al., 2011; Koh and Chung, 2012].

How PINK1 regulates cell cycle and proliferation is only beginning to be unveiled. For instance, the expression of Parkin is diminished or absent in breast and ovarian cancer [Cesari et al., 2003]. Recently, Parkin has been proposed as a tumour-suppressor protein that decreases cancer progression as well as the levels of cyclin E in MCF-7 cells [Tay

et al., 2010]. Therefore, PINK1 anti-proliferative or tumour-suppressor activity could be mediated through Parkin. Next frontiers lie in how PINK1 protein and its different binding partners can exert different functions in several compartments as mitochondria, cytosol, and, perhaps, nucleus.

7.5 Expression of PD genes/mRNA in breast cancer tissues

We have found that PINK1 is widely expressed in normal human tissues, suggesting that its function is not limited to the nervous system [Berthier et al., 2011]. The mRNA levels of PINK1 have been found to be over-expressed in different cancers with high metastatic potential, as well as in cells forced to express ectopically the PTEN tumour suppressor, making possible the involvement of PINK1 in the unbalanced cell growth- or apoptotic-processes of cancer. In our study, we detected up-regulation of PINK1 mRNA levels in breast cancer compared to control breast. Moreover, we detected that the subcellular distribution of PINK1 in breast tumours change from punctate cytoplasmic to more membranous localisation. This suggests that PINK1 expression levels and subcellular distribution could be associated to breast cancer progression. Together with PINK1, other PD-associated genes have been linked to cancer. Our analysis concluded that mRNA expression of Htra2/omi, ATP13A2, LRRK2, GBA, DJ-1 and TRAP1 is up-regulated in breast tumours whereas mRNA expression of PTEN and SNCAIP is down-regulated (**Figure 7.5**). Moreover, the high expression of PINK1 in some of the tumours was associated with high expression of Htra2/omi, ATP13A2, GBA, DJ-1 and TRAP1. Interestingly, it has been described that breast cancer patients have elevated levels of serum DJ-1 and circulating anti-DJ-1 auto-antibodies, compared to healthy and non-breast cancer patients [Le Naour et al., 2001]. Together, these findings suggest the existence of a network of PD-related protein interactions that could be relevant in breast cancer. Further studies are required to ascertain if over-expression of PINK1 or other PD-related proteins correlates with breast cancer subtypes or with response to breast cancer therapies.

In favour to the relationship between PD genes expression in cancer progression, it has been described common determinants to both diseases, such as mitochondrial DNA mutations and complex I deficiency [Plak et al., 2008]. The mitochondrial bioenergetic metabolism has been widely linked to both diseases. In addition, mitochondria dysfunction has been implicated in carcinogenesis since the 1930s where Otto Wanburg suggested that "respiration damage" is a pivotal feature of cancer cells [Wallace, 2005; Gogvadze et al., 2008] and in PD pathogenesis [Schapira et al., 1990; Schapira, 2008]. The bioenergetic metabolism is important for both diseases and our results contributed to this thought where PD-linked genes could be associated to breast cancer. Furthermore, decrease in autophagy/mitophagy and fail in ubiquitin-proteasome system have been documented in both diseases [McNaught et al., 2001; Mitchell, 2003; Cuervo, 2004].

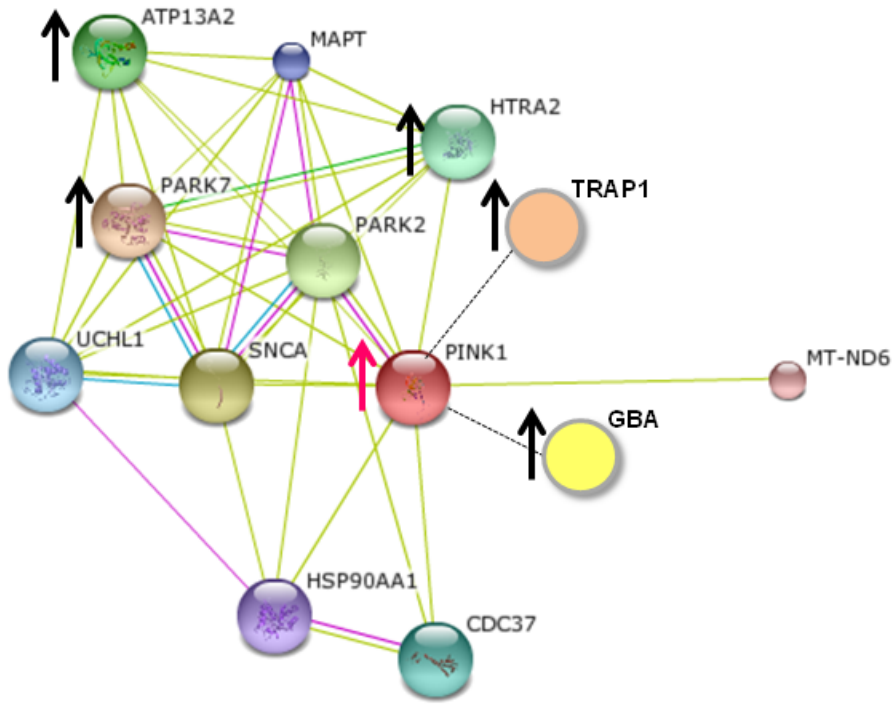

Figure 7.5: PINK1, PINK1-related genes and their linkage expression in breast cancer samples. PINK1-related genes provided by STRING software and by reported functional association. The up-regulation of PINK1 in breast cancer samples is associated with up-regulation of Htra2/omi, ATP13A2, DJ-1, GBA and TRAP1 genes associated to PD and protein quality control. Modified from `http://string-db.org/`.

7.6 PINK1: an anti-apoptotic and anti-proliferative protein

From our analysis, we propose that PINK1 has several functions and mainly working as an anti-apoptotic protein under certain stress situations and as an anti-proliferative protein in breast cancer progression, regulating important processes involved or associated to cancer and PD pathogenesis **Figure 7.6**.

PINK1 function is associated to mitochondria homeostasis and integrity. Mitochondria integrity and mitochondria, play an important role in regulating both apoptosis and cell proliferation. Small and fragmented mitochondria, together with changes in mitochondrial density and distribution, have been associated to cancer processes, and inhibition of fission prevents the cell cycle progression in lung cancer [Olichon et al., 2006; Rehman et al., 2012]. Alterations in oxidative phosphorylation and increase in oxidative stress, resulting from mitochondrial dysfunction, have long been hypothesised to be involved in tumourigenesis. Specifically, it has been postulated that the switch in ATP production from mitochondrial oxidative phosphorylation to glycolysis is one of the characteristics of cancer cells, a process known as the "Warburg effect" [Wallace, 2005]. Remarkably. PINK1 KO mice present increased glycolisis [Yao et al., 2011]. More recently, it has been reported that

mitochondrial respiratory complex I dysfunction promotes tumourigenesis through ROS alteration and AKT activation [Sharma et al., 2011]. Our data corroborates that mitochondrial dysfunction and increase in oxidative stress, showed in cells over-expressing PINK1 PD-linked mutations, could be associated to tumourigenic progression (**Figure 7.6**).

PINK1 has been associated to Beclin-1-inducted autophagy and Beclin-1 activity is controlled by Bcl-2 activity. Bcl-2 and Beclin-1 has been associated to proliferation and cell death. Low levels of Bcl-2 correlates with poor prognosis in patients with breast cancer [Chang et al., 2003] and increase in cell growth in MCF-7 cells [Oh et al., 2010], inversely Bcl-2 expression correlates with low proliferation index and favourable prognosis [Friedrich et al., 1995; Luna-More et al., 1996]. Beclin-1 down-regulation leads to bad prognosis in cancer [Mutee et al., 2009]. Therefore, PINK1 can mediate its anti-apoptotic and anti-proliferative functions through Bcl-2 and/or Beclin-1 [Clarke et al., 2011].

Together, all our results prompt PINK1 as a new modulator controlling important pathways involved in the progression of PD and cancer. PINK1 can control pro-survival mechanism against mitochondria-mediated apoptosis by keeping the integrity of mitochondria, and can block the uncontrolled division of cancer cells. PINK1 impedes neuronal cell death, blocks abnormal entry into the cell cycle, and inhibits excessive proliferation. The analysis of PINK1 mutations can provide new evidences on a positive link between cancer and PD. In this line, it has been documented that PD patients have increased risk of breast cancer [Möller et al., 1995; Minami et al., 2000; Olsen et al., 2004, 2006; Lo et al., 2010], and we have described here that MCF-7 breast cancer cells over-expressing a PD-linked PINK1 mutation show increased cell death and apoptosis, proliferation and abnormal entry into the cell cycle.

The fact that PINK1 functions as an anti-apoptotic and anti-proliferative protein is not an unique example of a protein with opposite functions (**Figure 7.6**). The Bcl-2 protein is known to have dual anti-proliferative and anti-apoptotic roles depending upon cellular and developmental context [Borner, 1996]. Besides, the transcription factor FHL2 exhibits anti-proliferative and anti-apoptotic activities in liver cancer cells [Ng et al., 2011]. Retinoblastome (Rb) regulates tumour suppression and anti-apoptotic function [Ma et al., 2003]. In these cases, the phosphorylation status, the protein levels, the subcellular localisation, as well as the association to other proteins may trigger one function or the other. Remarkably, PINK1 is a short life Ser/Thr kinase protein which levels are regulated by uncoupling agents and oxidative stress and is associated to different proteins localised in is associated to different proteins localised in distinct cellular compartments. Further studies need to be done to understand how these regulatory processes may drive the dual function of PINK1 in physiology and human disease (**Figure 7.6**).

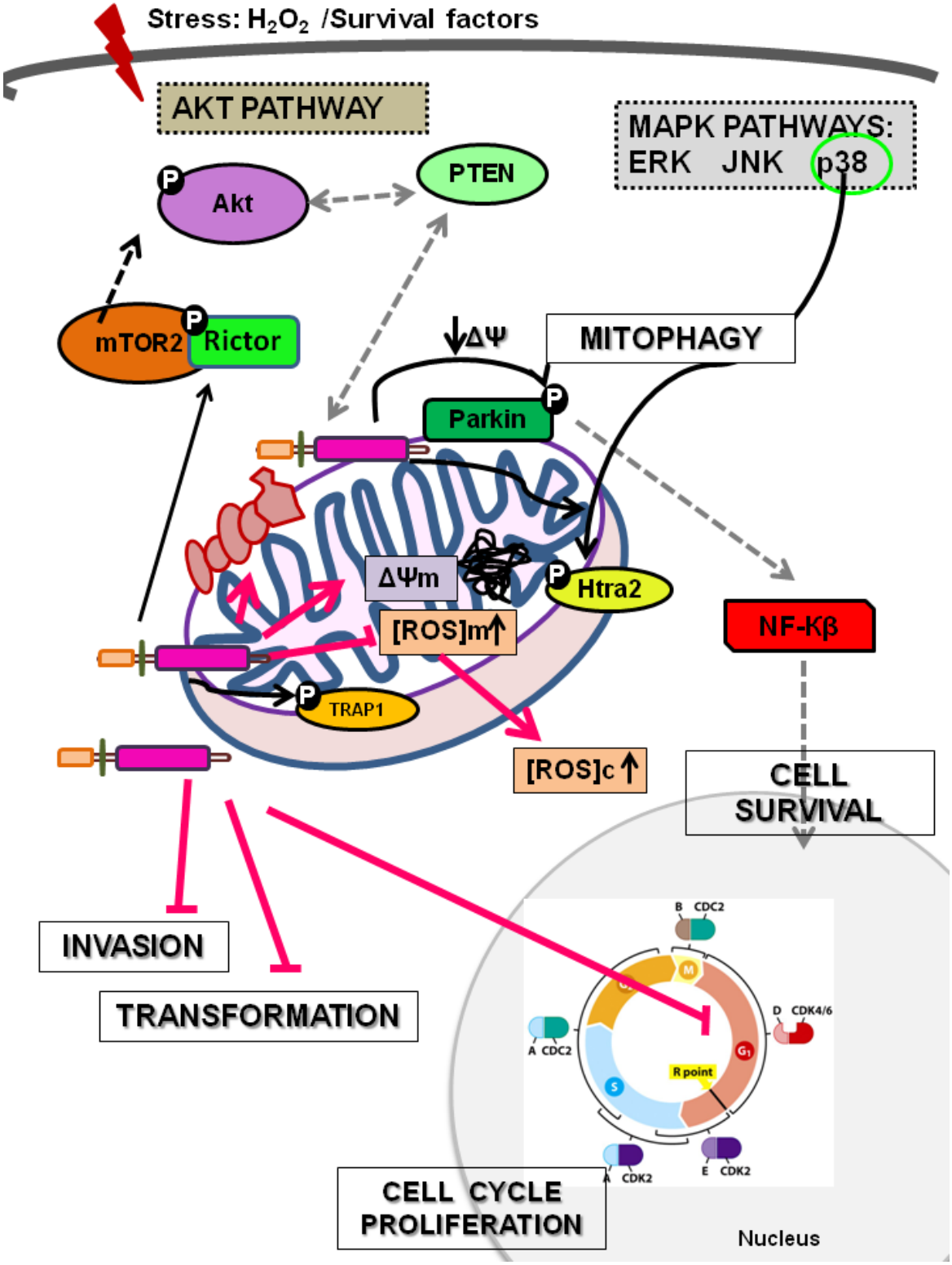

Figure 7.6: PINK1 as anti-apoptotic and anti-proliferative protein. PINK1 responds to stress signals that activates MAPKs and AKT pathways are activates the mitochondrial homeostasis. PINK1 inhibits apoptosis and controls the ATP production, the $\Delta\Psi$m, the ROS production and the mtDNA content. PINK1 exerts its anti-apoptotic function interacting and controlling the phosphorylation status of TRAP1, Htra2/omi and Rictor, which subsequently activates pro-survival genes and controls the protein misfolding. Upon mitochondrial depolarization, PINK1 binds and phosphorylates Parkin, and together with Beclin-1, activates mitophagy and the clearance of damaged mitochondria. Black lines: phosphorylation processes; Dash grey line: associated documented processes; Pink line: PINK1 processes described in this study.

Chapter

8 Conclusions

1. PINK1 localises between mitochondria and cytosol compartments under basal conditions. PD-linked and catalytic inactive missense mutations do not show changes in subcellular localisation, whereas PINK1 C-terminal truncations are slightly more mitochondrial, and PINK1 N-terminal truncations are mainly cytosolic.

2. PINK1 wt, but not missense mutations or truncations, accumulates at the mitochondria upon mitochondrial-uncoupling CCCP treatment.

3. PINK1 missense mutations and C-terminal truncations lead to mitochondrial dysfunctions in neuroblastoma cells, with ATP production impairment, decrease in mitochondrial membrane potential, increase oxidative stress and decrease mtDNA content.

4. PINK1 wt, but not PINK1 missense mutations or truncations, increases the protein levels of the autophagosome marker LC3-II, and decreases mitochondrial content.

5. PINK1-mediated mitophagy required accumulation and stabilisation of the PINK1 protein at the mitochondria, which is disrupted in PINK1 missense or truncated mutations.

6. PINK1 wt and N-terminal truncation (ΔN), but not PINK1 missense mutations or C-terminal truncations, protect against mitochondrial-mediated apoptosis trigerred by H_2O_2.

7. PINK1 wt and the N-terminal truncation ΔN, but not PINK1 mutations or C-terminal truncations, inhibit the colony formation capacity and invasion of breast cancer cells. The PINK1 L347P mutation inhibits colony formation and increases invasion.

8. PINK1 wt, but not the PD-linked mutation G309D, decreases proliferation and delays cell cycle progression at G_0/G_1-S phase of breast cancer cells.

9. PINK1 protein and mRNA are widely expressed in human normal tissue, and PINK1 expression is altered in neoplastic tissues.

10. PINK1 mRNA, together with other PD-linked genes or related partners, is upregulated in breast cancer tumours.

11. PINK1 is a pivotal protein controlling mitochondrial homeostasis, as well as survival, proliferation and cell death. PINK1 mutations impair these processes, which may be linked to cancer and PD pathogenesis.

Conclusiones

1. PINK1 se localiza en condiciones basales en la mitocondria y el citosol. PINK1 cataliticamente inactivo y las mutaciones asociadas a PD no muestran cambios en la localización subcelular, mientras las formas C-terminales truncadas de PINK1 son ligeramente más mitocondriales, y las formas N-terminales truncadas de PINK1 son principalmente citosólicas.

2. PINK1 wt, pero no las mutaciones con cambio de aminoácido o las formas truncadas, se acumula en la mitocondria tras el tratamiento de desacoplamiento mitocondrial con CCCP.

3. Las mutaciones de cambio de aminoácido y las formas C-terminales truncadas de PINK1 producen disfunción mitocondrial en células de neuroblastoma, con desregulación en la producción de ATP, disminución del potencial de membrana mitocondrial, incremento de estrés oxidativo y disminución del contenido de mtDNA.

4. PINK1 wt, pero no las mutaciones de cambio de aminoácido o las formas truncadas, aumenta los niveles de la proteína marcadora de autofagosomas LC3-II, y disminuye el contenido mitocondrial.

5. La mitofagia mediada por PINK1 requiere acumulación y estabilización de la proteína en la mitocondria, lo cual está afectado en las mutaciones y las formas truncadas de PINK1.

6. PINK1 wt y la forma N-terminal truncada (ΔN), pero no las mutaciones de cambio de aminoácido o las formas C-terminales truncadas, protegen de la apoptosis mediada por la mitocondria inducida por H_2O_2.

7. PINK1 wt y la truncación N-terminal (ΔN), pero no mutaciones de cambio de sentido o truncaciones C-terminal, inhiben la capacidad de formación de colonias y la invasión de células de cáncer de mama. La mutación L347P inhibe la formación de colonias e incrementa la invasión.

8. PINK1 wt, pero no la mutación asociada a PD G309D, disminuye la proliferación y retrasa la progresión del ciclo celular en fase G_0/G_1-S en células de cáncer de mama.

9. El mRNA y la proteína de PINK1 están ampliamente expresados en téjidos normales humanos, y la expresión de PINK1 se encuentra alterada en téjidos neoplásicos.

10. La expresión del mRNA de PINK1, junto con la expresión de otros genes asociados con PD o proteínas relacionadas, está aumentada en tumores de cáncer de mama.

11. PINK1 es una proteína central que controla la homeostasis mitocondrial, asi como supervivencia, proliferación y muerte celular. Las mutaciones de PINK1 afectan estos procesos, lo que podría estar asociado con la patogénesis del cáncer y PD.

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First appendix

Table 8.1: Studies on the association between PD and cancer.

Study		All cancers,% or RR	Smoking related	Non smoking related	Breast	Skin	Melanoma	Comments
Doshay, 1954	—	—	—	—	—	—	—	—
Westlund and Hougen, 1956	CB	6.3% obs-3.2% exp.	—	—	—	—	—	—
Barbeau and Joly, 1963	CB	1.8% obs-5.8%-15.9%exp.	—	—	—	—	—	—
Hoehn et al., 1998	CB	12% obs-20.6% exp.	—	—	—	—	—	—
Pritchard III and Netsky, 1973	CB	26% cases -11.5% controls	—	—	—	—	—	—
Harada et al., 1983	PB	10% cases-18.8% controls	—	—	—	—	—	—
Jansson and Jankovic, 1985	CB	RR=0.36 before PD onset; RR=0.46 after PD onset	—	—	—	—	—	Cancer, before and after PD pathology, less frequent than expected
Rajput et al., 1987	CB	—	—	—	—	—	—	—
•Before PD onset		16.1% obs-13.1% exp.	—	—	—	—	—	Cancer, before PD pathology, more common in PD than controls
•After PD onset		7.2% obs-10.9% exp.	—	—	—	—	—	Cancer less common in PD than controls
Möller et al., 1995	RB	Rr=0.9 (0.8-1)	0.5 (0.4- 0.6)	1.3 (0.9- 1.5)	1.2 (1-1.5)	2 (1-3.2)	1.6 (0.9- 2.7)	Cancer less common in PD than in Danish population; melanoma incidence increased
Minami et al., 2000	RB	SIR=0.8 (0.5-1.4)	—	—	5.5 (1.1-16)	—	—	Cancer less common in PD than Japan population
Elbaz et al., 2002 (before PD onset)	RB	OR=0.8 (0.5-1.3)	0.8 (0.3-2.2)	—	—	0.2 (0.02-1.7)	—	Cancer, before PD pathology, less common in PD than controls, except melanoma

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Table 8.1 – Studies on the association between PD and cancer.

Study		**All cancers,% or RR**	**Smoking related**	**Non smoking related**	**Breast**	**Skin**	**Melanoma**	**Comments**
Olsen et al., 2004	RB	SIR=0.9 (0.8-0.9)	0.6 (0.5-0.6)	1.2 (1.0-1.5)	1.3 (1.1-1.4)	2 (1.4-2.6)	1.3 (0.9-1.9)	Cancer less common in PD than in Danish population; melanoma incidence increased
D'Amelio et al., 2004 (before PD onset)	RB	OR=0.5 (0.1-1.9)	—	—	—	—	—	Cancer, before PD pathology, less common in PD than controls
Elbaz et al., 2005	RB	OR=1.6 (1.2-2.4)	0.8 (0.3-2.0)	—	—	1.8 (1.1-2.9)	—	Cancer more common in PD than controls
Bertoni et al., 2006	RB	—	—	—	—	—	2.2 (1.2-4.2)	—
Olsen et al., 2006 (before PD onset)	RB	OR=1 (1-1.1)	0.7 (0.6-0.8)	1 (0.9-1.1)	1.1 (0.9-1.3)	1.3 (1.1-1.4)	1.4 (1-2)	No association of overall cancer with PD; melanoma prevalence increased in PD
Constantinescu et al., 2007	RB	—	—	—	—	—	3.3 (1.1-7.8)	—
Driver et al., 2007a (before PD onset)	RB	OR=0.87 (0.6-1.3)	0.8 (0.4-1.6)	0.9 (0.6-1.4)	—	—	—	Cancer, before PD pathology, less common in PD than controls
Olsen et al., 2007	RB	—	—	—	—	RR= 1.21 (1.1-1.4)	RR= 1.9 (1.4-2.5)	—
Driver et al., 2007b	RB	RR=0.8 (0.6-1.2)	0.7 (0.4-1.4)	0.9 (0.6-1.4)	—	—	6.2 (1.8-21.4)	Cancer less common in PD than controls; melanoma incidence increased
Lo et al., 2010	—	—	—	—	—	—	—	—
•Before PD onset	RB	OR=0.9 (0.6-1.3)	0.5 (0.3-1.2)	1 (0.6-1.4)	0.9 (0.4-1.9)	—	1.3 (0.4-4.4)	—
•After PD onset	RB	RR=0.9 (0.7-1.1)	0.6 (0.4-1)	1 (0.7-1.3)	1.2 (0.5-2.7)	—	1.4 (0.7-3.0)	—

PD=Parkinson diseae; obs=observed; exp=expected; RR=relative risk; Rr= risk ratio; SIR=standard incidence ratio; OR=odds ratio; PB=population based; CB=clinic based; RB=registry based.

Second appendix

Table 8.2: Mutations identified in PINK1 gene.

PINK1 mutation	Exon	Protein Domain	Mutation type	Patient origin	References
Complete gene delPINK1	1-8	Complete protein	Deletion	Italian	Marongiu et al., 2007
Splice variant affecting exon 7 and 8	7-8	Kinase and C-terminus	Deletion	Italian	Marongiu et al., 2007
ex3-8del (breakpoints not mapped)	ex3-8del	Kinase and C-terminus	Deletion	Chinese	Nuytemans et al., 2010
ex4-8del (breakpoints not mapped)	ex4-8del	Kinase and C-terminus	Deletion	Sudanese	Cazeneuve et al., 2009
ex6-8del (breakpoints not mapped)	ex6-8del	Kinase and C-terminus	Deletion	Japanese	Li et al., 2005; Atsumi et al., 2006
ex7del (g.170750-171044del295)	ex7del	Kinase	Deletion	Spanish	Samaranch et al., 2010
ex7del (breakpoints not mapped)	ex7del	Kinase	Deletion	Brazilian	Camargos et al., 2009
c.-82G>A (g.155274G>A)	5'UTR	—	Missense	Italian	Bonifati et al., 2005
c.-21G>A (g.155335G>A)	5'UTR	—	Missense	Italian	Klein et al., 2005
c.-20C>T (g.155336C>T)	5'UTR	—	Missense	Italian	Bonifati et al., 2005
Lys24fs (g.155425-155456del32)	EX1	N-terminus (Transit)	Frameshift	French	Ibanez et al., 2006
Gly32Arg (g.155449G>A)	EX1	N-terminus (Transit)	Missense	Korean	Choi et al., 2008
Pro52Leu (g.155510C>T)	EX1	N-terminus (Transit)	Missense	American	Brooks et al., 2009
Arg58Val59 ins-GlyArg	EX1	N-terminus (Transit)	Frameshift	Japanese	Funayama et al., 2008
Leu63 (g.155544C>T)	EX1	N-terminus (Transit)	Synonymous	Caucasian	Valente et al., 2004b; Groen et al., 2004; Schlitter et al., 2005
Leu67Phe (g.155554C>T)	EX1	N-terminus (Transit)	Missense	Italian	Marongiu et al., 2008

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Table 8.2 – Mutations identified in PINK1 gene.

PINK1 mutation	Exon	Protein Domain	Mutation type	Patient origin	References
Arg68Pro (g.15558-15559GC>CT)	EX1	N-terminus (Transit)	Missense	Italian	Valente et al., 2004b; Marongiu et al., 2008
Ala78Val (g.155588C>T)	EX1	N-terminus (L1)	Missense	Japanese	Kumazawa et al., 2008
Cys92Phe (g.155630G>T)	EX1	N-terminus (L1)	Missense	Italian	Valente et al., 2004b
Arg98Trp (g.155647C>T)	EX1	N-terminus (TM)	Missense	Italian	Marongiu et al., 2008
Ile111Ser (g.155687T>G)	EX1	N-terminus (L2)	Missense	Italian	Marongiu et al., 2008
Gln115Leu (g.155699A>T)	EX1	N-terminus (L2)	Missense	Italian	Bonifati et al., 2005; Schlitter et al., 2005; Ishihara Paul et al., 2008
Ala124Val (g.155726C>T)	EX1	N-terminus (L2)	Missense	Italian	Marongiu et al., 2008
Cys125Gly (g.155728T>G)	EX1	N-terminus (L2)	Missense	Italian	Ibanez et al., 2006
Gln126Pro (g.155732A>C)	EX1	N-terminus (L2)	Missense	German	Prestel et al., 2008
Gln129fs (g.155740delC)	EX1	N-terminus (L2)	Frameshift	Tunisian	Ishihara Paul et al., 2008
Gln129Stop (g.155740C>T)	EX1	N-terminus (L2)	Nonsense	Tunisian	Ishihara Paul et al., 2008
IVS1+14G>A (g.155756G>A)	IVS1	—	Insertion	Indian	Biswas et al., 2010
IVS1-7A>G (g.159642A>G)	IVS1	—	Insertion	Italian	Valente et al., 2004b
Thr145Met (g.159695C>T)	EX2	N-terminus (L2)	Missense	Italian	Marongiu et al., 2008; Ishihara Paul et al., 2008
Arg147His (g.159701G>A)	EX2	N-terminus (L2)	Missense	Irish	Healy et al., 2004
Arg152Trp (g.159715C>T)	EX2	N-terminus (L2)	Missense	Italian	Ishihara Paul et al., 2008
Ala168Pro (g.159763G>C)	EX2	Kinase	Missense	Italian	Valente et al., 2004b; Bonifati et al., 2005
Val170Gly (g.159770T>G)	EX2	Kinase	Missense	Norwegian	Toft et al., 2007
Pro179 (g.159798C>T)	EX2	Kinase	Missense	Indian	Biswas et al., 2010
Lys186Asn (g.159819G>C)	EX2	Kinase	Missense	Serbian	Djarmati et al., 2006; Marongiu et al., 2008
Gly189 (g.159828G>A)	EX2	Kinase	Missense	American	Brooks et al., 2009
Gly193Arg (g.159838G>A)	EX2	Kinase	Missense	Taiwanese	Weng et al., 2007
Pro196fs (g.159848delC)	EX2	Kinase	Frameshift	Japanese	Kumazawa et al., 2008
Pro196Leu (g.159848C>T)	EX2	Kinase	Missense	Italian	Bonifati et al., 2005
Gly197 (g.159852T>C)	EX2	Kinase	Missense	Indian	Biswas et al., 2010

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Table 8.2 – Mutations identified in PINK1 gene.

PINK1 mutation	Exon	Protein Domain	Mutation type	Patient origin	References
Pro209Ala (g.159886C>G)	EX2	Kinase	Missense	Taiwanese	Lee et al., 2009
Pro209Leu (g.159887G>T)	EX2	Kinase	Missense	Serbian	Djarmati et al., 2006
Pro209Pro (g.159887G>A)	EX2	Kinase	Missense	—	Devine et al., 2011
Pro215Leu (g.159904C>T)	EX2	Kinase	Missense	—	Devine et al., 2011
Ala217Asp (g.159911C>A)	EX2	Kinase	Missense	Sudanese	Leutenegger et al., 2006
Gly227Arg (g.161702G>C)	EX3	Kinase	Missense	Tunisian	Ishihara Paul et al., 2008
Glu231Gly (g.161715A>G)	EX3	Kinase	Missense	North American	Rogaeva et al., 2004
Asn235Ile (g.161727A>T)	EX3	Kinase	Missense	North American	Rogaeva et al., 2004
Thr236 (g.161731A>C)	EX3	Kinase	Synonymous	North American	Rogaeva et al., 2004
Met237Val (g.161732A>G)	EX3	Kinase	Missense	Dutch	Macedo et al., 2009
Glu239Stop (g.161738C>T)	EX3	Kinase	Nonsense	Taiwanese	Hatano et al., 2004; Weng et al., 2007
Glu240Lys (g.161741G>A)	EX3	Kinase	Missense	North American	Rogaeva et al., 2004; Ibanez et al., 2006
Ala244Gly (g.161754C>G)	EX3	Kinase	Missense	Italian	Gelmetti et al., 2008
Arg246Stop (g.161759C>T)	EX3	Kinase	Nonsense	Taiwanese	Hatano et al., 2004; Tan et al., 2006; Ephraty et al., 2007
Arg246Gln (g.161760G>A)	EX3	Kinase	Missense	Indian	Biswas et al., 2010
Thr257Ile (g.161793C>T)	EX3	Kinase	Missense	Italian	Marongiu et al., 2008
Tyr258Stop (g.161797C>A)	EX3	Kinase	Nonsense	Asian	Tan et al., 2006; Keyser et al., 2010
Arg263Gly (g.166307A>G)	EX4	Kinase	Missense	North American	Rogaeva et al., 2004
Leu268Val (g.166322C>G)	EX4	Kinase	Missense	Asian	Tan et al., 2005; Marongiu et al., 2008
Leu268 (g.166324A>G)	EX4	Kinase	Missense	Taiwanese	Weng et al., 2007
His271Gln (g.166333C>A)	EX4	Kinase	Missense	Japanese	Hatano et al., 2004
Arg276Gln (g.166347G>A)	EX4	Kinase	Missense	Asian	Marongiu et al., 2008; Biswas et al., 2010
Leu278Val (g.166352C>G)	EX4	Kinase	Missense	Chinese	Guo et al., 2010
Arg279His (g.166356G>A)	EX4	Kinase	Missense	Italian	Klein et al., 2005; Choi et al., 2008
Arg279 (g.166357C>T)	EX4	Kinase	Synonymous	Norwegian	Toft et al., 2007

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Table 8.2 – Mutations identified in PINK1 gene.

PINK1 mutation	Exon	Protein Domain	Mutation type	Patient origin	References
Ala280Thr (g.166358G>A)	EX4	Kinase	Missense	Taiwanese	Tan et al., 2006
Thr282 (g.166366C>G)	EX4	Kinase	Synonymous	Indian	Biswas et al., 2010
Ser284 (g.166372C>T)	EX4	Kinase	Synonymous	Italian	Bonifati et al., 2005
Val293 (g.166399C>A)	EX4	Kinase	Missense	Brazilian	Godeiro-Junior et al., 2009
Pro296Leu (g.166407C>T)	EX4	Kinase	Missense	Italian	Valente et al., 2004b; Marongiu et al., 2008
Pro296 (g.166408T>G)	EX4	Kinase	Synonymous	North American	Rogaeva et al., 2004
Asp297fs (g.166409delG)	EX4	Kinase	Frameshift	Japanese	Kumazawa et al., 2008
Pro305Leu (g.166434C>T)	EX4	Kinase	Missense	Irish	Healy et al., 2004
Gly309Asp (g.166446G>A)	EX4	Kinase	Missense	Spanish	Valente et al., 2004a
Arg312 (g.166456G>A)	EX4	Kinase	Synonymous	Italian	Valente et al., 2004b
Thr313Met (g.116458C>T)	EX4	Kinase	Missense	Asian	Chishti et al., 2006; Kumazawa et al., 2008; Guo et al., 2008
Leu316 (g.166468C>T)	EX4	Kinase	Synonymous	North American	Rogaeva et al., 2004
Val317Ile (g.166469G>A)	EX4	Kinase	Missense	Caucasian	Abou-Sleiman et al., 2006; Marongiu et al., 2008; Gelmetti et al., 2008
Met318Leu (g.166472A>T)	EX4	Kinase	Missense	North American	Rogaeva et al., 2004; Djarmati et al., 2006; Brooks et al., 2009
IVS4-5G>A (g.167362G>A)	IVS4	—	Insertion	Italian	Valente et al., 2004a,b; Schlitter et al., 2005; Godeiro-Junior et al., 2009
Pro322Leu (g.167372C>T)	EX5	Kinase	Missense	Italian	Marongiu et al., 2008
Arg337 (g.167418C>T)	EX5	Kinase	Synonymous	American	Brooks et al., 2009
Ala339Thr (g.167422G>A)	EX5	Kinase	Missense	North American	Rogaeva et al., 2004; Abou-Sleiman et al., 2006; Marongiu et al., 2008
Ala340Thr (g.167425G>A)	EX5	Kinase	Missense	Italian	Valente et al., 2004b; Groen et al., 2004
Met341Ile (g.167430G>A)	EX5	Kinase	Missense	Taiwanese	Lee et al., 2009
Met342Val (g.167431A>G)	EX5	Kinase	Missense	Japanese	Kumazawa et al., 2008
Met342Ile (g.167433G>A)	EX5	Kinase	Missense	Chinese	Nuytemans et al., 2010

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Table 8.2 – Mutations identified in PINK1 gene.

PINK1 mutation	Exon	Protein Domain	Mutation type	Patient origin	References
Leu347Pro (g.167447T>C)	EX5	Kinase	Missense	Filipino	Hatano et al., 2004; Rogaeva et al., 2004
Gln355 (g.167472A>G)	EX5	Kinase	Synonymous	Italian	Valente et al., 2004b
Asp362His (g.167491G>C)	EX5	Kinase	Missense	Noth American	Rogaeva et al., 2004
Ser365 (g.167502C>T)	EX5	Kinase	Synonymous	Caucasian	Valente et al., 2004b; Brooks et al., 2009
Asn367Ser (g.167507A>G)	EX5	Kinase	Missense	Korean	Choi et al., 2008
Leu369Pro (g.167513T>C)	EX5	Kinase	Missense	French	Ibanez et al., 2006
Ala383Thr (g.170335G>A)	EX6	Kinase	Missense	Caucasian	Ibanez et al., 2006; Abou-Sleiman et al., 2006; Marongiu et al., 2008
Phe385Leu (g.170341T>C)	EX6	Kinase	Missense	Korean	Choi et al., 2008
Gly386Ala (g.170345G>C)	EX6	Kinase	Missense	French	Ibanez et al., 2006
Cys388Arg (g.170366T>C)	EX6	Kinase	Missense	Japanese	Li et al., 2005; Kumazawa et al., 2008
Gly395Val (g.170372G>T)	EX6	Kinase	Missense	Italian	Marongiu et al., 2008
Asp391 (g.170377T>C)	EX6	Kinase	Synonymous	Caucasian	Rogaeva et al., 2004; Myhre et al., 2008
Pro399Leu (g.170384C>T)	EX6	Kinase	Missense	Chinese	Tang et al., 2006
Tyr404 (g.170400C>T)	EX6	Kinase	Synonymous	Taiwanese	Weng et al., 2007
Arg407Gln (g.170408G>A)	EX6	Kinase	Missense	Asian	Fung et al., 2006; Guo et al., 2010
Gly409Val (g.170414G>T)	EX6	Kinase	Missense	French	Ibanez et al., 2006
Asn410 (g.170418C>T)	EX6	Kinase	Synonymous	Brazilian	Godeiro-Junior et al., 2009
Gly411Ser (g.170419G>A)	EX6	Kinase	Missense	Irish-German	Abou-Sleiman et al., 2006; Toft et al., 2007; Brooks et al., 2009
Pro416Arg (g.170435C>G)	EX6	Kinase	Missense	Jordanian	Myhre et al., 2008
Glu417Gly (g.170438A>G)	EX6	Kinase	Missense	Japanese	Hatano et al., 2004
23bp del ex7 (g.170800-170822del23)	EX7	Kinase	Deletion	Italian	Marongiu et al., 2007
Ser419Pro (g.170805T>C)	EX7	Kinase	Missense	Jordanian	Myhre et al., 2008
Thr420 (g.170810G>A)	EX7	Kinase	Synonymous	American	Brooks et al., 2009
Pro425Ser (g.170823C>T)	EX7	Kinase	Missense	North American	Rogaeva et al., 2004

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Table 8.2 – Mutations identified in PINK1 gene.

PINK1 mutation	Exon	Protein Domain	Mutation type	Patient origin	References
Ala427Glu (g.170830C>A)	EX7	Kinase	Missense	American	Brooks et al., 2009
Tyr431His (g.170841T>C)	EX7	Kinase	Missense	Caucasian	Abou-Sleiman et al., 2006
Trp437Arg (g.170859T>C)	EX7	Kinase	Missense	Turkish	Kumazawa et al., 2008
Trp437Stop (g.170861G>A)	EX7	Kinase	Nonsense	Italian	Valente et al., 2004a; Bonifati et al., 2005
Gly440Glu (g.170869G>A)	EX7	Kinase	Missense	Tunisian	Ishihara Paul et al., 2008
Ile442Thr (g.170875T>C)	EX7	Kinase	Missense	Italian	Valente et al., 2004b
Asn451Ser (g.170902A>G)	EX7	Kinase	Missense	Caucasian	Abou-Sleiman et al., 2006
Tyr454 (g.170912C>T)	EX7	Kinase	Synonymous	Taiwanese	Weng et al., 2007
Gln456Stop (g.170932C>T)	EX7	Kinase	Nonsense	Italian	Bonifati et al., 2005
Arg464His (g.170941G>A)	EX7	Kinase	Missense	Italian	Valente et al., 2004b
Glu476Lys (g.170976G>A)	EX7	Kinase	Missense	Italian	Valente et al., 2004b
Val482Met (g.170994G>A)	EX7	Kinase	Missense	Japanese	Kumazawa et al., 2008
Leu489Pro (g.171016T>C)	EX7	Kinase	Missense	North American	Rogaeva et al., 2004
Arg492Stop (g.171024C>T)	EX7	Kinase	Nonsense	Taiwanese	Hatano et al., 2004
IVS7+1G>A (g.171039G>A)	IVS7	—	Insertion	Spanish	Samaranch et al., 2010
IVS7+14C>G (g.171052C>G)	IVS7	—	Insertion	Italian	Valente et al., 2004b
Pro498Leu (g.172245C>T)	EX8	Kinase	Missense	Norwegian	Toft et al., 2007
Lys520fs (g.172309delG)	EX8	C-terminus	Frameshift	Korean	Choi et al., 2008
Asn521Thr (g.172314A>C)	EX8	C-terminus	Missense	Italian	Valente et al., 2004b
Asp525Asn (g.172325G>A)	EX8	C-terminus	Missense	Italian	Valente et al., 2004b
Asp525fs (g.172325-172326insTTAG)	EX8	C-terminus	Frameshift	Italian	Bonifati et al., 2005
534-535insQ (g.172354-172355insCAA)	EX8	C-terminus	Insertion	Italian	Klein et al., 2005
Asp537Thr (g.172361G>A)	EX8	C-terminus	Missense	Italian	Marongiu et al., 2008
Asn542Ser (g.172377A>G)	EX8	C-terminus	Missense	Japanese	Kumazawa et al., 2008

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Table 8.2 – Mutations identified in PINK1 gene.

PINK1 mutation	**Exon**	**Protein Domain**	**Mutation type**	**Patient origin**	**References**
Cys549fs (g.172399-172402delTGTG)	EX8	C-terminus	Frameshift	French	Ibanez et al., 2006
Cys575Arg (g.172475T>C)	EX8	C-terminus	Missense	Caucasian	Abou-Sleiman et al., 2006
Ser576 (g.172496A>G)	EX8	C-terminus	Synonymous	North American	Rogaeva et al., 2004
Stop582Leu (g.172497G>T)	EX8	C-terminus	Frameshift	Norwegian	Toft et al., 2007
c.*37T>A (g.172535T>A)	3'UTR	—	—	North American	Rogaeva et al., 2004; Schlitter et al., 2005
c.*40G>A (g.172538G>A)	3'UTR	—	—	North American	Rogaeva et al., 2004; Biswas et al., 2010

Data from Deas et al. and `http://www.molgen.ua.ac.be` Deas et al., 2009; Sobrido et al., 2012.
Putative signal peptide, Transit; N-terminus L1: after signal peptide and before TM;
N-terminus L2: after TM.

Publications

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2. A. Berthier, **J. Jiménez-Sáinz**, and R. Pulido: PINK1 regulates histone H3 trimethylation and gene expression by interaction with the polycomb protein EED/WAIT1. Submitted.
3. **J. Jiménez-Sáinz**, M. Gegg, A. Berthier, A. Abramov, M. Cooper, J-W. Taanman, R. Pulido, A. Schapira: PINK1 kinase inactive and C-terminal truncation are important for maintenement of mitochondrial function and mitophagy processes. Manuscript in preparation.
4. **J. Jiménez-Sáinz**, Berthier, R. Pulido: PINK1 functions as a tumor suppresor protein in breast carcinoma cells. Manuscript in preparation.

Mon ocupation préférée.
Aimer.
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