University of Cyprus
Department of Biological Sciences

The PINK1/Parkin-dependent mitophagy pathway and its role in Parkinson's disease

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A. ABSTRACT

Parkinson's disease is the second most prevalent neurodegenerative disease, surpassed only by Alzheimer's disease. The disease is relentless, progressive, and incurable, and there is a great need to understand its causes and develop new treatments. A distinctive pathology in most cases of Parkinson's disease is the loss of dopaminergic neurons in the Substantia Nigra. This depletion of dopaminergic neurons has been mostly part due to the appearance of Lewy bodies containing misfolded, toxic α-synuclein within. Most recently, Parkinson's disease has also been linked to the mitophagy pathway since genetic studies identified numerous genes linked to this disease. The processes that keep mitochondria networks healthy can be impaired in Parkinson's disease, and mitochondria are unable to sustain proper neuronal function. Mitophagy is a selective autophagy process which targets the mitochondria for degradation. Parkinson's disease has been shown to be linked with one of the three distinct pathways of mitophagy namely the PINK1/Parkin-dependent pathway. In this bibliographical review we will extensively discuss the work leading to delineation of this pathway as well as more recent work involving novel targeting approaches for the development of a potential new drug to halt the neurodegeneration of dopaminergic neurons and therefore treat Parkinson's disease.

Keywords: Autophagy, Mitophagy, Parkinson's disease, Neurodegenerative disorders, Neurodegeneration, PINK1, Parkin, ubiquitination, and intracellular signalling pathways.

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C. COMPOSITION OF THE EXAMINATION COMMITTEE

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D. SEMINAR ANNOUNCEMENT

University of Cyprus
Department of Biological Sciences

BIO 680 Scientific Methodology in Molecular Biology

Student Presentation

Wednesday, 13 December 2023 at 09:30
Building ΘΕΕ 02, Room B172, Panepistimioupoli Campus

This seminar is open to the public

Irene Theodosiou

Thesis Supervisor: Special Teaching Staff, Dr. Annita Charalambous

"The PINK1/Parkin-dependent mitophagy pathway and its role in Parkinson's disease"

Parkinson's disease is the second most prevalent neurodegenerative disease, surpassed only by Alzheimer's disease. The disease is relentless, progressive, and incurable, and there is a great need to understand its causes and develop new treatments. A distinctive pathology in most cases of Parkinson's disease is the loss of dopaminergic neurons in the Substantia Nigra. This depletion of dopaminergic neurons has been mostly part due to the appearance of Lewy bodies containing misfolded, toxic α-synuclein within. Most recently, Parkinson's disease has also been linked to the mitophagy pathway since genetic studies identified numerous genes linked to this disease. The processes that keep mitochondria networks healthy can be impaired in Parkinson's disease, and mitochondria are unable to sustain proper neuronal function. Mitophagy is a selective autophagy process which targets the mitochondria for degradation. Parkinson's disease has been shown to be linked with one of the three distinct pathways of mitophagy namely the PINK1/Parkin-dependent pathway. In this bibliographical review we will extensively discuss the work leading to delineation of this pathway as well as more recent work involving novel targeting approaches for the development of a potential new drug to halt the neurodegeneration of dopaminergic neurons and therefore treat Parkinson's disease.

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1. INTRODUCTION

1.1. Parkinson's Disease

With approximately 7 million cases worldwide, Parkinson's disease (PD) is the most prevalent neurodegenerative illness after Alzheimer's disease. This severe and progressive disease is age-related and most commonly observed in adults over the age of 50 but cases have been recorded in younger individuals. Patients with PD present damage in the midbrain region called Substantia Nigra pars compacta (SNpc) which is associated with motor symptoms. The SNpc is built by dopaminergic (DA) neurons and belongs to a significant brain circuit that is essential for enabling movement. As depicted in *Figure 1*, DA neurons found in the SNpc gradually die in PD leading to the malfunction of movement ability (1).

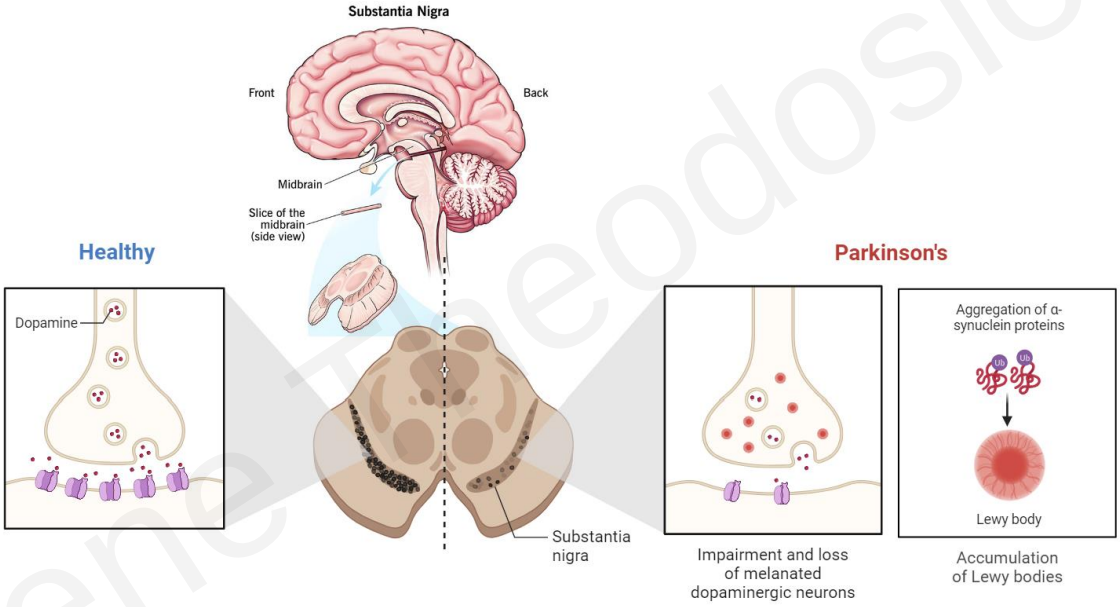


Figure 1. Lewy bodies in SNpc neurons. *Representation of the human brain and the position of the Substantia Nigra. In Parkinson's disease motor DA neurons develop Lewy bodies which are mostly composed of the accumulation of α-synuclein. Healthy DA neurons have high Dopamine neurotransmission in comparison with DA neurons in Parkinson's with lower Dopamine neurotransmission (BioRender illustration).*

1.1.1. Parkinson's Disease Pathology and Symptoms

PD is a complex disorder with its molecular aetiology poorly understood. The most distinctive characteristic in PD is the appearance of Lewy bodies in the DA neurons. Lewy bodies are deposits containing an abnormal, misfolded protein called α-synuclein (2). These molecules can assemble into small, repeated units called oligomers and then longer fibrils which are harmful to neurons

(3). More specifically, in DA neurons α-synuclein is responsible for dopamine homeostasis regulation (4). The physiological role of α-synuclein in neurons involves neurotransmitter release by participating in the vesicular trafficking machinery at the presynaptic terminal. The accumulation of abnormal α-synuclein in presynaptic terminals, however, is responsible for synaptic dysfunction and synaptotoxicity, leading to cell death by degeneration (5). The dysfunction of α-synuclein in the DA neurons is responsible for the impairment of vesicle docking and recycling, thus preventing the incorporation of dopamine into vesicles. This pathological event leads to cell death in PD (4) and over time more areas of the nervous system are affected through the transmission of the misfolded α-synuclein (3). As a result of neuronal loss, PD patients appear to have symptoms reflecting movement deficits, for example, tremor, rigidity, bradykinesia, and postural instability. These movement-related symptoms worsen when a person is at rest. Also, some non-motor symptoms affect PD patients like cognitive impairment, olfactory and sleep dysfunction, or psychiatric symptoms like anxiety and depression. One of the many challenges in PD is that by the moment of the clinical evaluation, 60-80% of the dopamine producing neurons in the SNpc are already degenerated (6).

1.1.2. Parkinson's Disease Causative Factors

The causes of PD are not fully understood but they can vary since a complex mix of aging, genetics, and environmental factors are responsible. These 3 factors converge to different degrees along a spectrum. At one end of the spectrum, genetic factors are the result of familial PD caused by mutations in different genes and constitute 10% of PD cases. On the other end, environmental factors (like the neurotoxin MPTP which inhibits the mitochondrial Complex I) are associated with a few cases of PD. However, most cases of PD are sporadic (90%) and result from a complex combination between genes and environment, played out against the background of age, which remains the greatest risk factor (4). Cellular ageing results from the accumulation of damage brought on by multiple mechanisms, including the dysregulation of the autophagic machinery (7).

The most frequent PD cases drop in the idiopathic category. Despite that, 22 genes (*Table 1*) have been associated with familial PD (8). Some of the mutated genes in PD are PARK2 and PARK6 which express Parkin and PTEN-induced kinase 1 (PINK1), respectively. These mutated forms of PARK2 and PARK6 have been shown to be responsible for mitochondria homeostasis and more precisely disrupting the process of mitophagy (9). Moreover, mutations found in genes encoding either PINK1 or Parkin are the main cause of autosomal recessive early onset PD (9).

Overall, the majority of these PD-causing genes either control or participate in the autophagic pathway, indicating the pathway's significance in neurodegeneration (8).

Loci	Gene	Protein	Position	Inheritance	Onset
PARK1	*SNCA*	Alpha-synuclein	4q21	AD, sporadic	Early or late
PARK2	*PRKN*	Parkin	6q25–q27	AR, sporadic	Early
PARK3	*Unknown*	Unknown	2p13	AD	Late
PARK5	*UCHL1*	Ubiquitin C-Terminal Hydrolase L1	4p14	AD	Late
PARK6	*PINK1*	PTEN-induced putative kinase 1	1p35–p36	AR	Early
PARK7	*DJ-1*	Protein DJ-1	1p36	AR	Early
PARK8	*LRRK2*	Leucine-rich repeat kinase 2	12q12	AD, sporadic	Early or late
PARK9	*ATP13A2*	ATPase 13A2	1p36	AR	Early
PARK10	*Unknown*	Unknown	1p32	Unknown	Unknown
PARK11	*GIGYF2*	GRB10 interacting GYF protein 2	2q36–q37	AD	Late
PARK12	*Unknown*	Unknown	Xq21–q25	Unknown	Unknown
PARK13	*HTRA2*	Serine peptidase 2	2p13	AD	Late
PARK14	*PLA2G6*	Phospholipase A2 Group VI	22q12–q13	AR	Early
PARK15	*FBX07*	F-Box protein 7	22q12–q13	AR	Early
PARK17	*VPS35*	Vacuolar protein sorting 35	16q11.2	AD	Late
PARK18	*EIF4G1*	Eukaryotic translation initiation factor 4 gamma, 1	3q27.1	AD	Late
PARK19	*DNAJC6*	DNAJ subfamily C member 6	1p31.3	AR	Early
PARK20	*SYNJ1*	Synaptojanin-1	21q22.11	AR	Early
PARK21	*DNAJC13*	DNAJ subfamily C member 13	3q22.1	AD	Early
PARK22	*CHCHD2*	Coiled coil-helix-coiled coil-helix domain 2	7p11.2	AD	Late
PARK23	*VPS13C*	Vacuolar protein sorting 13 homolog C	15q22.2	AR	Early
-	*GBA*	Glucocerebrosidase	1q21	AD	Unknown

Table 1. PD-related genes. *The table lists the genes related to PD pathogenesis together with the locus of genes, expressed proteins, chromosome position, hereditary properties, and the onset of the disease (8).*

1.1.3. Mitophagy in Parkinson's Disease

Dopaminergic neuronal loss caused by mitochondrial dysfunction has been recognised in PD for over 30 years, but the exact mechanisms that drive the disease progression remain unclear. More specifically it has been shown that the pathogenic α-synuclein binds to mitochondria, and more specifically to the translocase of the outer mitochondrial membrane 20 (TOMM20). This inhibits mitochondrial protein import leading to mitochondrial dysfunction (10). Furthermore, mitochondria cannot maintain appropriate neuronal function in PD due to abnormalities in the processes of mitochondria dynamics, including biogenesis, fission, fusion, and mitophagy. The mitophagy pathway replaces lost mitochondria with new ones when they age or sustain damage. Nevertheless, the recycling purpose of mitophagy is disrupted in PD leading to the accumulation of dysfunctional mitochondria (2). While it is challenging to fully comprehend the multifactorial nature of PD progression, there are a lot of viable therapeutic targets available for reducing neurodegeneration. One example of such pharmacological effort is to enhance the clearance of damaged mitochondria by boosting the respective autophagic pathway called mitophagy (11).

1.2. Mitophagy in pathology

Given the critical role that mitophagy plays in preserving mitochondrial homeostasis, it is not surprising that a variety of disorders are associated with mitochondrial malfunction. Such disorders include neurodegenerative diseases like Parkinson's disease (PD), Alzheimer's disease, Huntington's disease (HD) and Amyotrophic lateral sclerosis (ALS). For most neurodegenerative diseases cognitive function declines with aging (7, 12). This is partially explained by the post-mitotic nature of neurons that are unable to undergo cytokinesis and thus aggregated proteins cannot be diluted by cell division and are accumulated (11). Protein aggregations are aggregate structures composed by abnormal proteins which tend to be insoluble (13). The autophagy mechanisms help remove the accumulation of these abnormal protein aggregates and is therefore considered a primary target for neurodegenerative therapeutics (14). However, autophagy-dependent protein aggregate removal in neuronal cells becomes a challenge as people age and these processes become less effective in the brain (11). Other disorders linked with abnormal mitophagy are cancer, heart diseases (cardiomyopathy) and liver diseases (diabetes). Mitophagy is also intensively associated with aging as reactive oxygen species (ROSs) and mitochondrial DNA (mtDNA mutations) increase (12).

1.3. Autophagy

Cells use a process known as autophagy, to break down unwanted material, like intracellular components or organelles. Autophagy, which means self-eating, is a highly conserved eukaryotic recycling process that is important for normal cell growth and development (15). The decomposition of protein or organelle aggregates takes place through the formation of autophagosomes which are double-membraned vesicles that direct the respective cargo to the lysosomes. In the lysosomes specific enzymes, called hydrolases, catalyse the targeted cellular components (9). Autophagy is considered a quality control guardian as it regulates cell homeostasis and cell survival by degrading intracellular components and provides the respective degradation products (16). It can be distinguished into three groups: chaperone mediated autophagy (CMA), microautophagy and macroautophagy (*Figure 2*). The mechanisms of the distinct types of autophagy differ but the goal of all three is mutual (15).

Macroautophagy can be further divided as either a non-selective degradation process or a highly selective one. What characterizes selective autophagy is the ability to recognize specific cargo by a set of soluble or membrane-bound cargo receptors called selective autophagy receptors (SARs)

(17, 18). There are many diverse types of selective autophagy depending on the targeted cargo (*Table 2*) (9, 11). Mitophagy, which is the main topic of this paper, carries out the targeted degradation of attenuated mitochondria in lysosomes via the autophagy machinery.

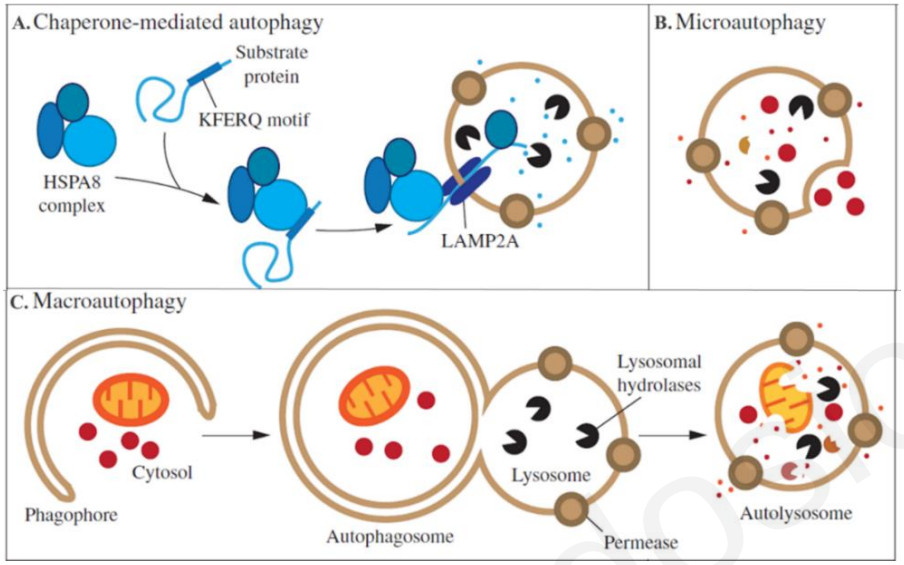


Figure 2. The three groups of autophagy: A. Chaperone-mediated autophagy (CMA): *targets proteins that contain KFERQ-motif which is recognized by the HSPA8 complex. The complex drives the targeted protein to the lysosome and is then transfused into the lysosome via the receptor LAMP2A,* ***B. Microautophagy:*** *invagination of the lysosomal membrane, which allows for direct cargo uptake,* ***C. Macroautophagy:*** *initial formation of the phagophore which closes to form the autophagosome and it fuses with the lysosome in order to degrade the cargo (15).*

Selective Autophagy Type	Target Cargo
Mitophagy	Mitochondria
Glycophagy	Glycogen
ER-phagy	Endoplasmic reticulum
Lysophagy	Lysosome
Nucleophagy	Nucleus
Pexophagy	Peroxisomes
Xenophagy	Cellular pathogens
Aggrephagy	Abnormal protein aggregates
Ribophagy	Ribosomes

Table 2. Types of selective autophagy. *List of selective autophagy types and their target cargo (9).*

1.3.1. Macroautophagy Pathway

Macroautophagy starts with the phagophore formation. The main players that initiate the engulfment of the targeted cargo include the ATG12-5-16L1 complex (responsible for phagophore elongation), WIPI2, LC3 and ATG4/3/7. The autophagy-related proteins (ATG) and WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) are responsible for the LC3 (ubiquitin-like ATG8 family) conjugation with phosphatidylethanolamine (PE) on the initial stages of phagophore formation. For this step to take place the double membrane needs to be marked with RAB11A (RAB11A positive compartment). This process continues until the closure of the phagophore and the formation of the autophagosome. The autophagosome then carries the respective cargo to the lysosome for degradation. In the case of non-specific macroautophagy (in bulk, *Figure 3i*) the triggering signal can be either nutrient or energy deprivation. The pathway of selective macroautophagy (*Figure 3ii*) is mediated by specific sequestration of substrates via adaptor proteins (surface proteins which are ubiquitinated) and components of the developing autophagosome, like LC3 and GABARAP (gamma-aminobutyric-acid-receptor-associated protein). SARs (like p62) are also important for recognising their cargo and through LC3-interacting region (LIR) motifs they connect the cargo protein with the autophagosome which can then transport this cargo to the lysosomes for degradation (11).

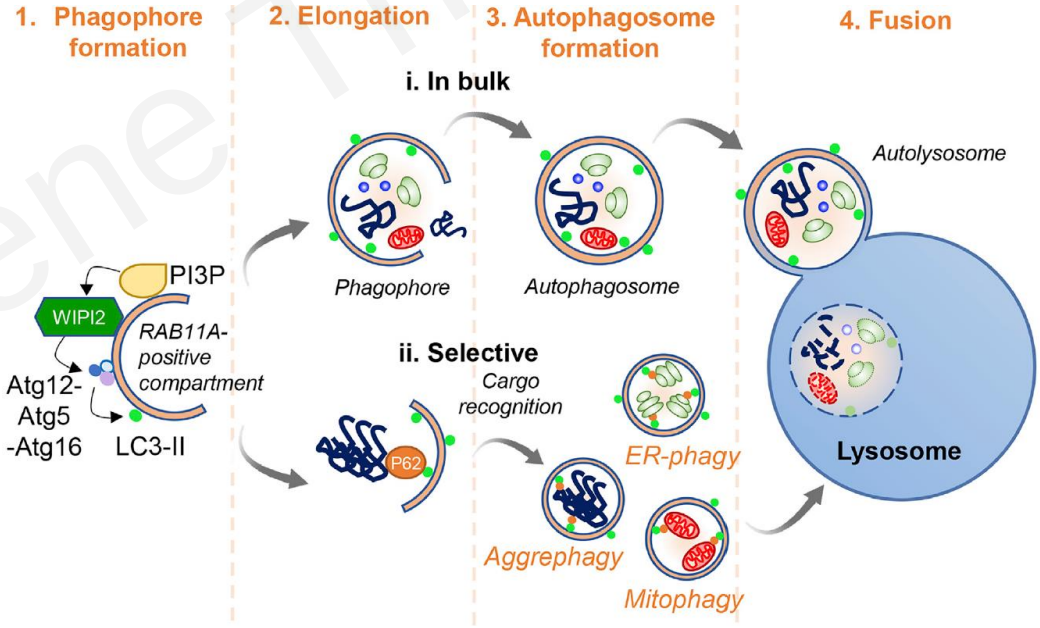


Figure 3. Schematic representation of macroautophagy steps i: non-selective and ii: selective. ***1.*** *The step of phagophore formation which is a double-membraned structure that engulfs cell components targeted for degradation.* ***2.*** *The phagophore's edges elongate and* ***3.*** *close to form a*

structure called autophagosome. ***4.*** *After the autophagosome formation, it fuses with the lysosomal membrane for cargo degradation. The presence of autophagy cargo receptors makes the autophagy process a selective one. Here the example of p62 receptor is shown (11).*

1.4. Mitophagy

The process of mitophagy involves the selective degradation of a cell's damaged mitochondria. This type of selective macroautophagy is conserved among species (yeast to humans). It is essential in mammalian cells for mitochondrial cell preservation and the clearance of impaired mitochondria (19). It works as a neuroprotective machinery as well by controlling the quality and quantity of the mitochondria. Also, mitophagy is a critical mechanism for cell differentiation and such an example are the red blood cells which in order to mature, need to remove the mitochondria via the mitophagy pathway (15).

Mitochondria are organelles of utmost importance in the cell. They play a vital role in supplying energy through the cellular respiration system and the production of adenosine triphosphate (ATP), phospholipid biosynthesis, and apoptosis induction. Because of their extreme dynamism, mitochondria can unite or split into smaller forms in response to the energy needs of the cell, especially in neurons. They can also be transported to areas of a cell that need the most function (19).

For cells to maintain a healthy network of mitochondria several steps need to be followed and these are Biogenesis, Fission, Fusion, and Mitophagy (*Figure 4*). Mitochondrial membrane depolarization caused by stress or damage results in dysfunctional mitochondria which are separated (Fission) from the healthy ones and targeted for degradation. This process requires a protein called Dynamin-related protein 1 (DRP1) which is responsible for the above separation and the isolation of the damaged mitochondria away from the healthy pool. The healthy mitochondria maintain their network when they fuse together and they mix their membranes, proteins, and mtDNA (Fusion). The damaged mitochondria are separated from healthy ones and degraded in a specialized type of autophagy called Mitophagy (20). Mitochondria besides being the cell's powerhouse, they also produce ROSs which derive from the electron transport chain. Damaged mitochondria can build up and eventually become a source of ROSs when mitophagy is disrupted. An increase in ROSs can result in oxidative stress, which is responsible for protein degradation, apoptosis, and mutations in mtDNA (21).

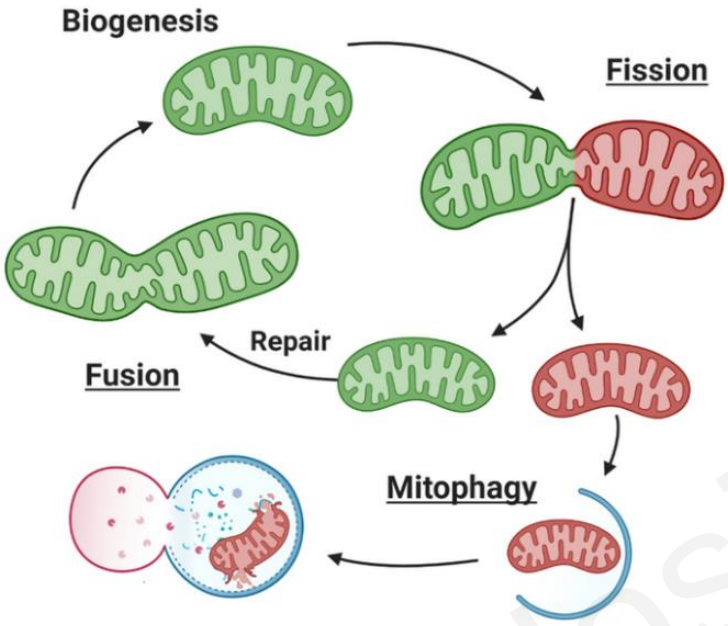


Figure 4. Mitochondrial Homeostasis. *Mitochondria follow a cycle of events which include the steps of Biogenesis, Fission, Fusion and Mitophagy. Damage or stress-induced depolarization of the mitochondrial membrane produces dysfunctional mitochondria (red), which are isolated by Fission events from the healthy ones (green) and destined for destruction via mitophagy (20).*

1.4.1. Molecular Pathways of Mitophagy

Depending on the method used to attract the phagophore membrane to mitochondria, there are diverse types of mitophagy. There is the receptor-mediated pathway, the lipid-mediated pathway, and the ubiquitin-mediated pathway. The pathway most recently found in cells is the specific ubiquitin pathway called PINK1/Parkin-dependent mitophagy (22). In the receptor-mediated mitophagy pathway the link of mitochondria to the autophagosome is mediated by direct contact of specific receptors like FUNDC1, AMBRA1 and NIX receptors (*Figure 5A*). In the lipid-mediated mitophagy pathway the link between mitochondria and autophagosome is mediated by lipids like cardiolipin and ceramide (*Figure 5B*). In the ubiquitin-mediated mitophagy pathway the connection between mitochondria and the autophagosome is mediated by ubiquitin and E3 ligases like SIAH1 and MUL1 (*Figure 5C*). The specific PINK1/Parkin ubiquitin-dependent mitophagy pathway is closely related to the development of PD (9) since mutations in their respective genes have been found to cause loss of protein function, disruption of mitophagy and early onset PD (*Figure 5D*) (23).

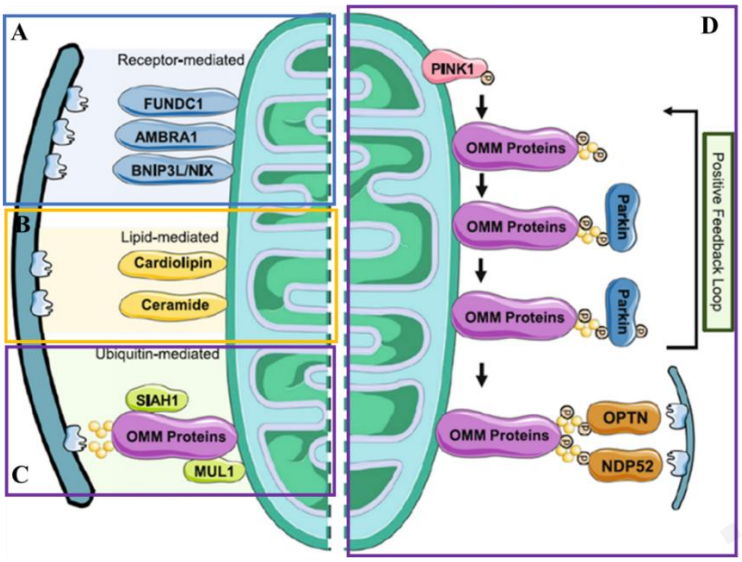


Figure 5. Mitophagy pathways. ***A.*** *Receptor-mediated pathway via receptors like FUNDC1, AMBRA1 and NIX,* ***B.*** *Lipid-mediated pathway via lipids like cardiolipin and ceramide,* ***C.*** *Ubiquitin-mediated pathway via ubiquitin and E3 ligases like SIAH1 and MUL1,* ***D.*** *PINK1-Parkin-dependent mitophagy via a specific kinase, PINK1, and a specific E3 ligase, Parkin. After the ubiquitination of the OMM proteins the receptors OPTN or NDP52 bring mitochondria in contact with the autophagosome (23).*

1.4.2. PINK1/Parkin-dependent Mitophagy

The most frequent pathway that cells use for mitophagy is called PINK1/Parkin-dependent mitophagy. For depolarized mitochondria, PINK1/Parkin-dependent mitophagy is the primary regulator of turnover (24).

Parkin protein, an E3 ubiquitin ligase, and PINK1 kinase protein work together for the ubiquitin-dependant pathway of mitophagy (25). Normally functioning mitochondria have an electrochemical H^+ gradient in their inner mitochondrial membrane that drives ATP synthesis. Physiologically in mitochondria, PINK1 is sent into the mitochondrial inner membrane via the TOM (translocase of outer membrane) / TIM (translocase of inner membrane) target sequence complexes, and the mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL) cleave and eventually degrade PINK1. Otherwise PINK1 if not cleaved, is inserted on the outer mitochondrial membrane (OMM) and leads to mitophagy (25). However, damaged mitochondria (by stress resulting in depolarization) cannot maintain the H^+ gradient and consequently block mitochondria mechanisms. The phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1)/Parkin complex have the role of phosphorylating ubiquitin on

the OMM to induce mitophagy upon unwanted mitochondria (26). First the reduction in the mitochondrial membrane potential ($\Delta\psi_m$) attracts PINK1 at the OMM and becomes activated after its dimerization and autophosphorylation (*Figure 6*). PINK1 then phosphorylates Ubiquitin chains on OMM proteins such as TOM complex members which are formed by another E3-ubiquitin ligase (March5). Ser65 phosphorylated Ubiquitin chains (p-Ser65-Ub) enhance the recruitment of Parkin to OMM where it is phosphorylated by PINK1 at Ser65 and becomes fully activated (27). With Parkin now active and placed on the mitochondria, it can further ubiquitylate multiple OMM proteins (such as VDAC1) to target them for recognition by the autophagy machinery. PINK1 together with Parkin can generate a self-amplifying feedback loop. Adaptor/Receptor proteins, like nuclear dot protein-52 (NDP52) or optineurin (OPTN), are the connection between LC3-coated vesicles and the OMM proteins tagged with p-Ser65-Ub chains. The LC3 interaction leads to sequestration of the phagophore and the formation of the autophagosome. The autophagosome then carries the mitochondria cargo to the lysosomes for the final degradation (15, 27).

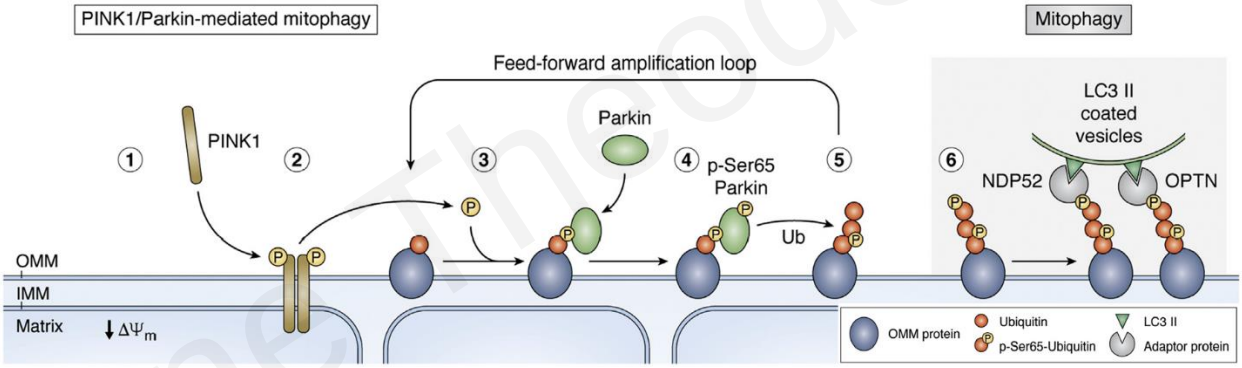


Figure 6. PINK1/Parkin-dependent mitophagy. *Damaged mitochondria are recognized by PINK1 which then recruits Parkin on previously p-Ser65-Ub OMM proteins. Parkin further ubiquitinates OMM proteins which are then recognised by receptors like NDP52 or OPTN. These receptors recognise LC3-coated vesicles via their LIR motifs and drive the targeted mitochondria for sequestration by the autophagosome which is driven to the lysosome for degradation (27).*

It is a fact that the life expectancy of the world population is rising and aging has become the biggest risk factor for neurodegeneration, which explains the number of patients with neurodegenerative disorders. In order to understand the aetiology of Parkinson's disease, there is a need for detail description of the PINK1/Parkin-mediated mitophagy pathway which might be the future key for a potential treatment. The aim of this review is to describe the link between PD

and mitophagy and how can various targets help in the successful therapy or even cure of this devastating disorder.

2. OVERVIEW

The intricate relationship between mitophagy, a selective autophagic process responsible for the removal of damaged mitochondria, and PD, a yet uncurable disease, represents a critical avenue of investigation (28). Initially the genetic association of PD with mitochondrial dysfunction was discovered by clinical genome-wide association studies (GWAS). These studies have associated specific genes that are known to modulate mitochondrial functions and cause clinical PD symptoms (29, 30). By combining recent advancements in the genetics of mitochondrial disease with improvements in the scale and analysis of PD GWAS data, the aim of more recent work was to assess the role of mitochondrial function in sporadic PD comprehensively (31). Genetic studies first indicated the link between PD pathogenesis and mitochondrial function which was further explored by *in vitro* research (30).

By examining the molecular mechanisms within the PINK1/Parkin pathway and their association with the pathogenesis of Parkinson's disease, this review aims to shed light on the potential therapeutic interventions and diagnostic strategies for this debilitating neurodegenerative disorder.

2.1. *In vitro* delineation of the PINK1/Parkin-dependent mitophagy

Starting off, it is important to go through scientific literature that delineates the PINK1/Parkin-mediated mitophagy pathway to better understand the association with PD pathogenesis (1). Since the 1980s, there has been evidence linking not only familial but also sporadic PD to abnormalities in the mitochondria (32). The PARK6 (PINK1 expression) and PARK2 (Parkin expression) genes were identified as causal genes for hereditary early onset autosomal recessive PD and are extensively researched in many *in vitro* based articles (28, 33). The tiggers of mitophagy found in mammalians are stress and developmental factors which cause mitochondrial depolarization. To create the same cell state in experimental studies mitochondrial depolarizing agents are used such as CCCP (Carbonyl cyanide m-chlorophenyl hydrazone, a proton-selective ionophore) or antimycin A (an inhibitor of the respiratory complex III) to cause mitochondrial depolarization and explore the mitophagy pathway (28).

2.1.1. Link of Parkin to mitophagy

Initial work in human cell lines showed that Parkin is translocated to depolarized mitochondria and attracts the autophagy machinery to degrade the mitochondria via the lysosome. This fundamental research by Narendra *et. al.* 2008 (34) showed that Parkin promotes mitophagy *in vitro*. They demonstrated, in two different cell lines, that Parkin is specifically drawn to malfunctioning mitochondria where it mediates the autophagosomes' eventual engulfment and degradation of the mitochondria via the lysosome. The initial evidence show that Parkin accumulates on depolarized mitochondria both on HEK293 cells - a cell line that uses the PRK8 monoclonal antibody to express Parkin at high levels-(*Figure 7a, b, c)* and on rat cortical neurons (*Figure 7d*). This indicated that Parkin is predominantly located in the cytosol, but also colocalizes with a subset of small and fragmented mitochondria (34).

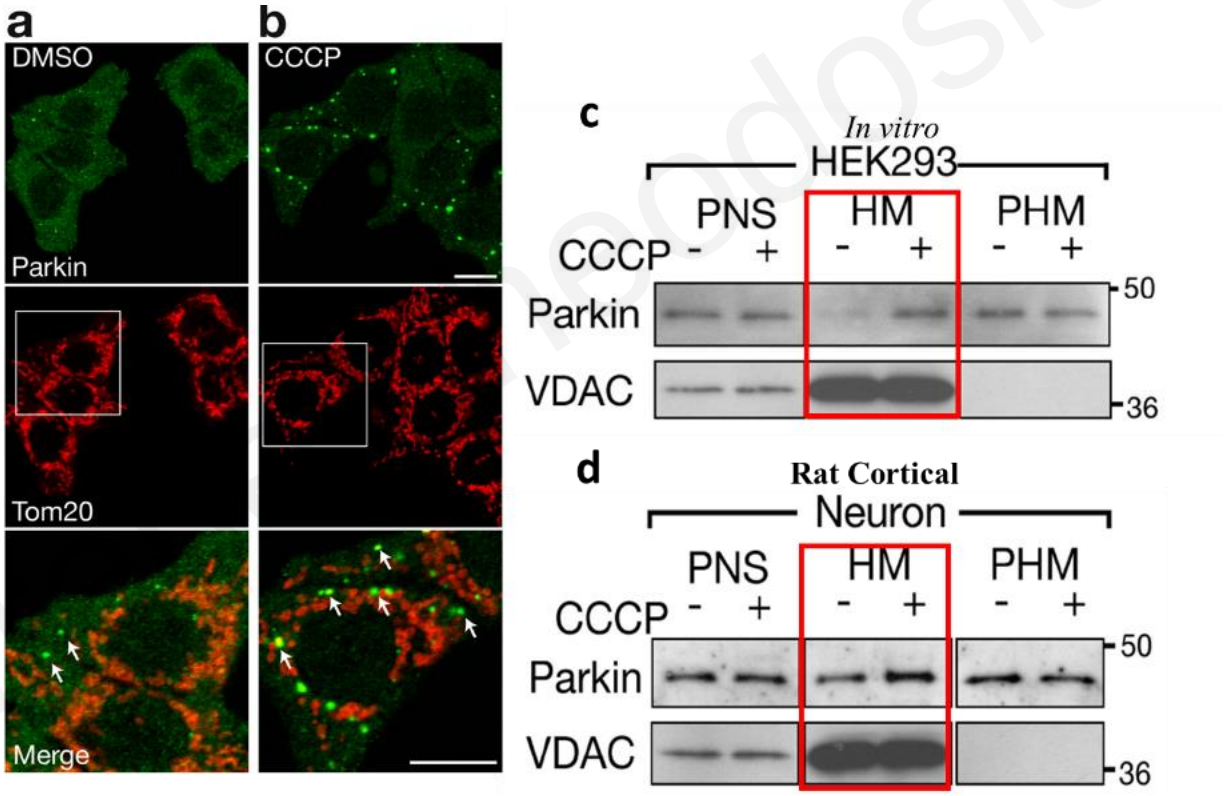
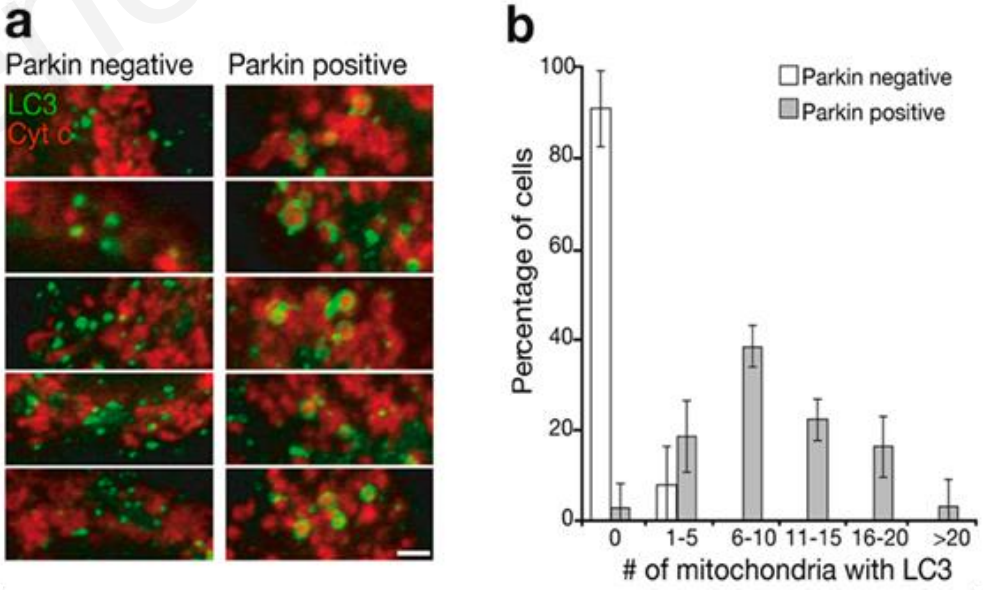


Figure 7. Colocalization of Parkin with depolarized mitochondria. *Subcellular localization of endogenous Parkin (a) in HEK293 cells treated with DMSO (control) and in HEK293 cells treated with CCCP. Green marks endogenous Parkin and red marks the mitochondria (Tom20 marker). Merging the Parkin and mitochondrial images shows their colocalization (arrows). Immunoblots for (c) HEK293 cells and (d) rat cortical neurons for endogenous Parkin and VDAC as a*

mitochondrial control. PNS=post-nuclear supernatant, HM=mitochondrial-rich heavy membrane pellet, PHM=post – heavy membrane supernatant, respectively (34).

Moreover, they indicated that HeLa cells which express Parkin (Parkin-positive) have more overlap between autophagosomes and mitochondria, compared to Parkin-negative cells, indicating the potential role of Parkin in mediating the engulfment of mitochondria by autophagosomes (*Figure 8a*). A chart was also created (*Figure 8b*) to show in how many Parkin-positive or Parkin-negative HeLa cells there are mitochondria which were engulfed by autophagosomes containing LC3. The results showed that nearly all cells with no Parkin had no association between mitochondria and autophagosomes, but for a significant number of cells expressing Parkin there was an overlap between mitochondria and the autophagosome machinery. In *Figure 8d & 8e*, it was shown that mouse embryonic fibroblasts (MEFs) lacking ATG5, which is a key component of the autophagy pathway, keep Parkin-targeted mitochondria after treatment with CCCP in contrast to wild type MEFs (WT MEFs) which had no sign of the damage mitochondria because of mitophagy. Additionally, *Figure 8f* shows that bafilomycin, a lysosomal inhibitor, and 3-methyl adenine (3MA), an autophagy inhibitor, block Parkin-induced mitophagy in HeLa cells. This further supports the hypothesis that Parkin promotes the autophagic degradation of impaired mitochondria. The findings provide a new molecular link between mitochondrial membrane depolarization and autophagy *in vitro* by identifying Parkin as a mediator of mitophagy downstream of mitochondrial depolarization. This suggests that loss of Parkin activity may allow the accumulation of dysfunctional mitochondria, leading to neuron loss in Parkinson's disease (34).



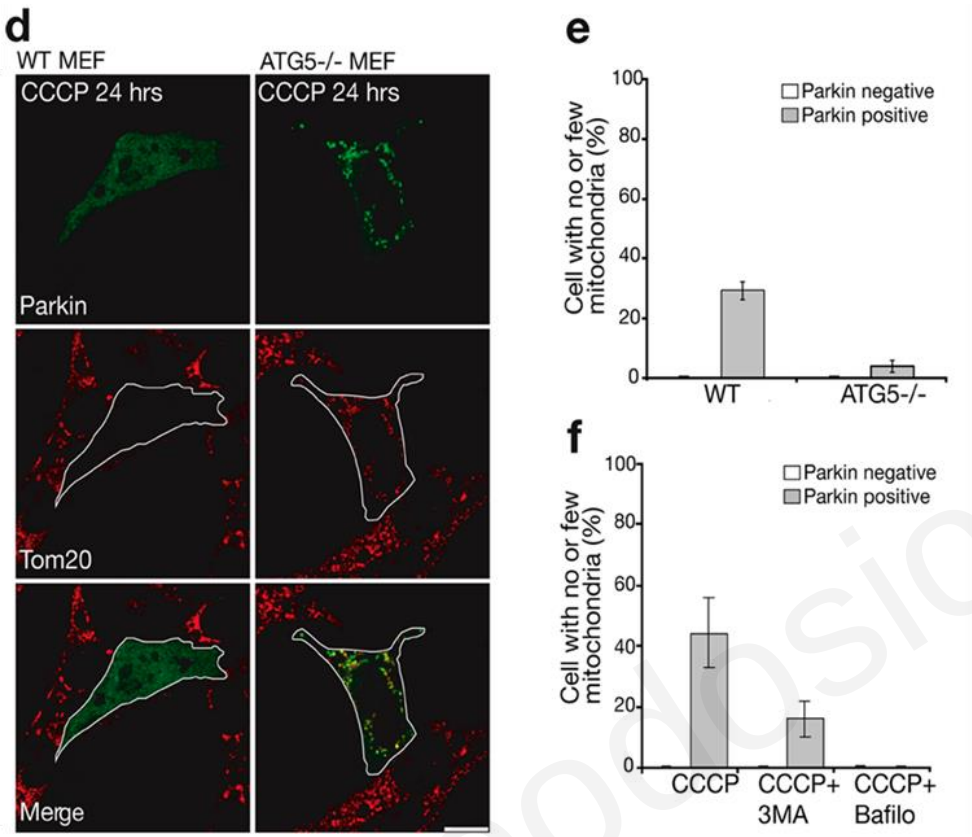


***Figure 8*. Parkin induces mitophagy via the autophagy machinery.** *(a) Parkin-negative (left) and Parkin-positive (right) HeLa cell that have achieved stable expression of GFP-LC3 (green fluorescence) and treated with CCCP. Autophagosomes and mitochondria (red) overlap less in Parkin-negative cells than in Parkin-positive cells. (b) Chart showing the number of encapsulated mitochondria in LC3-positive autophagosomes in cells (percentage). (d) After 24 hours of exposure to CCCP, the YFP-Parkin (green)-induced mitochondrial removal seen in WT Mouse embryonic fibroblasts (MEFs) (left), did not occur in ATG5 $^{-/-}$ MEFs (right). The boundaries of cells expressing YFP-Parkin are indicated by outline. (e) Chart showing the percentage of cells with no or few mitochondria in both WT MEFs and in ATG5 $^{-/-}$ MEFs. (f) Chart shows the percentage of cells with no or few mitochondria in cells treated only with CCCP (control), CCCP + Bafilomycin and CCCP + 3MA. The cells treated with either of the two inhibitors have more mitochondria which shows the impaired parkin-mediated mitophagy in HeLa cells (34).*

2.1.2. PINK1 recruits Parkin to damaged mitochondria

How Parkin differentiates between depolarized and healthy mitochondria was initially not evident. A study by Matsuda *et. al.* 2010 (35) demonstrated that PINK1 recruits Parkin from the cytoplasm to damaged mitochondria in low membrane potential conditions, initiating the

autophagic degradation of damaged mitochondria. The paper also highlights the etiological importance of pathogenic mutations of PINK1 and Parkin in interfering with these processes.

In *Figure 9* they showed that Parkin is recruited from the cytoplasm to damaged mitochondria by PINK1. In the PINK1$^{+/+}$ control cells, Parkin was specifically enlisted to the mitochondria following CCCP administration. However, in PINK1$^{-/-}$ cells, mitochondria are shown to accumulate. Also, it was observed that Parkin's E3 activity is suppressed in the cytoplasm in steady state conditions, but it is activated by PINK1-dependent mitochondrial localization. All together this study managed to characterize PINK1 as 'a Parkin recruitment factor', in a manner that is dependent on membrane potential and facilitates mitochondrial degradation (35).

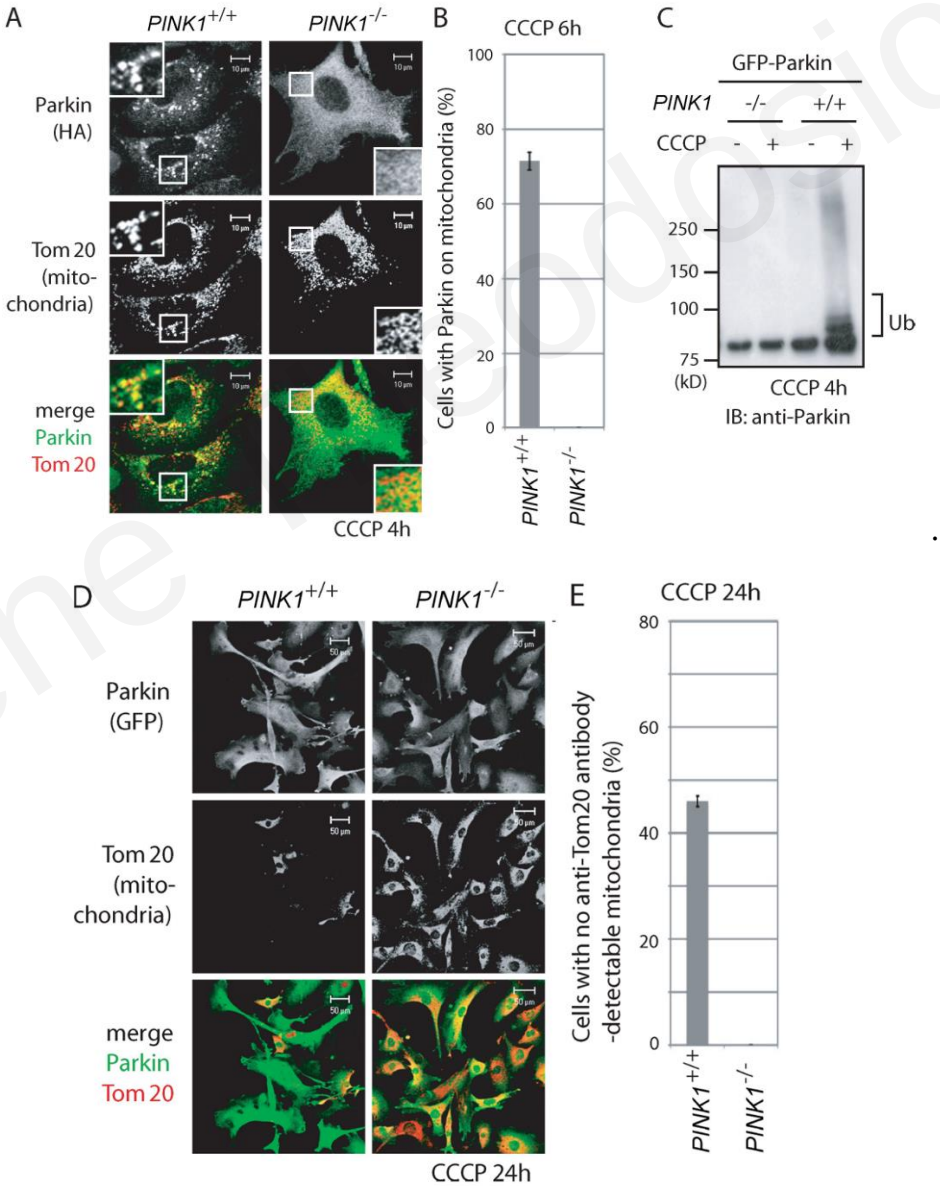


Figure 9. The recruitment of cytoplasmic Parkin to damaged mitochondria is facilitated by PINK1. *(A) Transfected PINK1 knockout (PINK1$^{-/-}$) or control (PINK1$^{+/+}$) MEFs with HA-Parkin were treated with CCCP and immunocytochemically probed. The insets display views of the boxed areas at higher magnification. (B) Quantification of MEFs with Parkin localised to the mitochondria was counted. In PINK1$^{-/-}$MEFs, neither Parkin activation nor mitochondrial degradation was seen. MEFs that were stably expressing GFP-Parkin were either treated with CCCP for 4 hours, after which they underwent immunoblotting (C) or immunocytochemistry (D) for 24 hours. (E) MEFs lacking mitochondria detectable by the anti-Tom20 antibody were tallied and in PINK1$^{-/-}$ cells there were more mitochondria compared to the control. HA: Human influenza hemagglutinin tag, IB: immunoblot, Ub: ubiquitylation (35).*

Similarly, Narendra *et. al.* 2010 (36) wanted to show what is upstream of Parkin and found that PINK1 is strictly required for Parkin recruitment to uncoupled mitochondria. In PINK1 KO MEFs, the YFP-parkin had a dispersed expression and mitochondria (Tom20 marker) accumulated compared to WT MEFs. However, after PINK1 restoration in PINK KO MEFs Parkin levels appear more concentrated in the mitochondria locations (*Figure 10*). This is evidence that PINK1 controls Parkin localization on depolarized mitochondria (36).

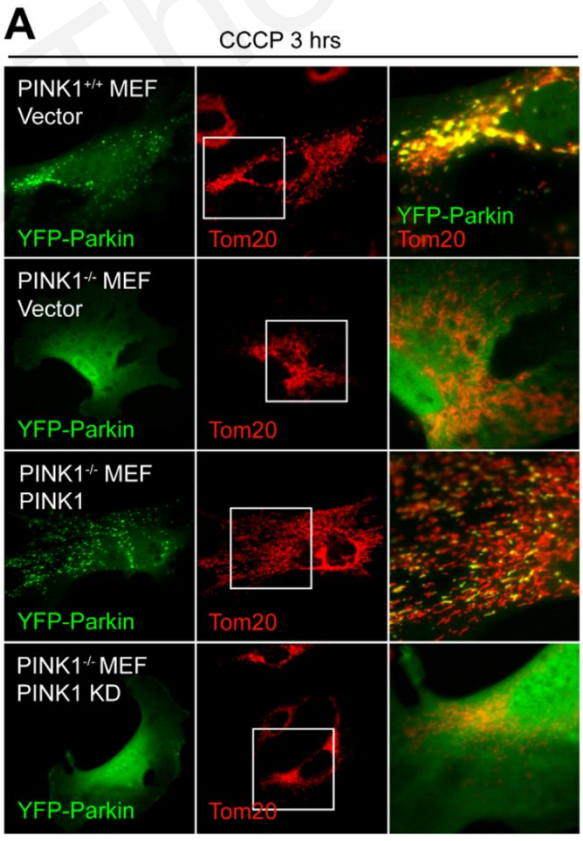


Figure 10. Parkin recruitment to depolarized mitochondria requires PINK1. *MEFs treated with CCCP in four different conditions: PINK1 $^{+/+}$ MEFs, PINK1 $^{-/-}$ MEFs, PINK1 restoration of PINK1 $^{-/-}$ MEFs, PINK1 KD in PINK1 $^{-/-}$ MEFs. YFP tagged fluorescence of Parkin is shown in the first column of microscopy images, Tom20 tagged fluorescence of mitochondria is shown in the middle column of microscopy images and merge of Parkin and mitochondria localization is depicted in the third column (36).*

The essential role of PINK1 in not only the mitophagy pathway but also in the PD pathogenesis. In their experiments Narendra *et. al.* 2010 (36) also illustrated that disease-causing mutations in Parkin disrupt the protein's recruitment to mitochondria and/or Parkin-induced mitophagy (*Figure 11*). Specifically, the R275W mutation of Parkin had similar effects with the WT meaning that the Parkin expression was normal while the other mutations expressed dysfunctional Parkin. All the mutations however had a high number of mitochondria present. This indicates the role of Parkin in clearing abnormal mitochondria and that Parkin's malfunction disrupts the mitophagy pathway (36).

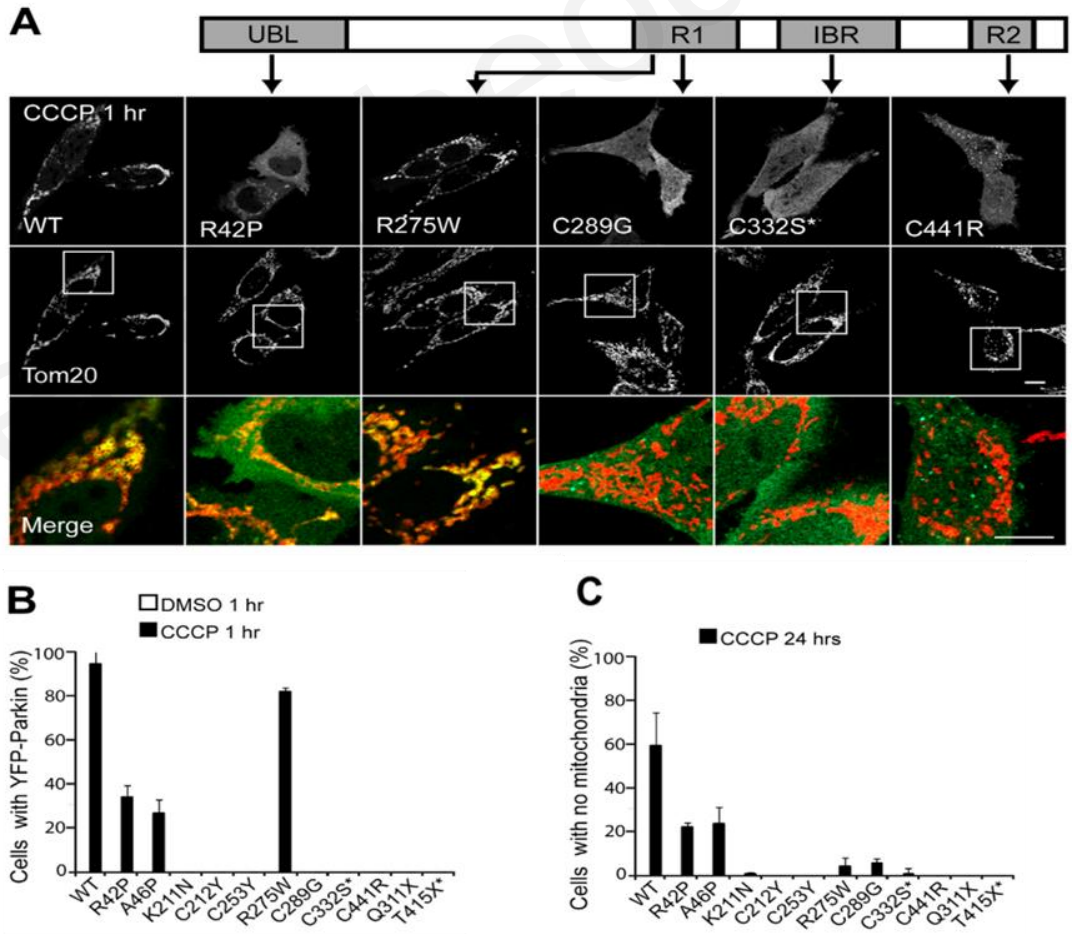


Figure 11. Mutations in Parkin that cause PD impair Parkin-induced mitophagy and Parkin recruitment to mitochondria. *(A) HeLa cells containing the indicated mutations in the respective Parkin domains were treated with CCCP for one hour after transfecting them with YFP-Parkin (green) and anti-Tom20-labeled mitochondria (red). The regions denoted by the boxes in the middle row are expanded upon in the images in the bottom row. (B) Chart showing the number of cells with Parkin in every mutation condition. (C) Chart showing the number of cells with no mitochondria in every mutation condition (36).*

2.1.3. Ubiquitination and phosphorylation mechanisms in PINK1/Parkin-dependent mitophagy

Two main molecular mechanisms are found in the PINK1/Parkin-dependent mitophagy: phosphorylation and ubiquitination (37). After finding that PINK1 is implicated in this pathway a question raised: What does PINK1 phosphorylate? The answer was given by Kazlauskaite et. al. 2014 (38) because they found through their experiments that Ubiquitin is a substrate of PINK1. They also depicted the specific amino acid phosphorylation which was Ser65 *(Figure 12).* This is an indication that PINK1 directly phosphorylates Ubiquitin on Ser65 (38).

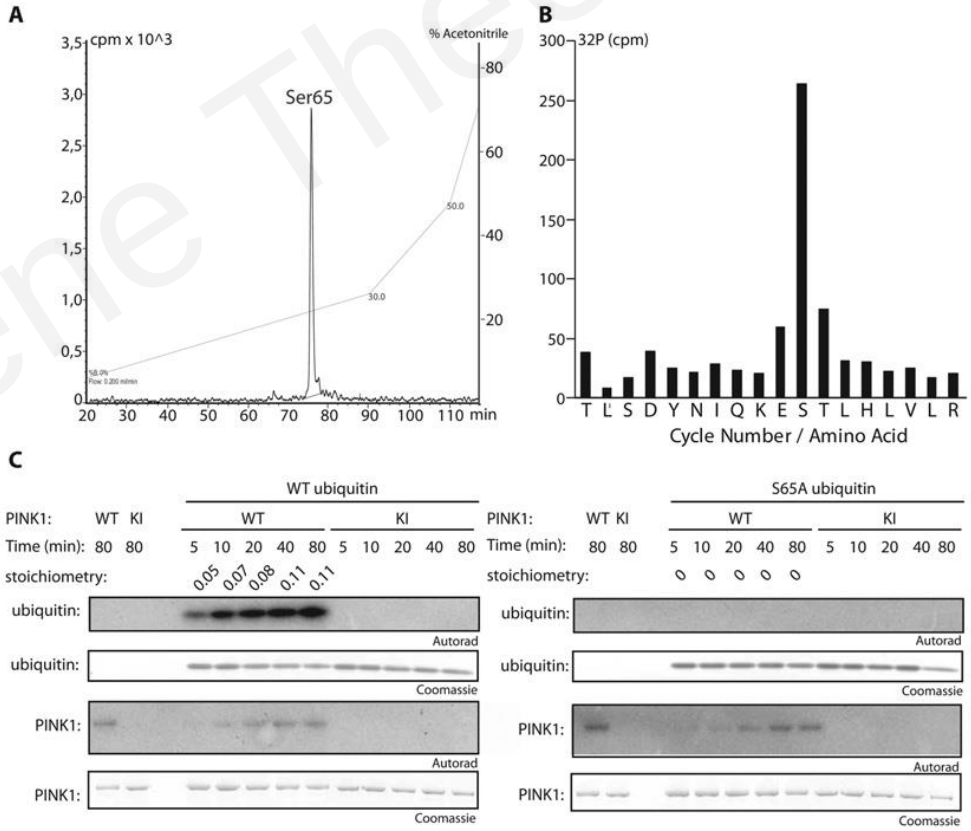


Figure 12. Ubiquitin phosphorylation at S65 by PINK1. *(A) Chromograph on reverse-phase HPLC Vydac C18 column for the mapping of phosphopeptides on ubiquitin after phosphorylation by either wild-type PINK1 or kinase-inactive PINK1 (D359A) in vitro. (B) Solid-phase Edman sequencing and mass spectrometry were used to analyze the phosphopeptide found in (A). A single-letter amino acid code is used to represent the amino acid sequence inferred from the single phosphopeptide observed in the LC–MS/MS analysis. (C) PINK1's ubiquitin phosphorylation is eliminated by the S65A mutation resulting in PINK1 with either inactive kinase or wild-type and cultures were made with ubiquitin that was either wild-type or mutant. Samples underwent SDS/PAGE, and the incorporation of [γ -32P] ATP was detected by autoradiography (top panels) and proteins by Colloidal Coomassie Blue staining (bottom panels). KI, kinase-inactive; WT, wild-type (38).*

Kane *et. al.* 2014 (39) further revealed that phospho-Ub activates Parkin activity *in vitro* The ubiquitin phosphorylation by PINK1 is necessary in order for Parkin be translocated on the depolarized targeted mitochondria and get activated by PINK1 via direct phosphorylation. Fluorescent tagged imaging via microscopy showed that YFP-Parkin is colocalizing with HA-Ub WT, but mutated Ub has less affinity with Parkin *(Figure 13)*.

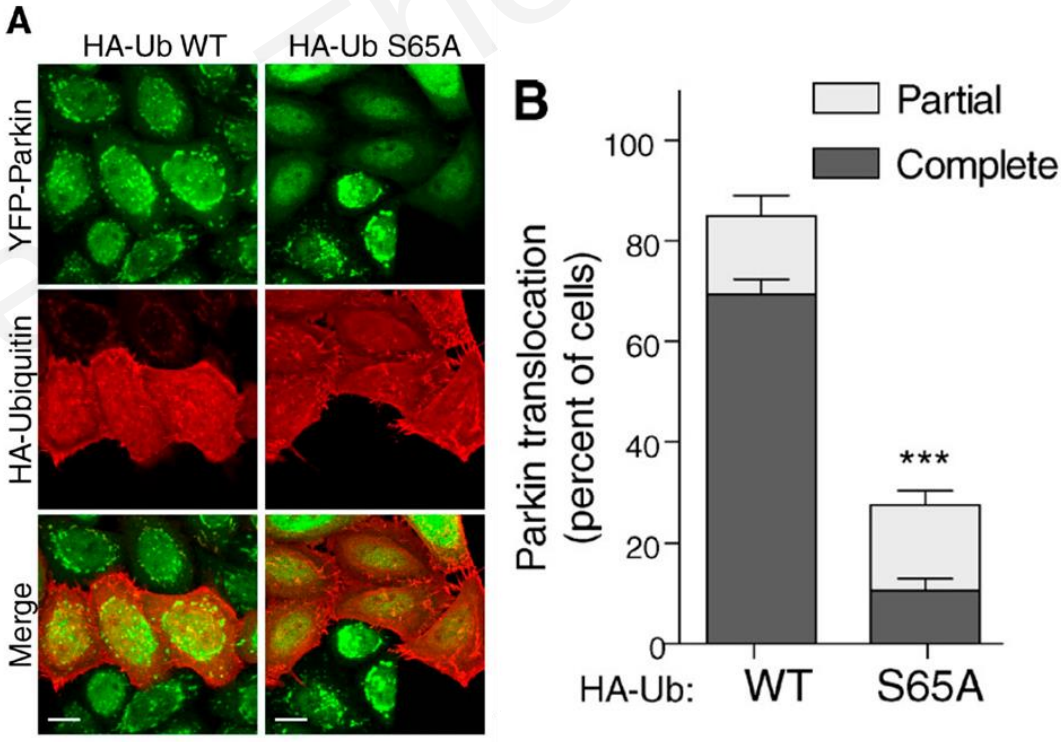


Figure 13. Activation of Parkin via phospho-ubiquitin. *(A) Following CCCP treatment, in cells expressing elevated levels of WT HA-Ub, YFP-Parkin WT translocates to damaged mitochondria (red, HA immunostaining). In contrast HA-UbS65A-expressing cells exhibit reduced YFP-Parkin translocation. (B) Quantification of A showing the cells with Parkin translocation in either WT HA-Ub or in HA-UbS65A (39).*

2.1.4. Recruitment of receptors by PINK1

Downstream of the PINK1/Parkin-mediated mitophagy a set of receptors have been noted to be involved. The study by Lazarou et. al. 2015 (40) investigates the role of PINK1 in recruiting autophagy receptors (NDP52 and OPTN) for mitophagy. To track mitophagy the induced mitochondrial damage with oligomycin and antimycin A (OA) and used cytochrome C oxidase subunit II (COXII), a mitochondrial DNA (mtDNA)-encoded inner membrane protein, to measure its degradation or accumulation. Accumulation of COXII represents blocked mitophagy and degradation of COXII shows induced mitophagy. In order to find which receptors, participate in mitophagy, they created a series of cell lines: PentaKO (NDP52/OPTN/TAX1BP1/NBR1/p62 knock out receptors), NDP52 KO, OPTN KO and NDP52/OPTN DKO (N/O DKO). It was observed that in NDP52 or OPTN knockout alone mitophagy was not affected, whereas the N/O DKO inhibited mitophagy (*Figure 14*) (40).

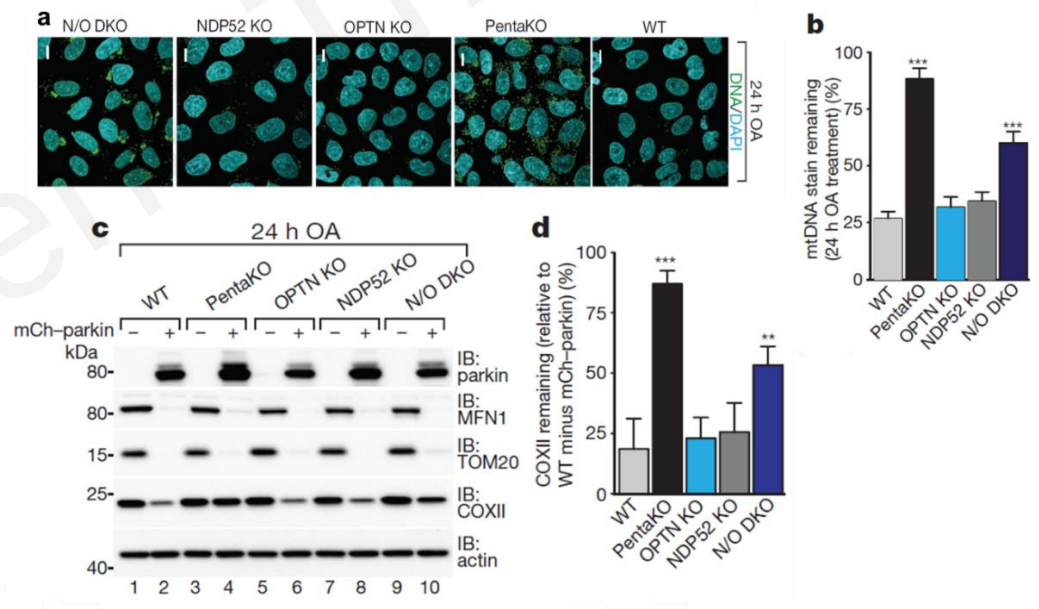


Figure 14. PINK1/parkin mitophagy recruits OPTN and NDP52. (*a) After OA-induced mitochondrial damage in wild-type, penta receptor KO, OPTN knockout, NDP52 knockout and NDP52/OPTN DKO cells that express mCh–parkin immune-stained with anti-DNA (green) the*

respective microscopy images were captured, (b) quantification of images for mitophagy, (c) wild-type, penta receptor KO, OPTN knockout, NDP52 knockout and NDP52/OPTN DKO cell lines immunoblotted, (d) COXII levels were quantified respectively (40).

To further investigate the role of the receptors NDP52 and OPTN in the PINK1/Parkin-dependent mitophagy, they wanted to find the downstream interactors of these two receptors which most possibly are autophagosome proteins. In order to do that, they examined the recruitment of all LC3 and GABARAP family members to mitochondria in wild-type, PentaKO and NDP52/OPTN DKO cells. First for the LC3 proteins, they showed that the localization of GFP–LC3s in wild-type cells induced by OA was not observed in PentaKO cells, whereas only the recruitment of GFP–LC3B was hindered in NDP52/OPTN DKOs. (*Figure 15a*). Secondly for the GABARAP proteins now association was observed towards mitophagy (not shown). Lastly, the involvement of WIPI1 and DFCP1, two proteins that facilitate the process of phagophore biogenesis upstream of LC3, in the phenomenon of mitophagy was also investigated. In wild type cells after OA induction, foci of both GFP-WIPI1 and GFP-DFCP1, primarily localized on or in close proximity to mitochondria. However, in cells lacking the NDP52/OPTN proteins, the foci of GFP–WIPI1 and GFP–DFCP1 were diminished, reaching a state of near-undetectability in cells lacking all five receptors (PentaKO cells) (*Figure 15 b, c*). Taking all the above into consideration, they showed that NDP52 and OPTN after their recruitment to the mitochondria, they further recruit autophagy factors such as LC3B, DFCP1, and WIPI1 (*Figure 15*) (40).

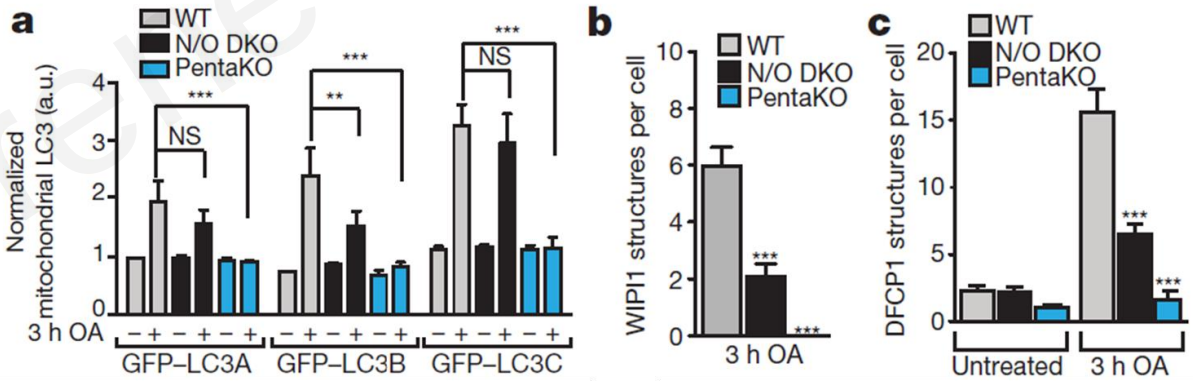


Figure 15. Autophagy receptor interactions with autophagy machinery during mitophagy. *(a) Quantification charts showing GFP–LC3A, GFP–LC3B and GFP–LC3C translocation to mitochondria in wild type, NDP52/OPTNDKO and PentaKO cells expressing mCh–parkin. (b) Quantification charts showing GFP–WIPI1 translocation to mitochondria in wild type,*

NDP52/OPTNDKO and PentaKO cells expressing mCh–parkin. (c) Quantification charts showing GFP–DFCP1 translocation to mitochondria in wild type, NDP52/OPTNDKO and PentaKO cells expressing mCh–parkin (40).

2.2. PINK1/Parkin-dependent mitophagy *in vivo*

Although significant progress has been made in the PINK1/Parkin-dependent mitophagy description, the exact role of Parkin and PINK1 in dopaminergic neurons remains unknown because of the poor contribution of *in vivo* studies. However, a few studies revealed some unexpected findings about mitophagy in organisms (*Drosophila melanogaster*, *Mus musculus*).

2.2.1. PINK1/Parkin-dependent mitophagy in *D. melanogaster*

Evidence of PINK1 and Parkin role in mitophagy *in vivo* is still scarce, but findings using *Drosophila* models in the Lee *et. al.* 2018 (41) paper provide novel evidence. They produced fluorescent mitophagy reporter-expressing transgenic *Drosophila* flies to assess the effect of PINK1/Parkin mutations on basal mitophagy in physiological settings. The mito-QC images revealed that in DA neurons with mutant PINK1 ($Pink1^{B9}$) or mutant Parkin ($park^{25}$) the effect on mitophagy was no different compared to the controls *(Figure 16)*. Their results showed that PINK1 and Parkin proteins are not essential for bulk basal mitophagy in *Drosophila* (41).

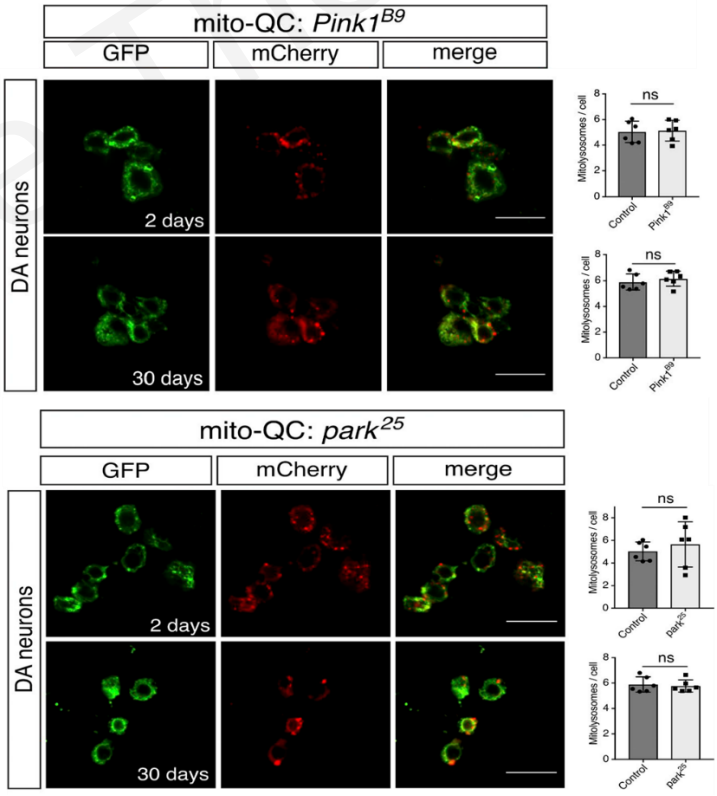


Figure 16. Basal mitophagy is minimally affected in PINK1 and PARKIN mutants. *Confocal microscopy analysis of mito-QC reporter in Pink1^{B9} mutant (top) and park^{25} mutant (bottom) in DA neurons at 2 and 30 days old, as indicated. Mito-lysosomes are evident as GFP-negative/mCherry-positive (red-only) puncta. Charts show quantification of mito-lysosomes per cell in control and PINK mutant flies (41).*

2.2.2. PINK1/Parkin-dependent mitophagy in *M. musculus*

A study in 2018 used mouse models in order to observe the Parkin expression in neurons and whether Parkin participates in mitophagy (42). They characterised PINK1 wild-type (WT) and knockout (KO) mice expressing the mito-QC transgene, concentrating on tissues of high metabolic dependence and cell types of clinical importance to Parkinson's disease (PD) in order to examine the role of PINK1 in controlling basal mitophagy *in vivo*. DA neurons are among the highly metabolic cell subsets *in vivo* that they reported to exhibit basal mitophagy. In addition, they showed that basal mitophagy *in vivo* happens independently of PINK1 *(Figure 17)*. Their results lead to the assumption that PINK1 knockout mice do not replicate the degenerative phenotypes of dopaminergic neurons in humans with Parkinson's disease, and these mice show normal *in vivo* mitophagy measurements. Numerous mitophagy pathways are present in mammalian cells, and under certain circumstances, these pathways can be activated in response to a variety of stimuli. Thus, it is possible that in mammals, the activation of PINK1-dependent mitophagy is highly context-dependent and happens in reaction to a particular kind of stress. (42).

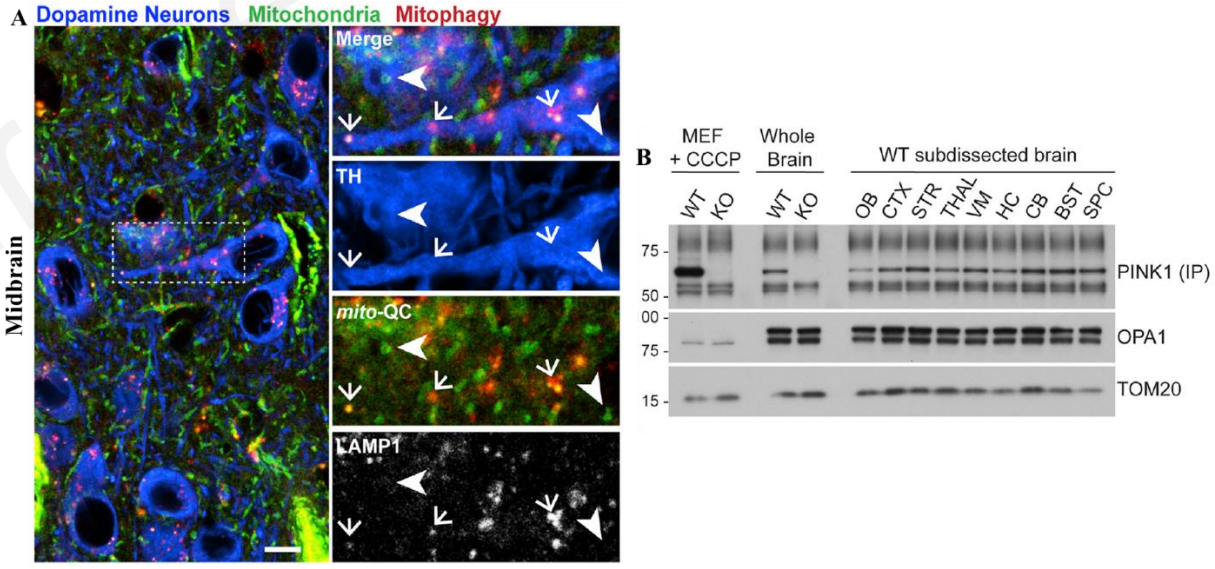


Figure 17. Basal Mitophagy continues in the mammalian dopaminergic system without PINK1. (A) In vivo basal mitophagy of immunolabeled midbrain dopaminergic neurons is depicted in a representative micrograph. Antibodies against the DA markers tyrosine hydroxylase (TH) and LAMP1 were used to label sections of the mito-QC mouse ventral midbrain (inset: arrows indicate mitolysosomes and arrowheads show mitochondria). (B) IP-immunoblot showing expression of PINK1 in the adult mouse nervous system under basal conditions. OPA1 as and TOM20 as mitochondrial controls. OB, olfactory bulb; CTX, neocortex; STR, striatum; Thal, thalamus; VM, ventral midbrain; HC, hippocampus; CB, cerebellum; BST, brainstem; SPC, spinal cord (42).

2.3. PINK1/Parkin mutations have low pSer65Ub in post-mortem human brains

Autosomal recessive early onset Parkinson disease is caused by mutations in PINK1 and Parkin. More specifically, loss of function or dysfunction of PINK1 and Parkin can impair mitophagy by impacting various pathways stages including inhibition of ubiquitin phosphorylation leading to the accumulation of damaged mitochondria in dopaminergic neurons (43).

When loss-of-function PINK1 and/or Parkin mutations occur, they cause decreased pSer65Ub and consequently decreased mitophagy. An indication of the above was shown by studying post-mortem human PD brains and discovered diminished pSer65Ub structures in patients with PINK1/Parkin mutations, indicating the relevance of this pathway in disease *(Figure 18).* This could serve as a possible mitophagy marker. However, how these observations apply to the general sporadic PD population and the upstream or downstream role of pSer65Ub in PD pathology remain important questions (44).

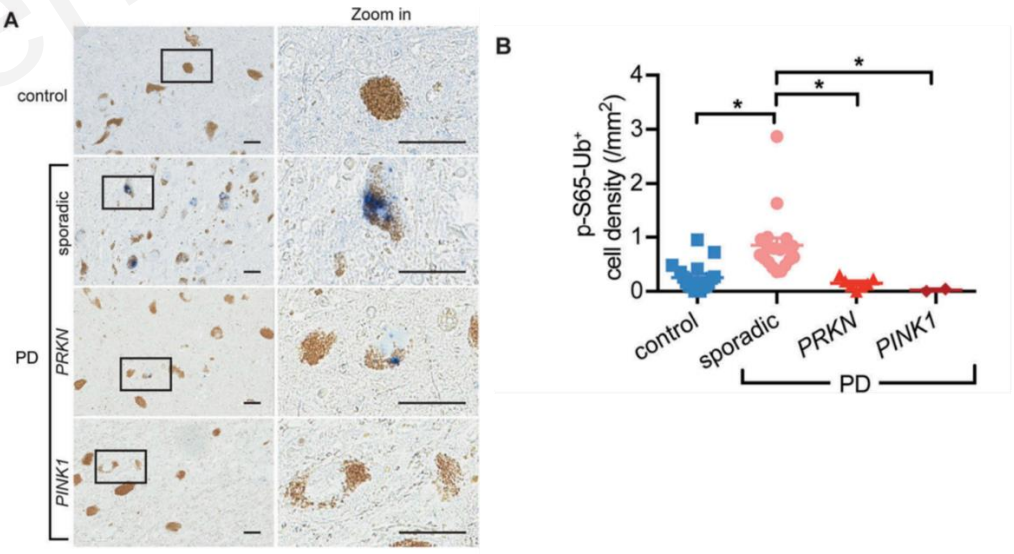


Figure 18. Brains of PRKN or PINK1 mutation carriers show less or no p-S65-Ub staining.
(A) Substantia nigra sections stained with p-S65-Ub antibody were obtained from controls, cases with PINK1 or PRKN mutations, and sporadic LBD cases. The stained sections exhibit elevated levels of p-S65-Ub in cases of sporadic PD, while the signal in the SN of PRKN or PINK1 mutant cases is either absent or reduced. To the right is an enlarged picture of the boxed area. (B) Measuring the p-S65-Ub signal in carriers of mutations, age-matched controls, and sporadic PD cases. (44).

2.4. Therapeutic approaches

The description of the PINK1/Parkin-dependent mitophagy pathway is important in order to be targeted for a therapeutic purpose of PD. The pathophysiology of Parkinson's disease (PD) is linked to loss-of-function mutations in important mitophagy effectors, such as PINK1, Parkin and OPTN. These effectors can be targeted to restore the impaired mitophagy in PD by either activating positive regulators such as PINK1 and Parkin, and/or inhibiting negative regulators such as phosphatases and deubiquitinating enzymes (DUBs) like USP30 (43). Screening for small molecules that would bind and induce a conformational change in parkin to expose its catalytic domain would mimic overexpression of parkin and promote mitophagic turnover of damaged mitochondria and prevent cell death (43, 45).

2.4.1. Identification of therapeutic agents via screening

Screening tests of small molecules/inducers aim to broaden the PD drug spectrum in respect to the patients cause of disease. A high-throughput system was introduced by the team of Yamaguchi *et. al.* 2020 (45). In their procedure they used iPSCs derived DA neurons from patients with PD caused by *PINK1* or *Parkin* mutations (Parkin-Ex2-4 homozygous deletion (PA), Parkin-Ex6, 7 homozygous deletion (PB) and PARK6 (PINK1-c.1162T>C heterozygous mutation (PKB)). They evaluated a total of 320 substances by monitoring their effect on mitochondrial clearance and apoptosis of the candidate cells. In *Figure 19*, the four most promising substances were picked for mitochondrial clearance assessment. The substances MRS1200, Tranylcypromine, Bromocriptine, and Flunarizine showed increased elimination of mitochondria in PARK2 and PARK6 neurons treated with CCCP in a partially dose-dependent manner. Moreover, by using lysosomal inhibitors such as E64d and pepstatin A they concluded that the four compounds promoted mitochondrial degradation through the lysosome (*Figure 19*) (45).

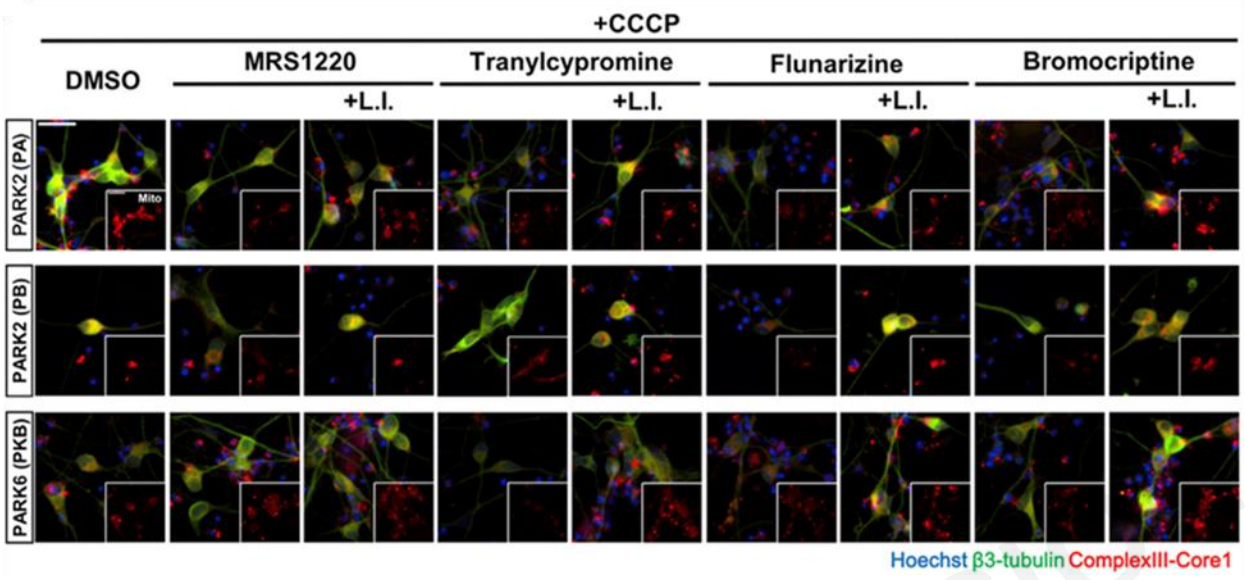
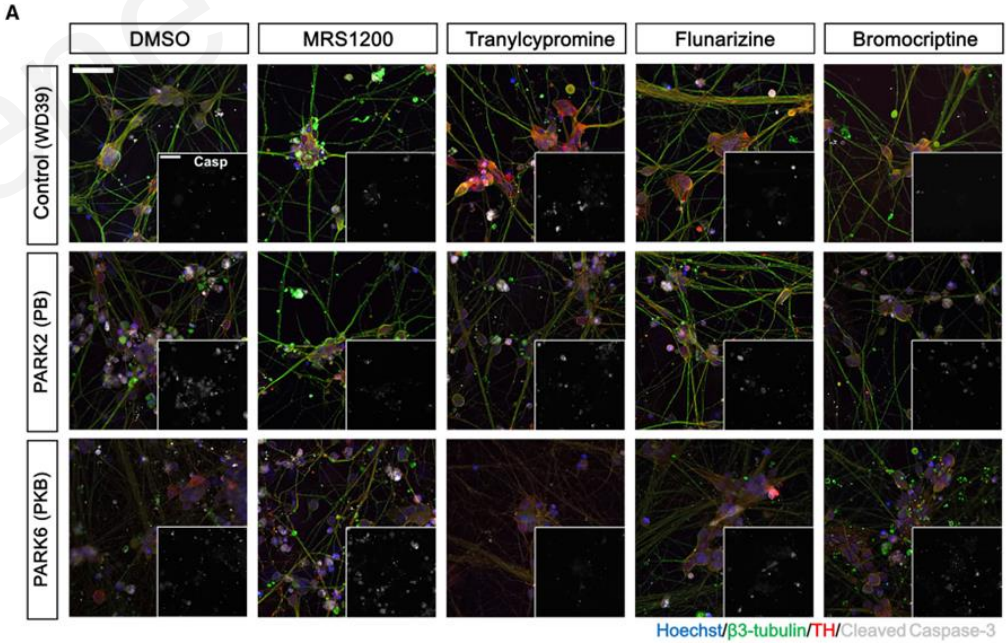


Figure 19. Mitochondrial clearance effect of MRS1200, Tranylcypromine, Bromocriptine, and Flunarizine in PARK2 and PARK6 neurons. *Images of the mitochondrial clearance analysis using the substances MRS1200, Tranylcypromine, Bromocriptine, and Flunarizine in cells with the 3 different PINK1 or Parkin mutations. Also, a lysosomal inhibitor (L.I.) was added after drug induction (45).*

In the apoptosis assessment, Tranylcypromine, Bromocriptine, and Flunarizine decreased the neuron's death in comparison to MRS1200 *(Figure 20).* This was demonstrated by the decreased fluorescence intensity of cleaved caspase-3 in the compound-treated neurons (45).



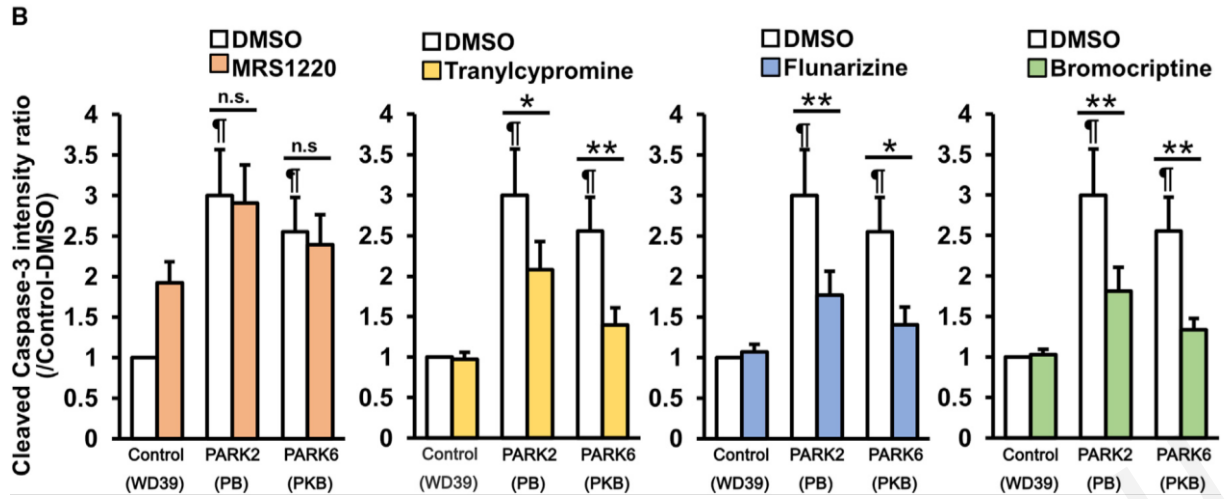


Figure 20. Cell apoptosis assessment in MRS1200, Tranylcypromine, Bromocriptine, and Flunarizine in PARK2 and PARK6 neurons. *(A) Images of the cell apoptosis analysis using the substances MRS1200, Tranylcypromine, Bromocriptine, and Flunarizine in cells with the 3 different PINK1 or Parkin mutations. (B) Quantitative data of the apoptosis analysis. The fluorescence intensity of cleaved caspase-3 in DMSO-treated control (WD39) neurons and that in compound-treated neurons in the 3 different PINK1 or Parkin mutations (45).*

Four out of the 320 drugs showed to be effective for PD-derived neurons with impaired mitochondrial clearance, suggesting the potential of the high-throughput system for identifying effective drugs for familial PD and providing a step towards developing therapeutic agents (45).

2.4.2. Silibinin targets mitophagy for PD therapy

While the targeting of specific molecules in the PINK1/Parkin pathway of mitophagy that can lead to a PD therapy can be challenging it also broadens the PD drug spectrum. A study by Liu *et. al.* 2021 (46), aimed to research a specific molecule called silibinin as a new potential target for PD therapy.

In this study Silibinin was used with the aim to observe its' potential as a protective substance against PD using PD-model mice induced by MPTP. Silibinin is a flavonoid and derives from the plant *Silybum marianum*. While injection of MPTP in mice causes both dopaminergic neuronal loss in the SN, and the decrease of the striatal tyrosine hydroxylase (dopamine synthesis), subsequent oral administration of silibinin, protected the PD-mouse models against dopaminergic neuronal loss. More specifically, there was an increase of PINK1 and Parkin proteins, indicating the activation of mitophagy (*Figure 21*). These results point to the importance of mitophagy in DA neuron protection and the possibility of Silibinin's continued development as a Parkinson's disease

treatment candidate due to the fact that silibinin diminished MPTP-induced movement disorder in mice behavioural tests. In *Figure 22* it is notable that in all tests there is an improvement in movement abilities in mice treated with Silibinin as the dose increases. Especially for the high silibinin test (red) the results were as good as the control mice. This shows the possibility of Silibinin as a treatment for PD (46).

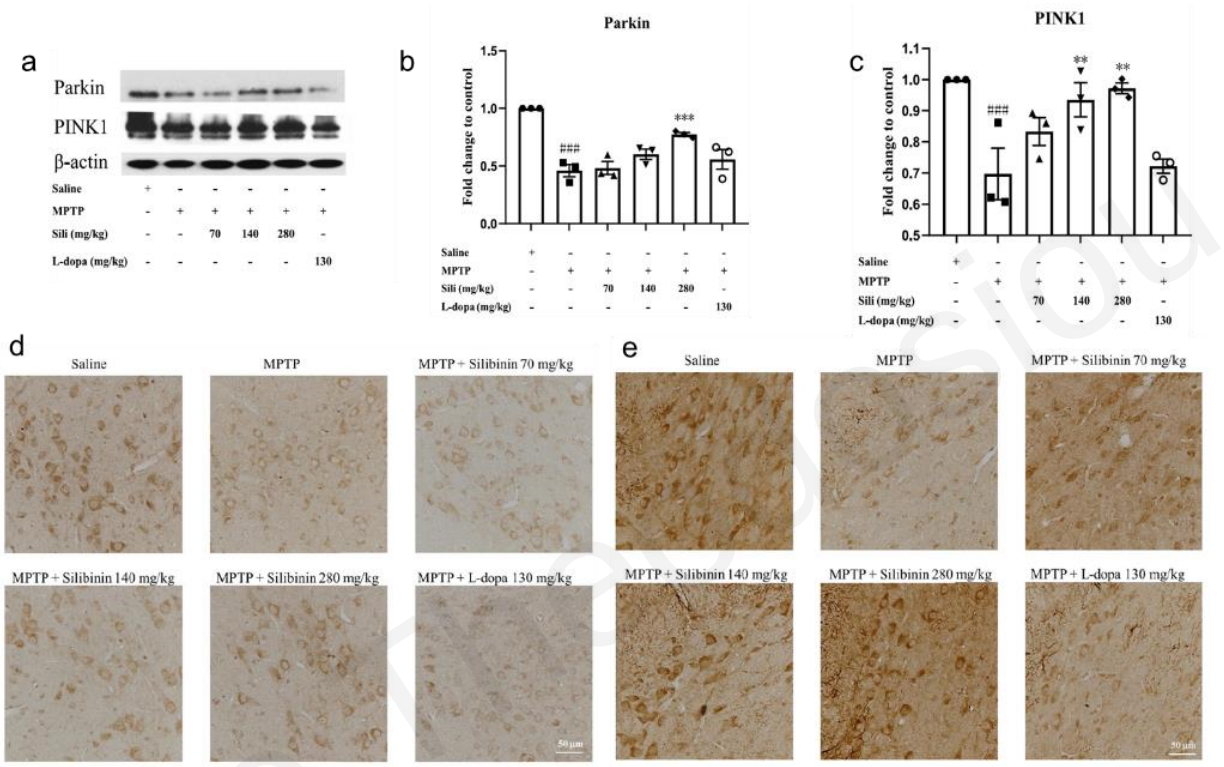


Figure 21. PINK1 and Parkin are affected by Silibinin. *(a) Western blotting analysis showing the expression levels of PINK1 and Parkin, the mitophagy-related proteins, in the striatum and substantia nigra. (b) Chart showing the Parkin expression in the different conditions. (c) Chart showing the Parkin expression in the different conditions. (d) Immunohistochemical staining of Parkin in substantia nigra in different conditions. (e) Immunohistochemical staining of PINK1 in substantia nigra in different conditions (46).*

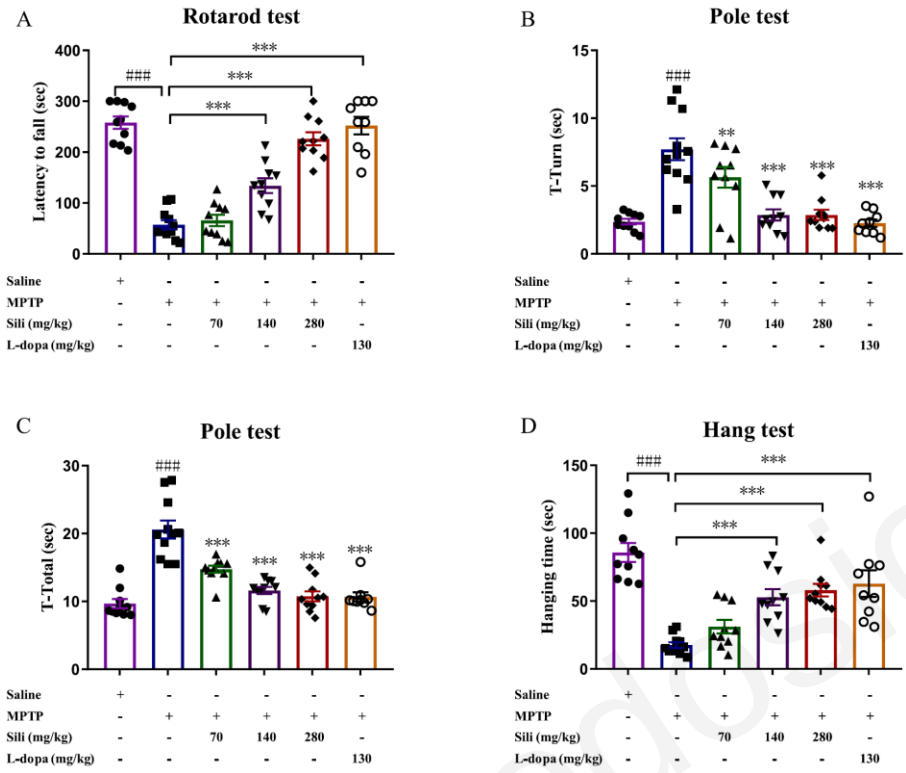


Figure 22. Behavioral tests in mise testing the effect of silibinin. *Mice after various conditions were placed under A) Rotarod tests to check coordination skills B) & C) Pole test to evaluate bradykinesia degree & locomotor balance and D) Hang test to evaluate neuromuscular strength and motor function. Conditions-Purple: control-treated only with saline, Blue: MPTP-treated mice to cause PD pathogenesis without any treatment molecules, Green: MPTP-treated mice to cause PD pathogenesis with low silibinin treatment, Dark purple: MPTP-treated mice to cause PD pathogenesis with medium silibinin treatment, Red: MPTP-treated mice to cause PD pathogenesis with high silibinin treatment, and Orange: MPTP-treated mice to cause PD pathogenesis with L-dopa treatment (46).*

2.4.3. Cannabinol modulation of Mitophagy in PD

Another emerging therapeutic approach for the treatment of neurodegenerative disorders are Cannabinoids (CBN). In the present investigation by Gugliandolo *et. al.* 2021 (47), the objective was to assess the potential shielding impacts of CBN in an *in vitro* MPP^+-induced PD model, specifically retinoic acid-differentiated SH-SY5Y neuroblastoma cells. They have showed that the expression of the genes associated with PINK1/Parkin mitophagy was enhanced following the administration of MPP^+ (an MPTP metabolite). Conversely, the expression of these genes was

diminished after pre-treatment with CBN. These findings implied that CBN possesses the capability to confer a safeguard against the detrimental effects on mitochondria caused by MPP^+. Western blot analysis was done to evaluate the expression of PINK1 in cells treated with MPP^+ and cells with MPP^+ and CBN treatment (*Figure 23*). The treatment with CBS has improved the expression of PINK1 in comparison with the control but did not reach the MPP^+ induced mitophagy levels (47).

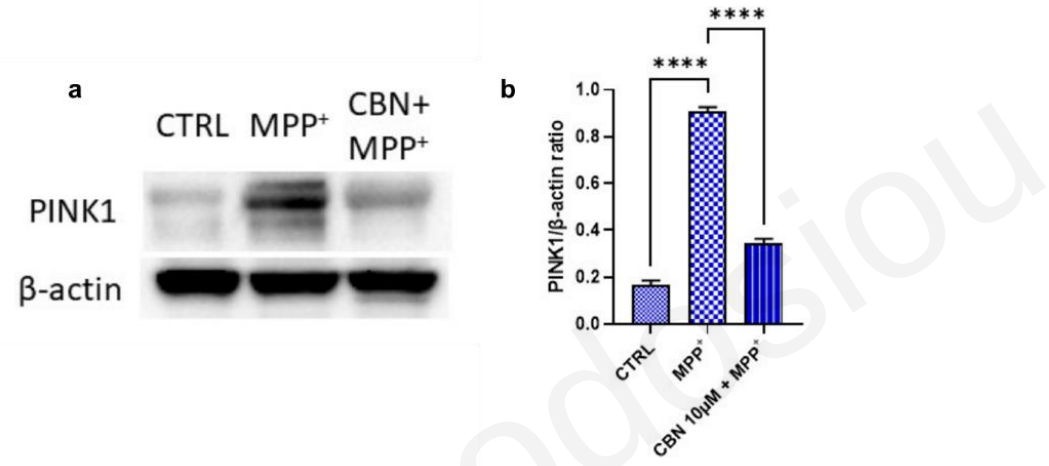


Figure 23. PINK1 expression after CBN treatment. *(a) Western blot of 3 different cell lines: control, MPP^+, MPP+ & CBN. (B) Quantified chart showing the PINK1/β-actin ratio in these 3 different conditions (47).*

2.4.4. Engineered strain of *Clostridium butyricum*-GLP-1 promotes mitophagy via PINK1/Parkin pathway in PD mouse models.

In another study by Wang *et. al.* 2023 (48), using the *C. butyricum* (a probiotic), they constructed it to expresses glucagon-like peptide-1 (GLP-1, a peptide-based hormone with neurological advantage) creating the *Clostridium butyricum*-GLP-1 strain. Their aim was to investigate the neuroprotective mechanism of *C. butyricum*-GLP-1 on MPTP-induced PD mice models. The findings suggested that the administration of *C. butyricum*-GLP-1 exhibited potential for improving motor impairment and also the neuroprotective effects of *C. butyricum*-GLP-1 are exerted through the promotion of PINK1/Parkin mediated mitophagy and the reduction of oxidative stress. They noticed in the immunoblots that PD-models treated with *C. butyricum*-GLP-1 have reverted expression levels of key proteins involved in PINK1/Parkin mitophagy such as PINK1, Parkin and LC3B in comparison to PD models *(Figure 24).* This study demonstrates that

C. butyricum-GLP-1 enhances the process of mitophagy, thereby offering an alternative therapeutic approach for the treatment of Parkinson's disease (48).

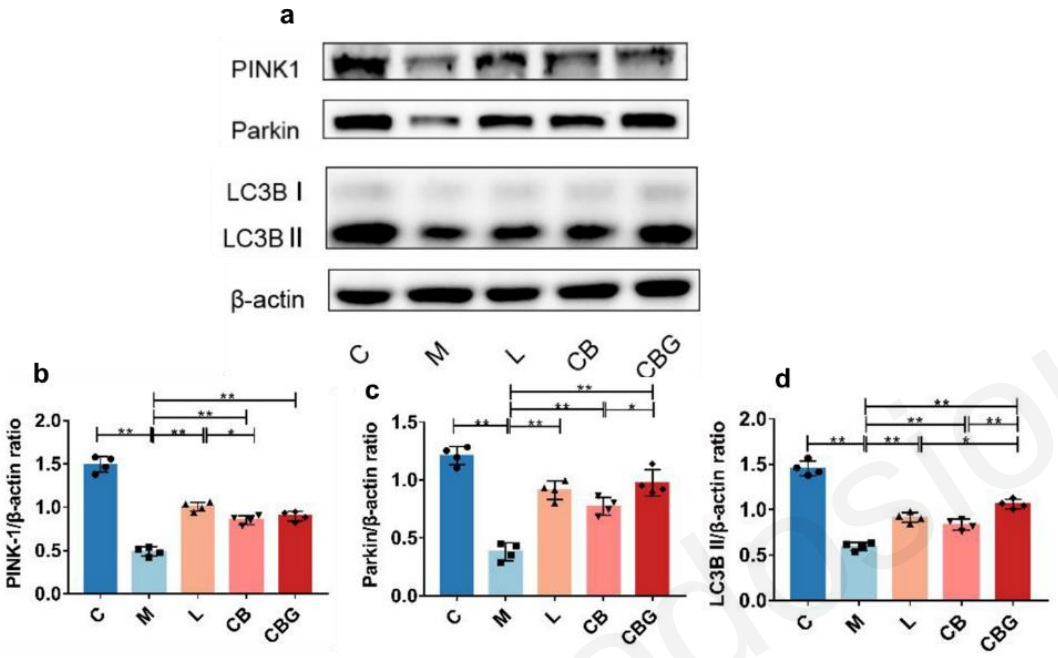


Figure 24. Expression levels of mitophagy related proteins after C. butyricum-GLP-1 treatment. *(a) Immunoblotting of PINK, Parkin, LCB3 (I & II) mitophagy related proteins in different conditions: C=control group, M=MPTP group, L= MPTP + liraglutide group, CB= MPTP + CB group, CBG=MPTP + CB-GLP-1 group. (b) Chart of quantified levels of PINK1 in the 5 different conditions. (c) Chart of quantified levels of Parkin in the 5 different conditions. (d) Chart of quantified levels of LC3BII in the 5 different conditions. CB=Clostridium butyricum, CBG=C. butyricum-GLP-1 (48).*

Therapeutic strategies aimed at enhancing mitophagy or mitigating mitochondrial dysfunction are being explored as potential approaches to slow down or halt the progression of Parkinson's disease. However, more work remains to fully understand the complex interactions between mitochondrial quality control mechanisms and the development of neurodegenerative diseases like Parkinson's especially in *in vivo* models.

3. DISCUSSION

Parkinson's disease is one of the most devastating diseases among humans and there is an urgent need for detailed analysis of its cause. Besides the genetic causes, age is also a part of the disease progression which is always inevitable. From GWAS studies, specific genes were found that are linked to PD and two of them also participate in the mitophagy pathway (PARK2 and PARK6). Mitophagy is a crucial step for mitochondria clearance to keep a healthy pool of functioning mitochondria. This recycling mechanism of mitochondria involves various pathways that cooperate to regulate the dynamics of mitochondria, including fitness and populations. Not too long-ago, PD was strongly associated with the PINK1/Parkin-dependent mitophagy pathway (28). Even though susceptibility loci have been found, a single, cohesive molecular mechanism causing Parkinson's disease has not yet been put forth. While the exact mechanism behind the pathogenesis of Parkinson's disease is still unknown, mitochondrial dysfunction is thought to play a significant role. Since mitochondrial dysfunction and PD are closely related, this disease is now referred to as a "mitochondrial disease" (30). This review provides an opportunity to clarify how PD therapies can be developed by providing a thorough description of PINK1/Parkin-dependent mitophagy pathway.

The collection of research papers about the PINK1/Parkin pathway agree to the specific steps that need to take place in order for mitophagy to occur (34-40). PINK1/Parkin-mediated mitophagy is a cellular process that plays a crucial role in maintaining the health and quality of mitochondria, the energy-producing organelles within cells. This mechanism is essential for the removal of damaged or dysfunctional mitochondria, which can be harmful if left unchecked (28, 30). Mitochondria are susceptible to damage due to a range of factors, such as oxidative stress, DNA mutations, or impaired membrane potential. When mitochondria become damaged, they are recognized as targets for removal (28). Three major players of mitophagy have been characterized as follows: PINK1 as the mitochondrial damage sensor, Parkin as the signal amplifier and ubiquitin chains as the signal effectors. In other words, the sensor of damaged mitochondria that have low membrane potential is PINK1 and in turn Parkin is activated by PINK1 for ubiquitin labelling to target mitochondria for degradation (49).

The damaged mitochondria are recognized by PINK1, which accumulates and becomes activated on the mitochondrial outer membrane (34). When PINK1 accumulates on the damaged mitochondria, it phosphorylates ubiquitin (38) and other proteins, leading to the recruitment and

activation of Parkin (39), an E3 ubiquitin ligase enzyme, and its activation is a key step in mitophagy. Activated Parkin tags various mitochondrial proteins with ubiquitin, a small protein that acts as a signal for degradation. This ubiquitination marks the damaged mitochondria for selective removal. The ubiquitinated mitochondria then interact with autophagy receptors which bridge the damaged mitochondria to autophagosomal membranes which are double-membrane structures that engulf and isolate cellular components for degradation (40). The autophagosomes containing the damaged mitochondria fuse with lysosomes, forming autolysosomes. Lysosomes contain enzymes that break down the contents of the autophagosomes, including the damaged mitochondria. Within the autolysosome, the damaged mitochondria are degraded, releasing their components for recycling, and preventing the release of harmful molecules that can trigger cell damage and inflammation. The collaboration of Ser/Thr ubiquitin kinase PINK1 with E3 ubiquitin ligase Parkin, mark injured mitochondria for lysosome fusion-mediated degradation, resulting in the formation of autophagosomes (50). Overall, the most important molecular mechanisms in mitophagy are phosphorylation, ubiquitination and autophagosome formation (10, 27, 28).

Defective mechanisms can result in the accumulation of defective mitochondria, which causes neuronal cytotoxicity. These mechanisms include disruption of the mitochondrial electron transport chain (ETC), especially complex I, decreased mitochondrial membrane potential (depolarization), and impaired organelle turnover (25). By removing damaged mitochondria through this PINK1/Parkin-mediated mitophagy process, cells can maintain their energy production and prevent the accumulation of harmful ROSs and other toxic byproducts. This helps ensure overall cellular health and contributes to cellular homeostasis. PINK1/Parkin-mediated mitophagy is a vital quality control mechanism that plays a critical role in cellular health and is particularly important in tissues with high energy demands, such as the brain and muscles. Dysregulation of this process has been associated with various neurodegenerative diseases, including Parkinson's disease, highlighting its significance in maintaining cellular and organismal health (28).

A sizeable portion of our understanding of the PINK1/Parkin-dependent mitophagy process has come from *in vitro* research using cell lines that knock-out Parkin or PINK1 and that have been subjected to high concentrations of mitochondrial toxins to totally collapse the mitochondrial network's membrane potential. With the dynamic nature of the mitochondrial network and coordinated activity of fission–fusion and mitochondrial biogenesis processes sufficient to

maintain most mitochondrial units in a polarised state, PINK1/Parkin-dependent mitophagy is an uncommon event under healthy basal conditions (41, 42). This does not, however, lessen the significance of Parkin and PINK1 for the survival of dopaminergic neurons. A possible explanation is that the induction of this type of mitophagy in mammals is highly context-dependent and occurs in response to a specific type of stress (42). Although noteworthy progress has been made, the exact role of Parkin and PINK1 in dopaminergic neurons remains unknown, as does their contribution to mitophagy *in vivo* under normal physiological conditions (51). The challenge of researching both endogenous PINK1/Parkin signalling and mitophagy in mammalian tissues (*in vivo*) is a major factor in the lack of available data (42). Another piece of data that was collected form researching the PINK1/Parkin mutations and role in mitophagy, was the pointing of pSer65Ub as a mitophagy marker (44).

In PD, mutations in PINK1 and Parkin genes, cause the respective proteins to be less efficient in their role in mitophagy, making cells more susceptible to oxidative stress and inclusion bodies, including Lewy bodies (52). Genetic analyses followed by functional studies supported the relevance between mitophagy and familial PD. Besides that, it is still unclear whether mitochondrial dysfunction is associated with idiopathic PD (27). Another event that needs to be taken into consideration is that, even though loss of mitophagy is harmful for maintaining mitochondrial homeostasis, it is possible that aberrantly excessive mitophagy can have negative effects as well, possibly even resulting in cell death. As such, both accelerators and brakes need to be considered for controlling mitophagy (28).

In addition to the investigation of the PINK1/Parkin mitophagy pathway, there have been scientific inquiries that adopt a more therapeutic orientation. Several publications have focused on distinct molecules within the PINK1/Parkin pathway of mitophagy that exhibit potential for PD therapy (43). The objective of these articles is to expand the range of pharmaceutical options available for the treatment of PD, taking into consideration the underlying causes of the illness. A variety of treatments is available from deep brain stimulation (surgery) to several synthetic and natural compounds which have been demonstrated to stimulate mitophagy. Pharmacological screening is still a current procedure to find chemical agents that modulate mitochondrial elimination (45). Other substances that were researched as possible PD therapies are Silibinin (46) and Cannabinol (or CBN) (47). Another approach that seems promising are probiotic contracts expressing key molecules that enhance specific pathways. Such an example is the *C. butyricum*-

GLP-1 that showed significant reversed up-regulation of the associated mitophagy proteins (PINK1, Parkin, LC3B) which makes it another potential drug approach (48).

Understanding the precise roles of mitophagy in neurodegenerative diseases is an active area of research, and therapeutic strategies are researched as potential interventions for modulating mitophagy. However, it is essential to note that the relationship between mitophagy and disease is complex, and further research is needed to fully elucidate the mechanisms involved. Research into mitophagy and PD is ongoing, and scientists are exploring various strategies to enhance or restore mitophagy as a potential therapeutic approach. By promoting the removal of damaged mitochondria and reducing oxidative stress, it may be possible to slow down the progression of PD. The research could not have reached this level without the help and constant evolution of mitophagy reporter assay systems such as Mito-QC and Mt-keima (27). With the help of these techniques and with further development of *in vivo* PD-induced models (mouse or rats) researchers will be able to explore mitophagy even more and find PD treatments. However, it is important to note that the exact mechanisms and potential therapeutic interventions are still a subject of active investigation and have not yet yielded a definitive treatment for the disease (53).

F. ABBREVIATIONS

ABBREVIATION	MEANING
AD	Autosomal dominant
ALS	Amyotrophic lateral sclerosis
AR	Autosomal recessive
ATG	Autophagy-related protein
ATP	Adenosine triphosphate
CB	*Clostridium butyricum*
CBG	*C. butyricum*-GLP-1
CBN	Cannabinol
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CMA	Chaperone-mediated autophagy
DA	Dopaminergic
DFCP1	double FYVE-containing protein 1
DMSO	Dimethyl sulfoxide
DRP1	Dynamin-related protein 1
DUBs	Deubiquitinating enzymes
ER	Endoplasmatic Reticulum
ETC	Electron transport chain

FUNDC1	FUN14 domain-containing protein 1
GABARAP	Gamma-aminobutyric-acid-receptor-associated protein
GFP	Green Fluorescence Protein
GLP-1	Glucagon-like peptide-1
GWAS	Genome-wide association study
HA	Human influenza hemagglutinin
HD	Huntington's disease
HM	Mitochondrial-rich heavy membrane pellet
HSPA8	Heat shock 70 kDa protein 8
IB	Immunoblot
KO	Knock-out
LAMP2A	Lysosomal-associated membrane protein 2A
LC–MS/MS	Liquid Chromatography with tandem mass spectrometry
LIR	LC3-interacting region
MEFs	Mouse embryonic fibroblasts
MPP	Mitochondrial processing peptidase
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial deoxyribonucleic acid

MUL1	Mitochondrial E3 ubiquitin protein ligase 1
NDP52	Nuclear dot protein-52
OA	Oligomycin and Antimycin A
OMM	Outer Mitochondrial Membrane
OPTN	Optineurin
PARL	Presenilin-associated rhomboid-like protease
PD	Parkinson's disease
PE	Phosphatidylethanolamine
PHM	Post – heavy membrane supernatant
PINK1	PTEN-induced kinase 1
PNS	Post-nuclear supernatant
PTEN	Phosphatase and Tensin Homolog
ROS	Reactive Oxygen Species
SARs	Selective autophagy receptors
SNpc	Substantia Nigra pars compacta
TBK1	TANK-binding kinase 1
TIM	Translocase of inner membrane
TOM	Translocase of outer membrane
TOMM20	Translocase of the outer mitochondrial membrane 20

Ub	Ubiquitylation / Ubiquitin
ULK1	Unc-51- like autophagy-activating kinase 1
USP30	Ubiquitin specific peptidase 30
VDAC	Voltage-dependent anion channel
WT	Wild type
WIPI1 or WIPI2	WD repeat domain phosphoinositide-interacting protein 1/2
YFP	Yellow fluorescent protein
3MA	3-methyl adenine

G. AMINO ACID CODE

Amino acid or residue	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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