Cysteine mediated interaction of MIA40 with PINK1; implications in PINK1 stability, and mitophagy

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that this thesis entitled **"Cysteine mediated interaction of MIA40 with PINK1; implications in PINK1 stability, and mitophagy"** submitted to the University of Hyderabad by **Ms. Vandana Bisoyi**, bearing the **Reg. No. 17LBPH04** for the degree of Doctor of Philosophy in Biochemistry, is based on the studies carried out by her under my supervision. To the best of my knowledge, this work has not been submitted earlier for the award or diploma from any other University or Institution, including this University.

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DECLARATION

I, Vandana Bisoyi, hereby declare that the work presented in this thesis entitled **"Cysteine mediated interaction of MIA40 with PINK1; implications in PINK1 stability, and mitophagy"** is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of **Prof. Naresh Babu V Sepuri.** I further declare that this work has not been submitted earlier for the award of a degree or diploma from any other University or Institution.

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This thesis is free of plagiarism and has not previously been submitted in part or whole for the award of a degree or diploma from this or any other University or Institution. Furthermore, prior to submitting the thesis/monograph for adjudication, the student had the following publication(s) and proof for it in the form of reprints in the relevant field of this study.

1. Fareed Mohammed, Madhavi Gorla, **Vandana Bisoyi**, Prasad Tammineni, Naresh Babu V Sepuri. **Rotenone-induced reactive oxygen species signal the recruitment of STAT3 to mitochondria**. FEBS Letters. 2020 May; 594(9):1403-1412.

2. Venkata Ramana Thiriveedi, Ushodaya Mattam, Prasad Pattabhi, **Vandana Bisoyi**, Noble Kumar Talari, Thanuja Krishnamoorthy, Naresh Babu V. Sepuri. **Glutathionylated and Fe–S cluster containing hMIA40 (CHCHD4) regulates ROS and mitochondrial complex III and IV activities of the electron transport chain**. Redox Biology. 2020, 37, 101725

The student has attended the following conferences during her Ph.D program:

1. Presented a poster entitled **"Role of MIA40 in PINK1 import, stability and Mitophagy"** at the **42nd Mahabaleshwar Seminars: Mitochondria Network Meeting**

held at the IISER, Pune, India, during the period February 13-15, 2023.

2. Flash Talk entitled **"Role of MIA40 in PINK1 import, stability and Mitophagy"** in **42nd Mahabaleshwar Seminars: Mitochondria Network Meeting** held at the IISER, Pune, India, during the period February 13-15, 2023.
3. Presented a poster entitled **"Role of MIA40 in PINK1 import, stability and Mitophagy"** at the **International Conference on Virus Evolution, Infection, and Disease Control** held on 15th-17th December 2022, organized by the University of Hyderabad, Hyderabad.
4. Presented a poster entitled **"Role of MIA40 in PINK1 import, stability and Mitophagy"** at **90th Annual Meeting of SBC(I) "Metabolism to Drug Discovery: Where Chemistry and Biology Unite"** organized in virtual mode from 16th to 19th December 2021 by Amity Institute of Biotechnology and Amity Institute of Integrative Sciences and Health, Amity University, Haryana (AUH), Gurugram.
5. Participated in the conference **XI International Conference on Biology of Yeasts and Filamentous Fungi** held on 27th-29th November 2019, organized by the University of Hyderabad and Centre for DNA Fingerprinting and Diagnostics, Hyderabad.

Furthermore, the student has completed the following courses to fulfill the coursework requirement for the Ph.D.

Course Code	Name	Credits	Pass/Fail
BC801	Research Methodology/Analytical Techniques	4	Pass
BC802	Research ethics, Biosafety, Data Analysis and Biostatistics	4	Pass
BC803	Scientific Writing and Research Proposal	4	Pass

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Vandana Bisoyi

Table of Contents

Abbreviations

°C	degree Celsius
AD	Alzheimer's Disease
Ala (A)	Alanine
ALR	Augmenter of liver regeneration
ALS	Amyotrophic Lateral Sclerosis
AMPK	AMP-activated protein kinasPAM
APAF-1	Apoptotic Peptidase Activating Factor 1
ATP	Adenosine triphosphate
BCL2	B-cell leukemia/lymphoma 2 protein.
BNIP	BCL2 interacting protein
BSA	Bovine Serum Albumin
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CO_2	Carbon dioxide
COX	Cytochrome c oxidase
CPC	Cysteine-Proline-Cysteine
CQ	Chloroquine
Cys (C)	Cysteine

DAMP	Damage Associated Molecular Patterns
DAPI	4’ 6-diamidino-2phenylindole
DIABLO	Direct Inhibitor of Apoptosis-Binding protein with low pI
DNA	Deoxyribonucleic acid
DRP1	Dynamin-related protein 1
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ER	Endoplasmic Reticulum
Erv1	Enzyme essential for respiration and viability 1
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
Fe-S	Iron-Sulfur cluster
FUNDC1	FUN14 domain-containing 1
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescence Protein
GPX4	Glutathione peroxidase
GSH	Glutathione
GTP	Guanosine triphosphate

H_2O_2	Hydrogen peroxide
HD	Huntington's Disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hr	Hour(s)
HRP	Horseradish peroxidase
IMM	Inner Mitochondrial Membrane
IMS	Intermembrane Space
ITS	Internal Targeting Sequence
kb	Kilo bases
KCl	Potassium chloride
kD	Kilo Dalton
KOH	Potassium hydroxide
LB	Luria-Bertani
LC3	Microtubule-associated protein 1A/1B-light chain 3
MAMs	Mitochondria-associated endoplasmic reticulum membranes
MARCH5	Membrane Associated Ring-CH-Type Finger 5
MCU	Mitochondrial calcium uniporter
MDV	Mitochondrial Derived Vesicles
MFN	Mitofusin

$MgCl_2$	Magnesium Chloride
MIA	Mitochondrial Intermembrane space Import and Assembly
Min	minute(s)
ml	Milliliter
mm	Millimeter
mM	Millimolar
MPP	Mitochondrial Processing Peptidase
MQC	Mitochondrial Quality Control
MSR	Methionine Sulfoxide reductase
mtDNA	Mitochondrial DNA
MTS	Mitochondrial Targeting Signal
MUL1	Mitochondrial E3 ubiquitin protein ligase 1
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NDP52	Nuclear Dot Protein 52
NDUFS4	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4
ng	Nanogram
nm	Nanometer

nM	Nanomolar
NRF 1 and 2	Nuclear Respiratory Factor 1 and 2
OD	Optical Density
OMM	Outer Mitochondrial membrane
OPA1	Optic Atrophy 1
OPTN	Optineurin
PAGE	Polyacrylamide gel electrophoresis
PAM	Pre-sequence translocase-associated motor
PARL	presenilin-associated rhomboid-like protein
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PD	Parkinson's Disease
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator
PINK1	PTEN Induced Kinase 1
PRKN	Parkin
RIPA	Radio immunoprecipitation assay
ROS	Reactive oxygen species
rpm	Rotations per minute
SAM	Sorting and Assembly Machinery

SDHB	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, CII
SDM	Site-directed mutagenesis
SDS	Sodium dodecyl sulfate
Ser	Serine
SM	Skimmed milk
SMAC	Second mitochondria-derived activator of caspase
SOD	Superoxide dismutase
TBS	Tris-buffered saline
TCA	*Tricarboxylic acid cycle/ Trichloroacetic acid*
TFAM	Mitochondrial transcription factor A
Thr	Threonine
TIM	Translocase of Inner Membrane
TMD	Transmembrane Domain
TOM	Translocase of Outer Membrane
TRIS	Tris(hydroxymethyl)aminomethane
TRX-R	Thioredoxin reductase
Ub	Ubiquitin
UPS	Ubiquitin-proteasome system
VDAC	Voltage-dependent anion channel

WT	Wild type
ΔΨm	Membrane potential
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar

CHAPTER 1

Introduction

1.1 Mitochondria

More commonly referred to as the “Powerhouse of a cell”, mitochondria are membrane-bound organelles found in most eukaryotic cells. Its capacity to produce ATP through a process known as cellular respiration is the reason behind its given name. The term ‘mitochondrion’ stemmed from two Greek words “*mitos*” and “*chondrion*” which respectively mean “thread” and “granules-like”. Apart from energy production, it performs various multifaceted yet interdependent functions such as ROS generation, lipid and amino acid metabolism, Fe-S cluster biogenesis, quality control, calcium homeostasis, and apoptosis. Mitochondria have their own genome, which is separate from the nuclear genome found in the nucleus of eukaryotic cells. Mitochondrial DNA codes for 13 mitochondrial proteins that are the components of the ETC. The nuclear genome encodes the remaining mitochondrial proteins, which are then transported into the mitochondria. Numerous human diseases, such as cancer, metabolic diseases, and neurodegenerative disorders, are linked to mitochondrial dysfunction.

1.2 Evolution of Mitochondria

The endosymbiotic theory proposes that mitochondria have originated approximately 1.5 billion years ago through an endosymbiotic relationship between a eukaryotic cell and an α-proteobacterium (Margulis, 1970; Lane, 2010). The theory suggests that a nucleus-containing host cell engulfed an α-proteobacterium, which evolved into the mitochondria over a period of time. This symbiotic relationship provided the host cell with a constant supply of ATP while the endosymbiont received protection and a stable environment (Martin et al., 2016). Mitochondria evolution brought numerous significant changes in the mitochondria. One of the major changes is the loss of genes from the mitochondrial genome (Gray et al., 2015). This is hypothesized to be caused by endosymbiotic gene transfer, a process by which mitochondrial genes are transferred from the host cell's mitochondria to its nuclear genome (Smith and Keeling, 2015; Adams and Palmer, 2003). The other significant change is the development of cristae, which increased the surface area of the IMM. The cristae provided more surface area allowing a more efficient ATP production (Davies et al, 2012). Detailed evidence in favor of the notion that mitochondria and plastids evolved through endosymbiotic relationships can be found in Lynn Margulis' 1970 book "Origin of Eukaryotic Cells," (Margulis, 1970). The endosymbiotic concepts of mitochondrial origin are based on two distinct themes: the Archezoan scenario and the Symbiogenesis scenario. The Archezoan scenario suggests that the

primitive mitochondrial eukaryote acquired the endosymbiont while the latter proposes that the endosymbiont was acquired by the archeal cell, leading to the formation of the nucleus and further cell compartmentalization (Lane, 2010). The endosymbiotic process rendered mitochondria reliant on the host cell's nucleus for the proteins necessary for their activity. Because of this reliance on the host's nucleus, specialized protein import machinery has evolved across the outer and inner membranes (Neupert and Herrmann, 2007).

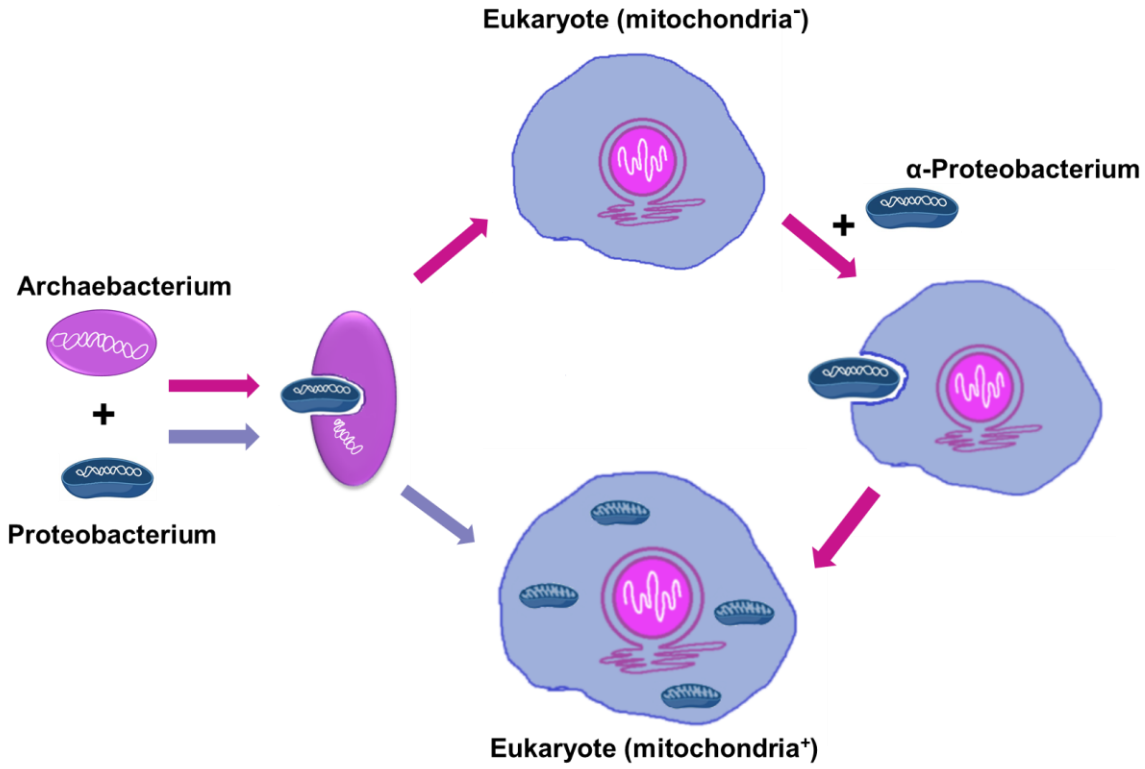


Figure 1.1: Endosymbiotic Theory

The formation of the eukaryotic mitochondrion and nucleus is indicated by the purple arrows that indicate the fusion between an archaebacterium (host) that needs hydrogen and a hydrogen-producing proteobacterium (symbiont). A proteobacterium and an archaebacterium fuse to generate an amitochondriate eukaryote, which later acquires a mitochondrion through endosymbiosis with an α-proteobacterium (pink arrows).

1.3 Structure of Mitochondria

Mitochondria are double membrane-bound organelles found in almost all eukaryotic cells. These are responsible for generating ATP for various cellular processes. The structure of mitochondria comprises of an outer membrane, an inner membrane, an intermembrane space, and a matrix. The different compartments of mitochondria have their own distinguished function. In addition to their structure, mitochondrial size and genome also play an essential role in their proper functioning.

With respect to the type of the cell and its energy requirements, the size of the mitochondria can vary. Normally, mitochondria are 0.5 to 1 μm in diameter and 2 to 10 μm in length. However, they can range in size from 0.2 μm to 10 μm.

Mitochondria generally appear as bean or rod-shaped structures but they can also exist as flattened discs, elongated ovals or long branched structures. The shape and size of mitochondria can vary with changes in mitochondrial dynamics as well. Mitochondria undergoing fission appear punctate and mitochondria undergoing fusion appear tubular in structure. Fusion and fission of mitochondria are crucial in maintaining their optimal size and shape (Nunnari et al., 2012).

Depending on their metabolic requirements, different cell types have different levels of mitochondria. Muscle, liver, brain, and heart cells, amongst others, have high energy requirements, thus they have an abundance of mitochondria to generate ATP and supply energy for their functions. On the other hand, cells that require less energy, like RBCs, skin cells, and fat cells, have fewer mitochondria. Amongst these, RBCs are devoid of mitochondria because they get their energy through anaerobic glycolysis. Since storing energy rather than using it is the major purpose of skin and fat cells, these cells possess very few mitochondria. (Mootha et al., 2003).

Two membranes separate mitochondria from the cytoplasm and divide them into four compartments: the IMM, OMM, IMS, and matrix.

The inner mitochondrial membrane and outer mitochondrial membranes are the two membranes that distinguish mitochondria from the cytosol and separate them into these four compartments discussed in detail below.

1.3.1 Outer membrane:

The outer membrane of mitochondria is a semi-permeable membrane that serves as a protective barrier. It comprises of proteins, such as porins and VDAC, that allow the entry of small molecules, ATP, and ions into the intermembrane space. The OMM is associated with the ER through the mitochondria-associated ER membrane (MAM). MAMs are essential for the transport of calcium and lipids between ER and mitochondrial membranes. Additionally, the OMM is home to protein complexes involved in lipid metabolism, including those that create cardiolipin, a special phospholipid found only in the MIM. Simple phospholipids make up the outer membrane, and their phospholipid-to-protein ratio (1:1) is comparable to that of the plasma membrane.

1.3.2 Inner membrane:

The IMM has a high protein-to-phospholipid ratio (3:1) making it highly impermeable compared to the OMM. Because of its impermeable nature, it requires transporter proteins to transport molecules across it. The TIM complex transports proteins that are targeted to the matrix or the inner membrane. The IMM also harbors mitochondrial respiratory chain complexes, which play a crucial role in ATP synthesis and the electron transport chain. The IMM folds to form cristae, which increases the surface area for ETC complexes that are involved in oxidative phosphorylation, the process that generates ATP. In the process of generating ATP, the ETC also forms a proton gradient across the IMM. The IMM serves as the principal site for ATP generation in the mitochondria due to the inner membrane's impermeability and the presence of a large number of respiratory complexes. (Mannella et al., 2006).

1.3.3 Intermembrane space:

The region that lies between the IMM and the OMM is known as the IMS. It is a narrow space that accommodates a number of proteins and enzymes important in controlling apoptosis (programmed cell death) and oxidative stress, and various other cellular processes.

1.3.4 Matrix:

The innermost compartment of the mitochondria is known as the matrix. it contains a variety of enzymes, ribosomes, and mtDNA. The apparatus required for the replication and transcription and translation of mtDNA, which is crucial for the production of new mitochondria and the preservation of mitochondrial function, is also found in the mitochondrial

matrix. The two processes crucial for ATP synthesis namely, the TCA cycle and β-oxidation of fatty acids, also occur in the mitochondrial matrix.

All mitochondrial compartments work in tandem to enable the mitochondria to efficiently carry out cellular respiration and generate ATP for the cell's energy requirements.

1.3.5 Genome:

A circular DNA molecule with a size of roughly 16.6 kb makes up the human mitochondrial genome. There are 22 transfer RNAs, 2 ribosomal RNAs, and 37 genes present that code for the 13 oxidative phosphorylation-related proteins. Unlike nuclear DNA, mtDNA is inherited from the mother and has a greater probability of mutation than nuclear DNA. Each mitochondrion contains multiple copies of the mtDNA (Wallace et al., 2013).

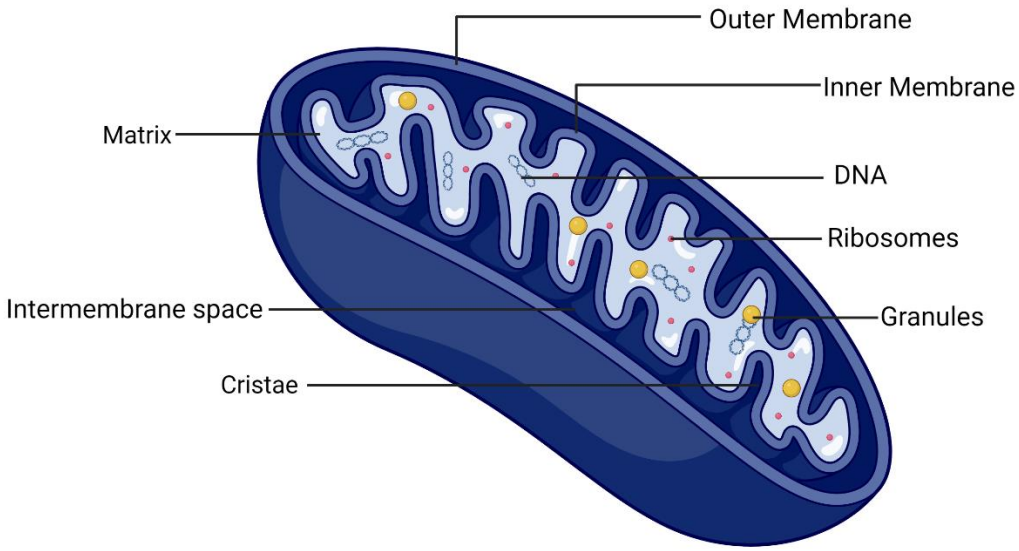


Figure 1.2: Structure of Mitochondria

1.4 Functions of Mitochondria

Mitochondria perform various functions but its principal function is the generation of ATP through a process called oxidative phosphorylation. In this process a proton gradient is generated across the mitochondrial membrane, which is utilized for producing ATP, by transferring electrons from substrates like glucose and fatty acids to molecular oxygen.

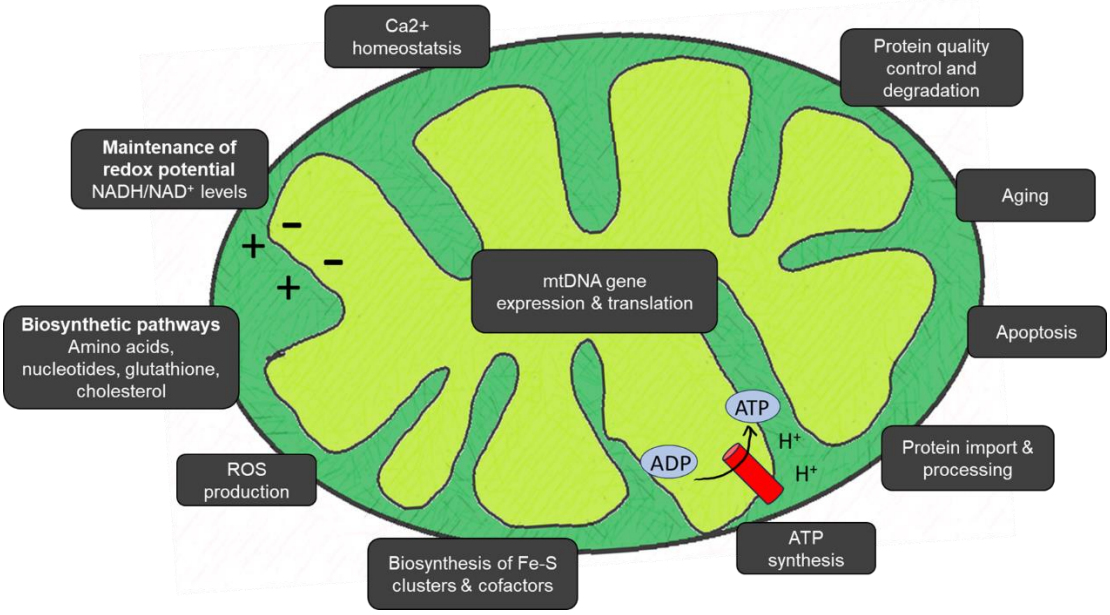


Figure 1.3: Functions of mitochondria

Diagrammatic representation of various functions that are regulated by mitochondria

Other functions of mitochondria include the following

1.4.1 Calcium regulation:

Mitochondria are involved in regulating calcium levels within cells, playing a crucial role in preventing excessive calcium signaling that can be detrimental to the cell. By taking up calcium ions from the cytoplasm, mitochondria help to maintain proper calcium levels that are vital for cellular development, proliferation, and cell death. In order to regulate the activity of the enzymes involved in the TCA cycle and the ATP synthase complex, the calcium levels within mitochondria need to be regulated (Mishra et al., 2016; Vandecasteele et al., 2001). The crucial protein in this process is called mitochondrial calcium uniporter (MCU), which is found in the IMM (Kirichok et al., 2004; Kamer et al., 2015).

1.4.2 ROS production:

The formation of ROS in mitochondria can be impacted by a multitude of factors, including the electron flow through the ETC, the electric potential of the mitochondrial membrane, and the concentrations of antioxidants in the mitochondria. The generation of ROS is strictly regulated under normal circumstances and can actually contribute to cellular signaling which boosts cell survival and stress adaption (Sies et al., 2017). Dysregulation of the mentioned

factors can lead to increased ROS production and oxidative stress, which are linked to a number of diseases such as cancer, cardiovascular disease, and neurodegenerative disorders (Ježek & Dlasková, 2015)

1.4.3 Apoptosis:

"Programmed cell death" or apoptosis is a process in which mitochondria play a role. Mitochondria release certain proteins, such as cytochrome c, which activates caspase and causes the cell to undergo apoptosis. BCL-2 proteins, which have the ability to either stimulate or inhibit cytochrome c release from the mitochondria, control this process. Following its release from the mitochondria, cytochrome c binds to APAF-1, which oligomerizes to form the apoptosome, a large complex that activates caspase-9. (Green et al., 2014; Martinou et al., 2011). The activation of subsequent effector caspases like caspase-3 and caspase-7 by caspase-9 causes them to cleave a range of intracellular substrate proteins, ultimately causing apoptosis. Besides cytochrome c, mitochondria can also release other proteins that can promote or inhibit apoptosis (Vander Heiden, 1999). For example, the release of SMAC/DIABLO can enhance the activation of caspases and promote apoptosis by inhibiting certain caspase inhibitors. Mitochondrial dysfunction can also contribute to cell death by other mechanisms, such as necrosis or necroptosis, which can cause tissue damage by activating inflammatory pathways through the release of DAMPs.

1.4.4 Lipid metabolism:

Mitochondria are involved in the biosynthesis of lipids. Many lipids, including phosphatidylglycerol, phosphatidylcholine, cardiolipin, and to a lesser extent, phosphatidylethanolamine, phosphatidic acid, and CDP-diacylglycerol, can be synthesized by mitochondria on their own in the IMM (Horvath et al.,2013). Additionally, mitochondria are also involved in lipid metabolism, which breaks down fatty acids via the carnitine shuttle system. Lipid metabolism leads to the subsequent generation of ATP through the TCA cycle. The lipids are converted into fatty acids and glycerol by lipases. The glycerol formed is used as a substrate to generate pyruvic acid which is subsequently used for the generation of ATP. A malfunction of mitochondrial phospholipid biosynthesis can result in failure of cellular respiration, affects the assembly and stability of the machinery required for the import of mitochondrial proteins, and result in aberrant mitochondrial morphology or even death (Mayr et al., 2015).

1.4.5 Inter-organelle communication between mitochondria and other organelles

Mitochondria interact with several organelles through direct physical contact, including the endosomes, ER (Copeland and Dalton, 1959), Golgi apparatus (Nagashima et al., 2020), lipid droplets (Rambold et al., 2015), lysosomes/vacuoles (John Peter et al., 2017), melanosomes, nucleus (Eisenberg et al., 2016), peroxisomes (Fan Li et al., 2016), and plasma membrane. These interactions play a crucial role in regulating various cellular processes, such as calcium signaling, lipid metabolism, mitochondrial dynamics, and autophagy. The mechanisms responsible for tethering these organelles and the specific cellular processes regulated by these interactions are still not fully understood. However, proteins that act as direct tethers between organelles are crucial in mediating these interactions and maintaining mitochondrial homeostasis (Vafai & Mootha, 2012). Studies on the functional relevance of these interactions date back to the 1950s, and research in the last two decades has identified and extensively studied the communication between mitochondria and other organelles (Berridge, 2016).

Additionally, mitochondria also produce heme, contribute to the generation of heat in brown adipose tissue, and control cell proliferation by modifying ATP levels.

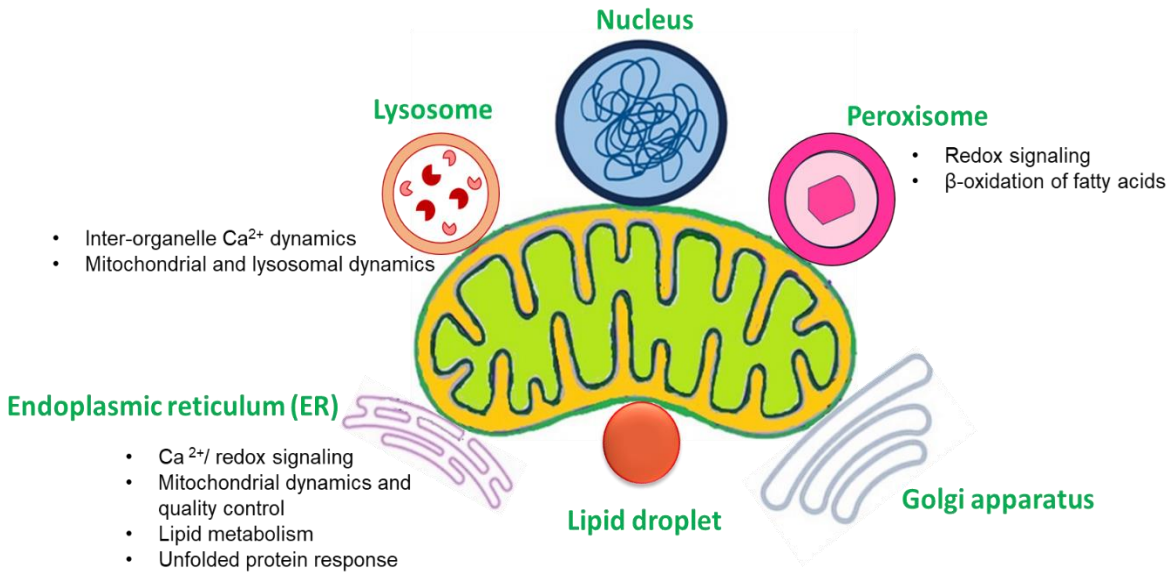


Figure 1.4: Inter-organellar communication of mitochondria

Diagrammatic illustration of mitochondrial connections with several organelles, including the vacuole/lysosome, endoplasmic reticulum, lipid droplets, peroxisomes, and Golgi apparatus.

1.5 Mitochondrial Biogenesis

Mitochondrial biogenesis is the process by which new mitochondria are formed in cells. It involves the growth and division of pre-existing mitochondria to increase the mitochondrial mass and copy number in the cell. This process is tightly regulated and involves several molecular factors. These can be divided into three categories: transcription factors, co-activators, and signaling molecules.

1.5.1 Transcription factors:

Mitochondrial biogenesis is regulated by several transcription factors such as PGC-1α, NRF-1 and NRF-2, and TFAM (Scarpulla, 2011; Handschin & Spiegelman, 2008). PGC-1α increases mitochondrial biogenesis and is activated in response to physiological stress, such as exercise and exposure to cold (Puigserver & Spiegelman, 2003). TFAM, NRF1 and NRF2 are upregulated by PGC-1α, which increases the replication of mitochondrial DNA and its gene transcription (Scarpulla, 2002; Scarpulla, 2008).

1.5.2 Co-activators:

The co-activators involved include the PGC-1α co-activator PGC-1β and the histone acetyltransferase P300/CBP-associated factor (PCAF). PGC-1β and PGC-1α have a comparable role in mitochondrial biogenesis. PCAF is a histone acetyltransferase that acetylates histones, altering the chromatin structure and facilitating the binding of transcription factors to DNA (Handschin & Spiegelman, 2006; Scarpulla, 2008).

1.5.3 Signaling molecules:

AMPK, sirtuins, and IGF-1 are some of the signaling molecules involved in mitochondrial biogenesis. All of them signal for the activation of PGC-1α. AMPK is a cellular energy sensor that is activated upon low cellular energy levels. After becoming active, AMPK phosphorylates and activates PGC-1, which in turn promotes mitochondrial biogenesis. Similarly, Sirtuins are deacetylases that deacetylate SIRT1 to activate PGC-1α (Austin & Pierre, 2012; Scarpulla, 2011). IGF-1 promotes PGC-1α activation and mitochondrial biogenesis via activating the PI3K/Akt signaling pathway (Wenz, 2013; Handschin, 2009).

1.6 Mitochondrial Protein Import

Mitochondria have their own genome and are capable of synthesizing some of their proteins. However, the nuclear DNA encodes for the maximum mitochondrial proteins and are directed to mitochondria by certain signal peptides or transit peptides (Pfanner et al., 2009; Pfanner et al., 2019). A series of protein complexes are involved in mitochondrial protein import. The first step of mitochondrial protein import involves the recognition of the targeting sequence by the TOM complex. Numerous subunits make up the 400 kDa TOM complex, which creates a large protein pore in the OMM (Pfanner et al., 2019). The protein is then translocated into the IMS. The TIM complex present in the inner membrane aids in the subsequent stage of importing the protein into the IMM or the matrix. The TIM complex also consists of a number of subunits. (Endo & Kohda et al.,2002).

The mitochondrial targeting sequence or the MTS is usually positioned at the N-terminus of the protein. It is a short peptide that can range between 3-70 amino acids in length. It is mostly rich in positively charged or basic amino acids.

There are now five significant protein import pathways known, each of which is identified by a distinct targeting signal.

1.6.1 Pre-sequence Pathway:

The most prevalent pathway for the import of mitochondrial proteins is the pre-sequence pathway. A significant proportion of mitochondrial matrix proteins are imported via this pathway and contain an N-terminal pre-sequence. The TOM complex recognizes the pre-sequence, which is then transported by the TOM and TIM complex through the outer and inner membranes. In the end, the PAM complex helps these proteins enter the matrix (Pfanner et al., 2019). The MPP protease cleaves off the pre-sequence when the protein enters the matrix, leaving the mature protein behind.

The remaining four primary protein import pathways involve internal targeting signals (ITS) rather than a cleavable pre-sequence. Nevertheless, the TOM complex acts as the entrance for both cleavable and non-cleavable precursors (Rehling et al., 2004).

1.6.2 Carrier Pathway:

Mitochondrial carrier proteins belong to a class of proteins responsible for transporting small hydrophobic molecules, such as metabolites, nucleotides, and amino acids across the IMM.

These proteins contain an ITS or the internal targeting sequence that guides them to the IMM. The carrier protein is transferred to the TIM22 complex after being recognized by the TOM complex, which enables its insertion into the inner membrane.

1.6.3 Outer Membrane (OM) or β-barrel Pathway:

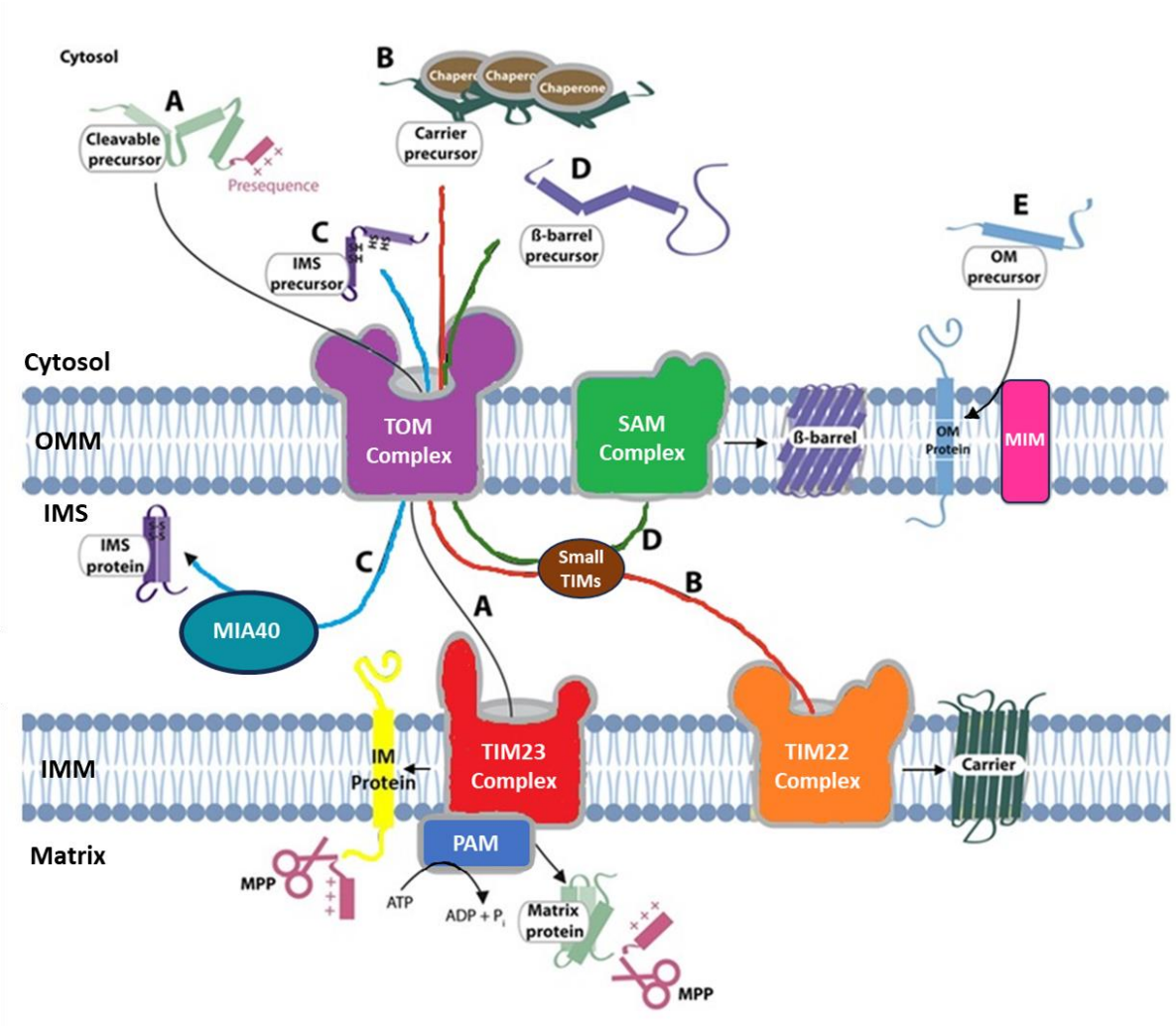
Certain proteins found in the OMM, such as porins and the SAM complex, possess a specific signal that guides them to the TOM complex for insertion. The third pathway for importing proteins is the β-barrel pathway. The TOM complex and the chaperones of the TIM complex are used in this process to import β-barrel proteins from the OM. Afterward, the SAM complex located in the OM incorporates these proteins into the OM.

1.6.4 Intermembrane Space (IMS) or MIA Pathway:

Precursor proteins with cysteine residues are recognized by the MIA40 pathway and imported into the IMS. These proteins are first imported via the TOM complex and then retained in the IMS with the help of MIA machinery present in the IMS. MIA40 enables the proper folding of proteins through the formation of disulfide bonds. The mature folded protein is released into the IMS to carry out its specific cellular function. Proteins that reside in the IMS, such as cytochrome c and the small Tim proteins, are imported via this pathway (Truscott et al., 2019).

1.6.5 Inner Membrane (IM) or MIM Pathway:

The internal targeting signal in proteins such as cytochrome b2 and the ADP/ATP carrier directs them to the TIM22 complex for incorporation into the IMM. The MIM pathway is responsible for transporting proteins across the IMM and requires the cooperation of the TIM and PAM complexes (Chacinska et al., 2009). This pathway is also used for OM proteins that have an α-helical transmembrane domain. The MIM complex facilitates the efficient import of multispanning OM proteins (Pfanner et al., 2009; Pfanner et al., 2019).



(Modified from Harbauer et al., 2014)

Figure 1.5: Major Mitochondrial Protein Import Pathways

The TOM complex imports precursors made in the cytosol, where the maximum mitochondrial proteins are synthesized. A) Pre-proteins having N-terminal cleavable pre-sequences can go through this pathway. to be transported from TOM to the TIM23 complex. (B) The TOM channel is used to transport hydrophobic metabolite carriers with non-cleavable precursors. By attaching to TIM chaperones in the IMS, the TIM22 complex inserts the precursors into the membrane. (C) The MIA machinery imports IMS proteins with high cysteine residues and connects them by disulfide bonds after passing the proteins through the TOM channel in a reduced form. (D) The tiny TIM chaperones import the OM β-barrel protein precursors, which are subsequently introduced into the OM via the SAM complex. E) OM proteins with α-helical transmembrane domains are introduced into the membrane by the MIM complex.

1.7 MIA40 and its Role in Electron Transport Chain

The MIA pathway that resides in the IMS of mitochondria plays a critical role in importing cysteine-rich proteins into the IMS (Chacinska et al., 2004; Hofmann et al., 2005). IMS contains a redox-active environment that aids in the oxidative folding of substrate proteins to create disulfide bonds. Two thiol groups present in the two cysteine residues are oxidized during this process, creating an intramolecular disulfide bond that is covalently bonded.

MIA40, the key protein in the MIA pathway is either soluble in higher eukaryotes (CHCHD4) or insoluble as in the case of primitive eukaryotes such as fungi and yeast, where it is attached to the IM with the help of its N-terminal (Chacinska et al., 2004; Hofmann et al., 2005). In contrast to yeast's membrane-bound MIA40, CHCHD4 is missing the MTS and TMD from its N-terminal region. However, CHCHD4 retains its characteristic CPC and twin CX9C motifs present in its C- terminal (Terziyska et al., 2009; Banci et al., 2009; Modjtahedi et al., 2016; Hofmann et al., 2005; Chacinska et al., 2004).

MIA40 is an oxidoreductase that possesses six conserved cysteine residues arranged as a CPC motif and twin CX9C motifs. These CX9C motifs form structural disulfides that contribute to the protein's stability. These also create a hydrophobic cleft that facilitates substrate-protein binding. The CPC motif in MIA40 is sensitive to the redox environment of the IMS. It accepts electrons from the reduced substrates in the IMS and transfers them to molecular oxygen or other electron acceptors, generating a disulfide bond in the process (Sideris et al., 2009). To restore its capacity to import more substrate proteins, MIA40's reduced CPC motif must go through re-oxidation. (Banci et al., 2009; Manganas et al., 2017). Apart from this, it has also been implicated that the CPC motif in MIA40 facilitates the export of iron-sulfur clusters across the mitochondrial membrane into the cytosol (Murari et al, 2015). With the use of disulfide bonds, MIA40 switches between an oxidized and a reduced state to ensure the proper import of IMS proteins. (Naoé et al., 2004; Kawano et al., 2009; Banci et al., 2009).

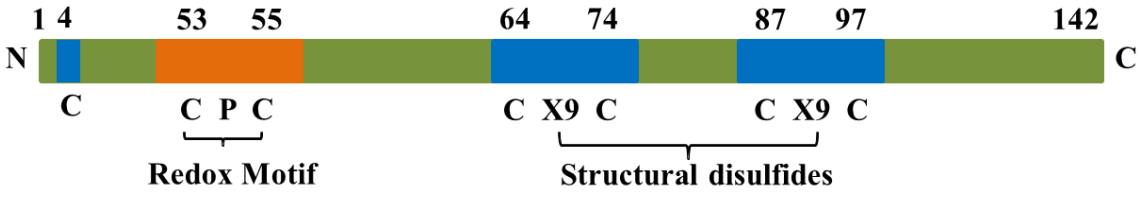


Figure 1.6: Structure of MIA40

ALR (Erv1 in yeast) is the next crucial part of the MIA machinery. It is a sulfhydryl oxidase that accepts electrons from MIA40 to re-oxidize it for the next round of protein import by forming disulfide bonds with the reduced imported protein (Banci et al., 2013, Banci et al., 2011). ALR has the ability to *de novo* transfer these electrons to oxygen, leading to H_2O_2 production. It can also transfer these electrons to cytochrome c and ETC complex IV. As the reduced imported protein transfers electrons to MIA40, then to ALR, and finally to a terminal electron acceptor, MIA40 undergoes reoxidation (Bihlmaier et al., 2007; Allen et al., 2005; Daithankar et al., 2009; Dabir et al., 2007; Kojer et al., 2012).

Twin CX3C or CX9C motifs and a hydrophobic IMS targeting signal (ITS) are common features of MIA40 substrates. Other MIA40 substrates, however, can have various arrangements of cysteine residues (Vogtle et al., 2012), like Mix23 contain a CX13C motif (Modjtahedi et al., 2016). In order for MIA40 to be imported into the IMS, it must interact with endogenous MIA40 (Murray et al., 2021; Chatzi et al., 2013).

These series of redox reactions involved in the protein import via MIA pathway produce ROS, which when present in low levels have a beneficial role in cell signaling but can be harmful in excessive amounts. Signaling molecules like H_2O_2 and glutathione (GSH) are essential for initiating cellular redox responses. H_2O_2 acts as an oxidizing agent, while GSH acts as a reducing agent. Recently we have demonstrated that MIA40 undergoes glutathionylation, which regulates the levels of ROS generated by ETC complexes III and IV (Thiriveedi et al., 2020).

The ETC relies heavily on MIA40 as it facilitates the import and assembly of several ETC subunits. A few examples are the NDUFS4 subunit of Complex I, the SDHB subunit of Complex II, and the COX10 and COX17 subunits of Complex IV. Apart from its role in protein import, recent investigations have also implied that MIA40 may have a direct role in regulating mitochondrial complex activities. MIA40 has been shown to interact with the Complex III of ETC. It has also been shown that glutathionylation of human MIA40 enables MIA40 to directly transfer electrons to cytochrome c (Thiriveedi et al., 2020). As a result, it is possible that MIA40 functions in ways other than protein import and assembly that are more directly related to the regulation of ETC activity.

Defects in mitochondrial protein import can be a result of mutations in the conserved cysteines in MIA40 which in turn affects ATP synthesis. Multiple mitochondrial illnesses, such as

mitochondrial myopathy, lactic acidosis, encephalopathy, and MELAS syndrome, have been linked to MIA40 mutations (Imai et al., 2019).

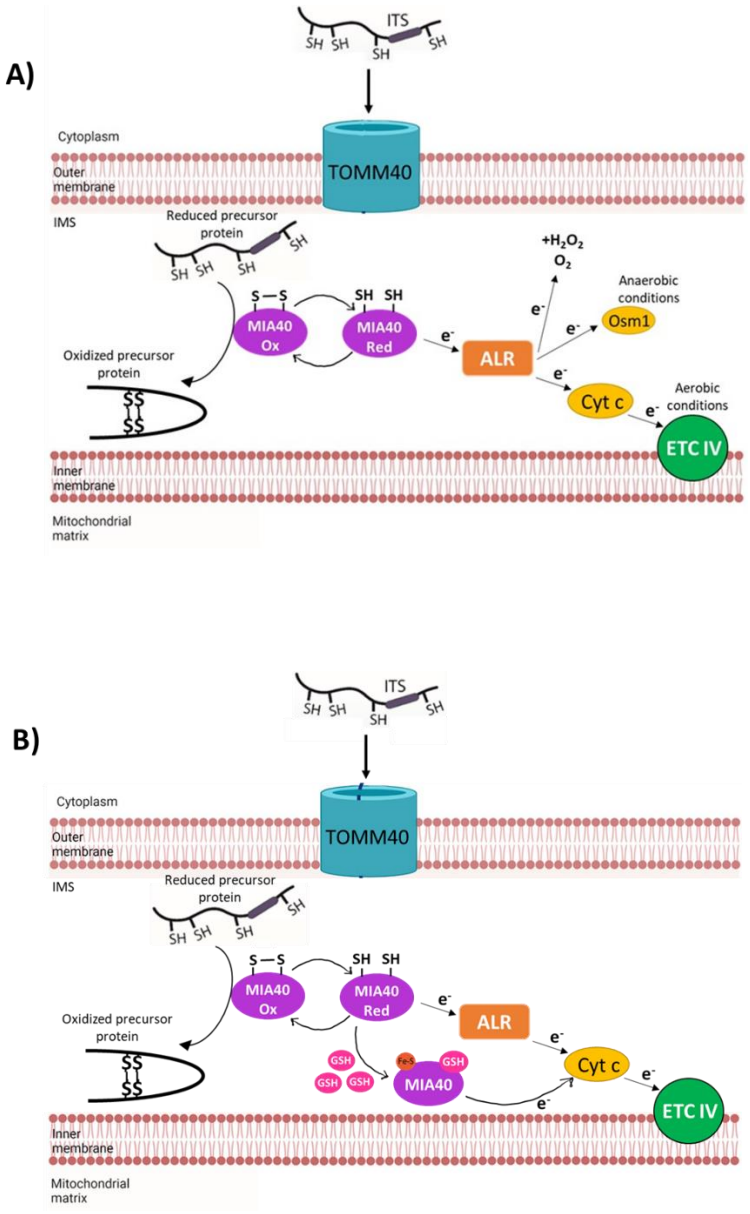


Figure 1.7: Role of MIA40 in Electron Transport Chain

A) MIA40, a thiol oxidoreductase converts the reduced unfolded imported proteins to their oxidized folded forms by forming disulfide bonds. ALR which is a sulphydry oxidase accepts electrons from MIA40 to maintain MIA40 in its oxidised form. these electrons are further transferred directly to molecular O_2, or to cytochrome c and complex IV (Murray et al., 2021). B) Glutathionylated MIA40, with the help of Fe-S cluster, is able to transfer electrons directly to cytochrome c (Thiriveedi et al.,2020).

1.8 Mitochondrial Diseases

1.8.1 Neurodegenerative diseases

The intricate process of mitochondrial biogenesis renders them highly susceptible to damage, and their dysfunction has been linked to various neurodegenerative diseases. When mitochondrial damage persists, it disrupts energy metabolism, resulting in reduced ATP production, elevated levels of ROS, and impaired calcium regulation. Additionally, abnormalities in mitochondrial fission and fusion, cytochrome c release, and the initiation of apoptosis further contribute to dysfunctional mitochondria (Van der Bliek et al.,2013; Meyer et al., 2017). Collectively, these factors contribute to neuronal loss, a characteristic feature observed in both acute as well as chronic neurodegenerative disorders (Liu et al., 2018; Fatokun et al., 2014).

Here are some neurodegenerative diseases associated with mitochondrial dysfunction:

1.8.1a. Parkinson's Disease: In PD, dopaminergic neurons in the substantia nigra area of the brain are known to deteriorate (Tysnes at al., 2017). Mitochondrial dysfunction, including impaired oxidative phosphorylation and increased production of ROS, is believed to contribute to neuronal cell death in PD. Familial types of PD have been associated with mutations in mitochondrial genes like PINK1 and Parkin (Lucking et al., 2000; Klein & Westenberger, 2012). PD patients with PINK1 mutations that result in loss of function have impaired complex I activity. Some of the proteins imported by MIA40 are directly involved in the biogenesis and construction of mitochondrial respiratory complex I. Some of these proteins are NDUFS5, NDUFAF8, NDUFA8, NDUFB10, NDUFS8, and NDUFB7 (Reinhardt et al., 2020; Brunelli et al., 2020).

1.8.1b. Alzheimer's Disease: AD is the most common type of dementia that is characterized by the increased number of tau tangles and beta-amyloid plaques in the brain. Mitochondrial dysfunction, including decreased ATP production, impaired mitochondrial dynamics, and increased oxidative stress, are characteristics of Alzheimer’s (Swerdlow et al, 2009: Lane et al, 2018; Querfurth et al., 2010).

1.8.1c. Huntington's Disease: HD is a hereditary neurological condition marked by huntingtin gene mutations. Neuronal malfunction and cell death in the brain are attributed to mitochondrial dysfunction, which includes increased oxidative stress, and dysregulation of mitochondrial dynamics, and poor energy metabolism. (Quintanilla et al., 2009; Milakovic et al., 2005).

1.8.1d. Amyotrophic Lateral Sclerosis: Motor neurons are impacted by the progressive neurodegenerative disease ALS. Mutations in genes such as SOD1 and C9orf72, associated with familial ALS, affect mitochondrial function (Smith et al., 2019).

1.8.2 Cancer:

In addition to neurodegenerative diseases, mitochondrial dysfunction has a substantial impact on the onset and progression of cancer. The Warburg effect or aerobic glycolysis, a change in metabolism seen in cancer cells, is a key variable in cancer. Cancer cells significantly rely on glycolysis to meet their high energy demands and promote rapid proliferation even when oxygen is present. This phenomenon along with mitochondrial dysfunction, leads to increased production of ROS. Prolonged exposure to ROS causes chronic oxidative stress and DNA damage, contributing to instability in the genome of cancer cells. This instability promotes the accumulation of genetic alterations that leads to tumour development. Additionally, dysfunctional mitochondria can impair apoptotic signaling pathways, reducing the ability of cancer cells to undergo apoptosis. This resistance to apoptosis enhances cell survival, facilitates tumour growth, and confers resistance to conventional cancer therapies (DeHart et al., 2018).

1.8.3 Mitochondrial Diabetes:

Certain mitochondrial mutations can lead to impaired insulin secretion, resulting in diabetes mellitus. The condition may manifest as either type 1 or type 2 diabetes.

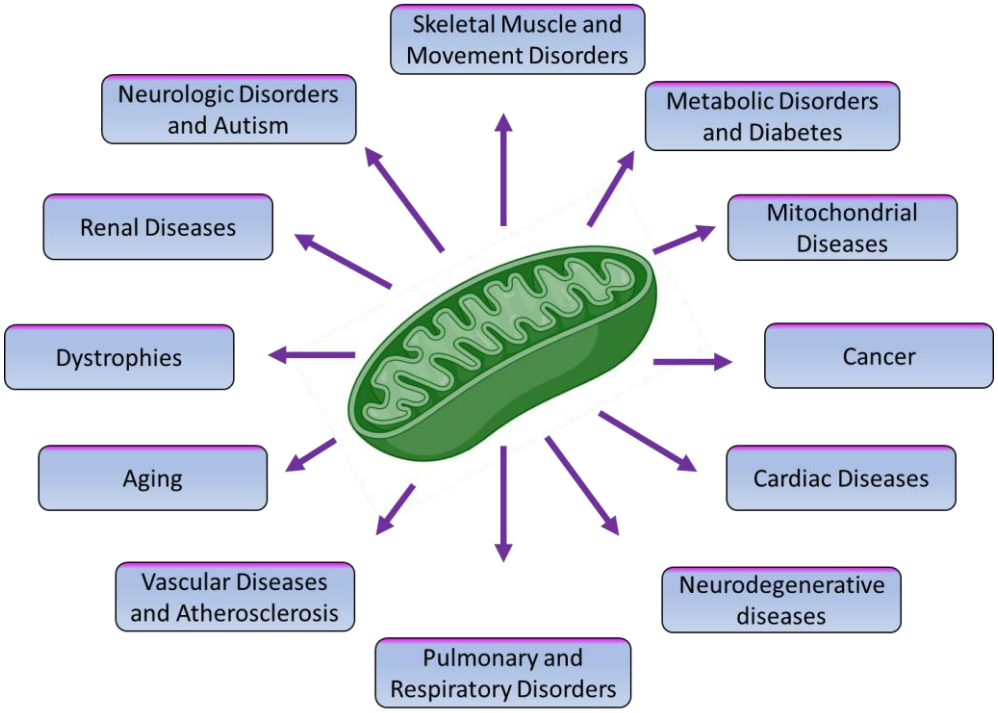


Figure 1.8: Mitochondrial dysfunction causes a myriad of diseases

1.9 Mitophagy

A special type of autophagy known as mitophagy targets damaged or malfunctioning mitochondria for degradation. It is essential for preserving the health of the mitochondria and cellular homeostasis. Mitophagy involves the recognition and tagging of damaged mitochondria, followed by their sequestration into autophagosomes and subsequent fusion with lysosomes for degradation. When mitochondria is damaged or has lost its membrane potential, they can generate ROS leading to oxidative stress within cells. When the mitochondrial damage is beyond repair, cells have developed a mechanism to selectively eliminate and degrade damaged mitochondria through mitophagy (Youle., 2008). Dysfunction in mitophagy has been implicated in a number of diseases, including neurological disorders such as Parkinson's disease (Lucking et al., 2000; Valente et al., 2004; Kitada et al., 1998;), Alzheimer's (Lane et al, 2018; Ye, Sun et al., 2015), and Huntington's disease (Khalil et al., 2015). Impairment in mitochondrial function can also cause cancer (DeHart et al., 2018; Bernardini et al., 2017) as well as metabolic disorders such as diabetes.

In mammals, there are three major types of mitophagy These different types of mitophagy involve various molecular pathways and mechanisms.

1.9.1 Ubiquitin-Mediated Mitophagy

1.9.1a. PINK1/Parkin-mediated mitophagy: This is one of the most well-studied types of mitophagy (Youle, 2008; Gautier, 2008). In damaged mitochondria, this pathway involves the stabilization of PINK1 protein on the OMM. Upon stabilization, PINK1 recruits Parkin, an E3 ubiquitin ligase, to the mitochondria. Subsequently, PINK1 and Parkin work together to phosphorylate and ubiquitinate OMM substrates (Yoshii et al., 2011). These phosphorylated and ubiquitinated proteins serve as signals for the recruitment of autophagy machinery to recognize and engulf the damaged mitochondria by the formation of autophagosomes.

1.9.1b. Other E3 Ubiquitin Ligases: Besides PINK1-Parkin-mediated mitophagy, other E3 ubiquitin ligases can also regulate mitophagy independently of PINK1 and Parkin (Villa et al., 2018). One example is MUL1, which shares similar substrates with Parkin. MUL1 can directly interact with GABARAP and facilitate the autophagic engulfment of damaged mitochondria (Szargel et., 2016).

1.9.2. OMM Receptor-Mediated Mitophagy

In addition to PINK1/Parkin-mediated mitophagy, other autophagy receptor proteins are anchored on the OMM and play a role in initiating mitophagy. These receptors include NIX (also known as BNIP3L) (Novak et al., 2010), BCL2-L13 (Murakawa et al., 2015), FUNDC1 (Liu et al., 2012), and FKBP8 (Bhujabal et al., 2017). By attaching to LC3, FUNDC1 or NIX/BNIP3L can promote the sequestration of mitochondria into autophagosomes for destruction. (Wang et al., 2017).

1.9.3. Lipid-Mediated Mitophagy:

The movement of cardiolipin, a phospholipid typically present in the IMM, to the OMM, is the characteristic feature of this mitophagy mechanism. (Chu et al., 2013). Cardiolipin directly interacts with LC3, initiating mitophagy through lipid-mediated signaling (Cai & Jeong, 2020)

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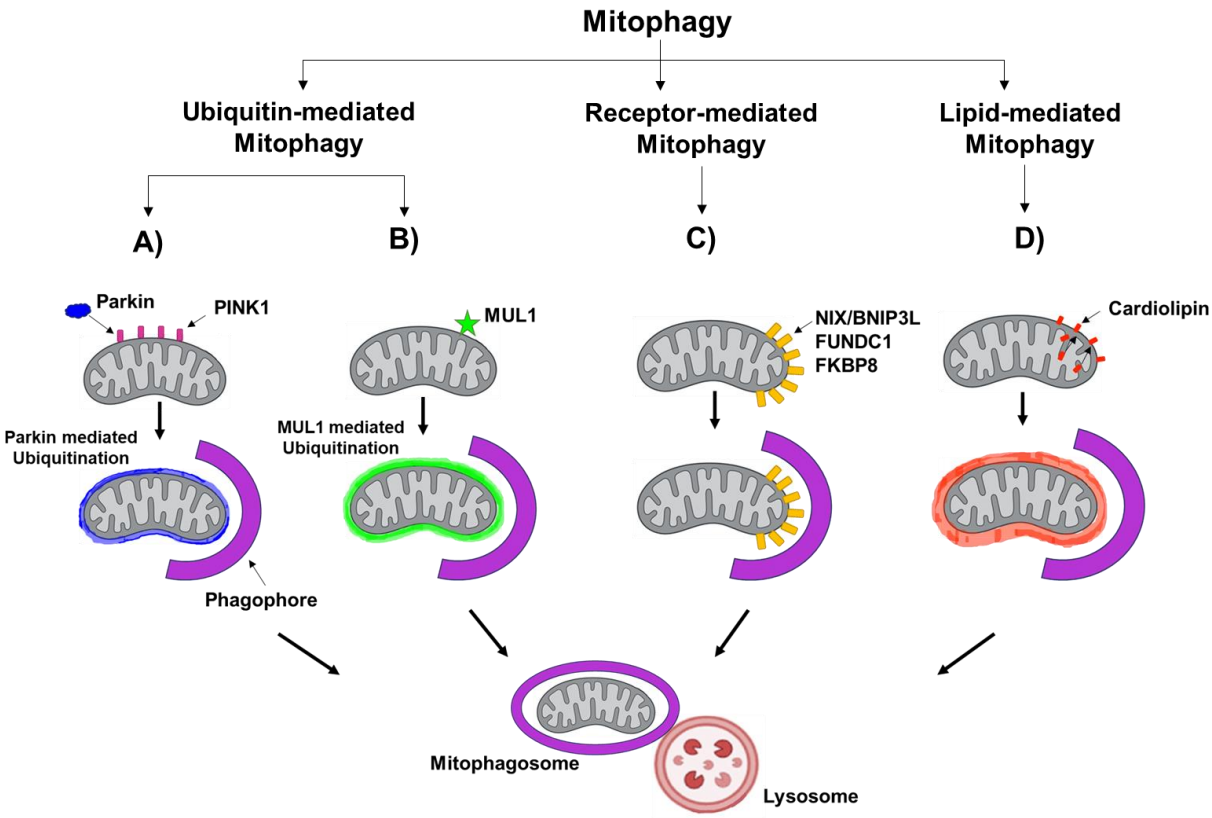


Figure 1.9: Three major types of mitophagy in mammals

Three main pathways can be used to trigger mitophagy in response to mitochondrial damage: A & B) Mitophagy mediated by ubiquitin, C) Mitophagy mediated by OMM receptors, and D) Mitophagy mediated by lipids.

1.10 PINK1 as a sensor of the health status of mitochondria

PINK1 is one of many mitochondrial proteins that are translated as their precursors located in the cytosol and then trafficked into the mitochondria. With a molecular weight of 63 kD, PINK1 is a Ser/Thr kinase. (Valente et al., 2004). PINK1 is continually imported into the IMM in a healthy mitochondria across the TOM and TIM23 complexes in a membrane potential ($\Delta\Psi m$) dependent way across the inner membrane. (Greene et al., 2012; Lazarou & Youle, 2012). The N-terminal MTS of PINK1 is cleaved off by MPP in the matrix, and then further cleaved by PARL, a rhomboid family protease, at Ala103 to generate a less stable 54kD form. The TOM complex then retro-translocates the cleaved/processed PINK1 back into the cytosol where it is then targeted for proteasomal destruction. (Deas et al., 2011; Yamano & Youle, 2013).

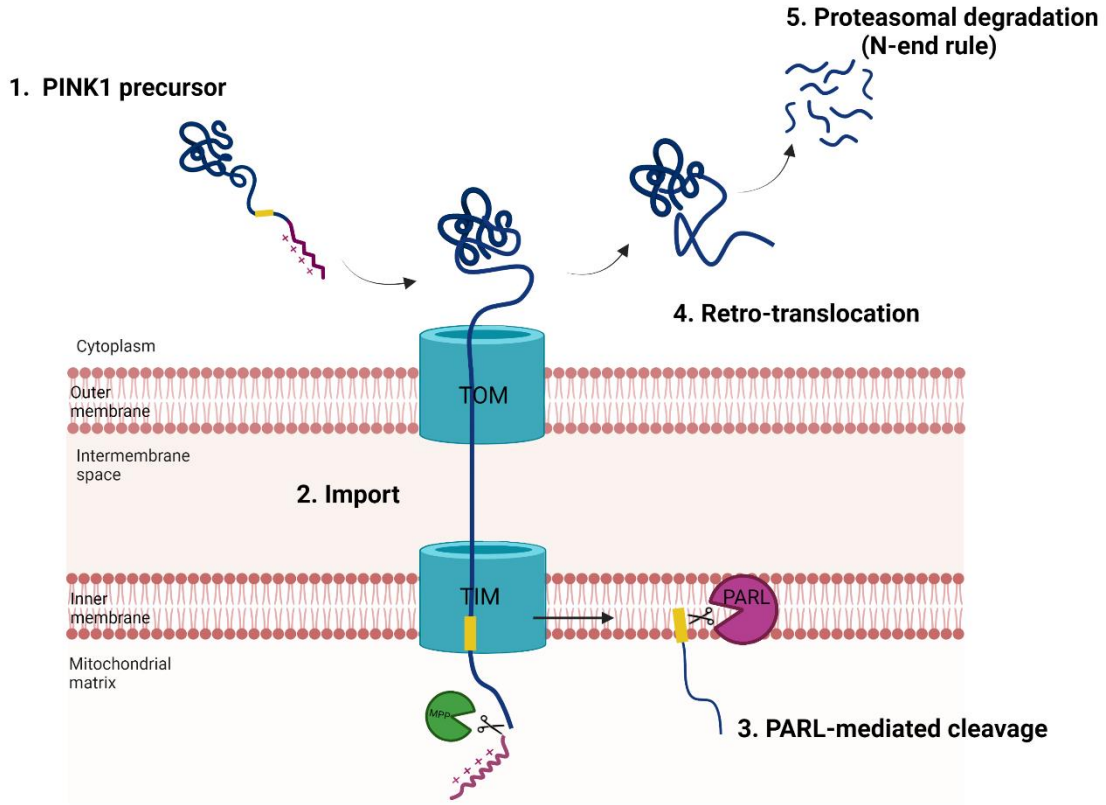


Figure 1.10: PINK1 import into healthy mitochondria

In a damaged mitochondria that has lost its ΔΨm, PINK1 import is stalled, resulting in the stabilization of 63kD PINK1 on the OMM (Narendra et al., 2010). This accumulated PINK1 on the OMM forms a HMW complex of around 700 kD with the TOM complex (Okatsu et al., 2013; Lazarou et al., 2012). The TOM complex plays a role in the proper positioning of the dimeric PINK1. PINK1 autophosphorylates itself and recruits Parkin, an E3 ubiquitin ligase, to the mitochondria (Okatsu et al., 2015). Once recruited to the damaged mitochondria, Parkin catalyzes the ubiquitination of outer membrane proteins (Koyano et al., 2015). Ubiquitination of mitochondrial proteins by Parkin acts as a signal for autophagy receptors to recognize damaged mitochondria and induce the formation of autophagosomes. Phospho-Ubiquitin signal promotes the binding of autophagy receptors such as NDP52, Optineurin, and p62/SQSTM1, to the ubiquitin chains on the mitochondria. These autophagy receptors interact with LC3, facilitating the engulfment of the damaged mitochondria into autophagosomes (Onishi et al.,2012). The autophagosomes containing the damaged mitochondria then fuse with

lysosomes, forming autolysosomes, where the mitochondria is degraded by lysosomal enzymes. The breakdown products, including amino acids and lipids, are then recycled back into the cytosol for cellular energy production and biosynthesis.

In addition to its role in mitophagy, PINK1 also regulates other cellular processes such as the regulation of mitochondrial transport, maintenance of calcium homeostasis, and control of crosstalk between ER and mitochondria (Brunelli et al., 2020).

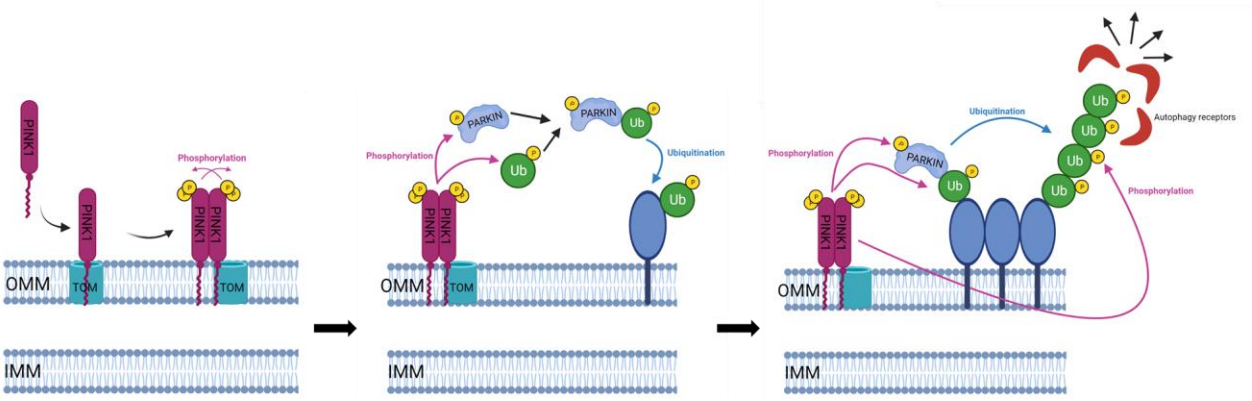


Figure 1.11: PINK1/Parkin-mediated mitophagy

PINK1 import into the IMM is prevented in mitochondria that have lost their membrane potential. On the OMM, PINK1 accumulates. The dimeric PINK1 is positioned correctly because of the TOM complex. Parkin is recruited to the mitochondria by PINK1, which then autophosphorylates itself. Proteins in mitochondria are ubiquitinated by parkin. Enrichment of phospho-Ubiquitin chains on the surface of a damaged mitochondria recruits autophagy machinery.

1.11 Rationale and Hypothesis

We know that PINK1 gets accumulated on the mitochondria under stress conditions or when the membrane potential is disrupted. But how it gets accumulated is an area that is still being extensively studied. The exact topology of PINK1 accumulation is not known.

PINK1 has an N-terminal MTS signal and a kinase domain. While studying its structure we observed that PINK1 has twin Cx3C and one Cx9C motif in its structure. These are the motifs usually present in a cysteine-rich protein that is imported into the mitochondria with the help of the MIA40 protein. Hence, we hypothesized that MIA40 might be interacting with PINK1 to help its import and stabilization onto the mitochondria thereby regulating mitophagy.

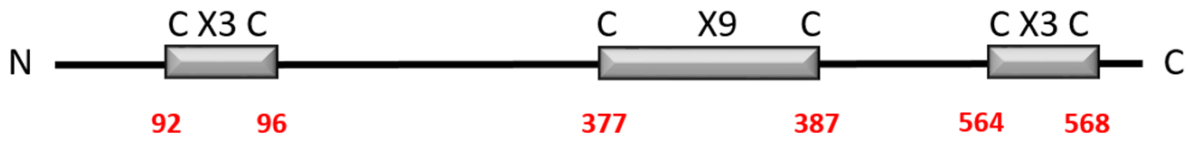


Figure 1.12: Structure of PINK1 highlighting cysteine motifs

To test our hypothesis, we formed two objectives

1. MIA40 interacts with PINK1 and regulates mitophagy; characterization of the role of MIA40 cysteine motifs in mitophagy

2. Identification and characterization of the cysteine motifs of PINK1 and their involvement in mitophagy

CHAPTER 2

Investigating the interaction between MIA40 and PINK1; characterizing the role of MIA40 cysteine motifs in mitophagy

2.1 Introduction

Six preserved cysteine residues grouped as a CPC motif and dual CX9C motifs are present in MIA40. The CX9C motif forms structural disulfides that contribute to the protein's stability. The CPC motif in MIA40 accepts electrons from the reduced unfolded proteins in the IMS and transfers them to molecular oxygen or other electron acceptors, generating a disulfide bond in the process and assisting its retention in the IMS region (Banci et al., 2009; Manganas et al., 2017; Sideris et al., 2009). Twin CX3C or CX9C motifs can be commonly found in MIA40 substrates. (Vogtle et al.,2012; Modjtahedi et al.,2016).

PINK1 is a Ser/Thr kinase that acts as a sensor of the health status of mitochondria by accumulating on the OMM of depolarised or damaged mitochondria. The molecular factors assisting the same are still not well studied. While analyzing its structure we observed that it possesses the twin CX3C or CX9C motifs that are present in typical MIA40 substrates.

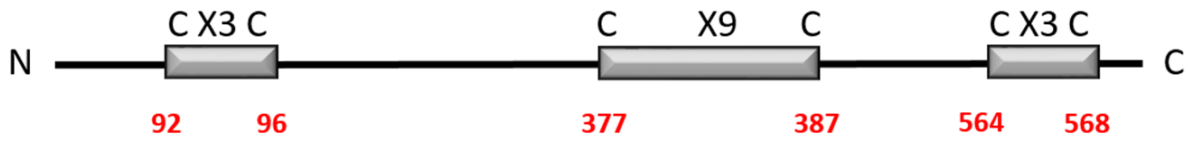


Figure 2.1: Structure of PINK1 highlighting cysteine motifs

Hence, in this study, we aimed to establish an interaction between MIA40 and PINK1. We also wanted to check the effect of MIA40 cysteines on mitophagy.

We have used stabilization of 63 kDa PINK1, increase in phospho-ubiquitination, and increase in LC3-II flux as the parameters to study mitophagy.

LC3 is a 17 kDa protein that can be used as a marker of mitophagy as well (Lazarou et al., 2015; Onishi et al., 2021; Guerroue et al., 2017). LC3 can be detected as two bands on western blot. LC3-I which is present in the cytosol is usually detected around a molecular weight of 16 kDa. Whereas, LC3-II which is conjugated with PE is usually detected at approximately 14 kDa. Phosphatidylethanolamine conjugated LC3-II is found to be associated with autophagosomes (Bhujabal et al., 2017).

Since the amount of LC3-II and the number of autophagosomes are strongly connected, it can be used to predict the formation of autophagosomes. However, LC3 immunoblotting is occasionally interpreted incorrectly since LC3-II itself is degraded by autophagy. An increased level of LC3-II can be indicative of either enhanced autophagy, suggesting an increase in autophagosomes, or it can be interpreted as reduced autophagy due to an inhibition of autophagosome degradation. Also, the amount of LC3 at a given moment does not necessarily represent the amount of autophagic flux, making it crucial to measure the LC3-II turnover by measuring the difference in the levels of LC3-II in presence and absence of lysosomal inhibitors

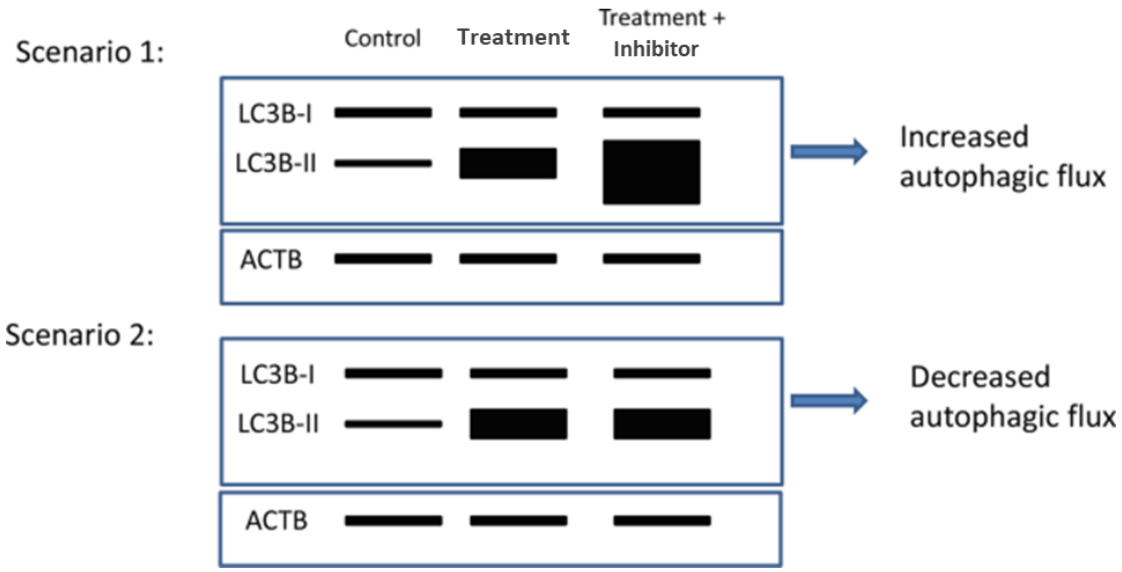


Figure 2.2: Measurement of autophagic flux

An increase in LC3-II levels upon treatment can be either due to an increase in the production of autophagosomes or a restriction of autophagic degradation. To clear that out, Lysosome inhibitors, such as Chloroquine or Bafilomycin A, are used to treat the cells. If the amount of LC3-II continues to increase when lysosomal inhibitors are present, this would suggest an increase in the autophagic flux (Scenario 1). On the other hand, if the level of LC3-II remained constant, it is likely that autophagosome build-up happened as a result of the suppression of autophagic degradation (Scenario 2).

2.2 Experimental Procedures and Requirements

2.2.1 Bacterial Transformation

To begin, 100µL of DH5α ultra-competent cells taken and were thawed on ice. The cells were then treated with up to 200 ng of the required plasmid for transformation for 30 minutes while being kept on ice. The tube was subsequently subjected to a thermal shock at 42°C lasting 90 seconds, and then it was immediately submerged in ice for three minutes. After the heat shock, the tube was filled with 1 mL of autoclaved LB media devoid of any antibiotics, and it was then incubated in a shaker incubator for an hour at 37°C. In order to transform the ligated product, cells were pelleted down at 8000 rpm for 4 mins, mixed in 100µL of LB, followed by spreading the cells on 100µg/mL of Ampicillin or Kanamycin (25µg/mL) containing plates. 100 µL of the 1 mL of the culture was plated in case of plasmid transformation. The plates were then kept at 37°C incubation overnight for the growth of transformants.

2.2.2 Plasmid construction

HeLa cells were used to extract total cellular RNA and synthesize the cDNA for hMIA40. The resulting cDNA formed was subsequently used as a template to clone hMIA40 gene into a pcDNA3.1 Myc-His vector to produce a plasmid that can express MIA40 WT having a C-terminal His tag (Murari et al., 2015). Cysteine mutants CPC (MIA40 C53S,55S) and QM (MIA40 C4,64,74,97S) were generated using SDM for expression in mammalian cells (Thiriveedi et al., 2020). Additionally, the plasmid pCMVTNT PINK1 C-myc was obtained from Addgene.

2.2.3 DNA Transfection and Cell Culture

The ATCC Cell bank was used to procure the HEK293T cell lines that were used in the experiments. Complete DMEM medium (Invitrogen) formulated with 10% FBS, and 1% Antibiotic-Antimycotic (Gibco™), was used to cultivate the cells and maintained at 37°C with 5% CO_2. The cells were transfected with the appropriate plasmid with Lipofectamine 2000 (Invitrogen) once they had reached 60–70% confluency.

2.2.4 Immunoblotting

Cells were harvested and for lysis, either RIPA buffer or NP-40 buffer was used supplemented with PIC (Roche, Switzerland) to prepare samples for western blot analysis. The Bradford reagent was used to determine the protein content, and the lysates made were resolved on SDS

polyacrylamide gel before being transferred to the NC membrane at 4°C. Ponceau stain was used to verify the correct transfer of proteins onto the membrane. Prior to incubation with the specific antibody, to prevent non-specific binding, the membrane was blocked with a 5% SM powder prepared in TBST for 1 hour. The specified primary antibody was then added to the blots after blocking, and they were left to sit on a rocker at 4°C overnight. The following morning, the blots were incubated with an HRP-tagged secondary antibody at RT for one hour after being washed thrice with TBST for five minutes each. An ECL detection kit (Advansta Western-Bright) was used to develop the blots, using Bio-Rad VersaDoc imaging system.

NP-40 Buffer	50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, and 1% IGEPAL or NP-40
RIPA Buffer	50 mM Tris-HCl pH 8, 1% deoxycholic acid, 1% Triton X-100, 150 mM NaCl, 0.25 mM EDTA, and 0.1% SDS
TBST	20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20

2.2.5 Antibodies and Reagents

The antibodies used in this study were anti-MIA40 (Proteintech 21090-1-AP), anti-PINK1 (CST bc100-494; D8G3), anti-TOMM20 (CST, D8T4N), anti-β-Tubulin (10068-1-AP), anti-LC3 (CST), anti-Myc tag (Proteintech 16286-1-AP), anti-pSer65Ub (CST, E2J6T), anti-GAPDH (Proteintech 60004-1-Ig), Veriblot HRP for IP (ab131366) and HRP-tagged secondary antibodies anti-Rabbit or anti-Mouse were obtained from Jackson ImmunoResearch. Except when otherwise noted, all compounds were bought from Sigma-Aldrich.

2.2.6 Co-Immunoprecipitation

For the co-immunoprecipitation study, HEK293T cells grown in a 100mm dish were transfected with the indicated plasmid constructs and their respective controls. The cells were grown in DMEM devoid of serum for six hours and then substituted with complete DMEM. Cells were treated with 10μM CCCP for 4 hours after 36-hour transfection. Cells were further lysed in NP-40 buffer containing 1X PIC (Roche) after which they were washed with PBS. Total cell lysates were pelleted down at 13000rpm for 20 minutes at 4 °C. To perform the co-immunoprecipitation, 500 μg of the lysates were precleared with 10 μL Protein A or Protein G agarose beads (SC-2003) for 1 hour at 4 °C to get rid of non-specific interactions. 1 μg of the desired antibody was incubated overnight with the pre-cleared lysate on an end-over-rotator at 4 °C. Protein A or Protein G Plus beads were added and incubated for 4 hours at 4ºC. The

antibody-protein interactome was then washed four times to avoid any non-specific binding. with NP-40 buffer at 5000rpm for 2 mins at 4 °C. Samples were incubated in SDS sample buffer dye at 95°C for 5 mins, resolved on reducing SDS polyacrylamide gel, and processed by western blotting.

2.2.7 Pull-down with Ni-NTA beads

The Ni-NTA pull-down assay is a technique that enables the isolation of polyhistidine-tagged proteins from cell lysates using an immobilized metal affinity chromatography resin. In this experiment, HEK293T cells were co-transfected with PINK1 WT along with pcDNA-MIA40 c-Myc-His plasmids. To ensure plasmid uptake, the cells were grown in DMEM devoid of serum for six hours and then switched to complete DMEM. Cells were treated with 10μM CCCP for 4 hours after 36-hour transfection. Cells were further lysed in NP-40 buffer containing 1X PIC (Roche) after which they were washed with PBS. Total cell lysates were pelleted down at 13000rpm for 20 minutes, after which 500μg of the supernatant was incubated with Ni-NTA beads containing 1X PIC for 4 hours at 4°C on an end-over-rotator. The Ni-NTA beads were taken and subsequently washed four times with NP-40 buffer. The precipitated complex was incubated in SDS sample buffer dye at 95°C for 5 mins, resolved on reducing SDS polyacrylamide gel, western blotted, and probed with hMIA40 antibody.

2.2.8 Densitometric Quantification and statistical analysis

The signals in the western blots were quantified using ImageJ software. Protein bands from a minimum of three independent experiments were quantified for each assay. GraphPad Prism7 was used for the statistical analysis and plotting of graphs. The results were presented as the mean ± SEM. Two-way ANOVA was used for calculating the statistical significance of grouped data. Statistical significance was defined as a p-value less than or equal to 0.05.

2.2.9 AlphaFold2 Prediction and PyMOL Alignment

The protein sequence was obtained from NCBI and analyzed using AlphaFold2 Colab with all the parameters selected. The 3D structure thus predicted was downloaded in PDB format. PDB format structures of the WT protein and its mutant protein were structurally aligned using PyMOL and studied for their similarities or differences.

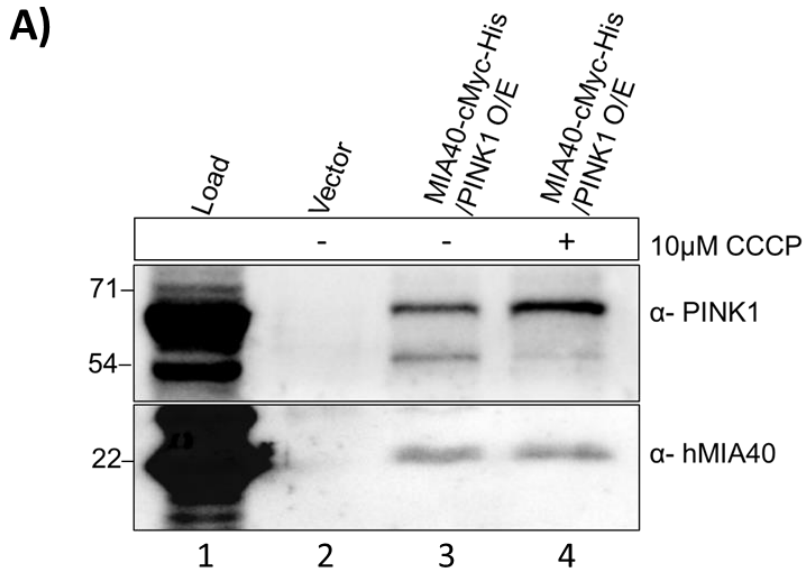
2.3 Results

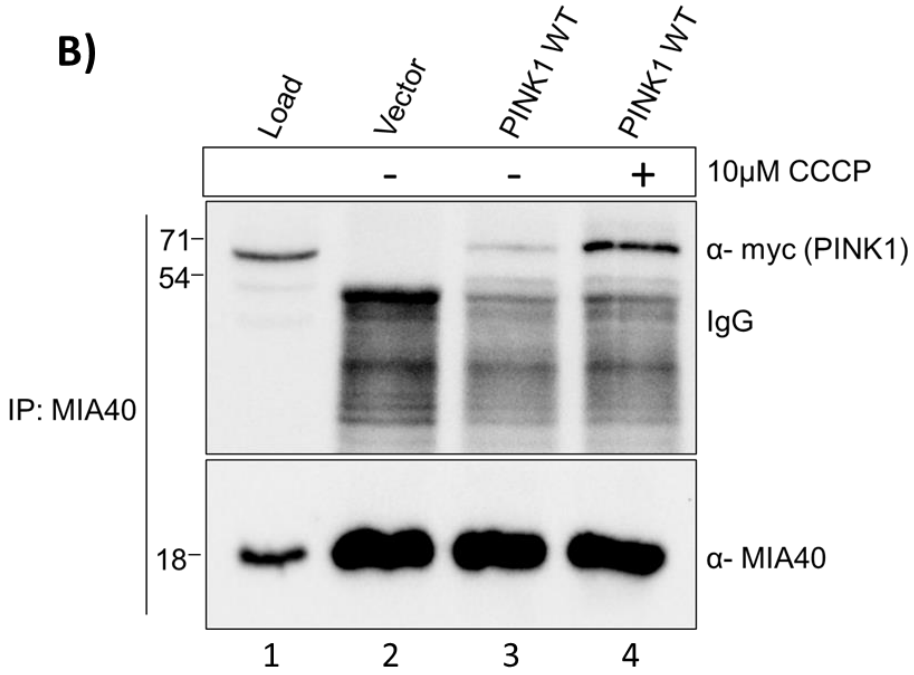
2.3.1 Interaction between MIA40 and PINK1

We performed a Ni-NTA pull-down assay on HEK293T cells that had been co-transfected with MIA40 WT c-myc-His and PINK1 WT c-myc to investigate our hypothesis that MIA40 plays a critical role in the import and stabilization of PINK1 through its interaction. The eluted proteins were then analyzed by western blotting using an anti-PINK1 antibody. The co-expressed cell lysate exhibited the full-length 63kDa PINK1 band (Fig 2.3A, Lane 3), which was not present in the vector control lysate (Fig 2.3A, Lane 2). Furthermore, the intensity of this PINK1 band was observed to be notably higher in the samples that were subjected to CCCP treatment (Fig 2.3A, Lane 4), a mitochondrial uncoupler that induces mitophagy by stabilizing PINK1 on the OMM. This finding suggests that the interaction between PINK1 and MIA40 might be more pronounced under conditions of mitochondrial stress (Figure 2.3 A).

To further establish the interaction between PINK1 and MIA40, immunoprecipitation was performed with a MIA40 antibody. To perform an IP with MIA40 antibody, whole-cell extracts were prepared from HEK293T cells expressing PINK1 WT. The lysates were then incubated with an anti-MIA40 antibody. The proteins that were bound to the beads were eluted and subjected to western blotting analysis after non-specifically bound proteins had been removed by washing. Notably, in the lane with the PINK1 WT overexpressed cell lysate, we detected the stabilized 63kDa PINK1 band (Fig 2.3B, Lane 3), whereas there was none in the lane with the vector control lysate (Fig 2.3B, Lane 2). The addition of CCCP, a mitochondrial uncoupler, which has been shown to stimulate mitophagy by ensuring the stability of PINK1 on the OMM, enhanced the intensity of this band and was more pronounced in Fig 2.3B, Lane 4. Therefore, we concluded that MIA40 interacts with PINK1 in stressed conditions (Figure 2.3B).

Since endogenous PINK1 levels are really low and are continuously prone to degradation making it difficult to be detected in a western blot, we have used PINK1 overexpressed cell lysate for our studies. But to establish MIA40 and PINK1 interaction at endogenous levels, we also immunoprecipitated HEK293T cell lysate with MIA40 antibody and probed with PINK1 antibody and find the interaction of PINK1 with MIA40 (Fig 2.3C, Lane 2). These results suggest that MIA40 may interact with PINK1 and be more pronounced under stressed conditions (Figure 2.3C).


A)
Load
Vector
MIA40-cMyc-His /PINK1 O/E
MIA40-cMyc-His /PINK1 O/E
-
-
+
10μM CCCP
71
54
22
α- PINK1
α- hMIA40
1
2
3
4


B)
Load
Vector
PINK1 WT
PINK1 WT
-
-
+
10μM CCCP
71
54
18
IP: MIA40
α- myc (PINK1)
IgG
α- MIA40
1
2
3
4

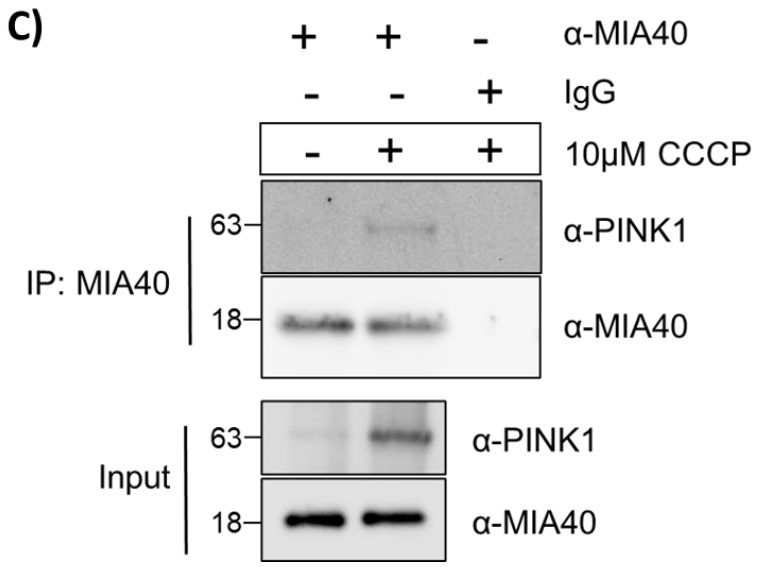


Figure 2.3: Interaction of MIA40 with PINK1. A) Ni-NTA pull-down was performed with cell lysates co-transfected with hMIA40 c-myc-His and PINK1 c-myc. The lysates were pulled down with Ni-NTA beads, western blotted, and probed with an anti-PINK1 antibody. B) Cells transfected with PINK1 WT were lysed and subjected to immunoprecipitation using the anti-MIA40 antibody. The resulting immune complexes were then western blotted and probed with an anti-PINK1 antibody. C) HEK293T cell lysates were immunoprecipitated using an anti-MIA40 antibody. The immunoprecipitates were western blotted and probed with an anti-PINK1 antibody.

2.3.2 MIA40 cysteine mutants and their predicted 3D structures

As mentioned earlier, MIA40 possesses six cysteine residues that are conserved across different organisms. These cysteine residues are arranged into three distinct motifs: a CPC motif and dual CX9C motifs. The CPC motif facilitates the formation of disulfide bonds between MIA40 and its substrate proteins. It has also been found that MIA40 undergoes glutathionylation to regulate ROS levels and mitochondrial complex activities of the electron transport chain. Cysteines at positions C4, C64, C74, and C97 are responsible for the maximum glutathionylation of MIA40 (Thiriveedi et al., 2020).

Hence, to study the molecular mechanism of cysteines involved in PINK1 import, we created two distinct mutants using site-directed mutagenesis. One with a mutated CPC motif (CPC mutant) and another mutant called QM, where four glutathionylated cysteines (C4, C64, C74, and C97) were replaced with serine.

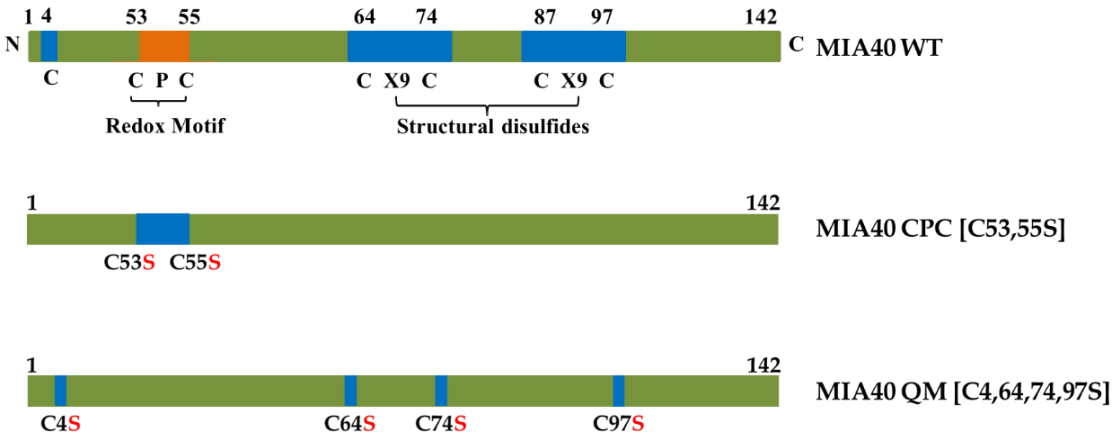


Figure 2.4: Generation of MIA40 cysteine mutants

In our study, we utilized AlphaFold2 to predict the 3D structure of MIA40 WT, MIA40 CPC mutant, and MIA40 QM mutant. AlphaFold2 analyzes the amino acid sequence of the protein and predicts the distances between these amino acids. These predictions are used to generate a 3D structure using a variety of computational techniques.

To assess the structural changes between the wild-type and the mutant, their predicted protein structure obtained from AlphaFold2 was loaded onto PyMOL in PDB format. The protein structures to be compared were superimposed and aligned in a way that RMSD is minimal.

Our analysis confirmed that the 3D structure of the MIA40 CPC mutant aligns closely with the MIA40 WT protein (Figure 2.5A). However, the MIA40 QM mutant did not align with the MIA40 WT structure (Figure 2.5B). This outcome was anticipated because the cysteine residues at positions C4, 64, and 97 in MIA40 form crucial structural disulfide bonds, and their mutation would inevitably disrupt the overall protein structure.

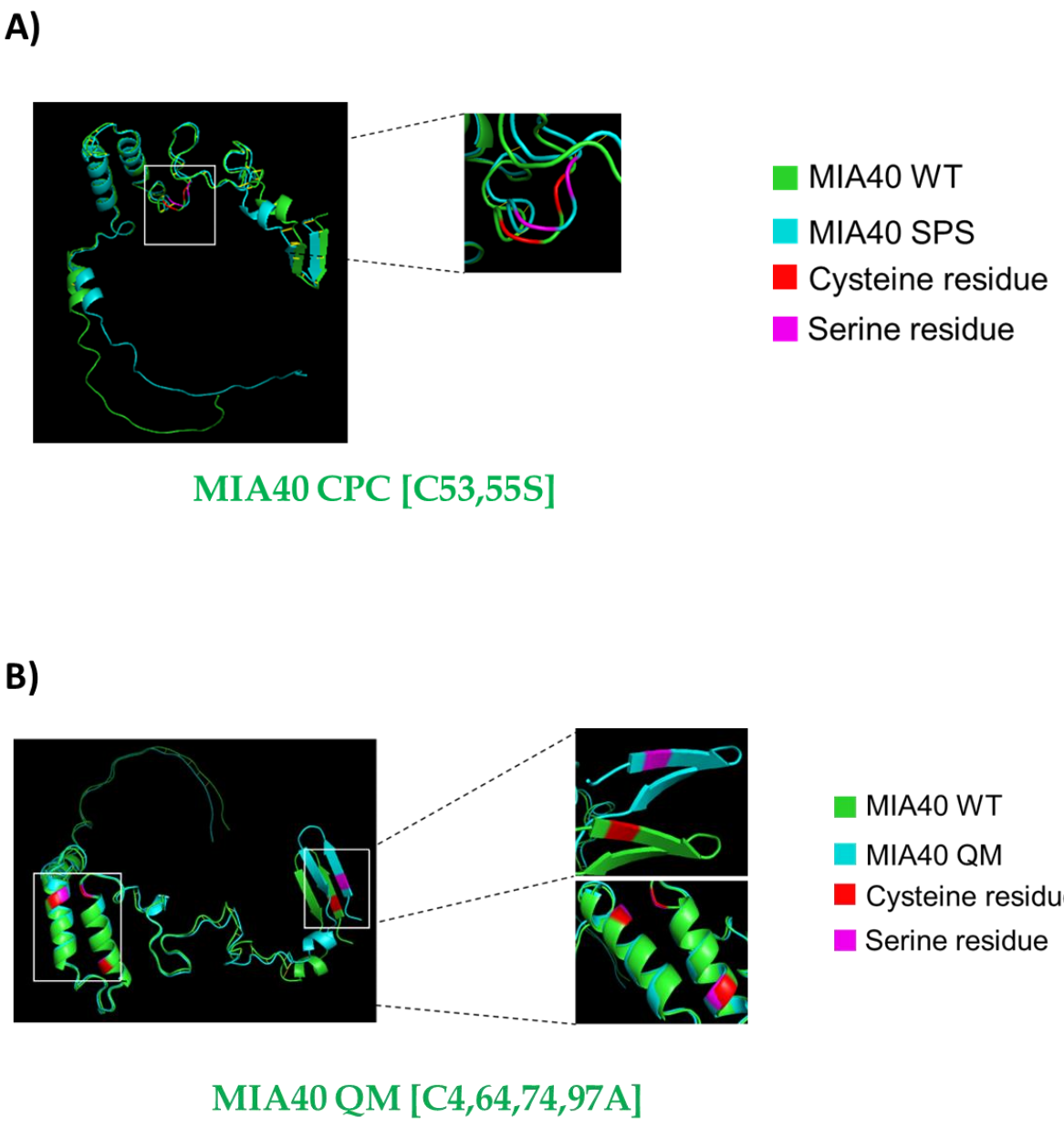


Figure 2.5: Predicted 3D structure of MIA40 cysteine mutants using AlphaFold2. 3D structures of MIA40 mutants were aligned with the 3D structure MIA40 WT using PyMOL. A) MIA40 CPC mutant aligned with PINK1 WT B) MIA40 QM mutant exhibited some structural differences compared to MIA40 WT.

2.3.3 MIA40 WT and its cysteine mutants modulate the basal level of mitophagy

To investigate the potential role of these cysteine residues in mitophagy, HEK293T cells were co-transfected with PINK1 WT and either MIA40 WT or its mutants. Cell lysates were then probed with an anti-PINK1 antibody to determine the level of PINK1 stabilization following treatment with mitochondrial uncoupler CCCP. Results showed that the stabilization of PINK1

significantly increased upon overexpression of MIA40 WT (Fig 2.6A, lanes 3&4), but this increase was not observed in cells expressing the CPC mutant (Fig 2.6A, lanes 5&6) and the QM mutant (Figure 2.6A, lanes 7&8 and Figure 2.6B).

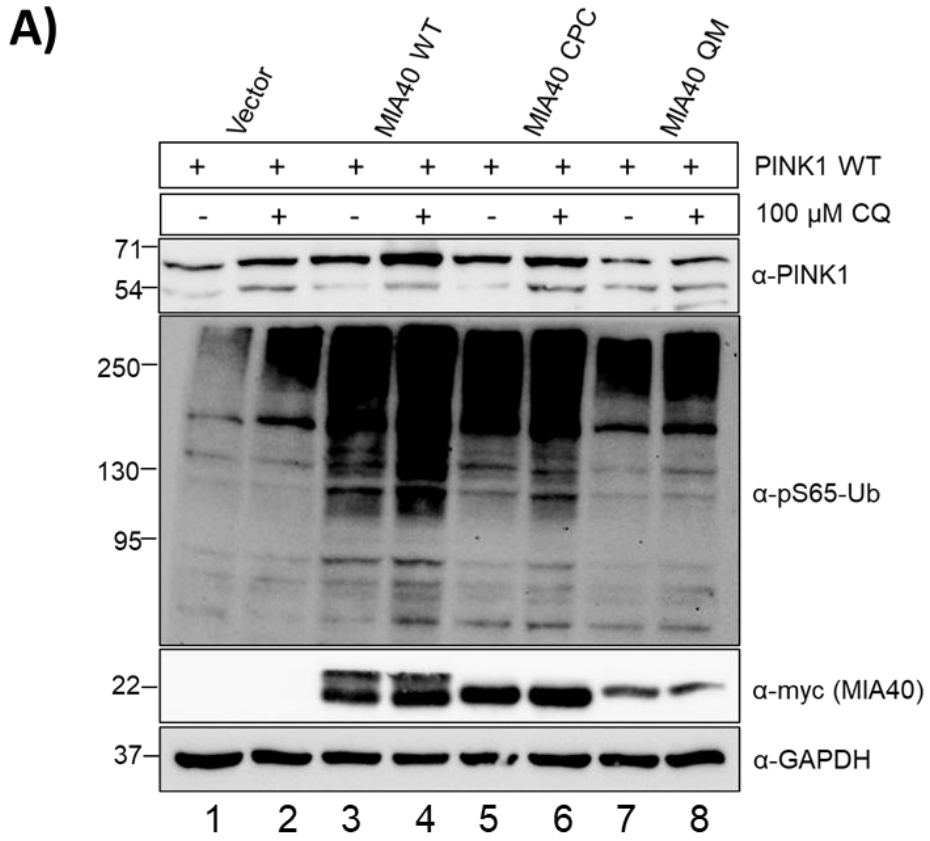
As mentioned earlier, phosphorylation of ubiquitin at serine 65 is a crucial step in mitophagy. Therefore, we checked for changes in the levels of phosphorylated ubiquitin. Consistent with our previous observations, we found that overexpression of MIA40 WT led to an increase in the levels of phosphorylated ubiquitin (Fig 2.6A, lanes 3&4). However, these levels did not show an increase when cells were transfected with the CPC (Fig 2.6A, lanes 5&6) and the QM mutant (Fig 2.6A, lanes 7&8). Overall, these findings suggest that overexpression of MIA40 WT enhances mitophagy, likely through its interaction with PINK1 (Figure 2.6A and C) whereas the cysteines mutated in the CPC and the QM mutant might be crucial for regulating mitophagy.

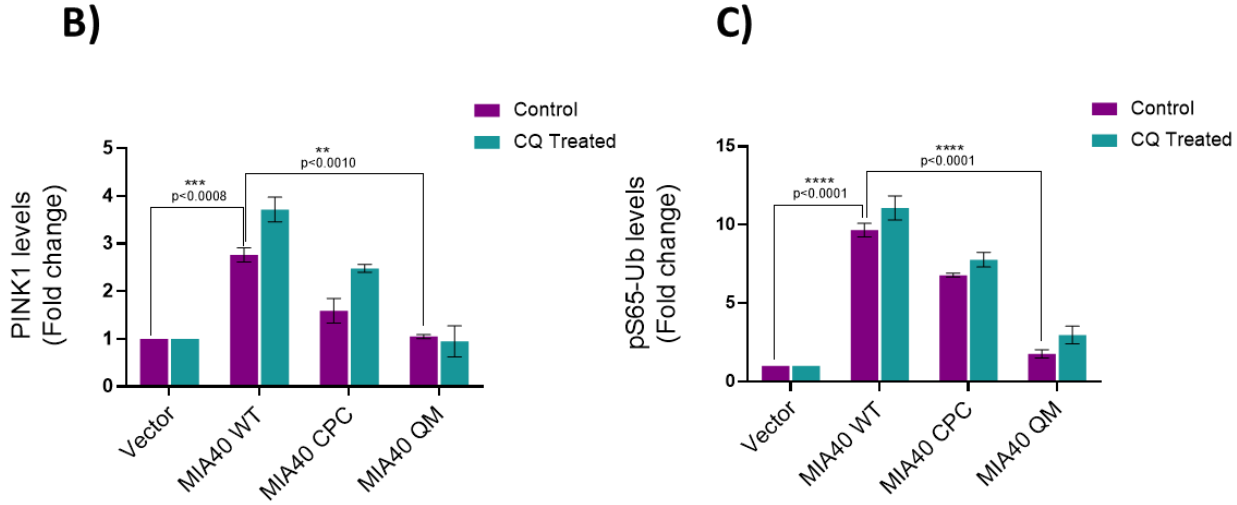
Another parameter for measuring mitophagy is to measure LC3-II flux (Lazarou et al., 2015; Onishi et al., 2021; Bhujabal et al., 2017; Guerroue et al., 2017). Hence, to quantify LC3 flux, we treated the samples with chloroquine, an autophagosome-lysosome fusion inhibitor, and calculated flux by subtracting the values obtained from CQ-treated from untreated samples after normalizing with GAPDH. Consistent with our other observations, overexpression of MIA40 WT resulted in a significant increase in mitophagy flux (Fig 2.6D, difference between lane 3 and lane 4), indicating that it enhances mitophagy, whereas, this increase was not observed in the cells expressing MIA40 QM (Figure 2.6D Lane 7 & 8 and Figure 2.6 E). Cells expressing the MIA40 CPC mutant exhibited a slight decrease in the LC3-II flux (Fig 2.6D, difference between lane 5 and lane 6).

Our results suggest that overexpression of MIA40 WT enhances mitophagy, likely through its interaction with PINK1. The MIA40 CPC mutant appears to decrease mitophagy to an extent however, the MIA40 QM mutant shows a more significant decrease in mitophagy. Our results indicate that the CPC mutant also might have a role in stabilizing PINK1 as the CPC domain is involved in forming disulfide bonds with the incoming substrate proteins. It also plays an important role in the assembly of ETC subunits (Sideris et al., 2009).

Furthermore, we noted that the intensity of the MIA40 QM band in Fig 2.6A, lanes 7&8 was noticeably lower compared to MIA40 WT in Fig 2.6A, lanes 3&4. This decrease in intensity can be attributed to the high instability and susceptibility to degradation of the MIA40 QM mutant caused by the mutations in the structural disulfide bonds. As a result, the protein levels

of MIA40 QM are diminished. Interestingly, despite its reduced abundance, the MIA40 QM mutant exhibits a functional alteration, as demonstrated in the study by Thiriveedi et al. in 2020. This unique characteristic classifies it as a dominant positive mutant, capable of exerting its effects despite its lower protein levels.





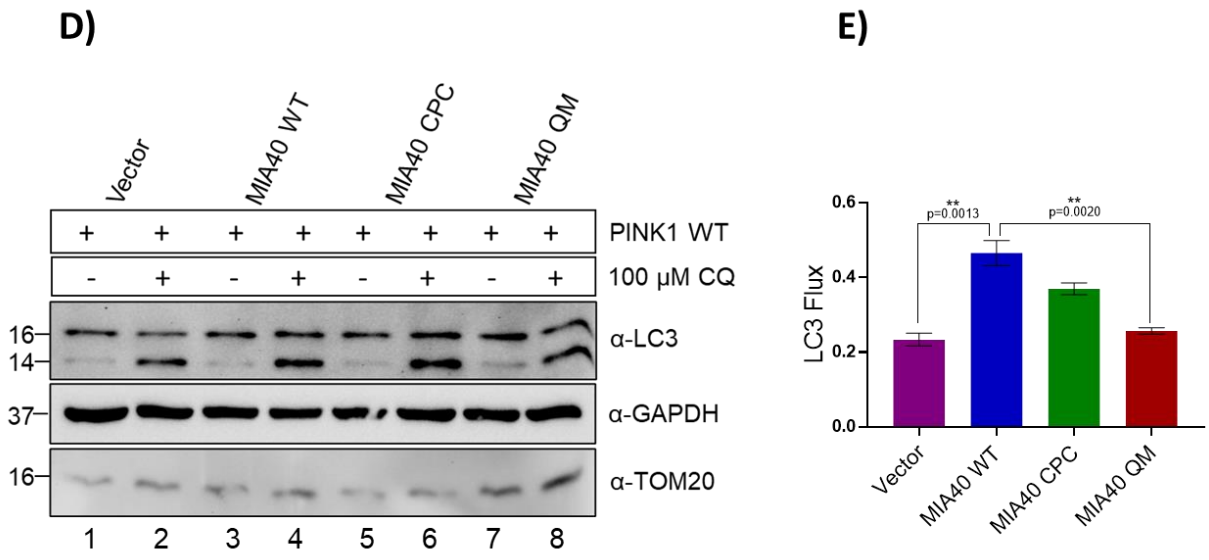


Figure 2.6: MIA40 regulates the basal level of Mitophagy. A) The cells were co-transfected with PINK1 WT and either MIA40 WT or its mutants. The level of phosphorylated ubiquitin and PINK1 stabilization was analyzed in healthy mitochondria. B & C) The band intensities of PINK1 and pS65-Ub from more than three independent experiments were quantified and normalized to GAPDH, respectively. D) Cells transfected with the indicated plasmid were treated with 100µM CQ to measure LC3-II flux. E) The band intensities of CQ untreated were subtracted from CQ treated after being normalized to GAPDH.

2.3.4 MIA40 WT and its cysteine mutants modulate mitophagy under stress conditions

As we observed that the interaction between PINK1 and MIA40 is stronger in dysfunctional mitochondria, targeted for mitophagy, we hypothesized that MIA40 might be playing a strong role in regulating mitophagy under stress conditions.

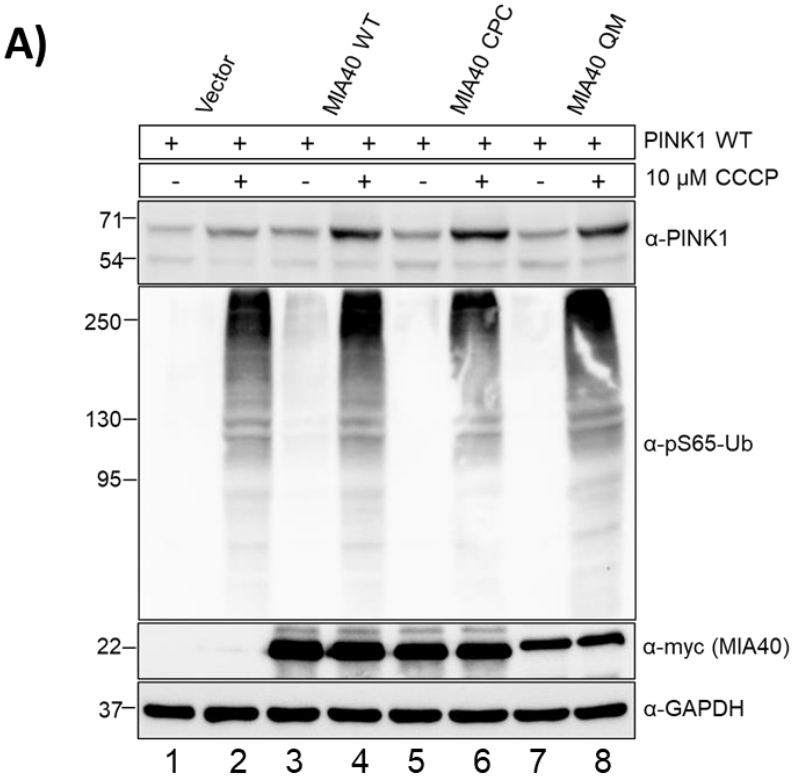
During our investigation under CCCP stress, we interestingly found an increase in the stabilization of PINK1 in cells overexpressing MIA40 WT (Fig 2.7A, lanes 3&4). However, we found that these levels did not show an increase when cells were transfected with QM mutants (Figures 2.7A, lanes 7&8, and Figure 2.7B) indicating that they might be playing a role in the stabilization of PINK1. It was also observed that cells transfected with the MIA40 CPC mutant (Fig 2.7A, lanes 5&6, and Figure 2.7B) did not show any change in PINK1

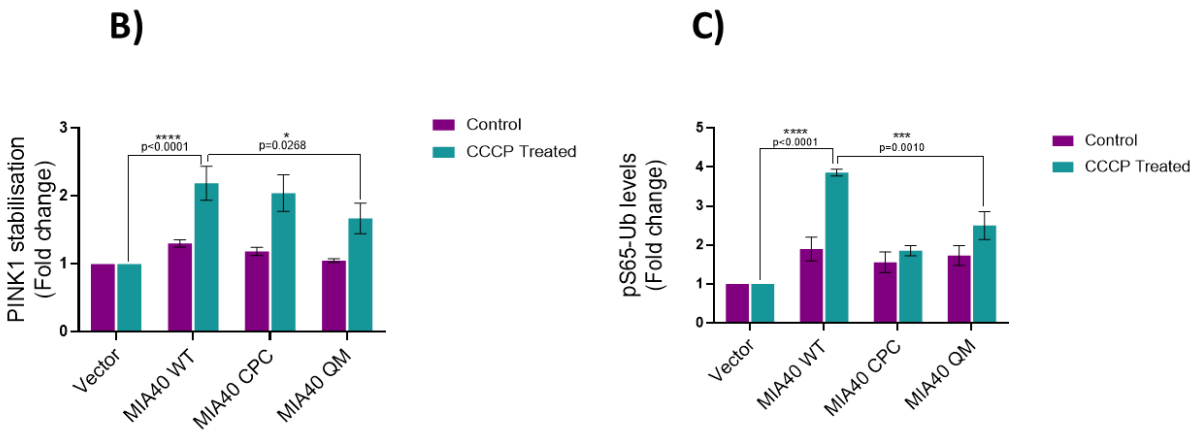
stabilization upon CCCP stress indicating that it might not play a direct role in PINK1's stability.

In the same line, phosphorylation of ubiquitin at Ser65 exhibited an increase in cells overexpressing MIA40 WT (Fig 2.7A, lanes 3&4). However, we found that these levels decreased when cells were transfected with either the CPC (Fig 2.7A, lanes 5&6) or the QM mutants (Figures 2.7A, lanes 7&8, and Figure 2.7C). This observation led us to hypothesize that MIA40 WT overexpression promotes mitophagy even in depolarised mitochondria. It also signifies that the cysteines mutated in the QM mutant might have an effect on mitophagy. On the other hand, cysteines mutated in the CPC mutant did not have a significant impact on PINK1's stabilization in stressed conditions, however, it did lead to a decrease in phospho-ubiquitination. This suggests that these cysteines might be involved in other pathways associated with ubiquitination.

To further quantify LC3 flux, we treated the CCCP-treated samples with chloroquine, an autophagosome-lysosome fusion inhibitor, and calculated flux by subtracting the values obtained from CQ-treated from untreated samples after normalizing with GAPDH (Figures 2.7D and E). Overexpression of MIA40 WT resulted in a significant increase in mitophagy flux (Fig 2.7D, difference between lane 5 and lane 6), indicating that it enhances mitophagy. Whereas, a decrease in mitophagy flux was observed in the cells expressing MIA40 QM (Figure 2.7D Lane 11 & 12 and Figure 2.7 E) or the MIA40 CPC mutant (Fig 2.7D, difference between lane 8 and lane 9), highlighting the role of the mutated cysteines in mitophagy.

Based on the experiments conducted, it appears that the cysteines mutated in the QM mutant have a more significant role in regulating mitophagy compared to the cysteines mutated in the CPC mutant, under stressed conditions. This conclusion is drawn from the observation that only the QM mutant is the only mutant that exhibited changes in all the analyzed parameters. However, the role of cysteines mutated in the CPC mutant in mitophagy cannot be neglected completely.


A)
Vector
MIA40 WT
MIA40 CPC
MIA40 QM
PINK1 WT
10 μM CCCP
71
54
250
130
95
22
37
α-PINK1
α-pS65-Ub
α-myc (MIA40)
α-GAPDH
1 2 3 4 5 6 7 8


B)
PINK1 stabilisation (Fold change)
****
p<0.0001
*
p=0.0268
Control
CCCP Treated
Vector
MIA40 WT
MIA40 CPC
MIA40 QM
C)
pS65-Ub levels (Fold change)
****
p<0.0001
***
p=0.0010
Control
CCCP Treated
Vector
MIA40 WT
MIA40 CPC
MIA40 QM

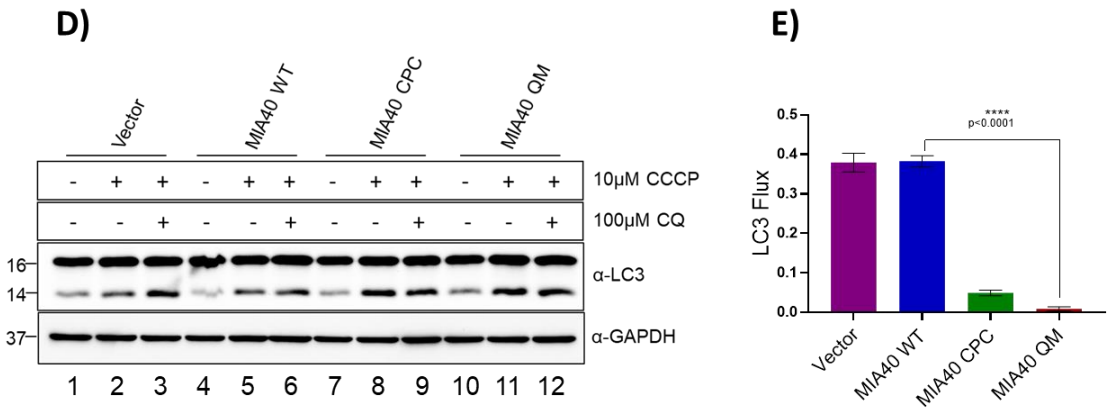


Figure 2.7: MIA40 regulates CCCP induced Mitophagy. A) The cells were co-transfected with PINK1 WT and either MIA40 WT or its mutants and treated with CCCP. The level of phosphorylated ubiquitin and PINK1 stabilization was analyzed in depolarised mitochondria treated with CCCP. B & C) The band intensities of PINK1 and pS65-Ub from more than three independent experiments were quantified and normalized to GAPDH, respectively. D) Cells transfected with the indicated plasmid were treated with 100μM CQ to measure LC3-II flux. E) The band intensities of CQ untreated were subtracted from CQ treated after being normalized to GAPDH.

2.3.5 Interaction of MIA40 cysteine mutants with PINK1

Now that we know PINK1 WT interacts with MIA40 WT and that cysteines at position C4, C64, C74 and C97 are crucial for mitophagy; their absence in QM affects mitophagy. Hence, we wanted to check the interaction of PINK1 with MIA40 mutants.

To establish the interaction between PINK1 and MIA40 mutants, immunoprecipitation was performed with a PINK1 antibody. To perform an IP with PINK1 antibody, whole-cell extracts were prepared from HEK293T cells co-expressing PINK1 WT and MIA40 WT and mutants and treated with CCCP. The lysates are then incubated with an anti-PINK1 antibody. The proteins that were bound to the beads were eluted from the beads and then subjected to western blotting analysis after non-specifically bound proteins had been removed by washing. The blots were probed with anti-Myc antibody. Interestingly, we observed the presence of the MIA40 band in all the lanes except in the lane expressing MIA40 QM lysate (Fig 2.8, lanes 7&8).

Therefore, we concluded that cysteines present at positions C4,64,74 and C97 of MIA40 interact with PINK1 in both normal and stressed conditions and hence regulate mitophagy (Figure 2.8).

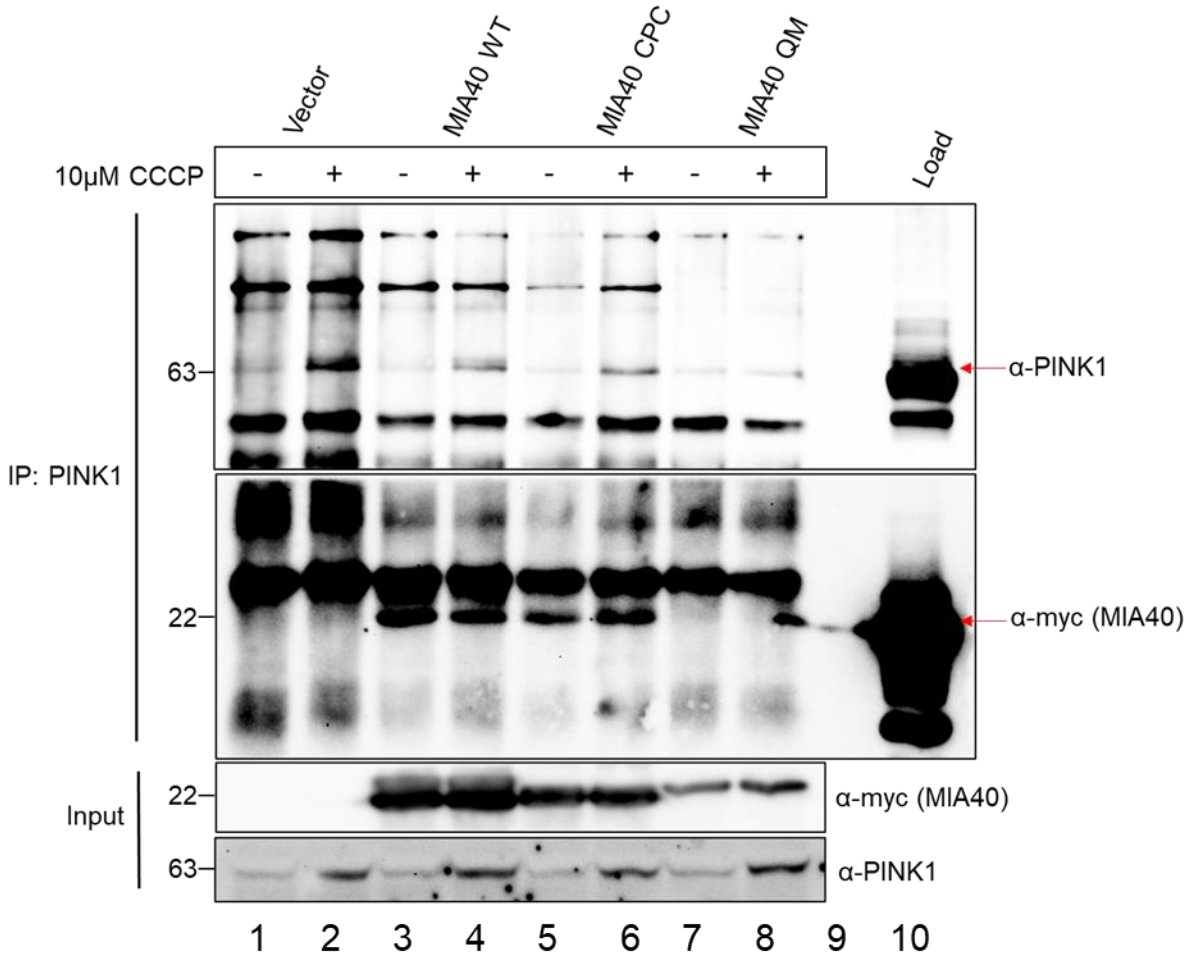


Figure 2.8: Cysteines present at positions C4,64,74 and 97 of MIA40 regulate mitophagy. To perform Co-IP, HEK293T cell lysates co-transfected with PINK1 WT along with MIA40 WT and its mutants were subjected to immunoprecipitation using an anti-PINK1 antibody. Subsequently, the resulting immune complexes were analyzed by western blotting and probed with an anti-myc antibody for the detection of MIA40.

2.4 Conclusion

Our research in his chapter leads us to the conclusion that MIA40 plays a crucial role in interacting with PINK1 under stress conditions, thereby regulating its stability and mitophagy. We validated this through Ni-NTA and immunoprecipitation studies, which demonstrated the physical association between MIA40 and PINK1. Moreover, our immunoprecipitation experiments revealed that the cysteine residues located at positions C4, C64, C74, and C97 of MIA40 are essential for mediating its interaction with PINK1.

Furthermore, our investigations also showed the significance of these cysteine residues in mitophagy. Notably, the absence of these cysteines in the MIA40 QM mutant resulted in a decrease in PINK1 stability, phospho-ubiquitination at Ser65, and LC3-II flux. This impairment was observed in both basal levels and stressed mitochondria induced by the mitochondrial uncoupler CCCP. These findings highlight the crucial role of the identified cysteine residues in regulating proper mitophagy functioning.

Based on the experiments conducted, it appears that even though the MIA40 QM mutant exhibits a more pronounced decrease in mitophagy, the MIA40 CPC mutant also appeared to decrease mitophagy to an extent, especially in the basal level of mitophagy. Considering that the CPC domain is involved in forming disulfide bonds with the incoming substrate proteins (Sideris et al., 2009), it would be reasonable to say that the cysteines mutated in the CPC mutant might have a role in stabilizing PINK1 that cannot be entirely disregarded. The study published by Gao et al., in 2020 also demonstrated that the deletion mutant of PINK1 lacking the amphipathic helix (PINK1 Δ166-171) led to a decreased PINK1 accumulation on mitochondria in the cells that were treated with CCCP by decreasing its interaction with CHCHD4. Hence, it can be inferred that the interaction between PINK1 and MIA40 is governed by not just one but multiple factors.

CHAPTER 3

Identification and characterization of the cysteine motifs of PINK1 and their involvement in mitophagy

3.1 Introduction

Mitochondria play a crucial role in coordinating various biological processes within cells. They are involved in ATP production, calcium homeostasis, phospholipid biosynthesis, iron-sulfur cluster biosynthesis, and even cell death regulation (Spinelli and Haigis, 2018). However, mitochondrial function can be compromised due to damage or dysfunction, which can have detrimental effects on cellular health. Based on the intensity of the mitochondrial damage, cells have deployed different mitochondrial quality control or safeguard mechanisms to maintain mitochondrial homeostasis. MQC can be broadly divided into two categories: regulation at the molecular level and regulation at the organelle level.

The first line of defense against protein damage caused by ROS consists of antioxidant enzymes, including SODs, GPX4, TRX-R, MSR-A, and MSR-B (Napolitano, Fasciolo, et al., 2021). These enzymes play a crucial role in neutralizing ROS and protecting proteins from oxidative damage.

The second line of defense involves mitochondrial proteases responsible for degrading damaged proteins within the mitochondria. For instance, mislocalized proteins on the OMM are removed, ubiquitinated, and delivered by AAA+ ATPase to the proteasome for destruction prior to mitochondrial protein import (Xu et al., 2011). Mitoproteases such as LON protease, m-AAA protease, and PARL protease also contribute to protein degradation within mitochondria (Deshwal et al., 2020; Leonhard et al., 1999).

In addition to these proteases, MARCH5 is an E3 ubiquitin ligase that ubiquitinates proteins that are folded incorrectly on the OMM, targeting them for degradation by the proteasome (Sugiura et al., 2011; Yonashiro et al., 2009). The proteasome primarily degrades proteins that are ubiquitinated at positions Ser29 and Ser48, while proteins that are ubiquitinated at Ser63 are targeted for degradation through autophagy or mitophagy (Kwon and Ciechanover, 2017).

Another mode of defense in MQC operates at the organelle level, specifically by preserving the equilibrium between mitochondrial fusion and fission in order to repair damaged mitochondria. The OMM's MFN1 and MFN2 and the IMM's OPA1 control mitochondrial fusion in mammals (Giacomello et al., 2020). Whereas fission is regulated by DRP1 (Dnm1 in yeast), MFF, and FIS1 (Cipolat et al., 2004).

MDVs, which have a dimension of 70–100 nm, are tiny vesicles that are pinched off the OMM. They contain selected fragments of mitochondrial protein that are meant to be eliminated by

lysosomes or peroxisomes. MDVs can be enriched with either TOMM20 or MAPL proteins, which participate in their formation and cargo selection (Neuspiel et al., 2008). These serve as the fourth line of defense in MQC.

When mitochondrial damage is beyond repair by any of the above-mentioned pathways, the cell resorts to the removal of the whole mitochondria by a process called mitophagy. Mitophagy in mammals can be categorized into two ways: PINK1/Parkin independent and PINK1/Parkin dependent mitophagy. The former mitophagy pathway is induced by receptors attached to the OMM such as BNIP3L (Schwarten et al., 2009), BNIP3 (Quinsay et al., 2010), FUNDC1 (Chen et al., 2016), BCL2-L13 (Murakawa et al., 2015), and FKBP8 (Bhujabal et al., 2017). It can also be lipid-mediated which is induced by cardiolipin in the IMM that acts as a mitophagy receptor to interact with LC3.

In PINK1/Parkin-dependent mitophagy pathway, PINK1 import into the mitochondria is hindered upon depolarization of the mitochondria. As a result, PINK1 accumulates on the OMM and forms a complex with The TOM complex (Lazarou et al., 2012). This is followed by the recruitment of PARKIN to the OMM, which ubiquitinated the mitochondrial proteins. The enhancement of the phospho-ubiquitin chain on the OMM surface signals for the recruitment of autophagy receptors such as OPTN and NDP52. Then, the mitochondria are targeted for engulfment by autophagosomes. Many proteins like SAM50 (Jian et al., 2018), TOM70 (Kato et al., 2013), and TIM23 (Akabane et al., 2023) are known to be involved in the stability of PINK1 to regulate mitophagy. Yet, multiple molecular factors that can regulate PINK1's stability remain unidentified and requires extensive research.

While analyzing PINK1's structure, we observed that it possesses the twin CX3C or CX9C motifs present in typical MIA40 substrates.

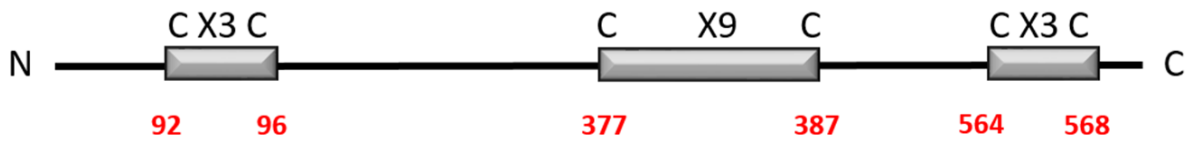


Figure 3.1: Structure of PINK1 highlighting cysteine motifs

In our previous objective, we found that MIA40 interacts with PINK1, and this interaction is cysteine mediated. Hence, we mutated the cysteine motifs present in PINK1 which may assist PINK1's interaction with MIA40. The following mutants were generated.

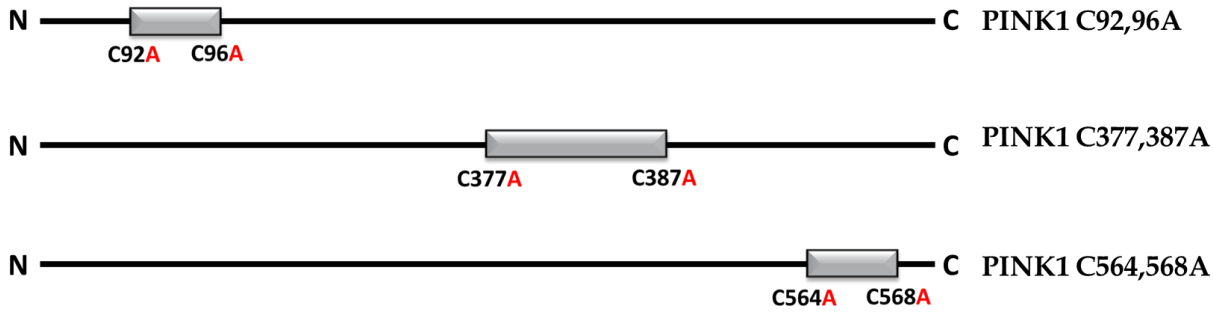


Figure 3.2: Generation of PINK1 cysteine mutants

3.2 Experimental Procedures and Requirements

3.2.1 Bacterial Transformation

To begin, 100µL of DH5α ultra-competent cells taken and were thawed on ice. The cells were then treated with up to 200 ng of the required plasmid for transformation for 30 minutes while being kept on ice. The tube was subsequently subjected to a thermal shock at 42°C lasting 90 seconds, and then it was immediately submerged in ice for three minutes. After the heat shock, the tube was filled with 1 mL of autoclaved LB media devoid of any antibiotics, and it was then incubated in a shaker incubator for an hour at 37°C. In order to transform the ligated product, cells were pelleted down at 8000 rpm for 4 mins, mixed in 100µL of LB, followed by a spread-plating with 100µg/mL of Ampicillin or Kanamycin (25µg/mL) containing plates. Whereas, 100 µL of the 1 mL of the culture was plated in case of plasmid transformation. The plates were then kept at 37°C overnight for incubation.

3.2.2 Plasmid construction

The pCMVTNT PINK1 c-myc plasmid, obtained from Addgene, served as a template for sub-cloning PINK1 WT into a pcDNA3.1 myc vector. Cysteine mutants PINK1 C92.96A; PINK1 C377A, C387A; and PINK1 C564A, C568A were generated using SDM for expression in mammalian cells.

3.2.3 DNA Transfection and Cell Culture

The ATCC Cell bank was used to procure the HEK293T cell lines that were used in the experiments. Complete DMEM medium (Invitrogen) formulated with 10% FBS, and 1% Antibiotic-Antimycotic (Gibco™), was used to cultivate the cells and maintained at 37°C with 5% CO_2. The cells had been transfected with the appropriate plasmid making use of Lipofectamine 2000 (Invitrogen) once they had reached 60–70% confluency.

3.2.4 Immunoblotting

Cells were harvested and for lysis either RIPA buffer or NP-40 buffer was used supplemented with PIC (Roche, Switzerland) to prepare samples for western blot analysis. The Bradford reagent was used to determine the protein content, and the lysates made were resolved on SDS polyacrylamide gel before being transferred to the NC membrane at 4°C. Ponceau stain was used to verify the correct transfer of proteins onto the membrane. Prior to incubation with the specific antibody, to prevent non-specific binding, the membrane was blocked with a 5% SM

powder prepared in TBST for 1 hour. The specified primary antibody was then added to the blots after blocking, and they were left to sit on a rocker undisturbed at 4°C overnight. The following morning, the blots were incubated with an HRP-conjugated 2° antibody at RT for one hour after being washed thrice with TBST for five minutes each. An ECL detection kit (Advansta Western-Bright) was used to develop the blots, using Bio-Rad VersaDoc imaging system.

NP-40 Buffer	50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, and 1% IGEPAL or NP-40
RIPA Buffer	50 mM Tris-HCl pH 8, 1% deoxycholic acid, 1% Triton X-100, 150 mM NaCl, 0.25 mM EDTA, and 0.1% SDS
TBST	20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20

3.2.5 Antibodies and Reagents

The antibodies used in this study were anti-MIA40 (Proteintech 21090-1-AP), anti-PINK1 (CST bc100-494; D8G3), anti-Parkin (Sigma, 05-882), anti-TOMM20 (CST, D8T4N), anti-β-Tubulin (10068-1-AP), anti-LC3 (CST), anti-Myc tag (Proteintech 16286-1-AP), anti-pSer65Ub (CST, E2J6T), anti-GAPDH (Protrintech 60004-1-Ig), Veriblot HRP for IP (ab131366) and HRP-tagged 2° antibodies anti-Rabbit or anti-Mouse were obtained from Jackson ImmunoResearch. Except when otherwise noted, all compounds were bought from Sigma-Aldrich.

3.2.6 Co-Immunoprecipitation

For the co-immunoprecipitation study, HEK293T cells grown in a 100mm dish were transfected with the indicated plasmid constructs and their respective controls. The cells were grown in DMEM devoid of serum for six hours and then substituted with complete DMEM. Cells were treated with 10μM CCCP for 4 hours after 36-hour transfection. Cells were further lysed in NP-40 buffer containing 1X PIC (Roche) after which they were washed with PBS. Total cell lysates were pelleted down at 13000rpm for 20 minutes at 4 °C. To perform the co-immunoprecipitation, 500 μg of the lysates were precleared with 10 μL Protein A or Protein G agarose beads (SC-2003) for one hour at a temperature of 4 °C to get rid of non-specific interactions. 1 μg of the desired antibody was incubated overnight with the pre-cleared lysate

on an end-over-rotator at a temperature of 4 °C. Protein A or Protein G Plus beads from Santa Cruz were added and incubated for 4 hours at 4ºC. The antibody-protein interactome was then washed four times to avoid any non-specific binding. with NP-40 buffer at 5000rpm for 2 mins at 4 °C. Samples were incubated in SDS sample buffer dye at 95°C for 5 mins, resolved on reducing SDS polyacrylamide gel, and processed by western blotting.

3.2.7 Confocal Microscopy

HEK293T cells were cultured on coverslips placed in a 35mm dish. When the cells reached approximately 60% confluency, they were co-transfected with Parkin-mcherry along with PINK1 WT or its mutants. After 36 hours post-transfection cells were treated with CCCP for the indicated time interval. Prior to cell retrieval, cells were incubated with MitoTracker for 30 mins. Following treatment, cells were rinsed with 1X PBS and then fixation was performed with methanol that had been pre-chilled at -20°C for a duration of 20 minutes. Subsequently, the cells were washed with 1X PBS, each washed for 5 minutes. The coverslips were mounted onto a glass slide with an antifade reagent containing DAPI (ab104139). Imaging was performed using a Leica SP8 confocal microscope, and Image J software was employed for image processing.

3.2.8 Isolation of Mitochondria from HEK293T cells

The procedure to isolate mitochondria from cultured HEK293T cells involved washing the cells twice with 1X PBS. The washed cells were then re-suspended in mitochondria isolation buffer. The cell suspension was plunged into ice for 1 hour with gentle swirling every 15 min to swell up the mitochondria. For proper homogenization of the cell suspension, two 3-sec pulses were applied using Polytron 1600 homogenizer at 15 rpm, followed by a Glass-glass Dounce homogenizer. To remove cell debris and unbroken cells, the homogenate was then pelleted down at a speed of 1000 g for ten minutes. The supernatant was collected and further centrifuged at a high speed of 10,000 g and a duration of 15 min. All the centrifugations should be performed at 4°C. The pellet obtained after centrifugation was the mitochondria. Following two washes the mitochondrial pellet was resuspended in a Mitochondrial resuspension buffer.

Mitochondria Isolation Buffer	20 mM HEPES pH 7.5, 1 mM EDTA pH 8.0, 1.5 mM $MgCl_2$, 1 mM EGTA, 70 mM Mannitol, and 210 mM Sucrose
Mitochondria Resuspension Buffer	250 mM sucrose, 10 mM HEPES-KOH pH 7.4, and 5 mM $MgCl_2$.

3.2.9 Densitometric Quantification and statistical analysis

The signals in the western blots were quantified using ImageJ software. Protein bands from a minimum of three independent experiments were quantified for each assay. GraphPad Prism7 was used for the statistical analysis and plotting of graphs. The results were presented as the mean ± SEM. Two-way ANOVA was used for calculating the statistical significance of grouped data. Statistical significance was defined as a p-value less than or equal to 0.05.

3.2.10 AlphaFold2 Prediction and PyMOL Alignment

The protein sequence was obtained from NCBI and analyzed using AlphaFold2 Colab with all the parameters selected. The 3D structure thus predicted was downloaded in PDB format. PDB format structures of the WT protein and its mutant protein were structurally aligned using PyMOL and studied for their similarities or differences.

3.3 Results

3.3.1 PINK1 cysteine mutants and their predicted 3D structures

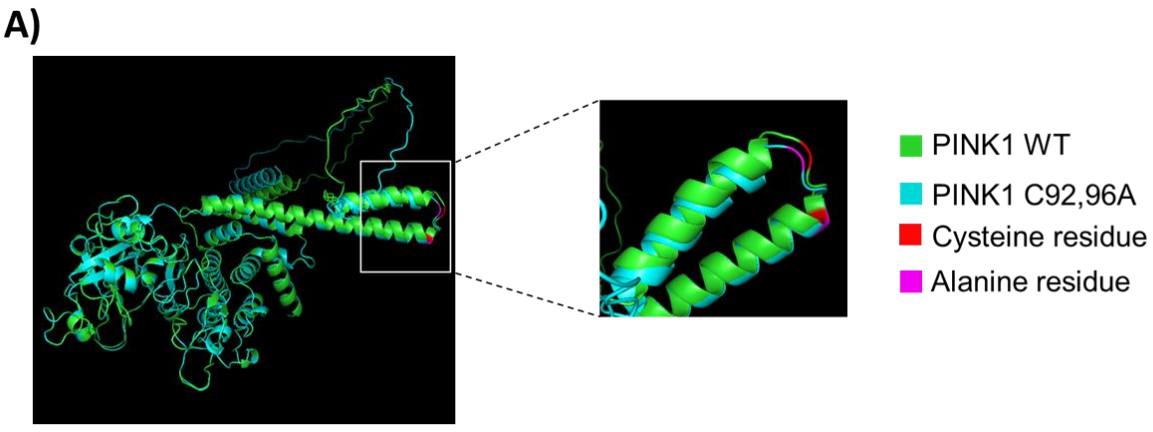
As mentioned earlier, PINK1 possesses distinctive cysteine motifs, Cx3C or Cx9C, that are required for the protein's import via the MIA pathway. In our previous chapter, we have also shown that MIA40 interacts with PINK1, and this interaction is cysteine-mediated.

Hence, to study the PINK1 cysteines involved in PINK1s interaction with MIA40, we created three distinct double mutants using site-directed mutagenesis. Two with a mutated Cx3C motif (C92A, C96A and C564A, C568A) and another mutant called C377A, C387A, where cysteines of the Cx9C motif were replaced with alanine.

In our study, we utilized AlphaFold2 to predict the 3D structure of PINK1 WT, PINK1 C92A, C96A; PINK1 C377A, C387A; and PINK1 C564A, C568A mutant. AlphaFold2 analyzes the amino acid sequence of the protein and predicts the distances between these amino acids. These predictions are used to generate a 3D structure using a variety of computational techniques.

To assess the structural changes between the wild-type and the mutant, their predicted protein structure obtained from AlphaFold2 was loaded onto PyMOL in PDB format. The protein structures to be compared were superimposed and aligned in a way that RMSD is minimal.

Our analysis confirmed that the 3D structure of all the PINK1 mutants aligned closely with the PINK1 WT protein (Figure 3.3). This outcome gave us an idea that these cysteine to alanine mutations did not cause any significant changes to alter the PINK1 protein structure.



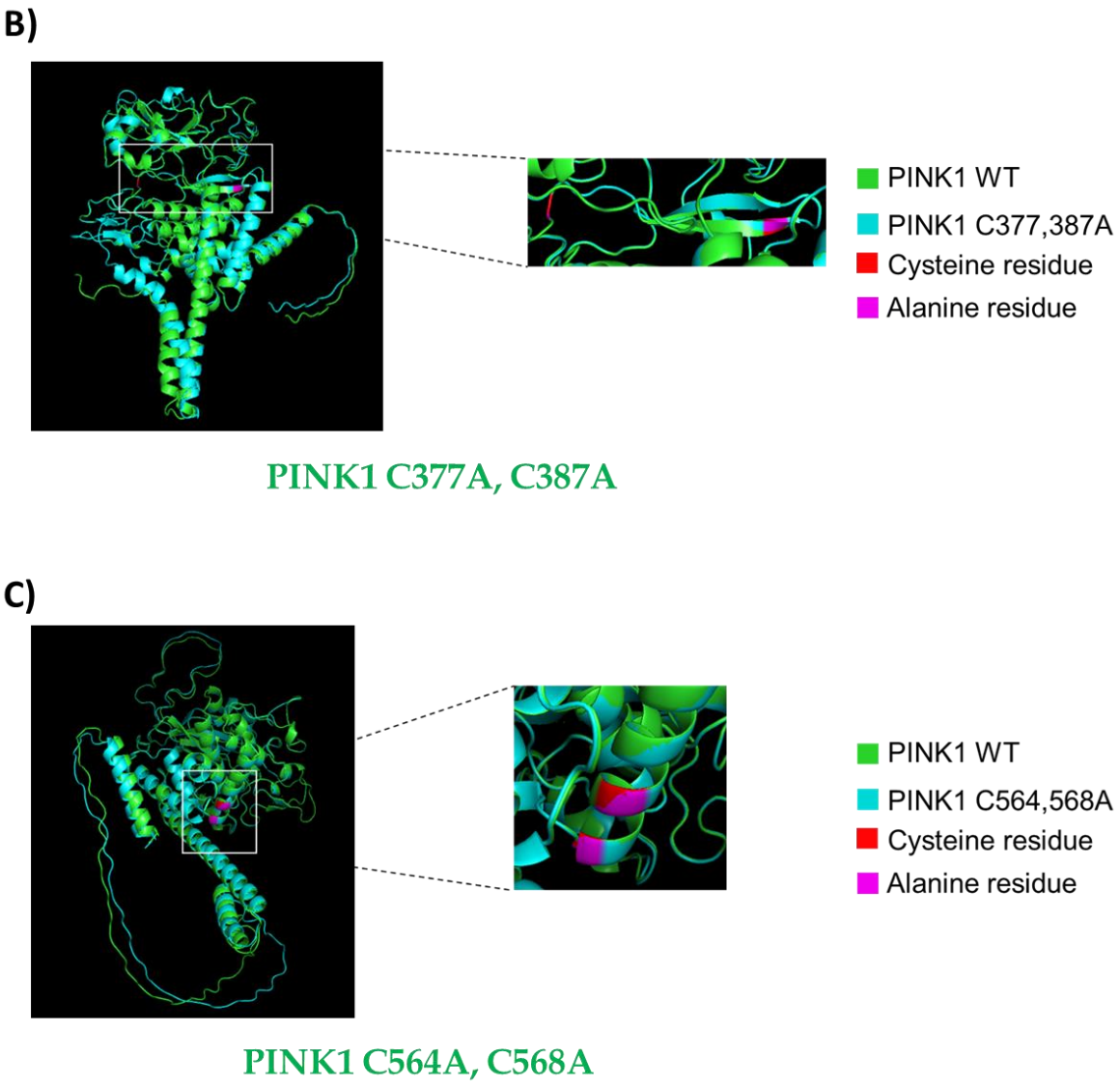


Figure 3.3: Predicted 3D structure of PINK1 cysteine mutants. 3D structures of PINK1 mutants were aligned with the 3D structure PINK1 WT using PyMOL. A) PINK1 C92A, C96A aligned with PINK1 WT B) PINK1 C377A, C387A aligned with PINK1 WT C) PINK1 C564A, C568A aligned with PINK1 WT.

3.3.2 Interaction of PINK1 cysteine mutants with MIA40

To investigate whether the mentioned cysteine motifs are involved in PINK1's interaction with MIA40, we generated three cysteine double mutants C92A, C96A; C377A, C387A; and C564A, C568A. To further determine the role of these cysteine motifs in PINK1-MIA40 interaction, we transfected HEK293T cells with PINK1 WT and the different mutants followed by treatment with 10µM CCCP for 4 hours, 36 hours post-transfection. Next, we prepared cell lysates using NP-40 buffer and immunoprecipitated them with an anti-hMIA40 antibody before

probing with an anti-myc antibody. These experiments aimed to elucidate the role of the cysteine motifs in the interaction between PINK1 and MIA40 during mitophagy. We observed that both the PINK1 bands were detected in all the lanes except the lane containing the PINK1 C564A, C568A mutant (Fig 3.4, lanes 9&10). This finding suggests that the cysteine residues at positions 564 and 568 may be crucial for PINK1's interaction with MIA40, as well as for its stabilization on the OMM upon depolarization of the mitochondria (Figure 3.4).

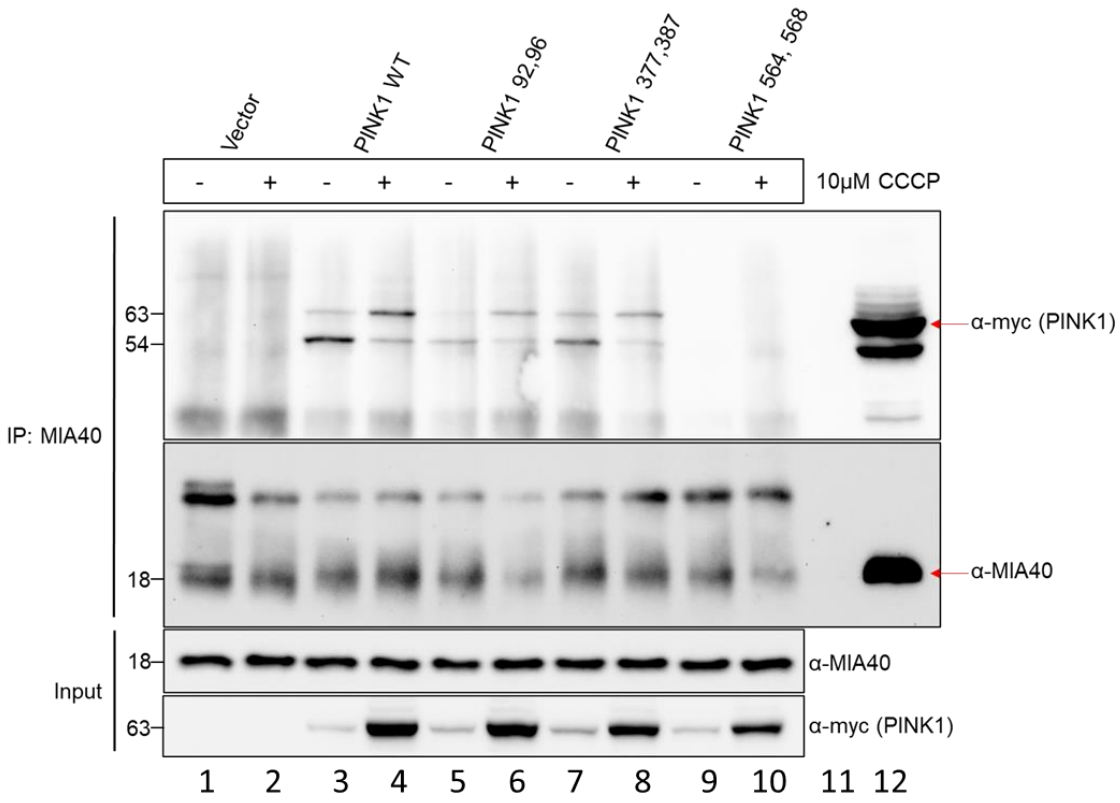


Figure 3.4: Cysteines present at positions 564 and 568 of PINK1 regulates mitophagy. To perform Co-IP, HEK293T cell lysates transfected with PINK1 WT and mutants were immunoprecipitated using an anti-MIA40 antibody. The resulting immunoprecipitates were analyzed by western blotting and probed with an anti-myc antibody for the detection of PINK1. This experiment suggests that the cysteines at positions 564 and 568 of PINK1 might be interacting with MIA40

3.3.3 PINK1 cysteine mutants modulate mitophagy

A critical step in the initiation of mitophagy is the stabilization of PINK1 on the OMM of mitochondria, which acts as a reliable marker for detecting damaged or malfunctioning

mitochondria that need to be eliminated from the cell. In order to study the effect of PINK1 cysteine mutants on mitophagy, we thought of examining the PINK1 stabilization in HEK293T cells expressing PINK1 WT; PINK1 C92A, C96A; PINK1 C377A, C387A; and PINK1 C564A, C568A, with and without treatment with CCCP as described in the methods. An increase in PINK1 stabilization was observed in the cells expressing the PINK1 C92A, C96A mutant (Fig 3.5A lane 6) whereas no significant change was observed in cells expressing C377A, C387A mutant (Fig 3.5A lane 8). We also observed that the PINK1 stabilization upon CCCP treatment was significantly decreased in the PINK1 C564A, C568A mutant (Fig 3.5A, lane 10) compared to PINK1 WT-expressing HEK293T cells (Fig 3.5A, lane 4). This observation suggests that cysteines at positions 377 and 387 may not significantly impact PINK1's stability and that mutation of cysteines at 92 and 96 might be causing more stress that led to the increase in PINK1 accumulation. It also suggests that the cysteine residues at positions 564 and 568 are critical for PINK1 stabilization during mitophagy, and their mutation may impair the mitophagy process (Figure 3.5A and B).

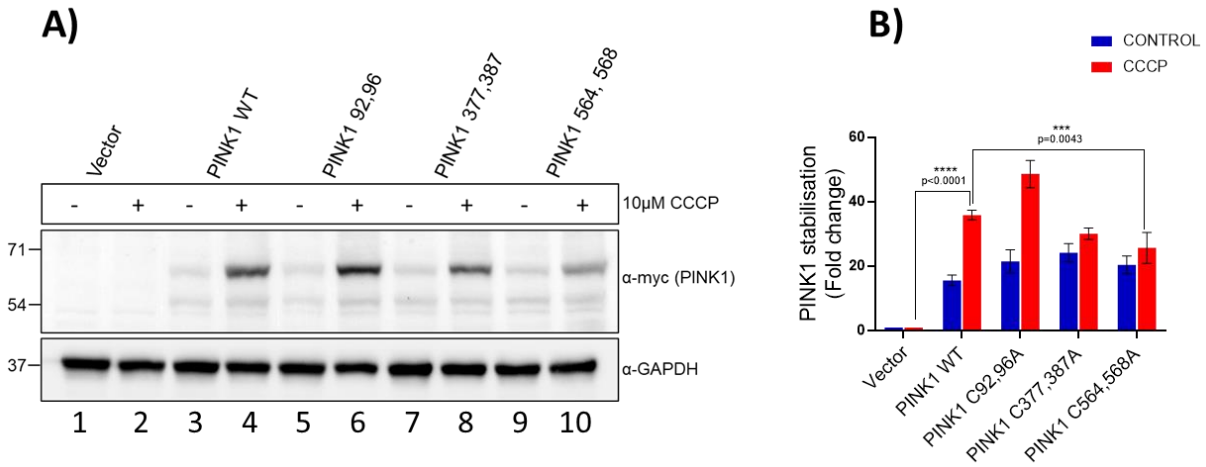


Figure 3.5: A) Western blot analysis showing the level of PINK1 stabilization in HEK293T cells transfected with PINK1 WT or its different cysteine mutants (C92A, C96A; C377A, C387A; C564A, C568A) with or without treatment of 10μM CCCP for 4 hours. B) The band intensities were quantified and normalized to GAPDH. The results indicate that the stabilization of PINK1 upon CCCP treatment in PINK1 C564A, C568A mutant was significantly decreased compared to that of PINK1 WT-expressing HEK293T cells.

3.3.4. pSer65-Ub levels in cysteines mutants of PINK1

Phosphorylation of ubiquitin has emerged as a critical post-translational modification involved in the regulation of mitophagy. Several studies have shown that phosphorylation of ubiquitin by various kinases, including PINK1 can modulate the efficiency and selectivity of mitophagy. PINK1 is known to phosphorylate ubiquitin at Ser65, which recruits the autophagy receptor proteins like p62, NDP52, and OPTN, to ubiquitinated mitochondria. These adaptor proteins recruit the autophagosome to initiate the engulfment of mitochondria and their delivery to lysosomes for degradation. This process is thought to be essential for the efficient clearance and prevention of the accumulation of dysfunctional mitochondria, which can lead to cell damage and disease. We observed that the PINK1 C564A, C568A mutant displays decreased PINK1 stabilization upon mitochondrial depolarization (Figure 3.5). To investigate further downstream processes of mitophagy, western blots of PINK1 WT and mutant transfected cell lysates were probed with pS65-Ub. Phosphorylation of ubiquitin is moderately decreased, indicating a decrease in mitophagy in cells expressing the PINK1 C377A, C388A and the C564A, C568A mutant (Fig 3.6A, lanes 8&10 respectively when compared to lane 4). Whereas no significant change was observed in cells expressing PINK1 C92A, C96A mutant (Fig 3.6A, lane 6). These results suggest that the decreased phosphorylation of ubiquitin at Ser65 may be contributing to altered mitophagy in the PINK1 C564A, C568A mutant (Figures 3.6A and B). whereas the decreased phospho-ubiquitination in PINK1 C377A, C387A mutant could be a result of other cellular processes associated with phosphorylation of ubiquitin as PINK1 stabilization remained unaltered as depicted in Figure 3.5. Even though stabilization of PINK1 was enhanced in cells expressing PINK1 C92A, C96A mutant (Figure 3.5), the subsequent process of mitophagy i.e., phosphorylation of ubiquitin remained unaltered. This observation indicates that cysteines at positions 92 and 96 may have additional functions other than mitophagy.

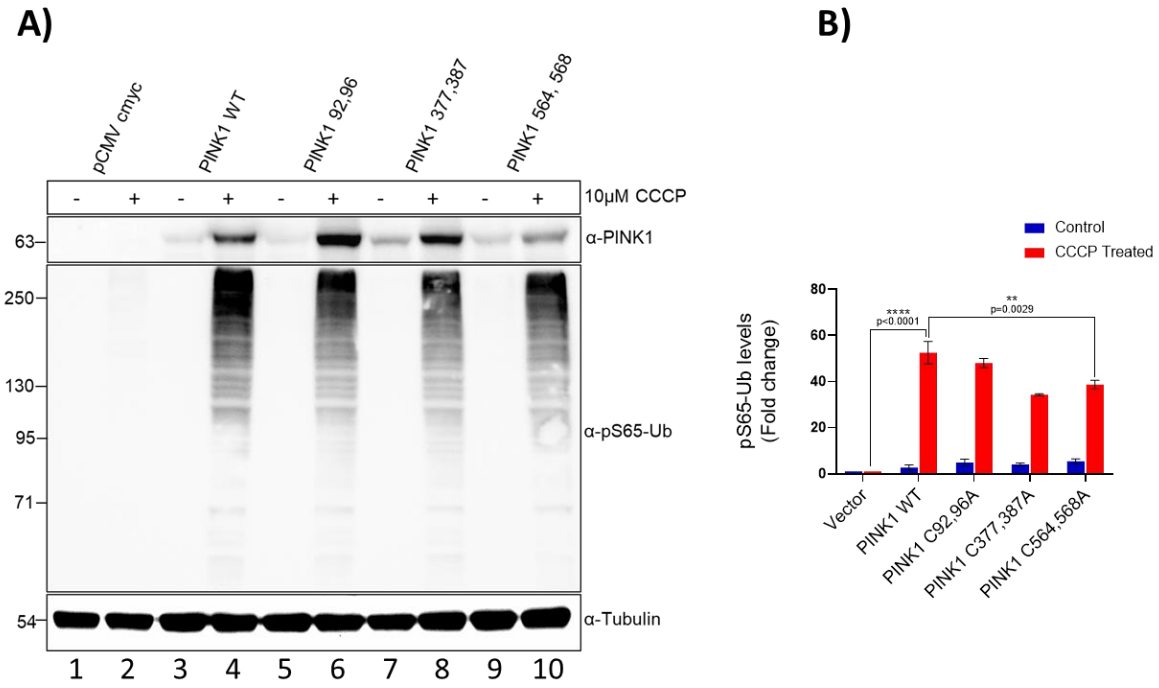
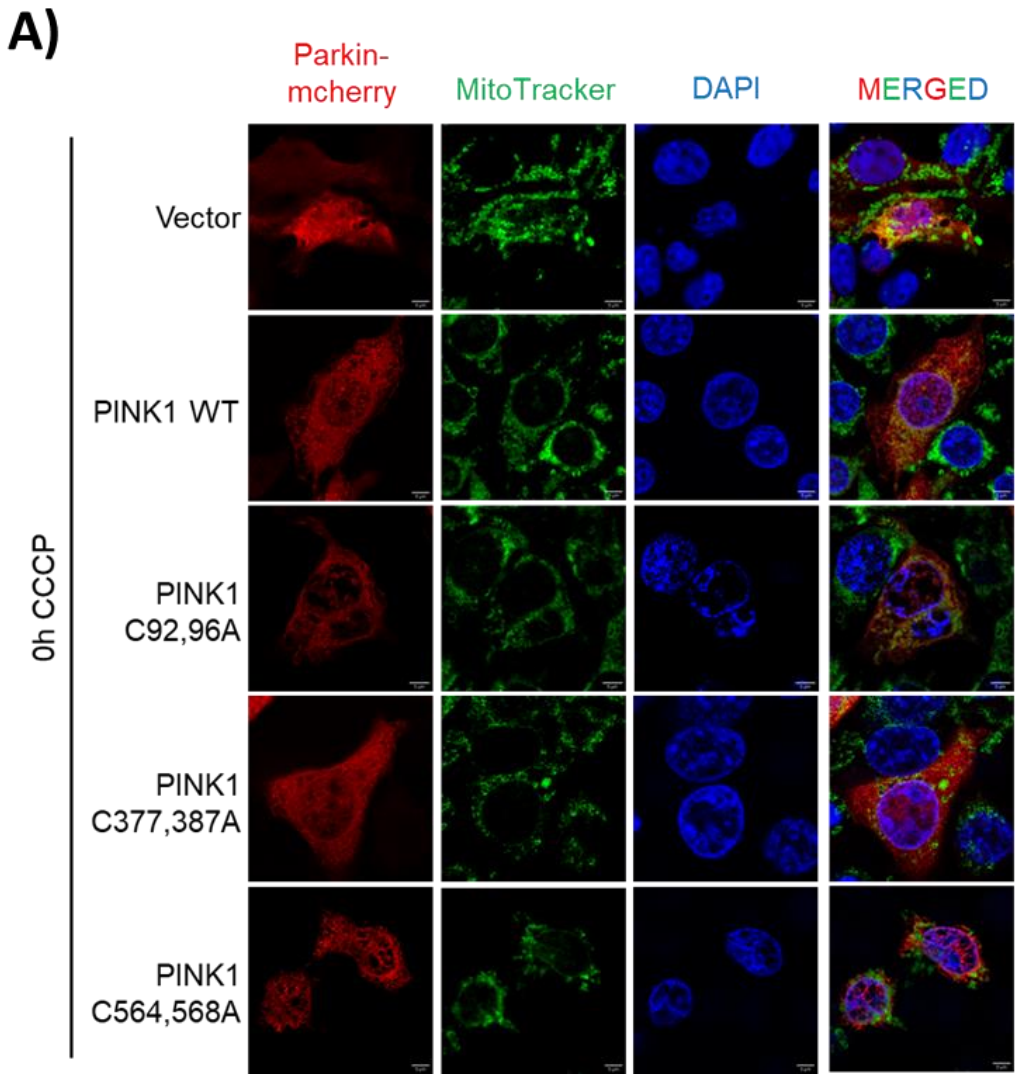


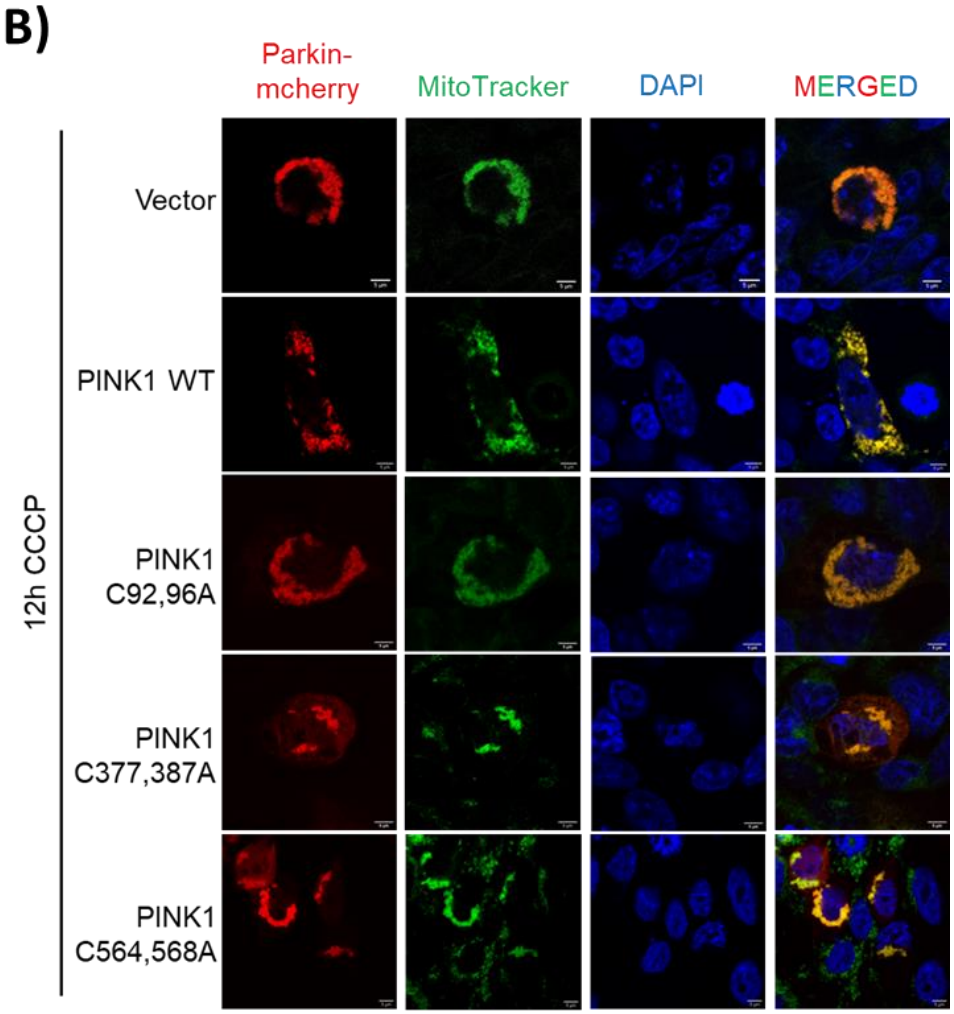
Figure 3.6: A) Western blot analysis showing the level of pS65-Ub in HEK293T cells transfected with PINK1 WT or its different cysteine mutants (C92A, C96A; C377A, C387A; C564A, C568A) with or without treatment of 10μM CCCP for 4 hours. B) The band intensities were quantified and normalized to Tubulin. The results indicate a decrease in phosphorylation of ubiquitin at Ser65 in the PINK1 C564A, C568A mutant compared to that of PINK1 WT-expressing HEK293T cells.

3.3.5. Parkin recruitment to mitochondria in cysteines mutants of PINK1

The stabilized PINK1 on OMM signals the recruitment of Parkin. Parkin is an E3 Ubiquitin Ligase that ubiquitinates mitochondrial proteins. Hence, Parkin recruitment to mitochondria is also considered a parameter to measure mitophagy. Hence, in order to analyze the co-localization of Parkin with mitochondria, HEK293T cells co-transfected with Parkin-mcherry and PINK1 WT or its mutants, 36 hours post-transfection cells were treated with CCCP and then stained with MitoTracker to stain the mitochondria as described in the Methods (Li et al., 2018; Lee et al., 2021; Lin et al., 2019; Kubohara et al., 2013). Slides were prepared as per the protocol mentioned and examined under Leica confocal microscope at 63X magnification. The analysis revealed a noticeable decrease in Parkin co-localization with mitochondria in both C377A, C387A and C564A, C568A mutant upon CCCP treatment, indicating a reduction in mitophagy (Figure 3.7B). However, no change was observed in Parkin co-localization with mitochondria in PINK1 C92A, C96A mutant. To quantify this observation, Mander's

coefficient was calculated using the JACoP plugin in ImageJ. The resulting values were plotted, confirming our previous observation (Figure 3.7C).




B)
Parkin-mcherry
MitoTracker
DAPI
MERGED
12h CCCP
Vector
PINK1 WT
PINK1 C92,96A
PINK1 C377,387A
PINK1 C564,568A

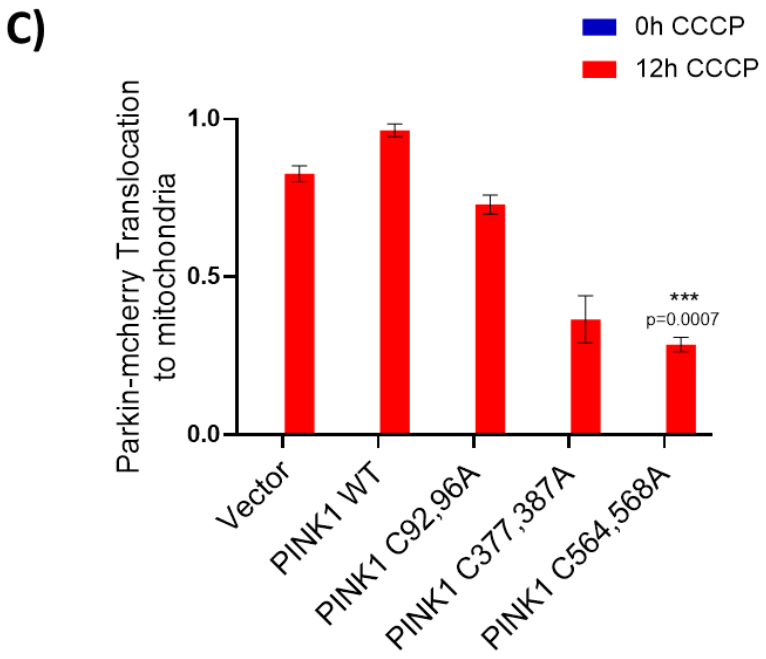

C)
0h CCCP
12h CCCP
Parkin-mcherry Translocation to mitochondria
1.0
0.5
0.0
***
p=0.0007
Vector
PINK1 WT
PINK1 C92,96A
PINK1 C377,387A
PINK1 C564,568A

Figure 3.7: Parkin localization to mitochondria A) Confocal microscopy images showing Parkin-mcherry localization to mitochondria (green) in control cells transfected with PINK1 WT and mutants. B) Confocal microscopy images showing Parkin-mcherry localization to mitochondria (green) in cells treated with 10μM CCCP for 12 hours. C) Representative graph for co-localization of Parkin with mitochondria. Scale bars correspond to 5μm.

To provide additional validation regarding the co-localization of Parkin to mitochondria, mitochondrial were isolated from cells expressing PINK1 WT and PINK1 C564A, C568A mutant, as per the protocol mentioned in section 3.2.8. Upon treatment with CCCP, which induces mitochondrial depolarization, Parkin was observed to be localized to the mitochondria in both PINK1 WT and PINK1 C564A, C568A mutant. However, the extent of localization was slightly reduced in the PINK1 C564A, C568A mutant (Fig 3.8A, lane 6 compared to lane 4). This finding further supports the conclusion that mitophagy is diminished in the presence of the PINK1 C564A, C568A mutant (Figure 3.8A).

3.3.6. Levels of mitochondrial proteins in PINK1 cysteine mutants

During mitophagy, mitochondrial proteins are selectively targeted for degradation. Several studies have shown that mitophagy leads to changes in the levels of specific mitochondrial proteins. For example, the mitochondrial membrane protein, TOMM20, and mitochondrial fusion protein, MFN2 are selectively degraded during mitophagy. Other mitochondrial proteins that have been shown to be degraded during mitophagy include the electron transport chain (ETC) complex subunits, such as NDUFS1, SDHA, and COXIV. In addition to protein degradation, mitophagy is also known to stimulate mitochondrial biogenesis as an adaptive response. The selective degradation of specific mitochondrial proteins, along with changes in mitochondrial biogenesis, ensures that the cell maintains a healthy pool of functional mitochondria while removing damaged or unwanted mitochondria.

To check the levels of mitochondrial proteins in cells expressing PINK1 WT and mutants, HEK293T cells were transfected with PINK1 WT and PINK1 C564A, C568A mutant constructs, followed by treatment with 10μM CCCP for a duration of 4 hours. Mitochondria were isolated from these cells and probed with the mitochondrial membrane protein TOMM20. Interestingly, as anticipated, mitochondria isolated from cells expressing PINK1 WT exhibited a reduction in TOMM20 levels (Fig 3.8A, lane 4 is reduced compared to lane 3). However, this

decline in TOMM20 levels was not observed in mitochondria isolated from cells expressing PINK1 C564A, C568A mutant, as depicted in Figure 3.8A (lane 6 is not reduced compared to lane 5). This further supports the conclusion that mitophagy is diminished in the presence of the PINK1 C564A, C568A mutant.

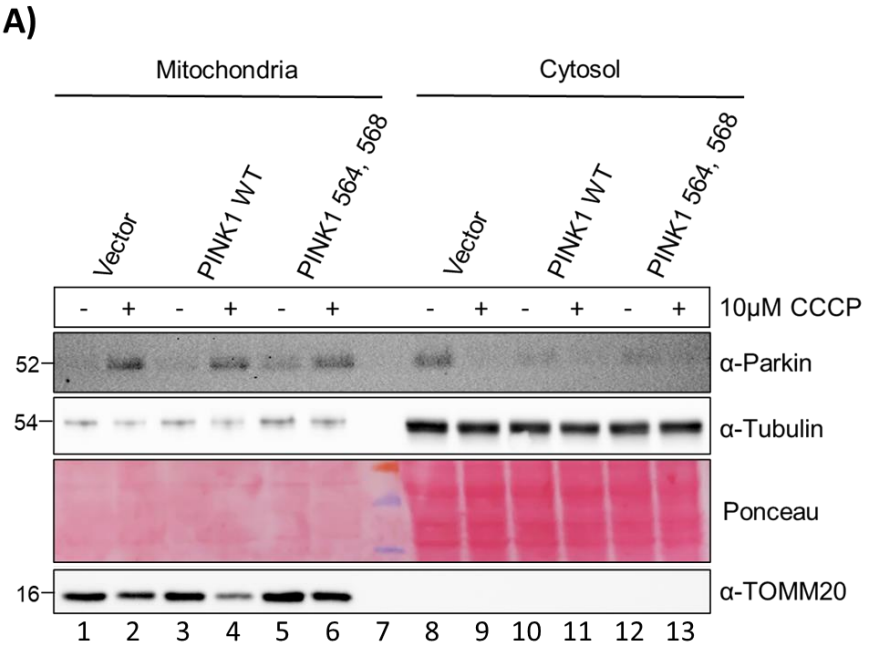


Figure 3.8: Effect of PINK1 WT and cysteine mutants on mitochondrial proteins following CCCP treatment. A) Mitochondria were isolated from the indicated sample and probed with the mentioned mitochondrial proteins.

3.3.7. LC3 Flux in PINK1 cysteine mutants

Another parameter for measuring mitophagy is to measure LC3-II flux. To quantify LC3 flux, we transfected HEK293T cells with PINK1 WT and mutant plasmids and treated them with CCCP for 4 hours. The CCCP-treated samples were treated with chloroquine, an autophagosome-lysosome fusion inhibitor, and LC3-II flux was calculated by subtracting the values obtained from CQ-treated from untreated samples after normalizing with GAPDH. It was observed that overexpression of PINK1 WT resulted in a significant increase in mitophagy flux (Fig 3.9A, difference between lane 5 and lane 6) and overexpression of PINK1 C564A, C568A mutant decreased the mitophagy flux (Fig 3.9A, difference between lane 14 and lane 15) which was consistent with all the other results, indicating that cysteines at positions 564 and 568 of PINK1 might play a crucial role in regulating mitophagy (Figure 3.9).

A decrease in mitophagy flux was also observed in the mutants PINK C92A, C96A as well as PINK1 C377A, C387A. However, a consistent alteration pattern across all mitophagy steps was observed only in the case of PINK1 C564A, C568A mutant. Hence it was concluded that cysteines at positions 564 and 568 of PINK1 might play a crucial role in regulating mitophagy.

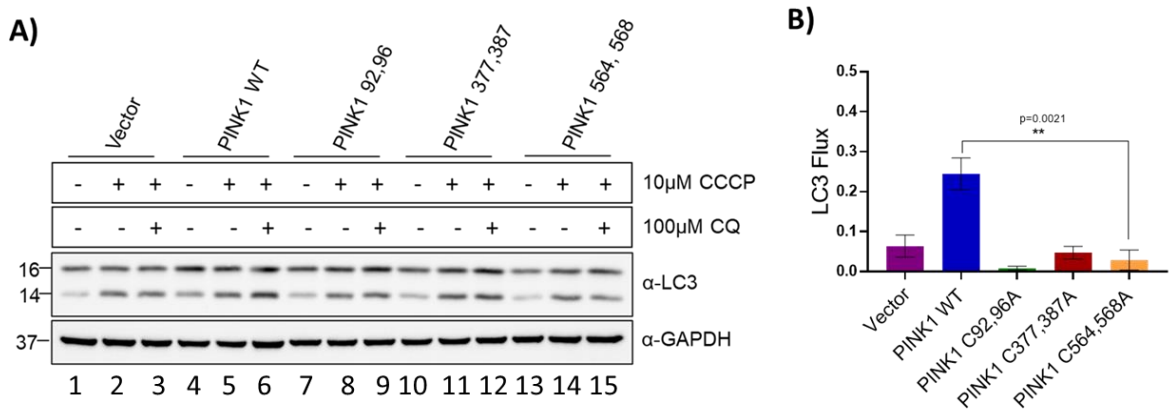


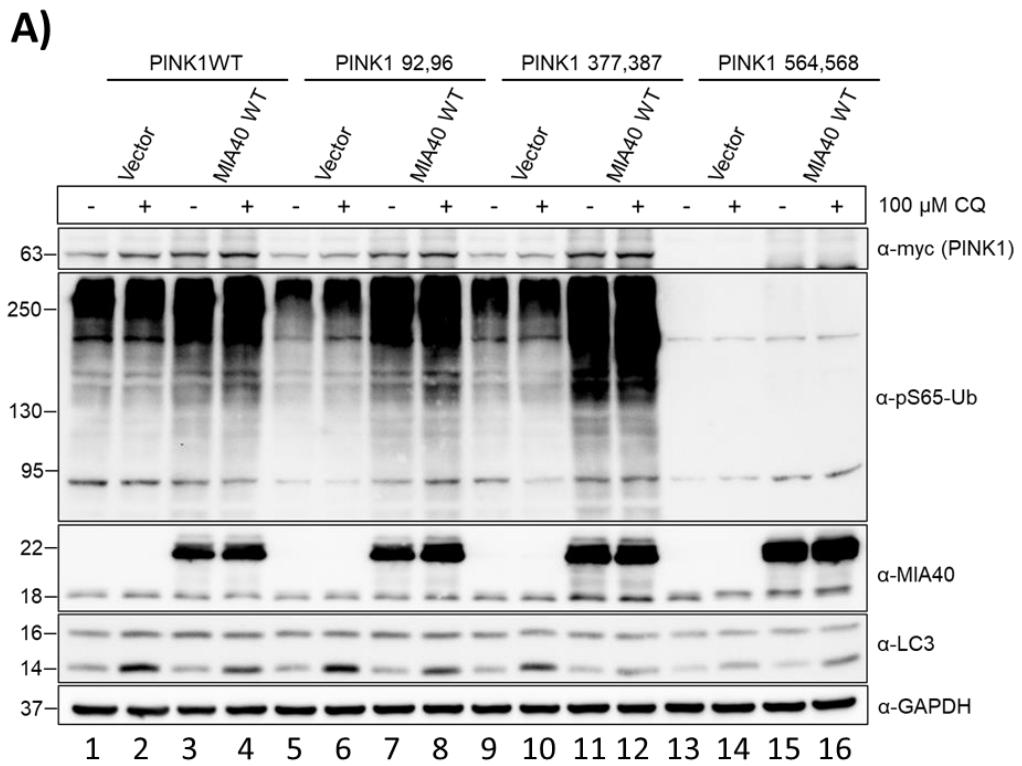
Figure 3.9: LC3 Flux in of PINK1 WT and cysteine mutants. A) Western blot analysis showing the level of LC3-II flux in HEK293T cells transfected with PINK1 WT or its different cysteine mutants (C92A, C96A; C377A, C387A; C564A, C568A) and treatment with 10μM CCCP. B) The band intensities of CQ untreated were subtracted from CQ treated after being normalized to GAPDH.

3.3.8. Phospho-Ubiquitination is lower in PINK1 C564A, C568A mutant

In our previous chapter, we have shown that overexpression of MIA40 WT in PINK1 WT transfected cells increases mitophagy. So next we thought of checking if overexpressing MIA40 WT in PINK1 mutants has an effect on mitophagy. To study the impact of MIA40 WT overexpression on cells expressing PINK1 WT and cysteine mutants, HEK293T cells were co-transfected with MIA40 WT and either PINK1 WT or cysteine double mutants. The cells were then lysed in RIPA buffer and subjected to western blot analysis. Our observations showed a significant increase in ubiquitin phosphorylation, as detected by the pS65-Ub antibody, with MIA40 WT overexpression, regardless of the PINK1 plasmid transfected (Fig 3.10A, lanes3-4, 7-7,11-12). However, there was a significant decrease in ubiquitin phosphorylation in cell lysates expressing the PINK1 C564A, C568A mutant (Fig 3.10A, lanes 13-16). A similar pattern was observed in LC3-II levels as well i.e., LC3-II levels in cells expressing PINK1

C564A, C568A mutant are lower compared to all the other lanes (Fig 3.10A, lanes 13-16). This finding is consistent with our previous results, which demonstrate that the PINK1 C564A, C568A mutant fails to interact with MIA40 and induce mitophagy, resulting in reduced ubiquitination and ultimately decreased mitophagy (Figure 3.10A).

To further explore this phenomenon, we sought to determine if the expression of PINK1 C564A, C568A in combination with MIA40 WT and its two mutants, CPC and QM mutant, would have a synergistic effect on ubiquitin phosphorylation. To our surprise, we observed an overall decrease in ubiquitin phosphorylation in cells expressing the PINK1 C564A, C568A mutant (Fig 3.10B, lanes 10-17) compared to cells expressing PINK1 WT irrespective of the MIA40 plasmid transfected (Fig 3.10B, lanes 1-8). However, ubiquitin phosphorylation in cells co-expressing MIA40 QM and PINK1 C564A, C568A mutant was significantly diminished compared to all other samples (Figure 3.10B, lanes 16&17). These results suggest that the cysteines present at positions 564 and 568 of PINK1; as well as positions 4, 64, 74, and 97 of MIA40, play a crucial role in the regulation of mitophagy. Substituting these residues has a significant impact on various stages of the mitophagy process.



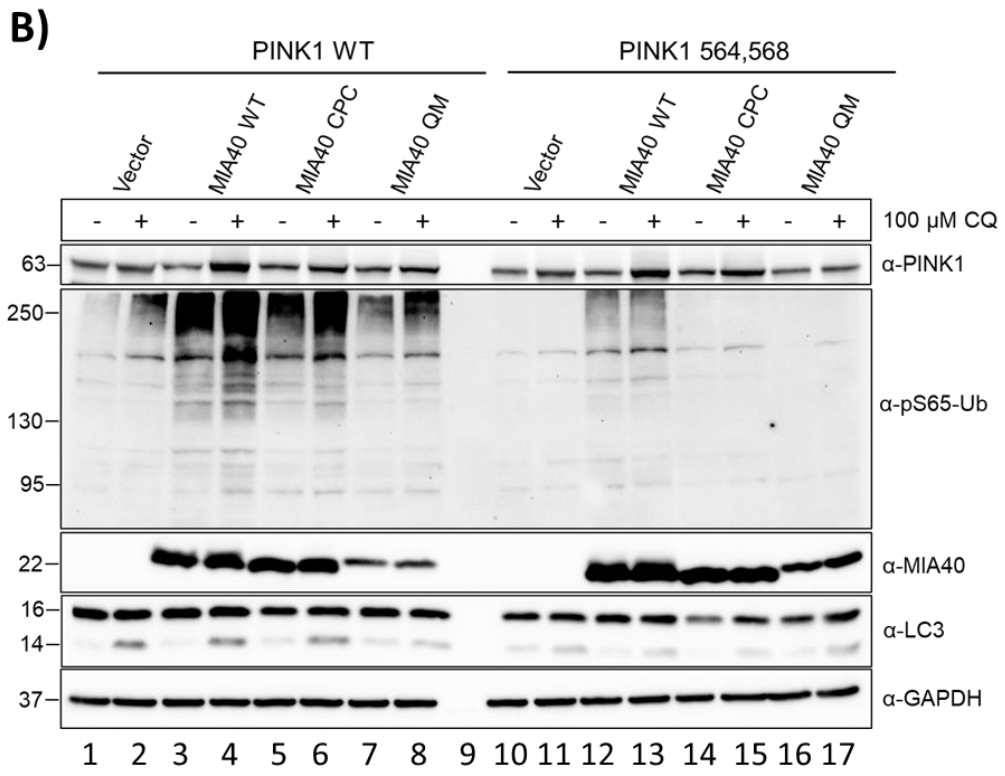


Figure 3.10: LC3 Flux in of PINK1 WT and cysteine mutants. A) MIA40 WT overexpression increases ubiquitin phosphorylation regardless of the PINK1 plasmid transfected. An overall decrease in ubiquitin phosphorylation in cell lysates expressing the PINK1 C564A, C568A mutant was observed. B) The blot shows the levels of ubiquitin phosphorylation in cells expressing PINK1 WT or PINK1 C564A, C568A mutant, in combination with MIA40 WT, MIA40 CPC mutant, or MIA40 QM mutant. We observed an overall decrease in ubiquitin phosphorylation in cells expressing the PINK1 C564A, C568A mutant compared to cells expressing PINK1 WT.

3.4 Conclusions

Our research in this chapter leads us to the conclusion that cysteines at positions 564 and 568 of PINK1 play a crucial role in mediating its interaction with MIA40 under stress conditions, thereby regulating its stability and mitophagy. We validated this through immunoprecipitation studies, which demonstrated the physical association between MIA40 and PINK1 is mediated by cysteines.

Furthermore, our investigations also showed the significance of these cysteine residues in mitophagy. Notably, the absence of these cysteines in the PINK1 C564A, C568A mutant resulted in a decrease in PINK1 stability, phospho-ubiquitination at Ser65, Parkin recruitment to mitochondria, and LC3-II flux. These findings highlight the crucial role of the identified cysteine residues in regulating proper mitophagy functioning.

In addition to the PINK1 C564A, C568A mutant, the PINK1 C377A, C387A mutant also exhibited a slight decrease in PINK1 stability, phospho-ubiquitination at Ser65, Parkin recruitment to mitochondria, and LC3-II flux. However, this mutant was also observed to interact with MIA40. Hence, it can be inferred that cysteines at positions 377 and 387 might be regulating mitophagy through an alternative pathway. Furthermore, the PINK1 C92A, C96A mutant exhibited an increase in PINK1 stabilization and a decrease in LC3-II flux, whereas, no change was observed in phospho-ubiquitination or Parkin recruitment. This suggests that the increased PINK1 stabilization observed in this mutant may be attributed to other functions of PINK1 apart from mitophagy.

It can also be observed that the co-expression of PINK1 C564A, C568A mutant along with the MIA40 QM mutant resulted in an additional reduction in the phosphorylation of ubiquitin and LC3-II flux. This finding suggests that the identified cysteines in both proteins can have an additive effect when mutated. Therefore, it can be concluded that these cysteines play a significant role in mitophagy.

CHAPTER 4

Discussion

4.1 Discussion

Mitochondria play a crucial role in coordinating various biological processes within cells. They are involved in ATP production, calcium homeostasis, phospholipid biosynthesis, iron-sulfur cluster biosynthesis, and even cell death regulation (Spinelli and Haigis, 2018). However, mitochondrial function can be compromised due to damage or dysfunction, which can have detrimental effects on cellular health. When mitochondrial damage is beyond repair, the cell resorts to the removal of the whole mitochondria by a process called mitophagy. It is well known that in PINK1 dependent mitophagy pathway, PINK1's import into the mitochondria is hindered upon depolarization of the mitochondria. As a result, PINK1 accumulates on the OMM and forms a complex with the TOM complex (Lazarou et al., 2012). This is followed by the recruitment of Parkin to the OMM which ubiquitinated the mitochondrial proteins. The enhancement of the phospho-ubiquitin chain on the OMM surface signals for the recruitment of autophagy receptors such as OPTN and NDP52. Then, the mitochondria are targeted for engulfment by autophagosomes. Many proteins like SAM50 (Jian et al., 2018), TOM70 (Kato et al., 2013), and TIM23 (Akabane et al., 2023) are known to be involved in the stability of PINK1 to regulate mitophagy. In a recent study by Gao et al. in 2020, it was demonstrated that along with the disruption of mitochondrial membrane potential, the activation of the mitochondrial disulfide relay system within the IMS upon oxidative stress is also critical for PINK1 accumulation. Due to the complex nature of the process, multiple molecular factors that potentially influence PINK1's stability are yet to be identified.

In this study, we have shown that cysteines in both PINK1 and MIA40 interact with each other to regulate PINK1's stability upon disruption of membrane potential. Through Ni-NTA and immunoprecipitation studies, we validated that MIA40 may interact with PINK1 under stressed conditions. Moreover, our immunoprecipitation experiments revealed that the cysteine residues located at positions C4, C64, C74, and C97 of MIA40 are essential for mediating its interaction with PINK1. Various parameters of measuring mitophagy such as PINK1 stabilization, levels of phosphorylated ubiquitin, and mitophagy flux showed a significant increase upon overexpression of MIA40 WT, but this increase was not observed in cells expressing MIA40 QM mutant. Overall, these findings highlight the crucial role of the cysteine residues at positions C4, C64, C74, and C97 of MIA40 in regulating proper mitophagy functioning.

Even though the MIA40 QM mutant exhibits a more pronounced decrease in mitophagy, the MIA40 CPC mutant also appeared to decrease mitophagy to an extent, especially in the basal

level of mitophagy. Considering that the CPC domain is involved in forming disulfide bonds with the incoming substrate proteins (Sideris et al., 2009), it would be reasonable to say that the cysteines mutated in the CPC mutant might have a role in stabilizing PINK1 that cannot be entirely disregarded. The study published by Gao et al., in 2020 also demonstrated that the deletion mutant of PINK1 lacking the amphipathic helix (PINK1 Δ166-171) led to a decreased PINK1 accumulation on mitochondria in the cells that were treated with CCCP by decreasing its interaction with CHCHD4. Hence, it can be inferred that the interaction between PINK1 and MIA40 is governed by not just one but multiple factors.

PINK1 possesses distinctive cysteine motifs, Cx3C or Cx9C, that are required for the protein's import via the MIA pathway. Hence, to study the PINK1 cysteines involved in PINK1's interaction with MIA40, we created three distinct double mutants. Two with a mutated Cx3C motif (C92,96A and C564,568A) and another mutant called C377,387A, where cysteines of the Cx9C motif were replaced with alanine. Immunoprecipitation studies showed that the cysteine residues at positions 564 and 568 are crucial for PINK1's interaction with MIA40, as well as for its stabilization on the OMM upon depolarization of the mitochondria. The absence of these cysteines in the PINK1 C564,568A mutant resulted in a decrease in PINK1 stability, phospho-ubiquitination at Ser65, Parkin recruitment to mitochondria, and LC3-II flux. Consistent with all other results PINK1 WT exhibited a reduction in mitochondrial protein, TOMM20. However, this decline in TOMM20 levels was not observed in cells expressing the PINK1 C564,568A mutant. Overall, these findings highlight the crucial role of the cysteine residues at positions 564 and 568 of PINK1 in regulating proper mitophagy functioning.

To further explore this phenomenon, we sought to determine if the expression of PINK1 C564,568A in combination with MIA40 QM mutant, would have a synergistic effect on ubiquitin phosphorylation. We observed that ubiquitin phosphorylation in cells co-expressing MIA40 QM and PINK1 C564,568A mutant was significantly diminished compared to all other samples. These results suggest that the cysteines present at positions 564 and 568 of PINK1; as well as positions 4, 64, 74, and 97 of MIA40, play a crucial role in the regulation of mitophagy. Substituting these residues has a significant impact on various stages of the mitophagy process.

In summary, we reveal the importance of cysteines in regulating a complex process such as mitophagy. The dependence of PINK1 on cysteines for its stability represents a small drop in the ocean of the molecular factors involved in regulating PINK1's stability. Nevertheless,

additional studies are required to identify the exact cysteine residues of MIA40 responsible for its interaction with PINK1. Furthermore, it remains to be determined whether these interactions have an impact on the formation of the high HMW complex with TOM.

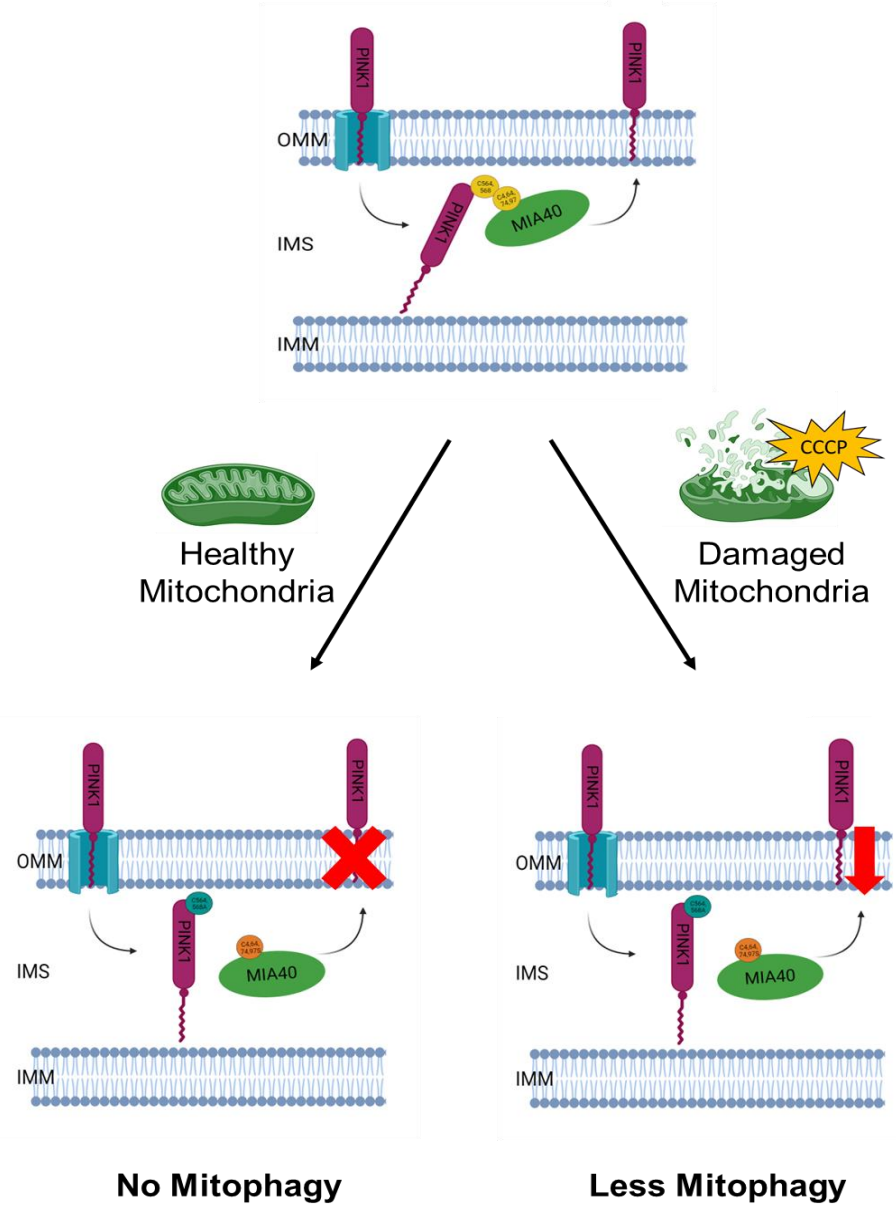


Figure 4.1: Overall summary of the work

Cysteines present at positions 564 and 568 of PINK1; as well as positions 4, 64, 74, and 97 of MIA40, play a crucial role in the regulation of mitophagy. Mutation of these cysteines results in the absence of basal mitophagy in healthy mitochondria and a reduction in mitophagy in dysfunctional mitochondria.

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Publications

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Rotenone-induced reactive oxygen species signal the recruitment of STAT3 to mitochondria

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STAT3, a transcription factor involved in various physiological and pathological processes, is also present in mitochondria. Mitochondrial STAT3 regulates complex I activity and reactive oxygen species (ROS) production, yet the mechanisms governing its translocation to mitochondria remain poorly understood. In this study, we show that rotenone-induced ROS triggers the Ser727 phosphorylation of STAT3 and its increased mitochondrial localisation. Furthermore, we show that STAT3-depleted cells display increased ROS levels during rotenone treatment. Targeted expression in mitochondria of wild-type STAT3 – but not S727A mutant – lowers ROS levels, indicating the importance of Ser727 phosphorylation, both in rotenone-induced mitochondrial targeting and quenching of ROS levels. Together, our results demonstrate a novel STAT3-mediated feedback mechanism to maintain redox homeostasis during stress.

Keywords: mitochondria; oxidative stress; ROS; STAT3

STAT3 is a latent transcription factor which responds to various stimuli, including cytokines and growth factors [1]. Activated STAT3 integrates external stimuli to nuclear gene expression by undergoing post-translational modifications, such as phosphorylation, oxidation, acetylation and methylation [2–5]. The STAT3 C-terminal domain harbours two important phosphorylation sites – Tyr705 and Ser727. Phosphorylation on these two residues is crucial for transcriptional activation and DNA-binding activity of STAT3 in the nucleus [6,7]. STAT3-targeted genes are involved in a diverse array of physiological processes, and aberrant activation of STAT3 often leads to various pathological conditions, such as cancer, compromised immune response and cardiac failure. The complex signaling pathways that are associated with nuclear STAT3 are well studied. However, the discovery of STAT3 pools in other subcellular compartments makes it a much more complicated signaling mechanism.

Mounting evidence suggests that a significant level of STAT3 is present in the mitochondria and regulates its function independent of transcription. Mitochondrial STAT3 (MitoSTAT3) regulates the electron transport chain, thereby ATP production [8]. MitoSTAT3 also supports Ras-dependent cellular transformation [9] and the growth of breast cancer [10]. MitoSTAT3 additionally preserves mitochondrial function during ischemia [11–13] and has been linked to

Abbreviations
ATP, adenosine triphosphate; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; FBS, fetal bovine serum; NAC, *N*-acetyl cysteine; NAD, nicotinamide adenine dinucleotide; PBS, phosphate buffered saline; RIPA, radioimmunoprecipitation assay; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; WT, wild-type.

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Research Paper

Glutathionylated and Fe–S cluster containing hMIA40 (CHCHD4) regulates ROS and mitochondrial complex III and IV activities of the electron transport chain

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ABSTRACT

Human MIA40, an intermembrane space (IMS) import receptor of mitochondria harbors twin CX9C motifs for stability while its CPC motif is known to facilitate the import of IMS bound proteins. Site-directed mutagenesis complemented by MALDI on *in vivo* hMIA40 protein shows that a portion of MIA40 undergoes reversible S-glutathionylation at three cysteines in the twin CX9C motifs and the lone cysteine 4 residue. We find that HEK293T cells expressing hMIA40 mutant defective for glutathionylation are compromised in the activities of complexes III and IV of the Electron Transport Chain (ETC) and enhance Reactive Oxygen Species (ROS) levels. Immunocapture studies show MIA40 interacting with complex III. Interestingly, glutathionylated MIA40 can transfer electrons to cytochrome C directly. However, Fe–S clusters associated with the CPC motif are essential to facilitate the two-electron to one-electron transfer for reducing cytochrome C. These results suggest that hMIA40 undergoes glutathionylation to maintain ROS levels and for optimum function of complexes III and IV of ETC. Our studies shed light on a novel post-translational modification of hMIA40 and its ability to act as a redox switch to regulate the ETC and cellular redox homeostasis.

1. Introduction

The intermembrane space (IMS) of mitochondria is teeming with proteins enriched in disulphide bonds [1]. The disulphide bonds of these proteins enable them to be locked in the IMS. Unlike the proteins bound to the mitochondrial membranes or matrix by dedicated import machinery constituting a plethora of proteins, the IMS proteins are brought in and retained in the IMS by a unique pathway called the disulphide relay pathway [2,3]. The import of the IMS targeted proteins is coupled to their folding and oxidation by the disulphide relay pathway [4,5]. MIA40 (Mitochondrial intermembrane space import and assembly protein 40)/CHCHD4 (coiled-coil-helix-coiled-coil-helix-domain containing 4) and ALR (Augmenter of liver regeneration) are the two important known proteins of this pathway [2,3].

MIA40 is an oxidoreductase that facilitates the import and folding of the IMS targeted proteins by introducing disulphide bonds. ALR (Erv1 in yeast) recycles reduced MIA40 to its oxidized form to initiate another import cycle. The downstream trail of the electrons from ALR reaches the ETC *via* cytochrome C [3,4]. The hydrophobic binding cleft of MIA40 recognizes the substrates to introduce the disulphide bonds and to trigger subsequent folding of the precursor proteins. Additionally, there is a tight coupling between the efficacy of oxidative folding of IMS proteins and their accumulation in the IMS [3,6]. The disulphide relay system prevents the leakage of mature proteins from the IMS [7,8].

MIA40 is a conserved and essential protein. The highly conserved region in human MIA40 (hMIA40) contains six conserved cysteine residues aligned in the order of CPC and two CX9C motifs. The cysteine pair in the CPC motif is implicated in the formation of disulphide bond during the run of the disulphide relay system [9]. At the end of the disulphide relay cycle, ALR, a flavoprotein re-oxidizes the CPC motif of hMIA40 to facilitate a new disulphide relay cycle [10]. The ternary complex formed between a substrate, hMIA40, and ALR is efficient in executing the electron transfer critical for the import, folding, and retention of the substrate i.e., an IMS protein. ALR acts as a redox switch

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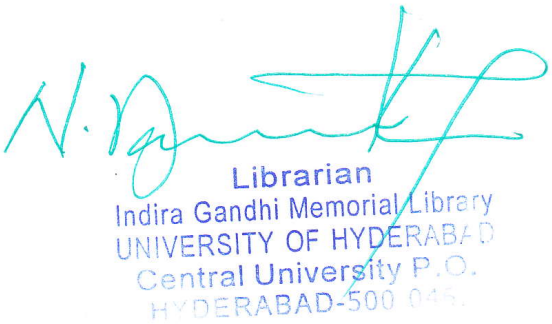
Cysteine mediated interaction of MIA40 with PINK1:implications in PINKI stability, and mitophagy

by Vandana Bisoyi



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