Characterization of mitochondrial localized novel protein Cux1 (p75) and its role in ER stress and mitophagy

A thesis submitted for the degree of

Doctor of Philosophy in Biochemistry

By

Arun Kumar Paripati

(15LBPH03)

Under the supervision of

Prof. Naresh Babu V Sepuri

Department of Biochemistry

School of Life Sciences

University of Hyderabad

Hyderabad, India-500046

Department of Biochemistry,
School of Life Sciences
University of Hyderabad,
Hyderabad-500046, Telangana, India

CERTIFICATE

This is to certify that this thesis entitled **"Characterization of mitochondrial localized novel protein Cux1(p75), and its role in mitochondrial quality control and ER stress"** submitted to the University of Hyderabad by **Mr. Arun Kumar Paripati**, bearing the **Reg. No. 15LBPH03** for the degree of Doctor of Philosophy in Biochemistry, is based on the studies carried out by him 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.

S. V. Naresh B

Prof. Naresh Babu V. Sepuri

Supervisor

NARESH B SEPURI, Ph.D
Professor
Dept. of Biochemistry
School of Life Science
University of Hyderabad
Hyderabad - 500 046.

Head

Department of Biochemistry

HEAD
Dept. of Biochemistry
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046.

Dean

School of Life Sciences

DEAN
School of Life Sciences
University of Hyderabad
Hyderabad-500 046.

Department of Biochemistry,
School of Life Sciences
University of Hyderabad,
Hyderabad-500046, Telangana, India

DECLARATION

I, Arun Kumar Paripati, hereby declare that the work presented in this thesis entitled **"Characterization of mitochondrial localized novel protein Cux1(p75), and its role in mitochondrial quality control and ER stress"** 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 degree or diploma from any other University or Institution.

S.V. Naresh Babu

Prof. Naresh Babu V Sepuri

Supervisor

NARESH B SEPURI, Ph.D
Professor
Dept. of Biochemistry
School of Life Science
University of Hyderabad
Hyderabad - 500 046.

Arun Kumar Paripati

Student

Date:

Place: **Department of Biochemistry,**

University of Hyderabad - 500046

Department of Biochemistry, School of Life Sciences,
University of Hyderabad,
Hyderabad-500046, Telangana, India

CERTIFICATE

This is to certify that the thesis entitled **"Characterization of mitochondrial localized novel protein Cux1(p75), and its role in mitochondrial quality control and ER stress"** submitted by Mr. Arun Kumar Paripati bearing **Reg. No.15LBPH03**, in partial fulfilment of the requirements for the Doctor of Philosophy in Biochemistry, is genuine work done by him under my supervision.

This thesis is free of plagiarism and has not previously been submitted in part or whole for the award of 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. Mattam, U., N. K. Talari, **A. K. Paripati**, T. Krishnamoorthy and N. B. V. Sepuri (2021). "Kisspeptin preserves mitochondrial function by inducing mitophagy and autophagy in aging rat brain hippocampus and human neuronal cell line." Biochim Biophys Acta Mol Cell Res 1868(1): 118852.
2. Karri, S., S. Singh, **A. K. Paripati**, A. Marada, T. Krishnamoorthy, L. Guruprasad, D. Balasubramanian and N. B. V. Sepuri (2019). "Adaptation of Mge1 to oxidative stress by local unfolding and altered Interaction with mitochondrial Hsp70 and Mxr2." Mitochondrion 46: 140-148.
3. Sepuri, N. B. V., P. Tammineni, F. Mohammed and **A. Paripati** (2017). "Nuclear Transcription Factors in the Mitochondria: A New Paradigm in Fine-Tuning Mitochondrial Metabolism." Handb Exp Pharmacol 240: 3-20.

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

1. Presented a poster entitled **"Role of Mge1 in chaperone-mediated autophagy"** in **"International Congress of Cell Biology (ICCB)"** held on 27th to 31st January 2018, organized by Centre for Cellular and Molecular biology, Hyderabad.
2. Presented a poster entitled **"Role of Mge1 in chaperone-mediated autophagy"** in **90th Annual Meeting of SBC(I) "Metabolism to Drug Discovery: Where Chemistry and Biology Unite"** organized in virtual mode during 16th to 19th December 2021 by Amity Institute of Biotechnology and Amity Institute of Integrative Sciences and Health, Amity University, Haryana (AUH), Gurugram.
3. Participated in **XI International Conference on Biology of Yeasts and Filamentous Fungi** held on 27 - 29 November 2019, organized by University of Hyderabad and Centre for DNA Fingerprinting and Diagnostics, Hyderabad.
4. Participated in the **10th Conference on Yeast Biology** organized by School of Life Sciences, Jawaharlal Nehru University, New Delhi & Amity University, Gurugram, during February 8-11, 2018.

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

Course Code	Name	Credits	Pass/Fail
BC801	Analytical Techniques	4	Pass
BC802	Research ethics, Data Analysis and Biostatistics	3	Pass
BC803	Lab Seminar and Record	3	Pass

Supervisor

NARESH B SEPURI, Ph.D
Professor
Dept. of Biochemistry
School of Life Science
University of Hyderabad
Hyderabad - 500 046.

Head

Department of Biochemistry

HEAD
Dept. of Biochemistry
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046.

Dean

School of Life Sciences

DEAN
School of Life Sciences
University of Hyderabad
Hyderabad-500 046.

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Arun Kumar Paripati

Table of Contents

Abbreviations

ANT	Adenine nucleotide translocase
ATF	Activating transcription factor
ATG	Autophagy-related protein
ATP	Adenosine triphosphate
BNIP	BCL2 interacting protein
BN-PAGE	Blue native PAGE
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CDK	Cyclin-dependent kinase
CUX1	Cut Like Homeobox 1
DAPI	4’ 6-diamidino-2phenylindole
DRP1	Dynamin-related protein 1
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ER	Endoplasmic reticulum
ETC	Electron transport chain
FADH	Flavin adenine dinucleotide
FBS	Fetal Bovine Serum
FIS1	Fission 1
FUNDC1	FUN14 Domain-Containing Protein 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRIM19	Genes associated with Retinoid–IFN-induced Mortality-19
GRP	Glucose-regulated protein

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTRA2	High-Temperature Requirement Protein A2
IMM	Inner mitochondrial membranes
IMS	Intermembrane space
KCl	Potassium chloride
KOH	Potassium hydroxide
MAM	Mitochondria-associated endoplasmic reticulum membrane
MCU	Mitochondrial calcium uniporter
MFF	Mitochondrial
MFN	Mitofusin
MgCl2	Magnesium Chloride
MIA	Mitochondrial Intermembrane Space Import and Assembly Protein
Mid	Mitochondrial Dynamics Protein
MARCH5	Membrane Associated Ring-CH-Type Finger 5
ml	Milliliter
mm	Millimeter
NADH	Nicotinamide adenine dinucleotide
NRF2	NF-E2-Related Factor 2
OMA1	Overlapping Activity With M-AAA Protease
OPA1	Optic Atrophy 1
PARP	Poly (ADP-Ribose) Polymerase 1
PBS	Phosphate Buffered Saline
PHB2	Prohibitin 2
PINK1	PTEN Induced Kinase 1

PVDF	Polyvinylidene fluoride
RIPA	Radio immunoprecipitation assay
ROS	Reactive oxygen species
rpm	Rotations per minute
SAM50	Sorting and Assembly Machinery Component 50
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TFAM	Transcription Factor A, Mitochondrial
TG	Thapsigargin
TIM	Translocase of Inner Membrane
TMHMM	Tied Mixture Hidden Markov Model
TN	Tunicamycin
TOM	Translocase of Outer Membrane
TRIS	Tris(hydroxymethyl)aminomethane
VDAC	Voltage-dependent anion channel
µg	Microgram
µl	Microliter
µM	Micro mole

Chapter 1

Introduction

1.1 Mitochondria

The history of mitochondria started nearly three decades ago. In the quest to mine architecture inside the cell, around the 1840s, the structures representing mitochondria were recorded. Later on, around 1890, Altman noticed the ubiquitous structures present in the cell, and he called them "Bioblasts," which were thought to be the elementary organisms present inside for requisite functions. The term mitochondria were given by Benda, in Greek, Mitos (Thread) and Chondros (Granule). Researchers have paid more attention to mitochondria in the last three decades and explored their vital functions in eukaryotic cells.

Mitochondria are the imperative organelles in all eukaryotic cells. Mitochondria are double-membrane, thread-like structures that reside in the cytoplasm. The skeletal structure of the mitochondria is divided into four parts: outer mitochondrial membrane (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM), and matrix (Figure 1.1). Each compartment has a different composition and function.

1.1.1 Outer Mitochondrial Membrane

The outer mitochondrial membrane is the smooth surface that is the outermost layer of the mitochondria. It separates the inside of mitochondria from the cytosol. The outer membrane acts as a doorman for the entry of proteins and ions inside the mitochondria. VDAC, or porin, is the nineteen-beta barrel outer membrane protein, one of OMM's major proteins. It is an access gate for the metabolites and ions inside the mitochondria. The translocase of the outer mitochondrial membrane (TOM) complex acts as a gatekeeper for the entry of proteins into mitochondria. The TOM complex is composed of different subunits; out of them, TOMM40 forms a central pore for the entry of proteins. TOMM20 and TOMM70 are the tail-anchored proteins that recognize the localization sequences within the protein and en route towards the TOM pore for entry into mitochondria (Wiedemann and Pfanner 2017). The outer membrane can decide the cell's fate by recruiting apoptotic proteins Bax and Bad under death stimuli (Bock and Tait 2020). It is also the site for the proteins that regulate mitochondrial dynamics and inter-organelle interaction (Xian and Liou 2021).

1.1.2 Inter-Membrane Space

The space between the outer and inner membrane is the intermembrane space. It is crucial for the folding and recruitment of inner membrane proteins. It provides an oxidative environment for

cysteine-rich proteins folded by the disulfide relay system. MIA40 in IMS acts as a chaperone and does di sulfide bond formation by extracting electrons from the SH group of the cysteine-rich substrate (Stojanovski, Bragoszewski et al. 2012). The reduced MIA40 transfers electrons via Erv1 or directly to complex III. This is the major site for the proton gradient.

1.1.3 Inner Membrane

The inner membrane is heavily invaginated. These invaginations in the membrane (cristae) provide greater space for the electron transport chain (ETC) complex. The inner membrane is mainly home to the closely packed OXPHOS protein complexes. Nearly 80% of the mass consists of protein in the inner membrane. The inner membrane functions as a barrier for the movement protons into the matrix, results in the proton gradient across the inner membrane. The protein complexes that are involved in energy generation in the inner membrane are NADH dehydrogenase (complex I), receiver of electrons from the NADH; succinate dehydrogenase (complex II), receiver of electrons from the FADH2 and complex I; ubiquinol–cytochrome c oxidoreductase (complex III) transfers electrons from ubiquinol to cytochrome c; cytochrome c oxidase (complex IV) transfers electrons to the oxygen and forms water molecule; ATP synthase complex (complex V) generates ATP in the mitochondrial matrix by allowing the movment of protons from inter membrane space to matrix

1.1.4 Matrix

The matrix is the innermost compartment of the mitochondria. It is the site for many biochemical reactions including, TCA cycle, beta-oxidation of fatty acids, and Fe-S cluster biosynthesis; it acts as a reservoir for the cellular energy, ATP. Besides these functions, the matrix also contains mitochondrial DNA, which encodes a few subunit proteins of ETC.

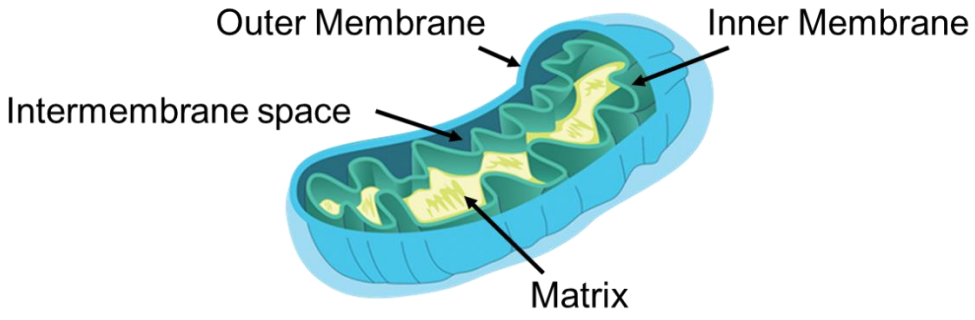

Figure 1.1: The architecture of Mitochondria. Mitochondria are double membrane organelles compartmentalized into the outermost layer of the outer membrane, an invaginated inner membrane, intermembrane space, and the innermost matrix.

1.2 Functions of Mitochondria

Mitochondria are the hub for many cellular functions other than being a source of cellular fuel, including cellular signaling, cell death regulation, Fe-S cluster biosynthesis, and a source for nucleotide and amino acid biosynthesis precursors (Figure 1.2).

1.2.1 Mitochondria in Energy Generation

ATP is the energy fuel of living cells. A significant portion of the cellular ATP is generated from mitochondria by the oxidative phosphorylation of ADP in the mitochondrial matrix. Mitochondria use carbohydrates, fatty acids, and amino acid catalysis by-products as raw materials and generate ATP as a product. Pyruvate, the end product of glycolysis (catabolism of glucose), moves into the mitochondrial matrix, further enters into the TCA cycle, and generates the fuel-generating precursors such as NADH and $FADH_2$ (Walsh, Tu et al. 2018). The mitochondrial matrix is also a major site for fatty acid oxidation. Acetyl-CoA, the terminal product of the fatty acid oxidation enters into the TCA cycle and generates NADH and $FADH_2$. Further, these two products enter the electron transport chain to generate the energy (Ahn and Metallo 2015). The active movement of electrons in the electron transport chain facilitates the transfer of H^+ ions from the matrix to intermembranous space, resulting in the formation of a proton gradient. Protons return to the matrix via the ATP synthase complex to produce ATP. The minute amount of ATP comes from amino acid-derived intermediates such as alpha-ketoglutarate, succinyl CoA, and oxaloacetate (Wei, Liu et al. 2020).

1.2.2 Mitochondria in Cell Signaling

Apart from playing a central role in ATP biogenesis, mitochondria also play an essential role in various cellular communication pathways. Mitochondria regulate metabolic and cell death signaling by altering cellular ATP and ROS levels. Recent research shows that the mitochondrial-associated membrane (MAM) is the main site for the formation of autophagosomes (Nakatogawa 2020). Mitochondria also play crucial role in activation of INF inducing pathway and mitophagy (Refolo, Vescovo et al. 2020). Also, the damaged mitochondria elicit the signals for NLRP3

recruitment and assembly, which eventually triggers inflammatory cell death (Zhou, Yazdi et al. 2011).

1.2.3 Mitochondria in Cell Death Regulation

Mitochondria also play an administrative role in cell death pathways. Pro-apoptotic (Bax, Bad, etc.) and anti-apoptotic (Bcl-2) proteins are recruited onto the outer mitochondrial membrane. In response to death-related signals, they induce the release of cytochrome c into the cytoplasm. (Tait and Green 2013). Mitochondria are also linked to pyroptosis (Rogers, Erkes et al. 2019) through Gasdermin D (GSDMD), ferroptosis (Gao, Yi et al. 2019), and initiates necrotic cell death via excess ROS production (Redza-Dutordoir and Averill-Bates 2016).

1.2.4 Mitochondria in Biosynthetic Pathways

Mitochondria supply critical building blocks for different biosynthetic pathways. Mitochondria supply purines and pyrimidines for nucleotide biosynthesis and various intermediates like citrulline, glutamate, and aspartate for amino acid synthesis. Acetyl CoA and malonyl CoA are precursors for fatty acid, cholesterol biosynthesis, and gluconeogenesis (Spinelli and Haigis 2018).

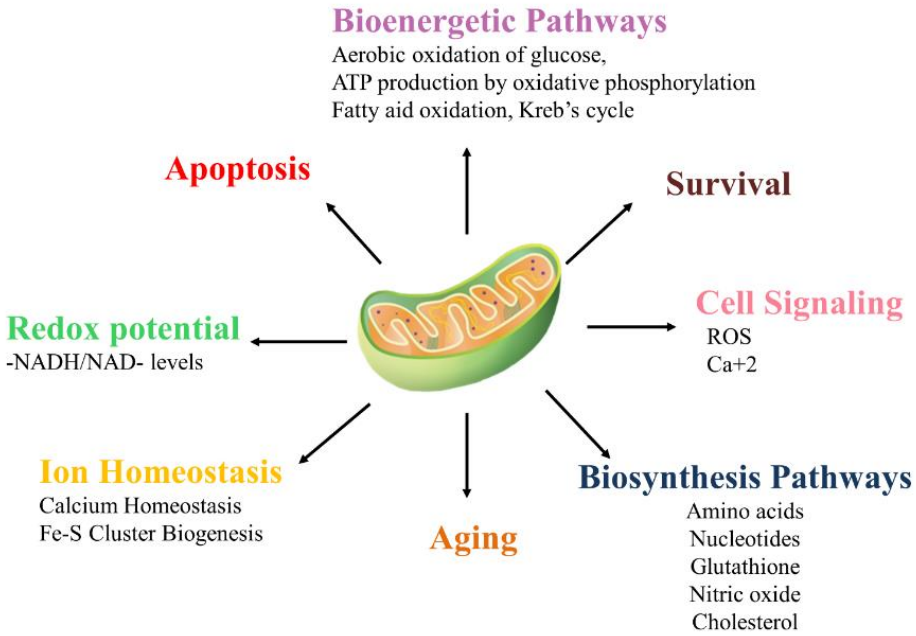

Figure 1.2: Functions of Mitochondria. Mitochondria function in different cellular processes, including biosynthetic pathways (ATP, nucleotides, amino acids, glutathione, nitric oxide, and cholesterol), cellular signaling (ROS and Ca^{+2}), iron homeostasis, apoptosis, and aging.

1.3 Mitochondrial Quality Control

Mitochondria are specialized double-membrane organelles that are involved in a plethora of cellular functions such as ATP generation, fatty acid metabolism, nucleotide biosynthesis, regulation of cell death, and calcium signaling. Because it performs critical cellular processes, cells employ various safeguard mechanisms depending on the degree of damage. Among them, the major quality control mechanisms are through anti-oxidant enzymes, mitochondrial proteases, mitochondrial dynamics, mitochondrial-derived vesicles, and mitophagy (Figure 1.3).

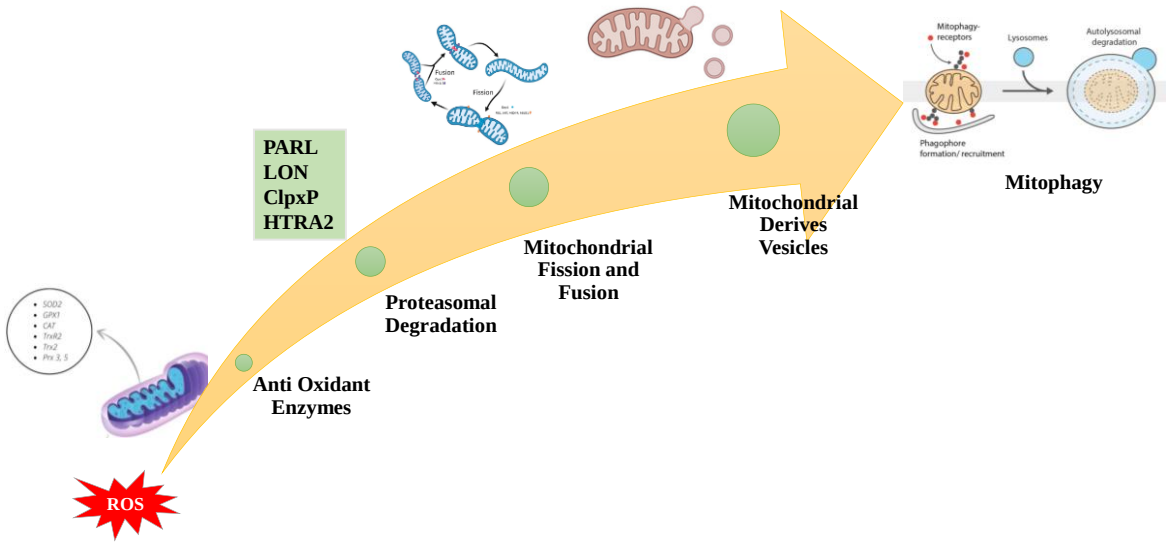

Figure: 1.3: Different levels of Mitochondrial quality control mechanisms. First, the repair of oxidized proteins by antioxidant enzymes and chaperones. Second, proteasomal degradation of irreversibly damaged proteins. Third, mitochondrial fission and fusion. Fourth, pinching off the damaged portion by mitochondrial derived vesicles (MDVs) and last, mitophagy.

1.3.1 Anti-oxidant enzymes and chaperones

An imbalance in the mitochondrial proteome or an insult with toxins leads to ROS accumulation in the mitochondrial matrix. ROS damages the proteins by oxidizing them. Cysteine and methionine are the primary victims of ROS-mediated oxidation of proteins. Reverting these oxidized proteins into reduced form is essential to restore their function. Antioxidant enzymes like superoxide dismutase, glutathione peroxidase, thioredoxin reductase, and methionine-sulfoxide reductase act as the first defense against ROS-mediated damage to the proteins by removing various types of ROS and by reversing the oxidized proteins into reduced form (Napolitano, Fasciolo et al. 2021). Moreover, the Hsp70 and Hsp60 chaperones bind to the damaged unfolded proteins and refold them into their functional form (Jadiya and Tomar 2020).

1.3.2 Mitochondrial Proteases

Mitochondrial proteases are an integral part of the mitochondrial quality control mechanism. Around 45 mito proteases have been identified in mammals that localize to the OMM, IMM, IMS, and matrix to ensure protein quality in each compartment (Quiros, Langer et al. 2015). They are involved in critical regulatory processes such as protein trafficking, activation, processing, and turnover (Deshwal, Fiedler et al. 2020).

Membrane proteins are synthesized in the cytosol and destined to different organelles, including peroxisomes, endoplasmic reticulum, lysosomes, plasma membrane, and mitochondria. Msp1 degrades the mistargeted tail-anchored proteins on OMM in yeast and ATAD1 in humans (Chen, Umanah et al. 2014). Mislocalized proteins don’t match the dimensions of the OMM. Consequently, the Msp1 hexameric complex recognizes the hydrophobic patches, extracts them from the membrane, and transports them toward the ER for ubiquitination by Doa1 (Matsumoto, Nakatsukasa et al. 2019). Subsequently, Cdc48 degrades the ubiquitinated protein in the cytosol. Another report shows that misfolded proteins on the OMM are ubiquitinated by MITOL (MARCH 5) and then broken down by proteasomal machinery (Nagashima, Tokuyama et al. 2014).

The inner membrane is protein-rich, with metabolite carriers and respiratory complexes involved in ATP synthesis. The catalytic domain of the i-AAA proteases (YEM1L, OMA1, and ATP23) faces toward the IMS. YME1L degrades the TIM23 complex under stress conditions to regulate protein import into mitochondria (Song, Herrmann et al. 2021). OMA1 and YME1L control the

mitochondrial dynamics by proteolysis of OPA1 (Anand, Wai et al. 2014). ATP23 regulates the phospholipid composition and electron transport chain assembly (Deshwal, Fiedler et al. 2020). HTRA2 is involved in protein quality control in the IMS region apart from cell death regulation (Verhagen, Silke et al. 2002).

LON and CLPXP are the major proteases that maintain the matrix proteome and misfolded protein degradation. CLPXP regulates 28S ribosome assembly by proteolysis of ERLA1, which involves 12S RNA stability (Szczepanowska, Maiti et al. 2016). Besides degrading misfolded proteins, LON protease degrades phosphorylated complex IV subunits under oxygen deprivation and myocardial ischemia (Sepuri, Angireddy et al. 2017). LON modulates the mitochondrial DNA content by regulating the turnover of TFAM (Matsushima, Goto et al. 2010).

1.3.3 Mitochondrial dynamics

Depending on cellular energy needs and to adapt to changing cellular conditions, mitochondria undergoes continuous fusion and fission events. The cell undergoes fission and fusion events to minimize the adverse effect of the damaged portion of the mitochondria.

Mitochondrial fusion occurs in both the outer and inner membranes. Mfn1 and Mfn2 are the two primary dynamin-related GTPase proteins involved in mitochondrial outer membrane fusion. Homo or hetero-oligomers of Mfn1 and Mfn2 initiate the fusion events (Naon, Zaninello et al. 2016). OPA1 is the inner mitochondrial dynamin-related GTPase involved in inner membrane fusion. Yme1 cleavage generates two OPA1 isoforms, L-OPA1 in the inner membrane, whereas S-OPA1 resides in the intermembranous space (Anand, Wai et al. 2014). L-OPA1 prevents mitochondrial fusion, resulting in mitochondrial fragmentation under depolarization.

1.3.4 Mitochondrial-derived vesicles (MDV)

One of the recently discovered mitochondrial quality control mechanisms is mitochondrial-derived vesicles (MDVs), where the damaged portion of mitochondria is pinched off and targeted towards the lysosomes or late endosomes for degradation. Recent findings suggest that MDVs enriched in phosphatidic acid are formed by the Miro1/2 dependent formation of protrusions, followed by Drp1 recruitment, leads to the scission of MDVs (Konig, Nolte et al. 2021). PINK1 and Parkin were shown to involve in MDV formation under oxidative stress (McLelland, Soubannier et al. 2014), and these are equipped with Syntaxin17 to fuse with the lysosome

(McLelland, Lee et al. 2016). Recently, TOMM20 enriched MDV proteomic analysis revealed selective protein degradation of the TOM complex by targeting cargo to lysosomes.

1.3.5 Mitophagy

Autophagy is an evolutionary conserved process where double membrane autophagosomes packed with cytosolic cargo fuses with lysosomes for degradation. Quality control of mitochondria achieved by autophagy is called mitophagy, by which the damaged mitochondria are targeted for degradation (Lemasters 2005). Mitophagy is crucial for maintaining mitochondrial quality in response to environmental cues such as nutrient deprivation, oxidative stress, hypoxia, oocyte maturation, the pluripotency of stem cells, and exposure to toxins (Ng, Wai et al. 2021). Dysregulation in mitophagy leads to neurological disorders like Huntington's disease (Khalil, El Fissi et al. 2015), Alzheimer's disease (Ye, Sun et al. 2015), Parkinson's disease (Kitada, Asakawa et al. 1998, Valente, Abou-Sleiman et al. 2004), and cancer (Bernardini, Lazarou et al. 2017). Mitophagy in mammals is classified into two types based on the presence of the PINK1 and the Parkin (E3 ubiquitin ligase) (Figure 1.4).

1.3.5 a. PINK1/Parkin-dependent mitophagy

In a healthy state, PINK1 gets imported into the mitochondria via the TOM/TIM complex and simultaneously cleaved by PARL protease in the IMS (Jin, Lazarou et al. 2010). Subsequently, PINK1 translocate back into the cytosol and undergoes degradation by the N-end rule pathway (Yamano and Youle 2013). In contrary, upon depolarization of mitochondria, PINK1 gets stabilized on outer mitochondrial membrane and forms the PINK1-TOM complex (Lazarou, Jin et al. 2012). Stabilized PINK1 phosphorylates Mfn2, and this leads to the recruitment of E3 ubiquitin ligase Parkin and ubiquitinates OMM proteins. Further, PINK1 phosphorylates ubiquitin chains to recruit additional Parkin to mitochondria (Lazarou, Jin et al. 2012). Formation of these phospho-ubiquitin chains results in the recruitment of autophagy receptors such as optineurin (Wong and Holzbaur 2014) and NDP52 (Lazarou, Sliter et al. 2015) to the surface of mitochondria, which is eventually engulfed by an autophagosome.

1.3.5 b. PINK1/Parkin-independent mitophagy

In mammals, PINK1/Parkin-independent mitophagy is triggered by the receptor on the OMM such as BNIP3 (Quinsay, Thomas et al. 2010), BNIP3L (Sandoval, Thiagarajan et al. 2008),

BCL2-L13 (Murakawa, Yamaguchi et al. 2015), FUNDC1 (Chen, Chen et al. 2016), and FKBP8 (Bhujabal, Birgisdottir et al. 2017). Under hypoxia and iron depletion, BNIP3 and BNIP3L are upregulated by the stabilized HIF1 (Bellot, Garcia-Medina et al. 2009). Affinity of mitophagy receptors (FUNDC1, BNIP3, and BNIP3L) towards ATG8 proteins is regulated by their phosphorylation at the LIR domain. Upon rupturing the outer mitochondrial membrane, inner membrane protein PHB2 (Wei, Chiang et al. 2017) and inner membrane enriched cardiolipin can interact directly with LC3 and act as mitophagy receptors (Chu, Ji et al. 2013).

In yeast, ATG32 acts as a mitophagy receptor and interacts with other autophagy related genes, ATG8 and ATG11 to initiate the mitophagy (Okamoto, Kondo-Okamoto et al. 2009). Yme1 is known to regulate mitophagy by proteolytic cleavage of ATG32 (Wang, Jin et al. 2013).

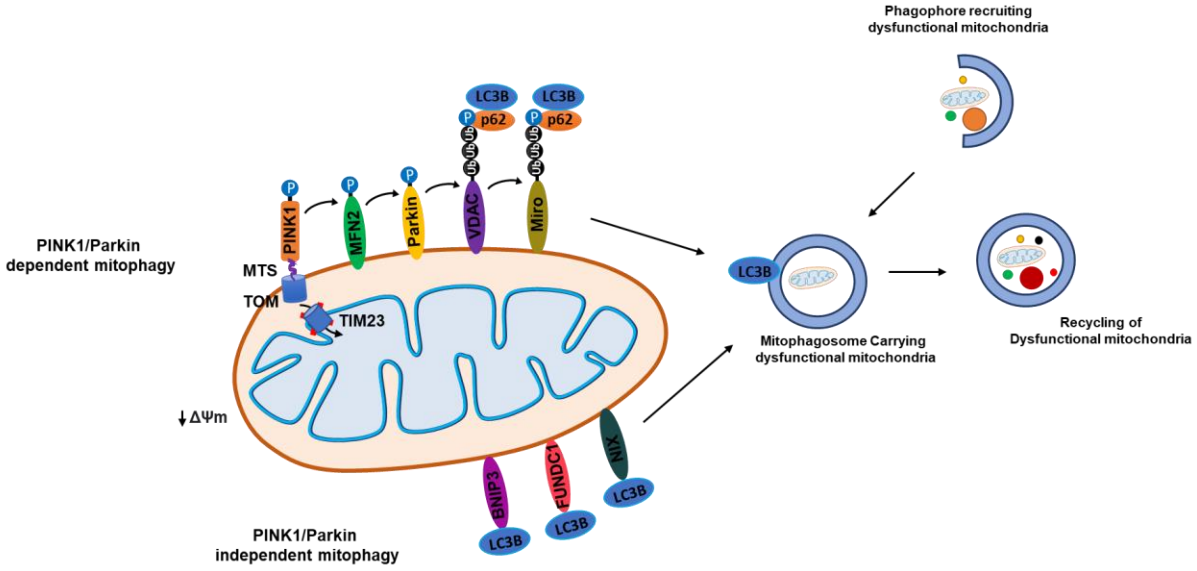

Figure 1.4: PINK1/Parkin dependent and independent mitophagy. In the PINK1/Parkin dependent mitophagy, when mitochondria is in healthy state, PINK1 is imported into mitochondria via TOM/TIM complex and is processed by matrix metalloprotease (MMP) and PARL, then retro-translocates into the cytosol for degradation. In unhealthy mitochondria, PINK1 gets stabilized on the outer mitochondrial membrane (OMM) and forms PINK1/TOM complex. After stabilization, PINK1 phosphorylates Mitofusin 2 (Mfn2), which acts as a docking site for the recruitment of Parkin to the OMM. Parkin ubiquitinates several OMM proteins, including VDAC and Miro, which leads to the recruitment of p62 to the OMM. Finally, p62 interacts with LC3B and

triggers the formation of mitophagosome. In PINK1/Parkin-independent pathway, mitophagy receptors FUNDC1, NIX, and BNIP3 interacts directly with LC3B.

1.4 Inter-Organelle Communication

Eukaryotic cells are composed of different intracellular organelles with defined functions, and they are interdependent to sustain intracellular and extracellular changes. Recent reports suggest extensive inter-organelle communication between the organelles through the membrane contact sites. The evidence suggests that every organelle interacts with the other organelles, such as ER-nucleus, ER-mitochondria, Mitochondria-lysosome, Mitochondria-lipid droplets, etc. (Petkovic, O'Brien et al. 2021).

Mitochondria communicate by forming a tethering complex with lysosomes, lipid droplets, and endoplasmic reticulum. Out of these, the most studied one is the ER-mitochondrial interaction. ER-mitochondria contact sites (ERMCSs) are dynamic in nature and help keep close association within a 10–30 nm distance. In the last few decades, different pairs of protein complexes that link the ER to the mitochondria have been found (Figure 1.5).

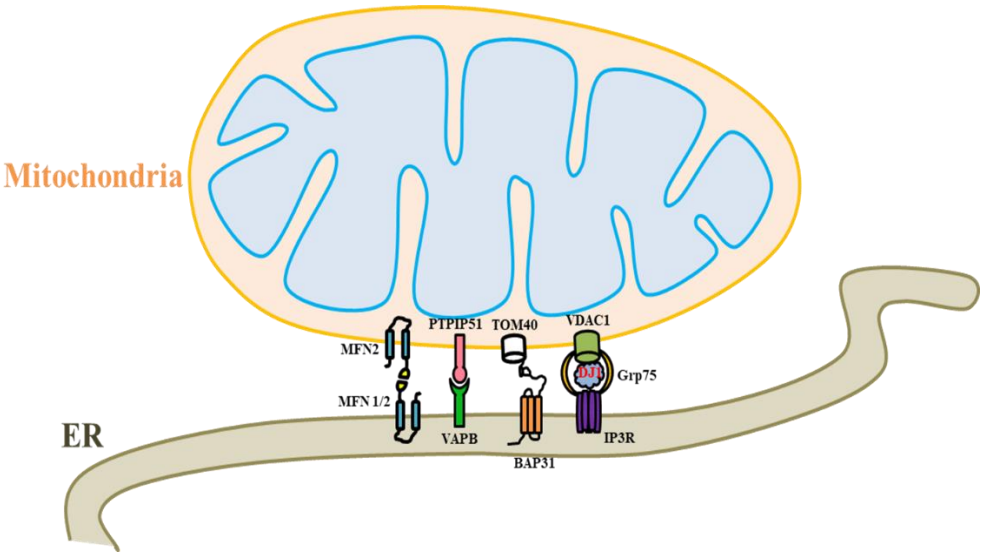

Figure 1.5. Different tether proteins mediate the formation of the ERMCS complex. BAP31 interacts with TOMM40 and is known for regulating cell death and mitochondrial functions. This complex is involved in transporting calcium (Ca^{2+}) to the mitochondria. Mfn2 can homodimerize with itself or heterodimerize with Mfn1 to form ERMCS, which is known to play a role in UPR in

ER. Lastly, VAPB, an ER protein directly engages with the OMM resident PTPIP51 to form ERMCS.

1.4.1 Endoplasmic reticulum and mitochondrial contact sites (ERMCSs)

1.4.1 a. IP3R–DJ-1–Grp75–VDAC Complex

The ER protein inositol 1,4,5-triphosphate receptor (IP3R) and the mitochondrial outer membrane protein VDAC1 tethering complex is the first known ERMCS. Grp75 mediates this interaction. Later, it was found that DJ1 also acts as a bridge between VDAC1 and IP3R (Szabadkai, Bianchi et al. 2006, Basso, Marchesan et al. 2020). This tetrameric complex regulates the calcium transfer from ER to the mitochondria. Interestingly, getting rid of IP3R doesn't change the ERMCS completely. However, loss of Grp75 and DJ1 causes a calcium imbalance in the mitochondria (Csordas, Renken et al. 2006).

1.4.1 b. Mitofusins

Mitofusin-2 is the well-studied ERMCS tether, yet it is controversial. Mfn2 is mainly associated with mitochondrial fusion. However, it is also localized to the endoplasmic reticulum and mediates the ER-mitochondrial interaction by forming homo or hetero dimers with Mfn1 on OMM. The initial report of Mfn2 mediated ER-mitochondrial tether was reported by Scorrano group (de Brito and Scorrano 2008). In contrast, Mfn2 knockdown increased the ERMCS and calcium transfer to mitochondria (Leal, Schreiner et al. 2016). Most recently, studies that used split fluorescent protein found that Mfn2 is important for making ER-mitochondrial contacts (Naon, Zaninello et al. 2016).

1.4.1 c. VAPB and PTPIP51

Protein tyrosine phosphatase interacting protein-51 (PTPIP51) is an OMM-residing protein that interacts with an ER protein vesicle-associated membrane protein-associated protein B (VAPB) (De Vos, Morotz et al. 2012). Loss of these proteins affects the portion of mitochondrial contact with ER, thus resulting in changes in ERMCS formation. Loss in either of these proteins results in disturbed calcium handling or delayed calcium transfer to mitochondria. Loss of VAPB is associated with mitochondrial aggregation (Stoica, De Vos et al. 2014, Stoica, Paillusson et al. 2016).

1.4.1 d. BAP31

ER-localized protein B-cell receptor-associated protein 31 (BAP31) interacts with Fis1 (Mitochondrial fission 1), which transmits the apoptotic signals from mitochondria to the ER (Iwasawa, Mahul-Mellier et al. 2011). In addition, recently, BAP31 was reported to regulate autophagy and mitochondrial homeostasis by interacting with TOMM40 (Namba 2019). Together, these results suggest that BAP31 acts as an ERMCS tether and transmits apoptotic signals from the mitochondria to the ER.

1.4.2 ERMCS Functions and Pathophysiology

ERMCS is vital for the lipid exchange between the mitochondria and endoplasmic reticulum. The conversion of phosphatidic acid (PA) to phosphatidylserine (PS) takes place in the ER. PS is transferred to the mitochondrial intermembrane space by the ERMCS complex for the synthesis of phosphatidylethanolamine (PE). The synthesized PE shuttles back from the mitochondria to the ER through ERMCS (Rowland and Voeltz 2012).

Low cytosolic calcium is important for Ca^{2+} signaling. Calcium exits the ER through IP3R and enters the mitochondrial matrix via VDAC1 and MCU (Giacomello, Drago et al. 2010, Baughman, Perocchi et al. 2011). Mitochondria act as a hotspot for calcium, and it is required for matrix-localized metabolic enzymes, isocitrate dehydrogenase, oxoglutarate dehydrogenase, and pyruvate dehydrogenase (Denton 2009). Apart from this, ERMCS also decides the site of mitochondrial fission by marking the sites for Drp1 recruitment and fusion by direct involvement of Mfn1 and Mfn2 (Wilson and Metzakopian 2021). Mitochondrial autophagy is also linked to the ERMCS complex, and pre-autophagosome markers ATG5 and ATG14L localize to the ERMCS complex for autophagosome biogenesis (Bravo, Vicencio et al. 2011, Hamasaki, Furuta et al. 2013). It has been shown that UPR in ER raises Ca^{2+} levels in mitochondria by improving ERMCS to speed up the production of ATP for the ER chaperones. Several neurological diseases have been linked to changes in ERMCS genes or the loss of the ERMCS complex (Wilson and Metzakopian 2021).

1.5 ER Stress-Induced Cell Death

The synthesis of membrane and secretory proteins takes place in the endoplasmic reticulum. It also involves different functions, including cholesterol and phospholipid biosynthesis and Ca^{2+} buffering. Disturbance of many homeotic processes causes the deposition of unfolding proteins in the ER (ER stress). ER stress turns on the unfolded protein response (UPR) signaling pathway to make more chaperones and stop general protein synthesis (Bravo, Parra et al. 2013, Hetz, Chevet et al. 2015) or to break down unfolded proteins through autophagy or ER-associated degradation. Three UPR sensors are orchestrated in the ER membrane and bind with Grp78/BiP in normal conditions (Figure 1.6). BiP gets released during ER UPR from sensors and engages with unfolded proteins. Freed sensors are autoactivated by transphosphorylation and trigger the signals to resolve UPR (Tabas and Ron 2011). All three pathways are known to lead to different levels of cell death caused by unresolved ER stress.

First, inositol-requiring enzyme 1 (IRE1) is a multidomain protein kinase with RNAse activity. IRE1 inhibits the protein synthesis by degrading the mRNA; this process is called IRE1-induced mRNA decay (RIDD) (Hollien and Weissman 2006). Under minimal activation conditions, IRE1 degrades specific mRNAs. Typically, X box-binding protein 1 (XBP1) is transcribed in its unspliced, untranslated form (uXBP1). IRE1 activation removes the intron in uXBP1 and turns it into the spliced form sXBP1 (Cawley, Deegan et al. 2011). This sXBP1 not only acts as a marker of UPR but also activates several genes to restore the ER folding capacity (Acosta-Alvear, Zhou et al. 2007). IRE1 can trigger the apoptotic pathway via activation of ASK1 and JNK1 (Urano, Wang et al. 2000). Under ER stress, JNK can activate apoptotic pathways and necrotic cell death (Saveljeva, Mc Laughlin et al. 2015).

Second, PERK phosphorylates eukaryotic translation initiation factor 2a (eIF2a) to attenuate the translation. mRNA with IRES (internal ribosomal entry site) and ATF4 can translate under these conditions (Harding, Zhang et al. 2003, Han, Back et al. 2013). ATF4 stimulates CHOP expression, which contributes to ER stress-induced cell death. Primary hippocampal neurons under hypoxia and pancreatic cell loss in the diabetic model are mediated by CHOP (Oyadomari, Koizumi et al. 2002, Tajiri, Oyadomari et al. 2004). CHOP and ATF4 are known to form dimers and increase the protein synthesis of UPR genes, which leads to cell death (Han, Back et al. 2013).

Third, ATF6 mediates cell death by increasing the expression of CHOP. CHOP activates Bim transcriptionally while suppressing Bcl-2 expression (Iurlaro and Munoz-Pinedo 2016).

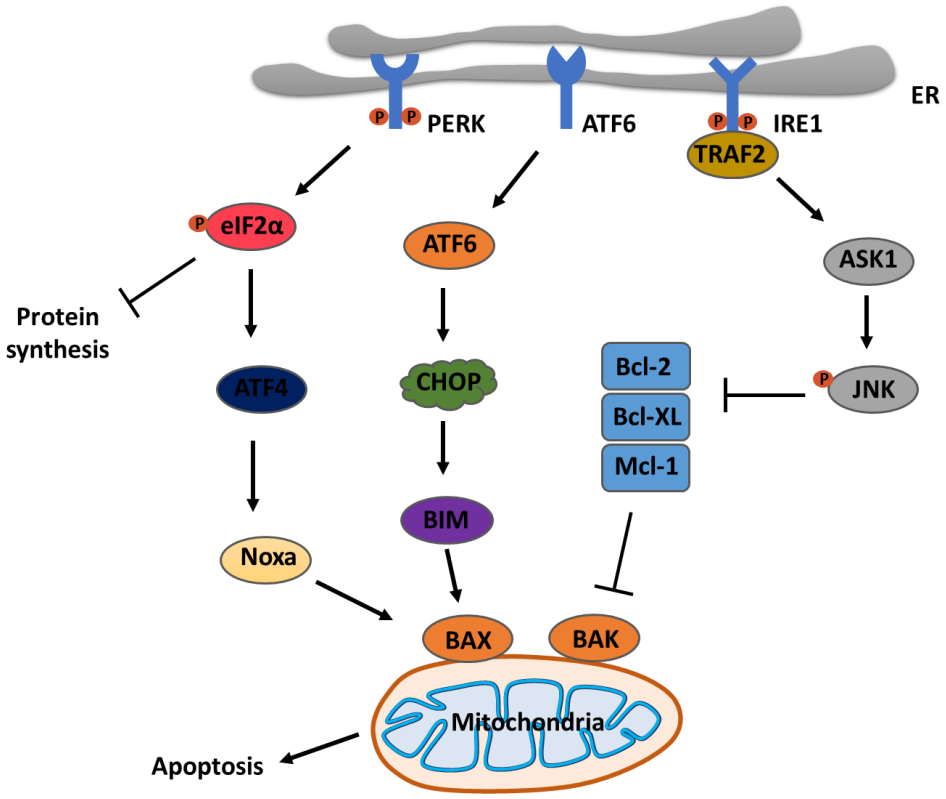

Figure 1.6. Pathways of ER stress-induced cell death. Under ER stress, PERK induces the cell death by activation of ATF4, CHOP, and Noxa. ATF6 can induce the apoptosis via CHOP and Bax. IRE1 activates the apoptosis by JNK mediated inactivation of Bcl-2 and Bcl-xL.

1.6 Cux1

Cux1 was previously named CDD (CCAAT displacement protein). also called "Cux1" or "CUTL1". Cux1 is a homeodomain transcription factor that was discovered to be a transcription repressor for the sea urchin sperm H2B gene (Liu, Sun et al. 2020). It is reported as a transcriptional activator and a repressor in a promoter-dependent manner. Cux1 regulates wide variety of functions including cell proliferation, differentiation, and migration. Additionally, Cux1 is also reported to have a role in tumorigenesis.

1.6.1 Cux1 Structure and Isoforms

The largest isoform of Cux1 contains DNA-binding domain, CUT repeats and one homeodomain. In addition, it also possesses a couple of repression domains at the C terminus and one auto-inhibitory domain at the N terminus (Figure 1.7). CUX1 exists in different isoforms either by the alternate splicing or proteolytic cleavage of the full-length protein. These isoforms are named according to their molecular weight from p200 (full length) to the smallest isoform p75 (Hulea and Nepveu 2012). Among them, 150 kDa, 110 kDa, 90 kDa, and 80 kDa isoforms are the results of proteolytic cleavage of the full-length protein. (Goulet, Baruch et al. 2004, Goulet, Truscott et al. 2006).

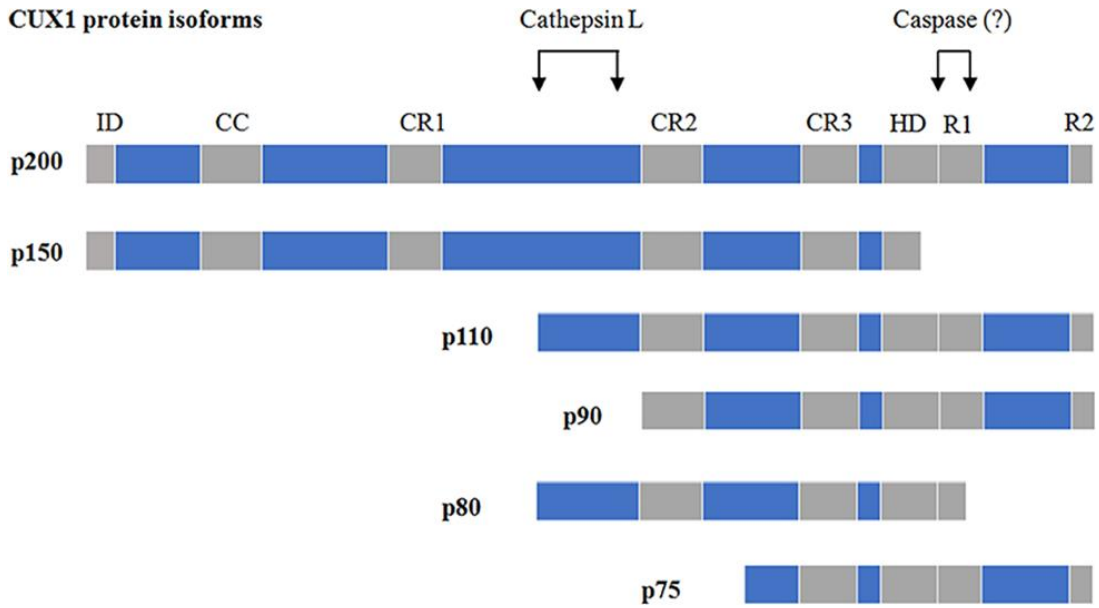

Figure 1.7. The Cux1 and their isoforms formation. ID, autoinhibitory domain; CR, cut repeats; HD, homeodomain; R1/R2, two active repression domains.

1.6.2 Functions of Cux1

1.6.2 a. Cux1 in nervous system development

In developing human neocortices, layers II to V have shown the Cux1 expression. (Saito, Hanai et al. 2011). During the *Drosophila* developmental process, Cux1 expressed only in external sensory organs and repressed in internal sensory organs. The lethal *cut* mutants transform external sensory organs into internal sensory organs (Bodmer, Barbel et al. 1987, Merritt, Hawken et al. 1993). Moreover, Cux1 and its homolog Cux2 have been shown to regulate dendritic branching,

synapsis formation, and spine development in the cerebral cortex (Cubelos and Nieto 2010, Cubelos, Sebastian-Serrano et al. 2010).

1.6.2 b. CUX1 in cell proliferation, migration, and differentiation

Cux1 is well known for its DNA binding activity in a cell cycle-dependent manner. In the G0 and early G1 phases, Cux1 DNA binding activity is minimal. It becomes detectable in the late G1 phase, and it peaks in the S phase. (Coqueret, Berube et al. 1998). Cdc25a dephosphorylates full length Cux1 at its homeodomain followed by generation of active DNA binding p110 isoform by proteolytic cleavage of full-length Cux1 (Goulet, Baruch et al. 2004). In the G2 phase, CyclinA-Cdk1 mediated phosphorylation attenuates the Cux1-DNA binding activity, and hyperphosphorylation by Cyclin B/Cdk1 resets the Cux1 DNA binding activity to zero level (Brendstrup and Biering-Sorensen 1987). In addition, accelerated S phase entry was reported with p110 Cux1 overexpression. Also, fibroblasts from mice with the *Cux1*$^{z/z}$ mutant gene had a more extended G1 phase (Sansregret, Goulet et al. 2006).

Recently, a high-throughput RNAi screening reveals that inhibition of Cux1 expression reduces cell migration and invasion in various cancer cell lines (Michl, Ramjaun et al. 2005). The Cux1 knockout cell line shows defective migration and invasion compared to the wild type. Interestingly, p110 overexpression could rescue the migration (Kedinger and Nepveu 2010). Altogether, these *in vitro* and *in vivo* studies demonstrate the role of Cux1 in the cell cycle, cell proliferation, migration, and invasion.

1.6.2 c. Cux1 in DNA damage repair.

The accumulating evidences supports the Cux1 importance in DNA damage repair (DDR). Several DDR-related genes, including ATM and ATR, were found to be the targets of 110 kDa Cux1 (Harada, Vadnais et al. 2008, Vadnais, Davoudi et al. 2012). Direct interaction of Cux1 with OGG1 can enhance its activity (Ramdzan, Pal et al. 2015). Moreover, to expedite DNA damage repair, Cux1 recombinant protein with CUT1 and CUT2 domains is sufficient. This demonstrates the importance of the CUT domain in DDR (Pal, Ramdzan et al. 2015, Kaur, Coulombe et al. 2016).

1.6.2 d. Cux1 in tumor development

In addition to its function in neuronal development, the cell cycle, and repair of damaged DNA, Cux1 is implicated in tumor development. Cux1 is reported to function as a tumor suppressor and an oncogene. Its role as a tumor suppressor or oncogene still remains controversial.

1.6.2 e. Cux1 as an Oncogene

Numerous cancers, including pancreatic cancer, melanoma, and glioma, had elevated expression of Cux1. (Liu, Sun et al. 2020). In addition, glioblastoma, pancreatic cancer, breast cancer, and colorectal cancer, Cux1 expression has been connected to a poor prognosis. (Michl, Ramjaun et al. 2005, Kaur, Ramdzan et al. 2018, Wu, Feng et al. 2019). Interestingly, CUX1 enhances the tumor progression in neuroblastoma by generation of circular RNA (Li, Yang et al. 2019). In addition, Cux1 modulates the expression of MMP9 and promotes the aggressiveness of pancreatic neuroendocrine tumors (Jiao, Zeng et al. 2020). Interestingly, Cux1 expression was found to be higher in tumor-associated macrophages (TAMs) and inactivated NF-kB signaling by interacting with NF-kB/p65, thereby decreasing T cell attraction and increasing angiogenesis in pancreatic cancer (Jiao, Zeng et al. 2020).

1.6.2 f. Cux1 as a Tumor Suppressor

The deletion of the 7th chromosome long arm has been reported in several cancers, including pancreatic carcinoma, kidney carcinoma, myeloid leukemia, and ovarian carcinoma (Liu, Sun et al. 2020). Interestingly, a comprehensive study of 7,651 genomic sequences of different tumor types revealed that mutations in CUX1 found to activate PI3K-AKT signaling (Wong, Martincorena et al. 2014).

1.7 Rationale and Hypothesis

A previous study from our lab has shown that the BAR domain protein Pil1 in *Saccharomyces cerevisiae* regulates the mitochondrial dynamics and cell death by specifically interacting with fission proteins (Pal, Paripati et al. 2022). We also observed that Pil1 regulates the mitochondrial-specific autophagy by directly interacting with ATG32. When we searched for the potential Pil1 homologue in humans through PSI-BLAST analysis, we found Cux1 (p75 isoform) as a partial homologue.

Most of the Cux1 functions were established on knockdown or knockout of full-length protein. However, the individual isoforms functions were not studied. A recent report suggests that the smallest isoform p75 is associated with tumorigenesis in breast cancer (Goulet, Watson et al. 2002). Additionally, it is also implicated in polycystic kidney disease development (Cadieux, Harada et al. 2008). However, p75 isoform of Cux1 is not very well characterized. In this study, we intended to characterize the Cux1 (p75) by studying its localization, role in mitophagy, and ER-mitochondrial communication.

Chapter 2

Characterization of Cux1 (p75)

2.1. Introduction

Cux1 is a nuclear-localized homeodomain transcription factor. It binds to the CCAAT region in the promoter and represses the gene expression. CUX1 exists in different isoforms by alternate splicing or proteolytic cleavage of the full-length protein. The full-length isoform is p200 and other isoforms are p150, p110, p90, p80, and p75 (Hulea and Nepveu 2012). Studies have shown that the p110 isoform of Cux1 binds to the DNA and accelerates the entry into the S phase. Moreover, genome-wide knockdown studies have revealed its role in cell proliferation in different cancer cell lines (Michl, Ramjaun et al. 2005). It is also implicated in DNA damage repair by directly interacting with the OGG1 by enhancing its enzymatic activity. Additionally, the genome-wide analysis revealed 18 DNA damage repair genes as Cux1 targets. Cux1 is also reported to have oncogenic and tumor suppressor functions (Liu, Sun et al. 2020). Furthermore, Cux1 is also involved in regulating dendritic branching, synapsis formation, and spine development in the cerebral cortex (Blochlinger, Bodmer et al. 1990, Cubelos, Sebastian-Serrano et al. 2010). Cux1 (p75) was reported arising from an alternate transcription start site in the intron 20. A recent report suggests that the p75 isoform is associated with tumorigenesis in breast cancer (Goulet, Watson et al. 2002). However, this isoform of Cux1 is not well characterized, and its existence is also questioned (Krishnan, Senagolage et al. 2022).

Most of the reported functions of the Cux1 were established based on the knockdown or knockout of the full-length gene. However, the functions of the individual isoforms have not yet been examined. In PSI-BLAST search, we found that the 75 kDa isoform of Cux1 is the partial homologue of yeast Pil1, which localizes to the outer mitochondrial membrane and regulates the mitochondrial dynamics and mitophagy (Pal, Paripati et al. 2022). Since Cux1 (p75) is a partial homologue of Pil1 and an uncharacterized isoform of Cux1, we intended to characterize its functions. In this study, we established the localization and topology of the p75 isoform of Cux1.

2.2 Methodology

2.2.1 Animals and Organs isolation

Male Wistar young, adult, and old rats were sacrificed by decapitation, and different organs were collected. The collected organs were ground to powder in $LiqN_2$, and further lysates were prepared for western blot analysis. All procedures are carried out according to the Institutional Animal Ethics Committee guidelines.

2.2.2 Cell culture and DNA transfection

HEK293T and HeLa cell lines used in this study were acquired from ATCC. Cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and Antibiotic-Antimycotic (Thermo fisher scientific) in a 5% CO_2 incubator at 37°C. Lipofectamine 2000 (Invitrogen) was used to transfect the required expression vectors.

2.2.3 Cloning and plasmid construction

cDNA prepared from HEK293T cells was used for amplifying the CUX1 coding sequence using NB1346 and NB1347 primers containing the HindIII site and XhoI sites, respectively. PCR product subjected to restriction digestion and ligated into pCDNA3.1-Myc.His vector to generate pCDNA3.1-CUX1-Myc/His plasmid. pLKO.1 puro and pLKO.1-shCUX1 puro were purchased from Dr. Subba Rao Gangishetty (IISc, India). For constructing pCDNA3.1-2XFLAG-CUX1, the CUX1 coding sequence was amplified using forward primer NB1546 and reverse primer NB1347 containing HindIII and XhoI restriction sites, respectively. The PCR amplified product was digested with respective enzymes and ligated into pCDNA3.1-Myc.His vector. For the construction of truncated CUX1 expression vectors, CUX1 lacking IMS region was amplified using primers pair NB1346/NB1515, and CUX1 lacking transmembrane region and IMS region was amplified using primers pair NB1346/NB1514. The amplified products were digested with HindIII and XhoI and cloned into pCDNA3.1-Myc.His vector. For generating pCDNA3.1-FLAG-CUX1-Myc/His plasmid, CUX1 coding sequence amplified with forward primer NB1547 containing FLAG-tag at 5' end and reverse primer NB1347 and digested with HindIII and XhoI enzymes and cloned into pCDNA3.1-Myc.His. Table 2.1 lists the primers used in the current study.

Table 2.1. Primers

S.No	Primer	Primer Sequence	Restriction site
1	NB1346	CTTTAAGCTTGCACCATGGCGGCCAATGTGGGATCG	HindIII
2	NB1347	CTTTCTCGAGCTGCCACAAGTCACCAGCCGCA	XhoI
3	NB1514	CTTTCTCGAGGGCCATCTTGTTGGAGAGAATCAGAC GGC	XhoI
4	NB1515	CTTTCTCGAGACTCTCACTCCATGCCAGCTTGTACAG CA	XhoI
5	NB1546	CTTTCTCGAGTTACTTGTCGTCATCGTCTTTGTAGTC CTTGTCGTCATCGTCTTTGTAGTCGTCGACCTGCCAC AAGTCACCAGCCGCA	XhoI
6	NB1547	CTTTAAGCTTGCACCATGGACTACAAAGACGATGAC GAC AAGGCGGCCAATGTGGGATCG	HindIII

2.2.4 Stable cell line generation by lentiviruses

When 50-60% confluent, HEK293T cells were transfected with shCTRL or shCUX1 plasmids along with lentiviral packaging plasmids (pVsVg, pΔR, pRev). 48 hrs after transfection, the viral soups were collected and filtered using a 0.45 µm filter. Filtered viral soup and fresh complete media were added in a 1:1 ratio to the desired cell line and incubated for 24 hrs. Two rounds of infections were performed to improve efficiency. Puromycin (1 mg/ml) was used for the selection of stable cells. The knockdown of the Cux1 was confirmed by western blot.

2.2.5 Immunoblotting

Cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% deoxycholic acid, 1% Triton X-100, 150 mM NaCl, 0.25 mM EDTA, and 0.1% SDS) containing a cocktail of protease inhibitors (Complete Cocktail, Roche). Protein concentrations were measured by Bradford reagent. An equal amount of protein was taken from each sample and boiled at 95°C with 1 X Laemmli buffer for 5 min. Using a Bio-Rad transfer unit, the proteins separated by SDS-PAGE were transferred onto the nitrocellulose membrane. The protein transfer onto the membrane was confirmed with ponceau stain. The membrane was blocked for 1 hr with 5% skimmed milk powder in 1XTBST before incubating with the specific antibody overnight at 4°C on the rocker. After the incubation, the blots were washed with 1XTBST three times, each wash for 5 min then incubated for 1 hr with HRP-conjugated secondary antibody at room temperature. The blots were developed by using an ECL detection kit (advansta) and captured using Bio-Rad Versadoc.

2.2.6 Antibodies and Reagents

The antibodies used in this study were anti-Cux1(11733-1-AP), anti-PINK1 (bc100-494), anti-TOMM40 (ab185543), anti-TOMM20(ab56783), anti-βTubulin(10068-1-AP), anti-TOMM70 (ab83841), anti-ANT2(ab195630), anti-GRIM19(ab110240), anti-Cytc (ab133504), anti-GRP75 (ab2799), anti-TFAM (ab131607), anti-Myc tag (16286-1-AP), anti-TOMM22 (ab179826), anti-GAPDH (60004-1-Ig), and anti-Flag (F2555). Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich.

2.2.7 Immunoprecipitation

The indicated constructs and their corresponding controls were transfected into a 100mm dish of HEK293T cells. After 48 hours of transfection, cells were collected and lysed in NTEN buffer (0.5% NP40, 20 mM Tris pH 8.0, and 100 mM NaCl) containing a cocktail of protease inhibitors (Complete Cocktail, Roche) by incubating in ice for 20 min. The lysate was centrifuged at 12,000 rpm at 4 °C for 10 min to remove cell debris. Preclearing was done to remove the non-specific interaction by incubating with Protein A/G agarose beads (sc-2003) for 1hr at 4°C. Precleared lysates were incubated overnight in an end-over-end rotator at 4°C with the desired antibody. Resultant immunocomplexes were collected and then washed with NTEN buffer three times by spinning at 2500 rpm, each wash for five min at 4°C. The immunocomplexes were separated by SDS-PAGE and analyzed by western blot.

2.2.8 FLAG Pull-down

Cells were lysed in NTEN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) by incubating on ice for 30 min, followed by removal of cell debris by spinning at 12000 rpm for 10 min. Pre-washed anti-FLAGM2 (M8823) beads were added to the cell lysate and incubated for 2 hrs on end-over-end rotator at 4°C. After incubation, cell lysate was removed, and washed the beads thrice with NTEN buffer. Finally, the interacting proteins were eluted with 3X FLAG peptide (A6001).

2.2.9 Confocal microscopy

HeLa cells were grown on coverslips in a 35mm dish and fixed for 20 min at room temperature (RT) using 4% paraformaldehyde (PFA) prepared in 1X PBS, followed by three washes with 1X TBS, each for 5 minutes. Pre-chilled acetone and methanol (1:3) were used to permeabilize the cells by incubating for 20 min, then three washes with 1XTBS, each wash for 10 min. Blocking

was done by incubating for 30 min with 3% BSA made in 1x TBS. Cells were incubated with primary antibody for 2 hours, then three washes for 15 minutes each in TBS, TBST, and TBS buffer. Secondary antibodies labeled with fluorescence were incubated at room temperature for 1 hour, then three washes for 15 min each in TBS, TBST, and TBS buffer. The coverslips were adhered on a glass slide with an antifade reagent containing DAPI (ab104139). Images were captured with a Leica microscope and processed with image J software.

2.2.10 Isolation of mitochondria from cell line

Cells were collected, and the residual medium was removed by washing twice with 1XPBS buffer. Cells were resuspended in mitochondria isolation buffer (70 mM mannitol, 210 mM sucrose, 1.5 mM $MgCl_2$, 20 mM HEPES pH 7.5,1 mM EDTA pH 8.0 and 1 mM EGTA) and incubated on ice for 1 hour with swirling in every 15 min. The cell suspension was homogenized thrice with a polytron homogenizer, with a 15 rpm pulse, then subjected to dounce homogenization. To pellet down the cell debris, the cell suspension was centrifuged at 2700 rpm for 10 min. Supernatant was collected and centrifuged for 15 min at 9500 rpm at 4°C to pellet down the mitochondria. After washing twice, the mitochondrial pellet was resuspended in mitochondrial resuspension buffer (10 mM HEPES-KOH pH 7.4, 5 mM magnesium acetate, and 250 mM sucrose).

2.2.11 Trypsin digestion and carbonate extraction of mitochondria

Mitochondria were digested with 1 µg trypsin (Sigma-Aldrich) for 10 µg of mitochondria for the indicated time points. Inhibited digestion by adding a 5-fold trypsin inhibitor (trypsin inhibitor from Soybean, Roche). Digestion of inner membrane and matrix proteins was achieved by the addition of digitonin (2 µg/µl) or Triton X-100 (3%) to the digestion reaction. Insoluble and soluble mitochondrial proteins were obtained by salt treatment and carbonate extraction of mitochondria. Briefly, mitochondria were treated with 0.2 M Na_2CO_3 or 0.4 M NaCl for 20 min on ice, and separated into supernatant and pellet fractions by centrifuging at 10,000 rpm for 10 min. The resultant fractions were analyzed by western blot.

2.2.12 Blue-Native PAGE (BN-PAGE)

Blue native PAGE of mitochondria was performed as described previously (Wittig, Braun et al. 2006). Briefly, mitochondria were solubilized in a buffer containing digitonin (final concentration of 60.0 g/g) and centrifuged at 20,000 g for 20 min. Solubilized membrane complexes in the supernatant were collected and separated on 4-13% gradient native gel. The complexes were

transferred onto the PVDF membrane and analyzed for the protein of interest using western blot.

2.3 Results

2.3.1 Expression of p75 Cux1 isoform

To find the Pil1 homologue in humans, we performed PSI-BLAST analysis with the Pil1 protein sequence. We found the 75 kDa isoform (Isoform 3) of Cux1 protein with 26% identity and 42% similarity (Figure 2.1A). In order to characterize this protein, we started by checking the expression of this protein in different tissues of rat. Interestingly, the p75 isoform was readily detected in all the tissues except the lung and spleen (Figure 2.1B), demonstrating that Cux1 (p75) is a widely expressed and stable protein. In general, homeodomain proteins are known to be involved in crucial regulatory developmental processes such as patterning, regional specification, and differentiation (Duverger and Morasso 2008). Notably, Cux1 has been shown to be extremely important for the neocortex and sensory organ development (Blochlinger, Bodmer et al. 1990, Saito, Hanai et al. 2011). Considering its role in developmental regulation, we sought to look into the age-dependent expression of Cux1. To elucidate this, the expression of Cux1 in rat tissue lysates from the young, adult, and old rats was analyzed by western blot. Interestingly, Cux1 expression varied with age in different tissues. In lung, it found to be expressed only in the young age, whereas we didn't find any expression in the spleen (Figure 2.1C).

A

```
>protein CASP isoform X7 [Homo sapiens]
Sequence ID: XP_006715918.1 Length: 765
Range 1: 452 to 644

Score:31.6 bits(70), Expect:9.3,
Method:Compositional matrix adjust.,
Identities:50/196(26%), Positives:83/196(42%), Gaps:41/196(20%)

Query  62   VKIEKNVLRSMELTANE---RRDAAKQLSIWGLENDDDV-----------SDITDKLGVL  107
            VK E N+L+SME   +E    +DAAK L +  LE +  +           SD++ +   L
Sbjct  452  VKKELNILKSMEFAPSEGAGTQDAAKPLEVLLLEKNRSLQSENAALRISNSDLSGRCAEL  511

Query  108  IYEVSEL------DDQFIDRYDQYRLTLKSIR--DIEGSVQPSRDRKDKITDKI--AYLK  157
              ++E         + I R +Q    ++SI+  D EG+ +    R +KI + I  A
Sbjct  512  QVRITEAVATATEQRELIARLEQDLSIIQSIQRPDAEGAAE---HRLEKIPEPIKEATAL  568

Query  158  YKDPQSPKIEVL--------------EQELVRAEAESLVAEAQLSNITRSKLRAAFNYQF  203
            +  P +P    L              ++E  RA  + L AE +L+  T   L++  +
Sbjct  569  FYGPAAPASGALPEGQVDSLLSIISSQRERFRARNQELEAENRLAQHTLQALQSELDSLR  628

Query  204  DSIIEHSEKIALIAGY  219
              I+  EKI  +  Y
Sbjct  629  ADNIKLFEKIKFLQSY  644
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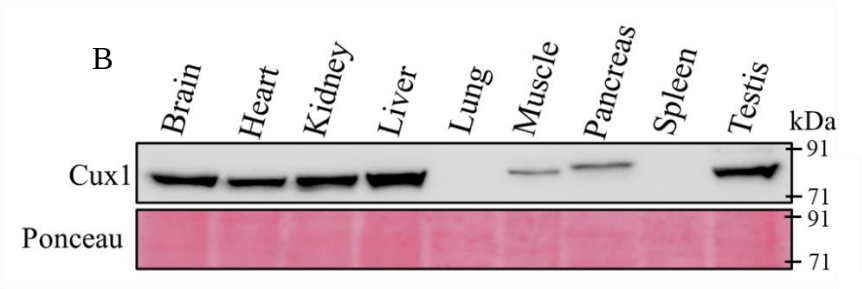


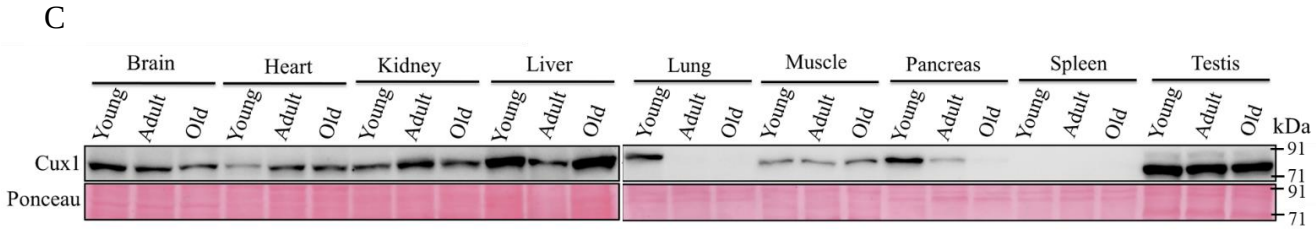


Figure 2.1 Expression of Cux1. (A) PSI-BLAST analysis of Pil1 protein sequence to show a homologue protein in humans. (B) Western blot of different adult rat tissues. Analysis of endogenous Cux1 expression. (C) Immunoblot of different aged rat tissues to assess age-dependent expression of Cux1. Ponceau stain used as a loading control.

2.3.2 Cux1 is a Mitochondrial Protein

Subcellular localization of a protein is important to understand an individual protein's function and the organization of the cell in whole (Scott, Calafell et al. 2005). Subcellular localization studies using immunofluorescence demonstrated that Cux1 localizes to the nucleus (Luong, van der Meijden et al. 2002). However, the studies were conducted on the full-length protein, and there was no evidence for the localization of individual isoforms. We decided to check the subcellular localization of Cux1 as it could provide some insight into its function. To achieve this, we performed subcellular fractionation of mitochondria from HEK293T and HeLa cells as detailed in the methods. The whole-cell lysate, cytosol, and mitochondrial lysates were subjected to western blot and analyzed for Cux1 with the specific antibody. Surprisingly, Cux1 (p75) was detected specifically in the mitochondrial fraction (Figure 2.2A). The purity of the mitochondrial and cytosolic fractions was confirmed by the absence of tubulin in the mitochondrial fraction and TOMM40 in the cytosolic fraction. To validate the localization of Cux1 to the mitochondria by imaging, we carried out colocalization studies in HeLa cells by immunofluorescence using specific antibodies against Cux1 and mitochondrial protein TOMM20 as mentioned in the methods (Figure 2.2 B). We observed complete colocalization of Cux1 with mitochondrial marker

TOMM20. Together, subcellular fractionation and colocalization studies suggest that Cux1 specifically localizes to mitochondria.

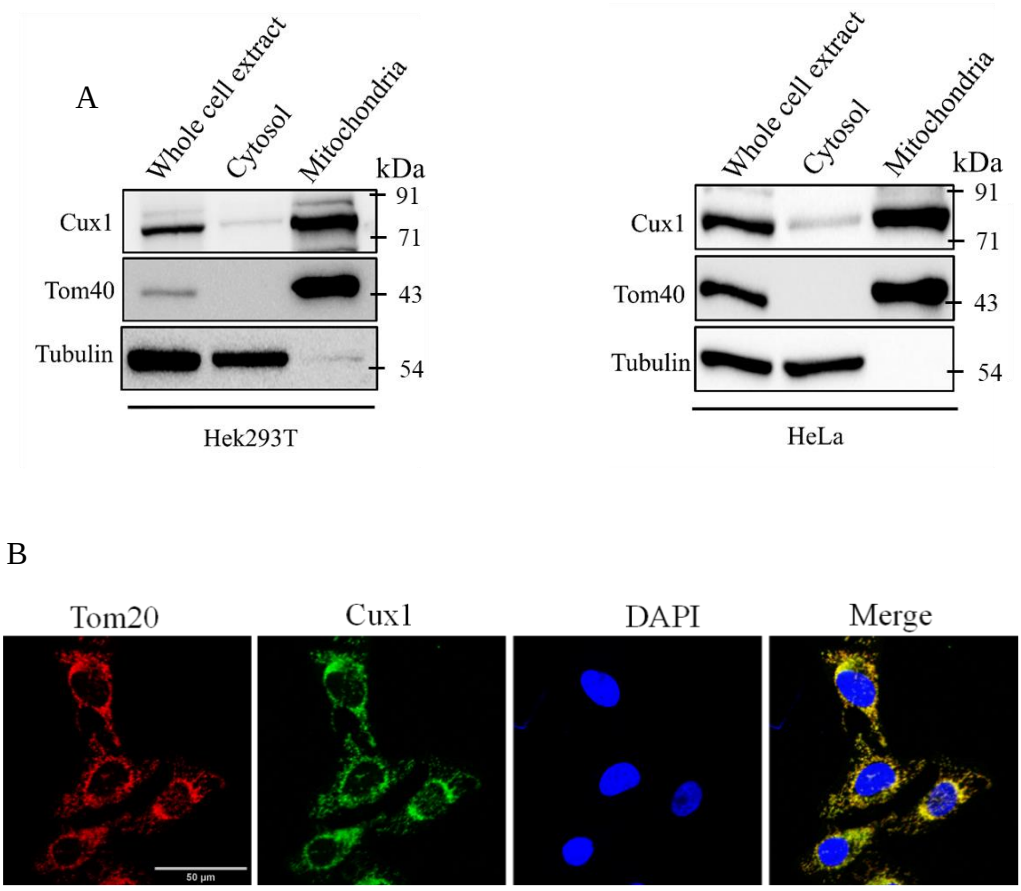


Figure 2.2. Cux1 localizes to mitochondria. (A) Whole-cell, cytosol, and mitochondrial fractions were prepared from HEK293T and HeLa cells and analyzed for Cux1 expression by western blot. Tubulin was used as a cytosolic and TOMM40 used as mitochondrial loading control (B) Immunofluorescence. HeLa cells were analyzed for Cux1 localization. TOMM20 served as a mitochondrial marker and DAPI was used to stain nuclei.

2.3.3 Cux1 is an Outer Mitochondrial Membrane Protein

The sequence analysis of the Cux1 protein by TMHMM revealed that it contains a single-pass transmembrane region at its C-terminus between 619 and 641 amino acids (Figure 2.3 A). Given this, we aim to determine whether Cux1 is a membrane or a soluble protein. To examine whether Cux1 is a membrane or soluble protein, mitochondria were subjected to salt and carbonate extraction, as detailed in the methods. With salt treatment, the loosely bound proteins with electrostatic interactions on the mitochondrial surface get detached into the supernatant, and the

remaining mitochondria separate into the pellet. Whereas high pH carbonate treatment disrupts the whole mitochondrial membrane and leaves the soluble proteins in the supernatant and membrane or insoluble proteins in the pellet. Following treatment, supernatant and pellet fractions were examined by western blot with specific antibodies against Cux1 and mitochondrial soluble and membrane proteins. Interestingly, Cux1 was retrieved exclusively in the pellet fraction in both treatments, indicating that Cux1 is a membrane protein or is tightly bound to the membrane (Figure 2.3 B). TOMM40, TOMM70, and ANT2 act as positive controls for the membrane proteins, and PDH serves as a control for soluble protein.

Considering the mitochondrial membrane localization of Cux1, we attempted to understand its sub-mitochondrial localization. To achieve this, mitochondria isolated from HEK293T cells were treated with trypsin in the presence and absence of detergent in a time-dependent manner. Solubilizing the membrane with digitonin gives mild access to the proteins for trypsin digestion, whereas TritonX-100 is a harsh detergent that solubilizes the membrane completely; thereby, integral membrane proteins get exposed for trypsin digestion (Figure 2.3 C). Following trypsin treatment, mitochondrial lysates were analyzed for Cux1 and mitochondrial outer, inter membrane space, inner and matrix proteins by western blot. The outermost mitochondrial protein TOMM20 was digested completely in the initial time point itself, whereas intermembrane protein cytochrome c and integral outer mitochondrial membrane protein TOMM40 got digested partially in the presence of digitonin. The inner membrane proteins Grim19 and matrix protein Hsp70 were digested completely in the presence of Triton X-100. Unexpectedly, Cux1 got digested in a time-dependent manner, similar to the outer mitochondrial membrane protein TOMM40 (Figure 2.3 C). However, some fractions of Cux1 didn’t undergo trypsin digestion even in the presence of TritonX-100. This could be due to the presence of proteolytically resistant region in Cux1 or due to its localization to the inner membrane. Together, these findings suggest that Cux1 is a membrane protein that primarily localizes to the outer mitochondrial membrane.

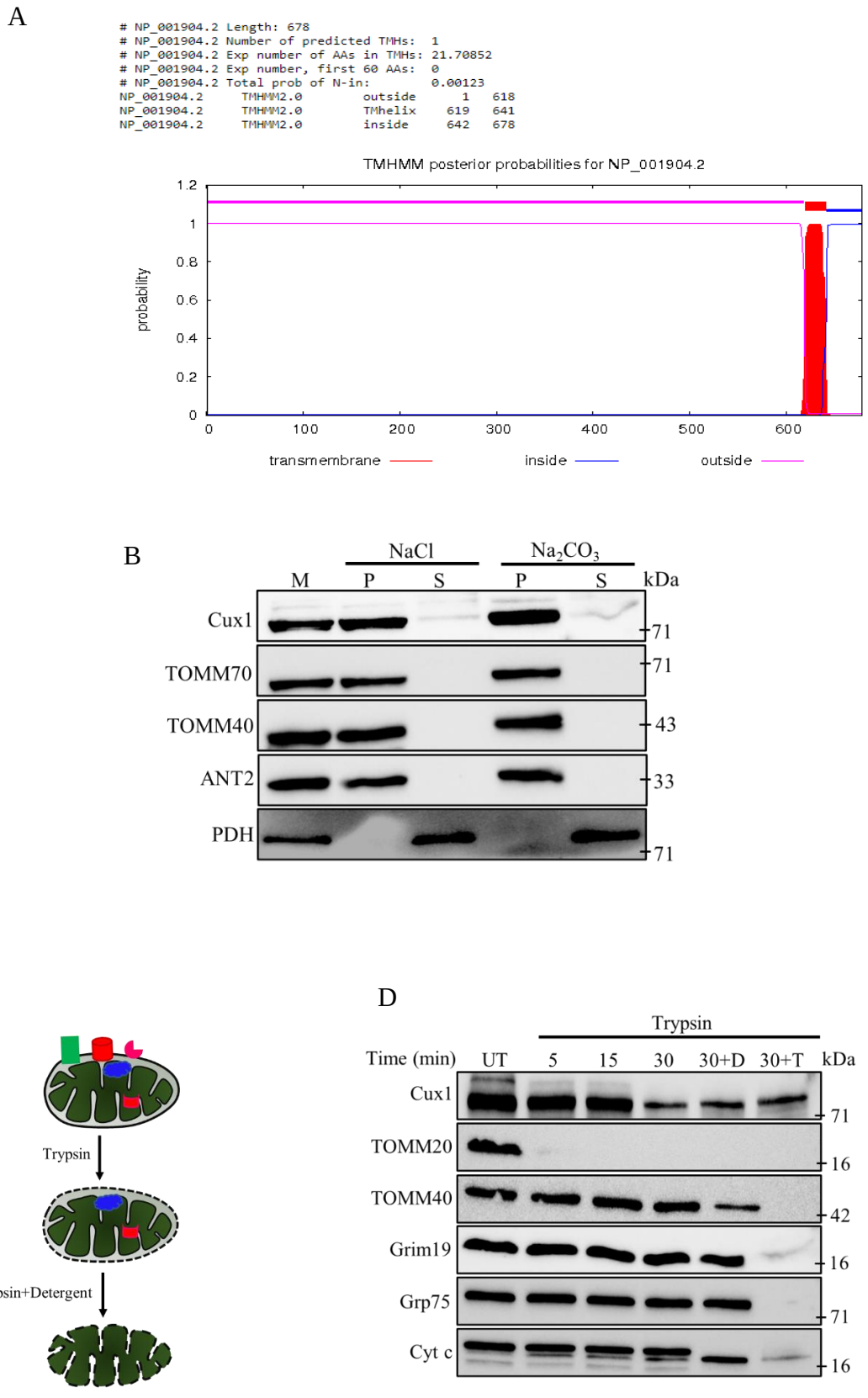


Figure 2.3. Sub-mitochondrial localization of Cux1. (A) Prediction of the transmembrane region in Cux1 (p75) by TMHMM tool. (B) Salt and carbonate extraction of mitochondria.

Mitochondria isolated from HEK293T cells were treated with 0.4 M NaCl and 0.2 M Na_2CO_3. Cux1, TOMM70, TOMM40, ANT2, and PDH were analyzed by immunoblot. TOMM70, TOMM40, and ANT2 were used as membrane protein controls. PDH was used as soluble protein control. P (Pellet), S (Supernatant). (C) The trypsin treatment of mitochondria is illustrated schematically. (D) Mitochondria digested with trypsin for the indicated time points with and without the detergents and subjected to immunoblotting. TOMM70, TOMM40, Grim19, and Grp75 were used as outer membrane, integral outer membrane protein, inner membrane protein, and matrix protein controls, respectively. D (Digitonin), T (Triton X-100).

2.3.4 Understanding the topology of Cux1

Understanding the protein topology provides valuable information about the region responsible for its functions. To investigate Cux1 topology, HEK293T cells were transiently transfected with dual tagged CUX1 construct containing FLAG-tag at its N-terminus and Myc-tag at its C-terminus. After 48 hrs of post-transfection, cells were fractionated into cytosol and mitochondria. Western blot analysis of whole cell, cytosol, and mitochondrial extracts confirms its expression and mitochondrial-specific localization (Figure 2.4 A). Tubulin and TOMM40 were used as cytosolic and mitochondrial markers, respectively.

Following verification of dual-tagged Cux1 expression and localization, mitochondria isolated from cells transfected with dual-tagged Cux1 were subjected to trypsin digestion in a concentration-dependent manner, as detailed in the methods. Cytosolic localization of N terminus FLAG causes its early trypsin digestion, followed by delayed C terminus Myc digestion. Cytosolic localization of C terminus Myc causes its early trypsin digestion, followed by delayed N terminus FLAG digestion (Figure 2.4.B). Following treatments, mitochondrial fractions were analyzed for the FLAG and Myc tag along with different mitochondrial compartment proteins by immunoblotting. Interestingly, the FLAG tag degraded rapidly, indicating that the FLAG-tagged N-terminus faces toward the cytosol and are readily accessible for trypsin digestion. On the contrary, the Myc tag degraded gradually in a concentration-dependent manner. This suggests that Myc-tagged C-terminus faces toward the intermembrane space where trypsin digestion has limited access (Figure 2.4 C). Here we used TOMM22 and TOMM40 as outer membrane markers, and ANT2 as inner membrane markers. This data suggests that the Cux1 N-terminus faces towards the cytosol and the C-terminus towards the intermembrane space.

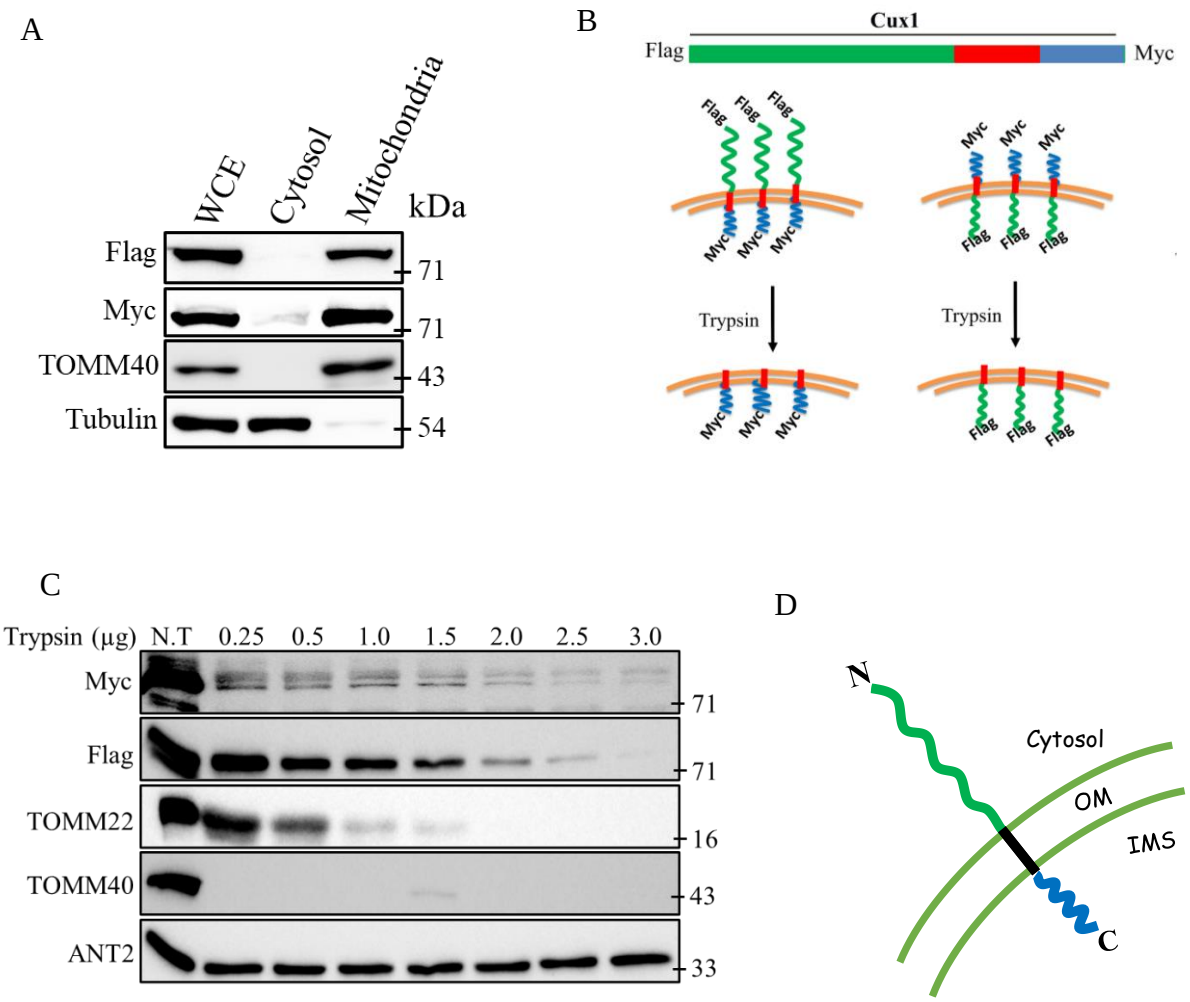


Figure 2.4. Topology of Cux1. (A) HEK293T cells were transiently transfected with dual-tagged CUX1 (N'-FLAG and C'-Myc), and whole cell, cytosol, and mitochondria fractions were prepared. Analyzed for Cux1 by immunoblot. TOMM40 used as mitochondria and tubulin used cytosol positive controls. (B) Schematic representation of trypsin digestion working principle (C) HEK293T cells were transiently transfected with dual-tagged CUX1 (N'-FLAG and C'-Myc), isolated mitochondria, and treated with trypsin in a concentration-dependent manner. Analyzed for Myc, Flag using immunoblot. TOMM22, TOMM40, and ANT2 were used as controls. (C) Schematic representation of biochemically validated topology of Cux1. D) Predicted topology of Cux1.

2.3.5 Cux1 is a Dimeric Protein

Since Cux1 (p75) has several cysteine residues, we intend to check whether Cux1 exists as a monomer or any other oligomeric state. To determine the oligomeric state of Cux1, HEK293T cells were transiently transfected with Myc tagged CUX1 expression plasmid or vector. After 48

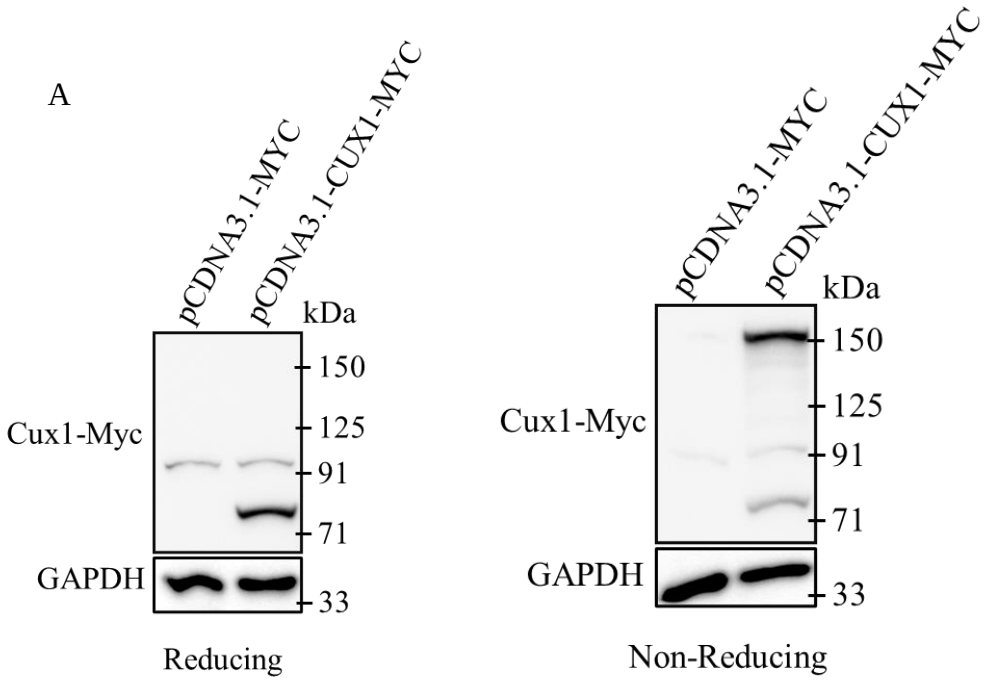
hrs of transfection, we examined for the Cux1 migration by SDS-PAGE and western blot using Myc tag specific antibody as detailed in the methods. Interestingly, under non-reducing conditions, Cux1 migrates as a dimer with around 150 kDa molecular weight. Whereas under reducing conditions, it migrates as a monomer (Figure 2.5A). In reducing conditions, the cysteine-mediated disulfide interactions between two polypeptides are disturbed and hence it migrates as 75 kDa form, whereas the interactions are intact under non-reducing conditions.

To further validate the dimerization of Cux1, HEK293T cells were co-transfected with CUX1-FLAG and pCDNA3.1 Myc vectors or CUX1-Myc and pCDNA3.1 Flag vectors, or CUX1-FLAG and CUX1-Myc vectors. After 48 hrs of transfection, immunoprecipitation was performed using anti-FLAG-M2 beads and then subjected to western blot. The presence of Myc-tagged Cux1 in the FLAG pull down confirms the homodimerization of Cux1 (Figure 2.5 B). These results further confirm the homodimerization of Cux1.

Cysteine residues are majorly responsible for the oxidative dimerization of the protein. Since Cux1 undergoes cysteine-mediated dimerization, we aimed to pin-point the cysteine residues that are responsible for its dimerization. Cux1 contains four cysteines; two of them are situated in the intermembrane space region at the 647^{th} and 651^{st} position, one is in the transmembrane region at the 628^{th} position, and the last one is in the cytosolic domain at the 419^{th} position. To test the importance of cysteines in the IMS, we constructed two plasmids: one without the IMS and transmembrane region and one without IMS region (Figure 2.5 C).

HEK293T cells were transiently transfected with either of these two truncated constructs and the full-length Cux1 plasmids. Cells were harvested 48 hrs after transfection, cell lysates were treated either with an oxidizing agent (H_2O_2) or a reducing agent (DTT) or left untreated. Resultant lysates were separated on reducing and non-reducing gels and analyzed by immunoblotting for Cux1 migration by probing against Myc tag-specific antibody. Interestingly, under non-reducing conditions, with or without H_2O_2 treatment causes dimerization of full-length Cux1, however, in presence of DTT, it migrates as a monomer. We observed that the truncated proteins fail to dimerize even after treating with H_2O_2, suggesting that the cysteines in the IMS region (647^{th} and 651^{st}) are responsible for the dimerization of Cux1 (Figure 2.5 D).

Altogether, these results suggest that Cux1 is a dimeric protein, and cysteines at 647^{th} and 651^{st} residues are responsible for its dimerization.


A
pCDNA3.1-MYC
pCDNA3.1-CUX1-MYC
kDa
150
125
91
71
33
Cux1-Myc
GAPDH
Reducing
pCDNA3.1-MYC
pCDNA3.1-CUX1-MYC
kDa
150
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91
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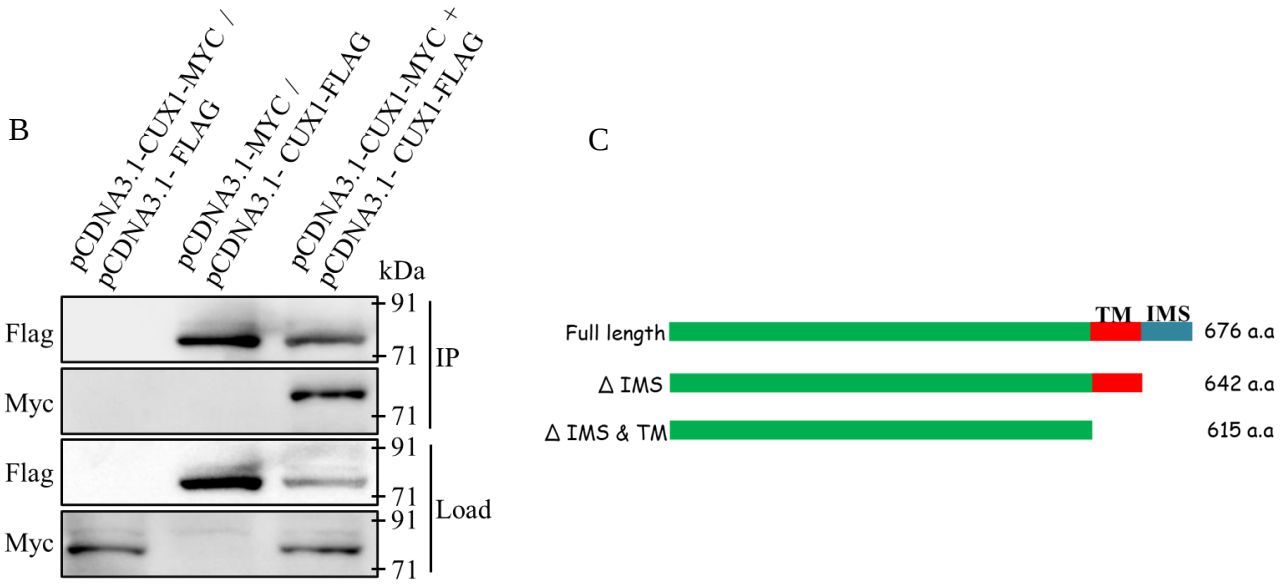
33
Cux1-Myc
GAPDH
Non-Reducing


B
pCDNA3.1-CUX1-MYC / pCDNA3.1- FLAG
pCDNA3.1-MYC / pCDNA3.1- CUX1-FLAG
pCDNA3.1-CUX1-MYC + pCDNA3.1- CUX1-FLAG
kDa
Flag
Myc
IP
Flag
Myc
Load
91
71
C
TM
IMS
Full length
676 a.a
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642 a.a
Δ IMS & TM
615 a.a

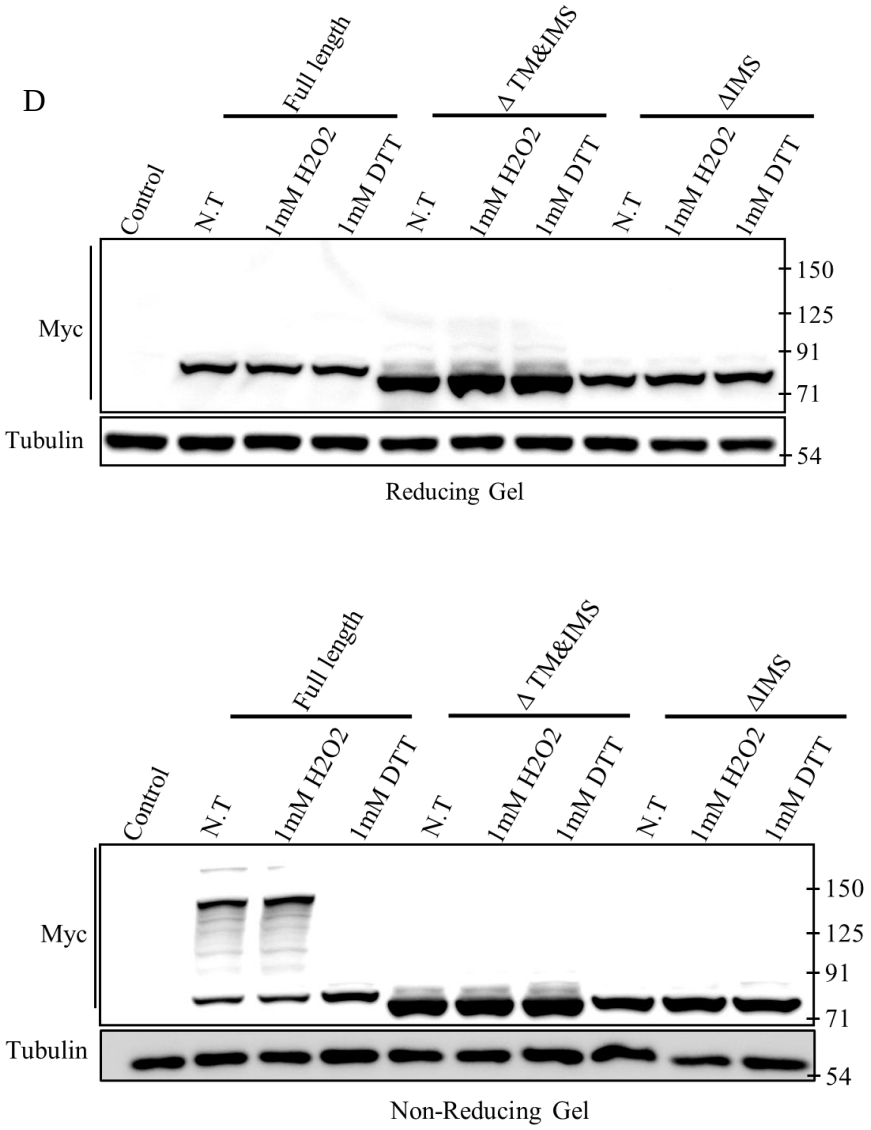


Figure 2.5. Cux1 exists as a dimer. (A) HEK293T cells were transiently transfected with pCDNA3.1-CUX1-MYC. Under reducing and non-reducing conditions Cux1 migration was analyzed by immunoblot. (B) HEK293T cells were transiently transfected with CUX1-FLAG or CUX1-MYC or both together and subjected to FLAG pull-down using anti-FLAG M2 beads, analyzed for Myc and FLAG tag with specific antibodies by immunoblot. (C) Schematic representation of full-length and truncated Cux1 proteins. (D) HEK293T cells were transiently transfected with CUX1 full-length and truncated plasmids. Lysates were treated with an oxidizing or reducing agent and analyzed for Cux1 migration with Myc-tag specific antibody using western blot.

2.3.6 Cux1 forms a higher molecular weight complex on mitochondria

Given the localization of Cux1 to the mitochondrial outer membrane, we wanted to know if Cux1 forms any high molecular weight complexes on mitochondria. To test this hypothesis, we generated Cux1 knockdown stable cell lines as described in the methods. Mitochondria isolated from HEK293T control and Cux1 knockdown cells were subjected to Blue native PAGE followed by Coomassie staining and western transferred onto the PVDF membrane as described in the methods and probed with Cux1 antibody. Surprisingly, we observed that Cux1 forms a higher molecular weight complex on the mitochondria with a molecular weight of ~450 kDa, which was absent in Cux1 knockdown cells (Figure 2.6). Coomassie stained gel shows the presence of equal amount of protein in control and Cux1 knockdown cells.

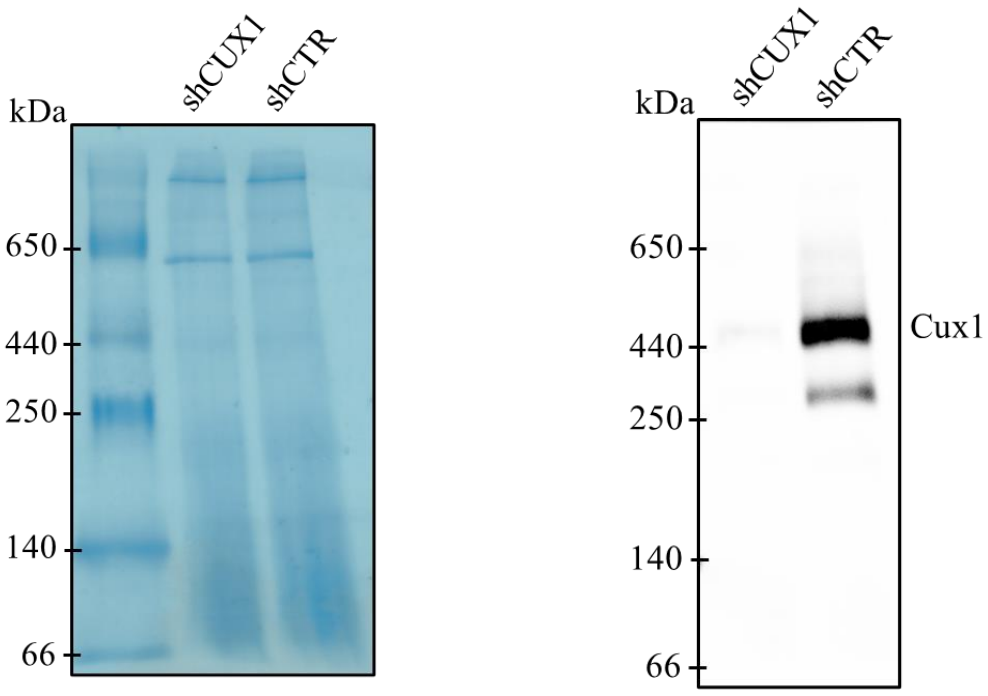


Figure 2.6. Cux1 forms a higher molecular weight complex on mitochondria. Blue native PAGE of mitochondrial isolated from shCTRL and shCUX1 was performed. Cux1 complex formation was analyzed by immunoblot.

2.4 Discussion

Mitochondria are complex organelles with a wide variety of roles in cellular functions. Changes in the mitochondrial architecture, enzyme activity, respiratory capacity, and ROS generation play essential roles in cancer and cardiac failure, Alzheimer's, Parkinson's, and Huntington's disease pathogenesis. Around 1098 genes encoding proteins were reported to localize to the mitochondria (Pagliarini, Calvo et al. 2008). Surprisingly, more than 300 of these proteins remain uncharacterized. About 20% of the predicted human mitochondrial proteins were implicated in several diseases. Therefore, it is important to characterize the uncharacterized mitochondrial localized proteins.

Using PSI-BLAST analysis, we found Cux1 (p75) is the homologue of Pil1 in humans. We confirmed the expression of this particular isoform in different rat tissues. Interestingly, subcellular localization studies by Immunofluorescence and cellular sub-fractionation have shown that Cux1 localizes specifically to the mitochondria. Further, the bioinformatics analysis of Cux1 (p75) by TMHMM revealed the presence of a single-pass transmembrane region in its C-terminus. Using salt and carbonate extraction of mitochondria confirmed that Cux1 is a membrane protein. Sub-mitochondrial localization studies by trypsin digestion of isolated mitochondria, we found that Cux1 majorly localizes to the outer mitochondrial membrane with some fraction possibly located in the inner membrane as well. Topology studies with trypsin digestion of mitochondria isolated from the cells transfected with dual-tagged Cux1 (N-terminus FLAG and C-terminus Myc) found that N-terminus localizes towards the cytosol and C- terminus towards the intermembrane space. We also observed that Cux1 undergoes dimerization under non-reducing conditions, which is mediated by inter-membrane space localized (647^{th} and 651^{st}) cysteines. Additionally, in BN-PAGE analysis of isolated mitochondria, we Cux1 forms a higher molecular weight complex on mitochondria around 450 kDa.

Overall, our findings show that Cux1 (p75) is an outer mitochondrial membrane protein, and its N-terminus faces toward the cytosol, whereas its C-terminus is towards the intermembrane space. Moreover, it also undergoes cysteine-mediated dimerization. Surprisingly, we also found that it forms a higher molecular weight complex in mitochondria, and further studies are needed to find the protein composition of this complex.

Chapter 3

Role of Cux1 (p75) in ER-Stress Induced Cell Death

3.1 Introduction

Eukaryotic cells are composed of different intracellular organelles with defined functions. They are interdependent to sustain the intracellular and extracellular challenges. Recent evidence suggests that every organelle interacts with the other organelles, such as ER-nucleus, ER-mitochondria, Mitochondria-lysosome, Mitochondria-lipid droplets, and mitochondria-nucleus (Petkovic, O'Brien et al. 2021). Mitochondria communicate with the different organelles, including the endoplasmic reticulum, lysosomes, peroxisomes, and nucleus. Mitochondria form tethering complexes to communicate with other organelles. Among them, extensively studied are the ER-mitochondrial tethering complexes. ER-mitochondria contact sites (MCS) are dynamic in nature and keep them close. Different pairs of protein complexes that link the ER to the mitochondria have been analyzed (Figure 1.5). The ER localized inositol 1,4,5-triphosphate receptor (IP3R) and the mitochondrial outer membrane protein VDAC1 form the first known ERMCS tethering complex. This interaction is mediated by GRP75 and DJ1 (Szabadkai, Bianchi et al. 2006, Basso, Marchesan et al. 2020). Later it was found that the PTPIP51 is an OMM-residing protein that also mediates the formation of MCS by interacting with VAPB (De Vos, Morotz et al. 2012). Recently, it was discovered that the ER-resident protein BAP31 interacts with Fis1 and TOMM40 and is involved in the formation of MCS (Iwasawa, Mahul-Mellier et al. 2011, Namba 2019). Apart from these, the Mfn2-mediated ER-mitochondrial contact site has received the most attention. Genetic ablation of Mfn2 attributed to elevated ER stress results in ER stress-induced apoptosis (Ngoh, Papanicolaou et al. 2012).

Mitochondrial morphology is maintained through the events of fission and fusion. The fusion at the outer membrane is mediated by Mfn1 and Mfn2, whereas Opa1 contributes to the inner membrane fusion. The fission is assisted by various GTPase family proteins, including, Drp1, Mff1, Fis1, Mid49, and Mid51 (Chiu, Lin et al. 2021).

Defects in the regulation of mitochondrial dynamics and ER mitochondrial communication lead to different pathophysiological conditions such as Parkinson’s, Alzheimer’s, cardiomyocyte hypertrophy, cancer, and diabetes (Chiu, Lin et al. 2021). It is important to understand the detailed molecular mechanism and unidentified regulators of these events. As Cux1 localizes to the mitochondrial outer membrane, which is the main site for inter-organelle contact of mitochondria, we were also curious about its role in mitochondrial morphology and ER mitochondrial

interaction, if any. In this objective, we studied the role of Cux1 in mitochondrial morphology and ER stress-induced cell death.

3.2 Methodology

3.2.1 Cell culture and DNA transfection

The cell lines HEK293T and U2OS used in this work were acquired from ATCC. Cells were cultured in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, and Antibiotic-Antimycotic (Thermo fisher scientific) in a 5% CO2 incubator at 37°C. Lipofectamine 2000 (Invitrogen) was used to transfect the required expression vectors.

3.2.2 Cloning and plasmid construction

Performed as mentioned in section 2.2.3.

3.2.3 Stable cell line generation by lentiviruses

At 50-60% confluence of HEK293T cells, they were transfected with shCTRL or shCUX1 plasmids along with lentiviral packaging plasmids (pVsVg, pΔR, pRev). 48 hrs after transfection, the viral soups were collected and filtered using a 0.45 µm filter. Filtered viral soup and fresh complete media were added in a 1:1 ratio to the U2OS cell line and incubated for 24 hrs. Two rounds of transfections were performed to improve efficiency. Puromycin (1mg/ml) was used for the selection of stable cells. The knockdown of the Cux1 was confirmed by western blot.

3.2.4 Immunoblotting

Cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% deoxycholic acid, 1% Triton X-100, 150 mM NaCl, 0.25 mM EDTA, and 0.1% SDS) containing a cocktail of protease inhibitors (Complete Cocktail, Roche). Protein concentrations were measured by Bradford reagent. An equal amount of protein was taken from each sample and boiled at 95°C with 1 X Laemmli buffer for 5 min. Using a Bio-Rad transfer unit, the proteins separated by SDS-PAGE were transferred onto the nitrocellulose membrane. The protein transfer onto the membrane was confirmed with ponceau staining. The membrane was blocked for 1 hr with 5% skimmed milk powder in 1XTBST before incubating with the specific antibody overnight at 4°C on the rocker. After the incubation, the blots were washed with 1XTBST three times, each wash for 5 min and then incubated for 1 hr with HRP-conjugated secondary antibody at room temperature. The blots were developed by using an ECL detection kit (advansta) and captured using Bio-Rad Versadoc.

3.2.5 Antibodies and Reagents

The primary antibodies used in this study were anti-CUX1(11733-1-AP), anti-PINK1 (bc100-494), anti-β-Tubulin (10068-1-AP), anti-TOMM40 (ab185543), anti-TOMM20 (ab56783), anti-TOMM70 (ab83841), anti-ANT2(ab195630), anti-GRP75 (ab2799), anti-TFAM (ab131607), anti-Myc tag (16286-1-AP), anti-GAPDH (60004-1-Ig), anti-Mfn2 (D2D10), anit-Drp1 (D6C7), anti-BiP (C50B12), anti-PARP (46D11), and anti-Flag(F2555). Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich.

3.2.6 Immunoprecipitation

Cells were collected and lysed in NTEN buffer (0.5% NP40, 20 mM Tris pH 8.0, and 100 mM NaCl) containing a cocktail of protease inhibitors (Complete Cocktail, Roche) by incubating in ice for 20 min. The lysate was centrifuged at 12,000 rpm at 4°C for 10 min to remove cell debris. Pre-clearing was done to remove the non-specific interaction by incubating with Protein A/G agarose beads (sc-2003) for 1 hr at 4 °C. Pre-cleared lysates were incubated overnight in an end-over-end rotator at 4°C with the desired antibody. Resultant immunocomplexes were collected and washed with NTEN buffer 3 times by spinning at 2500 rpm for 5 min at 4°C. SDS-PAGE was used to separate the immunocomplexes, which were then blotted with the appropriate antibodies.

3.2.7 FLAG Pulldown

Cells were lysed in NTEN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) by incubating on ice for 30 min, followed by removal of cell debris by spinning at 12,000 rpm for 10 min. Pre-washed anti-FLAGM2 (M8823) beads were added to the cell lysate and incubated for 2 hr on end-over-end rotator at 4°C. After incubation, cell lysate was removed, and washed the beads thrice with NTEN buffer. Finally, the interacting proteins were eluted with 3X FLAG peptide (A6001).

3.2.8 Confocal microscopy

HeLa cells were grown on coverslips in a 35mm dish and fixed for 20 min at room temperature (RT) using 4% paraformaldehyde (PFA) prepared in 1X PBS, followed by a wash with 1X PBS. The coverslips adhered to a glass slide with an antifade reagent containing DAPI (ab104139). Images were captured with a Leica microscope and processed with image J software.

3.2.9 Isolation of mitochondria from cell line

Performed as mentioned in section 2.2.10.

3.2.10 Caspase 3 activity assay

20 µM caspase 3 substrates (AC-DEVD-AFC) in caspase assay buffer (10% Sucrose, 100 mM NaCl, 20 mM HEPES pH 7.4,10 mM DTT, 0.1% CHAPS, and 1 mM EDTA) were incubated with 150 µg of lysate for 30 min at 30°C, followed by fluorescence intensity measurement at excitation 400 nm and emission at 510 nm wavelength.

3.3 Results

3.3.1 Cux1 alters the mitochondrial morphology

Outer membrane-localized proteins such as Drp1, Mfn2, and Mfn1 are the major regulators of fission and fusion events. Since Cux1 homologue in yeast Pil1 alters the mitochondrial morphology, we intend to check whether Cux1 affects mitochondrial morphology. We transfected the Mito-RFP plasmid either into control or CUX1 knockdown cells and observed any morphological changes in mitochondria by confocal microscopy. Interestingly, we noticed the elongated mitochondrial network in Cux1 knockout cells (Figure 3.1) indicating the involvement of Cux1 in mitochondrial morphology.

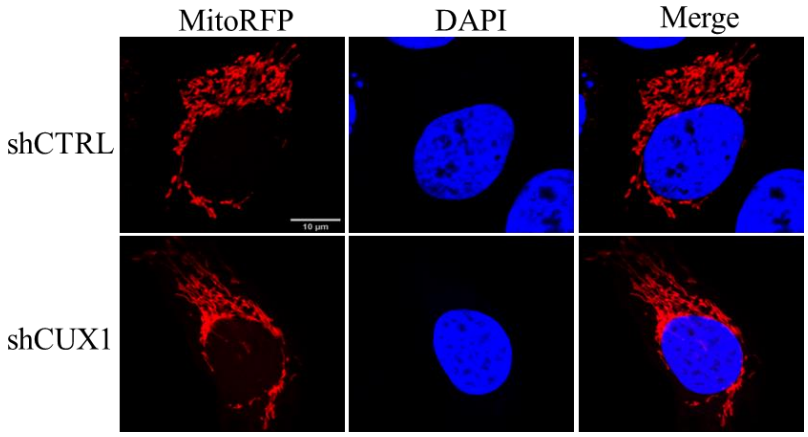


Figure 3.1 Inhibition of CUX1 expression alters the mitochondrial morphology. HeLa, shCTRL, and shCUX1 cells were transfected with Mito-RFP and mitochondrial morphology by fluorescence microscopy was observed (Scale bar: 10µm).

3.3.2 Cux1 regulates the Mfn2 levels

As Cux1 is involved in regulating mitochondrial morphology, we wanted to know whether it is due to altered mitochondrial proteome in the absence of Cux1. To determine the steady state levels of the mitochondrial proteins in the absence of Cux1, cell lysates were prepared either from HEK293T cells or Cux1 knockdown stable cell lines. Immunoblotting was performed using antibodies that were specific for various mitochondrial compartments after cell lysates were separated on SDS-PAGE. Interestingly, we didn't find any difference in TOMM70 (OMM), ANT2 (IMM), TFAM and HSP70 (matrix) protein levels, indicating that there is no change in the mitochondrial proteome. We further isolated mitochondria from control and CUX1 knockdown cells, separated on SDS-PAGE and immunoblotted with different sub-compartmental mitochondrial proteins (Figure 3.2 A). We find there is no reduction in the protein levels of outer membrane TOMM20, VDAC, and TOMM40

Since Mfn2 is involved in alteration of mitochondrial morphology, we also examined the expression of Mfn2 along with other mitochondrial dynamics regulatory protein- Drp1. Surprisingly, inhibition of CUX1 expression drastically reduced the Mfn2 levels, whereas Drp1 levels were not altered. Mfn2 is known to present in the mitochondria and endoplasmic reticulum. Since Cux1 localizes to mitochondria, we speculated that reduction in total Mfn2 is due to mitochondria localized Mfn2 rather than ER-localized Mfn2. To test the decrease in mitochondrial Mfn2, we isolated mitochondria from control and CUX1 stably knockdown HEK293T cells and examined the Mfn2 and Dpr1 levels by immunoblotting. Surprisingly, Mfn2 and Drp1 levels were not altered in isolated mitochondria (Figures 3.2 B &C). Collectively, these findings suggest that CUX1 knockdown alters the levels of Mfn2 in whole cell lysates. However, it doesn't alter the mitochondrial localized Mfn2 protein levels.

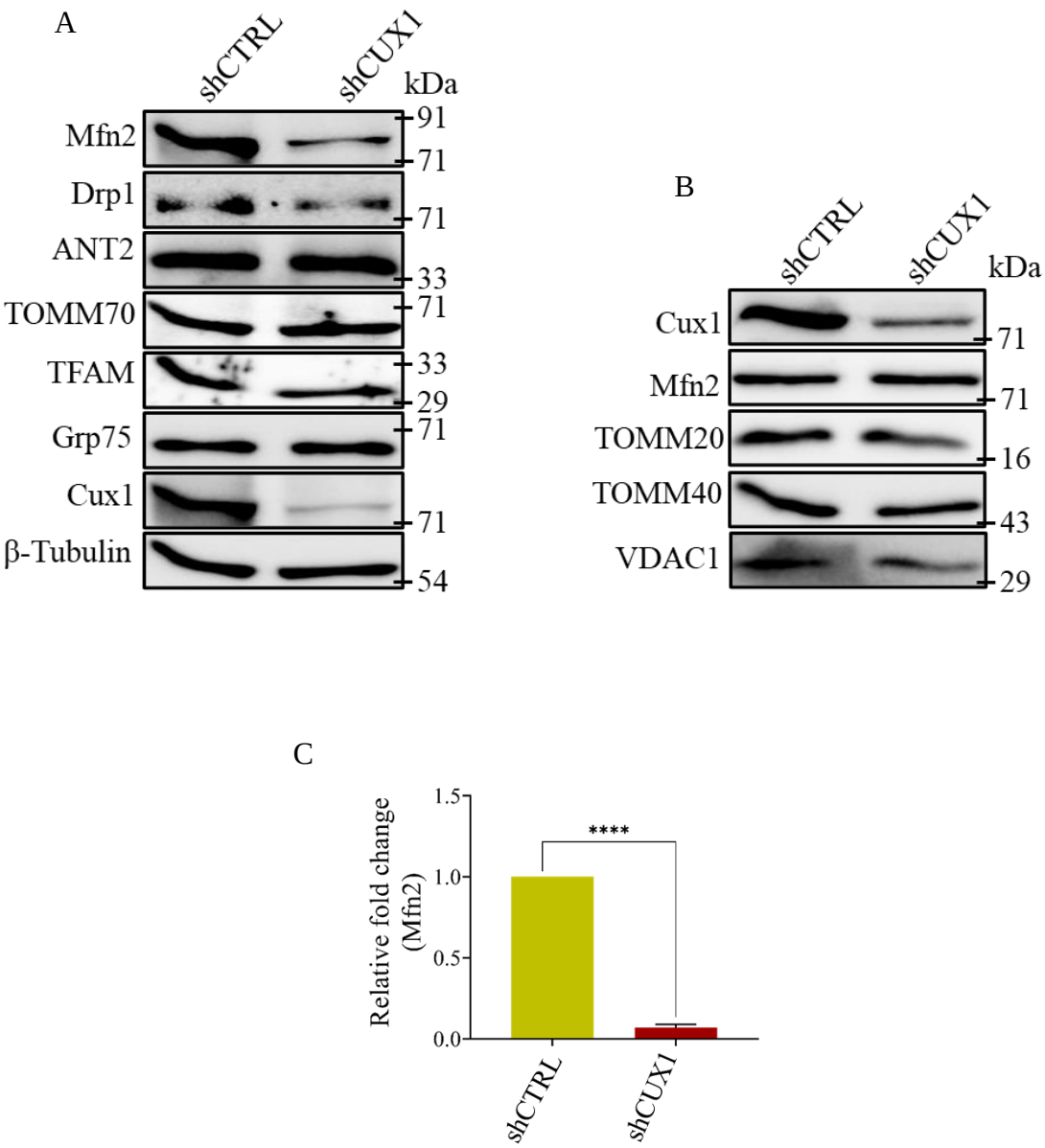


Figure 3.2 Cux1 modulates the Mfn2 expression. (A) Different mitochondrial protein steady levels in shCTRL and shCUX1 were assessed by immunoblotting. (B) Different mitochondrial protein levels were analyzed in shCTRL and shCUX1 by immunoblotting. (C) Representative graph for the quantification of Mfn2 from three independent experiments of B. CUX1 knockdown (shCUX1) and control (shCTRL) sample sets were compared using Student t-test. (*, p< 0.05; **, p<0.01; ***, p<0.001).

3.3.3 Cux1 Interacts with Mfn2

Given the depleted levels of Mfn2 in Cux1 knockdown cells, we intended to know whether Cux1 interacts with Mfn2. To test this phenomena, HEK293T cells transfected either with pCDNA3.1-

2XFlag or pCDNA3.1-CUX1-2XFLAG. After 48 hr of post-transfection, cells were processed for the FLAG pull-down and analyzed for the Mfn2 by immunoblotting. We find the presence of Mfn2 protein in FLAG pull-down assay indicating the interaction of Mfn2 with Cux1(Figure 3.3 A). Further, we also carried out co-immunoprecipitation with the Cux1 specific antibody and find the presence of Mfn2 in the immunoprecipitant indicating the interaction of Mfn2 with Cux1 (Figure 3.3 B). Together, the FLAG pull-down and co-immunoprecipitation studies suggest that mitochondrial protein Cux1 interacts with Mfn2.

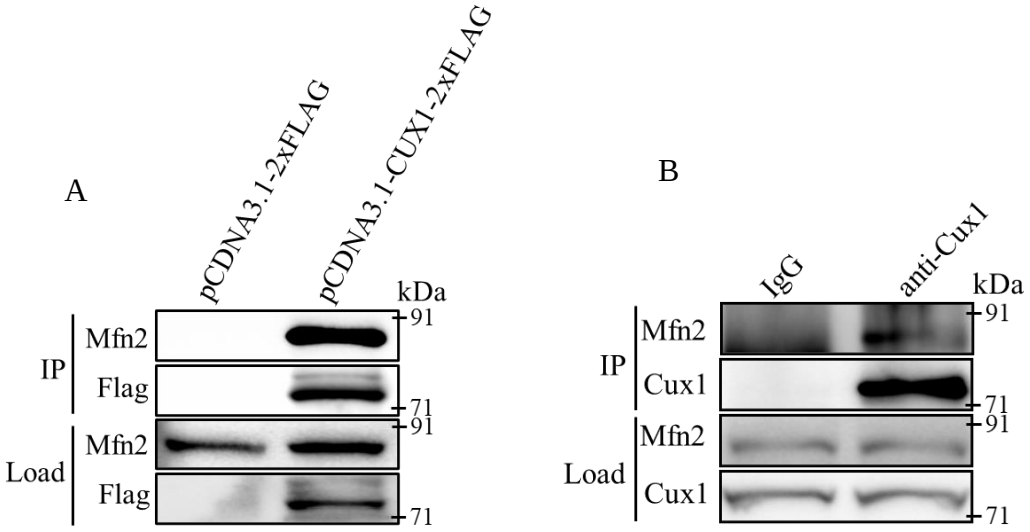


Figure 3.3 Cux1 interacts with Mfn2 in vivo. (A) HEK293T cells were lysed and subjected to immunoprecipitation using an anti-Cux1 antibody. Immunocomplexes were analyzed by immunoblotting using anti-Cux1 and anti-Mfn2 antibodies. (B) HEK293T cells were transiently transfected with control and CUX1-FLAG vector. Cell lysates were subjected to Flag pulldown using anti-FLAG M2 magnetic beads. Eluants were analyzed by immunoblotting using Flag and Myc tag-specific antibodies.

3.3.4 Cux1 is an ER Stress-responsive gene

Mfn2 is known to regulate the ER-mitochondrial interaction by forming homodimer or heterodimer with Mfn1. Reduced Mfn2 expression disrupts ER-mitochondrial communication and induces ER stress, which results in ER stress-induced cell death (Ngoh, Papanicolaou et al. 2012). Since Cux1 interacts and regulates the levels of Mfn2, we asked whether Cux1 also responds to ER stress. To elucidate this hypothesis, U2OS cells were treated with ER stress inducer tunicamycin (TN) in a dose-dependent and time-dependent manner and evaluated the

Cux1 levels by western blotting. Surprisingly, Cux1 levels were elevated in both time and dose-dependent manner in presence of tunicamycin (Figure 3.4 A & B). These results indicate that Cux1 responds to ER stress and it is probably an ER stress-responsive gene.

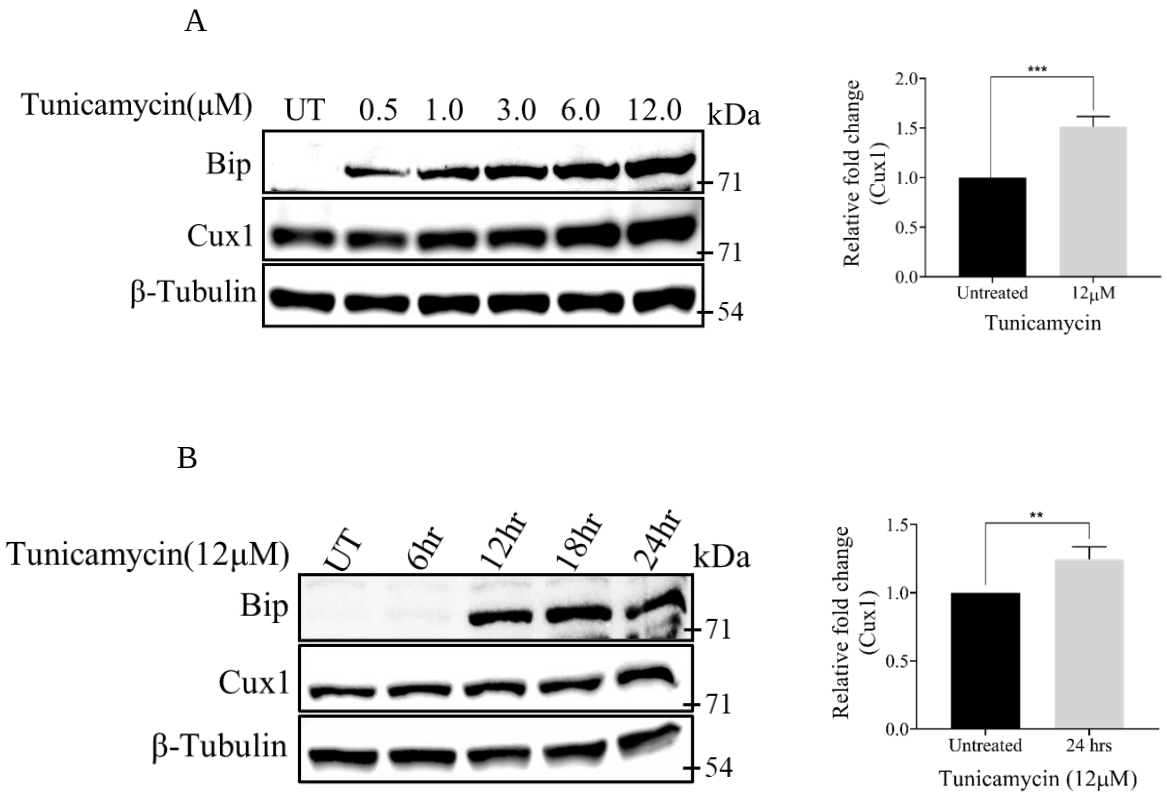
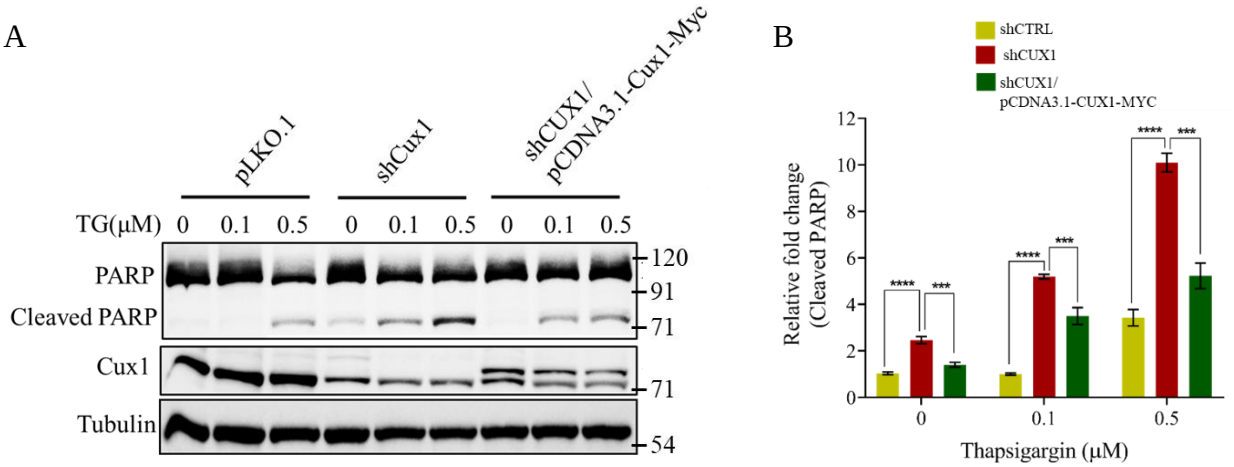


Figure 3.4 Cux1 is an ER stress-responsive gene. (A) U2OS cells were treated with tunicamycin in a concentration-dependent manner for 8 hr. Cux1 and Bip levels were analyzed by immunoblotting. Representative graph for the quantification of three independent experiments. Untreated and 12 μM treated sample sets were compared using a student t-test (*, p< 0.05; **, p<0.01; ***, p<0.001). (B) U2OS cells were treated with tunicamycin (12 μM) in a time-dependent manner for 24 hr. Cux1 and Bip levels were analyzed by immunoblotting. Representative graph for the quantification of three independent experiments. Untreated and 24 hrs timepoint sample sets were compared using Student t-test (*, p< 0.05; **, p<0.01; ***, p<0.001).

3.3.5 Cux1 regulates ER stress-induced cell death.

Mfn2 has been linked to ER-mitochondrial interactions, and its levels rise in response to ER stress. (Ngoh, Papanicolaou et al. 2012). The abrogated ER-mitochondrial interaction in Mfn2 knockout leads to increased ER stress sensitivity, which eventually triggers apoptotic cell death (Ngoh, Papanicolaou et al. 2012). As Cux1 interacts and influences the levels of Mfn2, we questioned its role in ER stress-induced cell death as well. To understand this, we examined the cleaved PARP levels, which is also an indicator of apoptotic cell death. Control and CUX1 knockdown cells were treated with Thapsigargin (TG) for 27 hr and checked for the cleaved PARP levels by immunoblotting as indicated in methods. Interestingly, we observed elevated levels of cleaved PARP in CUX1 knockdown, whereas CUX1 overexpression reduced the PARP cleavage (Figure 3.5 A & B). These results suggest that Cux1 knockdown aggravates ER stress-induced cell death. PARP is one of the many substrates of caspase-3, which acts as a marker for caspase activity. To validate the apoptotic cell death, we performed a fluorescent substrate-based caspase-3 activity assay. Consistent with the previous result, we observed that inhibition of CUX1 expression enhanced the caspase-3 activity, whereas over-expression reduced the activity (Figure 3.5 C). These findings suggest that inhibition of CUX1 expression elevates ER stress-induced caspase-3 activity.

Collectively, these findings suggest that CUX1 knockdown amplifies the ER stress induced apoptotic cell death.



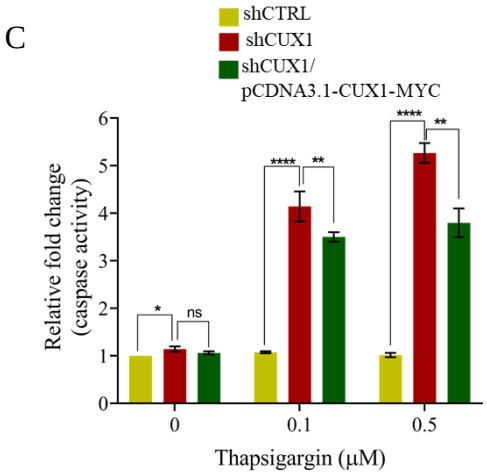


Figure 3.5 Cux1 regulates ER stress induced cell death. (A) shCTRL (control), shCUX1 (CUX1 knockdown), and shCUX1/CUX1-MYC (Endogenous CUX1 knockdown and ectopic CUX1 over expression) U2OS cells were treated with Thapsigargin at indicated concentrations for 27 hr. Cux1 and cleaved PARP levels were analyzed by immunoblotting. (B) Representative graph for the quantification of three independent experiments. Student t-test performed between shCTRL (control), shCUX1 (CUX1 knockdown), and shCUX1/CUX1-MYC (Endogenous CUX1 knockdown and ectopic CUX1 over expression) sample sets (*, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$). (C) shCTRL (control), shCUX1 (CUX1 knockdown), and shCUX1/CUX1-MYC (CUX1 knockdown along with CUX1 over expression) U2OS cells were treated with Thapsigargin at indicated concentrations for 27 hr. Lysates were subjected to caspase-3 activity using Ac-DEVD-AFC fluorescent substrate. Representative graph for the quantification of three independent experiments. Student t-test performed between shCTRL (control), shCUX1 (CUX1 knockdown), and shCUX1/CUX1-MYC (Endogenous CUX1 knockdown and ectopic CUX1 over expression) sample sets (*, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$).

3.4 Discussion

It is a well-known fact that mitochondria interact with many other organelles to exchange materials between the organelles., The well-studied inter-organellar interaction is between ER and mitochondria. ER-mitochondrial interaction is mediated by different tethering complexes, which help in the exchange of lipids, ATP, and calcium. Disruption of these interactions is well documented in various diseases such as cancer, neurological disorders, and diabetes. Understanding of such interactions are important to understand the pathophysiology of a disease.

Here, we found, that Cux1 (p75) involved in tethering of ER and mitochondria. We found that Cux1 knockdown shows elongated mitochondrial morphology A similar phenotype is observed in Mfn2 knockout cells as well. Then we went on to check the steady-state levels of different mitochondrial proteins. In CUX1 knockdown cells, we didn't observe any significant difference in the protein levels except TFAM. In addition, we checked the two prominent mitochondrial dynamics proteins, Mfn2 and Drp1. Surprisingly, the Mfn2 levels were drastically reduced in whole cell extracts. However, the purified mitochondrial fraction didn't show the reduction in Mfn2 levels. This may be due to the depletion of ER-localized Mfn2, but not the mitochondrial localized Mfn2 as purified mitochondrial fraction did not show any reduction in Mfn2. Further, using co-immunoprecipitation and pull-down studies, we found that interaction of Cux1 with Mfn2. It is known that ER stress elevates the Mfn2 levels, similarly we find the upregulation of Cux1 levels when cells were challenged with thapsigargin. These results show that Cux1 is an ER stress-responsive gene. Mfn2 depletion or genetic ablation in mouse embryonic fibroblasts elevated the ER stress and aggravated ER stress-induced apoptosis (Ngoh, Papanicolaou et al. 2012). Cux1 responds to ER stress and plays a role in the regulation of Mfn2 levels. We were intrigued about its role in ER stress-induced cell death. Interestingly, Cux1 knockdown aggravated the ER stress-induced cell death, examined by increased caspase-3 activity and PARP cleavage in response to ER stress. On the other hand, CUX1 overexpression reduced PARP cleavage and caspase-3 activity.

Collectively, these results suggest that Cux1 interacts with Mfn2 (possibly ER localized Mfn2) and its knockdown enhances ER stress-induced cell death. Further studies are required to understand the role of ER localized Mfn2 and its interaction with Cux1 and their role in ER-mitochondrial tethering and ER stress induced cell death.

Chapter 4

Role of Cux1 (p75) in Mitophagy

4.1 Introduction

Mitochondria are versatile organelles that serve as a hub for metabolic and signaling platforms and are involved in a variety of critical cell processes, including ATP generation, fatty acid oxidation, calcium buffering, iron-sulfur cluster biosynthesis, and ROS generation and maintenance (Spinelli and Haigis 2018). On account of its pivotal role, cells have evolved various safeguard mechanisms to reduce or eliminate the damaged mitochondria.

Antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, thioredoxin reductase, and methionine-sulfoxide reductase serve as the first line of defense against ROS-mediated protein damage (Napolitano, Fasciolo, et al. 2021). Second, the damaged proteins in the mitochondria are degraded by different mitochondrial proteases situated in each compartment. For instance, mislocalized proteins on OMM are degraded by Msp1 in yeast and ATAD1 in mammals (Matsumoto, Nakatsukasa et al. 2019). Apart from this, E3 ligase MITOL (MARCH 5) ubiquitinates misfolded proteins on OMM for degradation (Nagashima, Tokuyama et al. 2014). The protein-rich inner membrane region contains i-AAA proteases such as YEM1L, OMA1, and ATP23 to maintain quality control (Song, Herrmann, et al. 2021). In the matrix, Lon and CLPXP are the major proteases involved in the removal of damaged proteins. Third, mitochondria undergo constant fusion and fission events to repair damaged regions of the mitochondria (Ren, Chen et al. 2020). Fourth, the damaged portion of mitochondria can bud off and form mitochondria-derived vesicles (MDV), which further fuse with lysosomes for degradation (McLelland, Soubannier et al. 2014).

Lastly, mitophagy is the ultimate safeguard for mitochondrial homeostasis and maintenance of the healthy pool of mitochondria in the cell (Pickles, Vigie et al. 2018). In mammals, mitophagy happens in two ways. First, PINK1/Parkin-independent mitophagy, which is mediated by the receptor proteins BNIP3, BNIP3L, BCL2-L13, FUNDC1 and FKBP8. Upon rupture of the outer mitochondrial membrane, PHB2 (Wei, Chiang, et al. 2017) and inner membrane enriched cardiolipin is capable of interacting directly with LC3 and acting as mitophagy receptors (Chu, Ji et al. 2013). Second, in PINK1/Parkin-dependent mitophagy, upon depolarization, PINK1 accumulates on mitochondria and forms the PINK1-TOM complex (Lazarou, Jin, et al. 2012). These events lead to the recruitment of E3 ubiquitin ligase PARKIN which ubiquitinates OMM, followed by the recruitment of autophagy receptors Optineurin and NDP52, then eventually engulfment by autophagosomes.

Perturbation in the PINK1 stability leads to many pathophysiological conditions, such as aging, cancer, cardiovascular diseases, and neurodegenerative diseases. Thus, it is necessary to understand the factors regulating PINK1 stability. Recent reports suggest a possible link between Alzheimer's disease and Cux1 (Jaiswal 2022). Mitochondrial outer membrane proteins such as TOM complex, Sam50, and VDAC1 are known to involve directly or indirectly in the regulation of PINK1 mediated mitophagy (Geisler, Holmstrom et al. 2010, Jian, Chen et al. 2018). Since Cux1 localizes to the outer mitochondrial membrane like Pil1, which has been shown to regulate mitophagy in yeast via direct interaction with ATG32, we sought to determine the role of Cux1 in PINK1 stability in this study.

4.2 Methodology

4.2.1 Cell culture and DNA transfection

The cell lines HEK293T and HeLa used in this study were purchased from ATCC. Cells were cultured in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, and Antibiotic-Antimycotic (Thermo fisher scientific) in a 5% CO_2 incubator at 37°C. Cells were transfected with desired expression vectors using Lipofectamine 2000 (Invitrogen) at 60% confluence, according to the manufacturer's instructions.

4.2.2 Cloning and plasmid construction

Performed as described in the section 2.2.3.

4.2.3 Stable cell line generation by lentiviruses

At 50-60% confluence of HEK293T cells were transfected with shCTRL or shCUX1 plasmids along with lentiviral packaging plasmids (pVsVg, pΔR, pRev). 48 hr after transfection, the viral soups were collected and filtered using a 0.45 µm filter. Filtered viral soup and fresh complete media were added in a 1:1 ratio to the desired cell line and incubated for 24 hr. Two rounds of transfections were performed to improve efficiency. Stable cells were selected using puromycin (1mg/ml). The knockdown of the Cux1 was evaluated by western blot.

4.2.4 Immunoblotting

Cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% deoxycholic acid, 1% Triton X-100, 150 mM NaCl, 0.25 mM EDTA, and 0.1% SDS) containing a cocktail of protease inhibitors (Complete Cocktail, Roche). Protein concentrations were measured by Bradford

reagent. An equal amount of protein was taken from each sample and boiled at 95°C with 1 X Laemmli buffer for 5 min. Using a Bio-Rad transfer unit, the proteins separated by SDS-PAGE were transferred onto the nitrocellulose membrane. The protein transfer onto the membrane was confirmed with ponceau staining. The membrane was blocked for 1 hr with 5% skimmed milk powder in 1XTBST before incubating with the specific antibody overnight at 4 °C on the rocker. After the incubation, the blots were washed with 1XTBST three times, each wash for 5 min and then incubated for 1 hr with HRP-conjugated secondary antibody at room temperature. The blots were developed by using an ECL detection kit (advansta) and captured using Bio-Rad Versadoc.

4.2.5 Antibodies and Reagents

The primary antibodies used in this study were anti-Cux1(11733-1-AP), anti-PINK1 (bc100-494), anti- Sam50 (ab133709) and anti-GAPDH (60004-1-Ig). Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich.

4.2.6 Isolation of mitochondria from cell line

Cells were collected, and the residual medium was removed by washing twice with 1XPBS buffer. Cells were resuspended in mitochondria isolation buffer (70 mM mannitol, 210 mM sucrose, 1.5 mM MgCl2, 20 mM HEPES pH 7.5,1 mM EDTA pH 8.0 and 1 mM EGTA) and incubated on ice for 1 hour with swirling in every 15 min. The cell suspension was homogenized thrice with a polytron homogenizer, with a 15 rpm pulse, then subjected to dounce homogenization. To pellet down the cell debris, the cell suspension was centrifuged at 2700 rpm for 10 min. Supernatant was collected and centrifuged for 15 min at 9500 rpm at 4°C to pellet down the mitochondria. After washing twice, the mitochondrial pellet was resuspended in mitochondrial resuspension buffer (10 mM HEPES-KOH pH 7.4, 5 mM magnesium acetate, and 250 mM sucrose).

4.2.7 Blue-Native PAGE (BN-PAGE)

Blue native PAGE of mitochondria was performed as described (Wittig, Braun et al. 2006). Briefly, mitochondria were solubilized in a buffer containing digitonin (final concentration of 6.0 g/g) and subjected to centrifugation at 20,000 g for 20 min. Solubilized membrane complexes in the supernatant were collected and separated on 4-13% gradient native gel. The complexes were transferred onto the PVDF membrane and analyzed for the protein of interest using western blot.

4.3 Results

4.3.1 Cux1 Regulates the Pink1 Stability.

The stability of the PINK1 on damaged mitochondria is the key event for triggering mitophagy in mammals (Matsuda, Sato et al. 2010). Being a mitochondrial outer membrane protein and a partial homologue of Pil1, which is involved in mitophagy, we asked if Cux1 has any role in the regulation of PINK1 stability and mitophagy. To test this hypothesis, control and Cux1 knockdown stable cell lines were treated with 10 µM CCCP (mitochondrial uncoupler) in a time-dependent manner. Post-treatment cells were harvested, and lysates were subjected to western blot as described in the methods. Surprisingly, immunoblot analysis showed that the PINK1 (~63 kDa) stability drastically reduced in Cux1 knockdown cells (Figure 4.1 A). These results suggest the possible role of Cux1 in PINK1 stabilization under mitochondrial depolarization.

If Cux1 is involved in the regulation of PINK1 stability, the overexpression of CUX1 should restore the PINK1 levels. To test this hypothesis, control, Cux1 knockdown, and Cux1 overexpressed cells were treated with 10 µM CCCP for 4 hr. Following CCCP treatment, cells were subjected to immunoblot analysis for the PINK1 levels. Cux1 overexpression rescued the PINK1 levels; indeed, the PINK1 levels were dramatically increased (Figure 4.1 B). Further it establishes Cux1's contribution to PINK1 stability during mitochondrial depolarization. Together, these findings suggest that Cux1 regulates the PINK1 stability during mitochondrial depolarization.

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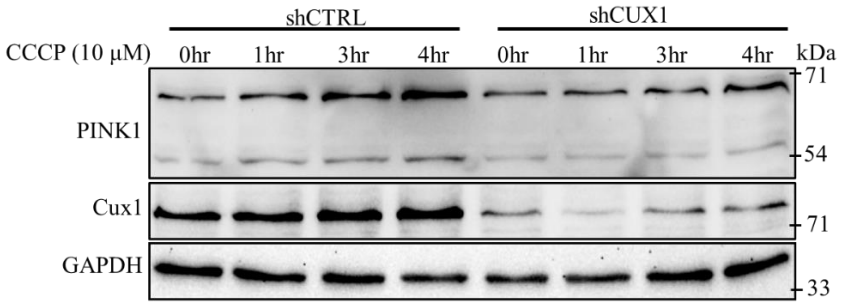


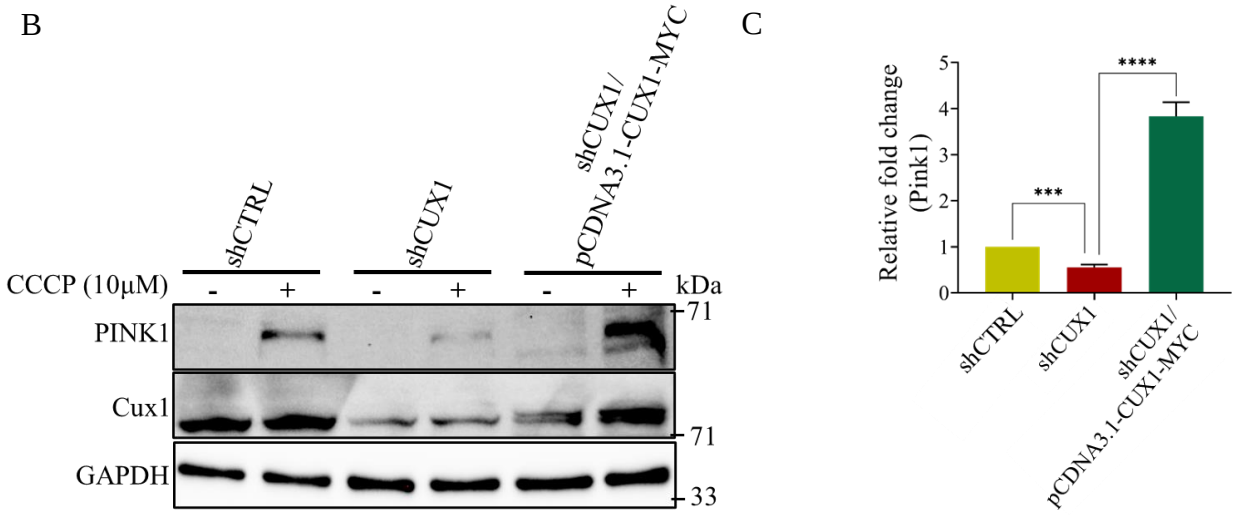


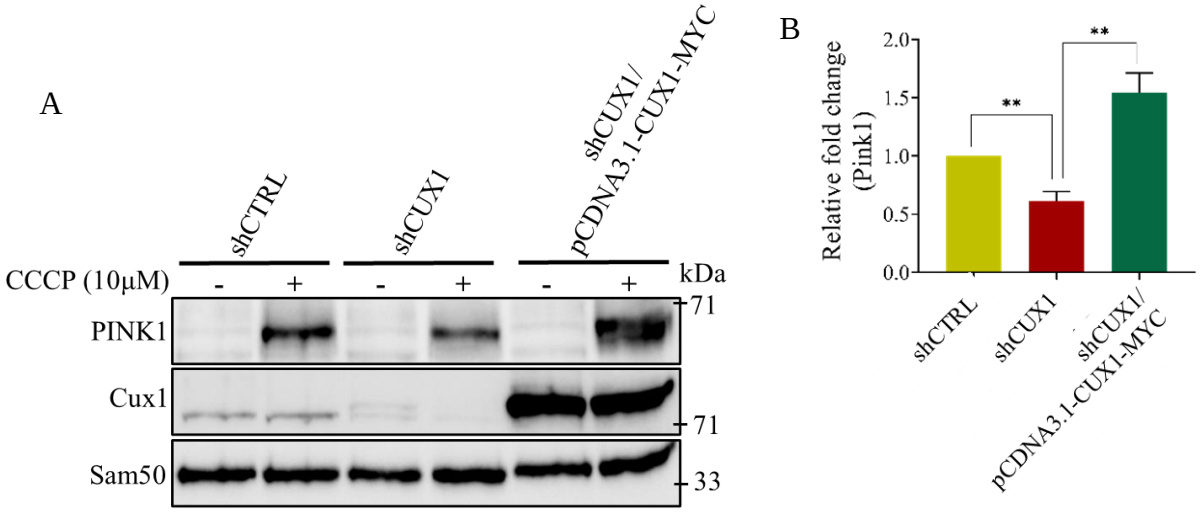
Figure 4.1. Cux1 regulates the PINK1 stability. (A) HEK293T, shCTRL and shCUX1 cells were treated with 10μM CCCP in time dependent manner. Cux1 and PINK1 levels were analyzed by immunoblot. (B) HEK293T, shCTRL (control), shCUX1 (CUX1 knockdown) and shCUX1/CUX1-MYC (Endogenous CUX1 knockdown and ectopic CUX1 over expression) were treated with 10μM CCCP for 4hrs. Cux1 and PINK1 levels were analyzed by immunoblotting. (C) Representative graph for the quantification of three independent experiments of B. Student t-test performed between shCTRL (control), shCUX1 (CUX1 knockdown), and shCUX1/CUX1-MYC (Endogenous CUX1 knockdown and ectopic CUX1 over expression) sample sets (*, p< 0.05; **, p<0.01; ***, p<0.001).

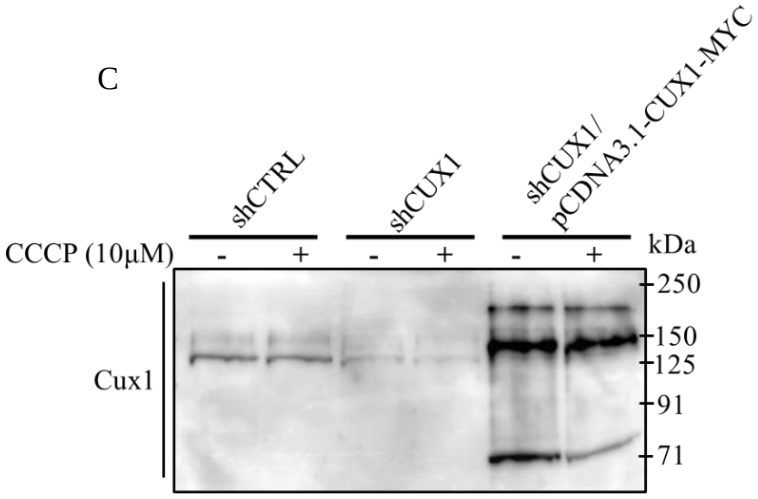
4.3.2 Cux1 Regulates the PINK1 Stability on Mitochondria.

Though PINK1 stabilizes at the cellular level, its recruitment to the mitochondria is the key factor for triggering mitophagy. To elucidate if Cux1 stabilizes PINK1 on depolarized mitochondria, HEK293T control, CUX1 knockdown, and overexpression cells were treated with 10 μM CCCP for 4 hrs. Cells were harvested and mitochondria isolated as detailed in the methods. The mitochondrial fractions were analyzed for PINK1 stability (~63-kDa) using western blot as described in the methods. Cux1 depletion significantly reduced PINK1 stability on mitochondria after CCCP treatment. Additionally, Cux1 overexpression could rescue the PINK1 (~63-kDa) levels in mitochondria (Figure 4.2 A& B). We also checked for the difference in Cux1 monomeric and dimeric forms under non reducing conditions, but we didn't observe any significant difference (Figure 4.2 C).

To validate further stabilization of PINK1 on the mitochondria, we performed the carbonate extraction of the isolated mitochondria from control, Cux1 knockdown, and overexpression cells treated with or without CCCP for 4 hr. The carbonate extraction of mitochondria enriches the mitochondrial membrane proteins. PINK1 levels were examined by western blot as detailed in the methods. In line with the previous observations, Cux1 knockdown significantly reduced the membrane-stabilized PINK1 on mitochondria, whereas Cux1 overexpression restored the stability (Figure 4.2 D& E).

Collectively, these results indicate that Cux1 controls the stability of PINK1 on the mitochondrial membrane during depolarization.





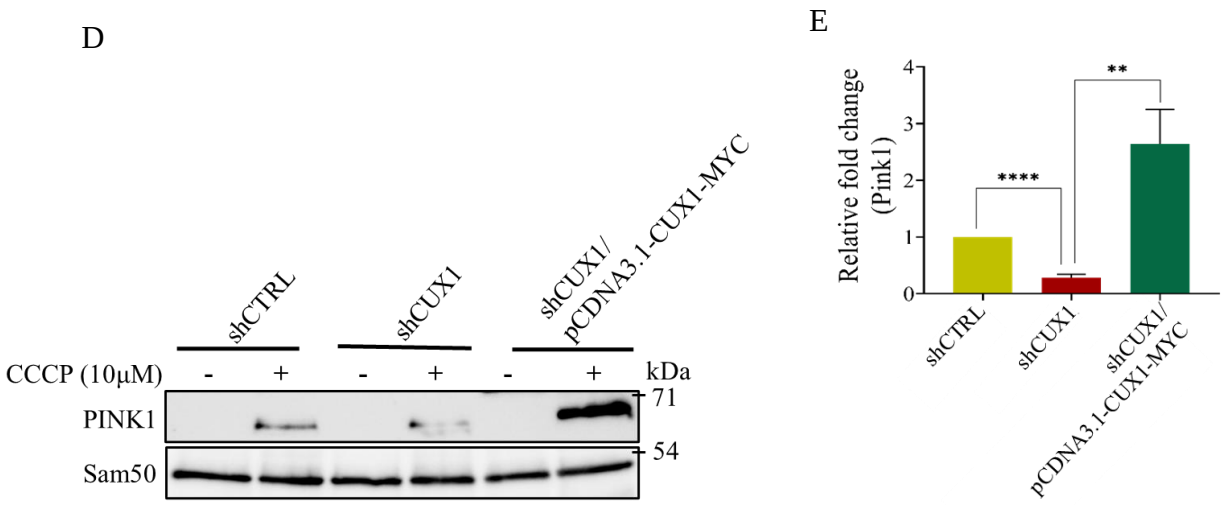


Figure 4.2. PINK1 stability on mitochondria is regulated by Cux1. (A) HEK293T, shCTRL (control), shCUX1 (CUX1 knockdown) and shCUX1/CUX1-Myc (endogenous CUX1 knockdown and ectopic CUX1 overexpression) were treated with 10 μM CCCP for 4 hr and isolated the mitochondria. Mitochondrial lysates were analyzed for PINK1 and Cux1 by immunoblotting. Sam50 used as loading control. (B) Representative graph for the quantification of three independent experiments of A. Student t-test performed between shCTRL (control), shCUX1 (CUX1 knockdown), and shCUX1/CUX1-MYC (endogenous CUX1 knockdown and ectopic CUX1 overexpression) sample sets (*, p< 0.05; **, p<0.01; ***, p<0.001). (C) Samples mentioned in A were separated on non-reducing gel and analyzed for Cux1 migration by immunoblotting with Cux1 specific antibody. (D) HEK293T, shCTRL (control), shCUX1 (CUX1 knockdown) and shCUX1/CUX1-MYC (endogenous CUX1 knockdown and ectopic CUX1 overexpression) were treated with 10 μM CCCP for 4 hr. Mitochondria were isolated and subjected to carbonate extraction and PINK1 levels were monitored by immunoblotting. Sam50 was used as a loading control. (E) Representative graph for the quantification of three independent experiments of A. Student t-test performed between shCTRL (control), shCUX1 (CUX1 knockdown), and shCUX1/CUX1-MYC (endogenous CUX1 knockdown and ectopic CUX1 overexpression) sample sets (*, p< 0.05; **, p<0.01; ***, p<0.001).

4.3.3 Cux1 regulates the higher molecular weight PINK1 complex formation

The stabilized Pink1 is known to form a higher molecular weight complex around 850 kDa on depolarized mitochondria to activate the downstream signaling cascade (Okatsu, Uno et al. 2013). We wondered if Cux1 is also involved in the regulation of 850 kDa PINK1 complex formation on mitochondria. To address this hypothesis, HEK293T control, Cux1 knockdown, and Cux1

overexpression cells were treated with or without CCCP for 4 hr. Post-treatment, mitochondria were isolated, and BN-PAGE was used to separate membrane complexes, which were then transferred to the PVDF membrane as detailed in the methods. The levels of PINK1 and Cux1 complexes were examined by immunoblotting. Surprisingly, Cux1 knockdown drastically reduced the 850 kDa PINK1 complex formation under mitochondrial depolarization (Figure 4.3).

In conclusion, this finding suggests that Cux1 positively regulates the formation of the 850 kDa PINK1 complex on the mitochondrial membrane.

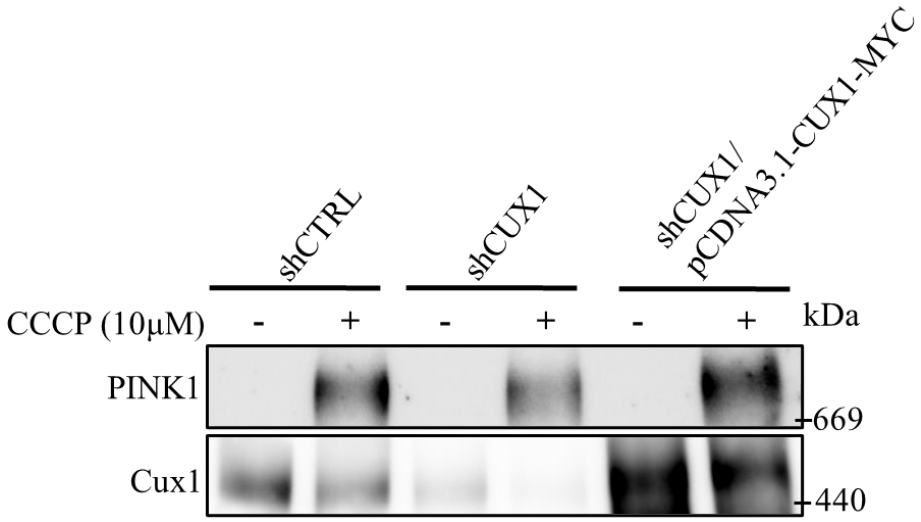


Figure 4.3. Cux1 regualtes the PINK1 complex formation. (A) HEK293T, shCTRL (control), shCUX1 (CUX1 knockdown) and shCUX1/CUX1-MYC (endogenous CUX1 knockdown and ectopic CUX1 overexpression) were treated with 10 μM CCCP for 4 hr and proceeded for isolation of mitochondria. Mitochondrial fractions were subjected to Blue native PAGE. PINK1 and Cux1 levels were analyzed by immunoblotting.

4.4 Discussion

Mitophagy plays a vital role in maintaining a healthy pool of mitochondria in the cell. The molecular mechanism and regulators of mitophagy in mammals are well-reported. In mammals, mitophagy occurs mainly through the PINK1/Parkin-dependent pathway, where PINK1 gets stabilized on depolarized mitochondria and recruits Parkin to mark the mitochondria by ubiquitinylation, which is further engulfed by the autophagosome and targeted to the lysosome for degradation (Dikic and Elazar 2018). The stabilization of PINK1 on mitochondria is the crucial step in triggering mitophagy. PINK1 stability on mitochondria is regulated by different mitochondrial proteins, including SAM50, TOMM40, and MIA40 (Jian, Chen et al. 2018, Gao, Zhang et al. 2020, Ng, Wai et al. 2021). Though it is well studied, it is an unavoidable fact that the unidentified PINK1 stability regulators are yet to be explored.

Here, we identified one of the isoforms of the homeodomain transcription factor Cux1 which regulates the stability of PINK1 under mitochondrial depolarization. Under Cux1 knockdown, the stability of the PINK1 was reduced with CCCP treatment in a time-dependent manner. In addition, the overexpression of Cux1 could rescue the PINK1 stability in Cux1 knockdown cells. Further, carbonate extraction of isolated mitochondria from Cux1 knockdown and overexpression clearly shows the differential stability of PINK1 on depolarized mitochondria. We also confirmed the reduction in the 850 kDa complex of PINK1 under Cux1 knockdown, which is rescued by overexpression of CUX1.

Altogether, these results suggest that Cux1 stabilizes PINK1 under mitochondrial depolarization. Further studies are required to understand the role of Cux1 in mitophagy.

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Publications

JBC RESEARCH ARTICLE

Eisosome protein Pil1 regulates mitochondrial morphology, mitophagy, and cell death in *Saccharomyces cerevisiae*

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Amita Pal, Arun Kumar Paripati, Pallavi Deolal, Arpan Chatterjee, Pushpa Rani Prasad, Priyanka Adla, and Naresh Babu V. Sepuri*

From the Department of Biochemistry, University of Hyderabad, Hyderabad, Telangana, India

Edited by Ursula Jakob

Mitochondrial morphology and dynamics maintain mitochondrial integrity by regulating its size, shape, distribution, and connectivity, thereby modulating various cellular processes. Several studies have established a functional link between mitochondrial dynamics, mitophagy, and cell death, but further investigation is needed to identify specific proteins involved in mitochondrial dynamics. Any alteration in the integrity of mitochondria has severe ramifications that include disorders like cancer and neurodegeneration. In this study, we used budding yeast as a model organism and found that Pil1, the major component of the eisosome complex, also localizes to the periphery of mitochondria. Interestingly, the absence of Pil1 causes the branched tubular morphology of mitochondria to be abnormally fused or aggregated, whereas its overexpression leads to mitochondrial fragmentation. Most importantly, *pil1*Δ cells are defective in mitophagy and bulk autophagy, resulting in elevated levels of reactive oxygen species and protein aggregates. In addition, we show that *pil1*Δ cells are more prone to cell death. Yeast two-hybrid analysis and co-immunoprecipitations show the interaction of Pil1 with two major proteins in mitochondrial fission, Fis1 and Dnm1. Additionally, our data suggest that the role of Pil1 in maintaining mitochondrial shape is dependent on Fis1 and Dnm1, but it functions independently in mitophagy and cell death pathways. Together, our data suggest that Pil1, an eisosome protein, is a novel regulator of mitochondrial morphology, mitophagy, and cell death.

Organelles have evolved a variety of stress response processes to maintain their proteostasis and cellular homeostasis. Mitochondrion, one of the essential organelles that carry out several important cellular processes, ensures its quality control through various pathways at the molecular, organellar, and cellular levels (1). Mitochondrial fission and fusion, generally referred to as mitochondrial dynamics, maintain mitochondrial integrity by regulating its size, shape, distribution, and connectivity. Mitochondrial dynamics regulate mitochondrial quality control, metabolism, apoptosis, mitophagy, and other essential processes. Fusion of mitochondria is required to mitigate the damage and nonfunctionality by mixing components and protecting them from autophagic degradation during starvation (2, 3). Fission helps produce new mitochondria and ensures quality control by removing damaged or unwanted mitochondria through mitophagy (4). A coordinated balance of fission and fusion is critical for maintaining mitochondrial biology and therefore a plethora of important cellular processes (5, 6). In *Saccharomyces cerevisiae*, Fzo1, Mgm1, and Ugo1 facilitate mitochondrial fusion. Outer membrane–anchored proteins, Fzo1p and Ugo1p, carry out the outer membrane fusion of adjacent mitochondria and inner membrane–anchored Mgm1 forms transcomplexes to tether the apposing inner membranes together (7–9). Dnm1, a dynamin-related GTPase, is the major protein in mitochondrial fission (10, 11). It is predominantly present in the cytosol and recruited to mitochondria *via* Fis1 (12). Dnm1 assembles into oligomers which form rings and spirals at the outer membrane of mitochondria. Recruitment of Dnm1 to the mitochondrial surface is mediated through two adaptor proteins, Mdv1 and Caf4 (13, 14). These four proteins together constitute the core proteins of the mitochondrial fission machinery. However, there is a possibility that other unidentified factors still exist that take part in mitochondrial dynamics (15). Several studies have shown that any dysregulation in mitochondrial dynamics leads to neuronal disorders like Alzheimer's, Parkinson's, and Huntington's (16–18). When the damage is beyond repair, mitochondria undergo mitophagy. Mitophagy is a selective autophagy where autophagosomes engulf the entire mitochondria and deliver them to the vacuole for their degradation (19–21). Mitophagy is required to eliminate the bad mitochondria from cells. Any kind of aberration in mitochondrial dynamics or mitophagy is harmful to cells. Mitochondrial dysfunction has also been linked with protein aggregation and reactive oxygen species (ROS) generation in cells which in turn leads to cell death.

Cellular organelles communicate with each other in order to cope with stress. Since the plasma membrane is positioned at the frontline to combat the external stress stimuli, it requires high degree of organization as it carries out a diverse array of functions and forms the protective barrier around the cell. The fungal plasma membrane is organized in lateral domains with specialized functions like cell wall synthesis, environmental sensing, nutrient uptake, secretion, and endocytosis (22–24). High-resolution electron microscopy of freeze-etched

* For correspondence: Naresh Babu V. Sepuri, nareshuohyd@gmail.com, nbvssl@uohyd.ernet.in.

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Kisspeptin preserves mitochondrial function by inducing mitophagy and autophagy in aging rat brain hippocampus and human neuronal cell line

Ushodaya Mattam[a,*,1], Noble Kumar Talari[a,1], Arun Kumar Paripati[a], Thanuja Krishnamoorthy[b], Naresh Babu V. Sepuri[a,**]

[a] Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500046, T.S., India
[b] Vectrogen Biologicals Pvt Ltd, Hyderabad, BioNEST, School of Life Sciences, University of Hyderabad, Hyderabad 500046, T.S., India

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ABSTRACT

It has become amply clear that mitochondrial function defined by quality, quantity, dynamics, homeostasis, and regulated by mitophagy and mitochondrial biogenesis is a critical metric of human aging and disease. As a consequence, therapeutic interventions that can improve mitochondrial function can have a profound impact on human health and longevity. Kisspeptins are neuropeptides belonging to the family of metastasis suppressors that are known to regulate functions like fertility, reproduction, and metabolism. Using SKNSH cell line, hippocampus explant cultures and hippocampus of aging Wistar rat models, we show that Kisspeptin-10 (Kp) induces autophagy and mitophagy *via* calcium, Ca^{2+}/CaM-dependent protein kinase kinase β (CaMKKβ), AMP-activated protein kinase (AMPK), and Unc-51 like autophagy activating kinase (ULK1) signaling pathway that is independent of mammalian target of rapamycin (mTOR). Intriguingly, Kp administration *in vivo* also results in the enhancement of mitochondrial number, complex I activity, and Adenosine Triphosphate (ATP) levels. This study uncovers potential effects of Kp in protecting mitochondrial health and as a possible therapeutic intervention to hippocampus associated impairments such as memory, cognitive aging, and other diseases linked to mitochondrial dysfunction.

1. Introduction

Autophagy is an evolutionary conserved intracellular catabolic process to eliminate protein aggregates that can trigger neurodegeneration, damaged organelles such as mitochondria, endoplasmic reticulum, and cellular pathogens. Autophagy was initially characterized as a bulk or non-selective degradative pathway that is induced by a myriad of stress conditions such as nutrient deprivation [1,2], oxidative stress [3], and hypoxia [4]. It is now apparent that autophagy is not restricted to stressed cells alone to conserve resources for survival but plays a central role in the maintenance of cellular homeostasis in normal cells. Autophagy is dedicated to the continuous enrichment and restoration of the intracellular pool of proteins, carbohydrates, and cellular organelles to maintain cellular homeostasis during opulent conditions. It also has a housekeeping function as it eradicates cells of damaged mitochondria (mitophagy), excess peroxisomes (pexophagy), and disease-causing pathogens (xenophagy) known as selective autophagy [5]. Autophagy is initiated by autophagosome formation induced by a serine-threonine protein kinase, unc-51 like kinase (ULK1) complex. The protein kinase activity of ULK1 is regulated by its upstream sensors, such as AMPK (the key energy sensor) and mTOR (the cell growth sensor). Upon starvation, AMPK phosphorylates ULK1 at position ser 555 to trigger autophagy while under nutrient rich conditions mTOR phosphorylates ULK1 at 757 position to inhibit autophagy. Selective autophagy requires the sequestration of specific cargo (p62 in case of mitophagy) by the organelle and its interaction with autophagy receptor protein, LC3, on the autophagosome. Autophagy utilizes the ubiquitin modifications as the degradation signal, and so p62 recognizes the poly-ubiquitylated mitochondria destined for autophagosome degradation during mitophagy [5]. Defects in the autophagy pathway are associated with various pathological conditions that include cancer, aging, infections, and neurodegenerative diseases like Parkinson's and Alzheimer's [6–9]. Several studies have demonstrated a substantial nexus between decreased autophagic activity, aging, and

* Corresponding author.
** Correspondence to: N.B.V. Sepuri, Laboratory of Mitochondrial Biology, Department of Biochemistry, School of Life sciences, University of Hyderabad, Hyderabad 500046, India.
E-mail addresses: mattamusha@gmail.com (U. Mattam), nbvssl@uohyd.ernet.in (N.B.V. Sepuri).
[1] Equally contributed to this work.

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Adaptation of Mge1 to oxidative stress by local unfolding and altered Interaction with mitochondrial Hsp70 and Mxr2

Srinivasu Karri[a], Swati Singh[b], Arun Kumar Paripati[a], Adinarayana Marada[a,c,1], Thanuja Krishnamoorthy[a], Lalitha Guruprasad[b], Dorairajan Balasubramanian[d], Naresh Babu V. Sepuri[a,*]

[a] Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India
[b] School of Chemistry, University of Hyderabad, Hyderabad 500046, India
[c] Institute for Biochemistry and Molecular Biology, University of Frieburg, Germany
[d] L.V Prasad Eye Institute, Banjara Hills Rd. No. 2, Hyderabad 500034, India

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ABSTRACT

Perturbations in mitochondrial redox levels oxidize nucleotide exchanger Mge1, compromising its ability to bind to the Hsp70, while the Mxr2 enzyme reduces the oxidized Mge1. However, the effects of persistent oxidative stress on Mge1 structure and function are not known. In this study, we show that oxidation-induced selective and local structural adaptations cause the detachment of Mge1 from Hsp70. Notably, persistent oxidative stress causes monomeric Mge1 to aggregate and to generate amyloid-type particles. Mxr2 appears to protect Mge1 from oxidative stress induced aggregation. We conclude that the Mxr2-Mge1-Hsp70 protein triad is finely regulated through structural alterations of Mge1 mediated by redox levels.

1. Introduction

The chaperone properties of two mitochondrial proteins, Hsp70 and Ssq1p are aided by their interaction with a mitochondrial matrix protein, a “co-chaperone”, called Mge1 (Schmidt et al., 2001). This molecule is the functional homolog in *Saccharomyces cerevisiae*, of the bacterial co-chaperone GrpE and the human GRPEL1. There are representative homologs in archaea, eubacteria and in chloroplasts as well (Ellis, 1993; Georgopoulos and Welch, 1993; Welch, 1993). The amino acid sequence of Mge1 is given in Fig. 1A. The first 45 residues represent the mitochondrial localization sequence and they are enzymatically cleaved, inserting the remaining chain in the matrix of the organelle (Yet, the individual residues are numbered based on the entire sequence). Given that aerobic metabolism occurs in the mitochondrion, oxidative stress to its components can lead to modification in the biochemical pathways therein (Schieber and Chandel, 2014; Meng et al., 2002; Allu et al., 2015). In one of our earlier papers, we had shown how Mge1 undergoes such an oxidative modification, particularly through the oxidation of its lone methionine residue in position 155 (Met155), leading the formation of methionine sulfoxide (or sulfone), and how this process is effectively reversed by the endogenous enzyme Methionine Sulfoxide Reductase 2 (abbreviated as Mxr2), both *in vivo* and *in vitro* (Allu et al., 2015).

Studies from our laboratory and of others have shown that thermal or oxidative stress inactivates the active dimeric form of Mge1/GrpE, which slows down the Hsp70 chaperone cycle (Moro and Muga, 2006; Marada et al., 2013). Gaining insight into the structure of Mge1 is of value in understanding the underlying mechanism behind the adaptation of Mge1 to oxidative stress and its interaction with Hsp70, and Mxr2.

In this study, we investigate the effect of varying levels of oxidative stress on the conformational features of the molecule Mge1. Towards this, we have used H_2O_2 as the oxidant, and analyzed its effect on the molecular structure of Mge1, using biophysical and biochemical methods, molecular dynamics simulations, atomic force microscopy, and co-immuno-precipitation methods to detect the structural alterations that direct and regulate the binding of Mge1 to Mxr2 and also to Hsp70. These studies reveal that oxidized Mge1 has an open conformation with an increase in surface exposure of its otherwise compactly packed residues. Oxidation-induced weakening of the structure occurs, and selective and local structural adaptations are noticed, allowing the detachment of Mge1 from Hsp70. In addition, we show that sequestration of Mxr2 by oxidized Mge1, or *vice versa*, is a protective mechanism, which precludes the aggregation of Mge1, thus

* Corresponding author.
E-mail address: nbvssl@uohyd.ernet.in (N.B.V. Sepuri).
[1] Present address

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Nuclear Transcription Factors in the Mitochondria: A New Paradigm in Fine-Tuning Mitochondrial Metabolism

Naresh Babu V. Sepuri, Prasad Tammineni, Fareed Mohammed, and Arunkumar Paripati

Contents

Abstract

Noncanonical functions of several nuclear transcription factors in the mitochondria have been gaining exceptional traction over the years. These transcription factors include nuclear hormone receptors like estrogen, glucocorticoid, and thyroid hormone receptors: p53, IRF3, STAT3, STAT5, CREB, NF-kB, and MEF-2D. Mitochondria-localized nuclear transcription factors regulate mitochondrial processes like apoptosis, respiration and mitochondrial transcription albeit being nuclear in origin and having nuclear functions.

N.B.V. Sepuri (✉) • P. Tammineni • F. Mohammed • A. Paripati
Department of Biochemistry, School of Life Sciences, University of Hyderabad, Telangana 500046, India
e-mail: nareshuohyd@gmail.com

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Charectorization of Mitochondrial localized novel protein Cux1(p75) and its role in ER stress and mitophagy

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