Role of hMIA40 (CHCHD4) in Mitochondrial electron transport chain biogenesis, protein import, and cellular iron homeostasis

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

T.VENKATARAMANA

Department of Biochemistry

School of Life Sciences

University of Hyderabad

Hyderabad-500046

INDIA

Enrolment No: 13LBPH09 JUNE 2018

University of Hyderabad

School Of Life Sciences

Department of Biochemistry

Hyderabad-500046, India

CERTIFICATE

This is to certify that this thesis entitled **"Role of hMIA40(CHCHD4) in Mitochondrial electron transport chain biogenesis, protein import, and cellular iron homeostasis"** submitted to the University of Hyderabad by **Mr. T. VENKATA RAMANA**, for the degree of Doctor of Philosophy, is based on the studies carried out by him under my supervision. I declare to the best of my knowledge that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

A. published as following research papers:

1. Anjaneyulu Murari, **Venkata Ramana Thiriveedi**, Fareed Mohammad, Viswamithra Vengaldas, Madhavi Gorla, Prasad Tammineni, Thanuja Krishnamoorthy and Naresh Babu V.Sepuri* *Human Mitochondrial MIA40 (CHCD4) is a component of Fe/S cluster export machinery*. Biochem J. 2015 Oct 15;471(2):231-41. doi: 10.1042/BJ20150012. Epub 2015 Aug 14. PMID: 26275620.

2. Adinarayana Marada, Praveen kumar Allu, Anjaneyulu Murari, **Venkata Ramana Thiriveedi**, Jayasree Danduprolu and Naresh Babu V.Sepuri* *Mge1, a nucleotide exchange factor of Hsp70, acts as an oxidative sensor to regulate mitochondrial Hsp70 function*. Mol Biol Cell. 2013 Mar;24(6):692-703. doi: 10.1091/mbc.E12-10-0719. Epub 2013 Jan 23. PMID: 23345595.

B. Presented in the following national and international conferences:

1. Poster presentation in ICCB-2018 conference on The Dynamic Cell: Molecules and network to form and function -2018 held at CCMB, Hyderabad, India.
2. Oral presentation in Mitochondria and Metabolism Network meeting held at IISER Pune Nov 2017.
3. Oral presentation Bio Quest 2017 held at University of Hyderabad, India.
4. Poster presentation in 8th international conference on Photosynthesis and Hydrogen energy Research for Sustainability -2017 held at University of Hyderabad, India

Further, the student has passed the following courses towards fulfilment of course work requirements for the award of Ph.D.

Course code	Name	Pass/ Fail	Credits
BC 801	Analytical Techniques	Pass	3
BC 802	Research Ethics and Management	Pass	2
BC 803	Biostatistics	Pass	2
BC 804	Lab Work	Pass	4

Prof.Naresh Babu. V. Sepuri

Supervisor

Head

Department of Biochemistry

Dean

School of Life Sciences

University of Hyderabad

School Of Life Sciences

Department of Biochemistry

Hyderabad-500046, India

DECLARATION

I hereby declare that the work presented in my thesis is entirely original, plagiarism free 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.

T.VENKATA RAMANA

Date

Prof. Naresh Babu V Sepuri

Supervisor

“This work is dedicated to my beloved Parents and family”

“Imagination is the highest form of research.”

Albert Einstein

Acknowledgements

I thank **ALMIGHTY GOD** for giving me a nice, caring and kind supervisor, **Prof. Naresh Babu V Sepuri** who provided an opportunity to work in his lab. I am grateful to my supervisor, for his immeasurable patience, invaluable guidance, constant support, and timely help during entire course of my research work. My sincere appreciation goes to him for his continuous help in all stages of the thesis preparation despite of his busy schedule. No words can adequately express my deep gratitude to my supervisor for all his guidance and kindness.

I am thankful to former Deans Prof. P. Reddanna, Prof. R. P. Sharma, Prof. Aparna Dutta Gupta, Prof. M. Ramanadham and, present Dean, School of Life Sciences, Prof. KVA. Ramaiah; former Heads of the department, Prof. N.Siva kumar, Prof. K. V. A. Ramaiah, Prof. O. H. Setty and present Head, department of Biochemistry, Prof. M. K. Bhattacharyya, for allowing me to utilize facilities at the School and department level.

I thank my Doctoral committee member's prof. KVA. Ramaiah and Dr. G. Ravi kumar, for their valuable suggestions in carrying out my work.

I express my gratitude to Prof. S. Rajagopal, Dr. M. Brahmanandam and Dr. Mahadev kalyankar, for moral support, inspiration and encouragement.

I thank all faculty members of school of life sciences, I also thank all the non-teaching staff especially S.B.Chary, Prabhu Srinivas, and Venkateswara rao for their timely help.

I acknowledge Mrs. Monika for MS/MS analysis.

I am happy to mention my Lab members M.Anjaneyulu, M. Aadinarayana, G. Madhavi, T. Prasad, A. Praveen, A. Chandra Sekhar, M.Fareed, B. Yerranna, Dr. Pulla Reddy, Dr.Usha, Dr.Samule, Dr.Aritra Simlai K. Srenivasulu, P.Arun, Amita Pal, Vandana.B, Arphan.C, Vandana, shilpa, P.Prasad, Priyanka.A, Bharati, Nayana, and lab attender G. Narasimha for providing congenial atmosphere in

the lab that led to smooth conduction of my research work. I feel very much happy to have such a nice lab mates.

I heart fully acknowledge the financial support of DST-SERB,CSIR, ICMR, UGC, DBT, CREBB, DST-FIST, UPE-II and PURSE which helped me directly or indirectly in the school and university for carrying out my research work properly.

I cannot forget to mention fruitful discussions with Dr.B.Suresh, Dr. M.Ushodyal, and Dr. T. Noble kumar and Dr.G.Vijay.N Reddy in personal, academic and research matters.

I will be failing my duty if I don't mention my Friends B.Suresh, G.Vijay.N. Reddy, Raghav, Chaitnya, M.Aadi, M.Usha, T.Noble, M.Narendra, A.Suman, Deepa, Varsha, Mr.Ch.Mohan and M.Prasanth for cheerful environment and charging with happiness during my interactions with them. A simple word 'thank you' cannot suffice how much their friendship means to me.

I thank all my Friends for their love and affection which cannot be explained in words.

My greatest appreciation goes to my evergreen closest Friends Suresh,Vijay and Raghav. who was always in great support to me in all my struggles and frustrations. I would like to register my affection to my Friends for his conversations and support.

I am kindful to my family members Mr T.Pedda Bapanaiah, Mrs T.Subbaratnamma (Father and Mother),Dhanalakshmi,Satyam,Bhargavi,Kiran,T.VenkataRatnam,Bhagyalakshmi,Jaswanth,Sreeja and T.Satya Narayana, for unstinting love and affection during my research work.

T. VenkataRamana

CONTENTS

Figures

ABBREVIATIONS

ABCB7	ATP binding cassette sub family member 7
ADP	Adenosine diphosphate
APS	Ammonium persulphate
ATM1	ABC transporter of mitochondria
ATP	Adenosine triphosphate
Bp	Base pair
BSA	Bovine serum albumin
BSO	L-Buthionine-(S,R)-sulfoximine
CD	Circular Dichroism
cDNA	Complementary DNA
CIA	Cytosolic Fe/S assembly
COX	Cytochrome oxidase
Cyt c	Cytochrome c
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPYD	Dihydropyrimidine dehydrogenase
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol tetra acetic acid
ETC	Electron transport chain

ER	Endoplasmic reticulum
et al	et alii (Latin: and others)
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FC	Ferrochelatase
Fe-S	Iron Sulfur cluster
FMN	Flavin mononucleotide
Ft	Ferritin
FRDA	Friedreich's ataxia
G	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGT	γ-glutamyl- transpeptidase
GPAT	Glutamate Phosphoribosyl pyrophosphate amidotransferase
GR	Glutathione reductase
GRX	Glutaredoxin
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
H2DCFDA	2′,7′-Dichlorodihydrofluorescein diacetate
HEK	Human embryonic kidney
HEPES	(N-(2-Hydroxyethyl)-piperizine-N'-(2-ethane sulfonic acid)
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSP70	Heat Shock Protein 70

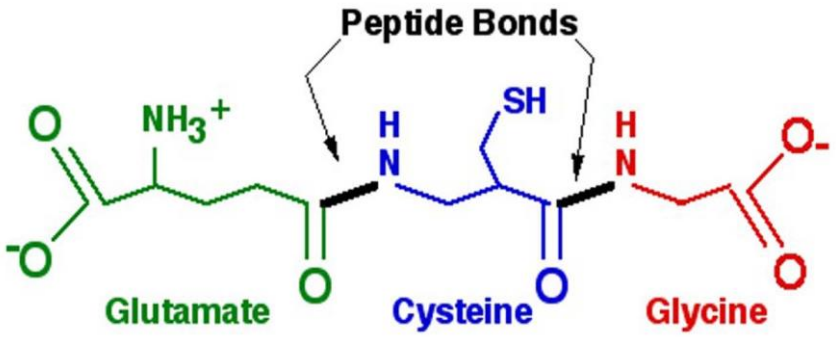
IAA	Iodoacetamide
IgG	Immunoglobulin G
IMS	Inter membrane space
IN	Inner membrane
IPTG	Isopropyl β-D-thiogalactopyranoside
IRE	Iron responsive element
IRP	Iron regulatory proteins
ISC	Iron-sulfur cluster
Kb	Kilo base pair
KCl	Potassium chloride
kDa	Kilo Dalton
KOH	Potassium hydroxide
LB	Luria Bertani
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
M	Molar
MALDI –TOF	Matrix assisted laser desorption/ionization- time-of-flight
MES	2-(N-morpholino) ethanesulfonic acid
MgCl2	Magnesium chloride
MIA	Mitochondrial inter membrane space assembly
Min	Minute
MS/MS	Mass spectrometers
μM	Micro meter
mM	milli molar
mL	milli litre
MPP	Mitochondrial processing peptidase

mtDNA	Mitochondrial deoxyribonucleic acid
MW	Molecular weight
NAC	N-acetylcysteine
NaCl	Sodium chloride
Na2S2O3	Sodium thiosulphate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAN3	Sodim azide
NC	Nitrocellulose
ND	NADH dehydrogenase
NIF	Nitrogen fixation
Ni-NTA	Nickel nitrilotriacetic acid
Nm	Nanometer
nM	Nano molar
OD	Optical density
OM	Outer membrane
PAGE	Polyacrylamide gel electrophoresis
PAM	Presequence translocase associated motor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	-log (H+) concentration
PPAT	Phosphoribosyl pyrophosphate amidotransferase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Rotations per minute

RT	Reverse transcriptase
SAM	Sorting and assembly machinery
SDS	Sodium dodecyl sulphate
SUF	Sulphur-mobilization
Taq	Thermophilus aquaticus
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween20
TCA	Tricarboxylic acid
TEMED	N,N,N‘,N‘-tetramethylethylene diamine
Tf	Transferrin
TfR	Transferrin receptor
TIM	Translocase of inner mitochondrial membrane
TOM	Translocase of outer mitochondrial membrane
Tris	Tris-(Hydroxymethyl) aminoethane
tRNA	Transfer ribonucleic acid
U	Units

CHAPTER I

INTRODUCTION



Glutathione (GSH)

1.1 Introduction

1.1. Mitochondria

Mitochondria is a double membranous organelle found in all eukaryotic organisms (Henze & Martin, 2003). Mitochondria differ in their size, shape and number that depends on tissue type and organism. The size of the Mitochondrion ranges from 0.5-10 μm and their number differs from 1 to 1000. These are called powerhouse of the cell. They are vital organelles for the survival under aerobic conditions. Besides the production of energy, they are involved in number of other fundamental functions of a cell that includes lipid metabolism, metal homeostasis, hormonal signaling, heme biosynthesis, amino acid metabolism, urea cycle, cellular metabolism, apoptosis and autophagy. To perform its multiple functions, mitochondria depends upon the products of 10-15% of genes found in eukaryotes. Mitochondria can encode 13 out of serval proteins produced by its individual genome. Majority of the mitochondrial proteins are encoded by nuclear DNA and imported to the mitochondria through well-characterized protein translocases of outer and inner membrane called as Translocase of Outer Membrane (TOM) & Translocase of Inner Membrane (TIM).

1.2. Architecture of Mitochondria

A typical mitochondrion has two detached membranes, outer and inner membrane made up of phospholipid bilayers and proteins. The outer membrane creates a fence between the organelle and the cytosol. The two membranes acquire different properties owing to their membrane composition, as a result of this double membrane structural organization, mitochondria have four different sub-compartments (Figure:1.1), Outer membrane, Intermembrane space, Inner mitochondrial membrane, and the matrix. These four compartments of mitochondria diverge in their structure and function. Mitochondria peeled off their outer membrane are labelled as mitoplast. The mitochondrial outer membrane wraps the entire organelle and is of 60 to 75 angstroms (Å) thick. It consists of phospholipids and protein in the proportion related to that of the eukaryotic plasma membrane (1:1). Porin channel proteins make the outer membrane accessible to the small molecules. Molecules around 5-10 kDa pass through the outer membrane passively via porin, whereas, the high molecular weight proteins pass into mitochondria through the TOM complex (Herrmann & Neupert, 2000). The intermembrane space is a sub-compartment in between outer membrane and inner (Frey & Mannella, 2000; Frey *et al*, 2002). The concentration of minute molecules like ions and sugars in the

intermembrane space are identical to cytosol, as the outer membrane is permeable to minute molecules. Proteins that have targeting information can only pass through the TOM complex and so the protein content in the intermembrane space is distinct from the cytosolic protein content. The mitochondrial inner membrane is rich with protein and has a proportion of protein to phospholipids is nearly 3:1. The mitochondrial inner membrane is impermeable to maximum number of the molecules, due to the presence of a special phospholipid known as cardiolipin (Bruce, Johnson et al. 1994). As the impermeable texture of the mitochondrial inner membrane, maximum number of ions and molecules depend upon exclusive transporters to go in or to go out from the matrix. Additionally, movement of enzymes create a membrane potential across the inner membrane of the electron transport chain. The mitochondrial inner membrane proteins are elaborated in metabolite transport, protein import, mitochondrial fusion, fission, oxidative phosphorylation and ATP synthesis. The space enveloped by the inner membrane is called matrix which consists of around 2/3 of total mitochondrial proteins. The mitochondrial matrix consists of a variety of enzymes, mitochondrial ribosomes, tRNA and several copies of circular DNA. The mitochondrial matrix enzymes are involved in citric acid cycle, oxidation of pyruvate and fatty acids, amino acid metabolism, heme and Fe-S cluster biosynthesis, and the urea cycle (Bruce, Johnson et al. 1994).

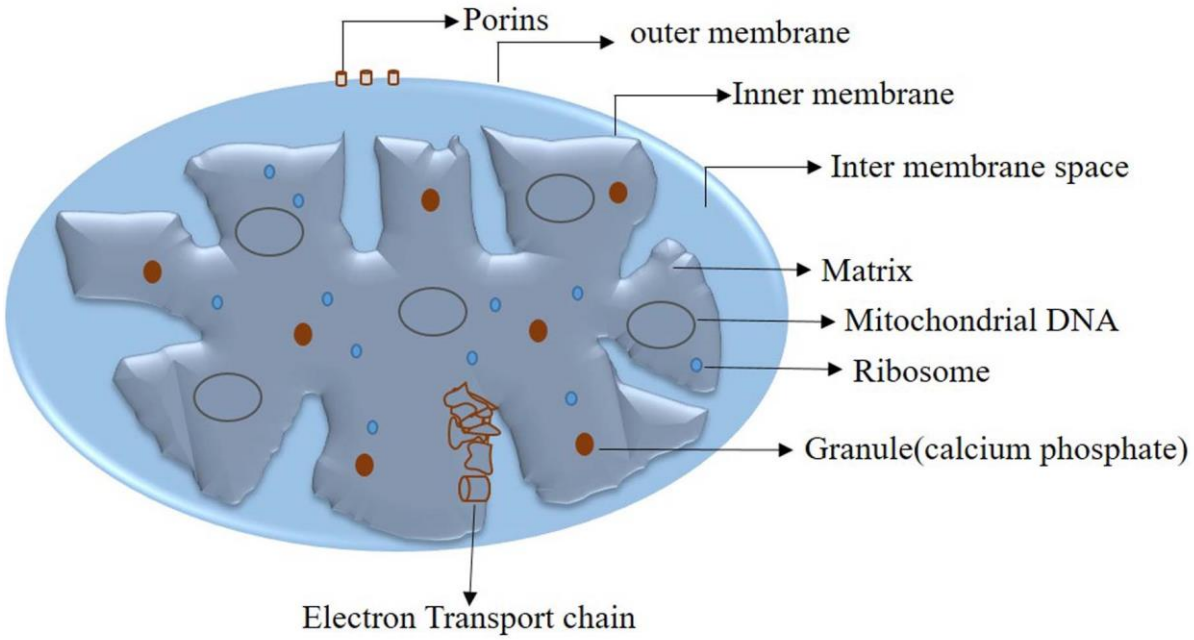


Figure: 1.1 Architecture of Mitochondria: - Mitochondria are double membrane-bound organelle. The outer membrane wraps the entire organelle. The wrinkle of the inner mitochondrial membrane is known as cristae where most of ATP is produced. The inner membrane encloses the fluid-filled compartment called the mitochondrial matrix. The mitochondrial matrix has ribosomes and DNA.

1.3 Functions of mitochondria

The prominent role of mitochondria is to produce energy. Mitochondria have a well-organized energy generating system known as the oxidative phosphorylation which utilizes NADH and oxygen to generate ATP (Figure: 1.2). It is very important to keep proper concentrations of calcium ions within compartments of various cell organelles (Newmeyer & Ferguson-Miller, 2003) for various biological functions. Mitochondria also act as storage reservoir for calcium ions. The mitochondria in liver cells bear enzymes that detoxify ammonia. Other than energy production, mitochondria are also studied as central integration sites for biological signals that promote cell death or cell survival. As mitochondria have various proteins involved in the apoptotic signaling cascade, it is now accepted that mitochondria play a key governing role in deciding whether a cell has to live or die following a death signal-factually a "license to kill" (Regula *et al*, 2003). In counter to apoptotic stimulation, one of the mitochondrial intermembrane space protein, Cytochrome c releases into the cytosol to begin the cascade of events that leads to the activation of caspase-3 and caspase-7 that finally degrades many cellular proteins and causes cell death (Regula *et al*, 2003; Gorla & Sepuri, 2014). Other than apoptosis, mitochondria are also involved in alternative forms of programmed cell death (Galluzzi *et al*, 2012). One such example is necroptosis (necrosis-like cell death pathway) which is mediated by RIPK-3 (Receptor-interacting serine/threonine Protein Kinase-3) (Vandenabeele *et al*, 2010). RIPK-3 dependent necrosis plays a crucial role in embryonic development and host antiviral immunity (Kaiser *et al*, 2011; Upton *et al*, 2010). Mitochondria serve as platforms to initiate cell signaling in multiple processes (Tait et al. 2012). For example, outer mitochondrial membrane behaves as a major signaling platform for antiviral innate immunity and regulated generation of mitochondrial reactive oxygen species (ROS) which serves as a signaling platform for anti-microbial activity during phagocytosis (Seth *et al*, 2005). Defects in mitochondrial regulation of cell signaling may cause many diseases and age-related pathologies (Tait et al. 2012).

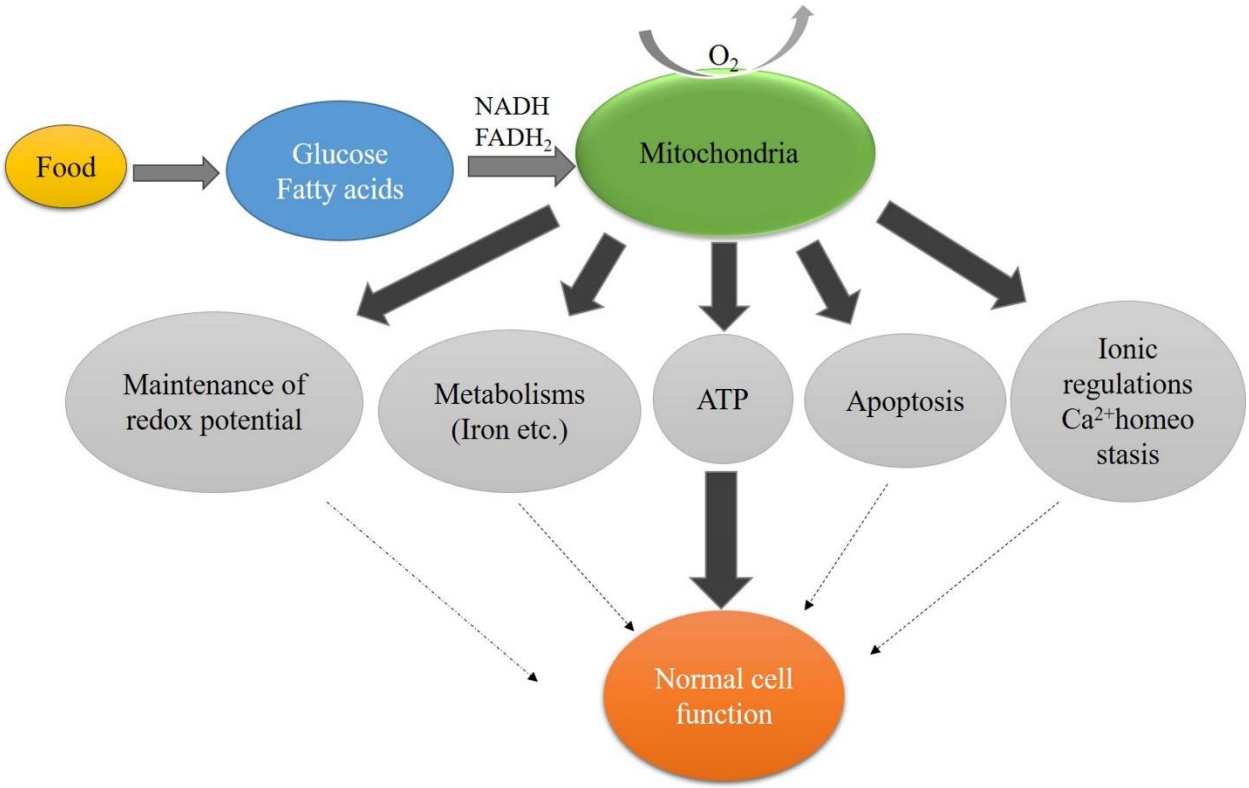


Figure: 1.2 Functions of Mitochondria:- Mitochondria are essential organelle involved in distinct functions which include energy production, various oxidative metabolisms, apoptosis, Ca homeostasis as well as the integration of most of the signaling pathways in response to external stimulus.

1.4 Evolution of mitochondria

Eukaryotic cells (Nucleated) are larger and highly structured than prokaryotic cells with sufficient genomes and proteomes (Lane et al. 2005). Acquisition of mitochondria and chloroplasts is one of the essential events in the evolution of eukaryotic cells as they perform the roles of energy generators and biosynthetic industry of the cell. The origin of Mitochondria in eukaryotic cells is best explained by an endosymbiotic theory which postulates that eukaryotic cells were invaded by eubacteria nearly a billion years ago which later entered into symbiotic association wherein complex molecular events triggered by evolutionary processes transformed the endosymbionts to present powerhouses of the cell (Dyall *et al*, 2004).

1.4.1 Endosymbiotic theory

The endosymbiotic theory proposes that the mitochondria are originated from α-proteobacterium (Lang *et al*, 1999; Gray *et al*, 2001). Phylogenetic analysis of small ribosomal RNAs proves that the monophylogenetc origin of mitochondria from α-proteobacterium (Yang *et al*, 1985). Two scenarios were proposed to explain the mitochondrial origin, the Symbiosis scenario, and the Archezoan scenario (Figure: 1.3) (Gray *et al*, 2012).

According to Symbiosis scenario, endosymbiont was buried by an archaeal cell (Martin & Müller, 1998), whereas Archezoan theory advocates that host of the endosymbiont was substantially compartmentalized as amitochondriate of the eukaryotic cell (CAVALIER- SMITH, 1987). However, growing evidence supports the Symbiosis theory due to the absence of any amitochondriate eukaryotic ancestry in eukaryotes (Embley & Hirt, 1998). The evolution of the ancestral bacterial endosymbiont to current mitochondria has been escorted by many changes such as reduction of mitochondrial genome due to loss or relocation of bacterial genome to the host genome (Adams & Palmer, 2003; Berg & Kurland, 2000). Thus, most of the genes coding for mitochondrial functions are in fact placed in the nucleus.

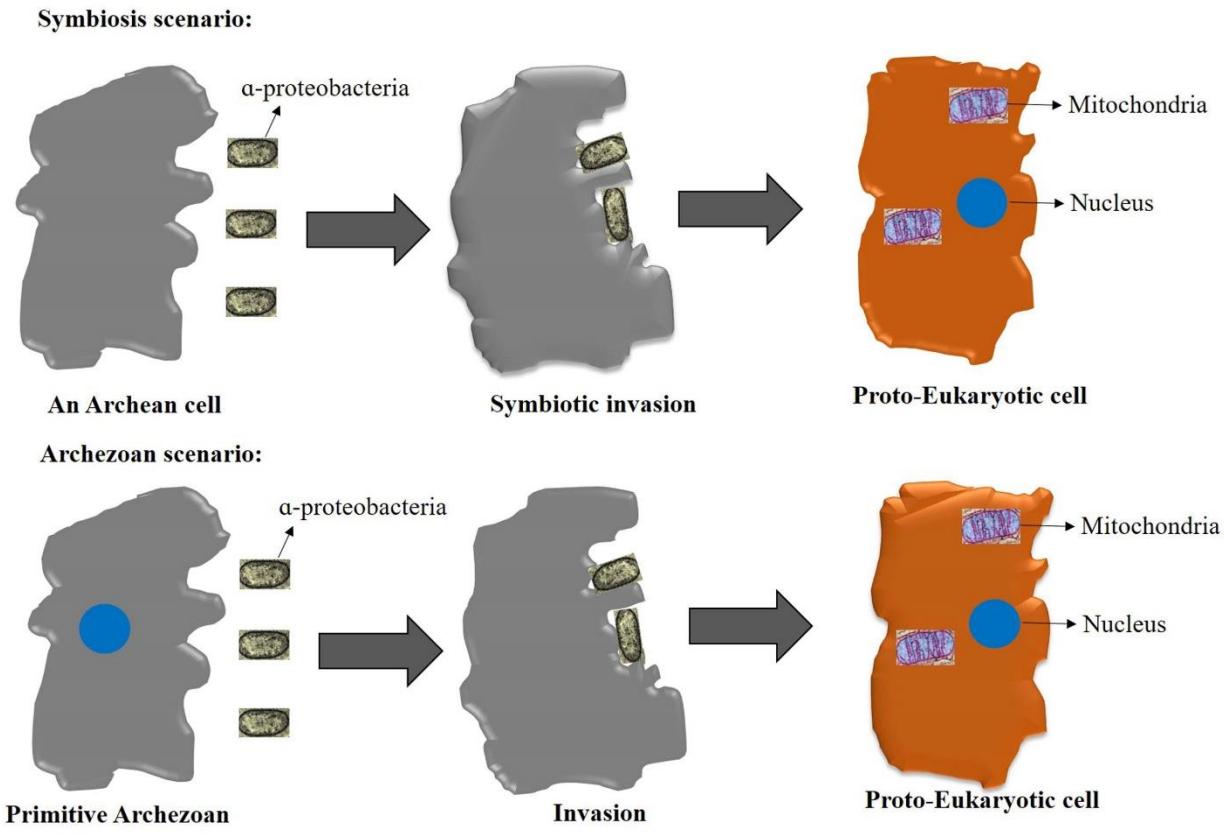


Figure: 1.3 Endosymbiotic theory:- The Simplified representation that denotes the bacterial origin of mitochondria. Two scenarios (Symbiosis scenario and Archezoan scenario) were proposed to demonstrate the endosymbiotic origin of mitochondria.

1.5 Mitochondrial biogenesis

It is a process through which cells gain mitochondrial mass and copy number to increase the production of ATP as feedback to higher energy consumption. It can be described as growth and subdivision of pre-existing mitochondria, and mitochondria don't originate *de novo* (Jornayvaz & Shulman, 2010). The symmetry, diameter, and the total number of mitochondria differ greatly in different cell types based on energy requirements, physiological and environmental conditions. The mitochondrial count per a cell is precisely connected to the biogenesis of the organelle. Its biogenesis per cell is firmly governed by the stimulation of different transcription factors and signaling pathways.

These are the absolute product of an α-proteobacteria endosymbiont that eventually is established in a host cell. Because of their bacterial ancestor, mitochondria carry their individual genome and can

auto replicate. Even though mitochondria consist of their individual genome, it encodes only a limited number of proteins, and maximum number of the proteins and enzymes that are located in the mitochondria are nuclear gene-encoded. Proper mitochondrial biogenesis depends on the coordinated synthesis and import of relatively 1000–1500 proteins encoded by the nuclear genome and synthesized on cytosolic ribosomes. Mitochondrial DNA replication, as well as mitochondrial fusion and fission, must also be coordinated. Import of macromolecules, proteins, and tRNA, represents a primary aspect of mitochondrial biogenesis.

The mitochondrial and nuclear-encoded proteins are well-organized and execute a function in the synthesis of ATP over an electron transport chain (ETC) and oxidative phosphorylation. Another duty of nuclear-encoded mitochondrial proteins includes maintenance of organelle structure, pyrimidine and heme biosynthesis, components of mitochondrial genetic information processing. The mitochondrial proteins encoded by nucleus are imported to the various sub-compartments of mitochondria by a complex import protein machinery present in the outer and inner mitochondrial membrane. Additionally, 13 proteins are encoded by Mt DNA are mostly consist of 7 subunits of Complex I /NADH dehydrogenase (ND1-ND6), single subunit of complex III /cytochrome c oxidoreductase (cytochrome B) and 3 subunits of Complex IV /cytochrome c oxidase (CoxI - CoxIII), two subunits of Complex V/ F0F1 ATPase (ATPase6 & ATPase8). Mammalian mt DNA further encodes for 2 rRNAs and the whole set of 22 tRNAs that are indispensable for protein synthesis in mitochondria. The functioning and assembly of the ETC enzymes in mitochondria depend upon combined expression and cooperation among mitochondrial and nuclear gene products.

Further, the mitochondria import phospholipids from the cytoplasm to preserve their membranes. Hydrophobic phospholipid & cardiolipin are essential for mitochondrial proteins such as cytochrome c oxidase.

The master governor of mitochondrial biogenesis appears to be peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1α) (Puigserver *et al*, 1998). PGC-1α is a co-transcriptional regulatory factor that activate mitochondrial biogenesis (Figure: 1.4) by activating the nuclear respiratory factors, NRF-1&NRF-2. The NRFs, consecutively, activate the mitochondrial transcription factor (Tfam) that transcribes the mitochondrial genome (Virbasius & Scarpulla, 1994). Mitochondrial biogenesis is also altered under environmental stresses like oxidative stress, exercise, low temperature, caloric restriction, cell division, renewal, & differentiation.

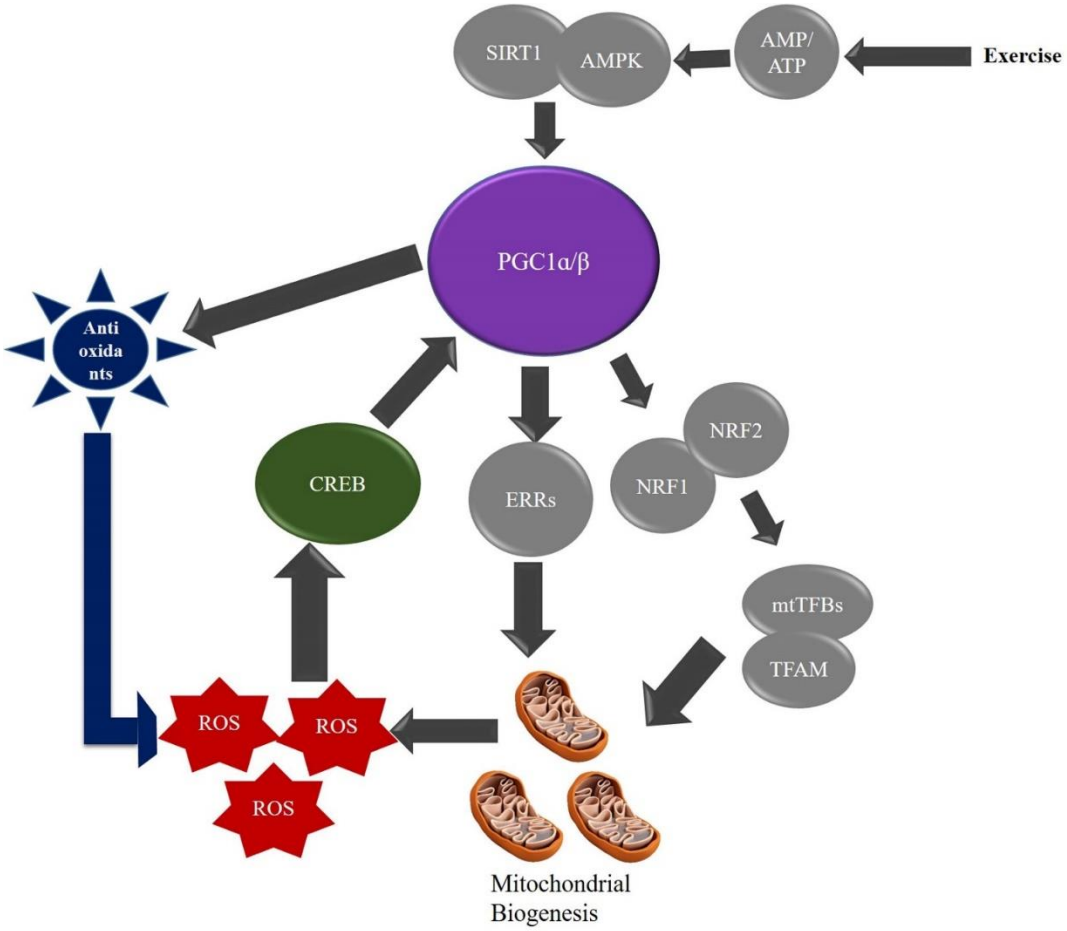


Figure: 1.4: Signaling pathway for mitochondrial biogenesis:- Mitochondrial biogenesis governed by PGC-1α through nuclear receptor family members, NRF-1/2 and ERR-α in response to increasing in energy demand. Reactive oxygen species generated by mitochondria increases PGC-1α by feedback mechanism through CREB gene-regulatory factor. ROS induced PGC-1α is crucial for stimulation and the expression of host antioxidant proteins such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and Catalase.

The majority of proteins required for mitochondrial structural integrity, functional diversity and biogenesis are imported from the cytosol.

1.6. General protein import machinery in the mitochondria

Cells have derived with mature import machinery at the OM, IM, and IMS of mitochondria to import nuclear-encoded proteins into various sub compartments of the mitochondria (Bolender *et al*, 2008). Most of the mitochondrial matrix-targeted proteins contain pre-sequence at N-terminus. These proteins traverse OM and IM mitochondrial membranes through TOM and TIM complexes

correspondingly in an unfolded conformation (Figure: 1.5A). Proteins are imported to the innermost compartments of mitochondria, known as the matrix, usually carry import signals at the N-terminus. The imported protein into the mitochondria is correspondingly known as pre-protein or precursor protein. The pre-protein N-terminal directing sequences generally contains 10-80 amino acid residues and it forms an amphipathic α-helices in the membrane ambience. These helices have positively charged amino acids on one side & hydrophobic amino acids on another side. The translocation of precursor proteins into mitochondria is directed by mitochondrial membrane potential and mitochondrial matrix Hsp70. After import of pre-protein into the matrix, the N-terminal targeting sequence is cleaved by the mitochondrial processing peptidases to generate a mature protein. In contradiction, most of the pre-proteins that are targeted to the mitochondria don't have any N-terminal directing sequence, and their targeting sequence is present within the protein. Almost all mitochondrial pre - proteins are imported through TOM complex. Tom20, Tom22, and Tom70 are the various receptor proteins for the detection of the mitochondrial targeted pre-proteins and rest of the other proteins create the translocation pore (Dekker, 1998; Künkele *et al*, 1998; Terziyska *et al*, 2007).

The cytosolic Hsp90 & Hsp70, binds and obstruct the accumulation of freshly synthesized pre-proteins in the cytosol and transport them to the OM. Hsp70 conversely binds to the pre-proteins having N-terminal pre-sequence and transport them to the TOM complex in an ATP dependent machinery. Tom20 is the first recognition place for pre-proteins having pre-sequences at N-terminus (Saitoh, Igura et al. 2007). Tom20 binds and handovers the pre-proteins to the Tom22. Hsp90 binds to the pre- proteins with internal targeting sequences and transport to the Tom70. Tom70 delivers the precursor proteins to Tom22 and then introduced into Tom40 channel. Later passing over the channel of TOM complex, pre-proteins can go after one of 4 key import pathways (Koehler, 2004; Zong *et al*, 2014); (i) The proteins with a pre sequence are transported to the pre-sequence translocase of the IM, known as TIM23 complex. TIM23 forms a channel in the inner membrane and connects to mtHsp70. mtHsp70 is the core of the pre-sequence translocase accompanying motor (PAM) which pushes the end of protein transport to the mitochondrial matrix. (ii) A few hydrophobic IM proteins use chaperone-like machineries in the IMS and the protein insertion mechanism of the inner membrane transporter translocase was known as TIM22 complex (iii) The precursor of OM proteins like Tom40 and Porin is incorporated into the OM of mitochondria by the sorting and assembly machinery(SAM). (iv) The sum of precursor proteins that are directed to the IMS use oxidative folding mechanism having Mia40 and Erv1 or TIM23 complex.

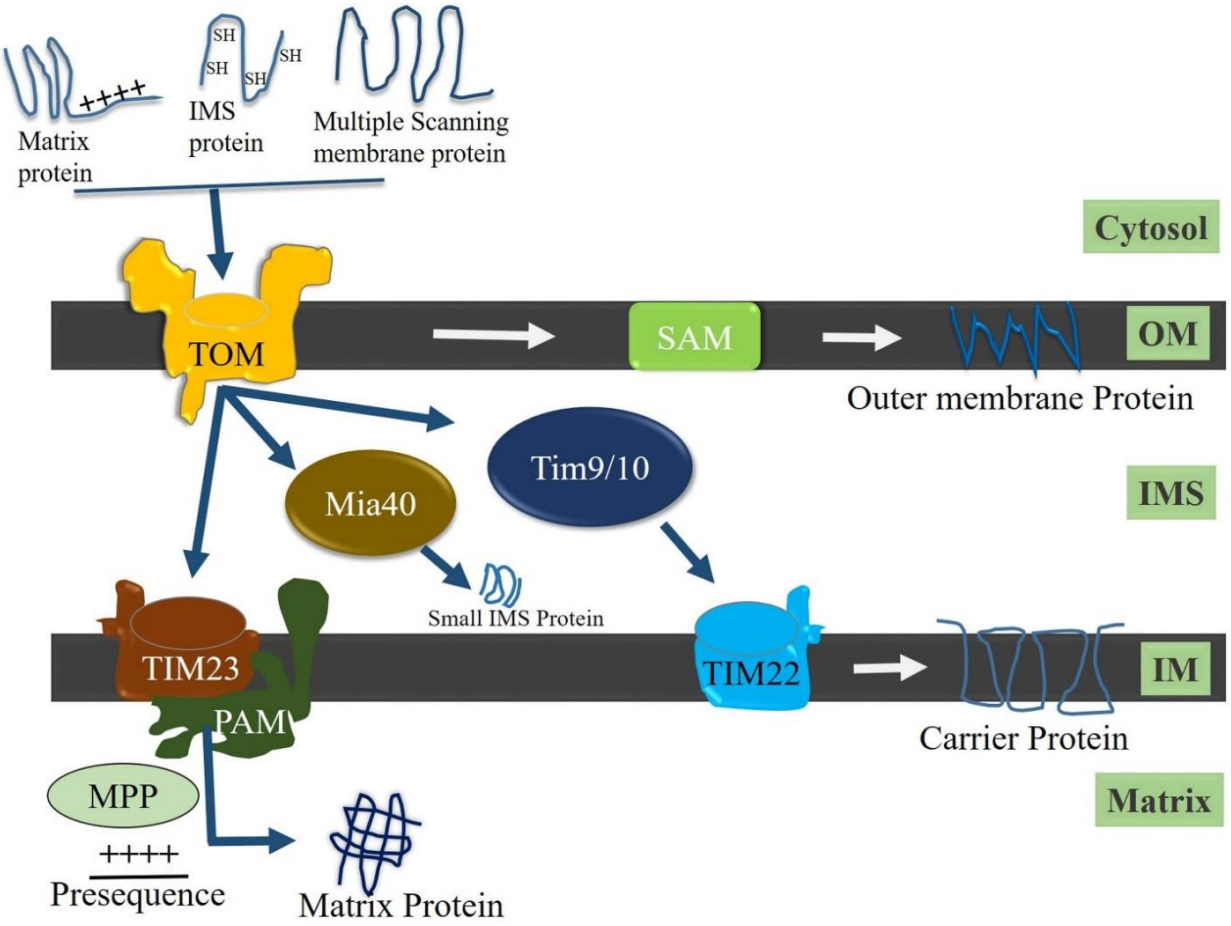


Figure: 1.5.A. The mitochondrial protein import pathway:- Nuclear-encoded mitochondrial targeting proteins are imported through the translocase of the outer membrane (TOM) complex. Precursor proteins they follow the different sorting pathways. (i) Pre-sequence containing proteins are translocated through the TIM23 complex and PAM complex into the matrix, mitochondrial processing peptidase (MPP) cleaves off the pre-sequences in the matrix. (ii) Small-scale proteins of the intermembrane space (IMS) are imported through the mitochondrial inter membrane space assembly machinery (MIA). (iii) β-barrel precursor proteins of the outer membrane (OM) are transported from TOM to SAM complex. (iv) Precursor proteins of the inner membrane (IM) carriers use Tim9-Tim10 and transfer to the TIM22 complex that drives infusion into the inner membrane (Wiedemann et al. 2004) .

1.6.1. Intermembrane Space (IMS)

Physico-chemical properties of the IMS

The IMS is linked to the cytosol via pores, typically formed through porins in the outer membrane, that permit the free passage of molecules of around 5–10 kDa (Benz, 1994). Due to unrestricted diffusion of GSH, ions, and protons, the intermembrane space is usually considered as a compartment with physicochemical properties undistinguishable to those of the cytosol. Unlike to the cytosol, cysteine residues in IMS almost completely reduced. However, current literature have shown that the existence of various disulfide bonds in isolated inter membrane space proteins like Cox11 (Banci *et al*, 2004), Erv1 (Levitan *et al*, 2004), Rieske iron-sulfur protein (Iwata *et al*, 1998), CCS [Lamb et al. 1999], Sod1 (Frazzon & Dean, 2003), and small Tim proteins (Lu *et al*, 2004; Curran *et al*, 2002). Although IMS contains a distinct number of important proteins, it is an intensely small part of mitochondria. IMS also have metal ions, electron transporters, set of pro-apoptotic proteins and enzymes vital for metabolic reactions. IMS targeted proteins don't hold any canonical mitochondrial targeting signals. Intermembrane space targeted proteins can be sub-divided into 3 classes based on their energy requirement, structural considerations, & sorting directions (Figure: 1.5B) (Terziyska *et al*, 2007).

Class I proteins have N-terminal mitochondrial targeting sequences followed by the hydrophobic transmembrane domain. They use the TOM and TIM23 complex for IMS targeting and require ATP and membrane potential. The targeting sequence at N-terminus translocates over the TIM23 complex. Mature proteins released into the inter membrane space by the action of inner membrane peptidase removes the introduced hydrophobic domain from the IMS domain (Glick *et al*, 1992). Class II proteins are usually low molecular weight, around 7-15 kDa, and they don't have any prominent pre-sequences. They consist of conserved cysteine and histidine those are necessary in the binding of other cofactors and / metal ions. The cysteines existing in these proteins permit them to form intra disulfide bonds to support mature protein. This kind of disulfide-mediated compact of proteins in inter membrane space stops them from diffusing back into the cytosol (Lutz *et al*, 2003; Mesecke *et al*, 2008). Class III proteins of the intermembrane space don't require ATP nor the membrane potential for their translocation. They diffuse into the IMS after cytosol and fix to high-affinity sites present on inter membrane space proteins. The energy released at the time of tie-up with IMS is the dynamic power for the import of third class proteins into inter membrane space (Steiner *et al*, 1995).

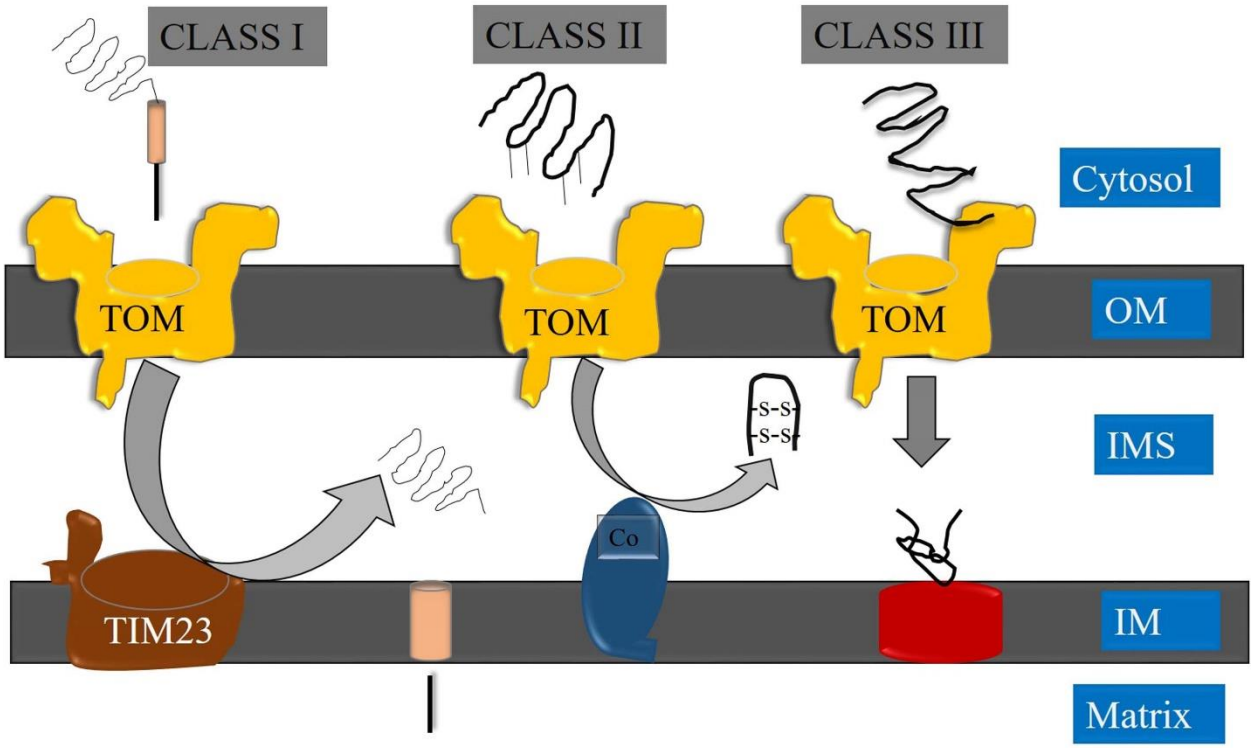
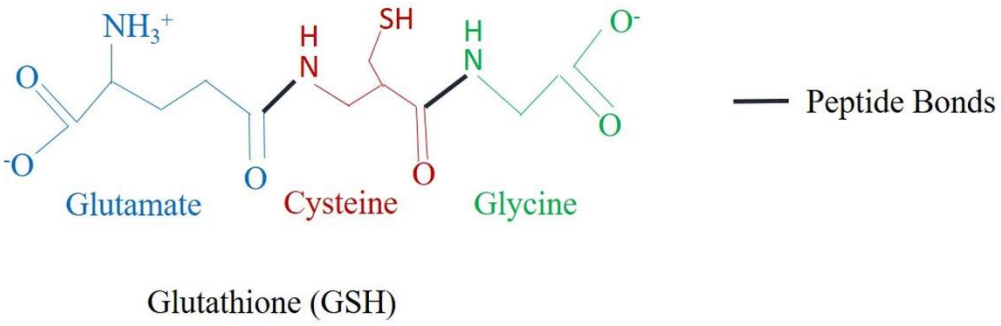


Figure: 1.5.B. Distribution of intermembrane space proteins: Intermembrane space proteins are divided into 3 classes depending on their import signals. 1) Class I proteins have two types of import signal, like a small helix and a hydrophobic anchoring domain. These proteins are processed after import. 2) Class II proteins obtain their native confirmation by forming disulfide bonds or binding to cofactors. 3) Class III proteins bind to high affinity site in the intermembrane space. (TOM - Translocase of the Outer Membrane, TIM - translocase of the Inner Membrane, OM - Outer Membrane, IMS - Intermembrane Space, IM - Inner Membrane,) (Adapted from Herrmann and Hell 2005).

1.7 Glutathione

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) (Figure: 1.6) is the amplest low molecular weight thiol in a eukaryotic cell (Meister et al. 1983). Glutathione is a tripeptide had gamma peptide linkage between the carboxyl (COOH) group of the glutamate side chain and the amine (NH2) group of cysteine, and the carboxyl (COOH) group of cysteine is attached by a normal peptide linkage to a glycine (Figure: 1.6). It is present in millimolar concentrations (1 to 10mM) in most mammalian cells, generally conserved among species throughout evolution, from microorganisms to plants and mammals. It is an important antioxidant in animals, plants, fungi, bacteria, and archaea. GSH gives the first line of defense against ROS, as it can protect reduced cysteine thiols on the surface of proteins, reduce disulfides to thiols or scavenging free radicals and reduce H_2O_2. Further GSH-dependent enzymes are able to detoxify ROS products and prevent generation of free radicals. The normal mechanism of this protection gets from the capacity of GSH to form a disulfide (GSSG) when oxidized, and that can then be reduced back to GSH by glutathione reductase (GR). This mechanism makes GSH a recyclable antioxidant molecule. The GSH/GSSG ratio reflects the intracellular redox status of the cell; typically this ratio is 100:1(Kosower & Kosower, 1978; Gilbert, 1995) in resting cells. However, the equilibrium may change to 10:1 in response to the cellular redox potential. This may result in the formation of protein-GSH mixed disulfides or protein disulfides and leads to alterations in the structure of the protein. Reduction of disulfides and mixed disulfides, and reversion to the original protein confirmation is typically mediated by redox enzymes such as thioredoxin, glutaredoxin and protein-disulfide isomerases. The GSH/GSSG ratio is maintained *de novo* by synthesis of nascent GSH, cellular excretion of GSSG, and reduction of GSSG back to GSH by glutathione reductase (GR) at the expense of NADPH. GR activity is found in cytosol as well as in mitochondria.

Apart from scavenging free radicals and maintaining the redox balance of the cell, GSH is involved in redox reactions, detoxification of xenobiotics, carcinogens, in the biosynthesis of DNA, protein, and leukotrienes. Further, protein glutathionylation may have a regulatory post-translational effect on protein function or activity. Glutathionylation or deglutathionylation of key proteins may be a control point for certain redox-sensitive gene expression.



Glutathione (GSH)

Figure: 1.6 Glutathione:- It is a tripeptide that is synthesized by forming one peptide bond between the γ-carboxyl group of glutamate and the amino group of cysteine and a peptide bond between the COOH group of cysteine and the NH2 group of glycine (γ-glutamyl cysteine) .

1.7.1 The role of glutathione

The oxidative stress is involved in the pathogenesis of a sum of human diseases, including neurodegenerative diseases and cancer. Glutathione has been shown to play a critical role in several diseases, for instance, Parkinson's disease, cystic fibrosis and, HIV (Wu *et al*, 2004). The brain is more sensitive to oxidative stress as it has a relatively low antioxidant defense, and the levels of glutathione play a key role in determining the way brain cells combat stressful situation (Mates, 2000). Other than protecting cells from oxidative stress, glutathione is also involved in a number of metabolic processes (Wu *et al*, 2004).

During oxidative stress, in addition to its radical scavenging properties, GSH can convert hydro peroxides into their corresponding alcohols through the action of glutathione peroxidase (Figure:1.7A). This reaction can also be catalyzed by peroxiredoxins, although their rate constants for the reaction with hydro peroxides in mammals is believed to be comparatively slow (Hofmann et al. 2002). If peroxides are not removed, they could produce toxic radicals in the presence of reduced metal ions such as Fe^{2+} and Cu^{+} (Halliwell & Gutteridge, 1985).

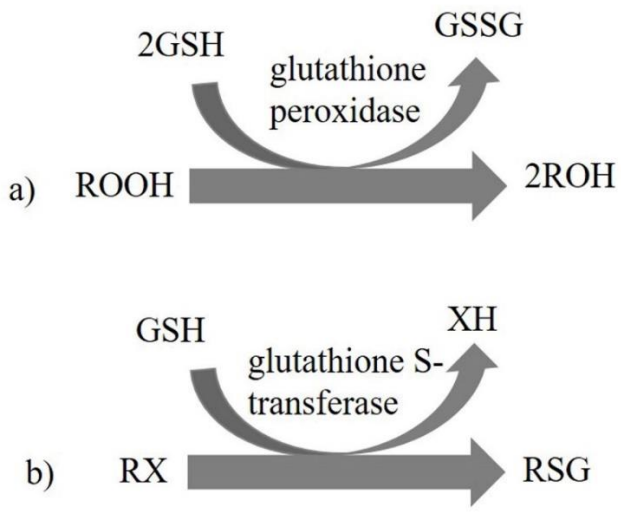


Figure: 1.7A Actions of glutathione peroxidase and glutathione S- transferases: ROOX is a peroxide converted into alcohol with the help of glutathione (GSH) and glutathione peroxidase. GSH is oxidized to glutathione disulphide (GSSG). RX is a different compound that is enzymatically conjugated with GSH forming RSG.

Glutathione can also form conjugates with electrophilic compounds through the action of glutathione S- transferases (Figure.1.7B). This will protect the cell against the redox cycling of quinones and oxidative stress that otherwise might result in the accumulation of these metabolites (Mates, 2000; Eaton & Bammler, 1999). The glutathione conjugates are excreted in the urine in the form of processed mercapturates, and the mechanism is a part of the coordinated detoxification machinery of the cell. The liver, which is particularly rich in glutathione S-transferases, often excretes glutathione conjugates into bile (Halliwell *et al*, 1995). Reduced glutathione is regenerated from glutathione disulfide by glutathione reductase, in an NADPH dependent reaction (Figure.1.7B). The reducing equivalents of NADPH are supplied predominantly by the pentose phosphate pathway (Dickinson & Forman, 2002).

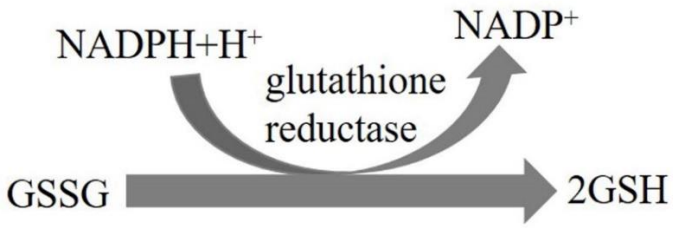


Figure: 1.7B:- The regeneration of reduced glutathione (GSH) from glutathione disulfide (GSSG).

The levels of GSH may vary diurnally in some cells, especially in liver cells, where they are affected by detoxification processes and oxidative stress. During this process, glutathione levels are decreased due to the formation of GSSG (via radical scavenging or oxidation via glutathione peroxidases), protein mixed disulfides, efflux of glutathione-S-conjugates and direct efflux of GSSG. When GSSG levels rise, it is exported from the liver into bile in order to avoid oxidative effects (i.e. oxidative stress). Thus the efflux is directly related to the hepatic GSSG levels (Brigelius *et al*, 1983). In other cells, such as erythrocytes and heart cells, transport of GSSG across the plasma membrane into the extracellular space is used to alleviate stress and recycle glutathione (Dickinson & Forman, 2002).

Deficiency of glutathione compromises the glutathione peroxidase detoxification system. This leads to accumulation of H_20_2 and lipid hydro peroxides and subsequent oxidative damage. This may overpower the cell's defense system and affect mitochondrial membrane permeability and enzymes function (Fernández-Checa, 2003). Redox imbalance has long been associated with cell death. In fact, glutathione efflux plays an integral part in the damage-induced apoptotic pathway, and GSH depletion causes cytochrome c release from mitochondria. Depending on the strength of the oxidative stimulus, this may lead to activation of the apoptotic pathway via caspase 3 (Coppola & Ghibelli, 2000).

Depletion of glutathione can either be counteracted by the cells through GSSG reduction by glutathione reductase (Figure: 1.7B) or by the release of GSH from cellular proteins by the action of glutaredoxin or thioredoxin or through glutathione recycling and *de novo* synthesis. Glutathione recycling involves the import of GSH from the extracellular space, where it is degraded, firstly by the membrane-bound enzyme γ-glutamyl-transpeptidase (GGT) (Figure: 1.7C), the only enzyme capable of breaking the γ-glutamyl bond in GSH and GSH conjugates. The Cys-Gly dipeptide is then cleaved by dipeptidases, and the constituents can be imported as amino acids, which are used in glutathione synthesis. This recycling of glutathione preserves the amino acid cysteine, which is often limiting in *de novo* synthesis (Dickinson & Forman, 2002; Griffith, 1999). The liver actively exports GSH and is the main source of circulatory GSH. Circulatory GSH may also play a role in the extracellular antioxidant defense (Smith *et al*, 1996).

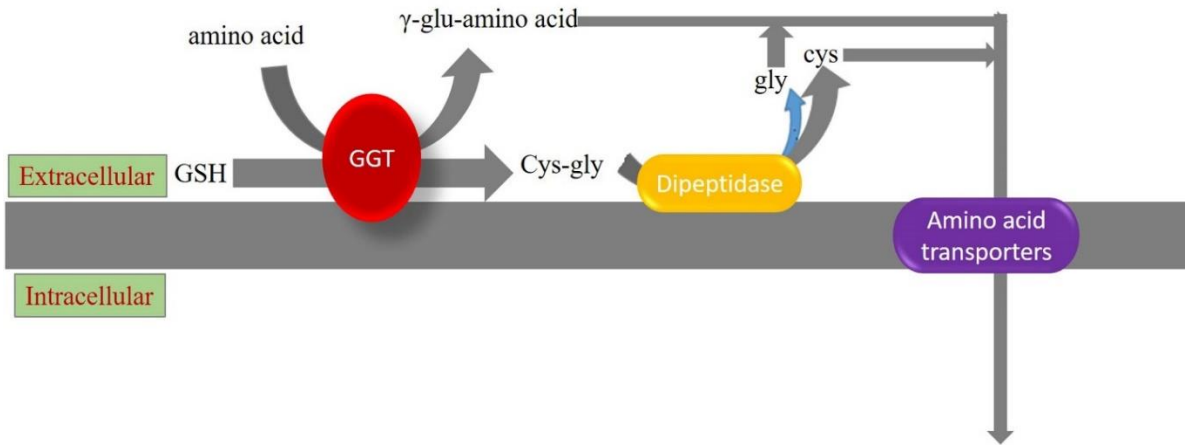


Fig1.7C:- Recycling of glutathione (GSH), and import of its amino acid constituents into the cell through the action of γ-glutamyl- transpeptidase (GGT), dipeptidases and amino acid transporters. (Placeholder1) Adapted from Akerboom et al. 1990.

1.8 GSH metabolism

Glutathione is synthesized from glutamate, cysteine, and glycine by the sequential actions of the ATP-dependent enzymes γ- glutamylcysteine synthetase and glutathione synthetase (Figure: 1.8). Glutathione is degraded by γ-glutamyltranspeptidase and dipeptidases. The most generally used inhibitor of glutathione synthesis is buthionine sulfoximine (BSO) that binds to γ- glutamylcysteine synthetase. Some cells may also export GSH. Liver is the leading organ for synthesis and export of GSH into the plasma. Further, when cells /tissues are exposed to oxidative stress, GSSG efflux has been observed. A putative reason for the export of GSSG may be maintenance of GSSG/GSH ratio and thus sustain a favourable redox environment in the cells.

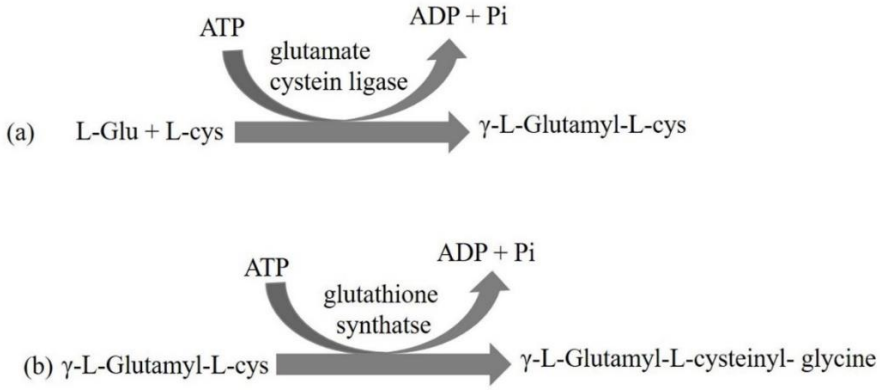


Figure: 1.8: The two enzymatic steps of glutathione *de novo* synthesis.

1.9 Protein S- glutathionylation

It is the posttranslational adaptation of protein cysteines by the addition of glutathione, the greater ample & essential low-molecular-mass thiol in utmost cell categories. Protein S-glutathionylation is involved in oxidative stress, nitrosative stress, and block irreversible oxidation of protein thiols and regulation of cell-signaling pathways by modifying protein function

1.9.1 Reversible S- glutathionylation

Reversible S- glutathionylation of protein thiols is an enzyme-catalyzed reaction, catalyzed by the enzyme Glutaredoxin (Grx), in both directions (Figure: 1.9) (Mannervik & Axelsson, 1975, 1980; Axelsson & Mannervik, 1980).

Figure: 1.9:- Reversible S- glutathionylation of S-glutathionylated proteins, catalyzed by glutaredoxin. Adapted from (Mannervik & Axelsson, 1975).

1.9.2 Glutaredoxin

It is a small redox enzyme of about one hundred amino acid residues that uses glutathione as a cofactor. This gets oxidized through substrates and reduced non-enzymatically by glutathione. It is an enzyme clearly ubiquitous to mammalian cells (Rozell *et al*, 1993; Fernández-Checa, 2003). Its structural relative, thioredoxin, may have a small capacity to aid Grx in the reduction of mixed disulfides *in vitro (*Jung et al. 1996). Glutaredoxin has an *in vitro* efficiency of about 5000 times that of thioredoxin in deglutathionylation reactions, and a broad specificity for various S-glutathionylated proteins. Hence, glutaredoxin appears to be the most physiologically relevant candidate for deglutathionylation reactions *in vivo* (Chrestensen *et al*, 2000). Glutaredoxin also has the capacity to reduce intramolecular disulfides, and therefore function as a backup for thioredoxin in some situations (Fernandes & Holmgren, 2004; Holmgren, 1979; Prinz *et al*, 1997).

The catalytic site for glutaredoxin typically contains a CXXC motif (generally CPYC), a solvent-accessible hydrophobic area and a binding site for GSH. To reduce a mixed disulfide, the enzyme recognizes and binds to the GSH moiety of the mixed disulfide via its N-terminal cysteine thiol,

forming a mixed intermediate. The protein is reduced and released, and the second intermediate glutaredoxin-SG is then in turn reduced by glutathione (Figure: 1.9A). This mechanism is referred to as a "monothiol mechanism", and it is functionally separate from its "dithiol mechanism", in which the enzyme utilizes both its catalytic site cysteines to reduce intramolecular disulfides (Fernandes & Holmgren, 2004; Nordstrand *et al*, 1999).

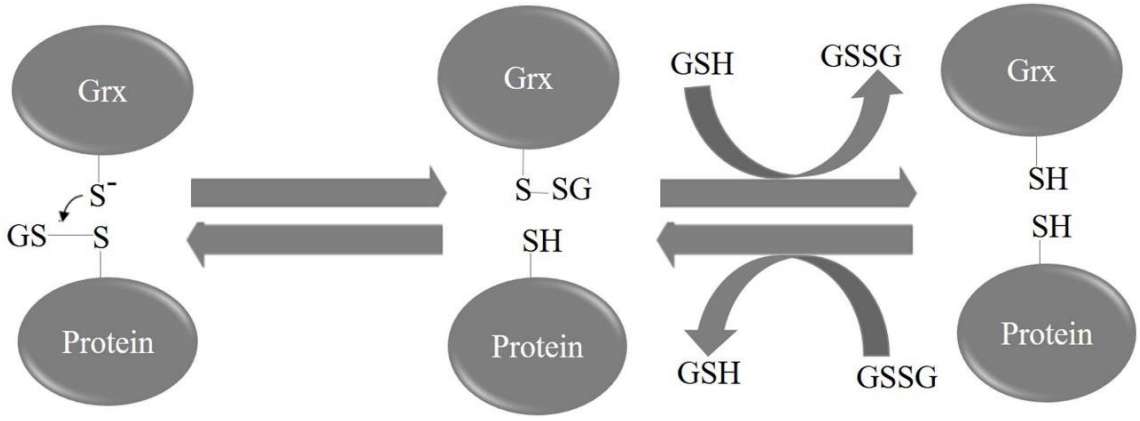


Fig1.9A The glutaredoxin monothiol mechanism:- The grey arrows show the initiation of deglutathionylation (left to right). The S-glutathionylation from right to left. Adapted from (Fernandes & Holmgren, 2004).

Glutaredoxin has been demonstrated to be susceptible to redox status of the cell. Wild-type *E. coli* Grx3 has a tendency to form mixed disulfides with glutathione (Lind *et al*. 1996) that causes a decrease in its catalytic activity (Åslund et al. 1998). A mutant construct of *E. coli* Grx3, with Tyr replacing a conserved Cys^{65} doesn't have this tendency, suggesting that S-glutathionylation of Cys^{65} regulates wild-type enzyme's activity. This particular cystein is S-glutathionylated *in vitro* during H_2O_2 induced stress, and it is possible that S-glutathionylation of residue Cys^{65} regulates Grx activity towards S-glutathionylation of protein thiols under oxidative stress (Lind et al. 1996).

Diverse factors may influence the process of S-glutathionylation of proteins *in vivo*. Different mechanisms have been proposed for S-glutathionylation, i.e. the development of mixed disulfides between protein and glutathione (GSH). A decrease in the cellular GSH/GSSG ratio due to the action of oxidants such as diamide, H_2O_2, t- butyl-hydro peroxide and redox cycling of quinones such as menadione, leads to the formation of mixed disulfides (Brigelius *et al*, 1983; Collison *et al*, 1986).

The properties and thermodynamic possibilities of the reversible glutathione – disulfide exchange reaction in the absence of enzymatic catalysis have been (Figure: 1.9B) thoroughly discussed (Gilbert, 1995).

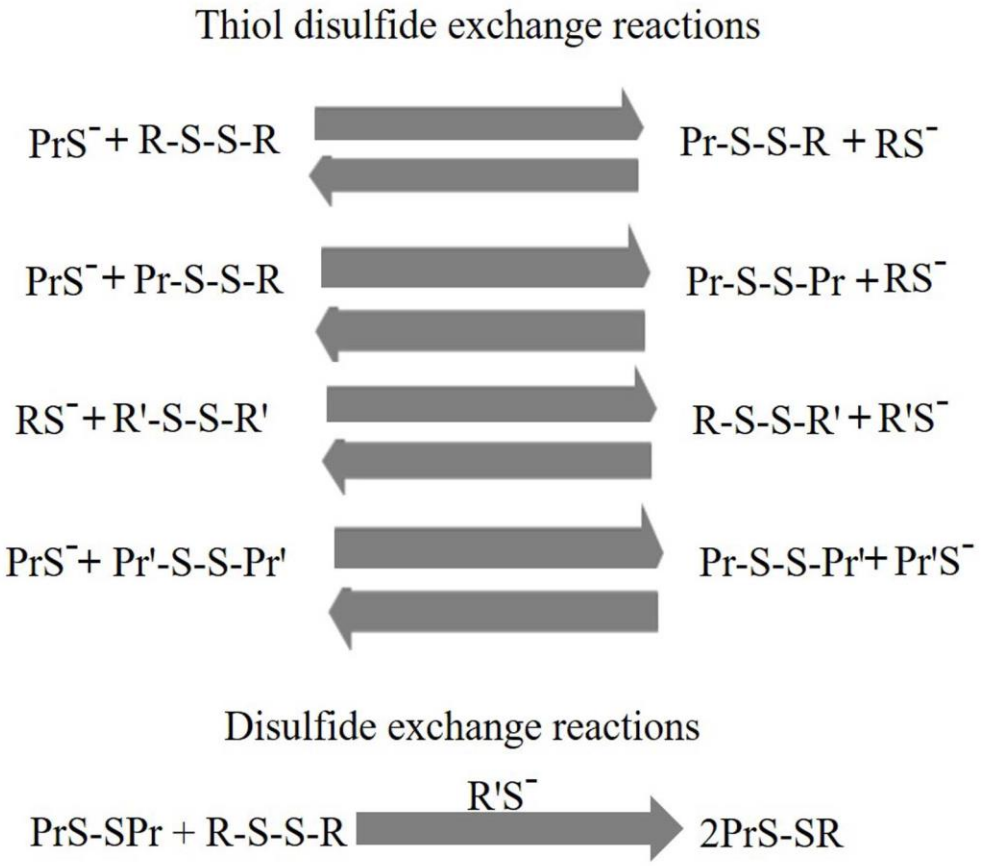


Fig 1.9B:- Proposed possible non-enzymatic thiol-disulfide exchange (4 different types) and disulfide exchange reactions: Pr, protein; R; low molecular weight thiols (Cotgreave, Atzori and Moldéus, 1989).

Typically, the formation of thiolate anion is necessary for thiol-disulfide and disulfide-disulfide exchange reactions under physiological conditions. Furthermore, the thiol-disulfide oxidation potentials of proteins are hard to predict. Further, a large variation between those potentials, one may presume that many proteins are not functionally affected simply by a physiological change in the GSH/GSSG ratio (Gilbert, 1995). There are proteins whose function can be non-enzymatically modified *in vitro* by a change in GSH redox status such as transcription factor c-Jun (Klatt *et al,* 1999). Further, the oxidative stress-induced formation of GSH-mixed-disulfides on proteins is not necessarily

dependent on perturbation of the cellular GSH/GSSG redox status(Chai *et al*, 1994; Schuppe-Koistinen *et al*, 1994).

Enzyme-catalyzed formation of GSH-mixed-disulfides is more rapid in the presence of free radicals (Starke et al. 2003). The thiyl-radical intermediate mechanism is may be one of the possible reason for formation of mixed disulfides as suggested by Thomas and co-workers (park et al. 1987; Thomas et al. 1995). Free radicals in the cell, like OH^-, induce the formation of reactive protein sulfhydryl intermediates such as thiyl-radicals, which then react rapidly with GSH to form mixed disulfides (Figure: 1.9C). Grx stabilizes a GS- radical in its active site and that the transfer of this radical to protein sulfhydryls is particularly rapid(Starke *et al*, 2003). Thus, the local redox environment will influence the enzymatic catalysis of S-glutathionylation and de-glutathionylation and the rate and direction of Grx-catalyzed reversible glutathionylation is often influenced by several factors.

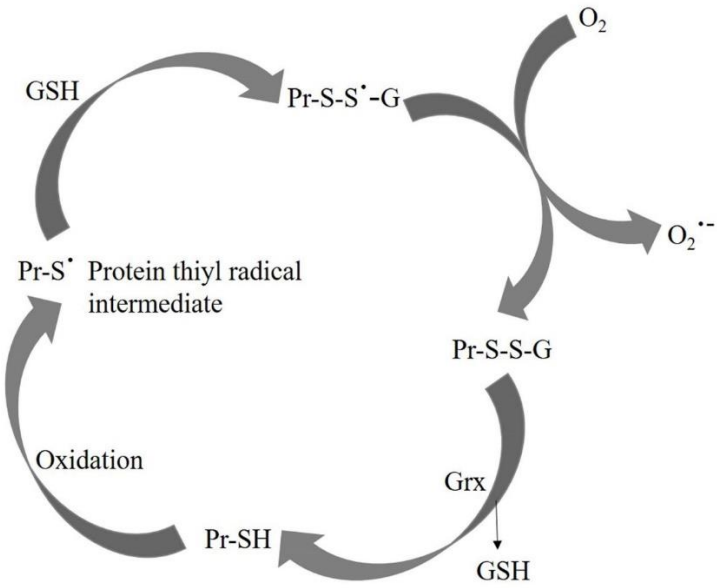


Figure: 1.9C:- Proposed mechanism of reversible S- glutathionylation via a thiyl-radical intermediate:Pr, protein (Thomas *et al*, 1995).

1.10 Functional significance of S-glutathionylation in proteins

Cellular stress can affect diverse functions such as apoptosis, proliferation, ion channel function, cytoskeleton apparatus arrangement, signal transduction pathways, gene regulation, carbohydrate metabolism, neurotransmitter release and proteolysis. It has been shown that S-

glutathionylation of GAPDH, actin, certain heat shock proteins and peroxiredoxins in various cell types in response to different types of stresses (Chai *et al*, 1994; Ravichandran *et al*, 1994). These studies indicate that the binding of glutathione to these proteins is not a random process. Further, oxidative stress-mediated regulation of actin polymerization with involvement of glutaredoxin, influence of GSH/GSSG perturbations on transcription factors like NFkB and AP-1 (Klatt *et al*, 1999; Pineda-Molina *et al*, 2001) (non-enzymatic redox regulation), and production of NADPH by stress-induced regulation of GAPDH and other glycolytic enzymes (Lind *et al*, 1998) demonstrates the importance of S-glutathionylation.

1.11 Electron Transport Chain (ETC)

Electron Transport Chain which is present in the inner mitochondrial membrane, is the most important step of cellular respiration. It is organized into five different complexes (Fig.1.11). 1) Complex I (NADH- coenzyme Q reductase) comprising of 46 subunits. 2) Complex II (succinate dehydrogenase–ubiquinone oxidoreductase) comprising of 4 subunits. 3) Complex III (ubiquinone–cytochrome c oxidoreductase) comprising of 11 subunits. 4) Complex IV (cytochrome c oxidase) comprising of 13 subunits and 5) Complex V (ATP synthase) comprising of 16 subunits. Apart from these membrane-bound complexes, two small mobile electron carriers, cytochrome c and coenzyme Q are also required. In this reaction, electrons are transferred along ETC complexes to oxidise NADH or $FADH_2$ and reduce molecular oxygen to water. The energy released during this process is used to transfer protons from matrix to the intermembrane space. Influx of these protons back into the mitochondrial matrix produces ATP via ATP synthase (complex V) (Elston *et al*, 1998; Noji & Yoshida, 2001). This pathway is often called the "respiratory chain" as it is the major reason for respiration in animal cells.

Complex I (NADH dehydrogenase)

Complex I contains an Fe-S cluster and an FMN. In Complex I, NADH is oxidised to NAD^+ by reducing FMN to $FMNH_2$. Electrons are then transferred from $FMNH_2$ to Ubiquinone via Fe-S cluster. During this process, each pair of electron pumps 4 protons from the mitochondrial matrix to the intermembrane space thus generating a proton gradient. Coenzyme Q located in IMM can accept electrons from Complex I and II and transfer it to Complex III. The main source of superoxide generation in ETC is Complex I due to its premature electron leakage to O_2.

Complex II (Succinate Dehydrogenase)

Succinate dehydrogenase is a TCA cycle exclusive enzyme present in inner mitochondrial membrane. It facilitates conversion of succinate to fumarate via reduction of FAD to $FADH_2$. Electrons are further tranferred from succinate to Coenzyme Q during oxidation of $FADH_2$. Succinate dehydrogenase does not pump protons, and therefore less energy is produced in this step.

Complex III (Coenzyme Q-dependent cytochrome c reductase)

Complex III accepts electrons from Coenzyme Q and sequentially tranfers it to Cytochrome c. Cytochrome c is a water soluble electron carrier present in the intermembrane space. Akin Complex I, Complex III also generates a proton gradient. Complex III may also contribute to ROS generation in mitochondria by leaking electrons directly to oxygen (Turrens, 2003).

Complex IV (Cytochrome c oxidase)

Cytochrome c oxidase takes electrons from Cytochrome c and transfers them straight to oxygen (O_2). Akin Complexes I and III, Complex IV also generates a proton gradient. Approximately 10 protons per pair of electron are pumped from mitochondrial matrix by these three proton pumps (Complex I, III, and IV).

Complex V (ATP Synthase)

ATP synthase is required for the synthesis of ATP. One molecule of ATP is synthesised for every 4 proton, that moves down the proton gradient generated by the proton pumps. The ATP synthase is a protein complex comprising of two subunits: F_0 (a transmembrane pore that facilitates (H^+) proton movement down the gradient) and F_1 (the part of the complex that performs ATP synthesis). Because of their rotational motor mechanism, ATP is produced.

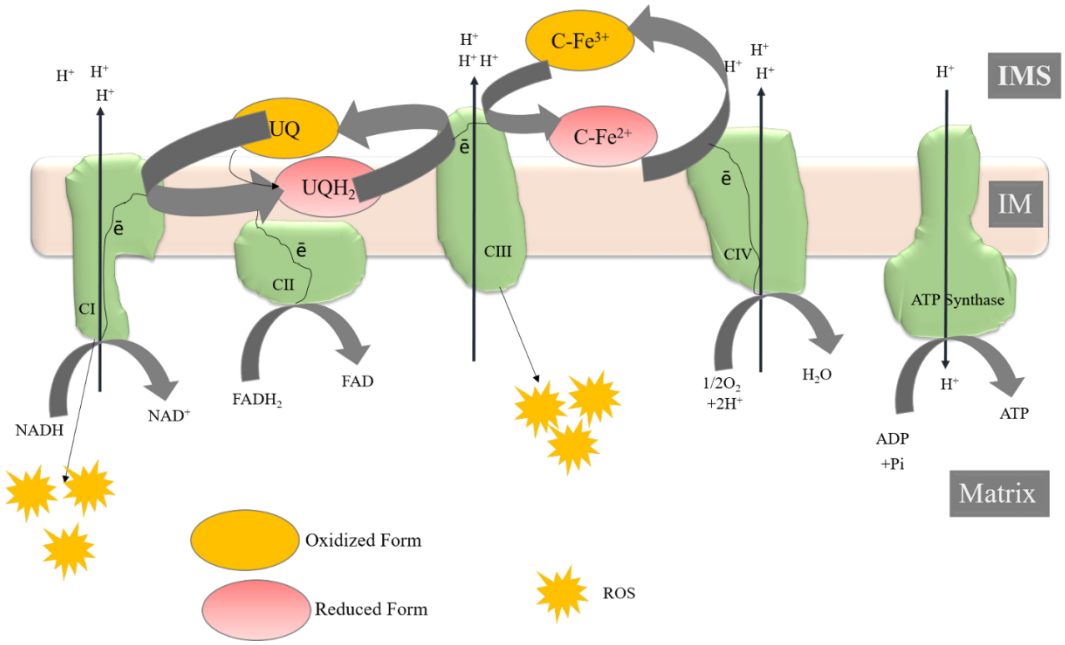


Figure: 1.10 Schematic Representation of electron transport chain:- It is composed of five enzyme complexes which are Complex I (NADH-ubiquinone oxidoreductase), Complex II (succinate ubiquinone oxidoreductase), Complex III (ubiquinol: cytochrome c oxidoreductase), Complex IV (cytochrome c oxidase) and ATP synthase (Complex V). NADH and $FADH_2$ enter the chain via complex I and complex II, proceeding with electron transfer through molecular oxygen and result in H_2O formation in complex IV. H_2O formation changes proton gradient and membrane potential resulted in ADP phosphorylation in complex V .

1.11.1Mitochondrial Electron transport chain- Diseases

Mitochondria have their own DNA (mtDNA) and machinery for RNA and protein synthesis. But mtDNA encodes for only 3% of mitochondrial gene products, the remaining 97% are encoded by nuclear DNA (nDNA) and are imported from the cytoplasm. Any defect in mitochondrial pathways may lead to mitochondrial diseases. Mitochondrial disorders affecting brain and skeletal muscle are referred to as mitochondrial encephalomyopathies whereas the ones caused due to defects in electron transport chain are called LHON, which is under both Mendelian and mitochondrial genetics. Yet, these diseases are not as rare as commonly accepted neurologic diseases, like amyotrophic lateral sclerosis and the muscular dystrophies (Chinnery & Turnbull, 2001).

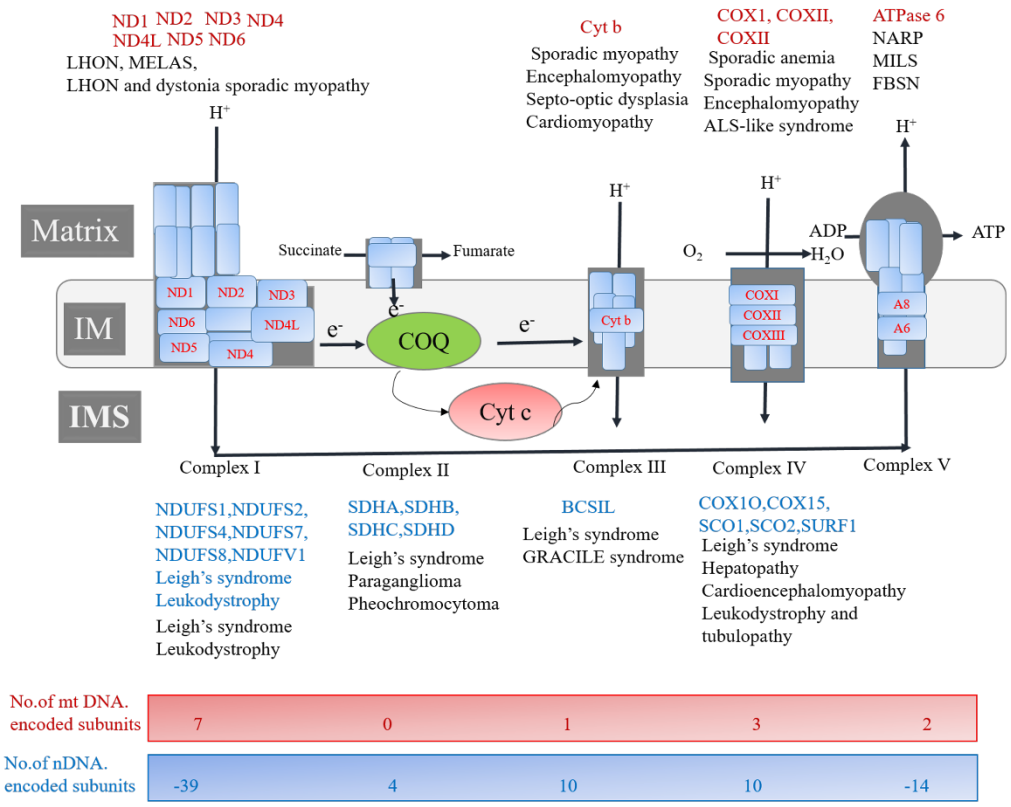


Figure: 1.11. The mitochondrial electron transport chain- Diseases: - The subunits of the respiratory chain encoded by nuclear DNA (nDNA) in blue and the subunits encoded by mtDNA in red. At the time of electrons (e^-) flow along the ETC, H^+ (protons) are pumped from the matrix to the intermembrane space over complexes I, III, and IV & again back into the matrix through complex V, to generate ATP. CoQ and Cyt *c* are electron-transfer carriers. Genes responsible for the indicated respiratory-chain (ETC) disorders are also shown. *ATPase 6* stand for ATP synthase 6; *BCS1L* Cytochrome *b–c* complex assembly protein (complex III); *NDUF* NADH dehydrogenase–ubiquinone oxidoreductase; *SCO* synthesis of cytochrome oxidase; *SDHA, B, C, & D* succinate dehydrogenase subunits; *SURF1* surfeit gene 1; FBSN familial bilateral striatal necrosis; LHON Leber's hereditary optic neuropathy; MELAS mitochondrial encephalomyopathy, lactic acidosis, and stroke like episodes; MILS maternally inherited Leigh's syndrome; NARP neuropathy, ataxia, and retinitis pigmentosa; GRACILE growth retardation, aminoaciduria, lactic acidosis, and early death; and ALS amyotrophic lateral sclerosis. Adapted from (Dimauro & Schon, 2003).

1.12 Iron (Fe)

From single cell bacterium to human's iron is the vital molecule. Iron can occur in 3 different oxidation states, 1) Fe^{2+}, 2) Fe^{3+}, and 3) Fe^{4+}. Iron plays a role in e^- transfer and flexible binding to the

ligands because of their inter exchange of oxidative states. So, the e^- spin state and redox potential of Fe are important for its chemical activity. Oxygen, nitrogen, and sulfur are favoured biological ligands for Fe. Hence, iron is an essential for some the key biological molecules those participate in oxidation-reduction (ferredoxin), oxygen transport (hemoglobin), and electron transfer (cytochromes).

1.13 Major routes for cellular iron uptake

Iron homeostasis is firmly controlled in a cell to evade extra iron toxicity or iron scarcity (Hentze *et al*, 2004; De Domenico *et al*, 2008; Ganz, 2008). There are 2 mechanisms to transport non-heme iron into cells, transferrin and non-transferrin bound iron uptake (Tf and NTBI respectively). In circulation, iron is completely bound to transferrin under normal physiological conditions. Transferrin saturation with iron and surplus of plasma iron occurring as NTBI are usually seen in diseased conditions that results from iron load. Specific uptake route(s) for NTBI remain uncertain, however in some cases it was shown that it might involve more than one cellular surface ferrireductases. Nevertheless, the main iron uptake route is through the iron which is bound to the transferrin. This transferrin bound iron is adopted by receptor-mediated endocytosis once it binds to transferrin receptor 1 (TfR1). The ferric state of iron, free from transferrin within the endosome is later acted upon by an endosomal ferrireductase (STEAP3), or by a new machinery involving cellular ascorbate to decrease its acidification. Similar to NTBI uptake, ZIP14 or DMT1 mediate the transport of the ferrous iron through the endosomal membrane. This promising cytosolic iron then becomes part of an unwell characterized chelatable or LIP (labile iron pool) that can be used for metabolism, deposited in ferritin or released back to the extracellular space. The released iron can be gathered into Fe-S clusters for the maturation of cytosolic and nuclear proteins (Rouault 2006) for example ribonucleotide reductase (Liu and Graslund 2000). Mitochondria, the key iron consuming sub-cellular organelle, has a central role in iron metabolism. IRP1& IRP2, 2 iron regulatory proteins are involved in regulating the iron homeostasis in cellular systems. (Rouault, 2006; Wallander *et al*, 2006).

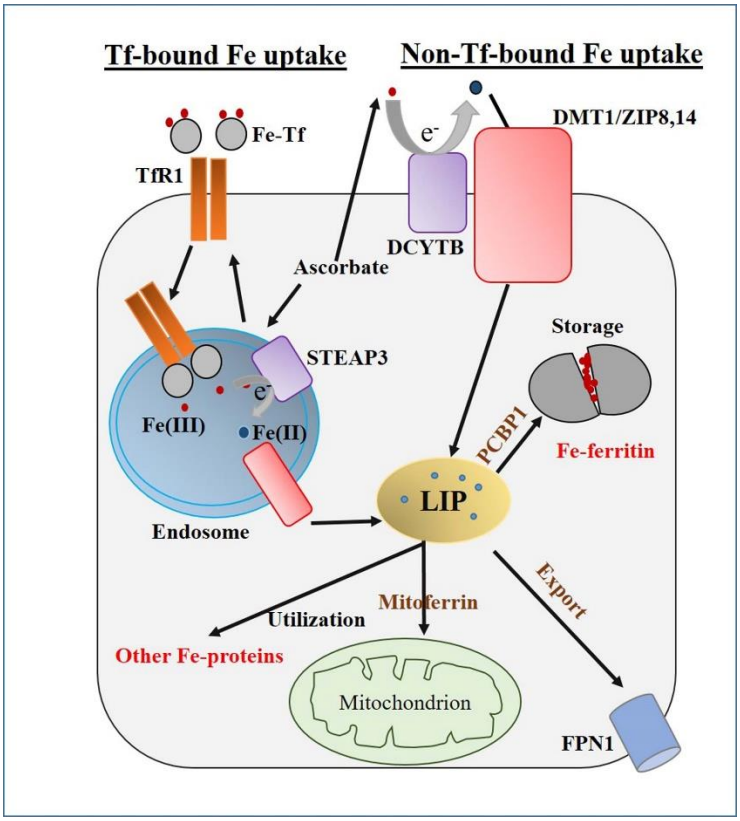


Figure 1.12 Mechanism of iron transport in human cells:- Two types of cellular iron (Fe) uptake pathways, i.e. transferrin (Tf)-bound Fe uptake and non-Tf-bound Fe (NTBI) uptake. Under physiological conditions, most Fe is bound to Tf which binds to the transferrin receptor 1 (TfR1) on the cell surface that is then involved in receptor-mediated endocytosis with the Fe being released from Tf by a decrease in endosomal pH and reduction by an endosomal reductase [e.g., six transmembrane epithelial antigens of the prostate 3 (STEAP3)] or, potentially, by ascorbate. The Fe (II) is then transported across the endosomal membrane by divalent metal transporter 1 (DMT1) where it then becomes part of the poorly characterized labile iron pool (LIP) in the cytosol. Iron in the LIP acts as an intermediate and can be utilized for storage in the iron storage protein, ferritin, or used for the synthesis of heme and iron-sulfur clusters in the mitochondrion or cytosol. Iron can also be exported from the cell by ferroportin 1 (FPN1). In conditions of iron overload, NTBI exists in the blood, and may be taken up by processes that include cell surface reduction by ferrireductases such as duodenal cytochrome b (DCYTB), or by effluxed reductants, such as ascorbate. Intracellular ascorbate can also supply electrons for DCYTB-dependent ferrireduction. Such enzymes reduce ferric NTBI to its ferrous state, which can be imported from the plasma membrane by transporters such as DMT1. Adapted from(Lane *et al*, 2015).

1.14 Fe-S clusters

Iron is a module of iron- sulfur clusters (Fe-S), it is vital for many essential processes together with replication and repair of DNA processes, and processes regulating gene expression, redox catalysis, electron transfer, and cellular respiration (Stephens et al. 1996; Beinert 2000; Ryter and Tyrrell 2000; Walden et al. 2006; Boal et al. 2007; Netz et al. 2012; Rouault 2012). They are evolutionarily antique and are typically found in all organisms. The most familiar iron sulfur clusters found in eukaryotic proteins are 2Fe-2S and 4Fe-4S. These iron sulfur clusters are made by tetrahedral coordination of iron atoms with sulphide and ligated to the protein over the cysteine residues.

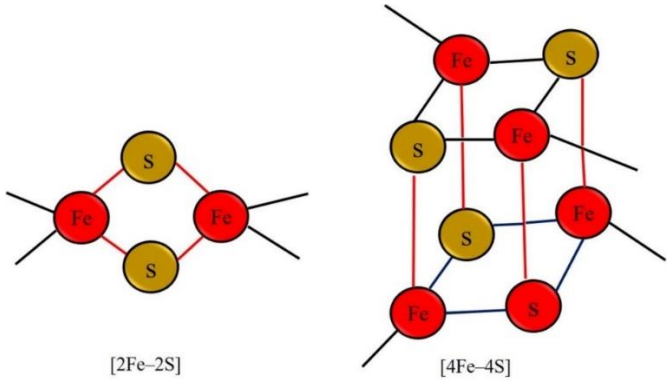


Figure 1.13 Structure of Fe-S clusters: - The most familiar iron sulfur clusters are the 2Fe-2S and 4Fe-4S.

1.15 Fe-S cluster biogenesis

The investigation work on bacteria elucidates that 3 diverse biosynthetic types of machinery are present to make iron-sulfur proteins (Frazzon and Dean 2003). 1st machinery, the nitrogen fixation machinery is involved in the association of the complex iron-sulfur protein nitrogenase. Nitrogenase is important for the conversion of nitrogen (N_2) to ammonia (NH_3) in nitrogen-fixing bacteria (Rees and Howard 2000; Frazzon and Dean 2002). 2) The 2nd machinery, iron-sulfur cluster assembly mechanism that is required for the synthesis of cellular iron-sulfur proteins (Zheng et al. 1998). 3) The 3rd machinery, sulphur-mobilization mechanism that is an autonomous system and mostly utilizes under iron-restrictive and/ or oxidative conditions (Fontecave et al. 2005; Johnson, Dean et al. 2005). Mitochondria have the mechanisms similar to ISC machinery, although plastids have machineries similar to SUF machinery of bacteria (Lill and Kispal 2000; Balk and Lobreaux 2005).

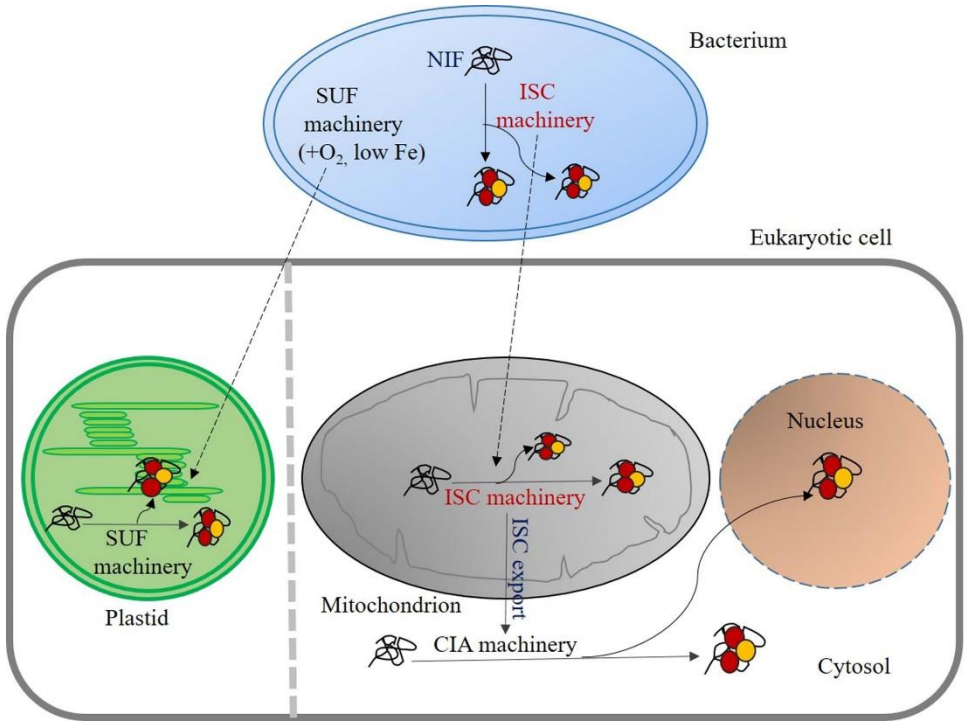


Fig 1.14 Biogenesis and the evolutionary origin of Fe-S cluster proteins:- In eukaryotes, Fe-S cluster proteins are present in the cytosol, mitochondria, and nucleus. The ISC assembly machinery of mitochondria was originated from the evolutionary ancestor, α-proteobacteria. The SUF machinery of plastids in plant cells may be transferred to the eukaryotic cell by endosymbiosis of a photosynthetic bacterium. The mitochondrial ISC assembly machinery, the ISC export system and the CIA machinery are required for the maturation of both nuclear and cytosolic Fe-S proteins. These three systems are highly conserved from lower to higher eukaryotes. The bacterial NIF machinery is principally required for the assembly of nitrogenase in bacteria. A small red and yellow circle denotes the Fe/S clusters . Adapted from Ronald Lill et al. 2006.

1.16 Role of Fe-S proteins in metabolism

Aconitase, residing in both mitochondria and cytosol, is an iron-sulfur cluster enzyme. The mitochondrial enzyme contains a 4Fe-4S cluster wherein the cysteines on the enzyme backbone are bound by three Fe atoms and the fourth is ligated to sulfur of the inactive 3Fe-4S cluster giving rise to a free coordination site that binds substrates (Gardner and Fridovich 1992; Gardner 1997). The cytosolic enzyme is a bifunctional enzyme which may or may not have a 4Fe-4S cluster. The holo form (4Fe-4S) has aconitase activity while the Apo form lacking the enzyme activity regulates the levels of iron intracellularly.

In humans, there are three different kinds of iron sulfur proteins involved in the metabolism of nucleotides. 1) Dihydropyrimidine dehydrogenase (DPYD) consist of four 4Fe-4S clusters that function as a channel for e^- transport between the FAD and FMN groups of the enzyme (Schnackerz et al. 2004). It participates in the catalysis of initial rate-determining step in pyrimidine metabolism, i.e. degradation. 2) Glutamate phosphoribosyl pyrophosphate amidotransferase (GPAT) containing 4Fe-4S cluster catalyses the rate- determining step of purine biosynthesis. Remarkably, iron sulfur cluster in *Bacillus subtilis* associated with GPAT is essential for the structural stability and N-terminal processing of the protein. (Makaroff et al. 1986; Grandoni et al. 1989). The requirement of iron sulfur cluster integration in to GPAT for protein stability has also been exposed in human cell lines.

1.17 Medical Impact

Iron- sulfur cluster biogenesis is necessary for cellular development. These Fe-S proteins are vital for functioning of electron transport chain, TCA pathway enzymes like aconitase, and succinate dehydrogenase and some of the functions in mitochondria, cytosol and nucleus. Hence, it isn't very surprising that defects in iron sulfur cluster biogenesis can lead to numerous disorders (Table 1.1).

Table 1.1. Diseases caused by defects in Fe-S cluster biogenesis

Disease	Cause	Clinical phenotype and incidence	Tissues affected
Friedreich's ataxia (FRDA)	Decreased expression of frataxin due to expansion of intronic GAA repeat	Ataxia, loss of sensation in extremities, heart failure; incidence: 1/50,000 births	Primerly affects dorsal root ganglia, cerebellum and heart
GLRX5 deficient sideroblasticanemea	Low expression of GLRX5 due to mis splicing	Sideroblasticanemia	Red blood cells
ISCU myopathy	Splicing defect in ISCU that diminishes expression	Exercise induced lactic acidosis and muscle weakness	Skeletal muscle and heart
Mitochondrial encephalomyopathy	Mutations in NUBPL impair the function of respiratory complex I	Developmental delay, myopathy ataxia	Neurons, skeletal muscle
Multiple mitochondrial Dysfunctions syndrome (NFU type)	Mis-splicing of the NFU1, causes reduced expression	Weakness, lethargy progressing to death at 4 weeks of age, lactic acidosis, elevated blood levels of branched chain amino acids and glycine	No tissue specificity, possibly because of early onset severe systemic illness
Multiple Mitochondrial dysfunctions syndrome (BOLA type)	Single exonic base pair duplication leads to frame shift and a premature stop codon in BOLA3	Hyper glycinemia, acidosis, dilated cardiomyopathy, epileptic encephalo myopathy, death at 11 months of age	Majorly affects central nervous system and heart

Adapted from (Rouault, 2012a).

1.18 Scope of the present study investigation

Human MIA40 contains seven cysteine residues. Six cysteines residues are conserved and are aligned in the order of one CPC (C represents cysteine and P proline) and two CX9C motifs. In a disulfide relay system, oxidized CPC motif introduces a disulfide bond in the substrate molecule by accepting electrons from the incoming polypeptide chain (Stojanovski *et al*, 2012). The reduced MIA40 needs to be reoxidized to perform another round of substrate oxidation and import. Reoxidation of CPC motif in MIA40 is mediated by Erv1 (in mammals ALR). The ternary complex that is formed in-between substrate, hMIA40 and ALR is able to introduce efficient electron transfer during disulfide relay system (Bottinger *et al*, 2012). Further, electrons from ALR are transferred to molecular oxygen via cytochrome c of the electron transport chain.

Recently, we and others have shown that MIA40 binds Fe-S clusters and is involved in the export of Fe-S from mitochondria to the cytosol (Daithankar et al. 2010; Spiller et al. 2013; Murari et al. 2015). In yeast ATM1 (ABCB7 in mammals), an inner membrane protein has been recognized as a Fe-S cluster exporter from mitochondria. The exact module that is exported by ATM1 is still not known, but, few studies suggest that it is maybe involved in the export of sulfur to cytosol through some anonymous mechanism. The extra components of Fe-S export machinery that are perhaps working beside with ATM1 includes Erv1, MIA40, and GSH (Ronald et al. 2006), as depletion of these proteins leads to increase of iron in mitochondria and defect in maturation of cytosolic Fe-S containing proteins. Still, the specific physiological importance of Fe-S cluster in MIA40 is yet to be understood.

In addition, it has been shown that hMIA40 interacts with AIF and regulates respiratory chain biogenesis. Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein. Apart from its apoptotic function, it is required for the normal expression of major respiratory chain complexes. Depletion of AIF causes a down-regulation of hMIA40 protein probably by reduction of its mitochondrial import. Restored hMIA40 levels by overexpressing MIA40 restores the respiratory chain function in AIF deficient cells (Hangen *et al*, 2015). Hence the potency of the MIA pathway influences the functional state of the respiratory chain complex (Allen *et al*, 2005). However, the precise mechanism of the MIA40 role in electron transport chain function is still to be explored. Further, the importance of GSH on MIA40 mediated import suggests that a possible potential role of GSH in electron transport chain biogenesis and function, protein import and Fe-S cluster biogenesis.

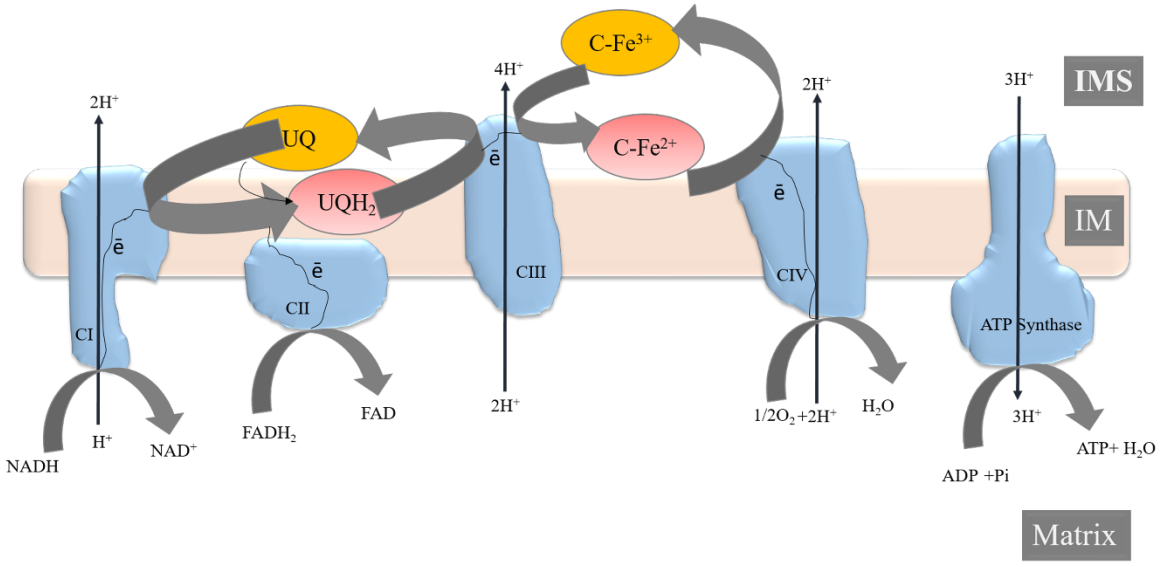
Based on the above rationale, the outline of the present study is as follows:

1. Characterization of hMIA40 function in electron transport chain biogenesis.

2. Functional characterization of glutathionylated MIA40 in protein import and iron homeostasis of the cell.

CHAPTER II

Characterization of human MIA40 function in electron transport chain Biogenesis



2.1 Introduction:-

The intermembrane space (IMS) of mitochondria has been found to be teeming with proteins enriched in disulphide bonds (Herrmann & Hell, 2005). It is thought that the presence of disulphide bonds locks these proteins in the IMS. Unlike the proteins that are present in the mitochondrial membranes or matrix by a dedicated import machinery constituting a plethora of proteins, the IMS proteins are brought in and retained in the IMS by a unique pathway called the disulphide relay pathway. The disulphide relay pathway depends on two proteins: MIA40 and ALR/Erv1. MIA40 is an oxidoreductase that facilitates the import and folding of the IMS targeted proteins by introducing disulphide bonds. Reduced MIA40 is recycled to oxidized form for initiating another import cycle by its partner, ALR (in yeast Erv1). The downstream trail of the electrons from ALR has been shown to reach the electron transport chain. The hydrophobic binding cleft of MIA40 is thought to recognize the substrates for introducing the disulphide bonds, to trigger subsequent folding of precursor proteins. Additionally, the efficacy of oxidative folding of IMS proteins has been shown to be tightly coupled to their accumulation in the IMS (Mesecke *et al*, 2005; Müller *et al*, 2008). Thus, disulphide relay system prevents the leakage of mature proteins from the IMS (Chacinska *et al*, 2009; Sideris & Tokatlidis, 2010).

Maintenance of mitochondrial redox homeostasis is a tightly regulated and controlled process as any alterations affect mitochondrial function. Generally, cells use glutathione (GSH) and oxidized glutathione (GSSG) to tweak redox imbalances that frequently occur in the mitochondria that house the electron transport chain, the main contributor of Reactive Oxygen Species (ROS). Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinylglycine, GSH) that serves as an easily accessible cellular antioxidant. More recently, it has been re-discovered to S-glutathionylated proteins, a covalent post-translational modification (PTM) that is competing with other PTMS for its ability to modulate proteins reversibly in diverse cellular pathways like cell growth, cell differentiation, transcription and metabolism during both physiological and mild oxidative stress conditions. Interestingly, the IMS import process can be accelerated by the presence of GSH (Bien *et al*, 2010). Glutathione reduces the formation of long-lived, partially oxidized import intermediates and functions as a proof-reader during mitochondrial disulphide relay system (Bien *et al*, 2010). However, it is not clear whether GSH directly interacts with MIA40 or employs glutaredoxin.

MIA40 is an evolutionally conserved and essential protein. The highly conserved region in human MIA40 (hMIA40) harbours seven cysteines, out of which six conserved cysteine residues aligned in the order of CPC and two CX9C motifs besides present as C4 (Fig:2.3.4A). The cysteine pair in the CPC motif has been implicated in the formation of disulphide bond during the run of the disulphide relay system (Stojanovski *et al*, 2012). At the end of the disulphide relay cycle, ALR, a flavoprotein re-oxidizes the CPC motif of hMIA40 so as to facilitate a new cycle of disulphide relay cycle. The ternary complex formed between substrate, hMIA40 and ALR is efficient in executing the electron transfer, critical for the import, folding and retention of IMS proteins. ALR is thought to act as a redox switch as it accepts an electron pair from MIA40 and transfers two single electrons to two molecules of cytochrome c (Fig: 2.1).

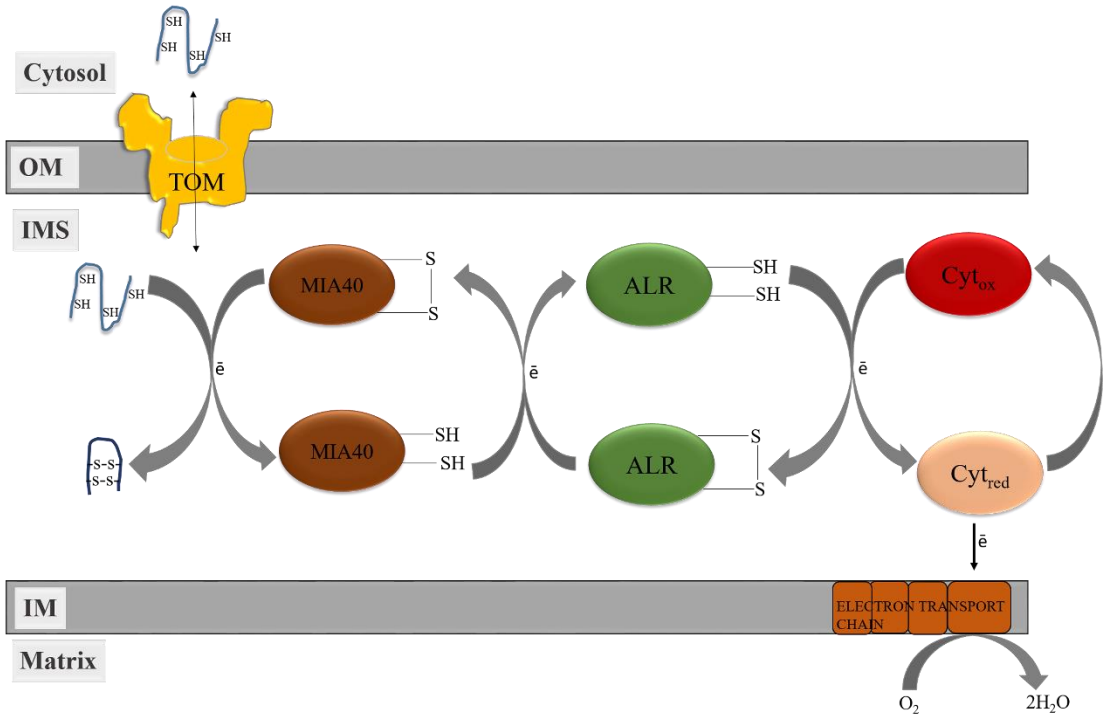


Figure: 2.1 Schematic representation of the MIA40-Erv1/ALR disulfide relay system: Simplified image of the protein import into intermembrane space (IMS) of mitochondria. The oxidized active state of MIA40 interacts with newly imported pre-proteins by intermolecular disulfide bonds, and subsequently, the substrate proteins getting into folded state. For the next cycle of protein import, MIA40 interacts directly with sulfhydryl oxidase Erv1/ALR and gets converted into active oxidized MIA40. The sulfhydryl oxidase Erv1/ALR is a dimeric FAD-binding protein that maintains an oxidized state by transferring electrons to cytochrome c and or molecular oxygen. Adapted from (Hell, 2008).

Recently, it has been shown that Apoptosis-inducing factor (AIF), a known mitochondrial apoptotic factor, is also required for biogenesis of respiratory chain complex I. AIF interacts with hMIA40 and regulates respiratory chain biogenesis by acting upstream to hMIA40. Depletion of AIF reduces hMIA40 protein levels in mitochondria by affecting its mitochondrial import. Overexpression of hMIA40 in AIF depleted cells restores the respiratory chain function, in particular, Complex I (Hangen *et al*, 2015). Thus MIA40 mediated pathway can influence the functional state of the respiratory chain complexes (Allen *et al*, 2005). However, the precise mechanism is not known. Besides the role of MIA40 in the disulphide relay system and in the functioning of Complex I, we have additionally shown that the CPC motif in MIA40 is capable of binding iron and thereby helps in the export of Fe-S clusters from mitochondria (Murari *et al*, 2015).

2.1.2 *In vivo* redox status of hMIA40:

It has been shown that 70% of steady state levels of hMIA40 in oxidized state under normal physiological condition, and it appears that local glutathione pool offers reducing equivalents to maintain redox levels of MIA40 (Kojer *et al*, 2012a). But it is not clear whether glutathione directly interacts with MIA40 or not. To test the redox status of MIA40 *in vivo*, we overexpressed hMIA40 in Human Embryonic Kidney (HEK293) T cells and isolated the mitochondria. 50 µg of mitochondria were treated with or without 10 mM DTT and kept for 15 min at 60°C and then cooled to room temperature following by addition of 20 mM IAA for 1 hr at room temperature to block free cysteines. Next, mitochondria were resolved on 12%SDS-PAGE and immunoblotted with the antibodies specific for hMIA40 (Figure: 2.12). As expected, most of the MIA40 was found to be in oxidized form. However, some portion of MIA40 was converted to the reduced form in presence of DTT. It is an *in vitro* experiment as we have treated mitochondria with DTT, but *in vivo* GSH may play a similar role.

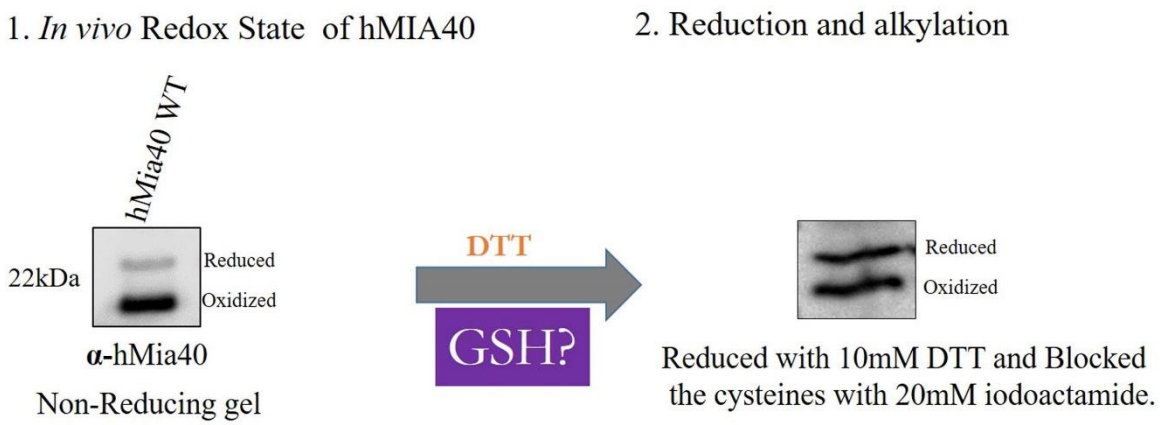


Figure: 2.1.2: *In vivo* redox status of hMIA40:- hMIA40 overexpressing mitochondria were treated with 10 mM DTT& blocked free cysteines with IAA and immunoblotted with antibodies against hMIA40.

In view of the importance of GSH during disulfide relay system, we speculate that chances of MIA40 undergoes glutathionylation are considerably high. In such case, it should also influence the electron transport chain as GSH plays a role in redox homeostasis. The present study was, therefore, undertaken with the following objectives:

1. Does MIA40 undergo post-translational modification, like glutathionylation?

2. Does glutathionylated MIA40 influence the function of electron transport chain?

2.2. Materials and Methods

2.2.1. Materials

N-Acetyl-L-cysteine (NAC) and L-Buthionine-sulfoximine (BSO) were bought from sigma to induce and inhibit glutathione synthesis respectively in cells. TOM40, OXPHOS, AIF, Hsp70, Aco1, Aco2, TOM20, c-Myc, GAPDH antibodies were procured from Abcam (USA). To knockdown humanMIA40, MIA40 specific shRNA was procured from ORIGENE. Glutathione Sepharose and Ni-NTA Sepharose beads were bought from GE health care. Cytochrome c, decylubiquinone, NADH, rotenone, BSA, sodium azide and DCFDA for mitochondrial complex activities and ROS measurement respectively were purchased from Sigma. DTT and IAA for protein reduction and alkylation studies were obtained from Sigma-Aldrich. Cell culture components like DMEM, Pen-strep, FBS, and lipofectamine for preparation of complete media and for transfection studies

respectively were purchased from Invitrogen. Cell culture 35mm, 60mm and 100mm dishes and T-25, T-75 and T-175flasks were purchased from Eppendorf India.

2.2.2. Methods

2.2.2A Plasmid constructions

From total RNA of HeLa cells, cDNA encoding hMIA40 was generated by following the manufacturer protocol (Bangalore GeNei, India). Open reading frame (ORF) of hMIA40 was amplified from cDNA by using primers MIA40-Fwd1 (5'-"CCCAGAATTCACCATGTCCTATTGCCGGCAGGAA"-3') and MIA40-Rev1 (5'-"CCACTCGAGTTAACTTGATCCCTCCTCTTCTTT"-3'). hMIA40 ORF was cloned into pET28 (a+) vector to generate plasmid pNB130 carrying hMIA40 with a His tag at the N terminus as described earlier (Murari *et al*, 2015). As per manufacturer's protocol site-directed mutagenesis (Fermentas) was performed with plasmid pNB130 containing wild type hMIA40 to create plasmids pNB309 (hMIA40 C53S&C55S), pNB388 (hMia40 C4-97S), pNB389 (hMIA40 C4S, C74S&C97S) and pNB390 (hMIA40 C4S, C64S, C74S&C97S). All mutants were cloned into pcDNA3.1 Myc-His vector with *EcoR1* and *XhoI* restriction sites to generate plasmids pNB202 (WThMIA40) and pNB314 (hMIA40 C53S&C55S), pNB379 (hMIA40 C4S&C97S (DM)), pNB380 (hMIA40 C4S, C74S&C97S(TM)), pNB391 (hMIA40 C4S, C64S, C74S&C97S (QM)) for expression in mammalian cells.

2.2.2B Site-Directed Mutagenesis

For generation of cysteine to serine mutation (Cysteine-Serine) in hMIA40, pcDNA-MIA40/ pET28a-hMIA40 plasmid was used as a template to amplify the DNA using Pfu /Taq DNA polymerase (Invitrogen) and exact primers as defined below. After amplification the PCR product was treated with the enzyme *DpnI* at 37°C for 1 hr. The enzyme treated PCR/plasmid product was transformed into E.coli DH5α Cells. Finally, clones were confirmed by the restriction digestion and plasmid sequencing. The list of the primers used for the generation of Cysteine to Serine was listed below.

GB1 FWP	5'-AAC TGG AAC TCC CCA TGC CTT -3'	MIA40 C53S
GB2 Rev	5'-AAG GCA TGG GGA GTT CCA GTT-3'	MIA40 C53S
GB3 FWP	5'-AAC TGC CCA TCC CTT GGG GGA-3'	MIA40 C55S
GB4 Rev	5'-TCC CCC AAG GGA TGG GCA GTT-3'	MIA40 C55S
GB13 FWP	5'-TGG AAC TCC CCA TCC CTT GGG-3'	MIA40 C5355S
GB14 Rev	5'-CCC AAG GGA TGG GGA GTT CCA-3'	MIA40 C5355S
GB5 FWP	5'-AGC GGT CCC TCT GGA GAA CAG-3'	MIA40 C64S
GB6 Rev	5'-CTG TTC TCC AGA GGG ACC GCT-3'	MIA40 C64S
GB7 FWP	5'-GCC TTT TCC TCC TTC CAC TAT-3'	MIA40 C74S
GB8 Rev	5'-ATA GTG GAA GGAGGA AAA GGC-3'	MIA40 C74S
GB9 FWP	5'-GGG TCA GAC TCT GTA GAC CAG-3'	MIA40 C87S
GB10 Rev	5'-CTG GTC TAC AGA GTC TGA CCC-3'	MIA40 C87S
GB11 FWP	5'-ATG CAG GAA TCC ATG CAG AAA-3'	MIA40 C97S
GB12 Rev	5'-TTT CTG CAT GGA TTC CTG CAT-3'	MIA40 C97S
NB 695FWP	5'-CCCAGAATTCACCATGTCCTATTCCCGGCAGGAA-3'	MIA40 C4S

2.2.2C Restriction Digestion

The PCR amplified hMIA40 Cysteine to Serine mutants and pcDNA3.1 c-myc-His/ pET28 (a+) vectors were exposed to double digestion with E.coRI & XhoI restriction endonucleases. In a 20 µl reaction {(Plasmid /PCR amplified product 10 µl; 10X Tango Buffer 4 µl (Fermentas); double distilled water 5.4 µl; E.coRI 0.3 U; XhoI 0.3 U) and pcDNA3.1 c-myc-His vector /pET 28 (a+) 30 µl; 10X Tango Buffer 4 µl; double distilled water 5.4 µl; E.coRI 0.3 U; XhoI 0.3 U)} at 37°C for12-16hrs. The digested products were visualized by agarose gel electrophoresis.

2.2.2D Bacterial Transformation

DH5α *E. coli* cells / *E. coli* Rosetta gami competent cells were used for transformation of plasmids and/ ligated products. Around 10 µl of PCR amplified product was added to DH5α cells and incubated on ice for 20- 30 min; heat shock was given at 42°C for 90 seconds and cool on ice for 2 min. After 2min 1 ml of LB medium was added and incubated at 37°C shaker incubator for 1hr, After 1hr 100µl culture was plated on LB agar plate containing an antibiotic (Ampicillin/ Kanamycin). Finally clones were confirmed with restriction digestion and sequencing.

2.2.2E Bacterial expression and protein purification of recombinant proteins

Plasmids pNB130, pNB388, pNB389 and pNB390 were transformed into *E. coli* Rosetta gami strain. Expression and purification of all recombinant cysteine mutant proteins were done as described earlier (Murari *et al*, 2015). Briefly, with 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) recombinant protein expression was induced when bacterial cultures attained an OD_{600nm} equal to 0.6-0.8. Ni-NTA affinity column (Clontech) was used to purify recombinant proteins with Buffer- A. Buffer -A containing 10 mM imidazole was used to wash the column. Buffer- A containing 400 mM imidazole was used for elution.

2.2.2E1 Expression of His-hMIA40

The plasmid pET28 (a^+) harbouring His-hMIA40 and His-hMIA40 mutants (Cysteine-Serine) were transformed into Rosetta gami *E. coli* cells. A colony having the pET28-His-hMIA40 plasmid was grown for12-16hr in LB medium containing kanamycin at 37°C shaker incubator. The principal culture was diluted to 1:100 in 500 ml LB medium, full-grown with dynamic agitation to reach OD600 nm: 0.6 and kept at 37°C by addition of 1 mM IPTG for 3-4 hrs. Afterwards, the culture was spin at 8000 rpm for 15 min to pellet down the cells. The bacterial pellet was resuspended in 50 mM Tris-HCl pH8.0 of $1/20^{th}$ of culture. Further, the frozen bacterial cells were burst by sonication. Next, the supernatant (soluble) and inclusion (insoluble bodies) portions were separated by spin at 8000 rpm for 15 min. The recombinant protein was present in supernatant (soluble) form. The soluble hMIAA40 recombinant protein was purified by using Ni-NTA Sepharose beads (Clontech).

2.2.2E2 Recombinant His-hMIA40 protein purification by Ni-NTA Sepharose beads

To the soluble / supernatant protein portion having His-MIA40, an equivalent volume of Buffer-B was added. The sample was then passed through the Ni-NTA column which was equilibrated with Buffer-A. Then the column washed thrice with Buffer-A containing 10 mM Imidazole pH7.2, and the bound protein was eluted by using elution buffer.

Buffer A	Buffer B	Elution buffer
100Mm Nacl, 20Mm Tris-Hcl pH 7.5 and 10Mm β-Me	200Mm Nacl, 10Mm Tris-Hcl-pH-7.5, and10Mm β-Me	0.4M Imidazole,50mMTris-Hcl-pH7.5,10mMNacl and 5mMDTT

2.2.2F SDS-PAGE analysis

Purified proteins or cell lysate were subjected to reducing and/ non- reducing SDS-PAGE (Sds-page & Sds, 1970). Further, to analyse the redox states of hMIA40, mitochondria were treated with or without DTT and subjected to non-reducing SDS-PAGE. For MALDI study GSH-Sepharose pulldown and/ recombinant hMIA40 protein was subjected to non-reducing SDS-PAGE and further stained with Coomassie brilliant blue R250.

2.2.2G MALDI-TOF/LC-MS/MS

MALDI-TOF and LC-MS/MS were implemented to identify cysteine modifications of hMIA40. Glutathionylated peptide peaks can be distinguished in MALDI-TOF spectra by the corresponding 306 Da increase in molecular weight. Trypsin-digested peptides were separated by BECH18 (2.1mmx150mmx1.7um dimension) column and were run on Synapt G2 QTOF instrument for MS/ MS analysis and the data received from the instrument was analysed by PLGS (protein lynx global server) software.

2.2.2H Western blot examination

The proteins were subjected to SDS-PAGE as defined earlier and proteins were transferred to a NC membrane/PVDF membrane (Pall/Millipore) 12hrs at 45 volts. The NC/PVDF membrane was blocked with TBST (“150 mM NaCl, 20 mM Tris-HCl pH 7.5, and 0.05% Tween-20”) having 5%

milk powder for 60 min at room temperature on rocker. The NC/PVDF membrane was probed with primary antibody in TBST at 4ºC for 12-16hrs on rocker. The NC/PVDF membrane was washed 3 times (each 10-15 min) with TBST solution and probed with Horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hr at room temparature on rocker. The NC/PVDF membrane was washed three times (each 10-15 min) with TBST and developed the blot using Western Bright ECL HRP substrate (advansta, USA) and Chemi Doc Imaging system (Bio-Rad). Cell lysate (50 µg), Mitochondria (30 µg) and recombinant proteins were resolved on SDS-PAGE and electroblotted onto Nitrocellulose membrane (PALL). The blots were probed with primary antibodies such as hMIA40 (human mitochondrial intermembrane space import and assembly protein 40), GAPDH (glyceraldehydes-3-phosphate dehydrogenase), ACO1 (Aconitase1), ACO2 (Aconitase2), Myc, TOMM40 (translocase of outer mitochondrial membrane 40), AIF (apoptosis-inducing factor) and Total OXPHOS (Abcam) followed by rabbit or mouse secondary antibody conjugated with HRP (Abcam) and developed by Western Bright ECL HRP substrate (advansta, USA) and Chemi Doc Imaging system (Bio-Rad).

2.2.2I Antibodies production

Against the hMIA40 protein polyclonal antibodies were raised up in the rabbit. The MIA40 protein was grained with either Freund complete adjuvant or Freund incomplete adjuvant (Bangalore GeNei) for primary and subsequent booster dose respectively (20 days break for each booster) and introduced into the rabbit. Serum was collected from the rabbit blood after following booster doses. With the help of antigen coupled Sepharose beads (GE healthcare) human MIA40 monospecific antibodies were purified. Before inducing antibodies, Pre-immune serum was collected from the rabbit blood

2.2.2J hMIA40 Polyclonal Antibodies Purification

The purified antigen (hMIA40) was dialyzed against coupling buffer (0.5 M NaCl and 0.1 M NaHCO3 pH 8.0) for 12-16hrs at 4°C. CNBr activated Sepharose 4B beads were mixed with the dialyzed antigen in coupling buffer and kept on the rotator for 60 to 90 mins at room temperature or 12-16hrs at 4°C. Further, beads were washed thrice with coupling buffer, and the free active groups were blocked with the 100mM Tris-HCl, pH 8.0 and incubated at room temperature for 60-120mins. Afterwards the column was washed thrice with different pH cycles. Every cycle consists of a wash

with 100mM Acetate buffer pH 4.0 having 500mM Na Cl followed by a wash with 100mM Tris-HCl, pH 8.0 containing 500mM Na Cl. Lastly, the beads were stored at 4°C in PBS buffer pH 7.2. The dialyzed serum against 1X PBS pH 7.2 buffer was mixed with the ligand coupled CNBr activated Sepharose beads and kept on a rotator for 2-4 hrs at 4°C, followed by spin at 5000 xg for 5 min to pellet down the Sepharose beads. Next the beads were washed thrice with PBS. Finally, the Mono-specific antibodies bound to beads were eluted with 100mM Glycine pH 2.5. The antibodies were neutralized with tris base, concentrated and store at -20°C or -80°C.

2.2.2K UV-Visible Absorption Spectroscopy

UV-Visible absorption spectra were documented in 10 mm path length quartz cuvettes with a HITACHI U-2910 spectrophotometer at RT in the range 200 nm-600 nm (Gorla & Sepuri, 2014).

2.2.2L Circular Dichroism Spectroscopy

For recombinant hMIA40 and cysteine mutants of hMIA40 Circular Dichroism were performed in a Jasco J-815 spectrophotometer with quartz cuvettes of 0.1 cm path length. Spectra was documented at 200ºC from 200 to 260 nm wavelength with a determination of 1.0 nm and an acquisition time of 50 nm/min. The ultimate spectra were got by averaging three repeated scans. CD scans were adjusted for background noise by deducting spectra of protein-free samples documented under same conditions.

2.2.2M Cell culture, transfection and Treatments

HEK 293T cells were cultured in complete Dulbecco's Modified Eagle's Medium (Invitrogen) having 10% (v/v) Foetal Bovine Serum at 37°C under an atmosphere of 5% CO_2. Cells grown in 100 mm/T-175 mm flasks were transfected with either 8 µg /15 µg respectively of scrambled shRNA or human MIA40 shRNA plasmid (ORIGENE) by using lipofectamine transfectant agent (Invitrogen). HumanMIA40 was overexpressed in HEK 293T cells by transfecting with mammalian expression vector pcDNA3.1c-myc-His vector containing human MIA40 ORF (pNB202) pNB314 (hMIA40 C53S-C55S), pNB380 (hMIA40 C4S, C74S&C97S(TM)) and pNB391 (hMIA40 C4S, C64S, C74& C97S (QM)). Co-expression was done in HEK 293T cells by co-transfecting with 15 µg plasmid each of MIA40 shRNA/pNB314 c-myc-His and MIA40 shRNA/ pNB380 c-myc-His, and MIA40 shRNA/ pNB391c-myc-His. After 24hrs of transfection DMEM medium was substituted with fresh DMEM

medium and cells were incubated in the presence or absence of NAC (1 & 5 mM) and / H_2O_2, (25, 50 & 100 µM) for 12hrs / 30 mins respectively at 37ºC under an atmosphere of 5% CO_2.

2.2.2N Mitochondria isolation from HEK 293T cells

Isolation of mitochondria from HEK 293T cell lines was performed as described earlier (Murari *et al*, 2015). Briefly, Cells were washed twice with 1XPBS and harvested in mitochondria isolation buffer. The cell suspension was further homogenized using Polytron 1600 at 5 sec X 2 pulses and 15 rpm. The cell suspension was subjected to Dounce homogenization and spin at 1000 X g for 10 min at 4°C. Pellet fraction contains a nucleus and unbroken cells. The supernatant was spin again at 10,000 X g for 15 min at 4°C. Pellet fraction containing mitochondria were washed twice and suspended in a Mito solubilization buffer.

Mito isolation buffer	Mito solublization buffer
20 mM HEPES pH 7.5, 1.5 mM MgCl2, 1mM EDTA pH 8.0, 210 mM sucrose and 70 mM mannitol	250 mM sucrose, 5 mM magnesium acetate,40 mM potassium acetate, 10 mM sodium succinate, 1 mM DTT and 20 mM HEPES-KOH pH 7.4.

2.2.2O Immunoprecipitation

Transfection was done with mammalian expression vector pcDNA3.1c-Myc-His or vector (Invitrogen) containing hMIA40 ORF to HEK293T cells. Cells were grown for 6h in serum-free DMEM medium and later replaced with complete media. 24h of post transfection cells were treated with 10mM NAC for 12h. Cells were washed with PBS and lysed in NP-40 buffer (150 mM Na Cl ,50 mM Tris-HCl-pH 8.0, 10% NonidetP-40 (NP40) and 1X protease inhibitor cocktail). Immunoprecipitation (IP) was done with Glutathione antibody for overnight, and later Protein A-Sepharose beads were added to lysates and incubated for 3h at 4ºC on a rotator. Washing of beads was done with NP40 buffer and elution with 2× Laemmli's sample buffer. Elutes were subjected to reducing SDS PAGE and immunoblotted with the hMIA40 antibody, true blot secondary rabbit –HRP antibody was used to detect hMIA40 band specifically.

2.2.2P GSH-Sepharose Pulldown Assay

Transfection was performed with mammalian expression vector pcDNA3.1c-myc-His or vector (Invitrogen) containing hMIA40 ORF in HEK29T cells. For co-expression studies, HEK 293T cells were co-transfected with 15 µg of each plasmid MIA40 shRNA/pNB314 c-myc-His and MIA40 shRNA/ pNB380 c-myc-His, and MIA40 shRNA/ pNB391 c-myc-His. Cells were allowed to grow for 6h in serum-free DMEM medium and later replaced with complete media. 24h of post transfection cells were treated with 10mM NAC for 12h. Next, cells were lysed with NP-40 buffer and thereafter lysates (1mg) with 1X protease inhibitor cocktail (Roche) were incubated with Glutathione Sepharose beads for 1hr at room temperature on a rotator. Beads were washed thrice with NP40 buffer and elution was done with 2× Laemmli's sample buffer and subjected to reducing SDS PAGE and immunoblotted with the hMIA40 antibody.

2.2.2Q Detection of GSS-hMIA40 on GSH-Sepharose *in vitro*

50µg of recombinant hMIA40 protein was pre-treated with different concentrations of 1, 5, 10, and 20mM DTT and/ or 1, 2, and 5mM H_20_2 at room temperature for 1hr. To block free thiols/ free cysteines we added alkylating agent (20mM iodoacetamide (IAA)). After 30 mins pre-treated (DTT/H_20_2) recombinant hMIA40 protein was mixed with 30µl of GSH-Sepharose beads for 1hr at room temperature on a rotator. Beads were extensively washed and elution was done with buffer containing 10mMDTT. Buffer containing 10mMDTT released proteins binds to GSH Sepharose beads by disulfide bonds. We identified GSS-hMIA40 by probing with antibodies against hMIA40.

2.2.2R *In Vitro* GSH-Sepharose Pulldown Assay

Recombinant WThMIA40, hMIA40 DM, hMIA40 TM and hMIA40 QM (200ug) proteins were incubated with glutathione beads for 1h at room temperature on a rotator. Beads were washed thrice with 1mM reduced glutathione and beads were eluted with 10mMDTT and/or 20mM reduced glutathione and subjected to reducing SDS PAGE and immunoblotted with the hMIA40 antibody.

2.2.2S Mitochondrial complex activities

a) NADH oxidation to NAD^+ at 340 nm was calculated for complex I activity (Spinazzi *et al*, 2012). Briefly, 30 µg of 0.05% Triton X 100 treated mitochondria were incubated with 100 µM NADH in a

buffer containing 50 mM potassium phosphate (KP) pH 7.5, 5 mM $MgCl_2$ and 0.25% BSA for 2 min. By adding 60 µM decylubiquinone the activity assay was initiated, and the decrease in absorbance at 340 nm was measured. With the help of velocity of reaction (Δabsorbance/min) and the molar extinction coefficient of NADH (3.4 mM-1cm-1 at 340 nm with reference wavelength) Complex I activity was calculated.

b) Mitochondrial complex II activity was monitored spectrophotometrically at 600 nm (Spinazzi *et al*, 2012). Briefly, mitochondria (30µg) was solubilized in 0.05% Triton X 100 in 25mM potassium phosphate buffer and 20mM succinate incubated at 37°C for 10 min. The activity of complex II was initiated by addition of 80µM 2, 6-dichlorophenolindophenol (DCPIP) and the decrease in absorption was measured at 600 nm. The blue color of oxidized DCPIP turns colourless when it accepts an electron from complex II. Complex II activity is determined with the velocity of reaction and the molar extinction coefficient of DCPIP ($19.1 mM^{-1}cm^{-1}$).

c) Mitochondrial complex III activity was analysed by measuring the cytochrome c reduction at 550 nm (Spinazzi *et al*, 2012). Briefly, 30 µg of 0.05% Triton X 100 treated mitochondria were incubated with 25mM Potassium phosphate buffer pH7.4, 2mM NaN3 and 50µM cytochrome c. By the addition of 50µM reduced decylubiquinone (DBH2), the activity was initiated, and the increase in absorption at 550 nm for 2 min was measured. Cytochrome c is brown in colour in its oxidized state, and that turns orange-pink when it accepts an electron from complex III. Complex III activity was calculated with the velocity of reaction (Δabsorbance/min) and the molar extinction coefficient of cytochrome c ($18.5\ mM^{-1}cm^{-1}$ at 550 nm with reference wavelength). Beer-Lambert law equation was used to calculate the complex III activity.

d) Mitochondrial complex IV activity was analysed by measuring cytochrome c oxidation at 550 nm (Spinazzi *et al*, 2012). Briefly, 50 µM of reduced cytochrome c was incubated in 50 mM Potassium phosphate buffer pH7.4. The activity of complex IV was initiated by addition of 30 µg of 0.05% Triton X 100 treated mitochondria, and the decrease in absorbance at 550 nm for 2min was measured. Cytochrome c reduction was performed by adding tiny amounts of sodium dithionate until the absorbance of reduced cytochrome c at 550 nm is between 1.8 and 1.9. Complex IV activity was calculated with the velocity of reaction (Δabsorbance/min) and the molar extinction coefficient of

cytochrome c (18.5 $mM^{-1}cm^{-1}$ at 550 nm with reference wavelength). Complex IV activity was calculated by using the Beer-Lambert law equation.

2.2.2T Cytochrome c Reduction by hMIA40 (CHCHD4)

Recombinant WT hMIA40, (or) hMIA40 DM, (or) hMia40 TM, (or) & hMIA40 QM (10µM) were incubated with 50mM potassium phosphate buffer pH7.4, 0.5mM EDTA and 40µM oxidized cytochrome c for 5min (Bihlmaier *et al*, 2007). Reduction of cytochrome c was measured with UV-Visible spectra from 200 to 700 nm and recorded in a HITACHI U-2910 spectrophotometer at room temperature using a quartz cuvette of 1cm path length. Cytochrome c will be brown in colour in its oxidized state and becomes orange-pink colour upon electron acceptance from MIA40 protein.

2.2.2U ROS measurement

The generation of ROS was measured using H_2DCFDA. Post-transfection, cells were incubated with 25µM DCF-DA for 45 min at 37ºC. Then cells were washed and harvested in PBS. The emission of fluorescence was measured by using fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm respectively.

2.2.2V Statistical analysis

All statistical studies were done using Jandel Scientific Sigma stat software by one-way ANOVA followed by Post hoc Dunkan's test for multiple comparisons and alterations between two groups with Student's *t*-test.

2.3 Results

2.3.1 MIA40 undergoes glutathionylation *in vitro*

Recombinant human MIA40 was purified as described in the Methods and separated on non-reducing and reducing SDS-PAGE (Figure: 2.3.1 A) and stained with Coomassie. As expected, in the absence of reducing agent, MIA40 was found to be in two major forms, a fast migrating oxidized and a slow migrating reduced form. Also we find other high molecular weight forms and they likely represent the dimer, trimeric and oligomeric forms of MIA40 as described (Figure: 2.3.1A Lane1). (Hofmann *et al*, 2005; Murari *et al*, 2015; Erdogan *et al*, 2018). However, majority of MIA40 migrates as a reducing form in the presence of reducing agent (Figure: 2.3.1A Lane 2). To test either

recombinant hMIA40 protein has any glutathione, increasing concentration of recombinant MIA40 protein (0.5, 1, &1.5µg) was separated on non-reducing SDS PAGE and probed with antibodies specific for glutathione and MIA40 (Figure: 2.3.1B&C). We find that several prominent glutathione antibody cross reacting bands of M.W 22 kDa, 35 kDa and 50 kDa along with some high molecular weight bands. These protein bands likely represent the monomeric, dimeric, trimeric and high molecular weight oligomeric form of MIA40 as described (Figure: 2.3.1 Lane 1). However, when we treated the similar amount of protein with increasing concentration of DTT (1mM, 2mM & 5mM), we find that a single glutathione antibody cross reacting band at 22 kDa that likely represent the monomeric hMIA40 (Figure:2.3.1B Lane 4-6). These results suggest that both monomeric and oligomeric forms of hMIA40 may contain glutathione moiety in its structure. Further, similar kind of pattern was observed when we probed with MIA40 antibodies except a prominent oxidized form under non-reducing conditions (Figure: 2.3.1C). It's well known that proteins undergo glutathionylation under oxidative conditions (Dalle-Donne *et al*, 2009). To test whether enhanced glutathionylation of MIA40 under oxidative conditions, recombinant MIA40 (50µg) incubated with increasing amount of H_2O_2 (1mM, 2mM&5mM) and 5 µg of protein was separated on non-reducing gel and immunoblotted with glutathione antibody. We find that increasing concentration of H_2O_2 treatment enhances the oligomeric form of glutathionylated MIA40 while decrease in dimeric form (Figure: 2.3.1D lane 3&4). Similar kind of pattern was observed when recombinant MIA40 incubated with 2mM & 5mM of H_2O_2 and probed with MIA40 antibody (Figure:2.3.1E). However, majority of H_2O_2 treated hMIA40 resolves as monomeric and oligomeric form in presence of glutathione (Figure: 2.3.1D). This pattern is indeed similar when we probed with glutathione antibody as well (Figure: 2.3.1D). These results suggest that altered confirmation of MIA40 with peroxide treatment and presence of glutathionylated moiety in MIA40 (Figure: 2.3.1D). Protein S-glutathionylation is a reversible process and oxidative agents enhance S-glutathionylation while reducing agents reverse this process. Intriguingly, we still detect glutathione moiety on MIA40 in presence of DTT (1-5 mM) or GSH (1-5mM). This observation likely suggests that a strong –S-S-P bond due to multiple glutathionylation of available cysteines and probably require strong reducing agent to reduce the protein. To test this hypothesis, equivalent amount of recombinant MIA40 protein was treated with increasing concentration of DTT (1mM, 5mM &10mM) and/or increasing concentration of β-Me (100 mM,150 mM & 200 mM) and analysed on the non-reducing SDS-PAGE and immunoblotted with glutathione antibody and hMIA40 (Figure: 2.3.1F&G). Glutathione probed blots show that a lower level of glutathionylated MIA40 with

10 mM DTT (Figure: 2.3.1F Lane 4). However, β-Me treatment completely reduces the MIA40 protein as glutathione antibody fails to detect any protein band. Further, MIA40 antibody probed blots shows the presence of equivalent amount of protein in DTT or β-Me treated samples (Figure: 2.3.1G). These results suggest that with increasing concentration of either DTT/ β-Me reverses S-glutathionylation of MIA40.

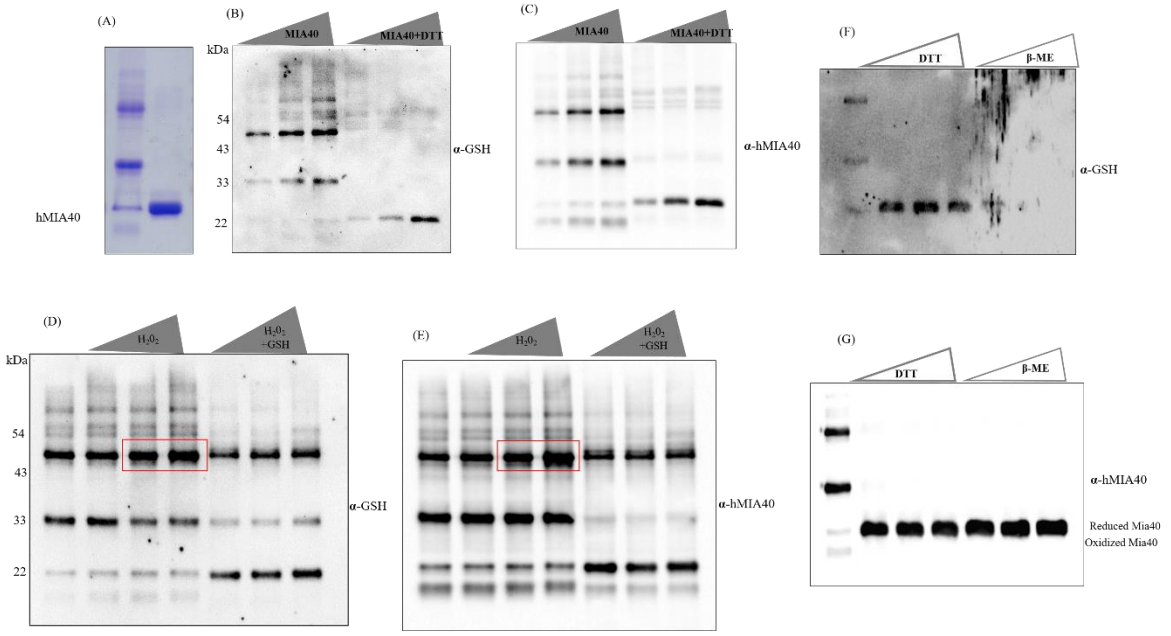


Figure: 2.3.1 MIA40 undergoes glutathionylation *in vitro*: (A&B) Recombinant purified MIA40 subjected to Non-reducing (Lane 1) and reducing SDS-PAGE (Lane 2). (B&C) Increasing concentration of recombinant hMIA40 was treated with or without DTT was analysed on non-reducing SDS-PAGE and immunoblot was performed with antibodies against GSH and hMIA40. (D&E) Recombinant hMIA40 protein was treated with increasing concentration of H_20_2 and/H_20_2&GSH, thereafter analysed on non-reducing SDS-PAGE, and immunoblot was performed with antibodies against GSH and hMIA40. (F&G) Recombinant hMIA40 protein was treated with increasing concentration of DTT and/β-ME thereafter analysed on non-reducing SDS-PAGE and immunoblot was performed with antibodies against GSH and hMIA40.

2.3.2 Glutathionylation of hMIA40

GSH-Sepharose beads has been used successfully to identify the glutathionylated proteins. Treatment of proteins either with peroxynitrate or peroxide induces the formation of reactive cysteines. Reactive cysteines that are prone to glutathionylation can form disulphide bond with GSH-Sepharose. The bound proteins eluted with reducing agents like DTT by disrupting the disulphide bond in between protein and GSH-Sepharose beads. We have shown that recombinant MIA40 undergoes glutathionylation and it enhances with peroxide treatment (Figure: 2.3.1E) indicating that the presence of reactive cysteines. To further analyse the glutathionylation profile and presence of reactive cysteine on MIA40, recombinant purified MIA40 was pre-treated with either increasing concentrations of DTT (1 to 20 mM) to reduce the glutathionylated cysteines/ reactive cysteines (Figure: 2.3.2A) or H_2O_2 (1 to 5 mM) (Figure: 2.3. 2B) prior to binding to GSH Sepharose beads as described in the methods section. Beads were washed, eluted with 10 mM DTT and loaded on SDS-PAGE, immunoblotted with antibodies against MIA40. Recombinant MIA40 is observed to harbour glutathionylated cysteines/ reactive cysteines as it binds to GSH Sepharose beads, however, this binding decreases with increasing concentration of DTT pre-treatment while H_2O_2 has the opposite effect. We repeated this experiment using lysates from HEK293Tcells that were transiently transfected with the Myc-His-hMIA40 construct or vector control. Cells were pre-treated with H_2O_2 (25 to 100 µM) as described in the methods. An equivalent amount of lysates were used (Figure: 2.3.2D) for binding studies with GSH Sepharose beads (Figure: 2.3.2C). Two forms of hMIA40 are observed, a reduced and probably an oxidized form (Figure: 2.3.2D). The binding of native MIA40 is faint, however, with over-expression, we detect binding that is further augmented with H_2O_2 pre-treatment. This result is consistent with the assessment using recombinant MIA40 that MIA40 has a mixture of reactive and latent cysteines and that H_2O_2 can activate the latter.

N-acetyl-L-cysteine (NAC) can act as a precursor for GSH synthesis intracellularly. If indeed MIA40 is getting glutathionylated under normal physiological conditions, we should expect an increase in its glutathionylation with an increase in GSH levels. Hence, to confirm glutathionylation of MIA40, we initially grew HEK293T cells in the presence of increasing concentration of NAC for 12 hrs. We used HEK293T cells that were transiently transfected with Myc-His-hMIA40 construct or vector alone. Transfected cells were used instead of native cells so as to improve the sensitivity of the immunoprecipitation assay with an antibody against glutathione. Upon immunoprecipitation of cell

lysates with glutathione antibody, the resolution on SDS-PAGE and immunoblotted with antibodies against MIA40. We find MIA40 being pulled down with glutathione antibody and its level increases with increase in NAC treatment (Figure: 2.3.2E).

The transfected cell lysates were additionally monitored for steady-state expression level of hMIA40, mitochondrial and cytosolic proteins by western blot after exposure to NAC. The protein levels were comparable except for MIA40 that had increased expression in Myc-hMIA40 transfected cells (Figure: 2.3.2F). The aforementioned results clearly show that MIA40 undergoes glutathionylation.

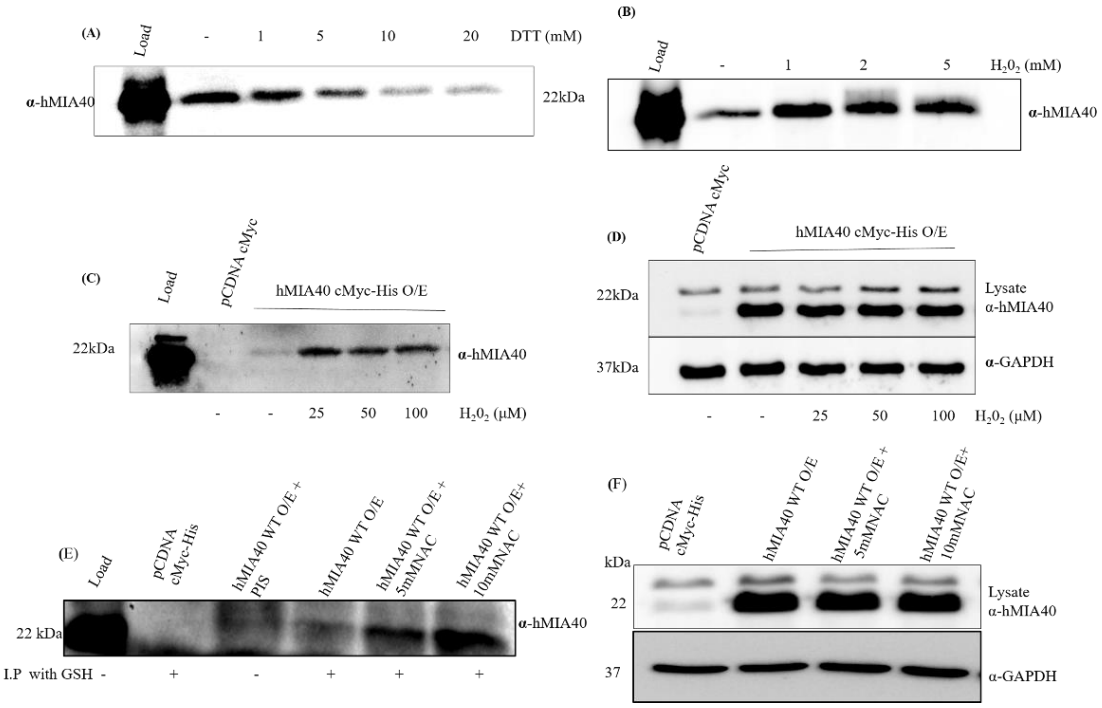


Figure: 2.3.2 Glutathionylation of hMIA40: (A) Recombinant hMIA40 protein was treated with increasing concentration of DTT (1, 5, 10 and 20mM) followed by pulldown with GSH-Sepharose beads and immunoblot was performed with antibodies against hMIA40. (B) Recombinant hMIA40 protein was treated with increasing concentration of H_2O_2 (1, 2, and 5mM) followed by pulldown with GSH-Sepharose beads and immunoblot was performed with antibodies against hMIA40 (C) hMIA40 was overexpressed in HEK293T cells and treated with increasing concentration of H_2O_2 (25, 50 and 100μM) lysate was pulldown with GSH-Sepharose beads and immunoblot was performed with antibodies against hMIA40. (D) hMIA40 was overexpressed and treated with increasing concentration of H_2O_2 (25, 50 and 100μM) as described in the Methods. Cell lysates were immunoblotted with antibodies against hMIA40 and GAPDH. (E) hMIA40 was overexpressed and treated with increasing concentration of NAC (1, 5 and 10mM) and the cell lysates were either immunoprecipitated with GSH antibody and immunoprobed with antibodies against hMIA40 or (F) immunoblotted with antibodies against hMIA40 and GAPDH.

2.3.3: Identification of Glutathionylation sites on hMIA40

Human MIA40 harbours seven cysteines clustered in the CPC (C53, C55) and in two CX9C (C64, C74, C87, C97) motifs besides present as C4. MALDI-TOF-MS/MS (hereafter, MALDI) analysis was used to ascertain the reactive cysteines and glutathionylation in hMIA40. Recombinant hMIA40 or HEK293T cells over-expressing Myc-His-hMIA40 were harvested for carrying out GSH pull-down assay as described in the methods. The proteins bound to GSH-Sepharose were resolved on a non-reducing SDS-PAGE along with recombinant His-hMIA40 and the gel Coomassie stained (Figure: 2.3.3A). The ~22 kDa band corresponding to over-expressed Myc-His-hMIA40 (using recombinant hMIA40 as reference), was excised out, trypsin digested and subjected to MALDI analysis as described in the Methods (Figure 2.3.3). C4, C64, C74 and C97 appear to be glutathionylated while the cysteines in CPC motif and C87 in CX9C motif appear to be insensitive to glutathionylation.

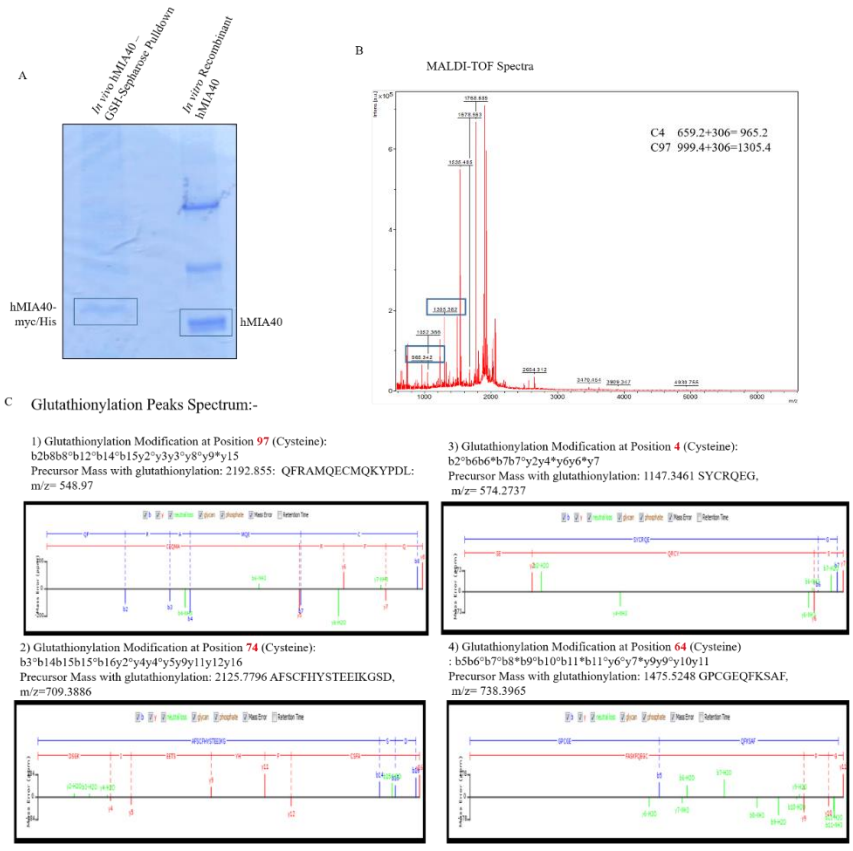


Figure: 2.3.3: Four cysteines in MIA40 are prone to Glutathionylation: hMIA40 c-Myc-His was overexpressed in HEK293T cells in presence of 10 mM NAC. (A) GSH-Sepharose pulldown was performed with lysates overexpressing hMIA40, eluted samples were subjected to non-reducing SDS-PAGE. Recombinant

purified MIA40 was used as a control to excise 22 kDa band. The protein band was subjected to MALDI analysis. (B) MS/MS spectra of hMIA40 showing an increase of 306 Da at multiple Cysteine sites. (C) LC-MS/MS analysis of glutathionylated Cysteine peaks spectrum.

2.3.4 Validation of cysteines involved in hMIA40 glutathionylation

To further establish that C4, C64, C74 and C97 are indeed glutathionylated, we resorted to site-directed mutagenesis studies to generate double (pNB388 C4S, C97S, henceforth MIA40 DM), triple (pNB389, C4S, C74S, C97S, henceforth MIA40 TM) and quadruple (pNB390, C4S, C64S, C74S, C97S, MIA40 QM) MIA40 mutants (Figure:2.3.4A). *E.coli* Rosetta gami cells were transformed with the plasmids, and the overexpressed recombinant His-MIA40 wild type and mutants were affinity purified using Ni-NTA beads as described in the methods. An equal amount of recombinant MIA40 proteins was used for carrying out affinity pull down using GSH Sepharose beads as described above. Western blots were probed with the MIA40 antibody. Recombinant wild type His-MIA40 having all the reactive cysteines is able to bind GSH beads efficiently. However, the binding capacity of MIA40 decreases with increase in substitutions at the reactive cysteines/glutathione prone cysteines. Despite the presence of intact cysteines in the CPC motif and of C87, binding of His-MIA40 QM to GSH Sepharose is almost abolished (Figure: 2.3.4B). Together, these results clearly demonstrate that hMIA40 is glutathionylated *in vivo* at four cysteine sites and it is imperative that C4, C64, C74 and C97 be present for maximum glutathionylation.

We replicated the *in vitro* GSH-Sepharose binding studies using the whole repertoire of cysteine to serine hMIA40 mutants expressed in HEK293T cells. Endogenous expression of hMIA40 in HEK293T was silenced partially by transfecting cells with shRNA specific against endogenous hMIA40 while cells were concomitantly co-transfected with pcDNA3.1 plasmids hosting Myc-His-hMIA40 wild type or hMIA40 TM and MIA40 QM mutants. After 24 hrs post-transfection, an equal number of cells were treated with 10 mM NAC for 12 hrs followed by cell lysis as described in the methods section. Lysates were resolved on SDS-PAGE, western blotted and probed with hMIA40 antibody to measure the shRNA mediated reduction in endogenous hMIA40. Around 30 to 40% reduction in endogenous expression of hMIA40 was achieved compared to control cells that were transfected with random shRNA (Figure: 2.3.4D). Most importantly, a three to four fold over-expression of wild type and DM (C53S-C55S) mutant of Myc-His-MIA40 was observed compared to endogenous control level (Figure:2.3.4D). However, cells transfected with triple and quadruple Myc-His-MIA40 construct had a relatively lower expression (Figure 3D). A plausible reason for the dip in

the over-expression of the triple and quadruple mutants maybe increased degradation triggered by structural instability (Figure: 2.3.5) (Hofmann *et al*, 2005), which infers the importance of MIA40 glutathionylation in its stability. GSH-Sepharose pulldown assay using the above lysates essentially re-confirmed the MALDI and *in vitro* results. hMIA40 wild type has the innate ability to bind to GSH-Sepharose as a result of its glutathionylated cysteines/reactive cysteines. Cysteine residues at positions 4, 64, 74 and 97 are instrumental in conferring this capability to MIA40 while the cysteines in the CPC motif are not involved (Figure: 2.3.4C). In addition, we also monitored the steady-state protein levels of several mitochondrial proteins in the lysates to ensure that there are no non-specific effects (Figure: 2.3.4D). Taken together, the GSH assays using hMIA40 mutants provide convincing evidence that indeed hMIA40 is glutathionylated *in vivo* and more specifically four of the seven cysteines within it -are glutathionylated *in vivo*.

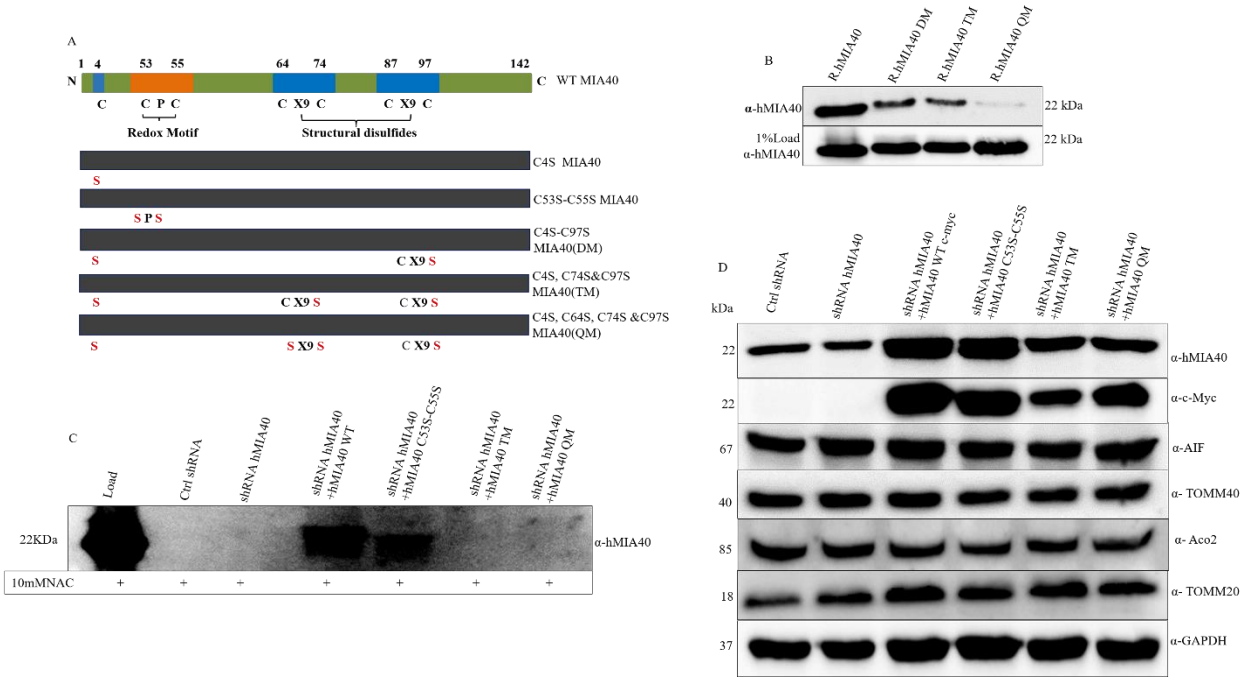


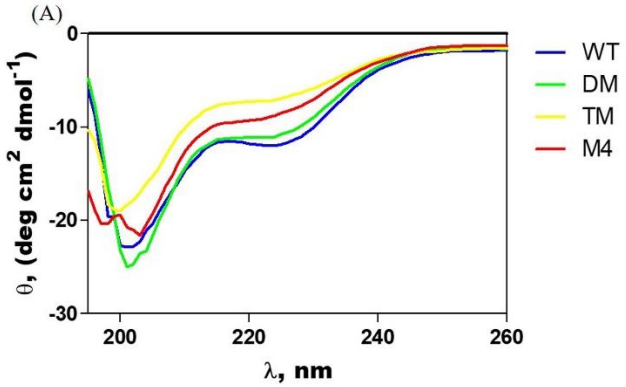
Figure: 2.3.4: Validation of glutathionylated Cysteines in hMIA40:- (A) Schematic representation of hMIA40 showing all seven cysteines and generation of, double (DM), triple (TM) and quadruple (QM) Cysteine to Serine mutants at C4, C64, C74, C97 and CPC motif. (B) GSH-Sepharose pulldown was performed with recombinant WT hMIA40 His, double (DM), triple (TM) and quadruple mutants (QM), immunoblotted with antibodies against hMIA40. (C) HEK293T cells were transfected with scrambled shRNA and shMIA40, and co-transfected with shMIA40/ WThMIA40 c-Myc-His, shMIA40/DM hMIA40, shMIA40/TM hMIA40 and shMIA40/QM hMIA40. After 24h of post transfection cells were treated with 10mM NAC for 12h and lysates were pulldown with GSH-Sepharose beads and immunoblotted with antibodies against hMIA40. (D)

Immunoblot analysis of few cytosolic and mitochondrial proteins as shown with whole cell lysates expressing WT hMIA40 and mutants in HEK293T cells.

2.3.5 Role of cysteine residues on the secondary structure of hMIA40

In our previous experiments, we find that cells transfected with triple and quadruple Myc-His-MIA40 construct had a relatively lower expression (Figure: 2.3.4D). We reasoned that triple and quadruple mutants might have structural instability (Figure: 2.3.5) as cysteine residues play a key role in proper folding and structural stability of proteins. We examined whether cysteine mutants have any effect on folding and structural stability of hMIA40 by analysing the secondary structure of recombinant WT hMIA40 and mutants of MIA40 using Circular Dichroism. These results show that WT hMIA40, DM hMIA40, TM hMIA40 and QM hMIA40 mutants predominantly contain α-helical structure of 84.27%, 84.27%,56.82% 80.68% respectively. Further, WT hMIA40, DM hMIA40 and QM hMIA40 has very low β-barrel structure of 1.24%, 1.24% and 1.47% respectively while hMIA40TM has a moderately high β-barrel structure of 5.09%. Except TM hMIA40, no major deviation in secondary structure was observed in cysteine mutants of human MIA40. However, considerable deviation in the alpha helix and β-Sheet percentage was observed only in case of TM MIA40. This altered structure may be one of the reason for the instability of TM MIA40 *in vivo*. However, it does not explain about the instability of QM hMIA40 *in vivo* as we observed very slight deviation in alpha helix and β-Sheet content from that of WT. We assume that lower expression of TM and QM MIA40 may be a combination of altered alpha helix, β-Sheet content and instability of mRNA and or lower level of translation.

(A)



(B)

Proteins	Wave length 200-260 nm	
	α-Helix (%)	β-Sheet (%)
hMia40 WT	84.27	1.24
hMia40 C4-97S	84.27	1.24
hMia40 C4,97&74S	56.82	5.09
hMia40 C4,97,64&74S	80.68	1.47

Figure: 2.3.5 Secondary structure analysis of hMIA40 and cysteine mutants by Circular dichroism:- (A). CD spectrum (200-260 nm) study of hMIA40 and cysteine mutants. (B)The secondary structure of hMIA40 and cysteine mutants was measured from 200 nm to 260 nm by CD. The content of α-helix and β-sheet was calculated using web-based CDNN 2.1 analysis.

2.3.6 Glutathionylated hMIA40 regulates the ETC Biogenesis and ROS production

For the first time, we show that hMIA40 is getting glutathionylated *in vivo*. To gain insights into the physiological consequences of this post-translational modification. One of the other primary function of MIA40 is its chaperonic role in the assembly of inner mitochondrial membrane protein complexes of the vital ETC. We speculated that glutathionylation of the cysteines in MIA40 might improve its effect on the ETC complex assembly. To check if this is correct, we monitored the steady-state levels of the various complex proteins of ETC in HEK293T cells over-expressing Myc-His-MIA40 wild type and mutants (CPC motif mutant; triple mutant and quadruple mutant). Mitochondrial samples from the shRNA and pcDNA3.1 co-transfected cells were resolved on SDS-PAGE, western blotted and probed with the OXPHOS antibody. OXPHOS antibody is a combination of antibodies that are able to detect five significant components of complexes I, II, III, IV and V of ETC. Components of complex 1 (NDUFB8), II (SDHB), IV (MTCO1) have reduced steady-state levels in the mitochondria isolated from cells expressing hMIA40 TM, QM besides control cells wherein hMIA40 is partially silenced compared to cells expressing wild type MIA40, or it's CPC mutant version (Figure: 3.2.6A).

This outcome depicts a direct correlation between the glutathionylation of hMIA40 and the protein level of the components of ETC complexes. As a follow up to this finding, we were curious to look at the efficiency and activity of the individual complexes that constitute the ETC in the mitochondrial samples. Surprisingly, in case of complexes I and II activities, all the samples had comparable activity despite the over-expression of wild type and mutant hMIA40 or partial silencing of endogenous hMIA40 (Figure: 3.2.6B, C and Figure:3.2.8A). However, Complex III activity is sensitive to partial silencing of endogenous hMIA40 as the activity decreases by half in cells transfected with only MIA40 shRNA (Figure:2.3.6D). Correspondingly, complex III activity is augmented in cells over-expressing hMIA40 wild type or the CPC mutant. However, cells over-expressing hMIA40 TM or QM have compromised complex III activity compared to cells over-expressing wild type hMIA40 (Figure:2.3.6D). The pattern of complex IV activity mimics that of complex III except that there is a dramatic reduction of this activity in cells over-expressing all the

mutants of hMIA40 including the CPC mutant (Figure:2.3.6E). These results provide evidence that hMIA40 modulates complex III and complex IV activity to influence the ETC. Most importantly, glutathionylation of cysteines in MIA40 is crucial for both complex III and complex IV activities while the latter is additionally sensitive to the overall structure of hMIA40. Further, alteration of steady-state levels may not affect the activities of ETC as in case of Complex I and II activities (Figure: 2.3.6 A&B). As ETC is the production site for ROS and hMIA40 regulates the complexes of ETC, we measured ROS in the cells over-expressing hMIA40 wild type and its mutants. A highly significant increase in ROS is observed when endogenous hMIA40 is partially silenced (Figure 4F). It is notable that about 30 to 40% reduction in hMIA40 is enough to evoke such a dramatic increase in ROS. In corollary, over-expression of hMIA40 wild type decreases the level of ROS to a level that is lower than in control cells. Over-expression of hMIA40 CPC, TM and QM mutants increase the ROS. Our results disclose for the first time a hitherto unappreciated link between hMIA40 and ROS production that impacts cellular homeostasis.

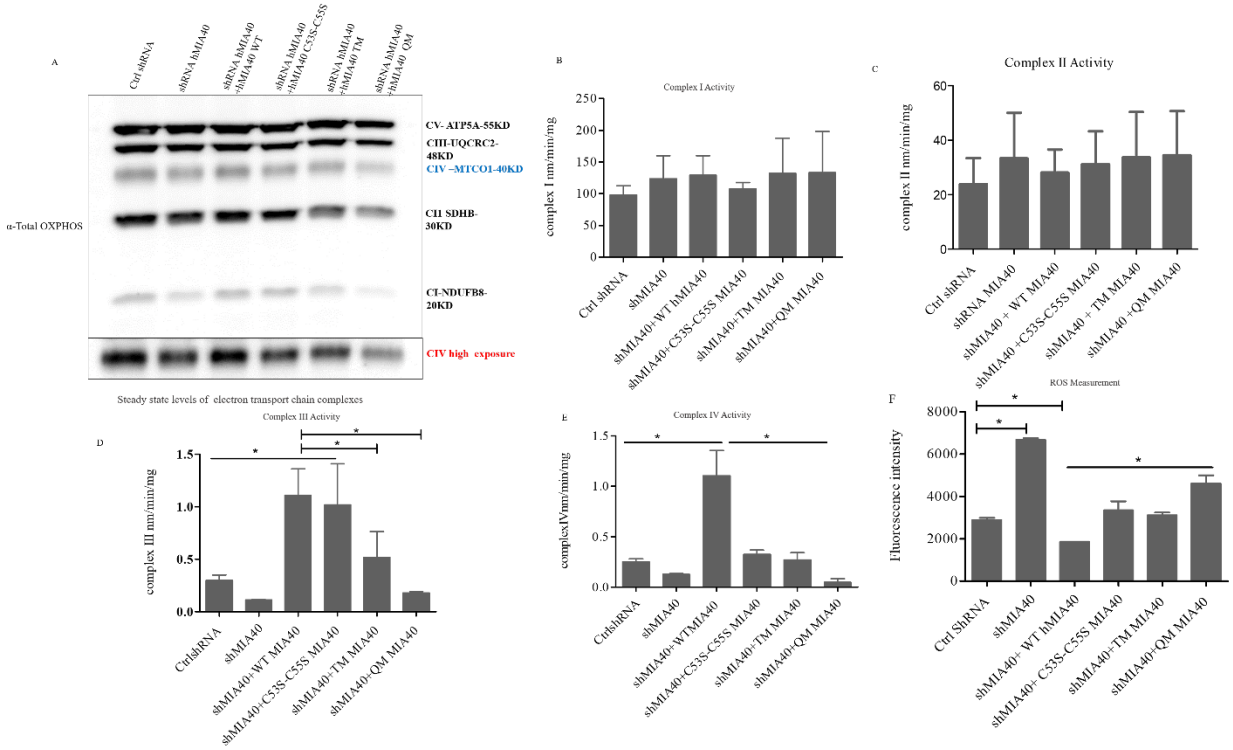


Figure: 2.3.6 Glutathionylated hMIA40 regulates the ETC Biogenesis and ROS production: - HEK293T cells were transfected either with scrambled shRNA or shMIA40, or co-transfected with shMIA40/WThMIA40, shMIA40/ C53S-C55S MIA40, shMIA40/TM hMIA40 and shMIA40/QM hMIA40 mutants. (A) Mitochondria were isolated and immunoblot was performed with OXPHOS subunits antibodies that can detect major components of electron transport chain such as Complex I (NDUFB8, 20 KDa), Complex II (SDHB, 30 KDa), Complex III (UQCRC2, 48 KDa), Complex IV (MTCO1, 40 kDa) and Complex V (ATP5A, 55 kDa). (B, C, D and E) Electron transport chain Complex I, II, III and IV activities was analyzed spectrophotometrically as described in the Methods. (F) Reactive Oxygen Species (ROS) levels were measured using H2DCFDA in cells after 24 hrs of post-transfection as described in the Methods. All results were plotted with mean ± S. E. (n=3), $^{*}P \leq 0.05$.

2.3.7.1 Glutathionylated hMIA40 transfers electrons to cytochrome c

Cytochrome c is the intermediate molecule accepts electrons from complex III and mitochondrial intermembrane disulphide relay system and gives to complex IV. As complex III and IV activity is decreased in mutants, we further estimated cytochrome c (intermediate molecule between complex III and IV) levels in mitochondrial (Fig: 2.3.7.1A) and whole cell lysates of WT hMIA40 and mutants in HEK 293 T cells (Fig: 2.3.7.1B). We found cytochrome c levels were found to be decreased in all mutants compared to wild type as well with controls. Since we observed the decreased levels of cytochrome c in all mutants (Mitochondria and whole cell lysate), we interpret that glutathionylated hMIA40 may regulate the steady state or the stability of cytochrome c.

In the ETC assembly line, cytochrome c accepts electrons from complex III and mitochondrial intermembrane relay system to deliver them to complex IV. We reasoned that since the activities of both complex III and IV were decreased in the MIA40 mutants, the activity of cytochrome c could also have been compromised as a result. To validate this reasoning, we assessed the activity of cytochrome c using purified recombinant hMIA40 wild type and mutant proteins *in vitro*. Oxidized cytochrome c was incubated with reduced hMIA40 proteins, and the reduction of oxidized cytochrome c was followed by monitoring the UV-visible spectra from 200 to 700nm. Cytochrome c reduction can be monitored by the appearance of the prominent α and β peaks and the subtle movement of the gamma peak to the right. Addition of sodium dithionate, a known reducing agent, efficiently reduces cytochrome c while GSH cannot (Figure: 2.3.7.1C). It has been reported that hMIA40 requires ALR/Erv1 to act as an intermediate for transfer of electrons to cytochrome c. Curiously, we find reduced recombinant wild type hMIA40 is capable of reducing cytochrome c efficiently in the presence of DTT or GSH and does not require ALR. However, all the mutants of hMIA40 that were tested (DM, TM, QM and CPC) have significantly reduced activity with the CPC mutant being

completely defective in reducing oxidized cytochrome c (Figure: 2.3.7.1D). Interestingly we could not find cytochrome c reduction when oxidized cytochrome c is incubated with recombinant ALR protein alone ((Figure: 2.3.7.1E). Cytochrome c reduction was decreased significantly when the assay was performed with WT hMIA40 and ALR, suggesting the sharing of electrons between ALR and cytochrome c from hMIA40 *in vitro*. (Fig: 2.3.7.1D).

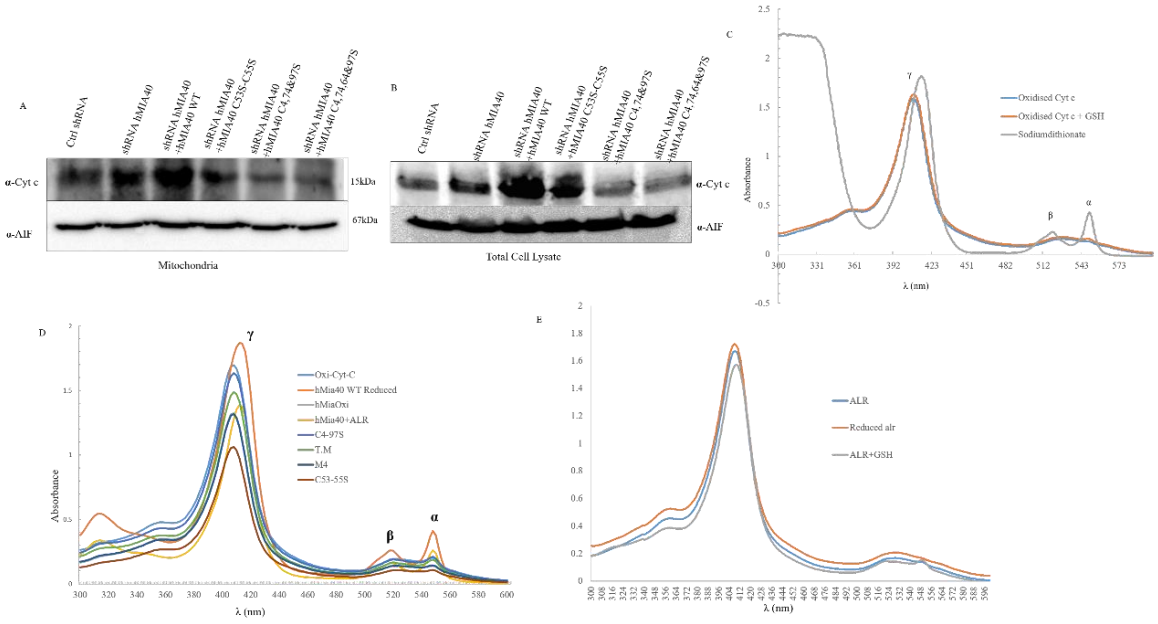


Figure: 2.3.7.1 Glutathionylated hMIA40 transfers electrons to cytochrome c: (A) HEK293T cells were transfected either with scrambled shRNA or hMIA40 shRNA, and or co-transfected with hMIA40 shRNA/ WThMIA40, hMIA40 shRNA /TM hMIA40 and hMia40 shRNA/QM hMIA40. After post transfection, isolated mitochondria (A) and/ cell lysates (B) were prepared and immunoblotted with antibodies against hMIA40, Cytochrome c and AIF. (C) Oxidized cytochrome c was incubated with GSH/Sodium dithionate and scanned in the range of 200 to 700 nm wavelength using spectrophotometer. (D) WT hMIA40 and mutants were overexpressed in *E. coli* Rosetta gami and purified with Ni-NTA beads as described in the Methods. Recombinant WThMIA40 and mutant proteins were incubated with oxidized cytochrome c for 5 min and performed scanned wavelength spectroscopy (200 to 700 nm). (E) Recombinant purified ALR protein was incubated with oxidized cytochrome c for 5 min and performed wavelength spectroscopy as mentioned (200 to 700 nm).

2.3.7.2 Glutathionylated hMIA40 transfers electrons to cytochrome c

Further chased the reduction of oxidized cytochrome c on SDS-PAGE in a temporal manner. Reduced recombinant hMIA40 and the above mutants were incubated with oxidized cytochrome c to different time points in the presence of GSH or DTT as shown (Figure: 2.3.7.2). The free cysteines in

MIA40 were blocked with iodoacetamide (IAA) to preserve the reduction status prior to the separation of samples on a non-reducing SDS-PAGE and staining with Coomassie to detect for the presence of oxidized (lower) and reduced (upper) forms of MIA40. Sample with wild type MIA40 and cytochrome c shows its reduced form decreasing with time while its oxidized form makes its presence with an increase in time (Figure:2.3.7.2A). The reduced form is clearly visible while the oxidized form, especially at later time points, is diffused probably to degradation through over-oxidation (Pajares *et al*, 2015; Mitchell *et al*, 1985). Both the QM and CPC mutants of hMIA40 remain in the reduced form (Figure: 2.3.7.2B&C) consistent with the above *in vitro* activity assay. Notably, the oxidized and reduced temporal pattern of MIA40 directly implicates it in the direct transfer of electrons to cytochrome c. Importantly, both glutathionylation and CPC motif appear to be essential for reducing cytochrome c.

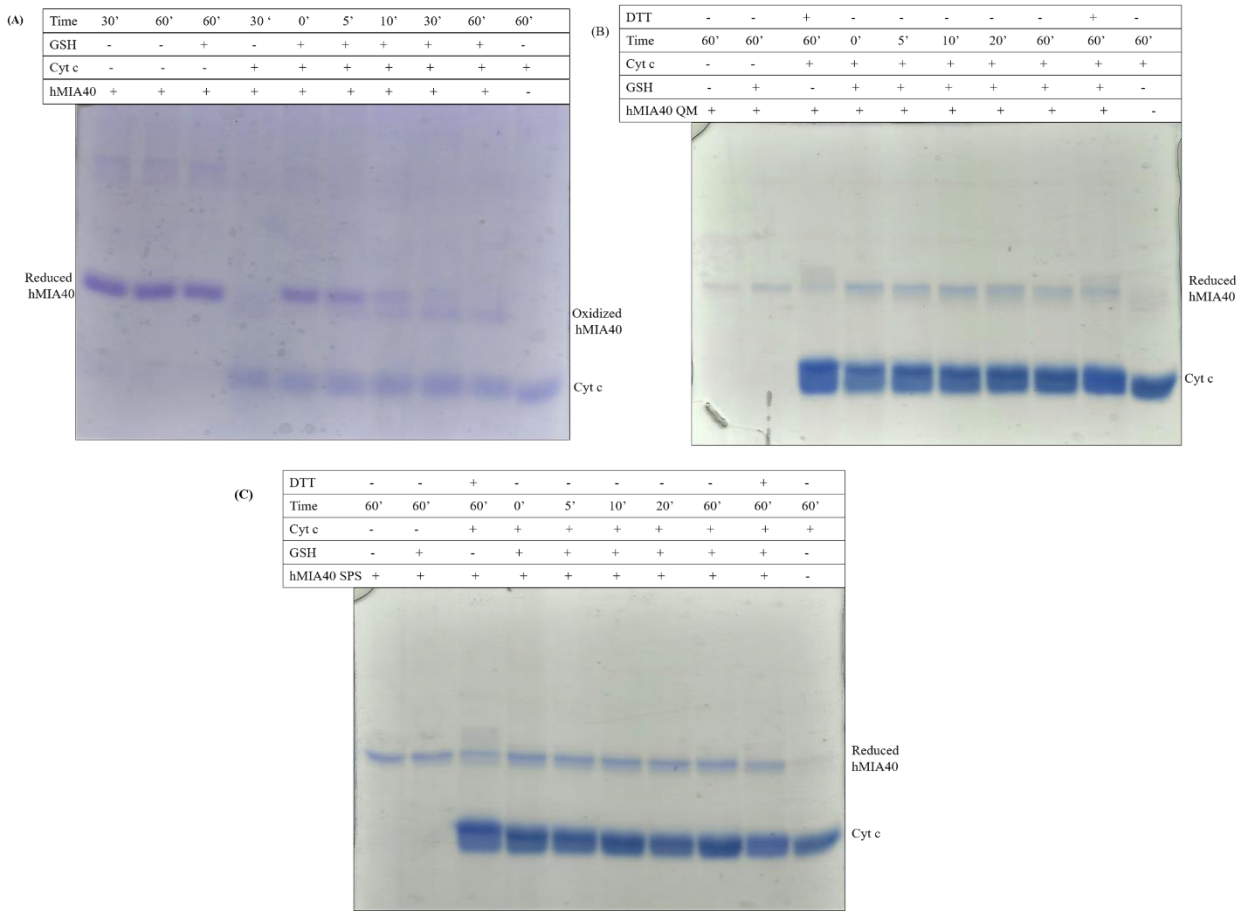


Figure: 2.3.7.2 Glutathionylated hMIA40 transfers electrons to Cytochrome c: (A) Recombinant WT hMIA40 protein was incubated with cytochrome c from zero to 60 mins and samples were subjected to non-reducing SDS-PAGE and further stained with Coomassie brilliant blue. (B) Recombinant hMIA40 QM mutant protein was incubated with cytochrome c from zero to 60 mins and separated on non-reducing SDS page and stained with Coomassie brilliant blue. (C) Recombinant hMIA40 C53S-C55S protein was incubated with cytochrome c from zero to 60 mins and samples were subjected to SDS PAGE and stained with Coomassie brilliant blue.

2.3.8 hMIA40 interacts with cytochrome c

To test whether MIA40 is directly interacts with Cytochrome c, we carried out immunoprecipitations and Ni-NTA pull down studies. We transfected the HEK293T cells either with scrambled shRNA or shMIA40, and/or co-transfected with shMIA40/WThMIA40c-Myc-His, shMIA40/MIA40 C53S-C55S c-Myc-His, shMia40/TM hMIA40c-Myc-His and shMIA40/QM hMIA40c-Myc-His. After 48hrs of post-transfection, cells were harvested, lysed and the cell lysates were resolved on SDS-PAGE and immunoblotted with MIA40, Myc and GAPDH (Figure:2.3.8A), these results suggest that the over-expression of wild type and mutant hMIA40 or partial silencing of endogenous hMIA40.GAPDH used as internal loading control. Further cell lysates were either subjected to immunoprecipitation with hMIA40 antibody (Figure: 2.3.8B)) or pulldown with Ni-NTA Sepharose beads (Figure: 2.3.8C) and immunoblotted with cytochrome c antibody. In MIA40 knockdown and in glutathionylated cysteine mutants (TMhMIA40&QM hMIA40) (Figure: 2.3.8B & C) the cytochrome c levels were decreased, while they were increased in MIA40 WT and C53S-C55S hMIA40 over-expression conditions (Figure: 2.3.8B & C). These results support that MIA40 may interact with cytochrome c.

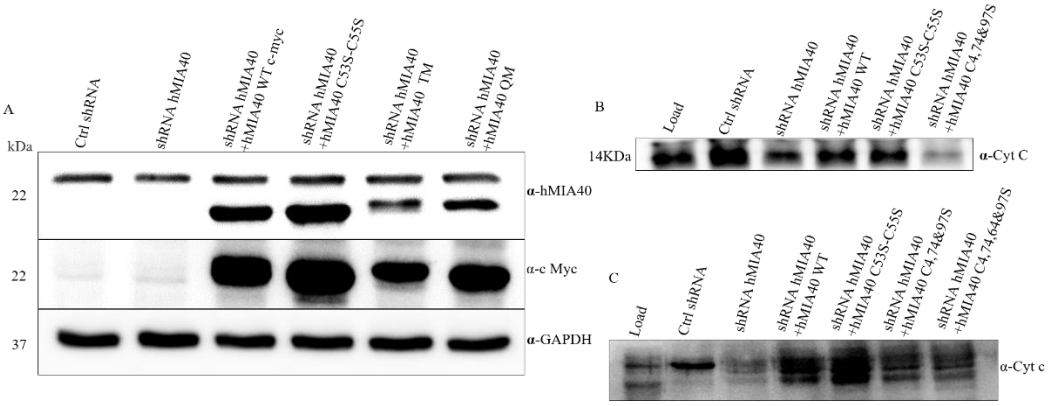


Figure: 2.3.8 MIA40 interacts with cytochrome c: - HEK 293T cells were transfected with scrambled shRNA and shMIA40, and co-transfected with shMIA40/WThMIA40 c-Myc-His, shMIA40/MIA40 C53-55S, shMIA40/TM hMIA40 and shMIA40/QM hMIA40 mutants. (A) Cell lysates were analysed on SDS-PAGE and immunoblotted with hMIA40, Myc and GAPDH. (B) Cell lysates were immunoprecipitated with MIA40 and immunoblotted with cytochrome c antibody. (C) Cell lysates were pulldown with Ni-NTA Sepharose beads and immunoblotted with cytochrome c antibody.

2.3.9 Glutathionylated hMIA40 may be essential for the stability of the electron transport chain components:-

It is known that hMIA40 acts as a chaperone and contributes to the assembly of inner mitochondrial membrane complexes, including some components of the (ETC). To further elucidate whether glutathionylation of hMIA40 has any role in regulation ETC components, we have probed the mitochondrial samples of WT hMIA40 and mutants isolated from HEK 293 T cells with total OXPHOS antibody that can detect five significant components of Complex I, II, III, IV, and V of ETC. Interestingly we found NDUFB8 (a component of complex I), SDHB (a component of complex II) and MTCO1 (a component of IV) was found to be decreased TM hMIA40, QM hMIA40 mutants and in endogenous hMIA40 silenced condition and increased in hMIA40WT (Fig:2.3.6A). Further to elucidate either glutathionylated MIA40 is necessary for import and /or the stability of ETC components, we have probed the total cell lysate samples of WT hMIA40 or mutants with total OXPHOS (Figure: 2.3.9) and β-Tubulin used as an internal loading control. Interestingly we found NDUFB8, SDHB and MTCO1 were found to be decreased in TM hMIA40, QM hMIA40 mutants and hMIA40 silenced condition, and increased in WT hMIA40. NDUFB8 and SDHB genes were nuclear encoded, whereas MTCO1 is a mitochondrial encoded OXPHOS protein. In general, protein import defect associated with decrease in steady state levels of proteins in mitochondria. Since MTCO1 is a mitochondrially encoded protein and the levels of MTCO1 may not decrease due to defect in import. Surprisingly, we find that decrease in protein levels of nuclear encoded NDUFB8, SDHB and mitochondrial encoded MTCO1 in total cell extracts. It probably indicates that glutathionylated hMIA40 is necessary for the stability of ETC components rather than import.

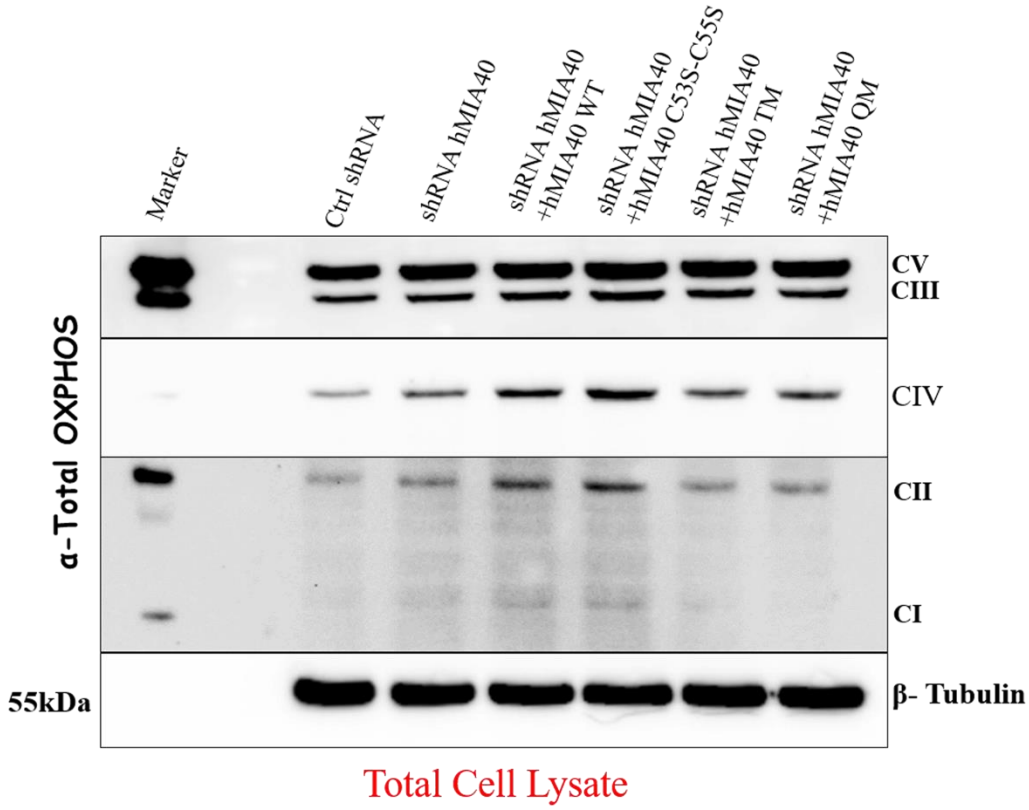
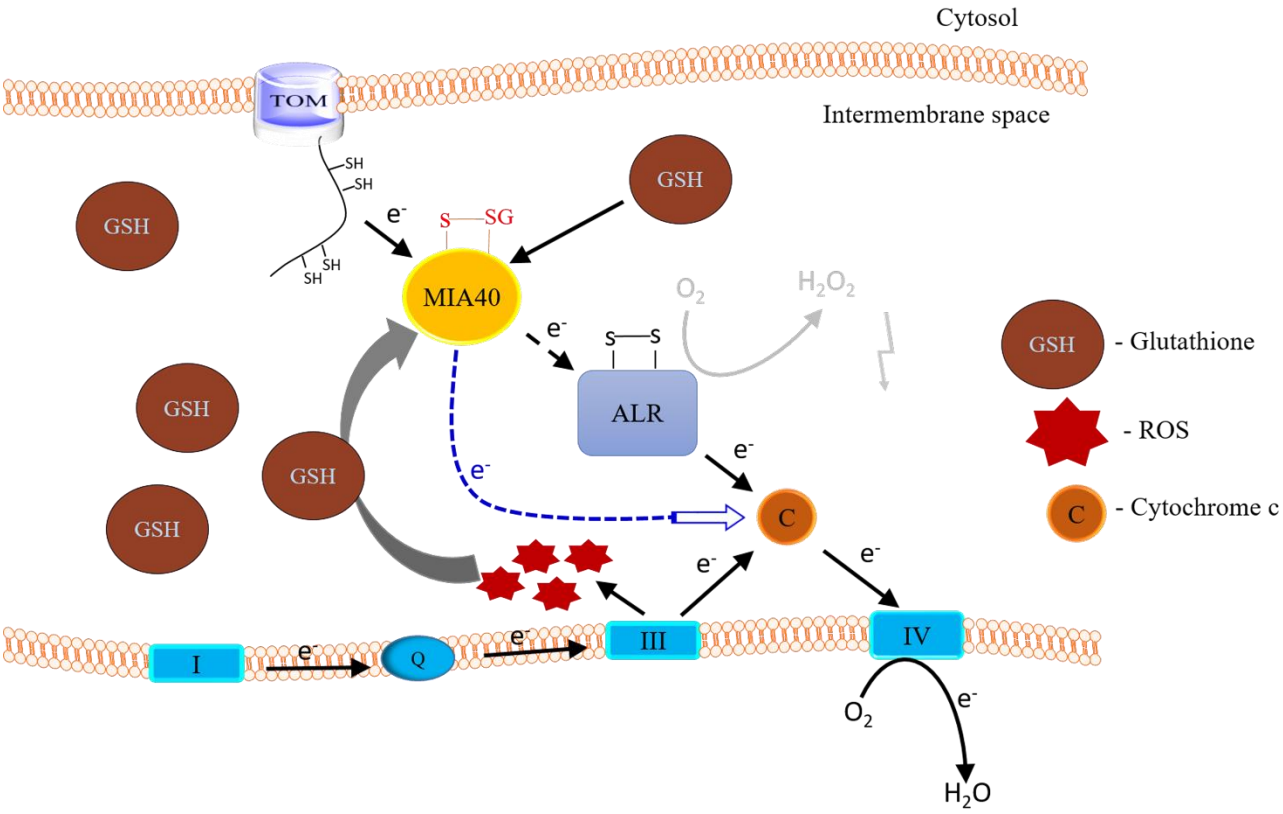


Figure: 2.3.9 Glutathionylated hMIA40 may be essential for the stability of the electron transport chain components: - HEK 293T cells were transfected with scrambled shRNA and shMIA40, and co-transfected with shMIA40/WThMIA40 c-Myc-His, shMIA40/MIA40 C53-55S, shMIA40/TM hMIA40 and shMIA40/QM hMIA40 mutants. Total cell lysates were immunoblotted with OXPHOS antibody which can detect major components of electron transport chain such as Complex I (NDUFB8, 20 kDa), Complex II (SDHB, 30 kDa), Complex III (UQCRC2, 48 kDa), Complex IV (MTCO1, 40 kDa) and Complex V (ATP5A, 55 kDa).

2.3.10: Schematic representation of the inter membrane space disulfide relay system and the regulation of ETC and ROS production: -

In this study we have identified hMIA40 undergo glutathionylation, and /elevated ROS may increase the hMIA40 glutathionylation due to which ROS levels may get regulated. Glutathionylated MIA40 transfer electrons directly to cytochrome c and /or share electrons with the FAD-containing sulfhydryl oxidase Erv1 and regulates cytochrome c mediated electron transport chain biogenesis. Gt MIA40 directly transfer the electrons to cytochrome c. Given the importance to all these observations, this study explains the pleiotropic roles of Gt MIA40 during disulfide relay system.



2.3.10: Schematic representation of the inter membrane space disulfide relay system and the regulation of ETC and ROS production: - Proteins in the IMS compartment enter through the TOM complex. They are typical of small size and contain several reduced cysteines resides. The IMS receptor/oxidoreductase MIA40 is able to form mixed disulfides with these proteins and promotes their oxidation. MIA40 undergo glutathionylation and /elevated ROS may increase the hMIA40 glutathionylation due to which ROS levels may get regulated. Glutathionylated hMIA40 transfer electrons directly to cytochrome c and/or share electrons with the FAD-containing sulfhydryl oxidase Erv1 and regulates cytochrome c mediated electron transport chain biogenesis. The flow of electron (e^-) from the imported proteins to the final electron acceptor oxygen (O_2) is indicated. The cytochrome *c*–independent side reaction of Erv1 with oxygen is shown in light grey. Cytochrome *c* reductase and oxidase complexes are indicated as complexes III and IV, respectively. Q indicates the ubiquinone pool .

2.3.4 Discussion:-

MIA40, a synonym for CHCHD4 (Coiled-coil-helix-coiled-coil-helix domain-containing protein 4) is imported from the cytoplasm into the mitochondrial IMS. It has been assigned the function of folding small cysteine containing mitochondrial IMS bound proteins for their import and retention. We have shown earlier that it has an essential role as a component of the mitochondrial Fe-S cluster export machinery. In this study, we show that hMIA40 undergoes post-translational modification by getting glutathionylated at four cysteines C4, C64, C74 and C97. We demonstrate that glutathionylation of

hMIA40 has critical consequences that affect cellular respiration and redox homeostasis. hMIA40 defective in glutathionylation increases ROS decreases the steady-state levels of components of complexes III and IV of the ETC and is unable to reduce oxidized cytochrome c (Figure: 2.3.7.1D). MIA40 resides in the IMS that is populated with proteins of the respiratory chain proteins and IMS import machinery with possible overlap in functions and dependency (Allen *et al*, 2005; Bihlmaier *et al*, 2007; Dabir *et al*, 2007). We demonstrated for the first time that, hMIA40 undergoes glutathionylation. We have shown employing several approaches that GSH has direct interaction with hMIA40 (Figure: 2.3.1& Figure: 2.3.2). Earlier studies could not prove this direct interaction as it was thought that glutaredoxin was acting as a catalyst with GSH being its cofactor. Over-expressed cytoplasmic hMIA40 was always found to be in an oxidized and folded state, and their entry into the mitochondrial IMS was facilitated by glutaredoxin (Banci *et al*, 2009; Kojer *et al*, 2012b; Durigon *et al*, 2012). In the current study, we show that GSH can directly interact with hMIA40 and glutathionylated it. Intriguingly, the CPC motif is bereft of any glutathionylation while three cysteine residues in the twin CXC9 motifs are glutathionylated.

Interestingly, the CPC motif of MIA40 contributes a redox potential of -200 mV (Banci *et al*, 2009) while CXC9 motif containing proteins have -300 mV redox potential (Voronova *et al*, 2007; Lu & Woodburn, 2005). Based on the possible redox dynamics within the hMIA40 protein, we believe that the CXC9 motifs get glutathionylated by transferring electrons to the CPC motif which in turn transfers electrons directly to cytochrome c or ALR based on the redox environment. The cycle of glutathionylation on MIA40 may be continuous on and off cycle to regulate mitochondrial electron transport chain activities, in particular, Complex III and Complex IV. Based on the above explanation, we should have observed a decrease in Complex III and Complex IV activities in the CPC mutant and not just in the QM and TM mutants of hMIA40. As we don't see the CPC mutant being compromised in complex III activity, it is possible that there is another layer of regulation that we are yet to understand. Erv1/ALR is a Flavin oxidoreductase that is known to re-oxidize hMIA40 and transfer electrons to cytochrome c (Stoganovski et al.2012)

There have been reports suggesting that complex I was compromised in MIA40 silenced cells. However, in our studies, we did not see this effect. We could not achieve greater than 30% reduction in MIA40 levels as depletion of MIA40 greater than 50% was lethal in our hands. Nevertheless, with 30% reduction itself, we were able to observe a dramatic increase in ROS and decrease in complex III

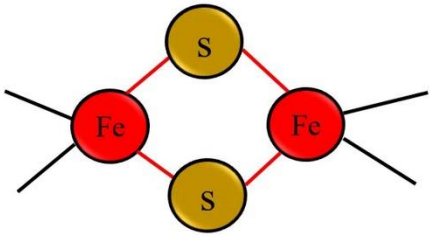
and IV activities. Given that hMIA40 affects complex III and IV activities *in vivo* and is able to reduce cytochrome c *in vitro* without the requirement of ALR, the obvious question of its physiological significance in the presence of cytochrome c and ALR arises. It is well known that redundancy of pathways provides flexibility and a failsafe mechanism to sustain critical processes in the face of potential damage. ETC is crucial for energy production, and cells must be employing multiple electron acceptors and intermediates. Interestingly, Erv1/ALR has been shown to transfer electrons directly to oxygen form H_2O_2 (Fraga & Ventura, 2013). However, during anaerobic conditions, fumarate reductase (Osm1) transfers the electrons from Erv1 to fumarate in the IMS (Neal *et al*, 2017). Based on our investigations, we strongly believe that the function of hMIA40 is not limited to import of proteins into IMS or to the export of Fe-S cluster but undergoes post-translational modification to sustain the ETC.

Mitochondria act as a hub for production of ROS which is a physiological by-product for many of the metabolic activities. The cell efficiently uses it as a signalling molecule when it is at a low level. However, it succumbs to it when its level increases. The mitochondria continually adapt to the dynamic redox environment as a result of flux changes in ROS by modulating the function of its proteins through oxidation of protein cysteine thiols. The importance of glutathionylation as a PTM has recently gained more traction. It is now considered as a '*bona fide*' redox signal as it can modulate protein function *in vivo* by modifying it reversibly, swiftly and in a highly specific manner, fulfilling all the criteria for an efficient and important PTM (Shelton & Mieyal, 2008). Several proteins from varying biological pathways including metabolic and mitochondrial protein import have been identified to undergo glutathionylation. Reactions involving glutathionylation are susceptible to both oxidized and reduced glutathione levels that are driven by the cellular ROS. Glutathionylation of proteins is now considered as a hallmark for stress response and any defects in this can lead to pathological conditions. MIA40 could act as a repository for ROS by getting glutathionylated and transferring back the electrons to the ETC to mitigate the toxic effects of ROS. 30% reduction in MIA40 increases ROS several folds (Figure: 2.3.6F). Because of the biological outcomes of protein glutathionylation associated with stress, they are now being viewed as potential biomarkers for stress-related human diseases. The concept of cells having a 'redoxome' wherein protein cysteine thiols act as sensors for the surrounding redox environment is being appreciated. Jones and Sies have recently suggested the existence of a 'redox code' determined by the redox flux generated by the cellular metabolic activities, bioenergetics and the health of the anti-oxidant systems (Jones and Sies 2015).

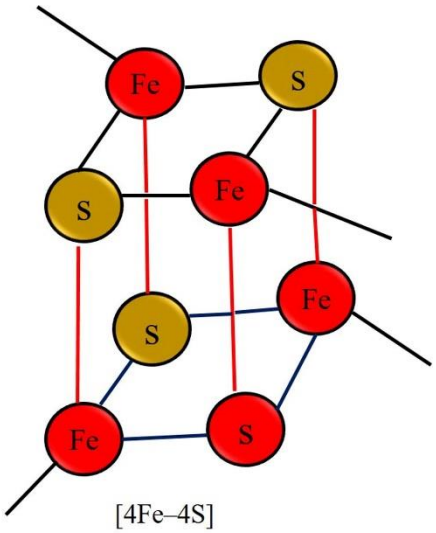
Given that proteins can undergo a multitude of redox modifications, glutathionylation being just one; and the paucity of information on the molecular mechanism behind these modifications, further studies are required to unravel the redox world. Increased ROS production was associated with several pathological conditions such as cancer, diabetes mellitus, atherosclerosis, ischemia and other mitochondria-related abnormalities (Balaban *et al*, 2005; Dröge, 2002; Ha *et al*, 2000; Baynes, 1991; Kinscherf *et al*, 1999; Kushnareva *et al*, 2002; Chen *et al*, 2003). Hence, glutathionylation of hMIA40 could be an important mechanism as it is protecting ETC complex components, ETC activities and ROS generation.

CHAPTER III

Functional characterization of glutathionylated MIA40 In protein import and cellular iron homeostasis



[2Fe–2S]



[4Fe–4S]

3.1 Introduction

Mitochondria are essential for distinct cellular processes that includes energy metabolism, amino acid, lipid metabolism & programmed cell death (apoptosis). Further, mitochondria also play a major part in iron metabolism that includes Fe-S clusters biogenesis and heme synthesis. Iron is a vital molecule in all organisms from single cell bacteria to humans. Besides, iron is a module of the Fe-S cluster, an ancient biological molecule that is necessary for many essential processes including DNA replication, DNA repair, heme synthesis, redox catalysis, regulation of gene expression, and electron transfer (Beinert, H. (1954). BIOLOGICAL OXIDATIONSl, 2 *et al*, 2007; Netz *et al*, 2012; Boal *et al*, 2007; Stephens *et al*, 1996). Generation, maturation and transfer of Fe-S clusters to substrates is a very complex processes and require protein components that are present particularly in mitochondria (Rouault, 2012b). Based on research in yeast, it was proposed that three different systems are intricate in the biogenesis of Fe-S clusters known as cytosol iron-sulfur assembly system (CIA), iron-sulfur cluster assembly system (ISC), and iron-sulfur exporter system (Roland et al. 2006). The above two systems (ISC and Fe-S export) are present in mitochondria, and the CIA is present in the cytosol. Homologues for most of the yeast proteins that are involved in Fe-S cluster biogenesis are present in higher eukaryotes. Although the low amounts of few components of ISC machinery were detected in nucleus and cytosol, there are some differences in Fe-S cluster biogenesis in higher eukaryotes related to yeast. Anyway, the overall Fe-S system in mammals is functionally identical to yeast counterpart.

The mitochondrial matrix localized iron-sulfur cluster assembly (ISC) system is actually involved in the formation of Fe-S cluster, which occurs in three different steps (Figure: 3.1). In the first step, a desulfurase (Nfs1) in combination with Isd11 withdraws sulfur from cysteine and transfer it to a scaffolding protein Isu1, and it also receives Fe from frataxin (Yfh1) and forms a transient Fe-S cluster. In the next step, this cluster will be transferred to Apo protein with the help of Hsp70 isoform (SSQ) and glutaredoxin (Grx5) ((Rouault, 2012b)). Mitochondria also contain Fe-S export machinery. In yeast ATM1 (ABCB7 in mammals), an inner membrane protein has been identified as an iron-sulfur exporter from mitochondria (Ronald et al. 1997). The exact module that is exported by ATM1 is still not known, but, few studies suggest that it is possibly involved in the export of sulfur to cytosol through some unknown mechanism. The additional modules of the Fe-S export machinery that are perhaps working along with ATM1 includes Erv1, a sulfhydryl reductase, and GSH (Figure:3.1) (Ronald et al. 2001), as depletion of these proteins replicates the comparable phenotype like ATM1

deficiency like an increase of iron in mitochondria and defect in maturation of cytosolic Fe-S containing proteins. Erv1 has been shown to play a role in grouping with MIA40, a mitochondrial IMS protein in the import of various cysteine-rich intermembrane space proteins by an oxidative folding machinery (Bottinger *et al*, 2012).

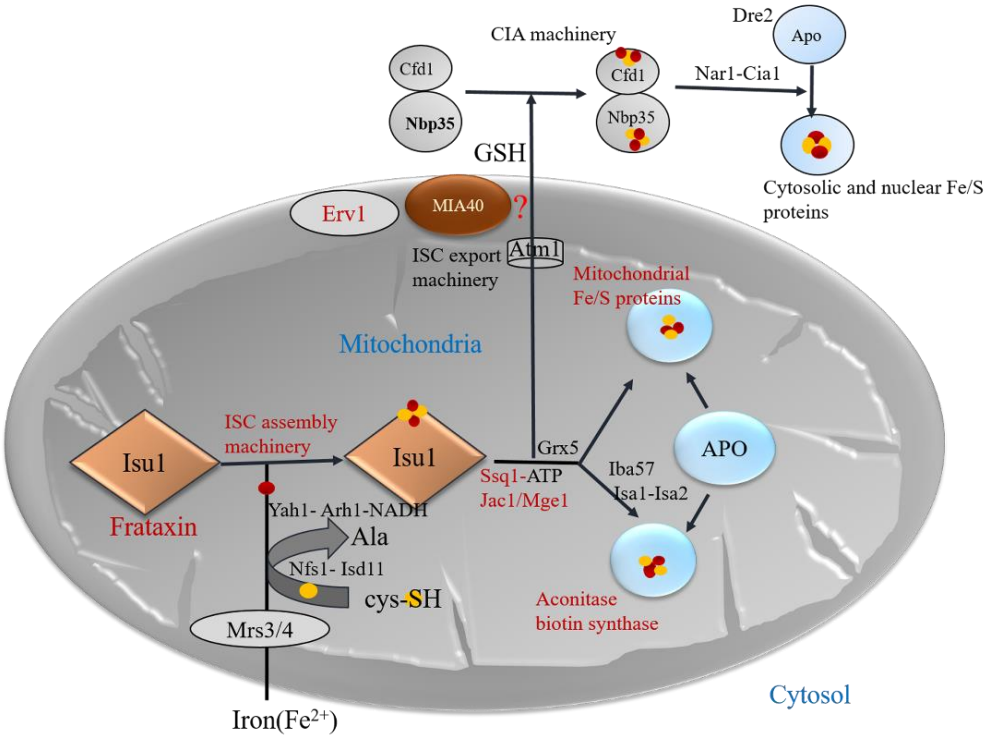


Figure: 3.1 Schematic representation of Fe-S cluster biogenesis in eukaryotes:- The Fe-S clusters are synthesized in mitochondrial matrix by the ISC machinery. Cytosolic and nuclear Fe-S cluster proteins depend on the mitochondrial ISC assembly and also on the ISC export machinery for their maturation and function. Previous literature suggested that the yeast Atm1 is involved in the export of Fe-S clusters over the inner membrane of mitochondria. Though, it is not established that how these Fe-S clusters are exported to the cytosol from the inter membrane space and outer membrane of mitochondria .

The CPC motif of MIA40 is involved in drawing the electrons from substrate proteins to Erv1/ALR and thereby facilitates the oxidative folding of substrate proteins in the intermembrane space of mitochondria (Figure: 2.1). The human MIA40 (CHCHD4) and yeast Mia40 contains the α-helical structure and is stabilized by two intra disulfide bonds. However, in yeast, Mia40 is stably fixed to the inner membrane of mitochondria over its N-terminal hydrophobic domain whereas essential domain open to the intermembrane space. In contrast to yeast Mia40, human MIA40 lacks the transmembrane domain.(Hofmann *et al*, 2005).

Recently, we and others have shown that MIA40 binds Fe-S clusters and is involved in the export of Fe-S from mitochondrial to the cytosol (Daithankar *et al*, 2010; Spiller *et al*, 2013b; Murari *et al*, 2015). However, the specific physiological significance of Fe/S cluster in MIA40 is yet to be understood. Further, the importance of GSH on MIA40 mediated import suggests that a possible potential role of GSH in protein import and Fe-S cluster biogenesis. Based on the above explanation, the outline of the current work is as follows:

(1) Functional characterization of glutathionylated MIA40 in protein import and iron homeostasis of the cell.

3.2. Materials and Methods

3.2.1. Materials

His60 Ni super flow resin was purchased from Clontech. L-Glutathione (reduced) was purchased from Sigma-Aldrich. Decylubiquinone, NADH, rotenone, BSA, sodium azide for complex I, and 1,4-Dithiothreitol (DTT) were obtained from Sigma. Cell culture components like Dulbecco's Modified Eagle's Medium (DMEM), Pen-strep, foetal bovine serum (FBS), and lipofectamine for preparation of complete media and for transfection studies respectively were purchased from Invitrogen. Cell culture 35mm, 60mm and 100mm dishes and T-25, T-75 and T-175flasks from Eppendorf India.

Bathophenanthroline was bought from Sigma Aldrich to determine the mitochondrial non-heme iron. Decylubiquinone and isocitrate were bought from Sigma Aldrich to quantify the complex I cytosolic & aconitase activity respectively. Radiolabel ^{55}Fe was bought from American Radio Chemicals (USA) for quantifying the iron uptake. Mouse polyclonal GPAT (Glutamate phosphoribosyl pyrophosphate amido transferase), a c-myc tag, GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) antibodies were bought from Abcam (USA).

3.2.2. Methods

Details of cloning, protein expression, and protein purification by His60 super flow resin, complex 1 activity were described in 2.2 Methods.

3.2.2A Fe-S cluster measurement with UV Absorption Spectroscopy

Recombinant WT hMIA40 protein was incubated with or without 5mM GSH and / 5Mm GSSG for 0, 15, and 30 mins. Fe-S cluster was measured with UV-Visible spectra from 200 to 700 nm and recorded in a HITACHI U-2910 spectrophotometer at room temperature using a quartz cuvette of 1cm path length.

3.2.2B Cell culture, transfection and Treatments

HEK 293T cells were grown in complete Dulbecco's modified Eagle's medium (Invitrogen) containing 10% (v/v) foetal calf serum at 37°C under an atmosphere of 5% CO_2. Cells grown in 100 mm / T-75 mm flasks, were transfected with either 8 µg /12 µg respectively of scrambled shRNA or human MIA40 shRNA plasmid (ORIGENE) by using lipofectamine transfectant agent (Invitrogen). HumanMIA40 was overexpressed in HEK 293T cells by transfecting with mammalian expression vector pcDNA3.1c-myc-His vector containing human MIA40 ORF (pNB202) pNB314 (hMIA40 C53S-C55S), pNB380 (hMIA40 C4S, C74S&C97S(TM)) and pNB391 (hMIA40 C4S, C64S, C74& C97S (QM)). Co-expression was done in HEK 293T cells by co-transfecting with 12 µg plasmid each of MIA40 shRNA/pNB314 c-myc-His and MIA40 shRNA/ pNB380 c-myc-His, and MIA40 shRNA/ pNB391c-myc-His. After 24hrs of post transfection DMEM medium was substituted with fresh DMEM medium and cells were incubated with or without of 500 nM ^{55}Fe, NAC (1 & 5 mM) and /or BSO (100, 250 & 500 µM) for 12hrs at 37ºC under an atmosphere of 5% CO_2.

3.2.2C Aconitase activity

The mitochondrial/ cytosolic Aconitase activity was determined spectrophotometrically. The reaction mixture holds, 5 mM $MnCl_2$, 50 mM Tris-HCl pH 7.5, and 1 mM isocitrate as a substrate, and a cytosolic/mitochondrial fraction. The conversion of isocitrate to cis-aconitate was measured as an increase in absorbance at A240 nm. One unit of enzyme activity was determined as the absorbance equivalent of 1 µmole of cis-aconitate released per min per mL of the enzyme solution under experimental conditions.

3.2.2D Measurement of mitochondrial iron

The non-heme iron present in the mitochondria was determined by the bathophenonthroline method (Tangerås *et al*, 1980). Concisely, mitochondria were resuspended in medium having 10 mM

MES pH 4.5, 1 % sodium dodecyl sulphate and 0.5 mM dithionite. To this reaction mixture, 50 μM bathophenonthroline was added, and the Fe (II)-chelate formation was determined in a dual-wavelength HITACHI spectrophotometer at 540 nm and 575 nm. For the measurement of mitochondrial ^{55}Fe content (specific Activity-10.18 mCi/mg), 60% - 80% confluent cells were incubated with or without 500 nM ^{55}Fe for 24 hrs. Mitochondria were wash away with cold 500 μM bathophenonthroline to eliminate membrane-bound iron. Radioactivity was counted by using a Beckman scintillation counter.

3.2.2E *In vitro* protein import into isolated mitochondria

Precursor proteins were produced by coupled transcription/translation in rabbit reticulocyte lysate (Promega) in the presence of ^{35}S (radio labelled methionine) and imported into mitochondria isolated from HEK293T cells. The radiolabeled Signals of the proteins were identified by autoradiography.

For *in vitro* protein import studies, ^{35}Slabeled TIMM9, TIMM8 and SU9-DHFR protein was incubated with purified mitochondria in import buffer (250 mM Sucrose, 5 mM Mg acetate, 80 mM K acetate, 10 mM Na-Succinate pH-7.2, 1 mM DTT, 20 mM HEPES-KOH pH 7.4, 0.1mMADP,2 mM ATP, 2 mM GTP) at 37°C for 2 to 10 min. The samples were then subjected to 25 μg/ml trypsin treatment for 15 mins on the ice and 100μg /ml trypsin inhibitor treatment on ice for 5mins. To remove the non-imported proteins, mitochondria were reisolated by passing through a sucrose cushion (0.8 M sucrose in 10 mM HEPES, pH-7.2). Mitochondria were rewashed with buffer containing 0.25 M Sucrose, 1 mM EDTA, 10 mM HEPES, pH-7.2 and pellet down by centrifugation at 12,000 rpm for 10 min at 4°C and resuspended in 2x Laemmli sample buffer**.** The mitochondrial proteins were subjected to SDS-PAGE and examined the labelled protein by autoradiography.

3.3 Results:

3.3.1 The CPC motif in hMIA40 is essential for Fe–S cluster export from mitochondria

We have shown earlier (Murari A thesis) that MIA40 is a component of Fe-S cluster export machinery of mitochondria. However, it is not clear wheter CPC motif or other cysteine motif of MIA40 are involved in the export of Fe-S from mitochondria. We evaluate the role of the hMIA40 CPC motif in binding iron *in vivo*. HEK-293T cells were transfected with plasmids pNB202 and pNB314, which express hMIA40 wild-type or S53-55S (M1) respectively.

To determine the importance of the CPC motif in Fe–S export *in vivo*, we overexpressed either wild-type hMIA40 or hMIA40 CPC motif mutant (hMIA40 S53S&C55S) in HEK293T cell lines that are depleted in endogenous hMIA40. We used hMIA40 shRNA that is specific to the non-coding region of hMIA40 to deplete the steady-state levels of endogenous hMIA40. HEK-293T cells were transfected with either hMIA40 shRNA or co-transfected with hMIA40 shRNA/wild-type hMIA40 or hMIA40 shRNA/hMIA40 S53S&C55S to overexpress wild-type and mutant CPC motif respectively. In shRNA hMIA40-transfected samples, hMIA40 levels were reduced by 50% compared with scrambled shRNA-transfected samples (Figure: 3.3.1A). However, both WT hMIA40 and mutant hMIA40 protein levels are high in co-transfected samples as shRNA fails to inhibit the plasmid-borne hMIA40 translation. Next, we checked the steady state levels of GPAT, a cytosolic Fe-S containing enzyme and its activity and stability depends on the presence of Fe-S clusters. As expected, GPAT levels were reduced by around 50% in cell lysates expressing shRNA. However, overexpression of WT hMIA40, but not mutant, rescues the steady-state levels of GPAT in hMIA40-depleted cell lines (Figure: 3.3.1A). In addition, we found an accumulation of ^{55}Fe in the mitochondria samples isolated from shRNA hMIA40 or shRNA hMIA40 co-transfected with mutant, but not with wild-type hMIA40, co-expressing cell lines (Figure: 3.3.1B). Overexpression of wildtype hMIA40, but not SPS hMIA40 mutant, rescues the cytosolic aconitase activity in hMIA40-depleted cell lines (Figure: 3.3.1C). The transfected samples were probed with Myc to confirm the equivalent overexpression of plasmid-borne wild-type and mutant proteins and GAPDH to confirm the equivalent amount of lysate in all samples (Figure: 3.3.1A). Besides the comparable amount of wild-type and mutant hMIA40 proteins, we observed no significant difference in the mitochondrial Complex I and aconitase activity in all samples (Figures: 3.3.1D&E). These results absolutely incriminate the CPC motif in hMIA40 to be necessary for effective binding to iron both *in vitro* and *in vivo*.

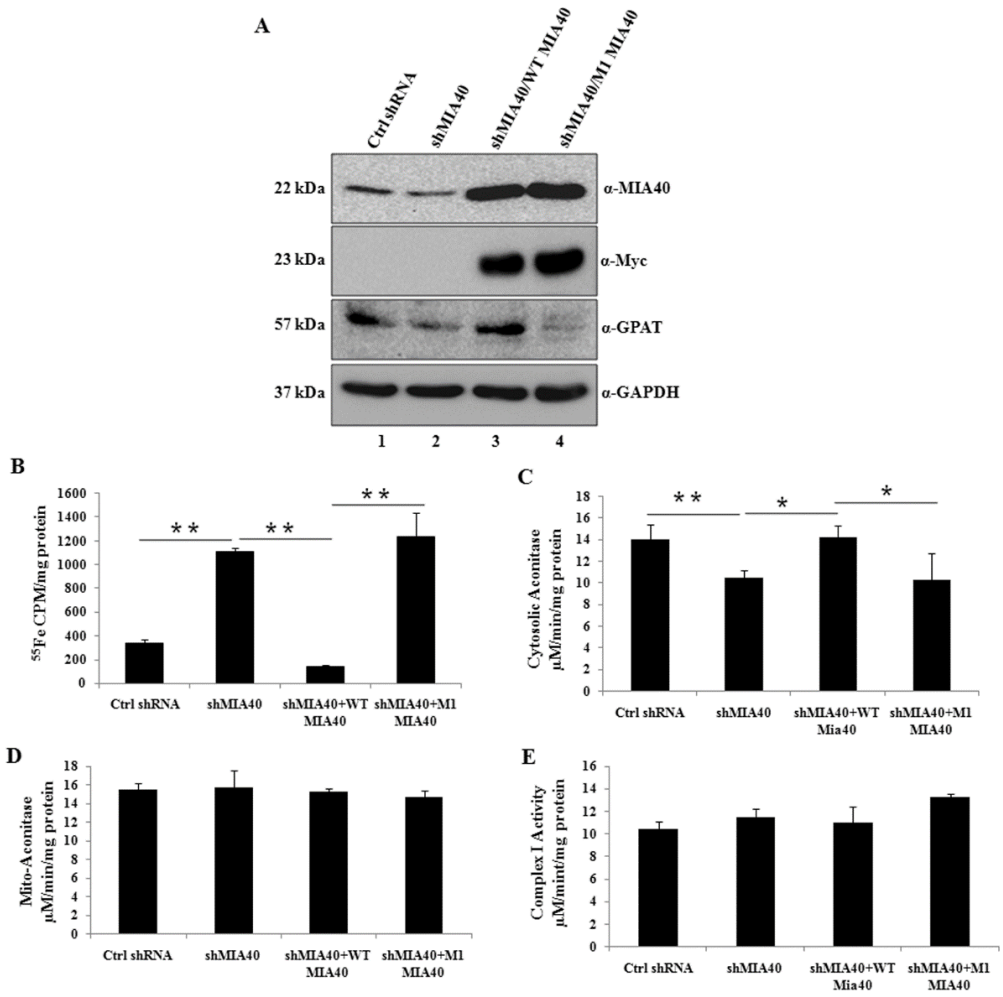


Figure 3.3.1 CPC motif in hMIA40 is essential for Fe-S export from mitochondria: - HEK293T cells were transfected with MIA40 shRNA specific to a noncoding region or scrambled shRNA vector control or co-transfected with shRNA MIA40/WT MIA40 or shRNA MIA40/S53-55SMIA40 and isolated the mitochondria or cytosolic fraction as described in the methods. (A) Western blot analysis of total cell extract of HEK293T cells. The blot was probed with antibodies against GPAT, MIA40, Myc and GAPDH. GAPDH was used as an internal loading control. (B) Mitochondrial accumulated radiolabelled ^{55}Fe was measured from the isolated mitochondrial fractions of transfected cells as described in the methods section and shown here as CPM/mg of mitochondrial protein. (C) Cytosolic aconitase activity was measured at 240 nm and shown here as μM/min/mg protein. (D) Mitochondrial aconitase activity was measured at 240 nm and represented as units/mg protein. (E) Complex I activity was measured in transfected cells spectrophotometrically at 340 nm and presented as μM/min/mg protein. Bar graphs represent the means of three independent experiments, $P<0.001$. Statistical analysis was performed using t- test .

3.3.2 Glutathione has an effect on hMIA40 iron binding

We have shown in Chapter II about the importance of glutathionylated MIA40 in ETC function. Given the importance of glutathione, we would like to explore the role of glutathione in Fe-S synthesis and export. It is well known that N-acetylcysteine (NAC) is a pro-glutathione drug and DL-Buthionine (S, R)-sulfoximine (BSO) is an inhibitor of glutathione synthesis(Akan *et al*, 2005). Our *in vitro* and *in vivo* studies evidently show that hMIA40 binds to iron. Based on these results, we were interested to see the iron binding to hMIA40 protein in the presence and /or absence of NAC and BSO (Glutathione). To enhance hMIA40 *in vivo*, cells were transfected with a plasmid containing pcDNA-*MIA40 cmyc-His* and incubated with or without ^{55}Fe and then treated with NAC and /or BSO for 12 h. Thereafter, cell lysates were subjected to pulldown with Ni-NTA Sepharose beads. The amount of radioactivity in the pull-down fraction was counted in a scintillation counter (Figure: 3.3.2A). With increasing concentration of NAC, the amount of ^{55}Fe binding to MIA40 is decreased, and no significant change was observed with BSO treatment (Figure: 3.3.2B). Our results indicate that glutathione may have an effect on the binding of iron to hMIA40. Further, cell lysates were separated on non-Reducing SDS-PAGE, immunoblotted with hMIA40 antibody to test the redox status of hMIA40 in the presence and absence of NAC and / or BSO treatment (Figure: 3.3.2C&D). Oligomeric forms of MIA40 decreased while monomeric form or reduced form of MIA40 increased with increasing concentration of NAC treatment (Figure: 3.3.2C). However, oligomeric forms of MIA40 increased with increasing concentration of BSO (Figure: 3.3.2D). Our results suggest that NAC and BSO has an opposing effect on MIA40 redox status. These results also suggest that glutathione also influences the binding of iron to MIA40.

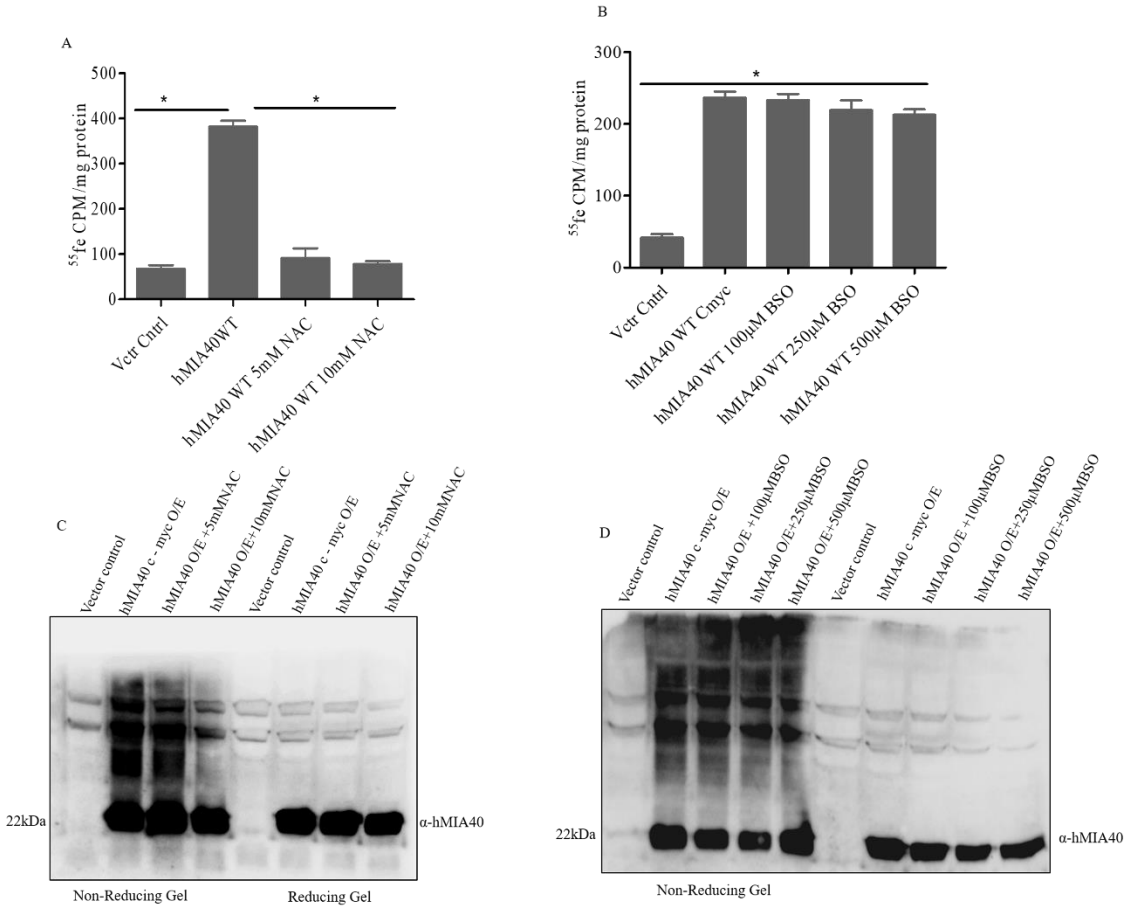


Figure 3.3.2 Glutathione have an effect on the redox status of hMIA40 and iron binding:- hMIA40 transfected HEK293T cells were treated with increasing concentration of NAC or/and BSO and incubated with ^{55}Fe. (A&B) Cell lysates were pull-down with Ni-NTA Sepharose beads and amount of radiolabeled iron present in the pulldown fraction was determined in the scintillation counter. (C&D) Cell lysates were analyzed on reducing and non - reducing gels and immunoprobed with antibodies against hMIA40. All results were plotted with mean ± S. E. (n=3), $^{*}P \leq 0.05$.

3.3.3 Glutathione affects the binding of iron to Recombinant hMIA40 *in vitro*

Earlier we have shown that an oxidative sensitive labile iron bound to hMIA40 *in vitro* (Murari A et al. 2005). To test whether the glutathione has any effect on Fe-S clusters binding to hMIA40, the recombinant hMIA40 protein was incubated with or without glutathione (GSH), oxidized glutathione (GSSG) and exposed to air and at different time points UV-VIS spectrum was monitored for the presence of Fe-S as described in the Methods. We have shown earlier that MIA40 shows a characteristic Fe/S cluster peak either at 410 or 460 nm or both depending on the redox status (Murari A Thesis, 2014). As expected, all samples at 0 min time point showed an absorption peak at 460nm indicating the presence of Fe-S cluster. Later, we observed a decrease in absorption in a time

dependent manner whether hMIA40 in presence or absence of GSH/GSSG. However, in the presence of GSH/GSSH, there is rapid decrease in absorption at 460 nm indicating the loss of Fe/S. These results suggest that MIA40 may be glutathionylated in presence GSH/GSSG in exchange of Fe/S clusters (Fig 3.3.3 A, B, &C).

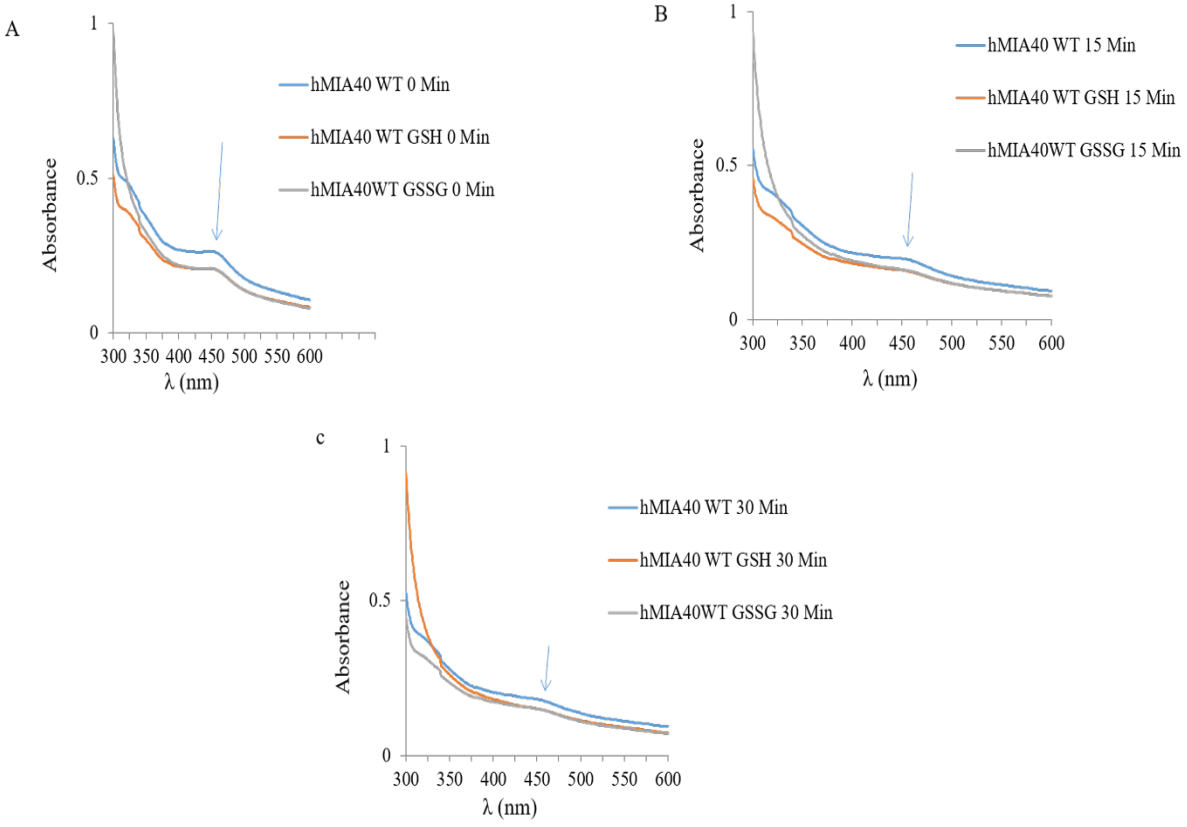


Fig 3.3.3 Glutathione affects binding of iron to Recombinant hMIA40 *in vitro*:- UV-Vis spectra (300 nm-600 nm) of purified recombinant His-hMIA40 was carried out in presence and absence of GSH/GSSG for different time points. Arrows indicate the characteristic iron peak at 460 nm, (A, B&C).

3.3.4 Glutathionylated MIA40 mutants do not affect the binding of iron

Our *in vitro* and *in vivo* studies clearly show that hMIA40 binds to iron and also sensitive to GSH/GSSG. Based on these results, we were curious to know wheather iron binds to the glutathione cysteine mutants of hMIA40 (hMIA40 DM, hMIA40 TM& hMIA40 QM). Equivalent amount of purified recombinant hMIA40 and glutathione cysteine mutants hMIA40 (hMIA40 DM, hMIA40 TM&hMIA40 QM) were subjected to UV-VIS absorption spectrum for the presence Fe/S cluster. Interestingly, WT and all cysteine mutants displayed an absorption peak at 410 nm and 460 nm in the UV-Visible absorption spectrum, a typical spectral feature of a protein harbouring Fe-S clusters (Fig 3.3.4A). These results suggest that glutathione cysteine mutant's in hMIA40 does not affect the binding of Fe-S clusters *in vitro*. Further, our results strongly suggest that CPC motif of hMIA40 is critical for iron binding.

To test the effect of glutathionylated mutants on binding of iron to MIA40, we overexpressed hMIA40 or glutathionylated (TM, QM) or CPC mutants by co-transfecting HEK293T cells with specific shRNA that selectively depletes endogenous MIA40. After 12 hrs of transfection, cells were incubated with or without ^{55}Fe for 12 hrs. Cells were harvested with NP40 buffer and lysates were subjected to pulldown with Ni-NTA Sepharose beads as described in the Methods. The amount of radioactivity present in the pull-down fraction was counted using scintillation counter (Fig3.3.4B). WT and glutathione cysteine mutants of hMIA40 (hMIA40 TM&hMIA40 QM) were more enriched in ^{55}Fe compared to ctrl shRNA, shRNA MIA40 or hMIA40 CPC motif mutants. These results indicate that glutathione cysteine mutants hMIA40 (hMIA40 TM&hMIA40 QM) does not affect the binding of Fe-S clusters *in vivo*. It also confirms that CPC motif of hMIA40 is critical for binding of Fe-S clusters.

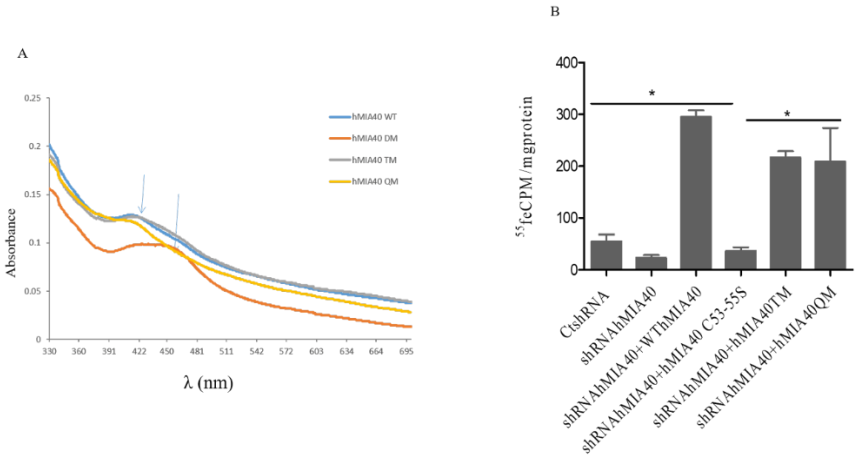


Figure 3.3.4 Glutathionylated MIA40 mutants also bind to Fe-S clusters :- (A) UV-Vis spectra (300 nm-700 nm) of purified recombinant His-hMIA40 and glutathionylated cysteine mutants were carried out as described in the Methods. Arrows indicate the characteristic Fe/S cluster peak at 410 and 460 nm. B) HEK293T cells were transiently transfected either with shRNAMIA40 or shRNAMIA40 / Myc-His-hMIA40 or shRNAMIA40 / mutants of MIA40 followed by incubation with labelled ^{55}Fe. The cell lysate was prepared by using NP40 buffer and Ni-NTA pulldown was carried out as described in the methods. Labelled ^{55}Fe present in Ni-NTA pulldown fractions was measured in a scintillation counter and shown here as CPM/mg protein. All results were plotted with mean ± S. E. (n=3), $^{*}P \leq 0.05$.

3.3.5 Glutathionylated hMI40 is important for Fe–S cluster export from mitochondria

To demonstrate the importance of glutathionylation of MIA40 in in Fe–S export *in vivo,* we overexpressed either wild-type hMIA40 or hMIA40 CPC motif mutant (hMIA40 C53-55S) and glutathione cysteine mutants of MIA40 (hMIA40 TM, hMIA40 QM) in HEK293T cells that are depleted in endogenous hMIA40. HEK-293T cells were transfected with either hMIA40 shRNA or co-transfected with hMIA40 shRNA/wild-type hMIA40, hMIA40 shRNA/hMIA40 S53-C55S, hMIA40 shRNA/ hMIA40 TM, and hMIA40 shRNA/ hMIA40 QM. Cell lysates or mitochondria were isolated as described in the Methods. Cell lysates were analysed on SDS-PAGE and immunoblotted with antibodies against MIA40, GAPDH and GPAT. hMIA40 levels were 30% reduced in ShRNA for MIA40 transfected samples when compared to scrambled shRNA-transfected samples (Figure: 3.3.5B). However, both WT and CPC mutant of hMIA40 protein levels are high in co-transfected samples as shRNA fails to inhibit the plasmid-borne hMIA40 translation. As expected, GPAT levels were reduced in cell lysates expressing shRNA and CPC mutant of MIA40. However, overexpression of WT hMIA40, but not mutant, rescues the steady-state levels of GPAT in hMIA40-depleted cell lines (Figure: 3.3.5B). In addition, we found an accumulation of ^{55}Fe in the mitochondria samples isolated from shRNA hMIA40 or shRNA hMIA40 co-transfected with mutant (hMIA40 SPS, hMIA40 TM and hMIA40 QM) but not with wild-type hMIA40 (Figure: 3.3.5A). The transfected samples were probed with Myc to confirm the overexpression of plasmid-borne wild-type and mutant proteins and GAPDH used as a control to confirm the equivalent amount of lysate in all samples (Figure: 3.3.5B). Further, overexpression of wildtype hMIA40, but not SPS mutant hMIA40, TM MIA40 and QM MIA40, rescues the cytosolic aconitase activity in hMIA40-depleted cell lines (Figure: 3.3.5C). Our results clearly show that the CPC motif in hMIA40 to be important for effective binding to iron both *in vitro* and *in vivo*. However, glutathionylated hMIA40 may be important for the Fe-S cluster export.

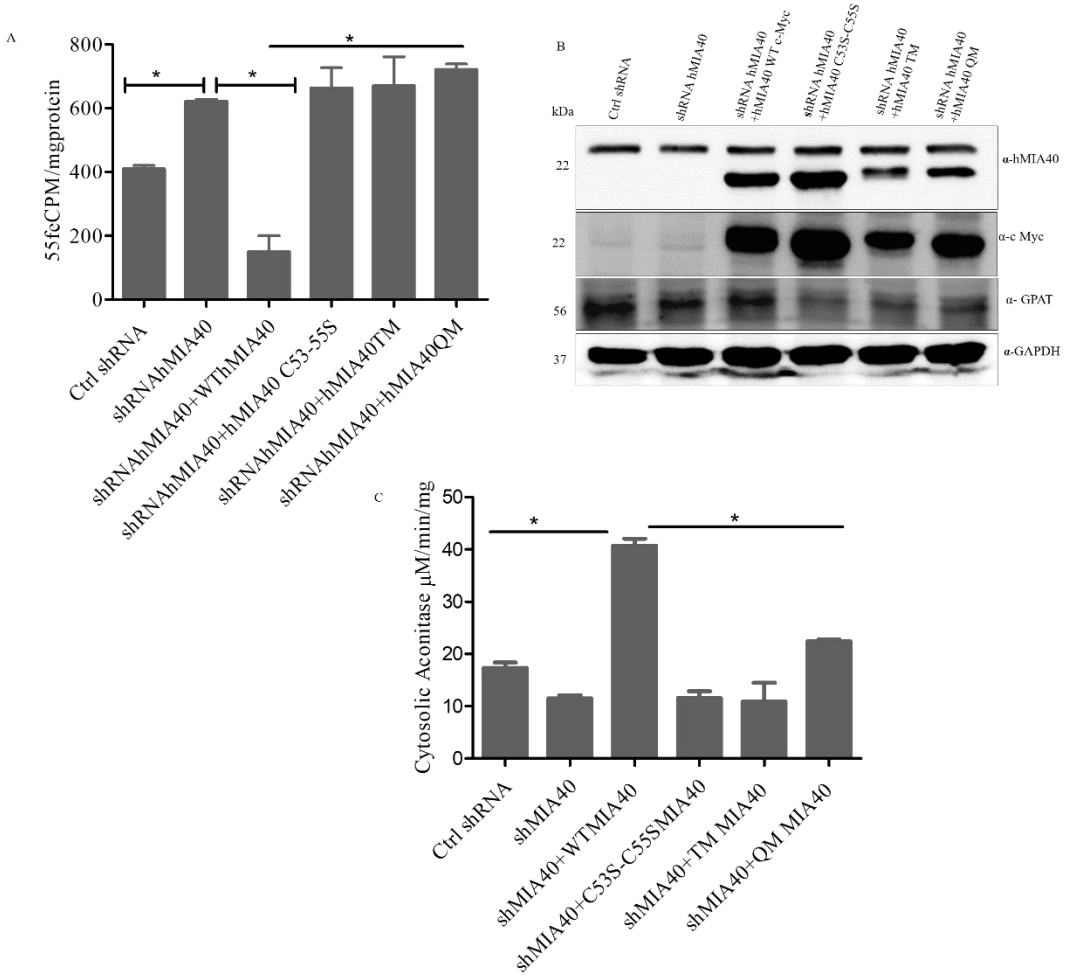


Figure 3.3.5 Glutathionylated hMIA40 is important for Fe/S export from mitochondria:- HEK293T cells were transfected with MIA40 shRNA specific to a noncoding region or scrambled shRNA vector control or co-transfected with shRNA MIA40/WT MIA40 or shRNA MIA40/S53-55S MIA40,shMIA40/TM MIA40 and shMia40/QM hMIA40 and isolated the mitochondria or cytosolic fraction. (A) Mitochondrial accumulated radiolabelled ^{55}Fe was measured from the isolated mitochondrial fractions of transfected cells as described in the methods section and shown here as CPM/mg of mitochondrial protein. (B) Western blot analysis of total cell extract of HEK293T cells. The blot was probed with antibodies against GPAT, MIA40, Myc and GAPDH. GAPDH was used as an internal loading control. (C) Cytosolic aconitase activity was measured at 240 nm and shown here as µm/min/mg protein. All results were plotted with mean ± S. E. (n=3), $^{*}P \leq 0.05$.

3.3.6 Glutathionylated MIA40 have no defect in IMS protein import

To examine whether the glutathionylated MIA40 is essential for the import of MIA40 0substrates into mitochondria, we performed import studies of known MIA40 substrates like T0IMM8 and TIMM9. We isolated the mitochondria from HEK293T cells overexpressing either WT or 0glutathionylated mutants of MIA40 (hMIA40 DM and hMIA40 TM) and import of radiolabelled TIMM8 and TIMM9 was carried out as described in the Methods. Radiolabeled TIMM8 and TIMM9 is ef0ficiently imported into WT and mutants of MIA40 mitochondria (Fig 3.3.6A &B). To test the qualit0y of mitochondria, we carried out the import of matrix targeted radiolabelled SU9-DHFR into WT an0d mutant MIA40 mitochondria. The amount of imported SU9-DHFR increased considerably from 2 0min to 10 min (Fig 3.3.6C). In addition, immunoblot analysis of the WT and mutant MIA40 mitochondria with complex III core1, TIMM9A andTIMM10 antibodies reveals that no defect in the import of these IMS substrates (Fig3.3.6D). It's known that CPC motif of Mia40 is critical for import of intermembrane space (IMS) proteins. These results suggest that glutathionylated MIA40 have no defect in IMS protein import.

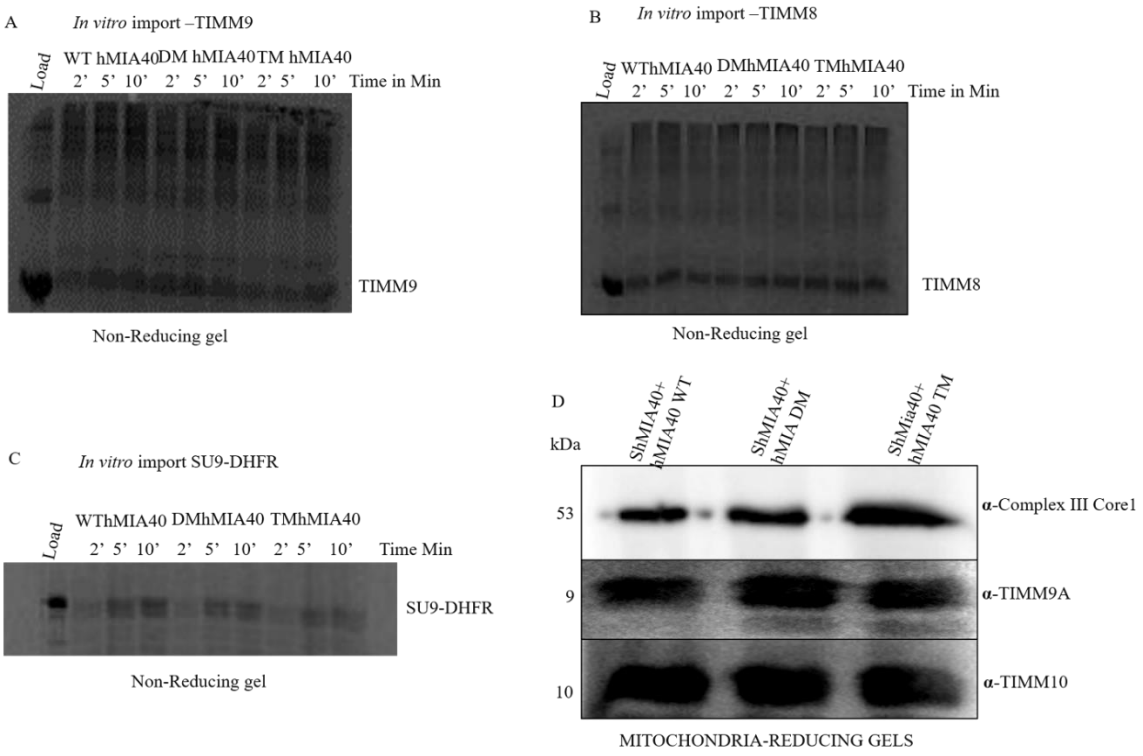


Fig 3.3.60 Role of glutathionylated hMIA40 in protein import. (A, B, & C) Radiolabelled precursors TIMM8, TI0MM9 and SU9-DHFR were imported *in vitro* into WT hMIA40 and mutants MIA40 mitochondria isolated from0 HEK293T for the indicated time periods at 37°C. Samples were analysed by SDS-PAGE and autoradiography. (D) WThMIA40 and mutant's hMIA40 mitochondria were prepared, and equal amounts of protein (50 mg00) were immunoblotted with TIMM9A, TIMM10 and complex III core1 antibodies.

3.4 Discussion

Being the fundamental organelle of the cell, mitochondria is involved in diversified functions. Regulating iron homeostasis in the cell is one of the most significant roles of mitochondria. It is the core for the production and export of iron-sulfur clusters. The enzyme machineries of the iron-sulfur cluster synthesis pathway of the mitochondria have been well categorised genetically and biochemically in bacteria and yeast (Frazzon & Dean, 2003; Lill & Mühlenhoff, 2006). In higher eukaryotes, they have their own machinery for the iron sulfur cluster assembly in both nucleus and cytoplasm (CIA). The mechanism underlying the maturational defects of cytoplasmic and nuclear enzymes caused by mitochondrial iron sulphur pathway is yet to understood. This specifies the significance of mitochondrial iron-sulfur cluster system in the iron homeostasis of the cell. The mechanism by which mitochondrial iron-sulfur cluster system exports Fe-S to the cytoplasm is yet to be clarified.

Ultraviolet-Visible (UV-Vis) spectrum of recombinant hMIA40 and glutathione hMIA40 mutants (hMIA4 DM, hMIA4 TM and hMIA4 QM) displayed an absorption spectrum peak at 410 and 460 nm (Fig: 3.3.3 A, B, C&3.3.4A) which are typical iron peaks for 4Fe-4S and 2Fe-2S clusters correspondingly. We assume that 4Fe-4S and 2Fe-2S forms might be coordinated to the CPC motif of hMIA40. Our Atomic Emission Spectroscopy (AES) experiments by exhausting hMIA40 protein strongly suggest0 that hMIA40 binds to Fe-S cluster (Murari *et al*, 2015). In addition spectral analysis, and gel filtration of hMIA40 protein strongly recommend that majority of hMIA40 is in a tetrameric state (Murari *et al*, 2015) and possibly contains a cubane 4Fe-4S cluster as it displays a typical 4Fe-4S spectrum peak at 410 nm while dimeric form of hMIA40 displayed a 460 nm absorption spectrum peak which is representative for 2Fe-2S cluster (Murari *et al*, 2015; Cai *et al*, 2013). The hMIA40 2Fe-2S cluster may involve in oxidoreductase activity, 4Fe-4S cluster may involve in the iron-sulfur export.

The biological importance of iron-sulfur clusters found in MIA40 is anonymous. We have tried to elucidate t0he function of hMIA40 in iron homeostasis of the cell. We assessed the ^{55}Fe binding to hMIA40 WT, CPC hMIA40 mutant and glutathione hMIA40 mutants (hMIA40TM and hMIA40QM). These results show a significant defective in ^{55}Fe binding in CPC hMIA40 mutant compared to WT hMIA40 and glutathione hMIA40 mutants (hMIA40 TM and hMIA40 QM) (Fig: 3.3.4B). These results strongly suggest that CPC motif of hMIA40 is critical for Fe binding. Since the CPC motif is required for protein import and Fe–S binding, it is possible that the microenvironment of the intermembrane space and its redox status may influence hMIA40's function either in protein import or/and Fe–S export.

We used shRNA to deplete the levels of MIA40 and overexpression of glutathione hMIA40 mutants hMIA40TM, and hMIA40QM HEK293T cell lines (Fig: 3.3.1A&3.3.5B). To measure the iron present in mitochondria, we have followed 2 different methods, namely bathophenanthroline and ^{55}Fe method. These results display significant increase of mitochondrial iron load when we knockdown the hMIA40 and/ overexpression of glutathione hMIA40 mutants (hMIA40TM and hMIA40QM) in HEK293T cell lines (Fig: 3.3.6A &3.3.1B). In addition, we have studied the activity and maturation of cytosolic iron-sulfur cluster containing enzymes in MIA40 knockdown and overexpression of glutathione hMIA40 mutants (hMIA40TM and hMIA40QM) in HEK293T cell lines. The knockdown of hMIA40 and overexpression of glutathione hMIA40 mutants (hMIA40TM and hMIA40QM) significantly decreased the activity of cytosolic iron-sulfur cluster containing enzymes Aconitase (Fig: 3.3C& 3.3.6C), the stability of GPAT (Glutamate Phosphoribosyl pyrophosphate Amido transferase) is also decreased in the hMIA40 knockdown cells and overexpression of glutathione hMIA40 mutants (hMIA40TM and hMIA40QM) (Fig: 3.3.6B& 3.3.1A). These results strongly propose that hMIA40 CPC motif is key for Fe binding and glutathione binding cysteines are critical for Fe-S cluster export. Therefore, these outcomes strongly propose that hMIA40 exports Fe-S clusters from mitochondria to cytosol.

Related information has been published when the amount of ABCB7 (humans)/ Atm1 (yeast), an inner mitochondrial membrane protein levels were decreased & so that Atm1/ ABCB7 was proposed to be a part of Fe-S export device of mitochondria (Kispal *et al*, 1997). Albeit the actual module that is exported by Atm1 from mitochondria is arguable. The defect in Atm1 leads to iron load in mitochondria followed by defects in iron-sulfur cluster biogenesis that arise in nucleus & cytosol.

Yet, no significant effect was detected on the stability of mitochondrial enzymes associated with Fe-S clusters or mitochondrial ISC pathway(Kispal *et al*, 1997). Additionally, ABCB7/Atm1, the iron-sulfur export machinery most likely contains ALR/Erv1 in mammalians or yeast respectively, a sulfhydryl oxidase and GSH as knockdown of these proteins effects in a phenotype comparable to that of Atm1 deficiency.

It is a known fact that MIA40 and Erv1/ ALR cooperate in the import of various cysteine contain intermembrane space (IMS) proteins by an oxidative folding machinery (Allen *et al*, 2005) from this we have hypothesised that MIA40 may work in association with Erv1/ALR to export iron-sulfur cluster from mitochondria to cytosol.. The mutations in these proteins that are involved in iron-sulfur cluster biogenesis lead to certain human disorders. The particular diseases are ranging from ataxia like Friedreich's ataxia (FRDA), anaemia & myopathies (Mochel, Knight et al., 2008; Ye, Jeong et al., 2010; Koeppen 2011). Deeper insight into iron-sulfur cluster synthesis and export from mitochondria to cytosol would lead to the development of improved remedy against genetic disorders caused by mutations in iron-sulfur cluster biogenesis.

Bibliography

Alberts, Bruce; Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, Peter Walter (1994). Molecular Biology of the Cell. New York: Garland Publishing Inc. ISBN 0-8153-3218-1.

Adams KL & Palmer JD (2003) Evolution of mitochondrial gene content: Gene loss and transfer to the nucleus. *Molecular Phylogenetics and Evolution* **29:** 380–395

Akan I, Akan S, Akca H, Savas B & Ozben T (2005) Multidrug resistance-associated protein 1 (MRP1) mediated vincristine resistance: Effects of N-acetylcysteine and Buthionine sulfoximine. *Cancer Cell International* **5:** 1–9

Allen S, Balabanidou V, Sideris DP, Lisowsky T & Tokatlidis K (2005) Erv1 mediates the Mia40-dependent protein import pathway and provides a functional link to the respiratory chain by shuttling electrons to cytochrome c. *Journal of Molecular Biology* **353:** 937–944

Axelsson K & Mannervik B (1980) General specificity of cytoplasmic thioltransferase (thiol:disulfide oxidoreductase) from rat liver for thiol and disulfide substrates. *BBA - Enzymology* **613:** 324–336

Balaban RS, Nemoto S & Finkel T (2005) Mitochondria, oxidants, and aging. *Cell* **120:** 483–495

Banci L, Bertini I, Cantini F, Ciofi-Baffoni S, Gonnelli L & Mangani S (2004) Solution structure of Cox11, a novel type of β-immunoglobulin-like fold involved in CuB site formation of cytochrome c oxidase. *Journal of Biological Chemistry* **279:** 34833–34839

Banci L, Bertini I, Cefaro C, Ciofi-Baffoni S, Gallo A, Martinelli M, Sideris DP, Katrakili N & Tokatlidis K (2009) MIA40 is an oxidoreductase that catalyzes oxidative protein folding in mitochondria. *Nature Structural and Molecular Biology* **16:** 198–206

Baynes JW (1991) Role of Oxidative Stress in Development of Complications in Diabetes. *Diabetes* **40:** 405–412 Available at: http://diabetes.diabetesjournals.org/cgi/doi/10.2337/diab.40.4.405

Beinert, H. (1954). BIOLOGICAL OXIDATIONSl, 2 (1)., Altman, F. P. (1974). Studies on the reduction of tetrazodium salts - III. The products of chemical and enzymic reduction. Histochemistry, 38(2) 155–171. http://doi.org/10.1007/BF00499663, Hopkins, T. J. (1980). Sites of Electron 235(9)., Alföldy, P., & Lemmel, E. M. (1979). Reduction of nitroblue tetrazolium for functional evaluation of activated macrophages in the cell-mediated immune reaction. Clinical Immunology and Immunopathology, 12(3) 263–270. http://doi.org/10.1016/0090-1229(79)90029-1, Mourier, A., & Larsson, N. G. (2011). Tracing the trail of protons through complex i of the mitochondrial respiratory chain. PLoS Biology, 9(8) 8–11. http://doi.org/10.1371/journal.pbio.1001129, Gammage, P. A., Rorbach, J., Vincent,

A. I., Rebar, E. J., & Minczuk, M. (2014). Mitochondrially targeted ZFNs for selective degradation of pathogenic mitochondrial genomes bearing large-scale deletions or point mutations. EMBO Molecular Medicine, 6(4) 458–466. http://doi.org/10.1002/emmm.201303672, Kann, O., & Kovacs, R. (2006). Mitochondria and neuronal activity. AJP: Cell Physiology, 292(2) C http://doi. org/10. 1152/ajpcell. 00222. 200., Nass, M. M. K. K., & Nass, S. (1963). Intramitochondrial fibers characteristics: I. Fixation and Electron Staining Reactions. The Journal of Cell Biology, 19(3) 593–611. http://doi.org/10.1083/jcb.19.3.593, Tinel, H., Cancela, J. M., Mogami, H., Gerasimenko, J. V., Gerasimenko, O. V., Tepikin, A. V., & Petersen, O. H. (1999). Active mitochondria surrounding the pancreatic acinar granule region prevent spreading of inositol trisphosphate-evoked local cytosoli 4999–5008. http://doi.org/10.1093/emboj/18.18.4999, Yang, F., He, X. P., Russell, J., & Lu, B. (2003). Ca2+ influx-independent synaptic potentiation mediated by mitochondrial Na+-Ca2+ exchanger and protein kinase C. Journal of Cell Biology, 163(3) 511–523. http://doi.org/10.1083/jcb.200307027, Tang, Y. G., & Zucker, R. S. (1997). Mitochondrial involvement in post-tetanic potentiation of synaptic transmission. Neuron, 18(3) 483–491. http://doi.org/10.1016/S0896-6273(00)81248-9, Friel, D. D. (2000). Mitochondria as regulators of stimulus-evoked calcium signals in neurons. Cell Calcium, 28(5–6) 307–316. http://doi.org/10.1054/ceca.2000.0172, Rizzuto, R., & Pozzan, T. (2006). Microdomains of Intracellular Ca 2+ : Molecular Determinants and Functional Consequences. Physiological Reviews, 86(1) 369–408. http://doi.org/10.1152/physrev.00004.2005., Dolmetsch, R. E., Xu, K., & Lewis, R. S. (1998). Calcium oscillations increase the efficiency and specificity of gene expression. Nature, 392(6679) 933–936. http://doi.org/10.1038/31960, Hajnóczky, G., Robb-Gaspers, L. D., Seitz, M. B., & Thomas, A. P. (1995). Decoding of cytosolic calcium oscillations in the mitochondria. Cell, 82(3) 415–424. http://doi.org/10.1016/0092-8674(95)90430-1, Yang, S., Tang, Y., & Zucker, R. (1999). Selective induction of LTP and LTD by postsynaptic i elevation. Journal of Neurophysiology, 81 781–787. http://doi.org/10.1152/jn.1999.81.2.781, Gómez-Durán, A., Pacheu-Grau, D., López-Gallardo, E., Díez-Sánchez, C., Montoya, J., López-Pérez, M. J., & Ruiz-Pesini, E. (2010). Unmasking the causes of multifactorial disorders: OXPHOS differences between mitochondrial haplogroups. Human Molecular Gene 3343–3353. http://doi.org/10.1093/hmg/ddq246, Burgoyne, R. D. (2007). UKPMC Funders Group Neuronal Calcium Sensor Proteins :

Generating Diversity in. Nature Reviews Neuroscience, 8(3) 1–25. http://doi.org/10.1038/nrn2093.Neuronal, Rizzuto, R., De Stefani, D., Raffaello, A., & Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. Nature Reviews Molecular Cell Biology, 13(9) 566–578. http://doi.org/10.1038/nrm3412, Wang, J. Q., Chen, Q., Wang, X., Wang, Q. C., Wang, Y., Cheng, H. P., … Tang, T. S. (2013). Dysregulation of mitochondrial calcium signaling and superoxide flashes cause mitochondrial genomic DNA damage in Huntington disease. Journal of Biological Chemist 3070–3084. http://doi.org/10.1074/jbc.M112.407726, et al (2007) Regulation of mitochondrial oxidative phosphorylation through cell signaling. *Biochimica et Biophysica Acta - Molecular Cell Research*

Benz R (1994) Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins. *BBA - Reviews on Biomembranes* **1197:** 167–196

Berg OG & Kurland CG (2000) Why mitochondrial genes are most often found in nuclei. *Molecular Biology and Evolution* **17:** 951–961

Beynnon BD, Johnson RJ, Abate JA, Fleming BC & Nichols CE (2005) Treatment of Anterior Cruciate Ligament Injuries, Part 2. *The American Journal of Sports Medicine*

Bien M, Longen S, Wagener N, Chwalla I, Herrmann JM & Riemer J (2010) Mitochondrial Disulfide Bond Formation Is Driven by Intersubunit Electron Transfer in Erv1 and Proofread by Glutathione. *Molecular Cell* **37:** 516–528 Available at: http://dx.doi.org/10.1016/j.molcel.2010.01.017

Bihlmaier K, Mesecke N, Terziyska N, Bien M, Hell K & Herrmann JM (2007) The disulfide relay system of mitochondria is connected to the respiratory chain. *Journal of Cell Biology* **179:** 389–395

Boal AK, Yavin E & Barton JK (2007) DNA repair glycosylases with a [4Fe-4S] cluster: A redox cofactor for DNA-mediated charge transport? *Journal of Inorganic Biochemistry* **101:** 1913–1921

Bolender N, Sickmann A, Wagner R, Meisinger C & Pfanner N (2008) Multiple pathways for sorting mitochondrial precursor proteins. *EMBO Reports* **9:** 42–49

Bottinger L, Gornicka A, Czerwik T, Bragoszewski P, Loniewska-Lwowska A, Schulze-Specking A, Truscott KN, Guiard B, Milenkovic D & Chacinska A (2012) In vivo evidence for cooperation of Mia40 and Erv1 in the oxidation of mitochondrial proteins. *Molecular Biology*

of the Cell **23:** 3957–3969 Available at: http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E12-05-0358

Brigelius R, Muckel C, Akerboom TPM & Sies H (1983) Identification and quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochemical Pharmacology* **32:** 2529–2534

Cai K, Frederick RO, Kim JH, Reinen NM, Tonelli M & Markley JL (2013) Human mitochondrial chaperone (mtHSP70) and cysteine desulfurase (NFS1) bind preferentially to the disordered conformation, whereas co-chaperone (HSC20) binds to the structured conformation of the iron-sulfur cluster scaffold protein (ISCU). *Journal of Biological Chemistry* **288:** 28755–28770

CAVALIER- SMITH T (1987) The Simultaneous Symbiotic Origin of Mitochondria, Chloroplasts, and Microbodies. *Annals of the New York Academy of Sciences* **503:** 55–71

Chacinska A, Koehler CM, Milenkovic D, Lithgow T & Pfanner N (2009) Importing Mitochondrial Proteins: Machineries and Mechanisms. *Cell* **138:** 628–644

Chai YC, Ashraf SS, Rokutan K, Johnston RB & Thomas JA (1994) S-thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: Evidence against a role for glutathione disulfide. *Archives of Biochemistry and Biophysics* **310:** 273–281

Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL & Lesnefsky EJ (2003) Production of Reactive Oxygen Species by Mitochondria . *Journal of Biological Chemistry* **278:** 36027–36031 Available at: http://www.jbc.org/content/278/38/36027.abstract

Chinnery PF & Turnbull DM (2001) Epidemiology and treatment of mitochondrial disorders. *American Journal of Medical Genetics* **106:** 94–101 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11579428

Chrestensen CA, Starke DW & Mieyal JJ (2000) Acute cadmium exposure inactivates thioltransferase (Glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis. *Journal of Biological Chemistry* **275:** 26556–26565

Collison MW, Beidler D, Grimm LM & Thomas JA (1986) A comparison of protein S-thiolation (protein mixed-disulfide formation) in heart cells treated with t-butyl hydroperoxide or diamide. *BBA - Molecular Cell Research* **885:** 58–67

Coppola S & Ghibelli L (2000) GSH extrusion and and the mitochondrial pathway of apoptotic signalling. *Biochemical Society transactions* **28:** 56–61

Curran SP, Leuenberger D, Schmidt E & Koehler CM (2002) The role of the Tim8p-Tim13p

complex in a conserved import pathway for mitochondrial polytopic inner membrane proteins. *Journal of Cell Biology* **158:** 1017–1027

Dabir D V., Leverich EP, Kim SK, Tsai FD, Hirasawa M, Knaff DB & Koehler CM (2007) A role for cytochrome c and cytochrome c peroxidase in electron shuttling from Erv1. *EMBO Journal* **26:** 4801–4811

Daithankar VN, Schaefer SA, Dong M, Bahnson BJ & Thorpe C (2010) Structure of the human sulfhydryl oxidase augmenter of liver regeneration and characterization of a human mutation causing an autosomal recessive myopathy. *Biochemistry* **49:** 6737–6745

Dalle-Donne I, Rossi R, Colombo G, Giustarini D & Milzani A (2009) Protein S-glutathionylation: a regulatory device from bacteria to humans. *Trends in Biochemical Sciences* **34:** 85–96

Dekker PJT (1998) Protein Complexes Involved in Import of Mitochondrial Preproteins. **II:**

Dickinson DA & Forman HJ (2002) Glutathione in defense and signaling: Lessons from a small thiol. In *Annals of the New York Academy of Sciences* pp 488–504.

Dimauro S & Schon EA (2003) Mitochondrial Respiratory-Chain Diseases. **:** 2656–2668

De Domenico I, McVey Ward D & Kaplan J (2008) Regulation of iron acquisition and storage: Consequences for iron-linked disorders. *Nature Reviews Molecular Cell Biology* **9:** 72–81

Dröge W (2002) Free Radicals in the Physiological Control of Cell Function. *Physiological Reviews* **82:** 47–95 Available at: http://physrev.physiology.org/lookup/doi/10.1152/physrev.00018.2001

Durigon R, Wang Q, Ceh Pavia E, Grant CM & Lu H (2012) Cytosolic thioredoxin system facilitates the import of mitochondrial small Tim proteins. *EMBO Reports* **13:** 916–922

Dyall SD, Brown MT & Johnson PJ (2004) Ancient Invasions: From Endosymbionts to Organelles. *Science* **304:** 253–257

Eaton DL & Bammler TK (1999) Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicological Sciences* **49:** 156–164

Elston T, Wang H & Oster G (1998) Energy transduction in ATP synthase. *Nature* **391:** 510–513 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9461222

Embley TM & Hirt RP (1998) Early branching eukaryotes? *Current Opinion in Genetics and Development* **8:** 624–629

Erdogan AJ, Ali M, Habich M, Salscheider SL, Schu L, Petrungaro C, Thomas LW, Ashcroft M, Leichert LI, Prates L & Riemer J (2018) Redox Biology The mitochondrial oxidoreductase CHCHD4 is present in a semi-oxidized state in vivo. *Redox Biology* **17:** 200–206 Available at:

https://doi.org/10.1016/j.redox.2018.03.014

Fernandes AP & Holmgren A (2004) Glutaredoxins: Glutathione-Dependent Redox Enzymes with Functions Far Beyond a Simple Thioredoxin Backup System. *Antioxidants & Redox Signaling* **6:** 63–74 Available at: http://www.liebertonline.com/doi/abs/10.1089/152308604771978354

Fernández-Checa JC (2003) Redox regulation and signaling lipids in mitochondrial apoptosis. *Biochemical and Biophysical Research Communications* **304:** 471–479

Fraga H & Ventura S (2013) Oxidative folding in the mitochondrial intermembrane space in human health and disease. *International Journal of Molecular Sciences* **14:** 2916–2927

Frazzon J & Dean DR (2003) Formation of iron-sulfur clusters in bacteria: An emerging field in bioinorganic chemistry. *Current Opinion in Chemical Biology* **7:** 166–173

Frey TG & Mannella CA (2000) Mannella - The Internal Structure of Mitochondria. *Trends Biochem Sci* **0004:** 1–6 Available at: papers2://publication/uuid/07A16125-F493-43E7-8CEE-EF344B83E701

Frey TG, Renken CW & Perkins GA (2002) Insight into mitochondrial structure and function from electron tomography. *Biochimica et Biophysica Acta - Bioenergetics* **1555:** 196–203

Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny M V., Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, et al (2012) Molecular definitions of cell death subroutines: Recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death and Differentiation* **19:** 107–120

Ganz T (2008) Iron Homeostasis: Fitting the Puzzle Pieces Together. *Cell Metabolism* **7:** 288–290

Gilbert HF (1995) Thiol/disulfide exchange equilibria and disulfide bond stability. *Methods in Enzymology* **251:** 8–28

Glick BS, Brandt A, Cunningham K, Müller S, Hallberg RL & Schatz G (1992) Cytochromes c1 and b2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism. *Cell* **69:** 809–822 Available at: http://linkinghub.elsevier.com/retrieve/pii/009286749290292K

Gorla M & Sepuri NB V (2014) Perturbation of apoptosis upon binding of tRNA to the heme domain of cytochrome c. *Apoptosis* **19:** 259–268

Gray MW, Burger G & Lang BF (2001) The origin and early evolution of mitochondria. *Genome biology* **2:** REVIEWS1018 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=138944&tool=pmcentrez&renderty

pe=abstract

Gray MW, Stehling O, Lill R, Gasparre G, Porcelli AM, Narendra D, Walker JE, Youle R, Stojanovski D & Bohnert M (2012) Mitochondrial Evolution. *Cold Spring Harb Perspect Biol.* **4:** a011403

Griffith OW (1999) Biologic and pharmacologic regulation of mammalian glutathione synthesis. In *Free Radical Biology and Medicine* pp 922–935.

Ha HC, Thiagalingam a, Nelkin BD & Casero R a (2000) Reactive oxygen species are critical for the growth and differentiation of medullary thyroid carcinoma cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **6:** 3783–3787 Available at: http://clincancerres.aacrjournals.org/content/6/9/3783.abstract%5Cnhttp://clincancerres.aacrjournals.org/content/6/9/3783.full.pdf

Halliwell B, Aeschbach R, Löliger J & Aruoma OI (1995) The characterization of antioxidants. *Food and Chemical Toxicology*

Halliwell B & Gutteridge JMC (1985) Free radicals in biology and medicine. *Journal of Free Radicals in Biology & Medicine* **1:** 331–332 Available at: http://linkinghub.elsevier.com/retrieve/pii/0748551485901400

Hangen E, Féraud O, Lachkar S, Mou H, Doti N, Fimia GM, Lam N vy, Zhu C, Godin I, Muller K, Chatzi A, Nuebel E, Ciccosanti F, Flamant S, Bénit P, Perfettini JL, Sauvat A, Bennaceur-Griscelli A, Ser-Le Roux K, Gonin P, et al (2015) Interaction between AIF and CHCHD4 Regulates Respiratory Chain Biogenesis. *Molecular Cell* **58:** 1001–1014

Hell K (2008) The Erv1-Mia40 disulfide relay system in the intermembrane space of mitochondria. *Biochim Biophys Acta*

Hentze MW, Muckenthaler MU & Andrews NC (2004) Balancing acts: Molecular control of mammalian iron metabolism. *Cell* **117:** 285–297

Henze K & Martin W (2003) Essence of mitochondria. *Nature* **426:** 127–128

Herrmann JM & Hell K (2005) Chopped, trapped or tacked - Protein translocation into the IMS of mitochondria. *Trends in Biochemical Sciences* **30:** 205–212

Herrmann JM & Neupert W (2000) Protein transport into mitochondria. *Current Opinion in Microbiology* **3:** 210–214

Hofmann S, Rothbauer U, Mühlenbein N, Baiker K, Hell K & Bauer MF (2005) Functional and

mutational characterization of human MIA40 acting during import into the mitochondrial intermembrane space. *Journal of Molecular Biology* **353:** 517–528

Holmgren A (1979) Glutathione-dependent synthesis of deoxyribonucleotides. Characterization of the enzymatic mechanism of Escherichia coli glutaredoxin. *Journal of Biological Chemistry* **254:** 3672–3678

Iwata S, Lee JW, Okada K, Lee JK, Iwata M, Rasmussen B, Link TA, Ramaswamy S & Jap BK (1998) Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1complex. *Science* **281:** 64–71

Jornayvaz FR & Shulman GI (2010) Regulation of mitochondrial biogenesis. *Essays in biochemistry* **47:** 69–84

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T & Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* **471:** 368–373

Kinscherf R, Wagner M, Kamencic H, Bonaterra GA, Hou D, Schiele RA, Deigner HP & Metz J (1999) Characterization of apoptotic macrophages in atheromatous tissue of humans and heritable hyperlipidemic rabbits. *Atherosclerosis* **144:** 33–39

Kispal G, Csere P, Guiard B & Lill R (1997) The ABC transport Atm1p is required for mitochondrial iron homeostasis. *FEBS Letters* **418:** 346–350 Available at: http://dx.doi.org/10.1016/S0014-5793(97)01414-2

Klatt P, Molina EP, De Lacoba MG, Padilla C a, Martinez-Galesteo E, Barcena J a & Lamas S (1999) Redox regulation of c-Jun DNA binding by reversible S-glutathiolation. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **13:** 1481–90 Available at: http://www.ncbi.nlm.nih.gov/pubmed/10463938

Koehler CM (2004) NEW DEVELOPMENTS IN MITOCHONDRIAL ASSEMBLY. *Annual Review of Cell and Developmental Biology* **20:** 309–335 Available at: http://www.annualreviews.org/doi/10.1146/annurev.cellbio.20.010403.105057

Kojer K, Bien M, Gangel H, Morgan B, Dick TP & Riemer J (2012a) Glutathione redox potential in the mitochondrial intermembrane space is linked to the cytosol and impacts the Mia40 redox state. *file:///C:/Users/RAMS/Desktop/RAMANA THESIS/A novel CHCHD10 mutation implicates a Mia40.pdf* **31:** 3169–3182 Available at: http://dx.doi.org/10.1038/emboj.2012.165

Kojer K, Bien M, Gangel H, Morgan B, Dick TP & Riemer J (2012b) Glutathione redox potential in

the mitochondrial intermembrane space is linked to the cytosol and impacts the Mia40 redox state. *EMBO Journal* **31:** 3169–3182 Available at: http://dx.doi.org/10.1038/emboj.2012.165

Kosower NS & Kosower EM (1978) The Glutathione Status of Cells1. *International Review of Cytology* **54:** 109–160

Künkele K, Heins S, Dembowski M, Nargang FE, Benz R, Thieffry M, Walz J, Lill R, Nussberger S & Neupert W (1998) of the Outer Membrane of Mitochondria. *Cell* **93:** 1009–1019

Kushnareva Y, Murphy AN & Andreyev A (2002) Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. *The Biochemical journal* **368:** 545–553

Lane DJR, Merlot AM, Huang MLH, Bae DH, Jansson PJ, Sahni S, Kalinowski DS & Richardson DR (2015) Cellular iron uptake, trafficking and metabolism: Key molecules and mechanisms and their roles in disease. *Biochimica et Biophysica Acta - Molecular Cell Research* **1853:** 1130–1144 Available at: http://dx.doi.org/10.1016/j.bbamcr.2015.01.021

Lang BF, Gray MW & Burger G (1999) MITOCHONDRIAL GENOME EVOLUTION AND THEORIGIN OF EUKARYOTES. *Annu. Rev. Genet.* **33:** 351–97

Levitan A, Danon A & Lisowsky T (2004) Unique Features of Plant Mitochondrial Sulfhydryl Oxidase. *Journal of Biological Chemistry* **279:** 20002–20008

Lill R & Mühlenhoff U (2006) Iron-Sulfur Protein Biogenesis in Eukaryotes: Components and Mechanisms. *Annual Review of Cell and Developmental Biology* **22:** 457–486 Available at: http://www.annualreviews.org/doi/10.1146/annurev.cellbio.22.010305.104538

Lind C, Gerdes R, Schuppe-Koistinen I & Cotgreave I a (1998) Studies on the mechanism of oxidative modification of human glyceraldehyde-3-phosphate dehydrogenase by glutathione: catalysis by glutaredoxin. *Biochemical and biophysical research communications* **247:** 481–486

Lu H, Allen S, Wardleworth L, Savory P & Tokatlidis K (2004) Functional TIM10 Chaperone Assembly Is Redox-regulated in Vivo. *Journal of Biological Chemistry* **279:** 18952–18958

Lu H & Woodburn J (2005) Zinc binding stabilizes mitochondrial Tim10 in a reduced and import-competent state kinetically. *Journal of Molecular Biology* **353:** 897–910

Lutz T, Neupert W & Herrmann JM (2003) Import of small Tim proteins into the mitochondrial intermembrane space. *EMBO Journal* **22:** 4400–4408

Mannervik B & Axelsson K (1975) Reduction of Disulphide Bonds in Proteins and Protein Mixed Disulphides Catalysed by a Thioltransferase in Rat Liver Cytosol. *Biochem. J.* **149:** 785–788

Mannervik B & Axelsson K (1980) Role of cytoplasmic thioltransferase in cellular regulation by thiol-disulphide interchange. *Biochemical Journal* **190:** 125–130 Available at: http://www.ncbi.nlm.nih.gov/pubmed/7447929%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1162070

Martin W & Müller M (1998) The hydrogen hypothesis for the first eukaryote. *Nature* **392:** 37–41

Mates JM (2000) EFFECTS OF ANTIOXIDANT ENZYMES IN THE MOLECULAR CONTROL OF REACTIVE OXYGEN SPECIES TOXICOLOGY. *Biogenic Amines (VSP International Science Publishers)* **16:** 53 Available at: http://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=4944115&site=ehost-live&scope=site

Mesecke N, Bihlmaier K, Grumbt B, Longen S, Terziyska N, Hell K & Herrmann JM (2008) The zinc-binding protein Hot13 promotes oxidation of the mitochondrial import receptor Mia40. *EMBO Reports* **9:** 1107–1113

Mesecke N, Terziyska N, Kozany C, Baumann F, Neupert W, Hell K & Herrmann JM (2005) A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. *Cell* **121:** 1059–1069

Mitchell HK, Petersen NS & Buzin CH (1985) Self-degradation of heat shock proteins. *Proceedings of the National Academy of Sciences of the United States of America* **82:** 4969–73 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=390479&tool=pmcentrez&rendertype=abstract

Müller JM, Milenkovic D, Guiard B, Pfanner N & Chacinska A (2008) Precursor oxidation by Mia40 and Erv1 promotes vectorial transport of proteins into the mitochondrial intermembrane space. *Molecular biology of the cell* **19:** 226–36 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17978092%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2174169

Murari A, Thiriveedi VR, Mohammad F, Vengaldas V, Gorla M, Tammineni P, Krishnamoorthy T & Sepuri NB V. (2015) Human mitochondrial MIA40 (CHCHD4) is a component of the Fe-S cluster export machinery. *Biochemical Journal* **471:** 231–241 Available at: http://biochemj.org/cgi/doi/10.1042/BJ20150012

Neal SE, Dabir D V, Wijaya J, Boon C & Koehler CM (2017) Osm1 facilitates the transfer of electrons from Erv1 to fumarate in the redox-regulated import pathway in the mitochondrial

intermembrane space. *Molecular biology of the cell* **3:** mbc.E16-10-0712 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28814504

Netz DJA, Stith CM, Stümpfig M, Köpf G, Vogel D, Heide M, Stodola JL, Lill R, Burgers PMJ, Pierik AJ & Marburg P (2012) Eukaryotic DNA polymerase require an iron-sulfur cluster for the formation of active complexes. *Nature Chemical Biology* **8:** 125–132

Newmeyer DD & Ferguson-Miller S (2003) Mitochondria: Releasing power for life and unleashing the machineries of death. *Cell* **112:** 481–490

Noji H & Yoshida M (2001) The rotary machine in the cell, ATP synthase. *Journal of Biological Chemistry* **276:** 1665–1668

Nordstrand K, Åslund F, Holmgren A, Otting G & Berndt KD (1999) NMR structure of Escherichia coli glutaredoxin 3-glutathione mixed disulfide complex: Implications for the enzymatic mechanism. *Journal of Molecular Biology* **286:** 541–552

Pajares M, Jim??nez-Moreno N, Dias IHK, Debelec B, Vucetic M, Fladmark KE, Basaga H, Ribaric S, Milisav I & Cuadrado A (2015) Redox control of protein degradation. *Redox Biology* **6:** 409–420 Available at: http://dx.doi.org/10.1016/j.redox.2015.07.003

Pineda-Molina E, Klatt P, Vázquez J, Marina A, García de Lacoba M, Pérez-Sala D & Lamas S (2001) Glutathionylation of the p50 subunit of NF-κB: A mechanism for redox-induced inhibition of DNA binding. *Biochemistry* **40:** 14134–14142

Prinz WA, Åslund F, Holmgren A & Beckwith J (1997) The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the Escherichia coli cytoplasm. *Journal of Biological Chemistry* **272:** 15661–15667

Puigserver P, Wu Z, Park CW, Graves R, Wright M & Spiegelman BM (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92:** 829–839

Ravichandran V, Seres T, Moriguchi T, Thomas JA & Johnston RB (1994) S-thiolation of glyceraldehyde-3-phosphate dehydrogenase induced by the phagocytosis-associated respiratory burst in blood monocytes. *Journal of Biological Chemistry* **269:** 25010–25015

Regula KM, Ens K & Kirshenbaum LA (2003) Mitochondria-assisted cell suicide: A license to kill. *Journal of Molecular and Cellular Cardiology* **35:** 559–567

Rouault TA (2006) The role of iron regulatory proteins in mammalian iron homeostasis and disease. *Nature Chemical Biology* **2:** 406–414

Rouault TA (2012a) Biogenesis of iron-sulfur clusters in mammalian cells: new insights and

relevance to human disease. *Disease models & mechanisms* **5:** 155–64 Available at: /pmc/articles/PMC3291637/?report=abstract

Rouault TA (2012b) Biogenesis of iron-sulfur clusters in mammalian cells: new insights and relevance to human disease. *Disease Models & Mechanisms* **5:** 155–164 Available at: http://dmm.biologists.org/cgi/doi/10.1242/dmm.009019

Rozell B, Barcena JA, Martinez-Galisteo E, Padilla CA & Holmgren A (1993) Immunochemical characterization and tissue distribution of glutaredoxin (thioltransferase) from calf. *Eur J Cell Biol* **62:** 314–323

Schuppe-Koistinen I, Moldéus P, Bergman T & Cotgreave I a (1994) S-thiolation of human endothelial cell glyceraldehyde-3-phosphate dehydrogenase after hydrogen peroxide treatment. *European journal of biochemistry / FEBS* **221:** 1033–7 Available at: http://www.ncbi.nlm.nih.gov/pubmed/8181459

Sds-page L & Sds T (1970) Laemmli buffer Background Purpose of the Laemmli buffer Laemmli Buffer Recipe Preparation Recommended Storage Temperature of Laemmli buffer Laemmli buffer References. *Nature*

Seth RB, Sun L, Ea C-K & Chen ZJ (2005) Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122:** 669–682

Shelton MD & Mieyal JJ (2008) Regulation by reversible S-glutathionylation: molecular targets implicated in inflammatory diseases. *Molecules and cells* **25:** 332–346

Sideris DP & Tokatlidis K (2010) Protein Secretion. **619:** 411–423 Available at: http://link.springer.com/10.1007/978-1-60327-412-8

Smith C V., Jones DP, Guenthner TM, Lash LH & Lauterburg BH (1996) Compartmentation of glutathione: Implications for the study of toxicity and disease. *Toxicology and Applied Pharmacology* **140:** 1–12

Spiller MP, Ang SK, Ceh-Pavia E, Fisher K, Wang Q, Rigby SE & Lu H (2013a) Identification and characterisation of mitochondrial Mia40 as an iron-sulphur protein. *The Biochemical journal* Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=23834247&retmode=ref&cmd=prlinks

Spiller MP, Ang SK, Ceh-Pavia E, Fisher K, Wang Q, Rigby SEJ & Lu H (2013b) Identification and

characterization of mitochondrial Mia40 as an iron-sulfur protein. *The Biochemical journal* **455:** 27–35 Available at: http://www.biochemj.org/bj/455/0027/bj4550027.htm

Spinazzi M, Casarin A, Pertegato V, Salviati L & Angelini C (2012) Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. *Nature Protocols* **7:** 1235–1246

Starke DW, Chock PB & Mieyal JJ (2003) Glutathione-thiyl radical scavenging and transferase properties of human glutaredoxin (thioltransferase): Potential role in redox signal transduction. *Journal of Biological Chemistry* **278:** 14607–14613

Steiner H, Zollner A, Haid A, Neupert W & Lill R (1995) Biogenesis of mitochondrial heme lyases in yeast. Import and folding in the intermembrane space. *Journal of Biological Chemistry* **270:** 22842–22849

Stephens PJ, Jollie DR & Warshel a. (1996) Protein Control of Redox Potentials of Iron−Sulfur Proteins. *Chemical Reviews* **96:** 2491–2514 Available at: http://pubs.acs.org/doi/abs/10.1021/cr950045w

Stojanovski D, Bragoszewski P & Chacinska A (2012) The MIA pathway: A tight bond between protein transport and oxidative folding in mitochondria. *Biochimica et Biophysica Acta - Molecular Cell Research* **1823:** 1142–1150

Tangerås A, Flatmark T, Bäckström D & Ehrenberg A (1980) Mitochondrial iron not bound in heme and iron-sulfur centers. Estimation, compartmentation and redox state. *BBA - Bioenergetics* **589:** 162–175

Terziyska N, Grumbt B, Bien M, Neupert W, Herrmann JM & Hell K (2007) The sulfhydryl oxidase Erv1 is a substrate of the Mia40-dependent protein translocation pathway. *FEBS Letters* **581:** 1098–1102

Thomas JA, Poland B & Honzatko R (1995) Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. *Archives of Biochemistry and Biophysics* **319:** 1–9

Turrens JF (2003) Mitochondrial formation of reactive oxygen species. *The Journal of Physiology* **552:** 335–344 Available at: http://doi.wiley.com/10.1111/j.1469-7793.2003.00335.x

Upton JW, Kaiser WJ & Mocarski ES (2010) Virus inhibition of RIP3-dependent necrosis. *Cell Host and Microbe* **7:** 302–313

Vandenabeele P, Galluzzi L, Vanden Berghe T & Kroemer G (2010) Molecular mechanisms of necroptosis: An ordered cellular explosion. *Nature Reviews Molecular Cell Biology* **11:** 700–

714

Virbasius J V & Scarpulla RC (1994) Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **91:** 1309–1313 Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=8108407&retmode=ref&cmd=prlinks%5Cnpapers2://publication/uuid/4B80EAB7-68E7-4F26-9919-4CCBCB8BD1A1

Voronova A, Meyer-Klaucke W, Meyer T, Rompel A, Krebs B, Kazantseva J, Sillard R & Palumaa P (2007) Oxidative switches in functioning of mammalian copper chaperone Cox17. *The Biochemical journal* **408:** 139–148

Wallander ML, Leibold EA & Eisenstein RS (2006) Molecular control of vertebrate iron homeostasis by iron regulatory proteins. *Biochimica et biophysica acta* **1763:** 668–89 Available at: http://www.sciencedirect.com/science/article/pii/S016748890600111X

Wu G, Fang Y-Z, Yang S, Lupton JR & Turner ND (2004) Glutathione Metabolism and Its Implications for Health. *The Journal of Nutrition* **134:** 489–492 Available at: https://academic.oup.com/jn/article/134/3/489-492/4688681

Yang D, Oyaizu Y, Oyaizu H, Olsen GJ & Woese CR (1985) Mitochondrial origins. *Proc Natl Acad Sci U S A* **82:** 4443–4447 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=391117&tool=pmcentrez&rendertype=abstract

Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, Shulman GIG, Wrutniak-Cabello C, Casas F, Cabello G, Ventura-Clapier R, Garnier A, Veksler V, Valsecchi F, Ramos-Espiritu LS, Buck J, Levin LR, Manfredi G, Schreiber SN, Emter R, et al (2014) Regulation of mitochondrial biogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **23:** 459–466 Available at: http://dx.doi.org/10.1038/nature06322%5Cnhttp://www.nature.com/nature/journal/v450/n7170/suppinfo/nature06322_S1.html%5Cnhttp://dx.doi.org/10.1016/j.mito.2011.03.121%5Cnhttp://www.hmg.oupjournals.org/cgi/doi/10.1093/hmg/ddh109%5Cnhttp://www.ncbi.nlm.nih.g

Role of hMIA40 (CHCHD4) in Mitochondrial electron transport chain biogensis,protein import and celluar iron homeostasis

by Venkat Ramana Thiriveedi

Role of hMIA40 (CHCHD4) in Mitochondrial electron transport chain biogensis,protein import and celluar iron homeostasis

ORIGINALITY REPORT

13%	3%	7%	7%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to University of Hyderabad, Hyderabad Student Paper	5%
2	"Encyclopedia of Signaling Molecules", Springer Nature, 2018 Publication	<1%
3	essays.biochemistry.org Internet Source	<1%
4	Salvatore DiMauro. "Mitochondrial Respiratory-Chain Diseases", New England Journal of Medicine, 06/26/2003 Publication	<1%
5	Lane, D.J.R., A.M. Merlot, M.L.-H. Huang, D.-H. Bae, P.J. Jansson, S. Sahni, D.S. Kalinowski, and D.R. Richardson. "Cellular iron uptake, trafficking and metabolism: Key molecules and mechanisms and their roles in disease", Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2015. Publication	<1%

PUBLICATIONS

1. Anjaneyulu Murari, **Venkata Ramana Thiriveedi**, Fareed Mohammad, Viswamithra Vengaldas, Madhavi Gorla, Prasad Tammineni, Thanuja Krishnamoorthy and Naresh Babu V.Sepuri* *Human Mitochondrial MIA40 (CHCD4) is a component of Fe/S cluster export machinery*. Biochem J. 2015 Oct 15;471(2):231-41. doi: 10.1042/BJ20150012. Epub 2015 Aug 14. PMID: 26275620.

2. Adinarayana Marada, Praveen kumar Allu, Anjaneyulu Murari, **Venkata Ramana Thiriveedi**, Jayasree Danduprolu and Naresh Babu V.Sepuri* *Mge1, a nucleotide exchange factor of Hsp70, acts as an oxidative sensor to regulate mitochondrial Hsp70 function*. Mol Biol Cell. 2013 Mar;24(6):692-703. doi: 10.1091/mbc.E12-10-0719. Epub 2013 Jan 23. PMID: 23345595.

3. **Venkata Ramana Thiriveedi**, Ushodaya.Mattam and Naresh Babu.V.Sepuri* Glutathionylated hMIA40 (CHCHD4) is involved in regulation of mitochondrial electron transport chain activities. (Submitted to Cell Reports)

4. Ushodaya Mattam, Noble Kumar Talari, **Venkata Ramana Thiriveedi**, Fareed Mohammed, Sathya Velmurugan, Kalyankar Mahadev, Naresh babu Venkata Sepuri* Aging declines kisspeptin receptor (GPR54) levels in hypothalamus and extra-hypothalamic brain regions of male Wistar rats" (Submitted to Biogerontology; BGEN-D-18-00051).

5. **Venkata Ramana Thiriveedi**, Ushodaya.Mattam and Naresh Babu.V.Sepuri* Glutathionylated human mitochondrial MIA40 (CHCHD4) is critical for *Fe/S* cluster export from mitochondria. (Manuscript under preparation)

Human mitochondrial MIA40 (CHCHD4) is a component of the Fe–S cluster export machinery

Anjaneyulu Murari*, Venkata Ramana Thiriveedi*, Fareed Mohammad*, Viswamithra Vengaldas*, Madhavi Gorla*, Prasad Tammineni*, Thanuja Krishnamoorthy* and Naresh Babu V. Sepuri*[1]

*Department of Biochemistry, University of Hyderabad, Gachibowli, Hyderabad 500046, India

Mitochondria play an essential role in synthesis and export of iron–sulfur (Fe–S) clusters to other sections of a cell. Although the mechanism of Fe–S cluster synthesis is well elucidated, information on the identity of the proteins involved in the export pathway is limited. The present study identifies hMIA40 (human mitochondrial intermembrane space import and assembly protein 40), also known as CHCHD4 (coiled-coil–helix–coiled-coil–helix domain-containing 4), as a component of the mitochondrial Fe–S cluster export machinery. hMIA40 is an iron-binding protein with the ability to bind iron *in vivo* and *in vitro*. hMIA40 harbours CPC (Cys-Pro-Cys) motif-dependent Fe–S clusters that are sensitive to oxidation. Depletion of hMIA40 results in accumulation of iron in mitochondria concomitant with decreases in the activity and stability of Fe–S-containing cytosolic enzymes. Intriguingly, overexpression of either the mitochondrial export component or cytosolic the Fe–S cluster assembly component does not have any effect on the phenotype of hMIA40-depleted cells. Taken together, our results demonstrate an indispensable role for hMIA40 for the export of Fe–S clusters from mitochondria.

Key words: CIA, GPAT, hMIA40, iron export, iron–sulfur (Fe–S) cluster, mitochondria.

INTRODUCTION

Fe–S (iron–sulfur) clusters are essential inorganic structures required by all organisms across evolution from bacteria to humans to perform various cellular functions [1]. Several enzymes that are involved in a variety of biological processes such as electron transfer, redox catalysis, DNA replication and repair, and regulation of gene expression contain Fe–S clusters as prosthetic groups [2–6]. The synthesis, maturation and transfer of Fe–S clusters to apoproteins are very complex processes requiring multiple protein components present in mitochondria and cytosol.

Several studies in yeast suggest the existence of three different assembly systems for the biogenesis of Fe–S clusters. These are the ISC (iron–sulfur cluster) assembly system, the ISE (iron–sulfur exporter) system and the CIA (cytosolic iron–sulfur assembly) system [7–10]. The first two systems are present in the mitochondria, whereas the last-named system is present in the cytosol. Homologues of yeast proteins that have been implicated in the biogenesis of Fe–S clusters exist in higher eukaryotes as well. The Fe–S cluster systems in mammals are functionally equivalent to the yeast systems despite some of the components of ISC system being found in cellular organelles other than the mitochondria [11].

The ISC system is required for the maturation and functioning of mitochondrial enzymes that contain Fe–S clusters [12]. The components required for the ISC system are present in the mitochondrial matrix. Besides the assembly and insertion of Fe–S clusters, the ISC system of mitochondria is also essential for the maturation of cytosolic and nuclear proteins that contain Fe–S clusters [13,14]. This process probably uses the Fe–S clusters that are exported through the ISE machinery that is present in the mitochondria. Atm1, an inner membrane protein of yeast mitochondria, and its mammalian homologue ABCB7 (ATP-binding cassette transporter B7), and Erv1, an intermembrane space thiol oxidase, and its mammalian homologue ALR have been identified as components of the ISE machinery of mitochondria [13,15,16]. The phenotype that is associated with deficiency of Atm1 or Erv1 includes accumulation of iron in mitochondria and defects in maturation of cytosolic proteins that contain Fe–S clusters. Although there are reports suggesting that it may be exporting sulfur to the cytosol, the exact component that is exported by Atm1 or Erv1 and the export mechanism are not known [17,18]. Interestingly, Erv1 is also a member of the mitochondrial protein import machinery. Erv1 is specially required for the import of intermembrane space proteins of mitochondria. Erv1, along with Mia40, an intermembrane space protein of mitochondria, functions in the import of numerous cysteine-rich intermembrane space proteins by an oxidative folding mechanism [19,20]. Mia40 harbours six conserved cysteine residues that are clustered in the form of one CPC (Cys-Pro-Cys) and two CX_9C (Cys-Xaa_9-Cys) motifs. Mia40, through its CPC motif, promotes the oxidative folding of precursor proteins. In contrast, the CX_9C motifs are involved in creating intramolecular disulfide bonds for stabilizing the structure of Mia40 [21,22]. hMIA40 (human mitochondrial intermembrane space import and assembly protein 40), also known as CHCHD4 (coiled-coil–helix–coiled-coil–helix domain-containing 4), a homologue of yeast Mia40 contains similar cysteine motifs and is also involved in the import of intermembrane space-targeted proteins [23,24].

Abbreviations: ABCB7, ATP-binding cassette transporter B7; CIA, cytosolic iron–sulfur assembly; DCPIP, 2,6-dichlorophenol-indophenol; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPAT, glutamine phosphoribosylpyrophosphate amidotransferase; HEK, human embryonic kidney; hMIA40, human mitochondrial intermembrane space import and assembly protein 40; ISC system, iron–sulfur cluster system; ISE system, iron–sulfur exporter system; mtHsp70, mitochondrial heat-shock protein 70; Ni-NTA, Ni^{2+}-nitrilotriacetate; NUBP1, nucleotide-binding protein 1.

[1] To whom correspondence should be addressed (email nareshuohyd@gmail.com or nbvssl@uohyd.ernet.in).

Mge1, a nucleotide exchange factor of Hsp70, acts as an oxidative sensor to regulate mitochondrial Hsp70 function

Adinarayana Marada, Praveen Kumar Allu, Anjaneyulu Murari, BhoomiReddy PullaReddy, Prasad Tammineni, Venkata Ramana Thiriveedi, Jayasree Danduprolu, and Naresh Babu V. Sepuri
Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India

ABSTRACT **Despite the growing evidence of the role of oxidative stress in disease, its molecular mechanism of action remains poorly understood. The yeast *Saccharomyces cerevisiae* provides a valuable model system in which to elucidate the effects of oxidative stress on mitochondria in higher eukaryotes. Dimeric yeast Mge1, the cochaperone of heat shock protein 70 (Hsp70), is essential for exchanging ATP for ADP on Hsp70 and thus for recycling of Hsp70 for mitochondrial protein import and folding. Here we show an oxidative stress–dependent decrease in Mge1 dimer formation accompanied by a concomitant decrease in Mge1–Hsp70 complex formation in vitro. The Mge1-M155L substitution mutant stabilizes both Mge1 dimer and Mge1–Hsp70 complex formation. Most important, the Mge1-M155L mutant rescues the slow-growth phenomenon associated with the wild-type Mge1 strain in the presence of H_2O_2 in vivo, stimulation of the ATPase activity of Hsp70, and the protein import defect during oxidative stress in vitro. Furthermore, cross-linking studies reveal that Mge1–Hsp70 complex formation in mitochondria isolated from wild-type Mge1 cells is more susceptible to reactive oxygen species compared with mitochondria from Mge1-M155L cells. This novel oxidative sensor capability of yeast Mge1 might represent an evolutionarily conserved function, given that human recombinant dimeric Mge1 is also sensitive to H_2O_2.**

Monitoring Editor
Ramanujan S. Hegde
National Institutes of Health

Received: Oct 5, 2012
Revised: Jan 8, 2013
Accepted: Jan 14, 2013

INTRODUCTION

Mitochondria are essential organelles involved in many cellular processes, such as energy metabolism and apoptosis. Although the mitochondrion has its own genome, it depends on the nucleus for optimal functioning (Chacinska *et al.*, 2009). Based on their signal sequence, mitochondrial proteins encoded by nuclear DNA are targeted to different subcompartments of mitochondria through a translocase system present on outer and inner mitochondrial membranes known as the translocase of outer membrane (TOM) and translocase of inner membrane (TIM) complexes, respectively (Schulke *et al.*, 1997, 1999; Endo *et al.*, 2003; Kutik *et al.*, 2007; Neupert and Herrmann, 2007). Targeting of precursor protein to the matrix involves an interplay among many proteins; however, the final step of this process is mediated by Tim44 and a translocation motor that contains mitochondrial heat shock protein 70 (mHsp70), Pam16, Pam18, and the nucleotide exchange factor Mge1 (Azem *et al.*, 1997; Mokranjac *et al.*, 2007; Stojanovski *et al.*, 2007; Schiller *et al.*, 2008). Hsp70, in combination with Tim44, binds to the emerging end of the transit peptide from the TIM channel in an ATP-dependent manner, and the ATPase cycle of mHsp70 leads to pulling or vectorial translocation of preproteins across the inner mitochondrial membrane (Matouschek *et al.*, 2000; Okamoto *et al.*, 2002; Liu *et al.*, 2003). Mge1, a component of this translocation motor, accelerates the exchange of ATP for ADP on mHsp70 and promotes a change from the high–substrate affinity conformation of mHsp70 to a lower–substrate affinity form with a concomitant release of precursor protein from mHsp70 to begin the next round of translocation

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E12-10-0719) on January 23, 2013.

Address correspondence to: Naresh Babu V. Sepuri (nareshuohyd@gmail.com or nbvssl@uohyd.ernet.in).

Abbreviations used: Ccpo, cytochrome *c* peroxidase; DHFR, dihydrofolate reductase; HSP 70, heat shock protein 70; ROS, reactive oxygen species; Tim, translocase of inner membrane; Tom, translocase of outer membrane.