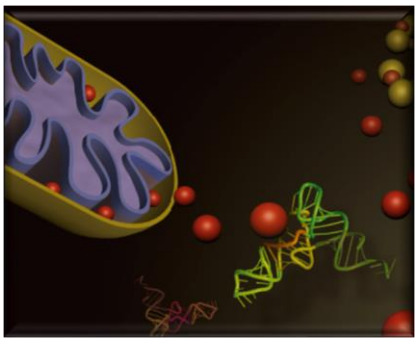
Identification and characterization of novel guanine nucleotide regulatory factors in mammalian mitochondrial tRNA import



Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that this thesis entitled **"Identification and Characterization of Novel Guanine Nucleotide Regulatory Factors in Mammalian Mitochondrial tRNA Import"** submitted to the University of Hyderabad by Ms. Madhavi Gorla, 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.

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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 **Dr. 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.

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…… Madhavi Gorla

Table of contents

Abbreviations

ABRA	Actin Binding Rho GTPase Activating protein
ADP	Adenosine diphosphate
$AgNO_3$	Silver Nitrate
ATP	Adenosine triphosphate
ATPase	ATP synthase
BAX	Bcl-2-associated X protein
BCL2	B-cell lymphoma 2
BIP	Binding Immunoglobin Protein
BSA	Bovine Serum Albumin
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
COX	Cytochrome C Oxidase
cpm	counts per minute
CREB	Cyclic AMP Response Element Binding protein
CypD	Cyclophilin D
DAPI	4' 6-diamidino-2phenylindole
DEPC	Diethylpyrocarbonate
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothritol

EDTA	Ethylene diamine tetraacetic acid
EGTA	Ehtylene glycol tetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
EtBr	Ethidium Bromide
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
Fe-S	Iron Sulfur cluster
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GB	Guanine nucleotide Binding protein
GMPPNP	5'-Guanylyl imidodiphosphate
GTP	Guanosinetriphoshate
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
HSP70	Heat Shock Protein 70
KCl	Potassium chloride
kDa	Kilodalton
KOH	Potassium hydroxide
MALDI-TOF	Matrix-assisted laser desorption/ionization- time-of-flight
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes

MERRF	Myoclonic Epilepsy with Ragged Red Fibers
Mg (**OAC2**)	Magnesium acetate
$MgCl_2$	Magnesium Chloride
ml	milliliter
mm	millimeter
mmole	millimole
MP	Mitoplast
mRNA	Messenger RNA
MS/MS	Mass spectrometers
MtDNA	Mitochondrial DNA
$Na_2S_2O_3$	Sodium thiosulphate
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide
NC	Nitrocelluolose
ND	NADH-ubiquinone oxidoreductase
Ni-NTA	Nickel Nitrilotriacetic acid
ORF	Open reading frame
OXPHOS	Oxidative phosphorylation system
PAGE	Ployacrylamide gel electrophoresis
PARP	Poly ADP ribose polymerase
PBS	Phosphate Buffered Saline

PMSF	Phenyl methyl sulfonyl fluoride
RhoGEF	Rho Guanine nucleotide Exchange Factor
RIPA	Radio immunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
rRNA	Ribosomal RNA
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium dodecyl sulphate
SU9-DHFR	Subunit 9 – Dihydrofolatereductase
TCA	Tricarboxylic acid
TIM	Translocase of Inner mitochondrial membrane
TOM	Translocase of outer mitochondrial membrane
TRIS	tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
µCi	microcurie
µg	microgram
µl	microliter
µM	micromolar

Chapter 1

INTRODUCTION

1.1 Mitochondria: Structure

Mitochondria are essential organelles present in the cytoplasm of nearly all eukaryotic cells [1]. Most of the cellular energy in the form of ATP is being generated by mitochondria, earning it the nick name "the powerhouse of the cell". Although mitochondria are present in every cell, their number varies depending on the energy requirement of a cell. Further, the distribution of mitochondria in a cell also depends on the energy requirement. For example, in mammalian sperm or flagellated protozoa, mitochondria are abundant around the base of the flagella. In cardiac muscle, mitochondria are abundant near the contractile elements [1].

Mitochondria are membrane bound organelles present in plant and animal cells, fungi and many other eukaryotes [1]. Mitochondria measure about 0.5-1.0 μm in diameter and structures are enclosed in double membranes-the outer membrane and the inner membrane. The membranes are made up of phospholipids and proteins. Because of this double-membrane structural organization, mitochondria are sub divided into four different sub-compartments: Outer membrane, Intermembrane space, Inner membrane and Matrix. These four sub compartments of mitochondria differ in their structure and function.

The outer mitochondrial membrane is a simple phospholipid bilayer. The outer mitochondrial membrane is smooth unlike the inner mitochondrial membrane and it contains large number of integral channel forming proteins called porins. Porins present in the outer mitochondrial membrane form channels that are permeable to the molecules of 5000 Da or less. The outer membrane of mitochondria is absolutely permeable to nutrients, ions, ATP and ADP molecules. The high molecular weight proteins can only translocated to mitochondria through the multi protein subunit TOM (Translocase of Outer Membrane) complex [2].

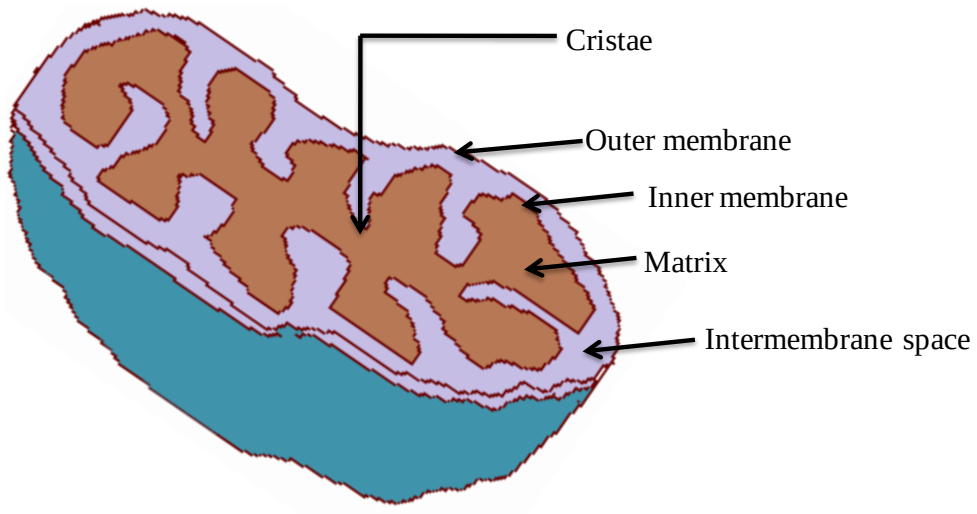


Fig.1.1 Structure of Mitochondria: Mitochondria are surrounded by a double membrane. The outer mitochondrial membrane encloses the entire organelle. The folds of inner mitochondrial membrane are called cristae where majority of ATP is produced. The inner membrane encloses the fluid filled compartment called mitochondrial matrix.

The intermembrane space is a small sub compartment that separates the outer and inner membranes of mitochondria and it has the same composition of metabolites like cytoplasm. However, the protein content of intermembrane space is different from that of cytoplasm. The intermembrane space plays an important role in the primary function of mitochondria, related to oxidative phosphorylation [3].

The structure of the inner membrane is highly complex compared to the outer membrane as it contains the electron transport chain complexes, ATP synthase complex and other transporters. The inner membrane of mitochondria is impermeable to most of the molecules due to the presence of unusual phospholipid known as cardiolipin. The numerous infoldings of the inner membrane are called cristae. Cristae increase the surface area of the inner membrane to accommodate the complexes and proteins that are involved in the generation of ATP.

The mitochondrial matrix is enclosed by the inner membrane and contains complex mixture of enzymes that are necessary for the biogenesis of ribosomes, tRNAs and replication and maintenance

of mitochondrial DNA. The mitochondrial matrix enzymes are also majorly involved in oxidation of pyruvate and fatty acids, aminoacid metabolism, citric acid cycle, urea cycle, heme and Fe/S cluster biogenesis.

1.2 Functions of mitochondria

1.2.1 Mitochondria: The dynamo of the cell

Mitochondria play a critical role to support all the cellular functions as they carry out energy yielding oxidative reactions that generate vast majority of ATP. Mitochondria have efficient energy generating system called the oxidative phosphorylation which consumes NADH and oxygen to generate ATP [4].

1.2.2 Mitochondria: The calcium storage sink

Mitochondria are remarkably important as storage tanks for calcium ions. It is very important to maintain the proper concentrations of calcium ions within the various compartments of the cell. In a cell, mitochondria achieve this goal by acting as storage sinks for calcium ions [4].

1.2.3 Mitochondria: The death squad

Apart from energy production, mitochondria are also considered as fundamental integration sites for biological signals that promote cell death or cell survival. Since mitochondria contain numerous proteins involved in the apoptotic signaling cascade, it is now believed that mitochondria play a key decision making role whether a cell to live or die following a death signal-factually a "license to kill" [5]. In response to apoptotic stimuli, one of the mitochondrial intermembrane space protein, Cytochrome c releases into the cytosol to initiate the cascade of events that leads to the activation of caspase-3 and caspase-7 that eventually degrades many cellular proteins and cause cell death [5-6].

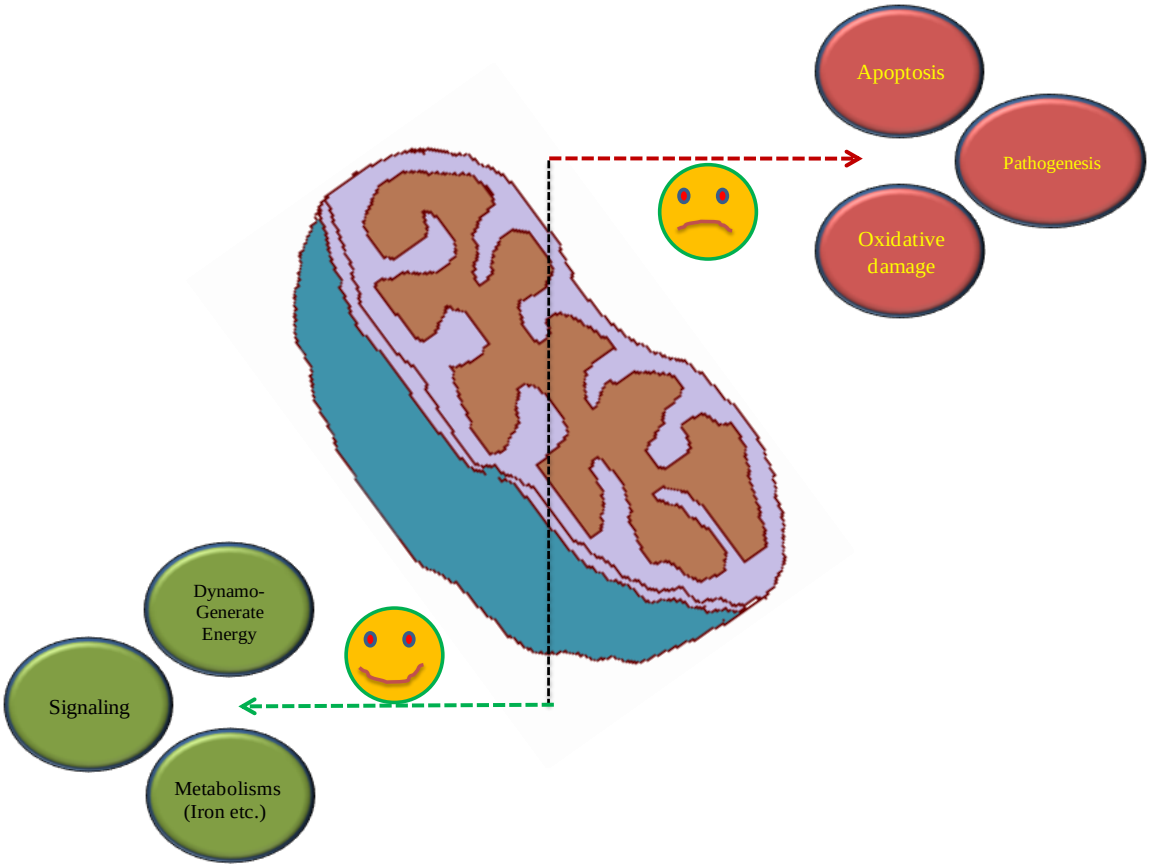


Fig.1.2 Functions of Mitochondria: Mitochondria are fundamental organelles involved in diverse functions which include energy production, various oxidative metabolisms, apoptosis, pathogenesis as well as integration of most of the signaling pathways in response to external cues.

In addition to apoptosis, mitochondria are also involved in other forms of programmed cell death [7]. One such example is necroptosis (necrosis-like cell death pathway) which is mediated by RIPK-3 (Receptor Interacting serine/threonine Protein Kinase-3) [8]. RIPK-3 dependant necrosis plays an important role in embryonic development and host anti-viral immunity [9-11].

1.2.4 Mitochondria: The cue of signaling

It is becoming increasingly apparent that mitochondria are intimately implicated in cell signaling pathways. Mitochondria serve as platforms to initiate cell signaling in multiple processes [12]. For examples, outer mitochondrial membrane serves as major signaling platform for anti-viral innate

immunity [13] and regulated generation of mitochondrial reactive oxygen species (ROS) serves as signaling platform for anti-microbial activity during phagocytosis [14]. Defects in mitochondrial regulation of cell signaling may lead to many diseases like age related pathologies [12].

1.3 Evolution of mitochondria

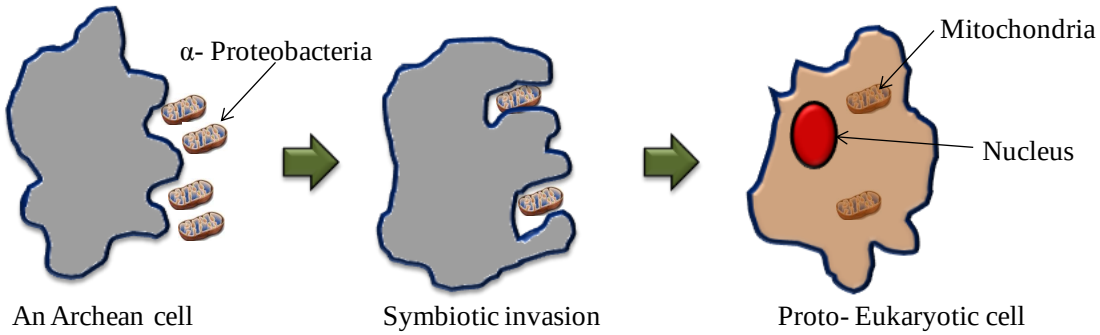
All complex life composed of eukaryotic (nucleated) cells. Eukaryotic cells are generally larger and highly structured than prokaryotic cells, with much bigger genomes and proteomes [15]. Acquisition of mitochondria and chloroplasts is one of the important events in the evolution of eukaryotic cells as they represent energy generating and biosynthetic factories of the cell [16].

1.3.1 Endosymbiotic theory

The endosymbiotic theory suggests that the mitochondria are originated from α- proteobacteria [17-19]. Phylogenetic analysis of small ribosomal RNAs demonstrates the monophylogenetc origin of mitochondria from α-proteobacterium [20]. Two scenarios were proposed to explain the mitochondrial origin, the Symbiosis scenario and the Archezoan scenario [21]. According to Symbiosis scenario, endosymboint was taken up by an archeal cell [22] where as Archezoan theory suggests that host of the endosymboint was essentially compartmentalized as amitochondriate of eukaryotic cell [23].

However, growing evidence supports the Symbiosis scenario theory, as absence of any amitochondriate eukaryotic ancestry till date [24]. The transition from the ancestral bacterial endosymboint to modern mitochondria has been escorted by many changes such as shrinkage of mitochondrial genome due to loss or transfer of bacterial genome to the host genome [25-27]. Thus the majority of genes coding for mitochondrial functions are in fact located in the nucleus.

Symbiosis scenario:



Archezoan scenario:

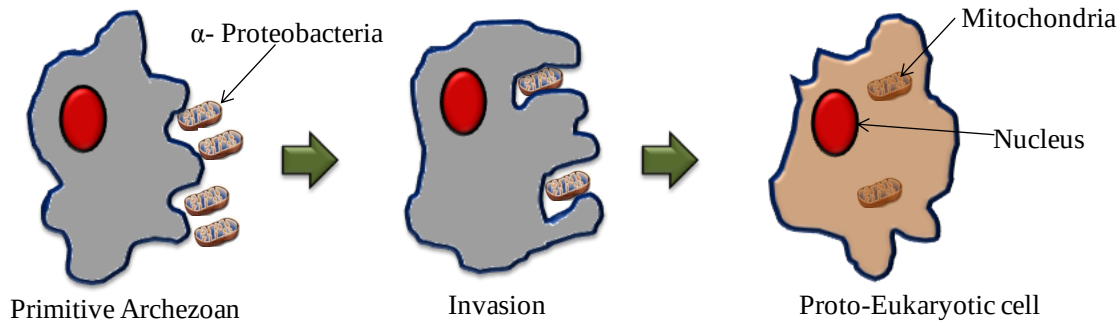


Fig.1.3 Endosymbiotic theory: Schematic representation which implies the bacterial origin of modern mitochondria. Two scenarios (Symbiogenesis scenario and Archezoan scenario) were proposed to explain the endosymbiotic origin of mitochondria.

1.4 Mitochondrial biogenesis

Mitochondrial biogenesis can be defined as growth and division of pre-existing mitochondria [28]. Due to their bacterial origin, mitochondria have their own genome and can autoreplicate. Although mitochondria have their own genome, most of the proteins and enzymes that reside in mitochondrial sub-compartments are nuclear encoded. As most of the mitochondrial proteins are encoded in the nucleus, the mechanism of protein targeting, import and their correct assembly are important for proper mitochondrial function and shape [28].

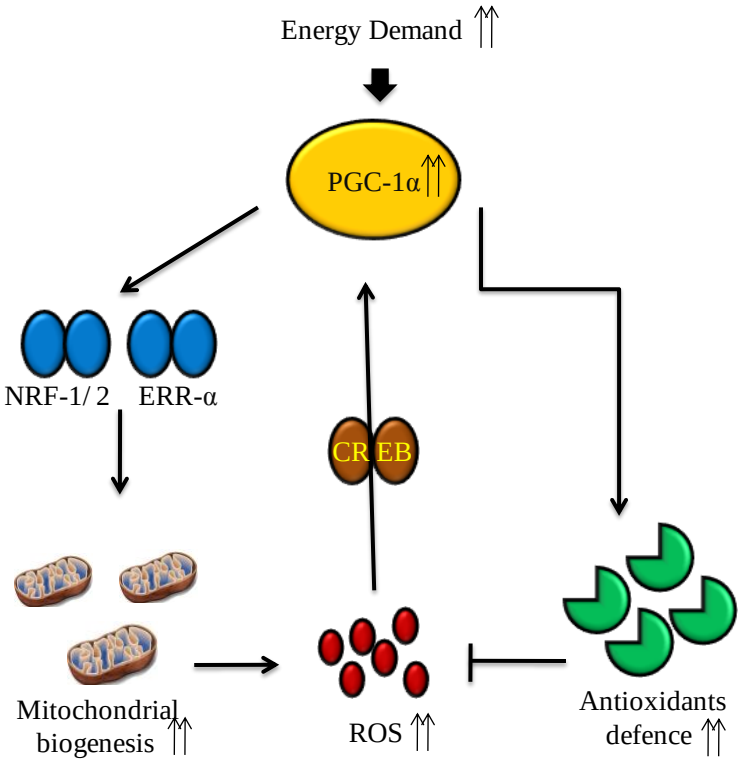


Fig.1.4 Regulation of mitochondrial biogenesis: Mitochondrial biogenesis regulated by PGC-1α through nuclear receptor family members, NRF-1/2 and ERR-α in response to increase in energy demand. Reactive oxygen species generated by mitochondria further increases PGC-1α by feedback mechanism through CREB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

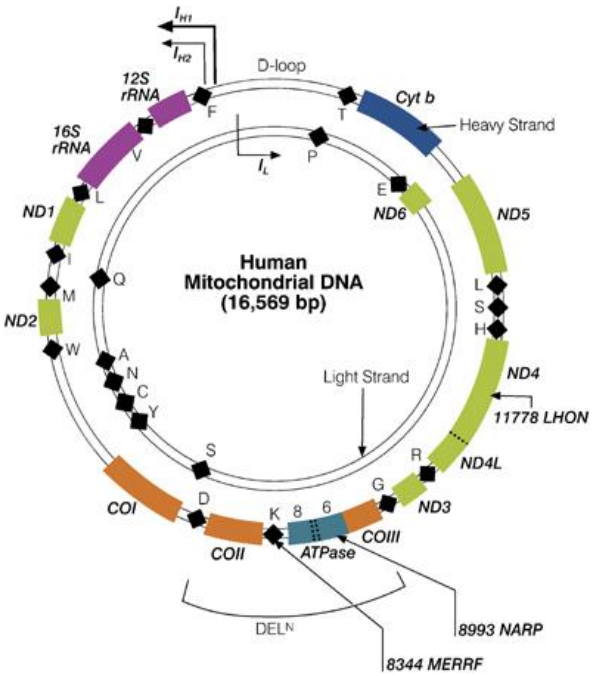
The master regulator of mitochondrial biogenesis appears to be peroxisome proliferator activated receptor gamma co activator 1- α (PGC-1α) [29]. PGC-1α is a co-transcriptional regulatory factor that induces mitochondrial biogenesis by activating the nuclear respiratory factors, NRF-1 and NRF-2. The NRFs in turn activate the mitochondrial transcription factor (Tfam), which transcribes the mitochondrial genome [30].

Most of the proteins that are required for mitochondrial function are nuclear encoded and imported from cytosol [31-32]. However, mitochondrial also import cytosolic tRNAs. Although mitochondrial protein import has been characterized very well, very little is known about the import of tRNAs into mitochondria [33]. Since inner membrane is impermeable to most of the ions, specialized

transporters facilitate the translocation of biomolecules. Import of macromolecules, proteins and tRNAs therefore represents a key aspect of mitochondrial biogenesis [34].

1.5 Mitochondrial DNA

In humans, each mitochondrion harbors 2-10 copies of mtDNA, which is a circular, double stranded molecule codes for 37 genes and contains approximately 16,600 base pairs. The heavy strand of mitochondrial DNA encodes 28 genes where as the light strand encodes 9 genes. Of the 37 genes, 13 genes encode polypeptides (ND1, ND2, ND3, ND4, ND4L, ND5, ND6, COI, COII, COIII, ATPase 6, ATPase 8 and Cytochrome b), 22 genes encode tRNAs and remaining two encode ribosomal RNAs (12S and 16S) [35]. The assembly and functioning of the respiratory enzyme complexes in mammalian cells require coordinated expression and interaction between gene products of the mitochondrial and nuclear genomes.



(Gene Therapy, 2008)

Fig.1.5 The human mitochondrial genome: Schematic depiction of human mtDNA with two continuous units, the heavy and light strands. The genome encodes 22 mt-tRNA (black diamonds), 2 mt-rRNA genes (purple) and 13 protein-coding genes (ND1-ND6, subunits of NADH dehydrogenase subunits 1-6 (ETC complex I), COXI-III, subunits of cytochrome c oxidase subunits 1-3 (ETC complex IV), ATPase 6 and ATPase 8, mitochondrial ATPase subunits (ETC complex V) and Cyt b, cytochrome b (ETC complex III). Point mutations in mitochondrial tRNA encoding genes were also shown.

1.6 General protein import mechanism in mitochondria

Eukaryotic cells have evolved with a sophisticated import machineries at the outer, inner, and intermembrane space of mitochondria to import nuclear encoded mitochondrial proteins into different sub-compartments of mitochondria [36]. After the activation of nuclear genome, mRNAs of mitochondrial precursor proteins are translated in the cytoplasm. Most of the mitochondrial matrix targeted proteins contain presequence at N-terminus. These proteins traverse outer and inner mitochondrial membranes through TOM (Translocase of Outer Membrane) and TIM (Translocase of Inner Membrane) complexes respectively in an unfolded conformation. The translocation of precursor proteins into mitochondria is driven by mitochondrial membrane potential and mitochondrial matrix Hsp70. The presequence of protein is then cleaved by metalloprotease present in the mitochondrial matrix. Then the imported protein is folded with the aid of molecular chaperones.

Currently, four major mitochondrial membrane protein translocases are known to be involved in the protein import. The TOM (translocase of outer membrane) is the universal gateway for all the proteins that are imported into mitochondria at the outer membrane. After traverse through the TOM complex, different protein pathways diverge at this point based on the signal sequence present in the precursor [37]. The mitochondrial inner membrane protein translocase complex, TIM (Translocase of Inner Membrane) sorts matrix targeted precursors where as the PAM (Presequence translocase Associated Motor) regulates the activity of Hsp70 to pull the precursors cross inner

membrane and into the matrix [38]. Finally, the outer membrane, SAM (Sorting and Assembly Machinery) complex inserts β-barrel proteins into outer membrane [34].

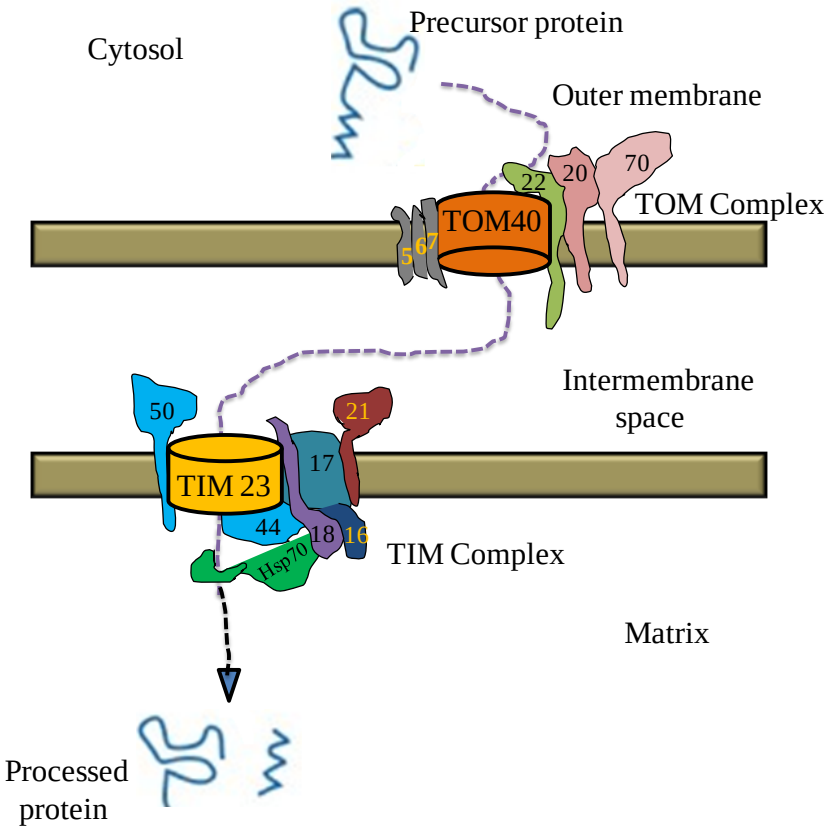


Fig.1.6 Mitochondrial protein import machinery: The import of nuclear encoded mitochondrial proteins into mitochondria is assisted by TOM complex (Translocase of Outer Membrane) (in orange) and TIM complex (Translocase of Inner Membrane) (in yellow). Both TOM and TIM23 complexes consist of multiple subunits and serves as molecular machines during protein import.

1.7 RNA: the versatile molecule

RNA performs a remarkable range of functions in all cells. The RNA molecule plays a central role in carrying the genetic information from DNA to protein. Although RNA is single stranded, it can still form complementary base pairs. RNA molecule can fold into a complex structure due to formation of base pairs in complementary sections within the single strand RNA. Further, base changes in the RNA strand will determine the molecule shape and its function. This flexibility makes RNA as a

versatile molecule [39]. There are three major types of RNA molecules: mRNA carries the genetic information from the nucleus to the cytoplasm, rRNA directs the translation of mRNA into proteins and tRNA transfers aminoacid to the ribosome that correspond to the triplet codon of mRNA.

1.8 tRNA: The genetic interpreter

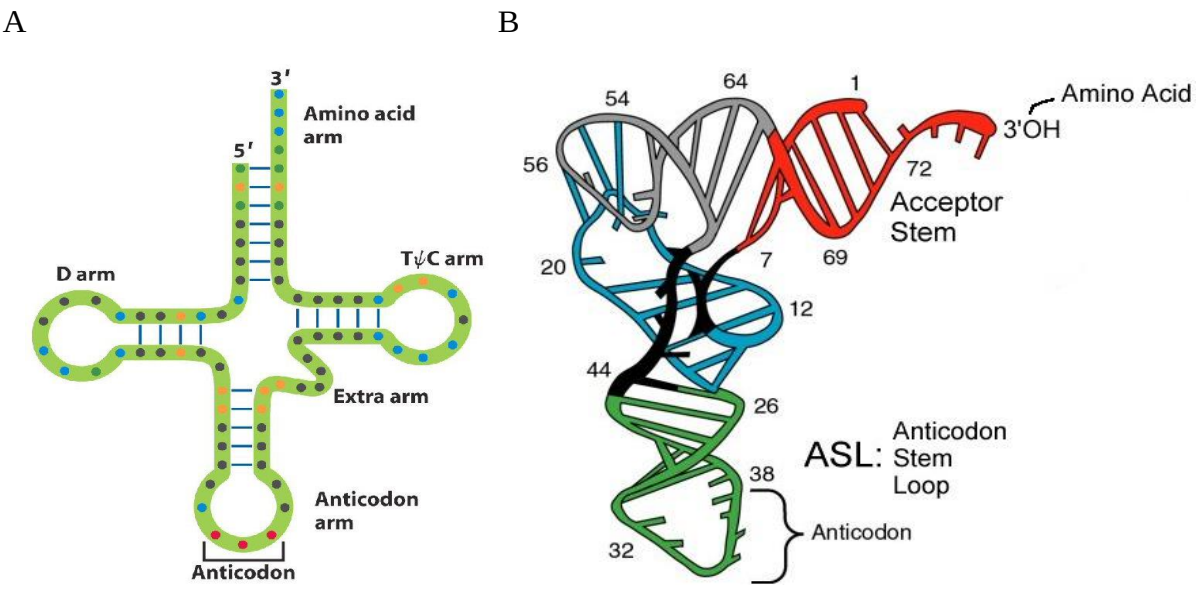
Because of its central role in gene expression and its relative abundance, tRNA is one of the best understood, most scrupulously studied biological macromeolcule. tRNAs are generally 75-90 nucleotides in length. Besides the usual bases A, U, G, and C, all tRNAs have significant number of modified bases such as pseudouridine, dihydrouridine, methylguanosine, inosine etc. In general, tRNAs show a typical secondary cloverleaf structure with defined sizes of loops and stems and an L-shaped tertiary structure with intricate tertiary interactions between conserved nucleotides. The secondary and tertiary structure of tRNA is highly conserved across the species and organelles.

1.8.1 Secondary structure

Based on base-base complementarity, the secondary structure of tRNA can be drawn as familiar cloverleaf structure with four branches; each consists of 4 to 7 Watson-Crick base pairs: the acceptor arm is terminated by CCA sequence at 3'-region, to which aminoacid is attached, the D-arm and TΨC arm (contains the modified bases dihydrouridine and pseudouridine respectively) and the anicodon arm containing anticodon triplet.

1.8.2 Tertiary structure

The tertiary structure of tRNA is L-shaped compact structure held together by base pairing of unpaired regions of the stems and unique staking interactions. tRNA in its tertiary structure folds back on itself through interactions between unpaired bases in the D-arm and TΨC arm results in formation of two segments of double helix.



(http://biology.kenyon.edu/)

Fig.1.7 Structure of tRNA: (A) Secondary cloverleaf structure of tRNA **(B)** Tertiary L-shaped structure of tRNA. Acceptor stem with CCA tail (in red) to accept the incoming tRNA, variable loop on black, anticodon arm (in green) with triplet codon complementary to mRNA, T-arm (in gray) with modified uridine, pseudouridine and D-loop (in blue) with dihydrouridine.

1.8.3 Diverse functions of tRNA in cellular system

tRNA biology has come of age, revealing an exceptional level of understanding and unexpected discoveries along the way. tRNAs are ancient molecules and they arose early in the evolution as the adaptors in the translation of genetic information into protein sequences. tRNA must interact with the cellular proteins transiently rather stably to function in protein synthesis. This exceptional obligation of tRNA may permit them additional functional versatility than other non-coding RNAs [40]. Besides the role of tRNA as an adaptor molecule in protein synthesis [41], it is also involved in many other functions, called extra-translational or non-canonical functions of tRNA [42].

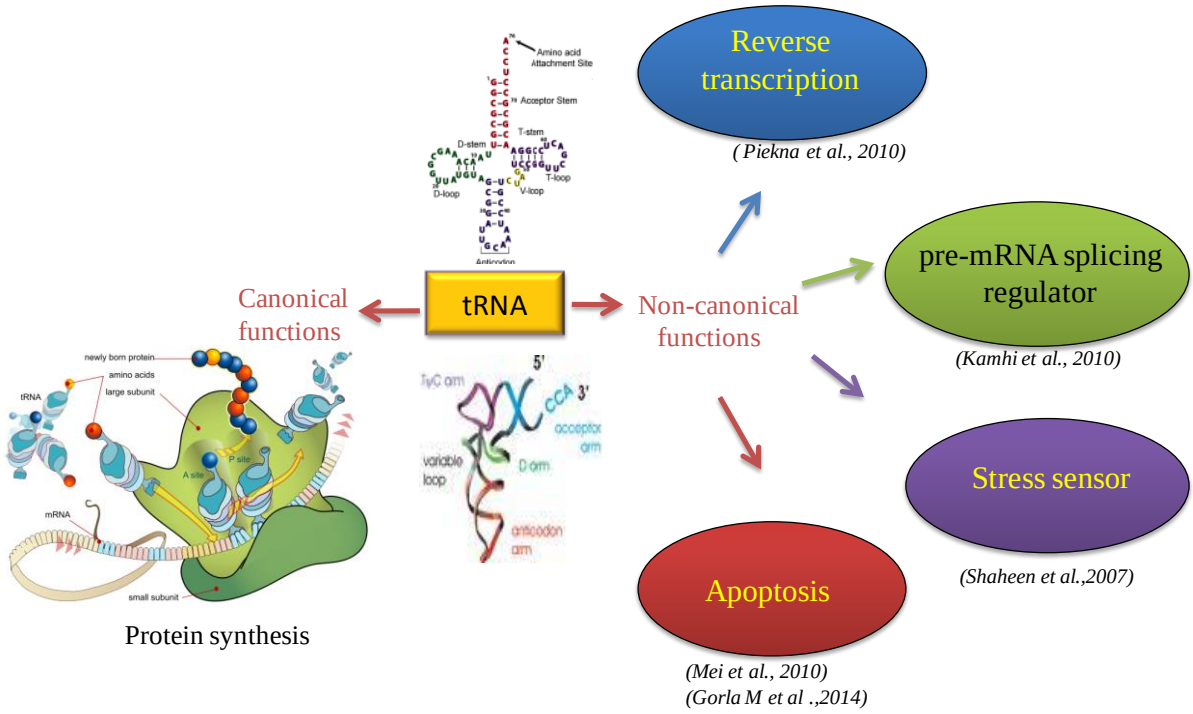


Fig.1.8 Diverse functions of tRNA in a cellular system: Besides the conventional role of tRNA in protein synthesis, it is also known to be involved in reverse transcription, pre-mRNA splicing regulation, stress signaling through GCN2 and also in regulation of apoptosis.

1.8.3.1 tRNA: As a primer in reverse transcription

The replication of RNA genome of human immunodeficiency virus-1(HIV-1) is known to be depends on the tRNA. In particular, the CCA sequence of the human host $tRNA^{Lys}_3$ serves as a primer for the initiation of replication cycle [43-44]. The specific $tRNA^{Lys}_3$, along with other $tRNA^{Lys}$ isoforms and the enzyme lysyl tRNA synthetase (LyRS) are required for the packaging of HIV virus. The molecular interactions studies towards understanding the assembly of $tRNA^{Lys}$ / LyRS packaging complex provides a prospective area for developing novel anti-viral agents [45].

1.8.3.2 tRNA: As a pre- mRNA splicing regulator

Apart from the canonical role of $tRNA_i^{Met}$ in the initiation of protein synthesis, it has the ability to acts as a pre- mRNA splicing regulator in a manner independent of its function in protein synthesis

[46]. In particular, the alternative splicing events due to translational initiation of AUG codon are suppressed by $tRNA_i^{Met}$ carrying anticodon mutations that compensate for AUG mutations. This mechanism of regulation of splicing maintains the quality control of splicing in cell nucleus and prevents the generation of premature termination codons [46].

1.8.3.3 tRNA: As a stress sensor

tRNA is a sensor for stress and nutrient deprivation. In response to these stresses, tRNA translocate from cytosol to the nucleus in a retrograde fashion. This retrograde movement of tRNAs from cytosol to the nucleus reduces the availability of tRNAs in cytosol, possibly minimizing the energy expenditure for protein synthesis [47]. Under nutrient deprivation conditions, uncharged tRNAs get accumulated and activates GCN2 kinase pathway [48]. The accumulated uncharged tRNAs interact with GCN2, the protein kinase that phosphorylates translation initiation factor eIF2α. The phosphorylated eIF2α reduces the general translation, but increases the translation of other transcription regulator GCN4, which in turn results in transcription of numerous genes involved in amino acid and nucleotide biosynthesis [49-50]. This mechanism reduces the overall rate of protein synthesis, limiting amino acid consumption while permitting the cell to translate appropriate stress-response genes.

1.8.3.4 tRNA: As a regulator in apoptosis

The apoptotic stimuli induce the release of Cytochrome c, a pro-apoptotic protein present in the intermembrane space of mitochondria [51-52]. The released Cytochrome c binds to the apoptotic protease activating factor-1 protein (Apaf-1) in the cytoplasm [53]. This binding induces the oligomerization of Apaf-1 that leads to the formation of apoptosome complex [54]. This apoptosome recruits and activates caspase-9, which in turn cleaves and activates caspase-3 [55]. Apoptosis is known to be regulated by several pro-apoptotic proteins (Bax, Bak and Bid), anti-

apoptotic proteins (Bcl-2 and Mcl-1) and a range of cellular factors (HSP90, HSP70 and HSP27) [42]. Interestingly, recent reports have shown the unexpected role of tRNA in regulation of apoptosis. tRNA binds to Cytochrome c and disrupts the interaction of Cytochrome c with Apaf- 1 thereby prevents the formation of apoptosome complex [56]. Further, our group has also shown that tRNA specifically binds to the heme domain of Cytochrome c and inhibits the pro-apoptotic functions of Cytochrome c [6].

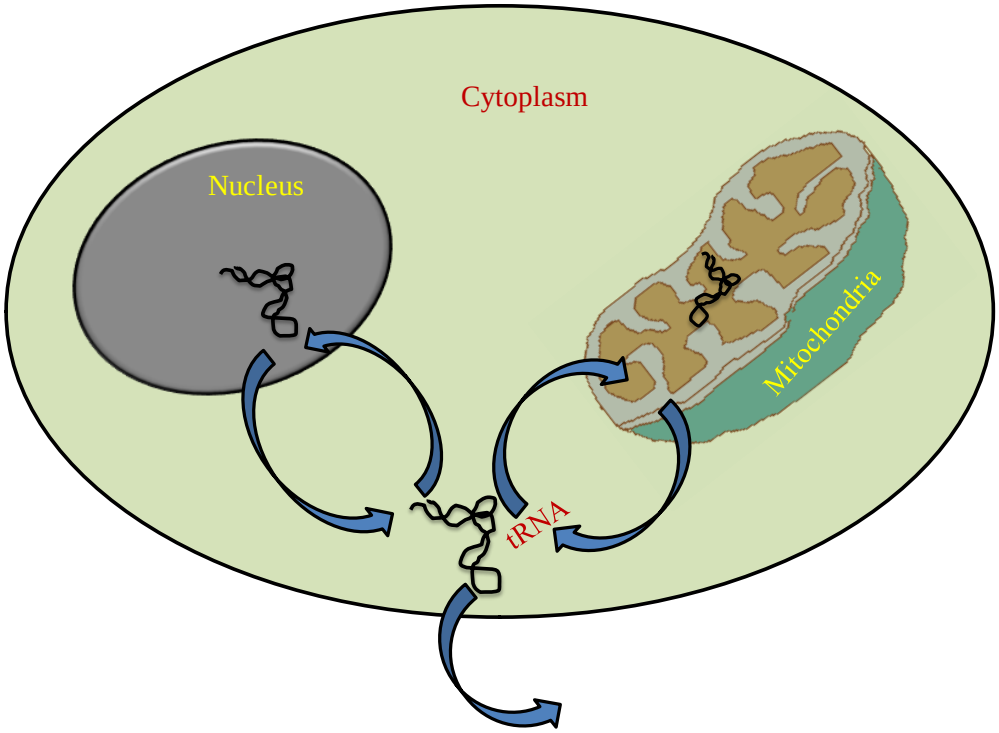
Recently we have also shown that Angiogenin (ANG) protects the cells during cellular stress by generating the tiRNAs or tRNA halves. During hyperosmotic stress, ANG cleaves tRNA at its anti codon arm results in the accumulation of tiRNAs in cytoplasm. The proposed cytoprotective mechanism is due to the interaction of accumulated tiRNAs with Cytochrome c and forms a Cyt c-tiRNA complex that inhibits the apoptosome formation and activity [57].

1.9 The Travels of tRNA in a cell

To execute multiple and diverse functions, tRNAs have to be mobile in between cellular compartments. Majorly, there are three compartments in a cell requires tRNA function: Nucleus, Cytoplasm and Mitochondria. There is a well defined mechanism for the export of newly synthesized and partially processed tRNAs from nucleus to the cytoplasm in a Ran-GTPase-exportin-t (Xpo-t) dependent pathway. Xpo-t directly binds tRNA in the presence of Ran-GTP. The heterotrimeric Ran-GTP-Xpo-t.tRNA complex moves through the nuclear pores to the cytoplasm in which Ran-GTP gets hydrolyzed by Ran-GAPs results in dissociation of complex and release of tRNA [58].

tRNA can also travel from cytoplasm to mitochondria. Early evidences suggested that some of RNA content of mitochondria could be transcribed from non-mitochondrial DNA [59]. It is now well

established that a variable number of tRNA species present in the some eukaryotic mitochondria are indeed nuclear encoded.



(EMBO reports, 2007)

Fig.1.9 The travels of tRNA: tRNAs are either nuclear encoded or mitochondrial encoded. Several tRNA transport systems are present in a cell to execute diverse functions. tRNAs can be translocated either from the nucleus or the mitochondria to the cytoplasm; from the cytoplasm to the nucleus or the mitochondria; and export out of the cell.

1.9.1 Necessity of mitochondrial tRNA import in cell

The number of tRNA encoding genes in numerous mitochondrial genomes is insufficient for proper mitochondrial translation. For example, in protozoans such as *Trypanosoma brucei* and *Leishmania tarentolae*, the mitochondrial genome is completely devoid of tRNA genes. The mitochondrial genome of *Arabidopsis thaliana* encodes six tRNAs where as the green algae *Chlamydomonas reinhardtii* mitochondrial genome encoded only three tRNAs. Thus, in these species, the import of nuclear

encoded tRNAs into mitochondria is necessary for mitochondrial protein synthesis [60]. However, in some organisms, a limited number of imported tRNAs appear to be redundant with regard to the set of tRNAs expressed from their mitochondrial genome [61-62]. In *S. cerevisiae*, the import of nuclear encoded $tRNA^{Lys}_{CUU}$ into mitochondria is demonstrated, although its mitochondrial encoded $tRNA^{Lys}_{UUU}$ recognize both AAA and AAG codons. It is shown that this redundant imported tRNA performs a crucial role in the conditional adaptation of mitochondrial protein synthesis in *S. cerevisiae* [61].

Mammalian mitochondria, with the exception of a few marsupials, encode a complete set of mitochondrial tRNAs. Initially, the import of lysyl tRNA is observed in marsupial animals [63]. However, growing evidence suggests that the presence of cytosolic $tRNA^{Gln}$ in human mitochondrial fractions. It has been shown that cytosolic $tRNA^{Gln}_{CUG}$ and yeast $tRNA^{Lys}_{CTT}$ imports naturally into human and rat mitochondria in an ATP dependent manner [64]. The reason for the dependence of mammalian mitochondria on cytosolic tRNAs probably due to the rate of point mutations in mitochondrial tRNA genes. Since mitochondrial DNA is in close proximity towards the ROS generating system, it is easily susceptible for mutations. Thus, to compensate the mitochondrial tRNA gene mutations, mammalian mitochondria depends on nuclear encoded cytosolic tRNAs for effective translation.

1.9.2 Mitochondrial tRNA gene mutations and diseases

Ineffective mtDNA repair mechanisms and high oxidative stress are accountable for high rate of mutations in mtDNA. Particularly, tRNAs appear to be frequently affected by pathogenic mtDNA mutations. More than 200 mutations in human mtDNA have been associated with neurodegenerative and muscular pathogenesis. Out of them, approximately 150 mutations are

shown to be located in tRNA sequences, although tRNA genes comprise 9% of the mitochondrial genome [65].

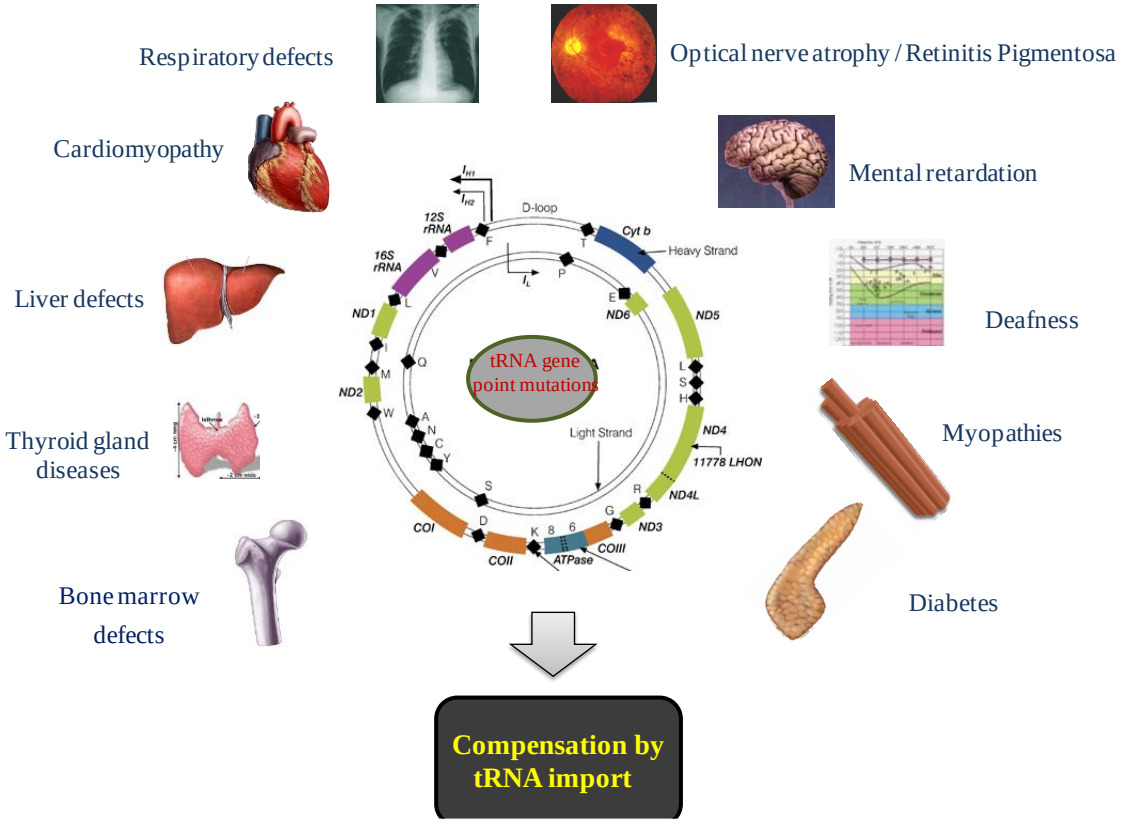


Fig.1.10 Importance of mitochondrial tRNA import in a cell: Reactive oxygen species generated by mitochondria may induce the point mutations in mitochondrial DNA encoded tRNA genes that leads to numerous disorders as mentioned in the figure. To perform the proper mitochondrial functions, it is essential for the cell to depend on the import of nuclear encoded cytosolic tRNAs.

As the main function of tRNA is to transfer the correct amino acid to the growing polypeptide chain, pathogenic tRNA mutations consequently affect the mitochondrial translation, leading to impairment of complex I, III, IV or V activity or a combined failure [66]. Pathogenic tRNA mutations can affect the stability, structure and aminoacylation properties of tRNA [67-68]. The mutations can appear in a homoplasmic or heteroplasmic state with diverse clinical manifestations depending on the mutation rate, position of the mutation and the affected tissue. Although the mutations have been identified in all 22 mitochondrial tRNAs, $tRNA^{Lys}_{TTT}$ and $tRNA^{Leu}_{UUR}$ are

considered as the hot spots for pathogenic mutations. The most common mutation found in 80% of individuals with MELAS is an A to G transition at the nucleotide position 3243 in the D-loop of $tRNA^{Leu}_{UUR}$ gene. An additional 7.5% have a heteroplasmic T to C point mutation at the nucleotide position 3271 in the anticodon stem of $tRNA^{Leu}_{UUR}$ gene. These mutations alter the structure and prevent modification of the wobble position resulting in an impaired aminoacylation [69]. The A to G transition at the nucleotide position 8344 in the T- loop of $tRNA^{Lys}$ leads to another distinctive mitochondrial disease called MERRF. This mutation cause poor aminoacylation of mutant $tRNA^{Lys}$, hypomodification of its anticodon wobble position, premature termination at some lysine codons [70]. These pathogenic mutations leading to various disorders ranking from mild (exercise intolerance, limb weakness) to severe (even lethal) including myopathies, encephalomyopathies, cardiopathies, diabetes, deafness, retinitis pigmentosa, bone marrow defects etc [71].

1.9.3 Mitochondrial tRNA import in protozoa

The two best studied protozoan systems, that examined the mitochondrial import of nuclear encoded tRNA are *Trypanasoma* and *Leishmania*.

Recent evidence shows that the involvement of eukaryotic elongation factor 1a (eEF1a) in mitochondrial tRNA import in *Trypanasoma*. eEF1a is a cytosolic protein interacts with all the elongated tRNAs except for selenocysteine tRNA ($tRNA^{Sec}$) and initiation methionine tRNA ($tRNA^{Met}_{i}$). Further, all tRNAs that interact with eEF1a are imported into mitochondria but $tRNA^{Sec}$ and $tRNA^{Met}_{i}$ remains in cytosol [72]. Further, i*n vivo* import studies revealed that mitochondrial tRNA import is dependent on mitochondrial membrane potential. Although, cytosolic protein eEF1a identified as a tRNA recruiting factor in *T. brucei*, littile is known about the mitochondrial membrane protein that is involved in the translocation process. However, it has been shown recently by using *in vivo* tRNA import and RNAi studies, Tim17, a component of the inner

membrane protein complex and mitochondrial matrix localized Hsp70 are involved in tRNA import [73].

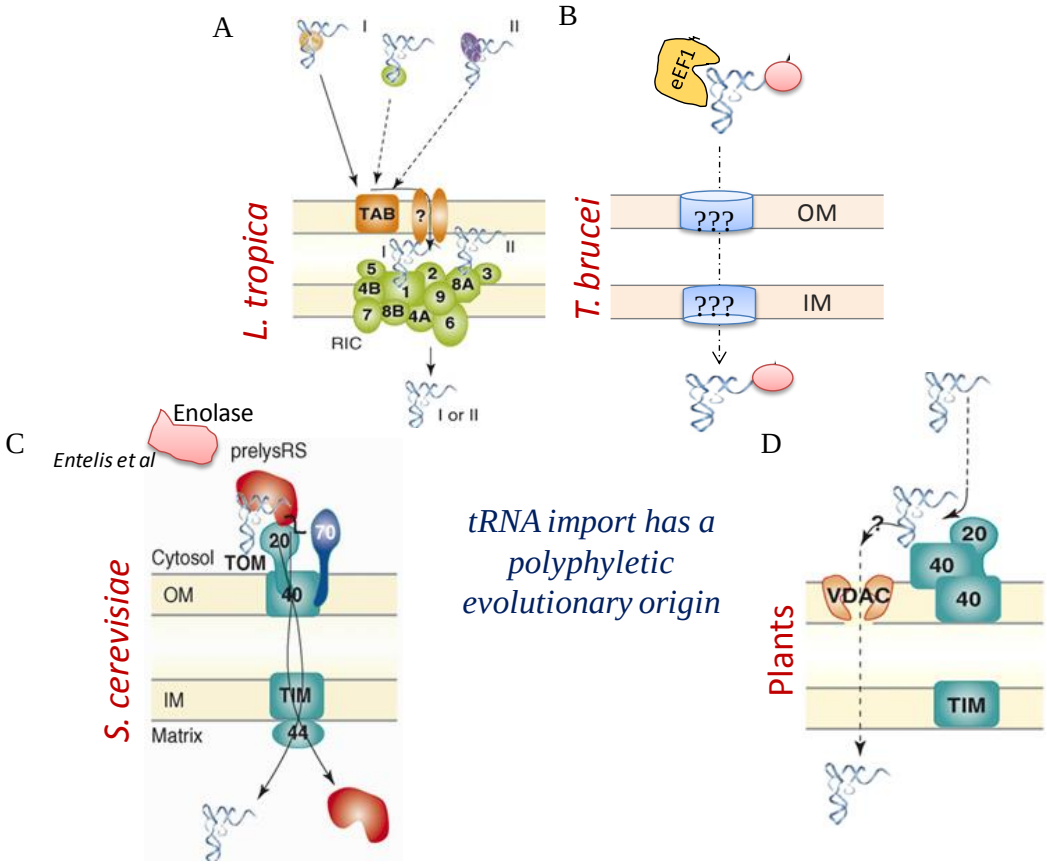
Recent evidence shows that the involvement of inner mitochondrial membrane resident multi-subunit RIC (RNA import complex) complex in *Leishmania* mitochondrial tRNA import [74]. Among the 11 fractionated and identified RIC components (8 nuclear encoded and 3 mitochondrial encoded), the RIC1 and RIC8A subunits known to interact with tRNA and translocate through the pore constituted by RIC6 and RIC9. Further it has been shown that the interaction of tRNA with different receptors present in RIC complex depends on the import determinants present in tRNA [74]. However, the receptors and the actual mechanism of tRNA translocation at the outer mitochondrial membrane are not yet explored in *Leishmania*.

1.9.4 Mitochondrial tRNA import in *S. cerevisiae*

The mechanism of mitochondrial tRNA import in *S. cerevisiae* is extensively studied. Although yeast mitochondrial genome encodes complete set of mitochondrial tRNAs, it has been shown that import of two cytosolic tRNAs ($tRNA^{Lys}_{CUU}$ and $tRNA^{Gln}_{CUG}$) into yeast mitochondria [75]. The import of $tRNA^{Lys}_{CUU}$ is redundant under standard growth conditions but becomes essential for conditional adaptation for the cells that are grown at elevated temperatures [61]. However, the exact function of imported $tRNA^{Gln}_{CUG}$ in yeast mitochondria is presently unknown.

Mitochondrial targeting of $tRNA^{Lys}_{CUU}$ requires specific binding to the glycolytic enzyme, Eno2p [76]. Enolase delivers tRNA on to the mitochondrial surface, where tRNA can binds to pre-MSK (precursor of mitochondrial lysyl-tRNA synthetase). The pre-MSK translated in the vicinity of mitochondria and acts as a carrier for tRNA import [77]. The specific binding of $tRNA^{Lys}_{CUU}$ to these two proteins is determined by the nucleotide present in its acceptor stem and anticodon loop [78]. However, we have shown that pre-Msk1 is not essential for import of tRK1 into mitochondria

from the cytoplasm *in vivo* [79]. Further, i*n vitro* import studies showed that mitochondrial tRNA import in yeast is dependent on ATP, mitochondrial membrane potential and functional components of mitochondrial membrane protein import machinery. It is found that a Tom20 (component of the Translocase of the Outer Membrane) as well as Tim44 (component of the Translocase of the Inner Membrane) are required for mitochondrial tRNA and protein import *in vitro* [80].



(Annu.Rev.Biochem. 2011)

Fig.1.11 Mitochondrial tRNA import is widespread among species: (A) In *Leishmania tropica*, tRNAs are first bound to outer mitochondrial membrane (OM) receptors (TAB). The bound tRNAs are translocated through the outer membrane through unknown translocator. At Inner membrane (IM), tRNAs reach a large multi-subunit complex called RNA-import complex (RIC) and then translocate through the pore constituted by RIC6 and RIC9. The imported tRNAs contain distinct import motifs in the anticodon or D arms for type-I tRNAs or in the variable loop and T arm for type-II tRNAs. **(B)** In *Trypanasoma brucei*, aminoacylated

tRNAs translocated to the mitochondrial matrix in the presence of elongation factor 1a (eEF1a) through unidentified translocator. **(C)** In *Saccharomyces cerevisiae*, the cytosolic factors enolase and precursor form of the mitochondrial lysyl-tRNA synthetase (prelysRS) are involved in the import of the nuclear-encoded $tRNA^{Lys}_{CUU}$ through the protein-import channel. The protease-sensitive receptor Tom20 of the TOM complex and the major component Tim44 of the TIM complex are essential for tRNA import. **(D)** In *Solanum tuberosum*, tRNA translocated through the pore of voltage-dependent anion channel (VDAC) present on the outer membrane. However, the proteins that are involved in the translocation of tRNA at the inner membrane still unknown.

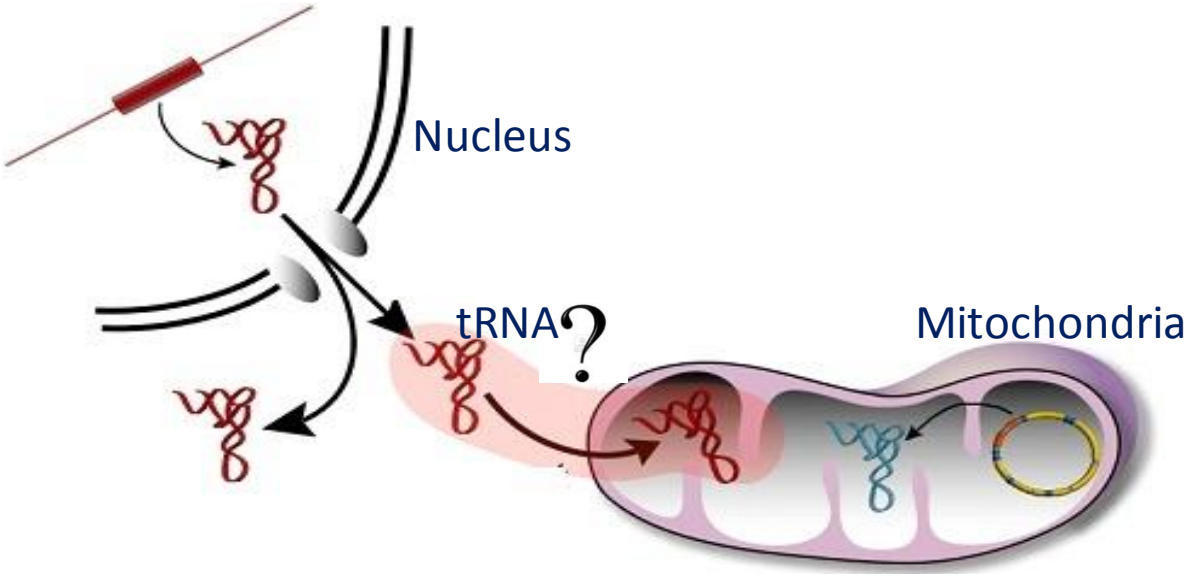
1.9.5 Mitochondrial tRNA import in plants

The tRNA encoding genes of plant mitochondrial genomes vary in number, ranging from 3 to 29 [81]. *Solanum tuberosum* is the only plant system in which a mitochondrial membrane dependent tRNA import system has been identified. By using immuno blocking studies, the involvement of voltage-dependent anion channel (VDAC), the metabolite transporter of the outer mitochondrial membrane in tRNA import. tRNAs and other nucleic acid substrates are able to interact with VDAC *in vitro* [82]. The import of tRNAs is inhibited in the presence of antisera against Tom20 and Tom40, two conserved components of the mitochondrial outer membrane translocation complex. Based on these results, it was suggested that VDAC may be the major channel for tRNA import where as Tom20 and Tom40 are the import receptors in *S. tuberosum* [82].

In contrast to protein import, mitochondrial tRNA import has a polyphyletic origin. In agreement with the postulated polyphyletic origin of mitochondrial tRNA import, the membrane dependent translocation mechanisms of tRNAs are not conserved between different organisms [60]. In fact, there is no evidence to show the presence of a dedicated mechanism for tRNA import in any system. Moreover, the cytosolic factors that are targeting tRNA onto mitochondrial surface appear to be housekeeping components.

1.9.6 Mitochondrial tRNA import in mammals

Mammalian mitochondrial genome encodes complete set of mitochondrial tRNAs. However, recent evidence suggests the fraction of the cytosolic $tRNA^{Gln}$ is imported into rat and human mitochondria in ATP dependant manner [64]. The mitochondrial tRNA import was also observed in marsupial mitochondria [63]. In addition to tRNA import, the import of RNA subunit of RNase P [83], MRP RNase P [84] and 5S rRNA [85-86] has been shown in humans. However, their presence in mitochondrial fraction remains controversial. The function of imported RNA subunit of RNase P is remains unanswered as mammalian mitochondrial RNase P lack RNA subunit [87].



http://mitocross.unistra.fr/partners/gmgm/team-1-intracellular-traffic-of-rna-and-mitochondrial-pathologies/

Fig.1.12 Overview of the fundamental question in mammalian mitochondrial tRNA import: Although it is known that mammalian mitochondria have the inherent ability to import nuclear encoded cytosolic tRNAs, the precise mechanism and the cytosolic factors that are involved in the regulation of tRNA translocation are yet to be explored.

On the other side, the import of 5S rRNA is also surprising as no 5S rRNA has been found in mammalian mitochondrial ribosomes [88]. Recent evidence suggests that the importance of mitochondrial intermembrane space protein, polynucleotide phosphorylase (PNPASE) in the import

of RNA subunits of RNase P, MRP RNase P, 5S rRNA and tRNAs. This import has been shown to be dependent on ATP, mitochondrial membrane protein and membrane potential [89]. However, another independent report suggests that the import of cytosolic $tRNA^{Gln}$ into human mitochondria does not require any mitochondrial membrane potential [64]. These results suggest that alternate mechanisms may be used for different RNAs translocation into mitochondria.

1.10 tRNA import: A novel strategy to treat mitochondrial diseases

Mitochondrial tRNA import offers a novel concept for the therapy of many human diseases that are associated with the mutations in mitochondrial encoded tRNA genes [90]. If it is possible to induce the import of cytosolic tRNA that can complement the mutated mitochondrial tRNA, mitochondrial dysfunction caused by the impairment of organellar translation might be alleviated. Recent experiments have been remarkably successful in testing this concept in both *in vitro* and *in cell culture* models [91-95].

It has been shown that mammalian mitochondria have the inherent ability to import tRNAs. However, the cytosolic factors that are involved in recruiting tRNAs and the precise mechanism of tRNA import into mammalian mitochondria are not known. Hence the present study was designed to investigate the mechanism involved in the targeting of nuclear encoded cytosolic tRNAs into mammalian mitochondria.

Chapter 2

Identification of tRNA recruitment factor(s) at the mitochondrial outer membrane

2.1 INTRODUCTION

Mitochondria are essential organelles of eukaryotes and play a role in a variety of essential cellular processes including respiration, oxidative phosphorylation-mediated ATP production, cell metabolism and apoptosis [4]. Mitochondria contain their own genome and encode a limited number of proteins that are required for proper mitochondrial respiratory chain functions [96]. However, in several evolutionary divergent eukaryotes, mitochondrial genome encodes none or partial set of tRNA genes to support the protein synthesis in mitochondria. Hence, these organisms have evolved to import cytosolic tRNAs into mitochondria to compensate the lack of tRNAs in their mitochondria [33].

Import of cytosolic tRNAs into mitochondria has been well characterized in different phyla that include *Saccharomyces cerevisiae*, protozoa, plants and marsupials [97]. However, the mechanism for import of tRNA into mitochondria is distinctly regulated in different organisms. For instance, eukaryotic translation elongation factor 1a (eEF1a) in *Trypanasoma* [72], RNA import complex (RIC) in *Leishmania* [74] and voltage dependant anion channel (VDAC) in plants [82] were shown to be required for the import of cytosolic tRNAs into mitochondria. In budding yeast, *Saccharomyces cerevisiae,* one cytosolic $tRNA^{Lys}_{CUU}$ (tRK1) gets translocated into mitochondria although its mitochondrial genome encodes all tRNAs that are vital for translation [75, 77, 79]. The import of cytosolic tRNA into yeast mitochondria can occur without the requirement of any cytosolic factors [79]. However, several reports suggest that the possible involvement of cytosolic factors in regulation of tRNA import in yeast [98]. These factors include precursor form of mitochondrial lysyl tRNA synthetase [77], 26S proteasome [99] and glycolytic enzyme enolase [76]. In contrast, the import of tRNAs into the mitochondria of either plants, mammalians or protozoans is independent of cytosolic factors [33, 60]. However, degradation of mitochondrial surface exposed proteins by

limited proteolysis inhibited translocation of tRNAs into mitochondria in many organisms [34]. This result indicates that tRNA import requires mitochondrial membrane bound or integral protein(s). Mitochondrial membrane bound proteins may include membrane bound cytosolic factors.

In mammals, mitochondrial genome encodes 22 tRNAs that are sufficient for efficient translation of mtDNA-encoded proteins [35]. However, mammalian mitochondria are able to import nuclear encoded cytoplasmic $tRNA^{Gln}$ and yeast $tRNA^{Lys}$ in mammals both *in vitro* and *in vivo* [64, 91]. Cytosolic $tRNA^{Gln}_{CUG}$ is imported naturally into human and rat mitochondria in an ATP dependent manner [64]. Later, *Wang et al.,* (2010) have implicated the role of PNPase in mitochondrial tRNA import [89]. Nevertheless, a comprehensive mechanistic insight into mammalian mitochondrial tRNA import is yet to be elucidated. The present study was undertaken to identify the putative tRNA recruiting factors that are regulating the tRNA import into mammalian mitochondria.

To gain an understanding of the innate ability of mitochondria to import tRNA, we monitored the endogenous import of twenty one cytoplasmic tRNAs into mitochondria. Our result suggested that the mitochondria has an inherent capacity to import multiple tRNAs and is not limited to the known import of $tRNA^{Gln}$ or $tRNA^{Lys}$. We find that mitochondria import of twenty one cytoplasmic tRNA albeit at various efficiencies. In this report, we show that salt extractable soluble factors of mitochondria enhance the efficiency of tRNA import into mitochondria. Specifically, we identify Actin Binding Rho Activating Protein (ABRA) present in the salt extracts as contributing to this stimulation in tRNA import. Recombinant ABRA stimulates tRNA import into mammalian mitochondria in a dose dependent manner *in vitro*. Further, we show the presence of imported tRNA in mitochondrial matrix fraction by using selective permiabilization studies with digitonin. Together, our results disclosed a novel role of cytosolic Actin Binding Rho Activating Protein in augmenting the import of cytosolic tRNAs into mammalian mitochondria.

2.2 METHODOLOGY

2.2.1 Antibodies and Reagents

Antibodies used in this study are either purchased, ABRA (Sigma Aldrich), Porin, COXIV, BIP, PARP-1, Aconitase-2, Tom20, β-Tubulin, ND1, Hsp70 (Abcam) or in-house raised (Tom40 and Mia40). All the chemicals used for this study were obtained from Sigma Aldrich, Amresco Himedia, Merck and Sisco Research Laboratories (SRL). Plasmid constructs pCMV-XL5-ABRA was purchased from OriGene.

2.2.2 Isolation of mitochondria from rat liver

Mitochondria were isolated from rat liver using differential centrifugation as described [100]. About 1.0-1.5 gm of rat liver was excised and pulverized in 0.9% saline. The excised liver tissue was subjected to homogenization in 10 ml ice-cold medium A (220 mM Mannitol, 70 mM Sucrose, 0.2 mM EDTA, 2 mM HEPES, pH-7.2, add BSA 0.36 mg/ml before use). The liver tissue homogenate was then diluted to 40 ml with medium B (medium A without BSA) and centrifuged at 1800 rpm for 10 min at 4^0C. The upper three quarters of supernatant was separated from pellet fraction (Nucleus) and centrifuged at 7000 rpm for 10 min at 4^0C. The resulting pellet contains mitochondria and it was washed with 10 ml of ice cold medium B followed by centrifugation at 1800 rpm for 10 min. The supernatant was again centrifuged at 7000 rpm for 10 min to collect purified mitochondria. The purified mitochondrial pellet was then suspended in import buffer (0.25 mM sucrose, 1.5 mM $MgCl_2$ and 10 mM HEPES, pH-7.2) and used for *in vitro* tRNA import and protein import studies. To obtain a highly purified mitochondrial fraction, the mitochondrial suspension was layered on top of a 2.5 M sucrose-percoll gradient and centrifuged at 46,000 x g at 4˚C for 45 min and purified mitochondria were collected as described [101-102]. Purity of isolated mitochondria was assessed by immunoblotting with specific organelle marker antibodies.

2.2.3 Isolation of mitochondria from HEK293T cells

Mitochondria were isolated from HEK293T cells using differential centrifugation [103]. In brief, HEK293T cells were grown as monolayers and suspended in mitochondria isolation buffer (10 mM HEPES-KOH, pH-7.2, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 210 mM sucrose, 70 mM Mannitol) and incubated for 30 min on ice with frequent tapping of cells. The cell suspension was subjected to homogenization by using polytron 1600 at 3 sec X 2 pulses at 15 rpm. The suspension was further subjected to dounce homogenization. The homogenate was centrifuged at 1000 x g for 10 min at 4°C to remove the nucleus and the supernatant was again centrifuged at 10,000 x g for 20 min at 4°C. The resultant mitochondrial pellet and cytosolic supernatant was used for the isolation of total RNA. Prior to RNA extraction, isolated mitochondria were treated with RNase A (100 μg/ml) to remove any cytosolic RNA contamination. To obtain a highly purified mitochondrial fraction, the mitochondrial suspension was layered on top of a 2.5 M sucrose-percoll gradient and centrifuged at 46,000 x g at 4°C for 45 min and purified mitochondria were collected as described [101-102].

2.2.4 Northern blot analysis

Total mitochondrial RNA, total RNA and cytosolic RNA were extracted by TRIZOL (Invitrogen) method [104]. Approximately, 1 μg of each set of RNA was separated on 7 M urea-10% polyacrylamide gel electrophoresis in Tris-Borate EDTA (1x TBE) buffer. The gels were then electroblotted onto a positively charged Hybond Nylon membrane (Amersham) by using Semi-Dry Blotting apparatus (Bio-Rad) for 1 hr at 10 V in 1x TBE buffer. After the semi dry transfer, RNA was immobilized onto the nylon membrane by using UV Stratalinker 1800 System (Stratagene) followed by subjecting the nylon membrane for Baking at 80^0C for 1 hr. After fixation of RNA, nylon membrane was subjected to pre hybridization in pre hybridization buffer (0.5 M Sodium

Phosphate, dibasic pH-7.2, 7% (w/v) SDS, 1 mM EDTA, pH-8.0) at 65^0C for 2 hrs. Blot was then incubated with specific 5'-end [γ-^{32}P]-ATP-labeled oligonucleotide probes in hybridization buffer for 12-16 hrs at 50^0C in Hybridization Chamber (Amersham). After incubation, blot was washed with wash buffer (2 X SSC, 0.1% SDS) at 50^0C for 10 min x 3 times. The hybridization signal was analyzed by exposing the blot in phosphorImager (GE Healthcare) and Typhoon 9410 scanner (Amersham Biosciences). Quantification of density in each band was performed by Image J analysis software. Blots were stripped in stropping buffer (1% SDS, 1mM EDTA, pH-8.0) at 68^0C for 30 min and reprobed with another specific oligonucleotide probe.

Table 2.1: Primers used for Northern Hybridization analysis

S.No.	Gene Name	Primer Name	Primer Sequence
1.	5S rRNA_Rev	NB 629	5'- GCGGTCTCCCATCCAAGTA - 3'
2.	5.8S rRNA_Rev	NB 631	5'- GCGACGCTCAGACAGG - 3'
3.	18S rRNA_Rev	NB 425	5'- GGCATCGTTTATGGTCGGAA - 3'
4.	Mito. $tRNA^{Glu}_{TTC}$_Rev	NB 337	5'- CCACGACCAATGATATGAA - 3'
5.	Mito. $tRNA^{Lys}_{TTT}$_Rev	NB 336	5'-TCACTGTAAAGAGTTGGTTCTCTTA- 3'
6.	Cyto. $tRNA^{Phe}_{GAA}$_Rev	NB 626	5'- TGCCGAAACCCGGGATCGA - 3'
7.	Cyto. $tRNA^{Gln}_{CTG}$_Rev	NB 620	5'- AGGTTCCACCGAGATTTGA - 3'
8.	Cyto. $tRNA^{Ser}_{AGA}$_Rev	NB 647	5'- TCGGCAGGATTCGAACCTG - 3'
9.	Cyto. $tRNA^{Ala}_{TGC}$_Rev	NB 653	5'- AGGCCTCATACATGCAAAG - 3'
10.	Cyto. $tRNA^{Trp}_{CCA}$_Rev	NB 618	5'- TGACCCCGACGTGATTTGA - 3'
11.	Cyto. $tRNA^{Leu}_{TAG}$_Rev	NB 612	5'- CAGCGGTGGGATTCGAACC - 3'
12.	Cyto. $tRNA^{Ala}_{CGC}$_Rev	NB 616	5'- AGATGCCGGGGATCGAACC - 3'
13.	Cyto. $tRNA^{Lys}_{TTT}$_Rev	NB 608	5'- CGCCTGGACAGGGACTTGA - 3'
14.	Cyto. $tRNA^{Met}_{CAT}$_Rev	NB 624	5'-TAGCAGAGGATGGTTTCGA - 3'
15.	Cyto. $tRNA^{Glu}_{CTC}$_Rev	NB 614	5'-TTACATGACTGGGAATCAA - 3'
16.	Cyto. $tRNA^{Lys}_{CTT}$_Rev	NB 622	5'-TGTCCAATGTGGGGCTTGA - 3'
17.	Cyto. $tRNA^{Cys}_{GCA}$_Rev	NB 662	5'- AGGGACCTCTTGATCTGCA - 3'
18.	Cyto. $tRNA^{Gly}_{GCC}$_Rev	NB 610	5'- TGCATAGGCCAGGAAATGA - 3'
19.	Cyto. $tRNA^{Pro}_{GGG}$_Rev	NB 658	5'- CTGGGATCAGCAGCCCCAG - 3'
20.	Cyto. $tRNA^{His}_{GTG}$_Rev	NB 656	5'- GAGGTTGCTGCGGCCACAA - 3'
21.	Cyto. $tRNA^{Ser}_{GCT}$_Rev	NB 648	5'- AGCACAATGGATTAGCAGT - 3'
22.	Cyto. $tRNA^{Pro}_{AGG}$_Rev	NB 652	5'- CGTCCGGGATTTGAACCCG - 3'
23.	Cyto. $tRNA^{Asp}_{GTC}$_Rev	NB 646	5'- CGTCGGGGAATCGAACCCC - 3'
24.	Cyto. $tRNA^{Leu}_{AAG}$_Rev	NB 655	5'- AGAGACTGGAGCCTTAATC - 3'
25.	Cyto. $tRNA^{Asn}_{GTT}$_Rev	NB 651	5'- GTGGGCTCGAACCACCAAC - 3'
26.	Cyto. $tRNA^{Val}_{AAC}$_Rev	NB 654	5'- TCCGCCCGGTTTCGAACCG - 3'

2.2.5 In vitro transcription

^{32}p- Radiolabeled human $tRNA^{Lys}_{TTT}$ transcript was generated from pUC19 vector containing human $tRNA^{Lys}_{TTT}$ gene. Initially, pUC19 plasmid was completely linearized by *BstN1* (Fermentas) at 37^{0}C for overnight and extracted by phenol:chloroform. 1 μg of linearized plasmid was incubated in a transcription buffer containing 200 mM Tris-HCl pH-7.9, 30 mM $MgCl_2$, 50 mM DTT, 50 mM NaCl, 10 mM spermidine, 0.5 mM each of rATP, rGTP, rUTP, 12 μM rCTP, 10 μci [α-^{32}P] CTP, 10 U of RiboLock RNase inhibitor and 20 U of T7 RNA Polymerase (Fermentas) at 37^{o}C for 2 hrs. The reaction was stopped by cooling at -20^{0}C. Nuclease-free NucAway spin column (Ambion) was used to remove unincorporated labeled nucleotides from the transcription reaction. Briefly, the NucAway spin column was hydrated with DEPC treated water for 15 min at room temperature. After incubation, column was subjected to centrifugation at 750 x g for 2 min to remove the interstitial fluid. Then the transcription reaction was loaded onto the column and spun at 750 x g for 2 min to collect the labeled transcript. Quality of the transcript was monitored on 7 M urea / 12% acrylamide gel electrophoresis followed by autoradiogram.

2.2.6 In vitro tRNA import assay

In vitro tRNA import assays were performed by incubating isolated rat liver mitochondria (50 μg) with ^{32}p- radiolabeled human $tRNA^{Lys}_{TTT}$ transcript or unlabeled tRNA in 100 μl of import reaction buffer containing 0.6 M Sorbitol, 20 mM Tris-HCl pH-8.0, 2 mM ATP, 2 mM DTT, 20 mM $MgCl_2$, 2 mM EDTA at 30^{0}C for 15 min. After incubation, mitochondria were treated with RNase A and RNase T1 to final concentrations of 100 μg/ml and 750 U/ml at 37^{0}C for 30 min to digest the non-imported tRNAs. Then mitochondria were washed once with sucrose cushion (0.8 M sucrose in 10 mM HEPES, pH-7.2) and pellet down by centrifugation at 12,000 rpm for 10 min at 4^{0}C. Mitochondria were rewashed with buffer containing 0.25 M Sucrose, 1.5 mM $MgCl_2$, 10 mM

HEPES, pH-7.2 and pellet down by centrifugation at 12,000 rpm for 5 min at 4^0C. These mitochondria were used to extract the total RNA.

2.2.7 Extraction of total RNA from mitochondria

After import, mitochondria were solubilized in 100 µl of solubilization buffer containing 10 mM Tris-HCl pH-8.0, 1 mM EDTA, 0.1% SDS. To the solubilized mitochondria, 1/10 volume of 3 M sodium acetate pH- 4.5, equal volume (110 µl) of saturated phenol pH- 4.5, 20 µl chloroform / isoamyl alcohol (49:1) were added sequentially and subjected to vigorous shaking by hand for 10 sec. Samples were then cooled on ice for 15 min followed by centrifugation at 14,000 rpm for 20 min at 4^0C. After centrifugation, upper aqueous phase (which contains mostly RNA) was carefully transferred into fresh tube and added equal volumes of ice cold isopropanol and kept at -20^0C for overnight to precipitate RNA. After precipitation, samples were subjected to centrifugation at high speed for 20 min at 4^0C. The supernatant was discarded and RNA pellet was washed with 75% ice cold ethanol. RNA pellet was air dried at room temperature for 5-10 min and suspended in DEPC treated water [105]. Extracted ^{32}p-radiolabeled tRNA was separated on 7 M urea, 12% acrylamide gel and analyzed by phosphorimager.

2.2.8 In vitro protein import assay

Mitochondria were isolated from rat liver as described above. For *in vitro* protein import studies, ^{35}S-labeled SU9-DHFR protein was incubated with purified mitochondria in import buffer (250 mM Sucrose, 5 mM $MgCl_2$, 30 mM KCl, 10 mM Na-Succinate pH-7.2, 1 mM DTT, 10 mM MOPS pH 7.2, 2 mM Methionine, 2 mM ATP, 2 mM GTP, 1% BSA) at 37^0C for 60 min. The samples were then subjected to 100 mg/ml trypsin treatment to remove the non-imported proteins and pellet out mitochondria by passing through sucrose cushion (0.8 M sucrose in 10 mM HEPES, pH-7.2). Mitochondria were rewashed with buffer containing 0.25 M Sucrose, 1.5 mM $MgCl_2$, 10 mM

HEPES, pH-7.2 and pellet down by centrifugation at 12,000 rpm for 5 min at 4^0C and re suspended in SDS sample buffer**.** The mitochondrial proteins were resolved on 12% SDS-PAGE and analyzed the labeled protein by autoradiography [102].

2.2.9 Preparation of mitochondrial salt extract

Freshly isolated, percoll purified rat liver mitochondria were incubated with 500 mM KCl solution on ice for 20 min. Then the reaction mixture was centrifuged for 20 min at 15,000 x g for 30 min at 4^0C. The supernatant was collected and concentrated through centricon concentrator (Amicon) tubes as mentioned by manufacturer's protocol. The concentrated protein extract was estimated and used for further experiments as a mitochondrial salt extract.

2.2.10 Biotin pull down assay

To pull down the tRNA binding proteins from mitochondrial salt extract, full length 5'-biotin tagged $tRNA^{Lys}_{TTT}$ (5'-BiotinGCCTGGGTAGCTCAGTCGGTAGAGCATCA GACTTTTAATCTGAGGGTCCAGGGTTCAAGTCCCTGTCCAGGCG-3') was used as a ligand. Initially, Dynabeads M-280 Streptavidin (Invitrogen) was washed with 0.5X SSC buffer (0.75 M NaCl and 0.075 M sodium citrate) for 3 times and suspend the beads in 0.5X SSC buffer. To this reaction 5'-biotin tagged $tRNA^{Lys}_{TTT}$ at a final concentration of 0.2 μM was added and incubated at 65^0C for 10 min. After incubation, beads were washed with 0.1X SSC buffer for 3 times followed by equilibrating the beads in protein binding buffer (160 mM MOPS, 310 mM Sucrose, 6.25 mM $MgCl_2$, 100 mM KCl, 9 mM DTT, 3U/200 μl RNase inhibitor, 1 mg/ml BSA). The equilibrated dynabeads were incubated with the mitochondrial salt extract for 1 hr at 4^0C with gentle agitation. Beads were washed 3-5 times with protein binding buffer. Biotin tagged tRNA bound proteins were eluted by heating at 95^0C for 5 min in 2X SDS loading buffer [106] and separated on 12% SDS-PAGE and analyzed by either silver staining or immunoblotting with specific antibodies.

2.2.11 SDS-PAGE analysis

Purified recombinant proteins or mitochondrial fractions were separated on 12% SDS-PAGE [107]. Biotin tagged tRNA pull down proteins from the mitochondrial salt extract were resolved on 12% SDS-PAGE and analyzed by silver staining. Desired bands were excised and given for MALDI-TOF-TOF/MS/MS analysis.

2.2.12 Western blot analysis

Resolved proteins on SDS-PAGE were transferred to a Nitro Cellulose membrane (Pall) for overnight at 45 volts in western blot buffer (25 mM Tris base, 190 mM Glycine and 20% Methanol). The membrane was blocked with TBST (20 mM Tris-HCl pH-7.5, 150 mM NaCl, 0.05% Tween 20) containing 3% BSA for 1 hr at room temperature. The membrane was incubated with primary antibody in 3% BSA for overnight at 4^0C followed by three washes with TBST for 15 min. The membrane was incubated with HRP-conjugated secondary antibody for 2 hrs at room temperature followed by three washes with TBST for 15 min and developed the blot by using ECL reagents (GE-Healthcare) and VersaDoc (Bio-Rad).

2.2.13 Urea polyacrylamide gel electrophoresis

Equal amount of 2x urea loading dye (7 M Urea, 1x TBE, 0.01% Bromophenol Blue and 0.02% Xylene Cyanol FF) was added to RNA sample and subjected to boiling at 65^0C for 5 min. Samples were immediately cooled on ice and then resolved on 12% acrylamide (from 40% stock solution containing 38 gm acrylamide & 2 gm bis acrylamide), 7 M urea gel in Tris-borate-EDTA buffer. RNA Samples were resolved until the xylene cyanol reaches to the ¾ the gel. Gel was dried in gel dryer and analyzed by autoradiogram [108].

2.2.14 Cloning of human ABRA

2.2.14. 1 Polymerase Chain Reaction (PCR)

Full length ABRA (Actin Binding Rho Activating Protein) was amplified by polymerase chain reaction using pCMV-XL5-ABRA (from OriGene) as a template. Amplification reaction was carried out in a final volume of 50 µl. The reaction mixture contains 10 pM of each forward primer NB169 Fwp: 5'- CCCAGAATTCACCATGGCTCCGGGCGAAAAGGAA -3' (*E.CoRI*) and reverse primer NB170 Revp: 5'- AACCCTCGAGTCACTTGAGTAGCGTAATCACAAC -3' (*XhoI*) specific for *hABRA*, 2.5 mM each of four dNTPs, 1x PCR reaction buffer, 0.5 U of DNA polymerase enzyme and 500 ng of pCMV-XL5-ABRA. The reaction was performed with an initial denaturation step at 95 ^{0}C for 4 min, followed by 35 cycles of 94 ^{0}C for 45 sec, 59 ^{0}C for 1 min and 72 ^{0}C for 2 min; and a final extension at 72 ^{0}C for 10 min. The amplified product was resolved on 1% agarose gel electrophoresis and purified by gel extraction method (QIAGEN).

2.2.14. 2 Restriction Digestion

The amplified *hABRA* and pGEX4T1 vector were subjected to double digestion with *E.coRI* and *XhoI* restriction enzymes in a 50 µl reaction {(Insert 30 µl; 10x Tango Buffer 10 µl; autoclaved milli Q water 9 µl; *E.coRI* 0.5 U; *XhoI* 0.5 U) and (pGEX4T1 vector 30 µl; 10x Tango Buffer 10 µl; autoclaved milli Q water 9 µl; *E.coRI* 0.5 U; *XhoI* 0.5 U)} at 37 ^{0}C for overnight. The digested products were resolved on 1% Agarose gel electrophoresis and the digested products were excised and gel purified by QIAGEN gel extraction method.

2.2.14.3 Cloning of hABRA into pGEX41 vector

The double digested *hABRA* fragment was cloned into same sites of pGEX4T1 vector by using T4 DNA Ligase (Fermentas). The reaction was carried out in a final volume of 10 µl with 200 ng of vector, 3 fold excess of insert, 1 µl of T4 DNA Ligase and 1 µl of 10x T4 DNA Ligase Buffer. The

reaction mixture was incubated at 22^0C for overnight and ligated product was transformed into *E.coli* DH5α competence cells.

2.2.14.4 Bacterial transformation

The ligated product (10 µl) was incubated with *E.coli* DH5α competent cells for 30 min on ice. The cells were subjected to heat shock at 42^0C for 90 sec and chilled on ice for 5 min. To this reaction, 1 ml of LB medium was added and incubated at 37 ^{0}C shaker incubator for 1 hr and the culture was plated onto LB agar plate containing antibiotic (Ampicillin). The colonies were screened for the presence of cloned fragment by double digestion with *E.coRI* and *XhoI* restriction enzymes and sequence was confirmed by automated sequencer.

2.2.15 Bacterial expression and protein Purification

2.2.15.1 Expression of GST tagged ABRA

The plasmid pGEX4T1 harbouring *hABRA* were transformed into *E.coli* Rosetta gami strain. A colony carrying the pGEX4T1-*hABRA* plasmid was grown for overnight in LB medium containing Ampicillin at 37^0C shaker incubator. The primary culture was diluted to 1:100 in 500 ml fresh LB medium, grown with vigorous agitation until the growth reaches to $OD_{600\ nm:}$ 0.6. Then the bacterial cells were induced with 1 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) and incubated at 37^0C for 3 hrs. After induction, the bacterial cells were pellet down by centrifugation at 10,000 rpm for 10 min. The cell pellet was suspended in ice cold 1x PBS, pH-7.5 (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) by $1/20^{th}$ volume and subjected to sonication in the presence of 1mg/ml lysozyme and protease inhibitor (1 mM PMSF). After sonication, Triton X-100 was added to the final concentration of 1% and mixed gently for 30 min to solubilize the protein.

2.2.15.2 Purification of GST-ABRA by Glutathione sepharose 4B beads

The soluble fraction containing GST-hABRA was incubated with 1x PBS, pH-7.5 equilibrated glutathione sepharose 4B beads (GE Healthcare) for 2 hrs at room temperature with gentle agitation. After incubation, glutathione beads were passed through the column followed by washing with 10 bed volumes of 1x PBS, pH-7.5. The bound recombinant GST-ABRA was then eluted from the beads by incubating the beads with elution buffer containing 25 mM reduced glutathione in 1x PBS, pH-7.5.

2.2.16 Silver staining

Mitochondrial salt extract proteins were resolved on 12% SDS-PAGE and proteins were fixed on the gel by fixer buffer (50% Methanol, 12% Acetic acid and 0.05% Formalin) for overnight on rocker. After fixation, gel was washed with 35% Ethanol for 20 min x 3 times. Gel was then sensitized with 0.02% $Na_2S_2O_3$ for 2 min followed by 3 washes with autoclaved water for 5 min each. After washing, gel was incubated with staining solution (0.2% $AgNO_3$, 0.076% Formalin) for 45 min on rocker. Excessive staining was removed by washing the gel with autoclaved water for 1 min x 2 times. Finally, gel was developed with developer solution (6% Na_2CO_3, 0.05% Formalin, and 0.0004% $Na_2S_2O_3$) till the proteins get appeared and the staining reaction was stopped by keeping the gel in stopper solution (50% Methanol and 12% Acetic acid) for 5 min. Desired protein bands were excised from the gel and analyzed by MALDI-TOF-TOF/MS/MS analysis.

2.3 RESULTS

2.3.1 Purity and Integrity of isolated rat liver mitochondria

The purity of isolated mitochondrial fraction was corroborated by western blotting with organelle specific antibodies. Isolated mitochondrial, nuclear, ER, post mitochondrial and mitoplast fractions were separated on SDS-PAGE, western transferred and probed with antibodies specific for each

organelle fraction. As shown in Fig.2.1A, mitochondrial fraction was enriched with outer mitochondrial membrane protein, Porin and the inner mitochondrial membrane protein, COXIV but not with other organelle specific proteins, BIP (Endoplasmic Reticular marker) and PARP-1 (Nuclear marker) suggesting that isolated mitochondria was pure and substantially free of nuclear and microsomal components. The purified mitochondria were further examined by performing an *in vitro* protein import assay with positive control protein, [^{35}S] labeled Su9-DHFR which has a presequence of subunit 9 of F_1F_0 ATPase. As shown in Fig.2.1B, [^{35}S] labeled Su9-DHFR was taken up by purified mitochondria and processed by mitochondrial matrix metalloprotease. Upon import, Su9-DHFR was protected from the protease treatment alike mitochondrial matrix protein, Aconitase (Fig.2.1B, compare top panel with bottom panel). All these results suggest that the isolated mitochondrial fraction was indeed pure and functionally active.

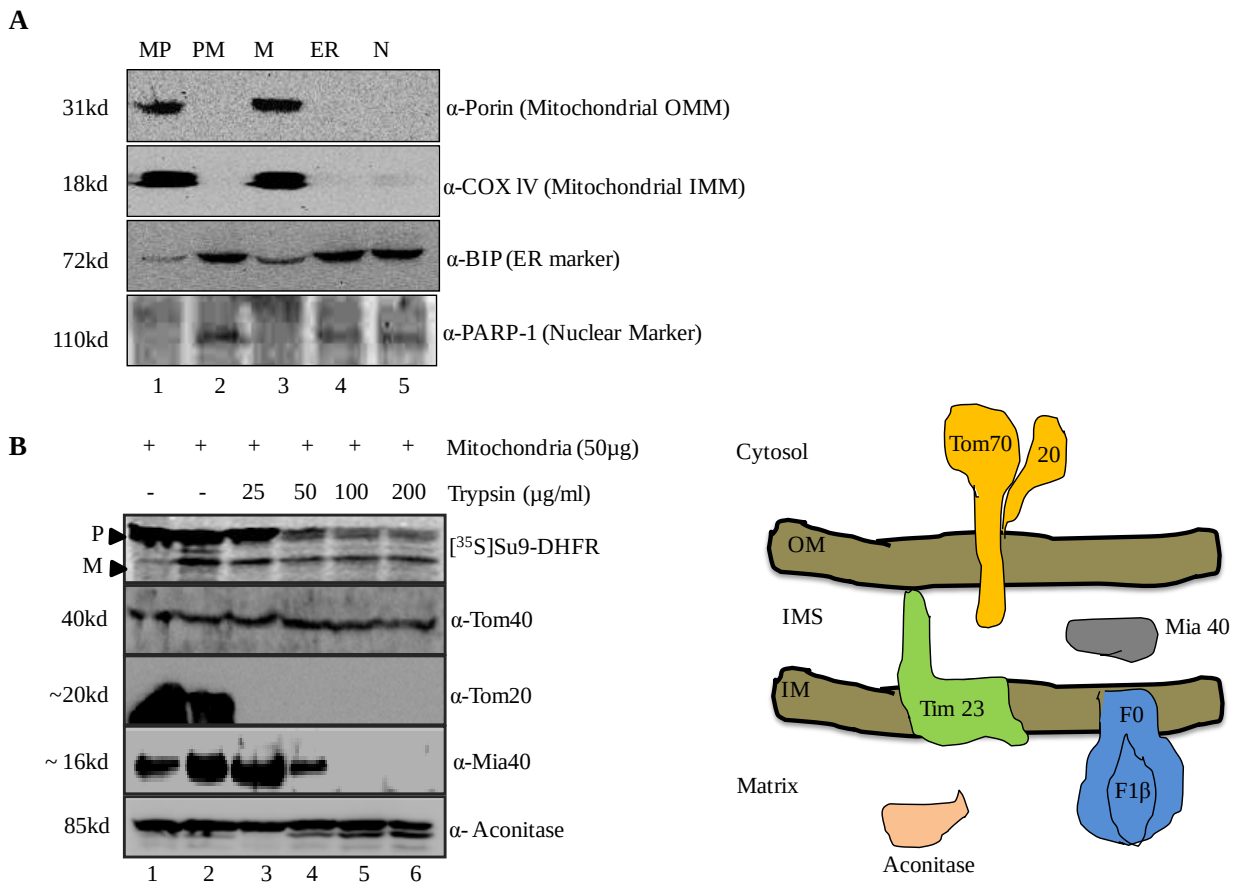


Fig.2.1 Checking the purity and integrity of isolated rat liver mitochondria: (A) Isolated mitochondrial, nuclear, ER, post mitochondrial and mitoplast fractions were separated on SDS-PAGE, western transferred and probed with antibodies specific for Porin, COXIV, BIP and PARP-1. **(B)** *In vitro* import of ^{35}S-Su9-DHFR was performed for 60 min as described in the methods. After import, samples were treated with increasing concentration of Trypsin. After inhibition of Trypsin with Trypsin inhibitor, samples were reisolated, resolved on SDS-PAGE (P: precursor, M: mature) and analyzed by phosphorimager. Fractions of mitochondria were also probed with mitochondrial membrane marker proteins as indicated in the figure.

2.3.2 Mammalian mitochondria have the innate ability to import multiple cytosolic tRNAs

It has been shown that isolated mammalian mitochondria can import tRNA^{Gln} and yeast tRNA ^{Lys}.

To test whether mammalian mitochondria have the inherent ability to import any externally

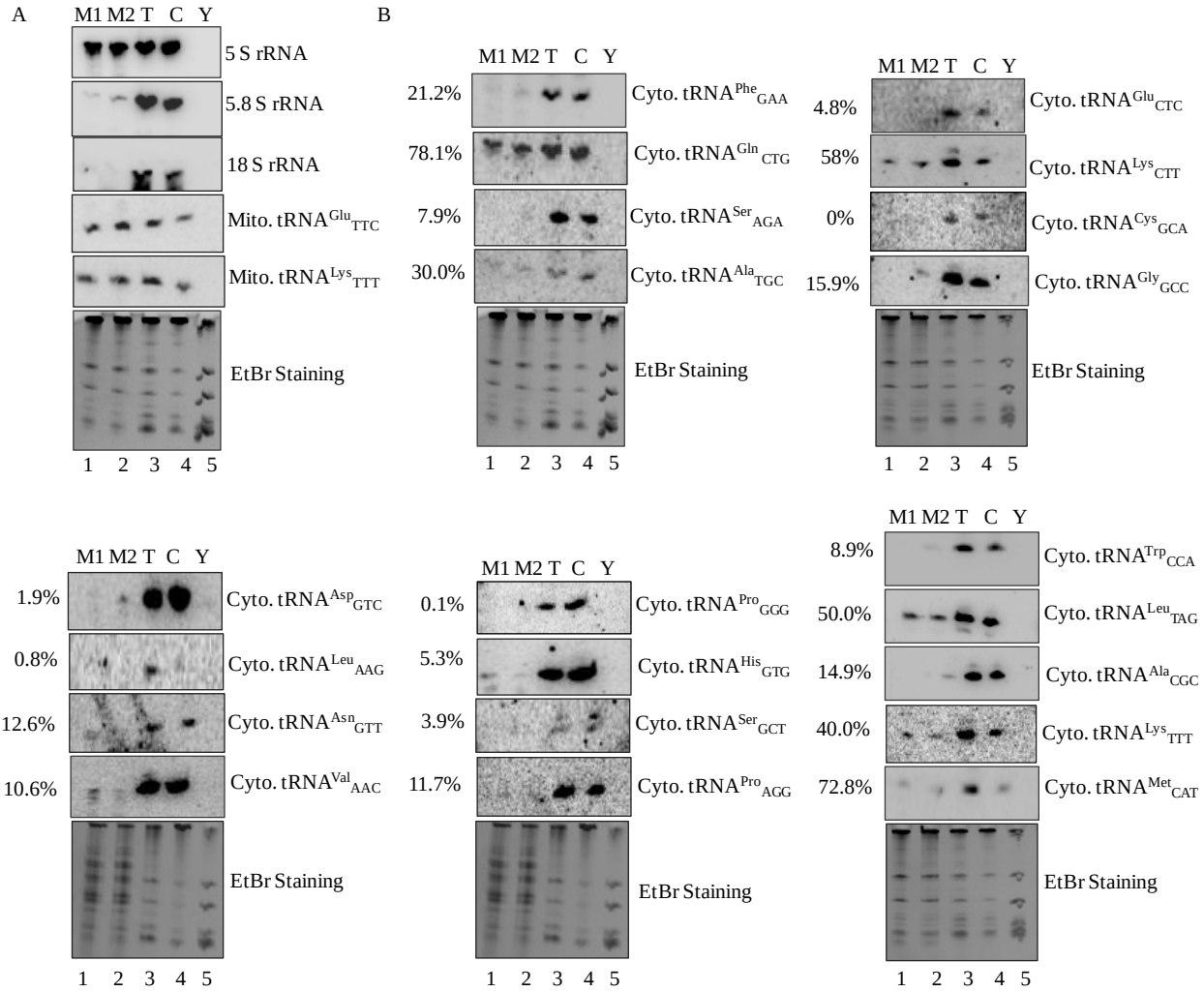


Fig.2.2 Purified mammalian mitochondria enriched with very few cytosolic tRNAs: Northern blots with two sets of 0.75 μg of each mitochondrial (pretreated with RNase A) (M1&M2), Total (T) cytosolic (C) and yeast(Y) total RNAs. The oligonucleotide probes specific for tRNAs were given in table 1. Hybridization signals were quantified, and the ratio between the mitochondrial (M) signal and the cytosol (C) signal was indicated. **(A)** Northern blotting was performed with rRNAs or mitochondrial tRNAs to check the cytosolic rRNA contamination or enrichment of mitochondrial tRNAs in mitochondrial fraction respectively. **(B)** Northern blot hybridization with 21 cytosolic tRNAs (also includes the isoacceptor for single tRNA). The total amount of RNA for each fraction used for northern blotting was shown by ethidium bromide staining.

added tRNAs, initially we examined the endogenous levels of various cytosolic tRNAs in highly purified mitochondria isolated from HEK293T cells. Isolated mitochondria were treated with RNase A and total RNA was extracted, resolved on 10% polyacrylamide gels and subjected to northern blot hybridizations as described in the methods section. Cytosolic 5S rRNA, 5.8S rRNA and 18S rRNA probes were used to monitor cytosolic contamination of mitochondrial RNA. High resolution northern blot hybridization for 21 cytosolic tRNAs including different isoacceptors for a single tRNA was carried out as described in the methods section. Of the 21 cytosolic tRNAs, we were able to detect the tRNAs in the mitochondria albeit at various levels ranging from 0% to 78% of their cytosolic level (Fig. 2.2B). Amongst the tRNAs that were detected in mitochondria, we find that five tRNAs ($tRNA^{Gln}_{CTG}$, $tRNA^{Leu}_{TAG}$, $tRNA^{Lys}_{TTT}$, $tRNA^{Met}_{CAT}$ and $tRNA^{Lys}_{CTT}$) are enriched at levels greater than 50% of their cytoplasmic levels. Intriguingly, we find correlation between the enrichment of most of the cytoplasmic tRNAs in the mitochondria to its codon usage by mitochondrial DNA for gene expression. However, one has to note that as the percent of tRNA enrichment is dependent on its cytoplasmic concentration, in certain cases although the enrichment may be higher, the actual concentration in the mitochondria may be lower than some of the tRNAs that had lower enrichment. Most importantly, the repertoire of tRNAs imported by mammalian mitochondria extends beyond two tRNAs that had been previously described and the aforementioned results show that mammalian mitochondria have an intrinsic ability to import cytoplasmic tRNAs.

2.3.3 Mitochondrial tRNA import is distinct from protein import

To explore the possibility of tRNA import into mammalian mitochondria, we have performed *in vitro* tRNA import assay by incubating ^{32}p-radiolabeled human $tRNA^{Lys}_{TTT}$ with isolated mitochondria. Import of radiolabeled $tRNA^{Lys}_{TTT}$ was confirmed by its resistance to externally added RNase A. As shown in Fig.2.3A (compare lanes 1 & 2), imported radiolabeled $tRNA^{Lys}_{TTT}$ was resistant to RNase A which suggests that mammalian mitochondria has inherent ability to take up externally added tRNAs. In all other known cases [62, 64, 98, 109-110], tRNA import into mitochondria is an ATP requiring process. We have tested the energy requirements in tRNA import into mammalian mitochondria. As shown in Fig.2.3A (compare lanes 3 & 4), the translocation of tRNA into rat liver mitochondria also depends on the energy (ATP and GTP).

It was shown that membrane potential is needed for the import of number of cytosolic tRNAs into isolated plant mitochondria [111] in addition to the import of $tRNA^{Lys}$ into yeast mitochondria. Interestingly, when we performed import assays in the presence of membrane potential dissipators, Valinomycin (Fig.2.3A, lanes 5-8) or CCCP (Fig.2.3B, lanes 1-4), there was no effect on tRNA import efficiency into mammalian mitochondria unlike protein import, where CCCP treated mitochondria lost their ability to import [^{35}S] labeled Su9-DHFR (Fig.2.3C, lanes 3-5). All these results suggesting that, tRNA import into mammalian mitochondria is facilitated by pathway distinct from protein import. Nevertheless, this conclusion does not rule out the possible involvement of protein factors in mitochondrial tRNA import.

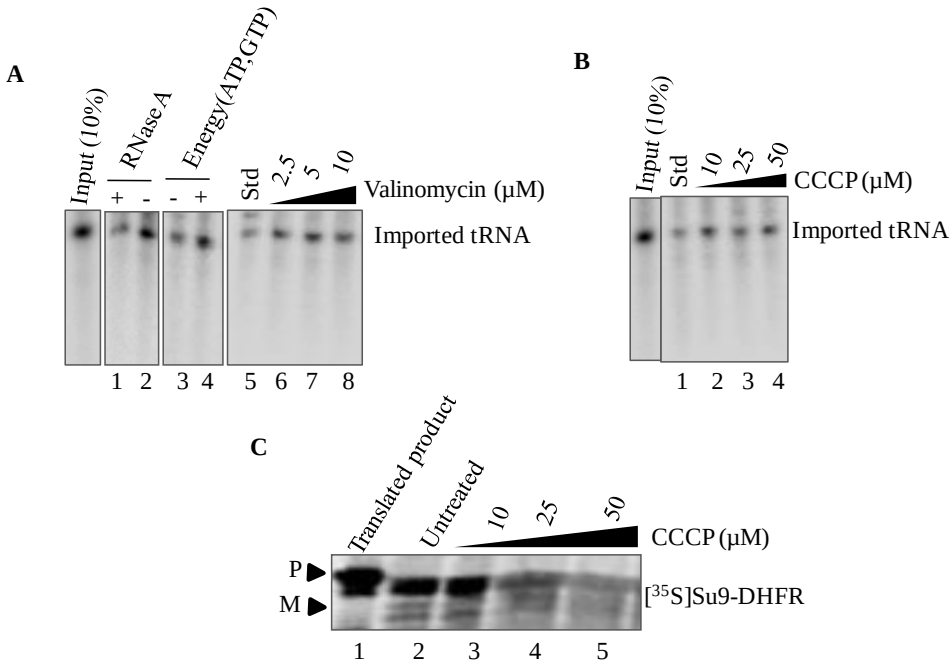


Fig.2.3 tRNA import depends on energy but not on membrane potential: (A & B) *In vitro* import of ^{32}p-radiolabeled tRNA was performed in the presence or absence of RNase A or energy (2 mM ATP & 2 mM GTP) or in the absence of membrane potential by incubating mitochondria with valinomycin or CCCP. **(C)** Import of ^{35}S-Su9-DHFR was examined in the absence of membrane potential by incubating mitochondria with increasing concentrations of CCCP in the import reaction. After incubation, mitochondrial samples were reisolated, resolved on SDS-PAGE (P: precursor, M: mature) and analyzed by phosphor imager.

2.3.4 Mammalian mitochondrial tRNA import requires outer mitochondrial membrane proteins

It has been shown that mitochondrial import of tRNA is independent of cytoplasmic factors but dependent on energy in the form of ATP [64]. We hypothesized that cytosolic factor(s) that are tightly associated with the mitochondrial membrane may influence the import of tRNAs into mitochondria. To examine the possible involvement of membrane bound protein factors in mammalian mitochondrial tRNA import, we obtained mitochondrial fraction from rat liver as described in the methods section and pre-treated it with proteinase K. An *in vitro* tRNA import assay was carried on the proteinase K treated mitochondria in the presence of ^{32}p-labeled $tRNA^{Lys}_{TTT}$ as described in the methods. An increase in proteinase K concentration during the pre-treatment of

mitochondria prior to tRNA import resulted in a decrease in the internalization of tRNA import in a dose dependent manner (Fig.2.4A, top panel, lanes 2-4). To test the efficacy of proteinase K treatment, mitochondrial fractions pre-treated with proteinase K were subjected to immunoblot analysis using antibodies to detect Tom20 and aconitase-2 proteins. A major portion of Tom20, an outer membrane protein of mitochondria is exposed to the cytosol while aconitase-2 is a mitochondrial matrix protein. Pre-treatment of mitochondria with proteinase K completely digests Tom20 as we fail to see Tom20 in the immunoblot (Fig.2.4A, top panel, lanes 2-4). However, acontiase-2 is still present in the pre-treated mitochondrial fraction (Fig.2.4A, bottom panel). Further, Ethidium bromide stained gel shows the equal amount of endogenous RNA in all the samples. Taken together, our results suggest that the mitochondrial membrane bound and/or cytosolic exposed protein component(s) of the mitochondrial outer membrane are required for import of cytosolic tRNAs into mitochondria.

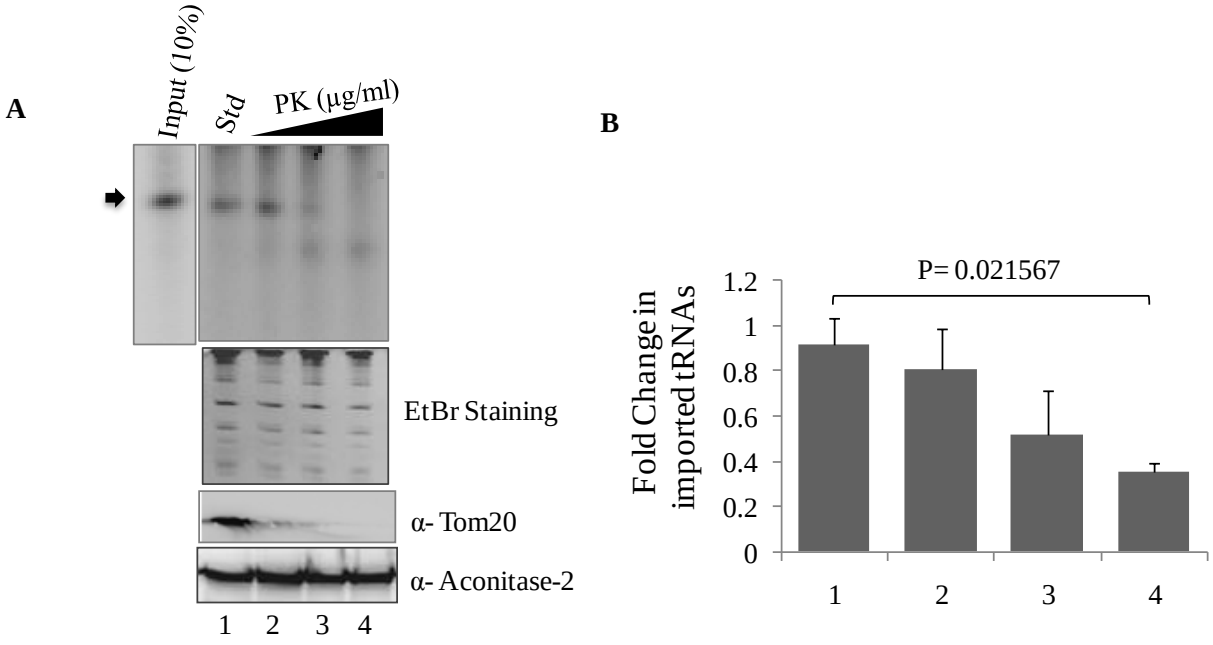


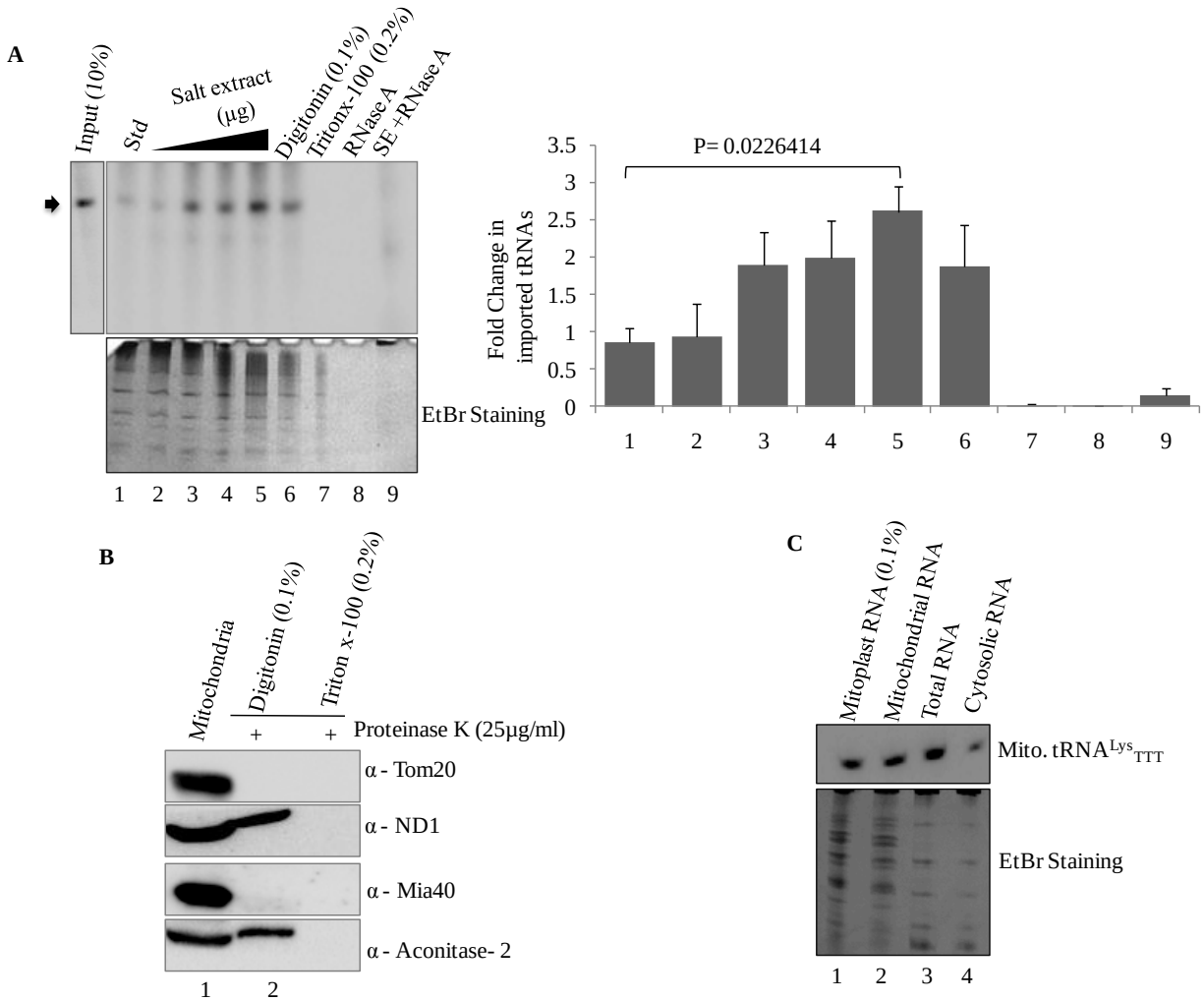
Fig.2.4 tRNA import requires outer mitochondrial membrane proteins: (A) Mitochondria were pretreated with proteinase K (10, 25 and 50 μg/ml) for 15 min on ice. After inhibition of proteinase k by 1 mM PMSF, mitochondria were reisolated and performed tRNA import assay by incubating with ^{32}p-radiolabeled tRNA in import reaction as described in methods. Total RNA was extracted, separated on urea-acrylamide gel, EtBr stained and analyzed by phosphorimaging. The other fraction of proteinase K treated mitochondria were separated on SDS-PAGE followed by immunoblotting with antibodies specific for Tom20 (outer mitochondrial membrane marker) and aconitase-2 (mitochondrial matrix marker). **(B)** Quantitative representations for band intensities of imported tRNAs were done by densitometry. Statistical analysis was performed using *t- test*.

2.3.5 Mitochondrial salt extract stimulates cytoplasmic tRNA import into mitochondria

As we observed the involvement of mitochondrial membrane bound and/or cytosolic exposed protein component(s) of the mitochondrial outer membrane in tRNA import, next we sought to determine the role of membrane bound factors in tRNA import. To remove membrane associated proteins, we treated highly purified mitochondrial fraction with 0.5 M KCl. The salt treated mitochondria were subjected to centrifugation and the resulting supernatant/salt extract was dialyzed and concentrated. An *in vitro* mitochondrial tRNA import assay was carried out in the presence and absence of salt extract and the imported tRNA was analyzed by phosphorimager as described in the methods section. Addition of increasing concentration of salt extract to the intact mitochondrial fraction stimulates the tRNA import in a dose dependent manner (Fig.2.5A, lanes 2-5). In the absence of mitochondrial fraction, tRNA was vulnerable to digestion with RNase A in the presence or absence of salt extract (Fig.2.5A, lanes 8 & 9). These results confirm that the observed enhancement in tRNA import in presence of salt extract is not due to any non-specific increase in stability of the tRNA in presence of salt wash.

To further confirm the presence of imported tRNA in the mitochondrial matrix fraction, the outer membrane of mitochondria was selectively permeabilized with digitonin prior to RNase A treatment. Digitonin treatment does not significantly reduce the imported tRNA levels (Fig.2.5A, compare lanes 5 & 6). However, the imported tRNA is susceptible to RNase A treatment when the

mitochondrial membranes have been solubilized with Triton X-100 (Fig.2.5A, compare lanes 6 & 7). To assess the permeabilization status of mitochondrial membranes, digitonin and Triton X-100 treated mitochondrial samples were further subjected to protease treatment, separated on SDS-PAGE and probed with antibodies against proteins that are hallmarks of mitochondrial outer membrane, inner membrane, inter membrane space and matrix. Digitonin treatment causes the degradation of Tom20 and Mia40 that are resident in the outer membrane and inter membrane space of the mitochondria respectively whereas the matrix localized aconitase-2 is seen to be protected. However, all the mitochondrial proteins that were tested are sensitive to protease digestion when the membranes were solubilized with Triton X-100 (Fig.2.5B, compare



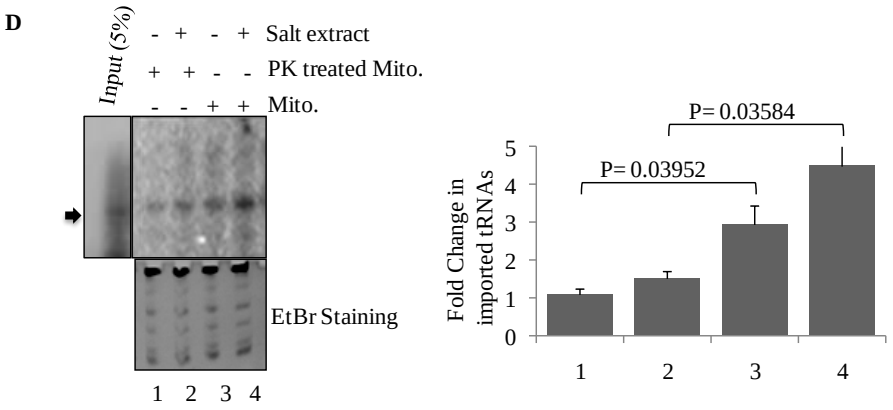


Fig.2.5 Outer mitochondrial membrane associated salt extractable factors stimulate the tRNA import into mammalian mitochondria: All *in vitro* tRNA import assays were performed as described under methods. **(A)** ^{32}p-radiolabeled tRNA was incubated with mitochondria in the presence of increasing concentrations of mitochondrial salt extract (0.5, 2, 4 and 6 μg). The internalization of tRNA was assayed in mitochondria samples after the import by treating with digitonin (0.1%) or triton x-100 (0.2%) for 10 min on ice followed by RNase A (100 μg/ml) treatment. **(B)** Digitonin or triton x-100 solubilized mitochondria were treated with proteinase K and re-isolated the mitochondria by centrifugation and separated on SDS-PAGE followed by immunoblotting with antibodies specific for Tom20, ND1 (inner mitochondrial membrane marker), Mia40 (Inter membrane space marker) and aconitase-2 (mitochondrial matrix marker). **(C)** Total RNA was extracted from RNase A treated mitochondria or digitonin (0.1%) solubilized mitochondria and run on 10% denaturing PAGE. Northern blot was performed with oligonucleotide probe specific for mitochondrial $tRNA^{Lys}_{TTT}$. **(D)** Proteinase k pretreated mitochondria (25 μg/ml) or untreated mitochondria were incubated with ^{32}P-radiolabeled tRNA in the presence or absence of mitochondrial salt extract. Quantitative representations for band intensities of imported tRNAs were done by densitometry. Statistical analysis was performed using *t- test*.

lanes 2 & 3 with lane 1). In addition, we studied the level of endogenous $tRNA^{Lys}$ in the mitochondria with and without digitonin by northern blot analysis. Our results show that digitonin treatment does not affect the integrity of the mitoplasts as comparable levels of $tRNA^{Lys}$ were detected in the presence and absence of digitonin (Fig.2.5C, lanes 1 & 2). The above results clearly show that mitochondrial associated cytosolic factor(s) are required to stimulate the import of tRNAs into mammalian mitochondria. These results also suggest that membrane associated factors are rate limiting for the mitochondria import of tRNA.

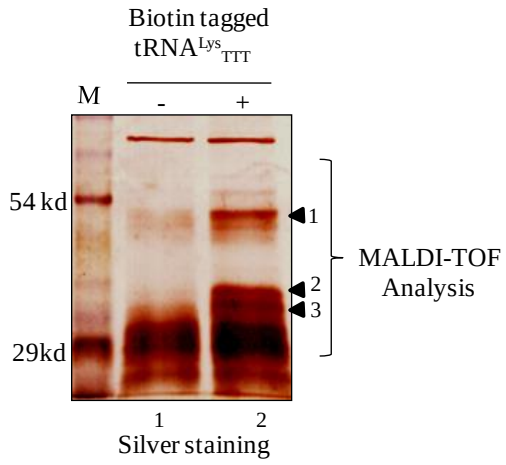
Interestingly, when we incubated the proteinase K treated mitochondria with mitochondrial salt extract, there was no enhancement in the tRNA import (Fig.2.5D, lanes 2 & 4). This result suggests

the possible involvement of mitochondrial surface receptors besides cytosolic protein factors for mammalian mitochondrial tRNA import.

2.3.6 Identification of tRNA recruitment factor(s) in the salt extract by biotin pull down assay

To identify the protein component(s) present in the salt extract that are responsible for stimulating mitochondrial tRNA import, biotin pull down assay was carried out. Mitochondrial salt extract was incubated with 5'-biotin tagged $tRNA^{Lys}_{TTT}$ followed by incubation with streptavidin magnetic beads as described in the methods to facilitate binding of biotin labeled tRNA to the beads and thereby pull down proteins bound to the tRNA. Proteins bound to the biotin tagged tRNA were eluted, separated on SDS-PAGE and silver stained. To monitor non-specific binding of the salt extract proteins to the beads, salt extract was incubated with the streptavidin beads and the eluate was used as an internal control (Fig.2.6A, compare lanes 1 & 2). We observed that three major proteins that were exclusively present in the eluate of the biotinylated tRNA-streptavidin beads (Fig.2.6A, lane 2). The proteins with molecular weight around 43 kDa, 37 kDa and 35 kDa were identified as an Actin Binding Rho Activating protein (ABRA), Guanine Nucleotide Binding protein subunit beta-4 (GB) and Rho Guanine Nucleotide Exchange Factor 39 (RhoGEF39) respectively by MALDI-TOF/MS/MS analysis.

A



B

S.No.	Name of the identified protein	Matched peptides
1	Actin Binding Rho Activating protein	5
2	Guanine Nucleotide Binding protein beta-4	4
3	Rho Guanine nucleotide Exchange Factor 39	4

Fig.2.6 Identification tRNA recruitment factors from mitochondrial salt extract: (A) Biotin pull down assay was performed by incubating the pre-cleared mitochondrial salt extract (500 μg) and full length biotin tagged $tRNA_{TTT}^{Lys}$ (1 μM) with streptavidin M-280 dynabeads as described in the methods. Eluted proteins were separated on SDS-PAGE followed by silver staining and subjected to MS/MS analysis. **(B)** Table representing the identified proteins and the number of matching peptides.

As we identified Actin Binding Rho Activating protein as one of the tRNA recruiting factor into mammalian mitochondria, we further investigated the possible involvement of ABRA in mammalian mitochondrial tRNA import. ABRA has been identified earlier as a component of protein translocation in between nuclear and cytosolic compartments and known to involve in up regulation of sequence-specific DNA binding transcription factor activity. ABRA is a Rho GTPase activating protein and acts as a positive regulator in Rho protein signal transduction [112].

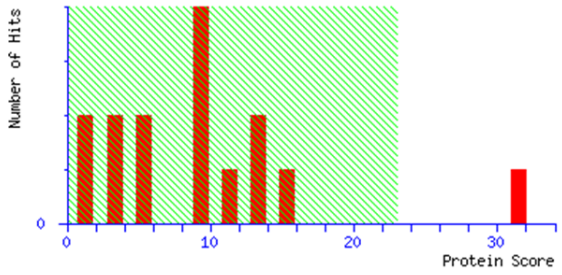
To confirm the MALDI-TOF/MS-MS result, we carried out western blot analysis of the eluate from the tRNA biotin pull down assay using an antibody that specifically recognizes ABRA (Fig.2.7B, lane 4). Our results clearly show the presence of ABRA in the eluate. Mitochondrial fraction and

mitochondrial salt extracts were probed for the presence of ABRA, Tom20 (mitochondrial outer membrane protein), Mia40 (mitochondrial intermembrane space protein) and Hsp70 (mitochondrial matrix protein) proteins by western blotting. Both mitochondrial fraction and mitochondrial salt fraction are found to be enriched in ABRA (Fig.2.7C). However, only the mitochondrial fraction contains Tom20, Mia40 and Hsp70 while the mitochondrial salt fraction is devoid of these mitochondrial proteins indicating that the mitochondria is intact and there was no leaching of mitochondrial proteins into the salt fraction (Fig.2.7C).

A

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 23 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Matched peptides for *ABRA* shown in *red*.

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  1 MAPGETVREA GPAKSALQKV RRATLVINLA RGWQQWANEN STRQAQEPAG WLPGATQDLP
 61 HTPKEPGPRQ HAPKPPSPKP DGDREGRGSE EATEVSHIKR KEVTRTVVSK AYERGGDVNY
121 LSHRYEHDGG VSEAVQPDND IDRILLSHDS PTRRRKCTNL VSKLTKGWKV MEQEEPKWKS
181 DSIDTEDSGY GGDMEERPEQ DVAQVAAARI KRPLHSQANR YSETLNCKAH RKYSQVDNLK
241 GRWQQWADEH IQSQKLNPFS DEFDYDLAMS TRLHKGDEGY GRPKEGSKTA ERAKRAEEHI
301 YREIMELCFV IRTMARHRRD GKIQVTFGEL FDRYVRISDK VVGILMRARK HGLVHFEGEM
361 LWQGKDDHVV ITLLE
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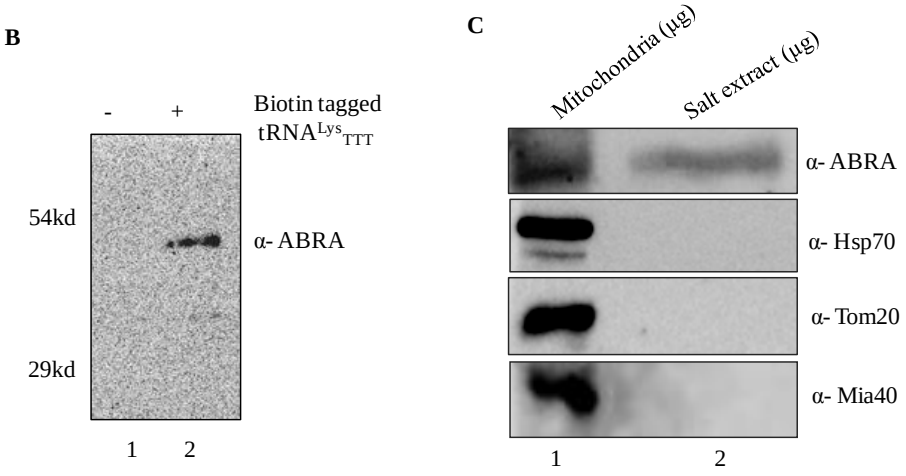


Fig.2.7 Identification of ABRA by MALDI-TOF analysis: (A) The protein band excised and performed in gel digestion with trypsin followed by peptide analysis using mass spectrometry. The peptide mass fingerprint (PMF) obtained by MALDI-TOF/MS identified as ABRA protein with the mascot search parameters. Peptides matched to ABRA are indicated in red. **(B)** Validating the presence of ABRA in the eluted fraction by immunoblotting with anti-ABRA antibody. **(B)** Total mitochondria (100 μg) and mitochondrial salt extract (100 μg) were lysed in SDS sample buffer and separated on SDS-PAGE and probed with specific markers of mitochondria as mentioned in the figure.

Having established that one of the prominent protein that binds to biotin labeled tRNA from the salt extract is ABRA, we wished to elucidate the importance of ABRA in stimulating mitochondrial tRNA import. Towards this end, we have cloned *hABRA* into pGEX4T1 vector. Briefly using pCMV-XL5-ABRA as a template, the *hABRA* was amplified by using *hABRA* ORF specific primers (NB169 and NB170) and the amplified product was cloned into pGEX4T1 vector for bacterial expression as described in the methods (Fig.2.8A). The expressed recombinant GST-ABRA was purified by Glutathione sepharose 4B beads (GE Healthcare) and resolved on SDS-PAGE. Purified GST-ABRA migrates as a 66 kDa protein on SDS-PAGE. Purity of the recombinant GST-ABRA was analyzed by coomassie staining and immunoblotting with anti-ABRA antibody (Fig.2.8B).

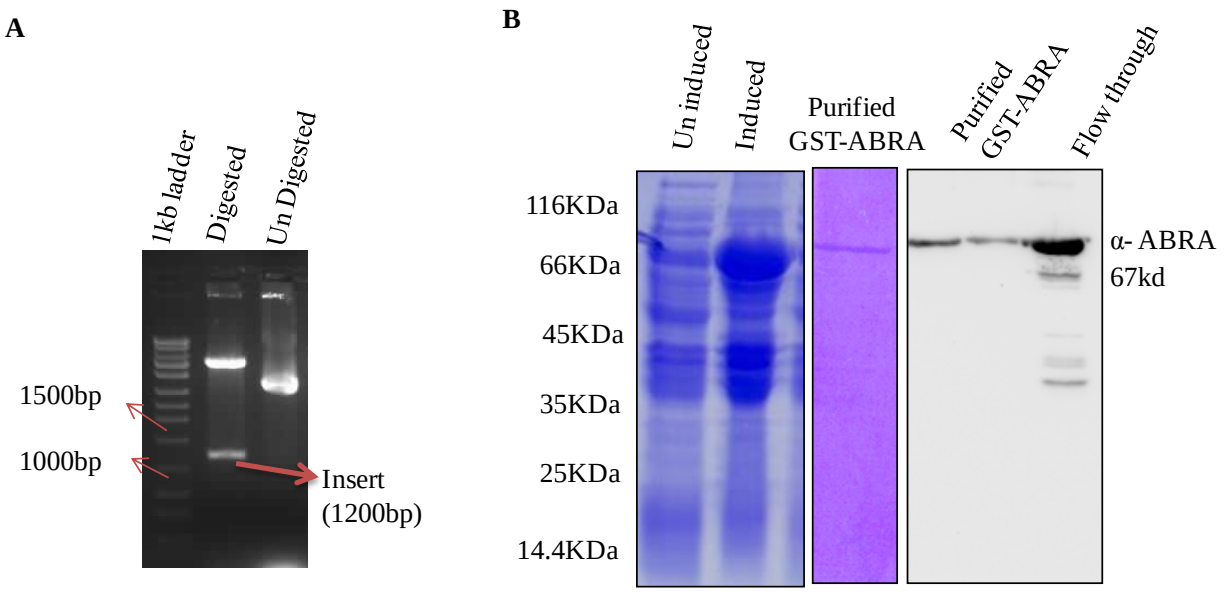


Fig.2.8 Cloning, expression and purification of hABRA: hABRA was cloned into bacterial expression vector pGEX4T1. **(A)** Restriction digestion of pGEX4T1-hABRA with *EcoRI* & *XhoI* enzymes followed by 1% Agarose gel electrophoresis to confirm the presence of amplified insert. **(B)** Bacterial expression of GST tagged ABRA upon induction of bacterial cells with 1 mM IPTG. GST-ABRA was purified to homogeneity as indicated in the methods (Middle Panel). Purity of GST-ABRA was examined by immunoblotting with anti-ABRA antibody (Right Panel).

2.3.7 ABRA stimulates mitochondrial tRNA import in vitro

Having confirmed that we have indeed purified GST-ABRA, we carried out a standard *in vitro* tRNA import assay using mitochondria from rat liver and ^{32}p-radiolabeled $tRNA^{Lys}_{TTT}$ in the presence and absence of recombinant GST-ABRA as mentioned in the methods. As shown in Fig.2.9A, GST-ABRA stimulates the import of tRNA into mitochondria in a dose dependent manner. Addition of equal concentration GST does not have any effect on tRNA import (Fig.2.9A, lanes 2 & 6).

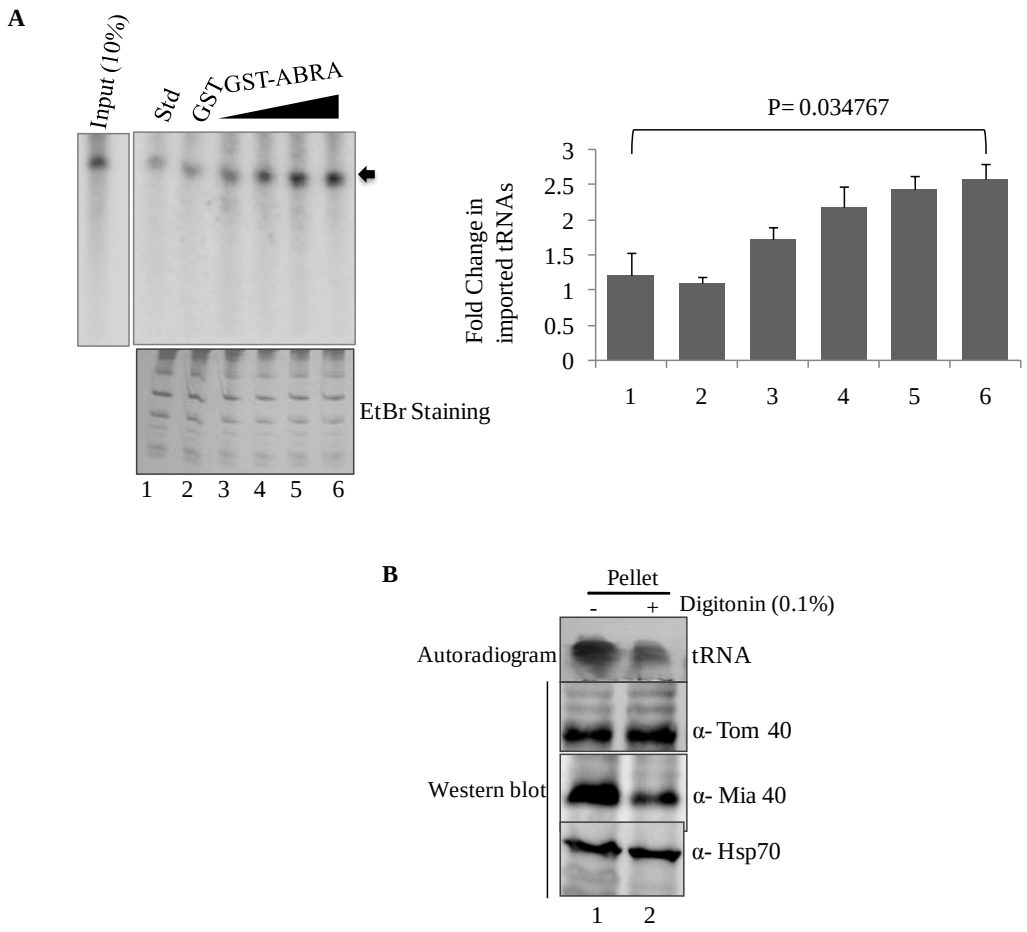


Fig.2.9 Recombinant ABRA stimulates the tRNA import into mitochondria. (A) *In vitro* tRNA import assay was performed by incubating purified mitochondria with ^{32}P-radiolabeled tRNA in the presence of increasing concentrations of recombinant GST-ABRA (0.2, 0.4, 0.6 and 1 μg) or GST (1 μg) as described in methods. After incubation, total RNA was extracted, resolved on denaturing PAGE and analyzed by phosphorimaging. Quantitative representations for band intensities of imported tRNAs were done by densitometry. Statistical analysis was performed using *t- test*. **(B)** The other fraction of mitochondria was solubilized in 0.1% digitonin and analyzed the presence of imported tRNA by autoradiogram (top panel). The integrity of digitonin solubilized mitochondria was analyzed by immunoblotting the mitochondrial fraction with specific mitochondrial markers indicated in the figure.

Further, the imported tRNA is present in the matrix fraction as permiabilization of mitochondrial outer membrane with digitonin prior to RNase treatment does not significantly reduce the imported tRNA levels (Fig.2.9B, lanes 1 & 2). These results implicate ABRA as one of the potential

cytoplasmic factors responsible for stimulating mitochondrial tRNA import as recombinant GST-ABRA is efficient in stimulating mitochondrial tRNA import *in vitro*.

2.4 DISCUSSION

It is well known that mitochondria in protozoans are totally dependent on cytosolic tRNAs for its protein synthesis [113]. It imports all the required tRNAs from cytoplasm. It has been generally assumed that the mitochondria in higher eukaryotes have lost their ability to import cytosolic tRNAs as the DNA in eukaryotic mitochondria had evolved to become mostly self sufficient. However, over the last couple of decades, research had revealed that mammalian mitochondria is able to still import tRNA but limited to just a couple [64, 91]. For the first time, our studies show that eukaryotic mitochondria are still endowed with its intrinsic ability to import a wide spectrum of cytosolic tRNAs based on requirement and at different efficiencies. Northern blot analysis on the import of twenty one cytosolic tRNAs into purified mitochondria revealed that more than 75% have been imported albeit to various extents (Fig. 2.1). Of the imported cytosolic tRNAs, we find that five are highly enriched in the mitochondria. Interestingly, we find a strong correlation between the highly enriched tRNAs and its codon usage in proteins synthesized within the mitochondria.

According to previous reports, the import of cytosolic tRNAs into mammalian mitochondria is independent of cytosolic factors [64]. Contrary to these findings, we provide evidence to show that cytoplasmic factors tightly associated with mitochondria stimulate tRNA import into mitochondria. This study identifies Actin Binding Rho Activating (ABRA) protein as one of the cytoplasmic factors that stimulate tRNA import into mammalian mitochondria (Fig.2.4A). Further, we also show the energy dependant and membrane potential independent import of tRNAs into mammalian mitochondria (Fig.2.3). We show that the purified, recombinant ABRA stimulates tRNA import into mammalian mitochondria in a dose dependent manner *in vitro*. In addition, by using selective

permeabilization studies with digitonin, we demonstrate the presence of imported tRNA in mitochondrial matrix fraction (Fig.2.9A & B). ABRA is a Rho GTPase activating protein, known to be involved in various functions such as protein translocation into nucleus, positive regulation on Rho protein signal transduction and co-activator of transcription [112]. In this study, we adhere another function to ABRA, that of recruiting and importing cytosolic tRNAs into mammalian mitochondria.

Mitochondria associated diseases are a heterogeneous group of disorders, which have been defined by deficits in the mitochondrial respiratory chain. A number of mitochondrial diseases MERFF [114], MELAS [115] etc., have been associated with the mutations in mitochondrial DNA encoded tRNA genes. It should be possible to complement defects associated with human mitochondrial tRNA gene mutations by importing a functional tRNA from the cytosol. Thus investigating the mechanistic details of tRNA import into mammalian mitochondria would provide insights towards therapeutic interventions. In this perspective, our current study identifies the novel role of mitochondrial peripherally associated cytosolic factor, ABRA in the regulation of tRNA import. However, additional experiments are required to pinpoint the precise role of ABRA in delivering cytosolic tRNAs into mammalian mitochondria.

Chapter 3

Functional characterization of tRNA recruitment factor(s)

3.1 INTRODUCTION

Mitochondria are essential organelles of eukaryotes and involved in many cellular processes including respiration, oxidative phosphorylation-mediated ATP production, cell metabolism and apoptosis. According to Endosymbiotic theory, the transition from the bacterial endosymboint to present mitochondria has been escorted by many changes such as shrinkage of mitochondrial genome as most of the bacterial genes have been either lost or transferred to the host genome [26]. Consequently, mitochondrial biogenesis mainly relies on the expression of nuclear genome and trafficking of macromolecules such as proteins and tRNAs from cytosol to mitochondria [33]. While there is an extensive knowledge regarding mechanisms of protein import, the import of tRNAs from cytosol to mitochondria is poorly understood.

Although, mitochondria from all the organisms retain the genes for all mitochondrial rRNAs, the loss of few or more mitochondrial tRNA genes has been shown in considerable number of species [116]. Unlike early eukaryotes, mammalian mitochondria encode all the tRNAs required for mitochondrial translation. Nevertheless, mammalian mitochondria still have inherent ability to import tRNAs both *in vitro* and *in vivo* [64, 92]. However, till now very little is known about the precise mechanism of tRNA import into mammalian mitochondria. As we discussed earlier in the chapter II, our studies show that the involvement of mitochondrial outer surface associated cytosolic factor, ABRA in tRNA import into mammalian mitochondria.

As point mutations in mitochondrial tRNA genes lead to many of the mitochondrial disorders such as MERRF, MELAS etc [71], investigating the mechanistic details of tRNA import into mammalian mitochondria would provide novel insights towards therapeutic interventions. So, the present study was designed to investigate the mechanistic details of the cytosolic factor, ABRA in the regulation of mitochondrial tRNA import.

In this chapter, we find that over expression of ABRA rescues the mitochondrial translational defect in partial mtDNA depleted (rho zero) cells by regulating the import of cytosolic tRNAs. Further, we identified GB (GTP binding protein) and GEF39 (Guanine nucleotide exchange factor 39) as novel ABRA interacting proteins from the salt extracts of mitochondria by using *in vitro* GST pull down assay. We further show that ABRA, GB and GEF39 dependent GTP hydrolysis is required in transferring cytosolic tRNA across the mitochondrial membranes. Taken together, our study identifies a novel GTPase cycle on outer mitochondrial surface regulates the recruitment of tRNA to the mitochondria.

3.2 METHODOLOGY

3.2.1 Antibodies and Reagents

Antibodies used in this study are either purchased {ABRA (Sigma Aldrich), COXI, Aconitase-2, Tom20, β-Tubulin, ND1, Myc, Hsp70 (Abcam), anti-His, anti-GST (Santa Cruz)} or in-house raised (Tom40 and Mia40). All the chemicals used for this study were obtained from Sigma Aldrich, Amresco and Himedia, Merck and Sisco Research Laboratories (SRL). MELAS cell lines were a kind gift from Prof. K Thangaraj, CCMB, Hyderabad. ABRA specific siRNA duplex and universal scrambled negative control siRNA duplex plasmid constructs were obtained from OriGene (USA). Methionine tRNA synthetase was a kind gift from Michael P. King, TJU.

3.2.2 Labeling of tRNA by aminoacyl tRNA synthetase

Total RNA was isolated from E.coli cells by TRIZOL (Invitrogen) method. Methionine tRNA population was specifically separated from bacterial total RNA by using methionine tRNA specific antisense primer with biotin tag (Table 3.1, Primer No. NB 353). Briefly, biotin tagged primer was incubated with Dynabeads M-280 Streptavidin beads (Invitrogen) for 15 min at room temperature.

Bacterial total RNA was then incubated with these beads for 1 hr at 4^0C. Beads were washed with wash buffer (1 mM EDTA, 0.2 M NaCl, 10 mM Tris-HCl, pH-7.5) and methionine tRNA was eluted by boiling the beads at 80^0C for 1 min in 0.1% SDS. The eluted E.coli methionine tRNA (5 μM) was incubated with labeling reaction mixture (30 mM HEPES pH-7.2, 30 mM KCl, 40 mM $Mgcl_2$, 2 mM ATP , 10 mM BME, 1 μM methionine tRNA synthetase, 5 μci ^{35}S-methionine) for 30 min at 37^0C [117-118]. Then, ^{35}S-methionine labeled $tRNA^{Met}$ was isolated by using NucAway spin columns (Ambion).

3.2.3 In organelle translation

In organelle translation was performed with isolated rat liver mitochondria as described [119]. Purified rat liver mitochondria (100 μg) was incubated with labeled ^{35}S-met-$tRNA^{Met}$ or 3'-biotin tagged $tRNA^{Lys}_{TTT}$ in the translation buffer (0.6 M Sorbitol, 150 mM KCl, 15 mM KH_2PO_4, 20 mM Tris-HCl pH-7.4, 13 mM $MgSO_4$, 2 mM ATP, 0.5 mM GTP, 0.15 mg/ml all amino acids, 150 μg/ml cycloheximide) at 30^0C for 1 hr. The mitochondrial translation reaction was stopped by adding 100 μg/ml chloromphenicol to the translational mixture. The incorporation of ^{35}S-methionine in mitochondrial translational products was measured by TCA precipitable counts in a liquid scintillation counter as described [120]. Total radioactivity or label ^{35}S-methionine present in the sample was measured without TCA precipitation in a Scintillation counter. The ratio of TCA-precipitable to non-TCA precipitable counts were calculated and reported as the percentage of ^{35}S-methionine incorporated into mitochondrial translated proteins. The incorporation of biotin tagged lysine in mitochondrial translational products was detected by immunoblotting with streptavidin HRP antibody.

3.2.4 Cell culture and transfections

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% (v/v) fetal bovine serum at 37^0C under an atmosphere of 5% CO_2 and maintained till the cell confluency reaches to 70%-80%. Cells were then transfected with 10 μg plasmid using Lipofectamine 2000 reagent (In vitrogen) in serum free DMEM. After 6 hrs of transfection, serum free DMEM was changed with complete DMEM and cells were harvested after 48 hrs. For depletion of ABRA, HEK293T cells were transfected with 10 nM ABRA specific siRNA duplex or universal scrambled negative control siRNA duplex (OriGene) for 36 hrs as described above.

3.2.5 Isolation of mitochondria from HEK293T cells

Mitochondria were isolated from HEK293T cells as described in the methods of chapter II.

3.2.6 Extraction of total RNA from mitochondria

Total RNA was extracted from isolated mitochondria as described in the methods of chapter II.

3.2.7 Immunoblotting

For immunoblot analysis, HEK293T cells were lysed in RIPA buffer (50 mM Tris-HCl pH-8.0, 150 mM NaCl, 1% deoxycholic acid and 0.1% SDS) with the protease inhibitor cocktail (Roche, Basel, Switzerland) and incubated for 30 min at 4°C. Samples were subjected to centrifugation at 14,000 rpm for 20 min at 4°C and cell lysates were collected. After measuring the protein concentration of the lysates using Bradford reagent (Amersco, USA), lysates were resolved on SDS-PAGE and transferred to NC membrane and probed with specific antibodies. After incubation with HRP conjugated secondary antibodies, the blots were developed using Versa doc imaging system (Bio-Rad).

3.2.8 Semi quantitative PCR

3.2.8.1 cDNA synthesis

Total RNA was extracted from purified HEK cell mitochondria and used as a template for cDNA synthesis. Initially, RNA was heated at 65°C for 5 min and immediately chilled on ice. Using this RNA as a template, the reaction was carried out to a final volume 10 µl. The reaction mixture contains 1x cDNA synthesis buffer, 0.5 mM dNTPs each, 20 pM of specific tRNA reverse primer, 1 µl RT enhancer, 1 µl Verso Enzyme mix and 0.1 µg of template RNA. The reaction was performed at 42°C for 50 min in PCR. The reaction was then stopped by heating at 95°C for 2 min.

3.2.8.2 Polymerase Chain Reaction (PCR)

The specific cytosolic and mitochondrial tRNAs were amplified using cDNA as a template. PCR was carried out in a final volume of 25 µl. The reaction mixture contains 10 pM of each forward and reverse primer specific to tRNA to be amplified, 2.5 mM of each of four dNTPs, 1x PCR reaction buffer, 0.25 U of Taq DNA polymerase and 1 µl of respective cDNA. The amplification was performed in 30 cycles with denaturation step at 95°C for 30 sec, annealing step at 55°C for 30 sec, elongation step at 72°C for 30 sec and final extension step at 72°C for 10 min. Controls include a mock reaction where the RT was left out of the reaction. PCR products were resolved on 2% agarose gel and analyzed by ethidium bromide staining. The primers used in this study are listed in Table 3.1.

3.2.9 Generation of partial mtDNA depleted cells by ethidium bromide treatment

Partial mtDNA depleted cell lines were generated as described [121]. In brief, HEK293T cells were grown in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 2.5 mM sodium pyruvate, 50 µg/ml uridine and ethidium bromide at the concentration of 50 ng/ml. The cells were maintained for 25-30 days by changing the DMEM medium containing EtBr for every 2 days. Cells

that were auxotropic for uridine and pyruvate for the viability are considered as ρ0 (rho zero) and these cells were pooled and maintained in complete DMEM medium without ethidium bromide for further analysis. To compare the mtDNA content between HEK and HEK EtBr cells, total cellular DNA was extracted and performed PCR amplification with human mtDNA specific primers upstream 5'-CCT AGG GAT AAC AGC GCA AT-3' and downstream 5'-TAG AAG AGC GAT GGT GAG AG-3'. The PCR conditions for mtDNA included an initial denaturing at 95°C for 4 min and then 35 cycles as follows: denaturing for 30 s at 94°C, annealing for 30 s at 60°C, and extending for 60 s at 72°C. PCR products were resolved on 1% agarose gel electrophoresis and analyzed by EtBr staining [122].

3.2.10 In vivo mitochondrial translation

In vivo mitochondrial translation was carried out as described [92]. In brief, HEK293T, HEK293T EtBr treated cells (ρ0) and MELAS cells were grown in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 2.5 mM sodium pyruvate, 50 μg/ml uridine to reach 80% confluency. Cells were washed twice with DMEM minus methionine medium for 2 X 5 minutes and incubated with DMEM minus methionine medium containing 150 μg/ml cycloheximide for 30 min at 37^0C in CO_2 incubator. Cells were then washed and incubated with DMEM minus methionine medium containing 2% dialyzed serum, 150 μg/ml cycloheximide and 200 μCi /ml ^{35}S-methionine for 3-4 hrs. Cells were washed with ice cold 1xPBS containing 150 μg/ml cycloheximide for 2 times and cell lysate was extracted by incubating cells with RIPA buffer (50 mM Tris-HCl pH-8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP 40, 0.1% SDS) containing protease inhibitor cocktail at 4°C. The lysate was centrifuged at 14,000 rpm for 15 min at 4°C and the supernatant fraction was separated on 8% SDS-PAGE, transferred to nitrocellulose membrane and analyzed by using phosphorimager.

3.2.11 Electrophoretic mobility shift assay (EMSA)

Purified GST-ABRA was incubated with yeast tRNA (Sigma Aldrich) in a binding buffer containing 20 mM Tris-HCl pH-7.0, 20 mM KCl, 5 mM $MgCl_2$, 5 mM DTT at 30°C for 45 min. Reaction was then quenched with 5x loading dye (50% glycerol and 1 mg/ml xylene cyanol in 5x TB buffer) and resolved on a 10% non denaturing PAGE followed by staining with methylene blue (0.1 M sodium acetate & 0.02% methylene blue) for 1 hr at room temperature. Gel was destained overnight with autoclaved milli Q water [6].

3.2.12 GST pull down assay

To examine the interacting partners of GST-ABRA from mitochondrial salt extract, we performed GST pull down assay [123]. Mitochondrial salt extract (500 µg) was incubated with 100 µl of glutathione-agarose beads for 2 h at 4°C with end-over-end mixing. The supernatant (pre cleared lysate) was transferred to a fresh tube after centrifugation at 5000 rpm for 2 min. Then equal amount of pre cleared lysate was added to two 100 µl of glutathione-agarose beads containing tubes either with ~10 µg of GST or ~10 µg of GST-ABRA. After incubation for 2 h at 4°C with end-over-end mixing, samples were centrifuged at 5000 rpm for 2 min. Beads were collected and washed with 1 ml of ice-cold GST lysis buffer (20 mM Tris-HCl pH-8.0, 200 mM NaCl, 1 mM EDTA pH-8, 0.5% NP40, 2 µg/µl aprotinin, 1 µg/µl leupeptin, 0.7 µg/ml pepstatin and 25 µg/ml PMSF) for 4-5 times. Bound proteins were eluted from the beads by heating with 2x SDS loading buffer. Eluted proteins were resolved on 12% SDS-PAGE and desired bands were analyzed by MALDI-TOF analysis.

3.2.13 Cloning of hGB and hRhoGEF39

3.2.13.1 cDNA synthesis

HEK cell total RNA was used for cDNA synthesis. Initially, RNA was heated at 65°C for 5 min and immediately chilled on ice. Using this RNA as a template, the reaction was carried out to a final volume 10 µl. The reaction mixture contains 1x cDNA synthesis buffer, 0.5 mM dNTPs each, 20 pM of RNA reverse primer, 1 µl RT enhancer, 1 µl Verso Enzyme mix and 0.5 µg of template RNA. The reaction was performed at 50°C for 30 min in a PCR machine. The reaction was then stopped by heating at 95°C for 2 min.

3.2.13.2 Polymerase Chain Reaction (PCR)

Full length *hGB* (Guanine nucleotide Binding protein) and *hRhoGEF39* (Rho Guanine nucleotide Exchange Factor) were amplified by polymerase chain reaction using HeLa cell cDNA as a template. Amplification reaction was carried out in a final volume of 50 µl. The reaction mixture contains 10 pM of each forward and reverse primer specific for *hGB and hRhoGEF39*, 2.5 mM each of four dNTPs, 1x PCR reaction buffer, 0.5 U of DNA polymerase enzyme and 500 ng of cDNA. The reaction was performed with an initial denaturation step at 95 ^{0}C for 4 min, followed by 35 cycles of 94 ^{0}C for 45 sec, 55 ^{0}C for 1 min and 72 ^{0}C for 2 min; and a final extension at 72 ^{0}C for 10 min. The amplified product was resolved on 1% agarose gel electrophoresis and purified by gel extraction method (QIAGEN). The primers used in this study are listed in Table 3.1.

3.2.13.3 Restriction Digestion

The amplified *hGB or hRhoGEF39* and pET28a vector were subjected to double digestion with *E.coRI* and *XhoI* restriction enzymes in a 50 µl reaction {(Insert 30 µl; 10x Tango Buffer 10 µl; autoclaved milli Q water 9 µl; *E.coRI* 0.5 U; *XhoI* 0.5 U) and (pET28a vector 30 µl; 10x Tango Buffer 10 µl; autoclaved milli Q water 9 µl; *E.coRI* 0.5 U; *XhoI* 0.5 U)} at 37 ^{0}C for overnight. The digested products were resolved on 1% Agarose gel electrophoresis and the digested products were excised and gel purified by NucleoSpin gel extraction method.

3.2.13.4 Cloning of hGB and hRhoGEF39 into pET28a vector

The double digested *hGB or hRhoGEF39* fragment was ligated into pET28a vector by using T4 DNA Ligase (Fermentas). The reaction was carried out in a final volume of 10 µl with 200 ng of vector, 3 fold excess of insert, 1 µl of T4 DNA Ligase and 1 µl of 10x T4 DNA Ligase Buffer. The reaction mixture was incubated at 22^0C for overnight and ligated product was transformed into *E.coli* DH5α competence cells.

3.2.13.5 Bacterial Transformation

The ligated product (10 µl) was incubated with *E.coli* DH5α competent cells for 30 min on ice. The cells were subjected to heat shock at 42 ^{0}C for 90 sec and chilled on ice for 5 min. To this reaction, 1 ml of LB medium was added and incubated at 37 ^{0}C shaker incubator for 1 hr and the culture was plated on to LB agar plate containing antibiotic (Kanamycin). The colonies were screened for the presence of cloned fragment by double digestion with *E.coRI* and *XhoI* restriction enzymes and sequence was confirmed by automated sequencer.

3.2.14 Bacterial expression and protein Purification

3.2.14.1 Expression of His tagged GB and RhoGEF

The plasmid pET28a harbouring *hGB or hRhoGEF* were transformed into *E.coli* Rosetta gami strain. A colony carrying the pET28a-*hGB or* pET28a-*hRhoGEF* plasmid were grown for overnight in LB medium containing Kanamycin at 37^0C in a shaker incubator. The primary culture was diluted to 1:100 in 500 ml fresh LB medium, grown with vigorous agitation until the growth reaches to $OD_{600\ nm}$: 0.6. Then the bacterial cells were induced with 1 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) and incubated at 37^0C for 3 hrs. After induction, the bacterial cells were pellet down by centrifugation at 10,000 rpm for 10 min. The cell pellet was suspended in 1/20th

volume of ice cold 50 mM Tris-HCl pH-8.0 by and subjected to sonication in the presence of 1 mg/ml lysozyme and protease inhibitor (1 mM PMSF). After sonication, soluble (supernatant) and insoluble (pellet / inclusion bodies) fractions were separated by centrifugation at 10,000 rpm 10 min. The expressed recombinant proteins were present in inclusion bodies. Further, inclusion bodies were solubilized with 50 mM Tris-HCl pH-8.0 containing 8 M Urea. The insoluble recombinant proteins were purified by Ni-NTA affinity column (Clontech).

3.2.14.2 Purification of His-GB and His-RhoGEF by Ni-NTA column

The inclusion bodies containing His-GB & His-RhoGEF were solubilized in urea buffer containing 50 mM Tris-HCl pH-8.0 and 8M urea. The solubilized inclusion bodies were subjected to centrifugation at 10,000 rpm for 10 min and the supernatant was mixed with buffer A (8 M Urea and 50 mM Tris-HCl pH-8.0) equilibrated Ni-NTA beads and kept for end over rotation at room temperature for 2 hrs. After incubation, Ni-NTA beads were washed with 10 bed volumes of wash buffer (8 M Urea, 50 mM Tris-HCl pH-8.0 and 10 mM Immidazole pH-7.0). The bound recombinant His-GB & His-RhoGEF proteins were eluted by using elution buffer (0.4 M Immidazole pH 7.0, 8 M Urea and 50 mM Tris-HCl pH-8.0).

3.2.15 Cloning of hABRA, hGB and hRhoGEF in pcDNA 3.1 c-myc vector

Full length *hABRA, hGB and hRhoGEF* were cloned into mammalian expression vector, pcDNA 3.1 c-myc as described in subchapter *3.2.13*. Briefly *hABRA, hGB and hRhoGEF* ORFs were amplified by using gene specific forward and reverse primers. The amplified fragment and pcDNA 3.1 c-myc vector were subjected to double digestion with *E.coRI* and *XhoI* restriction enzymes. The digested fragments were ligated into pcDNA 3.1 c-myc vector with T4 DNA Ligase followed by transformation of ligated products into *E.coli* DH5α competent cells. Colonies that were harbouring

Myc-ABRA or Myc-GB or Myc-RhoGEF were selected and confirmed their expression by confocal microscopy. The primers used for this study are listed in Table 3.1.

3.2.16 Confocal microscopy studies

HEK293T cells were transfected with Myc-ABRA or Myc-GB or Myc-RhoGEF using Lipofectamine 2000 reagent (Invitrogen). After 48 hrs of transfection, the cells on cover slip were washed with 1x PBS and fixed with ice cold Para-formaldehyde (4%) for 20 min at room temperature. Cells were then permeabilized with ice cold acetone and methanol (1:3) for 20 min followed by washing with TBS (20 mM Tris-HCl pH-7.5 and 150 mM NaCl) for 5 min x 3 times. The cells were blocked with 3% BSA in TBS buffer for 1 hr at room temperature. The permeabilized cells were incubated with primary antibody (c-Myc, 1:200 dilution) in 3% BSA solution at room temperature for 2 hrs followed by washing with TBS, TBST (20 mM Tris-HCl pH-7.5, 150 mM NaCl and 0.05% Tween 20) and TBS buffers for 15 min each on a rocker. Cells were incubated with secondary antibody (Alexa flour conjugated, 1:200 dilution) and 1x DAPI at room temperature in dark for 1 hr and washed with TBS, TBST and TBS buffers for 15 min each on a rocker. Cover slips were allowed for air dry at room temperature and mounted on glass slides with 50% glycerol.

3.2.17 In vitro protein-protein interaction studies

Initially, GST fusion protein (GST-ABRA) was immobilized on glutathione sepharose beads by incubating the purified recombinant GST-ABRA with glutathione sepharose beads that were equilibrated in binding buffer (20 mM Tris-HCl pH-7.5, 0.1 mM EDTA pH-8.0, 100 mM NaCl) at 4^0C for 2 hr in end over end rotator. In similar way, purified GST that was immobilized on glutathione sepharose beads used as a negative control. After incubation, beads were washed with binding buffer by centrifugation at 5000 rpm for 2 min to remove unbound proteins. Purified

protein of our choice (His-GB or His-RhoGEF) was incubated with immobilized GST or GST-ABRA at 4^0C for 2 hr in rotisserie. After incubation, beads were washed with wash buffer (20 mM Tris-HCl pH-7.5, 0.1 mM EDTA pH-8.0, 150 mM NaCl and 0.05% NP40) for 4-5 times. The bound proteins were eluted by boiling the beads in 2x SDS sample buffer and visualized by western blotting with anti-His and anti-GST antibodies [124].

3.2.18 In vitro tRNA binding affinity assay

In vitro tRNA binding affinity assay was performed by the addition of ^{32}p-radiolabeled human $tRNA^{Lys}_{TTT}$ transcript or unlabeled tRNA to the protein immobilized on beads [125-126]. Briefly, purified GST-ABRA was immobilized on glutathione sepharose beads (GE Healthcare) and purified His-GB/His-RhoGEF was immobilized on Ni-NTA affinity beads (Clontech). These beads were incubated with either ^{32}p-radiolabeled human $tRNA^{Lys}_{TTT}$ transcript or unlabeled tRNA in RSB-100 buffer (10 mM Tris-Hcl pH-7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% NP40) for 1 hr at 4^0C. After incubation, beads were washed carefully with RSB-100 buffer for 3-4 times. Bound radiolabeled $tRNA^{Lys}_{TTT}$ transcript or unlabeled tRNA was eluted from the beads by proteinase k (50 μg/ml) treatment followed by phenol-chloroform extraction method. The eluted radiolabeled $tRNA^{Lys}_{TTT}$ or unlabeled tRNA was analyzed either on 7 M urea-12% acrylamide gel followed by autoradiography or on 2% agarose gel electrophoresis followed by ethidium bromide staining.

3.2.19 Fluorescence spectroscopy analysis

The fluorescence emission spectra were monitored and recorded on a FluoroMax-3 Jobin-Yvon instrument from the range 300 to 400 nm, with excitation wavelength at 285 nm. The final concentrations of tRNA used for spectra were 0.1 μM, 0.3 μM, 0.5 μM, 0.7 μM, 1.0 μM and 1.2 μM with fixed concentration of recombinant His-GB protein at 10 μM in a binding buffer containing 20 mM Tris-HCl, pH-7.0 and 2.5 mM $MgCl_2$ [6].

3.2.20 In vitro tRNA import assay

In vitro tRNA import assay was performed as described in the methods of chapter II.

Table 3.1: Primers used in this study

S.No.	Gene Name	Primer Name	Primer Sequence
1.	Cyto. $tRNA^{Lys}_{TTT}$_Fwd	NB 420	5'- GCC TAT CGA GTC AGC CAT CTC - 3'
2.	Cyto. $tRNA^{Lys}_{TTT}$_Rev	NB 332	5'- CGC CCG AAC AGG GAC TTG AAC CCT G - 3'
3.	Cyto. $tRNA^{Leu}_{TAG}$_Fwd	NB 410	5'- GGT AGC GTG GCC GAG CGG TCT - 3'
4.	Cyto. $tRNA^{Leu}_{TAG}$_Rev	NB 335	5'- TGG CAG CGG TGG GAT TCG AAC CCA C - 3'
5.	Mito. $tRNA^{Glu}_{TTC}$_Fwd	NB 423	5'- GAA CAT CAA CTT TAT GTT GCT - 3'
6.	Mito. $tRNA^{Glu}_{TTC}$_Rev	NB 337	5'- TAT TCT CGC ACG GAC TAC AAC CAC G - 3'
7.	5S rRNA_Fwd	NB 628	5'- TACGGCCATACCACCCTGA - 3'
8.	5S rRNA_Rev	NB 629	5'- GCGGTCTCCCATCCAAGTA - 3'
7.	hGB _Fwd	NB 475	5'- CAAAGAATTCACCATGAGCGAACTGGAA-3'
8.	hGB _Rev	NB 476	5'- ACCCCTCGAGTTAATTCCAGATTCTAAG- 3'
9.	hRhoGEF _Fwd	NB 477	5'- CAAAGAATTCACCATGGAGCTCTCCTGC- 3'
10.	hRhoGEF _Rev	NB 478	5'- AACCCTCGAGCTAGTTTTTCTGGCTGCT- 3'
11.	hABRA _Fwd (c-myc)	NB 169	5'-CAGAATTCACCATGGCTCCGGGCGAAAAGGAA- 3'
12.	hABRA _Rev (c-myc)	NB 329	5'-AACCCTCGAGCTTGAGTAGCGTAATCACAACAT-3'
13.	hGB _Fwd (c-myc)	NB 475	5'- CAAAGAATTCACCATGAGCGAACTGGAA- 3'
14.	hGB _Rev (c-myc)	NB 509	5'- ACCCCTCGAGATTCCAGATTCTAAGAAA- 3'
15.	hRhoGEF _Fwd (c-myc)	NB 477	5'- CAAAGAATTCACCATGGAGCTCTCCTGC- 3'
16.	hRhoGEF _Rev (c-myc)	NB 510	5'- AACCCTCGAGGTTTTTCTGGCTGCTGAT- 3'
17.	Bacterial Methionine tRNA_ Rev	NB 353	5'TGGCTACGACGGGATTCGAACCTGTGACCCCAT CATTATGAGTGA-3'

3.3 RESULTS

3.3.1 ABRA stimulates mitochondrial translation by promoting tRNA import

To investigate the functional role of ABRA mediated imported tRNA, we chose to perform an *in organelle* translation assay. Isolated mitochondrial fraction was incubated with ^{35}S-methionine-tRNA^{Met} in the presence or absence of increasing concentrations of GST-ABRA in an in-organelle translation buffer as described in methods. *In-organelle* translation in the presence of GST can serve as an internal control. The incorporation of ^{35}S-methionine in mitochondrial translational products was measured by TCA precipitation counts as described in methods. In all the reactions, cycloheximide was used to inhibit cytosolic translation. As shown in Fig.3.1A, there was a moderate increase in the incorporation of ^{35}S-methionine in the presence of GST-ABRA compared to GST control (Fig.3.1A, compare lanes 2 & 6). In addition to this, we have also performed *in organelle* translation assay by incubating mitochondrial fraction with 3'- biotin labeled tRNA$^{Lys}_{TTT}$ (Fig.3.1B) in the presence or absence of increasing concentrations of GST-ABRA in an in-organelle translation buffer. Mitochondrial fractions were re isolated after translation, solubilized in SDS loading buffer, resolved on SDS-PAGE and the amount of biotin tagged lysine in mitochondrial translational products were analyzed by immunoblotting with streptavidin HRP antibody. As shown in Fig.3.1C, there was a moderate increase in the amount of biotinylated proteins in the presence of GST-ABRA (Fig.3.1C, top panel, compare lanes 3 & 4). Altogether these results likely indicate that salt extractable, mitochondrial peripherally associated cytosolic factor, ABRA stimulates the import of externally added tRNAs into mammalian mitochondria and imported tRNAs are functional in mitochondrial translation. Immunoblot with Aconitase - 2 (mitochondrial matrix protein) represent the equal mitochondrial loading control (Fig.3.1C, bottom panel).

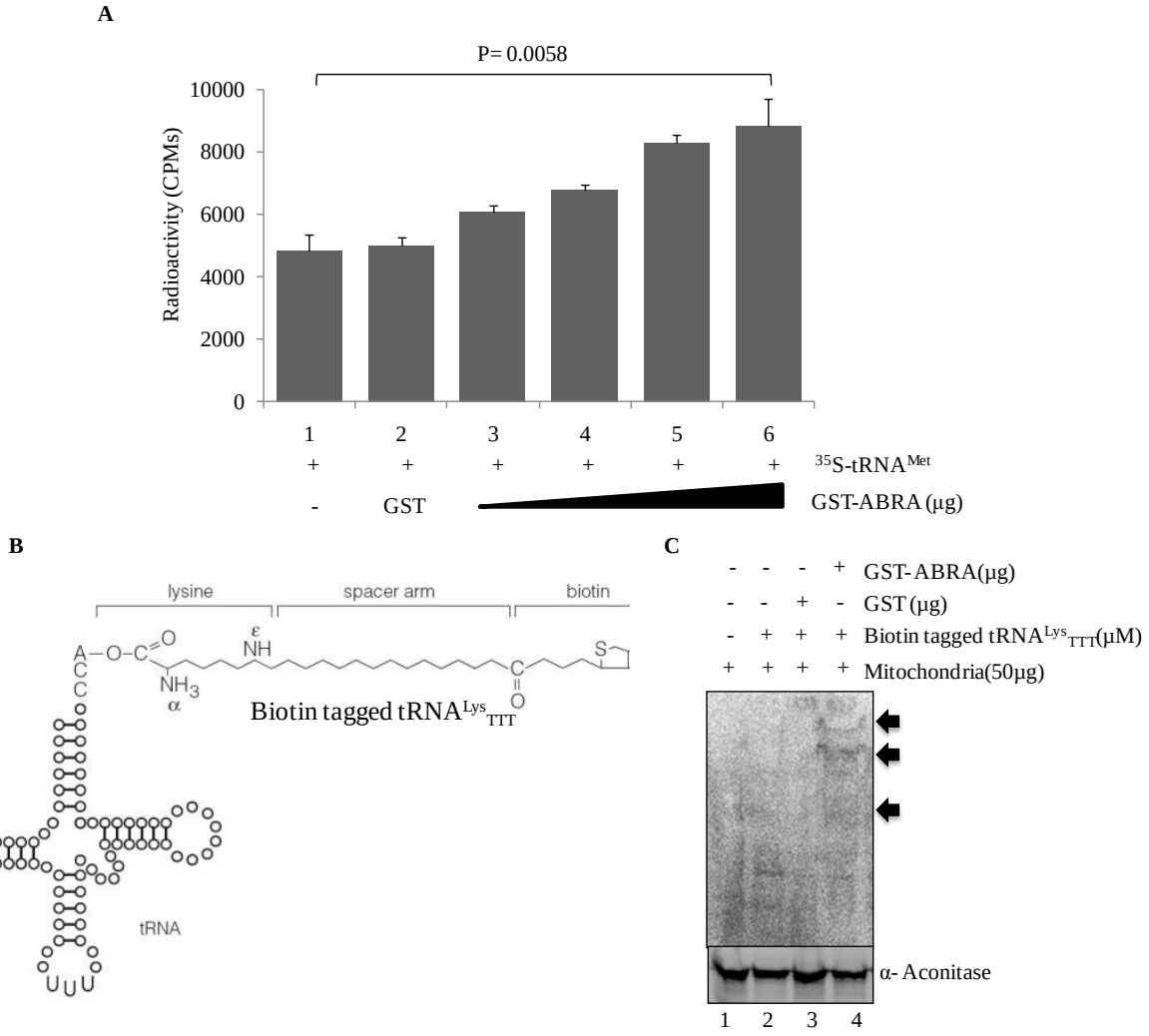


Fig.3.1 ABRA mediated imported tRNAs are functional in the mitochondria: (A) *In organelle* translation was performed by incubating radiolabeled ^{35}S-methionine labeled tRNA^{Met} with isolated mitochondria in the presence or absence of increasing concentrations of GST-ABRA (0.2, 0.4, 0.8 and 1 µg) as described under methods. Incorporation of **^{35}**S-methionine in the translational products was monitored by TCA precipitation. Histogram represents the percentage of **^{35}**S-methionine labeled proteins in cpms. Experiments were performed in triplicates statistical analysis was performed using *t- test*. **(B)** Schematic representation of 3'biotin tagged tRNA$^{Lys}_{TTT}$. **(C)** *In organelle* translation by incubating mitochondria with biotin tagged tRNA$^{Lys}_{TTT}$ in the presence of GST-ABRA (1µg). Mitochondria were re isolated, solubilized in SDS buffer and the incorporated biotinylated lysine in mitochondrial translation products were analyzed by immunoblotting with streptavidin HRP antibody. Black arrows denote mitochondrial translation products.

3.3.2 Over expression of ABRA induces mitochondrial tRNA import in vivo

To check the effect of ABRA on tRNA import *in vivo,* HEK293T cells were transfected with Myc-ABRA or Myc-pCDNA vector by using lipofectamine 2000 transfection reagent for 48 hrs as

described in the methods. After 48 hr post transfection, HEK293T cells were harvested and cell lysates were prepared. Expression level of ABRA was monitored by immunoblotting with anti-Myc antibody. As shown in Fig.3.2A, Myc levels represent the percentage of over expression of ABRA in HEK293T cells that were transfected with Myc-ABRA. Mitochondria were isolated from untransfected, Myc-ABRA or Myc-pCDNA transfected HEK293T cells as described in the methods and isolated mitochondrial fractions were treated with RNase A to eliminate the mitochondrial associated cytosolic contaminants. Total mitochondrial RNA was extracted from these samples and used as a template for a semiquanitative RT-PCR to quantity the accumulation of cytosolic tRNAs in the mitochondria. We chose two cytosolic tRNAs ($tRNA^{Lys}_{TTT}$ & $tRNA^{Leu}_{TAG}$) for RT-PCR. cDNA was prepared with antisense primers of cytosolic $tRNA^{Lys}_{TTT}$ (NB 332) or cytosolic $tRNA^{Leu}_{TAG}$ (NB 335) by using mitochondrial total tRNA as a template. Using this specific cDNA as a template, cytosolic $tRNA^{Lys}_{TTT}$ (NB 332 and NB 420) or cytosolic $tRNA^{Leu}_{TAG}$ (NB 335 and NB 410) were amplified by using gene specific primers as indicated. As a control, mock cDNA was prepared from total RNA where reverse transcriptase was left out of the reaction. The amplification reaction was performed in triplicates for each cytosolic tRNA. The band intensities of cytosolic $tRNA^{Lys}_{TTT}$ and cytosolic $tRNA^{Leu}_{TAG}$ from agarose gel were quantified by using Image J software and the relative fold change of tRNAs in transfected cells was represented in Histogram.

Consistent with *in vitro* data, we observed a moderate increase in cytosolic tRNAs accumulation in Myc-ABRA over expressed cells compared to untransfected and Myc-pCDNA transfected HEK293T cells (Fig.3.2B & C). As a control, we also quantified the levels of cytosolic 5S rRNA (NB 628 and NB 629) in these transfected cells.

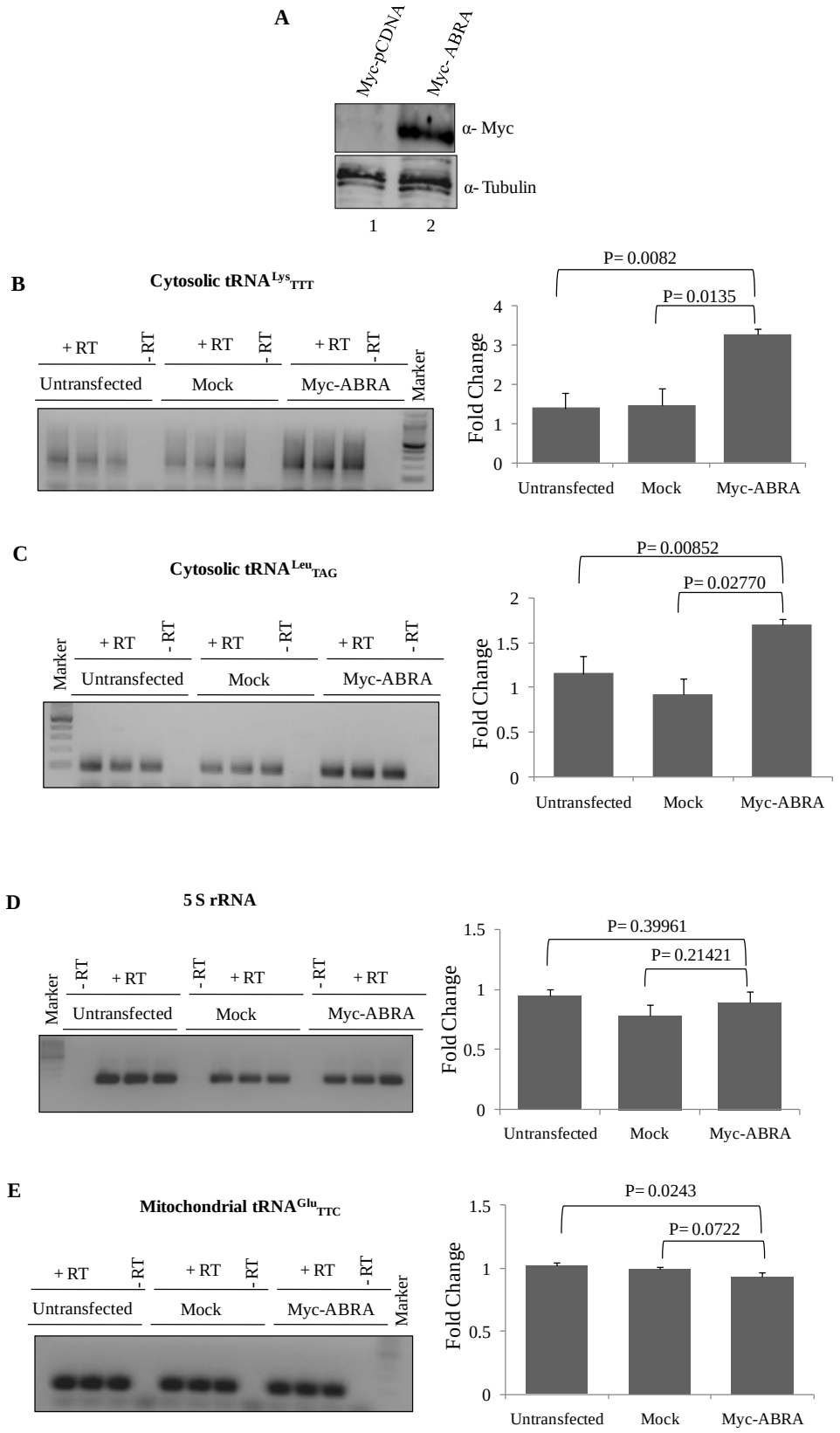

A
Myc-pCDNA
Myc-ABRA
α- Myc
α- Tubulin
1
2
B
Cytosolic tRNA Lys TTT
+ RT
- RT
Untransfected
Mock
Myc-ABRA
Marker
P= 0.0082
P= 0.0135
Fold Change
C
Cytosolic tRNA Leu TAG
P= 0.00852
P= 0.02770
D
5 S rRNA
P= 0.39961
P= 0.21421
E
Mitochondrial tRNA Glu TTC
P= 0.0243
P= 0.0722

Fig.3.2 ABRA over expression elevates the steady state levels of cytosolic tRNA in mitochondria: (A) Western blot analysis of Myc-pCDNA or Myc-ABRA transfected HEK293T cell lysates that were probed with anti-Myc and anti-Tubulin antibodies. **(B, C, D & E)** Ethidium bromide gels indicating the semi quantitative RT-PCR levels of cytosolic $tRNA^{Lys}_{TTT}$, $tRNA^{Leu}_{TAG}$, 5S rRNA & mitochondrial $tRNA^{Glu}_{TTC}$ in untransfected, Myc-pCDNA or Myc-ABRA transfected mitochondria. Experiments were performed in triplicates and band intensities of RNAs were quantified by densitometry. Statistical analysis was performed using *t- test*.

However, we did not observe any significant alteration in the levels of cytosolic 5S rRNA between untransfected and Myc-ABRA transfected cells (Fig.3.2D). All the transfected samples contain equivalent level of endogenous mitochondrial $tRNA^{Glu}_{TTC}$ (NB 337 and NB 423) (Fig.3.2E).

Further, we also used northern blot analysis to analyze the steady state levels of cytosolic tRNAs in mitochondria samples isolated from Myc-ABRA or Myc-pCDNA transfected HEK293T cell lines. Expression levels of ABRA were monitored by immunoblotting with anti-ABRA antibody (Fig.3.3A). Total mitochondrial RNA extracted from Myc-pCDNA or Myc-ABRA transfected HEK cells were resolved on urea PAGE and performed northern blot hybridization using radiolabeled oligonucleotide probe specific for cytosolic $tRNA^{Lys}_{TTT}$, $tRNA^{Gln}_{CTG}$, 5.8S and 5S ribosomal RNA as indicated in the Table 2.1.

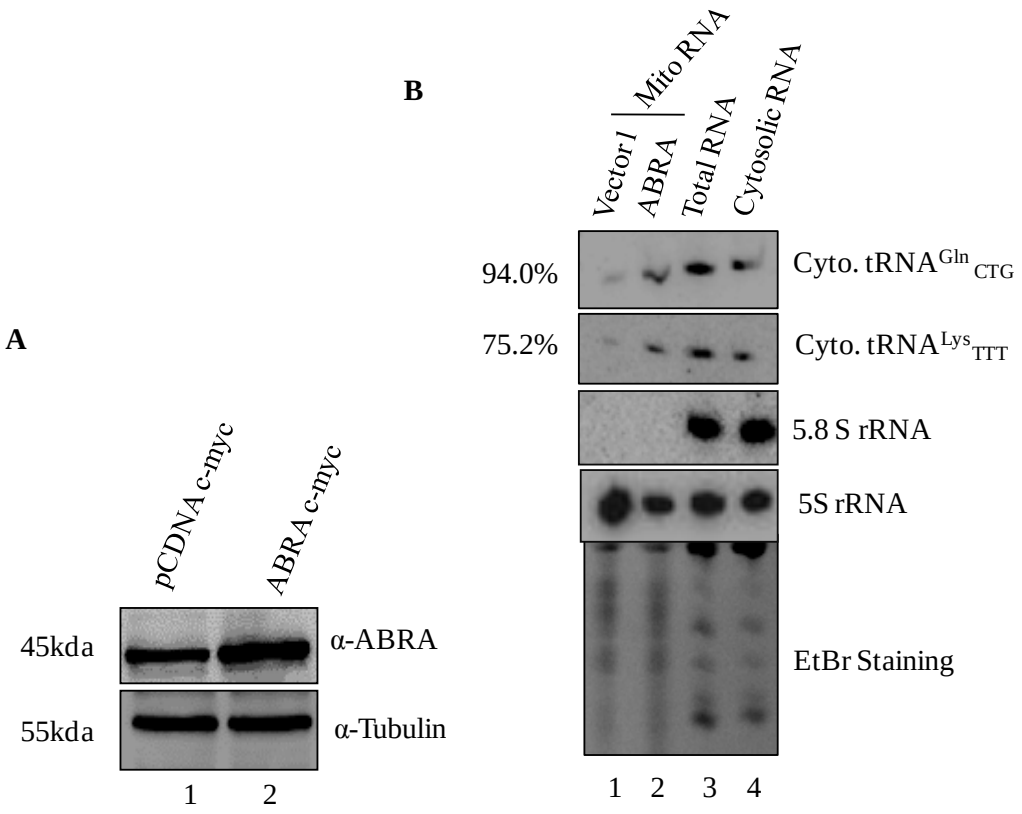


Fig.3.3 ABRA over expressed mitochondria enriched with cytosolic tRNAs: (A) Western blot analysis of Myc-pCDNA or Myc-ABRA transfected HEK293T cell lysates that were probed with anti-ABRA and anti-Tubulin antibodies. **(B)** Northern blots with Myc-pCDNA or Myc-ABRA mitochondrial (pretreated with 100 μg/ml of RNase A) (M), Total (T) and cytosolic (C) total RNAs. The oligonucleotide probes specific for tRNAs were given in table 3.1. Hybridization signals were quantified, and the ratio between the Myc-ABRA mitochondrial (M) signal and Myc-pCDNA signal was indicated. The total amounts of RNA for each fraction used for northern blotting were shown by ethidium bromide staining.

We observed the significant increase in the levels of cytosolic tRNA$^{Lys}_{TTT}$ & tRNA$^{Gln}_{CTG}$ in Myc-ABRA transfected cells compared to Myc-pCDNA transfected cells (Fig.3.3B, top 2 panels, compare lanes 1& 2) but not in cytosolic 5S rRNA & 5.8S rRNA levels (Fig.3.3B, bottom 2 panels, compare lanes 1 & 2). All these observations clearly indicate the involvement of cytosolic ABRA in the regulation of tRNA import into mammalian mitochondria.

3.3.3 Knockdown of ABRA reduces cytosolic tRNAs in mitochondria

Our results so far demonstrated that over expression of cytosolic protein, ABRA moderately stimulated the import of cytosolic tRNAs into mammalian mitochondria both *in vitro* and *in vivo*. To precisely understand the role of ABRA, we selectively depleted the ABRA in HEK293T cells by transfecting with ABRA specific siRNA duplex. HEK293T cells that were transfected with universal scrambled siRNA duplex serve as a negative control. Initially, we screened the depletion of ABRA in HEK293T cells by transfecting with three different ABRA siRNA duplex constructs. The cells were lysed and the cell extracts were probed with antibodies specific for ABRA. We find that ABRA siRNA-B construct depletes the steady state levels of ABRA by more than 50% when compared to scrambled siRNA or other ABRA siRNA constructs (Fig.3.4A). Mitochondria were isolated from untransfected, ABRA siRNA duplex transfected and scrambled siRNA duplex transfected cells as mentioned in the methods. Total mitochondrial RNA was extracted from these samples and used as a template for a semiquanitative RT-PCR to quantify the accumulation of cytosolic tRNAs in the mitochondria. We chose two cytosolic tRNAs ($tRNA^{Lys}_{TTT}$ and $tRNA^{Leu}_{TAG}$) for RT-PCR. cDNA was prepared with antisense primers of cytosolic $tRNA^{Lys}_{TTT}$ (NB 332) or cytosolic $tRNA^{Leu}_{TAG}$ (NB 335) by using mitochondrial total tRNA as a template. Using this specific cDNA as a template, cytosolic $tRNA^{Lys}_{TTT}$ (NB 332 and NB 420) or cytosolic $tRNA^{Leu}_{TAG}$ (NB 335 and NB 410) were amplified by using gene specific primers as indicated. As a control, mock cDNA was prepared from total RNA where reverse transcriptase was left out of the reaction. The amplification reaction was performed in triplicates for each cytosolic tRNA. The band intensities of cytosolic $tRNA^{Lys}_{TTT}$ and cytosolic $tRNA^{Leu}_{TAG}$ from agarose gel were quantified by using Image J software and the relative fold change of tRNAs in transfected cells was represented in Histogram. We observed the significant depletion in cytosolic tRNA levels in ABRA siRNA duplex transfected cells compared to scramble siRNA transfected and untransfected cells (Fig.3.4B & C). As a control, we also quantified

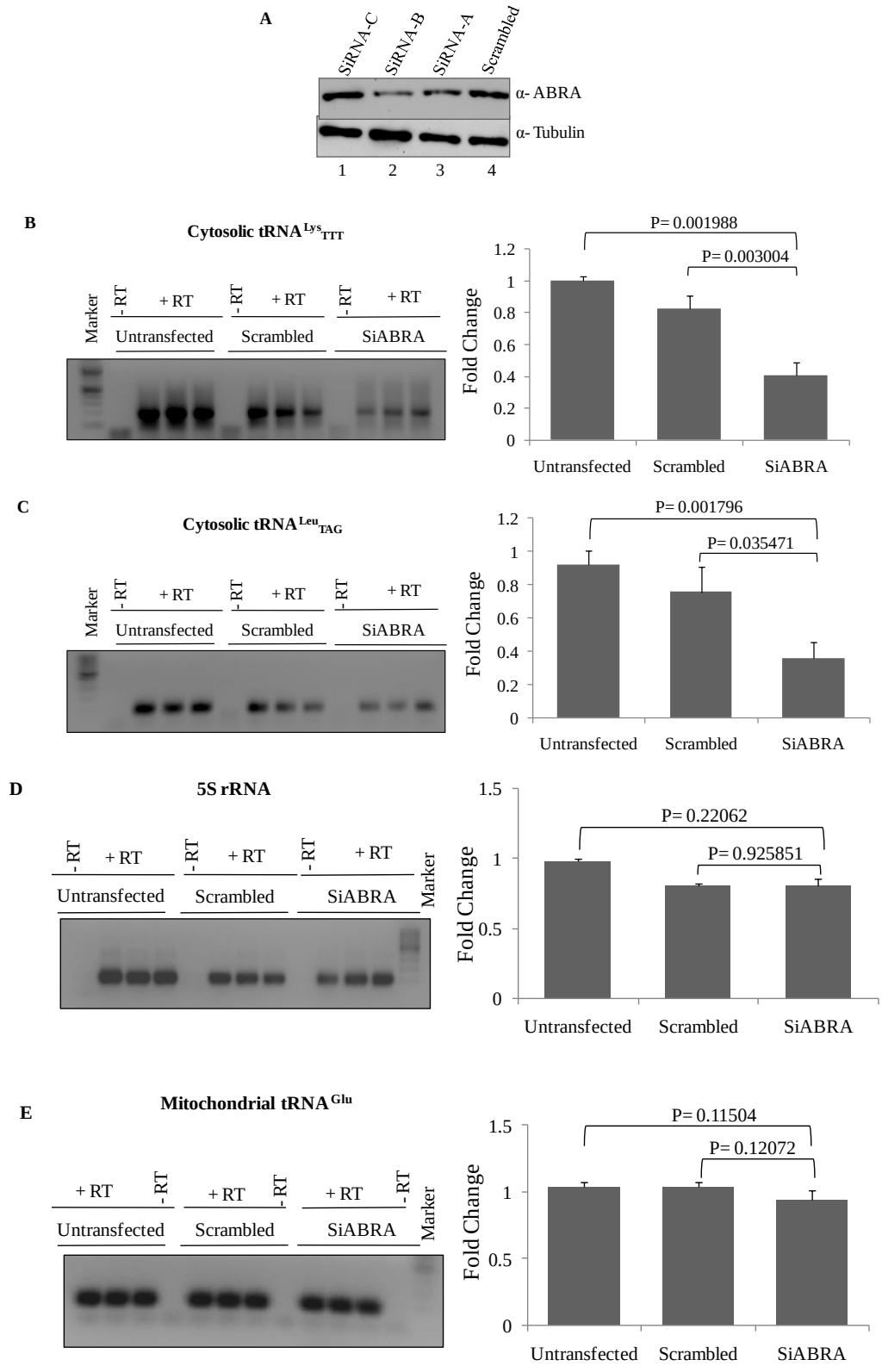

A
SiRNA-C
SiRNA-B
SiRNA-A
Scrambled
α- ABRA
α- Tubulin
1 2 3 4
B
Cytosolic tRNA Lys TTT
Marker
- RT
+ RT
Untransfected
Scrambled
SiABRA
P= 0.001988
P= 0.003004
Fold Change
C
Cytosolic tRNA Leu TAG
P= 0.001796
P= 0.035471
D
5S rRNA
P= 0.22062
P= 0.925851
E
Mitochondrial tRNA Glu
P= 0.11504
P= 0.12072

Fig.3.4 Depletion of ABRA impairs the accumulation of cytosolic tRNAs in mitochondria: (A) Western blot analysis of Scrambled RNA or ABRA siRNA transfected HEK293T cell lysates that were probed with anti-ABRA and anti-Tubulin antibodies. **(B, C, D &E)** Ethidium bromide gels indicating the semi quantitative RT-PCR levels of cytosolic $tRNA^{Lys}_{TTT,}$ $tRNA^{Leu}_{TAG,}$ 5S rRNA & mitochondrial $tRNA^{Glu}_{TTC}$ in untransfected, scrambled or SiABRA transfected mitochondria. Experiments were performed in triplicates and band intensities of RNAs were quantified by densitometry. Statistical analysis was performed using *t- test*.

the levels of cytosolic 5S rRNA (NB 628 and NB 629) in these transfected cells. However, we did not observe any significant alteration in the levels of cytosolic 5S rRNA between untransfected and ABRA siRNA duplex transfected cells (Fig.3.4D).

In addition, we also observed the equal levels of endogenous mitochondrial $tRNA^{Glu}_{TTC}$ (NB 337 and NB 423) in all the transfections (Fig.3.4E). Altogether, these results suggest that the possible involvement of ABRA in recruiting cytosolic tRNAs onto mammalian mitochondria.

3.3.4 Generation of partial mtDNA depleted cells by EtBr treatment

A number of point mutations in mitochondrial tRNA genes are associated with MERRF (Myoclonic Epilepsy with Ragged Red Fibers), MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke like episodes) and other disorders [71, 127]. These mutations lead to a severe reduction in the mitochondrial translation with a concomitant decline in mitochondrial respiration there by reduces the ability to generate ATP efficiently. Previous reports have shown that cytosolic tRNA could be delivered to the mitochondria of cytoplasmic hybrids from patients suffering from MERRF upon the incubation with putative RNA import complex (RIC) from *Leishmania* [94]. In light of findings illustrated here, we decided to examine the ability of ABRA to complement the mitochondrial translational defect. To study this possibility, we have generated partial mtDNA depleted HEK293T cell lines by ethidium bromide treatment as described in methods. To examine mtDNA depletion in HEK EtBr cells, PCR amplification was performed using human mtDNA specific primers. As shown in Fig.3.5A, there was a significant depletion of mtDNA in HEK EtBr

cells compared to HEK cells. Further, we examine the levels of mitochondrial encoded tRNAs in HEK293T EtBr treated cells. Mitochondria were isolated from HEK293T EtBr and HEK293T cells and the total RNA was extracted from these isolated mitochondria as described in the methods. Using mitochondrial total RNA as a template, we performed reverse transcription reaction with antisense primers specific for mitochondrial $tRNA^{Glu}_{TTC}$ (NB337) and mitochondrial $tRNA^{Lys}_{TTT}$ (NB336). Using particular tRNA specific cDNA as a template, mitochondrial $tRNA^{Glu}_{TTC}$ (NB423 and NB337) and mitochondrial $tRNA^{Lys}_{TTT}$ (NB336 and NB411) were amplified by using gene specific primers as indicated. As a control, mock cDNA was prepared from total RNA where reverse transcriptase was left out of the reaction. All the amplified products were resolved on 2% agarose gel and analyzed by ethidium bromide staining. We observed the significant decrease in the levels of mitochondrial tRNAs in the mitochondria of HEK293T EtBr cells compared to HEK293T mitochondria (Fig.3.5B).

Besides, we also measured the oxygen consumption rate of HEK EtBr cells using Strathkelvin Respirometry. It has been shown that rho zero cells exhibit reduced oxygen consumption rate compared to parental cells [128]. As shown in Fig.3.5D, we observed that reduced oxygen consumption in HEK EtBr cells when compared control HEK cells.

In addition, we also monitored the partial mtDNA depletion in HEK293T cells treated with ethidium bromide by immunoblotting with antibodies specific for mitochondrial DNA encoded proteins. The western blot analysis reveals that EtBr treatment causes the selective reduction in steady state levels of mtDNA-encoded proteins (COXI and ND1) and does not affect the nuclear encoded mitochondrial proteins (Mia40 and Hsp70) (Fig.3.5C).

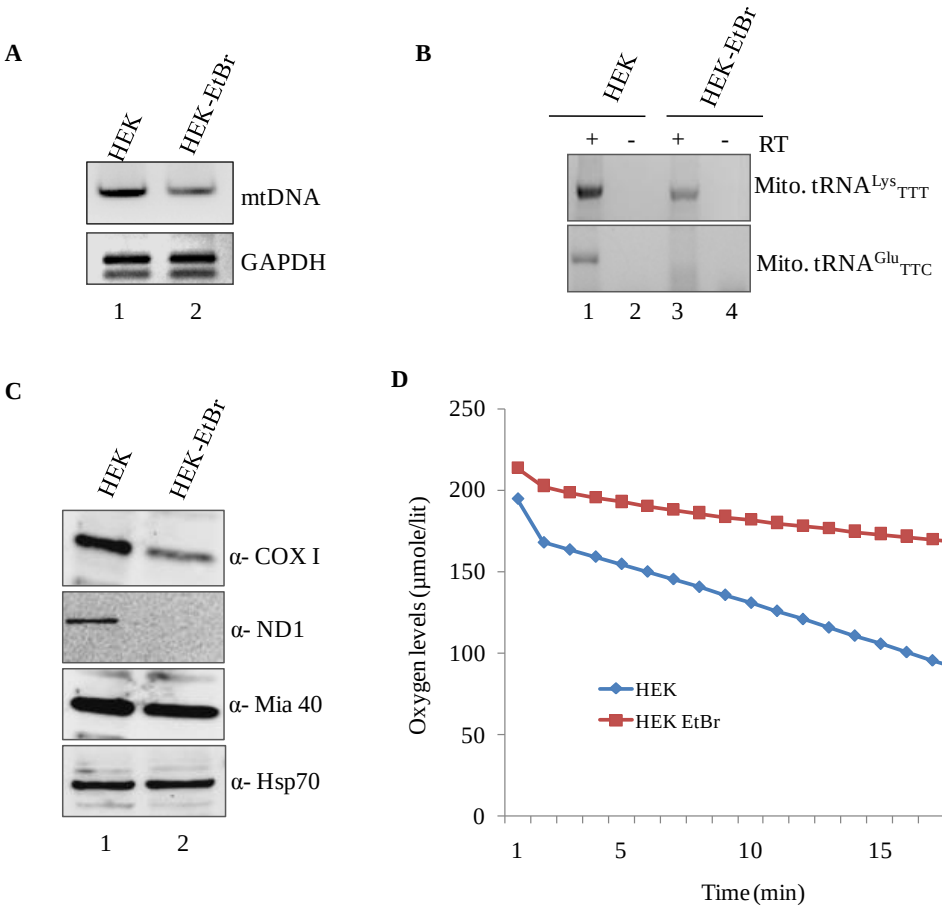


Fig.3.5 EtBr treatment partially depletes the mitochondrial DNA in HEK293T cells: (A) To verify the mtDNA depletion, total DNA was extracted and subjected to PCR using human mitochondrial DNA specific primers mentioned in the methods. As a control, we measured the expression levels of GAPDH. **(B)** To verify mitochondrial tRNA depletion, semi quantitative PCR was performed with mitochondrial tRNAs($tRNA^{Lys}_{TTT}$ & $tRNA^{Glu}_{TTC}$) using total mitochondrial RNA extracted from HEK293T and HEK EtBr cells as a template. **(C)** HEK293T and HEK EtBr cell lysates (100 µg) were resolved on SDS-PAGE and immunoblotted with specific antibodies as indicated in the figure. **(D)** Oxygen levels of wild type (HEK) and rho zero (HEK EtBr) cells were measured by Oxymeter and represented in a graph.

3.3.5 Over expression of ABRA moderately complement the translational defect in partial mtDNA depleted HEK and MELAS cells

To examine the physiological relevance of ABRA in mitochondrial tRNA import, we performed *in vivo* mitochondrial translation assay. Initially, we check the mitochondrial translational efficiency of mtDNA depleted and wild type cell lines by using ^{35}S-methionine as described in the methods. As

shown in Fig.3.6A, ethidium bromide treated HEK293T cells showed the mitochondrial translational defect compared to untreated HEK293T cells (Fig.3.6B, compare lane 3 with lane 4). Further, we over expressed the Myc-ABRA or Myc-pCDNA in EtBr treated HEK cell lines by transient transfection for 24 hrs prior to performing an *in vivo* mitochondrial translation assay (Fig.3.6A). Over expression of ABRA partially rescues the translational defect in EtBr treated cells when compared to vector control (Fig.3.6B, compare lane 2 with lane 3). Translation reaction with pCDNA c-myc serves as a vector control. Immunoblot with anti-Tom40 (nuclear encoded mitochondrial protein) serves as equal protein loading control (Fig.3.6B, bottom panel).

It is known that the patients with MELAS carry the heteroplasmic mutation (A to G transition at nucleotide position 3243) in mitochondrial $tRNA^{Leu}_{UUR.}$ This mutation leads to abnormal protein synthesis in mitochondria and affects the mitochondrial OXPHOS [129].

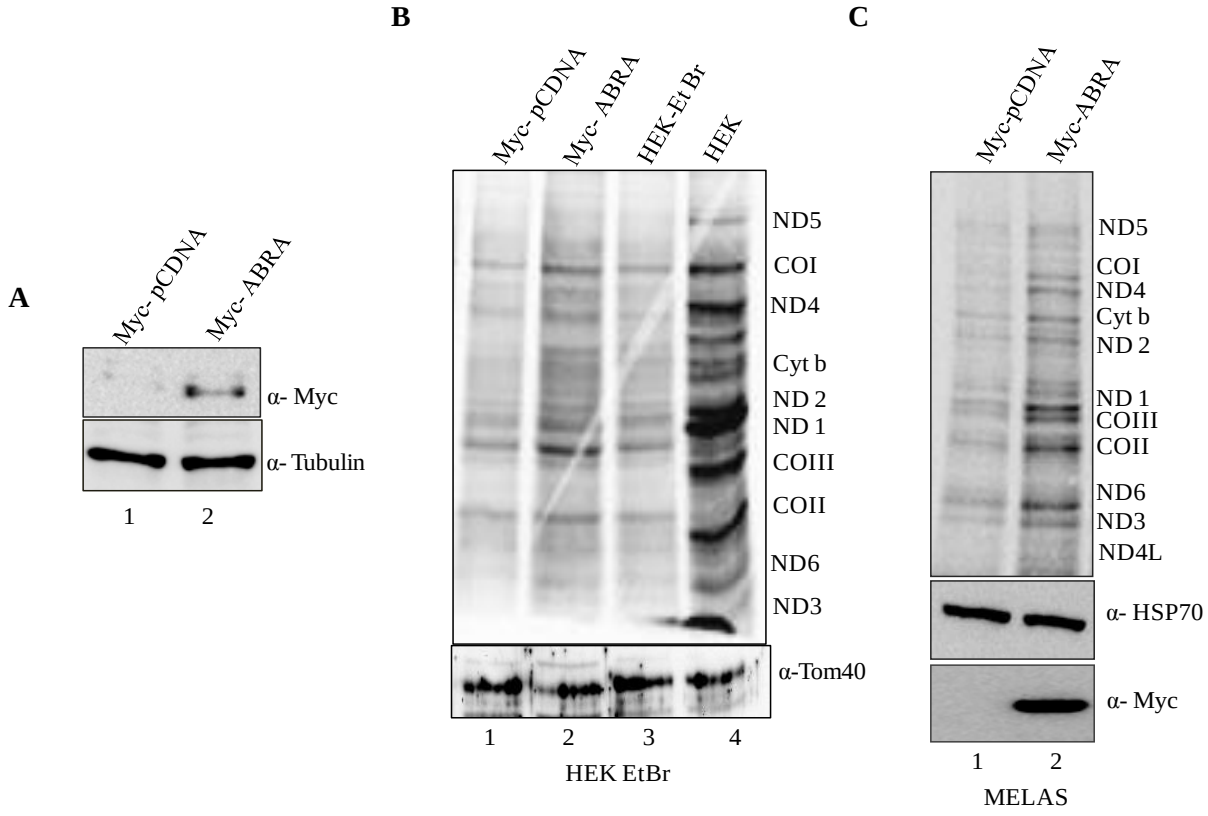


Fig.3.6 ABRA rescue mitochondrial translational defect *in vivo*: (A) Western blot analysis of Myc-pCDNA or Myc-ABRA transfected HEK EtBr cell lysates probed with anti-Myc and anti-Tubulin antibodies. **(B & C)** *In vivo* mitochondrial translation was performed in HEK293T and Myc-pCDNA or Myc-ABRA transfected HEK EtBr cells or in MELAS cells as described in methods. Cell lysates were resolved on 8% SDS-PAGE and analyzed by autoradiography.

We wanted to explore the importance of ABRA in mitochondrial tRNA recruitment using transmitochondrial cybrids MELAS harboring the point mutation. We check the mitochondrial translational efficiency of MELAS cells that were transiently transfected with Myc-ABRA by performing *in vivo* mitochondrial translation. As shown in Fig.3.6C, we observed the similar kind of rescue in mitochondrial translational defect in MELAS cells upon over expression of ABRA. Immunoblot with anti-Hsp70 (nuclear encoded mitochondrial protein) monitor as equal protein loading control (Fig.3.6C, middle panel). All these results likely indicate that mitochondrial associated cytosolic factor, ABRA has the ability to complement mitochondrial translational defect by importing functional tRNAs from cytosol to mammalian mitochondria.

3.3.6 ABRA does not interact with tRNA in vitro

It was shown that some of tRNA recruiting cytosolic factors bind to tRNA thereby induces tRNA import into mitochondria. For example, in *Saccharomyces cerevisiae*, aminoacylated tRK1 interacts with one of the glycolytic enzyme, enolase 2p in cytoplasm and targets the tRK1 on to the mitochondrial outer membrane surface. The targeted tRK1 enters to the mitochondria through the outer membrane pre-protein import complex, General Insertion Pore (GIP) [76, 80]. In *Trypanasoma brucei*, it was shown that interaction of eukaryotic elongation factor 1a (eEF1a) with the initiator $tRNA^{Met}$ is the prerequisite for the tRNA import into mitochondria [72]. As our *in vitro* and *in vivo* results suggest that the possible involvement of ABRA in mammalian mitochondrial tRNA import, we would like to examine the interaction between ABRA and tRNA. To analyze the interaction between ABRA and tRNA, we performed an electrophoretic mobility shift assay as described in the methods.

Surprisingly, we observed that there was no mobility shift of tRNA in the presence of ABRA (Fig.3.7A, lanes 3-5) unlike Cytochrome c, a known interacting partner for tRNA (Fig.3.7B, lanes 2-4) [56]. However, when we performed the biotin pull down assay with full length 5'- biotin tagged $tRNA^{Lys}_{TTT}$ from the mitochondrial salt extract and immunoblotted the pull down proteins with anti-ABRA antibody, we observed the presence of ABRA (Fig.2.7B, lane 2). This result was surprising and it may suggest that the involvement of additional components in association with ABRA to interact with tRNA and its targeting to mitochondria.

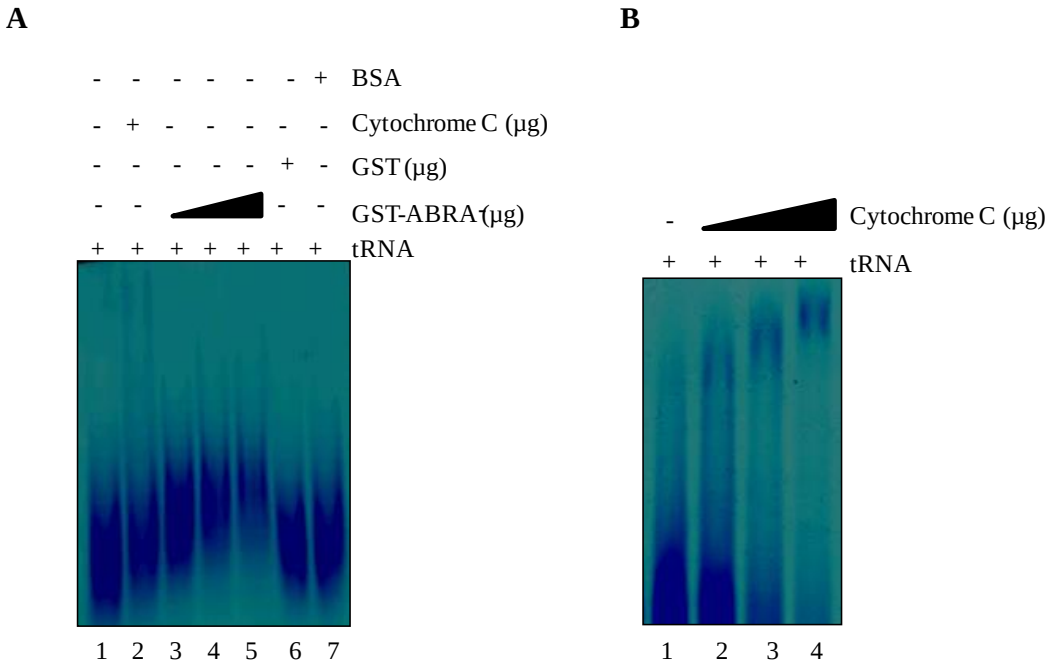


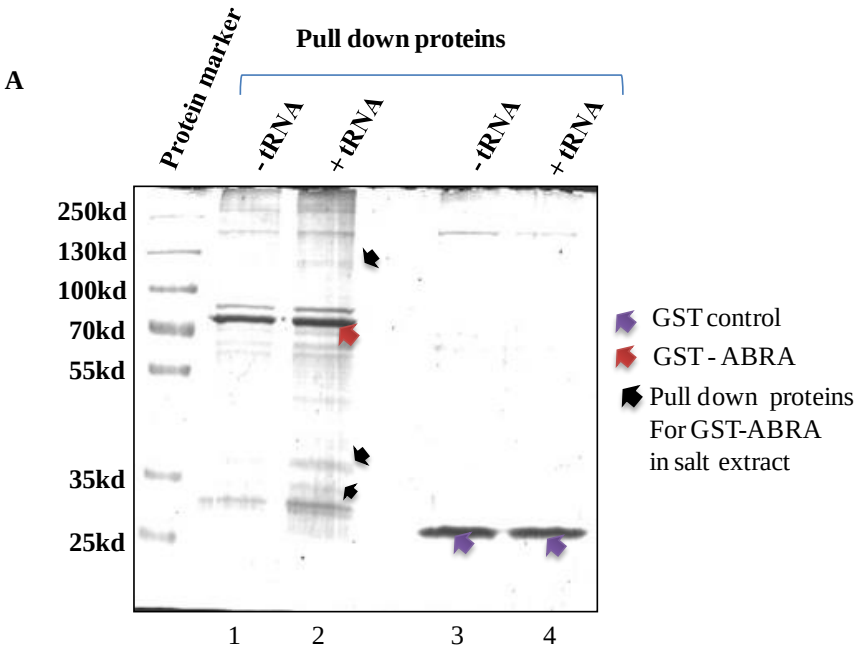
Fig.3.7 ABRA does not interact with tRNA *in vitro*. (A) Electrophoretic mobility shift assay of RNA binding reaction mixtures containing yeast tRNA (1 µM) and the indicated concentrations of GST-ABRA. RNA binding reactions with GST & BSA serves as a negative control. **(B)** Mobility shift assay with Cytochrome c and yeast tRNA serves as a positive control.

3.3.7 Identification of ABRA interacting partners from mitochondrial salt extract

Our previous results suggest that ABRA may be regulating tRNA import by interacting with other protein that may bind to tRNA. To explore this possibility, we have performed an *in vitro* GST pull

down assay to find the interacting partners of ABRA using recombinant GST-ABRA as a bait source and mitochondrial salt extract as a prey source. Initially, pre cleared mitochondrial salt extract was prepared by incubating with GST bound glutathione beads. The equal amount of pre cleared mitochondrial salt extract was incubated with GST-ABRA or GST immobilized beads in the presence or absence of tRNA. After incubation, glutathione beads were washed and the GST-ABRA or GST bound proteins were eluted by boiling glutathione beads in 2x SDS loading dye. The bound proteins were resolved on SDS-PAGE and coomassie stained.

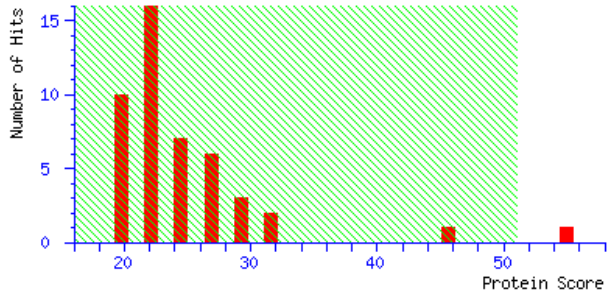
We observed that there were four major proteins that were exclusively pulled down in the presence of tRNA. These protein bands were excised from the gel and subjected to MALDI-TOF analysis (Fig.3.8A). These proteins had molecular weight ~130 kDa, ~37 kDa, ~35 kDa and ~25 kDa and were identified as KH domain containing RNA binding, signal transduction associated protein-1, Guanine Nucleotide Binding protein subunit beta-4 (GB), Rho Guanine Nucleotide Exchange Factor 39 (RhoGEF39) and Mitochondrial inner membrane translocase subunit (Tim23) respectively (Fig.3.8A).



B **Matched peptides for *GB* shown in *red*.**

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 51 are significant (p<0.05).

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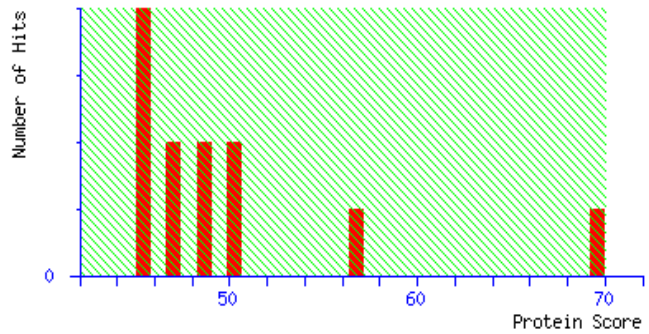
  1 MSELEQLRQE AEQLRNQIQD ARKACNDATL VQITSNMDSV GRIQMRTRRT LRGHLAKIYA
 61 MHWGYDSRLL VSASQDGKLI IWDSYTTNKM HAIPLRSSWV MTCAYAPSGN YVACGGLDNI
121 CSIYNLKTRE GNVRVSRELP GHTGYLSCCR FLDDSQIVTS SGDTTCALWD IETAQQTTTF
181 TGHSGDVMSL SLSPDMRTFV SGACDASSKL WDIRDGMCRQ SFTGHVSDIN AVSFFPNGYA
241 FATGSDDATC RLFDLRADQE LLLYSHDNII CGITSVAFSK SGRLLLAGYD DFNCNVWDTL
301 KGDRAGVLAG HDNRVSCLGV TDDGMAVATG SWDSFLRIWN
```

C **Matched peptides for *RhoGEF39* shown in *red*.**

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 70 are significant (p<0.05).

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  1 MESLGSSECC PVRQQRARWE RKRVCTAREL LETERRYQEQ LGLVATYFLG ILKAKGTLRP
 61 PERQTLFGTW ELIYAASLEL LPYLEEGQWG PGLQGFCPHL ELYTQFAANA ERSQTTLQEQ
121 LKKNKRFRRF VRLQEGRPEF RGLQLQDLLP LPLQRLQQYE NLVVALAENT VPNSSDYQQL
181 TRAARLVSET AQKVHAIGQN RKNEQHLLRV QALLSGRKAK GLTSGRWFLR QGWLLVVPPT
241 GEPRPRMFFL FSDALLMAKP RPPLHLLKSG TFVCRALYPM TQCQLSRVFG HSGGPCGGLL
301 SLSFPHEKLL LMSTDQEELS QWYHSLTQAI RSQKNSAHRS VPAT
```

Fig.3.8 Identification of ABRA interacting partners from mitochondrial salt extract: (A) Purified GST-ABRA (10 μg) or purified GST was incubated with pre cleared mitochondrial salt extract in the presence or absence of tRNA (1μg) and was followed by incubation with equilibrated glutathione agarose beads as described in methods. Beads were then washed with GST lysis buffer and proteins were eluted by heating the beads with 2xSDS loading dye and eluted proteins were analyzed by coomassie staining. **(B & C)** The protein bands excised and performed in gel digestion with trypsin followed by peptide analysis using mass spectrometry. The peptide mass fingerprint (PMF) obtained by MALDI-TOF/MS identified GB & RhoGEF39 with the mascot search parameters. Peptides matched to respective protein were indicated in red.

Among these, GB (Fig.3.8B) and RhoGEF39 (Fig.3.8C) gained our interest as these proteins also came up in our pull down assay with 5'-biotin tagged $tRNA^{Lys}_{TTT}$ along with ABRA (Fig.2.6A). All these observations led us to presume that probably ABRA, GB and RhoGEF39 may be exists in a complex and thereby these three proteins regulate the tRNA import into mammalian mitochondria.

3.3.8 ABRA interacts with GB but not with RhoGEF

Further, to validate the interaction of ABRA with GB and RhoGEF, we have purified bacterially expressed recombinant GB (Fig.3.9A) and RhoGEF (Fig.3.9B) as His-fusion proteins described in the methods. Using recombinant GST-ABRA, His-GB and His-RhoGEF, we performed an *in vitro* protein pull down assay. Briefly, GST-ABRA or GST immobilized sepharose beads were incubated with either His-GB or His-RhoGEF in a binding buffer as described in the methods. Bound proteins were collected by boiling the beads in 2X SDS loading dye. Eluted proteins were resolved on SDS-PAGE and immunoblotted with anti-His antibody. We find that His-RhoGEF was present in both eluates of GST-ABRA and GST sepharose beads (Fig.3.9C, top panel, compare lanes 1 & 3). This result likely indicates that the His-RhoGEF shows a non specific interaction with GST alone (Fig.3.9C, top panel, compare lane 1). However, this experiment does not exclude the possibility of Rho-GEF interaction with ABRA. Further, we find a specific interaction of His-GB with GST-ABRA as His-GB was exclusively present in the eluate when we used GST-ABRA as bait. (Fig.3.9C, top panel, compare lane 2 & 4).

Immunoblotting of eluates with anti-GST antibody confirms the presence of similar amount of immobilized bait proteins (GST-ABRA and GST) in all reactions (Fig.3.9C, bottom panel, lanes 1-4). Based on above observations, we hypothesize that Rho-GEF may be interacting with GB and thereby facilitate an indirect interaction between RhoGEF and ABRA. Nevertheless, these three proteins probably exist in a complex to perform a similar biological function such as delivering tRNA onto the mitochondria.

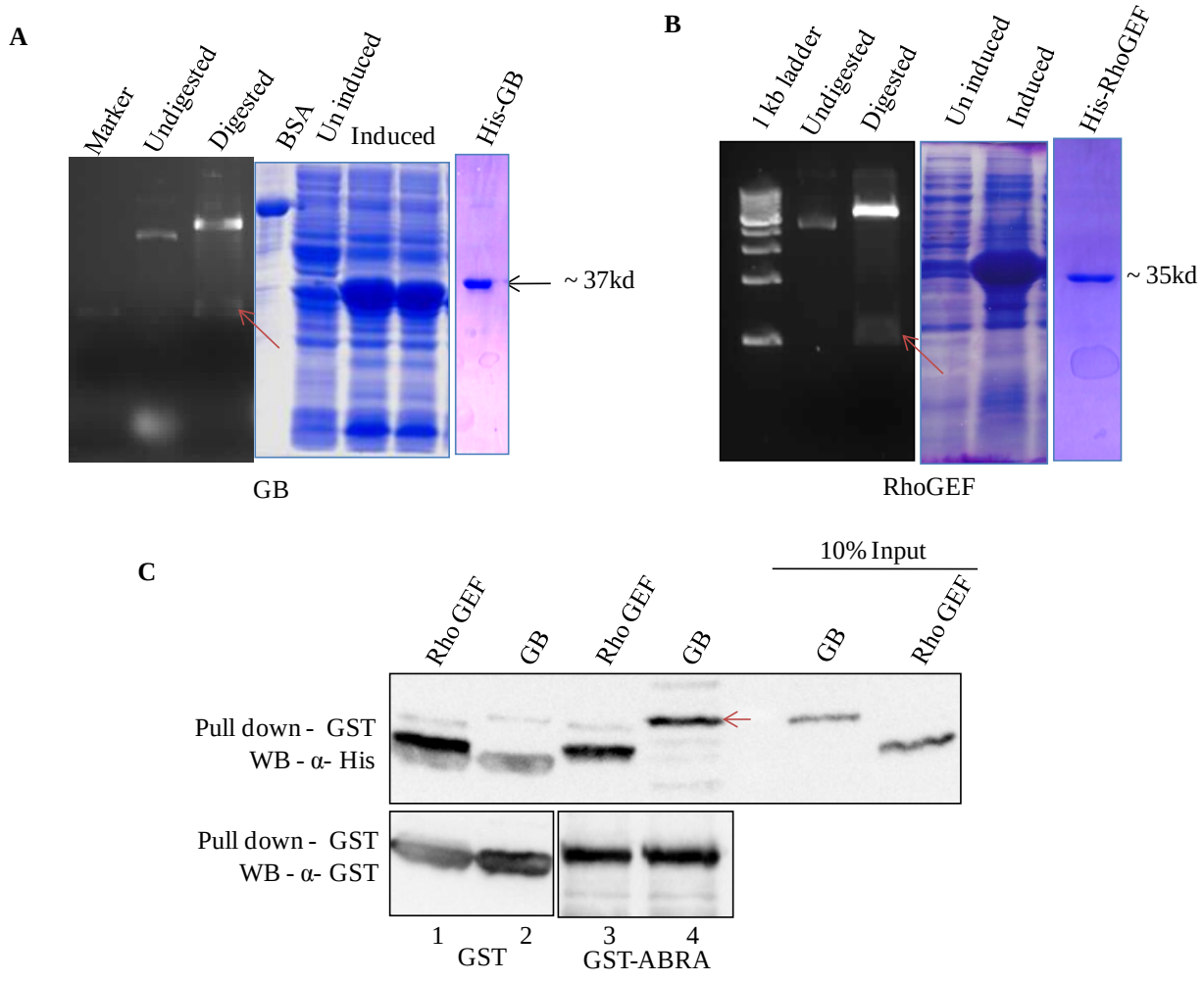

Fig.3.9 ABRA directly interacts with GB but not with RhoGEF *in vitro*: (A & B) Bacterial expression of GB or RhoGEF with His tag upon induction with 1 mM IPTG. Purity of His-GB and His-RhoGEF was examined by coomassie staining. **(C)** His-GB or His-RhoGEF were pull down with GST or GST-ABRA

immobilized beads and immunoblotted with anti-His and anti-GST antibodies. The loading inputs were 10% of the total purified His-GB and His-RhoGEF.

3.3.9 GB interacts with tRNA but not RhoGEF

Our *in vitro* protein pull down assays confirms that ABRA has a physical interaction with GB protein. Since ABRA does not have the ability to bind tRNA (Fig.3.7A), we speculated whether ABRA interacting partner GB protein can bind to tRNA. To address this question, we performed an *in vitro* tRNA binding assay as described in the methods subchapter *3.2.18*. Briefly, recombinant proteins were immobilized onto beads (His-GB or His-RhoGEF onto Ni-NTA beads and GST-ABRA onto glutathione sepharose beads) followed by incubation with either ^{32}p-radiolabeled $tRNA^{Lys}_{TTT}$ (Fig.3.10A) or unlabeled tRNA (Fig.3.10B). After incubation, washing, the bound tRNAs were eluted by incubating beads with proteinase K as described in the methods The eluted radiolabeled $tRNA^{Lys}_{TTT}$ or unlabeled tRNA was analyzed by autoradiogram (Fig.3.10A) or ethidium bromide staining (Fig.3.10B). We observed that tRNA was eluted from His-GB immobilized Ni-NTA beads indicating that GB can interacts with tRNA. However, tRNA fails to interact with ABRA and RhoGEF as tRNA was not eluted from the GST-ABRA immobilized glutathione beads or His-RhoGEF immobilized Ni-NTA beads (Fig.3.10A & B).

Further, we also analyzed the interaction of GB protein with tRNA by performing fluorescence spectroscopy analysis. The intrinsic fluorescence emission is mainly contributed by the tryptophan amino acids and to a lesser extent by phenylalanine and tyrosine amino acids resident in a protein. As shown in Fig.3.10C, recombinant His-GB gives a typical intrinsic tryptophan fluorescence emission peak at 340 nm. However, there is a gradual quenching of intrinsic fluorescence peak of tryptophan upon incubation of recombinant His-GB with increasing concentrations of tRNA (0.1 μM to 1.2 μM) indicating the binding of tRNA to GB protein. Altogether, these results suggest that

ABRA interacting partner GB protein binds to tRNA and may exhibit a possible role in regulating tRNA import into mammalian mitochondria.

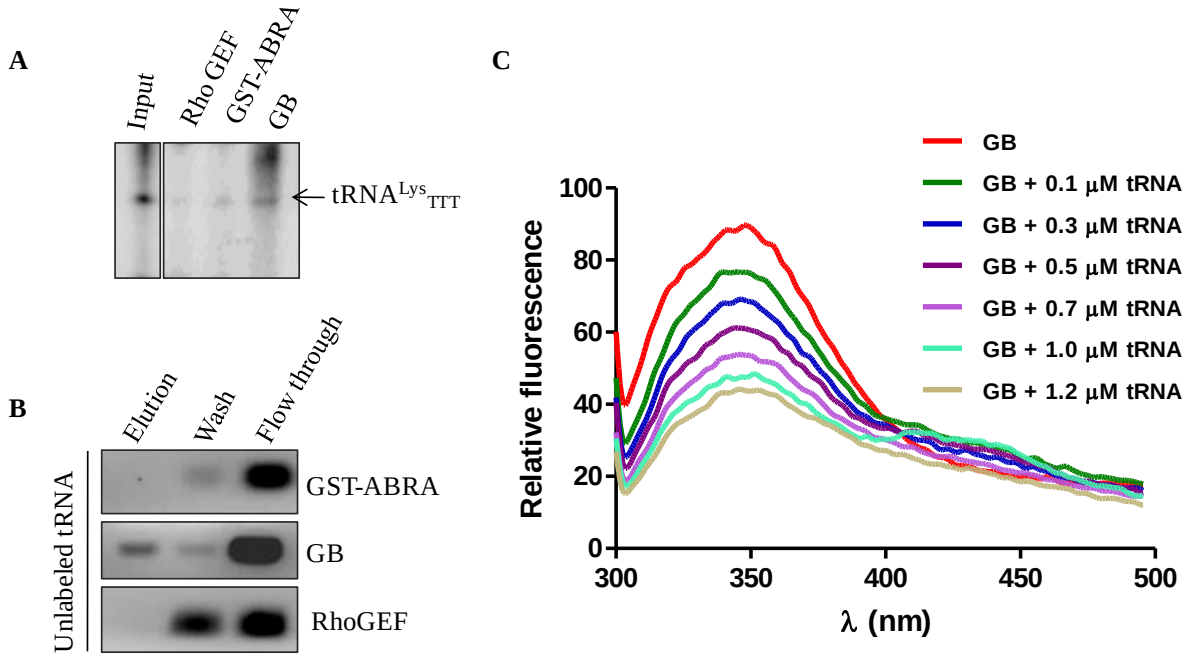

Fig.3.10 GB interacts with tRNA *in vitro*: (A & B) *In vitro* tRNA binding affinity assay was performed by incubating recombinant His-GB or His-RhoGEF or GST-ABRA with ^{32}P-radiolabeled tRNA$^{Lys}_{TTT}$ or with unlabeled yeast tRNA as described in methods. Bound tRNA was analyzed by either autoradiogram (A) or ethidium bromide staining (B). **(C)** Fluorescence emission spectra were recorded for His-GB (10 µg) in the absence and presence of indicated concentrations of tRNA.

3.3.10 ABRA, GB and RhoGEF resides on mitochondrial outer membrane

Since ABRA, GB and RhoGEF proteins were originally identified from the mitochondrial salt extract (Fig.2.6A), we wish to investigate the sub cellular localization of these proteins in HEK293T cells. To examine the localization of these proteins, we have cloned *hABRA*, *hGB* and *hRhoGEF* into mammalian expression vector Myc-pCDNA (Fig.3.11A). Using HeLa cell cDNA as a template, *hABRA*, *hGB* and *hRhoGEF* ORFs were amplified using *hABRA* specific primers (NB169 and NB329), *hGB* specific primers (NB475 and NB509) and *hRhoGEF* specific primers (NB477 and

NB510) respectively. The amplified products were cloned into Myc-pCDNA vector as described in the methods.

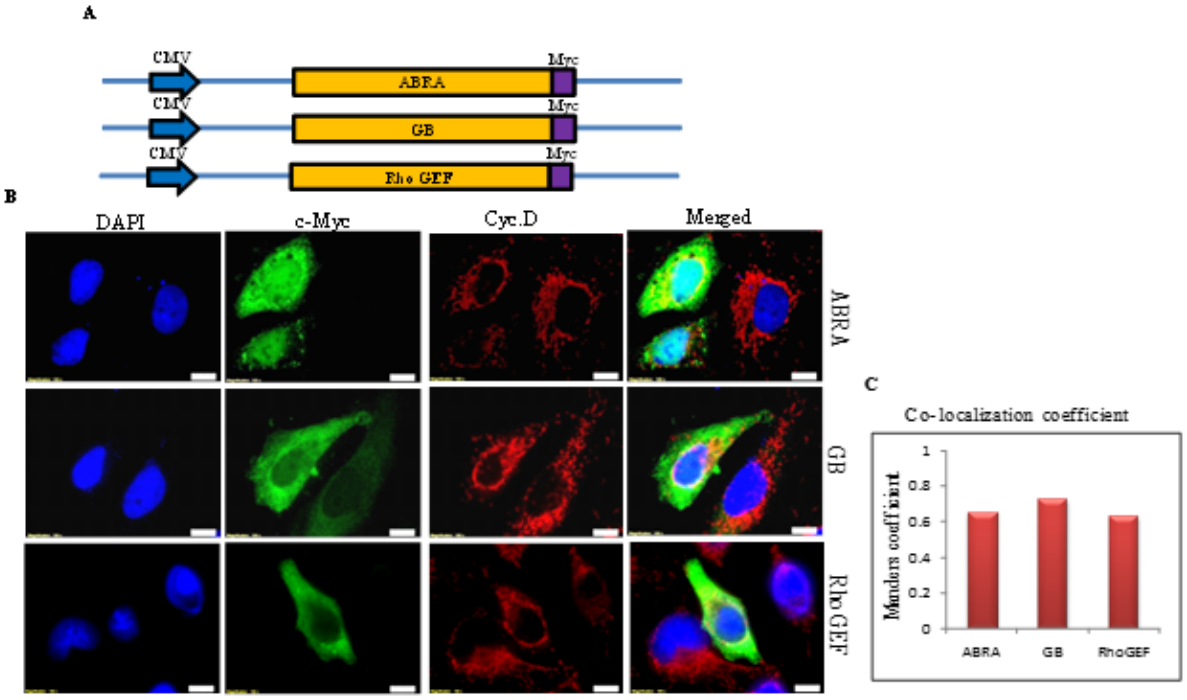

Fig.3.11 Localization of ABRA, GB and RhoGEF on mitochondrial outer surface: (A) *hABRA*, *hGB*, *hRhoGEF* genes were cloned into Myc fused mammalian expression vector (**B)** Subsequently, these Myc-fusion constructs were transfected into HEK293T cells. 48h post transfection cells were fixed and incubated with anti-Myc and anti-Cyclophilin D (mitochondrial marker) antibodies. Later, cells were incubated with Alexa labeled secondary antibodies to monitor the localization of Myc-fusion proteins as described in the methods. (**C)** Co-localization analysis was performed using Image J software and values of Mander's coefficient were plotted.

To check the sub cellular localization of ABRA, GB and RhoGEF, HEK293T cells were transfected with the plasmid harbouring *Myc-ABRA* or *Myc-GB* or *Myc-RhoGEF* for 48 hrs. The samples were processed for confocal microscopy as mentioned in the methods. As shown in Fig.3.11B, all three proteins ABRA, GB and RhoGEF were merged with mitochondrial marker, cyclophilin D. In addition, the localization pattern of ABRA, GB and RhoGEF in HEK293T cells clearly demonstrating that these proteins were probably localized to the mitochondrial outer membrane.

Further, the efficiency of co-localization of ABRA, GB and RhoGEF with mitochondria was quantified by calculating Mander's co-localization coefficient using Image J software (Fig.3.11C).

3.3.11 GTP enhances ABRA mediated tRNA import into mammalian mitochondria

Actin Binding Rho Activating Protein (ABRA) is an activator of Rho protein and known to induce the intrinsic GTPase activity of Rho protein, Guanine nucleotide binding protein (GB) is a GTPase and its activity is known to be regulated by the factors that control its ability to bind and hydrolyze GTP to GDP [130] and Rho guanine nucleotide exchange factor (RhoGEF) is a guanine nucleotide exchanger and it is known to stimulate the release of GDP and allow the binding of GTP to the GTPase [131]. Based on the known functions of these proteins, we hypothesized that the GTPase activity of GB may be regulating the tRNA import in the presence of co-activators ABRA and RhoGEF on the mitochondrial outer membrane surface (Fig.3.10). As GTP is the major molecule that is regulating the function of GB, we would like to check the role of GTP in mitochondrial tRNA import. It is known that addition of ATP induces the recruitment of tRNA into mammalian mitochondria [64]. Initially, we wished to investigate the role of GTP as a sole energy source on tRNA import into mammalian mitochondria. Addition of 5 mM GTP/ATP has a stimulatory effect on tRNA import when compared to 2 mM GTP/ATP (Fig.3.12A, compare lanes 3 & 5 with 2 & 4). However, the addition of non-hydrolyzable GTP analogue (GMPPNP) inhibits the tRNA translocation into mitochondria in 5 mM GTP/ATP supplemented samples (Fig.3.12A, lanes 6 and 7). These results suggest that the GTP hydrolysis is essential for efficient translocation of tRNA across mitochondrial membranes.

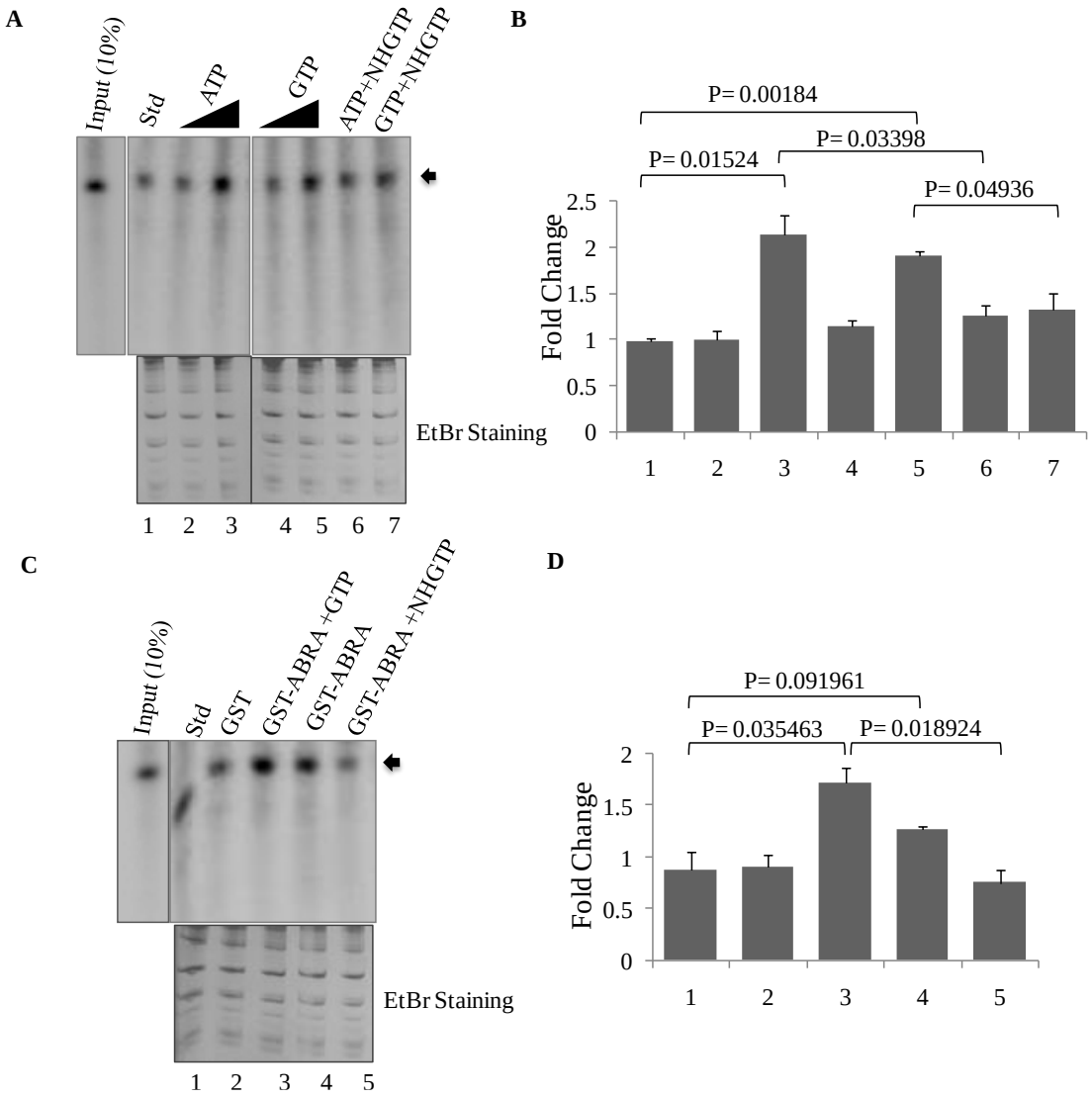

Fig.3.12 GTP stimulates ABRA mediated tRNA import: (A & B) *In vitro* tRNA import assay was performed by incubating mitochondria with ^{32}P- radiolabeled $tRNA^{Lys}_{TTT}$ in the presence of increasing concentrations of only ATP (2 mM and 5 mM) or only GTP (2 mM and 5 mM) and ATP/GTP with non hydrolyzable GTP analogue (5 mM). **(C & D)** Mitochondria were incubated with ^{32}P- radiolabeled $tRNA^{Lys}_{TTT}$ in the presence of GST-ABRA (1 μg) in an import buffer containing 2 mM ATP (lanes, 1, 2, 4 & 5) and GTP (5 mM, lane 3) or with non hydrolyzable GTP (5 mM, lane 5). Import reaction in the presence of GST (1 μg) serves as a negative control. All the experiments were performed in duplicates and the quantitative representations of band intensities of imported tRNAs were measured by densitometry. Statistical analysis was performed using *t- test*.

Further, we would like to check the role of GTP in the presence and absence of GST-ABRA on tRNA import into isolated, native mammalian mitochondria in which all the peripherally associated cytosolic proteins were intact. Addition of GTP to GST-ABRA containing samples further

stimulates the tRNA import by two fold when compared to the GST-ABRA containing sample (Fig.3.12B, compare lane 3 with 4). Further, the stimulatory effect on tRNA import mediated by ABRA is inhibited by non-hydrolyzable GTP analogue (Fig.3.12B, lane 5). All these results indicate that recombinant GST-ABRA stimulates the import of externally added tRNA into mammalian mitochondria in a GTP dependent manner *in vitro*.

3.3.12 ABRA acts in concert with GB and RhoGEF to regulate mammalian mitochondrial tRNA import

All the above observations prompted us to hypothesize that ABRA stimulates the mitochondrial tRNA import by inducing the intrinsic GTPase activity of GB bound to tRNA. Hydrolysis of GTP leads to conformation change in GB that may cause the release of bound tRNA onto the mitochondrial surface. For next round of tRNA import cycle, GDP bound from of GB may convert to GTP bound form of GB in the presence of RhoGEF. To test this hypothesis, we have performed an *in vitro* tRNA import assay with isolated mitochondria in the presence of indicated combinations of purified proteins GST-ABRA or His-GB or His-RhoGEF along with GTP (Fig.3.13A). As shown in the Fig.3.13A, we observed moderate increase in the levels of imported tRNAs into mitochondria that were incubated with only GST-ABRA (lane 4) or His-GB (lane 5) or His-RhoGEF (lane 6). However, the levels of imported tRNAs are further enhanced in mitochondria samples that were pre-incubated with the combination of GST-ABRA & His-GB (lane 7) but not with either of the combinations GST-ABRA & His-RhoGEF (lane 8) or His-GB & His-RhoGEF (lane 9).

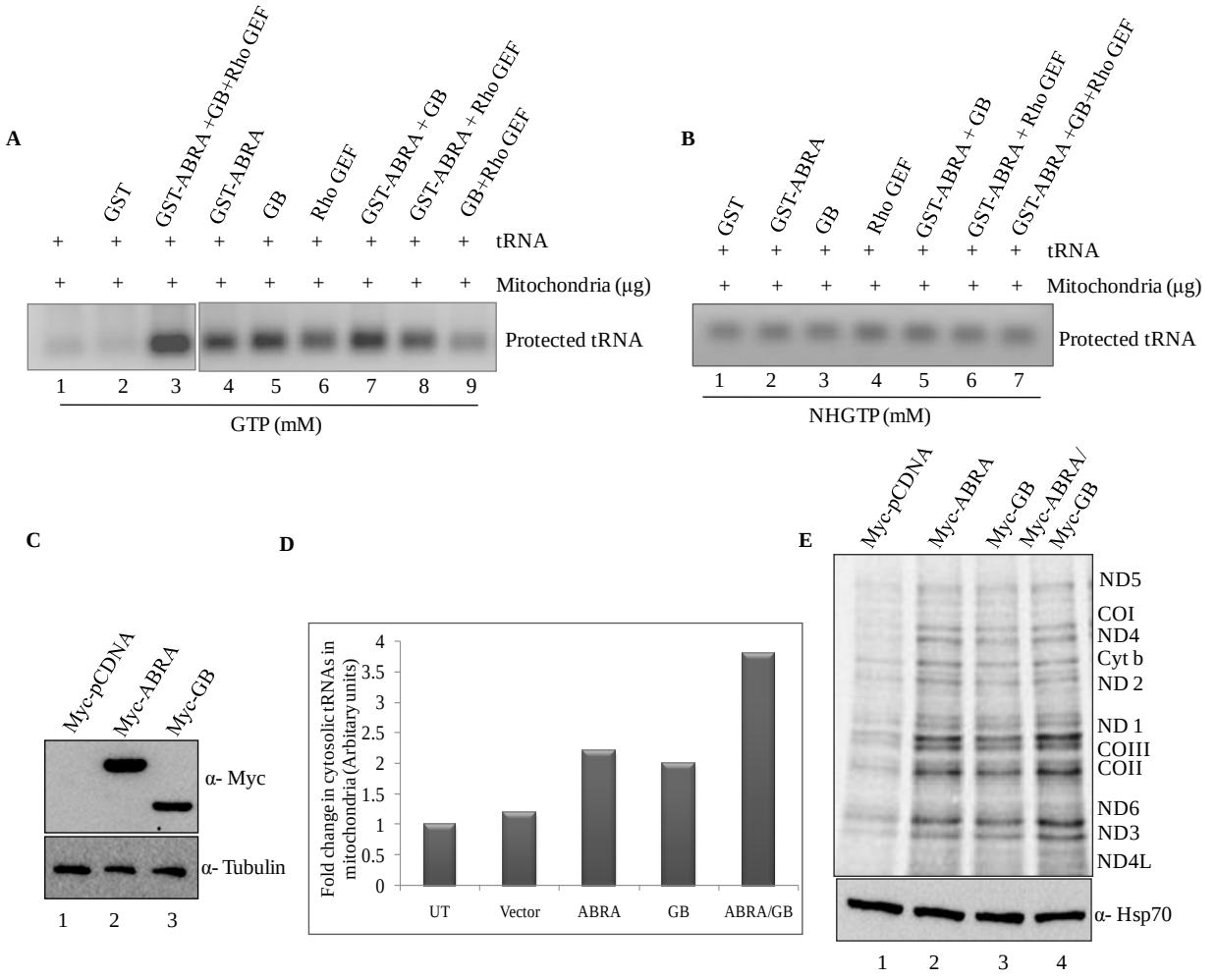

Fig.3.13 ABRA, GB and RhoGEF show additive stimulating effect on mitochondrial tRNA import: (A & B) *In vitro* tRNA import assay was performed by incubating mitochondria with $tRNA^{Lys}_{TTT}$ in the presence of indicated combinations of purified proteins in the presence of GTP (5 mM) or non-hydrolyzable GTP (5 mM) as described in methods. **(C)** Western blot analysis of Myc-pCDNA or Myc-ABRA/Myc-GB transfected MELAS cell lysates probed with anti-Myc and anti-Tubulin antibodies. **(D)** Graphical representation of relative levels of cytosolic $tRNA^{Lys}_{TTT}$ upon co-expression of Myc-fusion constructs. **(E)** *In vivo* mitochondrial translation was performed in Myc-pCDNA or Myc-ABRA/Myc-GB transfected MELAS cells as described in methods. Cell lysates were resolved on 8% SDS-PAGE and analyzed by autoradiography.

In the presence of GST-ABRA & GB, GTPase activity of GB is probably induced by ABRA and thereby releases the bound tRNA on to the mitochondrial surface. However, there is no stimulation in tRNA import in the presence of either combination of GST-ABRA & RhoGEF or GB & RhoGEF, due to the lower levels of GTPase (GB) or co-activator (ABRA) respectively. In addition,

we also observed an increase in the accumulation of cytosolic $tRNA^{Lys}_{TTT}$ levels in mitochondria of HEK cells (Fig.3.13D) and an efficient rescue in the translational defect in MELAS cells (Fig.3.13E) upon co-transfection with the plasmids harbouring Myc-ABRA/Myc-GB.

Interestingly, there was a prominent additive effect on tRNA import in the mitochondria that were incubated with ABRA, GB and RhoGEF together (Fig.3.13A, lane 3). However, this additive effect on mitochondrial tRNA import was decreased in the presence of non-hydrolyzable GTP (Fig.3.13B, lane 7). All these results suggest that ABRA may act in concert with GB and RhoGEF to regulate tRNA import into mammalian mitochondria in a GTP dependant manner. Taken together, our study identifies a novel GTPase cycle (consists of ABRA, GB and RhoGEF) on outer mitochondrial surface in the regulation of tRNA import in mammalian mitochondria.

3.4 DISCUSSION

In lower organisms, import of tRNAs from cytosol to mitochondria has revealed to maintain the mitochondrial translation, further mitochondrial biogenesis. The specificity and extent to which individual tRNAs are imported differs greatly between organisms and might reflect fundamental differences in mechanisms underlying tRNA import. The cytosolic factors and/or receptors involved in the regulation of tRNA import in lower organisms have extensively studied.

It has been shown that mammalian mitochondria can take up very few, specific cytosolic tRNAs post-transcriptionally in an energy dependant manner [64]. However, the cytosolic factors and/or receptors that are involved in the targeting of specific tRNAs onto mitochondria and their exact mechanism of regulation of tRNA import are not extensively studied. As most of the mitochondrial respiratory chain deficiencies associated with mutations in mitochondria encoded tRNA genes [127], finding the mechanism that facilitates the import of tRNAs from cytosol to mitochondria could provide the novel genetic therapies.

For the first time, our study identified the novel function of one of the mitochondrial associated, salt extractable soluble cytosolic protein factors, Rho GTPase activating protein (ABRA) in regulating the tRNA import into mammalian mitochondria. Recombinant ABRA stimulates tRNA import into mammalian mitochondria in a dose dependent manner *in vitro* (Fig.2.9A). Our studies show that there is an elevation in cytosolic tRNA levels in ABRA over expressed HEK293T cells (Fig.3.2B, C and Fig.3.3). In contrary, we observed that selective depletion of ABRA in mammalian HEK293T cells results in decrease in accumulation of cytosolic tRNAs in mitochondria (Fig.3.4B, C).

It has been shown that import of tRNAs from cytosol to mammalian mitochondria requires energy in the form of ATP [64]. Here our data demonstrate that GTP hydrolysis is also critical for translocation of tRNAs into mitochondria as addition of non-hydrolyzable GTP analogue inhibits the import of tRNAs in ATP or GTP supplemented samples *in vitro* (Fig.3.12A). Further, we find that GTP in conjunction with ABRA has a stimulating effect on tRNA import (Fig.3.12B). Our electrophoretic mobility shift assays demonstrate that ABRA does not interact with tRNA (Fig.3.7A). We speculate that other ABRA interacting tRNA binding proteins present in the extract are required for stimulation of tRNA import into mitochondria.

These observations prompted us to hypothesize that an indirect or sidewise interaction between ABRA and tRNA through other tRNA binding protein components of salt extract or mitochondria. It is known that proteins can bind directly to the RNA or indirectly through other RNA-bound proteins effectively. Proteins which do not directly bind to RNA molecules are known to be involved in various processes such as transcription and downstream mRNA processing etc. One such example is the large group of factors that interacts with C- terminal domain of RNA polymerase II, including chromatin remodeling complexes and histone modifying enzymes during transcription. Such factors can be cross linked to RNA via protein (RNAPII) bridges [132]. It has

been shown that transcriptional co activators, p300 and CREB binding protein (CBP) can serve as adapters for RNA binding protein (HIV-1 Tat) [133].

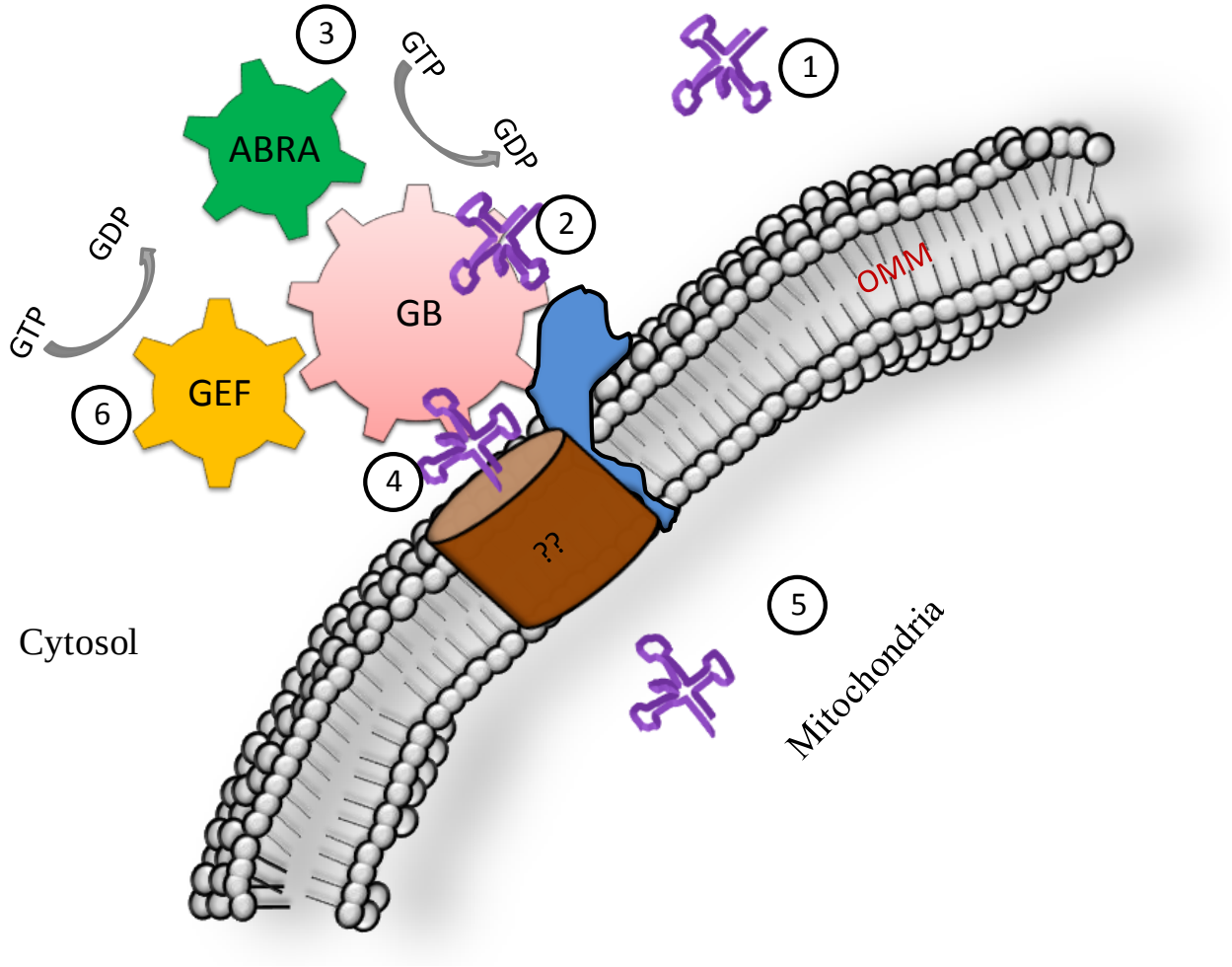

Fig.3.14 Mechanism of tRNA import into mammalian mitochondria by GTP regulatory proteins: Cytosolic tRNA comes **1)** and binds to mitochondrial outer surface resident protein, GB **2)**. The intrinsic GTPase activity of GB may be enhanced upon interacting with Rho GTPase activating protein, ABRA and converts GTP bound form of GB into GDP form of GB **3)**. This may induce the conformational change in GB and releases the bound tRNA onto mitochondrial surface **4)** which may enters to mitochondria through unidentified translocator **5)**. For next round of tRNA import, GDP bound GB converts to GTP bound GB in the presence of cytosolic nucleotide exchanger, RhoGEF **6)**.

Further, to determine the precise role of GTP and tRNA import mechanism mediated by ABRA, we have identified and characterized the interacting partners of ABRA from mitochondrial salt extract. Using *in vitro* GST pull down and *in vitro* protein - protein interaction studies, we identified Guanine nucleotide binding protein (GB) and Rho guanine nucleotide exchange factor 39 (RhoGEF39) as ABRA interacting partners (Fig.3.8 and Fig.3.9C). Further, using *in vitro* tRNA affinity binding and fluorescence spectroscopy studies, we have shown that GB directly binds to tRNA (Fig.3.10). Fluorescence microscopic studies suggest that these three proteins are indeed localized to mitochondrial outer membrane (Fig.3.11B).

With respect to localization of these proteins on mitochondrial outer surface, we also observed the collaborative effect of these three proteins in the stimulation tRNA import onto mitochondrial surface in GTP dependant manner (Fig.3.13A). Further, our *in vivo* mitochondrial translational studies show that co-expression of ABRA and GB stimulates the tRNA import (Fig.3.13D) and also rescue the translational defect in MELAS cells lines (Fig.3.13E). Together, all our observations suggest that, ABRA may act in concert with GB and RhoGEF to regulate tRNA import into mammalian mitochondria. Our studies provide a first clue that mammalian cells have a regulatory system (GTPase cycle) in cytosol prior to translocation of tRNAs into mitochondria (Fig.3.14). Further studies are needed to identify the translocators in mitochondrial outer membrane specifically involved in tRNA transport and the type of interaction between translocators and the cytosolic regulatory proteins that regulate the release of tRNAs into mitochondria.

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ORIGINAL PAPER

Perturbation of apoptosis upon binding of tRNA to the heme domain of cytochrome *c*

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Abstract In response to apoptotic stimuli, cytochrome *c*, an inter-membrane space protein is released from mitochondria to activate the cascade of caspases that leads to apoptosis. Recent evidence suggests that cytochrome *c* interacts with tRNA in the cytoplasm and this interaction was shown to inhibit the caspase mediated apoptotic process. Interestingly, cytochrome *c* does not contain any putative RNA binding domain. In this report, we sought to define the structural component of cytochrome *c* that is involved in binding of tRNA. By using gel mobility shift assays, we show that holocytochrome *c* can interact with tRNA but not apocytochrome *c* that lacks the heme domain suggesting that heme is essential for the interaction of cytochrome *c* to tRNA. In addition, using in vitro cross linking and circular dichroism spectroscopic studies, we show that cytochrome *c* can undergo heme mediated oligomerization. Prevention of heme mediated oligomerization of cytochrome *c* by potassium ferricyanide treatment prevents the binding of tRNA and promotes caspase activation. Our studies provide a novel regulation of apoptosis by heme dependent tRNA interaction to cytochrome *c*.

Keywords Cytochrome *c* · Transfer RNA (tRNA) · Apoptosis · Heme · Apocytochrome *c* · Hemin

Introduction

Apoptosis is the major regulatory multi-step pathway to maintain the cellular homeostasis in living organisms besides being an obstacle for diseases like cancer [1–3]. Apoptosis in mammalian cells is mediated by two major pathways known as extrinsic pathway and intrinsic pathway. In the extrinsic pathway, Fas and TNF-related apoptosis inducing ligand form death inducing signaling complex that facilitates the activation of some effector caspases to induce apoptosis [1, 4, 5]. In case of the intrinsic pathway also known as the mitochondrial pathway, apoptosis is mediated by cytochrome *c* dependent caspases [1, 6]. Cytochrome *c* is a heme containing soluble electron carrier that is present in the mitochondrial inner membrane and plays a crucial role in transferring electrons from complex III to complex IV [7]. In response to DNA damage, depletion of nutrients or activation of oncogenes, cytochrome *c* is released from the mitochondria into the cytosol [8]. In the cytosol, cytochrome *c* binds to apoptotic protease activating factor-1 (APAF-1) and forms a heptameric apoptosome complex [9]. Oligomerized APAF-1–cytochrome *c* complex activates procaspase-9 to active caspase-9 by auto proteolysis. Activated caspase-9 stimulates the subsequent effector caspases like caspase-3 and caspase-7 that eventually degrade many cellular proteins and cause cell death [9, 10].

Apoptosis is known to be regulated by several pro-apoptotic proteins (Bax, Bak and Bid), anti-apoptotic proteins (Bcl-2 and Mcl-1) and a range of cellular factors (HSP90, HSP70 and HSP27) [8, 11]. Interestingly, recent reports have shown that transfer RNA (tRNA) binds cytochrome *c* and inhibits apoptosis by preventing the formation of apoptosome complex [12]. Microinjection of tRNA into living cells prevents the cytochrome *c* induced caspase-9 activation whereas treating cells with tRNA specific RNase, onconase, results in the activation of cytochrome *c* mediated caspase-9 [12]. However, the mechanism of cytochrome *c*–tRNA interaction and the interacting residues or domains therein are yet to be elucidated.

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Mitochondrial Lysyl-tRNA Synthetase Independent Import of tRNA Lysine into Yeast Mitochondria

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Abstract

Aminoacyl tRNA synthetases play a central role in protein synthesis by charging tRNAs with amino acids. Yeast mitochondrial lysyl tRNA synthetase (Msk1), in addition to the aminoacylation of mitochondrial tRNA, also functions as a chaperone to facilitate the import of cytosolic lysyl tRNA. In this report, we show that human mitochondrial Kars (lysyl tRNA synthetase) can complement the growth defect associated with the loss of yeast Msk1 and can additionally facilitate the *in vitro* import of tRNA into mitochondria. Surprisingly, the import of lysyl tRNA can occur independent of Msk1 *in vivo*. This suggests that an alternative mechanism is present for the import of lysyl tRNA in yeast.

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Introduction

Aminoacyl-tRNA synthetases are a heterogeneous family of enzymes responsible for aminoacylating tRNAs with the appropriate amino acids. The budding yeast, *Saccharomyces cerevisiae*, contains two sets of lysyl-tRNA synthetases (Msk1 and Krs1) that are encoded by nuclear DNA. Krs1 participates in cytoplasmic protein synthesis while Msk1 in mitochondrial protein synthesis. Despite the structures of the Msk1 and Krs1 being different, functionally they are very similar. The yeast Msk1 has a bacterial ancestry, whereas the Krs1 represents the ancestral eukaryotic type [1,2].

Yeast Msk1 is a dual functional protein. In addition to aminoacylation of mitochondrial $tRNA^{Lys}$ (tRK3), it has been proposed that Msk1 plays an essential role in the import of cytosolic $tRNA^{Lys}_{CUU}$ (tRK1) into mitochondria [3]. There are two other lysine isoacceptors in yeast cells: the non-imported nuclear encoded $tRNA^{Lys}_{UUU}$ (tRK2) and the mitochondrial DNA encoded $tRNA^{Lys}_{UUU}$ (tRK3) [4]. Further, the imported tRK1 that was specifically mutagenized to alter amino acid specificity was functional in yeast mitochondrial translation both *in vivo* and *in vitro* [5]. However, for tRK1 to be eligible for mitochondrial import, it has to go through a complex set of reactions that includes aminoacylation of tRK1 by cytosolic lysyl-tRNA synthetase, interaction of tRK1 with glycolytic enzyme enolase 2 (Eno2) and binding to the precursor form of Msk1 (pre-Msk1) [3,6,7]. The subsequent translocation across the mitochondrial membranes requires intact protein import machinery and ATP [3,8]. The function of the imported tRNA is conditional and its import is also regulated by ubiquitin/26S proteosome [9,10]. Further, the translocation across the mitochondrial membranes requires intact protein import machinery, ATP and additional unidentified cytosolic factors. However, the charged tRK1 imported into mitochondria by the pre-Msk1p mediated mechanism is only utilized in one cycle of translation as it cannot be re-charged by Msk1. The utilization of tRK1 in mitochondria is dependent on continuous action of cytosolic and mitochondrial tRNA synthetases. Hence the utilization of imported tRK1 is limited by the activity and availability of these two synthetases besides its function being restricted to one round of translation.

Yeast tRK1 can also be imported into human mitochondria in the presence of yeast cytosolic factors and Msk1 [5]. In addition, human cytosolic factors can replace yeast cytosolic factors in the presence of Msk1 to drive the import of tRK1 into human mitochondria [5]. It was previously suggested that human Kars might play a similar kind of role in the import of tRK1 into mitochondria [11]. However, there was no direct evidence showing that human Kars is indeed involved in the import of tRK1 into either human or yeast mitochondria.

We previously have shown that human mitochondrial and cytoplasmic lysyl tRNA-synthetases are expressed from alternative spliced mRNAs from a single gene [12]. We are interested to see whether human mitochondrial tRNA synthetase mitigates the role of yeast tRNA synthetases in the import and aminoacylation of tRK1 since the evolution and structural relatedness of these enzymes has been a subject of intense research for many years. Unlike yeast synthetases, both human cytosolic and mitochondrial lysyl-tRNA synthetases (Kars) are capable of aminoacylating yeast tRK1. Human mitochondrial Kars can substitute for yeast Msk1 for protein synthesis and tRNA import functions. Further, our findings suggest that human Kars facilitates the import of tRK1 into isolated yeast, rat and human mitochondria. In addition, human *KARS* partially suppresses the growth defects that are associated with yeast *MSK1* deletion. Interestingly, *in vivo*

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Angiogenin-Cleaved tRNA Halves Interact with Cytochrome *c*, Protecting Cells from Apoptosis during Osmotic Stress

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Adaptation to changes in extracellular tonicity is essential for cell survival. However, severe or chronic hyperosmotic stress induces apoptosis, which involves cytochrome *c* (Cyt *c*) release from mitochondria and subsequent apoptosome formation. Here, we show that angiogenin-induced accumulation of tRNA halves (or tiRNAs) is accompanied by increased survival in hyperosmotically stressed mouse embryonic fibroblasts. Treatment of cells with angiogenin inhibits stress-induced formation of the apoptosome and increases the interaction of small RNAs with released Cyt *c* in a ribonucleoprotein (Cyt *c*-RNP) complex. Next-generation sequencing of RNA isolated from the Cyt *c*-RNP complex reveals that 20 tiRNAs are highly enriched in the Cyt *c*-RNP complex. Preferred components of this complex are 5′ and 3′ tiRNAs of specific isodecoders within a family of isoacceptors. We also demonstrate that Cyt *c* binds tiRNAs *in vitro*, and the pool of Cyt *c*-interacting RNAs binds tighter than individual tiRNAs. Finally, we show that angiogenin treatment of primary cortical neurons exposed to hyperosmotic stress also decreases apoptosis. Our findings reveal a connection between angiogenin-generated tiRNAs and cell survival in response to hyperosmotic stress and suggest a novel cellular complex involving Cyt *c* and tiRNAs that inhibits apoptosome formation and activity.

Cellular stress initiates a complex cascade of signaling pathways in an attempt to return the cell to its previous equilibrium. In most cases, the type, duration, and intensity of stress govern the outcome of the cellular response, resulting in either adaptation or cell death. Apoptosis is induced if the damage that occurred during stress exceeds the capacity of the repair mechanisms.

In vertebrates, apoptosis is orchestrated through one of two signaling cascades, termed the intrinsic and extrinsic apoptotic pathways (1). Both pathways converge at the activation of executioner caspase proteins 3 and 7 (1). These caspases signal the onset of apoptosis via cleavage of numerous cellular proteins, ultimately leading to the phagocytic recognition and engulfment of the dying cell (1). Mitochondria play a critical role in mediating the intrinsic apoptotic signal transduction pathway. During apoptosis mitochondria undergo biochemical and structural changes, which include swelling, depolarization, increased permeability of the outer membrane, and release of proteins from the intermembrane space, including cytochrome *c* (Cyt *c*) (2, 3). The released Cyt *c* binds the apoptotic protease activating factor 1 protein (Apaf-1) in the cytoplasm (4). This causes a conformational change and oligomerization of Apaf-1 that leads to the formation of the caspase activation platform, the apoptosome (5). The apoptosome recruits and activates initiating caspase 9, which in turn cleaves and activates caspase 3 (1).

The formation of the macromolecular complex apoptosome is a key event in the intrinsic apoptotic pathway. The binding of Cyt *c* to Apaf-1 is regulated through the action of various proteins (3), cations, and nucleotides (6, 7). Disruption of the binding of Cyt *c* to Apaf-1 can block the formation of the apoptosome and the subsequent activation of caspase 9, thus leading to inhibition of apoptosis (8, 9).

Apoptosis is the underlying mechanism of several diseases, such as organ failure and neurodegeneration as in amyotrophic lateral sclerosis (10, 11). Efforts have been directed toward the discovery of molecules that can attenuate apoptosis-mediated cell loss in disease states (10). Recent reports suggest that angiogenin (ANG) protects motor neurons from death induced by various cellular stresses, including excitotoxicity and hypoxia (12–14). The mechanism of this protective action of ANG in neurons remains largely unknown. ANG belongs to the pancreatic RNase A superfamily, a protein that cleaves single-stranded RNA (15). Widespread expression of ANG suggests that it may play a more universal role in the cell than stimulating angiogenesis (16, 17). An interesting function of ANG under certain stress conditions is tRNA cleavage at the anticodon loop, leading to an accumulation of small tRNA halves, known as tiRNAs (18–24).

We have previously shown that hyperosmotic stress induces apoptosis in wild-type mouse embryonic fibroblasts (MEFs) following the release of Cyt *c* from mitochondria (25). Hyperosmotic stress causes cell shrinkage, thus increasing macromolecular crowding and inducing a stress response (26). Regulation of cell volume and adaptation to hyperosmotic stress are fundamental processes required for the maintenance and well-being of all living cells (26). In a recent study, we showed that hyperosmotic stress induces tiRNA accumulation as early as 15 min after exposure to stress (20). The generation of tiRNAs during hyperosmotic stress

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