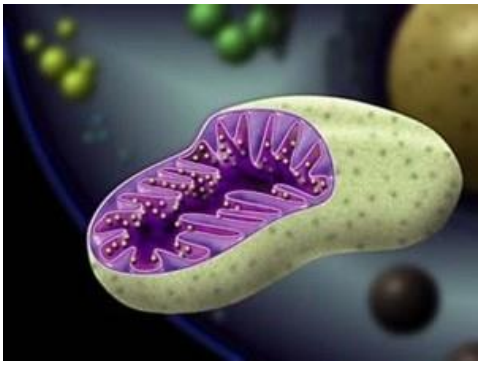
Delivering STAT3 into mitochondria and role of mitochondrial STAT3 in redox regulation



Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

Prasad Tammineni

(09LBPH14)

Department of Biochemistry

School of Life Sciences

University of Hyderabad-500046

India

Department of Biochemistry

School of Life Sciences

University of Hyderabad

Hyderabad 500 046

India

CERTIFICATE

This is to certify that this thesis entitled **"Delivering STAT3 into Mitochondria and Role of Mitochondrial STAT3 in Redox Regulation"** submitted to the University of Hyderabad by Mr Prasad Tammineni, 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.

Dr. Naresh Babu V Sepuri

Supervisor

Head

Department of Biochemistry

Dean

School of Life Sciences

Department of Biochemistry

School of Life Sciences

University of Hyderabad

Hyderabad 500 046

India

DECLARATION

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

Dr. Naresh Babu V Sepuri
Supervisor

Prasad Tammineni

Date:

Place: Department of Biochemistry
University of Hyderabad - 500 046

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BARC	Bhabha Atomic Research Centre
BAX	Bcl-2-associated X protein
BCL2	B-cell lymphoma 2
BN-PAGE	Blue Native Polyacrylamide gel electrophoresis
BSA	Bovine Serum Albumin
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	Cyclic Adenosine monophoshate
CCHL1	Cytochrome C Hemolyase
cIAP	Cellular Inhibitor of Apoptosis
COX	Cytochrome C Oxidase
cpm	counts per minute
CREB	cyclic AMP response element binding protein
Cyp D	Cyclophilin D
DAPI	4’ 6-diamidino-2phenylindole
DCFDA	Dichlorofluorescin diacetate
DHFR	Dihydrofolate reductase
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothritol
EDTA	Ethylene diamine tetraacetic acid

EGTA	Ehtylene glycol tetraacetic acid
ETC	electron transport chain
FBS	Fetal Bovine Serum
Fe-S	Iron Sulfur cluster
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRIM-19	Gene associated with IFN-beta/RA induced cell mortality
GTP	Guanosine triphoshate
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
HSP70	Heat Shock Protein 70
IFN-B	Interferon beta
IKB	inhibitor of kappa B
IMM	Inner mitochondrial membranes
IRF3	Interferon regulatory factor 3
KCl	Potassium chloride
kDa	Kilodalton
KOH	Potassium hydroxide
MAPK	Mitogen activated protein kinase
MEF2D	Myocyte enhancer factor 2D
$MgCl_2$	Magnesium Chloride
Mg (OAC2)	Magnesium acetate
Mito-GFP	Mitochondrial targeted Green Florescent protein
ml	milliliter

mm	millimeter
mmole	millimole
MMP+	1 methyl-4-phenylpyridinium
MnSOD	Manganese Super oxide dismutase
MP	Mitoplast
MtHSP70	Mitochondrial HSP70
Na_2CO_3	Sodium bicarbonate
NA_3O_4	Sodium Orthovanadate
NAC	N-AcetylCysteine
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide
NaF	Sodium Fluoride
NCCS	National Centre for Cell science
ND1	NADH-ubiquinone oxidoreductase 1
NF-KB	Nuclear Factor kappa-light chain- enhancer of activated B cells
Ni-NTA	Nickel Nitrilotriacetic acid
ORF	Open reading frame
OXPHOS	Oxidative phosphorylation system
PAGE	Ploy acrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PKA	Protein Kinase A
pMLS	Putative mitochondrial localization sequence
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride

RA	Retinoic acid
RBM3	RNA binding motif protein 3
RIPA	Radio immunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
RRL	Rabbit reticulo lysate
SDS	Sodium dodecyl sulphate
Ser	Serine
STAT	Signal transducer and activator of transcription
SU9-DHFR	Subunit 9 – Dihydrofolate reductase
TCA	Tricarboxylic acid
TIM	Translocase of Inner mitochondrial membrane
TOM	Translocase of outer mitochondrial membrane
TRIS	tris(hydroxymethyl)aminomethane
Tyr	Tyrosine
µCi	micro curie
µg	micro gram
µl	micro liter

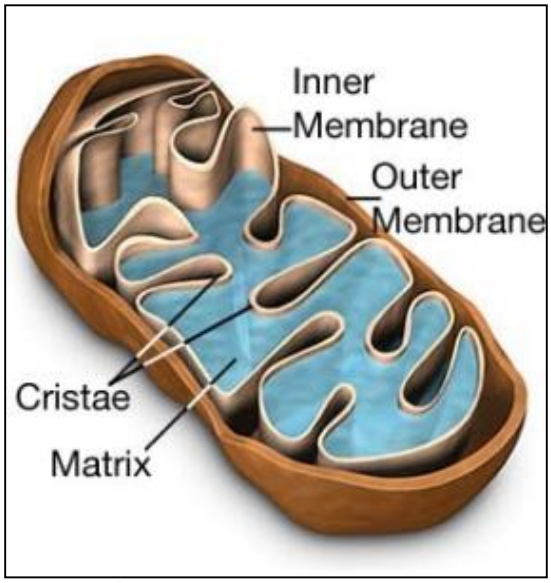
Chapter 1

INTRODUCTION

1.1 Mitochondria: Structure

Mitochondria are double membrane organelle consists of four compartments namely: Outer membrane, Intermembrane space, Inner membrane and Matrix. These four sub compartments are distinct in their structure and function.

The outer Mitochondrial membrane is relatively a simple phospholipid bilayer with less protein abundance and permeable to molecules approximately 10kDa or less. Ions, nutrients, ATP, ADP, etc. can easily pass through the outer membrane.



(https://confluence.crbs.ucsd.edu/display/CS/Mitochondria)

Fig.1.1 Structure of Mitochondria: Mitochondria are bounded by the double membrane system, consisting of Inner and outer membranes. Inner mitochondrial membranes fold called cristae extend into the mitochondrial matrix.

Intermembrane space separates the outer and inner mitochondrial membrane. It harbors H^+ ions coming from the matrix through the electron transport chain to create electrochemical gradient across the inner mitochondrial membrane to drive ATP synthesis.

The inner mitochondrial membrane is a complex of cardiolipin containing phospholipid

bilayer and serves an electrical insulator and chemical barrier. It is highly folded inwards into the matrix to form structures called cristae. Further inner membrane harbors protein complexes that are involved in electron transport, ATP synthesis and protein import.

The matrix contains mitochondrial genetic system as well as other enzymes required for the central reactions of oxidative metabolism, Fe-S biogenesis etc.

1.2 Mitochondria: Functions

The Mitochondrion is considered to be a cell within a cell and it executes varied functions apart from being the powerhouse of the cell. These functions include:

1.2.1 Mitochondria: The power generator

Mitochondria differ from its early eukaryotic cell in having an efficient energy generating system called the oxidative phosphorylation system. It utilizes the molecular oxygen and NADH to generate ATP. As most of the cellular processes depend on mitochondria for ATP, mitochondria are often considered as the power house of the cell. Further, most of the metabolic reactions like TCA cycle, oxidation of fatty acids and amino acids occurs in mitochondria.

1.2.2 Mitochondria: The decision maker

Apart from energy conversion, mitochondria also play a crucial role in deciding the cell fate. In response to external cues, mitochondria releases Cytochrome C into the cytosol to initiate a cascade of events which leads to the activation of caspase-3 and thereby promotes apoptosis.

1.2.3 Mitochondria: The major culprit of diseases

Though mitochondria produce most of the energy, they also generate Reactive Oxygen Species (ROS) as unavoidable by product. Since mitochondrial DNA is not protected and close proximity of mitochondrial DNA to the ROS generating system, it is easily susceptible

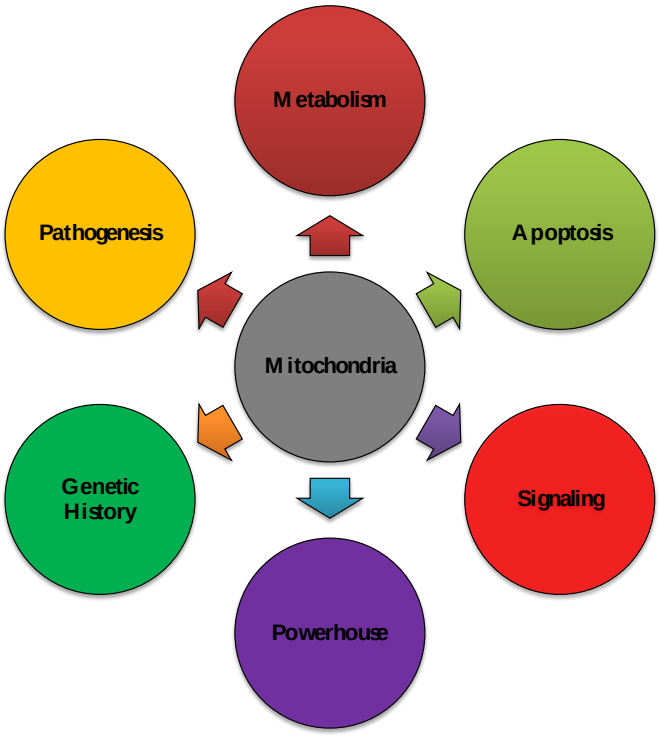


Fig.1.2 Mitochondria functions: Mitochondria are crucial organelle involved in various functions which includes energy generation, apoptosis, various oxidative metabolisms and pathogenesis as well as integration of most of the signalling pathways in response to external cues.

For mutations. These mutations often affect the high energy demanding areas such as brain and muscle that eventually leads to the pathophysiology. In addition, defects in mitochondrial dynamics and metabolism are also associated with various diseases.

1.2.4 Mitochondria: The taleteller of genetic history

Mitochondria have its own genome. Mitochondrial DNA is maternally inherited. Mitochondrial DNA of sperm is actively destroyed after its fusion with an egg. As mitochondrial DNA is directly inherited from maternal line, and never mixed up with male line, it has become a very good analytical tool for geneticists and anthropologists. Analysis of mitochondrial DNA reveals that we are descendants of a common ancestral group called "Mitochondrial Eves".

1.2.5 Mitochondria: The signalling platform

Growing evidence indicates that mitochondria are intimately involved in cellular signaling that includes being a platform to initiate cell signaling to act as either transducers or effectors towards the extracellular stimuli. For examples, mitochondrial outer membrane serve as a signaling platforms in antiviral immunity and mitochondrial ROS triggers activation of retrograde signaling pathways to cope up with the extracellular environment.

1.3 Mitochondria: Evolution

Eukaryotic cells fundamentally differ from their prokaryotic counterparts by having intra cellular membrane bound organelles. Acquisition of plastids and mitochondria was considered to be an important event in the evolution of the eukaryotic cell as they represent compartmentalized energy generating and biosynthetic factories of the cell [1]. However, the transition from the early endosymbiont to modern mitochondria accompanies major changes in its structure and content.

1.3.1 Endosymbiotic theory

According to the widely accepted endosymbiotic theory, mitochondria was thought to be derived from bacteria [2, 3]. Phylogenetic analysis of small ribosomal RNAs demonstrated the monophylogenetc origin of mitochondria from alpha-proteaobacterium [4]. To explain the mitochondrial symbiosis, two scenarios were proposed: Archezoan Scenario and Symbiogenesis scenario [5]. According to the Archezoan theme, host of the endosymbiont was essentially a eukaryote [6]. On the contrary, Symbiogenesis scenario states that endosymbiont was taken up by an archea that led to the symbiogenesis which is accompanied by the formation of nucleus and compartmentalization of eukaryotic cells [7].

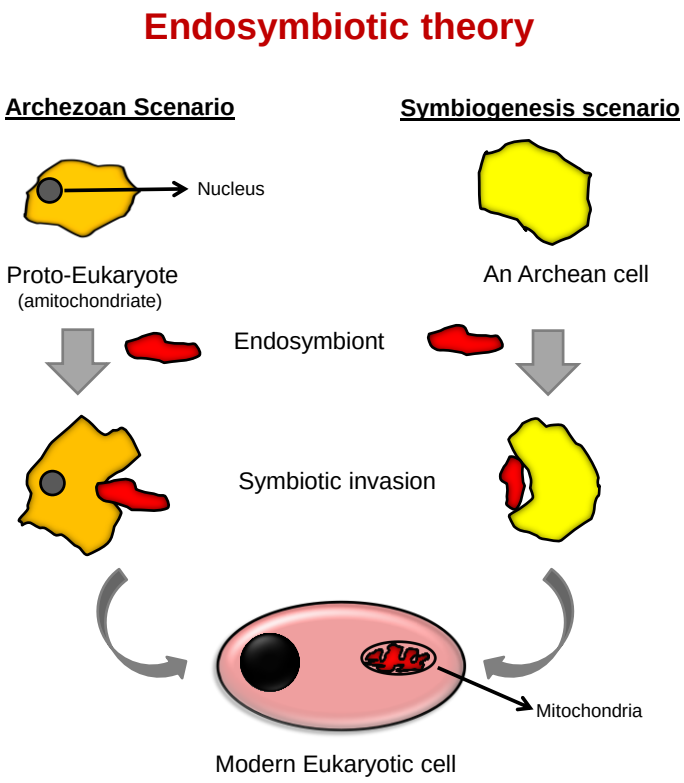


Fig.1.3 Endosymbiotic theory: Mitochondrial evolution is well explained by endosymbiotic theory which implies the bacterial origin of modern mitochondria. Two scenarios were proposed to explain the endosymbiotic origin of mitochondria. One being early in the formation of a nucleus (Symbiogenesis) and the other after the

compartmentalization of the early eukaryote (Archezoan).

A fundamental variation between these two theories is whether the host was a prokaryotic archeal cell (Symbiogenesis scenario) or compartmentalized amitochondriate eukaryotic cell (Archezoan scenario). Though both the theories have their limitations, increasing evidence suggests the symbiogenesis scenario because of the absence of any amitochondriate eukaryotic lineage till date [8].

Endosymbiotic theory proposes that mitochondria are evolved by two mechanisms: Gene loss and transfer of mitochondrial genes to the nucleus. Hence, we may not find most of the ancestral clusters of orthologous genes in modern mitochondria and some of the mitochondrial genes are found to be associated with nuclear DNA of the eukaryotic cell.

1.3.2 Mitochondrial gene loss

The endosymbiosis was followed by a rapid and significant loss of mitochondrial gene content [9]. Gene loss event could be either complete or partial. For example, hydrogenosomes and mitosomes have lost almost their entire mitochondrial genome [10]. However, in some species gene loss was not complete and they rely on oxidative phosphorylation for their survival and hence some of the OXPHOS subunits are encoded by mitochondrial DNA [11]. Gene loss is tolerated by endosymbiont due to three major reasons [12].

1. The function of the gene may not be necessary for survival and hence the endosymbiont lost the gene permanently. For Example, most of the genes required for various metabolisms and cell wall synthesis of the organism may not be necessary for endosymbiogenesis.

2. The function of the gene is substituted by other pre-existing gene in the host. These

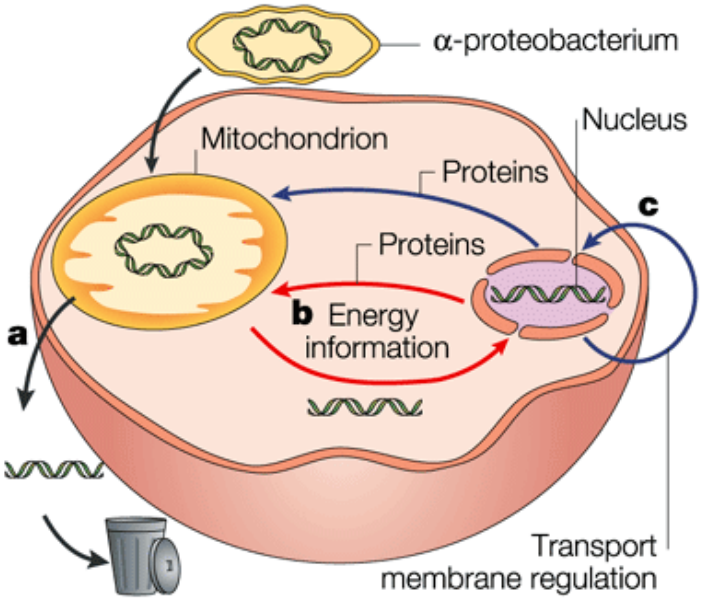
substitutions often occur by duplications of nuclear genes and the products of these genes, either with or without mitochondrial targeting sequences, play a similar role in mitochondria. For example, ribosomal proteins and enzymes of TCA cycle of mitochondria are encoded by nuclear genome and executes the functions in mitochondria.

3. Genes may also lost from the mitochondria because they were transferred to the nucleus as host lacks a functional counterpart of these genes. Genes of mitochondrial OXPHOS system are best examples of this possibility.

1.3.3 Mitochondrial gene transfer to the nucleus

Mitochondrial gene transfer is a predominant and a continuous pathway for establishing endosymbiosis. Mitochondrial DNA escapes from mitochondria through membrane disruption that may happen during lysosomal digestion or when the cell is under duress [13]. Organelle DNA is migrated to the nucleus either as DNA or cDNA intermediate and integrated into the nuclear genome by double strand break DNA repair or, chromosome end joining or other unknown mechanisms [14]. Based on the functional status of integrated genes, the gene transfer event can be divided into two types: Transfer of genes that results in generation of pseudogenes or functional proteins.

The presence of various pseudogenes were well documented in humans and plants. [15-17] *Pseudogenes* are generated as the transferred sequences do not possess any necessary regulatory elements required for their expression and targeting despite of their potential to encode a functional protein. Sometimes, transferred sequences to become non-functional due to differences in mitochondrial genetic code and the standard genetic code [17, 18].



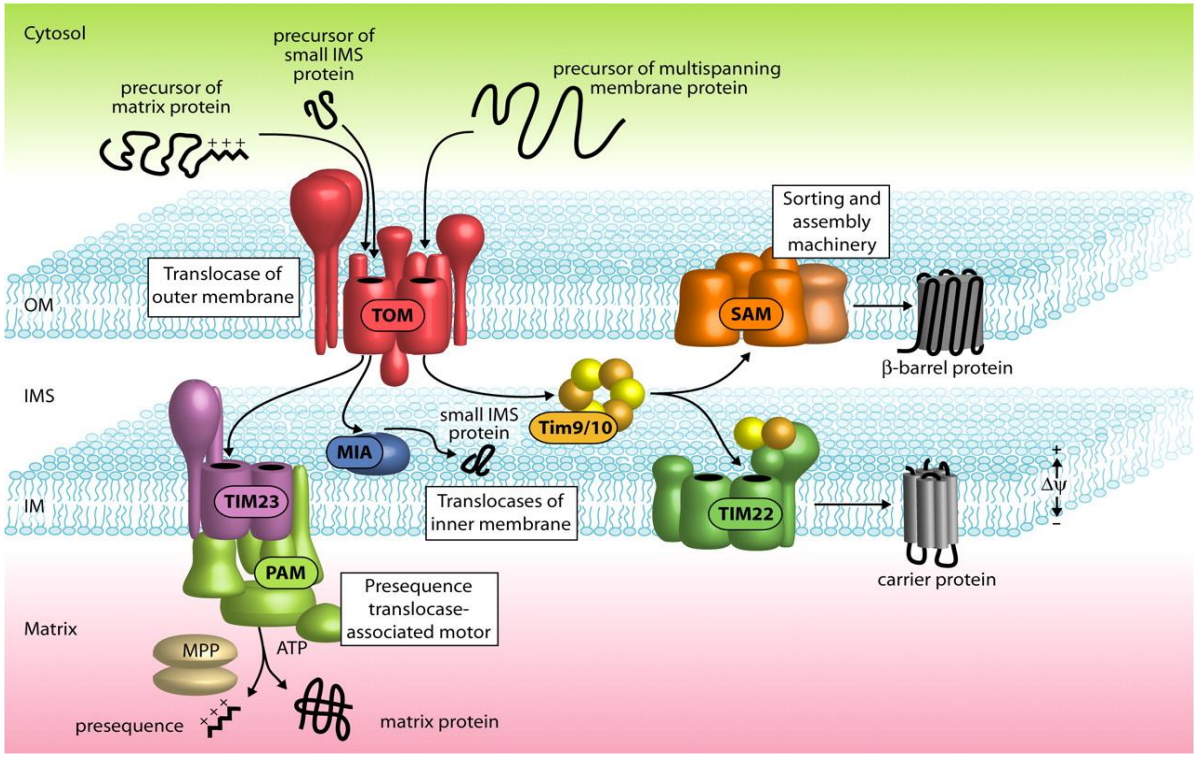
(Nature Reviews Genetics, 2003)

Fig.1.4 Mitochondrial gene loss and transfer: Endosymbiotic theory states that during evolution, most of the mitochondrial genes were lost **A)** Some genes are transferred to the nucleus and the products of these genes co-translationally imported into mitochondria **B)** Some nuclear genes are evolved in such a way that their translation occurs on the cytosolic ribosomes and imported into mitochondria post-translationally to regulate the mitochondrial functions.

Functional genes are generated as the transferred sequences gain regulatory elements required for their expression and targeting. Functional gene transfer and how these transferred sequences gain regulatory elements is well documented in plants [19, 20]. Sometimes, these regulatory sequences were also obtained through unknown mechanisms or synthesized *de novo [21]*.

As discussed earlier, most of the mitochondrial genes were either lost or transferred to the nucleus. To compensate for the genes that were lost or transferred, mitochondria have to import the proteins that were products of transferred genes. To enable this transition, a novel molecular machines have arisen at the outer and inner mitochondrial membranes to efficiently transport the proteins from cytosol into mitochondria.

1.3.4 Origin of Protein Import Machinery: To compensate the gene loss or transfer, endosymbiont probably underwent many transformations. Among them one important transformation was the acquisition of mechanism that facilitates the import of proteins encoded by transferred genes back into the mitochondria. Considering the fact that modern mitochondria unlike its autonomous ancestral counterpart depends on nuclear encoded proteins for its function. Origin of mitochondrial protein import machinery was considered to be the crucial step in mitochondrial evolution. However, it is difficult to discern whether transfer of mitochondrial genes to nucleus created an evolutionary pressure to enable the protein transport machinery or origin of protein import facilitated the massive gene transfer.



(J Cell Biol, 2007)

Fig.1.5 Mitochondrial protein import Machinery: To facilitate the import of nuclear encoded mitochondrial proteins, mitochondria acquired protein import machinery at the outer and inner mitochondrial membranes. Translocase of Outer mitochondrial membrane (TOM) serves as the common entry gate for the mitochondrial protein, whereas Translocase of inner mitochondrial membrane (TIM) assists in the transport of proteins through

inner mitochondrial membranes.

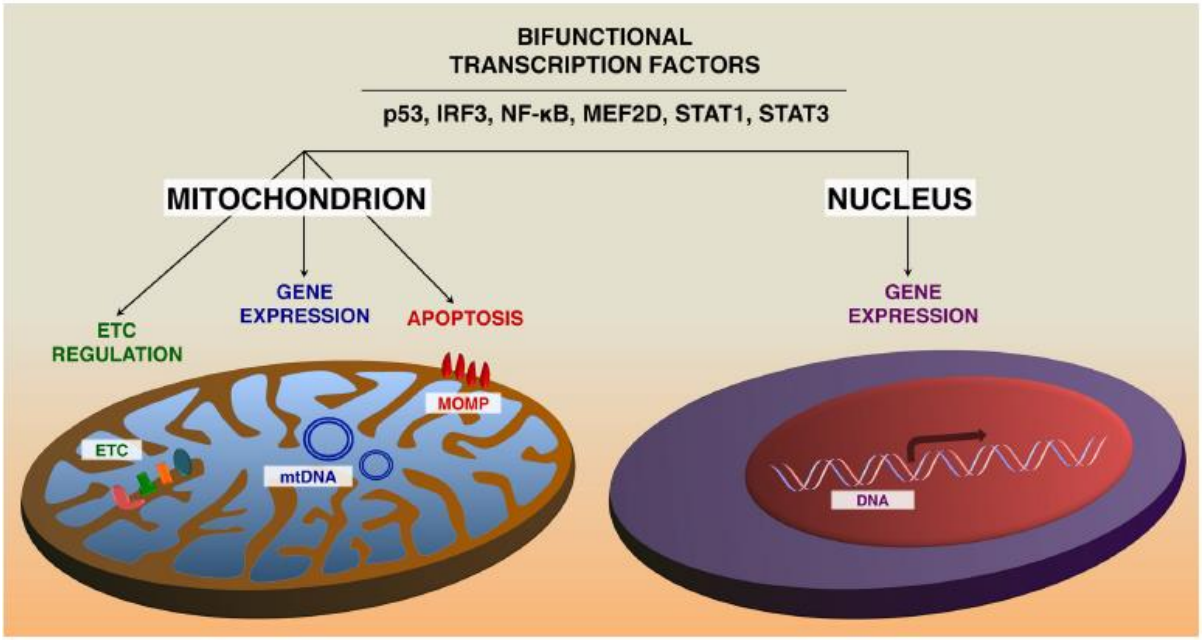
In addition, presence of bacterial homologs in modern mitochondrial import machinery suggests that the existence of primitive system in ancestor which may be evolved more rapidly to a sophisticated mechanism with high specificity and efficiency. Analogous to the evolution of transport machinery, mitochondrial targeting proteins have also acquired a targeting sequence that probably evolved with proteins that have inherent capacity to target to mitochondria. Later, it is possible that these sequences have been passed on to other genes by gene duplications and recombinant events. Therefore, evolution of mitochondrial protein import machinery provided a new dimension to the mitochondrial proteome by facilitating the expansion of the proteome as well as reduction of the genome. Together, these events make the modern mitochondria a highly evolved and sophisticated one.

Besides the acquisition of protein import machinery by mitochondria, host might also be employed some of the host derived factors to compensate the loss of mitochondrial genes during early stages of evolution. It is possible that some of these proteins remained obligate mitochondria proteins and remaining proteins were engaged in host functions but can be transported back to mitochondria when required. Nuclear transcription factors represent the latter group as they often regulate gene expression in the nucleus and also engaged in mitochondrial functions in response to external cues.

1.4 Nuclear Transcription factors in the mitochondria

The functions of transcription factors in the nucleus have been actively investigated. However, increasing evidence suggests that nuclear transcription factors are also present in the mitochondria and regulate mitochondrial functions. They are shown to regulate diverse functions like apoptosis, respiration and mitochondrial gene expression. It has been shown

that deregulation of these mitochondrial localized nuclear transcription factors associated with permeability transition and ROS generation [22]. Based on their sub-compartmental localization, mitochondrial localized transcription factors can be divided into two groups: Transcription factors that are associated with outer mitochondrial membranes and those localized within mitochondria.



(Trends in Cell Biol., 2012)

Fig.1.6 Distinct roles of nuclear transcription factors in mitochondria: Transcription factors regulate the gene expression in the nucleus. However, they were also reported to be present in mitochondria to regulate mitochondrial gene expression, apoptosis and the function of electron transport chain etc.

1.4.1 Transcription factors at the Outer mitochondrial membranes

1.4.1.1 P53

p53 is a tumor suppressor protein responds to a myriad of stresses that includes oxidative stress, DNA damage, nutrient stress and ischemia [23]. Though it was believed that p53 induced apoptotic changes were mediated by its transcriptional activation in the nucleus, recent reports suggest that non canonical role of p53 in mitochondria in regulation of

apoptosis. Most of the apoptotic signals, like gamma radiation and hypoxia, induce translocation of p53 to the outer mitochondrial membranes and permit its interaction with Bcl2 proteins to induce apoptosis [24]. P53 interacts with two of the Bcl2 family proteins BAX and BAK. During stress, p53 competes with mcl2 for its interaction with BAK to induce apoptosis [25]. Similarly, p53 also interacts with BAX, which disrupts sequestration of BAX by Bcl-XL, and thereby promotes oliogomerization of BAX on the outer mitochondrial membranes to promote the opening of permeability transition pore [26].

In addition to its influence on outer mitochondrial Bcl2 proteins, p53 is also present in other sub-compartments of mitochondria. For instance, p53 present in mitochondrial matrix and interacts with mtHSP70 and mtHSP60 [27]. Matrix localized p53 was also shown to sequester MnSOD to initiate apoptosis [28].

1.4.1.2 IRF3

IRF3 exerts its pro-apoptotic functions by regulating the Bcl-2 family members and these functions of IRF-3 are transcriptional independent [29]. Similar to p53, IRF3 recruitment to mitochondria is induced by RNA virus or synthetic dsRNA, poly (I: C). IRF3 also interacts with Bcl2-family proteins to activate the mitochondrial apoptotic pathway. IRF3 binds with BAK of outer mitochondrial membrane and facilitates mitochondrial membrane permeabilization thereby promote the release of pro-apoptotic proteins into the cytosol to initiate the activation of the caspase cascade [30].

1.4.2 Transcription factors present within mitochondria

Some of the transcription factors, like CREB, NF-kB, MEF2D and STATs, are shown to be present in IMS, IMM or matrix. Unlike outer mitochondrial membrane associated

transcription factors, these transcription factors regulate diverse functions of mitochondria, which includes mitochondrial gene expression and electron transport chain activity etc.

1.4.2.1 CREB

Cyclic-AMP response element binding protein (CREB) is a crucial regulator of cell survival and differentiation and responds to a diverse range of stimuli. It is activated by various kinases like PKA, MAPKs and CaMKs [31]. Recent reports have demonstrated that the existence of a self-contained CREB pathway in mitochondria comprising PKA and cAMP. This pathway promotes the binding of CREB to the D-loop of mitochondrial DNA to regulate mitochondrial transcription [32]. Over expression of CREB in mitochondria results in increased expression of mitochondrial mRNAs of complex I with a concomitant increase in complex I activity [33].

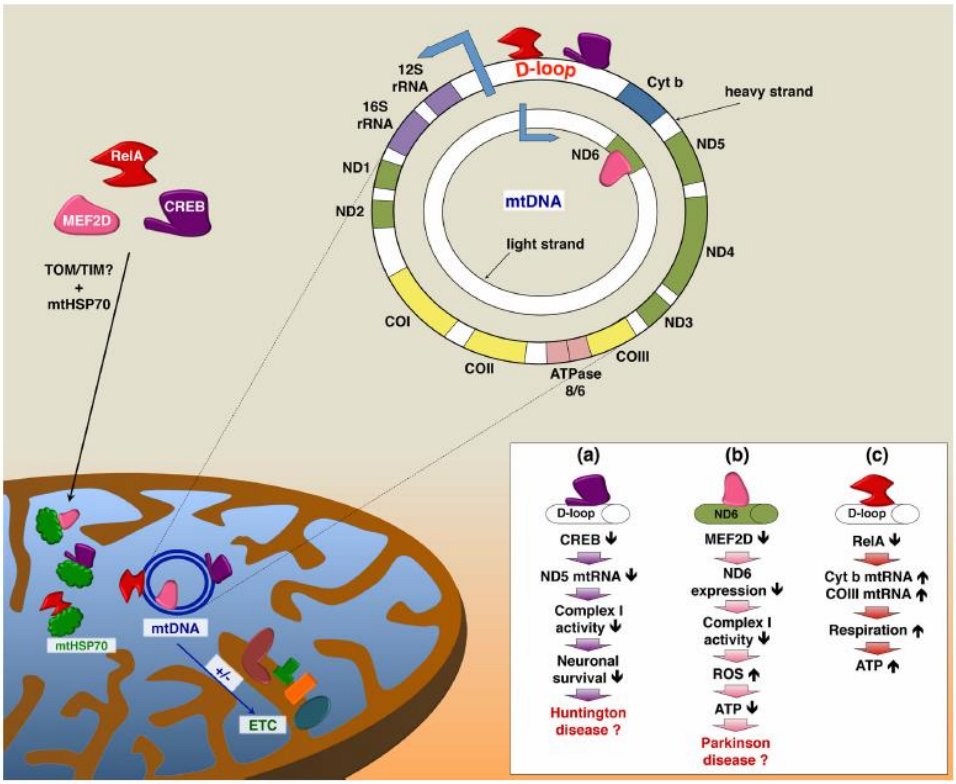
1.4.2.2 MEF2D

The Myocyte –specific enhancer factor-2 (MEF-2) family of transcription factors plays a major role in immune response, muscle differentiation and carbohydrate metabolism. Though the involvement of MEF-2 family protein in mitochondrial biogenesis was known since long time, a recent study demonstrated the localization of MEF-2D in the mitochondria [34]. MEF-2D binds to the consensus sequence present in the light strand of the mitochondrial DNA that encodes a complex I subunit called ND6. Disruption of MEF-2D resulted in decreased complex I activity, increased ROS production and decreased ATP levels. Rotenone treatment decreases the binding of MEF-2D to the ND6promoter [34]. In addition, MMP+ treatment resulted in decline in the levels of MEF-2D and ND6 which is associated with decreased neuronal viability in brains from MMP+ treated mice. Intriguingly, decreased

levels of ND6 and mitochondrial MEF-2D were documented in postmortem brains from PD patients. These results suggest the crucial role of MEF-2D in regulating the mitochondrial metabolism through modulation of ND6.

1.4.2.3 NF-kB

NF-kB family transcription factors responds towards diverse stimuli which results in the expression of genes involved in inflammation, metabolism, cancer and development. Along with its inhibitor, Ki-B, several members of NF-kB family present in mitochondria. NF-kB was shown to interact with ATP-ADP translocator-1 [35]. This interaction was shown to promote the mitochondrial recruitment of NF-kB with a concomitant decrease in its nuclear activity. This further correlates with decreased expression of known anti-apoptotic NF-kB target genes, Bcl-xL, c-IAP-2[36].



(Trends in Cell Biol., 2012)

Fig.1.7 Role of CREB, MEF2D and Reel A in mitochondria: Apart from their role in the nucleus, CREB, MEF2D and Rel A are also present in mitochondria. Though all the three transcription factor binds to the mitochondrial DNA, CREB (a) and MEF-2D (b) positively regulates mitochondrial gene expression whereas RelA (c) repress the expression of COXIII gene.

In addition, RelA, a NF-kB family member, was shown to present in mitochondria and binds with mitochondrial DNA and inhibits the expression of cytochrome c oxidase III (COXIII) [37]. Intriguingly, mitochondrial levels of p53 negatively correlate with levels of RelA in mitochondria. Over expression of p53 reverses the inhibitory effect of RelA on mitochondrial gene expression. It has been proposed that the actions of p53 and RelA on mitochondrial respiration influence the metabolic switch from OXPHOS to glycolysis [38].

1.4.2.4 STATs

Signal Transducer and Activator of Transcription (STAT) family proteins responds towards various cytokine stimuli, translocate to the nucleus to regulate target gene expression [39]. Despite their well-established nuclear functions, it has been reported that a pool of STATs are also present in mitochondria and regulate diverse mitochondrial functions [40-42]. Till now, only three of the STAT3 family members were reported to be present in mitochondria. These are STAT3, STAT5 and STAT1.

STAT3 was the first STAT family member to be found in the mitochondria. It was shown to regulate the activities of complex I and II [40]. Mitochondrial STAT3 was also shown to mediate the Ras-induced cellular transformation [43]. Though Tyr 705 phosphorylation is required for nuclear functions of STAT3, mitochondrial functions require phosphorylation at Ser 727 [40, 43]. Mitochondrial STAT3 was also shown to protect against ischemic injury by preventing the leakage of electrons from complex I of ETC [44]. Mitochondrial STAT3 was documented to interact with Cyp D suggesting the possible role of this transcription factor in

permeability transition [45]. A recent study also suggested the involvement of STAT3 in mitochondrial gene expression [46].

STAT5 is a transcription factor majorly involved in the growth and development of blood cells. However, recently it was also shown to be present in mitochondria. The Il-2 treatment increases mitochondrial recruitment of STAT5 to mitochondria [41]. It is also binds to D-loop of mitochondrial DNA and interacts with an E2 subunit of mitochondrial Pyruvate Dehydrogenase complex [41].

STAT1, a key regulator of antiviral immune response, is also localized to mitochondria. Though there is no function ascribed for this transcription factor, it may be repressing the mitochondrial gene expression as IFN-β inhibition activates STAT1 [42], mitochondrial RNAs as well as nuclear encoded mRNAs of ETC.

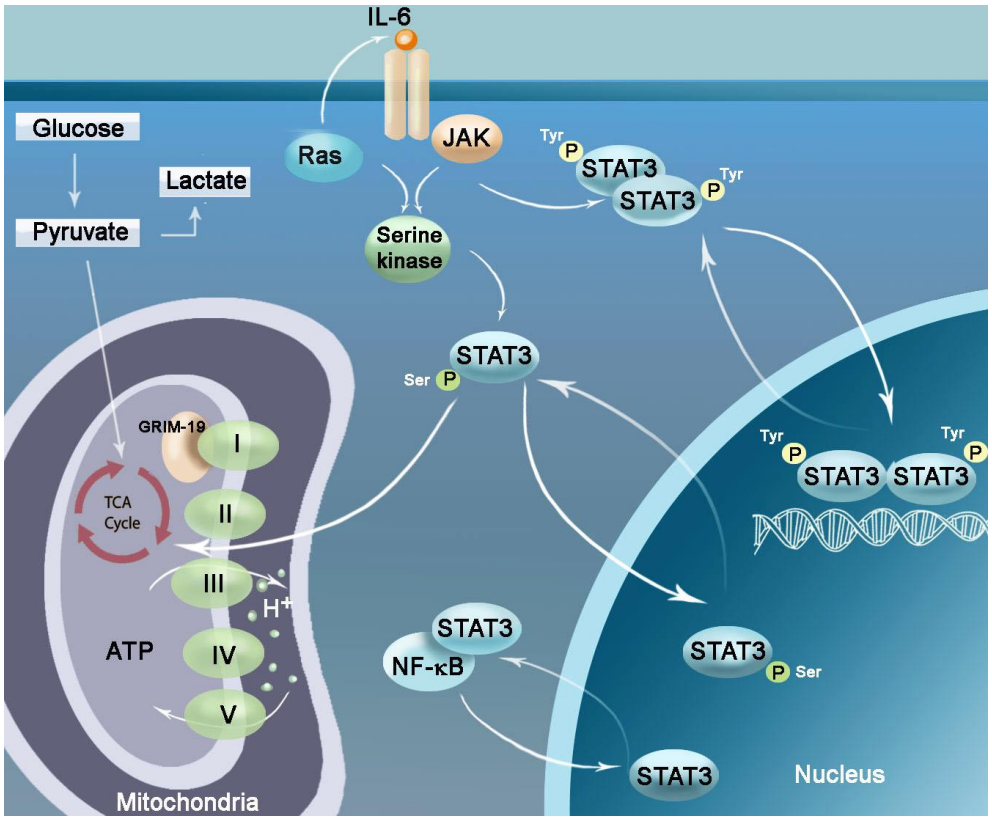


Fig.1.8 Role of STAT3 in mitochondria: Besides its role in the nucleus, STAT3 is also present in mitochondria and regulate the activities of complex I, reduces mitochondrial ROS. Permeability transition and apoptosis.

1.5 Travelling mechanisms of transcription factors to mitochondria

Mitochondria being a semi-autonomous organelle, imports majority of the proteins synthesized on the cytosolic ribosomes. Nuclear encoded mitochondrial proteins, generally, possess cleavable, N-terminal pre-sequence which targets them to mitochondria. Some mitochondrial proteins also contain non cleavable internal targeting sequence. In addition, the mitochondrial recruitment of these proteins requires an interplay between both cytosolic and mitochondrial chaperone system.

Nuclear transcription factors, despite of any canonical mitochondrial targeting sequence, were reported to be present in mitochondria and their mitochondrial functions are often associated with pathogenesis. Investigation of their mitochondrial recruitment has become an active area of research in cell biology. Though the attempts were made to study the role of HSP70, TOM complex and alternative translation initiation in mitochondrial recruitment of few transcription factors like, CREB, Rel A and p53, however, the exact mechanism of their recruitment remains poorly studied [28, 29, 34, 38, 47, 48].

Our lab, for the first time, identified a pool of STA3 in mitochondria and its role in cellular respiration. Subsequently, it was shown that mitochondrial STAT3 plays a very critical role in wide-array of cellular processes. However, mechanistic details of its mitochondrial recruitment remain elusive. Hence the present study was designed to investigate the mechanism underlie STAT3 targeting to mitochondria, given the fact that STAT3 does not have any canonical mitochondrial targeting sequence.

Chapter 2

Mechanistic details of STAT3 transport to mitochondria

2.1 INTRODUCTION

The molecular mechanisms by which transcription factors regulate nuclear gene expression have been actively pursued for decades. Likewise, the canonical signaling pathways that regulate transcription factors including the STATs are well understood. Recent reports suggested non canonical functions for these transcription factors in mitochondria that involve regulation of energy management by the mitochondria. For instance, researchers have identified STAT3 [40], estrogen receptors [49, 50], glucocorticoid receptors [51, 52], p53 [53], NF-kB [37] and CREB [32, 33, 54] in mitochondria regulating mitochondrial functions in a manner that is dependent or independent of transcription [55]. These findings suggest a paradigm shift in the functions of these transcription factors to target and modulate mitochondrial driven cellular functions.

Signal Transducer and Activator of Transcription-3 (STAT3) is a key transcription factor that is phosphorylated at tyrosine 705 and serine 727 in response to cytokines and growth factors. Phosphorylated STAT3 translocates into nucleus and regulates expression of genes associated with various cellular processes. We and others have recently shown that STAT3 is also localized to mitochondria [40, 43, 45, 56, 57]. Mitochondrial STAT3 increases activity of complex I and II of the ETC in a transcriptional independent manner [40]. In addition, Ras mediated cellular transformation is shown to be dependent on mitochondrial STAT3 [43]. Interestingly, phosphorylation of STAT3 on Ser727, but not Tyr705, seems to be integral for its mitochondrial activity [40, 43]. Recent reports also suggest involvement of mitochondrial STAT3 in cardioprotection during ischemia and reperfusion possibly by preventing leakage of electrons from complex I [44, 45]. Nevertheless, regulatory mechanisms involved in STAT3 import, integration into complex I and its role in respiration remain obscure.

GRIM-19 was identified as a principal mediator of IFN-β/RA induced cell death [58]. Subsequently, GRIM-19 was identified as a component in the electron transport chain, although low amounts have been detected in nuclei [58-60]. GRIM-19 is reported to be a negative transcriptional regulator of STAT3 [61-64]. Phosphorylation of STAT3 on Ser727 seems to be essential for its interaction with GRIM-19 [64]. The presence of STAT3 in mitochondria, requirement of Ser727 phosphorylation for its mitochondrial functions and its interaction with GRIM-19 suggested that STAT3-GRIM-19 interaction may be influencing the localization or function of each other.

In the study discussed in this chapter, we find that STAT3 anchors to the inner mitochondrial membrane. Using an *in vitro* import system, we demonstrate the involvement of GRIM-19 in the recruitment of STAT3 to mitochondria and integration into complex I. Import of STAT3 requires phosphorylation at Ser727 site as removal of the C-terminus of STAT3 or mutation of Ser727 reduces integration of STAT3 into the inner membrane of mitochondria. Together, our results disclosed a novel chaperonic role of GRIM-19 in STAT3 recruitment to mitochondria.

2.2 METHODOLOGY

2.2.1 Antibodies and Reagents

Antibodies used in this study were: STAT3C-20, TOM20 and ND1 (Santa Cruz biotechnology, USA), cytochrome C (Cell Signaling technology, USA), GRIM-19 (Mitosciences, USA), Aconitase 2 (Novus Biologicals) and Mia40 (in house generated and purified using CNBr-Sepharose). All chemicals were obtained from Sigma Aldrich Chemical Co, USA and Amersco, USA.

2.2.2 Plasmid constructs

Full length GRIM-19, TIM23 and RBM3 ORFs were amplified by polymerase chain reaction from total HeLa cell RNA using gene specific primers mentioned in the Table I. and cloned into a myc-tagged mammalian expression vector, pcDNA 3.1/myc using the restriction sites mentioned in the Table I to obtain myc-fusion protein. All STAT3 clones in pcDNA 3.1 [40] and pGEMT-SP6-Su9-DHFR have been described earlier [65] and kind gift from Andrew C Larner (Virginia Commonwealth University, Richmond, USA). Full length STAT3 was sub-cloned into Pet28 (a+) vector using the primers mentioned in the Table 2.1. The protein was expressed in bacteria and purified to homogeneity by using Ni-NTA column. pMLS-I, pMLS-II, pMLS-III, pMLS-IV, pMLS-V were cloned into pEGFP vector using pcDNA3.1-STAT3 WT/myc as a template and specific primer mentioned in the Table 2.1. His6-DHFR was amplified using Su9-DHFR as a template and cloned into pGEMT vector. pcDNA-ER-α was a kind gift from Dr. Bramanandam Manavathi (University of Hyderabad, Hyderabad, India). Mito-GFP was a generous gift from Sudipto Das (Lupin Pharmaceuticals Inc., USA).

Table 2.1: Primers used in the study

S.No.	Gene Name	Primer Name	Primer Sequence	Restriction sites
1	GRIM-19	NB91_Sense	5'CCCA *GAATTC* ACC ATG GCG GCG TCA AAG GTG 3'	EcoR I
		NB128_Antisense	5' CCAA *CTCGAG* CGT GTA CCA CAT GAA GCC 3'	Xho I
2	TIM23	NB49_Sense	5' CCCA *GAATTC* ACC ATG GAA GGA GGC GGG GGA 3'	EcoRI
		NB240_Antisense	5'CCCA *CTCGAG* GAG TGA CTG TTG GAG CAA GGA 3'	Xho I
3	RBM3	RBM3_Sense	5' CCAA *GAATTC* ACC ATG TCC TCT GAA GAA GGA 3'	EcoR I
		NB328_Antisense	5' CCCA *CTCGAG* GTT GTC ATA ATT GTC TCT GTA 3'	Xho I
4	STAT3	NB293_Sense	5' CCGA *GAATTC* ACC ATG GCT CAG TGG AAC CAG 3'	EcoR I
		NB294_AntiSense	5' CCGA *GGATTC* CAT GGG GGA GGT AGC ACA 3'	BamH I
5	pMLS-I	NB279_Sense	5' CCGA *GAATTC* ACC ATG TAT AGC CGA TTC CTG CAAG 3'	EcoR I
		NB280_Antisense	5' CCGA *GGATTC* GG GAT TCT TCG AAG GTT GTG 3'	BamH I
6	pMLS-II	NB281_Sense	5' CCGA *GAATTC* ACC ATG GAG AAG CAG CAG ATG TTG 3'	EcoR I
		NB282_Antisense	5' CCGA *GGATTC* GG CTT GAG GGT TTT GTA GTT 3'	BamH I
7	pMLS-III	NB283_Sense	5' CCGA *GAATTC* ACC ATG AAG ATG CAG CAG CTG GAAC 3'	EcoR I
		NB284_Antisense	5' CCGA *GGATTC* GG GCA CGC GAT CTG CTG CCG 3'	BamH I
8	pMLS-IV	NB285_Sense	5' CCGA *GAATTC* ACC ATG GAC CGT CTG GAA AAC TGG 3'	EcoR I
		NB286_Antisense	5' CCGA *GGATTC* GG GGC ACT CTT CAT TAA GTT 3'	BamH I
9	pMLS-V	NB287_Sense	5' CCGA *GAATTC* ACC ATG CCA AAT GCT TGG GCA 3'	EcoR I
		NB288_Antisense	5' CCGA *GGATTC* GG ATA CTT TTT CAC AAG GTC 3'	BamH I
10	ΔN-17 GRIM-19	NB295_Sense	5' CCGA *GAATTC* ACC ATG CCC ATC GAC TAC AAA CGG 3'	EcoR I
		NB128_Antisense	5' CCAA *CTCGAG* CGT GTA CCA CAT GAA GCC 3'	Xho I
11	STAT3 (1-470)	NB293_Sense	5' CCGA *GAATTC* ACC ATG GCT CAG TGG AAC CAG 3'	EcoR I
		NP9_Antisense	5' CCGA *CTCGAG* CAG AAC GCT GAG TCT TGG 3'	Xho I

2.2.3 Cell free synthesis of proteins

Full length STAT3 and mutants, GRIM-19, Su9-DHFR, RBM3, ER-α, TIM23 and DHFR proteins were synthesized using T7 or Sp6 *in vitro* coupled transcription and translation system (Promega, USA) according to the manufacturer's instructions. Each translation mix contains 20 µCi of [^{35}S] labeled methionine (1170 Ci/mmole, BARC, India). Translated products were analyzed using phosphor imaging and scintillation counting.

2.2.4 Isolation of Mitochondria

Mitochondria were isolated from rat heart using differential centrifugation [66, 67]. Briefly, excised tissues were minced and perfused in 0.9% saline and then homogenized in cold homogenization buffer (H medium: 220 mM Mannitol, 70 mM Sucrose, 0.2 mM EDTA, 2 mM HEPES, pH 7.2 and added 0.36 mg/ml BSA prior to use). Homogenates were centrifuged at 2,000 rpm for 10 min. Supernatants were centrifuged at 10,000 rpm for 10 min. The pellet was washed in H-medium twice and suspended in import buffer (0.25 M Sucrose, 1.5 mM $MgCl_2$, 2.5 mg/ml BSA and 10 mM HEPES, pH7.2). In order to obtain a highly purified mitochondrial fraction, the crude mitochondrial suspension was layered on top of a 2.5 M sucrose-percoll gradient and centrifuged at 46,000g at 4˚C for 45 min and mitochondria isolated as described [40].

2.2.5 Separation of Inner Mitochondrial Membrane (IMM) and matrix fraction of mitochondria

IMM and matrix fractions were generated from mitoplasts as described [68]. Mitochondria were re-suspended in 450 µl of hypotonic buffer (5 mM Tris-HCl and 1 mM EDTA pH 7.4) and incubated on ice for 15 minutes to generate mitoplasts (MPs). The solution was

centrifuged at 20,000g for 10 min at 4ºC to pellet mitoplasts. The resulting mitoplasts were resuspended in 450µl of hypotonic buffer and sonicated for 2 min (30 sec OFF and 30 sec ON at 150W, Branson Sonifier) on ice. The solution was then spun at 100,000g for 40 min. The resultant pellet contains the IMM-enriched fraction while the supernatant contains matrix-enriched fraction. For high salt treatment, MPs were incubated with 400 mM KCl on ice for 10 minutes followed by centrifugation (15,000 rpm for 15 min). For high pH treatment, MPs were incubated with 200 mM Na_2CO_3 (pH 11.5) for 10 minutes followed by centrifugation.

2.2.6 In vitro import assay

[^{35}S] labeled proteins of GRIM-19:6,000 cpm; STAT3:12,000 cpm; $STAT3_{1-470}$:12,000 cpm; $STAT3_{S727A}$:12,000 cpm; Su9-DHFR: 10,000 cpm; His6-DHFR: 10,000 cpm, RBM3: 10,000 cpm, ER-α: 10,000 cpm and TIM23:10,000 cpm were used in import assays unless otherwise described in the legends. Labeled proteins were incubated with 200 µg of isolated mitochondria in import buffer (0.25 M Sucrose, 1.5 mM $MgCl_2$, 2.5 mg/ml BSA and 10 mM HEPES, pH 7.2) supplemented with 2 mM ATP, 2 mM GTP, 5 mM Mg $(OAC)_2$, 20 mM KCl and 2 mM succinate at 37ºC for 60 min. After import, one half of each sample was directly used to assess the mitochondrial association, and the other half was treated with proteinase K on ice for 15 min to remove the non-imported protein. After inhibiting proteinase K with PMSF (1 mM), mitochondria were re-isolated by passing through a sucrose cushion (0.8 M sucrose in 10 mM HEPES, pH 7.2) at 12,000 rpm for 10 min. Mitochondria were separated on SDS-PAGE and the import was analyzed by using phosphor imaging. For mitoplasts preparation, after import mitochondria were resuspended in hypotonic buffer (20 mM KCl, 10 mM HEPES, pH 7.2) or 0.1% digitonin and incubated on ice for 20 min. The resulting mitoplasts were reisolated by centrifugation at 12,000 rpm for 10 min and processed as

mentioned above.

2.2.7 Immunoblotting

For western blot analysis, mitochondria were lysed in RIPA buffer (50 mMTris-HCl, pH 7.2, 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS and 0.25 mM EDTA) with the protease inhibitor cocktail (Roche, Basel, Switzerland) and centrifuged to remove the debris. Protein concentrations were measured using Bradford reagent (Amersco, USA). Then lysates were resolved on SDS-PAGE and transferred to poly vinylidene difluoride (PVDF) or nitrocellulose membranes and probed with specific antibodies. After incubation with HRP conjugated secondary antibodies, the blots were developed using Bio-Rad's Versa doc imaging system.

2.2.8 In vitro phosphorylation assay

Recombinant purified STAT3 protein was incubated with Rabbit reticulocyte lysate (RRL) in phosphorylation buffer (20 mM Tris-Cl pH 7.5, 1 mM DTT, 25 µM ATP, 10 mM $MgCl_2$, 1 mM NaF, 1 mM Na_3VO4, 40 mM KCl) containing [$^{32}\gamma$P] labeled ATP at 35°C for 30 min. Reaction was quenched by adding SDS and recombinant protein was pulled down using Ni-NTA resin and separated on SDS-PAGE and analyzed using phosphor imaging system.

2.2.9 2D-BNPAGE

For the first dimension BN-PAGE, mitochondrial pellets were resuspended in solubilization buffer (1% Dodecyl maltoside, 0.75 M Aminocaproic acid and 50 mM Bis-Tris, pH 7.2). Mitochondrial suspensions were cleared at 14,000 rpm for 30 minutes at 4°C and mixed with 5% serva blue G dye. Complexes were resolved on native 6-13% acrylamide gradient gels as described [69]. For the second dimension, excised bands from BN-PAGE were soaked in 1%

β-mercaptoethanol and 1% SDS for 1h at room temperature. Gel pieces were layered on top of a 12% Tricine–SDS gel and electrophoresis was performed at room temperature at 100 V [70].

2.210 *Protein purification by Ni-NTA column*

To the soluble protein fraction containing His-STAT3, an equal volume of buffer B (200 mM NaCl, 10 mM βME and 50 mM Tris-HCl pH 7.5) was added. The sample was then passed through the Ni-NTA column which was equilibrated with buffer A (100 mM NaCl, 5 mM βME and 25 mM Tris-HCl pH 7.5). The column was washed with Buffer A containing 10 mM imidazole and the bound protein was eluted by using elution buffer (0.4 M imidazole pH 7.0, 50 mM Tris-HCl pH 7.5, 100 mM NaCl and 5 mM βME).

2.3 RESULTS

2.3.1 Mitochondrial STAT3 is present in inner mitochondrial membranes

Though STAT3 was shown to be associated with mitochondria, the exact sub-mitochondrial localization remains ambiguous. Recognizing the fact that targeting of proteins to the different sub - compartment of mitochondria requires different protein receptor complexes, we tried to define the precise sub-mitochondrial localization of STAT3 anticipating to get some hints about its mitochondrial recruitment. To find out sub-mitochondrial localization of STAT3, highly purified rat heart mitochondriawere subjected to increasing concentrations of trypsin treatment (Fig.2.1A). Mitochondrial fractions were separated on SDS-PAGE, western transferred and probed with antibodies specific for each mitochondrial sub-compartment (Fig.2.1A). As shown in the Figure 2.1A (Top Panel), STAT3 is resistant to low concentrations of protease treatment like matrix localized protein, Aconitase (Bottom Panel)

whereas outer membrane protein, TOM20 (Fig.2.1A, lane 2) and the inter membrane space protein, Mia40 were completely digested (Fig.2.1A, lane 3). Further increase in the trypsin concentration to 100 µg/ml reduced the mitochondrial associated STAT3 by 50% when compared to Aconitase levels (Fig.2.1A, lane 4). However, solubilization of mitochondrial membranes by TritonX-100 prior to protease treatment completely degraded the STAT3 and Aconitase (Fig.2.1A, lane 6). A similar observation was made when mitochondria subjected to proteinase K treatment (not shown). These results show that a significant fraction of endogenous STAT3 is localized in the protease inaccessible compartment of mitochondria.

To further investigate the sub-mitochondrial localization of STAT3, mitochondrial outer membrane was solubilized with digitonin (Fig.2.1B). The resulting mitoplasts were spun at high speed to separate insoluble mitoplasts and soluble intermembrane space fraction. 40% Mia40, an intermembrane space marker, was detected in the supernatant fraction but not ND1, an inner memrbane marker (Fig.2.1B, lane2). Like ND1 most of the STAT3 was associated with mitoplast fraction (Fig.2.1B, lane 3) and also partially resistant to protease treatment (Fig.2.1B, lane 5). These results suggest that STAT3, like ND1, is not associated with soluble intermembrane space fraction rather it is likely to be associated with inner membrane or matrix fraction.

To ascertain the exact localization of STAT3, we further separated mitoplasts into IMM (Inner Mitochondrial Membrane) and matrix fractions as described in the Methods section. Separation of these fractions was confirmed by immunoblotting against specific marker proteins (Fig.2.1C). Immunoblotting of these fractions with STAT3 specific antibody revealed enrichment of STAT3 in IMM fraction (Fig.2.1C, lane 3). Treatment of mitoplasts with high salt (400 mM KCl) failed to dislodge STAT3 from membrane fraction like GRIM-

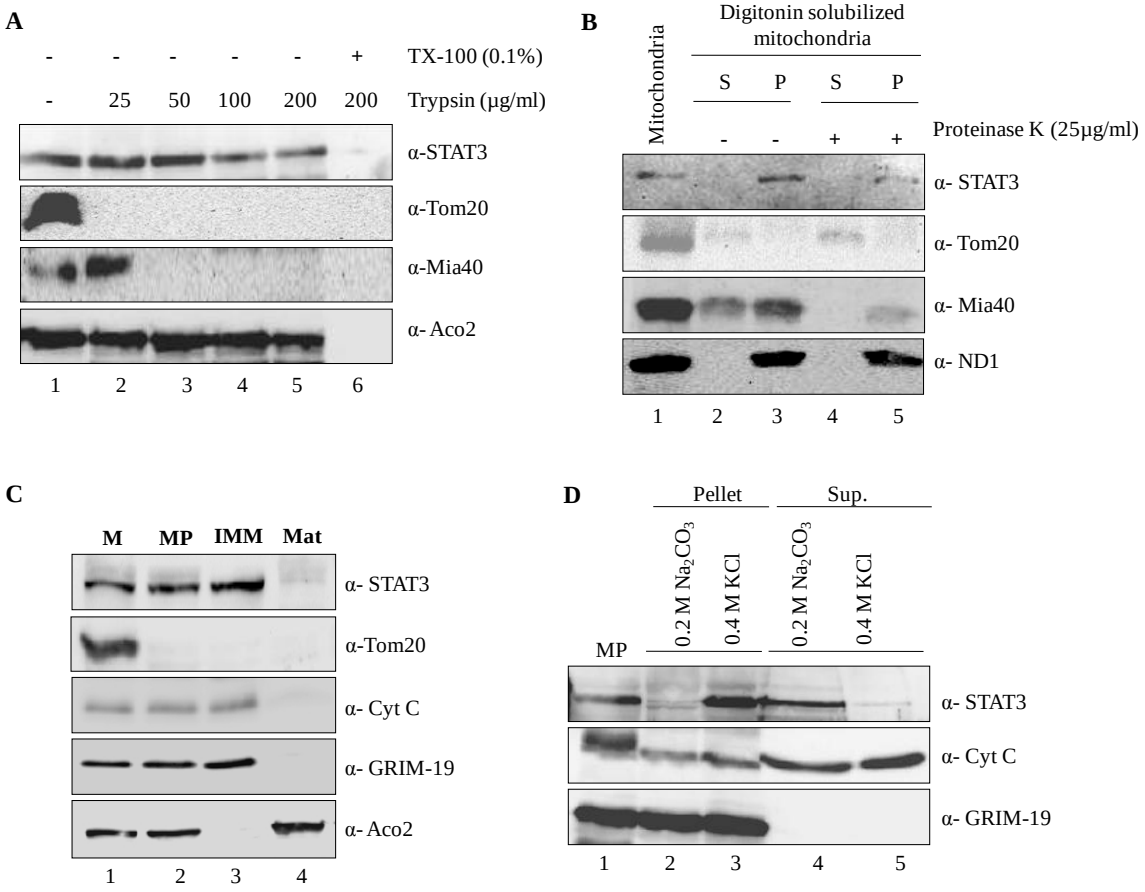


Fig.2.1 Mitochondrial Localization of STAT3: A) Purified rat heart mitochondria were subjected to different concentrations of trypsin (25 to 200 µg/ml, lanes 2-6) either in the absence (lanes 2-5) or presence (lane 6) of Triton X-100 for 20 minutes on ice. After inhibiting the trypsin by trypsin inhibitor, mitochondrial fractions were reisolated by centrifugation and separated on SDS-PAGE followed by immunobloting with antibodies specific for STAT3, Tom20 (outer membrane marker), Mia40 (inter membrane space marker) and Aconitase (matrix marker). B) STAT3 sub-compartmental localization: Mitochondrial outer membranes were solubilized with digitonin (0.1%) for 15 minutes on ice and resulting mitoplasts were reisolated by centrifugation at 20,000g for 10 min. Both soluble (Supernatant, S) and insoluble fractions (Pellet, P) were collected and treated or left untreated with proteinase K (25 µg/ml). After inhibition of proteinase K with PMSF (1 mM), all the fractions were separated on SDS-PAGE and probed with antibodies as specified in the figure. **C**) Rat heart mitochondria (M) were sub-fractionated into mitoplasts (MP), inner mitochondrial membranes (IMM) and matrix (Mat) fractionations as described in Methods and separated on SDS-PAGE and probed with specific markers as indicated in the figure. **D**) To show the tight association of STAT3 with membranes, isolated mitoplasts were subjected to high salt (400 mM KCl) and high pH (200 mM Na_2CO_3, pH 11.5) treatment for 15 minutes on ice. Mitoplasts were reisolated and equivalent fractions were resolved on SDS-PAGE and analyzed by western blot.

19 (an integral membrane protein) whereas Cytochrome C, a loosely associated inner membrane protein was released into soluble fraction (Fig.2.1D, compare lanes 3 & 5). However, high pH treatment (200 mM Na_2CO_3, pH 11.5) releases STAT3 into soluble fraction (Fig.2.1D, lane 4) indicating that STAT3 is tightly associated but not integrated into the membrane.

2.3.2 STAT3 is present in complex I of electron transport chain

Since STAT3 is known to increase the activities of complex I and II [40], we further evaluated the presence of STAT3 in the mitochondrial electron transport chain complexes. Mitochondria and mitoplasts fractions were subjected to blue native gel electrophoresis and probed for STAT3 and a complex I subunit, ND1 (Fig.2.2A). As shown in the Figure 2.2A, STAT3 was found to be associated with a high molecular weight complex above 700 kDa like a known complex I subunit, ND1.

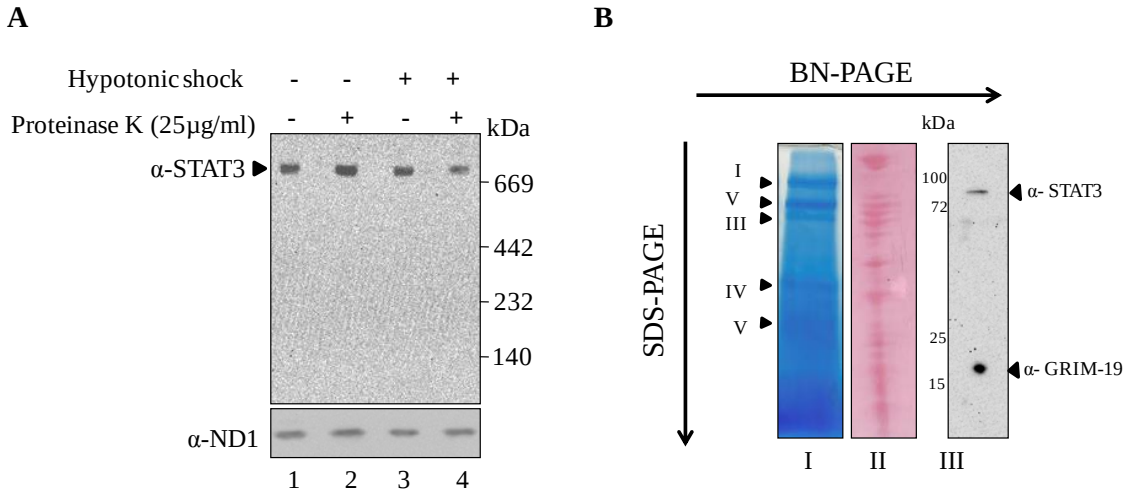


Fig.2.2 STAT3 is present in complex I of electron transport chain: A) Mitochondria (lanes 1 & 2) and mitoplasts (lanes 3 & 4) were treated without (1 & 3) or with 25 µg/ml proteinase K (lanes 2 & 4). After inhibiting proteinase K with PMSF, samples were solubilised in 1% dodecyl maltoside buffer as described in the Methods. The samples were subjected to BN-PAGE and probed for STAT3 and ND1. **B)** Isolated mitochondria

were solubilized in Solubilization buffer and ETC components were resolved by BN-PAGE in first dimension (panel I) and complex I specific band was excised to further separate in second dimension (panel II) and probed with specified antibodies (panel III).

Protease treatment of mitoplast fraction prior to BN-PAGE reduced STAT3 association with this high molecular weight complex by 50%. In contrast, the levels of ND1 did not change significantly (Fig.2.2A, lanes 3 and 4). Separation of this high molecular weight complex in second dimension showed the presence of STAT3 and GRIM-19, a known complex I subunit (Fig.2.2B). Taken together, our results indicate that STAT3 tightly associates with inner mitochondrial membrane complexes, likely to complex I, in a protease inaccessible manner.

2.3.3 Prediction of possible mitochondrial targeting sequences in STAT3

Despite of its mitochondrial localization, surprisingly, we did not find any cleavable pre-sequence in STAT3 protein sequence. As some mitochondrial proteins, which doesn't have the signal sequence, utilizes internal targeting sequence for their mitochondrial targeting. However, this sequence would be an amphipathic helix, rich in positive amino acids. As STAT3 has no signal sequences, we have either aligned STAT3 protein sequence with known internal targeting sequences containing mitochondrial proteins (BCS1, CCHL) or selected amphipathic helical regions of STAT3 that are rich in positive amino acids. We identified the region 197-233 and 482-537 of STAT3 similar to the internal targeting sequence of CCHL (177-202 and 243-276). Similarly, region 561-594 of STAT3 matches with the internal targeting sequence of BCS1 (91-126). In addition to this, we also found two regions of STAT3, 139-180 and 199-251, harbors

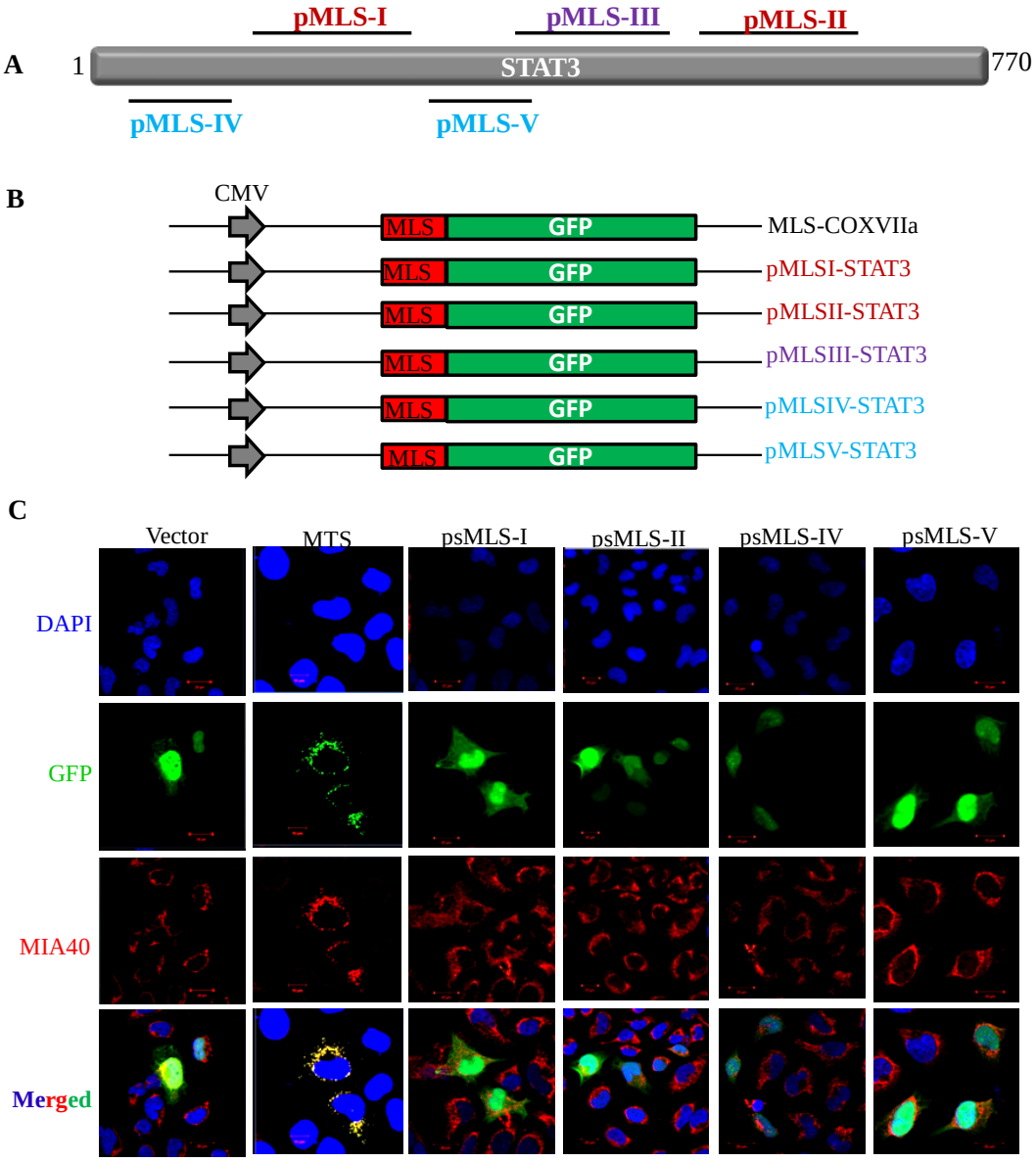


Fig.2.3 Prediction of putative mitochondrial targeting sequence in STAT3: A) STAT3 protein sequences either matched with CCHL (pMLS-I and II), BCS1 (pMLS-III) or which can form amphipathic helices, rich in positive amino acids (pMLS-IV and V) were selected and **B)** cloned into pEGFP-vector. **C)** Subsequently, these GFP-fusion constructs were transfected into HeLa cells. 48h post transfection cells were fixed and incubated with Mia 40 antibody. Later, cells were incubated with Alexa labeled secondary antibodies to monitor the localization of GFP-fusion proteins as described in the methods.

amphipathic helices that are rich in positive amino acids (Fig.2.3A). Further, we have cloned these sequences as GFP-fusion products and monitored their potential for mitochondrial

targeting (Fig.2.3B). As show in the Fig.2.3C, neither of these GFP-fusion proteins colocalized with mitochondria. However, mitochondrial targeting sequence from COXVIIIa drives the import of GFP to mitochondria and colocalized with mitochondrial marker, Mia40. Together, these results suggest that mitochondrial targeting sequence of STAT3 may not be linear rather it might be conformational. However, these results do not rule out the possibility of requirement of some other factors for mitochondrial targeting of STAT3.

2.3.4 In vitro import of STAT3 into isolated mitochondria

As our preliminary results suggest either conformational dependency or requirement of other protein factor for STAT3 recruitment to mitochondria, we adopted *in vitro* protein import assay to address the mechanistic details of STAT3 targeting to mitochondria. To investigate the translocation of STAT3 across mitochondrial membranes, *in vitro* translated and [^{35}S] labeled STAT3 was incubated with isolated mitochondria. Import of STAT3 was confirmed by its resistance to externally added proteinase K. As shown in the Figure 2.4A, STAT3 was resistant to proteinase K (Top Panel) which indicates the presence of STAT3 within mitochondria. Import of [^{35}S] labeled Su9-DHFR (Fig.4B) and His_6-DHFR proteins (Fig.2.4C) were used as positive and negative controls respectively. Upon import, Su9-DHFR which has a presequence of subunit 9 of F_1F_0 ATPase (Fig.2.4B) was protected from the protease treatment whereas His_6-DHFR protein, having no mitotargeting sequence was susceptible to protease treatment (Fig.2.4C). Most of the imported fragments were completely digested when the mitochondrial membranes were solubilized with Triton X-100 (Fig.2.4A, lane 6). These results clearly indicate that STAT3 could able to traverse the mitochondrial membranes *in vitro*.

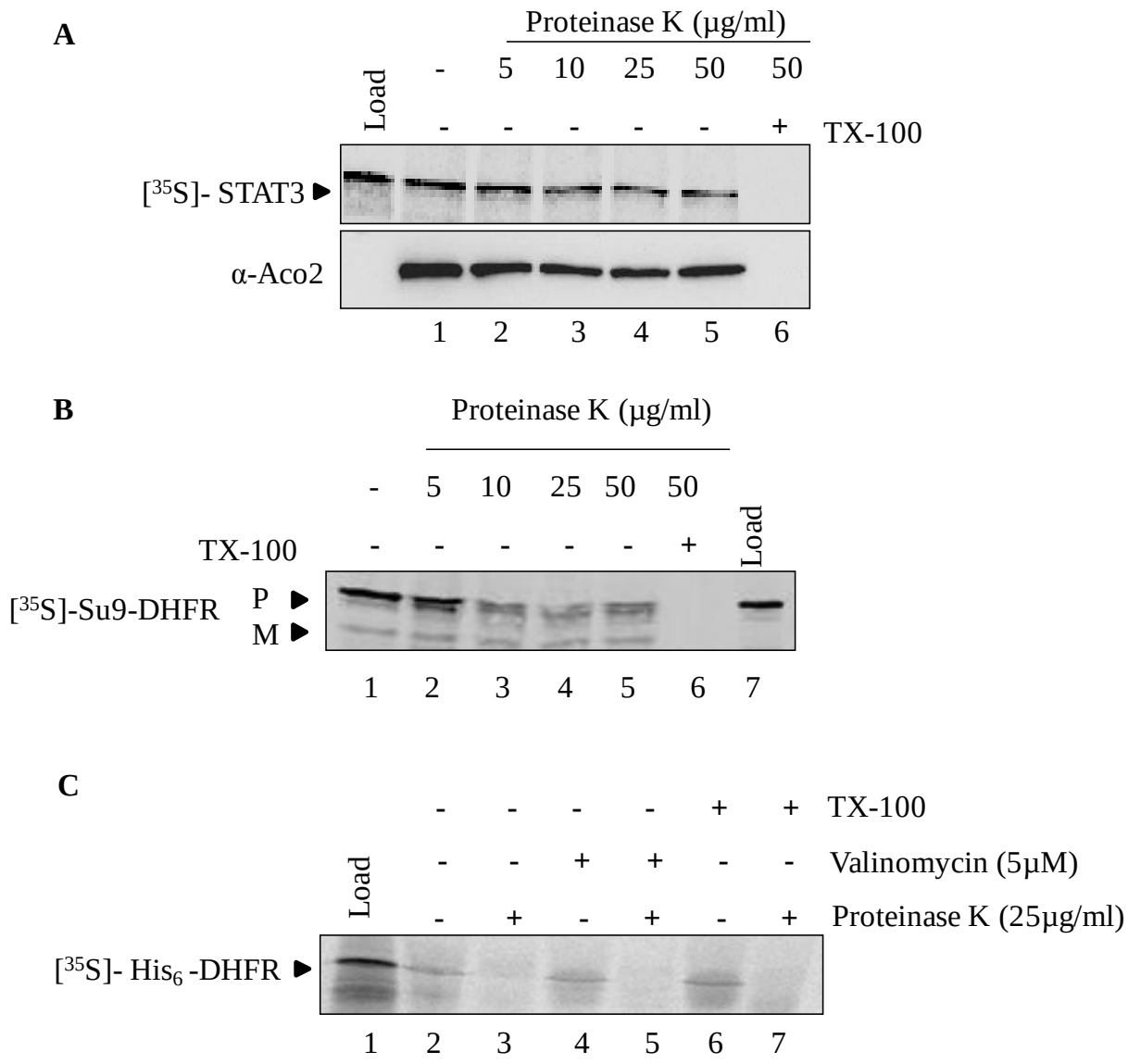


Fig.2.4 *in vitro* import of STAT3 into isolated mitochondria: A) *In vitro* import of STAT3 and **B)** Su9-DHFR was performed for 60 min as described in the Materials and Methods. After import, samples were treated with increasing concentration of proteinase K (lanes 2-6) either in the absence (2-5) or presence of Triton X-100 (lane 6). After inhibition of proteinase K with PMSF (1 mM), samples were reisolated and resolved on SDS-PAGE (p: precursor, m: mature).**C**) Import of His_6-DHFR was carried out as described in the Methods and the samples were treated with proteinase K (25 µg/ml) and analyzed by phosphor imager.

2.3.5in vitro import of labeled STAT3 requires membrane potential

Subsequently, we sought to determine the basal conditions required for import of labeled STAT3 *in vitro*. Primary criteria for translocation of proteins into mitochondria are the

requirement of energy in the form of ATP and GTP [71-73]. In addition, most of the matrix targeted proteins and some of the inner membrane targeted proteins require membrane potential to cross the inner

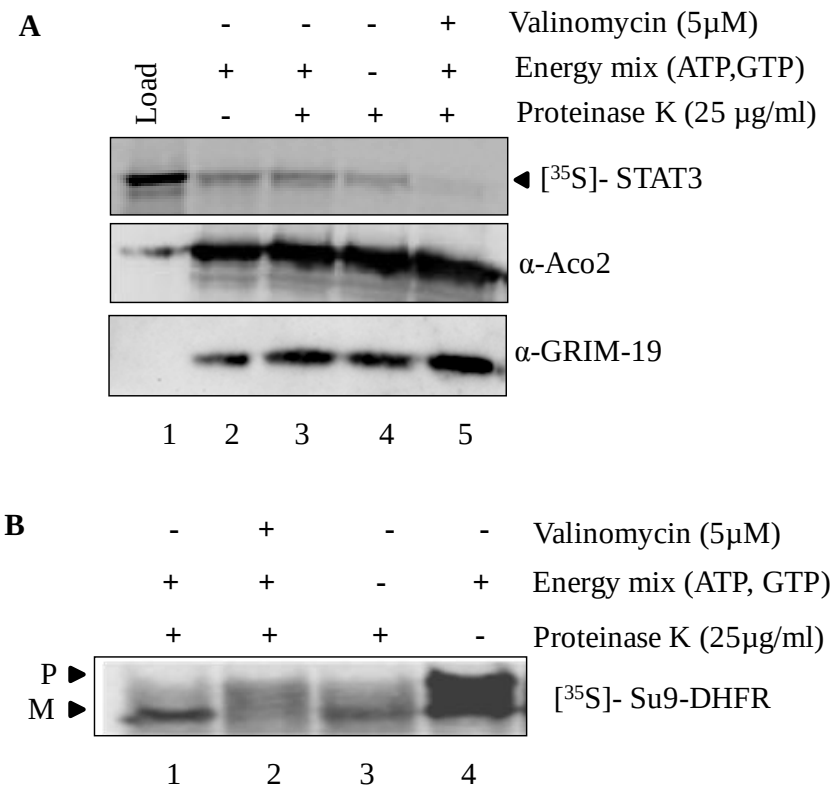


Fig.2.5 STAT3 requires membrane potential for its import: A) Import of STAT3 (Top Panel) and **B)** Su9-DHFR (Bottom Panel) was examined in the absence of membrane potential by incubating mitochondria with Valinomycin (5 µM) for 5 min (Top Panel, lane 5; Bottom panel, lane 2) or by excluding the external energy source in the form ATP, GTP and Succinate (Top Panel, lane 4; Bottom Panel, lane 3) in the import reaction.

membrane [74-76]. To analyze whether STAT3 translocation requires membrane potential and energy, the import was performed in the absence of membrane potential and external energy source and immunoblots were probed with Aconitase antibody to show equal amount of protein in all samples (Fig.2.5A). When membrane potential was dissipated with valinomycin, import of both Su9-DHFR (Fig.2.5B, lane 2) and STAT3 (Fig.2.5A, lane 5) was

abolished whereas import under energy deficit conditions resulted in 20-30% reduction of import (Fig.2.5A, lane 4). The persistent translocation of STAT3 in the absence of external energy could be due to ATP leaking out of the mitochondria [77] or mitochondria respiring on endogenous substrates (data not shown). These results indicate that STAT3 import and translocation across mitochondrial membrane requires membrane potential and likely requires energy.

2.3.6 Sub-compartmental localization of in vitro imported labeled STAT3

As our earlier results suggests that mitochondrial STAT3 resides in complex I of inner mitochondrial membranes, we asked whether *in vitro* imported labeled STAT3 also located in this protease inaccessible compartment. To examine the sub-compartmental localization, import was performed using labeled STAT3. Afterwards, mitochondria were subjected to hypotonic shock followed by proteinase K treatment to monitor the localization of *in vitro* imported protein. Unlike endogenous mitochondrial STAT3, *in vitro* imported STAT3 failed to resist the protease action in the mitoplast fraction. However, an inner mitochondrial membrane protein, GRIM-19 and a matrix protein, Su9-DHFR resists protease action upon their *in vitro* import into isolated mitochondria (Figure 2.6). Susceptibility of *in vitro* imported labeled STAT3, but not the endogenous mitochondrial STAT3, to protease in the mitoplast fraction indicates the requirement of some unknown factor (s) for the integration of labeled STAT3 into protease inaccessible fraction.

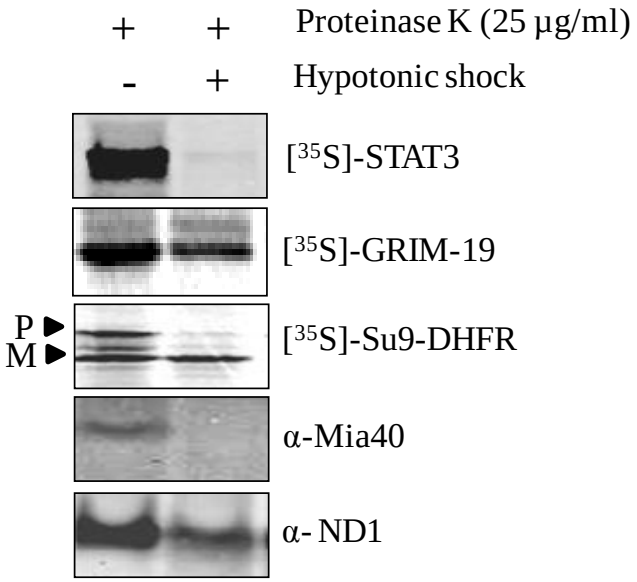


Fig.2.6 Localization of in vitro imported labeled STAT3 in isolated mitochondria: Import of labeled STAT3, GRIM-19 and Su9-DHFR was performed for 60 minutes with isolated mitochondria as indicated in the Methods. One fraction of imported sample was directly subjected to proteinase K (25 µg/ml), the other fraction was subjected to hypotonic shock followed by proteinase K treatment. After, inhibiting the proteinase K, samples were resolved on SDS-PAGE and analyzed by phosphor imaging. Fractions of mitochondria and mitoplasts were also probed with ND1 (inner membrane marker) and Mia40 (inter membrane space marker).

2.3.7Cloning, expression and localization of GRIM-19

As earlier results suggest the requirement of some other factors for recruitment of STAT3 to inner mitochondrial membranes, we reasoned the possible involvement of GRIM-19 because of the two reasons: One reason is, GRIM-19 physically interacts with STAT3. And the other reason is that it is an inner mitochondrial membrane protein. To evaluate the involvement of GRIM-19 in the recruitment of STAT3 to mitochondria, we have cloned GRIM-19 in a mammalian expression vector (Fig.2.7A) as myc fusion protein and expression was monitored by either over expression in HEK293 cell lines (Fig.2.7B) or *in vitro* coupled transcription and translation (Fig.2.7C). Immunofluorescence studies reveal that GRIM-19 co- localizes with mitochondria like a known mitochondrial marker, Mia40 (Fig.2.7D).

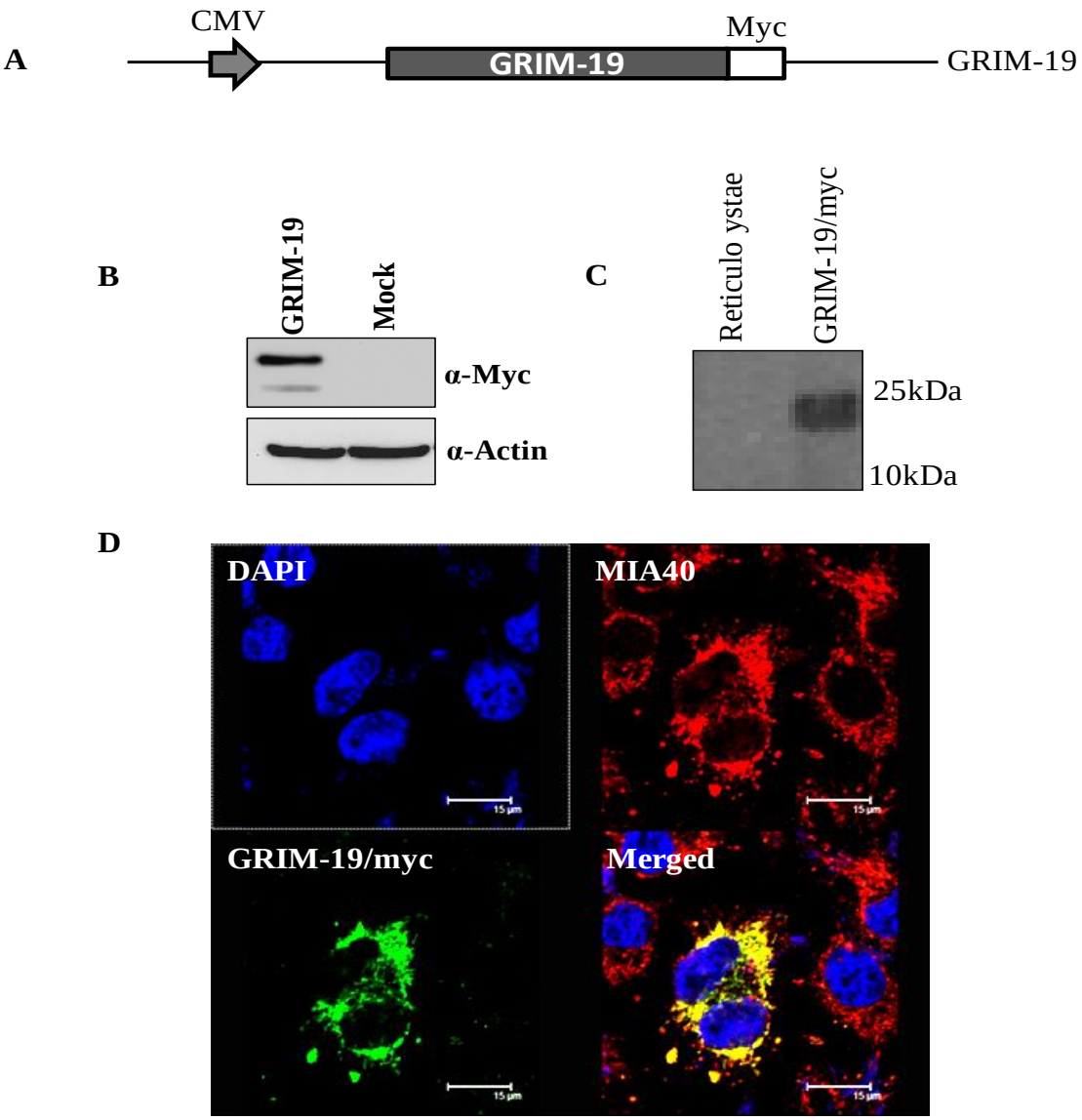


Fig.2.7 Cloning, expression and localization of GRIM-19: A) GRIM-19 cDNA amplified from HeLa cells total RNA was cloned into pcDNA3.1/myc vector. **B)**Expression of myc-fusion proteins was either confirmed by immunoblotting cell lysates prepared from HEK cells transfected with pCDNA3.1-GRIM-19/myc or **C)** by cell free synthesis of labelled protein using T7 coupled transcription, translation system or **D)** by immunolocalization of myc-fusion protein in cells transfected with pCDNA3.1-GRIM-19/myc.

2.3.8 GRIM-19 regulates STAT3 translocation into mitochondria

Since GRIM-19 is a negative transcriptional regulator of STAT3, we investigated whether GRIM-19 also regulates STAT3 import into mitochondria. Co-import of [^{35}S] labeled STAT3 and GRIM-19 was carried out as described in the Methods. Interestingly, STAT3 import was steadily enhanced up to certain concentrations of GRIM-19 and stimulatory effect was more profound at lower concentrations (Fig.2.8A). To rule out the possibility of interference from reticulocyte lysate components, we performed an *in vitro* import with increasing concentration of lysate but found no difference in STAT3 recruitment (Fig.2.8B). To show specificity, co-import was performed with another transcription factor, ER alpha, known to be associated with mitochondria. Most of mitochondrial localized ER alpha is accessible to externally added protease [78]. However, in our *in vitro* import studies, ER alpha neither increased its association with mitochondria (not shown) nor was protected from externally added protease even in the presence of GRIM-19 (Fig.2.8C). Most importantly, GRIM-19 doesn't have any effect on the import of a mitochondrial inner membrane protein, TIM23 (Fig.2.8D). Additionally, as a negative control, we also performed co-import of GRIM-19 with RBM3, a RNA binding motif protein, localized mostly in nucleus and cytosol. As shown in the Figure 2.8E, RBM3 failed to be internalized into mitochondria and GRIM-19 doesn't affect the import of this protein. In addition, we also performed co-import of STAT3 with increasing concentrations of another mitochondrial targeted protein, Su9-DHFR and found no significant difference in STAT3 recruitment to mitochondria (Fig. 2.8F). Together these results demonstrate the specific role of GRIM-19 in STAT3 recruitment to mitochondria.

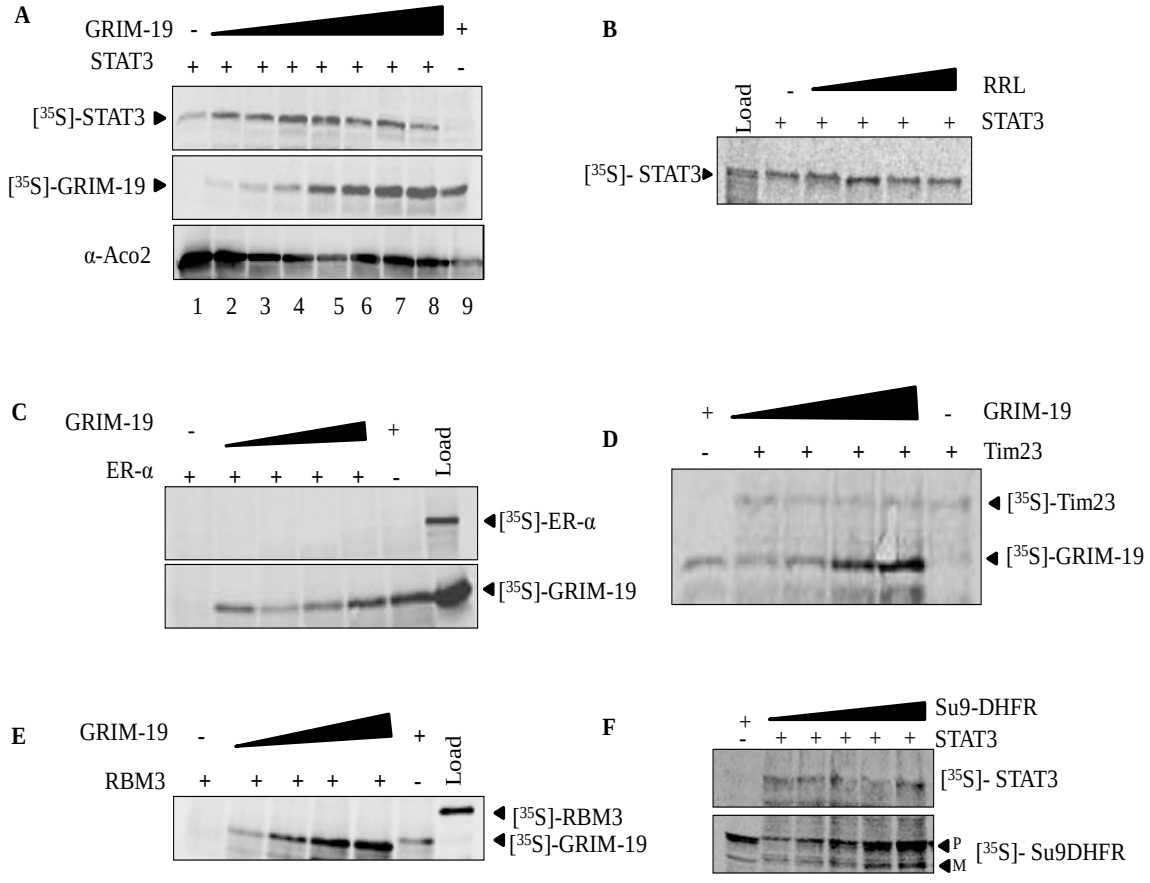


Fig.2.8 GRIM-19 stimulates the import of STAT3 into mitochondria: A) *in vitro* co-import of STAT3 ($6x10^3$cpm) was performed with increasing concentrations of GRIM-19 ($1x10^3$, $1.5x10^3$, $2x10^3$, $2.5x\ 10^3$, $3x10^3$, $4x10^3$ and $6x10^3$) of [^{35}S]-GRIM-19 for lanes 2-8 for 60 min at 37°C. After import, outer membrane associated proteins were digested with proteinase K (25 µg/ml). Then samples were resolved on SDS-PAGE and imported proteins were analysed by phosphor imager. **B)** STAT3 import was performed without (lane 1) or with increasing concentrations of reticulocyte lysates (2-5) for 60 min as indicated in the Methods. Samples were treated with 25 µg/ml proteinase K for 15 minutes. After inhibiting the proteinase K, samples were resolved on SDS-PAGE and analyzed by phosphor imager. As a negative control, co-import was also performed with ER alpha **C**), TIM23**D)** and RBM3 **E**) with increasing concentrations of GRIM-19. In another control experiment, STAT3 ($6x10^3$ cpm) was co-imported with increasing concentrations of Su9-DHFR ($3x10^3$, $5x10^3$, $8x10^3$& $10x10^3$cpm of Su9-DHFR for lanes 2-6 of Fig. **F**) as described above.

2.3.9 Over expression of GRIM-19 redistributes STAT3 localization in HeLa cells

To validate the physiological requirement of GRIM-19 for STAT3 recruitment to mitochondria, we have over expressed GRIM-19 in HeLa cells and monitored the STAT3 localization using immunofluorescence microscopy. As shown earlier, STAT3 exhibits predominant nuclear staining in HeLa cells (Fig.2.9E). However, in cells over expressing GRIM-19, STAT3 shows punctate cytosolic staining, most of which co-localized with GRIM-19 (Fig.2.9A).

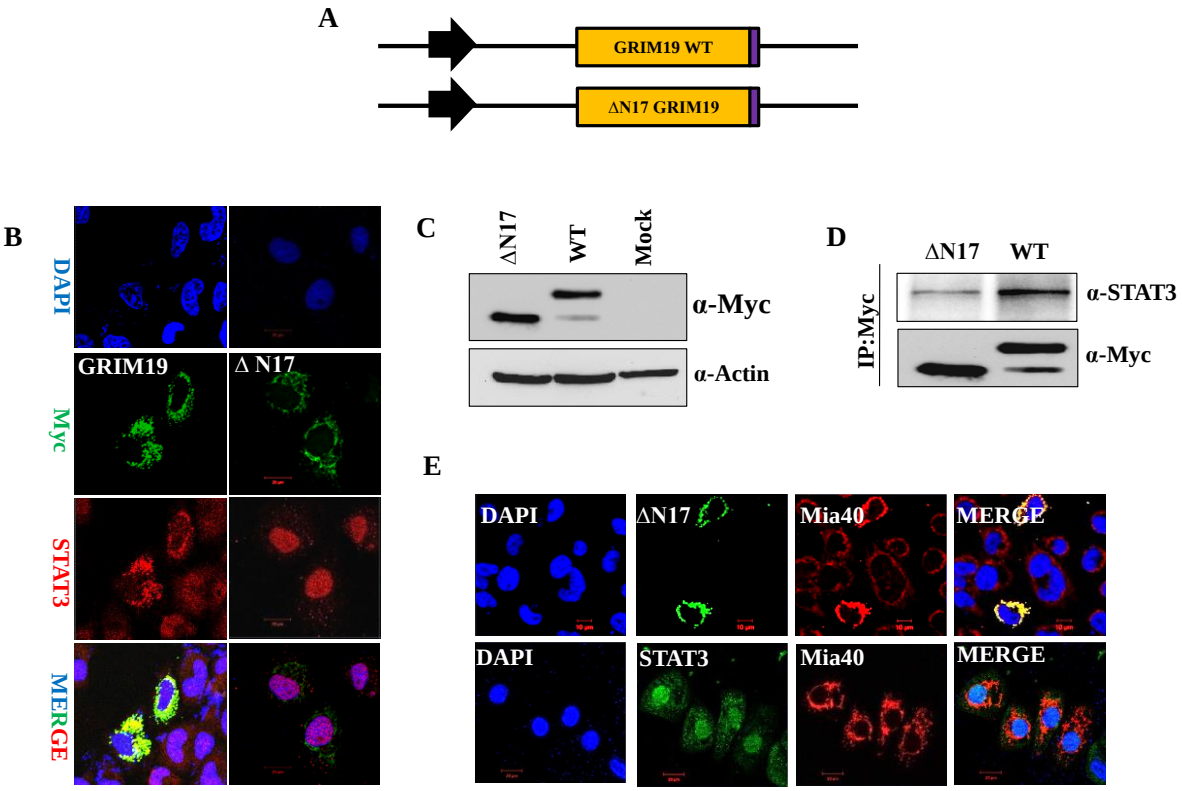


Fig.2.9 GRIM-19 redistributes intracellular STAT3 in HeLa cell lines: A) GRIM-19 wild type and D-N17-GRIM-19 mutant proteins cloned into pcDNA 3.1 myc vector were transfected into HeLa cell lines. **B)** 48h post transfection cells were immnuno-stained for STAT3 and myc-fusion proteins to monitor the intracellular localization. **C)** Cell lysates were also prepared from the same cell lines and either immunoblotted with the antibodies mentioned or **D)** immunoprecipitated with anti-myc and probed with myc and STAT3 specific

antibodies. **E)** To monitor the localization of STAT3 and D-N17 cells were also immunostained with myc and STAT3 specific antibodies.

As most of the GRIM-19 puncta stain co-localized with a mitochondrial marker, Mia40, we speculate that punctate STAT3 pools may represent the mitochondrial localized portion of STAT3. Further, we monitored the STAT3 distribution in cells over expressing ΔN17-GRM-19 mutant, which is known to poorly interact with STAT3 [79]. Interestingly, over expression of mutant protein fails to redistribute the intracellular STAT3 pools to mitochondria whereas wild type protein promotes mitochondrial recruitment of STAT3 (Fig.2.9B). Further, we performed the co-immunoprecipitation of cell lysates expressing Myc-GRIM19 or Myc-ΔN17-GRM-19 with Myc antibodies and probed with Myc and STAT3 antibodies. Though both wild type and mutant proteins expression levels were similar (Fig.2.9C) and localized to mitochondria (Fig. 2.9E), our immunoprecipitation studies confirm that the interaction of mutant with STAT3 was less efficient when compared to wild-type GRIM-19 (Fig.2.9D), Altogether, these results suggest that GRIM-19 positively regulates the mitochondrial recruitment of STAT3 and this process requires interaction between these two proteins.

2.3.10 GRIM-19 enhances integration of STAT3 into Complex I

As GRIM-19 was found to be a positive regulator of STAT3 import into mitochondria, we further evaluated whether GRIM-19 could influence STAT3 topology and its association with membranes or complex I. After *in vitro* import of [^{35}S] labeled STAT3 in the presence or absence of GRIM-19 (Fig.2.10A), mitochondria were subjected to hypotonic shock to generate mitoplasts. As expected, imported STAT3 was protected from protease treatment of mitochondria (Fig.2.10A, lane 2). However, most of the imported fraction is susceptible to protease treatment in mitoplasts (Fig.2.10A Right Panel, lane 3) indicating STAT3 is

translocated across the outer membrane and probably loosely associated with the inner membrane. Surprisingly, upon its co-import with GRIM-19 a significant fraction of STAT3 was protected from externally added protease even in mitoplasts (Fig.2.10A Right Panel, lane 5) indicating GRIM-19 dependent integration of STAT3 into protease inaccessible fraction that is likely matrix or to be inner mitochondrial membrane. Protection of *in vitro* imported GRIM-19 and Su9-DHFR in mitoplasts indicates the intactness of mitochondrial inner membrane and matrix (Fig.2.6). Immunoblots were also performed to confirm the formation of mitoplasts by protease susceptibility of Mia40, an intermembrane space marker, but not ND1, an inner membrane marker (Fig.2.6).

To precisely determine the localization of protease protected [^{35}S]-STAT3 in mitoplasts, we carried out the import of STAT3 in the presence of GRIM-19. Protease treated mitoplasts were separated into IMM and matrix fractions (Fig.2.10B). Separation of these fractions was demonstrated by immunoblotting with known marker proteins such as GRIM-19 (inner membrane) and Aconitase (matrix) (Fig.2.10B Bottom Panels). When STAT3 was imported alone, a small fraction of STAT3 was found to be associated with IMM and matrix fraction (Fig.2.10B Left Panel, lanes 1 & 2). However, upon its co-import with GRIM-19, most of the STAT3 was recruited to IMM (Fig.2.10B Right Panel, lane 1). These results demonstrate that GRIM-19 integrates STAT3 into the inner membrane of mitochondria. To further assess whether the co-import of STAT3 with GRIM-19 enhances the association of STAT3 with respiratory complexes, in particular complex I, *in vitro* co-imported samples of [^{35}S]-STAT3 with unlabeled GRIM-19 (cold) were separated by BN-PAGE and STAT3 association was monitored by phosphor imager.

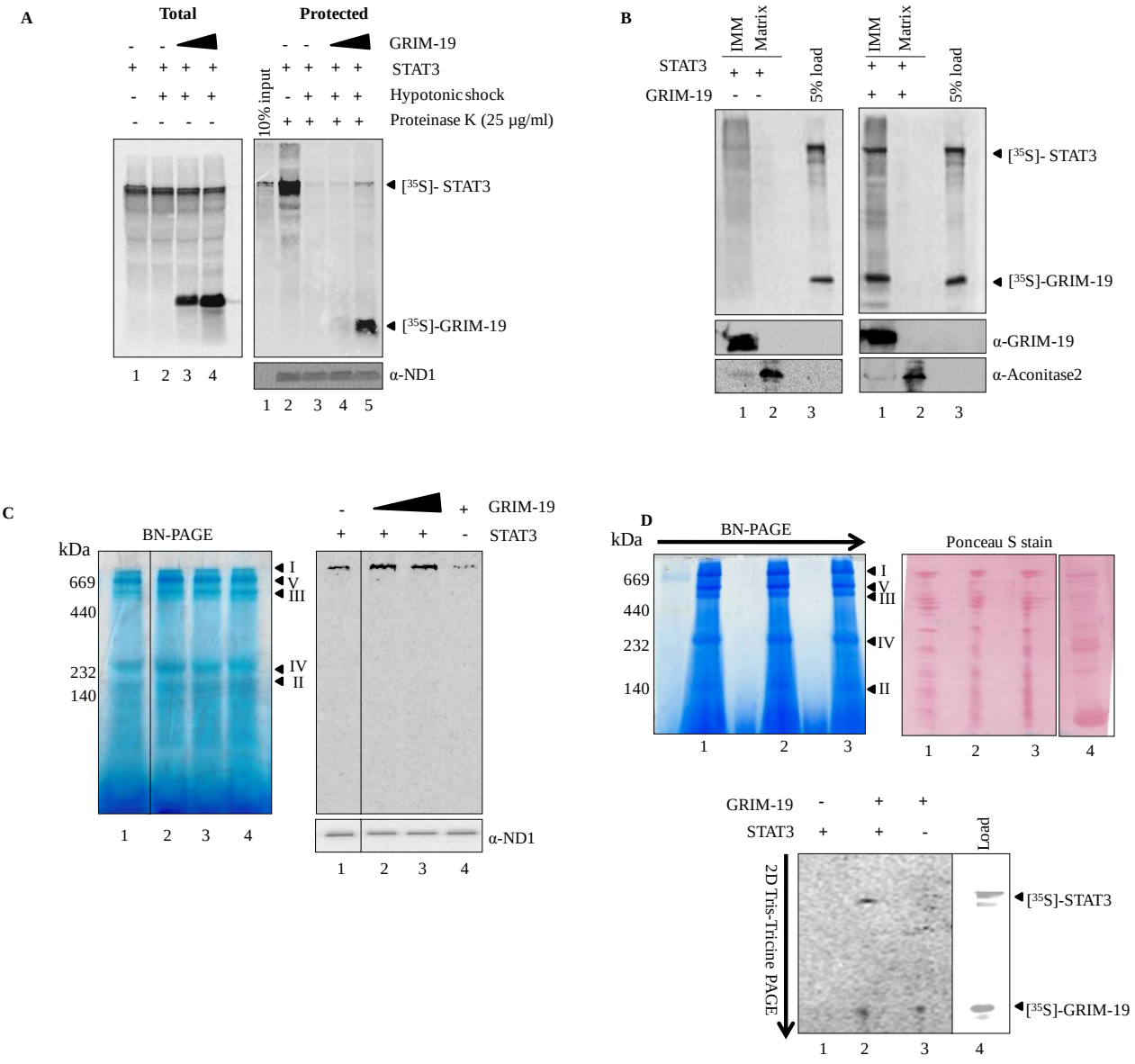


Fig.2.10 GRIM-19 mediated integration of STAT3 into Complex I: A) Labeled STAT3 was imported into mitochondria for 60 min in the absence (Left Panel lanes, 1 & 2; Right Panel lanes 2 & 3) or presence of GRIM-19 (Left panel, lanes 3 & 4; Right Panel, lanes 4 & 5) and mitoplasts were generated by hypotonic treatment of mitochondria. Mitochondria (Left Panel, lane 1; Right Panel, lane 2) and the resulting mitoplasts (Left Panel, lanes 2-4; Right Panel, lanes 3-5) were either left untreated (Left Panel) or treated with proteinase K (Right Panel). After inhibiting the protease, mitochondria and mitoplasts fractions were reisolated and separated on SDS-PAGE and analyzed by phosphor imager. As a loading control, the Right Panel fractions were probed with antibodies specific for ND1. **B**) To demonstrate the association of co-imported STAT3 (with GRIM-19) with IMM, mitoplasts were further separated into IMM and matrix. The equivalent fractions of IMM and matrix

were separated on SDS-PAGE and analyzed by phosphor imaging. Left and Right panels represent the IMM associated STAT3 in the absence and presence of GRIM-19 respectively. To confirm the separation of IMM and matrix fractions, these fractions were also probed with inner membrane specific antibody, GRIM-19 and matrix specific antibody, Aconitase (Bottom Panels). **C**) To analyze complex I association of STAT3, import of radio labeled STAT3 was performed without (lane 1) or with increasing concentrations of unlabeled GRIM-19 (lanes, 2 & 3). Import of labeled GRIM-19 (lane 4) used as a positive control. After import, mitochondria were re-isolated and solubilized in 1% dodecyl maltoside buffer and ETC components were separated by BN-PAGE. Association of STAT3 with the components of the ETC was monitored by using phosphor image (C, Right Panel). Immunoblot, with ND1 specific antibody, was performed to show the equal loading of protein in all the fractions. **D**) To provide direct evidence for STAT3 association with complex I, *in vitro* import of labeled STAT3 (lane 1) or STAT3/GRIM-19 (lane 2) or GRIM-19 (lane 3) was performed and these imported samples were protease treated (25 µg/ml). After inhibiting the protease, the mitochondria samples were solubilized in 1% dodecyl buffer and separated on BN-PAGE (Left Panel) in first dimension. For the second dimension, complex I bands were excised and separated on tris-tricine PAGE (Right Panel) and analyzed by phosphor imager. The ponceau S stained blot shows equal amount of protein in all lanes (Middle Panel). Lane 4 represents the *in vitro* translated [^{35}S]-labeled products of STAT3 and GRIM-19 to identify the STAT3 and GRIM-19 on 2D-PAGE.

As shown in the Figure 2.10C, STAT3 association with complex I was increased in the presence of GRIM-19 (Fig.2.10C, compare lanes 1 & 2) and any further increase in GRIM-19 had no effect on STAT3 association with complex I (Fig.2.10C, compare lanes 2 & 3). [^{35}S]-STAT3 or [^{35}S]-GRIM-19 when imported alone was associated with the complex I (Fig 2.10C, lanes 1 & 4). However, [^{35}S]-STAT3 that was associated with complex I was loosely bound (Fig.2.10D) and susceptible to protease treatment. This was further confirmed by separating labeled [^{35}S]-STAT3 alone (Fig.2.10D bottom Panel, lane 1) or co-imported [^{35}S]-STAT3 and [^{35}S]-GRIM-19 (Fig.2.10D, lane 2) or [^{35}S]-GRIM-19 alone (Fig.2.10D lane 3) by BN-PAGE (Fig.2.10D, Left Panel) followed by two dimensional tris-tricine gels (Fig.2.10D bottom Panel). The association was monitored by phosphor imaging of 2D gels. STAT3 was found only in co-imported fraction (Fig.2.10D bottom Panel, lane 2) but not in STAT3 alone fraction (Fig.2.10D bottom Panel, lane 1). GRIM-19, a known complex I subunit serves as positive control (Fig.2.10D bottom Panel, lane 3). Ponceau S stained 2D

gel shows equal amount of complex I proteins in all samples. Taken together, these results demonstrate that GRIM-19 probably alters topology of mitochondrial STAT3 and also promotes its integration into complex I.

2.3.11 Ser727 of STAT3 is required for GRIM-19 dependent import and assembly

The transcriptional activation domain lies in the carboxy terminus of STAT3. To test whether the C-terminus is required for the protein to be imported into the mitochondria, we generated a STAT3 truncation mutant, $STAT3_{\Delta C}$ ($STAT3_{1-470}$). We carried out an*in vitro* import of labeled $STAT3_{\Delta C}$ in the presence and absence of GRIM-19 (Fig.2.11). Import of $STAT3_{\Delta C}$ was absent even in the presence of GRIM-19 (Fig.2.11A, lanes 2-5). Further, the protein devoid of C-terminal domain failed to assemble into complex I even in the presence of GRIM-19 whereas full length STAT3 was significantly present in complex I (Fig.2.11B, Right Panel, compare lane 4 and 3). This clearly indicates that the C-terminus of STAT3 is required for GRIM-19 dependent import and integration of STAT3 into complex I. Ser727 of STAT3 is located in the C-terminal region of the protein and is known to be essential for STAT3 functions in mitochondria [40, 43] and also a prerequisite for GRIM-19/STAT3 interaction [64]. We evaluated the role of Ser727 on mitochondrial import of STAT3 in the presence of GRIM-19. Interestingly, mutation of serine 727 to alanine reduced the import of STAT3 into mitochondria even in the presence of GRIM-19 (Fig.2.11C, compare lane 1 with 2-4). This study indicates a requirement of Ser727 residue in C-terminus of STAT3 for its GRIM-19 dependent import into mitochondria. To further define, whether STAT3 was phosphorylated prior to import, we performed an *in vitro* phosphorylation assay using purified recombinant STAT3 and reticulocyte lysate (used as a kinase source). STAT3 was found to be phosphorylated by the reticulocyte lysate *in vitro* (Fig.2.11D, lane 4).

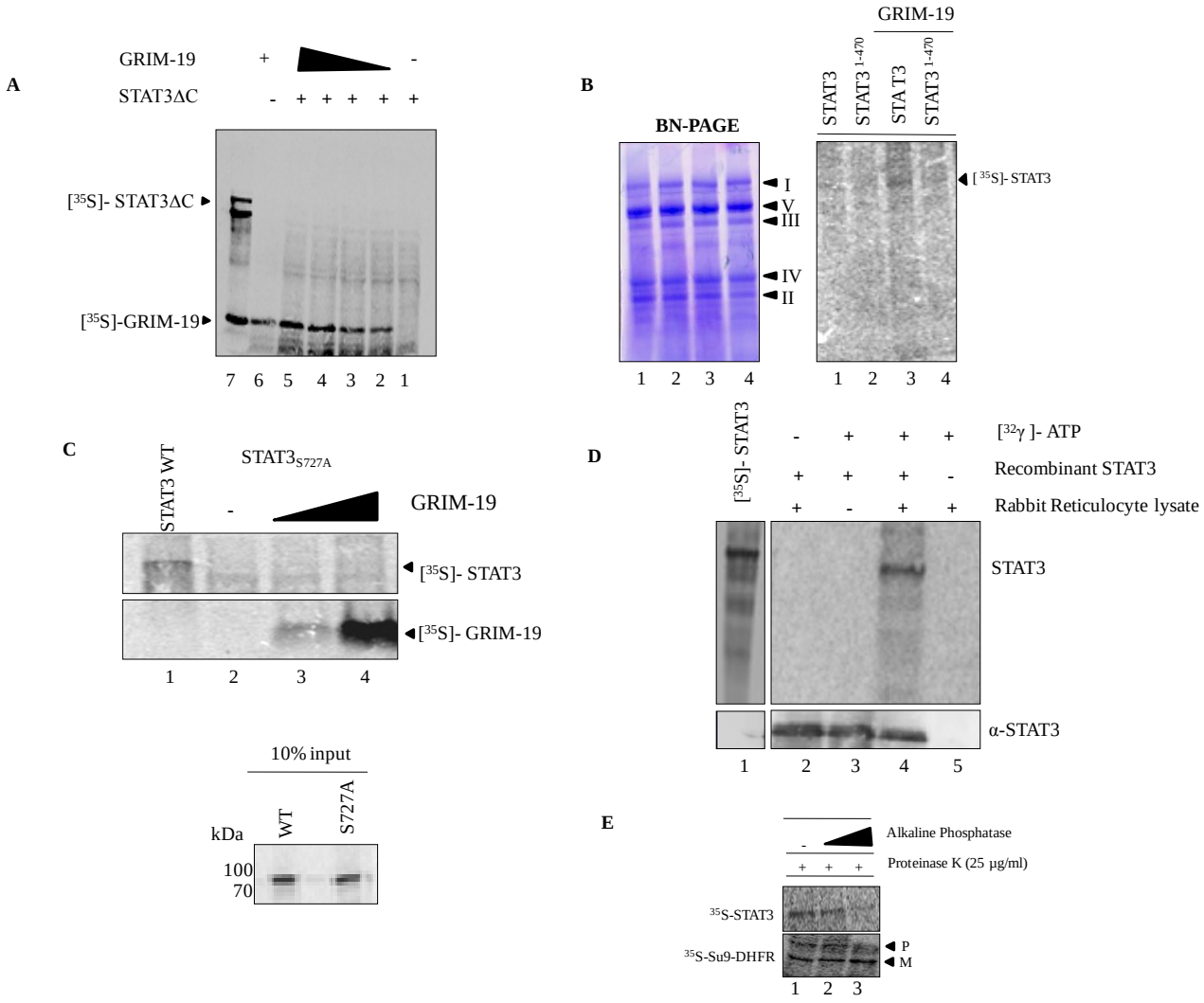


Fig.2.11 Requirement of Ser727 of STAT3 for GRIM-19 dependent import and integration: A) and B) *in vitro* import of $STAT3_{\Delta C}$ ($STAT3_{1-470}$) and $STAT3_{S727A}$ with increasing concentrations of GRIM-19 was performed. After proteinase K treatment, samples were resolved on SDS-PAGE and analyzed by phosphor imager (**A and C**). **B**) Import of full length STAT3 (lanes 1 & 3) and $STAT3_{(1-470)}$ (lanes 2 & 4) was carried out either in the absence (lanes 1 & 2) or presence (lanes 3 & 4) of unlabeled GRIM-19 for 60 minutes as indicated in the Methods. Samples were processed for proteinase K treatment and solubilized in 1% dodecyl maltoside buffer and resolved on BN-PAGE followed by analyzing on phosphor imaging system. **D)** To determine whether STAT3 is phosphorylated prior to import, *in vitro* phosphorylation was performed using rabbit reticulocyte lysate as kinase source. Recombinant purified His tagged STAT3 was incubated either in the presence (lane 2 & 4) or absence (lane 3) of rabbit reticulocyte lysate and in the presence of (lanes 3-5) or absence (lane 2) of [$^{32}\gamma$P]-ATP as indicated in the Methods. RRL (Rabbit Reticulocyte Lysate) incubated with [$^{32}\gamma$P]-ATP serves as a negative control (lane 5). Ni-NTA pulled down products were analyzed by phosphor

imager and also probed with STAT3 antibodies to show equal amount of protein. **E)** ^{35}S-STAT3 and Su9-DHFR were synthesized in reticulocyte lysate and subsequently treated without (lane 1) or with calf intestinal alkaline phosphatase (5 U and 10 U for lane 2 and 3 respectively) for 10 minutes at 37˚C. In vitro import was performed with AP treated samples were analyzed by phospho imager as described in the Methods.

In addition, alkaline phosphatase treatment of reticulocyte lysate containing labeled STAT3 prior to import reduces the import of STAT3 whereas no such inhibition was observed with Su9-DHFR (Fig.2.11E, compare lanes 1 with 2 & 3). These results likely indicate that phosphorylation at Ser727 of STAT3 may be required for its import into mitochondria.

2.4 DISCUSSION

Recent findings from us and others indicate that STAT3 is associated with mitochondria [40, 43, 45, 56, 57], where it modulates cellular respiration (5) and supports cellular transformation induced by the Ras oncogene [43]. The mechanism by which STAT3 is imported into mitochondria has not been addressed in detail. In this report, we demonstrate that a distinct mechanism is employed by STAT3 for its import and assembly into mitochondria and that GRIM-19 has a major influence on this process.

Using an *in vitro* import system we show that STAT3 efficiently traverses the mitochondrial membrane although it doesn't have any canonical mito-targeting sequence. We further demonstrate that the mitochondrial import of this protein requires membrane potential and may require energy (Fig.2.5). We also show that *in vitro* imported STAT3 is susceptible to proteases upon removal of the outer membrane by digitonin treatment (Fig.2.10, lane 3). However, the endogenous mitochondrial STAT3 is partially protected from externally added protease (Fig.2.1B and C). *In vitro* importedstat3, but not the endogenous protein, is accessible to protease treatment in mitoplasts, suggesting that unknown factor(s) may be required for stat3 transport in a protease-inaccessible mitochondrial compartment. This led us

to evaluate whether GRIM-19 has any role in import and assembly of STAT3 into mitochondria given that both the proteins have been shown to interact and are present in mitochondria (*24-27*). Interestingly, we find that low concentration of GRIM-19 facilitates transport of STAT3 into the mitochondria. However, further increase in GRIM-19 reduced internalization of STAT3 (Fig.2.8A). We speculate that the decrease in recruitment of STAT3 at high concentrations of GRIM-19 may be due to competition for the same receptor as both are devoid of cleavable mitochondrial targeting sequences and proteins having similar sequences utilize the same receptor. In support of this notion, co-import of STAT3 with a pre-sequence containing protein, Su9-DHFR, does not affect its mitochondrial recruitment, even at higher concentrations (Fig. 2.8F).

Further we also analyzed the role of GRIM-19 on sub compartmental localization of *in vitro* imported STAT3. In the absence of GRIM-19, most of the STAT3 is susceptible to protease upon opening of outer mitochondrial membrane. Surprisingly, upon its co-import with GRIM-19, it resists protease action even in mitoplasts, indicating its association with protease inaccessible portion of mitochondria (Fig.2.10, lane 5). We further demonstrate that this protease inaccessible portion of imported STAT3 is associated with inner mitochondrial membranes by separating IMM and matrix fractions. Absence of STAT3 in the matrix fraction and resistance towards the protease in mitoplasts suggest that STAT3 might be anchored to inner membrane facing towards the matrix and this organization seems to require GRIM-19. In support, we find that major portion of endogenous STAT3 also resides in inner mitochondrial membranes (Fig.2.1). Similar to GRIM-19, high salt treatment failed to dislodge STAT3 from mitochondrial inner membranes while high pH treatment releases most of the protein into the soluble fraction which implies strong association with mitochondrial membranes. Based on these *in vivo* and *in vitro* import studies, we propose that STAT3

tightly associates with the inner mitochondrial membrane and this association requires GRIM-19.

Studies indicate that 50% of mitochondrial proteins do not contain any canonical mitochondrial targeting sequences [80-82]. Generally, proteins having no typical mitochondrial targeting sequence utilize the chaperone system [83] to translocate into the mitochondria while some mitochondrial targeted proteins contain an internal targeting sequences [84]. It has been shown that phosphorylation of some proteins enhance their interaction with chaperones or exposes the cryptic mitochondrial targeting sequences to facilitate their mitochondrial recruitment [85, 86]. Despite the absence of mitochondrial targeting sequence, we found that STAT3 efficiently traverse the mitochondrial membranes and alkaline phosphatase treatment or S727A mutation diminishes the import of STAT3 into mitochondria. In addition, recently it has been shown that HSP22 is required for mitochondrial localization of STAT3. Based on these findings, we speculate that phosphorylation of STAT3 might be required for its interaction with chaperones and/or GRIM-19 to facilitate its mitochondrial localization.

In a parallel study, Shulga, N., and Pastorino [87] also showed that phosphorylation of STAT3 on Ser727 by RIPK1 enhances its interaction with GRIM-19 thereby its mitochondrial localization.

Chapter 3

Functional importance of mitochondrial STAT3

3.1 INTRODUCTION

Mitochondria are the energy generating organelles of the cell by virtue of coupling electrochemical gradient across inner membrane to ATP synthesis through the electron transport chain. Complex I (NADH-Ubiquinone oxidoreductase) is the largest protein complex of electron transport chain. It transfers electrons from NADH to ubiquinone and creates an electrochemical gradient across the inner mitochondrial membrane. Complex I is one of the major sources of ROS production in mitochondria [88, 89]. Genetic dysfunction of Complex I has been implicated in several diseases that includes Parkinson's [90, 91], cancer[92] and other neurological disorders [93-95]. In addition, ROS produced by Complex I is known to be involved in apoptosis[96] and aging[97]. A myriad of signaling networks converge on Complex I and hence not surprising that Complex I has been implicated in most of the mitochondrial disorders [98].

STAT3 is a latent transcription factor that translocates to the nucleus in response to various stimuli to regulate the expression of genes involved in various biological pathways. Several reports suggest the non-canonical functions for STAT3 in mitochondria and cytosol [40, 99, 100]. Recently, we and others have shown that STAT3 is required for regulation of mitochondrial function. Mitochondrial STAT3 is involved in diverse functions like cellular respiration [40], tumorigenesis [43], mitochondrial gene expression [46] and membrane permeability transition [45]. Phosphorylation of STAT3 at Ser727 is critical for its mitochondrial functions [40, 43, 101, 102]. It is now known that the phosphorylation of STAT3 at Ser727 is required for mitochondrial recruitment of STAT3especially on to Complex I of electron transport chain [101, 103]. In addition, targeted expression of STAT3 in mitochondria is thought to be cyto-protective during oxidative stress, possibly by

preventing the leakage of electrons from complex I [44]. However, the physiological relevance of the Complex I association of STAT3 remains poorly understood.

Rotenone is a Complex I inhibitor of electron transport chain (ETC) and is extensively used as a pesticide. Rotenone efficiently inhibits activity of Complex I by interfering with electron transfer results in generation of ROS [96]. Thus, rotenone is being extensively used to study mitochondrial dysfunction disorders. For instance, rotenone has been effectively used in generating parkinsonian models in mice as it promotes the degeneration of substantia nigra [98, 104] and the formation of Lewy bodies. Since rotenone also promotes apoptosis in dopamergic neurons, it has been used to understand the pathophysiology of Parkinson disease in cell culture model systems *in vitro*.

In this chapter, we evaluated the role of ROS generated by complex I inhibition to understand the physiological relevance of STAT3 association with Complex I. We find that ROS generated by complex I inhibitor, rotenone, re-distributes the STAT3 localization from cytosol to mitochondria. Further, STAT3 recruitment to mitochondria is mediated by phosphorylation of S727 and interaction with GRIM19. Mitochondrial localized STAT3 protects the Complex I function and cell death by absorbing leaked electrons. Together, our study provides a novel feedback control mechanism for cell survival by preventing the rotenone induced oxidative stress.

3.2 METHODOLOGY

3.2.1 Antibodies and Reagents

Antibodies specific to phospho Ser727 in STAT3, STAT3, GRIM-19, Cyclophilin D and Myc were purchased from Abcam whereas antibodies against Tubulin and GAPDH were

from Sigma Aldrich. All chemicals were obtained either from Sigma Aldrich or Amersco unless otherwise mentioned.

3.2.2 Plasmid constructs

Plasmids harboring STAT3 and GRIM-19 have been previously described [103]. STAT3 specific shRNA and pSIH-puro-STAT3 shRNA was purchased from Addgene [105]. For targeted expression of STAT3 in mitochondria, mitochondrial targeting sequence from COXVIIIa was fused to the N-terminus of STAT3 ORF in pCDNA-3.1 vector using the following primers; NB502_Sense: 5'ATAT*AAGCTT*ATGTCCGTCCTGACGCCGCTGC TGCTGCGGACAGGCTCGGCCCGGCGGCTCCCAGTGCCGCGCGCCAAGATCCATT CGTTGGGGAATTCATAT 3' and NB503_Antiense:5'ATAT*GAATTC*CCCAACGAATG GATCTTGGCGCGCGGCACTGGGAGCCGCCGGGCCGAGCCTGTCAAGCCCCGCAG CAGCAGCGGCGTCAGGACGGACATAAGCTTATAT3' with BamH I and EcoR I restriction sites. STAT3 C4S construct was a generous gift from Peter E Shaw, University of Nottingham, UK.

3.2.3 Cell culture and DNA transfection

Experiments were performed using HeLa and HEK293T cell lines that were obtained from the Cell repository at NCCS, Pune, India. Cell lines were cultured in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C in a 5% CO_2 incubator. At 60% confluence, cells were transfected with required expression vectors using Lipofectamine 2000 (Invitrogen) and following the manufacturer's instructions.

3.2.4 Isolation of Mitochondria from the cell lines

Cell lines grown as monolayers were suspended in mitochondria isolation buffer (20 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 1mM EDTA pH 8.0, 1 mM EGTA, 210 mM sucrose and 70 mM Mannitol). The cell suspension was subjected to homogenization using polytron 1600. The cells were homogenized using 2 sec pulses twice at 15 rpm followed by dounce homogenization. The homogenate was centrifuged at 1000 X g for 10 min at 4^oC and the resultant supernatant was again centrifuged at 10,000 X g for 15 min at 4^oC to obtain mitochondria. The mitochondrial pellet was washed twice and suspended in a buffer containing 250 mM sucrose, 5 mM magnesium acetate, 80 mM potassium acetate, 10 mM sodium succinate, 1 mM DTT and 20 mM HEPES-KOH pH 7.4

3.2.5 Immunoblotting

For western blot analysis, cells were lysed in RIPA buffer (50 mMTris-HCl, pH 7.2, 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS and 0.25 mM EDTA) with the protease inhibitor cocktail (Roche, Basel, Switzerland). After measuring the protein concentration of the lysates using Bradford reagent, lysates were resolved on SDS-PAGE, transferred to nitrocellulose membranes and probed with the required specific antibody. The blots were developed using Bio-Rad's Versa doc imaging system.

3.2.6 Confocal Microscopy

HeLa cells grown on cover slips were treated with rotenone for the indicated time points. Post treatment, cells were fixed in 4% paraformaldehyde and washed with PBS. After permeabilization with organic mixture containing acetone and methanol (1:3 respectively) for 15 min, cells were incubated with 3% bovine serum albumin in PBS followed by incubation

with primary antibody. After washing with PBS, cells were incubated with corresponding secondary antibody conjugated with Alexa flours having different excitation wavelengths. Subsequently, cells were mounted using Prolong gold antifade with DAPI and analyzed using a confocal microscope (Olympus).

3.2.7 Immunoprecipitation

Cells lysates prepared in NP-40 buffer (20 mMTris-HCl pH 8.0, 137 mM NaCl, 10% Glycerol and 1% Nonidet P-40) were pre-cleared and incubated with primary antibody overnight at 4^{o}C. Pre-washed protein A/G beads were added to facilitate binding of the antibody-protein complex to the beads. The antibody-protein complex beads were washed thrice with RIPA buffer and eluted using 2X SDS loading dye. The eluted proteins were separated by SDS-PAGE, blotted to nitrocellulose membrane and probed with the required antibodies.

3.2.8 Measurement of ROS

Post rotenone treatment, cells were incubated with 10µM H2DCF-DA (Sigma) for 30 min. Then cells were washed and re-suspended in PBS. Fluorescent stained cells were sorted using FACS caliber (BD biosciences) and analyzed using Cell Quest software.

3.2.9 Trypan Blue exclusion assay

Cells grown in DMEM were treated with rotenone for 24h. After, treatment, cells were detached using trypsin and incubated with 0.1% trypan blue. The number of trypan blue negative cells was counted using a microscopic cell counting chamber.

3.2.10 Complex I activity

The mitochondrial complex I activity was determined by measuring the oxidation of NADH to NAD^+ at 340 nm with 380 nm as the reference wavelength at 37^0C. 50 µg of mitochondria was incubated with 5 mM NADH in a buffer containing 25 mM potassium phosphate pH 7.4, 5 mM $MgCl_2$ and 0.25% BSA for 1 min at 37^0C. The activity assay was initiated by addition of 3 mM decylubiquinone and the decrease in absorbance at 340 nm was measured. Complex I activity was calculated by using the velocity of reaction (Δabsorbance/min) and the molar extinction coefficient of NADH (3.4 $mM^{-}1cm^{-}1$ at 340 nm with reference wavelength).

3.3 RESULTS

3.3.1 Over expression of GRIM-19 promotes ROS production

Complex I is one of the major source of cellular ROS generation. Any alteration in the stoichiometric ratio of subunit composition or inhibition of electron flow through the complex I results in production of ROS. Since GRIM-19 is a component of complex I, any alteration in GRIM-19 levels may affect the activity of complex I as well as intracellular ROS levels. To verify this hypothesis, we have over expressed GRIM-19 in HeLa cell lines and monitored the intracellular ROS levels. As shown in the Figure 3.1, over expression of GRIM-19 resulted in enhanced intracellular ROS levels. However, addition of a ROS scavenger, N-Acetylcysteine (NAC) reduced both GRIM-19 induced and basal ROS levels in these cell lines (Fig.3.1A).

Subsequently, we also measured complex I activity, to see whether increased ROS in GRIM-19 over expression corresponds to altered complex I activity. Interestingly, we found that ectopic expression of GRIM-19 promoted complex I activity when compared to mock

transfected cell lines (Fig.3.1B). Together, our data indicates that over expression of GRIM-19 leads to enhancement of complex I activity with a concomitant increase in intracellular ROS levels.

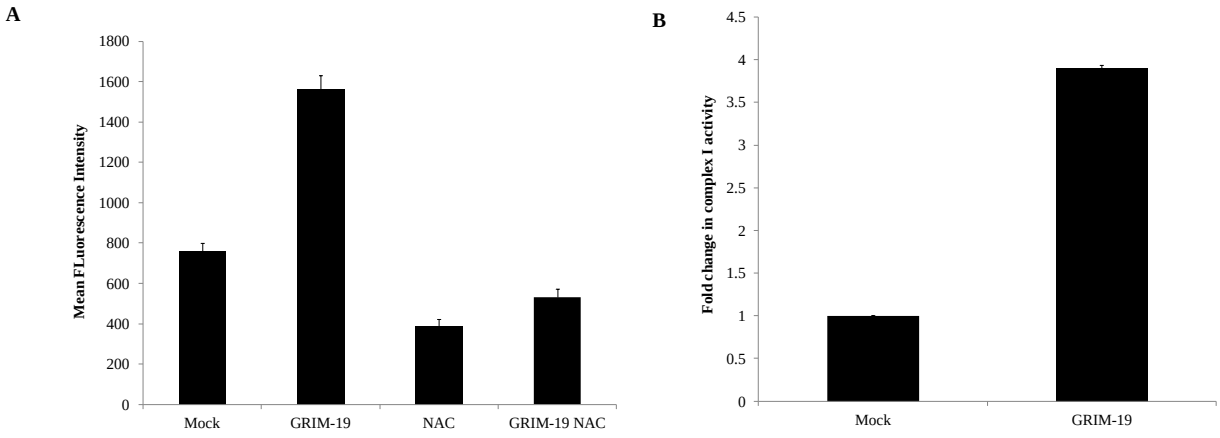


Fig.3.1 Over expression of GRIM-19 induces ROS production: **A)** HeLa cells were transfected with pcDNA3.1-GRIM-19/myc. 46h post transfection cells were incubated with or without NAC (50mMM) for 1 hour. Subsequently, cells were loaded with 10µM DCFDA for 30 minutes and then mean intensity of the dye measured by FACS analysis. **B)** Similarly, complex I activity was also measured in GRIM-19 over expression cell lines as described in the methods section.

3.3.2 Co-expression of STAT3 suppresses GRIM-19 induced ROS production

Although we have shown that GRIM-19 promotes integration of STAT3 into complex I of electron transport chain, the physiological relevance of this phenomenon remains to be poorly studied. Since over expression of GRIM-19 results in elevation of intracellular ROS levels, we sought to examine whether STAT3 has any role in GRIM-19 induced ROS generation. To test this hypothesis, we have co-expressed wild type STAT3 along with wild type GRIM-19 and monitored intracellular ROS levels. As expected, GRIM-19 transfected cell lines exhibits enhanced intracellular ROS levels relative to the mock transfected cell lines. However,

GRIM-19 induced ROS levels were significantly diminished upon co-expression of wild type STAT3.As we have shown that Ser727 phosphorylation of STAT3 is crucial for its mitochondrial targeting (chapter II) and functions, we also co-expressed GRIM-19 along with STAT3 Ser727 mutant and followed intracellular ROS levels. On the contrary to the above data, co-expression of STAT3 S727A mutant failed to diminish GRIM-19 induced ROS production rather we find that levels of ROS increased when compared to mock and GRIM-19 transfected cell lines (Fig.2.2A). We find that expression levels of both wild type and STAT3 Ser727 mutant remains similar (Fig.2.2B).

Further, to determine the role STAT3 in GRIM-19 induced ROS reduction, we also knock down STAT3 using gene specific shRNA (Fig.2.2D).

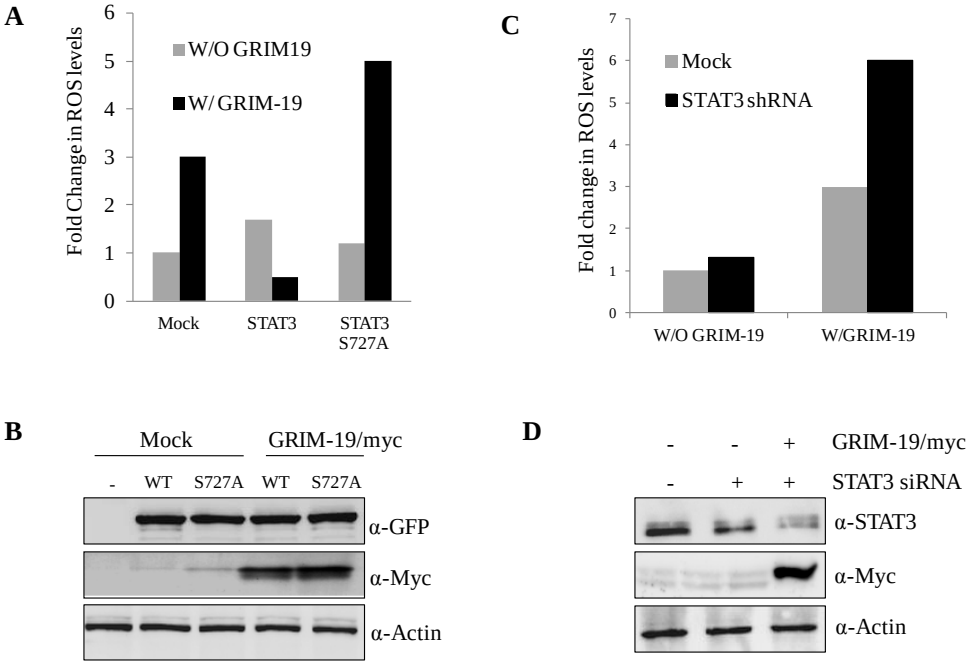


Fig 3.2 STAT3 reduces GRIM-19 induced ROS production: A) HeLa cells were transfected with or without GRIM-19 along with either STAT3 wild type or S727A mutant. 48h post-transfection cells were loaded with DCFDA and mean fluorescence intensity of the dye analyzed by FACS Caliber was normalized and plotted. **B)**

Lysates were prepared from the cell lines and immunoblotted using antibodies mentioned. **C)** Similarly, GRIM-19 was also over expressed in STAT3 knockdown HeLa cells. 48h post transfection intracellular ROS levels were monitored as mentioned and values were plotted. **D)** Cell lysates from the same cell lines were resolved on SDS-PAGE and immunoblotted using the antibodies mentioned.

When we over expressed GRIM-19 in STAT3 knock down cell lines (Fig.2.2D), contrary to the above data, over expression of GRIM-19 in STAT3 knock down cells resulted increased ROS levels when compared to either mock or GRIM-19 transfected cell lines (Fig.2.2C). Altogether, this data suggests thatSTAT3 reduces GRIM-19 induced ROS production in Ser727 dependent manner.

3.3.3 STAT3 suppresses Rotenone induced ROS production

Considering the fact that that STAT3 suppress GRIM-19 induced ROS production, we have asked whether STAT3 also responds in a similar fashion towards ROS induced by chemical inhibition of complex I. Towards this end, we have used rotenone, a chemical inhibitor of complex I, to induce mitochondrial ROS production. And to verify the hypothesis, HeLa cells were transiently transfected with empty vector (mock) or plasmids harboring wild type STAT3 or a phosphomimetic form of STAT3 (S727D Y705F) or a phosphomutant form of STAT3 (S727A, Y705F) and treated with 2.5 µM rotenone for 24 hrs to monitor the intracellular ROS levels as described in methods section. As a positive control, ROS levels were also measured in cells treated with rotenone either in the presence or absence of a ROS scavenger, N-Acetylcysteine (NAC). Consistent with our earlier results, rotenone treatment enhanced ROS levels and addition of a ROS scavenger, NAC, prior to rotenone treatment significantly reduced rotenone induced ROS production in HeLa cell lines. Likewise, cells over-expressing wild type STAT3 did not increase their intracellular ROS levels even in the presence of rotenone (Figure 3.3A). Most notably, over-expression of wild type STAT3 in

presence of rotenone brought down internal ROS to a level that is similar to NAC and rotenone treated cells and lower than its control cells that were not treated with rotenone. Further, over-expression of the phospho-mimetic form of STAT3 (S727D, Y705F) had an effect similar to that of wild type STAT3 and was able to effectively bring down the rotenone induced ROS levels (Figure 3.3A). In contrast to wild type and the phospho-mimetic mutant, over-expression of STAT3 phospho-mutant (S727A, Y705F) not only failed to diminish rotenone-induced ROS levels but had ROS levels that were much higher than that observed in case of rotenone treated mock cells (Figure 3.3A).

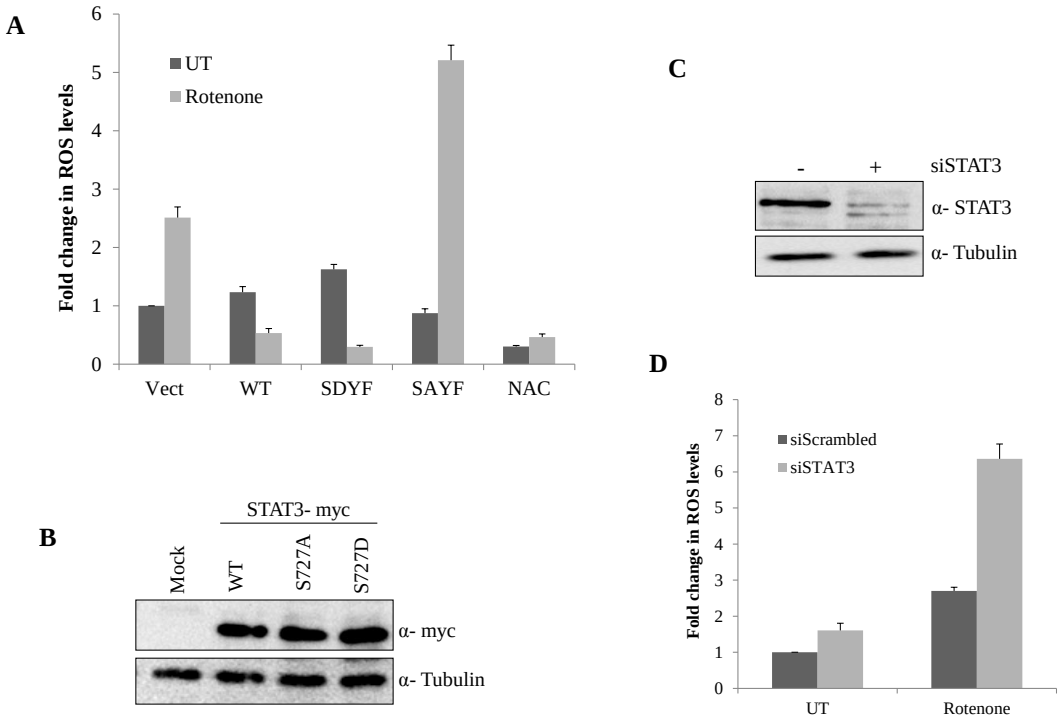


Fig.3.3 STAT3 suppress rotenone induced ROS production: A) HeLa cells were transiently transfected with pCDNA3.1/STAT3-myc or STAT3 S727A Y705F-myc or STAT3 Ser727D Y705F. After 24 hrs of transfection, cells were treated with rotenone for another 24h followed by incubation with DCFDA for 30' and the fluorescence intensity was measured using FACS. The results were further analyzed by Flowjo software and plotted along with standard deviation. **B**) Cell lysate from above samples were resolved on SDS-PAGE, immunoblotted and probed with anti-Myc and anti-tubulin. **C**) Cells were transfected with STAT3 specific

shRNA and 24h post transfection, cells were treated with rotenone for 24h. Then cell lysates separated on SDS-PAGE were immunoblotted and probed for STAT3 and tubulin. **D)** Intracellular ROS levels were measured in these cell lines by DCF-DA staining followed by FACS analysis.

To ensure the observed effects were not because of the differential expression, lysates of the cells transfected with plasmids harboring STAT3 or its mutant forms were probed with anti-myc and found that levels were comparable (Figure 3.3B). Together, this data clearly indicates that STAT3 also efficiently suppresses rotenone induced ROS production in a Ser727 dependent manner.

To further ascertain the role of STAT3 in regulating rotenone induced ROS production, we depleted internal STAT3 in HeLa cells by transfecting with STAT3 specific shRNA (Figure 3.3C). STAT3 levels were reduced by more than 50% in cells transfected with STAT3 shRNA compared to cells transfected with scrambled shRNA (Figure 3.3C). Consistent with the observations made with over-expressed wild type STAT3 in reducing ROS levels, we find that STAT3 knock down cells exhibit significant elevation in intracellular ROS levels (Figure 3.3D). Altogether, these results provide strong evidence that STAT3 controls rotenone induced ROS production from Complex I and this function is dependent on the phosphorylation status of Ser727 in STAT3.

3.3.4 Rotenone promotes STAT3 phosphorylation at Ser727

As STAT3 was found to reduce rotenone induced ROS levels, next we sought to investigate the mechanism underlie this phenomenon. Since STAT3 responds towards oxidative stress by undergoing post translational modifications [106-111], we examined the phosphorylation status of STAT3 at Ser727 and Tyr705 during rotenone treatment. As expected, rotenone augments intracellular ROS levels in a dose and time dependent manner (Figures 3.4A and 3.4B). To monitor the phosphorylation status of STAT3, HeLa cells treated with various

concentrations of rotenone and to various time points were collected and lysed. The lysates were analyzed by western blotting. The blots were probed with anti-pSer727 in STAT3, anti-pTyr705 in STAT3 and anti-STAT3 antibodies. Phosphorylation of Ser727 in STAT3 increased with rotenone treatment in a dose and time dependent manner (Figures 3.4C and 3.4D). However, loss of correlation at higher concentrations (25 µM and above) or later time

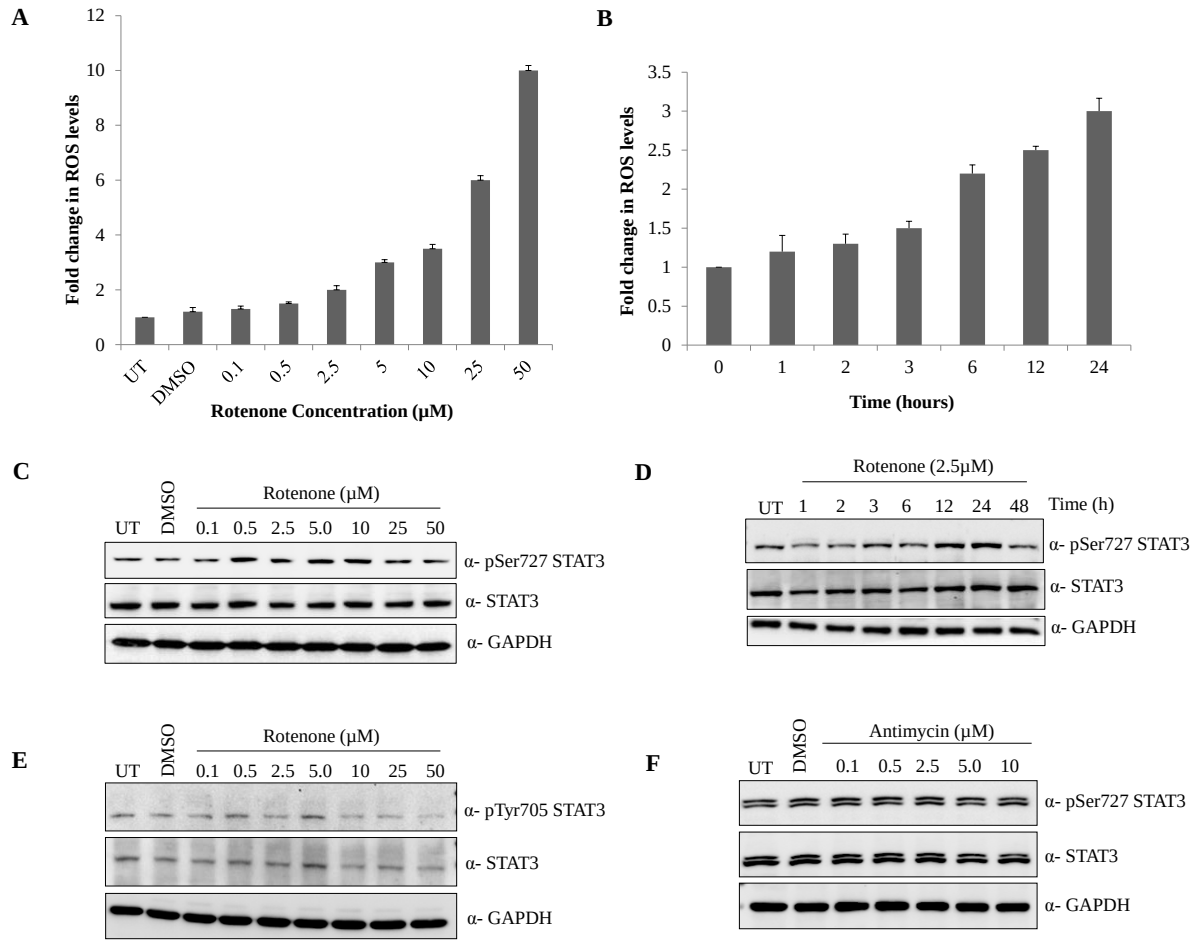


Fig.3.4 Effect of rotenone on STAT3 phosphorylation: A) HeLa cells were treated with different concentrations of rotenone for 24h. Then cells were stained with H_2DCFDA 30' prior to the analysis. The fluorescence intensity of DCFDA was measured using FACS canto (BD Biosciences) and results were analysed by FlowJo software. **B)** HeLa cells were treated with a 2.5 µM concentration of rotenone for the different time points indicated. After treatment, cells were stained with DCF-DA and fluorescence intensity was measured by FACS analysis. HeLa cells were treated with rotenone either by increased concentrations **C)** or different time

points **D)** and cell lysates were prepared and immunoblotted using either anti-pSer727 oranti-pTyr705 **E)**. Cells were also treated with Antimycin A and cell lysates were immunoblotted with antibodies mentioned **F)**.

points (48 hrs after 2.5 µM rotenone treatment) might be due to lethal actions of rotenone. Nevertheless, steady state levels of STAT3 remain similar in both the cases. On contrary, there was no discernible change in the phosphorylation status of Tyr705 in STAT3 (Figure 3.4E). To ascertain the specificity of the rotenone effect on STAT3 phosphorylation, we repeated the above experiment using Antimycin A, a complex III inhibitor, instead of rotenone. Antimycin A, however, did not have any effect on the phosphorylation status of Ser727 in STAT3 (Fig.3.4F). Together, these results demonstrates that STAT3 undergoes phosphorylation at Ser727 in response to chemical inhibition of Complex I.

3.3.5 Rotenone redistribute STAT3 into mitochondria

Phosphorylation of STAT3 at Ser727 is required for its mitochondrial functions. We and others have shown that pSer727 is also responsible for its recruitment to mitochondria [101, 103]. As rotenone treatment causes an increase in Ser727 phosphorylation, we hypothesized that the increased pSer727 may affect STAT3 abundance in mitochondria. To test this hypothesis, we initially treated HeLa cells with increasing amount of rotenone or antimycin A or left untreated. Subsequent to the treatment, cells were lysed, sub-fractionated into mitochondria and cytosol. These fractions were resolved on SDS-PAGE and subjected to immunoblotting with STAT3 antibody. As a loading control for mitochondrial and cytoplasmic fractions, the blots were, further, probed with cyclophilin D and tubulin antibodies respectively. As shown in the Fig. 3.5, we observed a steady increase in the levels of STAT3 in the mitochondrial fraction with an increasing amount of rotenone (Fig.3.5A).

However, we did not observe any discernable changes in mitochondrial STAT3 with increasing amount of Antimycin A (Figure 3.5B).

To more directly investigate if indeed STAT3 is re-localized to mitochondria in the presence of rotenone, we performed immunolocalization studies using antibodies specific to pSer727-STAT3 (pSTAT3) and Mia40, a mitochondrial marker. HeLa cells were treated with increasing concentrations of rotenone (0, 0.5 and 2.5 µM) and immunostained with pSer727 STAT3 antibodies. All the cells stained with DAPI and Mia40 antibodies to indicate the location of nucleus and mitochondria (Figure 3.5C). Cells incubated with rotenone strongly reacted with pSer727 STAT3 antibody (Figure 3.5C). Most significantly, rotenone treatment enhanced the co-localization of pSTAT3 with the mitochondrial marker, Mia40, indicating an influx of STAT3 pSer727 into mitochondria (Figure 3.5C). Further, the efficiency of co-localization of

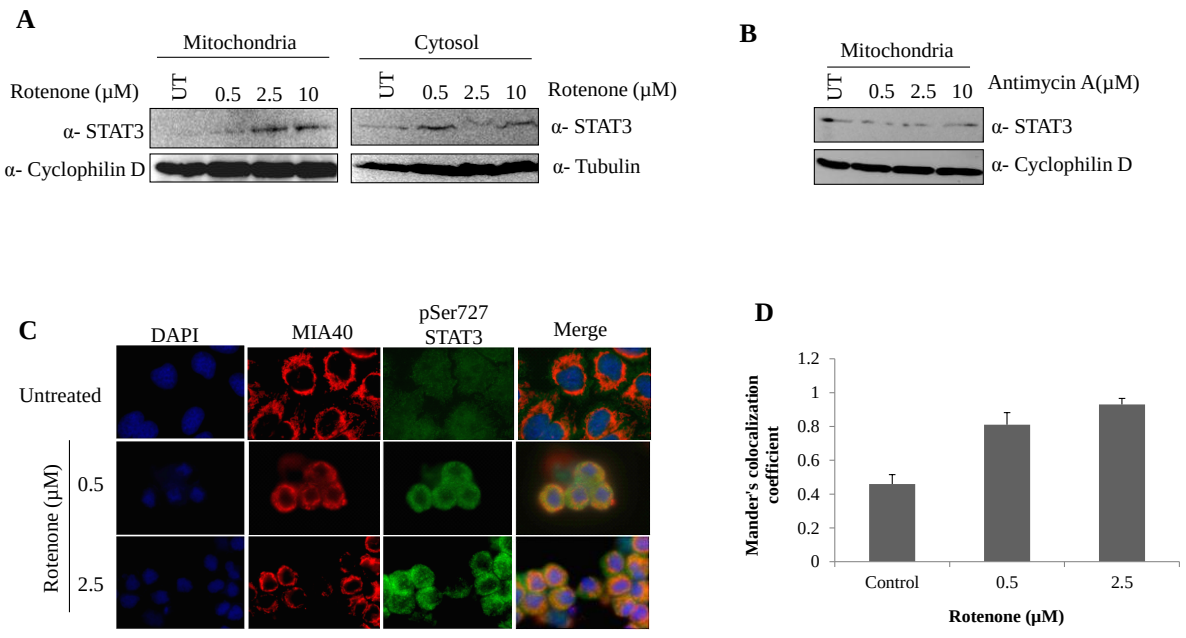


Fig.3.5 STAT3 recruitment to mitochondria during rotenone treatment: A) Cells were treated with indicated concentrations of rotenone for 24h followed by sub-cellular fractionation of cell lysates into

mitochondria and cytosol fractions. The fractions were resolved on SDS-PAGE and immunoblotted using antibodies mentioned. **B)** Mitochondria were isolated from the cells treated with antimycin A, and samples were resolved on SDS-PAGE followed by immunoblotting with antibodies mentioned. **C)** HeLa cells were treated with rotenone for 24h and immunofluorescence was carried out as described in the Methods using confocal microscopy (Carl Zeiss, USA). **D)** Co-localization analysis (n = 10) was performed using Image J software and values of Mander's coefficient were plotted.

pSer727 STAT3 with mitochondria was quantified by calculating mander's co-localization coefficient using Image J software (Figure 3.5D). However, we did not detect any significant change in pSTAT staining in un-treated cells and it poorly colocalized with mitochondrial marker, Mia40 (Figure 3.5C& D).

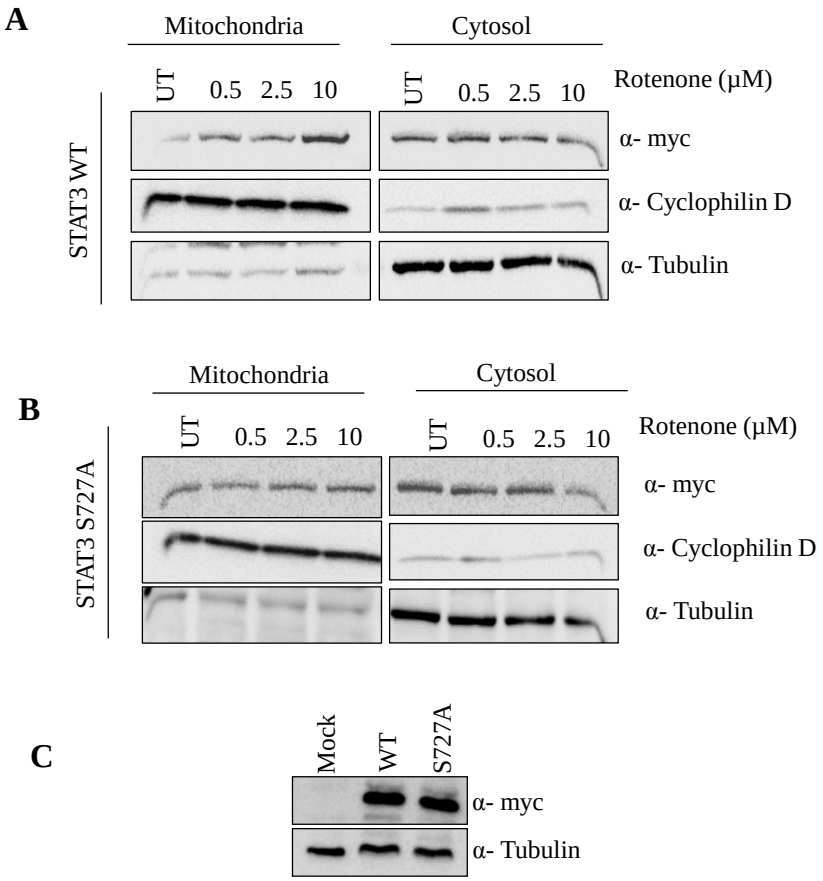


Fig 3.6 Rotenone induced mitochondrial recruitment of STAT3 requires Ser727: A) HeLa cells were transfected with either Myc-STAT3 WT or Myc-S727A Y705F mutant **B)** and treated with rotenone for 24h. After treatment, mitochondria and cytosol were isolated, resolved on SDS-PAGE and western blots were probed

with antibodies mentioned. **C)** Cell lysates prepared from cells over expressing Myc-STAT3 WT or Myc-S727A mutant were resolved on SDS-PAGE followed by immunoblotting with specific antibodies.

To further confirm the increased recruitment of STAT3 pSer727 into mitochondria in presence of rotenone, we ectopically expressed STAT3-myc wild type or STAT3 S727A-myc in HEK293T cells by transient transfection. The transiently transfected cells were treated with rotenone prior to fractionating the cells into mitochondrial and cytosolic fractions as described in the Methods section and probed with Myc antibodies. Cells ectopically expressing Myc-STAT3 wild type displayed increased abundance of STAT3 in mitochondria upon rotenone treatment (Figure 3.6A). In contrast, we did not observe any increase in the amount of STAT3 in mitochondria fractionated from cells ectopically expressing STAT3 S727A with rotenone treatment when compared to untreated or wild type (compare Figure 3.6A & B). However, expression levels of STAT3 wild type and mutant were found to be similar (Figure 3.6C). Taken together, these results strongly suggest that rotenone treatment increases the influx of STAT3 into mitochondria in a manner that is dependent on the phosphorylation status of Ser727 in STAT3.

3.3.6 Rotenone promotes interaction of STAT3 and GRIM-19

GRIM-19, a complex I subunit, is a negative transcriptional regulator of STAT3 [59, 63, 64]. Interaction of GRIM-19 with STAT3 requires phosphorylation of Ser727 in STAT3 and co-incidentally, we and others have recently shown that this interaction is required for the translocation of STAT3 to mitochondria. As our results clearly demonstrated that rotenone promotes mitochondrial recruitment of STAT3 in a Ser727 dependent manner, we were intrigued to investigate the role of GRIM-19 in the translocation of STAT3 to mitochondria during rotenone treatment. To address this query, we carried out immunoprecipitation studies

using cell lysates prepared from the cells treated with increasing concentrations of rotenone for 24 hrs. After treatment, cell lysates were immunoprecipitated with STAT3 antibody as described in the Methods section and the western blots were probed with STAT3 and GRIM-19 antibodies. The amount of GRIM-19 immunoprecipitated increased with increase in rotenone concentration suggesting that rotenone promotes the interaction of STAT3 with GRIM19 (Fig.3.7A).

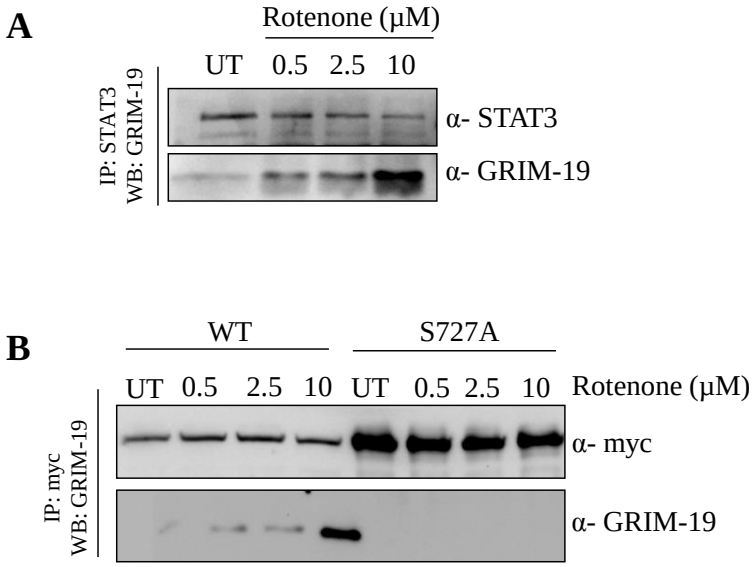


Fig.3.7 Role of GRIM-19 in rotenone induced STAT3 recruitment to mitochondria: A) Cell lysates were prepared from rotenone subjected cell lines for 24h. The lysates were immunoprecipitated with anti-STAT3 followed by immunoblotting with anti-STAT3 and anti-GRIM-19. **B)** Cells transfected with STAT3 WT and S727A mutant were treated with rotenone for 24h and then cell lysates were prepared. These lysates were immunoprecipitated with anti-myc and immunoblotted with anti-GRIM-19 and anti-myc.

To identify the importance of Ser727 phosphorylation in rotenone induced STAT3-GRIM-19 interaction, we repeated the experiment using HEK293T cells that were transiently transfected with plasmids harboring Myc-STAT3 wild type or Myc-STAT3 S727A mutant. The transiently transfected cells were subjected to increasing concentrations of rotenone for

24 hrs. Immunoprecipitation of cell lysates were carried out using Myc antibody and the western blots were probed with Myc and GRIM-19 antibodies. Consistent with the previous results, rotenone increases the interaction of ectopically expressed wild type STAT3 with GRIM-19 (Figure 3.7B) in cells transiently transfected with wild type STAT3. Most importantly, GRIM-19 failed to interact with STAT3 S727A mutant even at high concentrations of rotenone underscoring the functional importance of STAT3 Ser727 phosphorylation in the translocation of STAT3 to mitochondria (Figure 3.7B). Together, this data indicates that rotenone induced Ser727phosphorylation of STAT3 promotes its interaction with GRIM-19 to enhance its mitochondrial localization.

3.3.7 ROS is the key mediator for STAT3 activation and mitochondrial targeting

Rotenone increases ROS production in mitochondria and also promotes the translocation of cytoplasmic STAT3 to mitochondria. STAT3 in turn reduces the mitochondrial ROS production and protects the cell from the deleterious effects of high ROS levels. We were intrigued to identity the signaling intermediate that is coordinating rotenone and STAT3. Incidentally, earlier studies have shown that ROS can act as a signaling intermediate [112]. Hence, we further investigated if ROS is responsible for the downstream signaling of rotenone induced STAT3 activation and its subsequent recruitment to mitochondria. We incubated HeLa cells with rotenone in the presence and absence of NAC. Cells were harvested, lysed and subjected to western blotting. Blots were probed with the phospho-specific STAT3 pSer727 antibody besides STAT3 and GAPDH antibodies. As expected, rotenone induced the phosphorylation of STAT3 at Ser727 site in a concentration dependent manner. However, co-treatment of HeLa cells with rotenone and NAC efficiently suppressed the rotenone induced STAT3 phosphorylation (Figure 3.8A). This finding suggests that ROS

relays the rotenone effect to trigger STAT3 phosphorylation and lends support to the view of ROS as a signaling intermediate.

To determine if the translocation of STAT3 to mitochondria can also be attributed to ROS dependent, we monitored STAT3 level in mitochondria isolated from HeLa cells that were treated with rotenone in the presence or absence of NAC. Western blotting of the mitochondrial samples followed by immunoblotting with STAT3and cyclophilin D antibodies revealed that in the presence of NAC, the rotenone treatment failed to enhance mitochondrial abundance of STAT3 (Figure 3.8B Right panel).

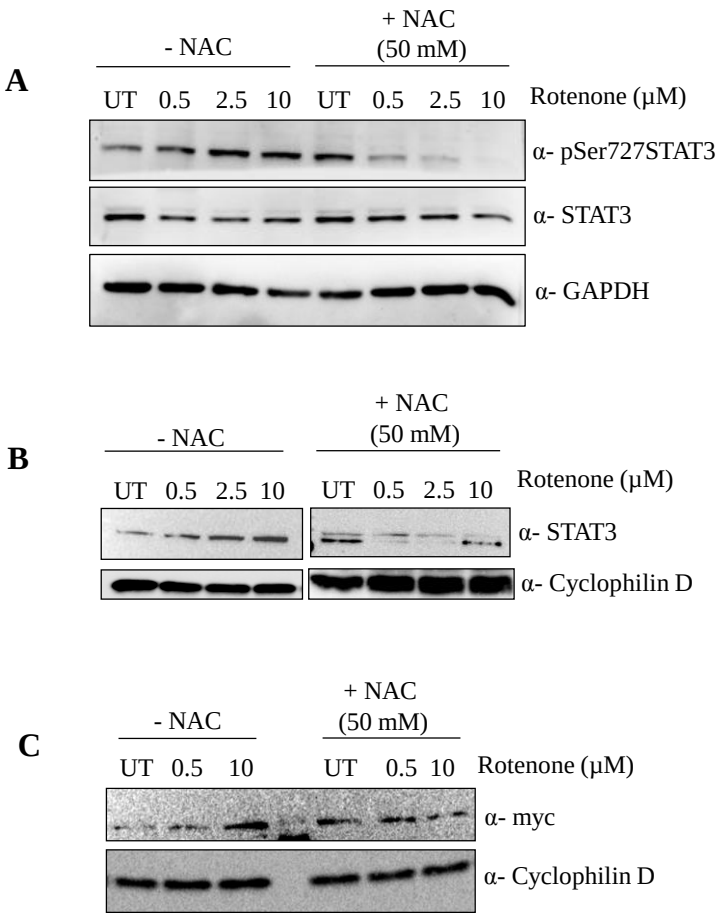


Fig.3.8 Role of ROS in the mitochondrial recruitment of STAT3 during rotenone challenge: A) Cells were co-treated with NAC (50 mM) and increasing concentrations of rotenone for 24h. Then cell lysates were prepared and resolved on SDS-PAGE followed by immunoblotting with antibodies specific to pSer727-STAT3, Total STAT3 and GAPDH. **B)** Mitochondrial fractions isolated from the cells treated with either rotenone alone

or along with NAC were separated on SDS-PAGE and immunoblotted with antibodies specific to STAT3 and Cyclophilin D. **C)** HeLa cells were transfected with pcDNA3.1 STAT3/myc and co-treated with or without rotenone/NAC. Isolated mitochondrial fractions from these cell lines were separated on SDS-PAGE and immunoblotted with antibodies as mentioned.

Further, co-treatment of HeLa cells with NAC and rotenone reduced the mitochondrial abundance of ectopically expressed STAT3 upon rotenone treatment (Figure 5C). In conclusion, the above data suggest that ROS acts as signaling intermediate in rotenone induced activation and recruitment of STAT3 to mitochondria.

3.3.8 Mitochondrial STAT3 exerts cytoprotective effects during rotenone induced oxidative stress

Some of the cytoplasmic synthesized proteins are targeted to mitochondria immediately upon their synthesis as they harbor a mitochondrial targeting sequence. However, there are few proteins that lack a mitochondrial targeting sequence but manage to enter the mitochondria during specific cell signaling processes. A typical example is STAT3 that enters mitochondria with help of GRIM19 when the ROS levels increase as shown above. To further unravel the precise role that mitochondrial STAT3 is playing during rotenone induced oxidative stress, we over-expressed STAT3 fused to a mitochondrial targeting sequence of subunit 9 of COXIV (MLS-STAT3) in HeLa cells [113]. It has been previously shown that over-expressed MLS-STAT3 is targeted to mitochondria efficiently and has no effect on olfactomedin M induced chromosomal STAT3 dependent gene expression [102]. HeLa cells over-expressing MLS-STAT3 were treated with rotenone and intracellular ROS levels were monitored. Targeted over-expression of STAT3 in mitochondria reduced rotenone induced ROS generation (Figure 3.9A). To evaluate the importance of phosphorylation, ROS levels were also monitored in cells expressing MLS-STAT3 S727A Y705F and STAT3 S727D

Y705F. Consistent with previous results, expression of MLS-STAT3 S727D mutant was able to reduce ROS production in presence of rotenone. In case of STAT3 phosphomutant, the ROS levels were higher than even the mock treated sample in presence of rotenone. Most intriguingly, the phosphomimetic STAT3 mutant displayed very high levels of ROS in absence of rotenone while the ROS levels

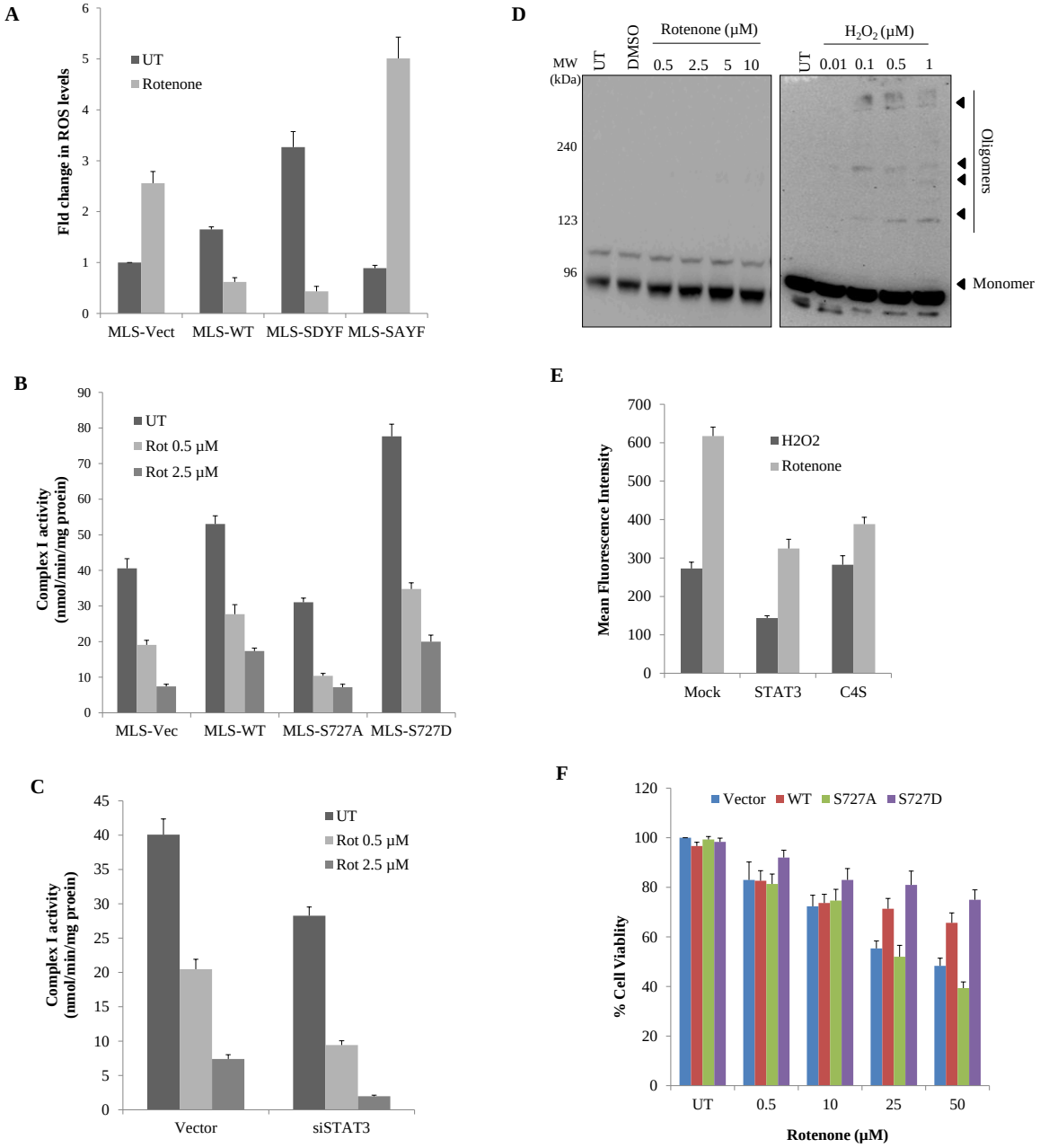


Fig. 3.9 Targeted expression of STAT3 ameliorates rotenone induced cellular events: A) Cells were transfected with MLS-STAT3 WT, MLS-STAT3 S727A Y705F and MLS-STAT3 S727D Y705F. 24h post transfection, cells were treated with rotenone and stained with DCFDA to measure the intracellular ROS or **B)** Mitochondria were isolated from the above samples to measure the complex I activity using decylubiquinone as terminal electron acceptor as mentioned in the methods section. **C)** Mitochondria also isolated from STAT3 knockdown cells treated with or without rotenone and assayed for complex I activity. **D)** Cells were treated with either H_2O_2 (Right panel) or rotenone (Left panel) and then cell lysates were prepared and separated in non-reduced conditions and immunoblotted with anti-STAT3. **E)** HeLa cells transfected with STAT3 wild type or STAT3 C4S mutant were treated with rotenone and intracellular ROS levels were monitored using DCFDA staining. **F)** To measure the cell survival, transfected HeLa cells were treated with rotenone for 24h and then viable cells were counted using trypan blue exclusion using haemocytometer.

decreased dramatically in presence of rotenone. An increase in intracellular ROS levels can reflect increased or decreased complex I activity. Hence, we measured Complex I activity in HeLa cells where there was over-expression of mitochondrial targeted STAT3 wild type or any of its mutants described. As expected, rotenone treatment significantly inhibited the complex I activity compared to control cells. Interestingly, over expression of MLS-STAT3 counteracted the complex I inhibition by rotenone (Fig.3.9B). Further, rotenone mediated Complex I inhibition was not rescued by over expression of STAT3 S727A, Y705F mutant. In contrast, over expression of phosphomimetic mutant, STAT3 S727D Y705F, restored the activity Complex I activity, even in the presence of rotenone (Fig.3.9B). Surprisingly, we found elevated complex I activity in cell lines expressing MLS-STAT S727D Y705F. Additionally, knockdown of STAT3 resulted in a 30 % decrease in complex I activity, which was further reduced to 60% by rotenone treatment (Fig. 3.9C). Together, these results indicate that STAT3 counteracts the rotenone inhibition by preserving the complex I activity thereby reduces ROS generation.

As STAT3 responds towards oxidative stress by forming cysteines dependent oligomers [111], we also investigated the role of cysteines in rotenone induced ROS reduction. As

shown previously [111], H_2O_2 treatment promoted the formation of higher order structures of STAT3 under non reducing conditions (Fig.3.9D, right panel). However, rotenone failed to promote the formation of STAT3 oligomers (Fig.3.9D, left panel). In addition to this, over expression of the STAT3 C4S mutant, a cysteine triple mutant, failed to reduce H_2O_2 induced ROS production, but abrogate the rotenone induced ROS production like that of wild type STAT3 (Fig.3.9E). Altogether, this data indicates that STAT3 mediated protection might be achieved by phosphorylation and preserving the activity of Complex I rather than redox modification.

To gain the physiological relevance of STAT3 function against rotenone treatment, we also examined the cell survival during rotenone treatment. As shown in the Figure 3.9F, rotenone treatment resulted in increased cell death at higher concentrations. Targeted expression of STAT3 in mitochondria protects cells from rotenone induced cell death (Figure 3.9F). However, over expression of MLS-STAT3 Ser727A mutant failed to protect the cells from rotenone induced cell death. In contrast, over expression of MLS-STAT3 Ser727D significantly reduced rotenone induced cell death (Figure 3.9F). Altogether, this data indicates that mitochondrial STAT3 improves rotenone induced cellular effects by counteracting the complex I inhibition thereby mitochondrial ROS generation.

3.4 DISCUSSION

STAT3 is an acute phase transcription factor, translocates to nucleus in response to cytokine stimuli to regulate gene expression. In addition to its well-known functions in the nucleus, STAT3 is also required for the regulation of a myriad of mitochondrial functions. Most of its mitochondrial functions require phosphorylation of STAT3 at Ser727 [40, 43, 44, 114]. Among the mitochondrial functions, STAT3 involvement in ETC regulation gained much

attention because of its implications on oxidative metabolism [106, 114, 115]. Earlier reports suggest the involvement of STAT3 in controlling cellular respiration by regulating Complex I and II activities (15). Subsequently, it was shown that targeted expression of STAT3 in mitochondria conferred cyto-protection to the cell against ischemia and reperfusion presumably by preventing the leakage of electrons from Complex I [44]. However, a recent study demonstrated that at basal conditions, the amount of STAT3 present in mitochondria is not sufficient to interact with complex I and II in stoichiometric ratio [116]. Nevertheless, cells deleted for STAT3 exhibit decreased Complex I activity concomitant with an increase in intracellular ROS [40, 117]. In addition, mitochondrial localized STAT3 has been speculated to reduce ROS levels through coupling of Complex I in ischemic models [44]. Hence, we studied the function of STAT3 in the background of ETC complex inhibition so that we can learn the relationship between ROS production, Complex I and III activities and STAT3. Most interestingly, we find that chemical inhibition of Complex I triggers phosphorylation of STAT3 specifically at Ser727. In addition to the varied effects of ROS, we find that ROS produced as a result of Complex I inhibition has a feedback inhibition on Complex I induced ROS through STAT3. ROS conveys the signal of Complex I inhibition by inducing the phosphorylation of cytoplasmic STAT3 at Ser727 results in its enhanced interaction with GRIM19 and subsequent transport to the mitochondria. The cells may use this feedback loop to maintain optimum activity of Complex I for their cell survival during stress conditions. On the other hand, absence of STAT3 activation when complex III is inhibited implies the existence of discrete response strategies for different stress conditions.

Ser727 phosphorylation in STAT3 has previously been shown to be an integral component of STAT3 recruitment to mitochondria and its functions [114]. In addition, it has been observed

that activation of upstream kinases that phosphorylate STAT3 on Ser727 also increase mitochondrial STAT3 levels[101]. Similarly, we find that treatment of HeLa cells with rotenone promotes recruitment of STAT3 to mitochondria. However, usage of a ROS scavenger or expression of STAT3 phosphomutant abrogated rotenone induced mitochondrial accumulation of STAT3. Together, this data demonstrates that the process of STAT3 entry to mitochondria appears to be regulated by ROS and dependent on phosphorylation of Ser727 in STAT3. Curiously, we find that HeLa cells over-expressing STAT3 phosphomutant harbors basal level of the phosphomutant in the mitochondria. As the mechanistic details of STAT3 transport to mitochondria are not clearly elucidated, it is possible that an alternate mechanism might be operating that is independent of STAT3 Ser727 phosphorylation. It is tempting to speculate that such a mechanism is also independent of ROS and is a part of the housekeeping processes to keep STAT3 dependent pathways to operate in mitochondria. However, the cell employs the rapid phosphorylation signaling mechanism to bring in STAT3 to mitochondria during any kind of duress that affects Complex I activity.

The role of STAT3 in oxidative metabolism was thought to be questionable as there were contradictory reports on its effect on ROS levels. Certain reports suggested that STAT3 negatively regulates intracellular ROS levels (22, 39-40) while it has also been shown that STAT3 stimulates the generation of ROS to promote necroptic cell death (19). To add to this contradiction, it has also been thought that higher Complex I activity or low Complex I activity trigger ROS production. Our site directed mutational studies have resolved this conundrum and prove that indeed Complex I activity that is higher or lower than optimum activity triggers ROS production. Over-expression of STAT3 phosphomimetic mutant

increases Complex I activity concomitantly increasing ROS production. However, in the presence of Complex I inhibition mediated by rotenone, the over-expressed STAT3 wild type and the phosphomimetic forms try to protect Complex I activity and thereby decrease ROS production. We believe that STAT3 maintains Complex I activity and the cell follows a feedback inhibition model to regulate Complex I activity.

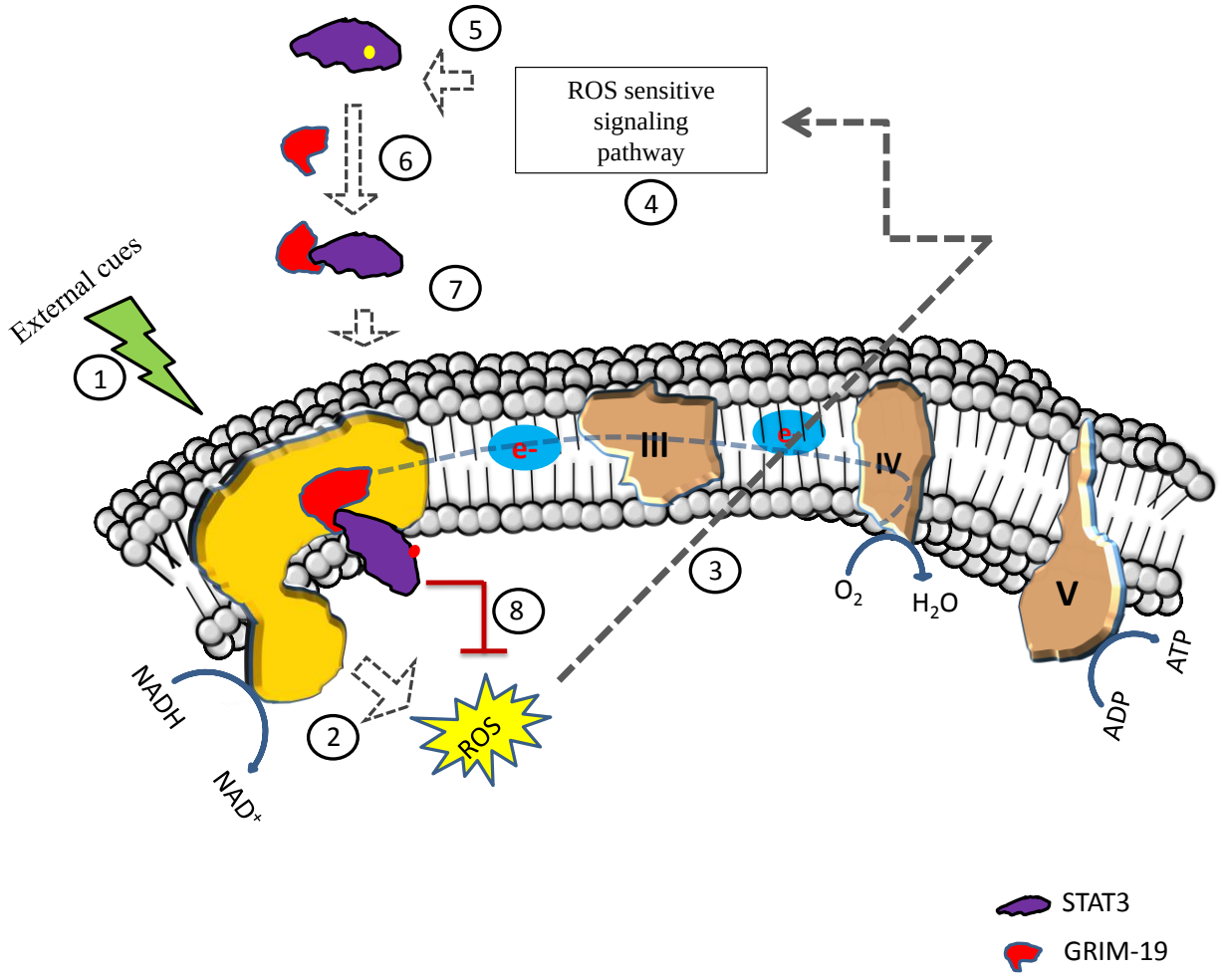


Fig.3.10 Feedback mechanism of mitochondrial STAT3 to maintain redox homeostasis: 1) Extracellular stress inducers, like rotenone, which **2)** promote mitochondrial ROS may activate **3 & 4)** retrograde ROS-sensitive signaling pathways in the cytosol. **5)** These pathways may further activate STAT3 and **6)** promotes its

interaction with GRIM-19 to **7)** facilitate its mitochondrial recruitment. Together, this pathway may represent a feedback loop to **8)** prevent the leakage of excessive ROS production from complex I.

It is essential for the cell to maintain certain levels of ROS production through Complex I for various signaling processes. In addition, viability of these cells in absence of rotenone is comparable to that of control cells or cells expressing wild type STAT3. We speculate that the kind of ROS being produced because of higher Complex I activity is different from the ROS that is being produced when Complex I activity is inhibited. Further studies are needed to unravel other players in the feedback inhibition loop that involves Complex I, ROS and STAT3. The current study indicates that Complex I inhibition triggers a possible ROS driven retrograde signaling pathway that employs STAT3 to maintain redox homeostasis and promote cell survival.

Chapter 4

General Discussion and Future Perspectives

4.0 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Mitochondria are essential organelles involved in many cellular processes like energy transduction, apoptosis, metabolism of lipids and amino acids. According to the endosymbiotic theory, mitochondria were thought to be derived from its proteobacterial ancestor. The transition from the primitive endo-symbiont to modern mitochondria has been accompanied by the cooperation and conflicts between the host and endosymbiont to achieve the harmony seen in modern eukaryotic cell. Among them, one of the features is shrinkage of mitochondrial genome as it transferred most of its genes to the nucleus. The other prominent feature is the changes in proteome over a time. These changes include complete loss, modification or complete gain of protein(s) like protein import machinery etc.,. These changes are so extensive that very less percentage of modern mitochondrial proteins can be drawn backwards to its antecedent. The rest of the evolved to proteins are divergently function in the organelle. One such group of proteins are nuclear transcription factors that are recruited to mitochondria and regulate function of this organelle thereby various cellular processes.

Number of transcription factors associated with mitochondria is increasing rapidly. Recently, our lab has identified a pool of STAT3 in mitochondria and its role in cellular respiration. Mitochondrial STAT3 was demonstrated to be involved in various cellular processes. However, mechanistic details of STAT3 recruitment to mitochondria remains poorly addressed at that time. Using very simple *in vitro* protein import assay, we have shown, for the first time that mitochondrial STAT3 resides in complex I of ETC. It requires the assistance of a complex I subunit called, GRIM-19 for its optimal import and integration into

complex I. Though GRIM-19 was proven to be a transcriptional negative regulator of STAT3, it is surprising to see that it positively regulates mitochondrial recruitment. Further, we also found that phosphorylation of STAT3 at Ser727 is required for its efficient translocation into mitochondria. However, future studies will uncover whether phosphorylation of STAT3 is required for its interaction with chaperones and/or GRIM-19 to facilitate its mitochondrial localization.

Subsequently, while understanding the physiological significance of STAT3 recruitment to mitochondria, we found that ROS generated by inhibition of complex I signals the recruitment of STAT3 to mitochondria. This process seems to require phosphorylation of STAT3 at Ser727, which facilities the interaction of STAT3 with GRIM-19 thereby its mitochondrial localization. However, ROS generated by complex III, neither activated nor induced mitochondrial recruitment of STAT3, indicating the specificity of mitochondrial STAT3 signaling towards complex I dysfunction. In line with this data, we found that mitochondrial STAT3 alleviates molecular changes associated with complex I inhibition by rotenone. Further, this process appears to be a feedback mechanism to prevent excessive ROS production from complex I. Contrary to our finding, Shulga N et al., demonstrated the pro-oxidant role of mitochondrial STAT3 during TNF-alpha treatment. As the upstream signaling pathways of mitochondrial STAT3 remains poorly understood, we speculate that investigation of these pathways may address how a single molecule can be efficiently used to deliver opposing functions in mitochondria.

Our findings also implicates the evolutionary importance of mitochondrial STAT3 during the early symbiotic events. As discussed earlier, primitive endosymbiont differs from the host in having respiratory chain, which produces ROS as the byproducts. As the continuous ROS

production from endosymbiont destabilizes synergetic relationship, regulation of respiration is too critical for the emergence of stable symbiosis. Hence, most of the respiratory chain gens have been transferred to then nucleus where their expression is under tight regulation. Nevertheless, this gene transfer may have taken a relatively long period of time to accomplish. So, inserting a host protein, early in the symbiosis, into the electron transport chain to allow host regulation of respiration may have been critical and strongly selected for. STAT3 is a plausible candidate for such a regulatory protein. In consistent with this notion we find that STAT3 regulates cellular respiration and also optimal functioning of complex I, which is a major source for ROS production, during oxidative stress.

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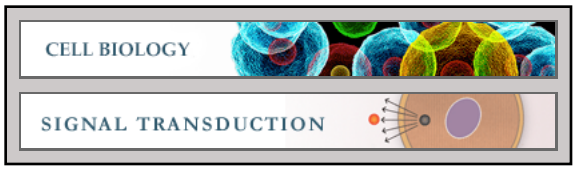
PUBLICATIONS

Cell Biology:

The Import of the Transcription Factor STAT3 into Mitochondria Depends on GRIM-19, a Component of the Electron Transport Chain



Prasad Tammineni, Chandrashekhar Anugula, Fareed Mohammed, Murari Anjaneyulu, Andrew C. Larner and Naresh Babu Venkata Sepuri

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

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

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

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INTRODUCTION

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

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

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

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Papers Submitted:

- **Tammineni P**, Mohammed F, Gorla M, Sepuri NB (2014). Reactive Oxygen Species generated by complex I inhibition signals the recruitment of STAT3 to mitochondria. ***J Biol Chem*** Submitted. ***(***Under review ***# JBC/2014/608885)***

- Murari A, Thiriveedi VR, Mohammed F, Vengaldas M, Gorla M, **Tammineni P**, Krishnamoorthy P, Sepuri NB (2014) Role of Human mitochondrial MIA40 (CHCD4) in the Mauration of Cytosolic Fe/S Cluster Proteins**.** ***J Biol Chem*** (Under review **#JBC/2014/583187**).