TARGETING OF STAT3 TO MITOCHONDRIA AND THE ROLE OF MITOCHONDRIAL STAT3 IN REDOX HOMEOSTASIS AND CELL CYCLE

A thesis submitted for the degree of
Doctor of Philosophy in Biochemistry

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CERTIFICATE

This is to certify that the thesis entitled **"Targeting of STAT3 to mitochondria and the role of mitochondrial STAT3 in redox homeostasis and cell cycle"** submitted to the University of Hyderabad by **Mr. Fareed MD**, bearing the Reg. No **14LBPH02** for the degree of Doctor of Philosophy in Biochemistry is based on the studies carried out by him under my supervision. To the best of my knowledge, this has never been submitted for an award or certificate from any other university or institution, including this institute.

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DECLARATION

I, **Fareed MD**, hereby declare that the work presented in this thesis entitled **"Targeting of STAT3 to mitochondria and the role of mitochondrial STAT3 in redox homeostasis and cell cycle"** is entirely original and that it was completed at the Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, India under the supervision of Professor Naresh Babu V Sepuri. I also declare that this work has not previously been submitted for the award of a degree or diploma from any other university or institution.

Signature of the student

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CERTIFICATE

This is to certify that the thesis titled **"Targeting of STAT3 to mitochondria and the role of mitochondrial STAT3 in redox homeostasis and cell cycle"** submitted by **Mr. Fareed MD** bearing Reg. No. **14LBPH02**, in partial fulfillment of the requirements for the Doctor of Philosophy in Biochemistry, is genuine work done by him under my supervision.

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

1. **Fareed Mohammed**, Madhavi Gorla, Vandana Bisoyi, Prasad Tammineni, Naresh Babu V Sepuri. **Rotenone-induced reactive oxygen species signal the recruitment of STAT3 to mitochondria. FEBS Letters**. 2020 May; 594(9):1403-1412. doi: 10.1002/1873-3468.13741. PMID: 31981230

Aside from his thesis publications, the student was also involved in other publications.

1. Chaitanya Gandikota, **Fareed Mohammed**, Lekha Gandhi, Deepti Maisnam, Ushodaya Mattam, Deepika Rathore, Arpan Chatterjee, Katyayani Mallick, Arcy Billoria, V S V Prasad, Naresh Babu Venkata Sepuri, Musturi Venkataramana. **Mitochondrial Import of**

Dengue Virus NS3 Protease and Cleavage of GrpEL1, a Cochaperone of Mitochondrial Hsp70. Journal of Virology. 2020 Aug 17; 94(17):e01178-20.

2. Naresh Babu V Sepuri, Prasad Tammineni, **Fareed Mohammed**, Arunkumar Paripati. **Nuclear Transcription Factors in the Mitochondria: A New Paradigm in Fine-Tuning Mitochondrial Metabolism.** Hand Book of Experimental Pharmacology. 2017 (Review)

3. Anjaneyulu Murari, Venkata Ramana Thiriveedi, **Fareed Mohammad**, Viswamithra Vengaldas, Madhavi Gorla, Prasad Tammineni, Thanuja Krishnamoorthy, Naresh Babu V. Sepuri. **Human mitochondrial MIA40 (CHCHD4) is a component of the Fe–S cluster export machinery.** Biochemical Journal Oct 02, 2015,

4. Prasad Tammineni, Chandrashekhar Anugula, **Fareed Mohammed**, Murari Anjaneyulu, Andrew C Larner, Naresh Babu Venkata Sepuri. **The import of the transcription factor STAT3 into mitochondria depends on GRIM-19, a component of the electron transport chain.** Journal of Biological Chemistry. 2013 Feb 15; 288(7):4723-32.

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

1. Presented a poster entitled "**Reactive Oxygen Species generated by Mitochondria Signals the Recruitment of STAT3 to Mitochondria**" by **Fareed MD,** Prasad Tammineni and Naresh Babu V Sepuri at conference "**International Congress of Cell Biology (ICCB)**" held on **27th-31st January 2018** organized by **Centre for Cellular and Molecular biology**, Hyderabad.
2. Oral presentation entitled "**The new perspective towards cellular power houses (Ion Channels)**" by **Fareed MD** at GIAN programme "**Ionic Signaling and Human Disease**" held on **18th July 2016** at **University of Hyderabad**, Hyderabad.
3. Participated in conference "**Mitochondria and metabolism network meeting**" held on 10th-11th November 2017 at IISER-Pune.

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

Course Code	Name	Credits	Pass/Fail
BC 801	Analytical Techniques	4	Pass
BC 802	Research ethics, Data Analysis, and Biostatistics	3	Pass
BC 803	Lab Seminar and Record	5	Pass

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..... *Fareed MD*

Table of contents

Abbreviations

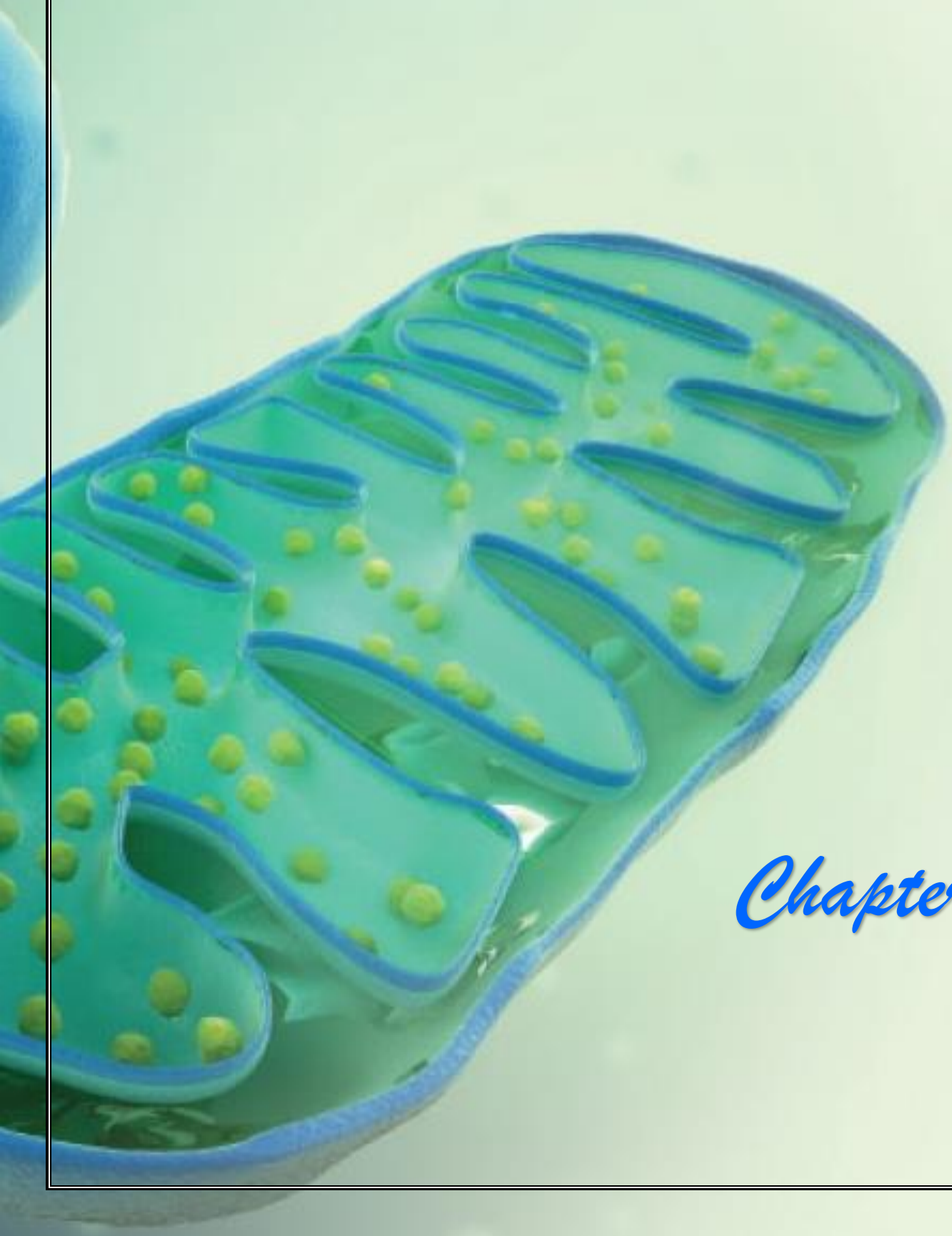
ADP	Adenosine diphosphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CDK	Cyclin dependent kinase
COX	Cytochrome C Oxidase
CREB	cyclic AMP response element binding protein
Cyt c	Cytochrome c
DAPI	4’ 6-diamidino-2phenylindole
DCFDA	Dichlorofluorescin diacetate
DMEM	Dulbecco’s Modified Eagle Medium
DRP1	Dynamin-related protein 1
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ETC	Electron transport chain
FBS	Fetal Bovine Serum
Fe-S	Iron Sulfur cluster
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

Gly	Glycine
GRIM-19	Gene associated with IFN-beta/RA induced cell mortality
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP	Heat Shock Protein
IMM	Inner mitochondrial membranes
IMS	Inter membrane space
IRF3	Interferon regulatory factor 3
KCl	Potassium chloride
KOH	Potassium hydroxide
MAMs	Mitochondria-associated endoplasmic reticulum membranes
MCU	Mitochondrial calcium uniporter
MFN	Mitofusin
Mg (OAC2)	Magnesium acetate
$MgCl_2$	Magnesium Chloride
ml	Milliliter
mm	Millimeter
MnSOD	Manganese superoxide dismutase
NAC	N-Acetyl Cysteine
NADH	Nicotinamide adenine dinucleotide

NF-KB	Nuclear Factor kappa-light chain- enhancer of activated B cells
ng	Nanogram
Noc	Nocodazole
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PI	Propidium iodide
RA	Retinoic acid
RIPA	Radio immunoprecipitation assay
RNase A	Ribonuclease A
ROS	Reactive oxygen species
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
Ser	Serine
STAT	Signal transducer and activator of transcription
Thy	Thymidine
TIM	Translocase of Inner Membrane
TOM	Translocase of Outer Membrane
TRIS	Tris(hydroxymethyl)aminomethane
Tyr	Tyrosine
VDAC	Voltage-dependent anion channel

µCi	Microcurie
µg	Microgram
µl	Microliter

INTRODUCTION



Chapter 1

1.1 Structure of Mitochondria

Mitochondria are membrane-bound organelles present in all eukaryotic cells that play a fundamental role in cellular energy production, thereby referred to as the "powerhouse of the cell," ranging in size from 0.1 to 1 μm and are round or oval. Mitochondria are enclosed by two membranes, outer mitochondrial membrane, and inner mitochondrial membrane. These two membranes are divergent in their composition and function; they further define the two aqueous sub-compartments, Inter membrane space, and Matrix.

The outer mitochondrial membrane comprises simple phospholipids [phospholipid to protein ratio (1:1) relatively similar to plasma membrane]. The outer membrane is porous, allowing the passage of ions, ATP, ADP, and metabolites, the porosity of the membrane is due to the presence of porins and VDAC. Because of this porosity, there is no membrane potential across the outer membrane. Larger proteins enter the mitochondria through multisubunit TOM complexes. It is also associated with endoplasmic reticulum through mitochondria-associated ER membrane (MAM); these are important in the transfer of lipids and calcium across ER and mitochondrial Inter membrane space (IMS), also known as perimitochondrial space, is between the outer and inner membrane. Metabolite content is similar to that of cytosol, but the protein composition is different from the cytosol. It acts as a store for protons generated by the electron transport chain, generating the electrochemical gradient across the inner membrane to drive the ATP synthesis. The protein to phospholipid ratio is high in the inner mitochondrial membrane (3:1) and contains the unusual phospholipid Cardiolipin. The inner membrane is highly impermeable when compared to the outer membrane. Proteins present in the matrix or inner membrane are transported by the translocase of the inner membrane (TIM). The inner membrane is highly infolded into the matrix;

these infoldings are known as Cristae and are home to the complex proteins responsible for synthesizing ATP by oxidative phosphorylation. The matrix contains the mitochondrial genome, mitochondrial ribosomes, tRNAs, and a mixture of enzymes. Primary functions performed by the matrix enzymes are the TCA cycle, Urea cycle, oxidation of fatty acids, and Fe-S cluster biogenesis.

Figure.1:

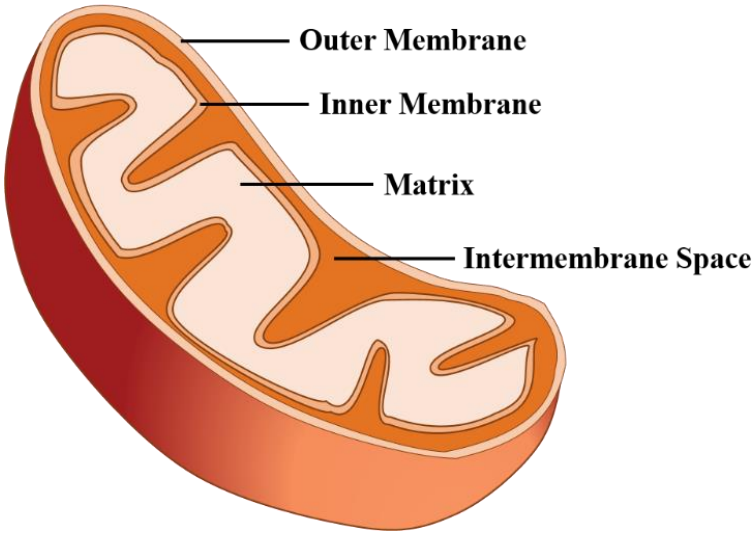


Figure 1.1: Structure of Mitochondria – Mitochondria are membrane-bound organelles composed of two membranes, Outer mitochondrial membrane and Inner mitochondrial membrane. These two membranes further compartmentalize mitochondria to Matrix (the fluidic compartment inside the inner membrane) and Intermembrane space (the space between the two membranes).

1.2 Mitochondrial functions:

The primary function of mitochondria is the generation of ATP. Besides generating ATP, mitochondria are also involved in various other functions. Some of these functions are as follows:

1.2.1 The Powerhouse:

Most of the cellular energy (ATP) is synthesized in mitochondria using NADH, $FADH_2$, and molecular oxygen through oxidative phosphorylation. Thus mitochondria are often referred to as the powerhouse of the cell.

1.2.2 The Gatekeeper of cell death:

Besides generating the cellular energy currency, mitochondria are also crucial in deciding the fate of the cell. Proteins like Cytochrome c (Cyt c), SMAC, Htra2, which are involved in cell death are present in mitochondria. Upon external stimuli, Cyt c is released into the cytosol and activates the caspases leading to apoptosis. Mitochondria are also involved in other forms of cell death like Necrosis and autophagy [1].

Figure.1.2:

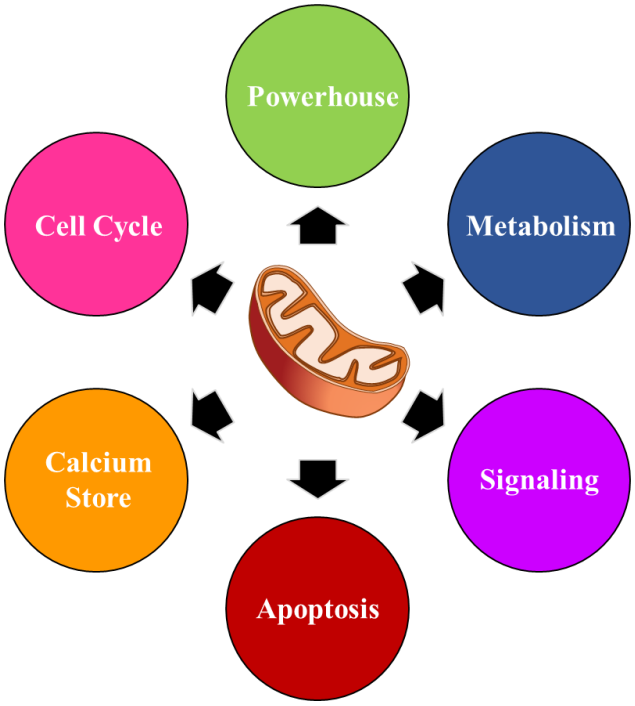


Figure 1.2: Mitochondria Functions – Mitochondria are essential eukaryotic organelles, the primary function is to generate the ATP required for the cellular processes. Besides producing energy, mitochondria are involved in various other functions like apoptosis, storage of calcium, regulation of cell cycle, and mediating different cellular signaling in response to external cues.

1.2.3 The Calcium store:

Calcium signaling is fundamental in regulating various cellular processes such as cellular development, proliferation, and cell death. The levels of calcium in mitochondria regulate some of the TCA cycle enzymes and ATP synthase complex [2-4]. Mitochondrial membrane potential generated to synthesize ATP is also responsible for calcium uptake. Mitochondrial Calcium Uniporter (MCU) present in the IMM of mitochondria is involved in the calcium uptake.

1.2.4 The Hub of Signaling:

Apart from being the source for the generation of ATP, mitochondria are also involved in various other cellular signaling processes by acting as initiators and transducers of cell signaling. Signaling through mitochondria is regulated in two ways, (a) by signaling through protein-protein interactions on the mitochondrial surface, (b) by controlling the levels of intracellular signaling molecules such as ROS and ATP [5]. Regulation of Autophagy by intracellular calcium and innate immune system are a few of the examples of mitochondrial signaling [6, 7].

1.2.5 Mitochondrial control of cell cycle:

The cell cycle and the other associated events are energetically demanding and probably require the cells to synthesize more ATP. The interplay between the metabolic status of the cell and the machinery of the cell cycle is indispensable for the initiation of the cell cycle. Growing evidence suggests that mitochondria also play a key role in regulating cell division. Mitochondria are dynamic organelles, which undergo constant fusion-fission reactions by constantly changing their

morphology. This constant change in mitochondrial morphology has been correlated with the different cell cycle stages, and it is reported to be regulating cell cycle progression. [8-12].

In addition to the above functions, mitochondria are also involved in the generation of heat in brown adipose tissue, synthesis of heme, and regulating cell proliferation by controlling the levels of ATP [8].

1.3 Evolution of Mitochondria:

The evolutionarily significant event of a eukaryotic cell was the acquisition of mitochondria and chloroplast, which formed the biosynthetic factories and gave the ability to generate energy. Mitochondria are thought to be originated from the alpha-proteobacteria by the endosymbiosis with the proto-eukaryote [13, 14]. Two different theories that define the origin of mitochondria are Archezoan and Symbiogenesis theory [15], which differ in respect to the host, capabilities of the endosymbiont, and the nature of their interaction. According to archezoan theory [14], the endosymbiont was acquired by the primitive mitochondrial eukaryote, whereas the symbiogenesis theory suggests the endosymbiont was acquired by the archeal cell and gave rise to mitochondria [16], which later transformed the cell into forming the nucleus and further compartmentalized the cell. During this endosymbiotic process, mitochondria have either lost some of their genes and/or have transferred their genes to the nucleus of the host cells, because of this gene loss or transfer mitochondria are dependent on the nucleus of the host cells for the proteins required for its function. This dependency of mitochondria on the host gave rise to the generation of specialized protein import machinery across the outer and inner mitochondrial membranes.

Figure.1.3:

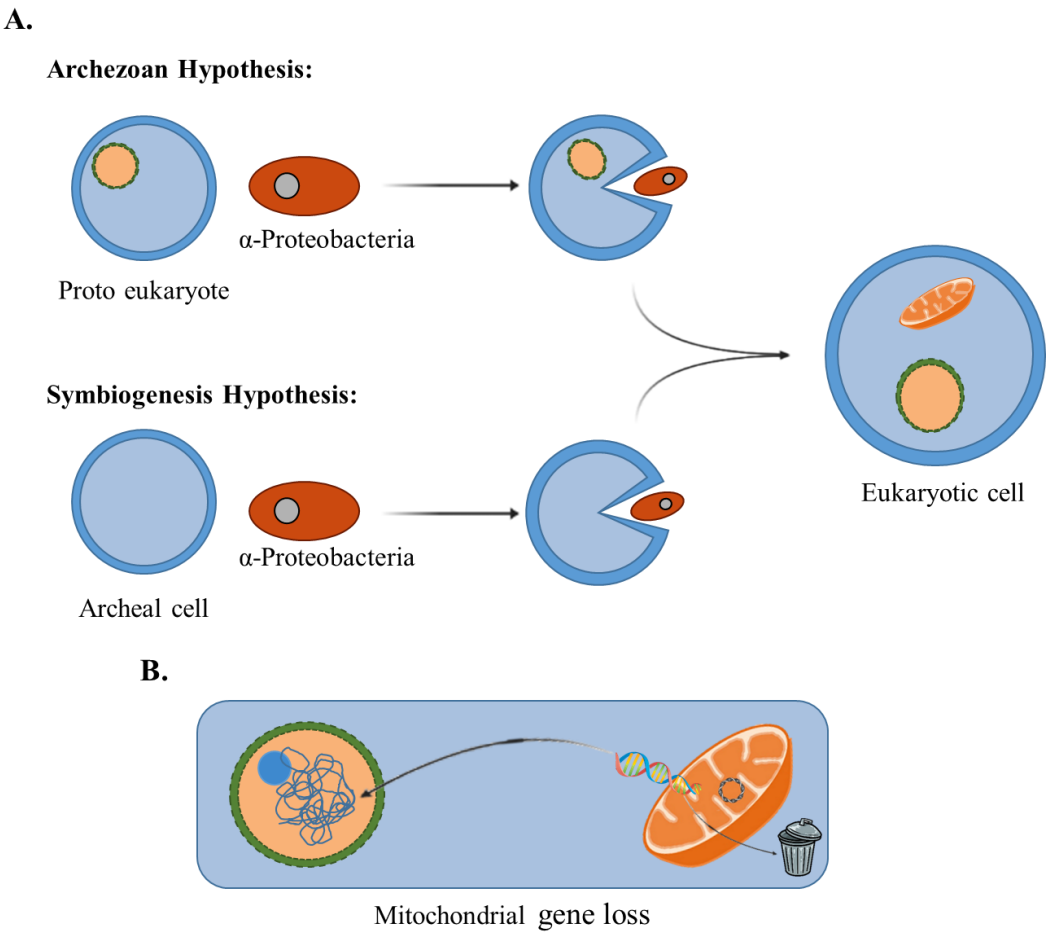


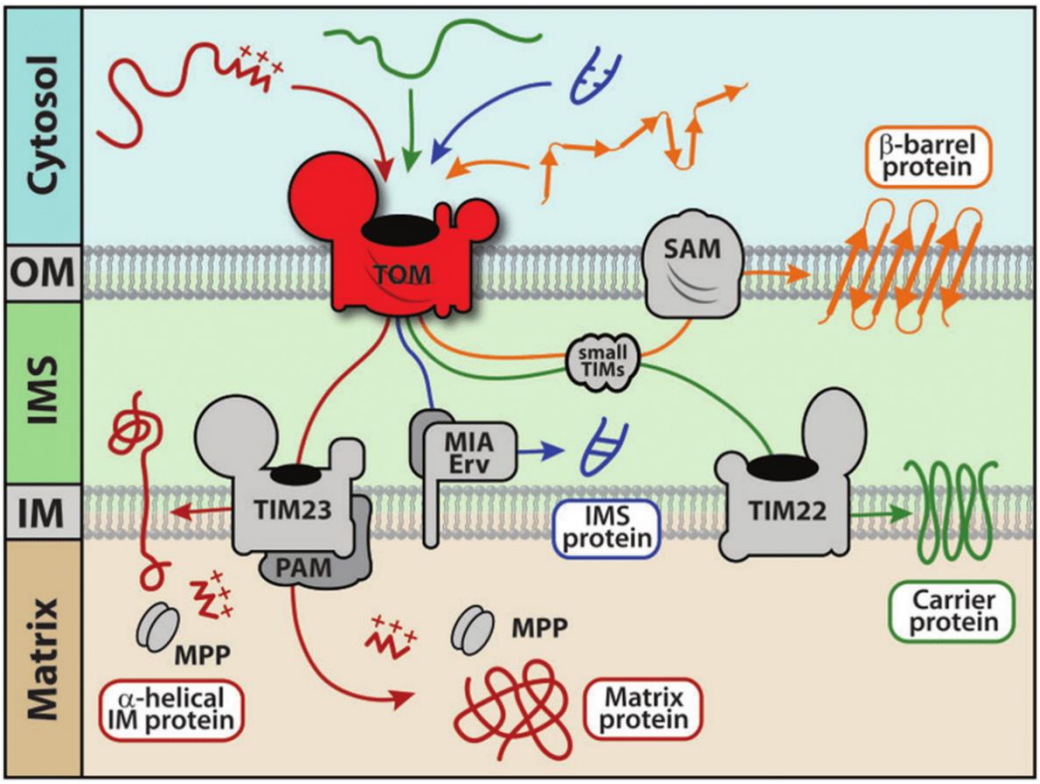
Figure 1.3: Evolution of Mitochondria – A. Endosymbiotic theory suggests that mitochondria were evolved by the symbiosis between the α-proteobacteria and early eukaryotic cell. Two widely accepted theories are Archezoan and Symbiogenesis theory, which essentially differ by the nature of the host that acquired the α-proteobacteria. **B.** During the evolution of mitochondria, mitochondria have lost their genome through evolutionary stress, or their genome was transferred to the host.

1.4 Mitochondrial Protein Import Machinery:

During the evolutionary process of origin of mitochondria and the tendency of endosymbiont to transfer their genes to host or due to gene loss, modern mitochondria are dependent on the nuclear-encoded proteins to shuttle back from the nucleus to mitochondria for proper function. Thus the transfer of proteins to the nucleus has imparted the control of mitochondria to the nucleus and also

facilitated the organellar communication with the nucleus [17]. Also, mitochondria acquired the machinery on their membranes to facilitate the import of nuclear-encoded proteins. The acquisition of mitochondrial protein import motors has been evolutionarily important in establishing the endosymbiont [18]. There are two main protein translocases, TOM complex (Translocase of Outer Membrane) and TIM complex (Translocase of Inner Membrane), present on the outer and inner mitochondrial membranes, respectively. In parallel to the evolution of protein translocases, the proteins destined to mitochondria have acquired the mitochondrial targeting sequences.

Figure.1.4:



(Jan Mani et. al., Molecular Biology and Evolution 2015)

Figure 1.4: Mitochondrial protein import pathway – To facilitate the intake of nuclear-encoded proteins, mitochondria have acquired the specialized import machinery. All proteins are primarily targeted through the TOM complex. Based on the signal sequence present in proteins, they are translocated to the different compartments of mitochondria. Presequence or Internal targeting sequences containing proteins are translocated to the inner mitochondrial membrane or matrix through the TIM22/23 complex. Proteins rich

in cysteines are targeted to intermembrane space through the MIA pathway. Outer mitochondrial membrane-localized proteins utilize the SAM complex for their targeting.

The targeting signals are recognized by the import receptors and target the proteins to different sub-compartments of mitochondria. Two types of targeting sequences are present on imported proteins, one at the N-terminal cleavable presequences, and the remaining are internal targeting sequences. Different precursors follow different import pathways. However, the TOM complex is the common entry gate for most of the import pathways. Majorly, there are four different import pathways. 1. Presequence pathway through which proteins are targeted to the mitochondrial matrix and inner membrane. 2. Carrier pathway targets the proteins containing the multiple membrane span domains to the inner membrane. 3. Oxidative folding pathway, which targets the proteins to the mitochondrial intermembrane space. 4. Outer membrane protein targeting pathways [19].

1.5 Nuclear transcription factors in the mitochondria:

To compensate for the mitochondrial gene loss during the evolution of mitochondria, the host probably transported some of its factors to mitochondria when required. One such class of host factors is nuclear transcription factors. The primary function of these factors is in the nucleus, but when required, they are transported to the mitochondria and regulate mitochondrial functions. These factors regulate the diverse mitochondrial functions like mitochondrial gene expression, mitochondrial respiration, and apoptosis. Defects in the functions of these transcription factors can increase ROS production and alter the mitochondrial permeability transition pore. Based on the localization of these transcription factors in the mitochondria, they can be categorized into two parts. Transcription factors that are linked to the mitochondrial outer membrane (IRF3 and p53) and transcription factors that are associated with inner compartments of mitochondria (CREB, NF-κB, STAT3) [20, 21].

Though several studies reveal the presence and targeting of transcription factors to mitochondria, the precise molecular mechanism of how these transcription factors translocate to mitochondria is not well understood.

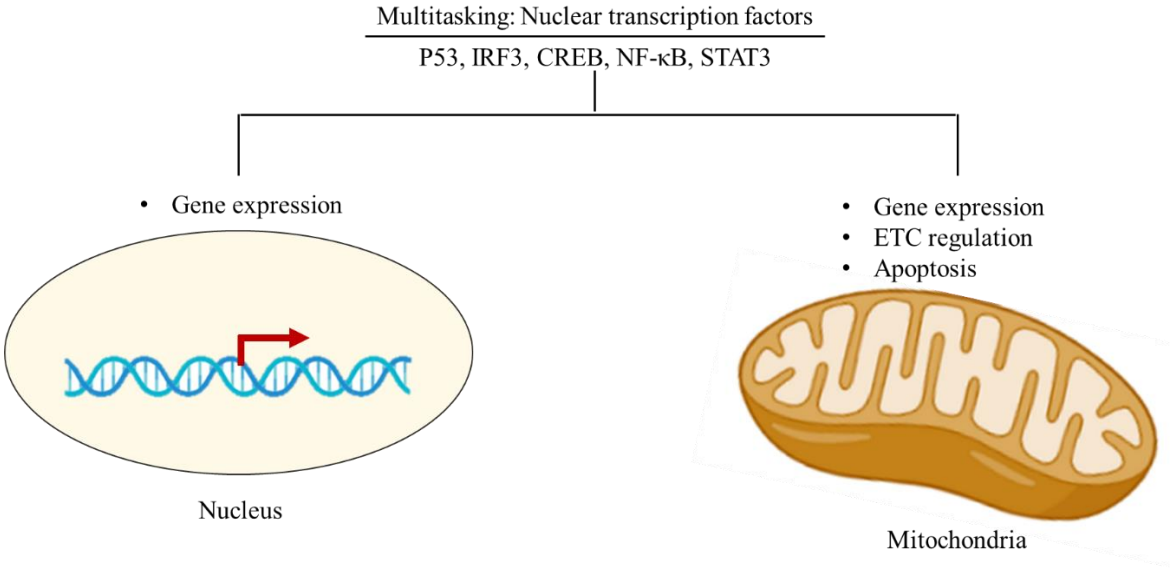
1.5.1 STAT3:

STAT3 is one of the nuclear transcription factors known to be present in mitochondria. STAT3 gets phosphorylated on two key residues at its c-terminus, Tyr705, and Ser727. Upon stimulation by external cues, STAT3 gets activated and translocates to the nucleus to perform the canonical nuclear transcription functions. Maximal transcriptional activity is obtained by STAT3 when it is phosphorylated both at Tyr705 and Ser727 [22]. Though STAT3 is not required for mitochondrial biogenesis or maintenance, it enhances mitochondrial function [23, 24]. Mitochondrial STAT3 regulates the complex-I activity of the electron transport chain by an independent transcriptional mechanism [24]. In the cells which acquired the potential to undergo an oncogenic transformation, MitoSTAT3 is shown to increase the function of Complex-V [23]. Mitochondrial STAT3 also protects the function of Complex-I during the Ischemia/reperfusion injuries [25-27].

MitoSTAT3 confers cardioprotection during Ischemia/Reperfusion injuries through interacting with Cyclophilin D and delaying the opening of permeability transition pore [28]. Further mitochondrial STAT3 is also important for Ras-dependent oncogenic transformation [23]. Treatment of pancreatic cancer with IL-6 increased the abundance of STAT3 in mitochondria thereby enhancing ATP production [29]. The IL-6 stimulated CD4 T cells increased STAT3 in mitochondria with no change in levels of ATP [30]. Thus the regulation of ATP by STAT3 might be cell type-dependent. Besides regulating mitochondrial respiration, mitoSTAT3 also contributes to Ca^{2+} homeostasis by regulating the mitochondrial membrane potential [30]. Ser727 phosphorylation and mitochondrial translocation of STAT3 enhance the growth of breast cancer

cells [31]. MitoSTAT3 is also present in Respiratory Chain Super complexes (RCS) which might contain the Complex-I and III or complexes-I, III, and IV and thereby reduce ROS production [30]. Though both the Tyr 705 and Ser727 phosphorylated STAT3 are present in mitochondria [23, 24], the reported functions of STAT3 in mitochondria impart the importance of Ser727 phosphorylation but not at Tyr705. Therefore phosphorylation of STAT3 at Ser727 might be indispensable for the functions of STAT3 in mitochondria. The functions of STAT3 in mitochondria have been studied but the molecular mechanism by which STAT3 translocates to mitochondria was not well explored.

Figure.1.5:



(Karol Szczepanek et. al., Trends in cell biology, 2012)

Figure 1.5: Bifunctional nuclear transcription factors – In addition to regulating nuclear gene expression, a pool of transcription factors were also reported to be present in mitochondria. These transcription factors regulate the key mitochondrial functions such as electron transport chain (ETC), apoptosis, and mitochondrial gene expression.

1.5.2 Mitochondrial translocation of STAT3 via GRIM-19:

Our lab has demonstrated that mitochondrial complex-I protein, GRIM-19, chaperones the STAT3 to mitochondria. GRIM-19 (Genes associated with Retinoid – Interferon-induced Mortality 19) gene encodes for a 16 kDa protein composed of 144 amino acids. GRIM-19 was identified in an anti-sense technical knockout screen (TKO) as one of the genes responsible for cell death. [32] Later, GRIM-19 was found in association with Complex-I (NADH – Ubiquinone oxidoreductase) of mitochondria [33]. It is an integral part of the functioning of mitochondrial complex-I and vital for early embryonic development. Knockdown of GRIM-19 enhanced the ROS production by disrupting the assembly of complex-I [34]. GRIM-19 interacts with STAT3 and negatively regulates the functions of STAT3. The interaction of GRIM-19 is dependent on the Ser727 phosphorylation of STAT3 [35, 36]. Other than being part of complex-I, GRIM-19 is also known to interact with other proteins. GRIM-19 binds with STAT3 where it regulates the cellular transformation and the localization of STAT3 to mitochondria. GRIM-19 interacts with the Htra2 to increase the rate of apoptotic induction, GW112, and p16INK4a to regulate the cell cycle, and NOD2 to regulate the innate immune response [37].

The Ser727 phosphorylation is crucial not only for the mitochondrial functions of STAT3 and its interaction with GRIM-19. *In vitro* mitochondrial import, assays showed that STAT3 is present in mitochondria and is associated with the Complex-I in the inner mitochondrial membrane and the import of STAT3 into mitochondria is energy and potential dependent. GRIM-19 was important in targeting STAT3 to protease inaccessible fraction of mitochondria. Ser727 phosphorylation of STAT3 is important for the translocation of STAT3 to mitochondria and further enhances its incorporation into mitochondrial Complex-I. Further mutating the STAT3 Ser727 residue to Ala reduced the amounts of STAT3 in mitochondria [38].

In addition to our findings, mitochondrial protein import receptor TOM20, chaperone HSP22 are also shown to mediate the translocation of STAT3 [28, 39] to mitochondria. Moreover, acetylation of STAT3 is also shown to increase the association of STAT3 to mitochondria [40]. It is still elusive how phosphorylation or acetylation of STAT3 affects its interactome or translocation to mitochondria.

Figure.1.6:

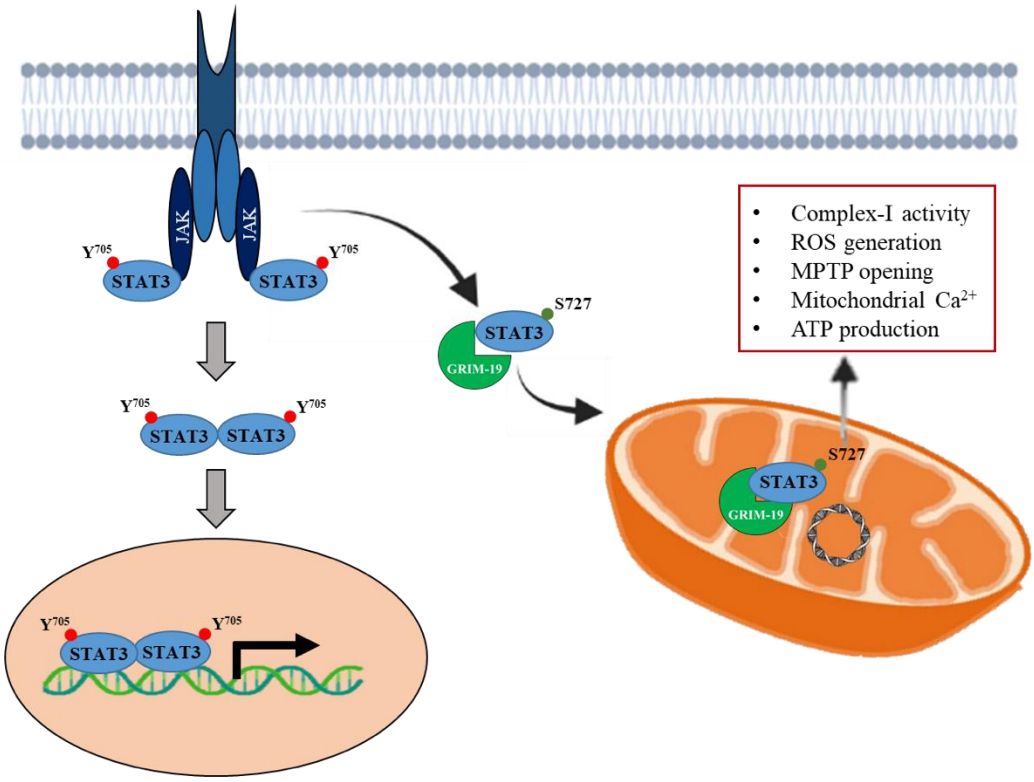


Figure 1.6: Role of STAT3 in mitochondria – Depending upon the stimulus, STAT3 can get phosphorylated either at Y705 or Ser727 residues. When STAT3 gets phosphorylated at Y705, STAT3 translocates to the nucleus and performs the canonical nuclear transcription functions. In contrast, STAT3 translocates to mitochondria if Ser727 gets phosphorylated. GRIM-19 drives the translocation of STAT3 to mitochondria. The mitochondrial pool of STAT3 regulates various functions like Complex-I activity, ROS production, the opening of transition pore, and ATP production.

1.6 The interplay between mitochondria and cell cycle:

Cell division / Cell cycle is a highly controlled and coordinated process, where a cell divides and produces two daughter cells. Cell division can be separated into various phases, G1 (Gap 1)-phase: where the cells grow in size by accumulating the nutrients required for the division and also increasing the number of organelles in a cell. S (Synthesis)-phase: DNA replication takes place and the DNA content in the cell gets doubled. G2 (Gap 2)-phase: the cell further grows by accumulating the nutrients and prepares the cell to progress through mitosis, M (Mitotic)-phase: the duplicated DNA condenses and separates equally into two daughter cells, this phase can further be divided into four stages based on the events that take place (Prophase, Metaphase, Anaphase, Telophase). Mitosis is followed by cytokinesis where the subcellular organelles, cytoplasm, and cell membranes are equally divided, generating two daughter cells.

The key proteins that regulate the cell cycle progression are cyclin-dependent kinases (CDKs) and their binding counterpart's cyclins. CDKs are serine/threonine-dependent protein kinases that regulate the cell cycle by phosphorylating different substrate proteins. CDKs are maintained in the inactive state, and they get activated when bound to the specific cyclin at the specific stage of the cell cycle. About 20 different CDKs (CDK1, CDK2, CDK3…) and 11 different cyclins (A, B, C, D…) have been identified in mammalian cells. Different CDKs bind with different cyclins to drive the particular phase of the cell cycle [41]. For instance, the early G1 phase is regulated by CDK4/6 and cyclin-D, the late G1 phase by CDK2 and cyclin-E, and S-phase is regulated by CDK2 and cyclin-A. CDK1 and cyclin-A control the early transition of cells in the G2/M phase, while CDK1 and cyclin-B regulate mitosis. During synchronous cell division, oscillations in CDKs activities and the levels of cyclins are observed. These oscillations are regulated by phosphorylation of CDKs, synthesis, and degradation of cyclins and CDKs

inhibitions by CDK-inhibitors. Proper progression of the cell cycle is further regulated by the cell cycle checkpoints, a) G1/S checkpoint or restriction- which checks whether the cell has required raw materials for progression into S-phase, b) G2/M checkpoint- checks whether the cell has enough materials (cytoplasm and phospholipids) for division, c) Spindle assembly checkpoint- checks whether the chromosomes are properly aligned to the spindles.

Figure.1.7:

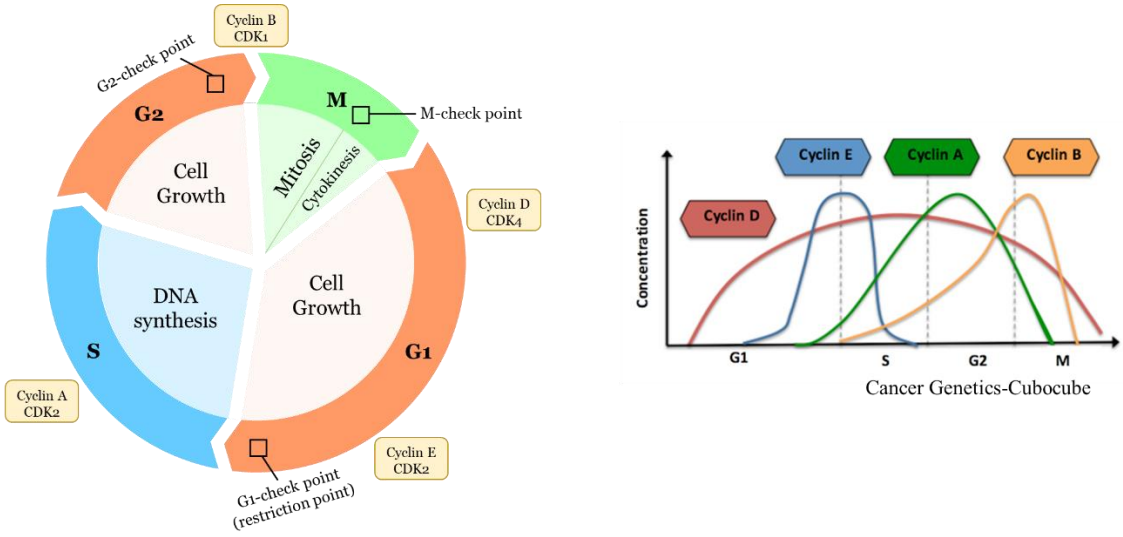


Figure 1.7: Cell Cycle and regulation – During Interphase (G1, S, and G2), the cell grows and duplicates its DNA in the cell cycle. During Mitosis, the distribution of DNA to daughter cells the followed by cytokinesis in which the cytoplasm is equally divided into two daughter cells. The levels of cyclins change during each phase of the cell cycle, and there is a correlation between the cell cycle phase and the level of cyclins. After each phase of the cell cycle, the cyclin associated with that particular phase is degraded.

Mitochondria, for long, were thought to be the stable organelles with the primary function to provide the energy (ATP) to cells. However, growing evidence suggests that mitochondria are not stable organelles, change their shape dynamically from the tubular to filamentous or fragmented form depending on the physiological or metabolic context of the cell. Many cell cycle regulators are shown to regulate mitochondrial function and dynamics. Similarly, mitochondria

are also shown to regulate the cell cycle progression or division. In addition to cell cycle regulators, different transcription factors that play a key role in cell cycle regulation are also known to be important for regulating the functions of mitochondria [42].

1.6.1 Effect of cell cycle regulators on mitochondria:

Different cell cycle regulators were shown to alter mitochondrial function or biogenesis. For instance, Cyclin D1 was shown to regulate mitochondrial biogenesis and activity through NRF1. The increase in expression of Cyclin D1 and NRF1 appears to be inversely correlated [43, 44]. In contrast, in Drosophila, increased mitochondrial biogenesis was correlated with enhanced levels of cyclin D1-CDK4 [45]. Cyclin B1/CDK1 phosphorylates Ser106 residue of MnSOD (Manganese superoxide dismutase) and gives the survival advantage to cells under genotoxic stress conditions [46]. CDC2 phosphorylates Bcl2 at Thr56 during the G2/M phase, thereby regulating the cell cycle without affecting the anti-apoptotic property [47]. Cyclin B1/CDK1 gives a cell pro-survival advantage by phosphorylating Ser315 of mitochondrial localized p53 resulting in elevated mitochondrial ATP production and membrane potential [48].

Mitochondrial structure and activity are also regulated by mitochondrial fusion and fission mechanisms. The equilibrium between fusion and fission of mitochondria is maintained by the fusion proteins (OPA1, MFN1, and MFN2) and fission proteins (DRP1, hFIS1) [42]. During the cell division cycle, mitochondria were found to be oscillating in different forms, tubular in the G1 phase to hyperfused in G1/S and fragmented in the G2/M phase. Mitochondrial depolarization arrested the cells at the G1/S phase, determining the importance of mitochondria for the cell cycle progression. An increase in Cyclin E levels tends to transform the mitochondria into a hyperfused tubular network. Also, the hyperfused mitochondria increase the levels of Cyclin E by limiting its degradation and committing the cells towards division.

Figure.1.8:

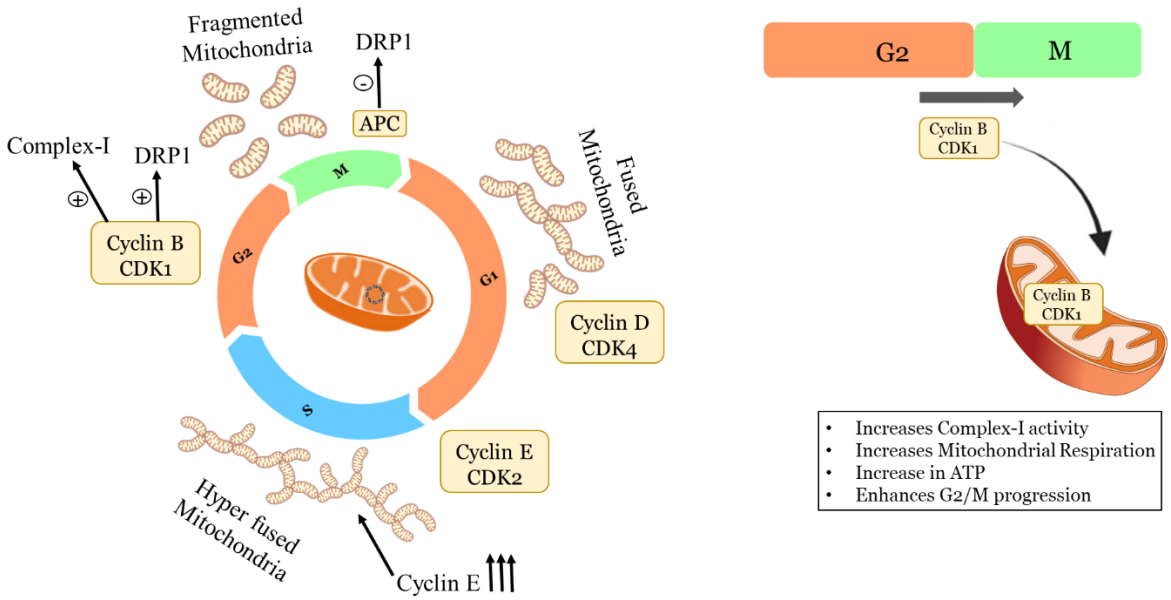


Figure 1.8: Cell Cycle regulation in correlation with mitochondrial function and dynamics – Activation of Cyclin D/CDK4 drives the cells through the G1 phase by inactivating pRb and activating E2F, followed by Cyclin E/CDK2 that drives the cells through the G1/S phase. Finally, activation of Cyclin B/CDK1 takes the cells through mitosis and division. Morphology of mitochondria changes during the cell cycle progression, and this change is correlated with activities of different Cyclins and CDKs. During the G1/S transition, with the increase in the levels of Cyclin E, the mitochondrial network becomes hyper-fused. In contrast, the fragmented network is predominant during the G2/M transition due to activation of DRP1 by the Cyclin B/CDK1. DRP1 is inactivated in the late mitotic phase by the APC complex. Cyclin B/CDK1 also facilitates the progression of cell G2/M phase by phosphorylating mitochondrial complex-I proteins, thereby increasing the mitochondrial activity (Positive circle for positive regulation and negative circle for negative regulation).

Unregulated hyperfusion of mitochondria leads to defects in the cell cycle. In addition, increasing the levels of Cyclin E results in the hyperfused mitochondrial network which is also important for mixing the mitochondrial DNA, increasing the levels of mitochondrial ATP, and importantly protecting the cells from apoptosis during the G1/S transition phase [10]. In contrast, the hyperfused mitochondrial network during the G1/S phase while the fragmented mitochondrial

network is predominant during the G2/M or early mitotic phase. CDK1/Cyclin B phosphorylates Drp1 at Ser616 (Ser585 of rat Drp1) during the G2/M phase to initiate mitochondrial fission. The fragmented mitochondria are arranged more or less equally throughout the cell. This process further mediates the equal distribution of mitochondria to the daughter cells. Mitochondria are not distributed equally to daughter cells when Drp1 is inactivated. Hence the mitotic phosphorylation of Drp1 is important, but it is not a checkpoint for the progression of cells through mitosis [49].

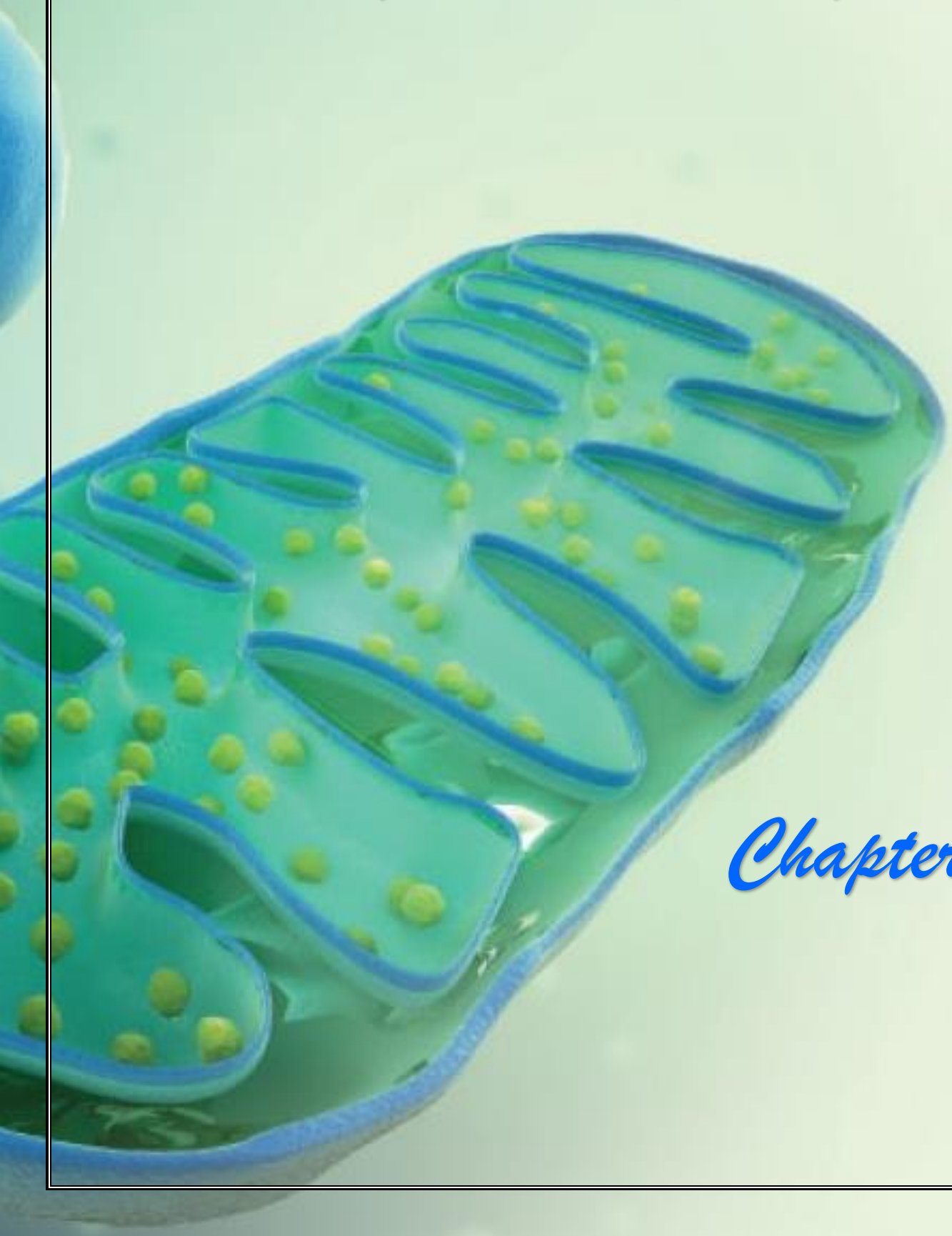
1.6.2 Effect of mitochondria on cell cycle

Cell division is a high-energy demanding process. For a cell to divide, it has to meet all the energy requirements needed to synthesize the raw materials. Mitochondria being the major sites for the production of energy (ATP), proper functioning of mitochondria is the prerequisite for successful cell division. If mitochondria are not functioning correctly, the energy demand will increase, which leads to building up the AMP/ATP ratio resulting in activation of AMPK and activated AMPK phosphorylates p53 at ser15. AMPK dependent ser15 phosphorylation of p53 leads to arresting of the cell cycle at the G1/S phase [50, 51]. Loss of function mutations in mitochondrial ETC complexes I and IV also arrest the cells in a G1/S phase [51]. TOM6, a component of outer mitochondrial protein translocase - TOM complex, is important for regulating the cell cycle. The levels of TOM6 are found to be elevated in mitosis and CDK1 mediated phosphorylation of TOM6 facilitates the proper translocation and integration of TOM6 into the TOM complex. It is essential to import fusion proteins required for the proper mitochondrial function to provide the energy required for cytokinesis [52]. hFis1, mitochondrial fission factor, is also shown to regulate the cell cycle machinery. Knockdown of hFis1 resulted in decreased expression of key Cyclin B, CDK1, Plk1, and several key factors required for G2/M transition [53]. Cyclin B/ CDK1 mediated phosphorylation of Complex-I sub-units is important for enhancing the activity of mitochondrial

complex-I during G2/M progression. Thus linking the Cyclin B/CDK1 with bioenergetics of mitochondria is essential for a successful transition of cells from the G2/M phase [54].

Our lab has identified and demonstrated that a fraction of STAT3 is present in mitochondria, and GRIM-19, a sub-unit of mitochondrial Complex-I, chaperones the STAT3 to mitochondria. Though STAT3 is present in mitochondria, the molecular mechanism by which STAT3 is targeted to mitochondria and the function of mitochondrial STAT3 is not well understood. The present study was designed to decipher the residues in GRIM-19 that interact with STAT3 and to find out if other chaperones, other than GRIM-19, have any role in targeting STAT3 to mitochondria. Our study also aims at understanding how mitochondrial STAT3 regulates the generation of reactive oxygen species and the effect of mitochondrial STAT3 on cell cycle regulation.

DISSECTING THE PATHWAY OF STAT3 ENTRY INTO MITOCHONDRIA



Chapter 2

2.1 Introduction

Mitochondria are commonly referred to as the cell's powerhouse as they generate ATP through oxidative phosphorylation. Mitochondria are also involved in various other metabolic pathways, calcium storage, regulation of cell proliferation, and cell death [55]. Though mitochondria are involved in many cellular pathways, only a few proteins are encoded by the mitochondrial genome. Most of the mitochondrial functions depend on the nuclear-encoded proteins. Mitochondria have acquired specialized protein complexes to facilitate the import of proteins across mitochondrial membranes through the evolutionary process. These protein complexes are referred to as mitochondrial protein import complexes [56].

One class of nuclear-encoded proteins translocated to mitochondria are nuclear transcription factors. Though the functions of these transcription factors in regulating nuclear transcription functions are widely studied, how these transcriptional factors regulate mitochondrial functions and whether these functions are dependent or independent of their transcriptional function are yet to be explored [20, 21]. STAT3 is one such transcription factor shown to be present in mitochondria [24]. The C-terminus of STAT3 harbors two important residues (Tyr705 and Ser727) that undergo phosphorylation upon external stimuli. Canonical nuclear functions of STAT3 are widely studied and are owed towards the phosphorylation of STAT3 at Tyr705. However, mitochondrial translocation or functions of STAT3 are mediated through the phosphorylation at Ser727 [57, 58]. Mitochondrial STAT3 regulates the activities of Complex-I and II of the ETC complex [24]. By preventing the leakage of electrons from complex-I and blocking the opening of the permeability transition pore, STAT3 regulates the RAS-induced cellular transformation and protects the cell from ischemia or reperfusion [23, 25, 26, 28, 39].

Though the pool of STAT3 is known to be associated with the mitochondria, the molecular mechanism by which STAT3 translocates to mitochondria is not well known. One of the reports from our lab has shed light on how STAT3 translocated to mitochondria. Through *in vitro* mitochondrial import assays, we have shown that GRIM-19, a subunit of mitochondrial complex-I chaperones STAT3 to mitochondria, was found to be both energy and potential-dependent. Moreover, GRIM-19 enhances the incorporation of STAT3 into mitochondrial complex-I. Interestingly, this study showed that STAT3 could translocate to mitochondria without GRIM-19, but it was loosely attached to the inner membrane and susceptible to proteases [38]. In addition to GRIM-19, small heat shock protein (HSP22) was shown to activate STAT3 by enhancing the phosphorylation of STAT3 at Ser727 and the depletion of HSP22 reduced the mitochondrial STAT3 pool, indicating the importance of HSP22 in maintaining the mitochondrial STAT3 [39]. HSP90, a general chaperone for the import of proteins with internal targeting sequences, is also shown to interact with STAT3 [59, 60].

In this study, we wanted to address whether, in addition to GRIM-19, other chaperones (HSP22 and HSP90) play a role in recruiting STAT3 to mitochondria. Further, we would like to address the stage-specific requirement of GRIM-19 in translocation of STAT3, and the residues in GRIM-19 that are important for STAT3 translocation to mitochondria. Here, we find that HSP22 and/or HSP90 are required for initial STAT3 translocation to mitochondria while GRIM-19 is needed at a later stage STAT3 integration into mitochondria. GRIM-19 Y33 residue might be important in interacting and integrating STAT3 into the mitochondrial inner membrane.

2.2 Methodology

2.2.1 Antibodies and Reagents

Antibodies specific to STAT3 (ab119352), TOM40 (ab185543), HSP22 (ab79784), HSP90 (ab13492), and Tubulin (ab6046) were purchased from Abcam-Cambridge, United Kingdom. All chemicals were obtained from Sigma Aldrich Chemical Co - USA and Ameresco - USA. ^{35}S-Methionine was obtained from American Radiolabeled Chemicals, Inc. USA. 17-AAG was a gift from Prof. Mrinal K Bhattacharyya (University of Hyderabad).

2.2.2 Cloning of STAT3, GRIM-19 Wild type, and Phospho-mutants:

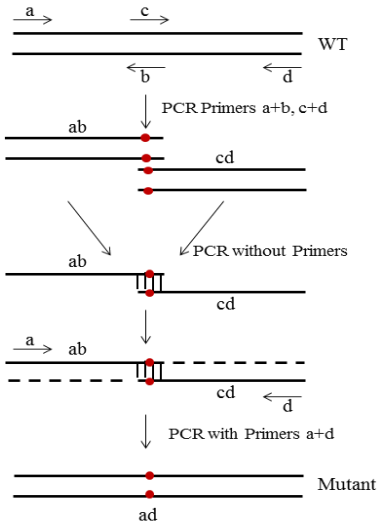
GRIM-19 was amplified using the polymerase chain reaction from HeLa cell cDNA using the forward primer NB-91 and reverse primer NB-128 containing EcoRI and XhoI sites respectively. The amplified fragment was digested with the respective enzymes and cloned into mammalian (pcDNA 3.1A+) expression vector. STAT3 pcDNA 3.1 construct was a kind gift from Andrew C Larner (Virginia Commonwealth University, Richmond, USA).

The GRIM-19 phospho-mutants were generated using the overlap PCR method (Represented schematically). In brief two individual PCR reactions were kept using the full length and mutation primers listed in Table-I. The two PCR amplicons were separated on a 1.5% agarose gel. After extracting the DNA from the agarose, equal moles of each amplicon were used as a template for another PCR reaction to generate the full-length product, After 15 cycles of PCR reaction without addition of primers, full-length primers were included and PCR reaction continued for another 15-18 cycles. On a 1.5 percent agarose gel, the PCR product was separated,

extracted, and digested using EcoRI and XhoI enzymes. The digested insert was cloned into pcDNA 3.1 A+ vector. Primers used in the study are mentioned in Table-I.

Table 1.

S.No	Primer Name	Primer	Restriction Sites	Remarks
1	NB91	5'CCCAGAATTCACCATGGCGGCGTCAAAG GTG 3'	EcoR1	Sense
2	NB128	5' CCAA CTCGAG CGT GTA CCA CAT GAA GCC 3'	Xho1	Antisense
3	NB 469	5' CTG TCG GGC TTC AGC ATG CTG GCC 3'	Y33F	Sense
4	NB 470	5' CAG CAT GCT GAA GCC CGA CAG TCC 3'	Y33F	Antisense
5	NB 489	5' GGG GGC TAT GCG CCC ATC GAC 3'	G17A	Sense
6	NB 490	5' GAT GGG CGC ATA GCC CCC CGG 3'	G17A	Antisense
7	NB 493	5' CCC ATC GAC TTC AAA CGG AAC 3'	Y21F	Sense
8	NB 494	5' CCG TTT GAA GTC GAT GGG CCC 3'	Y21F	Antisense



Representation of Overlap PCR

2.2.3 *In vitro* protein synthesis:

STAT3, GRIM-19 WT, and mutant proteins were synthesized according to the manufacturer's protocol using T7 *in vitro* coupled transcription and translation kit (Promega, USA). Briefly, 500 ng of respective plasmid DNA was incubated with 20 µl of T7 lysate and 20 µCi of ^{35}S-Methionine

at 30ºC for 90 min. A phosphor imager scanner was used to analyze the lysate after SDS-PAGE separation. After *in vitro* protein synthesis, HSP22 was immunodepleted using HSP22 antibody where as HSP90 was depleted using specific inhibitor 17-AAG. Before mitochondrial protein import labeled reticulo-lysates were incubated with either HSP22 antibody (0.5 to 2 µg) or HSP-90 inhibitor, 17-AAG (2 µM) for 20 min. These depleted lysates were used for the import studies.

2.2.4 Isolation of mitochondria:

Mitochondria were isolated from liver or heart tissue excised from rats. Liver or heart tissue was washed with 0.9% saline to remove the bloodstains and chopped into small pieces. 1 gm of tissue in cold medium-A (10 mM HEPES pH-7.4, 220 mM Mannitol, 70 mM Sucrose, 1 mM EGTA) containing 10 mg/ml BSA was homogenized for 3 sec in Polytron 1600 E homogenizer. Then the volume was adjusted to 50 ml with medium-A, and the homogenate was spun at 1800 rpm for 10 min. The supernatant was collected in a fresh tube and centrifuged at 7000 rpm for 10 min. The resultant pellet containing mitochondria was suspended in resuspension buffer (250 mM Sucrose, 10 mM HEPES pH-7.4, and 1.5 mM $MgCl_2$).

2.2.5 Separation of IMM and Matrix:

Isolated mitochondria were resuspended in 450 µl hypotonic buffer (5 mM TRIS–HCl and 1 mM EDTA, pH 7.4), incubated on ice for 15 minutes, and centrifuged at 20,000 x g for 10 minutes at 4°C to generate mitoplasts. The pellet containing mitoplasts was further resuspended in 450 µL of hypotonic buffer and sonicated. The resulting solution was spun for 40 minutes at 100,000 x g, yielding a pellet and supernatant fraction enriching IMM and matrix, respectively.

2.2.6 Mitochondrial Protein Import:

Freshly isolated mitochondria (100 µg) were incubated with ^{35}S -Methionine labelled proteins at 30º C for 60 min in import buffer (0.25 M sucrose, 1.5 mM $MgCl_2$, 2.5 mg/ml BSA, and 10 mM

HEPES, pH 7.4) and energy mix (2 mM ATP, 2 mM GTP, 5 mM $Mg(OAC)_2$, 20mM KCl, and 2mM succinate). After the import, samples were treated with trypsin (25 µg/ml) on ice for 15 min and inhibited by trypsin inhibitor (100 µg/ml). Protease treated and untreated samples were passed through the sucrose cushion (0.8 M Sucrose, 10 mM HEPES pH-7.4) by spinning at 12000 rpm for 10 min. The samples were separated on SDS-PAGE and analyzed using a phosphor-imager.

2.3 Results

2.3.1 Prediction of GRIM-19 residues that possibly interact with STAT3.

Our earlier studies have shown that GRIM-19 interacts with STAT3 and acts as a chaperone to recruit STAT3 to mitochondria [38]. GRIM-19 was shown to interact with the DNA Binding-Linker domain and the transactivation domain of STAT3, but the residues/domains of GRIM-19 that interact with STAT3 are not known [35, 36]. Therefore we sought to determine the residues of GRIM-19 that interact with STAT3. Molecular docking studies using the available docking software {(GRAMM, HEX, and PATCHDOCK) GRAMM uses only the atomic coordinates of the two molecules to predict the structure of a complex. The program conducts a comprehensive 6-dimensional search of the molecules' relative translations and rotations. Hex is an interactive molecular graphics program for calculating and displaying possible docking modes of pairs of protein and DNA molecules The PatchDock algorithm is based on computer vision algorithms for object detection and image segmentation. Molecular Shape Representation, Surface Patch Matching, and Filtering and Scoring are the three primary stages of this technique}. Three residues in the N-terminus of GRIM-19 Gly-17, Tyr-21, and Tyr-33 were found to be common in the three software's used. These residues are likely to be interacting with STAT3 [docking studies were performed by Pushpalatha, MTech student] (Figure. 2.1 A). To determine whether these residues

had any effect on GRIM-19 import itself, mutants of GRIM-19 (G17A; Y21F and Y21F; Y33F) were generated (Figure. 2.1 B). ^{35}S-labelled GRIM-19 WT and mutants were incubated with isolated mitochondria in import buffer at 30^{o}C for one hour. After the import, mitochondria were treated with protease and re-isolated as indicated in the methods. GRIM-19 WT and mutants were present in the protease protected fraction, suggesting that mutation in GRIM-19 does not affect their import into mitochondria (Figure. 2.1 C).

Figure.2.1:

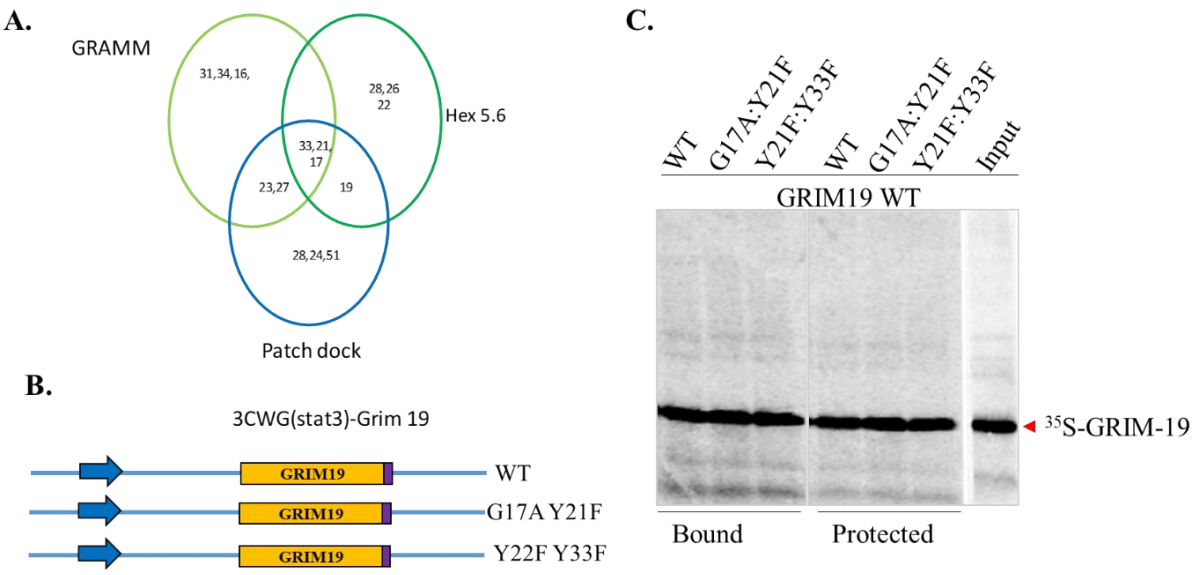


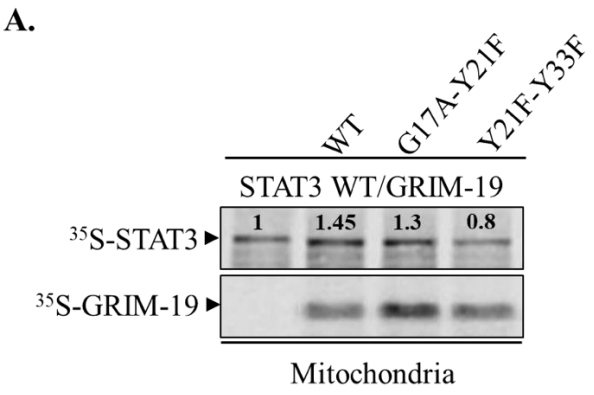
Figure 2.1: Prediction of GRIM-19 residues that possibly interact with STAT3: A. Pie chart showing the common interacting residues between GRIM-19 and STAT3, predicted by the three different molecular docking software's GRAMM, Hex, and Patch dock. **B.** Schematic representation of GRIM-19 WT and mutants cloned into pCDNA3.1A+ myc/His vector. C. Import of GRIM-19 WT and mutants was performed as described in methods, after trypsin treatment and inhibition, SDS-PAGE was used to separate the samples, which were then examined using a phosphor imager.

2.3.2 GRIM-19 Y33 residue important for STAT3 import into mitochondria.

It has been shown by Tammineni et al., that GRIM-19 interacts with STAT3 and recruits STAT3 to mitochondria [38]. In this study, we used WT and GRIM-19 mutants, which may not interact

with STAT3, thereby regulating the import of STAT3 into mitochondria. As higher concentrations of GRIM-19 were shown to decrease the import of STAT3 to mitochondria [38], a limiting amount of ^{35}S-labelled GRIM-19 was used in our experiments. Co-import of either ^{35}S-labelled GRIM-19 WT or mutants along with ^{35}S-labelled STAT3 were carried out as indicated in the methods. The import of STAT3 into mitochondria was reduced in the presence of GRIM-19 Y21F:Y33F compared to GRIM-19 WT or GRIM-19 G17A:Y21F mutant (Figure.2.2 A). Further, the association of STAT3 with inner mitochondrial membrane was reduced, when STAT3 co-imported with GRIM-19 Y21F:Y33F mutant (Figure.2.2 B). These results suggest that GRIM-19 Y33 residue (as GRIM-19 Y21 is present in both mutants) might be important for interaction with STAT3 and regulate the translocation of STAT3 to the inner mitochondrial membrane.

Figure.2.2:



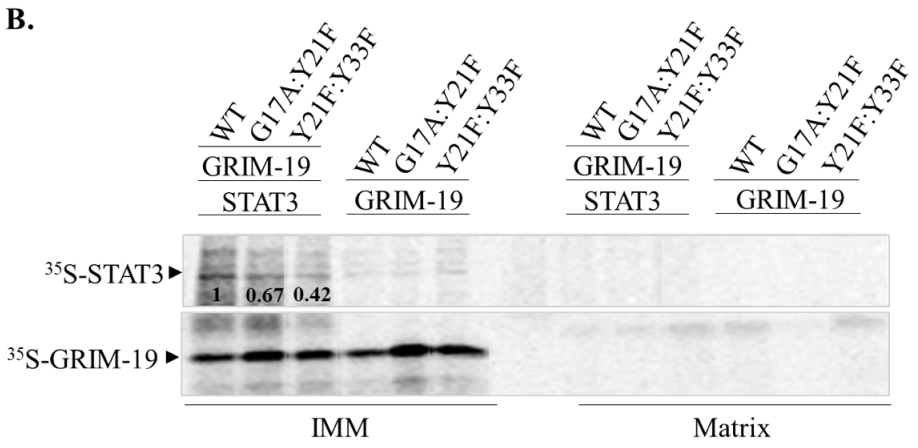


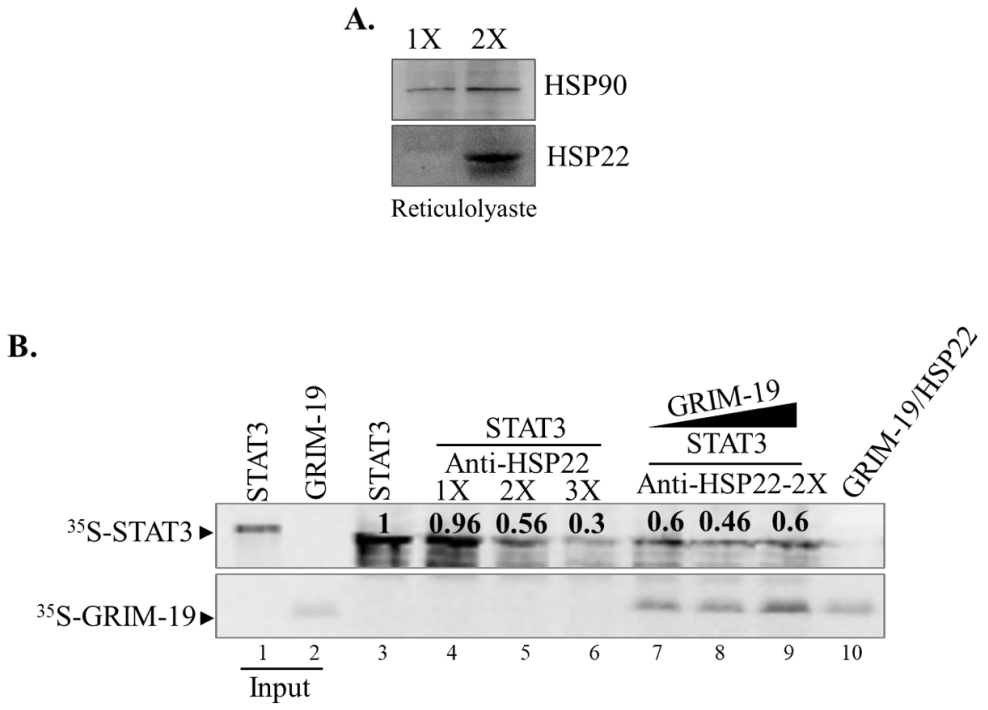
Figure 2.2: GRIM-19 Y33 residue regulates the STAT3 import into mitochondria: A. *In vitro* co-import of STAT3 with GRIM-19 WT or mutants was conducted at 30º C for 60 min. After import, peripherally attached proteins were digested with trypsin and re-isolated the mitochondria. SDS-PAGE was used to separate the samples, which were then examined using a phosphor imager. **B.** After co-import with STAT3 and GRIM-19 WT or mutants, mitochondria was fractionated to IMM and matrix as described in the methods section. The resultant fractions were separated on SDS-PAGE then analyzed using a phosphor imager.

2.3.3 Role of HSP22 and HSP90 in import of STAT3 to mitochondria

We had shown that imported STAT3 is present in protease accessible fraction of mitochondria (loosely attached to mitochondria) when an *in vitro* import was performed without co-import of GRIM-19. In this study, we wanted to find out other molecular factors involved in the translocation of STAT3 to mitochondria. We have evaluated whether HSP22 and HSP90 could regulate the STAT3 translocation. HSP90 was shown to interact with STAT3 [59], and HSP90 plays a critical role in importing non-cleavable mitochondrial proteins [60]. HSP22 is known to affect the functions of mitochondrial STAT3, and depletion of HSP22 decreased the mitochondrial abundance of STAT3 [39]. We asked whether inhibiting these chaperones has any effect on STAT3 transport. Both chaperones were present in reticulo-lysates, HSP22 was immunodepleted using HSP22 specific antibody, and HSP90 was inhibited by specific HSP90 inhibitor 17-AAG.

As expected, in our *in vitro* import assays, STAT3 alone was imported into mitochondria. However, depletion of HSP22 decreased the import of STAT3 significantly (Figure.2.3 B lane 4 to 6), and co-import of GRIM-19 does not restore this inhibition (Fig.2.3 B lane 7 to 9). Further, inhibition of HSP22 does not affect the import of GRIM-19 (Figure.2.3 B lane 10). Similarly, a decrease in STAT3 import to mitochondria is observed by the depletion of HSP90 from the lysates (Figure.2.3 C lane 4) and this decrease is not reversed by GRIM-19 (Figure.2.3 C lane 8). Further, depletion of HSP90 does not affect the import of GRIM-19 (Figure.2.3 C lane 2). This data suggests that HSP22 and HSP90 facilitate the import of STAT3 into mitochondria, and GRIM-19 probably regulates the downstream integration of STAT3 into the mitochondrial inner membrane.

Figure.2.3:



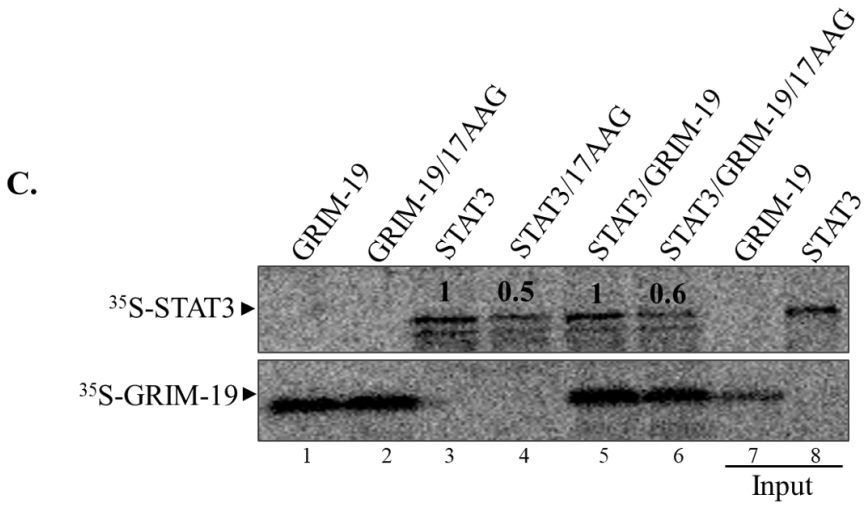


Figure 2.3: Role of HSP22 and HSP90 in the import of STAT3 to mitochondria: A. Representative western-blot indicating the presence of HSP22 and HSP90 in the T7-TNT reticulocyte lysates used for the *in vitro* import assays. **B.** To demonstrate the role of HSP22 in STAT3 import to mitochondria, HSP22 was immunodepleted from the lysates, and the STAT3 import was performed, either in the absence (lanes 4, 5, and 6: increasing amount of HSP22 antibody was used) or presence of GRIM-19 (lanes 7, 8 and 9). After import, mitochondria were protease treated and reisolated. The samples were separated on SDG-PAGE and analyzed by phosphor imager **C.** To show the importance of HSP90 in STAT3 import, HSP90 from the lysates was depleted using 17-AAG, and the import of STAT3 was performed, with (lanes 5 and 6) GRIM-19 or without GRIM-19 (lanes 4 and 5). After import, mitochondria were protease treated and re-isolated, separated on SDG-PAGE, and analyzed by phosphor imager

2.4 Discussion

Emerging evidence indicates that a pool of STAT3 is associated with mitochondria [23, 24, 28]. The mitochondrial pool of STAT3 is important for regulating mitochondrial functions, cellular respiration, and RAS-induced cellular transformation [23, 26]. Though the mitochondrial functions were studied, the mechanism by which STAT3 is targeted to mitochondria is unknown. Our lab has shown that STAT3 is targeted to the inner membrane of mitochondria, more specifically to mitochondrial complex-I. Further, the translocation of STAT3 to mitochondria

requires membrane potential and energy. Moreover, GRIM-19, a component of mitochondrial Complex-I, was shown to chaperone STAT3 to mitochondria, and phosphorylation of STAT3 at ser727 is important for mitochondrial translocation. In addition, imported STAT3 was accessible to proteases when imported alone, and conversely, STAT3 was inaccessible to proteases when co-imported with GRIM-19 [38]. In this study, we wanted to address the precise molecular mechanism of STAT3 translocation to mitochondria. Here, we show that the initial translocation of STAT3 to mitochondria is regulated by chaperones HSP22 and HSP90, whereas GRIM-19 mediates the final translocation and integration into the inner mitochondrial membrane.

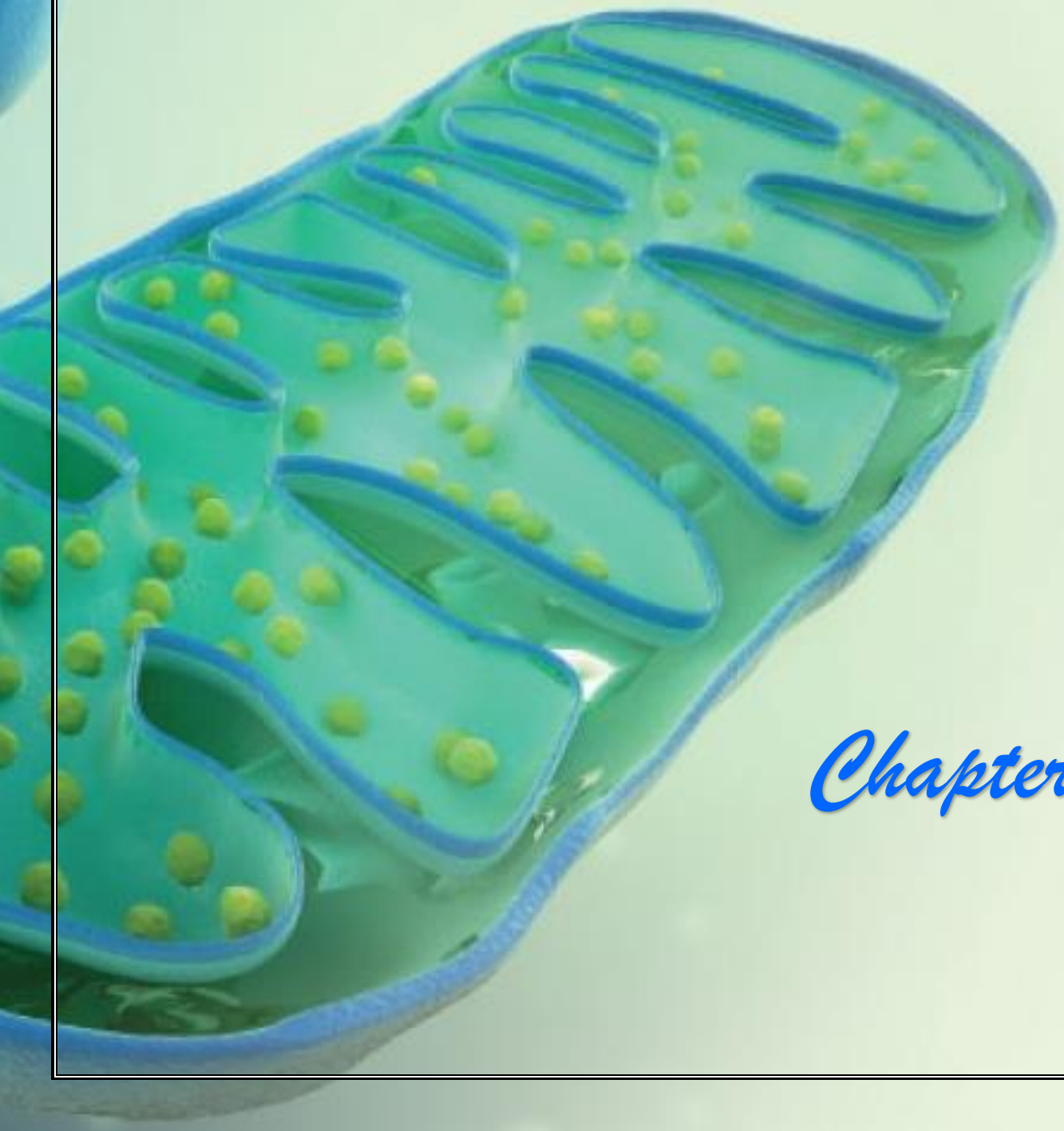
GRIM-19 was shown to interact with STAT3 and also chaperone the STAT3 translocation to the mitochondrial inner membrane, but the exact residues or domains of GRIM-19 that interact with STAT3 were unknown. In this study, using molecular docking, we show that probably residues G17, Y21, and Y33 interact with STAT3. To support our findings, a more recent study from another group using alpha fold has shown that N-terminal residues of GRIM-19 interact with N-terminal domains of STAT3 [61]. Further, using *in vitro* mitochondrial import assay, we show that Y-33 residue of GRIM-19 is important for importing STAT3 to mitochondria. In addition, we also show that GRIM-19 Y33 is important for the integration of STAT3 to the inner membrane of mitochondria.

Interestingly, STAT3 alone can translocate to mitochondria but, is found to be loosely associated with mitochondria and accessible to protease. However, co-import of STAT3 with GRIM-19 facilitates the translocation of STAT3 to protease inaccessible position, complex-I in the inner mitochondrial membrane [38]. The molecular factors that regulate the initial translocation of STAT3 to mitochondria are unknown. Here, we looked at two chaperones, HSP90 and HSP22, for their possible role in STAT3 translocation to mitochondria. HSP90 was shown to be important

in regulating the import of proteins that do not have canonical mitochondrial import sequences. Further, HSP90 also directly interacts with STAT3. Deletion of HSP22 led to a decrease in the abundance of STAT3 in mitochondria, and HSP22 was also shown to regulate the STAT3 mediated functions of mitochondria. Our *in vitro* mitochondrial import assays have revealed that HSP90 and HSP22 are necessary for translocation of STAT3 to mitochondria, and GRIM-19 could not target STAT3 to mitochondria when either HSP90 or HSP22 were inhibited. Our results suggest that HSP90 and HSP22 might directly or indirectly facilitate the initial translocation, and GRIM-19 is important for late-stage translocation and integration of STAT3 into the mitochondrial inner membrane.

Overall our data revealed GRIM-19 Y33 residue is important for translocation and integration of STAT3 in mitochondria, and in addition to GRIM-19, chaperones HSP90 and HSP22 are important for targeting STAT3 to mitochondria.

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



Chapter 3

3.1 Introduction

STAT3 is a latent transcription factor that responds to various stimuli including cytokines and growth factors [62]. Activated STAT3 integrates external stimuli to nuclear gene expression by undergoing post-translation modifications such as phosphorylation, oxidation, acetylation, and methylation [63-66]. STAT3 C-terminal domain harbors two important phosphorylation sites; Tyr 705 and Ser 727. Phosphorylation on these two residues is crucial for transcriptional activation and DNA binding activity of STAT3 in the nucleus [57, 58]. STAT3 targeted genes are involved in a diverse array of physiological processes, and aberrant activation of STAT3 often leads to various pathological conditions like cancer, compromised immune response, and cardiac failures. The complex signaling pathways that are associated with nuclear STAT3 are well studied. However, the discovery of STAT3 pools in other subcellular compartments makes it a much more complicated signaling mechanism.

Mounting evidence suggests that significant levels of STAT3 is present in the mitochondria and regulate its function independent of transcription. Mitochondrial STAT3 regulates the electron transport chain, thereby ATP production [24]. MitoSTAT3 also supports Ras-dependent cellular transformation [23] and the growth of breast cancer [31]. MitoSTAT3 also preserves mitochondrial function during ischemia [25, 26, 39] and has been linked to cardio protection through the regulation of mitochondrial permeability transition pore [28]. Upon NGF stimulation STAT3 localizes to mitochondria and promotes neurite outgrowth [67]. STAT3 is also shown to bind with mitochondrial DNA and TFAM to regulate mitochondrial gene expression [68]. Most of the reported mitochondrial functions appear to be contingent on phosphorylation of STAT3 on Ser727, as S727A mutation resulted in decreased cellular respiration and failed to protect against ischemic-

perfusion injuries [26, 28]. In addition, recent reports shed light on the importance of phosphorylation and acetylation in mitochondrial transport of STAT3 in response to external stimuli [40]. Despite phosphorylation of STAT3 by many kinases on Ser727, signaling pathways responsible for mitochondrial localization and/or functions remains poorly studied.

Mitochondrial metabolic, redox and respiratory state and function play a significant role to maintain cellular homeostasis. Thus, mitochondrial dysfunction is associated with activation of the adaptive response pathway either by altering the expression of the nuclear genome or altering the mitochondrial proteome. Rotenone, a pesticide, is known to generate ROS by interfering with the function of mitochondrial complex I and it has been widely used in understanding the mitochondrial associated dysfunctions. In this study, we wanted to understand the role of oxidative stress in STAT3 translocation to mitochondria and function. Here we report that rotenone treatment enhances phosphorylation of STAT3 on Ser727 and thereby its mitochondrial localization. In contrast, co-treatment with N-Acetyl Cysteine (NAC), a ROS quencher, decreased phosphorylation and mitoSTAT3 levels. We also demonstrated that STAT3 ablation results in increased oxidative burden upon rotenone treatment, while over expression of STAT3 wildtype, but not S727 mutant, alleviates rotenone-induced ROS production. Our results provide new insights into STAT3 mediated feedback loop to maintain redox homeostasis during mitochondrial insults.

3.2 Methodology

3.2.1 Antibodies and Reagents

Antibodies specific to phospho Ser727 (CST 9134) was purchased from CST, Tyr 705 in STAT3, STAT3 (ab119352), Tom20 (ab56783), Myc (ab9106), and Tubulin (ab6046) were purchased

from Abcam whereas antibody against GAPDH (CST 8884) was from CST. All chemicals were obtained either from Sigma Aldrich or Ameresco unless otherwise mentioned.

3.2.2 Plasmid constructs and shRNA

Plasmids harboring STAT3 WT and mutants were a kind gift from Andrew C Larner (Virginia Commonwealth University, Richmond, USA). These plasmids were used as templates to sub-clone STAT3 WT and STAT3 Y705F:S727A into pcDNA3.1 A+ vector. For targeting STAT3 to mitochondria, mitochondrial targeting sequence (MTS) from COX VIIIa [24] was cloned to the N-terminus of STAT3 cDNA sequence using primer annealing. Primers used for cloning are given in Table II. STAT3-specific shRNA – pSIH-puro-STAT3 shRNA – was purchased from Addgene (Watertown, MA, USA) (Cat-26596) [69].

Table 2.

S.No	Gene Name	Primer Number	Primer sequence	Restriction Sites
1	STAT3	NB293 Sense	5’ CCGAGAATTCACCATGGCTCAGTGGAA CCAG 3’	EcoR I
2	STAT3	NP 4 Antisense	5’ CCGACTCGAGCATGGGGGAGGTAGCA CA 3’	Xho I
3	MTS	NB502 Sense	5’ ATATAAGCTTATGTCCGTCCTGACGCCG CTGCTGCTGCGGGGCTTGACAGGCTCGGC CCGGCGGCTCCCAGTGCCGCGCGCCAAGA TCCATTCGTTGGGGAATTCATAT 3’	Hind III
4	MTS	NB503 Antisense	5’ ATATGAATTCCCCAACGAATGGATCTTG GCGCGCGGCACTGGGAGCCGCCGGGCCG AGCCTGTCAAGCCCCGCAGCAGCAGCGGC GTCAGGACGGACATAAGCTTATAT 3’	EcoR I
5	STAT3 shRNA	Cat-26596	gatccGCATCTGCCTAGATCGGCTATTCAAG AGATAGCCGATCTAGGCAGATGTTTTTTg	Addgene

3.2.3 Cell culture and DNA transfection

Experiments were performed using HeLa and HEK293 cell lines that were obtained from the Cell repository at ATCC, 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% CO2 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 MgCl2, 1 mM 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^{o}C and the resultant supernatant was again centrifuged at 10,000 X g for 15 min at 4^{o}C to obtain mitochondria. The mitochondrial pellet was washed twice and suspended in a buffer containing 250 mM sucrose, 5 mM magnesium acetate, and 10 mM HEPES-KOH pH 7.4

3.2.5 Immunoblotting

For western blot analysis, cells were lysed in RIPA buffer (50 mM Tris-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 the 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

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 5% bovine serum albumin in PBS followed by incubation with primary antibody. After washing with PBS, cells were incubated with corresponding secondary antibodies conjugated with Alexa fluor having different excitation wavelengths. Subsequently, cells were mounted using Prolong Gold antifade with DAPI, and images were captured using a confocal microscope (Zeiss LSM NLO 710).

3.2.7 Measurement of ROS

Post rotenone treatment, cells were incubated with 10 µM 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.

3.2.8 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 $MgCl_2$, 0.05% Triton X-100 and 0.25% BSA for 1 min at 37^0C. The activity assay was initiated by the 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 (6.2 $mM^{-}1cm^{-}1$ at 340 nm with reference wavelength).

3.3 Results

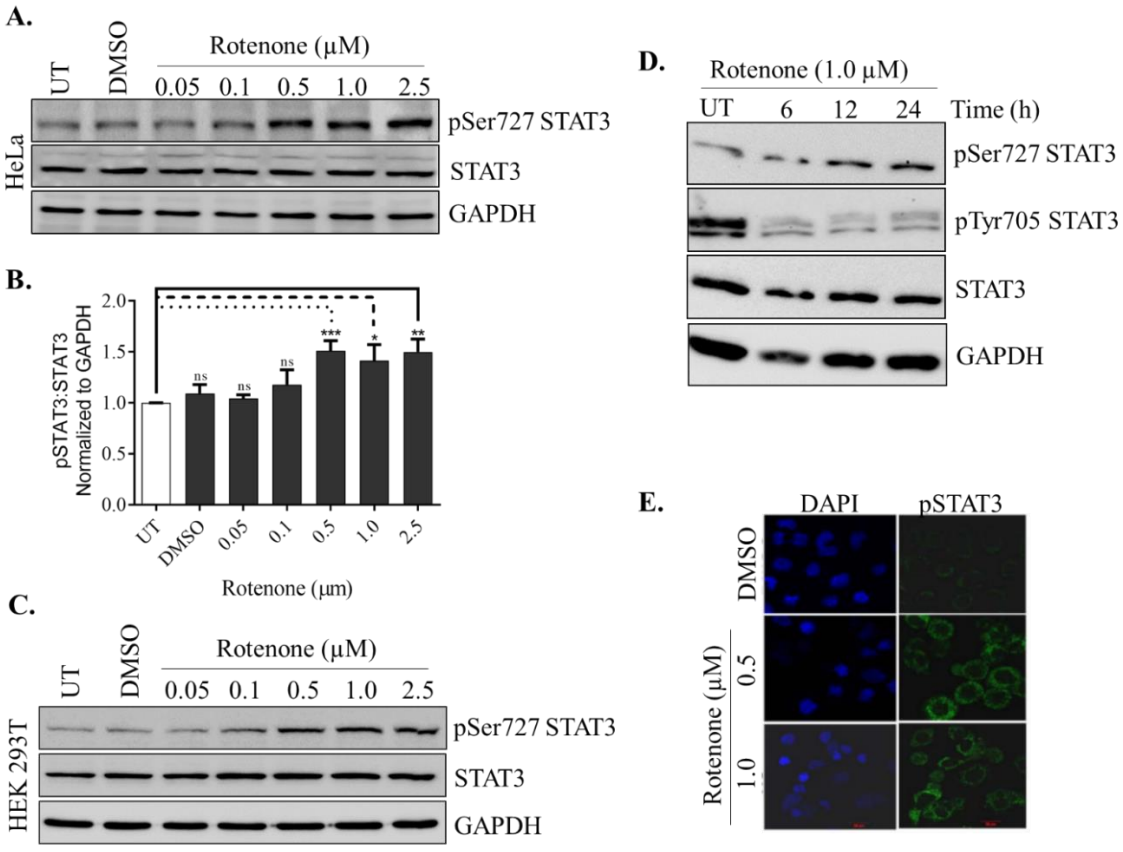
3.3.1 Rotenone promotes STAT3 phosphorylation on Ser727

STAT3 responds to various stimuli by undergoing phosphorylation on Tyr 705 or Ser 727 in the C-terminal domain [62]. In addition, STAT3 reacts towards oxidative stress by undergoing oxidation [70-74]. Therefore, we hypothesized that STAT3 function and localization is regulated by oxidative stress. We used rotenone, a complex I inhibitor, to induce oxidative stress in HeLa cells [75]. HeLa and HEK293 cells were treated with increasing concentrations of rotenone for 18h and cell lysates were probed with phosphoserine specific STAT3 and STAT3 antibodies. We observed a spike in phosphorylation of STAT3 on Ser727 from 0.5 µM concentration (Figure 3.1 A, B, and C). When we treated the cells with 1 µM rotenone for various time points, we found that 12h is sufficient to trigger Ser727 phosphorylation. Interestingly, phosphorylation on Tyr705 was reduced within 6h of rotenone treatment (Figure 3.1 D). However, no changes were noted in total STAT3 and GAPDH levels. Consistently, we saw increased immunostaining with phospho-Ser727 (pS727) STAT3 antibody in rotenone treated cells compared to vehicle-treated cells (Figure 3.1 E) indicating the enhanced phosphorylation of STAT3. Together these results suggest that rotenone treatment triggers STAT3 phosphorylation on Ser727.

Figure 3.1. STAT3 Ser727 phosphorylation is increased during rotenone treatment: Representative blot **(A)**, quantification **(B),** and **(C)** showing increased Ser727 phosphorylation of STAT3 upon rotenone treatment in HeLa cells and HEK cells. Cells were treated with different concentrations of rotenone for 18 h **(A and C)** or **(D)** with 0.5µM of rotenone for different time points and cell lysates were probed with pSerSTAT3, pTyrSTAT3, STAT3, and GAPDH. GAPDH was used as a loading control. **(E)** Representative immunofluorescence images of HeLa cells showing increased immunostaining of Ser727 phosphorylation specific antibody (pSTAT3) with increased concentration of rotenone. GAPDH was used as a loading control. Data were quantified from three independent experiments. Error bar represent S.E and

Student's t-test was performed between control and treated sample sets (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$)

Figure.3.1:



3.3.2 Rotenone activated STAT3 is targeted to mitochondria

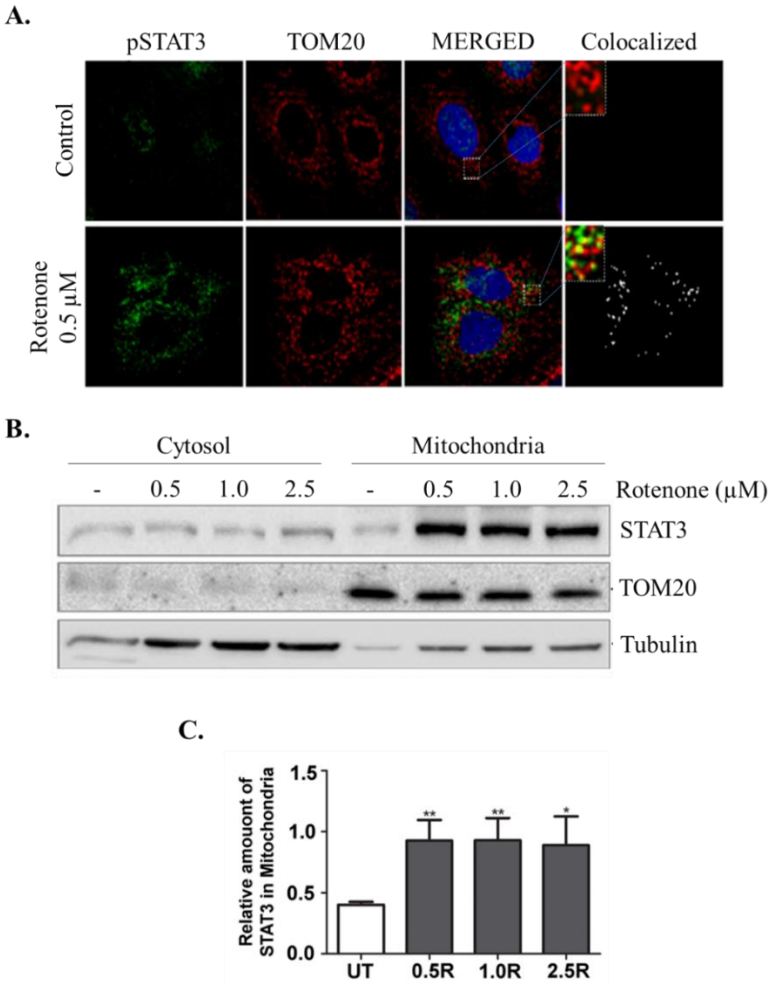
We and others have shown that pSer727 is also responsible for STAT3 recruitment to mitochondria [38]. As rotenone treatment enhanced Ser727 phosphorylation, we hypothesized whether rotenone treatment promotes mitochondrial targeting of STAT3. To test this hypothesis, we treated HeLa cells with 0.5 μM of rotenone and performed immunostaining with pS727 antibody. Cells were counterstained with Tom20 and DAPI to visualize mitochondria and the nucleus. pSTAT3 signal was increased in rotenone treated samples and the majority of pS727 immunostaining was

observed in cytosol compared to weak nuclear staining in vehicle-treated cells. Moreover, a significant fraction of this cytosolic pS727 staining was colocalized with the mitochondrial marker, Tom20 indicating mitochondrial localization of pSTAT3 upon rotenone treatment (Figure 3.2 A).

To further confirm this, we isolated mitochondria from the cells treated with rotenone and monitored STAT3 levels. Mitochondria isolated from rotenone-treated cells displayed increased STAT3 levels. However, STAT3 association did not show dose dependency, as levels of STAT3 increased dramatically with 0.5 μM rotenone and remains stable until 2.5 μM rotenone while Tom20 levels, used as a negative control, remain unaltered (Figure 3.2 B and C) with or without rotenone treatment

Figure 3.2. Mitochondrial STAT3 levels increased upon rotenone treatment: A) Representative immunofluorescence images of HeLa cells treated with rotenone (0.5 μM), showing colocalization of S727 phosphorylated STAT3 with mitochondria. Cells were treated with rotenone and immunostained with anti-pS727 STAT3 (Green) and anti-Tom20 (Red). The colocalized pixels were indicated in white. **(B-C)** Representative blots **(B)** and quantification **(C)** showing increased STAT3 levels in mitochondria enriched fractions in rotenone treated samples. Mitochondria enriched fractions were prepared from both vehicle (control) and rotenone treated cells by differential centrifugation and probed with antibodies to mitochondrial (Tom20) and cytosolic (Tubulin) compartments. Note that rotenone treated samples display STAT3 abundance in mitochondrial enriched fractions. Data were quantified from three independent experiments. Error bar represent S.E and Student's t-test was performed between control and treated sample sets (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$)

Figure.3.2:



3.3.3 ROS is involved in Ser727 phosphorylation of STAT3 and its mitochondrial targeting

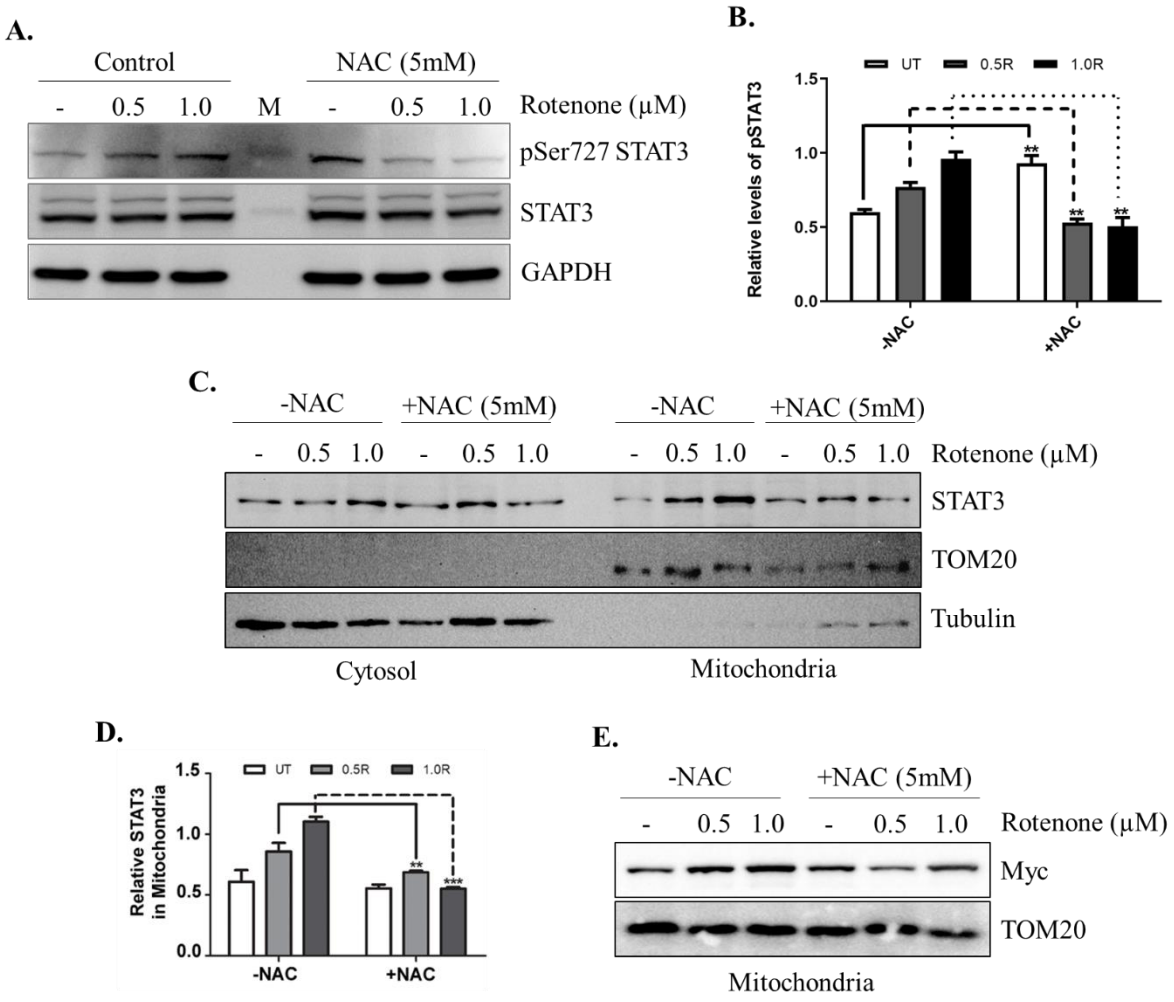
Rotenone increases mitochondrial ROS production by inhibiting complex I function and is also known to be associated with other oxidative stress phenotypes [75]. Mitochondrial STAT3 reduces the ROS production from Complex I and protects the cell from the deleterious effects of high ROS levels. Moreover, earlier studies have shown that ROS can act as a signaling intermediate [76]. Hence, it is intriguing to see whether ROS is involved in rotenone-induced STAT3 phosphorylation and its mitochondrial localization. We used N-Acetyl Cysteine (NAC) to quench ROS. First, we sought to determine whether NAC influence the phosphorylation of STAT3 on

Ser727. Cell lysates were prepared from HeLa cells co-treated with rotenone and NAC and probed with the phospho-specific STAT3 pSer727 antibodies. As expected, rotenone-induced the phosphorylation of STAT3 at Ser727 (Figure 3.3 A). However, co-treatment of HeLa cells with rotenone and NAC suppressed the rotenone-induced STAT3 phosphorylation (Figure 3.3A and B), indicating the importance of ROS in rotenone-induced STAT3 phosphorylation. Surprisingly, we find that NAC increased basal level phosphorylation of STAT3 (Figure 3.3A, Right Panel). Next, to determine whether NAC treatment also influence rotenone-induced mitochondrial translocation of STAT3, we monitored STAT3 levels in mitochondria isolated from HeLa cells treated with rotenone and NAC (Figure 3.3C). Immunoblotting of the mitochondrial samples with STAT3 and TOM20 antibodies showed that the NAC treatment reduced rotenone-induced accumulation of STAT3 in mitochondria, further providing evidence for the involvement of ROS (Figure 3.3C and D). Consistently, co-treatment of HeLa cells with NAC and rotenone reduces the mitochondrial abundance of ectopically expressed Myc-STAT3 upon rotenone treatment (Figure 3.3 E). Together our data provide compelling evidence that rotenone-induced ROS promotes Ser727 phosphorylation and mitochondrial localization of STAT3, possibly by activation of specific signaling pathways.

Figure 3.3. Rotenone-induced ROS promotes mitochondrial STAT3 accumulation: A-B) Representative image **(A)** and quantitative analysis **(B)** showing reduced Ser727 phosphorylation of STAT3 upon co-treatment with NAC and rotenone. HeLa cells pre-incubated with N-Acetyl Cysteine for 1hr were treated with rotenone and samples were immunoblotted with anti-pS727 STAT3 (pSTAT3) antibodies and Total STAT3. GAPDH was used as the loading control. Data were represented from three independent experiments. **C-D)** Co-treatment of NAC with rotenone also reduced rotenone mediated mitochondrial accumulation of STAT3. Mitochondrial and cytosolic fractions were prepared from HeLa cells treated with or without NAC and rotenone and immunoblotted with STAT3 specific antibodies. Antibodies to TOM20 and Tubulin were used as a loading control for mitochondria and cytosolic fraction respectively. Note that rotenone treatment increased mitochondria levels of STAT3 while quenching ROS levels by NAC co-treatment reduced mitochondrial STAT3 levels. **E)** Mitochondria was also isolated from cells expressing

wildtype-STAT3 myc and treated with only rotenone or with NAC to monitor the levels of ectopically expressed STAT3 in mitochondria. Error bar represent S.E and Student's t-test was performed between control and treated sample sets (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$)

Figure.3.3:



3.3.4 Cytoprotective effects of STAT3 during rotenone treatment

Rotenone promotes ROS production by inhibiting the activity of Complex I. It also exerts non-mitochondrial effects at higher concentrations. STAT3, on the other hand, was shown to protect mitochondria against oxidative stress and confers cytoprotective effects during ischemic injury. STAT3 also responds to oxidative stress by undergoing modification on cysteine residues [74].

Since rotenone promotes phosphorylation of STAT3 and its mitochondrial localization, we asked whether STAT3 has any role in rotenone-induced oxidative stress. To address this question, we knockdown endogenous STAT3 using STAT3 specific shRNA. STAT3 levels were reduced by more than 70% in cells transfected with STAT3 shRNA, while STAT3 levels remain unaltered in vector (scRNA) transfected cells (Figure 3.4 A). STAT3 knockdown cells display increased total ROS levels compared to control cells. Rotenone treatment further exacerbated this phenotype, as ROS levels are increased further in STAT3 knockdown cells (Figure 3.4 A). This suggests that STAT3 is required to maintain ROS levels under basal and rotenone treatment conditions.

To test the role of S727 in rotenone-induced ROS production, we measured ROS levels in HeLa cells expressing empty vector (mock) or plasmids harboring wild-type STAT3 or STAT3 S727A mutant after rotenone treatment. HeLa cells displayed increased ROS levels in the presence of rotenone, while cells expressing wild-type STAT3 did not show increased ROS levels with rotenone (Figure 3.4 B). However, over expression of the phospho-mutant form of STAT3 (S727A) failed to diminish rotenone-induced ROS levels, instead, had more ROS levels compared to rotenone treated cells (Figure 3.4 B). To ensure that the above observations were not due to differential expression of STAT3 constructs, we examined the steady-state level of the STAT3 from Wildtype and S727A expressing cells using Myc antibody. Expression of STAT3 remains the same in both wildtype and S727A mutant transfected cells (Figure 3.4 B). These results suggest the importance of STAT3 in handling rotenone-induced ROS production in a Ser727 dependent manner.

Since phosphorylation of STAT3 on Ser727 is required for its mitochondrial recruitment, we sought to determine whether the S727A mutant failed to recruit to mitochondria, thus could not mitigate rotenone-induced ROS production. To test this, cells expressing either wildtype or

S727A mutant were treated with rotenone, and levels of these proteins were monitored in mitochondria. As shown, earlier, the level of wild-type STAT3 was increased in mitochondria with rotenone treatment. Interestingly, S727A mutation, though did not affect its mitochondrial localization under normal conditions, it failed to display rotenone-induced accumulation (Figure 3.4 C), indicating the importance of S727 in rotenone-induced mitochondrial targeting.

Since rotenone is also associated with other aspects of oxidative stress at higher concentrations, we set out to understand whether mitochondrial localization of STAT3 can sufficiently exert cytoprotective effects during rotenone treatment. To address this, we over-expressed STAT3 fused to a mitochondrial targeting sequence of subunit 9 of COX VIIIa (MLS-STAT3) in HeLa cells. It has been previously shown that over-expressed MLS-STAT3 is targeted to mitochondria efficiently and has no effect on olfactomedin M induced gene expression [26]. HeLa cells over-expressing MLS-STAT3 were treated with rotenone and intracellular ROS levels were monitored. Targeted expression of STAT3 in mitochondria reduced rotenone-induced ROS generation (Figure 3.4 D). Since Ser727 was shown to be important for its effects, ROS levels were also monitored in cells expressing MLS-STAT3 S727A Y705F. Interestingly, the MLS-STAT3 S727A mutant also failed to reduce rotenone-induced levels, though it is still targeted to mitochondria even in the absence of MLS (Figure 3.4 C). This further indicates that Ser727 phosphorylation is indispensable for mitochondrial functions of STAT3 (Figure 3.4 D). Consistently, over expression of wildtype STAT3 rescued complex I activity in rotenone treated cells, while expression of STAT3 Ser727A offered no advantage to rotenone inhibited complex I activity (Figure 3.4 E). However, knockdown of STAT3 reduced complex I activity in both basal and rotenone treatment (Figure 3.4 F). Together, these results indicate that phosphorylation of

STAT3 is required for rotenone-induced mitochondrial targeting and alleviates rotenone-induced oxidative stress in a Ser727 dependent mechanism.

Figure.3.4:

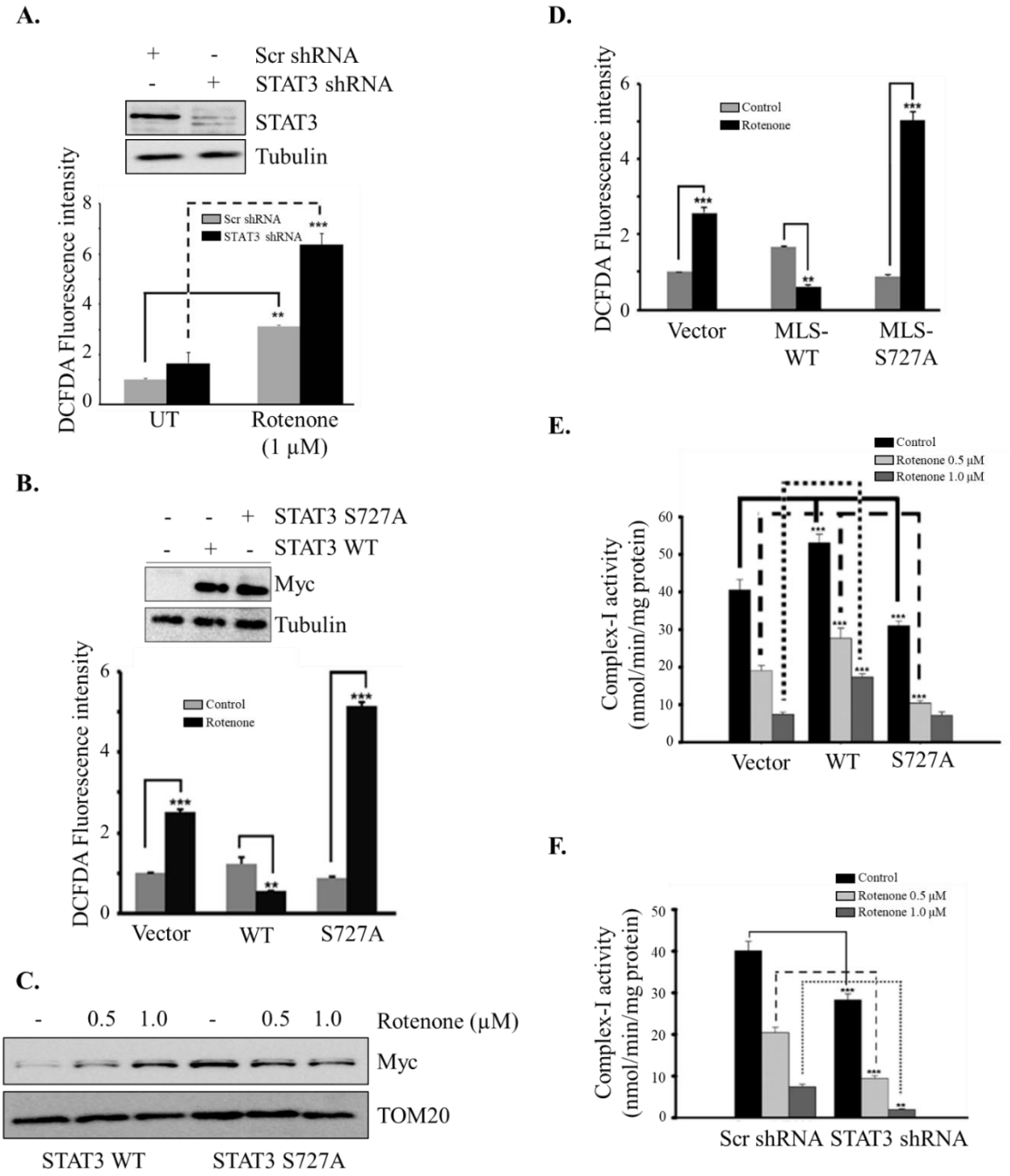


Figure 3.4. Over expression of STAT3 reduces rotenone-induced ROS production: (A) Quantitative analysis showing increased ROS levels in STAT3 knockdown cells compared to cells expressing Scr shRNA. HeLa cells were transfected with STAT3 specific shRNA or Scr shRNA. 24 h post-transfection, cells were treated with rotenone for 18 h. Intracellular ROS were measured using DCFDA followed by FACS analysis. . **(B)** Quantitative analysis showing ROS levels in cells expressing Wildtype STAT3 (WT) or S727A mutant. **(C)** Representative western blot showing the levels of WT and STAT3 Ser727A proteins in mitochondria enriched fractions upon rotenone treatment. **(D)** Quantitative analysis showing ROS levels in cells expressing Mitochondrial targeted wildtype STAT3 (MLS-WT) or S727A mutant (MLS-S727A) **(E-F)** Quantitative analysis showing STAT3 rescues rotenone mediated Complex I inhibition. Mitochondria were isolated from the cells expressing either STAT3 WT, S727A mutant, or STAT3 shRNA treated with or without rotenone to measure the complex I activity using decylubiquinone as terminal electron acceptor as mentioned in the methods section. **A, B, D, E,** and **F** are the representatives of three independent set of experiments showing the mean and standard deviation. Error bar represent S.E and Student's t-test was performed between control and treated sample sets (***, $p < 0.001$; **, $p < 0.01$; *, $p< 0.05$)

3.4 Discussion

STAT3 is an acute transcription factor, which responds to various external stimuli and translocates to the nucleus to regulate the expression of numerous genes involved in various cellular processes [62]. In addition to its well-known role in the nucleus, STAT3 is also required for the regulation of a myriad of mitochondrial functions. Mitochondrial STAT3 is involved in cellular respiration and is also important for cellular transformation. It was assumed that mitochondrial STAT3 confers cytoprotection, against ischemia and reperfusion, by preventing the leakage of electrons from complex I. Cells deleted for STAT3 exhibit decreased Complex I activity concomitant with an increase in intracellular ROS [24, 77]. In addition, mitochondrial localized STAT3 has been speculated to reduce ROS levels through the coupling of Complex I in ischemic models [26]. It has also been shown that mitochondrial STAT3 is required for super complex formation [30]. Most

of the mitochondrial functions of STAT3 seem to require phosphorylation of STAT3 on Ser727. Given the vast majority functions of STAT3 in mitochondria, signaling pathways converge on Ser727 to regulate its mitochondrial targeting and functions gained a lot of attention.

In this study, we found that rotenone promotes phosphorylation of STAT3 on Ser727 in a dose and time-dependent manner. Though phosphorylation of STAT3 at 0.5 μM rotenone increased dramatically, it marginally increases afterward. Interestingly, a negative correlation between pSer727 and pTyr705 was observed with rotenone treatment as shown before [78]. Reduced Ser727 phosphorylation of STAT3 is associated with a concomitant increase in Tyr705 phosphorylation, cell proliferation, and invasive properties of glioma cells [78]. Likewise, it is interesting to see whether rotenone-induced Ser727 phosphorylation compromises the nuclear functions of STAT3. Interestingly, rotenone treatment sensitizes NSCLC cells to TRAIL-induced apoptosis through mitochondrial ROS [79].

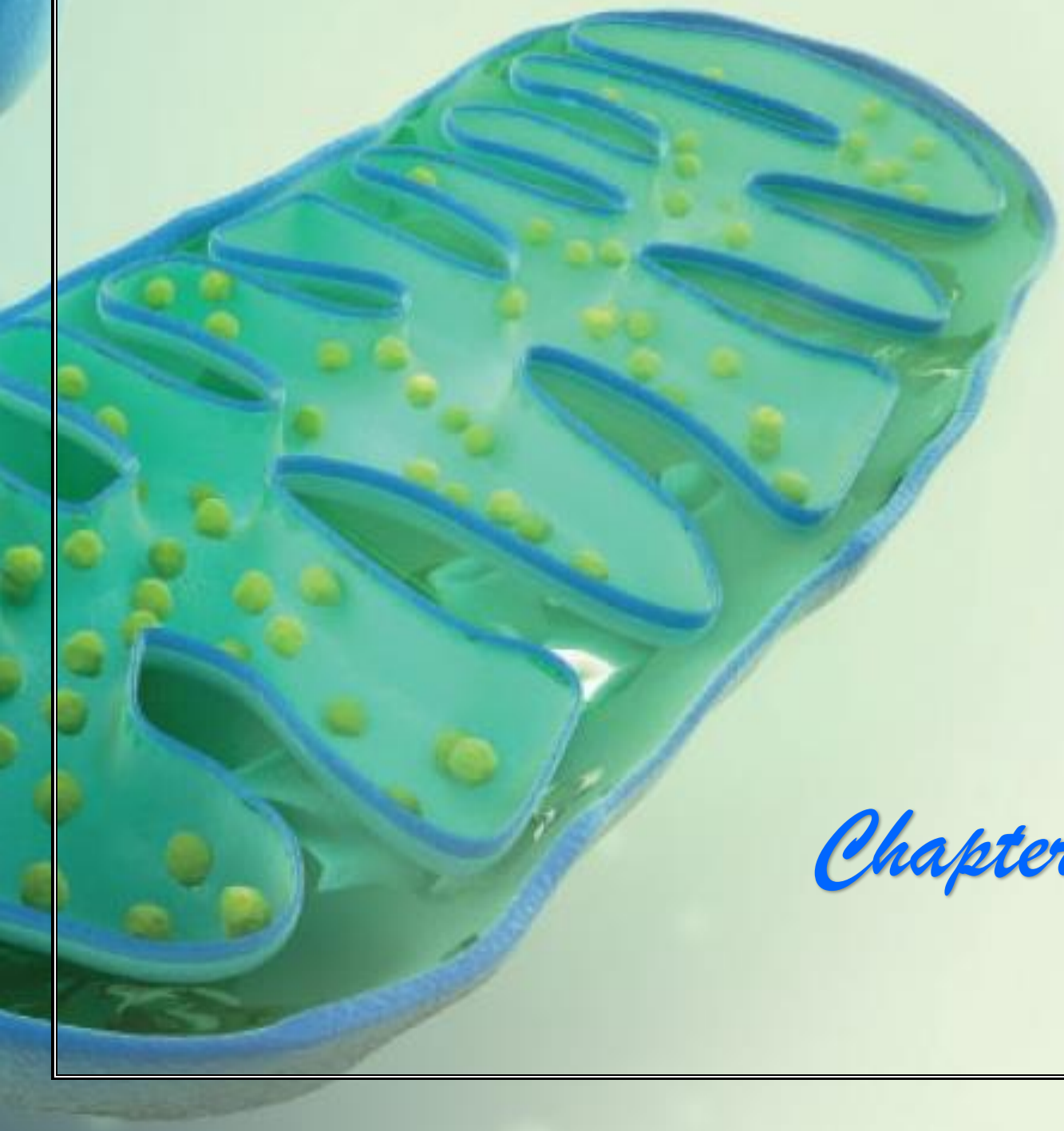
Given the involvement of STAT3 in diverse mitochondrial functions, dissecting targeting mechanisms would provide a better understanding of its function in this compartment. Our data suggest that rotenone treatment promoted mitochondrial targeting of STAT3, possibly through Ser727. Recent report suggests that oxidative stress and cytokine stimuli resulted in a rapid loss of mitochondrial STAT3. Nevertheless, recovery after stimulation requires Ser727 phosphorylation, indicating the importance of Ser727 in stress-induced mitochondrial targeting [80]. Consistently, our data also suggest that S727A mutation, though did not affect STAT3 mitochondrial localization, plays an important role in recruiting STAT3 to mitochondria during rotenone-induced oxidative insult. Since acetylation of STAT3 also promotes its mitochondrial localization [40], accumulation of S727A mutant in mitochondria, under normal conditions, likely suggests activation of alternative pathways to maintain mitochondrial STAT3 levels. However, more

studies are needed to address whether these pathways are operating parallel to each other or working together in a tightly regulated microcellular environment.

To address the functional importance of rotenone-induced mitochondrial targeting of STAT3, we monitored intracellular ROS levels. STAT3 seems to be important for handling rotenone-induced ROS production. STAT3 knockdown cells show higher levels of ROS, while over expression of STAT3 reduced ROS levels during rotenone treatment. Increased mitochondrial STAT3 is assumed to be important for redox homeostasis by regulating electron transport chain activity. Rotenone regulates intracellular ROS levels by inhibiting complex I activity and also perturbs microtubule dynamics at higher concentrations, independent of complex I. The concentrations required to disrupt microtubules vary among cell types. In our study, we did not observe microtubule alterations at 0.5-1 μM, while over 60% of cells are arrested in the mitotic phase at 2.5 μM (Data not shown). On the other hand, we observed increased mitochondrial localization of STAT3 with as low as 0.5 μM rotenone and levels sustained until 2.5 μM, suggesting mitochondrial targeting of STAT3 as an early event during rotenone treatment.

Complex I is involved in generating proton gradient and also is one of the main sites for the generation of ROS. It is known to be susceptible to a plethora of chemicals that include insecticides and pesticides present in the environment besides genetic changes in the nucleus and mitochondria. Our study suggests ROS generated by Complex I inhibition possibly induces downstream events that induces phosphorylation of STAT3 and its subsequent translocation to the mitochondria. Our study further suggests that STAT3 is part of a feedback loop to quench ROS and to maintain Complex I activity during cellular distress.

REGULATION OF G2/M PROGRESSION OF CELL CYCLE BY SER727 PHOSPHORYLATION AND MITOCHONDRIAL TRANSLOCATION OF STAT3



Chapter 4

4.1 Introduction

Cell cycle progression is a set of tightly regulated events regulated by the CDKs (Cyclin-Dependent Kinases) and their Cyclin counterparts [81]. The progression of the cell cycle demands high energy, and the cell has to produce the necessary amounts of ATP to cope with the division process. In addition, several studies suggest that the metabolic condition of the cell is also important for the cell to undergo the division process [82]. The cell cycle checkpoint machinery, CDKs, and the respective cyclins have been widely studied, but how these checkpoint machineries are interlinked with the biosynthetic demand of the cell is still needs to be further explored.

Mitochondria are the primary organelles that provide the ATP required for the cell. Accumulating evidence suggests that besides providing ATP, mitochondrial metabolic status and dynamics are intricately linked with the cell division process [9]. Mitochondria constantly undergo remodeling by the fusion and fission reactions; the rate of these fusion/fission reactions and the form of the mitochondria are determined by the cell cycle stage [83]. During the cell cycle, mitochondria change their shape from an interconnected network in the G1 phase to the hyperfused network in the G1/S phase and a fragmented network in the mitotic phase [42]. Changes in the mitochondrial respiration and metabolic status regulate the cell cycle, and the alteration in the regulators control the mitochondrial form and function. G1/S and G2/M phases of the cell cycle are the most energy-dependent phases of the cell cycle. To maintain the energetic requirements of Mitosis, mitochondrial mass and membrane potential are increased during the G1/S phase until the late mitotic phase [84]. In Drosophila, it has been shown that low amounts of ATP decrease Cyclin E expression and arrest the cells in the G1 phase. Overexpression of Cyclin E in cells with low energy levels relieved the G1/S arrest [50]. Cyclin E also controls a hyperfused tubular network of

mitochondria during the G1/S phase [10]. Cyclin D governs the mitochondrial metabolic status during the G1 phase [44]. CyclinB1/CDK1 phosphorylates DRP1, which modulated fission during the mitosis [49]. CyclinB1/CDK1 translocate to mitochondria and phosphorylates several complex-I proteins, enhancing the G2/M progression [54].

STAT3 is one of the STAT family members of transcription factors, which regulates various critical cellular processes upon activation by cytokines. Upon stimulation, STAT3 undergoes phosphorylation at two residues at its C-terminus (Tyr705 and Ser727). Tyr705 phosphorylation of STAT3 induces the translocation of STAT3 either in a homo or heterodimeric form to the nucleus and activates the genes involved in many cellular events like growth, development, and apoptosis [62]. The nuclear functions of STAT3 have been widely studied. On the contrary, non-nuclear functions of STAT3 attributed to the phosphorylation at Ser727 of STAT3. Ser727 phosphorylated STAT3 translocates to mitochondria and enhances the activity of mitochondrial complex-I [24, 26]. STAT3 phosphorylation at Ser727 by microtubule inhibitors is also important for the onset of mitotic arrest [85]. Further, downregulation of STAT3 causes mitochondrial-dependent G2/M arrest [86]. Though nuclear functions of STAT3 with respect to cell cycle regulation are well studied, significantly less is known about how the mitochondrial pool of STAT3 regulates the cell cycle proliferation.

In this study, we explored to understand the mitochondrial STAT3 in modulating the cell cycle progression, in particular the G2/M phase of the cell cycle. We found that STAT3 is phosphorylated at ser727 possibly through CDK1, and this phosphorylation is a mitotic event. In addition, a decreased cyclin B1 turnover in STAT3 knockdown cells arrested the cells at the G2/M phase. Our results suggest that STAT3 enhances the G2/M progression by probably regulating the functions of mitochondrial Complex-I.

4.2 Methodology

4.2.1 Antibodies and Reagents

Antibodies specific to phospho Ser727 in STAT3 (SAB4300034) and Actin (A3854) were purchased from Sigma Aldrich, USA; Cyclin B1 (#12231), pSer10 H3 (#3377), Cyclin A2 (#4656), Cyclin E2 (#4132), CDK1 and GAPDH (CST 8884) were purchased from Cell Signaling Technologies, USA; antibodies against STAT3 (ab119352), Myc (ab9106), TOM40 (ab185543) and Tubulin (ab6046) were purchased from Abcam. Thymidine, Nocodazole, RO-3306, TMRE, and Propidium Iodide were from Sigma Aldrich, Mito Tracker Red CMXRos, and CM-H2DCFDA were from Thermo Scientific. Protease and Phosphatase inhibitor cocktails were from Roche. Other chemicals used in this study were either obtained from Sigma Aldrich or Ameresco.

4.2.2 Plasmid constructs and shRNA

Plasmids used were described in methods of chapter II. Briefly, Plasmids containing STAT3 WT and STAT3 S727A:Y705F and STAT3 S727D:Y705 were cloned into pcDNA 3.1 A+ vector and to target STAT3 to mitochondria, mitochondrial targeting sequence of COX-VIIIa was tagged to N-terminus of STAT3 coding sequence in pCDNA 31. A+ vector. STAT3 specific shRNA pSIH-puro-STAT3 was purchased from Addgene [69] and pLKO.1 Puro STAT3 shRNA was purchased from Dr. Subba Rao Gangi Setty lab IISC.

Table 3.

S.No	Name	Cat. No	Sequence	
1	STAT3 shRNA	Cat-26596	gatccGCATCTGCCTAGATCGGCTATTCAAG AGATAGCCGATCTAGGCAGATGTTTTTTg	Addgene
2	STAT3 shRNA	GS Lab	CCGGCATCTGAAACTACTAACTTTGCTCG AGCAAAGTTAGTAGTTTCAGATGTTTTTG	IISC Banglore

4.2.3 Cell culture and DNA transfections

HeLa cells were grown in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and cultured at 37°C in a 5% CO2 incubator. HeLa cells were grown to 80% confluence in 60 mm dishes at 37°C in a humidified atmosphere of 5% CO_2. Cells were transiently transfected with plasmid by Lipofectamine T2000 reagent (Invitrogen) following the manufacturer's instruction. After 12-48 hours, expression was confirmed either by immunoblotting or indirect immunofluorescence.

4.2.4 Generation of STAT3 shRNA Stable cell lines

At 40 to 50 % confluency, HEK 293T cells were transfected with lentiviral STAT3 shRNA plasmids along with lentiviral packaging plasmids (pRev, pΔR, and pVsVg). After 24 hours, viral supernatant was collected, and then fresh complete medium was added. This process was repeated three times for 72 hours. Viral supernatants were passed through 0.45µM filters and added to HeLa cells at a ratio of 1:1 with complete medium containing polybrene (10 µg/ml). After transduction of the HeLa, stable cells were selected using puromycin (1 µg/ml). After selecting the stable cells, the knockdown of the gene was confirmed by western blotting.

4.2.5 Isolation of Mitochondria from the cell lines

HeLa cells were harvested and washed twice with 1X PBS. Cells were resuspended in mitochondria isolation buffer (20 mM HEPES pH 7.5, 1.5 mM MgCl2, 1 mM EDTA pH 8.0, 1 mM EGTA, 210 mM sucrose and 70 mM mannitol with protease inhibitors). The cell suspension was homogenized using a Dounce homogenizer for about 50 strokes and the homogenate was centrifuged at 600xg for 10 min. The resulting supernatant was centrifuged at 10,000xg for 15 min to obtain a mitochondrial pellet, Supernatant fraction was used as the cytosolic fraction. Pellet was

washed twice and resuspended in mitochondrial resuspension buffer (10 mM HEPES-KOH pH 7.4, 250 mM Sucrose, and 1.5 mM $MgCl_2$).

4.2.6 Cell synchronization by double thymidine or nocodazole block

To synchronize the cells in the G1/S phase, when HeLa cells attained 60% confluency, 2mM thymidine was added to the growth medium, and cells were further incubated for 18 hr. After 18 hours, cells were released from the first thymidine block for 9 hours in growth medium. A second thymidine block was initiated by adding 2 mM thymidine in growth medium and incubated for 15 hr. Cells were released from the double thymidine block and harvested for the indicated time points.

Nocodazole or CDK1 inhibitor (RO-3306) was used to synchronize the cells in the G2/M phase. For Nocodazole-mediated G2/M arrest, 2 mM thymidine was added to the growth medium, and cells were incubated for 24 hrs. Cells were released from the thymidine block after 3 hours and then nocodazole (100 ng/ml) was added to the growth medium and cells were further grown for 12 hr. Then the cells were released from the nocodazole block and harvested for the indicated time points. For RO-3306 mediated G2/M arrest, after treating the cells with 2 mM Thymidine, cells were released from thymidine block for 3 to 4 hours, and cells were further treated with 9 µM RO-3306 for 12 hr. The cells were then released and harvested at the given time points.

4.2.7 Cell cycle assay

The distribution of cells in various phases of the cell cycle (G0/G1, S, and the G2/M) was analyzed using a flow cytometer by quantifying the DNA content stained with PI. HeLa cells were harvested by trypsinization, washed with chilled phosphate-buffered saline (PBS) containing 4mmol/L EDTA, and fixed with 70% cold ethanol at 4°C for 30 min. After fixation, the cells were collected by centrifugation and washed once with PBS. The pellets were suspended and incubated in PBS

containing 20 µl/ml of PI and 40 µg/ml RNaseA at 4°C for at least 30 min. Finally, the cycle phase distribution was assessed by flow cytometry.

4.2.8 Immunoblotting

HeLa cells were lysed in RIPA lysis buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 1 mM PMSF, and protease inhibitor cocktail). Equal amounts of protein were subjected to SDS-PAGE (12%), then transferred onto the nitrocellulose membrane. Membranes were blocked using 3% milk in TBS (3% BSA for phospho-antibodies) and incubated overnight at 4°C with the appropriate primary antibody, followed by three 15min washes in 1x TBST buffer. Membranes were probed with secondary antibody labeled with HRP in 3% milk in TBS, followed by three 15min washes in 1x TBST buffer. The blots were developed by using an ECL detection kit (GE Healthcare), and the signal was detected using Bio-Rad Versadoc.

4.2.9 Confocal Microscopy

HeLa cells were seeded onto glass coverslips in a six-well tissue culture plate. Post-transfection, cells were fixed with 4% paraformaldehyde (PFA) in 1x PBS for 20 min at room temperature (RT), followed by three 5 minutes washes in 1x TBS. Cells were permeabilized at RT with pre-chilled acetone and methanol (1:3) for 20 minutes, followed by three 10 minutes washes in 1x TBS. Cells were blocked in 3% BSA in 1x TBS. Cells were probed with respective primary antibodies for 2 hours, followed by three 15 minutes washes in TBS, TBST, and TBS buffer. Fluorescent labeled secondary antibodies were added and incubated at room temperature for 1 hour, followed by three 15 minutes washes in TBS, TBST, and TBS buffer. The coverslips were mounted on a glass slide using antifade reagent with DAPI (Invitrogen). The images were taken using a Leica microscope. For live-cell imaging, cells were seeded in a 35 mm glass-bottom dish, and then after

synchronization, cells were imaged under a fluorescence microscope attached with live cell chamber. Images were captured at 10 min intervals and movies were made using Image J software.

4.2.10 Measurement of ROS and mitochondrial membrane potential

After the indicated treatment, HeLa cells were incubated with 10 µM CMH2DCF-DA for 30 min at 37ºC. Then cells were collected, washed, and re-suspended in PBS. For analyzing mitochondrial membrane potential, post-treatment cells were incubated with 400 nM TMRE (Tetramethylrhodamine, ethyl ester) for 20 – 30 min at 37ºC and harvested. Fluorescently stained cells were sorted using FACS caliber (BD biosciences) and analyzed using Flow Jo software.

4.2.11 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 were incubated with 5 mM NADH in a buffer containing 25 mM potassium phosphate pH 7.4, 5 mM $MgCl_2$, 0.05% Triton X-100 and 0.25% BSA for 1 min at 37^0C. The activity assay was initiated by adding 3 mM decylubiquinone and the decrease in absorbance at 340 nm was measured. Complex I activity was calculated by using the reaction velocity (Δabsorbance/min) and the molar extinction coefficient of NADH (6.2 $mM^{-1}cm^{-1}$ at 340 nm with reference wavelength).

4.3 Results

4.3.1 Rotenone arrests the cells at the G2/M phase of the cell cycle.

Rotenone is a mitochondrial complex-I inhibitor and has been widely used to develop and understand mitochondrial disorders. High concentrations of rotenone arrest cell cycle at the G2/M phase [87, 88]. We have shown that rotenone induces the phosphorylation of STAT3 at ser727

[89]. To check whether rotenone-induced phosphorylation of STAT3 at ser727 and cell cycle arrest have any correlation, we treated the HeLa cells with increasing concentrations of rotenone (0.05 – 2.5 µM) and analyzed the DNA content by FACS as described in the Methods. Results show an increase in the cell population at the G2/M state (Figure 4.1 A). To further see the correlation between phosphorylation of STAT3 and the G2/M cell cycle arrest, cells were treated with an increasing concentration of rotenone, and cell lysates were subjected to western blotting. We find that Ser727 phosphorylation of STAT3 increased with increasing concentrations of rotenone. Further, we find an increase in STAT3 phosphorylation with the increase in Cyclin B1 levels (Figure 4.1 B) (which is a marker for the G2/M phase of the cell cycle), suggesting a correlation between phosphorylation of STAT3 and the G2/M arrest of the cell cycle.

Figure.4.1:

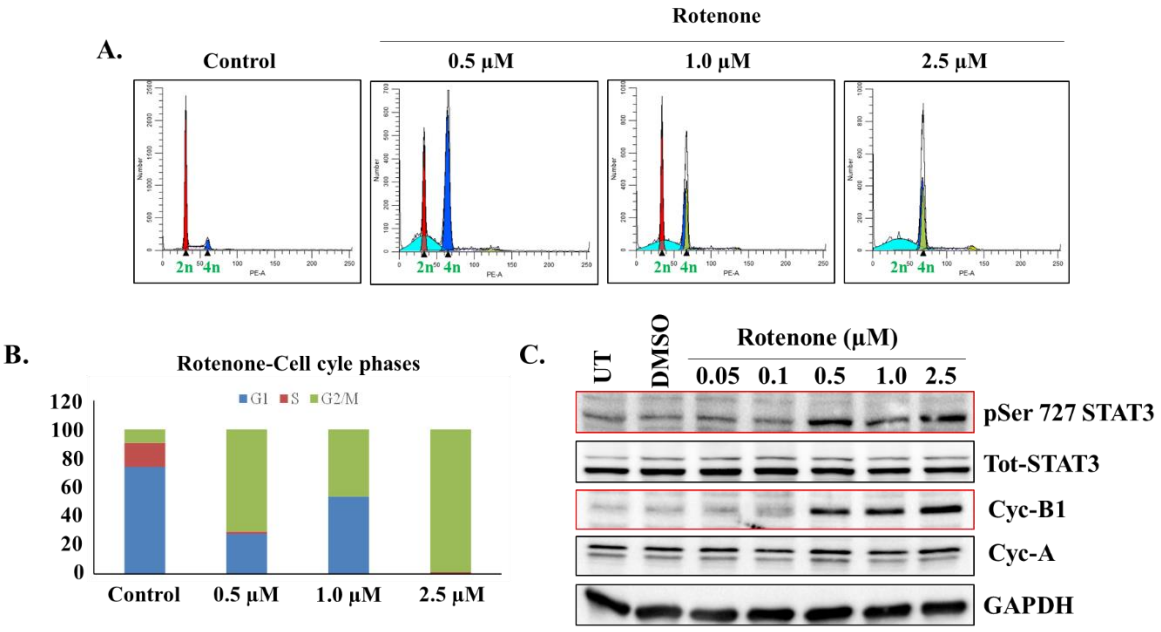


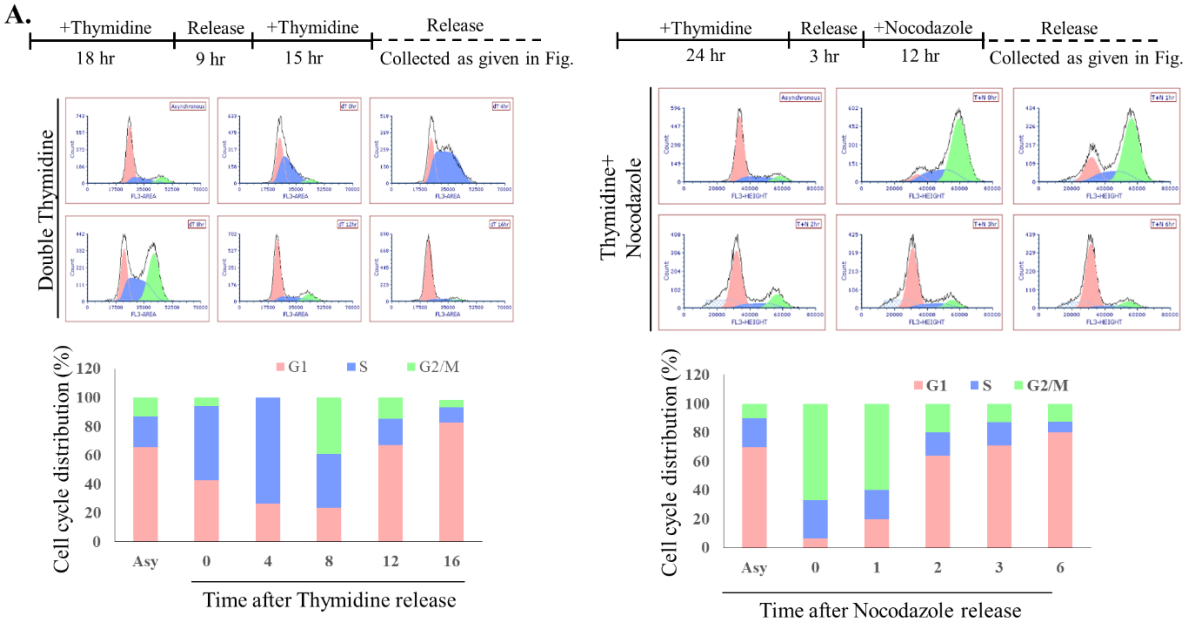
Figure 4.1. Rotenone arrests the cells at the G2/M phase of the cell cycle: A. HeLa cells were treated with indicated concentrations of rotenone for 18 hr. Cell cycle distribution of PI-stained HeLa cells was analyzed by flow cytometry (FACS). **B.** Represents the quantification of FACS analysis. **C.** HeLa cells

were treated with increasing concentrations of rotenone and subjected to western blotting and probed with the indicated antibodies.

4.3.2 Phosphorylation of STAT3 at ser727 is associated with the G2/M phase of the cell cycle.

As phosphorylation of STAT3 and cell cycle arrest at the G2/M is observed. To further ascertain whether Ser727 phosphorylation of STAT3 is a mitotic event, we synchronized the HeLa cells either with double thymidine block or thymidine-nocodazole block as described in the Methods (Figure 4.2 A). After synchronization, cells were released and harvested at indicated time points, and cell lysates were analyzed by western blotting. We find an increase in STAT3 Ser727 phosphorylation, Cyclin B1, and pH3 Ser10 levels at specific time points(8, 10 & 12 hr time points) However, there is no change in total STAT3 protein levels, indicating the phosphorylation of STAT3 is the G2/M associated cell cycle event (Figure 4.2 B and C). Interestingly, we also find an increase in Cyclin E levels at 0 hr time point where phosphorylation of STAT3 is observed (Figure 4.2 B). These results further suggest that a biphasic increase in STAT3 phosphorylation might be important to regulate both the G1/S and the G2/M phases of the cell cycle.

Figure.4.2:



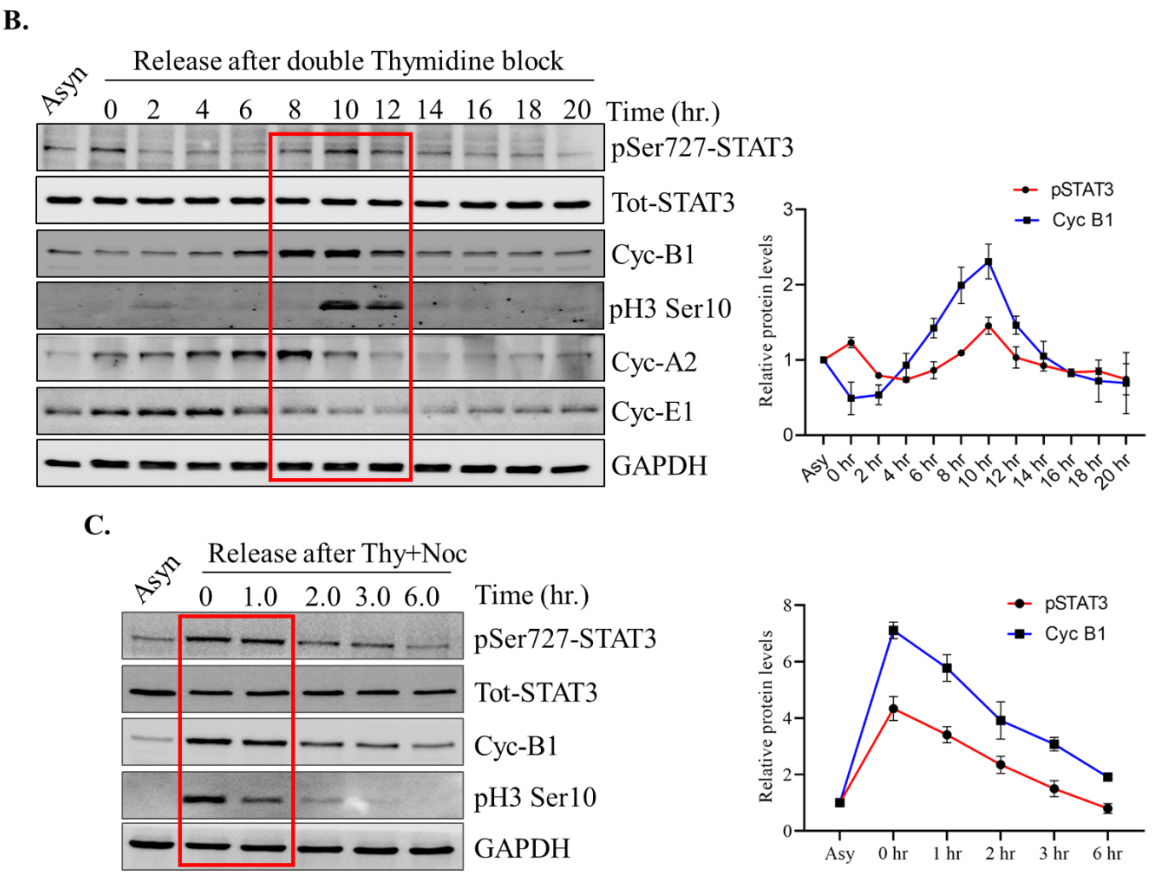


Figure 4.2. Phosphorylation of STAT3 at ser727 is associated with the G2/M phase of the cell cycle: A. HeLa cells were synchronized at the G1/S phase by double thymidine block or G2/M phase by using nocodazole and the cells were released into fresh medium and collected at different time points. Cell cycle distribution of synchronized cells was analyzed by collecting cells at indicated time points and subjected to flow cytometry. **B.** After Synchronizing, cells were released and collected at different time points, and the lysates were probed with indicated antibodies. **C.** Cells were synchronized with nocodazole and the lysates were probed with antibodies mentioned. The experiment was repeated three times independently and quantified. Error bars represent standard error and Student's t-test was performed to compare the samples.

4.3.3 Knockdown of STAT3 delayed G2/M progression of Cell cycle.

Previously, it was reported that STAT3 downregulation induces cell cycle arrests at the G2/M in esophageal carcinoma cells [86]. To further confirm this phenomenon in our system, we used two different STAT3 shRNAs to generate the STAT3 knockdown stable cell lines as described in the Methods. HeLa STAT3 shRNA stables were confirmed by western blotting, and the DNA content

was assessed using FACS. In support with previous reports, we find that knockdown of STAT3 increases the G2/M cell population (Figure 4.3 A). In addition, cell lysates were probed with two cell cycle markers proteins, Cyclin B1 (which accumulates during G2/M) and Cyclin E (which increases during the S phase). STAT3 knockdown cells displayed increased Cyclin B1 levels when compared to control cells (Figure 4.3 B). This increase in Cyclin B1 levels and FACS analysis suggests that knockdown of STAT3 arrests the cells at the G2/M transition.

Figure.4.3:

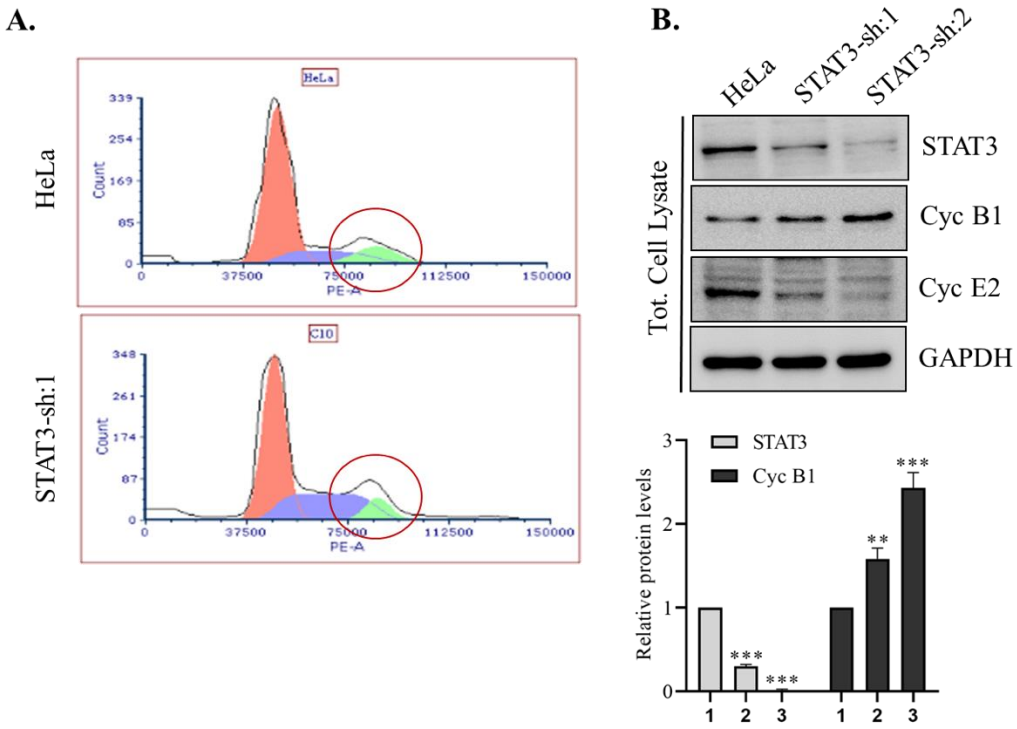


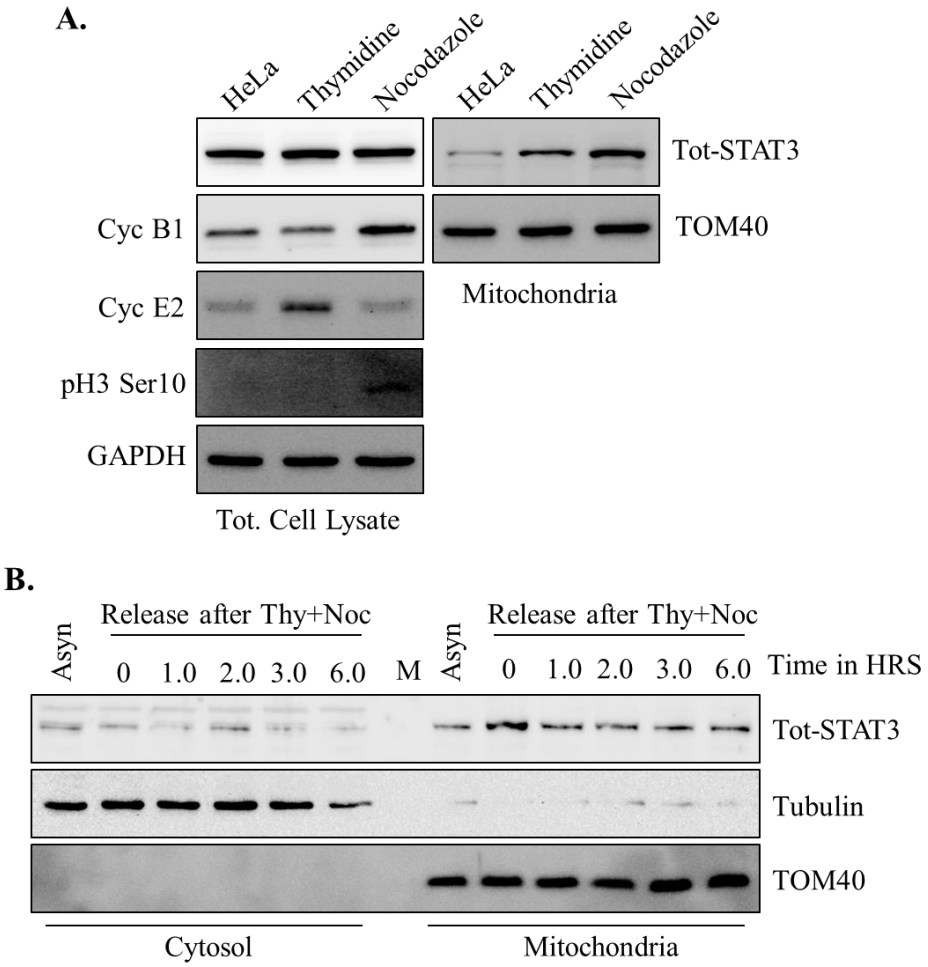
Figure 4.3. Knockdown of STAT3 delayed G2/M progression of Cell cycle. (Arrests cells at the G2/M): A. Stable cells of STAT3 shRNA-1 in HeLa cells were generated as described in methods. HeLa and STAT3 shRNA-1 cells were analyzed for DNA content using FACS. **B.** HeLa, STAT3 shRNA-1, and STAT3 shRNA-2 cells were lysed, western transferred, and probed with indicated antibodies. The experiment was repeated three times independently and quantified. Error bars represent standard error and Student's t-test was performed to compare the samples.

4.3.4 Translocation of STAT3 to mitochondria during the G2/M phase.

As our earlier results indicated, rotenone-induced phosphorylation of STAT3 at ser727 triggers STAT3 translocation to mitochondria [89]. We wanted to test whether the G2/M cell cycle phase associated phosphorylation of STAT3 at ser727 induces the translocation of STAT3 to mitochondria. HeLa cells were synchronized either with both double Thymidine block (G1/S phase) or with Nocodazole block (G2/M), and the cells were sub-fractionated into mitochondria and cytosol. These fractions and total cell lysates were subjected to western blotting and probed with cell cycle and mitochondrial markers. Steady-state levels of Cyclin A, B, E, and pH3 Ser10 in total cell lysates confirm the proper synchronization of cells either at the G1/S phase or the G2/M phase (Figure 4.4 A). We find an increase in STAT3 levels in mitochondria at the G2/M arrested cells compared to Asynchronous control cells (Figure 4.4 A). To further check if the G2/M phase mediated localization of STAT3 to mitochondria is specific, Thymidine + Nocodazole synchronized HeLa were released at different time points and subjected to sub fractionation. We find an increase in mitochondrial localization of STAT3 at the G2/M phase with further decrease in STAT3 levels in mitochondria as the cells released from the G2/M arrest (Figure 4.4 B). Taken together, these results suggest that STAT3 translocates to mitochondria during the G2/M cell cycle phase.

Figure 4.4. Translocation of STAT3 to mitochondria during G2/M phase: A. HeLa cells were synchronized either at G1/S phase (double thymidine block) or G2/M phase (Thymidine-Nocodazole block). After synchronization cells were collected, total cell lysates and mitochondria were isolated, western transferred, and probed with total STAT3, TOM40. CycB1 and pH3 Ser10 (G2/M) CycE2 (G1/S) were used as synchronization controls and GAPDH was used to show equal loading. **B.** HeLa cells were synchronized with Thymidine-Nocodazole block and released at indicated time points. Cells were harvested, sub-fractionated into cytosol and mitochondria, western transferred, and probed with the antibodies mentioned. Tubulin and Tom40 levels serve as cytosolic and mitochondria loading controls, respectively.

Figure.4.4:



4.3.5 Knockdown of STAT3 delays exit of cells from Mitosis.

As knockdown of STAT3 results in an increased population at the G2/M phase, we intend to explore the role of STAT3 in cell cycle progression. We synchronized the Hela and STAT3 knockdown cells with nocodazole block (G2/M arrest). These cells were released and assessed the cell cycle progression using Leica SP8 microscope. Our live-cell imaging results demonstrate a delay in the exit of the cells from mitosis in STAT3 knockdown cells compared to control cells (Figure 4.5).

Figure.4.5:

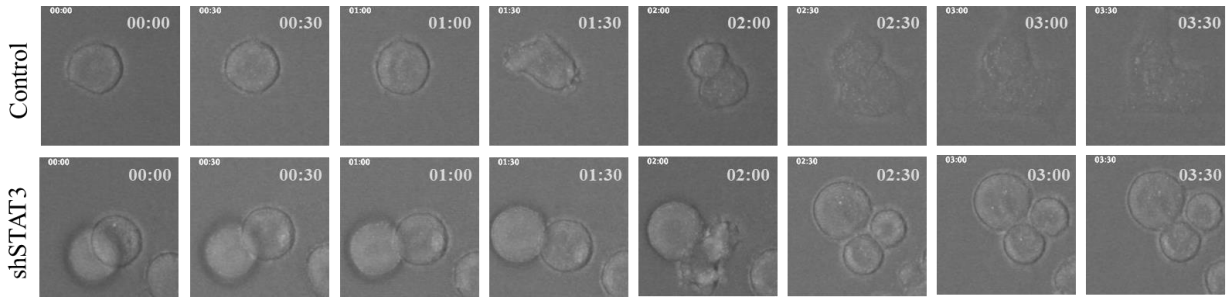


Figure 4.5. Knockdown of STAT3 delays exit cells from Mitosis: HeLa and shSTAT3 stable cell lines were synchronized at the G2/M phase (Thymidine-Nocodazole block) and released into fresh medium. Time-lapse imaging of synchronized cells after releasing into fresh media was performed at 60X magnification using Leica SP6 system attached with OKO live cell imaging set up and CO2 cylinder. Images were analyzed Image J (Fiji) software.

4.3.6 CDK1 phosphorylates STAT3 at ser727 and regulates its mitochondrial localization.

CDK1 (CDC2) and cyclin B1 regulate the entry of cells into the G2/M phase and the progression of cells through mitosis [90, 91]. Different CDKs control the cell cycle progression at different stages. Our results so far illustrated that STAT3 gets phosphorylated at Ser727 in the G2/M phase-dependent manner. We further wanted to explore whether CDK1 is involved in the phosphorylation of STAT3 and its subsequent mitochondrial localization. RO-3306 - a selective inhibitor of CDK1, known to arrest the cells at the G2/M phase [92], was used to determine whether CDK1 phosphorylates STAT3 at ser727. We find a decrease in Ser727 phosphorylation of STAT3 in RO-3306 synchronized cells compared to nocodazole treated cells. Further, a decrease in STAT3 Ser727 phosphorylation is also observed in the presence of both nocodazole and RO-3306 compared to cells treated with nocodazole alone (Figure 4.6 A). We further explored whether CDK1 dependent phosphorylation of STAT3 is required for the translocation of STAT3 to mitochondria. HeLa cells were synchronized either with nocodazole or RO-3306, or both nocodazole/RO-3306. Cells were further sub-fractionated into mitochondria and cytosol. We

found a decrease in STAT3 translocation to mitochondria when cells were treated with RO-3306 or both Nocoazole and RO-3306 compared to Nocodazole alone treated cells (Figure 4.6 B). These results suggest that CDK1 phosphorylates STAT3 at ser727 and translocates STAT3 to mitochondria during the G2/M phase.

Figure.4.6:

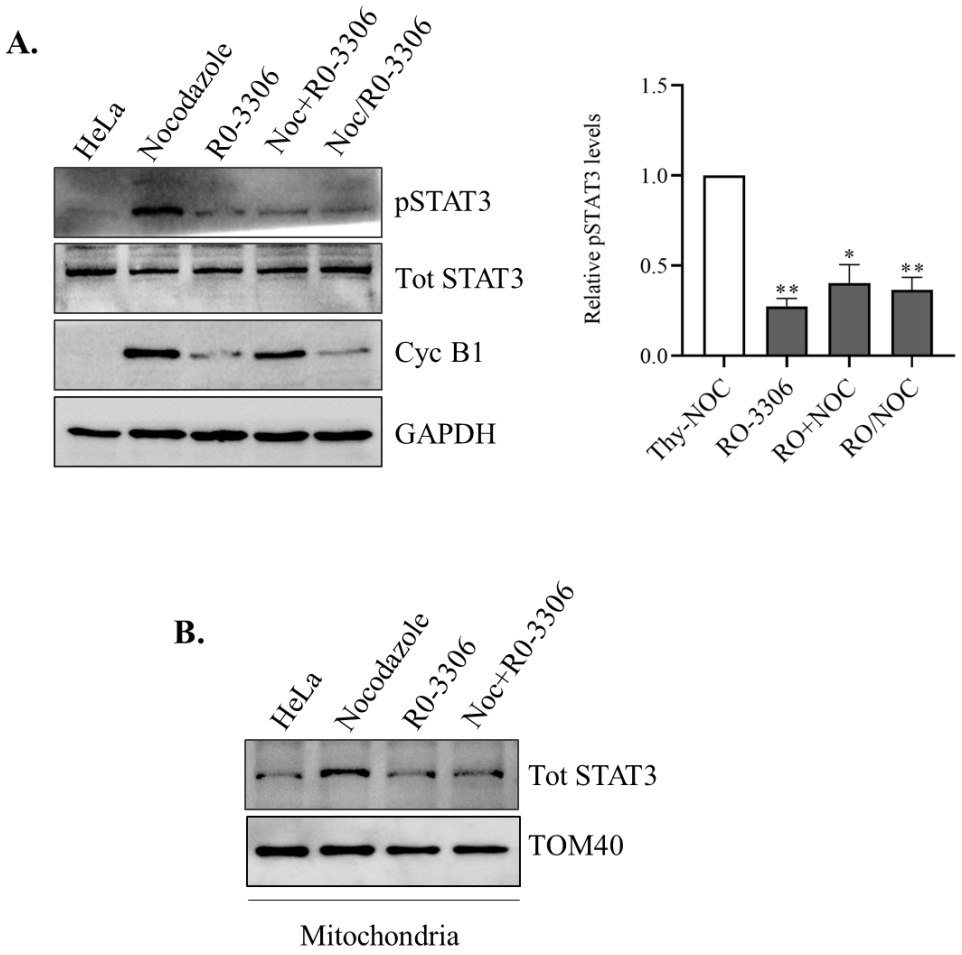


Figure 4.6. CDK1 phosphorylates STAT3 at ser727 and regulates its mitochondrial localization: A. HeLa cells were treated with Thymidine-Nocodazole or Thymidine-RO-3306 to synchronize cells at G2/M phase (in lane 4 Nocodazole and RO-3306 were added simultaneously, in lane 5 RO-3306 was added and after cells were treated with nocodazole). After synchronization cells were collected, lysed, western transferred, and immunoblotted with antibodies mentioned. The experiment was repeated three times independently and the data was quantified. **B.** After synchronization by either Nocodazole or RO-3306 and

both Nocodazole and RO-3306 (lane 4), cells were collected and sub-fractionated into mitochondria and after western transfer immunoblotting with the indicated antibodies was performed. The experiment was repeated three times independently and quantified. Error bars represent standard error and Student's t-test was performed to compare the samples.

4.3.7 Knockdown of STAT3 displays decreased Cyclin B1 and pH3 Ser10 turnover.

Activation of CDK1 and induction of Cyclin B1 as well are important for the successful onset of mitosis. Once the cells have entered into mitosis, CDK1 gets inactivated, and the degradation of Cyclin B1 takes place. In our case, we found a delay in mitotic exit in the STAT3 knockdown cells. We further explored the turnover rates of Cyclin B1 and pH3 Ser10 levels in WT and STAT3 knockdown cells after releasing from Nocodazole (Figure 4.7 A) or RO-3306 (Figure 4.7 B) block. We find a reduced Cyclin B1 degradation and dephosphorylation of pH3 Ser10 or turnover in STAT3 knockdown cells compared to control cells.

Figure.4.7:

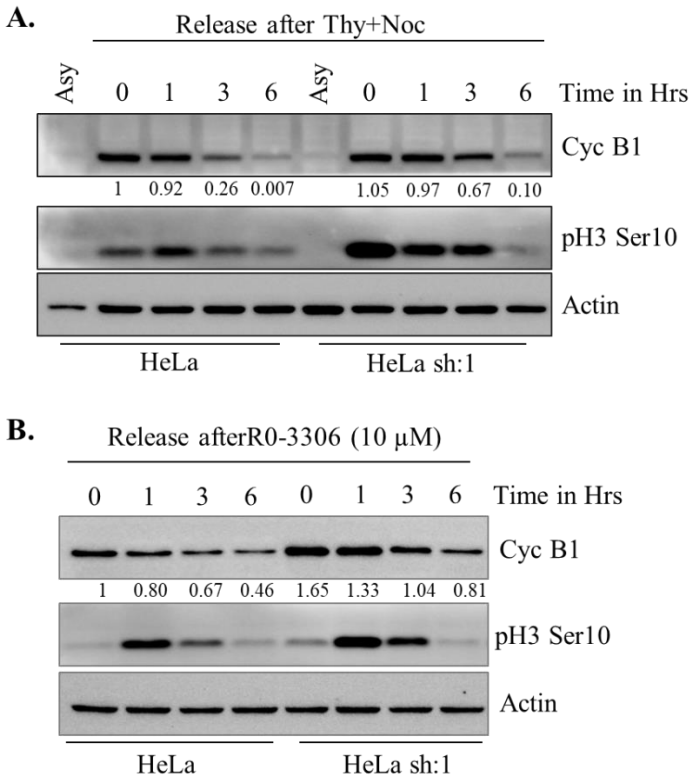


Figure 4.7. Knockdown of STAT3 displays decreased Cyclin B1 turnover: A. HeLa cells were synchronized by thymidine-nocodazole at the G2/M phase, released into fresh media, and collected at the given time points, lysed, western transferred, and probed with Cyc B1, pH3 Ser10, and actin antibodies. **B.** After synchronization of HeLa cells at the G2/M phase using thymidine-RO-3306 block, cells were collected at the given time points, lysed, and probed with the antibodies mentioned.

4.4 Discussion:

The nuclear transcription functions of STAT3 are well documented. Increasing evidence suggests that the pool of STAT3 is also present in mitochondria [24, 38, 89]. STAT3 is shown to be associated with the Complex-I of the electron transport chain [24, 26]. Downregulation of STAT3 reduced the mitochondrial complex-I activity and increased ROS production [89]. Mitochondrial STAT3 has been shown to reduce ROS levels. It is shown that there is a relation between clustered localization of STAT3 and cell cycle behavior [93]. Cells depleted with STAT3 were shown to inhibit the cell cycle at the G1/S phase in esophageal carcinoma cells (ECA109 cells) [94]. In another set of esophageal carcinoma cells (TE-1 cells), depletion of STAT3 arrested the cell cycle at the G2/M phase [86]. Nevertheless, in both studies, the inhibition of the cell cycle is shown to be mitochondrial-dependent.

Cell division is regulated at different stages and critical events are at G1/S or G2/M transition. This regulation in the cell cycle is mediated by the CDKs and their cyclin partners. The commitment of a cell to divide is an energy-dependent process, and cells have to generate enough energy (ATP) required to meet the division process [8, 9]. In a cell, mitochondria are the primary source of ATP production. Apart from generating ATP, studies have shown that the mitochondrial form (shape) and its metabolic status also regulate the cell division process [95, 96]. For instance, during the G1/S phase, mitochondria are shown to form the extended tubular networks [10].

Depletion of ATP arrested the cells in the G1 phase, and overexpression of Cyclin E relieves the ATP-dependent arrest at the G1 phase [50]. Much is known about the energetic requirements at the G1/S transition (Restriction point), but very little about the G2/M energy demands. In colon cancer, it is shown that the G2/M energy requirements are met by mitochondrial ATP, whereas in the G1 phase, Glycolysis meets most of the energy demands [97]. Interestingly, CDK1/Cyclin B1 was translocated to mitochondria and enhanced the mitochondrial complex-I functions during the G2/M transition [54].

As a fraction of STAT3 is present in mitochondria and phosphorylation of Ser727 STAT3 is important for the G2/M progression [85, 86], in this study, we tried to address whether (mitochondrial pool) mitochondrial translocation of STAT3 is important for the G2/M progression of the cell cycle. As reported previously, we found that STAT3 gets phosphorylated at ser727 during the G2/M progression of the cell cycle. Our results also suggest ser727 phosphorylation of STAT3 occurs at the G1/S phase indicating that Ser727 phosphorylation is probably essential in driving both the G1/S and the G2/M progression. STAT3 ser727 phosphorylation is indeed important for mitochondrial translocation and mitochondrial functions of STAT3. Our cell synchronization and sub-fractionation assays demonstrate that G2/M dependent phosphorylation of STAT3 at ser727 also drives the translocation of STAT3 to mitochondria. Depletion of STAT3 was found to be arresting the cell cycle at the G2/M phase [86]. The successful transit of the cell from the G2/M phase requires the activation of CDK1 and a concomitant increase in Cyclin B levels. After the successful G2/M transition, cells enter into mitosis, inactivation of CDK1, and initiation of Cyclin B degradation occurs. CDK1 is the major kinase during the G2/M cell cycle progression. We found that the addition of RO-3306, a specific CDK1 inhibitor, reduced the phosphorylation at ser727 of STAT3, indicating that STAT3 might be a substrate for CDK1.

Further, kinetic analysis of Cyclin B1 after release either from Nocodazole or RO-3306 block revealed that knockdown of STAT3 delays Cyclin B1 turnover. Similar results were observed in the pH3 Ser10 turnover. In addition, live-cell imaging data suggested that knockdown of STAT3 delayed the mitotic exit of cells. Our results pointed out that the G2/M-driven mitochondrial translocation of STAT3 is probably required for maintaining the mitochondrial functions to facilitate the G2/M progression of the cell cycle.

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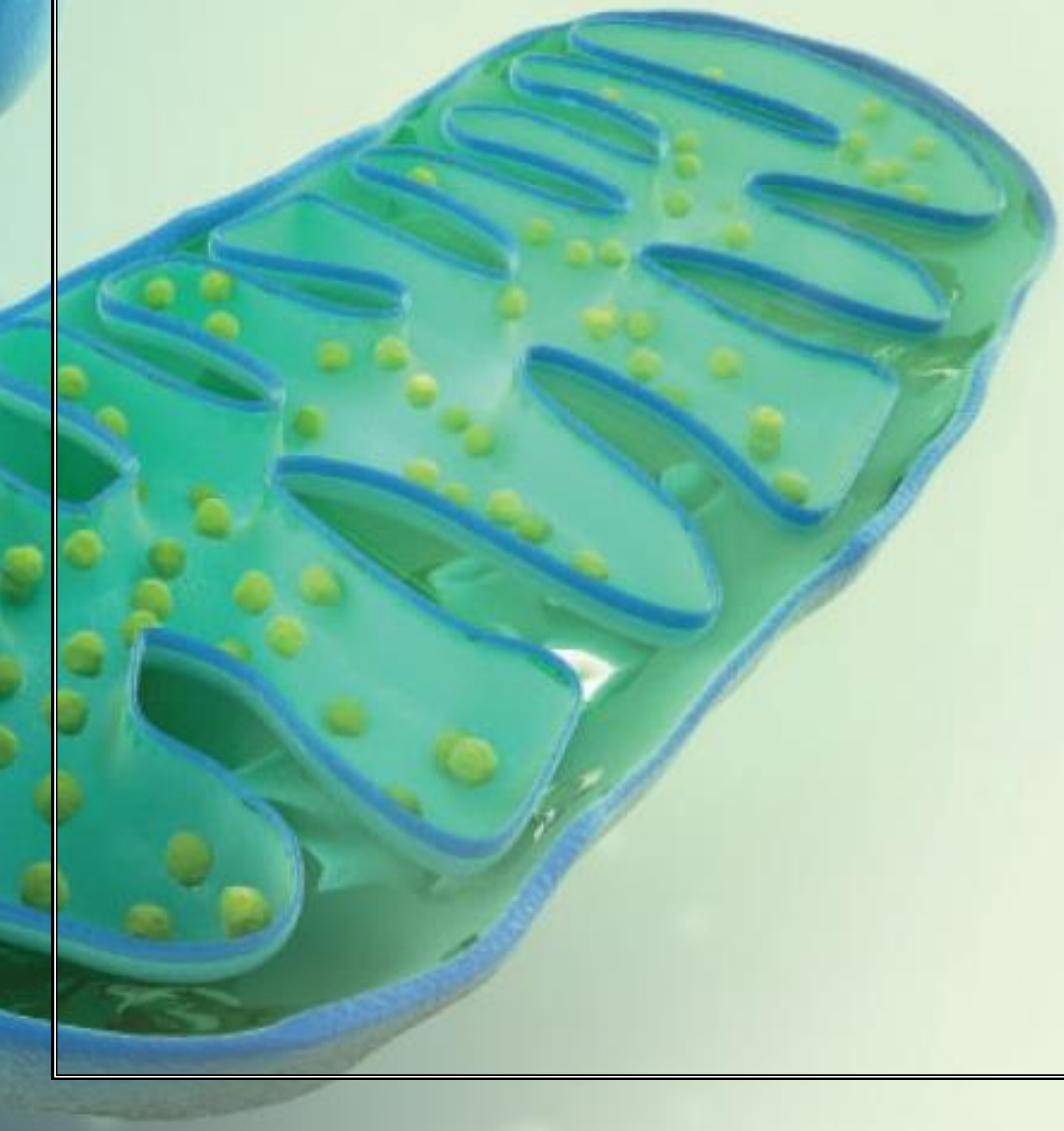

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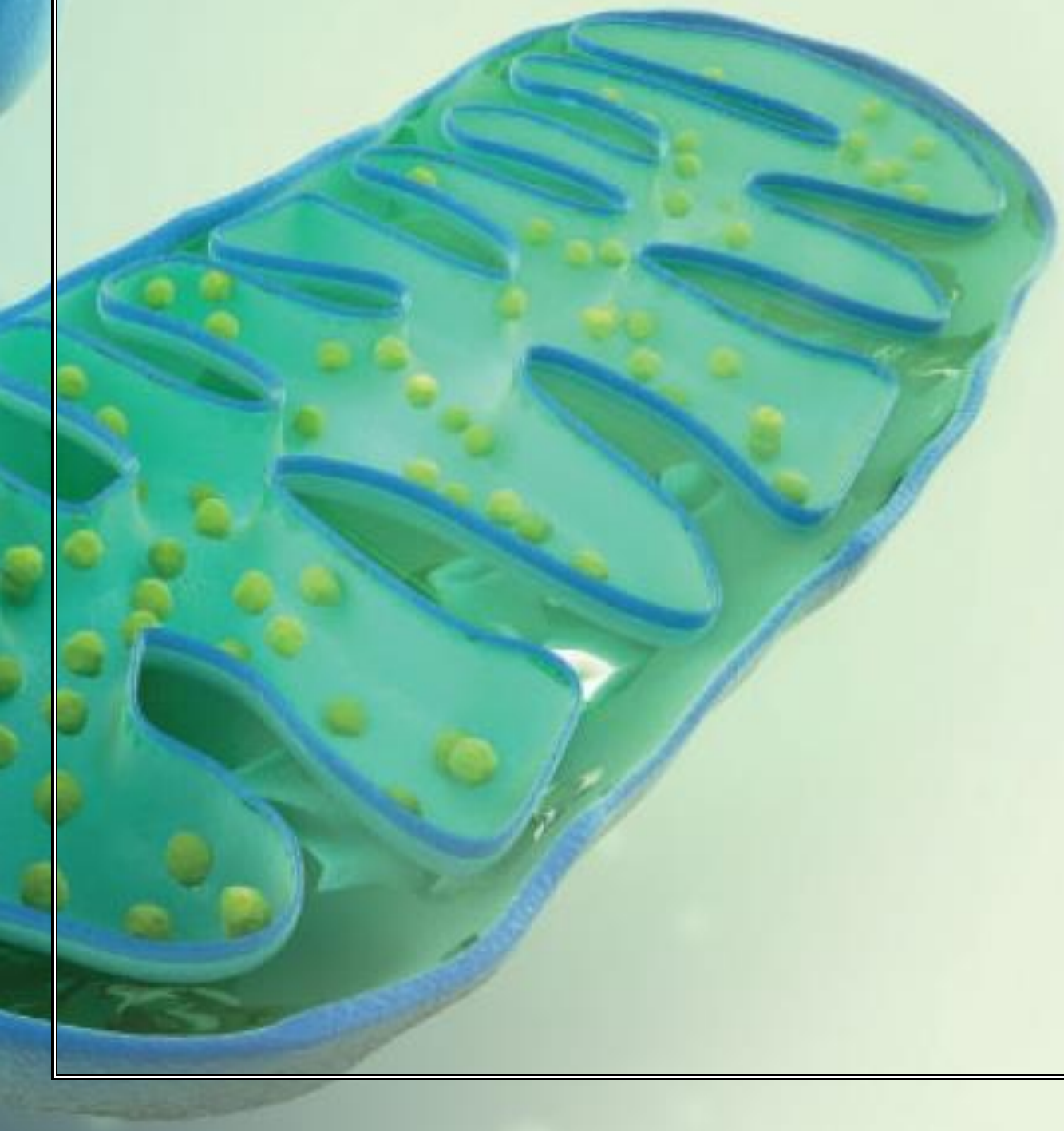

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Rotenone-induced reactive oxygen species signal the recruitment of STAT3 to mitochondria

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

Keywords: mitochondria; oxidative stress; ROS; STAT3

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

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

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

Aging reduces kisspeptin receptor (GPR54) expression levels in the hypothalamus and extra-hypothalamic brain regions

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Abstract. Aging leads to the diminished pulsatile secretion of hypothalamic gonadotropin-releasing hormone (GnRH). Kisspeptin (Kp), the upstream regulator of the hypothalamic-pituitary-gonadal (HPG) axis, regulates GnRH synthesis and release through its cognate receptor, G-protein coupled receptor 54 (GPR54). In turn, GnRH regulates GPR54 expression. GnRH administration into the third ventricle has been shown to induce neurogenesis in different brain regions in old age. However, aging-associated changes in hypothalamic and extra-hypothalamic GPR54 expression were unclear. Therefore, the expression levels of GPR54 were evaluated in various brain regions of adult (age, 3-4 months) and old (age, 20-24 months) male Wistar rats in the present study. In the hypothalamus, mRNA and protein levels of Kp and GPR54 were identified to be significantly decreased in old age. Furthermore, *GnRH1* expression in the hypothalamus was analyzed to observe the functional consequence of a reduced Kp-GPR54 system in the hypothalamus. It was found that hypothalamic *GnRH1* levels were significantly decreased in old age. As GnRH regulates GPR54 levels, GPR54 was examined in extra-hypothalamic regions. GPR54 levels were found to be significantly decreased in the hippocampus and medulla and pons in old-age rats when compared to adult rats. Notably, GPR54 expression was observed in the frontal lobe, cortex, midbrain and cerebellum of adult and old-age rats; however, the difference between the two groups was not statistically significant. To the best of our knowledge, this is the first study that provides the quantitative distribution of GPR54 in different brain regions during aging. Thus, the reduced levels of Kp and its receptor, GPR54 in the hypothalamus could be cumulatively responsible for reduced levels of GnRH observed in old age.

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Key words: aging, kisspeptin, G-protein coupled receptor 54, gonadotropin-releasing hormone, brain

Introduction

Aging is a complex process and is associated with several alterations, including a decline in the activity of the hypothalamic-pituitary-gonadal (HPG) axis, hormonal abnormalities, cognitive impairments and depression (1). Aging is accompanied by a decrease in pulsatile luteinizing hormone (LH) secretion due to a decline in the pulsatile secretion of gonadotropin-releasing hormone (GnRH)/LH (2-4). A recent study describes that aging could be accompanied by decreased expression of arcuate (ARC) nucleus kisspeptin (Kp) along with neurokinin B and dynorphins (5). However, neuronal projections expressing Kp from the hypothalamic anteroventral periventricular nucleus (AVPV) play a pivotal role in the regulation of GnRH along with Kp expressed in the ARC nucleus (6). Kps are a family of peptide products known to stimulate GnRH secretion and play an important role in fertility and reproduction by regulating the HPG axis. Kp acts through G-protein coupled receptor 54 (GPR54) and numerous studies reported the presence and functions of Kp or GPR54 in various types of tissue and organs (7-9). In the brain, the Kp-GPR54 system functions predominantly to control the reproductive process (10). In the hypothalamus, the increased GPR54 signaling initiates puberty, whereas its loss of function delays pubertal onset (11,12).

In addition to its major function as an upstream regulator of the HPG axis, the Kp-GPR54 system also triggers several other signaling pathways. The role of the Kp-GPR54 system in reproductive and non-reproductive functions was indicated in a previous study (13). Numerous studies reported that Kp induces phospholipase C enzyme activity through GPR54 (14,15). Additionally, it has been shown that mutation of GPR54 elicits prolonged activation of the ERK signaling pathway in response to Kp (16). Furthermore, loss of function of GPR54 is known to be associated with hypogonadotropic hypogonadism (17). Therefore, the loss or gain of function of the GPR54 receptor determines the specificity and importance of appropriate signaling of the Kp-GPR54 system (13).

The Kp-GPR54 system has been extensively investigated in the hypothalamus. Certain studies suggest that GPR54 is

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Mitochondrial Import of Dengue Virus NS3 Protease and Cleavage of GrpEL1, a Cochaperone of Mitochondrial Hsp70

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ABSTRACT Dengue virus infections, which have been reported in nearly 140 countries, pose a significant threat to human health. The genome of dengue virus encodes three structural and seven nonstructural (NS) proteins along with two untranslated regions, one each on both ends. Among them, dengue protease (NS3) plays a pivotal role in polyprotein processing and virus multiplication. NS3 is also known to regulate several host proteins to induce and maintain pathogenesis. Certain viral proteins are known to interact with mitochondrial membrane proteins and interfere with their functions, but the association of a virus-coded protein with the mitochondrial matrix is not known. In this report, by using *in silico* analysis, we show that NS3pro alone is capable of mitochondrial import; however, this is dependent on its innate mitochondrial transport signal (MTS). Transient-transfection and protein import studies confirm the import of NS3pro to the mitochondrial matrix. Similarly, NS3pro-helicase (amino acids 1 to 464 of NS3) also targets the mitochondria. Intriguingly, reduced levels of matrix-localized GrpE protein homolog 1 (GrpEL1), a cochaperone of mitochondrial Hsp70 (mtHsp70), were noticed in NS3pro-expressing, NS3pro-helicase-expressing, and virus-infected cells. Upon the use of purified components, GrpEL1 undergoes cleavage, and the cleavage sites have been mapped to $KR^{81}A$ and $QR^{92}S$. Importantly, GrpEL1 levels are seriously compromised in severe dengue virus-infected clinical samples. Our studies provide novel insights into the import of NS3 into host mitochondria and identify a hitherto unknown factor, GrpEL1, as a cleavage target, thereby providing new avenues for dengue virus research and the design of potential therapeutics.

IMPORTANCE Approximately 40% of the world's population is at risk of dengue virus infection. There is currently no specific drug or potential vaccine for these infections. Lack of complete understanding of the pathogenesis of the virus is one of the hurdles that must be overcome in developing antivirals for this virus infection. In the present study, we observed that the dengue virus-coded protease imports to the mitochondrial matrix, and our report is the first ever of a virus-coded protein, either animal or human, importing to the mitochondrial matrix. Our analysis indicates that the observed mitochondrial import is due to an inherited mitochondrial transport signal. We also show that matrix-localized GrpE protein homolog 1 (GrpEL1), a cochaperone of mitochondrial Hsp70 (mtHsp70), is also the substrate of dengue virus protease, as observed *in vitro* and *ex vivo* in virus-infected cells and dengue virus-infected clinical samples. Hence, our studies reveal an essential aspect of the pathogenesis of dengue virus infections, which may aid in developing antidengue therapeutics.

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

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

Contents

Abstract

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

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

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

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

INTRODUCTION

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

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

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

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

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

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Background: Apart from its mitochondrial localization, mechanistic details of STAT3 import and assembly in mitochondria remain elusive.
Results: Using an *in vitro* import assay, we show that STAT3 associates with the mitochondrial inner membrane in a GRIM-19-dependent manner.
Conclusion: GRIM-19 chaperones the recruitment of STAT3 into mitochondrial inner membrane complexes.
Significance: This study identifies a novel function of GRIM-19 and a mechanism for STAT3 import into mitochondria.

The signal transducer and activator of transcription 3 (STAT3), a nuclear transcription factor, is also present in mitochondria and regulates cellular respiration in a transcriptional-independent manner. The mechanism of STAT3 import into mitochondria remains obscure. In this report we show that mitochondrial-localized STAT3 resides in the inner mitochondrial membrane. *In vitro* import studies show that the gene associated with retinoid interferon induced cell mortality 19 (GRIM-19), a complex I subunit that acts as a chaperone to recruit STAT3 into mitochondria. In addition, GRIM-19 enhances the integration of STAT3 into complex I. A S727A mutation in STAT3 reduces its import and assembly even in the presence of GRIM-19. Together, our studies unveil a novel chaperone function for GRIM-19 in the recruitment of STAT3 into mitochondria.

Mitochondria are essential organelles involved in many cellular processes including energy transduction, apoptosis, and metabolism of lipids and amino acids. Even though they possess their own genome, most mitochondrial proteins are encoded by the nuclear genome and are imported into different sub-compartments of mitochondria by multisubunit protein import receptors (1–3). Understanding the mechanisms by which proteins are imported into the mitochondria will provide insights concerning the role of these proteins in mitochondrial respiration, biogenesis, and apoptosis (4).

The molecular mechanisms by which Stat transcription factors regulate nuclear gene expression have been actively pursued for decades. Recent reports suggested non-canonical functions for STAT3 and other nuclear transcription factors in mitochondria that involve regulation of energy management by the mitochondria. For instance, researchers have identified STAT3 (5), estrogen receptors (6, 7), myocyte enhancer factor 2D (8), glucocorticoid receptors (9, 10), p53 (11), NF-κB (12), and CREB (cAMP-responsive element binding protein) (13–15) in mitochondria regulating mitochondrial functions in a manner that is dependent or independent of transcription (16). These findings suggest a paradigm shift in the functions of these transcription factors to target and modulate mitochondrial driven cellular functions.

STAT3 is a key transcription factor that is phosphorylated on 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 (5, 17–20). Mitochondrial STAT3 increases activity of complex I and II of the electron transport chain in a transcriptional-independent manner (5). In addition, Ras-mediated cellular transformation is shown to be dependent on mitochondrial STAT3 (17). Interestingly, phosphorylation of STAT3 on Ser-727, but not Tyr-705, seems to be integral for its mitochondrial activity (5, 17). Recent reports also suggest involvement of mitochondrial STAT3 in cardioprotection during ischemia and reperfusion possibly by preventing leakage of electrons from complex I (18, 21). The 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/Retinoic acid-induced cell death (22). Subsequently, GRIM-19 was identified as a component of complex I in the electron transport chain. Small amounts of GRIM-19 have also been detected in nuclei (22–24) where it has been reported to be a negative transcriptional regulator of STAT3 (25–28). Phosphorylation of STAT3 on Ser-727 seems to be essential for its interaction with GRIM-19 (28). The requirement of Ser-727 phosphorylation for its mitochondrial functions and its interaction

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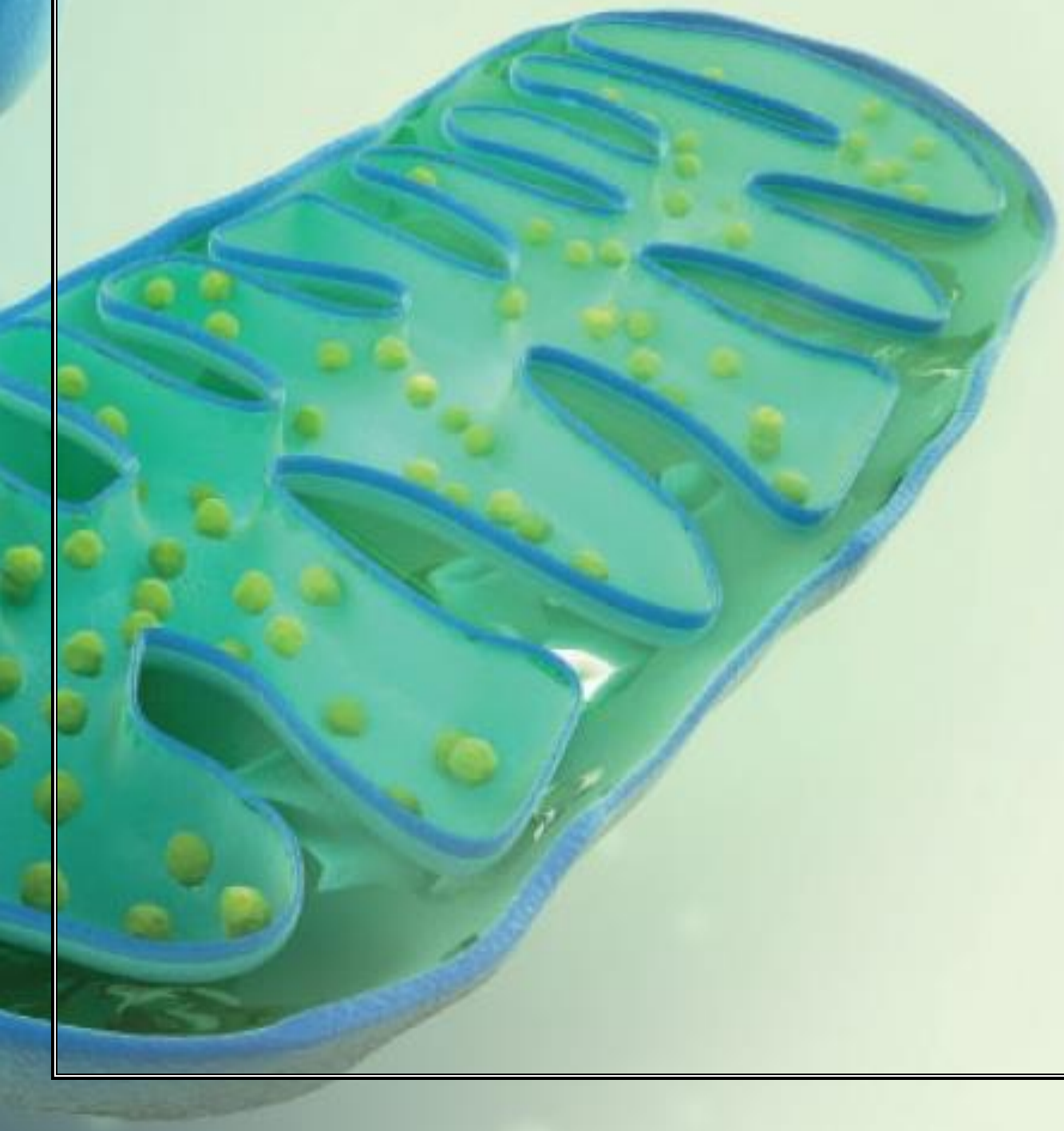

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