Pim1/Lon mediated regulation of PAM complex subunits, Mge1 and identification of novel inhibitors for Lon protease

A Thesis

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Department of Biochemistry, School of Life Sciences

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CERTIFICATE

This is to certify that the thesis entitled **" Pim1/Lon mediated regulation of PAM complex subunits , Mge1 and identification of novel inhibitors for Lon protease "** submitted by **Yerranna B** bearing registration number **13LBPH05** in partial fulfillment of the requirements for the award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

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Parts of this thesis have been:

A. Published in the following publications:

1. PK Allu, **Y Boggula**, S Karri, A Marada, T Krishnamoorthy, NBV Sepuri , A conserved R type methionine sulfoxide reductase reverses oxidized GrpEL1/Mge1 to regulate Hsp70 chaperone cycle, Scientific reports 8 (1), 1-12.
2. PK Allu, A Marada, **Y Boggula**, S Karri, T Krishnamoorthy, NBV Sepuri , Methionine sulfoxide reductase 2 reversibly regulates Mge1, a cochaperone of mitochondrial Hsp70, during oxidative stress, Molecular biology of the cell 26 (3), 406-419.
3. Marada, A., Karri, S., Singh, S., Allu, P. K., **Boggula, Y.**,T Krishnamoorthy, NBVSepuri, A Single Point Mutation in Mitochondrial Hsp70 Cochaperone Mge1 gains thermal stability and resistance. Biochemistry, 55(51), 7065-7072.

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2. Conference attended and presented a poster in International Congress Of Cell biology (ICCB) at Hyderabad from 27th to 30th January 2018.

Further, the student has passed the following courses towards the fulfillment of the coursework requirement for Ph.D.

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DECLARATION

I, Yerranna B hereby declare that this thesis entitled " **Pim1/Lon mediated regulation of PAM complex subunits,Mge1 and identification of novel inhibitors for Lon protease** " submitted by me under the guidance and supervision of **Prof. Naresh Babu V Sepuri** is an original and independent piece of research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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-Yerranna B

List of abbreviations

PAM	Pre-sequence translocase Associated Motor
5-FOA	5-Fluoroorotic acid
ATP	ADP Adeninosine diphosphate
ADP	Adeninosine triphosphate
AMP	Ampicillin
BSA	Bovine serum albumin
DAPI	4', 6-Diamidino-2-phenylindole
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetra acetic acid
GFP	Green fluorescent protein
HIS	Histidine
LB	Luria-Bertani broth
LEU	Leucine requiring
LINC	Linker of nucleoskeleton and cytoskeleton
LiOAC	Lithium Acetate
MAT	Mating type
$Mgcl_2$	Magnesium chloride
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NP-40	Nonyl phenoxypolyethoxylethano-40
OD	Optical density
ONM	Outer nuclear membrane
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Potential of Hydrogen
rpm	revolutions per minute
SC	Synthetic complete
ssDNA	Single-stranded DNA
TCA	Trichloroacetic acid
TES	Tris EDTA KOH
Tris-HCl	Tris (hydroxymethyl) aminomethane (THAM)
URA3	Uracil 3
UV	Ultraviolet
WT	Wild-type
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose
YPG	Yeast Peptone Glycerol
Hsp’s	Heat shock proteins
H2O2	Hydrogen peroxide
IMS	Inter membrane space proteins
DTT	Dithiothreitol
dNTPs	Deoxy nucleotide triphosphates
ETC	Electron transport chain
EDTA	Ethylene diamine tetra acetic acid
OXPHOS	Oxidative phosphorylation

PAM	Pre-sequence Associated Motor complex
PMSF	phenylmethylsulfonyl fluoride
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
µg	Microgram
µl	Microlitre
µM	Micomolar
ng	Nanogram
Kb	Kilobase
kDa	Kilodalton
KI	Potassium Iodide

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Chapter 1

Review of Literature

Chapter 1: Review of literature

1.1. Mitochondria structure and functions

Mitochondria are double-membrane organelles. They consist of the outer membrane, inner membrane, two aqueous spaces called inter membrane space, and matrix. The outer membrane is involved in non-selective ion and lipid exchange. It mainly comprises ion channels and protein translocation complexes. The inner membrane is impermeable to ions and it helps in maintaining the membrane potential. The inner membrane is the hub for oxidative phosphorylation (OXPHOS) complexes. The folding of the inner membrane protruding into the matrix forms cristae which help in increasing the surface area and hence enhance the chemical reactions. Although mitochondria lost most of their genes to the nuclear genome, it contains autonomous replicating DNA and encodes proteins that play a significant role in electron transport complex (ETC) assembly. Also, mitochondria produce ROS. Mitochondrial biogenesis requires coordination of several processes that include nuclear-encoded protein import and assembly, ETC assembly, mtDNA maintenance, and mitochondrial fusion-fission (Bauer, Sirrenberg et al. 1996, Chen and Chan 2009). In eukaryotic cells, mitochondria are important organelle involved in many fundamental processes. Mitochondria are called the power house of the cell because of their most noted function of ATP production. Apart from these, mitochondria are also essential for performing different cellular functions ranging from cell survival to cell death (Wallace 2012). It is a hub for various metabolic pathways like a β-oxidation system of fatty acids, citric acid cycle, lipid biosynthesis pathways, as well as amino acid metabolism (Figure 1.1). Metal metabolism is also a feature of mitochondria, especially iron-sulfur clusters and heme biosynthesis (Lill and Muhlenhoff 2008). Besides, apoptosis (Danial and Korsmeyer 2004)), calcium homeostasis and redox signaling involve mitochondria (Jacobson and Duchen 2004)).

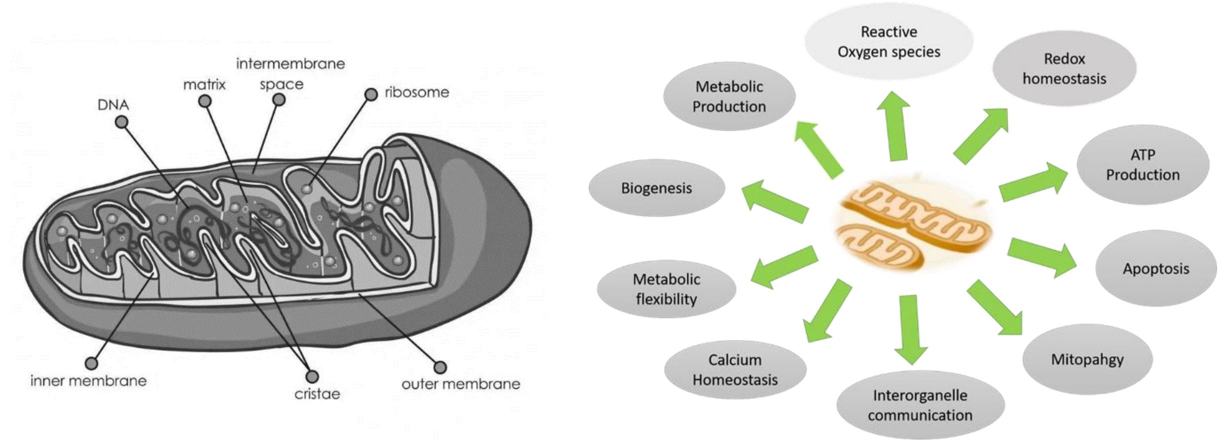


Figure 1.0. Mitochondria are involved in many functions: *Schematic representation of a mitochondrion illustrating the different functions.*

1.2. Evolution of mitochondria

Eukaryotic cells are normally larger and highly organized with much larger genomes and proteomes than prokaryotic cells (Lane and Martin 2010). The acquisition of mitochondria and chloroplasts is one of the significant events in eukaryotic cell evolution as they represent energy generating and biosynthetic factories of eukaryotes. As indicated by the endosymbiotic hypothesis, mitochondria are free-living, ancient alpha-proteobacteria that were occupied in the cytosol of the primitive eukaryotic cell (Henze and Martin 2003). During the evolutionary transition from symbiotic to organized eukaryotic cells, mitochondria progressively lost independence and rely on nuclear-encoded gene products for their biogenesis (Huang, Ayliffe et al. 2004)

Endosymbiotic theory

The endosymbiotic theory suggests that the mitochondria are originated from α- proteobacteria (Anand, Langer et al. 2013), (Gray, Burger et al. 2001). Phylogenetic analysis of small ribosomal RNAs demonstrates the non-phylogenetic origin of mitochondria from α-proteobacterium (Yang, Oyaizu et al. 1985). Two scenarios were proposed to explain the mitochondrial origin, the Symbiosis scenario and the Archezoan scenario (Gray, M.W.et al.2012). According to the Symbiosis scenario, endosymbiont was taken up by an archeal cell (Martin and Muller 1998)whereas Archezoan theory suggests that the host of the endosymbiont was essentially compartmentalized as amitochondriate of the eukaryotic cell (Cavalier-Smith 1987). However, growing evidence supports the Symbiosis scenario theory due to the absence of any amitochondriate eukaryotic ancestry to date (Embley and Hirt 1998), (Embley and Hirt 1998). The transition from the ancestral bacterial endosymbiont to modern mitochondria has been escorted by many changes such as shrinkage of the mitochondrial genome due to loss or transfer of bacterial genome to the host genome (Andersson, Karlberg et al. 2003). Thus the majority of genes coding for mitochondrial functions are located in the nucleus.

1.3. Mitochondrial functions under stress conditions

A living organism adapts to different stresses by regulating several cellular processes like signal transduction, transcriptional and post-transcriptional management, protein-targeting, accumulation of protectants, exaggerated activity of repair functions, and alteration of metabolic pathways. Several pieces of evidence support that the mitochondria in yeast cells are prime targets for many abiotic stress conditions.

Thermal stress is studied well in terms of protein folding and related diseases. Recent studies have been focused on changes in transcript levels to understand the pathophysiology of cells and the development of strains for industrial purposes (Gibson, Lawrence et al. 2007) (Nevoigt 2008). Thermal stress causes the highest change in transcripts levels compared to other stresses. Thermal stress causes a significant loss of biomass and increases the ATP demand. Thermal stress downregulates the mitochondria-based amino acid biosynthesis and translation. It also shares the pathways related to mitochondrial respiration and antioxidant inducing with other stresses like oxidative and osmotic stress (Morano, Grant et al. 2012). It also induces many genes involved in protein folding and their turnover along with the activation of specialized machinery called autophagy to eliminate the damaged proteins.

1.4.0 Mitochondrial import

1.4.1. TOM complex: common gateway for mitochondrial precursor proteins

Mitochondrial translocase outer membrane (TOM) is defined as a general import pore and adapter for many mitochondria precursor proteins after translation. It contains Tom40, which is an essential β-barrel protein that helps in pore-forming and is indispensable for cell survival (Hill, Model et al. 1998). Tom22 is a receptor protein that binds with multiple Tom40 proteins and forms the 440 kDa complex. The complex is associated with Tom5 and Tom6 (small Tom proteins).

1.4.2. TOB Complex

Mitochondrial membrane proteins translocation and assembly are driven by the TOB complex which is located in the outer membrane. The TOB complex comprises two basic components, Sam50, Sam35, and a non-essential peripheral membrane protein Mas37 (Figure 1.1). Like all other precursor proteins, the β-barrel precursors cross the external membrane via the TOM complex, then IMS where small Tim proteins guide them to TOB complex.

1.4.3 MIM — α-helical outer membrane proteins involved in import and insertion assembly complex

The MIM α helical outer membrane proteins involved in import and insertion assembly complex present in the mitochondrial outer membrane and comprises of Mim1 and Mim2, forming 200 kDa complex, dedicated for efficient import and assembly of single and multi-spanning α helices outer membrane proteins (Becker, Pfannschmidt et al. 2008, Becker, Wenz et al. 2011). MIM complex is associated with SAM complex, which contributes to membrane protein complex assembly.

1.4.4 TIM23 - inner membrane translocase for pre-sequence precursor proteins

Translocation of both matrix-targeted pre-proteins and certain internal membrane proteins is part of TIM23, a major complex inner-membrane translocase (Marom, Dayan et al. 2011). Import of pre-proteins required inner mitochondrial membrane potential and ATP as energy sources (Stuart, Gruhler et al. 1994). Outer membrane channel TOM complex is connected to TIM23 complex by Tim23 protein. Tim23 is a four helical spanning membrane protein forming cation-selective translocase channel which gets activated by pre-sequence and membrane potential (Bauer, Sirrenberg et al. 1996). Part of N terminus residues of Tim23 is embedded in the outer membrane and the remaining parts are associated with Tim50 proteins. Tim17 is an important protein in the Tim23 complex which serves multiple functions like stabilization and voltage sensing. Tim17 is also involved in lateral sorting of single-channel proteins and import of matrix target proteins (Martinez-Caballero, Grigoriev et al. 2007). Tim50 is a receptor protein having a huge C-terminal area exposed into the intermembrane space. This space helps in saving the potential by diverting Tim23 to the inert state.

1.4.5. PAM: motor complex associated with pre-sequence translocase

To import precursor mitochondrial protein into the mitochondrial matrix, PAM activity is necessary (Figure 1.1). The PAM complex is built around the mitochondrial Hsp70 protein. Hsp70 has a C terminal substrate-binding domain and an N terminal ATPase domain. The main component of the import engine is mtHsp70 (Ssc1 in *S. cerevisiae*), which aids in the import and folding of matrix-targeted proteins in ATP-based cycles (Mapa, Sikor et al. 2010).

Tim44 acts as a co-chaperone and helps by creating an ATP-dependent complex to recruit Hsp70 chaperones and co-chaperone (Hutu, Guiard et al. 2008) (Albrecht, Rehling et al. 2006). The mtHsp70 import chaperone is facilitated by the PAM complex. Tim44, a protein with a J-domain that is attached to the internal mitochondrial membrane, is a fundamental PAM complex protein. It binds to mtHsp70 just as Tim23 motor proteins and has been appeared to direct the J-complex to the TIM23 translocase (Schiller, Cheng et al. 2008).

Two J proteins affecting the ATPase activity of mtHsp70 are Pam16 and Pam18. Pam17 cooperates with the binding of the Pam16 and Pam18 to the TIM23 complex (Frazier, Dudek et al. 2004, Chacinska, Lind et al. 2005), (Hutu, Guiard et al. 2008). Another stage of the mtHsp70 ATPase cycle is promoted by the mitochondrial matrix nucleotide exchanger Mge1, which helps to efficiently import precursor proteins into mitochondria through an exchange of ADP for ATP on mtHsp70. (Voos, Gambill et al. 1994) (Westermann, Prip-Buus et al. 1995).

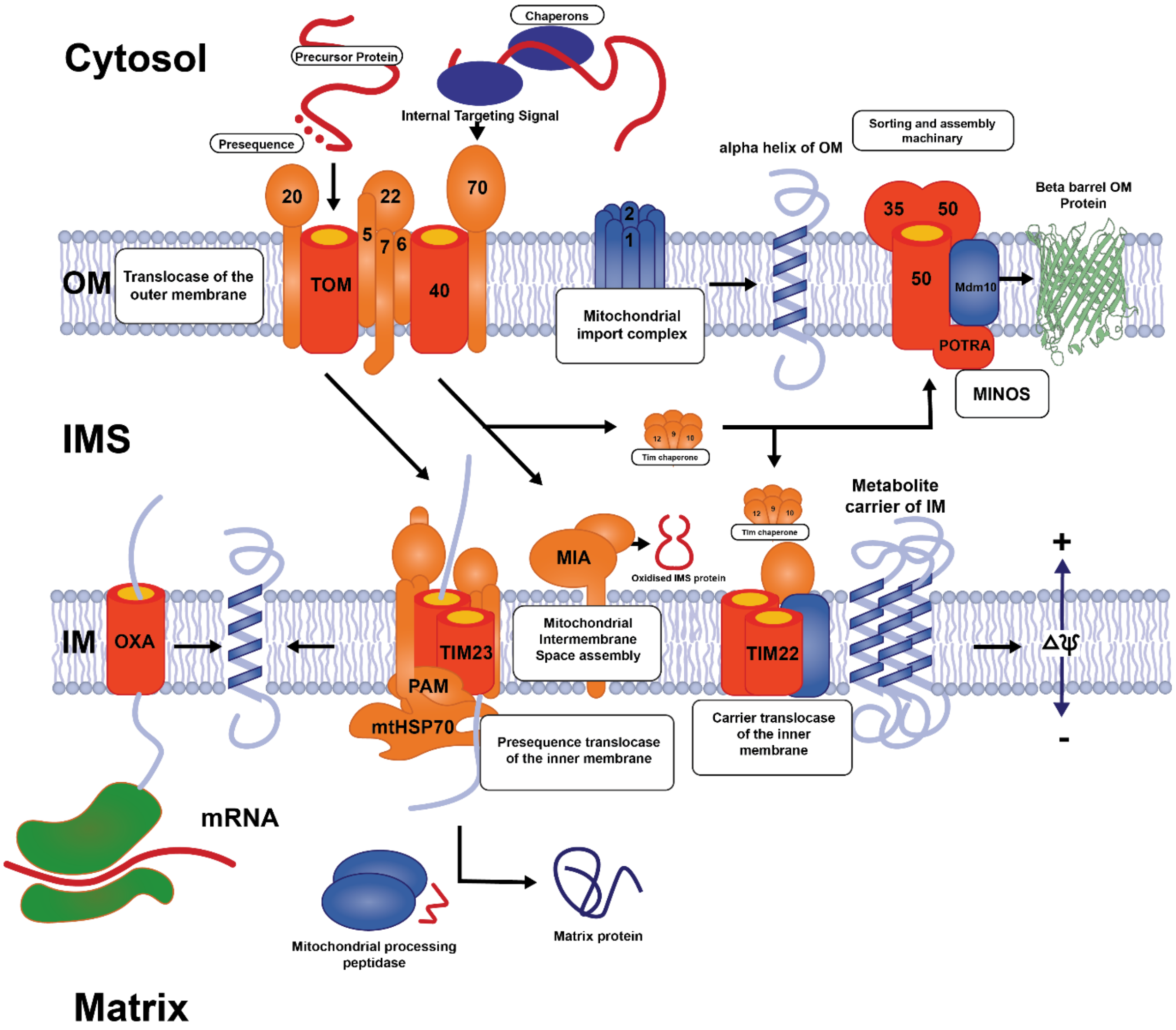


Figure 1.1: Mitochondrial import pathways. *Mitochondrial nascent proteins are translated into the matrix by the outer membrane complex (TOM). Pre-sequence proteins are transported from a TOM complex to a mitochondrial membrane translocase via a membrane potential driven by precursor proteins (TIM23). The PAM complex is involved in the import of matrix proteins with the help of ATP. MPP (mitochondrial peptidases) proteases are cleaved off by the pre-sequences. Outer membrane β-barrel proteins are incorporated by SAM into the outer membrane (sorting and assembly machinery). Tom70 carries non-cleavable proteins bound to the cytosolic chaperones in hydrophobic precursors. The complex insertion of small TIM chaperone proteins in the internal space membrane aid TIM22. The encoded mitochondrial proteins are inserted through the OXA machinery into internal mitochondrial proteins. The mitochondrial inner membrane space import and assembly (MIA) imports cysteine-rich proteins for internal membrane space (IMS) (Adapted from (Horvath, Rampelt et al. 2015)*

1.5.0 Mitochondrial import and Respiratory complex

The majority of precursor proteins containing hydrophobic transmembrane domains are imported across the inner membrane using membrane potential. For certain import events that are dependent on membrane potential, the TIM23 complex associates with Tim21, which is a single spanning membrane protein. The C-terminal domain of Tim21 is exposed to the inter membrane space and it has been known to interact with inter membrane space domain of Tim22 to promote the transition of precursors across the inner membrane from the TOM complex to the TIM23 complex. It also helps in maintaining the membrane potential (Albrecht, Rehling et al. 2006). Tim21 also participated in the interaction of the translocase of TIM23 with the super complex respiratory cytochrome c oxidase (COX) and cytochrome c reductase (complex III) (Dienhart and Stuart 2008). Furthermore, two import motor regulatory subunits, Pam16 and Pam18, are independently associated with the super complex respiratory chain (Wiedemann, van der Laan et al. 2007). Thus, respiratory super complexes and import machinery together maintain membrane potential.

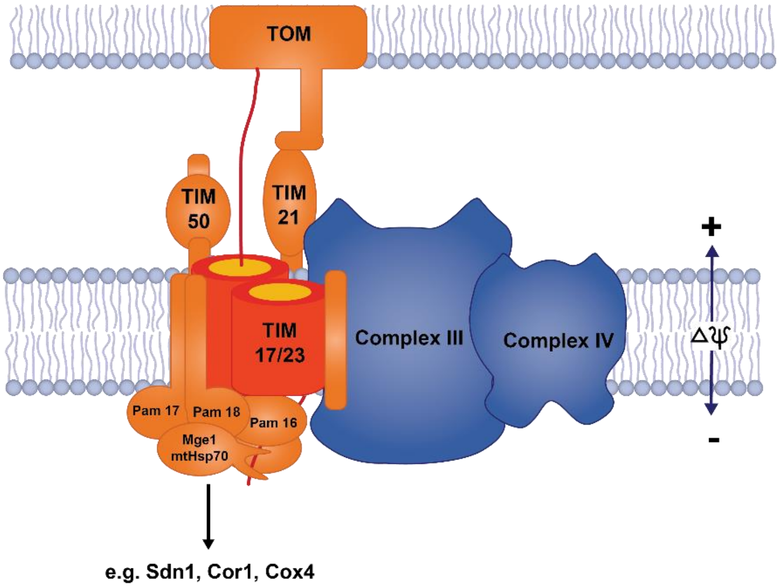


***Figure 1.2: Association of inner membrane translocase TIM23 with respiratory super chain complex**: Precursor proteins are imported through the outer membrane translocase (TOM) and handed to the inner membrane translocase, which then imports them into the matrix via the PAM complex. The association of the TIM23 complex with the RC super complex maintains mitochondrial potential. Membrane potential is required for the translocation of precursor proteins across the inner membrane. (From BoguszKulawiak et al. (2013)*

1.5.1. MPP: peptides cleaves the pre-sequences of matrix precursors

MPP processing in the matrix immediately cleaves the N-terminal sequence of the import precursor proteins, which is needed for further maturation and catalytic action of these proteins. The two homologous subunits of MPPs are Mas1 (Mif1, β-MPP) and Mas2 (Mif2, x-MPP) (Hawlitschek, Schneider et al. 1988), (Witte, Jensen et al. 1988). Extended conformation precursor proteins are cleaved N-terminally by Mas1 (zinc-metalloprotease) during or immediately after import via TIM23. MPP cleaves the precursor protein and arginine residue -2 from the site of cleavage (R-2motif: R-X-X) (Vogtle, Wortelkamp et al. 2009). Also, the matrix has two other aminopeptidases, Icp55 and Oct1 for further processing of the precursor proteins after cleavage by MPP.

1.5.2. Inner membrane protease complex (IMP)

The mitochondrial inner membrane proteases are involved in releasing precursor proteins into the intermembrane space. The release of specific soluble domains of inner membrane proteins was carried out by the inner membrane protease complex (IMP). Imp1 and Imp2 are two important inner membrane space proteases with different specificity and catalytic activity (Schneider, Behrens, et al. 1991). Som1 is another subunit that is catalytically inactive and interacts with Imp1 and helps in its activity (Jan, Esser, et al. 2000). Inner membrane soluble proteins are substrates of the TOM and TIM23 complex. They are inserted into the inner membrane. These soluble inner membrane space proteins are released into membrane space after cleavage by IMP and MPP proteases that act before the C terminal soluble domain.

1.5.3. Carrier precursor proteins assembly and import

Transporter proteins contain hydrophobic, hydrophilic, charged, or uncharged motifs as signal sequences that can be distributed across the entire polypeptide chain, unlike the import of N-terminal pre-sequence precursors (Zara, Ferramosca et al. 2007). Sequential steps occur in the import of carrier precursor proteins. After cytosolic protein translation, the hydrophobic carrier precursors are directed to the mitochondrial outer membrane. The Tom22 membrane protein is involved in translocation through the TOM complex. Tiny complexes of Tim9 and Tim10 bind unfolded proteins and help in preventing the aggregation of protein in aqueous inter-membrane space. By directing the carrier precursor to the TIM22 complex, which relies on the membrane potential, the Tim9-Tim10 complex promotes membrane incorporation. Finally, the mature precursor proteins are released to IMS space .

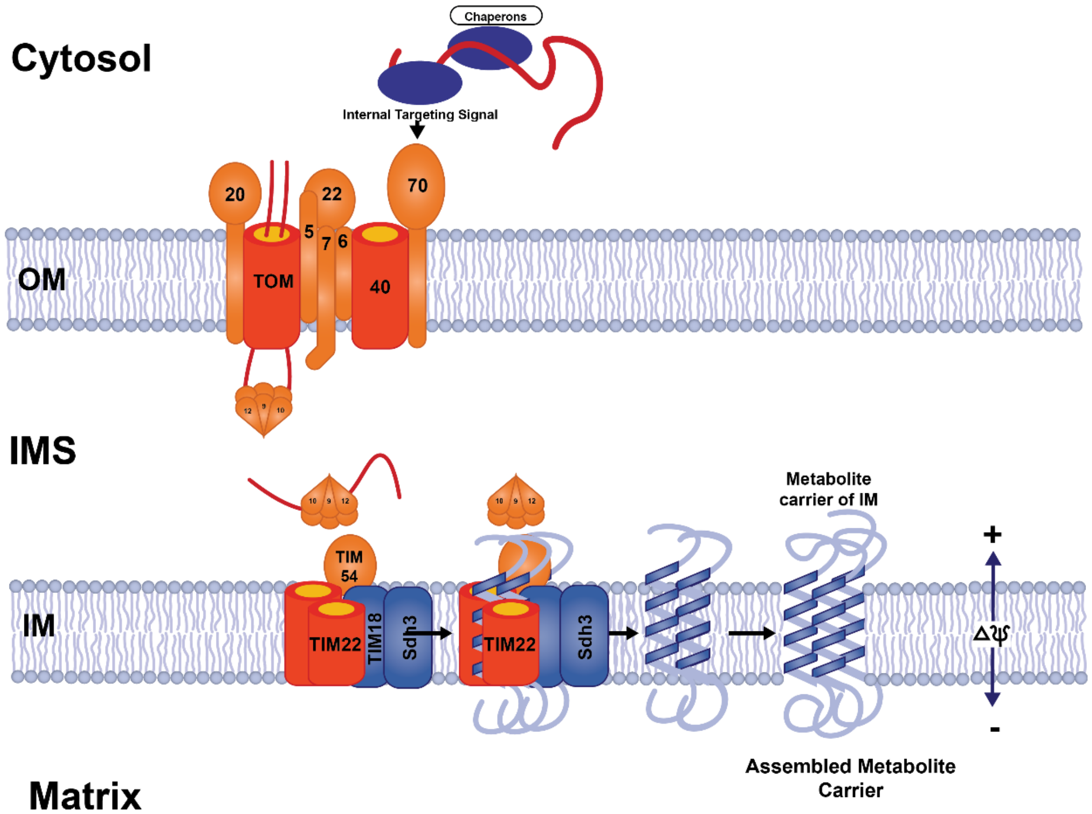


Figure.1.3: Metabolite carrier protein import pathway. *In the cytosol, carrier proteins for metabolites are formed & translocated through the outer membrane translocase TOM40 channel with help of cytosolic chaperones. After import into inner membrane space, precursors are bound to small TIM chaperones and transferred to inner membrane translocase TIM22. Lateral insertion of carrier proteins into the inner membrane of mitochondria is depended on membrane potential. Finally, carrier proteins are dimerized and stabilized with help of lipids. (Adapted from BoguszKulawiak et al., 2013).*

1.5.4. TIM22: Inner membrane mitochondrial carrier translocase complex

TIM22 complex composed of Tim9, Tim10, Tim12, Tim18 and Tim54 subunits. It was identified in 1996 which is homologues with N-terminus translocase subunitsTim17 Carrier translocase exclusively insert all the metabolite carrier proteins into the inner membrane.Tim22 is capable of pore-forming core subunit of the complex hence the complex named as TIM22 complex (Kovermann, Truscott et al. 2002). TIM23 is the complex having Tim9 and Tim10 subunits, capable to form hexameric chaperone complex in IMS. Tim12 is an essential subunit of complex which is peripherally associated with TIM22 complex (Bauer, Sirrenberg et al. 1996). The C terminus of Tim12 is associated with lipid of inner membrane, thus maintaining the sub-complex which is subsequently associated with other subunits to form TIM22 complex. TIM54 subunits have both N terminus sequence and larger C terminus inter membrane space domain (Kerscher, Holder et al. 1997). Deletion of Tim54 causes import defect of carrier proteins and growth defect Tim18 was discovered independently as multicopy suppressor of tim54 mutant and was shown to co-precipitation with TIM22 complex (Kerscher, Sepuri et al. 2000). Tim18 has three trans membrane helical structure similar to sdh4 but their function in respiration remains to be explored, since it is not necessary in import of carrier proteins (Yankovskaya, Horsefield et al. 2003).

2. Regulation of Mitochondrial Protein Import

Cytosolic kinases that can phosphorylate not only the precursor proteins but also the proteins involved in the import machinery regulate mitochondrial imports. Several kinases have been observed to control import machinery and are also involved in both positive and negative biogenesis of import components. Protein kinase A, a well-studied kinase phosphorylates TOM40, the pore-forming channel of the TOM complex. The early translocation stage of precursor proteins is inhibited by phosphorylated TOM40 (Rao, Schmidt et al. 2012). PKA phosphorylates TOM70 as well, thereby inhibiting the function of the receptor. TOM70 has both a receptor site and a chaperone binding site, so it recruits chaperones such as cytosolic Hsp70 and Hsp90 for proper folding and import of precursor proteins. PKA selectively phosphorylates TOM70 serine residues close to the chaperone binding site, thus inhibiting chaperone binding. The import of metabolite carrier proteins into mitochondria is therefore delayed by PKA (Schmidt, Harbauer et al. 2011). The MIM outer membrane complex is involved in the insertion of alpha-helical proteins, including TOM2O and TOM70 precursors. Cytosolic kinase 2 phosphorylates TOM22 and facilitates its binding with TOM20 by aiding imports. Phosphorylated TOM22 inhibition reduces the amount of TOM70 and TOM20, suggesting CK2's role in biogenesis (Gerbeth, Schmidt et al. 2013).

The regulation of mitochondrial import is closely linked to multiple cellular stress adaptations. It also provides the ability to sense the various grades of stress (lower to higher levels) by unknown mechanisms and distinguishes the degrees of stress. Import machinery, including Hsp70 and co-chaperones, specifically the TIM23 complex, interacts with respiratory complexes (complex III and IV) (van der Laan, Wiedemann et al. 2006). The respiratory complex is responsible for the production of ATP and maintenance of the membrane potential of the cell. Due to membrane potential, precursor proteins are translocated to some degree and further imported by ATP-driven

Hsp70 motor proteins. The energy production and respiratory complex of the cell are compromised by many stress signaling pathways. Besides, aggregated proteins that are mainly involved with Hsp70 chaperones, caused by stress, inhibit the import pathways.

Various groups studied mitochondrial unfolded protein response (Pellegrino, Nargund et al. 2013) (Jovaisaite, Mouchiroud et al. 2014) in great detail. The response helps to communicate the signals in a retrograde direction from the mitochondria to the nucleus. Recent research has shown a link between mitochondrial import proteins and mitochondrial stress in *Caenorhabditis elegans*. The stress-1-associated activated transcription factor (ATFS-1) has both nuclear and mitochondrial targeting signals (Nargund, Pellegrino et al. 2012). ATFS-1 is degraded by Lon protease when it gets completely imported into the matrix by the normal healthy mitochondria. The mitochondrial import machinery (TOM40, TIM23, Hsp70, or membrane potential) is disrupted under mild stress conditions affecting the import of ATFS-1 into the mitochondria, rerouted to the nucleus through its NLS resulting in activation of mitochondrial UPR.

Ssc1, Ssq1, and Ecm1 having bacterial Hsp70 (Dank) homology and are included in the Hsp70 class of chaperones. Ssc1 is an important chaperone of all three for cell surveillance and is involved in various functions such as protein import, protein folding, and mitochondrial biogenesis. Ssc1 has two domains, namely the domain binding to the C-terminal peptide (PBD) and the domain binding to the N-terminal nucleotide (NBD). In two functional states, Ssc1 exits to assist the opposing forms in the chaperone cycle. On the other hand, when Ssc1 is bound to ADP, the PBD is present in the closed conformation and has a high binding affinity for peptides. In recent research, Hsp70 has received enormous attention and studies show that oxidative stress is regulated by glutathionylation. It is also associated with the pathogenesis of neurological disorders such as Parkinson's, Huntington's, and Alzheimer's. Several advanced structural studies have shown that two unique cochaperones, Mdj1 and Mge1, are involved in regulating the Hsp70 chaperone cycle. Mdj1 is a non-essential protein in the J class that helps to activate ATP hydrolysis and is also involved in mitochondrial respiration. The protein of the GrpE family, Mge1, is an important protein that assists in nucleotide exchange on Hsp70.

3. Mitochondrial quality control

Several disorders, including cancer, neurodegeneration, and aging, are associated with a shift in mitochondrial function. Because of its inherent vulnerability to biochemical stress, elaborate quality control mechanisms have been developed to investigate, repair or remove damaged mitochondria. Mitochondrial quality control (MQC) mechanisms may interact at several levels, depending on the degree of damage. Network of evolutionary preserved mitochondrial proteases and chaperones spread throughout mitochondrial compartments and cytosolic compartments like the Ubiquitin-Proteasome System (UPS) (Livnat-Levanon and Glickman 2011),(Karbowski and Youle 2011). Table 1 summarizes the main molecules involved in protein mitochondrial quality control (PMQC). The mtHsp70 and Hsp60 heat-shock protein groups, which are dependent on the ATP, are responsible for sorting, folding, and dissolving of proteins in the matrix compartment (Neupert and Herrmann 2007) (Voos 2013). Chaperones of the cytosol-type Hsp70 and Hsp90 work in the same way as the aggregation and transport of newly developed or nascent polypeptides into mitochondria (Fan, Bhangoo et al. 2006) (Hartl, Bracher et al. 2011), (Young, Hoogenraad et al. 2003). Multiple proteases are part of the proteolytic facet of PMQC (Anand, Langer et al. 2013) (Baker, Tatsuta et al. 2011), (Rambold, Kostelecky et al. 2011). Proteases are spread through mitochondrial subsystems and can normally be classified into two groups: the ATP-dependent proteases (AAA+), which are often referred to as cellular activity-associated ATPases, and (ii) proteolytic enzymes which are ATP independent. Proteases present in the matrix, such as ClpXP and Lon/Pim1, degrade oxidatively damaged or aggregated polypeptides.

As another process for quality control, mitochondria usually undergo complex opposing functions called fusion and fission. For cell viability, fusion and fission events are important and help to sort non-functional mitochondria from functional, to promote their preservation or removal from their mother cells (Vevea, Swayne et al. 2014). Mitochondrial fission is needed to degrade the damaged mitochondria (Sathananthan and Trounson 2000). Fusion allows the mixture of mitochondria to complement pathogenic mtDNA mutations and other damages (Gilkerson, Schon et al. 2008). When the damage is beyond repair, specific autophagy (mitophagy) has been adapted by cells to destroy whole damaged mitochondria. During mitophagy, damaged mitochondria or fragmented mitochondria with the aid of receptors are sequestered into autophagosomal bodies and further transported for degradation to lysosomes. Finally, when quality control has failed or cells are subjected to extreme stress, cells undergo apoptosis. Mitochondria are the primary organelle involved in apoptosis (Kerr, Wyllie et al. 1972). Recently a new MQC mechanism was identified. Mitochondrial-derived vesicles (MDV) have been reported to facilitate MQC transporting a selected oxidative load and supplying this cargo to lysosomes (Neuspiel, Schauss et al. 2008), (Sugiura, McLelland et al. 2014). The MDV path seems to function under normal and oxidative stress and is independent of mitochondrial fission-fusion and mitophagy (Soubannier, McLelland et al. 2012) (Sugiura, McLelland et al. 2014).

4. Mitochondrial Proteases

Mitoproteases (proteases found inside mitochondria) are emerging as key regulators of mitochondrial proteostasis. Different mitoproteases remove damaged proteins and avoid their possible harmful effects as a primary shield from mitochondrial damage before mitophagy irreversibly harms the organelles (Sugiura, McLelland et al. 2014) (Youle and van der Bliek 2012). ATP-dependent mitochondrial proteases fine-tune the turnover of proteins imported from the cytosol to mitochondria. i-AAA, m-AAA are transmembrane proteases (Confalonieri and Duguet 1995, Venkatesh, Lee et al. 2012). Matrix soluble proteases are ClpXP and LonP1 (Fig. 1.5).

Mitochondria have a free proteolytic system that performs complete polypeptide degradation to amino acids in different mitochondrial compartments. 45 proteases are just found in mitochondria; 23 of these are discovered uniquely in mitochondria, while others transport between the cytosol and mitochondria. Pseudomitoproteases are five of the mitochondria-restricted proteases (Quiros, Langer et al. 2015). The remaining 18 mitoproteases are classified into four groups, as shown in Table 1. Adapted from (Deshwal, Fiedler et al. 2020).

Table 1: Intrinsic mitoproteases and associated functions

Category	***Protease***	***Localization***	*Regulatory functions*
ATP-dependent proteases	AFG3L2 AFG3L2/SPG7	Matrix/IM	Ribosome assembly MCU assembly
	CLPP	Matrix	Transcription/translation Ribosome assembly
	LONP1	Matrix	mtDNA maintenance
			mtDNA replication
			Adaptation to hypoxia
	YME1L	IM/IMS	Protein import Lipid trafficking Mitochondrial dynamics
Processing peptidases	ATP23	IMS	Protein maturation F_1F_O-ATP synthase assembly
	IMMP	IM/IMS	Protein maturation Apoptosis/senescence
	METAP1D	Matrix	Protein maturation
	MIP	Matrix	Protein maturation Coenzyme Q biosynthesis Complex III and IV activity

	OMA1	IMS/IM	Mitochondrial dynamics
	PARL	IM	Apoptosis Coenzyme Q biosynthesis Complex III assembly Lipid trafficking Mitophagy
	PMPCB	Matrix	Protein maturation
	XPNPEP3	Matrix	Protein maturation and stability
Oligo peptidases	MEP	IMS	ND
	PITRM1	Matrix	ND
Other mitochondrial proteases	HTRA2	IMS	Stress signaling Apoptosis
	LACTB	**IMS**	PE metabolism

Abbreviations: IM, inner membrane; IMS, intermembrane space; MCU, mitochondrial Ca2+ uniporter; NA, not applicable; ND, no data; PE, phosphatidylethanolamine

4.1.0 LON Protease

The Lon is conserved from archaic to eubacteria in every phylogenetic realm. In the mitochondrial network, Lon and m-AAA are major matrix proteases, though ClpXP is present in metazoan (Fig. 1.4).

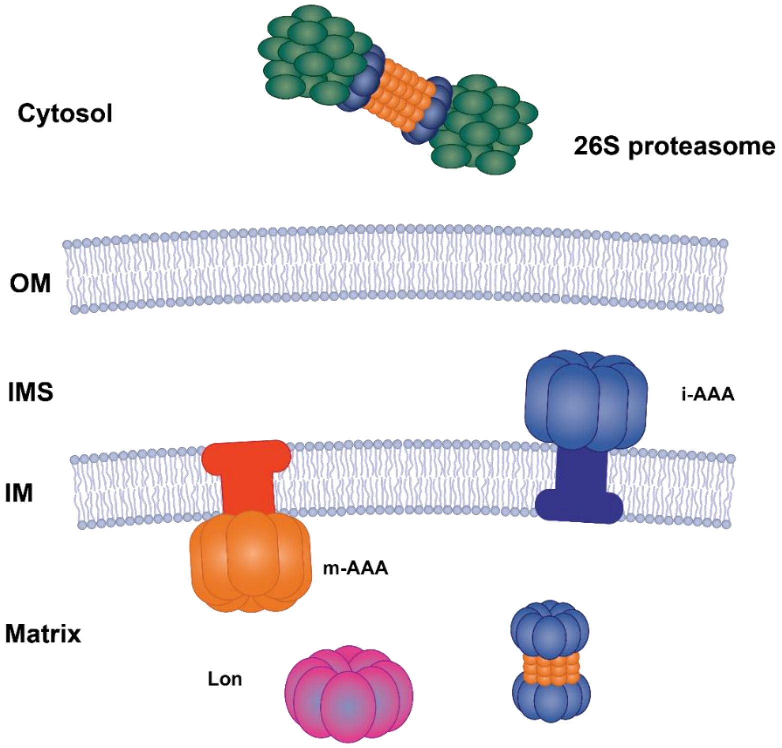


Fig.1.4: ATP dependent proteolytic machines inside metazoan cells: *The 26S proteasome is an energy-dependent non-mitochondrial protease in cytosol. The i-AAA and m-AAA are proteases containing ATP hydrolysis and proteolysis in their C-terminal regions found in inner membrane. The soluble matrix proteases inside the network are Lon and ClpXP. (Picture Adopted from Venkatesh et al. (2012)*

Lon crosses the mitochondrial external and internal layers. Lon's yeast homolog (Pim1) likewise has an autocatalytic cleavage of its amino end (Wagner, van Dyck et al. 1997). The N-terminal space (N-space), the ATPase space or AAA+ module, and the protease area (P-space) are the three zones found in each Lon subunit (Figuge.1.6). The N-terminal is believed to be engaged with substrate restricting. The Walker Box A and B in the AAA+ module are liable for ATP restricting and hydrolysis. The P-area contains the synergist serine in the proteolytic dynamic site.

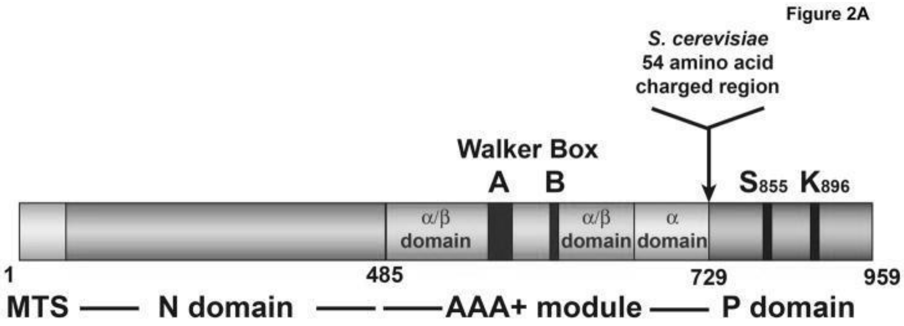


Fig. 1.5: Functional motifs and structural domains in Lon: *Lon has MTS and substrate binding N terminal domain. The AAA+ module contains walker boxes A and B for ATP restricting and hydrolysis. P area is comprised of serine (S) and lysine (K) that structure a synergist dyad at the dynamic site. Lon protease in S. cerevisiae has an extra 54 amino corrosive charged locale situated between AAA+ module and P Domain. (Picture Adopted from Venkatesh et al. (2012).*

4.1.1 Substrate recognition and catalysis functional mechanics by Lon

In an ATP-independent way, the holoenzyme's N-domain & AAA+ module initially recognizes a specific recognition determinant within a protein substrate (stage 1). ATP-restricting and hydrolysis cause conformational changes inside the protein complex that spread out substrate polypeptides (stage 2) and further impel denatured substrates into the proteolytic chamber (stage 3). Peptide security cleavage happens when the unfolded substrate is available to the proteolytic actions (stage 4). The rate-limiting steps of the reaction are proposed to be substrate unfolding through ATP hydrolysis that starts conformational changes inside the protease (Goldberg, Moerschell et al. 1994).

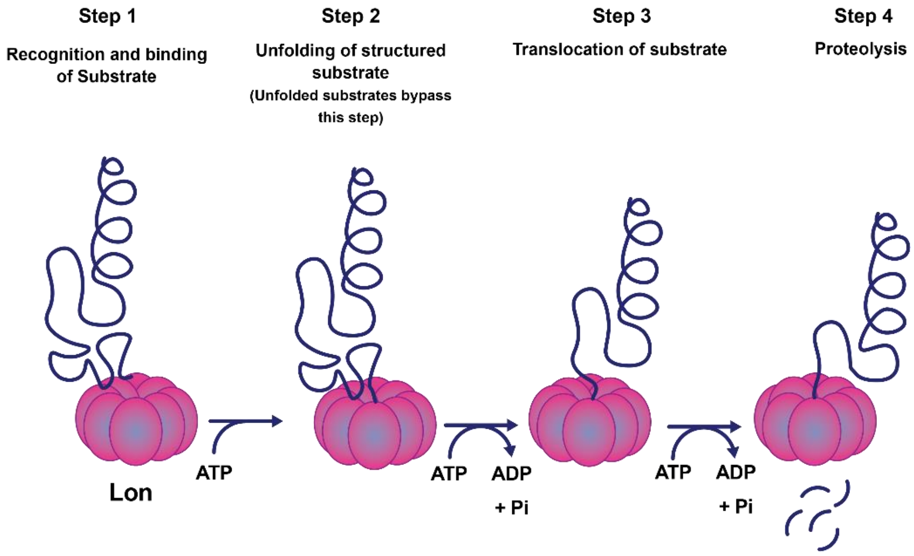


Fig. 1.6 .Schematic representation of proteolytic degradation of the target proteins by Lon protease. *Step 1. Lon protease recognizes and binds to a specific sequence of the target protein. Step 2. The AAA+ domains of the holoenzyme unfold the structured targets in an ATP-dependent manner. This is not seen in unfolded proteins. Stage 3. The unfurled peptides are moved into the debasement chamber, which is likewise an ATP-dependent process. Stage 4. Cleavage of peptide bonds.*

4.1.2 Mitochondrial Lon in diseases.

Mitochondrial proteins are more vulnerable to oxidative damage because they contain the respiratory chain, which generates ROS. Protein misfolding might lead to various kinds of human diseases and aging (Pedersen, Kolvraa et al. 2008). Mitochondrial Lon has a role in regulating mitochondrial metabolism, reducing free radical damage, and also maintaining mtDNA. Recent studies about the physiological significance of Lon functions have brought forth its role in various human disorders like cancer, neurodegeneration as well as aging. Table 2 (Adapted from (Bota and Davies 2016) sums up a portion of these findings.

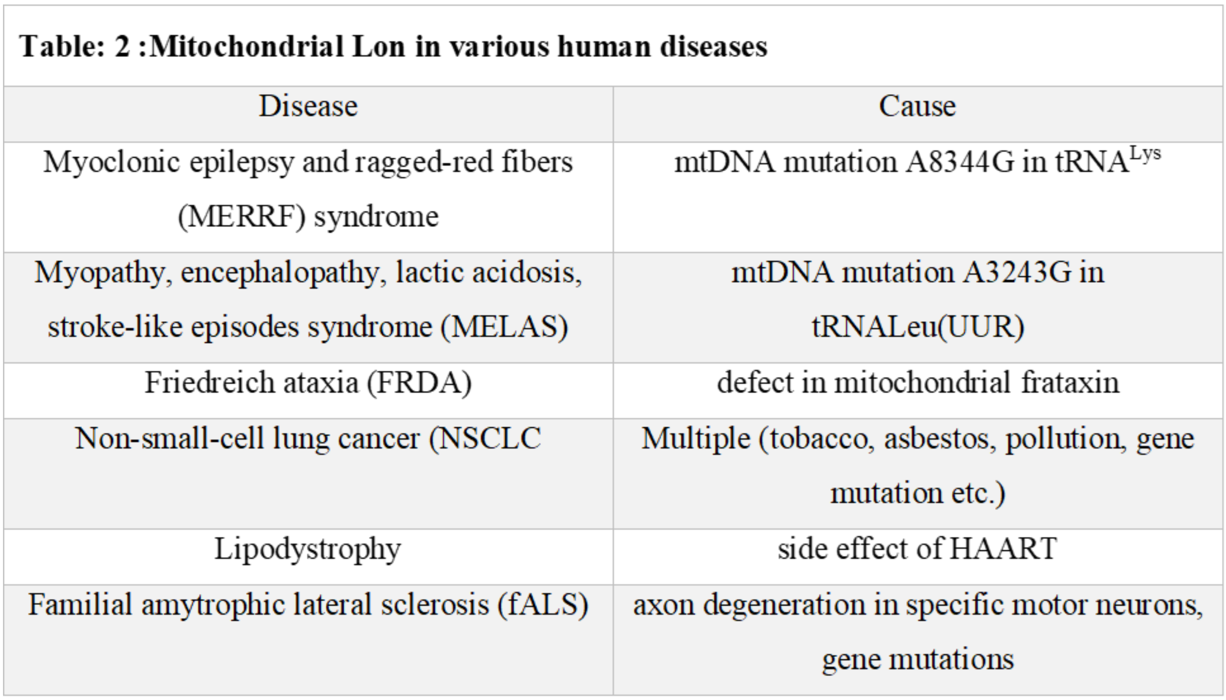


Table: 2 :Mitochondrial Lon in various human diseases

Disease	Cause
Myoclonic epilepsy and ragged-red fibers (MERRF) syndrome	mtDNA mutation A8344G in $tRNA^{Lys}$
Myopathy, encephalopathy, lactic acidosis, stroke-like episodes syndrome (MELAS)	mtDNA mutation A3243G in tRNALeu(UUR)
Friedreich ataxia (FRDA)	defect in mitochondrial frataxin
Non-small-cell lung cancer (NSCLC	Multiple (tobacco, asbestos, pollution, gene mutation etc.)
Lipodystrophy	side effect of HAART
Familial amytrophic lateral sclerosis (fALS)	axon degeneration in specific motor neurons, gene mutations

5. Aim of work

Cellular proteins are vulnerable to a variety of physical and chemical stresses, including heat, free radicals, and toxic compounds. These agents can cause protein malfunction or aggregation, resulting in cellular dysfunction, and adduct formation. Cells contain an extensive network of chaperones and proteases for protein homeostasis quality controls. The mitochondrial protein quality control network is critical for cellular protein and energy homeostasis.

Internal mitochondrial proteases appear to act as the first layer of quality control to maintain protein homeostasis. Any internal mitochondrial proteolysis dysfunction has serious implications for cell survival and may also lead to apoptosis. Many proteases help to completely degrade superfluous or damaged mitochondrial protein (Koppen and Langer 2007). ATP-dependent AAA proteases (ClpP, Lon...) are important tools to remove oxidatively damaged components from an internal mitochondrial membrane?

ClpP protein is absent in yeast S.cerevisiae (*Saccharomyces cerevisiae*). Pim1 is the yeast homolog of the Lon protease, involved in protein quality control by degrading the misfolded protein (von Janowsky, Knapp et al. 2005). According to these findings, Pim1 serves as the mitochondrial matrix's primary quality control protease. Furthermore, mitochondrial enzymes such as Ilv3 and aconitase are particularly susceptible to Pim1 degradation during oxidative stress, most likely because their Fe/S cluster cofactors become unstable after covalent oxidative modification (Bota and Davies 2002).

Lon and AAA family proteases help avoid the toxic aggregate formation and other negative effects by degrading denatured polypeptides (Gur and Sauer 2008). The existence of general chaperones and proteases indicates that cells differentiate between refolded proteins and damaged proteins bound for the proteolytic pathway. The mechanisms that regulate this crucial metabolic decision are unknown. Furthermore, the mechanism by which these enzymatic mechanisms differentiate between misfolded and correctly folded proteins is unknown. It’s been discovered that mtHsp70 works in tandem with ATP-dependent Lon proteases to recognize and remove damaged proteins (Voos 2013). The inner mitochondrial protein import and folding machinery include mtHsp70. The main inner membrane translocase complex, TIM23, is involved in the translocation of matrix-targeted preproteins as well as some inner membrane proteins (Marom, Dayan et al. 2011).

Preproteins import through the inner mitochondrial membrane necessitates energy from both the inner mitochondrial membrane potential and ATP (Stuart, Gruhler et al. 1994). Tim23 protein binds the outer membrane channel TOM complex. Tim23 is a membrane protein with four helices that forms a translocase channel that is cationic selective and activated by the pre-sequence and membrane potential (Bauer, Sirrenberg et al. 1996).

The PAM complex is important for precursor mitochondrial protein import into the mitochondrial matrix. Several chaperones & co-chaperone proteins that are conserved across eukaryotes make up the PAM complex. The core component of the PAM complex is mitochondrial Hsp70. Pam16 and Pam18 are two J proteins that influence mtHsp70's ATPase activity. Pam17 works together with Pam16 and Pam18 (Frazier, Dudek et al. 2004) (Hutu, Guiard et al. 2008). Mge1, a nuclear matrix exchanger, supports the mtHsp70 ATPase cycle that helps to efficiently import the mitochondrial proteins by exchanging the ATP for the ADP on mtHSp70 (Voos, Gambill et al. 1994) (Westermann, Prip-Buus et al. 1995). Since Hsp70 collaborates with Lon protease, the relationship between the PAM complex's components and Lon protease is fascinating. However, very less is known regarding the regulation and turnover rate of PAM complex subunits like Pam16 and Pam18. Similarly, the regulatory role of Lon on other Hsp70 co-chaperone like Mge1 is still to be explored. Lon/Pim1 has emerged in recent years as a key component in mitochondrial quality control and is increased in chronic illnesses and cancer. Several groups have generated Lon inhibitors for inhibition of Lon that has a potential effect in regression of tumors. Therefore, there is a need for the identification of additional inhibitors that may help cure diseases such as cancer. This study aims to learn more about how the Lon protease interacts with the Pam16 and Pam18 subunits of the PAM complex. Besides, novel inhibitors of Lon protease were identified. Regulation and turnover of Mge1 in relation to Lon role was also established in this study.

Chapter 2

Methods & Materials

Materials and methodology

2.0 Materials

2.1 Yeast strains construction

Table 2.1 indicates the strains of S. cerevisiae used and their genotypes in this thesis .W303 or BY4741 derivative strains were used in this analysis. Yeast strains were grown in either YPD (1 percent yeast extract, 2% peptone, and 2% glucose) or synthetic medium (SD; 0.67 percent yeast nitrogen base, 2 percent glucose, and auxotrophic amino acids and vitamins as required). Since MGE1.(MATa/α;hisΔ1/3his3Δ1;Leu2Δ0/leu2Δ0;lys2Δ0/LYS2;MET15/met15Δ0;ura3Δ0/ura3Δ0;OR232w::kanMX4/YOR232w),PAM16.(MATa/α;hisΔ1/3his3Δ1; leu2Δ0/leu2Δ0;lys2Δ0/LYS2;MET15/met15Δ0;ura3Δ0/ura3Δ0;YJL104w::kanMX4/YJL104w)andPAM18(MATa/α;hisΔ1/3his3Δ1;Leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; O YLR008c: kanMX4/YLR008c) are essential genes, the heterozygous deleted diploid strains were purchased from EUROSCARF, Germany. To generate haploid overexpression strains, wild type pTEF-Ura MGE1 (pNB45), pTEF-Leu PAM16 (yNB100), and pTEF-Leu PAM18 (yNB101) vectors were transformed to their respective heterozygous deleted diploid strains. Haploid colonies were selected through random sporulation to generate yNB65, yNB100, and yNB101 strains. For constructing Mge1 mutant strains, yNB 65 strain was transformed with plasmids pNB358 (pTEF-Leu MGE1 WT), pNB361 (pTEF-Leu Mge1 M155L), and pNB353 (pTEF-Leu Mge1 H167L), and subsequently URA3 plasmid was kicked out by growing on a 5-FOA plate to generate yNB67 (wild type MGE1), yNB69 (MGE1 M155L) and yNB70 (MGE1 H167L) strains respectively.

pim1Δ(MATa;ura3-1,ade2-1,trp1-1,his3-11,leu2-3,112,met2-1,lys2-2,Pim1::KanMX4/) strain is a gift from E. Craig, USA. The pNB5637 and pNB5638 plasmids were transformed into *pim1Δ* strains to create yNB374 and yNB375 strains, respectively. To create *pim1* deletion in yNB67 (wild type MGE1), yNB69 (MGE1 M155L), yNB70 (MGE1 H167L), yNB100 and yNB101 strains, we used PCR based homologous recombination method. pYM HisMX vector was used as a template to amplify HIS cassette using YB13 (Forward) and YB14 (Reverse) primers containing 50-52bp of homology to Pim1. The PCR amplified product was transformed to each strain using the conventional lithium acetate transformation method. Selectable markers were used to pick the colonies of interest and deletion was confirmed by full-length PCR primers of Pim1 (NB770, NB771).

W303 (yNB111) strain, i-AAA (yNB185), m-AAA (yNB186), and OMA1 (yNB187) deletion strains are a generous gift from Prof. Krishnaveni Mishra, University of Hyderabad, India. PNB123 plasmid was transformed to yNB111 and *yNB222* to create yNB333 and yNB444 strains respectively. Pim1 TAP-tagged strain was purchased from EUROSCARF, Germany.

2.2 Plasmids construction

pNB5637 (pRS414 ADH-Pim1 WT) and pNB5638 (pRS414 ADH-Pim1 mutant S855S) plasmids are a gift from E. Craig, USA. pNB123 (Mge1-GFP) plasmid is a gift from C.Z, USA. NS86 (Lon proEX WT) and NS87 (Lon S-A Mutant) plasmids are a gift from C.Suziki, Johns Hopkins University, USA. For the construction of pTEF-Pam16 and pTEF-Pam18 plasmids, complete coding sequences of yeast Pam16 and Pam18 were amplified with primer pairs NB862 Pam16_F/NB863 Pam16_R for Pam16 and NB864 Pam18_F /NB865 Pam18R for Pam18. The PCR amplified products were digested with BamH1/Xho1 restriction enzymes (Thermo Scientific). Restricted digested plasmids and amplified PCR products were separated on 0.8 % agarose gel electrophoresis and purified using nucleospin columns. The digested product was ligated with pTEF Leu vector, double digested with the respective restriction enzymes. Ligation was carried out by taking digested plasmid DNA and PCR product in a 1:3 ratio and incubation at 22 °C for 2-3 hours using T4 DNA ligase (Thermo Scientific).

2.3 Yeast strains and their genotypes

Table 2.1 Yeast strains and their genotypes

strain	Genotype	Source
yNB36	*MATa/alpha;hisΔ1/3his3Δ1;Leu2Δ0/leu2Δ0;lys2Δ0/LYS2;MET15/met15Δ0;ura3Δ0/ura3Δ0;OR232w::kanMX4/YOR232w*	EUROSCRAF

yNB37	*MATa/alpha;hisΔ1/3his3Δ1;Leu2Δ0/leu2Δ0;lys2Δ0/LYS2;MET15/met15Δ0;ura3Δ0/ura3Δ0;YJL104w::kanMX4/YJL104w*	EUROSCRAF
yNB38	*MATa/alpha;hisΔ1/3his3Δ1;Leu2Δ0/leu2Δ0;lys2Δ0/LYS2;MET15/met15Δ0;ura3Δ0/ura3Δ0;YLR008c::kanMX4/YLR008c*	EUROSCRAF
yNB65	*MATa,hisΔ1,leu2Δ0,lys2Δ0,ura3Δ0,YOR232w::KANMX4 pTEF-2μ-WT MGE1- URA*	This study
yNB67	*MATa,hisΔ1,leu2Δ0,lys2Δ0,ura3Δ0,YOR232w::KANMX4:pTEF-2μ-WT MGE1- LEU*	This study
yNB69	*MATa,hisΔ1,leu2Δ0,lys2Δ0,ura3Δ0,YOR232w::KANMX4: pTEF-2μ- M155L MGE1- LEU*	This study
yNB70	*MATa:hisΔ1,leu2Δ0,lys2Δ0,ura3Δ0,YOR232w::KANMX4 :pTEF-2μ- H167L MGE1- LEU*	This study
yNB100	*MATa:hisΔ1,leu2Δ0,lys2Δ0,ura3Δ0,YOR232w::KANMX4 :pTEF-2μ-WT PAM16-LEU*	This study
yNB101	*MATa,hisΔ1,leu2Δ0,lys2Δ0,ura3Δ0,YOR232w::KANMX4 pTEF-2μ-WT PAM18-LEU*	This study
yNB145	MATa;*leu2-3,112trp1-1,met2-1,ura3-1ade21,trp,his3-11,15,lys2-3 YBL022c::KAMX4/YBL022c*	Ref
yNB257	Strain YNB145 transformed with pTEF PAM16,2µ, LEU	This study
yNB258	Strain YNB145 transformed with pTEF PAM8,2µ, LEU	This study

yNB374	Strain YNB145 transformed with pRS414 –ADH-PIM1 WT	This study
yNB375	Strain YNB145 transformed with pRS414 –ADH-PIM1 MUT	This study
yNB358	Strain YNB145 transformed with pTEF-MGE1 WT, 2μ, LEU	This study
yNB361	Strain YNB145 transformed with pTEF-MGE1 M155L, 2μ, LEU	This study
yNB353	Strain YNB145 transformed with pTEF-MGE1 H167L, 2μ, LEU	This study
yNB111	W0303 *: MATa ;leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11::KANMX4*	Ref
yNB112	BY4741 *: MATa ;his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Ref
yNB365	PIM1 TAP: *MATa ;his3Δ1 leu2Δ0 met15Δ0 ura3Δ0:PIM1 TAP Tag:URA*	Ref
yNB333	Strain YNB111 transformed with pRS 316 MGE1*:GFP-URA*	This study
yNB444	Strain YNB145 transformed with pRS 316 MGE1*:GFP-URA*	This study
yNB185	i-AAA: *Mat a, hisΔ1, leu2Δ0, lys2Δ0, ura3Δ0, YPR034w::KANMX4*	Ref
yNB186	m-AAA*: Mat a, hisΔ1, leu2Δ0, lys2Δ0, ura3Δ0, YMR089l::KANMX4*	Ref

yNB187	OMA1:*Mat a, hisΔ1, leu2Δ0, lys2Δ0, ura3Δ0, YKR087c::KANMX4*	Ref

2.4 Plasmid list

Table 2.2 Plasmids list

Name	**Description**
pNB207	pET28a+ Pam16
pNB144	pET28a+ Pam18
pNB182	pET28a+ GRPEL1
pNB186	pET28a+ MGE1
pNB183	pET28a+ M155L MGE1
pNB208	pET28a+ H167L MGE1
pNB200	pETT28+ mt HSP70
pNB485	pETT28+ Mxr2
pNB488	pETT28+ mpp
pNB502	pTEF PAM16,2µ, LEU
pNB502	pTEF PAM18,2µ, LEU
pNB45	pTEF-MGE1, 2µ, URA
pNB358	pTEF-MGE1, 2µ, LEU
pNB361	pTEF-MGE1 M155L, 2µ, LEU
pNB353	pTEF-MGE1 H167L, 2µ, LEU
pNB111	Lon ProEX

pNB112	Lon ProEX S-A

2.5 Yeast and *E.coli* growth media composition

Table.2.3 Yeast and *E.coli* growth media composition:	
YPD	1% yeast extract,2% peptone,2% Dextrose
YPGal	1% yeast extract
YPG	*1% yeast extract,2% peptone.3% Glycerol*
Lactate media	3 g yeast extract,1 g KH$_2$PO$_4$,1 g NH4Cl,0.5 g CaCl2,0.5 g NaCl,0.8 g MgCl$_2$, 1 g glucose.100 ml 20% lactic acid, pH 5.5
YPL	1% yeast extract, 2% peptone, 2% Lactate pH 5.5

2.6 Primers list

Table 2.4 Primers list

Primer Number	**Sequence (5’---3’)**	**Restriction sites**	**Insert**
NB862	AAA GGATCC ATGGCTCACAGGGCTTTCATAC	Bam H1	PAM16 F TEF 2
NB863	AAA CTCGAG CTACTGATTGCTGCTTGCACTA	Xho1	PAM16 TEF
NB864	AAA GGATCC ATGAGTTCTCAAAGTAATACTG	BamH1	PAM18 F TEF 2
NB865	AAA CTCGAG GAAAAAAGGGGTATTAGCAAA	Xho1	PAM18 F TEF 2
NB435:	CGC CTC GAG ATG GAG CCG CAG TCA	Xho1	GrpeL1F PET28
NB436	CGC GAA TTC TCA GTC TGA GTC AGG CCC	EcoR1	GrpeL1F PET28
NB540	GGG GTT AGA TGT ACA AGA GAT 3’	Site Directed	YMGE1 M155C F
NB541	ATC TCT TGT ACA TCT AAC CCC 3’	-	YMGE1 M155C R
NB561	CCC AGA ATT CAC CAT GTT GAG ATC CGT T	EcoR1	HSP60F
NB562	CCCA CTC GAG TTA CAT ACC TGG CAT	Xho1	HSP60R
YB13	TTTTCTTTTGGTTTTCGAGGTGCTTGAACGAAAAGATTTGCAAATAGAGCCGGATCCCCGGGTTAATTAA	-	
YB14	AAATATTTACAGAATGTTTAAACAGGTATTTAATCCATTTAGATGAAAAGGAATTCGAGCTCGTTTAAAC	-	
NB770	CCACCGGAAGGAAATAAAGTCAGACG	-	
NB771	CTTTCGCCAGAATACTTTTGCCGC	-	

2.3.0 Methods used in the thesis

2.3.1 Cloning

Cloning was performed by digesting DNA using restriction enzymes (FERMENTAS). Usually, plasmid DNA (200-400 ng) was digested overnight in a total volume of 50 µl. Restricted digested plasmids and amplified PCR products were separated on 1% agarose gel electrophoresis and purified using nucleospin columns. Ligation was carried out by taking digested plasmid DNA and PCR product in a 1:3 or 1:5 ratio and kept incubation at 22 °C for overnight along with T4 DNA ligase (FERMENTAS).

2.3.2 Preparation of ultra-competent *E.coli* cells

E. coli cells were grown in 250 ml of bacterial LB medium (2% peptone, 0.5% yeast extract, 0.5% NaCl,) in a one-liter volumes flask. The cultures were grown at 18 °C, orbital shaker maintained at 220 rpm speed until OD600 reached approximately 0.6 nm (about 20 hrs.). The culture was allowed to cool on ice for 10 mins and centrifuged at 2,500 g for 10 mins at 4 °C. The pellet was then suspended in PIPES buffer (10 mm PIPES, 15 mm $CaCl_2$, 250 mM KCl; pH 6.7 adjusted with KOH). The cell suspension was kept on ice for 30 mins, later cells were spun at 4 °C with a speed of 2,400g. And the pellet was then suspended in 20 ml of PIPES buffer with 7% DMSO. Further, cells were kept on ice for 10 mins and aliquots were snap freeze with liquid N_2 before storage at - 80 °C.

2.3.3 Plasmid DNA purification

DH5α E. coli strains were used to amplify the plasmid copy number. A single colony was inoculated into a 10 ml LB medium containing either Ampicillin (100 µg/ml) or Kanamycin (25 µg/ml) incubated overnight at 37 °C with shaking 180 rpm speed. Cells were collected by spin at 13,000 rpm speed for 1 min on a tabletop centrifuge (Eppendorf, Germany). Plasmid DNA was purified by the QIAPrep Spin Miniprep Kit (QIAGEN, Germany).

2.3.4 Yeast transformation

Yeast strains were transformed by following the standard lithium acetate method. Yeast cells were inoculated in YPD (10 ml) media and grown by keeping culture on a shaker with speed 180 rpm at 30 °C and grown until OD reaches 0.6. The cells were centrifuged at 2,500 rpm for 5 mins, suspended in 1 ml of 100 mm LiOAc (Lithium Acetate). 50-100 µl cells were taken from suspension and centrifuged at high speed for 30 secs and transformation mixture was prepared by adding 240 µl 50 % PEG, 36 µl 1 M LiOAc, 50 µl single standard DNA, 1 µg of plasmid DNA in sequential order and vortexed vigorously. The transformation mixture was later incubated in a thermo mixture maintained at 30 °C with shaking (750 rpm) for 30 mins. Later yeast cells were subjected to heat shock by keeping the cells at 42 °C for 25 mins. Immediately after heat shock, cells were spun at high speed for 30 secs and suspended in 500 µl of mill Q water and plated on drop-out media and incubated at 30ºC for 2-3 days until colonies appeared.

2.3.5 Procedure for isolation of genomic DNA from yeast cells

Parental BY4741 yeast cells were inoculated into 10 ml of YPD media and grown overnight at 30ºC in a shaking incubator. Cells were then centrifuged at 5000 rpm followed by washing with water and suspended in 0.5 ml H_2O. Again, the cell suspension was spin at a high speed for 15 secs. The cell pellet was resuspended in 200 µl of breaking buffer (2% v/v Triton X 100, 1% v/v SDS, 100 mm NaCl, 10 mm Tris-Cl, pH8.0, 1 mm EDTA, pH 8.0) and cells were lysis by vortexing at high speed by adding 0.2 gm of 0.45 mm acid-washed glass beads (Sigma). 200 µl of TE buffer (10 mm Tris-Cl pH7.5/8.0, 1 mm EDTA, pH 8.0) was added and the lysate was again vortexed for 20 secs and spin at high speed for 5 mins using Eppendorf table centrifuge. The aqueous layer was transferred to an autoclaved microfuge tube, an equal volume of 100% ethanol was added and mixed gently by inversion. The Mixture was spin at 13,000 rpm for 3 mins at RT. The supernatant was discarded and the pellet was kept for drying at room temperature and resuspended in 100 µl TE buffer. To remove RNA contaminants, 30 µl of 1 mg/ml RNase A was added and incubated for 1 hrs.at 37 °C and 10 µl of 4M NH_4COOCH_3 and 1 ml 100% ethanol was added and gently mixed. The mixture was spin at high speed for 3 mins at RT. The supernatant was discarded and the pellet (DNA) was kept for drying at room temperature. Finally, the pellet was resuspended in 100 µl of TE buffer. The concentration of DNA was quantified by Nano drop (Thermos Scientific, USA) at a wavelength of 260 nm.

2.3.6 Yeast growth assays

Yeast cells were grown up to the mid-log phase at 30ºC. About 0.5 OD cells were suspended in sterile water and made serial dilutions by 10 folds. Each dilution (5-10 µl) was spotted on agar plates supplemented with selective media or glucose-containing rich media, incubated for 2- 3 days at indicted temperatures.

2.3.7 Yeast cell lysates preparation for Western blotting

Yeast strains were grown overnight in a 10 ml appropriate selective medium. Cultures were centrifuged at 5,000 rpm for 5 mins (Eppendorf, Germany) at room temperature and cells were washed with sterilized water and resuspended in 50 µl buffer (50 mm Tris pH 7.5, 100 mM NaCl, 1 mM PMSF (phenylmethylsulfonyl fluoride)) along with 0.2 gm acid wash glass beads. This mixture was vortex vigorously and centrifuged (Eppendorf, Germany) at 13,000 rpm for 10 min at 4ºC. The supernatant contains soluble proteins was estimated by the Bradford protein assay kit (AMRESCO). 2X SDS sample buffer was added to 50 – 100 µg of protein and kept for boiling at 95 ºC for 5 mins. These samples were run on SDS-PAGE and western analysis or can be stored at -20 ºC.

2.3.8 Procedure of protein purification from *E. coli* cells

Pam18, Pam16, and Mdj1 clones constructed into pET28a (+) vector were transformed to BL21 (DE3) bacterial competent cells. A single colony from transformed was inoculated into LB media and grown at 30ºC for 3-4 hrs.by shaking at 220 rpm speed. Cultures were centrifuged at 8,000 rpm for 10 mins and resuspended in buffer (25 mm Tris-HCl pH 8.0, 100 mm NaCl, and 2 mm DTT) and stored at -80 ºC. Cell suspensions were thawed to 4 ºC and the suspension was lysed by extensive sonication for 20 cycles (15 sec ON and 45 sec OFF) at 30% amplitude. The bacterial lysates were cleared at high speed for 30 mins and the supernatant was loaded to Ni- NTA charged beads (Clontech, USA). Beads were washed with 3-5 volumes of buffer containing 20mM imidazole. Finally, proteins were eluted with buffer containing 400mM imidazole and dialyzed against buffer containing 10% glycerol without imidazole. Protein aliquots were subjected to snap freeze using liquid nitrogen and stored at -80ºC.

2.3.9 Western blotting

Protein immuno-blotting was done by using yeast protein extract prepared by following protocol. An equal amount of proteins were separated by 12% SDS PAGE (PolyAcrylamide Gel Electrophoresis) and transferred to nitrocellulose membrane using buffer (Towbin buffer) overnight at 50 V. Transferred proteins were visualized by staining the membrane with Ponceau S stain (0.2% Ponceau S in 3% Acetic acid) followed by repeatedly washing with 1X TBST (0.1% Tween 20 in TBS: 25 mm Tris pH 7.4, 155 mm NaCl, with HCl). Blocking was done using 5% milk powder (non-fat) in 1X TBST for 1 hrs at room temperature. The membrane was probed with the appropriate primary antibody or antisera by incubating on a shaker overnight at 4ºC or 1 hour at room temperature. The membrane was further washed three times for every 15 mins with 1X TBST. Horse Radish Peroxidase (HRP) coupled rabbit anti-mouse or anti-rabbit IgG secondary antibodies were incubated with the membrane at room temperature for 1 hour followed by again washing for 3 times each 15 mins with 1X TBST. Finally, the membrane was developed by the Enhanced Chemiluminescence (ECL) reagent supplied by Bio-Rad or AMERSHAM.

2.3.10 Fluorescence microscopy analysis

Mid-log phase yeast cells, grown in selective media, cells were spin at 2000 rpm. Cells were suspended in sterile water, 10- 20 µl of cell suspension was spread on the glass slide and allowed to dry at RT. 5 µl of 50 % glycerol was added to avoid air bubbles and a coverslip was placed. Cells were viewed either with bright filed or GFP filter using a ZEISS microscope.

2.3.11 Yeast mitochondria isolation procedure

Yeast mitochondria were isolated using the standard procedure. Cells were grown in YPL or Semi-synthetic lactate media up to 1- 2 OD at 30ºC with shaking at 180 rpm speed. Cells were harvested by centrifuge (KUBOTA, Japan) at 5,000 rpm for 5 mins followed by washing with sterile water. The cells were resuspended in buffer containing 10 mm dithiothreitol (DTT) and 0.1 M Tris-SO4, pH 9.4, and incubated for 15 min with slow shaking. Again cells were harvested by centrifugation at 5000 rpm for 5 mins. Spheroplast was made lysing the cells by treating with Zymolyase (Lyticase Sigma) in 1.2 M sorbitol/20 mm phosphate buffer, pH 7.0 for 45 mins at 30ºC by slow shaking. Lysis was monitored by observing the OD of spheroplast 600 nm by UV-Visible spectrophotometer (HITACHI, Japan). After reaching half the OD without Zymolyase treated cells, spheroplast was gently washed with 1.2 M sorbitol two or three times at 3500 rpm in cold conditions. Now spheroplast was resuspended in SEM buffer (250 mm sucrose, 1 mm EDTA, 10 mm 3-(N-morpholino) propane sulfonic acid [MOPS] and homogenized using Dounce homogenizer by giving 15 strokes. The homogenate was centrifuged at 3500 rpm for 5 mins. Repeat the homogenization step was repeated once more and the supernatant fraction was collected. The supernatant was spun at 10,000 rpm for 10 min (Eppendorf, Germany) and the pellet fraction was saved. The pellet was resuspended in SEM buffer and the supernatant was saved by centrifugation at 3500 rpm for 5 mins. Finally, the supernatant was centrifuged at 10,000 rpm for 10 mins, and saved the pellet containing mitochondria, and washed three times with SEM buffer by centrifugation at the same speed. The mitochondria were solubilized in SEM buffer (without BSA) and made into aliquots (10 mg/ml), snap freeze the samples, and stored at -80 °C.

2.3.12 Identification of Pim1 Partners Using TAP:

The tagged complexes were purified from mitochondria isolated from two different yeast strains (from Euroscarf), SC0000 (control strain expressing the Pim1 protein) and SC0437 (strain expressing the Pim1-TAP fusion protein). The TAP purification was performed by sequential IgG affinity columns. Briefly, mitochondria were lysed with 0.25% NP-40 and cleared by low-speed centrifugation. The supernatant was further lysed with 1.25% NP-40, cleared by high-speed centrifugation, and incubated with IgG beads. The bound complexes were eluted by glycine

2.3.13 Preparation of H_2O_2 [1 M]

Hydrogen peroxide 30% purchased from Merck, chemicals. Molarity was estimated by taking absorbance at 240 nm using its extinction coefficient (E 240 nm=43.6 M^{-1} cm^{-1}). 1 M hydrogen peroxide was prepared by appropriate volume from 30% hydrogen peroxide and diluted to 1 ml with deionized water.

2.3.14 Expression of recombinant proteins in E.coli

In an E. coli Rosetta 2 (DE3) strain, a cDNA encoding human Lon with an amino-terminal hexa histidine a linker region (MGHHHHHHDYDIPTTENLYFQGAHM) fused to methionine 115 in a pProEx-1 vector (gift of C.Suzuki) was expressed (Novagen). Bacteria were grown at 37°C in 1 percent bactotryptone, 0.5 percent yeast extract, 1 percent NaCl, 100 g/ml ampicillin, and induced for 90 mints with 1 mm isopropyl thio galactoside (IPTG) at 30°C. The expressed protein is present in the soluble fraction.

2.3.15 Purification of recombinant proteins by Ni-NTA column

Bacterial cells expressing recombinant proteins were harvested and suspended in Tris buffer (pH 8.0) and sonicated on ice. The cell lysate was centrifuged for 10 min at 100 000 × g and the supernatant was equilibrated in buffer A (Tris Hcl, pH.7.5, NaCl, β-Mercaptoethanol) and added to the Ni Beads (Clonetech). The column was washed with buffer B (50mM TrisHCl pH 7.5 with 300 mm KCl, 5mM $MgCl_2$ and 10% glycerol) containing 50 mM Imidazole. The column was then eluted in five steps with buffer C containing 0.4 M Imidazole. Purified protein was analyzed by SDS-PAGE and western blotting.Proteins were snap freeze and stores at -80 °C till use.

2.3.16 Lon Degradation assays

To monitoring the ATP-dependent proteolytic activity of hLon is to follow the degradation of β-casein on SDS-PAGE using Coomassie Brilliant Blue staining. To do this, aliquots were taken at different time points (0, 30, 60 and 90 min) from a 50 µl reaction mixture containing 1µg Lon and 20 µg of β-casein (Sigma Aldrich) in 50Mm Tris HCl, 100 mm KCl, 40 mm $MgCl_2$, 1mM DTT, and 5mM ATP. Pure gene Pertained Protein Ladder was used as the molecular weight standard. Degradation assays were carried out at 37°C with Purified protein (5µg) was incubated with or without Lon (1µg). After the addition of the enzyme to the protein, immediately added 2X loading dye kept at 4°C. It is taken as 100 percent or zero time point. Protein was treated with 20 mm H_2O_2 for 3 hours then dialyze the protein used for proteolysis assay with Lon.

2.3.17 Yeast whole cell lysates preparation

0.1 OD_{600} units of whole-cell lysates prepared by bead beading after suspension in 1X RIPA Buffer (50 mM Tris-HCl (pH 7.5) ,150 mm NaCl, 0.5% Na_2EDTA,1% NP-40 1% sodium deoxy cholate ,0.1%SDS). Lysates were centrifuged at 10000 rpm for 10 min to remove cell debris.

The resulting supernatants protein concentration was measured by the Folin method. Supernatant suspended in 2x SDS loading dye, boiled at 95ºC for 5 min. An equal amount of cell lysate for immunoblotting.

2.3.18 Cell culture and drug treatment.

HeLa (human epithelial) cell was cultured in DMEM (Dulbecco's modified Eagle's medium) (Invitrogen) containing 10% (v/v) FBS at 37°C under an atmosphere of 5% CO_2. Cells were grown until confluence then treated with the 100 μM methyl Gallic acid and Gallic acid in time deponent (12,24,36,46 hrs.,) manner individually. After that cell extract was prepared and used 50 μg of protein lysate for Weston blotting.

2.3.19 Molecular Docking:

The structure of the human Lon proteolytic P-domain was (Protein Data Bank code 2X36) as the template structure. The active site was docked with the inhibitors by using the dock suite of DISCOVERY STUDIO 2.0 (Accelrys).PYMOL software (DeLano Scientific, http://www.pymol.org) was used to show the final blocking structure of human Lon homology with the inhibits.

2.3.20 Protein aggregation assay

For each protein aggregation assay, 50 μg of mitochondria were suspended in aggregation assay buffer (250 mM sucrose, 10 mM MOPS, 80 mM KCl, 5 mM MgCl2, 5 mM ATP, and 4 mM NADH) and treated with H2O2 for 30 min at room temperature (Bender et al., 2011). The mitochondria were centrifuged at 10,000 rpm for 10 min, and the pellet was solubilized in lysis buffer (0.5% Triton X-100, 200 mM KCl, 30 mM Tris-HCl, pH 7.5, 5 mM EDTA). The solubilized mitochondria were centrifuged at 25,000 rpm for 1 h. The supernatant fraction (soluble fraction) was collected, and the pellet (aggregated fraction) was resuspended in lysis buffer. Twenty percent of supernatant fraction and total pellet fraction was separated by SDS–PAGE, and the gel was silver stained.

Chapter 3

Lon/Pim1 mediated regulation of mitochondrial inner-membrane PAM complex subunits, Pam16 and Pam18

3.1 Introduction

Mitochondrial proteins that are synthesized in the cytosol are translocated into the matrix through a membrane channel. Mitochondrial function depends on these inner and outer membrane proteins. PAM which resides on the inner membrane of mitochondria (Neupert and Brunner 2002), is involved in the mitochondrial Hsp70 mediated import of precursor proteins. Pam16 and Pam18 are subunits of the PAM complex containing a J domain that influences the ATPase activity of mtHsp70. Pam17 cooperates with the binding of Pam16 and Pam18 to the TIM23 complex. (Frazier, Dudek et al. 2004) (Hutu, Guiard et al. 2008).The C-terminal matrix-localized J-domain of Pam18 protein has a single membrane region and an intermembrane space (IMS) N-terminal domain. (IMS) (Figure 1). The Pam18 IMS domain collaborates with Tim17 and also TIM23 (D'Silva, Schilke et al. 2003) (Truscott, Voos et al. 2003).

Pam18's J-domain collaborates with Pam16 (also called Tim14) protein whose N-end partners with the translocon, mostly through connection with Tim44 (D'Silva, Schilke et al. 2008). Pam18 communicates with the translocon in a variety of ways, including explicit interactions in the IMS and subtle interactions in the matrix. The J-like domain is also available in Pam16. Despite the similarity in structures and sequences to Pam18 J-domain, Ssc1/Hsp70's ATPase activity cannot be stimulated. Pam16 and Pam18 make up a stable heterodimer, which is essential for the translocation of proteins and the viability of cells via their J- and J-like domains (Truscott, Brandner et al. 2003).

Chaperones and proteases shape a complicated network of mitochondrial protein quality control. Inner mitochondrial proteases, which have developed over time, seem to work as the first line of defense inside the organelle. Large numbers of these proteases are found in eukaryotic cells and work together to degrade damaged mitochondrial proteins (Koppen and Langer 2007).

Malfunctions of the mitochondrial proteolytic pathway directly affect cell survival by inducing apoptosis thereby leading to neurodegeneration in mammalian cells. Inner mitochondrial membrane proteases, i-AAA proteases are specifically and selectively involved in eliminating oxidatively damaged components. Conserved from bacteria, mitochondria possess Lon protease and ClpP proteases localized in the matrix of mammalian cells. *Saccharomyces cerevisiae* is devoid of ClpP protease but has Lon protease homolog, Pim1 that functions to degrade misfolded proteins.

Interior mitochondrial proteolysis failures have severe effects on cell survival and lead to apoptosis and mammalian cell neurodegeneration. AAA proteases, which are found at the inner mitochondrial membrane and are ATP dependent, are critical tools for removing oxidatively damaged components from the inner membrane (Smakowska, Czarna et al. 2014).

When cells are stressed, proteins expand or misfold. Lon and related ATP-dependent AAA+ proteases help prevent the harmful aggregate formation and other negative effects by degrading denatured polypeptides, but how these degradative enzymatic machines differentiate between damaged and properly folded proteins is unknown (Gur and Sauer 2008). Hsp70 has been found to work in tandem with ATP-dependent Lon proteases to identify and remove damaged proteins (Voos 2013). The function of PAM critical subunits like Pam16 and Pam18 in these processes is unknown.

How do different PAM complex proteins of the mitochondria are subjected to specific degradation and turnover of PAM complex subunits such as Pam18 and Pam16 is poorly understood. Here we investigated Lon's role in regulating PAM complex subunits using budding yeast as a model system. We find that mitochondrial matrix Pim1 protease is involved in the degradation of inner-membrane proteins Pam16 and Pam18. We also find that stress-induced aggregation of Pam18 and Pam16 proteins are protected by Pim1 protease.

3.2.0 Results

3.2.1 Degradation of recombinant Pam16 and Pam18 proteins by Lon protease *in vitro*

We used purified (Figure: 3.1) Lon protease for *in vitro* degradation assays described in the Methods. We purified recombinant Pam18, Pam16, and Mxr2 proteins from bacterial cells as described in the Methods. Casein protein was purchased commercially. The proteins were found to be stable upon incubation either at 30°C or 37°C. Next, we incubated these proteins (5 µg each) with a sub-stoichiometric concentration of Lon protease (2 µg) either at 30ºC or 37ºC at different time points as mentioned in the figures. Casein is well known substrate for protease , so we used it as a substrate for Lon and perform degradation assay and found that time dependent degradation of it (Figure 3:2A). The amount of Pam16 or Pam18 present in the reaction mixture decreases with time (Figure3: 2C) and only 10% remains after 60 min of incubation. In contrast, the quantity of Mxr2, a mitochondrial matrix protein, remains unchanged. We also performed an additional control experiment with Mpp, a known substrate of Lon protease, and discovered that it is degraded in a time-dependent manner (Figure 3: 2B). Besides, we also expressed and purified the recombinant human catalytically inactive mutant Lon (Lon S_A variant) and observed that there is no degradation of Pam16 (Figure3: 2D). Taken together, these *in vitro* results suggest that Pam18 or Pam16 are possible the substrates for LON protease.

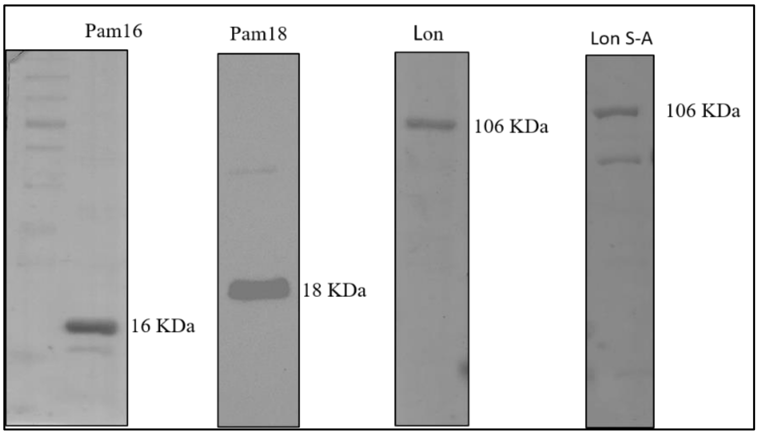


Figure3.1: Purification of recombinant Pam18, Pam16, and catalytically inactive LON (Lon S-A) proteins by Ni-NTA column: *Bacterial cells expressing recombinant proteins were purified by Ni-NTA column and separated on SDS–PAGE. Coomassie-stained gels are shown.*

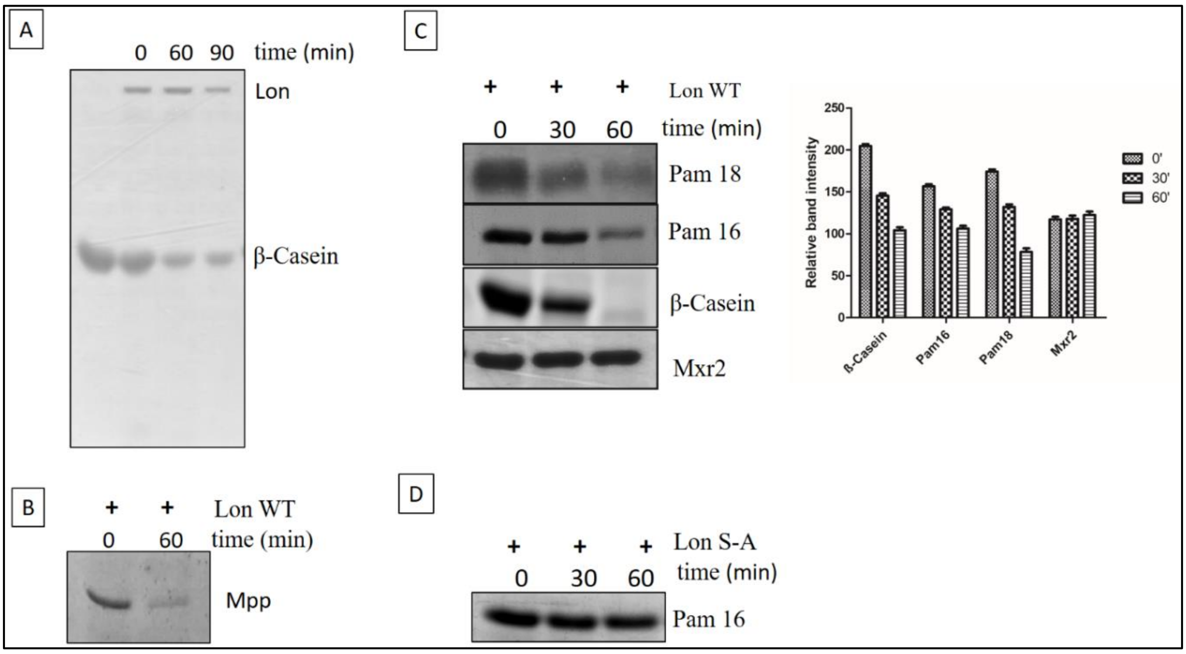


Figure 3.2: Degradation of Pam16 and Pam18 subunits by Lon protease in vitro*. (A-C): Casein (10 µM) (A), Mpp (B) (5 µM) Pam18, Pam16, Mxr2 (5 µM each) (C) were incubated with human recombinant Lon*

protease (5 µM) as described in the methods. At indicated time points, samples were removed. Latter on separated using SDS-PAGE & stained with Coomassie. The amount of protein present after degradation was quantified by ImageJ and plotted as relative band intensity. (B & D): Mpp was incubated with Lon, Pam16 (5 µM) was incubated with Lon mutant (LON S_A) at indicated times, aliquots of reactions were removed, separated on SDS–PAGE, and immunoblotted with Pam16 antibodies.

3.2.2 Pim1 regulates Pam18 or Pam16 levels *in vivo*

To test whether Lon protease regulates PAM complex subunits *in vivo*, we used *pim1Δ* and *pim1Δ*/ PIM1 yeast strains that lack a Lon protease homolog, Pim1, or presence of a plasmid-borne copy of PIM1 in *pim1* deletion strain. In whole-cell lysates or isolated mitochondria, we determined the steady-state levels of Pam16 and Pam18 in *Δpim1* strain. Pam16 and Pam18 levels are significantly more in *Δpim1* (Figure3.3A) than in wild-type (WT) and *Δpim1*/PIM1 strains in whole-cell lysates or isolated mitochondria. Hsp60, a known substrate for Lon protease, levels are markedly increased in the *Δpim1* strain while control proteins such as cytosolic Pgk1 or mitochondrial Hsp70 or Porin levels are not altered. Next, we wanted to check whether different proteases found in the inner membrane, known to have a wide scope of substrate specificities, could likewise be associated with Pam18 and Pam16 degradation. We estimated Pam18 and Pam16 levels in other membrane protease yta12, yme1, and oma1 cells. Pim1 deletion cells have higher levels of Pam18 and Pam16 than WT, yta12, oma1 or yme1 cells (Figure3: 3B), indicating the importance of Pim1 protease in the regulation of Pam18 and Pam16 abundance *in vivo*.

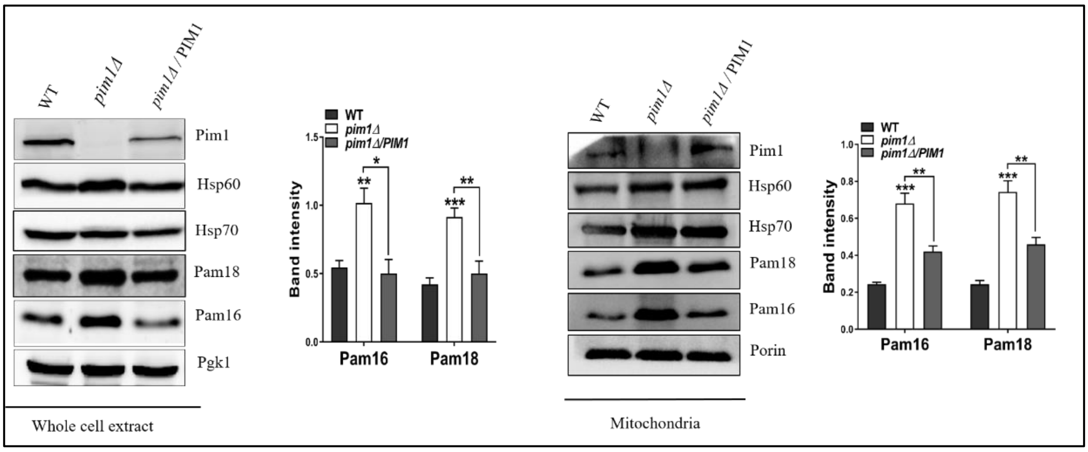


Fig3.3A. Accumulation of PAM complex subunits in pim1Δ strain: *equal amounts of whole-cell lysates (1.0 OD cells) or isolated mitochondria (60 µg) from WT, Δpim1,pim1 /PIM1 strains were taken and run on SDS-PAGE followed by western blotting with the indicated specific antisera. Positive loading control is provided by Hsp60, while negative loading control is provided by Pgk1 and Porin. Data represented as mean ± SD of 3 independent experiments. **p<0.1 and ***p<0.001.*

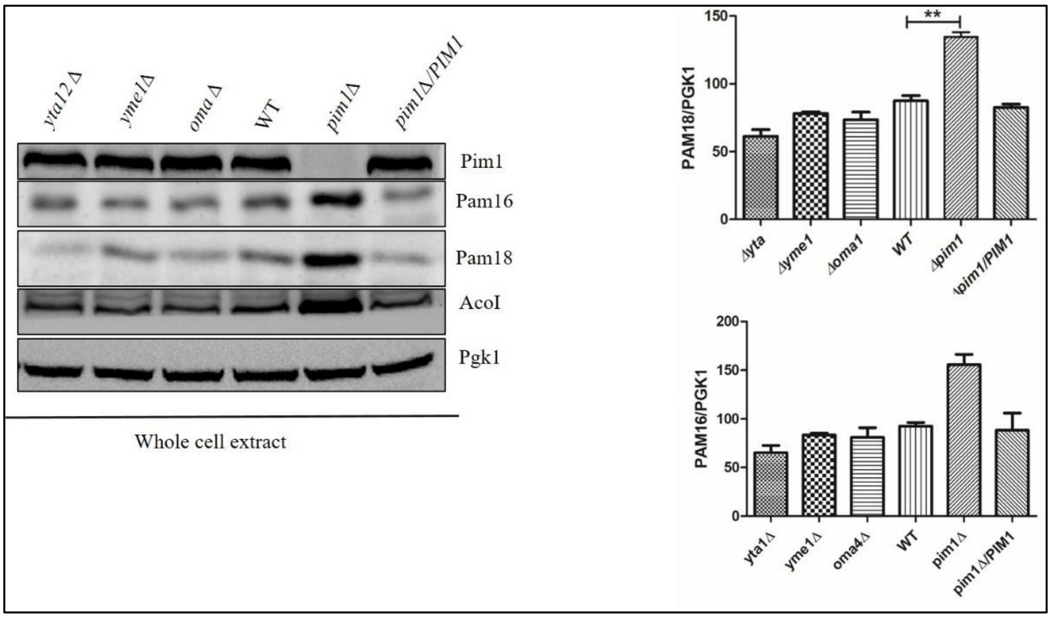


Figure 3.3B: Pim1 specifically regulates PAM complex proteins in vivo: *SDS-PAGE and western blotting with the specified unique antisera were used to evaluate equal quantities of whole-cell lysates (1.0 OD cells) from WT, Δpim1, and Δpim1/PIM1 strains. Pgk1 protein serves as a loading control. Amounts of Pam16 and Pam18 amount of protein after degradation was quantified by ImageJ and plotted as a relative band intensity. Data represented as mean ± SD of 3 independent experiments. **p<0.1.*

3.2.3 The proteolytic domain of Pim1 is critical for the proteolysis and regulation of Pam18 or Pam16 *in vivo*

In vitro results suggest that Pam18 and Pam16 undergo proteolysis by the Pim1/Lon protease. If the P domain (proteolytic domain) of Pim1 is critical then the catalytically inactive Lon mutant fails to degrade the substrates. We wanted to see if its proteolytic activity is also essential for the *in vivo* regulation of Pam18 and Pam16 levels. Alanine substituted for the catalytic serine, Ser-1015 (Pim1 S_A), serves as proteolytically inactive Lon but retains all of its other functions. So we used the Lon variant (Pim1 S_A) and compared the levels of Pam16 and Pam18 in all three strains (WT, *pim1Δ* and *pim1Δ/*PIM1). Pam16 and Pam18 expression resulted in lower levels of Pam18 in *pim1*Δ cells, as predicted, whereas Pim1 S_A expression did not (Figure3: 4D).

It is known that *pim1Δ* cells have growth defects in both rich and minimal media. Since the misfolded proteins are the substrates for Pim1, the elevated temperature might lead to the enhanced accumulation of substrates in *pim1Δ* strain background and hence the growth defect is further exacerbated. If the accumulation of Pim1 substrates causes the growth defect of *pim1Δ* cells, substrate overexpression also should lead to enhanced growth defect of *pim1Δ* strain, particularly at elevated temperatures. As expected, we observed slow growth of *pim1Δ cells either at 30*°C, 37, or 42 or on non-fermentable carbon sources (Fig3: 3A-C). Overexpression of Pam18 does not affect WT cell growth at 30, 37, or 42°C or with non-fermentable carbon sources (Fig 3. 3A-C). Pam18 overexpression, on the other hand, significantly hampered *pim1Δ* cell growth at 42°C or in YPGE media (Fig 3: 3A-C). In addition, Pam16 overexpression inhibited the growth of *Δpim1* cells at 42°C in a similar way (Fig 3. 3A-C) but relatively less. The cells carrying the plasmid-borne WT Pim1 or catalytically inactive Pim1 in *pim1Δ* cells show a growth phenotype similar to WT or *pim1Δ* strains respectively (Fig 3.3C). These findings suggest that proteolytically active Pim1 is required for the degradation of Pam18 or Pam16 and that accumulation of Pam18 and 16 may contribute to the *Δpim1* strain's growth defect phenotype.

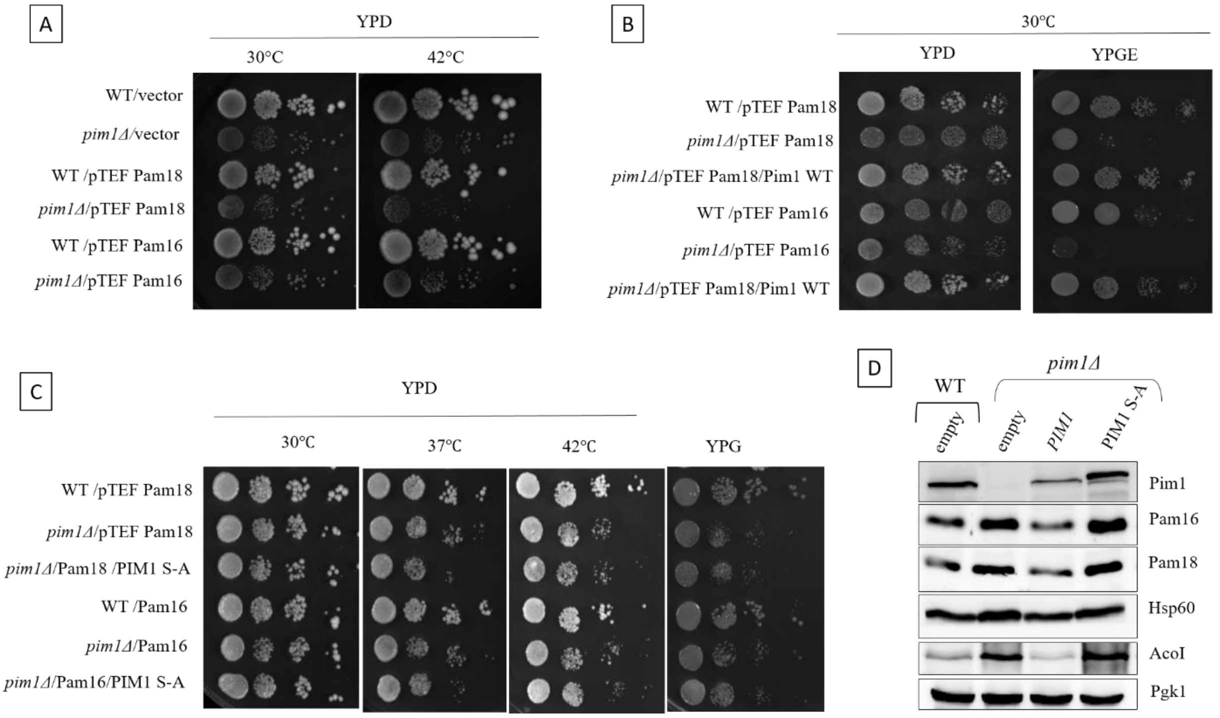


Figure 3.4: The proteolytic active Pim1 is critical for the degradation of Pam18 or Pam16*: (A-C) WT or pim1Δ in cells with empty vectors (V), or indicated Pam16, Pam18, WT PIM1, and PIM S-A variants plasmids were grown in glucose media till the log phase, then subjected to 10-fold serial dilution and plated on glucose (YPD) or glycerol (YPG) plates. Plates were incubated at the indicated temperature for 2-5 days. (D) Whole-cell extracts from the indicated strains were separated on SDS–PAGE and probed with antibodies specific for Pim1, Pam 16, Pam 18, Hsp60, Aco1, and Pgk1.*

3.2.4. Pam18 is an interacting Partner of Pim1 protease.

To further investigate whether Pam18 or16 proteins interact with Pim1, we used yeast strains carrying a genomic copy of either a normal or TAP-tagged Pim1. Extracts from the yeast cells expressing either WT or Pim1-TAP protein were subjected to TAP affinity purification and analyzed the eluted fractions by western immunoblotting. No protein was observed in the eluate fraction of W303 strain (wild type) cell extracts. In contrast, Pam18 protein is observed in the eluate fraction of Pim1-TAP (SC0437) cell extracts (Fig 3.5). similarly, we find Hsp60, a known interacting partner of Pim1 in the eluate fraction of Pim1-TAP cell extracts. These studies suggest that Pim1 interacts with Pam18.

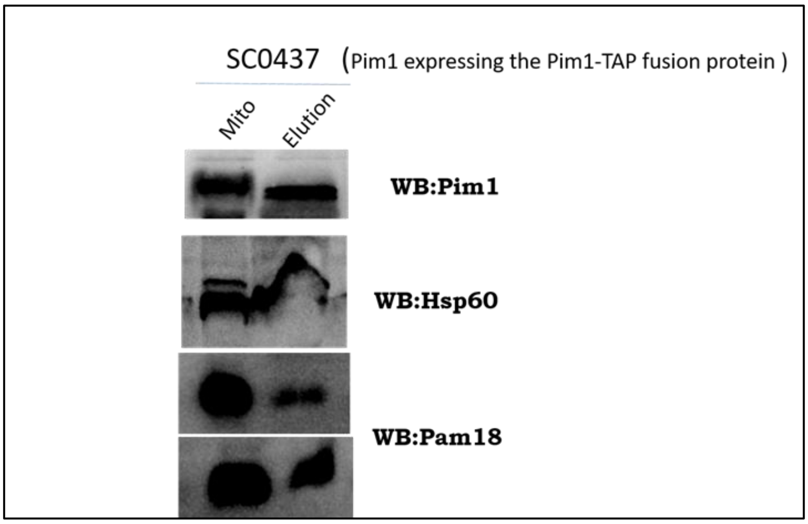


Figure 3.5: Pam18 is an interacting partner of Pim1 protease. *Extracts from a yeast strain expressing Pim1-TAP fusion protein were subjected to TAP purification and eluates are immunoblotted with the indicated antibodies.*

3.2.5. Pim1 mediated protection of Pam18 and Pam16 proteins from stress-induced aggregation

Tom Bender's (Bender, Leidhold et al. 2010) group has shown that under stress conditions, Pim1 substrates like Aco1 are prone to aggregation in the absence of Pim1. We would like to explore the kinetics of aggregation of proteins to test whether stress-induced aggregated proteins are protected by Pim1 protease. To test whether Pim1 shows its effect on Pam18 and Pam16 proteins, we subjected isolated mitochondria either from WT or *Pim1Δ* strains to thermal stress. Although 30°C is known to be physiological, 42°C provides moderate thermal stress.

To keep mitochondria completely energized during the stress therapy, they are supplemented with ATP and NADH as stated in the materials and methods section. Aggregated proteins were separated by detergent lysis followed by high-speed centrifugation. SDS-PAGE is used to separate the proteins in the pellet (aggregated) and supernatant (soluble) fractions, which are then either Coomassie-stained or immunoblotted.

We find that aggregated proteins are more in *pim1*Δ mitochondria than WT upon thermal stress (Figure 3.6A). Weston blot analysis shows that time-dependent accumulation of Pam16 and Pam18 aggregates are more in *pim1Δ* mitochondrial fraction (Figure 3.6B) than WT. Similarly, stress-dependent aggregation of Aco1, a known Lon substrate, is observed in *pim1Δ* mitochondrial fraction. These results indicate that Pim1 is probably regulating the Pam16 and Pam18 proteins by preventing their aggregation by stabilizing aggregation-prone polypeptides. In conclusion, our findings show that Pim1 protease, a key component of the proteolytic system, protects mitochondrial PAM complex proteins from aggregation by degrading them.

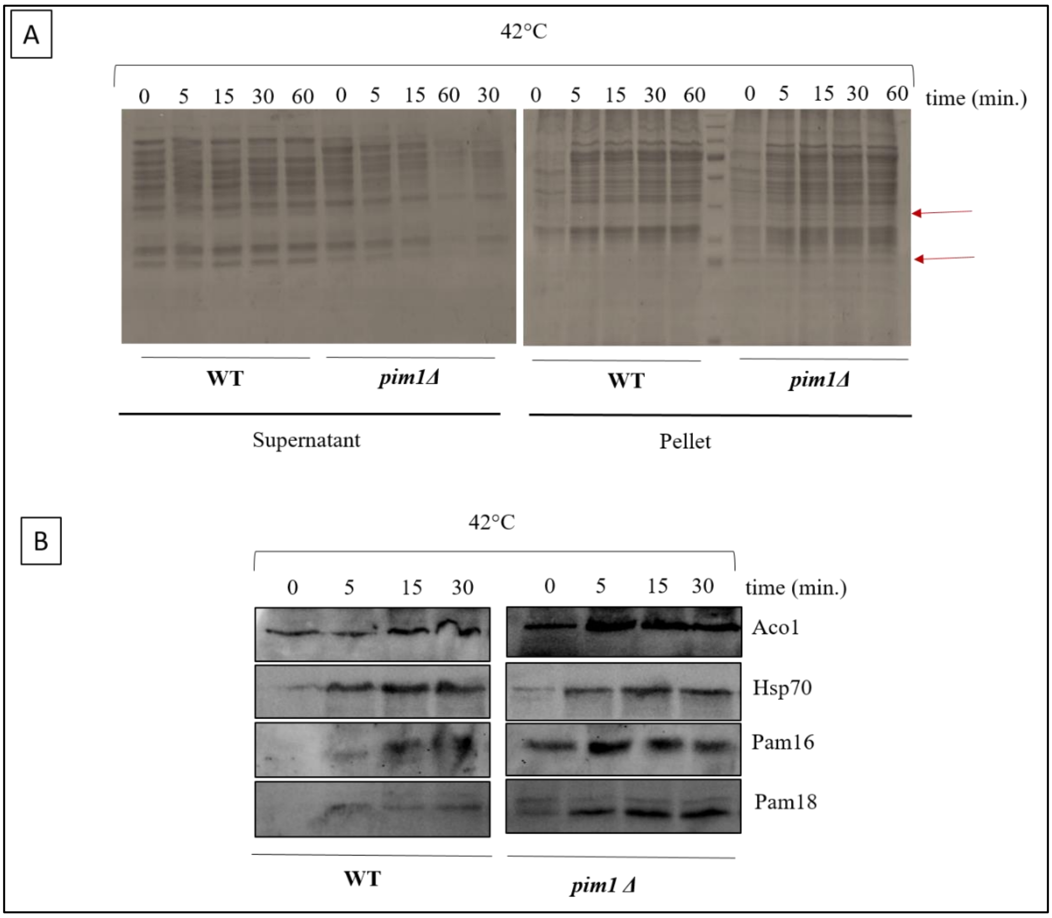


Fig3.6: Aggregation of Pam16 and Pam18 proteins during thermal stress in pim1Δ cells. *(A) Mitochondria isolated from WT or pim1Δ cells were exposed to thermal stress (42ºC) for different time points and aggregated fraction was separated by solubilizing and centrifuging at 22,000 × g for 1 hr. Supernatants and pellet fraction were separated on SDS–PAGE and Coomassie-stained. (B) Western blotting of the soluble and aggregated protein fractions of WT or pim1Δ with the indicated specific antisera.*

3.3 Discussion

ATP-dependent proteases are presently arising as key controllers of mitochondrial functions. Pim1 in *S.cerevisiae* controls protein turnover in the mitochondrial matrix (Bayot, Gareil et al.

2010). How turnover of PAM complex subunit proteins is regulated? The role of mitochondrial AAA proteases in the regulation of PAM complex subunits is not known. In my thesis, I studied the role of Pim1 protease in the proteolysis of mitochondrial PAM complex subunits. I have adopted two different experimental approaches to provide an overall picture of PAM complex homeostasis. First, since each protein may behave differently in its natural cellular environment, I attempted to study its behavior in its natural environment. Second, the dependence of PAM complex proteins on cellular protein quality control components, I wanted to find out how the Pim1 protease behaves in the stress conditions towards PAM subunit proteins.

The accumulation of misfolded proteins is directly related to progressions of a disease condition. Pim1 promotes protein folding and prevents aggregation by binding and shielding the client proteins' hydrophobic peptide segments. Pim1 decides the client protein's fate by binding and holding the misfolded protein and submit to the chaperone. However, under critical conditions, it degrades it. Here, we established the dual functionality of the Pim1 protease. Our first goal was to identify the turnover rate of Pam16 and Pam18 mitochondrial proteins and the role of Pim1 protease, if any, in this process. As expected we can see Pam16 and Pam18 accumulation in the absence of Pim1 protease. Also, *in vivo* results show that Pim1 could degrade the PAM complex subunit proteins. Further TAP tag pull-down studies show the interaction of Pim1 with Pam16.

The membrane protein degradation process is known to be carried out by m-AAA and i-AAA proteases (Arnold and Langer 2002). We couldn’t see any significant alteration in steady-state levels of Pam16 and Pam18 in the absence of membrane proteases. Pam16 and 18 act similarly to Isu, a well-known Pim1 substrate. However, Pam16 and Pam18 could be used as an interior membrane regulator to control the quality of membrane proteins. Indirectly, Pim1 could interact and stabilize unbound subunits of TIM23. We speculate that J domain is probably vital for interaction with Pim1 protease. Further studies in this direction may give more insight to understand the mitochondrial inner membrane protein regulation by proteases.

The absence of the Lon mammalian protease also results in the accumulation of oxidatively damaged proteins in mitochondria (Bezawork-Geleta, Brodie et al. 2015). The turnover of the mitochondrial energy-handling complex subunits is viewed as an important process to give the stoichiometric amounts of the various subunits which are vital for proper respiratory chain assembly.

The experiment was conducted by energized isolated mitochondria from *S.cerevisiae,* to maintain the natural environment. Aggregation assays in organelles have several advantages, including specified conditions, reasonable protein concentrations, and complete protein quality control system operation. In a technique previously used to study the aggregation of bacterial and yeast mitochondrial proteins, aggregated polypeptides are separated by high-velocity centrifugation from detergent-lysed mitochondria (von Janowsky, Knapp et al. 2005). The majority of mitochondrial PAM complex proteins remained soluble during heat or thermal stress, according to our findings. Pam18 and Pam16 proteins showed temperature-dependent aggregation in intact cells.

When misfolded proteins are present in a soluble confirmation before aggregation, Pim1 protease recognizes them. During proteolysis, molecular chaperone proteins perform a key role by preventing the combination of misfolded proteins, which enables them to be degraded by Pim1 protease. Pim1's chaperone activity probably prevents the Pam16 and Pam18 aggregation.

The functions of Pim1 depend on its capacity to distinguish misfolded portions of its substrates (von Janowsky, Knapp et al. 2005), which is initiated by its proteolytic movement on the substrate polypeptide. Furthermore, Pim1 has been shown to function in concert with Hsp70, meaning that if refolding fails, unfolded proteins are degraded by Pim1 (Wagner, Arlt et al. 1994). We found a proteolytic mechanism towards PAM complex misfolded proteins.

3.4 Summary

Targeting and degradation of proteins in bacteria and eukaryotes obey the same general concept. Eukaryotes and bacteria seem to have solved the problem of how to use a proteolytic mechanism that can hydrolyze nearly all proteins in a regulated and precise manner. Our data suggest that mitochondrial matrix Pim1 recognizes the inner membrane of Tim23 complex subunits Pam16 and Pam18. After binding to Pam16 and Pam18, the ATPase activity of Pim1 causes the unfolding and dislocation of the two proteins into the matrix for proteolysis. The following observations serve as the foundation for our hypothesis: (A) Pam16 and Pam18 are degraded in a Pim1-dependent manner (B) Pim1 P domain is required for PAM complex degradation.

In conclusion, we proposed that inner membrane PAM complex proteins are targeted for degradation by ATP-dependent Pim1 proteases and shielded from stress-induced aggregation.

More exploration is needed to study the mechanism and other possible interacting partners of Pim1 which are responsible for the regulation of the PAM complex.

Chapter 4

Mge1, a nucleotide exchange factor of Hsp70 is regulated by mitochondrial protease Pim1 /LON

4.1 Introduction

In recent researches, Hsp70 has received enormous attention, and studies show that oxidative stress is regulated by glutathionylation and is also linked with neurological disorders. Several advanced structural studies have shown that Hsp70 is regulated allosterically by both substrates and cochaperons. In controlling the chaperone cycle, two unique cochaperons, Mge1 & Mdj1 are involved. Mdj1 is a J class protein that helps activate the hydrolysis of ATP and is also involved in mitochondrial respiration. Mge1 is an essential protein of the GrpE family that helps to exchange nucleotides on Hsp70. Mdj1 is a co-chaperone with different roles, such as biosynthesis of the iron sulfur cluster, import, and folding of the protein.

Mge1 is a bacterial GrpE homologue that interacts with mtHsp70 to allow import across the inner mitochondrial membrane. Yeast Mge1 is 57% identical to bacterial GrpE1 and can functionally replace Escherichia coli GrpE, demonstrating the evolutionarily conserved mechanism of action. Mge1 is a dimer in its functional state, and this dimerization appears to be essential for its interaction with mHsp70. Since the monomeric form of Mge1 fails to interact with mHsp70, the ATPase cycle and chaperone function of mtHsp70 is delayed. Mge1 is an ATP–ADP exchanger that also functions as a thermosensor in bacteria and yeast. Mge1 exists as a monomer at high temperatures and does not interact with mHsp70. Furthermore, temperature-sensitive Mge1 mutants accumulate precursors in the cytosol, aggregate proteins in the matrix, and have decreased mHsp70 nucleotide-dependent dissociation from Tim44. It is also involved in mitochondrial maintenance during heat stress, protein sorting, and misfolded protein degradation, but its regulation is poorly understand.

Lon acts as a master regulator of mitochondrial proteins as it degrades the misfolded proteins of the mitochondrial matrix. We hypothesized that Lon might have a role to regulate oxidized or misfolded Mge1 protein. Here, using yeast as a model, we investigated how Mge1 proteins of the mitochondria are subject to specific degradation and the turnover by Pim1 protease.

The denatured Mge1 is selectively recognized and degraded by Lon protease according to this analysis. We also discovered that Pim1 protease protects Mge1 protein from stress-induced aggregation.

4.2 Results

4.2 1. Lon protease degrades Mge1 *in vitro* at 37^{0}C.

For in vitro degradation assays, we purified Lon protease, Hsp70, Mge1, and it mutants (Figure: 4.1). We added a sub-stoichiometric concentration of Lon protease (5 µg GrpEl1/Mge1/Mge1 mutants or Mge1 with Hsp70; 2µg Lon) and removed aliquots over a 90-120 min time course after confirming that Mge1, its mutants and Hsp70 were stable after incubation at 30°C.

The enzyme assay was carried out at 30°C and 37°C. There is no decrease in Mge1 or GrpEL1 at 30°C while casein, a known substrate of Lon is efficiently degraded (Figure: 4.2). The amount of Mge1 and its mutants present in the reaction decreased with time at 37°C (Figure 4.3A). Only 3% of substrate remained after 120 min incubation at 37°C (Figure: 4.3A). Further control experiments were conducted to confirm the decrease was caused by Lon dependent proteolysis. We tested the Mge1 degradation with LON S _A (catalytically inactive) and found no reduction in Mge1 (Fig 4.3B). Also, we experimented with the Hsp70 alone in presence of Lon and found no reduction in Hsp70 occurred over the 60 min. time course. In combination, these in vitro findings show that Mge1 is a Lon protease substrate.

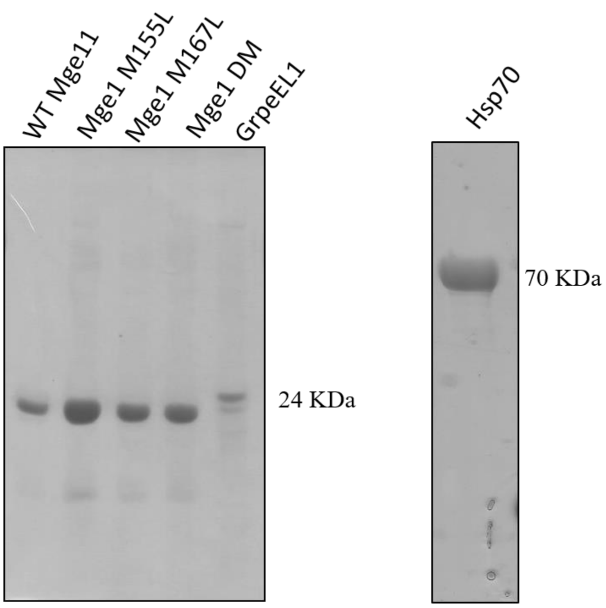


Fig-4.1: Purification of recombinant Hsp70, GrpEL1, Mge1, and its mutants using Ni-NTA column chromatography: *Bacterial cells expressing recombinant proteins were purified by Ni NTA & separated on SDS–PAGE, and coomassie stained.*

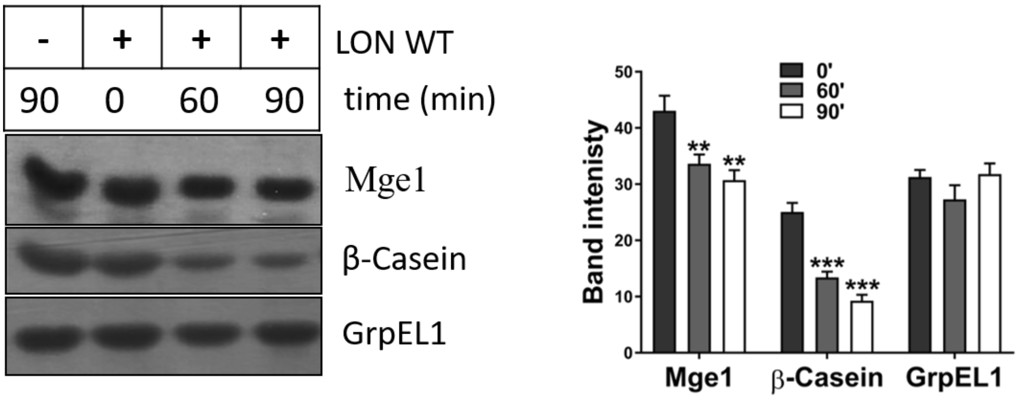


Fig-4.2: LON protease does not degrade Mge1 at 30°C in vitro: *In normal reaction conditions, Casein, Mge1, GrpEL1 were incubated with human Lon WT (5 µM). Samples have been collected at specified times, mixed with dye, and stained by SDS-PAGE. The relative amount of all proteins was quantified by ImageJ. Band intensity of Mge1, casein, GrpEL1. Data represented as mean ± SD of 3 independent experiments. **p<0.1 and ***p<0.001.*

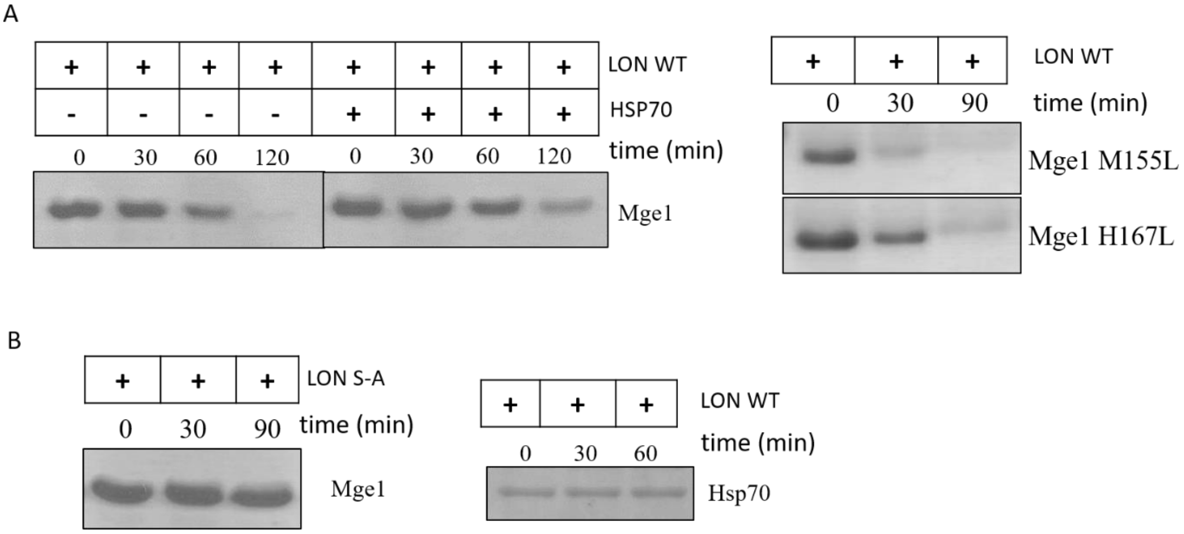


Fig-4.3: Lon protease degrades Mge1 and its mutants in vitro at 37°C. *(A) Mge1 and its mutants (Right Panel) incubated with human LON WT (5µM) in the presence and absence of Hsp70. At specified times, samples were taken, separated by SDS–PAGE, stained. (B); Human Lon Mutant was incubated with Mge1, or Human Lon incubated with Hsp70. Aliquots were taken at fixed intervals, separated on SDS–PAGE, and stained with Coomassie.*

4.2.2 Pim1 protease regulates Mge1 levels in vivo

Mge1, a major mitochondrial enzyme, is particularly sensitive to oxidative damage. Levels of Lon substrates are more in *pim1Δ* cells than the wild-type (WT) strain. We found that Mge1 is degraded at thermal stress (37°C) but not at a normal physiological temperature *in vitro*. To know further *in vivo* its regulation, we isolated mitochondria from WT, *pim1Δ*, *pim1Δ* /Pim1 OE strains, grown either in 2% lactate or 2% dextrose media as described in the methods. Only in the mitochondria that were isolated from lactate media, *pim1*Δ cells had elevated Mge1 levels compared to WT, but not in dextrose media. (Figure: 4.4). This result suggests the role of Pim1 protease in the regulation of Mge1 under stress conditions.

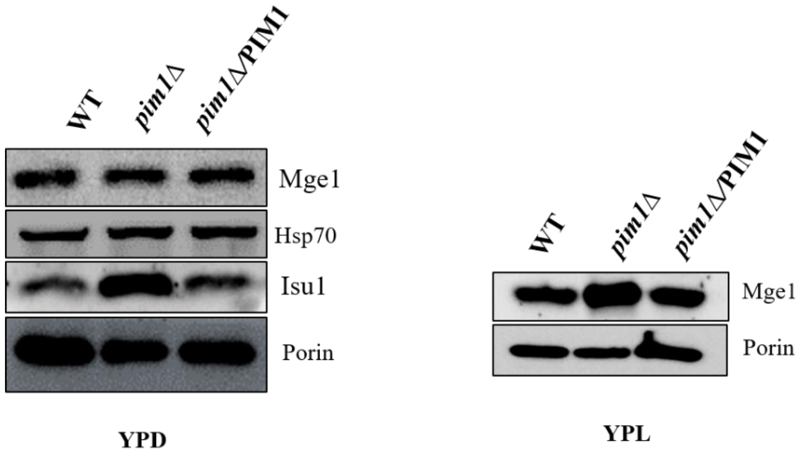


Fig-4.4. Accumulation of Mge1 proteins in pim1Δ strain: *Mitochondria (60µg) were isolated from WT, Δpim1, and pim1 /PIM1cells grown in lactate or dextrose media & subjected to SDS-PAGE & Western blotting with the indicated antibodies. Isu1 and Hsp70 serve as positive and negative controls, respectively, and Porin serves as a loading control.*

4.2.3 Binding of Hsp70 prevents degradation of Mge1 *in vitro*

Lon-type proteases ,a large focus on the degradation of misfolded and oxidized proteins as a feature of a quality control mechanism (Baker, Tatsuta et al. 2011) (Voos 2013). Very few of mitochondrial proteins have been identified *in vitro* as specific substrates under normal physiological conditions (Venkatesh, Lee et al. 2012). Mitochondrial processing protease (MPPα) subunit α is shielded from degradation through its cooperation with another protein subunit (MPP β) (Ondrovicova, Liu et al. 2005). Because Hsp70 is the binding partner of Mge1, the simplest hypothesis is that its interaction could physically prevent Mge1 from Pim1.

We tried to investigate the effect of Hsp70 on Mge1 degradation mediated by Lon in vitro. Lon was added to the reaction, the Mge1 was pre-incubated with a double molar excess of Hsp70. In the presence of Hsp70, degradation of Mge1 was inhibited (Figure: 4.3A). 50% of Mge1 was intact after 60 minutes, compared with 15% in control reactions. The findings reported here are significant because it describes Mge1 regulation. To get *in vivo* evidence towards that need to over express the Hsp70 in WT, *Δpim1* cells and check the Mge1 proteins levels.

4.2.4. The proteolytic active Pim1 is critical for the degradation of Mge1

So far our results suggest that Mge1 undergoes proteolysis by the Pim1. A possible mechanism is that substrate degradation happens through the P domains of Pim1. As a result, we wanted to know if its proteolytic activity is essential for Mge1 regulation in vivo. Mge1 levels in vectors containing WT, *Δpim1*, or Pim1 S_A cells were compared (Figure: 3D). In *Δpim1* cells, Mge1 expression is lower as compared to WT, whereas Pim1 S_A expression did not affect Mge1 levels.

We found a growth defect in *pim1Δ* cells caused by the accumulation of *pim1Δ* substrates *in vivo*. If a growth defect in *pim1*Δ cells is caused by Pim1 substrates accumulation, overexpression of Pim1 substrates should exacerbate the issue. Mge1 overexpression did not affect WT cell growth at 37°C (Fig. 4.5). Mge1 overexpression, on the other hand, significantly hampered *pim1*Δ cell growth at 42°C. Similarly, when we ectopically expressed Pim1 in *pim1Δ* cells, we observed an increase in growth (Fig. 4.5) but the expression of proteolytic inactive Pim1 in *pim1Δ* cells did not cause any increase in growth (Fig.4.5). These findings suggest that proteolytically active Pim1 is needed for Mge1 degradation.

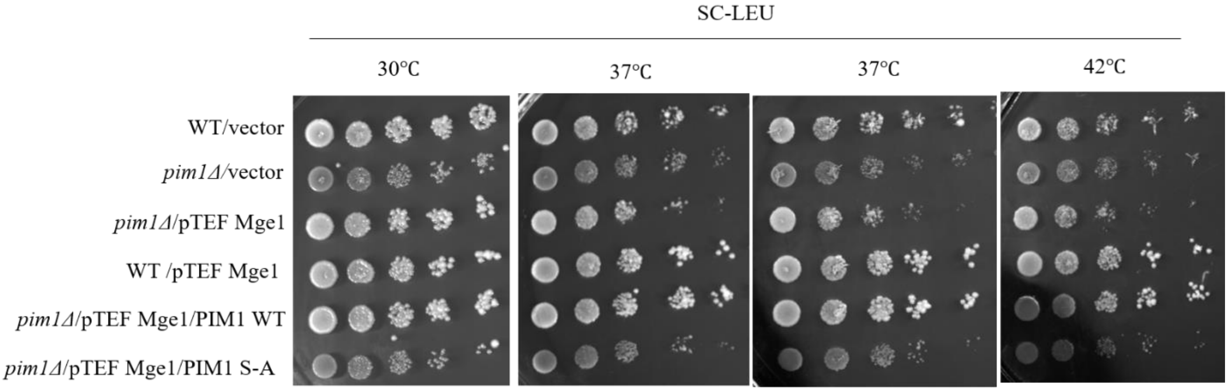


Fig-4.5: The proteolytic active Pim1 is critical for the degradation of Mge1*: WT or pim1Δ cells or the mentioned overexpression plasmid were grown in SC-LEU media until log phase, then spotted in a 10-fold serial dilution on SC-LEU media plates, and incubated for 2–5 days at the indicated temperature.*

4.2.5 Thermal resistance property of Mge1 mutants is influenced by Pim1

Many proteins are misfolded or unfolded during stress conditions, resulting in the formation of protein aggregates. Thermal stress can induce the aggregation of proteins in mitochondria. Previous research from our lab has shown that Mge1 serves as the first line of protection against oxidative and thermal stresses. Dimeric Mge1 monomerizes when exposed to oxidative or thermal stress that impacts the mitochondrial Hsp70 chaperone cycle. We identified single point mutations Mge1 M155L and Mge1 H167L in Mge1 which confer resistance to oxidative and thermal stresses respectively (Marada, Allu et al. 2013) (Marada, Karri et al. 2016). However, we find that Mge1 M155L, H167L (mentioned as Mge1 DM) is highly sensitive to both thermal and oxidative stress.

We speculated that the absence or deletion of the PIM1 gene would affect Mge1's thermal resistance property. To test this hypothesis, we generated yeast strains expressing Mge1 WT, Mge1M155L, and Mge1H167L in *pim1Δ* cells as described in the Methods section. We grew yeast strains expressing either Mge1 WT, Mge1 M155L, Mge1 H167L, or Mge1 DM in wild type and *pim1Δ* background stains. We exposed them to thermal stress (37ºC). Intriguingly, the thermal resistance property of Mge1 single mutants (Mge1 M167) is lost and gain of thermal stability property of Mge1 DM was observed in pim1 deletion condition.(Figure 4.6) These results suggest that Pim1 has a role for Mge1 functional adaptations towards thermal stress.

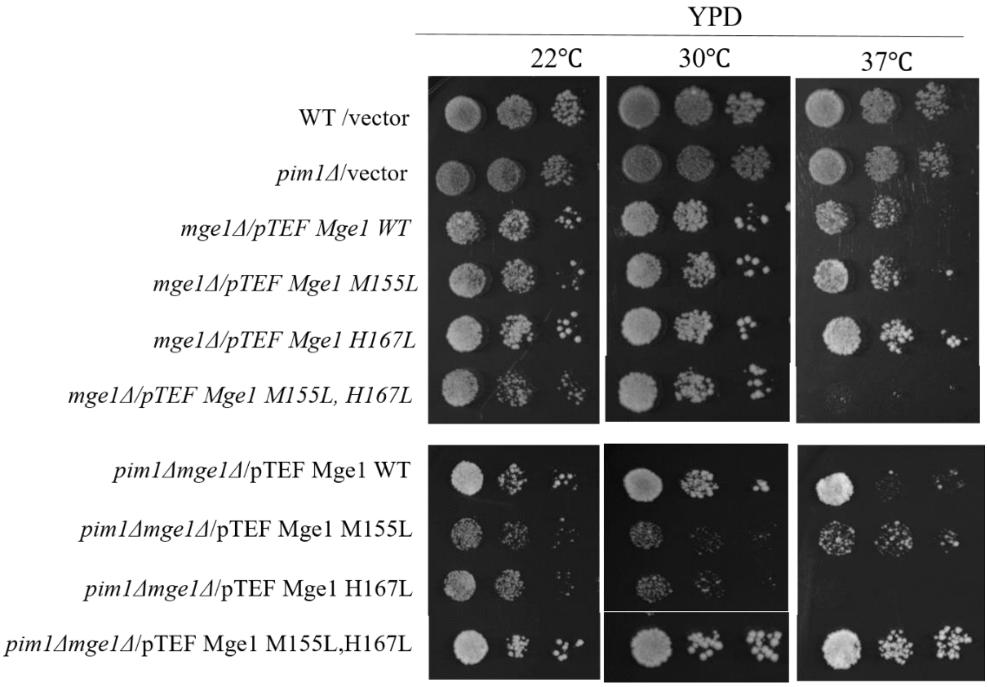


Fig-4.6: Thermal resistance property of Mge1 mutants is influenced by Pim1*: WT or pim1 cells, as well as the described overexpression plasmid, were grown in SC-LEU mid-log phase, then spotted in a 10-fold serial dilution on SC-LEU media plates, and incubated for 2–5 days at the specified temperature.*

4.2.6 Pim1 protease protects the Mge1 from stress-induced aggregation

To assess the impact of the stress on the aggregation of Mge1, we analyzed the stress foci formations at 42ºC in the absence and presence of PIM1gene in GFP-tagged Mge1 transformed cells. Through confocal microscopy, we analyzed the stress foci formation in WT and *pim1*Δ cells that are transformed with Mge1-GFP plasmid. The formation of stress foci is more in *pim1*Δ cells compared to WT upon heat stress (Fig 4.7).

Since we hypothesize that stress-induced aggregation is protected by Pim1 protease, we wished to study the kinetics of aggregation in the presence and absence of PIM1. Tom Bendera et al (2010) group has shown that under stress conditions Pim1 substrates such as Aco1 form aggregates and aggregated polypeptides accumulate in *pim1Δ* cells. Here, we subjected the isolated mitochondria from WT and *pim1Δ* cells to thermal stress. 30°C temperature is physiological, if raised to 37°C and 42°C constitutes a mild and extreme thermal shock, respectively. The separation by high-speed centrifugation of aggregated proteins after *in vitro* heat stress and subsequent lysis was done as described in materials and methods.

Pellet fraction was subjected to SDS-PAGE, Western blotted, and probed with Mge1 antibodies. We observed a time depend accumulation of aggregates is more in pim1 deletion cells (Figure: 4.8) compared to WT. These results suggest that Pim1 regulates Mge1 proteins by preventing protein aggregation probably by stabilizing aggregation-prone polypeptides and hence maintaining mitochondrial homeostasis.

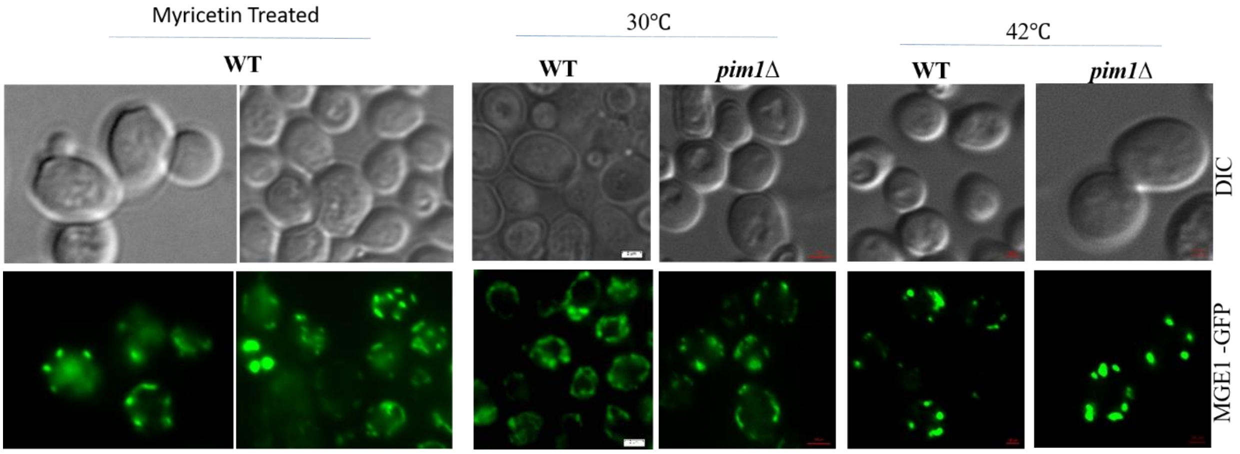


Fig-4.7: Live cell imaging for Mge1 GFP. *Mge1 expressing GFP was transformed into WT, pim1Δ and grown (0.8 OD) at 30°C. Grown cells are shifted to 42°C for 2 hrs and then GFP expression was observed under microscopy. DIC for whole-cell, GFP for Mge1.*

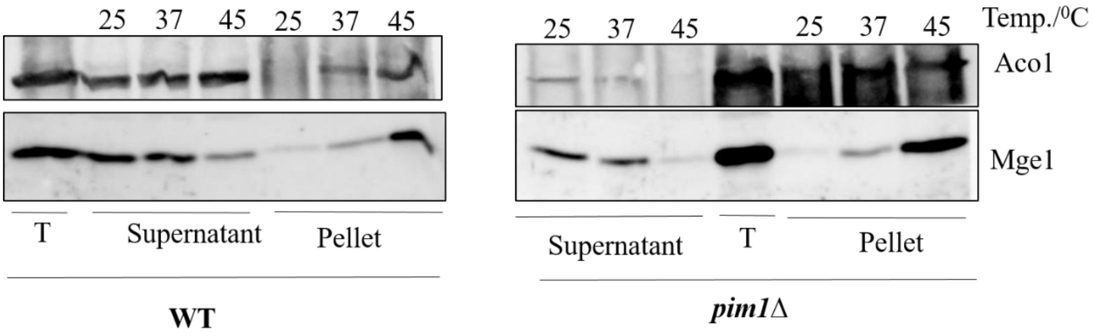


Fig-4.8. Mge1 Protein aggregation during thermal stress: *A). Mitochondria isolated from WT or delta pim1 cells were subjected to thermal stress (42^0C) for 0 to 30 minutes before being separated by ultracentrifugation. SDS PAGE and western blotted with Mge1 and Aco1 were analyzed for supernatants and pellets. As a positive control Aco1 is used.*

4.3 Discussion

mtHsp70 system is required for mitochondrial protein folding and degradation and it is an important component of TIM23. Molecular chaperones play a significant role in the proteolysis of misfolded proteins in mitochondria. Hsp70 and Pim1 cooperate in the degradation of misfolded protein. The period of binding to and releasing from Ssc1 (Hsp70) allows newly imported pre proteins to achieve their native state in the matrix.

Misfolded polypeptide proteolysis is dependent on the actions of Ssc1p and Mdj1p, both of which serve as chaperones and thus most likely prevent irreversible misfolding of degradation-prone polypeptides. This can enable protease to recognize the polypeptides and degrade them after they are released from the chaperone protein. The function of the mtHsp70 system in inner membrane protein folding remains unknown; however, findings suggest that soluble and at least some membrane-embedded proteins have the same effect on chaperone proteins.

There is a possibility of misfolding co-chaperones like Mge1 and Mdj1 under stress conditions, and hence regulation of turnover of these proteins is important. We conducted an in vitro degradation assay to determine whether Lon controls mge1 and discovered that it is degraded in a time-dependent manner. Mge1, like other recognized substrates such as Isu1 and Hsp60, accumulates in pim1 deletion conditions. Mge1's binding to Hsp70 prevents it from degradation. This outcome gives new bits of knowledge into how the interaction between Mge1 vulnerability and degradation from debasement may add to mitochondrial import regulation in *vivo.*

Earlier, Mge1 has been known to respond to both thermal and oxidative stress by changing its oligomer state to monomer state and it is known to detach from the Hsp70 chaperone (Moro

(Moro and Muga 2006) (Marada, Allu et al. 2013) Recently, we also reported that Mge1 is oxidized by peroxide levels and get repaired by methionine sulfoxide reductase (Allu, Marada et al. 2015), We identified single point mutation in Mge1 which confers its resistance to oxidative (Mge1 M155L) and thermal (Mge1 H167L) stresses (Marada, Karri et al. 2016).

Lon protease is needed for oxidative stress protection. Mitochondrial aconitase is vulnerable to oxidative stress and can aggregate and accumulate in several age-related diseases. After mild oxidative modification, Lon protease, an ATP-stimulated mitochondrial matrix protein, recognizes and degrades the oxidized, hydrophobic form of aconitase (Bota and Davies 2002).

Similarly, by recognizing minor oxidative changes to protein structure and rapidly degrading the mildly modified protein, Lon protease may prevent extensive oxidation, aggregation, and accumulation of Mge1 which could otherwise compromise mitochondrial Hsp70 function, protein import, and mitochondrial respiration. Mge1 is probably one of many mitochondrial matrix proteins that are preferentially degraded by Lon protease after oxidative damage.

Here, we show based on *in vitro* enzymatic assay and *in vivo* studies that the Mge1 thermal resistance property of Mge1 mutants (M155L, M167) is influenced by Pim1. Thermal resistance property of Mge1 single mutants (Mge1 M167) are lost and gain of thermal stability property of Mge1 double mutants (Mge1 DM) was observed in pim1 deletion. This could be helpful to understand the Hsp70 function and interplay of chaperones and protease during stress conditions.

We also establish that Pim1 also has the chaperone activity that prevents Mge1 from aggregation. The ability of Pim1 to identify unstructured segments of its substrates (von Janowsky, Knapp et al. 2005), which is the basis for the initiation of its proteolytic activity on the substrate polypeptide, is most likely the basis for its protective impact. Furthermore, functional cooperation of Pim1 with Hsp70 has been reported, suggesting that unfolded protein binding is followed by degradation if refolding fails (Wagner, Arlt et al. 1994). However, the function of chaperone and protease in the removal of misfolded and oxidized Mge1 needs to be investigated further. Our findings show that the Pim1 protease, a key component of proteolytic systems, prevents mitochondrial Mge1 proteins from aggregation by degrading them.

4.4 Summary

In this study, we observed: (A) Mge1 is degraded in a pim1-dependent manner. (B) P domain of Pim1 is essential for the degradation of Mge1. (C). Thermal resistance property of Mge1 single mutant (Mge1 M167) is lost and gain of thermal stability property of Mge1 double mutants (Mge1 DM) was observed in Pim1 deletion.Pim1's function in Mge1 processing under other stress and pathological conditions needs to be investigated further. Other mechanistic specifics of co-chaperone degradation and regulation have not been discovered to this point.

In summary, we proposed that the regulation of matrix Mge1 protein happens through degradation by ATP-dependent Pim1 proteases, and this way it is protected from stress-induced aggregation.

Chapter 5

Identification of NOVEL inhibitors of mitochondrial Lon protease

5.1 Introduction

Standard cell neoplastic transformation is a multi-step process that is caused by various genetic changes and then intensified by multiple cellular stresses associated with oncogenesis. Cancer cells grow in a microenvironment where stressors are greater than those found in normal cells, requiring many anti-stress pathways and defense mechanisms to be activated for them to survive. A variety of non-oncogenic proteins support these pathways and mechanisms, i.e. molecules which not initiate tumorigenesis, but participate in cell stress responses and increase the survival and proliferation of cancer-cell cells.

One of the mitochondrial quality control proteins in eukaryotes is Lon protease (Lon), which is strongly conserved from bacteria to mammals. It is encoded in the nucleus and localizes to the mitochondrial matrix where it controls mitochondrial functions, particularly under conditions of oxidative, hypoxic and metabolic stress. Lon recognizes and mediates proteolysis of degraded and oxidized proteins, acts as a chaperone, and participates in the maintenance of mitochondrial DNA. Aconitase and mitochondrial transcription factor A have known substrates for Lon proteolytic activity (TFAM) (Bota and Davies 2002, Matsushima, Goto et al. 2010)

Several lines of evidence point to Lon's position as a non-oncogenic protein that is important for cancer survival. To begin with, Lon's expression increases in response to a variety of stresses. Lon is activated in hypoxic cells and is responsible for the degradation of cytochrome c oxidase 4 subunit 1 (COX4-1) to increase respiration performance. Similarly, as cells are subjected to oxidative stress, Lon participates in the degradation of misfolded, oxidized, and carbonylated proteins, preventing their accumulation. Second, Lon is involved in the remodeling of respiratory chain complexes during metabolic reprogramming triggered by mitochondria in several cancer cells.

Lon deficiency activates AMP-activated protein kinase (AMPK), a central regulator of energy homeostasis under metabolic stress. Third, Lon deficiency disrupts mitochondrial function and structure, resulting in decreased proliferation and increased apoptosis in cancer cells. Lon is upregulated in many cancer cell lines, including RKO colon carcinoma, HepG2 hepatocarcinoma, and large cell lymphoma cell lines. Lon overexpression has also been related to cancer cell aggression (Gibellini, Pinti et al. 2015).

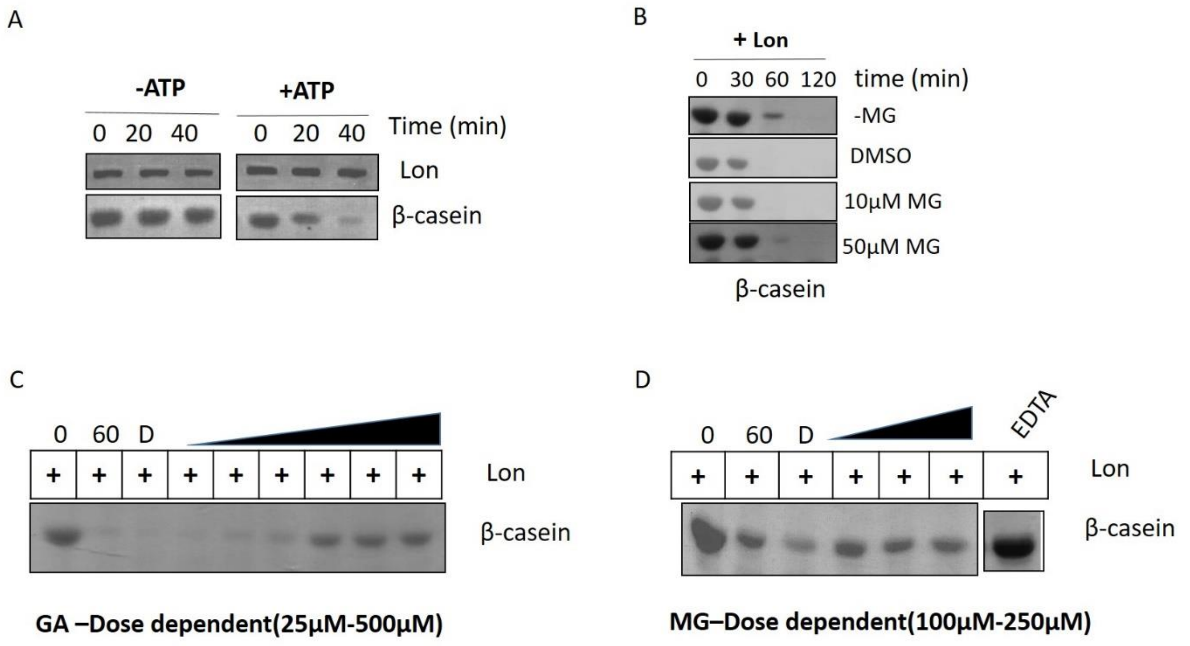
Although it is unknown how Lon's expression and functions are regulated, targeting its activity in cancer cells may be a novel and useful therapeutic strategy. Gallic acid (GA) and Methyl gallate (MG) are anti-inflammatory and anti-proliferative molecules (Fernandes and Salgado 2016).

Several mechanisms have been proposed for their anticancer effect, such as i) the formation of Michael adducts with reactive nucleophiles, including free thiols on target proteins, ii) the inhibition of mitogen-activated protein kinase (MAPK), iii) induction of apoptosis through the mitochondrial pathway. We show that both GA and MG may interact with Lon and form covalent adducts that irreversibly inhibit Lon activity, thereby inducing mitochondrial protein aggregation

5.2.0 Results

5.2.1 Lon-mediated degradation of β-casein is blocked by Gallic acid (GA) & Methyl Gallic acid (MG) in vitro.

β-casein is an unstructured protein that is degraded by a wide range of proteases. It is also degraded by Lon protease. Gallic acid (GA) structure is similar to Bortezomib, which is a well-known inhibitor of Lon protease. To examine the effect of GA & MG in inhibiting the proteolytic activity of Lon, we used β-casein as substrate. At first, the β-casein degradation was investigated in the presence and lack of ATP and the proteolytic activity based on ATP was conformed (Fig.5A). In the presence and absence of GA or MG, we studied the degradation of β-casein. We have observed that Lon-mediated *in vitro* β-Casein degradation is blocked by gallic acid (GA) and methyl gallic acid in a dose-dependent manner (Figure 5.2). This confirms that the degradation of casein by Lon protease is inhibited by GA or MG. Less than 50µM concentration of MG or GA doesn’t show any effect on human Lon protease activity.



GA –Dose dependent(25µM-500µM)

MG–Dose dependent(100µM-250µM)

Fig-5.1: Lon-mediated degradation of β-casein in vitro is blocked by Gallic acid (GA) & Methyl Gallic acid (MG) at 37°C *.A) In the presence or absence of ATP, Lon protease (2 µg) incubated with casein . B) Lon protease (2µg) incubated with casein in increasing concentration of Gallic Acid or Methyl Gallic acid at 37°C for 60 min. Protein samples were run in 10% SDS-PAGE and stained with Coomassie blue stain.*

5.2.2. Gallic and Methyl Gallic acid effect on Lon and its associated protein

The inhibition of Lon proteolytic activity leads to alternations of Lon substrates. To understand the effect of Gallic acid, Methyl Gallic acid on Lon and its physiological substrate levels *in vivo*, we treated HeLa cells with 100µM (IC 50=100 µM) of Gallic acid and methyl Gallic acid. After 48 hours of treatment, we observed a significant increase in Lon substrate Aco2 levels (Figure 5.2). This indicates Gallic acid and Methyl Gallic acid might interact with Lon and alters its function. Also, we found that GA and MG induce selective cytotoxicity in cancer (Hela) cell lines (Fig: 5.3).

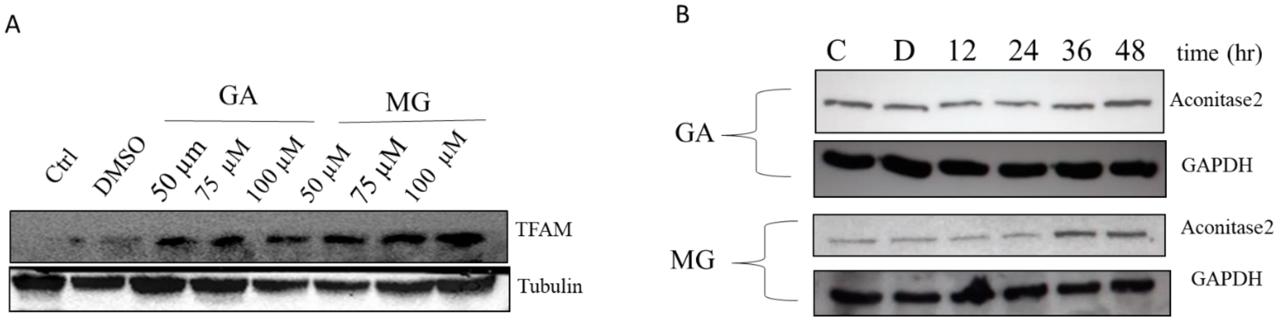


Fig-5.2: Gallic and Methyl Gallic acid effect on Lon and its associated protein: A) 100 µm Gallic acid-treated in time depend on manner B) 100 µm methyl Gallic acid-treated in time dependent manner. The HeLa cell extract (50µg) run 10% SDS PAGE probed with respective antibodies. Normalized GAPDH used as control.

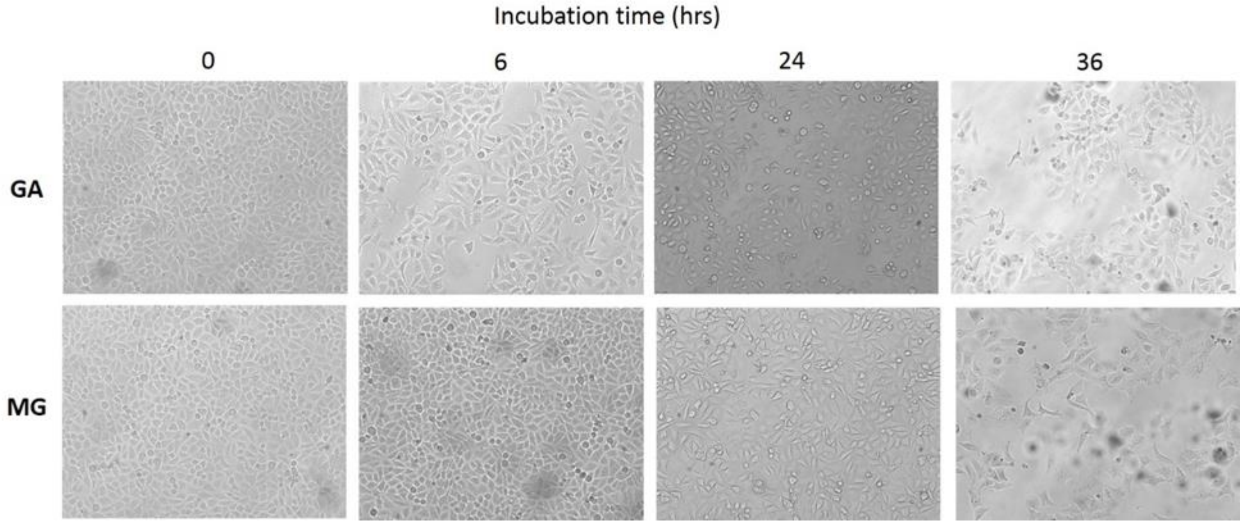
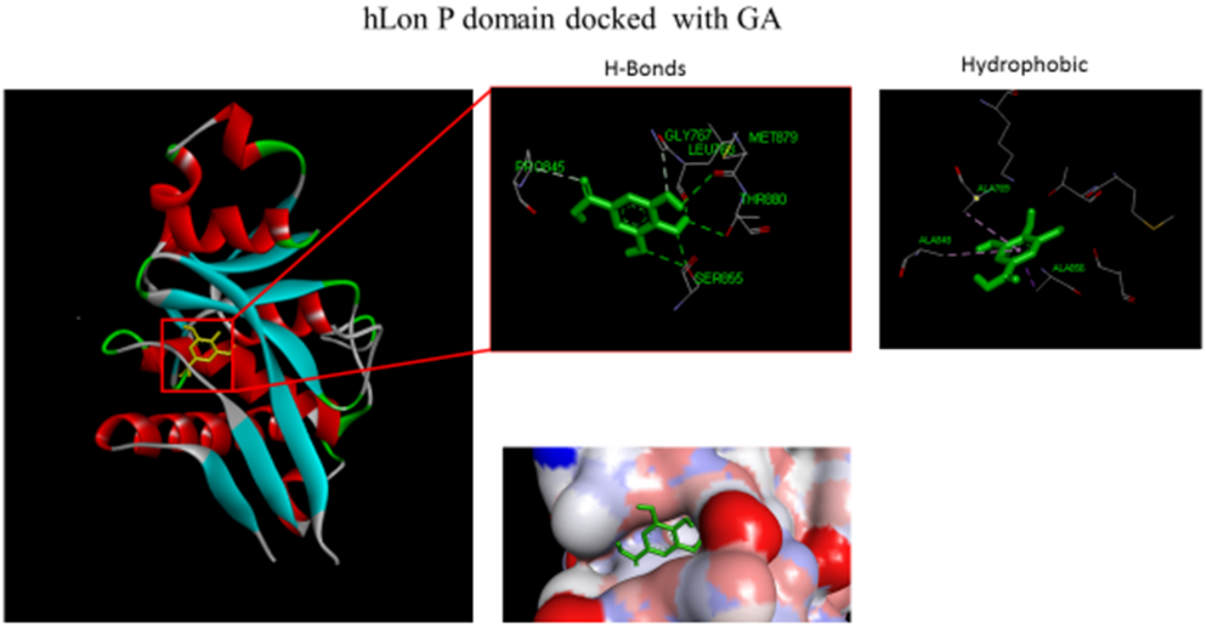


Fig-5.3: Cytotoxicity: cytotoxicity in HeLa cells treated with100 µM of GA, MG at the indicated incubation times. Cell images were captured using a Nikon inverted microscope.

5.2.3 GA & MG docks with the active site of P domain of human Lon protease

The anticancer agent like sesamin inhibits Lon by interacting with Ser855 and Pro845 positions. To understand further the mechanism of inhibition of Lon by GA and MG, we did *in silicon* studies as mentioned in materials and methods. Results show that GA and MG dock at the active site of the P domain of Lon. GA and MG hydroxyl groups interact with Ser855 and Pro845 (Fig: 5.4, Fig: 5.5). GA and MG molecules enter deep into the pocket because of their small size and ring-like structure.

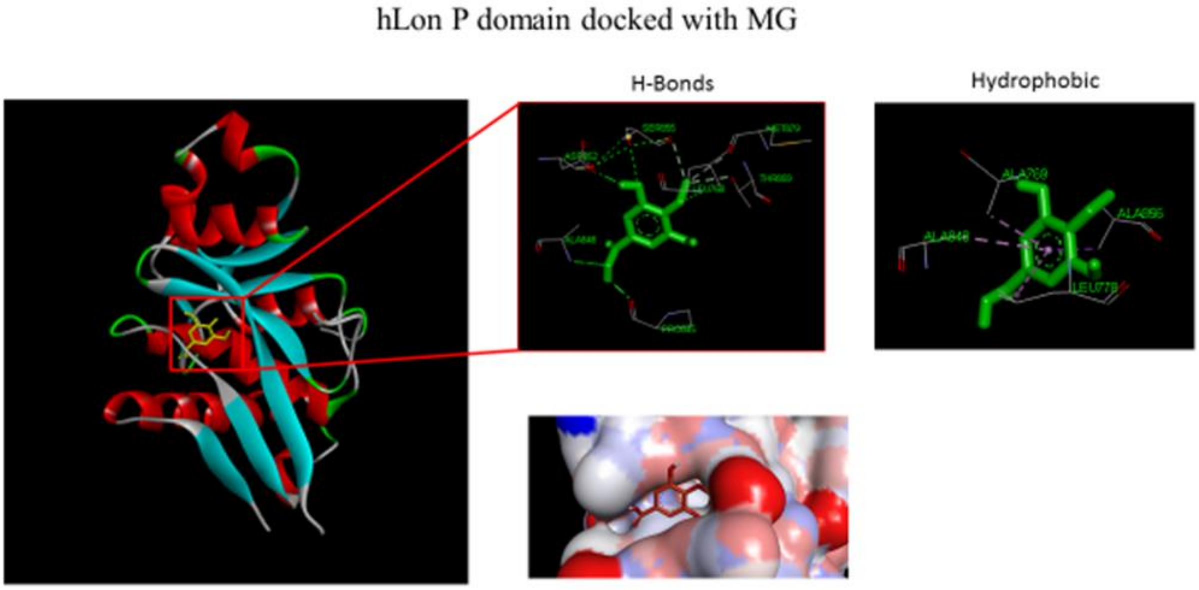


hLon P domain docked with GA

H-Bonds

Hydrophobic

Fig 5.4: Homology modelling*: The binding mode of GA in the active site of the human Lon proteolytic (P) domain was modeled using homology modeling.*



hLon P domain docked with MG

H-Bonds

Hydrophobic

Fig 5.5: Homology modelling*: The binding mode of MG in the active site of the human Lon proteolytic (P) domain was modeled using homology modeling.*

5.3 DISCUSSION and SUMMARY

Lon prevents the development of reactive oxygen species in hypoxic cells by degrading the COX4 subunit. The Lon protease enzyme is a key regulator of several mitochondrial functions. It is becoming known as one of the non-oncogenic proteins needed for cancer cell survival and proliferation. Lon is a promising candidate for anticancer drug production (Lu 2017) . To date, only a few synthetic Lon inhibitors have been identified: MG132, sesamin, synthetic triterpenoid CDDO, and its methyl ester derivative (Wang, Cheng et al. 2010, Gibellini, Pinti et al. 2015).

Most inhibitors of proteasomes are short peptides that have a reactive group, which forms a covalent bond with catalytic threonine, such as peptide aldehydes (MG132), peptide boronates (BG262), and a peptide vinyl sulfone (Velcade). The N-terminal nucleophilic N amino group does not exist in their active site for other proteases inhibited by aldehydes, vinyl sulfones, and derivatives of boronate. Non-covalent proteasome inhibitors were less thoroughly examined.

To date, no specific inhibitors of Lon protease are available. Certain inhibitors of proteasome such as peptidyl aldehyde MG132 can, however, diffuse into the mitochondrion and inhibit degradation of the endogenous mammalian Lon steroidogenic regulatory protein (StAR), indicating that the proteasome and Lon share certain similarities in their proteolytic mechanism (Granot, Kobiler et al. 2007). Since Lon resembles proteases of the proteasome family, the screening of commercially available peptide-based inhibitors led to the identification of proteasome inhibitor MG262 as a potent, ATP-dependent inhibitor of *Salmonella entera serovar typhimurium* Lon protease. MG262 is a peptide borate (Frase, Hudak et al. 2006). The sensitivity of the peptide moiety to proteolysis together with that of boronate group to oxidation decreases its metabolic stability as observed for the related peptide boronate bortezomib which is used for the treatment of multiple myeloma. Therefore, we sought to identify stable non- peptidic selective Lon protease inhibitors.

GA analogues (GA4) emerged as a potent drug candidate for HIV-1 Protease inhibition. Phytochemical Gallic Acid (GA) derivatives have been screened for protease inhibitor activity. Gallic acid is a natural phenolic compound found in several fruits and medicinal plants. It is reported to have several health-promoting effects. Structurally, Gallic acid and Bortezomib, which is a well-known inhibitor of Lon protease, belong to the same family. So we suspected that GA might serve as a natural compound that inhibits the Lon protease.

In this study, we identified novel natural molecular compounds Gallic Acid (GA) and Methyl Gallic acid (MG) as a potential blocker of Lon protease activity. GA and MG molecules inhibit the protease activity of purified recombinant Lon in vitro.

The addition of GA or MG to cultured cells induces a substantial accumulation in a time and dose-dependent manner of mitochondrial aconitase, TFAM a Lon substrates. GA and MG may changes mitochondrial morphology inhibition of Lon protease.

Furthermore, a study of molecular docking shows that MG and GA interact in a Lon protease active site with Ser855 and Pro845 residues. Further, the development analysis of these molecules would be an effective alternative drug for cancer treatment. The potential of GA and MG to inhibit hLon and the kinetic inhibition parameters should be further studied.

6. Summary of Thesis

Hundreds of proteins that are synthesized on cytosolic ribosomes and then translocated into the matrix through protein channels are required for mitochondrial function. The TIM23 import complex is involved in the import of major matrix proteins as well as the assembly of the respiratory complex. PAM (presequence translocase-associated motor complex) is a TIM23 complex that exists on the matrix side of the inner membrane. Pam16, Pam18, Mge1, and Hsp60 are important components of import motors (PAM).

Lon protease is critical in preventing damaged proteins from accumulating in the matrix. We researched to learn more about the role of the Lon protease concerning the TIM23 complex, specifically the import motor. In vitro, we identified the Lon substrates using recombinant and purified Tim23 import motor components.

In the first part, we investigated using a budding yeast model, how does different PAM complex proteins of the mitochondria are subjected to specific degradation. Results show that mitochondrial matrix PIM1 protease is indeed involved in the turnover rate of mitochondrial inner-membrane proteins, Pam16 and Pam18. The turnover of subunits of the PAM complex proteins Pam16 and Pam18 are specifically regulated by Pim1 protease but not by other membrane proteases of mitochondria. Further, results show that the ATP and proteolytic active site of Pim1 is essential for its substrate degradation. Besides, stress-induced aggregation of Pam18 and Pam16 proteins is probably protected by Pim1 protease. The results obtained in this study are discussed to the available literature in the field. In conclusion, we hypothesized that ATP-dependent Pim1 proteases target the regulation of inner membrane PAM complex proteins, protecting them from stress-induced aggregation.

In the second part, we investigated how Mge1 and its mutants resistant to oxidative and thermal stress are subjected to specific degradation by mitochondrial proteases. Previously, we reported that Mge1 both single mutations (M155L and H167L) shows thermal and oxidative resistance properties due to which functions like an increase in respiratory capacity and enhanced the mitochondria translation were observed. Our current results show that mitochondrial matrix Pim1 protease is involved in the degradation of thermally /oxidatively denatured Mge1. Loss of functional phenotype is observed in single mutants (M155L oxidative resistant; H167L thermal resistant) of Mge1 in *Δpim1*. Further, the proteolytic active site of Pim1 is essential for its substrate degradation. Also, results show that stress-induced aggregation of Mge1 protein is protected by Pim1 protease. In summary, we proposed that the regulation of Mge1 protein is

targeted for degradation by ATP-dependent Pim1 proteases under stress and protected from stress-induced aggregation.

In the third part of the work we investigated the inhibitors of mitochondrial Lon protease. Results show that Gallic and Methyl Gallate are efficiently inhibiting the Lon protease activity both *in vitro* and *in vivo*.

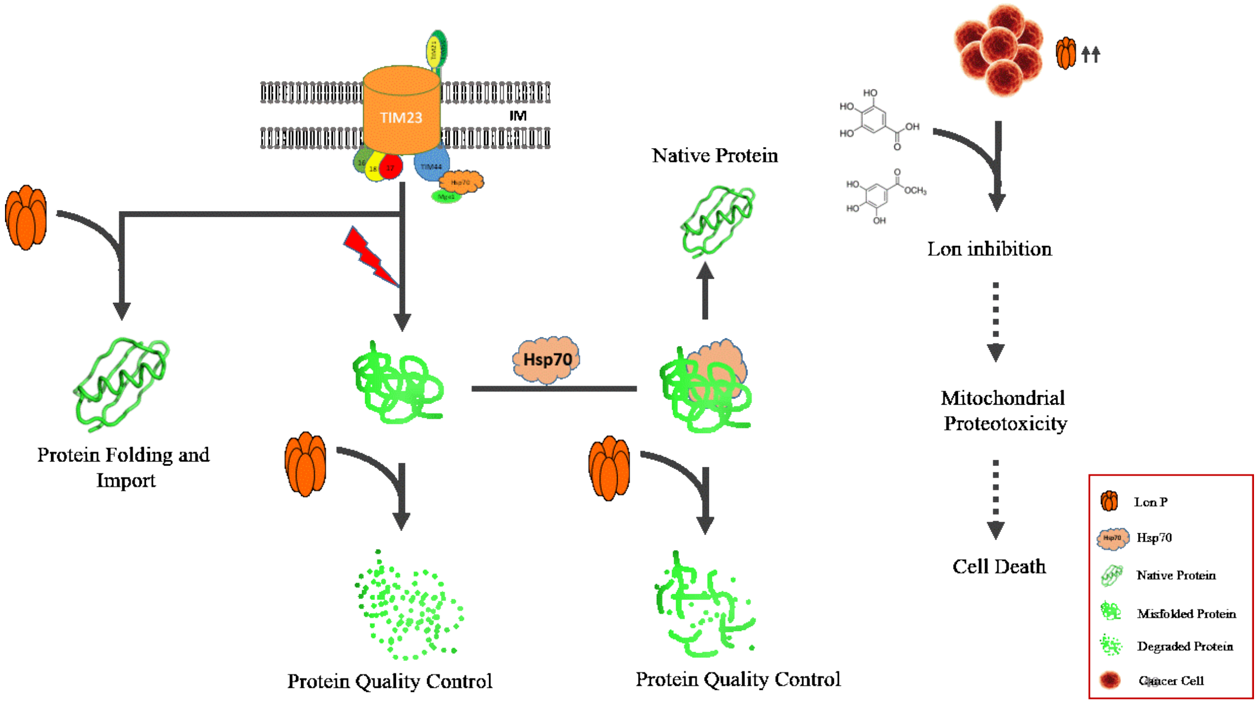


Fig 6: The Proposed model of the study

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SCIENTIFIC REPORTS

A conserved R type Methionine Sulfoxide Reductase reverses oxidized GrpEL1/Mge1 to regulate Hsp70 chaperone cycle

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Cells across evolution employ reversible oxidative modification of methionine and cysteine amino acids within proteins to regulate responses to redox stress. Previously we have shown that mitochondrial localized methionine sulfoxide reductase (Mxr2) reversibly regulates oxidized yeast Mge1 (yMge1), a co-chaperone of Hsp70/Ssc1 to maintain protein homeostasis during oxidative stress. However, the specificity and the conservation of the reversible methionine oxidation mechanism in higher eukaryotes is debatable as human GrpEL1 (hGrpEL1) unlike its homolog yMge1 harbors two methionine residues and multiple cysteines besides the mammalian mitochondria hosting R and S types of Mxrs/Msrs. In this study, using yeast as a surrogate system, we show that h*GRPEL1* and R type *MSRs* but not the S type *MSRs* complement the deletion of yeast *MGE1* or *MXR2* respectively. Our investigations show that R type Msrs interact selectively with oxidized hGrpEL1/yMge1 in an oxidative stress dependent manner, reduce the conserved hGrpEL1-Met146-SO and rescue the Hsp70 ATPase activity. In addition, a single point mutation in hGrpEL1-M146L rescues the slow growth phenotype of yeast *MXR2* deletion under oxidative duress. Our study illustrates the evolutionarily conserved formation of specific Met-R-SO in hGrpEL1/yMge1 and the essential and canonical role of R type Msrs/Mxrs in mitochondrial redox mechanism.

Redox imbalance is one of the key factors for myriad of diseases including metabolic and neurodegenerative disorders[1,2]. Enhanced levels of Reactive Oxygen Species (ROS) or alterations in antioxidant mechanisms affect the activities of biomolecules with reversible and irreversible modifications. Interestingly, ROS also has a beneficial side to it as they known to play as signaling molecule for various cellular functions including redox regulation of several transcription factors, kinases and enzymes[3,4]. Protein reversible oxidative modification, in particular at cysteine and methionine amino acid residues play an important role in many cellular functions. Methionine, upon oxidation forms reversible diastereomeric methionine sulfoxide (Met-S-SO or Met-R- SO) and these sulfoxides are specifically reduced by methionine sulfoxide reductase MsrA or MsrB respectively in a thioredoxin dependent mechanism[5,6]. Deletion of *MSRs* is known to enhance cellular ROS levels that have been implicated in several diseases including diabetes, neurodegeneration, and aging[7]. Methionine in proteins is known to act as an antioxidant and regulate several proteins that undergo Met-SO dependent structural destabilization. Recent studies predict that methionine oxidation could act like protein phosphorylation in cellular signaling to regulate several protein functions[8,9].

Chaperones play an important role in mitochondrial biogenesis through an efficient protein translocation, assembly, iron-sulfur cluster formation, mtDNA maintenance and protein homeostasis[10]. The indispensable Hsp70 chaperone system consists of several conserved components that include DnaK/DnaJ or J-complex and Mge1/GrpE proteins. Mge1/GrpEL1, a conserved nucleotide exchange factor, in its dimeric form interacts with Hsp70-ADP-substrate complex to facilitate the exchange of ADP for ATP so as to initiate another round of Hsp70 cycle. Consequently, it has been shown that GrpE enhances DnaK ATPase cycle by 5000 folds[11]. The stoichiometry between GrpE orthologs and Hsp70/DnaK is shown to be 2:1[12,13]. Mge1/GrpE protein in Hsp70 chaperone

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Methionine sulfoxide reductase 2 reversibly regulates Mge1, a cochaperone of mitochondrial Hsp70, during oxidative stress

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ABSTRACT Peptide methionine sulfoxide reductases are conserved enzymes that reduce oxidized methionines in protein(s). Although these reductases have been implicated in several human diseases, there is a dearth of information on the identity of their physiological substrates. By using *Saccharomyces cerevisiae* as a model, we show that of the two methionine sulfoxide reductases (MXR1, MXR2), deletion of mitochondrial MXR2 renders yeast cells more sensitive to oxidative stress than the cytosolic MXR1. Our earlier studies showed that Mge1, an evolutionarily conserved nucleotide exchange factor of Hsp70, acts as an oxidative sensor to regulate mitochondrial Hsp70. In the present study, we show that Mxr2 regulates Mge1 by selectively reducing MetO at position 155 and restores the activity of Mge1 both in vitro and in vivo. Mge1 M155L mutant rescues the slow-growth phenotype and aggregation of proteins of *mxr2*Δ strain during oxidative stress. By identifying the first mitochondrial substrate for Mxrs, we add a new paradigm to the regulation of the oxidative stress response pathway.

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INTRODUCTION

Persistent oxidative stress is responsible for several neurological disorders, heart failure, and ageing (Finkel and Holbrook, 2000; Orrenius *et al.*, 2007). To combat oxidative stress, cellular systems have evolved several antioxidant defensive mechanisms. Methionine sulfoxide reductases (Msrs) are among the important regulators of oxidative stress (Levine *et al.*, 2000). These enzymes reduce either free or protein-bound oxidized methionine in a thioredoxin and thioredoxin reductase–dependent mechanism to prevent the accumulation of oxidized proteins and amino acids. In yeast, they are three methionine sulfoxide reductases, known as fRMsr, Mxr1/MsrA, and Mxr2/MsrB. The first is responsible for reducing free methionine sulfoxide (free Met-SO), and the last two are involved in the reduction of oxidized methionine present in proteins (Boschi-Muller *et al.*, 2008; Le *et al.*, 2009). Oxidation of methionine can produce a diastereomeric mixture of R and S methionine sulfoxide forms. Mxr1 apparently plays a role in reducing the S form of sulfoxide, whereas Mxr2 acts specifically on R sulfoxides. The mechanism of action of these enzymes has been well characterized in vitro using reconstitution assays containing purified Mxrs (Boschi-Muller *et al.*, 2008; Tarrago *et al.*, 2012). Homologues of Mxr proteins are present across evolution and have been found to play an essential role in the prevention of oxidative damage to proteins mediated by reactive oxygen species (ROS) in bacterial, plant, and animal cells (Zhang and Weissbach, 2008). These enzymes have been implicated in ageing and age-related disorders such as Alzheimer and Parkinson disease, cataract development, and insulin resistance (Gabbita *et al.*, 1999; Kantorow *et al.*, 2004; Glaser *et al.*, 2005; Styskal *et al.*, 2013). Despite their clinical relevance, there is a paucity of information on the identity of the physiological substrates of Mxrs and their regulation. Several reports suggest NF-κBα, potassium channel, and calmodulin as possible substrates of Mxrs (Ciorba *et al.*, 1997; Midwinter *et al.*, 2006; Erickson *et al.*, 2008). In a recent report, SelR, a homologue of MsrB in *Drosophila*, was shown to reduce the oxidized R form of methionine in actin and thereby regulate actin polymerization (Hung *et al.*, 2013). In mammals, MsrA has two isoforms present in mitochondria and cytosol, whereas MsrB has multiple isoforms

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Abbreviations used: Hsp70, heat shock protein 70; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Mge1, mitochondrial GrpE; Mxr, methionine sulfoxide reductase; Tim23, translocase of inner membrane 23.

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A Single Point Mutation in Mitochondrial Hsp70 Cochaperone Mge1 Gains Thermal Stability and Resistance

Adinarayana Marada,[†] Srinivasu Karri,[†] Swati Singh,[‡] Praveen Kumar Allu,[†] Yerranna Boggula,[†] Thanuja Krishnamoorthy,[†] Lalitha Guruprasad,[‡] and Naresh Babu V Sepuri[*,†]

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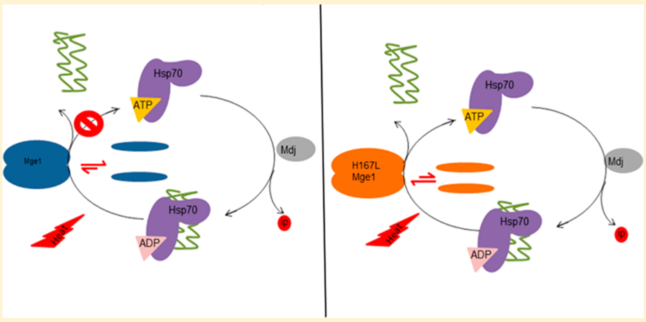

ABSTRACT: Mge1, a yeast homologue of *Escherichia coli* GrpE, is an evolutionarily conserved homodimeric nucleotide exchange factor of mitochondrial Hsp70. Temperature-dependent reversible structural alteration from a dimeric to a monomeric form is critical for Mge1 to act as a thermosensor. However, very limited information about the structural component or amino acid residue(s) that contributes to thermal sensing of Mge1/GrpE is available. In this report, we have identified a single point mutation, His167 to Leu (H167L), within the hinge region of Mge1 that confers thermal resistance to yeast. Circular dichroism, cross-linking, and refolding studies with recombinant proteins show that the Mge1 H167L mutant has increased thermal stability compared to that of wild-type Mge1 and also augments Hsp70-mediated protein refolding activity. While thermal denaturation studies suggest flexibility in the mutant, ionic quenching studies and limited proteolysis analysis reveal a relatively more rigid structure compared to that of the wild type. Intriguingly, *Thermus thermophilus* GrpE has a leucine at the corresponding position akin to the Mge1 mutant, and thermophilus proteins are well-known for their rigidity and hydrophobicity. Taken together, our results show that the yeast Mge1 H167L mutant functionally and structurally mimics *T. thermophilus* GrpE.

The Hsp70 class of proteins consists of evolutionarily conserved ATP-dependent molecular chaperones. These members are involved in protein folding, prevention of protein aggregation, stress response, and remodeling of protein complexes.[1,2] Hsp70 protein comprises a nucleotide binding N-terminal domain and a substrate binding C-terminal domain. These two domains are connected through a disordered loop.[3] The binding and hydrolysis of ATP result in a conformational change in Hsp70 that modulates its affinity for the substrate. In the ATP-bound state, the affinity of Hsp70 for the substrate is low with fast on/off kinetics due to formation of a tense state (T state), whereas in the ADP-bound state, the affinity of Hsp70 for the substrate is high with slow on/off kinetics due to formation of a relaxed state (R state).[4] Hsp70 has low intrinsic ATPase activity with slow ADP dissociation kinetics, and hence, it depends on cochaperones for stimulation of ATPase activity and recycling of ATP for ADP.[5]

Although individual components involved in the Hsp70 chaperone cycle have been well studied, the communication between these components still needs to be explored in detail. In general, thermal stress, a transient increase in temperature, induces the expression of chaperones and cochaperones.[6] In addition to overexpression, temperature-dependent structural modulation or alterations in chaperones have been observed.[4] GrpE, an evolutionarily conserved nucleotide exchange factor (NEF) of DnaK (Hsp70), has been identified as a thermal sensor in *Escherichia coli*.[7] In response to thermal stress, the active dimeric GrpE dissociates into a monomer and fails to interact with DnaK.[8] The NEF family is represented by candidates from archaea, eubacteria, and eukaryotic related organelles such as chloroplasts and mitochondria. Mge1, a yeast mitochondrial homologue of *E. coli* GrpE, has also been shown to undergo a reversible structural alteration during thermal stress.[8] The crystal structure of the *E. coli* GrpE−DnaK asymmetry complex reveals a GrpE homodimer attached to DnaK at its C-terminal end.[9] Each GrpE molecule in the homodimer consists of an N-terminal α-helix domain, central two-helix bundle domains, and a C-terminal β-sheet domain. The β-sheet domain interacts with the nucleotide binding domain of DnaK and is responsible for decreasing the affinity of DnaK for ADP. The temperature-dependent structural transition in *E. coli* GrpE and yeast Mge1 is attributed to the unfolding of long N-terminal helices followed by destabilization of the β-sheet.[10] However, in the case of *Thermus thermophilus* GrpE, melting of the β-sheet domain at permissive transition temperature induces the unwinding of N-terminal helices during thermal stress.[7]

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