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The role of p58IPK in connecting ER stress to mitochondriopathies

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Abstract

Mitochondria and Endoplasmic Reticulum (ER) are organelles which from a network between each other and maintain the cellular homeostasis in the cell. The close network between mitochondria and ER is observed via communication between these two compartments. A number of physiological processes, like Ca^{2+} signaling, lipid metabolism and cell death regulate communication between mitochondria and ER. ER stress leads to accumulation of Ca^{2+} signaling and increase Ca^{2+} uptake into the mitochondria, leads to cell death and eventually mitochondrial dysfunction. The accumulation of unfolded protein in the ER leads to a response which is known as unfolded protein response (UPR), which helps in rescuing the ER from the stress. $P58^{IPK}$ is a cytosolic protein which plays a major role in recruiting cytosolic chaperone and helps in the degradation the unfolded proteins. From recent studies in P58 -/- cells there is an accumulation of misfolded proteins observed, which leads to ER stress and elevated substrate burden on the ER. In this investigation I am working with human patients fibroblast were the cell are mutated in $P58^{IPK}$ protein, with the aim of understanding in understanding the effects of $P58^{IPK}$ on ER stress leading to mitochondrial dysfunction. The initial stage of the project is to standardize the experiment. To check if the mitochondria are functional, I had isolated mitochondria from fibroblast cell and performed *invitro* import studies. To understand bioenergetics properties like respiration rate, a new protocol was standardized by using Sea horse analyzer.

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Abbreviation

Bcl-2	B-cell lymphoma 2
BiP	Immunoglobulin heavy chain binding protein
BSA	Bovine serum albumin
CHOP	C/EBP homologous protein
CREB	cAMP response element binding protein
DAG	Diglyceride
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylendiamine tetraacetate
ER	Endoplasmic reticulum
ERAD	ER associated protein degradation
FA	Fatty acid
FCS	fetal calf serum
g	standard gravity
GFP	green fluorescent protein
GIP	general import pore
GTP	guanosine triphosphate
HEPES	N-2 hydroxyl piperazine-N´-2-ethane sulphonic acid
Hsp	heat shock protein
IM	inner membrane
IMM	inner mitochondrial membrane
IMS	intermembrane space
IRE-1	Inositol-requiring enzyme 1
JNK	c-Jun NH_2-terminal kinase
kDa	kilodalton
MIA	Mitochondrial Intermembrane space Assembly
MIM	mitochondrial inner membrane
Mim1	mitochondrial import 1
MnSOD	Manganese superoxide dismutase
MOM	mitochondrial outer membrane
mt	mitochondrial
MW	molecular weight
NP-40	Nonidet P-40
ODx	optical density at x nm
OMM	outer mitochondrial membrane
PERK	Double-stranded DNA-dependent protein kinase (PKR)-like ER kinase
PK	proteinase K
PMSF	phenylmethylsulfonyfluoride
PVDF	polyvinylidene difluoride
RNasin	ribonuclease inhibitor
ROS	reactive oxygen species

SDS-PAGE	Sodium dodecyl sulfate polyacrylamid gel electrophoresis
TCA	trichloroacetic acid
TEMED	N,N,N‘,N‘-tetramethylene diamine
TOM	translocase of the outer mitochondrial membrane
UPR	Unfolded protein response
VDAC	Voltage dependent anion selective channel
V/v	volume per volume
W/v	weight per volume
WCL	whole cell lysate

1 Introduction

1.1 Structural link between Mitochondria and ER

Mitochondria and endoplasmic reticulum (ER) are both organelles that together maintain the cellular homeostasis and determine the cell fate in stress conditions. A number of studies have shown the communication between the mitochondria and ER. The physical link between ER and mitochondria is been evidently observed in cosedimentation and microscopic results, association between the ER particle with mitochondria[1, 2].

1.1.1 Endoplasmic reticulum

The ER is a continuous membrane bound compartment present in all eukaryotic cells, which has an extensive network of cisternae and microtubules occupying greater than 10% of total cell volume or space. The ER plays a major role in translocation of newly synthesizes peptide across rough ER and correct post translational folding of these proteins [3]. ER plays a role in directing the proteins into their destination and lastly involving in physiological signaling of calcium, which is triggered by electrical and chemical cell stimulation.

The role of ER is strongly influenced by components of the Ca^{2+} signaling, its response in ER is controlled by inositol-1-4, 5triphosphate (IP_3) receptor and ryanodine receptors (RyRs). They are three types of RyRs and three types of IP_3Rs known so far [4, 5]. IP_3R and RyRs form very large pore complex by homotetramers of ~ 3000 amino acids. IP_3R are ligand gate channels, they co-ordinates the release of Ca^{2+} from ER. Storage of Ca^{2+} in response to inositol-1-4-5- phosphate (IP_3) is generated by an agonist. The interaction of the agonist and cell surface receptor leads to a response of IP_3 which generates Ca^{2+} storage [6]. Ca^{2+} signaling plays a fundamental role in modulating a range of cellular response, which includes proliferation, muscle contraction, exocytosis, motility and gene expression. Activation of IP_3R through its agonist and cell receptor interaction leads to physiological release of $Ca^{2+,}$ which serves in important signaling and maintains the “house keeping genes”. During the apoptotic stimulus, IP_3 can mediate Ca^{2+} release which can lead to apoptotic pathways by releasing pro-apoptotic factor from the mitochondria (figure 1) [7].

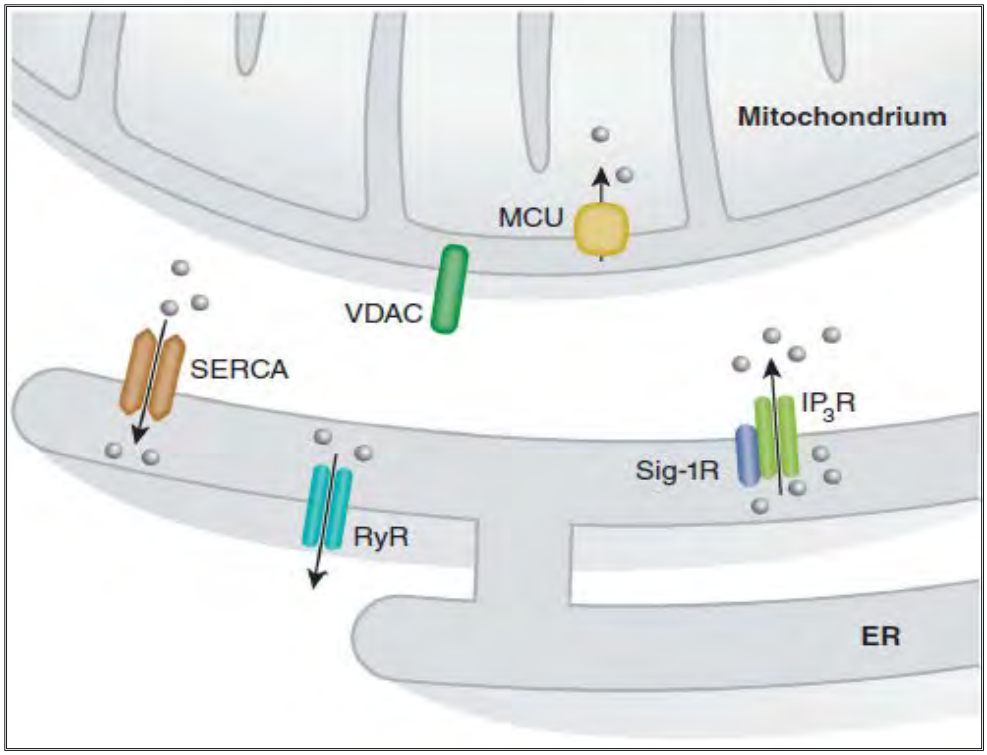

Figure 1: *Ca^{2+} signaling between ER and mitochondria. Ca^{2+} is mainly released from ER through IP3 and RyR, which is close contact to mitochondria (Adopted from Olga Martins de Brito and Luca Scorrano, EMBO review 2010[8]).*

1.1.2 Mitochondria

The cytoplasm of nearly all eukaryotes cells contains mitochondria, these are double membrane structure, separated by four distinct compartments i.e., the outer mitochondrial membrane (OMM), the inter membrane space, the inner mitochondrial membrane (IMM) and the matrix. The inner membrane is enclosed into the mitochondrial matrix forming cristae. The outer membrane of mitochondria is widely permeable to ions and larger molecules in comparison to the inner membrane which is less permeable to ions and small molecules[9].

The structural and functional interaction of ER-mitochondria plays an important role in controlling the Ca^{2+} signaling and other processes which are dependent on them. Cytoplasmic Ca^{2+} passes through OMM protein voltage dependent anionic channel (VDAC) into the matrix via inner mitochondrial membrane, which is driven by large electrochemical gradient [10].

1.2 ER and Mitochondria dynamics

ER and mitochondria are highly dynamic organelles which is crucial for the cell, they are tightly capable of modulating their structure and function during change in the environmental conditions. ER has a highly complex tubular structure, which constantly modifies its structure through tubule sliding, tubule branching and ring closure due to change in the environment of the cell[11].

Mitochondria are heterogeneous in shape in different cell types, they are short, rod shaped, continuous, elongated, tubular, highly dynamic and interconnected network. These phenotypes result in different complex equilibrium among organelles motility, fusion and fission events[12]. In eukaryotes, biogenesis of mitochondria predominantly occurs in peri-nuclear region and there is a highly evolved system to transport these organelles and require higher amount of energy for the transportation, as well as metabolic functions.

Microtubules play an important role in the movement of the mitochondria with help of actin, which is known as microtubules-driven process[13]. ER and mitochondria dynamics are regulated together, but in some cases, the cellular signaling is efficiently synchronized by the movements of these organelles. The movement of ER and mitochondria is influenced by secondary messenger Ca^{2+}, which actively triggers the cascade signaling. Ca^{2+} is an agonist which can effectively produce a rapid signaling of the movement of both organelles [14]. Ca^{2+} transmits from ER to mitochondria metabolism via contact between the two organelles.

1.3 ER and Mitochondria function

The structure membrane interaction forms a bridge between mitochondria and the ER which is known as mitochondria- associated membrane (MAM). The important function of MAM is to build a relationship between two compartments in phospholipids and its cellular functions. The most crucial function of MAM contact sites is the transmission of Ca^{2+} between the two organelles and function of this process during apoptosis [3, 15]. The contact between ER and mitochondria is also modulated by fission and fusion of mitochondria. Fission and fusion are regulated by the mitochondrial shaping proteins like dynamin- related protein 1 (DRP1), mitofusion-1 and mitofusion-2. Mitofusion-2 is a mitochondrial transmembrane GTPase which regulates the mitochondrial fusion and enhances the MAM. The binding of mitofusion-2 plays a role in controlling the Ca^{2+} flow between mitochondria and ER [16, 17].

1.4 Endoplasmic reticulum stress

The ER is the site of synthesis, folding and modification of secretory and cell-surface protein, as well as the resident protein of the secretory pathway. The ER has quality control machinery, which operates in conjugation to the protein folding pathway. A minor defect in the protein folding can cause rejection of nascent protein as misfolded, leading to accumulation or degradation of these proteins, and it is known as ER stress. During this process, ER has also a function in sensing misfolded protein and initiating changes in gene expression as well as the folding capacity if the ER.

During the ER stress, the cell triggers conserved strategies of ER-to-nucleus signaling which is known as unfolded protein response (UPR). This response down regulates the global translation and on reverse effect increases the misfolded protein degradation via autophagy[18-20]. The UPR cascade is involved in three different transduction mediated by ER transmembrane receptors in parallel : double- strand DNA- dependent kinase (PKR)- like ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6)(figure 2). During the resting phase of the cell, these receptors are bound to the ER chaperones, BiP also known as GRP7s, which is kept in inactive state. Upon the ER stress, accumulation of misfolded or unfolded proteins leads to dissociation of BiP and therefore three different transduction proteins are activated (PERK, IRE1 and ATF6)(figure 2)[21]. ER stress can occurred by physiological or pathological stimuli like cytokines inducing B cell differentiation and immunoglobulin's synthesis and expression of aggregation prone protein, as well as by pharmacological treatments [19].

PERK mediates eIF2α (eukaryotic translation initiation factor 2) is initiated by dimerization and autophosphorylation, resulting in phosphorylation of eIF2α that inhibits the protein translation [22]. Phosphorylation of eIF2α leads to inhibition of protein translation aids cell survival by decreasing the load of nascent protein on ER during the stress condition [23]. ATF-6 (activating transcription factor 6) is a transcription factor which dissociates from BiP and translocates to the golgi apparatus, where a cytosolic fragment is released by proteolytic cleavage. ATF4 up-regulates genes involved in stress response, like chaperones and as well as redox reaction and amino acid metabolism[22].IRE1 performs dual enzyme activity by oligomerization and removal of 26 nucleotide long intron of x-box binding protein 1 (XBP-1) mRNA with help of IRE1 endoribonuclease domain. The spliced products generated by frameshift splice variant (sXBP 1) encode a stable, active transcription factor which binds to

the promoter elements to activate transcription of ER chaperones, ER associated protein degradation (ERAD) factors and the HSP40 family member P58 ^{IPK} protein[24].

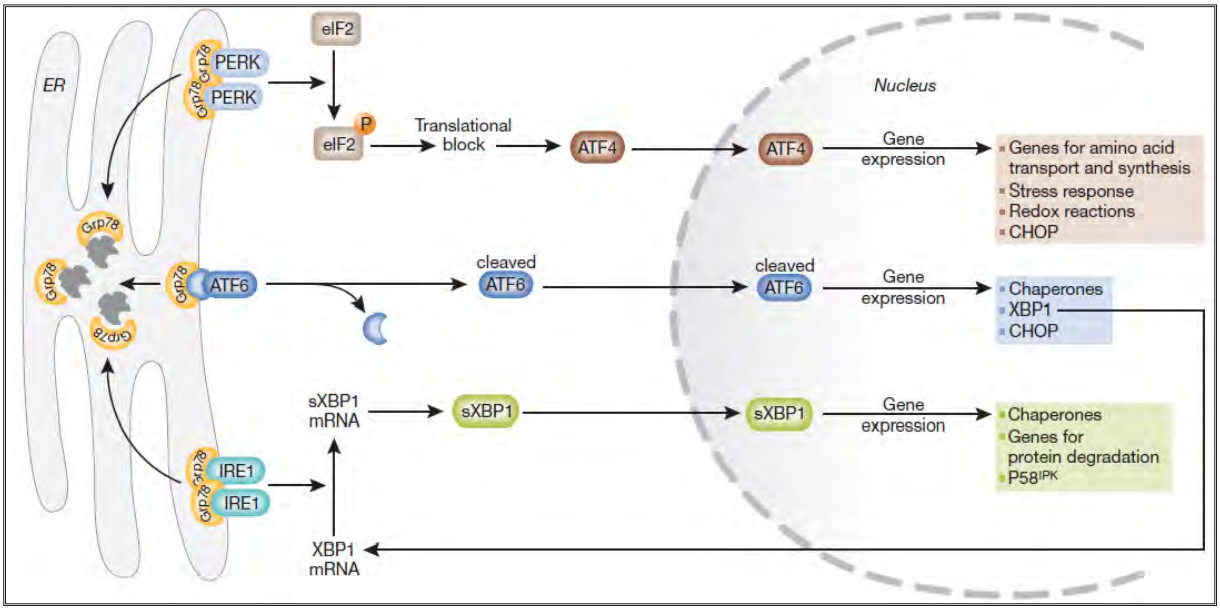

Figure 2: *Activation of ER stress pathway: The unfolded response – There are three endoplasmic reticulum (ER) stress receptors (a)PERK- pancreatic ER kinase- like ER kinase, (b)IRE1- inositol- requiring enzyme 1 and (c)XBP1- X box- binding protein 1. All the three receptors help the ER rescuing from stress (Adopted from Afshin Samali EMBO review 2006[22]).*

P58^{IPK} is HSP40- DNAJ like family protein which inhibits PERK, it provides the negative feedback loop by mediating PERK translation block [21, 25]. It has recently been shown that P58^{IPK} have an independent function in protecting ER from stress condition [26]. The upregulation of P58^{IPK} is not an immediate reaction in the ER stress cells; they generally take several hours to be activated after the phosphorylation of eIF2α and PERK. P58^{IPK} plays an important role in ER stress by attenuating protein synthesis and reducing substrate influx into ER during the stress condition. P58^{IPK} also terminates the unfolded protein response (UPR), in the successful condition the ER returns to the normal state and function in cell survival[22]. In absence of P58^{IPK} there is over expression of two ER stress inducible genes BiP and CHOP, which constantly express phosphorylated eIF2α and leads to blocking of protein translation[27]. During ER stress response, P58^{IPK} represses PERK and plays a major role in rescuing the ER from the stress. P58^{IPK}as well as acts as good biomarker for downstream regulator of PERK [26].

PERK, ATF6 and IRE1 can also trigger pro-apoptotic signaling during the ER stress condition. Apoptosis is mainly initiated by activation of downstream factors like transcription factor C/EBP homologous protein (CHoP) or JNK by further activation of downstream pathways. The molecular mechanism of CHOP mediating apoptosis is not well understood, However it is known that they reduces the expression of anti-apoptotic BCl-2 family proteins and Ca^{2+} related apoptotic signaling [28].

1.5 Mitochondrial dysfunction

Mitochondria plays an important role in maintain the cell metabolism pathways, the dysfunction of mitochondria affects the patients with severs clinical symptoms [29, 30]. Mitochondrial disorders mainly occur due to mutation in mitochondrial DNA and in the nuclear gene encoding mitochondrial proteins. The Mitochondrial disorders are been associated with pathological manifestation like CNS, leading seizures, strokes, auditory system, optic system and also with high metabolic energy demand such as nerves and muscles.

1.6 ER involvement in mitochondrial dysfunction or vice versa

Mitochondria dysfunction and ER stress have crucial role in the pathogenesis of type 2 diabetes mellitus, decreasing levels of adiponectin and developing hepatic insulin resistance [31]. Studies from E.H Koh et al showed that the ER stress induces mitochondria dysfunction and affects the physiological response within cells. In ER - mitochondria interaction, ER facilitate control of Ca^{2+} signaling and alters its homeostatic mechanism resulting in Ca^{2+} overload to apoptosis[32]. Transfer of Ca^{2+} from ER lumen into mitochondria associated membrane increases the levels of Ca^{2+} uptakes by mitochondria. The overload leads to an imbalance in buffer capacity of matrix and accumulation of Ca^{2+}. The accumulation of Ca^{2+} triggers the opening off mitochondrial permeability transition pore, resulting in swelling of mitochondria and rupturing the OMM, thereby releasing the pro-apoptotic protein into the cytosol [10].

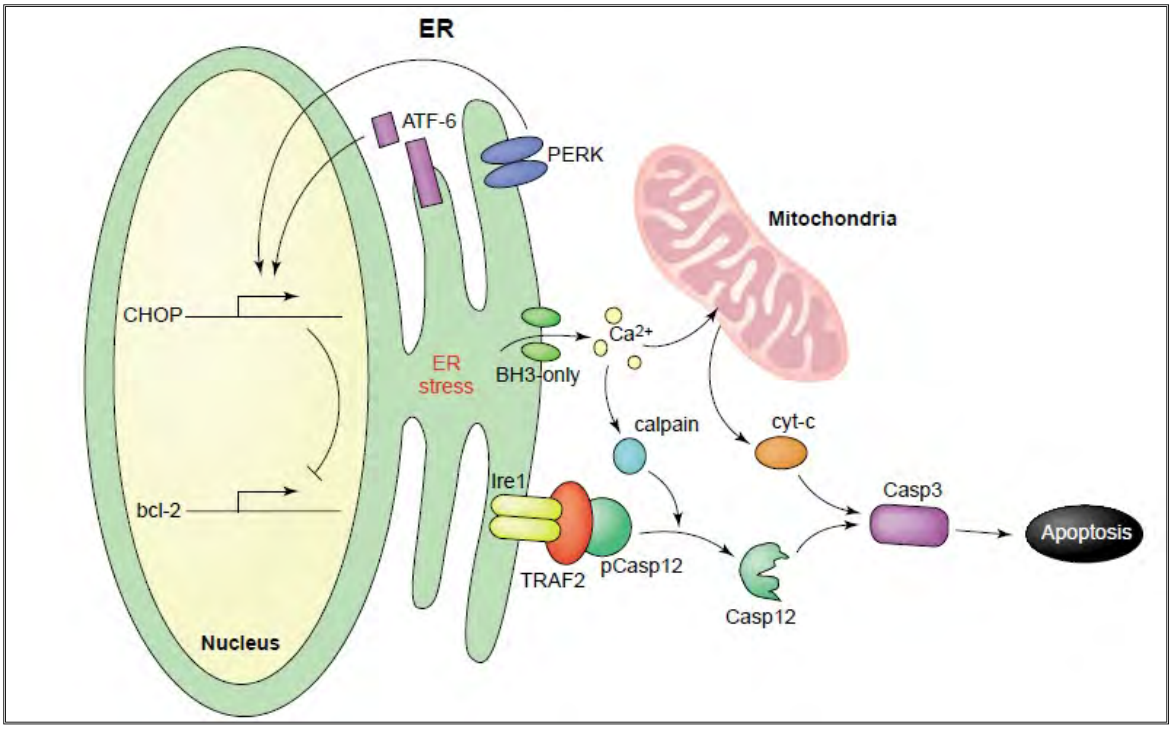

Figure 3: *ER stress response on mitochondria: The regulation of Ca2+ concentration from ER, mitochondria and cytoplasm trigging the apoptotic pathway.*

The synthesis of protein folding in ER and maintaining Ca^{2+} balance within ER requires large number of ATP. The depletion of ATP due to dysfunction on mitochondria induces ER stress. Due to nitric oxide, the disruption of mitochondrial respiratory chain can lead to ER stress response, is one-way process were only ER stress leads to mitochondrial dysfunction [31].

1.7 Aim

The aim of this study is to elucidate the function of $P58^{IPK}$ in ER stress induced mitochondrial dysfunction specialized in fibroblasts cells. One main point is to investigate the impact of deletion of $P58^{IPK}$ protein in the human patient family with specific emphasis on the effects of ER stress leading to the mitochondrial dysfunction. My aim was to standardize three assays by parallel measuring of oxygen consumption by Sea horse analyzer, steady- state level of whole cell lysate proteins and the morphology of mitochondria in the primary fibroblast.

2 Materials and methods

2.1 Cell biology methods

2.1.1 Cell lines

HeLa and primary fibroblast cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, PAA, Germany), low glucose (PAA, Germany) supplemented with 10 % (v/v) FBS-Gold (PAA, Germany), 2 mM glutamine, and 5 mg/l gentamycin. The cells were kept at 37°C under 5 % CO_2 atmosphere.

Cells were maintained in culture by growing them until 95 % confluency and passaged in 1:8 for HeLa cells and 1:3 dilutions for primary fibroblast cells.

2.1.2 Primary fibroblast cells

For culturing of primary fibroblasts derived from the patient's with$p58^{IPK}$mutation and healthy donor. HeLa cells were already cultured in the laboratory; DMEM high glucose (PAA, Germany) with stable glutamine was supplemented with 10 % (v/v) FCS-Gold and 5mg/l gentamycin. The cells were cultivated at 37°C under a 5 % CO_2atmosphere. For growth analysis, cells were maintained in DMEM without glucose (PAA, Germany), and glutamine was supplemented with 10 % (v/v) FCS-Gold, 10 mM HEPES, 2.25 mM uridine, 2 mM sodium pyruvate, 5 mg/l gentamycin and 10 mM galactose, 200 mg/l glucose or 4.5 g/l glucose.

2.1.3 Growth analysis of primary patient fibroblasts

For growth analysis, fibroblasts were cultivated for ~ 2 weeks in Dulbecco's Modified Eagle's Medium containing 4.5 g/l glucose, 200 mg/l glucose or 10 mM galactose according to previously described procedures[33]. Cells are counted by viability test using trypan blue staining.

2.1.4 High resolution live cell imaging

For the fluorescence microscopy, cells we plated into 8 well microscope plates a day before the imaging and the cells were incubated for 30 min with medium containing 50-100 nM MitoTracker, ER tracker (Molecular Probes), DAPI (Invitogen) and $P58^{IPK}$antibody(Cell Signaling). Cells were visualized on a Delta Vision microscope and image 3D segmentation and analysis was performed with Image J or Imaris software (Bitplane). For morphological

quantification, cells were observed into three different classes based on the appearance of the mitochondrial structure: volume, surface and aspect ratio with help of image J software.

2.1.5 Respiration analysis

Oxygen consumption analysis was performed on cells grown in DMEM medium without glucose (PAA, Germany), supplemented with 10 % (v/v) FCS-Gold, 10 mM HEPES, 2.25 mM uridine, 2 mM sodium pyruvate, 5 mg/l gentamycin and either 10 mM galactose or 4.5 g/l glucose. Respiration analysis was performed with Dulbecco's Modified Eagle's Medium, containing either 10 mM galactose or 4.5 g/l glucose, supplemented with 1 g/100 ml sodium pyruvate and 1 g/100 ml glutamine during measurement recordings.

Cellular bioenergetics measurement is performed in 96 wells Seahorse analyzer plate. A day before the analyses of O_2 consumption, cells were trypsinised and resuspended in to the growing media. Around 15,000 cells were plated into the Seahorse plated at 37°C in 5% CO_2. On the same day calibration plate is prepared with 200 µl of calibration buffer and is incubated in the Seahorse incubator. On the second day cells are washed with assay media and replaced by the growth media with assay media before the measurement (150µl per well). Cells are incubated for one hour in the Seahorse incubator without O_2. Ports were filled with 25 µl of compounds: port A: oligomycin, port B: FCCP, port C and D: rotenone and antimycin A, the ports are open, pipetting much be done gently with little pressure due to the ports are open. Protocol was started with the calibration and after the calibration; assay plate was exchange with calibration plate for the measurement of the cells. After measurement the cells were fixed with accustain for 45 to 60 seconds and washed the cells with PBS. The cells are stained by DAPI (1:5000) for 10 min in the dark. Wash the cells with PBS and count the cell by using high quantiant imager.

2.2 Biochemical methods

2.2.1 Protein extraction

Adherent cells were thoroughly washed once with 1x PBS, subsequently harvest the cell by cell scraper with RIPA buffer (Tris-HCl: 50mM PH 7.4, NP-40: 1%, Na-deoxycholate: 0.25%, NaCl: 150mM, EDTA: 1mM is prepared in 100ml of water) Cell suspension in RIPA buffer is constant agitation at 4°C for 15 min and lysates were centrifuged for 15 min at 14000 x g to remove unsolubilized cell debris. Immediately the supernatant is transferred into a fresh centrifuge tube and the pellet is discarded.

2.2.2 Determination of protein concentration

Protein concentrations were determined according to the method described by M. M. Bradford[34].The Roti-Quant Bradford reagent was used for determination of protein concentrations of the samples. Samples with 1 to 15 µg BSA content were used as internalstandards. The cell lysate were diluted to 1:10 before determining the protein concentration. Quantification was performed by measuring OD at 595 nM, using the standard curve feature of an Eppendorf BioPhotometer (Eppendorf, Germany).

2.2.3 SDS-PAGE and Western

Separation of proteins by denaturing Tris-glycine SDS-PAGE was carried out as Laemmli, 1970[35]. Samples were resuspendend in 2x Laemmli buffer (60 mM Tris-HCl, 1 % (w/v) SDS, 10% (v/v) glycerol, 5 % β-mercaptoethanol, 0.01 % (w/v) bromphenol-blue in purified water)and heated to 95°C for 10 min prior to loading. For casting of gels, glass plates of 130 mm width were used with 1 mm spacers. Stacking gels were typically 10 mm, separating gels 120 mm and bottom gels 8 mm in length. Gel mixtures were prepared in 1.0 M Tris-HCl, pH 8.8(resolving gel), 1.0 M Tris-HCl, pH 6.8 (stacking gel), 0.1 % N,N,N‟,N‟-29 tetramethylethane-1,2-diamine (TEMED) and 0.5 % (w/v) ammonium persulphate (APS) were added as polymerization starters. Gels were run in SDS-PAGE running buffer for1.5 h at 25 mA. Page Ruler pre-stained marker (Fermentas, Germany) was used as a protein molecular weight marker.

For protein transfer, the semi-dry blotting method was used for transfer of proteins separated via SDS-PAGE onto nitrocellulose membrane [36, 37]. The membrane, the gel and six sheets of Whatman filter paper (3MM) were incubated shortly in Blotting buffer (20 mM Tris, 150 mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol). The gel was placed on top of the membrane, and three filter papers were placed on a bottom and on a top of the blotting sandwich. Transfer was performed at 200 mA for 60 min. Membranes were blocked in 5% (w/v) skim dry milk in TBS for 1 h while shaking and then incubated with primary antibodies for overnight at 4°C. Membranes were washed for 5 min in TBS, then in TBS with 0.05% (v/v) Triton X-100 and finally in TBS again. As secondary antibodies, were incubated for 1 h at RT and the blot were washed 3 times with TBS. After brief incubation with ECL solution luminescence was detected with X-ray films (Fujifilm).

2.2.4 Isolation of mitochondria from adherent cells

Mitochondria were isolated from HeLa and primary fibroblast cells according to published procedures, Johnston and modified version [38].Cells were grown to ~ 95% confluency, washed with PBS, and harvested by the cell scraper. Cells were then pelleted at 800 × g for 5 min on2°C and resuspended in HBB buffer (20 mM HEPES, pH 7.6, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM PMSF) supplemented with 2 mg/ml BSA (is named as HBA buffer) followed by homogenization with a needle and centrifuge for 5 min at 800 × g on 2°C.Supernant was collected and centrifuged again at 10,000× g on 4°C for 10 min and mitochondrial pellet is resuspended with HBB buffer. Concentration of protein was determined by Bradford method described as above.

2.2.5 *Invitro* import of proteins into isolated mitochondria

For *invitro* import, the precursor proteins were synthesized by coupled transcription-translation in recticulocyte lysate in presence of [35S] methionine using SP6 RNA polymerase.Precursor proteins were synthesized by coupled transcription/translation in reticulocyte lysate (TNT Coupled Reticulocyte Lysate System, Promega) in the presence of [35S]methionine using SP6 RNA polymerase. For *invitro* import, isolated mitochondria (25-100 µg) were incubated with radiolabeled precursor proteins at 25°C for different time periods in import buffer. Samples were transferred on ice and treated with proteinase K (100 µg/ml) to remove non-imported material. The protease was inactivated by addition of 2 mM phenylmethylsulphonyl fluoride (PMSF) and then mitochondria were reisolated and washed with import buffer. Transcription of the mRNA using SP6 RNA Polymerase was performed at 37°C for 1 h by adding 5 µl of 10 M LiCl and 150 µl of absolute ethanol and subsequent incubation at -20°C for 2 to 3 hours. The transcribed material was centrifuged for 20 min at 2°C, 37.000 x g and pellet was washed with 70% fresh ethanol and was dried for 5 min followed by resuspension of 40 µl of water with 2 µl of RNasin. Translation of synthesized mRNA in radioactive labeled protein from rabbit reticulocyte lysate was used. Mixture for in vitro translation contained: 12.5 µlmRNA, 1.75 µl amino-acid mix (without methionine), 3.5 µl 15 mM Mg-acetate, 0.5 µlRNase inhibitor, 6 µl S35-Methionine (10 mCi/ml) and 50 µl rabbit reticulocyte lysate. Translation mixture was incubated at 30°C for 1 h. At the end ofthe translation reaction 6 µl of 58 mM methionine and 12 µl of 1.5 M sucrose were added. To remove ribosome from the lysate, samples were centrifuged in an ultracentrifuge (50 min,

90,000 g, 4°C); supernatant containing radioactively labeled protein was transferred in a new tube and used in import experiments.

For *invitro* import, isolated mitochondria (25-35µg) were incubated with radiolabelled precursor proteins at 25°C for different time periods in import buffer (250 mM sucrose, 80 mM KCl, 5 mM MgCl2, 3% (w/v) fatty acid free BSA, 10 mM MOPS, pH adjusted to 7.2 with KOH). Samples were transferred on iceand treated with proteinase K (100 µg/ml) to remove non-imported material. The protease was inactivated by addition of 2 mM phenylmethylsulphonyl fluoride (PMSF) and then mitochondria were re-isolated by centrifuging it for 15 min, 36.000 g at 4°C) and resuspended either in 30 µl of 2 x Lämmli solutions for further SDS-PAGE analyses.

3 Results

3.1 Standardization of the cell culture protocol

3.1.1 HeLa cells

To gain some hands-on experience before working with patient's primary fibroblast, I initiated my work with HeLa cells to understand the aseptic techniques and principle of mammalian cell culture. I performed the isolation of mitochondria from HeLa cell by using a modified version of Johnson et al protocol followed by an *invitro* import experiment[38]. *Invitro* import experiment was performed to assure the functionality of the isolated mitochondria. *Invitro* import was performed with pSu9-DHFR already existing construct in the laboratory, which is a fungal derived presequence of an ATPase subunit coupled to dihydrofolate reductase.

In figure 4, the import of pSu9-DHFR into isolated mitochondria from HeLa cells, I compared the import efficiency of mitochondria from yeast cell (received from Thomas Müller) with HeLa cells. Isolated mitochondria were incubated with radiolabelled presequence protein (pSu9-DHFR) and they were further analyzed on the SDS-PAGE followed by autoradiography. A significant increase of matured form of pSu9-DHFR import is observed in the samples 5 and 20 min in HeLa cell mitochondria as well as, in yeast mitochondria. It is the working model in the laboratory used as a control in this case to check the efficiency of the presequence import.

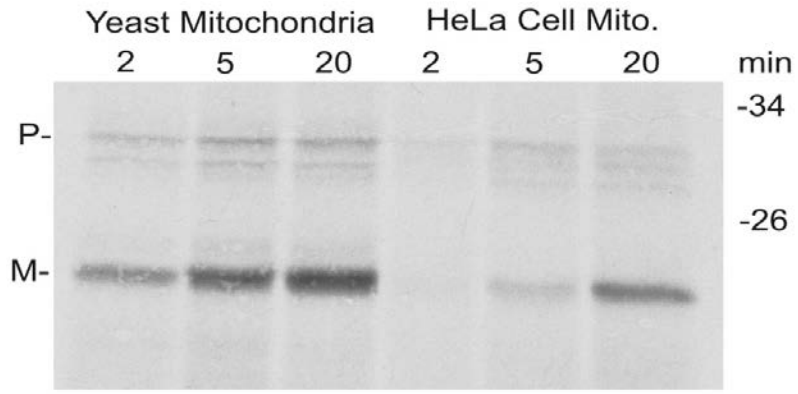

Figure 4: *Mitochondria isolated from HeLa cell were incubated at 25 C for 2', 5' and 20' minutes with radiolabelled precursor of pSU9-DHFR. The bands representing the import after PK treatment, the premature (P) and the matured band (M) show the import into the IMM. Yeast mitochondrion was used as a control to check the import efficiency in the HeLa cell mitochondria.*

In figure 5, the blot from autoradiography (blot after the *invitro* import studies) was used to decorate the antibody related to mitochondria and ER proteins like TOM40 (outer mitochondrial membrane protein), MnSOD (mitochondrial matrix protein) and $P58^{IPK}$ (ER stress related chaperone protein) to check the working condition of the antibodies.

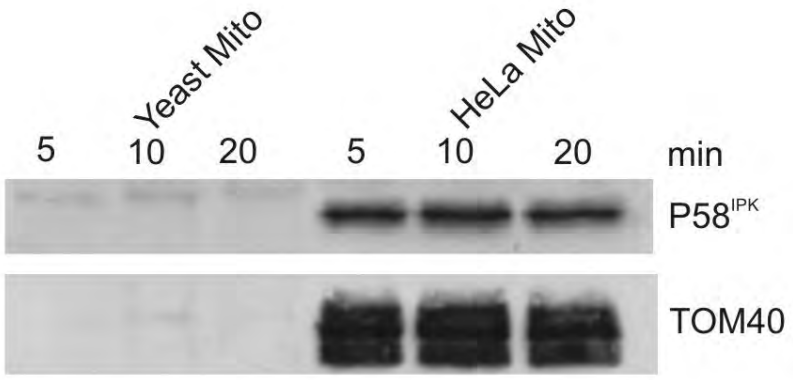

Figure 5: *Mitochondrial derived from HeLa cells were tested with the specific antibodies to check the concentration and specificity of the antibodies. In this blot $P58^{IPK}$ (cytosolic protein) and TOM40 (outer mitochondria membrane protein) antibodies were used. Yeast mitochondira was used as control to check the specificity of the antibody as these antiobody are raised for mammilan therefore there is no band observed in yeast mitochondria (This blot is from the invitro import experiment which is decorated with the specific antibodies).*

To the check the steady state levels of proteins ,the blot was decorated with TOM40, MnSOD, $P58^{IPK}$(figure 6) and α-tubulin to check the enrichment of mitochondrial proteins in HeLa cell mitochondria in compare to whole cell lysate (WCL) and α- tubulin to check the cystoloic containtmation and control for loading (figure 7).

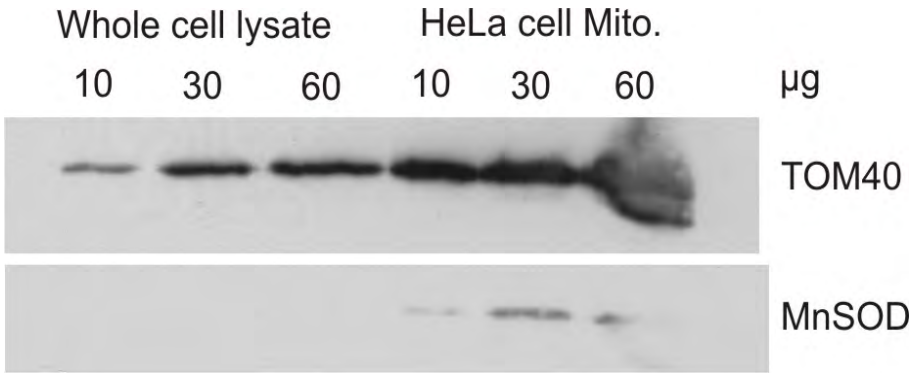

Figure 6: *Steady state levels of isolated mitochondria from HeLa cells were compared with whole cell lysate at 3 differenct concentration 10, 30 and 60 µg with specific mitochondrial related protein TOM40 and MnSOD.*

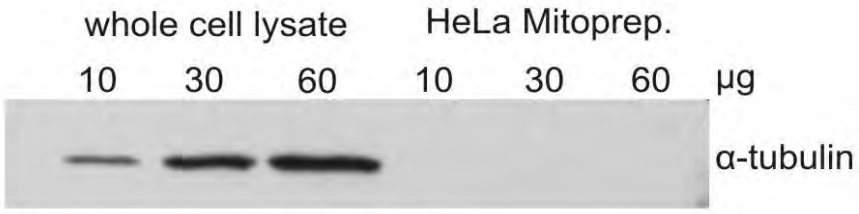

Figure 7: *Steady state levels of isolated mitochondria and WCL were compared in HeLa cells. α-tubulin was used as control to check the contamination in isolated mitochondria.*

3.1.2 Standarization of fibroblast

Fibroblast cells generally take longer time to grow than HeLa cell, after standardizing the protcol I switched to fibroblast to estimate the growth pattern and amount of cells required for the experiments before starting with the patient fibroblast. Isolation of mitochondria and *invitro* import was performed with similar protocol used for HeLa cells as mentioned above (figure 8). In figure 8, there is a clear import observed in yeast cell mitochondria and in the fibroblast cell mitochondria.

In figure 9, the blot from autoradiography was used to decorate the antibody related TOM40, MnSOD and P58^{IPK}is similar to HeLa cells, to check the specificity and working condition of the antibodies.

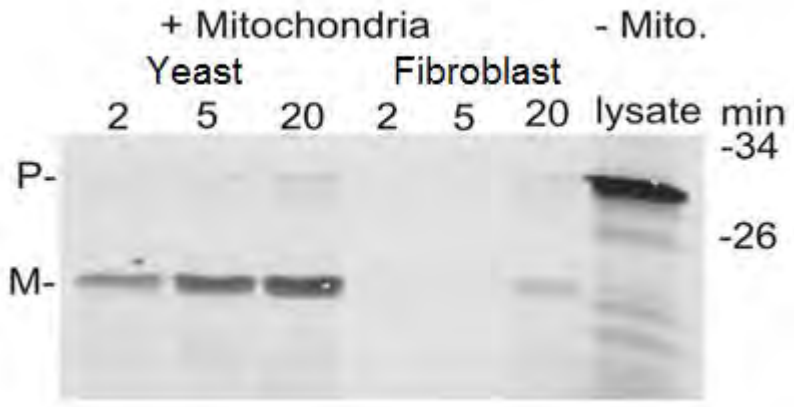

Figure 8*: Invitro import study: Isolated mitochondria from fibroblast were incubated at 25 C for 2, 5 and 20 mintues with radiolabelled precursor of pSU9-DHFR. After the import the mitochondria were subjected to PK treatment and analyzed on the SDS-PAGE. The premature (P) and the matured band (M) show the import into the IMM. Yeast mitochondria were used as a control to check the import efficiency in the HeLa cell mitochondria.*

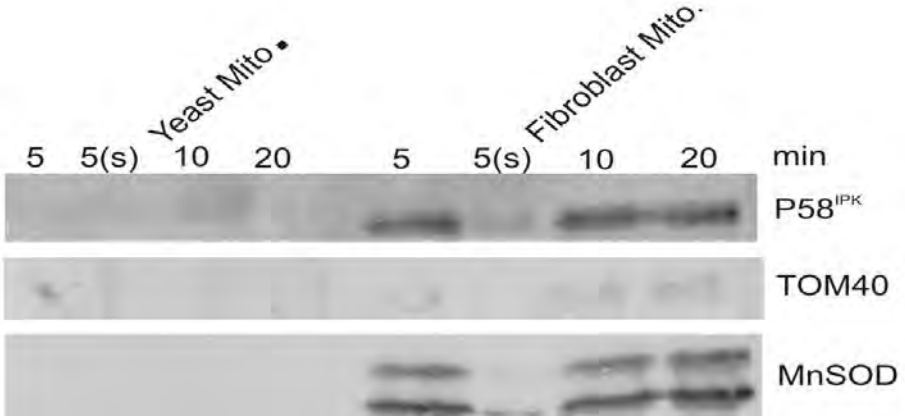

Figure 9: *Mitochondrial derived from fibroblast cells were tested with the specific antibodies to check the concentration and specificity of the antibodies. In this blot P58^{IPK} (cytosolic protein), MnSOD (Inner mitochondria matrix protein) and TOM40 (outer mitochondria membrane protein) antibodies were used. Yeast mitochondira was used as control to check the specificity of the antibody as these antiobody.*

3.1.3 Standardizing of patients fibroblast

$P58^{IPK}$ is 58 KDa DNAJ family proteins, which is involved in inhibition of eI2Fα and PERK signaling during ER stress [39].The patient family condition is a suffering from $P58^{IPK}$ mutation, there are three affected siblings, one unaffected sibling and the parents are unaffected and consanguinity is likely. A specific antibody $P58^{IPK}$ was used to detect the protein in western blot analysis and to estimate the expression levels in patient family affected by $P58^{IPK}$. As a control the primary fibroblast were collected from the healthy, unrelated donor patient.

The whole cell lysate (WCL) extracted was prepared from the patient fibroblast and control fibroblast which was decorated with $P58^{IPK}$, TOM40, MnSOD and α-tubulin. In control (CO-29) cell line, there is a specific band observed in $P58^{IPK}$ with an apparent Mw of 58, TOM40, MnSOD (figure 10) and α-tubulin which was used as loading control.

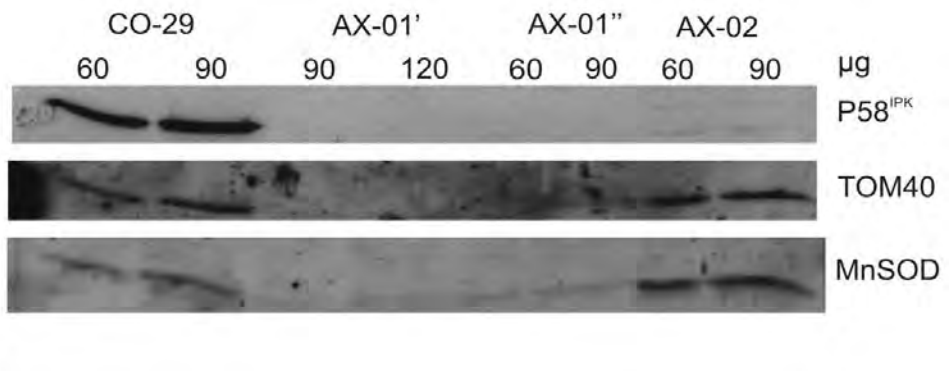

Figure 10: *Whole cell lysate derived from patient and control fibroblast: Steady state levels were checked in AX-01, Ax-02 and control fibroblast cells. In mutant form of AX-01 and AX-02 of P58IPK, there was no expression observed when the proteins were sunjected to specific antibody directed against P58IPK when compared with C0-29 control fibroblast. Ax-01' and AX-01'' are the same type of cells but isolated in two different times had no expression against TOM40 and MnSOD as well compared to AX-02 and C0-29.*

Interestingly, in the samples from patients; AX-01 and AX-02 cell had no signal observed in present of $P58^{IPK}$ specific antibody (figure 10). Whereas, in the mitochondrial related protein like TOM40 and MnSOD there was an equal expression seen in all the cell lines. In figure 11, there was one more interesting observation seen in the loading control. CO-29 and AX-02 there is a higher signal seen in present of α-tubulin, in comparison to AX-01' and AX-01''. In AX-01' the concentration of protein was increased to normalize the equal loading but there

was no difference seen in different concentration of AX-01 (60 and 90 µg). However when the blots were incubated in ponceus S, band pattern showed equal loading of WCL.

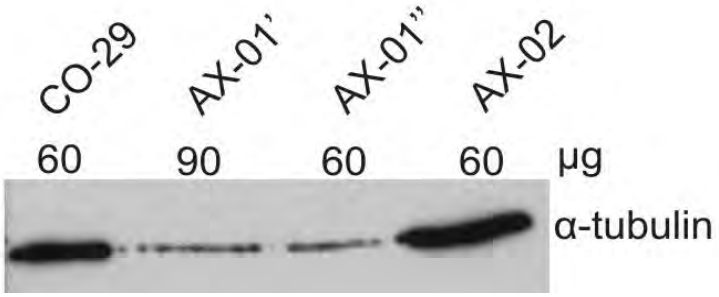

Figure 11: *Steady state levels of whole cell lysate: α-tubulin was used as loading control for the control and patient fibroblast.*

Invitro import studies were perfomed with the isolated mitochondria from patients and control fibroblast cells, as shown in figure 12, there is an equal import of pSu9-DHFR presequence observed in the patients and control fibroblast cells.

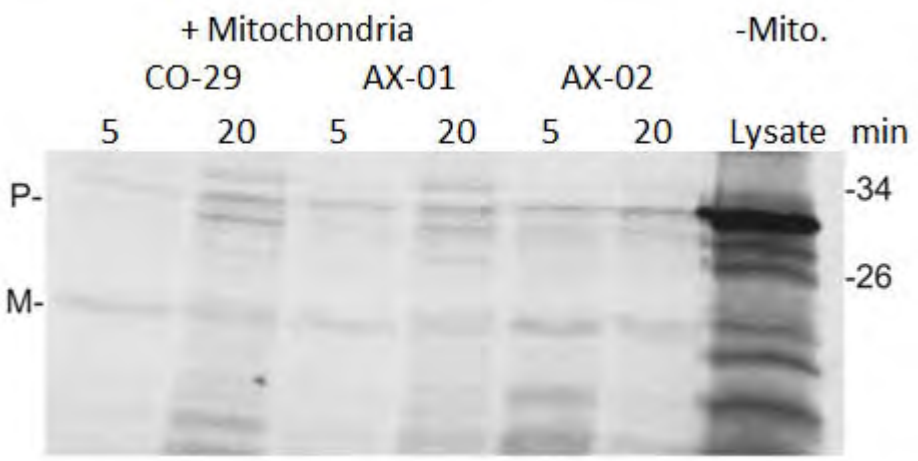

Figure 12: *Invitro import study: Isolated mitochondria from patient and control fibroblast were incubated at 25 C for 5 and 20 mintues with radiolabelled precursor of pSU9-DHFR. After the import the mitochondria were subjected to PK treatment and analyzed on the SDS-PAGE. The premature (P) and the matured band (M) show the import into the IMM.*

3.2 Bioenergetic characteristing of mitochondria in control and affected patients

The mutation observed in ER and mitochondria can affect the mitochondria function such as respiration and maintaing the membrane potiential. Primary fibroblast from control and

patients were used to monitor the respiration under normal growth condition and in growth media with glucose and $NaHCO_3$. For the assay the media used was with glucose, Na-pyruvate and $NaHCO_3$.Mitochondrial respiration was measured with help of different substrate like olgomycin, FCCP, rotenone and antimycin. Respiration assay was performed sequenqueiously injecting the substrate into the cell culture. The steps involved are ADP induced respiration, oligomycin copules the respiration, FCCP uncouples and induces the respiration, rotenone and antimycin are combined together and inhibits the respiration.

In figure 13, rate of oxygen compustion is measured before normalisation of the cells i.e, normalization by the total number of cells is before and after the assay. State A respiration was induced by ADP, state B was achieved by the oligomycin and FCCP, finial state C was achieved by antimycin and rotenone together inhibiting the complex 1 with increasing concentration of rotenone.

In figure 13, there is a increase of OCR in control cell (CO-29) compared to AX-01 and AX-02 (affected patient) before normalization. After normalization (figure 14), there is no significant difference observed in the patients compared to control cell line as seen in (figure 13) before normalization. These results indicate that the number of control cells are higher compare to the patient cell lines and it need to be normalized.

In figure 15, there is an observation in changes in oxygen tension (mm Hg of O_2) with respect to the time in individual wells. Larger amount of O_2 consumption is observed in control cell line after injection of FCCP compound compare to patient.

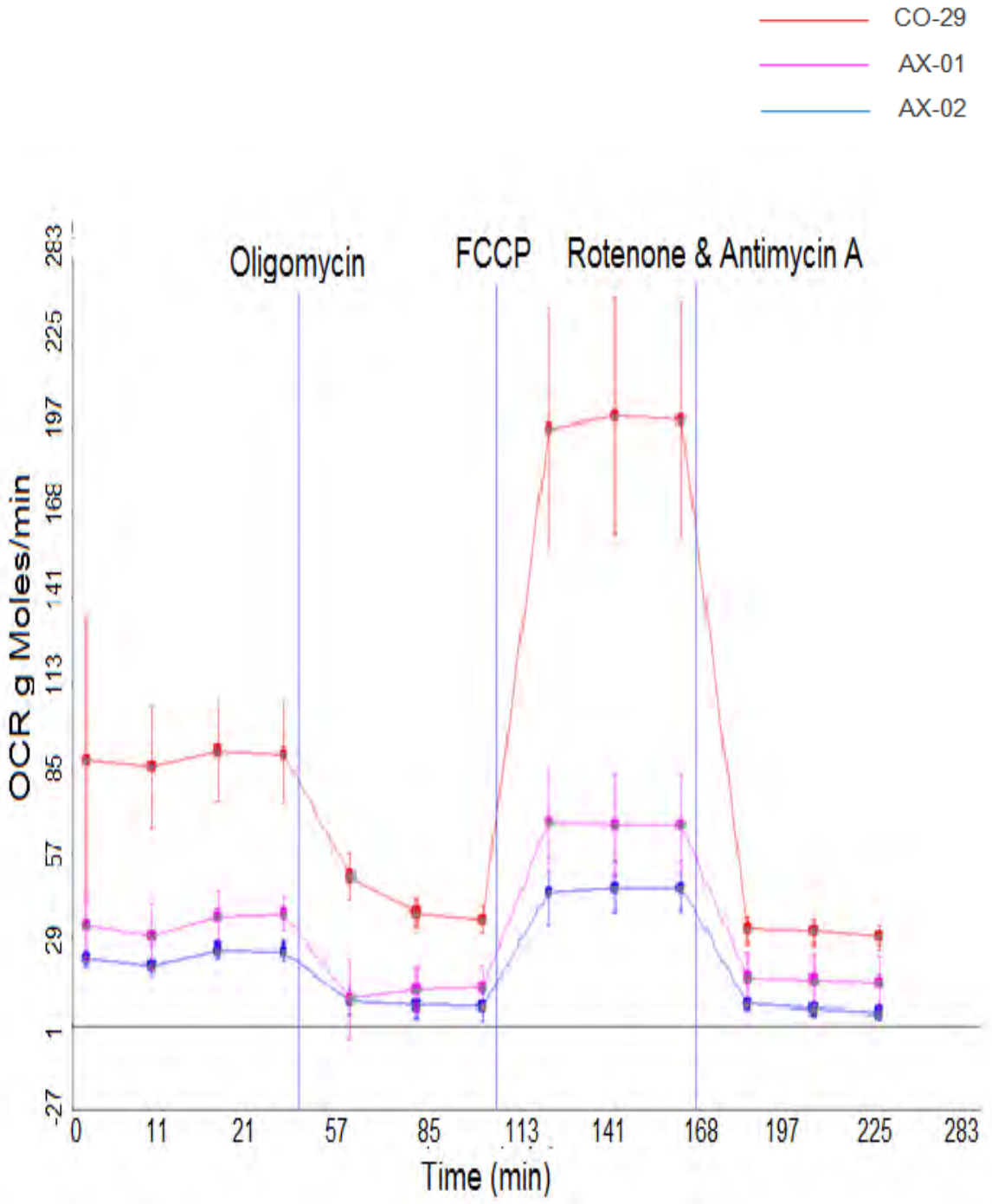

Figure 13: *Mitochondrial energetics: Fibroblast cells were cultured in the Sea Horse assay plate measuring the oxygen compustion in presence of oligomycin, FCCP, antimcin and rotenene. This graph shows the cellular respiration in CO-29 is in red, AX-01 is in purple and AX-02 in blue fibroblast cells which is before normalization of the cells.*

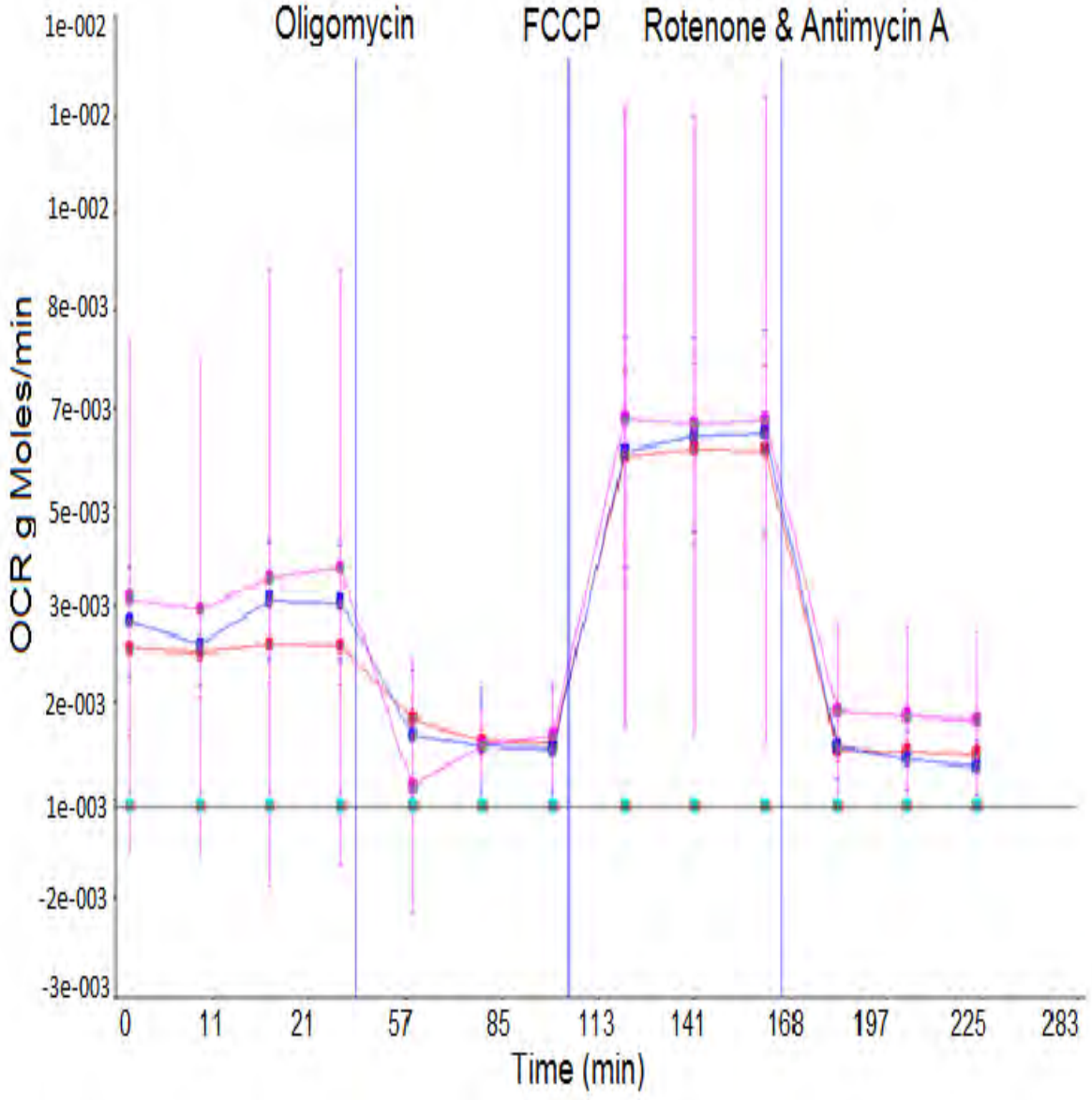

Figure 14: *Mitochondrial energetics: Fibroblast cells were cultured in the SeaHorse assay plate measuring the oxygen compustion in presence of oligomycin, FCCP, antimcin and rotenene. This graph shows the cellular respiration in CO-29, AX-01 and AX-02 fibroblast cells which is after normalization of the cells were is no significant difference between the control and patient fibroblast.*

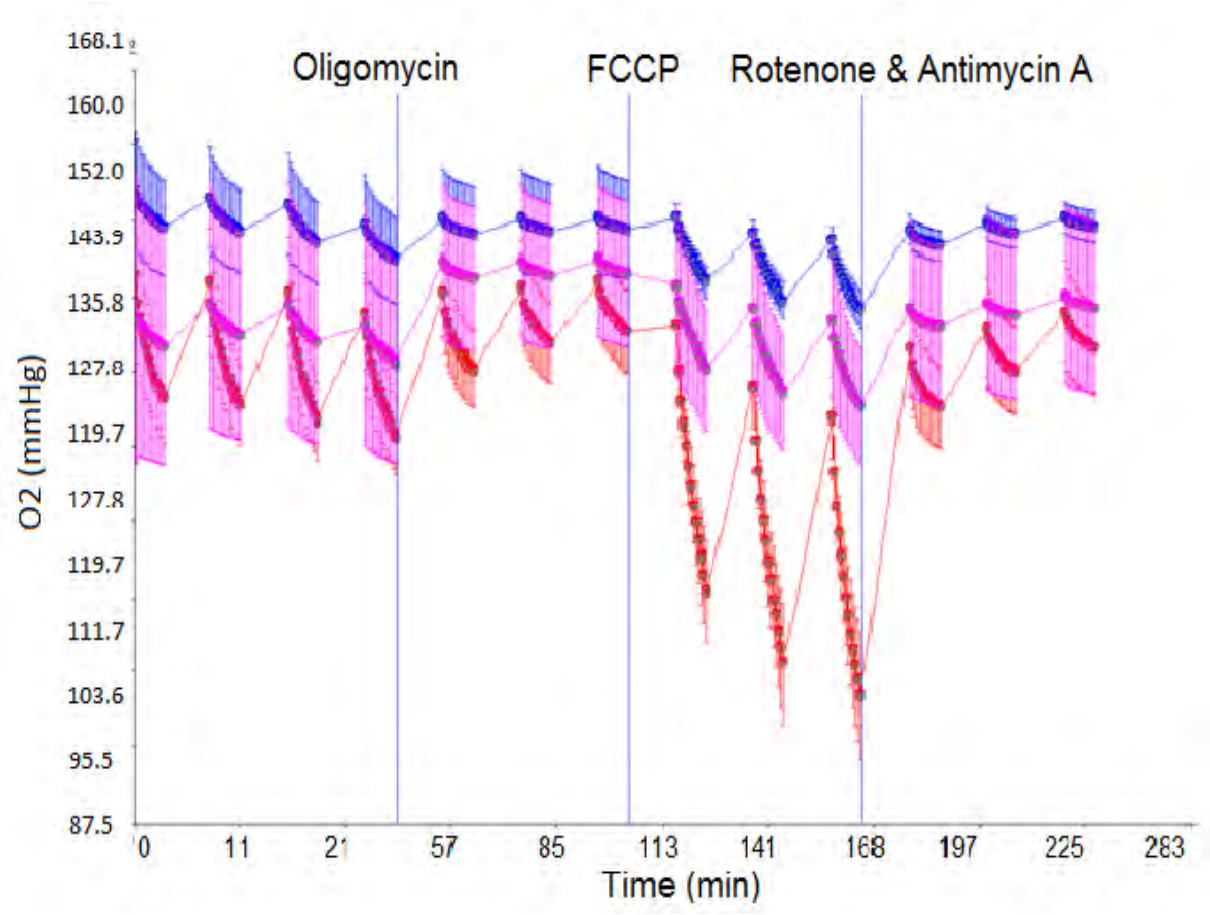

Figure 15: *Mitochondrial oxygen consumption: Rate of oxygen consumed during the respiration of mitochondria in different substrate (Color code for the graph is same as above).*

3.3 Estimating the levels of mitochondrial morphology

To understand in-depth we analyzed the mitochondrial morphology changes in control cells and patients cells. To understand the morphology, I performed live cell imaging of primary fibroblasts with control cells and patient's cells. To facilitate the visualization of the mitochondrial morphology morphometric analyses using 3D reconstruction of the mitochondrial network with help of Imaris software. In figure 16, all the cells look similar and there is no significant difference observed by just visualizing until further characterization is done by the Imaris software to calculate the aspect ratio, volume and shape of the cells.

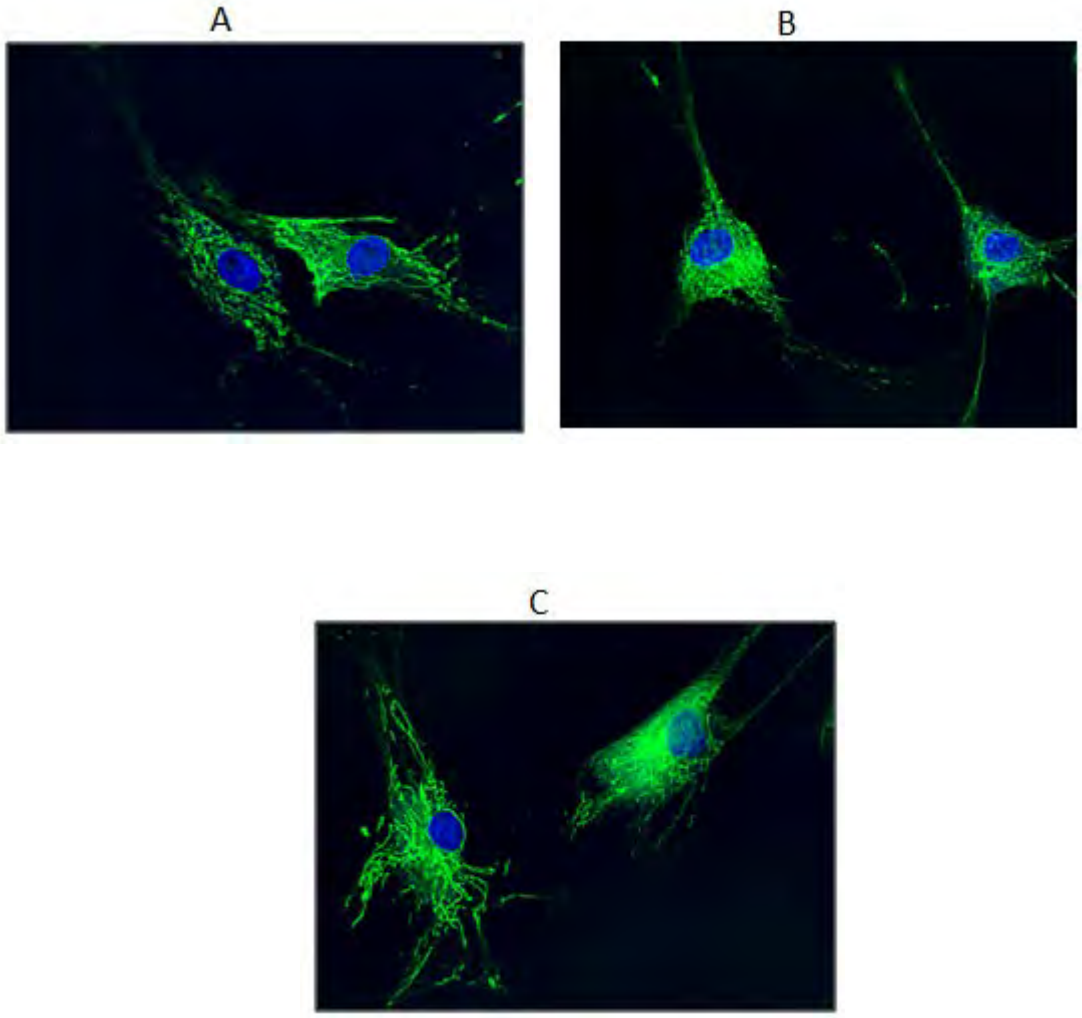

Figure 16: *Mitochondrial morphology: Live cell imaging was performed on primary fibroblasts cell which were subjected to MitoTracker Green for 3 minutes and observed under the live cell microscopy. Picture A, B & C are control fibroblast CO-29, Patient fibroblast AX-01 and AX-02 respectively.*

4 Discussion

ER and mitochondria together form a network, which maintains the cellular homeostasis in the cell. It plays a major role in resulting or determines the cellular fate under stress conditions [15].

To date, there are not many identified diseases which are related to ER stress and mitochondrial dysfunction. One of the examples of mitochondrial dysfunction occurring due to ER stress is type 2 diabetes mellitus were β- cell dysfunction and peripheral insulin resistance. These studies emphasize the relation between ER stress and mitochondrial dysfunction suggests ER stress plays a role on apoptosis signaling with means of Ca^{2+} and by ROS- dependent mechanism [31].

In our studies we are at the preliminary state of understanding of the interaction between ER stress and mitochondria dysfunction. The fibroblast derived from the family, patients are affected by the deletion of $P58^{IPK}$, which leads to autosomal recessive multisystemic disease with growth retardation, Insulin- dependent diabetes mellitus, hypoakusis, cerebellar and sensory ataxia. The clinical manifestation beginned during the childhood, there are three affected sibling, 1 unaffected sibling and parents are unaffected. In the fibroblast derived from the patients I am mainly interested in looking if there is any relation between ER related disorders to mitochondrial dysfunction. The phenotypes seen in the patients are closer towards mitochondrial dysfunction.

$P58^{IPK}$ is located in ER lumen, which interacts with J-domain of BiP in a dependent manner and influences the maturation of protein efficiently. Previous reflect the new functionality of $P58^{IPK}$ in acting as co-chaperone protein to maintain the folding of protein and in homeostasis in the ER [25]. In the absence of $P58^{IPK}$ in ER, there is a compromise of overall protein folding capacity in the ER lumen.P58-/- (Knockout) in the cell are affected by the greater levels of stress, by increasing the substrate burden on the ER and lowering the protein maturation capacity. In the longer term, the P58 -/- cells are accumulated and leading to misfolded proteins and aggregates [26]

Due to restricted assay and the availability of primary fibroblast from the affected patients and unaffected patients, I started to standardize the protocol on already existing HeLa cell. Once the protocol standardized in the HeLa cells; I switch to control fibroblast before working with

patient fibroblast. It is advantageous to use a model system for establishing a ferm protocol before using a real system.

HeLa cells were used to understand the growth pattern and standardizing the protocol for mitochondrial isolation and checking the functionality by *invitro* import of pSu9-DHFR presequence protein. There is difference observed in the growth pattern in HeLa cell compare to fibroblast. HeLa cells grow much faster and more confluent than fibroblast, so the standardization was first done in HeLa cell to estimate the amount of material required for the assay.

In the current study, I investigated the functionality of isolated mitochondria by invitro protein import experiments as well as steady state level using specific antibodies. To check the complete import in isolated mitochondria were treated to proteinase K.

The steady state level experiments revealed that in patients, there is no expression of $P58^{IPK}$ due to the deletion of this gene in the patients. In AX-01 there was a weaker expression of TOM40, MnSOD and α-tubulin seen compare to other patient AX-02. In AX-01 sample were loaded more than control and AX-02 samples to normalize the concentration of the protein equally in all wells, but despite the expression of the protein were weaker. In AX-01 the protein might be degrading during the extraction, so further quantification should be done to understand this pattern.

To understand the bioenergetics properties, control cell and patient's cell were subjected to Seahorse analyzer to check the respiration rate and membrane potential. In mitochondrial disorder more often there is a change in observed in bioenergetics properties. Seahorse analyzer is a major tool for our project to check if, the ER stress in these patients leads to mitochondrial disorder. The results from Seahorse in control and fibroblast cell showed no difference in the oxygen consumption in the normalized cells. Before normalization of the results to the total number of cells, there was a big difference observed in control fibroblast to patient fibroblast. In summary from Seahorse experiment, the cell number should be standardized again to reproduce the results.

Mitochondrial morphology is another important factor to analyze the characteristic of the regulating of the organelle. We examined the morphological implication by staining the cell with DAPI for nucleus and Mitotracker Green for mitochondria. From the current live imaging results there is no difference observed by just visualizing the images.

As this study is still in the preliminary stage, the protocol is standardized, and provides an initial hint that $P58^{IPK}$ gene is deleted in the patient. Further studies will help to understand the process in depth and to gain more knowledge about the correlation of between ER and mitochondria.

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6 References

1. Shore, G.C. and J.R. Tata, *Two fractions of rough endoplasmic reticulum from rat liver. I. Recovery of rapidly sedimenting endoplasmic reticulum in association with mitochondria.* J Cell Biol, 1977. **72**(3): p. 714-25.
2. Mannella, C.A., et al., *Electron microscopic tomography of rat-liver mitochondria and their interaction with the endoplasmic reticulum.* Biofactors, 1998. **8**(3-4): p. 225-8.
3. Chevet, E., et al., *The endoplasmic reticulum: integration of protein folding, quality control, signaling and degradation.* Curr Opin Struct Biol, 2001. **11**(1): p. 120-4.
4. Patel, S., S.K. Joseph, and A.P. Thomas, *Molecular properties of inositol 1,4,5-trisphosphate receptors.* Cell Calcium, 1999. **25**(3): p. 247-64.
5. Mikoshiba, K., *Inositol 1,4,5-trisphosphate IP(3) receptors and their role in neuronal cell function.* J Neurochem, 2006. **97**(6): p. 1627-33.
6. Patterson, R.L., D. Boehning, and S.H. Snyder, *Inositol 1,4,5-trisphosphate receptors as signal integrators.* Annu Rev Biochem, 2004. **73**: p. 437-65.
7. Joseph, S.K. and G. Hajnoczky, *IP3 receptors in cell survival and apoptosis: Ca2+ release and beyond.* Apoptosis, 2007. **12**(5): p. 951-68.
8. de Brito, O.M. and L. Scorrano, *An intimate liaison: spatial organization of the endoplasmic reticulum-mitochondria relationship.* EMBO J, 2010. **29**(16): p. 2715-23.
9. Schmidt, O., N. Pfanner, and C. Meisinger, *Mitochondrial protein import: from proteomics to functional mechanisms.* Nat Rev Mol Cell Biol, 2010. **11**(9): p. 655-67.
10. Giorgi, C., et al., *Structural and functional link between the mitochondrial network and the endoplasmic reticulum.* Int J Biochem Cell Biol, 2009. **41**(10): p. 1817-27.
11. Borgese, N., M. Francolini, and E. Snapp, *Endoplasmic reticulum architecture: structures in flux.* Curr Opin Cell Biol, 2006. **18**(4): p. 358-64.
12. Chan, D.C., *Mitochondria: dynamic organelles in disease, aging, and development.* Cell, 2006. **125**(7): p. 1241-52.
13. Anesti, V. and L. Scorrano, *The relationship between mitochondrial shape and function and the cytoskeleton.* Biochim Biophys Acta, 2006. **1757**(5-6): p. 692-9.
14. Brough, D., M.J. Schell, and R.F. Irvine, *Agonist-induced regulation of mitochondrial and endoplasmic reticulum motility.* Biochem J, 2005. **392**(Pt 2): p. 291-7.
15. Csordas, G., et al., *Structural and functional features and significance of the physical linkage between ER and mitochondria.* J Cell Biol, 2006. **174**(7): p. 915-21.
16. Mozdy, A.D. and J.M. Shaw, *A fuzzy mitochondrial fusion apparatus comes into focus.* Nat Rev Mol Cell Biol, 2003. **4**(6): p. 468-78.
17. Westermann, B., *Mitochondrial fusion and fission in cell life and death.* Nat Rev Mol Cell Biol, 2010. **11**(12): p. 872-84.
18. Deniaud, A., et al., *Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis.* Oncogene, 2008. **27**(3): p. 285-99.
19. Malhotra, J.D. and R.J. Kaufman, *Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword?* Antioxid Redox Signal, 2007. **9**(12): p. 2277-93.
20. Schroder, M., *Endoplasmic reticulum stress responses.* Cell Mol Life Sci, 2008. **65**(6): p. 862-94.
21. Rutkowski, D.T. and R.J. Kaufman, *A trip to the ER: coping with stress.* Trends Cell Biol, 2004. **14**(1): p. 20-8.
22. Szegezdi, E., et al., *Mediators of endoplasmic reticulum stress-induced apoptosis.* EMBO Rep, 2006. **7**(9): p. 880-5.

23. Zhang, K. and R.J. Kaufman, *The unfolded protein response: a stress signaling pathway critical for health and disease.* Neurology, 2006. **66**(2 Suppl 1): p. S102-9.
24. Rasheva, V.I. and P.M. Domingos, *Cellular responses to endoplasmic reticulum stress and apoptosis.* Apoptosis, 2009. **14**(8): p. 996-1007.
25. Rutkowski, D.T., et al., *The role of p58IPK in protecting the stressed endoplasmic reticulum.* Mol Biol Cell, 2007. **18**(9): p. 3681-91.
26. Oyadomari, S., et al., *Cotranslocational degradation protects the stressed endoplasmic reticulum from protein overload.* Cell, 2006. **126**(4): p. 727-39.
27. van Huizen, R., et al., *P58IPK, a novel endoplasmic reticulum stress-inducible protein and potential negative regulator of eIF2alpha signaling.* J Biol Chem, 2003. **278**(18): p. 15558-64.
28. Tabas, I. and D. Ron, *Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress.* Nat Cell Biol, 2011. **13**(3): p. 184-90.
29. Harman, D., *Prolongation of the normal life span by radiation protection chemicals.* J Gerontol, 1957. **12**(3): p. 257-63.
30. Raha, S. and B.H. Robinson, *Mitochondria, oxygen free radicals, disease and ageing.* Trends Biochem Sci, 2000. **25**(10): p. 502-8.
31. Leem, J. and E.H. Koh, *Interaction between mitochondria and the endoplasmic reticulum: implications for the pathogenesis of type 2 diabetes mellitus.* Exp Diabetes Res, 2012. **2012**: p. 242984.
32. Szabadkai, G. and M.R. Duchen, *Mitochondria: the hub of cellular Ca2+ signaling.* Physiology (Bethesda), 2008. **23**: p. 84-94.
33. Bonnet, C., et al., *Allotopic mRNA localization to the mitochondrial surface rescues respiratory chain defects in fibroblasts harboring mitochondrial DNA mutations affecting complex I or v subunits.* Rejuvenation Res, 2007. **10**(2): p. 127-44.
34. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.
35. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
36. Kyhse-Andersen, J., *Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose.* J Biochem Biophys Methods, 1984. **10**(3-4): p. 203-9.
37. Towbin, H., T. Staehelin, and J. Gordon, *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.* Proc Natl Acad Sci U S A, 1979. **76**(9): p. 4350-4.
38. Johnston, A.J., et al., *Insertion and assembly of human tom7 into the preprotein translocase complex of the outer mitochondrial membrane.* J Biol Chem, 2002. **277**(44): p. 42197-204.
39. Yan, W., et al., *Control of PERK eIF2alpha kinase activity by the endoplasmic reticulum stress-induced molecular chaperone P58IPK.* Proc Natl Acad Sci U S A, 2002. **99**(25): p. 15920-5.