

# **Role of SLMAP in Endoplasmic Reticulum Stress and Unfolded Protein Response**

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A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the M.Sc. Program in Cellular and Molecular Medicine

Cellular and Molecular Medicine

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## ABSTRACT

Cardiac function is regulated by the molecular components of the sarco/endoplasmic reticulum (ER/SR). Disruptions in homeostatic balance of these proteins and calcium regulation results in activation of ER stress response. Sarcolemmal membrane-associated proteins (SLMAPs) are found in cell membrane, SR/ER, and mitochondria. Overexpression of SLMAP in the myocardium has shown to impair excitation-contraction (E-C) coupling in the transgenic (Tg) mice. ER stress response was examined in Tg mice overexpressing SLMAP in the myocardium. In Tg hearts, changes observed in the expression of proteins involved in ER stress were dependent on the age and sex. SLMAP overexpression results in maladaptive ER stress response, as the mice age. Neonatal cardiomyocytes isolated from the Tg hearts showed decreased viability, upregulation of ER stress response proteins, which were sensitized to thapsigargin-induced stress, and desensitized to palmitate-induced oxidative stress. These findings suggest that normal SLMAP levels are important for proper cardiac function, and cell viability.

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## LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
Akt	Protein kinase B
ANF	Atrial natriuretic peptide
ATF	Activating transcription factor
ATP	Adenosine triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
BCA	Bicinchoninic acid
Bcl	B-cell lymphoma
Bim	Proapoptotic member of Bcl-2 protein family
BiP	Binding immunoglobulin protein
BrS	Brugada Syndrome
BSA	Bovine serum albumin
bZIP	Basic Leucine Zipper
CHOP	CCAAT/-enhancer-binding protein homologous protein
CSQ	Calsequestrin
C-tg	Cardiomyocytes from transgenic mouse
C-wt	Cardiomyocytes from wild-type mouse
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E-C	Excitation-contraction
ECM	Extracellular matrix

ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
eIF2	Translation initiation factor 2
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ERSR	Endoplasmic reticulum stress response elements
EtBr	Ethidium bromide
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FHA	Forkhead associated
F-tg	Fibroblasts from transgenic mouse
F-wt	Fibroblasts from wild-type mouse
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT-4	Glucose transporter-4
GRP	Glucose-regulated protein
GST	Glutathione S-transferase
HBSS	Hank's Buffer
hNav1.5	Human cardiac sodium channel
IP3R	Inositol trisphosphate receptor
IRE1	Inositol requiring transmembrane kinase/endonuclease 1
LC3	Light Chain 3
LV	Left ventricular
LZ	Leucine zipper
MAM	Mitochondria-associated endoplasmic reticulum membrane
MCU	Mitochondrial calcium uniporter

Met	Methionine
MHC	Myosin heavy chain
MOM	Mitochondrial outer membrane
MTOC	Microtubule organizing center
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NaCl	Sodium Chloride
NEAA	Non-essential amino acid
NMCM	Neonatal Mouse Cardiomyocytes
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
PERK	Pancreatic ER kinase
PKA	Protein kinase A
Plm	Palmitate
PPAR	Proliferator-activated receptor
PVDF	Polyvinylidene fluoride
RIPA	Radio-immunoprecipitation assay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RyR	Ryanodine receptor
S1P	Sphingosine-1-phosphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	Endoplasmic reticulum/sarcoplasmic reticulum calcium-ATPase

siRNA	Small interfering ribonucleic acid
SLMAP	Sarcolemmal membrane-associated protein
SR	Sarcoplasmic reticulum
Tg	Transgenic
Thg	Thapsigargin
TM	Transmembrane
tRNA	Transfer ribonucleic acid
UPR	Unfolded protein response
VDAC	Voltage-dependent anion channel
Wt	Wild-type
XBP	X-box binding protein

## **ACKNOWLEDGMENTS**

I am grateful to my supervisor, Dr. Balwant Tuana, for his support and encouragement throughout my studies. It was an honor to be a member of his lab. I would also like to thank my advisory committee members, Dr. Simon Lemaire, Dr. Erik Suuronen, and Dr. Patrick Burgon, for their helpful advice. I would like to express my gratitude to all current lab members for their help and guidance. A very special compliment goes to our laboratory manager, Maysoon Salih, whose help and advice was imperative. I would also like to thank Omar Hawari for his mentorship and friendship during my studies. Finally, I would like to thank my parents. Their constant support and encouragement motivated me during my graduate studies.

## **CHAPTER 1: INTRODUCTION**

### **1.1 Regulation of cardiac function**

The heart is a four-chambered organ whose continuous cycles of excitation-contraction (E-C) coupling pump blood through the circulatory system. Cardiac function depends on the proper expression, assembly, regulation of the molecular components, and the right balance of the ions involved in E-C coupling. Functional characterization of cardiac genes and proteins has improved our knowledge of cardiac function in normal and diseased states (Kamp & Hell, 2000). Myocardial contractility in the heart is regulated by changes in the interactions between the contractile proteins. The functional changes are initiated by membrane regulation of calcium transport across the cell membrane and endoplasmic reticulum/sarcoplasmic reticulum (ER/SR), which determines the efficiency of myocardium contraction-relaxation (Arai, 2000; Zima, Pabbidi, Lipsius, & Blatter, 2013).

### **1.2 Membrane biology and ER function**

The ER is a multifunctional organelle that supports many important processes required by the cells (Anelli & Sitia, 2008). Optimal function of ER is essential for all cellular activities like protein synthesis, transcriptional regulation, calcium regulation, energy metabolism, lipid synthesis, and steroid metabolism (Groenendyk, Sreenivasaiah, Kim, Agellon, & Michalak, 2010). ER is the site for most secreted and membrane protein synthesis, which makes the ER a major integration site of cell growth and signaling. ER membrane network is more extensive in cardiomyocytes due to role played by the SR in

calcium homeostasis, and muscle contraction-relaxation (Belmont et al., 2008; Doroudgar & Glembotski, 2011; Thuerauf, Marcinko, Belmont, & Glembotski, 2007). Overlap of function between the SR and the ER in protein synthesis and folding suggests that they play a critical role in cardiac physiology and pathology.

Proteins synthesized in the ER are delivered to the Golgi where they are modified, packed, and directed to their final destinations (Doroudgar & Glembotski, 2011). The ER in cardiac myocytes is in the peri-nuclear network that is contiguous with the nuclear envelop (Kaisto, 2003), and calcium can diffuse freely between SR and the peri-nuclear ER (Wu, 2006). The proteins involved in ER protein synthesis, folding, and quality control are found in perinuclear regions, and peripheral areas of the cardiac myocytes (McFarland, Milstein, & Cala, 2010).

The SR is a unique form of ER in the muscle cells, which regulates calcium homeostasis and myofilament shortening. In myocytes, calcium ions are stored inside the SR, which are used for contraction and other physiological activities (Kaisto, 2003). Precise regulation of calcium ions in muscle and cardiac myocytes is important for the maintenance of voluntary contractile activity of skeletal muscle, and for the rhythmic contraction of the heart (Zhao et al., 2011). The complex three-dimensional organization of internal membranes in myocytes, and the localization of proteins at specific sites of the SR play a crucial role in calcium release from the SR, following the depolarization of the plasma membrane (Rios, Ma, & Gonzalez, 1991).

The SR surrounds the myofilaments and associates with deep invaginations of the sarcolemma called transverse (t)-tubules to regulate myocyte contraction by releasing

calcium from the SR lumen into the cytoplasm (Sobie & Lederer, 2012). The increase in cytoplasmic calcium results in E-C coupling, which depolarizes sarcolemma and causes brief increase in intracellular calcium via opening of the ryanodine receptor (RyR) calcium release channel on the SR. Following contraction, the calcium ions are sequestered back in the SR by ER/SR calcium-ATPase 2a (SERCA2a). For optimum function of myocytes, low calcium level in the cytosol and sufficient calcium storage inside the SR is required (Zhao et al., 2011). Storage of calcium in the SR lumen is facilitated by calcium binding proteins, such as calsequestrin (CSQ) (Lanner, Georgiou, Joshi, & Hamilton, 2010).

The ER is not an isolated organelle because it has developed mechanisms of communication with many other cellular compartments, especially mitochondria, the plasma membrane, and the nucleus (Groenendyk et al., 2010). An elaborate signaling process involving calcium transfer between the ER and mitochondria is assisted by physical association of the two organelles (Garcia-Perez, Schneider, Hajnoczky, & Csordas, 2011). This structure is known as the mitochondria-associated ER membrane (MAM), which regulates ER-mediated energy metabolism (Raturi & Simmen, 2013). Calcium is released from the ER through RyR and inositol trisphosphate receptor (IP3R) into the ER/mitochondrial space (Marks, 1997). The calcium passes through voltage-dependent ion selective channel (VDAC) in the outer mitochondrial membrane, and then in the inner mitochondrial membrane through the mitochondrial calcium uniporter (MCU) (Stefani et al., 2011). Under certain conditions, e.g. ER stress, calcium release across MAM is increased, which enhances adenosine triphosphate (ATP) production (Garcia-Perez et al., 2011).

Proper folding of proteins in the ER is essential to carry out cellular functions, and for maintaining homeostasis. The synthesis, modification, folding, and processing of nascent polypeptide chains of all proteins occur in the rough ER (Kitakaze & Tsukamoto, 2010). In the process of protein folding, the linear polymer of amino acids must acquire the native conformation to perform its precise cellular functions. When proteins are folding in their unique three-dimensional structure, they interact with molecular chaperones that bind to the hydrophobic regions of incompletely folded proteins to facilitate proper folding (Araki & Nagata, 2012). The transmembrane proteins in the ER recognize a specific signal sequence in all the proteins destined for secretion. These polypeptides are translocated across the ER membrane and become part of the membrane, or are released into the ER lumen (Kitakaze & Tsukamoto, 2010).

The cells are often exposed to various stressors such as hypoxia, heat, osmotic imbalance, and oxidation (Wetzel, 1994). These changes can lead to aberrant structures in the damaged proteins, and consequently affect protein function. The chaperones monitor the presence of misfolded proteins in the ER, because accumulation of misfolded proteins can trigger a heat-shock response, which stimulates the transcription of genes in order to maintain homeostasis (Kaufman, 1999). This signaling cascade results in an increase of the protein folding capacity, refolding of the aberrant proteins, or marking them for degradation (Wang & Kaufman, 2012). Similarly, perturbation of ionic concentration, especially calcium, contributes to various pathologies (Lautenschlaeger, Prell, & Grosskreutz, 2012). This understanding of ER function and protein folding has led to evolution of many therapeutic treatments that counter various pathologies.

Altered post-translational modifications or mutations of SR proteins involved in calcium homeostasis are linked to several diseases, affecting skeletal and cardiac muscle tissues (Sorrentino, 2011). In Brody's disease for example, mutations in the SERCA1a results in delay in calcium reuptake from the cytosol and delayed relaxation, with skeletal muscle cramping (Brini & Carafoli, 2009). Mutations in RyR2 and CSQ2 genes are linked with severe stress-induced ventricular tachycardia (Csordás et al., 2010; Wehrens, 2007). Cardiac arrest and sudden death is observed in the patients due to aberrant calcium release from the SR during diastole, which triggers ventricular arrhythmia (Wehrens, 2007). The activity of several SR proteins like SERCA2a and RyR2s are regulated by  $\beta$ -adrenergic stimulation. Phosphorylation of RyR2 by protein kinase A (PKA) contributes to deterioration of the cardiac performance in the failing heart (Wehrens et al., 2003). Likewise, modifications in proteins involved in the organization of the SR can also cause muscular diseases. The understanding of molecular basis of SR structure and function has led to the search of novel therapies.

### **1.3 Regulation of ER stress**

ER is the major site of synthesis, modification, and delivery of proteins to their destinations within the cell or exocytosis. The folding of the proteins takes place in the lumen of this organelle after the protein enters the secretory pathway by translocation in the ER (Rutkowski & Kaufman, 2004). The proteins fold into their native conformation as dictated by the primary and secondary structure of the polypeptide, and usually undergoes post-translational modification (Schröder & Kaufman, 2005). It is very

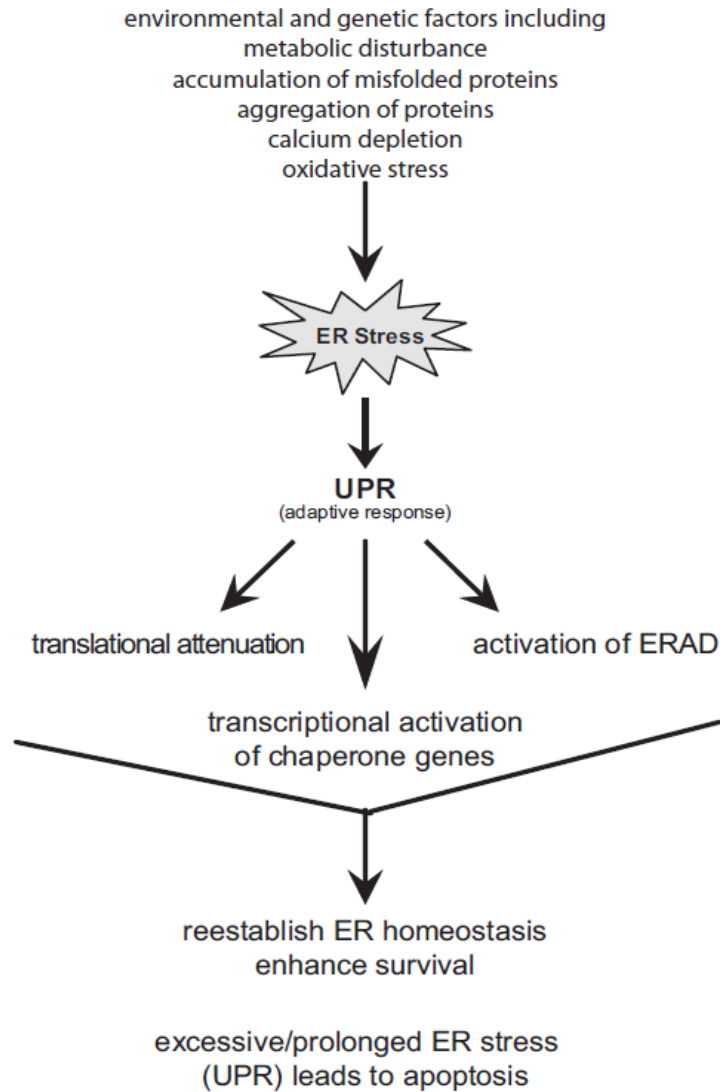
important that nascent proteins are properly folded and localize to their compartments in the cell.

The ER also functions to maintain the quality-control machinery so that only correctly folded proteins are exported to the Golgi complex. This role is played by the chaperones in the ER that help proteins to fold properly. Chaperones interact with partially folded proteins and selectively add carbohydrate moieties, which serves as a signal for protein folding (Bernales, Bernales, Papa, Walter, & Papa, 2006). The ER houses resident molecules that sense proteins misfolding, and accumulates them for degradation (Schröder & Kaufman, 2005). Properly folded proteins are very important for the cell because proteins relay important signals. Any changes in the ER disrupts homeostasis and affects metabolism, apoptosis, cell signaling, and gene expression (Rutkowski & Kaufman, 2004).

ER stress occurs when the cell faces situations that cause the protein folding demand to overwhelm the ER folding capacity (Szegezdi et al., 2006). The folding of secreted and membrane proteins can be impaired during various pathologies like heart failure (Okada, 2004), ischemia (Kuznetsov et al., 1996), diabetes (Oyadomari et al., 2001), hypertrophy (Brostrom, Mourad, & Brostrom, 2001), hypoxia (Price, Mannheim-Rodman, & Calderwood, 1992), and nutrient starvation (Doroudgar & Glembotski, 2013). The ER stress response can be activated by conditions that alter the ER environment in ways that impair nascent ER protein glycosylation, disulfide bond formation, or calcium levels (Doroudgar & Glembotski, 2013). Calcium buffering proteins in the ER strictly regulate calcium concentration in the ER lumen. When there is

imbalance in calcium concentration, chaperones detect this change and trigger ER stress response (Groenendyk et al., 2010).

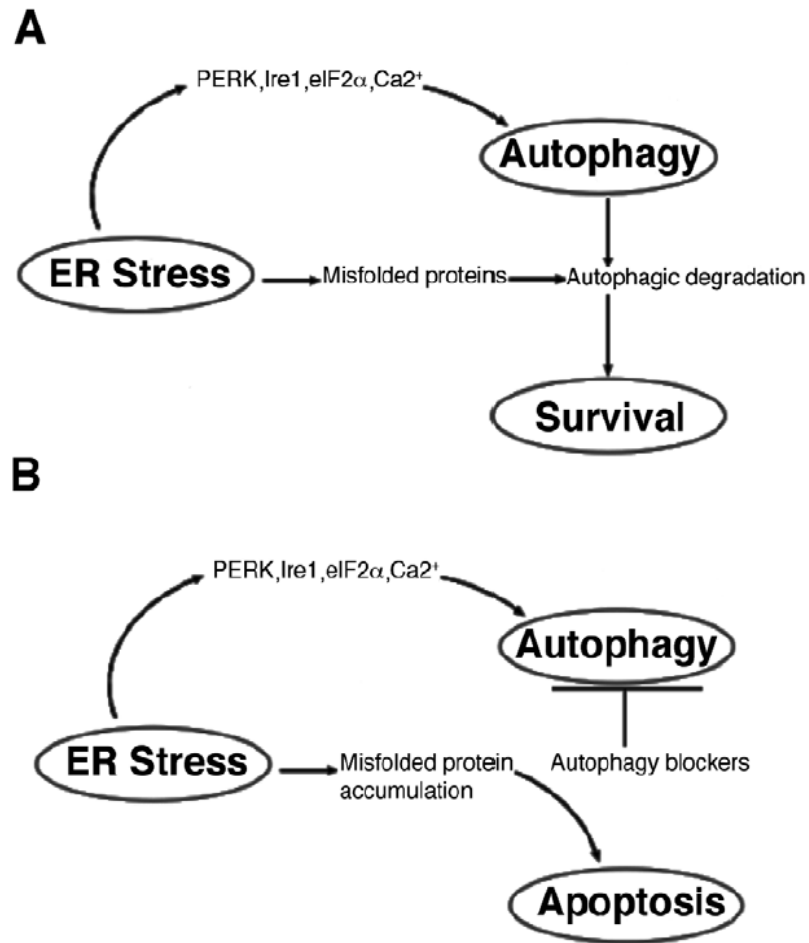
To counter ER stress, an intracellular signal transduction pathway termed the unfolded protein response (UPR) has evolved (Blond-Elguindi et al., 1993). The UPR triggers an adaptive response to reestablish the ER homeostasis by upregulating genes that encode for chaperones, prevent polypeptide aggregation, and aid polypeptide folding. The UPR also reduces the rate of protein synthesis and augments the ER-associated degradation (ERAD) system, leading to proteasome-mediated degradation of misfolded ER proteins to relieve ER stress (Bertolotti et al., 2000).



**Figure 1:** *ER stress activates UPR.* ER stress signaling leads to protein translational attenuation, activation of chaperones, and activation of ERAD to eliminate the accumulation of misfolded protein. Figure is taken from Groenendyk et al. (2010).

Initially, stress response aims to suppress protein synthesis, upregulates ER resident chaperones to resolve the stress, and restores homeostasis to enhance survival (Groenendyk et al., 2010). If the ER stress is prolonged due to limited ability to fold or degrade misfolded proteins, the UPR will eventually signal pathways leading to apoptosis (Wang & Kaufman, 2012). The cell eliminates the risk of producing and displaying malfunctioning proteins on its surface, and is eliminated from the organism (Bernales et al., 2006). This has been observed in various pathologies, especially hypoxia and ischemia/reperfusion injury (Groenendyk et al., 2010).

ER stress can be pro-survival or death-oriented, which depends on the strength and duration (Groenendyk et al., 2010). Certain conditions during ER stress response also promote autophagy (Bernales, Schuck & Walter, 2007), which is an energy-conserving catabolic process that promotes cell survival (Gustafsson & Gottlieb, 2008). This process benefits the cell by recycling proteins and fatty acids needed to maintain energy production and restore normal cell function. Severity of autophagy during ER stress can be detrimental, possibly through excessive self-digestion (Gustafsson & Gottlieb, 2008).



**Figure 2:** Regulation of ER stress pathway. **A)** ER stress induces autophagy pathway through PERK, IRE1, eIF2 $\alpha$  and increased calcium, which helps to degrade unfolded protein and alleviate ER stress, thus promoting survival. **B)** Blockage of autophagy results in ER stress leading to apoptosis. Figure is taken from Weston & Puthalakath (2010).

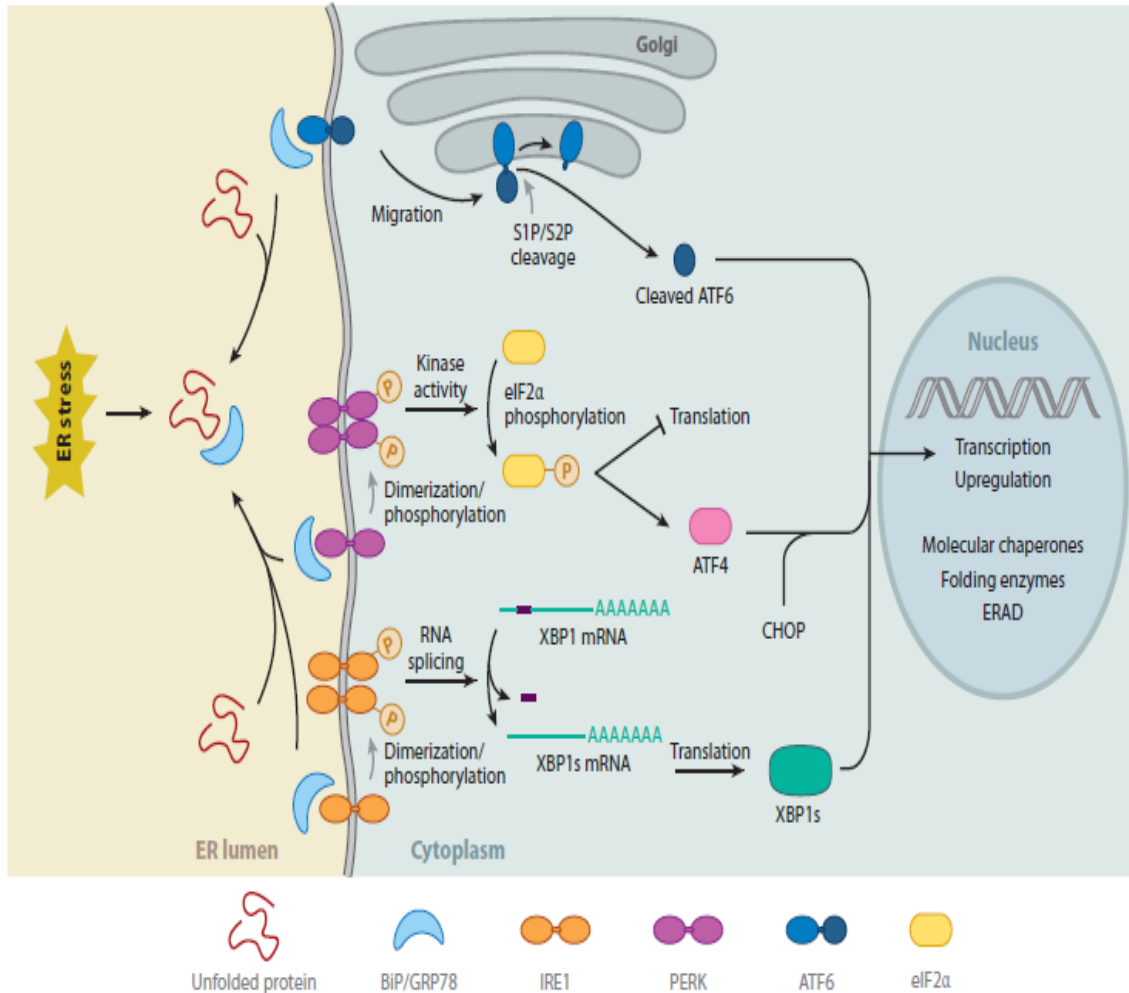
#### **1.4 The three main branches of ER stress**

The ER protein folding machinery is very important for cell survival. Chaperones and protein folding genes are activated during stress response, and they are conserved across many organisms (Kitakaze & Tsukamoto, 2010). The nascent proteins associate with the chaperones including binding immunoglobulin protein (BiP), calreticulin, calnexin, glucose-regulated protein 94 (GRP94), and protein disulphide isomerase (PDI) (Groenendyk et al., 2010).

Misfolded proteins activate a complex 3-branched UPR signaling pathway involving pancreatic ER kinase (PERK), inositol requiring transmembrane kinase/endonuclease 1 (IRE1), and activating transcription factor 6 (ATF6) (Kitakaze & Tsukamoto, 2010). These UPR sensors have N-termini in the lumen of the ER and C-termini in the cytosol. The luminal domains of these three sensors bind to the ER chaperone BiP (also called GRP78) under non-stressed conditions (Bertolotti et al., 2000).

Upon ER stress, BiP is released from these sensors and relocates misfolded proteins to either facilitate their folding or lead them to proteasome-mediated ERAD (Doroudgar & Glembotski, 2011). The disassociation of BiP from PERK and IRE1 allows their oligomerization, which then facilitate the activation of transcription factors ATF4, X-box binding protein (XBP1), and ATF6. The transcription factors activate ER stress response elements (ERSR) that encode for chaperones, calcium-binding proteins, and disulphide isomerases (Minamino, Komuro, & Kitakaze, 2010; Doroudgar & Glembotski, 2013). The main UPR transcription factors XBP1 and ATF6 protects the

heart from ischemic/reperfusion injury by regulating prosurvival pathways. On the other hand, activation of the PERK/ATF4 branch of the UPR triggers the signals that may lead to apoptosis (Groenendyk et al., 2010).



**Figure 3:** *ER stress response signaling.* BiP associates with the luminal domains of the 3 proximal effectors of ER stress; PERK, IRE1, and ATF6. During stress conditions, BiP is released and binds to misfolded proteins to facilitate their folding or degradation. The disassociation of BiP from PERK, IRE1 allows their oligomerization, which promotes trans-phosphorylation and activation of these effectors. Figure is taken from Groenendyk, Agellon, & Michalak (2013).

#### **1.4.1 BiP - Molecular chaperone with protective role in UPR**

BiP is a 78 kDa protein that was originally identified as immunoglobulin heavy chain binding protein (Haas & Wabl, 1983) and glucose-regulated protein (Pouysseburg, Shiu, & Pastan, 1977). It is also a member of HSP (heat shock proteins) family of molecular chaperones that helps in protein translocation and folding (Bernales et al., 2006). BiP is a monomeric, globular protein with an ATP binding domain and a peptide binding domain (Groenendyk et al., 2010). BiP plays an important role as a calcium buffer in the lumen of the ER (Lièvreumont et al., 1997), because its association with nascent polypeptides is stabilized by high calcium concentration (Suzuki et al., 1991). It is also involved in calcium transport from the ER to the mitochondria through MAM (Groenendyk et al., 2010). BiP recognizes nascent polypeptides with exposed hydrophobic regions and inhibits intramolecular or intermolecular aggregation, thus maintaining them in a state to prevent misfolding (Gething, 1999; Groenendyk et al., 2010). Studies have shown that UPR is not directly induced by accumulation of unfolded proteins; rather it is the decrease in the concentration of free BiP that occurs when BiP binds to unfolded proteins (Kohno et al., 1993; Gething, 1999).

In a study, when a high-salt diet is administered to Dahl salt-sensitive rats (Campese, 1994), BiP was significantly upregulated along with observed hypertension, cardiac hypertrophy, and subsequent heart failure (Isodono et al., 2010). Conditions like hypoxia and ischemia leads to glucose starvation, which regulate BiP levels in the cell (Kerbey, Vary, & Randle, 1985). Furthermore, upregulation of BiP is also observed during transaortic constriction, suggesting a protective role (Okada, 2004).

#### **1.4.2 PERK - Kinase that couples ER stress signals to translation inhibition**

PERK is an important ER stress sensor and a serine threonine kinase (Minamino et al., 2010). BiP interacts with ATPase binding domain of PERK in unstressed conditions (Gething, 1999). Upon release from BiP, PERK oligomerizes and causes phosphorylation of the  $\alpha$ -subunit of the translation initiation factor 2 (eIF2) (Bertolotti et al., 2000). The activated eIF2 $\alpha$  inhibits protein synthesis by binding the transfer ribonucleic acid-methionine (tRNA-met) responsible for initiating protein translation (Groenendyk et al., 2010), and inhibition of 80S ribosome assembly (Rutkowski & Kaufman, 2004). Activation of eIF2 also activates ATF4, which activates gene expression of certain chaperones, antioxidants, and CCAAT/-enhancer-binding protein homologous protein (CHOP) (Minamino et al., 2010). These pathways help to decrease the load of proteins translocated into the ER, and allowing the cell to focus to alleviate stress rather than on growth and division (Rutkowski & Kaufman, 2004).

#### **1.4.3 IRE1 - Proximal sensor for UPR that transmits signals across the ER membrane**

IRE1 is an ER stress sensor and transmembrane protein with a C-terminal endoribonuclease domain (Groenendyk et al., 2010). It is conserved from yeasts to humans (Minamino et al., 2010). Upon detection of ER stress, BiP is released from the ATPase domain of IRE1. The activation of IRE1 is caused by its dimerization and autophosphorylation, which exhibits endoribonuclease activity that catalyzes the removal of a small 26-nucleotide intron from the messenger ribonucleic acid (mRNA) of the gene

that encodes for XBP1 (Rutkowski & Kaufman, 2004). The splicing creates a translational frameshift in XBP1 mRNA to produce an active transcription factor. The active XBP1 binds to the promoters of numerous ERSR whose products help to fold or degrade proteins (Minamino et al., 2010). The endoribonuclease activity of IRE1 also cleaves 28S ribosomal ribonucleic acid (rRNA) and inhibits protein synthesis (Groenendyk et al., 2010).

#### **1.4.4 ATF6 - Transcription factor that activates ERSR**

ATF6 is associated with BiP in the ER under non-stressed conditions. The ATF6 transits to the Golgi complex when there is accumulation of misfolded proteins in the ER lumen (Groenendyk et al., 2010). In the Golgi complex, it is cleaved by sphingosine-1-phosphate (S1P) and S2P, yielding a free cytoplasmic domain that is an active transcription factor. This transcription factor is a soluble basic leucine zipper (bZIP) that binds to ERSR (Yoshida et al., 1998).

There are 2 isoforms of ATF6 (ATF6 $\alpha$  and ATF6 $\beta$ ) (Thuerauf, 2004), with ATF6 $\alpha$  being a stronger transcriptional factor than ATF6 $\beta$  (Thuerauf et al., 2007). The strength and duration of ATF6 branch of UPR is determined by relative abundance of these two isoforms. ATF6 $\beta$  represses transcriptional activity of ATF6 $\alpha$ , resulting in downregulation of BiP (Thuerauf et al., 2007). In transgenic mice with dominant-negative mutant of ATF6 $\alpha$ , increase in apoptosis was observed (Toko et al., 2010). This finding showed that ATF6 $\alpha$  has a role in maintaining cellular function under physiological conditions (Groenendyk et al., 2010).

A microarray study showed that in the mouse myocardium, ATF6 induces several genes encoding chaperones, ERSR, BiP, PDI, and calcium binding proteins, thus suggesting cardioprotective role of ATF6 *in vivo*. Activation of ATF6 in the heart downregulates glucose and fatty-acid metabolism genes (Thuerauf et al., 2007; Doroudgar & Glembotski, 2011). Besides upregulating many protective genes, ATF6 also downregulates several potentially damaging genes (Belmont et al., 2008). Activation of ATF6 in cardiomyocytes protects the heart from ischemic damage, and helps to conserve energy and limit growth during stress response (Doroudgar & Glembotski, 2013).

In a study done by Yoshida et al. (1998), ATF6 was converted from a 90 kDa protein to a 50 kDa protein upon treatment with ER stress-inducing reagents. Upon overexpression of ATF6 in HeLa cells, increase in 50 kDa protein also paralleled upregulation of BiP. The study suggested that 50 kDa isoform of ATF6 represents an active form of the protein that is transformed by a post-translational modification.

#### **1.4.5 CHOP - Mediates programmed cell death**

CHOP is a proapoptotic basic-leucine zipper transcription factor. Upregulation of the CHOP transcription factor is downstream of the ATF6 and PERK pathways (Rutkowski & Kaufman, 2004). CHOP regulates the apoptosis pathways by regulating the balance of proapoptotic and antiapoptotic B-cell lymphoma (Bcl)-2 family proteins. CHOP represses the expression of anti-apoptotic proteins Bcl-2 (Fu et al., 2010). Deletion of CHOP gene makes cell resistant to apoptosis induced by ER stressors

(Minamino et al., 2010). Downregulation of Bcl-2 expression by CHOP promotes mitochondrial cytochrome-c release, and activation of caspases that leads to cell death (Bernales et al., 2006).

Under ER stress, CHOP induces transcription of Bim, which is a proapoptotic member of Bcl-2 protein family (Minamino et al., 2010). It has been shown that in CHOP knockout mice, cardiac hypertrophy and dysfunction were reduced as compared to wild type mice (Minamino et al., 2010). The increased expression of CHOP is also linked to transition from cardiac hypertrophy to heart failure (Kitakaze & Tsukamoto, 2010).

#### **1.4.6 PDI - Chaperone that catalyze disulfide bond in newly synthesized proteins in ER lumen**

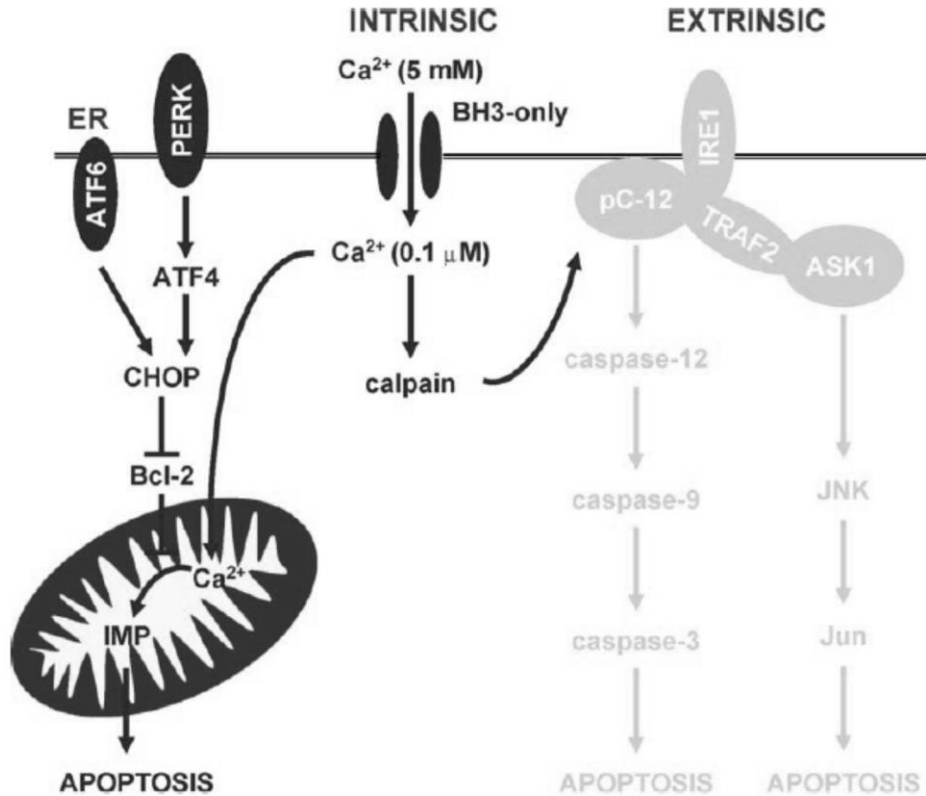
Post-translational modifications of newly synthesized proteins require the formation of intra- or inter-molecular disulphide bonds. This process is called oxidative protein folding, and PDI catalyzes the formation and isomerization of these disulfide bonds. However, hydrogen peroxide is generated as a byproduct of disulphide bond formation, which can cause oxidative stress (Turano, Coppari, Altieri, & Ferraro, 2002).

The oxidative state of heart directly influences PDI, because during hypoxic conditions, disulfide bonds are not formed properly leading to accumulation of misfolded proteins, thus executing ER stress response (Groenendyk et al., 2010). In human heart samples, PDI promotes survival after ischemic damage, and is upregulated 3-fold in the viable peri-infarct myocardial region (Severino et al., 2007). In a study, PDI gene transfer

to the mouse heart using adenovirus resulted in a smaller infarct size and reduced cardiomyocyte apoptosis in the peri-infarct region (Minamino et al., 2010).

#### **1.4.7 Caspase-3 - A critical executioner of apoptosis**

The activation of members of the caspase family of cysteine proteases has a central role in apoptosis. Caspases coordinate the demolition of the cell using proteolysis of several intracellular proteins (Creagh & Martin, 2003). They are present in cells as proenzymes but become activated during apoptosis. Caspase-3 is a critical executioner of apoptosis, and it is responsible for the proteolytic cleavage of many proteins. Activation of caspase-3 requires proteolytic cleavage of the proenzyme into activated p17 and p12 fragments (Fernandes-Alnemri, Litwack, & Alnemri, 1994). It is directly involved in activation of caspase-2 and caspase-6, which are required for execution of apoptosis (Creagh & Martin, 2003).



**Figure 4:** Regulation of apoptosis pathways by UPR. The intrinsic pathway of apoptosis responds to intracellular signals, and extrinsic pathway responds to extracellular stimuli. The extrinsic pathway is triggered by recruitment of caspases and initiation of a caspase cascade. The intrinsic pathway is controlled by a balance between proapoptotic proteins (e.g. Bcl-2-associated death promoter (Bad), Bcl-2 homologous antagonist killer (Bak), and Bcl-2-associated X protein (Bax)), and anti-apoptotic proteins (e.g. Bcl-2 proteins). Figure is taken from Schröder & Kaufman (2005).

#### **1.4.8 Akt - Promotes cell survival by inhibiting apoptosis**

Akt (protein kinase B) is protein kinase that regulates signaling pathways related to cell proliferation and survival. Overexpression of Akt in the heart has shown to reduce cardiac contractile dysfunction which is caused by ER stress (Dong et al., 2013). Akt promotes cell survival by inhibiting apoptosis via phosphorylation of several targets proteins that promote apoptosis (Cardone, 2013). Deregulation of Akt signaling under stress condition is linked to diseases like cancer and diabetes (Hotamisligil, 2010; Xu, 2005). ER stress causing drug thapsigargin also reduces phosphorylation activity of Akt (Chen et al., 2011).

#### **1.4.9 PARP - Involved in DNA repair in response to environmental stress**

Poly adenosine diphosphate (ADP) ribose polymerase (PARP) is a 116-kDa protein with an N-terminal DNA-binding domain (Luo & Kraus, 2012). PARP plays an important role in deoxyribonucleic acid (DNA) repair under stress conditions. It also has the ability to bind to intact and nicked DNA, resulting in activation of its enzymatic activity. PARP promotes cell viability, and its cleavage is an indicator of cellular disassembly, which serves as important marker for apoptotic cell death (Oliver, 1998).

#### **1.5 Role of mitochondria in ER stress response**

The role of mitochondria in cell metabolism and survival is controlled by calcium signals that are transmitted by the ER (Csordas et al., 2006). This dynamic physical and

functional association is commonly known as MAM (Hayashi, Rizzuto, Hajnoczky, & Su, 2009; Doroudgar & Glembotski, 2013). The flux of calcium into mitochondria is mediated by the voltage-dependent anion channel (VDAC) and the calcium uniporter, as a result of high cytoplasmic calcium concentration around MAM (Rizzuto, 2013). ER stress can increase the tethering of ER and mitochondria. These changes in the ER environment could affect metabolic function by transmitting signals to mitochondria using calcium transport (Csordas et al., 2006). Under ischemic conditions, decreased mitochondrial ATP production impairs ER protein folding by depleting calcium in the ER (Thuerauf, 2006). Decreased ER calcium also activates proximal sensors of ER stress, because ER chaperones require calcium to fold nascent proteins (Simmen, Lynes, Gesson, & Thomas, 2010). Another study reported that during simulated ischemia on endothelial cells, caspase-mediated apoptosis was induced due to calcium leak from the ER (Kumar et al., 2007).

## **1.6 Role of autophagy in ER stress response**

Autophagy is highly conserved cellular process that is used by the cell during both non-stress and stress conditions to maintain homeostasis (Gustafsson & Gottlieb, 2008). The process is responsible for degrading long-lived proteins and organelles, followed by their delivery to the lysosome for degradation and recycling (Groenendyk et al., 2010). Under stress conditions, autophagy can be activated by IRE1/ATF4 dependent pathways. Autophagy is upregulated in response to various stresses like hypoxia, mitochondrial dysfunction, nutrient deprivation, and infection (Gustafsson & Gottlieb,

2008). In heart, both beneficial and harmful roles of autophagy have been observed, and it depends on the level of autophagy (Decker et al., 1980).

Autophagy marker Light Chain 3 (LC3) is subunit of microtubule associated proteins 1A and 1B. Three human LC3 isoforms (LC3A, LC3B, and LC3C) undergo post-translational modifications during autophagy. Cleavage of LC3 at the carboxy terminus immediately following synthesis yields a cytosolic form called LC3-I. Some of LC3-I is further converted to LC3-II through lipidation process (Kabeya, 2004). The presence of LC3 in autophagosomes along with conversion of LC3 to the lower migrating form LC3-II indicates active autophagy (Kabeya, 2004).

## **1.7 Role of ER stress in pathology**

ER stress has been implicated in a number of diseases including ischemia/reperfusion of the brain and heart, hypertrophy, heart failure, diabetes, bipolar disorder, and neurodegenerative diseases (Groenendyk et al., 2010; Kitakaze & Tsukamoto, 2010). Activation of UPR in cardiac tissue is also caused by decrease in ATP and ER calcium, which is further linked to the development of cardiovascular diseases.

ER stress in the heart has received a great deal of attention because of its association with several cardiac pathologies. Pressure overload in mouse myocardium has shown to activate ER stress-mediated apoptosis (Okada, 2004). In cultured cardiomyocytes, ER stress has shown to contribute to ischemia-induced apoptosis (Dalal, Foster, Bhudev, Singh, & Singh, 2012). ER resident chaperone BiP has cardioprotective effects. Pharmacological induction of BiP has shown to attenuate cell death by

proteasome inhibition (Fu et al., 2008). Under mild hypoxia during an ischemic insult in the heart, the metabolic changes try to adopt. However severe hypoxia causes anaerobic metabolism, which is paralleled by dramatic increase in reactive oxygen species (ROS). This condition then leads to cell death and cardiovascular dysfunction (Groenendyk et al., 2010). The length of UPR induction also plays an important role to decide for survival or proapoptotic pathways. If the heart experiences stress for a long period, the UPR triggers autophagy and apoptosis as a last measure to deal with the problem. This may ultimately result in heart failure and death (Groenendyk et al., 2010).

### **1.8 SLMAPs are components of cardiac ER/SR membranes involved in E-C coupling**

Sarcolemmal membrane-associated proteins (SLMAPs) gene is assigned to human chromosome 3p14.3–21.2. (Wigle et al., 1997), and it is comprised of 24 exons spread over ~122 kb of DNA (Guzzo et al., 2004a). A unique feature of SLMAP is the ability to form extensive coiled-coil structure containing two leucine zipper (LZ) motifs (Wigle et al., 1997). The hydrophobic residues in the coiled-coil conformation give the  $\alpha$ -helix an amphipathic nature, which is important for protein-protein interactions (Wigle et al., 1997), and has a role in activation of transcription factors (Hollenberg & Evans, 1988). The transmembrane domain of SLMAP targets it to various membrane structures in the cell (Guzzo et al., 2004b).

The polypeptides resulted from alternative splicing of the 5' region in the SLMAP gene are expressed in developmental and tissue specific manner, which indicated that

variants have distinct functions (Wielowieyski, 2000). Northern blot experiment showed that SLMAP has three transcripts (5.9, 4.5, and 3.5 kb). These transcripts are generated by alternative splicing because SLMAP proteins are encoded by a single gene. Several SLMAP isoforms of varying molecular size (35, 45, 63, and 83-91 kDa) have been identified, with roles in myoblast fusion (Guzzo et al., 2004b), excitation-contraction coupling (Guzzo et al., 2005), and centrosomal organization (Guzzo et al., 2004a). The largest SLMAP isoform 91 kDa SLMAP3 is expressed in all tissues abundantly, while the short 35 kDa isoform SLMAP1 was predominantly expressed in cardiac and slow twitch skeletal muscle (Wigle et al., 1997).

Alternative splicing of the C-terminal gives two variants of SLMAP polypeptides, each having unique transmembrane (TM) domain, which target proteins to membrane systems (Guzzo et al., 2004b). Immunocytochemical localization and subcellular fractionation studies of SLMAP showed that TM domains are necessary for SLMAP integration into membranes. In the absence of exons that code for TM domains, the resulting protein is excluded from the membrane (Wielowieyski et al., 2000). All isoform of SLMAP have either TM1 or TM2 for membrane integration, suggesting that hydrophobic profile of a tail anchor is very important for determining subcellular localization (Byers et al., 2009). Furthermore, expression of TM1 and TM2 is mutually exclusive, because splicing of alternative exon TM1 introduces an in-frame stop codon which makes the TM2 nonfunctional (Guzzo et al., 2004a).

Protein-protein interaction analysis indicates that SLMAP proteins can self-assemble and bind to other proteins in the membrane structures. These interactions are mediated by the leucine-rich coiled-coil motifs (Guzzo et al., 2004b). The SLMAPs share

an overall structural similarity to other tail-anchored membrane proteins such as the syntaxins, which serve roles in membrane docking and fusion events (Kutay, Hartmann, & Rapoport, 1993). Many tail-anchored proteins are involved in diverse cellular functions, including docking and fusion of synaptic vesicles with the plasma membrane (Wielowieyski et al., 2000). In cardiac tissue, SLMAP is localized to sarcolemma and intracellular membrane, suggesting that it can interact with important effector proteins to regulate cell functions (Wigle et al., 1997).

A 91 kDa SLMAP variant (SLMAP3M1) was identified as a component of the microtubule organizing center (MTOC) (Guzzo et al., 2004a). N-terminal sequence analysis showed the presence of a conserved motif known as the forkhead associated (FHA) domain, which mediates protein's colocalization with Y-tubulin at the centrosomes at all phases of the cell cycle (Guzzo et al., 2004a). Deletion-mutant analysis showed that N-terminal sequence and overall structure of SLMAP was required for centrosomal targeting. Deregulation of SLMAP levels decreased cell viability (Guzzo et al., 2004). FHA domain is found in several proteins including kinases, phosphatases, kinesins, transcription factors, DNA- and RNA-binding proteins, and metabolic enzymes (Hofmann & Bucher, 1995; Durocher & Jackson, 2002). FHA domain also has a role in phosphoserine/threonine specific protein-protein interaction motifs (Guzzo et al., 2004a).

SLMAP is expressed in a regulated manner during the development of muscle tissue and plays a crucial role in myoblast fusion (Guzzo et al., 2004b). Expression starts during *in vivo* skeletal myogenesis and increases as the fusion process starts in the developing embryo (Furst, Osborn, & Weber, 1989). SLMAP proteins become distributed within specialized membrane systems involved in calcium signalling as

development progresses. The 80 kDa SLMAP isoform is expressed after the initiation of myotube formation (Guzzo et al., 2004b). It shows that precise levels of SLMAP are important for the morphology of skeletal muscle development.

The deregulation of SLMAP by ectopic expression in myoblasts affects the ability of these cells to fuse to myotubes during differentiation (Guzzo et al., 2004b). The overexpression of SLMAP deletion mutants were defective in homodimerization domain, and expression of SLMAP mutants without the C-terminal TM domain compromised the fusion of myoblasts (Guzzo et al., 2004b). The abnormal phenotype caused by the deregulation of SLMAP expression in myoblasts employs that normal levels and the temporal induction of SLMAP isoforms plays important role in the fusion of myoblasts, and normal muscle development and function (Guzzo et al., 2004b). The distribution of SLMAP in the SR and t-tubules of developing and adult skeletal muscles shows its importance in SR function, and regulation of calcium homeostasis (Guzzo et al., 2004b). These observations also suggest that SLMAPs may function as junctional membrane components that provide structural integrity (Guzzo et al., 2004b).

SLMAPs reside at the level of the E-C coupling apparatus of the cardiomyocytes, and in the SR (Guzzo et al., 2005). The SLMAP isoforms generated by alternative splicing are expressed in a tissue-specific manner. SLMAP appeared to be targeted to distinct subcellular membrane compartments depending on the C-terminal membrane anchor used (i.e. TM1 or TM2). Confocal imaging of SLMAP expression showed that it is localized with the SR and cell-surface membranes of the ventricular myocytes, and also with markers of the surface membrane such as caveolin and RyR (Guzzo et al., 2005).

Protein-protein interaction analysis indicated that cardiac specific SLMAP variant (SLMAP1) binds with the  $\alpha$ - and  $\beta$ -subunit of the myosin heavy chain in cardiac muscle (Guzzo et al., 2005). It has also shown to colocalize at the level of the M-line in adult cardiomyocytes. The analysis also showed that SLMAP can interact with the contractile apparatus via its N-terminal sequences. Furthermore, glutathione S-transferase-SLMAP (GST) fusion proteins bind with the 70 kDa and 35 kDa cardiac isoforms, implying that these molecules can form homodimers in cardiac muscle (Guzzo et al., 2005). SLMAPs can form homodimers via their leucine-rich coiled-coil motifs (Guzzo et al., 2004). The distribution of SLMAPs in various cardiac membranes, and their ability to self-assemble, suggests that SLMAPs regulate membrane function through homodimer formation (Guzzo et al., 2005).

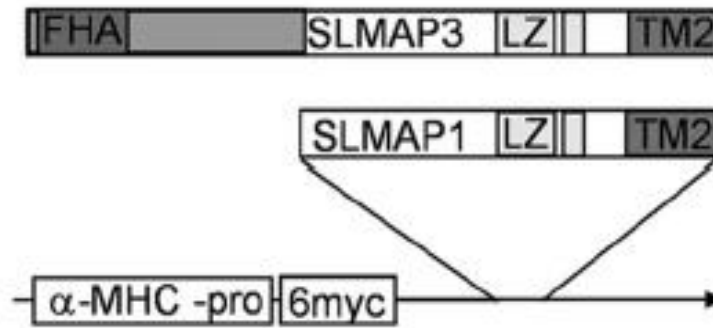
Earlier findings from our group revealed that both TM1 and TM2 target SLMAP1 to the ER, whilst TM2 also targets SLMAP1 to the mitochondrial outer membrane (MOM) (Byers et al., 2009). This difference in membrane targeting was due to the lower hydrophobic content of the trans-membrane helix in TM2, as compared to the TM1. Some reports have also shown that tail-anchored proteins can target to MOM (Habib et al., 2003) and peroxisomes (Lisenbee, Karnik, & Trelease, 2003). The presence of positively charged residues in the regions flanking the membrane spanning domain can cause TM to target the MOM (Kuroda et al., 1998).

Sequence analysis of SLMAP1 TM domain showed that only TM2 possesses positively charged residues (Byers et al., 2009). This suggested that TM2 may target the MOM, whilst TM1 could target the ER. Contrarily, some ER targeting TMs possess positively charged residues in the flanking regions (Beilharz, 2003). This suggested that

charge of amino acid residues, as well as overall hydrophobicity profile of TM domain determines membrane targeting (Byers et al., 2009).

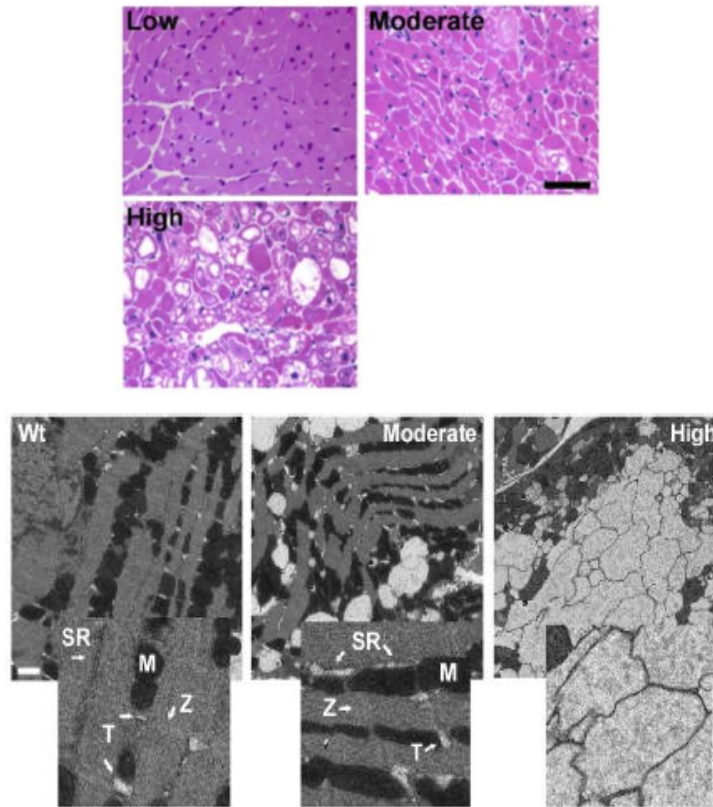
The presence of the two TM domains which target SLMAP1 to different locations suggests that SLMAP1 may perform multiple subcellular functions. The coiled-coil nature of the majority of the SLMAP protein may help to tether proteins into place on both the ER and MOM. On the other hand, it may be involved in tethering of the two membrane systems together through homo/heterodimerisation. Given SLMAP's varying levels of expression with different TM domains, the protein may have variety of subcellular roles in tissue specific manner.

Our group generated transgenic (Tg) mice with cardiac-restricted overexpression of the SLMAP1-TM2 sequence driven by  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter (Nader et al., 2012).  $\alpha$ -MHC restricts the expression of the gene only to cardiomyocytes (Gupta et al., 1998). It was speculated that overexpressed SLMAP1-TM2 protein would interfere with endogenous SLMAP homodimer formation and SR function. The overexpression of SLMAP1-TM2 negatively impacted heart function by inducing structural and functional changes in the SR membrane (Nader et al., 2012).



**Figure 5:** *The SLMAP1-TM2 construct.* The SLMAP1-TM2 construct was designed to be specifically expressed in mouse heart. SLMAP1 lacks the N-terminal stretch of SLMAP3, composed of the forkhead-associated domain (FHA). The SLMAP1 sequence containing leucine zipper (LZ), coiled-coil region and TM2 was tagged with a 6-myc epitope and cloned in-frame with  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter sequence. Figure is taken from Nader et al. (2012).

The overexpression caused vacuolated myocardium, which positively correlated with expression level of SLMAP1-TM2 isoform in myocardium (figure 6). Furthermore, it also caused dilation of ER/SR in the cardiomyocytes, along with inappropriate homodimerization of the overexpressed protein (Nader et al., 2012). Expression of SLMAP1-TM2 was associated with the dilation of the SR and activation of cardiac remodeling genes atrial natriuretic peptide (ANF) and  $\beta$ -MHC. The mRNA levels of cardiac fetal genes such as ANF and  $\beta$ -MHC were analyzed using quantitative polymerase chain reaction (PCR) in order to establish a link with structural changes observed in hearts from 5-week old Tg mice (Nader et al., 2012). In Tg hearts, up to 7-fold increase in mRNA levels was observed as compared to wild-type (Wt) hearts, which indicated presence of pathological remodeling (Nader et al., 2012).



**Figure 6:** *SLMAP1* overexpression disrupts ER/SR structure. Remodeling of the myocardium in *SLMAP1-TM2* Tg hearts is shown by membrane vesicle formation in low, moderate, and high expressers (5 week) of *SLMAP1-TM2*. Sarcoplasmic reticulum (SR); t-tubule (T); mitochondria (M); Z-line (Z). Scale bar = 10  $\mu$ m. Figure is taken from Nader et al. (2012).

Analysis of electrocardiogram (ECG) *in vivo* at age of 5 weeks in Tg mice did not show any change in contraction compared with age-matched Wt littermates. At 28-weeks, there was reduction in left ventricular (LV) systolic pressure in Tg hearts as compared to Wt hearts (Nader et al., 2012). Furthermore, the QT interval was also prolonged by ~15% in Tg hearts, which indicated defect in calcium recycling in Tg hearts at 28-weeks. The data suggested that overexpression of SLMAP1-TM2 can result in progressive deterioration of cardiac contractility through adulthood, from 5- to 28-weeks (Nader et al., 2012).

Western blot analysis of microsomes isolated from hearts of 5-week old Tg mice showed significant decrease in the expression of the SR calcium handling proteins including RyR2, SERCA2a, CSQ, and triadin (Nader et al., 2012). These observations indicated that SLMAP1-TM2 alters the expression of calcium handling SR proteins. When SERCA2a was stimulated using ATP in Tg SR vesicles, ~50% decrease in calcium uptake was observed, as compared to Wt vesicles. The reduction in calcium uptake was linked to decreased expression level of SERCA2a protein at SR membranes (Nader et al., 2012).

The SR in cardiomyocytes has a pivotal role in regulating intracellular calcium concentration, which regulates cardiac electrophysiology and contractile function (McPherson & Campbell, 1993; Oort et al., 2011). Disruption in expression level of calcium handling proteins or their activity sensitizes the heart to cardiac arrhythmias (Terentyev et al., 2005). Aberrant calcium recycling through ion transporters in SR membranes impairs cardiac electrophysiology, which leads to pulsus alternans (Schmidt et al., 2000).

SLMAP has a critical role in regulating E-C coupling at the SR level, and disturbing the normal level of the protein results in deterioration of cardiac electrophysiology and function. These findings highlight the role of SLMAP in the membrane biology of cardiac function, and that regulated levels of the protein are critical for E-C coupling (Nader et al., 2012).

In mouse model of type-2 diabetes, Ding and colleagues (2005) found that endothelial dysfunction is related to upregulation of the 35 kDa isoform of SLMAP in the vascular tissue. The cardiovascular complications and endothelial dysfunction are major risk factors in type-1 and type-2 diabetes (Bohlen, 2004). The deregulation of SLMAP expression in the microvasculature suggested the importance of normal SLMAP level in proper function of membrane biology, and thus functions of cells lining the blood vessels. This finding suggested that upregulation of SLMAP can be the indicator for microvascular disease associated with type-2 diabetes (Ding et al., 2005). In diabetic mouse, treatment with peroxisome proliferator-activated receptor (PPAR $\gamma$ ) agonist resulted in recovery from endothelial dysfunction, reduction in SLMAP expression, and correction of plasma glucose and triglyceride levels. This finding implied that SLMAP expression is regulated by PPAR $\gamma$ -related mechanism (Ding et al., 2005).

Ding and colleagues (2011) isolated adipocytes from the abdomen of *Tally Ho* mice, which is a polygenic mouse model of type-2 diabetes. The adipocytes exhibited upregulation of 45 kDa and 35 kDa isoforms of SLMAP, and lower expression of the membrane-associated glucose transporter-4 (GLUT-4), when compared with normoglycemic mice (Chen et al., 2011). Immunoprecipitation experiment showed association of GLUT-4 with 45 kDa SLMAP isoform, but this association was decreased

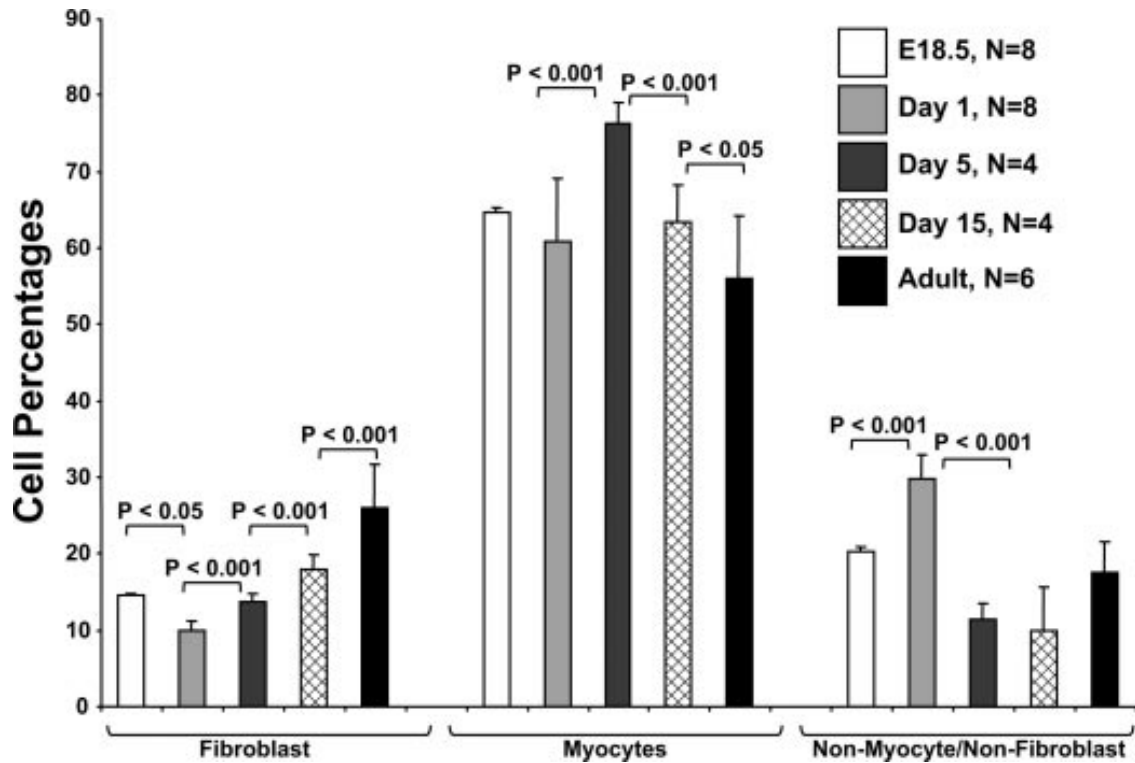
when adipocytes from *Tally Ho* mouse were subjected to hyperglycemic conditions. Knock down of SLMAP with small interfering ribonucleic acid (siRNA) in adipocytes resulted in a significant decrease in glucose uptake (Chen et al., 2011). Taken together, the experiments suggested an important role of SLMAP in regulation of glucose transporters.

Mutations in SLMAP gene has shown to impair intracellular trafficking of human cardiac sodium channel hNav1.5 (Ishikawa et al., 2012). SCN5A gene encodes for pore-forming  $\alpha$ -subunit of the cardiac sodium channel hNav1.5 (Tfelt-Hansen et al., 2010). Mutations in the gene reduce the availability of sodium channels, which is a known pathology in Brugada syndrome (BrS) (Mohler, 2004). Consequently, there is a decrease in inward sodium current during action potential due to loss of hNav1.5 function. Analysis of BrS patients for SLMAP mutations revealed two missense mutations, which were not found in healthy individuals. The mutations found in SLMAP were purposed to cause the observed decrease in surface expression of hNav1.5 and inward sodium current (Ishikawa et al., 2012). Although there was no direct physical interaction of SLMAP with hNav1.5, the data suggested that SLMAP is indirectly regulating function and localization of hNav1.5 in cardiomyocytes (Ishikawa et al., 2012). This finding further highlights that normal expression and function of SLMAP is important, because it resides at E-C coupling apparatus of muscle cells (Guzzo et al., 2005).

## **1.9 Myocardium structural composition**

The main cellular components of the mouse heart include fibroblasts, myocytes, endothelial cells, and vascular smooth muscle cells (Souders, Bowers, & Baudino, 2009). These cell types maintain the electrical and biomechanical responsive nature of the heart, which determines the overall function of the heart. Fluorescence-activated cell sorting (FACS) analysis shows that the adult murine myocardium is composed of approximately 56% myocytes, 27% fibroblasts, 7% endothelial cells, and 10% vascular smooth muscle cells (Banerjee, Fuseler, Price, Borg, & Baudino, 2007).

Fibroblasts in the heart produce variety of extracellular matrix components including collagen, growth factors, cytokines, and other signaling molecules. They are also involved in maintenance of cardiac function and remodeling during pathological conditions (Souders et al., 2009). During normal function of the heart, the cellular components interact with each other and respond to alterations in developmental, homeostatic, and pathological stimuli (Banerjee et al., 2007). Fibroblasts and myocytes are connected via gap junctions to aid electrical conduction in the heart (Baudino, 2006). Cardiac fibroblasts interact with other cells during cardiac development, and they also respond to chemical and mechanical signals. Disruptions of interaction via remodelling e.g. between myocytes and fibroblasts, can lead to pathological conditions (Baudino, 2006).



**Figure 7:** FACS analysis of cells population in the adult mouse heart. During development, cell population changes in the late embryonic, early neonate, and adult murine heart. Figure is taken from (Banerjee et al., 2007).

## 1.10 Statement of the problem

SLMAP has an important role in regulation of membrane biology, and thus cardiac function. Precise regulation in expression of SLMAP is important for proper functioning of E-C coupling and calcium regulation. Overexpression of SLMAP in myocardium of Tg mice has resulted in ER/SR remodeling and vacuolization during adulthood. The significant changes in the SR structure, volume, and protein load in the cardiomyocytes (Nader et al., 2012) lead us to hypothesize that overexpression of SLMAP may causes ER stress in the transgenic hearts, and the cells will exhibit UPR, impacting survival or death.

The primary objective was to use transgenic animal model in order to study how overexpression of the SLMAP in the heart is affecting the expression of key ER stress marker proteins. The analysis may indicate which ER stress pathways are activated, and what are the downstream effects. If cells under ER stress are trying to adopt pro-survival pathways, we will see upregulation of ER chaperones that will help the misfolded proteins to fold properly. Alternatively, if cells are unable to resolve the ER stress, does it lead to apoptosis?

In order to assess the hypothesis, the objectives are as follows:

- 1) The expression level of key ER stress marker proteins in the hearts of Tg versus Wt mice were examined. Since the strength of ER stress response is dependent on the intensity of the stressor (SLMAP overexpression) and duration of the stress (age of mice); modulation of ER stress will be explored as mice grow older from 1-day after birth, up to 16-weeks.

- 2) The overexpression of SLMAP is only targeted in cardiomyocytes due to presence of cardiac specific  $\alpha$ -MHC promoter. Changes in the expression of ER stress markers due to SLMAP overexpression in neonatal cardiomyocytes will be assessed, and compared with fibroblasts.
  
- 3) The changes in viability of neonatal cardiomyocytes from hearts of 1-day old Tg versus Wt mice will be assessed using MTT assay. The cells will also be challenged with stress causing reagents (thapsigargin and palmitate) to observe any effects of excess SLMAP on ER stress, and changes in viability.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Transgenic mice

**a) Mouse line:** Previously established transgenic mouse lines (Nader et al., 2012) were used and bred with B6C3F1 mice. All animals were handled according to protocols approved by the Institution Animal Care Committee.

**b) Genotyping:** Genomic DNA was extracted from the tail clips using Red Extract Tissue (Sigma). Transgenic mice were identified by polymerase chain reaction (PCR) using a forward primer: 5`-GAA AAG CCT ATC GAA ATC AAG TTG-3` corresponding to exon 18 sequence, and reverse primer: 5`-ACC TTC TTA AGC TCT TCT TGC AAA G-3` corresponding to exon 19. This resulted in a ~686 bp difference between endogenous (wild-type) and 6-myc-tagged SLMAP1-TM2 PCR products, which were visualized by ethidium bromide (EtBr) staining on a 1.2% agarose gel. Genotyping was routinely verified at the protein level by western blot using anti-myc antibody to detect 6-myc-tagged SLMAP1-TM2.

### 2.2 Protein isolation from mouse heart

Hearts of older mice were collected after CO<sub>2</sub> euthanasia, and immediately frozen in liquid nitrogen. Each single heart was later washed with ice-cold 1X phosphate buffered saline (PBS), and homogenized using Fisher handheld Maximzer homogenizer in ice-cold modified radio-immunoprecipitation assay (RIPA) buffer (0.25% deoxycholate, 1 mM ethylenediaminetetraacetic (EDTA), 50 mM Tris base, 1% Nodidet

P-40, 100 mM sodium chloride, and 1% complete mini EDTA-free protease inhibitor cocktail (Roche)). The suspension was centrifuged for 15 min at 12000g to separate the proteins from cell debris. The supernatant containing proteins was collected in eppendorf tubes and stored in freezer at -80 °C. In immunoblotting experiments, proteins from each heart were used in a single lane of 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### **2.3 Neonatal Mouse Cardiomyocytes (NMCM) Culture**

Hearts from 1-day old pups were collected by decapitating the head. The hearts were immediately washed with ice-cold Hank's Buffer (HBSS) containing no calcium or magnesium, and transferred to tubes containing HBSS with 0.1% trypsin. The tubes were left overnight on a slow shaker at 4 °C. The hearts collected from each pup were used for genotyping to identify Tg and Wt mice.

The Tg and Wt hearts were pooled in separate 15 mL falcon tubes containing HBSS solution. The hearts were washed twice with HBSS solution at room temperature. Following complete aspiration of HBSS solution from the tubes, the hearts were digested at 37 °C in 0.1% collagenase II dissolved in HBSS. Digestion process was done three times for 12 min each with gentle shaking. Each time the supernatant containing predominantly cardiomyocytes and fibroblasts was collected and centrifuged at 600g for 3 min. The supernatant after centrifugation was discarded and the pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% non-essential amino acid (NEAA) and 1% pen/strep (hereinafter referred as

cardiomyocyte media). The resuspended cells were kept at 37 °C and 5% CO<sub>2</sub>. The suspension resulting from three digestions were pooled together in 15 mL falcon tube and centrifuged at 600g for 3 min. The pellet was resuspended in desired amount of cardiomyocyte media. The cells were plated on uncoated culture dishes for 45 minutes at 37 °C and 5% CO<sub>2</sub> to allow the fibroblast to adhere (cardiomyocytes required gelatin coating). Using the media from culture plates, this step was performed again before a final plating of the cardiomyocytes on plates coated with 1% gelatin. The cells were incubated for 48 hours at 37 °C and 5% CO<sub>2</sub>.

In experiments using drug treatment (thapsigargin or sodium palmitate (Sigma)), the culture media with desired drug concentration was added and incubated for additional 24 hours. Cells were treated with thapsigargin (1 μM and 2 μM) or its diluent 0.065% dimethyl sulfoxide (DMSO) (v/v) as a control, and palmitate (0.25 mM and 0.50 mM) or its diluent 2.5% water (v/v) as a control. After incubation period, the media from culture dishes was completely aspirated and the dishes were washed with PBS, and followed by freezing at -80 °C. The culture dishes were scraped using modified RIPA buffer. The cells in the suspension were lysed by syringing at least 10 times, and followed by freezing at -80 °C. The suspension was later thawed on ice and centrifuged for 15 min at 12000g to separate the proteins from cell debris. The supernatant containing proteins was collected in eppendorf tubes and stored in freezer at -80 °C.

## **2.4 Protein measurements**

Bicinchoninic acid (BCA) protein assay kit (Pierce) was used as per the manufacturer's protocol to make standard curve, and for measuring protein concentration of the samples.

## **2.5 Western blotting**

For western blotting experiments, 25 µg of proteins from each heart was loaded in single well of a 10% SDS-PAGE gel. The gels were transferred overnight on a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) in a buffer containing 25 mM Tris, 190 mM Glycine, and 20% methanol. All membranes were blocked at room temperature for 1-hour in Tris-buffered saline (TBST) containing 1 M Tris, 290 mM NaCl, 0.1% Tween 20, pH 7.4, and with 5% non-fat dry milk. Primary antibodies (listed in Table 1) in 1:1000 dilution in TBST were incubated overnight at 4 °C with 5% bovine serum albumin (BSA). Membranes were washed 5 times for 5 min each in TBST before adding the appropriate horseradish peroxidase labeled secondary antibody (Jackson) in 1:5000 dilution in TBST with 5% non-fat dry milk, with slow shaking at room temperature for 1-hour. Membranes were washed 5 times for 5 min each in TBST. Membranes were treated with BM Chemiluminescence Western Blotting Kit (Roche) and developed using autoradiography films (HyBlot CL and GE Healthcare). Bands on the autoradiography films were scanned using Gel Doc System (Bio-Rad), and quantified by densitometry using Image Lab software v.4.0.1 (Bio-Rad). Membranes were stripped (25 mM glycine, 10% SDS and pH 2.2 in dH<sub>2</sub>O) and reprobbed with different antibodies.

**Table 1:** List showing antibodies used to detect proteins on western blotting membranes.

<b>Target Protein</b>	<b>Company/Source</b>	<b>Catalogue Number</b>
<b>LC3</b>	Cell Signaling	4108
<b>Caspase-3</b>	Cell Signaling	9662
<b>CHOP</b>	Cell Signaling	2895
<b>BiP</b>	Cell Signaling	3177
<b>Akt</b>	Cell Signaling	9272
<b>PDI</b>	Cell Signaling	3501
<b>GAPDH</b>	Invitrogen	AM4300
<b>PERK</b>	Cell Signaling	3192
<b>PARP</b>	Cell Signaling	9542
<b>ATF6</b>	Abcam	ab37149
<b>MYC</b>	Sigma	A7470

## **2.6 Cell Proliferation Assay (MTT)**

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a dye that is reduced to purple formazan in living cells. The cardiomyocytes were incubated for 48 hours in gelatin coated 96-well culture plate containing approximately equal number (~ 15,000/well) of cardiomyocytes in 100  $\mu$ L culture media. Following aspiration of media, the cells were incubated with control media (DMSO or water) or media containing drugs (thapsigargin: 1  $\mu$ M and 2  $\mu$ M; palmitate: 0.25 mM and 0.50 mM) for 24 hours before performing MTT assay following manufacturer's protocol. The absorbance was recorded at 570 nm wavelength using BioTek Synergy H1 reader.

## **2.7 Statistical analysis**

Statistical analysis of data was performed using 2010 Microsoft Excel software. P-value of less than 0.05 was considered to be significant.

## CHAPTER 3: RESULTS

### 3.1 ER stress response in the SLMAP overexpressing Tg hearts

In earlier findings from our group, overexpression of SLMAP-TM2 in mouse myocardium was associated with ER/SR remodeling and vacuolization as mice grew older after 5-weeks (Nader et al., 2012). The dilation of ER/SR in the cardiac myocytes is reminiscent of changes caused by ER stress due to disruption in the membrane architecture and biology (Uemura et al., 2009). Since overexpression of SLMAP is causing these changes in mouse myocardium, we reasoned that they may be caused by ER stress.

To determine whether ER stress is mediated by overexpression of SLMAP in cardiomyocytes, we examined the key proteins that are markers of ER stress in protein samples from homogenized full heart tissue. These ER stress proteins indicated which pathways of ER stress are activated. Upregulation of chaperones like BiP and PDI generally indicate activation of ER stress, whereas specific stress markers like caspase-3 and CHOP are upregulated during apoptosis. During activation of autophagy, LC3-I is converted to LC3-II, and PARP is cleaved when DNA in the cell is fragmented.

To study temporal changes in these protein markers as mice age, we analyzed the hearts from 1-day, 5-weeks, 8-weeks, 11-weeks, and 16-weeks old Wt and Tg mice. If the cells are facing mild ER stress, the chaperones can reestablish homeostasis by refolding or degrading the misfolded proteins. If the extent of ER stress is high due to significant disruptions in ER/SR homeostasis, or the stress is prolonged, the cells will activate pathways that lead to apoptosis or autophagy. We were interested to analyze

which pathways of ER stress are active at early age i.e. 1-day, and how the pathways change at onset of puberty i.e. 5-weeks, and beyond into adulthood up to 16-weeks.

### **3.2 ER stress response in the hearts of 1-day old mice**

Proteins samples were isolated from the heart tissue, and expression of ER stress proteins were compared by SDS-PAGE immunoblot analysis. After electrophoretic transfer of proteins to a membrane, the target proteins were detected by using specific antibodies. The intensity of bands on western blot films was quantified using densitometry. The bands on films with transfer bubbles were omitted from statistical analysis. Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein level was monitored as loading control in immunoblotting experiments.

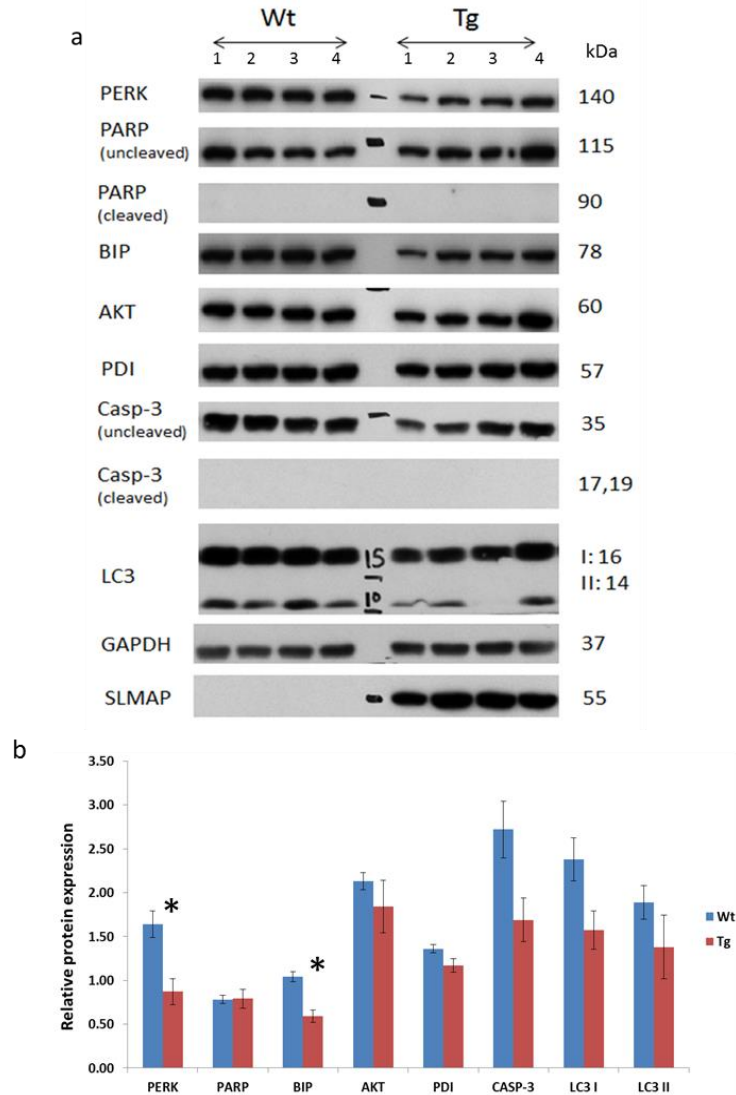
To compare the expression levels of the various ER stress markers between the Wt versus Tg hearts 1-day after birth, heart tissue samples were extracted from 4 Wt and 4 Tg mice, and examined via western blot (figure 8a). Each lane on the western blot represents an individual heart tissue sample. The band intensity for each ER stress protein was quantified via densitometry. The average expression for each protein in the Wt hearts was compared to the Tg hearts. The expression of target proteins for the Tg hearts was calculated relative to expression in the Wt hearts (control).

In 1-day old Tg hearts, there was a general trend in downregulation of ER stress marker proteins (figure 8). Anti-PERK antibody accurately identified 140 kDa PERK protein in immunoblotting. At the age of 1-day, the average expression of PERK in 4 Tg hearts was 47% less than the average expression in 4 Wt hearts (figure 8b). Similarly,

anti-BiP antibody identified BiP protein at 78 kDa. The average BiP expression was 43% less in the 1-day old Tg hearts. Caspase-3 also showed strong downregulation in Tg hearts, marked by 38% decrease. The active autophagy marker LC3 II exhibited downregulation by 27%. Both PDI and Akt protein showed a relatively small decrease (14%) in expression in Tg hearts. The expression of PARP protein remained unchanged. CHOP, cleaved caspase-3, and cleaved PARP were not detected. Antibodies to CHOP, cleaved caspase-3, and cleaved PARP correctly identified bands at right molecular weight in other experiments, showing that the antibodies are functioning as intended.

The downregulation of BiP and PERK in the hearts of Tg mice was statistically significant ( $P < 0.05$ ). Variations in expression of other proteins in the heart of individual mouse translated to high standard deviation values in statistical analysis (paired t-test). Consequently, the decrease in protein expression of ER stress marker proteins in Tg mice did not reach statistical significance ( $P > 0.05$ ) from Wt mice, despite downregulation of these markers (figure 8).

In Tg mice, the expression of ER stress markers did not correlate with level of expression of SLMAP. The expression of SLMAP in Tg mice varied between individuals from the same litter. For example, the expression of SLMAP in the heart of 2<sup>nd</sup> Tg mice is 38% more than the 1<sup>st</sup> Tg mice (figure 8a). The expression of stress markers in different Tg mice also varied considerably, implying that individual mouse is reacting differently to SLMAP overexpression in myocardium.



**Figure 8:** Downregulation of ER stress proteins in the hearts of 1-day old Tg mice.

**a)** Expression level of ER stress proteins was analyzed in the hearts of 4 Wt and 4 Tg 1-day old mice. The mice were not differentiated as males or females. Anti-myc antibody identifies the 6-myc tagged SLMAP protein which is only found in the hearts of Tg mice. GAPDH levels were monitored as loading control. **b)** Bar graphs represent quantification of densitometry analysis of the immunoblotted proteins represented as mean  $\pm$  standard error (SE) of N=4 animals. Asterisk (\*) shows changes in protein expression that were statistically significant ( $P < 0.05$ ).

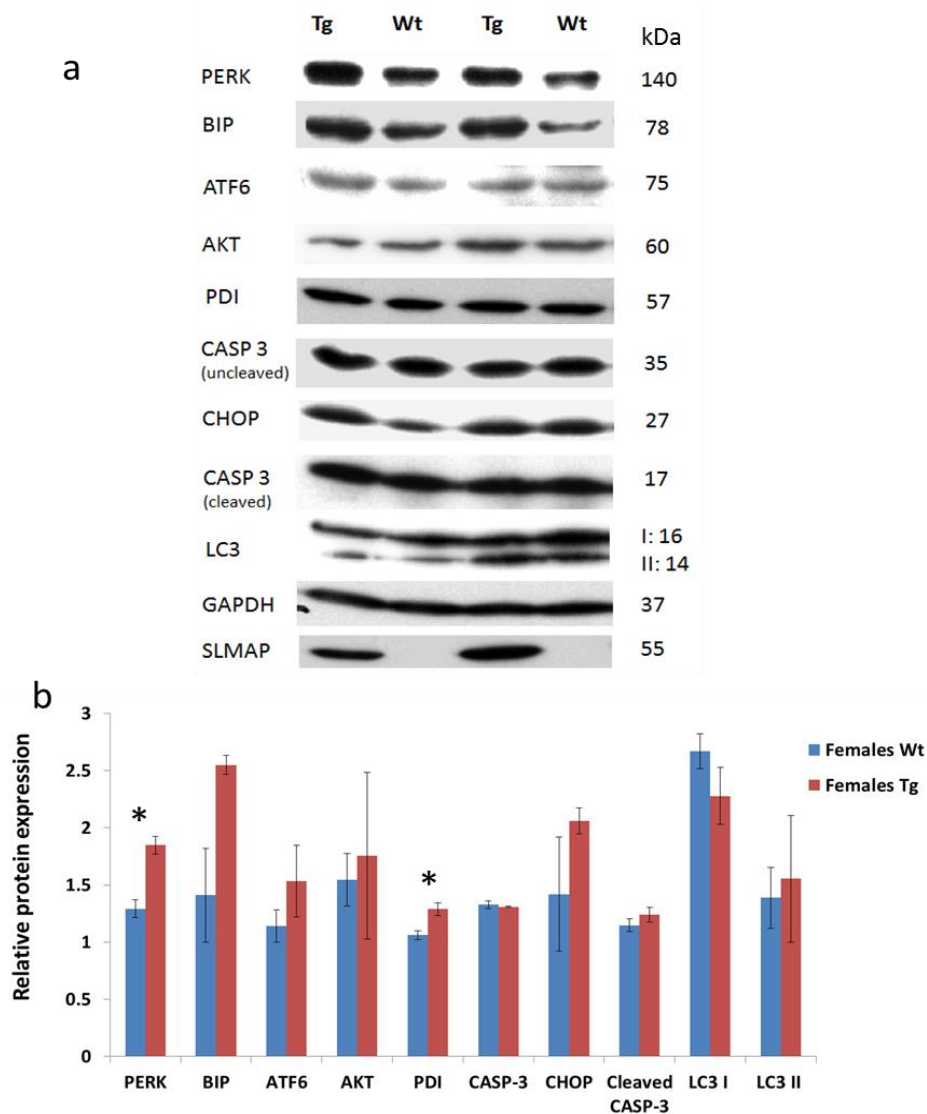
### 3.3 ER stress response in the hearts of 5-weeks old mice

Although the cellular architecture of a cardiomyocyte after birth has the complexity of an adult cardiomyocyte, the high degree of organization of ER/SR does not reach its peak until around 15 days after birth (Ostadal & Dhalla, 2013). The early cardiac development is also paralleled by age dependent maturation of E-C coupling apparatus and ER/SR structure. In the first 4 days of postnatal cardiac development, the cardiomyocytes exhibit hyperplastic growth. Thereafter, the growth of cardiomyocytes is predominantly hypertrophic (Piquereau et al., 2010). Environmental factors, as well as genetic alterations can profoundly affect the normal cardiac development (Dorn, 2005).

We wanted to explore how the overexpression of SLMAP in myocardium impacts the expression of ER stress marker proteins during and after full cardiac development. For that reason, we analyzed the expression of ER stress markers in 5-weeks old mice, in order to observe changes in protein expression as mice grew older. The experimental protocol was same as described for 1-day old hearts. To study sex-related differences, hearts from female and male mice were analyzed separately, because ER stress response and apoptosis pathways are dependent on sex differences (Sari et al., 2011). In both experiments, 3 Wt and 3 Tg hearts were used from 5-weeks old mice.

In hearts of Tg females, there was a trend in upregulation of ER stress proteins, as shown in figure 9. In the Tg hearts, PDI and PERK were significantly ( $P < 0.05$ ) upregulated by 22% and 43% respectively. Non-significant ( $P > 0.05$ ) upregulation in the Tg hearts was observed for BiP (81%), CHOP (45%), and ATF6 (35%). The change in expression of these proteins did not reach statistical significance, because the data was

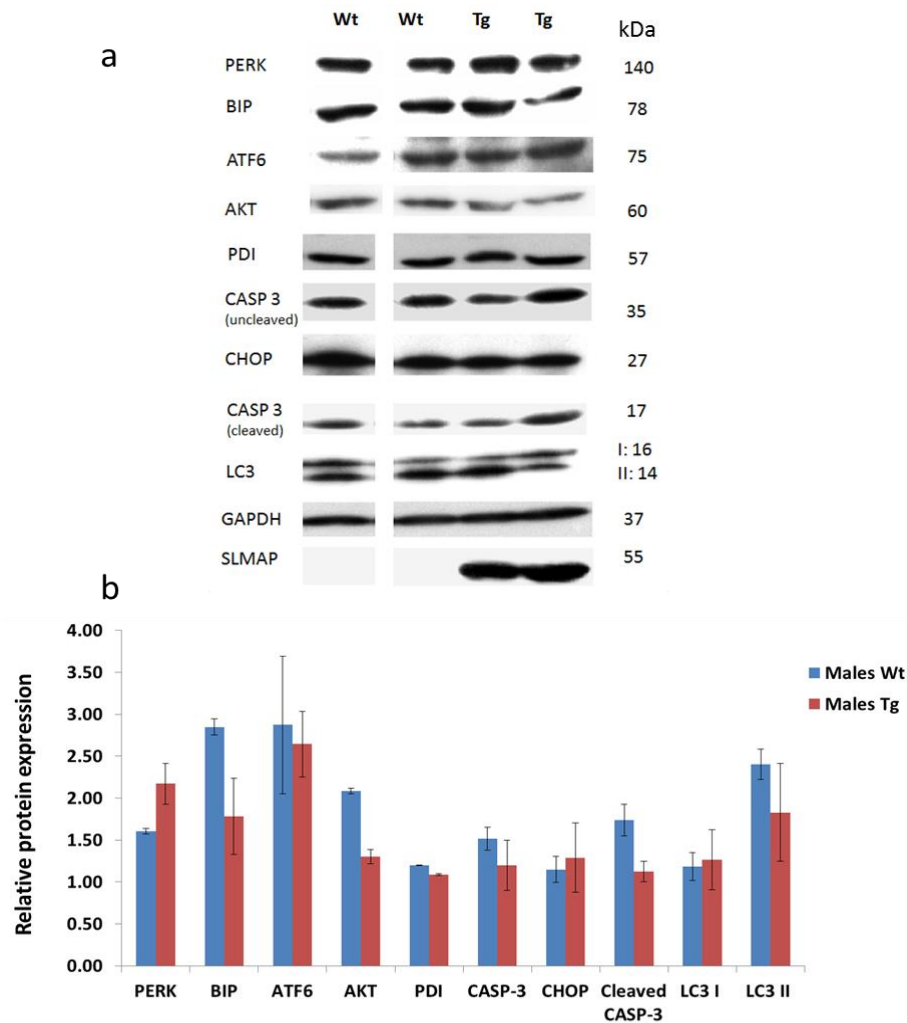
combined from different experiments, which resulted in high standard deviation values. The expression of caspase-3, cleaved caspase-3, LC3-II, and Akt remained unchanged (figure 9b).



**Figure 9:** Upregulation of ER stress proteins in the hearts of 5-weeks old female Tg mice.

**a)** Expression level of ER stress protein were analyzed in the hearts of 3 Wt and 3 Tg female mice of 5-weeks age. Anti-myc antibody identifies the 6-myc tagged SLMAP protein which is only found in the hearts of Tg mice. Only 2 Wt and 2 Tg hearts from same blot are shown here. GAPDH levels were monitored as loading control. **b)** Bar graphs represent quantification of densitometry analysis of the immunoblotted proteins represented as mean  $\pm$  SE of N=3 animals. Asterisk (\*) shows changes in protein expression that were statistically significant ( $P < 0.05$ ).

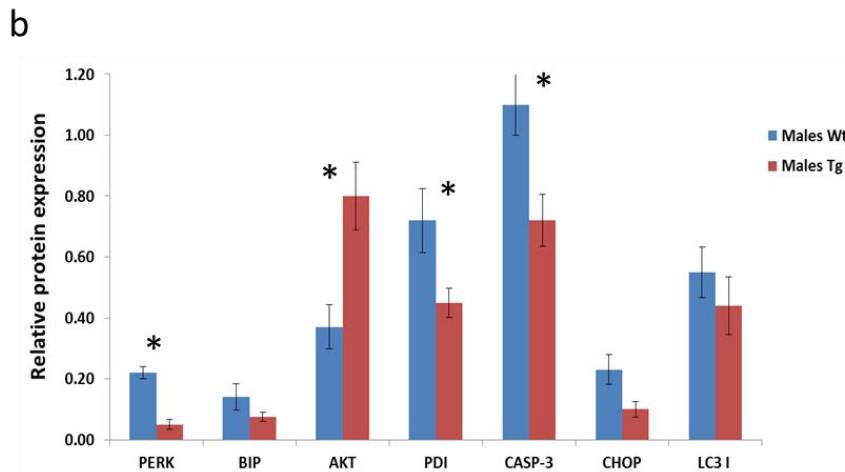
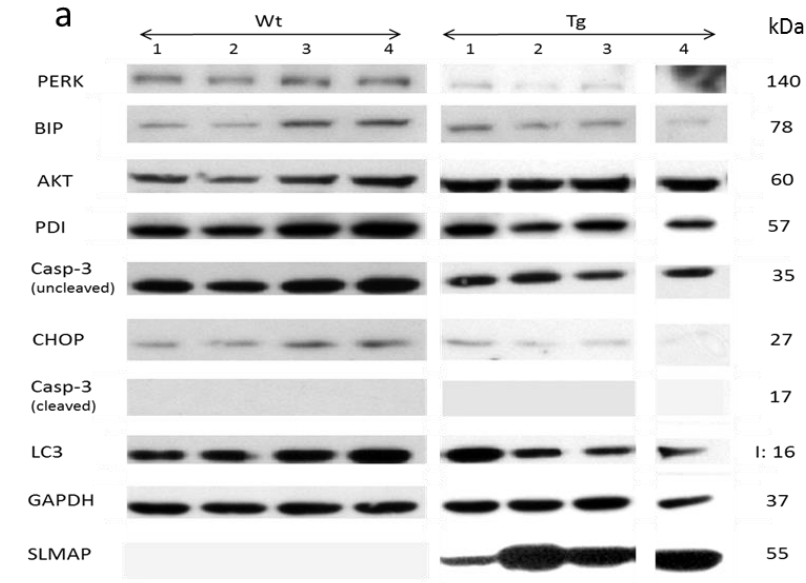
In hearts of Tg males, few ER stress proteins showed a trend in downregulation (figure 10). However none of the changes in this experiment were significant due to same reason explained above. Downregulation in the Tg hearts was observed for BiP (37%), caspase-3 (26%), cleaved caspase-3 (35%), LC3-II (24%), and Akt (38%). Only PERK was upregulated in the Tg hearts by 35%. The expression of PDI, CHOP, ATF6, and LC3-I remained unchanged (figure 10b).



**Figure 10:** A trend in downregulation of ER stress proteins in the hearts of 5-weeks old male Tg mice. **a)** Expression level of ER stress protein was analyzed in the hearts of 3 Wt and 3 Tg male mice of 5-weeks age. Anti-myc antibody identifies the 6-myc tagged SLMAP protein which is only found in the hearts of Tg mice. Only 2 Wt and 2 Tg hearts from same blot are shown here. GAPDH levels were monitored as loading control. Lane 1 (Wt) represents a distinct heart sample run on same gel. **b)** Bar graphs represent quantification of densitometry analysis of the immunoblotted proteins represented mean  $\pm$  SE of N=3 animals. Asterisk (\*) shows changes in protein expression that were statistically significant ( $P < 0.05$ ).

### **3.4 ER stress response in the hearts of 8-weeks old mice**

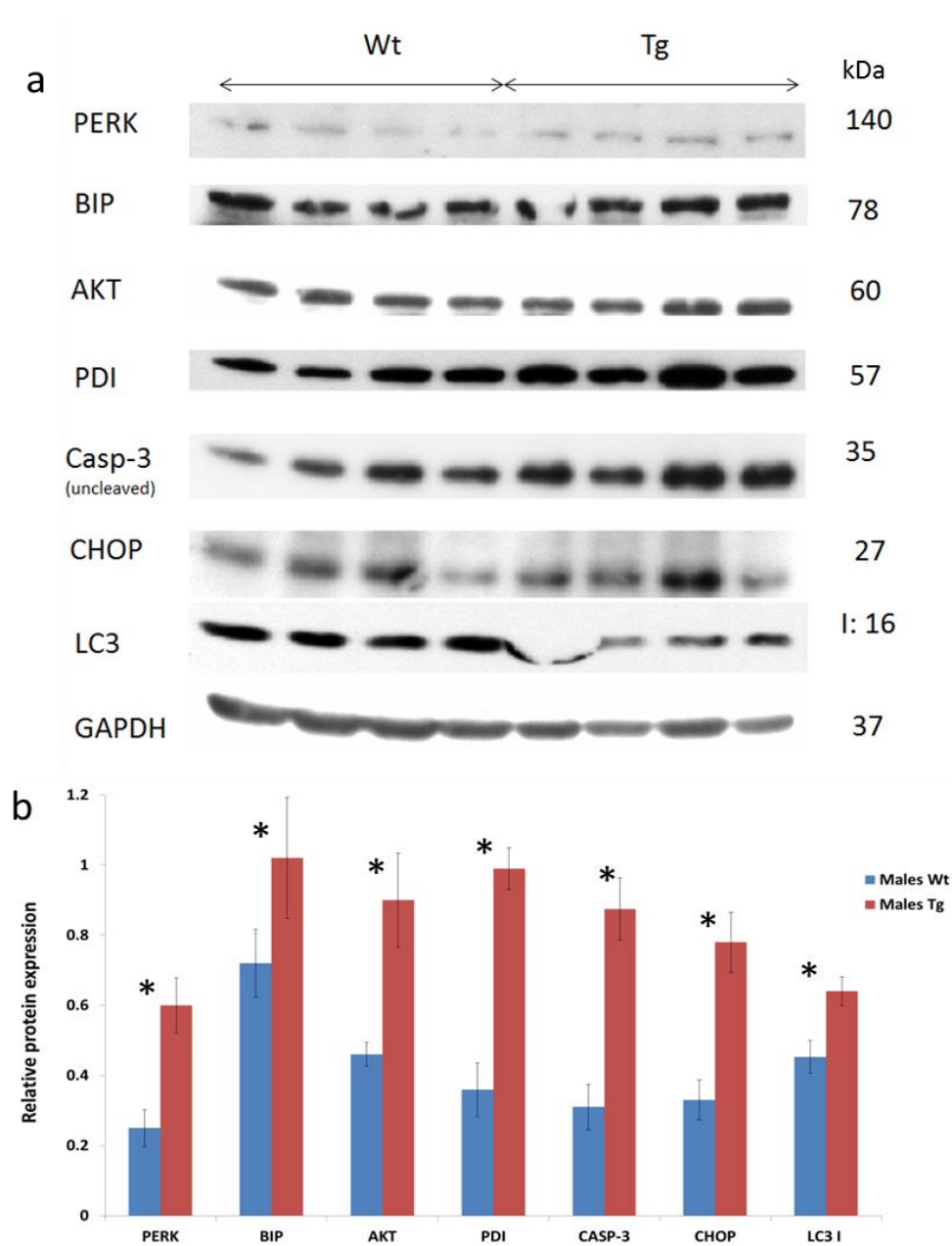
The ER stress proteins were analyzed in 8-weeks old male mice to observe any temporal changes in protein expression as the mice grew older. The experimental protocol was same as described for 1-day old hearts. Akt was upregulated by more than two-fold in the Tg hearts ( $P < 0.05$ ) as compared to the Wt hearts (figure 11a). A significant decrease ( $P < 0.05$ ) was observed in the expression of PDI (38%), PERK (75%), and caspase-3 (34%) in the Tg hearts (figure 11b). However, CHOP was downregulated by 62% in Tg mice, but the change was not significant ( $P > 0.05$ ). The chaperone BiP also showed 46% downregulation in Tg hearts, but the change did not reach statistical significance. In figure 11a, the band for PERK protein in fourth lane of Tg mice was omitted because of background signal.



**Figure 11: ER stress proteins in the hearts of 8-weeks old male Tg mice. a)** Expression level of ER stress protein was analyzed in 4 Wt and 4 Tg hearts from 8-weeks old male mice. Anti-myc antibody identifies the 6-myc tagged SLMAP protein which is only found in the hearts of Tg mice. GAPDH levels were monitored as loading control. Lane 4 (Tg) represents a distinct heart sample run on same gel. **b)** Bar graphs represent quantification of densitometry analysis of the immunoblotted proteins represented as a percentage of the mean  $\pm$  SE of N=4 animals. Asterisk (\*) shows changes in protein expression that were statistically significant ( $P < 0.05$ ).

### **3.5 ER stress response in the hearts of 11-weeks old mice**

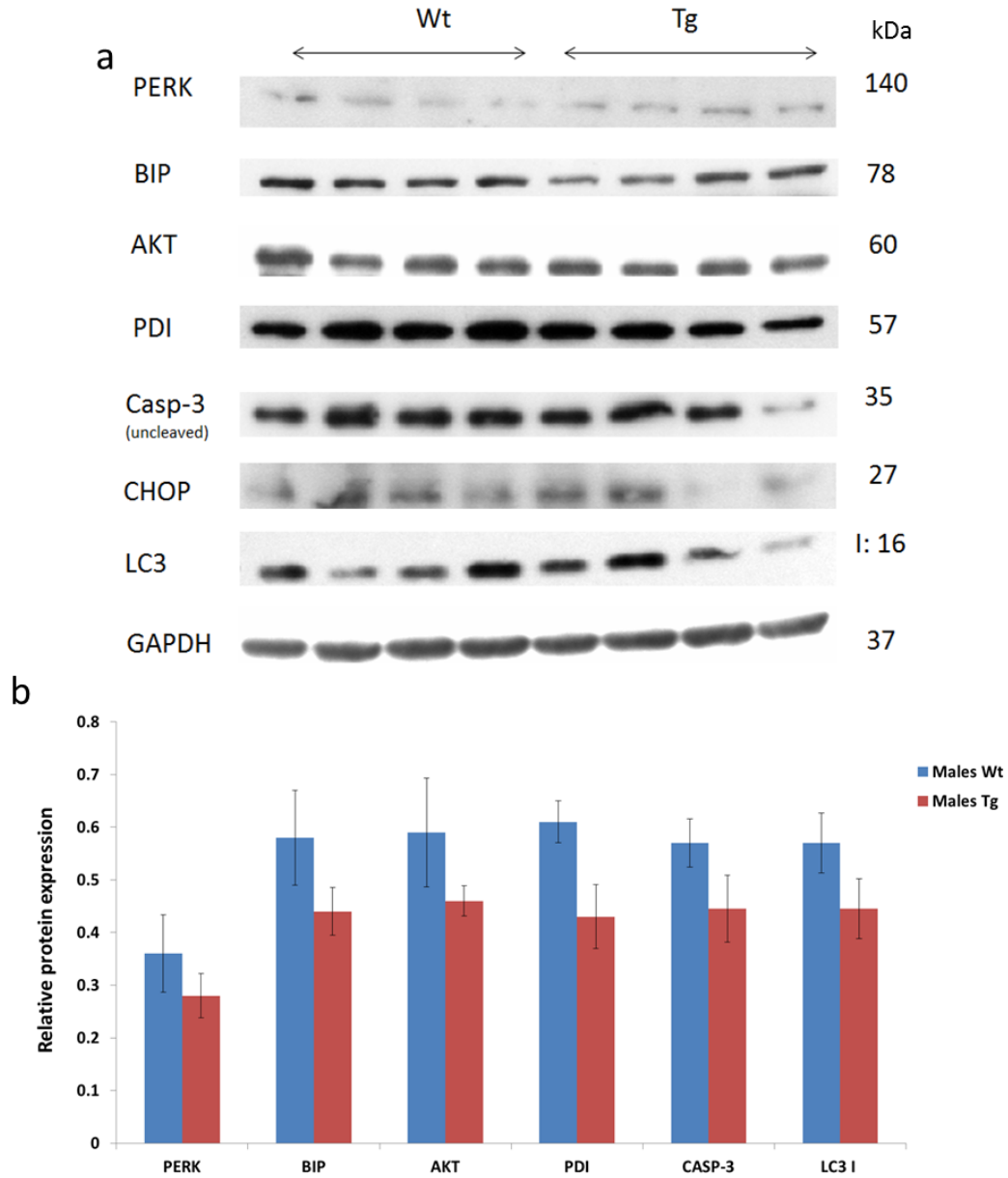
The ER stress proteins were analyzed in 11-weeks old male mice to see any changes in expression as the mice grew older. The experimental protocol was same as described for 1-day old hearts. The Tg hearts overexpressed ( $P < 0.05$ ) BiP (46%), PDI (176%), CHOP (140%), PERK (132%), uncleaved caspase-3 (186%), and Akt (95%) (figure12).



**Figure 12:** Upregulation of ER stress proteins in the hearts of 11-weeks old male Tg mice. **a)** Expression level of ER stress protein was analyzed in 4 Wt and 4 Tg hearts from 11-weeks old mice. GAPDH levels were monitored as loading control. **b)** Bar graphs represent quantification of densitometry analysis of the immunoblotted proteins represented as a percentage of the mean  $\pm$  SE of N=4 animals. Asterisk (\*) shows changes in protein expression, that were statistically significant (P<0.05).

### **3.6 ER stress response in the hearts of 16-weeks old mice**

The ER stress proteins were further analyzed in 16-weeks male old mice. The experimental protocol was same as described for 1-day old hearts. ER stress proteins downregulation in the Tg hearts; BiP (24%), PDI (30%), PERK (23%), uncleaved caspase-3 (32%), LC3-I (22%), and Akt (21%) (figure 13). None of the changes reached statistical significance. The bands for CHOP protein were difficult to quantify due to background signal, and were omitted for statistical analysis.



**Figure 13: Downregulation of ER stress proteins in the hearts of 16-weeks old male *Tg* mice.** **a)** Expression level of ER stress protein was analyzed in 4 Wt and 4 *Tg* hearts from 16-weeks old mice. GAPDH levels were monitored as loading control. **b)** Bar graphs represent quantification of densitometry analysis of the immunoblotted proteins represented as a percentage of the mean  $\pm$  SE of N=4 animals. Asterisk (\*) shows changes in protein expression, that were statistically significant ( $P < 0.05$ ).

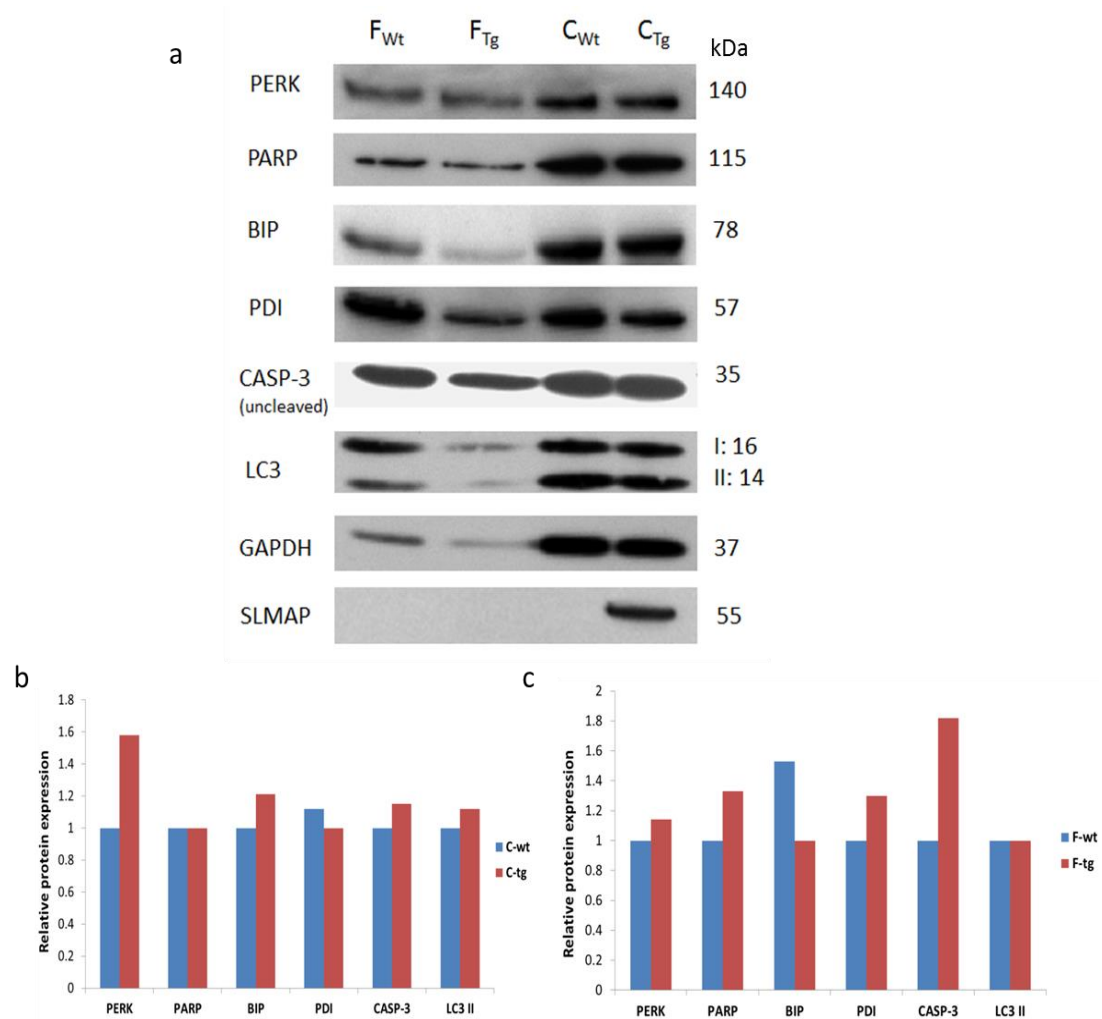
### **3.7 ER stress response in cardiomyocytes and fibroblasts of Tg hearts**

Disruption of homeostasis in the ER results in the accumulation of unfolded proteins, which leads to upregulation of ER stress genes, and activation of proteins that cope with the stress. It is known that the adult murine myocardium is mostly composed of myocytes (~60%) and fibroblasts (~30%) (Banerjee et al., 2007). In the analysis of ER stress proteins in the full heart tissue samples, we observed a trend in upregulation or downregulation in the Tg hearts, but often these changes did not reach statistical significance. In order to study precise effects of SLMAP overexpression in cardiomyocytes, we reasoned that separating the cardiomyocytes from fibroblasts in primary culture of neonatal mouse cardiomyocytes will give us a better understanding of the ER stress response. SLMAP1-TM2 is only expressed in the cardiomyocytes due to presence of cardiac muscle-specific  $\alpha$ -MHC promoter.

For each primary NMCM culture experiment, the hearts were collected from the litter 1-day after birth (~N=10). The pups were identified as Wt or Tg by genotyping (PCR) using tail clippings. The hearts from Wt and Tg pups were pooled separately for cell culture. The heart tissue was digested by collagenase enzyme to separate the cells. Sequential plating of the digested heart tissue suspension separated the fibroblasts from cardiomyocytes, because fibroblast-like cells adhere to culture plates very rapidly. After incubation period, the cells were scraped to collect the proteins. We were interested to see the expression level of various ER stress proteins in cardiomyocytes and fibroblasts from the Wt and Tg hearts. The primary NMCM culture experiment was done 3 times using the hearts from new litter each time.

Proteins samples were isolated from the fibroblasts and cardiomyocytes, and expression of ER stress proteins were compared by SDS-PAGE immunoblot analysis in a similar way as described for 1-day old hearts. The values obtained from quantification using densitometry were normalized for each cell culture experiment, and data for 3 experiments was combined (figure 14b and 14c). In bar graph from figure 14b, 14c, 15b, and 17b, error bars were not plotted because the data from multiple experiments was normalized and combined. Statistical analysis using paired t-test indicated that most of the ER stress proteins were upregulated in cardiomyocytes from Tg hearts (C-tg). C-tg overexpressed BiP (21%), caspase-3 (15%), LC3-II (12%), and PERK (58%). PDI was downregulated in C-tg by 11%. Cleaved caspase-3 or cleaved PARP were not detected. PARP expression remained unchanged in cardiomyocytes. Except BiP, the changes in proteins expression did not reach statistical significance ( $P>0.05$ ), because of the variations in different NMCM culture experiments.

The expression of BiP was decreased in fibroblasts from Tg hearts (F-tg) by 35% (figure 14). F-tg overexpressed PDI (30%), caspase-3 (82%), PERK (14%), and PARP (33%). However, the changes did not reach statistical significance ( $P>0.05$ ) due to previously mentioned reason. LC3 II expression remained unchanged in fibroblasts.



**Figure 14:** ER stress marker proteins in cardiomyocytes and fibroblasts from the Tg and Wt hearts. **a)** A representative western blot of fibroblasts and cardiomyocytes from 1-day old hearts shown by 1 NMCM culture experiment. The cells (F-wt, F-tg, C-wt and C-tg) were separated using collagenase digestion and sequential plating. Anti-myc antibody identifies the 6-myc tagged SLMAP protein which is only found in the cardiomyocytes of Tg mice. GAPDH levels were monitored as a loading control. **b)** Bar graph represents quantification of densitometry analysis of ER stress proteins expression between C-wt and C-tg. **c)** Bar graph represents proteins expression in F-wt and F-tg. The data from 3 experiments was normalized and presented here.

### **3.8 Effect of stress causing reagents on expression of ER stress proteins in cardiomyocytes and fibroblasts from the Tg and Wt hearts**

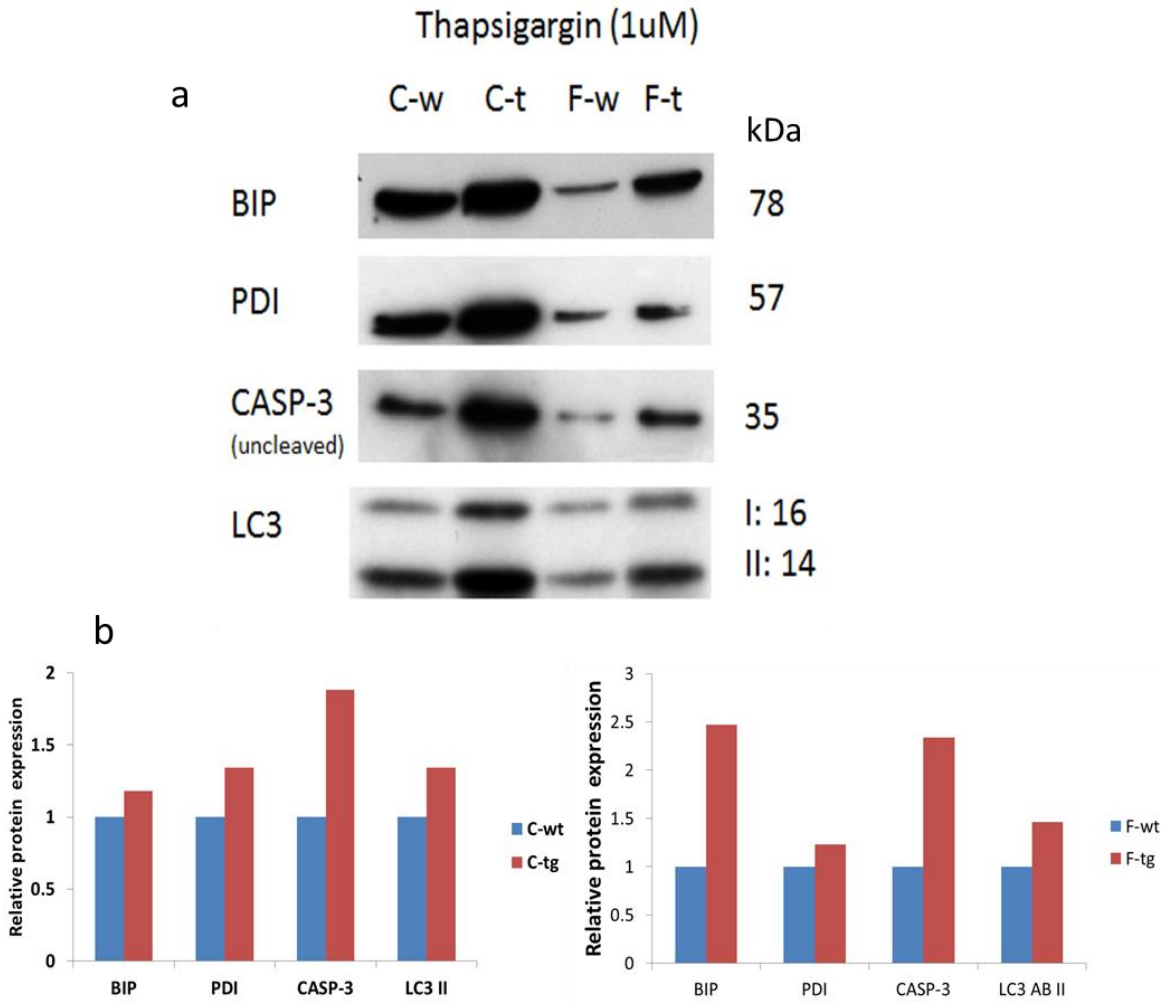
#### **3.8.1 Thapsigargin treatment**

Earlier findings from our lab have shown that SLMAP1-TM2 overexpression in Tg mice correlates with decrease in expression of SERCA2a (Nader et al., 2012). As a result, calcium uptake in SR is reduced, resulting in impaired cardiac electrophysiology. Other studies have also shown that altering SERCA2a expression impairs calcium homeostasis and cardiac function (Seth et al., 2004).

Thapsigargin is an inhibitor of SERCA (Cheng & Benton, 1994), and is known to cause ER stress and initiate UPR (McCormick, McColl, & Distelhorst). We were interested to see whether treatment of cells from primary NMCM culture with thapsigargin results in ER stress. It was reasoned that addition of thapsigargin will disrupt the calcium homeostasis to a greater extent in C-tg when compared to Wt counterparts, because calcium regulation has already been disrupted in C-tg due to overexpression of SLMAP.

The NMCM culture experiments with 1  $\mu$ M thapsigargin treatment were performed twice, yielding consistent results (figure 15). Treatment with drug resulted in overexpression of BiP (18%), PDI (34%), caspase-3 (88%), and LC3-II (34%) in C-tg. In F-tg, the ER stress maker proteins were upregulated, BiP (147%), PDI (23%), caspase-3 (134%), and LC3-II (46%). A suitable loading control was not found for this experiment. GAPDH,  $\beta$ -actin,  $\alpha$ -actin, Y-tubulin,  $\beta$ -tubulin, and histone H3 were monitored as potential loading controls, but the expression of these proteins changed dramatically

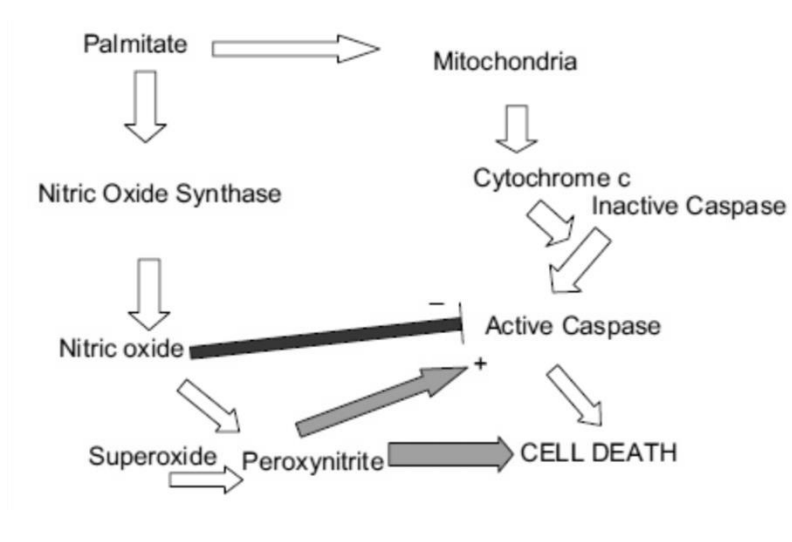
between similar cells. Consequently, the percentage change values presented here are ratio of protein expression in cells from the Tg versus Wt hearts (figure 15b). These were absolute values obtained from densitometry quantification.



**Figure 15:** *Effects of 1  $\mu$ M thapsigargin treatment on ER stress marker proteins in cardiomyocytes and fibroblasts from the Tg and Wt hearts. a)* A representative western blot of fibroblasts and cardiomyocytes from 1-day old hearts that were treated with 1  $\mu$ M thapsigargin for 24 hours. The expression levels of proteins related to ER stress response were measured. The cells (F-wt, F-tg, C-wt and C-tg) were separated using collagenase digestion and sequential plating. **b)** Bar graphs represent quantification of densitometry analysis of the immunoblotted proteins in fibroblasts and cardiomyocytes from the Wt and Tg hearts with 1  $\mu$ M thapsigargin treatment. The data from 2 experiments is normalized and presented here.

### **3.8.2 Palmitate treatment**

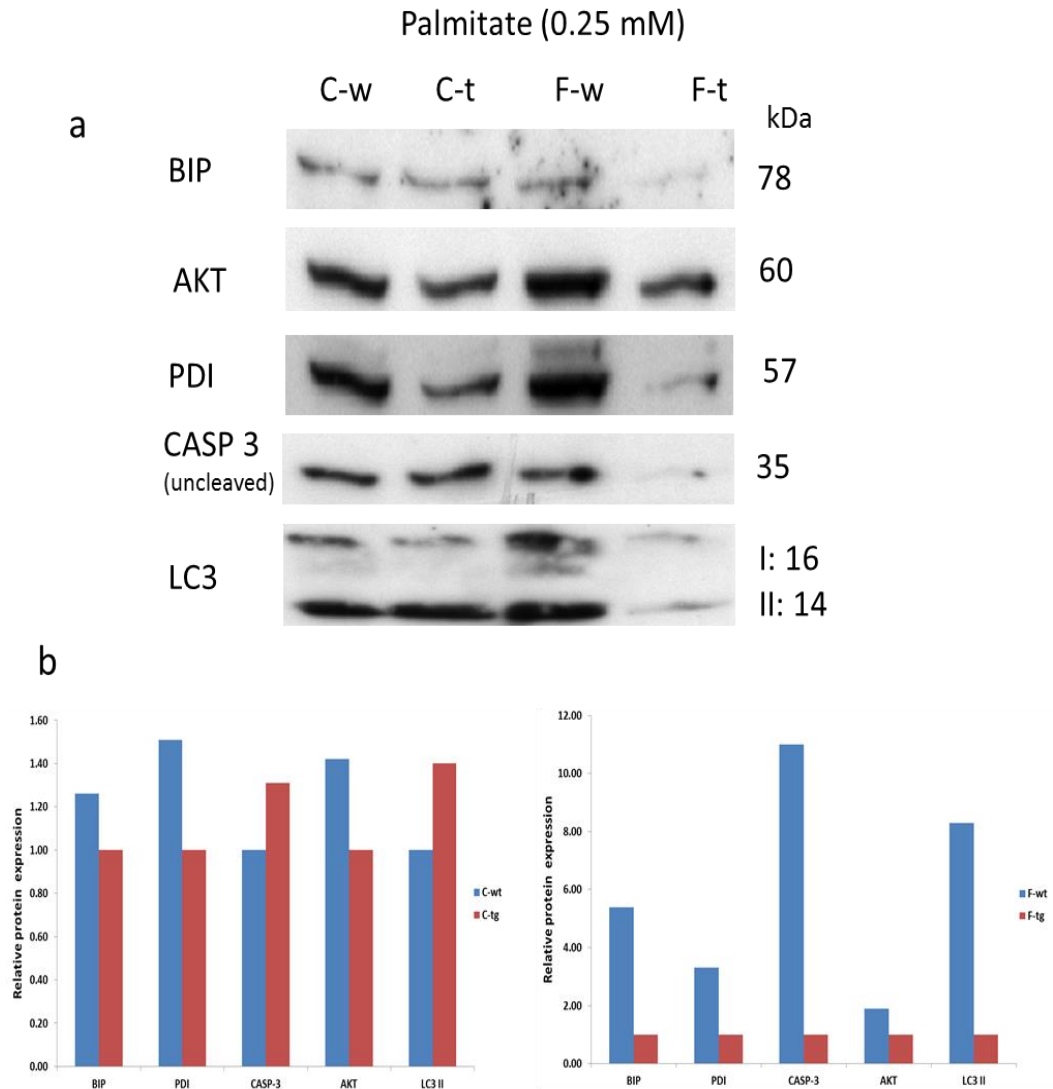
Palmitate is a saturated fatty acid which is known to induce oxidative and nitrosative damage (Rabkin & Klassen, 2008), and induces apoptotic cell death due to ER stress (Zhao et al., 2013). Palmitate induces production of ROS and reactive nitrogen species (RNS), which causes apoptotic cell death by activating caspase pathway. It is also known to increase the expression of BiP, CHOP, caspase-12, suggesting the induction of ER stress related cell death (Zhao et al., 2013). In other study, palmitate treatment induced a weak activation of ER stress via IRE1 and c-JUN NH2-terminal kinase (JNK) pathway in human muscle cells (Hassan et al., 2012).



**Figure 16:** *Scheme of palmitate-induced apoptosis.* Palmitate causes cell death by inducing cytochrome-c release from mitochondria and ROS/RNS generation, which results in activation of caspases. Figure is taken from Rabkin & Klassen (2008).

It was reasoned that addition of palmitate may cause ER stress, and cells from the Wt and Tg hearts may react differently to drug treatment; because overexpression of SLMAP in C-tg may enhance palmitate-induced ER stress response, or SLMAP may have a protective role against palmitate-induced ER stress.

The NMCM culture experiments using 0.25 mM palmitate was performed once (figure 17). Palmitate treatment resulted in overexpression of caspase-3 (31%) and LC3 II (40%) in C-tg. BiP (21%), PDI (22%), and Akt (30%) were downregulated in C-tg. In F-tg, the ER stress maker proteins were downregulated, BiP (81%), PDI (70%), caspase-3 (91%), Akt (47%), and LC3 II (88%). In NMCM culture experiment with palmitate treatment, a suitable loading control was not found. The percentage change values presented here are ratio of the protein expression in cells from the Tg and Wt hearts (figure 17b).

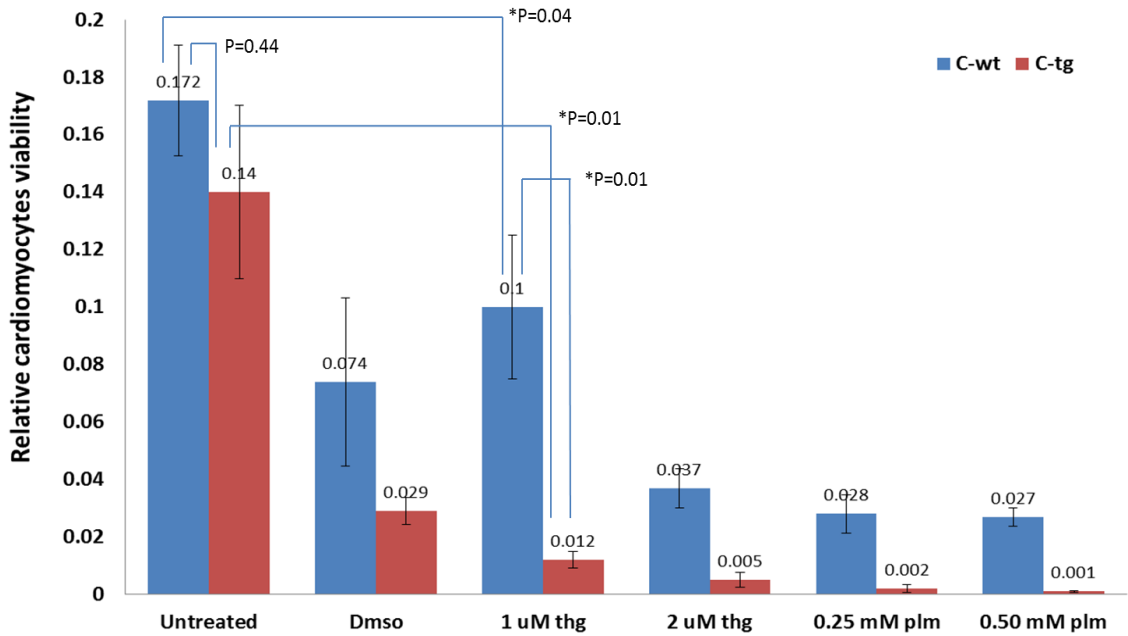


**Figure 17:** *Effects of 0.25 mM palmitate treatment on ER stress marker proteins in cardiomyocytes and fibroblasts from the Tg and Wt hearts. a)* A representative western blot of fibroblasts and cardiomyocytes from 1-day old hearts that were treated with 0.25 mM palmitate for 24 hours. The expression levels of proteins related to ER stress response were measured. The cells (F-wt, F-tg, C-wt and C-tg) were separated using collagenase digestion and sequential plating. **b)** Bar graphs represent quantification of densitometry analysis of the immunoblotted proteins in fibroblasts and cardiomyocytes from the Wt and Tg hearts with 0.25 mM palmitate treatment.

### **3.9 MTT assay measures viability of Tg and Wt cardiomyocytes**

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay measures number of viable cells. Besides measuring cytotoxic effect of different chemicals and apoptosis, the assay is commonly used for studying drug resistance and sensitivity in order to choose effective drug therapies in clinical settings. We wanted to explore the relation between SLMAP overexpression in C-tg and cell viability, and how the viability changes when the cells are challenged with stress causing agents (figure 18). This assay also served to validate that drug treatment in primary NMCM culture experiments is working effectively.

C-wt and C-tg were grown in multiwell microtiter plate. Cells were treated with thapsigargin (1  $\mu$ M and 2  $\mu$ M) or its diluent dimethyl sulfoxide (DMSO) as a control, and palmitate (0.25 mM and 0.50 mM) or its diluent water (2.5%) as a control. The assay is based on reduction of tetrazolium salt into a coloured formazan product, which is dependent on NAD(P)H-dependent oxidoreductase enzymes. Only living and metabolically active cells have the capability of converting the tetrazolium component of the dye into a coloured formazan product. The absorbance at 570 nm is directly proportional to number of viable cells.



**Figure 18:** Viability of the C-wt and C-tg. Viability of the cardiomyocytes was measured using MTT assay under untreated conditions, and using stress inducing agent: thapsigargin (thg: 1  $\mu$ M and 2  $\mu$ M), control for thapsigargin (DMSO: 0.065%), and palmitate (plm: 0.25 mM and 0.50 mM). Each value represented in the bar graph is average of readings from at least 8 wells of 96-well plate. The bar graph represents the average absorbance values combined from two different experiments. The cells attached in the well were treated with test compounds for 24 hours. Asterisk (\*) represents statistically significant P-values from paired t-test.

Student's paired t-test was used to compare the absorbance values between different groups. C-tg are 19% less viable than C-wt, but no statistical difference ( $P>0.05$ ) was found between them (figure 18). Upon treatment with stress inducing agents (thapsigargin and palmitate) for 24 hours, there was significant ( $P<0.05$ ) decrease in cell viability of both C-wt and C-tg, when compared with untreated conditions. For each treatment condition, the difference between viability of C-wt and C-tg was statistically significant ( $P<0.05$ ).

The decrease in viability of Wt cardiomyocytes under treatment relative to untreated conditions was DMSO (57%), 1  $\mu$ M thapsigargin (42%), 2  $\mu$ M thapsigargin (78%), 0.25 mM palmitate (84%), and 0.50 mM palmitate (84%). The decrease in viability of Tg cardiomyocytes under treatment conditions was DMSO (79%), 1  $\mu$ M thapsigargin (91%), 2  $\mu$ M thapsigargin (96%), 0.25 mM palmitate (99%), and 0.50 mM palmitate (99%). Under similar treatment conditions, the decrease in viability of Tg cardiomyocytes was greater, as compared to Wt cardiomyocytes.

Increasing the concentration of drugs treatment also decreased the cell viability. Treatment for cardiomyocytes with palmitate at 0.25 mM and 0.50 mM yielded almost same values for cell viability, with values of absorbance (hence viability) for Tg cardiomyocytes dropping to zero.

## CHAPTER 4: DISCUSSION

ER is a multi-function organelle that serves as a site for calcium regulation, lipid synthesis, protein folding and maturation. Failure to maintain tight regulation of calcium homeostasis, and protein folding leads to accumulation of unfolded proteins (Weston & Puthalakath, 2010). This disruption in homeostasis is known to be associated with ER stress, which activates an adaptive signaling pathway called UPR to help ER cope with the stress (Ozcan, 2012). ER stress is related to physiological and pathological states in the cardiovascular system (Groenendyk, Agellon, & Michalak, 2013).

SLMAP has been proposed to play a critical role in organization of the ER/SR structure. It impacts the molecular components of the E-C coupling apparatus, which are involved in calcium regulation. Overexpression of SLMAP in the myocardium of Tg mouse model has shown to affect the structure and function of the ER/SR. The increase in the SR volume and deregulation of calcium handling proteins caused impaired cardiac electrophysiology, and thus function (Nader et al., 2012). In the light of earlier findings from our group (Nader et al., 2012), we propose that abnormalities in the cardiac function due to overexpression of SLMAP leads to ER stress. In this study, we explored the relation between the overexpression of SLMAP in the myocardium and ER stress.

### **4.1 SLMAP overexpression impacts molecular components of the ER**

The myocardium is most vulnerable to stress right after birth, because slight changes in homeostasis due to insult (intrinsic and extrinsic factors) can trigger ER stress response (Groenendyk, Agellon, & Michalak, 2013). As the mouse grows older,

hypertrophic increase in the size of heart is required to meet greater blood circulation workload, which results in a better developed ER structure, and greater ability to cope with any stress (Martin & Blaxall, 2012).

Analysis of the 1-day old Tg hearts revealed a trend in downregulation of ER resident proteins, as compared to Wt hearts (figure 8). The male and female mice were not discriminated during hearts isolation for this experiment. SLMAP overexpression appears to downregulate the protein markers normally associated with ER stress at 1-day after birth. If the cells are exhibiting UPR, there should be an increase in expression of the ER resident molecular chaperones, which play a role in reestablishing homeostasis. Since SLMAP is overexpressed only after birth due to cardiac  $\alpha$ -MHC promoter, the changes caused due to SLMAP overexpression may not be significant enough to cause ER stress response at 1-day.

It has been previously shown that excess SLMAP is able to form dimers with endogenous SLMAP and affects the normal function of the protein (Nader et al., 2012). This happens due to presence of LZ domain in SLMAP, which helps the protein to interact and bind with other molecules in the vicinity. When SLMAP is being translated in excess, large amounts of proteins in the cytosol may bind to, and affect the function of other molecules including proteins, mRNAs, and transcription factors. As a result, the expression of some of the molecular components of the ER/SR may be affected. This may be the reason why a trend in decrease of some of the proteins related to ER stress response was observed at 1-day.

BiP is the principal regulator of ER stress and under normal development it plays an important role as a calcium buffer in the lumen of the ER (Lièvremon et al., 1997). Upregulation of BiP indicates disruption in ER/SR homeostasis, and activation of ER stress response (Sun et al., 2008). Since BiP was downregulated by 43% along with downregulation of other stress makers in the Tg hearts, SLMAP overexpression appears to impact the normal function of ER resident chaperones and calcium regulation, which disrupts the ER/SR homeostasis. This observation is supported by our lab's earlier findings (Nader et al., 2012) where downregulation of key calcium handling proteins was observed in the hearts of SLMAP overexpressing Tg mice.

The UPR is a prosurvival response in ER stress. The cell tries to eliminate misfolded proteins, conserve energy, suppress protein synthesis, and reestablishes homeostasis. ER stress only becomes maladaptive when homeostasis is not restored in the ER (Groenendyk et al., 2010). Overexpression of SLMAP may be negatively impacting the heart by downregulating the expression of ER resident proteins BiP and PERK. It may also prevent the activation of an adaptive coping response and interfere with normal cardiac development. Normal SLMAP levels appear to be important for appropriate expression of proteins involved in ER stress response.

At 1-day, overexpression of SLMAP appears to disrupt the normal expression of chaperones and ER resident proteins, which compromises normal cardiac function. Whether downregulation of these markers in Tg hearts compromises the ability of the ER to handle stress remains to be investigated. This observation suggests that normal expression of SLMAP is imperative for normal expression of ER components. We further analyzed older Tg and Wt mice in a similar way to examine the link between SLMAP

overexpression and altered expression of chaperones and molecular components of the ER.

Analysis of hearts from 5-weeks old mice shows that hearts of Tg females are undergoing ER stress, as compared to age-matched Wt females. This inference was made by observing upregulation of PERK, BiP, ATF6, and PDI (figure 9). BiP is a central regulator of ER function, and its upregulation is required to cope with ER stress in order to restore homeostasis (Sari et al., 2011). Overexpression of SLMAP seems to be inducing activation of BiP to initiate ER stress response. Upregulation of ATF6 and PERK in 5-weeks old Tg female mice shows that they are experiencing more stress than Wt counterparts. The PERK and ATF6 pathways of ER stress are adaptive, because they cause general translation attenuation, and upregulation of chaperones. The decline in proteins synthesis helps to conserve energy (ATP) and decrease protein folding load in the ER (Walter & Ron, 2011). The chaperones like BiP and PDI aid protein folding (Schrodera, Randal, & Kaufman, 2005).

CHOP is an important transcription factor that is activated downstream of the PERK pathway (Marciniak et al., 2004; Groenendyk, Agellon, & Michalak, 2013). CHOP target genes promote ER protein load and oxidative environment in the ER, which results in activation of apoptosis. Knockout of CHOP gene in mice renders significant protection against maladaptive ER stress response (Marciniak et al., 2004). Upregulation of CHOP protein by 45% further supports the view that overexpression of SLMAP is linked to ER stress in 5-week old Tg females. The disruption in homeostasis due to structural and functional changes caused by overexpression of SLMAP (Nader et al., 2012) may cause ER stress in 5-weeks old Tg females, as manifested by upregulation of

ER stress proteins. However, it needs to be further explored whether overexpression of SLMAP is directly causing the activation of ER stress, or the consequences of SLMAP overexpression are indirectly showing this effect.

Contrary to our observations in 5-weeks old female Tg mice, Tg males of same age exhibited a trend in downregulation of ER stress proteins in the hearts, when compared to Wt males. Downregulation of BiP, cleaved caspase-3, Akt, and active autophagy marker LC3 II indicated that SLMAP is involved in regulation of these ER resident molecules. These findings imply that ER stress due to overexpression of SLMAP is related to age and sex of mice

Autophagy is adaptive process that degrades and recycles unwanted macromolecules or organelles in the cell. The recycling process generates free amino acids and fatty acids which help to maintain energy production (Gustafsson & Gottlieb, 2008). During stress conditions autophagy is upregulated to protect cells; however, enhanced response can also lead to apoptosis due to excessive self-digestion (Lum et al., 2005). Downregulated of LC3 II by 24% also suggests that there no active ER stress response in Tg males at 5-weeks. The effects of SLMAP overexpression on chaperones and ER stress markers are different in males and females. To further explore how ER stress is modulated in Tg males during aging, the expression of proteins involved in ER stress response was analyzed in older males.

At the age of 8-weeks, upregulation of Akt in Tg males indicates activation of adaptive ER stress response. Akt has a protective role in ER stress response by repressing the cellular signals that lead to apoptosis (Zhao et al., 2013). The protective effect of Akt

upregulation in 8-weeks old Tg mice results in downregulation of proapoptotic marker CHOP and caspase-3. At this age, SLMAP overexpression seems to have a negative impact on ER/SR biology, resulting in activation of ER stress response. However, downregulation of CHOP and caspase-3 in Tg mice suggests that ER stress response due to SLMAP overexpression is relatively mild and adaptive.

During the aging process of mice, there is a shift in the balance from the adaptive ER stress response, to the maladaptive pathways that lead to apoptosis (Hussain & Ramaiah, 2007). The important chaperones in ER such as BiP and PDI, which are required for protein folding, are progressively oxidized and impaired during the aging process (Brown & Naidoo, 2012). Hence the efficiency of ER stress response to restore homeostasis is significantly declined. When UPR fails to restore homeostasis and the ER stress is prolonged, apoptotic pathways are activated (Schroder & Kaufman, 2005). Comparison of ER stress proteins at the age of 11-weeks in Wt males versus Tg male mice showed a significant increase in expression of ER stress proteins in Tg mice. Even though BiP was upregulated by 46%, it may have reduced efficacy, resulting in little protection against ER stress. Consequently, proapoptotic marker CHOP was upregulated by more than 2-fold in the Tg mice. In western blot analysis, cleaved caspase-3 was not detected, suggesting that caspase-dependent apoptotic pathway was not active. Since upregulation of PERK pathway also seems to be activated in Tg male mice at 11-weeks, it is reasonable to suggest that overexpression of SLMAP in the myocardium is resulting in long-term changes in ER/SR structure and function, ultimately leading to maladaptive ER stress response. A similar trend in upregulation of ER stress makers was seen in 5-weeks old Tg females; but in Tg males, this effect was not observed until mice reached the age

of 11-weeks. Excess of SLMAP in myocardium is specifically causing delayed launch of UPR and upregulation of these proteins in Tg males.

Surprisingly, a trend in downregulation of ER stress proteins was observed in 16-weeks old Tg male mice. The downregulation of chaperones (BiP and PDI) and PERK suggests that age dependent factors are specially impacting the expression of these proteins in the Tg hearts. The autophagy marker LC3 was also downregulated in the myocardium of Tg mice, which is consistent with the literature findings, where stress conditions reduce autophagy in older animals (Rajawat & Bossis, 2008). These findings suggest that the maladaptive ER stress pathways are active due to the natural aging process of the animal, and due to consistent overexpression of SLMAP. We suggest that overexpression of SLMAP is modulating the ER stress pathways by structurally and functionally disrupting the ER/SR structure in older Tg mice.

Oxidation and impaired function of chaperones in prolonged ER stress during aging has been linked to their reduced expression. With aging, an organism tends to produce more oxidation products (Brown & Naidoo, 2012). Downregulation of BiP protein and mRNA levels have been observed in various tissues as the mice become older (Naidoo et al., 2008; Gavilan et al., 2006). Additionally, PERK mRNA levels and activity of eIF2 $\alpha$  kinase enzyme has been reported to decrease in aged rat brain tissue. The decline in PERK signaling indirectly increases the expression of proapoptotic proteins like CHOP. Gavilan et al. (2006) observed an increase in CHOP and caspase-12 expression in aged rats that were stressed, but not in younger stressed animals; thus supporting the idea that animals are more prone to maladaptive ER stress pathways as they age.

## 4.2 ER stress response in neonatal cardiomyocytes and fibroblasts

In our Tg mouse model, the cardiac muscle-specific  $\alpha$ -MHC promoter derives the transcription of SLMAP1-TM2 in the C-tg (Molkentin, Jobe, & Markham, 1996; Nader et al., 2012). Since F-tg and F-wt do not have SLMAP overexpression, it was assumed that there will be no difference in expression of ER stress proteins in fibroblasts. At 1-day, fibroblasts compose approximately 30% of mouse heart (Banerjee et al., 2007). Considering that SLMAP is only overexpressed in C-tg, the changes in the ER/SR biology and related ER stress should only be impacting C-tg. Due to the presence of fibroblasts, it is possible that the changes in UPR pathways in C-tg may have been masked in the immunoblot analysis of full heart samples. It was speculated that separation of cardiomyocytes from fibroblasts in primary NMCM culture will give clear indication ER stress pathways that are active in cardiomyocytes.

Significant upregulation of BiP in C-tg as compared to C-wt suggests the activation of ER stress in C-tg, caused specifically by SLMAP overexpression. Furthermore, caspase-3, LC3 II, and PERK also exhibited overexpression in C-tg. Interestingly, 35% increase in the expression of BiP was observed in F-tg as compared to F-wt, suggesting that F-tg are indirectly responding to ER stress in C-tg. F-tg also had overexpression for other stress markers; PDI, caspase-3, and PERK. SLMAP overexpression is specifically implicated in ER stress response in C-tg. In addition, the findings indicate that cardiomyocytes may be communicating and affecting ER stress pathways in fibroblasts. The dynamic interaction between fibroblasts and cardiomyocytes through development is dependent on paracrine signaling and direct cell-cell communication (Kakkar & Lee, 2010). The cardiac fibroblasts are primarily responsible

for synthesis, deposition, and degradation of matrix proteins (Sullivan & Black, 2013). Due to their important role in the maintenance of extracellular matrix (ECM), their function is critical for the development and maintenance of heart function. Since fibroblasts are crucial for ECM maintenance, any disruptions in the homeostasis affects the function of cardiomyocytes, and thus contribute to cardiac pathology (Sullivan & Black, 2013). The findings further support the ability of cardiomyocytes to effectively communicate with fibroblasts under stress conditions.

#### **4.3 SLMAP sensitizes cardiomyocytes to thapsigargin induced stress**

Analysis of the expression of ER stress proteins in cardiomyocytes from Wt and Tg hearts revealed upregulation of these markers in C-tg. Since only C-tg overexpress SLMAP, we attributed upregulation of ER stress proteins to structural and functional changes in the ER/SR caused by SLMAP overexpression (Nader et al., 2012). To better understand the ER stress pathways in neonatal cardiomyocytes and fibroblasts from both Tg and Wt hearts, the cells were treated with known ER stress causing reagent thapsigargin, which disrupts calcium regulation in the cell by inhibiting SERCA2a (Cheng & Benton, 1994). We reasoned that if SLMAP is sensitizing the Tg heart to ER stress, then thapsigargin treatment would enhance the ER stress response notably in C-tg. Significant upregulation in of ER stress markers in both C-tg and F-tg implies that thapsigargin is augmenting the stress response in C-tg and F-tg. Previously in NMCM culture experiments without drug treatment, no change in active autophagy was detected in Wt versus Tg cardiomyocytes and fibroblasts. However thapsigargin treatment

increases the autophagy in C-tg and F-tg. Thapsigargin also increased the expression of caspase-3 in C-tg to near two-fold. Since cleaved caspase-3 and CHOP were not detected in immunoblot analysis, it seems that apoptosis pathways are not active at this concentration of thapsigargin (1  $\mu$ M). These findings imply that cells from Tg hearts are more prone to ER stress when challenged, as compared to Wt counterparts at 1-day. Additionally, more than two-fold increase in BiP and caspase-3 expression in F-tg suggests that cardiomyocytes are somehow affecting ER stress pathways in fibroblasts. The lack of a suitable loading control in western blot analysis makes the data difficult to quantify. However, two independent experiments yielded similar results.

#### **4.4 SLMAP overexpression protects cardiomyocytes from palmitate-induced ER stress**

The relation between SLMAP overexpression in C-tg and ER stress response was further explored by challenging the cells in NCMC culture with palmitate. Palmitate induces stress by causing oxidative and nitrosative stress (Rabkin & Klassen, 2008), while thapsigargin disrupts calcium homeostasis, as discussed above. The ER-resident chaperones BiP, PDI, and Akt were downregulated in C-tg, when cells were treated with 0.25 mM palmitate. The stress makers were also downregulated in F-tg. In this regard, Zhao et al., (2013) reported that 0.0625 mM palmitate treatment resulted in increased expression of BiP and CHOP in H9C3 rat cardiomyoblast, suggesting induction of ER stress. Overexpression of SLMAP may be desensitizing palmitate induced changes in ER stress by interfering with the pathways that result in chaperones expression. This analysis

suggests that increased SLMAP levels may dampen the ER stress response afforded by palmitate. Excess SLMAP seems to act like an anti-oxidant by inhibiting oxidative stress caused due to palmitate. However, mechanism of this effect needs further exploration.

#### **4.5 SLMAP overexpression reduces viability in cardiomyocytes**

The viability assay determines the percentage of cells that are alive or metabolically active. Our findings showed that C-tg from NMCM culture experiments were 20% less viable, indicating that SLMAP overexpression is reducing the viability of cardiomyocytes. Challenging cardiomyocytes with thapsigargin or palmitate not only reduced viability significantly, it also affected C-tg more than C-wt in a dose-dependent way. The control for thapsigargin treatment DMSO (0.065%) also reduced viability of cardiomyocytes more in Tg (79%) versus Wt (57%). Overexpression of SLMAP is making cardiomyocytes more inclined to cell death or arrested metabolic state. Since our data showed only a small increase in caspase-3 expression in C-tg (figure 14), and absence of cleaved caspase-3 and CHOP, it is very likely that SLMAP overexpression is affecting the metabolic state of cardiomyocytes, and/or compromising their survival through another mechanism. This may also explain changes in GAPDH (an enzyme involved in glycolysis), which we were unable to use as a suitable loading control in drug treated NMCM culture experiments.

## CONCLUSION

Collectively, the findings here suggest that normal levels of SLMAP in the heart are necessary for normal expression of chaperones, molecular components of the ER, and ER stress response proteins. Overexpression of SLMAP in the myocardium disrupts structure and function of the ER/SR, which consequently disrupts the homeostatic balance, and alters expression levels of these proteins. The changes observed in the expression of key ER stress proteins in the hearts of SLMAP transgenic mice were found to be dependent on the age and sex. Neonatal cardiomyocytes exhibited activation of ER stress response due to overexpression of SLMAP, and also illustrated the ability to effectively communicate with fibroblasts under stress conditions. Excess SLMAP sensitizes cardiomyocytes to thapsigargin induced stress, potentially via calcium deregulation. Furthermore, excess SLMAP is dampening ER stress response, potentially by inhibition of palmitate-induced oxidative stress. Viability of neonatal cardiomyocytes was also compromised due to SLMAP overexpression, and was further reduced due to drug treatment.

SLMAP plays an imperative role in membrane biology of myocardium, and thus cardiac function. It has exhibited unique expression patterns, ability to target membranes, roles in cell division, and signal transduction in membrane biology. According to our previous findings, normal levels of SLMAP are important for accurate MTOC function, muscle development, E-C coupling, and cardiac structure and function. In light of our current study, SLMAP is playing a role in modulation of key proteins that are usually associated with ER stress response. However, the mechanism by which these effects are exerted remains to be further explored.

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