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The Roles of Protein Disulfide-Isomerase Associated 6 and Alpha-B Crystallin in Chaperone-Mediated Cardioprotection

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

John Alan Vekich

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2013
The dissertation of John Alan Vekich is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego
San Diego State University
2013
DEDICATION

This dissertation, and all that I accomplish as a scientist, is for my loving wife, Michelle, son, Tyler and daughter, Alexa. Without them there would be no purpose behind the work that I do, no one to share in my successes, and no one to confide in my failures and frustrations. They make me who I am and without them I would be lost.
EPIGRAPH

When nothing seems to help, I go look at a stonecutter hammering away at his rock perhaps a hundred times without as much as a crack showing in it. Yet at the hundred and first blow it will split in two, and I know it was not that blow that did it, but all that had gone before.

- Jacob Riis

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'

- Sir Isaac Asimov
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LIST OF ABBREVIATIONS

AAV9 – adeno-associated virus serotype 9
AdV – adenoviral
ANP – atrial natriuretic factor
ATF4 – activating transcription factor 4
ATF6 – activating transcription factor 6
BNP – brain natriuretic peptide
bZIP – basic leucine zipper
CAD – coronary artery disease
CDP – CCAAT displacement protein
ChIP – chromatin immuno-precipitation
CHOP – CCAAT/enhancer-binding protein-homologous protein
COPII – coat protein II
cTNT – cardiac troponin t
CVD – cardio-cascular disease
Derl3 – derlin 3
DKO – double knock-out
eIF2α – eukaryotic initiation factor 2, α subunit
EMSA – electro-mobility shift assay
ER – endoplasmic reticulum
ERAD – endoplasmic reticulum-associated degradation
ERK – extracellular signal regulated kinase
ERO1 – ER oxidoreductin 1
ERp5 – ER protein 5
ERSE – ER-stress-response element
FAD – flavin adenine dinucleotide
GRP78 – glucose-regulated protein 78
GRP94 – glucose-regulated protein 94
GSH – glutathione reduced
GSSG - glutathione oxidized
HO-1 – heme-oxygenase 1
HSF1 – heat shock factor 1
HSP – heat shock protein
HSP70 – heat shock protein 70
HSPB2 – heat shock protein B2
I/R – ischemia reperfusion
IL-1 – interleukin 1
IP – immuno-precipitation
IRE1 – inositol-requiring protein-1
JNK – c-jun -terminal kinase
kD – kilodalton
KO – knock-out
LC-MS/MS – liquid chromatography-tandem mass spectrometry
LVDP – left ventricle developed pressure
MAPK – mitogen-activated protein kinase
MAPKAPK2 – MAPK activated protein kinase 2
MI – myocardial infarction
MKK – MAP kinase kinase
MKKK – MAP kinase kinase kinase
MTP – mitochondrial transition pore
NF-Y – nuclear factor Y-box
NO – nitric oxide
Nrf2 – nuclear factor (erythroid-derived 2) like 2
NRVMC – neonatal rat ventricular myocyte cultures
NTG – non-transgenic
OASIS – old astrocyte specifically induced substance
PDI – protein disulfide isomerase
PDIA6 – protein disulfide isomerase associated 6
PE – phenylephrine
PERK – protein kinase R (PKR)-like ER kinase
PI – propidium iodide
PKC – protein kinase C
PKN – protein kinasa C-related kinase 1
qRT-PCR - quantitative real time PCR
ROS – reactive oxygen species
S1P – site-1-protease
S2P – site-2-protease
si – simulated ischemia
si/R – simulated ischemia/reperfusion
siRNA – small interfering RNA
SR – Sarcoplasmic reticulum
TAC – trans-aortic constriction
TG – transgenic
TM – tunicamycin
TNF-α – tumor necrosis factor alpha
TRAF2 – tumor necrosis factor associated factor 2
TRAIL – TNF-related apoptosis-inducing ligand
TXNDC7 – thioredoxin domain containing 7
UPRE – unfolded protein response element
VEGF – vascular endothelial growth factor
VP16 – viral protein 16
WGA – wheat germ agglutinin
WT – wild-type
XBP1 – X-box binding protein 1
αBC – alpha-B crystallin
αMHC – alpha myosin heavy chain
βMHC – beta myosin heavy chain
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A portion of the text and figures in Chapter Two and Four is a reprint of the material as it appears in the Journal of Molecular Cellular Cardiology. I was primary author and the co-authors listed in this publication contributed to the research which forms a basis for Chapter Two and Four.

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ABSTRACT OF THE DISSERTATION

The Roles of Protein Disulfide Isomerase-Associated 6 and Alpha-B Crystallin in Chaperone-Mediated Cardioprotection

by

John Alan Vekich

Doctor of Philosophy in Biology

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San Diego State University, 2013

Professor Christopher Glembotski, Chair

Protein quality control has been shown to protect the heart from acute stresses such as ischemia and reperfusion (I/R). This dissertation explores two mechanisms by which protein quality control protects the heart from I/R-induced damage. The first study characterizes protein disulfide isomerase-associated 6 (PDIA6) as an endoplasmic reticulum (ER) stress response gene and its ability to
protect cultured cardiac myocytes from I/R damage. The second study elucidates the role of alpha-B crystallin (αBC) in protecting the myocardium from I/R injury using a novel knock-out (KO) mouse model.

I/R can impair ER protein folding and initiate the ER stress response. ER stress and I/R activate the transcription factor, activating transcription factor 6 (ATF6), which induces numerous genes, many of which have not been examined in the heart. In this study, we presented that, ATF6 induced the PDIA6 gene, which encodes an ER enzyme that catalyzes protein disulfide bond formation. Gain- and loss-of-function studies showed that PDIA6 protected cardiac myocytes against simulated I/R-induced death in a manner that was dependent on the catalytic activity of PDIA6. Thus, by facilitating disulfide bond formation, and enhancing ER protein folding, PDIA6 may contribute to the protective effects of ATF6 in the ischemic heart.

Overexpression studies have shown that the small heat shock proteins (sHSP) can protect from I/R damage. Previous studies have shown that hearts harboring the deletion of both αBC and HSPB2, both sHSP family members, were more susceptible to I/R damage. In this study, we generated and characterized a mouse line in which only αBC was deleted (αBC KO), in order to clarify the roles of αBC and HSPB2 in cardioprotection. Cardiac morphology and function of the αBC KO mice were indistinguishable from age-matched wild-type (WT) mice, although a few markers of cardiac pathology were elevated, suggesting an underlying phenotype. The function of isolated perfused hearts
from αBC KO mice were initially indistinguishable from those from WT mice, however, after I/R, αBC KO mouse hearts exhibited a 50% reduced functional recovery when compared to WT mouse hearts. Thus, αBC is required for maximal functional recovery from I/R-induced injury.
CHAPTER ONE

I. Introduction

Cardiovascular disease (CVD) continues to be the leading cause of death among Americans. Nearly, a quarter of all deaths in the United States are the result of CVD [1]. One of the most common forms of CVD is coronary artery disease (CAD), which is a build-up of plaque in the vessels that supply blood to the heart. This build of plaque can lead to atherosclerosis, a narrowing and hardening of the blood vessels, which can weaken the heart muscle resulting in arrhythmia, and ultimately heart failure. If the vessels that supply blood to the heart become occluded, tissue distal to the blockage will fail to receive nutrients and oxygen, resulting in a myocardial infarction (MI).

In 2008, nearly 1.5 million Americans suffered from a coronary event. Approximately, every 25 seconds an American suffered a coronary event and, on average one fatal coronary event every minute. Besides the social impact of CVD, nearly $300 billion were spent on direct and indirect costs associated with CVD and stroke [1]. Developing new, more effective, treatments for CVD is therefore paramount to reducing the impact of CVD on our society. The key to creating new and better treatments is to gain a more thorough understanding of
the mechanisms by which the heart protects itself during stress, and how to augment and enhance these natural, endogenous protective mechanisms.

A. Ischemia/Reperfusion Injury in the Myocardium

During a MI, the vessels supplying blood to the heart are blocked; thus, the tissue distal to the blockage is deprived of nutrients and oxygen, and a buildup of CO₂ occurs. This state of low nutrients and oxygen is called ischemia. During ischemia, myocyte survival and continued function depend on how quickly the blockage that created the ischemic condition can be cleared. Once the blockage is removed, and blood flow restored, an additional stress is placed on the tissue. This state when blood flow is restored is called reperfusion.

During ischemia, the ATP levels within cardiomyocytes drop rapidly, as the cell switches from aerobic to anaerobic metabolism. Along with the rapid drop in ATP, glycolytic metabolites, such as lactic acid, build-up [2]. The accumulation of lactic acid, along with the increased levels of CO₂ associated with ischemia, can lower cellular pH, creating a condition called acidosis [3]. Altering ATP levels and the pH can drastically change the environment in which the proteins function and fold. Even short exposure to these conditions can trigger the apoptotic cascade and result in cell death [4].

If the patient survives the initial ischemia, and the formerly blocked coronary vessel is reopened, whether by a natural passage of the obstruction, or
by some intervention, such as a stent, angioplasty, bypass, or thrombolytic drugs, the heart will face a new set of challenges initiated by the reinstatement of coronary blood flow during reperfusion [5]. The reinstatement of blood flow brings the necessary nutrients and oxygen back to the previously ischemic tissue, but along with the needed nutrients and oxygen is a burst of reactive oxygen species that is released during the initial moments of reperfusion. These reactive oxygen species can damage every macromolecule in the cell by attacking peptide bonds, protein side chains altering the protein’s structure and function which can lead to cellular dysfunction and eventual cell death [6].

B. Protein Folding

Protein quality control assists in the proper folding of nascent proteins, prevents mature proteins from denaturing and misfolding, and aids in the degradation of terminally misfolded proteins [7]. Under normal physiological conditions, the cells of the heart are under tremendous mechanical and energetic stress. During pathological conditions, such as ischemia and hypertension, extra stress is placed on the heart. This extra stress could impair the ability of the heart to maintain homeostasis in the cytosol and endoplasmic reticulum (ER), causing the unfolding and misfolding of proteins. In organs that have a limited regenerative capacity, such as the heart, this is even more critical, as there can be permanent tissue damage if the cells are unable to survive until the stress is resolved [8]. Two mechanisms which may allow the cell to survive are the ER
stress response and the intrinsic ability of molecular chaperones to prevent mature proteins from unfolding [8, 9].

C. Misfolded Proteins and Pathology

The accumulation of misfolded proteins can be damaging to the cell, and has been implicated in the pathogenesis of numerous diseases [10, 11]. The aberrant folded proteins can come from genetic disorders, such as diabetes mellitus or cystic fibrosis, or from external factors, such as myocardial infarction or stroke [8, 12]. The misfolding of proteins can damage the cell, cause pathological conditions, or even death [8, 12]. These protein-folding maladies affect many organ systems, for example: neurodegenerative diseases such as Alzheimer’s and Parkinson’s, hemopoietic diseases such as sickle cell anemia and hemophilia, cardiac diseases such as atherosclerosis and myocardial infarction. As such, a better understanding of how proteins fold and how the cell copes with conditions that cause the misfolding of proteins is needed to address these diseases.
CHAPTER TWO

I. Introduction

A. Protein Folding in the Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a specialized cellular compartment in which approximately 35% of cellular protein is produced, including the majority of transmembrane proteins and proteins destined for cellular export [13]. The cytosolic face of the ER is decorated with ribosomes and is involved in translation of both ER and cytosolic targeted proteins [14]. Those proteins destined for the ER are cotranslationally passed through the ER translocon and assume either an ER lumen, or transmembrane conformation [15, 16]. The lumen of the ER contains chaperones that aid the folding of nascent proteins, proteases which cleave the proteins into their functional units, isomerases which help form the correct disulfide linkages, and a myriad of other proteins involved in the post-translational modification of proteins such as N-linked glycosylation and acetylation [17]. Along with its role in protein folding, the ER also plays a critical role in protein quality control. Proteins that pass through the ER are only allowed to proceed to their final destination, whether it be for insertion into the cell membrane, or for secretion, if they are properly folded and carry the correct post-translational modifications [12, 18, 19].
B. Endoplasmic Reticulum Stress

1. The ER Stress Response

To prevent ER stress due to the misfolding of proteins, cells have a system for detecting and preventing the accumulation of misfolded protein and initiating a signal transduction pathway to restore proper function to the ER. This signal transduction cascade is called the ER stress response [20].

The ER stress response is mediated by three major ER transmembrane proteins that act as sensors and initiators: PKR double-stranded-RNA-dependent protein kinase-like ER kinase (PERK), inositol-requiring protein 1 (IRE1), and activating transcription factor 6 (ATF6). One mechanism by which the sensors are regulated is through their association with the chaperone glucose-regulated protein 78 (GRP78). Under basal conditions these proteins are anchored to the luminal side of the ER in their inactive form by GRP78. GRP78 is a well characterized molecular chaperone with a major role in detecting and re-folding misfolded proteins [7, 20]. During ER stress the amount of misfolded proteins increases in the ER, and GRP78 preferentially binds to the misfolded proteins in the ER and releases from the three ER stress sensors. Once GRP78 is released from the three sensors, this allows the three sensors to transition into their active forms [21].

Another mechanism by which the ER stress response may be activated is through the direct binding of misfolded proteins to the luminal domains of IRE1
and PERK. The association of the luminal domains of IRE1 or PERK with misfolded proteins is thought to provide a scaffold for the oligomerization of IRE1 or PERK. The oligomerization of IRE1 or PERK induces subsequent auto-phosphorylation and activation [22]. In this proposed mechanism, the association of GRP78 with IRE1 and PERK is believed to serve as a tuning mechanism, insuring that there are sufficient concentrations of the monomeric forms of each of the sensors available for activation [23]. Although the evidence for this mechanism is far stronger for IRE1 than PERK, since mutation to the GRP78 binding domain of PERK creates a constitutively active form of the protein [24], the interaction between PERK and GRP78 is evidently sufficient to regulate its activity.

Alternative mechanisms have been proposed for the regulation of ATF6. Calreticulin has been implicated in the retention of ATF6 in the ER [25]. During ER stress, under-glycosylated ATF6 cannot interact with calreticulin, which facilitates the translocation of ATF6 to the Golgi [25]. Another mechanism by which ATF6 may be regulated is through inter- and intra-molecular disulfide bonds between the luminal domains of ATF6 [26]. Under basal conditions, ATF6 retains these disulfide bonds and oligomerizes [26]. The multimeric form of ATF6 is unable to translocate to the Golgi, but during ER stress these disulfide bridges can be reduced, allowing ATF6 to form monomers to be transported to the Golgi [26].
Acute activation of the ER stress response can lead to the up-regulation of pathways that protect the cell, and resolve the overload of misfolded protein within the ER. Otherwise, chronic activation to the ER stress response, and the failure to resolve the condition that leads to the misfolding of proteins, can lead to the activation of apoptosis (Fig.1) [7, 20].

2. Activation of PERK

PERK homodimerization results in its autophosphorylation and activation [21, 27]. Phosphorylated PERK is able to phosphorylate the α subunit of eukaryotic initiation factor 2 (eIF2α), a ribosomal protein. Phosphorylation of eIF2α decreases ribosomal activity, resulting in general translational repression, and a decrease in global protein synthesis, including a decrease in the newly-synthesized protein load placed on the ER [28]. The lighter load of newly synthesized proteins is thought to ease the requirement of the ER to fold proteins and to facilitate the resolution of the stress and return the cell to homeostasis. Although the phosphorylation of eIF2α results in general translational repression, translation of a select group of genes is actually increased during ER stress; one of these genes is activated
Figure 1. Activation of the ER Stress Response

Represented are the three main regulators of the ER stress response, PERK, ATF6, and IRE1, in their inactive state (top), or in response to ischemic/reperfusion stress (bottom). Ischemia/reperfusion results in a decrease in ATP, an increase in CO₂, Ca²⁺, redox deregulation, and an inhibition of glycosylation. These perturbations can lead to an increase in misfolded proteins and activation of the three major sensors of ER stress. Acute activation can initiate protective signaling (black arrows), whereas chronic activation, when the stress remains unresolved, can induce a pro-apoptotic signaling cascade (red arrows).
transcription factor 4 (ATF4) [29]. The 5’ untranslated region of the ATF4 mRNA contains multiple short open reading frames prior to the real start of the gene [30]. Under basal conditions, the ribosome detects these short peptides and disengages before the true start is reached [31]. eIF2α is able to initiate ATF4 gene transcription at the true start site by inhibiting the assembly of the ribosomal complex, specifically the 40s scanning ribosome, which allows the ribosome to skip the false start sites and begin translation of ATF4 at the true start site [32]. ATF4 has been implicated in the transcription of protective ER stress response genes, but prolonged activation of ATF4 can lead to the transcription of pro-apoptotic genes, one of which is CCAAT/enhancer-binding protein-homologous protein (CHOP), a transcription factor which initiates apoptotic cell death [29].

3. Activation of IRE1

Like PERK, IRE1 also homodimerizes, this dimerization and phosphorylation results in activation [21, 27]. Unlike PERK, the activation of IRE1 results in a unique endoribonuclease activity, and the splicing of the x-box binding protein-1 (XBP1) mRNA, leading to the formation of an alternatively spliced variant of XBP1 [33, 34]. The spliced form XBP1 mRNA encodes for a potent transcription factor, which regulates the expression of a multitude of ER stress response genes [35]. Again, like PERK, the extended activation of IRE1 can lead to pro-apoptotic signal transduction. In the case of IRE1, prolonged
activation leads to tumor necrosis factor associated factor 2 (TRAF2) activation
and the activation of the pro-apoptotic C-Jun N-terminal kinase (JNK) pathway
[36].

4. Activation of ATF6

The unmasking of the Golgi localization sequence of ATF6, allows it to
translocate to the Golgi apparatus via coat protein II (COPII) mediated vesicles
[18, 37]. Once ATF6 is transported to the Golgi, it is cleaved by two proteases,
site-1 and 2 proteases (S1P, S2P). This cleavage event releases a 50-kilodalton
(kD), N-terminal fragment from the cytosolic side of the ER, which translocates to
the nucleus, where it acts as a potent transcription factor [38]. The N-terminal
fragment of ATF6 regulates the transcription of many ER stress related genes
[38]. In addition to a DNA-binding domain and a transactivation domain, ATF6
contains a domain that shares homology to the VN8 region of the herpes simplex
viral protein 16 (VP16) and like VP16, ATF6 is rapidly degraded after its
activation [39].

5. Activation of Novel Types of ER Stress Transducers

The old astrocyte specifically induced substance (OASIS) family of basic
leucine zipper (bZIP) transmembrane transcription factors are similar to ATF6
[40]. This family of transcription factors includes OASIS, Luman, BBF2H7,
CREBH and CREB4 [40]. Each is targeted to the ER and contains
transmembrane domains, which allows them to anchor to the ER [41-46]. They also have transcriptional activation domains and a bZIP domain. Additionally, they all have cleavage sites recognized by S1P in the Golgi [41, 47]. While release from the ER, translocation to the Golgi, and subsequent cleavage of OASIS, BBF2H7, and CREBH are ER stress dependent processes, the stimulus of activation for Luman and CREB4 is currently unclear [40]. Interestingly, the expression of the OASIS family members is more specific than that of ATF6, suggesting a more specialized function for these transcription factors. Indeed, there is a growing literature describing the role for OASIS in regulating the ER stress response in cell differentiation and maturation [48, 49]. Besides their differential tissue expression, the OASIS family members also recognize different transcriptional target elements, and as a result, regulate a different subset of genes than ATF6 [40]. OASIS and BBF2H7 have been shown to bind to CRE-like domains and to regulate Col1a1 and Sec23a, respectively [49, 50]. Luman and CREB4 have been shown to bind to the unfolded protein response element (UPRE) and ER stress response element-II (ERSEII) and to regulate Herp, RAMP4, and EDEM [51-53]. CREBH is able to recognize ER stress response element-I (ERSEI), ERSEII and CRE-like transcriptional elements, and has been shown to regulate CRP and SAP [54].
C. Gene Induction by ATF6

1. Promoter Elements Recognized by ATF6

The N-terminal portion of ATF6 is a potent transcription factor that is able to recognize specific DNA sequences, or elements, found in the 5' flanking region, or promoter, of a subset of ER stress inducible genes. The ER stress response element (ERSE, CCAAT-N9-CCACG), the ER stress response element-II (ERSEII, ATTGG-N-CCACG), and the unfolded protein response element (UPRE, TGACGTGGA) are recognized by the ATF6 as well as spliced XBP1, although XBP1 is thought to preferentially bind the UPRE [55-58]. Additionally, since both ATF6 and XBP1 are members of the basic leucine zipper (bZIP) family of transcription factors, they are thought to homo- and heterodimerize, which may facilitate their binding to the aforementioned elements [59]. ATF6 and XBP1 require strict fidelity to recognize the ERSE, ERSEII and UPRE where only certain substitutions are tolerated [57]. Furthermore, there may be other elements that have not been fully characterized as ATF6 and XBP1 binding sites that would classify the genes that contain them as ER stress response genes [60].

6. ATF6-Regulated Genes

After ATF6 is cleaved and translocated to the nucleus, it regulates the expression of specific genes, many of which encode proteins that help restore
the proper protein folding environment in the ER. These genes encode ER chaperones, such as GRP78 and glucose regulated protein 94 (GRP94), isomerase, such as protein disulfide isomerase (PDI), and proteins associated with ER associated protein degradation (ERAD), such as Derlin 3 (Derl3) [60]. Each class of proteins upregulated by ATF6 plays a role in restoring the folding environment of the ER. These proteins can aid in folding misfolded proteins, chaperones and isomerases, or degrading terminally misfolded proteins, and ERAD-associated proteins, all of which play a role in returning the ER folding environment back to homeostasis and maintain critical protein folding until the stress that caused the perturbation can be resolved [7, 20].

7. ER Stress Response in the Heart

To examine the function of the ATF6 branch of the ER stress response in the heart, we previously developed a line of transgenic (TG) mice that expresses a conditionally activated form of ATF6 in the myocardium [9]. We have shown that, when ATF6 is activated, the hearts of these mice are resistant to ischemia/reperfusion injury [9]. A microarray analysis of ATF6 TG mouse hearts showed that 381 genes were induced by ATF6. However, many of those genes were not previously known to be ATF6-regulated, and/or have not been previously studied in the heart [60]. Many of the ATF6-inducible genes in the heart have consensus, or near-consensus ERSEs in their regulatory regions,
suggesting mechanisms by which ATF6 might induce them [61]. One of the genes induced by ATF6 in the heart was protein disulfide isomerase-associated 6, or PDIA6, which has not been studied in the heart.

8. **PDI family of proteins**

The PDI proteins comprise a specialized family of ER-resident chaperones that facilitate the formation, isomerization and reduction of disulfide bonds [62]. PDI proteins are found in the greatest abundance in tissues with the highest collagen synthesis, but they are nearly ubiquitously found in all tissue and cell types. These proteins are highly abundant in the ER, and can constitute up to 1% of the total ER protein [63]. There are at least 17 members of this family, which are categorized by the presence of at least one or more domains that are homologous to thioredoxin. Many of these domains contain a cysteine active site (CXXC) which allows for the catalysis of disulfide bond formation [64].

The folding of reduced substrates is initially catalyzed by conserved oxidized cysteines in the active sites of the PDIs. The subsequent reduced PDI is reoxidized by ER oxidoreductin (Ero1), which then transfers electrons to molecular oxygen via flavin adenine dinucleotide (FAD) [65, 66]. Sometimes, this results in non-native disulfide bond formation. The non-native
Figure 2. Schematic of PDIA6-Facilitated Isomerization
The left panel depicts an improper disulfide bond being attacked by the thioredoxin domain of PDIA6. The middle panel depicts PDIA6 in a mixed disulfide state where the improper bond was broken and a new bond is formed. From the mixed disulfide, the proper disulfide bond can be formed (right panel) or another improper disulfide bond (left panel). If an improper disulfide bond is formed the isomerization process continues but if a proper disulfide bond is formed the client protein is released.
bonds require the disulfide bond to be broken and refolded in a process called isomerization. The PDIs carry out the isomerization reaction of multiple reductions and oxidations reactions using reduced glutathione as a co-reductant, until the correct conformation is obtained [67] (Fig. 2).

Along with their chaperone and isomerization functions, PDIs have been implicated in other functions as well. For example, PDI has been shown to play a major role in asymmetrical gene expression during development, where PDI-null zebrafish developed major heart defects and exhibited disrupted gene patterning [68]. PDIs have also been implicated in the protein loading onto MHC class I and the regulation of NAD(P)H oxidase [69-72]. Recent studies have also shown that over-expression of PDI in mouse hearts reduces infarct size and significantly reduce cardiomyocyte apoptosis in the perifarct zone [73].

The ER contains several different PDIs, which vary in size, catalytic potential, and substrate specificity. One ER-resident PDI is protein disulfide isomerase-associated 6 (PDIA6), also known as PDI P5, thioredoxin domain containing 7 (TXNDC7), or ER protein 5 (ERp5) [74]. The PDIA6 gene encodes a protein with an ER targeting signal sequence, a C-terminal KDEL motif, and two thioredoxin domains. PDIA6 is the smallest PDI, at 50kD in
Figure 3. PDI Family Protein Domain Structure
PDI family gene names listed (left). Blocks “a” indicate thioredoxin domains containing active reductive sites which facilitate protein isomerization. Blocks “b” represent thioredoxin domains without an active site, which are thought to contain client protein recognition motifs. Blocks “c” indicate high acidity domains, which currently have no ascribed function.
size, to contain two active thioredoxin domains, and a third catalytically inactive domain [62] (Fig. 3).

Disulfide bond formation is one of the rate-limiting steps in ER protein folding, and it is dependent on molecular oxygen [75]. Accordingly, hypoxia-mediated activation of the ER stress response during ischemia may up-regulate PDI family member genes, such as PDI and PDIA6, that increase ER-protein folding and disulfide bond formation [76, 77]. This increased ER-protein folding capacity may aid in cell survival and limit ischemic damage [73, 78, 79]. Moreover, PDIs have been linked to cardioprotection [79, 80]. While much is known about other members of the PDI family of proteins, PDIA6 is one of the least studied; moreover, to the best of our knowledge, it has not been studied in the heart.

PDIA6 was of interest, not only because of it was ATF6-inducible, but also because it encodes a protein with a predicted ER retention sequence, C-terminal KDEL (lys-asp-glu-leu), and its predicted molecular mass (50kD) matched that of a previously uncharacterized ER stress-inducible protein observed in previous studies [81].
D. Hypothesis: PDIA6 is responsible, in part, for the protection mediated by ATF6

During times of ER stress and activation of the ER stress response, both glucose regulated protein 94 (GRP94) and glucose regulated protein 78 (GRP78) are up-regulated and detectable by immunoblotting using an antibody raised against the C-terminal KDEL moiety [81, 82]. Along with GRP94 and GRP78, another unidentified protein is detected by the KDEL antibody. This unknown 50kD protein is co-regulated with GRP94 and GRP78 in terms of activation by the activating transcription factor 6 (ATF6) branch of the ER stress response (ERSR), and its identity has remained a mystery since the development of the KDEL antibody in 1990 [81, 82]. Other genes induced by ATF6 in the ER stress pathway, such as GRP94 and GRP78, have been shown to protect cardiac myocytes against IR damage; thus, this protein could contribute to the mechanism by which the ATF6 branch of the ERSR can protect cardiac myocytes during ER stress. The overall hypothesis is that the previously unknown, 50kD, KDEL-positive ER stress-inducible protein is PDIA6 and that it protects cardiomyocytes by enhancing disulfide bond formation during ischemia or ischemia reperfusion injury (Fig. 4).
Figure 4. Depiction of the Overall Hypothesis: PDIA6 mediated cardiac myocyte protection
ER stress, such as myocardial infarction, leads to activation and translocation of ATF6 to the nucleus, where it induces several protective genes, some of which aid in restoring the ER protein-folding environment. One of these genes is PDIA6, which can facilitate the folding of nascent and misfolded proteins in the ER through its isomerization capability, ultimately leading to recovery from the stress and cell survival.
II. Materials and Methods

A. Cultured Cardiac Myocytes

Neonatal rat ventricular myocytes (NRVMCs) were isolated as described [83] from 1-2-day-old Sprague-Dawley rat hearts digested with collagenase, and purified by passage through a Percoll gradient, as described [84].

B. Immunoblotting

Immunoblotting was carried out as previously described [39] using antibodies raised against KDEL (cat# SPA-827, Stressgen, Ann Arbor, MI), PDIA6 (cat# ab37756, Abcam, Cambridge, MA) or GAPDH (cat# RDI-TRK5G4-6C5, Fitzgerald Industries International, Concord, MA) at dilutions of 1:1000, 1:1000, and 1:1.5x10^5, respectively.

C. Immunopurification/Depletion

Ten µg of KDEL antibody (cat# SPA-827, Stressgen, Ann Arbor, MI) were combined with 40µl of a 50% slurry of protein A sepharose beads (Pierce Classic IP Kit, cat# 26146, Thermo Scientific, Rockford, IL) and conjugated to the beads using 10µl of disuccinimidylsuberate (DSS) (cat# S1855, Sigma-Aldrich, St Louis, MO) at 13 mg/ml in DMSO for 1hr at 22ºC. The KDEL antibody conjugated beads were then used as per manufacturer’s instructions (Pierce Classic IP Kit, cat# 26146, Thermo Scientific, Rockford, IL).
D. ATF6-MER TG Mice

All animal procedures were carried out in accordance with the institutional guidelines. The animal protocol used in this study was reviewed and approved by the San Diego State University Institutional Animal Care and use Committee. The generation of ATF6-MER (mutant mouse estrogen receptor) transgenic (TG) mice featuring cardiomyocyte-specific transgene expression was described previously [9]. Non-transgenic (NTG) and ATF6-MER transgenic (TG) mice were treated with vehicle or tamoxifen, which activates ATF6 only in the TG mouse hearts. Tamoxifen (cat# T5648, Sigma-Aldrich, St Louis, MO) was suspended at 10mg/mL in 100µL of 95% ethanol and 900µL of sunflower oil and sonicated until clarified. Animals were injected intra-peritoneal (IP) with 20 mg/kg tamoxifen, or with vehicle, once daily for 5 days. After 5 days, RNA was extracted from mouse heart ventricles, as described [9].

E. Tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS)

Protein extracts were subjected to SDS-PAGE and then silver stained; bands of interest were then excised and then digested with trypsin (10µg/mL) at 37°C overnight. The digest was then subjected to LC-MS/MS analysis, essentially as described in Shevchenko et al 1996 [85], using a linear quadrupole ion trap ThermoFinnigan LTQ mass spectrometer (San Jose, CA) equipped with
a Michrom Paradigm MS4 HPLC, a SpectraSystems AS3000 autosampler, and a nanoelectrospray source.

Mass spectrometric data were acquired by the Arizona Proteomics Consortium supported by NIEHS grant ES06694 to the SWEHSC, NIH/NCI grant CA023074 to the AZCC and by the BIO5 Institute of the University of Arizona.

F. Adenovirus Constructs

Recombinant adenovirus (AdV) encoding only GFP (AdV-Con), GFP and constitutively active ATF6α (GenBank™ accession number NM_001107196) (AdV-ATF6), or GFP and spliced form of XBP1 (GenBank™ accession number NM_001004210) were generated, as described [81], using the AdEasy System (cat# 240009, Aligent Technologies, La Jolla, CA) [86]. PDIA6 (GenBank™ accession number NM_001004442) was cloned from cDNA isolated from NRVMCs using primers in Table 1, cloning. PCR-based mutagenesis (QuikChange site-directed mutagenesis kit, cat# 200518, Stratagene, Santa Clara, CA) was used, as per the manufacturer’s instructions, to generate a catalytically inactive mutant of PDIA6 (PDIA6 CD) where the cysteine at positions 60, 63, 195, and 198 in PDIA6 were mutated to alanine. These forms of the PDIA6 coding region were then used to generate recombinant AdV, as described above.
<table>
<thead>
<tr>
<th>Method</th>
<th>Primers</th>
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<th>Reverse</th>
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</thead>
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<td>CCAAGCGATTTCAAGCAGCA</td>
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<tr>
<td>qRT-PCR: mouse PDIA6</td>
<td>TGCCACCATGATGAGGTCAAGGCAT</td>
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<tr>
<td>qRT-PCR: mouse GAPDH</td>
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<td>GTCATGACGCTCTCCACGAT</td>
<td></td>
</tr>
<tr>
<td>qRT-PCR: rat PDIA6</td>
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<td>CAGGAGACTGCGCTTTCTAGGA</td>
<td></td>
</tr>
<tr>
<td>qRT-PCR: rat GAPDH</td>
<td>CCTGCGCAAGGTCATCCAT</td>
<td>GTCATGACGCTCTCCACGAT</td>
<td></td>
</tr>
<tr>
<td>qRT-PCR: human ATF6(1-373)</td>
<td>CCAGTGTTCAGCAAAACCA</td>
<td>GCAAAACCGCTCGGCCTTTAG</td>
<td></td>
</tr>
<tr>
<td>ChIP: mouse PDIA6</td>
<td>CCTTACCGCGCCGCTT</td>
<td>TCGTGGACCTGCGCTATG</td>
<td></td>
</tr>
<tr>
<td>ChIP: mouse HO-1</td>
<td>GGGCTACTCCCGCTTCTTGT</td>
<td>CTTTCCAGACCCCTCCTCTACTCTCTCT</td>
<td></td>
</tr>
<tr>
<td>ChIP: mouse GAPDH</td>
<td>ATGCCTTCTAGGTTCAGC</td>
<td>ATGTTTCTGCTGGGTGCAGA</td>
<td></td>
</tr>
</tbody>
</table>
G. MicroRNA Constructs

Recombinant adenovirus encoding either miRNA targeted to ATF6α (miATF6), XBP1, (miXBP1), PDIA6 (miPDIA6) or a negative control (miCon) were created using the Gateway System (cat# 11828-029, Life Technologies, Inc., Carlsbad, CA). The generation of the adenovirus encoding miRNA targeted to ATF6 was previously described [76]. Two hairpin sequences to either ATF6α or PDIA6 were generated using Life Technologies's online miRNA designer and ATF6α cDNA (GenBank™ accession number NM_001107196), XBP1 cDNA (GenBank™ accession number NM_001004210), or PDIA6 cDNA (GenBank™ accession number NM_001004442) (Table 2). The negative control sequence (GTCTCCACGCGCATTACATTT) was provided by Life Technologies, and is not targeted toward any known gene.

H. Virus Generation

The recombinant adenovirus discussed in sections 2.6 and 2.7 were created using the AdEasy system, as described [87].

I. Simulated Ischemia/Simulated Reoxygenation

NRVMCs were maintained for 16 hours in 2% FBS-supplemented medium, then subjected to simulated ischemia (si) or simulated ischemia
**Table 2. Hairpin Sequences**

<table>
<thead>
<tr>
<th>miATF6-1</th>
<th>TAGCCAAGCTCAGCAAACAAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>miATF6-2</td>
<td>TTTGTTTCAGGACCAGTCAC</td>
</tr>
<tr>
<td>miPDIA6-1</td>
<td>ATCACTGGAGGAATAGAGAGC</td>
</tr>
<tr>
<td>miPDIA6-1</td>
<td>TTGTCAATGGGATTTTCAAGA</td>
</tr>
</tbody>
</table>


followed by reperfusion (sI/R), essentially as described [87]. Briefly, for sI, the medium was replaced with glucose-free Dulbecco's modified Eagle's medium/F-12 containing 2% dialyzed fetal bovine serum, and cultures were placed in a gas-tight chamber outfitted with a BioSpherix PROOX model 110 controller, which was used to set the \([O_2]\) to 0.1%. For sI/R, following sI, the medium was replaced with glucose-containing Dulbecco's modified Eagle's medium/F-12 supplemented with 2% fetal bovine serum albumin, and cultures were placed in an incubator at \(\sim\)20–21% \(O_2\).

J. qRT-PCR

RNA was extracted from NRVMCs or heart tissue using Quick-RNA MiniPrep Kit, as per the manufacturer's recommended instructions (cat# R1055, Zymo Research, Irvine, CA). cDNA was generated using Superscript III, as per the manufacturer’s instructions (cat# 18080-300, Life Technologies, Carlsbad, CA). Real-time quantitative PCR was performed as described in Martindale et al 2006 [9] using the Biopioneer 2x qPCR Master mix (cat# QPCR-10, Biopioneer, San Diego, CA) and the primers in Table 1, qRT-PCR.

K. Luciferase Reporter Constructs

The rat 5'-flanking sequence of the PDIA6 gene (GenBank\(^\text{TM}\) accession number NM_001004442) from nt -296 to +18 were cloned into the pGL2 luciferase reporter vector (cat# E1631, Promega, Madison, WI). Using PCR-based mutagenesis, as per manufacturer’s instructions (QuickChange site-
directed mutagenesis kit, cat# 200518, Stratagene, Santa Clara, CA), the CCAAT box at -142, and/or the endoplasmic reticulum stress response element (ERSE) at -109 were changed from ATTGG and CCAAT-N9-CCACG to TCCAG and GATCT-N9-AACAT, respectively.

**L. Luciferase Reporter Assay**

NRVMCs were transfected, as described [87], with 15µg of a plasmid encoding the luciferase reporter constructs generated above. The cultures were then plated and after various treatments, the cell extracts were assayed for luciferase activity, as described in Craig et al 2000 [87].

**M. Electro-Mobility Shift Assay**

Double-stranded synthetic oligonucleotides (Table 3) were labeled with $^{32}$P-dCTP (cat# BLU513Z250UC, Perkin-Elmer, Waltham, MA) using Klenow fragment, as per manufacturer’s instructions (Klenow Fragment, cat# EP0051, Thermo-Fisher, Glen Burnie, MD). The double-stranded synthetic oligonucleotides were then used as $^{32}$P-labeled probes, as described in the figure legends. Nuclear extracts were isolated from NRVMCs, as described in Dignam et al 1983 [88]. Nuclear extracts served as the source of nuclear proteins (e.g. NF-Y, YY1, and TFII-I) that are needed to observe ATF6 binding to ER stress response elements [89]. Binding assays were carried out as described in Doroudgar et al 2009 [76].
Table 3. EMSA Probes

<table>
<thead>
<tr>
<th>Probe Type</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCAAT box probe</td>
<td>ccgggGCCACTTTCAATTGGCCGTGCACATc</td>
<td>ccgggGCCACTTTCAATTGGCCGTGCACATc</td>
</tr>
<tr>
<td></td>
<td>oCGGTGAACGTAAACGGCGACGTGGTCTgggcc</td>
<td>oCGGTGAACGTAAACGGCGACGTGGTCTgggcc</td>
</tr>
<tr>
<td>ERSE probe</td>
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<tr>
<td>CCAAT box/ERSE probe</td>
<td>ccgggGCCACTTTCAATTGGCCGTGCACATGCGAATCCACAGACGCAGCGCCACCGCGTGGCAGGc</td>
<td>oCGGTGAAGTGTAACGGCGACGTGACTCGCTTTAGGTGCTCTGTGTACcTgccgggC6076CCACCGCTCgggcc</td>
</tr>
</tbody>
</table>
A. *In Vivo* Quantitative ChIP

Hearts from NTG and TG mice treated with tamoxifen were flash-frozen, and 25μg of tissue were processed, as per manufacturer's instructions (ChampionChIP One-Day kit, cat# 334471, SA Biosciences, Frederick, MD). Approximately 200μg of samples were used for pre-clearing, then 30μl of the subsequent supernatant (pre-cleared fraction) were used in the immunoprecipitation, using either 10μg of FLAG antibody (cat# F1804, Sigma-Aldrich, St Louis, MO), or non-immune IgG beads, which served as the non-immune control, as per manufacturer’s instructions (ChampionChIP One-Day kit, cat# 334471, SA Biosciences, Fredrick, MD).

Primer sets (Table 1, ChIP) that flank the ER stress response elements in the promoter regions of the genes-of-interest were designed. In the case of GAPDH, which does not contain ER stress response elements, primers were designed to overlap a randomly selected portion of the promoter.

B. Live/Dead Assay

Assessment of cell death in NRVMCs was performed using Hoescht (cat# H21486; Invitrogen) and propidium iodide (PI, cat# P1304MP, Invitrogen), as described [61].
C. Animals

Approximately 18 adult male C57/BL6 mice (6 NTG and 12 ATF6-MER TG mice), and 100 1-4 day-old Harlan Sprague-Dawley rats were used in this study. All procedures involving animals were carried out in accordance with the San Diego State University Institutional Animal Care and Use Committee.

D. Statistical Analyses

Data are reported as mean ± SEM and analyzed via Student’s-t test, or 1-way ANOVA with Newman–Keuls post-hoc analysis, when appropriate, using Graphpad Prism version 4. Unless otherwise stated in the figure legends, *, δ, ψ = p < 0.05, **, δδ = p < 0.01 and ***, δδδ, ψψψ = p < 0.001 different from all other values.
III. Results

A. Identification of PDIA6 in cardiac myocytes

We previously showed that, in cardiac myocytes, ER stresses, such as simulated ischemia (sI), or simulated ischemia/reperfusion (sI/R), increased the expression of numerous ER proteins, including some that assist in ER protein folding, such as chaperones and protein disulfide isomerases [9, 60, 61, 81]. Many of these proteins, such as glucose-regulated protein-78 (GRP78) and glucose-regulated protein-94 (GRP94) [90], have a C-terminal KDEL motif through which they bind to the KDEL-receptor, which facilitates their retrograde transport from the Golgi to the ER, and thus their retention in the ER [91, 92]. An antibody raised against KDEL (anti-KDEL) is often used to assess expression of such proteins in response to ER stress [82]. When anti-KDEL was used in immunoblots of extracts of cultured cardiac myocytes that had been treated with tunicamycin (TM), or subjected to sI, or sI/R, the expression of two major KDEL-cross-reactive proteins, known to be GRP78 and GRP94, was increased, as expected (Fig. 5A, GRP94 and GRP78). A previously unidentified anti-KDEL cross-reactive protein of about 50 kD was also induced (Fig. 5A, arrow), suggesting that it has a function in ER-stressed cardiac myocytes.
Figure 5. Identification of PDIA6 as a 50 kD anti-KDEL cross-reactive protein

Panels A and B- NRVMCs were treated with or without 10 µg/ml of TM for 24hrs, or subjected to 20 hrs of simulated ischemia (sl), or 20 hrs of sl followed by 24 hrs of simulated reperfusion (sl/R). Culture extracts were then analyzed by immunoblotting using anti-KDEL, anti-PDIA6, and anti-GAPDH antibodies. The arrow shows the location of the 50 kD anti-KDEL cross-reactive protein. n=3 cultures per treatment

Panel C- NRVMCs were infected with either a control adenovirus (AdV-Con), or an adenovirus encoding PDIA6 (AdV-PDIA6). Forty-eight hours later, culture extracts were analyzed by qRT-PCR to determine the levels of PDIA6 and GAPDH mRNAs (top). Values are mean PDIA6/GAPDH mRNA ± SE (n=3). Cultures were also analyzed by immunoblotting with anti-KDEL or anti-PDIA6 antibodies (bottom), n=3 cultures per treatment.

Panel D- NRVCMs were infected with an adenovirus encoding a control microRNA (AdV-miCon), or a microRNA designed to reduce the expression of PDIA6 (AdV-miPDIA6). Forty-eight hours later, culture extracts were analyzed by qRT-PCR to determine the levels of PDIA6 and GAPDH mRNAs (top). Values are mean PDIA6/GAPDH mRNA ± SE (n=3). Cultures were also analyzed by immunoblotting with anti-KDEL or anti-PDIA6 antibodies (bottom), n=3 cultures per treatment.

Panel E- NRVMCs were treated with or without 10µg/ml TM for 24hrs, and lysates were incubated with either a non-immune antibody, or with anti-PDIA6, followed by immune complex removal, then analysis of the supernatants by immunoblotting with anti-KDEL, n=3 cultures per treatment. The arrow shows the location of the 50 kD anti-KDEL cross-reactive protein.
In order to determine the function of the 50 kD anti-KDEL cross-reactive protein in cardiac myocytes, it was necessary to determine its identity. Accordingly, proteins in cardiac myocyte extracts with C-terminal KDEL motifs were isolated by anti-KDEL affinity purification; the 50 kD anti-KDEL cross-reactive material was analyzed by LC-MS/MS and shown to contain protein disulfide isomerase-associated 6 (PDIA6), a member of the protein disulfide isomerase (PDI) family of thiol oxidoreductases (**Table 4**). In order to verify this initial identification, immunoblots were carried out using a PDIA6-specific antibody (anti-PDIA6), and the results were compared to those obtained with anti-KDEL. In comparison to the 50 kD anti-KDEL cross-reactive protein, there was an increase in the 50 kD anti-PDIA6 cross-reactive band in response to TM (**Fig. 5B**). Moreover, when cultured cardiac myocytes were infected with an adenovirus (AdV) encoding native PDIA6 (AdV-PDIA6), there was a 275-fold increase in PDIA6 mRNA (**Fig. 5C, top**), as expected, as well as coordinate increases in the levels of 50 kD anti-KDEL, and anti-PDIA6 cross-reactive material (**Fig. 5C, bottom arrow and PDIA6**). Conversely, when cardiac myocytes were infected with an AdV encoding a microRNA targeted to endogenous PDIA6 mRNA (AdV-miPDIA6), there was a 70% decrease in PDIA6 mRNA (**Fig. 5D, top**), as well as coordinate decreases in the levels of the 50 kD anti-KDEL and anti-PDIA6 cross-reactive material (**Fig. 5D, bottom arrow and PDIA6**). Furthermore, when anti-PDIA6 was used to deplete
Table 4. LC-MS/MS Sequencing Results

Protein was isolated by immunoprecipitation using an anti-KDEL antibody, separated by size using SDS-PAGE and proteins of approximately 50 kD were excised. Isolated protein was then subjected to LC-MS/MS protein sequencing.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Sequence Coverage</th>
<th>Sequence Count 1</th>
<th>Sequence Count 2</th>
<th>MolWt</th>
<th>Descriptive Name</th>
<th>KDEL Containing Protein</th>
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<tr>
<td>Pdia6</td>
<td>21.1%</td>
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<td>7</td>
<td>48760</td>
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<tr>
<td>Basp</td>
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cardiac myocyte lysates, the 50 kD anti-KDEL cross-reactive material decreased (Fig. 5E, arrow). Taken together the results in Figure 5 and the LC-MS/MS protein sequencing (Table 4) demonstrates that the 50 kD anti-KDEL cross-reactive protein was PDIA6.

**B. Effects of ER stress on PDIA6 expression**

To characterize the mechanism of PDIA6 induction, the effects of the ER stress-activated transcription factor, ATF6, and X-box binding protein 1, XBP1, were examined. When cardiac myocytes were infected with a recombinant AdV encoding activated ATF6 (AdV-ATF6) or a recombinant AdV encoding XBP1 (AdV-XBP1) [93], ATF6 mRNA was increased about 50-fold and XBP1 mRNA was increased about 1600-fold (Fig. 6A and 6B). In cultures infected with AdV-ATF6, PDIA6 mRNA and protein increased by 8- and 13-fold, respectively (Fig. 6C, bar 2; Fig. 6B, bar 2). While, in culture infected with AdV-XBP1, PDIA6 mRNA and protein both increased by 3-fold (Fig. 6C, bar 3; Fig. 6B, bar 3). To explore the effect of endogenous ATF6 and XBP1 on PDIA6 induction, a recombinant AdV encoding a microRNA (miRNA) targeted to rat ATF6 (AdV-miATF6) or rat XBP1 (AdV-miXBP1) was used [76]. Cultures infected with AdV-miATF6 or AdV-miXBP1 exhibited a 70% reduction in the quantity of ATF6 mRNA or a 60% reduction in the quantity of XBP1 mRNA, respectively (Fig. 7A, bar 3 and 6), thus validating the utility of these
**Figure 6. Effect of AdV-ATF6 and AdV-XBP1 on PDIA6 expression**

Panel A- NRVMCs were infected with AdV-Con or AdV-ATF6. Twenty-four hours later, cultures were analyzed for ATF6 and GAPDH mRNAs. Values are mean ATF6/GAPDH mRNA ± SE, n=3 cultures per treatment.

Panel B- NRVMCs were infected with AdV-Con or AdV-XBP1. Twenty-four hours later, cultures were analyzed for XBP1 and GAPDH mRNAs. Values are mean XBP1/GAPDH mRNA ± SE, n=3 cultures per treatment.

Panel C- NRVMCs were infected with AdV-Con, AdV-ATF6, or AdV-XBP1. Forty-eight hours later, culture extracts were analyzed for PDIA6 and GAPDH mRNA. Values are mean PDIA6/GAPDH mRNA ± SE, n=3 cultures per treatment.

Panel D- NRVMCs were treated as described for Panel C, except extracts were analyzed for PDIA6 and GAPDH protein by immunoblotting. Relative blot intensities of PDIA6/GAPDH expressed as fold of control (bottom). Values are mean relative PDIA6/GAPDH ± SE, n=3 cultures per treatment.
Figure 7. Effect of knocking down endogenous ATF6 or XBP1 on PDIA6 expression

Panel A- NRVMCs were infected with adenovirus encoding AdV-miCon, AdV-miATF6 or AdV-miXBP1. Twenty-four hours later, cultures were analyzed for ATF6, XBP1 and GAPDH mRNAs. Values are mean ATF6 or XBP1/GAPDH mRNA ± SE, n=3 cultures per treatment.

Panel B- NRVMCs were infected with AdV-miCon, AdV-miATF6 or AdV-miXBP1. Twenty-four hours later, cultures were treated with or without 10µg/ml of TM for 24hrs, then analyzed for PDIA6 and GAPDH mRNA. Values are mean PDIA6/GAPDH mRNA ± SE, n=3 cultures per treatment.

Panel C- NRVMCs were infected with AdV-miCon, AdV-miATF6 or AdV-miXBP1. Twenty-four hours later, cultures were treated with or without 10µg/ml of TM for 24hrs, and then analyzed for PDIA6 and GAPDH by immunoblotting. Image quantification of the blots shown at the top of Panel C are plotted at the bottom. Values are PDIA6/GAPDH ± SE, n=3 cultures per treatment.
reagents for knocking down endogenous ATF6 and XBP1 in cultured cardiac myocytes. AdV-miATF6 reduced TM-mediated induction of PDIA6 mRNA and protein by about 50% and 80%, respectively (Fig. 7B, bar 5; Fig. 7C, bar 5). AdV-miXBP1 increased TM-mediated induction of PDIA6 mRNA by 50%, while reducing TM-mediated induction of PDIA6 protein by 50% (Fig. 7B, bar 6; Fig. 7C, bar 6). Taken together, the results shown in Figures 6 and 7 demonstrate that in cultured cardiac myocytes, ER stress-mediated PDIA6 induction was partly dependent upon transcriptional induction by ATF6 and to a lesser degree by XBP1. Since ER stressors, such as TM, activate ATF6 and XBP1, which increases transcription of certain ER stress response genes, the effects of ER stress and ATF6 and XBP1 on PDIA6 promoter activation were examined.

C. Regulation of PDIA6 promoter activation and ER stress response element identification

A construct composed of the nucleotides -296 to +18 of the PDIA6 promoter driving firefly luciferase (Fig. 8A) was transfected into cultured cardiac myocytes, which were then treated with TM, si, or AdV-ATF6. These treatments increased PDIA6 promoter activity by ~2- to 6-fold (Fig. 8B, bars 1-3; Fig. 8C, bars 1, 2). A search for ER stress response elements (ERSEs) revealed a region spanning nts -109 to -90 of the PDIA5 5’-flanking sequence that comprised a CCAAT box element, followed by 9 nts then a CCACG
Figure 8. Effect of TM, sl, AdV-ATF6 or AdV-XBP1 on the PDIA6 promoter

Panel A- The promoter and 5’-flanking region of the rat PDIA6 gene from -296 to +18 is shown, as are the putative CCAAT box and ERSE at -142 to -137 and 109 to -90, respectively. M1 and M2 denote mutations 1 and 2 that were made in the PDIA6 5’-flanking sequence. The sequences of the native CCAAT box and ERSE are shown, and the mutations are shown below. These mutations were prepared in a manner predicted to disrupt NF-Y, ATF6 or XBP1 binding to these elements.

Panel B- NRVMCs were transfected with either an empty vector control, or with wild-type or mutated PDIA6-luciferase constructs, as shown. After 24h, cells were subjected to 20hrs of sl, or treated with 10µg/ml of TM for 24hrs, and then analyzed for reporter activity. Values are the mean luciferase activity expressed as fold of empty vector control ± SE, n=3 cultures per treatment.

Panel C- NRVMCs were transfected as described in Panel B, and then infected with either AdV-Con, AdV-ATF6 or AdV-XBP1. Twenty four hours later, cell extracts were analyzed for reporter activity. Values are the mean luciferase expressed as fold empty vector control ± SE, n=3 cultures per treatment.
sequence, which fulfills the requirements of an ERSE [57, 89]. Transcriptional
induction via ERSEs by ATF6 requires that the nuclear protein, nuclear factor Y-
box (NF-Y), binds to the CCAAT box portion of the ERSE, and that ATF6 binds to
NF-Y and to the CCACG portion of the ERSE [89]. Thus, while NF-Y can bind
directly to ERSEs via CCAAT sequences, ATF6 binding to ERSEs requires that
ATF6 also binds to NF-Y. The search also revealed a CCAAT box about 30 nt 5’
of the ERSE, between nts -142 to -137, which was not part of a consensus
ERSE. NF-Y binding to such isolated CCAAT boxes can increase transcription of
some ER stress response genes [94, 95]. Since isolated CCAAT boxes and
ERSEs can mediate transcriptional induction upon ER stress, the effects of
mutating these two elements on PDIA6 promoter activity were examined.

Mutating the isolated CCAAT box alone (Fig. 8A, M1) slightly increased promoter
activation in response to TM or sI (Fig. 8B, bars 5, 6), while it slightly decreased
promoter activation in response to ATF6 (Fig. 8C, bar 5). However, mutations to
the ERSE that are predicted to disrupt NF-Y and ATF6 binding (Fig. 8A, M2)
decreased promoter activation by 3- to 4-fold in response to all of the treatments
(Fig. 8B, bars 8, 9; Fig. 8C, bar 8, 9). When the isolated CCAAT box and ERSE
were mutated (Fig. 8A, M1+M2), promoter activation was completely lost (Fig.
8B, bars 10-12; Fig. 8C, bars 10-12). Surprisingly, AdV-XBP1 was not able to
significantly induce promoter activity in any of the constructs, further suggesting
the minimal role XBP1 may play in the regulation of PDIA6 (Fig. 8C, bars 3, 6, 9,
Thus, while the ERSE seemed to be the dominant element, the isolated CCAAT box exerted inhibitory effects in the native promoter, but in the absence of the ERSE, it appeared to be capable of mediating some promoter activation, albeit relatively small.

To examine the mechanisms by which these elements regulate the PDIA6 promoter, electromobility shift assays (EMSAs) were performed using oligonucleotide probes that mimicked the region of the promoter containing either the isolated CCAAT box, or the ERSE (Fig. 9A, CCAAT box probe, ERSE probe). Since the binding of ATF6 to ERSEs requires NF-Y, nuclear extracts of untreated cardiac myocytes were used in the EMSAs as a source of NF-Y, as previously described [89, 96-98]. Incubation of nuclear extract with either the ERSE or CCAAT box probe resulted in formation of a complex (Fig. 9B, lanes 2 and 7), which is due to NF-Y binding to the CCAAT box region of each probe. Addition of ATF6 decreased the mobility of the ERSE probe (Fig. 9B, lane 3), and the addition of ATF6 and an ATF6 antibody further decreased the mobility of ERSE probe (Fig. 9B, lane 4), which verified ATF6 binding [99]. In contrast, neither ATF6, nor ATF6 antibody affected the mobility of the CCAAT box probe (Fig. 9B, lanes 8 and 9), demonstrating that ATF6 did not
Figure 9. Electromobility shift assays with NF-Y and ATF6

Panel A- Shown are diagrams of the probes containing the isolated PDIA6 ERSE, PDIA6 CCAAT box and a “double” probe containing both elements. $^{32}$P-labeled overhangs are shown in italics and the relevant elements are highlighted in gray.

Panel B- ERSE and CCAAT box Probes: EMSA reactions were carried out as described in the methods using radiolabeled ERSE or CCAAT box probes, as shown. Reactions analyzed in lanes 1 and 6 contained only the probes, while those in lanes 2-5 and 7-10 contained nuclear extract from NRVMCs, recombinant ATF6(116-373) obtained from in vitro transcription/translation reactions, and/or anti-ATF6, as shown.

Panel C- CCAAT box/ERSE Double Probe: Binding reactions were carried out using either wild-type probe (lanes 1-5), probe with the isolated CCAAT box mutated (lanes 6-10), or probe with the ERSE mutated (lanes 11-15). All other additions were made as described in the methods and in the legend to Panel A.

Panel D- Effect of NF-Y Antibody: Binding reactions were carried out using the CCAAT box/ERSE double probe and the other additions, as shown.
A. Diagram of PDIA6 Probes

CCAAT box probe

ccggtGCCACTTCCATTGGCCGTGACATcc

ERSE probe

ccggtTCCACAGAGACCAATGAACGGGCAGCCAGGCATGGCGAGcc

CCAAT box/ERSE probe (double probe)

ccggtGCCACTTCCATTGGCCGTGACATGAAGCGAAATCCACAGAGACCAATGAACGGGCAGCCAGGCATGGCGAGcc

B. CCAAT box and ERSE Probes

NE only

ATF6

ATF6 antibody

Probe only

C. CCAAT box/ERSE Double Probe

NE only

ATF6

ATF6 antibody

Probe only

D. NF-Y Binding

NE only

ATF6

NF-Y antibody

Probe only
bind to the isolated CCAAT box. The reduction of the intensity of the complex observed when ATF6 was added to the CCAAT box probe (Fig. 9B, lanes 8 and 9) is most likely due to the titration of NF-Y off the probe by ATF6, which can bind to NF-Y in the absence of DNA [89].

Since the promoter/luciferase experiments suggested that the isolated CCAAT box and the ERSE might both contribute to regulating the PDIA6 promoter, EMSAs were carried out using a probe that had both the isolated CCAAT box and the ERSE in their native relative positioning (Fig. 9A, CCAAT box/ERSE double probe). Incubation of nuclear extract with the double probe resulted in two complexes (Fig. 9C, lane 2). Mutations to either element in the double probe disrupted the slower-migrating complex, but not the faster-migrating complex (Fig. 9C, lanes 7 and 12), indicating that the slower complex was due to NF-Y binding to both sites, while the faster complex was due to NF-Y binding to either one of the sites. Addition of an NF-Y antibody disrupted both complexes (Fig. 9D, lanes 2 and 3) further demonstrating that it was responsible for the formation of both complexes. Addition of ATF6 to the double probe resulted in the formation of a new, slower migrating complex, (Fig. 9C, lane 3) that was sensitive to addition of ATF6 antibody (Fig. 9C, lane 4), indicating that the slowly migrating complex in Figure 9C, lane 3 was the result of ATF6 binding. The complexes formed using the double probe in which the CCAAT box was mutated (Fig. 9C, lanes 6-10) resembled those formed using the ERSE
probe (Fig. 9B, lanes 1-5), indicating that NF-Y and ATF6 bound to the ERSE in the double probe. Moreover, the complex formed using the double probe in which the ERSE was mutated (Fig. 9C, lanes 11-15) resembled that formed using the CCAAT box probe (Fig. 9B, lanes 6-10), indicating that NF-Y, but not ATF6, bound to the isolated CCAAT box in the double probe.

The antioxidant response element (ARE) and tandem CCAAT motifs are recognized by the transcription factors nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and CCAAT displacement protein (CDP), respectively [100, 101]. The ARE and tandem CCAAT motifs are structurally similar to the ERSE and the CCAAT box/ERSE, respectively. In order to determine whether Nrf2 and CDP can regulate PDIA6 additional experiments were carried out. As expected, ATF6 was able to induce the luciferase report gene driven by either the PDIA6 promoter or the GRP78 promoter by approximately 20- to 15-fold over their respective control, respectively (Fig. 10A, bars 1-2 and 4-5), while Nrf2 was unable to induce luciferase activity in these same constructs (Fig. 10A, bars 3 and 6). Additionally, the isolated CCAAT box and the ERSE were used in an EMSA to determine whether the transcription factors Nrf2 or ATF4, Nrf2 has been shown to heterodimerize with ATF4, were able to bind to the PDIA6 promoter [102]. Incubation of nuclear extract with either the ERSE or CCAAT box probe resulted in formation of a complex (Fig. 10B, lanes 2 and
Figure 10. Effect of Nrf2 on the PDIA6 promoter

Panel A- NRVMCs were transfected with either an empty vector control, wild type PDIA6 or GRP78-luciferase constructs and then infected with AdV-Con, AdV-ATF6 or AdV-Nrf2. Twenty four hours later, cell extracts were analyzed for reporter activity. Values are the mean luciferase expressed as fold empty vector control ± SE, n=3 cultures per treatment.

Panel B- ERSE and CCAAT box Probes: EMSA reactions were carried out as described in the methods using radiolabeled ERSE or CCAAT box probes, as shown. Reactions analyzed in lanes 1 and 6 contained only the probes, while those in lanes 2-5 and 7-10 contained nuclear extract from NRVMCs, anti-ATF6, ATF4 or Nrf2 antibodies as shown.
The addition of ATF6 antibody was able to retard the mobility of the complex bound to the ERSE probe but not the CCAAT box probe (Fig. 10B, lanes 3 and 8) confirming that ATF6 is indeed bound to the ERSE and not to the CCAAT box. The addition of ATF4 antibody and Nrf2 antibody were unable to change the mobility of either the CCAAT box or ERSE probe (Fig. 10B, lanes 4-5 and 9-10) suggesting that neither ATF4 nor Nrf2 is able to bind to the promoter of the PDIA6.

In order to determine whether CDP could possibly regulate the expression PDIA6 in cardiac myocytes we first had to determine whether CDP was expressed in the heart. Figure 11A and 11B confirms that CDP mRNA and protein expression was easily detected in cardiac myocytes. Interestingly, infection with AdV-ATF6 decreased CDP mRNA and protein expression by 75% and 50%, respectively (Fig. 11A and B). Since CDP is a negative regulator of gene transcription and not previously appreciated as an ATF6 responsive gene, this data suggests another mechanism by which ATF6 can regulate gene transcription. Traditionally, CDP recognizes tandem CCAAT motifs, a pair of CCAAT elements in close proximity to each other [101]. Since the promoter of PDIA6 a pair of CCAAT motifs, a CCAAT box and another CCAAT motif imbedded in the ERSE, the isolated ERSE and double probe were used in EMSAs in order to determine the ability of CDP to bind to the these elements. Expectantly, the addition of nuclear extract resulted in a single
Figure 11. Effect of CDP on the PDIA6 promoter

Panel A - NRVMCs were infected with AdV-Con or AdV-ATF6. Twenty-four hours later, cultures were analyzed for CDP and GAPDH mRNAs. Values are mean CDP/GAPDH mRNA ± SE, n=3 cultures per treatment.

Panel B - NRVMCs were treated as described for Panels A, except nuclear extracts were analyzed for CDP and Histone H3 protein by immunoblotting. Relative blot intensities of CDP/Histone H3 expressed as fold of control (bottom). Values are mean relative CDP/Histone H3 ± SE, n=3 cultures per treatment.

Panel C - ERSE and CCAAT box/ERSE Double Probe: EMSA reactions were carried out as described in the methods using radiolabeled ERSE or CCAAT box/ERSE double probe, as shown. Reactions analyzed in lanes 1 and 4 contained only the probes, while those in lanes 2-3 and 5-6 contained nuclear extract from NRVMCs, or anti-CDP antibody as shown.
complex formation with the isolated ERSE and two complexes with the double probe (Fig. 11C, lanes 2 and 5). The addition of CDP antibody was unable to neither change the mobility of the complex bound the isolated ERSE, nor change the mobility of either of the complexes bound to the double probe. The inability of the CDP antibody to change the mobility of any of the complexes indicates that CDP is not part of the complex formation. The results in Figures 10 and 11 suggest that the promoter of PDIA6 is not recognized or regulated by Nrf2 or CDP.

Taken together, these results showed that NF-Y bound to the isolated and ERSE-associated CCAAT box elements in the double probe, while ATF6 bound only to the ERSE (Fig. 12). Moreover, with the results shown in Figure 8 and 9 suggest that, in the native promoter, binding of NF-Y to the CCAAT box element may reduce promoter activity, perhaps because ATF6 may be titrated off the ERSE by the NF-Y on the isolated CCAAT box element, thus reducing the amount of ATF6 available to bind to the ERSE. However, since some promoter activity remained after mutation of the ERSE, and since this remaining activity was extinguished by mutations to both the ERSE and the CCAAT box, it is apparent that the CCAAT box alone confers some PDIA6 promoter activation in response to ER stress.
Figure 12. Schematic of NF-Y and ATF6 binding the PDIA6 promoter
Diagram of NF-Y and ATF6 binding to the CCAAT box and ERSE in the PDIA6 promoter.
A. ATF6 regulates PDIA6 expression, \textit{in vivo}

Since ATF6 was required for maximal PDIA6 promoter activation, and since it bound to oligonucleotides mimicking the ERSE in the promoter, we determined whether ATF6 bound to the PDIA6 promoter, \textit{in vivo}, using ATF6 transgenic (TG) mouse hearts. In a model in which a FLAG tagged tamoxifen-inducible form of ATF6 was expressed (ATF6 TG), ER stress response genes are activated in response to tamoxifen treatment \cite{9}. To determine whether PDIA6 was induced by ATF6 in this mouse model, non-transgenic (NTG) and ATF6 TG mice were treated with, or without tamoxifen, then RNA was isolated from their hearts and was examined for PDIA6 expression. PDIA6 mRNA levels increased by about 5-fold in tamoxifen-treated ATF6 TG mice, but were unaffected in the other groups (Fig. 13A), thus verifying that ATF6 induced PDIA6 in mouse hearts, \textit{in vivo}.

Chromatin immunoprecipitation (ChIP) showed that the PDIA6 promoter was enriched in tamoxifen-treated ATF6 TG mouse hearts, but not in NTG mouse hearts when DNA bound to ATF6 was isolated (Fig. 13B; PDIA6). ChIP analyses of the same samples for the ER stress response gene, heme-oxygenase 1 (HO-1) served as a negative control (Fig. 13B; HO-1), since its promoter lacks ERSEs, or any other known ATF6 binding sites \cite{60, 103}. These results demonstrate that activated ATF6 bound directly to the PDIA6 promoter in the mouse heart and regulated its activity, \textit{in vivo}.
Figure 13. PDIA6 expression in ATF6 TG mouse hearts and ATF6 binding to the PDIA6 promoter in mouse hearts, *in vivo*

*Panel A*- NTG and ATF6 TG mice were treated with or without tamoxifen for 5 days, after which hearts were extracted and analyzed for PDIA6 and GAPDH mRNA by qRT-PCR. Values are mean PDIA6/GAPDH mRNA ± SE, n=3 mice per treatment.

*Panel B*- Quantitative ChIP analysis was carried out to determine the ability of ATF6 to bind to the PDIA6 and hemoxygenase-1 (HO-1) promoters in the mouse heart, *in vivo*. Chromatin was isolated from NTG and ATF6 TG mouse hearts, and then subjected to FLAG IP. Isolated chromatin was then examined by quantitative PCR using primers targeted to the mouse PDIA6 and mouse HO-1 promoters. Shown is the quantitation of the PCR products obtained, n=3 mice per treatment.
Figure 14. Effect of PDIA6 on sI/R-mediated cell death

Panel A- Effect of PDIA6 or PDIA6 catalytically dead (CD) on sI/R-mediated cell death: NRVMCs were infected with a control adenovirus (AdV-Con), an adenovirus encoding PDIA6 (AdV-PDIA6), or a catalytically dead mutant of PDIA6 (AdV-PDIA6 CD) and then analyzed for PDIA6 and GAPDH by immunoblotting (top). NRVMCs infected with AdV-Con, AdV-PDIA6 or AdV-PDIA6 CD were subjected to 20hrs of sl followed by 24hrs of reperfusion (sI/R), and then examined for cell death (bottom). Shown are the results of a cell death assay, mean + SE, n=3 cultures per treatment.

Panel B- Effect of PDIA6 knockdown on sI/R-mediated cells death: NRVMCs were infected with AdV-Con or AdV-miPDIA6, subjected to 20hrs of sl followed by 24hrs of reperfusion (sI/R), then examined for cell death. Shown are the results of a cell death assay, mean + SE, n=3 cultures per treatment.
A. PDIA6 is cardioprotective

To examine the function of PDIA6, AdV-PDIA6 and AdV-miPDIA6 were used in cultured cardiac myocytes to assess the effects of PDIA6 gain- and loss-of-function, respectively. Compared to AdV-Con, cultures infected with AdV-PDIA6 (Fig. 14A, top) exhibited significantly less cell death when subjected to sI/R (Fig. 14A, bars 4 and 5). Furthermore, infection with an AdV that encodes a form of PDIA6 that is catalytically inactive (AdV-PDIA6-CD) (Fig. 14A, top) did not protect the cells from sI/R-induced cell death (Fig. 14A, bar 6). Conversely, knocking down PDIA6 using AdV-miPDIA6 (Fig. 5D) resulted in increased cell death under control conditions and upon sI/R (Fig. 14B). These results demonstrate that PDIA6 can protect cardiac myocytes from sI/R induced death and this protection is dependent on the oxidoreductase activity of PDIA6.
IV. Conclusion

In this study I identified PDIA6 as a previously uncharacterized 50 kD anti-KDEL cross-reactive protein that is induced by hypoxic and ischemic stress in various cells and tissues, including the heart. Moreover, this study showed that, upon simulated ischemia, PDIA6 was induced in cardiac myocytes in an ATF6-dependent manner. These findings are consistent with another recent study which showed that another member of the ER protein disulfide isomerization coupling reaction, ERO1, is also strongly induced in cultured cardiac myocytes by hypoxia [104]. Thus, the ER/SR thiol oxidoreductase enzyme system is likely to serve important roles in the biosynthesis of numerous secreted and membrane proteins that are required for proper cardiac myocyte and heart function, supporting the hypothesis that the induction of enzymes, such as PDIA6, may help protect the myocardium in response to pathological ER stresses, such as ischemia.

A portion of the text and figures in this chapter is a reprint of the material as it appears in the Journal of Molecular Cellular Cardiology. I was primary author and the co-authors listed in this publication contributed to the research.

John A. Vekich, Peter J. Belmont, Donna J. Thuerauf, and Christopher C. Glembotski: "Protein disulfide isomerase-associated 6 is an ATF6-inducible ER stress response protein that protects cardiac myocytes from
CHAPTER THREE

I.  Introduction

A. Signal transduction pathways activated by I/R

In response to ischemia/reperfusion (I/R) in cardiac myocytes a series of signal transduction pathways are activated that contributes to survival and, thus, maintenance of cardiac function [105]. The activation of these pathways may up- or down-regulate specific genes, reorganize signaling complexes, activate or deactivate proteins, change metabolism, alter contraction, and initiate autocrine and paracrine signaling.

These changes are used by the cell in order to survive I/R injury, or if the damage is insurmountable initiate apoptosis. Three of the most studied I/R-activated signaling cascades are the mitogen activated protein kinase (MAPK) family of proteins [105]. Each of these pathways are named after their end effector kinase, extracellular signal regulated kinase (ERK), c-jun n-terminal kinase (JNK), and p38. These pathways can modulate a host of cellular functions, including influencing both cell death and survival (Fig. 15).

1. ERK pathway

ERK is typically activated by extracellular signals, such as growth factors and hormones [106]. ERK is traditionally activated by the signaling
Figure 15. The MAPK signaling cascade
Activation of the different MAPK pathways have differential outcomes from the same stimulus.
cascade that starts with ras/RAF which in turn activates MAP kinase kinase 1/2 (MKK1/2). The activation of MKK1/2 culminates in the phosphorylation and activation of ERK, of which there are two isoforms, ERK1 and ERK2. MKK1/2 phosphorylates ERK at two sites, threonine 202 and tyrosine 204 for ERK1 and threonine 185 and tyrosine 187 for ERK2. Both MKK1/2 and ERK have been shown to be activated by I/R, while ischemia alone fails to activate ERK [107]. ERK can also be activated by nitric oxide (NO), a free radical produced during reperfusion [108]. Additionally, ERK has been shown to be activated by oxidative stress in a protein kinase C (PKC) dependent manner [109].

ERK is generally thought of as protective. The activation of the ERK before, or during I/R, has been shown to be protective in many in vitro cell culture models, resulting in reduced apoptosis and cell death [110]. Activating ERK by increasing NO in the heart improved functional recovery of hearts subjected to ex vivo I/R [108]. Preventing ERK activation with the MEK inhibitor PD98059 increase cardiac myocyte cell death following simulated I/R; additionally, the same compound reduced the functional recovery of mouse hearts subjected to ex vivo I/R [110]. These studies demonstrated the protective effects of ERK both in cultured cells and in the heart.

2. JNK pathway

JNK can be activated by inflammatory cytokines such as interlukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [111]. IL-1 and TNF-α are able to activate
extra cellular receptors that in turn activate the MAP kinase kinase kinases (MKKK) as well as ASK1 [112, 113]. These kinases in turn are able to phosphorylate and activate MKK4/7, which will ultimately result in the phosphorylation and activation of JNK [114, 115]. I/R and reactive oxygen species (ROS) have been shown to activate JNK in cardiac myocytes and an increase in JNK activation has been reported in the myocardium of hearts subject to MI [116-118]. ASK1, the upstream activator of MKK4/7 is kept inactive through its binding to reduced thioredoxin, but under oxidative stress, such as I/R or an increase in ROS, thioredoxin is oxidized and ASK1 is subsequently released [115]. Free ASK1 is then able to phosphorylate and activate downstream MKKs.

JNK activation in the heart is usually linked to an increase in apoptosis. JNK1/2 null mice exhibited a decrease in apoptosis seen after I/R injury when compared to their WT litter mates [119]. Additionally, mice overexpressing a dominant negative version of JNK exhibited similar results after I/R injury [119]. Conversely, mice expressing a constitutively active form of MKK7, the upstream activating kinase of JNK, resulted in a progressive cardiomyopathy [120].

3. p38 pathway

The cell fate associated with the activation of p38 has been controversial in the literature. There are large bodies of work correlating the activation of p38 with both cell death and survival [121]. This controversy may be attributed to the multiple isoforms of p38, of which two are ubiquitously expressed, p38α and
p38β [116, 122]. p38α, the predominant isoform in the heart, has been linked with apoptosis, while p38β is associated with hypertrophy and cell survival [123].

MKK3 and MKK6 are responsible for the phosphorylation and activation of p38 [124, 125]. MKK3 is thought to be a specific activator of p38α, while MKK6 has been shown to activate both of the predominant isoforms. p38 can be activated by extracellular signals such as growth factors and inflammatory cytokines. I/R can activate p38 through ASK1, also a regulator of JNK, as well as MKKK1, 3, and 4 [117].

I/R-mediated activation of p38 has been shown to be both protective and to induce apoptosis. Inhibition of p38 was shown to decrease infarct size in a pig model of MI [126]. Similarly, it has been demonstrated that activation of p38 reduced cardiac contractility and blocking the activation of p38 by either a dominant negative version of p38, or with SB203580, an inhibitor of p38, restored the contractility of the heart [127]. On the other hand, mice overexpressing the upstream activator of p38, MKK6, were protected from I/R injury [128]. Additionally, administration of SB203580 prior to I/R resulted in a lower functional recovery in an ex vivo model of I/R injury [129]. Finally, p38 activation has been shown to be necessary for the beneficial effect of ischemic preconditioning [125, 130].
One mechanism by which p38 can protect cardiac myocytes is through a downstream target, MAPK activated protein kinase 2 (MAPKAPK2) [131]. Activation of MAPKAPK2 by p38 results to the phosphorylation of a pair of heat shock proteins (HSP), alpha-B crystallin (αBC) and heat shock protein 27 (HSP27) [123]. The phosphorylation of both of these sHSPs has been demonstrated to protect cells from numerous stresses.

B. Chaperones

Chaperones are a class of proteins that help nascent proteins achieve their proper conformation and protect existing proteins during times of stress by keeping them from misfolding and aggregating [132]. During nascent protein formation, chaperones bind to the newly synthesized proteins, isolating them from other proteins before they have achieved their proper tertiary structure [132]. Chaperones also help facilitate the new protein fold into its final native conformation by assisting in the formation of disulfide bonds, post-translational modification, and cleavage of unnecessary peptides when needed [132]. During times of stress, the role of chaperones change from helping nascent proteins to establish their native conformation to preventing properly folded protein from misfolding so that proper cellular function can be maintained.

1. Heat shock proteins

One major class of chaperones is the HSPs [133]. Classically, HSPs were defined by properties of stress-inducible expression in response to diverse
stimuli, pathological or physiological [134]. Despite this definition, not all HSPs display stress-inducible expression. HSPs are better characterized by their low molecular weight, approximately 20-30 kD, and the presence of a conserved alpha-crystallin domain which is responsible for its chaperone capability [134]. HSPs are ubiquitously expressed through the body with differential location and temporal expression [134].

In the heart, HSPs play a critical role in the function and health of the myocardium. Vascular tone has been shown to be regulated, in some part, by two HSPs, HSPB1 and HSPB6. While the phosphorylation of HSPB1 leads to vasoconstriction, the phosphorylation of HSPB6 had the opposite effect and contributed to vasodilation [135, 136]. Independent studies using the overexpression of HSPs, specifically HSP70, HSP60, and HSP10, have shown that increased levels of these HSPs protected the cardiomyocytes from simulated ischemic injury and cell death [137, 138]. Transgenic mice that overexpress HSP20, HSP27, or both demonstrated an increase in functional recovery and decreased cell death following I/R [139-141]. Expectedly, the knock-out of HSP70 sensitized the heart ischemic damage, as shown by a reduced functional recovery following ex vivo I/R on a Langendorff apparatus [142]. The role of another HSP, alpha-B crystallin (αBC), in protecting the heart has well studied by our lab and others and is reviewed in the following sections.
2. Alpha-B crystallin

Alpha crystallin was first discovered in 1927 as a major component of the lens of eye [143]. Some 50 years later, alpha crystallin was characterized as two separate proteins, alpha-A crystallin and alpha-B crystallin, which share 53% homology, and their role in preventing the development of cataracts [144]. Unlike alpha-A crystallin, which seems to exhibit lens-specific expression, αBC is expressed in the lung, brain, kidney, skeletal muscle and the heart. In the heart, the expression levels of αBC are extremely high; in fact, it has been reported that αBC may constitute up 5% of the total protein in the heart [145]. This extremely high expression has spawned great interest in possible roles for αBC in the heart.

a) Gene structure and regulation

The αBC gene is located on chromosome 8, 9, and 11 in rat, mouse and human, respectively and αBC shares a promoter with another closely related HSP, HSPB2, as a result of their head to head orientation [146, 147]. The shared promoter preferentially favors the expression of αBC over HSPB2, which is consistent with the relative expression of these two genes.

The promoter of αBC contains a serum response element and a two heat shock elements that are required for its expression [131]. Multiple transcription factors have been shown to influence the expression of αBC. In vitro and in vivo, the activation of MKK6 has been shown to regulate the expression of αBC [128, 131]. Heat shock factor 1 (HSF1) has been also shown to regulate the
expression of αBC in the heart. HSF1 knock-outs exhibit a 40% reduction in αBC expression when compared to their wild-type litter mates [138].

Alpha-B crystallin is a 175 amino acid protein with a mass of approximately 22 kD. αBC consists of three main regions, the N-terminal domain, the alpha crystallin domain and the C-terminal extension. The alpha crystallin domain is conserved among most HSPs [134]. Point mutation analysis of the αBC protein has established that the chaperone activity of αBC lies mainly within the alpha crystallin domain and the C-terminal extension. Since no single mutation was able to inactivate all of αBCs chaperone capability, it is believed that αBC interacts with multiple proteins through different regions within these domains [148-150]. The N-terminal domain contains three phosphorylation sites, two of which greatly affect the chaperone activity of αBC (Fig. 16).
Figure 16. αBC protein structure and phosphorylation sites
Depiction of the three domains of the 175 amino acid protein αBC. The N-terminal domain contains three phosphorylation sites. Serines at positions 45 and 59 can be phosphorylated by MAPK activation, while the kinase that phosphorylates the serine at position 19 has yet to be uncovered. The crystallin domain is a conserved region present in all heat shock family proteins and is believed to be important for the chaperone activity of this family of proteins. The C-terminal domain has also been shown to be involved in chaperone activity, as well as oligomerization.
b) Phosphorylation

Stresses, such as heat shock, \( H_2O_2 \), and I/R have been shown to increase phosphorylate \( \alpha BC \) [151]. Under such stresses, \( \alpha BC \) is phosphorylated on serines 19, 45 and 59 [131, 152]. Both serine 45 and 59 have been shown to improve \( \alpha BC \)'s chaperone activity and this has been correlated to an increase in cell survival [131, 153]. The p38 pathway, through MAPKAPK2, is responsible for the phosphorylation of serine 59, but I/R-induced activation of protein kinase C-related kinase 1 (PKN) has also been shown to increase the phosphorylation of \( \alpha BC \) serine 59 and 45 with no increase in MAPKAPK2 activity [154, 155]. Serine 59 has been shown to be necessary and sufficient to mediate \( \alpha BC \) protective ability in the heart in response to I/R [153]. The ERK pathway has been shown to be the major contributor to the phosphorylation of serine 45 and, to the best of my knowledge, the kinase involved in the phosphorylation of serine 19 as well as the role it plays in \( \alpha BC \) function has yet to be uncovered (Fig. 16).

Like most phosphorylation events, the phosphorylation of \( \alpha BC \) is a reversible process. Cardiomyocytes treated with a phosphatase inhibitor exhibit an increase in phosphorylated \( \alpha BC \), which was associated with an increase in protection from ischemia induced cell death [156]. The phosphatase responsible for dephosphorylation of \( \alpha BC \) has yet to be identified.
c) Translocation during stress

Under basal conditions, αBC is detected mainly in the cytosol of cardiac myocytes; however, following ischemic stress, αBC translocates from the cytosol to other cellular regions [129, 157, 158]. Upon stress, αBC translocates to myofilaments and mitochondria of cardiac myocytes [129, 157, 158]. In other cell lines, this translocation has also been reported in response to heat stress [159].

The translocation of αBC to the myofilaments is thought to preserve contractile protein integrity and maintain myocyte function [160]. The translocation of αBC was first reported by Golenhofen, et al in an in vivo model of myocardial infarction [160]. Basally, αBC was located in the cytosol and showed little association with the cytoskeleton or sarcomeric proteins. Following myocardial infarction αBC translocated from the cytosol to myofilaments, to the z-line, actin, myosin, desmin, titin, and vimentin [160].

It is thought by stabilizing the structural integrity of the myofilaments and maintaining elasticity that αBC maintains cardiac function when it is located at the sarcomere. αBC is thought to maintain the elasticity of the sarcomere, in part, through its binding to the globular regions of titin [161]. This binding of αBC to titin helps maintain sarcomeric structure during ischemia, where unregulated contractions can strain the sarcomeres.
αBC also translocates to mitochondria during stress [129, 158]. This translocation to the mitochondria happens concurrent with its migration to the sarcomere and in a similar fashion the αBC at the mitochondria helps protect and stabilize proteins involved in mitochondrial function [158]. It is thought that when αBC translocates to the mitochondria, it stabilizes the mitochondrial transition pore (MTP) [129]. These studies have shown that αBC phosphorylated at serine 59 can prevent mitochondrial swelling, which correlates with the MTP opening and apoptotic cell death [129, 158].

d) Protein degradation

Besides αBCs functions reviewed in the above sections, it has also been implicated in many other cellular functions not associated with either the sarcomere or mitochondria. αBC has been implicated in the secretion of vascular endothelial growth factor (VEGF) from retinal pigment epithelial (RPE) cells and a breast cancer cell line [162, 163]. αBC and VEGF were both colocalized in the ER after hypoxia in the RPE cells. In αBC knock-out mice, VEGF protein expression and secretion were markedly reduced but VEGF mRNA levels remained steady [162]. This reduction in VEGF secretion significantly reduced the amount of angiogenesis after laser induced choroidal neovascularization. This suggested that αBC was responsible for posttranslational regulation of VEGF degradation. This was confirmed when VEGF protein expression and secretion were rescued in the αBC/HSPB2 double knock-out (DKO) mice when
treated with a proteasome inhibitor [162]. These studies suggest that αBC can protect VEGF from proteasome-mediated degradation and, thus, affect VEGF function by regulating its stability and secretion.

αBC has also been implicated in the post-translational regulation of muscle regulatory factor MyoD in C2C12 myoblasts [164]. MyoD is a regulator of the cell cycle and an important factor in myoblast differentiation [164]. Increased MyoD induces C2C12 differentiation and decreases the proliferative capability of the cell. C2C12 cells that over-express αBC are slower to differentiate and retain more of a proliferative phenotype [164]. This observation correlates with a decrease in MyoD protein expression in the αBC overexpressing cell line when compared to wild-type cells. The phenotypes observed were reversed with the treatment of a proteasome inhibitor suggesting that αBC plays a role in MyoD regulation by increasing its degradation in a proteasome-dependent manner [164].

e) Negative regulator of apoptosis

αBC, and more specifically, αBC phosphorylated at serine 59, have been shown to decrease apoptosis induced by oxidative, hyperosmotic, and hypoxic stress [153]. αBC directly interacts with the pro-apoptotic proteins, Bax and Bcl-Xs, and prevents their translocation from the cytosol to the mitochondria, where they are known to open the MTP and release the cytochrome-c that initiates apoptosis [129, 165]. Additionally, studies have shown that αBC is able to
mitigate receptor-induced activation of apoptosis through the inhibition of TNF-related apoptosis-inducing ligand (TRAIL) signaling [166]. Furthermore, αBC blocks caspase 3-mediated apoptosis through a direct interaction with pro-caspase 3 which, prevents its cleavage [167, 168]. These studies highlight the roles αBC plays in cell survival through its direct regulation of apoptotic signaling.

f) Cardiac functional studies in mouse models

The use of transgenic mouse models in which αBC was either over expressed or knocked-out have provided the most insight into αBC’s function in the heart. When wild-type αBC was globally overexpressed, the hearts of the αBC transgenic mice demonstrated a significant preservation of function, compared to wild-type hearts after ex vivo I/R [169]. Additionally, the αBC transgenic mice exhibited a significant reduction in the hypertrophic response after trans-aortic constriction (TAC), a model for hypertension induced cardiomyopathy. Although this reduction in hypertrophy was short lived, as the there was no difference in the ratio of heart weight to body weight when comparing the WT and transgenic animals ten weeks after the TAC procedure [170].

In order to study loss-of-function of αBC, a knock-out mouse line was created by a targeted deletion of the first two exons of αBC along with its promoter. Although this strategy succeeded in deleting αBC, due to the close proximity and the head-to-tail orientation of the αBC and the HSPB2 genes,
HSPB2 was also inadvertently deleted [147]. This αBC/HSPB2 double knock-out (DKO) mice were viable into adulthood and exhibited no major abnormalities during development [147, 171, 172]. The lack of development abnormalities in the HSPB2/αBC DKO mice suggests that the expression of these two chaperones is not critical during development or at the very least that a functional redundancy with other HSPs is able to overcome the lack of these two proteins.

The DKO mice had a significantly shorter life span, primarily due to malnutrition as they developed megaesophagus around 10 months of age, yet the hearts of the DKO animals had no overt phenotype [172]. Additionally, the hearts also exhibited a level of reduce glutathione (GSH) and a reduced level of oxidized glutathione (GSSG) when compared to wild-type mouse hearts suggesting that the hearts of the DKOs were under greater oxidative stress [172]. The sarcomeric structure of the DKO hearts was also altered, as they had a significantly larger I-band, compared to the sarcomeres of control hearts [172]. Hearts from the DKO mice showed significantly reduced cardiac functional recovery compared to the hearts from control animals after the hearts were subjected to global ischemia on an ex vivo Langendorff perfusion apparatus [172]. Additionally, this reduced cardiac functional recovery coincided with higher levels of apoptosis and necrosis [172].
g) Alpha-B crystallin and pathology

Pathological mutations in the αBC gene are rare; only 15 human mutations are currently known. The formation of cataracts is the most common phenotype, followed by cardiomyopathy and respiratory failure [173]. Of the 15 αBC mutations, the mutation in which arginine at amino acid 120 is changed to glycine (R120G) has been studied the most thoroughly.

The R120G mutation was first discovered in a French family that had an unusually high frequency of cardiomyopathy and cataracts. The R120G mutation in αBC causes the loss of chaperone function, and increases the propensity for αBC to form a desmin-rich aggresome. Introducing the same mutation into the mouse recapitulated the same phenotypes as in the human mutation. R120G mice exhibited aggregate formation in the myocytes, along with a pronounced disorganization of the myofilaments [174]. Cardiac function was significantly reduced, and by six months the mice were in the advanced stages of heart failure [174]. Interestingly, the aggregates also stained positive for pre-amyloid precursor protein, suggesting some similarity to those found in neurodegenerative diseases [175]. Additionally, the proteasome degradation in these mice was also impaired, but enzymatic activity of the proteasome system was actually increased, suggesting a defect in substrate delivery and not with the degradation machinery [176]. Augmenting the proteasomal machinery through the up-regulation of autophagy reduced the formation of aggresomes and
rescued many of the maladies induced by the R120G mutation [177]. These finding suggest that protein quality control and specifically αBC chaperone activity, are critical for proper cardiac myocyte function and survival.

C. Hypothesis: αBC is important in protecting the heart from ischemia/reperfusion injury

The literature is rich with studies demonstrating the protective effects of αBC and, specifically αBC phosphorylated at serine 59 [131, 153, 157, 158, 166-168, 172, 178-180]. It has been shown that αBC protects through a number of different mechanisms, such has sarcomeric and cytoskeletal stabilization, prevention of mitochondrial-induced apoptosis, and maintainance of protein quality control. However, many of the in vivo studies exploring the protective effects of αBC through the use of αBC knock-out mice have been confounded by the inadvertent co-knock-out of a related heat shock protein HSPB2. Additionally, other studies have uncovered other mechanism by which αBC influences protein quality control in other tissue types but have not been studied in the context of the heart.

Therefore, taking into account the current literature regarding αBC, I hypothesize: That αBC is an important protective protein in the heart and through several mechanisms, such as those incurred during ischemia and reperfusion, helps preserve and maintain cardiac function during stress. The mechanism by
which αBC protects the heart is, in part, through the stabilization of the sarcomeric structure during times of ischemia and reperfusion stress (Fig. 17).
Figure 17. Depiction of the overall hypothesis: αBC acts in multiple pathways to protect the myocardium

Alpha-B crystallin is an important protective protein in the myocardium that acts in multiple pathways to stabilize structure and preserve function of the heart. The mechanisms of protection by αBC include, but are not limited to stabilizing sarcomeric structure, preventing apoptosis directly and indirectly, influencing protein flux, and preserving mitochondrial integrity.
II. Material and Methods

A. Cultured Cardiac Myocytes

Neonatal rat ventricular myocytes (NRVMCs) were isolated as described [83] from 1-2-day-old Sprague-Dawley rat hearts digested with collagenase, and purified by passage through a Percoll gradient, as described [84].

B. Immunoblotting

Immunoblotting was carried out as previously described [39] using antibodies raised against αBC (ENZO, cat# ADI-SPA-223), HSPB2 (BD Biosciences, cat# 611298), HSP70 (Cell signaling, cat# 4872), p38 (Cell Signaling, cat# 9212), MKK3/6 (Upstate, cat# 07-417), troponin T (NeoMarkers, cat# MS-295-P1ABX), or GAPDH (Fitzgerald Industries International, cat# RDI-TRK5G4-6C5) at dilutions of 1:1000 for all antibodies specified except troponin T and GAPDH which were used at 1:4000 and 1:1.5x10^5, respectively.

C. Adenovirus Constructs

Recombinant adenovirus (AdV) encoding only GFP (AdV-Con), GFP and alpha-B crystallin (AdV-αBC) were generated, as described [153], using the AdEasy System (cat# 240009, Aligent Technologies, La Jolla, CA) [86]. PCR-based mutagenesis (QuikChange site-directed mutagenesis kit, cat# 200518, Stratagene, Santa Clara, CA) was used, as per the manufacturer’s instructions, to generate a phospho-inactive mutant of αBC (AdV-AAA) where the serines at positions 19, 45 and 59 in αBC were mutated to alanine or a phosphor-mimetic
mutant of αBC (AdV-AAE) where the serines at positions 19, and 45 were mutated to alanine and the serine at position 59 was mutated to glutamic acid [153]. These forms of αBC were then used to generate recombinant AdV, as described above.

D. Virus Generation

The recombinant adenovirus discussed in Section IIC were created using the AdEasy system, as described [87].

E. qRT-PCR

RNA was extracted from NRVMCs or heart tissue using Quick-RNA MiniPrep Kit, as per the manufacturer’s recommended instructions (cat# R1055, Zymo Research, Irvine, CA). cDNA was generated using Superscript III, as per the manufacturer’s instructions (cat# 18080-300, Life Technologies, Carlsbad, CA). Real-time quantitative PCR was performed as described in Martindale et al 2006 [9] using the Fishers Maxima SYBR Green/ROX Master mix (cat# FERK0223, Fisher, Hanover Park, IL) and the primers in Table 5, qRT-PCR.

F. Alpha-B crystallin Knock-out Mice

The αBC KO targeting vector was constructed by Marie Marcinko in our lab by replacing intron 1, exon 2 and a portion of intron 2 of αBC with a PGK/Neomycin cassette (Figure 18). The αBC KO targeting vector was linearized and transfected into embryonic stem cells. The embryonic stem cells were then screened for recombination by Northern blot analysis and positive cell lines were
expanded. The positive cell lines were then introduced to the embryo by routine
intra-nuclear injection at the San Diego State University transgenic mouse core
facility. Genetic transmission was screened using DNA isolated from tail biopsies
obtained from 3-4 week old mice by standard PCR using primers listed in Table
5, Genotyping. Since the genotyping primer sets for the WT and KO allele share
a common primer, PCR reactions are set up using a three primer system where
the shared primer is added in excess along with the two unique primers.
Heterozygous mice will display both PCR products, while the homozygous mice
will either display only the band corresponding to either the WT of KO allele.
Transgenic mice were maintained as heterozygotes in a SVJ129 background. In
order to generate experimental animals the mice were bred as either
homozygous WT or KO pairs. All experimental animals were F7 generation, or
later, and were 3+ months of age.

All animal procedures were carried out in accordance with the institutional
guidelines. The animal protocol used in this study was reviewed and approved by
the San Diego State University Institutional Animal Care and use Committee.
Approximately 100 10-36 week-old were use in this study.
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<th></th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>qRT-PCR: mouse ANF</td>
<td>TTGTGGTGAGTGACAGCAAGCT</td>
<td>TGTTCAACAGCCACACGTG</td>
</tr>
<tr>
<td>qRT-PCR: mouse BNP</td>
<td>AAGTCGGAAGGAAATGCCC</td>
<td>TTGTGAAGGCTTGTGCCTTC</td>
</tr>
<tr>
<td>qRT-PCR: mouse GAPDH</td>
<td>CCCTGGCAAGAGCTCATCCAT</td>
<td>GTCATGAGCCCTTCCACGAT</td>
</tr>
<tr>
<td>qRT-PCR: rat ANF</td>
<td>GCGAGATCAAGCTGCTCAGT</td>
<td>CTCTGGCTCCAAATCTGTC</td>
</tr>
<tr>
<td>qRT-PCR: rat BNP</td>
<td>GTGCTGGCCCAAGATGATTCT</td>
<td>CAGCGGGGACAGATTTAAGGA</td>
</tr>
<tr>
<td>qRT-PCR: rat GAPDH</td>
<td>CCCTGGCAAGAGCTCATCCAT</td>
<td>GTCATGAGCCCTTCCACGAT</td>
</tr>
<tr>
<td>qPCR: CMVβ+MLC000</td>
<td>AAGTCGGAAGGAAATGCCC</td>
<td>AAGGAGCCTGAAGCTTTTGATTTC</td>
</tr>
<tr>
<td>Genotyping: αβC WT</td>
<td>CACCTGTTTCTTTGTCCAT</td>
<td>AGTGGCGGCATTGTAAACTG</td>
</tr>
<tr>
<td>Genotyping: αβC KO</td>
<td>CACCTGTTTCTTTGTCCAT</td>
<td>CTTTGCTACCCGCTATT</td>
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Alpha-B crystallin was globally knocked-out by replacing the distal half of exon 1 and exon 2 using a targeting vector that contained PGK and a Neomycin resistance gene.

Figure 18. Diagram of the αBC KO transgenic mouse construction
G. Echocardiography

Transthoracic two-dimensional guided M-mode echocardiography of minimal (0.8%) isoproterenol anesthesia with 2.5% supplemented oxygen using a Vevo 770 sonogram, as previously described [181].

H. Hemodynamic Measurements

Hemodynamic measurements were carried out as previously described [181]. Briefly, hemodynamic measurements were accomplished in a closed chest preparation by inserting a pressure/volume catheter through the carotid artery, past the aortic valve, and into the left ventricle under chloral hydrate sedation. Data were collected and analyzed using a Scisense microtip pressure transducer attached to an A/D converter.

I. Electron Microscopy

Hearts were excised and immediately placed into 0.15M cacodylate buffer containing 100mM sucrose overnight. Then the heart was chopped into ~1 mm² pieces. The pieces were incubated in 2% glutaraldehyde 2% formaldehyde (EMS) in 0.15 cacodylate buffer for 4 hrs, washed and fixed again in 1% osmium (EMS) for 2 hrs. After being rinsed with dH2O, the tissue was dehydrated with acetone and infiltrated with epon (EMS) for polymerization. After sectioning was completed, grids were stained with uranyl acetate and lead citrate before being viewed on a Phillips IL410 transmission electron microscope.
J. Immunofluorescence Microscopy

Paraffin embedded tissue sections, cut at 5µm, were prepared from mouse hearts that had been processed as described previously [60]. Briefly, tissue sections were deparaffinized in xylene and rehydrated in a series of graded alcohols to distilled water. Antigen retrieval was then performed in 10 mmol/L citrate, pH 6.0, using 1100-W microwave oven for 3 mins at high power and 12 mins at 50% power. The slides were allowed to cool for 15 mins at 4ºC, then washed in 3 times in TN buffer (NaCl 150 mmol/L, Tris 100mmol/L, pH 7.5) and quenched with 3% hydrogen peroxide in TN buffer for 20 mins to remove endogenous peroxidase activity. Slides were then washed 3 times in TN buffer and blocked for 1 hr in TNB (TN buffer containing 0.5% Blocking Buffer, proprietary formula from TSA TM kit, Perkin Elmer/NEN). Slides were incubated at 4ºC overnight with primary antibodies diluted in TNB. Slides were then washed 3 times for 5 mins per wash in 1X TN and incubated for 2 hrs at room temperature in the dark with species-specific secondary antibodies conjugated to fluorophores in TNB. After secondary labeling, slides were washed 3 times in TN. Nuclei were stained for 20 mins with either To-pro-3-Iodide (Topro) at 1/10,000 in TN. Slides were mounted for viewing in Vectashield medium. Wheat germ agglutinin, flourescein conjugated stain was used (Molecular Probes, Cat# W834, Eugene, OR). Confocal images and counts were acquired using a Leica TCS SP2 confocal microscope.
K. Ex Vivo Ischemia-reperfusion

Global no-flow ex vivo I/R was performed on a Langendorff apparatus, as previously described [172]. Briefly, aged matched (12-14 week of age) WT and KO mice were treated with 500 U/kg heparin (Sigma, Cat# H3393-250KU, St. Louis, MO) 10 min prior to administration of 150 mg/kg of pentobarbital (Sigma, Cat# P-3761, St. Louis, MO), both via intraperitoneal injection. Animals were then sacrificed and the hearts were quickly removed and placed in ice-cold modified Krebs-Henseleit buffer (Sigma, Cat# K3753-10X1L, St. Louis, MO). The aorta was then cannulated and the heart was mounted on a Langenforff apparatus and perfused with oxygenated Krebs-Henseleit buffer at a constant pressure of 80 mmHg. Both atriums were removed and flash frozen in liquid nitrogen. A water filled balloon connected to a pressure transducer (Gould Stathem P23 ID) was inserted into the left ventricle and inflated in order to record left ventricle developed pressure (LVDP). Hearts were submerged in buffer warmed to 37°C at all times. Following a 30 min equilibration period, hearts were subjected to 25 min of ischemia followed by 90 min of reperfusion. Following the I/R time course the hearts were quickly removed from the apparatus and any remaining connective tissue was removed and subsequently flash frozen in liquid nitrogen. Frozen hearts were stored at -80°C until processed.
L. **Adeno-Associated Virus Serotype 9**

For generation of recombinant AAV vector pTRUF-CMV\textsubscript{enh}MLC800 was constructed by modifying pTRUF12 (a gift from Dr. Roger Hajjar) by first removing the region encoding GFP that was down-stream of the IRES. New restriction sites were inserted into the multiple cloning site to include Nhe1, Pme1, Xho1, and Mlu1. The CMV promoter was replaced with a composite promoter comprised of an 800 bp fragment of the MLC2v promoter downstream of a CMV enhancer (a gift from Dr. Oliver J. Muller) [182]. AAV9 vectors with wild-type capsids were generated by cotransfection the helper plasmid pDG-9 (a gift from Dr. Roger Hajjar).

pTRUF-CMV\textsubscript{enh}MLC800-\alpha BC was created by subcloning the mouse \alpha BC cDNA (accession NC\_000011) from pcDNA3.1-\alpha BC plasmid by excising with Xho1 and HindIII.

To prepare the recombinant AAV9, HEK293T cells were grown in DMEM/F12 containing 10% FBS, penicillin/streptomycin at 37°C and 5% CO\textsubscript{2}. HEK293T cells were plated at 8x10\textsuperscript{6} per T-175 flask. Twenty-four hours after plating, cultures were transfected using Polyethylenimine “Max” (MW 40,000; cat. No: 24765; Polysciences, Warrington, PA) as follows: 15\mu g of helper plasmid and 5 \mu g of pTRUF plasmid were mixed with 1ml of DMEM:F12 and 160 \mu l of polyethylenimine (0.517 mg/ml), vortexted for 30 secs, and incubated for 15 mins at room temperature. This mixture was then added to the cultures in a drop-wise
fashion. The cultures were then rocked intermittently for 15 mins before incubation. The culture medium was changed 6 to 18 hrs later. Three days after transfection, the cells were collected from six flasks and then centrifuged at 500xg for 10 mins. The cells were resuspended in 10 ml of lysis buffer (150 mmol/l NaCl, 50 mmol/l Tris-HCL). The resuspended cells were then subjected to three rounds of freeze-thaw followed by treatment with benzonase (1500 U of benzonase; Novagen cat# 71205) and 1 mM MgCl₂ at 37°C for 30 mins. The cell debris was collected by centrifugation at 3,400xg for 20 mins. The supernatant containing the AAV9 virus was then purified on an iodixanol gradient comprised of the following four phases: 7.3 ml of 15%, 4.9 ml of 25%, 4 ml of 40%, and 4 ml of 60% iodixanol (Optiprep; Sigma-Aldrich, cat# D1556) overlayed with 10 ml of cell supernatant. The gradients were centrifuged in a 70Ti rotor (Beckman Coulter, Brea, CA) at 69,000 rpm for 1 hr using OptiSeal Polyallomer Tubes (cat# 361625; Beckman Coulter). The virus was collected by inserting a needle 2 mm below the 40%-60% interphase and collecting 4 or 5 fractions (~4 ml) of this interphase and most of the 40% layer. The fractions were analyzed for viral content and purity by analyzing 10µl of each fraction on a 12% SDS-PAGE gel (BioRad, cat# 345-0119) followed by staining with InstantBlue (Expedeon; cat# ISB1L) to visualize the viral capsid proteins, VP1, VP2 and VP3. The virus was then collected from the fractions of several gradients and the buffer was exchanged with lactated Ringer’s using an ultrafiltration device, Vivaspin 20, 100
kD MWCO (GE Healthcare, cat# 28-9323-63). The final viral preparation was then fractionated on a 12% SDS-PAGE gel, stained with InstantBlue, and then compared with a similarly stained gel of a virus of a known titer. Alternatively, a qPCR was performed using primer which spans the CMV_{enh}MLC800 composite promoter (Table 5, qPCR). A pTRUF vector containing the CMV/MLC800 promoter was used as a standard to determine copy number.

**M. Statistical Analyses**

Data are reported as mean ± SEM and analyzed via Student’s-t test, or 1-way ANOVA with Newman–Keuls post-hoc analysis, when appropriate, using Graphpad Prism version 4. Unless otherwise stated in the figure legends, *, δ, ψ = p ≤ 0.05, **, δδ = p ≤ 0.01 and ***, δδδ = p ≤ 0.001 different from all other values.
III. Results

A. Generation of the αBC KO mice
Generating a line of mice with only αBC knocked-out proved to be more
difficult than expected. Of the 8 founders that were eventually obtained, only one
harbored the deletion for αBC. To further confound the study, the lone founder
was bred into a C57 mouse background before we could specify the background.
Accordingly, this line was backcrossed into a SVJ129 background for 7
generations using WT SVJ129 female mice obtained from Jackson Laboratories
in order obtain a homogenous SVJ129 background. After completing the
backcross, this line of mice starting with generation F7 was used for the present
study.

B. Physical characterization of αBC KO mice
As part of the initial characterization of the KO mice, a longevity study was
carried out. We found that, through 10 months of age, there was no difference in
the survival rates of the KO mice, when compared to WT. At 11 months of age,
the KO mice started to exhibit a significantly reduced survival rate when
compared to their WT litter mates and by 16 months all of the KO mice were
deceased with a mean survival age of 15.5 months, while 100% of the WT had
survived (Fig. 19). Upon postmortem examination, the KO mouse hearts
exhibited no obvious physical difference, compared to WT mouse hearts. Like
the αBC-HSPB2 DKO mice, the cause of premature death in the KO mice is
Figure 19. Life span of the αBC KO mice
Time course of wild-type (WT) and αBC knock-out (KO) mice. Equal numbers (n=10) of male WT and KO mice were observed for 20 months. Values are percentage of mice surviving at a given time point.
most likely malnourishment due to megaesophagus, and not a cardiac functional deficit [147, 172].

Physical examination of the KO mice at 16 weeks of age showed no significant change in heart, body or liver weight (Table 6). This result is in contrast to those observed in the DKO mice in which the body weight of the DKO mice was significantly lower than those or the WT mice which was believe to be due to malnutrition [172].

To further characterize the effect of αBC gene disruption, expression of several proteins involved in the phosphorylation of αBC and related a HSP family members were analyzed. As predicted, the expression of the αBC protein in the KO mouse heart was completely ablated (Fig. 20A and 20B). Conversely, the expression of HSPB2 was increased approximately 2-fold in the KO heart when compared to the WT expression level (Fig. 20A and 20C). This is not surprising, since both αBC and HSPB2 share a common promoter it is possible that the deletion of αBC may allow for the unrestricted expression of HSPB2. This increase may also be a compensatory up-regulation as αBC and HSPB2 have a greater than 50% homology and may share overlapping functions. Another related HSP, HSP70, exhibited no change in protein expression when the WT and KO mouse hearts were compared (Fig. 20A and 20D). Additionally, the two upstream kinases, MKK3/6 and p38, involved in the
Table 6. Physical Characteristics of αBC KO Mice
Values are means ± SE; WT n=9, KO n=8. Heart, liver, and body weights of 12 week old mice were obtained as described in the materials and methods. KO, αBC knock-out mice; WT, wild-type mice.

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<th>Parameter</th>
<th>WT</th>
<th>KO</th>
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<td>Heart weight, mg</td>
<td>175.6 ± 4.515</td>
<td>170.9 ± 8.056</td>
</tr>
<tr>
<td>Liver weight, mg</td>
<td>1031 ± 56.87</td>
<td>1067 ± 40.27</td>
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<tr>
<td>Body weight, g</td>
<td>29.67 ± 1.655</td>
<td>29 ± 0.9934</td>
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<tr>
<td>Heart/BODY weight, mg/g</td>
<td>6.028 ± 0.3255</td>
<td>5.943 ± 0.3813</td>
</tr>
<tr>
<td>Liver/BODY weight, mg/g</td>
<td>35.02 ± 1.709</td>
<td>36.99 ± 1.779</td>
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Figure 20. Effect of αBC KO on HSP and MAPK protein expression

Panel A- Extracts from WT and KO mouse heart ventricles were analyzed for αBC, HSPB2, HSP70, P38, MKK3/6, and GAPDH protein by immunoblotting.

Panels B-F- Relative blot intensities of indicated protein/GAPDH expressed as fold of control. Values are mean relative indicated protein/GAPDH ± SE, n=3 mice.
phosphorylation and activation of αBC were examined. The protein levels of MKK3/6 and p38 were the same in the KO and WT mouse hearts (Fig. 20A and 20E-F). The deletion of αBC did not affect the expression of HSP70 or of the two upstream kinases MKK3/6 and p38, whereas the expression HSPB2 is upregulated in response to αBC deletion which may be compensatory.

To determine the effect of knocking out αBC several cardiac genes that serve as markers of hypertrophy were analyzed by qRT-PCR. When compared to the WT mouse ventricles, the KO mice ventricles exhibited a 1.5-fold increase in atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC) mRNA. However, the levels of brain natriuretic peptide (BNP), α-myosin heavy chain (α-MHC), and cardiac troponin t (cTNT) mRNA were not changed when the ventricles from WT and KO mice were compared (Fig. 21). Though there was a small but significant increase in the ANF and β-MHC mRNA, this increase is negligible compared to a pathological heart where ANF and β-MHC expression can increase as much as 25-fold [183].

To determine if the deletion of αBC, had an effect on cardiac hypertrophy at the cellular level, cross sections of WT and KO mouse hearts from 12 week old male mice were stained with wheat germ agglutinin to determine average cross-sectional area of the myocytes. The average cross-sectional area of the myocytes in the WT hearts was approximately 305 μM²,
Figure 21. Cardiac gene expression
RNA was isolated from WT and KO mouse heart ventricles and analyzed for transcript levels, as described in materials and methods. Levels of each transcript were determined by qRT-PCR. Values are mean target/GAPDH mRNA ± SE, n=3 mice per group.
while in the KO hearts were approximately 260 µM² (Fig. 22). Though the cross-sectional areas of the KO myocytes tended to be lower, there was no statistically significant difference in area from the WT myocytes. Taken together with the physiological measurements, there appeared to be no overt signs of cardiac hypertrophy in the αBC KO mice (Table 6 and Fig. 21-22).

C. Function assessment of αBC KO mouse hearts

To assess the hemodynamic properties of the αBC KO mouse hearts, two-dimensional guided M-mode echocardiograph and in vivo hemodynamic measurements were obtained on 12 week old male mice. All of the parameters measured were the same in the KO and WT mouse hearts (Fig. 23). These data indicate that the deletion of αBC had no effect on normal hemodynamic cardiac function as determined by echocardiography and in vivo hemodynamics.

D. Myofilament structure of αBC KO mouse hearts

To assess the effect of αBC deletion on the structure of the myofilaments, 36 week old WT and KO mouse hearts were examined by electron microscopy. The WT mouse hearts appeared normal with well-organized sarcomeric structure, including distinct and sharp Z-lines and easily defined I-bands. The mitochondria appear well organized and evenly
Figure 22. Myocyte cell size
WT and KO mice hearts were sectioned for immunofluorescence confocal microscopy. Sections were co-stained with wheat germ agglutinin (WGA; green) and TOPRO (blue), as described in materials and methods (top). Quantification of myocyte cross-sectional area n=3 mice (bottom).
Figure 23. Functional assessment of αBC KO mouse hearts

Panels A-D- Echocardiography was performed on WT (n=11) and KO (n=10), 12-14 week old mice as described in materials and methods; left ventricle diastolic volume (LV vol d), left ventricle systolic volume (LV vol s), ejection fraction (EF%), fractional shortening (FS%) and left ventricle mass (LV mass) were measured.

Panels E-F- In vivo hemodynamics was performed on WT (n=7) and KO (n=6), 12-14 week old mice as described in materials and methods; systolic and diastolic pressure (dP/dt) was measured.
distributed (Fig. 24A). In contrast, in hearts of the KO mice the sarcomeric structure is less organized, as pointed out by the stars (Fig. 24B, *). Along with the myofilament disorganization, the Z-lines of the KO hearts appeared more diffuse and the I-bands were almost nonexistent when compared to the WT. Additionally, the mitochondria of the KO hearts appeared to be less organized in their distribution and their shape was less uniform (Fig. 24B).

The effect of αBC gene disruption on a select component of the sarcomere was assessed. Troponin T is a structural protein within the myofilament, and along with the other troponins, troponin I and troponin C, regulate the calcium sensitivity of the myosin actin interaction. Troponin T is also a marker for cardiomyopathy and can often be detected in the blood prior to any changes in cardiac function [184]. In the KO mouse hearts troponin T was 6-fold more expressed then in the WT hearts (Fig. 25). These data along with the abnormal sarcomeric structure, indicate an underlying abnormality not detected in baseline functional assessments.

E. Postischemic contractile recovery

Over-expression of αBC was shown to enhance functional recovery of isolated perfused hearts following global ex vivo ischemia [169]. Conversely, the disruption of αBC and HSPB2 impaired the function
Figure 24. Electron microscopy of αBC KO mouse hearts

Panels A-B: Left ventricular sections of WT and KO male mice were analyzed by electron microscopy and photographed at x5000 as described in materials and methods. Sarcomeric disorganization is indicated by the * symbol in the KO sections.
Figure 25. Analysis of Troponin T in αBC KO mouse hearts
Extracts from WT and KO mouse heart ventricles were analyzed for troponin T and GAPDH protein by immunoblotting (top). Relative blot intensities of indicated troponin T/GAPDH expressed as fold of control (bottom). Values are mean relative troponin T/GAPDH ± SE, n=3.
recovery of transgenic mouse hearts following global ischemia but the functional
deficit could not be attributed solely to either gene [172]. In order to attribute the
functional deficit to either αBC or HSPB2, αBC KO only mice were subjected to
ex vivo global ischemia/reperfusion. Hearts were subjected to 30 min of
equilibration. Equilibration was followed by 25 min of no-flow global ischemia and
90 min of reperfusion. Hearts from both the WT and KO exhibited similar left
ventricle developed pressure (LVDP) during equilibration and ischemia.
However, during reperfusion the KO mouse hearts exhibited lower functional
recovery when compared to the WT mouse hearts. Similar to the αBC/HSPB2
DKO, the difference in contractile recovery between the αBC KO and WT was
evident as early as 5 min, when the LVDP of the KO mouse hearts was
approximately 25% of the WT mouse hearts. This functional deficit continued
throughout the 90 min reperfusion, where the WT mouse hearts reached 60% of
maximal developed pressure whereas the KO mouse hearts only reached 25%
(Fig. 26A).

In an attempt to rescue the functional deficit after ischemia exhibited by
the αBC KO, adeno-associated virus serotype 9 (AAV9)-mediated gene transfer
was used to replace αBC in the KO background. Six week old αBC KO mice
were injected, via tail vein, with 5x10^{11} viral particles of either an AAV9 containing
a control vector (AAV9-Con) or with a vector encoding WT αBC (AAV9-αBC). Six
weeks after the injection with the AAV9 viruses, KO
Figure 26. Effect of ischemia and reperfusion (I/R) on ventricular function of isolated mouse hearts

Panel A- Age-matched, 12-14 week old WT (n=7), KO (n=7), AAV9-Con (n=5), and AAV9-αBC (n=5) male mouse hearts were subjected to ex vivo I/R as described in materials and methods. Values are % maximum developed left ventricular pressure, means ± SE.

Panel B- Extracts from WT, AAV9-Con, and AAV9-αBC mouse heart ventricles were analyzed for αBC and GAPDH protein by immunoblotting (top). Relative blot intensities of indicated αBC/GAPDH expressed as fold of control (bottom). Values are mean relative αBC/GAPDH ± SE, n=3.
animals injected with the AAV9-αBC expressed similar levels of αBC as their WT litter mates, whereas those injected with the AAV9-Con still were devoid of αBC expression (Fig. 26B). However, no statistical difference in functional recovery was observed between the AAV9-Con or AAV9-αBC injected animals, and neither of the AAV9 injected groups were significantly different from the normal αBC KO mice (Fig. 26A). The AAV9 data suggest that simply replacing the missing αBC does not rescue the observed deficit in functional recovery from ischemia, and that other developmental, or physiological abnormalities may still be present in the AAV9-αBC injected mice.

IV. Conclusion

In this study we have clarified the role αBC plays in protecting the myocardium from I/R damage. We have shown that the deletion of αBC in the heart decreases the ability of the heart to recover from I/R injury and that the sarcomeric structure of the myocytes in the αBC KO mice is disorganized and abnormal. Additionally, we have shown that some markers of hypertrophy, ANF, β-MHC, cardiac troponin T, are slightly but significantly up-regulated in the hearts of KO mice when compared to WT litter mates, suggesting a slight pathology even though there were no overt changes in cardiac function at baseline. These findings are consistent with the previously published work by Morrison et al, demonstrating the requirement of αBC and/or HSPB2 in protection from I/R [172]. Moreover, a recent study highlighting the dispensable role HSPB2 plays in the
pressure overload in mice heart further clarifies the role αBC plays in the hypertrophic response protecting the heart [185]. Thus, αBC has been shown to play a critical role in the protection of the myocardium in response to the ischemic stress whereas the role of HSPB2 in response to I/R is still unclear.
CHAPTER FOUR

I. Discussion

The misfolding of nascent proteins, or the unfolding of already synthesized proteins, can occur in response to numerous stresses, such as I/R, or mutations that de-stabilize protein structure. Cells have evolved a complex system that recognizes and refolds improperly folded proteins, or targets terminally misfolded proteins for degradation. One such quality control system is the ER stress response. The ER stress response is a highly conserved signal transduction system that is activated when cells are subjected to conditions that alter the ER in ways that impair the folding of nascent proteins in this organelle. Another protein quality control system is the HSPs. HSPs are molecular chaperones that recognize and bind to misfolded or denatured proteins, which leads to their correct folding or degradation. Accumulating evidence has implicated that maintaining proper protein quality control, particularly in experimental models of I/R, results better cardiac function after injury. Thus the study of these protein quality control systems is critical in understanding how to protect the heart from stress induced injury.

Secretory proteins, including growth factors, hormones, and stem cell homing factors, as well as membrane proteins, such as receptors and ion channels, are made in the ER, and sarcoplasmic reticulum (SR) of cardiac
myocytes [186]. In most cells, secretory and membrane proteins account for at least one-third of all proteins produced. Most secretory and membrane proteins contain disulfide bonds that are required for their functions [187]. Therefore, protein disulfide bond formation in the ER/SR is critical for a significant portion of protein synthesis and, thus, is required for proper function of all organs, including the heart. This emphasizes the essential role played by the proteins responsible for disulfide bond formation, the protein disulfide isomerases (PDIs), which have not been studied extensively in the heart.

In Chapter II of this dissertation, the PDI family member, PDIA6, also called ERp5, PDI P5, TXNDC7, was characterized in cultured cardiac myocytes, and in the mouse heart [188]. PDIA6 expression was increased by chemical ER stressors, as well as by simulated ischemia-mediated ER stress. The nodal ER stress-activated transcription factor, ATF6, which is cardioprotective [9] and activated by ischemia [76], was shown to induce PDIA6 in cultured cardiac myocytes, and in the myocardium, in vivo. Moreover, wild-type PDIA6 protected cardiac myocytes from ischemia-mediated cell death, while a mutant form of PDIA6 that lacks disulfide isomerase activity was not protective. These results suggest that by serving a central role in secretory and membrane protein folding, PDIA6 contributes to the mechanisms by which ATF6 protects the heart from damage during ischemia.
Although little is known about PDIA6, and it has not been studied in the heart, the PDI family has been extensively studied [74, 189]. The PDI family comprises 20 diverse members, ranging in molecular weight from about 18 to 80 kD. The PDIs have been shown to participate in oxidative disulfide bond formation of nascent proteins in the ER, but also many other functions, such as chaperone and, perhaps, regulation of calcium levels in the ER. PDI family members vary in domain arrangement, but have at least one domain that resembles thioredoxin, as well as -Cys-X-X-Cys- motifs that participate in the catalysis of disulfide bond formation in newly synthesized proteins. Like other PDI family members, PDIA6 is ubiquitously expressed in human tissue (BioGPS, 207668_x_at), albeit at different levels, depending upon the tissue [190], and, although each member can facilitate disulfide bond formation in numerous proteins, they appear to exhibit a certain degree of client selectivity [191]. Moreover, the various PDI family members exhibit cell- and stimulus-specific induction responses. For example, hypoxia was shown to induce PDIA6 in head and neck carcinoma cells, but not in cervical carcinoma cells [189].

Although the precise function of PDIA6 in cardiac myocytes is yet to be determined, it is safe to speculate that it most likely contributes to oxidative protein disulfide bond formation in nascent secretory and cell-membrane proteins made in the ER/SR of cardiac myocytes. Since PDI-mediated disulfide bond formation requires oxygen, the induction of PDIA6 by ischemia, which we
showed to be ATF6-dependent, likely represents a compensatory response to the ER stress that is generated upon the accumulation of misfolded proteins in the ER/SR of ischemic cardiac myocytes. It is of further interest to note that in the sole previous paper in which hypoxia was shown to induce PDIA6, compared to atmospheric oxygen levels of 21%, 1% oxygen resulted in minimal PDIA6 induction, while 0% oxygen maximally induced PDIA6 [192]. This further supports the hypothesis that PDIA6 is induced by hypoxia-mediated ER stress, because ER stress is not activated until oxygen levels decrease to about 0.1% [76]. Indeed, ER stress is activated during myocardial infarction and stroke [9, 76, 193] and some studies support the notion that under these conditions, ER stress promotes survival [9, 73, 81]. Moreover, we previously showed that the 50 kD anti-KDEL cross-reactive protein identified in this study as PDIA6, was strongly upregulated in cultured cardiac myocytes exposed to simulated ischemia [81].

In addition to its role in oxidative protein disulfide bond formation in the ER, PDIA6 has been shown to have other functions. For example, in agonist-treated platelets, PDIA6 translocates from the ER to the cell surface, where it participates in the final aspects of platelet activation, such as α-granule secretion and the binding of platelets to fibrinogen; these activities were shown to depend upon PDIA6 enzyme activity [194]. Interestingly, in the same study, cell-surface PDIA6 was found to associate with integrins, suggesting that PDIA6 may contribute to platelet activation by affecting the configuration of disulfide bonds of
several integrins. These intriguing effects were extended further when it was shown that PDIA6 was one of several other ER proteins that relocated to the cell surface upon plasma membrane wounding [195]. Cell-surface PDIA6 has also been shown to promote tumor immune evasion through the shedding of tumor associated ligands [196]. Additionally, PDIA6 has been found on the inner side of the inner mitochondrial membrane, but currently the function of PDIA6 in mitochondria is not known [197]. Thus, in the heart, PDIA6 may play diverse roles beyond ER/SR protein disulfide bond formation, including functioning on the cell surface and in mitochondria in ways that regulate cardiac myocyte signaling from both the inside and outside of the cell.

The major portion of the cellular proteins are made in the cytosol, including structural, signaling, and functional proteins. When nascent proteins are made in the cytosol they must be protected by a specialized class of proteins, chaperones, that bind to the still translating protein. These chaperones prevent the nascent protein from prematurely coming into contact and binding to the multitude of resident proteins before the nascent protein can be properly folded. One family of chaperones is the HSPs, of which αBC is a member. The HSPs proteins are classically known to help stabilize protein structure during times of stress but as with many other proteins other functions for these proteins are being appreciated.
Alpha-B crystallin is a major constituent, up to 5%, of the total myocyte cell protein [145]. Due to the high expression in the heart many studies have been carried out examining the role of αBC in the heart. One of the major studies highlighting the protective effects of αBC was carried out in our lab by Dr. Morrison in which she characterized a line of mice in which αBC and HSPB2 was knocked-out [172]. In this study, the mice were shown to be susceptible to I/R injury but due to the inadvertent knock-out of HSPB2 the susceptibility could not be attributed to either αBC or HSPB2 alone.

In Chapter III of this dissertation, a mouse line in which αBC was knocked-out but HSPB2 was left intact was characterized. While αBC was undetectable in this mouse the expression of HSPB2 was increase by 2-fold. This increase could possibly be due to compensatory up-regulation since αBC and HSPB2 share 33% homology at the amino acid level or that the deletion of αBC may allow for the unrestricted expression of HSPB2 [198]. Interesting, even though no overt cardiac phenotypes were observed in the unstressed heart, a select few markers of pathology were increased, ANF, β-MHC and cardiac troponin T. These markers being increased suggest an underlying pathology not easily discernible in the unstressed heart. Moreover, in the aged heart the sarcomeric structure of the myocytes has started to become disorganized indicating a role αBC may play in maintaining structural integrity of the sarcomere. When KO mouse hearts were subjected to I/R they exhibited a reduced functional recovery when compared to
WT mouse hearts. This result was similar to those found by Dr. Morrison [172]. A recent study highlighted the dispensable role of HSPB2 in the cardiac response to pressure overload, this study further emphasized the role αBC, while marginalizing the role of HSPB2, in the protecting the heart [185]. Thus, αBC has been shown to play a critical role in the protection of the myocardium in response to the ischemic stress, whereas the role of HSPB2 in response to I/R is still unclear.

Additionally, an attempt to rescue the αBC knock-out mice using AAV9-mediated gene transfer was carried out. Mice injected with the AAV9-αBC virus exhibit relatively comparable expression of αBC when compared to the WT mouse hearts. However, the AAV9-mediated rescue was unsuccessful in regaining the protection from I/R injury conferred by endogenous αBC. Even though protection from I/R was not regained with the AAV9, it is possible that other parameters that we did not measure were altered. Alternatively, the timing of the rescue may not have been optimal to confer the beneficial effects of reintroducing αBC into the myocardium. αBC is differentially regulated during ocular growth and shown to be involved in the assembly of the sarcomere [199, 200]. Administering the AVV9-αBC at an earlier age, during which the majority of post-natal growth occurs, may allow the gene replacement therapy to correct early developmental abnormalities that may occur with the lack of αBC in the heart. Additionally, increasing the duration of gene replacement therapy may also
allow for greater efficacy. Since sarcomeric proteins are extremely stable and have a long half-life of 15 days, the initial experiment may not have allowed for enough turnover of previously damaged structure [201, 202]. Thus, the length of time which the mice receive the treatment can be critical and affect the efficacy of the treatment.

In addition to its role in assisting nascent proteins fold, αBC has also been implicated in regulating protein stability and function. In the eye, αBC has been shown to regulate the degradation and secretion of vascular endothelial growth factor (VEGF). Isolated retinal epithelial cells from the αBC/HSPB2 knock-out mice exhibit a marked decrease in VEGF protein, both within the cells and in the media, but levels of VEGF mRNA remained constant when compared to retinal epithelial cells isolated from WT mice [162]. This suggests that αBC may be regulating the degradation and secretion of VEGF post translationally. Additionally, αBC has been show to interact with known secreted proteins, EGF, FGF, TGF-β, and insulin, further implicating a possible role αBC may play in regulating secretion [203]. Not known traditionally as an endocrine organ, the heart has been recently appreciated as a source of cytokines, especially under times of stress [186]. What has not been studied is whether αBC plays a role in the regulation of secretion from heart. Preliminary studies in our lab have revealed that αBC may play a role in regulating secretion of ANF. But these studies have been clouded by the fact that αBC also regulated ANF transcription
Therefore, the effects of αBC on ANF secretion and translation were difficult to separate.

The Z-line of cardiac myocytes along with its structural function has also been demonstrated as a major location of signal transduction [204, 205]. Z-lines can act as the interface between the sarcomeres and the cytoskeleton, and transduce signals from the outside of the cell to the inside. Additionally, the Z-line has also been described as the cardiac stretch sensor regulating growth [205]. αBC translocation to the Z-line increases following I/R and has been implicated in maintaining the sarcomeric structural integrity. In addition to maintaining structural integrity, αBC may also provide stability to the signaling complexes that use the sarcomere, and more specifically the Z-line, as their signaling node. The maintenance of the various signaling complexes at the Z-line may be critical to cell survival.

In conclusion, the present study has characterized two pathways in which protein quality control is maintained within the myocardium. We have shown that PDIA6 and αBC are essential for proper cellular function and survival after I/R injury. Though there have been many studies highlighting the essential role protein quality control plays in the health of the cell there is still much to learn as the protein quality control story unfolds.
A portion of the text and figures in this chapter is a reprint of the material as it appears in the Journal of Molecular Cellular Cardiology. I was primary author and the co-authors listed in this publication contributed to the research.

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