I, Guan-Sheng Liu, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Molecular, Cellular & Biochemical Pharmacology.

It is entitled:
Studies of Human Mutations in Phospholamban and Heat Shock Protein 20

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Committee member: David Wieczorek, Ph.D.

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Studies of Human Mutations in Phospholamban and Heat Shock Protein 20

A dissertation submitted to the

Division of Graduate Studies
of the University of Cincinnati

In partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

In the Department of Pharmacology and Cell Biophysics

2015

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BS, Shandong Normal University, 2007
MS, Second Military Medical University, 2010

Committee Chair: Evangelia G. Kranias
Co-Chair: Guo-Chang Fan
Abstract

In this dissertation, we identified two novel human mutations in the major Ca$^{2+}$-handling proteins, PLN and Hsp20, in patients with DCM. Specifically, exome sequencing identified a C73T substitution in the coding region of PLN in a family with DCM. This C73T substitution results in the change of arginine at position 25 into cysteine, which is called R25C. It was found that the 4 heterozygous family members had implantable cardiac defibrillators, and 3 developed prominent ventricular arrhythmias. The second mutation in Hsp20 is S10F, which was identified in DCM patients with a frequency of 2.8%, while there were no normal subjects carrying the S10F-Hsp20. To determine if there are correlative or causative relationships between the two mutations and the observed clinical phenotypes in human carriers, the effects of R25C and S10F mutations on cardiac function were investigated by utilizing adenoviral infected rat cardiomyocytes and transgenic mouse model respectively.

First of all, this dissertation reveals that overexpression of R25C-PLN in adult rat cardiomyocytes significantly suppressed the Ca$^{2+}$ affinity of SERCA2a, causing decreased SR Ca$^{2+}$ content, Ca$^{2+}$ transients and impaired contractile function, compared to WT-PLN. These inhibitory effects were associated with enhanced interaction of R25C-PLN with SERCA2a, which was prevented by PKA phosphorylation. However, R25C-PLN also elicited increases in the frequency of Ca$^{2+}$ sparks and waves as well as stress-induced aftercontractions. This was accompanied by increased CaMKII activity and hyper-phosphorylation of RyR2 at serine 2814.
Regarding the role of S10F-Hsp20 in cardiac function, *in vitro* studies in infected cardiomyocytes showed that the inotropic effects of Hsp20 were negated by S10F. Furthermore, S10F significantly abrogated the protective effects of WT-Hsp20 upon prolonged isoproterenol-stimulation. To investigate the *in vivo* impact of S10F-Hsp20, transgenic mice with cardiac-specific overexpression of this mutant were generated. Our results indicated that in contrast to the enhancing effects of WT-Hsp20, contractility and SR Ca$^{2+}$ cycling in myocytes from S10F-Hsp20 hearts were not different compared to those in NTG cells, consistent with the *in vitro* data. The underlying mechanisms involved reduced ability of mutant Hsp20 to interact with protein phosphatase 1 (PP1) and inhibit it, compare to WT-Hsp20.

Furthermore, the role of the S10F-Hsp20 in cardiac function and protection under stress conditions were determined. It was found that the protective effects of WT-Hsp20 against I/R-induced injury were abrogated by S10F-Hsp20. In addition, the S10F mutation was found to accelerate the progression of MI-induced cardiac remodeling and heart failure. More importantly, under the physiological stress of aging, there was severe contractile dysfunction and cardiomyocyte apoptosis in the S10F hearts, which eventually led to dilated cardiomyopathy, heart failure and accelerated death in the mutant mice. The main mechanisms associated with impaired recovery after I/R injury, detrimental remodelling after MI or upon aging in the mutant hearts included reduced interaction of S10F-Hsp20 with: a) Bax, leading to activation of caspase-3 and apoptosis; b) pAkt, associated with diminished Akt activation, reduction in p-Bad...
and increased apoptosis; and c) Beclin 1, resulting in decreased activation of autophagy and thus ability to remove damaged organelles under stress conditions.
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<tr>
<td>±dL/dt</td>
<td>Maximal rates of cardiomyocyte contraction (+) and relengthening (-)</td>
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<tr>
<td>±dP/dt</td>
<td>Maximal rates of left ventricular pressure change during contraction (+) and during relaxation (-)</td>
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<td>AA</td>
<td>Amino acids</td>
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<td>Aβ</td>
<td>amyloid-β peptide</td>
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<td>ACD</td>
<td>Alpha(α)-crystallin domain</td>
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<td>AD</td>
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</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia/reperfusion</td>
</tr>
<tr>
<td>Iso</td>
<td>Isoproterenol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>IVSd</td>
<td>Interventricular septal end diastole</td>
</tr>
<tr>
<td>IVSs</td>
<td>Interventricular septal end systole</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KHB</td>
<td>Krebs-Henseleit buffer</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LAD</td>
<td>Left anterior descending coronary artery</td>
</tr>
<tr>
<td>LCA</td>
<td>Left coronary artery</td>
</tr>
<tr>
<td>LTCC</td>
<td>L-type calcium channel</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricular</td>
</tr>
<tr>
<td>LVDP</td>
<td>Left ventricular developed pressure</td>
</tr>
<tr>
<td>LVEDD</td>
<td>Left ventricular end diastolic dimension</td>
</tr>
<tr>
<td>LVEDV</td>
<td>Left ventricular end diastolic volume</td>
</tr>
<tr>
<td>LVESD</td>
<td>Left ventricular end systolic dimension</td>
</tr>
<tr>
<td>LVESV</td>
<td>Left ventricular end systolic volume</td>
</tr>
<tr>
<td>LVIDs</td>
<td>Left ventricular internal diameter end systole</td>
</tr>
<tr>
<td>LVIDd</td>
<td>Left ventricular internal diameter end diastole</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LW</td>
<td>Lung weight</td>
</tr>
<tr>
<td>I-1</td>
<td>Inhibitor 1</td>
</tr>
<tr>
<td>I-2</td>
<td>Inhibitor 2</td>
</tr>
<tr>
<td>MCU</td>
<td>Mitochondrial Ca(^{2+}) uniporter</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MyBP-C</td>
<td>Myosin binding protein-C</td>
</tr>
<tr>
<td>NCX</td>
<td>Na- Ca(^{2+}) exchanger</td>
</tr>
<tr>
<td>NHE</td>
<td>Sodium hydrogen exchanger</td>
</tr>
<tr>
<td>NTG</td>
<td>Non-transgenic</td>
</tr>
<tr>
<td>OE</td>
<td>Over-expression</td>
</tr>
<tr>
<td>OMPs</td>
<td>Outer membrane proteins</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>PP1</td>
<td>Protein phosphatase 1</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>RyR</td>
<td>Cardiac ryanodine receptor</td>
</tr>
<tr>
<td>SERCA2</td>
<td>Sarco(endo)plasmic reticulum Calcium ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TG</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling</td>
</tr>
<tr>
<td>Vmax</td>
<td>Maximal velocity of calcium uptake</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Chapter I: Introduction

Section I: Cardiac contractile function

I.1 E-C coupling and Ca\(^{2+}\) cycling in the cardiomyocyte

The heart utilizes Ca\(^{2+}\) cycling to achieve E-C coupling, which is fundamental to myocyte contractile function. Specifically, upon a depolarizing signal during an action potential, a relatively small amount of Ca\(^{2+}\) enters the cell through the voltage-gated L-type Ca\(^{2+}\) channels (LTCC). This extracellular Ca\(^{2+}\) activates the ryanodine receptors (RyR) on the sarcoplasmic reticulum (SR) membrane, leading to release of Ca\(^{2+}\) stored in the SR. This process is termed Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR). Subsequently, Ca\(^{2+}\) diffuses to the myofilaments and binds to troponin C (TnC), which triggers a conformational alteration in the troponin complex, resulting in dissociation of troponin I (TnI) from tropomyosin and exposure of myosin binding sites on actin. Therefore, cross-bridges of myosin ATPase with actin filaments are formed, activating the ATP hydrolysis cycle and producing force for the myosin to move along the actin filaments towards the Z-line. This movement reduces the sarcomere length, the distance between two Z-lines, and leads to cardiomyocyte contraction. Collectively, this process enables the transduction of the electrical excitation to a mechanical result and is therefore termed excitation-contraction coupling (E-C Coupling).

For cardiomyocyte relaxation to occur, cytosolic Ca\(^{2+}\) must decline, allowing Ca\(^{2+}\) to dissociate from TnC. This requires Ca\(^{2+}\) transport out of the cytosol via four pathways. The first pathway is mediated by the SR Ca\(^{2+}\)-ATPase (SERCA2a), which is highly conserved across species and is critically regulated.
by phospholamban (PLN), a 52 amino acid protein. As stated above, increases in cytosolic Ca\(^{2+}\) level at the beginning of a contraction cycle are primarily mediated by SR Ca\(^{2+}\) release. Correspondingly, during relaxation, most of the cytosolic Ca\(^{2+}\) is re-uptaken into SR. In the murine heart, 92% of the Ca\(^{2+}\) is removed by the SR Ca\(^{2+}\)-ATPase, while in the human heart the SERCA2a removes approximately 70% of the systolic Ca\(^{2+}\).\(^1\) The second pathway is mediated by sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchanger (NCX). Notably, the NCX extrudes around 8% of systolic Ca\(^{2+}\) in the murine and 30% in the human hearts.\(^2\) The third and fourth pathways are facilitated by the mitochondrial Ca\(^{2+}\) uniporter (MCU) and the sarcolemmal Ca\(^{2+}\)-ATPase, which are responsible for overall removal of around 1% of the cytosolic Ca\(^{2+}\).\(^2\) Taken together, the SR plays a crucial role in Ca\(^{2+}\) cycling and is responsible for RyR2 induced Ca\(^{2+}\) release and SERCA2a/PLN complex mediated Ca\(^{2+}\)-uptake.

I.2 Beta-adrenergic signaling and Ca\(^{2+}\)-cycling

Under ‘fight-or-flight’ conditions, the rate and amplitude of Ca\(^{2+}\) cycling and cardiac performance are greatly increased with sympathetic stimulation, mediated mostly through activation of Gs (stimulatory G-protein )-coupled β-adrenergic receptors (β-ARs) on the sarcolemma. β-ARs, firstly identified by Ahlquist et al. in 1948\(^3\), belong to the ubiquitous family of guanine nucleotide (G)-protein-coupled receptors. They are broadly distributed throughout the heart, with approximately equal densities in human atrial and ventricular tissue.\(^4\) Myocardial β-ARs are classified into β1-AR and β2-AR, which are expressed at approximately 4:1 ratio.\(^4\) During activation of the sympathetic nervous system,
catecholamines (epinephrine or norepinephrine) binds to β-ARs and trigger a cascade of events, which eventually enhances cardiomyocyte contractility. Specifically, upon agonist binding, β1-AR coupled with the Gs is able to activate adenylate cyclase (AC), while the β2-AR is coupled to both Gs and to the inhibitory G protein, named Gi, which inhibits AC activation. As the β1-ARs predominate in number, catecholaminergic stimulation results in overall enhanced AC activity. Elevated AC activity leads to increased cyclic AMP (cAMP) formation, which in turn activates the cAMP dependent protein kinase A (PKA), leading to phosphorylation of several Ca2+ cycling proteins and promotion of maximal cardiac contractile function. In detail, PKA is able to phosphorylate: a) LTCC, resulting in a two- to four fold increase in Ca2+ current (ICa) in ventricular myocytes; b) ryanodine receptor, which modifies gating property of RyR and increases Ca2+ release from SR; c) myosin binding protein-C (MyBP-C), which increases myosin-actin crossbridge kinetics; d) phospholamban, which removes inhibitory effects of PLN on SERCA2a and increases SR Ca2+ uptake; and e) troponin I, which lowers myofilament Ca2+ affinity and leads to the more rapid relaxation, owing to faster Ca2+ dissociation from TnC. Collectively, these phosphorylation modifications result in enhanced Ca2+ cycling and therefore greater and faster contraction and relaxation of the cardiac muscle.

I.3 The role of phospholamban in cardiac function

Phospholamban (PLN), a 52 amino acid protein of 6.1 kDa, was first discovered in cardiac microsomes in the early 1970s, by A. Katz and M. Tada. Because it severs as the major substrate of cAMP-dependent protein kinase in cardiac SR
vesicles, it was named phospholamban, from the words phosphate and the Greek word “λαμβανειν” (meaning “to receive or to seize”), indicating that it is a “phosphate receptor”. PLN is able to form a homopentamer, which explains the original observed phosphorylated PLN with an apparent molecular weight of 22 kDa. PLN is present mainly in cardiac muscle and expressed at very low levels in smooth and slow-twitch skeletal muscles and endothelial cells. However, its regulatory effects on SERCA2a have been best studied in cardiac muscle. PLN is a membrane-spanning protein. The accurate structure of PLN is currently unknown. However, based on analysis of its amino-acid sequence, several models have been proposed. It is generally accepted that the protein is organized in three domains: cytosolic domain Ia (AA 1-20, indicating amino acid residues 1 to 20) which contains serine (Ser) 16, the site of phosphorylation by PKA, and threonine (Thr) 17, the site of phosphorylation by Ca²⁺/calmodulin-dependent protein kinase II (CaMKII); cytosolic domain Ib (AA 21–30), which is rich in amidated amino acids; and domain II (AA 31–52), which is anchored into the cardiac SR membrane. Domain Ia presumably forms an α-helical structure, and domain Ib is likely to exist as a random coil. The transmembrane domain II has been proposed to form an α-helical structure as well. Therefore, these domains form a helix (Ia)-turn (Ib)-helix (II) structure.

PLN has been shown as a critical regulator of cardiac contractility by modulating SERCA2a activity. As stated above, SERCA2a removes more than 70% of the Ca²⁺ during diastole in human cardiomyocytes, suggesting that it is an important modulator of Ca²⁺ cycling. Indeed, it has been shown that
SERCA2a ablation led to embryonic lethality, while partial deletion (35% reduction in SERCA2a levels) in heterozygous hearts caused impaired cardiac function.\textsuperscript{14, 15} Accordingly, cardiac overexpression or gene transfer of SERCA2a considerably improved cardiac contractility and relaxation.\textsuperscript{16, 17} SERCA2a is regulated by PLN. In the dephosphorylated form, PLN binds to SERCA2a (physical interaction between PLN and SERCA2a by crosslinking lysine (Lys) 3 in PLN domain Ia to Lys397 and Lys400 in the cytosolic nucleotide-binding domain of SERCA2a\textsuperscript{18} and inhibits Ca\textsuperscript{2+}-pump activity. However, phosphorylation of PLN at Ser16 by PKA or Thr17 by CaMKII disrupts the PLN–SERCA2a interaction, relieving SERCA2a inhibition and enhancing relaxation rates as well as myocyte contractility.\textsuperscript{19}

I.3.A The physiological role of PLN in cardiac function

Several studies by us and other researchers focused on elucidating the functional role of PLN in SR Ca\textsuperscript{2+} cycling and cardiac contractility by targeting the PLN gene in embryonic stem cells and producing heterozygous and homozygous PLN-knockout (PLN-KO) mice, in which PLN protein levels are decreased by 60% and 100%, respectively.\textsuperscript{20, 21} In addition, PLN-overexpressing (PLN-OE) mouse models with twofold and fourfold increases in the cardiac phospholamban protein levels were also generated.\textsuperscript{22, 23} It was found that, the the relative levels of PLN in the heart reflected a linear correlation with the affinity of SERCA2a for Ca\textsuperscript{2+} and cardiomyocyte contractile parameters including fractional shortening (FS\%) and the rates of cell contraction and relaxation.\textsuperscript{23} Alterations in cardiomyocyte contractility were associated with changes in the rates and
amplitude of Ca²⁺ transients in cardiomyocytes, which, in turn, mirrored the alterations in SR Ca²⁺ store in the PLN genetically altered mouse models. These results further supported the role of SR Ca²⁺ content as a principal determinant of cardiac contractility. Notably, the hyperdynamic cardiac function in PLN-KO mice was not diminished under the physiological stresses of exercise or aging and PLN ablation did not shorten the life span in these mice. Besides, the incidence of heart failure induced by transverse aorta constriction was similar between PLN-KO and wild-type mice. In contrast to PLN-KO mice, transgenic mice with cardiac specific PLN overexpression (two-fold) exhibited diminished fractional shortening and reduced rates of shortening and relengthening in isolated, unloaded cardiomyocytes compared with their wild-type littermates. These alterations reflected decreases in the amplitude of the Ca²⁺ signal and a prolonged time for the decay of the Ca²⁺ transient, which were associated with a remarkable decrease in the apparent affinity of the SERCA2a for Ca²⁺. Further in vivo analysis of left ventricular (LV) systolic function using echocardiography revealed a significant decrease in fractional shortening in transgenic mice compared to wild type mice. However, the differences in contractile mechanics and Ca²⁺ kinetics in cardiomyocytes and the suppressed LV systolic function in transgenic mice were completely abolished by the β-agonist isoproterenol (Iso), which phosphorylated PLN and relieved its inhibition on SERCA2a. Transgenic mice overexpressing phospholamban at four-fold of normal levels also demonstrated depressed cardiomyocyte mechanics, Ca²⁺ kinetics and ventricular contractility, which were also reversed by Iso stimulation. However, compared to
two-fold PLN overexpression transgenic mice with no phenotypic changes upon aging, transgenic mice with four-fold PLN overexpression had increased levels of adrenaline/noradrenaline as a compensatory adaptation, which ultimately became maladaptive in the long term, leading to development of overt heart failure and premature mortality. Notably, the phenotypes observed in the mice with no PLN, normal levels and twofold overexpression of PLN revealed a strong linear correlation between the Ca$^{2+}$ affinity of SERCA2a or the myocyte contractility and the relative PLN/SERCA2a ratio, suggesting that the PLN/SERCA2a ratio is a pivotal indicator of cardiac function.

In addition to PLN levels, its phosphorylation state is also critical for SR Ca$^{2+}$ cycling and cardiomyocyte contractility. In vitro studies with purified SR membranes have shown that PLN is able to be phosphorylated at three distinct sites, specifically Ser16 (by PKA/PKG), Thr17 (by CaMKII) and Ser10 (by PKC). Phosphorylation by each kinase relieves PLN’s inhibitory effects and increases the affinity of the SR Ca$^{2+}$ transport system for Ca$^{2+}$. However, in the intact heart, PLN phosphorylation only occurs on Ser16 (by PKA) and on Thr17 (by CaMKII). To elucidate the functional significance and interplay of dual-site phosphorylation of PLN at Ser16 and Thr17, transgenic mice carrying phosphorylation-site-specific PLN mutants were generated and characterized. In detail, cardiac specific overexpression (2.6 fold) of a non-phosphorylatable form of PLN (S16A,T17A) in transgenic mice resulted in maximal SERCA2a inhibition. Thus, functional saturation of SERCA2a inhibition occurs at a ratio of 2.6:1 of PLN: SERCA2a, suggesting that only 40% of the
SERCA2a are regulated by PLN in mouse heart. To determine the physiological role of PLN phosphorylation at its Ser16 site, a transgenic model with S16A-PLN introduced in the cardiac compartment of the PLN-KO mice was developed. It was found that Ser16A mutant hearts exhibited a blunted response to isoproterenol and lack of Thr17 phosphorylation, suggesting that phosphorylation at Ser16 is a prerequisite for Thr17 phosphorylation in PLN. On the contrary, both phosphorylation of PLN at Ser16 site and β-adrenergic responsiveness were successfully preserved in transgenic hearts expressing T17A-PLN in PLN null background. These results indicated that Ser16 can be phosphorylated independently of Thr17 in vivo and phosphorylation of Ser16 is sufficient to mediate the maximal contractile responses of the heart to β-adrenergic stimulation. However, it is important to note that Thr17 phosphorylation has been shown to be phosphorylated independently of the Ser16 site in: a) hearts perfused with elevated Ca\textsuperscript{2+}, b) hearts after an ischemic injury, and c) intact cardiomyocytes subjected to frequency-dependent increases of contractility. These studies suggest that PLN phosphorylation at Thr17 is critical for the heart in response to the direct stress, whereas phosphorylation at Ser16 is responsible for mediating the β-adrenergic responses in vivo. Collectively, these studies of PLN transgenic models indicate that PLN is a crucial regulator of basal cardiac Ca\textsuperscript{2+} cycling and contractile parameters and a principle determinant of β-adrenergic responses in vivo.

I.3.B The role of PLN in heart failure

It has been shown that abnormal SR Ca\textsuperscript{2+} cycling is associated with significant
decrease in SERCA2a levels in human heart failure, whereas PLN levels remain unaltered.\textsuperscript{40, 41} A reduction in the level of SERCA relative to PLN would be anticipated to increase the PLN/SERCA2a ratio, inhibition of the Ca\textsuperscript{2+} affinity of SERCA2a and prolonged cardiomyocyte relaxation time. In addition, there is a considerable amount of evidence that phosphorylation of PLN at Ser16 is reduced in animal models of heart failure and human failing hearts, indicating an increased inhibitory function by PLN, which further suppresses SR Ca\textsuperscript{2+} uptake activity and aggravates the already impaired cardiac contractile function.\textsuperscript{40, 42-45} Indeed, consistent with these findings, significant decreases in both $V_{\text{max}}$ and Ca\textsuperscript{2+} affinity for SR Ca\textsuperscript{2+} uptake were identified in the human failing hearts.\textsuperscript{40} In comparison, the contribution of Thr17-PLN phosphorylation to compromised cardiac function remains controversial.\textsuperscript{45-47} Notably, phosphorylation levels of PLN at Ser16 and Thr17 were found to be significantly increased at the end of ischemia and early stages of reperfusion in ischemia/reperfusion (I/R) studies, suggesting that PLN phosphorylation might be a critical mechanism attempting to improve contractile dysfunction under these conditions.\textsuperscript{38, 48, 49} Based on these discoveries, strategies to augment SR Ca\textsuperscript{2+}-uptake activity by increasing SERCA2a and/or attenuating inhibitory PLN levels might hold therapeutic promise in the treatment of heart failure. Indeed, adenoviral-mediated expression of SERCA2a transgene successfully reconstituted depressed endogenous SERCA2a levels, augmented SR Ca\textsuperscript{2+} uptake and enhanced contractility in isolated murine and human failing cardiomyocytes.\textsuperscript{50, 51} Furthermore, adenoviral gene transfer of SERCA2a significantly improved left-ventricular function, cardiac
metabolism and survival in rat models of heart failure. Moreover, overexpression of SERCA2a was also able to protect diabetic hearts from severe contractile dysfunction by improving the Ca\textsuperscript{2+} sequestration by the SR. Together, these studies support the idea that an increased SERCA2a level restores impaired intracellular Ca\textsuperscript{2+} cycling. Similarly, strategies aimed at suppressing PLN activity with expression of PLN antisense RNA, PLN dominant-negative mutants or expression of an antibody to PLN resulted in improved SR Ca\textsuperscript{2+} cycling, restored contractility and attenuated heart failure progression. More importantly, PLN ablation effectively restored the depressed function and prevented cardiac remodeling in cardiomyopathic mice that lack muscle Lim protein MLP. In addition, PLN ablation was also able to rescue the impaired contractile function in hearts overexpressing either calsequestrin or a mutant myosin heavy chain. However, PLN ablation failed to improve cardiac contractile function or hinder the progression to remodeling and heart failure in transgenic mice overexpressing G\textsubscript{q} and human familial cardiomyopathy mutant myosin binding protein C (MyBP-C(MUT)). This suggests that impaired SR Ca\textsuperscript{2+} cycling may represent one of the multiple modulators of the hypertrophic responses in these models and PLN ablation may not rescue all kinds of heart failure.

**I.4 Human PLN mutations and cardiomyopathy**

The crucial role of PLN in cardiac function prompted searches for human PLN genetic variants, which may be linked with cardiomyopathy. Indeed, a couple of human mutations have been identified in the coding region of PLN. The first
human PLN mutation, a T116G point mutation, which harbors a stop codon for Leu 39 (PLN-L39X) was discovered in two large Greek families with hereditary heart failure. This truncation mutation in the PLN gene leads to absence of detectable PLN protein, namely PLN null, in individuals homozygous for PLNL-39X.20, 62 However, in contrast to the benefits of PLN ablation in mouse, human carriers homozygous for PLNL-39X developed lethal cardiomyopathies requiring cardiac transplantation at an early age. Heterozygous carriers for this mutation developed delayed dilated cardiomyopathy, even without diminished contractile performance.63 This discrepancy between the cardiac phenotypes in mice and humans might be due to differences in cardiac reserves or myocyte Ca\(^{2+}\) fluxes between mouse and human and suggest that PLN modulation might be of more significance in humans. The second mutation is R9C, a missense mutation in PLN cytoplasmic domain that elicits severe dilated cardiomyopathy and premature death in humans.64 To further determine the effects of R9C-PLN in the heart, transgenic mice with cardiac specific overexpression of R9C-PLN were generated and it was found that R9C TG mice recapitulated human heart failure with early death.64 Cellular and biochemical studies discovered that unlike wild type PLN, R9C-PLN had no direct effects on SERCA2a activity under basal conditions but appeared to trap PKA, which blocked PKA-mediated phosphorylation of even wild-type PLN and resulted in superinhibition of SERCA2a activity. Thus, the long-term detrimental effects of such chronic superinhibition of SERCA2a activity are sufficiently deleterious to cause the onset of lethal dilated cardiomyopathy in R9C-PLN carriers.19 The third naturally occurring
mutation in the human PLN gene is associated with the deletion of Arginine-14 (PLN-R14Del) in the coding region. This mutation was discovered in a large family with lethal, hereditary cardiomyopathy. Within this family, no homozygous individuals were identified and heterozygous carriers developed severe cardiac dilation, contractile dysfunction and malignant ventricular arrhythmias and died by middle age. Transgenic mouse model with cardiac specific overexpression of the heterozygous PLN Arg14Del mutant recapitulated human phenotype, displaying similar histopathologic abnormalities and early death. Further mechanistic studies revealed that similarly to R9C-PLN, PLN Arg14Del results in superinhibition of SERCA2a activity, likely mediated by a disturbance in the structure of PLN. Particularly, this superinhibition on the pump could not be fully removed, even upon phosphorylation by protein kinase A. Therefore, this long-term nonreversible superinhibition on SERCA2a activity may be the main cause of dilated cardiomyopathy and premature death in both human carriers and transgenic mice. Finally, in addition to mutations in the coding region of human PLN gene, human mutations have also been discovered in the promoter region of this gene in patients with hypertrophic cardiomyopathy. It was found that these mutations resulted in either increased or decreased PLN promoter activity, which may alter the expression levels of PLN in the heart. However, alterations in PLN promoter activity have not been correlated with changes in protein expression levels of PLN in these studies. Overall, these findings, again establish that PLN is an important regulator of cardiac function and add to accumulating evidence that myocellular Ca²⁺
dysregulation caused by mutations in human \textit{PLN} is sufficiently detrimental to cause DCM and initiate heart failure.

Taken together, PLN is a critical determinant of the SR Ca$^{2+}$-ATPase, Ca$^{2+}$ cycling and contractility and a key regulator of $\beta$-adrenergic signaling pathway in the heart.

\textbf{Section II: Heat shock proteins (Hsps)}

\textbf{II.1 Stress response and heat shock proteins}

Heat shock response was first described in 1962, by an Italian geneticist, Ferruccio Ritossa, when he was interested in determining what type of nucleic acid was synthesized in chromosome puffs of the salivary glands of Drosophila.$^{68}$ One day, Ritossa observed a novel puffing pattern uncharacteristic of that particular stage of larval development. After realizing that something was incorrect, he found that one of his colleagues adjusted his incubator to a higher temperature, which delivered an inadvertent heat shock to the salivary glands of Drosophila kept in the incubator.$^{69}$ This observation finally resulted in the identification of the heat-shock proteins (Hsp), whose expression these puffs represented.$^{70}$ Starting from the 1970s, Heat shock proteins (Hsps) were recognized as a diverse group of molecular chaperones that have been reported in all organisms, from bacteria to plants and animals.$^{71}$ The expression of Hsps can be transiently upregulated in response to various stresses in addition to heat, such as inflammation, viral infection, exercise, starvation, water deprivation, toxic chemicals (glucose/amino acid analogues, heavy metals, arsenite, ethanol), ultraviolet light, oxidative and osmotic stresses, which play a pivotal role in
protecting the cell against different kinds of damage. Therefore, heat shock proteins are also referred to as stress proteins.

Mechanistically, upon stress (i.e., heat shock) on the cell, the outer membrane proteins (OMPs) can not be folded correctly and insert into the outer cell membrane. The unassembled OMPs accumulate in the periplasm, bind to and activate the sensor of envelope stress named DegS, which is an inner membrane protease. Activated DegS protease cleaves the transmembrane protein (RseA), which normally binds to and inhibits the $\sigma^E$ transcription factor. Thus, cleavage of RseA by DegS relieves its inhibition on $\sigma^E$, resulting in increased transcription of stress genes in the cytoplasm.

The predominant role of Hsps in the cell is to enhance correct protein folding and refolding, intracellular proteins trafficking and prevent protein aggregation. They function as intracellular “chaperones” for other proteins, assist the newly produced proteins to establish proper conformation, stabilize partially unfolded proteins, and prevent aggregation of denatured proteins through protein-protein interactions. Hsps are also able to shuttle proteins across membranes and transport old proteins to “garbage disposals” within the cell. In addition, Hsps play a pivotal role in the degradation of misfolded or mislocalized proteins by involving the cytoplasmic Ubiquitin-Proteasome System. These effects of Hsps are essential for maintaining the basic function of the cell and are of great significance for repairing the damage resulting from various harmful stimuli.

Hsps can be categorized by their molecular weight and function as
follows: a) high molecular weight Hsps (Hsp90, Hsp84, Hsp60 and Hsp70), which are best described; b) glucose-regulated proteins (GRP94, 78, 75, 56, 47, 34), which are induced under conditions of glucose deprivation and c) small heat shock proteins (HspB1-B10) whose molecular weight ranges from 12 to 43 kDa.77

II.2 Small Heat Shock Proteins (sHsps)

There are ten members (Hsp27/HspB1, HspB2, HspB3, alphaA-crystallin/HspB4, alphaB-crystallin/HspB5, Hsp20/HspB6, cvHsp/HspB7, H11/HspB8, HspB9 and ODF1/HspB10) within the sHsps' family. The monomer structure of sHsps is characterized by a highly conserved C-terminal region (sequence of 80–100 amino acid residues), which is termed a-crystallin domain (ACD), a variable N-terminal sequence, and a short and variable C-terminal tail.78, 79 The sHsps are able to assemble into mono- and poly-disperse oligomers, where the rate of disassembly affects their chaperone activities. The alpha-crystallin domain is composed of a number of beta-strands, which are organized into two beta-sheets and essential for dimer (the basic functional unit of sHsp) formation. The N-terminal extension primarily regulates oligomerization, subunit dynamics as well as substrate binding, whereas the C-terminal tail helps chaperoning and solubility.80, 81 Functionally, as ATP-independent molecular chaperones, sHsps play an important role in cellular proteostasis. Specifically, sHsps associate with nuclei, cytoskeleton and membranes and are able to prevent stress-induced aggregation of unfolding proteins by refolding them to native conformations or transporting them to proteasomes or lysosomes, the protein ‘garbage disposals’
inside the cell.\textsuperscript{79} This buffering mechanism is of particular importance under stress conditions, when the ATP-dependent refolding system is overloaded. Over the recent years, among the sHsp family, Hsp20 (HspB6) has emerged as an attractive cytoprotective mediator that is found to be implicated in multiple pathophysiological processes.\textsuperscript{82}

\textbf{II.3 Heat shock protein 20 (HspB6)}

\textbf{II.3.A Overviews of Hsp20}

Hsp20, a 17 kDa protein, was originally identified as a byproduct of the purification of HspB1 (Hsp27) and HspB5 (\(\alpha\)-B-crystallin) by Kato \textit{et al.} in 1994.\textsuperscript{83} It was shown that Hsp20 can be detected in all tissues but is most abundant in cardiac, smooth, and skeletal muscles. For instance, the content of Hsp20 in liver and different regions of the brain is 3~6 ng/mg cell protein, which represents the lowest Hsp20 content. In comparison, the quantity of Hsp20 in slow skeletal muscle (m. soleus), diaphragm, heart, and smooth muscles is 1,200~13,000 ng/mg cell protein, which reaches a maximal level of 1.3\% of total protein in these tissues.\textsuperscript{83-85}

The primary structure of human Hsp20 is composed of 160 amino acids and is highly homologous to that of \(\alpha\)-B-crystallin.\textsuperscript{79} Particularly, Hsp20 is the only member among the sHsp family that contains a consensus peptide motif (RRAS), which can be phosphorylated by cAMP-and cGMP-dependent protein kinases (PKA/PKG) at Ser16.\textsuperscript{86, 87} The secondary structure of Hsp20 contains a well conserved \(\alpha\)-crystallin domain (ACD), flanked by the unstructured N-terminal domain and short C-terminal tail. Similar to other sHSPs, the ACD domain is
composed of β-sheets, which mainly mediate dimer formation between individual Hsp20 molecules. Particularly, recent structural studies indicate that the dimer interface is formed by the symmetric antiparallel interaction of β7 strands, producing an extended β-sheet on one face of the ACD dimer.88 As for the N- and C-terminal extensions, they contain small quantities of α-helices and primarily dictate the chaperoning activity of Hsp20.88, 89

As shown in the early studies by Kato et al. Hsp20 is present in aggregated (200~300 kDa) and low molecular weight (~67 kDa) forms.83 Recent studies by van de Klundert et al. also confirmed that Hsp20 exists in the form of large (~470 kDa) and small (~43 kDa) oligomers, which are easily interconvertible.90 Notably, it was shown by Brophy et al. that, upon phosphorylation of Hsp20 at Serine 16, the large macromolecular aggregates formed by Hsp20 dissociate to oligomers of small mass (~158 kDa).91 However, more recent findings indicated that in standard buffers at neutral pH and physiological ionic strength, human recombinant Hsp20 predominately formed dimers, which were independent of protein concentration or phosphorylation by PKA.92-94 In addition, it was found that Hsp20 is able to interact with other small heat shock proteins (ie. HSP27 and αB-crystallin) to form high molecular mass hetero-oligomeric complexes.83, 95 In detail, mixing of human Hsp20 and Hsp27 leads to formation of hetero-oligomers (100 and 300 kDa), which contain approximately equal quantities of each small heat shock protein.92 Considering that skeletal, smooth and cardiac muscles contain a rather high quantity of Hsp20, Hsp27, and αB-crystallin83, 96, the probability of formation of hetero-
oligomers in these tissues is rather high, which makes it difficult to determine the exact location of Hsp20 within the cell and protein partners, involved in direct interaction with Hsp20. Actually, at present, the exact intracellular location of Hsp20 remains ambiguous.

As small chaperone proteins, sHsps play an important role in protecting organisms from stress induced injury. However, for a long time, Hsp20 was considered to be a special member of the sHsp family with very low chaperone activity, *in vitro* studies by van de Klundert *et al.* showed that recombinant rat Hsp20 has a much lower chaperone activity than αB-crystallin. More recently, this assumption was contradicted by Bukach *et al.* who demonstrated that the chaperone activity of recombinant human Hsp20 was similar to or higher than that of commercial alpha-crystallin, as exhibited by its ability to inhibit the reduction-induced aggregation of insulin or heat-induced aggregation of yeast alcohol dehydrogenase.

II.3.B The role Hsp20 in non-cardiac tissues.

Hsp20 has been implicated in many pathophysiological processes such as Alzheimer's disease, platelet aggregation, smooth muscle dilation, myocardial infarction and cancer development.

II.3.B.1 Alzheimer's disease

The pathological characteristic of Alzheimer's disease (AD) is the extracellular deposition of amyloid-like filaments which form neuritic plaques in the brain. The main component of amyloid plaques is a small 40–42 amino acid peptide, called
amyloid–β (Aβ), which are from sequential proteolytic cleavage of the amyloid precursor protein. The effects of sHsps on amyloid formation were firstly identified by Kudva et al. in 1997. By utilizing a thioflavine T fluorescence assay and electron microscopy, they found that human Hsp27 is able to inhibit in vitro amyloid formation by the Alzheimer’s Aβ (1-42) polypeptide. Furthermore, it was shown that Hsp27 not only inhibits the initial rate of amyloidogenesis but also decreases the amount of Aβ oligomers, which are formed prior to HSP27 addition. The role of Hsp20 in amyloidogenesis inhibition was first investigated by Lee et al. in a model system named Babesia bovis, a protozoan parasite of cattle. It was found that not only was B.bovis Hsp20 (isolated from Babesia bovis) able to dramatically reduce Aβ amyloid formation but it was able to do so at molar ratios of Hsp20 to Aβ of 1 to 1000. More importantly, it was shown that toxicity of Aβ to both SH-SY5Y and PC12 neuronal cells was considerably reduced by Hsp20 at similar molar ratios. Notably, it was discovered that Hsp20 displays its aggregation inhibition and toxicity attenuation properties only at low concentrations in which dimer is present. The mechanism of Hsp20 interaction with Aβ 1-40 was further determined by the same group. It was discovered that B. bovis Hsp20 is able to form large multimeric complexes around Aβ, when co-incubated. Particularly, compared to free Aβ tangles, the Hsp20–Aβ complex was non-toxic to cultured SH-SY5Y cells. This finding was the first indication that Hsp20 could interact with Aβ in its multimeric form, resulting in the solubilization and clearance of toxic Aβ oligomers. In human, extracellular expression of Hsp20 was found in pathological lesions from post-mortem AD brains. Hsp20
can also be detected in normal brain parenchyma, associated with reactive astrocytes, but is highly overexpressed and co-localized with Aβ in senile plaques of the neocortex and hippocampus, which allows Hsp20 to form part of an innate cellular response to increasing levels of Aβ. Moreover, studies in human brain pericytes (HBP) exhibited that co-incubation of Aβ with Hsp20 completely abolished the cytotoxic effect in comparison with incubations with Aβ alone. This finding was confirmed by immunocytochemistry studies showing a striking reduction in Aβ staining at the cell surface of HBPs, when co-incubated with Aβ and Hsp20.

II.3.B.2 Platelet aggregation

It has been also shown that Hsp20 has extracellular functions. In vitro and ex vivo studies by Matsuno et al. in 1998 revealed that Hsp20 is able to inhibit thrombin or botrocetin induced platelet aggregation in a dose-dependent manner, and to specifically bind human platelets with a Kd of 310 nM. Further mechanistic studies demonstrated that Hsp20 inhibited thrombin-induced Ca^{2+} influx without affecting Ca^{2+} release from intracellular Ca^{2+} stores. These data suggest that Hsp20 inhibits the receptor-mediated Ca^{2+} influx of platelets, resulting in its anti-platelet activity. Hsp20 is also capable of suppressing thrombin-induced phosphoinositide hydrolysis by phospholipase C in human platelets. It was found that Hsp20 binds to the surface of human platelets and there is a single class of binding sites. Importantly, results from sucrose density gradient assay suggest that only dissociated form of Hsp20 but not an aggregated form inhibits platelet aggregation. In addition, it has been
demonstrated by McLemore et al. that recombinant Hsp20, linked to the TAT protein transduction domain, significantly inhibited collagen-induced platelet aggregation in human citrated whole blood. Collectively, these studies suggest that Hsp20 binds to a specific receptor on platelets, which inhibits the activation of cascades implicated in platelet aggregation. Particularly, it was previously reported that Hsp20 is present in serum (0.06 ng/ml in hamsters), suggesting that it may be an endogenous regulator of platelet function.

II.3.B.3 Smooth muscle relaxation

As stated above, Hsp20 is highly expressed in vascular, airway and uterine smooth muscle. The role of Hsp20 as well as its phosphorylation at serine 16 by PKG/PKA in smooth muscle relaxation has been well studied over the last few years. The vasorelaxation effects of Hsp20 were initially recognized in human umbilical artery smooth muscle (HUASM), which is refractory to cyclic nucleotide-dependent vasorelaxation. It was found that after activation of the PKA signaling pathway by forskolin, the HUASM did not relax, which was accompanied with phosphorylation of Hsp20. It was later discovered that impaired relaxation of umbilical artery was associated with decreased expression of Hsp20. Moreover, it was found that in rabbit bladder smooth muscle, which is also refractory to cyclic nucleotide-dependent relaxation, and in precontracted cerebral arteries from a subarachnoid hemorrhage rat model with impaired relaxation, the levels and phosphorylation of Hsp20 were diminished. On the contrary, increases in the phosphorylation of Hsp20 were found to be associated with significant cyclic nucleotide-dependent vasorelaxation in bovine
carotid and airway, porcine carotid and coronary smooth muscles.\textsuperscript{117-120} Furthermore, elevated phosphorylation of Hsp20 was shown to be correlated with cyclic nucleotide-dependent inhibition of contraction and endothelial-dependent vasorelaxation in perfused carotid arteries.\textsuperscript{121} Collectively, these data suggest that Hsp20 mediates relaxation or inhibits contraction in smooth muscle primarily via changes in Hsp20 expression and phosphorylation levels.

To elucidate the potential mechanisms underlying the regulation of Hsp20 and its phosphorylation in smooth muscle relaxation, an Hsp20 phosphopeptide was generated.\textsuperscript{122} It was found that the Hsp20 phosphopeptide interacts with the intracellular scaffolding protein 14-3-3 which is ubiquitously expressed and binds to a number of proteins, including a large group of proteins that regulate cytoskeletal architecture.\textsuperscript{123} Specifically, the 14-3-3 protein recognizes serine 16, containing the motif RRApSAP, in Hsp20 and forms a tight complex with dimers of phosphorylated Hsp20. Importantly, nonphosphorylated Hsp20 peptides and Hsp20 peptides, in which the serine 16 is replaced with an alanine, are not able to interact with 14-3-3.\textsuperscript{124} Upon the binding of 14-3-3 protein to phosphorylated Hsp20, cofilin dissociates from 14-3-3 protein. Cofilin, as an actin-depolymerizing protein, has been shown to interact with 14-3-3 proteins in the phosphorylated form.\textsuperscript{122, 125} Displacement of cofilin from 14-3-3 results in dephosphorylation of cofilin by phosphatases and activation as an actin-depolymerizing protein, followed by disruption of actin stress fibers and smooth muscle relaxation.\textsuperscript{126} These findings indicate that there is a unique mechanism of the effects of Hsp20 on smooth muscle relaxation, in which phosphorylated Hsp20 modulates actin
cytoskeletal dynamics by competing with the actin-depolymerizing protein cofilin for binding to the scaffold protein 14-3-3.

II.3.B.4 Cancer

As a multifunctional protein, several lines of evidence shows that Hsp20 also acts as an inhibitor of neoplastic growth, suggesting that it may play an important role in tumor progression as well. Firstly, Noda et al. showed that expression levels of Hsp20 in a variety of hepatocellular carcinoma cell lines, isolated from 53 different patients, were significantly decreased when compared with adjacent non-tumor tissues and decreased temporally, in parallel with tumor progression. These data suggest that Hsp20 plays a role against the progression of human HCC.\(^{127}\) Hsp20 was also identified as a novel marker, associated with malignant transformation in a recent genome-wide integrative analysis of DNA methylation in different stages of human melanoma cells by Koga et al.\(^{128}\). Methylation of cytosine residues serves as a key regulator of gene expression and alterations in tumor DNA, leading to reduced genome stability and inactivation of genes implicated in tumor suppression. In this study, the promoter region of the Hsp20 gene was found to be hypermethylated in melanoma cells, which inversely correlated with Hsp20 transcript levels. Moreover, the level of promoter methylation was found to be significantly increased in advanced stage melanoma cells, while being only moderately augmented in early stage melanomas, suggesting a causative relationship between Hsp20 protein levels and malignant melanoma progression.\(^{128}\) In addition, using microarray technology, expression levels of Hsp20 have been found to be reduced in several other cancers, such as
cervical cancer, lung cancer and colorectal cancer (CRC). Particularly, in colorectal cancer, it was found that protein levels of Hsp20 were down-regulated by 2.5 fold in 20 resected CRC specimens, compared with their paired normal tissues. Importantly, it was recently discovered that overexpression of Hsp20 in HCT-116 cells (a human colorectal cancer cell line) induced substantial cell apoptosis by activating the pro-apoptotic signaling pathways. Taken together, these findings suggest that Hsp20 may be utilized as a valuable prognostic tumor marker and its overexpression may serve as a novel strategy for cancer therapy.

Section III: The role of Hsp20 in the heart

III.1 The role of Hsp20 in cardiac function

III.1.A The role of Hsp20 in ex vivo cardiac function

For a long time, it was considered that sHsps have little or no effects on the intracellular Ca^{2+} handling and contractile function of the heart under normal conditions. For instance, it was shown by Morrison et al. that double knock-out of α-B crystallin and HspB2 genes had almost no effect on mouse cardiac function. However, recent studies by Pipkin et al. showed that in cultured adult rat cardiomyocytes, immunoreactive Hsp20 is present in transverse bands together with sarcomere actin. This localization of Hsp20 to specific cytoskeletal domains suggests that Hsp20 may play a critical role in regulating cytoskeletal dynamics and cardiac function. Indeed, further studies from the same group indicated that incubation of transiently permeabilized rat cardiomyocytes with phosphopeptide analogues of Hsp20 (Hsp20 phospho-Serine 16 analogue N-WLRRASphos APLPGLK) leads to increased rates of
cell shortening and lengthening, which is associated with a more rapid decay of the Ca\(^{2+}\) transient. However, in comparison, the nonphosphorylated peptide (N-WLRRRAAPLPGK) did not change the shortening and lengthening rates in these cardiomyocytes.\(^{133}\) Furthermore, by employing adenoviral gene transfer of mouse Hsp20 into adult rat ventricular myocytes, Chu et al. also reported that overexpression of Hsp20 leads to significant increases in contractility and Ca\(^{2+}\) transient peak in infected cardiomyocytes.\(^{87,133}\)

III.1.B Hsp20 regulating in vivo cardiac function

III.1.B.1 PP1 activity and cardiac contractility

Protein phosphatase 1 (PP1), which belongs to a class of serine/threonine phosphatases is a ubiquitous enzyme that modulates several cellular processes by dephosphorylation of various substrates. PP1 contains the catalytic domain (PP1c, 30 kD), which possesses its phosphatase activity, and form a complex with various regulatory subunits. These regulatory subunits either act as targeting subunits, guiding PP1 to its substrate and to the target subcellular location or regulate its catalytic activity.\(^{134,135}\) In the early 1990s, two independent studies by MacDougall et al.\(^{136}\) and Steenaart et al.\(^{137}\) identified that PP1 is the major phosphatase responsible for dephosphorylating PLN in SR-enriched preparations. These findings indicate that PP1 may act as a critical regulator of SR Ca\(^{2+}\) cycling and contractile function in the heart. Actually, the first evidence demonstrating the potential physiological relevance of PP1 came from the use of okadaic acid, which is a protein phosphatase non-specific inhibitor and inhibits both PP1 and PP2A at low concentrations. It was found that
at concentrations above $10^{-5}$ M, okadaic acid caused a dose-dependent increase of contractile force in guinea-pig isolated ventricular muscles, which was accompanied with considerable increases in phosphorylation of PLN and TnI.\textsuperscript{138, 139} The positive inotropic effects of PP1 inhibition were also observed in isolated guinea pig ventricular myocytes incubated with calyculin A or cantharidin, the PP1 and PP2A inhibitors, respectively. Particularly, the enhanced contractile function was found to be associated with increased phosphorylation of several key regulatory phospho-proteins, including PLN, TnI, myosin light chain 2, MyBP-C and the LTCC.\textsuperscript{140, 141} The first direct evidence into the functional significance of PP1 in cardiac function was from the generation and characterization of transgenic mice with cardiac specific overexpression of PP1c-\alpha.\textsuperscript{142} In this study, briefly, overexpression of PP1 (3 fold) resulted in significant increases in PP1 activity and diminished phosphorylation of PLN at Ser16, which were associated with depressed cardiac function, dilated cardiomyopathy, and premature mortality, similar to observations in end stage human heart failure.\textsuperscript{142} In addition, genetic models, which either overexpress or lack the endogenous inhibitors of PP1 (ie. inhibitor-1 and inhibitor-2) also provided key insights into the physiological role of PP1 in the heart. Specifically, mice with ablation of the endogenous inhibitor 1 (I-1) exhibited significant increases in PP1 activity, impaired cardiac contractility and diminished PLN phosphorylation at both Ser 16 and Thr17 sites.\textsuperscript{142} On the contrary, expression of constitutively active forms of I-1 or inhibitor 2 (I-2) significantly enhanced contractile function and increased PLN phosphorylation in the heart.\textsuperscript{143, 144} Taken together, these data suggest that PP1 is an important
regulator of cardiac function.

III.1.B.2 Hsp20 and cardiac contractility

Accumulating evidence implies that cardiac contractility and Ca²⁺ cycling are closely related with proper functioning of the PLN/SERCA complex and regulated by a fine equilibrium of PLN phosphorylation enacted by the balance of protein kinases and protein phosphatases.¹⁰¹,¹⁴⁵,¹⁴⁶ As described above, ex vivo studies showed that Hsp20 is able to increase intracellular Ca²⁺ cycling and enhance cardiomyocyte contractility. The in vivo role of Hsp20 in cardiac function and the mechanisms underlying its regulatory effects in Ca²⁺ cycling were further determined by Qian et al. in 2011, by employing a transgenic mouse model with cardiac specific overexpression of Hsp20.¹⁰¹ In detail, chronic overexpression of Hsp20 in intact animals led to significant enhancement of cardiac function, associated with increased Ca²⁺ cycling and SR Ca²⁺ load in isolated cardiomyocytes. These positive inotropic effects were coupled with specific increases in phosphorylation of PLN at both Ser16 and Thr17, which relieve its inhibition of the apparent Ca²⁺ affinity of SERCA2a, resulting in increased SR Ca²⁺ uptake. Accordingly, the contractile enhancing effects of Hsp20 were completely abolished in cardiomyocytes expressing nonphosphorylatable PLN (S16A/T17A). Most interestingly, the activity of PP1, the major phosphatase responsible for dephosphorylating PLN was found to be substantially reduced by Hsp20 overexpression, suggesting that the Hsp20 stimulatory effects are mediated by the PP1–PLN axis. Further mechanistic studies discovered that Hsp20 specifically interacts with PP1 and inhibits its activity, resulting in
increased PLN phosphorylation, SR Ca\textsuperscript{2+} cycling and cardiomyocyte contractility. Briefly, cell fractionation, coimmunoprecipitation, and coimmunolocalization studies revealed a strong association between Hsp20, PP1, and PLN in cardiomyocytes. In fact, by using microcystin-affinity chromatography, in 1998, Damer \textit{et al.} purified PP1-binding proteins from the myofibrillar fraction of rabbit skeletal muscle and surprisingly discovered that Hsp20 is able to complex with the catalytic subunit of PP1 (PP1C), suggesting that Hsp20 may modulate the activity of PP1\textsuperscript{147}. Subsequent studies by Qian \textit{et al.} using recombinant proteins revealed a direct physical interaction between Hsp20 and PP1. The region implicated in this binding is within the C-terminal domains of both proteins, as exhibited by blot overlay assays.\textsuperscript{101} Taken together, these studies indicate that Hsp20 is a crucial modulator of SR Ca\textsuperscript{2+} cycling and heart contractile function, by regulating the PP1 activity.

\textbf{III.2 Hsp20 and cardiac protection}

\textbf{III.2.A Myocardial infarction and dilated cardiomyopathy}

Cardiovascular disease (CVD) is the primary cause of death (1 of every 3 deaths) in America, according to the 2014 report of the American Heart Association.\textsuperscript{148} An estimated 83 million American adults are affected by one or more types of CVD and more than 2200 of them die of CVD each day (an average of 1 death in every 39 seconds). Among all CVDs, myocardial infarction resulting from a coronary obstruction infarction and dilated cardiomyopathy characterized by ventricular dilation and contractile dysfunction are the two main causes of mortality.\textsuperscript{148}
III.2.A.1 Myocardial Infarction

Myocardial infarction is normally known as heart attack and it happens when the coronary artery that supplies oxygen and nutrients to a region of the heart are limited or blocked, resulting in cell loss and severe damage to the heart muscle. The resulting condition of low oxygen and nutrient supply is named ischemia. This ischemic condition induces rapid adenosine-5'-triphosphate (ATP) depletion, sustained glycolysis, lactic acid accumulation, resulting in cytosolic acidosis and cell death in the injured cardiomyocytes.\textsuperscript{149, 150} In addition, under ischemic conditions, sarcolemmal sodium hydrogen exchanger (NHE) is activated by accumulation of hydrogen ions, which leads to elevated cytosolic sodium ions and reversal of the sarcolemmal NCX, causing it to extrude sodium and bring in Ca\textsuperscript{2+}, as opposed of its normal direction.\textsuperscript{151, 152} Therefore, accumulation of cytosolic Ca\textsuperscript{2+} named Ca\textsuperscript{2+} overload is induced in ischemic cardiomyocytes. Elevated intracellular Ca\textsuperscript{2+} is able to activate Calpain, a protease that belongs to the family of Ca\textsuperscript{2+}-dependent, non-lysosomal cysteine proteases. Upon activation, calpain cleaves and activates caspase-12, which can initiate an apoptotic cell death signaling cascade.\textsuperscript{153} Furthermore, Ca\textsuperscript{2+} overload is able to induce the loss of mitochondrial membrane integrity, a main cause of cardiomyocyte death in ischemia-induced injury.\textsuperscript{150} Although treatment options including anticoagulants, thrombolytics, angioplasty, vasodilators and bypass surgeries can remove the obstruction and achieve reperfusion\textsuperscript{154}, paradoxically, reperfusion itself causes further damage and subsequent cell death to the myocardium, which is referred to as
ischemia/reperfusion (I/R) injury. The primary mediators of reperfusion injury are oxygen radicals, Ca\textsuperscript{2+} loading, and neutrophils. Upon reperfusion, oxygen radicals generated by injured cardiomyocytes, endothelial cells in the ischemic zone and neutrophils that enter the ischemic zone will become activated. Oxygen radicals have high chemical reactivity and are able to destroy peptide/protein structures and membrane lipids of various organelles, particularly the mitochondria. At the same time, exacerbated membrane damage by oxygen radicals leads to elevated intracellular Ca\textsuperscript{2+} loading in the injured myocytes. Increased mitochondrial membrane damage and Ca\textsuperscript{2+} overload further amplify the generation of reactive oxygen species and pushes cardiomyocytes into the vicious cycle of oxidative stress, leading to the direct activation of cell death mechanisms. In addition, accumulated neutrophils in the microcirculation release inflammatory mediators, which contributes to microvascular obstruction and the no-reflow phenomenon in the reperfused myocardium, exacerbating cardiomyocyte death. Overall, ischemia/reperfusion injury can induce a large amount of cardiac cell death, resulting in the loss of viable cardiac tissue which is difficult to replace. As cardiomyocytes possess a limited capacity of regeneration, augmented cardiac cell death can cause compromised cardiac contractile function, leading to heart failure in both the short and long term. In particular, as an organ independent of the brain, the heart has its own pacemaker and relies on regular propagation of electrical signal for proper function. Therefore, the presence of dead tissue which is not excitable can block the electrical signal transduction,
resulting in cardiac arrhythmia. Indeed, it has been shown that ischemia/reperfusion injury can induce significant ventricular arrhythmia. Cardiac arrest, caused by severe and continued arrhythmic events, is one of the key risk factors leading to sudden cardiac death in patients suffering from heart attacks.

III.2.A.2 Dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is marked by an enlarged heart, in which ventricular contractile function is damaged. It is a very common form of non-ischemic cardiomyopathy and particularly, around 1 in 3 cases of heart failure is caused by DCM. Furthermore, it has been shown by Grogan et al. that only 50% of patients with DCM survive >5 years after diagnosis.

Although in up to 50% of the cases the exact cause of DCM remains unknown (idiopathic DCM), numerous factors including genetics, congenital heart defects, viruses, fungi or parasite infections, drug or alcohol abuse, exposure to toxins, hypertension, late-stage pregnancy, diabetes and heart attack can cause the left ventricle to dilate and weaken. In regard to genetic factors, around 25–35% of DCM patients have familial forms of this disease, with genetic mutations affecting proteins involved in Ca\(^{2+}\) cycling regulation, force generation, transmission and energy production in cardiomyocytes. First of all, as described above, several missense mutations in PLN, a key regulator of SR Ca\(^{2+}\) cycling and cardiomyocyte contractility can induce inherited human dilated cardiomyopathy. Moreover, it was recently reported by Blaich et al. that mutation of IQ to EQ in the calmodulin binding motif of the L-type
Ca(v)1.2 Ca\(^{2+}\) channel induces dilated cardiomyopathy and death in murine models.\(^{168}\) In addition, recent studies by Kamisago et al. discovered that mutations in sarcomere protein genes are responsible for about 10\% of cases of familial dilated cardiomyopathy and are particularly prevalent in families with early-onset cardiac dilatation and dysfunction.\(^{169}\) For instance, missense mutations in cardiac \(\beta\) myosin heavy chain and an in-frame deletion of cardiac troponin T have been shown to cause early-onset ventricular dilatation (average age at diagnosis, 24 years), diminished contractile function and frequently resulted heart failure. Mechanistically, cardiac troponin T deletion is thought to destroy Ca\(^{2+}\)-sensitive troponin C interactions, which are crucial for maintaining actin-myosin ATPase activity. Reduction in this enzymatic activity results in the loss of energy to power sarcomere contraction. In addition, for the \(\beta\) cardiac myosin missense mutations, their location indicates that these may also reduce contractile force.\(^{169}\) To maintain cardiac function, contractile force produced by the sarcomere needs to be effectively transmitted to the extracellular matrix, which is enacted by multiple filamentous proteins that link the contractile apparatus to the sarcolemma. Among these filamentous proteins, actin is centrally located within the sarcomere, in which it participates in force generation and is connected to cytoskeletal components that transmit force. Actually, two mutations in cardiac actin that cause early onset of autosomal dominant dilated cardiomyopathy were reported by Olson et al. in 1998. It was found that the location of actin missense mutations alters amino acid residues involved in actin-cytoskeletal interactions, which reduced the force transmission from the
sarcomere to the cytoskeleton, leading to dilated cardiomyopathy. In addition, several other causative mutations in molecules involved in force transformation (ie. α-tropomyosin, desmin, dystrophin, δ-sarcoglycan) have also been identified in patients with dilated cardiomyopathy. Mutations in genes encoding transport proteins or enzymes implicated in cardiac fatty acid β-oxidation have also been shown to induce dilated cardiomyopathy. Beta-oxidation in mitochondria is of particular importance for energy production during periods of fasting and deficiency in this pathway impairs the heart either indirectly by insufficient supply of energy or directly by toxic effects of intermediary metabolites. It has been discovered that mutations in proteins involved in transportation of carnitine (required for entry of long-chain fatty acids into mitochondria) can prevent metabolism of long-chain fatty acids and are heritable causes of dilated cardiomyopathy.

### III.2.B Necrosis, apoptosis and autophagy

It has been found that under stress conditions (ie. prolonged β-agonist stimulation, myocardial infarction, I/R injury), both cell necrosis and apoptosis are elevated in the damaged myocardium. During necrosis, cells are normally under ATP crisis, as ischemic conditions are connected with, mitochondrial malfunction and depletion of ATP generation. In the absence of ATP, plasma membrane ATPase fails to maintain the membrane potential, resulting in imbalance of the electro-gradient across the membrane and loss of osmotic balance control. This allows inflow of water, resulting in organelle swelling or rupture of surface membranes and unrestrained release of cell death products.
into the extracellular space. This spillage of intracellular contents stimulates an inflammatory response in the neighboring tissue, inhibiting nearby phagocytes from locating and removing the dead cells via phagocytosis.\textsuperscript{177} Therefore, the debris and dead cells will not be removed and stiff scar will be formed in the injured myocardium, leading to compromised cardiac contractility. Particularly, after cardiac ischemia, it was found that necrosis occurs in a group of cells, contributing to the formation of a dead tissue zone, known as infarction area.\textsuperscript{178}

In contrast, apoptosis (also termed Type I programmed cell death) is a highly regulated, genetically encoded cell death mechanism, which secures the integrity of cell plasma membranes and therefore does not incite inflammatory responses.\textsuperscript{177} Moreover, as a highly regulated death process, apoptosis needs energy (ATP) to guarantee the proper operation of the whole signaling cascade, including a serial activation of cysteine-aspartic proteases (caspases). Cell apoptosis is characterized by chromatin condensation, DNA fragmentation (unit with 200 base pairs), cell shrinkage and membrane blebbing. Moreover, cell membrane structure is changed, which allows inner membrane proteins (ie. annexin V) to be expressed on the outer membrane. These alterations on the membrane serve as markers for apoptotic cells.\textsuperscript{179} Eventually, the cell will be broken into several apoptotic bodies with intact membranes, which will be recognized and eliminated by neighboring cells including cardiomyocytes, vascular smooth muscles or endothelial cells through phagocytosis. Since the plasma membrane of apoptotic body is intact, the release of cellular compounds
and the resulted inflammatory response will not be provoked throughout the apoptotic process.\textsuperscript{179}

Autophagy, also referred to as Type II programmed cell death, is a lysosomal driven pathway that is responsible for removing damaged organelles, such as mitochondria or endoplasmic reticulum and providing nutrients under starvation stress.\textsuperscript{180} In cardiomyocytes, continuous alterations of intracellular environments (ie. oscillation of Ca\textsuperscript{2+} levels) can induce damage to proteins and organelles. Autophagy, as a physiological mechanism is able to mediate fusion of lysosomes with damaged proteins/organelles for degradation, and recycle the building components. In addition, during nutrient deprivation situations, autophagy can be activated by low level of cellular ATP, which then recycles intracellular materials to generate energy for cell function.\textsuperscript{181} However, extensive autophagy is detrimental and can cause cell death. As excessive activation of this response can induce over-digestion of healthy organelles, compromising basic cardiomyocyte function.\textsuperscript{182}

\textbf{III.2.C Hsp20 and cardiac protection}

Over the recent years, the capacities of Hsp20 in the field of cardioprotection have received considerable attention. Studies by us and other researchers found that Hsp20 and its PKA-phosphorylation are able to protect the heart against I/R injury, hypertrophic remodelling and stress-induced apoptosis. Previous studies discovered that the expression of myocardial Hsp20 was significantly upregulated upon I/R injury. It was later reported that overexpression of Hsp20 in mouse heart improved cardiac function recovery and promoted cardiomyocytes
survival after I/R. Accordingly, knockdown of Hsp20 by miRNA-320 in the mouse heart led to increased infarct size after I/R, which further supported the protective role of Hsp20 against I/R injury. In addition, it was discovered that increased expression of Hsp20 was protective against β-agonist and I/R-induced apoptosis. Moreover, Hsp20 has been shown to attenuate cardiac hypertrophy and prevent apoptosis and fibrosis, which thereby delays the progression of Isoproterenol-mediated heart failure.

Accumulating evidence implies that Ser16 phosphorylation of HSP20 serves as a switch of the cardioprotective capability of Hsp20. Specifically, studies from Qian et al. revealed that Hsp20 protects hearts from ischemic injury in a phosphorylation dependent manner. In the mouse heart, the phosphorylation level of Hsp20 was augmented by 40% after I/R. Moreover, TG mice with cardiac specific overexpression of non-phosphorylatable Hsp20, where serine 16 was replaced with alanine (S16A-Hsp20), became more sensitive to I/R injury. Interestingly, further studies uncovered that autophagy, a physiological catabolic process whereby eukaryotic cells degrade and remove damaged proteins and organelles, was significantly suppressed in S16A-Hsp20 hearts. It was proposed that inhibition of autophagy may contribute to the impairment of cardiac contractile recovery in post-I/R S16A-Hsp20 hearts. Furthermore, the protective effects of Hsp20 against stress-induced apoptosis were found to be enhanced by a constitutively phosphorylated Hsp20 mutant, where serine 16 was replaced with aspartic acid (S16D-Hsp20) in infected cardiomyocytes. However, the non-phosphorylatable S16A-Hsp20 displayed no anti-apoptotic properties.
In addition, Sin et al. recently reported that a constructed peptide (‘Peptide 906’), which specifically augments the phosphorylation of Hsp20, considerably attenuated β-agonist-induced cardiomyocyte hypertrophy and remodelling, suggesting that phosphorylation of Hsp20 at Ser16 enhances the anti-hypertrophic capability of Hsp20.\textsuperscript{101}

Hsp20 has been recently reported to modulate multiple kinase signaling pathways, which are considered to be the underlying mechanisms of its anti-apoptotic and anti-hypertrophic effects. It was revealed that Hsp20 interacts with Bax, a key pro-apoptotic protein, and prevents its translocation to mitochondria, which subsequently blocks the initiation of apoptosis by suppression of caspase-3-mediated apoptotic pathway. Also, Hsp20 was reported to protect against doxorubicin-induced cardiomyopathy by sustaining the Akt-BAD signalling pathway. Indeed, Hsp20 was able to maintain the Akt activity, which retains the phosphorylation of Bad and thus represses caspase-3 mediated apoptosis.\textsuperscript{187} Additionally, Hsp20 is likely implicated in maintaining the filament integrity during heart failure progression.\textsuperscript{185, 188} Progressive cardiac remodeling is associated with the degradation of contractile filament proteins and loss of sarcomeric skeleton proteins in cardiomyocytes. Hsp20 has been reported to translocate to the filament meshwork in cardiomyocytes to stabilize the cytoskeleton and protect from further damage under stress conditions, which are associated with its phosphorylation at Ser16.\textsuperscript{185}

In summary, these studies imply that Hsp20 as well as its phosphorylation can protect the heart at multiple levels, which presents a promising therapeutic
target for ameliorating the function of failing cardiomyocytes.

### III.3 Human Hsp20 mutation

Recently, Nicolaou et al. identified a C59T substitution in the human Hsp20 gene by screening the coding region of this gene in 1347 patients, suffering from dilated cardiomyopathy, and 744 subjects with no cardiac disease. The prevalence of this mutation is 3/744 in non-cardiomyopathic and 1/1347 in the dilated cardiomyopathic population. All subjects were heterozygous for this mutation. Particularly, the Hsp20 mutation changes a proline residue, which is highly conserved in human, dog, mouse and rat, into leucine at position 20 (P20L), resulting in secondary structural alterations. Functional studies using adenoviral technology showed that compared to the protective effects of WT-Hsp20 against I/R-induced cardiomyocyte apoptosis, the P20L-Hsp20 mutant showed no protection against apoptosis. Subsequent studies found that the loss of cardioprotection by the mutant Hsp20 was associated with its diminished phosphorylation at Ser16 compared with WT-Hsp20. This compromised ability of the mutant Hsp20 to be phosphorylated at Ser16 was further confirmed by stimulation of cardiomyocytes with isoproterenol. These findings imply that the cardioprotective effects of Hsp20 were entirely abolished by the P20L substitution, which may damage the ability of human carriers to cope with cellular stress.189

### Section IV: Dissertation scope and objectives

As stated above, both PLN and Hsp20 are critical determinants of the SR Ca\(^{2+}\) cycling and cardiomyocyte contractility. In addition, Hsp20 is able to protect the
heart against stress-induced injuries at multiple levels. To determine whether additional genetic variants may exist in human PLN and Hsp20 genes, which may modify their functional significance in the heart, we teamed up with our collaborators and identified a novel human mutation in PLN (R25C) and another one in Hsp20 (S10F). The main objectives of this dissertation were to elucidate the roles of these two human mutations: PLN (R25C) and Hsp20 (S10F) in cardiac function and protection, uncovering new insights into the mechanisms by which PLN and Hsp20 regulate the heart. The rationale for these studies and the specific objectives are described below:

a) PLN is a prominent regulator of Ca\textsuperscript{2+} cycling and a primary mediator of the β-adrenergic effects resulting in enhanced cardiac output. In the dephosphorylated state, PLN inhibits SERCA2a and shifts its Ca\textsuperscript{2+} activation toward lower apparent Ca\textsuperscript{2+} affinity. However, upon PKA-mediated phosphorylation, the inhibition on SERCA2a by PLN is relieved and its Ca\textsuperscript{2+} affinity is increased. Thus, PLN plays a key role in regulation of Ca\textsuperscript{2+} reuptake by SERCA2a to induce relaxation and decrease diastolic Ca\textsuperscript{2+} levels. The important role of PLN in cardiac function has prompted searches for human PLN genetic variants, which may be associated with cardiomyopathy. Indeed, three mutations have been identified in the coding region of PLN and it was found that all three mutations caused myocellular Ca\textsuperscript{2+} dysregulation, which eventually led to dilated cardiomyopathy and heart failure. In this study, we report a new mutation (R25C) in the coding region of the human PLN gene, identified in a pedigree with DCM that also showed prominent ventricular arrhythmia and the need for implantable cardiac defibrillators (ICDs).
To further determine if there is a correlative or causative relationship between this mutation and the observed clinical phenotypes in human carriers, the effects of R25C mutation in cardiomyocyte mechanics and intracellular Ca$^{2+}$ kinetics were investigated by using adenoviral technology.

b) Previous studies have shown that increases in cardiac Hsp20 are associated with protection against apoptosis, elicited by I/R-injury or prolonged β-agonist stimulation. More importantly and surprisingly for a chaperone protein, we found that Hsp20 enhances contractility through its interaction and inhibition of protein phosphatase 1 (PP1), resulting in specific increases in phospholamban (PLN) phosphorylation. Thus, Hsp20 has dual benefits in the heart and may hold promise as a therapeutic target in heart failure. Recently, we identified a new human mutation in Hsp20, S10F, in patients with dilated cardiomyopathy. Importantly, this S10F-Hsp20 mutation was found exclusively in male, black DCM patients with the prevalence of 4/142. To determine the functional significance of S10F-Hsp20 mutation in cardiac function and protection under basal and stress conditions, a series of *in vitro* and *in vivo* studies were performed by utilizing infected rat cardiomyocytes as well as a transgenic mouse model with cardiac overexpression of S10F-Hsp20.

**Chapter II: Materials and Methods**

**Section I: Identification of a PLN Mutation in a Familial DCM Pedigree**

Written informed consent was obtained from research participants who provided a blood sample for genetic DCM research. Release of medical records was also sought to confirm and document diagnoses. Affected status was assigned with
LV enlargement and systolic dysfunction not attributable to coronary artery disease, cardiotoxic exposure, or other known detectable causes. Familial DCM was deemed present with more than one confirmed affected family member.

Genomic DNA was extracted using standard procedures. In this family, exome sequencing was performed in the two sisters (III.1 and III.3) (Fig. 1), who tested negative for the previously identified LMNA mutation\textsuperscript{190} at the University of Washington Genome Sciences with capture, sequencing, alignment, and data analysis as previously reported. Variants selected for analysis met the following criteria: 1) \(<0.5\%\) allele frequency in 5400 exomes from the Exome Variant Server; 2) predicted to alter an amino acid, affect splicing, or lead to coding insertion-deletions; 3) read depth \(\geq 5\) and quality scores \(\geq 40\); 4) Phastcons score \(>0.4\) or a Genomic Evolutionary Rate Profiling score \(>2\); and 5) shared among exomed samples from the same family. Sanger-validated variants were then assessed in family members who did not undergo exome sequencing.

**Section II: Infection of cardiomyocytes with recombinant adenoviruses**

**II.1 PCR Mutagenesis and Mammalian Expression Construct Generation**

The R25C mutant was produced by employing the Quik-Change-Site-Directed Mutagenesis II kit (Stratagene). The primers were sense: 5' CCATTGAAATGCCTCAACAAGCATGTCAAAAGCTACAGAATC, antisense: 5' GATTCTGTAGCTTTTGACATGCTTGTTGAGGCATTTCAATGG 3'. The conditions were: one cycle at 95°C for 30 sec, connected to 16 cycles at 95°C for 30 sec, 55°C for 1 min and 68°C for 12 min. The reaction mixture contains 5 μl reaction buffer, 50 ng of template, 1 μg sense and antisense primers,
correspondingly, 1 μl dNTP mix and 1 μl Pfu Turbo high-fidelity DNA polymerase, in a final reaction volume of 50 μl. Upon finishing point, the PCR reaction was cooled on ice for 2 min and then digested with Dpn I (1 μl) to eliminate methylated parental DNA. The mutated DNA was transformed into XL-1 supercompetent bacterial cells. Particularly, 50 μl of the bacteria were incubated with 10 μl of the Dpn I-treated DNA on ice for 30 min, followed by heat shock at 42°C for 30 sec. Then, the cells were cooled on ice for 2 min. 500 μl of SOC were then added to the transformed reaction mixture and grown at 37°C for 1 hr. 200 μl of this mixture were then plated on Amp-LB-agar plates. A number of colonies were picked and grown overnight at 37°C in Amp-LB broth. Plasmid DNA, isolated using a miniprep kit (QIAGEN), was sequenced to warrant correct mutagenesis and proper cloning.

II.2 Generation of adenoviral vectors

The Ad-Easy XL adenoviral system (Stratagene) was employed to generate adenoviruses carrying PLN-R25C (Ad.R25C). The various steps are described below.

II.2. A Cloning R25C-PLN into the pShuttle vector

The R25C-PLN from the plasmids described above were sub-cloned into the pShuttle-IRES-hrGFP-1 vector. Cloned genes are expressed under the control of the ubiquitous cytomegalovirus promoter. In addition, this vector also contains the green fluorescent protein (GFP) gene, which allows expression of GFP and monitoring of infection efficiency. To facilitate ligation, the plasmids containing R25C-PLN and the pGEX-6P-3 plasmid were digested in two separate reactions
with Sal I/Not I for 2 hrs at 37°C. The reaction mixture consisted of 5 μl of Sal I buffer, 2 μl Sal I, 2 μl Not I and 23 μl (~120 ng) R25C-PLN plasmids and 5 μl (~30 ng) of the pGEX-6P-3 plasmid in a final volume of 30 μl. The digested products were gel-purified (QIAGEN) and then the digested plasmids and fragments were ligated overnight at 4°C. The reaction mixture consisted of 5 μl of the plasmids (~5 ng), 9 μl of the R25C-PLN fragments (~40 ng), 2 μl ligase buffer, 2 μl rATP and 1 μl T4 ligase (Stratagene). The next day, the ligated plasmid was transformed into XL-10 bacterial cells, according to the manufacturer's instructions (Stratagene). Briefly, 100 μl of the bacteria were incubated with 4 μl of β-mercaptoethanol on ice for 10 min. Then, 10 μl of the ligation reaction was added to this mixture and allowed to stand on ice for 30 min, followed by heat shock at 42°C for 30 sec. The cells were cooled on ice for 2 min, 900 μl of SOC added and grown at 37°C for 1 hr. The cells were pelleted gently at 1,000 rpm for 10 min, resuspended in 200 μl of SOC and plated on Ampicillin (Amp)-LB-agar plates. More than a few colonies were picked and grown overnight at 37°C in Kan-LB broth. DNA was isolated using a miniprep kit (QIAGEN) and then sequenced to guarantee proper cloning.

II. 2. B Electroporation and identification of correctly recombined clones

The R25C-PLN cDNA cloned in the pShuttle vector was introduced into an adenoviral backbone plasmid vector by homologous recombination via electroporation into BJ5183-AD-1 cells. These cells were used because they are pre-transformed with the 33.5 kb AdEasy-1 adenoviral backbone vector, which is necessary for virus generation. Importantly, this vector contains three regions of
homology with the pShuttle vector, the left homology arm, the right homology arm and the origin of replication, which facilitate homologous recombination. The pShuttle vectors containing R25C-PLN were linearized, using the restriction enzyme, Pme I (New England Biolabs) overnight at 37°C. The reaction mixture consisted of 26 μl of plasmid DNA (obtained from minipreps of a single colony), 3 μl of NEB buffer 4 and 1 μl of the enzyme. The digested plasmids were incubated with the BJ5183-AD-1 electrocompetent cells on ice for 5 min. Electroporation was performed at 200 Ω, 2500 V and 25 μF in an electroporation cuvette (0.2 cm gap). The cells were then mixed with 1 ml SOC and allowed to grow for 1 hr at 37°C. 200 μl of this reaction mixture were streaked on Kan-LB-agar plates and allowed to grow overnight at 37°C. Three populations grew on the plates, consisting of small-, medium- and large-sized colonies. The small colonies, which were expected to have the highest frequency of homologous recombination, were picked the following day, amplified in LB-Kan broth and screened for the recombination event, using the Pac I restriction enzyme (New England Biolabs). The reaction mixture consisted of 26 μl of miniprep-DNA, 3 μl of NEB buffer 1 and 1 μl of the Pac I enzyme. The digestion reaction was run on a 0.8% agarose gel, to identify positive colonies. Homologous recombination results in a band pattern consisting of the plasmid backbone (>25kb) and the liberated vector (3 or 4 kb upon recombination at the origin of replication and homology arms, respectively).

II. 2. C Transfection of recombinant adenoviral plasmids into HEK293 cells and generation of adenoviruses
The correctly recombined colonies were transfected into HEK293 cells to allow viral synthesis. These cells were used because they express in trans the E1 gene necessary for replication of the new virus from the replication-deficient AdEasy-1 backbone vector. In particular, the correctly-recombined colonies were amplified in XL-10 cells, purified and digested with Pac I for 4 hours at 37°C. Upon completion of the digestion, the reaction was brought to a final volume of 250 μl with sterile water. 25 μl of 2.5 M CaCl₂ and 225 μl of 0.1 M BBS (N.N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid) transfection reagent were added to the digested plasmids. The reaction mixture was added slowly to HEK293 cells, in Dulbecco’s Modified Eagle Medium supplemented with 10% modified bovine serum, during their exponential growth phase (70% confluency). The media were replaced with media supplemented with fetal bovine serum after 3 hr and after 6 hr. Expression was monitored for 7-13 days, using a fluorescent microscope.

II. 2. D Purification of viruses

The media from flasks of successfully-generated viruses were applied to naïve HEK293 cells (100% confluent) for amplification. The cells were monitored daily and harvested once all of the cells had been infected with the virus, and were dislodged from the bottom of the flask. The virus was purified using the Adeno-X™ virus mini purification kit (Clonetech). The cells underwent three cycles of freeze/thaw and vortexing to lyse them and release the virus. The cells were pelleted by centrifugation at 800g for 10 min and discarded, while the supernatant was filtered through a 0.45μm syringe and applied to pre-equilibrated purification columns. The columns were washed twice with wash
buffer to remove any contaminants and non-specific binding. The virus was finally eluted in 400 μl of elution buffer and stored at -80°C in 20% cryopreservative.

II. 2. E Titer assay

The purified viruses were tittered, using the Adeno-X™ Rapid Titer kit (Clontech), according to the manufacturer’s instructions. Briefly, HEK293 cells were seeded in 12-well plates at 90% confluency and infected with the virus at various dilutions ranging from $10^{-2}$ to $10^{-6}$. After 48 hrs, the media were removed and the cells were dried in the air for 5 min. The cells were next fixed in 100% ice-cold methanol for 10 min at -20°C, followed by washing with PBS containing 7.5% BSA. The cells were then incubated with the anti-hexon primary antibody (1:1000 dilution) for 1 hr at 37°C. Following 3 washes with PBS supplemented with 7.5% BSA, the cells were incubated with the anti-rat secondary antibody provided with the kit (1:500 dilution) at 37°C for 1 hr. The cells were then washed as before. Finally, the cells were incubated with the DAB colorimetric solution for 10 min and the infected cells were counted in the dilutions which produced 5-50 infected cells per field, using the 20X objective. The viral titer was calculated, utilizing the formula: PFU= $[(\text{infected cells/field}) \times (\text{fields/well})] / [\text{volume virus (ml)} \times (\text{dilution factor})].$

II.3 Myocyte preparation and infection of myocytes

II.3.A Adult rat cardiomyocyte isolation and culture

Animals were handled based on the Institutional Animal Care and Use
Committee at the University of Cincinnati. Myocytes from adult male Sprague-Dawley rats (~300 grams) were isolated through collagenase digestion, as previously described. Briefly, the rat, anesthetized with sodium pentobarbital (90 mg/kg) was heparinized to reduce clot formation. The aorta of the removed heart was cannulated and the heart was perfused using Krebs-Henseleit buffer (KHB), consisting of 118 mM NaCl, 4.9 mM KCl, 25 mM HEPES, 1.25 mM MgSO4 and 1.25 M K2HPO4, pH=7.4 supplemented with 11 mM glucose, 5 mM taurine and 10 mM 2, 3-butanedione monoxime (BDM), until there were no blood drops in the perfusion buffer. Subsequently, the heart was digested, with 5000 U hyaluronidase and 263 U collagenase in KHB buffer supplemented with 0.1 mM CaCl2, until the heart became softer and the perfusion rate increased (25-30 min depending on the size of the heart). At the end of the digestion, the ventricles were excised, minced and agitated by suction with a Pasteur pipet. The myocytes were filtered using a mesh cloth and subjected to incremental Ca2+ increases (Ca2+ gradients) from 0.1 mM to 1.2 mM to remove Ca2+-intolerant or unhealthy cells. Finally, the myocytes were resuspended in modified culture medium (M199, Sigma) supplemented with 5 mM taurine, 5 mM L-carnitine, 5 mM creatine and 2% BSA, counted and plated on laminin-coated 6-well plates (4x10⁴ cells per well) or 10 cm dishes (1~2x10⁵ cells) for 2 hrs at 37°C in a humidified, 5% CO2 incubator. The cells were subsequently infected with the three adenoviruses (Ad.GFP, Ad.WT, and Ad.R25C) at a MOI of 500 for 2 hrs. The media was replaced after infection and after 24 hrs.

II.3.B Measurement of contractile parameters and Ca2+ transients

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Cell shortening and Ca\textsuperscript{2+} transients were examined in cardiomyocytes at room temperature. To get intracellular Ca\textsuperscript{2+} signals, cells were treated with the acetoxyethyl ester form of fura-2 (Fura-2/AM; 2\,\mu M) in 1mM Ca\textsuperscript{2+}-Tyrode solution for 30 minutes at room temperature. The cells were then resuspended in 1.8 mM Ca\textsuperscript{2+}-Tyrode solution. 300 \mu L of 1.8 mM Ca\textsuperscript{2+}-Tyrode solution and 100 \mu L of the myocyte suspension were placed in a Plexiglas chamber, which was positioned on the stage of an inverted epifluorescence microscope (Nikon Diaphot 200) at room temperature (22–23°C). Myocyte contraction was field-stimulated by a Grass S5 stimulator (0.5 Hz, square waves), and contractions were videotaped and digitized on a computer. Pulse duration was set at 4 milliseconds and voltage at 80 mV. A video edge motion detector (Crescent Electronics) was used to measure cardiomyocyte length and cell shortening, the percent fractional shortening (FS\%: (resting cell length-maximal cell shortening length) / resting cell length \times 100), and maximal rates of contraction (+dL/dt) and relaxation (-dL/dt) were calculated.

For Ca\textsuperscript{2+} signal measurements, the cells were excited at 340 and 380 nm by a Delta Scan dual-beam spectrophotofluorometer (Photon Technology International). Ca\textsuperscript{2+} transients were recorded as the 340 nm: 380 nm ratio of the resulting 510 nm emissions. For caffeine-induced Ca\textsuperscript{2+} release, field stimulation was stopped before 10 mM caffeine was rapidly (in 10 seconds) applied to the cells, and the following Ca\textsuperscript{2+} transients were recorded. Baseline and amplitude, estimated by the 340 nm: 380 nm ratio, and the time to 50% decay of the Ca\textsuperscript{2+} signal were acquired. 10-12 cells per heart were studied, and each animal was
analyzed as a single n. Data were acquired using Felix® computer software (Photon Technology International, Lawrenceville, New Jersey, USA) and analyzed using Ion Wizard® software (Ion Optix, Corp.)

II.4 Ca^{2+} uptake assay

Infected, cultured adult rat cardiomyocytes were washed twice with PBS to remove traces of culture medium. Next, 500 µl of ice-cold PBS was applied to the cells, and then the cells were dislodged from the plate by utilizing a cell scraper. The cells were next centrifuged at 3000 rpm for 5 min. Then, the cells were suspended in homogenization buffer (50 mM KH_{2}PO_{4} buffer, pH=7.0, 10 mM NaF, 1 mM EDTA, 0.3 M Sucrose, 0.3 mM PMSF, 0.5 mM Dithiothreitol) and homogenized at 4°C. Initial rates of SR Ca^{2+} uptake were measured in cell homogenates by using oxalate and ^{45}\text{CaCl}_{2}, as previously reported. Specifically, 250 µg of homogenates were incubated at 37°C in a reaction buffer having: 40 mM imidazole pH 7.0, 95 mM KCl, 5 mM NaN_{3}, 5 mM MgCl_{2}, 0.5 mM EGTA, and 5 mM potassium oxalate. In the cell homogenates, only SR vesicles contain the functional anion channel that facilitates the transportation of oxalate into SR and oxalate will precipitate all the Ca^{2+} up-taken by SERCA2a and not let Ca^{2+} release occur. At the same time, ruthenium red (1 µmol/l) was added, which will blocks all the Ca^{2+} channels and prevent Ca^{2+} release. The initial uptake rates were examined over a range of Ca^{2+} concentrations (pCa^{2+} 8 to 5). Ca^{2+} uptake into SR vesicles was initiated by addition of 5 mM ATP, and aliquots were filtered via a 0.45-μm Millipore filter after 0, 30, 60, and 90 s. The specific Ca^{2+}-uptake values, Ca^{2+} affinity (EC_{50}), and maximal Ca^{2+}-uptake rates (V_{max}) were analyzed
through nonlinear regression, utilizing the OriginLab 5.1 program.

II.5 HEK293 cell culture and Immunofluorescence Studies

HEK293 cells (ECACC, Salisbury, UK) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Invitrogen). The full length GFP-PLN and SERCA2 constructs that were used in these studies have been previously described. Plasmids were transiently transfected in HEK293 cells using Lipofectamine™ 2000 (Invitrogen) and immunofluorescence analysis was performed. Briefly, forty-eight hours after transfection, cells were fixed for 20 minutes with ice cold methanol and after three washes in phosphate-buffered saline (1x PBS), the cells were permeabilized for 30 minutes in PBS containing 0.1% Triton X-100. Following three washes, the cells were incubated with blocking buffer (1x PBS, 1 mg/ml BSA, 10 mM NaN3) for 1 hour. SERCA2 antibody (Affinity Bioreagents, Golden, USA) was then applied for 1 hour and samples were washed three times before counterstaining for 1 hour with the Alexa Fluor anti-mouse 568 secondary antibody (Invitrogen). Samples were mounted with Vectashield medium comprising DAPI (Vector Laboratories, Burlingame, CA) and analyzed via confocal microscopy.

II.6 Immunoprecipitation of PLN with SERCA2a

Transfected HEK293 cells (48 hours) were lysed in 50 mM Tris-HCl, 150 mM NaCl and 1% NP40 added with protease inhibitors. Cell lysates were precleared with 30 µl protein G-Sepharose (Amersham Biosciences Europe, Uppsala, Sweden) for 1 h at 4°C and then incubated with 4 µg of GFP antibody (Sigma-Aldrich) and 30 µl protein G-Sepharose at 4°C overnight. The beads were
washed three times with lysis buffer and were subsequently analyzed by Western blot using SERCA2 (Affinity Bioreagents) or GFP (Sigma-Aldrich) antibodies.

Phosphorylation of PLN was performed in HEK293 cell lysates using PKA catalytic subunit (New England Biolabs). Briefly, cell lysates were incubated with PKA Reaction Buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂), added with 200 μM ATP (Millipore) and 1,250 units of PKA catalytic subunit. Samples were incubated at 30 °C for 1 hour and were then used in immunoprecipitations.

II.7 Ca²⁺ sparks and waves measurement

Spontaneous Ca²⁺ sparks were acquired in quiescent cells loaded with Rhod-2 AM (10 μM, Invitrogen). Fluorescence images were recorded by employing a Zeiss LSM 510 inverted confocal microscope via a x40 water-immersion objective lens with excitation wavelength of 488 nm. Fluorescence signals were measured at greater than 515 nm with line-scan imaging at 3.07-ms intervals, with each line containing 512 pixels spaced at 0.056 μm. Ca²⁺ waves were recorded using similar methods as Ca²⁺ sparks. Ca²⁺ spark amplitude was normalized as F/F₀. Three dimensional images of Ca²⁺ sparks and waves were acquired by utilizing MATLAB 7.11.0 (R2010b).

II.8 Measurement of after contractions in cardiomyocytes

Rod-shaped rat ventricular myocytes, which showed no spontaneous activity at rest, were paced at 2 Hz in the presence of 1 μmol/L isoproterenol in 1.2 mM Ca²⁺-Tyrode solution at room temperature. After 2 or 3 trains of stimulation, pacing was stopped to allow the recording of spontaneous aftercontractions
within 2 to 5 seconds.

II.9 Measurement of diastolic SR Ca\(^{2+}\) leak in cardiomyocytes

Diastolic SR Ca\(^{2+}\) leak was measured by employing the tetracaine method.\(^{193, 194}\) Concisely, Fura 2-loaded myocytes were field stimulated at 0.5 Hz until they reached a steady-state Ca\(^{2+}\) transient height. Stimulation was then switched off, and the external solution was quickly changed to Tyrode’s solution 0 Ca\(^{2+}\), 0 Na\(^{+}\) to eliminate transsarcolemmal Ca\(^{2+}\) fluxes, causing a new steady state [Ca\(^{2+}\)]. When RyR2 channels are inhibited by 1 mM tetracaine (in Tyrode’s solution, 0 Ca\(^{2+}\), 0 Na\(^{+}\)), Ca\(^{2+}\) shifts from the cytosol into the SR. The tetracaine-stimulated drop in diastolic Fura-2 fluorescence ratio was used as an estimate of SR Ca\(^{2+}\) leak, which is insensitive to alterations in SR Ca\(^{2+}\) uptake. Then, the myocytes were exposed for 4 seconds to 0 Ca\(^{2+}\), 0 Na\(^{+}\) Tyrode’s solution containing 10 mM caffeine and 20 mM 2,3-butanedione monoxime (to prevent myocyte hypercontracture). The amplitude of caffeine-induced Ca\(^{2+}\) transient was used to estimate the total [Ca\(^{2+}\)], which included the Ca\(^{2+}\) leak.

II.10 CaM Kinase II activity assay

Cultured cardiomyocytes were lysed for 20 minutes at 4°C in lysis buffer, described above. The supernatants obtained after centrifugation at 8000 g for 10 minutes were processed for CaM Kinase II activity assay, using non-radiographic CaM Kinase II ELISA (Cyclex CaM Kinase II Assay Kit, MBL International, Woburn, MA). Briefly, uniform amounts of cell lysates were loaded onto micro titer wells coated with a specific peptide substrate for CaMKII-Syntide-2, along with kinase reaction buffer with or without Ca\(^{2+}/\)calmodulin. Purified CaMKII (30
milli units per reaction; CycLex Co Ltd) was used as positive control. CaMKII activity is expressed as spectral absorbance units at 450 nm.

Section III: Generation of S10F-Hsp20 transgenic mice

We generated S10F-Hsp20 TG mice carrying the mouse cardiac S10F-Hsp20 cDNA under the control of the α-myosin heavy chain (α-MHC) mouse promoter as described. A 0.5 kb mutant mouse Hsp20 cDNA was ligated with the mouse cardiac α-MHC promoter (5.5-kb). The care of all animals utilized in the present study was based on the University of Cincinnati animal care guidelines.

III.1 Generation of Hsp20-S10F transgenic constructs.

III.1.A Cloning of Hsp20 into the α-MHC vector

The mouse Hsp20 cDNA (Open Biosystems) was cloned into the α-MHC vector (from Dr. Guo-Chang Fan) by creating Sal I and Hind III sites at the 5’ and 3’ ends of the Hsp20 gene, respectively, using PCR. The sense primer was 5’ CAGAGTCGACATGGAGATCCCCGTG 3’ and the antisense primer was 5’ CAGAAAGCTTCTACTTGGCAGCAGGTG 3’. The conditions were: one cycle at 95°C for 2 min, followed with 35 cycles at 95°C for 1 min, 51°C for 1 min, 72°C for 1 min, next by a final extension at 72°C for 10 min. The reaction mixture contains 5 μl 5X buffer, 50 ng template DNA, 200 ng sense and antisense primers, respectively, and 1 μl Accuprime GC-rich DNA polymerase (Invitrogen) in a final reaction volume of 50 μl. The PCR fragment was then ligated into α-MHC vector. To facilitate ligation, the gel-purified PCR product and the α-MHC plasmid were digested in two separate reactions with Sal I/Hind III for 2 hrs at 37°C. The
reaction mixture consisted of 5 μl of Sal I buffer, 1 μl Sal I, 1 μl Hind III and either 23 μl of PCR-purified product or 5 μl (~30 ng) of the α-MHC plasmid in a final volume of 30 μl. The digested products were gel-purified (QIAGEN) and then the digested plasmid and the PCR-amplified Hsp20 gene were ligated overnight at 4°C. The reaction mixture consisted of 5 μl of the plasmid (~5 ng), 9 μl of the Hsp20 PCR product (~50 ng), 2 μl ligase buffer, 2 μl rATP and 1 μl T4 ligase (Stratagene). The next day, the ligated plasmid was transformed into XL-10 bacterial cells, according to the manufacturer's instructions (Stratagene). Briefly, 100 μl of the bacteria were incubated with 4 μl of β-mercaptoethanol on ice for 10 min. Subsequently, 10 μl of the ligation reaction was supplemented to this mixture and allowed to stand on ice for 30 min, followed by heat shock at 42°C for 30 sec. The cells were cooled on ice for 2 min, with 900 μl of SOC added and grown at 37°C for 1 hr. The cells were pelleted gently at 1,000 rpm for 10 min, resuspended in 200 μl of SOC and plated on Ampicillin (Amp)-LB-agar plates.

III.1.B Generation of the S10F-Hsp20 mutant

The S10F mutant was generated, using the Quik-Change-Site-Directed Mutagenesis II kit (Stratagene) by using the α-MHC-Hsp20 vector generated above as templates. The primers were sense: 5’ GTGCAGCCTTTTTGGCTGCGCCGTG 3’, antisense: 5’ CACGGCGCAGCCAAAAAGGCTGCAC 3’. The conditions were: one cycle at 95°C for 30 sec, linked to 16 cycles at 95°C for 30 sec, 55°C for 1 min and 68°C for 12 min. The reaction mixture has 5 μl reaction buffer, 50 ng of template, 1 μg sense and antisense primers, respectively, 1 μl dNTP mix and 1 μl Pfu Turbo
high-fidelity DNA polymerase, in a final reaction volume of 50 μl. Upon completion, the PCR reaction was immediately cooled on ice for 2 min and then digested with Dpn I (1 μl) to remove methylated parental DNA. The mutated DNA was transformed into XL-1 supercompetent bacterial cells. In particular, 50 μl of the bacteria were incubated with 10 μl of the Dpn I-treated DNA on ice for 30 min, followed by heat shock at 42°C for 45 sec. Subsequently, the cells were cooled on ice for 2 min. 500 μl of SOC were then added to the transformed reaction mixture and grown at 37°C for 1 hr in a shaker. 200 μl of this mixture were then plated on Amp-LB-agar plates. Several colonies were picked and grown overnight at 37°C in Amp-LB broth. Plasmid DNA, isolated using a miniprep kit (QIAGEN), was sequenced to ensure correct mutagenesis and proper cloning.

III.1.C S10F-Hsp20 construct microinjection

The completed construct was sequenced to ensure proper cloning and submitted to the Cincinnati Children Hospital Medical Center Transgenic Animal and Genome Editing Core for pronuclear microinjection.

III.2 Isolation of mouse tail DNA and PCR conditions

III.2.A DNA isolation

Mice were weaned at 21 days post-birth, tail-clipped and ear-marked appropriately. DNA was isolated from the tails, using a DNA purification kit (QIAGEN), according to the manufacturer’s instructions. Briefly, tails were lysed in 180 μl of buffer ATL, in the presence of 20 μl of proteinase K, overnight at 55°C. Subsequently, the cells were lysed in 400 μl of buffer AL/ethanol. The
lysate was added to a purification column and centrifuged at 8,000 rpm for 1 min to bind the DNA. The column was washed by wash buffer AW1 and centrifuged at 8,000 rpm for 1 min. The supernatant was removed and the column was washed with wash buffer AW2 and centrifuged at 13,000 rpm for 1 min. The solution was emptied and the column was centrifuged to remove excess wash buffer. The DNA was finally eluted in 150 μl of elution buffer (EB) and used for PCR analysis, as described below.

III.2.B Genotyping mice for the S10F-Hsp20 transgene

The isolated DNA was screened for the S10F-Hsp20 transgene, using Taq DNA polymerase. The primers used were: sense: 5’-CACATAGAAGCCTAGCCCACAC-3’ antisense: 5’-GCTTGTCCTGTGCAGCTGGGAC-3’. The conditions were as follows: one cycle at 95°C for 1 min, linked to 35 cycles at 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min. The reaction mixture contains 2.5 μl polymerase buffer, 5 μl template DNA, 250 ng forward and reverse primers, respectively, 6.25 mM dNTPs, and 0.5 μl of the DNA polymerase, in a final reaction volume of 25 μl. The PCR product was pictured on a 1% agarose gel. The PCR-amplified product was sequenced to confirm transgene expression.

Section IV: Morphological studies

Gravimetric analysis was performed by weighing anesthetized mice and/or their isolated heart and lungs on a Mettler Toledo, AT201 analytical scale. After hearts were excised, fat and connective tissues were dissected, and then the
hearts were dried on Kimwipe paper and weighed. Histological examination of hematoxylin and eosin (H&E), and Masson’s trichrome stained atria and ventricles were carried out by the Department of Pathology at the Cincinnati Children Hospital Medical Center. Briefly, the heart tissue was fixed in 10% formalin, dehydrated by graded alcohols, and embedded in paraffin. Longitudinal sections (5 µm) of the heart (cut at 50-µm intervals) were stained with hematoxylin and eosin or subject to trichrome staining.

The sarcolemma was labeled with wheat germ agglutinin (WGA, Invi). WGA selectively binds to N-acetylglucosamine and N-acetylneuraminic. Oregon Green®488 (Invitrogen)-conjugated WGA was dissolved in PBS (pH 7.4) to give a stock solution with a concentration of 1 mg/ml. The stock solution was separated into small aliquots and frozen at -18°C. An aliquot was thawed and further diluted with deionized water to provide a working solution with a concentration of 200 g/ml. Paraffin-embedded tissue was first deparaffinized and incubated with WGA working solution for 60 minutes. Immunofluorescent images were taken by a confocal microscope under a 40x water immersion objective lens.

Section V: Isolation of adult mouse cardiomyocyte from NTG or S10F-Hsp20 mice

First, the mouse was injected with 2.5% Avertin (150 mg/ kg, IP), and mouse hearts were cut out and cannulated by the aorta onto a 20-gauge needle at room temperature in ice-cold Ca²⁺-free Tyrode solution. The cannulated heart was mounted on a Langendorff perfusion apparatus and perfused with Ca²⁺-
free Tyrode solution (140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 10 mM glucose, and 5 mM HEPES, pH 7.4) at 37°C for 3 minutes. The initial perfusion pressure was maintained at 40 mmHg by adjusting the flow rate. Perfusion was then switched to the same solution containing 1 mg/mL type II collagenase (276 U/mg, Worthington), and the enzymatic digestion was ended immediately, when the heart became flaccid (~8-10 minutes, with perfusion pressure lowered to 28 mmHg). All solutions were constantly bubbled with 100% oxygen. Then the heart was quickly removed from the Langendorff system, and the atria and aorta were dissected away. The LV tissue was minced into small portions in a dish containing Ca²⁺-free Tyrode solution. The single myocytes were gently dispersed from these portions by a wide tipped pipette, and further filtered through a 240-μm screen. The cardiomyocyte suspension was sequentially washed in 25, 100, 200 μM and 1 mM Ca²⁺-Tyrode solution, and resuspended in 1.8 mM Ca²⁺-Tyrode solution for further analysis.

Section VI: In vivo assessment of cardiac function using echocardiography

Echocardiography was achieved to examine heart contractile function in a noninvasive manner. Briefly, mice were anesthetized with 2.5% Avertin (0.01 mL/g), and animals were allowed to breathe spontaneously. The animal's chest was shaved, its extremities were secured to the examining surface, and the animal was placed on a warming pad (37°C; Deltaphase isothermal pad, Braintree Scientific) to maintain normothermia. Two-dimensional targeted M-mode and color flow-directed pulsed wave Doppler studies were performed with a 9 mHz imaging and a 5-7.5 mHz Doppler transducer (Philips-2000). M-mode
measurements of the LV end diastolic dimension (LVEDD) and LV end systolic dimension (LVESD), posterior and anterior wall thickness (PW and AW) and ejection time, measured at end diastole, were calculated from original tracings by employing a commercially available image analysis system (Freeland Medical), according to the leading-edge convention established by the American Society of Echocardiography, and by using the steepest continuous endocardiac echoes. The onset of the QRS complex and the peak of the posterior wall motion served as indicators of end diastole and end systole, respectively. Doppler measurements of peak and integrated aortic velocities, acceleration, and ejection times (ET) were made from ½ inch videotapes. Calculated variables included: LV fractional shortening p; normalized mean velocity of circumferential fiber shortening corrected for heart rate: \( V_{cf_c} = \frac{FS}{ET/(R-R_{int})^{1/2}} \), where ET was taken from heart rate-matched aortic Doppler spectra. A minimum of three beats were averaged for each measurement.

Section VII: Ex vivo ischemia/reperfusion studies

VII.1 Basal cardiac function measurement

Our methods for isolation and perfusion of mouse hearts by the Langendorff method have been described before. Specifically, animals were injected intraperitoneally with heparin (10,000 U/kg) and anesthetized with pentobarbital (10 μg/kg, IP). The thorax was rapidly opened and the heart was excised and arrested in Krebs-Henseleit buffer. A short perfusion cannula was inserted into the aortic root to initiate retrograde perfusion. The hearts were perfused with Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7, CaCl\(_2\) 1.8, KH\(_2\)PO\(_4\) 1.2,
MgSO₄ 1.2, NaHCO₃ 24.0, and glucose 10.0) equilibrated with 95% O₂ /5% CO₂ to get a pH of 7.4. A ventricular balloon, composed of polyvinyl chloride film and connected to a polyethylene tube, was inserted into the LV via the mitral valve from pulmonary vein. The balloon was connected to a pressure transducer (Micro-Med, Ltd) for recording of LV pressures. The balloon was inflated with water to modify the end-diastolic pressure (EDP) to 5-8 mmHg, and the balloon volume was held constant for the duration of the experiment. Before treatment, hearts were perfused at constant pressure (65 cm H₂O) for a 30-minute stabilization period. Cardiac contractile performance was assessed with a fluid-filled intraventricular balloon connected to a pressure transducer (Micro-Med, Ltd). The end-diastolic pressure (EDP) was set to 5 to 10 mmHg. LV developed pressure (LVDP), EDP, maximal rates of contraction (+dP/dt) and relaxation (-dP/dt) were recorded online by using a commercially available data acquisition system (PowerLab AD Instruments). Developed pressure (the difference between systolic and diastolic pressures), ±dP/dt and EDP were utilized as indices of contractile and diastolic function, respectively. A bipolar electrode (NuMed) was inserted into the right atrium, and atrial pacing was performed at 400 bpm with a Grass S-5 stimulator. At the end of the perfusion period, hearts were either frozen rapidly in liquid nitrogen and stored at -80°C or immersed in 1.5% paraformaldehyde.

VII.2 Ischemia/reperfusion protocol

Global ischemia was applied by stop perfusion completely and reperfusion was introduced by restoring the perfusion. Several I/R protocols have been used in
wild type FVB/N male mice, such as 30 min ischemia/60 min reperfusion, 45 min ischemia/60 min reperfusion, and 60 min ischemia/60 min reperfusion. In the current study, the protocol of 45 min ischemia/60 min reperfusion was used based on preliminary results, upon which the functional recovery was around 80%. However 30 min ischemia/60 min reperfusion resulted in a full functional recovery, and less than 15% recovery was observed under 60 min ischemia/60 min reperfusion.

**Section VIII: In vivo myocardial infarction study**

Mice were anesthetized by use of pentobarbital (100 mg/kg IP), and the trachea was intubated perorally with a plastic tube. The animals were mechanically ventilated with room air. After left thoracotomy and exposure of the heart, the left anterior descending coronary artery (LAD) was ligated with 8-0 polypropylene just proximal to its main branching point. Mice were raised for 12 weeks after surgery. For each condition, at least 6 successfully operated animals were incorporated. Sham surgeries were identical, with the exception of the actual tying of the polypropylene suture. Infarct sizing was carried out as described in our previous studies. The heart was perfused with 1% TTC (37°C, 60 mmHg). Then, the occluder was tied again and the heart was perfused with 5% phthalo blue. Hearts were transversely cut into 5 to 6 sections, with 1 section made at the site of the ligature. Infarct sizes were examined and expressed as a percentage of the region at risk.

**Section IX: Apoptosis analysis**

For terminal dUTP nick end-labeling (TUNEL) assays, heart was removed from
the body, and the atrial tissue was dissected away. The ventricles were fixed in 10% buffered formalin and later embedded in paraffin according to standard procedures and 5 m-thick sections were obtained to perform TUNEL assays by employing the DeadEnd™ Fluorometric TUNEL system (Promega, Madison, WI), according to the manufacturer’s instructions. Green nuclei TUNEL-positive myocytes were examined by randomly counting 10 fields of the mid-ventricular section and were expressed as a percentage of the total cardiomyocyte population. Myocyte cytoplasm was detected by α-sarcomeric actin (1:50 dilution; Sigma) labeling; nuclei were stained by DAPI (Invitrogen). Sections were analyzed with a fluorescence microscope. Alterations in the caspase-3 activity were studied using Caspase-3 Colorimetric Assay Kit (BioVision). The presence of caspase-3 activation was also tested by the observation of a 17-kDa subunit that was derived from the cleavage of 32-kDa proenzyme caspase-3 by using Western blot analysis.

**Section X: Autophagy activity analysis**

Autophagy activity in S10F heats were determined by measuring the autophagy markers. The microtubule-associated protein light chain 3 (LC3) is known to remain associated with the completed autophagosome. As such, it is the most common marker used to follow autophagic induction. The increase in the levels of lipidated LC3, referred to as LC3-II, or LC3-II/LC3-I (soluble fraction), by Western blot, indicate the induction of autophagy. Levels of Beclin-1, another autophagy marker, were also detected by Western blot. Beclin 1, an autophagy related protein, is a critical player in the formation of
Section XI: Measurement of protein and mRNA transcript expression

XI.1 Quantitative immunoblotting analysis

For immunoblot analysis of cardiac homogenates, frozen hearts that were free of auricles were homogenized in 1x Cell Lysis Buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/mL leupeptin, Cell Signaling Technology) supplemented with phosphatase inhibitor cocktail set I and II (CalBiochem) and complete protease inhibitor cocktail (Roche Applied Science) using a glass homogenizer with a teflon pestle at 4 °C. The homogenate was aliquoted and stored at -80°C.

For immunoblot analysis of cell lysates, harvested cardiomyocytes were centrifuged at 4°C, using low speed (3000 rpm) for 10 minutes, to collect the cell pellet. 120 μL of 1x cell lysis buffer was added to the cell pellet and then vortexed for 30 minutes on ice to release the cell contents. The resultant lysate was collected and stored at -80°C after removal of debris and non-lysed cells by centrifugation at 13000 rpm for 10 minutes at 4°C.

Protein concentration in cardiac homogenates or cell lysates was measured using the Bio-Rad colorimetric protein assay kit. Equal amounts of protein were subjected to electrophoresis on a sodium-dodecyl sulfate-polyacrylamide gel in running buffer (25 mM Tris, 191.8 mM glycine, 1% SDS) at 130 Volts until the protein loading dye ran off the gel. Protein was then
transferred to a nitrocellulose membrane (BioRad Laboratories) at 4°C for 3 hours at 180 mA in blotting buffer, comprising 25 mM Tris, 191.8 mM glycine, and 20% methanol. After proper transfer was achieved by Ponceau-S staining, the membranes were incubated in blocking buffer having 5% non-fat dried milk dissolved in TBST (100 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.4) for one hour at room temperature. The membranes were subsequently incubated overnight with the corresponding primary antibody diluted in blocking solution at 4°C. The dilutions of primary antibodies are listed in Table 1. After four 15 minute washes with TBST, the membranes were incubated with mouse, rat or rabbit secondary antibody conjugated with horseradish peroxidase at a 1:5000 dilution in blocking buffer of 0.5% milk for 2 hours at room temperature. After four TBST washes, visualization was achieved using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) or the ECLPLUS Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). The intensities of the bands were determined by the AlphaEaseFC™ software. To quantify the protein level in cardiac homogenates, different amounts of protein pooled from NTG hearts was run in three lanes on the same blot as a standard linear range. A linear correlation was obtained from this range and a coefficient of above 0.95 was defined as an acceptable linear relationship. Protein levels in different hearts were quantified using this linear relationship.
Table 1. Primary antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>p-S16 PLN</td>
<td>Badrilla</td>
<td>1: 1000</td>
</tr>
<tr>
<td>p-T17 PLN</td>
<td>Badrilla</td>
<td>1: 1000</td>
</tr>
<tr>
<td>RyR</td>
<td>Sigma</td>
<td>1: 1000</td>
</tr>
<tr>
<td>p-S2809 RyR</td>
<td>Badrilla</td>
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</tr>
<tr>
<td>p-S2814 RyR</td>
<td>Badrilla</td>
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<td>Affinity Bioreagents</td>
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<td>Cell Signaling</td>
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<td>Thermo Scientific</td>
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<tr>
<td>LC3</td>
<td>Novus</td>
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</tr>
</tbody>
</table>
XI.2 Quantitative real-time PCR assay

Total RNA was extracted and purified from heart tissue with miRNeasy Mini Kit (QIAGEN 133220834). The first strand cDNA was generated from total RNA (1 μg) with the reverse transcriptase kit (Invitrogen). PCR was then performed with the Bio-Rad real time thermal cycler, using the following specific primer sequences: Mouse full length SERCA2a: (Forward) 5’-CTGTGGAGACCCCTGTGGTGT-3’, (Reverse) 5’-CAGAGCACAGATGGTGCTGCT-3’, Mouse ANP: (Forward) 5’-GAGAAGATGCCGGTAGAAGA-3’, (Reverse) 5’-AAGCACTGCGGTCTTCAGA-3’, Mouse Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Forward) 5’-TCAACAGCAACTCCCACTCTT-3’, (Reverse) 5’-ACCCTGTGGCTGTAGCCGTATTCA-3’.

Section XII: Immunoprecipitation

In this dissertation, we used immunoprecipitation to determine the interactions between Hsp20 and Bax, Akt, Beclin 1. Firstly, cardiac homogenates from NTG and S10F-Hsp20 hearts were lysed in extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na2VO4, 1 ug/ml leupeptin; supplemented with 1x protease inhibitor cocktail) using an end-over-end rotator at 4°C. Lysates were centrifuged at 13,000 x g for 10 minutes, and supernatants were collected. The cleared supernatant was then incubated overnight at 4°C with 10 μl (2 μg) of mouse monoclonal IgG against Hsp20 (RDI) or an equivalent amount of control IgG. Following precipitation with 100 μl of protein G-agarose for 2 h, the beads were washed five times with lysis buffer. The final pellet was
subjected to SDS-PAGE and immunoblotting as described above.

**Section XIII: Blot overlay assay**

Interactions of Hsp20 or Hsp20 mutant with PP1 were evaluated *in vitro* by blot overlay assays, as previously described.\(^{101}\) Briefly, MBP (maltose binding protein), MBP-PP1 recombinant proteins generated by Dr. Elizabeth Vafiadaki were separated by SDS-PAGE and transferred to nitrocellulose membranes. Retention of the MBP-tag in the fusion proteins enabled the use of the MBP protein, encoded by the empty vector, as a negative control in these binding assays. Following blocking, the membranes were incubated with glutathione-S-transferase (GST)-WT-Hsp20 or GST-S10F-Hsp20 fusion proteins (also generated by Dr. Elizabeth Vafiadaki). The blots were probed with anti-GST and the immunoreactive bands were visualized using ECL reagents. Western blot analysis with MBP antibody (New England Biolabs) determined changes in the level of PP1 binding to WT or human variant proteins.

**Section XIV: PP1 activity assay**

Protein phosphatase activity was measured using RediPlate™96 EnzChek® Serine/Threonine Phosphatase Assay Kit (Molecular Probes), according to manufacturer's instructions. Briefly, reactions were performed using heart homogenates from the S10F and NTG hearts in the presence of 1X Reaction Buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35), supplemented with 1 mM MnCl\(_2\). Following the addition of 10 nM okadaic acid to discern between PP1 and protein phosphatase-2A (PP2A) activities, samples were incubated at 37°C for 30 minutes. Reactions were then terminated and the
activity of PP1 on 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) substrate was determined by using a fluorescence microplate reader equipped with appropriate filters. DiFMU has excitation/emission maxima of approximately 358/452 nm.

Section XV: Statistical analysis

Data are expressed as mean ± SEM. For the studies using isolated myocytes, 8-12 cells per heart were studied, and each animal was analyzed as a single n. Comparison between two groups was evaluated with Student's t test. Comparison of the results from more than three groups was analyzed using ANOVA. Probability (P) values of <0.05 were considered to be significant.

Chapter III Results

Section I: A Novel Human R25C-Phospholamban Mutation Is Associated with Superinhibition of Ca Cycling and Ventricular Arrhythmia

I.1 Identification of a novel PLN mutation in a familial Dilated Cardiomyopathy (DCM) pedigree

Recently, through collaboration with Dr. Ray E. Hershberger,’s team, a novel mutation (R25C) in the coding region of the human PLN gene (PLN) was identified in a pedigree with DCM that also showed prominent ventricular arrhythmia and need for implantable cardiac defibrillators (ICDs).\textsuperscript{190, 197} Specifically, forty eight variants, meeting our established criteria for exome sequencing analysis\textsuperscript{190}, were identified in two sisters (III.1 and III.3) in our family pedigree (Fig. 1). Of these 48, 3 were in previously identified DCM genes,
including a novel heterozygous \textit{PLN} mutation that resulted in change of arginine into cysteine at position 25, and two missense mutations in \textit{TTN} (A3656S and P7178S). Because it is known that even rare (<0.5%) \textit{TTN} missense mutations are prevalent in control samples\textsuperscript{198}, these missense variants were considered unlikely to be the cause of DCM. The \textit{PLN} mutation had high conservation scores (Phastcons 1; GERP 4.31), and based on other heterozygous \textit{PLN} missense mutations causing DCM\textsuperscript{19, 63, 65, 67, 145}, it was hypothesized that this mutation may also alter \textit{PLN} activity. None of the genes harboring the remaining 45 variants were associated with known cardiovascular disorders. No additional \textit{PLN} variants were identified in 16 other families.\textsuperscript{190} A pedigree with the \textit{PLN} mutation status is shown in Figure 1. Sanger sequencing confirmed that the \textit{PLN} mutation was present in the sisters (III.1 and III.3), the proband (III.2) who also carried a previously identified \textit{LMNA} mutation, as well as in his mother (II.3), who had DCM but was not found to carry the \textit{LMNA} mutation.\textsuperscript{190}
**Figure 1.** Familial DCM Pedigree with a *PLN* mutation. Squares represent males and circles represent females. Slash denotes deceased. Darkened symbols indicate idiopathic DCM with implantable cardiac defibrillator (ICD) and gray symbols represent a significant cardiovascular abnormality. Open symbols represent negative cardiovascular history. The presence of the *PLN* mutation is denoted with (+) and the presence of the *LMNA* mutation\(^{197}\) with *. (*Data generated by collaborator Dr. Hershberger*)
I.2 Clinical characteristics of family members carrying the PLN mutation

Clinical characteristics of family members with the PLN mutation are provided in Table 1. The proband (III.2) presented with sudden cardiac arrest at age 47 and was diagnosed with non-ischemic DCM upon resuscitation. An implantable cardiac defibrillator (ICD) was placed from which he had received multiple appropriate shocks. About 10 years later, he required heart transplantation due to worsening heart failure. Two of his sisters were also found to harbor the R25C-PLN mutation. His older sister (III.1) was first screened clinically at age 51 and was found to have LV enlargement but preserved systolic function. Despite already receiving an angiotensin receptor blocker (ARB), 2 years later her ventricular function had deteriorated. Non-sustained ventricular tachycardia (NSVT) was found on Holter monitoring, and an ICD was placed prior to the onset of any heart failure. She was treated with full dose beta blockade and an ARB. Over the course of the following ten years, two cardioversions were required due to persistent atrial fibrillation. The proband’s younger sister (III.3), who also had NSVT and required an ICD, was diagnosed with DCM at age 45 after presenting with signs of heart failure. Their mother (II.3), who had complained of palpitations since age 47, had premature ventricular contractions at age 57. She was diagnosed with DCM at 71 and had multiple PVCs, couplets and NSVT for years, for which an ICD was placed at age 84 because of progressive pump dysfunction. She progressed to chronic atrial fibrillation and received multiple cardioversions, and died from heart failure at age 90.
TABLE 2 Clinical characteristics of family members with the R25C-PLN mutation

<table>
<thead>
<tr>
<th>Pedigree position</th>
<th>DCM</th>
<th>Age (Years)</th>
<th>LVEDD (Z-score)</th>
<th>EF (%)</th>
<th>ECG/Arrhythmia</th>
<th>Comments</th>
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<tr>
<td>II.3</td>
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<td>71</td>
<td>60 (4.1)</td>
<td>35</td>
<td>AF, CVN, PVCs</td>
<td>HF</td>
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<td></td>
<td></td>
<td></td>
<td>LBBB, ICD</td>
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<td>III.1</td>
<td>yes</td>
<td>53</td>
<td>58 (3.4)</td>
<td>40</td>
<td>AF, 1AVB, VT, ICD</td>
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<tr>
<td>III.2</td>
<td>yes</td>
<td>47</td>
<td>72 (4.9)</td>
<td>28</td>
<td>AF, RBBB, PMICD, VT, SCD, Heart transplant: 58 y, LMNA mutation +</td>
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<tr>
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<td>yes</td>
<td>45</td>
<td>60 (4.0)</td>
<td>17</td>
<td>NSVT, PMICD</td>
<td>HF</td>
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Table 2. Clinical characteristics of family members with the R25C-PLN mutation.

LVEDD = LV end-diastolic dimension by echocardiography; Z-score = number of standard deviations of the LVEDD above the population mean; EF = LV ejection fraction; AF = atrial fibrillation; PVCs = premature ventricular contractions; LBBB = left bundle branch block; ICD = implantable cardiac defibrillator; HF = heart failure; 1AVB = first degree atrioventricular block; VT = ventricular tachycardia; RBBB = right bundle branch block; PM = pacemaker; SCD = sudden cardiac death; NSVT = non-sustained ventricular tachycardia. (Data generated by collaborator Dr. Hershberger)
I.3 The C73T genetic variant changes a fully-conserved arginine residue at position 25 to a cysteine, resulting in alterations of PLN’s secondary structure

The C73T mutation changes an arginine at position 25 to cysteine (R25C). As shown in Figure 2 A, comparison of this site in human, rabbit, pig, dog, rat and mouse revealed that it is fully conserved among these species, suggesting a critical role in PLN’s function. Considering the structure difference between arginine and cysteine residues, it was important to examine the functional significance of this mutation on the secondary structure of PLN. Thus, protein structure analysis software was utilized to predict any potential alterations. Interestingly, the R25C mutation is predicted to increase the helical content (R25C 13.46% vs. WT 11.54%) of the secondary structure of PLN (Fig. 2 B). Therefore, it is possible that the R25C substitution is associated with structural changes which modify the basic function of PLN in the heart.

I.4 Expression of Mutant PLN in Adult Cardiomyocytes

To determine if the inhibitory effects of PLN on cardiac contractility are modified by the R25C mutation, recombinant adenoviruses encoding GFP, WT-PLN (generated by Dr. Kobra Haghighi) or R25C-PLN were transduced into adult rat ventricular myocytes. The protein levels of PLN in infected cardiomyocytes were assessed by SDS-PAGE and Western blots. Quantitative immunoblotting of cell lysates revealed a similar ratio of PLN pentamers to monomers in mutant PLN (85.8%) cardiomyocytes, compared with WT-PLN (86.6%) and GFP (84.9%) groups (Fig. 3A). Furthermore, upon boiling the cardiac homogenates prior to
SDS-PAGE, the mutant could be dissociated into monomers, similar to WT-PLN. There were no significant changes in the SERCA2a protein levels in the infected mutant cardiomyocytes (Fig. 3A). When the apparent PLN/SERCA2a ratio was calculated, it was found to be 1.9 fold higher in R25C myocytes, compared with the GFP group (1.0), and this ratio was similar to that for WT-PLN (1.93) (Fig. 3B).
FIGURE 2

A

<table>
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<th>21</th>
<th>25</th>
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<tbody>
<tr>
<td>Human</td>
<td>Pro Gln Gln Ala</td>
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<tr>
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B

<table>
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<tr>
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<th>WT-PLN</th>
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<tr>
<td></td>
<td>11.54%</td>
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**Figure 2.** The R25C mutation changes a fully-conserved arginine at position 25 to a cysteine, which is predicted to result in secondary structural alterations. A, PLN Ib domain (amino acids: 21-30) in human, rabbit, dog, pig, mouse and rat shows that Arg25 is fully conserved, suggesting a critical role in PLN’s function. B, Secondary structure predictions (PredictProtein program) revealed alterations in the helical content in PLN mutant.
FIGURE 3

A

<table>
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<tr>
<th></th>
<th>GFP</th>
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B

![Bar graph showing PLN/SERCA2a expression levels](image)
**Figure 3.** Quantitative immunoblots from infected cardiomyocytes. Cell lysates from cardiomyocytes infected with GFP (Ad.GFP), wild-type PLN (Ad.WT-PLN) or mutant PLN (Ad.R25C-PLN) adenoviruses were subjected to 13% SDS-PAGE and electroblotted on nitrocellulose membranes. A, Membranes were probed with PLN antibody or SERCA antibody and GAPDH was used as loading control. PLNp, pentameric PLN; PLNm, monomeric PLN; B, PLN protein levels in GFP, WT and R25C cardiomyocytes expressed as relative ratio of PLN/SERCA2a. n=6 hearts; Values are mean ± SE.*p<0.05, vs. GFP.
I.5 R25C-PLN is a super-inhibitor of the Ca affinity of SERCA2a

To examine the functional significance of the R25C substitution in PLN and its impact on SERCA2a regulation, the initial rates of ATP-dependent, oxalate-facilitated SR Ca\textsuperscript{2+} uptake were measured in cell lysates from infected cardiomyocytes. There were no significant differences in the maximal rates of SR Ca\textsuperscript{2+} uptake between GFP, WT or R25C groups. However, the EC50 value for Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} uptake was significantly higher in WT-PLN cells (0.40±0.03 µM), compared with the GFP group (0.22±0.02 µM), consistent with our previous findings.\textsuperscript{23} Interestingly, R25C-PLN further increased the SERCA2a EC50 value to 0.63±0.07 µM (Fig. 4), Thus, the mutant PLN is a super-inhibitor of the affinity of SERCA2a for Ca\textsuperscript{2+} compared with WT-PLN.

I.6 In vitro functional analysis of the R25C-PLN mutant

Overexpression of WT-PLN has been shown to lead to significant depression of cardiomyocyte function.\textsuperscript{23} To determine if the super-inhibitory effects of the R25C mutation on SERCA2a activity translated into alterations at the cellular level, the contractile parameters of infected cardiomyocytes were assessed. The resting cell length in mutant PLN infected myocytes was not altered, compared with the WT-PLN or GFP groups. However, the amplitude of basal cell contraction (fractional shortening, FS%) was more depressed in myocytes overexpressing R25C mutant PLN (60%) than in myocytes overexpressing WT-PLN (76%), compared with GFP control (100%) (Fig. 5 A, B). The maximal velocities of cardiomyocyte shortening and re-lengthening were also more depressed in myocytes with mutant PLN (+dL/dt: 52%; −dL/dt: 46%) than in cells with WT-PLN.
(+dL/dt: 67%; −dL/dt: 66%), compared to GFP controls (100%) (Fig. 5 C, D). Accordingly, the relaxation time was significantly prolonged in R25C cardiomyocytes with respect to WT-PLN and GFP groups (Fig. 5 E).

I.7 Overexpression of R25C-PLN significantly suppresses Ca\(^{2+}\) transients, delays the rate of Ca\(^{2+}\) removal and elevates intracellular diastolic Ca\(^{2+}\).

To determine whether the observed alterations in myocyte mechanics reflected similar alterations in Ca\(^{2+}\) handling, intracellular Ca\(^{2+}\) transients in infected cardiomyocytes were measured by use of the Fura-2/AM fluorescence indicator (2 µM). Our results demonstrate that the amplitude of Ca\(^{2+}\) transients was reduced by 55% in mutant-PLN and by 37% in WT-PLN, compared to GFP cardiomyocytes (Fig. 6 A, B). The time to 50% decay of the Ca\(^{2+}\) signal was prolonged by 65% in cardiomyocytes overexpressing mutant and by 32% in cardiomyocytes overexpressing WT-PLN, relative to GFP group (Fig. 6 C). In addition, intracellular diastolic Ca\(^{2+}\) was also analyzed and it was found to be increased by 18 % in R25C-PLN compared to WT-PLN or GFP cardiomyocytes (Fig. 6 D). Thus, the R25C-PLN mutant depressed mechanical and Ca\(^{2+}\) kinetic parameters and increased diastolic Ca\(^{2+}\) levels, consistent with the enhanced inhibition of SERCA2a.
**Figure 4.** Effects of wild-type and mutant R25C-PLN on the apparent Ca\textsuperscript{2+} affinity of SERCA2a. After 24-hours infection with adenoviruses, cardiomyocytes were homogenized and the initial rates of oxalate-supported SR Ca\textsuperscript{2+} uptake were measured. Data are expressed as % of maximal uptake rates in each group ($V_{\text{max}}$: 99±4 in GFP, 101±3 in WT, and 96±5 in R25C, nM/mg/min). Values are mean ±SE. *p<0.05, vs. GFP; †p<0.05, vs. WT. Inset: The average EC50 values for the 3 groups. n = 6 for GFP, 4 for WT and 4 for R25C independent experiments.
FIGURE 5

A

Cell length (μm)

105
95
85

GFP  WT  R25C

2 S

B

FS%

12
10
8
6
4
2
0

GFP  WT  PLN

*  *†
Figure 5. Contractile parameters in Ad.GFP, Ad.WT-PLN and Ad.R25C-PLN infected cardiomyocytes. After 24-h incubation with adenoviruses, maximum rates of contraction and relaxation in cardiomyocytes were measured. A, Representative cell-shortening traces of Ad.GFP, Ad.WT-PLN and Ad.R25C-PLN cardiomyocytes; B, Fractional shortening (FS%); C, Maximum rates of contraction (+dL/dt); D, Maximum rates of relengthening (-dL/dt); and E, Relaxation kinetics in cardiomyocytes paced at 0.5 Hz, expressed as the time from peak amplitude to 50% relaxation. (n=20~25 cells per group per experiment; each experiment was repeated at least 4 times using different hearts). Values are mean ± SE. *p<0.05, vs. GFP; †p<0.05, vs. WT.
FIGURE 6

A

```
Fura-2 Ratio
```

2s

GFP  WT  R25C

B

```
Ca Transient Peak (Fura-2 Ratio)
```

GFP  WT  R25C
Figure 6. Ca$^{2+}$ kinetics in Ad.GFP, Ad. WT-PLN, and Ad.R25C-PLN cardiomyocytes. Infected myocytes were incubated with Fura2-AM for half an hour and Ca$^{2+}$ transients were measured. A, Representative tracings of Ca$^{2+}$ transients; B, Ca$^{2+}$ transient amplitude in infected cardiomyocytes; C, Time to 50% decay (T50) of Ca$^{2+}$ signal; (n=20~25 cells per group per experiment; each experiment was repeated at least 3 times using different hearts). Values are mean ± SE *p<0.05, vs. GFP; †p<0.05, vs. WT.
FIGURE 7

A

Caffeine-Ca Transient Peak (Fura-2 Ratio)

0.5
1.0
1.5
2.0

5 S

B

C

Caffeine-Ca Transient Peak (Fura-2 Ratio)

GFP  WT  R25C

T50 (Sec)

GFP  WT  R25C

*  *†
Figure 7. Overexpression of R25C-PLN significantly suppresses SR Ca\textsuperscript{2+} load. A, Representative tracings of caffeine (10 mM)-induced Ca\textsuperscript{2+} transient; B, Caffeine-induced Ca\textsuperscript{2+} transient amplitude; C, Time to 50% decay (T50) of caffeine induced Ca\textsuperscript{2+} transient peak (n=20–25 cells per group per experiment; each experiment was repeated at least 3 times using different hearts). Values are mean ± SE. *p<0.05, vs. GFP; †p<0.05, vs. WT.
I.8 Overexpression of R25C-PLN significantly suppresses SR Ca load

The effects of mutant-PLN on intracellular Ca$^{2+}$ transients prompted further studies on the influence of R25C-PLN on SR Ca$^{2+}$ load. It was observed that the caffeine-induced Ca$^{2+}$ transient peak was reduced by 37% in R25C-PLN cardiomyocytes and by 20% in WT-PLN cardiomyocytes, compared with the GFP group (Fig. 7 A, B), indicating a superinhibitory effect of mutant-PLN on SR Ca$^{2+}$ content. However, the time constant for 50% decay of the caffeine-induced Ca$^{2+}$ transient (T$_{50}$), which mainly reflects Ca$^{2+}$ extrusion by the NCX, was similar between the three groups (Fig. 7 C).

I.9 Isoproterenol stimulation relieves the inhibitory effects of R25C-PLN

Phospholamban has been shown to be phosphorylated by β-adrenergic stimulation, leading to relief of its inhibitory effects on SERCA2a and cardiac function. To determine whether the superinhibitory effects of mutant PLN could be also relieved by β-agonists, cardiac myocytes infected with Ad.GFP, Ad.WT-PLN or Ad.R25C-PLN were stimulated with isoproterenol and their mechanical and Ca$^{2+}$ kinetic parameters were assessed. It is interesting to note that maximal stimulation, obtained at 100 nM isoproterenol, resulted in complete reversal of the inhibitory effects of wild-type or mutant PLN. Similarly, under maximal isoproterenol stimulation, the inhibition on the amplitude of systolic Ca$^{2+}$ transient and the rate of decay of this signal were fully reversed in cardiomyocytes overexpressing mutant PLN. The maximally stimulated values were similar among the three groups (Fig. 8).
FIGURE 8

A

B

C

D

E
Figure 8. Effects of isoproterenol (100 nM) on cardiomyocyte mechanics and Ca\textsuperscript{2+} kinetics. A, Fractional shortening (FS %); B, Maximum rates of contraction (+dL/dt); C, Maximum rates of re-lengthening (-dL/dt); D, Ca\textsuperscript{2+} transient amplitude; E, Time to 50% of Ca\textsuperscript{2+} decay. n=15~20 cells per group per experiment; each experiment was repeated at least 3 times using different hearts; Values are mean ± SE.
I.10 The mutant R25C-PLN exhibits enhanced association to SERCA2 and PKA abrogates this effect

To gain insights into the mechanisms associated with increased inhibition of SERCA2a by R25C-PLN, we co-expressed SERCA2 with either WT or mutant PLN in HEK cells to evaluate their interactions in the absence of endogenous proteins. For this, we generated a GFP-WT-PLN or GFP-R25C-PLN constructs and transiently co-transfected these along with SERCA2 in HEK 293 cells. Initial immunofluorescence studies indicated that, similar to GFP-WT-PLN, the GFP-R25C-PLN mutant exhibited co-localization with SERCA2 in ER (Fig. 9 A). Subsequent immunoprecipitation studies from co-transfected cells revealed a significant increase in the levels of SERCA2 in the R25C-PLN sample, compared to WT-PLN (Fig. 9 B, C), indicating enhanced formation of the SERCA2/R25C-PLN protein complex. Interestingly, parallel experiments in cell lysates, that had been previously phosphorylated by PKA, revealed that phosphorylation of R25C-PLN abolishes the increased interaction of this mutant with SERCA2, as similar levels of SERCA2 were observed in the phosphorylated R25C-PLN and WT-PLN protein complexes (Fig. 9 D, E). These findings are consistent with relief of the mutant PLN superinhibitory effects on contractility and Ca^{2+}-cycling upon Iso-stimulation of cardiomyocytes (Fig. 8).
Figure 9. A, The R25C-PLN mutant co-localizes with SERCA2 in transfected HEK 293 cells, similar to WT-PLN. Nuclei are stained with DAPI. Scale bar, 5 μm; B and C, R25C-PLN mutant exhibits enhanced association to SERCA2. Immunoprecipitation assays in HEK 293 cells that co-express GFP-PLN and SERCA2 were performed using GFP antibody. Quantification of SERCA2 levels revealed a significant increase in the SERCA2/R25C-PLN protein complex compared to WT-PLN (n=4; values are means ± SE; *P<0.05, compared to WT-PLN); D and E, enhanced binding of R25C-PLN with SERCA2a was abolished upon PKA phosphorylation. Immunoprecipitations were performed in lysates from HEK 293 transfected cells that had been previously phosphorylated with PKA. Western blot analysis (D) determined similar levels of SERCA2 in both WT-PLN and R25C-PLN samples and quantitative analysis (E) showed no difference in SERCA2 binding between WT-PLN and R25C-PLN (n=4). (Data generated by collaborator Dr. Elizabeth Vafiadaki)
I.11 Increased Ca sparks and waves in R25C-PLN cardiomyocytes.

As described above, all R25C affected members in the family pedigree developed cardiac arrhythmias (Fig. 1 and Table 2). The molecular trigger for arrhythmia is enhanced SR Ca\(^{2+}\) leak, evidenced by increases in Ca\(^{2+}\) sparks or waves at the cardiomyocyte level.\(^{200}\) To determine the effect of R25C on SR Ca\(^{2+}\) release, we examined Ca\(^{2+}\) spark properties in intact quiescent cells. The line-scan and three dimensional images for Ca\(^{2+}\) sparks are presented in Figure 10 A. It was observed that spark frequency was 2 fold higher in the mutant PLN cardiomyocytes, compared to WT-PLN or GFP cardiomyocytes (Fig. 10 B). Next, the frequency of spontaneous Ca\(^{2+}\) waves was examined in GFP, WT-PLN and R25C-PLN myocytes. It was found that Ca\(^{2+}\) waves were developed in 40% of R25C-PLN cardiomyocytes, compared with 4% of WT-PLN cells and 6% of GFP cells (Fig. 10 C, D). Thus, R25C-PLN increases the frequency of Ca\(^{2+}\) sparks and Ca\(^{2+}\) waves in cardiomyocytes.

I.12 Increased diastolic SR Ca\(^{2+}\) leak in R25C-PLN cardiomyocytes

Recent studies have suggested that RyR2-mediated Ca\(^{2+}\) leak occurs in part as Ca\(^{2+}\) sparks, although there is RyR-mediated and Ca\(^{2+}\) spark–independent leak.\(^{201}\) Therefore, total diastolic SR Ca\(^{2+}\) leak was also measured using the tertacaine protocol\(^{202}\) (Fig. 11 A, B). The ratio of SR Ca\(^{2+}\) leak to SR Ca\(^{2+}\) load was significantly larger in R25C cardiomyocytes, compared with GFP and WT cardiomyocytes (Fig. 11 C), suggesting that R25C-PLN increases the SR Ca\(^{2+}\) leak.

I.13 Increased stress-induced aftercontractions in R25C-PLN cardiomyocytes
cardiomyocytes

Next, we determined the role of R25C-PLN under stress conditions by measuring the frequency of after-contractions in GFP, WT-PLN and R25C cardiomyocytes at 2-Hz field stimulation in the presence of 1 μM Isoproterenol. Spontaneous after-contractions occurred in 74% of R25C cells within 5 seconds after pacing was stopped, compared with 17% of WT-PLN and 16% of GFP cells (Fig. 12 A, B). Taken together, these findings suggest that overexpression of R25C-PLN enhances the propensity for spontaneous Ca\(^{2+}\) release from the SR, resulting in increased susceptibility to arrhythmia.

I.14 Increased SR Ca leak and arrhythmias in R25C myocytes are associated with augmented phosphorylation of RyR2 at Serine-2814

We then investigated whether the increased SR Ca\(^{2+}\) leak in R25C cardiomyocytes was associated with increases in the phosphorylation of RyR2. It has been previously reported that enhanced phosphorylation of Ser2808 (PKA site) and Ser2814 (CaMKII site) in RyR2 can modulate RyR2 function. Thus, we examined the phosphorylation state of RyR2 in R25C cardiomyocytes, by performing Western blots with phospho-specific antibodies against the RyR2 Ser2808 and Ser2814 sites. It was observed that R25C cells had significantly increased phosphorylation levels of RyR at Ser2814, but not at Ser2808 (Fig. 13 A, B, C).
FIGURE 10

A

Ca\(^{2+}\) Spark Ca\(^{2+}\) Spark-3D

GFP

WT

R25C

B

Ca\(^{2+}\) Spark frequency (events/s/100 μm)

GFP WT R25C

*
Figure 10. Ca^{2+} sparks and waves in GFP, WT-PLN and R25C-PLN cardiomyocytes. A, Representative line-scan and three dimensional (3D) images of Ca^{2+} sparks acquired in infected cardiomyocytes; B, Cumulative data on Ca^{2+} spark frequency; C, Representative line-scan and 3D images of Ca^{2+} waves acquired in R25C-PLN cardiomyocytes; D, Percentage of cells showing Ca^{2+} waves (n=73 in R25C-PLN cells, n=66 in WT-PLN cells and n=67 in GFP cells per experiment; each experiment was repeated 6 times). Values are mean ± SE.*p<0.05, vs. GFP and WT.
FIGURE 11

A

GFP

WT

R25C

Load

30 S

SR Ca Leak

Tetracaine Caffeine

B

SR Ca Leak (Fura-2 Ratio)

0.12

0.09

0.06

0.03

0

GFP WT R25C

C

Leak/SR Load (%)

12

9

6

3

0

GFP WT R25C

*
Figure 11. Diastolic SR Ca\textsuperscript{2+} leak in GFP, WT-PLN and R25C-PLN cardiomyocytes. A, Representative traces of SR Ca\textsuperscript{2+} leak were obtained from the three groups. Ca\textsuperscript{2+} leak was determined as the tertacaine sensitive drop in diastolic Fura 2 ratio; B, Comparison of average diastolic SR Ca\textsuperscript{2+} leak; C, Quantification of Ca-leak/SR Ca\textsuperscript{2+}-load relationships in GFP, WT and R25C myocytes (ratio of Ca\textsuperscript{2+} leak/caffeine-induced Ca\textsuperscript{2+} transient, n=12-18 cells per experiment; each experiment was repeated at least 4 times); Values are mean ± SE. *p<0.05, vs. WT and GFP.
FIGURE 12

A

GFP

WT

R25C

B

% of cells with Acs

GFP  WT  R15C

*
Figure 12. Stress-induced aftercontractions (Acs) in GFP, WT-PLN and R25C-PLN cardiomyocytes. A, Representative traces of Acs; B, Percentage of the infected cardiomyocytes that developed Acs (n=61 in R25C-PLN cells, n=56 in WT-PLN cells and n=77 in GFP cells per experiment; each experiment was repeated 6 times). Values are mean ± SE.*p<0.05, vs. GFP and WT.
I.15 Increased CaMKII activation in R25C cardiomyocytes

Elevated phosphorylation of RyR2 at serine-2814 prompted us to determine the activity of CaMKII in cell lysates from GFP, WT-PLN and R25C cardiomyocytes, using a non-radiographic CaMKII ELISA. We found that the activity of CaMKII in R25C myocytes was increased to 2-fold compared to WT-PLN or GFP groups (Fig. 14 A), consistent with the increase in diastolic Ca$^{2+}$ in these cells (Fig. 5 D). To further confirm this finding, phosphorylation of CaMKII at Thr286 residue, which represents permanent activation of the CaMKII enzyme, was determined in Num>ern blots. It was observed that the level of phosphorylation of CaMKII at Thr286 was considerably higher in R25C cells, compared to the WT-PLN or GFP groups (Fig. 14 B, C). Interestingly, the increased CaM kinase activity did not reflect altered phosphorylation of PLN (Fig. 16) in the mutant cells, suggesting that mutant PLN either altered CaMKII binding or increased PLN-phosphatase activity.

I.16 Increased SR Ca$^{2+}$ leak in R25C myocytes is abolished by CaMKII inhibitor

To further examine the contribution of CaMKII mediated phosphorylation of RyR2 in the R25C-induced increases of Ca$^{2+}$ sparks and waves, KN93, the selective inhibitor of CaMKII was utilized in parallel studies with KN92 as a control. We found that KN93 completely abrogated the increased Ca$^{2+}$ spark frequency (Fig. 15 A), indicating that the aberrant SR Ca$^{2+}$ release was contributed by the increased CaMKII phosphorylation of RyR. Furthermore, KN93 fully abolished the Ca$^{2+}$ waves, elicited by R25C-PLN (Fig. 15 B). These data suggest that the
FIGURE 13

A

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B

**pS2814-RyR2/T-RyR2**

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C

**pS2808-RyR2/T-RyR2**

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Figure 13. Phosphorylation of RyR2. A, Representative blots of phosphorylation and total levels of RyR2. Cell lysates from infected cardiomyocytes were subjected to 6% SDS-PAGE and electroblotted on nitrocellulose membranes. The membranes were probed with anti-pSer2808, anti-pSer2814 RyR2 or anti-RyR2 antibodies; B and C, Percentage of phosphorylated Ser2814 (pSer2814) and Ser2808 (pSer2808) RyR2 in infected cardiomyocytes. (n= 6 hearts); Values are mean ± SE. *p<0.05, vs. WT-Basal.
Figure 14. A, CaMKII activity in GFP, WT and R25C cardiomyocytes (n=7 hearts); B, Representative blots of phosphorylation and total levels of CaMKII; C, Percentage of phosphorylated Thr286 (pT286) CaMKII in infected cardiomyocytes (n=4 hearts); Values are mean ± SE. *p<0.05, vs. WT-Basal.
aberrant SR Ca$^{2+}$ leak and resultant arrhythmia were associated with increased CaMKII activity and hyper-phosphorylation of RyR at Ser2814.

I.17 Phosphorylation of R25C-PLN

To determine if the ability of PLN to be phosphorylated is modified by the R25C mutation, cell lysates from infected cardiomyocytes with/without Isoproterenol (100 nM) treatment were subjected to SDS-PAGE and electroblotted on nitrocellulose membranes. The membranes were probed with anti-Ser16, anti-Thr17 PLN or PLN antibodies. It was found that R25C mutation did not alter the ability of PLN to be phosphorylated (Fig. 16).
FIGURE 15

A

Ca^{2+} Spark frequency (events/s/100 um)

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B

Ca^{2+} waves (% of cells)

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Figure 15. Increased SR Ca\textsuperscript{2+} leak in R25C myocytes is abolished by CaMKII inhibitor. A, and B, Ca\textsuperscript{2+} spark frequency and percentage of Ca\textsuperscript{2+} waves recorded in GFP, WT and R25C cardiomyocytes in the absence or presence of CaMKII inhibitor KN93 (1 µmol/L), with KN92 (1 µmol/L) used as a control (15~20 cells were measured per experiment or each heart; n=4 hearts for GFP, WT-PLN and R25C-PLN groups). Values are mean ± SE. *p<0.05, vs. WT-Basal.
FIGURE 16

A

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- pSer16-PLN
- pThr17-PLN
- PLN
- GAPDH

B

- pSer16-PLN/PLN (relative units)

- Basal
- Iso

C

- pThr17-PLN/PLN (relative units)

- Basal
- Iso

- WT
- R25C

* p < 0.05
† p < 0.01
**Figure 16.** Cell lysates from infected cardiomyocytes with/without Isoproterenol (100 nM) treatment were subjected to 13% SDS-PAGE and electroblotted on nitrocellulose membranes. The membranes were probed with anti-Ser16, anti-Thr17 PLN or PLN antibodies; GAPDH was used as a loading control. A, Representative blots of phosphorylation and total levels of PLN under basal and Iso conditions. B and C, Percentage of phosphorylated Ser16 (pSer16) and Thr17 (pThr17) PLN in WT and R25C cardiomyocytes. Values are mean ± SE. *p<0.05, vs. WT-Basal; †p<0.05, vs. R25C-Basal; n= 4 hearts;
Section II: The Role of Human Hsp20 Mutant (S10F) in Cardiac Function and Protection

II.1 Identification of a C29T substitution in the human Hsp20 gene

In the heart, Hsp20 has been shown to be a positive modulator in cardiac function and protection. Specifically, studies from our laboratories and other researchers indicate that Hsp20 and its phosphorylation can protect the heart against I/R injury, hypertrophic remodelling and stress-induced apoptosis. In addition, recent studies in our laboratory showed that overexpressing Hsp20 significantly enhanced cardiac contractile performance and calcium kinetics by inhibiting protein phosphatase-1 (PP1) activity. These studies suggest that Hsp20 has a dual beneficial effect on the heart and may serve as novel therapeutic target for the treatment of heart failure. Therefore, experiments were designed to identify possible genetic variants in the human Hsp20 gene, which may alter its cardiac contractile enhancing and cardioprotective properties.

Identification of genetic variants in the human Hsp20 gene was performed by Persoulla Nicolaou, a former Ph.D student in Dr. Kranias’ lab. In detail, the entire Hsp20 gene (NCBI Accession #: NM_144617), consisting of 3 exons, was analyzed in 470 DCM patients and in 282 individuals with no heart disease. This screen led to the identification of a C29T transversion in the coding region (exon1) of the Hsp20 gene. This C29T transversion entails substitution of a serine at position 10 to a phenylalanine (S10F). Notably, this mutation was found exclusively in male, black patients with DCM, with the prevalence of 2.82%.

II.2 The C29T genetic variant changes a fully conserved serine residue at
position 10 to a phenylalanine, resulting in alterations of Hsp20’s secondary structure

The C29T mutation changes a serine at position 10 to phenylalanine (S10F). As shown in Figure 17 A, comparison of this site in human, pig, dog, rat and mouse revealed that it is fully conserved among these species, suggesting a critical role in Hsp20’s function. Considering that the structure of serine residue is very different from that of phenylalanine, it was therefore important to examine the functional significance of this mutation on the secondary structure of Hsp20. As such, protein structure analysis software was utilized to predict any potential alterations. Interestingly, the S10F substitution is predicted to change the contents of all three components (helix strand and loop) of the secondary structure of Hsp20 (Fig. 17 B). Consequently, it is possible that the S10F substitution is associated with structural changes which may modify the basic function of Hsp20 in the heart.

II.3 In vitro studies: the beneficial effects of Hsp20 on cardiac function and protection are completely abolished by S10F substitution

To investigate the functional significance of the human S10F-Hsp20 variant, adult rat cardiomyocytes were infected with Ad.GFP (control), Ad.WT-Hsp20 (generated by Dr. Guo-Chang Fan) and Ad.S10F-Hsp20 (generated by Dr. Tracy Pritchard).

Since the viruses utilized in this study also express green fluorescent protein (GFP), adenoviral infection efficiency was determined at 48 hours post-infection by green fluorescence, which demonstrated that ~100% of the cells
FIGURE 17

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<tr>
<td>S10F-Hsp20</td>
<td></td>
<td>13.3%</td>
<td>18.7%</td>
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**Figure 17.** The C29T substitution changes a fully-conserved serine at position 10 to a phenylalanine (S10F), which is predicted to result in secondary structural alterations. A. Partial amino acid sequence alignment (amino acids: 1-20) in human, pig, dog, rat and mouse shows that serine 10 is fully conserved, suggesting a critical role in Hsp20’s function. B. Secondary structure predictions revealed alterations in the helix, strand and loop contents in the mutant Hsp20.
were infected in all of the groups. As exhibited in Figure 18 A and B, this resulted in similar levels of overexpression between the WT-Hsp20 and the S10F-Hsp20 groups (~3.5-fold, as compared to the GFP control) (Fig. 18 C). It has been previously reported that the cardiomyocyte mechanics and intracellular calcium kinetics are significantly enhanced by Hsp20. Thus, to examine if S10F substitution alters the effects of Hsp20 on cardiac contractile function, adult rat cardiomyocytes were infected with Ad. GFP, Ad. WT-Hsp20 or Ad. S10F-Hsp20 and stimulated by Grass S5 stimulator at a pacing rate of 0.5 Hz respectively.

Contractile parameters including FS%, +dL/dt and −dL/dt were monitored using video edge detection. As shown in Figure 19, the amplitude of basal cell contraction (FS%) was significantly increased in cardiomyocytes infected with Ad.WT-Hsp20 compared to GFP control cardiomyocytes (Fig. 19 A, B), which is consistent with our previously published data. However, the enhancing effects on contractility were completely abolished in S10F-Hsp20 cardiomyocytes, which showed similar value of FS% to the GFP cardiomyocytes. In addition, the maximal velocities of shortening and re-lengthening were also considerably reduced in cardiomyocytes overexpressing the mutant Hsp20 than those in the WT-Hsp20 cells (Fig. 19 C, D).

To determine whether the observed alterations in myocyte mechanics reflected similar alterations in Ca\(^{2+}\) handling, intracellular Ca\(^{2+}\) transients in infected cardiomyocytes were measured by use of the Fura-2 AM fluorescence indicator (2 µM). Consistent with the contractility data, our results showed that the amplitude of Ca\(^{2+}\) transients was significantly reduced in mutant-Hsp20 cells
Figure 18. Adenoviral-mediated expression of WT-Hsp20 (WT) and S10F-Hsp20 (S10F) in adult rat cardiomyocytes. A. Cardiomyocytes were infected with Ad.WT-Hsp20 and Ad.S10F-Hsp20. The Ad.GFP vector was used as a control. Images were captured, using the 20X magnification objective. Fluorescent image of adult rat cardiomyocytes at 48 hours post-infection, showing GFP expression, indicates ~100% infectivity. The scale bar represents 100 μm. B. Representative Western blot of Hsp20 expression at 48 hours post-infection. C. Quantification of the relative overexpression of WT- and S10F-Hsp20, normalized to calsequestrin (CSQ), revealed a 3.5-fold increase in both groups, as compared to the GFP control. Values represent mean ± SEM obtained from 4 independent cardiomyocyte lysates; *P<0.001 vs. GFP.
compared to WT-Hsp20 cardiomyocytes (Fig. 20 A, B). Accordingly, the time to 50% decay of the Ca\(^{2+}\) signal was found to be significantly prolonged in cardiomyocytes overexpressing mutant Hsp20, compared to the WT-Hsp20 cells (Fig. 20 C). Thus, these experiments indicate that the inotropic effects of Hsp20 in cardiomyocyte are completely abolished by the S10F mutation.

As the anti-apoptotic effects of Hsp20 in the heart are now well-recognized, it was critical for us to examine whether the mutant Hsp20 protein could elicit any potential alterations on programmed cell death. Thus, the extent of apoptosis was determined in the GFP, WT-Hsp20 and S10F-Hsp20 infected cardiomyocytes. We first determined the effect of the S10F substitution on basal levels of apoptosis by using Hoechst staining (Hoechst 33342) to visualize nuclei. Cells undergoing apoptosis have pyknotic nuclei, which look like round, while longitudinal nuclei are indicative of non-apoptotic cells. As demonstrated in Figure 21, no differences were detected between the three groups at basal levels. As Hsp20 has been previously shown to be cardioprotective against β-agonist induced apoptosis\(^{185, 186}\), infected myocytes were subjected to isoproterenol (10 µM) treatment for 24 hours, as described in Methods section, to assess any potential alterations caused by the S10F mutation on the beneficial effects of Hsp20. It was found that the percentage of pyknotic nuclei was significantly increased in the Ad.GFP-infected cells, following Iso treatment (Fig. 21). Nevertheless, the percentage of pyknotic nuclei in the WT-Hsp20 group was considerably lower than the GFP group after Iso treatment (Fig. 21), which was consistent with the cardioprotective effects of Hsp20.\(^{203}\) Interestingly, the mutant
FIGURE 19

A

Cell length (μm)

GFP  WT  S10F

B

FS%

GFP  WT  S10F

C

+dl/dt (μm/s)

GFP  WT  S10F

D

-dl/dt (μm/s)

GFP  WT  S10F
Figure 19. Contractile parameters in Ad.GFP, Ad.WT-Hsp20 and Ad. S10F-Hsp20 infected cardiomyocytes. After 24-h incubation with adenoviruses, maximum rates of contraction and relaxation in cardiomyocytes were measured. A, Representative cell-shortening traces of Ad.GFP, Ad.WT-Hsp20 and Ad. S10F-Hsp20 cardiomyocytes; B, Fractional shortening (FS%); C, Maximum rates of contraction (+dL/dt); D, Maximum rates of relengthening (-dL/dt); n=20~25 cells per experiment; each experiment was repeated at least four times; *p<0.05, vs. GFP; #p<0.05, vs. WT. Values are mean ± SE. (Data generated by collaborator Dr. Wen Zhao)
FIGURE 20

A

Ca Transient Amplitude (Fura2 ratio)

B

Ca Transient Peak (Fura 2 Ratio)

C

Tau

GFP WT S10F

GFP WT S10F

* #
Figure 20. Ca$^{2+}$ kinetics in Ad.GFP, Ad.WT-Hsp20 and Ad. S10F-Hsp20 cardiomyocytes. Infected myocytes were incubated with Fura2-AM for half an hour and Ca$^{2+}$ transients were measured. A, Representative tracings of Ca$^{2+}$ transients; B, Ca$^{2+}$ transient amplitude in infected cardiomyocytes; C, Time to 50% decay (T50) of Ca$^{2+}$ signal; n=20~25 cells per experiment; each experiment was repeated at least 3 times; *p<0.05, vs. GFP; #p<0.05, vs. WT. Values are mean ± SE. (Data generated by collaborator Dr. Wen Zhao)
S10F-Hsp20 showed no cardioprotection after Iso treatment compared to the WT-Hsp20 group. Thus, these results showed that the anti-apoptotic effect of Hsp20 is completely eliminated by the S10F mutation.

II.4 In vivo functional significance of S10F-Hsp20 in cardiac function and protection

II.4.A Transgenic mice with cardiac-specific overexpression of S10F-Hsp20

The findings from in vitro studies above suggested that both inotropic and protective effects of Hsp20 on the heart are abrogated by S10F substitution. To determine the in vivo functional significance of this mutation, we generated transgenic mice that carried the mouse cardiac Hsp20 cDNA, in which TCT encoding for Ser10 was substituted with TTT encoding for Phenylalanine (Fig. 22 A). To verify whether the S10F-Hsp20 cDNA was present in the mouse genome, PCR amplification of genomic DNA, followed by DNA sequencing was performed. This confirmed that the TCT codon, encoding Ser10, was mutated into TTT, which encoded the Phenylalanine residue (Fig. 22 B). Western blot analysis revealed that multiple transgenic lines of S10F-Hsp20 were identified and one line with 10 Fold overexpression of Hsp20, which is similar to the previously published WT-Hsp20 transgenic model was chosen for further characterization studies.
Figure 21. Isolated cardiomyocytes infected with Ad.GFP, Ad.WT-Hsp20 and Ad.
S10F-Hsp20 were subjected to isoproterenol (10 μM/L) treatment for 24 hours. A.
Cells were fixed and stained with Hoechst 33343 to detect nuclei (magnification
300X); B Pyknotic nuclei were counted and expressed as the percentage of total
nuclei. Values represent means ± SEM; n=20~25 cells per experiment; each
experiment was repeated 4 times. *: p<0.05, vs. GFP basal; #: p<0.05, vs. basal
in it’s respective group. $: p<0.05, vs. GFP Iso.
FIGURE 22

A

\[\text{ATGGAGATCCCGTGCCTGTGACGCCTTTTTAG}^{\text{Ser}} \quad \text{ATGGAGATCCCGTGCCTGTGACGCCTTTTTAG}^{\text{Phe}}\]

B

Ser10-Hsp20

\[\begin{array}{cccc}
G & C & A & G \\
T & T & T & T
\end{array}\]

Phe10-Hsp20

\[\begin{array}{cccc}
G & C & A & G \\
T & T & T & T
\end{array}\]
**Figure 22.** Generation of S10F-Hsp20 transgenic mouse model. A) Diagram of S10F-Hsp20 TG construct. The mutant mouse Hsp20 cDNA, in which Serine 10 encoded by codon TCT was mutated into TTT (encoding phenylalanine), was driven by the a-myosin heavy chain promoter (a -MHCp); B) DNA sequencing of PCR products from S10F-Hsp20 mouse genomic DNA confirmed the TCT to TTT mutation.
II.4.B Inotropic effects of Hsp20 are negated in cardiomyocytes from S10F-Hsp20 TG hearts

It has been shown that WT-Hsp20 overexpression in transgenic mouse hearts resulted in significant enhancement of contractility, augmented Ca\textsuperscript{2+} cycling and SR Ca\textsuperscript{2+} load in isolated cardiomyocytes.\textsuperscript{87, 101} To determine the effects of S10F-Hsp20 overexpression on cardiac contractile function, mechanical parameters were further assessed in isolated cardiomyocytes from non-transgenic (NTG) and S10F-Hsp20 transgenic hearts, which represent a load-independent preparation. In contrast to the enhanced contractility in WT-Hsp20 overexpressing cardiomyocytes\textsuperscript{87, 101}, the contractile parameters including FS\%, +dL/dt and -dL/dt were not altered in S10F-Hsp20 cardiomyocytes compared with the NTG cells (Fig. 23). To examine the effects of S10F-Hsp20 overexpression on SR Ca\textsuperscript{2+} cycling, isolated cardiomyocytes were loaded with Fura-2, and intracellular Ca\textsuperscript{2+} transient amplitude and Ca\textsuperscript{2+} kinetics were measured at 0.5 Hz. It was observed that the Ca\textsuperscript{2+} transient peak in S10F-Hsp20 cells was similar to the NTG group (Fig. 24 A, B). In addition, the time constant to 50% of Ca\textsuperscript{2+} transient decay (T50) was not changed in S10F-Hsp20 cells compared to the NTG group (Fig. 24 C). Again, consistent with the \textit{in vitro} findings, these data indicated that the cardiac contractile enhancing effects of Hsp20 were completely abrogated by S10F substitution.
FIGURE 23

A

Cell length (µm)

NTG  S10F

110
106
102
98

B

FS%

NTG  S10F

0  2  4  6  8  10

C

+dl/dt (µm/s)

NTG  S10F

0  15  30  45  60  75

D

-dl/dt (µm/s)

NTG  S10F

0  15  30  45  60  75
Figure 23. Mechanics of isolated non-transgenic (NTG) and S10F-Hsp20 TG cardiomyocytes. Isolated myocytes from 8 weeks old hearts were suspended in 1.8 mM Ca-Tyrode solution and field-stimulated at 0.5 Hz. A, Representative cell shortening traces of NTG and S10F-Hsp20 cells; B, Fractional shortening (FS); C, Maximum rates of contraction (+dL/dt); D, Maximum rates of relaxation (-dL/dt). 36~42 cells from 5 hearts for each group. Values represent means ± SEM.
FIGURE 24

A

Ca Transient Amplitude
(Fura2 ratio)

NTG
S10F

B

Ca Transient Peak
(Fura 2 Ratio)

NTG
S10F

C

T50 (sec)

NTG
S10F
Figure 24. Isolated mouse cardiac myocytes were stimulated at 0.5 Hz to obtain the Ca\textsuperscript{2+} transients. A, Representative tracings of Ca\textsuperscript{2+} transients in NTG and S10F-Hsp20 TG myocytes; B, Ca\textsuperscript{2+} transient amplitude in NTG and S10F-Hsp20 TG myocytes; C, Time to 50% of Ca\textsuperscript{2+} decay in NTG and S10F-Hsp20 TG myocytes. T50: time to 50% decay of Ca\textsuperscript{2+} transient. 30-45 cells from 3 hearts for each group. Values represent means ± SEM.
II. 4. C Effects of S10F-Hsp20 on cardiac function under the pathological stress of ischemia/reperfusion injury

II.4.C.1 Impaired functional recovery in S10F-Hsp20 hearts during ex vivo ischemia/reperfusion injury

Myocardial ischemia/reperfusion (I/R) injury is known as a primary cause of morbidity and mortality associated with coronary artery disease. Recent studies have shown that ischemia, followed by reperfusion activates the cascade of necrosis, apoptosis which contributes to ventricular dysfunction and end-stage failure.

Recently, studies from us and other laboratories revealed that Hsp20 and its subsequent phosphorylation can protect the heart against I/R injury, hypertrophic remodelling and stress-induced apoptosis. Previous studies found that the expression of myocardial Hsp20 was significantly upregulated upon I/R injury. It was later reported that overexpression of Hsp20 in mouse heart improved cardiac function recovery and promoted cardiomyocytes survival after I/R. Consistently, knockdown of Hsp20 in the mouse heart led to increased infarct size after I/R, which further supported the protective role of Hsp20 against I/R injury. Also, it was discovered that increased expression of Hsp20 was protective against β-agonist and I/R-induced apoptosis. In addition, Hsp20 has been shown to attenuate cardiac hypertrophy and prevent apoptosis and fibrosis, which delays the progression of Isoproterenol (Iso)-mediated heart failure.

To determine if these beneficial effects of Hsp20 on the heart, described above, are modified by S10F substitution, S10F-Hsp20 transgenic hearts were
isolated and subjected to ex vivo 45 minutes of no-flow global ischemia, followed by 1 hour of reperfusion, with NTG hearts as controls. It was found that under basal conditions, there were no differences in +dP/dt, -dP/dt and LVDP between the S10F-Hsp20 and NTG hearts (Fig. 25). More importantly, after 45 minutes ischemia, functional recovery of S10F-Hsp20 hearts was even worse than that of the NTG hearts, as determined by depressed +dP/dt (34% versus 60%; Fig. 25 A), -dP/dt (30% versus 50%; Fig. 25 B), LVDP (31% versus 50%; Fig. 25 C) and elevated end diastolic pressure (EDP) (51 mmHg versus 39 mmHg; Fig. 25 D). These results are in contrast to the beneficial effects of WT-Hsp20 overexpression, which improved the functional recovery upon I/R. Taken together, these data suggest that the protective effects of Hsp20 against I/R injury are abrogated by the S10F mutation.

II.4.C.2 Increased cardiomyocyte apoptosis in S10F-Hsp20 TG hearts under basal and I/R injury conditions

It is well recognized that preserving an adequate number of cardiomyocytes is crucial to the preservation of structural integrity and cardiac contractile function following ischemia/reperfusion. As stated above, Hsp20 is able to protect against β-agonist and I/R-induced apoptosis. To determine if these effects are modified by the S10F mutation, the extent of apoptotic cell death was examined in S10F-Hsp20 and NTG hearts under basal and I/R conditions by TUNEL staining assay. It was found that, even under basal condition, the number of TUNEL-positive nuclei was significantly increased in S10F hearts compared to the NTG hearts (0.38% versus 0.26%) (Fig. 26). Moreover, upon I/R injury, the
Figure 25. S10F-Hsp20 hearts are more sensitive to ischemia/reperfusion injury, evidenced by lower recovery of contractile function. After 20 minutes of stabilization, hearts were subjected to 45 minutes of global ischemia and 1 hour of reperfusion. During reperfusion, recovery was significantly depressed in S10F-Hsp20 hearts compared with NTG hearts, as evidenced by ±dP/dt (A and B), LV developed pressure (LVDP) (C) and end diastolic pressure (EDP) (D). Values represent means ± SEM; *: p<0.05, vs S10F; n=8 hearts for NTG and 9 hearts for S10F.
number of TUNEL-positive nuclei in S10F-Hsp20 hearts was 2.6-fold higher than that in control hearts (5.73% versus 2.19%) (Fig. 26). These results indicate that overexpression of S10F-Hsp20 promotes cellular disruption in the myocardium under basal and ischemia/reperfusion-induced stress conditions and suggest that the anti-apoptotic effects of Hsp20 are totally negated by the S10F human mutation.
FIGURE 26

(A)

NTG  S10F

Basal

I/R

(B)

TUNEL Positive Nuclei (% of Total)

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<th>I/R</th>
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<tr>
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<td>1.5</td>
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<tr>
<td>S10F</td>
<td>0.3</td>
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* p < 0.05
# p < 0.01
**Figure 26.** Increased cardiomyocyte apoptosis in S10F-Hsp20 TG hearts under basal and I/R injury conditions. A, Triple-staining with anti-sarcomeric actin antibody (red, to reveal cardiomyocyte), DAPI (blue, to reveal all nuclei), and TUNEL (bright green, to determine apoptosis) in NTG or S10F-Hsp20 TG cardiomyocytes under basal and I/R injury conditions (arrows indicate TUNEL-positive nuclei); B, quantitative analysis of the numbers of TUNEL-positive nuclei in NTG and S10F TG hearts under basal and I/R conditions. Values represent mean ± SEM; *: p<0.05, vs NTG; #: p<0.05, vs. NTG; ; n=3 hearts for NTG and 3 hearts for S10F, with 3 sections from each heart.
II.4.D Effects of S10F-Hsp20 on cardiac function after myocardial infarction (MI) in vivo

To determine the functional significance of mutant Hsp20 on the progression of cardiac remodeling and heart failure, transgenic mice carrying S10F-Hsp20 mutant were subjected to chronic left coronary artery ligation surgery to induce heart failure, along with NTG mice as controls. As shown in Figure 27, ligation was first performed on spot ‘a’ of the main left coronary artery (LCA), which resulted in infarction of the LV wall, involving around 50% of LV circumference. It was found that, with this infarct size, 10 out of 16 (62%) NTG mice and all of 14 (100%) S10F-Hsp20 mice died in the first 24 hours after surgery (Fig. 27 B). Thus, the LCA ligation site was switched to spot ‘b’, which led to approximately 30% infarct size and it was observed that 4 out of 13 (30%) NTG mice and 11 out of 14 (79%) S10F-Hsp20 mice died 24 hours post surgery (Fig. 27 C). To further reduce the mortality rate in the S10F-Hsp20 group after surgery, we continued to lower the LCA ligation site to spot ‘c’, which gave rise to infarcts of around 20% of the LV circumference, named mild myocardial infarction (MI). By performing this mild MI, only 1 out of 9 (10%) NTG mice and 6 out of 14 (43%) S10F-Hsp20 mice died within the first 24 hours after surgery (Fig. 27 D). Since the survival rate in the S10F TG mice was significantly improved with 20% infarct size, compared to those with infarcts of 30% and 50% respectively, these mice with mild MI were used (from both NTG and S10F TG) for further studies as described below. In parallel, 10 NTG and 9 S10F-Hsp20 TG mice were subjected to sham surgery (experienced the same surgery except that the LCA was not occluded).
C

Survival of 30% infarct size

- NTG
- S10F

D

Survival of 20% infarct size

- NTG
- S10F
**Figure 27.** Myocardial infarction surgery was performed on the S10F-Hsp20 TG mice, with NTG mice as controls. A, LAD ligation sites a, b, c and survival curves for the NTG and S10F TG mice with 50% (B), 30% (C) and 20% (D) infarct size in the first 24 hours post surgery.
There were no animal deaths observed in either NTG or S10F TG mice in the first 24 hours after sham surgery.

**II.4.D.1 Depressed cardiac contractility in S10F-Hsp20 hearts under stress of mild chronic effects of ischemia injury**

After infarction or sham surgery, M-mode echocardiography were performed to monitor cardiac function in NTG and S10F TG mice at 1, 2, 3, 4, 6, 8, 10, and 12 weeks post-infarction. Among all indices, LV fractional shortening (FS%) and ejection fraction (EF%) are the most commonly used parameters to determine LV systolic performance. As shown in Figure 28, no differences in FS% and EF% were observed between NTG sham and S10F-Hsp20 sham mice at different time points post surgery. However, it was noted that starting at 3 weeks post mild MI surgery, EF% (S10F 46.1% versus NTG 56.5%) and FS% (S10F 22.9% versus NTG 30%) were significantly reduced in S10F-Hsp20 hearts, compared to the NTG hearts (Fig. 28). Particularly, at 12 weeks, cardiac EF% and FS% in S10F-Hsp20 infarcted mice were reduced by 43% and 50%, respectively, compared to S10F-Hsp20 sham mice (Fig. 28). However, no difference in cardiac contractility (both EF% and FS%) was observed between NTG infarcted hearts and NTG sham hearts at 12 weeks post surgery (Fig. 28). These data suggest that mild chronic ischemic injury results in significantly depressed *in vivo* function of S10F-Hsp20 hearts, compared to NTG hearts.
Figure 28. Depressed cardiac contractility in S10F-Hsp20 TG hearts under the stress of chronic ischemic injury. Starting at 3 weeks post MI surgery (mild), EF% (A) and FS% (B) were significantly reduced in S10F-Hsp20 hearts compared to NTG hearts. Values represent means ± SEM; *: p<0.05 vs NTG; n=5 for NTG and 5 for S10F TG mice.
II.4.D.2 Increased cardiac remodeling in S10F-Hsp20 hearts under the stress of chronic ischemic injury

Starting in the first week post mild MI surgery, significant cardiac dilation was observed in the S10F-Hsp20 TG mice, as evidenced by increased LV end systolic volume (LVESV) (S10F 45.3 µl versus NTG 31.8 µl), LV end diastolic volume (LVEDV) (S10F 94.5 µl versus NTG 77.2 µl), LV end systolic dimension (LVESD) (S10F 3.3 mm versus NTG 2.8 mm) and LV end diastolic dimension (LVEDD) (S10F 4.5 mm versus NTG 4.1 mm) compared to the NTG infarcted hearts (Fig. 29). Notably, the LVESV and LVEDV in S10F-Hsp20 infarcted hearts were increased by 83% and 72% respectively, compared to the S10F-Hsp20 sham hearts at 12 weeks post surgery (Fig. 29). Nevertheless, no difference in LVESV and LVEDV between NTG infarcted hearts and NTG sham hearts were found at 12 weeks after surgery (Fig. 29). In addition, no alterations in LV end systolic/diastolic volumes or left ventricle chamber size were discovered between NTG sham hearts and S10F-Hsp20 sham hearts at different time points post surgery (Fig. 29). After completion of functional measurements at 12 weeks post surgery, the animals were anesthetized and sacrificed. Body weight, heart weight and lung weight were then obtained. When the ratios of heart weight over body weight (HW/BW) or lung weight over body weight (LW/BW) were analyzed, there were no differences detected between NTG sham and S10F-Hsp20 sham mice (Fig. 30 A, B). However, for animals that underwent infarction surgeries, the ratios of HW/BW and LW/BW were found to be increased by 36% and 19%, respectively, in the S10F-Hsp20 mice Fig. 30 A, B). There were, no significant
differences in the ratios of HW/BW and LW/BW between the NTG infarcted mice and NTG sham mice (Fig. 30). These data suggest that overexpression of S10F-Hsp20 accelerates the onset and progression of cardiac remodeling and heart failure under the stress of chronic ischemic injury. Overall, the depressed contractility and increased hypertrophy and remodeling in the S10F-Hsp20 hearts after mild chronic MI suggest that the protective effects of WT-Hsp20 against ischemia injury were entirely abrogated by S10F human mutation. In addition, this mutation accelerated cardiac remodeling and heart failure development.
**FIGURE 29**

**A**

- **LVESV (ul)**
- Lines represent different groups:
  - Green: NTG-Sham
  - Brown: S10F-Sham
  - Blue: NTG-MI
  - Purple: S10F-MI
- **X-axis:** Time points (1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 8 weeks, 10 weeks, 12 weeks)
- **Y-axis:** LVESV (ul)
- Significant differences indicated by asterisks (*)

**B**

- **LVEDV (ul)**
- Same groups as in A
- **X-axis:** Time points (1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 8 weeks, 10 weeks, 12 weeks)
- **Y-axis:** LVEDV (ul)
- Significant differences indicated by asterisks (*)
Figure 29. Increased cardiac hypertrophic remodeling in S10F-Hsp20 hearts under the stress of chronic ischemic injury. Compared to NTG mice, cardiac dilation parameters including LVESV (A), LVEDV(B), LVESD (C) and LVEDD (D) were considerably increased in the S10F-Hsp20 TG mice after MI surgery. Values represent means ± SEM; *: p<0.05 vs NTG; n=5 for NTG and 5 for S10F TG mice.
**Figure 30.** Heart weight and lung weight were significantly increased in the S10F-Hsp20 TG mice after mild chronic MI surgery. After measurements of cardiac function at 12 weeks post surgery, the animals were sacrificed and the ratios of HW/BW (A) and LW/BW (B) were obtained. Values represent mean ± SEM; *: p<0.05, vs NTG, n=5 for NTG, and 4 for S10F TG mice.
II.4.E Effects of S10F-Hsp20 on cardiac function under the physiological stress of aging

As shown in the *in vitro* and *in vivo* studies above, the enhanced contractile effects as well as the cardioprotective effects of WT-Hsp20 in the heart were abolished by the S10F mutation. Since loss of cardioprotection and chaperone-like activity by the Hsp20 mutant may result in remodeling under the physiological stress of aging, it was important to extend these studies to aging mice. Therefore, starting at 2 months, echocardiography was used to assess integrated LV performance and geometry in S10F hearts at bimonthly intervals. NTG mice were used as controls.

II.4.E.1 Depressed cardiac contractility in S10F-Hsp20 hearts under the physiological stress of aging

In the aging studies, it was observed that FS% and EF% were not altered in S10F-Hsp20 hearts at the age of 2 months and 4 months, compared to those of NTG hearts (Fig. 31). However, starting from the 6th month, EF% (S10F 48.9% versus NTG 54%) and FS% (S10F 24% versus NTG 28%) were found to be significantly decreased in the S10F-Hsp20 TG hearts, compared to NTG hearts (Fig. 31). Particularly, at the age of 16 months, cardiac EF% and FS% in S10F TG mice were reduced by 49% and 43%, respectively, compared to the NTG mice (Fig. 31). These data suggest that *in vivo* function of the S10F-Hsp20 hearts was significantly depressed under physiological stress of aging, compared to the NTG hearts.
Figure 31. Cardiac contractility was significantly decreased in the aging S10F-Hsp20 mice, as evidenced by reduced EF% (A) and FS% (B). Values represent means ± SEM; *: p<0.05, vs NTG, n=6 mice for NTG, and 5 mice for S10F.
II.4.E.2 Increased cardiac remodeling in S10F-Hsp20 hearts under the physiological stress of aging

Moreover, as stated above, using echocardiography, LV end systolic/diastolic volumes or left ventricle chamber size were also measured in S10F-Hsp20 and NTG mice along with aging. At 2, 4, 6 and 8 months of age, no differences in parameters such as LVESV, LVESD, LVEDV and LVEDD were observed between NTG and S10F-Hsp20 mice (Fig 32). However, starting from the 10th month, significant cardiac dilation was pronounced in the S10F-Hsp20 mice, as evidenced by increased LVESV (S10F 58 µl versus NTG 41 µl), LVEDV (S10F 104 µl versus NTG 91 µl) and LVESD (S10F 3.2 mm versus NTG 3 mm) compared to the NTG hearts (Fig. 32). Particularly, at ages of 16 months, the LVESV, LVEDV, LVESD and LVEDD in S10F-Hsp20 hearts were found to be increased by 99%, 33%, 44% and 18% respectively compared to those in the NTG hearts (Fig. 32 A, B, C, D). Moreover, LV internal diameter end systole (LVIDs) and end diastole (LVIDd), interventricular septal end systole (IVSs) and end diastole (IVSd) were also augmented by 36%, 20%, 40% and 53% in the S10F hearts respectively compared to the NTG hearts at ages of 16 months (Fig. 32 E, F, G, H). These data exhibited above suggest that overexpression of S10F-Hsp20 accelerates the onset and progression of cardiac remodeling and heart failure under the physiological stress of aging.
**Figure 32.** Cardiac hypertrophic remodeling was greatly increased in S10F-Hsp20 aging mice. Cardiac remodeling parameters including LVESV (A), LVEDV (B), LVESD (C), LVEDD (D), LVIDs (E), LVIDd (F), IVSs (G) and IVSd (H) were significantly augmented in S10F-Hsp20 aging mice, compared to the NTG mice. Values represent means ± SEM; *: p<0.05, vs NTG, n=6 for NTG, and 5 for S10F TG mice.
II.4.E.3 Increased heart weight and lung weight in S10F aging hearts

After completion the measurement of cardiac function, animal body weight, heart weight as well as lung weight were obtained from NTG and S10F mice at 2, 8 and 14 months of age, followed with analysis of ratios of HW/BW or LW/BW. As shown in Figure 33, at the ages of 2 months and 8 months, there were no differences in HW/BW and LW/BW between NTG and S10F-Hsp20 mice. However, at 14 months of age, the ratios of HW/BW and LW/BW were found to be increased by 67% and 38%, respectively, in the S10F-Hsp20 mice compared to the NTG mice (Fig. 33 B, C).

II.4.E.4 Increased cardiac cell size, expression of atrial natriuretic peptide (ANP) and decreased expression of SERCA2a in S10F aging hearts

To determine cardiac hypertrophy and remodeling at the cellular level, heart sections from NTG and S10F TG mice were stained with a fluorescence Oregon Green 488–labeled wheat germ agglutinin and myocyte cross-sectional area was measured, as described in the method section. As shown in Figure 34, at ages of 2 months and 8 months, cardiomyocyte size was not altered in S10F hearts, compared to those of NTG hearts. However, at 14 months of age, consistent with increased HW/BW ratio in S10F-Hsp20 mice, cardiomyocyte size was increased by 61% compared to the NTG cardiomyocyte (Fig. 34 A, B). Importantly, the mRNA level of the hypertrophy marker atrial natriuretic peptide (ANP) was found to be increased by 3 fold in S10F hearts, compared to the NTG hearts at 14 months of age (Fig. 35 A). No difference in the expression of this marker was noted between NTG and S10F hearts at 2 months and 8 months of age,
FIGURE 33

A

B

C

Heart Weight/Body Weight ($\times 10^{-3}$)

Lung Weight/Body Weight ($\times 10^{-2}$)

2 months  8 months  14 months

NTG  S10F  NTG  S10F  NTG  S10F

*
Figure 33. Increased heart weight and lung weight in S10F aging hearts. A, H&E staining of the mouse hearts from NTG and S10F-Hsp20 mice at ages of 2, 8, and 14 months; significant increases in ratios of HW/BW (B) and LW/BW (B) in 14 months old S10F TG mice compared to the NTG mice. Values represent means ± SEM; *: p<0.05, vs NTG, n=6 for NTG and S10F TG mice.
FIGURE 34

A

2 months   8 months   14 months

NTG  

S10F  

B

Cardiomyocyte Cross-Sectional Area (µM)

NTG  

S10F  

2 months   8 months   14 months

*
Figure 34. Cardiomyocyte size was found to be significantly increased in aging S10F-Hsp20 hearts. A, Immunostaining of heart sections from 2, 8 and 14 month-old NTG and S10F-Hsp20 TG mice with fluorescence labeled wheat germ agglutinin; B, individual myocyte size was assessed using ImageJ (National Institutes of Health). The mean myocyte area was evaluated by measurement of 300 cells per heart. Values represent means ± SEM;*: p<0.05, vs NTG; n=4 hearts for NTG and 4 hearts for S10F, 3 sections from each heart.
respectively (Fig. 35 A). Over the last decade or more, reduced expression of SERCA2a has been widely recognized as a hallmark of late stage heart failure.\textsuperscript{206} We therefore, determined the mRNA levels of SERCA2a in the aging S10F TG hearts, with NTG hearts as controls. As shown in Figure 35B, at ages of 2 months and 8 months, expression of SERCA2a was not altered in the S10F TG hearts in comparison with the NTG hearts. However, at 14 months of age, SERCA2a mRNA level was reduced by 30% compared to the NTG hearts (Fig. 35 B). These data further support that S10F-Hsp20 overexpression accelerates the onset and progression of heart failure during the aging process.

**II.4.E.5 Increased cardiomyocyte apoptosis in the aging S10F-Hsp20 TG hearts**

As described above the number of cardiomyocytes that underwent apoptosis was significantly increased in S10F hearts compared to the NTG hearts, even under basal conditions. To determine the effects of S10F-Hsp20 overexpression on cardiomyocyte survival under the physiological stress of aging, heart sections from the 2, 8 and 14 months old S10F TG mice were subjected to TUNEL staining assay, a photometric enzyme immunoassay for the quantitative determination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) of programmed cell death. NTG heart sections from similar ages were used as controls. It was found that, consistent with the data shown in the I/R studies at age of 2 months, the number of TUNEL-positive nuclei was increased by 46% in S10F hearts compared to the NTG hearts (Fig. 36). At ages of 8 and 14 months, the numbers of TUNEL-
FIGURE 35

A

![Chart A showing ANP mRNA levels for NTG and S10F groups at 2, 8, and 14 months.](image)

B

![Chart B showing SERCA2a mRNA levels at 2, 8, and 14 months.](image)
Figure 35. Increased cardiac expression of atrial natriuretic peptide (ANP) and decreased expression of SERCA2a in S10F aging hearts. Ventricular mRNA levels of ANP (A) and SERCA2a (B) in 2, 8, and 14 month-old NTG and S10F mice. Values represent means ± SEM; *: p<0.05, vs NTG; n=4 hearts for NTG and 4 hearts for S10F.
FIGURE 36

A

2 months  8 months  14 months

NTG

S10F

B

TUNEL Positive Nuclei (% of Total)

2 months  8 months  14 months

NTG  S10F
**Figure 36.** Increased cardiomyocyte apoptosis in aging S10F-Hsp20 TG hearts. A, Triple-staining with an anti-sarcomeric actin antibody (red, to reveal cardiomyocyte), DAPI (blue, to reveal all nuclei) and TUNEL (bright green, to determine apoptosis) in NTG or S10F-Hsp20 TG cardiomyocytes at 2, 8 and 14 months of age (arrows indicate TUNEL-positive nuclei); B, quantitative analysis of the numbers of TUNEL-positive nuclei in NTG and S10F TG hearts at 2, 8 and 14 months of age. Values represent means ± SEM; *: p<0.05, vs NTG; n=4 hearts for NTG and 4 hearts for S10F, with 3 sections from each heart.
positive nuclei in S10F-Hsp20 TG hearts were increased by 97% and 118%, respectively, compared to those in the NTG hearts (Fig. 36).

II.4.E.6 Increased interstitial fibrosis in the S10F-Hsp20 TG hearts under physiological stress of aging

As shown above, S10F-Hsp20 hearts developed significant cardiac hypertrophy and remodeling upon aging. Thus, we assessed the existence of fibrosis in these transgenic hearts, using Masson’s Trichrome staining of histological sections, using NTG heart sections as controls. Interstitial cardiac fibrosis serves as an important feature of cardiac remodeling. It reduces ventricle compliance, hampers oxygen diffusion to cardiomyocytes and alters the transmission of force from individual cells to global chamber contraction, all of which decrease the cardiac contractile performance. Masson’s Trichrome stain shows the fibrosis areas by blue color. It was observed that in the S10F hearts, fibrosis occurred as early as 8 months of age and was mostly pronounced at 14 months of age (Fig. 37). However, in comparison, no fibrosis was found in 2, 8 and 14 months old NTG hearts (Fig. 37).

II.4.E.7 Accelerated death in S10F-Hsp20 mice upon the physiological stress of aging

As shown above, cardiac contractility was severely depressed and remodeling was considerably increased in the S10F-Hsp20 TG mice upon aging. Thus, the survival rate was analyzed in aging NTG and S10F-Hsp20 mice. As shown in Figure 38, S10F-Hsp20 transgenic mice died sporadically and suddenly,
compared with their wild type littermates. Actually, the S10F-Hsp20 mice demonstrated 100% (n=21) mortality by 18 months of age, with survival declining rapidly after 11 months of age. In comparison, only 1 out 19 (5%) NTG mice died (Fig. 38) during that time-course. About 33% of the TG mice died at 14 months of age and 76% of the TG mice died at 16 months of age. The mean life span of S10F-Hsp20 mice was found to be 13.7 months, which was significantly shorter than the mean life span of 19.7 months in NTG mice.
FIGURE 37

<table>
<thead>
<tr>
<th></th>
<th>2 months</th>
<th>8 months</th>
<th>14 months</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<td><img src="S10F_8_months.jpg" alt="Image" /></td>
<td><img src="S10F_14_months.jpg" alt="Image" /></td>
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</table>
**Figure 37.** Representative sections of Masson’s Trichrome stained cardiac sections from 2, 8, and 14 month old NTG and S10F-Hsp20 TG mice. Interstitial fibrosis stained in blue was observed in myocardium from 8 and 14 month old S10F TG hearts, but not in NTG hearts.
Figure 38

Survival of NTG and S10F TG mice

Percent survival

Time (months)
**Figure 38.** Accelerated mortality in S10F TG mice. Percentage of survival rate was plotted against age (in-months) for S10F-Hsp20 TG and NTG mice (n=19 for NTG and 21 for S10F TG mice). All S10F TG mice died by 18 months of age while only 1 NTG mouse died. The mean life span was 13.7 months for S10F-Hsp20 mice and 19.7 months for NTG mice.
II. 5 Mechanisms of the effects of S10F-Hsp20 on cardiac function and protection

As stated above, studies in cardiac muscle revealed that overexpression of Hsp20 protected the heart against stress-induced apoptosis, hypertrophic remodeling and I/R injury. The underlying mechanisms involved the interaction of Hsp20 with Bax and prevention of its translocation to mitochondria, which subsequently blocked the initiation of apoptosis by suppression of the caspase-3-mediated apoptotic pathway. Also, Hsp20 can protect against doxorubicin-induced cardiomyopathy by interacting with Akt, thereby maintaining Akt activity, which retains the phosphorylation of Bad and thus represses caspase-3 mediated apoptosis. Moreover, Hsp20 was reported to inhibit the activation of p38 and SAPK/JNK by suppressing the ASK1 activity through its association with ASK1, and thereby attenuating β-agonist-induced cardiac remodeling and apoptosis. In addition, Hsp20 was found to translocate to the actin filament in cardiomyocytes under stress conditions, which stabilizes the cytoskeleton and protects from further damage. Besides, as described above, overexpression of Hsp20 in the mouse heart improved cardiac functional recovery and promoted cardiomyocytes' survival after I/R. It was later discovered that phosphorylated Hsp20 enhanced protection against I/R-induced damage by associating with autophagy-related Beclin1 and thus preserving autophagy activity, a physiological catabolic process whereby eukaryotic cells degrade and remove damaged proteins and organelles.

Recent studies from our lab indicate that the salutary effects of Hsp20 are
not limited to cardioprotection. We found that Hsp20 is also able to enhance cardiac contractile performance. Specifically, Hsp20 suppresses PP1 activity through its direct interaction with PP1. Down-regulated activity of PP1 leads to specific increases in the phosphorylation of PLN, which relieves its suppression on SERCA2a activity and subsequently increases SR Ca^{2+} reuptake as well as Ca^{2+} cycling and thereby enhances cardiomyocyte contraction.\textsuperscript{101}

To investigate if the regulation of Hsp20 on these signaling pathways is modified by the S10F mutation, mouse hearts from 2 month old S10F and NTG mice were harvested and processed for further studies.

\textbf{II. 5.A Increased Caspase-3 activation in 2 month old S10F-Hsp20 TG mice}

Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspase-3 (CPP-32) is a critical executioner of apoptosis, as it is responsible for the proteolytic cleavage of many key apoptotic proteins, such as caspases 6 and 7. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments. In particular, elevated level of a fragment p17 of caspase-3 in the heart and the bloodstream is recognized as a sign of cardiomyopathy.\textsuperscript{175} Therefore, hearts harvested from the S10F TG and NTG mice were homogenized to determine the protein levels of cleaved p17 fragment of caspase-3 using western blots. As shown in Figure 39 (A and B), the level of p17 fragment was increased by 24% in S10F TG hearts compared to the NTG hearts. To confirm these results, caspase-3 activity was also measured in heart homogenates from S10F and NTG mice using the Caspase-3 Activity Assay Kit (Cell Signaling). Indeed, Caspase-3 activity was
FIGURE 39
Figure 39. Increased Caspase-3 activation in 2 months old S10F-Hsp20 TG mice. A, Representative immuno-bLOTS of cleaved p17 fragment of caspase-3 from NTG and S10F TG hearts, with CSQ as a control; B, quantitative analysis of the levels of cleaved p17 fragment of caspase-3; C, caspase-3 activity measured in heart homogenates from S10F TG and NTG hearts using Caspase-3 Activity Assay Kit. Values represent means ± SEM; *: p<0.05, vs NTG; n=7 hearts for NTG and 7 hearts for S10F.
augmented by 34% in S10F-Hsp20 hearts compared to the NTG hearts (Figure 39C).

II. 5.B Increased Caspase-3 activation is associated with reduced interaction of Hsp20 with Bax in S10F TG hearts

To elucidate the potential mechanisms behind increased cardiomyocyte apoptosis in the S10F TG hearts, we first assessed the cardiac protein levels of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic), since alterations in these proteins have been reported in cardiomyocytes undergoing apoptosis.\textsuperscript{208-210} It was found that the expression level of Bax was significantly increased (by 25%), whereas the level of Bcl-2 was greatly reduced (by 52%) in 2 month old S10F TG hearts compared to NTG hearts (Fig. 40, A, B, C). When the relative ratio of Bcl-2 to Bax expression was analyzed, it was found that this ratio was reduced by 61% in S10F-Hsp20 hearts compared with the NTG hearts (Fig. 40 D).

As described above, Hsp20 was reported to interact with Bax and prevent its translocation to mitochondria, which blocks caspase-3 initiated apoptosis.\textsuperscript{183} To examine if the ability of Hsp20 to interact with Bax is compromised by the S10F mutation, heart homogenates from S10F TG and NTG mice were immunoprecipitated with anti-Hsp20. Proteins were separated on SDS-PAGE gels and probed with the anti-Bax antibody. As shown in Figure 41, the interaction of Hsp20 with Bax was considerably reduced in the S10F TG hearts, compared with NTG hearts.
FIGURE 40

A

[Image of Western blot showing bands for Bax, Bcl-2, and CSQ in NTG and S10F groups]

B

[Bar graph showing relative protein density values for Bax in NTG and S10F groups]

C

[Bar graph showing relative protein density values for Bcl-2 in NTG and S10F groups]

D

[Bar graph showing relative ratio (Bcl-2/Bax) in NTG and S10F groups]
**Figure 40.** Decreased ratio of Bcl-2/Bax in 2 month old S10F-Hsp20 TG mice. A, Representative immuno-blots of Bax and Bcl-2 in 2 month old NTG and S10F TG hearts, with CSQ as a control. B, Quantitative analysis of expression levels of Bax, Bcl-2 (C) and ratio of Bcl-2/Bax (D). Values represent means ± SEM; *: p<0.05, vs NTG; n=6 hearts for NTG and 6 hearts for S10F.
**Figure 41.** Decreased interaction of Hsp20 with Bax in 2 months old S10F-Hsp20 TG mice. Mouse heart homogenates from NTG and S10F TG hearts were immunoprecipitated with anti-Hsp20, proteins were separated on SDS-PAGE gels and probed with anti-Bax or anti-Hsp20 antibodies. A, Representative immuno-blots of Bax and Hsp20. B, Quantitative analysis of levels of Bax associated with Hsp20 and ratio of Bax/Hsp20. Values represent means ± SEM; *: p<0.05, vs NTG; n=4 hearts for NTG and 4 hearts for S10F.
II. 5.C Down-regulated Akt-Bad signaling pathway in S10F TG hearts

Hsp20 was shown to interact with phosphorylated Akt, thereby maintaining Akt activity, which retains the phosphorylation of Bad and thus represses caspase-3 mediated cardiomyocyte apoptosis. To determine if this anti-apoptotic effect of Hsp20 is altered by S10F mutation, the interaction of Hsp20 with Akt and the phosphorylation levels of Akt and Bad were examined in S10F-Hsp20 TG hearts using immunoprecipitations and western blotting, along with NTG hearts as controls. As shown in Figure 42, immunoprecipitation experiments exhibited greatly reduced interaction of Hsp20 with phosphorylated Akt in the S10F hearts compared to NTG hearts. Accordingly, phosphorylation levels of Akt and Bad were also found to be decreased by 29% and 32% respectively in S10F TG hearts in comparison with the NTG hearts (Fig. 43).

II. 5.D Impaired autophagy activity in the S10F TG hearts

Autophagy is an intracellular degradation process whereby cytosolic proteins and organelles are degraded and recycled. The primary role of autophagy is to supply nutrients for survival. In addition, in order to maintain cell function, a low level of constitutive autophagy is also critical for controlling the quality of proteins and organelles. It has been well accepted that constitutive autophagy in the heart under basal conditions is a homeostatic mechanism for preserving myocyte size, cardiac structure as well as function. Moreover, recently, more and more studies have shown that autophagy plays an important role in several types of cardiomyopathy. As stated above, Hsp20 is able to protect the heart against I/R-induced damage by preserving
FIGURE 42

A

<table>
<thead>
<tr>
<th></th>
<th>IP with Hsp20 Ab</th>
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<tbody>
<tr>
<td></td>
<td>+    -  NTG  S10F</td>
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<tr>
<td>p-Akt</td>
<td>![Image]</td>
</tr>
<tr>
<td>Hsp20</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

B

![Bar chart showing relative protein density values for p-Akt and p-Akt/Hsp20 with NTG and S10F conditions.](chart)
**Figure 42.** Decreased interaction of Hsp20 with p-Akt in 2 month old S10F-Hsp20 TG mice. Mouse heart homogenates from NTG and S10F TG hearts were immunoprecipitated with anti-Hsp20, proteins were separated on SDS-PAGE gels and probed with anti-phospho-Akt or anti-Hsp20 antibodies. A, Representative immuno-blots of phospho-Akt and Hsp20. B, Quantitative analysis of levels of phospho-Akt associated with Hsp20 and ratio of p-Akt/Hsp20. Values represent means ± SEM; *: p<0.05, vs NTG; n=4 hearts for NTG and 4 hearts for S10F.
FIGURE 43

A

<table>
<thead>
<tr>
<th></th>
<th>NTG</th>
<th>S10F</th>
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<tbody>
<tr>
<td>p-Akt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total-Akt</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

![Bar graph showing p-Akt/Total Akt comparison between NTG and S10F](image_url)
Figure 43. Decreased phosphorylation levels of Akt and Bad in 2 month old S10F-Hsp20 TG mice. A, Representative immuno-blots of phospho-Akt; B, quantitative analysis of phosphorylation level of Akt; C, representative immuno-blots of phospho-Bad; D, quantitative analysis of phosphorylation level of Bad. Values represent means ± SEM; *: p<0.05, vs NTG; n=6 hearts for NTG and 6 hearts for S10F.
autophagy activity. To investigate if this beneficial effect is modified by S10F mutation, we examined the autophagy activity in S10F-Hsp20 hearts by measurement of the microtubule-associated protein light chain 3 (LC3)-II/LC3-I ratio and Beclin 1 protein levels, using NTG hearts as controls. Alteration of cytosolic LC3-I to membrane-conjugated LC3-II is associated with the number of autophagosomes which are hallmarks of autophagic activity\textsuperscript{196}. Beclin 1, an autophagy related protein, is also a critical player in the formation of autophagosomes\textsuperscript{196}. As shown in Figure 44, the ratio of LC3-II/LC3-I was reduced by 30% in the 2 month old S10F-Hsp20 TG hearts compared to NTG hearts. Alteration (50% decrease) of another autophagy-related protein, Beclin1 (Fig. 45) paralleled the change of the LC3-II/LC3-1 ratio in S10F-Hsp20 TG hearts. Taken together, these data indicate that autophagy activity was significantly impaired in the S10F-Hsp20 hearts under baseline conditions. Since Hsp20 was reported to be able to interact with Beclin1 and thus preserve autophagy activity\textsuperscript{86}, we examined if this interaction is altered by the S10F substitution. Heart homogenates from S10F and NTG mice were immunoprecipitated with anti-Hsp20 and probed with anti-Beclin 1 antibody. As shown in the Figure 46, the interaction between Hsp20 and Beclin was greatly reduced in S10F hearts compared to the NTG hearts.

II.5.E Decreased autophagy activity is associated with increased levels of ubiquitinated protein in the 2 month old S10F-Hsp20 TG hearts

It has been demonstrated that reducing autophagy by Beclin1 haploinsufficiency induced greater accumulation of polyubiquitinated proteins in cardiomyocytes,
Figure 44. Decreased ratio of LC3-II/LC3-I in 2 months old S10F-Hsp20 TG mice. A, Representative immuno-blots of LC3-I and LC3-II, CSQ as a control; B, quantitative analysis of ratio of LC3-II/LC3-I. Values represent means ± SEM; *: p<0.05, vs NTG; n=4 hearts for NTG and 4 hearts for S10F.
**Figure 45.** Decreased level of Beclin1 in 2 months old S10F-Hsp20 TG mice. A, Representative immuno-blots of Beclin1 and Hsp20, CSQ as a control; B, quantitative analysis of protein level of Beclin1. Values represent means ± SEM; *: p<0.05, vs NTG; n=6 hearts for NTG and 6 hearts for S10F.
FIGURE 46

A

IP with Hsp20 Ab

+ - NTG S10F

Beclin

Hsp20

B

Relative Protein Density Value

NTG

S10F

Beclin

Beclin/Hsp20
**Figure 46.** Decreased interaction of Hsp20 with Beclin1 in 2 month old S10F-Hsp20 TG hearts. Mouse heart homogenates from NTGs and S10F TGs were immunoprecipitated with anti-Hsp20, proteins were separated on SDS-PAGE gels and probed with anti-Beclin1 or anti-Hsp20 antibodies. A, Representative immuno-blots of Beclin1 and Hsp20. B, Quantitative analysis of levels of Beclin1 associated with Hsp20 and ratio of Beclin1/Hsp20. Values represent means ± SEM; *: p<0.05, vs NTG; n=4 hearts for NTG and 4 hearts for S10F.
which accelerated heart failure progression and caused early mortality in mice expressing a human CryABR\textsuperscript{120G,212}. Therefore, the levels of ubiquitinated proteins in the 2 month old S10F hearts were examined using Western Blotting technique, with NTG hearts as controls. It was found that the levels of ubiquitinated proteins in S10F hearts were greatly increased compared to the NTG hearts (Fig. 47).

II. 5.F Diminished interaction S10F-Hsp20 with PP1

Cardiac contractile function and Ca\textsuperscript{2+} cycling are strictly regulated by a good equilibrium of PLN phosphorylation achieved by the balance between protein kinases and protein phosphatases, including PP1.\textsuperscript{213} As stated above, Hsp20 is able to tilt this fine-tuned balance by regulating PP1 activity. Specifically, Hsp20 suppresses PP1 activity through its direct interaction with PP1. Down-regulated activity of PP1 leads to specific increases in the phosphorylation of PLN, which relieves its suppression on SERCA2a activity and subsequently increases Ca\textsuperscript{2+} cycling and cardiomyocyte contractility. To determine if the interaction between Hsp20 and PP1 is altered by S10F substitution, recombinant PP1 in fusion with the maltose-binding protein (MBP) and WT or mutant-Hsp20 in fusion with the glutathione-S-transferase (GST) were generated, followed with blot overlay assays using anti-MBP or anti-GST antibodies. Our results indicated that the binding of Hsp20 mutant to PP1 was significantly decreased under both basal and PKA-stimulated conditions compared to WT-Hsp20 (Fig. 48).
FIGURE 47
Figure 47. The levels of ubiquitinated protein were greatly increased in the 2 month old S10F-Hsp20 TG hearts. Heart homogenates from NTG and S10F mice were separated in SDS-PAGE gel and probed with anti-ubiquitin antibody. Representative immuno-blots of ubiquitinated proteins from NTG and S10F hearts are shown. n=3 hearts for NTG and 3 hearts for S10F.
FIGURE 48

A

<table>
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<tr>
<th>Protein</th>
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<th>26kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-Hsp20-WT</td>
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<td>[image of Coomassie gel]</td>
</tr>
<tr>
<td>GST-Hsp20-S10F</td>
<td>[image of Coomassie gel]</td>
<td>[image of Coomassie gel]</td>
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<tr>
<td>GST</td>
<td>[image of Coomassie gel]</td>
<td>[image of Coomassie gel]</td>
</tr>
</tbody>
</table>

B

Overlay: MBP-PP1_{aai-330}
WB: MBP ab

C

Protein binding (arbitrary units)

<table>
<thead>
<tr>
<th>Protein</th>
<th>WT-Hsp20</th>
<th>S10F-Hsp20</th>
</tr>
</thead>
</table>

*
**Figure 48.** Diminished interaction between Hsp20 mutant and PP1. A, SDS-gel stained with Coomassie blue showing purified GST-Hsp20-WT and mutant recombinant protein; B, Blot overlay assay using MBP-PP1 protein and immunodetection with the MBP antibody shows that the Hsp20 mutant has reduced binding to PP1. C, Quantification of PP1 protein binding to Hsp20-WT and mutant as shown in Fig. B. Values represent means ± SEM; *p<0.05 vs WT-Hsp20, n=4. *(Data generated by collaborator Dr. Elizabeth Vafiadaki)*
II. 5.G PP1 activity is not altered in S10F-Hsp20 hearts

The decreased binding of S10F-Hsp20 to PP1 suggests that the ability of Hsp20 to inhibit PP1 activation may be compromised by the S10F mutation. To further confirm this, heart homogenates from 2 months old S10F and NTG mice were subjected to the PP1 activity assay using RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit as described in the method chapter. It was found that the PP1 activity was not changed in the S10F hearts compared to the NTG group (Fig. 49).

II. 5.H The ability of Hsp20 to be phosphorylated is not altered by S10F mutation

As described above, the protective effects of Hsp20 against I/R induced injury and β-agonist induced apoptosis in a phosphorylation dependent manner. To examine if the capability of Hsp20 to be phosphorylated is modified by S10F mutation, levels of phosphorylation of Hsp20 at Serine 16 in S10F heart were measured using western blots, with NTG heart as a control. Our results showed that phosphorylation levels of Hsp20 were significantly higher than that of the NTG group. However, when the ratio of phospho-Hsp20/Total-Hsp20 was analyzed, it was discovered that this ratio is not altered between the S10F and NTG groups (Fig. 50), suggesting that S10F substitution does not alter the ability of Hsp20 to be phosphorylated.
**Figure 49.** PP1 activity in 2 month old S10F-Hsp20 and NTG hearts. Mouse heart homogenates from S10F TGs and NTGs were subjected to the PP1 activity assay using RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit. Values represent means ± SEM; n=6 hearts for NTG and 6 hearts for S10F.
FIGURE 50

A

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
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<td></td>
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<tr>
<td>Total-Hsp20</td>
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</table>

B

![Bar graph showing](image)

Phospho-Hsp20/Total-Hsp20 (Relative units)
Figure 50. Phosphorylation of Hsp20 at serine 16 in 2 month old S10F-Hsp20 and NTG hearts. Mouse heart homogenates from S10F TGs and NTGs were subjected to the western blots using anti-Ser16 Hsp20 or Hsp20 antibodies. A, Representative blots of phosphorylation and total levels of Hsp20; B, Percentage of phosphorylated Hsp20 in S10F and NTG hearts (Normalized to the NTG group). Values represent means ± SEM; n=5 hearts for NTG and 5 hearts for S10F.
Chapter IV: Discussion

Section I: Dissertation Summary

Human mutations in Ca-cycling genes have been associated with dilated cardiomyopathy and arrhythmias. In this study, our first objective was to determine the functional significance of a novel human PLN mutation (R25C) in cardiomyocyte Ca\(^{2+}\)-handling and contractility. Briefly, exome sequencing identified a C73T substitution in the coding region of PLN in a family with DCM. The 4 heterozygous family members had implantable cardiac defibrillators, and 3 developed prominent ventricular arrhythmias. Overexpression of R25C-PLN in adult rat cardiomyocytes significantly suppressed the Ca\(^{2+}\) affinity of SERCA2a, resulting in decreased SR Ca\(^{2+}\) content, Ca\(^{2+}\) transients and impaired contractile function, compared to WT-PLN. These inhibitory effects were associated with enhanced interaction of R25C-PLN with SERCA2a, which was prevented by PKA phosphorylation. Accordingly, isoproterenol stimulation relieved the depressive effects of R25C-PLN in cardiomyocytes. However, R25C-PLN also elicited increases in the frequency of Ca\(^{2+}\) sparks and waves as well as stress-induced aftercontractions. This was accompanied by increased CaMKII activity and hyper-phosphorylation of RyR2 at serine 2814. Collectively, these findings demonstrate that human R25C-PLN is associated with super-inhibition of SERCA2a and Ca\(^{2+}\) transport as well as increased SR Ca\(^{2+}\) leak, promoting arrhythmogenesis under stress conditions. This is the first mechanistic evidence that increased PLN inhibition may impact both SR Ca\(^{2+}\) uptake and Ca\(^{2+}\) release activities and suggests that the human R25C-PLN may be a prognostic factor for
increased ventricular arrhythmia risk in DCM carriers.

The second objective of our study was to determine the role of a novel human S10F-Hsp20 mutation in cardiac function and protection. We identified a S10F-Hsp20 in DCM patients with a frequency of 2.8%, while there were no normal subjects carrying the S10F-Hsp20. In vitro studies in infected cardiomyocytes showed that the inotropic effects of Hsp20 were negated by S10F, which was coupled with suppressed SR Ca\textsuperscript{2+} cycling. Furthermore, S10F significantly abrogated the protective effects of WT-Hsp20 upon prolonged isoproterenol-stimulation. To investigate the in vivo impact of S10F-Hsp20, transgenic mice with cardiac-specific overexpression of this mutant were generated. Our results indicated that in contrast to the enhancing effects of WT-Hsp20\textsuperscript{101}, contractility and SR Ca\textsuperscript{2+} cycling in myocytes from S10F-Hsp20 hearts were not different than those in NTG cells, consistent with the in vitro data. The underlying mechanisms involved reduced ability of mutant Hsp20 to interact with PP1 and inhibit it, compare to WT-Hsp20.

Furthermore, the role of the S10F-Hsp20 in cardiac function and protection under stress conditions were determined. It was found that the protective effects of WT-Hsp20 against I/R-induced injury were abrogated by S10F-Hsp20, as evidenced by diminished recovery of contractile function, which was even lower than that of NTG hearts. In addition, the S10F mutation was found to accelerate the progression of MI-induced cardiac remodeling and heart failure, as indicated by significantly impaired contractile function and severe dilation in these hearts. More importantly, under the physiological stress of aging,
there was severe contractile dysfunction and cardiomyocyte apoptosis in the S10F hearts, which eventually led to dilated cardiomyopathy, heart failure and accelerated death in the mutant mice. The main mechanisms associated with impaired recovery after I/R injury, detrimental remodelling after MI or upon aging in the mutant hearts included reduced interaction of S10F-Hsp20 with: a) Bax, leading to activation of caspase-3 and apoptosis; b) pAkt, associated with diminished Akt activation, reduction in p-Bad and increased apoptosis; and c) Beclin 1, resulting in decreased activation of autophagy and thus ability to remove damaged organelles under stress conditions. These findings suggest that both the enhanced contractility and cardioprotection, elicited by WT-Hsp20, were abrogated by the S10F mutation. Thus, it is intriguing to speculate that patients carrying S10F-Hsp20 may have weaker heart function coupled with intrinsic compromise in coping with various cardiac stress conditions.

Overall, in this dissertation, we identified two novel human mutations in the major Ca-handling proteins, PLN and Hsp20, in patients with dilated cardiomyopathy. De novo results showed that both R25C and S10F mutations altered the basic function of PLN and Hsp20 respectively, resulting in disrupted SR Ca cycling and impaired cardiomyocyte contractility. In addition, the chaperone activity of Hsp20 was negated by S10F mutation, resulting in cardiomyocyte apoptosis under basal condition. These findings suggest that both R25C and S10F mutations are disease-associated mutations in human carriers and may potentially serve as prognostic or diagnostic markers for dilated cardiomyopathy.
Section II: SR Ca\textsuperscript{2+} cycling and dilated cardiomyopathy

II.1 SR Ca\textsuperscript{2+} cycling regulation by PLN and Hsp20

The heart utilizes Ca\textsuperscript{2+} cycling to achieve E-C coupling, which is vital for myocyte contractile function. Specifically, upon a depolarizing signal during an action potential, extracellular Ca\textsuperscript{2+} enters the cell through L-type Ca\textsuperscript{2+} channels. Increased intracellular Ca\textsuperscript{2+} binds and activates ryanodine receptors on the SR membrane, resulting in the release of a larger amount of Ca\textsuperscript{2+} from the SR, which is called Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release. The Ca\textsuperscript{2+} diffuses to the myofilaments and initiates cardiomyocyte contraction. After contraction, Ca\textsuperscript{2+} is re-uptaken into the SR by SERCA2a to maintain SR Ca\textsuperscript{2+} content, which is crucial for the next cycle of cardiac contraction. Ca\textsuperscript{2+} reuptake by SERCA2a is critically regulated by PLN, a 52 amino acid protein. In the dephosphorylated state, PLN inhibits SERCA2a and shifts its Ca\textsuperscript{2+} activation toward lower apparent Ca\textsuperscript{2+} affinity. However, upon it’s phosphorylation by either PKA at Ser16 or CaMKII at Thr17, the inhibition on SERCA2a by PLN is relieved and its Ca\textsuperscript{2+} affinity is increased.\textsuperscript{199} Thus, PLN plays a key role in regulation of Ca\textsuperscript{2+} reuptake by SERCA2a to induce relaxation and decrease diastolic Ca\textsuperscript{2+} levels.

It has been well recognized that cardiac contractility and Ca\textsuperscript{2+} cycling are strictly regulated by the PLN/SERCA2a complex. Phosphorylation of PLN reveals the fine balance of the activities of protein kinases and protein phosphatases.\textsuperscript{19, 199} Particularly, PP1 is the major phosphatase responsible for dephosphorylating PLN and its activity was found to be significantly upregulated in heart failure.\textsuperscript{142} Recent studies by the Kranias lab have shown that Hsp20 is able to enhance
cardiac contractile performance by targeting PP1. Specifically, Hsp20 suppresses PP1 activity through its direct interaction with PP1. Decreased PP1 activity causes increases in the phosphorylation of PLN, relieving its suppression on SERCA2a activity, which subsequently increases SR Ca\textsuperscript{2+} reuptake and Ca\textsuperscript{2+}-cycling and thereby enhances cell contraction.\textsuperscript{101} These effects of Hsp20 in the heart indicate that, similar to PLN, Hsp20 is also a critical player involved in the regulation of SR Ca\textsuperscript{2+}-cycling and cardiomyocyte contractility.

**II.2 Dilated cardiomyopathy and human mutations in SR Ca\textsuperscript{2+} cycling genes**

Accumulating evidence from human and experimental animal studies has demonstrated that SR Ca\textsuperscript{2+} cycling is critical to normal myocardial physiology and point to the crucial role of disturbed SR Ca\textsuperscript{2+} uptake, storage, and release in the process of cardiomyopathy and heart failure. For instance, during congestive heart failure, expression levels of SERCA2a are considerably reduced, which results in abnormal Ca\textsuperscript{2+} handling and diminished contraction in the heart.\textsuperscript{1, 214} Accordingly, cardiac overexpression or gene transfer of SERCA2a in rat models of heart failure not only significantly improved SR Ca\textsuperscript{2+} cycling and cardiac contractile function, but increased survival and cardiac metabolism.\textsuperscript{16, 17} Similarly, inhibition of PLN activity resulted in enhanced SR Ca\textsuperscript{2+} cycling and improved cardiac contractility.\textsuperscript{19} Moreover, suppression of PLN restored contractility in human failing ventricular myocytes and attenuated heart failure progression in cardiomyopathic hamsters.\textsuperscript{56, 215} In addition, RyR2 inhibition by calstabin considerably improved cardiac function in failing hearts.\textsuperscript{200} These studies suggest that disturbed SR Ca\textsuperscript{2+} cycling may serve as a root cause of heart failure and this
notion has been well supported by recent studies in human patients.\textsuperscript{216} Thus, proteins involved in SR Ca\textsuperscript{2+} cycling are key regulators of cardiac contraction, and disturbances in their function may result in cardiac pathology.

Dilated cardiomyopathy (DCM), a common cause of heart failure, is characterized by dilation and impaired contraction of the left ventricle. Multiple etiologies may underlie DCM and genetics is a common one, with the disease often affecting several individuals within a family. To date, genetic variants in more than 30 genes were identified, which initiate diverse pathophysiologic mechanisms leading to LV dysfunction or arrhythmia and increased morbidity and mortality.\textsuperscript{217} Particularly several disease-causing mutations have been identified in the genes encoding proteins involved in SR Ca\textsuperscript{2+} cycling regulation over the recent years. First of all, human mutations in RyR2 (P2328S, Q4201R, V4653F, G1885E and G1886S, S2246L, N4104K, and R4497C) were recently reported, which were found to cause SR Ca\textsuperscript{2+} leak and disruption of Ca\textsuperscript{2+} homeostasis in the cardiomyocyte, leading to cardiomyopathies combined with life-threatening arrhythmias.\textsuperscript{218} Another important SR Ca-cycling protein is CSQ, which is the most abundant Ca\textsuperscript{2+}-buffering protein located in the lumen of SR. It provides a store of Ca\textsuperscript{2+} for release during systole and also allows the RyR2 to sense luminal Ca\textsuperscript{2+}. Studies have shown that missense mutations in CSQ (R33Q and D307H) are able to induce lethal catecholaminergic polymorphic ventricular tachycardia in human carriers.\textsuperscript{219, 220} Junctin and triadin are CSQ-binding proteins that regulate SR Ca\textsuperscript{2+} release by interacting with the ryanodine receptor at the luminal side of the SR. It was found that null mutations in either Junctin or triadin
impaired SR Ca$^{2+}$ release and overall excitation-contraction (EC) coupling, which caused ventricular arrhythmias and sudden death in human.$^{221, 222}$ Furthermore, as described in the Introduction section, a couple of human mutations in PLN, a key regulator of SR Ca$^{2+}$ cycling and cardiomyocyte contractility, were identified and all of them were associated with inherited dilated cardiomyopathy and heart failure.$^{63, 65, 67, 145}$ In addition, a mutation was also identified in the human Hsp20 (P20L), which was shown to negate the protective effects of Hsp20 in cardiomyocytes.$^{189}$ These findings add to accumulating evidence that myocellular Ca$^{2+}$ dysregulation, caused by mutations in SR Ca$^{2+}$ handling proteins, is sufficiently deleterious to cause DCM and initiate heart failure.

In this dissertation, we identified two novel human mutations in the major Ca-handling proteins, PLN and Hsp20, in patients with dilated cardiomyopathy. The functional significance of the two human mutations, R25C-PLN and S10F-Hsp20 in the regulation of SR Ca$^{2+}$ cycling and cardiac function are addressed below.

Section III: A novel human R25C-PLN mutation is associated with super-inhibition of Ca$^{2+}$ cycling, dilated cardiomyopathy and ventricular arrhythmia

III.1 The role of R25C-PLN in SR Ca$^{2+}$ cycling

A common clinical characteristic, contributing to the reduced contractile function in human and experimental failing hearts, is impaired Ca$^{2+}$ sequestration into the SR, which reflects prolonged decay time of intracellular Ca$^{2+}$ transient and changes in systolic and diastolic Ca$^{2+}$ levels.$^{168, 214, 223}$ The prolonged Ca$^{2+}$-
transient decay time and increased diastolic Ca\textsuperscript{2+} levels may result from deceased SERCA2a levels or augmented SERCA2a inhibition by PLN.\textsuperscript{224} Notably, while determining the functional significance of the R25C-PLN in cardiomyocyte Ca\textsuperscript{2+}-handling and contractility, no significant changes in the SERCA2a protein levels were identified between WT-PLN and mutant-PLN cardiomyocytes. However, when measuring the initial rates SR Ca\textsuperscript{2+} uptake in WT-PLN or mutant-PLN cardiomyocytes, the EC50 value for Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} uptake was found to be 1.5 fold higher in R25C-PLN cells compared with the WT-PLN group, suggesting that the mutant PLN is a super-inhibitor of SERCA2a, compared with WT-PLN. Further functional studies revealed that the super-inhibitory effects of the R25C mutation on SERCA2a activity were translated into alterations at the cellular level, as evidenced by significantly suppressed contractile parameters including FS\%, +dL/dt and −dL/dt in R25C-PLN cardiomyocytes, compared with the WT-PLN cells. Moreover, the depressed contractile mechanics were found to be associated with remarkably reduced intracellular Ca\textsuperscript{2+} kinetics, as exhibited by decreased Ca\textsuperscript{2+} transients, SR Ca\textsuperscript{2+} load and prolonged time of diastolic Ca\textsuperscript{2+} decay in R25C-PLN cardiomyocytes, compared with the WT-PLN group. Particularly, the intracellular diastolic Ca\textsuperscript{2+} was found to be increased by 18% in R25C-PLN cardiomyocytes, compared with WT-PLN. Thus, the R25C-PLN mutant depressed mechanical and Ca\textsuperscript{2+} kinetic parameters and increased diastolic Ca\textsuperscript{2+} levels, consistent with the enhanced inhibition of SERCA2a. Subsequent studies on the mechanisms associated with increased inhibition of SERCA2a by R25C-PLN revealed that the mutant R25C-
PLN exhibits enhanced association to SERCA2, compared to the WT-PLN.

It is worth noting that the super-inhibitory effects of R25C-PLN on cardiomyocyte contractility and intracellular Ca^{2+} cycling could be completely relieved upon isoproterenol stimulation. Moreover, the increased interaction between the mutant PLN and SERCA2a could be abolished by PKA phosphorylation. These findings suggest that the R25C mutation did not alter the ability of PLN to get phosphorylated. Indeed, western blotting data demonstrated that both Ser16 and Thr17 sites on R25C-PLN could be phosphorylated to the same extent as those of WT-PLN under both basal and Iso stimulated conditions.

The mechanisms associated with the superinhibitory effects of R25C-PLN are likely mediated through a disturbed conformational structure of PLN. Phospholamban, as an integral membrane protein, has been predicted to contain three domains: cytosolic domain Ia (residues 1-20), cytosolic domain Ib (residues 21–30) and transmembrane domain II (31-52). These domains form a helix (Ia)-turn (Ib)-helix (II) structure. The Arg25 residue is located in the hinge region Ib of PLN, and earlier studies showed that deletion of domain Ib (AA 21-30) in the PLN molecule resulted in a significant decrease in the apparent Ca^{2+} affinity of SERCA2a, when the truncated PLN was co-expressed with SERCA2a.\textsuperscript{225} Additionally, three PLN mutations in domain Ib (N27A, Q29A, and N30A) were discovered to be associated with gain of inhibitory function.\textsuperscript{226, 227} Particularly, Arg25 is highly conserved across species and this residue in region Ib supplies a positive charge, which is important for maintaining the hinge region of PLN in the hydrophilic cytosolic environment. Thus, the Arg25 replacement with cysteine, a
non-polar amino acid, could destabilize domain Ib and alter the hinge angle between domain Ia and domain II, which may lead to conformational changes that enhance the association between PLN and SERCA2a. Future studies on the three-dimensional structure of mutant PLB and SERCA2a in native SR membranes will provide further insight into the mechanisms by which R25C-PLN exerts its inhibitory effects. Such insight may contribute to the design of appropriate molecules to relieve the PLB inhibition of SERCA2a and improve function in DCM patients.

III.2 SR Ca\textsuperscript{2+} leak and ventricular arrhythmia

During cardiac relaxation, ideally, the SR Ca\textsuperscript{2+} release channel RyR2 would be fully closed, which would allow relaxation to proceed efficiently and limit the amount of energetically futile SERCA2a activity to keep diastolic Ca-levels low. However, although most of SR Ca\textsuperscript{2+} release is shut off during relaxation, there is still a finite level of SR Ca\textsuperscript{2+} leak, such as the localized spontaneous SR Ca\textsuperscript{2+} release events known as Ca\textsuperscript{2+} sparks, which are observed by confocal microscopy. The diastolic Ca\textsuperscript{2+} sparks arise from the activation of a cluster of RyR2, and the frequency relates directly to the finite open probability of individual cardiac RyR2. SR Ca\textsuperscript{2+} leak can be explosive (as Ca\textsuperscript{2+} sparks) or effectively invisible (not detectable by confocal microscopy). The amount of local Ca\textsuperscript{2+} released is high during Ca\textsuperscript{2+} sparks because multiple RyR channels within a junction are activated by Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release. In comparison, during invisible Ca\textsuperscript{2+} leak, only single RyR2 opening occurs.

Accumulating evidence has shown that the frequency of Ca\textsuperscript{2+} sparks is
significantly increased during heart failure\textsuperscript{230} and this increased SR Ca\textsuperscript{2+} leak can result in the following detrimental consequences in the heart: i) Augmented SR Ca\textsuperscript{2+} leak leads to diminished SR Ca\textsuperscript{2+} content and therefore to reduced systolic function during heart failure; ii) Enhanced Ca\textsuperscript{2+} leak is able to induce elevated diastolic Ca\textsuperscript{2+} and slow diastolic Ca\textsuperscript{2+} decline, which contributes to diastolic dysfunction; iii) SR Ca\textsuperscript{2+} leak, when synchronized as Ca\textsuperscript{2+} waves, can drive inward NCX current and lead to both delayed and early afterdepolarizations (DADs/EADs) respectively, which initiate triggered ventricular arrhythmias; iv) SR Ca\textsuperscript{2+} leak is energetically costly, as the leaked Ca\textsuperscript{2+} has to be re-uptaken into the SR by the Ca\textsuperscript{2+}-ATPase, transported out of the cell by NCX, which relies indirectly on Na/K-ATPase, or into mitochondria, utilizing the proton motive force that generates ATP. It has been shown that Ca\textsuperscript{2+} and Na transport utilizes a substantial fraction of cardiomyocyte ATP and the extent of such consumption is second only to contractility use. Therefore, during heart failure, when the hearts are already energetically compromised, SR Ca\textsuperscript{2+} leak may add an extra burden to cellular energy generation or demand\textsuperscript{229}.

Ryanodine receptor-mediated SR Ca\textsuperscript{2+} leak can be increased through phosphorylation of RyR2 by PKA or CaMKII. In human heart failure, RyR2 is found to be hyper-phosphorylated by PKA at Serine 2808 (S2808), which causes enhanced RyR2 opening and SR Ca\textsuperscript{2+} leak, leading to ventricular arrhythmias. RyR2 gating activity can also be influenced by CaMKII, which phosphorylates RyR2 at Serine 2814. Researchers discovered that expression and activity of CaMKII and RyR2 phosphorylation at S2814 are significantly increased during
Further studies from Currie et al. demonstrated that CaMKII-dependent RyR2 phosphorylation significantly activates RyR2 openings, resulting in enhanced Ca²⁺ sparks, Ca²⁺ leak, and DADs. Whereas, CaMKII inhibition dramatically inhibits SR Ca²⁺ leak and arrhythmogenic events in heart failure. These studies suggest that CaMKII may serve as a major culprit in the enhanced SR Ca²⁺ leak observed during heart failure.

III.3 The role of R25C-PLN in SR Ca²⁺ leak and ventricular arrhythmia

Clinical characteristics of family members with the R25C mutation indicated that all R25C affected members developed cardiac arrhythmias. As described above, the molecular trigger for arrhythmia is enhanced SR Ca²⁺ leak, evidenced by increases in Ca²⁺ sparks or waves at the cardiomyocyte level. To determine the effect of R25C on SR Ca²⁺ release, we examined Ca²⁺ spark properties in intact quiescent cells. It was observed that spark frequency was 2 fold in the mutant PLN cardiomyocytes, compared to WT-PLN cardiomyocytes. Next, the frequency of spontaneous Ca²⁺ waves was examined in WT-PLN and R25C-PLN myocytes. It was discovered that Ca²⁺ waves were developed in 40% of R25C cardiomyocytes, compared with 4% of WT-PLN cells. Thus, R25C-PLN increases the frequency of Ca²⁺ sparks and Ca²⁺ waves in cardiomyocytes. Recent studies have suggested that RyR2-mediated Ca²⁺ leak occurs in part as Ca²⁺ sparks, although there is RyR-mediated and Ca²⁺ spark-independent leak. Therefore, total diastolic SR Ca²⁺ leak was also measured using the tertacaine protocol. The ratio of SR Ca²⁺ leak to SR Ca²⁺ load was significantly larger in R25C cardiomyocytes, compared with WT cardiomyocytes, suggesting that R25C-PLN
increases the SR Ca\(^{2+}\) leak. Next, we determined the role of R25C-PLN under stress conditions by measuring the frequency of aftercontractions in WT-PLN and R25C cardiomyocytes at 2-Hz field stimulation in the presence of 1 µM Isoproterenol. Spontaneous aftercontractions occurred in 74% of R25C cells within 5 seconds after pacing was stopped, compared with 17% of WT-PLN cells. Taken together, these findings suggest that R25C-PLN enhances the propensity for spontaneous Ca\(^{2+}\) release from the SR, resulting in increased susceptibility to arrhythmia.

We then investigated whether the increased SR Ca\(^{2+}\) leak in R25C cardiomyocytes was associated with increases in the phosphorylation of RyR2. As stated above, enhanced phosphorylation of Ser2808 (PKA site) and Ser2814 (CaMKII site) in RyR2 can regulate RyR2 function. Thus, we examined the phosphorylation state of RyR2 in R25C cardiomyocytes, by performing Western blots with phospho-specific antibodies against the RyR2 Ser2808 and Ser2814 sites. It was observed that R25C cells had significantly increased phosphorylation levels of RyR at Ser2814, but not at Ser2808. This prompted us to determine the activity of CaMKII in cell lysates from WT-PLN and R25C cardiomyocytes, using a non-radiographic CaMKII ELISA. We found that the activity of CaMKII in R25C myocytes was increased to 2 fold compared to WT-PLN group, consistent with the increase in diastolic Ca\(^{2+}\) in these cells. To further confirm this finding, phosphorylation of CaMKII at Thr286 residue, which represents permanent activation of the CaMKII enzyme, was determined in cell lysates from WT-PLN and R25C cardiomyocytes using Western blots. It was
observed that the level of phosphorylation of CaMKII at Thr286 was considerably higher in R25C cells, compared to the WT-PLN. Interestingly, the increased CaMKII activity did not reflect altered phosphorylation of PLN in the mutant cells, suggesting that mutant PLN either altered CaMKII binding or increased PLN-phosphatase activity. To further examine the contribution of CaMKII mediated phosphorylation of RyR2 in the R25C-induced increases of Ca\(^{2+}\) sparks and waves, KN93, the selective inhibitor of CaMKII was utilized in parallel studies with KN92 as a control. We found that KN93 completely abrogated the increased Ca\(^{2+}\) spark frequency, indicating that the aberrant SR Ca\(^{2+}\) release was contributed by the increased CaMKII phosphorylation of RyR. Furthermore, KN93 fully abolished the Ca\(^{2+}\) waves, elicited by R25C-PLN. These data suggest that the aberrant SR Ca\(^{2+}\) leak and resultant arrhythmia were associated with increased CaMKII activity and hyper-phosphorylation of RyR at Ser2814.

Collectively, the R25C-PLN was shown to increase Ca\(^{2+}\) sparks, waves and stress-induced after-contractions, which may have contributed to the clinical features of cardiac arrhythmia in R25C carriers at the cellular level. As summarized in Figure 51, the underlying pathways appear to involve increased inhibition of SERCA2a by mutant PLN, resulting in elevated diastolic Ca\(^{2+}\) and activation of CaMKII.\(^{235}\) Actually, several studies in neuron cells and cardiomyocytes have shown that sustained intracellular Ca\(^{2+}\) elevation enhances CaMKII auto-phosphorylation and increases Ca\(^{2+}\)/calmodulin-independent kinase activity.\(^{236-238}\) The activation of CaMKII reflected enhanced phosphorylation of RyR2 at Ser2814, leading to increases in SR Ca\(^{2+}\) leak and arrhythmia,
(From Michael et al. Cardiovascular Research, 2015)
Figure 51. Schematic representation of the mechanisms underlying the proarrhythmic effects of R25C-PLN in infected cardiomyocytes. Na\textsubscript{v}, fast, voltage-dependent Na\textsuperscript{+} channel; NCX, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger; CaMKII, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase; PLN, phospholamban; RyR, ryanodine receptor 2; SERCA, sarco/endoplasmatic reticulum Ca\textsuperscript{2+} ATPase; SR, sarcoplasmic reticulum; P, phosphate.
consistent with previous studies.\textsuperscript{239} Indeed, inhibition of CaMKII activity by KN93 abrogated the increases in Ca\textsuperscript{2+}-sparks and Ca\textsuperscript{2+}-waves, elicited by R25C-PLN. Notably, overexpression of WT-PLN in cardiomyocytes, which resulted in a smaller degree of SERCA2a inhibition than R25C-PLN, did not significantly alter diastolic Ca\textsuperscript{2+} levels, CaMKII activity or phosphorylation of RyR2 at Ser2814. These findings indicate that inhibition of SERCA2a beyond a threshold point has detrimental effects through increased diastolic Ca\textsuperscript{2+} levels and CaMKII activation.

Taken together, in this study we identified a novel R25C-PLN mutation in familial DCM, which acts as a superinhibitor of SERCA2a and results in decreased SR Ca\textsuperscript{2+} content, Ca\textsuperscript{2+} transients as well as damaged contractile function. The depressed SR Ca\textsuperscript{2+} resequestration is associated with increased CaMKII activity and hyper-phosphorylation of RyR2 at Ser2814, leading to aberrant Ca\textsuperscript{2+}-leak and after-contractions under stress conditions. This is the first evidence that an experiment by nature to alter PLN function results in increased SR Ca\textsuperscript{2+}-leak and propensity to arrhythmia, a significant component of the cardiovascular phenotype observed in the carriers of the PLN mutation.

In addition, our findings provide valuable new insights into the established paradigm of DCM gene/phenotype relationships. We\textsuperscript{217, 240} and others have suggested that the vast majority of DCM cases and families, shown to have genetic cause, appear monophenotypic-a classic ‘pure’ DCM with no atypical cardiovascular or syndromic features. For these families, ventricular or supraventricular arrhythmias have been attributed to a generic progression towards advanced heart failure with its manifold cellular abnormalities, including
increased susceptibility to arrhythmia rather than a gene-specific abnormality. Dissecting between the DCM elicited arrhythmias and those resulting from a specific molecular defect remains challenging, although the exception has been the category of “DCM with prominent arrhythmia” 240, assigned predominantly to variants in LMNA, (lamin A/C) but also observed with variants in SCN5A (sodium channel, voltage gated, type V alpha subunit) or DES (desmin).241, 242 Our current work supports this established gene/phenotype paradigm, as previously reported rare variants in PLN have been associated with predominantly monophenotypic DCM, while the R25C PLN variant leads to prominent arrhythmias, which we suggest may be due in part to novel, specific mechanisms. These PLN-mutation carriers presented with arrhythmia in middle-age and in the setting of DCM, characterized by an “adult-onset”, as commonly observed in most of genetic cardiomyopathy. Another recent report with the R14del-PLN variant, a founder mutation in a large number of Dutch individuals, also suggested a propensity to arrhythmia, although a key predictor of arrhythmia was reduced systolic function (<45%).243 Thus, while gene/phenotypic generalizations may be valid for the most part, specific variants, particularly those of key hub proteins such as PLN, that interact with a variety of other critical partners, may have yet unknown functions that when disrupted lead to highly specific, but otherwise unexpected, phenotypic characteristics that would not usually be anticipated for the gene in question.

This new insight has two corollaries. The first validates ongoing cellular and subcellular mechanistic studies in genetic DCM. That is, ongoing
investigations such as this one are essential to understand gene/phenotype relationships, and more specifically the genotype/phenotype variability within any one gene, as the generalization of the past decade is not likely to hold going forward. Even though extensive studies have already been conducted on PLN physiology, including several prior PLN rare variants in association with DCM reviewed herein, the meticulous investigation of even more variants is clearly warranted. The second is applicable to clinical genetics: while we must use prior gene/phenotype and genotype/phenotype data to infer genetic diagnoses, studies such as this one suggest that we should be more circumspect, when applying gene/phenotype relationships derived from our current knowledgebase.

Section IV: The role of human S10F-Hsp20 mutation in cardiac function and protection

In this study, we elucidated the functional significance of a newly identified human Hsp20 mutation, S10F, in cardiac contractile function and protection under both basal and stress conditions. Briefly, this human S10F-Hsp20 mutant was discovered only in DCM patients and is associated with a change of a serine at position 10 into phenylalanine (S10F). As stated above, Serine 10 is fully conserved among species, suggesting a critical role in Hsp20’s function. Particularly, secondary structural prediction revealed that all three components (helix strand and loop) of the structure of Hsp20 are altered by the S10F mutation, implying that this mutation is associated with structural changes, which may modify the basic function of Hsp20 in the heart. Therefore, our hypothesis is that S10F results in conformational changes in Hsp20, which renders it incapable of
enhancing cardiac contractility and protecting against cell apoptosis. Thus, S10F-expressing hearts exhibit accelerated onset and progression of pathologic hypertrophy, leading to heart failure under stress conditions.

**IV.1 Hsp20 enhances cardiac contractility**

The principal function of the heart is to pump blood carrying oxygen and nutrients to all tissues of the body via the circulatory system. The inability of the heart to provide sufficient blood to meet the body's needs is termed heart failure. As described above, maintaining the intracellular Ca^{2+}-homeostasis and normal SR Ca^{2+} cycling is of great significance for the cardiomyocyte contractility and heart function. Accumulating evidence has shown that cardiac contractility and Ca^{2+} cycling are closely regulated by a fine equilibrium of PLN phosphorylation enacted by the balance of protein kinases and protein phosphatases^{19, 136}. In heart failure, PP1, the major phosphatase responsible for dephosphorylating PLN, was found to have significantly increased activity, which resulted in PLN dephosphorylation and depressed cardiac contractility.^{142} Recent studies by us revealed that Hsp20 constitutes a novel regulator of PP1 activity through its direct interaction and inhibition of PP1. Importantly, inhibition of PP1 leads to increased PLN phosphorylation, which relieves its suppression on SERCA2a activity and subsequently increases SR Ca^{2+}-cycling and cardiomyocyte contractility.^{101} These effects of Hsp20 in the heart indicate that Hsp20 is a negative regulator of PP1 activity.

**IV.2 S10F-Hsp20 mutation abrogates the contractile enhancing effects of Hsp20**
To investigate the effects of the human S10F-Hsp20 variant on cardiomyocyte contractility and intracellular Ca\(^{2+}\) cycling, we first overexpressed the S10F-Hsp20 and WT-Hsp20 in adult rat cardiomyocytes, using adenoviral technology, with GFP as a control. Then infected cardiomyocytes were stimulated by Grass S5 stimulator at a pacing rate of 0.5 and contractile parameters were monitored using video edge detection. Consistent with previous findings, myocyte contractility was significantly increased in WT-Hsp20 cardiomyocytes. However, these enhancing effects were completely abolished in the S10F-Hsp20 cardiomyocytes, which exhibited similar contractile parameters as the GFP cardiomyocytes. Moreover, the amplitude of Ca\(^{2+}\) transients was significantly reduced and time to 50% decay of the Ca\(^{2+}\) signal was considerably prolonged in the mutant-Hsp20 cells, compared to WT-Hsp20 infected cardiomyocytes. Furthermore, subsequent studies in cardiomyocytes isolated from the S10F transgenic hearts indicated that both contractile mechanics and intracellular Ca\(^{2+}\) kinetics were not altered compared to the NTG group, which are consistent with the \textit{in vitro} findings. More importantly, under basal conditions, no differences in the contractile performance of intact hearts were observed between the transgenic mice overexpressing S10F-Hsp20 and NTG mice, as determined by either \textit{ex vivo} Langendorff perfusion system or \textit{in vivo} Echocardiography. Taken together, these data suggest that the cardiac contractile enhancing effects of WT-Hsp20 overexpression are completely abrogated by the S10F substitution.

As described above, Hsp20 is able to increase cardiac contractility by inhibiting the activity of PP1, which is achieved through its direct interaction with
To determine if the interaction between Hsp20 and PP1 is altered by S10F substitution, recombinant PP1 in fusion with maltose-binding protein (MBP) and WT or mutant-Hsp20 in fusion with glutathione-S-transferase (GST) were generated, followed with blot overlay assays using anti-MBP or anti-GST antibodies. Our results indicated that the binding of Hsp20 mutant to PP1 was significantly decreased, compared to the WT-Hsp20. Further studies in TG hearts revealed that, compared to the suppressed cardiac PP1 activity in WT-Hsp20 overexpression mice\textsuperscript{101}, the activity of PP1 was not altered in the S10F-Hsp20 hearts, in comparison with the NTG hearts. Thus, these findings suggest that the abolished contractile enhancement in S10F-Hsp20 hearts may result from the inability of Hsp20 mutant to interact with PP1 and inhibit its activity.

**IV.3 Hsp20 and myocardial ischemia induced injury**

Myocardial ischemia, also called coronary heart disease (CHD), occurs when blood flow to the heart is reduced, preventing it from receiving enough oxygen, leading to cell loss and severe damage to the heart muscle. Coronary reperfusion is the primary therapeutic strategy in CHD, but reperfusion itself induces necrosis and apoptosis, leading to further damage to the myocardium, which is referred to as I/R injury\textsuperscript{155}.

The CHD is rather common in the United States and is associated with approximately one third of all deaths in men and one quarter of all deaths in women\textsuperscript{244}. Currently, there are no effective therapies for CHD and the mechanisms underlying this pathological process have not been fully elucidated. Recently, studies by us and other researchers revealed that a small heat shock
protein, Hsp20, is capable of protecting the heart against ischemia and reperfusion induced injury, which may hold promise as a therapeutic target in CHD. Specifically, in 2005, Fan et al. generated a TG mouse model with cardiac-specific overexpression of Hsp20 and then subjected the TG hearts to global no-flow I/R (45 minutes/120 minutes), using the Langendorff preparation. It was discovered that increased Hsp20 expression in the heart protects against IR injury, resulting in full functional recovery and significantly reduced infarction size. This improvement was accompanied by considerably decreased cardiomyocyte necrosis and apoptosis in the TG hearts after I/R injury.\(^{183}\) Consistently, studies from the same group showed that knockdown of Hsp20 using microRNA-320 in the mouse heart led to remarkably reduced contractile functional recovery and increased infarct size after I/R, which further supported the protective role of Hsp20 against I/R injury.\(^ {184}\) In addition, \textit{in vitro} studies by Islamovic et al. demonstrated that overexpression of Hsp20 in neonatal and adult rat cardiomyocytes considerably reduced simulated I/R injury induced cardiomyocyte apoptosis.\(^ {89}\) More importantly, in 2009, Qian et al. discovered that Hsp20 protects the heart against I/R injury by preserving autophagy activity, a physiological process whereby cells degrade and remove damaged proteins and organelles.\(^ {86}\) Thus, these findings suggest that Hsp20 may constitute a new therapeutic target for ischemic heart diseases.

**IV.4 The S10F mutation abrogates the protective effects of Hsp20 against myocardial ischemia-induced injury**

To determine if the beneficial effects of Hsp20 against I/R injury are modified by
S10F substitution, S10F TG hearts were subjected to ex vivo 45 minutes of no-flow global ischemia, followed by 1 hour of reperfusion, with NTG hearts as controls. It was found that after 45 minutes ischemia, unlike the previously reported full functional recovery in the WT-Hsp20 TG hearts\textsuperscript{183}, the contractile function recovery of S10F-Hsp20 hearts was even worse than that of the NTG hearts. As stated above, Hsp20 is able to protect against I/R-induced apoptosis.\textsuperscript{183} To determine if these protective effects are altered by the S10F mutation, the extent of apoptotic cell death was examined in S10F and NTG hearts under basal and I/R conditions. It was found that, even under basal condition, the number of myocytes undergoing apoptosis was significantly increased in S10F hearts, compared to the NTG hearts. Moreover, upon I/R injury, the number of apoptotic cells in S10F hearts was 2.6-fold higher than that in NTG hearts. These data suggest that the protective effects of Hsp20 against I/R injury and the resultant apoptosis are abolished by the S10F mutation.

To investigate the effects of S10F-Hsp20 on cardiac function and protection after chronic myocardial ischemia injury, transgenic mice carrying S10F-Hsp20 mutant were subjected to mild myocardial infarction surgery. It was discovered that the mortality rate within the first 24 hours after surgery was significantly increased in the S10F-Hsp20 mice, compared with the NTG mice. The mice that survived from both S10F and NTG groups were then subjected to echocardiography to monitor cardiac function at 1, 2, 3, 4, 6, 8, 10, and 12 weeks post-infarction. It was observed that starting at 3 weeks post mild MI surgery, EF\% and FS\% were significantly reduced in the S10F-Hsp20 hearts, compared to the
NTG hearts. Particularly, this contractile dysfunction in the S10F hearts was associated with severe cardiac dilation and remodeling, as evidenced by remarkable increases in LV end systolic/diastolic volumes, left ventricle chamber size and ratio of HW/BW. Taken together, the reduced survival, depressed contractility and increased hypertrophy and remodeling in the Hsp20 mutant hearts after MI suggest that the protective effects of Hsp20 against ischemia injury were entirely negated by the S10F mutation.

IV.5 The effect of S10F-Hsp20 on cardiac function under the physiological stress of aging

IV.5.A Aging stress and cardiovascular system

Aging is found to be associated with a progressive decline in many physiological processes, causing an increased risk of health complications and disease. Particularly for the cardiovascular system, which delivers oxygen and nutrients to maintain the health of every tissue and longevity of the whole organism, aging has a significant effect on it, leading to an increase in cardiovascular diseases, such as atherosclerosis, myocardial infarction, and hypertension. Briefly, with age, the function of the heart is primarily affected by the decrease in elasticity and the capability to respond to alterations in pressure of the arterial system. The resultant increase in the resistance to the pumping action of the heart increases the work needed to drive the blood to different parts of the body. Therefore, with age, the LV contractility, sympathetic modulation of heart rate, and response to β-adrenergic stimulation decrease. Moreover, during aging, a significant decrease in absolute number of cardiomyocytes has been observed, which
results from apoptosis and necrosis and a reduction in repopulation of cardiomyocytes from cardiac stem cell reserves. In addition, with age, a decrease in the number and function of sinoatrial nodal pacemaker cells and a concomitant increase in conduction abnormalities were also observed, resulting in reduced heart rate variability and maximum heart rate in the aging heart. Besides, it was found that aging of the vasculature leads to augmented arterial thickening and stiffness as well as endothelium dysfunction, which causes increased systolic pressure and presents major risk factors for progress of atherosclerosis, hypertension, cardiac hypertrophy and arterial fibrillation.

IV.5.B Cardiac dysfunction and remodeling in the aging S10F-Hsp20 hearts

As shown in the aforementioned *in vitro* and *in vivo* studies, the enhanced contractile effects as well as the cardioprotective effects of WT-Hsp20 in the heart were abolished by the S10F mutation. Since loss of cardioprotection and chaperone-like activity by the Hsp20 mutant may result in remodeling under the physiological stress of aging, it was important to extend these studies to aging mice. Therefore, starting at 2 months, echocardiography was used to assess integrated LV performance and geometry in S10F hearts at bimonthly intervals. Our results showed that starting from the 6th month, EF% and FS% were significantly decreased in the S10F hearts, compared to NTG hearts. Particularly, at ages of 16 months, cardiac EF% and FS% in S10F mice were reduced by 49% and 43%, respectively, compared to the NTG mice. More importantly, starting from the 10th month, significant cardiac dilation was pronounced in the S10F-Hsp20 mice. At ages of 16 months, the LVESV, LVEDV, LVESD and LVEDD in
S10F-Hsp20 hearts were found to be increased by 99%, 33%, 44% and 18% respectively compared the NTG group. Notably, at 14 months of age, the ratios of HW/BW and LW/BW were also considerably increased in the S10F-Hsp20 mice compared to the NTG mice.

To determine cardiac hypertrophy and remodeling at the cellular level, cardiomyocyte cross-sectional area in S10F and NTG hearts was measured. It was found that, at 14 months of age, consistent with the increased HW/BW ratio, cardiomyocyte size was increased by 61% compared to the NTG cardiomyocytes. More importantly, the mRNA level of ANP was increased by 3 fold and the expression level of SERCA was reduced by 30% in the 14 months old S10F hearts, compared to the NTG hearts with same age.

These data above suggest that overexpression of S10F-Hsp20 remarkably depressed cardiac contractile function and accelerated the onset and progression of cardiac remodeling and heart failure under the physiological stress of aging.

**IV.5.C Increased cardiomyocyte apoptosis, cardiac fibrosis and accelerated death in the aging S10F-Hsp20 mice**

As described above, even under basal conditions, cardiomyocyte apoptosis was significantly increased in the S10F hearts compared to the NTG hearts. To determine the effects of S10F mutation on cardiomyocyte survival under the stress of aging, heart sections from the 2, 8 and 14 months old S10F mice were subjected to TUNEL staining assay. Surprisingly, increased cell apoptosis in S10F hearts was found to occur as early as 2 months of age, compared to the
NTG group. At ages of 14 months, the numbers of apoptotic cells in S10F hearts were increased to 2.2 fold, compared to the NTG hearts. Moreover, Masson’s Trichrome staining assay revealed that in the S10F hearts, fibrosis occurred as early as 8 months of age and was mostly pronounced at 14 months of age.

As shown above, severe cardiac dysfunction and remodeling were developed in the S10F mice, which were associated with increased cell death and fibrosis in the hearts. Thus, the survival rate was analyzed in the aging S10F and NTG mice. It was discovered that S10F mice died sporadically and suddenly, compared with their wild type littermates. The mean life span of S10F mice was found to be 13.7 months, which was significantly shorter than the mean life span of 19.7 months in NTG mice.

**IV.6 Hsp20 and cardiomyocyte apoptosis.**

**IV.6.A Cardiomyocyte apoptosis in heart failure**

Apoptosis (programmed cell death) is a critical physiological process involved in tissue development, differentiation and homeostasis, resulting in the removal of unwanted or damaged cells. Since the heart is a highly differentiated organ with limited regenerative capacity, initiation of this cell death mechanism is therefore strictly controlled. Under normal conditions, cardiomyocytes have a relatively high ability to resist the initiation of apoptosis, and thus exhibit a very low number of apoptotic events. However, under stress conditions, this cell death response is significantly increased and plays a detrimental role in cardiac injury. For instance, as stated above, during ischemia and reperfusion damage, ATP deprivation, oxygen radical accumulation and intracellular Ca$^{2+}$ overload can
induce a large amount of cardiac cell death, resulting in the loss of viable cardiac tissue which is difficult to replace. Under the physiological stress of aging, cardiomyocytes become more susceptible to oxidative stress resulting from the increased reactive oxygen species production with age, leading to increased rate of cardiomyocyte apoptosis in the aging hearts.

As cardiomyocytes possess a limited capacity of regeneration, augmented cardiac cell death can cause compromised cardiac contractile function, leading to heart failure in both the short and long term. Indeed, apoptosis has been observed in several animal models of heart failure and in failing human hearts. More importantly, strong scientific evidence from recent studies demonstrated that slight elevation of apoptotic events can lead to a lethal, dilated cardiomyopathy, suggesting that apoptotic cell death plays a pivotal role in the development of heart failure. Therefore, inhibiting the loss of the terminally differentiated cardiomyocytes is critical for the maintenance of normal cardiac function and the treatment of heart failure.

IV.6.B Hsp20 protects against stress-induced cardiomyocyte apoptosis

As a chaperone protein, Hsp20 has been shown to protect the heart against various stress (ie. I/R injury, β-agonist)-induced cardiomyocyte apoptosis by regulating multiple signaling pathways. Specifically, overexpression of Hsp20 in mouse heart improved cardiac functional recovery and promoted cardiomyocytes’ survival after I/R induced injury. Subsequent studies revealed that Hsp20 is able to interact with Bax, a key pro-apoptotic protein and prevent its translocation to mitochondria, which preserves the integrity of mitochondria, restricts the release
of cytochrome c, and represses activation of caspase-3. Therefore, the initiation of apoptosis mediated by caspase-3 is blocked. More importantly, further studies discovered that Hsp20 enhanced protection against I/R-induced damage also by associating with autophagy-related Beclin1 and thus preserving autophagy activity, which degrades and removes damaged mitochondria, therefore preventing activation of apoptosis. Moreover, Hsp20 was shown to protect against doxorubicin-induced apoptosis by interacting with Akt, thereby maintaining Akt activity, which retains the phosphorylation of Bad and thus represses caspase-3 mediated apoptosis.

IV.7 The S10F mutation abrogates the anti-apoptotic effects of Hsp20

IV.7.A Increased apoptosis in S10F cardiomyocytes

As the anti-apoptotic effects of Hsp20 in the heart are now well-recognized, it was critical for us to examine whether the mutant Hsp20 protein could elicit any potential alterations on programmed cell death. Thus, we overexpressed the S10F-Hsp20 and WT-Hsp20 in adult rat cardiomyocytes, using adenoviral technology and measured the number of these infected cardiomyocytes undergoing apoptosis induced by sustained β-agonist activation. It was found that, WT-Hsp20 protected cardiomyocytes from apoptosis, consistent with previous findings. However, this protection was significantly abrogated by the S10F mutation. Furthermore, the number of cardiomyocytes that underwent apoptosis was remarkably increased in S10F hearts compared to the NTG hearts, even under basal conditions. In addition, while determining the effects of S10F-Hsp20 overexpression on cardiomyocyte survival under the physiological stress
of aging, increased cardiomyocyte apoptosis in S10F hearts was observed to occur as early as the age of 2 months. At age of 14 months, the number of cardiomyocytes undergoing apoptosis in S10F hearts was increased to 2 fold compared to the NTG hearts. These data suggest that the anti-apoptotic effects of Hsp20 are completely abolished by the S10F mutation.

IV.7.B Mechanisms behind the effects of S10F-Hsp20 on cardiomyocytes apoptosis

IV.7.B.1 Deregulated apoptotic signaling cascades in the S10F-Hsp20 cardiomyocytes

Caspase-3 is a critical executioner of apoptosis, as it is responsible for the proteolytic cleavage of many key apoptotic proteins, such as caspases 6 and 7. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments. Particularly, elevated level of a fragment p17 of caspase-3 in the heart and the bloodstream is recognized as a sign of cardiomyopathy\textsuperscript{175}. Therefore, the protein levels of cleaved p17 fragment of caspase-3 were examined in heart homogenates from 2 months old S10F mice. It was discovered that the level of p17 fragment was increased by 24% in S10F hearts, compared to the NTG hearts. To confirm these results, caspase-3 activity was also measured in S10F and NTG heart homogenates. Indeed, Caspase-3 activity was augmented by 34% in S10F-Hsp20 hearts compared to the NTG hearts.

To further elucidate the mechanisms associated with increased apoptosis in S10F hearts, we assessed the cardiac protein levels of Bcl-2 (anti-apoptotic)
and Bax (pro-apoptotic), since both of these two proteins play key roles in regulating cardiomyocytes apoptosis. Briefly, studies have shown that cardiac specific overexpression of Bcl-2 or ablation of Bax significantly improved contractile function and attenuated cardiomyocyte apoptosis in the heart after I/R injury. Moreover, delayed ischemic preconditioning, on phenylephrine treatment of rabbit hearts, led to an increase in Bcl-2/Bax ratio and a substantial reduction of apoptosis\textsuperscript{208-210}. These findings imply that preserved Bcl-2 and reduced Bax protein levels are important mechanisms of inhibiting cardiomyocyte apoptosis. In this study, it was discovered that the expression level of Bax was significantly increased, whereas the level of Bcl-2 was greatly reduced in 2 month old S10F hearts, compared to NTG hearts. When the relative ratio of Bcl-2 to Bax expression was analyzed, it was found that this ratio was reduced by 61% in S10F hearts compared with the NTG group.

As described above, Hsp20 is able to interact with Bax and block the caspase-3 initiated apoptosis.\textsuperscript{183} To examine if the ability of Hsp20 to interact with Bax is modified by the S10F mutation, immunoprecipitation assay was performed in heart homogenates from S10F and NTG mice. Our results showed that the interaction of Hsp20 with Bax was considerably reduced in the S10F hearts, compared with the NTG group.

Akt, a serine/threonine kinase, is at the central hub of signaling pathways that regulates several cellular functions, such as survival, growth, and metabolism. A number of studies have shown that activation of Akt is cardioprotective and reduces cardiac dysfunction and stress-induced
apoptosis.\textsuperscript{187, 253} Actually, Akt has been shown to promote cardiac cell survival via its ability to phosphorylate the Bcl-x inhibitor BAD at Ser136, which inhibits the caspase-3 cleavage, resulting in attenuation of apoptosis.\textsuperscript{187, 253} Hsp20 was shown to interact with phosphorylated Akt, thereby maintaining Akt activity, which retains the phosphorylation of Bad and thus represses caspase-3 mediated apoptosis.\textsuperscript{187} To determine if the regulation of Hsp20 on this signaling pathway is altered by S10F mutation, the interaction of Hsp20 with Akt and the phosphorylation levels of Akt and Bad were examined in S10F-Hsp20 hearts using immunoprecipitations and western blotting. It was discovered that the interaction of Hsp20 with phosphorylated Akt was greatly reduced in the S10F hearts, which was associated with remarkable decrease in phosphorylation levels of Akt and Bad in comparison with the NTG hearts.

\textbf{IV.7.B.2 Impaired autophagy activity in the S10F TG hearts}

Autophagy is a process whereby cytosolic proteins and organelles are degraded and recycled. The common form of autophagy (macroautophagy) is a double membrane cytoplasmic vesicle, called autophagosome, which engulfs cellular components (ie. proteins, organelles). Then the autophagosomes fuse with lysosomes, forming autolysosomes, followed by degradation. The mechanism of autophagosome formation involves a type III PI3-kinase complex that contains the Vps34 kinase, Beclin 1 (Atg6), and the ultraviolet irradiation resistance–associated tumor suppressor gene (UVRAG).\textsuperscript{195} The Ambra1 (activating molecule in Beclin1-regulated autophagy) activates autophagy by interacting with Beclin 1, which stimulates Vps34, followed with the binding of Bif-1 (BAX-
interacting factor 1) to UVRAG, which prompts autophagic vesicle formation. The maturation of autophagosome is mediated by two ubiquitin-like conjugation systems (Atg12–Atg5 and LC3–phosphatidylethanolamine). The autophagosome travels via the cytoplasm of the cell to a lysosome, and the two organelles fuse to create autolysosome. While vesicle fusing occurs, lipid conjugation alters the soluble form of LC3 (LC3-I) to the autophagic vesicle-associated form (LC3-II). Therefore, LC3-II is recognized as a golden marker of autophagosome maturation, and Beclin as a marker of initiation. Together, the increase in both markers strongly supports increased levels of the process of autophagy.

The primary role of autophagy is to supply nutrients for survival. In addition, in order to maintain cell function, a low level of constitutive autophagy is also critical for controlling the quality of proteins and organelles. It has been well documented that constitutive autophagy in the heart under baseline conditions is a homeostatic mechanism for preserving cell size, cardiac structure and function. A defect in this pathway will lead to severe damage to the heart. For example, severe cardiac dysfunction and dilation were found in human patients with defective autophagic degradation, caused by a deficiency of the lysosomal-associated membrane protein-2. During cellular stress conditions, autophagy functions predominantly as a pro-survival pathway, which protects the heart against stress-induce injuries. Indeed, several lines of evidence have shown that autophagic activity was significantly elevated in cardiomyocytes under various stress conditions, including chronic ischemia, I/R injury, pressure
overload, dilated cardiomyopathy, and heart failure. Upregulation of autophagy is beneficial to the cell by recycling of proteins to produce free amino acids and fatty acids necessary for energy production, by clearing damaged organelles, and by avoiding accumulation of protein aggregates. Notably, excessive autophagy activation may induce cell death through self-digestion. It has been observed that many autophagosomes are formed in dying cells, however it is not clear whether autophagy directly causes cell death or is augmented as an effort to inhibit it.

As stated above, Hsp20 is able to protect the heart against stress-induced damage by preserving autophagy activity. To investigate if this beneficial effect is altered by S10F mutation, we examined the autophagy activity in S10F-Hsp20 hearts by measurement of the LC3-II/LC3-I ratio (conversion of cytosolic LC3-I to membrane-conjugated LC3-II is linked with the number of autophagosomes) and Beclin 1 protein levels, using NTG hearts as controls. It was observed that the ratio of LC3-II/LC3-I and expression levels of Beclin1 were significantly reduced in the 2 month old S10F hearts, compared to NTG hearts. These findings indicate that autophagy activity was considerably impaired in the S10F-Hsp20 hearts under baseline conditions. Since Hsp20 was reported to be able to regulate autophagy activity by interacting with Beclin1, we therefore examined if this interaction is altered by the S10F mutation. Thus, heart homogenates from S10F and NTG mice were immunoprecipitated with anti-Hsp20 and probed with anti-Beclin 1 antibody. It was found that the interaction between Hsp20 and Beclin1 was greatly reduced in S10F hearts compared to the NTG hearts.
In addition, the direct consequence of autophagy deficiency is found to be the intracellular accumulation of ubiquitinated proteins, which impairs cell function.\textsuperscript{257} For instance, it was demonstrated that reducing autophagy induced greater accumulation of polyubiquitinated proteins in cardiomyocytes, leading to cell apoptosis, heart failure and early mortality in mice, expressing a human CryAB\textsuperscript{120G212}. Therefore, the levels of ubiquitinated proteins in the 2 month old S10F hearts were also examined using western blotting. Indeed, it was observed that the levels of ubiquitinated proteins in S10F hearts were greatly increased compared to the NTG hearts.

These studies demonstrated that the ability of Hsp20 to interact with Bax, Akt and Beclin1 was considerably compromised by the S10F mutation, leading to deregulated apoptotic signaling cascades, suppressed autophagy activation and the resultant apoptosis even under basal conditions.

Taken together, our findings indicate that the S10F mutation alters conformational structure and the basic function of Hsp20, which renders it incapable of enhancing cardiac contractility and protecting against cell apoptosis. These defects contribute to the accelerated onset and progression of pathologic hypertrophy and heart failure in the S10F-expressing hearts under different stress conditions.

These studies are of high clinical significance, since our results suggest that patients carrying the S10F mutation may have weaker heart function and thus, may be intrinsically compromised in coping with various cardiac stresses. Elucidating the mechanisms underlying the potential role of S10F-Hsp20 in
cardiac function and protection may contribute to the development of novel clinical therapy for the treatment of cardiomyopathy in Hsp20 mutant carriers.

Section V: Future directions

The major limitation of the first study regarding the role of R25C-PLN in cardiomyocyte contractility and SR Ca$^{2+}$ cycling is that all experiment were performed in isolated cardiomyocytes or HEK293 cells, which are an ex vivo system. Therefore, expression of R25C-PLN in vivo will not only allow extension of our ex vivo findings to the intact heart level but may also yield insights into its potential role in cardiac pathogenesis. Based on our findings, hearts with expression of R25C-PLN may be more susceptible to cardiac dysfunction and ventricular arrhythmia. Therefore, these in vivo experiments may further advance our understanding of the role of the R25C-PLN mutant and its implications for human carriers.

For the second study regarding the role of S10F-Hsp20 in cardiac function and protection, it will be important to determine the effects of S10F substitution on the chaperone activity of Hsp20. This can be achieved by testing the ability of recombinant S10F-Hsp20 protein to inhibit the reduction-induced aggregation of insulin or heat-induced aggregation of yeast alcohol dehydrogenase, using WT-Hsp20 protein as a control. Furthermore, it will be important to determine infarct size in the S10F and WT hearts after ex vivo I/R injury and in vivo MI surgery (after 24 hours) and examine the mechanisms underlying the abolished protection against I/R and MI-induced injury in the S10F hearts. In addition, for the cardiac effects of S10F-Hsp20 under the physiological stress of aging,
studies should be extended to early time points (ie. one month old S10F mice) to elucidate the primary insult of S10F-Hsp20 in the heart. Moreover, another limitation is that transgenesis was utilized and the effects of the mutant were investigated in the presence of the endogenous Hsp20 protein. This may have complicated the observed phenotype although very useful and new information concerning the role of S10F mutation in cardiac function was obtained. To solve this problem, a tissue specific or global knock-in mouse model may be generated in the future. Furthermore, as a multifunctional stress protein, Hsp20 was recently found to be significantly increased in the hearts from exercise-trained rats, suggesting a critical role of Hsp20 in the exercise-induced remodeling and cardioprotection.\textsuperscript{258} Therefore, in our future studies, the role of S10F-Hsp20 in exercise-trained hearts may be investigated by conducting a tread mill running protocol\textsuperscript{259} on the S10F-Hsp20 mice. These studies will further provide valuable insights regarding the role of S10F-Hsp20 in mammalian hearts.

More importantly, to investigate the cardiac effects of R25C-PLN and S10F-Hsp20 in human carriers, induced pluripotent stem cells (iPSCs) may be generated from the patients harbouring the R25C or S10F mutations and differentiated into cardiomyocytes (iPSC-CMs). The patient-specific iPSC-CMs could recapitulate the disease phenotype and provide opportunities for novel insights into the underlying mechanisms of DCM pathogenesis in R25C or S10F carriers.

In addition, gene therapy approaches to reverse the cardiac dysfunction in R25C or S10F patients may be developed in the future. Particularly, as recently
reported by Karakikes et al.\textsuperscript{260}, combinatorial adeno-assisted vector (AAV)-mediated gene therapy to knockdown the endogenous \textit{PLN} or Hsp20 while expressing a codon-optimized human \textit{PLN} or Hsp20 could be utilized to reverse the abnormal phenotypes observed in patients affected by the R25C or S10F mutations.

\textbf{Section VI: Conclusion of the dissertation}

In this dissertation, we identified two novel human mutations in the major \ce{Ca^{2+}}-handling proteins, PLN and Hsp20, in DCM patients. De novo results suggest that both R25C and S10F mutations altered the basic function of PLN and Hsp20 respectively, resulting in disrupted SR \ce{Ca^{2+}} cycling and impaired cardiomyocyte contractility. Moreover, impaired \ce{Ca^{2+}}-handling by R25C-PLN also activates other detrimental pathways, leading to arrhythmias. In addition, the chaperoning activity of Hsp20 was also negated by S10F mutation, resulting in cardiomyocyte apoptosis under basal conditions. These findings suggest that both R25C and S10F mutations may be disease modifying mutations in human carriers and may be utilized as prognostic or diagnostic markers for dilated cardiomyopathy. In addition, in this dissertation, these “experiments by nature” not only further advanced our understanding of the basic functional significance of PLN and Hsp20 in the heart respectively, but also yielded insights into the roles of R25C and S10F mutations in cardiac pathogenesis. This may contribute towards better personalized treatment of cardiomyopathy in the PLN and Hsp20 mutation carriers.
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