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MOLECULAR MECHANISMS OF MYOSIN LIGHT CHAIN MUTATION-INDUCED CARDIOMYOPATHIES

By
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MOLECULAR MECHANISMS OF MYOSIN LIGHT CHAIN MUTATION-INDUCED CARDIOMYOPATHIES

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In this proposal, I have studied the HCM (hypertrophic cardiomyopathy) and DCM (dilated cardiomyopathy) disease causing mechanisms associated with mutations in the myosin regulatory (RLC) and essential (ELC) light chains. Specifically, four HCM mutations, RLC-A13T, RLC-K104E, ELC-A57G and ELC-M173V and one DCM mutation, RLC-D94A were studied. The RLC-A13T, RLC-K104E and ELC-A57G mutations were primarily investigated in transgenic (Tg) mice using in vitro and in vivo approaches, while RLC-D94A and ELC-M173V were studied in reconstituted system because of no Tg mice available in the laboratory (Specific Aims 1&2). In addition, the effects of RLC/ELC serine phosphorylation on the structure and function of the heart were examined (Specific Aim 3). Our studies indicated that RLC and ELC mutations lead to cardiomyopathy disease through different mechanisms and therefore resulted in different disease phenotypes. Specially, RLC mutations (exclude D94A) resulted in a classic HCM phenotype as left ventricular (LV) / septum hypertrophy and diastolic dysfunction. RLC mutations caused HCM or DCM through altering the secondary structure of the RLC, which further affected the structure (and function) of the lever arm domain imposing changes in the cross bridge cycling rate, myosin force generation ability and muscle relaxation. On the other hand, ELC mutations (e.g. A57G) caused a rare HCM phenotype as eccentric hypertrophy and
systolic dysfunction. As for the RLC, ELC mutations also exert their detrimental effects through altering the structure (and function) of the ELC, especially its N-terminus. These changes further affect the N-ELC-actin interactions and the cross talk between the thin and thick filaments resulting in altered force generation and the calcium sensitivity of force (Special Aim 1&2). In Specific Aim 3, we have examined the rescue effects of Serine15 phosphorylation in the RLC and Serine 195 phosphorylation in the ELC. For RLC, we observed a myosin light chain kinase (MLCK)-induced phosphorylation was able to rescue the abnormally high IFS observed in the K104E fibers. However, compromised force generation observed in K104E myocardium was not restored upon K104E phosphorylation. The effects of phosphorylation on the A13T and D94A RLC mutants are still to be studied. For ELC, since no ELC specific kinase exists, Serine195 was mutated to Aspartic Acid (S195D) to mimic ELC phosphorylation. The S195D-ELC protein partially restored the reduced \( V_{\text{max}} \) observed in M173V-ELC exchanged myosin and “corrected” the abnormally high calcium sensitivity of force in A57G-S195D and M173V-S195D exchanged porcine cardiac muscle fibers, to the level near WT. However, no effect of S195D on force generation was observed in ELC-exchanged fibers, presumably due to a harsh treatment with chemicals during ELC exchange. Thus, despite of some promising results, the effects of MLCs phosphorylation on the rescue of abnormal RLC and ELC function are still inconclusive. New strategies need to be applied to further investigate this MLC phosphorylation-mediated rescue mechanism.
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# TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... ix

LIST OF TABLES ........................................................................................................... xiii

ABBREVIATIONS ......................................................................................................... xiv

BACKGROUND .................................................................................................................1

SPECIFIC AIMS .................................................................................................................3

CHAPTER 1. INTRODUCTION ........................................................................................8

  Familial Hypertrophic Cardiomyopathy (HCM) ..............................................................8

  Dilated cardiomyopathy (DCM) ......................................................................................9

  Sarcomere structure and muscle contraction ..................................................................10

  Myosin and its light chains .............................................................................................11

  HCM/DCM-linked mutations in myosin RLC ...............................................................13

  HCM-linked mutations in myosin ELC .........................................................................15

  Significance of this Proposal ..........................................................................................16

CHAPTER 2. MATERIALS AND METHODS ...................................................................17

  Experimental Methods for Specific Aim 1 ....................................................................17

    Methods for experiments associated with human recombinant RLC proteins .........17

    Methods for experiments associated with Tg mice expressing RLC mutations ........23

  Experimental Methods for Specific Aim 2 ....................................................................33

    Methods for experiments conducted on Tg ELC mice .............................................33
Methods for experiments associated with human recombinant ELC proteins ..........37

Experimental Methods for Specific Aim 3 ...............................................................39

Methods for experiments associated with RLC Serine15 phosphorylation ..........39

Methods for experiments associated with ELC Serine195 pseudo-phosphorylation .40

Statistical Analysis: ...............................................................................................41

CHAPTER 3. RESULTS ............................................................................................42

Specific Aim 1: Disease causing mechanisms of RLC linked cardiomyopathies ....42

RLC-K104E linked HCM .......................................................................................42

RLC-A13T linked HCM .......................................................................................51

RLC-D94A linked DCM .......................................................................................54

Specific Aim 2: Disease causing mechanisms of ELC-linked cardiomyopathies ....58

ELC-A57G linked HCM .......................................................................................58

ELC-M173V linked HCM .....................................................................................66

Specific Aim 3: Does phosphorylation work as a common rescue mechanism for both
RLC and ELC mutants? .......................................................................................67

Phosphorylation-mediated mechanisms in myosin RLC ....................................67

Phosphorylation-mediated mechanisms underlying myosin ELC mutants ..........70

CHAPTER 4. DISCUSSION .....................................................................................73

Disease causing mechanisms of RLC-linked HCM and DCM ..........................73

RLC-K104E linked HCM .....................................................................................73
RLC-A13T linked HCM.............................................................................................77
RLC-D94A linked DCM ............................................................................................79
Disease causing mechanisms of ELC-linked HCM .................................................81
ELC-A57G linked HCM ...........................................................................................81
ELC-M173V linked HCM..........................................................................................86
Myosin light chain phosphorylation mediated rescue mechanisms ......................86
RLC Phosphorylation at Serine 15 ..........................................................................86
ELC phosphorylation at Ser 195 ..............................................................................89
CHAPTER 5. SUMMARY AND CONCLUSIONS .........................................................90
Comparison of the disease causing mechanisms for RLC and ELC linked
   cardiomyopathies ...........................................................................................................90
Comparison of the effects of serine phosphorylation on RLC and ELC mutations......94
CHAPTER 6. FUTURE DIRECTIONS .....................................................................97
Future Directions for Specific Aim 1 ...........................................................................97
Future Directions for Specific Aim 2 ..........................................................................98
Future Directions for Specific Aim 3 ..........................................................................98
LITERATURE CITED ....................................................................................................100
FIGURES .........................................................................................................................113
TABLES ..........................................................................................................................157
LIST OF FIGURES

Figure 1. Morphology of healthy, hypertrophic and dilated cardiomyopathy hearts. .....113

Figure 2. Structure of the sarcomere and the Ca^{2+}-Tn-Tm dependent muscle activation.................................................................................................................................114

Figure 3. Structure of myosin, myosin head (S1) and the lever arm domain. .............115

Figure 4. Amino acid sequences of myosin light chains and point mutations associated with HCM and DCM................................................................................................................116

Figure 5. Determination of protein expression in transgenic WT and K104E mice...........117

Figure 6. Effects of the K104E mutation on the CD spectra of the RLC. ......................118

Figure 7. Small angle X-ray diffraction measurements conducted in skinned papillary muscle fibers from Tg-WT and Tg-K104E mice..........................................................119

Figure 8. Steady state force (A), calcium sensitivity of force (B), muscle relaxation kinetics (C), and passive tension measurements (D) in skinned papillary muscle strips from Tg-WT and Tg-K104E (K104E) mouse hearts.................................................................120

Figure 9. Actin-activated myosin ATPase activity assay. ............................................121

Figure 10. Frictional load in vitro motility assay of Tg-WT and Tg-K104E mouse myosin. .................................................................................................................................122

Figure 11. Electron microscopy imaging and mitochondrial content assessment in Tg-WT and Tg-K104E mouse hearts.........................................................................................123

Figure 12. Histopathological assessment of left ventricular tissue from transgenic mice.................................................................................................................................124
Figure 13. Representative images of mitral inflow of 6 month-old Tg-K104E (A) and Tg-WT (B) mice. ..........................................................125

Figure 14. The Adrenergic Signaling in cardiomyocytes. ..............................................126

Figure 15. Scheme of the disease causing mechanism of RLC-K104E linked HCM. ....127

Figure 16. Far UV CD spectrum of recombinant human ventricular WT and A13T RLC under no metal conditions (Apo state). .................................................................128

Figure 17. Expression of A13T-RLC in transgenic mice. ..............................................129

Figure 18. Maximal steady-state force (A) and force-pCa measurements (B) in Tg-A13T, Tg-WT and NTg skinned muscle fibers.................................................................130

Figure 19. Actin-activated myosin ATPase activity. ...................................................131

Figure 20. Histopathological studies on~ 6 month-old A13T transgenic mouse hearts..132

Figure 21. Pedigree of a Caucasian family with DCM which carries the D94A mutation. ..........................................................................................................................133

Figure 22. Effect of the D94A mutation on the CD spectra of RLC. ..............................134

Figure 23. Modeled structure of human ventricular D94A-RLC using I-TASSER. ......135

Figure 24. The interaction of WT and D94A with the myosin heavy chain..................136

Figure 25. Actin-activated ATPase activity of WT and D94A reconstituted porcine cardiac myosins. .............................................................................................................137

Figure 26. Maximal force generation (A) and the force-pCa relationship (B) in skinned papillary muscle strips reconstituted with WT and D94A RLCs. .........................138
Figure 27. Analysis of protein expression in left ventricular extracts from different lines of Tg-WT and Tg-A57G mice. .................................................................139

Figure 28. Rigor and Relaxed stiffness measured in skinned Tg ELC-WT and Tg ELC-A57G mice fibers. .................................................................140

Figure 29. Measurements of steady-state force in skinned papillary muscle fibers from Tg-A57G vs. Tg-WT mice. .................................................141

Figure 30. Actin-activated myosin ATPase activity assay performed on Tg-WT-ELC and Tg-A57G mouse myosins. ......................................................142

Figure 31. Assessment of cardiac morphology and hypertrophy in Tg-A57G and Tg-WT mice..............................................................................143

Figure 32. Quantification PCR for detecting gene expression of HCM markers in both sedentary and exercised groups of WT and A57G. ..........144

Figure 33. Steady state force generation and pCa50 measurements in both sedentary and exercised groups of WT and A57G. ..........................145

Figure 34. Actin-activated myosin ATPase activity assay of WT, A57G and M173V ELC-exchanged porcine cardiac myosin preparations. ......146

Figure 35. Steady state force measurements in WT, A57G and M173V ELC exchanged skinned porcine cardiac papillary muscle fibers. ..............147

Figure 36. Scheme of the disease causing mechanism of ELC-A57G linked HCM. ......148

Figure 37. Determination of endogenous RLC phosphorylation level in Tg-WT and Tg-K104E hearts and the time course of RLC phosphorylation of WT and K104E RLCs. .149
Figure 38. Small angle X-ray diffraction measurements in phosphorylated and non-phosphorylated Tg-WT and Tg-K104E mouse papillary muscle fibers .......................150

Figure 39. Maximal force and the force-pCa relationship in non-phosphorylated and phosphorylated WT and K104E skinned papillary muscle fibers. ...............................151

Figure 40. Analysis of RLC phosphorylation in LV of Tg-WT and Tg-A13T mice.......152

Figure 41. Time course of RLC phosphorylation with human recombinant WT and D94A RLCs. ..............................................................................................................................153

Figure 42. Generation and purification of phospho-mimic (S195D) ELC proteins. ......154

Figure 43. The actin-activated myosin ATPase activity assay of ELC exchanged porcine myosin .........................................................................................................................155

Figure 44. Relative force and force-pCa relationship of WT, mutated and phospho-mimic (S195D) ELCs - exchanged skinned porcine papillary muscle fibers. .......................156
LIST OF TABLES

Table 1. Echocardiography and hemodynamic parameters in 6 month-old Tg-K104E vs. Tg-WT mice .........................................................................................................................................................................................157

Table 2. Echocardiography indices in senescent Tg-K104E vs. Tg-WT mice .............158

Table 3. Differential expression of important genes in various biological processes (WT vs. K104E, number of mice = 2 for each group) .............................................................................................................................159

Table 4. List of important pathways which was significantly affected (enrichment p value <0.05) (WT vs.K104E) .........................................................................................................................................................................................160

Table 5. Echocardiography parameters in Tg-A13T vs. Tg-WT mice .....................161

Table 6. Clinical information of pedigree with D94A-DCM ..................................162

Table 7. IFS and I_{1,1}/I_{1,0} intensity ratio measured on Tg-WT and Tg-A57G fibers under rigor state .........................................................................................................................................................................................163

Table 8. Echo analysis of Tg-A57G vs. Tg-WT mice ..............................................163

Table 9. Hemodynamic parameters derived from PV relations in Tg-A57G and Tg-WT mice .........................................................................................................................................................................................164

Table 10. IFS (in nm) and Intensity ratio (I_{1,1}/I_{1,0}) for short and long SL (average ±SE) of WT and K104E phosphorylated and non-phosphorylated fibers .................................................................165

Table 11. Statistical analysis of IFS and Intensity ratios for short and long SL (average ±SE) of WT and K104E phosphorylated and non-phosphorylated fibers ..................165

Table 12. Summary of structural and functional effects and disease-causing mechanisms for RLC and ELC mutations studied in this thesis proposal .........................................................166
ABBREVIATIONS

CaM-calmodulin
CD-circular dichroism
DCM-dilated cardiomyopathy
ECG-echocardiography
EF-ejection fraction
ELC—essential light chain of myosin
EM-electron microscopy
HCM-hypertrophic cardiomyopathy
IFS-interfilament lattice spacing
IVS-inter-ventricular septa
H&E-hematoxylin and eosin
KPr-potassium propionate
LV-left ventricle
MHC—myosin heavy chain
MT-mitochondria
MLCs—myosin light chains
MLCK-myosin light chain kinase
MyBP-C-myosin binding protein C
NTg-non transgenic
PKA-protein kinase A
PTM-post translational modification
P-V-pressure volume

RCM-restrictive cardiomyopathy

RLC- regulatory light chain of myosin

RV-right ventricle

SCD- sudden cardiac death

SERCA: sarcoplasmic reticulum Ca²⁺-ATPase

smMLCK: smooth muscle myosin light chain kinase

SR: sarcoplasmic reticulum

TFP: 10-\([3-(4-Methyl-Piperazin-1-YL)-Propyl]-2-Trifluoromethyl-10H-\)

Phenothiazine

Tg- transgenic

Tm- tropomyosin

Tn- troponin

WT- wild-type

3D –three dimensional
BACKGROUND

This proposal is focused on mutations in the myosin regulatory (RLC) and essential (ELC) light chains associated with hypertrophic (HCM) and dilated (DCM) cardiomyopathy. To date, more than 15 mutations in the RLC gene (MYL2) (Poetter, Jiang et al. 1996, Flavigny, Richard et al. 1998, Andersen, Havndrup et al. 2001, Kabaeva, Perrot et al. 2002, Morner, Richard et al. 2003, Richard, Charron et al. 2003, Richard, Charron et al. 2004, Hougs, Havndrup et al. 2005, Caleshu, Sakhuja et al. 2011, Luis Álvarez-Acosta 2014) and about 10 mutations in the ELC gene (MYL3) have been associated with HCM (Poetter, Jiang et al. 1996, Lee, Hwang et al. 2001, Olson, Karst et al. 2002, Morita, Seidman et al. 2005, Andersen, Hedley et al. 2012). In addition, clinical studies of Dr. Ray E. Hershberger from Ohio State University, identified a novel single point mutation in myosin RLC, D94A, as DCM causing mutation (Huang et al. FEBS J, under revision). The D94A-RLC mutation is the first and only one in MLC associated with DCM. Although mutations in both myosin light chains are rare, they are often associated with distinctive phenotypes and some of them lead to malignant outcomes. It is therefore of great importance to investigate the underlying mechanisms of MLC-linked cardiomyopathies. In addition to the knowledge of MLC-induced cardiac disease, this research aimed to provide basic science information regarding the role of both MLCs in cardiac muscle contraction.

The goal of my Ph.D. proposal was to determine the molecular roles of both light chains in cardiac muscle contraction and regulation, and to elucidate the mechanisms underlying the specific disease phenotypes. I focused on HCM-linked mutations (A13T, K104E), and DCM-linked mutation (D94A) in the RLC; and on HCM-linked mutations in the ELC (A57G, M173V). Finally, knowing more about the mechanisms of MLC-related
cardiomyopathies, I aimed to elaborate on potential rescue strategies to reverse or alleviate MLC induced cardiac disease. I hypothesized that mutations in both light chains significantly alter their molecular structures and consequently their specific functions leading to abnormally working sarcomeres and defective muscle contraction. As a consequence, hypertrophy/dilation of the ventricles and compromised heart performance could be observed. Furthermore, I hypothesized that all five MLC mutations may have an effect on MLC-dependent phosphorylation and used this mechanism in as potential rescue strategy to mitigate the mutant-elicited changes of structural and functional properties of the heart. Exogenous MLC phosphorylation and/or pseudo-phosphorylation were explored as potential rescue tools to treat hypertrophy-related cardiac phenotypes.
SPECIFIC AIMS

The cardiac muscle sarcomere is composed of the thick and thin filaments consisting mainly of myosin and actin–tropomyosin (Tm)-troponin (Tn), respectively. The myosin molecule is formed by two heavy chains (MHC) and two types of light chains: essential light chain (ELC) and regulatory light chain (RLC). Most mutations identified in both RLC and ELC have been implicated in familial Hypertrophic Cardiomyopathy (HCM), while latest clinical studies from our collaborator, Dr. Ray E. Hershberger’s lab, identified a novel mutation in RLC, D94A, as DCM (dilated cardiomyopathy)-causing mutation (Huang et al. FEBS J, under revision). The objective of my proposal is to elucidate and compare the mechanisms by which specific mutations in both types of myosin light chains compromise cardiac function and lead to distinctive disease phenotypes. Our ultimate goal is to look for potential strategies to rescue both HCM/DCM phenotypes. To achieve this goal, I pursued the following specific aims:

Specific Aim 1: Determine the functional consequences of HCM/DCM-linked mutations located in two distinct domains of myosin RLC and delineate the RLC-specific phenotypes of disease. The molecule of RLC contains one functional EF-hand Ca\(^{2+}\)-Mg\(^{2+}\) binding site, shown to be important for the Ca\(^{2+}\)-dependent regulation of contraction, and one MLCK-dependent phosphorylation site located at serine 15 (Ser15). Alterations in both sites might be associated with abnormalities in heart morphology and function. In this specific aim, I propose to study two HCM-linked mutations in myosin RLC, the N-terminal A13T (Alanine to Threonine) mutation and the C-terminal K104E (Lysine to Glutamic acid) mutation. In addition, I propose to study the DCM-linked D94A
(Aspartic Acid to Alanine) mutation in myosin RLC. Since all three mutations are localized in close proximity to the RLC’s two functional domains it is very likely that they may result in discrete changes in the RLC properties. These alterations may further result in abnormal structure of the lever arm and consequently changes in muscle sarcomere leading to changes in cardiac muscle contraction and HCM/DCM.

*I hypothesize that these RLC mutations may lead to cardiomyopathy through different mechanisms involving changes in RLC’s secondary structure, binding of RLC to MHC, changes in RLC phosphorylation (at Ser15) and/or changes in the interaction of the mutant myosin with actin at various levels of system complexity (e.g. myofibrils, muscle fibers, intact heart).* To test this hypothesis, I propose to: 1) Titrate the RLC-depleted porcine β-myosin with increasing concentrations of bacterially expressed RLC-WT (human ventricular RLC-wild type; Swiss-Prot: P10916), or HCM/DCM mutants to study the RLC-MHC binding properties (affinity, stoichiometry, kinetics); 2) Study the effect of these mutations on the binding of mutant myosin to actin under rigor conditions by fluorescence and light scattering (LS) measurements. 3) Examine their effects on force generation and cross bridge kinetics by conducting the force-pCa and muscle activation and relaxation measurements, and ATPase activity assays. 4) Examine the effects of these mutations on endogenous RLC phosphorylation at Ser15 by SDS-electrophoresis and immunoblotting using phospho-RLC-specific antibodies. These experiments are performed on transgenic mouse cardiac muscle preparations or in porcine β-myosin, myofibrils and fibers reconstituted with bacterially expressed RLC-WT and mutants.
Specific Aim 2: Examine the effects of N- and C-terminal HCM mutations in myosin ELC on the structure and function of muscle sarcomere and decipher the ELC-specific mechanisms of HCM. Cardiac myosin ELC (Swiss-Prot: P08590) does not bind Ca\(^{2+}\); however, its N and C termini have distinctive functions. The long and cardiac specific N-terminus of ELC was shown to regulate contraction by its direct interactions with actin (Kazmierczak, Xu et al. 2009), while the C-terminal region, containing Ser195 phosphorylation site, was implicated as functionally important for the ELC-mediated contractility in Zebrafish (Meder, Laufer et al. 2009). Based on these data, I propose to study two representative HCM ELC mutations, one located at its N-terminus, A57G (alanine to glycine); and the other at the C-terminus, M173V (Methionine to Valine). Since both N- and C-termini are located in close proximity to each other in the 3D structure of ELC, it is very important to determine whether they will cause similar or different functional phenotypes. I will first look at how these mutations affect a direct ELC-actin interaction and then the interaction of the mutant-myosin with actin, using different systems of complexity (actomyosin, myofibrils, muscle fibers, intact heart). In addition, similar to RLC, both mutations in the ELC are expected to affect its binding to the immediate binding partner, the MHC. An impaired ELC-MHC interaction may potentially lead to the abnormal sarcomere assembly and myofilament disarray, one of the features of HCM. I aimed to perform the following: 1) Execute direct ELC-actin binding experiments using F-actin in co-sedimentation assays or by fluorescence measurements using pyrene labeled F-actin; 2) Study of the ELC-MHC binding by incubation of porcine cardiac myosin with increasing concentrations of ELC-WT or ELC mutants to monitor the effect of mutations on the degree of exchange for endogenous ELC protein; 3) Use transgenic
mouse cardiac myosin/myofibrils/fibers to perform functional assays described in Specific Aim 1. As for the M173V mutant, the transgenic mice have not yet been generated in our lab. Therefore, the experiments were conducted on ELC-exchanged porcine cardiac myosin, myofibrils and skinned cardiac muscle fibers.

Specific Aim 3: Does phosphorylation work as a common rescue mechanism for both RLC and ELC mutants? Previous studies from our group have suggested that myosin light chain kinase (MLCK)-dependent RLC phosphorylation (at Ser15) is able to rescue or reverse several pathologic functional phenotypes introduced by HCM-linked RLC mutations. Moreover, phosphorylation of RLC was shown to increase the proximity of myosin heads to actin leading to an increased acto-myosin interaction, ATPase activity and myofilament Ca²⁺ sensitivity (Colson, Locher et al. 2010). Recent reports also showed that RLC phosphorylation can transfer myosin heads from the super-relaxed (SRX) state into the relaxed state increasing thermogenesis and having cardioprotective function (Hooijman, Stewart et al. 2011). Latest work from Dr. James Stull’s lab revealed that cardiac MLCK-dependent RLC phosphorylation in vivo may prevent the development of hypertrophic phenotype (Ding, Huang et al. 2010, Kamm and Stull 2011). All these data strongly suggest that RLC phosphorylation may indeed play a protective and/or rescuing role in cardiac muscle contraction in HCM-mutated hearts. For DCM, recent report from Dr. Ju Chen’s lab demonstrated that the non phosphorylatable RLC mimic (Ser mutated to Ala) resulted in a typical DCM phenotype in Tg mice, suggesting that RLC phosphorylation may also be important in DCM disease (Sheikh, Ouyang et al. 2012). Similar to RLC, it has been shown that ELC can also be phosphorylated. Proteomic studies from the Van Eyk’s group
have demonstrated two potential phosphorylation sites in myosin ELC (Thr64 and Ser194 or 195) determined in pharmacologically preconditioned myocardium in vivo (Arrell, Neverova et al. 2001). Although the ELC-specific kinase has not yet been identified, work from our lab and others have shown that ELC can be phosphorylated with the Ca\(^{2+}\) -calmodulin (CaM) activated MLCK (Morano, Rosch et al. 1990). In the recent study by Meder et al., it was also seen that a pseudo-phosphorylated ELC mimic (Ser195Asp) was able to restore impaired contractility in the C-terminally-ELC truncated cardiomyocytes (Meder, Laufer et al. 2009). Interestingly, the lack of recovery of contractility was observed in cardiomyocytes transfected with non-phosphorylated ELC-WT. Based on these results, I hypothesize that phosphorylation may work as a common mechanism for both MLCs to reverse or alleviate the mutation induced HCM/DCM phenotype(s). To test this hypothesis, transgenic mouse cardiac muscle preparations (myosin, myofibrils or fibers) were phosphorylated in vitro by Ca\(^{2+}\)-CaM-MLCK and functional studies from Specific Aims 1 and 2 were performed to examine whether impaired functions due to all studied HCM/DCM mutations in RLC and ELC could be rescued by MLCs phosphorylation. We also aimed at elucidating the underlying mechanisms.
CHAPTER 1. INTRODUCTION

**Familial Hypertrophic Cardiomyopathy (HCM)**

HCM is an autosomal dominant disease characterized by ventricular hypertrophy (Fig. 1), myofibrillar disarray (Maron, Bonow et al. 1982) and is considered the leading cause of sudden cardiac death (SCD) among athletes and young adults under the age of 30 (Maron, Gardin et al. 1995, Marian and Roberts 1998). Knowing the impact of HCM, it is of great importance to discover therapeutic approaches to treat this disease. Genetic studies have linked HCM to the mutations in genes encoding for all major sarcomeric proteins, including β-myosin heavy chain (44% of all mutations), myosin binding protein C (35%), troponin T (7%), troponin I (5%), α-tropomyosin (2.5%), regulatory light chain (RLC) (2%), essential light chain (ELC) (1.6%) troponin C (~1%), α-actin (1%) and titin (<1%) (Reviewed in (Alcalai, Seidman et al. 2008)). To date, more than 15 mutations in the RLC gene (**MYL2**) (Poetter, Jiang et al. 1996, Flavigny, Richard et al. 1998, Andersen, Havndrup et al. 2001, Kabaeva, Perrot et al. 2002, Morner, Richard et al. 2003, Richard, Charron et al. 2003, Richard, Charron et al. 2004, Hougs, Havndrup et al. 2005, Caleshu, Sakhuja et al. 2011, Luis Álvarez-Acosta 2014) and about 10 mutations in the ELC gene (**MYL3**) have been associated with HCM (Poetter, Jiang et al. 1996, Lee, Hwang et al. 2001, Olson, Karst et al. 2002, Morita, Seidman et al. 2005, Andersen, Hedley et al. 2012). Previous studies from our lab as well as others have shown that mutations in both, myosin RLC (Szczesna, Ghosh et al. 2001, Szczesna-Cordary, Guzman et al. 2004, Szczesna-Cordary, Guzman et al. 2005, Dumka, Talent et al. 2006, Wang, Xu et al. 2006, Abraham, Jones et al. 2009, Greenberg, Watt et al. 2009, Kerrick, Kazmierczak et al. 2009, Muthu, Mettikolla et al. 2010) and ELC (Hernandez, Jones et al. 2007, Kazmierczak, Xu et al. 2009, et al. 2010).
2009) can disrupt normal interactions between mutant myosin with actin and other sarcomeric proteins, thus causing dysfunction of myofilaments and ultimately the heart leading to HCM.

**Dilated cardiomyopathy (DCM)**

Unlike HCM, DCM is characterized by ventricular dilatation (Fig. 1) and diminished contractile function (Schonberger and Seidman 2001). The clinical symptom of DCM is heart failure (HF), which is often associated with arrhythmia and sudden death (Hershberger, Hedges et al. 2013). DCM is one of the leading causes of HF with high morbidity and mortality and the prevalence is 1/2500 individuals (Millat, Bouvagnet et al. 2011). DCM can be caused by a variety of factors, including ischemia, alcohol toxicity, viral infections, as well as genetic factors (Gomes, Venkatraman et al. 2005, Szczesna-Cordary, Morimoto et al. 2012). Among these factors, it is estimated that 25% to 48% of all DCM cases are due to familial and genetic factors (Kamisago, Sharma et al. 2000, Millat, Bouvagnet et al. 2011). Parvari and Levitas have reported that ~ 35% of DCM patients have a family history of autosomal dominant inheritance, although some familial cases could be explained by autosomal recessive or X-linked recessive traits (Parvari and Levitas 2012). Up to date, more than 30 genes encoding sarcomeric, cytoskeletal, and nuclear proteins are associated with DCM (Dellefave and McNally 2011). Among those are mutations in at least eight genes encoding for sarcomeric proteins: β-MHC, α-MHC, MyBP-C, titin, cardiac actin, α-Tm and troponins (Perriard, Hirschy et al. 2003, Piran, Liu et al. 2012).
Sarcomere structure and muscle contraction

The sarcomere, a basic unit of muscle contraction comprises of two different filament types, the thick filaments and the thin filaments (Fig. 2A). The thick filaments are composed of myosin and structural proteins such as myosin binding protein C and titin. The thin filaments are made of polymerized actin (F-actin) and the regulatory proteins, including troponin C (TnC), troponin T (TnT), troponin I (TnI) and α-tropomyosin (α-Tm). Contraction of cardiac muscle is initiated by binding of calcium released from the sarcoplasmic reticulum (SR) to TnC, initiating multiple interactions between TnI, TnT, α-Tm and actin. These interactions cause an axial translocation of the Tn-Tm complex on the actin filament to facilitate the binding of myosin heads and force generation (Fig. 2B). This process is coupled with the hydrolysis of ATP within the catalytic site of the myosin head and formation of the actin-myosin (AM)-ADP-Pi complex (weakly attached cross-bridges). It is postulated that the power stroke occurs at the step of inorganic phosphate (Pi) release, when myosin cross-bridges bind to actin with high affinity (AM-ADP) (strongly attached cross-bridges) (Rayment, Rypniewski et al. 1993, Geeves and Holmes 2005) (Fig. 2). Release of the second hydrolysis product, ADP, leads to rigor acto-myosin (AM) bonds (Fig. 2). Binding of a new ATP molecule dissociates myosin heads from actin and the cross bridge cycle repeats (Fig. 2). Together, muscle contraction mainly depends on the interaction between two major sarcomeric proteins, myosin and actin and it is regulated by the tropomyosin-troponin complex in the Ca$^{2+}$ dependent manner. The energy released from the hydrolysis of ATP (at the myosin head) is used for the mechanical work and muscle contraction.
Myosin and its light chains

The role of myosin in muscle contraction is to form thick filaments and interact with the thin filaments, hydrolyze ATP and produce force and movement. Myosin molecule is composed of three major structural domains (Rayment, Rypniewski et al. 1993): 1) the motor domain, containing actin and ATP binding sites; 2) the lever arm domain, containing myosin ELC and RLC (both domains form the myosin head also called S1); and 3) the tail region, which is responsible for filament formation (Fig. 3). Myosin lever arm, also called the regulatory domain is composed of an 8.5 nm long α-helix containing two IQ motifs (IQX3 RGX3-4R) and is linked to the motor domain by a short converter region (Rayment, Rypniewski et al. 1993). Myosin light chains, ELC and RLC are attached to the two consecutive IQ motifs within the lever arm (Fig. 3). Considering the localization of both MLCs in the myosin head, it is conceivable that any mutations in either RLC or ELC may obstruct the function of the lever arm to amplify small conformational changes originated in the myosin motor domain into large movements needed to produce motion and force (Geeves 2002).

The RLC molecule attaches to the MHC IQ motif and structurally supports the lever arm domain (Rayment, Rypniewski et al. 1993, Geeves 2002). As an EF-hand Ca\(^{2+}\)-binding protein, RLC contains its one Ca\(^{2+}\)-Mg\(^{2+}\) binding site with the helix-loop-helix motif, which can be occupied by either Ca\(^{2+}\) or Mg\(^{2+}\). It has been postulated that during cardiac muscle contraction, this site could act as a delayed Ca\(^{2+}\) buffer helping the SR to sequester Ca\(^{2+}\) during diastole (Wang, Xu et al. 2006, Szczesna-Cordary, Jones et al. 2007). Furthermore, the conformation of the RLC and presumably the RLC-MHC interface can be affected by the binding of metal to this Ca\(^{2+}\)-Mg\(^{2+}\) site (Lowey and Risby 1971, Alexis and Gratzer...
Another functionally important domain of RLC is the myosin light chain kinase (MLCK) dependent phosphorylation site at Ser15 (Fig. 3C and 4). Numerous studies have shown that RLC phosphorylation can regulate the inotropic state of the heart and therefore any change in RLC phosphorylation is expected to cause abnormal heart performance, presumably through morphological and/or functional alterations (change in force, myofilament calcium sensitivity, ATPase activity, etc.) (Sweeney, Bowman et al. 1993, Morano 1999, Huang, Shelton et al. 2008). Recent reports from many have revealed that cardiac MLCK-dependent RLC phosphorylation in vivo may prevent the development of hypertrophic phenotype (Ding, Huang et al. 2010, Kamm and Stull 2011, Sheikh, Ouyang et al. 2012, Warren, Briggs et al. 2012). In summary, both the calcium binding domain and the phosphorylation site are crucial for the RLC function and it is anticipated that HCM-linked mutations in RLC could exert their action through alterations in the working of these two important RLC domains leading to dysfunctional RLC.

Myosin essential light chain (ELC) is also attached to the MHC IQ motif (Fig. 3C) and together with myosin RLC provides the structural stability to the myosin lever arm domain (Rayment, Rypniewski et al. 1993). However, the function of ELC in the regulation of muscle contraction is still not well understood. Although ELC also belongs to the group of EF-hand calcium binding proteins, its muscle isoform does not bind Ca$^{2+}$. While, the C-terminus of ELC shares a high sequence homology with other ELC proteins, its N-terminus is highly variable (Xie, Harrison et al. 1994, Timson 2003, Hernandez, Jones et al. 2007, Kazmierczak, Xu et al. 2009). The cardiac specific ELC isoforms contain a unique N-terminal extension present in both the ventricles and atria. It has been suggested by various biochemical and structural studies that this positively charged N-terminus of ELC makes
contact with the negatively charged C-terminus of actin and that this interaction may be important in muscle contraction (Xie, Harrison et al. 1994, Timson 2003, Hernandez, Jones et al. 2007, Kazmierczak, Xu et al. 2009). We hypothesize that the N-terminus of ELC acts as a tether and aids in pre-positioning the myosin heads for attachment to actin and facilitates the thin filament cooperative transitions and strong cross-bridge formation. The C-terminal domain of ELC contains the MHC binding site, and it may also interact with RLC. In addition, it has been postulated that threonine and serine phosphorylation of ELC may also have an impact on the ELC structural and functional properties. Proteomic analysis from Van Eyk’s lab has revealed that the residues Thr64 and Ser194 and/or Ser195 of ELC can be phosphorylated in pharmacologically preconditioned cardiomyocytes although no specific ELC-kinases have been found responsible for this phosphorylation (Arrell, Neverova et al. 2001). It is speculated that the Ca^{2+}-CaM activated MLCK could be involved in phosphorylation of both RLC and ELC (Morano, Rosch et al. 1990). In another study in Zebrafish, it was shown that a pseudo phosphorylation mimic of ELC (Ser195Asp) could rescue the lost contractility in a C-terminal ELC truncated Zebrafish cardiomyocytes (Meder, Laufer et al. 2009), which suggested a potential beneficial effects of ELC phosphorylation.

**HCM/DCM-linked mutations in myosin RLC**

There are two different phenotypes found in HCM-patients harboring the RLC mutations. The first rare type (A13T, E22K and P95A) is associated with a particular subtype of cardiac hypertrophy characterized by mid left ventricular obstruction (Poetter, Jiang et al. 1996, Andersen, Havndrup et al. 2001, Kabaeva, Perrot et al. 2002, Hougs, Havndrup et al. 2005) The second more “classical” HCM phenotype is caused by F18L, N47K, R58Q,
K104E and D166V-RLC mutations and is manifested by thickening of the left ventricular (LV) wall and abnormal ECG findings (Flavigny, Richard et al. 1998, Andersen, Havndrup et al. 2001, Kabaeva, Perrot et al. 2002, Morner, Richard et al. 2003, Richard, Charron et al. 2003, Richard, Charron et al. 2004, Hougs, Havndrup et al. 2005). Two RLC mutations, A13T and K104E were studied in this proposal (Fig. 4). Though present at opposite ends of the regulatory light chain in the 3D structure, these two mutations lie in close proximity to each other and to the calcium binding and phosphorylation sites of RLC (Fig. 3C). Studies from our lab show that HCM RLC mutations could cause changes in RLC secondary structure, Ca$^{2+}$ binding, phosphorylation (Szczesna, Ghosh et al. 2001) as well as MHC-RLC binding, acto-myosin interactions and ultimately cause changes in muscle contraction (Szczesna-Cordary, Guzman et al. 2004, Wang, Xu et al. 2006, Szczesna-Cordary, Jones et al. 2007). For A13T, it was shown that unphosphorylated A13T had a 3-fold lower $K_{Ca}$ than WT, while its phosphorylation led to a 6-fold increase in $K_{Ca}$ (Szczesna, Ghosh et al. 2001). For the K104E mutant of RLC, the unpublished data from the lab showed no binding affinity to the 22 amino acid peptide derived from the sequence of MHC containing the IQ-RLC binding motif (Szczesna-Cordary, Jones et al. 2004). However, the comprehensive mechanism as to how these mutations affect the RLC properties, acto-myosin interaction and ultimately lead to HCM is still lacking, and this proposal is aimed to further investigate this.

Clinical studies of Dr. Ray E. Hershberger from Ohio State University, identified a novel single point mutation in myosin RLC, D94A, as DCM causing mutation (Huang, et al. FEBS J, under revision). The D94A-RLC mutation is first and only one in MLCs associated with DCM. The probands were from a Caucasian family with typical DCM phenotype as
enlarged LVs and systolic dysfunction (characterized as decreased EF), and all carriers are heterozygous and showing phenotypes, implying D94A causing DCM through an autosomal dominant fashion. It is was noting that other two RLC mutations: P95A (only one amino acids away from D94 site) (Poetter, Jiang et al. 1996), and D166A (also D to A mutation) (Luis Álvarez-Acosta 2014) both causes HCM, which suggests that the negatively charged Aspartic Acid at the site 94 playing a very special role in regulating RLC structure and function.

_HCM-linked mutations in myosin ELC_

The majority of the HCM-linked ELC mutations (Poetter, Jiang et al. 1996, Lee, Hwang et al. 2001, Olson, Karst et al. 2002, Morita, Seidman et al. 2005) are located in the C-terminus of the ELC (E143K (which is RCM causing), M149V, E152K, R154H, H155D and M173V) while four (E56G, A57G, V79I, R81H) are located near its N-terminus. In this application, I focused on two representative ELC mutations, A57G and M173V (Fig. 4) located on the two opposite termini of ELC. The A57G mutation was discovered in Korean and Japanese patients (Lee, Hwang et al. 2001) and was shown to cause a classic asymmetric septal hypertrophy and SCD. The M173V mutation was discovered in an adult proband who was diagnosed with HCM as a child, but the clinical information on the specific disease phenotype is very limited (Morita, Seidman et al. 2005). This proposal will investigate the functional consequences of these two ELC mutations and how they may lead to the development of HCM.
Significance of this Proposal

This study contributes to a better understanding of how mutations in different regions of two myosin light chains can manifest into potentially different functional and clinical phenotypes. It also shows the role of phosphorylation as a potential common rescue mechanism for the RLC and ELC induced cardiomyopathy that could ultimately lead to the development of target-specific treatments.
CHAPTER 2. MATERIALS AND METHODS

Experimental Methods for Specific Aim 1

Methods for experiments associated with human recombinant RLC proteins

Cloning, expression and purification of recombinant human ventricular WT and mutated RLC proteins

The cDNA for wild-type (WT) human cardiac RLC (hcRLC) was cloned by reverse transcription-PCR using primers based on the published cDNA sequence (GenBankTM accession number AF020768) and standard methods (Szczesna, Ghosh et al. 2001). The A13T, D94A and K104E-RLC mutants were generated using overlapping sequential polymerase chain reaction as described earlier (Szczesna, Ghosh et al. 2001). The cDNAs of WT and mutant RLCs were transformed into BL21 expression host cells, and proteins expressed in 16-liter large cultures. Expressed proteins were purified using S-Sepharose column followed by Q-Sepharose column. S-Sepharose column was equilibrated with 2M urea, 20mM Citrate, 0.1mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.02% NaN₃, pH 6.0. For Q-Sepharose purification the following buffer was used: 2M urea, 25 mM Tris-HCl, pH 7.5, 0.1 mM PMSF, 1 mM DTT and 0.02% NaN₃. The proteins were eluted with an 800 ml salt gradient of 0–450 mM KCl. The final purity of the proteins was tested using 15% SDS-PAGE. Protein were stored in Q-Sepharose buffer in -20°C for further use.

Circular dichroism (CD) measurements for secondary structure of RLCs

Far-UV circular dichroism (CD) spectra were obtained using a 1-mm-path quartz cell in a Jasco J-720 Spectropolarimeter. Spectra were recorded at 190–250 nm with a bandwidth
of 1 nm at a speed of 50 nm/min and a resolution of 0.5 nm as described in Szczesna et al. (Szczesna, Ghosh et al. 2001). The measurements were performed at 22 °C in 30 mM NaCl, 0.3 mM EGTA, 0.7 mM MgCl₂, and 3 mM Tris-HCl buffer at pH 7.4. Analysis and processing of data were done using the Jasco system software (Windows standard analysis, version 1.20). Ten scans were averaged, baselines were subtracted, and no numerical smoothing applied. Spectra were presented as mean residue ellipticity and the values of mean residue ellipticity ([θ]_{MRE}, in degree × cm²/dmol) were calculated as shown previously (Szczesna, Ghosh et al. 2001). The α-helical content was calculated using [θ]_{222}:

$$[\theta]_{222} = -30,300 f_H - 2340 \quad (\text{Eq. 1})$$

where $f_H$ is the fraction of α-helix (in 0-1 scale). $f_H \times 100$ indicated the percent of α-helical in WT and mutated proteins.

**Secondary structure prediction of RLCs**

The secondary structure prediction was conducted with the I-TASSER online server from Zhanglab at the University of Michigan, following instructions on: http://zhanglab.ccmb.med.umich.edu/I-TASSER/. In brief, the amino acid sequences of WT and mutated RLC were compared against template proteins of similar structure chosen from the PDB library. The full length protein was assembled from excised fragments and simulated into the lowest energy model using specific algorithms. The confidence of each predicted model structure was presented as C-score, ranging from -5 to 2. The quality of prediction was proportional to the C-score (Zhang 2008, Roy, Kucukural et al. 2010, Roy, Yang et al. 2012). The predicted structures were then modeled using PyMOL molecular visualization system allowing determination of the distances, in Angstroms (Å), between C-α of the neighboring amino acids. Predicted structures WT and mutated RLCs were also
compared and superimposed using PyMOL to look into detail the conformational changes caused by RLC mutations.

**Depletion of native RLC from porcine cardiac myosin and reconstitution with increasing molar ratios of WT- and mutated-RLCs**

Porcine cardiac myosin was purified as described in Pant et al. (Pant, Watt et al. 2009). Endogenous RLC was depleted from porcine myosin by incubation of myosin (~1.5mg/ml) with 1% Triton X-100 and 5 mM CDTA (1, 2-cyclohexylenedinitrilotetraacetic acid), pH 8.5 for 30 minutes at room temperature. The Triton/CDTA-treated myosin was precipitated in 1 mM ice cold dithiothreitol (DTT) and collected by centrifugation at 8000xg in 4°C for 10 min. Myosin depleted of endogenous RLC was then re-suspended in RLC reconstitution buffer (0.4M KCl, 50mM MOPS, pH 7.0, 2mM MgCl₂, and 1mM DTT) and titrated with increasing concentrations of WT or D94A. The molar ratio of RLC to depleted myosin ranged from 0.1 to 5. Titrations were performed in the presence of BSA to prevent nonspecific RLC binding. The mixtures were incubated for 30 minutes at room temperature and left to precipitate in 13 volumes of ice-cold 1mM DTT for 30 min. Protein complexes were then pelleted by centrifugation (8000 x g at 4°C for 10 minutes) and dissolved in small volumes of 3M KCl (~20 µl) and ultracentrifuged at 200,000 x g in 4°C for 45 min. The supernatant was mixed 1:1 ratio with SDS-PAGE sample buffer with ¼ volume of β-ME, heated at 95°C for two minutes and loaded into SDS-PAGE. Coomassie stained gels were scanned and the bands were quantified using the Odyssey Infrared Imaging System. The essential light chain of myosin (ELC), not extracted under experimental conditions used (Pant, Watt et al. 2009), served as a loading control and the ELC/RLC band ratios

between the reconstituted and native (untreated) myosin preparations were used to
determine the degree of reconstitution with WT or mutated RLC proteins.

**Human recombinant RLC mutant- reconstituted myosin**

Rabbit skeletal F-actin was prepared according to Kazmierczak et al. (Kazmierczak, Xu et
al. 2009). The RLC-depleted porcine myosin, obtained as described above, was incubated
with 3 molar excess of human recombinant ventricular WT or mutated RLCs and the
mixtures were dialyzed for 2 h against the reconstitution buffer (composition as above).
The protein complexes were dialyzed overnight in the cold room against 5 mM DTT. The
reconstituted myosins were collected by centrifugation at 8000 x g for 10 minutes and the
pellets were resuspended in the myosin ATPase buffer (0.4 mM KCl, 10 mM MOPS, pH
7.0, 1 mM DTT). This procedure yielded myosin fully reconstituted with WT- and mutated
RLCs. The myosins were further dialyzed into myosin ATPase buffer overnight preparing
for actin activated myosin ATPase assay.

**Actin activated (RLC reconstituted porcine cardiac) myosin ATPase activity assay**

Actin-activated RLC exchanged porcine cardiac myosin ATPase activity was measured as
a function of actin concentration and the data analyzed as described in detail in
(Kazmierczak, Xu et al. 2009). Briefly, myosin at ~10 µM starting concentration was added
to the 96-well microplate and then mixed with increasing concentrations of F-actin (in µM):
0.1, 2.5, 5, 7.5, 10, 15, 20 and 25. The assay was performed in a 120 µl reaction volume in
a buffer consisting of 25 mM imidazole, pH 7.0, 4 mM MgCl₂, 1 mM EGTA, and 1 mM
DTT. The final KCl concentration was 107 mM. The final myosin concentration is ~1.7uM.
Protein mixtures were first incubated on ice for 10 minutes and then for another 10 minutes
at 30°C. The reactions (run in triplicates) were initiated with the addition of 2.5 mM ATP
with mixing in a Jitterbug incubator shaker (Boekel), allowed to proceed for 20 min at
30°C and then terminated by the addition of 5% trichloroacetic acid. Precipitated proteins
were cleared by centrifugation and the inorganic phosphate was determined using the Fiske
Subbarow method (Fiske and Subbarow 1925). Data were analyzed using the Michaelis–
Menten equation yielding the V_max and K_m parameters (Hanson, Ling et al. 1967, Trybus
2000).

_Depletion of native RLC from porcine cardiac muscle strips and reconstitution with WT-
and mutated-RLCs_

Freshly isolated porcine hearts were placed in oxygenated physiological salt solution of
140 mM NaCl, 4 mM KCl, 1.8 mM CaCl_2_, 1.0 mM MgCl_2_, 1.8 mM NaH_2PO_4_, 5.5 mM
glucose, and 50 mM Hepes buffer, pH 7.4. The papillary muscles of the left ventricles were
isolated, dissected into muscle bundles of about 20x3 mm, and chemically skinned in a 50%
glycerol, 50% pCa 8 solution (10^{-8} M [Ca^{2+}], 1 mM [Mg^{2+}], 7 mM EGTA, 5 mM [MgATP],
20mM imidazole, pH 7.0, 15 mM creatinine phosphate; ionic strength=150 mM adjusted
with potassium propionate (KPr)) containing 1% Triton X-100 for 24 h at 4 °C. Then the
strips were transferred to the same solution without Triton X-100 and stored at -20 °C for
about 2 months.

Depletion of endogenous RLC from porcine cardiac muscle preparations was achieved in
RLC depletion buffer, with 5 mM CDTA, 40 mM Tris, 50 mM KCl, 1 µg/ml pepstatin A,
0.6 mM NaN_3_, 0.2 mM PMSF, and 1% Triton X-100, pH 8.4. Briefly, small muscle strips
of approximately 1.4 mm in length and 100 µm in diameter were attached by tweezer clips
to a force transducer. The strips were placed in a 1 ml cuvette and freshly skinned in 1%
Triton X-100 dissolved in pCa 8 buffer for 30 min. They were rinsed 3 times x 5 min in
pCa 8 buffer and then incubated in RLC depletion buffer for 5 min at room temperature and transferred to the fresh solution of the same composition for another 30 min for RLC depletion. The extent of RLC extraction was tested by SDS-PAGE.

Reconstitution of the RLC-depleted muscle strips with WT or mutants (and porcine cardiac TnC) was performed in pCa 8 solution containing 40 µM RLC and 15 µM TnC. The solution of TnC was included in the reconstitution protein mixture during the first 30 min of fiber incubation followed by a 30 min incubation with fresh 40 µM RLC at room temperature. The addition of cTnC was to assure that the strips were not deficient of TnC, because its partial extraction could affect the Ca$^{2+}$ sensitivity of force development. Reconstituted strips were then washed in pCa8 solution for 3 times (5 min each) and subjected to force measurements.

**Steady-state force development**

The RLC-mutant/WT-reconstituted muscle strips were tested for maximal steady state force development in pCa 4 solution (composition is the same as pCa 8 buffer except the [Ca$^{2+}$] =10$^{-4}$ M). Maximal force readings (in pCa 4) were taken and the tension per cross-section of muscle strip was calculated and expressed in kN/m$^2$. The cross sectional area of the muscle strip was assumed to be circular and the diameter of the muscle strip was taken at 3 points along the fiber and averaged.

**The Ca$^{2+}$ dependence of force development**

After the initial steady state force was determined, RLC exchanged muscle strips were relaxed in pCa 8 buffer and exposed to solutions of increasing Ca$^{2+}$ concentrations from pCa 8 to pCa 4 (Dweck, Reyes-Alfonso et al. 2005). The level of force was measured in each "pCa" solution. Data were analyzed using the Hill equation (Hill 1980), where
"[Ca\textsuperscript{2+}]_{50} or pCa_{50}" is the free Ca\textsuperscript{2+} concentration which produces 50% of the maximal force and n_H is the Hill coefficient. The pCa\textsubscript{50} represents the measure of Ca\textsuperscript{2+} sensitivity of force and the n_H is the measure of myofilament cooperativity.

**Methods for experiments associated with Tg mice expressing RLC mutations**

*Generation of transgenic K104E (Tg-K104E) mice*

All animal studies were conducted in accordance with institutional guidelines. The University of Miami has an Animal Welfare Assurance (A-3224-01, effective November 23, 2011) on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. We have generated transgenic mouse models for the wild-type human ventricular RLC (Wang, Xu et al. 2006) and the A13T or K104E-mutated RLC. The cDNA of the mutant was cloned into the unique SalI site of the plasmid, \(\alpha\)-MHC clone 26 (generously provided by Dr. J. Robbins, Cincinnati Children's Hospital Medical Center, Cincinnati, OH). The resulting constructs contained about 5.5 kb of the mouse \(\alpha\)-MHC promoter, including the first two exons and part of the third, followed by the human recombinant RLC cDNA (498 bp) and a 630 bp 3' untranslated region from the human growth hormone transcript as described earlier for Tg-WT mice (Wang, Xu et al. 2006). All of the founders were bred to non-transgenic B6SJL mice. Multiple crosses of Tg-mice with B6SJL/F1 mice were performed before the animals were used for experiments.

*Analysis of transgenic protein expression*

The \(\alpha\)-MHC-driven expression of the human ventricular WT or mutated-RLC proteins in mouse hearts was determined as described previously (Szczesna-Cordary, Guzman et al. 2005, Wang, Xu et al. 2006, Kerrick, Kazmierczak et al. 2009, Kazmierczak, Muthu et al.
2012), both heart extracts and the atrial and ventricular myofibrils were used. Briefly, the hearts from transgenic and non-transgenic mice were excised and the ventricles and atria were immediately separated and frozen in liquid nitrogen. Prior to the experiment, ventricles and atria were thawed in CMF (cardiac myofibril) buffer consisting of 5 mM NaH₂PO₄, 5 mM Na₂HPO₄ (pH 7.0), 0.1 mM NaCl, 5 mM MgCl₂, 0.5 mM EGTA, 5 mM ATP, 5 nM microcystin, 0.1% Triton X-100, 20 mM NaF (phosphatase inhibitor), 5mM DTT and 1 μl/ml protease inhibitor cocktail. Tissue samples were homogenized for 2 min in a Mixer-Mill MM301 at 30 Hz, chilled on ice and homogenized again for 2 min. Homogenates were then centrifuged for 4 min at 1800 g and the supernatants were discarded. The pellets were re-suspended in the CMF buffer and the myofibrils were subsequently dissolved in SDS-PAGE sample buffer and loaded on 15% SDS-PAGE at 30 μg per lane for Coomassie staining and at 20 μg per lane for Western blotting. The expression level was assessed on the basis of the faster gel mobility of the human ventricular RLC (18.789 kDa) vs. mouse atrial RLC (19.450 kDa) (Wang, Xu et al. 2006) detected utilizing a rabbit polyclonal RLC CT-1 antibody (produced in this lab (Szczesna-Cordary, Guzman et al. 2005)) followed by a secondary goat anti-rabbit antibody conjugated with the fluorescent dye, IR red 800 (Szczesna-Cordary, Guzman et al. 2005, Wang, Xu et al. 2006). The myosin ELC was used as a loading control and detected with the monoclonal ab680 antibody (Abcam) followed by a secondary goat anti-mouse antibody conjugated with the fluorescent dye, Cy 5.5. Blots were scanned and respective bands were quantified using the Odyssey Infrared Imaging System (LI-COR Inc.). The transgenic protein expression level was calculated as following:
% RLC\textsubscript{transgenic} expression level = \frac{RLC\textsubscript{transgenic}}{RLC\textsubscript{endogenous} + RLC\textsubscript{transgenic}} \times 100 
(Eq. 2)

**Small Angle X-ray diffraction measurements:**

The X-ray diffraction studies were performed on the small-angle instrument on the BioCAT beamline 18-D at the Advanced Photon Source, Argonne National Laboratory (Fischetti, Stepanov et al. 2004). For X-ray experiments, the papillary muscle from the Tg-WT and Tg-mutant mouse hearts were isolated into large bundles and skinned at room temperature for 1h, then the large bundles were further isolated into small muscle strips in pCa8 solution (composition same as described before) with BDM and mounted in a simple plexiglas X-ray chamber in the same solution. Sarcomere length was adjusted to 2.1 μm (short sarcomere length) using a light microscope (Nikon Diaphot with a 40x ELWD objective) and a digital image analysis system (model 900B VSL, Aurora Scientific). X-ray exposures were 1 s at an incident flux of \( \sim 1 \times 10^{12} \) photons/s with 12 keV photon energy. Camera length was 2.8 m. Diffraction patterns were collected on a CCD-based X-ray detector (Mar 165, Rayonix Inc. Evanston II, USA) and the 1,0 and 1,1 equatorial reflections were acquired (Irving, Konhilas et al. 2000, Colson, Locher et al. 2010). The fiber was then stretched to a long sarcomere length (2.3 μm) for the second measurements, and the 1,0 and 1,1 equatorial reflections at this sarcomere length was also recorded. The distance between the 1,0 and 1,1 reflections were converted to the d\textsubscript{1,0} lattice spacing using Bragg’s law and converted to the inter-thick filament spacing (IFS) depicting center-to-center distance by multiplying by \( \frac{2}{\sqrt{3}} \) (Irving and Millman 1989). Intensities of the 1,0 and 1,1 equatorial reflections were determined from non-linear least square fits to one-dimensional
projections of the integrated intensity along the equator (Colson, Locher et al. 2010). All data were analyzed independently by three people and the results were averaged.

**Steady state force development and force-pCa relationship measurements on transgenic mouse papillary muscle strips**

The papillary muscles of the left ventricles from transgenic mice were isolated, dissected into muscle bundles in the buffer containing pCa 8 solution \((10^{-8} \text{ M } [\text{Ca}^{2+}], 1 \text{ mM free [Mg}^{2+}] (\text{total MgPr (propionate) }= 3.88 \text{ mM}), 7 \text{ mM EGTA, } 2.5 \text{ mM [Mg-ATP}^2-], 20 \text{ mM MOPS, pH 7.0, } 15 \text{ mM creatine phosphate and } 15 \text{ units/ml of phosphocreatine kinase, ionic strength } = 150 \text{ mM adjusted with KPr), } 15\% \text{ glycerol and } 30 \text{ mM BDM. Muscle bundles were then skinned in } 50\% \text{ pCa 8 solution and } 50\% \text{ glycerol containing } 1\% \text{ Triton X-100 for 24hr at } 4^\circ\text{C. Muscle bundles were then transferred to the same solution without Triton X-100 and stored at } -20^\circ\text{C for experiments within } \sim 5 \text{ days (Szczesna-Cordary, Guzman et al. 2005). The steady state force development and force-pCa relationship were measured the same as described in previous section.**

**Muscle relaxation kinetics**

To monitor the muscle relaxation rate, a photolabile derivative of BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), diazo-2, was used. Diazo-2 is able to rapidly chelate Ca\(^{2+}\) upon photolysis converting from a low affinity \((K_d = 2.2 \mu\text{mol/l})\) to a high affinity \((K_d = 0.073 \mu\text{mol/l})\) for Ca\(^{2+}\). The experiment was performed as described in Kazmierczak et al. (Kazmierczak, Paulino et al. 2013). Briefly, after testing the steady state force, papillary muscle strips were immersed in the solution of 2 mM diazo-2, 0.5 mM CaCl\(_2\), 60 mM TES, pH 7.0, 5 mM MgATP, 1 mM [Mg\(^{2+}\)] and 10 mM creatine phosphate along with 15U/ml creatine phospho kinase, ionic strength = 200 mM. At the ratio of total
added Ca²⁺ to diazo-2 given above, the resulting average initial force was around 80% of the maximum (determined in pCa 4 solution). When the force level reached equilibrium, the strips were exposed to a UV flash from a Xenon lamp. The photolysis-induced relaxation isotherms were fitted to a double exponential decay equation (Sigma Plot 11.0) yielding the relaxation rates in (s⁻¹) for Tg-K104E vs. Tg-WT muscles.

**Passive force measurements**

The measurement of passive force (in pCa 8 solution) in response to muscle stretch was performed as described in Kazmierczak et al. (Kazmierczak, Paulino et al. 2013). The strip was first released and stretched until it began generating tension. This point was set as zero for both the passive force and starting length of the muscle strip. Then, the strip was stretched by 10% of its length x 4 consecutive times, and the passive force per cross-section of muscle (in kN/m²) was determined.

**Actin-activated Tg-mouse myosin ATPase activity assays**

Mouse myosins isolated from the hearts of Tg-mice were purified as described previously (Szczesna-Cordary, Jones et al. 2007, Kazmierczak, Xu et al. 2009). The protocol is the same as the RLC reconstituted porcine cardiac myosin ATPase assay except for two differences: the concentrations of rabbit skeletal F-actin (in μM) were slightly different 0.1, 0.5, 3, 5, 7.5, 10, 15; and the reaction time with ATP is 15 min at 30°C.

**Frictional loading in vitro motility assays**

To assess the functionality of Tg mouse purified cardiac myosins, a frictionally-loaded in vitro motility assay was performed using Tg-K104E vs. Tg-WT myosin preparations. In vitro motility assays were conducted as previously described (Greenberg, Watt et al. 2009) with some modifications. Before motility experiments, actin was mixed with myosin in an
approximately equal molar concentration and incubated in the absence of ATP for 5 min at room temperature. Then ATP was added to 1.5 mM and the mixture incubated for 60-90 s to allow the “active” heads to release the actin. Inactive actomyosin complexes were removed by centrifugation at ~135,000 g x 25 min (30 psi Airfuge; Beckman-Coulter, Inc.). The concentration of myosin in the resulting supernatant was determined using a Bradford Assay (Bio-Rad, Hercules, CA) and adjusted to ~100 µg/ml with high salt buffer containing: 300 mM KCl, 25 mM Imidazole, 1 mM EGTA, 4 mM MgCl₂, 10 mM DTT, pH 7.3.

To examine the impact of increased load on the motility of actin filaments the actin binding protein α-actinin was added, in varying amounts, to the diluted myosin. Sliding velocity of unregulated actin was calculated using a plugin for imageJ called wrMTrck (Jesper Søndergaard Pedersen: http://www.phage.dk/plugins/wrmtrck.html), which is an automated centroid-based filament-tracking program. For analysis, movies were generated at 5 frames per second and a threshold was applied to improve contrast. The wrMTrck algorithm identifies filaments by position and tracks the motion by determining the closest filament in successive images. Therefore a maximum velocity threshold was set to prevent tracks from switching between different filaments from one frame to the next. To remove background noise particles smaller than < 0.5 µm were removed. Because of variation of filament centroid calculation between frames, filaments that moved less than one quarter pixel were considered stalled. Only filaments that moved greater than 75% of the total frames in a movie were quantified. Filaments from 4 preparations were pooled (average 5 movies per experiment, 3-4 experiments per α-actinin, 0-6 µg/ml, while 7 and 8 µg/ml had 2 and 1 experiments, respectively) and were averaged to determine the relationship between velocity and frictional load applied by α-actinin.
**Electron microscopy imaging**

Transmission EM imaging was conducted in the EM Core Facility at the University of Miami Miller School of Medicine. After euthanasia, the hearts of age matched Tg-WT or Tg-mutated RLC mice were excised and immediately perfused in a solution containing 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 overnight at 4 °C. The hearts were then fixed with 2% glutaraldehyde and subsequently separated into the left ventricle and septum. Left ventricles from Tg-mutants and Tg-WT mice were sectioned longitudinally for imaging. The slides were examined using a Philips CM-10 electron microscope with 10,500 x magnification.

**Analysis of mitochondrial content**

The hearts from age matched Tg-WT and Tg-mutants mice were homogenized at 25 Hz for 2 min in a buffer containing 8 M urea, 10 mM Tris-HCl, pH 7.0, 1% SDS, 1% β-mercaptoethanol (β-BME), 1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail (PIC) (Sigma-Aldrich). At least two mice per group were used to prepare heart extracts. The heart extracts were clarified by centrifugation at 18,000 g x 5 min and quantified by a Coomassie-Plus Assay (Pierce). Supernatants were loaded at 20 μg per lane, subjected to 15% SDS-PAGE and electrophoresed for Western blotting. The mitochondrial Voltage-dependent anion channel (VDAC/porin) protein was detected using anti-VDAC antibodies (V2139, Sigma-Aldrich) followed by a secondary goat anti-rabbit antibody conjugated with the fluorescent dye, IR red 800. The myosin ELC was used as a loading control (detected as described above). Respective protein bands were quantified using the Odyssey Infrared Imaging System (LI-COR Inc.) (Kazmierczak, Muthu et al. 2012). The normalized VDAC
ELC band intensity ratio was used to assess the relative mitochondrial content in Tg-WT and Tg-mutants hearts.

**Histopathological staining**

For histological assessment of heart morphology Tg mutants and Tg-WT mice of various ages were used. After sacrificing, the hearts were excised and immersed in 10% buffered formalin. Slides of whole hearts were prepared by American Histolabs Inc. The paraffin-embedded longitudinal sections of the hearts stained with H&E (hematoxylin and eosin) and Masson’s trichrome were examined for overall morphology and fibrosis using a Dialux20 microscope, × 40/0.65 NA (numerical aperture) Leitz Wetzlar objective and an AxioCam HRc camera (Zeiss).

**Echocardiography assessment**

Mice were anesthetized in a Plexiglas box with isoflurane 4%, weighted and placed on a heating pad to maintain body temperature of 37°C monitored with a rectal thermometer. Ventilation and anesthesia were provided via a nose cone and isoflurane was reduced to 1.0-1.5% in order to minimize any effect on cardiac function. Heart rate was monitored with ECG recording. *In vivo* cardiac function, pulse-wave Doppler images and Tissue Doppler Imaging (TDI) were assessed in age matched WT and mutated RLC Tg mice. Images were obtained using a Vevo 2100 ultrasound machine (Visualsonics Inc.) with a 40MHz transducer, which was mounted on the Vevo Imaging Station (Visualsonics Inc.) for improved micromanipulation. Anterior and posterior wall thicknesses, left ventricular (LV) end-diastolic and end-systolic dimensions were measured in short axis (SAX) at papillary muscle level using M-mode images. LV mass, LV volumes and ejection fraction (EF) were calculated from these measurements (Manning, Wei et al. 1994, Tanaka, Dalton
et al. 1996). Apical four chamber view was used for Pulsed-Wave (PW) Doppler measurement of mitral inflow, and TDI measurements at the annular level of the septal wall. With PW-Doppler we measured early (E) and late (A) mitral inflow and deceleration time and with TDI we measured systolic velocity s’ and early diastolic velocity e’. We evaluated diastolic function with E/A-ratio, deceleration time, e’ and E/e’ (Schaefer, Klein et al. 2003).

**Invasive hemodynamics**

*Surgical preparation and instrumentation:* Age matched Tg-WT and Tg mutated RLC animals were used in this study. After the animal had been placed in a Plexiglas box with isoflurane 4 % for several seconds, the chest and neck were shaved and a tracheostomy was performed. Anesthesia was maintained with mixture of oxygen 100 % and isoflurane 1-1.5 % and mechanical ventilation was provided with a MiniVent ventilator for mice (Harvard apparatus). For a 30g intubated mouse we used a respiratory rate of 130-135 / min and tidal volume of 180 μl. The mouse was placed on a heating pad to maintain the body temperature at 37 °C, monitored with a rectal thermometer. The left jugular vein was accessed and cannulated with a 30 G needle and a constant infusion of albumin 12.5 % in normal saline was administered at 5 μl/min after a 50 μl bolus to counteract peripheral vasodilatation and hypotension induced by anesthesia. The heart was accessed via a subxyphoid incision and diaphragm exposure. Then, the LV apex was punctured with a 26 G needle and a pressure-volume catheter (Millar Instruments) was introduced into the LV cavity aligned with the LV longitudinal axis (Pacher, Nagayama et al. 2008, Cingolani and Kass 2011). Baseline pressure and volume signals were recorded and consecutive pressure-volume loops under varying loading conditions were taken with progressive compression
of the inferior vena cava (IVC) with a cotton tip. Since the animal was heavily sedated with an open chest, an intra-cardiac injection of 50 mg/kg sodium pentobarbital was administered for euthanasia (AVMA Guidelines for Euthanasia). Catheter Calibration: Pressure calibration was performed before starting the experimental procedure according to the manufacturer’s guidelines.

Hemodynamic measurements: LV pressure and volume signals were recorded during the experiment. All hemodynamic parameters along with indices of systolic and diastolic function were calculated with Labchart 7 Pro software (AdInstruments). Systolic indices consist of maximum derivative of LV pressure (dp/dtmax), the ratio of dp/dtmax to pressure at dp/dtmax [(dp/dtmax)/(P@dp/dtmax)] and Preload Recruitable Stroke Work (PRSW) calculated as the slope of the linear correlation between Stroke Work (SW) and End-Diastolic Volume (EDV). Diastolic indices consist of minimum dp/dt (dp/dtmin) and time constant (Tau) representing the isovolumic relaxation time.

Microarray for differential gene expression, enrichment signaling pathways and biological processes analysis in mice

The total RNA was extracted from flash frozen tissue from left ventricle of the transgenic mouse hearts with QIAGEN® RNeasy Fibrous Tissue Mini kit (same protocol as qPCR) and then RNA samples ~30ng in total amount were subjected to microarray assay using GeneChip® Mouse Gene 2.0 ST Array. The raw data obtained from the assay were transformed to .cel file format and were further analyzed in Partek® Genomics Suites software. The gene expression ratio between WT and mutants was automatically calculated by the software, and genes which the absolute fold change (+ or -) between WT and mutants was larger than 1.2 were sorted out to obtain the differential gene expression list. Pathway
analysis was conducted based on the differential gene expression lists and significantly affected pathways were sorted (low to high) based on enrichment p value (p<0.05 considered as significant, automatically calculated by the software). The number of gene which are upregulated or downregulated in the pathway was given, and the percentage of gene affected in the pathway (number of affected genes divided by total number of genes in the pathway) was also calculated.

Experimental Methods for Specific Aim 2

Methods for experiments conducted on Tg ELC mice

Human WT and mutant ELC transgenic mice generation

The transgenic ELC mice generation was following the same protocol as described above in Tg RLC mice generation.

Determination of ELC protein expression in Tg-ELC mice

About 30-60 mg of left ventricular tissue from Tg-WT and Tg-mutant mice were minced in a solution consisting of 8 M Urea, 10 mM Tris-HCl, pH 7.0, 1% SDS, 1% β-mercaptoethanol (β-ME), 1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail, and then homogenized in the same buffer for two minutes at 25Hz. The homogenates were centrifuged at 18,000 x g for 10 minutes and the supernatants quantified for protein expression by Pierce Coomassie-Plus Assay. The extracts were loaded (~5 μg per lane) and run on 15% SDS-PAGE for Western Blotting. Proteins were transferred from the gel to nitrocellulose membrane over 1 hour on ice at a constant voltage of 95 V and 260-500 mA. Membranes were washed 5 minutes at room temperature in TBS (50 mM Tris, 0.2 M
NaCl, pH 7.55) and blocked for 1 hour in the 1:1 mixture of TBS/Odyssey blocking buffer. All subsequent washes and antibody incubations were performed in the 1:1 mixture of 0.05% Tween 20-TBS/Odyssey blocking buffer. Specific detection of ELC proteins was accomplished using 1:2000 mouse monoclonal antibody, ab680 (Abcam). The myosin RLC was utilized as a loading control and visualized with 1:20,000 rabbit polyclonal antibody CT-1 (against the C-terminus of human cardiac RLC) developed in this lab (Wang, Xu et al. 2006). Differential detection of primary antibody bound to the ELC and RLC was performed by use of affinity purified polyclonal Infrared Cy5.5 (700 nm) labeled goat anti mouse IgG H&L (Rockland Antibodies) and a parallel polyclonal Infrared IR 800 Red Dye (800 nm), both at 1:4,000 (Fig. 3). Percent ELC transgene expression was defined as:

\[
\frac{ELC_{\text{transgenic}}}{(ELC_{\text{endogenous}} + ELC_{\text{transgenic}})} \times 100 \quad (\text{Eq. 3})
\]

**Small angle X-ray diffraction measurements on skinned Tg-ELC mouse papillary muscle strips**

The protocol was the same as described in RLC part except for the measurements was conducted under rigor conditions (100 mM KPr; 1 mM MgPr; 20mM MOPS, pH 7; and 1 mM DTT; ionic strength 107 mM) rather than pCa8 relaxation condition, and only one sarcomere length (2.2 µm) was studied.

**Rigor and relaxed stiffness measurements**

Papillary muscle fibers from Tg-ELC WT and Tg-ELC mutants mice were dissected into ~100 µm in diameter and ~2 mm in length small bundles and attached to the experimental apparatus by a small amount of nail polish at both ends (Bai, Weis et al. 2011). The preparation was relaxed in a solution consisting of: 7 mM Na₂ATP, 6 mM K₂EGTA, 2 mM MgAc₂ (Ac=acetate), 8 mM K₃Pi, 41 mM NaAc, 71 mM KAc, 10 mM MOPS, 19 mM
KOH, and pH adjusted to 7.00. The preparations were subjected to two protocols where [MgATP] and [Pi] were varied. Before and after each protocol, a control activation was performed in order to assess run down of the preparation. The solution for the control activation consisted of: 6 mM CaEGTA, 6.1 mM Na₂ATP, 15 mM Na₂CP (CP=creatine phosphate), 6.6 mM MgAc₂, 8 mM K₁.₅Pi, 13 mM NaAc, 54 mM KAc, 12 mM KCl, 18 mM KOH, 10 mM MOPS, and 320 units/ml creatine kinase (pCa 4.55, pH 7.00, 5 mM [MgATP²⁻], 1 mM Mg²⁺, 200 mM ionic strength). If tension reproducibility was not greater than 85% of control, the data were not used for analysis. Toward the end of studies, rigor was induced by removing ATP from the control activation, and rigor stiffness data were collected (Lu, Heeley et al. 2010). Stiffness during relaxation and after rigor induction was collected at 100Hz.

*Steady state force development, calcium sensitivity of force, muscle relaxation rate and passive tension measurements*

Papillary muscle was isolated from Tg ELC mouse hearts, dissected into large bundles and skinned overnight at 4°C. The skinned fibers were further dissected into smaller bundles (~100 microns in diameter) and subjected to functional studies. The protocol used was the same as described in RLC methods part.

*Actin activated ATPase activity assay on Tg ELC-WT and ELC-A57G mouse myosin*

Cardiac myosin was purified from Tg ELC-WT and Tg ELC-mutant mouse hearts and subjected to ATPase activity using the same protocol as described in RLC methods part.
**Histopathological evaluation**

Histopathological studies, including echocardiography and invasive hemodynamics measurements on Tg-ELC WT and mutant mice was performed using the same protocol as described in RLC methods part.

**Swimming exercise training of Tg-WT and Tg-A57G mice**

Chronic 4 week-long training by swimming of Tg-WT and Tg-A57G mice was performed in water at 30°C (to avoid thermal stress). Initial swim time was set as 10 min, thereafter gradually increasing until 90 min sessions were reached. The 90-min training schedule was continued twice a day (separated by 4–5 h), 7 days a week, for 4 weeks. This protocol was demonstrated to be highly effective in promoting physiological hypertrophy in mice (Evangelista, Brum et al. 2003, Galindo, Skinner et al. 2009).

**Quantification of gene expression of HCM makers in sedentary and exercised Tg-WT and Tg-A57G mouse hearts**

Total RNA was isolated from ventricles of sedentary and exercised Tg-WT and Tg-A57G mice and converted to double stranded cDNAs using Random Primers and a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as described earlier (Kazmierczak, Xu et al. 2009). Quantitative PCR was conducted using SYBR Green I chemistry with gene-specific primer sets for the atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), β-myosin heavy chain and sarcoplasmic reticulum Ca²⁺-ATPase 2a (SERCA2a). All reactions were performed in triplicate and run using BIO-RAD iQ5 Multicolor Real-Time PCR Detection System with the following cycle parameters: cycle of 50 °C (2 min) followed by 95°C (10 min), 40 cycles of 95°C (15 s) followed by 60°C (1 min). Raw data were analyzed using the BIO-RAD CFX Manager Software, and fold change in expression
of each gene was calculated using the relative quantification (RQ) ΔΔCt method with the levels of GAPDH as the normalizer gene (Kazmierczak, Xu et al. 2009).

**Maximal force and calcium sensitivity of force measurements in sedentary and exercise Tg WT and Tg A57G mouse fibers**

Protocols are same as described in Chapter 2. Experimental Methods for Specific Aim 1.

**Methods for experiments associated with human recombinant ELC proteins**

*Cloning, expression and purification of human cardiac ELC (WT, A57G and M173V):*

The WT and mutated A57G and M173V ELCs proteins were cloned and expressed using the same protocol as described in experimental methods for specific aim 1, however, the composition of buffer used for ELC purification was different: the ELC S-Sepharose buffer comprise 6 M Urea, 50 mM Citrate, 2 mM EDTA, 1 mM DTT, 0.02% NaN₃, pH 4.0 and the ELC Q-Sepharose buffer consist of 2 M Urea, 25 mM Tris, 0.1 mM PMSF, 1 mM DTT, 0.02% NaN₃, pH 7.5.

*Exchange human recombinant ELC into porcine cardiac myosin and actin activated ELC-exchanged myosin ATPase activity assay*

Recombinant human cardiac WT and mutant ELC proteins were bacterially expressed and purified as described above. Native porcine cardiac myosin (2mg/ml, 4uM) was mixed together with 10 molar excess of recombinant ELC proteins (40uM) in the buffer containing 200 mM KCl, 10 mM MgCl₂, 0.5 mM TFP and 10μM native porcine cardiac RLC, 1mM DTT, pH 6.8, and incubated for 1 h in dark at 30°C with shaking. The mixtures were precipitated with 14 volumes of ice-cold water containing 1 mM DTT and centrifuged at 8000 × g for 10 min (4°C). Myosin pellets were resuspended and then dialyzed in myosin
ATPase buffer (buffer composition is the same as described in Chapter 2. Experimental Methods for Specific Aim 1) and subjected to actin activated ATPase activity assay, the assay condition is the same as the one for RLC-reconstituted PC myosin.

**Exchange of human recombinant ELC into porcine muscle fibers and steady state force development and pCa-force relationship measurements in ELC-exchanged fibers**

A bundle of approximately 3-5 single fibers isolated from a batch of glycerinated skinned porcine papillary muscle fibers was attached to a force transducer and skinned for 30 minutes in a 1 ml cuvette in a solution containing pCa 8 buffer and 1% Triton X-100. The fibers were then washed 3 times, 5 minutes each in pCa 8 before undergoing the ELC protein exchange protocol. The reaction was performed in 200 µl solution containing 30 µM of recombinant human cardiac ELC-WT or mutant and 150 mM KCl, 10 mM KH₂PO₄, 10 mM imidazole (pH 6.5), 5 mM MgATP, 5 mM DTT and 1mM TFP at room temperature in dark for 1 hr. The ELC exchanged fibers were washed 3 x 5 minutes in pCa 8 buffer, and further reconstituted with 15 µM of human cardiac myosin RLC and 15 µM of human cardiac TnC in 200 µl solution containing pCa 8 buffer and 2 mM DTT at room temperature for 30 minutes. ELC exchanged and RLC/TnC reconstituted fibers were then washed 3 x 5 minutes in pCa 8 buffer and subjected to force and pCa₅₀ measurements same as described above, except for utilizing the ΔpCa₅₀ (pCa₅₀ after exchange minus pCa₅₀ before exchange) instead of pCa₅₀ to represent the calcium sensitivity of force of ELC-exchanged fibers.
Experimental Methods for Specific Aim 3

Methods for experiments associated with RLC Serine15 phosphorylation

Analysis of protein phosphorylation

RLC phosphorylation was determined in the ventricles of Tg mutants vs. Tg-WT mice. Ventricular myofibrils were prepared as described in detail in supplement materials in Huang, et al. (Huang, Liang et al. 2014). Myofibrillar samples were dissolved in SDS-PAGE sample buffer and loaded on 15% SDS-PAGE. Phosphorylated RLC was detected with phospho-specific RLC antibodies (produced in this laboratory (Kazmierczak, Muthu et al. 2012), which recognize the phosphorylated form of the RLC followed by a secondary goat anti-rabbit antibody conjugated with the fluorescent dye, IR red 800. The total RLC protein was detected with CT-1 and served as a loading control. The endogenous RLC phosphorylation level was calculated as the band intensity ratio between phosphorylated RLC and total RLC.

MLCK phosphorylation time curve of WT and mutated-RLCs

Skeletal muscle myosin light chain kinase (skMLCK) was prepared as described in Greenberg et al. (Greenberg, Mealy et al. 2009). WT and mutated proteins were first dialyzed into phosphorylation buffer containing 30 mM KCl, 20 mM PO₄, pH 8.0 and the concentration adjusted to 2 mg/ml. The proteins (1mg/ml) were mixed with 0.5 μM myosin light chain kinase (MLCK), 5 μM calmodulin (CaM), 0.1mM CaCl₂ and 12.5 mM MgCl₂. The reaction was started with the addition of 5mM ATP. RLCs were phosphorylated at room temperature and the samples taken at eight different time points (0.5 min, 1 min, 1.5 min, 2 min, 2.5 min, 3 min, 3.5 min, 4 min and 5 min) for further analysis. Briefly, 100 μl
samples were mixed with 70 mg of ultrapure urea, 10 µl β-ME and 5 µl of Bromophenol Blue and run on 10% Precise™ Tris-Glycine gels (Thermo Scientific) at 120 Volts for 90 min. The bands of non-phosphorylated and phosphorylated (P) RLCs, separated by charge differences, were scanned and quantified using Li-Cor Odyssey software and the level of RLC phosphorylation was calculated as:

$$\frac{\text{P-RLC}}{\text{RLC} + \text{P-RLC}} \quad \text{(Eq. 4)}$$

Phosphorylation isotherms for WT and mutated RLCs were fitted to Eq. 3 (exponential rise to maximum, two parameters): 

$$f = a \times (1-e^{-bx}) \quad \text{(Eq. 5)}$$

where a is a constant and b is phosphorylation rate in min⁻¹.

**MLCK phosphorylation of skinned WT and mutated mouse papillary muscle fibers**

The skinned muscle strips (~100 microns in diameter) was mounted on the force transducer and submerged into 200 µl of pCa8 solution plus MLCK, CaM for phosphorylation at room temperature for 1h. The phosphorylated fibers were washed three times in pCa8 solution and then subjected to steady state force and pCa₅₀ measurements as described in earlier sessions.

**Methods for experiments associated with ELC Serine195 pseudo-phosphorylation**

**Molecular cloning, protein expression and purification of pseudo phosphorylated S195D ELCs**

The S195D mutation was introduced by the site directed mutagenesis kit. The protein expression and purification was followed the same protocol as described in Chapter 2.
Exchange S195D ELCs into myosin or fibers

The exchange experiments were conducted using the same protocol as described in ELC part.

Functional studies on ELC exchanged porcine myosin and fibers

These experiments were conducted using the same protocol as described in Chapter 2. Experimental Methods for Specific Aim 3.

Statistical Analysis:

All values are shown as means ±SEM (standard error of the mean). Statistically significant differences between two groups were determined using an unpaired Student’s t-test (Sigma Plot 11; Systat Software, San Jose, CA), with significance defined as P<0.05. Comparisons between multiple groups were performed using ANOVA and/or ANOVA for repeated measures using IBM® SPSS® Statistics 21 software (Chicago, IL). Actin sliding velocity curves were fit using Prism 5 (GraphPad7825, La Jolla, CA 92037 USA) and the quality of the fit was determined based on the R² value derived from the fit. Significance between the curves was determined using the extra sum-of-squares F-test. The enrichment p value (the significance of a specific group of genes associated with the whole cluster of genes in the process / pathway) of biological processes and signaling pathways in microarray analysis was calculated automatically through Partek Genomic Suite® software.
CHAPTER 3. RESULTS

Specific Aim 1: Disease causing mechanisms of RLC linked cardiomyopathies

RLC-K104E linked HCM

Generation of Tg-K104E mice

To study the effects of the K104E mutation in animals, we have successfully produced multiple lines of Tg-K104E mice expressing high levels of mutant RLC in mouse hearts (Fig. 5). A substantial difference in gel mobility was observed between Tg human ventricular RLC (WT or K104E) and the endogenous mouse atrial RLC (GenBank accession no. Q9QVP4: MW 19.450 kDa) (Fig. 5), making quantification of transgene expression in the atria useful and efficient. On the other hand, no differential gel mobility due to no difference in molecular weights was observed between the endogenous mouse (GenBank accession no. P51667: MW 18,864 kDa) and Tg human P10916: MW 18,789 kDa) ventricular RLC (Fig. 5). We have previously generated Tg WT mice with ~100% protein expression (Wang, Xu et al. 2006), and similar to Tg-WT, all three lines of Tg-K104E mice showed high levels of mutant expression (Tg-K104E L2: ~94%, L3: ~97% and L7: ~99%) and therefore they were all used in functional assays.

Assessment of K104E induced changes in secondary structure of RLC

Far-UV CD spectroscopy was used to analyze the effect of the K104E mutation on the secondary structure of the RLC. As shown in Fig. 6, the mutation significantly changed the CD spectrum of the RLC, and the calculated α-helical content (at wavelength 222 nm at 22°C) was 15.2 ± 0.4% (n=11) in the K104E mutant versus 16.8 ± 0.1% determined for WT (n=11) (p<0.01). A decrease in α-helical content due to Lysine to Glutamic Acid
replacement indicates that the mutation imposed conformational changes in the RLC structure. These changes may further affect the interaction of RLC with its immediate binding partner, the MHC, alter the structure of the lever arm and the sarcomere, and ultimately trigger pathologic cardiac remodeling in K104E positive patients.

**Assessment of K104E-induced changes in sarcomere structure**

We further conducted small angle X-ray diffraction measurements on freshly skinned mouse papillary muscle fibers to look at the effect of K104E mutation on the structure of the sarcomere. Data from the measurements demonstrated a mutation-induced increase in the IFS at both short (2.1 µm) and long (2.3 µm) sarcomere lengths (SL) compared with Tg-WT. The equatorial reflections intensity ratio (I_{1,1}/I_{1,0}) in WT fibers increased upon stretch (from SL=2.1 to SL=2.3 µm) indicating a shift in the cross-bridge mass distribution toward the thin filaments. In contrary, the K104E fibers demonstrated no change in I_{1,1}/I_{1,0} on stretch (Fig. 7). Increased IFS and no change in I_{1,1}/I_{1,0} ratio correlate with reduced force generation with no changes in the pCa_{50} in steady state force measurements (Fig. 8AB).

**Assessment of K104E-induced changes in contractile properties**

Assessment of contractile function in skinned papillary muscle strips from Tg-K104E vs. Tg-WT mice (maximal force, calcium sensitivity, muscle relaxation and passive tension)

To investigate how changes observed in the structure could manifest on the function *in vitro*, measurements of steady-state force generation were performed in skinned papillary muscle strips from 5-6 month-old male and female Tg-K104E and Tg-WT mice (5-7 mice per group). A significant reduction in maximal (at pCa 4) isometric force was observed in Tg-K104E compared with Tg-WT mice (p<0.01) (Fig. 5A). The values of tension per
cross-sectional area of muscle (in kN/m² ± SEM) were: Tg-K104E: 36.8 ± 0.7 vs. Tg-WT: 52.8 ± 0.78 (Fig. 8A). The average diameter of muscle strip (in µm ± SEM) was 102 ± 2 for Tg-K104E and 97 ± 2 for WT. There was a slight but not statistically significant increase in pCa₅₀ of the force-pCa dependence: 5.72 ± 0.01 (SEM) for Tg-K104E and 5.70 ± 0.01 (SEM) for Tg-WT (Fig. 8B). Likewise no significant differences in the Hill coefficient were seen between the groups: 2.55 ± 0.11 (SEM) for Tg-K104E and 2.59 ± 0.07 (SEM) for Tg-WT. All these data were derived from n=66 papillary muscle strips from Tg-K104E and n = 58 strips from Tg-WT hearts (Fig. 8).

Since a phenotype of diastolic filling abnormalities was reported in K104E-positive patients, we further assessed the muscle relaxation kinetics in skinned papillary muscle strips from Tg-K104E and Tg-WT mice (three 7 month-old mice per group). A significant decrease in muscle relaxation rates were observed in Tg-K104E vs. Tg-WT myocardium (Fig. 8D) (p < 0.01). The relaxation rate values (in s⁻¹ ± SEM) were: 15.5 ± 0.6 for Tg-K104E (n = 36 strips) compared to 23.7 ± 1.2 for Tg-WT (n = 32 strips). These results indicated a mutation-induced impairment in the muscle relaxation kinetics suggesting that Tg-K104E hearts might be prone to diastolic disturbance.

Diastolic dysfunction is often associated with alterations in relaxed muscle stiffness, and therefore we also proceeded to measure the level of passive tension (at pCa 8) in response to muscle stretch using two 6 month-old mice per group (Fig. 8C). The following values of passive tension (in kN/m² ± SEM) were measured as a function of muscle stretch: Tg-K104E: 10%, 1.45±0.21; 20%, 5.17±0.37; 30%, 10.81±0.50; and 40%, 18.47 ± 0.40 (n=25 strips) compared to Tg-WT: 10%, 1.14 ± 0.18; 20%, 4.07 ± 0.25; 30%, 8.17 ± 0.40; and 40% 13.88 ± 0.66 (n=19 strips). The p value between Tg-K104E and Tg-WT groups was <
0.001 as determined by ANOVA for repeated measurements (Fig. 8C). In conclusion, Tg-K104E preparations displayed significantly increased levels of passive tension compared to Tg-WT, suggesting a higher muscle stiffness in the mutant myocardium.

**The effect of K104E on myosin ATPase and myosin’s force production capacity**

Since myosin and actin need to undergo the cross bridge cycle in order to generate force and muscle contraction, the myosin ATPase activity assay was performed to measure the cross bridge cycling rate. Two age groups (~6 month-old and ~11 month-old) of mice were used to obtain myosin from Tg-K104E and Tg-WT mice. No age-dependent differences within each group were noted in solution experiments using various batches of myosin (data not shown). Actin-activated myosin ATPase activity was plotted as a function of F-actin concentration (in μM) with data points expressed as average ± SEM of n=13-16 experiments. The data were fitted to the Michaelis-Menten equation yielding the $V_{\text{max}}$ and $K_{\text{m}}$ parameters. The $V_{\text{max}}$ represents the rate constant of the detachment step and the transition from the weakly (A·M·ATP ↔ A·M·ADP·Pi) to strongly (A·M·ADP ↔ A·M) (A depicts actin and M depicts Myosin) bound cross bridges. The $K_{\text{m}}$ determines the concentration of actin needed to produce half of the activation of the myosin heads and stands for the binding affinity of actin to myosin. **Fig. 9A** shows a significant ($p<0.01$) increase in $V_{\text{max}}$ in Tg-K104E mice (0.62 ± 0.03 s⁻¹, n=13) compared to Tg-WT (0.27 ± 0.04 s⁻¹, n=16). The $K_{\text{m}}$ values were no different between Tg-WT (5.0 ± 2.4 μM, n=16) and Tg-K104E (6.6 ± 0.9 μM, n=13) mice. These results demonstrate that while the K104E mutation does not change the affinity of binding (no change in $K_{\text{m}}$), it facilitates the detachment state and thus produces the quicker ATPase cycle (increase in $V_{\text{max}}$) compared to WT (Fig. 9).
Since steady state force measurements were conducted under loaded conditions, in order to assess the acto-myosin interaction in the presence of a frictional load, the in vitro motility assays were performed in the presence of α-actinin, a low affinity actin binding protein. The use of α-actinin was to slow down the actin filament motion and served as an indicator of the force generation capacity of myosin molecules. Compared with WT, K104E-myosin produced a higher actin sliding velocity under no load (no α-actinin added) (Fig. 10). And sliding velocity observed with K104E-myosin decreased drastically with increasing amounts of α-actinin, indicating that the K104E mutation inhibited myosin force production (Fig. 10). This result is consistent with reduced steady state force observed in Tg-K104E vs. Tg-WT skinned muscle preparations (Fig. 8A), and suggests that the K104E mutation may affect, at least in part, the myosin's force production capacity.

**Cardiac function in vivo, morphology, ultrastructure and differential gene expression patterns of Tg-WT and Tg-K104E mouse hearts**

To investigate how abnormalities observed in vitro could manifest at the heart level, histopathological studies, electron microscopy imaging, in vivo echocardiography and hemodynamics measurements were performed.

**Mutation induced abnormalities in sarcomere structure and mitochondrial content in vivo**

To determine whether the K104E mutated RLC incorporates normally into the cardiac muscle sarcomeres, we have performed electron microscopy assessment of LV tissue from Tg-K104E vs. Tg-WT mice (Fig. 11). No significant defects in the sarcomeric ultrastructure were observed in the myocardium of ~6 month-old Tg-K104E mice compared to age and gender matched Tg-WT controls and the cardiac muscle of both groups of mice demonstrated normally arranged and aligned in series sarcomeres (Fig. 11).
However, in contrast to Tg-WT myocardium, the mutant hearts displayed visibly abundant mitochondria with the preserved overall architecture (Fig. 11). To quantify the mitochondrial content we have run SDS-PAGE on the heart extracts and probed for the mitochondrial protein VDAC1 (porin). The myosin ELC was used as a loading control. Fig. 11B shows a representative Western blot of heart samples from 4 and 14 month-old Tg-K104E mice (lanes 2 and 4) compared to samples from 4 and 14 month-old Tg-WT mice (lanes 1 and 3). At least two mice per group were tested. Both young and old Tg-K104E mice showed significantly higher mitochondrial content compared to age matched Tg-WT controls and a 1.6-fold difference was measured in the VDAC content between Tg-K104E and Tg-WT hearts (p=0.042) (Fig. 4B, lower panel). These data indicate that more mitochondria have been biosynthesized by the Tg-K104E vs. Tg-WT hearts suggesting their higher energetic demands during force generation and cardiac muscle contraction.

Development of cardiomyopathic phenotype in Tg-K104E mice

Histopathological evaluation of the hearts from mice of different ages: ~4 (young), ~8 (intermediate) and ~15 (old) month-old Tg-K104E and Tg-WT showed no major abnormalities and the lack of myofilament disarray in either group of mice (Fig. 12A). However, occurrences of mild fibrosis were noted in 8 month-old Tg-K104E animals, which severely intensified in 15 month-old mice compared to Tg-WT hearts (Fig. 12B). Myocardial fibrosis is a hallmark of hypertrophic cardiomyopathy and the activation of profibrotic genetic pathways can take place even before hypertrophic remodeling (Ho, Lopez et al. 2010). Accordingly, gradually increasing fibrotic lesions were seen in the hearts of Tg-K104E animals implying that these changes did precede the development of LV hypertrophy (Fig. 12).
Cardiac hypertrophy in senescent mutant mice

We have not observed any morphological HCM features in 6 month-old Tg-K104E mice (Table 1) but the hypertrophic phenotype was evident in senescent (aged >13 months) mice (Table 2). Old Tg-K104E and Tg-WT mice had similar body weights, but the mutant displayed significant hypertrophy of both anterior and posterior wall and consequently a ~1.6-fold higher LV mass compared to Tg-WT mice. The hypertrophic phenotype in old Tg-K104E animals showed concentric pattern manifested by reduced LV diameter in systole and diastole compared to senescent Tg-WT mice (Table 2). The EF was higher in Tg-K104E compared to Tg-WT mice, indicating that the observed hypertrophy in mutant animals did not compromise their systolic function. The increased EF in HCM is well-established (Carasso, Yang et al. 2008), but it's mostly due to reduced volumes as observed in our senescent mutant mice (Table 2). In addition, it has been demonstrated that even though HCM patients may have increased EF, they usually show reduced LV function (Afonso, Bernal et al. 2008, Carasso, Yang et al. 2008). Consistently with our results, impaired myofilament contractile function was suggested to be the most important mechanism in HCM, accounting for compensatory hypertrophy and diastolic dysfunction, forming two of the hallmarks of the clinical HCM phenotype (Frey, Luedde et al. 2012). These results confirmed a late onset feature of RLC-K104E linked HCM.

Mutation induced diastolic disturbance in mice

In vitro studies on Tg-K104E muscle strips suggested that the K104E mutation may be involved in alterations of muscle relaxation rates. Diastolic function was then assessed in vivo using pulse-wave Doppler images of the transmitral filling pattern with the early transmitral filling wave followed by the late filling wave due to atrial contraction and by
TDI. Even though, the mutant mice displayed no sign of LV hypertrophy at 6 month with similar anterior and posterior walls and LV-mass, they showed signs of diastolic disturbance with significantly lower E/A-ratios and a prolonged deceleration time compared with WT (Fig. 13 and Table 1). The TDI-measurements including e’ and E/e’ were similar between the two groups of mice indicating no effect on LV filling pressure and therefore suggesting mild diastolic dysfunction in Tg-K104E if classified according to human guidelines (Redfield, Jacobsen et al. 2003).

Invasive hemodynamics evaluation of 6 month-old mutant vs. control mice showed preserved systolic function in Tg-K104E compared to Tg-WT mice. However, diastolic function represented by dp/dtmin was slightly reduced showing a slower rate of pressure decline. Most importantly, Tau (time of isovolumic relaxation) was prolonged in the Tg-K104E vs. Tg-WT mice with the difference being borderline significant (p=0.059). The time needed for passive LV filling in Tg-K104E mice was 58% longer than in Tg-WT (Table 1).

Microarray characterization of gene expression and enrichment pathways/biological processes in Tg-K104E mice

To further look into the molecular mechanisms of RLC-K104E linked HCM, we conducted the microarray analysis for differential gene expression, enrichment pathways and biological processes utilizing total RNA samples extracted from Tg WT and Tg K104E mouse hearts. Table 3 lists several important genes implicated in major biological processes. The results demonstrate that most significantly changed gene groups were in the structure, protein synthesis and metabolism. No significant differences were observed between WT and K104E in calcium handling genes. In addition, the hypertrophic markers,
ANP, BNP and Serpina3a’s were drastically upregulated in K104E mice, indicating the HCM disease phenotype. Results from enrichment pathway analysis showed five signaling pathways that were significantly affected by K104E mutation (characterized as enrichment p value less than 0.05) (Table 4) Among them, the adrenergic pathway in cardiomyocytes was of most interest, since this pathway plays a pivotal role in regulating cardiac function in response to environment, and altered adrenergic signaling in vivo is associated with cardiac hypertrophy and heart failure (Barki-Harrington, Perrino et al. 2004). Fig. 14 depicts the differential expression of genes involved in this pathway. Genes upregulated at least 20% (1.2 fold) in K104E vs. WT mice were colored in red, genes downregulated at least 20% (1.2 fold) were colored in green, and the genes whose expression was similar between WT and K104E were colored in gray. As seen in Fig. 14, PKA expression was down while PP2A expression was up, suggesting potential disruption in the sarcomeric protein phosphorylation, in particular TnI that is phosphorylated with PKA in response to beta adrenergic stimulation. PKA can also phosphorylate phospholamban (PLN) and further activate SERCA2a, a critical regulator of calcium homeostasis in the heart (MacLennan and Kranias 2003). Other significantly impaired genes were clustered in ion transmembrane transportation and the PI3K-Akt signaling pathway (Fig.14).

**Schematic summary of disease causing mechanisms for K104E-RLC**

Fig. 15 shows the scheme of the disease causing mechanisms of K104E-linked HCM. Briefly, at the sarcomere level, the K104E mutation led to compromised force generation, increased maximal ATPase activity, and disrupted the myosin load-dependent mechanochemistry. These alterations were manifested at the heart level as: 1) significantly increased mitochondrial content, and 2) inefficiency in energy usage (implicated by
increased ATP hydrolysis with a decrease in maximal force generation). On the other hand, the K104E mutation decreased muscle relaxation rate and increased the passive tension. These changes were manifested at the heart level as a decrease in the E/A ratio, prolonged muscle relaxation constant (Tau) and severe fibrosis. All these alterations could potentially lead to diastolic dysfunction in mice. More importantly, inefficiency of energy usage and diastolic disturbance are closely correlated with each other. The increased energy usage means the heart consumes more ATP than under the normal condition. This may affect calcium reuptake into the SR by SERCA and/or calcium transported out of the cytosol by the NCX (both of them are ATPases). Therefore, one can expect disruption of the calcium homeostasis and diastolic dysfunction. In addition, aging also plays an important role in progression of the K014E-HCM phenotype. All these abnormalities trigger the development of K104E-RLC induced heart disease.

**RLC-A13T linked HCM**

*Generation of Tg-A13T mice and analysis of protein expression*

Two transgenic mouse lines of Tg-A13T were produced with 11.4±1.8% expression (n=8) in L1, and 0 expression in L2 (Fig. 17) (Kazmierczak, Muthu et al. 2012). Due to the low expression of A13T achieved in mice (L1), two WT controls were used: ~100% WT (L2) and ~40% WT (L4). No differences in functional measurements were observed between WT L2 and WT L4 (Kazmierczak, Muthu et al. 2012). In addition, because of zero expression of Tg in A13T L2 (Fig. 17), the non-transgenic (NTg) littermates were included as additional control group in all performed experiments (Kazmierczak, Muthu et al. 2012).

*Assessment of the A13T induced changes in the secondary structure of RLC*
We have shown previously that the A13T mutation leads to a significant increase in the α-helical content of the RLC (Fig. 16 and (Szczesna, Ghosh et al. 2001)). We also speculated that these intra-conformational changes in RLC molecule change the interaction of RLC with the MHC (Szczesna, Ghosh et al. 2001, Kazmierczak, Muthu et al. 2012). The in vitro experimental results on recombinant human cardiac A13T mutant RLC (Szczesna, Ghosh et al. 2001, Kazmierczak, Muthu et al. 2012) suggested that the A13T mutation may elicit functional changes in force generation and cross bridge kinetics, and ultimate lead to HCM.

**Assessment of the A13T-induced changes in contractile properties**

**Force development in skinned papillary muscle fibers from mice**

To understand the effect of the A13T induced changes in myos RLC on force generation, we measured Ca\(^{2+}\)-dependent steady state force development in skinned papillary muscle fibers from Tg-A13T vs. Tg-WT and NTg fibers. As shown earlier (Wang, Xu et al. 2006), the papillary muscle fibers were least affected by any HCM-linked RLC mutations as evaluated by histopathological changes in the mutated myocardium, and therefore were used in the functional studies. Any remaining extracellular collagen deposits were removed from the fibers during the process of skinning with 1% Triton X-100 (see Materials and Methods). A large increase in maximal tension at pCa 4 was observed for Tg-A13T fibers (76.56±2.51 kN/m\(^2\), n=11 fibers) compared with controls, Tg-WT (57.43±0.16 kN/m\(^2\), n=11) or NTg (52.49±0.93 kN/m\(^2\), n=5) muscle fibers (Fig. 18). The increase in maximal tension was not accompanied by any changes in myofilament Ca\(^{2+}\) sensitivity and, as demonstrated in Fig. 18, no significant differences in the pCa\(_{50}\) values of the force-pCa dependence were observed between Tg-A13T fibers compared with Tg-WT and NTg fibers (p>0.05). The midpoint pCa\(_{50}\) values were: Tg-A13T: pCa\(_{50}\)=5.60±0.01 (n=10 fibers), Tg-
WT: pCa50 = 5.59±0.01 (n=11), and NTg: pCa50 = 5.56±0.01 (n=5). The Hill coefficients were nH=3.16±0.15 for Tg-A13T, nH=2.95±0.08 for Tg-WT, and nH=3.69±0.15 for NTg fibers showing the lowest cooperativity in the mutated Tg-A13T papillary muscle fibers (Kazmierczak, Muthu et al. 2012).

Actin-activated myosin ATPase activity assays
Since myosin is an ATPase that converts chemical energy into directed movement powering force generation and muscle contraction, the ATPase activity assay was performed using Tg WT and Tg-A13T mouse myosins. The results showed a significant decrease in Vmax in Tg-A13T mice (0.376±0.016 s⁻¹) compared to Tg-WT (0.508±0.012 s⁻¹) or NTg (0.627±0.039 s⁻¹) mice (data points expressed as averages ± SEM of 4-5 experiments) (Fig. 19). Vmax represents the rate constant of the transition from the weakly \((A\cdotM\cdotATP \leftrightarrow A\cdotM\cdotADP\cdotPi)\) to strongly \((A\cdotM\cdotADP \leftrightarrow A\cdotM)\) bound myosin cross-bridges with phosphate release being rate limiting (Rayment 1996). Therefore, the A13T mutation decreased the rate of the weak to strong actin binding transition by 25% and 40% compared with Tg-WT and NTg myosins, respectively (Fig. 19). The Km (dissociation constant) values between Tg-A13T (Km=2.35±0.21 μM) and Tg-WT (Km=2.00±0.05 μM) or NTg (Km=1.75±0.32 μM) myosins were slightly different, but the differences were not statistically significant (Fig. 19). Since the actin activated hydrolysis of Mg·ATP by myosin fuels muscle contraction, these results suggest that the A13T mutation may affect the ability of the mutated cross-bridges to hydrolyze ATP decreasing chemical energy that could be used to produce mechanical work.

Assessment of A13T-induced changes in heart morphology and function
To further verify the defects seen in vitro experiments at the heart level, histopathological staining and in vivo echocardiography measurements were performed.

**Heart morphology in Tg-A13T mice**

Histopathological evaluation of the hearts from Tg-A13T, Tg-WT and NTg mice is presented in Fig. 20. The upper panel shows longitudinal sections of the whole hearts and the lower panel high magnification left ventricular sections from representative 6 months old male mice. As indicated with arrows, a significantly larger inter-ventricular septal mass was observed for Tg-A13T mice compared with Tg-WT or NTg littermates (Fig. 20). In addition, A13T animals demonstrated severe fibrotic lesions in their left ventricular walls compared with controls suggesting exaggerated activation of extracellular collagen (Fig. 20). These findings demonstrate that the human phenotype of septal hypertrophy observed in patients harboring this mutation (Poetter, Jiang et al. 1996, Andersen, Havndrup et al. 2001, Hougs, Havndrup et al. 2005) could be recapitulated in mice.

**Echocardiography studies on Tg-A13T mouse hearts**

Echocardiography measurements were conducted on ~16 month-old WT and A13T animals. Compared to WT, surprisingly, no significant differences were seen in heart morphology between the WT and A13T, except for EF, the A13T’s EF was pronounced lower than WT (Table 5).

**RLC-D94A linked DCM**

*Identification of novel DCM mutation in the MYL2 gene*

Twenty two variants, meeting our established criteria for exome sequencing analysis (Norton, Li et al. 2011, Norton, Li et al. 2013), were identified in the three affected family
members of the pedigree (Fig. 21). Of these 22, only one variant, a missense mutation in TTN (Trp7406Cys), was identified in a gene previously associated with DCM. This variant was not prioritized due to the uncertain relevance of TTN missense mutations in DCM given their relatively high prevalence in control samples. A PKP2 variant (Val587Ile) was also identified; however, its pathogenicity is uncertain given a 0.46% allele frequency among control chromosomes of European ancestry (Norton, Robertson et al. 2012). The MYL2 mutation, on the other hand, is absent in the exome variant server database and had a GERP score of 5.07. Therefore, we hypothesized that this variant may explain DCM in this family (Norton, Robertson et al. 2012). None of the genes harboring the remaining 22 variants were associated with known cardiovascular disorders. No additional MYL2 variants were identified in the 16 other families. Sanger sequencing confirmed the MYL2 variant was present in all three affected family members (I.2, II.1, and II.2). Clinical characteristics of family members with the MYL2 mutation are provided (Table 6). The proband (II.2) and his sister (II.1) were diagnosed at ages 24 and 29 after preventive cardiovascular screening revealed mild global left ventricular systolic dysfunction. Medical therapy was initiated upon evidence of decreased ejection fraction. Their mother (I.1) was diagnosed with peripartum cardiomyopathy (PPCM) at age 21. Septal wall thickness measurements were within normal range in all affected family members.

Characterization of human recombinant D94A-RLC mutant

Effect of D94A mutation on the secondary structure of RLC

Far-UV CD spectroscopy was used to analyze the effect of the D94A mutation on the secondary structure of the RLC. As shown in Fig. 22, the mutation significantly changed the CD spectrum of the RLC, and the calculated \( \alpha \)-helical content (at wavelength 222 nm
at 22°C) was 13.3±0.5% (n=12) in the mutant versus 16.8±0.1% determined for WT (n=11) (p<0.05). A decrease in α-helical content due to Aspartic Acid to alanine substitution indicates that the D94A mutation imposed some significant conformational changes in the RLC structure that may be sufficient to affect the inter-domain organization within the RLC molecule and to further affect the interaction of RLC with its immediate binding partner, the MHC. This altered protein-protein interaction may ultimately trigger pathologic cardiac remodeling in D94A positive patients.

Using I-TASSER software, we have analyzed five lowest energy secondary structures of human ventricular RLC. The structures were computed using protein templates extracted from the PDB library. The following PDB structures of high similarity to the human ventricular RLC were used: 3jvtB, 1prwA, 4ik1A, 2mysA, 4i2yA and 2w4aB. The resulting model of the WT and D94A mutant structure is presented in Fig. 23. Interestingly, the position of the amino acid 94 site was predicted to be closer to the Ser15 phosphorylation site after the Asp to Ala mutation. The superimposed WT and D94A structures suggested that the D94A mutation indeed result in significant changes in the secondary structure of the RLC (Fig. 23).

Effect of D94A on the RLC-MHC interaction

To test whether the alterations in the RLC structure due to D94A may affect the assembly of the RLC into the lever arm domain of MHC, we studied the binding profiles of the WT and D94A proteins to the RLC-depleted porcine cardiac myosin. The CDTA/Triton-based treatment yielded ~90% RLC-free myosin, which was then titrated with increasing concentrations of recombinant WT- or D94A-RLCs. The degree of RLC reconstitution was calculated based on the ELC/RLC band ratio of reconstituted versus native (untreated)
myosin obtained from SDS-PAGE images (Fig. 24A). Fig. 24B demonstrates averaged apparent K_d constants derived from the binding isotherms of D94A vs. WT to porcine RLC-depleted myosin. There were no differences in the binding affinity between two groups with K_d = 3.17 ± 5.20 µM (n=10) for WT and K_d = 4.92 ± 7.48 µM (n=10) for the D94A mutant reconstituted myosin (Fig. 24B, p>0.05). However, the maximal level of reconstitution was largely decreased in D94A (0.72 ± 0.05, n=10) compared WT (0.88 ± 0.11, n=10) (Fig. 24B, p>0.05), and the differences of maximal RLC exchange level at experimental points of molar ratio 2.8 (WT: 0.69 ± 0.03 (n=10) vs. D94A: 0.52 ± 0.04 (n=10), p<0.01) and 3 (WT: 0.70 ± 0.05 (n=10) vs. D94A: 0.57 ± 0.03 (n=10), p<0.05) are significant. This result implies that a mutation induced changes in the secondary structure of the RLC seen in CD measurements may be responsible for this reduced ability of D94A to stoichiometrically bind to the MHC in the mutant reconstituted myosin.

**Functional assessment of D94A mutation in porcine reconstituted cardiac muscle preparations**

**Actin activated WT- and D94A-myosin ATPase activity**

To further investigate the effect of the D94A substitution on RLC function, WT- and D94A-RLCs were reconstituted into RLC-depleted porcine cardiac myosin and subjected to actin activated myosin ATPase activity assays. Fig. 25 shows a significant (p<0.01) increase in V_max for D94A myosin (0.22 ± 0.004 s⁻¹, n = 13) compared to WT myosin (0.19 ± 0.004 s⁻¹, n = 13). The K_m values were no different between the WT (0.45 ± 0.10 µM, n = 13) and D94A (0.50 ± 0.10 µM, n = 13) myosin. These results demonstrate that while the D94A mutation does not change the affinity of binding (no change in K_m), it facilitates
the detachment state and thus produces the quicker ATPase cycle (increase in $V_{\text{max}}$) compared to WT (Fig. 25).

**Steady state force and the force-pCa relationship in WT- and D94A- reconstituted porcine papillary muscle strips**

To further study the effect of D94A at the sarcomeric level, WT- and D94A- RLCs were reconstituted into the RLC-free skinned porcine papillary muscle strips are the steady-state force generation and force-pCa relationship were measured. As shown in Fig. 26, a slight decrease in maximal force generation was observed in D94A-reconstituted strips compared to WT. The values of tension per cross sectional area of muscle strip (in kN/m² ± SEM) were: D94A: 35.65 ± 1.11 (n = 25) vs. WT: 37.13 ± 1.09 (n = 25) (Fig. 26A, p >0.05). There was no significant alterations in pCa$_{50}$ of the force-pCa dependence and pCa$_{50}$=5.67 ± 0.01 (n=25) for D94A, and 5.69 ± 0.01 (n=25) for WT (Fig. 26B, p >0.05) or in the Hill coefficient, $n_H$=2.99 ± 0.10 (n = 25) for D94A and 2.93 ± 0.10 (n = 25) for WT (Fig. 26B, p >0.05). These results indicate that the D94A mutation did not impose any significant changes at the level of sarcomere force generating conditions.

*Specific Aim 2: Disease causing mechanisms of ELC-linked cardiomyopathies*

**ELC-A57G linked HCM**

**Generation of Tg-A57G-ELC mice and analysis of protein expression**

We have generated three lines of WT-ELC and three lines of A57G-ELC transgenic mice with different protein expression levels. Transgenic mouse papillary muscle fibers expressing these ELC mutants were used, and the results are: Tg-WT mice expressing ∼77% (L4), ∼30% (L3) and ∼88% (L1) of human ventricular ELC (Kazmierczak, Xu et al. 2009)
Tg-A57G mice, L1, L5 and L2 expressing ~80%, ~75% and ~55% of A57G mutant protein, respectively (Fig. 27) were used in all experiments.

Assessment of A57G-induced changes in sarcomeric structure and stiffness

ELC-A57G mutation decreases the IFS with no change in I\textsubscript{1,1}/I\textsubscript{1,0} ratio in rigor

We have assessed the effect of ELC-A57G mutation on the Interfilament lattice spacing and the I\textsubscript{1,1}/I\textsubscript{1,0} equatorial reflection intensity ratio from the small-angle X-ray diffraction patterns in papillary muscle fibers from Tg-A57G compared with Tg-WT mice. As shown in Table 7, the intensity ratios I\textsubscript{1,1}/I\textsubscript{1,0} from Tg-A57G fibers were similar to those of Tg-WT fibers. Therefore, the A57G mutation had no effect on the mass distribution between the thin and thick filaments (Table 7). However, the IFS of A57G was significantly decreased compared to WT (Table 7) (Muthu, Wang et al. 2011). It has to be noted that the measurements were performed under rigor conditions when the majority of myosin cross-bridges in Tg-WT, Tg-A57G fibers were attached to actin. The decrease in IFS under rigor conditions suggested the tighter packing of the filament, which may further indicated increase in rigor stiffness.

Effects of A57G mutation on rigor and relaxed stiffness

The rigor and relaxed stiffness were then measured in our collaborator, Dr. Masataka Kawai’s lab at the University of Iowa. Based on their results, the A57G mutation significantly increased stiffness under rigor conditions compared to WT (rigor stiffness, in kPa: WT: 410±40 (n=26); A57G: 710±70 (n=18), p<0.01) (Fig. 28), while under relaxed condition, A57G also largely increased relax stiffness, although not reaching significance (relaxed stiffness in kPa ±SEM): WT: 44±5 (n=26); A57G: 57±9 (n=18), p=0.093) (Fig. 28) (Muthu, Wang et al. 2011). Change in the rigor stiffness suggested the mutation-elicited
stiffness of the cross bridge, most likely the lever arm domain. The change in relaxed stiffness can be attributed to either fibrosis or alterations in titin properties, e.g. phosphorylation or isoform switch.

Assessment of A57G-induced changes in contractile properties

Interaction of mouse cardiac myosin with F-actin assessed by actin-activated myosin ATPase activity

Actin-activated ATPase activity assay was performed using myosins prepared from Tg WT and Tg A57G mouse hearts. No change in either V\text{max} (in s^{-1}) (Tg-A57G (0.340±0.032) vs. Tg-WT (0.364±0.031)) or K\text{m} (Michaelis-Menten constant) values (in µM) (Tg-A57G (6.26±1.54) vs. Tg-WT (7.43±1.52)) were observed between WT and A57G myosins. Data were expressed as averages ± SEM of 6-7 experiments performed on different myosin preparations (Fig. 30).

Force and kinetic measurements in skinned mouse papillary muscle strips

Measurements of steady state force generation and muscle relaxation rates were performed on skinned papillary muscle strips from age and gender matched Tg-A57G vs. Tg-WT mice. Five to eight mice per group were used with each ventricle yielding 6-9 papillary muscle strips that were subjected to mechanic measurements. A large (20-35%) decrease in maximal tension per cross section of muscle strip was observed in all tested lines of Tg-A57G mice compared to age and gender matched Tg-WT mice (Fig. 29A). Interestingly, the mutation also affected the Ca^{2+} sensitivity of force and a small (ΔpCa_{50}~0.07) but significant (p < 0.01) increase in the Ca^{2+} sensitivity of force was observed between Tg-A57G animals compared with Tg-WT (Fig. 29B). No change in the Hill coefficient of the
force-pCa dependence was noted between all groups of mice (nH = 3.9 ± 0.3) (Kazmierczak, Paulino et al. 2013).

Since our previous mechanical study demonstrated a significantly increased rigor stiffness in Tg-A57G vs. Tg-WT mice (Muthu, Wang et al. 2011), also Fig. 28, we then pursued measurements of passive tension (in pCa 8 solution) in response to fiber stretch (Fig. 29C). As demonstrated, the level of passive tension was significantly increased in Tg-A57G strips compared with Tg-WT (p<0.001) suggesting higher stiffness (increased resistance to stretch) in Tg-A57G myocardium (Kazmierczak, Paulino et al. 2013). It is important to note that the mutation did not cause any differences in muscle strip’s resting length allowing for direct comparison of the effects elicited by Tg-A57G and Tg-WT in response to stretch (Muthu, Wang et al. 2011). After steady state characterization, we then examined the effect of the A57G mutation on the kinetics of muscle relaxation using Diazo-2, a chelator of Ca^{2+} upon photolysis (Fig. 29D). A small (but not significant) decrease (by ~8%) in the rate of force relaxation was observed in Tg-A57G preparations compared with Tg-WT (Fig. 29D) (Kazmierczak, Paulino et al. 2013).

Assessment of A57G-induced changes in heart morphology and function

Assessment of cardiac hypertrophy and morphology in Tg-A57G mice

Representative H&E and Masson’s trichrome stained left ventricular sections from Tg-A57G and Tg-WT mouse hearts are presented in Fig. 31. The heart tissue morphology pictured in the H&E stained sections showed no mutation-induced abnormalities however, the Masson trichrome’s stained A57G heart sections manifested severe fibrosis in animals as young as 6 months of age (Fig. 31). Histopathology data collected in this study are in
accord with those observed previously and obtained on older (~12 months of age) animals (Muthu, Wang et al. 2011).

**Echocardiographic characterization of Tg-A57G mice**

Baseline echocardiography documented similar parameters of left ventricle posterior (LVPW) and anterior (LVAW) wall dimensions in diastole and systole in Tg-A57G and Tg-WT mice (Table 8). However, LV volumes in diastole and systole were significantly larger in Tg-A57G mice, indicating a mutation induced chamber enlargement and the development of eccentric hypertrophy in Tg-A57G mice (Table 8) (Kazmierczak, Paulino et al. 2013).

**Impact of A57G on cardiac performance in vivo**

Table 4 summarizes the analyses of hemodynamic parameters derived from pressure volume loops at a steady state and following the inferior vena cava occlusion (Kazmierczak, Paulino et al. 2013). The concentration of the anesthetic (1-2% isoflurane) was controlled to maintain the same heart rates in both groups of mice (Tg-A57G and Tg-WT). Load-dependent parameters of systolic function such as stroke work (SW), stroke volume (SV) and cardiac output (CO) were significantly increased in Tg-A57G mice compared to Tg-WT (Table 9). However, ejection fraction (EF) and cardiac efficiency were decreased in Tg-A57G vs. Tg-WT mice, but the differences between the groups were not statistically significant (Table 9). Consistently with the relationship between the LV filling volume, which produces the stretch of the heart fibers and a subsequent contraction of the next cycle, we observed increased LVEDV with higher LVESP in Tg-A57G mice compared with Tg-WT. (Table 9). In addition, Ees, the load-independent end-systolic elastance, Ees, defined as the slope of the end-systolic pressure–volume relationship, was also higher in Tg-A57G
An increase in LVEDV, LVESP and Ees indicate higher heart contractility in Tg-A57G mice. The time constant of ventricular relaxation Tau (in ms) was 2-fold less in Tg-A57G mice but the difference compared with Tg-WT did not reach statistical significance (Table 9). Because end-systolic pressure was higher in Tg-A57G vs. Tg-WT mice, Tau was expected to be smaller in the mutant mice (Gilbert and Glantz 1989). These changes were most likely associated with the type of cardiac remodeling in A57G animals manifested by eccentric hypertrophy and sphericalization of the left ventricle leading toward systolic dysfunction.

**Assessment of the effect of exercise in A57G animals**

**Effects of exercise on HCM related gene expression in Tg-WT and Tg-A57G mouse hearts**

The quantitative real time PCR was conducted to measure expression of HCM-related genes in the hearts of sedentary vs. exercised A57G mice (Kazmierczak, Yuan et al. 2014). While in sedentary group only SERCA2a was significantly upregulated in A57G vs. WT mice, in exercised animals there were several HCM related markers (ANP, BNP, Collagen VIIIa) significantly upregulated in A57G mouse hearts compared to WT (Fig. 32), suggesting that exercise was capable of exacerbating the A57G’s disease phenotype (Kazmierczak, Yuan et al. 2014).

**Effects of exercise on steady state force and pCa50 in papillary muscle fibers from Tg-WT and Tg-A57G mouse hearts**

The steady state force and pCa50 were measured in muscle fibers from sedentary and exercised WT and A57G mice. As shown in Fig. 33, similar to the data on sedentary mice (Kazmierczak, Paulino et al. 2013, Kazmierczak, Yuan et al. 2014), the maximal force generation in A57G mice was still significantly lower than WT after exercise. The pCa50 of
A57G was reduced after exercise, and was significantly lower than WT. This result suggests that exercise may significantly increase the risk of heart failure in A57G animals.

**Assessment of the functional effects of A57G in ELC-exchanged porcine cardiac fibers**

**ELC-exchanged porcine papillary muscle strips**

To assess the effect of A57G mutation on force generation in β-MHC-containing porcine papillary muscle strips, we have replaced the endogenous ELC protein with A57G or WT using an ELC-exchange protocol, shown schematically in Fig. 35A. This experiment was critical to assure that the effect of HCM-linked mutation can be examined in the background of β-MHC, the isoform of cardiac myosin heavy chain (MYH7) that is observed in humans. Following ELC-protein exchange, porcine papillary muscle strips were subsequently reconstituted with exogenous TnC and RLC to secure the functionality of the reconstituted system (Fig. 35A). The amino acid sequence of porcine cardiac ELC (UniProtKB: F1SNW4) is 95% identical to the human ELC, especially in the vicinity of the A57G mutation. Unexpectedly, there was a difference in the level of A57G vs. WT reconstitution assessed in 7 individual exchange experiments showing 1.6-fold higher yield for the mutant (Fig. 35E). The extent of A57G-exchange in porcine strips was in accord with the degree of A57G-exchange observed previously in porcine myosin (Muthu, Wang et al. 2011), but WT-exchange efficiency was lower in porcine muscle strips (Fig. 29E) compared to myosin (Muthu, Wang et al. 2011).

**Ca^{2+}-sensitivity of force in ELC-exchanged porcine papillary muscle strips**

Similar to the results observed for Tg mouse papillary muscle strips, the Ca^{2+} sensitivity of force in A57G-exchanged porcine strips was increased compared with WT-exchanged muscles (Fig. 35D). The pCa_{50} values were: pCa_{50}=5.75 ± 0.02 for A57G-exchanged strips
(derived from n = 12 individual experiments) compared to WT-reconstituted with pCa$_{50}$=5.64 ± 0.01 (n = 9). A slight and not statistically significant decrease in maximal force was also noted in A57G-exchanged (30.6 ± 1.2 kN/m$^2$) vs. WT-exchanged (32.4 ± 2.1 kN/m$^2$) porcine cardiac muscle strips (Fig. 35C). These changes are not expected to be due to a higher level of reconstitution with A57G vs. WT proteins as the same increase in the Ca$^{2+}$ sensitivity of force was observed in transgenic preparations expressing similar amounts of human ventricular ELC (WT or A57G) protein in mice.

**Scheme of the disease causing mechanism for ELC-A57G**

As shown in Fig. 36, the A57G specific phenotypes (not seen in the RLC mutants) were depicted in yellow. Similar to RLC-K104E, the A57G mutation decreased maximal force generation in mice, however, unlike for RLC mutants, which altered the ATPase activity, the maximal ATPase activity of ELC-A57G was not affected. At the heart level, the A57G’s contractility was significantly increased. The SW, SV, and CO were significantly larger in A57G than WT. All of these *in vivo* parameters suggested the A57G hearts need to work much harder than WT to produce the same or similar effect to WT. Given the compromised force generation and no change in ATPase activity, the hyper systolic function seen in A57G hearts strongly indicate inefficiency in energy usage. On the other hand, the A57G mutation significantly increased calcium sensitivity of force, which is not seen in RLC mutants. The increased pCa$_{50}$ of force may result in disruption of calcium homeostasis in the heart and ultimately cause arrhythmia and SCD in A57G positive patients. The A57G mutation also increased passive tension and relaxed stiffness (at the sarcomere level), and fibrosis (at the heart tissue level), which also can contribute to arrhythmia and SCD. In addition, pCa$_{50}$ of force was significantly reduced after exercise.
in A57G animals, and the steady state force generation of exercised A57G was still lower than WT, suggesting a potential risk of heart failure. At the same time, the HCM related genes ANP, BNP and Col VIIIa were upregulated in exercised A57G, which confirmed that the disease phenotype in A57G was exacerbated after exercise. All three aspects described above comprise the ELC A57G linked HCM (Fig. 36).

**ELC-M173V linked HCM**

*Clinical information of ELC M173V mutation*

The M173V mutation was reported in a human patient as an HCM causing mutation, but the clinical phenotype and family linkage information were not known (Morita, Seidman et al. 2005). Therefore, functional with the use of recombinant M173V ELC mutant were conducted to test whether M173V mutation could indeed cause HCM or whether it was a polymorphic mutation.

*Functional characterization of M173V-ELC exchanged porcine cardiac muscle preparations*

*Actin activated ELC-exchange porcine cardiac myosin ATPase activity assay*

The human recombinant WT or M173V-ELC was exchanged into porcine cardiac myosin and undergone the ATPase activity assay. The M173V mutation significantly decreased both the maximal ATPase activity ($V_{\text{max}}$) and $K_m$ compared to WT. The $V_{\text{max}}$ in mole Pi/sec ± SEM were: WT: 0.17 ± 0.01 (n = 12); M173V: 0.09 ± 0.01 (n = 6); and $K_m$ values: WT: 4.64 ± 0.98 (n = 12); M173V: 1.65 ± 0.58 (n = 6). These results suggest that the M173V mutation could potentially cause some changes in myofilament function in M173V hearts (Fig. 34).
Steady state force and pCa\textsubscript{50} measurements in ELC-exchanged porcine papillary muscle fibers

Similar to A57G, the M173V mutation also significantly increased the calcium sensitivity of force compared to WT (ΔpCa\textsubscript{50}: WT: -0.06 ± 0.01 (n = 16) vs. M173V: -0.05 ± 0.01 (n = 17), p < 0.05), however, the maximal force generation was not affected (force in kN/m\textsuperscript{2} ± SEM): WT: 31.51 ± 1.24 (n = 16) vs. M173V: 31.88 ± 0.87 (n = 17) (p > 0.05) (Fig. 35C-D).

Specific Aim 3: Does phosphorylation work as a common rescue mechanism for both RLC and ELC mutants?

Phosphorylation-mediated mechanisms in myosin RLC

Phosphorylation related studies on RLC-K104E

Examination of endogenous RLC phosphorylation in Tg-K104E mouse hearts

A direct correlation between compromised RLC phosphorylation and the development of cardiomyopathic phenotype was noted in our previous studies on Tg mouse models of HCM (Abraham, Jones et al. 2009, Kerrick, Kazmierczak et al. 2009), and it was also reported by Sheikh et al. (Sheikh, Ouyang et al. 2012). Therefore, we have examined the hearts of Tg-K104E mice for a mutation-induced effect on myosin RLC phosphorylation. Fig. 37 demonstrates a 1.8-fold reduction in endogenous RLC phosphorylation in Tg-K104E mice compared to Tg-WT, as determined by SDS-PAGE of myofibrils (n=10 preparations from each group of mice) isolated from left ventricles of rapidly frozen 3 and 17 month-old Tg-K104E (lanes 2 and 4) vs. 3 and 18 month-old Tg-WT (lanes 1 and 3) hearts (Fig. 37). The extent of RLC phosphorylation was evaluated using our generated phospho-specific RLC
antibody (Kazmierczak, Muthu et al. 2012), which recognizes the phosphorylated form of the RLC (lane 6). As observed previously (Kazmierczak, Muthu et al. 2012), there was no reaction of the antibody with the unphosphorylated RLC (lane 5). Total RLC protein was probed with the CT-1 antibody as described in our earlier publications (Szczesna-Cordary, Guzman et al. 2005, Wang, Xu et al. 2006, Kerrick, Kazmierczak et al. 2009, Kazmierczak, Muthu et al. 2012). This result confirms our earlier observations of compromised RLC phosphorylation in cardiomyopathic myocardium, especially in those mouse models which show strong HCM phenotypes (Abraham, Jones et al. 2009, Kerrick, Kazmierczak et al. 2009).

Time dependent phosphorylation of WT and K104E RLC proteins is shown in Fig. 37C. No difference in the rate of RLC phosphorylation was observed between WT and K104E, suggesting that the decrease in endogenous RLC phosphorylation in Tg-K104E mice could be due to a decrease in MLCK expression (Table 3) or steric alterations occurring in situ that prevent the MLCK from reaching the Ser15 site in K104E myocardium.

Effects of RLC phosphorylation on sarcomere structure

The MLCK treated and untreated WT or K104E skinned muscle strips were mounted in the X-ray chamber and undergone small angle X-ray diffraction measurements at both short (2.1 nm) and long (2.3 nm) sarcomere lengths. The IFS and I_{1,1}/I_{1,0} intensity ratios were obtained. The MLCK phosphorylation significantly increased I_{1,1}/I_{1,0} at both short and long sarcomere lengths compared to untreated K104E (K104E untreated: short SL: 0.40 ± 0.03 (n = 9, fibers); Long SL: 0.44 ± 0.04 (n = 8, fibers); K104E phosphorylated: short SL: 0.58 ± 0.06 (n = 8, fibers), long SL: 0.65 ± 0.07 (n = 6, fibers); untreated WT: short SL: 0.35 ± 0.02 (n = 10, fibers), long SL: 0.46 ± 0.04 (n = 10, fibers)) (Table 11). IFS was decreased
in K104E after phosphorylation compared with WT. IFS (at long sarcomere length) was restored due to MLCK treatment to the level near the untreated WT with the difference statistically significant (p < 0.05). K104E untreated: short SL: 46.99 ± 0.33 nm (n = 11, fibers); Long SL: 44.90 ± 0.41 nm (n = 10, fibers); K104E phosphorylated: short SL: 46.28 ± 0.23 nm (n = 7, fibers), long SL: 43.01 ± 0.60 nm (n = 5, fibers); untreated WT: short SL: 45.60 ± 0.32 nm (n = 12, fibers), long SL: 43.19 ± 0.46 nm (n = 10, fibers) (Table 10, Fig. 38). The decreased IFS may be associated with increased maximal force generation while increased I_{1,1}/I_{1,0} ratio may be linked to increased calcium sensitivity of force.

**Effects of RLC phosphorylation on maximal force generation and calcium sensitivity of the force**

We further investigated how the alterations in sarcomere structure caused by RLC phosphorylation could reflect on contractile function by conducting steady state force and pCa_{50} measurements on either untreated control or MLCK-phosphorylated skinned mouse papillary muscle fibers. **Figure 39** shows that the RLC phosphorylation have no effect on either maximal force production or calcium sensitivity of force (**Fig. 39BC**). Western blots on fibers show that both WT and K104E fibers were successfully phosphorylated by MLCK. However, the maximal RLC phosphorylation level only reached ~60% compared to phosphorylated (+P) RLC standard (**Fig. 39A**).

**Phosphorylation related studies on RLC-A13T**

**Endogenous RLC phosphorylation in Tg-A13T mouse hearts**

**Fig. 40** demonstrates the level of RLC phosphorylation determined in left ventricular extracts of the rapidly frozen hearts from Tg-A13T vs. Tg-WT and NTg mice, by CT-1 for total RLC, and phospho-specific RLC antibody to detect +P-RLC. As in **Fig. 40A**, total
RLC protein content in all mouse models shown in Fig. 40B was assessed by CT-1 antibody (red bands). Total ELC probed with the ELC antibody (green bands) was used as a loading control. As indicated, compared with NTg and Tg-WT mice, the endogenous RLC phosphorylation in Tg-A13T mice was not affected by the A13T mutation (Fig. 40, lane 2 vs. lanes 1&3). This result however, was somewhat expected after consideration of the very low level of A13T expression.

Phosphorylation related studies on RLC-D94A

Time-dependent MLCK phosphorylation of WT and D94A

To investigate the effect of D94A on RLC phosphorylation, the time-dependent MLCK treatment was conducted on WT- and D94A-RLCs (Fig. 41A). 100% of RLC phosphorylation could be achieved for WT (1.00±0.02, n=6) compared to ~50% for D94A (0.48±0.01, n=6) following 3 min treatment with MLCK (Fig. 41B, P<0.01). Even though phosphorylation was compromised in D94A mutant, the rate of phosphorylation was significantly higher in D94A compared with WT (D94A: 2.26±0.39 min⁻¹, n=6) versus WT: 1.06±0.02 min⁻¹, n=6) (Fig. 41B p<0.05). These results suggest that a mutation induced alteration in the secondary structure may impair the phosphorylation of the RLC.

Phosphorylation-mediated mechanisms underlying myosin ELC mutants

Cloning and purification of ELC pseudo (S195D) phosphorylation mimic proteins

The Serine (AGC) to Aspartic Acid (GAT) mutation at amino acid 195 was introduced and the SD-ELCs were cloned in the pET3d vector and expressed in BL21 bacteria. The proteins were purified using S and Q Sepharose columns and the purity of recombinant
ELCs were tested using SDS-PAGE. As seen, ~95% of purity was achieved in SD-ELC preparations (Fig. 42).

**Functional studies in porcine cardiac ELC-mutant reconstituted preparations**

**Actin activated ELC-exchanged myosin ATPase activity assay**

The human recombinant WT, A57G, M173V, and respective SD ELCs were exchanged into porcine cardiac myosin and the actin activated myosin ATPase activity assay was performed. Compared to WT, neither A57G nor A57G-S195D ELC significantly changed the maximal ATPase activity ($V_{\text{max}}$ in mol Pi/sec: WT: 0.17±0.01 (n=12); A57G: 0.16±0.01 (n=11);) or $K_m$ (WT: 4.64±0.98 (n=12); A57G: 4.69±1.13 (n=11), while the A57G-S195D significantly decreased $V_{\text{max}}$ and $K_m$ ($V_{\text{max}}$ in mol Pi/sec: A57G-S195D: 0.13±0.01 (n=9); $K_m$: A57G-S195D: 1.98±0.45 (n=9)). On the other hand, the M173V mutation significantly decreased maximal ATPase activity and M173V-S195D ELC was able to partially restore $V_{\text{max}}$ to the level similar to WT ($V_{\text{max}}$ in mol Pi/sec: M173V: 0.09±0.01 (n=6) M173V-S195D:0.12±0.01 (n=6), p<0.05). However, we observed a decrease in $K_m$ caused by M173V that was not restored by the S195D phospho-mimic ($K_m$: M173V: 1.65±0.58 (n=6); M173V-S195D: 2.56±0.77 (n=6), p>0.05) (Fig. 43).

**Maximal force and pCa50 measurements of SD-ELCs exchanged porcine muscle fibers**

The maximal force and pCa50 measurements on ELC exchanged fibers showed no effects of S195D phospho-mimics on the steady state force generation (maximal force in kN/m² ± SEM: WT: 31.51 ± 1.24 (n = 16); A57G: 31.66 ± 1.53 (n = 12); A57G-SD: 30.02 ± 1.67 (n = 15); M173V: 1.88 ± 0.87 (n = 17); M173V-SD: 31.25 ± 1.02 (n = 19), all p < 0.05). However, S195D mutation did restore the abnormally high calcium sensitivity of force of A57G or M173V to the level similar to WT ($\Delta$pCa50: WT: -0.06 ± 0.01 (n = 16); A57G: -
0.01 ± 0.01 (n = 12); A57G-SD: -0.08 ± 0.02 (n = 15); M173V: -0.05 ± 0.01 (n = 17);
M173V-SD: -0.09 ± 0.01 (n = 19); A57G vs. A57G-SD, p < 0.05; M173V vs. M173V-SD,
p < 0.05). This suggested a potential rescue effect of S195D-induced pseudo
phosphorylation of ELC (Fig. 44).
CHAPTER 4. DISCUSSION

*Disease causing mechanisms of RLC-linked HCM and DCM*

**RLC-K104E linked HCM**

The K104E mutation resulted in some structural changes in the RLC molecule and in the sarcomere. The Far UV CD measurements showed a significant decrease in the $\alpha$-helical content in K104E-RLC. This change in structure may suggest ultimate changes in the RLC properties, such as the ability of Ser15 to be phosphorylated or the binding affinity of RLC to the MHC. At the sarcomere level, the small angle X-ray diffraction measurements on skinned WT and K104E mouse muscle strips showed a significantly increased IFS with no change in $I_{1,1}/I_{1,0}$ ratio. These rearrangements in sarcomere structure were closely correlated with mutation-induced changes in the heart function. More specifically, increased IFS would cause the thick and thin filaments to move away from each other and therefore a decrease in force generation would be expected. On the other hand, the increased $I_{1,1}/I_{1,0}$ ratio indicates that the cross bridge mass is shifted from the myosin backbone toward the thin filament part, which would make the thin filament activated at lower calcium concentrations resulting in an increase in the calcium sensitivity of force. Functional studies showed the K104E mutation significantly reduced maximal force generation (correlates with increased IFS) and that the calcium sensitivity of force was not affected (agrees with no change in $I_{1,1}/I_{1,0}$ ratio). These results confirmed a strong link between the change in sarcomere structure and alterations in muscle function.

Functional studies on fibers also showed that the K104E mutation significantly increased passive tension under relaxed conditions. This change can be attributed to either
occurrences of fibrosis or alterations in the titin properties. Since the passive tension was increased in the fibers from ~6 month-old K104E mice, where no severe fibrosis was seen in histopathological studies, it is very likely that the changes in the mega-protein titin, also called the ruler of the sarcomere, is the main contributor responsible for increased passive tension. There are two major isoforms of titin: the more compliant N2BA isoform and the stiffer N2B isoform. The N2BA to N2B ratio in the normal human heart is 0.56; however, the stiffness between these two isoforms is so different that even a slight change in the N2BA/N2B ratio can significantly shift the passive tension (LeWinter 2004). Warren, et al. reported that the N2BA/N2B ratio was decreased drastically (increase in more stiff N2B isoform) in the pressure-overload hypertrophic rat hearts (Warren, Jordan et al. 2003). On the other hand, the N2B isoform is also a substrate for protein kinase A (PKA), and the PKA-dependent phosphorylation of titin may result in a decrease of N2B stiffness (Yamasaki, Wu et al. 2002). Thus increased passive tension could also be attributed to less phosphorylated N2B titin isoform. Future studies should include the examination of the phosphorylation level of titin, especially N2B.

The K104E mutation also decreased the relaxation rate in fibers, which was manifested at the heart level as decreased E/A ratio and increased Tau. Many factors could affect the muscle relaxation rate, such as cytosolic calcium concentration, muscle stiffness, and post translational modifications (PTMs) of thin filament proteins, e.g. TnI. The real time quantitated PCR results showed no pronounced changes in mRNA expression of RyR and SERCA2a suggesting that other factors may affect muscle relaxation in K104E myocardium. On the other hand, our observed increase in passive tension measured under relaxation conditions suggests an increase in muscle stiffness that at the heart level would
make the LV chamber difficult to be filled during the relaxation phase. Prolonged relaxation would ultimately lead to diastolic dysfunction. In addition, PTMs of the thin filament proteins, in particular TnI, would also affect relaxation. The PKA-dependent phosphorylation of Ser23/24 on TnI has been well known to play a role in muscle relaxation-contraction and diminished Ca\textsuperscript{2+} sensitivity of contraction. Many reports showed that Ser23/24 phosphorylation can enhance muscle relaxation in vivo (Takimoto, Soergel et al. 2004, Tong, Gaffin et al. 2004), and thus decreased relaxation rates observed in K104E mice can be caused by lower TnI-Ser23/24 phosphorylation.

Given the unique position of the RLC within the lever arm domain of the myosin head (Rayment, Rypniewski et al. 1993), one can understand why a structural change brought about by the Lysine residue to Glutamic Acid substitution leads to alterations in cross-bridge function and somewhat abnormal myosin ATPase cycling kinetics. As we observed, the ATPase activity of the mutant myosin was largely increased compared to WT. The $V_{\text{max}}$ of actin activated myosin ATPase can be determined by two parameters, the cross bridge cycling rate and the number of myosin cross bridges participating in the cycle. The higher $V_{\text{max}}$ noted for K104E myosin suggests that either myosin cross bridge cycling rates were augmented or there were more K104E vs. WT cycling cross-bridges interacting with actin. On the other hand, the steady state maximal force generation was significantly lower in Tg-K104E compared to Tg-WT muscle strips, suggesting that the former explanation is true.

Higher ATP cycling rates (increase in myosin ATPase activity) and a lower force generated by Tg-K104E hearts maybe suggestive of potentially low efficiency of contraction. In line with this, the EM results showed more mitochondria recruited by Tg-K104E myocardium
compared to Tg-WT, confirming higher energy demands in the mutant. In the *in vitro* motility assay performed under α-actinin-induced frictional load, Tg-K104E myosin was more sensitive to applied load compared with Tg-WT, suggesting that the K104E mutation may affect, at least in part, the myosin's force production capacity. Noteworthy, as determined for the other RLC-HCM mutations, N47K and R58Q, we observed that the K104E disease phenotype might be manifested by the disrupted load-dependent kinetics (Greenberg, Kazmierczak et al. 2010). Taken together, the higher ATP consumption, altered load dependence, and lower force generated by Tg-K104E hearts may suggest low efficiency of contraction in the mutant.

Histopathological studies showed minor fibrosis in 6 month-old Tg-K104E hearts and severe fibrotic lesions in 13 month-old hearts, which suggested the K104E’s HCM phenotype may be age dependent. The echocardiography assessment showed no HCM in 6 month-old Tg-K104E vs. Tg-WT hearts, but pronounced HCM phenotype in 13 month-old K104E hearts. This result confirmed the late onset of the RLC-K104E linked HCM. The next step will include the microarray analysis to look at the overall gene expression profiles at different age stages in order to establish the age dependent development of the phenotype and explore the gene expression patterns and signaling pathways in K104E mice.

In summary, we confirmed that RLC-K104E is a disease causing mutation, and that it may lead to HCM through the mechanism of inefficiency in energy usage and diastolic disturbance. The mutation-induced alterations in the structure of the RLC and the sarcomere clearly add to the many molecular causes of disease.
RLC-A13T linked HCM

Our earlier far UV CD measurements showed that the A13T mutation significantly increased the α-helical content of the RLC (Szczesna, Ghosh et al. 2001). Given the unique position of the RLC within the lever arm domain and its active participation in the acto-myosin ATPase cycle, it is conceivable that the structural change brought about by the Alanine to Threonine mutation could result in an abnormal myosin ATPase activity, decreased rates of inorganic phosphate release and potential changes in the power stroke (Muthu, Wang et al. 2011, Kazmierczak, Muthu et al. 2012). Since the hydrolysis of ATP by myosin fuels muscle contraction, it is likely that the A13T mutation in the RLC interferes with the ability of myosin to hydrolyze ATP providing inadequate energy to support mechanical work. The slower rates of Pi release by the cycling A13T-mutated myosin cross-bridges coincided with higher force measured at maximum activation (pCa 4) in Tg-A13T skinned papillary muscle fibers compared with controls (Kazmierczak, Muthu et al. 2012). Higher force produced by the A13T cross-bridges was not accompanied by any increase (or decrease) in the Ca²⁺-sensitivity of force. One can hypothesize that the structural change that occurs in the myosin lever arm is communicated to the myosin catalytic domain leading to a decreased rate of cross-bridge transition from their weakly to strongly bound configuration: $\text{A} \cdot \text{M} \cdot \text{ATP} \leftrightarrow \text{A} \cdot \text{M} \cdot \text{ADP} \cdot \text{Pi} \leftrightarrow \text{A} \cdot \text{M} \cdot \text{ADP} \leftrightarrow \text{A} \cdot \text{M}$ (Rayment 1996). According to Huxley (Huxley 1957), the transition from the non force-generating states ($\text{A} \cdot \text{M} \cdot \text{ATP} \leftrightarrow \text{A} \cdot \text{M} \cdot \text{ADP} \cdot \text{Pi}$) to the force-generating states ($\text{A} \cdot \text{M} \cdot \text{ADP} \leftrightarrow \text{A} \cdot \text{M}$) in muscle can be characterized by the cross-bridge attachment rate, $f$ and the return to the non force-generating states by the cross-bridge detachment rate, $g$. Consequently, the fraction of force generating myosin cross-bridges attached at maximal Ca²⁺ activation can be
characterized by \( f / (f + g) \) (Brenner 1988). For the ratio \( f / (f + g) \) to remain constant or increased (higher pCa 4 force with no change in force-pCa) with potentially decreased \( f \) in the A13T myocardium, the rate of cross-bridge dissociation \( g \) would have to decrease. We believe that the A13T mutation affects the myosin power stroke generation by changing the kinetic properties of the cross-bridges (Kazmierczak, Muthu et al. 2012). Future studies using single molecule detection, as we have done for other RLC mutations (Mettikolla, Luchowski et al. 2009, Muthu, Mettikolla et al. 2010), are necessary to directly determine the effect of A13T mutation on the kinetics of cycling cross-bridges in Tg-A13T muscle preparations.

It is also worth noting that we were only able to achieve \(~10\%\) A13T mutant protein expression in our Tg mouse models. It is likely that the mutation induced alteration in the secondary structure of RLC affects the binding affinity of A13T RLC to the MHC, and thus contribute to the low recombinant protein incorporation in mice, but it is also possible that the A13T RLC is lethal that only those mice which have less than 10\% mutant RLC expression can survive. On the other hand, with only \(~10\%\) protein expression, the A13T animals have already shown strong disease phenotypes such as upregulated force production and decreased maximal ATPase activity. This result suggested that the A13T mutation led to HCM through a so called dominant negative (or poison peptide) fashion (Roberts and Sigwart 2001). In Tg-A13T mouse myocardium both proteins are expressed, the endogenous RLC (normal allele) and the mutant RLC (A13T-transgenic allele); however, the mutant protein functions as a poison peptide that changes the function of the normal protein, leading to disease. Similar poison peptide effects were observed with other
HCM-linked sarcomeric mutations expressed in mice, e.g. TnT (Oberst, Zhao et al. 1998) and TnI (Wen, Xu et al. 2009), where despite relatively low levels of expression (below 10%), the incorporation of mutant protein induced myofilament disarray, profound fibrotic lesions and functional abnormalities. As with our A13T mutation, the observed phenotypes associated with these troponin mutations were essentially the same as that observed in humans. We can conclude then that A13T-RLC exerted a dominant-negative effect on cardiac myocyte structure and function in patients carrying this mutation (Poetter, Jiang et al. 1996) and in transgenic mice.

**RLC-D94A linked DCM**

The far UV CD measurement showed a significant decrease in $\alpha$-helical content of D94A vs. WT RLC. The I-TASSER structure prediction analysis showed that the D94A mutation may lead to shorter distance between the 94 site and Ser15 phosphorylation site in the RLC with the latter being physiologically important for the RLC function. Quite drastic differences were seen in the superimposed WT-RLC and D94A-RLC structures (Fig. 23), suggesting that the D94A mutation imposes significant changes on the secondary structure of the RLC. The proper conformation of the RLC is important for its function and its ability to interact with the MHC. The proper conformation supported by intact secondary structure is also important for the MLCK to access phosphorylatable Ser15. Therefore, the mutation may lead to impaired MHC-RLC interaction and cause alterations in RLC phosphorylation properties.

As with other RLC mutations, the D94A is expected to alter the force production and cardiac muscle contraction. However, functional studies utilizing skinned porcine cardiac muscle preparations reconstituted with the human recombinant D94A and/or WT showed
no mutation-elicited changes in force generation or in myofilament Ca$^{2+}$-sensitivity. Both of these parameters have been reported to be diminished in the DCM hearts (Gomes, Venkatraman et al. 2005, Spudich 2014). Perhaps the system used in this study, where the papillary muscle strips were reconstituted with the mutant protein, was not sensitive enough to detect subtle differences between the phenotypes. Another possibility includes the lack of Ca$^{2+}$ dependent changes in force generation by D94A in the same manner as observed for HCM causing mutations in the RLC. However, our observed enhancement in the actin-activated myosin ATPase activity for D94A myosin and a small reduction in maximal tension may indicate the inefficiency in energy use as a potential mechanism of disease (Huang, Liang et al. 2014).

In summary, we have characterized here the properties of a novel single point mutation in the myosin RLC to be associated with DCM. The D94A mutation is the first disease causing mutation in the RLC that is linked to the DCM phenotype. The mutation significantly altered the N-terminal α-helical region of the RLC, which further led to impaired RLC-MHC interaction and diminished Ser15 phosphorylation. These abnormalities combined with potential inefficiency in energy usage as manifested by elevated ATPase activity with slightly lower force production may contribute to the D94A mediated DCM phenotype.
**Disease causing mechanisms of ELC-linked HCM**

**ELC-A57G linked HCM**

Structural studies using small angle X-ray diffraction showed that the A57G mutation resulted in decreased IFS with no change in $I_{1,1}/I_{1,0}$ ratio under rigor condition (Muthu, Wang et al. 2011). This suggested a tighter packing of A57G filaments compared to WT-ELC, indicating increased rigor stiffness. Measurements of stiffness conducted by our collaborator Dr. M. Kawai at the University of Iowa showed that the A57G mutation indeed led to increased rigor and relaxed stiffness. Since under rigor condition, in which no ATP is present, myosin and actin are strongly bound with each other, increase in rigor stiffness suggests that the stiffness of the myosin head, presumably the lever arm domain, is elevated (Seebohm, Matinmehr et al. 2009). The force generation capacity of each cross bridge, the unitary force, depends on two parameters: the step size and the stiffness of the myosin head. Since the length of the lever arm was unlikely to be altered by the mutation, the step size remained the same, and the increased stiffness of the myosin head resulted in increased unitary force of each cross bridge (Muthu, Wang et al. 2011). This result, together with a decrease in overall maximal force generation in the fibers (Kazmierczak, Paulino et al. 2013) implied that the number of force-generating myosin cross bridges is decreased in A57G mice. On the other hand, the A57G mutation also increased relaxed stiffness. Fiber experiments showed that A57G mutation significantly increased passive tension under relaxed condition, which agrees with the relaxed stiffness measurement results. There are two potential explanations for the increased relaxed stiffness: increased fibrosis or potential alterations in titin. Severe fibrosis seen in 6 month-old Tg-A57G hearts suggested the former may be the cause; however, the possibility of titin involvement cannot be ruled out.
Investigating the isoform switch from titin N2BA (a more compliant isoform) to N2B (a stiffer isoform) (LeWinter 2004) or phosphorylation of titin (PKA-induced phosphorylation of N2BA isoform has been found to decrease passive tension (Yamasaki, Wu et al. 2002)) will shed more light on this issue.

On the other hand, we observed that the calcium sensitivity of force in Tg-A57G mouse fibers was significantly higher than in WT. The calcium sensitivity of force depends on two parameters: the \( k_{on} \) and \( k_{off} \) rate of calcium to TnC and the cross bridge dissociation/association to actin kinetics (Robinson, Wang et al. 2002). Since no differences were seen in the actin-activated myosin ATPase activity (which is a measurement of the cross bridge cycling rate) between WT and A57G, the alteration in the pCa\(_{50}\) of force was most likely due to a change in the \( k_{on} \) and/or \( k_{off} \) rate of TnC. The rate of calcium binding/releasing from TnC can be affected by various parameters, such as intracellular calcium concentration (Pan and Solaro 1987), TnI phosphorylation (Solaro, Rosevear et al. 2008), or alterations in other thin filament proteins such as TnT, Tm or actin (Gordon, Regnier et al. 2001). Quantitated real-time PCR results showed that SERCA2a expression was significantly upregulated in sedentary Tg-A57G hearts. Since SERCA2a is an ATPase located on the SR membrane that mainly functions to reuptake cytosolic calcium back to SR, the increase in SERCA expression may suggest a disruption of calcium homeostasis in the heart and therefore may contribute to alterations in calcium sensitivity.

On the other hand, TnI phosphorylation may also play an important role in regulating the calcium sensitivity of force. For example, the β-adrenergic stimulation-induced PKA phosphorylation of TnI Ser 23/24 sites rightward shifts the force-pCa relation, while PKC phosphorylation of TnI Ser 42/44 sites causes the opposite effect increasing the calcium
sensitivity of force (Solaro, Rosevear et al. 2008). Although there is no effect of A57G on the overall phosphorylation level of TnI (Kazmierczak, Paulino et al. 2013), the phosphorylation level of each individual Ser/Thrs site of TnI in Tg-A57G mouse hearts remains unknown, and thus requires further investigation. In addition, since the N-terminus of ELC was reported to interact with actin, and the A57G mutation is located in the N-terminal part of ELC (Hernandez, Jones et al. 2007), it is likely that the N-terminus ELC and actin interaction is disrupted by A57G and therefore leads to altered calcium sensitivity of force through the cross talk between the thick and thin filaments.

Increased calcium sensitivity of force may also increase the risk of arrhythmia, which is one of the leading causes of SCD. The disease-causing mechanisms of arrhythmias are not fully understood, but the evidence supports a prominent role for abnormal cycling of intracellular calcium ions (Knollmann and Roden 2008). Baudenbacher et al. showed that in mice expressing HCM-linked mutations in TnT, the risk of developing ventricular tachycardia was directly proportional to the degree of Ca$^{2+}$ sensitization caused by the TnT mutation (Baudenbacher, Schober et al. 2008). In another study using cardiomyocytes isolated from mice expressing TnT mutants, the authors demonstrated that myofilament Ca$^{2+}$ sensitization altered cytosolic Ca$^{2+}$ buffering, resulting in arrhythmogenic changes in overall myocyte Ca$^{2+}$ homeostasis (Schober, Huke et al. 2012). Based on these reports, we speculate that by altering Ca$^{2+}$ sensitivity of contraction the A57G mutation may increase the propensity of A57G-positive patients to cardiac arrhythmias.

The *in vivo* echocardiography and hemodynamics measurement results suggested cardiac remodeling and systolic dysfunction in Tg-A57G mouse hearts. The echocardiography results showed significantly increased HW/BW ratio with increased LV end systolic and
diastolic volumes with no change in LV wall thickness, suggesting an eccentric hypertrophy phenotype. Hemodynamics measurements showed a significant increase in SW, SV, and CO, which agrees with the eccentric hypertrophy phenotype seen by echocardiography. In addition, the slope of ESPVR (end systolic pressure volume relationship), Ees, which is the measurement of contractility of the heart, was also significantly increased. The increase in Ees may be attributed to the compensatory effects of increased chamber size, such as increased LVVed, but it is also influenced by passive myocardium stiffness (Borlaug and Kass 2008). Thus, the increased passive tension and stiffness in Tg-A57G mouse fibers may also contribute to the increase in Ees. In addition, the increased SW, SV, CO and the contractility of the heart suggested that the diseased A57G hearts have to work harder than the WT. However, the maximal ATPase activity remained the same and force generation per cross-section of muscle strip decreased, which may also result in inefficiency in energy usage in A57G mouse hearts. The increase in contractility with potential decrease in cardiac efficiency indicate the potential for systolic dysfunction, rather than diastolic dysfunction in A57G patients.

The A57G mutation was reported to cause SCD in humans (Lee, Hwang et al. 2001). Since sudden cardiac death often happens after intense exercise, the effects of exercise of Tg-A57G vs. Tg-WT mice were investigated. Compared to the sedentary group, in which only SERCA2a was upregulated, the HCM-related genes ANP, BNP, and Col VIII were all upregulated in exercised A57G mice (Fig. 32). Upregulation of ANP and BNP transcripts is considered a highly conserved feature of ventricular hypertrophy (Lee, Bloch et al. 1988, Dagnino, Lavigne et al. 1992, Nakagawa, Ogawa et al. 1995). ANP and BNP are released by the heart, usually in response to heart failure (cardiac injury). Both natriuretic peptides
are useful as sensitive markers of cardiac pathology/stress as well as of pathological cardiac hypertrophy. The Col VIIIa is the gene coding for type VIII collagen, and is the hallmark of fibrosis (Teekakirikul, Eminaga et al. 2010). The increased expression of ANP, BNP, and Col VIIIa suggested that exercise induced or worsened the HCM phenotype in Tg-A57G mouse hearts. On the other hand, fibers from exercised Tg-A57G hearts showed a significantly decreased calcium sensitivity of force with lower-than-WT mice maximal force generation (Fig.33). Decreased pCa50 with low force production in the exercised animals might be indicative of progressing toward heart failure (HF) as observed in HF animal models (Cazorla, Szilagyi et al. 2005, Belin, Sumandea et al. 2007). Together with the result of increased Collagen VIII expression, abnormalities in force development and pCa50 in exercised mice suggest reduced contractility of A57G hearts and increased risk of heart failure.

Compared to the disease-causing mechanisms of RLC-K104E, in which the mutation led to a classical HCM phenotype characterized by LV and septum wall hypertrophy with inefficiency in energy usage and diastolic dysfunction, the ELC-A57G caused HCM through different mechanisms and therefore resulted in a distinct phenotype. The ELC-A57G led to a rare HCM phenotype characterized by eccentric hypertrophy with no change in LV and septum wall thickness and systolic dysfunction. Besides lowering the efficiency of energy usage, the A57G mutation also increased the calcium sensitivity of force and upregulated SERCA2a expression, indicating disruption of Ca2+ homeostasis in the heart (Periasamy and Huke 2001). In addition, the calcium sensitivity of force dropped drastically after exercise, increasing the risk of heart failure and ultimately SCD. Therefore, we can conclude that the ELC-A57G caused HCM through a different mechanism than the
RLC-K104E. These results support clinical findings and the fact that A57G led to malignant phenotype compared to the phenotype of K104E, which was shown to be benign.

**ELC-M173V linked HCM**

The M173V mutation was reported in one human patient with HCM; however, detailed clinical information and family history are not available. In reconstitution studies, M173V significantly lowered the maximal ATPase activity as well as Km and increased calcium sensitivity of force generation with no change in maximal force. These changes suggested that this mutation could be a disease causing mutation. The increase in calcium sensitivity is one of the important characteristics of HCM, and the lower maximal ATPase activity with no change in maximal force suggest, as for other HCM mutations, inefficiency in energy usage. Although other ELC mutation, A57G, also led to increased calcium sensitivity of force, unlike M173V, it resulted in no change in ATPase activity. This result alone suggests that M173V leads to HCM through a different mechanism compared to A57G.

*Myosin light chain phosphorylation mediated rescue mechanisms*

**RLC Phosphorylation at Serine 15**

The MLCK-dependent phosphorylation of Ser15 has been shown to be important for the normal function of the heart in health and in disease (Sweeney, Bowman et al. 1993, Davis, Satorius et al. 2002, Scruggs, Hinken et al. 2009, Toepfer, Caorsi et al. 2013). A significant role of RLC phosphorylation was shown in cardiogenesis (Seguchi, Takashima et al. 2007) and proper myofibrillogenesis (Terry, Walker et al. 2006). Depressed levels of RLC phosphorylation were observed in patients with heart failure (van der Velden, Papp et al.
Finally, the inability of RLC to become phosphorylated was shown to result in a DCM phenotype in mice (Sheikh, Ouyang et al. 2012). Interestingly, our studies with HCM causing mutations in the myosin RLC suggest a correlation between the severity of cardiomyopathy phenotype and the level of RLC phosphorylation \textit{in vivo} (Kerrick, Kazmierczak et al. 2009, Muthu, Kazmierczak et al. 2012, Huang, Liang et al. 2014).

Increase in RLC phosphorylation was reported to be beneficial for the function of the heart. Results from Dr. Kasahara’s lab showed that overexpression of cMLCK, the kinase which is responsible for RLC phosphorylation in cardiomyocytes, could prevent the heart from developing the pressure-overload induced HCM in mice (Warren, Briggs et al. 2012). Functional studies from our lab on other HCM-linked RLC mutations not studied in this proposal (D166V and R58Q) also showed that Ser15 phosphorylation or the S15D phospho-mimic proteins act as protective or rescue entities to restore the detrimental effects caused by these mutation (e.g. compromised force generation or ATPase activity) to the level near WT (Szczesna, Ghosh et al. 2001, Greenberg, Kazmierczak et al. 2010, Muthu, Kazmierczak et al. 2012, Muthu, Liang et al. 2014). All these data strongly suggest that RLC phosphorylation may play an important preventive or rescue role in RLC-related cardiomyopathies.

For RLC mutations, I first checked whether the endogenous RLC phosphorylation was altered by HCM-causing mutations. If the answer was positive, I then phosphorylated these mutant proteins to test whether RLC-mutant phosphorylation was able to restore their functional defects. For K104E mutation, we did observe that endogenous RLC phosphorylation was lower than in WT hearts. However, we did not see any difference in
time dependent phosphorylation *in vitro* between the WT and K104E. On the other hand, microarray results showed a significant decrease in cMLCK gene expression in K104E hearts, which may contribute to decreased endogenous RLC phosphorylation.

Although phosphorylation of RLC significantly increased the $I_{1,1}/I_{1,0}$ intensity ratio and decreased IFS in Tg-K104E mouse fibers, the maximal steady state force and calcium sensitivity of force generation were not changed in response to RLC phosphorylation. However, Western blot data suggested that neither Tg-WT nor Tg-K104E mouse fibers were fully phosphorylated, which could be the reason for the lack of functional effects of RLC phosphorylation in these fibers. Future studies have to be performed utilizing the cardiac specific MLCK, the kinase which is responsible for RLC phosphorylation in the heart (Ding, Huang et al. 2010), rather than the rabbit skeletal MLCK that was available for these studies. The alternative approach would include generating the S15D pseudo phosphorylation mimetic proteins to simulate constitutive phosphorylation of RLC mutants.

For A13T mutation, we did not observe alterations in endogenous RLC phosphorylation level in Tg-A13T mouse hearts. This could be because the A13T mutation has no effects on RLC phosphorylation or due to a very low level (10%) of A13T expression in mice. In order to further investigate this, we have to either increase the A13T-RLC expression in mouse hearts or utilize the S15D-A13T pseudo-phosphorylation mimic of RLC. Alternatively, computation modeling can be employed to model the effects of phosphorylation at Ser15 in the background of A13T mutation.

For D94A mutation, the time dependent phosphorylation results, combined with data obtained from far UV CD measurements and I-TASSER secondary structure predictions suggested that the D94A mutation may impair RLC phosphorylation through changes in
the secondary structure of RLC. The next step would be generating Tg-D94A mice and assessing the effects of this mutation on RLC phosphorylation in the mouse heart *in vivo*.

**ELC phosphorylation at Ser 195**

The knowledge on the functional role of ELC phosphorylation is limited, and although reports were showing that ELC can be phosphorylated *in vitro* by MLCK, the ELC specific kinase remains unknown. Proteomics studies showed that ELC at Thr69 and Ser195 can be phosphorylated *in vivo* (Arrell, Neverova et al. 2001). In addition, the pseudo phosphorylated S195D ELC was able to restore the contractility of the ELC C-terminally truncated cardiomyocytes in Zebrafish (Meder, Laufer et al. 2009). This report suggested the potential rescuing effects of Ser195 phosphorylation on ELC function. To test the effect of ELC phosphorylation, the S195D-ELC pseudo phosphorylation mimic ELC proteins were produced for this research.

The S195D pseudo phosphorylation was able to restore the abnormally high calcium sensitivity of force of A57G or M173V ELCs to the level similar to WT. The significantly compromised maximal ATPase activity of M173V-ELC exchanged myosin was also partially recovered after introducing the S195D mutation in the background of M173V. These results revealed some potential for the S195D phospho-mimics in rescuing the ELC-linked HCM.
CHAPTER 5. SUMMARY AND CONCLUSIONS

Comparison of the disease causing mechanisms for RLC and ELC linked cardiomyopathies

The important question addressed here pertains to the underlying causes of different disease mechanisms observed for the RLC vs. ELC mutations in humans and in experiments utilizing animal models of human disease. The RLC and ELC mutations share common mechanisms by which they result in detrimental HCM/DCM phenotypes; however, due to the structural and functional differences between the RLC and ELC proteins and between the N- and C-termini of the protein itself, distinct disease phenotypes among certain mutations are observed.

For RLC, both A13T and K104E mutations cause HCM, while the D94A mutation leads to DCM in humans. In our studies, compared to control WT mice, the N-terminal A13T RLC mutation increased maximal force generation, decreased maximal ATPase activity and did not affect endogenous RLC phosphorylation, while the C-terminal K104E RLC mutation decreased maximal force generation, increased maximal ATPase activity, slowed down muscle relaxation and decreased endogenous RLC phosphorylation (Table 12). These results suggest that the N- and C-RLC mutations may lead to HCM through different mechanisms and the manifestation of disease phenotypes between A13T and K104E differ. The A13T’s phenotype is seen as early as in 6 month-old mice even though only 10% transgenic protein is expressed. The disease phenotype of K104E is age dependent, and can be clearly manifested only in animals older than 13 months of age (Table 12). Thus, we conclude that compared to K104E, which is shown to cause a relatively benign phenotype, the A13T mutation may lead to a more malignant HCM phenotype.
For the DCM causing D94A mutation, most alterations are seen in the RLC structure. The D94A mutation changed the $\alpha$-helical content and led to impaired MHC-RLC interaction. It also decreased the overall MLCK-induced RLC phosphorylation. However, functional defects, except for elevated maximal ATPase activity, were not manifested in D94A exchanged porcine cardiac muscle preparations (Table 12). The lack of a clear phenotype could be due to the RLC mutant reconstitution system that was utilized in the experiments compared to the native Tg mouse cardiac muscle studied for the A13T and K104E phenotypes. Tg-D94A mouse colonies have been successfully generated in the laboratory and the work in vivo and in vitro on disease phenotypes is in progress allowing for future conclusions on the D94A-induced DCM.

At the molecular level, all three RLC mutations alter the secondary structure of the RLC in different ways and these changes may differently affect the cross bridge cycling rates (ATPase activity) and the interaction of mutant myosin with actin during force generation and muscle contraction (Table 12). For A13T mutation, the $\alpha$-helical content was significantly increased by 11% compared to WT (Szczesna, Ghosh et al. 2001), but the maximal ATPase activity was significantly reduced. For K104E and D94A mutations, the $\alpha$-helical content was decreased, but the maximal ATPase activity was significantly higher than WT (Table 12). Differences in the ATPase and cross bridge cycling rates could also be seen at the myofilament level, in force generation. The A13T mutation significantly increased maximal force generation (lower cycling rates), while K104E reduced maximal force (higher cycling rates) (Table 12). These changes further underlie different disease phenotypes observed at the heart level.
Interestingly, the calcium sensitivity of force was not affected in any of the three studied RLC mutations (Table 12). This might be due to no direct interaction between the RLC and the troponin complex-containing thin filaments. It is also possible that calcium binding to RLC is not being affected by the mutations. As shown earlier, RLC can work as a delayed calcium buffer helping sarcoplasmic reticulum to sequester calcium after contraction (Szczesna-Cordary, Jones et al. 2007). RLC mutations that affect calcium binding to RLC were shown to cause delayed calcium and force transients (Wang, Xu et al. 2006). However, as reported earlier (Szczesna-Cordary, Guzman et al. 2004), the calcium binding affinity of A13T RLC was significantly lower than WT-RLC (higher Kd), but the calcium binding to RLC exchanged myofibrils or fibers was similar between WT and A13T. This suggests that alterations in calcium binding to A13T-RLC are not large enough to affect the overall calcium sensitivity of the fibers. The calcium binding properties of K104E and D94A RLCs are still under investigation.

For the ELC, the A57G ELC mutation, similar to K104E-RLC, significantly decreased maximal force generation and increased passive tension. However, unlike RLC mutations, it significantly left shifted the calcium sensitivity of force, with no changes in the ATPase activity or muscle relaxation rates (Table 12). In histopathology studies, fibrosis was observed as early as in 6 month-old mice. The in vivo studies using echocardiography and invasive hemodynamics showed a rare eccentric hypertrophy phenotype with signs of systolic dysfunction. These abnormalities were not observed in Tg-RLC animal models studied to date (Huang, Liang et al. 2014) (Table 12). More interestingly, A57G mutation was reported to cause SCD in human patients while no cases of sudden cardiac death were reported in neither of studied RLC mutations (Poetter, Jiang et al. 1996, Andersen,
For the C-terminal ELC mutation M173V, similar to A57G, this mutation also showed higher (left-shifted) calcium sensitivity of force compared with WT. In addition, the maximal ATPase activity was reduced (Table 12). It is worth mentioning that the results on M173V were obtained in reconstituted porcine muscle preparations due to no Tg-M173V mice were generated. Therefore, to conclude on this ELC M173V mutation, more structural and functional studies need to be conducted in the future.

Unlike RLC proteins, the ELC interacts with actin through its N-terminus (Winstanley, Trayer et al. 1977, Sutoh 1982, Trayer and Trayer 1985), and this interaction is expected to be the molecular target for the ELC disease causing mechanisms. We have previously shown that the N-terminally truncated Δ43-ELC was able to shift the cross bridge mass distribution toward the thin filament. This result suggested that the N-terminus of ELC may work as a tether to regulate the thin and thick filament interaction (Muthu, Wang et al. 2011). Since RLC does not interact with the thin filaments, I suspect that it is the direct interaction between the N-ELC and thin filaments that causes phenotypic differences between the RLC and ELC mutations. For example, the tethering effect of the N-terminus of ELC was manifested as increased rigor stiffness in Tg-A57G mice (Muthu, Wang et al. 2011). The increased rigor stiffness most likely reflected increased stiffness of the myosin head, and the lever arm domain in particular further increased the unitary force produced by A57G myosin (Muthu, Wang et al. 2011). This together with decreased maximal force generation in fibers (Table 12), suggests that ELC mutations may lead to a reduced number of force generating myosin cross bridges. These phenotypes were not observed for the RLC mutants. Interestingly, even though the ELC does not bind calcium, A57G and M173V both left shifted the calcium sensitivity of force in fibers (Table 12), suggesting the
possibility of the N-ELC mediated cross talk between the thick and thin filaments that results in alterations in pCa50. Alterations in calcium sensitivity are most likely induced by the changes of calcium binding to TnC, the major intracellular calcium buffer, e.g. increased $k_{on} / k_{off}$ ratio causing a leftward shift in the force-pCa relationship (Robinson, Wang et al. 2002).

In conclusion, different phenotypes observed between myosin light chains mutations are most likely caused by different properties of the two light chains, RLC vs. ELC, and the differences between their N- and C-termini domains.

*Comparison of the effects of serine phosphorylation on RLC and ELC mutations*

Regarding the myosin light chain phosphorylation on the RLC, the three studied mutations behaved differently. The A13T mutation has not altered the endogenous RLC phosphorylation, but this could be due to low expression of A13T in Tg mouse hearts. The K104E mutation showed a significantly lower endogenous RLC phosphorylation compared to WT; however, the time dependent phosphorylation curve of K104E overlapped with that of WT. For D94A, we observed differences in the rate of phosphorylation and in the maximal level of phosphorylation between the mutant and WT. D94A was phosphorylated faster than WT but the maximal phosphorylation level was lower than for WT protein (*Table 12*). The endogenous RLC phosphorylation level in D94A mouse hearts remain to be investigated. These results suggested that the observed changes in RLC phosphorylation in K104E were most likely due to alterations in myosin light chain kinase (MLCK) or phosphatase properties as manifested by a decrease in MLCK gene expression in Tg K104E hearts (*Table 3*). For D94A, the effects were mainly
due to alterations in the secondary structure of RLC, but the causative role of MLCK/phosphatase on D94A-RLC phosphorylation requires further investigation.

In X-ray diffraction measurements, K104E phosphorylation resulted in decreased IFS and increased I_{1,1}/I_{1,0} in Tg-K104E fibers indicating structural rearrangements in response to K104E phosphorylation. The change in IFS is expected to be negatively correlated with change in force, while the change in I_{1,1}/I_{1,0} ratio positively correlated with changes in pCa_{50}. However, none of these effects of phosphorylation was observed in Tg-K104E fibers in contractile force or pCa_{50}. The lack of phosphorylation-dependent functional effects in Tg-K104E fibers could be due to the smooth or skeletal muscle MLCK (smMLCK / skMLCK) used in the experiments, and Western blots showed that both WT and K104E fibers could only reach ~60% phosphorylation after smMLCK treatment. Future experiments are needed utilizing cardiac specific MLCK that was not available at the time of experiments. For A13T and D94A mutations, the effects of RLC phosphorylation are yet to be studied.

For A57G and M173V ELC mutations, we used S195D pseudo phosphorylation as a tool to mimic ELC phosphorylation. Both A57G-S195D and M173V-S195D restored abnormal (high) calcium sensitivity of force generation to the level similar to WT. M173V-induced low maximal ATPase activity was also partially restored by S195D. However, no effects of S195D on maximal force generation in ELC-exchanged fibers were observed (Table 12). For A57G mutation, no effect of S195D on the low level of force was observed. This lack of effect of S195D pseudo-phosphorylation on force recovery in either A57G or M173V exchanged muscle strips suggests that the harsh treatment of fibers with TFP during ELC-exchange may be the cause.
In summary, I explored whether myosin light chain phosphorylation could work as a common rescue mechanism for both RLC and ELC mutations linked to cardiomyopathies. Although positive results were obtained for both light chains, the true effects of phosphorylation are still inconclusive. More experiments are required to conclude on this issue (please see Chapter 6. Future Directions).
CHAPTER 6. FUTURE DIRECTIONS

Future Directions for Specific Aim 1

For K104E-RLC, our results indicated that changes in titin properties may be involved in elevated passive tension observed in Tg-K104E mice (Yamasaki, Wu et al. 2002, LeWinter 2004). Future examination of titin isoform switch or phosphorylation is therefore necessary. On the other hand, K104E’s disease phenotype exacerbates with age, and it is therefore important to examine the gene expression and signaling pathways in K104E mice at different life stages. Only then one can understand how K104E-linked HCM progresses in an age-dependent manner. For A13T-RLC, the transgenic protein expression level could only reach around 10%, and although the A13T mutation showed a dominant negative phenotype in mice, the detrimental effects of this mutation may become clearer with higher protein expression (e.g. effect of mutation on endogenous RLC phosphorylation). The alternative approaches include generating the knock-in mutant mice or engineering A13T-RLC adenovirus vector (AAV). AAV could be injected into NTg mouse hearts leading to higher mutant expression. It is worth mentioning that AAV-9 is known as heart specific and of high efficiency that can last for at least 1 year (Vassalli, Bueler et al. 2003, Yang and Xiao 2013). This approach is expected to yield higher expression of A13T–RLC mutant in the heart. For RLC-D94A, the Tg-D94A animals with ~100% protein expression have been successfully generated, and the effects of the D94A mutation can and will be assessed in vitro and in vivo utilizing this Tg-D94A mouse model.
**Future Directions for Specific Aim 2**

For the ELCs, since A57G’s passive tension was significantly elevated compared to WT, the involvement of titin isoform switch and its phosphorylation need to be examined. After intense exercise, HCM markers such as ANP, BNP and Collagen VIIIa were significantly upregulated in Tg-A57G vs. WT hearts. On the other hand, A57G’s calcium sensitivity of force and maximal tension were significantly lower than in WT, suggesting a risk for heart failure, especially after exercise (**Table 12**). Thus, future work needs to be conducted *in vivo* by echocardiography or hemodynamics to verify the potential for exercise-induced heart failure in Tg-A57G animals. The overall gene expression profile will also be analyzed in order to further understand the molecular mechanisms underlining the exercise induced heart dysfunction. Regarding the M173V mutation, the clinical information for this mutation is very limited. The M173V significantly decreased maximal ATPase activity and increased calcium sensitivity of force suggesting that it is a disease causing mutation. However, the effects of M173V on force generation were not seen in the M173V-ELC-exchanged fibers. This may due to harsh treatment of fiber preparations during ELC-mutant exchange. Future work will include generating ELC-M173V transgenic mice or utilizing the AAV system to specially express M173V-ELC in NTg mouse hearts rendering further research on this mutation.

**Future Directions for Specific Aim 3**

For RLC mutants, as mentioned in Chapter 5, we were not able to reach an optimal RLC phosphorylation level in K104E with smMLCK. Since the cMLCK is currently unavailable, the alternative strategy would be utilizing the S15D phospho mimics. The rescue effects of
S15D-pseudo phosphorylation have been demonstrated for another HCM causing RLC mutation D166V, in which S15D-D166V RLC showed powerful recovery of function that was compromised by the D166V alone (Muthu, Liang et al. 2014). AAV expression of S15D-RLC in the mouse myocardium would be a good strategy to look at the potential rescuing effects of phospho mimics of K104E. The other two RLC mutation, A13T and D94A, may also be examined using the same strategy; however, the effects of both mutations on RLC phosphorylation \textit{in vitro} (time dependent phosphorylation of RLCs) and \textit{in vivo} (endogenous RLC phosphorylation level) need to be first tested.

For ELC mutants, we have already tested the phosphomimetic S195D constructs expressed in the background of ELC mutations. However, the harsh treatment with TFP during ELC exchange process belied the true effects of S195D. Future work would be utilizing the AAV expression system to yield S195D-ELC expression in mouse hearts to facilitate further research on the beneficial effects of ELC phosphorylation.
LITERATURE CITED


Figure 1. Morphology of healthy, hypertrophic and dilated cardiomyopathy hearts. Illustrations depicting (A) a healthy heart with normal wall thicknesses and chamber volumes and (B) hypertrophic cardiomyopathy. Asymmetric hypertrophy of the interventricular septum as well as the left ventricular posterior wall and apex is present. (C) Dilated cardiomyopathy. Thinning of the ventricular walls enlarges the interior dimensions of the ventricular chamber. The left and right atria are also enlarged due to impaired ventricular relaxation. Ao, aorta; LA, left atrium; LV, left ventricle; RV, right ventricle (Harvey and Leinwand 2011).
Figure 2. Structure of the sarcomere and the Ca\textsuperscript{2+}-Tn-Tm dependent muscle activation. (A) The sarcomere is composed of two types of filaments, the thick filaments consisting of myosin and structural proteins such as myosin-binding protein C; and the thin filaments, which comprise of polymerized actin and the regulatory proteins, such as troponin and tropomyosin. (B) Cardiac muscle contraction is generated by binding of Ca\textsuperscript{2+} to troponin C (TnC) causing conformational changes in the Tn-Tm complex, so that the myosin binding sites on the thin filament are exposed. As a result, the myosin will attach to actin, hydrolyze ATP, and the energy released in this process will cause filament sliding and generation of force. Modified from (de Tombe 2003).
Figure 3. Structure of myosin, myosin head (S1) and the lever arm domain. (A) The myosin molecule is composed of one pair of coil-coiled heavy chains and two pairs of light chains. The C-terminus of myosin molecule forms the tail and this region is responsible for thick filament formation. The N-termini form two globular structures called myosin heads, or S1 domains. (B) The myosin head is made of two different domains, the motor domain, which contains one ATP binding site and one actin binding site. The lever arm domain, is made of a part of MHC, and two light chains, RLC and ELC. (C) RLC belongs to EF-hand calcium-binding proteins and contains one calcium binding site. In addition, it contains one functional phosphorylation site at serine 15. The ELC, although the EF-hand protein, does not bind calcium, however, its N-terminus is believed to interact with actin, while its C-terminus mainly interacts with MHC and possibly RLC. It was shown that it can be phosphorylated at Serine 195.
**Human Ventricular RLC – amino acid sequence**

MAPKKAKKRA GCNSNVFSM EFTQIQEFK EAFTIMDONRDGFIDKNDLR 50
DTFAALGRVN VKNEEIDEMI KEAPGPINFT VFLTFGEKLGADPEETIL 100
NAFVFDPKGKVVLKADYVR EMLTTQAERF SKEVDQMFA AFPPDVTGNL 150
DYKNLVHIT HGEEKD 166

**Human Ventricular ELC - amino acid sequence**

MAPKKPEPKK DDAAAPKAAPAPAPPEPE RPKEVEFAS KIKIEFTPEQ 50
IEEFKEAFFML FDRTPKCEMK ITYGQCGDVGL RALGNQNTQAVLRVLGKPR 100
QEELNTKMDM FETFLPMLQH ISKNKTDTGY EDFVEGLRVDKENGTVMG 150
AEELRHLVTLGLERLTEDEVE KLMAGQEDSN GCINYEAFVK HIMSS 195

**Figure 4. Amino acid sequences of myosin light chains and point mutations associated with HCM and DCM.** The bold and underlined letters represent amino acids depicted a disease-causing mutation. The areas highlighted in green stand for single point mutations which were studied in this proposal. Highlighted in yellow are the phosphorylation sites in RLC and ELC. Highlighted in cyan is the calcium binding loop of the RLC. The amino acid sequences of myosin light chains were taken from Uniprot P10916 (RLC) and P08590 (ELC).
Figure 5. Determination of protein expression in transgenic WT and K104E mice. RLC protein incorporation in mouse myocardium is determined in atria due to lower MW of transgenic (ventricular) vs. endogenous (atrial) RLC. Both ventricular RLC proteins (mouse and human) have similar MW preventing analysis of protein expression in ventricles. The Tg-protein expression was calculated as the ratio between band intensity of Tg-RLC and total RLC bands. The result showed both Tg-WT and Tg-K104E mice reach >95% protein expression. A: Atrium; LV: Left Ventricle
Figure 6. Effects of the K104E mutation on the CD spectra of the RLC. (A) Effect of the K104E RLC mutation on the CD spectra of RLC. Far-UV CD was performed utilizing a 1-mm path quartz cell in a Jasco J-720 spectropolarimeter. Spectra were recorded at 190–250 nm with a bandwidth of 1 nm. (B) The $\theta_{222}$ value was used to calculate the $\alpha$-helical content ($f_H$) using the following equation: $\theta_{222} = -30,300 f_H - 2,340$. Note significant reduction in $\alpha$-helical content in K104E mutant compared to WT. The values in (B) was shown as ± SEM, and the n = number of experiments, in which each experiment included ten individual curves.
Figure 7. Small angle X-ray diffraction measurements conducted in skinned papillary muscle fibers from Tg-WT and Tg-K104E mice. (A) The cartoon description of the cross section of the fibers. The large and small dots represent thick and thin filament respectively. $d_{1,0}$ stands for the lattice spacing and the interfilament spacing (IFS), which is the center to center distance between two thick filaments, is calculated as $d_{1,0}/\sin (60^\circ)$. (B) A representative picture of the equatorial reflection patterns of the cardiac fibers. We calculated the band intensity ratio between the 1,1 and 1,0 reflection to obtain the $I_{1,1}/I_{1,0}$ intensity ratio, which reflects the cross bridge distribution between the thick and thin filament. (C) Compared to WT, no significant change in $I_{1,1}/I_{1,0}$ intensity ratio was observed in K104E fibers (for values and statistical results please refer to table 10&11). (D) The K104E fiber’s IFS is significantly increased at both short and long sarcomere length compared to WT (for values and statistical results please refer to table 10&11).
Figure 8. Steady state force (A), calcium sensitivity of force (B), muscle relaxation kinetics (C), and passive tension measurements (D) in skinned papillary muscle strips from Tg-WT and Tg-K104E (K104E) mouse hearts. (A) Maximal tension assessment at saturating (pCa 4) calcium concentrations. Note Tg-K104E skinned papillary muscle strips developed a 1.4-fold lower maximal force (per cross-sectional area of muscle strip) compared with Tg-WT (p < 0.01). Error bars represent SEM. (B) Calcium sensitivity of force in skinned papillary muscle strips from Tg-WT vs. Tg-K104E mice. No significant (P>0.05) changes in the Ca\(^{2+}\) sensitivity of force or Hill coefficient values were observed between the strips from Tg-K104E animals compared with Tg-WT. (C) Assessment of passive tension (passive stiffness) under relaxation conditions (pCa 8). Note significantly increased levels of passive tension in Tg-K104E compared to Tg-WT mice (p < 0.001, as determined by ANOVA for repeated measurements). (D) Muscle relaxation dependence in Tg-K104E and Tg-WT papillary muscle strips. A significant reduction in relaxation rates was observed in Tg-K104E vs. Tg-WT muscle strips (p<0.01). All data were shown in ± SEM and n = number of experiments. 5-7 mice per group of WT or K104E were used in experiments (Huang, Liang et al. 2014).
Figure 9. Actin-activated myosin ATPase activity assay. Measurements were performed on myosin isolated from left and right ventricles of 5-11-month-old Tg-K104E (K104E) and Tg-WT mice. A pool of approximately six or seven hearts from each group was used to obtain myosin preparations used in (WT n = 16; K104E n = 13) independent experiments run in triplicates. Note a significantly higher V_{max} in K104E mouse myosin compared with WT myosin. The K104E’s K_{m} value, which represents the binding affinity of myosin to actin, is not significantly different from WT (Huang, Liang et al. 2014).
Figure 10. Frictional load in vitro motility assay of Tg-WT and Tg-K104E mouse myosin. Actin sliding velocity as a function of $\alpha$-actinin concentration. Tg-K104E (K104E) myosin propelled actin at a higher unloaded sliding velocity. However, once $\alpha$-actinin was applied to the flow cell the velocity dropped until reaching a plateau of $\sim 0.25 \ \mu m/s$. Conversely Tg-WT myosin had a slower unloaded velocity that dropped less steeply with $\alpha$-actinin concentration until $\sim 4 \ \mu g/ml$ $\alpha$-actinin. At higher $\alpha$-actinin concentrations velocity declined to a plateau similar to Tg-K104E (Huang, Liang et al. 2014).
Figure 11. Electron microscopy imaging and mitochondrial content assessment in Tg-WT and Tg-K104E mouse hearts. (A) Transmission electron micrographs showing sarcomeric ultrastructure of LV tissue from 6 month-old transgenic mice. The magnification is $10,500 \times$. Bar = 1 $\mu m$. Black arrows depict the Z-discs and white arrows the mitochondrial structures. Note, more mitochondria are recruited in the mutated hearts compared to WT controls. (B) Assessment of mitochondrial content in Tg-K104E vs. Tg-WT mice. Upper panel: SDS-PAGE of heart extracts probed for VDAC (porin) protein and normalized for the myosin ELC, used as a loading control. Lanes 1 and 3, Tg-WT 4 and 14 month-old, respectively; lanes 2 and 4, Tg-K104E, 4 and 14 month-old, respectively. Lower panel: Band intensity ratios derived from gel samples run for young and old Tg-WT and Tg-K104E extracts. 14 myocardial extracts were used for each group of mice. Note that the mitochondrial content was significantly ($p<0.05$) higher in young and old Tg-K104E vs. Tg-WT hearts (Huang, Liang et al. 2014).
Figure 12. Histopathological assessment of left ventricular tissue from transgenic mice. (A) Hematoxylin and eosin (H&E), and (B) Masson’s trichrome stained LV sections from 4.6 (young), 8.6 (intermediate) and 15.8 (old) month-old Tg-WT and 3.9 (young), 8 (intermediate) and 14.6 (old) month-old Tg-K104E mice. Note the progression of fibrosis in Tg-K104E hearts in mice of 8 to 15 months of age however, no disarray of myofilaments were observed through age (Huang, Liang et al. 2014)
Figure 13. Representative images of mitral inflow of 6 month-old Tg-K104E (A) and Tg-WT (B) mice. Notice a reduced E/A ratio in Tg-K104E compared with Tg-WT, and the difference was significant (p < 0.05 vs. WT, detailed data please refer to table 1). The average heart rate of all tested mice was ~475 bpm (Huang, Liang et al. 2014).
**Figure 14. The Adrenergic Signaling in cardiomyocytes.** The red color depicted genes which were upregulated in K104E compared to WT, the green color represented those downregulated, and grey for those whose expression was not changed. As seen in the figure, the expression of kinase PKA was down while the phosphotase PP2A expression was up, suggesting potential disruption in the sarcomeric protein phosphorylation, in particular TnI, which is phosphorylated with PKA in response to beta adrenergic stimulation. PKA was also responsible for PLN phosphorylation, which can further activate SERCA2a and thus regulate the calcium reuptake from the cytosol back into the Sakroplasmic Reticulum (SR). Other significant impaired genes were clustered in ion transmembrane transportation and PI3K-Akt signaling pathways.
Figure 15. Scheme of the disease causing mechanism of RLC-K104E linked HCM. Integrated molecular effects of the K104E (K104E) mutation in the myosin RLC depicted at the level of sarcomere and the whole heart that contribute to the development of hypertrophic cardiomyopathy (HCM) in transgenic mice. At the sarcomere level, the K104E mutation leads to a decrease in contractile force, increased maximal ATPase activity, and a decrease in muscle relaxation rate. It also results in elevated passive tension. These molecular events further trigger morphological and functional remodeling of the heart manifested by abundant mitochondria, development of fibrosis and altered diastolic indices. These changes are anticipated to result in diastolic disturbance and inefficiency in energy usage in Tg-K104E mice. In addition, aging also plays an important role in RLC-K014E linked HCM. All these aspects can trigger the development of K104E-RLC induced heart disease. Modified from (Huang, Liang et al. 2014).
Figure 16. Far UV CD spectrum of recombinant human ventricular WT and A13T RLC under no metal conditions (Apo state). Notice a pronounced change in CD spectrum due to the A13T mutation, which resulted in a large increase in the $\alpha$-helical content from 18% in WT (n = 4) to 29% in A13T (n = 2), n = number of experiments, and each experiment combined 10 individual curves (Szczechna, Ghosh et al. 2001).
Figure 17. Expression of A13T-RLC in transgenic mice. Upper panel: expression in atria, lower panel: in left ventricles (LV). Lanes 1-2, NTg extract; lane 3, Tg-WT L2 expressing ~100% of the human ventricular myosin RLC; lane 4, Tg-A13T L2 with 0% protein expression and lane 5, Tg-A13T L1 with ~10% protein expression.
Figure 18. Maximal steady-state force (A) and force-pCa measurements (B) in Tg-A13T, Tg-WT and NTg skinned muscle fibers. A 30% increase in maximal force (expressed per cross-sectional area of muscle fibers) was observed for Tg-A13T mice compared with controls, Tg-WT or NTg (A). The increase in maximal force was not accompanied by any changes in the force-pCa dependence and no significant differences in the pCa50 values were observed between Tg-A13T and control fibers (B). The data were derived from measurements on 5-11 individual skinned muscle fibers. Values shown as ± SEM: Maximal force: A13T 76.56 ± 2.51 kN/m² (n = 11), WT 57.43 ± 0.16 kN/m² (n = 11), NTg 52.49 ± 0.93 kN/m² (n = 5). pCa50: A13T 5.60 ± 0.01 (n = 10), WT: 5.59 ± 0.01 (n = 11), NTg: 5.56 ± 0.01 (n = 5).
Figure 19. Actin-activated myosin ATPase activity. Measurements were performed on myosin isolated from left and right ventricles of 3-5 months old Tg-A13T, Tg-WT and NTg mice. A pool of about 6 - 7 hearts from each group was used to obtain myosin preparations used in 4 - 5 independent experiments run in triplicate. Note a significantly lower $V_{\text{max}}$ for A13T mouse myosin compared with WT or NTg mouse cardiac myosins. Values: $V_{\text{max}}$: A13T $0.38 \pm 0.02$ s$^{-1}$ (n = 5), WT $0.51 \pm 0.01$ s$^{-1}$ (n = 4), NTg $0.63 \pm 0.04$ s$^{-1}$ (n = 4). $K_{m}$: A13T $2.35 \pm 0.21$µM (n = 5), WT: $2.00 \pm 0.05$ µM (n = 4), NTg $1.75 \pm 0.32$µM (n = 4). Data was shown in ± SEM and n = number of experiments.
Figure 20. Histopathological studies on~ 6 month-old A13T transgenic mouse hearts. (A) H&E staining on the whole heart slides from 6 month-old A13T, WT and NTg mice. Note the pronounced increase of IVS wall thickness and decrease in LV cavity in A13T hearts. (B) Less number of nuclei per area was seen in microscopy images taken from H&E slides of A13T animals, also suggesting IVS hypertrophy. (C) Masson’s trichrome staining showed a severe myofilament disarray in 6 month-old A13T mice, which was not seen in same age group of WT and NTg mice.
Figure 21. Pedigree of a Caucasian family with DCM which carries the D94A mutation. Solid symbols denote DCM, open symbols stand for unaffected, “+” denotes heterozygous for D94A mutation, arrow points to the proband. Individuals who underwent exome sequencing are denoted “exome”.
Figure 22. Effect of the D94A mutation on the CD spectra of RLC. (A) Far-UV CD was performed utilizing a 1-mm path quartz cell in a Jasco J-720 spectropolarimeter. Spectra were recorded at 190–250 nm with a bandwidth of 1 nm. (B) The $[\theta]_{222\text{nm}}$ value was used to calculate the $\alpha$-helical content ($f_\text{H}$) using the following equation: $[\theta]_{222} = -30,300 f_\text{H} - 2,340$. Note significant reduction in $\alpha$-helical content in D94A mutant compared to WT. Values: WT=0.168 ± 0.001 (n = 11) vs. D94A=0.133 ± 0.005 (n = 12), * states p < 0.01. Data was shown in ± SEM and n = number of experiments.
Figure 23. Modeled structure of human ventricular D94A-RLC using I-TASSER. The Ser15 phosphorylation site was labeled in cyan while the Amino acid 94 site in green. The distance between the C-α of Ser15 and C-α of Asp94 in WT is 21.9Å while the same distance in D94A was drastically decreased to 13.8 Å. And the superimposed structures showed D94A mutation significantly changed the secondary structure of RLC compared to WT. The predicted structure of WT and D94A was based on PDB structures: 3jvtB, 1prwA, 4ik1A, 2mysA, 4i2yA and 2w4aB.
Figure 24. The interaction of WT and D94A with the myosin heavy chain. (A) Representative 15% SDS-PAGE gel picture of titration experiments of RLC-depleted porcine cardiac myosin with increasing concentrations of WT or D94A RLCs. ELC that remain intact during the depletion reconstitution procedures was used as a loading control. (B) Binding isotherms of WT or D94A to RLC-depleted porcine myosin. The data points were fitted into the ligand binding equation to generate the maximal reconstitution level and the Kd, the apparent dissociation constant. Compared to WT, the D94A mutation large decrease in maximal RLC reconstitution level (WT: 0.88 ± 0.11 (n = 10) vs. D94A: 0.72 ± 0.05 (n = 10)), and the differences of maximal RLC exchange level at experimental points of molar ratio 2.8 (WT: 0.69 ± 0.03 (n=10) vs.D94A: 0.52 ± 0.04 (n = 10), p < 0.01) and 3 (WT: 0.70 ± 0.05 (n = 10) vs.D94A: 0.57 ± 0.03 (n = 10), p < 0.05) are significant. The Kd between WT (3.17 ± 5.20 (n = 10) and D94A (4.92 ± 7.48 (n = 10) p > 0.05) were not different. Data was shown in ± SEM and n = number of experiments.
Figure 25. Actin-activated ATPase activity of WT and D94A reconstituted porcine cardiac myosins. Data points were fitted to Michaelis-Menten equation to generate $V_{\text{max}}$ and $K_m$. Note that the D94A mutation significantly increased the maximal level of ATPase activity (WT: $0.19 \pm 0.004 \text{ s}^{-1}$ (n = 10) vs. D94A: $0.22 \pm 0.004 \text{ s}^{-1}$ (n = 13) p<0.01) with no change in $K_m$ compared with WT (WT: $0.45 \pm 0.10 \mu\text{M}$ (n = 10) vs. D94A: $0.50 \pm 0.10 \mu\text{M}$ (n = 13), p > 0.05). Data was shown in ± SEM and n = number of experiments.
Figure 26. Maximal force generation (A) and the force-pCa relationship (B) in skinned papillary muscle strips reconstituted with WT and D94A RLCs. (A) The difference in maximal tension per cross section of muscle strip was ~3 kN/m² (p > 0.05). (B) The pCa-force curve of D94A was overlapping that of WT, indicating no changes in the calcium sensitivity or Hill coefficient. The values are the following: Maximal force generation: WT: 37.13 ± 1.09 kN/m² (n=25) vs. D94A: 35.65 ± 1.11 kN/m² (n = 25) (p > 0.05), pCa50: WT: 5.69 ± 0.01 (n = 25) vs. D94A: 5.67 ± 0.01 (n = 25) (p>0.05), nH: WT: nH=2.93 ± 0.10 (n = 25) vs. D94A: nH=2.99 ± 0.10 (n = 25) (p > 0.05). Data was shown in ± SEM and n = number of fibers.
Figure 27. Analysis of protein expression in left ventricular extracts from different lines of Tg-WT and Tg-A57G mice. Myosin ELC protein (endogenous mouse or transgenic human) was detected with the monoclonal ab680 antibody (Abcam) followed by a secondary goat anti-mouse antibody conjugated with the infrared dye, Cy 5.5 (green bands). Total myosin RLC was probed with polyclonal CT-1 antibodies followed by a secondary goat anti-rabbit antibody conjugated with the infrared dye, IR red 800 (red bands). The ELC exchange percentage was calculated as the ratio between the band intensity of Tg-ELC and the total ELC (Tg-ELC plus mouse ELC). Two high expression lines and one low expression line of Tg-WT (L1: ~76%, L3: ~36%, L4: ~71%) and Tg-A57G ELC (L1: ~80%, L2: ~50%, L5: ~74%) were successfully generated (Muthu, Wang et al. 2011).
Figure 28. Rigor and Relaxed stiffness measured in skinned Tg ELC-WT and Tg ELC-A57G mice fibers. As shown in the figure, the A57G mutation significantly increased stiffness under rigor conditions (no ATP, actomyosin strongly bound) compared to WT (rigor stiffness, in kPa: WT: 410 ± 40 (n = 26); A57G: 710 ± 70 (n = 18), p < 0.01) while under relaxed condition (with ATP, actomyosin dissociated), A57G also largely increased relaxed stiffness, although not reaching significance (relaxed stiffness in kPa ± SEM: WT: 44 ± 5 (n = 26); A57G: 57 ± 9 (n = 18), p = 0.093) (Muthu, Wang et al. 2011). Change in the rigor stiffness suggested the mutation-elicited stiffness of the cross bridge, most likely the lever arm domain. The change in relaxed stiffness can be attributed to either fibrosis or alterations in titin properties, e.g. phosphorylation or isoform switch. n = number of measurements.
Figure 29. Measurements of steady-state force in skinned papillary muscle fibers from Tg-A57G vs. Tg-WT mice. (A) Maximal tension assessment. Note a 20% significant decrease in maximal force (pCa4) per cross-sectional area of fiber in the mutant mice. (B) Calcium sensitivity of force. Small but significant increase in the Ca\(^{2+}\) sensitivity of force was observed in Tg-A57G vs. Tg-WT fibers. (C) Assessment of passive tension (stiffness). Tg-A57G fibers demonstrated significantly increased levels of passive tension (pCa 8) at all points of stretch (in kN/m\(^2\)): 10%, 1.81 ± 0.17; 20%, 4.92 ± 0.34; 30%, 10.33 ± 0.49 and 40%, 19.93 ± 0.90 for n = 38 compared to Tg-WT fibers: 10%, 1.07 ± 0.09; 20%, 3.42 ± 0.16; 30%, 7.10 ± 0.22 and 40%, 13.29 ± 0.37 for n = 39 fibers. WT vs. A57G’s p < 0.01 by ANOVA with repeated measurements. (D) Relaxation rates in Tg-A57G vs. Tg-WT fibers assessed with Diazo-2. A mutation induced 8% decrease in relaxation rates was seen but the difference between relaxation kinetics in Tg-A57G and Tg-WT fibers was not statistically significant (p > 0.05) (Kazmierczak, Paulino et al. 2013).
Figure 30. Actin-activated myosin ATPase activity assay performed on Tg-WT-ELC and Tg-A57G mouse myosins. Neither the maximal ATPase activity ($V_{max}$) nor binding affinity to actin ($K_m$) was different from WT to A57G, suggesting this mutation had no effect on actomyosin interactions with the presence of ATP (WT vs. A57G $p > 0.05$ in both $V_{max}$ and $K_m$). Data was shown in ±SEM and $n =$ number of experiments.
Figure 31. Assessment of cardiac morphology and hypertrophy in Tg-A57G and Tg-WT mice. Histopathology images of heart samples from representative Tg-A57G and Tg-WT mice stained with hematoxylin and eosin (H&E) and Masson’s trichrome (Masson). Left ventricular sections from the hearts of 2 lines of Tg-A57G were compared with Tg-WT mice. Note extensive collagen stained fibrotic lesions in 6-mo-old Tg-A57G vs. 9-mo-old Tg-WT mouse hearts (bottom panels), however, no myofilament disarray was seen in either group of hearts (top panels) (Kazmierczak, Paulino et al. 2013).
Figure 32. Quantification PCR for detecting gene expression of HCM markers in both sedentary and exercised groups of WT and A57G. In Sedentary group, except for SERCA, which was upregulated in A57G, expression of rest genes was similar between WT and A57G. However, in the exercised group, gene expression of HCM markers ANP, BNP and fibrosis marker ColVIIIa all significantly increased in A57G, suggesting that exercise may result in more severe HCM phenotype in A57G animals. (Data was shown as mean ± SEM) (Kazmierczak, Yuan et al. 2014).
Figure 33. Steady state force generation and pCa$_{50}$ measurements in both sedentary and exercised groups of WT and A57G. Exercise seems to have minimal effects on the maximal force generation in both WT and A57G mouse fibers. There is a slight increase in maximal tension in A57G after exercise, but the amount of force in exercised A57G was still significantly lower than exercised WT. On the other hand, the pCa$_{50}$ of A57G significantly decreased after exercise, while the pCa$_{50}$ was increased in exercised WT. The low force and decreased calcium sensitivity in A57G suggest high risk of heart failure in A57G animals. The values are: force (kN/m$^2$ ± SEM, n = number of fibers): Sedentary: WT: 54.80 ± 1.13 (n = 15); A57G: 42.48 ± 1.46 (n = 11); Exercised: WT: 57.73 ± 1.27 (n = 15); A57G: 47.62 ± 1.97 (n = 8) pCa$_{50}$ (± SEM, n = number of fibers): Sedentary: WT: 5.63 ± 0.01 (n = 15); A57G: 5.67 ± 0.01 (n = 11); Exercised: WT: 5.66 ± 0.01 (n = 15); A57G: 5.59 ± 0.02 (n = 8) (Kazmierczak, Yuan et al. 2014).
Figure 34. Actin-activated myosin ATPase activity assay of WT, A57G and M173V ELC-exchanged porcine cardiac myosin preparations. The maximal ATPase activity (Vmax) and Michaelis-Menten constant (Km) were obtained from the assay. Compared with the WT exchanged myosin, A57G mutation did not affect either Vmax or Km, which agreed with the experimental results on Tg-A57G myosin. On the other hand, the M173V mutation significantly decreased Vmax (p < 0.01 vs. WT) and reduced Km (p < 0.05) compared to WT. Data was shown in ± SEM and n = number of experiments.
Figure 35. Steady state force measurements in WT, A57G and M173V ELC exchanged skinned porcine cardiac papillary muscle fibers. (A) Experimental protocol of force measurements in ELC-mutant exchanged porcine β-MHC papillary muscle fibers; (B) Representative SDS-PAGE picture of ELC-mutant exchanged fibers; (C-E) Maximal force generation, ∆pCa50 and the degree of ELC exchange in A57G, M173V and WT-porcine fibers; (C) No changes were observed in maximal force generation in either A57G or M173V ELC exchanged fibers compared to WT; (D) A57G significantly increases the Ca2+ sensitivity of force (decrease in ∆pCa50), while M173V does not significantly affect Ca2+ sensitivity compared to WT exchanged fibers; (E) Degree of ELC exchange in WT- and A57G exchanged fibers. Both A57G and M173V ELCs were able to incorporate into skinned porcine cardiac papillary muscle fibers with higher efficiency than WT. Part of this data were published in (Kazmierczak, Paulino et al. 2013)
As shown in the figure, the A57G specific phenotypes (not seen in the RLC mutants) were depicted in yellow. Similar to RLC-K104E, the A57G mutation decreased maximal force generation in mice, however, unlike RLC mutants, which altered the ATPase activity, the maximal ATPase activity of ELC-A57G was not affected. At the heart level, the A57G’s contractility was significantly increased. The SW, SV, and CO were significantly larger in A57G than WT. All of these *in vivo* parameters suggested the A57G hearts need to work much harder than WT to produce the same or similar effect to WT. Given the compromised force generation and no change in ATPase activity, the hyper systolic function seen in A57G hearts strongly indicate inefficiency in energy usage. On the other hand, the A57G mutation significantly increased calcium sensitivity of force, which is not seen in RLC mutants. The increased pCa50 of force may result in disruption of calcium homeostasis in the heart and ultimately cause arrhythmia and SCD in A57G positive patients. The A57G mutation also increased passive tension and relaxed stiffness (at the sarcomere level), and fibrosis (at the heart tissue level), which also can contribute to arrhythmia and SCD. In addition, pCa50 of force was significantly reduced after exercise in A57G animals, and the steady state force generation of exercised A57G was still lower than WT, suggesting a potential risk of heart failure. At the same time, the HCM related genes ANP, BNP and Col VIIIa were upregulated in exercised A57G, which confirmed that the disease phenotype in A57G was exacerbated after exercise. All three aspects described above comprise the ELC A57G linked HCM.
Figure 37. Determination of endogenous RLC phosphorylation level in Tg-WT and Tg-K104E hearts and the time course of RLC phosphorylation of WT and K104E RLCs. (A) Examination of endogenous protein phosphorylation in flash frozen hearts and in myofibrillar preparations from Tg-WT and Tg-K104E mouse hearts. The CT-1 antibody was used to detect total RLC while +P RLC antibody was used to detect only phosphorylated RLC. The ELC bands served as loading controls. (B) The endogenous RLC phosphorylation was calculated as the band intensity ratio between +P RLC and total RLC. The quantitative results show a significant decrease in endogenous RLC phosphorylation in Tg-K104E hearts. (C) Representative native gel picture of time course phosphorylation of WT and K104E RLCs with Ca$^{2+}$-CaM activated MLCK. No difference was observed between WT and K104E mutant. Part of data published in (Huang, Liang et al. 2014)
Figure 38. Small angle X-ray diffraction measurements in phosphorylated and non-phosphorylated Tg-WT and Tg-K104E mouse papillary muscle fibers. (A) Equatorial reflections’ intensity ratio in WT fibers did not significantly change after phosphorylation while it significantly increased in K104E fibers at both short and long sarcomere length. (B) The IFS of nonphosphorylated K104E (K104E-p) was significantly higher than nonphosphorylated WT (WT-p) at both short and long sarcomere lengths. RLC phosphorylation restored the IFS of K104E to the level near WT. (For details in value of each points and statistical results please refer to table 10&11.)
Figure 39. Maximal force and the force-pCa relationship in non-phosphorylated and phosphorylated WT and K104E skinned papillary muscle fibers. (A) Western blot results demonstrated that after MLCK treatment, both WT and K104E fibers reached similar levels of RLC phosphorylation. (B) Maximal force generation at pCa4 of non-phosphorylated and phosphorylated WT and K104E fibers. No significant differences were seen before and after phosphorylation in WT or K104E fibers. (C) The force-pCa relationship and pCa50 values for K104E vs. WT fibers. Noticed no effect of RLC phosphorylation on pCa50 (calcium sensitivity) of force generation.
Figure 40. Analysis of RLC phosphorylation in LV of Tg-WT and Tg-A13T mice. Upper panel: total ventricular RLC (t-RLC
ventr) detected with CT-1 RLC; lower panel, phosphorylated ventricular RLC (+P-RLCventr) detected with +P-RLC antibody. Lane 1, NTg extract; lane 2, Tg-A13T L1; lane 3, Tg-WT L2; lane 4-5, phosphorylated (+P) and non-phosphorylated (-P) myosin protein standard. Note no change in endogenous RLC phosphorylation level caused by A13T mutation.
Figure 41. Time course of RLC phosphorylation with human recombinant WT and D94A RLCs. (A) Representative native gel picture of the time course of phosphorylation of WT and D94A RLCs. Note a charge based separation of the phosphorylated (P-RLC) and non-phosphorylated RLC bands. (B) Time course of MLCK-dependent phosphorylation of WT and D94A RLCs (0 to 5min). Data points were fitted to exponential rise to maximal, single, 3 parameters equation to generate the phosphorylation rate kinetics (k). Note that compared to WT, the rate of RLC phosphorylation was significantly increased in D94A but the maximal level of RLC phosphorylation was only 0.48 of the WT. The values are: Max ratio: WT =1.00 ± 0.02 (n = 6) vs. D94A = 0.48 ± 0.01 (n = 6) (p<0.01), k: WT=1.06 ± 0.02 min⁻¹ (n = 6) vs. D94A = 2.26 ± 0.39 min⁻¹ (n=6) (p < 0.05). Data was shown in ± SEM and n = number of experiments.
Figure 42. Generation and purification of phospho-mimic (S195D) ELC proteins.  (A) Generation of single point mutations in ELC proteins. I used human recombinant WT-ELC DNA sequence as a template. For A57G mutant, we changed Alanine (GCC) into Glycine (GGC) through primer design and PCR. Similar missense mutations, M173V (ATG→GTT) and S195D (AGC→GAT) were also generated. All S195D-ELC cDNAs are available in the lab. All six ELC recombinant proteins: WT, A57G, M173V, WT-S195D, A57G-S195D and M173V-S195D are expressed and purified.  (B) Representative 15% SDS-PAGE gel picture of purified human recombinant ELC. Purification was executed using S-Sepharose and then followed by Q-Sepharose columns.
Figure 43. The actin-activated myosin ATPase activity assay of ELC exchanged porcine myosin. (A) No differences in $V_{\text{max}}$ and $K_m$ were seen among WT-ELC, A57G exchanged myosin, however, A57G-S195D significantly decreased $V_{\text{max}}$ and $K_m$ compared to A57G. (B) M173V-ELC exchanged myosin displayed a large decrease in maximal ATPase activity compared to WT-ELC exchanged myosin, and M173V-S195D was able to partially rescue the compromised maximal ATPase activity seen in M173V-exchanged myosin. Data was shown in $\pm$ SEM and $n =$ number of experiments.
Figure 44. Relative force and force-pCa relationship of WT, mutated and phospho-mimic (S195D) ELCs - exchanged skinned porcine papillary muscle fibers. (A) No differences were seen among all ELC exchanged fibers. (B) The ΔpCa50 of all ELC exchanged fibers. The ΔpCa50 was calculated as pCa50 after exchange minus pCa50 before exchange, the higher the number is, the higher the calcium sensitivity of force is. Note S195D pseudo phosphorylation restored the pCa50 of A57G and M173V to the level similar for WT. The values are shown in (± SEM) (n = number of fibers): force: WT = 31.51 ± 1.24 (n = 16), A57G=31.6 ± 61.53 (n = 12), A57G-SD = 30.02 ± 1.67 (n = 15), M173V = 31.88 ± 0.87 (n = 17), M173V-SD = 31.25 ± 1.02 (n = 19). ΔpCa50: WT = -0.06 ± 0.01 (n = 16), A57G = -0.006 ± 0.012 (n = 12), A57G-SD = -0.082 ± 0.015 (n = 15), M173V = 0.045 ± 0.008 (n = 17), M173V-SD = 0.087 ± 0.013 (n = 19).
## TABLES

**Table 1.** Echocardiography and hemodynamic parameters in 6 month-old Tg-K104E vs. Tg-WT mice

<table>
<thead>
<tr>
<th><strong>Echo/Doppler</strong></th>
<th><strong>Tg-K104E</strong> (n=10 males)</th>
<th><strong>Tg-WT</strong> (n=10 males)</th>
<th><strong>P-value</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate (bpm)</td>
<td>480±16</td>
<td>471±14</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>32±1.3</td>
<td>36±0.8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Ant. wall (mm)</td>
<td>0.86±0.05</td>
<td>0.87±0.03</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Post. wall (mm)</td>
<td>0.72±0.04</td>
<td>0.71±0.02</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>EF (%)</td>
<td>64±1.7</td>
<td>64±2.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Dec. time (ms)</td>
<td>25±2.2</td>
<td>21±1.8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>E/A</td>
<td>1.5±0.05</td>
<td>1.8±0.16</td>
<td>0.011</td>
</tr>
<tr>
<td>E/e’</td>
<td>26±2</td>
<td>26±2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>e’ (mm/s)</td>
<td>-31±2</td>
<td>-30±2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>s’ (mm/s)</td>
<td>27±0.9</td>
<td>28±1.3</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Invasive hemodynamics</strong></th>
<th><strong>Tg-K104E</strong> (n= 5 males)</th>
<th><strong>Tg-WT</strong> (n= 6 males)</th>
<th><strong>P-value</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>dp/dtmax (mmHg/s)</td>
<td>9,319±613</td>
<td>10,840±1,244</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>(dp/dtmax)/(P@dp/dtmax) (1/s)</td>
<td>162.0±19.7</td>
<td>174.4±11.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>PRSW (mmHg)</td>
<td>79.8±7.7</td>
<td>94.9±6.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>dp/dtmin (mmHg/s)</td>
<td>-7,911±1,188</td>
<td>-11,024±947</td>
<td>0.068</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>7.1±1.3</td>
<td>4.5±0.3</td>
<td>0.059</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Abbreviations: Ant., anterior; Post., posterior; EF, Ejection Fraction; Dec., deceleration; E, early mitral inflow; A, late mitral inflow; e’, early diastolic velocity; s’, systolic tissue velocity; dp/dtmax, maximum derivative of LV pressure; (dp/dtmax)/(P@dp/dtmax), ratio of dp/dtmax to pressure at dp/dtmax; PRSW, Preload Recruitable Stroke Work; dp/dtmin, minimum derivative of LV pressure; Tau, time constant (isovolumic relaxation time); Red color indicates significant difference between Tg-WT and Tg-K104E. (Huang, Liang et al. 2014)
Table 2. Echocardiography indices in senescent Tg-K104E vs. Tg-WT mice

<table>
<thead>
<tr>
<th></th>
<th>Tg-K104E</th>
<th>Tg-WT</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>12 (9 males)</td>
<td>15 (11 males)</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>36.58±2.64</td>
<td>33.60±1.56</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>406±52</td>
<td>483±19</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Ant. wall (mm)</td>
<td>1.19±0.07</td>
<td>0.70±0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Post. wall (mm)</td>
<td>1.12±0.09</td>
<td>0.72±0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LV diameter in diastole (mm)</td>
<td>3.65±0.13</td>
<td>4.22±0.10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LV diameter in systole (mm)</td>
<td>2.12±0.08</td>
<td>2.84±0.10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LVmass (mg/g weight)</td>
<td>4.9±0.33</td>
<td>3.3±0.12</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LVvol-end-diastole (µl)</td>
<td>57±5</td>
<td>86±4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LVvol-end-systole (µl)</td>
<td>15±1</td>
<td>33±3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>EF (%)</td>
<td>73.02±2.33</td>
<td>62.12±1.99</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Abbreviations: Ant., anterior; Post., posterior; LVmass, mg of LV weight divided by body weight; LVvol, LV volume; LVvol-end-systole, LV volume at the end of systole; EF, Ejection Fraction. Red color indicates significant difference between Tg-WT and Tg-K104E (Huang, Liang et al. 2014).
Table 3. Differential expression of important genes in various biological processes (WT vs. K104E, number of mice = 2 for each group)

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fold change (K104E vs. WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structural</strong></td>
<td></td>
</tr>
<tr>
<td>β-myosin</td>
<td>myh7</td>
</tr>
<tr>
<td>cMLCK</td>
<td>Mylk3</td>
</tr>
<tr>
<td>atrial ELC</td>
<td>Myl4</td>
</tr>
<tr>
<td>Collagen VIII</td>
<td>Col8a1</td>
</tr>
<tr>
<td>skeletal alpha actin</td>
<td>Acta1</td>
</tr>
<tr>
<td>matrix metallopeptidase 2</td>
<td>Mmp2</td>
</tr>
<tr>
<td>Titin</td>
<td>Ttn</td>
</tr>
<tr>
<td>MyBP-C</td>
<td>Mybpc3</td>
</tr>
<tr>
<td>Troponin C, cardiac/slow skeletal</td>
<td>Tnnc1</td>
</tr>
<tr>
<td>Troponin I, cardiac</td>
<td>Tnni3</td>
</tr>
<tr>
<td><strong>Cytoskeletal</strong></td>
<td></td>
</tr>
<tr>
<td>Four-and-a-half LIM protein 1</td>
<td>FHL1</td>
</tr>
<tr>
<td>Skeletal muscle α 2 actinin</td>
<td>ACTN2</td>
</tr>
<tr>
<td>Nebulin-related protein</td>
<td>NRAP</td>
</tr>
<tr>
<td>Desmin</td>
<td>DES</td>
</tr>
<tr>
<td><strong>Protein synthesis</strong></td>
<td></td>
</tr>
<tr>
<td>Transcription elongtation factor 1α1</td>
<td>EEF1A1</td>
</tr>
<tr>
<td>A+U-rich element RNA binding factor</td>
<td>Hnrpdl</td>
</tr>
<tr>
<td>Mitochondrial ribosomal protein L52</td>
<td>Mrpl52</td>
</tr>
<tr>
<td>Ribosomal protein L10</td>
<td>Rpl10</td>
</tr>
<tr>
<td>Ribosomal protein L13</td>
<td>Rpl13</td>
</tr>
<tr>
<td><strong>Redox system</strong></td>
<td></td>
</tr>
<tr>
<td>NADH ubiquinone oxidoreductase</td>
<td>Ndubfb3</td>
</tr>
<tr>
<td>Ubiquinol-cytochrome c reductase hinge protein</td>
<td>Uqcrh</td>
</tr>
<tr>
<td>NADH dehydrogenase Fe-S protein 6</td>
<td>Ndufs6</td>
</tr>
<tr>
<td><strong>Ca²⁺ handling</strong></td>
<td></td>
</tr>
<tr>
<td>SERCA2a</td>
<td>Atp2a2</td>
</tr>
<tr>
<td>RyR2</td>
<td>Ryr2</td>
</tr>
<tr>
<td>NCX</td>
<td>Slc8a1</td>
</tr>
<tr>
<td>L-type Channel Channel, cardiac</td>
<td>Cacna1c</td>
</tr>
</tbody>
</table>
**Phospholamban**

**Metabolism**

- Apoptosis-inducing factor, mitochondrion-associated, 1 (Aifm1) - 1.22
- Porin (Vdac1) - 1.04
- Medium-chain specific acyl-CoA dehydrogenase, mitochondrial (Acadm) - 1.13
- PPARalpha (Ppara) - 1.07
- acetoacetyl-CoA synthetase (Aacs) - 1.00
- cAMP-dependent protein kinase catalytic subunit alpha (Prkaca) - 1.07
- Glucose transporter 4 (slc2a4) - 1.16
- L-type pyruvate kinase (Pkm) - 1.24

**Other biomarkers for hypertrophy/heart failure and unknowns**

- Atrial natriuretic peptid (Nppa) - 1.32
- Brain natriuretic peptide (Nppb) - 1.33
- Serine protease inhibitor A3A (Serpina3a) - 1.24
- Heat Shock 70kD protein 8 (Hspa8) - 1.05

Red color indicates fold change larger than 1.2 between WT and K104E

---

**Table 4.** List of important pathways which was significantly affected (enrichment p value <0.05) (WT vs. K104E)

<table>
<thead>
<tr>
<th>Signaling pathways (K104E vs. WT)</th>
<th>number of genes up</th>
<th>number of genes down</th>
<th>gene %* in the pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine metabolism</td>
<td>6</td>
<td>3</td>
<td>51.72</td>
</tr>
<tr>
<td>Glyoxylate and dicarboxylate metabolism</td>
<td>5</td>
<td>5</td>
<td>44</td>
</tr>
<tr>
<td>Toll-like receptor signaling pathway</td>
<td>20</td>
<td>9</td>
<td>31.68</td>
</tr>
<tr>
<td>Protein digestion and absorption</td>
<td>6</td>
<td>9</td>
<td>32.18</td>
</tr>
<tr>
<td>PI3K-Akt signaling pathway</td>
<td>14</td>
<td>20</td>
<td>26.2</td>
</tr>
<tr>
<td>Adrenergic signaling in cardiomyocytes</td>
<td>6</td>
<td>9</td>
<td>33.3</td>
</tr>
</tbody>
</table>

*gene % in the pathway was calculated as number of larger than 1.2 fold changed genes divided by total number of genes in the pathway
Table 5. Echocardiography parameters in Tg-A13T vs. Tg-WT mice

<table>
<thead>
<tr>
<th></th>
<th>A13T</th>
<th>WT</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals n</td>
<td>9</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>36</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Age (month)</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.67±0.08</td>
<td>0.7±0.08</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>4.28±0.40</td>
<td>4.22±0.38</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.67±0.09</td>
<td>0.72±0.06</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>3.11±0.26</td>
<td>2.84±0.38</td>
<td>0.052</td>
</tr>
<tr>
<td>EF (%)</td>
<td>52.96±5.32</td>
<td>61.3±7.58</td>
<td>0.005</td>
</tr>
<tr>
<td>LV Mass (g)</td>
<td>105.87±27.53</td>
<td>111.67±25.47</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>LV Mass Corrected (g)</td>
<td>84.7±22.03</td>
<td>89.34±20.37</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>E Velocity (mm/s)</td>
<td>807.1±115.31</td>
<td>892.69±179.77</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

The numbers highlighted in red show significant differences compared to WT (p<0.05).

IVSd: Interventricular septum diameter at the end of diastole; LVIDd: Left ventricle inner diameter at the end of diastole; LVPWd: Left ventricle posterior wall diameter at the end of diastole; LVIDs: Left ventricle inner diameter at the end of systole; EF: Ejection Fraction; E Velocity: E-wave Velocity.
Table 6. Clinical information of pedigree with D94A-DCM.

<table>
<thead>
<tr>
<th>Pedigree position</th>
<th>Age of diagnosis, years</th>
<th>ECG / Arrhythmia</th>
<th>LVEDD, mm (Z-score)</th>
<th>LVEDD Framingham percentile</th>
<th>EF, %</th>
<th>PWd (mm)</th>
<th>SWd (mm)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>NA</td>
<td>none</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>normal ECG at age 56</td>
</tr>
<tr>
<td>I-2</td>
<td>21</td>
<td>AVB</td>
<td>56 (3)</td>
<td>&gt; 99th</td>
<td>33</td>
<td>11</td>
<td>10</td>
<td>PPCM</td>
</tr>
<tr>
<td>II-1</td>
<td>29</td>
<td>NSSTT, ectopic atrial rhythm, PVCs, PACs</td>
<td>47.2 (0.44)</td>
<td>&lt; 95th</td>
<td>40</td>
<td>7.9</td>
<td>5.9</td>
<td>DCM assignment by systolic dysfunction only. Medical therapy initiated at earliest evidence of decreased ejection fraction with ongoing periodic clinical screening of at-risk relatives.</td>
</tr>
<tr>
<td>II-2</td>
<td>24</td>
<td>PACs</td>
<td>55 (1.3)</td>
<td>&lt; 95th</td>
<td>49</td>
<td>NA</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>III-3</td>
<td>NA</td>
<td>NSR</td>
<td>49 (-0.9)</td>
<td>&lt; 95th</td>
<td>58</td>
<td>9</td>
<td>9</td>
<td>normal echo and ECG at age 31</td>
</tr>
</tbody>
</table>

LVEDD = left ventricular end diastolic dimension; EF = ejection fraction; PWd = posterior wall diastole; SWd = septal wall diastole, 1AVB = first degree atrioventricular block; PPCM = peripartum cardiomyopathy; NSSTT = non-specific ST-T wave abnormality; PVCs = premature ventricular contractions; PACs = premature atrial contractions; NSR = normal sinus rhythm.
Table 7. IFS and I₁₁/I₁₀ intensity ratio measured on Tg-WT and Tg-A57G fibers under rigor state

<table>
<thead>
<tr>
<th>Transgenic mice</th>
<th>IFS (nm)</th>
<th>I₁₁/I₁₀ intensity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>49.18±0.42 (n=17)</td>
<td>3.53±0.14 (n=15)</td>
</tr>
<tr>
<td>A57G</td>
<td>47.70±0.20 (n=10)</td>
<td>3.96±0.24 (n=10)</td>
</tr>
</tbody>
</table>

p value: 0.017 (WT) 0.109 (A57G) (NS)

Published in (Muthu, Wang et al. 2011)

Table 8. Echo analysis of Tg-A57G vs. Tg-WT mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tg-A57G</th>
<th>Tg-WT</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (number of mice)</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>BW/HW</td>
<td>5.4 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.84 ± 0.04</td>
<td>0.83 ± 0.04</td>
<td>0.753</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>1.24 ± 0.07</td>
<td>1.20 ± 0.04</td>
<td>0.629</td>
</tr>
<tr>
<td>LVAWd (mm)</td>
<td>0.95 ± 0.05</td>
<td>1.02 ± 0.05</td>
<td>0.396</td>
</tr>
<tr>
<td>LVAWs (mm)</td>
<td>1.52 ± 0.09</td>
<td>1.58 ± 0.04</td>
<td>0.526</td>
</tr>
<tr>
<td>LVVd (μl)</td>
<td>74.6 ± 3.3</td>
<td>57.1 ± 3.6</td>
<td>0.003</td>
</tr>
<tr>
<td>LVVs (μl)</td>
<td>31.4 ± 3.0</td>
<td>20.9 ± 2.9</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Abbreviations: LV, left ventricle; PW, posterior wall; AW, anterior wall; LVV, LV end-diastolic (d) and end-systolic (s) endocardial volumes. Red color indicates significant difference between WT and A57G. (Kazmierczak, Paulino et al. 2013)
Table 9. Hemodynamic parameters derived from PV relations in Tg-A57G and Tg-WT mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tg-A57G</th>
<th>Tg-WT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (number of mice)</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>463 ± 20</td>
<td>478 ± 22</td>
<td>0.648</td>
</tr>
<tr>
<td><strong>Integrated performance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF (%)</td>
<td>57.9 ± 3.2</td>
<td>64.6 ± 3.0</td>
<td>0.147</td>
</tr>
<tr>
<td>SW (mmHg x μl)</td>
<td>3,435 ± 294</td>
<td>2,233 ± 214</td>
<td>0.005</td>
</tr>
<tr>
<td>SV (μl)</td>
<td>43.3 ± 2.4</td>
<td>33.4 ± 1.4</td>
<td>0.002</td>
</tr>
<tr>
<td>CO (ml/min)</td>
<td>20.2 ± 1.7</td>
<td>15.9 ± 0.9</td>
<td>0.033</td>
</tr>
<tr>
<td>Cardiac efficiency</td>
<td>0.286 ± 0.062</td>
<td>0.428 ± 0.068</td>
<td>0.170</td>
</tr>
<tr>
<td><strong>Afterload</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVESV (μl)</td>
<td>34.0 ± 3.6</td>
<td>27.3 ± 3.1</td>
<td>0.183</td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>104.7 ± 2.9</td>
<td>91.4 ± 4.0</td>
<td>0.032</td>
</tr>
<tr>
<td>Ea (mmHg/μl)</td>
<td>2.45 ± 0.12</td>
<td>2.78 ± 0.18</td>
<td>0.186</td>
</tr>
<tr>
<td><strong>Preload</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDV (μl)</td>
<td>69.4 ± 3.2</td>
<td>52.6 ± 2.9</td>
<td>0.002</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>11.5 ± 2.1</td>
<td>12.6 ± 1.9</td>
<td>0.720</td>
</tr>
<tr>
<td><strong>Contractility</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dt_{max} (mmHg/s)</td>
<td>8073 ± 793</td>
<td>6951 ± 589</td>
<td>0.267</td>
</tr>
<tr>
<td>Ees (slope)</td>
<td>66.1 ± 7.4</td>
<td>44.9 ± 3.4</td>
<td>0.020</td>
</tr>
<tr>
<td><strong>Relaxation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-dP/dt_{min} (mmHg/s)</td>
<td>8576 ± 934</td>
<td>6873 ± 582</td>
<td>0.125</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>8.5 ± 0.9</td>
<td>17.1 ± 4.8</td>
<td>0.175</td>
</tr>
</tbody>
</table>

Abbreviations:  HR, heart rate; EF, ejection fraction; SW, stroke work; SV, stroke volume; CO, cardiac output; LVESV and LVESP, left ventricular (LV)-end systolic volume and pressure, respectively;  Ea, arterial elastance: ratio of LV-end systolic pressure to stroke volume (ESP/SV); LVEDV and LVEDP, LV-end diastolic volume and pressure; dP/dt_{max}, the peak rate of rise in LV pressure; Ees, measure of myocardial contractility defined by the slope of the end-systolic pressure–volume relationship (ESPVR); Tau, isovolumic relaxation constant. Red color indicates significant difference between WT and A57G. (Kazmierczak, Paulino et al. 2013)
**Table 10.** IFS (in nm) and Intensity ratio ($I_{1,1}/I_{1,0}$) for short and long SL (average ±SE) of WT and K104E phosphorylated and non-phosphorylated fibers

<table>
<thead>
<tr>
<th>Protein</th>
<th>IFS short</th>
<th>IFS long</th>
<th>p value (short vs.long)</th>
<th>$I_{1,1}/I_{1,0}$ short</th>
<th>$I_{1,1}/I_{1,0}$ long</th>
<th>p value (short vs.long)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>45.60±0.32</td>
<td>43.19±0.46</td>
<td>p&lt;0.01</td>
<td>0.35±0.02</td>
<td>0.46±0.04</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>50 shots</td>
<td>41 shots</td>
<td>12 fibers</td>
<td>46 shots</td>
<td>10 fibers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>43.19±0.46</td>
<td>41 shots</td>
<td>10 fibers</td>
<td>46 shots</td>
<td>10 fibers</td>
<td></td>
</tr>
<tr>
<td>WT+p</td>
<td>45.72±0.51</td>
<td>44.39±1.10</td>
<td>p&gt;0.05</td>
<td>0.43±0.06</td>
<td>0.54±0.14</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>26 shots</td>
<td>8 shots</td>
<td>9 fibers</td>
<td>16 shots</td>
<td>7 shots</td>
<td></td>
</tr>
<tr>
<td></td>
<td>44.39±1.10</td>
<td>8 shots</td>
<td>7 fibers</td>
<td>16 shots</td>
<td>2 fibers</td>
<td></td>
</tr>
<tr>
<td>K104E</td>
<td>46.99±0.33</td>
<td>44.90±0.41</td>
<td>p&lt;0.01</td>
<td>0.40±0.03</td>
<td>0.44±0.04</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>40 shots</td>
<td>32 shots</td>
<td>11 fibers</td>
<td>33 shots</td>
<td>27 shots</td>
<td></td>
</tr>
<tr>
<td></td>
<td>44.90±0.41</td>
<td>32 shots</td>
<td>10 fibers</td>
<td>33 shots</td>
<td>8 fibers</td>
<td></td>
</tr>
<tr>
<td>K104E+p</td>
<td>46.28±0.23</td>
<td>43.01±0.60</td>
<td>p&lt;0.01</td>
<td>0.58±0.06</td>
<td>0.65±0.07</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>22 shots</td>
<td>16 shots</td>
<td>7 fibers</td>
<td>23 shots</td>
<td>12 shots</td>
<td></td>
</tr>
<tr>
<td></td>
<td>43.01±0.60</td>
<td>16 shots</td>
<td>5 fibers</td>
<td>23 shots</td>
<td>6 fibers</td>
<td></td>
</tr>
</tbody>
</table>

Significant difference (p<0.05) was depicted in red color.

**Table 11.** Statistical analysis of IFS and Intensity ratios for short and long SL (average ±SE) of WT and K104E phosphorylated and non-phosphorylated fibers

<table>
<thead>
<tr>
<th>System</th>
<th>IFS Short SL</th>
<th>IFS Long SL</th>
<th>$I_{1,1}/I_{1,0}$ Short SL</th>
<th>$I_{1,1}/I_{1,0}$ Long SL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT vs WT+p</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>K104E vs.K104E+p</td>
<td>p&gt;0.05</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>K104E vs.WT</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>K104E+p vs.WT+p</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>p&lt;0.01</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>

Significant difference (p<0.05) was depicted in red color.
Table 12. Summary of structural and functional effects and disease-causing mechanisms for RLC and ELC mutations studied in this thesis proposal

<table>
<thead>
<tr>
<th></th>
<th>RLC</th>
<th>ELC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A13T</td>
<td>K104E</td>
</tr>
<tr>
<td><strong>Sarcomere level</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Light chains</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-helical content ↓</td>
<td>Endogenous RLC</td>
</tr>
<tr>
<td></td>
<td>endogenous RLC</td>
<td>phosphorylation level (NC)</td>
</tr>
<tr>
<td></td>
<td>Time dependent RLC</td>
<td>phosphorylation:</td>
</tr>
<tr>
<td></td>
<td>phosphorylation (NC)</td>
<td>maximal level ↓; rate ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myosin</td>
<td>Cross bridge cycling rate (ATPase) ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endogenous RLC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phosphorylation level (NC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time dependent RLC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phosphorylation (NC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fibers</td>
<td>Force ↑ pCa50 (NC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Force ↓ pCa50 (NC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relaxation rate ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Histopathology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe IVS</td>
<td>Fibrosis exacerbates with age; no myofilament disarray. High mitochondrial content seen in 6 month-old LV</td>
</tr>
<tr>
<td></td>
<td>hypertrophy and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fibrosis seen in 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>month-old mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Heart Level</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>In vivo measurements</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal cardiac</td>
<td></td>
</tr>
<tr>
<td></td>
<td>morphology and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>function</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LV and septum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hypertrophy not seen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in 6 month-old K104E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>but clearly manifested</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diastolic dysfunction;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tau and E/A ratio ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as early as 6 months of age.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCM related gene</td>
<td>NCX and L-type</td>
</tr>
<tr>
<td></td>
<td>expression</td>
<td>calcium channel ↑; but do not significant effect on calcium homeostasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effect through the N-ELC and actin interaction; S195D ELC able to partially restore decreased ATPase activity in M173V
<table>
<thead>
<tr>
<th>Disease level</th>
<th>Morphology</th>
<th>Function</th>
<th>Possible disease causing mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe septum hypertrophy with decrease in LVV seen in the hearts of mice</td>
<td>Late onset of hypertrophy phenotype: LV hypertrophy and LVV↑ seen in &gt;13 month-old animals</td>
<td>Alteration in cross bridge cycling rate, particularly dissociation rate (g) may be due to change in A13T RLC structure. Disease phenotype manifested with only 10% protein expression, suggesting a possible poison peptide mechanism.</td>
</tr>
<tr>
<td></td>
<td>Increase in LVEDD seen in patients carrying D94A mutation</td>
<td>Decrease in EF was seen in DCM patients</td>
<td>Diastolic disturbance and inefficiency in energy usage, both of which could be caused by alterations in structure of RLC; changes in endogenous RLC phosphorylation in the heart working as potential mechanism of action</td>
</tr>
<tr>
<td></td>
<td>Eccentric hypertrophy</td>
<td>Systolic dysfunction with high risk of sudden cardiac death after intense exercise in mice</td>
<td>Mainly through alterations in RLC structure and phosphorylation; inefficiency in energy usage may be another contributor to the disease mechanism</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td>Increased ATPase activity and increased calcium sensitivity of force may underlie disease mechanism; possible alterations in N-ELC-actin interaction</td>
</tr>
</tbody>
</table>