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Haploinsufficiency of Cardiac Myosin Binding Protein-C in the Development of Hypertrophic Cardiomyopathy

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LOYOLA UNIVERSITY CHICAGO

HAPLOINSUFFICIENCY OF CARDIAC MYOSIN BINDING PROTEIN-C IN THE DEVELOPMENT OF HYPERTROPHIC CARDIOMYOPATHY

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

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DOCTOR OF PHILOSOPHY

PROGRAM IN CELL AND MOLECULAR PHYSIOLOGY

BY

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CHICAGO, IL

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For My Wife and Parents
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LIST OF ABBREVIATIONS

Ala         Alanine
α-MHC       Cardiac α-myosin heavy chain protein
α-TM        α-tropomyosin
Asp         Aspartic Acid
β-AR        β Adrenergic Signaling
β-MHC       Cardiac β-myosin heavy chain protein
BP          Basepair
CASQ2       Calsequestrin 2 gene
cMyBP-C     Cardiac myosin binding protein-C protein
cTnC        Cardiac troponin C protein
cTnI         Cardiac troponin I protein
cTnT         Cardiac troponin T protein
E/A         Ratio of the early (E) to late (A) ventricular filling velocities
E'/A'       Ratio of early (E') to late (A') mitral annulus motion
FMAX        Force developed at saturating calcium concentrations
GAPDH       Glyceraldehyde 3-phosphate dehydrogenase gene
HCM         Hypertrophic cardiomyopathy
Het         Heterozygous genotype
HF          Heart Failure
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>$k_{Tr}$</td>
<td>Rate of force redevelopment</td>
</tr>
<tr>
<td>MYBPC3</td>
<td>Cardiac myosin binding protein-C gene</td>
</tr>
<tr>
<td>MYH6</td>
<td>Cardiac α-myosin heavy chain</td>
</tr>
<tr>
<td>MYH7</td>
<td>Cardiac β-myosin heavy chain gene</td>
</tr>
<tr>
<td>nH</td>
<td>Hill Coefficient</td>
</tr>
<tr>
<td>NPPA</td>
<td>Atrial natriuretic factor gene</td>
</tr>
<tr>
<td>pCa</td>
<td>Calcium concentration in $-\log_{10}$</td>
</tr>
<tr>
<td>pCa$_{50}$</td>
<td>Calcium concentration required for 50% maximal force</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SL</td>
<td>Sarcomere length, in μm</td>
</tr>
<tr>
<td>S2</td>
<td>Myosin Subfragment 2</td>
</tr>
<tr>
<td>TAC</td>
<td>Transverse Aortic Constriction</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>+/-</td>
<td>Wild-type MYBPC3 mouse</td>
</tr>
<tr>
<td>+/t</td>
<td>Heterozygous MYBPC3 truncation mutant mouse</td>
</tr>
<tr>
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CHAPTER ONE

INTRODUCTION

Heart Failure (HF) is one of the leading causes of morbidity and mortality in the human population and represents a common endpoint for several diseases including inherited cardiomyopathies (Niimura, Bachinski et al. 1998). Hypertrophic Cardiomyopathy (HCM) is characterized by left ventricular wall thickening, diastolic dysfunction, and sarcomere disarray (Watkins, MacRae et al. 1993, Maron, Gardin et al. 1995, Watkins, Conner et al. 1995, Spirito, Seidman et al. 1997). Mutations in sarcomeric protein encoding genes have been linked to HCM although the mechanisms by which these mutations act remain unclear. Therefore, functional characterization of frequent mutations would help define the mechanisms of pathogenesis and provide novel therapeutic targets.

The gene MYBPC3, encoding cardiac myosin binding protein-C (cMyBP-C), has been established as the second most commonly mutated gene in HCM cases. As a majority of these mutations have been determined to result in a null allele which does not produce any protein, it has been suggested that haploinsufficiency (i.e. the inability for one allele to express enough protein) of the MYBPC3 gene is responsible for the development of HCM (Bonne, Carrier et al. 1995, Watkins, Conner et al. 1995). In symptomatic heterozygous patients
with null MYBPC3 mutations, reduced cMyBP-C levels have been shown (van Dijk, Dooijes et al. 2009). However, as protein levels in asymptomatic carriers have not been assessed, it is unclear if reduced protein is causative for or occurs as a result of the onset of HCM. Therefore, in this work I tested whether heterozygous (Het) mice carrying a knock-in C’ truncation mutation that expresses normal levels of cMyBP-C had alterations in function, and whether stress-induced hypertrophy resulted in decreased levels of cMyBP-C.

The results of this study show that Het mice have dysfunction under normal conditions and show significantly reduced MYBPC3 expression in Het hearts with preserved cMyBP-C levels. Significant impairments were observed in cardiomyocyte force generation and whole-organ diastolic function in naïve Het mice compared to WT controls. Following transverse aortic constriction (TAC) pressure-overload cardiac stress Het mice show significantly greater hypertrophy and reduced cMyBP-C levels compared to WT controls. Force generation is reduced both in TAC and sham Het cardiomyocytes and significant Ca\textsuperscript{2+} sensitization is also observed. Finally, significant deficits in systolic function in vivo are evident in the Het TAC hearts compared to WT TAC.

These findings demonstrate that Het MYBPC3 truncation mutant mice have baseline dysfunction that leads to exacerbated development of hypertrophy and dysfunction following stress. These results suggest that human carriers may have functional deficits prior to the onset of remodeling and are predisposed to developing exacerbated hypertrophy with worsened cardiac function.
CHAPTER TWO

LITERATURE REVIEW

2.1 Cardiovascular Physiology in Health and Disease

Hypertrophic cardiomyopathy (HCM) is a genetically based pathological enlargement of the heart that occurs in the human population with a frequency of 1 in 500 individuals and can result in the development of heart failure (HF) (Watkins, MacRae et al. 1993, Bonne, Carrier et al. 1995, Watkins, Conner et al. 1995, Niimura, Bachinski et al. 1998). HF is clinically defined as the inability of the heart to pump a sufficient amount of blood to meet the needs of the body. HF is also a common end-point for a variety of cardiovascular diseases; it is estimated to affect approximately eighty million adults per year in the United States and is one of the leading causes of death in the human population (Go, Mozaffarian et al. 2013).

HCM has been described as a disease of the cardiac sarcomere, as mutations in genes encoding sarcomeric proteins have been identified as causative in the majority of HCM cases (Arad, Seidman et al. 2002). Cardiac myosin binding protein-C (cMyBP-C) is a sarcomeric trans-filament protein that is involved in the regulation of sarcomere structure and function. Mutations in the
cMyBP-C gene (*MYBPC3*) are associated with ~34% of all cardiomyopathy cases, of which 70% are predicted to produce C'-truncated proteins (Spirito, Seidman et al. 1997, Morita, Rehm et al. 2008). Strikingly, one specific *MYBPC3* mutation has been estimated to occur in over 60 million people worldwide, underscoring the necessity of determining its mechanism in the pathogenesis of HF (Dhandapany, Sadayappan et al. 2009).

*MYBPC3* mutations are associated with incomplete disease penetrance and/or late disease onset and are highly associated with HF (Niimura, Bachinski et al. 1998, Moolman, Reith et al. 2000, Dhandapany, Sadayappan et al. 2009). The functional and pathological consequences of *MYBPC3* mutations are not fully characterized, as the majority of these mutations are expected to generate C'-truncated cMyBP-C that is often not found in cardiac tissue from affected HCM patients (Moolman, Reith et al. 2000, Marston, Copeland et al. 2009, van Dijk, Dooijes et al. 2009). This indicates that the truncated protein is either not expressed or is rapidly degraded. Furthermore, in symptomatic human patients carrying truncating *MYBPC3* mutations, cMyBP-C levels have been shown to be decreased (van Dijk, Dooijes et al. 2009). These observations suggest that the development of HCM could be caused by insufficient cMyBP-C production due to haploinsufficiency of the *MYBPC3* gene, meaning that a single functional copy of the gene is insufficient to achieve a wild-type (WT) phenotype. However, reduced cMyBP-C levels have not been established to be causative for, or as a result of the development of HCM in human carriers.
2.1.1 The Healthy and Sick Heart

The pump function of the heart supplies the human body with oxygen and nutrients through the circulation of blood. Normal cardiac function requires dynamic regulation in order provide proper blood supply in response to the changing needs of the body. The contraction and relaxation of the heart is achieved through an exquisitely tuned molecular machine and disruption of any of the constitutive parts can result in aberrant function and development of disease. Perturbations in cardiac function can develop into life threatening situations which must be addressed immediately in order to restore proper organ function and preserve the life of the individual. Due to the essential role of the heart and its complexity, cardiovascular disease is the leading cause of death in the human population, accounting for approximately 1 in 3 deaths (Go, Mozaffarian et al. 2013).

Cardiovascular disease can occur for a host of reasons, with each distinct pathological situation presenting different challenges for effective treatment. HCM is a general term for pathological enlargement of the heart that occurs in the absence of an externally identifiable cause. This distinction differentiates HCM from other diseases that cause hypertrophy, such as chronic high blood pressure, diabetes, or autoimmune disease (Maron, Gardin et al. 1995). Due to this clinical definition, HCM is diagnosed by excluding other causes of hypertrophy, and it wasn’t until the mid-1990’s that a genetic cause for the disease was defined (Watkins, MacRae et al. 1993, Bonne, Carrier et al. 1995).
Originally, this form of HCM was termed Familial Hypertrophic Cardiomyopathy, as it followed Mendelian inheritance patterns and persisted in families (Bonne, Carrier et al. 1995).

Populations with ancient founder mutations typically have a high prevalence of these mutations. In the human population as a whole, the prevalence of HCM is 1 case in every 500 people, making it one of the more common forms of heart disease (Go, Mozaffarian et al. 2013). The discoveries that defined the genetic cause of HCM established the general understanding that HCM is a disease of the sarcomere (Bonne, Carrier et al. 1995, Watkins, Conner et al. 1995). Furthermore, HCM typically results in a gradual decline of cardiac function towards HF, which in some cases can include a transition to dilated cardiomyopathy (DCM). HF is a common end point for a variety of cardiovascular diseases including HCM, DCM, myocardial ischemia and infarction, and is estimated to affect over 5 million Americans over 20 years of age (Go, Mozaffarian et al. 2013). However, in order to understand the pathological development of HCM, it is important to understand how specific mutations affect sarcomere function and how these changes perturb total cardiovascular physiology.

2.1.2 Cardiac Anatomy and Function

The role of the heart is to circulate blood to supply the body's tissues with oxygen and nutrients and to transport waste products for removal. In order to
accomplish this task, the heart functions as a unidirectional pump that pressurizes blood in order to perfuse the body. The pump function of the heart can be explained by examining the anatomy of the organ.

The mammalian heart is made up of four chambers, two atria and two ventricles, attached to the systemic venous and arterial systems. Blood enters the right atrium from the venous system via the vena cava. During cardiac relaxation, known as diastole, blood passively flows through the right atrium into the right ventricle due to the slightly higher blood pressure in the venous system than in the diastolic right atrium and ventricle. During atrial systole, the right atrium contracts to assist in right ventricular filling. When the right ventricle is full and begins to contract, the tricuspid valve separating the right atrium and ventricle closes due to the higher pressure in the contracting ventricle. The right ventricle contracts until the blood inside has been sufficiently pressurized to open the pulmonary valve (typically around 25mmHg) that separates the right ventricle from the pulmonary artery. Blood is then ejected into the pulmonary circulation where gas exchange occurs in the alveolar capillaries. Following contraction the pressure in the right ventricle drops below the pressure in the pulmonary artery, causing blood to flow backwards; however, this backwards flow is prevented by the closing of the pulmonary semilunar valve that separates the right ventricle from the pulmonary artery.

The left side of the heart mirrors what occurs on the right, except the pressures exerted are much higher. Oxygenated blood returns from the lungs via
the pulmonary vein and empties into the left atrium and passively fills the left ventricle during diastole. Passive left ventricular filling slows as the ventricle fills with more blood due to the elastic properties of the left ventricle. During atrial systole the contraction of the left atrium ejects an additional amount of blood into the left ventricle, which in a healthy heart amounts to approximately 5% of total left ventricle peak diastolic volume. This relationship can be defined by the early to atrial ratio (E/A ratio) of LV filling (Figure 1). An additional parameter of diastolic function is the early E’ and atrial A’ (recoil) of the heart, measured as the movement of the tissue surrounding the mitral valve. This relationship can also be expressed as a ratio of E’/A’ (Nag, Appleton et al. 2009).

After filling the left ventricle contracts causing increased blood pressure that closes the mitral (or bicuspid) valve, which separates the left atrium from the ventricle. This prevents backwards flow of blood during ventricular systole. Isovolumic left ventricular contraction occurs until the blood reaches sufficient pressure to open the aortic valve, which is a pressure equal to diastolic blood pressure (typically around 80mmHg in a healthy adult). Blood rapidly ejects into the aorta and peripheral arteries until the end of ventricular systole, at which point the left ventricle begins to relax. High systemic blood pressure forces the aortic valve to close when pressure inside the left ventricle drops following contraction, preventing retrograde flow. During a typical heart beat the human left ventricle will eject 65-75% of the total LV volume, a value known as ejection fraction (Kass, Yamazaki et al. 1986).
The heart pumps blood to circulate oxygen and nutrients to the peripheral tissues. However, the heart must also perfuse itself, as the blood within the atria and ventricles is not useable by the cardiac tissue. To this end coronary arteries branch off of the aorta immediately superior to the aortic valve. These arteries branch several times, providing oxygenated blood to the whole heart. Occlusions in these arteries from plaque deposits or thrombosis can lead to ischemia localized downstream of the blockage. If perfusion is not restored by clot-dissolving drugs or angioplasty then the ischemic area will become necrotic, resulting in a myocardial infarction (Go, Mozaffarian et al. 2013).

Hypertrophic remodeling alters the anatomy of the heart. As the left ventricle enlarges the internal diameter of the ventricle gets smaller, reducing the amount of blood that can fill the ventricle in diastole. As the myocardium thickens in hypertrophy, it also becomes stiffer, which causes additional resistance to diastolic filling. A hallmark of this diastolic dysfunction is observed in the E/A ratio, where early filling becomes a smaller component of total diastolic filling, and the atrial component becomes more important. When this occurs the E/A ratio decreases (Figure 1) (Nagueh, Appleton et al. 2009).

Cardiac systolic function is typically compensated in early hypertrophy, with normal ejection fraction and maintained cardiac output. However, if cardiac function begins to decompensate, congestive heart failure can develop, with blood pooling in the pulmonary and venous circulation, typically resulting in distal edema (Go, Mozaffarian et al. 2013). At this stage the E/A ratio can be altered in
a variety of ways, however one potential observation is a merged E and A peak, when the pressure in the pulmonary circulation increases enough that the opening of the mitral valve is followed by one large influx of blood to the LV, instead of two distinct filling phases (Nagueh, Appleton et al. 2009).

As diastolic function can be an early indication of cardiac dysfunction, prior to other overt manifestations, in this study the E/A, and E'/A' parameters were measured to assess functional decline in naïve heterozygous mice as well as mice developing pressure-overload induced hypertrophy.
The early (E) and atrial (A) diastolic filling peaks are represented above in a typically normal ratio (left) and in a situation where the ratio is decreased (right). The deflections below the 0 blood flow velocity line represent aortic ejection velocity, with the periods between the diastolic filling and aortic eject representing the isovolumic filling and isovolumic ejection.

Figure 1. Schematized Depiction of the E/A Ratio.
2.1.3 The Pressure-Volume Relationship of the Left Ventricle

Understanding the anatomy of the heart provides an understanding of its function; however, cardiac function in terms of the contraction and relaxation of the ventricles can be better described using the four phases of the pressure-volume relationship (Figure 2A) (Suga and Sagawa 1974, Sagawa 1981, Kass, Yamazaki et al. 1986).

During ventricular diastole the left ventricle passively fills with blood, causing the volume to increase, but due to the compliance of the ventricle the pressure only increases at the end of diastolic filling (phase I). Pressure will start to rise if the heart is filled to the point where the compliance drops and the elastic elements start to resist additional filling. The pressure exerted by the total amount of blood filling the ventricle at the end of diastole is considered the preload on the heart. At the start of systole, the ventricle begins to contract, which closes the mitral valve (point A) and seals the ventricular chamber. As the aortic valve is also closed, the volume within the ventricle remains constant, with increasing pressure as the heart exerts force on the blood. This phase is termed isovolumic contraction (phase II).

The ventricle exerts force on the preload until the pressure in the LV is greater than the diastolic blood pressure on the distal side of the aortic valve, at which point the aortic valve opens (point B) and the rapid ejection phase begins (phase III). The level of diastolic blood pressure the heart is pumping against is considered the afterload. The heart continues to eject blood until the ventricle
stops contracting, after which the ventricular pressure begins to fall. Once the pressure in the heart falls below the systolic blood pressure, the aortic valve closes (point C) which marks the end of systole. The left ventricle then relaxes, with pressures still high enough to keep the mitral valve closed, meaning this phase has no change in volume, with a rapid decline in pressure and is termed isovolumic relaxation (phase IV). Once pressure in the left ventricle falls below the pressure in the left atrium, the mitral valve opens (point D) and again allows filling of the ventricle and the cardiac cycle repeats.
Figure 2. The Cardiac Pressure-Volume Relationship.

A. Schematized depiction of the pressure-volume relationship for left ventricular function. Letters denote closing or opening of valves (A: mitral valve closes; B: aortic valve opens; C: aortic valve closes; D: mitral valve opens) and numerals identify stages of the cardiac cycle (I: ventricular filling; II: isovolumic contraction; III: ventricular ejection; IV: isovolumic relaxation). B. Diagram of a normal loop (black) compared to a loop from a heart pumping against a high afterload (grey) and a failing heart in dilation (red). C. Diagram depicting the ESPVR and EDPVR during a reduction in cardiac pre-load.
During hypertension or aortic stenosis the heart is required to work harder to open the aortic valve and eject blood (Figure 2B). Following prolonged periods of increased work the heart will begin to hypertrophy which can result in the eventual death of cardiomyocytes, replacement with fibroblasts, potential thinning of the ventricle wall and the development of heart failure (Gonzalez, Fortuno et al. 2003). In cases where the ventricular walls thin and the heart dilates, the LV enlarges and the total ventricular volume in diastole and systole increases (Figure 2B). Importantly, as cardiac function is decreased the amount of blood ejected in each cycle decreases, meaning a reduction in the amount of cardiac output. The inability to maintain pump function is the definition of the transition to HF (Kannel and Belanger 1991, Go, Mozaffarian et al. 2013).

Several important aspects of cardiac function can be studied using the pressure-volume relationship including two critical concepts: the end-systolic and end-diastolic pressure-volume relationships (ESPVR, EDPVR) (Figure 2C) (Sagawa 1981, Kass, Yamazaki et al. 1986). The ESPVR is a linear relationship that describes the contractile function of the heart. ESPVR is determined by plotting the pressure-volume relationship at the end of systolic ejection (Point C) in a series of cardiac cycles with alterations in preload or afterload. The slope of the ESPVR line describes cardiac function by relating the preload volume (reduced by occluding venous return) with how much blood was ejected (i.e. contractility). In disease models with decreased contractility (e.g. HF without preserved ejection fraction), the ESPVR is expected to have a shallower slope,
meaning that the amount of blood pumped out during systole is lower than would occur with a normal ESPVR for a given preload. Additionally, positive inotropic drugs and signaling pathways that increase contractility result in a steeper ESPVR slope and an increase in blood ejection (Suga and Sagawa 1974, Sagawa 1981, Kass, Yamazaki et al. 1986).

The EDPVR describes how resistant the heart is to passive diastolic filling by measuring how alteration in total filling volume of the ventricle at peak diastole is related to the passive pressure found at peak diastole (Point A on the PV-Loop). This is intuitive when thinking about how additional filling of the heart will stretch the ventricle, with increased volume causing an increase in pressure as the heart becomes less distensible. In disease states like dilated cardiomyopathy, the EDPVR has a rather flat slope, as increased filling of the dilated and distensible ventricle causes an increase in volume with a smaller increase in pressure. In contrast, during hypertrophy the increased fibrosis in the myocardium results in decreased compliance and results in a steeper EDPVR, as increased filling is met with greater resistance and causes pressure to rise more rapidly (Sagawa 1981, Kass, Yamazaki et al. 1986).

2.1.4 Electrical Regulation of Contraction

The contraction of the right and left atria and ventricles occurs concomitantly, with the coordinated contraction of the heart controlled by the propagation of electrical signals through the myocardium. These signals begin in
a specialized group of cells in the right atrium known as the sinoatrial (SA) node. This node is comprised of pacemaker cells that set the heart rate autonomously by initiating depolarization, and can be modulated by sympathetic and parasympathetic inputs. When the SA node fires, it initiates a wave of depolarization that spreads across the right and left atria, that causes their contraction. As this depolarization spreads, it also travels from the SA node to the atrioventricular (AV) node, which is located near the junction of the four chambers, superior to the interventricular septum.

The AV node coordinates the contraction of the ventricles and has its own latent pacemaker activity, such that if the SA node is injured or ablated the heart will still rhythmically contract at the rate of AV node firing. The action potential propagates from the AV node down specialized conduction fibers known as the His-Purkinje system. These fibers run inferiorly through the interventricular septum and then run from apex to base up the free walls of the left and right ventricle. This conduction pattern allows both ventricles to contract in concert, with the endocardium contracting slightly prior to the epicardium, and the apex contracting before the base of the ventricle. This orientation and sequence of contraction causes the ventricles to contract from the apex up, allowing efficient ejection of blood from the ventricle. Action potentials also spread from cell-to-cell due to the electrical coupling of cardiomyocytes via gap junctions (typically Connexin 43 in ventricular tissue) that allow propagation of depolarization through cells (Kreuzberg, Willecke et al. 2006).
Hypertrophic remodeling of the heart can alter the electrical properties of the myocardium and lead to arrhythmias and sudden cardiac death. As the tissue thickens the wave of depolarization must travel further to activate all areas of the heart. Furthermore the electrical properties of the tissue change in hypertrophy, with alterations in gap junctions and ion channels influencing the propagation of excitation (Sepp, Severs et al. 1996). Increased fibrosis in hypertrophy also decreases the propagation of electrical current by increasing resistance. These changes, along with certain alterations in the structure of the heart, such as asymmetric hypertrophy, can create an environment that is arrhythmogenic. These situations have been speculated to be one of the contributing factors in sudden cardiac death in patients with mild hypertrophy (Swynghedauw 1999, Maron, Doerer et al. 2009).

2.1.5 Regulation of Cardiac Function

Regulation of heart rate (chronotropy), contractility (inotropy), and relaxation (lusitropy) occurs via innervation of the heart as well as through endocrine signaling. Regulation of the SA node is controlled by innervation from sympathetic and parasympathetic neurons and alters heart rate, with sympathetic innervation causing the heart to beat at an increased rate (Olshansky, Sabbah et al. 2008). This is accomplished by increasing the rate of depolarization of the SA node cells by increasing the funny channel (I_f) current, which allows more Na⁺ influx into the cell (DiFrancesco and Tortora 1991). In contrast parasympathetic
innervation causes the SA node cells to depolarize more slowly by reducing the If current, therefore reducing the heart rate.

Positive inotropy and lusitropy is regulated primarily through sympathetic innervation or pharmacological stimulation of the adrenergic signaling pathway which involves agonist binding to the \(\beta\)-AR G-protein coupled receptor, production of cyclic-AMP by adenylyl cyclase and activation of protein kinase A (PKA). This kinase phosphorylates several targets in the cardiomyocytes including numerous \(\text{Ca}^{2+}\) handling proteins, as well as several sarcomeric proteins which will be discussed later. PKA phosphorylation of Ryanodine Receptor (RyR) increases \(\text{Ca}^{2+}\) release into the cytosol, providing increased contractility, although this remains controversial (Bers 2000, Marks 2000). Phosphorylation of phospholamban relieves an inhibition on the sarcoplasmic reticulum ATPase (SERCA), which allows SERCA to pump \(\text{Ca}^{2+}\) back into the sarcoplasmic reticulum (SR) at a higher rate, which facilitates faster relaxation. This also causes a positive inotropic effect as the SR is loaded with more \(\text{Ca}^{2+}\) for release in subsequent beats (MacLennan and Kranias 2003).

### 2.1.6 Excitation-Contraction Coupling

The sarcomeric machinery does not function without a cytosolic rise in free \(\text{Ca}^{2+}\). Normal diastolic levels of \(\text{Ca}^{2+}\) in cardiomyocytes are in the range of \(\sim 100\text{nM}\), whereas \(\text{Ca}^{2+}\) concentrations outside the cell are around 2mM, and in the sarcoplasmic reticulum during diastole are in the 1mM range (Bers 2000).
The series of events leading from cardiac action potential to the generation of force is termed excitation-contraction (EC) coupling. This process begins with depolarization of the cardiomyocyte, with depolarization spreading along the sarcolemma and down the T-tubules, which are specialized invaginations of the sarcolemma that penetrate into the cardiomyocyte. Depolarization of the T-tubule membrane causes opening of L-type Ca\(^{2+}\) channels which allows an influx of calcium into the cell. RyR Ca\(^{2+}\) channels are positioned in the sarcoplasmic reticulum opposite of the L-Type Ca\(^{2+}\) channels. When calcium binds to the cytosolic side of RyR, the channel opens and releases large amounts of Ca\(^{2+}\) from the sarcoplasmic reticulum. This event is known as calcium-induced calcium release (CICR) (Figure 3).

During hypertrophic remodeling there is a general increase in β-AR signaling, resulting in hyperphosphorylation of PKA targets (Heineke and Molkentin 2006). These signaling changes cause greater Ca\(^{2+}\) release in the cell during systole. However, if systolic free Ca\(^{2+}\) is increased for too long, diastolic relaxation can be disrupted, leading to diastolic dysfunction as will be discussed in regards to myofilament protein properties. Prolonged increases in diastolic Ca\(^{2+}\) can also lead to arrhythmias as Ca\(^{2+}\) is extruded from the cell and alters repolarization (Schober, Huke et al. 2012). Following chronic β-AR signaling, desensitization can occur resulting in hypophosphorylation of many PKA targets. This situation can result in a decline in contractility and further aberrations in Ca\(^{2+}\) handling.
A schematic diagram showing Ca$_{2+}$ entry into the cardiomyocyte following membrane depolarization. Calcium-induced calcium release (CICR) starts from Ca$_{2+}$ entry via L-Type Ca$_{2+}$ channels which activates RYR Ca$_{2+}$ release. This activates the myofilaments (green and red lines). Following contraction Ca$_{2+}$ is taken back up into the SR via SERCA or extruded from the cell via NCX.

Figure 3 Simplified Excitation-Contraction Diagram.
2.1.7 Molecular Mechanisms of Sarcomere Contraction

Excitation-contraction coupling links the rise in intracellular Ca\(^{2+}\) to development of force in the sarcomere. As a general overview, as these proteins will be discussed later in greater detail, the sarcomere is comprised of thick filaments containing myosin heavy chain (MHC), cardiac myosin binding protein-C (cMyBP-C), and titin. The sarcomeric thin filaments are composed of actin, α-tropomyosin, and the troponin complex containing troponin C (TnC), troponin T (TnT), and troponin I (TnI).

Excitation-contraction begins with the entrance of Ca\(^{2+}\) into the cell and the release of calcium from the SR cause a rise in cytosolic Ca\(^{2+}\) as previously discussed. The SR runs along the myofibrils and allows local Ca\(^{2+}\) concentrations to increase. When Ca\(^{2+}\) binds to TnC it causes conformational changes in the protein that transmit through TnT to actin and α-tropomyosin. This initiates conformational changes that cause α-tropomyosin to alter its position along the actin filaments, unmasking myosin binding sites on actin. The myosin motor head is then able to bind weakly to the thin filament, establishing a cross-bridge (Solaro 2009).

Sarcomere contraction is mediated by myosin’s ATPase activity that occurs in a cyclic progression, using ATP for the energy of crossbridge cycling and force generation (Rayment 1996). When the myosin heads have an empty nucleotide binding site they remain tightly bound to the thin filaments. These locked cross bridges are the molecular basis for rigor mortis following death as
ATP levels are depleted, and binding of ATP to myosin causes dissociation from the thin filament. The myosin ATPase then hydrolyzes ATP into ADP and inorganic phosphate which causes a conformational change that allows myosin to bind strongly with actin, as well as “priming” the myosin head for delivery of the power-stroke. Upon acto-myosin crossbridge formation the Myosin ATPase ejects the ADP and Pi from their binding sites, causing a conformational change that generates the power-stroke. The myosin head remains attached until another ATP molecule is bound, at which point the cycle repeats. The concerted effort of crossbridge cycling results in sarcomere contraction and shortening, which is the basis for cardiomyocyte contractility. A subset of HCM-causing mutations in myosin have been identified; these mutations alter ATPase activity and acto-myosin interaction, causing a direct impairment in force generation and ATP usage (Sommese, Sung et al. 2013, Bloemink, Deacon et al. 2014).

Following force generation during systole, rapid relaxation of the sarcomeres must occur to allow the heart to relax and fill with blood for the subsequent beat. As the heart fills with blood during diastole, the left ventricle distends and the cardiomyocytes are stretched. Increased stretch of cardiomyocytes causes an increase in contractility due to the Frank-Starling Law of the Heart (de Tombe, Mateja et al. 2010). This is a functionally useful property for the heart, as it allows the heart to contract harder when it is filled with more blood, regulating cardiac function on a beat-to-beat basis without changes in heart rate or requiring alterations in signaling pathways. The molecular basis for
the Frank-Starling Law is due to the length-dependent increases in sarcomere Ca\(^{2+}\) sensitivity which causes increased force development at a given Ca\(^{2+}\) concentration. This is observed in single cells where a stretched sarcomere will generate more force than an un-stretched sarcomere (Konhilas, Irving et al. 2002). This effect has an upper limit, where additional stretch will produce no additional force, and indeed excess stretch results in decreased force generation due to physical deformation of the sarcomeric machinery. Despite the importance of length dependent activation in regulating cardiac function, the mechanism by which it alters Ca\(^{2+}\) sensitivity and regulates force generation is still being determined.

Due to the importance of length-dependent changes in Ca\(^{2+}\) sensitivity of force development, the work outlined in this study used skinned cardiomyocytes to assess this effect. As will be discussed later, alterations in myofilament proteins can alter Ca\(^{2+}\) sensitivity of force development, and haploinsufficiency of cardiac myosin binding protein-C may alter these parameters.

### 2.1.8 Etiology of Hypertrophic Cardiomyopathy

In the previous sections the many physiological functions of the heart have been described, as well as some of the perturbations that occur during the development of cardiomyopathy. While some functional alterations that occur in HCM have been highlighted in the previous sections, the etiology, prognosis, and treatment of HCM must be examined in order to understand the complexity of
Hypertrophic cardiomyopathy is a prevalent inherited pathological enlargement of the heart that was originally described in 1958 (Teare 1958) (Maron, Gardin et al. 1995). This disease involves hypertrophy of the left ventricle, in some cases with disproportionate hypertrophy of the interventricular septum, termed asymmetric hypertrophy. A diagnosis of HCM requires an absence of causes of hypertrophic remodeling such as diabetes, high blood pressure, or infarcted tissue (Spirito, Seidman et al. 1997, Go, Mozaffarian et al. 2013). As hypertrophy in HCM does not occur in response to an external stimulus it was classically defined as idiopathic cardiomyopathy. In the 1990’s this idiopathic heritable HCM, also known as familial hypertrophic cardiomyopathy was determined to be a disease of the cardiac sarcomere, with mutations in several sarcomeric genes identified as the heritable causes of the disease, which will be described later.

At the level of the whole organ, physiological hypertrophy, as occurs following aerobic training, involves growth of the heart muscle outwards with a preserved ventricular internal diameter and increased contractile performance (Swynghedauw 1999). However, in pathological hypertrophy the myocardium hypertrophies inward, reducing the volume of the ventricle. During hypertrophy, the heart typically retains the ability to pump blood, with a maintained ejection fraction and normal cardiac output.

One hallmark of HCM is the development of diastolic dysfunction that can
occur for various reasons, including reduction in left ventricular compliance due to increased wall thickness and increased fibrotic remodeling. Diastolic dysfunction prevents normal relaxation, which, along with the reduced ventricular volume, causes reduced filling of the ventricle (Nagueh, Appleton et al. 2009). Following pathological hypertrophy in mice, and in a subset of human HCM cases (approximately 10%), progression to DCM can occur. This typically accompanies the onset of HF, with morphological changes including thinning of the ventricle walls, resulting in reduced force generation, and dilating of the left ventricle (Figure 4).

In mice the progression from pathological hypertrophy to dilation is an important morphological indicator of development of HF, despite the fact that this transition from HCM to DCM to HF is not commonly observed in humans (Kannel and Belanger 1991, Olivotto, Cecchi et al. 2012). Therefore, in the work detailed in this dissertation periodic monitoring of ventricular morphology was done using echocardiography to assess the progression of disease.
This schematic illustrates that during physiological hypertrophy the heart enlarges proportionally, adding myocardium with preserved luminal space. Pathological hypertrophy results in an increased wall thickness and reduced internal diameter. During dilation the walls of the heart thin and begin to lose the ability to generate force for sufficient ejection of blood, while the ventricular volume increases.

Figure 4. Cardiac Morphology in Health and Disease.
At the cellular level remodeling of the cardiomyocyte contractile machinery also occurs in HCM. During hypertrophy the cardiomyocytes start to produce additional sarcomeres. However, unlike physiological hypertrophy, these new sarcomeres are added in deranged patterns which result in less coordinated contraction and less efficient force development (Arad, Seidman et al. 2002). Despite the inefficiency of hypertrophic remodeling, hypertrophic hearts can maintain pump function for prolonged periods. However, for reasons that are still being studied, functional decompensation can occur, leading to heart failure with preserved or impaired ejection fraction and insufficient cardiac output. These hearts are unable to efficiently exert force on the blood in the ventricle, which results in reduced ejection fraction and impaired cardiac output. The transition to dilation in mice and human patients with dilation often accompanies the transition to heart failure (Teare 1958, Olivotto, Cecchi et al. 2012).

During the development of hypertrophy, fibroblasts increase the deposition of extracellular matrix, causing increased fibrosis of the heart. The mechanisms governing fibrotic remodeling are still being deciphered; however, as cardiomyocytes die they are replaced by fibroblasts (Varnava, Elliott et al. 2000). Increased collagen and connective tissue deposition increases the fibrous nature of the myocardium and remodeling of normal extracellular matrix increases the proliferation of fibroblasts (Ho, Lopez et al. 2010). This reduces the compliance of the ventricle as a whole, and has adverse effects on cardiac performance as diastolic filling becomes more difficult (Nagayama, Takimoto et al. 2007).
Another common complication of HCM is sudden death, which tends to occur in younger carriers of HCM mutations and typically occurs during high levels of exertion (Maron, Roberts et al. 1980, Maron, Doerer et al. 2009). Victims of sudden death often have a mildly hypertrophic phenotype and in many cases were unaware of any pathology (Maron, Doerer et al. 2009). The cause of sudden death in these patients is unclear, but it has been suggested that alterations in conduction pathways in the hypertrophied myocardium provide the opportunity for arrhythmias to develop, especially at high heart rates, and can lead to ventricular fibrillation and death (Schober, Huke et al. 2012). While this provides a general explanation for how hypertrophy causes sudden cardiac death, some specific HCM-causing mutations in the troponin complex have been established to cause alterations in Ca\textsuperscript{2+} handling in the myocyte and can cause the development of arrhythmia directly (Knollmann, Kirchhof et al. 2003, Schober, Huke et al. 2012).

Effective treatment of HCM is complicated due to the diverse causes of hypertrophy, especially in treating genetic HCM compared to treating a hypertrophic stimulus, such as blood pressure. In cases of HCM caused by mutations in sarcomeric proteins, interventions are currently only available to treat the phenotype, while the primary cause is unaddressed. In cases of asymmetric hypertrophy, where septal enlargement has partially occluded the aortic outflow tract, use of septal ablation has been recently shown to improve function (Olivotto, Ommen et al. 2007). This technique removes portions of the
septal myocardium by surgical excision or by destroying the tissue with ethanol injection (Olivotto, Ommen et al. 2007).

The dilemma for producing a viable treatment for familial cardiomyopathy is that each mutation may have a unique mechanism of action and require a different treatment strategy. The difficulty of viable treatment is demonstrated by the different causes of HCM in patients with myosin heavy chain or cMyBP-C mutations, with myosin mutation carriers developing HCM typically with a more severe phenotype and worse prognosis (Arad, Seidman et al. 2002). The majority of myosin heavy chain mutations result in a protein with aberrant function, whereas cMyBP-C mutations predominantly result in a lack of viable protein. This necessitates different strategies to address these two types of mutations at the molecular level. Gene therapy has been employed in mouse models carrying mutations in both myosin and cMyBP-C and has shown some positive results (Cheng, Wan et al. 2013, Jiang, Wakimoto et al. 2013). These strategies focus on either expressing a gene that is not expressed in suitable quantity (i.e. to treat a haploinsufficiency-based disease) or to knock-down a mutant protein product that exerts a pathological effect (i.e. to treat a poison polypeptide). Therefore, it is imperative that the mechanisms by which these mutations exert a pathological phenotype are determined in order to guide any potential future therapies.
2.1.9 Heart Failure

Heart failure is a common end point for a number of cardiovascular diseases. Clinically, heart failure is defined by the inability of the heart to supply enough blood to meet the body’s demands (Go, Mozaffarian et al. 2013). HF is a life threatening condition and despite the alarming and acute sounding nature of the disease’s name, a patient can live with clinical heart failure for years with various reductions in quality of life. In fact, a patient with early heart failure may show few signs of any physical abnormalities when resting. However, a typical feature of HF is exercise intolerance, when an individual’s heart may be able to perform suitably under resting conditions, but upon increased cardiac demand the heart is unable to provide sufficient perfusion for proper oxygen delivery. HF patients typically suffer from fatigue due to this reason, and as the disease progresses, more routine tasks become impossible. During the progression of HF cardiac function will decline until even the most basic demands on the heart are unable to be met, leading to death (Go, Mozaffarian et al. 2013).

Heart failure defines a condition that can come about as the result of several pathologies. As discussed previously, familial hypertrophic cardiomyopathy can cause reduced cardiac function that can ultimately result in HF. This is also true of other pathologies that cause a reduction in cardiac function such as hypertensive hypertrophy, diabetic cardiomyopathy, and necrosis of myocardial tissue caused by ischemia-reperfusion injury or myocardial infarction. All of these diseases can cause decreases in cardiac
function until HF develops (Swynghedauw 1999).

Despite the high prevalence and poor prognosis of HF, effective treatments for this disease remain elusive. Research has focused on determining the molecular mechanisms which lead to the development of HF with the intention to mitigate or reverse the progression of the disease. Unfortunately, once HF has developed treating this disease and reversing the dysfunction has proven difficult. The optimal clinical approach to treating HF is to prevent its onset in the first place. This is obviously difficult to accomplish, and realistically approaching the management of HF in this way requires a firm understanding of the risk factors and etiology of the diseases leading to HF (Kannel and Belanger 1991).

One of the major treatments for end-stage HF is heart transplantation, which is not an ideal treatment strategy due to the paucity of viable donor hearts and issues with donor-recipient compatibility (Hunt, Baker et al. 2001). Recent advances made in the development of cheaper, more effective left-ventricular assist devices (LVADs) have proved effective in treating HF by reducing the work-load on the heart. Patients receiving LVADs have shown improved energy levels and quality of life (Slaughter, Rogers et al. 2009). Interestingly, a subset of patients who have received an LVAD and subsequently had the device removed have shown reversal of some of the adverse remodeling of the heart, with increased cardiac function and patient quality of life (Slaughter, Rogers et al. 2009). While this finding is fascinating, the efficacy of LVAD as actual treatment
for HF is unclear and the high cost of the devices make them an unfeasible treatment for the millions of patients suffering from HF.

The clinical treatment of HF has focused on managing symptoms and increasing the patient’s physical capacity (Hunt, Baker et al. 2001). Therapies such as using diuretics to reduce blood volume, or β-AR signaling blockade (β-Blockers) to help reduce blood pressure by reducing the contractility of the heart, both act to treat symptoms of HF, such as congestion. Although pharmacological treatments can improve cardiac function and symptoms in the short-term, they do not serve as treatments of the underlying pathology, and patients will still progress into worsened HF with time unless the underlying causes can be addressed (Hunt, Baker et al. 2001).

A treatment for HF that has shown promise in clinical trials is the use of gene therapy. Virally mediated gene therapy using recombinant SERCA protein in human myocardium has been effective for increasing cardiac performance by increasing the rate of Ca\(^{2+}\) reuptake into the SR. This also improves systolic function due to increased systolic SR Ca\(^{2+}\) release causing increased contractility (Miyamoto, del Monte et al. 2000, del Monte, Williams et al. 2001). While the long-term efficacy of this and similar gene therapy approaches have yet to be demonstrated, efficacious treatment for advanced HF may have tremendous potential as technical limitations such as maintaining gene expression and efficacious delivery routes are overcome. The potential for using these techniques for treating HCM makes identifying the molecular mechanisms that
lead to hypertrophy and HF critical, as this knowledge will be immediately applicable to in the development of future genetic therapy.

2.2 HCM: A Disease of the Cardiac Sarcomere

As sarcomere dysfunction and mutations in genes encoding sarcomere proteins have been shown to cause HCM, it is critical to understand the composition, function, and regulation of the cardiac sarcomere. The functional unit of the cardiomyocyte contractile machinery is the sarcomere. Single sarcomeres are arranged end-to-end to form long myofibrils that run the length of the cardiomyocyte. The concerted shortening of the sarcomeres causes contraction of the cell which in turn results in contraction of the heart. The single sarcomere is made up of numerous thin and thick filaments arranged parallel to each other that contain all the proteins responsible for contraction and relaxation of the sarcomere. Structures called Z-discs bookend the contractile machinery and demarcate a single sarcomere (Figure 5). From the Z-discs, thin filaments extend inwards towards the M-line, the midline of the sarcomere, while the myosin thick filaments originate at the M-line and extend outwards towards the Z-discs. This arrangement gives polarity to both filament types, and when actomyosin crossbridge cycling occurs, both Z-discs are brought closer together.
Depiction of parallel interdigitated thick and thin filaments located between Z-disks with mirrored orientation on either side of the M-line. Actomyosin overlap occurs in the A band, with cMyBP-C occupying axial stripes in a subset of the A band, termed the C-zone (Barefield and Sadayappan 2010).
2.2.1 The Z-Disks

The Z-disks of the sarcomere are structurally comprised of antiparallel dimers of α-actinin, although numerous other regulatory proteins also are located in this structure (Sjoblom, Salmazo et al. 2008). The α-actinin proteins in the Z-disks bind to the actin thin filaments, attaching them to the Z-disks where the actin filament is capped. The giant sarcomere protein titin is also connected to the Z-disks through α-actinin binding. The sarcomeres are anchored to the cell via the intermediate filament desmin, which is thought to bind to the Z-disks (Witt, Burkart et al. 2006). Desmin also links to ankyrin proteins in the sarcolemma and helps stabilize and organize the sarcomeres into well-ordered bundles (Tonino, Pappas et al. 2010). The Z-Disks also serve as localization points for a variety of signaling molecules. This includes anchoring of PKA and PKC, as well as the Ca^{2+} responsive phosphatase calcineurin (Pyle and Solaro 2004).

The Z-disk has also been implicated as a sensor for sarcomere strain and is thought to play a role in the development of hypertrophy. While several pro-hypertrophic signaling molecules, such as PKA, are localized at the Z-disk, the presence of calcineurin and its repressor protein four and a half LIM domains 2 (FHL2) in the Z-disk has been suggested as a critical component of hypertrophic signaling following stress (Nordhoff, Hillesheim et al. 2012). Indeed, when activated by Ca^{2+}/calmodulin, calcineurin dephosphorylates the pro-hypertrophic transcription factor NFAT, leading to its nuclear localization and activation of hypertrophic remodeling (Molkentin, Lu et al. 1998).
2.2.2 The Thick Filament

The sarcomeric thick filaments are primarily composed of the motor protein myosin heavy-chain, with the slow β isoform predominant in human hearts and the fast α isoform as the main isoform in mouse hearts. The myosin molecule has a long filamentous tail region (light meromyosin, or LMM) and forms a coiled coil dimer that incorporates to form the body of the thick filament. Groups of myosin heavy-chain dimers are bundled together to form the thick filament backbone, resulting in a filament that has a staggered helix of myosin heads every 14.3 nm, with a 43 nm helical turn distance (Craig, Lee et al. 2014).

Myosin also has a motor head domain that extends away from the LMM backbone of the thick filament and contains the ATPase activity-containing subunit responsible for force generation. The neck and head region of myosin also bind the accessory proteins myosin essential light chain (ELC) and myosin regulatory light chain (RLC), such that each functional myosin dimer consists of two myosin heavy chains (LMM and the head region), two ELCs and two RLCs (Poetter, Jiang et al. 1996).

The protein titin is attached to the myosin thick filament, which is anchored to the Z-disk protein, α-actinin, and to the actin thin filament at its N′-terminal region and runs to the M-line of the sarcomere (Pyle and Solaro 2004). Titin provides the major elastic component of the sarcomere and is one of the determinants of the level of resting tension in a cardiomyocyte (LeWinter and Granzier 2010). Titin’s C′-terminal region occupies the A band and is composed
of 11 non-elastic immunoglobulin and fibronectin like super repeats. The periodicity of these super repeats along the barrel of the thick filament has been suggested to help organize sarcomere proteins along the thick filament and act as a molecular ruler for sarcomere assembly (Labeit and Kolmerer 1995). In the cardiac sarcomere titin can be found in three splice variants. The N2B titin isoform contains normal elastic components and is the most widely expressed in adult sarcomeres. The larger N2BA titin has additional elastic elements and is considerably longer than N2B titin, resulting in lower passive tension in experimental models with this isoform predominantly expressed. The third splice variant category consists of several variants that are predominantly expressed in fetal development and do not normally occur in adult cardiomyocytes (Bang, Centner et al. 2001).

Another major thick-filament protein is cMyBP-C, which will be discussed in detail later. In brief, cMyBP-C interacts with the thick filament proteins LMM and titin via binding sites at its C'-terminus (Figure 6) (Okagaki, Weber et al. 1993). The exact organizational arrangement of cMyBP-C's binding to the thick filament is currently unclear. A body of evidence supports a strut model, where the C'-terminal residues of cMyBP-C run parallel along the thick filament, with their N'-terminal residues extending out into the interfilament space where they can interact with the myosin S2 region and actin. Other evidence suggests that the C'-terminal domains of cMyBP-C wrap around the thick filament like a collar, with three cMyBP-C molecules forming a ring around the thick filament and also
interacting with each other. This model also posits that the N’ regions of the protein extend into the interfilament space and interact with Myosin S2 and actin. However, direct evidence has demonstrated that cMyBP-C’s C’ residues are 10 nm closer to the M-line than its C0-C5 domain, which is consistent with three C’ residues running parallel down the thick filament and supports the strut model (Craig, Lee et al. 2014).

Alterations to the thick filament, often from a genetic basis, have been well established to cause cardiomyopathy. Specific mutation effects on sarcomere function will be discussed in detail later; however, it is important to note that alterations in thick filament assembly, regulation by phosphorylation, and myosin motor activity can cause deficits in relaxation and force development. An example of this dysfunction has been observed in a well-studied MHC mutation (R403W) that exhibits an enhanced ATPase activity without a concomitant increase in force, meaning that this mutation causes the myosin motor to work less efficiently (Keller, Coirault et al. 2004).
Myosin/titin thick filaments run parallel to decorated actin thin filaments, with the myosin head extending into the interfilament space. Cardiac MyBP-C interacts with the LMM domain of myosin and Titin via its C'-terminal domains and interacts with the myosin head and actin via its N'-terminal domains. Phosphorylation of identified sites on cMyBP-C have been proposed to modulate its interaction with its N'-terminal binding partners by altering the affinity of the N'-terminal region for myosin S2 and Actin, regulating crossbridge cycling (Barefield and Sadayappan 2010).

Figure 6. Molecular Schematic of a Single Crossbridge.
2.2.3 The Thin Filament

The thin filaments of the sarcomere are principally comprised of long helical actin chains. Actin is a globular monomeric protein that can polymerize into filaments of various lengths. These filaments form a coiled-coil with two actin filaments coiled around each other, with seven actin monomers per single actin filament per half-turn. In the sarcomere these filaments are anchored at the M-line via the structural protein α-actinin (Sjoblom, Salmazo et al. 2008).

Sarcomeric actin filaments are decorated by several other proteins that regulate sarcomere function. The regulatory protein α-Tropomyosin lies in the cleft between actin monomers and runs along the thin filament in this groove. α-tropomyosin also exists as a coiled-coil dimer and one dimer runs along the actin filament for half a helical turn. Chains of α-tropomyosin are formed along the thin filament by head-to-tail binding to other α-tropomyosin dimers (Mun, Previs et al. 2014).

Along the thin filament the heterotrimeric troponin protein complex binds to α-tropomyosin and actin. The troponin complex is composed of three troponin proteins, all with an individual function. Troponin T (TnT) binds to α-tropomyosin and actin, and anchors the troponin complex to the thin filament. Troponin I (TnI) has an inhibitory function on thin filament activation, which will be discussed later. Troponin C (TnC) contains the Ca\textsuperscript{2+} binding domain of the troponin complex and is responsible for initiating molecular changes that occur following Ca\textsuperscript{2+} binding (Parmacek and Solaro 2004).
2.2.4 Regulation of Sarcomere Function

The function of the sarcomere can be dynamically regulated by a number of signaling pathways. The β-adrenergic pathway begins at the plasma membrane with the binding of norepinephrine (or a β-AR agonists) to the β-AR G-Protein coupled receptor, causing the activation of adenylyl cyclase and the production of cyclic AMP (cAMP), which activates protein kinase A (PKA) (Dorn and Force 2005). PKA is able to phosphorylate several sarcomeric proteins with the concerted effect of increasing inotropy and lusitropy. PKA targets cTnI by phosphorylating serine 22/23 in humans (23/24 in mouse), which alleviates the inhibitory effects of cTnI, resulting in a decrease in Ca$^{2+}$ sensitivity of the myofilaments. This allows faster displacement of Ca$^{2+}$ during diastole, leading to faster relaxation (Frazier, Ramirez-Correa et al. 2011). Phosphorylation of serine 273, 282, and 302 (mouse) of cMyBP-C regulates crossbridge cycling rates, allowing for faster contraction and relaxation kinetics and increased force generation (Tong, Stelzer et al. 2008). Titin phosphorylation by PKA is associated with reduced stiffness of the myofilaments, facilitating faster relaxation during diastole (Kotter, Gout et al. 2013).

Alterations in signaling occurring through increased mechanical stress or signaling via various pathways (e.g. angiotensin, norepinephrine) can cause a rise in cardiomyocyte Ca$^{2+}$ and have been associated with both physiological and pathological hypertrophic remodeling (Dorn and Force 2005). This increase in Ca$^{2+}$ triggers the calcineurin pathway (as mentioned previously) and can activate
protein kinase C (PKC) through Ca\(^{2+}\) binding or via diacylglycerol from phospholipase-C activity (Nishizuka 1986). In the myofilaments, it has been observed that PKC-mediated phosphorylation of cTnT and cTnl reduces contractility, and treatment of skinned cardiomyocytes with PKC results in decreased force generation and decreased Ca\(^{2+}\) sensitivity of force development (Belin, Sumandea et al. 2007).

Another kinase that has been established to play a role in myofilament signaling and contractility is CaMKII. The isoform predominantly expressed in the heart is calcium-calmodulin kinase II delta (CaMKII\(\delta\)), with a nuclear-localized variant responsible for hypertrophic gene expression and a cytosol-localized variant that is responsible for phosphorylation of the myofilament proteins (Zhang, Maier et al. 2003). CaMKII has been suggested to act in response to increased Ca\(^{2+}\) concentrations by activating a number of transcription factors, including myocyte enhancer factor 2 (MEF2), that are pro-hypertrophic (Passier, Zeng et al. 2000). CaMKII has been reported to target SERCA/phospholamban, L-type Ca\(^{2+}\) channels and RyR, with a net effect of causing increased SR Ca\(^{2+}\) leak, although the effect of these regulatory modifications remain controversial (Zhang, Maier et al. 2003). Interestingly, CaMKII has been shown to phosphorylate cMyBP-C \textit{in vitro}, with decreased CaMKII phosphorylation associated with decreased contractility (Tong, Gaffin et al. 2004).
2.2.5 Sarcomere Protein Mutations

Hundreds of mutations in sarcomeric proteins have been linked to the development of HCM and DCM (Table 1). Even with this number of mutations described, roughly a third of cardiomyopathies remain idiopathic, with no currently described mutations. Of the mutations that result in cardiomyopathy, mutations in myosin (MYH7) and cMyBP-C (MYBPC3) each comprise around 30-40%. The other described mutations mostly occur in other myofilament proteins. It has been suggested that Titin, due to its enormous size may be a significant contributor to HCM and DCM mutations, although mutations in this gene have not been studied until recently (Golbus, Puckelwartz et al. 2012, Herman, Lam et al. 2012). The exact mechanisms by which these mutations exert a pathological effect are still under investigation and vary depending on the specific mutation. However, while most of these effects are currently poorly understood, these mutations can be grouped into a few general categories.
Table 1. Currently Identified HCM and DCM Associated Sarcomere Gene Mutations

This table reports the number of identified mutations associated with HCM and DCM found in genes encoding proteins of the cardiac sarcomere. Updated from (Barefield and Sadayappan 2010) with (Harris, Bartley et al. 2002, Herman, Lam et al. 2012).

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene</th>
<th>HCM</th>
<th>DCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYH7</td>
<td>β-cardiac Myosin Heavy Chain</td>
<td>194</td>
<td>13</td>
</tr>
<tr>
<td>MYBPC3</td>
<td>Cardiac Myosin Binding Protein-C</td>
<td>197</td>
<td>2</td>
</tr>
<tr>
<td>TNN</td>
<td>Titin</td>
<td>3</td>
<td>72</td>
</tr>
<tr>
<td>TNNT2</td>
<td>Cardiac Troponin T</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td>TNNI3</td>
<td>Cardiac Troponin I</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>TPM1</td>
<td>α-Tropomyosin</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>ACTC</td>
<td>Cardiac α-Actin</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>MYL2</td>
<td>Cardiac Regulatory Myosin Light Chain</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>MYL3</td>
<td>Cardiac Essential Myosin Light Chain</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total Mutations</strong></td>
<td></td>
<td><strong>485</strong></td>
<td><strong>98</strong></td>
</tr>
</tbody>
</table>
2.2.6 The Poison Polypeptide Mechanism

Mutations in the gene for myosin heavy chain (MHC, predominantly encoded by the MYH7 gene in human adults) have been identified to be missense mutations that result in the substitution of one amino acid for another. Due to the requirement of functional myosin for the development of a viable organism, it is understandable why drastic mutations, such as mutations causing exon skipping or frame shifts, would be lethal in the myosin gene. As such, the identified MHC mutations are typically amino acid substitutions that cause a mechanistic impairment in myosin function that does not prohibit myosin from functioning in sarcomere assembly. The point mutations studied in MHC have shown that slight alterations in myosin’s ability to interact with regulatory proteins or in functionally critical regions can cause a decline in function, development of cardiomyopathy and progression to HF. Indeed, mutations in MYH7 are often associated with a more severe phenotype and poorer outcomes for the patients than are mutations in other sarcomeric proteins (Bloemink, Deacon et al. 2014).

In these mutations a protein is expressed that exerts a dominant negative effect that acts as a poison polypeptide. As this disease mechanism is based on the expression of a mutant protein, treating this pathology may be possible by knocking down the mutant allele specifically. Some interesting progress has been made on this front recently by the Seidman lab, where allele-specific siRNA was employed in a heterozygous mouse model of the well-studied myosin heavy chain R403Q mutation (studied in the mouse MYH6 gene instead of the human
MYH7 gene) via adeno-associated virus delivery to good effect (Jiang, Wakimoto et al. 2013). However, this approach has applicability only in mutations with an expressed protein product, and is difficult in that specific targeting of a mutant allele is challenging, but does show promise for treating cardiomyopathy as well as other diseases caused by missense mutations.

2.2.7 The Haploinsufficiency Mechanism

In contrast, mutations in the gene MYBPC3 have been suggested to cause cardiomyopathy through a different mechanism. Of the currently identified cardiomyopathy-associated MYBPC3 mutations, approximately 70% are expected to code for a protein that is missing its C’ region (Barefield and Sadayappan 2010). This occurs in most cases due to mutations that cause exon skipping and frame shifts, with the inclusion of novel stop codons. As the C’-terminal region of cMyBP-C is required for incorporation into the sarcomere, these mutant proteins are not typically detected in tissue from mutant carriers. As the pathological mechanism in this case may involve insufficient expression of cMyBP-C, gene therapy approaches have been tried to correct this deficit. Recently, work from the Stelzer lab described a promising experiment using adenoviral expression of MYBPC3 in a heterozygous mouse with decreased cMyBP-C content, resulting in some functional improvements (Cheng, Wan et al. 2013).
2.2.8 Other Pathological Mechanisms in Sarcomere HCM Mutations

Studies aiming to understand the mechanism by which mutations in other sarcomeric proteins exert a pathological effect are also ongoing. These mutations depend on the specific protein and the type of mutation. For example, a group of mutations in troponin T that cause HCM and cardiac sudden death have been established to act via calcium sensitization of the myofilament, causing disruption of normal calcium cycling and increasing the propensity for arrhythmias (Knollmann, Kirchhof et al. 2003, Schober, Huke et al. 2012).

Due to its enormous size, mutations in the gene encoding the sarcomeric protein titin (TTN) have been understudied. Recent analysis has revealed that mutations in TNN are found in 25% of cases of idiopathic familial DCM. These findings provide an onus to examine the possibility of TNN mutations in HCM, as nearly a quarter of familial HCM cases remain idiopathic with no causative mutations identified, although currently titin mutations seem to only result in DCM phenotypes (Herman, Lam et al. 2012).

2.2.9 Discovery of New HCM Linked Mutations

Following the discovery that familial HCM was often caused by mutations in certain sarcomere proteins, screening for known and novel mutations in these genes became routine. Initially, screening panels were limited to known HCM associated genes, such as MYH7 and MYBPC3, with the addition of some novel genes. Eventually screening for these mutations began to use arrays, which
investigated 50 or so known and novel genes (Puckelwartz and McNally 2014).

An understandable consequence to screening in this manner is that novel HCM associated genes are unlikely to be sampled by such a limited panel. Also, known HCM genes that have been screened routinely for over a decade will have numerous new variants identified. A complication of this approach is that discovery of a potentially pathogenic single-nucleotide polymorphism (SNP) in a known HCM related gene may not be the responsible, or the only responsible mutation in the development of HCM. However, once a SNP in a known cardiomyopathy-associated gene is discovered, further screening has not historically been performed (Christodoulou, Wakimoto et al. 2014, Puckelwartz and McNally 2014).

With the development of next-generation sequencing technology, the sequencing of whole genomes, exomes, and transcriptomes has become considerably more feasible in clinical practice and basic science laboratories. Screening patients with family histories of HCM, using whole-exome or whole-genome techniques, allows identification of potentially pathogenic mutations in known HCM-associated genes, as well as mutations in novel genes (Puckelwartz and McNally 2014, Golbus, Puckelwartz et al. 2012). In addition, recent use of these techniques has allowed identification of novel modifiers of cardiomyopathies (Christodoulou, Wakimoto et al. 2014). In this study, the use of whole-transcriptome RNA-sequencing methods were employed to assess novel changes in gene expression in heterozygous MYBPC3 mutant-carrying mice
(Christodoulou, Gorham et al. 2011). The power of this technique is its ability to assess changes in the transcriptome without needing to identify a specific candidate \textit{a priori}.

\subsection*{2.3 Cardiac Myosin Binding Protein-C}

Cardiac MyBP-C is a 140 kDa sarcomeric thick-filament protein that binds to myosin. This protein was originally described by Offer et al, 1973 when it was discovered as a major contaminant in myosin extracts. It is found exclusively in the vertebrate heart and is encoded by the gene \textit{MYBPC3}. It is localized in the inner two-thirds of the A band, and gives rise to the name for its zone of localization: the C-zone (Yamamoto and Moos 1983, Fougerousse, Delezoide et al. 1998, Niimura, Bachinski et al. 1998). Antibody staining shows that cMyBP-C is restricted to the outer 7 to 9 axial bands, depending on the tissue type, with 9 bands found in the heart, spaced approximately 43 nm apart in each C-zone \textbf{(Figure 5)} (Luther, Bennett et al. 2008).
There are eight immunoglobulin-type (Ig) and three fibronectin-type (Fn3) domains numbered C0 to C10. The cardiac isoform of MyBP-C has a specific domain (C0) at the N-terminus and a specific sequence insertion in C5. The Pro-Ala-rich domain, M-domain, and interacting sites with actin, myosin and titin are shown. The M-domain contains 3 sites that can be phosphorylated by various kinases including PKA, PKD, RSK, CAMKII, and PKC. These amino acid sequences are conserved between mice and humans. The red arrow shows the major proteolytic cleavage site of the calpain protease. The truncation mutant used in this proposal contains an insertion of novel residues in domains C9 and C10, which disallow strong interaction with myosin and titin, preventing proper incorporation into the sarcomere (Barefield and Sadayappan 2010).
2.3.1 Molecular Structure and Localization of cMyBP-C

Cardiac MyBP-C consists of 11 domain modules, labeled C0 to C10 from the N to the C-terminus, (Figure 7) and is composed of repeating immunoglobulin and fibronectin type-3 domains. Cardiac MyBP-C interacts with the S2 fragment of myosin via its M-domain in a phosphorylation dependent manner; such that when cMyBP-C is dephosphorylated it binds tightly to the myosin S2 region, whereas after phosphorylation the M-domain does not interact with myosin S2 and instead has been shown to be able to interact with actin (Gruen and Gautel 1999, Sadayappan, Osinska et al. 2006, Shaffer, Kensler et al. 2009). Cardiac MyBP-C is strongly anchored to LMM via its C10 domain, (Gilbert, Cohen et al. 1999) and titin via the C8-C10 domains (Freiburg and Gautel 1996). In addition, a potential actin-binding sequence exists in the Pro-Ala-rich linker sequence located between the C0 and C1 domains, (Shaffer, Kensler et al. 2009) and there is evidence for in vitro interaction between cMyBP-C and actin via the C0 domain, (Kulikovskaya, McClellan et al. 2003) C1 and M-domain, (Shaffer, Kensler et al. 2009) and C5 domain (Rybakova, Greaser et al. 2011). It has also been shown that cMyBP-C can interact and alter the action of α-Tropomyosin (Mun, Previs et al. 2014).

Cardiac MyBP-C modulates myosin assembly (Offer, Moos et al. 1973) and stabilizes the thick filaments, (Moos, Mason et al. 1978, Squire, Luther et al. 2003) which is important for the precise arrangement of actin-myosin filaments in the sarcomere. These studies indicate that the N’-terminal region of cMyBP-C is
critical for sarcomere stability. It has been shown that the N'-regions are severely degraded during myocardial injury, which will be further addressed later (Decker, Decker et al. 2005, Sadayappan, Osinska et al. 2006).

2.3.2 Phosphorylation Mediated Regulation

A feature of the cardiac specific isoform of MyBP-C is its multiple phosphorylation sites (Yuan, Guo et al. 2006). Cardiac MyBP-C is extensively phosphorylated under basal conditions in mice and humans; however, the level of cMyBP-C phosphorylation is altered in mouse models during development of HF, I-R injury, pathologic hypertrophy, (Sadayappan, Gulick et al. 2005) myocardial stunning (Squire, Luther et al. 2003, Yuan, Guo et al. 2006) and in human patients with atrial fibrillation (El-Armouche, Boknik et al. 2006). Three of the serine residues in cMyBP-C (mouse Ser-273, Ser-282 and Ser-302) are differentially phosphorylated by the enzymes PKA, PKC, protein kinase D (PKD), ribosome s6 kinase (RSK) and CaMKII (Fig. 7) (Gautel, Zuffardi et al. 1995, Mohamed, Dignam et al. 1998, Bardswell, Cuello et al. 2010, Cuello, Bardswell et al. 2011). Recent evidence also established that glycogen synthase kinase 3 beta (GSK3β) phosphorylates cMyBP-C in the Pro-Ala-rich linker region on Ser-133 (Ser-128 in mice) (Kuster, Sequeira et al. 2013). During the development of HF, total phosphorylation of cMyBP-C is decreased, with a major loss of tri-phosphorylated protein (Decker, Decker et al. 2005, Sadayappan, Gulick et al. 2005). Reduced phosphorylation is accompanied by contractile dysfunction,
reduced force generation, and increased cleavage of cMyBP-C (Decker, Decker et al. 2005, Sadayappan, Osinska et al. 2006).

Previous studies have shown that cMyBP-C phosphorylation by PKA regulates myocardial function (Sadayappan, Gulick et al. 2005, Sadayappan, Osinska et al. 2006, Nagayama, Takimoto et al. 2007, Sadayappan and Robbins 2008, Sadayappan, Gulick et al. 2009) and confers resistance to proteolysis and protection against I-R injury (Sanada, Asanuma et al. 2004, Sadayappan, Osinska et al. 2006, Sadayappan, Gulick et al. 2009). It has also been shown that degradation of cMyBP-C correlates well with contractile dysfunction (Decker, Decker et al. 2005, Sadayappan, Osinska et al. 2006). Transgenic expression of phospho-ablated (Ser substitution to Ala mutations) cMyBP-C<sup>AlIP-</sup> causes functional deficits at the whole-heart level and leads to impaired hemodynamics (Sadayappan, Gulick et al. 2005). Conversely, the phospho-mimetic (Ser substitution to Asp) cMyBP-C<sup>AlIP+</sup> replacement acts as effectively as wild-type transgenic cMyBP-C in rescuing the <i>MYBPC3<sup>(t/t)</sup></i> phenotype (Sadayappan, Osinska et al. 2006), showing the necessity of phosphorylation for normal cardiac function.

While evidence for alterations in cMyBP-C phosphorylation as a modifier of disease has been growing for the last decade, new insights into how cMyBP-C phosphorylation alters sarcomere function have added to the mechanistic understanding of sarcomere regulation. Phosphorylation of cMyBP-C has been proposed to alter the structure of the M-domain. These findings fit nicely with
observations regarding the hierarchal phosphorylation relationship of the major M-domain serine residues. Phosphorylation has been proposed to cause rearrangement of the structure of the M-domain may expose additional serine residues for phosphorylation by kinases, establishing a hierarchy of phosphorylation (Previs, Beck Previs et al. 2012, Weith, Sadayappan et al. 2012, Bezold, Shaffer et al. 2013, Karsai, Kellermayer et al. 2013).

Phosphorylation of cMyBP-C has been shown to directly alter the actomyosin crossbridge cycling rate. In a series of experiments measuring the speed of actin filament propagation along a myosin-coated surface, regions with dephosphorylated cMyBP-C were shown to slow actin sliding velocity significantly compared to areas with no cMyBP-C. However, additional phosphorylation of the major serine sites in the M-domain showed step-wise increases in actin sliding velocity, such that when cMyBP-C was maximally phosphorylated actin sliding velocity was equal to its velocity in the absence of cMyBP-C. These data illustrate the ability of cMyBP-C to regulate sarcomere shortening velocities based on the level of phosphorylation. Due to the complexity of kinase signaling that regulates the M-domain of cMyBP-C, these interactions are still being explored (Previs, Beck Previs et al. 2012, Weith, Sadayappan et al. 2012).

The ability of cMyBP-C to regulate cardiac function relies on several factors that are altered in hypertrophy, such as phosphorylation. In addition to these modifications, sarcomere occupancy of cMyBP-C has been shown to alter
contractile kinetics, with regions lacking cMyBP-C regulating actin sliding differently than regions with cMyBP-C (Weith, Sadayappan et al. 2012, Witayavanitkul, Ait Mou et al. 2014). Therefore, any reduction in cMyBP-C level or functional capacity can cause disruptions in sarcomere function. Due to the importance of cMyBP-C content and modifications on sarcomere function, in this study levels of cMyBP-C were carefully assessed as well as alterations in phosphorylation levels.

In addition to regulating acto-myosin interactions by directly interacting with those proteins, cMyBP-C has also been reported to interact with α-tropomyosin. This interaction was first described in skeletal muscle (Yamamoto 1986) by demonstrating that cMyBP-C could bind to actin/tropomyosin thin filaments. Further evidence suggests that cMyBP-C can alter α-Tropomyosin function and activate the thin filament. Structural studies of cMyBP-C have suggested that the C0-C1 domains can interact with the low-Ca$^{2+}$ α-Tropomyosin site on actin, displacing α-tropomyosin from this blocked position and activating the thin filament independent of cytosolic Ca$^{2+}$ levels by exposing myosin binding sites on actin (Orlova, Galkin et al. 2011, Mun, Previs et al. 2014).

Proteolytic degradation of cMyBP-C, as seen during I-R injury, causes cleavage of the full-length 140-kDa cMyBP-C and the production of a 40-kDa N'-fragment (Sadayappan, Osinska et al. 2006, Sadayappan, Gulick et al. 2009). Calpain is a Ca$^{2+}$-activated protease that regulates myofilament proteins (Gao, Atar et al. 1997, Galvez, Diwan et al. 2007) and plays a role in myofibril
degradation and is activated during I-R injury and myocardial stunning. Developing data from the Sadayappan lab show that cMyBP-C is a substrate for μ-calpain \textit{in vitro}. The removal of the N'-terminal residues by a Ca\textsuperscript{2+} activated protease raises many interesting questions, especially when taken with the evidence that the major regulator of acto-myosin interaction is found within the M-domain, which is also the location of the cleavage site. It may be that cleavage of cMyBP-C may provide a short-term advantage in terms of increased contractility, although the long-term effects of this modification may be deleterious. Also, cleavage of the N'-terminal region of cMyBP-C may serve as a biomarker for ischemic injury, as this fragment has been shown to be released into the circulation (Kuster, Cardenas-Ospina et al. 2014).

In addition to regulatory modifications and proteolysis, recent studies have begun to implicate oxidative modifications, such as glutathionylation, as playing a role in pathological regulation of sarcomeric proteins. In a hypertensive mouse model hypertrophy and sarcomeric dysfunction were shown to be modified by cMyBP-C glutathionylation, and these deficits can be attenuated by restoring the redox state of the cardiomyocyte, and reversing glutathionylation of cMyBP-C (Jeong, Monasky et al. 2013, Patel, Wilder et al. 2013). Further studying the detrimental effect of oxidative modifications on sarcomeric proteins has the potential to provide novel targets to regulate myocardial function following oxidative stress.

The necessity of cMyBP-C for cardiac development and function was
reported by the Seidman lab in 1999, (McConnell, Jones et al. 1999) who also showed that heterozygous \( \text{MYBPC3}^{(+/-)} \) mice were phenotypically similar to WT under basal conditions. Furthermore, the truncation mutation in \( \text{MYBPC3}^{(t/t)} \) is a good model to study typical \( \text{MYBPC3} \) familial hypertrophic cardiomyopathy mutations, as the majority of \( \text{MYBPC3} \) mutations reported are expected to produce a C'-terminal truncated protein product which are rarely detected in patient tissue (Dhandapany, Sadayappan et al. 2009, van Dijk, Dooijes et al. 2009). In these \( \text{MYBPC3}^{(t/t)} \) mice, there is no detectable cMyBP-C and hypertrophy develops, similar to other knockout mice (Harris, Bartley et al. 2002). This lack of protein incorporation in the sarcomere contributes to a phenotype of hypertrophy, dilation, and increased fibrosis and necrosis in \( \text{MYBPC3}^{(t/t)} \) mouse hearts. These data underscore the importance of cMyBP-C for normal cardiac function, and demonstrate that under basal conditions the heterozygote is phenotypically normal, but they do not address whether the heterozygote exhibits any deficits following stress.

### 2.3.3 Experimental Investigation of \( \text{MYBPC3} \) Mutations in HCM

Diagnostic criteria state that HCM is a disease that causes hypertrophy in the absence of an external stimulus (Go, Mozaffarian et al. 2013). This criterion requires that other causes of hypertrophy must be ruled out, as the genetic basis of familial HCM had not been elucidated until the mid-1990’s. In 1995 the Seidman lab and the Schwartz lab both published in Nature Genetics the first
evidence of mutant MYBPC3 genes involved in HCM, and that the pathological mechanism may be improper splicing, leading to a truncated protein (Bonne, Carrier et al. 1995, Watkins, Conner et al. 1995). The role of MYBPC3 in HCM is now well established, with a common trend among identified mutations involving improper splicing, exon skipping, frame shifts, and the introduction of a premature stop codon. Of the over 200 identified mutations in MYBPC3 associated with inherited HCM, 70% are suggested to result in one of these truncated proteins.

The pace of MYBPC3 mutation identification picked up speed tremendously in the first decade of the 21st century. As mutations were discovered, the extent to which they were carried in various populations became apparent. Many of the populations carrying familial HCM mutations result from specific founder mutations, resulting in widespread distribution of particular mutations. Notable among these are Dutch (Michels, Soliman et al. 2009) and Italian (van Dijk, Dooijes et al. 2009) founder mutations, which are highly abundant in their respective countries, and are also prevalent in the United States due to the country’s diverse ethnic background.

Perhaps the most prevalent mutation in MYBPC3 was identified in 2003 (Waldmuller, Sakthivel et al. 2003, Dhandapani, Sadayappan et al. 2009). This mutation is a 25 base pair deletion that results in skipping of exon 33, a frame shift, and a novel stop codon in the 3’ untranslated region that ultimately results in a predicted protein product missing several C’ residues. Functionally, the result
of this mutation is likely similar to many other MYBPC3 mutations that cause truncations or incorporation of novel C’ residues in the myosin binding domains. These mutations are thought to remove key myosin binding residues on the C’ of the protein, disallowing proper incorporation in the sarcomere. Although the pathogenic mechanism of this mutation is still under investigation the impact of this mutation is severe as it is thought to be carried by 60 million people of South Asian descent (Kuster, Sadayappan 2014).

Homozygous carriers of most HCM-causing MYBPC3 mutations develop severe hypertrophy at a young age and have a poor prognosis (Morita, Rehm et al. 2008). However, heterozygous carriers typically start showing a mild phenotype in early adulthood, and it is common for many carriers to not show any symptoms until middle age, with some never demonstrating pathology. Because of the late onset of these symptoms, it is difficult for carriers to know of their condition until after their reproductive years, making the transmission of these mutations common. In families with a history of HCM or sudden cardiac death, it has become clinically important to screen for known or novel mutations in order for carriers to make more informed life decisions regarding their own health and reproductive options (Dhandapany, Sadayappan et al. 2009).

Since the identification of MYBPC3 as a cardiomyopathy-related gene, increasing amounts of work have been done to identify the mechanisms by which mutations exert pathology. This effort has used tissue collected from human carriers, pedigree analysis of affected families, and mouse models carrying
common human mutations. The result of nearly 20 years of work has established that about 70% of MYBPC3 mutations are C'-truncations and are never detected by Western blot in myocardial tissue samples from HCM patients (Harris, Lyons et al. 2011). These MYBPC3 mutations are predicted to produce C'-truncated protein products of cMyBP-C that are unable to incorporate into the sarcomere due to the loss of key titin and myosin-binding residues (Harris, Lyons et al. 2011).

As a result, homozygous C'-truncations often result in total lack of cMyBP-C, effectively acting as a null allele, not producing any cMyBP-C. Conversely, some strains of asymptomatic heterozygous MYBPC3(+/t) mice show normal cMyBP-C stoichiometry, (McConnell, Jones et al. 1999, McConnell, Fatkin et al. 2001) suggesting that haploinsufficiency is singularly responsible for development of the HCM phenotype under normal conditions. However, it is unclear if asymptomatic human heterozygous mutation carriers have normal levels of cMyBP-C, and whether reduced cMyBP-C levels observed in symptomatic carrier myocardium is causative for the transition to a hypertrophic phenotype, or is a result. Therefore, it is critical to determine the effect of cardiovascular stress as a contributing factor in a haploinsufficiency mechanism for the development of hypertrophy.
2.3.4 Mouse models of MYBPC3 mutations

In the initial effort to determine the role of cMyBP-C in cardiac function, numerous cMyBP-C mutation carrying mouse models have been developed. Most of these models were originally designed to study the absence of cMyBP-C in the homozygote; however, the heterozygous genotype is also quite interesting as it mirrors the clinical situation often found in the human population. In the Moss lab in 2002, a MYBPC3 knockout mouse model was generated that is referred to in the literature as the “null” mouse (Harris, Bartley et al. 2002). This model was designed with exons 3-10 removed, resulting in the absence of cMyBP-C expression in the homozygous mouse. In initial reports, the heterozygous genotype of this model does not exhibit any reported deficits, with normal levels of cMyBP-C, despite reduced mRNA levels. However, more recent evaluation of this heterozygous model does show a 32% decrease in cMyBP-C levels, and mild functional impairments consistent with the early onset of HCM (Desjardins, Chen et al. 2012).

Two MYBPC3 mutant mouse models were developed by Lucie Carrier, one of which is a knockout mouse lacking exon 1 and 2 that results in a null allele (Carrier, Knoll et al. 2004). This heterozygous mouse model does show a reduction in cMyBP-C levels (25% of WT), with a reduction in MYBPC3 mRNA (from ~80% to ~50% of WT levels from 6 to 11 months of age) and develops symptoms of HCM at 10 to 11 months of age. The other model is a knock-in mouse carrying a common human mutation that results in several mRNA splice
products. This results in the expression of one potential poison polypeptide and an overall 50% reduction in \textit{MYBPC3} mRNA which translates into a 20% reduction of cMyBP-C in the heterozygote. Interestingly, this model does not appear to develop HCM; however, these mice do show increased Ca\textsuperscript{2+} sensitivity at the skinned fiber level and alterations in Ca\textsuperscript{2+} handling were observed in intact cardiomyocytes (Vignier, Schlossarek et al. 2009).

A consistent theme found in all these models is that homozygous mutant carriers show a severe hypertrophic or dilated phenotype, whereas the heterozygous models are more variable in their pathology, showing asymptomatic or mild phenotypes with the development of age-dependent HCM. These observations parallel those made in human carriers of HCM causing \textit{MYBPC3} mutations. The study of these various heterozygous mouse models underscores the subtle mechanistic differences between specific \textit{MYBPC3} mutations, illustrating the complexity of the heterozygous condition in mouse models and human patients.

The Seidman lab generated one of the first cMyBP-C null mouse models that demonstrated the importance of cMyBP-C for integrity of sarcomeric structure and its involvement in normal contractility of the heart (McConnell, Jones et al. 1999, Palmer, Georgakopoulous et al. 2004, Palmer, McConnell et al. 2004, Palmer, Noguchi et al. 2004). These mice model a human mutation reported by the Seidman lab (Watkins, Conner et al. 1995) carrying a G to C mutation at nucleotide 3367, causing an altered 5’ splice site and skipping of
exon 30, resulting in a frame shift and the inclusion of novel predicted C’-terminal amino acids. This cMyBP-C truncation mouse demonstrated that cMyBP-C is not essential for development, although its absence causes hypertrophic cardiomyopathy. This homozygous truncation (t/t) MYBPC3\(^{t/t}\) mouse model was extensively characterized to demonstrate that lack of cMyBP-C leads to development of ventricular dysfunction, ventricular arrhythmia and severe dilated cardiomyopathy (McConnell, Jones et al. 1999, McConnell, Fatkin et al. 2001, Palmer, Georgakopoulous et al. 2004, Palmer, McConnell et al. 2004, Palmer, Noguchi et al. 2004). These studies showed that heterozygous MYBPC3\(^{+/t}\) and homozygous MYBPC3\(^{t/t}\) mice are viable, but homozygous MYBPC3\(^{t/t}\) mice display progressive HF following hypertrophy and dilation (McConnell, Jones et al. 1999, McConnell, Fatkin et al. 2001). Using MYBPC3\(^{+/t}\) mice is intended to model a situation where one MYBPC3 allele codes for a mutant cMyBP-C peptide that cannot properly incorporate into the sarcomere, therefore recapitulating the conditions expected in human heterozygous carriers of similar mutations.

The initial reports on the MYBPC3\(^{+/t}\) genotype showed normal ECG intervals and sinus node, atrial, and ventricular conduction and refractory time, and showed mild HCM only after 125 weeks of age (McConnell, Fatkin et al. 2001). The suitability for using this mouse to model human disease is supported by the observation that human heterozygote carriers of MYBPC3 mutations also show an age-dependent phenotype. In one large study, young human
heterozygote carriers of a specific MYBPC3 mutation are mostly asymptomatic or mildly symptomatic, whereas ~90% of the heterozygote carriers over 50 years of age were mildly symptomatic (Dhandapany, Sadayappan et al. 2009).

Using this heterozygous model for the experiments detailed in this dissertation, it is possible to determine whether decreased MYBPC3 expression causes any detrimental effects under normal conditions, and whether hypertrophic stress results in either a reduction of cMyBP-C or worsened phenotype. These questions are clinically relevant due to the number of people carrying these mutations and the relatively small amount known about the mechanism by which these mutations exert a pathological effect.
CHAPTER THREE

AIMS AND HYPOTHESIS

Based on the observation that human heterozygous carriers of MYBPC3 mutations have variable penetrance and are often asymptomatic until middle age, I investigated whether haploinsufficiency of MYBPC3 predisposed carriers to the development of HCM and HF. To accomplish this I used a mouse model developed by McConnell et al (1999) with one functional allele of MYBPC3 and one allele containing a truncating mutation that results in a null allele. I hypothesize that mice with heterozygous MYBPC3 truncation mutations (MYBPC3^+/t) have subtle functional deficits and these mice will respond worse to cardiac stress than WT controls. Therefore, the overall objectives of this proposal were to 1) determine if the MYBPC3^+/t mice, initially reported as asymptomatic, show subtle functional deficits that may presage the development of hypertrophy; 2) demonstrate that insufficient expression of cMyBP-C contributes to the development of HCM in MYBPC3^+/t mice following stress.

Specific Aim 1. To assess molecular and morphological differences between WT and heterozygous mice under basal conditions. Previous studies established that MYBPC3^+/t mice express normal levels of cMyBP-C and exhibit a benign phenotype until extreme old age. Also, it has been shown that
human heterozygous carriers of similar mutations develop cardiomyopathies in adulthood, with delayed onset compared to homozygous carriers. Therefore, I hypothesized that the heterozygous genotype exhibits subtle functional impairments that facilitate the development of HCM. To test this hypothesis, levels of cMyBP-C transcript and protein were assessed and alterations in protein localization and phosphorylation status were evaluated. Force-pCa measurements of skinned cardiomyocytes and echocardiography analysis evaluated whether the heterozygous mice show any deficiencies under normal conditions.

**Specific Aim 2. To determine if the MYBPC3 heterozygous mice are more susceptible to the development of HCM following cardiovascular stress.**

Previous studies using this mouse strain have shown normal levels of cMyBP-C under baseline conditions, whereas studies using HCM symptomatic MYBPC3 mouse models and in human HCM patients have shown a reduction in cMyBP-C levels. I hypothesized that an increased demand for protein synthesis results in reduced cMyBP-C levels following cardiac insult, and this reduction in cMyBP-C exacerbates the onset of HCM. To test this hypothesis, transverse aortic constriction was used to induce hypertrophy. Echocardiography and skinned cardiomyocyte force measurements were used to assess function. Protein and mRNA levels were measured to evaluate haploinsufficiency, and RNA-Seq was performed to assess alterations in signaling pathways.
CHAPTER FOUR

CONTRACTILE DYSFUNCTION IN HETEROZYGOUS MYBPC3 MICE

4.1 Abstract

The etiology of hypertrophic cardiomyopathy (HCM) has been ascribed to mutations in genes encoding sarcomere proteins. In particular, mutations in MYBPC3, a gene which encodes cardiac myosin binding protein-C (cMyBP-C), have been implicated in over one-third of HCM cases. Of these mutations, 70% are predicted to result in C'-terminal truncated protein products which are undetectable in tissue samples. Heterozygous carriers of these truncation mutations exhibit varying penetrance of HCM, with symptoms often occurring later in life. We hypothesize that heterozygous carriers of MYBPC3 mutations, while seemingly asymptomatic, have subtle functional impairments that precede the development of overt HCM. This study compared heterozygous (+/t) knock-in MYBPC3 truncation mutation mice with wild-type (+/+) littermates to determine if functional alterations occur at the whole-heart or single-cell level prior to the onset of hypertrophy. The +/t mice show ~40% reduction in MYBPC3 transcription with no changes in cMyBP-C level, phosphorylation status, or cardiac morphology. However, cardiomyocytes from +/t mice show significantly decreased maximal force development at sarcomere lengths of 1.9 µm and 2.3
μm. In addition, heterozygous mice show significant reductions in vivo in the early/after (E/A) and E'/A' ratios, indicating diastolic dysfunction. These results suggest that seemingly asymptomatic heterozygous MYBPC3 carriers do suffer impairments that may presage the onset of HCM.

4.2 Introduction

Protein analysis of heart tissue from symptomatic heterozygous carriers of MYBPC3 mutations have shown reduced cMyBP-C levels compared to human non-failing donor hearts (Moolman, Reith et al. 2000, van Dijk, Dooijes et al. 2009, Marston, Copeland et al. 2012). This observation is paralleled in certain mouse models of MYBPC3 mutations, where symptomatic heterozygous mice have shown a decreased amount of cMyBP-C in the heart (Carrier, Knoll et al. 2004, Vignier, Schlossarek et al. 2009, Desjardins, Chen et al. 2012, Cheng, Wan et al. 2013). However, certain other heterozygous mouse models have demonstrated normal cMyBP-C levels with generally asymptomatic phenotypes (McConnell, Jones et al. 1999, Harris, Bartley et al. 2002). Therefore, it is currently unclear whether reduction in cMyBP-C content initiates the development of HCM via haploinsufficiency or if other less direct mechanisms are involved in the early stages of the pathology.

The functional impairments observed in heterozygous carriers of MYBPC3 truncation mutations have been suggested to result from reduced cMyBP-C levels (Moolman, Reith et al. 2000, van Dijk, Dooijes et al. 2009, Marston,
Copeland et al. 2012). However, transition to symptomatic HCM with reduced cMyBP-C levels remains poorly understood based in no small part on the uncertainty of whether reduced cMyBP-C levels are causative for this transition or are a result. Accordingly, this study investigated the physiological consequences exhibited by a mouse model previously described as having preserved cMyBP-C levels, but carrying only one functional allele of MYBPC3.

To accomplish this, a MYBPC3 truncation mutant mouse model generated by McConnell et al. was used (McConnell, Jones et al. 1999, McConnell, Fatkin et al. 2001, Palmer, McConnell et al. 2004). This model carries a knock-in mutation in MYBPC3 that results in the skipping of exon 30, a frame shift, and inclusion of a premature stop codon. As previously reported, the homozygous (t/t) mouse has no detectable cMyBP-C, increased fibrosis, dilation of the left ventricle, and decreased cardiac function leading to the development of heart failure (McConnell, Jones et al. 1999, Palmer, Georgakopoulous et al. 2004, Palmer, McConnell et al. 2004, Palmer, Noguchi et al. 2004). The original characterization of this model focused primarily on the homozygous genotype. However, the heterozygous mouse (+/t) was shown to be asymptomatic, with a level of cMyBP-C equal to that of wild-type (+/+). Also, hypertrophy was not reported in the initial characterization until the animals were over two years of age (McConnell, Jones et al. 1999, McConnell, Fatkin et al. 2001). Information about gene expression, myofilament performance and in vivo function were not reported for the heterozygote. As this heterozygous mouse appears to be
asymptomatic and maintains normal cMyBP-C levels, it is ideal for testing whether subtle deficits in cellular and cardiac function are present in the heterozygous mouse prior to the development of overt HCM.

4.3 Results

4.3.1 Heterozygous Express an Intermediate Level of MYBPC3 Transcript with Preserved cMyBP-C Content

Myofilament protein fractions isolated from three months old +/+ and +/t contained an equal amount of cMyBP-C, while no cMyBP-C was present in t/t hearts (Figure 8A). Furthermore, quantification of cMyBP-C in whole-heart homogenates showed that cMyBP-C levels normalized to actin are unchanged between the +/+ and +/t groups, consistent with previous reports (Figure 8B) (McConnell, Jones et al. 1999, Palmer, McConnell et al. 2004). In contrast to the protein data, levels of MYBPC3 transcript measured by qPCR were significantly reduced in the +/t group compared to +/+ (64% of +/+ levels), and t/t levels were significantly reduced compared to +/+ and +/t (16% of +/+ levels) using primers that recognize WT and truncated transcript (Figure 8D).

Protein levels of the pathological hypertrophy marker β-myosin heavy chain (β-MHC) resolved by SDS-PAGE and stained with Sypro-Ruby showed no change between +/+ and +/t, whereas the t/t hearts showed a significant elevation in β-MHC (Figure 8C). Analysis of the transcript levels of hypertrophic markers via qPCR showed no difference in either β-MHC (MYH7) or atrial
natriuretic factor (NPPA) between +/+ and +/t, with a significant elevation in the t/t mouse hearts (~6 fold increase in both MYH7 and NPPA) (Figure 8E & F).
Figure 8. Expression of MYBPC3 and hypertrophic markers.

A, Myofilament protein fraction resolved with SDS-PAGE and stained with Coomassie blue. B, Western blot showing levels of cMyBP-C and actin. Western blot quantification shows no cMyBP-C present in the t/t samples and no significant change in cMyBP-C levels between +/+ and +/t. (n=5) C, Total heart homogenate used for analysis of myosin heavy chain isoforms (α-MHC and β-MHC) resolved on a Hoefer format SDS-PAGE and stained with Sypro-Ruby. The +/+ and +/t hearts show no changes in α-MHC isoform levels, with t/t hearts showing a significant increase in the hypertrophic marker β-MHC. (N=11, 8, 9) D, Relative MYBPC3 expression is reduced in +/t hearts compared to +/+ hearts, and t/t hearts show an even further reduction in MYBPC3 expression, normalized to GAPDH expression. E & F, Relative transcript levels of hypertrophic markers MYH7 and NPPA are unchanged in +/+ and +/t hearts, but they show a significant increase in the t/t hearts normalized to GAPDH expression. (N=6,7,3) *P<0.05 vs. +/+, #P<0.05 vs. +/t. (Barefield, Kumar et al. 2014).
4.3.2 Phosphorylation of cMyBP-C Regulates Sarcomere Function.

In order to determine whether the phosphorylation status of cMyBP-C was altered in the +/t hearts, phosphorylation-dependent, site-specific antibodies to pS273, pS282, and pS302 were used. Phospho-cMyBP-C levels were normalized to total cMyBP-C (as shown in Figure 1B) and normalized to actin for a loading control. No changes in the phosphorylation status of cMyBP-C at serine 273, 282, or 302 were detected between +/+ and +/t groups (Figure 9A). Additionally, phosphorylation levels of cardiac troponin I (cTnI) at serine 23 and 24 showed no difference between +/+ and +/t or t/t, although a higher variability of phospho-cTnI was observed compared to phospho-cMyBP-C (Figure 9B).
Figure 9. Phosphorylation of cMyBP-C and cTnl.

A, Western blot analysis detecting cMyBP-C phosphorylated at serine-273, -282, and -302. No significant change in phosphorylation of cMyBP-C was observed at any of the phosphorylation sites between the +/+ and +/t groups. (n=5) B, The phosphorylation status of cTnl at serine-23 and -24, as determined by Western blot, shows no significant changes in any of the groups. (n=5) (Barefield, Kumar et al. 2014).
4.3.3 Heterozygous Mice do not Exhibit Gross Morphological Changes

Analysis of gross morphology and coronal sections of +/+ and +/- hearts showed no obvious pathology, whereas t/t hearts showed dilation as previously reported (Figure 10A & B) (McConnell, Jones et al. 1999). Heart weight to body weight ratios showed no significant difference between +/- (4.58 ± 0.32 mg/g HW/BW) and +/- (4.62 ± 0.08 mg/g HW/BW) (n=4). Similarly, H&E and Trichrome staining revealed no disparities between +/- and +/- samples (Figure 10C & D). Immunostaining of isolated cardiomyocytes showed doublet patterning consistent with properly localized C-band incorporation of cMyBP-C (green) between the α-actinin-stained Z-disks (red) in both the +/- and +/- groups (Figure 10E). These observations agree with the original characterization of this model and show that the +/- heart appears to have proper cMyBP-C incorporation with no grossly apparent phenotype.
Figure 10. Gross morphology and protein incorporation.

A, Representative whole heart images and B, coronal sections show no overt hypertrophy in the +/t hearts, whereas t/t hearts display a hypertrophic phenotype. C, Cross-sectional H&E and D, Trichrome staining reveal deranged muscle arrangement and fibrosis, respectively, in the t/t heart, with no notable differences in +/- and +/- t. E, Confocal microscopy immunofluorescence of isolated cardiomyocytes stained for cMyBP-C (Green) and α-actinin (Red). The cMyBP-C signal shows the characteristic C-zone doublet pattern between each Z-disk, stained for α-actinin in both +/- and +/- t cardiomyocytes. Staining of the t/t cells reveals only minimal cMyBP-C signaling and no structural localization. (Barefield, Kumar et al. 2014).
4.3.4 Heterozygous Cardiomyocytes Show Reduced Force Generation

Force-pCa analysis was performed to assess disparities in myofilament function between +/+ and +/t myocytes. Skinned myocytes showed a reduction in maximal developed force (F_{MAX}) at pCa 4.5 (3.16µM Ca^{2+}) in both +/t (68.5 ± 4.1 mN/mm^2 at SL 1.9; 79.2 ± 3.1 mN/mm^2 at SL 2.3, P=0.007 SL 1.9, P=0.004 SL 2.3) and t/t (69.3 ± 3.3 mN/mm^2 at SL 1.9; 81.1 ± 3.1 mN/mm^2 at SL 2.3, P=0.0241 SL1.9, P=0.009 SL2.3) groups compared to +/+ (82.2 ± 3.2 mN/mm^2 at SL 1.9; 95.5 ± 2.4 mN/mm^2 at SL 2.3) (Figure 11A-C). We also examined force generation at pCa 5.9 (0.13µM Ca^{2+}) to assess tension development at low Ca^{2+} concentrations. The force development of the +/+ myocytes (15.5 ± 3.0 mN/mm^2 at SL 1.9; 22.8 ± 3.0 mN/mm^2 at SL 2.3) was not significantly different than +/t (12.8 ± 1.5 mN/mm^2 at SL 1.9; 17.7 ± 1.8 mN/mm^2 at SL 2.3), although a significant (P=0.041) treatment effect was observed between +/+ and t/t (11.8 ± 1.3 mN/mm^2 at SL 1.9; 16.1 ± 1.1 mN/mm^2 at SL 2.3). Ca^{2+} sensitivity of force development, expressed as pCa_{50}, was significantly increased only in t/t hearts at SL 2.3 µm compared to +/+ (Figure 11D). The +/+ hearts show a significant change in pCa_{50} (P=0.01) with changes in sarcomere length, whereas this change is not observed in +/t or t/t (Table 2).
Figure 11. Heterozygous Cardiomyocytes Exhibit Impaired Force Generation.

A-C. Maximum development of force is significantly decreased in +/t and t/t skinned cardiomyocytes compared to +/+ at both short (SL 1.9 µm) and long (SL 2.3 µm) sarcomere lengths. D. Relative force comparisons show a significant decrease in Ca\(^{2+}\) sensitivity of force development in t/t hearts compared to +/+ at SL 2.3 µm. No significant changes in Ca\(^{2+}\) sensitivity were seen between +/t and +/+. (N=6, 7, and 7 hearts per group, with 3-4 cells averaged per heart) *P<0.05 vs. +/+, #P<0.05 vs. +/t. (Barefield, Kumar et al. 2014).
4.3.5 MYBP3 Heterozygous Mice Have Altered Cardiac Function in vivo

Echocardiography was performed to assess morphology, systolic, and diastolic function. M-mode parasternal long axis echocardiography showed no difference between +/t and +/- in interventricular septum or posterior wall thickness (Table 3), no dilation of the left ventricle in systole or diastole, and no changes in relative wall thickness compared to total heart thickness (Figure 12A, C-F). The +/- mice showed a non-significant (P=0.114) trend towards reduced % fractional shortening (27.4 ± 1.5%) compared to +/- (32.0 ± 2.1%), with a significant (P<0.001) reduction in fractional shortening in the t/t mice (11.53 ± 0.79) (Figure 12B).

Additionally, M-mode derived % ejection fraction followed the same non-significant trend between +/- (60.6% ± 3.0) and +/- (53.6 % ± 2.5), with a significant (P<0.001) reduction in systolic function in the t/t group (24.9% ± 1.6). Power Doppler measurements of left ventricular filling revealed a significant reduction (P=0.003) in the early/after (E/A) ratio in +/- (1.74 ± 0.12) compared to +/- (2.58 ± 0.53) (Figure 12G). Tissue Doppler analysis of mitral valve motion also showed a significant decrease (P=0.025) in the early movement of the myocardium around the mitral valve (E’) compared to the tissue motion associated with atrial contraction (A’), known as the E’/A’ ratio in the +/- mice (1.18 ± 0.05) compared to +/- (1.52 ± 0.15) (Figure 12H). While these diastolic parameters showed significant impairments in +/-, other parameters, such as the E’ and E velocities, as well as the E/E’ ratio, comparing blood flow velocity of
early filling compared to tissue movement, were not significantly altered (Table 3). Diastolic function was not assessed in t/t mice because that group was not available at the time diastolic parameters were measured.

In order to assess any additional whole-heart functional deficits, 2-dimensional echocardiography strain analysis was performed on B-mode long-axis images (Table 4). In both +/+ and +/t mice the left ventricle contracted synchronously, and no difference was detected in peak % strain, or time to peak. Furthermore, the strain rate and time to peak strain rate were unchanged.
Figure 12. Diastolic Dysfunction in Heterozygous Hearts

A, Representative parasternal long-axis M-mode echocardiography tracings from 10- to 12-week-old +/-, +/t, and t/t mouse hearts. B, Percent of left ventricular fractional shortening shows that +/t hearts trend towards reduced pump function compared to +/-, whereas t/t hearts show a significant deficit compared to both +/- and +/t groups. C & D, Left ventricular internal diameter (LVID) at peak systole and diastole shows significant dilation in the t/t hearts with no changes between +/- and +/t. E & F, Relative wall thickness (anterior + posterior wall / total heart thickness) at peak systole and diastole shows no change between +/t and +/-, whereas the wall-to-heart ratio is reduced in t/t. G & H, Representative power and tissue Doppler images depicting the early (E) and late (A) blood filling of the left ventricle and mitral valve motion. E/A and E’/A’ ratios were significantly reduced in +/t hearts compared to +/-, with t/t hearts showing a significant deficit compared to both groups. (N= 7, 10, 9) *P<0.05 vs. +/-, #P<0.05 vs. +/t. (Barefield, Kumar et al. 2014).
Table 2. Summary of force-pCa analysis of skinned cardiomyocytes.

Significant reductions in $F_{MAX}$ are observed in +/t and t/t compared to +/+ skinned cardiomyocytes. Significant alteration in calcium sensitivity of force development was only detected in the t/t skinned cardiomyocytes at SL 2.3 µm. No significant changes in Hill coefficient or length-dependent activation were observed. (n=3 +/+ hearts, 4 +/t and t/t hearts per group, with 4 cells averaged per heart) *$P<0.05$ vs. +/+, #P<0.05 vs. +/t. (Barefield, Kumar et al. 2014).

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SL 1.9

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SL 2.3

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<td>FS (%)</td>
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<td>E (mm/s)</td>
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Table 3. Summary of Echocardiography Results.

Significant reduction in fractional shortening (FS) was observed between t/t and +/-, E/A and E’/A’ ratios were significantly reduced in +/- compared to +/-.

*P<0.05 vs. +/-, #P<0.05 vs. +/- (Barefield, Kumar et al. 2014).
Table 4. Summary of Echocardiography Strain Analysis.

Radial and longitudinal strain peak % and strain rate show no significant differences between +/+ and +/t hearts. Also, time to peak strain and strain rate showed no significant alterations between groups. N=6

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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endo</td>
<td>26.7 ± 2.9</td>
<td>29.5 ± 2.6</td>
<td>29.0 ± 0.39</td>
<td>32.8 ± 3.2</td>
</tr>
<tr>
<td>Epi</td>
<td>26.7 ± 2.9</td>
<td>29.5 ± 2.6</td>
<td>41.7 ± 3.1</td>
<td>40.2 ± 4.2</td>
</tr>
</tbody>
</table>
4.4 Discussion

This study aimed to detect functional deficits in a MYBPC3 heterozygous mutant mouse with preserved cMyBP-C levels. While this heterozygous model has normal cMyBP-C levels, proper protein incorporation, and normal morphology, the results of these experiments demonstrate that functional deficits do exist at the whole-heart and single myocyte levels. Here we show a 36% decrease in MYBPC3 transcription in the heterozygote, while cMyBP-C protein levels remain normal. The preservation of cMyBP-C level in the heterozygote could be explained by reduced myofilament degradation or a longer half-life of cMyBP-C in the sarcomere compared to normal turnover levels in a WT heart. This suggests that WT levels of transcript are in excess under normal conditions, or the heterozygote maintains cMyBP-C levels by other compensatory mechanisms, such as reduced cMyBP-C turnover. However, in spite of proper cMyBP-C stoichiometry, maximal force development is reduced in heterozygous myocytes, indicating that these mice have impairments in contractility.

In addition to these deficits, the heterozygous mice demonstrate a trend towards reduced fractional shortening, which is consistently measured on the low-normal side, even in our FVB/N wild-type mice, as well as significantly reduced E/A and E’/A’ ratios, indicating the development of diastolic dysfunction. On the other hand, force development in skinned myocytes did not show any significant difference at diastolic calcium levels, leaving the cause of diastolic dysfunction unclear. The myofilament abnormalities and alterations in organ
function do not appear to manifest morphologically, as the heterozygote does not show any signs of hypertrophy at the whole organ level. Nor does the heterozygote display an increase in levels of hypertrophic markers. These findings suggest that seemingly asymptomatic heterozygous MYBPC3 mutant carriers do have functional impairments, which may cause the development of HCM with increased age or when subjected to additional cardiac stress.

The mechanism by which heterozygous truncation mutations of MYBPC3 initiate the development of HCM is unclear. Human carriers of these mutations exhibit variable disease penetrance, often with no expression of the mutant protein (Rottbauer, Gautel et al. 1997). Heterozygous carriers of various MYBPC3 mutations are typically asymptomatic until adulthood and often develop a mild phenotype compared to homozygous MYBPC3 mutant carriers (Charron, Dubourg et al. 1998, Niimura, Bachinski et al. 1998, Moolman, Reith et al. 2000, Dhandapany, Sadayappan et al. 2009). The variable penetrance of these mutations is evident in pedigree analysis of mutation-carrying families, where the same mutation can result in asymptomatic or symptomatic phenotypes (Charron, Dubourg et al. 1998, Niimura, Bachinski et al. 1998, Moolman, Reith et al. 2000, Dhandapany, Sadayappan et al. 2009).

The role that MYBPC3 haploinsufficiency plays in these cases is also unclear. In symptomatic heterozygous mutation carriers, a reduced level of cMyBP-C has been identified (Moolman, Reith et al. 2000, Marston, Copeland et al. 2009, van Dijk, Dooijes et al. 2009). This reduction in protein has been linked
to a reduction in force development, although this may be attributed to the HCM phenotype, as these alterations are also observed in HCM patients with non-
MYBPC3 mutations and normal cMyBP-C levels (van Dijk, Paalberends et al. 2012, Sequeira, Wijnker et al. 2013). However, since samples from asymptomatic mutation carriers are unavailable, it is unclear whether reduction of cMyBP-C initiates the development of HCM or merely accompanies it, as the result of an unknown primary effect. A better understanding of the early changes in these pathways would provide additional opportunity for diagnosis and intervention (Germans, Russel et al. 2010).

Several cMyBP-C knockout mouse models have been developed over the last 15 years. The model developed by the Seidman laboratory in 1999, which was used for this work, was generated with a knock-in mutation resulting in a gene encoding a predicted truncated protein, which is undetectable in the heart. Heterozygous mice with this mutation do not show a hypertrophic phenotype, whereas the homozygous mouse develops dilated cardiomyopathy, decreased cardiac function, and a reduction in force development (McConnell, Jones et al. 1999, McConnell, Fatkin et al. 2001, Palmer, Georgakopoulous et al. 2004, Palmer, McConnell et al. 2004, Palmer, Noguchi et al. 2004). The MYBPC3 knockout “null” mouse model was generated in the Moss laboratory in 2002. This model has exons 3-10 removed, resulting in the absence of cMyBP-C expression in the homozygous mouse (Harris, Bartley et al. 2002). In initial reports, the heterozygous genotype of this model does not exhibit any reported deficits, with
normal levels of cMyBP-C, despite reduced mRNA levels (Harris, Bartley et al. 2002). However, more recent evaluation of this heterozygous model does show a 32% decrease in cMyBP-C levels, as well as mild functional impairments consistent with the onset of HCM (Cheng, Wan et al. 2013), (Desjardins, Chen et al. 2012).

Two MYBPC3 mutant mouse models were developed in the Carrier lab (Carrier, Knoll et al. 2004, Vignier, Schlossarek et al. 2009), one of which is a knockout mouse lacking exon 1 and 2, resulting in a null allele (Carrier, Knoll et al. 2004). In contrast to the Seidman model, this heterozygous mouse model does show a reduction in cMyBP-C levels (25% of WT), with a reduction in MYBPC3 mRNA (from ~80% to ~50% of WT levels from 6 to 11 months of age), and develops HCM symptoms at 10-11 months of age (Carrier, Knoll et al. 2004). The other model is a knock-in mouse carrying a common human mutation that results in several mRNA splice products. This causes the expression of one potential poison polypeptide and an overall 50% reduction in mRNA, in turn resulting in a 20% reduction of cMyBP-C in the heterozygote (Vignier, Schlossarek et al. 2009). While this model does not appear to develop HCM, these mice do show increased calcium sensitivity at the skinned fiber level, and alterations in calcium handling were observed in intact cardiomyocytes (Fraysse, Weinberger et al. 2012).

A consistent theme found in all these models is that homozygous mutant carriers show a severe dilated or eccentric hypertrophy phenotype, whereas the
heterozygous models are more variable in their pathology, typically showing no hypertrophy or mild hypertrophy, with the development of age-dependent HCM. These observations parallel those made in human carriers of MYBPC3 mutations that are linked to HCM. The study of these various heterozygous mouse models underscores the subtle mechanistic differences among specific MYBPC3 mutations, illustrating the complexity of the heterozygous condition in mouse models and human patients. The mechanism that drives the development of dysfunction in heterozygous mutant mice is currently unclear. A haploinsufficiency model suggests that reduction of cMyBP-C levels from insufficient expression from one wild-type allele causes the initial insult for the development of HCM. The poison polypeptide model proposes that mutant MYBPC3 genes express a protein that is potentially unable to incorporate properly into the sarcomere, causing disruption of cellular processes. However, in cases where cMyBP-C levels are preserved and little mutant MYBPC3 transcript is present, other mechanisms may be involved. For example, it has been suggested that chronic expression of mutant transcript or misfolded protein may overwhelm and impair the ubiquitin-proteasome system or the nonsense-mediated mRNA decay pathway over an individual’s lifetime and may account for the late onset of hypertrophy (Vignier, Schlossarek et al. 2009, Schlossarek, Englmann et al. 2012, Schlossarek, Schuermann et al. 2012).

In this study, it was observed that heterozygous truncation in mice results in reduced mRNA levels, a full cMyBP-C complement, and only minor functional
disparities. Thus, even in non-hypertrophic carriers, some perturbations may be occurring. The identity of these alterations and whether they are compensatory processes or the result of decomposition remain to be clarified. Elucidating the mechanism by which lower levels of gene expression can lead to a disease phenotype, while protein levels are maintained, is an interesting problem. One line of inquiry into this problem might examine the effect of MYBPC3 gene dosage relative to normal cMyBP-C protein turnover in the sarcomere such that lower gene expression could lead to an increased half-life of cMyBP-C. Another potential explanation previously reported implicates expression of mutant RNA or protein as a mechanism, but not through the traditional poison polypeptide model (Vignier, Schlossarek et al. 2009, Schlossarek, Englmann et al. 2012, Schlossarek, Schuermann et al. 2012). This hypothesis asserts that an increased need to degrade aberrant mRNA via nonsense-mediated decay, or misfolded protein via proteasome or autophagic pathways, exerts a stress on the cardiomyocytes and prevents normal cellular housekeeping, leading to dysfunction. It is also not absolutely clear if the truncated gene is able to express small amounts of mutant cMyBP-C, which could result in a functional impairment of the sarcomere.

As the results of this study indicate that the this heterozygous mouse is already functionally compromised, it would be beneficial to determine if these animals have an increased risk for hypertrophy following cardiovascular stress,
as this may provide direct insight into the development of HCM and heart failure in human carriers of heterozygous MYBPC3 mutations.
CHAPTER FIVE

HAPLOINSUFFICIENCY OF MYBPC3 IN THE DEVELOPMENT OF HYPERTROPHIC CARDIOMYOPATHY

5.1 Abstract

Mutations in MYBPC3, encoding for cMyBP-C, account for nearly 40% of identified HCM causing mutations and often results in an absence of protein (i.e. haploinsufficiency). Heterozygous human carriers and mouse models have variable disease penetrance and late onset of HCM. Our objective was to determine if heterozygous MYBPC3 mutant carriers with preserved cMyBP-C levels under normal conditions are at a greater risk for HCM after cardiac stress. To investigate this question transverse aortic constriction or sham surgery was performed on 10-12 week old wild-type (WT) and MYBPC3 heterozygous (Het) mice, followed by four or 12 weeks to allow the development of hypertrophy. Heart weight/body weight ratios were significantly elevated 12 weeks post-TAC in WT and Het compared to sham controls, with Het TAC hearts showing significantly more hypertrophy than WT TAC. Echocardiography derived ejection fraction was significantly decreased in WT and Het mice post-TAC compared to sham controls. Force-pCa measurements on skinned myocytes showed
significant reduction in maximal force generation in both TAC groups, and also showed significant disparities in Het sham in force development and Ca^{2+} sensitivity. Levels of cMyBP-C assessed by Western blot show significant reductions at 4 weeks in both Het groups, which are no longer seen at 12 weeks. These observations suggest that haploinsufficiency of MYBPC3 does hasten the pathogenesis of HCM after cardiac insult. Further exploration of how heterozygous mutations in MYBPC3 predispose the heart to the development of maladaptive hypertrophic remodeling could provide novel therapeutic targets for the large number of human carriers of similar mutations.

5.2 Introduction

Recent reports have shown that cMyBP-C protein levels are decreased in symptomatic heterozygous carriers of several MYBPC3 truncating mutations (van Dijk, Dooijes et al. 2009, Copeland, Sadayappan et al. 2010). For obvious reasons examining cMyBP-C levels in the hearts of asymptomatic mutation carriers is nearly impossible, and therefore it remains unclear if the development of HCM occurs concomitantly with a reduction in cMyBP-C levels, or whether either event is causative for the other. Several mouse models of MYBPC3 truncation mutations currently exist and in most of these models the heterozygous genotype has been shown to be mildly symptomatic with reduced cMyBP-C levels (Fraysse, Weinberger et al. 2012, Cheng, Wan et al. 2013).
However, one heterozygous model has been shown to have normal cMyBP-C stoichiometry (McConnell, Jones et al. 1999). This model was initially thought to be asymptomatic but recent work from our group shows that despite normal levels of cMyBP-C, subtle functional deficits begin to manifest prior to the development of HCM (Barefield, Kumar et al. 2014). Using these mice I tested the hypothesis that heterozygous carriers are more susceptible to pressure-overload induced cardiovascular stress and are predisposed to the development of HCM. I also tested whether this stress causes haploinsufficiency in the heterozygous state, resulting in decreased cMyBP-C levels and additional dysfunction.

5.3 Results

5.3.1 Increased Hypertrophy in Heterozygous Mice Following TAC

In order to initiate hypertrophic remodeling, 10-12 week old WT and Het mice underwent a transverse aortic constriction (TAC) pressure-overload procedure or a sham surgery used as a control. The success of the TAC surgery was confirmed by assessing transverse aortic blood flow velocities at four and 12 weeks. Sham WT and Het mice show no alterations in this parameter at either four or 12 weeks, with velocities consistent with naïve mice. WT and Het TAC groups show a significant increase in aortic blood flow velocity compared to genotype controls at both four and 12 weeks. Furthermore, the aortic blood flow velocity was not significantly altered between WT and Het TAC groups,
suggesting that both groups received an equal amount of aortic constriction (Figure 13). As an additional verification, this parameter was measured over the 12 week time course of the study. During this time none of the groups showed significant differences in blood flow velocity between the four and 12 week time point, confirming that the TAC suture remained tightly in place for the duration of the experiment.

Cardiac hypertrophy was assessed by measuring the heart weight: body weight ratio which showed significant increases in both WT and Het post-TAC at both four and 12 weeks compared to sham controls (Figure 14). The Het TAC hearts did not show a significant difference compared to WT TAC at 4 weeks, but did show a significant increase compared to WT following at 12 weeks post-TAC. As an additional measure of hypertrophy, analysis of the relative percentage of α and β-MHC revealed a significant increase in the hypertrophic marker β-MHC in the WT and Het hearts four and 12 weeks post-TAC compared to their respective sham controls. However, levels of β-MHC were not altered between WT and Het TAC (Figure 14).
Figure 13. Hypertrophic Remodeling following TAC.

Transverse aortic blood flow velocity is significantly increased in both WT and Het TAC groups, and to the same extent, establishing the severity of the TAC procedure was similar and remained constant between four and 12 weeks. *P<0.05 vs. Sham N= 5-13
Hypertrophy measured by HW:BW and the Sypro stained gels of separated myosin isoforms to detect the hypertrophic marker β-MHC showed both TAC groups underwent hypertrophic remodeling. These effects were similar at four weeks, however at 12 weeks the Het TAC showed significantly greater hypertrophy than the WT TAC (B). N= 5-13 (A), 5 (B) *P<0.05 vs. Sham, #P<0.05 vs. WT.

Figure 14. Het Mice Show Greater Hypertrophic Remodeling Following TAC
5.3.2 Gene Expression Profile Shows Hypertrophy Post-TAC

MYBPC3 gene expression was measured with qPCR. Samples from Het hearts from both sham and TAC groups showed a significant reduction in MYBPC3 expression compared to wild-type sham and TAC controls. This was observed at both four and 12 weeks post-surgery. The reductions in MYBPC3 transcript were similar to the 37% reduction observed in the Het naïve hearts compared to WT naïve hearts as previously reported (Barefield, Kumar et al. 2014). This suggests that there is no significant increase in MYBPC3 gene expression in the Het hearts during hypertrophic remodeling at four and 12 weeks (Figure 15).

Transcript levels of hypertrophic markers NPPA (atrial natriuretic factor) and MYH7 showed significant increase in both WT and Het post-TAC at four and 12 weeks. The levels of these hypertrophic markers measured with qPCR confirmed the results from RNA-Seq, with good agreement between these two methods (Figure 15). Also, while there were no differences reported in the levels of MYH7 and NPPA between WT and Het TAC at 12 weeks from qPCR analysis, NPPA levels assessed by RNA-Seq showed significant increase in Het TAC compared to WT TAC (Figure 15).
Expression levels of MYBPC3, NPPA, and MYH7 were quantified by RNA-Seq and qPCR. Significant reduction in MYBPC3 transcript levels were observed in both Het samples, consistent with reduced transcript observed at baseline. Hypertrophic markers show significant increase in both TAC groups. (N=4) *\( P<0.05 \) vs. Sham, #\( P<0.05 \) vs. WT, †\( P<0.001 \) false discovery rate test.

Figure 15. Gene Expression Profiles of MYBPC3 and Hypertrophic Markers.
5.3.3 Decreased cMyBP-C Content in Heterozygous Sarcomeres

Protein levels of cMyBP-C assessed by western blot showed a significant reduction in both Het sham and Het TAC compared to WT controls (~30% of WT) four weeks post-surgery. However, by 12 weeks post-surgery no significant changes between WT and Het samples were detectable in either Sham or TAC treatments, although Het TAC mice showed a non-significant reduction in cMyBP-C compared to WT TAC (Figure 16).

Phosphorylation of myofilament proteins plays a role in regulation of cardiac function. Phospho-specific antibodies were used to assess the phosphorylation status of cMyBP-C on serine 273, 282, and 302, as well as the phosphorylation status of cTnI on serines 23/24. Four weeks following TAC, WT and Het hearts showed a significant interaction effect (2-way ANOVA reported a significant difference between the response of the two genotypes to the two treatments) in the increase in cMyBP-C phosphorylation at all three serine sites (Figure 17). Surprisingly, the Het sham hearts also showed a significant increase in phosphorylation at Ser-273 and 282 compared to WT sham, which differs from the equal phospho-cMyBP-C levels observed under baseline conditions between WT and Het. This finding complicates the analysis by statistical methods. By 12 weeks following surgery, phosphorylation was significantly reduced at Ser-273 in both WT and Het TAC hearts compared to sham, with no significant differences observed in Ser-282 in any group, and a significantly increased level of phosphorylation at Ser-302 in Het TAC compared to WT TAC. Also, by 12 weeks
the several-fold increase observed at four weeks had decreased to levels similar to WT Sham (Figure 17).

In addition to alterations in cMyBP-C phosphorylation, levels of phosphorylation of cTnI on Ser-23/24 were assessed at four and 12 weeks. Myofilament enriched protein fractions showed a significant increase in phospho-cTnI in both WT and Het TAC mice at four weeks compared to their respective sham controls, consistent with expected hypertrophic signaling and hyperphosphorylation of cMyBP-C. However, the elevation in phosphorylated cMyBP-C residues in Het Sham was not observed in cTnI at four weeks. Twelve weeks following TAC the large fold increase in cTnI phosphorylation had subsided, returning closer to WT Sham levels. However, Het Sham showed significantly higher levels of phosphor-cTnI compared to WT Sham. There also was a significant interaction effect detected by two-way ANOVA, which describes the non-significant trend towards increased phosphorylation in both TAC groups at 12 weeks (Figure 18).
Western blot analysis reveals significant reduction in cMyBP-C content in both Sham and TAC Het hearts compared to WT at four weeks. No alterations were observed 12 weeks following surgery. (N=4-6) *P<0.05 vs. Sham, #P<0.05 vs. WT.

**Figure 16. Het Hearts Show Reduced cMyBP-C.**
Phospho-cMyBP-C, normalized to total cMyBP-C showed increased trends at four weeks, with a notable increase in Het Sham as well. Levels of phospho-cMyBP-C were reduced at 12 weeks at Ser 273, consistent with hypophosphorylation reported during the development of HF. Significant interaction effects were noticed in Ser-273 and S-282 phosphorylation at four weeks. (N=4-6) *P<0.05 vs. Sham, #P<0.05 vs. WT.

Figure 17. Phosphorylation Status of cMyBP-C.
Significant elevation in cTnI phosphorylation was observed in both TAC groups at 4 weeks. Phosphorylation of cTnI at 12 weeks shows reduced phosphorylation levels with significant elevation in Het Sham. Significant interaction effects were reported at 12 weeks. (N=4-6) *P<0.05 vs. Sham, #P<0.05 vs. WT.

Figure 18. Phosphorylation of cTnI Ser-23/24.
5.3.4 Reduced Contractility in Het Cardiomyocytes

In order to assess contractile performance at the level of the cardiomyocyte, force-pCa analysis was performed. Skinned cardiomyocytes from Het sham animals four weeks post-surgery showed a significantly reduced maximal force generation at saturating Ca$^{2+}$ concentrations at both short (Figure 19) and long SL (Figure 20). This observation is consistent with the reduction in maximal force development observed in Het Naïve mice (Figure 11). The WT and Het TAC mice also show a reduction in maximal force generation, although this change is statistically confounded by interaction effects of genotype and treatment (Figure 19, 20). At 12 weeks post-surgery Het sham cardiomyocytes continue to exhibit a significantly decreased force generation compared to WT sham, but only at short sarcomere length. At 12 weeks both WT and Het TAC show a significantly reduced maximal force generation compared to sham controls, indicating a loss of contractility at this time point which was not evident at four weeks. Also, a significant genotype main effect was observed by 2-way ANOVA showing significant reduction in maximal force generation in the Het genotype compared to WT at long sarcomere length at 12 weeks.

In contrast to the trend that Het cardiomyocytes generate less force at saturating Ca$^{2+}$ levels, at diastolic Ca$^{2+}$ levels (pCa 5.75) Het myocytes four weeks post-TAC have a significantly elevated development of force at SL 1.9 vs. WT TAC ($22.1 \pm 2.2$ mN/mm$^2$ vs. $15.1 \pm 0.9$ mN/mm$^2$) (Table 5). This effect at
pCa 5.75 failed to reach significance at SL 2.3, and was not significantly altered at either sarcomere length at 12 weeks.

Length dependent increase in force development ($\Delta F_{\text{MAX}}$) was significantly attenuated in the Het TAC cardiomyocytes compared to WT TAC. There was also a significant genotype main effect by 2-way ANOVA showing a reduction in $\Delta F_{\text{MAX}}$ in the heterozygous genotype compared to WT at four weeks (Figure 22). In cardiomyocytes 12 weeks following surgery a significantly reduced $F_{\text{MAX}}$ was observed in both WT and Het TAC groups at long and short SL. Significant reduction in force observed in Het Sham cardiomyocytes was still observed at short SL compared to WT sham, with this effect not reaching significance at long SL. No changes in $\Delta F_{\text{MAX}}$ were observed in any of the groups at 12 weeks.

Comparing force generation at SL 1.9 there was a significant reduction in force in both TAC groups from four weeks to 12 weeks. At long sarcomere lengths there was a reduction in force generation over time in WT TAC cells, although this time-course was not observed in Het TAC. Furthermore, there was an increase in force generation capability in Het Sham between four and 12 weeks. Changes in $\Delta F_{\text{MAX}}$ between four and 12 weeks show restoration of length-dependent increase in force development observed in both Het groups at 12 weeks from the deficits observed at four weeks.
Force pCa tracings at SL 1.9 for four week and 12 week groups show significantly depressed maximal force in Het sham, as well as reduced force in TAC hearts at 12 weeks. (N=4-6 Hearts, 3 Cells/Heart) * = p<0.05 v. Sham control, # = p<0.05 v. WT control.

**Figure 19. Force Deficits in Het and TAC Cardiomyocytes at SL1.9.**
Figure 20. Force Deficits in Het and TAC Cardiomyocytes at SL2.3.

Force pCa tracings at SL 2.3 for four week and 12 week groups show significantly depressed maximal force in Het sham, as well as reduced force in TAC hearts at 12 weeks. (N=4-6 Hearts, 3 Cells/Heart) * = p<0.05 v. Sham control, # = p<0.05 v. WT control.
Force pCa tracings at SL 1.9 and SL2.3 for four week and 12 week groups. Graphs depict change in maximal force generation following stretch. At four weeks a significant genotype main effect was observed by 2-way ANOVA. (N=4-6 Hearts, 3 Cells/Heart) * = p<0.05 v. Sham control, # = p<0.05 v. WT control.

Figure 21. Deficits in Length-Dependent Increase in Force Development in Heterozygous Cardiomyocytes.
Alterations in force development can be explained by changes in myofilament Ca\(^{2+}\) sensitivity of force development. At four weeks post-surgery increased Ca\(^{2+}\) sensitivity was observed in the cardiomyocytes from both TAC groups compared to their respective sham controls at SL 1.9 and 2.3. Both Het groups also showed a significant increase in Ca\(^{2+}\) sensitivity compared to WT sham and TAC controls (Fig 22). The length-dependent change in pCa\(_{50}\) was significantly blunted in Het sham compared to WT, and a significant genotype main-effect was also observed.

At 12 weeks following surgery a significant genotype main-effect reduction in Ca\(^{2+}\) sensitivity was observed in Het cardiomyocytes at SL 1.9. At SL 2.3 both WT and Het cardiomyocytes were significantly less sensitive to Ca\(^{2+}\) compared to sham controls. A significant genotype main effect was observed in ΔpCa\(_{50}\), with Het cardiomyocytes showing impaired stretch mediated increase in Ca\(^{2+}\) sensitivity.

Longitudinal comparisons show that at SL 1.9 WT sham cardiomyocytes became more sensitive to Ca\(^{2+}\) between four and 12 weeks, whereas Het sham had no alterations over time. Hey Sham cardiomyocytes also exhibited a significantly attenuated increase in Ca\(^{2+}\) sensitivity compared to WT sham. While WT TAC did not show any time-dependent changes at SL 1.9, Het TAC became less sensitive to Ca\(^{2+}\) from four to 12 weeks, and was less sensitive than either Het sham or WT TAC cardiomyocytes. A similar trend was observed at SL 2.3 for these parameters. Interestingly, a significant genotype effect was observed in the
change in ΔpCa50 between four and 12 weeks in the Het genotype which showed an increase in length-dependent Ca$^{2+}$ sensitization effect. This suggests that at 12 weeks Het cardiomyocytes recovered a significant amount of length-dependent Ca$^{2+}$ sensitivity.
Calcium sensitivity of force development reported in at SL 1.9, 2.3 and ΔpCa_{50}) for four week (Left Column), and 12 week (right column). (N=4-6 Hearts, 3 Cells/Heart) * = p<0.05 v. Sham control, # = p<0.05 v. WT control, and † = p<0.05 in single group between 4 and 12 weeks.

Figure 22. Calcium Sensitivity of Force Development 4 and 12 Weeks Post-TAC.
Table 5. Summary of TAC and Sham Force-pCa Experiments

<table>
<thead>
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<th>Four Week Post-Surgery</th>
<th>Twelve Week Post-Surgery</th>
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<tbody>
<tr>
<td></td>
<td>WT Sham</td>
<td>Het Sham</td>
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<tr>
<td>( F_{pCa5.75} ) (mN/mm²)</td>
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<td>18.3 ± 1.5</td>
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<tr>
<td>( F_{MAX} ) (mN/mm²)</td>
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<td>60.1 ± 3.7</td>
</tr>
<tr>
<td>( pCa_{50} )</td>
<td>5.45 ± 0.03</td>
<td>5.56 ± 0.02</td>
</tr>
<tr>
<td>( \Delta F_{MAX} ) (mN/mm²)</td>
<td>33.8 ± 3.2</td>
<td>28.1 ± 2.6</td>
</tr>
<tr>
<td>( \Delta pCa_{50} )</td>
<td>0.12 ± 0.03</td>
<td>0.05 ± 0.02</td>
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5.3.5 Reduced Systolic Function in Heterozygous Hearts Post-TAC

*In vivo* cardiac function was assessed via echocardiography four and 12 weeks post-TAC. Systolic function measured by ejection fraction showed significant reduction in both WT and Het TAC mice compared to sham controls at both four and 12 week time points. Also, Het TAC mice showed a significant reduction in ejection fraction compared to WT TAC, which was also observed at both time points (*Figure 23*). The diastolic functional parameter of the E/A ratio showed no significant changes between groups at four weeks, although by 12 weeks both TAC groups showed a significantly increased E/A ratio. E’/A’ ratio showed an elevation in both TAC groups at four and 12 weeks (*Figure 23*). This suggests that left atrial and pulmonary pressures are higher in the TAC groups, causing increased early filling.

Septum thickness was significantly increased in both TAC groups at peak diastole at four and 12 weeks compared to sham controls. A significant interaction effect of treatment and genotype was observed in systolic and diastolic septal thickness parameters at both time points, meaning that genotype had opposite effects between both treatments, making statistical analysis difficult. LV posterior wall thickness was also significantly increased after TAC in diastole in both four and 12 week groups, with a significant increase in thickness occurring at peak systole in both TAC groups at four weeks but not at 12 weeks (*Figure 24*). At four weeks peak systolic LVID was significantly larger in the TAC groups; with the peak diastolic LVID not altered suggesting normal chamber
filling with decreased contractility, which is reflected in the ejection fraction (Table 6). Oppositely, diastolic internal diameter was significantly increased in both TAC hearts at 12 weeks, suggesting dilation (Table 7).
Figure 23. Reduced Systolic Function in Heterozygous Hearts Post-TAC.

Increased hypertrophy of the septum and posterior wall was observed in both WT and Het TAC, with increased chamber internal diameter at peak systole. TAC hearts show a significant reduction in function, with Het TAC showing reduced EF and FS compared to WT. The E'/A' ratio shows alterations in the TAC groups compared to sham. (N=5-13) *P<0.05 vs. Sham, #P<0.05 vs. WT.
Echocardiography measurements of ventricle wall thickness at four and 12 weeks show hypertrophied posterior wall (LVPW) and septum (IVS) thicknesses. The differences in diastolic and systolic IVS measurements are likely due to the significant interaction effects from two-way ANOVA. (N=5-13) *P<0.05 vs. Sham, #P<0.05 vs. WT.

Figure 24. Increased LV Wall Thickness Post-TAC.
<table>
<thead>
<tr>
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<th>Four Week Post-Surgery</th>
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<tbody>
<tr>
<td></td>
<td>WT Sham</td>
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<tr>
<td>Aortic Blood Flow (mm/sec)</td>
<td>840 ± 44</td>
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<td>Heart Rate (beats/min)</td>
<td>472 ± 7</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>4.00 ± 0.11</td>
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<td>MV E (mm/sec)</td>
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<td>MV E/A</td>
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<td>E' (mm/sec)</td>
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<td>A' (mm/sec)</td>
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</tr>
<tr>
<td>LVPW;s (mm)</td>
<td>1.15 ± 0.04</td>
</tr>
<tr>
<td>EF (%)</td>
<td>67.2 ± 1.6</td>
</tr>
<tr>
<td>FS (%)</td>
<td>34.9 ± 1.1</td>
</tr>
</tbody>
</table>

Table 6. Summary of Four Week Echocardiography Data.
Table 7. Summary of 12 Week Echocardiography Data.

<table>
<thead>
<tr>
<th></th>
<th>WT Sham</th>
<th>Het Sham</th>
<th>WT TAC</th>
<th>Het TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic Blood Flow (mm/sec)</td>
<td>915 ± 48</td>
<td>943 ± 27</td>
<td>3299 ± 102</td>
<td>3499 ± 153</td>
</tr>
<tr>
<td>Heart Rate (beats/min)</td>
<td>438 ± 15</td>
<td>446 ± 14</td>
<td>454 ± 23</td>
<td>447 ± 24</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.93 ± 0.12</td>
<td>3.96 ± 0.012</td>
<td>5.76 ± 0.39</td>
<td>6.99 ± 0.79</td>
</tr>
<tr>
<td>MV E (mm/sec)</td>
<td>717 ± 27</td>
<td>784 ± 41</td>
<td>798 ± 23</td>
<td>876 ± 52</td>
</tr>
<tr>
<td>MV A (mm/sec)</td>
<td>438 ± 29</td>
<td>428 ± 26</td>
<td>406 ± 69</td>
<td>316 ± 32</td>
</tr>
<tr>
<td>MV E/A</td>
<td>1.68 ± 0.09</td>
<td>1.98 ± 0.21</td>
<td>2.19 ± 0.45</td>
<td>3.02 ± 0.39</td>
</tr>
<tr>
<td>E' (mm/sec)</td>
<td>22.3 ± 1.4</td>
<td>23.3 ± 1.3</td>
<td>26.8 ± 2.5</td>
<td>27.2 ± 3.0</td>
</tr>
<tr>
<td>A' (mm/sec)</td>
<td>20.8 ± 1.1</td>
<td>21.7 ± 1.2</td>
<td>16.5 ± 1.9</td>
<td>16.7 ± 2.5</td>
</tr>
<tr>
<td>E/A'</td>
<td>1.09 ± 0.06</td>
<td>1.13 ± 0.10</td>
<td>1.69 ± 0.24</td>
<td>1.76 ± 0.19</td>
</tr>
<tr>
<td>MV E/E'</td>
<td>32.8 ± 1.5</td>
<td>34.8 ± 2.3</td>
<td>30.4 ± 2.5</td>
<td>33.9 ± 2.6</td>
</tr>
<tr>
<td>AET (ms)</td>
<td>54.9 ± 2.3</td>
<td>53.3 ± 1.4</td>
<td>63.8 ± 3.1</td>
<td>59.7 ± 3.6</td>
</tr>
<tr>
<td>IVS;d (mm)</td>
<td>1.02 ± 0.03</td>
<td>0.95 ± 0.03</td>
<td>1.04 ± 0.07</td>
<td>1.24 ± 0.08</td>
</tr>
<tr>
<td>IVS;s (mm)</td>
<td>1.52 ± 0.04</td>
<td>1.42 ± 0.05</td>
<td>1.40 ± 0.10</td>
<td>1.61 ± 0.07</td>
</tr>
<tr>
<td>LVID;d (mm)</td>
<td>3.93 ± 0.07</td>
<td>4.04 ± 0.07</td>
<td>4.26 ± 0.13</td>
<td>4.11 ± 0.09</td>
</tr>
<tr>
<td>LVID;s (mm)</td>
<td>2.51 ± 0.06</td>
<td>2.67 ± 0.06</td>
<td>3.06 ± 0.15</td>
<td>3.14 ± 0.09</td>
</tr>
<tr>
<td>LVPW;d (mm)</td>
<td>0.98 ± 0.06</td>
<td>0.92 ± 0.04</td>
<td>1.26 ± 0.08</td>
<td>1.22 ± 0.07</td>
</tr>
<tr>
<td>LVPW;s (mm)</td>
<td>1.3 ± 0.06</td>
<td>1.30 ± 0.05</td>
<td>1.57 ± 0.07</td>
<td>1.43 ± 0.07</td>
</tr>
<tr>
<td>EF (%)</td>
<td>66.2 ± 1.1</td>
<td>63.3 ± 1.0</td>
<td>55.1 ± 2.6</td>
<td>47.6 ± 1.5</td>
</tr>
<tr>
<td>FS (%)</td>
<td>36.0 ± 0.9</td>
<td>34.0 ± 0.7</td>
<td>28.5 ± 1.6</td>
<td>23.7 ± 0.9</td>
</tr>
</tbody>
</table>
5.3.6 Alterations in Gene Expression in Heterozygous Hearts.

RNA-Seq analysis revealed differential gene expression patterns in the four groups. Genes were included in analysis if the copy number detected reached significance following a false discovery rate test (P<0.001) and had a fold change of 1.5 fold increase or 0.6 fold decrease. Pathway analysis was performed using NCBI’s DAVID bioinformatics resource. A suite of 386 genes were co-regulated in both WT and Het TAC compared to their sham controls that contained numerous hypertrophic pathway-related genes as expected.

The initial purpose of RNA-Seq analysis was to evaluate if the ubiquitin-proteasome or nonsense-mediated RNA degradation pathways were altered due to the expression of the truncated MYBPC3 allele, as those pathways have been hypothesized to potentially contribute to dysfunction. However, Het expression patterns showed no significant changes in genes from these pathways in either sham or TAC treatments, suggesting that if these pathways are altered in disease, they are modified post-translationally.

The expression profile of Het sham samples compared to WT sham samples revealed 20 genes to be up-regulated and 23 down-regulated (Figure 25, Table 8). The genes that were differentially regulated had various functions, and no specific pathway was determined to be overly affected by these changes. Some specific genes that were altered in Het sham compared to WT sham included an up-regulation of calcipressin-1 (1.5 fold-increased in Het sham vs. WT sham, and 1.7 fold-increased in Het TAC vs. WT TAC), which was also
significantly up-regulated in Het TAC compared to WT TAC and a similar down-regulation of atrial myosin regulatory light chain. Calcipressin-1 has anti-hypertrophic effects by negatively regulating calcineurin signaling.

Comparison of Het TAC gene expression with WT TAC yielded 43 up-regulated and 20 down-regulated genes (Figure 26, Table 9 and 10) that were significantly changed compared to WT TAC. Some of these genes clustered into specific pathways related to skeletal muscle tissue development (2-fold increase in follistatin-related precursor), immune response (up-regulation of cytokine receptors), and fibrotic pathways (up-regulation of keratin and fibroblast growth factors). The hypertrophic marker NPPA was significantly up-regulated in Het TAC compared to WT TAC, consistent with qPCR data discussed previously. Also, several pathways were significantly enriched with genes down-regulated in Het TAC compared to WT TAC including reduction in pathways promoting transcription, and reduction in cell signaling pathways (0.4 fold expression in TNF, 0.3-fold expression in ATF-3, and 0.2-fold expression of Fos in Het vs. WT TAC). Furthermore, examining the changes between Het sham and WT sham transcript shows several altered extracellular matrix remodeling genes such as thrombospondin-1 and a disintegrin and metalloprotease-like extracellular protease. In order to confirm the results on the RNA-Seq data, qPCR was used to measure levels of genes identified by RNA-Seq with high, medium, and low levels of expression (Figure 27).
In addition to comparing total levels of gene expression between groups, identification of RNA splice variants is also possible with this technique. RNA-Seq data was consistent with qPCR data from both four and 12 weeks, as well as from baseline samples and further confirmed that Het hearts express only ~60% as much *MYBPC3* transcript as WT (Figure 14). From this reduced amount of total transcript in the Het hearts, both Het sham and Het TAC samples showed that *MYBPC3* transcript expressed from the truncated allele comprised approximately 10% of total transcript. Also, TAC caused a ~20% increase in *MYBPC3* transcript compared to Het sham levels, with an equal increase in both wild-type and truncated allele. This data indicates that *MYBPC3* expression is not able to increase much beyond its baseline level in Het hearts in response to hypertrophic remodeling. The data also shows that the truncated allele is expressed, although it is expressed as a minor component of total *MYBPC3* transcript level.
Figure 25. Heat Map of Genes Differentially Regulated in Het Sham vs. WT Sham.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Up-Regulated in Het Sham vs. WT Sham</th>
<th>Normalized Counts per Million</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrna2</td>
<td>neuronal acetylcholine receptor subunit alpha-2</td>
<td>WT Sham: 1.03, Het Sham: 3.15</td>
<td>TAC: 2.36, Het TAC: 1.40</td>
</tr>
<tr>
<td>Nr4a3</td>
<td>nuclear receptor subfamily 4 group A member 3</td>
<td>WT Sham: 4.46, Het Sham: 12.82</td>
<td>TAC: 2.59, Het TAC: 1.18</td>
</tr>
<tr>
<td>Zbtb16</td>
<td>zinc finger and BTB domain-containing protein</td>
<td>WT Sham: 53.98, Het Sham: 123.30</td>
<td>TAC: 47.12, Het TAC: 47.41</td>
</tr>
<tr>
<td>Map3k6</td>
<td>mitogen-activated protein kinase kinase kinase</td>
<td>WT Sham: 8.14, Het Sham: 17.96</td>
<td>TAC: 5.89, Het TAC: 6.56</td>
</tr>
<tr>
<td>Atf3</td>
<td>cyclic AMP-dependent transcription factor ATF-3</td>
<td>WT Sham: 11.82, Het Sham: 25.10</td>
<td>TAC: 9.82, Het TAC: 3.65</td>
</tr>
<tr>
<td>Fkbp5</td>
<td>peptidyl-prolyl cis-trans isomerase FKBP5</td>
<td>WT Sham: 16.19, Het Sham: 34.32</td>
<td>TAC: 13.43, Het TAC: 12.29</td>
</tr>
<tr>
<td>ASK2</td>
<td>apoptosis signal-regulating kinase 2</td>
<td>WT Sham: 3.94, Het Sham: 8.29</td>
<td>TAC: 3.06, Het TAC: 3.14</td>
</tr>
<tr>
<td>Ms4a6b</td>
<td>membrane-spanning 4-domains subfamily A member</td>
<td>WT Sham: 2.83, Het Sham: 5.68</td>
<td>TAC: 3.22, Het TAC: 2.92</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>cytochrome P450 2B10</td>
<td>WT Sham: 3.94, Het Sham: 7.91</td>
<td>TAC: 2.98, Het TAC: 2.30</td>
</tr>
<tr>
<td>Thbs1</td>
<td>thrombospondin-1</td>
<td>WT Sham: 20.91, Het Sham: 40.00</td>
<td>TAC: 24.58, Het TAC: 10.27</td>
</tr>
<tr>
<td>Adamt9</td>
<td>disintegrin-like metalloprotease</td>
<td>WT Sham: 15.17, Het Sham: 28.02</td>
<td>TAC: 15.86, Het TAC: 16.05</td>
</tr>
<tr>
<td>Ptkna2</td>
<td>plexin-A2 precursor</td>
<td>WT Sham: 37.10, Het Sham: 63.64</td>
<td>TAC: 41.38, Het TAC: 37.87</td>
</tr>
<tr>
<td>Arsb</td>
<td>arylsulfatase B precursor</td>
<td>WT Sham: 440.67, Het Sham: 751.29</td>
<td>TAC: 347.25, Het TAC: 357.39</td>
</tr>
<tr>
<td>PGC-1v</td>
<td>PPAR gamma coactivator variant</td>
<td>WT Sham: 95.79, Het Sham: 161.53</td>
<td>TAC: 62.74, Het TAC: 64.35</td>
</tr>
<tr>
<td>Ppargc1a</td>
<td>peroxisome proliferator-activated receptor gamma</td>
<td>WT Sham: 95.79, Het Sham: 161.53</td>
<td>TAC: 62.74, Het TAC: 64.35</td>
</tr>
<tr>
<td>Fam107a</td>
<td>downregulated in renal cell carcinoma</td>
<td>WT Sham: 12.34, Het Sham: 20.65</td>
<td>TAC: 6.52, Het TAC: 8.14</td>
</tr>
<tr>
<td>Gm12250</td>
<td>predicted gene 12250</td>
<td>WT Sham: 22.62, Het Sham: 36.24</td>
<td>TAC: 3.30, Het TAC: 3.20</td>
</tr>
<tr>
<td>Scd4</td>
<td>stearoyl-CoA desaturase 4</td>
<td>WT Sham: 12.08, Het Sham: 19.19</td>
<td>TAC: 13.86, Het TAC: 9.20</td>
</tr>
<tr>
<td>Rnf144b</td>
<td>E3 ubiquitin-protein ligase RNF144B</td>
<td>WT Sham: 34.96, Het Sham: 55.12</td>
<td>TAC: 35.02, Het TAC: 32.93</td>
</tr>
<tr>
<td>Dusp1</td>
<td>dual specificity protein phosphatase 1</td>
<td>WT Sham: 91.96, Het Sham: 56.12</td>
<td>TAC: 159.10, Het TAC: 94.59</td>
</tr>
<tr>
<td>Cyp26b1</td>
<td>cytochrome P450, family 26, subfamily b</td>
<td>WT Sham: 8.40, Het Sham: 3.92</td>
<td>TAC: 9.50, Het TAC: 6.62</td>
</tr>
<tr>
<td>Hba-a2</td>
<td>hemoglobin alpha, adult chain 2</td>
<td>WT Sham: 937.46, Het Sham: 399.67</td>
<td>TAC: 939.10, Het TAC: 835.18</td>
</tr>
<tr>
<td>Dkk3</td>
<td>dickkopf-related protein 3 precursor</td>
<td>WT Sham: 4.03, Het Sham: 1.69</td>
<td>TAC: 5.88, Het TAC: 8.86</td>
</tr>
<tr>
<td>Apol1</td>
<td>apolipoprotein L domain-containing protein 1</td>
<td>WT Sham: 35.73, Het Sham: 14.59</td>
<td>TAC: 63.61, Het TAC: 31.36</td>
</tr>
<tr>
<td>Egr1</td>
<td>early growth response protein 1</td>
<td>WT Sham: 200.84, Het Sham: 63.87</td>
<td>TAC: 332.17, Het TAC: 117.65</td>
</tr>
<tr>
<td>Lrrc55</td>
<td>leucine-rich repeat-containing protein 55</td>
<td>WT Sham: 2.66, Het Sham: 0.84</td>
<td>TAC: 2.59, Het TAC: 3.03</td>
</tr>
<tr>
<td>Gm16517</td>
<td>tubulin polyglutamylase complex subunit 1</td>
<td>WT Sham: 2.83, Het Sham: 0.77</td>
<td>TAC: 2.28, Het TAC: 3.70</td>
</tr>
<tr>
<td>Tnf</td>
<td>tumor necrosis factor</td>
<td>WT Sham: 2.31, Het Sham: 0.61</td>
<td>TAC: 5.10, Het TAC: 2.08</td>
</tr>
<tr>
<td>Fos</td>
<td>proto-oncogene c-Fos</td>
<td>WT Sham: 18.94, Het Sham: 3.07</td>
<td>TAC: 36.44, Het TAC: 7.74</td>
</tr>
<tr>
<td>Myl7</td>
<td>myosin regulatory light chain 2, atrial</td>
<td>WT Sham: 46.44, Het Sham: 5.99</td>
<td>TAC: 0.86, Het TAC: 0.73</td>
</tr>
</tbody>
</table>

Table 8. Genes Up and Down Regulated in Het Sham vs. WT Sham.
Figure 26. Genes Differentially Regulated in Het TAC vs. WT TAC.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>WT Sham</th>
<th>Het Sham</th>
<th>WT TAC</th>
<th>Het TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psca</td>
<td>prostate stem cell antigen precursor</td>
<td>0.00</td>
<td>0.00</td>
<td>0.24</td>
<td>21.94</td>
</tr>
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<td>Spon2</td>
<td>spondin-2 precursor</td>
<td>1.97</td>
<td>1.54</td>
<td>0.55</td>
<td>3.42</td>
</tr>
<tr>
<td>Arc</td>
<td>activity-regulated cytoskeleton-associated</td>
<td>1.29</td>
<td>1.38</td>
<td>2.12</td>
<td>8.75</td>
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<td>CRLM3</td>
<td>cytokine receptor like molecule 3</td>
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<td>0.71</td>
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<td>0.61</td>
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<td>1.02</td>
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<td>S100a9</td>
<td>protein S100-A9</td>
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<td>6.37</td>
<td>3.06</td>
<td>9.76</td>
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<td>Pdlim3</td>
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<td>2.98</td>
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<td>9.83</td>
<td>9.11</td>
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<td>292.81</td>
<td>2631.20</td>
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<td>calcipressin-1</td>
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<td>132.40</td>
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<td>28.33</td>
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<td>nicalin precursor</td>
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<td>Tspo</td>
<td>translocator protein</td>
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<tr>
<td>Zmym6</td>
<td>Zmym6 protein</td>
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</tr>
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<td>Dkk3</td>
<td>dickkopf-related protein 3 precursor</td>
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<td>1.69</td>
<td>5.58</td>
<td>8.86</td>
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<td>2.15</td>
<td>8.72</td>
<td>13.86</td>
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<td>semaphorin-3F</td>
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<td>Protein associated with Tlr4</td>
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</tr>
<tr>
<td>Arghap27</td>
<td>rho GTPase-activating protein 27</td>
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<td>7.14</td>
<td>6.83</td>
<td>10.72</td>
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<td>7.68</td>
<td>7.70</td>
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<td>U6 snRNA-associated Sm-like protein LSm6</td>
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<td>8.44</td>
<td>9.74</td>
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<td>Pura</td>
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<td>64.49</td>
<td>64.31</td>
<td>98.58</td>
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<td>7.06</td>
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<td>P16</td>
<td>peptidase inhibitor 16</td>
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<td>22.57</td>
<td>42.01</td>
<td>63.68</td>
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<td>24.11</td>
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<td>12.01</td>
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<td>Supt3h</td>
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<td>7.83</td>
<td>8.64</td>
<td>13.02</td>
</tr>
<tr>
<td>Gpx1</td>
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**Table 9. Genes Up-Regulated in Het TAC vs. WT TAC.**
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>WT Sham</th>
<th>Het Sham</th>
<th>WT TAC</th>
<th>Het TAC</th>
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<tr>
<td>Dusp1</td>
<td>dual specificity protein phosphatase 1</td>
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<td>56.12</td>
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<td>Ccdc63</td>
<td>coiled-coil domain-containing protein 63</td>
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<td>Kcnv2</td>
<td>potassium voltage-gated channel subfamily V</td>
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<td>Ier2</td>
<td>immediate early response gene 2 protein</td>
<td>15.59</td>
<td>7.22</td>
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<td>11.67</td>
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<tr>
<td>Rn4.5s</td>
<td>4.5S RNA</td>
<td>22.62</td>
<td>10.82</td>
<td>10.99</td>
<td>5.78</td>
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<td>Ddx3y</td>
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<td>0.00</td>
<td>47.98</td>
<td>25.19</td>
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<td>ABC transporter A subfamily member</td>
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<td>BC157982</td>
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<td>Tnf</td>
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<td>0.61</td>
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<td>3.07</td>
<td>36.44</td>
<td>7.74</td>
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</table>

Table 10. Genes Down-Regulated in Het TAC vs. WT TAC.
Levels of transcript were assessed by qPCR to confirm the findings of RNA-Seq using primers that recognized high-copy number genes with 100+ counts/million reads (Fhl1, Rcan1), medium copy number genes with 20 – 100 counts/million reads (Tgfb2, Pi16), and low copy number genes with <1 – 20 counts/million reads (Atf3, Fos, Gss). Relative gene expression in qPCR normalized against GAPDH expression. N=4 for qPCR experiments. * P<0.05 vs. Sham, # P<0.05 vs. WT, † P<0.001 false discovery rate test sham vs. TAC, ‡ P<0.001 false discovery rate test WT vs. Het.

Figure 27. Validation of RNA-Seq Reads with qPCR.
5.4 Discussion

In this study I aimed to determine if heterozygous carriers of MYBPC3 truncation mutations are predisposed to more severe hypertrophy following cardiovascular stress induced by TAC. These experiments were also designed to interrogate the effect of haploinsufficiency of MYBPC3 on the development of cardiac dysfunction. Previous work using this strain of mouse has shown that the heterozygous animal has a normal cMyBP-C compliment, despite reduced transcript levels, with some dysfunction in force development and diastolic function (McConnell, Jones et al. 1999, Barefield, Kumar et al. 2014). Other heterozygous MYBPC3 truncation mutation models do show a reduction in cMyBP-C and phenotypes showing more profound dysfunction than what has been shown in this model (Harris, Bartley et al. 2002, Vignier, Schlossarek et al. 2009). Therefore, we aimed to determine if additional stress would unmask haploinsufficiency of MYBPC3 resulting in reduced cMyBP-C levels and worsened prognosis. The results of this study show various impairments in the heterozygous genotype following stress, suggesting that these mice do exhibit a differential response to TAC induced pressure-overload stress. Furthermore, we have identified a transient reduction in cMyBP-C levels that may play a role in worsening of cardiac function.

The results from this study show that WT and heterozygous mice were subjected to an equal amount of pressure overload as assessed by transverse aortic blood flow velocity. However, HW:BW measurements show that Het hearts
were significantly more hypertrophied than WT 12 weeks following TAC, while both TAC groups showed significant hypertrophy compared to sham control. Additional evidence for the hypertrophic phenotype and verification of the TAC procedure via qPCR showed significantly elevated transcript levels of MYH7 and NPPA in both TAC groups compared to Sham, but did not show any changes between WT and Het TAC hearts. These findings were supported by measurement of protein levels of β-MHC that show a similar significant increase in both TAC groups compared to sham. This data concludes that Het hearts develop more robust hypertrophy following pressure-overload stress.

Despite the significantly heavier heterozygous hearts, left ventricular septum and posterior wall thickness were not significantly altered between WT and Het following TAC. In both TAC groups internal diameter was significantly increased at peak systole at four weeks, suggesting contractile deficits which are reflected in the reduced ejection fraction, whereas at 12 weeks diastolic internal diameter was significantly increased compared to sham, suggesting the onset of dilation. Importantly a significant decrease in contractile function was observed in the Het mice compared to WT at both four and 12 weeks following TAC, with reduced ejection fraction. These reductions were in addition to already significantly reduced systolic function in both WT and Het TAC compared to their sham controls at both time points. These findings suggest that the heterozygous heart is more susceptible to the onset of hypertrophy and dysfunction following cardiac stress.
MYBPC3 transcript and cMyBP-C levels were assessed to determine if haploinsufficiency occurred following cardiac stress. Levels of MYBPC3 transcript measured by qPCR in Het sham and TAC mice were approximately 40% lower compared to respective WT controls, but did not differ significantly between Het sham and Het TAC groups. This alteration in transcript is similar to the reduced transcript level in the Het mouse under naïve conditions compared to WT (McConnell, Jones et al. 1999, Barefield, Kumar et al. 2014). These findings suggest that there is no significant increase in MYBPC3 expression in the Het following TAC. Also, the level of MYBPC3 transcript was not elevated in WT TAC compared to WT sham at four or 12 weeks post-surgery, suggesting that either the hypertrophic response was reduced at this time point, or that WT mice do not require additional MYBPC3 expression during hypertrophy.

The analysis of cMyBP-C levels in the myofilaments of each group by western blot showed that there is a significant reduction in cMyBP-C at the four week time point, and this reduction occurs to the same extent in both sham and TAC operated heterozygous mice. However, 12 weeks after surgery cMyBP-C levels return to WT levels in heterozygous myofilaments, with no significant changes between groups, similar to baseline. This suggests that cMyBP-C is transiently reduced from a normal stoichiometry at baseline, and this reduction is recovered sometime between four and 12 weeks. The fact that this reduction in protein occurs in both TAC and sham heterozygous mice suggest that hypertrophy is not responsible for this decrease. The reduced cMyBP-C content
may be due to alterations in protein turnover in response to surgical inflammatory stress, but this remains enigmatic. Phosphorylation of cMyBP-C also differs with time, whereas phosphorylated species of cMyBP-C tended to be higher at four weeks, by 12 weeks they were unchanged or reduced compared to their sham controls. This is likely due to increased β-adrenergic signaling early in hypertrophy, causing hyperphosphorylation of cMyBP-C and cTnI at four weeks, and reduced PKA-target phosphorylation has been well established in the development of HF (Tong, Gaffin et al. 2004). Unexpectedly, Het sham hearts at four weeks showed a trend towards elevated phosphorylated cMyBP-C, which provides a confounding factor for statistical analysis. This alteration in phosphorylation status at Ser-273 and S-282 of cMyBP-C was not seen under baseline conditions and may be associated with other changes seen in Het sham at four weeks that were not seen at baseline, such as decreased cMyBP-C content.

The results of the force-pCa experiments provide several interesting insights into the dysfunction occurring in heterozygous cardiomyocytes. We have shown in the baseline experiments that cardiomyocytes from heterozygous hearts have reduced maximal force generation while retaining normal cMyBP-C levels (Barefield, Kumar et al. 2014). Results from the current study show expected deficits in force generation in both TAC groups. However, impaired force generation persists in Het sham cells, consistent with the previous study. Heterozygous hearts also show blunted length-dependent increases in force
development at four weeks, and this alteration is absent by 12 weeks. This suggests that despite the greater impairment of force generation 12 weeks post-surgery, the heterozygous genotype regains the ability to increase force generation following stretch. One notable difference in the Het cells between four and 12 weeks is their cMyBP-C content recovery. The role of cMyBP-C in length-dependent activation is a new and active area of inquiry, and the data from this experiment demonstrate the potential for cMyBP-C to regulate stretch sensitivity of cardiomyocytes.

Other notable alterations occur in Ca$^{2+}$ sensitivity of force development, with both heterozygous groups showing a significant Ca$^{2+}$ sensitization at short sarcomere lengths at four weeks as well as a blunted length-dependent increase in sensitivity at four weeks. This deficit in length dependent Ca$^{2+}$ sensitization was mildly attenuated by 12 weeks. These observations are again different from the data obtained at baseline, where Het cardiomyocytes did not show any significant difference in Ca$^{2+}$ sensitivity or length-dependent activation. Understanding why this change occurs is difficult, as at four weeks cMyBP-C phosphorylation is altered with normal cTnl phosphorylation in the Het sham cardiomyocytes. However, at 12 weeks Het cardiomyocytes show normal cMyBP-C levels and phosphorylation, but do show an increase in cTnl phosphorylation which would suggest a Ca$^{2+}$ desensitization.

The RNA-Seq experiment was performed to assess changes in the ubiquitin proteasome system and nonsense-mediated RNA degradation
pathways, as well as to identify novel changes in gene expression the Het hearts in order to identify alterations that occur as a result of haploinsufficiency of *MYBPC3*. The hypothesis that alterations in nonsense-mediated RNA degradation or the ubiquitin-proteasome system cause dysfunction in the Het was not supported by this data, as no changes in proteasome associated genes or in pathways associated with RNA degradation were identified. Interestingly, one of the common Het up-regulated genes in both sham and TAC compared to WT controls was calcipressin, the anti-hypertrophic regulator of calcineurin. Also interesting were the alterations observed in Het sham compared to WT sham. Specifically, altered regulation was observed in several extracellular matrix remodeling genes including the up-regulation of thrombospondin and matrix metaloproteases. These observations indicate a role for remodeling prior to the onset of hypertrophy, which may suggest a future direction looking at the role of fibroblasts and infiltrating immune cells in the early stages of pathology in Het hearts.

The results of this study clearly demonstrate that following pressure-overload stress heterozygous *MYBPC3* truncation mutation carriers develop more severe hypertrophy with worsened cardiac function compared to wild type. These deficits can be explained by a transient reduction in cMyBP-C levels in the heart due to haploinsufficiency in the heterozygote. However, due to the evidence that under naïve conditions these mice have subtle defects in
cardiomyocyte contractility, it is likely that another mechanism is partly responsible for these impairments.

The data from these experiments confirm our hypothesis that heterozygous mice are at a greater risk for cardiomyopathy following stress, and this is likely due to the initial dysfunction under naïve conditions which is exacerbated following stress. It remains unclear if reduced cMyBP-C protein level precedes the development of hypertrophy or follows it. We have shown here that heterozygous mice have a reduction in cMyBP-C levels under both Sham and TAC conditions four weeks post-surgery. This is an interesting observation and may suggest that stress from sham surgery alone, which is a considerably invasive surgical procedure, is able to exert a stress that causes reduced cMyBP-C expression. Whether this is mediated through inflammatory pathways or disruptions in protein turnover mechanisms remains unclear.

In the human population heterozygous HCM carriers typically develop symptomatic HCM later in life suggesting a change that triggers the onset of disease. Although samples from symptomatic HCM carriers have shown a reduction in total cMyBP-C levels, it is unknown if asymptomatic HCM mutation carriers have a deficit of cMyBP-C. Understanding whether reduced cMyBP-C is a cause or result of the onset of HCM in heterozygous truncation mutant carriers has implications on the mechanism of pathogenesis and targets for therapy.
CHAPTER SIX

TURNOVER OF cMyBP-C

6.1 Introduction

Under naïve conditions heterozygous MYBPC3 mice show normal levels of cMyBP-C yet also exhibit functional impairments at the single cell and whole-organ level (Barefield, Kumar et al. 2014). The only reported molecular deficit in these Het hearts is that MYBPC3 gene expression level is significantly lower than WT. The mechanism by which reduced gene dosage may lead to the onset of this observed dysfunction may provide a novel and early therapeutic target for carriers of this type of mutation. Therefore, it is important to determine what mechanisms are responsible for this dysfunction as they may be responsible for the early development of functional impairments and the onset of HCM.

To assess the mechanism of Het dysfunction two approaches were attempted. The first was to look at the effect of small reductions in cMyBP-C level, as had been shown in human MYBPC3 carrier hearts. Assessing this function requires knocking down cMyBP-C in a functioning adult cardiomyocyte, in order to measure functional impairments caused by reduced cMyBP-C.
However, turnover of cMyBP-C proved to be too slow in adult rat cardiomyocytes to use this system.

The second avenue of inquiry builds on the efforts to knock down MYBPC3. Sarcomere proteins assemble with well-defined stoichiometry and have a defined turnover rate. Large proteins that are integral to sarcomere structure have very long half-life (15 days for myosin heavy chain), with smaller proteins typically having shorter half-life in the sarcomere (3-5 days for the troponins) (Boateng and Goldspink 2008). As demonstrated in the attempted knock-down experiments, cMyBP-C has a long half-life. However, examining protein turnover in the context of altered MYBPC3 expression, as seen in the Het mice, may provide a mechanism for the dysfunction observed under naïve conditions and post-TAC.

As exogenous cMyBP-C has been shown to exchange passively with endogenous cMyBP-C in *in vitro* exchange assays, I hypothesized that synthesis of additional cMyBP-C will increase turnover of sarcomeric cMyBP-C. Turnover is assessed using $^{35}$S labeled amino acid media to label all proteins synthesized in a certain time frame followed by viral overexpression or knock-down of MYBPC3 to modulate gene dosage and protein turnover. Data supporting this hypothesis was expected to show that knocking down MYBPC3 transcript causes new cMyBP-C synthesis to reduce, resulting in less new protein exchanging with endogenous labeled protein. Also, MYBPC3 overexpression was hypothesized to
increase the amount of cMyBP-C synthesized, causing additional exchange and a reduction in the amount of labeled protein present in the sarcomere.

6.2 Results

6.2.1 Knock-Down of MYBPC3

In order to study the effect of altered cMyBP-C stoichiometry in the sarcomere, MYBPC3 was knocked down in adult rat ventricular cardiomyocytes and neonatal rat cardiomyocytes. To accomplish this, lipid transfection of siRNA or adenoviral delivery of siRNA was used to knock down cMyBP-C. The initial experimental design called for performing functional measurements of unloaded shortening using an IonOptix system following cMyBP-C knock-down.

Rat ventricular cardiomyocytes were isolated as described in the methods section and were treated with adenovirus expressing siRNA or an adenovirus with an empty vector, and were cultured for up to 48 hours. Protein and mRNA were isolated as described. Levels of MYBPC3 were significantly reduced in adenovirus-treated cardiomyocytes compared to vector-treated, normalized to GAPDH expression; however, protein levels were not significantly altered at this time point (Figure 28). As the function and viability of adult cardiomyocytes are already in decline at 48 hours, it was not deemed prudent to continue these experiments in this system. The resistance of cMyBP-C to knockdown is not surprising due to the long half-life of sarcomere proteins.
In order to assess how long it would take to knock-down cMyBP-C, neonatal rat cardiomyocytes were cultured with siRNA for seven days to knock down MYBPC3 expression. In this model, cMyBP-C levels normalized to actin were significantly reduced in the siRNA adenovirus treated cells compared to empty vector at seven days post-infection (Figure 28). These findings reinforce the notion that cMyBP-C would be difficult to knock down within 48 hours in order to study functional effects of reduced protein level in adult cardiomyocytes.
Figure 28. Knock-Down of MYBPC3.

A, Transcript level of MYBPC3 normalized to GAPDH expression assessed by qPCR shows that at two days post infection adult rat cardiomyocyte MYBPC3 expression was virtually abolished. B, Despite this efficient transcript knock-down levels of cMyBP-C normalized to α-tropomyosin were unchanged in adult rat cardiomyocytes 48 hours post-infection. C, However, cMyBP-C levels normalized to α-tropomyosin were significantly reduced 7 days post-infection in neonatal rat cardiomyocytes. UI: uninfected; EV: empty vector; GPF: green fluorescent protein expressing; siRNA: MYBPC3 siRNA. (N=4) *P<0.05, t-test.
6.2.2 Determination of Gene Expression on Protein Turnover

In order to determine if gene dosage plays a role in turnover of cMyBP-C adenoviral knock-down and overexpression of MYBPC was performed to reduce and increase the gene dosage, respectively (described fully in the methods section). These studies were originally performed in neonatal rat cardiomyocytes with a one day pulse of $^{35}$S labeled Met/Cys amino acids, as it was assumed long-culture periods would be required for proper turnover. Labeling of myofilament proteins with 12 hours of $^{35}$S media was successful and this labeling persisted for the length of the culture period (four days). Initial experiments demonstrated that the MYBPC3 overexpressing cells showed an increase in a larger labeled band of cMyBP-C (not shown), which indicates that new synthesis of labeled protein was occurring following removal of the labeled media, potentially resulting from liberation of labeled Met/Cys amino acids from natural protein degradation and recycling mechanisms. Therefore, following the initial pulse of labeled media, a wash-out period was added prior to treating with virus. Isolation of myofilaments from these cells showed good incorporation of the label; however, due to the small amount of myofilaments present in neonatal cardiomyocytes, levels of cMyBP-C were difficult to measure in any group (Figure 24). Due to this complication, this pulse-chase protocol has been adapted for rat ventricular cardiomyocytes which were shown to be labeled with short (1-4 hour) pulses (data not shown). Virally-mediated turnover experiments are currently ongoing.
Adult rat cardiomyocytes were treated either with empty adenovirus (empty), adenovirus containing shRNA for MYBPC3 (shMYBPC3), or carrying MYBPC3. Myofilament proteins from neonatal cells show low levels of myosin and actin compared to adult, with cMyBP-C too low to accurately quantify.

Figure 29. Phosphor-Storage Screen Image of 35S Labeled Cardiomyocytes.
6.2.3 **MYBPC3 Gene Dosage Directly Alters Force Development**

Despite the technical difficulties in calculating protein turnover by pulse-chase, the hypothesis that MYBPC3 gene expression has a direct link on cardiomyocyte contractility can be tested using a transgenic mouse model. A mouse model carrying transgenically expressed MYBPC3 crossed into the (t/t) background with no endogenous protein was previously generated and available for these experiments, named the WT(t/t) mouse. This mouse has two truncated alleles that as previously shown do not produce any protein. The transgene encodes MYBPC3 and expresses normal cMyBP-C which rescues the dilated phenotype observed in the t/t mouse. Also, the MYBPC3 is expressed approximately 5-fold higher than non-transgenic WT mice (Sadayappan Lab, Data in submission). Although cMyBP-C is overexpressed, these mice are normal, with cMyBP-C stoichiometry equal to non-transgenic WT. Using this model it is possible to test whether the truncated allele alone is sufficient for the reduction in force observed in the Het mice under normal conditions, as the WT(t/t) mouse has two of these alleles. Also, the WT(t/t) and the Het mice both have normal cMyBP-C levels. If reduced gene dosage is able to directly cause the reduction in force observed in the Het cardiomyocytes, then the WT(t/t) should show no significant reduction. However, if the truncated allele acts as a poison polypeptide, then the WT(t/t) should show significant dysfunction compared to non-transgenic WT controls.
To test this hypothesis force-pCa measurements were performed on WT(t/t) cardiomyocytes which were compared to non-transgenic WT and Het values. The results of this experiment demonstrated that transgenic WT cardiomyocytes show normal maximal force development despite carrying two truncated alleles, with Het cardiomyocytes showing significantly reduced force generation compared to both transgenic and non-transgenic WT groups.
Figure 30. Gene Dosage and Cardiomyocyte Force Generation.

WT(NTG) and WT(TG) cardiomyocytes showed equal maximal force generation at both sarcomere lengths. Het cardiomyocytes generated a significantly lower amount of force compared to both NTG and TG WT groups. N=3 hearts, 3 cells/heart, * P<0.05 vs. WT(NTG); # P<0.05 vs. WT(TG).
6.3 Discussion

The experiments described in this section aimed to explain how cMyBP-C content in the sarcomere contributes to the development of force in single cardiomyocytes. It became clear that knocking down cMyBP-C in cultured rat cardiomyocytes was not a viable approach as the time required to reduce the level of cMyBP-C was too long to perform the experiments originally proposed. However, the long-lived nature of cMyBP-C in the sarcomere is an interesting property that could be regulated by expression levels of MYBPC3, and therefore may be a mechanism by which to explain the dysfunction observed under baseline conditions.

Attempting to probe this mechanism with a pulse-chase assay was unable to be completed. However, assessing the relationship between transcript level and cardiomyocyte function was possible using a transgenic mouse model overexpressing WT MYBPC3 in the t/t background. The results of that experiment show that the presence of the t/t alleles do not cause contractile dysfunction as observed in the Het cardiomyocytes. This observation provides additional evidence against the poison polypeptide hypothesis that has remained persistent in the literature despite lack of evidence for truncated protein expression. Also, with WT transgenic, non-transgenic, and Het sarcomeres all containing the same amount of cMyBP-C, the results from this experiment reinforce the results from baseline analysis showing that there is something fundamentally wrong with Het cardiomyocytes, independent of cMyBP-C content.
CHAPTER SEVEN

FUTURE DIRECTIONS AND CONCLUSIONS

The data reported in this work support the initial hypothesis that carriers of MYBPC3 truncation mutations are predisposed to the development of cardiomyopathy due to subtle deficits under normal conditions and exacerbated pathology following stress. While these findings add insight into the current state of this area of research, several additional questions have arisen from this line of inquiry regarding the mechanisms underlying these observed functional deficits.

7.1 Altered Function with Normal Protein Level

As previously discussed, the observation that naïve Het mice exhibited contractile abnormalities was surprising, given the normal cMyBP-C stoichiometry observed in those hearts. In order to explain this observation, additional work is required to assess whether 1) the truncated allele produces a potentially pathogenic fragment that has yet to be identified; or 2) decreased transcript levels of MYBPC3 in the Het cardiomyocyte causes a deficit that is not related to absolute protein stoichiometry, such as altered turnover and/or post-translational modifications. While the work proposed and completed in this
project focused on evaluating the outcome of these mice following stress, the questions that have been raised from these results provide some interesting avenues for future research.

**7.2 Haploinsufficiency vs. Poison Polypeptide**

The mechanisms by which these heterozygous truncation mutations cause dysfunction have been hotly debated and have focused primarily on either haploinsufficiency or a poison polypeptide model. The results of this study, taken together with other recent findings, suggest that the poison polypeptide is unlikely to be responsible. Fragments of mutant cMyBP-C have rarely been identified in human patients or mouse models of MYBPC3 truncation mutant carriers, but despite this lack of evidence the possibility of a small amount of mutant protein exerting a pathological effect remains. The lack of identified protein fragments is certainly strong evidence against this model, but until recently it has been unclear how much fragmented cMyBP-C protein would be required to cause contractile deficits.

In a recent paper, Witayavanitkul et al. administered recombinant N'-terminal fragments of cMyBP-C to permeabilized human myocardial tissue (Witayavanitkul, Ait Mou et al. 2014). The rationale for this study was to determine the pathological potential of the cleavage and release of the N'-terminal residues of cMyBP-C following calpain cleavage that have been observed by the Sadayappan lab. In the Witayavanitkul et al. study, reduction in
force was observed with an EC$_{50}$ of 4.41μM of cMyBP-C C0-C1f (the first two N'-terminal domains of cMyBP-C, with a short fragment of the M domain) and a maximal reduction in force at ~30μM. The concentration of cMyBP-C in the sarcomere is estimated to be ~15μM (Witayavanitkul, Ait Mou et al. 2014). Using this information to understand the heterozygous poison polypeptide model, it becomes clear that the amount of cMyBP-C fragment required to impair contractility is too high to have remained undetected in patient biopsies and mouse models. As this truncated protein or similar proteins have not been detected by Western blot, these observations are unlikely to be explainable by a poison polypeptide mechanism. However, these observations alone are not sufficient to distinguish between haploinsufficiency, alterations in signaling pathways, or dysfunction in RNA and protein degradation pathways.

Another potential explanation for dysfunction has been proposed over the years and has been recently studied by the Carrier lab (Schlossarek, Schuermann et al. 2012). This model suggests that misfolded proteins or mutant transcripts may overwhelm degradation pathways, causing dysfunction. The RNA-Seq experiments reported in this work were initially planned in order to study any underlying alterations in these pathways. However, analysis of this data did not show any alterations in Het hearts compared to WT in either the ubiquitin proteasome pathway or in genes encoding for RNA degradation pathways. However, improper protein degradation may still play a role in the
pathogenesis in these mutant carriers and certainly warrants additional investigation.

In the current work, decreased levels of cMyBP-C were shown at four weeks, with return to normal levels by 12 weeks. Clearly the temporal resolution of the experimental design used in this study is not sufficient to capture the time-course of reduction and restoration of cMyBP-C levels. Therefore, it can still be asked whether reduced cMyBP-C levels initiate hypertrophy, or if hypertrophy causes reduced cMyBP-C levels. Recently the Moss lab used an inducible knock-out MYBPC3 mouse model to show that reduced cMyBP-C initiates DCM (Chen, Patel et al. 2012). In this model, cMyBP-C levels are reduced to approximately 50% of untreated after 2 weeks, with an almost complete knock-out 8 weeks following tamoxifen induction. Despite the efficient knock-down, systolic function remained unaltered 20 weeks post-treatment although hypertrophy and diastolic dysfunction were evident (Chen, Patel et al. 2012). This supports the idea that reduced cMyBP-C can be causative for the development of hypertrophy. It remains to be seen whether this reduction of cMyBP-C is causative for HCM in human heterozygous MYBPC3 carriers.

7.3 Decreased Transcript as a Mechanism of Dysfunction

Following the observation that Het naïve mice showed dysfunction despite normal cMyBP-C levels, several experiments were performed to assess whether gene dosage could directly explain this phenomenon. I hypothesized that
reduced MYBPC3 gene expression causes reduced cMyBP-C production, which leads to slower protein turnover in the sarcomere. Following the observations that MYBPC3 transcript was decreased, with preserved cMyBP-C levels and a reduction in force generation in Het naïve cardiomyocytes, the role of gene dose on protein turnover was assessed. To assess this, a pulse-chase experiment was performed using $^{35}$S labeled amino acids in neonatal and adult rat cardiomyocytes. A consequence of reduced protein turnover which would increase protein half-life and could allow cMyBP-C to become more heavily modified by functionally deleterious PTMs, particularly irreversible oxidative modifications.

If gene dose regulation of protein turnover proves to be a viable mechanism for contractile dysfunction, it still remains to be seen how these changes cause alterations in cardiomyocyte function. One possibility and future direction is to examine whether expression levels of MYBPC3 influences the rate of protein turnover in the sarcomere, with the hypothesis that reduced expression would lead to a longer cMyBP-C half-life. This hypothesis is supported from the cardiomyocyte force generation data outlined in chapter six, where decreases in force generation in Het mice were reversed with overexpression of MYBPC3 despite the presence of truncated alleles in the genetic background. Recent evidence from the Solaro lab has shown that cMyBP-C and other sarcomere proteins can be post-translationally regulated with oxidative modifications such as glutathionylation (Patel, Wilder et al. 2013). As cMyBP-C has been well
established as a regulatory protein, these oxidative modifications may cause dysfunction. If cMyBP-C did have a longer half-life in the sarcomere in heterozygous MYBPC3 truncation carriers, these oxidative modifications may become more deleterious as new cMyBP-C is not produced in sufficient amounts. These oxidative modifications could interfere with native cMyBP-C incorporation in the sarcomere, its ability to be targeted by kinases, and the ability of cMyBP-C to interact with its N'-terminal regulatory targets. This mechanism by which reduced transcript leads to dysfunction would be supportive of the notion that haploinsufficiency is occurring and would provide an explanation for dysfunction with preserved cMyBP-C levels.

7.4 Mutations as Modifiers of Cardiovascular Disease

As HCM associated mutations are relatively common in the human population (1:500) there is a possibility that carriers of HCM associated mutations have hypertrophic comorbidities. Indeed, in a recent large-scale human genome screening, the incidence of HCM associated mutations was much higher in the human population than the predicted 1:500 frequency (Golbus, Puckelwartz et al. 2012). This is interesting in terms of diagnosis of HCM, and the detection of pathogenic mutations in patients with diabetes, hypertension, ischemic injury, or other cardiac pathologies.

As most of these morbidities are also relatively common in the human population (Go, Mozaffarian et al. 2013), there is a possibility that HCM mutation
carriers that develop cardiomyopathy also share one or more of these comorbidities. In this case, diagnosis of HCM may not be attributed to the mutation, as a more obvious cause of cardiomyopathy is evident. Therefore, genetic screening for HCM causing mutations in patients with an identifiable cause for hypertrophy may be clinically useful, especially if these comorbidities are predictive of more severe cases of HCM and require more aggressive treatment. An investigation of the genetic background of cardiomyopathy patients suffering from non-genetic causes may be beneficial to establish if there is an association between undetected mutations and disease severity.

7.5 Conclusion

The results of this study demonstrate that the pathophysiological response to cardiovascular stress is altered in heterozygous MYBPC3 truncation mutant carriers. Due to the high prevalence of similar mutations in the human population, understanding the mechanism by which these mutations cause pathology is imperative. In addition, characterization of the effects of common cardiovascular disease risk factors in this genetic background may provide an understanding of why this disease shows such variable penetrance in families carrying similar mutations, and what steps might be taken to minimize risk of developing cardiomyopathy in the human population.
CHAPTER EIGHT

METHODS AND MATERIALS

8.1 Mouse model of *MYBPC3* haploinsufficiency

For this study non-transgenic WT mice were used as well as a knock-in model generated in the Siedman lab (McConnell, Jones et al. 1999) carrying a truncation mutation in the *MYBPC3* gene with either homozygous (t/t) or heterozygous (+/t) genotypes. All mice were in the FVB/N background and all experiments used mice between 10-12 weeks of age, including the TAC experiments which used this age as the time period in which the surgery was conducted. All the experiments using animals detailed in this work were approved by the Institutional Animal Care and Use Committees at Loyola University Chicago and followed the policies of the *Guide for the Use and Care of Laboratory Animals* published by the National Institutes of Health.

8.2 In vivo TAC in Mice

This study used a transverse aortic constriction (TAC) model of pressure-overload hypertrophy. The surgical procedure was very generously taught to me by Jop Van Berlo, MD, PhD, from the lab of Jeff Molkentin, PhD at Cincinnati Children’s Hospital in Cincinnati, OH. Development of this technique required
considerable practice, and I was given an excellent chance to practice this surgery and validate my results while collaborating on a project for Rajesh Kishore, PhD at Northwestern University (Verma, Krishnamurthy et al. 2012).

**Preparation**

Mice were anesthetized in an induction chamber with 5% vaporized isoflurane with 100% oxygen. Animals were weighed and transferred to a nose cone in the preparation area where their fur from neck to the level of the 6-7th rib was shaved with an electric razor to expose the surgical site. The animal was then transferred to a nose cone on a heated surgical table (Harvard Apparatus 50-1239) in a supine position with the forelimbs restrained using 3M Durapore™ tape. The site of incision was cleaned three times using Betadine in ethanol and gauze pads.

**Intubation**

A cut down the midline was made between the level of the clavicle and the chin with a pair of scissors (Harvard Apparatus PY2 72-8442). The skin was gently retracted laterally, exposing the salivary glands. Using two forceps (Harvard Apparatus PY 72-8599) the salivary gland was gently blunt dissected down its midline, separating into a left and right lobe. The muscle layer covering the trachea was visible, and using the same forceps was blunt dissected down the midline exposing the trachea. The animal was repositioned for intubation and using a pair of curved forceps the trachea was isolated, without applying any force to the trachea, and an intubation tube (BD 20Ga Shielded IV Catheter
381534) with blunted needle guide was introduced into the trachea. Visualization of the trachea ensured proper intubation is successful, as accidental intubation of the esophagus is immediately detectable using this method. The intubation tube was then attached to a ventilator (Hugo Sachs 845) and the mouse was ventilated at a rate and volume that establishes proper chest inflation without the animal gasping. Ventilator settings were determined animal-to-animal, but a guide following a body weight range of 20g – 40g, with a tidal volume of 120µl – 240µl and respiratory rate of 148 – 124 breaths per minute was used to avoid over-ventilation.

**Thoracotomy**

With the animal positioned on the ventilator in a supine position with the animal's head oriented toward the surgeon, the limbs were taped down, with care taken that the intubation tube remained inserted at a proper depth. The midline cut through the skin was continued to the level of the 4-5th rib using scissors. The skin was spread away from the midline, exposing the ribs and chest wall. It was important that at this stage ribs 1-3 were visible along the sternum. A pair of blunt forceps was used to firmly grasp the left clavicle, lifting up slightly. A pair of angled spring scissors with a blunted lower blade (Fine Science Tools 15033-09) was inserted under the ribs, at the midline. A short cut was made with these scissors, deviating slightly to the left, cutting through the left clavicle and the 1st rib on the left side of the sternum. This cut was then extended parallel to and very close to the sternum. Staying along this course, the cut was continued up to
the third rib, but not through the third rib. It was imperative that this cut remained as close to the sternum as possible, and the third rib is not cut, as both of these errors typically resulted in massive blood loss. As this step was the most painful, the isoflurane was reduced from the initial 5% level to 2% following this step. The midline dissection of the muscle layer covering the trachea was continued, which opened the cavity containing the thymus under the first two ribs. Blunt retractors (custom made bent dental wire) were used to pull the ribs laterally. This exposed the trachea, the right and left carotid arteries, the thymus, and the jugular veins.

**Transverse Aortic Constriction**

The left and right lobes of the thymus were blunt dissected with forceps and spread apart to expose the aortic arch and the base of the heart. Depending on the strain of mouse, extensive cleaning of the aorta was required, as the thymus was occasionally strongly attached to the aorta. Failure to clean the aorta properly can change the diameter of the constriction and introduce variability. A pair of hooked fine point forceps (Harvard Apparatus PY2 72-8611) was introduced under that aorta, and then the tips were angled upwards so that the aorta was cradled in the curve of the forceps. A 4-6cm long piece of 7-0 silk (Teleflex 103-S), which was soaked in sterile saline prior to the procedure, was grasped by the hooked forceps, which are then carefully pulled back under the aorta. This resulted in the piece of silk positioned beneath the aortic arch between the left and right carotid arteries. A blunted 27 gauge needle (Harvard Apparatus PY2 72-2304) was positioned parallel to the transverse aorta and then
a knot was tied tightly around this needle and the aorta. If done properly the needle pulsed in pace with the heart. While grasping one of the free silk threads with a pair of forceps for counterweight, the needle was removed. Visually evaluating whether the pulsing of blood through the right carotid artery was more severe than the left carotid artery was used as a qualitative indication of the success of the TAC.

**Closing and Recovery**

The lobes of the thymus were put back together and pushed under the ribs, and the retracted muscle layer was moved back to the midline. A 4-0 silk suture connected to a curved taper point needle (Ethicon K871H) was used to pierce the right side of the chest wall, just inside the joining of the sternum with the right clavicle. The needle was then brought through the left chest wall, avoiding the jugular vein lateral to the incision site. The suture was drawn through and tied tightly to close the ribs. Additionally, a 6-0 silk suture (Cat K802H) was used to make one or two interrupted sutures on the muscle layer between the clavicle and the third rib. These sutures ensured that the chest was sealed. Under normal conditions, the lungs did not need any additional steps to re-inflate, as the ventilator maintained them in an inflated state throughout the procedure. The 6-0 suture was then used to close the skin using a continuous suture pattern. The incision site was washed again with Betadine in ethanol, and the animal was turned over to lay prone. Buprenorphine (Reckitt Benckiser Pharmaceuticals) at a dose of 0.5mg/kg and ampicillin (AuroMedics) at a dose of
50 mg/kg were administered subcutaneously behind the head. The isoflurane was turned off and the animal was allowed to regain ambulation before it was removed from the ventilator and extubated. Animals were kept in a cage half on a warming pad in an incubator with 100% oxygen treatment for 1-2 hours following surgery.

_Echocardiographic Verification of TAC Procedure_

Proper placement and execution of the TAC procedure was evaluated using echocardiography as soon as the animal was well enough to withstand another procedure (typically 1 week post-op was sufficient in wild-type mice). Blood flow velocity across the transverse aorta was measured using a Visual Sonics Vevo 2100 echocardiography system with a 13-24 MHz transducer (MS-250) normally used for large rodent echocardiography. By positioning the probe parallel to the long axis of the heart on the animal’s right side, a full view of the aortic arch and three major vessels was easily obtained. Using the power-Doppler imaging modality, blood flow velocities were measured. A normal mouse under baseline conditions or following sham surgery had a transverse aortic blood flow velocity between 600-900 mm/s, which correlated to a pressure gradient of 3 mmHg across the transverse aorta. In an animal with a successful TAC in place, this velocity was required to be >3000 mm/s, corresponding to a pressure gradient of 36 mmHg. These measurements were repeated longitudinally, and were performed at the terminal time point to ensure that the suture had remained tied throughout the experimental period.
8.3 Echocardiography

Echocardiography was performed using a VisualSonics Vevo 2100 imaging system (VisualSonics, FujiFilm, Toronto, Canada) with an MS550S 21-56 MHz or MS250 13-24 MHz imaging transducer. Under isoflurane anesthesia (5% for knock-down and 1.5% for sedation), the hearts were monitored for changes in structure and morphology. Animals were measured one day prior to surgery, and then monthly for four to 12 weeks following surgery. WT and Het mouse hearts were subjected to analyses using noninvasive M-mode echocardiography, pulse-wave Doppler imaging for measuring blood flow, and tissue Doppler imaging to measure mitral valve performance.

Chamber dimension and wall thickness during systole and diastole were measured using M-mode images. From these values, % fractional shortening (FS) (measured as LVID;d – LVID;s / LVID;d x 100) and % ejection fraction (EF) were determined. Power Doppler and Tissue Doppler analysis was used to measure diastolic function via the E/A and E’/A’ ratio. Using the MS250 probe transverse aortic velocity was measured, as previously discussed which determined the efficacy of the TAC procedure and was used as an inclusion criterion. Parasternal long axis images were used to measure strain, which was assessed using the Vevo2100 image processing.

8.4 Steady-state force measurements

All force-pCa experiments were performed by Mohit Kumar, MS in the de Tombe lab at Loyola University Chicago. Cells were prepared and analyzed for
force-pCa relationship as previously described (van der Velden, Papp et al. 2003, Barefield, Kumar et al. 2014). Frozen left ventricular tissue was homogenized and filtered through a 70μm cell strainer, followed by centrifugation at 120g for 1 minute at 4°C. The cells were skinned by re-suspending the pellet in relaxing solution (97.92 mM KOH, 6.24 mM ATP, 10 mM EGTA, 10 mM Na$_2$CrP, 47.58 mM Kprop, 100 mM BES and 6.54 mM MgCl$_2$) with 1% Triton-X100 and then incubating for 10-15 minutes at room temperature on a rocking table. The Triton was washed out by two centrifugation steps at 120g for 1 minute at 4°C with resuspension in relaxing solution. The skinned myocytes were transferred to a culture dish coated with 0.1% BSA. Using an inverted microscope (Leica DM IRB) under bright field at 40x magnification, skinned myocytes were attached to two metal micro-needles using UV-sensitive glue (Norland, Cranbury, NJ). The micro-needles were attached to a high-speed piezo translator (ThorLabs, Newton, NJ) and a force transducer. Myocytes were selected based on uniformity of the cell and clear striation patterns. Myocytes were perfused via a closely placed perfusion pipette with relaxing solution. Subsequent perfusion used a mixture of relaxing and activating solutions with varying calcium concentrations (pCa 10.0 – pCa 4.5) to measure force development at sarcomere lengths of 1.9 μm and 2.3 μm. Maximal activating calcium solution was administered at the start of the experiment to ensure proper cell attachment. Sarcomere length (SL) was measured using FFT analysis of video images using custom-made LabView software (National Instruments, Austin, TX). Force-pCa
curves were fit using a modified Hill equation \( \frac{P}{P_o} = \frac{[Ca^{2+}]^n}{(k^n+[Ca^{2+}]^n)} \), where \( n \) is the Hill-slope and \( k \) is the pCa50. Cell cross-sectional elliptical area was calculated by buckling the cell, followed by measurement using a calibrated screen monitor. Developed force was measured at both SLs at each activating cycle, with the baseline value of developed force subtracted from subsequent measures. Data were not considered valid if total rundown was greater than 20% after final maximal activation at the end of each activating cycle at both SLs. All data were acquired by custom-made LabView software and analyzed using Origin Pro 8.0.

8.5 Histopathology and Immunohistochemistry

To define any gross hypertrophy that resulted from deleterious effects of \( MYBPC3^{(+/t)} \) haploinsufficiency during myocardial stress, HW/BW ratio was determined after TAC and isoproterenol treatment as described previously. Gross morphology and the presence of fibrosis was assessed using Hematoxylin & Eosin staining and Masson’s Trichrome staining of 5µm thick heart slices. Hearts were perfused with cold cardioplegic buffer via retrograde perfusion from insertion of a needle into the abdominal aorta. Hearts were then perfused with a 10% formalin solution via the same needle. Hearts were processed into sections by Loyola’s histological core sample processing facility. Localization and integration of cMyBP-C into the sarcomere was evaluated using immunostaining and confocal microscopy (Leica TCS SP5) using the departmental core facility. Cryosections were used for immunofluorescence analysis with custom anti-
cMyBP-C\textsuperscript{2-14} antibodies (N’-specific) and commercial anti-α-actinin antibodies.

### 8.6 Quantification of Protein Levels

Protein analysis was performed using homogenized whole-heart tissue, or with myofilament enriched fractions. Homogenization was performed using a mechanical bead beater, with urea buffer (50 mM Trish-HCL, pH 7.5, 4 M urea, 1 M thiourea, 0.4% CHAPS, 20 mM spermine, 20 mM DTT) for whole heart homogenate or F-60 buffer (60 mM KCl, 30 mM Imidazole, 2 mM MgCl\textsubscript{2}) with protease (Roche) and phosphatase (Sigma, cocktail I and II) inhibitors for myofilament isolations. Most protein assays were resolved on 4-15% SDS-PAGE gel cassettes from Bio-Rad (Cat# 456-1083). SDS-PAGE gels were assessed using Coomassie Brilliant Blue or SYPRO Ruby staining for total protein, or Pro-Q Diamond staining for phosphorylation. Western blot was performed using a Bio-Rad transfer system. All western blots were evaluated using the ECL+ reagent and imaged using the Typhoon Trio scanner (GE Health Systems). Western blot protein levels were quantified using band density measurements using ImageJ, with the target protein normalized to α-tropomyosin or actin.

Levels of cMyBP-C were measured using a multitude of site-specific antibodies, as well as a series of phospho-site specific antibodies to assess phosphorylation of cMyBP-C. Myofilament proteins derived from \textit{MYBPC3\textsuperscript{+/t}} and WT hearts in the TAC and sham groups were used to measure phosphorylation levels of Ser-273, Ser-282, and Ser-302 by Western blot using site-specific phospho-antibodies. SDS-PAGE with large format 6.25% acrylamide gels with
protein samples homogenized in RIPA buffer (500mM NaCl, 50mM Tris, pH 7.5, 0.1% SDS, 0.5% NaDeocycholate, 1% Triton X-100) with protease inhibitor (Roche) and phosphatase inhibitors (Sigma, cocktail I and II) will be used to assess the shift in the myosin heavy chain isoforms that often accompanies hypertrophic remodeling.

8.7 Isolation of mRNA and qPCR Analysis

Minced heart tissue was homogenized in 1mL of TriZol (BioRad) using a bead homogenizer, and mRNA was isolated using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Cat. 732-6820, BioRad). Synthesis of cDNA from the mRNA samples was performed using the iScript cDNA synthesis kit (Cat. 170-8891, BioRad) with 1μg of mRNA template used in each reaction. Templates for qPCR analysis were also generated using no reverse transcriptase, which were used as negative controls. Gene expression levels were analyzed using TaqMan primers recognizing MYBPC3 with FAM dye(Cat. Mm.PT.53a.2930640, IDT), MYH7-FAM, NPPA-FAM, GAPDH-VIC Dye, and CASQ2-VIC (Cat. Mm01319006g1; Mm01255748g1; Mm00486742m1; 4352339E, Applied Biosystems) with iTaq Probes Master Mix (Cat. 172-5131, BioRad) and analyzed using a BioRad CFX96 thermocycler. The cycle protocol was 94°C for 10 minutes, followed by 40 cycles at 94°C for 15 seconds and 60°C for 60 seconds. Analysis of qPCR data was performed using the ΔΔCq method, normalized to the geometric mean of GAPDH and CASQ2 Cq values and corrected for primer efficiency as described previously (Pfaffl 2001).
8.8 RNA-Seq

Thanks to the generous collaboration from Drs. Jonathan and Christine Seidman and work done by Joshua Gorham from Harvard Medical School's Department of Genetics, RNA-Seq analysis was able to be performed for this study. Analysis and sequencing of the transcriptome of WT and Het mice following sham and TAC surgery was performed as described previously by the Seidman laboratory (Christodoulou, Gorham et al. 2011). Briefly, RNA was isolated from three hearts per group using the same isolation method as described for qPCR analysis, and were used to make cDNA samples using the same reverse transcriptase as noted above. The three samples from each group were pooled prior to sequencing and used to create a cDNA library for sequencing. These steps were performed by me at Loyola, and samples were shipped to the Seidman lab for further processing and sequencing. For details of the 5'-RNA-Seq procedure see (Christodoulou, Gorham et al. 2011). Transcript reads were matched to their corresponding genes and a list was compiled of 30,000+ unique identified RNA species. This list was processed by excluding all genes that were not over 1.5 fold up-regulated or 0.6 fold down-regulated compared to specific controls. All transcripts reported that did not pass the Benjamini-Hochberg false discovery rate test with a p value <0.001 were considered non-significant. Gene array images were made in collaboration with Loyola’s Bioinformatics department, with thanks to Dr. Michael Zilliox for his time and suggestions with data analysis. Pathway analysis was performed using
NCBI’s DAVID Bioinformatics Tool (http://david.abcc.ncifcrf.gov/).

8.9 Rat Ventricular Cardiomyocyte Isolation

Adult rat cardiomyocytes were isolated by excising the heart followed by retrograde perfusion on a modified Langendorff apparatus. All cardiomyocyte isolations for this work were performed by Jollyn Tyryfter in the Department of Cell and Molecular Physiology at Loyola University. Heart digestion procedure used a perfusion buffer (120.4mM NaCL, 14.7mM KCl, 0.6mM KH2PO4, 0.6mM Na2HPO4, 1.2mM MgSO4, 10mM Na-HEPES, 4.6mM NaHCO3, 30mM Taurine, 10mM BDM, 5.5mM Glucose) for five minutes to stabilize the heart and wash out all red blood cells. Isolation of the cardiomyocytes was performed using 100mg bovine serum albumin (Sigma A6003-25G), 100mg collagenase type 2 (Worthington 41B12520), and 10mg Protease (Sigma P5147) per 100mL perfusion buffer. This was perfused into the rat heart for two minutes, following recirculation for 20-25 minutes. The heart was then triturated and minced followed by filtration through a 200µm screen. Cells were pelleted by gravity or a 500 rpm centrifugation for 1 minute. Pellet was re-suspended in a 1:1 BSA solution and Perfusion buffer. Cells were brought back up to calcium by slow addition of 200µl of 1mM Ca²⁺ (3x), 10mM Ca²⁺ (3x), and 100mM Ca²⁺ (2x) with five minute pauses between each addition (O’Connell, Rodrigo et al. 2007).

8.10 MYBPC3 Knock-down

In order to knock-down cMyBP-C in cultured rat cardiomyocytes, an
adenovirus was created by Jody Martin, PhD, Department of Cell and Molecular Physiology at Loyola, containing a sequence taken from work published by the Robbins lab which had previously been used to knock-down MYBPC3 expression in neonatal cardiomyocytes using a construct with the sequence 5'-GCA UGU UCU GCA AGC AGG GAG UAU U-3' (Bhuiyan, Gulick et al. 2012). This virus was administered to cardiomyocytes followed by 48 hours of culturing before protein and mRNA was isolated and evaluated as mentioned previously.

8.11 Statistical Analysis

All data are represented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using Graph Pad Prism (version 6.0). Baseline data were analyzed using one-way ANOVA with a Bonferroni post-hoc test. For force-pCa data at two sarcomere lengths, two-way ANOVA was performed, followed by a Bonferroni post-hoc test. For comparison of 4 and 12 week force-pCa parameters, two-way ANOVA was performed on the changed values between each time point to compared groups to each other, and a t-test was used to compare how single groups changed between time points. Experiments using the TAC model used a two-way ANOVA with a Bonferroni post-hoc test. Diastolic echocardiography parameters under baseline conditions were compared using an unpaired t-test. Statistical significance was defined as \( P<0.05 \). For RNA-Seq data analysis, significant fold changes were set \textit{a priori} as 1.5 fold increase and 0.6 fold decrease. The Benjamini-Hochberg false-discovery
rate test (Benjamini and Hochberg 1995) was used to evaluate significance in the relative abundance of a particular gene, with the threshold for significance set at $p \leq 0.001$. 
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VITA

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