Heart and Sole: The Functional Role of Fast-Skeletal Myosin Binding Protein-C in Cardiac and Skeletal Muscle

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LOYOLA UNIVERSITY CHICAGO

HEART AND SOLE: THE FUNCTIONAL ROLE OF FAST-SKELETAL MYOSIN BINDING PROTEIN-C IN CARDIAC AND SKELETAL MUSCLE

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

CELL AND MOLECULAR PHYSIOLOGY

BY
BRIAN LEEI LIN
CHICAGO, IL
AUGUST 2016
ACKNOWLEDGEMENTS

I would like to first thank my PhD mentor, Dr. Sakthivel Sadayappan, for providing me a fantastic opportunity to work in cardiac physiology. It is through his lab that I have found a home away from home, a passion for science, and a love/hate relationship with moderately spicy Indian cuisine. I would also like to thank the department chair of Cell and Molecular Physiology, Dr. Pieter de Tombe, whose efforts in shaping me into a real scientist is second to none. I have been extremely fortunate to have landed such an excellent opportunity to work with these gentlemen and will be forever grateful for their mentorship.

The support of my family and friends have also greatly contributed to my success. In particular, my wife, Tiffany Liu; my aunt and uncle, Jean and Alan Vliet; and my parents, Tang Wen Hsuan and Lin Chang Chueng.

The members of my lab and department, both past and present, have also been crucial in helping me develop as a scientist and a person. Over the past several years, the current members of my lab (Thomas Lynch, Aravindakshan Jagadeesan, Sangeetha O Kandoi, Angela Taylor, Mohit Kumar), as well as former members of the lab (Suresh Govindan, David Barefield, Diederik Kuster, and Xiang Ji) have been instrumental in providing extraordinary support and snacks on the food cart. I also greatly appreciate the support of Dr. Jody Martin, Dr. Seth Robia, Dr. Jonathan Kirk, Pete Carron, and Jollyn Tyryfter.
Furthermore, I would like to thank the collaborators that I have had the good fortune to meet and work with over the years: Drs. Ji Young Mun, Kyounghawn Lee, Roger Craig; Drs. Mike Previs, Amy Li, David Warshaw; Drs. Brett Colson, David Thomas; Dr. Stuart Campbell.

Finally, I would like to thank my committee members for their guidance over the last few years: Drs. Xun Ai, Elizabeth McNally, and Renzhi Han. To have learned so much from so many different people has truly been a privilege.
For our grandparents and grandparents everywhere
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<td>ARVM</td>
<td>adult rat ventricular myocytes</td>
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<tr>
<td>CB</td>
<td>C-bridge</td>
</tr>
<tr>
<td>cMyBP-C</td>
<td>cardiac myosin binding protein-C</td>
</tr>
<tr>
<td>DA1</td>
<td>distal arthrogryposis, type 1A</td>
</tr>
<tr>
<td>DA2</td>
<td>distal arthrogryposis, type 2</td>
</tr>
<tr>
<td>DCM</td>
<td>dilated cardiomyopathy</td>
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<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
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<td>DSKO</td>
<td>Double-Skeletal myosin binding protein-C Knock-Out</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>F&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximal force</td>
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<tr>
<td>FSKO</td>
<td>Fast-Skeletal myosin binding protein-C Knock-Out</td>
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<td>fsMyBP-C</td>
<td>fast-skeletal myosin binding protein-C</td>
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<td>HF</td>
<td>heart failure</td>
</tr>
<tr>
<td>HCM</td>
<td>hypertrophic cardiomyopathy</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>k&lt;sub&gt;tr&lt;/sub&gt;</td>
<td>rate of tension redevelopment</td>
</tr>
<tr>
<td>LCCS</td>
<td>lethal congenital contracture syndrome</td>
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<td>LGMD</td>
<td>limb-girdle muscular dystrophy</td>
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<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MyBP-C</td>
<td>myosin binding protein-C</td>
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<tr>
<td>NTF</td>
<td>native thin filament</td>
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<tr>
<td>pCa</td>
<td>-log[Ca$^{2+}$]</td>
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<td>pCa$_{50}$</td>
<td>calcium sensitivity</td>
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<tr>
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<td>actin subdomain 1</td>
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<td>SEM</td>
<td>standard error mean</td>
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<tr>
<td>SL</td>
<td>sarcomere length</td>
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<td>SSKO</td>
<td>Slow-Skeletal myosin binding protein-C Knock-Out</td>
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<tr>
<td>ssMyBP-C</td>
<td>slow-skeletal myosin binding protein-C</td>
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<tr>
<td>TAC</td>
<td>trans-aortic constriction</td>
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<tr>
<td>Tm</td>
<td>tropomyosin</td>
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<td>Tn</td>
<td>troponin</td>
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<td>TnC</td>
<td>troponin C</td>
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<td>troponin I</td>
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<td>TnT</td>
<td>troponin T</td>
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<td>XB</td>
<td>cross-bridge</td>
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ABSTRACT

The goal of my dissertation was to compare and contrast the function of all three major isoforms of Myosin Binding Protein-C (MyBP-C): slow-skeletal, fast-skeletal, and cardiac (ssMyBP-C, fsMyBP-C, and cMyBP-C, respectively), with a focus on the least characterized isoform, fsMyBP-C. Using a variety of ex vivo, in vitro, and in silico methods, my research demonstrated that the N-terminal region of all MyBP-C isoforms bind to actin and shift tropomyosin, thus activating the thin filament during contraction. Furthermore, each isoform differentially activated the thin filament over isoform-specific ranges of Ca\(^{2+}\): slow-skeletal activates at low Ca\(^{2+}\), fast-skeletal activates at higher Ca\(^{2+}\), and cardiac activates over the full spectrum of Ca\(^{2+}\). I propose that different expression of MyBP-C isoforms allow striated muscles to fine tune its function. For example, the cardiac muscle sees the full range of Ca\(^{2+}\) on a beat-to-beat basis, and therefore cMyBP-C needs to operate over the full spectrum of Ca\(^{2+}\) with much higher frequency. Conversely, various skeletal muscles have vastly different roles: the demands of postural muscles are different than that of phasic muscles; thus, varied expression of slow-skeletal and fast-skeletal MyBP-C can ideally regulate the function of different muscles.
CHAPTER ONE

INTRODUCTION

The clinical significance of understanding the role of Myosin Binding Protein-C (MyBP-C) is clear: mutations in all three major isoforms of MyBP-C can cause cardiac and skeletal muscle diseases. Notably, mutations in the cardiac isoform is one of the most common causes of hypertrophic cardiomyopathy (HCM) (Bonne, Carrier, Richard, Hainque, & Schwartz, 1998; Schlossarek, Mearini, & Carrier, 2011; van Dijk et al., 2009; Watkins et al., 1995), with an estimated 60 million people affected worldwide (Dhandapany et al., 2009). cMyBP-C mutations also cause dilated cardiomyopathy (DCM) (Ehlermann et al., 2008; Hershberger et al., 2010). In addition, mutations in the slow-skeletal and fast-skeletal isoforms have been implicated in distal arthrogryposis, type 1 (DA1) (Bayram et al., 2016; Gurnett et al., 2010), distal arthrogryposis, type 2 (DA2) (X. Li et al., 2015), and lethal congenital contracture syndrome, type 4 (LCCS4) (Markus et al., 2012) (Figure 1).

The timeline of research into each of these isoforms is marked by the discovery of links between the MyBP-C isoform and human disease. These links demonstrate a clear and clinically relevant rationale to investigate its function, but these links have been unearthed over a period of over two decades. Mutations in cMyBP-C and myosin have long been implicated as the two major factors in the development of HCM in patients.
(Geisterfer-Lowrance et al., 1990; Watkins et al., 1995), and has since spurred increasing interest in the role of the cardiac isoform (Harris, Lyons, & Bezold, 2011; Sadayappan & de Tombe, 2012). Within the past 6 years, studies have implicated the ssMyBP-C in skeletal muscle disease, followed by a subsequent rise in interest in the slow-skeletal isoform (Ackermann & Kontrogianni-Konstantopoulos, 2013; Gurnett et al., 2010; Ha et al., 2013). Most recently, fsMyBP-C has been implicated in human disease (Bayram et al., 2016). Just like the renewed interest in the slow-skeletal isoform and the cardiac isoform before that, the discovery of a genetic link between fsMyBP-C and muscle dysfunction in human patients is likely to stimulate attention in the last of the three major MyBP-C isoforms.

Therefore, I focused my present dissertation on the biophysical mechanics of fsMyBP-C. While the emphasis is on this overlooked isoform, the scope of my dissertation research includes the slow-skeletal and cardiac isoforms. In doing so, comparisons can be made to existing knowledge of MyBP-C, but more importantly, this expanded scope allows a more comprehensive analysis of the MyBP-C as a whole. Indeed, the research I have done has provided greater insight than the sum of its parts on individual isoforms, and has led to both exciting and novel concepts regarding the role of MyBP-C in muscle contraction.
Figure 1. **MyBP-C mutations cause muscle disease.** The clinical significance of studying Myosin Binding Protein-C (MyBP-C): mutations in MyBP-C cause hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), distal arthrogryposis type 1 (DA1), distal arthrogryposis type 2 (DA2), lethal congenital contracture syndrome type 4 (LCCS4). Furthermore, MyBP-C is dysregulated in diseases such as Duchenne muscular dystrophy (DMD) and limb-girdle muscular dystrophy (LGMD). However, the function of MyBP-C has yet to be fully elucidated.
CHAPTER TWO

MYBP-C AND THE SARCOMERE

Introduction

Striated muscles consist of cardiac and skeletal muscles with vastly different roles in the body, but its molecular mechanisms share some incredible similarities. Striated muscle proteins (myofilaments) do not exist in isolation and have evolved into a highly sophisticated ensemble with many contributing pieces. To affect contraction at the whole muscle level, myofilament proteins work together in concert with one another at the molecular level. At this level, the smallest functional unit is known as a sarcomere. The sarcomere consists of thick and thin filaments that slide across one another. This sliding action pulls the edges of the sarcomere together and is the basis for contraction. Each thick and thin filament consists of its own subset of myofilaments that operate via intricate intrafilament and interfilament interactions during the contraction process. Thus, the goal of this chapter is to introduce the myofilaments as they come into play during striated muscle contraction.
A Brief History

Major advancements have been made in our understanding of muscle contraction at the molecular level since the initial discovery and isolation of the first muscle protein, in 1864 by the German physiologist Wilhelm Kühne, which he called myosin (Kühne, 1864). Subsequent work by Vladimir Engelhardt and Militza Lyubimova in the late 1930s and 1940s demonstrated that myosin had enzymatic activity capable of hydrolyzing ATP (Engelhardt, 1939). This ATPase activity drove the interaction of myosin with the second major muscle protein, actin (Straub, 1943; Szent-Györgyi, 1943), discovered by Brunó Straub and Albert Szent-Györgyi. Then, in the 1950s, the work led by Andrew Huxley and Rolf Niedergerke, and Hugh Huxley and Jean Hanson demonstrated the sliding filament theory (A. F. Huxley & Niedergerke, 1954a; H. Huxley & Hanson, 1954; H. E. Huxley, 1953), in which contraction resulted from the interaction of myosin and actin filaments. Other elements of these myofilaments were discovered around the same time or shortly after: Kenneth Bailey and tropomyosin (Bailey, 1946, 1948), Setsuro Ebashi and troponin (Ebashi & Endo, 1968; Ebashi & Kodama, 1965), and Gerald Offer and Roger Starr (Offer, Moos, & Starr, 1973). The work by these and other pioneers have laid the foundation for our understanding of striated muscle function.

Striated Muscles

Striated muscles consist both cardiac and skeletal muscle, which composes 30% and 38% of total body mass in women and men, respectively (Janssen, Heymsfield, Wang, & Ross, 2000). Cardiac muscles in the heart are responsible for
circulating blood throughout the body (Friedland, 2009; Harvey, 1628), and are controlled by the autonomic nervous system (Robinson, Epstein, Beiser, & Braunwald, 1966). The heart consists of cardiac muscle cells, or cardiomyocytes, that are linked via intercalated discs in order to contract as a syncytium (Severs, 2000). These cardiomyocytes are usually bi-nucleated, and excitation-contraction coupling (ECC) (Bers, 2002) is induced by a calcium-induced calcium release (CICR) (Fabriato, 1983). Briefly, an electrical system travels via the T-tubules resulting in an initial burst of Ca\(^{2+}\) from L-type voltage-dependent Ca\(^{2+}\)-channels known as dihydropyridine receptors (DHPR), which is detected by ryanodine receptors (RyR) that release Ca\(^{2+}\) stores from sarcoplasmic reticulum (SR) (Fill & Copello, 2002). Calcium is then able to initiate contraction, which will be explained later in greater detail and can be summarized in Online Video 1 (https://vimeo.com/148663469). In contrast, skeletal muscle is responsible for both support and voluntary movement of the body, controlled primarily by the somatic nervous system. Skeletal muscle fibers are generally much larger than cardiomyocytes, consist of different fiber types, and a single fiber can stretch from tendon to tendon, from which force transmission is conducted (Schiaffino & Reggiani, 2011). Skeletal muscle cells are multi-nucleated, having hundreds of nuclei that dot the fiber from one end to another (Holtzer, Marshall, & Finck, 1957; Okazaki & Holtzer, 1966). Contraction of individual skeletal muscle fibers depend on the innervation of each fiber (Hughes, Kusner, & Kaminski, 2006). Activation of different subsets of muscle fibers may not affect activation of adjacent muscle fibers. Unlike in
cardiomyocytes, the electrical signal is transduced via neuromuscular junctions and RyR are physically coupled to DHPR. In addition, skeletal muscle cells have known regenerative capability, stemming from a reserve of satellite cells (Morgan & Partridge, 2003; Yin, Price, & Rudnicki, 2013). While the role of cardiac and skeletal muscles are distinct from one another, there are striking similarities in the molecular mechanisms of contraction that regulate striated muscles. Specifically, striated muscles have the same functional unit of contraction, known as the sarcomere.

The Sarcomere is the Functional Unit of Muscle Contraction

The functional unit of muscle contraction at the molecular level is the sarcomere, which comes from Greek sarx (flesh) and meros (part). (A. F. Huxley & Niedergerke, 1954b; H. Huxley & Hanson, 1954). The sarcomere gives striated muscles the striations that give them its name. These striations are visible by light microscopy, and its highly organized and specific architecture is due to the alignment of Z-disc proteins that delineate individual sarcomeres (Figure 2). Z-discs are named from the German word “Zwischenscheibe,” meaning “between discs.” In the middle of the sarcomere is the M-line, from the German word “Mittelscheibe,” meaning “center disc.” Using electron microscopy, two distinct bands are clearly visible: a light band (I-band) and a dark band (A-band), because these regions of the sarcomere are isotropic and anisotropic to polarized light, respectively. Within the darker A-band is a region called the H-zone, after the German word “heller.” These various regions are populated by thin and thick filaments that overlap one another
to create the varying densities that make up each region. The thin and thick filament proteins run parallel to one another and perpendicular to the Z-discs. The thin and thick filaments overlap and slide one another to affect contraction at the molecular level. This event is triggered by Ca$^{2+}$ activation of the thin filament, a process in which Ca$^{2+}$-binding to the troponin (Tn) complex shifts tropomyosin's (Tm) position along the thin filament from a “blocked” to a “closed” position, which exposes myosin-binding sites on actin (Kobayashi & Solaro, 2005). Once the myosin head binds to actin, myosin and actin undergo a transition from weak to strong binding, further shifting tropomyosin to the “open” position, thus fully activating the thin filament. During this process, myosin hydrolyzes ATP (ATPase), converting chemical energy to mechanical energy, thus providing the power to slide thin filaments over the thick filaments (H. Huxley & Hanson, 1954; Spudich, 2001). Due to its central location between thick and thin filaments, MyBP-C is thought to modulate contraction through its effects on both thin filament activation and cross-bridge power generation.
Figure 2. **MyBP-C and the sarcomere.** Top-down schematic of the location of Myosin Binding Protein-C (MyBP-C) in muscle at the molecular level. Muscles consist of bundles of muscle fibers, known as fascicles (top). Muscle fibers, or myofibrils, are composed of sarcomeres, individual functional units of contraction (middle). MyBP-C is localized specifically within the C-zone of the sarcomere in 7-9 transverse stripes flanking the M-line. Each sarcomere is delineated by the Z-disc, with thick and thin filaments that overlap in the center. The thick filament primarily consists of myosin, and thin filaments are composed of actin, troponin, and tropomyosin (bottom). MyBP-C interacts with both the thick and thin filaments, with its C-terminus anchoring on the myosin LMM region and titin, and N-terminus interacting with myosin S2 region and actin.
The Thin and Thick Filaments of the Sarcomere

Thin filaments consist primarily of actin that form a helical rope attached at the Z-discs. The actin filament is capped at the Z-discs by CapZ proteins and tropomodulin towards the M-line. Spanning the length of the thin filament is the giant protein, nebulin. Along the length of actin are tropomyosin, which also forms a helical structure, and periodically dotted by troponin complex proteins. Thick filaments consist primarily of myosin, which consists of multiple subfragments. Myosin is the motor that physically interacts with actin to slide the filaments across one another. Myosin itself consists of several distinct regions, including the head domain, neck domain, and tail domain. Spanning the length of the thick filament is the giant protein, titin. Within the A-band is an even darker region, known as the C-zone. The C-zone consists of Myosin Binding Protein-C (MyBP-C). This chapter will highlight aspects of these myofilament proteins in the order they come into play during contraction.

Troponins: TnC, TnI, TnT

In both cardiac and skeletal muscles, molecular contraction at the sarcomeric level is initiated by Ca$^{2+}$. The influx of Ca$^{2+}$, and the signal it carries, is detected and transduced by the troponin complex, which consists of three subunits Troponin C, Troponin I, and Troponin T (TnC, TnI, TnT, respectively) (Figure 2). As previously mentioned, initial discovery is largely attributed to the work by Setsuro Ebashi (Ebashi & Endo, 1968), and much has since been learned regarding their function.
**TnC binds Calcium**

After Ca\(^{2+}\) is released into the sarcomere, the first troponin to come into play is TnC, the 18 kDa troponin subunit that binds Ca\(^{2+}\), specifically within the N-terminal regulatory domains. TnC structure consists of two globular domains at the N- and C-terminal ends (Herzberg & James, 1988; Satyshur et al., 1988) connected via \(\alpha\)-helical bridge (Vassylyev, Takeda, Wakatsuki, Maeda, & Maeda, 1998). The N-terminal globular domain is thought to be the primary regulation region, capable of binding Ca\(^{2+}\) via one of four EF hand motifs (van Eerd & Takahashi, 1975), while the C-terminal domain is thought to serve a structural role. EF hand motifs are numbered Sites I – IV, with N-terminal sites (I and II) having relatively low affinity to Ca\(^{2+}\) compared to C-terminal sites (III and IV). However, sites III and IV are occupied by Mg\(^{2+}\) under physiological conditions (Zot & Potter, 1987). Interestingly, the C-terminus also has the EF hand motif, and evidence suggests some contribution to regulatory function (Biesiadecki, Kobayashi, Walker, Solaro, & de Tombe, 2007). The N-terminal domain of TnC interacts with the C-terminal region of TnI dubbed the “regulatory head” (Kleerekoper, Howarth, Guo, Solaro, & Rosevear, 1995; Takeda, Yamashita, Maeda, & Maeda, 2003), and the binding of Ca\(^{2+}\) to the N-terminal globular domain alters its interaction with TnI. The conformational change in the regulatory head transduces the Ca\(^{2+}\) signal.
**TnI is the inhibitory subunit**

TnI undergoes a conformational change, induced by Ca\textsuperscript{2+}-binding to TnC, and this structural change is a vital step in thin filament activation. TnI is a 21 kDa troponin subunit known to be an important inhibitor of sarcomeric activation due to its interactions with the other troponin subunits, as well as tropomyosin and actin (Ohtsuki, Maruyama, & Ebashi, 1986; Zot & Potter, 1987). TnI inhibits actomyosin Mg-ATPase, and this inhibition is enhanced by tropomyosin (Greaser & Gergely, 1971; Potter & Gergely, 1974). TnI has been demonstrated to be a potent regulator of Ca\textsuperscript{2+}-sensitivity that is also highly regulated by phosphorylation (Layland, Solaro, & Shah, 2005; Solaro & Van Eyk, 1996; Westfall & Metzger, 2001; Westfall, Turner, Albayya, & Metzger, 2001), with the central region of TnI primarily for both actin binding and inhibitory capacity (Syska, Wilkinson, Grand, & Perry, 1976; Talbot & Hodges, 1981), with some contributions to inhibitory activity from the N-terminal region (Sheng, Pan, Miller, & Potter, 1992), as well as the C-terminal region (Farah et al., 1994). In its inactivated state, N-terminal region of TnI loosely binds TnC and TnT, while its C-terminal region tightly binds actin. Its binding affinities change upon activation by Ca\textsuperscript{2+}: TnC binding to Ca\textsuperscript{2+} causes TnI to tightly bind TnC and TnT, while loosening its binding to actin (Potter & Gergely, 1974; Syska et al., 1976). This change is thought to transduce the signal down to the last troponin subunit, TnT.
**Troponin T binds tropomyosin**

TnT is the largest of the troponin subunits at 36 kDa, and TnT anchors the other troponin subunits to the actin filament, as well as binding to tropomyosin (Tm) (Hitchcock, 1975a, 1975b; Hitchcock & Lutter, 1975). The structure of TnT consists of two helical structures (T1 and T2), as well as an unstructured flexible region known as the hypervariable domain (Takeda et al., 2003). The C-terminal T2 structure interacts with TnI, TnC, and actin (Heeley, Golosinska, & Smillie, 1987; Schaertl, Lehrer, & Geeves, 1995), as well as the central region of Tm (Morris & Lehrer, 1984; Pearlstone & Smillie, 1982). The central T1 region interacts with C-terminus of Tm; however, the function of the N-terminal hypervariable domains (H1 and H2) remains unclear, though these regions may allow TnT to adapt to specific isoforms, muscles, or species (Biesiadecki, Chong, Nosek, & Jin, 2007; Jin, Chen, Ogut, & Huang, 2000). The primary role for TnT is thought to be structural, but recent studies suggest TnT has several phosphorylation sites that regulate Ca\(^{2+}\)-sensitivity and force generation (Jideama, Crawford, Hussain, & Raynor, 2006; Noland & Kuo, 1992; Sumandea, Pyle, Kobayashi, de Tombe, & Solaro, 2003). The interaction between Tm and TnT is likely the primary mechanism behind its regulation of thin filament activation. (Pato, Mak, & Smillie, 1981; White, Cohen, & Phillips, 1987)TnT loosens its interaction with Tm, which allows Tm to move its position relative to actin in such a way that exposes the myosin-binding site on actin (Potter, Sheng, Pan, & Zhao, 1995).
**Tropomyosin**

Tropomyosin (Tm) is a 37 kDa, 42nm-long protein that, much like actin, consists of double α-helices that form a coiled-coil that intertwines with the actin filament (Woods, 1966, 1967). There are multiple isoforms of Tm, such as α-Tm, β-Tm, and slow α-Tm, encoded by *TPM1*, *TPM2*, and *TPM3*, respectively (Perry, 2001). These isoforms are structurally similar, but variations in structural homology suggest variations in their capacity to regulate contraction at the molecular level (Muthuchamy et al., 1995). Tm polymers can form both homodimers and heterodimers, though in striated muscles Tm is arranged in heterodimers (Jancso & Graceffa, 1991; Lehrer, Qian, & Hvidt, 1989) that overlap by 8-9 amino acids from the N-terminus of one Tm polymer with the C-terminus of another (McLachlan & Stewart, 1975). Similar to one of its binding partners, TnT, Tm is thought to stabilize thin filaments: its interactions in conjunction with tropomodulin reduce depolymerization of the thin filament. Functionally, Tm works in tandem with Tn to regulate thin filament activation, and thus, myosin-actin interaction. Tm isoforms dimerizes and polymerizes end-to-end, fitting into the major groove within the actin double α-helix (Perry, 2001). In this groove, Tm physically blocks the myosin-binding site on actin, thus preventing contraction by interfering with myosin and actin interactions (Fraser & Marston, 1995). The conformational shift induced by Ca²⁺-bound troponin causes Tm to shift from the blocked to closed position, which exposes the myosin-binding sites on actin (McKillop & Geeves, 1993). The binding of myosin to actin further shifts the position of Tm on actin from the closed to open
position (Rosol et al., 2000).

Actin

The thin filament is primarily composed of actin filaments which, like tropomyosin, consists of double α-helices that form a coiled-coil within the sarcomere (Holmes, Popp, Gebhard, & Kabsch, 1990; Lorenz, Poole, Popp, Rosenbaum, & Holmes, 1995). Globular actin (G-actin) polymerizes into filamentous actin (F-actin) in a spontaneous manner, which then forms the major element of the thin filament. G-actin consists of 4 subdomains (SD1-SD4) (Kabsch, Mannherz, Suck, Pai, & Holmes, 1990; McLaughlin, Gooch, Mannherz, & Weeds, 1993; Schutt, Lindberg, Myslik, & Strauss, 1989), which forms a binding pocket for a nucleotide (ADP and ATP) (Egelman & Orlova, 1995; Kinosian, Selden, Estes, & Gershman, 1993a, 1993b) and for a divalent ion (Mg$^{2+}$ or Ca$^{2+}$) (Orlova & Egelman, 1995; Orlova, Prochniewicz, & Egelman, 1995). There are 7 actin monomers per half-helical turn of the actin filament, which interact with its opposing strand via subdomains (SD) 3 and 4, while subdomains 1 and 2 can interact with other myofilament proteins, such as myosin and MyBP-C (Figure 3). Subdomains on the actin monomer also interacts with other contractile proteins (von der Ecken et al., 2015). Vertebrate actin is composed of three main isoforms that are highly homologous (α, β, and γ), and skeletal, cardiac, and smooth variations of the α isoform (Vandekerckhove & Weber, 1979). The skeletal and cardiac isoforms are differentially expressed, and expression varies on muscle type and species. However, actin isoforms are highly conserved, with only minimal variation at the N-
terminal region (Vandekerckhove & Weber, 1979), though differences of just 15 amino acids can result in structural and functional variations (Brault, Reedy, et al., 1999; Brault, Sauder, Reedy, Aebi, & Schoenenberger, 1999). The importance of actin function is clear: actin is the primary component of the thin filament that serves to anchor troponins and tropomyosin. In addition, actin functions to transduce force generation via interactions with thick filament proteins, most notably, myosin. Specifically, actin subdomain 1 (SD1) binds to the myosin head (also known as the myosin subfragment 1 or myosin S1) primarily via two known ionic interactions: 1) the negative charge of SD1 interacts with the positively-charged lysine myosin 50k/20k loop 2) an adjacent SD1 interacts with myosin amino acid residues 567-578 (Milligan, 1996; Milligan, Whittaker, & Safer, 1990; Rayment, Holden, et al., 1993). A third interaction occurs between myosin S1 (amino acids 404-415) with the proline residues in between actin SD1 and SD3.
Figure 3. Actin has four subdomains. Actin monomer is the base unit of the actin filament (F-actin), which is a helical coiled-coil. There are 4 primary subdomains (SD) on the actin monomer that interact with other thick and thin filament proteins. SD1 is the primary binding site for myosin S1 region (represented by the green circle), which also is known to interact with the pocket between SD1 and SD3. SD3 and SD4 are primarily responsible for actin-actin interactions.
Myosin

Myosin is a superfamily of proteins with many classes and functional roles (Foth, Goedecke, & Soldati, 2006; Krendel & Mooseker, 2005; Woolner & Bement, 2009). This section will focus on the class 2 myosin in striated muscles that form the cross-bridge, which is responsible for muscle contraction. Myosin M2 was originally discovered in 1864 by Wilhelm Kühne (Kühne, 1864). The thick filament primarily consists of myosin, a large 220 kDa protein responsible for binding to actin and pulling the thin and thick filaments across one another to affect contraction. Myosin itself consists of several distinct regions: the head, neck, and tail regions. The N-terminal region has two heads, each known as the S1 subfragment (Dominguez, Freyzon, Trybus, & Cohen, 1998; Rayment, Holden, et al., 1993; Rayment, Rypniewski, et al., 1993). The S1 subfragment projects outward from the thick filament at 14.3 nm intervals and physically interacts with actin to form cross-bridges. The catalytic head is the motor of sarcomeric muscle contraction (Toyoshima et al., 1987) and is primarily responsible for the sliding of the thick and thin filaments across one another, and has nucleotide binding pocket capable of converting chemical energy (ATP) into mechanical energy (Holmes, Angert, Kull, Jahn, & Schroder, 2003). In the rigor state, myosin is attached to actin, with ADP and inorganic phosphate attached. The release of ADP and inorganic phosphate results in the myosin head to shift in such a way that it pulls on the actin thin filament, in what is known as the powerstroke. As ATP binds to the myosin head, myosin detaches from actin. Myosin MgATPase activity hydrolyzes the ATP
into ADP and inorganic phosphate, repositioning the myosin head in its original position, and the cycle begins anew (Holmes et al., 2003) (Figure 2). Just below the S1 subfragment is the myosin neck, S2 subfragment, and the S2 region links the myosin head to the rest of the thick filament. In addition, the S2 region interacts with two myosin light chains (MLC) thought to fine-tune myosin function: the essential light chain (ELC) and regulatory light chain (RLC). Further towards the C-terminus is the light meromyosin (LMM), and the LMM forms the coiled coil that anchor myosin to the thick filament, where it interacts with titin and Myosin Binding Protein-C.
Interaction between myosin and actin is regulated by Myosin Binding Protein-C (MyBP-C), which was first discovered as a myosin impurity in the 1970s (Craig & Offer, 1976; Offer et al., 1973). The three distinct MyBP-C isoforms: slow-skeletal, fast-skeletal, and cardiac, are each encoded by a distinct gene (*MYBPC1, MYBPC2, MYBPC3*, respectively) (Carrier et al., 1993; Weber, Vaughan, Reinach, & Fischman, 1993) (Figure 4). MyBP-C isoforms are localized within the C-zones (Offer et al., 1973), a region within the sarcomere flanking the M-line (Gilbert,
Cohen, Pardo, Basu, & Fischman, 1999), arranged in 7-9 transverse stripes at 43 nm intervals (Bennett, Craig, Starr, & Offer, 1986; Dennis, Shimizu, Reinach, & Fischman, 1984; Flashman, Redwood, Moolman-Smook, & Watkins, 2004; Rome, Offer, & Pepe, 1973) (Figure 2). As the names suggest, slow-skeletal and fast-skeletal isoforms (ssMyBP-C and fsMyBP-C, respectively) are expressed in skeletal muscles, whereas the cardiac isoform (cMyBP-C) predominates in the heart (Gautel, Furst, Cocco, & Schiaffino, 1998), though complexities of expression are further discussed in Chapter Three. The 43 nm interval of MyBP-C and 14.3 nm interval of myosin heads correlates to 1:3 ratio between MyBP-C and myosin within the C-zone (Craig & Offer, 1976; Luther & Craig, 2011). MyBP-C structure consists of seven immunoglobulin and three fibronectin III domains, numbered C1 through C10, from the N-terminus to the C-terminus (Luther et al., 2011). In addition, there is a proline/alanine-rich (PA) region that precedes the C1 domain, as well as a M motif between the C1 and C2 domains (Barefield & Sadayappan, 2010; Gautel, Zuffardi, Freiburg, & Labeit, 1995; Kuster et al., 2012). Despite these similarities, MyBP-C isoforms are structurally distinct and there are many key differences within the N-terminal region. Cardiac MyBP-C has an additional immunoglobulin-like domain, the C0 domain (Ratti, Rostkova, Gautel, & Pfuhl, 2011), as well as five known phosphorylation sites: four within the M motif (Copeland et al., 2010; Mohamed, Dignam, & Schlender, 1998) and one in the PA region (Kuster et al., 2013). Conversely, slow-skeletal MyBP-C has one known phosphorylation site in the M
motif and three phosphorylation sites in the PA region (Ackermann & Kontrogianni-Konstantopoulos, 2011a). Currently, no phosphorylation sites have been identified in fast-skeletal MyBP-C, but evidence suggest the presence of phosphorylation sites due to changes in the phosphorylation state of both ssMyBP-C and fsMyBP-C during disease (Ackermann, Kerr, King, C, & Kontrogianni-Konstantopoulos, 2015; Ackermann et al., 2013; Ackermann, Ward, Gurnett, & Kontrogianni-Konstantopoulos, 2015).

MyBP-C primarily operates via protein-protein interactions due to the lack of evidence of enzymatic activity, nor interactions with nucleic acids and sugars (Pfuhl & Gautel, 2012), though a low-affinity zinc binding site has been reported in the C1 domain of MyBP-C (Ababou et al., 2008), the vast majority of studies conducted in MyBP-C focuses on its interactions with other myofilament proteins.

The C-terminal region of MyBP-C has been demonstrated to interact with both the light meromyosin (LMM) region of myosin (Miyamoto, Fischman, & Reinach, 1999; Moos, Offer, Starr, & Bennett, 1975; Okagaki et al., 1993) and titin (Freiburg & Gautel, 1996; Furst, Vinkemeier, & Weber, 1992) of the thick filament, with the C8 through C10 domains being vital for localization to the A-band of the sarcomere (Gilbert et al., 1999). MyBP-C interaction with titin in particular is thought to be important for determining localization and periodicity of MyBP-C within the sarcomere (Furst et al., 1992; Koretz, Irving, & Wang, 1993): titin PEVK super repeats (a series of immunoglobulin and fibronectin III domains) act as a
scaffold for MyBP-C within the sarcomere (Labeit, Gautel, Lakey, & Trinick, 1992; Trinick, 1992, 1994). The C-terminal domains may also participate in the organization and stability of thick filaments during development (Nyland et al., 2009). However, cMyBP-C knock-out mouse models of cardiomyopathies have demonstrated sarcomere structures are still conserved (Harris et al., 2002; McConnell et al., 1999). Normal sarcomere structure is observed in human patients with MyBP-C mutations that result in hypertrophic cardiomyopathy and distal arthrogryposis type 1 (Vydyanath, Gurnett, Marston, & Luther, 2012), suggesting that MyBP-C role in sarcomeric assembly is not absolutely necessary. Though the MyBP-C C-terminal domains anchors MyBP-C to the thick filament, the MyBP-C N-terminal binding partners are more varied and functional implications more complex.
**Function of MyBP-C**

MyBP-C has been known as the braking mechanism in cardiac muscle contraction (Previs, Beck Previs, Gulick, Robbins, & Warshaw, 2012; Razumova et al., 2006), due to its ability to reduce thin filament sliding velocities. Furthermore, the N-terminal region of MyBP-C have been demonstrated to bind to actin and shift tropomyosin from the blocked to closed position, in a similar manner to Ca$^{2+}$-bound Tn complex (Mun et al., 2014). Studies using recombinant proteins representing the cMyBP-C N-terminus have demonstrated that cMyBP-C has a significant effect in promoting Ca$^{2+}$-sensitivity and rate of tension redevelopment (Razumova, Bezold, Tu, Regnier, & Harris, 2008; Witayavanitkul et al., 2014). The functional mechanism of MyBP-C is likely due to interactions with actin (Shaffer & Harris, 2009; Squire, Luther, & Knupp, 2003; Whitten, Jeffries, Harris, & Trewhella, 2008), myosin subfragment-2 (S2) (Gruen & Gautel, 1999; Gruen, Prinz, & Gautel, 1999; Moos et al., 1975; Offer et al., 1973; Starr & Offer, 1978), and regulatory light chain of myosin (Ratti et al., 2011). The primary interaction MyBP-C with the thin filament is via actin: the C1 domain and M-motif are required for interaction with actin (Bhuiyan, Gulick, Osinska, Gupta, & Robbins, 2012; Squire et al., 2003; Whitten et al., 2008), although the cardiac-specific C0 domain of cMyBP-C has been demonstrated to be sufficient to interact with actin and elicit some functional effects (Herron et al., 2006). This indicates MyBP-C interaction with actin likely involves several N-terminal domains. In contrast, MyBP-C interaction with myosin...
subfragment-2 appears to be limited to the M-motif, specifically the N-terminal 126 residues of S2 (Gruen & Gautel, 1999), and may be mutually exclusive to MyBP-C interaction with actin (Bhuiyan et al., 2012; Gruen & Gautel, 1999). However, there is some discrepancy within the literature on what this entails at the functional level. Kunst et al. 2000 demonstrated that the C0 domain alone does not interact with myosin (Kunst et al., 2000), while others demonstrated C0 does interact with the regulatory light chain (RLC) of myosin (Ratti et al., 2011). Interestingly, Herron et al. 2006 demonstrated C0 domain alone is able to elicit some functional effects (Herron et al., 2006), suggesting the C0 domain regulates function via interactions with actin.

Studies demonstrating MyBP-C N-terminus binding to actin and myosin suggest a unique orientation between the thin and thick filaments, as well as promiscuity with multiple binding partners that is regulated in a phosphorylation-dependent manner (Barefield & Sadayappan, 2010; Sadayappan & de Tombe, 2012) (Figure 4). Kulikovskaya et al. (2003) demonstrated that phosphorylation of cMyBP-C ablates its interaction with myosin subfragment-S2, thus resulting in greater force production by allowing myosin to bind to actin (Kulikovskaya, McClellan, Flavigny, Carrier, & Winegrad, 2003). Specifically, phosphorylation of MyBP-C regulatory sites (S276, S285, S304 in humans) results in the N-terminal region to favor binding with actin versus myosin. By this mechanism, cMyBP-C is thought to provide regulation at multiple levels during contraction. First, MyBP-C interaction with myosin is hypothesized to act as a mechanical load on myosin, and thus reduce its ability to form cross-bridges. Phosphorylation of MyBP-C releases
this load on myosin, thereby promoting cross-bridge cycling kinetics. Recent studies demonstrated phosphorylation of the M-motif of cMyBP-C confers a specific structural stiffness to the region and results in a conformation change in the N-terminal region (Colson, Thompson, Espinoza-Fonseca, & Thomas, 2016; Previs et al., 2016). In these studies, phosphorylation causes the N-terminal region of cMyBP-C to fold on itself, perhaps shielding interacting residues within the M-motif that are responsible for myosin binding. This would subsequently free the MyBP-C N-terminal for interaction with actin, to facilitate contraction. However, this conformational effect is ablated at high Ca^{2+} (Previs et al., 2016), suggesting that MyBP-C N-terminus would once again favor myosin binding. This model allows for binding of MyBP-C to both myosin and actin, at different stages of contraction (low and high Ca^{2+}), but whether the binding interactions are mutually exclusive has yet to be resolved.
CHAPTER THREE

EXPRESSION PROFILE OF THE THREE MYBP-C

2.1 Structures of MyBP-C isoforms are both similar and distinct

MyBP-C is a family of thick filament contractile proteins with three predominant isoforms: slow-skeletal, fast-skeletal, and cardiac (ssMyBP-C, fsMyBP-C, and cMyBP-C, respectively), which are expressed by individual genes (MYBPC1, MYBPC2, MYBPC3, respectively). It is worth mentioning the existence of an additional isoform, MyBP-H (encoded by MYBPH gene), whose structure resembles that of the C-terminal half of MyBP-C (Gilbert et al., 1999; Vaughan, Weber, Einheber, & Fischman, 1993). As mentioned in the Chapter Two, MyBP-C isoforms share several conserved structural elements, including seven immunoglobulin and three fibronectin III domains, numbered C1 through C10, from the N-terminus (Luther & Vydyanath, 2011), a proline/alanine-rich (PA) sequence preceding the C1 domain, and a conserved M domain between the C1 and C2 domains (Figure 4). Where the notable differences exist between MyBP-C isoforms is within their N-terminal regions. For example, cMyBP-C has an additional immunoglobulin-like “C0” domain of approximately 100 amino acids, which binds myosin light regulatory chain and actin in vitro (Gautel et al., 1995; Ratti et al., 2011). In addition, cMyBP-C has 4 phosphorylation sites in the M domain (Barefield & Sadayappan, 2010) and one in the PA region (Kuster et al., 2013), whereas ssMyBP-C has one phosphorylation site
in the M domain and three in the PA region (Ackermann & Kontrogianni-Konstantopoulos, 2011a) and no phosphorylation sites have yet to be identified in fsMyBP-C. Sequence identity between isoforms is also reduced in this N-terminal region (Table 1). Further differences are notable in the C5 domain of cardiac, in which cMyBP-C has an additional flexible insert of unknown function (Cecconi, Guardiani, & Livi, 2008; Guardiani, Cecconi, & Livi, 2008a, 2008b; Idowu, Gautel, Perkins, & Pfuhl, 2003), though more recent studies have suggested that this confers the ability to bend at particular hinge points centering around the C5 domain (Previs et al., 2016)
2.2 Skeletal isoforms of MyBP-C are present in the heart

As the names suggest, cardiac MyBP-C predominates in the heart, while slow- and fast-skeletal MyBP-C predominates in skeletal muscles. Contrary to common belief, the expression profile of MyBP-C isoforms is much more complicated in development versus adult stages and healthy versus disease states. For example, avian species temporarily expresses cMyBP-C in skeletal muscles during embryonic development (Bahler, Moser, Eppenberger, & Wallimann, 1985; Kawashima, Kitani, Tanaka, & Obinata, 1986; Obinata et al., 1984), though cMyBP-C expression is not observed during mammalian embryonic development at the transcript nor protein level (Gautel et al., 1998; Kurasawa et al., 1999). However, an embryonic isoform is

<table>
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<th>MyBP-C N-termini</th>
<th>ssC1C2 (a.a. 1-341)</th>
<th>fsC1C2 (a.a. 1-337)</th>
<th>C0C2 (a.a. 1-448)</th>
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<td>70%</td>
<td>2%</td>
</tr>
<tr>
<td>fsMyBP-C</td>
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</table>

Table 1. N-terminal homology between N-terminal MyBP-C proteins (ssC1C2, fsC1C2, C0C2) and full-length MyBP-C.
expressed in mammalian cardiac and skeletal muscles (Gautel et al., 1998), after which the cardiac and skeletal MyBP-C isoforms begin to predominate their respective muscles. The authors argued that during adulthood, cardiac and skeletal MyBP-C were specifically expressed in cardiac and skeletal muscles, respectively, though later studies demonstrated that was not necessarily the case. Adding further complication to our understanding of MyBP-C expression patterns, ssMyBP-C was detected in the heart (Dhoot & Perry, 2005), albeit in small quantities in the right atrium and interatrial septum. Perhaps most surprisingly, the cardiac samples used in this study were from healthy tissues and not diseased hearts.

This led us to suspect the expression of MyBP-C isoforms may be altered due to different conditions during disease states. To test this, we used a murine mouse model of heart failure, the cMyBP-C/− knock-out mouse known as the (t/t) mouse (McConnell et al., 1999). The t/t mouse expresses a truncated cMyBP-C, which is not incorporated properly within the sarcomere and degraded, creating a functional knock-out. Using hearts from these t/t mouse, we demonstrated the presence of fsMyBP-C in the heart during heart failure (B. Lin et al., 2013), indicating expression patterns of MyBP-C can shift due to physiological or pathophysiological conditions in the heart. To further investigate whether expression of fsMyBP-C was specific to this transgenic mouse model in which cMyBP-C was completely absent, I conducted preliminary investigations in other transgenic mouse models of HF, as well as a pressure-overload mouse model of HF. In addition to the t/t mouse heart samples, our lab studies other mouse models of heart failure, known as the 110 and C10 mice,
which have truncations in the N-terminal and C-terminal regions of cMyBP-C, respectively. In addition, I analyzed the hearts from mice that have undergone TAC (transaortic constriction), in which the aortic opening is sutured to induce pressure-overload hypertrophy of the ventricle. In the TAC mouse model, we have previously demonstrated that fsMyBP-C expression is increased at the transcript level (Figure 5). While this suggests fsMyBP-C may be upregulated, it is not a foregone conclusion that fsMyBP-C protein levels increase. The hearts from mouse models were compared to non-transgenic controls, while hearts from TAC mice were compared with sham surgery controls. Our preliminary results revealed that insult to the heart, in general, can result in the upregulation of fsMyBP-C expression in the heart at the protein level (Figure 6). Western blot analysis determined the presence of fsMyBP-C in all mouse models of HF that we tested. In addition to previously published evidence demonstrating the presence of small amounts of fsMyBP-C in the t/t mice, 110 mice and C10 mice also expressed comparable quantities of fsMyBP-C (Figure 6). Furthermore, fsMyBP-C was also detected in TAC mouse hearts, suggesting that the upregulation of fsMyBP-C was related to the diseased condition of the heart rather than a specific compensatory upregulation due to transgenic modification of cMyBP-C. I theorize that upregulation of fsMyBP-C in the heart in disease may be reflective of a reversion to fetal programming, commonly observed in the diseased heart in which genetic programming reverts to conditions similar to embryonic development gene programming (Olson, 2004; Razeghi et al., 2001). Regulation of this phenomena is highly regulated, (Dirkx, da Costa Martins,
De Windt, 2013; Dirkx, Gladka, et al., 2013), though much has yet to be elucidated. Some studies suggest the reversion to fetal programming is initially compensatory (Olson, 2004; Razeghi et al., 2001), but continued activation of fetal gene programming is detrimental to the long-term health of the heart (Olson, 2004). It should be noted that mouse hearts from even sham-surgery also exhibited upregulation of fsMyBP-C, suggesting even slight insults to the heart may result in fsMyBP-C upregulation and/or strain differences in the mouse models used (FVB/N versus C57BL background).
**Figure 5.** Increased fsMyBP-C transcript levels in pressure-overload mouse model, as induced by TAC surgery. RNA Seq (top row) and qPCR (bottom row) demonstrate increased relative expression of *MYBPC2*, the gene encoding fast-skeletal MyBP-C, but not *MYBPC3*, the gene encoding cardiac MyBP-C. *MYH7*, the gene encoding β-myosin heavy chain was also elevated. Housekeeping genes *GAPDH* and *CASQ2* were also analyzed as controls and were unchanged. TAC mice data courtesy of Dr. Dave Barefield.
**Figure 6. Skeletal MyBP-C may be present in the heart.** Hearts from multiple mouse models of heart failure were examined for possible expression of ssMyBP-C and fsMyBP-C. The t/t mouse model is a functional cMyBP-C knock-out, C10 mouse lacks the C-terminal region of cMyBP-C, and the 110 mouse lacks the N-terminal region of cMyBP-C. TAC mice are pressure-overload models of HF. Heart lysates courtesy of Thomas Lynch and Dave Barefield.
2.3 Expression profile of ssMyBP-C and fsMyBP-C in skeletal muscles

The expression profile of ssMyBP-C and fsMyBP-C is also complicated in skeletal muscles. The literature provides little insight into ssMyBP-C (Ackermann & Kontrogianni-Konstantopoulos, 2013; Gurnett et al., 2010; Ha et al., 2013) and even less so concerning fsMyBP-C (Colson, Rybakova, Prochniewicz, Moss, & Thomas, 2012; M. Li, Andersson-Lendahl, Sejersen, & Arner, 2016). Based on the name “slow-skeletal” and “fast-skeletal,” it was often assumed was that ssMyBP-C was expressed in “slow-type” muscles and fsMyBP-C was expressed in “fast-type” muscles. Like the assumptions made regarding MyBP-C expression profile in the heart, the reality is much more complicated. Employing the same methods to determine expression profile in the heart, I determined the expression profile of ssMyBP-C and fsMyBP-C in skeletal muscles. Despite its expression in skeletal muscles of avian species during development (Bahler et al., 1985; Gautel et al., 1995; Obinata et al., 1984), I did not determine cMyBP-C expression in skeletal muscles, because it has been convincingly demonstrated not to be expressed at all in skeletal muscles of mammalian species (Gautel et al., 1998; B. Lin et al., 2013). This endeavor was complicated by several factors. First, skeletal muscles encompass a variety of different muscles that all have very distinct roles in providing postural and locomotor support for the body. Second, within a specific type of skeletal muscle, slow- and fast-type muscle fibers can co-exist adjacent to one another, or even slow and fast isoforms of the same protein within the same fiber. Third, there are
different methods of classifications of skeletal muscle fiber types, such as myosin ATPase activity, myosin fiber types, and biochemical analysis.

Therefore, to determine the expression profile of ssMyBP-C and fsMyBP-C, several different skeletal muscles were selected for analysis, particularly those used previously in the skeletal literature to represent slow, fast, and mixed fiber types. These muscles included the SOL (soleus), a slow-oxidative muscle and commonly used to represent slow-type muscles (Lutz, Ermini, & Jenny, 1978; Rubinstein & Kelly, 1981); EDL (extensor digitorum longus), often used to represent fast glycolytic muscles, but is actually a mixed muscle type; TA (tibialis anterior), a larger muscle consisting of mixed muscle fiber types; and finally FDB (flexor digitorum brevis), a fast-glycolytic muscle located in the sole of the foot in mammals (Figure 7). Dual-color western blot analysis confirmed that ssMyBP-C was expressed in all skeletal muscles tested, as well as additional, large mixed muscles, such as the quadriceps and the gastrocnemius (data not shown). Interestingly, fsMyBP-C was only expressed in the EDL and TA skeletal muscles, but not the FDB nor SOL. This was confirmed using traditional Western blot (Figure 8), using alternative antibodies for fast- and slow-skeletal MyBP-C. Perhaps most surprisingly, fsMyBP-C was not expressed in the FDB, which is considered a fast-type skeletal muscle, warranting some caution when considering the nomenclature of the skeletal isoforms. Indeed, earlier researchers also found the naming conventions to be unsatisfactory (Dhoot, Hales, Grail, & Perry, 1985) There were no muscles tested with only fsMyBP-C, though there are muscles in avian species that do exclusively express fsMyBP-C
isoform in adulthood (Obinata & Shinbo, 1987). Overall, the expression profile suggests ssMyBP-C is the baseline MyBP-C isoform necessary for normal function throughout skeletal muscles, while fsMyBP-C expression may augment particular muscle function. Notably, data on function of both isoforms are lacking.
Figure 7. Expression profile of skeletal MyBP-C in FVB/N WT mice. Four skeletal muscles were chosen to represent different types of muscles. *Extensor digitorum longus* (EDL) and *flexor digitorum brevis* (FDB) are fast-type skeletal muscles, *soleus* (SOL) is slow-type, and *tibialis anterior* (TA) is composed of both slow- and fast-type skeletal muscles. fsMyBP-C is expressed in the EDL and TA, while ssMyBP-C is expressed in all skeletal muscle tested.
**Figure 8.** Skeletal MyBP-C expression quantified. Densitometry scan of traditional Western blots validate expression profile demonstrated in dual-color immunoblots (Figure 7).
2.4 Summary: expression of MyBP-C isoforms is complex

Cardiac and skeletal MyBP-C isoforms are the predominant isoforms expressed in cardiac and skeletal muscles, respectively. However, contrary to commonly held preconceptions, MyBP-C isoforms often co-express with one another in health and disease, cardiac and skeletal muscle (Figure 9). Both skeletal isoforms (ssMyBP-C and fsMyBP-C) are expressed in the heart at the protein level: ssMyBP-C is expressed in the atrial tissues of the heart and fsMyBP-C is upregulated in the heart during disease conditions. The differential expression of ssMyBP-C and fsMyBP-C in various skeletal muscles adds another layer of complexity. While ssMyBP-C is expressed in every skeletal muscle we tested, fsMyBP-C is expressed only within mixed skeletal muscles we tested. Further complications arise when one considers that fsMyBP-C is not expressed in skeletal muscles that are considered ‘fast’-type (e.g. FDB), warranting some caution when considering the nomenclature.

It is worth noting that the naming convention of slow-skeletal, fast-skeletal, and cardiac MyBP-C provides a degree of insight into where they are expressed. Nevertheless, the expression profile of MyBP-C isoforms in cardiac and skeletal muscles depicts a complex and nuanced contractile protein whose function warrants detailed investigation. In particular, the expression of skeletal MyBP-C practically begs the question: what are skeletal MyBP-C isoforms doing in the heart?
Figure 9. Summary of skeletal MyBP-C Expression. MyBP-C are differentially expressed in health and disease, cardiac and skeletal muscles. Therefore, the two primary goals of my dissertation are to determine the function of fsMyBP-C in the context of cardiac muscle and fsMyBP-C function in skeletal muscle.
In the previous chapter, the combination of expression profile studies (Figure 7) and previously published literature (Figure 9) (Dhoot & Perry, 2005; B. Lin et al., 2013) clearly demonstrates the presence of skeletal isoforms of MyBP-C in the heart in health and disease, contrary to what the names would suggest. The co-expression of various MyBP-C isoforms prompted the question: What are the skeletal MyBP-C isoforms doing in the heart? This question really then prompted another question: What are the skeletal MyBP-C isoforms doing in skeletal muscles? The literature is not particularly helpful in either regard, with only one study comparing the function of all three MyBP-C (Colson et al., 2012), one study focusing specifically on fsMyBP-C (M. Li et al., 2016), and few labs focused on ssMyBP-C (Ackermann & Kontrogianni-Konstantopoulos, 2013; Gurnett et al., 2010). The majority of the research on MyBP-C is solely dedicated to cMyBP-C, from which my dissertation must draw certain intuitive insights. However, this provides a fantastic opportunity to meticulously compare and contrast fsMyBP-C, the least characterized isoform, with its slightly better known relative, ssMyBP-C, and most well-known isoform, cMyBP-C. Therefore, my objective is to comprehensively characterize the function of fsMyBP-C in the context of cardiac and skeletal muscles.
**Aim 1. Determine the steady-state function of fsMyBP-C in the cardiac system**

Contrary to conventional wisdom, fsMyBP-C is demonstrated to be expressed in the heart in murine models of heart failure. The function of fsMyBP-C in the cardiac system is completely unknown, though distinct structural differences at the N-terminal region of fsMyBP-C suggest functionally distinct regulation of muscle contraction compared to cMyBP-C. In the case of the cardiac isoform, the N-terminal region, from the C0 domain through the C2 domain, has been demonstrated to be necessary and sufficient for the normal function of cMyBP-C. **Therefore, I hypothesized that fsMyBP-C N-terminal region up to the C2 domain differentially regulates steady-state properties of cardiac muscle contraction.**

To test this hypothesis, recombinant proteins were generated representing the N-terminal region of ssMyBP-C, fsMyBP-C, and cMyBP-C up to and including the C2 domain, and were thusly named ssC1C2, fsC1C2, and C0C2, respectively. These recombinant MyBP-C N-termini were utilized for a battery of extensive tests based primarily using cardiac contractile fibers and systems. The primary functional experiment used was the steady-state fiber force-ATPase assay, capable of simultaneously measuring force-pCa, force-ATPase, and force-stiffness relationships, as well as rate of tension redevelopment ($k_{tr}$). Actin cosedimentation with ssC1C2, fsC1C2, and C0C2 was used to determine differential affinities and capacities of MyBP-C isoforms to bind actin. These results will determine the mechanism that may underlie differences in the regulation of steady-state function.
Aim 2. Determine the dynamic function of fsMyBP-C in the cardiac system

Steady-state measurements capture detailed snapshots of a dynamic process. Expression of fsMyBP-C in the heart may directly impact the dynamics of the heart at the cellular level. Previous studies have suggested diseased hearts may revert to a fetal gene programming to initially compensate for dysfunction (Rajabi, Kassiotis, Razeghi, & Taegtmeyer, 2007), giving rise to re-expression of skeletal isoforms in the heart. Given that cMyBP-C mutations are the leading cause of HCM, a condition in which abnormally thickened ventricular walls may impair relaxation (Bonne et al., 1998; Marston, Copeland, Gehmlich, Schlossarek, & Carrier, 2012), I hypothesized that fsMyBP-C expression in cardiac cells would facilitate relaxation kinetics.

To test the effects of dynamic contraction on function, unloaded shortening was examined in adult rat ventricular myocytes (ARVM) overexpressing full-length, myc-tagged ssMyBP-C, fsMyBP-C, and cMyBP-C. In addition, to elucidate potential mechanisms that underlie dynamic contractile regulation, I applied computer modeling previously used to simulate unloaded shortening of cardiac cells (Campbell, Haynes, Kelsey Snapp, Nava, & Campbell, 2013; Kuo et al., 2014). Computer modeling of unloaded shortening revealed thin filament activation as a unifying mechanism behind both steady-state and dynamic contraction experiments.
Aim 3. Determine the steady-state function of fsMyBP-C in skeletal muscles

Expression of fsMyBP-C was specific to skeletal muscles with mixed fiber types (Figure 7,8), in contrast to ssMyBP-C, which was expressed throughout all skeletal muscles. This expression profile suggests fsMyBP-C exists to augment skeletal muscle function in which it is expressed. The only study to compare fsMyBP-C function with other MyBP-C isoforms suggests fsMyBP-C regulates by restricting actin torsional dynamics (Colson et al., 2012). In the cardiac isoform, actin binding has been demonstrated to directly promote thin filament activation (Mun et al., 2014). Therefore, I hypothesized that fsMyBP-C regulates thin filament activation in skeletal muscle system. To test this hypothesis, I utilized the force-ATPase assay once to determine the skeletal muscle fiber function of EDL muscles in a novel mouse model dubbed FSKO (Fast-Skeletal MyBP-C Knock-Out) mice. EDL tissues were selected for three reasons: 1) EDL muscle normally expresses fsMyBP-C. The absence of fsMyBP-C in FSKO mice would thus elicit more pronounced skeletal muscle deficiencies in our functional assays 2) the fiber size of EDL is ideal for use in force-ATPase assay, allowing for direct comparison with force-ATPase assay 3) EDL is commonly used to represent ‘fast-type’ skeletal muscles in the literature, allowing for comparisons with previously published literature. Within the scope of my dissertation, I focused on the steady-state functional aspects of the EDL of FSKO mice. However, it is worth noting additional biochemical and functional characterization of FSKO mice are planned to fully characterize this mouse model.
CHAPTER FIVE

FIBER FORCE-ATPASE ASSAY: N-TERMINAL REGION OF MYBP-C DIFFERENTIALY REGULATES MUSCLE CONTRACTION BY ACTIVATING THE THIN FILAMENT

5.1 Abstract

Skeletal and cardiac isoforms of MyBP-C are co-expressed in the heart during health and disease, but how ssMyBP-C, fsMyBP-C and cMyBP-C differ in their regulation of function is unknown. Previous studies demonstrated the necessity of the N-terminal region of cMyBP-C, but each MyBP-C isoform is structurally distinct within this region. Therefore, I hypothesized that the **fsMyBP-C N-terminal region up to the C2 domain differentially regulates steady-state properties of cardiac muscle contraction.** To test this hypothesis, I utilized the force-ATPase assay to determine the function of recombinant proteins representing the critical N-terminal region of MyBP-C (ssC1C2, fsC1C2, C0C2) in adult rat papillary muscle fibers (**Figure 10**), which are sufficiently large for experiments and share homology with mice. The results demonstrated that fsC1C2 and C0C2 increased Ca\(^{2+}\)-sensitivity of force development, as well as rate of tension redevelopment at submaximal Ca\(^{2+}\) levels, suggesting that these N-terminal regions affect thin filament activation. While ssC1C2 was not significantly different from controls, it was also not significantly different from fsC1C2, suggesting this isoform may operate at an intermediary modality and/or at even lower ranges of Ca\(^{2+}\).
**Figure 10. MyBP-C N-termini.** Recombinant proteins representing the N-terminal functional region of MyBP-C isoforms were generated up to, and including, the C2 domain. SDS-PAGE gels demonstrate the relative size and purity of each of the MyBP-C recombinant proteins, encompassing the N-terminal region, up to and including the C2 domain. The N-terminal fragments for ssMyBP-C, fsMyBP-C, and cMyBP-C are named ssC1C2, fsC1C2, and C0C2, respectively. Previous studies have demonstrated that this region is necessary and sufficient to recapitulate the function of cMyBP-C.
Figure 11. Papillary muscle fiber preparation. Papillary muscles were excised from rats and permeabilized overnight. Muscles were trimmed down to size and t-clipped. The fibers need to be this size to ensure penetration by recombinant MyBP-C N-termini, as well as Ca$^{2+}$.
5.2 Results

5.2.1 Absolute Force: fsC1C2 and C0C2 promotes submaximal force generation, and ssC1C2 trend suggests graded regulation

Similar studies have demonstrated cMyBP-C N-terminal fragments do not alter maximal force production in the heart (Herron et al., 2006; Razumova et al., 2008). At maximal activation states, permeabilized fibers are exposed to pCa 4.5, in which Ca$^{2+}$ levels are in excess of what is physiologically experienced by the sarcomere. At pCa 4.5, maximal force generation was unchanged between fibers incubated with ssC1C2, fsC1C2, and C0C2, reflecting the previous functional assays using N-terminal fragments of cMyBP-C. However, our studies demonstrated that fsC1C2 and C0C2 conferred significant force generation at submaximal Ca$^{2+}$ levels to different degrees (Figure 12). Interestingly, at most submaximal Ca$^{2+}$ levels, ssC1C2 was not significantly different from controls nor other MyBP-C fragments, suggesting ssC1C2 may operate at an intermediary step or at even lower Ca$^{2+}$ levels that are outside precision of the force-ATPase assay. Changes in submaximal force generation suggested MyBP-C isoforms confer Ca$^{2+}$-sensitization of force development and/or regulate contraction via isoform-specific mechanisms.
Figure 12. Force-pCa relationship. fsC1C2 and C0C2 promoted greater submaximal, but not maximal, force generation. Graphs represented as mean ± SEM, *p<0.05 vs. controls, **p<0.01 vs. controls, #p<0.05 vs. ssC1C2/fsC1C2, n=8-9 animals
5.2.2 Force-pCa: fsC1C2 and C0C2 confers Ca\textsuperscript{2+}-sensitization of force development

To determine Ca\textsuperscript{2+}-sensitivity of force development, submaximal force was normalized to $F_{\text{max}}$ for each fiber. That is, maximal force was set to 1, and subsequent submaximal activations of the fiber was normalized as a percentage, providing a force-pCa curve (Figure 13). The force-pCa relationship reveals a leftward shift in fsC1C2 and C0C2, indicating less Ca\textsuperscript{2+} is required to generate the same amount of force. Conversely, a relative rightward shift would signify a desensitizing effect. By convention, quantification of Ca\textsuperscript{2+}-sensitivity was calculated by determining the pCa\textsubscript{50}, which is the pCa value at which half-maximal force is generated. This revealed greater pCa\textsubscript{50} values, and thus Ca\textsuperscript{2+}-sensitizing effects, by both fsC1C2 and C0C2 compared to controls (Figure 13). Once again, the pCa\textsubscript{50} value for ssC1C2 was between controls and fsC1C2/C0C2, further demonstrating MyBP-C isoforms are not functionally equivalent and perhaps operated via unique mechanisms.
Figure 13. $Ca^{2+}$-sensitivity of force development. Relative force-pCa curves demonstrate fsC1C2 and C0C2 causes a leftward shift of the curve compared to ssC1C2 and control. Quantification of pCa$_{50}$ values demonstrate fsC1C2 and C0C2 increase $Ca^{2+}$-sensitivity. Graphs represented as mean $\pm$ SEM, *p<0.05 vs. controls, **p<0.01 vs. controls, #p<0.05 vs. ssC1C2/fsC1C2, n=8-9 animals.
5.3.3 Stiffness-Force

Muscle fiber stiffness is simultaneously recorded during force recordings and are a measure of bound cross-bridges. Therefore, stiffness would be indicative of whether MyBP-C isoforms differentially regulated myosin binding to actin. At maximal levels, stiffness is not significantly different between ssC1C2, fsC1C2, and C0C2, suggesting the maximal number of XB that can be bound is not affected by MyBP-C N-termini (Figure 14). When stiffness-force relationship is analyzed, one obtains a linear relationship that is one measure of the duty ratio (Figure 14). The slope of which is the amount of XB required to generate a certain amount of force. Quantification of the slopes (stiffness/force) for the stiffness-force relationship reveals no significant difference between groups. The closest to significance was fsC1C2’s trend towards decreased stiffness/force values. Interestingly, two inferences can be made from these observations. First, lack of regulation in stiffness suggests MyBP-C N-termini may not be the primary regulators of myosin during contraction. This does not preclude MyBP-C regulation of myosin during relaxation, MyBP-C regulation of myosin in other conditions (e.g. post-translational modification, disease, etc.), nor the possibility that the assay used lacked the necessary sensitivity. Thus, MyBP-C isoforms may regulate contraction via interactions with actin. Second, the trend in fsC1C2 observed suggested potential that fsC1C2 may regulate another aspect of contractile function.
Figure 14. Stiffness-force relationship. fsC1C2 and C0C2 trend towards decreased stiffness, suggesting they may reduce the number of cross-bridges required per unit of muscle contraction. However, changes were not significantly different. Graphs represented as mean ± SEM, *p<0.05 vs. controls, **p<0.01 vs. controls, #p<0.05 vs. ssC1C2/fsC1C2, n=8-9 animals.
5.2.4 ATPase-Force

ATPase activity of muscle fibers during steady-state contraction was measured using spectrophotometric recording of NADH conversion to NAD⁺, which is enzymatically linked to ATPase activity of the myosin head. A previous trend in fsC1C2 stiffness/force values suggested fewer bound XB are required to generate force, prompting the hypothesis that fsC1C2 improves the efficiency of contraction. If so, tension cost, or the amount of ATP required to generate force, would also be reduced. To determine tension cost, the ATPase-force relationship was plotted for individual fibers, generating a linear relationship whose slope would represent tension cost (Figure 15). The steeper the slope, the higher the tension cost, and vice versa. Analysis of ATPase-force relationship demonstrated fsC1C2 alone significantly reduced tension cost compared to controls (Figure 15), suggesting fsC1C2 regulation of contraction reduced the demand for ATP required to generate force. None of the MyBP-C N’ fragments altered maximal ATPase activity. The results presented thus far demonstrate MyBP-C differentially facilitates contraction, but not necessarily a mechanism by which contraction is regulated. The literature points to two powerful interaction partners, myosin and actin, but stiffness data (or lack thereof) suggests MyBP-C N-termini may function via actin interactions. Differential binding to thin filament proteins may also explain why fsC1C2 alone decreased tension cost in the fiber studies, which is explored in the next chapter.
Figure 15. fsC1C2 decreases tension cost. (left) ATPase-force relationship (binned data) demonstrate energy expenditure per unit force. (right) Slope from the ATPase-force relationship was then calculated to determine tension cost. Graphs represented as mean ± SEM, *p<0.05 vs. controls, **p<0.01 vs. controls, #p<0.05 vs. ssC1C2/fsC1C2. n=8-9 animals.
5.2.5 Rate of tension redevelopment

I hypothesized the mechanism for MyBP-C regulation of contraction is due to interactions between MyBP-C N-termini and actin, the rate of tension redevelopment ($k_{tr}$) of the muscle fibers was analyzed. The $k_{tr}$ is a measure of XB kinetics and thin filament activation. To determine $k_{tr}$ values, a rapid release and restretch maneuver is applied to the muscle fibers at the end of contraction. This maneuver breaks XB by physically disrupting actomyosin interactions by rapidly shortening the muscle fiber sarcomere length. Subsequently, the restretch back to the original sarcomere length will allow for myosin to once again bind to actin, in which the rate of force redevelopment, $k_{tr}$, can be measured (Figure 16). Since XB interactions have been broken, the primary affecter of $k_{tr}$ is the activation state of the thin filament. Results demonstrate that at maximal activation levels, $k_{tr}$ is unchanged between groups (Figure 17). However, at submaximal activation levels (pCa 6), fsC1C2 confers higher $k_{tr}$ relative to controls, and C0C2 confers greater $k_{tr}$ relative to both controls and ssC1C2, indicating MyBP-C N-termini have graded capacity to activate the thin filament (C0C2 > fsC1C2 > ssC1C2). Interestingly, these results correlate with the force generation data at their respective activation levels (Figure 17). Specifically, $F_{max}$ and $k_{tr}$ (pCa 4.5) demonstrate no change between all groups, but both fsC1C2 and C0C2 demonstrate increased submaximal force and $k_{tr}$ (pCa 6), suggesting thin filament activation is how MyBP-C differentially regulates contraction at these ranges of Ca$^{2+}$. 
**Figure 16. Rapid release and restretch maneuver.** By rapidly shortening the sarcomere to 80%, attached cross-bridges are broken. The following restretch back to resting sarcomere length allows the detached cross-bridges to re-attach, thus regenerating force. The rate of tension redevelopment ($k_{tr}$) is a measure of thin filament activation, because the state of thin filament is the primary determinant after cross-bridges are removed.
Figure 17. fsC1C2 and C0C2 increase cross-bridge cycling kinetics at submaximal Ca\(^{2+}\) levels. The rate of force redevelopment (k_tr) was determined following a rapid release and restretch maneuver (Figure 16). Graphs represented as mean ± SEM, *p<0.05 vs. controls, **p<0.01 vs. controls, #p<0.05 vs. ssC1C2/fsC1C2, n=5-9 animals.
<table>
<thead>
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<th></th>
<th>Control</th>
<th>+ssC1C2</th>
<th>+fsC1C2</th>
<th>+C0C2</th>
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<tbody>
<tr>
<td>pCa&lt;sub&gt;50&lt;/sub&gt;</td>
<td>5.2 ± 0.46</td>
<td>5.47 ± 0.06</td>
<td>5.71 ± 0.06*</td>
<td>5.6 ± 0.16*</td>
</tr>
<tr>
<td>Tension Cost</td>
<td>4.94 ± 0.45</td>
<td>3.95 ± 0.30*</td>
<td>2.7 ± 0.40**</td>
<td>4.1 ± 0.36*</td>
</tr>
<tr>
<td>k&lt;sub&gt;tr&lt;/sub&gt; (pCa 6)</td>
<td>1.97 ± 0.98</td>
<td>3.70 ± 1.81</td>
<td>4.23 ± 0.50*</td>
<td>6.22 ± 0.53***</td>
</tr>
<tr>
<td>Force (pCa 6)</td>
<td>0.62 ± 0.32</td>
<td>3.27 ± 1.70</td>
<td>9.48 ± 2.40**</td>
<td>9.50 ± 2.09**</td>
</tr>
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**Table 2.** Effects of MyBP-C N-termini as determined by Force-ATPase assay. Units for tension cost (pmol/s/fiber volume), k<sub>tr</sub> (s<sup>-1</sup>), force (mN/mm<sup>2</sup>). Values are mean ± SEM. ***p<0.0001, **p<0.01, *p<0.05 vs. control; #p<0.05 vs. ssC1C2 (n=5-9 animals)
5.2.6 Changes in steady-state function were not due to changes in ionic strength nor presence of random exogenous protein

Ionic strength has been previously demonstrated to alter steady-state myofilament properties (April, Brandt, Reuben, & Grundfest, 1968; Iwamoto, 2000; Julian & Moss, 1981; Martyn & Gordon, 1988; Thames, Teichholz, & Podolsky, 1974). In the present study, recombinant proteins were dialyzed in PBS and subsequently lyophilized. Briefly, this means the recombinant proteins in solution were freeze-dried (see Chapter 12). The lyophilized recombinant proteins (at 10 µM) were reconstituted with relaxing or preactivation solution prior to the beginning of the fiber assays. It was possible that the residual salts that remained after the lyophilization, however minimal, may influence functional results by increasing ionic strength. A second possible source of aberrant results may arise from the presence of any random large protein. That is to say, the results were from random large proteins interacting with the myofilament and not due to specific interactions of MyBP-C fragments. Therefore, to determine whether the additional salts from the lyophilization process was sufficient to induce changes in force production, stiffness, and ATPase activity, I generated recombinant proteins (ssC1C2, fsC1C2, and C0C2) dialyzed in PBS and diH2O, followed by lyophilization and reconstitution in relaxing and preactivation solutions to a concentration of 10 µM (the concentration used in the previously presented fiber studies) and 30 µM (Figure 18). Because proteins dialyzed in diH2O would not have residual salts, these recombinant proteins would
not contribute to ionic strength. An additional control, chicken egg albumin, was processed in parallel with MyBP-C proteins to control for the presence of a random large protein in the system. Chicken egg albumin was chosen as a control, because its size is approximately the average of the MyBP-C fragments (~45 kDa).

Baseline activation data for fibers were first gathered in the absence of recombinant proteins, followed by incubation with recombinant proteins and a subsequent maximal activation. The differences between the second activation and initial activation were calculated as ΔForce, ΔStiffness, and ΔATPase activity (Figure 19). Results demonstrate no significant changes in these functional parameters, regardless of whether 10 μM recombinant proteins were dialyzed in PBS and diH₂O. This suggests that the functional studies conducted with recombinant proteins at 10 μM were not influenced significantly by increases in ionic strength. Furthermore, the observed changes in function in previous results (e.g. increase in force) are opposite of what one would predict if ionic strength were increased, as increases in ionic strength have been previously demonstrated to depress force.

To determine whether even higher concentrations of recombinant proteins dialyzed in PBS can be used, fibers were also tested in the presence of 30 μM recombinant proteins. Results demonstrate that even when recombinant proteins were lyophilized and reconstituted to a final concentration of 30 μM, the contribution of the residual salts to ionic strength were not sufficient to significantly alter the steady-state functional parameters, with the exception of fsC1C2 dialyzed in PBS,
which drastically reduced force production (Figure 19). Because recombinant fsC1C2 dialyzed in PBS was at such a low concentration initially, this required significantly larger volume to be lyophilized to reach a final concentration 30 µM. Thus, the lyophilization process drastically increased the quantities of residual salts in the lyophilized form. In support of this, fibers incubated with comparable quantities of lyophilized salts also reflected the decrease in force production observed in fsC1C2 in PBS (30 µM), but fibers incubated with fsC1C2 in diH₂O at 30 µM did not alter functional parameters. Thus, a high concentration is not recommended when using low-yield recombinant proteins dialyzed in PBS. Even slight variations in initial concentrations of recombinant proteins can greatly amplify the effects of ionic strength or possibly protein stability. Given these results, careful consideration must be given to the process of generating recombinant proteins to ensure the validity of assays where they are used in the future.
Figure 18. Protocol to determine whether changes in ionic strength affected force development. Papillary muscles were excised and initially activated to obtain baseline force, stiffness, and ATPase measurements, followed by incubation with MyBP-C N-termini dialyzed in PBS or diH₂O (10 and 30 µM). This was followed by a second activation and changes in parameters were determined.
Figure 19. Ionic strength changes do not alter force production at 10 µM. Chicken egg albumin (Alb), ssC1C2 (slow), fsC1C2 (fast), C0C2 (cardiac) dialyzed in PBS or diH2O were incubated with muscle papillary fibers using the protocol in Figure 18. (left) No changes were detected when 10 µM of proteins were used, demonstrating ionic strength did not affect force-ATPase data. (right) There were also no difference using 30 µM of proteins, with the exception of fsC1C2 dialyzed in PBS (fast PBS 30), suggesting that the ionic concentration was sufficiently increased to cause a significant depression in force. This was due to higher concentration of salts, as demonstrated by an included group of just the salts (PBS 30). Graphs represented as mean ± SEM, *p<0.01, n=3-9
5.3 Discussion

MyBP-C N-termini have graded ability to regulate muscle fiber contraction

The primary aim of the experiments in this chapter is to determine the steady-state regulation of contraction by the N-terminal region of fsMyBP-C (fsC1C2) in the context of the heart, because skeletal MyBP-C isoforms may be present in cardiac muscles (Figure 6). In addition, I compared the function of fsC1C2 to the N-terminal region of ssMyBP-C and cMyBP-C, the latter of which had been previously demonstrated to increase Ca\textsuperscript{2+}-sensitivity and submaximal force generation (Herron et al., 2006; Razumova et al., 2008; Razumova et al., 2006; Walcott, Docken, & Harris, 2015). Due to the distinct structural differences between MyBP-C isoforms within the N-terminal region, I had hypothesized each isoform has distinct regulatory profiles. The N' fragments (ssC1C2, fsC1C2, and C0C2) were applied to cardiac papillary muscle fibers in the force-ATPase assay.

The fiber force-ATPase assay is an incredibly powerful, albeit time-consuming, experimental assay, capable of measuring several parameters simultaneously from a single mounted fiber. In reality, the assay is an amalgamation of several experiments that analyze multiple relationships including force-ATPase, force-pCa, stiffness-force, and k_{ir}-pCa. Furthermore, modifications can be easily made to experiment protocols to test for additional effects that were not tested for in the present study. Here, permeabilized rat papillary muscle fibers were incubated with 10 μM ssC1C2, fsC1C2, and C0C2, and analyzed the resultant relationships
between Force, Ca\(^{2+}\) (pCa), ATPase activity, stiffness, and \(k_{tr}\) (Figure 12 - 17).

The presence of exogenous N-terminal fragments had no effect on maximum force development and fiber stiffness at pCa 4.5 (Figure 12, 14); both serving as measures of the number of attached cross-bridges. However, as reported previously (Razumova et al., 2008), exogenous C0C2 increased Ca\(^{2+}\) sensitivity of force development as evidenced by a leftward shift in the normalized force-pCa curve and thus a significant increase in the pCa\(_{50}\) (Figure 13). This, too, was the case for fsC1C2, but not ssC1C2, strongly suggesting the various MyBP-C N-termini are not functionally equivalent. The differences between fsC1C2 and C0C2 versus ssC1C2 are further emphasized by analyzing kinetic parameters of cross-bridge cycling (Figure 17).

Previous studies have demonstrated cMyBP-C can activate the thin filament independently of Ca\(^{2+}\) (Mun et al., 2014), which may underlie its ability to promote submaximal force (Herron et al., 2006) (Figure 12). To determine whether this may be a unifying mechanism by which fsC1C2 and C0C2 regulate contraction, we applied a rapid release and immediate restretch of the fiber’s length to detach cross-bridges. As expected, at maximal activation (pCa 4.5), \(k_{tr}\) was not different than control for all N-terminal fragments (Figure 17 and Table 2). However, at submaximal Ca\(^{2+}\) levels (pCa 6.0), both fsC1C2 and C0C2 increased \(k_{tr}\) relative to controls, suggesting that, in addition to Ca\(^{2+}\)-sensitization of the thin filament, these fragments can also modulate cross-bridge kinetics by activating the thin filament in a Ca\(^{2+}\)-dependent manner to promote submaximal force generation (Figure 12).
The effect of MyBP-C N-termini is summarized in Online Video 2 (https://vimeo.com/148663470). Interestingly, ssC1C2 was not significantly different from control nor fsC1C2, suggesting the slow-skeletal isoform may regulate function at an intermediate modality or at even lower Ca\(^{2+}\) levels. My working theory is that ssC1C2 regulates contraction at low Ca\(^{2+}\), while fsC1C2 regulates function at high Ca\(^{2+}\), while C0C2 regulates contraction over the full spectrum of Ca\(^{2+}\). 

**Comparison to the literature provides unique insight into molecular mechanisms by which MyBP-C regulates contraction**

The objective of this experiment was to parse out differences between ssC1C2, fsC1C2, and C0C2, with the latter fragment acting as an additional control in order to compare it with the literature. It is important to note that this study utilized a concentration (10 \(\mu\)M) that was much higher than some previously published studies using cMyBP-C fragments (Hofmann, Hartzell, & Moss, 1991; Previs et al., 2012; A. Weith et al., 2012; A. E. Weith et al., 2012), comparable to others (Razumova et al., 2008; Razumova et al., 2006; Witayavanitkul et al., 2014), and much lower than others (Herron et al., 2006). When comparing results with previously published research, one must consider the variability in concentrations used (from nM concentrations – 80 \(\mu\)M), species used (mouse, rat, chicken, human), exogenous tags (biotin, cMyc, His, TEV-cleaved), and experimental assays (laser-trap orbital, motility assay, force-ATPase). Further complicating matters is the fact that within the same group, different species of recombinant cMyBP-C were used in
different systems (e.g. mouse cMyBP-C fragment in chicken skeletal muscle) (A. Weith et al., 2012; A. E. Weith et al., 2012). That being said, there were many similarities and differences between the present results and previously published literature, some of which are worth highlighting for discussion.

As previously mentioned, MyBP-C promotion of Ca\textsuperscript{2+}-sensitivity and submaximal force generation from our results is in agreement with similar studies in the literature (Herron et al., 2006; Razumova et al., 2008; Razumova et al., 2006), though some disagreement exists within the literature (Kulikovskaya et al., 2003). The latter study did conduct its experiments at a longer SL, which has been demonstrated to eliminate the effect of cMyBP-C (Witayavanitkul et al., 2014), suggesting cMyBP-C contributes to length-dependent activation (Cazorla & Lacampagne, 2011; de Tombe et al., 2010; Kumar et al., 2015; Mamidi, Gresham, Verma, & Stelzer, 2016). Whether cMyBP-C is physically altering myofilament properties to activate LDA or transducing a signal to its myofilament neighbors requires further analysis in future studies.

One particular study was of particular interest for comparison (Witayavanitkul et al., 2014), because it was the closest to the present study and the similarities and differences yielded novel insights into my own data. In the aforementioned study, the fragment used is known as hC0C1f, which is a human cMyBP-C fragment that is cleaved after a major cardiac insult (Govindan et al., 2013; Govindan et al., 2012). This cMyBP-C fragment demonstrated reduction in F\textsubscript{max}, as well as other pathogenic qualities, in contrast to observations in the present study.
and other studies that demonstrate no change in $F_{\text{max}}$, but increases in submaximal force (Herron et al., 2006). Importantly, the source of this fragment, hC0C1f, is from a pathogenic source and cleaves at a critical region within the M-motif, which has been demonstrated to be vital for cMyBP-C function (Razumova et al., 2008), though not necessarily binding (Shaffer, Kensler, & Harris, 2009). Specifically, the first 17 amino acids of the M-motif are absolutely necessary which is absent in the hC0C1f (Witayavanitkul et al., 2014). Therefore, the hC0C1f fragment would have been able to bind to the thin filament, but due to the lack of this critical region in the M-motif, hC0C1f also was unable to promote force generation. One reason why the present study used N-terminal fragment that included up, and including, the C2 region was because of the numerous studies that demonstrated that this entire N-terminal region was necessary to recapitulate cMyBP-C function and that missing pieces were only able to partially regulate contraction. Thus, the hC0C1f acts as competitive inhibitor of force generation: it binds to the thin filament, but lacks the critical region of the M-motif and C2 domain that are required to help promote force generation.

But the hC0C1f also promoted $k_{fr}$ and $Ca^{2+}$-sensitivity, reflecting our results and much of the literature. How does it do so, considering it is missing a specific region that I just mentioned was critical to cMyBP-C function? The answer lies within the data set itself: promotion of $k_{fr}$ implicates the hC0C1f as an agent capable of influencing thin filament activation, which is closely related to $Ca^{2+}$-sensitivity. This suggests that the fragment hC0C1f, while lacking the region critical for force
generation, still has the critical regions responsible for actin binding and thin filament activation. When considering the results from the present study and Witayavanitkul et al. 2014, there appears to be a division of labor between the domains within the N-terminal region of MyBP-C: particular domains are responsible for certain aspects of MyBP-C function and/or binding to different locations on the thin filament. Overall, these results suggested that MyBP-C N-termini interaction with the thin filament underlies its differential regulation, and the next chapter expounds on my working theory through experiments that examine actin binding.
CHAPTER SIX

ACTIN BINDING STUDIES

6.1 Abstract

The fiber force-ATPase assays from Chapter Five demonstrated that the MyBP-C N-terminal fragments are not functionally equivalent. C0C2 and fsC1C2 had significantly greater activation of the thin filament and submaximal force production, while ssC1C2 only trended towards significant regulation of contraction. These results suggested that MyBP-C binding to the thin filament may underlie functional regulation of steady-state contraction. To investigate MyBP-C binding effects, three experiments with these recombinant proteins were employed: 1) actin cosedimentation 2) 3D reconstructions 3) TPA assays. My results with the actin cosedimentation suggests that fsC1C2 and C0C2 have greater binding capacity to bind actin. In support of this, 3D reconstructions of actin binding demonstrate fsC1C2 and C0C2 bind two subdomains of actin (SD1 and SD2), while ssC1C2 bind only one subdomain (SD1). Interestingly, SD1 is the binding site for myosin, and thus may be why ssC1C2 is unable to regulate contraction at higher Ca$^{2+}$. However, at low Ca$^{2+}$, 3D reconstructions of thin filaments demonstrated C0C2 and ssC1C2 had greater capacity to activate the thin filament than fsC1C2. Finally, TPA assays demonstrated each MyBP-C uniquely regulated actin torsional movement, with C0C2 and ssC1C2 exhibiting increased resilience relative to fsC1C2.
6.2 Results

6.2.1 Actin binding – cosedimentation assays

To assess the binding affinities and capacities of MyBP-C N-termini to actin, I applied actin cosedimentation assays (Figure 20) in which increasing concentrations of ssC1C2, fsC1C3, and C0C2 (1-30 µM) were incubated with F-actin (5 µM) and rapidly spun down. Combined data from high-speed cosedimentation of MyBP-C fragments (Figure 21) revealed affinity (K\text{d}) to actin (Table 3) and binding capacity (B\text{max}) for each isoform. Previous reports have independently determined the K\text{d} and B\text{max} values for C0C2 (Shaffer et al., 2009), and comparisons with our C0C2 cosedimentation results reveal similar binding values (Shaffer et al. 2009: K\text{d} 13.7 ± 5.5 and B\text{max} = 0.92 ± 0.11). Interestingly, comparisons with the ssC1C2 and fsC1C2 reveals unique actin binding profiles. The ssC1C2 binding curve yielded the lowest K\text{d}, suggesting significantly higher binding affinity relative to fsC1C2 and C0C2. However, fsC1C2 and C0C2 exhibited approximately twice the B\text{max} value relative to ssC1C2, suggesting greater binding capacity. This last result suggested fsC1C2 and C0C2 may bind have two binding sites on actin, while ssC1C2 only had one. However, cosedimentation assays alone could not determine whether binding was the case. Therefore, we next collaborated with the lab of Dr. Roger Craig to confirm binding capacity and more specifically determine localization of binding.
Figure 20. Cosedimentation binding data for C0C2. A representative high speed cosedimentation assay. (left) C0C2 (1-30µM) was mixed with 5 µM F-actin and spun for 30 min. at 390,000 x g. (right) Quantification of binding curves required comparison with standard curve that was run on each gel, consisting of varying known molar ratios of C0C2 to actin. This was done for multiple experiments using ssC1C2, fsC1C2, and C0C2.
Figure 21. fsC1C2 and C0C2 have higher F-actin binding capacity. (left) Summary data of actin cosedimentation data demonstrates fsC1C2 and C0C2 has approximately twice the B_{max} (binding capacity) of ssC1C2 (see Table 3). (right) actin monomer diagram showing the four subdomains. The higher capacity suggests possible binding to twice the number of actin subdomains. (n=6-12)
<table>
<thead>
<tr>
<th>MyBP-C isoform</th>
<th>n</th>
<th>$K_d$ (µM)</th>
<th>$B_{max}$ (mol/mol actin)</th>
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<tr>
<td>ssC1C2</td>
<td>7</td>
<td>2.08 ± 0.76*</td>
<td>0.45 ± 0.15*#</td>
</tr>
<tr>
<td>fsC1C2</td>
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<td>10.51 ± 4.75</td>
<td>1.14 ± 0.25</td>
</tr>
<tr>
<td>C0C2</td>
<td>12</td>
<td>16.62 ± 2.88</td>
<td>1.11 ± 0.12</td>
</tr>
</tbody>
</table>

**Table 3.** Summary of dissociation constants ($K_d$) and molar binding ratios ($B_{max}$) calculated from binding of recombinant MyBP-C N-terminal proteins to F-actin. Values are mean ± SEM. (p<0.05 significant change compared *vs. C0C2 #vs. fsC1C2)
6.2.2 Actin binding – 3D reconstructions

In collaboration with Dr. Ji Young Mun and Dr. Roger Craig, we investigated the effects of ssC1C2, fsC1C2, and C0C2 on actin binding. While the ideas and conclusions made in this section are my own, the figures in this section represent the work of Dr. Mun and are reproduced here with permission.

In the previous cosedimentation assay, the $B_{\text{max}}$ value for fsC1C2 and C0C2 were approximately twice that of ssC1C2, suggesting the fsC1C2 and C0C2 has twice the binding capacity as ssC1C2. Thus, fsC1C2 and C0C2 may bind to twice as many locations on actin as ssC1C2. Specifically, fsC1C2 and C0C2 may bind to two subdomains on actin, while ssC1C2 only binds to one subdomain. To visualize binding interactions of recombinant MyBP-C N-termini, we utilized 3D reconstructions of computer-aligned actin filaments decorated with ssC1C2, fsC1C2, and C0C2. Both filamentous actin (F-actin) and reconstituted thin filaments were decorated with MyBP-C N-terminal fragments and imaged by negative stain electron microscopy (EM) at low Ca$^{2+}$ (Figure 22). The increase in filament diameter for F-actin in the presence of each MyBP-C N-termini confirms the fragments’ ability to bind actin (data not shown). Similarly, all fragments bound to reconstituted thin filaments, consisting of actin (A), tropomyosin (Tm), and troponin (Tn) (Figure 23). We propose reconstituted thin filaments represents the state of the thin filament at low Ca$^{2+}$, because Tn and Tm are present on the actin filament, blocking the myosin-
binding site. Conversely, F-actin alone represents the state of the thin filament at high Ca\(^2+\), because the myosin-binding sites on actin are not obscured.

In agreement with the actin cosedimentation assay results, 3D reconstructions indicate fsC1C2 and C0C2 decoration on the actin filament creates a greater space-filling density (Figure 22), correlating roughly with actin subdomains 1 (SD1) and subdomain 2 (SD2). In contrast, ssC1C2 decoration creates a much smaller density that correlates with subdomain 1 of actin. Interestingly, subdomain 1 of actin is also known to be the primary binding site for the myosin S1 region. At higher Ca\(^2+\), myosin S1 region would compete for the SD1 region and displace ssC1C2, which would explain why its regulatory function is limited at higher Ca\(^2+\). However, because fsC1C2 and C0C2 bind to both SD1 and SD2 of actin, these isoforms are capable of continued regulation of contraction at high Ca\(^2+\).

Previous studies reported that binding of cMyBP-C N-terminal fragments (C0C2, C0C3) to thin filaments, in the absence of Ca\(^2+\), shifts Tm from the “blocked” position to “closed” position, which then favors weak cross-bridge formation (Mun et al., 2014). 3D reconstruction of the thin filament was used to determine the degree of Tm shift by ssC1C2, fsC1C2, and C0C2. To visualize relative difference in MyBP-C-mediated Tm shift, the position of Tm in the blocked and closed positions are indicated by the red and green helices, respectively (Figure 23). Densities representing Tm position demonstrate that all MyBP-C N-terminal fragments can shift Tm position at least from the blocked to closed position, and thus activate the thin filament in a manner similar to that initiated by Ca\(^2+\). However, C0C2 and
ssC1C2 mediated a greater Tm shift compared to the closed Tm position (green helix), while fsC1C2 only shifted Tm equal to the closed position. Furthermore, C0C2 had a greater capacity to shift Tm relative to ssC1C2, revealing a graded thin filament activation capacity (C0C2 > ssC1C2 > fsC1C2) at low Ca\(^{2+}\). The unique capacity of each isoform to shift Tm strongly suggests differential thin filament activation may be one mechanism by which MyBP-C isoforms regulate contraction at low Ca\(^{2+}\).
Figure 22. 3D reconstruction of F-actin and MyBP-C visualize binding sites. F-actin were decorated with MyBP-C N-termini and computer-assisted alignment of EM images was used to create 3D reconstructions. (left) Actin filament alone was used as a reference for modeling with MyBP-C N-termini, and the actin monomer diagram was overlaid to identify approximate locations of subdomain (SD) 1 – 4. Densities representing ssC1C2 (red circle), fsC1C2 (blue circle), and C0C2 (black circle) are highlighted. Original image courtesy of Dr. Ji Young Mun and Dr. Roger Craig.
Figure 23. 3D reconstructions of reconstituted thin filament and MyBP-C demonstrate thin filament activation. Reconstituted thin filament consist of actin, tropomyosin (Tm), and troponin (Tn). The red helix is the Tm in the blocked position, when it is blocking the actin SD1 where myosin binds. The green helix is Tm in the closed position, with actin SD1 exposed. Decoration with ssC1C2 and C0C2 are able to shift Tm beyond the closed position, while fsC1C2 shifted Tm to the closed position. Original image courtesy of Dr. Ji Young Mun and Dr. Roger Craig.
6.2.3 Actin binding - TPA assays

In collaboration with Dr. Brett Colson and David Thomas, we investigated the effects of ssC1C2, fsC1C2, and C0C2 on actin dynamics. While the ideas and conclusions in this section are my own, the figures in this section represent the work of Dr. Brett Colson and are reproduced with permission.

To test whether MyBP-C isoforms alter the nature of interaction with actin at low and high Ca\(^{2+}\), we utilized time-resolved phosphorescence anisotropy (TPA) to determine how MyBP-C isoforms influence the torsional dynamics of actin filaments (Figure). We propose that the F-actin model allows representation of the state of the thin filament at high Ca\(^{2+}\), when the myosin-binding sites on actin are exposed. More importantly, TPA assay provides data on actin dynamics, which becomes important when considering actin filaments are not stationary players during contraction: the helical coils of actin filaments are rapidly twisting, with variable amplitude and rates of torsion. Differential actin binding by the N-terminal region of MyBP-C isoforms would uniquely restrict the amplitude and rates of actin, potentially in a manner to assist or resist myosin binding.

To determine intrafilament torsional motions with the TPA assay, actin filaments were uniformly labelled at Cys374 with a phosphorescent dye (Figure 24A). MyBP-C N-termini decreased amplitude of labelled actin TPA decay (Figure 24B), and all three MyBP-C N-termini had similar effects in increasing final anisotropy at low and high occupancy (Figure 24C). TPA amplitude was greatly reduced by all three MyBP-C N-termini (Figure 24D), with C0C2 having the greatest
amount of restriction on the amplitude of actin motion, followed by fsC1C2 and ssC1C2. Interestingly, while C0C2 increased the rate of actin torsional dynamics, fsC1C2 decreased this torsional rate (Figure 24E and Table 4). Because C0C2 restricted the range of motion of actin, the rates of torsion were increased as expected, a trend also observed in ssC1C2-bound actin. Both increased rate and decreased amplitude is indicative of an actin filament whose movement is constrained, but is moving very rapidly within its limitations by ssC1C2 and C0C2. However, fsC1C2 reduced both amplitude and rate of actin filament torsion, suggesting fsC1C2 binding may result in an actin filament that is less dynamic and have a reduced effect on the resilience of the actin filament. Resilience is an indicator of stored elastic energy, and is determined by Rate/Amplitude (A. Y. Lin et al., 2012). We observed ssC1C2 and C0C2 confer higher resilience relative to fsC1C2 (Figure 24F), and higher resilience has been previously shown to increase tension cost (Jones et al., 1999). Interestingly, our fiber studies also demonstrate ssC1C2 and C0C2 exhibit higher tension cost relative to fsC1C2 (Figure 15), suggesting fsC1C2 reduces tension cost through isoform-specific regulation of actin resilience. Furthermore, fsC1C2 also reduced tension cost relative to control fibers, suggesting fsC1C2 is an active, rather than passive, participant in the regulation of actin resilience. The unique restrictions on actin torsional motion by each MyBP-C isoform all indicate unique binding interactions with actin.
Figure 24. **MyBP-C differentially regulates actin torsional dynamics.** Actin binding was determined by time-resolved phosphorescence anisotropy (TPA) and actin co-sedimentation assays. 1 μM of MyBP-C N-termini binding to actin alters actin amplitude, rate of twisting, and resilience. (A) Schematic of intrafilament torsional dynamics. (B) MyBP-C N-termini results in the decay of ErlA-actin (black trace) compared to actin alone (grey trace) and (C) Dependence of final anisotropy of C0C2, fsC1C2, and ssC1C2 on actin binding. (D) Final anisotropy of actin as a function of the fraction bound to MyBP-C N-termini. All three MyBP-C N-termini restricted the amplitude of actin torsional dynamics, but C0C2 significantly reduced the amplitude relative to fsC1C2 and ssC1C2. (E) fsC1C2 decreased the rate of actin torsion, but C0C2 increased the rate of actin torsion. (F) Maximal relative resilience was measured as rate/amplitude compared to actin alone. C0C2 and ssC1C2 both significantly increased resilience, but fsC1C2 did not. Source data shown in Table 4. Original image courtesy of Dr. Brett Colson and Dr. David Thomas.
<table>
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<th>$\phi_1$</th>
<th>$\phi_2$</th>
<th>$r_1$</th>
<th>$r_2$</th>
<th>$r_\infty$</th>
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<tr>
<td>None</td>
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<tr>
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<tr>
<td>fsC1C2</td>
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<td>21±3</td>
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<td>0.059±0.005*</td>
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**Table 4.** Effect of Saturating Concentration of MyBP-C N-terminal regions on the Parameters of Actin TPA Decay. Values are mean ± SEM (n > 5). (*p<0.05 significant change compared to control)
6.3 Discussion

The mechanism by which MyBP-C isoforms regulate at low and high Ca$^{2+}$ is explored in this section. These results collectively demonstrate distinct actin profiles by each MyBP-C N-termini, suggesting its functional role is rooted in changes at the molecular level. The higher affinity ($K_d$) and lower capacity ($B_{max}$) of ssC1C2 to actin (Figure 21 and Table 3) may explain why ssC1C2 only activates during the early stages of contraction at low Ca$^{2+}$ (Figure 23), while modestly regulating contraction at higher Ca$^{2+}$, as observed in fiber studies (Figure 17). Conversely, fsC1C2 and C0C2 had approximately twice the capacity to bind to actin, suggesting these isoforms may bind to an additional site on actin (Figure 22). This additional interaction may confer the greater ability to regulate contraction at higher Ca$^{2+}$ levels later in the contraction process. Indeed, 3D reconstructions of F-actin demonstrates fsC1C2 and C0C2 interact with actin SD1 and SD2, while ssC1C2 only binds SD1. Assuming binding to the same actin monomer, this makes perfect sense why ssC1C2 is no longer able to regulate contraction at later stages of contraction, because actin SD1 is also a well-known myosin-binding site for the myosin S1. As the contractile process progresses, additional myosin S1 will directly compete with MyBP-C for actin SD1. Therefore, the lone actin-binding site for ssC1C2 will be occupied while fsC1C2 and C0C2 can still regulate contraction during these later stages of contraction via its interaction with SD2.
Some questions arise when considering the other actin subdomains and myosin interaction with actin. First, why does MyBP-C not bind to actin subdomains 3 and 4? Second, how might myosin binding to actin SD1 disrupt fsC1C2 and C0C2? The literature provides clues that may answer both in the future. First, MyBP-C is unlikely to bind to SD3 and SD4: these subdomains are thought to be responsible for intrafilament interactions (Chapter Two) (Kabsch et al., 1990). The helical structure of F-actin requires tight interactions between its actin monomers, which provides a major groove in which another helical thin filament, Tm, and SD3 and SD4 mediate that function.

Second, myosin would certainly compete with fsC1C2 and C0C2 binding to actin SD1, though evidence of myosin binding to other regions of actin indicate myosin S1 interaction may actually be further away from where fsC1C2 and C0C2 bind to actin. Our data does not preclude MyBP-C or myosin binding on different sites on multiple actin, nor binding to inter-subdomain pockets. While SD1 is thought to be the primary binding site for myosin, an unresolved loop region of myosin head is known to interact with the SD1 and SD3 junction (Milligan, 1996; Milligan et al., 1990). However, this junction is further away from SD2 and less likely to interfere with fsC1C2 and C0C2 binding to SD2 (Figure 21), though myosin S1 and MyBP-C are still in close proximity and our data does not preclude this possibility. Furthermore, myosin S1 and MyBP-C may interact on different actin
monomers along the F-actin filament and not necessarily be in direct competition. Interestingly, the actin-binding affinities of MyBP-C N-termini versus myosin S1 have not been compared. As an aside, it may very well be that MyBP-C N-termini have a higher affinity for actin SD1 compared to myosin and its role is to block actomyosin interaction. This is unlikely, as fiber studies performed with fragments in the present study and elsewhere would have shown significant suppression of force production, especially in light of the high concentrations used. Furthermore, even if this was the case in vivo, this effect would be localized to every third myosin within the C-zone and not throughout the sarcomere.

MyBP-C isoforms exhibit different capacity to shift Tm (C0C2 > ssC1C2 > fsC1C2) (Figure 23). Remarkably, ssC1C2 and C0C2 were able to shift Tm position beyond the ‘closed’ position, suggesting the greater capacity to shift Tm by C0C2 and ssC1C2 at low Ca\textsuperscript{2+} is important for initiating the contraction process. At higher Ca\textsuperscript{2+}, fsC1C2 and C0C2 are the predominant activators of the thin filament (Figure 17), suggesting these isoforms are responsible for regulation in the later stages of contraction. Increased thin filament activation by fsC1C2 and C0C2 would expose the myosin-binding site on actin, promoting actomyosin interaction and thus cross-bridge duration. This supports the previous research that suggests MyBP-C contributes to the maintenance of longer cross-bridge duration (Palmer et al., 2011), and may thus explain why we observe greater submaximal force production (Figure 12). Enhancement of cross-bridge duration almost assuredly rests on the ability of MyBP-C to regulate actomyosin interaction via direct interaction.
In support of this, MyBP-C/actin binding confers structural stability to actin akin to myosin/actin binding during cross-bridge formation (Figure 24), suggesting maintenance of actin resilience structural stability may be necessary to facilitate optimal force production, which is often disrupted in disease states (A. Y. Lin et al., 2012). MyBP-C stabilization of actin may therefore be an important aspect of its role at high [Ca\(^{2+}\)], and provides evidence of a so-called “C-bridge,” in which MyBP-C binding to actin creates a second thick-to-thin filament interaction to modulate the cross-bridge. This MyBP-C/actin interaction may compete with myosin for binding site or place a load on actin, thus limiting contractility (Walcott et al., 2015; A. Weith et al., 2012; A. E. Weith et al., 2012). Alternatively, MyBP-C may place a load on myosin, limiting cross-bridge formation via a tethering mechanism (Barefield & Sadayappan, 2010; Kampourakis, Yan, Gautel, Sun, & Irving, 2014). Indeed, recent studies show MyBP-C may favor myosin binding at high [Ca\(^{2+}\)] (Kampourakis et al., 2014; Previs et al., 2016), further highlighting the Ca\(^{2+}\)-dependent effects of MyBP-C. Whether MyBP-C acts on myosin or actin at high [Ca\(^{2+}\)], these constraints suggest an important physiological role for MyBP-C in preventing muscle hypercontractility (Spudich, 2014). Although our studies focused on MyBP-C N-terminal interactions with actin, they certainly do not preclude MyBP-C interaction with myosin, and may indeed suggest interactions both myosin and actin, possibly even simultaneously.
CHAPTER SEVEN

UNLOADED SHORTENING IN VITRO: FROM STEADY-STATE TO DYNAMIC CONTRACTION

7.1 Abstract

How does MyBP-C regulation of steady-state contractile properties translate to dynamic contraction? To answer this question, unloaded shortening kinetics experiments were applied to determine how MyBP-C isoforms may differentially regulate dynamic contraction. Using cultured adult rat ventricular myocytes (ARVM) as our baseline model, we overexpressed full-length, cMyc-tagged ssMyBP-C, fsMyBP-C, and cMyBP-C using adenoviral constructs (Figure 25). After 48H in culture, biochemical and unloaded shortening analysis were used to determine expression, localization, and function. Western blot demonstrated partial replacement of the endogenous cMyBP-C in cardiac cells, and immunofluorescence demonstrated localization of exogenously-expressed MyBP-C isoforms approximately within the C-zone. Surprisingly, functional data demonstrates no change in contraction kinetics, but ARVM overexpressing ssMyBP-C and fsMyBP-C exhibited faster relaxation kinetics relative to cMyBP-C. This suggests that cMyBP-C expression may prolong relaxation kinetics and/or ssMyBP-C and fsMyBP-C facilitate faster relaxation kinetics. To parse out the potential mechanisms behind this unexpected result, computer modeling was employed next.
Figure 25. Adenoviral-mediated overexpression of MyBP-C in ARVM. Adult rat ventricular myocytes (ARVM) were isolated, infected with full-length, cMyc-tagged MyBP-C isoforms, and cultured for 48 hours. Replacement levels, localization, and unloaded shortening function was then measured.
7.2 Results

7.2.1 Replacement Levels

ARVM were infected with adenoviral constructs (MOI: 1000) overexpressing full-length, Myc-tagged ssMyBP-C, fsMyBP-C, and cMyBP-C, and cultured for 48 hours. After 48 hours, ARVM were prepared for Western blot analysis to determine the amount of replacement of endogenous cMyBP-C (Figure 26). ARVM groups, including an uninfected control (UI), were probed with antibodies for ssMyBP-C, fsMyBP-C, cMyBP-C, and cMyc antibodies to determine the amount of exogenously expressed MyBP-C isoforms present in ARVM. Results demonstrate that ARVM infection was specific to the adenoviral construct, as well as cMyc. Due to limitation of culture time (48 hours), replacement levels were low, though still significantly higher compared to UI controls and not significantly different between groups.
**Figure 26. Adenoviral constructs partially replaced endogenous MyBP-C.** ARVM infected with adenoviral constructs overexpressing full-length, myc-tagged MyBP-C isoforms were cultured for 48 hours (MOI 1000). Uninfected ARVM were included as controls. (left) Western blots demonstrate specific expression of ssMyBP-C and fsMyBP-C. Due to the presence of endogenous cMyBP-C in ARVM, adenoviral-mediated MyBP-C expression was determined by cMyc tag detection. All analyses are shown normalized to β-actin. (right) Adenoviral-mediated overexpression of ssMyBP-C (red stripes), fsMyBP-C (blue stripes) and cMyBP-C (black stripes) partially replaced endogenous cMyBP-C (black solid), as demonstrated by densitometry analysis. There were no significant differences in replacement levels between adenoviral-mediated ssMyBP-C, fsMyBP-C, and cMyBP-C.
7.2.2 Localization determined by IF

Infected ARVM were also imaged using immunofluorescence, and included immunolabeling with α-actinin, which detected the Z-disc locations (Figure 27). This allowed visualization of the exogenously expressed MyBP-C within the sarcomere. IF data demonstrates that exogenously-expressed MyBP-C isoforms were localized specifically within the sarcomere and probably within the C-zone, as determined by doublets located within the Z-discs. Resolution of the IF images were not precise enough to determine whether adenovirally-mediated expression of MyBP-C caused localization into the adjacent D-zone.
Figure 27. Exogenous MyBP-C isoforms localized within the sarcomere. ARVM infected with adenoviral constructs overexpression of full-length, myc-tagged ssMyBP-C, fsMyBP-C and cMyBP-C were cultured for 48 hours. Localization of exogenous (A-F) ssMyBP-C, (G-L) fsMyBP-C, and (M-R) cMyBP-C was detected using immunofluorescence. Staining for α-actinin delineates the location of cardiomyocyte Z-discs, the borders of the sarcomere (A, D, G, J, M, P). Localization of adenoviral-mediated overexpression of MyBP-C was detected using antibodies for ssMyBP-C (B, E) fsMyBP-C (H, K) and cMyc (N, Q). Merged images demonstrated MyBP-C expression was limited specifically within the sarcomere (C, F, I, L, O, R).
7.2.3 Unloaded Shortening

After 48 hours in culture, ARVM were paced (2.0 ms, 1 Hz, 20V), and contractile kinetics were analyzed. Baseline parameters were not significantly different, such as resting sarcomere length nor percent shortening. Combined traces of each group demonstrated no significant changes in contraction kinetics, but suggested MyBP-C isoforms altered relaxation kinetics (Figure 28). Time to %Baseline is a measure of how fast a cell returns to resting sarcomere length (Baseline). Thus, time to 50% baseline is how long the cell takes from peak contraction to 50% of its resting sarcomere length. Quantification of time to 10% baseline, 50% baseline, and 90% baseline reveals cMyBP-C prolongs relaxation kinetics. Fitting of the relaxation constant, tau, also demonstrates cMyBP-C extends the amount of time the cell takes to relax.
Figure 28. cMyBP-C prolongs relaxation kinetics. (far left) Unloaded shortening was measured by changes in sarcomere length (SL) during dynamic contraction and relaxation (ARVM paced at 1Hz, 20V, 2ms). Trace images of combined unloaded shortening demonstrates changes to relaxation kinetics (error bars removed for clarity). Relaxation kinetics were quantified by (top left) time to % baseline, how fast the cell returns to a % of resting SL, and (top right) relaxation constant tau, a logarithmic fit of the relaxation curve. Both relaxation parameters, combined with composite traces of unloaded shortening, demonstrate cMyBP-C prolongs relaxation kinetics. (graphs represented as mean ± SEM, *p<0.05 vs. uninfected controls, #p<0.05 vs. cMyBP-C). (bottom left) Resting sarcomere length of ARVM were unchanged suggesting similar starting points of contraction. (bottom right) Percent shortening was also not different between groups suggesting similar contractility. (n=42-78 cells, n= 4-6 animals)
7.3 Discussion

To our surprise, MyBP-C overexpression in ARVM resulted in changes to relaxation kinetics, while no changes were detected in contraction kinetics. This may have been due to limitations of the experimental parameters and/or the ARVM model system. Several scientific and practical considerations were taken when designing the experiment to accommodate the ARVM model. For example, unlike previous steady-state experiments that used just the N-terminal region, full-length MyBP-C isoforms were used, because our studies have previously demonstrated abnormal localization and function due to the lack of the C-terminal region in the ARVM system (Kuster et al., 2015), although the N-terminal region may still localize within the A-band (Herron et al., 2006). Immunofluorescence imaging revealed adenoviral-mediated MyBP-C isoforms expressed and localized properly to the sarcomere A-band where myosin thick filaments reside (Figure 27), and doublets reveal the exogenously-expressed MyBP-C may even have localized specifically within the C-zone, though IF images were not at sufficient resolution to preclude possible expression in the D-zone as well. Similarities between the MyBP-C isoforms in the C-terminal region suggest potential for such proper C-zone localization. However, the PEVK repeats of cardiac and skeletal isoforms of titin (to which MyBP-C C-terminus purportedly anchors to the thick filament) have distinctive binding properties (Linke et al., 2002), and one can not presume skeletal MyBP-C isoforms would bind to cardiac titin PEVK with the same affinity, and therefore affect contractile kinetics.
In addition, only partial replacement of the endogenous cMyBP-C occurred after 48 hours, suggesting the changes in relaxation kinetics were a combination of the contribution of endogenous cMyBP-C and exogenous MyBP-C isoforms. While percentage of cells that contract in the adult rat cardiomyocyte system is considered greater than mouse (Pavlovic, McLatchie, & Shattock, 2010), cellular viability is still limited post-isolation (Westfall, Rust, & Metzger, 1997) due to its lack of regenerative and proliferative ability (van Berlo et al., 2014). This allows adenoviral-mediated expression from 30% to 90% replacement of smaller proteins, but in those instances, structural integrity is compromised, as shown in their IF images (Fink et al., 2001; Westfall et al., 1997). Furthermore, these functional experiments were in permeabilized cells rather than intact cells, and cell sarcomere lengths were mechanically set. In my preparations for intact cardiomyocytes, reliable cell viability could only be maintained for 48 – 72 hours. After this time period, detectable sarcomere lengths were considerably shorter. In addition, cardiomyocyte structure had either noticeably deteriorated, maintained their structure but were no longer responsive to electrical stimulation, or responded to stimulation initially followed by rapid deterioration in cell structure.

Isolation and culture techniques appear to be as varied as the labs that implement primary cardiomyocyte systems (Wolska & Solaro, 1996). Therefore, longer culture times were attempted via changing several parameters that have
been suggested to work, including, but not limited to, changing media more frequently (every 12 hours) and adding ascorbic acid to remove oxygen radicals and cellular waste, cellular pacing (0.1 Hz, 2.0ms) during culture to maintain contractility, and addition of 2,3-butanedioxome (BDM) to inhibit contraction during culture. The most successful protocol tested was an adaptation of a long-term mouse cardiomyocyte isolation protocol (O’Connell, Rodrigo, & Simpson, 2007) that utilized a custom HMEM-based media that contained BDM, which was then carefully and slowly washed out with Tyrode’s solution just prior to experimentation. This was a compromise of several systems we tested, and is not without its own advantages and disadvantages, as discussed previously. However, this method yielded the most consistently usable cells and was therefore implemented for the in vitro unloaded shortening.

Regarding the fact that only relaxation kinetics were altered and not contraction kinetics, the limitations in experimental parameters (1 Hz, 2.0 ms) could have limited what changes could be detected. Voltage was most commonly set at 20V, but was adjusted such that the minimal amount was required to stimulate the ARVMs. At a frequency of 1 Hz, the contraction rate of ARVMs would closer reflect that of a human heart. However, these conditions do not truly reflect that of a human heart nor rodent heart, because the cardiac system would not have the same MHC composition as humans (Miyata, Minobe, Bristow, & Leinwand, 2000), and is paced much slower than the frequency experienced in a rat heart (Azar, Sharp, & Lawson, 2011). Furthermore, the MyBP-C isoforms used in all steady-state and
dynamic experiments were that of mouse isoforms, though mouse and rat MyBP-C isoforms share similar homology. While there is much to be said regarding the caveats of choosing particular conditions, the mix-and-match of different systems in the literature have yielded many answers as well as more questions.

The question originally proposed was: how does MyBP-C regulation of steady-state contractile properties translate to dynamic contraction? The answer from unloaded shortening in intact myocytes turned out to be: cMyBP-C prolongs relaxation kinetics relative to ssMyBP-C and fsMyBP-C. The question now becomes: by what mechanism does cMyBP-C facilitate prolonged relaxation kinetics relative to ssMyBP-C and fsMyBP-C?
CHAPTER EIGHT

UNLOADED SHORTENING IN SILICO:
COMPUTER MODELING DEMONSTRATES THIN FILAMENT ACTIVATION BY MYBP-C ISOFORMS CONTRIBUTES TO REGULATION OF RELAXATION

8.1 Abstract

To determine whether our steady-state observations on thin filament activation can explain changes in relaxation kinetics during dynamic contraction, we utilized an online muscle simulator (OMS) that had been previously established to simulate unloaded shortening of a single cardiomyocyte (Campbell et al., 2013; Kuo et al., 2014). I applied parameters based on our steady-state experiments to the OMS that accounted for differential capacity to activate the thin filament (Figure 29 and Table 5). The steady-state studies suggest that ssMyBP-C activates the thin filament at low \([\text{Ca}^{2+}]\), fsMyBP-C at high \([\text{Ca}^{2+}]\), and cMyBP-C activates at both low and high \([\text{Ca}^{2+}]\). When accommodating for the greater capacity of cMyBP-C to activate the thin filament, in silico unloaded shortening traces demonstrate increased thin filament activation capacity results in prolonged relaxation kinetics (Figure 29). The right-shift of cMyBP-C’s relaxation trace in silico reflects the in vitro unloaded shortening data, in which cMyBP-C prolongs relaxation kinetics relative to ssMyBP-C and fsMyBP-C (Figure 28, 29). Combined, these studies indicate that thin filament activation capacity can directly contribute to relaxation kinetics.
8.2 Results

8.2.1 Thin filament activation

In collaboration with Dr. Stuart Campbell, we investigated the effects of ssMyBP-C, fsMyBP-C, and cMyBP-C using a muscle simulation program. While alterations to the simulations were conducted by myself and conclusions made in this section are my own, the muscle simulation program itself was written and made available for use by Dr. Campbell. The results from the simulations reproduced are here with permission.

The muscle simulation (OMS) applied in the present study was previously used to model unloaded shortening of intact cardiomyocytes (Campbell et al., 2013; Kuo et al., 2014; Sheikh et al., 2012), and adapted for the purpose of modeling how different MyBP-C isoforms may alter contractile kinetics. Previous implementations of this simulation did not account for contribution of cMyBP-C, and thus provided an opportunity to model how all three isoforms regulate contractile function. Based on the results from steady-state experiments, I concluded that MyBP-C isoforms differentially regulated thin filament activation: ssMyBP-C activates the thin filament at low [Ca²⁺], fsMyBP-C activates at high [Ca²⁺], and cMyBP-C activates at both low and high [Ca²⁺] (Online Video 3 https://vimeo.com/148663467). The online muscle simulation has several parameters relating to various contractile properties, and the closest parameter that fit changes in thin filament activation is $k_{on}$, or the rate constant for Ca²⁺ association (Kreutziger, Gillis, Davis, Tikunova, & Regnier, 2007) (Table 5). Due to the complexities of the simulation, only one
parameter was altered to minimize variability in the output. Simulation results demonstrate that increased ability for thin filament activation, the relaxation kinetics of the unloaded shortening trace is clearly prolonged with no such changes in contraction kinetics. This data suggests that the expanded capacity of thin filament activation by cMyBP-C accounts for the prolonged relaxation kinetics demonstrated by in vitro unloaded shortening experiments (Figure 29).
Figure 29. Computer modeling of unloaded shortening demonstrate thin filament activation can affect relaxation kinetics. To determine whether steady-state regulations (Figure 12,17,23) can explain observed dynamic contraction, computer simulations of unloaded shortening were run accommodating the differential capacity of MyBP-C isoforms to activate the thin filament. See Table 5 for source values. (bottom) Traces of in silico unloaded shortening suggest the increased capacity of cMyBP-C to activate thin filament contributes mechanistically to relaxation kinetics.
Table 5. Computational modeling parameters account for differential thin filament activation ($k_{on}$) capacities of MyBP-C. ($k_b$: rate of Ca$^{2+}$ binding to troponin C; $k_u$: rate of Ca$^{2+}$ dissociation from troponin C; $k_{on}$ rate of tropomyosin transition from blocked to closed state; $k_{off}$: rate of tropomyosin transition from closed to blocked; $f_{app}$: rate of XB attachment; $g_{app}$: rate of XB detachment from pre-powerstroke state; $h_f$: rate of forward powerstroke; $h_b$: rate of reverse powerstroke; $g_{xb}$: rate of XB detachment from the post-powerstroke state; gammaB: cooperative coefficient representing tropomyosin-tropomyosin interactions; Temp: temperature in degrees Kelvin).
8.3 Discussion

Our data suggests thin filament activation can directly contribute to relaxation kinetics. I theorize this effect occurs because MyBP-C activation of the thin filament would favor cross-bridge attachment. Because cMyBP-C has a greater capacity to activate the thin filament, the actin-myosin interaction would presumably be stronger and longer. The longer duration of these cross-bridges would prolong relaxation, which is observed in both in silico and in vitro unloaded shortening. This effect is summarized in Online Video 4, and has been suggested previously (Davis et al., 2016; Moss, 2016). However, important limitations should be addressed to maintain perspective when interpreting these results. It is worth noting that our modeling only accounted for differential MyBP-C thin filament activation to focus on how steady-state experiments from the present study translates to dynamic contraction and by what mechanism.

The simulation does not account for the presence of endogenous cMyBP-C present in the ARVM system, and assumes the only contribution to relaxation kinetics is that of the particular MyBP-C isoform. This is distinctly different from in vitro experiments, as endogenous cMyBP-C is present in ARVM. One alternative was to utilize cMyBP-C knock-out mice models that lack cMyBP-C incorporation in the sarcomere, two of which are available (Harris et al., 2002; McConnell et al., 1999). As the first experiment conducted to determine differences between the MyBP-C isoforms, this was not the route chosen due to complications from the development
of HCM and DCM in these mice that may mask or obscure isoform-specific changes. In addition, mouse cardiomyocytes are much less resilient than rat cardiomyocytes; thus, the adenoviral-mediated strategy of gene transfer would be significantly more difficult to implement or an alternative in vivo delivery approach would have been required. Therefore, as previously mentioned, the in vitro ARVM express endogenous cMyBP-C that was only partially replaced by exogenous ssMyBP-C, fsMyBP-C, and cMyBP-C after 48H in culture. The differences in MyBP-C regulation of relaxation kinetics is thus likely under estimated by experimental data.

The steady-state experiments that were used to draw conclusions regarding thin filament activation are also worth considering. Steady-state experiments are like snapshots of a dynamic process, and the variety of experiments used capture the process from different vantage points. Both of which lend insight into MyBP-C regulatory function, but highlight considerations when translating the results. Besides the fact that the changes to the simulations were made based on steady-state and not dynamic experimental data and that several steady-state experiments were utilized, the most discernable difference is the use of N-terminal versus full-length MyBP-C isoforms in the steady-state and full-length. As MyBP-C localization is highly regulated within the C-zone, the effects using N-terminal proteins is likely representative of an enhanced effect of MyBP-C. This indicates that our steady-state experiments exaggerate MyBP-C isoform function while dynamic experiments underestimates MyBP-C function. Thus, the relaxation data, however congruent, must be interpreted cautiously.
In both the *in silico* and *in vitro* unloaded shortening studies, the stimulation parameters of cardiomyocytes were not altered, which allowed for comparison, but also limited the scope of what we could ascertain between MyBP-C isoforms. The simulator did not yet have function to alter the stimulation parameters, which would then therefore allow for greater flexibility in potential comparisons with *in vitro* data. Changes in frequency, pulse duration, and voltage could be altered to more clearly parse out differences between MyBP-C isoforms, such as kinetics-frequency relationships. Permeabilized ARVM experiments can also be pursued to determine force-frequency and force-pCa relationships, bridging the gap in understanding from the steady-state fiber function data and the dynamic intact myocytes data. These experiments would provide greater evidence that thin filament activation can contribute to changes in relaxation kinetics, and also determine whether this mechanism precludes alternative roles for MyBP-C in regulating contraction.

**Do relaxation results have any physiological implications?**

Why does MyBP-C regulate relaxation? The answer may lie in what we know regarding the expression profile of skeletal MyBP-C. In a healthy, adult heart, fsMyBP-C is not expressed, but is present after some insult to the heart, such as HF (B. Lin et al., 2013). HF is defined by the American Heart Association as: ‘... a complex clinical syndrome that results from any structural or functional impairment of ventricular filling or ejection of blood.’ (Yancy et al., 2013)
Because mutations in the cMyBP-C are a primary cause of HCM, the ventricular or septal wall thickens, and the ventricular chamber has difficulty filling with blood. In order to compensate for the lack of ability to relax, fetal isoforms of MyBP-C that facilitate relaxation kinetics are expressed. This reversion to fetal gene programming has been suggested to be cardioprotective (Depre et al., 1998; Rajabi et al., 2007). Conversely, disruption of the normal signaling pathways that regulate gene expression have been suggested to be maladaptive (Dirkx, da Costa Martins, et al., 2013; Hannenhalli et al., 2006). The expression of fsMyBP-C in the heart during HF is therefore an artifact of these changes. The truth is likely somewhere in the middle: the expression of fetal gene programming may express contractile proteins that are initially compensatory, but long-term re-expression of these non-native isoforms eventually becomes disadvantageous and harmful.

The skeletal isoforms are expressed in the heart only conditionally and in relatively low quantities, and thus the physiological impact of unloaded shortening results may be limited in scope. The question then is: do changes in relaxation kinetics reflect a physiological phenomenon in the context of skeletal muscles? The key to answering this question may rest within what we know about skeletal disease-causing mutations in ssMyBP-C and fsMyBP-C. Currently, mutations in the skeletal MyBP-C isoforms are known to cause three distinct skeletal myopathies: 1) distal arthrogryposis, Type 1 (DA1) 2) distal arthrogryposis, Type 2 (DA2) and 3) lethal congenital contracture syndrome, type 4 (LCCS4). In these diseases, patients experience the inability or extreme contractures of the hand and feet, a congenital
deformity known as ‘club foot’ or ‘club hand’. Patients with DA2 also exhibit distinct contractures of craniofacial muscles. These abnormalities suggest skeletal MyBP-C is responsible for relaxation of these muscles and important in the structural development of these skeletal muscles. However, there is a noticeable lack of skeletal muscle deformities in other muscles in these human patients, such as large locomotor muscles in the leg and vital diaphragm muscles required for breathing. This may indicate that MyBP-C expression is more critical for muscles that require fine motor skills, or these known mutations only affect the molecular function of MyBP-C that are essential in the hands and feet.

8.5 Alternative molecular mechanisms

As previously suggested, MyBP-C may also act as a viscous load (Palmer et al., 2011; A. Weith et al., 2012; A. E. Weith et al., 2012) to put a brake on contraction during the later stages of contraction (Previs et al., 2012). MyBP-C N-termini binding to actin may act as a drag on actin (Walcott et al., 2015) or form a so-called “C-bridge,” in which MyBP-C binding to actin creates a second thick-thin filament interaction, though the latter mechanism may be more pronounced had full-length MyBP-C been used. Whether MyBP-C enhances cross-bridge duration, viscous load, or actin drag, unloaded shortening data suggests relaxation kinetics can be influenced as a result. Further complicating matters, other assays have suggested changes in functional effects by MyBP-C can be explained by a tethering mechanism in which MyBP-C acts as a load on myosin, limiting cross-bridge formation (Barefield & Sadayappan, 2010). In support of this mechanism, high Ca^{2+} causes a
conformational change in cMyBP-C N-terminal region that may favor myosin interaction (Previs et al., 2016). Regardless of mechanism, these constraints on the sarcomere at higher Ca\textsuperscript{2+} suggest a role for MyBP-C in limiting muscle hypercontractility: which is often observed in HCM, of which cMyBP-C mutations are a major cause (Spudich, 2014). As such, our experiments do not preclude MyBP-C interaction with myosin, or other regulatory effects on the thin filament (Walcott et al., 2015). Indeed, though our mechanistic studies features MyBP-C interactions with actin, our data also suggests further studies are required into different MyBP-C isoforms' interactions with myosin to fully understand the function of each MyBP-C isoform. More specifically, focus needs to be directed towards the function of skeletal isoforms of MyBP-C where they are predominantly expressed: in skeletal muscles.
CHAPTER NINE

FSKO MICE: FAST-SKELETAL MYBP-C KNOCK-OUT

SKELETAL MUSCLE DEFICIENCES OFFSET ONE ANOTHER

9.1 Abstract

FSKO (fast-skeletal MyBP-C knock-out) mice are the first mouse model designed to investigate the function of fsMyBP-C. EDL (extensor digitorum longus) muscle fibers from non-transgenic (NTG), heterozygous (FSKO-HET), and homozygous (FSKO-HOM) were selected for analysis by force-ATPase assay. EDL were selected due to robust expression of fsMyBP-C in NTG muscles and would therefore exhibit the greatest alterations to molecular function. Our results demonstrate that FSKO mice do not alter force generation nor Ca$^{2+}$-sensitivity of force development. Stiffness is decreased in FSKO-HOM mice, suggesting that the absence of fsMyBP-C releases a load on myosin that would facilitate myosin binding to actin and is therefore predicted to promote force production. In support of this, the ATPase activity in FSKO-HOM mice is also decreased, suggesting less energy expenditure is required to form an attached cross-bridge. However, rate of tension redevelopment ($k_r$) is significantly reduced in FSKO-HOM mice, which is predicted to decrease force production. Together, this suggests that absence of fsMyBP-C results in offsetting deficiencies in the FSKO mice, though the contribution of ssMyBP-C needs to be considered.
9.2 Results

9.2.1 Expression profile in FSKO mice

Western blot analysis was used to determine the protein expression profile of NTG, HET, and HOM FSKO mice that were 5 – 6 months old. FSKO mice were generated by targeting exons 2-4, and 20-22 of the MYBPC2 gene. The expression of fsMyBP-C and ssMyBP-C of NTG FSKO mice (Figure 31), which is C57BL6 background, matches that of FVB/N WT mice (Figure 7). Four commonly-studied muscles were selected to represent different types of skeletal muscles: extensor digitorum longus (EDL), flexor digitorum brevis (FDB), a slow-type muscle, soleus (SOL), and tibialis anterior (TA). The EDL and FDB are considered fast-type skeletal muscles, SOL is a slow-type, and TA is a mix of fast- and slow-type muscle fibers. The EDL and TA are on analogous to muscles on your shin, the FDB is located on the soles of your feet, and the SOL is located beneath your calf muscles. In NTG and HET mice, ssMyBP-C is present in all skeletal muscles tested and fsMyBP-C is expressed only in EDL and TA. However, HOM mice demonstrated a complete lack of fsMyBP-C in all muscles, and ssMyBP-C expression levels are unchanged in response to the lack of fsMyBP-C in these studies (Figure 32).
Figure 30. Targeting strategy for FSKO mice. (top) wild-type fsMyBP-C gene (bottom) targeted fsMyBP-C gene. Exons 2-22 were replaced with a neomycin resistance gene.
Figure 31. FSKO mice lack fsMyBP-C. Dual-color western blot was employed to

determine expression profile of FSKO (fast-skeletal MyBP-C knock-out) mice,

which are of C57BL6 background. NTG and HET FSKO mice expression profile

reflects that of FVB/N WT mice (Figure 7)
Figure 32. FSKO mice do not significantly alter ssMyBP-C expression. (left) Knock-out of fsMyBP-C does not alter the expression of ssMyBP-C, as determined by quantification of Western blots. (right) fsMyBP-C is not present in FSKO-HOM mice, though there may be partial dysregulation in HET mice.
9.2.2 Force Generation and Ca\textsuperscript{2+}-sensitivity of force-development

For comparison with previous steady-state experiments, the force-ATPase assay used was essentially the same with some distinct changes to accommodate the skeletal muscle. EDL muscle fibers were prepared as previously described (Wood, 1978; Wood, Zollman, Reuben, & Brandt, 1975). Briefly, muscles were excised tendon-to-tendon and tied to wooden sticks with silk surgical suture such that the muscle was taut to prevent damage during the permeabilization/cryopreservation process. The muscle were skinned over a period of at least 2 weeks in 1:1 glycerol/relax solution at -20°C, after which the fibers were mechanically separated, t-clipped and attached to a force transducer and length controller. The sarcomere length of EDL fibers were set to a slack length of 2.5 µm (Hofmann, Greaser, & Moss, 1991)

Force-pCa relationships demonstrate no significant differences between NTG, HET, and HOM FSKO mice (Figure 33). While the result was expected between NTG and HET mice, the lack of change in FSKO-HOM mice was surprising. Once the force was normalized, there was no observable shift in force-pCa curves between all groups, and quantification of the pCa\textsubscript{50} confirmed there were no changes in Ca\textsuperscript{2+}-sensitivity of force development (data not shown).
Figure 33. FSKO mice do not exhibit changes in force nor Ca²⁺-sensitivity. Knock-out of fsMyBP-C did not significantly alter force-pCa relationship (left), nor relative force-pCa relationship (right). Graphs represented as mean ± SEM, *p<0.05 vs. NTG, n=5-6 animals.
9.2.3 Stiffness-Force Relationship and ATPase-Force

Myosin S1 interaction with actin is a key event in the contraction process, and MyBP-C regulation of this process is likely due to either interaction with actin and/or myosin. Therefore, I analyzed parameters that would elucidate how fsMyBP-C regulates myosin and actin by analyzing stiffness and $k_{tr}$, respectively. It should be noted that these measurements are influenced by several factors. Stiffness is a measure of attached cross-bridges and is proportional to tension. Stiffness is measured by applying rapid, oscillating length changes and recording the change in force ($\Delta$force/$\Delta$length) (Ford, Huxley, & Simmons, 1977). Results demonstrate no changes in maximal stiffness levels (Figure 34), but when stiffness was normalized to force, FSKO-HOM mice demonstrated significant decrease stiffness-force relationship. This suggests fsMyBP-C may place a load on myosin, and this load is released due to the absence of fsMyBP-C in FSKO-HOM mice.

The lack of inhibitory activity of fsMyBP-C in FSKO-HOM mice would predict increased force generation, but this was not the case (Figure 33). However, the ATPase-force relationship of FSKO-HOM mice suggest that less energy expenditure is required per unit of force (Figure 35). Quantification of ATPase-force slopes determined tension cost, but only demonstrates a trend towards decreased energy expenditure and is not significantly different.
Figure 34. FSKO mice exhibit reduced stiffness. (left) knock-out of fsMyBP-C did not alter maximal stiffness levels. (right) However, reduced stiffness per unit force demonstrates fewer cross-bridges are required to generate force. Graphs represented as mean ± SEM, *p<0.05 vs. NTG, n=5-6 animals.
Figure 35. FSKO mice trend towards reduced ATPase activity. (left) ATPase-force relationship demonstrates the energy expenditure required to generate force. (right) Quantification of slopes of the ATPase-force relationship gives tension cost, which trends towards significance, but is not significantly different. Graphs represented as mean ± SEM, *p<0.05 vs. NTG, n=5-6 animals.
9.2.4 Rate of Tension Redevelopment

The rate of tension redevelopment \( k_{tr} \) is a measure of thin filament activation, as previously described in Chapter Five. After the previous experiments were completed, \( k_{tr} \) was analyzed following a rapid release-restretch maneuver. Results demonstrate that, at high \( \text{Ca}^{2+} \) (pCa 4.5), FSKO-HOM mice have significantly decreased \( k_{tr} \) relative to NTG, but not FSKO-HET (Figure 36). However, at low \( \text{Ca}^{2+} \) (pCa 6), \( k_{tr} \) is not significantly different between all groups. First, this suggests fsMyBP-C also regulates thin filament activation in skeletal muscles. Second, regulation of fsMyBP-C regulation of thin filament activation seems to limited to higher \([\text{Ca}^{2+}]\). The lack of fsMyBP-C in FSKO-HOM mice thus would partially diminish thin filament activation in the sarcomere, and therefore, one would predict reduced force production and this is not the case (Figure 33). Recall that stiffness data suggest FSKO mice exhibit reduced myosin inhibition, and therefore the opposing effects may cancel one another out.
Figure 36. FSKO mice are deficient in thin filament activation at high Ca\textsuperscript{2+}. (left) FSKO mice exhibit reduced ability to activate the thin filament, as demonstrated by reduced $k_\text{tr}$. (right) At submaximal Ca\textsuperscript{2+}, there was no difference, suggesting fsMyBP-C regulates thin filament activation specifically at higher Ca\textsuperscript{2+} levels in striated muscle. Graphs represented as mean ± SEM, *p<0.05 vs. NTG, n=4-6 animals.
9.3 Discussion

Collectively, the current data on FSKO mice demonstrate two intriguing effects due to the lack of fsMyBP-C. First, FSKO-HOM mice exhibit decreased stiffness-force ratios, suggesting that fsMyBP-C normally places a load on myosin. One would predict the release of this inhibitory load would result in increased force, but the data showed no change in force production (Figure 33). Second, FSKO-HOM mice have decreased $k_r$, suggesting fsMyBP-C also activates the thin filament. Therefore, the diminished activation capacity would thereby also result in reduced force production, but again force was unchanged. At face value, these data sets do not make any sense on their own. Together, these data suggest that fsMyBP-C has dual effects on myosin and actin that offset one another and is therefore why no change in force nor Ca$^{2+}$-sensitivity of force development was observed. Moreover, these effects may be Ca$^{2+}$-dependent and occur at different stages of contraction. For example, inhibition of myosin by fsMyBP-C may occur during relaxation, while activation of actin thin filament may occur during contraction. The $k_r$ data certainly suggest that fsMyBP-C activation capacity occurs at higher [Ca$^{2+}$] and is congruent with previous data in the cardiac muscle system that suggested fsMyBP-C operates at higher, but not lower [Ca$^{2+}$] (Figures 17). However, this range is shifted towards maximal [Ca$^{2+}$].

One noticeable observation from the ATPase-force relationship is that FSKO-HOM mice trends towards a decrease in ATPase activity, but the quantification of
the ATPase-force slopes to determine tension cost is not significantly different. This brings up an important stipulation regarding the muscle fibers used. Closer inspection revealed the possibility of distinct subpopulations within the data set. In other words, the fibers in the EDL fiber bundles may have consisted of distinct types of fibers (Augusto, 2004). When manually dissecting the fibers under the scope, one can not accurately distinguish the difference between fiber types, and thus the EDL fiber bundles used were of unknown composition. In combination with the presence of ssMyBP-C, the unknown percentage of fast- vs. slow-type fibers may contribute to the relatively muted effects observed in the functional assays. Therefore, to fully elucidate fsMyBP-C function, other studies must be disseminated to piece together a comprehensive functional story.

Intriguingly, there is one recent study that also focused on fsMyBP-C, though in a zebrafish model (M. Li et al., 2016). In this study, morpholino antisense nucleotides were used to knockdown approximately 50% of fsMyBP-C, variant 2B. Despite vastly different methodologies, this study is worth discussing, because it is currently the only study that focuses on fsMyBP-C. Their results demonstrated structural abnormalities, such as shorter sarcomere lengths, as well as functional changes, such as increased maximal shortening velocity and decreased active force. The latter functional effect reflects our own data suggesting fsMyBP-C places a load on myosin. The removal of this load would thereby also promote shortening velocity in both FSKO mice and the zebrafish morphants. However, limitations must be
considered when comparing these very different studies. One significant difference between the FSKO mice and zebrafish was how drastic the difference in deficiencies observed. In the zebrafish study, just 50% knockdown of fsMyBP-C, variant 2B resulted in gross morphological shrinkage, contractile changes in shortening velocity, as well as elevated apoptosis and protein-degradation factors. In the FSKO mice I studied, there is absolutely no fsMyBP-C present, though the functional changes were mild by comparison. The difference in species may explain this dichotomy. First, fsMyBP-C variant 2B is the dominant isoform in zebrafish, with some fsMyBP-C variant 2A, and very little ssMyBP-C. Conversely, in mice, ssMyBP-C is by far the predominant MyBP-C isoform, being expressed in all skeletal muscles and fsMyBP-C is only expressed in mixed-type muscles. Therefore, we would expect a stronger phenotype in a ssMyBP-C knock-out mouse model rather than the current fsMyBP-C knock-out mice. Second, there are several known splice variants of ssMyBP-C in mammals and only one fsMyBP-C isoform (Ackermann, Kerr, et al., 2015; Ackermann & Kontrogianni-Konstantopoulos, 2011b, 2013). In contrast, the opposite is true in zebrafish: there are two fsMyBP-C variants and only one ssMyBP-C variant. Third, cMyBP-C is present in zebrafish skeletal muscle during development (Chen et al., 2013; Cheng et al., 2013), but cMyBP-C is not present at all in mouse skeletal muscle during at any time (Gautel et al., 1998; B. Lin et al., 2013). The last limitation that I will discuss is that while their structural assays demonstrate that sarcomere length is diminished after fsMyBP-C knockdown (M. Li et al., 2016), their active force measurements to not normalize to the lower
sarcomere length. Therefore, the higher force production observed in controls may simply be due to changes in length (de Tombe et al., 2010).

These limitations are not meant to diminish the results from the zebrafish study. On the contrary, these simply illustrate the need for more in depth investigation into the function of fsMyBP-C. Clearly, deficiency in MyBP-C can directly affect structure and function. This becomes particularly vital as mutations in the skeletal MyBP-C isoforms are discovered as the cause for skeletal myopathies. (Bayram et al., 2016; Gurnett et al., 2010; X. Li et al., 2015).
CHAPTER TEN
FUTURE DIRECTIONS

Abstract

There are many avenues of research to pursue in the relative short-term to elucidate more about the MyBP-C family of proteins. Even within the research specific to cMyBP-C, much has yet to be determined. It is very exciting to think about the numerous and intriguing avenues of research that can be conducted in the near future.

Recombinant Proteins with phosphomimetic sites and PKA challenge

It is well known that cMyBP-C has several phosphorylation sites that alter its regulation of actin and myosin (Barefield & Sadayappan, 2010; Sadayappan & de Tombe, 2012), and studies are always being conducted on the mechanism of cMyBP-C phosphorylation (Colson et al., 2016; Previs et al., 2016). However, there is little known about how skeletal MyBP-C isoforms are phosphorylated. ssMyBP-C has several known phosphorylation sites (Ackermann & Kontrogianni-Konstantopoulos, 2011a), and circumstantial evidence suggests fsMyBP-C also has phosphorylation sites (Ackermann, Ward, et al., 2015), which will have to be clearly identified using mass spectrometry. Isoproterenol challenge to intact cells or incubation of PKA in permeabilized fiber studies would be able initially highlight how skeletal MyBP-C phosphorylation modulates their regulation. To more clearly delineate how skeletal
MyBP-C phosphorylation sites alter regulation of function, phosphomimetic and phosphoablated recombinant proteins can be utilized to determine how they affect interactions with actin and myosin, as well as function in a variety of single myocyte and fiber bundle assays. Future studies could also incorporate partial phosphomimetic/phosphoablated sites to determine hierarchy of regulation of these sites. Finally, transgenic models expressing phosphomimetic or phosphoablated MyBP-C may be necessary to demonstrate a physiological consequence of skeletal MyBP-C PTM.

**Sufficiency of N-terminal domains (e.g. C0-P/A-C1-C2)**

Studies have painstakingly demonstrated which domains of cMyBP-C are required for regulation of function (Bezold, Shaffer, Khosa, Hoye, & Harris, 2013; Colson et al., 2012; Herron et al., 2006; Kensler, Shaffer, & Harris, 2011; Razumova et al., 2008). Presumably, skeletal MyBP-C functional domains are similar, but there are distinct structural differences between MyBP-C isoforms. Determination of analogous or distinct binding and functional regulation may yield greater insights into MyBP-C function. Just within the context of the FSKO mice, determining whether the addition of recombinant fsC1C2 is sufficient to recover changes in function would be an important first step and relatively simple experiment, given the materials and baseline data are already there. Incubation with various lengths of MyBP-C N-termini in skeletal fibers or adenoviral-mediated expression in skeletal muscles may further yield interesting functional regulation, though the latter
strategy is difficult given the resistance of adult skeletal muscle fibers to adenoviral infection due to downregulation of adenoviral receptors.

**What does the C0 domain do?**

One of the most stark differences between cMyBP-C and its skeletal counterparts is the presence of an additional C0 domain on cMyBP-C: an N-terminal sequence of approximately 100 amino acids (Harris, Belknap, Van Sciver, White, & Galkin, 2016; Ratti et al., 2011). The function of this distinct structure in cMyBP-C is still debated in the literature (Herron et al., 2006). One possible experiment is to create a recombinant skeletal MyBP-C protein with the C0 domain artificially added to the N-terminus to investigate whether this would render skeletal MyBP-C binding and function more similar to that of cMyBP-C. In the same vane, the C0 domain can be specifically removed and used to demonstrate whether this would render cMyBP-C more similar in binding and function to that of skeletal MyBP-C. Removal of the C0 domain has previously been investigated, but not specifically conducted to differentiate functional regulation between skeletal and cardiac MyBP-C.

**MyBP-C, the Myosin Mesa, and the Super-Relaxed State**

Recently, research has demonstrated specific interactions of myosin head and MyBP-C (Spudich, 2015), whose formation entails unknown consequences. The proposed interaction site between a flat region of the myosin S1 region and the M-motif of cMyBP-C suggests charged residues are responsible for regulating their interaction. This interaction may very well be one mechanism that stabilizes the myosin head in the relaxed state (Woodhead et al., 2005), as well as the super-
relaxed state (McNamara et al., 2016). The physiological implication of this are completely unknown.

**MyBP-C, Actin, and the ‘C-bridge’**

Myosin S1 interaction with actin forms a cross-bridge that is central to the sliding filament theory of sarcomeric contraction (Spudich, 2001). However, MyBP-C activation of the thin filament via binding to actin (Mun et al., 2014) (Figure 23) would indicate the presence of a second thick-to-thin filament interaction, which is colloquially known as the ‘C-bridge.’ The C-bridge would presumably not contribute to active force production directly, but its formation could affect passive tension and stiffness. Therefore, to elucidate the potential presence or impact of such a C-bridge, one could simply study function in the presence of a myosin inhibitor, such as BDM or blebbistatin.

**ssMyBP-C splice variants**

One factor of complication regarding ssMyBP-C are the numerous splice variants known, which result ssMyBP-C that have unique N-terminal regions and C-terminal regions (Ackermann, Kerr, et al., 2015; Ackermann & Kontrogianni-Konstantopoulos, 2011b). While in mouse, only two variants have been identified, the possibility of specific roles and differential expression of ssMyBP-C variants may drastically alter known function of ssMyBP-C. Whether ssMyBP-C variations result in subtle changes in regulation or have completely different regulation is unknown, and may further elucidate why we express multiple isoforms of MyBP-C.
in vivo function

The biophysical assays of FSKO mice in the present study sheds light on the mechanics at the fiber level. However, experiments are necessary to determine whether fsMyBP-C is necessary in vivo, such as treadmill function, grip strength, voluntary wheel run, gait/locomotion analysis. More specific analysis can be done on anesthetized FSKO mice by analyzing torque injury, fatigability, and nerve-evoked muscle function. There are two possible scenarios that I predict, both of which are opposite one another: first, given the mild changes in fiber studies, the in vivo studies may demonstrate only subtle changes in function. This would require challenging the animal with stressors, such as forced exercise. Second, the opposite may occur, where in vivo function is severely diminished. The expression profile of fsMyBP-C demonstrates its presence in mixed skeletal muscles, such as the EDL and TA (Figure 7,30), as well as gastrocnemius and quadriceps (data not shown). These latter muscles suggest fsMyBP-C regulates function of very large muscle groups and therefore, FSKO mice would exhibit significant in vivo functional deficits.

SSKO mice

Concurrently with the development of the FSKO mice, SSKO mice (slow-skeletal MyBP-C knock-out) are now available for study. SSKO mice are not to be confused with liver-specific squalene synthase knock-out mice, L-SSKO (Nagashima et al., 2015). In contrast, the SSKO mice exhibit higher rates of early death relative to the FSKO mice (data not shown), suggesting a greater necessity for ssMyBP-C which
is expressed in all muscle types. The unique advantage of using SSKO mice is that the targeting strategy removes all variants of ssMyBP-C, both known and unknown. Therefore, the functional data from this mouse model would define the collective function of ssMyBP-C.

**DSKO mice as the baseline**

Crossing SSKO mice with FSKO mice yields the DSKO mice (double-skeletal MyBP-C knock-out), which would lack both skeletal MyBP-C isoforms. This is the baseline that should be used to compare both SSKO and FSKO mice. In FSKO mice, ssMyBP-C present and therefore, FSKO mice actually demonstrates the contribution of ssMyBP-C to function. Conversely, SSKO mice still express fsMyBP-C, and therefore SSKO mice would actually demonstrate the contribution of fsMyBP-C to function. Thus, comparison to a DSKO mouse model will provide a much clearer understanding of skeletal MyBP-C.

**Length-Dependent Activation**

The mechanism behind length-dependent activation (LDA) is still erroneously taught in medical texts as due to optimal overlap of thick- and thin-filaments (Berne, Koeppen, & Stanton, 2010). However, studies have demonstrated the precise mechanism may lie within cellular and myofilament properties (Allen & Kentish, 1985; Kentish, ter Keurs, Ricciardi, Bucx, & Noble, 1986; Kobayashi, Jin, & de Tombe, 2008; Konhilas, Irving, & de Tombe, 2002a; Kumar et al., 2015; ter Keurs, Rijnsburger, van Heuningen, & Nagelsmit, 1980), rather than purely structural explanations (Farman, Walker, de Tombe, & Irving, 2006). More recently, cMyBP-C
has been demonstrated to specifically regulate length-dependency (Kumar et al., 2015; Mamidi, Gresham, & Stelzer, 2014; Mamidi et al., 2016; Witayavanitkul et al., 2014). It should be noted that LDA effects are relatively muted in skeletal muscle systems (Konhilas, Irving, & de Tombe, 2002b; Konhilas et al., 2003). In these studies, the partial replacement with the ssTnI significantly blunted LDA, suggesting isoform-specific and muscle-specific regulation of contraction. However, other studies have demonstrated that fast-skeletal muscles and cardiac muscles exhibit more length-dependent effects than slow-skeletal (Konhilas et al., 2002b). This correlates with the present data that demonstrates fsC1C2 and C0C2 have similar function at higher [Ca$^{2+}$]. Therefore, I hypothesize fsMyBP-C has a greater contribution to LDA relative to ssMyBP-C in cardiac and skeletal muscles. The experiments presented in this study can be replicated at both low (2.0 µm) and high (2.3 µm) sarcomere lengths to determine whether the MyBP-C N-termini could regulate LDA and whether to the same degree. This was not pursued due to purely experimental reasons: the fragility of the papillary fibers and the time-consuming nature of the experiment precluded additional experiments at high SL. Switching to trabeculae or skeletal muscle fibers, which are more stable, may allow us to conduct serial measurements of the same fiber at low and high SL. Furthermore, narrowing the focus to just ssMyBP-C or fsMyBP-C would also free up time in a day in which to do experiments.

**Removing the effects of endogenous MyBP-C**

Because the experiments used WT rats as the baseline model system, the
presence of endogenous cMyBP-C in our studies complicates interpretation of the results. The alternative is to use papillary muscles from one of two cMyBP-C knock-out mouse models that originated from the Moss and Seidman group (Harris et al., 2002; McConnell et al., 1999), and continue pursuing experiments involving the FSKO, SSKO, and DSKO mice. Our objective was to determine the baseline physiological function of MyBP-C N-termini, and thus we did not pursue using these knock-out models as the baseline model several reasons. First, both the Moss and Seidman mouse models exhibit cardiac disease phenotype, specifically HCM and DCM, respectively. The contribution of fibrosis alone could mask several parameters that we measured. Second, the mouse model presents particular challenges. Mouse papillary muscles are smaller and would not have yielded as many usable fibers as rat papillary muscles. Given the context of our study with four experimental groups, there would have been very slim margin of error for the trimming of papillary muscles. In addition, mouse ventricular myocytes are also not nearly as robust as rat ventricular myocytes. As such, our culture experiments would have required additional time and expertise, with comparatively little payoff. Rat and mouse cardiomyocyte culture experiments were pursued side-by-side, and our mouse cardiomyocyte experiments were much less fruitful. Finally, the SSKO and FSKO mice are simply so new that we do not know whether the absence of either ssMyBP-C nor fsMyBP-C cause changes in morphology or exhibit a disease phenotype that may complicate function. All of these together indicated a simpler system using WT animals for our initial experiments. That is not to say that these experiments are not
worth pursuing. On the contrary, they would be very informative on two fronts. They would validate whether the functional results we observed in WT systems were accurate and not obfuscated by endogenous MyBP-C. Moreover, these experiments would be interesting in whether N-termini are sufficient to rescue function in disease states.
CHAPTER ELEVEN

FINAL THOUGHTS

Based on my data, ssMyBP-C and fsMyBP-C regulation of contraction is limited to low and high ranges of Ca\(^{2+}\), respectively, while cMyBP-C regulation extends over the full spectrum of Ca\(^{2+}\). This interpretation is supported by the role of cardiac versus skeletal muscles: the cardiac sarcomere is exposed to a wide and dynamic range of Ca\(^{2+}\) on a beat-to-beat basis. Conversely, skeletal muscles are not contracting and relaxing every second and therefore not exposed to the same range of Ca\(^{2+}\) with the same frequency. Various skeletal muscles also have different purposes: postural muscles have different roles than locomotor muscles. Therefore, skeletal muscles can fine-tune the role of specific muscles by expressing different amounts of slow- and fast-skeletal MyBP-C to accommodate the role of specific skeletal muscles. cMyBP-C has been demonstrated to be highly tunable via post-translational modification (Colson et al., 2012; Colson et al., 2016; Jia, Shaffer, Harris, & Leary, 2010), and such modifications can potentially act on actin and myosin at different contractile stages as either facilitator, inhibitor, or both. Such modifications to each MyBP-C isoform structure and function deserve further studies, particularly in light of the vast amount of information that can be elucidated from the study of multiple isoforms together. The convergence of steady-state and dynamic experiments in the present study, as well in vitro and in silico methods all suggest MyBP-C isoforms are distinctive in their regulation of muscle contraction.
CHAPTER TWELVE
MATERIALS AND METHODS

Expression and Purification of Recombinant MyBP-C proteins

Recombinant proteins representing the N-terminal domains up to the C2 domain of slow-skeletal, fast-skeletal, and cardiac (ssC1C2, fsC1C2, and C0C2, respectively) were generated, purified, and analyzed with SDS-PAGE (Govindan et al., 2012) (Figure 10). Each MyBP-C isoform was expressed using a pET28 Expression system (Millipore 70777) in E. coli, and included an N-terminal His-tag consisting of six histidine residues. Protein expression in BL21 was induced by IPTG and purified through a nickel-nitrilotriacetic acid affinity column in which eluted fractions were combined and dialyzed in PBS. Recombinant proteins were used in all following steady-state biophysical studies to determine the functional differences between the slow-skeletal, fast-skeletal, and cardiac isoforms of MyBP-C. Details regarding the transcript and amino acid sequence of N-terminal peptides can be found in Table 1. Recombinant proteins were quantified and the volume required for the final desired concentration was calculated. Proteins were then aliquoted, lyophilized, and stored at -80°C until ready for use in fiber experiments.
**Papillary Muscle Fiber Bundle Preparation**

Male Sprague-Dawley rats (250g) (Harlan) were euthanized using Beuthanasia in accordance to Institutional Animal Care and Use Committee protocol at Loyola University Chicago. The whole heart of the rat was rapidly excised underwent retrograde perfusion with Krebs-Henseleit Buffer (KHB) via proximal aorta. The heart was cut to carefully expose the papillary muscles in the left ventricle. Papillary muscles were carefully excised under a dissecting scope (Zeiss Discover.V8 Stereo, PlanAPO S 0.63x FWD 81mm) and permeabilized overnight in 1% Triton X-100 (Sigma) in mounting relaxing solution (6.3 mM ATP, 6.48 mM Mg Cl₂, 10mM EGTA, 10 mM Na₂CrP, 49.76 mM Kprop, 100 mM BES, pH 7) at 4°C, removing cell membrane and membrane-bound proteins. After overnight permeabilization, papillary muscles were further trimmed into fiber bundles approximately 1 mm in length under the dissecting microscope (Figure 11). Straight and parallel fiber bundles were selected based on uniformity and attached at each end with aluminum t-clips. Images were taken under the dissecting scope using an attached digital camera (Canon EOS Rebel T3i). Each fiber bundle was gently cleansed by transferring between a series of 4 washes containing fresh relaxing solution on ice and used within 12 hours. The t-clipped fibers were attached to a force transducer (BAM21-LC; World Precision Instruments, Sarasota, FL) and high speed length controller (Aurora Scientific, Inc.). Muscle dimensions (cross-sectional area, length, and volume) were determined using an ocular micrometer mounted in the dissection microscope (resolution, ~10 μm). These
muscle dimensions were used to normalize contractile force, sarcomere length (SL) and ATPase activity. SL was measured in passive relaxed condition by laser diffraction as previously described (de Tombe & ter Keurs, 1990), adjusted to and maintained at 2μm. Briefly, a helium-neon laser beam was directed through the isolated muscle fiber bundle, in which muscle striation pattern diffract the laser beam into a diffraction pattern. The striations of the muscle fiber bundle act as a grating, with known angles of diffraction, and the projected pattern can therefore be used to measure average SL.

**Skeletal Muscle Fiber Preparation**

Skeletal muscles were permeabilized as previously described (Wood et al., 1975). *Extensor digitorum longus* (EDL) and *soleus* (SOL) muscles were excised from the legs of FSKO mice. Care was taken to limit the damage to the muscle fibers by cutting at the tendons, followed by submergence in 4°C relax solution (67.93 mM KOH, 6.24mM ATP, 10mM EGTA, 10mM Na₂CrP, 47.58 Kprop, 100 mM BES, 6.54 mM MgCl₂, pH 7). Fiber muscles were attached to short sticks by tying surgical sutures around the muscle tendons, under a dissecting scope (Zeiss Discover.V8 Stereo, PlanAPO S 0.63x FWD 81mm). These ‘muscles-on-a-stick’ were then submerged in 1:1 glycerol/relax solution in a 5 mL conical tube and stored at -20°C for at least two weeks prior to use in functional assays. The purpose was two-fold: 1) to ensure optimal permeabilization and 2) to cryoprotect the muscle fibers for long-term storage. After at least two weeks, fibers were taken out and split into smaller fiber bundles of approximately 1 mm in length and t-clipped. Images were taken under
the dissecting scope using an attached digital camera (Canon EOS Rebel T3i). Each fiber bundle was gently cleansed by transferring between a series of 4 washes containing fresh relaxing solution on ice and used within 12 hours. The t-clipped fibers were attached to a force transducer (BAM21-LC; World Precision Instruments, Sarasota, FL) and high speed length controller (Aurora Scientific, Inc.). Muscle dimensions (cross-sectional area, length, and volume) were determined using an ocular micrometer mounted in the dissection microscope (resolution, ~10 μm). These muscle dimensions were used to normalize contractile force, sarcomere length (SL) and ATPase activity. SL was measured in passive relaxed condition by laser diffraction as previously described (de Tombe & ter Keurs, 1990), adjusted to and maintained at 2.5 μm. Briefly, a helium-neon laser beam was directed through the isolated muscle fiber bundle, in which muscle striation pattern diffract the laser beam into a diffraction pattern. The striations of the muscle fiber bundle act as a grating, with known angles of diffraction, and the projected pattern can therefore be used to measure average SL.

**Incubating with MyBP-C N-termini**

The permeabilized fibers were incubated with 10 μM of the ssC1C2, fsC1C2, and C0C2 recombinant proteins or unincubated for 3 minutes in relaxing solution, followed by 3 min in preactivating solution, before measuring force in activating solution (Witayavanitkul et al., 2014). Activating solution contained 20 mM Ca\(^{2+}\)-EGTA, 1.55 mM potassium propionate, 6.59 mM MgCl\(_2\), 100 mM N,N-bis (2-hydroxyethyl) taurine; N,N-Bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid, 5 mM
sodium azide, 1 mM DTT, 10 mM phosphoenolpyruvate, 0.01 mM oligomycin, 0.1 mM PMSF, and 0.02 mM A2P5, and a commercial protease inhibitor cocktail (Sigma). Relaxing solution was the same as activating solution, but contained 20 mM EGTA, 21.2 mM potassium propionate, 7.11 mM MgCl₂, and no calcium. Preactivating solution was the same as the activating solution, but contained 0.5 mM EGTA, 19.5 mM 1,6-diaminoehexane-N,N,N,N'-tetraacetic acid, 21.8 mM potassium propionate, and no calcium. All solutions contained 0.5 mg/ml pyruvate kinase and 0.05 mg/ml lactate dehydrogenase (Sigma) and had an ionic strength of 180 mM, 5 mM free ATP, and 1 mM free magnesium. Recombinant proteins were present in relaxing and preactivation solutions for subsequent measurements. Activation solution did not have recombinant proteins present to avoid potential complications with changes in Ca²⁺ levels. Isometric tension and ATPase activity were measured at various levels of Ca²⁺ activation as previously described (de Tombe & Stienen, 1995; Rundell, Manaves, Martin, & de Tombe, 2005). Briefly, the isolated muscle was exposed to a range of calcium solutions obtained by proportional mixing of activating and relaxing solutions. The force generated and ATP consumed were measured simultaneously during the contraction.

**Force-pCa, Force-ATPase, and rate of tension redevelopment**

Force-pCa relationship was determined by titrating the activation and relaxing solutions (Witayavanitkul et al., 2014). Maximal isometric tension was measured at 100% activation solution, followed by sequential washes in relaxing and preactivating solutions for 5 minutes each. However, subsequent measurements
of isometric tension occurred in solutions titrated with decreasing ratio of activation-to-relaxation solutions: 95%, 90%, 85%, 80%, 70%, and 0% activation solution, which corresponded to pCa values of approximately 4.50, 5.11, 5.42, 5.61, 5.77, 6.00, and 10.00, respectively, as determined by the Fabiato program (van der Velden, Moorman, & Stienen, 1998). The integrity of the fibers was tested afterwards by measuring maximal tension after the experiment. Any fibers that did not maintain 80% or greater maximal tension were excluded from analysis. Force-ATPase relationship was determined using an optical absorbance enzyme assay (Guth & Wojciechowski, 1986). Briefly, ATP consumption was determined by measuring the absorbance of UV light at wavelength 340 nm. ATP hydrolyses into ADP and inorganic phosphate inside the fiber, which is stoichiometrically coupled to the oxidation of NADH to NAD+. This oxidation reaction is catalyzed by pyruvate kinase and lactate dehydrogenase in our activating solutions. NADH, but not NAD+, absorbs light specifically at 340 nm. A series of 50 nl of 10 mM ADP were injected into the measuring chamber. Each injection of ADP induced a rapid reduction in fluorescence and allowed the calculation of the rate of ATP consumption, determined by measuring the fluorescent decay rate at 340 nm. After completion of the Force-ATPase assay, papillary muscles underwent a rapid release-restretch maneuver in order to break previously formed cross-bridges and allow cross-bridges to reform. The rate constant of tension redevelopment (k_tr) was then measured at maximal activation, as previously described (Rundell et al., 2005; Swartz & Moss, 1992). Force-pCa and Force-ATPase relationships were fitted with a
modified Hill equation (Chandra et al., 2001; Rundell et al., 2005; Sumandea et al., 2003). Stiffness and tension costs were fitted linearly to the Force-stiffness and Force-ATPase data, respectively. (Figure 14,15,33,34) Stiffness is a measure of attached cross-bridges and is proportional to tension. Stiffness is measured by applying rapid, oscillating length changes and recording the change in force (Δforce/Δlength) (Ford et al., 1977).

**Actin cosedimentation**

Assay cosedimentation was conducted on recombinant MyBP-C proteins, as previously described (Kuster et al., 2015; Shaffer et al., 2009). Increasing concentrations of ssC1C2, fsC1C2, and C0C2 (1 – 30 µM) was added to 5µM F-actin (Cytoskeleton AFK99A) in cosedimentation buffer that contained 1mM ATP and DTT. Reactions were allowed to equilibrate for 30 minutes at room temperature. Samples were spun in an ultracentrifuge (Beckman) for 30 minutes, 100,000 RPM, using a TLA 100 rotor at 4°C. Supernatants were removed and pellets gently washed with cosedimentation buffer, followed by an additional spin. Samples were then dissolved in a 1:1 cosedimentation/urea buffer for 5 minutes at room temperature. 4x Laemmli buffer was added to the samples and run on a 10% SDS-PAGE gel. Along with the sample preparations, standard curves were run along side, consisting of increasing amounts of MyBP-C N-termini (4-40 pmol) and F-actin (40 pmol) for a molar ratio of 0.10, 0.20, 0.35, 0.50, 0.65, 0.80, 1.00 (Figure 20). Gels were stained with coomassie blue R-250 solution (0.05% in 25% isopropanol, 10% acetic acid) and imaged using ChemiDoc imager (Bio-Rad). Densitometry analysis of sample
bands was conducted with ImageJ (NIH) and the intensity ratio of MyBP-C to F-actin was converted to a molar ratio using the standard curve. Cosedimentation sample curves were fit to a one-site, specific binding nonlinear regression. Combined ssC1C2, fsC1C2, and C0C2 cosedimentation curves were generated (Figure 21). B\text{max} and K\text{d} values were obtained from this fit and given in Table 4.

**Computational modeling**

We used an adapted version of computational model that had been previously demonstrated to predict changes in unloaded shortening of individual cardiomyocytes (Campbell et al., 2013). The Online Muscle Simulation (OMS) was made available online: http://onlinemusclesim.org/ and the myofilament model accounts for length-dependent activation and strain-dependent XB kinetics (Razumova, Bukatina, & Campbell, 1999; Rice, Wang, Bers, & de Tombe, 2008), but not the contribution of MyBP-C. Since our goal is to model unloaded shortening, in which individual cardiomyocytes are isolated, components accounting for force (Rice et al., 2008; Sheikh et al., 2012) were set to zero.

Computer modelling accounts for results suggesting differential MyBP-C capacities on thin filament activation, as defined as k\text{on}, or the rate of tropomyosin shift from blocked to closed position (see Table 6). All other parameters were unchanged to limit variability in our data and specifically determine whether thin filament activation accounted for changes in relaxation kinetics.
**Isolation of adult rat cardiomyocytes**

Cardiomyocytes from adult Sprague-Dawley rats (250g) were isolated as previously described (33). After isolation, cardiomyocytes were gradually switched into plating media, which contained HMEM supplemented with 100U/mL penicillin/streptomycin (pen/strep), 10% fetal bovine serum, and 10mM BDM. For functional measurements, cardiomyocytes were plated on laminin-coated 25mm coverslips. For protein analysis, cells were directly plated onto laminin-coated 35mm circular dishes. After 1-hour incubation, plating media was removed, and culture media was added. Culture media consisted of HMEM, supplemented with 0.1% BSA, 100U/mL pen/strep, 2mM glucose, 10mM BDM, and 5ug/mL insulin/transferrin/seleunium (ITS) supplement. Cells were kept in a 2% CO₂ incubator to maintain a pH of 7.0 and maintained for 48 hours.

**Adenovirus Infection and Cell Culture**

Commercially purchased slow and fast skeletal MyBP-C clones from Origene (NM_175418.3 and NM_004533, Rockville, MD) were used to construct the adenoviruses used to overexpress skeletal isoforms of MyBP-C. Cardiac MyBP-C adenoviral vectors were generated by the core facilities at Loyola University Chicago. Adenoviral constructs were created by cotransfection of MyBP-C cDNA into DH5α *E. coli*. Adenoviruses containing slow, fast, or cardiac MyBP-C included a CMV promoter and c-Myc tag to distinguish between endogenous and adenovirus-mediated MyBP-C protein expression. Uninfected cells and c-Myc-tagged cMyBP-C overexpressing cells were used as experimental controls.
overexpressing slow, fast, and cardiac MyBP-C (Multiplicity of Infection MOI: 1000) were added to the culture media 1 hour after incubation and allowed to infect cardiomyocytes for 24 hours. Culture media was changed after 24 hours, removing the adenovirus and waste, and replenishing nutrients in the media.

**Immunofluorescence Imaging**

Cultured adult rat cardiomyocytes were plated in chamber slides (Lab-Tek II, Thermo Scientific) for immunofluorescence imaging. After 48 hours in culture, cells were rinsed twice with PBS and fixed with cold (4°C) 4% paraformaldehyde for 3 minutes, followed by 1 minute in ice cold (-20°C) methanol. Cells were then permeabilized in 0.5% Triton X-100 in PBS (20 minutes), 0.1% Triton X-100 twice (10 minutes), and antigen-retrieval solution (0.1M glycine, pH 7.4) (30 minutes), and rinsed three times with PBS. Cells were blocked with 0.1% BSA, 0.1% gelatin, 0.1% tween-20, 0.0001%NaN₃, and incubated with primary antibodies for ssMyBP-C (ProSci 6679), fsMyBP-C (ProSci 5651), and rabbit c-Myc (1:500 Roche) and mouse α-actinin (1:500 Sigma) overnight. After rinsing with PBS, corresponding secondary antibodies (AlexaFluor rabbit 488 at 1:50 dilution and mouse 568 at 1:50 dilution) were added for 1 hour at room temperature, rinsed, coverslipped with VectaShield mounting medium (Vector Labs H-1500 10mUL 1.5ug/mL) for imaging with confocal microscopy. Images were taken using a Leica TCS SP5 and processed using ImageJ (NIH).
Western blot analyses

Detection of endogenous and de novo MyBP-C protein in cardiomyocytes was determined by immunoblotting. Adenovirus infected cardiomyocytes were rinsed with sterile PBS to remove culture media, followed by the addition of 60uL of urea buffer. Cardiomyocytes were scraped off using a cell lifter (Fisher Scientific) and pipetted into 0.6mL tubes. 25uL of 4x loading dye (0.4% bromophenol blue, 10% β-mercaptoethanol) was then added. Samples were heated at 100°C for 5 minutes, separated by gel electrophoresis in 10% SDS-PAGE gels, and blotted onto nitrocellulose membrane at 300 mA for 3 hours. Detection of protein was determined by using antibodies for c-Myc, ssMyBP-C, fsMyBP-C, and cMyBP-C (Sigma C3956, ProSci 6679, ProSci 5651, Santa Cruz sc-137180, respectively)(B. Lin et al., 2013) (Figure 26). Quantification of replacement levels was determined by densitometry analysis normalized to β-actin loading controls, using ImageJ (NIH). Replacement levels were calculated by signal of cMyc/(cMyc + cMyBP-C), as previously described (Metzger, Michele, Rust, Borton, & Westfall, 2003).

Unloaded shortening in cultured adult rat ventricular cardiomyocytes

After 48 hours in culture, HMEM media was incrementally replaced with Tyrode’s solution (135 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM d-glucose, 10 mM HEPES) to limit damage to the cells. Coverslips with attached cardiomyocytes were transferred to a custom stimulation chamber containing platinum electrodes. The chamber was then fitted onto a Nikon microscope stage and stimulated (2.0 ms pulse, 1 Hz, and 20V). Sarcomere length and SL shortening
were measured by a video-based detection system (Ionoptix, Milton, MA). Analysis of contractile function was determined from an average of 10 contractions of individual cardiomyocytes, with 10-20 cardiomyocytes measured per coverslip preparation.

**Determination of fsMyBP-C expression in heart failure samples**

Surgical procedures for TAC (transverse aortic constriction) and sham FVB/N mice were performed (Verma et al., 2012), and TAC mice developing pressure-overload hypertrophy. The transverse aorta was constricted by 7-0 silk suture, with a blunt 27 GA needle to standardize the size of constriction. After 12 weeks, RNA from hearts of TAC and sham mice (n=3 per group) were isolated and purified to make cDNA using the iScript cDNA synthesis kit (BioRad 1708891). cDNA libraries were generated, sequenced, and aligned (Christodoulou, Gorham, Herman, & Seidman, 2011). Number of reads aligned were normalized per million. Significant changes in gene expression was determined as ≥ 1.5-fold increase for upregulation and ≤ 0.6 fold decrease for downregulation, using the Benjamini-Hochberg false discovery rate test at p < 0.001. Validation of RNA-Seq data was determined by analysis of mRNA levels using qPCR. Samples for qPCR used TaqMan primers against *MYBPC2* with *GAPDH* as a reference gene (Applied Biosystems Mm00525419m1 and Mm00486742m1, respectively). The qPCR analysis was performed on a thermocycler (BioRad CFX96) with the following protocol: 10 minutes at 94°C, 40 cycles of 15 seconds at 94°C and 60 seconds at 60°C. Analysis of normalized gene expression was then computed with an amplification efficiency
corrected ΔΔCq equation (Pfaffl, 2001). (see Figure 5)

**Statistical Analysis**

Data were analyzed using Benjamini-Hochberg False Discovery-Rate Test (Reiner, Yekutieli, & Benjamini, 2003). For this test, a fold change 0.6 fold down or 1.5 fold up, and p < 0.001 was considered significant. TPA experiments were analyzed using an unpaired t-test. All other data are presented as means ± SEM, unless otherwise indicated. Statistical significance for all other experiments were set at p<0.05.


Ha, K., Buchan, J. G., Alvarado, D. M., McCall, K., Vydyanath, A., Luther, P. K., ... Gurnett, C. A. (2013). MYBPC1 mutations impair skeletal muscle function in


VITA

Brian Lee Lin was born on October 26, 1985 to Lin Chang Cheung and Tang Wen Hsuan in Taipei, Taiwan. His maternal grandmother, Chang Yuan Fang, brought him to the United States when he was eight years old, and was raised by his aunt Jean Vliet (née Tang Wen Jian) and later joined by his uncle Alan D. Vliet. Brian married his high-school sweetheart, Tiffany Chia-Shih Liu, on December 22, 2012.

Brian began conducting undergraduate muscle physiology research in the lab of Dr. Paul J. Schaeffer at Miami University beginning in 2006. After graduating in 2008, Brian worked in the neurophysiology lab of Dr. Charles Vorhees at Cincinnati Children’s Hospital Medical Center. While his future mentor, Dr. Sakthivel Sadayappan, was a post-doctoral fellow at the same institution, it would be a few years before they teamed up in 2012. Brian continued his scientific career at Northwestern University with the late Dr. Aryeh Routtenberg beginning in 2009.

Brian joined the Program of Cell and Molecular Physiology at Loyola University Chicago in August of 2011, chaired by Dr. Pieter de Tombe, and joined the lab of Dr. Sakthivel Sadayappan January 2012. His project focused on the biophysical function of the slow-skeletal, fast-skeletal, and cardiac isoforms of Myosin Binding Protein-C. Upon completion of his PhD, Brian will begin a post-doctoral position in the lab of Dr. David A. Kass at Johns Hopkins University in Baltimore, MD where he will continue to work on the biophysical mechanics of muscle proteins in heart failure.