Effects of cardiomyopathy-related mutations in alpha-cardiac actin on the actin-myosin complex

by

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ABSTRACT
EFFECTS OF CARDIOMYOPATHY-RELATED MUTATIONS IN ALPHA-CARDIAC ACTIN ON THE ACTIN-MYOSIN COMPLEX

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University of Guelph, 2012
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Cardiomyopathies are a distinct class of heart disease and are the third most common cause of heart failure. These conditions are generally characterized by remodeling of the left ventricle; however, the molecular mechanisms leading to the pathological states are still not fully understood. Mutated genes encoding for sarcomeric proteins have been shown to influence the development of cardiomyopathies. Missense mutations in alpha-cardiac actin (ACTC) have been associated with both hypertrophic and dilated cardiomyopathies (HCM and DCM respectively). Twelve of these mutations contribute to the onset of HCM: E99K, P164A, A331P, Y166C, A230V, A295S, M305L, S271F, H88Y, R95C, F90del and R312C while two contribute to the onset of DCM: E361G and R312H. These mutations are proposed to have detrimental effects on the structure and function of alpha-cardiac actin, thus leading to the diseased state. A295S ACTC did not exhibit intrinsic defects in protein folding and polymerization as seen with other ACTC variants with subdomain 3 mutations. Actin-activated ATPase rates measured showed the E99K ACTC variant increased the ATPase rate of skeletal myosin by approximately 30% compared to WT ACTC. E99K ACTC also exhibited decreased filament motility approximately 40-50% slower than WT ACTC at all ionic and ATP conditions examined demonstrating a reduction in force generation. These results demonstrate deficiencies in myosin binding of the E99K ACTC variant, providing insight into the primary molecular disruptions leading to the development of cardiomyopathies.
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<td>Actin binding proteins</td>
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<td>α-cardiac actin</td>
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<td>ATP</td>
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<td>Baculovirus expression system</td>
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<td>Cc</td>
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<td>DCM</td>
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<td>β-Myosin heavy chain Ocč⁺</td>
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Chapter 1 – Introduction

1.1 Heart disease: An overview

In recent years, public awareness of cardiovascular disease has increased. Presently, we are witnessing progress in comprehension of the multifaceted pathophysiology of heart failure and subsequent improvement in modern treatments of patients with this clinical syndrome. Still, numerous pathophysiological mechanisms remain unclear, especially in the development of heart failure at the very early stages. Despite this overall awareness and technological advances, heart failure remains a major medical problem affecting as many as 5.7 million individuals with over 670,000 new cases diagnosed each year in the United States ultimately leading to billions of dollars in annual costs (Hiroyuki et al., 2010).

Heart failure is defined as a physiological state in which a perturbation or defects in cardiac muscles does not allow for adequate delivery of blood and nutrients to surrounding tissues and almost always arises due to underlying cardiovascular disease, such as atherosclerosis, myocarditis, congenital malformations, valvular disease or cardiomyopathy (Dellefave and McNally, 2008). Among these, the term “cardiomyopathies” is a very general description for a diseased state of the heart. They are typically defined by the presence of abnormal myocardial structure and/or function and clinically reveal themselves as a range of symptoms affecting the muscle of the heart (Jacoby and McKenna, 2012). Conventionally, cardiomyopathies are distinguished according to these pathologic changes; usually diagnosed via cardiac imaging and invasive methods (Marian, 2010). The earliest physical consequence of cardiomyopathies is remodeling of the left ventricle, of which two distinct classes exist: hypertrophy and dilation. The interest in hypertrophic cardiomyopathy is largely due to its unusual clinical and pathological manifestations with
echocardiography becoming the most commonly used imaging method in assessing patients with hypertrophic cardiomyopathy (Marian, 2010).

1.2 Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is primarily a disease of the myocytes and is mainly distinguished by the thickening of the ventricular wall. Other morphological characteristics include asymmetrical cardiac hypertrophy, a non-dilated left ventricle and typical or enhanced global systolic function (Debold et al., 2010). Pathological characteristics of HCM include gross cardiac hypertrophy, myocyte hypertrophy and disarray, interstitial fibrosis and mitral valve leaflet anomalies; with cardiac myocyte disarray being the standard pathological characteristic of HCM. However, the classic clinical diagnostic feature of HCM is remodeling of the left ventricle with the inner-ventricular septum being the predominant site of involvement, where asymmetric septal hypertrophy causes a resting or induced left ventricular outflow tract obstruction in about 25% of affected individuals (Chung et al., 2003). This cardiac remodeling typically occurs through increased size of cardiac myocytes rather than hyperplasia (Seidman and Seidman, 2001). Due to the stiff nature of the hypertrophic left ventricle, ventricular relaxation is also impaired and diastolic pressures tend to be high (Hiroyuki et al., 2010)

HCM is the most common genetic cardiovascular disease and is hereditary in about 60% of cases (Olson et al., 2000; Otsuka et al., 2012) The estimated prevalence of HCM within the general population is approximately 0.2 % (Rodrigo et al., 2010) with
an estimated annual incidence of new HCM cases between 0.24 and 0.47 per 100,000 cases (Moak and Kaski, 2012). This estimate is primarily based upon the visualization of a left ventricular wall thickness equal to or greater than 15 mm in a fairly young population. The actual prevalence is thought to be higher due to genetic penetrance, with incomplete expression being common in the autosomal dominant forms, even between individuals carrying the same mutations within a family (Jacoby and McKenna, 2012; Marian, 2010). Since this disorder has such a broad clinical spectrum, diagnosis is not always simple and straightforward with symptoms ranging from asymptomatic to heart failure. Patient outcomes range from lack of symptoms and normal longevity to chronic progressive heart failure (Van Driest et al., 2003; Keren et al., 2008). Clinical diagnosis is typically by 2-dimensional echocardiograph identification, illustrating otherwise unexplained left ventricular wall thickening and limited chamber cavity size (Maron et al., 2002). The most serious complication of HCM manifests itself as sudden cardiac death (SCD), which is common in affected individuals under 35 years of age, namely young athletes (Maron et al., 1983). The major challenge in the prevention of this extreme outcome is to identify those at risk, which can prove to be extremely difficult due to the heterogeneity of this disease state. Unfortunately SCD may be the only sign of disease, especially in young and athletic individuals (Maron et al., 2009). Since SCD due to HCM remains a major concern, the essential focus of both researchers and physicians is prevention by assessing the risk of SCD and recognizing those patients at high risk to intervene.
1.3 Dilated cardiomyopathy

Although there are severe consequences associated with HCM, dilated cardiomyopathies (DCM) are the most common form of cardiomyopathy. It is the third most common cause of heart failure, subsequent to coronary artery disease and hypertension (Osterziel and Perrot, 2005; Vang et al., 2005). The incidence of familial DCM is most likely underestimated, as disease expression within family members is often subclinical. Also, it has been recognized that DCM is an initially subtle, slowly progressive disease (Jacoby and McKenna, 2012). Clinically, DCM is characterized by dilated ventricles and systolic dysfunction. The ventricular walls become thin and stretched, thus disturbing cardiac contractility and ultimately left ventricular function (Karkkainen and Peuhkurinen, 2007). Hearts of DCM patients can weigh two to three times that of a normal heart (Luk et al., 2009). The formation of thrombi can take place during the terminal stages of the disease thus leading to stroke or SCD (Luk et al., 2009).

Histological characteristics of DCM include thickened myocytes with enlarged nuclei. Some myocytes appear hypertrophic while others can appear thin and elongated, with the nucleus appearing to occupy the entirety of the cell (Luk et al., 2009). In accordance with the thinned appearance of the ventricular wall, the total number of intracellular contractile myofibrils is reduced, leaving the myocyte with an empty appearance. Regions of myocyte death may also be evident, and subsequently get replaced with collagen and become fibrotic (Luk et al., 2009; Olsen et al., 1998). Inheritance can be via autosomal dominance with incomplete penetrance due to modifier genes and environmental factors, autosomal recessive and X-linkage, where 20-30% of all cases are caused by known genetic mutations (Perrot et al., 2007). Individuals affected
by DCM can show symptoms as early as childhood. However, most physical manifestations are exhibited during middle life. Unfortunately, the disease generally reveals itself after it has progressed to the end-stages where significant myocardial fibrosis has occurred (Olsen et al., 1998).

1.4 Mutations in sarcomeric proteins associated with cardiomyopathies

The initial causes of HCM and DCM disease states are currently being determined, however it is clear that there is an undeniable genetic basis (Perrot et al., 2007). While identification and comprehension of all the genes that remodel the heart is still incomplete, current literature suggest numerous molecular pathways initiating heart failure. It has become apparent that there are numerous genes encoding for sarcomeric proteins involved in the development of HCM, DCM or both of these pathological states, with over 600 mutation in a variety of sarcomeric genes already identified (Jacoby and McKenna, 2012). Although the causes of heart failure cannot be solely attributed to genetic components, especially due to the complex interplay with environmental factors, research has shown the importance of single sarcomeric gene mutations that are linked to the development of HCM or DCM (Liew and Dzau, 2004). The sarcomere is the functional unit of muscular contractile force. If execution of force generation or its transmission to the extracellular matrix (ECM) between cardiomyocytes is inadequate cardiac remodeling may be induced (Perrot et al., 2007). Therefore, examining mutations in sarcomeric proteins is essential in the understanding and prevention of heart failure as a result of cardiomyopathy.
Hypertrophic and dilated cardiomyopathies are distinct disorders when it comes to diagnosis; however, genetically speaking they may share more than was previously known. It was formerly thought that mutations in genes encoding for sarcomeric proteins of the thick and thin filaments were exclusive to familial HCM, whereas cytoskeleton protein mutations caused familial DCM (Morgensen et al., 1999; Chen and Chien, 1999). However, recent studies have proved that this simplistic view of genetic segregation between these two forms of ventricular remodeling is far from correct and have showed a substantial overlap in genes causing perturbations in heart function, making attributing mutations in specific genes to distinct phenotypes a challenge for researchers (Vang et al., 2005).

The MYH7 gene encodes for the β-Myosin heavy chain and is the leading genetic cause of HCM responsible for up to 35% of known cases, where associated mutations have been found in exons 8 and 9 (Chung et al., 2003). These mutations exhibit a high degree of penetrance, as most affected individuals show significant myocardial hypertrophy by their early twenties (Chung et al., 2003).

Myosin binding protein-C (MyBP-C) is another important sarcomeric protein, which plays a major role in cardiac contraction regulation via phosphorylation and thick filament structure. Mutations in this gene are the second most common cause of HCM, accounting for about 30% of known cases (Marian et al., 2010). However, unlike MYH7, mutations in the MyBP-C gene often show incomplete penetrance and its detrimental consequences are not revealed until late in adult life (Chung et al., 2003).

Mutations in the thin filament regulatory protein troponin T (TNNT2) have also been found to cause up to 15% of all known cases of HCM and can be missense
mutations, small deletions or mutations in splice signals. Compared to mutations in myosin, the phenotype of these mutations shows much less hypertrophy and there is reduced penetrance. However, most reported cardiac TNNT2 mutations account for significantly reduced survival (Chung et al., 2003).

Mutations in the α-tropomyosin gene account for approximately 5% of all known HCM cases, and in contrast to the mutations mentioned above, there appears to be a narrow range of mutations. One known mutation in tropomyosin is D175N, which appears to reflect a mutational “hot spot” within the gene due to its independent presence in multiple families (Chung et al., 2003). There seems to be a great degree of variation in hypertrophy between individuals carrying this specific mutation, thus implying the role of possible modifier genes and the role of environmental factors. Unlike mutations in TNNT2 or MYH7, survival is not significantly implicated with mutations in tropomyosin (Lakdawala et al., 2010).

Although there tends to be a great deal of overlap of disease causing genes between the two forms of cardiomyopathies, possibly the most dramatic example of varying disease phenotype due to mutations within the same sarcomeric protein is the α-cardiac actin gene (ACTC). Among individuals affected with DCM, mutations in the α-cardiac actin gene were the first to be linked to this pathological state (Mogensen et al., 1999). It was later found that multiple mutations in ACTC were linked to the onset of HCM as well (Olsen et al., 2000).
Figure 1: Sarcomeric genes carrying mutations leading to HCM and DCM. A. Top five sarcomeric genes carrying mutations found in patients with HCM and DCM with prevalence in disease state. B. Venn diagram showing degree of overlap of sarcomeric genes carrying mutations linked to HCM and DCM.
1.4.1 Mutations in ACTC causing HCM and DCM

Actin is a highly abundant eukaryotic protein responsible for a myriad of functions within the cell. These include the integrity and dynamic nature of the cytoskeleton, motor-based organelle transport, receptor-mediated responses of the cell to external signals among others (Holmes, 2009). One of the most critical functions of actin is its involvement in muscle cells where it comprises up to 20% of the total mass (Holmes, 2009) and acts as the main component of the thin filament of the sarcomere. Actin comprises a highly conserved family of proteins consisting of three broad classes: α, β, and γ. Six actin isoforms exist in vertebrates all encoded by different genes: 2 striated muscle isoforms (α-skeletal and α-cardiac), 2 smooth muscle isoforms (α-smooth and γ-smooth) and 2 cytoplasm isoforms (β-cytoplasmic and γ-cytoplasmic). During embryonic development, five of the six isoforms are expressed in myocytes; however in mature cardiac myocytes, both skeletal and cardiac actins are expressed, with cardiac actin present as the major isoform (approximately 80%) (Olsen et al., 1998).

Actin is one of the most highly conserved proteins in the sarcomere, suggesting that mutations causing changes in residues within cardiac actin are likely to have detrimental effects on its essential functions or its interactions with other sarcomeric proteins. The α-cardiac actin gene (ACTC) has been long recognized to carried mutations leading to both HCM and DCM (Olsen et al., 2000) and is in the top five genes carrying mutations for both types of cardiomyopathies (Figure 1) (Perrot et al., 2007). Missense mutations identified in subdomains 1 and 3 of the actin monomer of ACTC lead to altered actin function ultimately causing a defect in force transmission to neighboring sarcomeres as well as the ECM (Olsen et al., 1999).
To date, there are fourteen naturally occurring ACTC mutations associated with cardiomyopathies: twelve linked to HCM: E99K, P164A, A331P, Y166C, A230V, A295S, M305L, S271F, H88Y, R95C, F90del and R312C. With two linked to DCM: E361G and R312H (Figure 2). HCM mutations have been thought to disrupt normal sarcomere contraction, while mutations leading to DCM are thought to affect force transmission from the sarcomere to the surrounding syncytium (Mogensen et al., 2004).

Individuals who possess these mutations show a phenotype varying from asymptomatic to severe cardiac disease, illustrating the complexity of the regulation of ACTC and the interplay by many other factors. Many ACTC mutations have been characterized and via in vitro studies much insight has been provided into the intrinsic and binding properties of these mutant proteins. The Trybus group at the University of Vermont have studied the HCM causing mutation E99K and hypothesized that this mutation has detrimental effects on myosin binding since it is located in subdomain one of the cardiac actin monomer where important ionic interactions with myosin take place. Glu-99 is one of three acidic residues thought to be part of this binding to myosin (along with Glu-93 and Glu-100) therefore, a plus two-charge reversion via a lysine substitution is believed to disrupt specific actomyosin interactions (Bookwalter and Trybus. 2006).
Figure 2: Actin monomer with mutations. Monomeric actin is comprised of four subdomains: Subdomain I (violet), subdomain II (yellow), subdomain III (green), subdomain IV (teal). Bound nucleotide and divalent cation are shown in the centre of the molecule (gray). 14 mutations in ACTC monomeric protein represented as red spheres: H88Y, F90, R95C, E99K, P164A, Y166C, A230V, S271F, A295S, M305L, R312H, R312C, A331P and E361G. Representation was generated using PyMol Viewer (PBD:1ATN). The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.
Song et al. (2011), generated a transgenic mouse model expressing the ACTC E99K mutation at 50% of total heart mass. This percentage is proposed to be the pathological distribution of mutant to WT cardiac-actin protein within the hearts of individuals affected with this autosomal dominant disorder (Bottinelli et al., 1998; Nier et al., 1999; Dyer et al., 2009) and was compared to patients carrying the same mutation (Song et al., 2011). The E99K mutation was found to cause a higher Ca^{2+} sensitivity compared to WT within reconstituted thin filaments by means of the in vitro motility assay. This mutation also eliminated the change in Ca^{2+} sensitivity generally associated with troponin I phosphorylation. More dramatically, E99K ACTC mice exhibited a high death rate between 28 and 45 days of age compared with WT mice (Song et al., 2011).

In addition, Debold et al. (2010) investigated the influence of the R312H and the E99K ACTC mutations on the actomyosin interaction, with particular focus on contractility and regulation. These defective proteins were examined in the presence of sarcomeric regulatory proteins troponin and tropomyosin. Using the in vitro motility assay, it was discovered that E99K and R312H ACTC have reduced velocities under high calcium concentrations. In optical trapping experiments, E99K ACTC protein showed reduced binding frequency to myosin in the presence of regulatory proteins (Debold et al., 2010). Recently Muller et al. (2012) expressed the M305L ACTC and Y166C ACTC proteins in insect cells using the baculovirus expression system. They found that M305L ACTC showed a reduced rate and extent of polymerization compared to WT ACTC. M305L ACTC and Y166C ACTC proteins also demonstrated a 50% reduced ability to stimulate the cardiac β-myosin ATPase. The Y166C ACTC protein was also expressed in newborn rat cardiomyocytes (NRC’s) using adenovirus constructs and incorporated into sarcomeric
thin filaments. Seventy-two hours post infection, the sarcomeres of NRC’s appeared shorter compared to WT infected cells (Muller et al., 2012) These results illustrate the perturbations in the actin-myosin interactions associated with the ACTC mutations and allude to their effect on the regulation and contractile abilities in the heart muscle contributing to cardiomyopathy development.

1.5 Monomeric actin

The structure of monomeric or globular actin (G-actin) has been studied via co-crystals of actin complexed with a variety of actin binding proteins (Schutt et al., 1993; McLaughlin et al., 1993), with the uncomplexed structure of actin bound to a nucleotide solved (Dominguez et al., 2001). G-actin is composed of 375 amino acid residues, with a molecular mass of 42 kDa (Kabsch et al., 1990). It is divided into two major domains, the large and small domain which can be further divided into four subdomains (Figure 2). The small domain is divided into subdomain 1 and subdomain 2. The large domain is divided into subdomains 3 and 4 (Dominguez et al., 2001). The DNase-I binding loop in subdomain 2 of the actin monomer binds with high affinity to the DNase-I enzyme forming a 1:1 complex, consequently inhibiting DNase-I activity (Kabsch et al., 1990). The actin monomer also contains a nucleotide-binding cleft located within the center of the molecule between the large and small domains. Due to differences in affinity, the nucleotide binding site is usually occupied by ATP ($K_{eq} = 10^{10} \text{ M}^{-1}$) or ADP-P$_i$ rather than ADP ($K_{eq} = 10^{8} \text{ M}^{-1}$) (Neidl and Engel, 1979). The adjacent high-affinity metal binding site, also located within the deep interdomain cleft is typically occupied by Mg$^{2+}$ in vivo ($K_{eq} = 10^{10} \text{ M}^{-1}$) due to its high Mg$^{2+}$ intracellular concentrations.
However, due to the high concentration of CaCl₂ in buffers used in actin preparation, Ca²⁺ is the normally bound cation in vitro (Gershman et al., 1986). This nucleotide-cation complex interacts with both domains of actin, restricting their motion at a flexible hinge region formed by an α-helix.

1.6 Actin polymerization

Actin is a very dynamic protein within the cell and carries out many functions via its ability to polymerize into filaments. Under appropriate conditions such as high ionic strength (salt concentration ≥50 mM) and temperature close to physiological (Dos Remedios et al., 2003), actin will exist naturally as polymers. To make the transition from G to F-actin states, the concentration of G-actin must exceed the critical concentration (Cc). The Cc is defined as the concentration of actin monomers that coexist with polymers at a steady state of polymerization (Asakura and Oosawa, 1975; Carlier et al., 1994). Therefore levels of actin below the Cc will fail to polymerize and remain in the monomeric state. The Cc is a function of the rate constants for monomer association and dissociation and is dependent on the conditions required for polymerization (Cc ↓ as ionic strength ↑, Cc ↓ as pH ↓, Cc ↓ as temperature ↑) (Grazi and Trombetta, 1985; Wang et al., 1989). These conditions allow actin to undergo the polymerization process, which consists of three main steps: activation, nucleation and elongation (Pollard 1990; Gaszner et al., 1999).

Actin activation is achieved by the binding of a divalent cation to the intermediate-affinity cation-binding site. This activation induces a conformation change in the actin monomer that facilitates the nucleation process (Cooper et al., 1983; Gaszner
et al., 1999). Actin nucleation is thermodynamically unfavorable and the rate limiting step of actin polymerization. The process of forming a stable actin dimers and trimers is unfavorable since the interactions between monomers are weak and unstable (Figure 3B) (Wegner and Engel, 1975; Pollard, 1990). After the arrangement of nuclei in the form of an actin trimer, the addition of more monomers becomes more favorable and elongation proceeds (Figure 3C) locking the newly added monomers via a conformational change (elongation).

The F-actin polymer is a polar molecule with a plus (or barbed) end and a minus (or pointed) end, based on the appearance of myosin decoration. These ends have different rate constants with association predominantly occurring at the barbed end ($K_a$ of $\sim 10^7 \text{M}^{-1}\text{s}^{-1}$) and dissociation at the pointed end of the filament ($K_a$ of $1-2 \times 10^6 \text{M}^{-1}\text{s}^{-1}$) (Pollard, 1983). There is a constant addition and removal of actin monomers from the ends of the actin polymers and steady state is reached when the rate of assembly equals that of disassembly. At steady state, ATP hydrolysis drives treadmilling of actin monomers where monomers are added to the plus or barbed end of the filament then move towards the minus or pointed end for dissociation. This process continues, with dissociated monomers migrating back towards the plus end (Bugyi and Carlier, 2010). ATP hydrolysis by F-actin is thought to happen in two major steps following monomer addition. The first being the cleavage of the gamma-phosphoester bond followed by the slower release of $P_i$, where only the second reaction appears to be reversible (Asai and Asai, 1976). The rate of ATP hydrolysis is slow (to the order of $0.01 \text{s}^{-1}$) and the rate of phosphate dissociation is about one-tenth as fast as ATP hydrolysis (Pollard, 1990).
Figure 3. Ribbon diagrams of monomeric and filamentous actin A. X-ray crystal structure of G-actin with ATP bound (grey). Individual subunits shown: subdomain I (violet), subdomain II (yellow), subdomain III (green) and subdomain IV (teal). Representation was generated using PyMol Viewer (PDB ID: 1ATN) The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC. B. Oda model of F-actin consisting of three monomers, determined by X-ray fiber diffraction. This trimer acts as the nuclei for actin polymerization C. Growth of the actin filaments by addition of actin monomers largely to the barbed end leads to elongation. Once steady state is reached, treadmilling of monomers from barbed end to pointed end and back occurs via ATP hydrolysis (shown by bi-directional arrow).
1.7 Filamentous Actin

Actin is one of the most abundant and well-studied proteins within the cell, however the definitive high-resolution structure of F-actin remains unclear (Fujii et al., 2010), this uncertainty is due to the heterogeneity and flexibility of actin filaments. The past few decades have been key in advancing our understanding of actin polymerization and its molecular interactions with binding partners. Holmes et al. (1990) generated the first low-resolution F-actin model by solving the crystal structure of G-actin bound to DNase-I and comparing it to the F-actin x-ray diffraction patterns. Oda et al. (2009) then built on this further by generating a higher resolution model. Currently the highest resolution model of the F-actin structure exists at 6.6 Å due to advances in electron cryomicroscopy by Fujii et al. (2010). This model reveals important inter and intra molecular contacts within the double helical structure of F-actin, which will be discussed further.

F-actin is a helical polymer with a maximum diameter of 90-95 Å and consists of 13 actin monomers per six left handed turns with a repeat of 360 Å down the length of the axis (Lorenz et al., 1993). The rotation per subunit is about 166°; this rotation allows F-actin to appear as both a single start left-handed helix or two right-handed helices winding around one another. The twisted F-actin helix has a single right-handed helical pitch of 51 Å, and a single left handed helical pitch of 59 Å (Oda et al., 2009).

The key difference between G-actin and F-actin, is the relative rotation of the large and small major domains by approximately 20°, giving the F-actin subunit a flat conformation (Oda et al., 2009). This flat conformation and domain rotation also includes a 5° anticlockwise rotation around the nucleotide within the plane of the actin molecule,
opening the nucleotide-binding pocket and the space between subdomains 1 and 3. This change in conformation has been proposed to allow a specific interaction with N137 to shift closer towards the position of the $\gamma$-phosphate, thereby allowing hydrolysis to take place (Oda et al., 2009; Fujii et al., 2010).

Important intra- and inter-filament contacts exist which are critical for stable F-actin formation (Figure 4). Extensive subunit interactions exist longitudinally between actin subunits, with subdomains 1 and 3 of the upper monomer enclosing subunits 2 and 4 of the lower monomer similar to a ball in socket. The inter-filament contacts are formed by two protrusions from subdomain 4. The carboxy terminus of the $\alpha$-helix from subdomain 4 of the lower monomer from one strand, contacts the amino terminus of the $\alpha$-helix of a monomer from the adjacent strand. The other contact is between the hydrophobic plug of one monomer to four regions in the opposite strand between two monomers (Oda et al., 2009; Fujii et al. 2010).

1.8 Actin-protein interactions

Currently there are over 160 proteins known to interact with actin and regulate its function within the cell (dos Remedios et al., 2003). Some actin-binding proteins (ABPs) carry out functions that are required to maintain or change cell structure while others specifically influence actin organization. *In vivo*, ABPs regulate the assembly and disassembly of actin filaments and their organization into higher-order networks allowing actin to be a highly dynamic protein (dos Remedios et al., 2003). A great deal of ABPs use actin as a scaffold or track rather than affecting actin dynamics or structure.
Figure 4. Oda model of the F-actin structure. The two actin monomers within the oval are magnified in b, and the three actin monomers in the triangle are magnified in c. Residues contributing to the intra-strand contacts between subunits b, and those contributing to the inter-strand contacts c, are highlighted. Black and red numbers represent the residue and subdomain numbers, respectively, whereas \( n, n + 1 \) and \( n + 2 \) are actin monomer numbers. d. The three-dimensional map independently reconstructed from cryoelectron micrographs is superposed with the F-actin model (resolution 13.8 Å). Obtained with permission from Nature Publishing Group.
1.8.1 Actin-myosin interaction

One such class of ABPs and possibly actin’s most important binding partner is myosin. Myosins are actin-dependent molecular motors, which generate movement (and force) through the hydrolysis of ATP. Many are familiar with the two-headed myosin II required for muscle contractility and tension, but there are currently over 24 different classes of myosins carrying out diverse functions (Syamaladevi et al., 2012). The remainder of this thesis however will focus specifically on myosin II involved in muscle contraction.

Myosin II is a large asymmetric hexameric protein around 520 kDa, consisting of two globular head regions and a rod-like tail formed by α-helices (Figure 5). These rod portions allow myosin to assemble and form the functional “thick filament”. The globular heads contain the enzymatic active site and the actin-binding region. The head region also consists of two noncovalently bound regulatory and essential light chains, which are proposed to be involved in contraction regulation. The enzymatic head and associated light chains are referred to as the subfragment-1 (S1), and although S1 has basal ATP hydrolysis activity, this is dramatically increased upon actin binding (Rayment et al., 1993). The interaction between myosin and actin are fundamental to the contractile process. The cycle of S1 attachment to and detachment from actin filaments along with the hydrolysis of ATP is referred to as the cross-bridge cycle (Geeves and Holmes., 1999) (Figure 6). Myosin is able to bind and hydrolyze ATP to ADP-Pi in the absence of actin, but release of inorganic phosphate is very slow. Once ATP is hydrolyzed, binding to actin accelerates this release of P_i (referred to as strong binding state) and this is thought...
Figure 5. Schematic of myosin structure. Myosin S1 heads serves as the catalytic motor domain, binding to actin filaments and upon release of Pi allows the power stroke. Myosin S2 acts as a lever arm for force transduction generated by the catalytic motor domain. S2 domain may also serve as a binding site for myosin light chains. Together the S1 and S2 domains are referred to as: Heavy Meromyosin (HMM) The tail domain referred to as: Light Meromyosin (LMM) generally mediates interaction with cargo molecules and contains phosphorylation sites.
to allow the working stroke (Behrmann et al., 2012). ADP release is thought to be the rate-limiting step of the cross-bridge cycle, where rebinding of ATP (referred to as the weak binding state) causes dissociation of the actomyosin complex and the cycle begins again (Murphy et al., 2001; Sun et al., 2008). The actin-binding site of S1 has been proposed to be located at the periphery of the broad head and extends into the long-pitch helical groove of the actin filament (Figure 7) (Goody et al., 1985; Taylor and Amos, 1985).

Recently, Lorenz and Holmes, (2010) described the actomyosin complex at 13-Å resolution. Using a novel molecular dynamic technique constrained by an EM map of the actomyosin complex, an atomic model of the rigor actomyosin interface was obtained. It was found that S1 not only makes major contacts with an actin monomer, but it also interacts with the monomer below the major monomer in the actin helix with a total contact area of 1980 Å² for both monomers (Lorenz et al., 2010). Figure 7B describes possible electrostatic interactions and H-bonds between S1 and the upper (AC1) and lower (AC3) monomer in the actin helix. E99K and R95C are mutations found in α-cardiac actin and are both associated with HCM. These specific residues are thought to make important electrostatic contacts with S1 with R95 also being involved in a number of H-bonds as well. In addition, a number of ACTC mutations are found within close proximity to potential interaction residues. Residues E167 and D311 are involved in electrostatic contacts located nearby to ACTC mutations Y166C and R312H respectively. Such mutations causing substitutions of amino acids required for specific molecular contacts in α-cardiac actin can perturb these proposed interactions, thereby disrupting actin-myosin interactions and contributing to the development of cardiomyopathy.
Figure 6: The actin-myosin ATPase cycle. Schematic diagram highlighting the major states of the actin-myosin ATPase cycle and the proposed states of the myosin lever arm. Myosin (shown in red) binds to ATP in the absence of actin binding, however phosphate release is slow. Binding of S1 to the actin filament (shown in blue) accelerates phosphate release, which in turn promotes a conformational change in the myosin lever arm causing the working stroke (represented with black arrow). Rebinding of ATP to myosin brings about actin-myosin dissociation and the cycle repeats again. Adapted from Murphy et al. (2001).
Figure 7: Actomyosin complex with proposed molecular contacts.

A. 3-D structure of S1 myosin heads (pink) bound to an actin trimer in the rigor state. The myosin head is proposed to interact with two monomers at a time down the actin filament. The upper monomer (AC1) is represented in purple while the lower monomer (AC3) is represented in blue. Residues involved in actin-myosin contacts that are within a mutation in α-cardiac actin are shown as yellow spheres.

B. Proposed electrostatic interactions and H bonds between myosin S1 head and upper (AC1) and lower (AC3) actin monomers down actin filament. (http://pymol.sourceforge.net/). Bolded red font indicates interaction is located at a naturally occurring mutated residue in α-cardiac actin. Bolded font indicated interaction is located near a mutated residue in α-cardiac actin. Representation was generated using PyMol Viewer (PDB ID: 1M8Q)
1.9 Research objectives

Actin is one of the most abundant and highly conserved proteins in the cell. Therefore changes within actin expressed in cardiac cells are thought to lead to detrimental protein structure and function within the heart. Specific mutations in α-cardiac actin have been linked to the development of both HCM and DCM. Cardiomyopathy has been known to cause heart failure remaining one of the leading requirements for heart transplants in the developed world (Seidman and Seidman, 2001). There are a myriad of factors though to influence the onset of cardiomyopathy leading to heart failure, however there is a strong genetic basis to the development of this disease. Specific mutations in the ACTC gene have been associated with the development of both HCM and DCM and are proposed to cause deficient myosin binding, resulting in perturbed heart contraction and force production in patients with cardiomyopathy. However, the molecular mechanisms leading to the disease state and ventricular remodeling is still unclear (Moak and Kaski, 2012). Namely, how mutations in the same protein and in certain cases the same subdomain of actin lead to the two distinct phenotypes of cardiomyopathy? The first research aim of the Dawson lab is to determine deficiencies present in the ACTC protein itself caused by each mutation. The second aim is to determine how these deficiencies lead to the development of disturbed protein interactions within the sarcomere causing a diseased state of the heart.

Previous work in the Dawson lab has described specific intrinsic deficiencies within several of the ACTC variants, especially those located within subdomain 3 of the actin monomer (Mundia et al., 2012). We hypothesize that the A295S mutation will demonstrate similar deficiencies in intrinsic characteristics due to its location in
subdomain 3. In addition, the effect of each ACTC mutation on myosin binding is currently not well understood. Since it is perhaps actin’s most important binding partner, it is hypothesized that mutations in ACTC will have a detrimental effect on myosin binding and the function of the actomyosin complex.

This thesis focuses on investigating the effects of the ACTC mutations as they relate to protein stability and function, especially their effects on myosin binding. The specific research goals of this thesis are to examine the novel intrinsic effects of the A295S mutation on protein stability and the effects of each ACTC mutation on actin-myosin binding to increase understanding regarding the molecular mechanisms of the development of cardiomyopathy.

The subdomain 3 mutation A295S will be examined in detail and its affect on overall actin structure will be assessed. Subsequently the consequences of several ACTC mutations on actin activated myosin ATPase activity and filament motility as it pertains to myosin binding will be tested.
Chapter 2- Materials and Methods

2.1 Reagents

DEAE Sepharose fast flow columns were obtained from Amersham (GE Healthcare, Piscataway, NJ). DNase-I affinity chromatography columns were produced in the laboratory with DNase-I obtained from Worthington Biochemical (Lakewood, NJ).

All restriction enzymes were obtained from New England Biolabs (Ipswich, MA).

All buffer reagents were obtained from Fisher Scientific (Mississauga, ON) or Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

2.2 Standard protocols

2.2.1 Quantification of protein concentration

The protein concentration of $\alpha$-cardiac actin was quantified by absorption spectroscopy at 290 nm with an extinction coefficient of 0.62 M$^{-1}$ cm$^{-1}$ using a Beckman Coulter DU 800 Spectrophotometer (Mississauga, ON). Protein concentration of skeletal myosin (full length and S1 subfragment) was quantified by absorption spectroscopy at 280 nm with an extinction coefficient of 0.58 M$^{-1}$ cm$^{-1}$ and 0.83 M$^{-1}$ cm$^{-1}$, respectively.
2.2.2 Polyacrylamide gel electrophoresis

All gels were prepared with Bio-Rad 29:1 bisacrylamide solution (Hercules, CA). SDS-PAGE gels were prepared using 5% polyacrylamide stacking and 10% polyacrylamide resolving gel. 2x Laemmli buffer solution (50 mM Tris, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 1.3 M βME) (Laemmli et al., 1970) was mixed in a 1:1 ratio with protein samples before they were run on gels. Gels were run on a Bio-Rad Mini PROTEAN Tetra cell system with 1x running buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS) at 70 V through the stacking gel and 140 V through the resolving gel. Gels were stained by means of Coomassie brilliant blue dye (R-250) and destained using destain buffer (40% methanol and 10% acetic acid) to visualize proteins.

2.2.3 Cell Culture

*Spodoptera frugiperda* (*Sf*9) cells (BD Bioscience, San Diego, CA) were maintained in suspension in Wheaton glass spinner flasks (Millville, NJ) or monolayer in T-75 flasks (Costar, Corning Inc., NY) in a 27°C incubator. Cells were cultured in supplemented Grace’s Insect Medium (1x) with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Mississauga, ON). Density of cells was determined via staining with trypan blue stain and counting using a hemocytometer as described (Mather al., 1998).

2.2.4 Amplification and titering of recombinant baculoviruses

Recombinant baculoviruses containing ACTC inserts were amplified by seeding a 120 mm plate (Costar, Corning, Inc., NY) with 2 x 10⁶ *Sf*9 cells and allowing them to
attach. A total volume of 30 mL was obtained by addition of supplemented Grace’s Insect Medium and 100 µl of plaque-purified recombinant baculovirus from Dawson lab stock was added to the cells and gently rocked to ensure proper mixing and equal exposure of cells to virus. The plate was incubated for 2 weeks at 27°C shielded from light. The recombinant baculoviruses were harvested by centrifugation for 10 minutes at 1000 rpm using a JS5.2 rotor (Beckman Coulter, Mississauga, ON) and the media containing virus particles was stored in T-75 flasks at 4°C protected from light. Recombinant baculoviruses were usually amplified to a third passage to achieve a higher titer values (~ 2 x10^7 to 1 x 10^8 plaque forming units (pfu)/mL). Recombinant ACTC baculoviruses were titered following the TCID$_{50}$ method as described (O’Reilly et al., 1994).

2.3 Generation of recombinant baculoviruses

2.3.1 Molecular cloning of α-cardiac actin variants

Mutant ACTC gene variants with the exception of A295S and P164A were previously cloned into the pAcUW2Bmod expression vector. This work was complete by past graduate student Maureen Mundia. A295S and P164A ACTC gene variants were cloned into pTOPO2 vector using TOPO-TA cloning (Invitrogen, Carlsbad, CA) by past graduate student, Ryan Demers. The ACTC gene was subsequently BamHI-digested out of the TOPO vector and ligated into BglII-digested pAcUW2Bmod vector. The presence and orientation of insert was verified by NaeI digestion due to non-directional cloning. Absence of other PCR-induced errors and mutations were verified by direct DNA sequencing.
2.3.2 Production and plaque purification of recombinant baculovirus

pAcUW2Bmod vector containing A295S or P164A ACTC insert was co-transfected with linear viral DNA (BacPAK5 viral DNA and Clontech, Mountain View, CA) to produce a recombinant ACTC baculovirus as described (Mundia et al., 2012). Passage 2 or higher recombinant ACTC baculoviruses were plaque-purified as described by O'Reilly et al. (1994). However, the agarose mixture used contained 4% agarose and 2x Grace’s Insect Medium supplemented with 20% fetal bovine serum and 2% penicillin/streptomycin (Gibco, Mississauga, ON). Neutral red stain was overlaid for WT and A295S recombinant ACTC baculoviruses only as a means of comparing infectivity. The BacPAK System uses the baculovirus Autographa californica nuclear polyhedrosis virus (AcMNPV) which is occlusion body positive (occ⁺). Occ⁺ baculoviruses express occlusion bodies or polyhedral inclusion bodies (PIBs) through the late infection phase and allow for visual conformation of viral infection and activity. The activities of all other recombinant ACTC baculoviruses were therefore visually confirmed by the presence of PIBs and development of viral plaques. Plaques were picked and resuspended in Grace’s insect medium to isolate a pure clonal stock of virus.

2.3.3 Verification of ACTC protein expression by western blotting

For detection of ACTC protein expression by infected Sf9 cells, 2 x 10⁶ cells were seeded into a T-75 flask and allowed to adhere. Cells were then infected with passage 3 plaque purified recombinant ACTC baculovirus at an MOI (multiplicity of infection) of
1, incubated at 27°C, and harvested at 72 hours post infection (p.i) by centrifugation for
10 minutes at 1000 rpm using a JS5.2 rotor (Beckman Coulter, Mississauga, ON). A295S
recombinant ACTC baculovirus-infected cells were harvested at 48 hours post
infection due to higher titer values and overall rate of infection. Cells were lysed using
lysis buffer (62.5 mM Tris, 2% SDS) for 5 minutes and lysate was mixed in a 1:1 ratio
with 2x Laemmli buffer solution and incubated for 5 minutes at 95°C before running on
10% SDS gels. Purified ACTC protein samples of equal concentration were also mixed
with Laemmli buffer solution and run on 10% SDS gels. Gels were transferred onto PVDF
membrane using Bio-Rad Mini Trans-Blot cell system at 100 V and blocked overnight at
4°C in 5% skim milk powder in TBST (0.1% Tween-20 in 1X Tris-buffered saline, pH
7.4). The membrane was probed using mouse monoclonal anti α-cardiac actin primary
antibody (5C5, Sigma-Aldrich, Oakville, ON) in a 1 in 1000 dilution and incubated for 1.5
hours at room temperature. The membrane was washed 5 times for 3 minutes with TBST
and probed with goat anti-mouse secondary antibody (Promega, Invitrogen, Burlington,
ON) in a 1 in 5000 dilution and incubated for 1 hour. The membrane was washed 3 times
for 5 minutes with TBST and developed using chemiluminescent detection reagent
(Amersham, GE Healthcare, Piscataway, NJ).

2.4 Protein expression and purification

2.4.1 Purification of α-skeletal actin

α-skeletal actin was prepared from acetone power from chicken pectoralis muscle as
described (Spudich and Watt, 1971).
2.4.2 Expression and purification of ACTC mutant proteins from Sf9 cells

Sf9 cells in cultured in suspension at cell density of ~1 x 10^6 cells/mL were infected at an optimal MOI of 1, with recombinant ACTC baculovirus. The volume of virus added to cells was determined based on titer value of virus, volume of cells, density of cells and desired MOI. Infected cells were harvested after 72 hours p.i. with the exception of A295S infected cells which were harvested after 48 hours p.i. Harvesting was by centrifugation for 10 min at 3000 rpm using the J25.2 rotor (Beckman Coulter, Mississauga, ON). The pellet was lysed with a high Tris-buffer containing a cocktail of protease inhibitors and vortexed as described (Mundia et al., 2012). Cell lysates were spun for 35 min at 45 000 rpm using a TLA 110 rotor (Beckman Coulter, Mississauga, ON).

To remove lipids, the supernatant was filtered twice through glass wool (Costar, Corning Inc., NY) in a 60 mL syringe (BD Biosciences, San Deigo, CA) equilibrated with G-buffer (10 mM Tris-HCl, pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.2 mM βME, and a protease inhibitor cocktail (antipain, aprotinin, BAEE, benzamidine, E-64, leupeptin, pepstatin, PMSF, TLCK, and TPCK and PMSF) at 0.5 µg/mL. The filtered lysate was purified by DNase-I affinity chromatography as described (Rutkevich et al., 2006). Purified α-cardiac actin protein was dialyzed overnight in 2 L G-buffer, and was centrifuged in Amicon 10 000 MWC0 concentrators (Millipore, Etobicoke, ON) to reduce volume. The purified α-cardiac actin was store at 4°C on ice and used within 3 days of purification.
2.4.3 Purification of skeletal myosin

Full-length skeletal myosin and S1 sub fragment were prepared as described (Margossian et al., 1982). Following skeletal myosin purification, subfragment-1 (S1) was prepared by papain digestion to produce EDTA•S1. Full-length skeletal myosin was stored in 50% glycerol at -20°C. EDTA•S1 was flash frozen and stored at -80°C.

2.5 Pyrene polymerization assay

The polymerization activities of ACTC variants were observed by the incorporation of pyrene labeled α-skeletal actin and monitoring the pyrene fluorescence signal over time. α-skeletal actin prepared from chicken pectoralis muscle was covalently labeled with pyrene (N-(1-pyrene) iodoacetamide) on cysteine-374 as described (Cooper et al., 1982) to produce pyrene actin. ACTC variant protein samples (final concentrations 2.5, 5, 10, 15, 20 µM ) in G-buffer were mixed with 2.5% pyrene labeled α-skeletal actin and then equilibrated at room temperature for 20 minutes. ACTC protein samples were carefully added to ultramicro fluorescence cuvettes with a 3 mm pathlength (Helma Inc., Concord, ON) and baseline readings were taken for at least two minutes using Varian Cary Eclipse Fluorescence Spectrophotometer (Varian Canada, Mississauga, ON). 10x polymerization buffer was added to the ACTC protein samples (final buffer concentration, 25 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EGTA, 2 mM MgCl₂ and 0.1 mM ATP) to initiate the formation of actin filaments. The formation of filaments induces a conformation change of actin monomers causing an increase in fluorescence (Cooper et al., 1982). The increase in pyrene fluorescence was monitored at 347 nm excitation with a 2.5 nm slit width, and 407 nm emission with a 5 nm slit width for 600
minutes as filaments were produced. The photomultiplier tube voltage was held at 1000 V. The critical concentration was determined by plotting the actin sample’s change in fluorescence signal against the actin concentration (2.5, 5, 10, 15, 20 µM). Linear regression of the last fifty fluorescence readings for each concentration were taken using Microsoft Excel 2008 (Redmond, WA). The x-intercept value was determined to be the critical concentration (C_c).

2.6 Circular Dichroism

The thermal stability of A295S protein was determined using circular dichroism (CD) spectrometry. Actin protein samples were dialyzed overnight in HEPES G-buffer (2 mM HEPES, pH 8.0, 0.2 mM CaCl₂, 0.2 mM ATP, and 0.2 mM BME) with at least 2 buffer exchanges. Following dialysis, the concentration of the A295S samples were determined using absorption spectroscopy at 280 nm and the protein was subsequently diluted to 0.16 mg/mL using HEPES G-buffer. The negative ellipticity of the A295S samples from three separate protein purifications were measured in capped, quartz cuvettes with a 1 mm pathlength (Hellma, Concord, ON) using a JASCO J-815 Chiro-optical spectrometer (JASCO, Easton, MD). An increasing thermal gradient from 20-85°C was applied to the samples at a rate of 1°C/minute using a JASCO PTC-424S Peltier temperature control unit (JASCO, Easton, MD) and the loss of negative ellipticity was monitored at 222 nm. To gain information regarding the secondary structure of the actin protein samples, the far-UV CD spectra of the protein samples were obtained from 195-250 nm.
using the Spectra Manager II software (JASCO, Easton, MD) before and after the application of the thermal gradient.

The melting temperature ($T_m$) of the actin protein samples was defined as the point at which 50% of the actin protein is unfolded, as described previously in (Pollard et al., 1988). To calculate the $T_m$ values for each actin protein, the rate of change in ellipticity at 222 nm was plotted as a function of temperature. A Weibull fit was then applied to this curve using SigmaPlot 11 (Systat Software, San Jose, CA) and the minima taken to be the $T_m$.

### 2.7 Fluorescent microscopy: In vitro motility assay

The velocities of mutant ACTC protein filaments were examined when bound and released by skeletal myosin in the presence of saturating ATP. Monomeric ACTC protein was polymerized overnight at 4°C with the addition of 10x polymerization buffer and equimolar rhodamine phalloidin (Cytoskeleton, Denver, CO). Skeletal myosin was diluted to 0.5 mg/mL using Assay buffer (25 mM KCl, 25 mM imidazole•HCl, pH 7.5, 1 mM K•EGTA, 4 mM MgCl$_2$, 10 mM DTT) and flowed over nitrocellulose-coated cover slips to bind for two minutes. Assay buffer containing Bovine serum albumin (1mg/mL) was then flowed over cover slip to block the surface. Fluorescently labeled actin filaments, diluted with assay buffer (to about 0.1µM) were then flowed over cover slip and allowed to bind for two minutes.

Movement was initiated upon the addition of motility buffer (25 mM KCl, 25 mM imidazole•HCl, pH 7.5, 1 mM K•EGTA, 4 mM MgCl$_2$, 10 mM DTT, 2 mM ATP) and
an oxygen-scavenging system to retard photobleaching (25 µg/mL glucose oxidase, 45 µg/mL catalase and 1% glucose). Visualization of filaments was by Zeiss Axiovert 200 inverted scope (Zeiss, Jena, Germany) with a TX2 (510 nm) Fluorescent filter. Videos were captured using an Andor Luca camera (Andor Technology, Belfast, Ireland) with Zeiss plan NEOFLUOR 100/1.30 oil objective and Andor Luca software at a frame rate of 0.12711 frames/second. Individual actin filaments, which moved in a linear pattern, were selected for measurement. Velocities for these filaments were determined by measuring total distance traveled over frame length using a calibration of 5.6 pixels/µm. Twenty-five to thirty filaments were measured for each ACTC variant protein per condition from at least two separate protein purifications. Filaments were selected based on linear movement over a minimum of 6 frames.

2.8 NADH coupled ATPase assay

The ATPase activity of myosin S1 upon activation by filamentous actin was examined using the NADH-coupled ATPase assay adapted from Kiianitsa et al. 2003. This assay utilizes the hydrolysis of ATP when phosphoenol pyruvate is converted to pyruvate by pyruvate kinase, and subsequently the oxidation of NADH when pyruvate is converted to lactate by lactate dehydrogenase. Provided with an ATP regenerating system, it is possible to observe the decline of NADH upon ATP hydrolysis. ACTC protein was polymerized overnight at 4°C with the addition of polymerization buffer (pH 7.5) to a final concentration of 6.25 µM. Filamentous actin was then placed into UV- plastic cuvettes and the sample was blanked. An ATP regenerating system (Lactate Dehydrogenase 4 U, Pyruvate Kinase 20 U, 500 µM Phosphoenol Pyruvate) (Sigma Aldrich, St. Louis, MO) was then added with
NADH (356 µM). Upon the addition of myosin S1 in solution with ATP (0.625 µM and 1 mM, respectively), readings were taken at 340 nm every 30 seconds for 1 hour to observe the decline in NADH over time. Slopes of the linear portion of each set of readings were taken and multiplied by the inverse slope of the NADH standard curve with an $R^2$ value of 0.99 (determined experimentally) to determine S1 ATPase rates.

2.9 Statistical analysis

Standard deviations for all results were calculated using Microsoft Excel, (Microsoft Office, 2008, Redmond WA). Statistical differences were determined using unpaired students t-test via Graph pad quick calcs (Graph pad software, San Diego, CA).
Figure 8. Determination of S1 ATPase rates from NADH coupled assay. A. Decline of NADH upon ATP hydrolysis measured at 340 nm. WT ACTC incubated with S1 in the presence of saturating ATP with regeneration system. B. NADH standard curve, determined experimentally at 340 nm, $R^2=0.99$. 

Chapter 3- Results

3.1 Production and maintenance of recombinant baculoviruses

The baculovirus expression system was used to produce quantities of active ACTC proteins around 1-2 mg per billion cells. Seven of the fourteen currently known ACTC recombinant baculoviruses had been previously made. However to increase infectivity and ensure viruses were still infectious, they were amplified to a higher viral passage by infecting Sf9 cells and monitoring the infection process and production of PIBs. All recombinant ACTC baculoviruses showed high expression of ACTC protein and the production of viral PIBs. For the production of the remaining two ACTC variants, the A295S ACTC and P164A ACTC genes were BamHI-digested out of TOPO vectors (Figure 9A) and sub-cloned into the pAcUW2Bmod transfer vector. The presence and orientation of ACTC insert in pAcUW2Bmod transfer vector were verified by NaeI digest where the appearance of bands at ~ 750 bp showed the ACTC insert in the correct orientation (Figure 9B). Direct sequencing ensured the presence of A295S and P164A mutations and no other mutation. The ACTC genes in pAcUW2Bmod transfer vector were cotransfected with linear viral DNA into Sf9 cells and incubated for a minimum of one week to produce passage 1 ACTC recombinant baculovirus. The A295S baculovirus was then amplified twice to a passage 3 (P3) viral stock. The P164A virus repeatedly failed to show infection of Sf9 cells and after re-cloning and re-making the P164A recombinant baculovirus various times, it only showed very weak infection.

To confirm that ACTC protein was being expressed, Sf9 cells were infected with A295S recombinant baculovirus and harvested 72 hours p.i. Actin was purified using
A. Isolation of ACTC insert was achieved by *BamHI* digest. Lanes 1 and 2 show presence of ACTC variant P164A, Lanes 3 and 4 show presence of ACTC variant A295S at approximately 1125 bp in TOPO vectors after digestion with *BamHI*. B. Presence and orientation of ACTC cDNA in pAcUW2Bmod expression vector confirmed with *NaeI* digestion. Lanes 1 and 2 show the presence of ACTC variant A295S while lane 5 shows presence of ACTC variant P164A in the correct orientation in pAcUW2Bmod at 741 bp. Lane 4 shows unsuccessful digestion of pAcUW2Bmod containing of ACTC variant P164A. Lanes 3 and 6 are empty.
DNase-I affinity chromatography and purified product was run on 10% SDS-PAGE, showing prominent bands at 42 kDa (Figure 10C). A295S ACTC protein was run on an additional gel with equal amounts of WT ACTC and α-skeletal actin as controls, and subsequently transferred onto nitrocellulose membrane and probed with mouse monoclonal anti-α-cardiac primary antibody (Figure 10A). Although the antibody bound with some affinity to α-skeletal actin, it bound with greater affinity to both WT and A295S ACTC protein showing intense bands compared to α-skeletal actin. To then verify that α-cardiac actin was being expressed and purified actin was not a result of endogenous insect actin, WT ACTC, A295S ACTC and endogenous insect actin were run on 10% SDS-PAGE in equal quantities along with ACTC recombinant baculovirus-infected cell lysate harvested 72 hours p.i. Protein was transferred to nitrocellulose membrane and probed with anti-α-cardiac primary antibody (Figure 10B). Again WT and A295S ACTC actin showed prominent bands, and ACTC protein expressed in infected cell lysate. Endogenous insect actin did not show affinity to anti-α-cardiac primary antibody and no band was present.
Figure 10: Confirmation of α-cardiac actin expression and purification.
A. α- Skeletal actin, baculovirus-expressed A295S and WT α-cardiac actin of equal quantity (6.5 µg) were loaded onto 10% SDS-PAGE and transferred to nitrocellulose membrane and probed using mouse monoclonal anti α-cardiac actin primary antibody. B. Baculovirus-infected Sf9 cells were lysed 72 hours p.i and lysate was analyzed by SDS- PAGE and western blot for expression of α-cardiac actin. Purified A295S ACTC, WT ACTC and endogenous Sf9 actin of equal quantity were loaded to verify lack of non-specific binding of α-cardiac actin monoclonal antibody to endogenous insect actin. C. Purified ACTC protein. Lane 1 and 2 show purified WT and A295S ACTC protein respectively.
3.3.1 A295S ACTC recombinant baculovirus is hyper-infective

During the amplification of the A295S ACTC recombinant baculovirus to higher viral passages, infected Sf9 cells showed increased infection and more severe cell morphology by 48 hours p.i and death by 72 hours p.i. With typical ACTC recombinant baculovirus infection, cells begin to show signs of infections by 36 hours p.i and full infection by 72 hour p.i, with cell lysis and death occurring around 96 hours p.i. These morphological changes include cell spreading, nuclei enlargement and the production of viral PIBs. Monolayers of cells of equal density were infected by WT and A295S ACTC recombinant baculoviruses, and were examined at 24, 36, 48 and 72 hours p.i (Figure 11B). WT ACTC baculovirus-infected cells showed enlarged nuclei and development of PIBs as early as 36 hours p.i, while A295S ACTC baculovirus-infected cells showed enlarged nuclei but never developed visible PIBs. A295S ACTC baculovirus-infected cells did show more cell spreading compared to WT ACTC baculovirus-infected cells, and demonstrated characteristics of cells lysis and death after 48 hours p.i. To compare viral infectivity between WT and A295S ACTC baculoviruses, plaque purification was completed. Monolayers of 2.0 x 10^6 Sf9 cells were infected with baculovirus diluted to 10^{-5} pfu/mL and the virus was allowed to adhere to cells. The cells were then overlaid with media containing 4% agarose and incubated for a week. The cells were subsequently stained with neutral red solution (Figure 11A). Live cells are stained red while dead cells demonstrating viral infection and subsequent cell death were unstained. At 10^{-5} pfu/mL dilution of virus, WT ACTC baculovirus-infected monolayers show clear viral plaques among live cells, while the A295S ACTC baculovirus-infected monolayers show no live cells. To obtain a quantitative value of infectivity, viral titer values were determined for
**Figure 11: A295S ACTC baculovirus is hyper-infective.**

**A.** Monolayer of $2.0 \times 10^6$ Sf9 cells infected with WT ACTC baculovirus (left panel) and A295S ACTC baculovirus (right panel) at an MOI of 1. Cells were stained with neutral red solution five days post infection, live cells are stained red while viral plaques (lysed cells) are shown as white. A295S ACTC baculovirus-infected monolayer shows increased cell lysis and death compared to WT.

**B.** After 72 hours p.i, cells infected with WT ACTC show formation of viral PIBs and typical infected cell morphology. A295S ACTC baculovirus-infected cells show spread cell morphology with beginning of cell lysis. Both WT and A295S ACTC infected cells show nuclear enlargement by 48 hours p.i.

<table>
<thead>
<tr>
<th>Virus Stock</th>
<th>Titer value: pfu/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$1.37 \times 10^8$</td>
</tr>
<tr>
<td>A295S</td>
<td>$1.61 \times 10^{10}$</td>
</tr>
</tbody>
</table>
the WT and A295S ACTC baculovirus. WT ACTC baculovirus had a titer value of $1.37 \times 10^8$ pfu, while A295S ACTC baculovirus had a titer value of $1.61 \times 10^{10}$ pfu. The A295S ACTC baculovirus titer is about 100 times higher than those generally obtained from baculoviruses.

### 3.2. Characterization of the intrinsic properties of ACTC variant protein

Due to the conserved nature of actin in eukaryotic cells, it is thought that mutated residues in the ACTC monomer can be detrimental in terms of protein structure and function. As previously stated, ACTC mutations have been known to be linked to the development of both hypertrophic and diluted cardiomyopathy (Song et al., 2010) with the molecular mechanisms leading to these disease states still not fully understood.

Several intrinsic properties of ACTC protein were examined to better understand the effect of each mutation using an array of techniques. The seven previously produced ACTC variants were tested by previous graduate students to investigate if defects were present in the either monomeric actin protein stability and folding, or in polymerization and filament production (Mundia et al., 2012).

ACTC protein folding and intrinsic stability were examined by obtaining the melting temperature ($T_m$) for each ACTC variant. This was achieved by means of circular dichromism, monitoring the decline of secondary structure while a temperature ramp was applied to each sample from 20-85°C at 1 °C /min scan. DNase-I inhibition was also examined, since properly folded actin has the ability to inhibit DNase-I enzymatic activity. The concentration of each ACTC variant at which 50% of DNase-I inhibition
was achieved; (IC$_{50}$ values) were used as an indication of protein folding (Morrison and Dawson, 2007).

Actin structure and function is in part maintained by its ability to bind an intrinsic nucleotide within the core of the monomer. Because of this important characteristic, previous work was done in order to determine the rates of intrinsic nucleotide release for each ACTC mutant. Whereas nucleotide binding is critical for protein stability, hydrolysis of bound ATP and inorganic phosphate release are significant for actin polymerization. ATP hydrolysis occurs at considerably higher rates during polymerization than basal ATPase rates for monomeric actin (Waechter and Engel, 1975); therefore, the rates of P$_i$ release during polymerization were also measured for each ACTC mutant in order to gather information regarding the intrinsic ATPase, and polymerization ability. The polymerization ability of each ACTC variant was also examined by determining the critical concentration (C$_c$) of each ACTC mutant protein required for polymerization.

The results of all previously determined intrinsic properties for several known ACTC mutations are given in Table 1 (Mundia et al., 2012). WT ACTC protein had a T$_m$ of 56.8 ± 0.35 °C while α-skeletal actin had a T$_m$ of 58.7 ± 0.40 °C. The lowest T$_m$ was obtained from the R312H mutant, which measured 53.6 ± 1.7 °C. The Y166C and A230V ACTC mutants also showed low T$_m$ values, while all other ACTC mutants had reported T$_m$ values comparable to WT ACTC.

The R312H, A331P, E99K and Y166C ACTC proteins had the highest DNase-I IC$_{50}$ values respectively compared to WT ACTC protein. R312H, A331P and Y166C ACTC also showed the highest concentration of monomeric actin required for
polymerization, while A230V, M305L and E99K ACTC proteins had moderately high $C_c$ values. R312H and M305L had similarly fastest intrinsic $P_i$ release rates, with Y166C ACTC demonstrating reduced rates.

Table 1. Summary of the intrinsic properties of ACTC variant proteins related to the development of cardiomyopathies in humans. All values are the average of at least three biological replicates. Standard deviations for each are shown.

<table>
<thead>
<tr>
<th>Actin Protein</th>
<th>$C_c$ (µM)</th>
<th>$T_m$ (°C)</th>
<th>$IC_{50}$ (nM)</th>
<th>$P_i$ Release (s$^{-1}$ × 10$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.28 ± 0.13</td>
<td>56.8 ± 1.3</td>
<td>13.2</td>
<td>1.24 ± 0.02</td>
</tr>
<tr>
<td>A230V</td>
<td>2.11 ± 1.23</td>
<td>53.9 ± 1.9</td>
<td>15.3</td>
<td>1.51 ± 0.24</td>
</tr>
<tr>
<td>A331P</td>
<td>2.95 ± 1.70</td>
<td>57.4 ± 0.23</td>
<td>26.0</td>
<td>0.88 ± 0.06</td>
</tr>
<tr>
<td>E361G</td>
<td>0.58 ± 0.48</td>
<td>55.6 ± 1.7</td>
<td>16.8</td>
<td>0.66 ± 0.05</td>
</tr>
<tr>
<td>E99K</td>
<td>1.67 ± 0.43</td>
<td>56.7 ± 0.23</td>
<td>25.7</td>
<td>1.25 ± 0.02</td>
</tr>
<tr>
<td>M305L</td>
<td>2.21 ± 1.78</td>
<td>55.5 ± 1.7</td>
<td>17.0</td>
<td>2.43 ± 0.03</td>
</tr>
<tr>
<td>R312H</td>
<td>4.75 ± 0.27</td>
<td>53.6 ± 2.0</td>
<td>36.7</td>
<td>2.59 ± 0.05</td>
</tr>
<tr>
<td>Y166C</td>
<td>2.67 ± 0.45</td>
<td>54.1 ± 1.9</td>
<td>24.6</td>
<td>0.38 ± 0.02</td>
</tr>
</tbody>
</table>

(Adapted from Mundia et al. 2012)

$C_c$: Critical concentration

$T_m$: Thermal Melting temperature

$IC_{50}$: The concentration of actin at which 50% of DNase-I is inhibited

$P_i$ release: Rate of intrinsic inorganic phosphate release of F-actin
3.3 Characterization of intrinsic properties of A295S ACTC variant protein

To understand the molecular mechanism of the cardiomyopathy disease states, the intrinsic characteristics of the A295S ACTC variant protein were examined. It is thought that intrinsic defects in ACTC may contribute to overall protein instability and perturb important contacts within actin-actin binding, thus leading to deficiencies in polymerization and therefore contribute to the development of disease. The A295S substituted residue of the ACTC monomer is located in subdomain 3, and is in close proximity to the intrinsic nucleotide and divalent cation binding sites. These inherent features of the ACTC monomer are essential for its structure and function. (Oda et al., 2009). The substitution of a hydrophobic amino acid for a polar amino acid may disrupt important contacts in this region of the ACTC monomer.

3.3.1 Characterization A295S ACTC variant protein folding and stability

The thermal stability of the A295S ACTC variant protein was measured using circular dichroism. Using values obtained from the loss of negative ellipticity as a temperature gradient (20-85°C) was applied to A295S ACTC protein samples, a denaturation curve was generated to show the unfolding of each protein sample as the temperature increased (Figure 12). Protein samples were analyzed within 48 hours of purification to ensure lack of inactivity or unfolding (Mundia, unpublished data). The T_m for the A295S ACTC mutant was determined to be 55.72 ± 0.22 ºC. Using an unpaired student t-test, the P value obtained was 0.2220; therefore the T_m of A295S ACTC was not significantly different from the measured T_m of WT ACTC at 56.80 ± 1.3 ºC.
**Figure 12. A295S ACTC variant protein folding:** Circular dichroism spectroscopy was used to determine the thermal melting temperature of A295S ACTC protein. A thermal gradient was applied to protein samples at a rate of 1°C per minute (from 20°C to 80°C). Averages of A295S ACTC protein samples were plotted with standard error at each temperature. The denaturation curve is shown with sigmoidal fit in blue. By identifying the midpoint of the denaturation curve, the melting temperature ($T_m$) of A295S ACTC was determined as 55.72 ± 0.22°C. The A295S ACTC $T_m$ is not significantly different from WT ACTC, 56.84 ± 1.3°C. (N= 3 biological replicates).
3.3.2 Characterization of A295S ACTC variant protein polymerization activity

To test the effects of the A295S substitution on actin-actin binding and rates of polymerization, the critical concentration of A295S ACTC variant protein was determined. Samples of 2.5, 5, 10, 15 and 20 µM A295S ACTC with 2.5% pyrene actin incorporation were measured at baseline fluorescence for two minutes, then upon the addition of polymerization salts, pyrene fluorescence was monitored as it increased over time (Figure 13A). A295S ACTC variant protein does not show polymerization deficiencies based on a rapid increase of pyrene fluorescence within twenty minutes of the addition of polymerization salts. Figure 13B shows the linear regression of the average maximum fluorescence for each concentration of A295S ACTC variant protein, with an $R^2$ value of 0.96. The x-intercept was taken as the critical concentration for A295S ACTC polymerization; and was determined to be 0.36 ± 0.06 µM. Pyrene polymerization assays were performed within 24 hours of purification to ensure all proteins used were stable and active. The measured $C_c$ of A295S ACTC protein was very similar to the measured $C_c$ of WT ACTC protein at 0.28 ± 0.1. Using an unpaired students t-test, the determined $p$-value was 0.3005; therefore, there does not appear to be significant differences in polymerization activities between WT ACTC and A295S ACTC proteins under these conditions.
Figure 13: Pyrene actin polymerization of A295S ACTC protein. A. Polymerization activities of A295S ACTC variant protein were examined by incorporating pyrene labeled actin and monitoring the pyrene fluorescence signal for 600 minutes. A295S ACTC protein samples of 2.5 µM, 5 µM, 10 µM, 15 µM and 20 µM contained 2.5% pyrene labeled actin. With the addition of polymerization salts there was an increase in the pyrene fluorescence signal as actin filaments were formed. B. Averages of the last fifty points for each protein concentration were taken and plotted as the maximal fluorescent values at each concentration. The x-intercept of the linear regression was taken as the minimum concentration of actin required for A295S ACTC protein polymerization. A295S ACTC does not show significant difference in critical concentration compared to WT ACTC. \( R^2 \) value = 0.96 , N=3 for each ACTC protein.
3.4 Characterization of ACTC variant proteins interaction with skeletal myosin

The molecular basis by which mutations in ACTC alter thin-filament function and lead to cardiovascular disease is currently unknown. While it is important to understand the effects of ACTC mutations on protein structure and function itself, the result of some mutations may in fact be impaired binding to a number of different sarcomeric proteins. Since a sarcomere is the most basic unit of muscle, perturbations in important protein-protein interactions can be detrimental to muscle function and stability and bring about a pathological state. Among these sarcomeric interactions, perhaps the most essential to muscle function and contractility is the actomyosin interaction (Behrman et al., 2012).

Mutations in MYH7, a gene encoding a myosin heavy chain beta (MHC-β) isoform expressed mainly in the heart are the primary cause of genetically inherited HCM and DCM (Curila et al., 2012). Therefore, mutations that specifically affect the mechanics, kinetics and regulation of the actomyosin complex could interfere with the basic contractile protein machinery (Debold et al., 2010). Comparing enzymatic activities, motility and force generation between WT and mutant ACTC variant proteins will determine the effects of ACTC mutations on actin-myosin binding and can contribute to our understanding of the molecular mechanisms leading to the diseased heart state.

3.4.1 Average activities of actin activated myosin ATPase

The ATPase activity of the S1 catalytic domain of skeletal myosin was measured when bound and released from cardiac actin. This was achieved by utilizing an NADH coupled assay in which the decline of NADH is proportional to ATP hydrolysis by
myosin fragment S1 (Kiianitsa et al., 2003). This assay is based on two reactions; the first being the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase with the regeneration of ATP from ADP. The second reaction is the reduction of pyruvate to lactate by lactate dehydrogenase with the oxidation of NADH to NAD⁺. ACTC mutant proteins were polymerized and mixed with S1. Immediately upon the addition of ATP, absorbance readings were taken every 20 seconds for an hour for each ACTC protein bound to S1. The rate of actin-activated S1 ATP hydrolysis was determined from the slope of the linear decline of NADH.

Rates of actin-activated S1 ATPase activities are shown in Figure 13. R312H ACTC consistently showed decreased ATPase rates compared to WT ACTC at 13.1 ± 0.63 µM/min, and 15.3 ± 1.58 µM/min, respectively (Figure 14A) (*p<0.05, student t-test unpaired). To ensure this difference was significant, R312H actin-activated S1 ATPase rates were compared to other ACTC mutant protein rates using a unpaired student t-test. Rates of R312H ACTC activated S1 ATPase activity were significantly different from A295S, E361G and M305L ACTC variants with p values of 0.0025, 0.0136 and 0.0019 respectively. The S1 ATPase rates from R312H ACTC were not compared to E99K ACTC rates, due to its divergence from all other ACTC protein rates. In contrast, E99K actin-activated S1 ATPase rates at 20.1 ± 1.46 µM/min were significantly higher than WT ACTC rates (p <0.01 student t-test unpaired). Again these rates were compared to other ACTC mutant rates. Actin-activated S1 ATPase rates of E99K ACTC were significantly different from all ACTC mutants.
Figure 14. ACTC activated ATPase activity of myosin. A. ATPase rates of S1 skeletal myosin were measured when bound to ACTC variant protein under saturating ATP concentration. The measured rates for E99K and R312H ACTC variants were significantly different than WT ACTC (*p <0.01, p<0.05 respectively, student t-test unpaired). Runs were done in triplicate, N=3 biological replicates (Y166C; N=2) for each ACTC variant. B. Summary of actin activated ATPase rates of S1 catalytic domain of skeletal myosin with ACTC variant protein. All values are the average of at least three biological replicates (Y166C; N=2) (*p<0.05, student t-test unpaired)
3.4.2 Motility of ACTC mutant filaments under increasing ionic strength

The effects of the ACTC mutations on actin-myosin binding were also examined by measuring the velocity of individual filaments of each ACTC mutant protein in an *in vitro* motility assay. This assay allows the measurement of the distance travelled by individual fluorescently labeled actin filaments, as it binds and releases from myosin under sufficient ATP conditions. The velocities of unregulated ACTC variant filaments propelled by rabbit skeletal myosin were determined under a range of ionic concentrations (Figure 15). All ACTC variant proteins showed a linear decline in velocity as ionic strength increased from 25 mM to 75 mM KCl. Measurements were taken at 100 mM KCl; however, no movement was observed for all ACTC mutant proteins, and actin filaments displayed dissociation from the myosin-bound surface. A295S and R312H mutants were not examined by this assay due to inability to form filaments of appropriate length after flash-freezing with liquid nitrogen for approximately 30 seconds and thawing upon usage. A230V and E99K mutants exhibited significantly reduced velocities compared to WT ACTC at all ionic concentrations tested (*p*<0.005). A331P and M305L ACTC showed decreased filament velocities compared to WT ACTC, but only at ionic strength at 50 mM KCl and higher (*p*<0.05). Y166C ACTC filaments also showed a decline in velocity compared to WT ACTC at higher ionic strength, but also showed formation of aggregates consistent with polymerization deficiencies associated with this mutant.
Figure 15: Average filament velocities of ACTC variant protein. Gliding filament assays were performed for each ACTC mutant labeled with rhodamine-phalloidin gliding over a bed of rabbit myosin-II at varying ionic strength (25 mM, 50 mM, and 75 mM KCl). For reference, the linear regression line for WT ACTC is shown in each panel. No movement was observed for all ACTC filaments at 100 mM KCl. The average and standard deviation of 25 filament velocities for each condition is plotted. A230V and E99K ACTC filaments were significantly slower at all ionic strengths. A331P, M305L and Y166C were significantly slower at higher ionic strength ($p<0.02$, student t-test, unpaired) E361G ACTC slower at 75 mM ionic strength. N=2 biological replicates per ACTC variant.
Table 2: Summary of the average velocities of ACTC variant filaments moving over immobilized rabbit skeletal myosin under increasing ionic strength.

<table>
<thead>
<tr>
<th>ACTC Variant</th>
<th>Filament Velocity (µm/sec) 25 mM KCl</th>
<th>Filament Velocity (µm/sec) 50 mM KCl</th>
<th>Filament Velocity (µm/sec) 75 mM KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.1 ± 0.69</td>
<td>2.7 ± 0.77</td>
<td>2.6 ± 0.88</td>
</tr>
<tr>
<td>A230V*</td>
<td>2.06 ± 0.46</td>
<td>1.36 ± 0.33</td>
<td>1.27 ± 0.43</td>
</tr>
<tr>
<td>A331P**</td>
<td>3.0 ± 0.72</td>
<td>1.37 ± 0.3</td>
<td>1.25 ± 0.54</td>
</tr>
<tr>
<td>E361G**</td>
<td>2.57 ± 0.66</td>
<td>2.67 ± 0.65</td>
<td>1.6 ± 0.77</td>
</tr>
<tr>
<td>E99K*</td>
<td>1.8 ± 0.56</td>
<td>1.50 ± 0.37</td>
<td>1.17 ± 0.36</td>
</tr>
<tr>
<td>M305L**</td>
<td>2.54 ± 0.62</td>
<td>2.18 ± 0.61</td>
<td>1.5 ± 0.52</td>
</tr>
<tr>
<td>Y166C**</td>
<td>2.51 ± 0.77</td>
<td>1.58 ± 0.42</td>
<td>1.25 ± 0.29</td>
</tr>
</tbody>
</table>

* Reduced velocity at all ionic concentrations, ** Reduced velocity at high ionic concentrations (*p<0.02).

3.4.3 Effects of increasing ATP concentrations on ACTC filament motility

Actin filament velocity is dependent on myosin detachment (Huxley et al., 1990) with detachment rate dependent on ADP release and subsequent binding of ATP to the myosin active site (Siemankowski et al., 1985). To understand the effect of ACTC mutations on these kinetic parameters, gliding assays were performed with WT, E99K and M305L ACTC variants under a range of ATP concentrations, keeping ionic strength at 25 mM KCl. Due to time constraints, other ACTC mutants were not measured under these conditions. The E99K ACTC variant was selected for this analysis because of its drastic reduction in velocity compared to WT ACTC under standard conditions and high ionic strength. Moreover, the E99 residue is located in a
proposed myosin-binding domain (Song et al., 2010). The M305L ACTC variant was selected as a positive control, since under standard conditions its velocity is comparable to WT ACTC. It has also been found to behave in a similar way to WT ACTC in several assays assessing intrinsic properties (Mundia et al., 2012).

As demonstrated in Figure 16 filament velocity increased hyperbolically with ATP concentration. By fitting these data sets to a rectangular hyperbola fit, maximum velocities ($V_{\text{max}}$) and Michaelis-Menten constants ($K_M$) were determined. (Figure 16). Consistent with reduced filament velocity, E99K ACTC had an increased $K_M$ compared to WT ACTC as well as significantly decreased $V_{\text{max}}$ ($p<0.0002$). Interestingly; M305L ACTC had a similar $K_M$ value to E99K however its maximal velocity was parallel to WT ACTC. This could suggest impaired affinity of the M305L ACTC variant protein for myosin at low ATP concentrations.
Figure 16: Average activities of ACTC variant proteins under increasing ATP concentrations. A. Gliding filament assays were performed for WT, E99K and M305L ACTC protein. Filaments were labeled with rhodamine-phalloidin and flowed over a bed of rabbit myosin-II at varying ATP concentrations with ionic strength kept constant at 25 mM KCl. No movement was observed at 0.02 mM ATP for all ACTC variants. The average and standard deviations of 25 filaments velocities for each ATP concentration are plotted and fitted to rectangular hyperbola equation describing Michaelis-Mentan kinetics. E99K ACTC filaments were significantly slower than WT and M305L ACTC at all ATP concentrations. ($p$<$0.0001$, student t-test, 2-tailed,unpaired). N=2 biological replicates per ACTC variant. B. Summary of the maximal velocity velocity ($V_{max}$) and Michaelis constant ($K_{M}$) for the binding of ACTC mutants to skeletal myosin using ATP as a substrate.

<table>
<thead>
<tr>
<th>ACTC Variant</th>
<th>$K_{m}$ (mM)</th>
<th>$V_{max}$ (μm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.068 ± 0.01</td>
<td>3.31 ± 0.12</td>
</tr>
<tr>
<td>E99K</td>
<td>0.086 ± 0.02</td>
<td>1.93 ± 0.1</td>
</tr>
<tr>
<td>M305L</td>
<td>0.088 ± 0.01</td>
<td>3.39 ± 0.11</td>
</tr>
</tbody>
</table>
Chapter 4-Discussion

4.1 Production of the A295S ACTC recombinant baculovirus

The baculovirus expression system (BEVs) was used to over-express α-cardiac actin protein. This system was utilized since human actin cannot be expressed in bacteria due to the absence of the appropriate eukaryotic chaperonin protein complex. *Saccharomyces cerevisiae* was not a suitable expression host since yeast actin only shares 87% homology to human actin. The BEVs was used due to its ability to produce milligrams of overexpressed human α-cardiac actin per billion cells, while shutting down the host’s endogenous actin production (Yates et al., 2007). The A295S and P164A ACTC producing baculoviruses were not constructed with the other ACTC recombinant baculoviruses due to problems with virus production and maintenance. To ensure the co-transfections with these ACTC genes were done accurately, the sub-cloning into pAcUW2Bmod was repeated. Co-transfection of the A295S ACTC gene in pAcUW2Bmod with linear baculovirus DNA was successful due to the unhealthy morphology of the *Sf9* cells after the virus was passaged to a p3. Unfortunately, the P164A ACTC virus showed lack of infectivity since “infected” cells never showed unhealthy cell morphology and never developed PIBs, which is typical of baculovirus infection in the late stage (Ohkawa et al., 2010), even after the virus was passaged to p4. Production of P164A ACTC recombinant baculovirus was attempted three times however a successful virus was never generated. This could be due to multiple factors influencing viral production and infectivity. The initial co-transfection of the recombinant baculovirus could have been very weak with a low rate of infection and titer value. Also,
the passaging to p3 values and higher to increase infectivity could have resulted in the production of mutant viruses with few to no PIBs (de Rezende et al., 2009).

4.1.1 Hyperactivity of A295S ACTC recombinant baculovirus

Interestingly, while the P164A ACTC seemed to produce a weak virus or lack of virus at all, the A295S ACTC recombinant virus appeared to be hyper-infective compared to the other ACTC recombinant baculoviruses. Upon reaching a p2 passage, cells infected with this A295S ACTC baculovirus appear unhealthy after 48 hours and were completely lysed by 72 hours p.i. This was strikingly different than the other ACTC recombinant viruses, which were passaged to a p3 and showed signs of late viral infection and expression of genes under the late viral promoter and the production of PIBs by 72 hours p.i. To investigate this difference, cells infected with either WT ACTC or A295S ACTC baculovirus were examined. At 48 hours p.i, cells infected with A295S ACTC baculovirus showed accelerated signs of viral infection such as enlarged cell nuclei and signs of cells lysis; however, cells never developed the PIBs typical of baculovirus infection. Also, when A295S ACTC virus was plaque-purified to ensure viral stocks were isolated from a single virus and reducing the likelihood of viral genetic diversity, viral plaques grew exceptionally quickly and merged into one another even at dilutions down to $10^{-5}$ (Figure 11A). This phenomenon with the A295S ACTC recombinant baculovirus was also seen by a previous graduate student; however, other mutations were present within the A295S ACTC gene of that viral stock.

The first notion of the cause of the accelerated cell death with A295S ACTC baculovirus was possible cytotoxicity released either from the cells into the media or
from the media itself. A fourth year project student in the Dawson lab tested this
hypothesis, and it was found that cytotoxicity was not the primary cause of the cell lysis
and death (data not shown). Unfortunately, the exact reason for the hyper-infectious state
of this virus is still unclear; however, it is speculated that the A295S ACTC mutation is
specifically affecting the viral sequestering of host actin and nuclear entry. Viruses
generally utilized microtubule-based mechanisms to enter and exit the host nucleus.
Baculoviruses are a remarkable exception to this, using the actin cytoskeleton during the
early phase of infection for the production of the virions released into the cytoplasm
responsible for cell to cell infection, known as budded virus (BV) (Volkman and Zaal,
1990; Ohkawa at al., 2010).

Since the ACTC gene is under the control of the very late phase \( p10 \) viral promoter
(Joel et al. 2004), a clear explanation of how the A295S ACTC mutation is affecting the
virus and its ability to hijack the host’s actin cytoskeleton for rapid nuclear entry and BV
production is not presented and further experiments will have to be carried out to
understand this baculoviruses’ mode of infection. The visible lack of PIBs with A295S
ACTC baculovirus infection may be explained by the control of the \( \text{polh} \) promoter. PIBs
contain baculovirus occluded virus (OV), which are baculovirus virions embedded in a
polyhedral matrix and are responsible for horizontal gene transfer and are produced in the
very late phase of infection under the baculovirus polyhedrin promoter (\( \text{polh} \)). The
Dawson lab has previously discovered that actin expression under the control of \( \text{polh} \) is
inefficient and suggested a model where active actin protein down-regulates the \( \text{polh} \)
promoter (Yates et al., 2007). Whether over-expression of this specific A295S
ACTC mutant protein is affecting the \( \text{polh} \) promoter in a similar method, or PIBs and
thus OV production is reduced indirectly by an over-production of BV still needs to be determined. Future experiments will be directed to the examination of the A295S ACTC recombinant baculovirus and the mechanism of hyper-infectivity.

4.2 Characterization of ACTC variant protein intrinsic stability and function

The intrinsic properties of the newly produced A295S ACTC variant protein were examined and compared to previous results with other ACTC variants. Generally, mutations located in subdomain 3 tended to affect the stability and polymerization ability of actin protein (Mundia et al., 2012). The R312H ACTC protein appeared to be severely affected, demonstrating reduced stability, increased polymerization critical concentration and inorganic phosphate release rates and a dramatic increase in nucleotide release rates compared to WT ACTC. The Y166C ACTC protein exhibited a higher critical concentration and reduced filament formation, leading to a decreased inorganic phosphate release rate (Mundia at al., 2012). Thus, the A295S ACTC substitution located in the lower region of subdomain 3 in the actin monomer was hypothesized to have similar affects on intrinsic actin stability and function.

First, the intrinsic folding of A295S ACTC protein was assessed using circular dichroism. The determined T_m of 56.84 ± 0.32 °C did not differ significantly from WT ACTC protein, indicating the variant protein was properly folded. The effects of the A295S mutation on actin polymerization was also examined by determining the critical concentration required for actin polymerization. Again the A295S ACTC variant did not exhibit differences in C_c values compared to WT ACTC, with these values being almost identical. This is consistent with finding by Vang et al. (2005), who tested the hypothesis that certain mutations in the ACTC gene cause misfolded polypeptides resulting in a loss
of function or the elimination of the protein leading to the development of disease. Using a cell-free coupled translation assay, the association of the A295S ACTC protein with the chaperonin TCP-1 ring was examined. The chaperonin TCP-1 ring complex/chaperonin containing TCP-1 (TriC/CCT) is required for complete actin folding in the cytoplasm. It was determined that the A295S ACTC protein did not have altered chaperone interaction kinetics (Vang et al., 2005). COS-7 cells overexpressing A295S ACTC protein were stained with fluorescent phalloidin to visualize its colocalization with cytoskeleton actin fibers. The A295S ACTC protein colocalized with actin filament similar to the wild-type (Vang et al., 2005), indicating proper folding.

The lack of similarities between A295S ACTC protein and the other subdomain 3 ACTC mutants in terms of detrimental actin stability and polymerization is likely due to the specific location of the A295S mutation in subdomain 3. The A295S mutation is located at the lower periphery of the actin monomer in contrast to the R312H mutation located in the centre of the molecule closer to the nucleotide binding cleft required for actin stability (Vang et al., 2005). Also, the Y166C ACTC variant may show more pronounced polymerization deficiencies compared to A295S ACTC protein since the Y166 residue is involved in important and inter- and intra-filament contacts within the actin helix (Oda et al., 2009). Furthermore, in patients carrying the A295S mutation, low penetrance, diverse phenotypes and relatively low morbidity with no sudden death were observed, illustrating the non-drastic effects of this specific mutation (Mogensen et al., 2004). In contrast, the P164A mutation is also located within the interior of subdomain 3 which may affect internal contacts and monomer stability more than mutations located on
the exterior. Future studies examining the P164A ACTC mutation may provide more insight into the effect of mutation locations on actin protein stability.

4.3 Understanding the effects of the ACTC mutations on skeletal myosin binding

To determine the impact of mutated residues in the ACTC protein on myosin binding, various *in vitro* assays were performed. Specific residues (especially those located in subdomain one of the actin monomer) are proposed to make important contacts with myosin (Lorenz and Holmes, 2010). Effects on myosin binding were investigated by determining the actin-activated ATPase of myosin subfragment S1 as it bound and released from each ACTC variant. These values were compared to filament velocities of each ACTC variant in the *in vitro* motility assay, to determine if effects in actin’s ability to activate the intrinsic ATPase of myosin correlated to force production and filament motility. It is important to note that the degree of effects seen with individual ACTC mutants filaments in these *in vitro* motility assays may be either more pronounced or subtle, than the actual pathological state due to the simplicity of the assay and the lack of regulatory and structural sarcomeric proteins and the control of regulatory ions. This reflects the many levels of control and compensation present physiologically within the cardiomyopathy diseased state of the heart. However, my results help identify the molecular deficiencies of each ACTC mutation and will provide insight to the basic molecular mechanisms involved in the onset of HCM and DCM.
4.3.1 Effect of ionic strength on ACTC mutant protein binding to myosin

The effects of several ACTC mutations were assessed by the *in vitro* motility assay over a range of ionic concentrations. Interactions between actin and myosin have been shown to weaken with increasing salt concentration (Bookwalter and Trybus, 2006). I also observed this effect, as the majority of filaments for all ACTC variants diffused away from the myosin-coated surface above 75 mM KCl; therefore, velocities could not be gathered at higher ionic strength. This assay was also performed in the absence of methylcellulose, which is typically used as an additive to prevent diffusion of actin filaments away from the myosin surface. This approach was taken so any results observed were directly due to the mutated ACTC residue and were not masked by the effects of methylcellulose.

The E99K ACTC mutant protein illustrated the most drastic difference in filament velocity compared to WT ACTC. E99K ACTC moved on average 50% slower than WT ACTC under all ionic concentrations measured. These data are consistent with results observed by Bookwalter et al. (2006) where baculovirus-expressed E99K ACTC protein velocities were approximately 20% slower than WT ACTC near physiological ionic strength. The magnitude of difference seen between their E99K ACTC filament velocity and the velocity reported here may be due to the use of methylcellulose to get near physiological ionic concentrations; the viscous substance helped to keep the actin filaments within close proximity to the myosin surface and possibly masked some of the effects of the E99K mutation on important electrostatic interactions. Song et al. (2010) also found that E99K ACTC filaments generated with the regulatory sarcomeric proteins troponin and tropomyosin showed reduced velocity and higher Ca$^{2+}$ sensitivity.
An unpublished study by Kawai et al. demonstrated similar results with the E99K ACTC variant, confirming that the E99K ACTC exhibits higher Ca\(^{2+}\) sensitivity with a pCa\(_{50}\) value for E99K ACTC (5.78±0.03) compared to recombinant WT ACTC (5.69±0.02). In addition, Kawai et al. investigated the effects of the E99K mutation on the actomyosin complex. Rigor stiffness of the E99K ACTC mutant was determined to be much lower at 0.65 ± 0.16 MPa compared to 2.07 ±0.53 MPa of WT ACTC. The isometric tension was also measured and was found to be 9.4±0.8 kPa for E99K ACTC (N=4) versus 19.6±4.1 kPa for WT ACTC (N=5) (personal communication). Debold et al. (2010) also reported that the E99K ACTC mutation increased the K\(_M\) of myosin to actin almost 4-fold. The initial weak interaction between myosin and the actin filament is essentially ionic in nature, later transitioning to a stronger interaction involving hydrophobic contacts. As stated previously, each myosin head interacts with two actin monomers along the actin helix with the primary binding site being with subdomain 1 of the upper monomer and the secondary binding site being with subdomain 1 of the lower monomer. The E99 actin residue is thought to play a role in this secondary binding, where important electrostatic interactions are made between three acidic residues (E93, E100 and E99) and a positively charged loop in the lower domain of the myosin motor domain (Rayment et al., 1993; Volkmann et al., 2000). The consequence of a glutamate substitution from an acidic amino acid to a basic lysine is expected to have detrimental effects on actin-myosin interactions. The effects of such charge reversions on binding were also seen with HCM- associated E40K and E45K mutations in α-tropomyosin, where both mutations demonstrated reduced affinity to actin (Mirza et al., 2007).
A230V ACTC protein also showed deficiencies in filament motility and myosin binding exhibiting filament velocities 45% slower than WT ACTC under all ionic conditions. This was surprising since the A230 residue is located in subdomain 4 of the actin monomer, and although it is in a proposed tropomyosin binding site, it is not believed to play any important role in actin-myosin binding. Subdomain 4 however, is a primary site for actin-actin binding during polymerization (Hori and Morita, 1992) and previous results with A230V ACTC protein have shown subtle intrinsic property defects. In the monomeric state, A230V ACTC protein demonstrated minor folding deficiencies with elevated DNase-I IC$_{50}$ values when compared to WT ACTC protein. Also A230V ACTC protein exhibited some polymerization deficiencies with moderately amplified C$_c$ values (Mundia et al., 2012). These effects within the actin filament could decrease the strength and stability of filaments and impair filament velocity. Since the A230 residue is located in a proposed tropomyosin site on the actin monomer, future studies using the in vitro motility assay with the addition of regulatory proteins troponin and tropomyosin may provide insight into the effect of this specific mutation.

ACTC variants A331P, M305L and Y166C also displayed reduced filament velocities compared to WT; however, only at ionic strengths of 50 mM or higher. Since actin-myosin binding grows weaker with increased ionic strength, it is expected that subtle effects by mutated ACTC residues may not manifest unless under these conditions. The A331P ACTC mutation resides in close proximity to several residues that are proposed to make important electrostatic and H-bonds with myosin. E334, R335 and K336 all make electrostatic interactions with S1 with E334 involved in H-bonds as well (Rayment et al., 1993). A hydrophobic region on actin (residues 329-334) have also been
proposed to make contact with myosin; therefore, a mutation in this area of the actin monomer could very well affect actin-myosin interactions and resulting force generation (Behrmann et al., 2012).

The M305L and Y166C ACTC variants are associated with symptoms such as pronounced hypertrophy and reduced exercise capacity; however, these mutated residues do not seem to be within important myosin binding regions (Mogensen et al., 2004). Interestingly, under standard conditions, both M305L and Y166C ACTC filaments had very similar velocities to WT ACTC filaments (Figure 15, Table 2) but again when ionic strength is increased to 50 mM and higher, there is an ionic effect. This effect may be explained by preferential binding of these ACTC variants and/or myosin to specific ions instead of one another. These mutations may not directly affect the actin-myosin binding interface; however, under certain pressures (such as increased ionic strength) the detriment of the M305L and Y166C mutations on actin-myosin binding becomes apparent, especially in the absence of sarcomeric structural proteins such as α-actinin and titin providing stability and anchoring for actin and myosin, respectively. These observations therefore are likely caused by the M305L and Y166C mutations affecting the actin protein intrinsically, indirectly implicating the actin-myosin interaction and leading to perturbed force production. The M305L mutation is located in subdomain 3 in close proximity to the nucleotide-binding cleft of the actin monomer; specifically underneath the adenine ring binding region. This mutation also resides in a prominent loop-helix-loop structure in subdomain 3 (Muller et al., 2012). M305L ACTC displayed disturbed intrinsic ATPase activity and nucleotide release in various assays (Muller et al., 2012; Mundia et al., 2012) and M305L filaments stimulated β-cardiac myosin ATPase to only 50% of WT cardiac F-actin (although this
was in contrast to ATPase results reported here). Although the M305L ACTC protein only displayed minor folding defects (Vang et al., 2005) the elevated intrinsic ATPase activity and nucleotide release rates could be a result of the mutations location so near to the nucleotide-binding cleft. If ATP hydrolysis and P_i release is uncoupled from actin polymerization, this may result in inefficient filament production and/or atypical F-actin regulation.

The Y166C mutation leads to a removal of an aromatic ring. This residue has long been recognized to participate in F-actin formation in the interface of the long pitch actin-actin contacts (Lorenz and Holmes, 1993). In addition this residue is within a sequence of actin called the “W-loop”, which has been proposed to act as a nucleotide state-sensing region of actin (Kudryashow et al., 2010). Upon ATP hydrolysis, this region undergoes minor conformational changes resulting from the formation of backbone H-bonds between Y166 and Y169 (Muller et al., 2012). Polymerization studies in the Dawson lab have previously shown that Y166C ACTC has reduced polymerization compared to WT ACTC and has the tendency to form insoluble aggregates (Mundia et al., 2012). These results suggest intrinsic defects in both M305L and Y166C ACTC protein, leading to unstable monomers and fragile filaments. Such deficiencies in actin stability may weaken the F-actin helix itself, reducing the force produced when bound and released by myosin, especially under conditions that weaken the initial actin-myosin interaction. These results may explain in part the classic remodeling and thickening of the left ventricle seen in the HCM condition, whereby cardiac myocytes may compensate for impaired myosin binding and reduced force generation by adopting a hyperfunctional state directly
inducing hypertrophy or up regulation of certain sarcomeric proteins indirectly causing cardiac hypertrophy (Bonne et al., 1998).

4.3.2 Effect of ATP concentration on ACTC variant protein binding to myosin

To further examine the effects of ACTC mutations on myosin binding, the *in vitro* motility assay was carried out under standard ionic concentrations (25mM KCl) and various ATP concentrations. Since ATP is a substrate for myosin’s ATPase and is required for actin release and re-binding, various concentrations of ATP were examined to evaluate effects on the actomyosin interaction by measuring actin motility. Velocities for WT, E99K, and M305L ACTC filaments were obtained under increasing ATP concentrations and were plotted and fitted using a Michealis-Menten equation. The velocity of M305L ACTC filaments was similar to WT ACTC filaments, with a V_max of 3.11 ± 0.11 µm/sec and 3.31 ± 0.12 µm/sec, respectively. E99K ACTC filaments exhibited a maximal filament velocity of 1.93 ± 0.1 µm/sec, almost half that of WT. Surprisingly, M305L and E99K ACTC had actomyosin K_M values of 0.088 ± 0.02 µM and 0.086 ± 0.01 µM, values increased from the WT ACTC K_M at 0.68 ± 0.01 µM. E99K ACTC consistently demonstrated poor myosin binding under various conditions; therefore, an elevated K_M is expected. However the similarly high K_M could suggest poor utilization of ATP by myosin at low concentrations due to impaired affinity of M305L ACTC variant protein for myosin under these conditions.
4.3.3. Myosin ATPase rates in the presence of ACTC variant proteins

Myosin hydrolyzes ATP in the absence of actin; however, this intrinsic S1 ATPase is activated upon actin binding (Rayment et al., 1993). S1 ATPase activity was measured in the presence of each ACTC variant. Many ACTC variants behaved much like WT ACTC; however, myosin S1 in the presence of R312H ACTC filaments exhibited decreased ATPase activity compared to all other ACTC variants. This could be due to the reduced intrinsic stability; decreased $T_m$, elevated nucleotide and $P_i$ release rates and polymerization deficiencies exhibited by the R312H ACTC protein (Mundia et al., 2012). R312H ACTC filaments also showed enhanced calcium activation and reduced filament velocity in the \textit{in vitro} motility assay compared to WT ACTC filaments (Debold et al., 2010). The DCM-associated R312H ACTC mutant may affect the stability and overall number of $\alpha$-cardiac actin filaments, thereby providing inefficient and insufficient amounts of actin for myosin to interact with. This explanation may help describe the phenotype of DCM, having a thin myocardium with decreased contractility (Kärkkäinen et al., 2007). The R312H ACTC mutation is also located in an important tropomyosin binding site, and could impair actin-tropomyosin binding and leave actin in a constant open state, consistent with its increased Ca$^{2+}$ sensitivity when in the presence of regulatory proteins (Debold et al., 2010).

The E99K ACTC variant elicited elevated S1 ATPase rates almost one third greater than WT and any of the ACTC mutants (Figure 14). Given the lowered filament velocities exhibited by the E99K ACTC filaments in the \textit{in vitro} motility assay under various ionic and ATP conditions, this result was unexpected. When various amounts of actin were incubated with full-length cardiac myosin, the $K_M$ for E99K actin was 4-fold
higher than for WT actin; however, the $V_{\text{max}}$ values were not significantly different (Bookwalter and Trybus, 2006). Since the $K_M$ describing myosin’s affinity for actin were not determined in my work, a comparison can not be made; however, the similar $V_{\text{max}}$ values seen between WT and E99K ACTC in the Bookwalter study previously described could be explained by the fact that full-length $\beta$-cardiac myosin was used with increasing amounts of actin, while the S1 catalytic fragment of skeletal myosin and a constant saturating concentration of actin were used for values reported here. Rabbit skeletal myosin has been reported to go through its ATPase cycle approximately 3 times faster than rabbit $\beta$-cardiac myosin (Barany et al., 1964). In addition, a study by Deacon et al. (2012) demonstrated the kinetic differences between $\alpha$ and $\beta$-myosin in terms of ATP hydrolysis. It was reported that $\alpha$-S1 exhibits a more rapid ADP release than $\beta$-S1 comparatively (Deacon et al., 2012). The proposed reasoning for this increased actin-activated S1 ATPase in the presence of E99K ACTC filaments will be explained below.

The E99K ACTC protein displayed a myriad of defects in terms of myosin binding and force production, implying that this mutation is harmful to important contacts necessary for the actomyosin complex. This effect is illustrated by the phenotype displayed specifically by patients harboring the E99K ACTC mutation such as severity of symptoms including ventricular arrhythmia and cardiac arrest in humans (Mogensen et al., 2004) and high morbidity as well as increased interstitial fibrosis, significant apical hypertrophy and sarcomeric disarray seen in mice expressing this mutant actin protein (Song et al., 2011). A loss of affinity for myosin could be the underlying molecular mechanism leading to heart failure and other cardiac dysfunctions seen with the HCM-
causing E99K ACTC mutation, with the hypertrophied appearance of the left ventricle being a compensatory mechanism of the heart.

4.3.4. E99K ACTC protein uncouples ATPase rates and filament motility

The capability to convert chemical energy into mechanical energy is fundamental to muscular contraction. The interaction and release of actin and myosin to produce force and contractility has been well characterized, but the mechanism behind ATPase activity and motility coupling is still unclear. It is generally believed that myosin hydrolyzes ATP in the absence of actin and that phosphate release from myosin is triggered by actin binding (Murphy et al., 2001; Sun et al., 2008, Behrmann et al., 2012). The light-chain binding region of myosin S1 subfragment is known as the “lever arm” and its rotation is responsible for the force-generating power stroke upon phosphate release. ADP dissociation is the rate-limiting step in the actin-myosin ATPase cycle, and once detached allows ATP to re-bind to the myosin catalytic head and the cycle can recommence (Murphy et al., 2001; Behrmann et al., 2012). An essential characteristic of this model is that ATP is not consumed unnecessarily by associating with phosphate release. Phosphate release is triggered by actin binding; therefore, myosin ATPase activity is tightly coupled to motility (Murphy et al., 2001). The E99K ACTC mutation however, seems to cause uncoupling of the actin-activated myosin ATPase and filament motility, yielding filaments velocities almost half that of WT ACTC filaments with S1 ATPase activity almost 30% greater than with WT ACTC. This disturbance of a highly regulated cycle could be extremely detrimental to the cell and cause inefficient usage of energy.
Murphy et al., (2001) describe the S456L mutation of Dictyostelium myosin-II whose consequences on myosin binding are similar to the E99K ACTC mutant. The S456L mutation in myosin causes a substitution from a polar serine to a nonpolar leucine and exhibits an increased rate of phosphate release and reduced step-size. This change leads to kinetic and mechanical deficiencies resulting in uncoupling of its typical actin-activated ATPase activity and reduced motility causing an over consumption in ATP and decreased motor ability of the myosin.

The two issues to address are firstly, how an increase in ATP consumption can be affected by poor actin-myosin binding and therefore decreased force production; and secondly, how the E99K ACTC mutation specifically disrupts the association between myosin ATP hydrolysis, phosphate release and the power stroke. As stated previously, the E99 residue along with two other negatively charged residues are proposed to play important roles in myosin binding by interacting with specific lysines on myosin. The loss of a negative charge within this region and substitution with a positive charge causes electrostatic repulsion disrupting an essential contact between the two proteins (Rayment et al., 1993). The weakening of actin-myosin binding by the E99K mutation could impact filament motility and force production upon P_i release, since a weak interaction cannot support the force of rotation of the lever arm. Another proposal is if there is a perturbation of ionic interactions in the binding interface between actin and myosin, initial binding may not sufficiently trigger the release of P_i that is normally associated with the lever arm movement resulting in low force and reduction in motility. However, this does not explain the excessive use of ATP seen with E99K ACTC and myosin S1 interaction compared to WT ACTC (Figure 14). A potential explanation of the effect of the E99K
ACTC mutation may be a “slipping” model of weak or disturbed actin and myosin binding. Given that actin-myosin dissociation is dependent on completion of the cross-bridge cycle as well as ATP re-binding to the myosin head, it may be possible that weak and unstable interactions between actin and myosin might disrupt the cycle by detaching or dissociating during the cycle before completion or without the regulation of ATP causing an overall reduction in strong-binding events. This is consistent with results reported by Bookwalter et al. (2006) showing a decreased affinity of E99K ACTC to cardiac S1 in the absence of ATP. The rate of association of S1 to E99K ACTC filaments was 60% of that reported for WT ACTC filaments.

Weak interactions caused by the E99K ACTC mutation may make the myosin head susceptible to slipping or simply falling off the actin filament during the cycle upon P_i release and lever arm rotation. This would result in an incomplete actin-myosin ATPase cycle (Figure 6), essentially not fulfilling the rate limiting dissociation of ADP by myosin while still bound to actin and the dissociation of the actomyosin complex regulated by ATP binding. A consequence of this incomplete cycle would be the increase in myosin heads not bound to actin and essentially available for ATP binding, without the regulation of ATP hydrolysis producing actin dissociation. Furthermore, this slipping could explain the lack of coordination between the working stroke of the lever arm upon P_i release and the reduced motility consistently observed with the E99K ACTC protein here and by Bookwalter et al. (2006). Consistent with the hypothesis that the E99K ACTC mutation disrupts the actin-myosin ATPase cycle, results observed by Debold et al. (2010) reported a reduction with the frequency of E99K ACTC-myosin strong binding events and its cooperative activation of regulated thin filaments being reduced. Although
further studies are required to fully understand the detrimental effects of the E99K ACTC mutation, its drastic effects are observed in *in vitro* systems and help to increase understanding of its role in the HCM disease state. These results demonstrate that specific mutations in ACTC can have detrimental effects on protein-protein interactions within the sarcomere, leading to dysfunctional heart muscles and development of disease.
Chapter 5 – Conclusions and future directions

5.1 Conclusions

The overall goal of this research was to determine the effects of point mutations in ACTC associated with cardiomyopathy development as they relate to the molecular mechanisms leading to the cardiomyopathy disease state. Examining both intrinsic α-cardiac actin protein characteristics and effects on important protein-protein interactions within the sarcomere, namely myosin, may aid in increasing understanding and diagnosis of both HCM and DCM patients. The intrinsic properties of the A295S ACTC variant protein were first investigated to assess how this mutation influences monomeric actin protein and its transition to the filamentous state by using diverse biochemical assays. Various intrinsic characteristics of several ACTC variant proteins were previously described by past students; therefore, my research focused on the production and characterization of the A295S ACTC variant. A295S ACTC protein did not demonstrate significant differences from WT ACTC and did not support the hypothesis that its location in subdomain 3 would cause disruptions in the overall structure and function. These results, however, may shed light on the subtle nature of A295S ACTC and reflect the late HCM onset associated with this mutation.

When effects on myosin binding were investigated, several ACTC mutants revealed deficiencies under various conditions (R312H, A230V and E99K under standard conditions and A331P, M305L and Y166C under extreme conditions). In the various assays performed, the E99K ACTC protein consistently stood out as having the most
severe effect on myosin binding. This was attributed to its location in subdomain 1 of the actin monomer, proposed to make critical electrostatic interactions with myosin. Overall, several of the ACTC mutations demonstrated consequences on myosin-binding, with some displaying more drastic effects than others supporting the hypothesis that mutations in ACTC can have a detrimental effects on myosin binding and the overall function of the actomyosin complex.

While the complex mechanism leading from genetic mutation to cardiomyopathy remains ambiguous, these results help describe the primary disturbance at the molecular level ultimately leading to the cardiomyopathy disease phenotype.

5.2 Future Work

Future work may include the production of the newly discovered ACTC mutations (H88Y, F90del, R95C, S271F and R312C) and the successful production and purification of the P164A ACTC protein. Investigation of the effects of these mutations on intrinsic characteristics such as $T_m$, DNase-I inhibition, intrinsic nucleotide release, $P_i$ release and critical concentration will be assessed. The influence of these mutations on important protein-protein interactions, specifically myosin-binding, will also be examined, with particular interest on the R95C ACTC protein to determine if its location within close proximity of an important actin-myosin interface will cause perturbations in the cross-bridge cycle. This interaction can be further examined by performing the *in vitro* motility assay with reconstituted thin filaments in the presence of regulatory sarcomeric proteins tropomyosin and tropinin to determined the mutation’s effects on other sarcomeric protein binding and calcium regulation in a more physiologically
relevant system. *In vivo* experiments using mammalian cardiomyocytes to express ACTC variant proteins would aid in understanding the harmful role of ACTC variant proteins in a whole cell system and the development of phenotypic characteristics of HCM leading to a heart diseased state (Muller et al., 2012). Further investigation using mice or other mammalian models can bring about increased understanding and developments in screening and diagnosis for this disease and potential treatments in humans (Richard et al., 2003)

The different effects of individual ACTC mutations on protein structure and function illustrate the complexity of molecular mechanisms responsible for HCM and DCM. By examining sarcomeric gene mutations associated with the development of cardiomyopathies, defects in the molecular machinery of the heart can be realized and targeted. Increasing our understanding of the initial molecular factors contributing to the development of cardiomyopathy and incorporating this knowledge with external factors influencing heart health, such as diet and lifestyle, could lead to valuable improvements in medical care and quality of life for those suffering with cardiomyopathy.
References


O’Reilly, D.R., Miller, L.K., & Luckow, V.A. (1994) Baculovirus expression vectors: A


Wang, L., Seidman, J. G., & Seidman, C. E. (2010). Narrative review: Harnessing molecular genetics for the diagnosis and management of hypertrophic
cardiomyopathy. *Annals of Internal Medicine, 152*(8), 513-20, W181.
