UNIVERSITY OF CALIFORNIA, SAN DIEGO

Cytoskeletal Regulation of Form and Function in Aging Myocardium

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Gaurav Kaushik

Committee in charge:

Professor Adam J. Engler, Chair
Professor Sanford I. Bernstein
Professor Rolf Bodmer
Professor Pedro Cabrales
Professor Andrew McCulloch
Professor Robert Ross

2015

The Dissertation of Gaurav Kaushik is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

_____
Chair

University of California, San Diego

2015

DEDICATION

I dedicate this dissertation to:

CSK, RK, ERP, and HDB.

EPIGRAPH

Science is the belief in the ignorance of experts. – Richard P. Feynman

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AFM	Atomic Force Microscopy
FS	Fractional Shortening
HF	Heart Failure
HP	Heart Period
HR	Heart Rate
VCL/Vcl/Vinc	Vinculin
MHC	Myosin Heavy Chain
RV	Relaxation Velocity
SV	Shortening Velocity
WT	Wild-type

LIST OF FIGURES

ACKNOWLEDGEMENTS

The content of Chapter 1 is nearly identical to a book chapter entitled "From Stem Cells to Cardiomyocytes: The Role of Forces in Cardiac Maturation" by Kaushik, G. and Engler, A.J., 2014. It contains minor revisions and formatting changes for this dissertation.

Chapter 2 is modified from a peer-reviewed journal article published in 2012, entitled: "Measuring Passive Myocardial Stiffness in *Drosophila melanogaster* to investigate diastolic dysfunction" Journal of cellular and molecular medicine 16, 1656-1662. The author of this dissertation was the lead author.

Chapter 3 is modified from a peer-reviewed journal article published in 2011, entitled: "*In situ* mechanical analysis of myofibrillar perturbation and aging on soft, bilayered Drosophila myocardium." Biophysical journal *101*, 2629-2637.

Chapter 4 is adapted from an unpublished work in review.

Chapter 5 is original in its entirety.

The author would like to thank his committee for helpful guidance throughout his graduate career and with this dissertation. In addition to co-authors in publications listed in this dissertation, the author would also like to acknowledge Emily R. Pfeiffer, Gretchen Meyer, Yu Suk Choi, Ludovic Vincent, Matt Ondeck, Kyle Kretchmer, Jennifer Young, Andrew Holle, Nikhil Rao, Alan Gilman, Brian Meckes, Margie Mathewson, Meera Viswanathan, and Anna Blice-Baum for their general help and guidance during the development of this dissertation.

This work was supported by the National Institutes of Health (NIH) National Heart, Lung, and Blood Institute (NHLBI), the NIH National Institute on Aging (NIA), the American Federation for Aging Research, and an American Heart Association Predoctoral fellowship award.

VITA

2004 - 2007 Adelphi University
Bachelor of Arts, Physics

2007 - 2009 Columbia University
Bachelor of Science, Biomedical Engineering

2009 - 2015 University of California, San Diego
Doctor of Philosophy, Bioengineering
American Heart Association Predoctoral Fellow
Howard Hughes Medical Institute Med-into-Grad Fellow
Graduate Researcher, Stem Cell Biology and Bioengineering Lab

TEACHING EXPERIENCE

University Of California, San Diego
Teaching Assistant, Department of Bioengineering
BENG 112A: Biomechanics I
BENG 112B: Biomechanics II
BENG 221: Mathematical Methods for Bioengineering

PUBLICATIONS

Kaushik G., Fuhrmann A., Cammarato A., and Engler A.J. (2011). In situ mechanical analysis of myofibrillar perturbation and aging on soft, bilayered Drosophila myocardium. Biophysical journal *101*, 2629-2637.

Kaushik G., Zambon A.C., Fuhrmann A., Bernstein S.I., Bodmer R., Engler A.J., and Cammarato A. (2012). Measuring passive myocardial stiffness in Drosophila melanogaster to investigate diastolic dysfunction. Journal of cellular and molecular medicine *16*, 1656-1662.

Viswanathan M.C., Kaushik G., Engler A.J., Lehman W., and Cammarato A. (2014). A Drosophila melanogaster model of diastolic dysfunction and cardiomyopathy based on impaired troponin-T function. Circulation research *114*, e6-17.

Nishimura M., Kumsta C., Kaushik G., Diop S.B., Ding Y., Bisharat-Kernizan J., Catan H., Cammarato A., Ross R.S., Engler A.J., Bodmer R., Occor K. (2014). A dual role for integrin-linked kinase and beta1-integrin in modulating cardiac aging. Aging cell.

Kaushik G., Spenlehauer A., Sessions A.O., Trujillo A., Fuhrmann, A., Fu Z., Venkatraman V., Pohl D., Tuler J., Wang M., Lakatta E.G., Ocorr K., Bodmer R., Bernstein S.I., Van Eyk J., Cammarato A. and Engler A.J. (in review). Vinculin-mediated cytoskeletal remodeling regulates contractile function in aging myocardium.

ABSTRACT OF THE DISSERTATION

Cytoskeletal Regulation of Form and Function in Aging Myocardium

by

Gaurav Kaushik

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2015

Professor Adam J. Engler, Chair

Heart failure associated with advanced age is accompanied by molecular and cellular structural remodeling events within cardiomyocytes. One such event is remodeling of the cortical cytoskeleton by actin-binding molecules. Once thought to be a static compartment, the cardiac cytoskeleton is now known to be populated by mechanically-sensitive structural and signaling molecules. This dissertation concerns the role of the mechanosensitive protein vinculin in remodeling of cardiac cortical mechanics and explores its potential role in regulating contractile function with age. Age-related cardiac cytoskeletal reinforcement is shown to be an evolutionarily-conserved mechanism in simians, rats, and *Drosophila melanogaster*.

Mechanistic studies show that cytoskeletal remodeling is correlated with preserved shortening velocity. Finally, cardiac-specific induction of cytoskeletal remodeling through overexpression of vinculin in both juvenile wildtype and myosin heavy chain knockdown flies led to enhanced shortening function and increased hemodynamic stress tolerance. Cytoskeletal reinforcement may play a protective role during aging in multiple cardiac systems by regulating myofilament organization and myocyte shortening.

Chapter 1

Structural and Mechanosensitive Compartments of the Cardiomyocyte

Introduction

Mechanics play an essential role in developing and maintaining function at every stage of the heart's lifespan, from differentiation and maturation to regulation of cardiac structure with advancing age. Cells not only respond to external stresses but are capable of affecting them through h internal and external restructuring. This remarkable ability is due to the abundance of mechanosensitive molecules and mechanisms populating the cardiac tissue, which form a closed feedback loop in which mechanics regulate mechanics.

Mechanotransduction, the process by which cells sense external forces and translate them into biochemical signals that can change cell function, is regulated in the heart by a diverse array of factors operating at different length scales. Externally, arterial blood pressure, valve compliance, passive stiffness and adhesivity of the cellular niche, and ventricular wall stress have all been shown to impact form and function of the heart. Through intracellular mechanosensitive pathways, cardiac cells can sense these changes and remodel themselves and their surroundings in order to achieve and maintain a level of function that meets physiological demand (McCain & Parker, 2011). Evidence also suggests that "inside-out" mechanical signaling is crucial for tissue morphogenesis, maintenance of homeostasis, and prolonging function over decades of life (Heisenberg & Bellaiche, 2013; Mammoto, Mammoto, & Ingber, 2013; Patwari & Lee, 2008).

In this chapter, I describe the establishment of cardiac fate from stem cells and subsequent morphogenesis of the heart and highlight several major mechanosensitive subcompartments of the heart, noting the way in which they engage in mechanical and biochemical cross-talk. Throughout the chapter, I also discuss how mechanical signaling helps establish cardiac fate, construct the contractile apparatus, shape cardiac morphogenesis, regulates force transmission between myocytes and their niche, and underlines multi-scale remodeling during aging and altered mechanical loads. I will also argue that establishment and

long-term heart maintenance is highly dependent upon the cardiomyocyte's ability to remodel its intracellular structure in order to adapt to changing mechanical loads and physiological demand. In dissecting the effector and affected pathways of cardiac mechanotransduction, the reader will appreciate how mechanics regulates cardiac differentiation and how physical parameters help engineer the function of adult cardiac myocytes in addition to developing a better understanding of the pathophysiology of genetic and age-related cardiomyopathies.

Cardiac Morphogenesis During the Lifespan of the Heart

Specification, Differentiation, and Heart Morphogenesis

The cells that eventually become the myocardium are derived from the mesoderm within the primitive streak (Lopez-Sanchez & Garcia-Martinez, 2011; Lopez-Sanchez, Garcia-Masa, Ganan, & Garcia-Martinez, 2009). Early cardiogenesis is driven by time-dependent biochemical signaling, such as BMP and suppression of WNT signaling (Marvin, Di Rocco, Gardiner, Bush, & Lassar, 2001; Yuasa et al., 2005). At this stage, cardiac progenitors begin to migrate and form two populations of cells, one of which will eventually become the early, beating heart tube and the other the outflow tract and portions of the right heart (Lopez-Sanchez & Garcia-Martinez, 2011; Tzahor & Lassar, 2001). It is shortly after formation of the heart tube that contractions begin and underline further growth and remodeling to loop and subdivide into a primitive four-chambered heart. Morphogenesis can continue in embryonic mice hearts *ex vivo* (Dyer & Patterson, 2013) as it is guided by an internal mechanism: the forces created by interactions of myosin and actin (Granados-Riveron & Brook, 2012).

Cell Maturation and Maintenance

For the remainder of embryogenesis, growth is caused by hyperplasia or proliferation of the early cardiomyocyte population (Hirschy, Schatzmann, Ehler, & Perriard, 2006; Mollova

et al., 2013). Postnatal cardiomyocytes continue remodeling in a process dubbed maturation, which includes both hyperplasia and hypertrophy that considered *adaptive* as the growth contributes to improved function (Mollova et al., 2013). Post-maturation myocardial remodeling, either through concentric or eccentric hypertrophy, is underlined by the addition of sarcomeres, remodeling of cortical ultrastructure and protein expression, altered cell morphology, and is associated with age-related dysfunction such as impaired fractional shortening (McCain & Parker, 2011; McCain, Sheehy, Grosberg, Goss, & Parker, 2013; Schaper et al., 2002). While primarily composed of terminally-differentiated adult cardiomyocytes, cardiac stem cells have also been recently identified (Ferreira-Martins et al., 2012; Hosoda, 2012; Torella, Ellison, & Nadal-Ginard, 2014); despite the presence of these progenitor cells however, the adult heart is still thought to have limited regenerative potential compared to other tissue systems given that the heart does not repair itself like other muscles. Therefore, adult cardiomyocytes must be extremely responsive to these changing mechanical environments (e.g. elevated arterial pressure, fibrosis) to maintain function over several decades, and mechanosensitive molecules provide a convenient feedback mechanism to maintain cardiac function.

Mechanosensitive Compartments in Cardiomyocytes

Cardiomyocytes are composed of several subcompartments involved in mechanotransduction, including the contractile sarcomeres, the cytoskeletal filament networks, transmembrane cell-cell and cell-matrix junctions, stretch-sensitive membrane structures, and calcium-handling machinery as shown in Figure 1.1. Each of these is involved in providing structural integrity as well as generation, sensation, transmission, and/or modulation of forces in the heart. In the following sections, I will summarize the basic structure and highlight known and hypothesized functions of each of these subcompartments.

The Sarcomere

The primary function of the heart is to pump blood throughout the circulatory system, dispersing oxygen, nutrients, and biochemical factors to both distal organs as well as itself (Sonnenblick, Spotnitz, & Spiro, 1964). This contraction is contingent upon the sarcomere, a dynamic protein complex that serves as the basic mechanical unit of muscle. A sarcomere is composed of actin-based thin filaments and myosin-based thick filaments as illustrated in Figure 1.1; these filaments slide past one another to create contraction. These structures are cross-linked together by Z-disks, and as they slide past one another, they create an electron-dense region known as the M-line. Each of these sarcomeric components is critical in regulating how muscle performs mechanical work, maintains passive tension (H. L. Granzier & Irving, 1995), undergoes biochemical signaling (Nicol, Frey, & Olson, 2000), and facilitates mechanotransduction (Kresh & Chopra, 2011). In vertebrates, the expression of these molecules during development is highly-coordinated (Ehler & Gautel, 2008) and mutations or alternative slicing of genes encoding these proteins are associated with a variety of congenital defects and cardiomyopathies (Swank, Wells, Kronert, Morrill, & Bernstein, 2000) that manifest in aberrant molecular, cellular, or organ-level structure.

Cardiac Structure and Mechano-signaling

Sarcomeric contraction and subsequent force production begins with an action potential. Depolarization of the cardiac membrane results in calcium-influx from the extracellular space via L-type calcium channels located in structural membrane invaginations known as the transverse-tubules (Turczynska, Hellstrand, Sward, & Albinsson, 2013). Local increases in calcium concentration are detected by proximal ryanodine receptors in the sarcoplasmic reticulum (SR), causing the latter to release additional calcium ions into the cytosolic space. This event is known as a “calcium spark” (Rovetti, Cui, Garfinkel, Weiss, &

Qu, 2010; Stern, Rios, & Maltsev, 2013) and is part of a calcium-induced calcium-release mechanism central to cardiac excitation-contraction coupling. Though each spark is spatiotemporally constrained, it can result in a significant elevation of global cytoplasmic calcium concentration when summed (~>10^4 sparks). This elevation is transient, as sarco/endoplasmic reticulum ATP-ase (SERCA) pump and the sodium-calcium exchanger (NCX) will transport calcium back into the SR and interstitium, respectively. This rise and fall of calcium is referred to as the calcium transient.

Myosin thick filaments bind to actin-based thin filaments transiently, and as they bind and unbind, they undergo a power stroke where the myosin head moves forward relative to the actin filament. Myosin's cyclic binding is known as cross-bridge cycling and this process creates the net contraction of the sarcomere. Binding can be blocked in the absence of calcium by the troponin-tropomyosin complex (Chalovich, Chock, & Eisenberg, 1981) where troponin C can cover up myosin's binding site on the thin filament. The probability of acto-myosin binding is therefore associated with increased intracellular calcium concentration (Bogeholz, Muszynski, & Pott, 2012). Changes in the calcium transient directly affect contraction and relaxation dynamics and short and long-term power output of the heart, making it a potent regulator of transient and long-term cardiac mechanotransduction (Judice, Marin, & Franchini, 2009).

In addition to perturbations in calcium handling, sarcomeric function is also modulated by changes in absolute gene expression (O'Neill, Holbrook, Fargnoli, & Lakatta, 1991), alternative splicing (Swank et al., 2000; Wiesner, Ehmke, Faulhaber, Zak, & Ruegg, 1997), impaired protein homeostasis (Douglas & Dillin, 2010; Kiriazis & Kranias, 2000), decreased protein quality (Schips et al., 2011), and altered ultrastructure of its subcomponents. Biochemical and mechanical cell stress can affect the quantity, quality, and integrity of the sarcomeres. In kind, molecules in the sarcomere are also capable of acting as stress sensors that

are capable of nuclear signaling, allowing the myocyte to respond (Bos et al., 2006; Le Guennec, Cazorla, Lacampagne, & Vassort, 2000).

The Z-disc was once thought to be a static architectural support for the myofilaments, responsible for anchoring and transversely cross-linking adjacent thin filaments. However in recent years, an intricate complex of force-sensing and signaling molecules have been identified within Z-discs, and many of these proteins have critical roles in development and disease (Judice et al., 2009). To briefly outline the contents of the Z-disc, it is primarily composed of alpha-actinin, a spectrin-family protein required for actin-filament anchoring to the Z-disc (Hein, Kostin, Heling, Maeno, & Schaper, 2000; Sorimachi et al., 1997). Non-sarcomeric clustering of alpha-actinin is a marker of sarcomere degeneration and is associated age-related dilated cardiomyopathy (Mohapatra et al., 2003).

In addition to alpha-actinin, Z-discs also contain a significant amount of titin, which helps maintain resting passive tension and longitudinal stiffness in the cell. Titin also acts as a molecular spring which spans the length of the Z-disk to the M-line (H. Granzier & Labeit, 2002; LeWinter, Wu, Labeit, & Granzier, 2007), (H. Granzier, Labeit, Wu, & Labeit, 2002). Embryonic titin is considerably shorter than adult titin, suggesting that transverse elasticity interplays with the changes in cardiac morphology and mechanics during development (Kruger, Babicz, von Frieling-Salewsky, & Linke, 2010). On the other hand, maladaptive cross-linking and differential splicing of titin have been shown to modulate resting sarcomere length and perturb sarcomere force production (Lewinter & Granzier, 2013). In addition to its mechanical properties, titin has several binding sites for ankyrin-repeat proteins (ARPs) (Miller et al., 2003). ARP signaling proteins are capable of translocating to the nucleus, where they presumably alter the transcriptome of the cell (Miller et al., 2003). In addition to binding alpha-actinin at its N-terminus, titin forms a complex with muscle LIM protein (MLP), which has been hypothesized to act as a stress and/or length tensor (Bos et al., 2006; Clark, Bland, & Beckerle, 2007). MLP-

null myocytes experience decreased longitudinal passive stiffness and muted response to stretch in the form of BMP expression (Buyandelger et al., 2011; Gunkel, Heineke, Hilfiker-Kleiner, & Knoll, 2009) as well as decreased power output (Clark, Lesage-Horton, Zhao, Beckerle, & Swank, 2011). MLP mutations are also associated with diastolic dysfunction followed by dilated cardiomyopathy, suggesting time-dependent remodeling (Lorenzen-Schmidt et al., 2005). Other LIM-domain-containing proteins, including myopodin (Faul, Dhume, Schecter, & Mundel, 2007) and zyxin (Linnemann et al., 2010), localize at the Z-disc and further suggest a critical sensory role for these types of proteins. During myoblast differentiation, myopodin is localized to the nucleus but translocates to the nascent Z-disk as sarcomerogenesis progresses (Weins et al., 2001). Acute, short-term heat shock of differentiated myotubes results in reverse translocation of myopodin from mature Z-discs to the nucleus, suggesting that myopodin plays a role in stress-response signaling (Faul et al., 2007; Weins et al., 2001). Nebulin, a hypothesized sarcomeric "ruler"(Fowler, McKeown, & Fischer, 2006; Kontrogianni-Konstantopoulos, Ackermann, Bowman, Yap, & Bloch, 2009; Littlefield & Fowler, 2008), is associated with nebulin-related anchoring protein (N-RAP), another well-known LIM protein and member of the Z-disk associated mechanosensitive network (Ehler et al., 2001; Panaviene & Moncman, 2007). Myomesin is another sarcomeric mechanosensor that is thought to bind titin near its C-terminal and act as a molecular spring (Agarkova & Perriard, 2005; Schoenauer et al., 2005). In addition to its contributions to mature sarcomere stability, it is associated with myosin during the earliest stages of sarcomerogenesis, suggesting that it is crucial for performing mechanical work given the few proteins present in these early contractile structures (Lange, Agarkova, Perriard, & Ehler, 2005).

Sarcomere Mutations and Their Impact

Mutations in vital myofilament proteins are associated with congenital defects and adult-onset cardiomyopathies and can arise due to increased or decreased sensitivity to calcium (Bos, Ommen, & Ackerman, 2007; Bos et al., 2006; Chang, Parvatiyar, & Potter, 2008; Cheng et al., 2013; Davis et al., 2012; Geske et al., 2013; Landstrom & Ackerman, 2012; Palmiter & Solaro, 1997; Parvatiyar, Pinto, Dweck, & Potter, 2010; Song et al., 2010; Tian, Liu, Zhou, & Song, 2013; Witjas-Paalberends et al., 2013). These changes reflect a potential role of the myofilaments in cardiogenesis and maintenance of structure. A missense mutation in α-MHC can result in maladaptive hypertrophy, myofibrillar disarray, and fibrosis due to impaired calcium homeostasis in the SR (Blanchard, Seidman, Seidman, LeWinter, & Maughan, 1999; Kim et al., 1999). This process can be ameliorated by inhibition of L-type calcium channel activity upstream (Semsarian et al., 2002) and potentially other negative-inotropic agents, suggesting that the mutation results in a maladaptive gain-of-function. Certain mutations in cardiac troponins are associated with restrictive cardiomyopathy (Akhter, Bueltmann, Huang, & Jin, 2013; Davis et al., 2012; Parvatiyar et al., 2010), a condition in which diastolic and systolic dimensions dramatically decline. Recent studies show that such mutations in critical regions can expose the myosin-binding site on thin filaments without the need for calcium influx, resulting in cross-bridge formation independent of calcium release, elevated diastolic tension, cortical stiffening, and impaired fractional shortening (Chang et al., 2008; Davis et al., 2012; Viswanathan, Kaushik, Engler, Lehman, & Cammarato, 2014). A mutation in troponin C resulting in calcium-independent sarcomeric perturbation has been shown to be rescued by an engineered molecule (B. Liu, Lee, Biesiadecki, Tikunova, & Davis, 2012).

Sarcomeres are also known to respond to alterations in the mechanical forces being presented to the heart, i.e. “outside-in” mechanotransduction. For example, application of stretch on cardiomyocytes *in vitro* results in increased sarcomerogenesis and hypertrophic

signaling (De Deyne, 2000; Dorn, Robbins, & Sugden, 2003; Frank et al., 2008). The addition of new sarcomeres appears to occur at the intercalated disc, suggesting synergy between the two compartments in this phenomena (Wilson, Schoenauer, Ehler, Agarkova, & Bennett, 2014). Developing embryonic chick cardiomyocytes have increased sarcomerogenesis from when plated on time-dependent, stiffening hydrogels as compared to static elastic substrates (Young & Engler, 2011; Young et al., 2013). The rate of MHC and thin filament actin turnover is stretch-sensitive (Simpson, Majeski, Borg, & Terracio, 1999; Simpson et al., 1996). Thus, sarcomerogenesis and maintenance of sarcomeric protein quality may depend upon the ability of the sarcomeres to act as a stress/strain sensor; perturbations of this structure could impair the resultant signaling.

Other Intracellular Mechanosensitive Structures

In order for sarcomeric shortening to translate into cellular contraction, and for the contractile apparatus to sense changes in external loads, they require mechanical coupling to the membrane. This is achieved via the sarcolemma, which consists of highly-ordered junctions and a cortical cytoskeletal network. Cardiomyocytes are coupled longitudinally by an electromechanically-active junctional complex known as the intercalated disc (ICD) and transversely at the Z-disc by special focal adhesion plaques known as costameres (Hirschy et al., 2006). Sensation and application of external loads by the sarcomeres is enabled by these transmembrane contacts and their associated cytoskeletal networks, making them the primary responders to forces leading to and from the contractile apparatus. Here, I will discuss these structures, their influence on downstream signaling, and its impact on contractile function.

Actin-associated Intercalated Disc and Costameric Proteins

Recent work has identified the intercalated disc and costameres, shown in Figure 1.1 at the cell membrane facilitating cell-cell and cell-ECM connections, as being remarkably mechanosensitive, i.e. capable of responding to and producing forces and enabling biochemical signaling through a variety of sensing molecules (Borghi et al., 2012; Holle et al., 2013; le Duc et al., 2010; Leerberg & Yap, 2013; Margadant et al., 2011; Spanjaard & de Rooij, 2013). Most of these studies have focused on *in vitro* systems (Spanjaard & de Rooij, 2013) or non-cardiac cells (Borghi et al., 2012; Holle et al., 2013), which have the same components but are organized differently. For example, Le Duc and coworkers applied cyclic stress to E-cadherin-coated magnetic beads bonded to endothelial cells and measured changes in bead deflection as the cell cytoskeleton responded (le Duc et al., 2010). They found that cadherin-mediated binding is mechanosensitive and results in local stiffening, potentially driven by reinforcement actin of the cortical cytoskeleton. The adhesions eventually asymptote to a new stiffness, the rate and magnitude of which depends on the presence of the actin-binding, mechanosensitive molecule vinculin (le Duc et al., 2010; Leerberg & Yap, 2013). In this way, the actin cytoskeleton downstream of cadherin-cadherin bonds react similarly to those proximal to integrin-ECM bonds (Choquet, Felsenfeld, & Sheetz, 1997; Margadant et al., 2011). It is reasonable to assume that cadherin contacts perform similarly in cardiomyocytes as all the critical machinery exists and cell-cell contacts in cultured myocytes are known to remodel during cyclic and static stretch (Zhuang, Yamada, Saffitz, & Kleber, 2000). However the mechano-sensing machinery is not always present or highly organized. In early development, cadherins are expressed ubiquitously throughout the membrane (Hirschy et al., 2006). It is only as sarcomerogenesis advances and cells elongate and hypertrophy that cadherins become polarized and constrained to the longitudinal ends. Once it is fully formed, the intercalated disc does not remain static; advancing age and dilation associates with convolution of the ICD ultrastructure and enrichment with

mechanotransductive molecules (Perriard, Hirschy, & Ehler, 2003; Schaper et al., 1991). Remodeling of actin-binding mechanosensors is hypothesized to result in cortical or transverse stiffening during aging (Hein et al., 2000), though the ultimate impact of this event on cardiac function remains unclear.

Beyond normal changes with development and age, studies suggest that cortical remodeling from disease or mutation may play a functional role in modulating sarcomeric function. For example, Tangney and coworkers showed that vinculin-null neonatal mouse cardiomyocytes experience decreased cortical stiffness and increased interfilament spacing, the distance between thin and thick filaments in the myofilament lattice (Tangney et al., 2013). Interfilament spacing impacts the calcium-sensitivity and power output of the sarcomeres (Konhilas, Irving, & de Tombe, 2002; McDonald & Moss, 1995; Y. Wang & Fuchs, 2001), perhaps due to a lack of cortical transverse compression upon the myofilaments. Cortical compression through osmotic loading *in vitro* has been shown to affect contractile function in rat myocardium (Farman, Walker, de Tombe, & Irving, 2006; McDonald & Moss, 1995).

Lastly, it is important to consider mechanotransduction at focal adhesions, which play a leading role in contractility (Bendig et al., 2006; Hannigan, Coles, & Dedhar, 2007) and mechanical-induction of myogenic differentiation (Holle et al., 2013). In cardiomyocytes specifically, integrin expression and signaling, as well as focal adhesion and integrin-linked kinases, have been the focus of understanding age-related hypertrophic signaling (Bettink et al., 2010; Brancaccio et al., 2006; de Jonge, Dekkers, Houtsmuller, Sharma, & Lamers, 2007; DiMichele et al., 2009; Franchini, 2012; H. Lu et al., 2006; Umar et al., 2009). Integrin expression is regulated by both passive and active mechanical forces as well as the ligands and ligand density presented to the cell (McCain, Lee, Aratyn-Schaus, Kleber, & Parker, 2012). Integrin clustering and isoform expression depends upon the extracellular matrix proteins and their arrangement (Bendall, Heymes, Ratajczak, & Samuel, 2002; Chopra et al., 2012; Dabiri,

Lee, & Parker, 2012). Another crucial component in lateral coupling to the membrane is the actin-binding dystrophin molecule, which is thought to stabilize the membrane and provide additional mechanical coupling to the cortical actin cytoskeleton (Ervasti & Sonnemann, 2008; Kabaeva, Meekhof, & Michele, 2011; Kaprielian & Severs, 2000). Dystrophin knockout or expression of a mutated isoform results in impaired sarcomeric force transmission to the membrane. Furthermore, without dystrophin as a structural support, conformational changes in stretch-activated channels are thought to occur, resulting in pathological intracellular leakage of calcium and eventual cell death (Allen, Gervasio, Yeung, & Whitehead, 2010; Carlson, 1999; Williams & Allen, 2007).

Intermediate Filament and Microtubule Networks

While actin plays a dominant role in regulating cardiomyocyte development and function, the intermediate filament (IF) and microtubule networks both play important supporting roles. For example, intermediate filaments (IFs) are extremely deformable, capable of being stretched to several times their slack length (Kreplak, Herrmann, & Aebi, 2008; Steinert, North, & Parry, 1994; Wagner et al., 2007). One of these IFs, desmin, provides scaffolding around the Z-disk and links them laterally, binds to desmosomes at the intercalated disc, and bridges the nucleus to adjacent sarcomeres (Pawlak, Gil, Walczak, & Seweryniak, 2009). Desmin is also believed to localize to costameres, perhaps conferring additional coupling between sarcomeres and the extracellular matrix (Kaprielian & Severs, 2000). It is an early marker of cardiogenesis (Auda-Boucher et al., 2000; van der Loop, Schaart, Langmann, Ramaekers, & Viebahn, 1995). Desminopathies result from mutations in the desmin gene and subsequent cytoplasmic aggregation of desmin and impaired myofibrillar assembly (Brodehl et al., 2013; Chang & Potter, 2005; Paulin, Huet, Khanamyrian, & Xue, 2004). Patients with arrhythmogenic right ventricular cardiomyopathy (ARVC) have been shown to have desmin

mutations, suggesting that it may play a crucial role in EC-coupling via longitudinal load-bearing along the sarcomeres or by stabilizing gap junctions at the intercalated disc (Lorenzon et al., 2013; Otten et al., 2010; Tsatsopoulou, Protonotarios, & McKenna, 2006).

Another IF protein prevalent in cardiomyocytes is lamin, which as with desmin, is also extremely compliant. For example during lineage specification, stem cells, lamin expression in stem cells scales with substrate stiffness (Swift et al., 2013) and is a determinant of nuclear cytoskeleton deformability (Capo-chichi et al., 2011; De Vos et al., 2010). Moreover, it may act as a universal transducer of mechanical signals into the nuclear to regulate gene expression (Talwar, Jain, & Shivashankar, 2014). Thus defects in Lamin A/C can adversely affect signal transduction, making them less efficient. Defects in Lamin are associated with advanced aging (Smith, Kudlow, Frock, & Kennedy, 2005), presumably due to genetic instability and accumulated DNA damage from external stressors (Gonzalez-Suarez & Gonzalo, 2010), and dilated cardiomyopathy (Broers et al., 2004; Schaper et al., 2002; Takeda, 2003). The existence of mechanical couplings between the nucleus and the cytoskeletal network implies that some form of communication is required during development (Li, Wang, & Gerdes, 1997).

Note that there exists structural overlaps in the actin and IF networks; ICD-localizing vinculin is also though to stabilize gap junction (Zemljic-Harpf et al., 2014) and perturbations of actin-binding nesprin protein, found at the nucleus, are also known to induce cardiovascular dysfunction (Banerjee et al., 2014; Stroud, Banerjee, Veevers, & Chen, 2014). These suggests functional overlap in both cytoskeletal networks and may also be accompanied by cross-talk.

On the other hand, microtubules are crucial in guiding cytokinesis (Lee, Davies, & Mishima, 2012) and directing vesicular transport. However, the microtubule network also serves several load-bearing and signaling functions which impact cardiac function (Garcia et al., 2012; Webster, 2002; Zhao et al., 2010). Much focus has been placed on how signal transduction is altered following application of colchicine, a pharmacological agent that induces

deconstruction of microtubule filaments, as well as how microtubule dysregulation occurs during cardiac hypertrophy (Gomez, Kerfant, Vassort, & Pappano, 2004; H. Liu et al., 2003; Malan et al., 2003; Palmer, Valent, Holder, Weinberger, & Bies, 1998). While it is unclear whether mechanical perturbation, impaired vesicular transport, or both are responsible for alterations in function, what is known is that microtubules contribute to passive stiffness (H. L. Granzier & Irving, 1995) and are remodeled during age (Hein et al., 2000); both of these observations point to microtubules as being important regulators of cardiomyocyte function.

The Cardiomyocyte Membrane

In addition to intracellular and transmembrane mechanosensitive compartments, the cardiac myocyte membrane is also enriched with stretch-sensitive structures (Akay & Craelius, 1993; Bett & Sachs, 1997; Bustamante, Ruknudin, & Sachs, 1991; Iribe & Kohl, 2008; Lammerding, Kamm, & Lee, 2004; McCulloch, Pfeiffer, Tangney, & Omens, 2013). Stretch-activated channels have been implicated in modulation of calcium handling and rhythmicity, although the strains required to observed stretch-activation are often superphysiological (Senatore, Rami Reddy, Semeriva, Perrin, & Lalevee, 2010). The membrane is partially buffered from stretch via caveolins, cytoskeletally-regulated invaginations which can add additional material to the membrane through rapid disassembly under osmotic loads (Sinha et al., 2011) or stretch (Albinsson, Nordstrom, Sward, & Hellstrand, 2008; Bellott, Patel, & Burkholder, 2005; Kawabe, Okumura, Lee, Sadoshima, & Ishikawa, 2004; Zhang et al., 2007). Caveolin is known to alter its expression with age (Fridolfsson & Patel, 2013; Zou, Stoppani, Volonte, & Galbiati, 2011). Deletion of Cav3 (Hnasko & Lisanti, 2003; Woodman et al., 2002) is associated with progressive dilated cardiomyopathy while Cav1 appears to play a greater role in endothelial function (Chow, Daniel, & Schulz, 2010; Murata et al., 2007).

Extracellular Matrix and Mechanosensing

The cardiac interstitium provides cardiomyocytes with avenues of mechanical and biochemical communication with their environment (Bendall et al., 2002; Borg, Rubin, Carver, Samarel, & Terracio, 1996; Fan, Takawale, Lee, & Kassiri, 2012; Goldsmith & Borg, 2002; Jourdan-Lesaux, Zhang, & Lindsey, 2010; Kresh & Chopra, 2011; Ma et al., 2014; McCurdy, Baicu, Heymans, & Bradshaw, 2010; Zamilpa & Lindsey, 2010). In particular, the insoluble extracellular matrix (ECM) is secreted by cardiac fibroblasts (Fan et al., 2012; Ma et al., 2014), which are the most abundant cells in the heart by number (Nag, 1980). This ECM plays a crucial role in providing architectural support for cardiomyocytes and allowing for efficient transmission of forces during contraction. ECM-organization is known to direct integrin assembly and signaling and vice versa (Coyer et al., 2012). This can influence cell morphology and sarcomere alignment (Grosberg, Alford, McCain, & Parker, 2011; McCain & Parker, 2011; Parker, Tan, Chen, & Tung, 2008). This interstitium, like the cardiomyocytes, remodels during development (Hirschy et al., 2006) and disease (Fan et al., 2012; Ma et al., 2014; Patterson et al., 2013). Deposition of fibrillar ECM proteins, such as Collagen, Laminin, and Fibronectin (Konstandin, Toko, et al., 2013; Konstandin, Volkers, et al., 2013), are vital for the initiation of cardiogenesis and wound healing but are also associated with maladaptive hypertrophic growth. In addition to ECM deposition and paracrine signaling, cardiac fibroblasts are known to directly communicate with cardiomyocytes via connexins (Doble & Kardami, 1995; D. Lu, Soleymani, Madakshire, & Insel, 2012) and N-cadherin (Thompson et al., 2014; Thompson, Copeland, Reich, & Tung, 2011), allowing for electromechanical coupling and modulation of action potential propagation (Thompson et al., 2011). Cardiac fibroblasts also respond to external forces by altering their internal cytoskeletal expression (Leslie, Taatjes, Schwarz, vonTurkovich, & Low, 1991), extracellular biochemical signaling (Dalla Costa et al., 2010; Herum et al., 2013; Thompson et al., 2011; J. Wang, Chen, Seth, & McCulloch, 2003), and

increasing matrix production, which can lead to the induction of a smooth muscle phenotype (Lefebvre, Nusgens, & Lapiere, 1994). These "myofibroblasts" (Figure 1.1, top) experience calcium-dependent contractility (Pipelzadeh & Naylor, 1998), elevated ECM deposition (de Haas, Arbustini, Fuster, Kramer, & Narula, 2014; Fan et al., 2012), and influence cardiac conduction (Kizana et al., 2007; Thompson et al., 2014). Increased substrate stiffness can also increase differentiation of fibroblasts into myofibroblasts (Atance, Yost, & Carver, 2004; Guvendiren, Perepelyuk, Wells, & Burdick, 2013; Solon, Levental, Sengupta, Georges, & Janmey, 2007), suggesting a positive feedback loop following ECM-deposition.

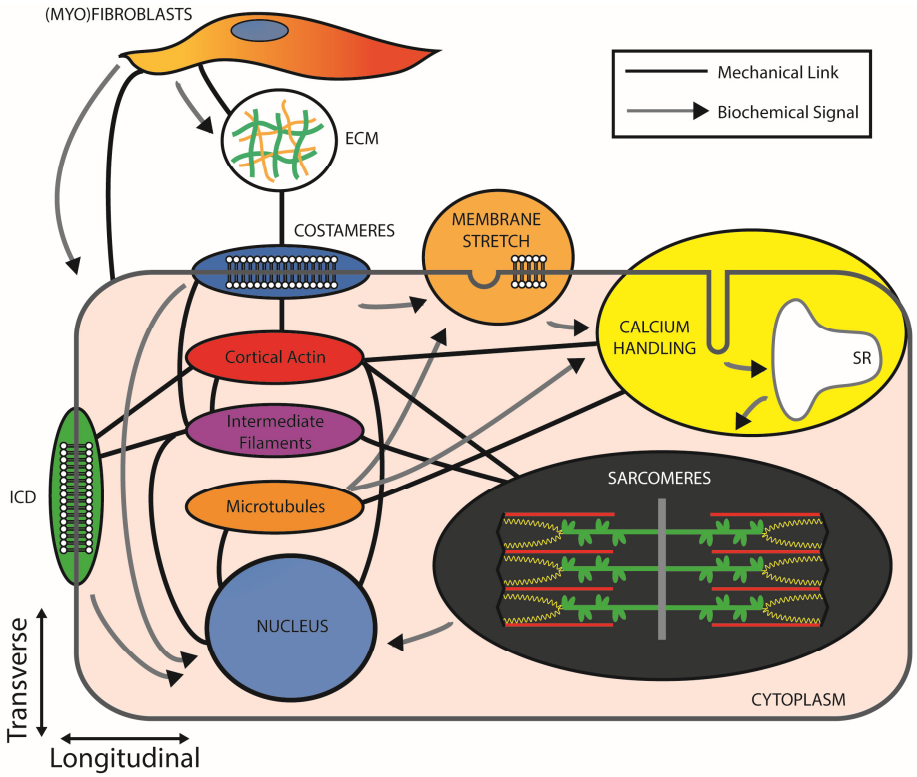

Figure 1.1: Mechanical signaling in the cardiomyocyte and its surrounding niche. Solid lines indicate known mechanical connections. Grey arrows indicate known biochemical signaling. The interconnectedness of individual molecular structures in the myocyte suggests that remodeling of one region is likely to affect both proximal and distal compartments.

The Influence of Mechanotransduction on Applications of Cardiac Regeneration

In this chapter, I have broadly discussed how maintenance of cardiac structure and function are underlined by spatiotemporal patterning of biochemical and mechanical signaling. We have seen cases in which mutation, alternative splicing, maladaptive post-translational modifications, and altered cytoskeletal and ECM assembly can dramatically alter morphology and function from the cell to the organ. One final consideration to note is how these changes, induced by development or disease, guide or can be used to guide regeneration.

The numerous heart shapes and sizes in the animal kingdom suggest that biology knows of many ways to build a heart. However, the sensitivity of the developing and aging human heart to genetic perturbations and externals stresses further suggests that there is a narrow window to form a human tissue that is competently functional. Minor alterations in calcium handling or cytoskeletal ultrastructure and organization appear to have broad impacts on both basal state as well as response to mechanical and biochemical or pharmacological stress. For example, the use of time-dependent soluble cues appears to be sufficient for initial differentiation of cardiac lineage but insufficient for maturation (Klaus, Saga, Taketo, Tzahor, & Birchmeier, 2007; Ren et al., 2011; Yuasa et al., 2005). The latter can be assisted by the application of external loads which in turn promote intracellular remodeling (Feinberg et al., 2012; Feinberg et al., 2013; McCain et al., 2013; Sheehy, Grosberg, & Parker, 2012). These mechanical cues may need to be dynamic; the limitations of static mechanical cues has become apparent in recent years. Attempts to revive cardiac function post-infarct via stem cell injection into the stiffened, infarcted niche, for example, results in differentiation into an osteogenic lineage instead of cardiac (Yoon, Park, Tkebuchava, Luedeman, & Losordo, 2004). Bulk hydrogels do little to provide mechanical support and may introduce arrhythmogenic defects by disrupting electrical coupling (Rane et al., 2011). Adult-like phenotype can be induced in developing cells through micropatterning of extracellular matrix in 2D(Bray, Sheehy, & Parker,

2008; Grosberg, Kuo, et al., 2011). In this way, a symmetry-breaking event can be used to guide integrin-clustering and sarcomerogenesis downstream. However, this kind of ECM-mediated "boundary condition" guidance in cardiomyocytes is also dynamic *in vivo*, suggesting that the same will be required in 3D cultures of mature cardiac tissue, which cannot be created through current micropatterning technologies. All of these concerns are ameliorated if it is shown that, for a given concern, the response and function of engineered tissues is similar to those observed *in vivo*. Such criteria has held for the use of simpler animal models with otherwise limited homology to human structure and function (Bier & Bodmer, 2004; Fink et al., 2009; Hinits et al., 2012; Kaushik et al., 2012; Viswanathan et al., 2014).

Conclusion

What is most evident from a review of current literature is that our understanding of precisely how the cardiomyocyte closes its mechanical feedback loop remains unknown; what are the basic signals that induce hypertrophy and self-assembly into a mature organ and what pathways signal the heart to cease or undergo aberrant growth? In the coming years, additional mechanosensitive molecules in cardiomyocytes will likely highlight cross-talk between subcompartments, such as cell-cell and cell-matrix cooperation or myocyte-fibroblast communication. What is likely more important, however, is improving our understanding of the precise timing of mechanotransduction and its downstream pathways during the lifespan of the heart. Improved understanding of cardiac differentiation from stem cells and the mechanotransductive signaling that enables this may reveal indirect therapeutic targets and/or enable better direct engineering of cells and tissue for repair and regeneration.

Acknowledgements

This chapter is adapted from a book chapter written by the author: Kaushik, G. and Engler, A.J. "From Stem Cells to Cardiomyocytes: The Role of Forces in Cardiac Maturation", Mechanotransduction: Progress in Molecular Biology and Translational Science, Elsevier (2014).

References

Agarkova, I., & Perriard, J. C. (2005). The M-band: an elastic web that crosslinks thick filaments in the center of the sarcomere. *Trends Cell Biol, 15*(9), 477-485. doi: 10.1016/j.tcb.2005.07.001

Akay, M., & Craelius, W. (1993). Mechanoelectrical feedback in cardiac myocytes from stretch-activated ion channels. *IEEE Trans Biomed Eng, 40*(8), 811-816. doi: 10.1109/10.238466

Akhter, S., Bueltmann, K., Jr., Huang, X., & Jin, J. P. (2013). Restrictive cardiomyopathy mutations demonstrate functions of the C-terminal end-segment of troponin I. *Arch Biochem Biophys*. doi: 10.1016/j.abb.2013.12.001

Albinsson, S., Nordstrom, I., Sward, K., & Hellstrand, P. (2008). Differential dependence of stretch and shear stress signaling on caveolin-1 in the vascular wall. *Am J Physiol Cell Physiol, 294*(1), C271-279. doi: 10.1152/ajpcell.00297.2007

Allen, D. G., Gervasio, O. L., Yeung, E. W., & Whitehead, N. P. (2010). Calcium and the damage pathways in muscular dystrophy. *Can J Physiol Pharmacol, 88*(2), 83-91. doi: 10.1139/Y09-058

Atance, J., Yost, M. J., & Carver, W. (2004). Influence of the extracellular matrix on the regulation of cardiac fibroblast behavior by mechanical stretch. *J Cell Physiol, 200*(3), 377-386. doi: 10.1002/jcp.20034

Auda-Boucher, G., Bernard, B., Fontaine-Perus, J., Rouaud, T., Mericksay, M., & Gardahaut, M. F. (2000). Staging of the commitment of murine cardiac cell progenitors. *Dev Biol, 225*(1), 214-225. doi: 10.1006/dbio.2000.9817

Banerjee, I., Zhang, J., Moore-Morris, T., Pfeiffer, E., Buchholz, K. S., Liu, A., Ouyang, K., Stroud, M. J., Gerace, L., Evans, S. M., McCulloch, A., & Chen, J. (2014). Targeted ablation of nesprin 1 and nesprin 2 from murine myocardium results in cardiomyopathy, altered nuclear morphology and inhibition of the biomechanical gene response. *PLoS Genet, 10*(2), e1004114. doi: 10.1371/journal.pgen.1004114

Bellott, A. C., Patel, K. C., & Burkholder, T. J. (2005). Reduction of caveolin-3 expression does not inhibit stretch-induced phosphorylation of ERK2 in skeletal muscle myotubes. *J Appl Physiol (1985), 98*(4), 1554-1561. doi: 10.1152/japplphysiol.01070.2004

Bendall, J. K., Heymes, C., Ratajczak, P., & Samuel, J. L. (2002). Extracellular matrix and cardiac remodelling. *Arch Mal Coeur Vaiss, 95*(12), 1226-1229.

Bendig, G., Grimmler, M., Huttner, I. G., Wessels, G., Dahme, T., Just, S., Trano, N., Katus, H. A., Fishman, M. C., & Rottbauer, W. (2006). Integrin-linked kinase, a novel component of the cardiac mechanical stretch sensor, controls contractility in the zebrafish heart. *Genes Dev, 20*(17), 2361-2372. doi: 10.1101/gad.1448306

Bett, G. C., & Sachs, F. (1997). Cardiac mechanosensitivity and stretch-activated ion channels. *Trends Cardiovasc Med, 7*(1), 4-8. doi: 10.1016/S1050-1738(96)00119-3

Bettink, S. I., Werner, C., Chen, C. H., Muller, P., Schirmer, S. H., Walenta, K. L., Bohm, M., Laufs, U., & Friedrich, E. B. (2010). Integrin-linked kinase is a central mediator in angiotensin II type 1- and chemokine receptor CXCR4 signaling in myocardial hypertrophy. *Biochem Biophys Res Commun, 397*(2), 208-213. doi: 10.1016/j.bbrc.2010.05.086

Bier, E., & Bodmer, R. (2004). Drosophila, an emerging model for cardiac disease. *Gene, 342*(1), 1-11. doi: 10.1016/j.gene.2004.07.018

Blanchard, E., Seidman, C., Seidman, J. G., LeWinter, M., & Maughan, D. (1999). Altered crossbridge kinetics in the alphaMHC403/+ mouse model of familial hypertrophic cardiomyopathy. *Circ Res, 84*(4), 475-483.

Bogeholz, N., Muszynski, A., & Pott, C. (2012). The physiology of cardiac calcium handling. *Wien Med Wochenschr, 162*(13-14), 278-282. doi: 10.1007/s10354-012-0102-3

Borg, T. K., Rubin, K., Carver, W., Samarel, A., & Terracio, L. (1996). The cell biology of the cardiac interstitium. *Trends Cardiovasc Med, 6*(2), 65-70. doi: 10.1016/1050-1738(96)00005-9

Borghi, N., Sorokina, M., Shcherbakova, O. G., Weis, W. I., Pruitt, B. L., Nelson, W. J., & Dunn, A. R. (2012). E-cadherin is under constitutive actomyosin-generated tension that is increased at cell-cell contacts upon externally applied stretch. *Proc Natl Acad Sci U S A, 109*(31), 12568-12573. doi: 10.1073/pnas.1204390109

Bos, J. M., Ommen, S. R., & Ackerman, M. J. (2007). Genetics of hypertrophic cardiomyopathy: one, two, or more diseases? *Curr Opin Cardiol, 22*(3), 193-199. doi: 10.1097/HCO.0b013e3280e1cc7f

Bos, J. M., Poley, R. N., Ny, M., Tester, D. J., Xu, X., Vatta, M., Towbin, J. A., Gersh, B. J., Ommen, S. R., & Ackerman, M. J. (2006). Genotype-phenotype relationships involving hypertrophic cardiomyopathy-associated mutations in titin, muscle LIM protein, and telethonin. *Mol Genet Metab, 88*(1), 78-85. doi: 10.1016/j.ymgme.2005.10.008

Brancaccio, M., Hirsch, E., Notte, A., Selvetella, G., Lembo, G., & Tarone, G. (2006). Integrin signalling: the tug-of-war in heart hypertrophy. *Cardiovasc Res, 70*(3), 422-433. doi: 10.1016/j.cardiores.2005.12.015

Bray, M. A., Sheehy, S. P., & Parker, K. K. (2008). Sarcomere alignment is regulated by myocyte shape. *Cell Motil Cytoskeleton, 65*(8), 641-651. doi: 10.1002/cm.20290

Brodehl, A., Dieding, M., Klauke, B., Dec, E., Madaan, S., Huang, T., Gargus, J., Fatima, A., Saric, T., Cakar, H., Walhorn, V., Tonsing, K., Skrzipczyk, T., Cebulla, R., Gerdes, D., Schulz, U., Gummert, J., Svendsen, J. H., Olesen, M. S., Anselmetti, D., Christensen, A. H., Kimonis, V., & Milting, H. (2013). The novel desmin mutant p.A120D impairs filament formation, prevents intercalated disk localization, and causes sudden cardiac death. *Circ Cardiovasc Genet, 6*(6), 615-623. doi: 10.1161/CIRCGENETICS.113.000103

Broers, J. L., Peeters, E. A., Kuijpers, H. J., Endert, J., Bouten, C. V., Oomens, C. W., Baaijens, F. P., & Ramaekers, F. C. (2004). Decreased mechanical stiffness in LMNA-/- cells is caused by defective nucleo-cytoskeletal integrity: implications for the development of laminopathies. *Hum Mol Genet, 13*(21), 2567-2580. doi: 10.1093/hmg/ddh295

Bustamante, J. O., Ruknudin, A., & Sachs, F. (1991). Stretch-activated channels in heart cells: relevance to cardiac hypertrophy. *J Cardiovasc Pharmacol, 17 Suppl 2*, S110-113.

Buyandelger, B., Ng, K. E., Miocic, S., Piotrowska, I., Gunkel, S., Ku, C. H., & Knoll, R. (2011). MLP (muscle LIM protein) as a stress sensor in the heart. *Pflugers Arch, 462*(1), 135-142. doi: 10.1007/s00424-011-0961-2

Capo-chichi, C. D., Cai, K. Q., Smedberg, J., Ganjei-Azar, P., Godwin, A. K., & Xu, X. X. (2011). Loss of A-type lamin expression compromises nuclear envelope integrity in breast cancer. *Chin J Cancer, 30*(6), 415-425.

Carlson, C. G. (1999). Spontaneous changes in acetylcholine receptor and calcium leakage activity in cell-attached patches from cultured dystrophic myotubes. *Pflugers Arch, 437*(3), 371-380.

Chalovich, J. M., Chock, P. B., & Eisenberg, E. (1981). Mechanism of action of troponin . tropomyosin. Inhibition of actomyosin ATPase activity without inhibition of myosin binding to actin. *J Biol Chem, 256*(2), 575-578.

Chang, A. N., Parvatiyar, M. S., & Potter, J. D. (2008). Troponin and cardiomyopathy. *Biochem Biophys Res Commun, 369*(1), 74-81. doi: 10.1016/j.bbrc.2007.12.081

Chang, A. N., & Potter, J. D. (2005). Sarcomeric protein mutations in dilated cardiomyopathy. *Heart Fail Rev, 10*(3), 225-235. doi: 10.1007/s10741-005-5252-6

Cheng, Y., Wan, X., McElfresh, T. A., Chen, X., Gresham, K. S., Rosenbaum, D. S., Chandler, M. P., & Stelzer, J. E. (2013). Impaired contractile function due to decreased cardiac myosin binding protein C content in the sarcomere. *Am J Physiol Heart Circ Physiol, 305*(1), H52-65. doi: 10.1152/ajpheart.00929.2012

Chopra, A., Lin, V., McCollough, A., Atzet, S., Prestwich, G. D., Wechsler, A. S., Murray, M. E., Oake, S. A., Kresh, J. Y., & Janmey, P. A. (2012). Reprogramming cardiomyocyte mechanosensing by crosstalk between integrins and hyaluronic acid receptors. *J Biomech, 45*(5), 824-831. doi: 10.1016/j.jbiomech.2011.11.023

Choquet, D., Felsenfeld, D. P., & Sheetz, M. P. (1997). Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages. *Cell, 88*(1), 39-48.

Chow, A. K., Daniel, E. E., & Schulz, R. (2010). Cardiac function is not significantly diminished in hearts isolated from young caveolin-1 knockout mice. *Am J Physiol Heart Circ Physiol, 299*(4), H1183-1189. doi: 10.1152/ajpheart.01195.2009

Clark, K. A., Bland, J. M., & Beckerle, M. C. (2007). The Drosophila muscle LIM protein, Mlp84B, cooperates with D-titin to maintain muscle structural integrity. *J Cell Sci, 120*(Pt 12), 2066-2077. doi: 10.1242/jcs.000695

Clark, K. A., Lesage-Horton, H., Zhao, C., Beckerle, M. C., & Swank, D. M. (2011). Deletion of Drosophila muscle LIM protein decreases flight muscle stiffness and power generation. *Am J Physiol Cell Physiol, 301*(2), C373-382. doi: 10.1152/ajpcell.00206.2010

Coyer, S. R., Singh, A., Dumbauld, D. W., Calderwood, D. A., Craig, S. W., Delamarche, E., & Garcia, A. J. (2012). Nanopatterning reveals an ECM area threshold for focal adhesion assembly and force transmission that is regulated by integrin activation and cytoskeleton tension. *J Cell Sci, 125*(Pt 21), 5110-5123. doi: 10.1242/jcs.108035

Dabiri, B. E., Lee, H., & Parker, K. K. (2012). A potential role for integrin signaling in mechanoelectrical feedback. *Prog Biophys Mol Biol, 110*(2-3), 196-203. doi: 10.1016/j.pbiomolbio.2012.07.002

Dalla Costa, A. P., Clemente, C. F., Carvalho, H. F., Carvalheira, J. B., Nadruz, W., Jr., & Franchini, K. G. (2010). FAK mediates the activation of cardiac fibroblasts induced by mechanical stress through regulation of the mTOR complex. *Cardiovasc Res, 86*(3), 421-431. doi: 10.1093/cvr/cvp416

Davis, J., Yasuda, S., Palpant, N. J., Martindale, J., Stevenson, T., Converso, K., & Metzger, J. M. (2012). Diastolic dysfunction and thin filament dysregulation resulting from excitation-contraction uncoupling in a mouse model of restrictive cardiomyopathy. *J Mol Cell Cardiol, 53*(3), 446-457. doi: 10.1016/j.yjmcc.2012.05.018

De Deyne, P. G. (2000). Formation of sarcomeres in developing myotubes: role of mechanical stretch and contractile activation. *Am J Physiol Cell Physiol, 279*(6), C1801-1811.

de Haas, H. J., Arbustini, E., Fuster, V., Kramer, C. M., & Narula, J. (2014). Molecular imaging of the cardiac extracellular matrix. *Circ Res, 114*(5), 903-915. doi: 10.1161/CIRCRESAHA.113.302680

de Jonge, H. W., Dekkers, D. H., Houtsmuller, A. B., Sharma, H. S., & Lamers, J. M. (2007). Differential Signaling and Hypertrophic Responses in Cyclically Stretched vs

Endothelin-1 Stimulated Neonatal Rat Cardiomyocytes. *Cell Biochem Biophys, 47*(1), 21-32.

De Vos, W. H., Houben, F., Hoebe, R. A., Hennekam, R., van Engelen, B., Manders, E. M., Ramaekers, F. C., Broers, J. L., & Van Oostveldt, P. (2010). Increased plasticity of the nuclear envelope and hypermobility of telomeres due to the loss of A-type lamins. *Biochim Biophys Acta, 1800*(4), 448-458. doi: 10.1016/j.bbagen.2010.01.002

DiMichele, L. A., Hakim, Z. S., Sayers, R. L., Rojas, M., Schwartz, R. J., Mack, C. P., & Taylor, J. M. (2009). Transient expression of FRNK reveals stage-specific requirement for focal adhesion kinase activity in cardiac growth. *Circ Res, 104*(10), 1201-1208. doi: 10.1161/CIRCRESAHA.109.195941

Doble, B. W., & Kardami, E. (1995). Basic fibroblast growth factor stimulates connexin-43 expression and intercellular communication of cardiac fibroblasts. *Mol Cell Biochem, 143*(1), 81-87.

Dorn, G. W., 2nd, Robbins, J., & Sugden, P. H. (2003). Phenotyping hypertrophy: eschew obfuscation. *Circ Res, 92*(11), 1171-1175. doi: 10.1161/01.RES.0000077012.11088.BC

Douglas, P. M., & Dillin, A. (2010). Protein homeostasis and aging in neurodegeneration. *J Cell Biol, 190*(5), 719-729. doi: 10.1083/jcb.201005144

Dyer, L. A., & Patterson, C. (2013). A novel ex vivo culture method for the embryonic mouse heart. *J Vis Exp*(75), e50359. doi: 10.3791/50359

Ehler, E., & Gautel, M. (2008). The sarcomere and sarcomerogenesis. *Adv Exp Med Biol, 642*, 1-14.

Ehler, E., Horowits, R., Zuppinger, C., Price, R. L., Perriard, E., Leu, M., Caroni, P., Sussman, M., Eppenberger, H. M., & Perriard, J. C. (2001). Alterations at the intercalated disk associated with the absence of muscle LIM protein. *J Cell Biol, 153*(4), 763-772.

Ervasti, J. M., & Sonnemann, K. J. (2008). Biology of the striated muscle dystrophin-glycoprotein complex. *Int Rev Cytol, 265*, 191-225. doi: 10.1016/S0074-7696(07)65005-0

Fan, D., Takawale, A., Lee, J., & Kassiri, Z. (2012). Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. *Fibrogenesis Tissue Repair, 5*(1), 15. doi: 10.1186/1755-1536-5-15

Farman, G. P., Walker, J. S., de Tombe, P. P., & Irving, T. C. (2006). Impact of osmotic compression on sarcomere structure and myofilament calcium sensitivity of isolated rat myocardium. *Am J Physiol Heart Circ Physiol, 291*(4), H1847-1855. doi: 10.1152/ajpheart.01237.2005

Faul, C., Dhume, A., Schecter, A. D., & Mundel, P. (2007). Protein kinase A, Ca2+/calmodulin-dependent kinase II, and calcineurin regulate the intracellular trafficking of myopodin

between the Z-disc and the nucleus of cardiac myocytes. *Mol Cell Biol, 27*(23), 8215-8227. doi: 10.1128/MCB.00950-07

Feinberg, A. W., Alford, P. W., Jin, H., Ripplinger, C. M., Werdich, A. A., Sheehy, S. P., Grosberg, A., & Parker, K. K. (2012). Controlling the contractile strength of engineered cardiac muscle by hierarchal tissue architecture. *Biomaterials, 33*(23), 5732-5741. doi: 10.1016/j.biomaterials.2012.04.043

Feinberg, A. W., Ripplinger, C. M., van der Meer, P., Sheehy, S. P., Domian, I., Chien, K. R., & Parker, K. K. (2013). Functional Differences in Engineered Myocardium from Embryonic Stem Cell-Derived versus Neonatal Cardiomyocytes. *Stem Cell Reports, 1*(5), 387-396. doi: 10.1016/j.stemcr.2013.10.004

Ferreira-Martins, J., Ogorek, B., Cappetta, D., Matsuda, A., Signore, S., D'Amario, D., Kostyla, J., Steadman, E., Ide-Iwata, N., Sanada, F., Iaffaldano, G., Ottolenghi, S., Hosoda, T., Leri, A., Kajstura, J., Anversa, P., & Rota, M. (2012). Cardiomyogenesis in the developing heart is regulated by c-kit-positive cardiac stem cells. *Circ Res, 110*(5), 701-715. doi: 10.1161/CIRCRESAHA.111.259507

Fink, M., Callol-Massot, C., Chu, A., Ruiz-Lozano, P., Izpisua Belmonte, J. C., Giles, W., Bodmer, R., & Ocorr, K. (2009). A new method for detection and quantification of heartbeat parameters in Drosophila, zebrafish, and embryonic mouse hearts. *Biotechniques, 46*(2), 101-113. doi: 10.2144/000113078

Fowler, V. M., McKeown, C. R., & Fischer, R. S. (2006). Nebulin: does it measure up as a ruler? *Curr Biol, 16*(1), R18-20. doi: 10.1016/j.cub.2005.12.003

Franchini, K. G. (2012). Focal adhesion kinase -- the basis of local hypertrophic signaling domains. *J Mol Cell Cardiol, 52*(2), 485-492. doi: 10.1016/j.yjmcc.2011.06.021

Frank, D., Kuhn, C., Brors, B., Hanselmann, C., Ludde, M., Katus, H. A., & Frey, N. (2008). Gene expression pattern in biomechanically stretched cardiomyocytes: evidence for a stretch-specific gene program. *Hypertension, 51*(2), 309-318. doi: 10.1161/HYPERTENSIONAHA.107.098046

Fridolfsson, H. N., & Patel, H. H. (2013). Caveolin and caveolae in age associated cardiovascular disease. *J Geriatr Cardiol, 10*(1), 66-74. doi: 10.3969/j.issn.1671-5411.2013.01.011

Garcia, L., Verdejo, H. E., Kuzmicic, J., Zalaquett, R., Gonzalez, S., Lavandero, S., & Corbalan, R. (2012). Impaired cardiac autophagy in patients developing postoperative atrial fibrillation. *J Thorac Cardiovasc Surg, 143*(2), 451-459. doi: 10.1016/j.jtcvs.2011.07.056

Geske, J. B., Bos, J. M., Gersh, B. J., Ommen, S. R., Eidem, B. W., & Ackerman, M. J. (2013). Deformation patterns in genotyped patients with hypertrophic cardiomyopathy. *Eur Heart J Cardiovasc Imaging*. doi: 10.1093/ehjci/jet234

Goldsmith, E. C., & Borg, T. K. (2002). The dynamic interaction of the extracellular matrix in cardiac remodeling. *J Card Fail, 8*(6 Suppl), S314-318. doi: 10.1054/jcaf.2002.129258

Gomez, A. M., Kerfant, B. G., Vassort, G., & Pappano, A. J. (2004). Autonomic regulation of calcium and potassium channels is oppositely modulated by microtubules in cardiac myocytes. *Am J Physiol Heart Circ Physiol, 286*(6), H2065-2071. doi: 10.1152/ajpheart.00933.2003

Gonzalez-Suarez, I., & Gonzalo, S. (2010). Nurturing the genome: A-type lamins preserve genomic stability. *Nucleus, 1*(2), 129-135. doi: 10.4161/nucl.1.2.10797

Granados-Riveron, J. T., & Brook, J. D. (2012). The impact of mechanical forces in heart morphogenesis. *Circ Cardiovasc Genet, 5*(1), 132-142. doi: 10.1161/CIRCGENETICS.111.961086

Granzier, H., Labeit, D., Wu, Y., & Labeit, S. (2002). Titin as a modular spring: emerging mechanisms for elasticity control by titin in cardiac physiology and pathophysiology. *J Muscle Res Cell Motil, 23*(5-6), 457-471.

Granzier, H., & Labeit, S. (2002). Cardiac titin: an adjustable multi-functional spring. *J Physiol, 541*(Pt 2), 335-342.

Granzier, H. L., & Irving, T. C. (1995). Passive tension in cardiac muscle: contribution of collagen, titin, microtubules, and intermediate filaments. *Biophys J, 68*(3), 1027-1044. doi: 10.1016/S0006-3495(95)80278-X

Grosberg, A., Alford, P. W., McCain, M. L., & Parker, K. K. (2011). Ensembles of engineered cardiac tissues for physiological and pharmacological study: heart on a chip. *Lab Chip, 11*(24), 4165-4173. doi: 10.1039/c1lc20557a

Grosberg, A., Kuo, P. L., Guo, C. L., Geisse, N. A., Bray, M. A., Adams, W. J., Sheehy, S. P., & Parker, K. K. (2011). Self-organization of muscle cell structure and function. *PLoS Comput Biol, 7*(2), e1001088. doi: 10.1371/journal.pcbi.1001088

Gunkel, S., Heineke, J., Hilfiker-Kleiner, D., & Knoll, R. (2009). MLP: a stress sensor goes nuclear. *J Mol Cell Cardiol, 47*(4), 423-425. doi: 10.1016/j.yjmcc.2009.07.012

Guvendiren, M., Perepelyuk, M., Wells, R. G., & Burdick, J. A. (2013). Hydrogels with differential and patterned mechanics to study stiffness-mediated myofibroblastic differentiation of hepatic stellate cells. *J Mech Behav Biomed Mater*. doi: 10.1016/j.jmbbm.2013.11.008

Hannigan, G. E., Coles, J. G., & Dedhar, S. (2007). Integrin-linked kinase at the heart of cardiac contractility, repair, and disease. *Circ Res, 100*(10), 1408-1414. doi: 10.1161/01.RES.0000265233.40455.62

Hein, S., Kostin, S., Heling, A., Maeno, Y., & Schaper, J. (2000). The role of the cytoskeleton in heart failure. *Cardiovasc Res, 45*(2), 273-278.

Heisenberg, C. P., & Bellaiche, Y. (2013). Forces in tissue morphogenesis and patterning. *Cell, 153*(5), 948-962. doi: 10.1016/j.cell.2013.05.008

Herum, K. M., Lunde, I. G., Skrbic, B., Florholmen, G., Behmen, D., Sjaastad, I., Carlson, C. R., Gomez, M. F., & Christensen, G. (2013). Syndecan-4 signaling via NFAT regulates extracellular matrix production and cardiac myofibroblast differentiation in response to mechanical stress. *J Mol Cell Cardiol, 54*, 73-81. doi: 10.1016/j.yjmcc.2012.11.006

Hinits, Y., Pan, L., Walker, C., Dowd, J., Moens, C. B., & Hughes, S. M. (2012). Zebrafish Mef2ca and Mef2cb are essential for both first and second heart field cardiomyocyte differentiation. *Dev Biol, 369*(2), 199-210. doi: 10.1016/j.ydbio.2012.06.019

Hirschy, A., Schatzmann, F., Ehler, E., & Perriard, J. C. (2006). Establishment of cardiac cytoarchitecture in the developing mouse heart. *Dev Biol, 289*(2), 430-441. doi: 10.1016/j.ydbio.2005.10.046

Hnasko, R., & Lisanti, M. P. (2003). The biology of caveolae: lessons from caveolin knockout mice and implications for human disease. *Mol Interv, 3*(8), 445-464. doi: 10.1124/mi.3.8.445

Holle, A. W., Tang, X., Vijayraghavan, D., Vincent, L. G., Fuhrmann, A., Choi, Y. S., del Alamo, J. C., & Engler, A. J. (2013). In situ mechanotransduction via vinculin regulates stem cell differentiation. *Stem Cells, 31*(11), 2467-2477. doi: 10.1002/stem.1490

Hosoda, T. (2012). C-kit-positive cardiac stem cells and myocardial regeneration. *Am J Cardiovasc Dis, 2*(1), 58-67.

Iribe, G., & Kohl, P. (2008). Axial stretch enhances sarcoplasmic reticulum Ca2+ leak and cellular Ca2+ reuptake in guinea pig ventricular myocytes: experiments and models. *Prog Biophys Mol Biol, 97*(2-3), 298-311. doi: 10.1016/j.pbiomolbio.2008.02.012

Jourdan-Lesaux, C., Zhang, J., & Lindsey, M. L. (2010). Extracellular matrix roles during cardiac repair. *Life Sci, 87*(13-14), 391-400. doi: 10.1016/j.lfs.2010.07.010

Judice, C. C., Marin, T. M., & Franchini, K. G. (2009). Calcium and the mechanotransduction in cardiac myocytes. *Front Biosci (Elite Ed), 1*, 189-199.

Kabaeva, Z., Meekhof, K. E., & Michele, D. E. (2011). Sarcolemma instability during mechanical activity in Largemyd cardiac myocytes with loss of dystroglycan extracellular matrix receptor function. *Hum Mol Genet, 20*(17), 3346-3355. doi: 10.1093/hmg/ddr240

Kaprielian, R. R., & Severs, N. J. (2000). Dystrophin and the cardiomyocyte membrane cytoskeleton in the healthy and failing heart. *Heart Fail Rev, 5*(3), 221-238. doi: 10.1023/A:1009805419285

Kaushik, G., Zambon, A. C., Fuhrmann, A., Bernstein, S. I., Bodmer, R., Engler, A. J., & Cammarato, A. (2012). Measuring passive myocardial stiffness in Drosophila

melanogaster to investigate diastolic dysfunction. *J Cell Mol Med, 16*(8), 1656-1662. doi: 10.1111/j.1582-4934.2011.01517.x

Kawabe, J., Okumura, S., Lee, M. C., Sadoshima, J., & Ishikawa, Y. (2004). Translocation of caveolin regulates stretch-induced ERK activity in vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol, 286*(5), H1845-1852. doi: 10.1152/ajpheart.00593.2003

Kim, S. J., Iizuka, K., Kelly, R. A., Geng, Y. J., Bishop, S. P., Yang, G., Kudej, A., McConnell, B. K., Seidman, C. E., Seidman, J. G., & Vatner, S. F. (1999). An alpha-cardiac myosin heavy chain gene mutation impairs contraction and relaxation function of cardiac myocytes. *Am J Physiol, 276*(5 Pt 2), H1780-1787.

Kiriazis, H., & Kranias, E. G. (2000). Genetically engineered models with alterations in cardiac membrane calcium-handling proteins. *Annu Rev Physiol, 62*, 321-351. doi: 10.1146/annurev.physiol.62.1.321

Kizana, E., Chang, C. Y., Cingolani, E., Ramirez-Correa, G. A., Sekar, R. B., Abraham, M. R., Ginn, S. L., Tung, L., Alexander, I. E., & Marban, E. (2007). Gene transfer of connexin43 mutants attenuates coupling in cardiomyocytes: novel basis for modulation of cardiac conduction by gene therapy. *Circ Res, 100*(11), 1597-1604. doi: 10.1161/CIRCRESAHA.106.144956

Klaus, A., Saga, Y., Taketo, M. M., Tzahor, E., & Birchmeier, W. (2007). Distinct roles of Wnt/beta-catenin and Bmp signaling during early cardiogenesis. *Proc Natl Acad Sci U S A, 104*(47), 18531-18536. doi: 10.1073/pnas.0703113104

Konhilas, J. P., Irving, T. C., & de Tombe, P. P. (2002). Myofilament calcium sensitivity in skinned rat cardiac trabeculae: role of interfilament spacing. *Circ Res, 90*(1), 59-65.

Konstandin, M. H., Toko, H., Gastelum, G. M., Quijada, P., De La Torre, A., Quintana, M., Collins, B., Din, S., Avitabile, D., Volkers, M., Gude, N., Fassler, R., & Sussman, M. A. (2013). Fibronectin is essential for reparative cardiac progenitor cell response after myocardial infarction. *Circ Res, 113*(2), 115-125. doi: 10.1161/CIRCRESAHA.113.301152

Konstandin, M. H., Volkers, M., Collins, B., Quijada, P., Quintana, M., De La Torre, A., Ormachea, L., Din, S., Gude, N., Toko, H., & Sussman, M. A. (2013). Fibronectin contributes to pathological cardiac hypertrophy but not physiological growth. *Basic Res Cardiol, 108*(5), 375. doi: 10.1007/s00395-013-0375-8

Kontrogianni-Konstantopoulos, A., Ackermann, M. A., Bowman, A. L., Yap, S. V., & Bloch, R. J. (2009). Muscle giants: molecular scaffolds in sarcomerogenesis. *Physiol Rev, 89*(4), 1217-1267. doi: 10.1152/physrev.00017.2009

Kreplak, L., Herrmann, H., & Aebi, U. (2008). Tensile properties of single desmin intermediate filaments. *Biophys J, 94*(7), 2790-2799. doi: 10.1529/biophysj.107.119826

Kresh, J. Y., & Chopra, A. (2011). Intercellular and extracellular mechanotransduction in cardiac myocytes. *Pflugers Arch, 462*(1), 75-87. doi: 10.1007/s00424-011-0954-1

Kruger, M., Babicz, K., von Frieling-Salewsky, M., & Linke, W. A. (2010). Insulin signaling regulates cardiac titin properties in heart development and diabetic cardiomyopathy. *J Mol Cell Cardiol, 48*(5), 910-916. doi: 10.1016/j.yjmcc.2010.02.012

Lammerding, J., Kamm, R. D., & Lee, R. T. (2004). Mechanotransduction in cardiac myocytes. *Ann N Y Acad Sci, 1015*, 53-70. doi: 10.1196/annals.1302.005

Landstrom, A. P., & Ackerman, M. J. (2012). Beyond the cardiac myofilament: hypertrophic cardiomyopathy- associated mutations in genes that encode calcium-handling proteins. *Curr Mol Med, 12*(5), 507-518.

Lange, S., Agarkova, I., Perriard, J. C., & Ehler, E. (2005). The sarcomeric M-band during development and in disease. *J Muscle Res Cell Motil, 26*(6-8), 375-379. doi: 10.1007/s10974-005-9019-4

le Duc, Q., Shi, Q., Blonk, I., Sonnenberg, A., Wang, N., Leckband, D., & de Rooij, J. (2010). Vinculin potentiates E-cadherin mechanosensing and is recruited to actin-anchored sites within adherens junctions in a myosin II-dependent manner. *J Cell Biol, 189*(7), 1107-1115. doi: 10.1083/jcb.201001149

Le Guennec, J. Y., Cazorla, O., Lacampagne, A., & Vassort, G. (2000). Is titin the length sensor in cardiac muscle? Physiological and physiopathological perspectives. *Adv Exp Med Biol, 481*, 337-348; discussion 348-351.

Lee, K. Y., Davies, T., & Mishima, M. (2012). Cytokinesis microtubule organisers at a glance. *J Cell Sci, 125*(Pt 15), 3495-3500. doi: 10.1242/jcs.094672

Leerberg, J. M., & Yap, A. S. (2013). Vinculin, cadherin mechanotransduction and homeostasis of cell-cell junctions. *Protoplasma, 250*(4), 817-829. doi: 10.1007/s00709-012-0475-6

Lefebvre, P., Nusgens, B. V., & Lapiere, C. M. (1994). Cultured myofibroblasts display a specific phenotype that differentiates them from fibroblasts and smooth muscle cells. *Dermatology, 189 Suppl 2*, 65-67.

Leslie, K. O., Taatjes, D. J., Schwarz, J., vonTurkovich, M., & Low, R. B. (1991). Cardiac myofibroblasts express alpha smooth muscle actin during right ventricular pressure overload in the rabbit. *Am J Pathol, 139*(1), 207-216.

Lewinter, M. M., & Granzier, H. L. (2013). Cardiac Titin and Heart Disease. *J Cardiovasc Pharmacol*. doi: 10.1097/FJC.0000000000000007

LeWinter, M. M., Wu, Y., Labeit, S., & Granzier, H. (2007). Cardiac titin: structure, functions and role in disease. *Clin Chim Acta, 375*(1-2), 1-9. doi: 10.1016/j.cca.2006.06.035

Li, F., Wang, X., & Gerdes, A. M. (1997). Formation of binucleated cardiac myocytes in rat heart: II. Cytoskeletal organisation. *J Mol Cell Cardiol, 29*(6), 1553-1565. doi: 10.1006/jmcc.1997.0403

Linnemann, A., van der Ven, P. F., Vakeel, P., Albinus, B., Simonis, D., Bendas, G., Schenk, J. A., Micheel, B., Kley, R. A., & Furst, D. O. (2010). The sarcomeric Z-disc component myopodin is a multiadapter protein that interacts with filamin and alpha-actinin. *Eur J Cell Biol, 89*(9), 681-692. doi: 10.1016/j.ejcb.2010.04.004

Littlefield, R. S., & Fowler, V. M. (2008). Thin filament length regulation in striated muscle sarcomeres: pointed-end dynamics go beyond a nebulin ruler. *Semin Cell Dev Biol, 19*(6), 511-519. doi: 10.1016/j.semcdb.2008.08.009

Liu, B., Lee, R. S., Biesiadecki, B. J., Tikunova, S. B., & Davis, J. P. (2012). Engineered troponin C constructs correct disease-related cardiac myofilament calcium sensitivity. *J Biol Chem, 287*(24), 20027-20036. doi: 10.1074/jbc.M111.334953

Liu, H., Ma, A., Wang, C., Liu, Y., Tian, H., & Bai, L. (2003). Variation and significance of microtubules in rat volume overload cardiac hypertrophy. *Chin Med J (Engl), 116*(3), 337-340.

Lopez-Sanchez, C., & Garcia-Martinez, V. (2011). Molecular determinants of cardiac specification. *Cardiovasc Res, 91*(2), 185-195. doi: 10.1093/cvr/cvr127

Lopez-Sanchez, C., Garcia-Masa, N., Ganan, C. M., & Garcia-Martinez, V. (2009). Movement and commitment of primitive streak precardiac cells during cardiogenesis. *Int J Dev Biol, 53*(8-10), 1445-1455. doi: 10.1387/ijdb.072417cl

Lorenzen-Schmidt, I., Stuyvers, B. D., ter Keurs, H. E., Date, M. O., Hoshijima, M., Chien, K. R., McCulloch, A. D., & Omens, J. H. (2005). Young MLP deficient mice show diastolic dysfunction before the onset of dilated cardiomyopathy. *J Mol Cell Cardiol, 39*(2), 241-250. doi: 10.1016/j.yjmcc.2005.05.006

Lorenzon, A., Beffagna, G., Bauce, B., De Bortoli, M., Li Mura, I. E., Calore, M., Dazzo, E., Basso, C., Nava, A., Thiene, G., & Rampazzo, A. (2013). Desmin mutations and arrhythmogenic right ventricular cardiomyopathy. *Am J Cardiol, 111*(3), 400-405. doi: 10.1016/j.amjcard.2012.10.017

Lu, D., Soleymani, S., Madakshire, R., & Insel, P. A. (2012). ATP released from cardiac fibroblasts via connexin hemichannels activates profibrotic P2Y2 receptors. *FASEB J, 26*(6), 2580-2591. doi: 10.1096/fj.12-204677

Lu, H., Fedak, P. W., Dai, X., Du, C., Zhou, Y. Q., Henkelman, M., Mongroo, P. S., Lau, A., Yamabi, H., Hinek, A., Husain, M., Hannigan, G., & Coles, J. G. (2006). Integrin-linked kinase expression is elevated in human cardiac hypertrophy and induces hypertrophy in transgenic mice. *Circulation, 114*(21), 2271-2279. doi: 10.1161/CIRCULATIONAHA.106.642330

Ma, Y., de Castro Bras, L. E., Toba, H., Iyer, R. P., Hall, M. E., Winniford, M. D., Lange, R. A., Tyagi, S. C., & Lindsey, M. L. (2014). Myofibroblasts and the extracellular matrix network in post-myocardial infarction cardiac remodeling. *Pflugers Arch*. doi: 10.1007/s00424-014-1463-9

Malan, D., Gallo, M. P., Bedendi, I., Biasin, C., Levi, R. C., & Alloatti, G. (2003). Microtubules mobility affects the modulation of L-type I(Ca) by muscarinic and beta-adrenergic agonists in guinea-pig cardiac myocytes. *J Mol Cell Cardiol, 35*(2), 195-206.

Mammoto, T., Mammoto, A., & Ingber, D. E. (2013). Mechanobiology and developmental control. *Annu Rev Cell Dev Biol, 29*, 27-61. doi: 10.1146/annurev-cellbio-101512-122340

Margadant, F., Chew, L. L., Hu, X., Yu, H., Bate, N., Zhang, X., & Sheetz, M. (2011). Mechanotransduction in vivo by repeated talin stretch-relaxation events depends upon vinculin. *PLoS Biol, 9*(12), e1001223. doi: 10.1371/journal.pbio.1001223

Marvin, M. J., Di Rocco, G., Gardiner, A., Bush, S. M., & Lassar, A. B. (2001). Inhibition of Wnt activity induces heart formation from posterior mesoderm. *Genes Dev, 15*(3), 316-327. doi: 10.1101/gad.855501

McCain, M. L., Lee, H., Aratyn-Schaus, Y., Kleber, A. G., & Parker, K. K. (2012). Cooperative coupling of cell-matrix and cell-cell adhesions in cardiac muscle. *Proc Natl Acad Sci U S A, 109*(25), 9881-9886. doi: 10.1073/pnas.1203007109

McCain, M. L., & Parker, K. K. (2011). Mechanotransduction: the role of mechanical stress, myocyte shape, and cytoskeletal architecture on cardiac function. *Pflugers Arch, 462*(1), 89-104. doi: 10.1007/s00424-011-0951-4

McCain, M. L., Sheehy, S. P., Grosberg, A., Goss, J. A., & Parker, K. K. (2013). Recapitulating maladaptive, multiscale remodeling of failing myocardium on a chip. *Proc Natl Acad Sci U S A, 110*(24), 9770-9775. doi: 10.1073/pnas.1304913110

McCulloch, A. D., Pfeiffer, E., Tangney, J., & Omens, J. (2013). Biomechanics of Cardiac Electromechanical Coupling and Mechanoelectric Feedback. *J Biomech Eng*. doi: 10.1115/1.4026221

McCurdy, S., Baicu, C. F., Heymans, S., & Bradshaw, A. D. (2010). Cardiac extracellular matrix remodeling: fibrillar collagens and Secreted Protein Acidic and Rich in Cysteine (SPARC). *J Mol Cell Cardiol, 48*(3), 544-549. doi: 10.1016/j.yjmcc.2009.06.018

McDonald, K. S., & Moss, R. L. (1995). Osmotic compression of single cardiac myocytes eliminates the reduction in Ca2+ sensitivity of tension at short sarcomere length. *Circ Res, 77*(1), 199-205.

Miller, M. K., Bang, M. L., Witt, C. C., Labeit, D., Trombitas, C., Watanabe, K., Granzier, H., McElhinny, A. S., Gregorio, C. C., & Labeit, S. (2003). The muscle ankyrin repeat proteins: CARP, ankrd2/Arpp and DARP as a family of titin filament-based stress response molecules. *J Mol Biol, 333*(5), 951-964.

Mohapatra, B., Jimenez, S., Lin, J. H., Bowles, K. R., Coveler, K. J., Marx, J. G., Chrisco, M. A., Murphy, R. T., Lurie, P. R., Schwartz, R. J., Elliott, P. M., Vatta, M., McKenna, W., Towbin, J. A., & Bowles, N. E. (2003). Mutations in the muscle LIM protein and alpha-

actinin-2 genes in dilated cardiomyopathy and endocardial fibroelastosis. *Mol Genet Metab, 80*(1-2), 207-215.

Mollova, M., Bersell, K., Walsh, S., Savla, J., Das, L. T., Park, S. Y., Silberstein, L. E., Dos Remedios, C. G., Graham, D., Colan, S., & Kuhn, B. (2013). Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc Natl Acad Sci U S A, 110*(4), 1446-1451. doi: 10.1073/pnas.1214608110

Murata, T., Lin, M. I., Huang, Y., Yu, J., Bauer, P. M., Giordano, F. J., & Sessa, W. C. (2007). Reexpression of caveolin-1 in endothelium rescues the vascular, cardiac, and pulmonary defects in global caveolin-1 knockout mice. *J Exp Med, 204*(10), 2373-2382. doi: 10.1084/jem.20062340

Nag, A. C. (1980). Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution. *Cytobios, 28*(109), 41-61.

Nicol, R. L., Frey, N., & Olson, E. N. (2000). From the sarcomere to the nucleus: role of genetics and signaling in structural heart disease. *Annu Rev Genomics Hum Genet, 1*, 179-223. doi: 10.1146/annurev.genom.1.1.179

O'Neill, L., Holbrook, N. J., Fargnoli, J., & Lakatta, E. G. (1991). Progressive changes from young adult age to senescence in mRNA for rat cardiac myosin heavy chain genes. *Cardioscience, 2*(1), 1-5.

Otten, E., Asimaki, A., Maass, A., van Langen, I. M., van der Wal, A., de Jonge, N., van den Berg, M. P., Saffitz, J. E., Wilde, A. A., Jongbloed, J. D., & van Tintelen, J. P. (2010). Desmin mutations as a cause of right ventricular heart failure affect the intercalated disks. *Heart Rhythm, 7*(8), 1058-1064. doi: 10.1016/j.hrthm.2010.04.023

Palmer, B. M., Valent, S., Holder, E. L., Weinberger, H. D., & Bies, R. D. (1998). Microtubules modulate cardiomyocyte beta-adrenergic response in cardiac hypertrophy. *Am J Physiol, 275*(5 Pt 2), H1707-1716.

Palmiter, K. A., & Solaro, R. J. (1997). Molecular mechanisms regulating the myofilament response to Ca2+: implications of mutations causal for familial hypertrophic cardiomyopathy. *Basic Res Cardiol, 92 Suppl 1*, 63-74.

Panaviene, Z., & Moncman, C. L. (2007). Linker region of nebulin family members plays an important role in targeting these molecules to cellular structures. *Cell Tissue Res, 327*(2), 353-369. doi: 10.1007/s00441-006-0305-2

Parker, K. K., Tan, J., Chen, C. S., & Tung, L. (2008). Myofibrillar architecture in engineered cardiac myocytes. *Circ Res, 103*(4), 340-342. doi: 10.1161/CIRCRESAHA.108.182469

Parvatiyar, M. S., Pinto, J. R., Dweck, D., & Potter, J. D. (2010). Cardiac troponin mutations and restrictive cardiomyopathy. *J Biomed Biotechnol, 2010*, 350706. doi: 10.1155/2010/350706

Patterson, N. L., Iyer, R. P., de Castro Bras, L. E., Li, Y., Andrews, T. G., Aune, G. J., Lange, R. A., & Lindsey, M. L. (2013). Using proteomics to uncover extracellular matrix interactions during cardiac remodeling. *Proteomics Clin Appl, 7*(7-8), 516-527. doi: 10.1002/prca.201200100

Patwari, P., & Lee, R. T. (2008). Mechanical control of tissue morphogenesis. *Circ Res, 103*(3), 234-243. doi: 10.1161/CIRCRESAHA.108.175331

Paulin, D., Huet, A., Khanamyrian, L., & Xue, Z. (2004). Desminopathies in muscle disease. *J Pathol, 204*(4), 418-427. doi: 10.1002/path.1639

Pawlak, A., Gil, R. J., Walczak, E., & Seweryniak, P. (2009). Desmin expression in human cardiomyocytes and selected clinical and echocardiographic parameters in patients with chronic heart failure. *Kardiol Pol, 67*(9), 955-961.

Perriard, J. C., Hirschy, A., & Ehler, E. (2003). Dilated cardiomyopathy: a disease of the intercalated disc? *Trends Cardiovasc Med, 13*(1), 30-38.

Pipelzadeh, M. H., & Naylor, I. L. (1998). The in vitro enhancement of rat myofibroblast contractility by alterations to the pH of the physiological solution. *Eur J Pharmacol, 357*(2-3), 257-259.

Rane, A. A., Chuang, J. S., Shah, A., Hu, D. P., Dalton, N. D., Gu, Y., Peterson, K. L., Omens, J. H., & Christman, K. L. (2011). Increased infarct wall thickness by a bio-inert material is insufficient to prevent negative left ventricular remodeling after myocardial infarction. *PLoS One, 6*(6), e21571. doi: 10.1371/journal.pone.0021571

Ren, Y., Lee, M. Y., Schliffke, S., Paavola, J., Amos, P. J., Ge, X., Ye, M., Zhu, S., Senyei, G., Lum, L., Ehrlich, B. E., & Qyang, Y. (2011). Small molecule Wnt inhibitors enhance the efficiency of BMP-4-directed cardiac differentiation of human pluripotent stem cells. *J Mol Cell Cardiol, 51*(3), 280-287. doi: 10.1016/j.yjmcc.2011.04.012

Rovetti, R., Cui, X., Garfinkel, A., Weiss, J. N., & Qu, Z. (2010). Spark-induced sparks as a mechanism of intracellular calcium alternans in cardiac myocytes. *Circ Res, 106*(10), 1582-1591. doi: 10.1161/CIRCRESAHA.109.213975

Schaper, J., Froede, R., Hein, S., Buck, A., Hashizume, H., Speiser, B., Friedl, A., & Bleese, N. (1991). Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy. *Circulation, 83*(2), 504-514.

Schaper, J., Kostin, S., Hein, S., Elsasser, A., Arnon, E., & Zimmermann, R. (2002). Structural remodelling in heart failure. *Exp Clin Cardiol, 7*(2-3), 64-68.

Schips, T. G., Wietelmann, A., Hohn, K., Schimanski, S., Walther, P., Braun, T., Wirth, T., & Maier, H. J. (2011). FoxO3 induces reversible cardiac atrophy and autophagy in a transgenic mouse model. *Cardiovasc Res, 91*(4), 587-597. doi: 10.1093/cvr/cvr144

Schoenauer, R., Bertoncini, P., Machaidze, G., Aebi, U., Perriard, J. C., Hegner, M., & Agarkova, I. (2005). Myomesin is a molecular spring with adaptable elasticity. *J Mol Biol, 349*(2), 367-379. doi: 10.1016/j.jmb.2005.03.055

Semsarian, C., Ahmad, I., Giewat, M., Georgakopoulos, D., Schmitt, J. P., McConnell, B. K., Reiken, S., Mende, U., Marks, A. R., Kass, D. A., Seidman, C. E., & Seidman, J. G. (2002). The L-type calcium channel inhibitor diltiazem prevents cardiomyopathy in a mouse model. *J Clin Invest, 109*(8), 1013-1020. doi: 10.1172/JCI14677

Senatore, S., Rami Reddy, V., Semeriva, M., Perrin, L., & Lalevee, N. (2010). Response to mechanical stress is mediated by the TRPA channel painless in the Drosophila heart. *PLoS Genet, 6*(9), e1001088. doi: 10.1371/journal.pgen.1001088

Sheehy, S. P., Grosberg, A., & Parker, K. K. (2012). The contribution of cellular mechanotransduction to cardiomyocyte form and function. *Biomech Model Mechanobiol, 11*(8), 1227-1239. doi: 10.1007/s10237-012-0419-2

Simpson, D. G., Majeski, M., Borg, T. K., & Terracio, L. (1999). Regulation of cardiac myocyte protein turnover and myofibrillar structure in vitro by specific directions of stretch. *Circ Res, 85*(10), e59-69.

Simpson, D. G., Sharp, W. W., Borg, T. K., Price, R. L., Terracio, L., & Samarel, A. M. (1996). Mechanical regulation of cardiac myocyte protein turnover and myofibrillar structure. *Am J Physiol, 270*(4 Pt 1), C1075-1087.

Sinha, B., Koster, D., Ruez, R., Gonnord, P., Bastiani, M., Abankwa, D., Stan, R. V., Butler-Browne, G., Vedie, B., Johannes, L., Morone, N., Parton, R. G., Raposo, G., Sens, P., Lamaze, C., & Nassoy, P. (2011). Cells respond to mechanical stress by rapid disassembly of caveolae. *Cell, 144*(3), 402-413. doi: 10.1016/j.cell.2010.12.031

Smith, E. D., Kudlow, B. A., Frock, R. L., & Kennedy, B. K. (2005). A-type nuclear lamins, progerias and other degenerative disorders. *Mech Ageing Dev, 126*(4), 447-460. doi: 10.1016/j.mad.2004.10.006

Solon, J., Levental, I., Sengupta, K., Georges, P. C., & Janmey, P. A. (2007). Fibroblast adaptation and stiffness matching to soft elastic substrates. *Biophys J, 93*(12), 4453-4461. doi: 10.1529/biophysj.106.101386

Song, W., Dyer, E., Stuckey, D., Leung, M. C., Memo, M., Mansfield, C., Ferenczi, M., Liu, K., Redwood, C., Nowak, K., Harding, S., Clarke, K., Wells, D., & Marston, S. (2010). Investigation of a transgenic mouse model of familial dilated cardiomyopathy. *J Mol Cell Cardiol, 49*(3), 380-389. doi: 10.1016/j.yjmcc.2010.05.009

Sonnenblick, E. H., Spotnitz, H. M., & Spiro, D. (1964). Role of the Sarcomere in Ventricular Function and the Mechanism of Heart Failure. *Circ Res, 15*, SUPPL 2:70-81.

Sorimachi, H., Freiburg, A., Kolmerer, B., Ishiura, S., Stier, G., Gregorio, C. C., Labeit, D., Linke, W. A., Suzuki, K., & Labeit, S. (1997). Tissue-specific expression and alpha-

actinin binding properties of the Z-disc titin: implications for the nature of vertebrate Z-discs. *J Mol Biol, 270*(5), 688-695. doi: 10.1006/jmbi.1997.1145

Spanjaard, E., & de Rooij, J. (2013). Mechanotransduction: vinculin provides stability when tension rises. *Curr Biol, 23*(4), R159-161. doi: 10.1016/j.cub.2013.01.020

Steinert, P. M., North, A. C., & Parry, D. A. (1994). Structural features of keratin intermediate filaments. *J Invest Dermatol, 103*(5 Suppl), 19S-24S.

Stern, M. D., Rios, E., & Maltsev, V. A. (2013). Life and death of a cardiac calcium spark. *J Gen Physiol, 142*(3), 257-274. doi: 10.1085/jgp.201311034

Stroud, M. J., Banerjee, I., Veevers, J., & Chen, J. (2014). Linker of nucleoskeleton and cytoskeleton complex proteins in cardiac structure, function, and disease. *Circ Res, 114*(3), 538-548. doi: 10.1161/CIRCRESAHA.114.301236

Swank, D. M., Wells, L., Kronert, W. A., Morrill, G. E., & Bernstein, S. I. (2000). Determining structure/function relationships for sarcomeric myosin heavy chain by genetic and transgenic manipulation of Drosophila. *Microsc Res Tech, 50*(6), 430-442. doi: 10.1002/1097-0029(20000915)50:6<430::AID-JEMT2>3.0.CO;2-E

Swift, J., Ivanovska, I. L., Buxboim, A., Harada, T., Dingal, P. C., Pinter, J., Pajerowski, J. D., Spinler, K. R., Shin, J. W., Tewari, M., Rehfeldt, F., Speicher, D. W., & Discher, D. E. (2013). Nuclear lamin-A scales with tissue stiffness and enhances matrix-directed differentiation. *Science, 341*(6149), 1240104. doi: 10.1126/science.1240104

Takeda, N. (2003). Cardiomyopathy: molecular and immunological aspects (review). *Int J Mol Med, 11*(1), 13-16.

Talwar, S., Jain, N., & Shivashankar, G. V. (2014). The regulation of gene expression during onset of differentiation by nuclear mechanical heterogeneity. *Biomaterials, 35*(8), 2411-2419. doi: 10.1016/j.biomaterials.2013.12.010

Tangney, J. R., Chuang, J. S., Janssen, M. S., Krishnamurthy, A., Liao, P., Hoshijima, M., Wu, X., Meininger, G. A., Muthuchamy, M., Zemljic-Harpf, A., Ross, R. S., Frank, L. R., McCulloch, A. D., & Omens, J. H. (2013). Novel role for vinculin in ventricular myocyte mechanics and dysfunction. *Biophys J, 104*(7), 1623-1633. doi: 10.1016/j.bpj.2013.02.021

Thompson, S. A., Blazeski, A., Copeland, C. R., Cohen, D. M., Chen, C. S., Reich, D. M., & Tung, L. (2014). Acute slowing of cardiac conduction in response to myofibroblast coupling to cardiomyocytes through N-cadherin. *J Mol Cell Cardiol, 68*, 29-37. doi: 10.1016/j.yjmcc.2013.12.025

Thompson, S. A., Copeland, C. R., Reich, D. H., & Tung, L. (2011). Mechanical coupling between myofibroblasts and cardiomyocytes slows electric conduction in fibrotic cell monolayers. *Circulation, 123*(19), 2083-2093. doi: 10.1161/CIRCULATIONAHA.110.015057

Tian, T., Liu, Y., Zhou, X., & Song, L. (2013). Progress in the molecular genetics of hypertrophic cardiomyopathy: a mini-review. *Gerontology, 59*(3), 199-205. doi: 10.1159/000346146

Torella, D., Ellison, G. M., & Nadal-Ginard, B. (2014). Adult c-kit(pos) cardiac stem cells fulfill Koch's postulates as causal agents for cardiac regeneration. *Circ Res, 114*(4), e24-26. doi: 10.1161/CIRCRESAHA.113.303313

Tsatsopoulou, A. A., Protonotarios, N. I., & McKenna, W. J. (2006). Arrhythmogenic right ventricular dysplasia, a cell adhesion cardiomyopathy: insights into disease pathogenesis from preliminary genotype--phenotype assessment. *Heart, 92*(12), 1720-1723. doi: 10.1136/hrt.2005.081679

Turczynska, K. M., Hellstrand, P., Sward, K., & Albinsson, S. (2013). Regulation of vascular smooth muscle mechanotransduction by microRNAs and L-type calcium channels. *Commun Integr Biol, 6*(1), e22278. doi: 10.4161/cib.22278

Tzahor, E., & Lassar, A. B. (2001). Wnt signals from the neural tube block ectopic cardiogenesis. *Genes Dev, 15*(3), 255-260.

Umar, S., van der Valk, E. J., Schalij, M. J., van der Wall, E. E., Atsma, D. E., & van der Laarse, A. (2009). Integrin stimulation-induced hypertrophy in neonatal rat cardiomyocytes is NO-dependent. *Mol Cell Biochem, 320*(1-2), 75-84. doi: 10.1007/s11010-008-9900-8

van der Loop, F. T., Schaart, G., Langmann, H., Ramaekers, F. C., & Viebahn, C. (1995). Rearrangement of intercellular junctions and cytoskeletal proteins during rabbit myocardium development. *Eur J Cell Biol, 68*(1), 62-69.

Viswanathan, M. C., Kaushik, G., Engler, A. J., Lehman, W., & Cammarato, A. (2014). A Drosophila melanogaster model of diastolic dysfunction and cardiomyopathy based on impaired troponin-T function. *Circ Res, 114*(2), e6-17. doi: 10.1161/CIRCRESAHA.114.302028

Wagner, O. I., Rammensee, S., Korde, N., Wen, Q., Leterrier, J. F., & Janmey, P. A. (2007). Softness, strength and self-repair in intermediate filament networks. *Exp Cell Res, 313*(10), 2228-2235. doi: 10.1016/j.yexcr.2007.04.025

Wang, J., Chen, H., Seth, A., & McCulloch, C. A. (2003). Mechanical force regulation of myofibroblast differentiation in cardiac fibroblasts. *Am J Physiol Heart Circ Physiol, 285*(5), H1871-1881. doi: 10.1152/ajpheart.00387.2003

Wang, Y., & Fuchs, F. (2001). Interfilament spacing, Ca2+ sensitivity, and Ca2+ binding in skinned bovine cardiac muscle. *J Muscle Res Cell Motil, 22*(3), 251-257.

Webster, D. R. (2002). Microtubules in cardiac toxicity and disease. *Cardiovasc Toxicol, 2*(2), 75-89.

Weins, A., Schwarz, K., Faul, C., Barisoni, L., Linke, W. A., & Mundel, P. (2001). Differentiation- and stress-dependent nuclear cytoplasmic redistribution of myopodin,

a novel actin-bundling protein. *J Cell Biol, 155*(3), 393-404. doi: 10.1083/jcb.200012039

Wiesner, R. J., Ehmke, H., Faulhaber, J., Zak, R., & Ruegg, J. C. (1997). Dissociation of left ventricular hypertrophy, beta-myosin heavy chain gene expression, and myosin isoform switch in rats after ascending aortic stenosis. *Circulation, 95*(5), 1253-1259.

Williams, I. A., & Allen, D. G. (2007). Intracellular calcium handling in ventricular myocytes from mdx mice. *Am J Physiol Heart Circ Physiol, 292*(2), H846-855. doi: 10.1152/ajpheart.00688.2006

Wilson, A. J., Schoenauer, R., Ehler, E., Agarkova, I., & Bennett, P. M. (2014). Cardiomyocyte growth and sarcomerogenesis at the intercalated disc. *Cell Mol Life Sci, 71*(1), 165-181. doi: 10.1007/s00018-013-1374-5

Witjas-Paalberends, E. R., Piroddi, N., Stam, K., van Dijk, S. J., Oliviera, V. S., Ferrara, C., Scellini, B., Hazebroek, M., ten Cate, F. J., van Slegtenhorst, M., dos Remedios, C., Niessen, H. W., Tesi, C., Stienen, G. J., Heymans, S., Michels, M., Poggesi, C., & van der Velden, J. (2013). Mutations in MYH7 reduce the force generating capacity of sarcomeres in human familial hypertrophic cardiomyopathy. *Cardiovasc Res, 99*(3), 432-441. doi: 10.1093/cvr/cvt119

Woodman, S. E., Park, D. S., Cohen, A. W., Cheung, M. W., Chandra, M., Shirani, J., Tang, B., Jelicks, L. A., Kitsis, R. N., Christ, G. J., Factor, S. M., Tanowitz, H. B., & Lisanti, M. P. (2002). Caveolin-3 knock-out mice develop a progressive cardiomyopathy and show hyperactivation of the p42/44 MAPK cascade. *J Biol Chem, 277*(41), 38988-38997. doi: 10.1074/jbc.M205511200

Yoon, Y. S., Park, J. S., Tkebuchava, T., Luedeman, C., & Losordo, D. W. (2004). Unexpected severe calcification after transplantation of bone marrow cells in acute myocardial infarction. *Circulation, 109*(25), 3154-3157. doi: 10.1161/01.CIR.0000134696.08436.65

Young, J. L., & Engler, A. J. (2011). Hydrogels with time-dependent material properties enhance cardiomyocyte differentiation in vitro. *Biomaterials, 32*(4), 1002-1009. doi: 10.1016/j.biomaterials.2010.10.020

Young, J. L., Tuler, J., Braden, R., Schup-Magoffin, P., Schaefer, J., Kretchmer, K., Christman, K. L., & Engler, A. J. (2013). In vivo response to dynamic hyaluronic acid hydrogels. *Acta Biomater, 9*(7), 7151-7157. doi: 10.1016/j.actbio.2013.03.019

Yuasa, S., Itabashi, Y., Koshimizu, U., Tanaka, T., Sugimura, K., Kinoshita, M., Hattori, F., Fukami, S., Shimazaki, T., Ogawa, S., Okano, H., & Fukuda, K. (2005). Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. *Nat Biotechnol, 23*(5), 607-611. doi: 10.1038/nbt1093

Zamilpa, R., & Lindsey, M. L. (2010). Extracellular matrix turnover and signaling during cardiac remodeling following MI: causes and consequences. *J Mol Cell Cardiol, 48*(3), 558-563. doi: 10.1016/j.yjmcc.2009.06.012

Zemljic-Harpf, A. E., Godoy, J. C., Platoshyn, O., Asfaw, E. K., Busija, A. R., Domenighetti, A. A., & Ross, R. S. (2014). Vinculin directly binds zonula occludens-1 and is essential for stabilizing connexin-43-containing gap junctions in cardiac myocytes. *J Cell Sci, 127*(Pt 5), 1104-1116. doi: 10.1242/jcs.143743

Zhang, B., Peng, F., Wu, D., Ingram, A. J., Gao, B., & Krepinsky, J. C. (2007). Caveolin-1 phosphorylation is required for stretch-induced EGFR and Akt activation in mesangial cells. *Cell Signal, 19*(8), 1690-1700. doi: 10.1016/j.cellsig.2007.03.005

Zhao, Y., Xue, T., Yang, X., Zhu, H., Ding, X., Lou, L., Lu, W., Yang, B., & He, Q. (2010). Autophagy plays an important role in sunitinib-mediated cell death in H9c2 cardiac muscle cells. *Toxicol Appl Pharmacol, 248*(1), 20-27. doi: 10.1016/j.taap.2010.07.007

Zhuang, J., Yamada, K. A., Saffitz, J. E., & Kleber, A. G. (2000). Pulsatile stretch remodels cell-to-cell communication in cultured myocytes. *Circ Res, 87*(4), 316-322.

Zou, H., Stoppani, E., Volonte, D., & Galbiati, F. (2011). Caveolin-1, cellular senescence and age-related diseases. *Mech Ageing Dev, 132*(11-12), 533-542. doi: 10.1016/j.mad.2011.11.001

Chapter 2

The Role of Stiffness in Cardiac Aging

Introduction

Cardiac output is determined by the volume of blood ejected with each heartbeat and by the heart rate. While ventricular contractile force helps determine ejection fraction during systole, proper myocardial relaxation during diastole is required for appropriate filling between contractions. Both pumping and filling properties of the heart can affect cardiac output and overall cardiac performance and can independently lead to heart failure when perturbed (Borlaug and Redfield, 2011).

The symptoms of heart failure observed in nearly half of all patients are associated with impaired ventricular relaxation or diastolic dysfunction (Borlaug and Redfield, 2011; van der Velden, 2011). Relaxation and filling abnormalities associated with diastolic dysfunction often result in elevated left ventricular end-diastolic pressures and can compromise overall work output of the heart (Kass et al., 2004; Kazik et al., 2010). Various pathogenic mechanisms are believed to underlie diastolic dysfunction. In addition to impaired relaxation, these include perturbations that decrease myocardial distensibility and/or that increase left ventricular end-diastolic stiffness. The molecular basis of these altered diastolic indices encompass altered Ca^{2+} handling, extracellular matrix modifications, and myofilament dysfunction (Borbely et al., 2009; van der Velden, 2011; Ouzounian et al., 2008). In failing hearts, perturbed Ca^{2+} homeostasis can contribute to diastolic dysfunction (Figure 2.1). Slowed Ca^{2+} transients can attenuate myocardial relaxation rates, prolong active force generation, and hinder ventricular filling. Changes in Ca^{2+} handling result from altered expression of Na/Ca^{2+} exchangers, the sarcoplasmic reticulum Ca^{2+} uptake protein SERCA, phospholamban, a SERCA regulator, and ryanodine receptors (Borbely et al., 2009; van der Velden, 2011; Ouzounian et al., 2008). Furthermore, post-translational modification of these proteins by numerous protein kinases can

influence their activity. Elevated diastolic Ca^{2+} levels can also influence overall ventricular distensibility by potentially increasing the active tone of resting myocytes (Kass et al., 2004).

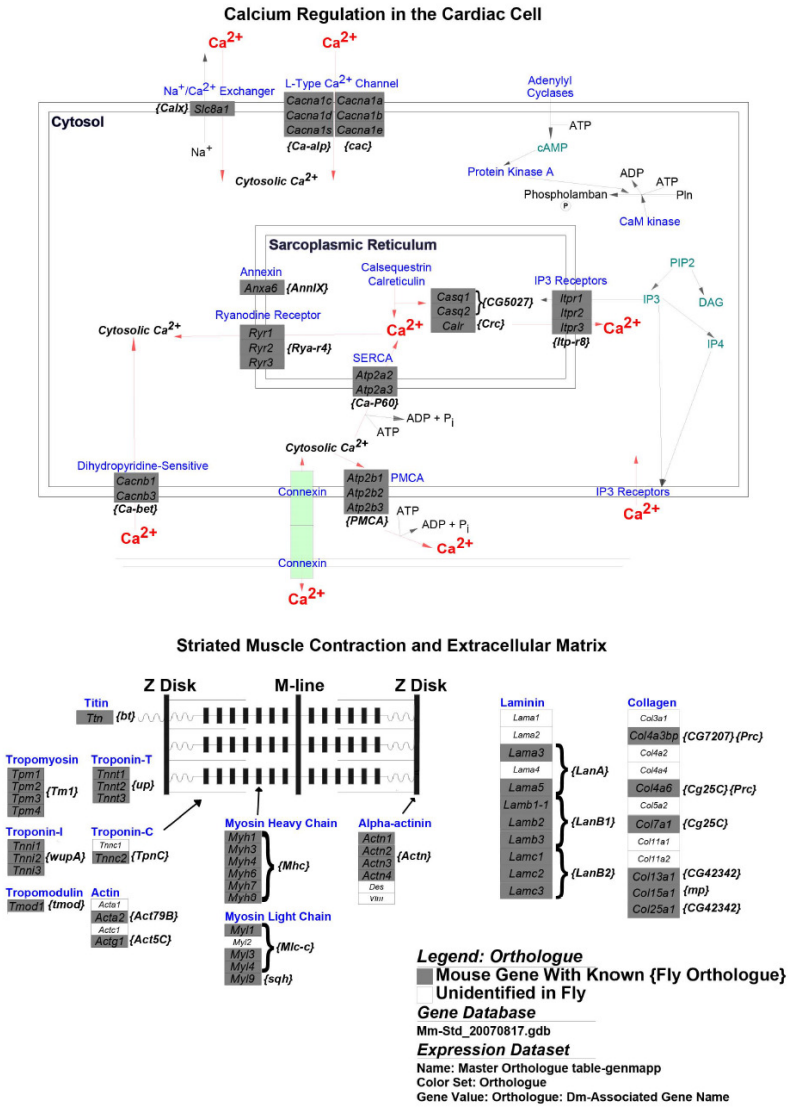

Figure 2.1: Cardiomyocyte gene pathways associated with diastolic dysfunction. Top: In failing hearts, perturbed Ca^{2+} homeostasis can contribute to diastolic dysfunction. Many genes involved with Ca^{2+} handling within cardiomyocytes are conserved between Drosophila and mammals. Here we compiled a gene orthology database from NCBI homologene (Geer et al., 2010), Ensembl (Vilella et al., 2009), and InParanoid (used by FlyBase to assign orthologues- (Remm et al., 2001)). This database assigns Drosophila orthologues to 12,304 unique mouse genes and was used to visualize orthologues related to Ca^{2+} homeostasis. Examples of orthologous genes are shown in grey boxes with the fly gene listed next to each box. Bottom: Modifications in cytoarchitectural components are also critical determinants of diastolic performance and diastolic heart failure. Examples of orthologues related to striated muscle contraction and to extracellular matrix are shown.

Modifications in cytoarchitectural components are likewise considered critical determinants of diastolic performance and failure (Figure 2.1). For example, the mechanical attributes of the extracellular matrix (ECM) that surrounds individual myocytes impact the overall compliance of the myocardium (Engler et al., 2008). ECM material properties are largely influenced by the absolute quantity and distribution of collagen, the ratio of different collagen types and isoforms and the extent of post-translational modification (Borbely et al., 2009; van der Velden, 2011; Ouzounian et al., 2008). Furthermore, deposition of advanced glycation end products can augment collagen crosslinking, altering the physical properties of the ECM and increase left ventricular diastolic stiffness (Kass et al., 2004; Borbely et al., 2009; Ouzounian et al., 2008).

Dysfunction in the myofilamentous components of the cytoarchitecture can additionally initiate abnormal cardiomyocyte compliance and relaxation (Figure 2.1). Passive properties of titin are believed to impart the majority of the longitudinal passive tension characteristics of the ventricles. Hence, alterations in the connecting (titin) filaments and in proteins of the thick and thin filament complexes can result in impaired diastolic function (van der Velden, 2011; Kass et al., 2004).

Thus, a multitude of mechanisms appear to be associated with the development of diastolic dysfunction. Many aspects of diastole can be altered either alone or in combination to elicit irregular performance (Kass et al., 2004). Comprehending the factors that predispose one to diastolic dysfunction and developing new genetic models that permit detailed quantitative analysis and descriptions of its biochemical and biophysical characteristics should provide important insights into diastolic heart failure and potentially facilitate the development of targeted treatments.

Diastolic Dysfunction: Effects of Age and Models for Investigation

Diastolic dysfunction is a major cardiac deficit that can result in diastolic heart failure. There appear to be several predisposing factors for diastolic dysfunction: female gender, obesity, coronary artery disease, hypertension, diabetes mellitus, and age (Kass et al., 2004). Geriatric hearts exhibit both impaired relaxation and increased myocardial stiffness (Ouzounian et al., 2008). Aging is associated with increased interstitial fibrosis and collagen crosslinking, disturbed Ca^{2+} homeostasis, and altered expression and modification of myofilamentous components, which, as outlined above are major contributors to impaired diastolic function. However, the specific causes of age-dependent changes in diastolic performance remain difficult to study. This can be attributed to a paucity of animal models that recapitulate human diastolic dysfunction and particularly myocardial stiffening, which is often unaffected in murine models despite extensive matrix, myofibrillar or signaling abnormalities (Kass et al., 2004; Borbely et al., 2009). Furthermore, the life span of vertebrate models is often several years, making gerontological studies prohibitively lengthy. Conversely, *Drosophila melanogaster*, the fruit fly, is a relatively inexpensive, rapidly aging, genetically tractable organism that is gaining acceptance as a viable alternative for investigating heart development, cardiac pathophysiology, and cardiac aging (Bier and Bodmer, 2004; Nishimura et al., 2011).

Evidence of Age-associated Diastolic Dysfunction in Flies

The advent of new dissection techniques, imaging assays, and analysis protocols have provided a much broader understanding of and appreciation for the utility of the bilayered *Drosophila* heart tube (Figure 2.2A-C) as a model for studying conserved responses to mutations in cardiac components and to age (Taghli-Lamallem et al., 2008). Using a semi-

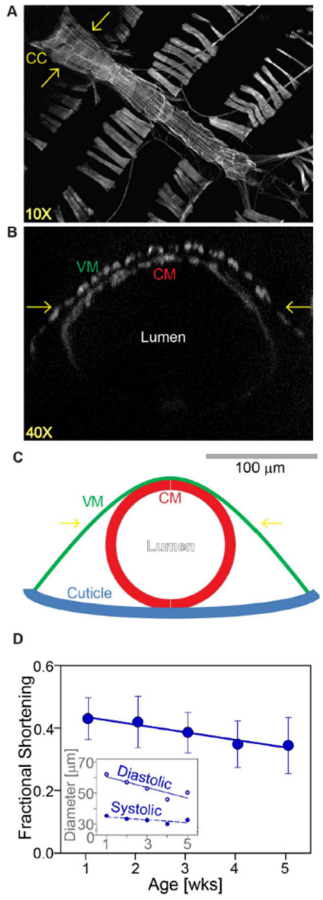

Figure 2.2: The *Drosophila* heart and evidence for age-associated diastolic dysfunction. (A) The *Drosophila* heart lies along the dorsal midline of the abdomen. It consists of a simple linear tube composed of a single layer of contractile cardiomyocytes covered by a thin ventral longitudinal muscle layer. Fluorescent image modified from (Cammarato et al., 2011). The conical chamber (CC) is the most pronounced muscular region of the heart and is likely a primary determinant of circulatory flow. (B) Cross section through the CC revealing the location of the ventral muscle layer (VM) and underlying cardiomyocytes (CM). (C) Illustration depicting the layers of the myocardium shown in (B). (D) Fractional shortening of the Drosophila heart tube declines with age due to an accelerated decline of diastolic diameters. Panel adapted from (Cammarato et al., 2008).

automated heart analysis program, Cammarato and co-authors (Cammarato et al., 2008) showed that wild-type *Drosophila* hearts exhibit a steady decrease in mean diastolic and systolic diameters with age (Figure 2.2D). Furthermore, progressive decline in diastolic dimensions was significantly greater than that for systolic dimensions. This highlights deterioration in contractile performance with age as indicated by significantly reduced percent fractional shortening. It is clear that in geriatric (five week old) flies, there is a substantial attenuation of

diastolic performance relative to that of adult (one week old) flies. This senescent-dependent response is indicative of the impaired myocardial relaxation and possibly of cardiac chamber stiffening as normally observed during age-associated human diastolic dysfunction.

Conservation of Components Involved in Diastolic Dysfunction in Flies

The responses to mutations and age observed in the *Drosophila* heart suggest the fly may serve as a powerful tool for studying cardiac molecular control mechanisms and basic physiological processes that have been conserved during evolution (Bier and Bodmer, 2004; Nishimura et al., 2011). These fundamental responses and processes depend upon conserved transcriptomic and proteomic networks. The extent of conservation with higher organisms has been described by Bier and Bodmer (2004), who suggested that nearly 75% of human disease genes, which include those that cause cardiovascular disorders, have homologues in *Drosophila*. Cammarato and co-authors (Cammarato et al., 2011) extended this finding to the proteome, which revealed a substantial degree of conservation between fly and murine hearts, particularly among cytoarchitectural components.

As an example of pathway-level conservation found between *Drosophila* and mammals, we compiled a gene orthology database from three independent publicly available sources: NCBI homologene (Geer et al., 2010), Ensembl (Vilella et al., 2009), and InParanoid (used by FlyBase to assign orthologues,(Remm et al., 2001)) (Figure 2.1). This database assigns *Drosophila* orthologues to 12,304 unique mouse genes and was used to visualize orthologues of three signaling networks related to passive myocardial stiffness and diastolic performance: calcium regulation in the cardiac cell, focal adhesion and striated muscle contraction (Figure 2.1). These pathways were downloaded from wikipathways (Pico et al., 2008) and colored with the GenMAPP program (Salomonis et al., 2007). Conservation and reduced redundancy of most major pathway components in all three networks are clearly evident. Ongoing cardiac-specific

microarray experiments have confirmed the expression of the majority of these orthologous genes (unpublished). Subsequent extension of this analysis will identify differentially expressed cardiac gene candidates with age and provide therapeutic targets that can be rapidly and systematically manipulated in flies to determine novel roles in diastolic relaxation and in cardiac stiffness *in situ*.

Measuring Passive Myocardial Stiffness *in situ*

Mechanical analyses have given remarkable insight into the role myocardial stiffness plays during development (Zamir et al., 2003; Jacot et al., 2008) as well as during aging and pathogenesis (Stedman et al., 1991; Fung, 1993). In particular, atomic force microscopy (AFM)-based indentation approaches, in which the tissue of interest is indented and the reaction forces are measured, offer the ability to investigate myocardial micromechanical properties (Kirmizis and Logothetidis, 2010). AFM-based analyses have revealed that cardiomyocytes stiffen with age (Lieber et al., 2004), suggesting that age-associated diastolic dysfunction may not only be due to structural (ECM-based) changes in the heart. Explanted mouse and avian epicardium have been shown to stiffen during pre- and post-natal development stages (Jacot et al., 2010; Young and Engler, 2011). Stiffening also occurs as a result of remodeling in post-myocardial infarct tissue (Berry et al., 2006) and with age and disease in vasculature (Sazonova et al., 2011). Finally, AFM has helped resolve a stiffness disparity between juvenile and senescent rat myocytes (Lieber et al., 2004; Azeloglu and Costa, 2010). Mechanical discrepancies with age were attributed to differences in myofibrillar content.

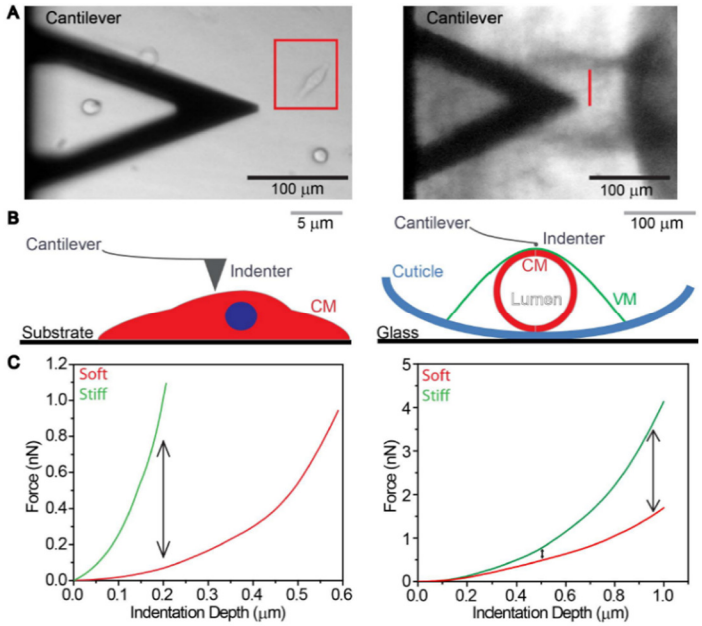

Figure 2.3: Direct mechanical comparison of murine myocytes and fly hearts. (A) Brightfield images of an AFM cantilever positioned over a mouse myocyte (left) and the *Drosophila* heart tube (right). Box on the left highlights probed region where the myocyte is attached and the line on the right image highlights the midline to lateral edge of the fly heart tube that is typically probed using our analysis method. (B) Schematics of a cross-section of the biological specimens analyzed by AFM (mouse on left, fly on right). (C) Left: representative AFM force-indentation curves plotted for mouse cardiomyocytes cultured on a stiff glass coverslip (green) versus a soft hydrogel (red). Arrow indicates an increased reaction force at 200 nm indentation depth consistent with a more rigid remodeling response due to substrate stiffness. Right: force-indentation plots (red vs. green curve) for fly heart tubes indicating overall differences in myocardial stiffness. Left arrow depicts a difference in reaction force response at shallow indentation depth (<500 nm) while the right arrow reveals a difference in force response at deep indentation depth (>1 µm), reflecting disparity in ventral muscle layer and cardiomyocyte stiffness respectively.

Although these results have advanced our understanding of myocyte mechanics, they have largely been limited to isolated myocytes *in vitro* (Figure 2.3, left) or to *ex vivo* tissue explants lacking normal "pre-stress", the tensile stress imparted by external entities *in vivo*. Experiments using isolated cultured cells can produce inconsistencies in mechanical data. For example, culture conditions such as substrate stiffness can drastically alter neonatal rat myocyte morphology, cytoarchitecture, and ultimately stiffness as indicated by force-indentation curves acquired by AFM (Jacot et al., 2008) (Figure 2.3C, left). Prolonged culture can also increase myocyte stiffness during contraction (Azeloglu and Costa, 2010). Furthermore, while matching

ECM cues such as stiffness helps mimic the *in vivo* environment, these artificial approaches do not completely recapitulate it. Cardiomyocytes fail to reorganize and reorder their myofilaments and cellular junctions as seen *in vivo*. To address this problem, *ex vivo* tissue explants are sometimes employed, however discrepancies exist between stiffness reported for cultured cells versus explants. The latter sample type lacks the pre-stress typically created by adjacent tissue contracting against it which can have profound effects on cells and even explants (Berry et al., 2006; Wang et al., 2002). Thus, unless pharmacological treatment establishes that measurements were indeed performed without pre-stress, partial contracture of the explant tissue may unknowingly skew data (Azeloglu and Costa, 2010). In order for *in vitro* mechanical studies to more authoritatively offer insight on cardiac development, maturation, and senescence, experimental conditions should rigorously approximate the physiological environment.

In light of the potential artifacts induced when cardiomyocytes are removed from their normal environment for *in vitro* studies, we have developed an *in situ* indentation method for the bilayered *Drosophila* heart tube (Figure 2.3A and B, right). Due to its length scale, the fly heart tube is especially well-suited for indentation studies as it can be investigated anywhere longitudinally along its surface, removing the need for explantation or isolation (Figure 2.3A, right). Since the tube is unperturbed and is spontaneously beating only seconds to minutes before indentation, this method is the closest an AFM indentation experiment may come to an *in vivo* study of quantifiable single cardiomyocyte and subcellular stiffness. By indenting along the transverse axis of the tube, we are able to measure stiffness both at the midline, upon the intercalated discs, and along the cortex or costameres.

Before comparing cultured myocytes and heart tubes, it is important to note that one complication of *in situ* analysis is tissue geometry. For the fly specifically, a ventral layer of muscle covers the tube, and dissection methods cannot remove it without damaging the tube.

Conventional mechanical analysis relies on the material being infinitely continuous (Hertz, 1882), and corrections to account for layered materials have been limited to thin coatings with a high degree of stiffness mismatch (Dimitriadis et al., 2002; Clifford and Seah, 2009). From the AFM's force-indentation plot, we can clearly observe differences in mechanical behavior based on experimental conditions (Figure 2.3C). At any given indentation depth (x-axis) into a sample, a greater reaction force response (y-axis) indicates a stiffer material (see arrows in Figure 2.3C). As shown previously by Jacot and co-workers, isolated neonatal mouse cardiomyocytes exhibited greater forces and were therefore stiffer when indented on glass coverslips as compared to those plated on a soft hydrogel tuned to ~8 kPa (Figure 2.3C, left), indicating cellular remodeling in response to the substrate. In fly myocardium, there is no such artifact and we are able resolve differences in the force response accurately and at both shallow and deep indentation depths, corresponding to the ventral muscle and cardiomyocytes, respectively (Figure 2.3C, right).

Both cultured myocytes and heart tubes can be probed with modest throughput. However, a major benefit of *in situ* measurement is that intact tissues undergo comparatively little remodeling during characterization relative to cultured cells; while probing cultured cells, their motile nature makes repetitive probing near impossible. Given spatial variation in populations, this would make attempts to measure age-related differences in murine myocardium in the same way very time consuming and difficult. Therefore, we propose that in situ AFM-based analysis of the fly heart in conjunction with established myocardial-specific genetic manipulations can serve as a robust screening tool for the mechanical consequences of age and genetic modification of mammalian-conserved genes in cardiomyocytes.

Future Perspectives

While a plethora of techniques to monitor cardiac function and structure in flies have been developed, the methodology is based predominantly on optical analysis such that myocardial mechanics are therefore inferred (Wessells and Bodmer, 2007; Nishimura et al., 2011). Here, for the first time, we have developed the capacity to directly evaluate passive myocardial mechanical parameters, in situ, in *Drosophila*. These parameters play a significant role in dictating cardiac output in all organisms. Knowledge of the heart's material properties and how these change with age are central to developing effective therapies directed against age-associated diastolic dysfunction and diastolic heart failure. The ability to employ bioinformatics networking approaches, genome-wide screening techniques and genetic manipulation of any gene of interest in rapidly aging hearts generates enormous potential for high-throughput analysis of senescent-related changes in passive cardiac mechanics, a main determinant of diastolic function. Understanding the genetic basis of these changes should greatly expedite the development of effective treatments. Thus, our pioneering approach in flies will yield rapid and potentially translatable findings regarding cardiac aging and will facilitate the testing of novel mechanical models in higher organisms.

Acknowledgements

This article is a modification of a previously-published article: Kaushik G., Zambon A.C., Fuhrmann A., Bernstein S.I., Bodmer R., Engler A.J., and Cammarato A. (2012). Measuring passive myocardial stiffness in Drosophila melanogaster to investigate diastolic dysfunction. Journal of cellular and molecular medicine *16*, 1656-1662. The author of this dissertation was lead author on this publication.

References

Azeloglu, E.U., and Costa, K.D. (2010). Cross-bridge cycling gives rise to spatiotemporal heterogeneity of dynamic subcellular mechanics in cardiac myocytes probed with atomic force microscopy. American journal of physiology Heart and circulatory physiology 298, H853-860.

Berry, M.F., Engler, A.J., Woo, Y.J., Pirolli, T.J., Bish, L.T., Jayasankar, V., Morine, K.J., Gardner, T.J., Discher, D.E., and Sweeney, H.L. (2006). Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. American journal of physiology Heart and circulatory physiology 290, H2196-2203.

Bier, E., and Bodmer, R. (2004). Drosophila, an emerging model for cardiac disease. Gene 342, 1-11.

Borbely, A., Papp, Z., Edes, I., and Paulus, W.J. (2009). Molecular determinants of heart failure with normal left ventricular ejection fraction. Pharmacol Rep 61, 139-145.

Borlaug, B.A., and Redfield, M.M. (2011). Diastolic and systolic heart failure are distinct phenotypes within the heart failure spectrum. Circulation 123, 2006-2013; discussion 2014.

Cammarato, A., Ahrens, C.H., Alayari, N.N., Qeli, E., Rucker, J., Reedy, M.C., Zmasek, C.M., Gucek, M., Cole, R.N., Van Eyk, J.E., Bodmer, R., O'Rourke, B., Bernstein, S.I., Foster, D.B. (2011). A mighty small heart: the cardiac proteome of adult Drosophila melanogaster. PloS one 6, e18497.

Cammarato, A., Dambacher, C.M., Knowles, A.F., Kronert, W.A., Bodmer, R., Ocorr, K., and Bernstein, S.I. (2008). Myosin transducer mutations differentially affect motor function, myofibril structure, and the performance of skeletal and cardiac muscles. Molecular biology of the cell 19, 553-562.

Clifford, C.A., and Seah, M.P. (2009). Nanoindentation measurement of Young's modulus for compliant layers on stiffer substrates including the effect of Poisson's ratios. Nanotechnology 20, 145708.

Dimitriadis, E.K., Horkay, F., Maresca, J., Kachar, B., and Chadwick, R.S. (2002). Determination of elastic moduli of thin layers of soft material using the atomic force microscope. Biophysical journal 82, 2798-2810.

Engler, A.J., Carag-Krieger, C., Johnson, C.P., Raab, M., Tang, H.Y., Speicher, D.W., Sanger, J.W., Sanger, J.M., and Discher, D.E. (2008). Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. Journal of cell science 121, 3794-3802.

Fung, Y.C. (1993). Biomechanics: mechanical properties of living tissues, 2nd edn (New York: Springer-Verlag).

Geer, L.Y., Marchler-Bauer, A., Geer, R.C., Han, L., He, J., He, S., Liu, C., Shi, W., and

Bryant, S.H. (2010). The NCBI BioSystems database. Nucleic Acids Res 38, D492-496.

Hertz, H. (1882). Über die Berührung fester elastischer Körper. Mathematik 92, 156-171.

Jacot, J.G., Martin, J.C., and Hunt, D.L. (2010). Mechanobiology of cardiomyocyte development. Journal of biomechanics 43, 93-98.

Jacot, J.G., McCulloch, A.D., and Omens, J.H. (2008). Substrate stiffness affects the functional maturation of neonatal rat ventricular myocytes. Biophysical journal 95, 3479-3487.

Kass, D.A., Bronzwaer, J.G., and Paulus, W.J. (2004). What mechanisms underlie diastolic dysfunction in heart failure? Circulation research 94, 1533-1542.

Kazik, A., Wilczek, K., and Polonski, L. (2010). Management of diastolic heart failure. Cardiology journal 17, 558-565.

Kirmizis, D., and Logothetidis, S. (2010). Atomic force microscopy probing in the measurement of cell mechanics. Int J Nanomedicine 5, 137-145.

Lieber, S.C., Aubry, N., Pain, J., Diaz, G., Kim, S.J., and Vatner, S.F. (2004). Aging increases stiffness of cardiac myocytes measured by atomic force microscopy nanoindentation. American journal of physiology Heart and circulatory physiology 287, H645-651.

Nishimura, M., Ocorr, K., Bodmer, R., and Cartry, J. (2011). Drosophila as a model to study cardiac aging. Experimental gerontology 46, 326-330.

Ouzounian, M., Lee, D.S., and Liu, P.P. (2008). Diastolic heart failure: mechanisms and controversies. Nature clinical practice Cardiovascular medicine 5, 375-386.

Pico, A.R., Kelder, T., van Iersel, M.P., Hanspers, K., Conklin, B.R., and Evelo, C. (2008). WikiPathways: pathway editing for the people. PLoS biology 6, e184.

Remm, M., Storm, C.E., and Sonnhammer, E.L. (2001). Automatic clustering of orthologs and in-paralogs from pairwise species comparisons. Journal of molecular biology 314, 1041-1052.

Salomonis, N., Hanspers, K., Zambon, A.C., Vranizan, K., Lawlor, S.C., Dahlquist, K.D., Doniger, S.W., Stuart, J., Conklin, B.R., and Pico, A.R. (2007). GenMAPP 2: new features and resources for pathway analysis. BMC Bioinformatics 8, 217.

Sazonova, O.V., Lee, K.L., Isenberg, B.C., Rich, C.B., Nugent, M.A., and Wong, J.Y. (2011). Cell-cell interactions mediate the response of vascular smooth muscle cells to substrate stiffness. Biophysical journal 101, 622-630.

Stedman, H.H., Sweeney, H.L., Shrager, J.B., Maguire, H.C., Panettieri, R.A., Petrof, B., Narusawa, M., Leferovich, J.M., Sladky, J.T., and Kelly, A.M. (1991). The mdx

mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. Nature 352, 536-539.

Taghli-Lamallem, O., Bodmer, R., Chamberlain, J.S., and Cammarato, A. (2008). Genetics and pathogenic mechanisms of cardiomyopathies in the Drosophila model Drug Discovery Today: Disease Models 5, 125-134.

van der Velden, J. (2011). Diastolic myofilament dysfunction in the failing human heart. Pflugers Archive: European journal of physiology 462, 155-163.

Vilella, A.J., Severin, J., Ureta-Vidal, A., Heng, L., Durbin, R., and Birney, E. (2009). EnsemblCompara GeneTrees: Complete, duplication-aware phylogenetic trees in vertebrates. Genome Res 19, 327-335.

Wang, N., Tolic-Norrelykke, I.M., Chen, J., Mijailovich, S.M., Butler, J.P., Fredberg, J.J., and Stamenovic, D. (2002). Cell prestress. I. Stiffness and prestress are closely associated in adherent contractile cells. American journal of physiology Cell physiology 282, C606-616.

Wessells, R.J., and Bodmer, R. (2007). Age-related cardiac deterioration: insights from Drosophila. Front Biosci 12, 39-48.

Young, J.L., and Engler, A.J. (2011). Hydrogels with time-dependent material properties enhance cardiomyocyte differentiation in vitro. Biomaterials 32, 1002-1009.

Zamir, E.A., Srinivasan, V., Perucchio, R., and Taber, L.A. (2003). Mechanical asymmetry in the embryonic chick heart during looping. Annals of biomedical engineering 31, 1327-1336.

Chapter 3

Mechanical Analysis of *Drosophila* Myocardium

Introduction

Adult *Drosophila melanogaster* possess an open circulatory system with an abdominally located pulsatile heart tube (Miller, 1950). The simple linear heart contains bilateral rows of contractile cardiomyocytes that form the cardiac lumen, which is aligned with the Drosophila body axis (Figure 1a). The anterior conical chamber is the most pronounced muscular region of the heart tube (Miller, 1950; Wasserthal, 2007) and is likely a primary determinant of circulatory flow. Covering the tube is a thin, longitudinal, and ventral muscle layer that extends from the conical chamber and runs nearly the entire length of the heart, creating a bilayered structure (Figure 1b) (Miller, 1950; Wasserthal, 2007). The ventral muscle layer tightly associates with the tube (Miller, 1950), making it difficult to remove without compromising underlying cardiomyocyte integrity. Although genetic tools and various imaging techniques (Ocorr et al., 2007; Taghli-Lamallem et al., 2008; Wolf et al., 2006) make perturbing and observing cardiac structure and function possible, corresponding biophysical analyses to monitor how genetic perturbations and aging alter the mechanical environment of soft bilayers do not exist. Since mechanics directly impacts both form and function across numerous animal models (Engler et al., 2009), the lack of a biophysical description of the Drosophila conical chamber severely limits our understanding of cardiovascular dynamics in such a genetically tractable system.

Atomic force microscopy (AFM) has been the standard biophysical method used to obtain force-indentation curves, which when fit with a mathematical model of a sphere indenting a material assumed to be homogeneous and thick as compared to indentation depth (Hertz, 1882; Radmacher, 2002) can determine tissue elastic moduli, or ‘stiffness’ (measured in Pascal, Pa). For biological materials, such assumptions are not generally valid since the material’s composition can be very heterogeneous and are often composed of bonded layers such as the Drosophila heart tube or skin or blood vessels in mammals. When indentations are small, i.e.

<10% global strain throughout the bulk material, it is possible to obtain elastic properties of the top layer of such materials as the strain field is not overly influenced by subsequent layers (Engler et al., 2004b; Reinhart-King et al., 2003; Roduit et al., 2009). In cases where the top layer is too thin to dissipate the strain from indentation before being influenced by underlying layer(s), correction models need to be applied. Two prominent correction methods include those introduced by Dimitriadis and co-workers (Dimitriadis et al., 2002) and Clifford and Seah (Clifford and Seah, 2009). The former method requires that juxtaposed layer(s) have sufficient moduli mismatch such as with cells adhering to a glass coverslip (Mahaffy et al., 2004; Rotsch et al., 1999). The latter method establishes an estimate for when the underlying layer's indentation influences measurement of the top layer based on a ratio of both moduli. These thin coating analysis methods may not pose a problem for isolated cells such as cardiomyocytes in vitro, which show stiffness changes with maturation (Collinsworth et al., 2002) and age (Lieber et al., 2004), since isolation simplifies the complex mechanical environment that the cells inhabit in vivo. However, for more representative in situ measurements of intact cells where heterogeneous muscle layers potentially exist, this poses a substantial problem for current analysis methods.

Our goal was to establish a straightforward analysis method for thin biphasic materials, especially those that are biological, where layer properties need not vary dramatically, within a well-characterized model system. The Linearized-Hertz method has been previously described for such occasions (Guo and Akhremitchev, 2006) where the elastic moduli of juxtaposed soft layers can be determined from a single force-indentation curve. Similar analyses have been applied to cell indentation where the cell is assumed to be a bilayer of soft cytosol and rigid cytoskeletal filaments (Carl and Schillers, 2008; Kim et al., 2011). However, previous descriptions lack rigorous verification in (synthetic?) polymer models and layered tissues and do not explicitly attribute depth-dependent findings to specific layers. Straightforward

methodological explanation and convenient analysis software also appear lacking. Here, we perform an analysis on model bilayered systems consisting of polydimethylsiloxane (PDMS) cast in layers and in situ indentation of the conical chamber of the Drosophila heart tube. We hypothesized that RNA interference (RNAi)-mediated myofibrillar disruption and aging would decrease and increase Drosophila cardiac fiber stiffness, respectively. Cardiomyocyte-specific RNAi targeted against myosin heavy chain (MHC), a major myofibrillar component, softened the cardiomyocyte layer of the fly heart without eliciting any significant stiffness change of the ventral layer. Age-induced stiffening in both layers was observed, which suggests wide applicability of our approach to models of cardiovascular aging. Together these data suggest that this analysis method enables direct in situ measurements of dysfunction in diverse, multi-layered, biological specimens.

Methods and Materials

Drosophila Husbandry and Preparation

Flies were maintained at 25˚C on standard cornmeal-agar medium. Control (yw) adult female flies were transferred to fresh food every 2–3 days and aged for 1 or 5 weeks. UAS-MHC RNAi flies (VDRC transformant ID 105355 KK) were crossed to Hand-Gal4 (II) driver flies (Han et al., 2006). The progeny express hairpins targeted against MHC transcripts, to reduce MHC expression, in a cardiomyocyte restricted manner. The parental Drosophila RNAi line has an “interfering RNA” coding region of a transgene located just downstream of the yeast “Upstream Activating Sequence”. The gene cassette containing the RNAi hairpin remains inactive in the absence of the yeast GAL4 transactivating protein. When flies carrying the UAS-MHC RNAi construct are crossed with flies carrying the GAL4 transcriptional activator, the progeny inherit both genes and will express the RNAi in the same pattern as GAL4. Transgenic RNAi expressed via the UAS/Gal4 system allows manipulation of gene expression in a highly

precise spatial fashion with heart specific Hand drivers. The adult progeny expressing cardiomyocyte-restricted RNAi targeted against myosin heavy chain (MHC) were aged for 1 week (see Figure 3.4). Myosin-GFP flies (from http://flytrap.med.yale.edu, flytrap name: YD0783) were used to illustrate heart tube structure (see Figure 1b).

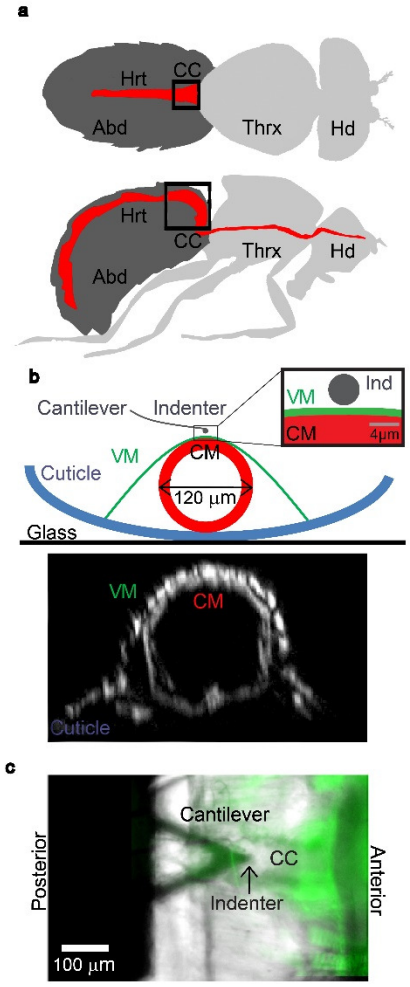

Figure 3.2. Drosophila heart structure and sample geometry. A. Ventral and orthogonal view of fly. B. Indentation of bilayered heart. C. GFP labeling of fly heart with indenter placed over ventral surface.

Microsurgery and Imaging of Drosophila Heart

Beating cardiac tubes for each genotype or age group were exposed via microsurgery ($n \geq 20$) according to Ocorr and Vogler (Vogler and Ocorr, 2009). All procedures were

performed at room temperature (18-22°C), under oxygenated hemolymph as previously described (Cammarato et al., 2008; Ocorr et al., 2007). Flies were anesthetized before mounting (dorsal side down) on 25 mm diameter coverslips and removal of the head, ventral thorax and abdominal cuticle (Vogler and Ocorr, 2009). All ventral tissues except the heart were excised. Conical chambers were carefully cleaned of all extraneous debris, e.g. adipose and nervous tissue. Exposed hearts typically exhibited rhythmic contractions for up to four hours, though immediately before nanoindentation heart tube contractions were inhibited by incubation in oxygenated hemolymph containing 10mM EGTA, resulting in heart tube relaxation including a cessation of flow and pressure gradients and an opening of ostia, i.e. inlet valves on the side of the heart. Motion-mode (M-mode) kymographs were generated from 30-sec brightfield movies of beating hearts taken at rates of 100–200 frames/sec using a Hamamatsu EM-CCD digital camera on a Leica DM LFSA microscope with a 10X immersion lens. M-modes were created by a previously described Matlab-based image analysis program (Fink et al., 2009).

To fluorescently label *Drosophila* heart tubes, rhythmic contractions were arrested by hemolymph containing 10mM EGTA. Hearts were fixed in phosphate buffer solution (PBS) containing 4% formaldehyde for 20 min, rinsed three times for 10 min with PBSTx (PBS containing 0.1% Triton-X-100), incubated with Alexa584-phalloidin in PBSTx (1:1000) for 20 min, and washed again three times with PBSTx for 10 min, all at 25oC with gentle agitation. The specimens were mounted on microscope slides with Vectashield and viewed at 10X magnification using a Zeiss Imager Z1 fluorescent microscope equipped with an Apotome sliding module.

Fabrication of PDMS Bilayers

Polydimethylsiloxane (PDMS) made from Sylgard® 184 Silicone Elastomer Base (Dow Corning, Midland, MI) was fabricated with varying elastic modulus and thickness by

changing the ratio of curing agent to elastomer (1:45 for stiffer and 1:60 for softer PDMS) (Goffin et al., 2006) and the angular velocity (1000 – 12000 RPM) at which the uncured PDMS was spun, respectively. PDMS was placed on a spincoater (Laurell Technologies; North Wales, PA) and spun for 80 sec at 1000 and 12000 RPM to achieve film heights of 75.0 ± 8.0 μm and 8.5 ± 1.5 μm, respectively, as measured by Dektak 3030 Surface Profiler (Veeco; Santa Barbara, CA). PDMS with a curing ratio of 1:45 and 1:50 was thermo-set for 30 min at 60-70°C and PDMS with a curing ratio of 1:60 was thermo-set for either 30 min at 60-70°C (1000 RPM) or 24 hrs at room temperature (12000 RPM). A bilayer material was made from spinning a 12000 RPM film (1:60 curing ratio) onto a pre-cured 1000 RPM layer (of indicated curing ratio) and subsequently thermo-setting the bilayer for 24 hrs at room temperature. During thermosetting, the second layer will bind to the primary layer resulting in a bonded, bilayer material. 75 μm thick, 1:45 curing ratio PDMS was also respun at 12000 RPM and thermo-set for 24 hrs at room temperature to mimic the multiple spins required for bilayer fabrication.

Atomic Force Microscopy Indentation and Force Curve Analysis

All nanoindentation was performed with an MFP-3D Bio Atomic Force Microscope (Asylum Research; Santa Barbara, CA) mounted on a Ti-U fluorescent inverted microscope (Nikon Instruments; Melville, NY). 120 pN/nm silicon nitride or 7500 pN/nm silicon cantilevers with pre-mounted borosilicate spheres (2μm radius, Novascan Technologies; Ames, IA) were used to indent Drosophila heart tubes or PDMS, respectively. Probes were calibrated using a thermal noise method provided by the MFP-3D Bio software.

For Drosophila heart indentation, *yw* adult female flies were immobilized on glass supports. Myogenic contractions of surgically-expose heart tubes were arrested and the AFM probe was aligned with the centerline of the conical chamber of the heart tube (Figure 1c). A 1 μm2 area was then probed in a 4x4 grid of indentations. Both Drosophila and PDMS were

indented up to a 100 nm cantilever deflection using approach and retraction speeds of 1 μm/s unless otherwise indicated. Probe deflection and position were monitored during indentation to produce plots of force, F, versus indentation depth, δ. Sample F-δ curves are shown for single and bilayered PDMS. All measurements were performed in liquid using hemolymph (Drosophila) or deionized water containing 2% w/v bovine serum albumin (PDMS). Drosophila nanoindentation was performed only on freshly isolated specimens. No significant probe-sample adhesion was observed.

Force-indentation curves made from a spherical indenter pressing into a sufficiently thick material with uniform properties are commonly analyzed by a method from Hertz (Hertz, 1882). In this model, the elastic modulus, E, is directly proportional to the load, F, distributed over the contact area and inversely proportional to indentation depth, δ:

$$F=4/3E/((1-\nu^2))\ \sqrt{(R\delta^3)}$$

where R is the radius of the sphere and ν is the Poisson ratio of the surface. Hertz analysis is highly dependent on accurately choosing the probe-material contact point, which is difficult for soft materials, and produces only a single elastic modulus (Engler et al., 2007). Equation 1 was transformed so that Equation 2 is now linearly dependent on indentation depth changes and the slope is directly proportional to $E^{2/3}$ (see Figure 2b) (Guo and Akhremitchev, 2006):

$$\Delta F^{2/3}/\Delta\delta=(4/3\ E/((1-\nu^2))\ \sqrt{R})^{2/3}$$

From this transformation, elastic modulus can be directly calculated from the slope without the need to determine the contact point. Linearized-Hertz and conventional force-

indentation curves (labeled as Hertz) were analyzed using custom written software in MATLAB (Mathworks, Natick, MA) that automates linear regression and least squares fitting analysis of the raw data to Equations 1 and 2, respectively. This software is available for download at http://ecm.ucsd.edu/AFM.html, as well as example force curves used in this analysis.

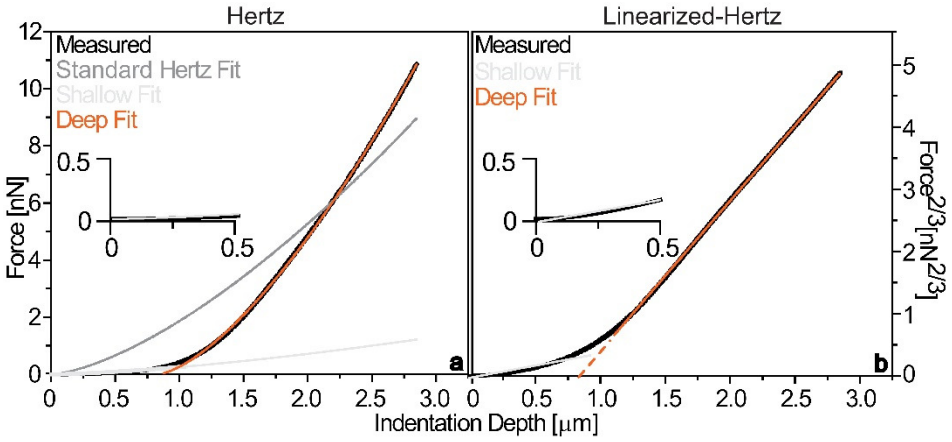

Figure 3.3. Nanoindentation analysis of *Drosophila* myocardium. Fitting of Hertz and linearized-Hertz transform equation.

To outline the software's approach, the $F^{2/3}$-δ curve is fit from maximum indentation depths backward toward the contact point in an iterative fashion until the coefficient of determination falls below 0.99. This isolates the fit slope of the deep indentation, which can be converted to an elastic modulus using Equation 2. The software then begins a second shallow-indentation fit of the $F^{2/3}$-δ curve from progressive indentation depths back toward the contact point until the coefficient of determination falls below 0.95, isolating the portion of the curve for which only the top layer is 'felt'. Note that the contact point does not need to be explicitly calculated as this transformation approach focuses on fitting linear regions of the $F^{2/3}$-δ curve. The first linear region will always be the approach portion of the curve and at least one other

linear region will be present and correspond to the material. The difference in slope between these lines will clearly delineate the regions of contact and non-contact.

In Hertzian analysis of F-δ curves from bilayers, we employ two new boundary conditions: 1) the indentation limit of the Hertzian shallow fit will be the same as for the Linearized-Hertz method and 2) the $F^{2/3}$-δ curve's deep fit x-intercept (Figure 2b; dashed orange line) will be used as a 'virtual contact point' to determine the starting point for a Hertzian deep fit. Using these modification produces two fits that mirror the Linearized-Hertz method by providing two moduli using coefficients of determination of 0.9 (Figure 2a; black data versus light gray and orange curve fits; black data versus green and red curve fits). Due to error susceptibility differences in curve shape, i.e. F vs $F^{2/3}$, as well as errors in contact point determination, the modified Hertz fit could not always be computed for each curve fit with the Linearized-Hertz method, hence the differences in the number of data points for Figures 5 and 6.

Sample Size and Statistical Analysis

Indentation analysis was performed approximately at the centerline of the conical chamber for all flies, with typically16 F-δ curves per fly. Measurements were performed on 22, 20, and 21 flies per group yielding 301, 244, and 301 total analyzable curves for 1 week old yw, 5 week old yw, and 1 week old MHC RNAi-expressing heart tubes, respectively. Due to variations between PDMS samples, single samples were indented at least three different locations on the sample with 192, 128, 74, and 64 curves analyzed for indentations of bilayered, 75 μm thick/1:45 curing ratio, 75 μm thick/1:60 curing ratio, and 8.5 μm thick/1:60 curing ratio PDMS, respectively. PDMS loading rate experiments were analyzed from indentations at at least three different locations on the sample with 64 curves each. All PDMS samples within each plot were made simultaneously from the same solutions. All box-and-whisker plots show

and have statistics computed for all data pooled together, whether from multiple samples or single samples as indicated above. Statistical significance was determined by Wilcoxon rank-sum test of the stiffness distributions, where statistical significance was assigned when p was at least less than 0.05.

Results

Indentation Analysis

The F-δ behavior of Drosophila myocardium was found not to match Hertz model behavior for indentation of a single material, i.e. Equation 1 (Figure 2a; black versus gray curves). This suggests the heterogeneous muscle layers of the Drosophila heart likely exhibit distinct biophysical properties. A power-law transformation was performed to linearize Equation 1 (F-δ data) into Equation 2 (F2/3-δ), referred to as the Linearized-Hertz equation since Hertzian material behavior is not represented by a straight line on the plot. Using an algorithm to detect these linear regimes with fitting tolerances at least exceeding $R2 > 0.95$ in the transformed curve, the plot of force to the 2/3 power versus indentation (F2/3-δ) was found to become linear in two distinct regions with different moduli (Figure 2b). We hypothesized that this was due to the distinct bilayered structure of the Drosophila heart where, up to indentations <10% of heart thickness, mechanics are dominated by one material or another depending on indentation depth. The ventral layer is only ~1-2 μm thick (Figure 1b), so linear fits of shallow indentations represent small strains (Figure 2b; inset) and produce moduli that agree with previous Hertzian analysis (Engler et al., 2004b; Roduit et al., 2009). When the ventral layer is completely compressed at deep indentations, cardiomyocyte layer properties should appear and represent the second linear portion of the plot as previously predicted by computational finite element analysis (Roduit et al., 2009). The transition region between the two layers is likely dependent on moduli and thickness differences of the layers, as predicted both from indentation

and modeling (Dimitriadis et al., 2002; Engler et al., 2004b; Reinhart-King et al., 2003; Roduit et al., 2009). However, the model set forth here is focused on linear regions of the transform and not the transition as the former indicates bulk material stiffness of the single tissue layer bearing the brunt of that strain at that point of indentation.

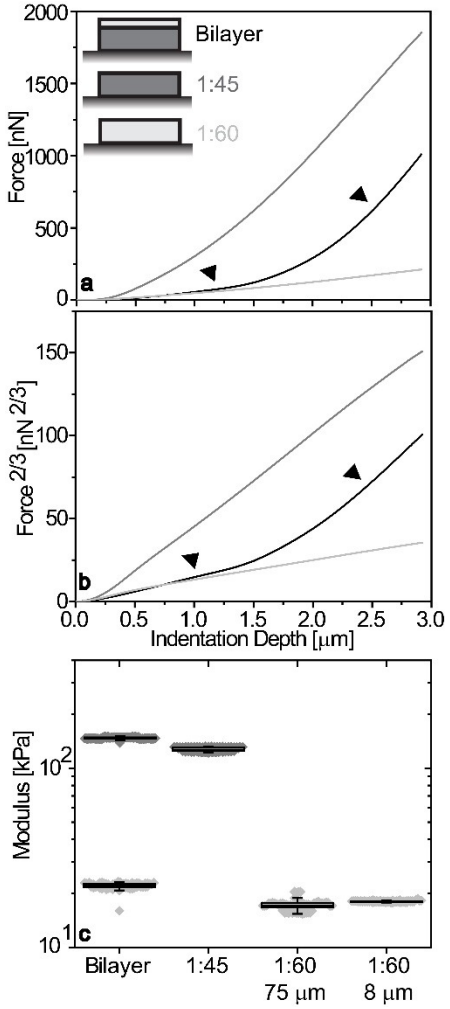

Figure 3.4. Indentation of single and bilayer polymer materials. Depth-dependent mechanics and extraction of moduli in bilayered PDMS.

To verify this interpretation and analysis, polydimethylsiloxane (PDMS) was fabricated as a synthetic model system with either one or two layers. Each layer contained PDMS of a different polymer curing ratio to modulate stiffness and differed in thickness. Samples were indented by AFM, and to confirm linearity, force and force to the 2/3 power were plotted versus cantilever z-position (F-z-position and $F^{2/3}$-z-position, respectively) to include the approach region before indentation. For linear regions in the $F^{2/3}$-z-position plot, its derivative, $dF^{2/3}/dz$, should result in a straight, flat line. $F^{2/3}$-z-position plots of bilayered PDMS exhibit 3 linear regions. These correspond to moduli describing tip approach (which is zero), the top layer, and the second layer (indicated by dashed lines) with the latter two linear regions separated by a non-linear transition. This analysis does not rely on directly determining the contact point between the material and the AFM tip. As with Drosophila, transformation of PDMS force-indentation curves (Figure 3a) again shows linear behavior that permits the determination of an elastic modulus over that linear indentation depth range (Figure 3b). Single layer PDMS exhibited a near 10-fold difference in elastic modulus as a function of polymer curing ratio, independent of PDMS thickness (Figure 3c). For bilayer PDMS, the slope of the force-indentation curve and its transform is quite shallow at first as the indentation depth of the transition between top and bottom layers was found to depend on thickness and moduli differences. Increasing thickness of the top layer allowed for increased compression before the effects of the bottom layer were observed. On the other hand, increasing bottom layer modulus allowed for less compression before onset of the modulus transition. This result is consistent with thin film AFM models where thicker and less mismatched layers require less correction due to lower strains (Clifford and Seah, 2006; Dimitriadis et al., 2002; Engler et al., 2004b; Roduit et al., 2009). Most importantly, the elastic modulus of the top layer is nearly identical to the single layer modulus of the same curing ratio, irrespective of its thickness (Figure 3c). After a transition region where both layers are likely compressed resulting in non-linear behavior,

transformation using Equation 2 again linearizes data and permits the determination of a second modulus (Figure 3c), which is not possible for thin film AFM models (Clifford and Seah, 2006; Dimitriadis et al., 2002; Engler et al., 2004b; Roduit et al., 2009). For bilayered PDMS, when the bottom layer was compared to single layered PDMS of the same curing ratio, layer elastic moduli were nearly identical. It should be noted that PDMS is a loading rate-sensitive material (Sun and Walker, 2005), and when indented at different loading rates, both single and bilayered materials had proportionately increased moduli as a function of PDMS curing ratio. For this reason, *Drosophila* measurements were made at the same loading rate.

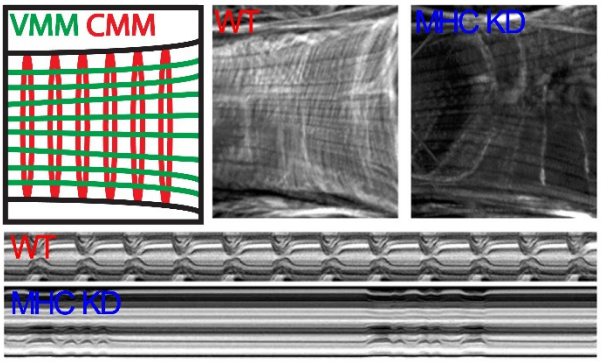

Figure 3.5. Physiological effects of MHC knockdown in the *Drosophila* heart tube. MHC KD results in loss of cardiac myofibrils and impaired contraction.

Cardiac-Specific MHC Knockdown

Myofibrillar disassembly likely leads to changes in myocardial stiffness. To verify that our analysis can determine independent mechanical changes in situ in a multilayered system such as the adult Drosophila heart tube, cardiac-specific (Han et al., 2006) RNAi for myosin heavy chain (MHC) was used to genetically modify cardiomyocytes and not ventral muscle fibers. Cardiomyocyte-restricted MHC knockdown induced a marked reduction in myofibrillar density in 1 week old flies relative to control (Figure 4; top). Moreover, severely depressed wall

motion and loss of rhythmic contractions confirmed extensive functional impairment of the heart tube (Figure 3.4; bottom). Both F-δ and F2/3-δ plots measured from ethylene glycol tetraacetic acid (EGTA)-arrested hearts indicated that there was no difference in slope at shallow indentation depths, corresponding to a lack of change in passive mechanics of the thinner ventral muscle. Interestingly, there was an appreciable difference between RNAi-treated and control flies at deep indentation depths, corresponding to differences in the mechanical properties of the cardiomyocytes (Figure 3.S6). Fits from the Linearized-Hertz and the Hertz analysis method based on Equation 1 confirmed that cardiac-specific RNAi did not significantly change median ventral muscle stiffness, i.e. < 10% (Figure 3.5a), but did cause a significant 2-fold reduction in median cardiomyocyte stiffness (Figure 3.5b). Despite the RNAi treatment, it is important to note that the cardiomyocytes remained an order of magnitude stiffer than the ventral muscle, ensuring the accuracy of our detection technique.

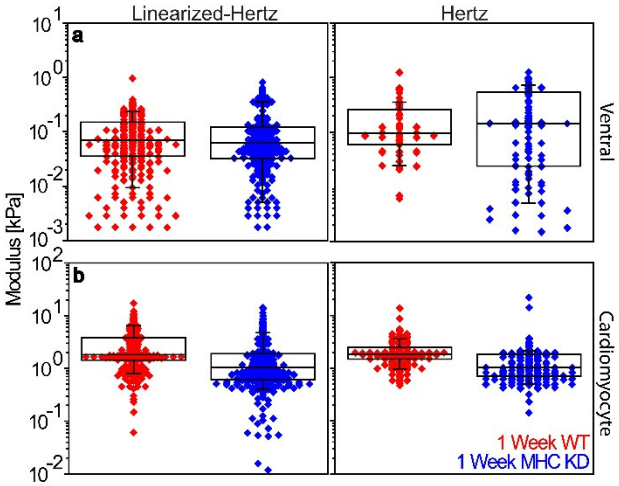

Figure 3.6. MHC knockdown decreases cardiomyocyte cortical stiffness. Cardiac stiffness decreases with MHC KD while ventral muscle is unchanged.

Cardiac Stiffness Increases with Age

In addition to robust genetic tools that make the organism a highly tractable system for investigating cardiac biology, Drosophila ages rapidly and therefore serves as an efficient model for studying senescent-dependent changes in myocardial properties. Hence we monitored heart tube stiffness as a function of age. Contrary to the effect of cardiac-restricted genetic manipulation of MHC (Figure 3.S6; compare red and blue curves), F-δ and F2/3-δ plots indicated an increase in slope for both shallow and deep indentation depths of EGTA-arrested hearts in 5 week old relative to 1 week old control flies (compare green and red curves, respectively). Using the Linearized-Hertz method, 5 week old flies were found to exhibit a statistically significant, 2.5-fold increase in median cardiomyocyte stiffness versus 1 week old flies (Figure 3.6a). Ventral stiffness increased 50% though remained an order of magnitude softer than cardiomyocytes at 5 weeks of age (Figure 3.6b).

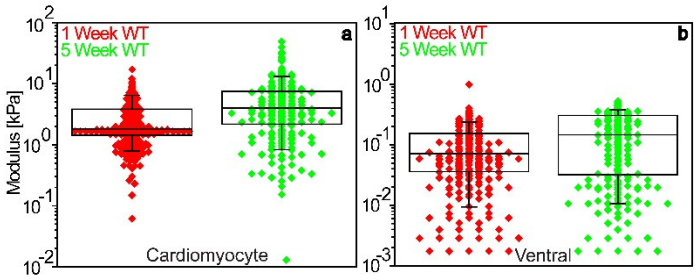

Figure 3.7. Aging increases cortical myocardial stiffness in wildtype Drosophila. Aging increases stiffness of both muscle layers with age.

Discussion

Analysis Advantages for Soft Bilayered Materials

Analysis of force-indentation curves from soft materials is often complicated by difficulties in determining the "contact point," the point at which the AFM probe contacts the

material, e.g. the F-δ curve slope may only be slightly higher than the thermal noise associated with probe fluctuations. The softness of many biological materials and the possibility for multiple layers of different materials further complicate the analysis (Engler et al., 2007). For layered materials, previous corrections to the Hertz model are useful specifically where the stiffness of one substrate is orders of magnitude greater than the other (Dimitriadis et al., 2002).

To date, there have only been limited observations of the phenomenon we observed in Drosophila myocardium, where the underlying materials of interest are both soft. Linearized-Hertz analysis of cell indentation reveals two separate depth-dependent moduli (Carl and Schillers, 2008) and varying stiffness by ablating cytoarchitectural proteins has been shown to induce changes in these depth-dependent moduli (Kim et al., 2011). The most complete analysis of this phenomenon has involved finite element modeling where force-indentation curves were identical at shallow indentations depths but increased with stiffening of in the underlying layer for deep indentation (Roduit et al., 2009). Though their work gave critical insight in how bilayers behave during nanoindentation qualitatively, no rigorous quantification of this phenomenon was performed. By using a Linearized-Hertz fit with sufficient indentation depth for both layered PDMS and the Drosophila heart tube, we further demonstrate the ability to determine stiffness of separate layers with similar mechanical properties but do so within well-characterized soft, bilayered materials in situ. While this Linearized-Hertz model sufficiently describes indentations of 2-3microns, other well-established hyperleastic indentation models may be more appropriate for deeper indentation or more complex cytoarchitectures (Lin et al., 2009).

It is important to note three considerations for this analysis method as it relates to either model system. First, the conical chamber of Drosophila is slightly rounded with a lateral pitch change of < 1o per µm (Figure 3.1b). By using a 2 µm radius sphere at the centerline of the tube, this curvature likely has negligible effects. Second, for all of the indentations performed,

the Linearized-Hertz fits converged to a solution for all curves given fitting tolerances. Even with adjustments made to the Hertz approach, e.g. fitting a deep indentation region with a "virtual" second contact point, fewer than half of the data could be fit by standard Hertzian analysis, accounting for differences in scatter plot density (Figures 5 and 6). This indicates that linearization produces a much more robust determination of biophysical parameters for bilayers since AFM measures relative z-position and force, which is the only measure needed for the linearized method.

Finally, the Hertz and Linearized-Hertz models are mathematically identical, so they should yield similar results, although Hertz fitting requires three fitting parameters (z-position and force at contact and the elastic modulus for which the fit is calculated) versus one parameter for Linearized-Hertz. Since AFM software displays relative indenter z-position and force, one must use algorithms to calculate the contact point, which is often done over a single range of z-position and applied to a single Hertz model (Engler et al., 2007). For a monolayer, model differences are negligible as only one contact point is needed. This is directly tested here for shallow indentations into the ventral muscle where both methods yield identical median moduli (Figure 3.5a).

To accurately measure bilayered materials with Hertzian analysis, a "contact point" for each layer must be obtained from separate analysis ranges. Otherwise, single contact point analysis will lead to a single, inaccurate modulus and poorly fit data (Figure 3.2a, gray versus black). Since imprecise contact-point-dependent analysis can introduce errors up to several fold even for monolayers (Azeloglu and Costa, 2011), this multi-"contact" approach for bilayers is likely to be increasingly error prone. Though methods that diminish the error introduced by a poorly calculated contact point have been established, these still require such a calculation (A-Hassan et al., 1998). In comparison, the Linearized-Hertz method relies only on the slope of the curve and thus is a truly "contact-point-independent" analysis method. Proper analysis only

requires that the user indent the material to an appropriate depth and then fit data based on relative differences within the force-indentation curve. In the case of bilayered materials, linearization of a sufficiently deep force-indentation curve reveals two straight lines both visually (Figure 3.2b, black) and through fitting (Figure 3.2b, orange and light grey). For materials consisting of three or more sufficiently thick layers, as compared to the indenter radius, Linearized-Hertz analysis would reveal three lines in the same way. Thus the advantage of this analysis method is that the $F2/3$-δ plots of Linearized-Hertzian analysis give straight lines that are easy to fit, making this fitting method more user-friendly and intuitive to novice biological AFM users.

Analyzing the Origins of Biological Stiffness

Direct application of sufficient force to ventrally-exposed, beating Drosophila myocardium results in the attenuation of rhythmic beating. A 50 μm indentation reportedly causes a total occlusion of the larval Drosophila heart tube (Senatore et al., 2010). This level of myocyte deformation is known to open the stretch-sensitive, calcium-permeable transient receptor potential (TRP) channel Painless, which would increase intracellular free calcium and potentially augment force production (Senatore et al., 2010). It is critical to note that the AFM indentation performed here is more than an order of magnitude smaller than indentation that induced cardiomyocyte arrest. For example, a 2μm-radius sphere indenting the adult Drosophila heart tube would create no more than 0.151% strain when examined in cross-section (Figure 3.S7). Thus nominal indentation of a relaxed heart with open ostial valves and a sufficiently thick wall (Figure 3.1b) would produce negligible global tube compression and thus not require a more complicated indentation model accounting for fluid compression within the tube. Moreover, this small amount of strain is below previously observed strain required for channel activation (Charras and Horton, 2002) and emphasizes that this AFM method seeks to minimize

strain to measure an intrinsic, passive material property and not a mechanobiological response. If, however, our indentation caused Painless or additional stretch sensitive channels to open in adult hearts, rapid myocyte stiffening should have been observed with the linearized F2/3-δ curve becoming non-linear, which was not the case (Figure 3.S2) and suggests active stiffening is not transpiring.

How Genetics and Aging Affects Drosophila Heart Stiffness

Due to its short life-cycle and genetic tractability, Drosophila is an ideal model organism for investigating senescent-dependent changes in cardiac biology with relatively high temporal throughput. A recent Drosophila genome-wide screen found that expression of a significant number of adhesion-related genes was vital to heart performance (Neely et al., 2010). Age-related differences in expression of these genes would likely affect cardiac function. The analysis method described here would thus serve as a useful biophysical method to complement current microscopy analyses used to identify the passive mechanical role played by these proteins, e.g. myofibril disassembly and decreased fractional shortening–the difference in heart tube diameter at systole relative to diastole (Cammarato et al., 2008; Taghli-Lamallem et al., 2008). This is especially true for age-related studies considering the significant age-related increase in myocardial stiffness observed by AFM (Figure 3.6). Our method could also act as a screen and/or confirm the function of mammalian-conserved, mechanically-sensitive proteins, such as has been implicated with the cell adhesion molecules faint sausage and DE-cadherin and the extracellular matrix protein laminin A (Haag et al., 1999). These proteins are essential for tube formation and maintenance, respectively, and their ablation, which is known to cause breaks in the heart tube during embryonic development (Haag et al., 1999), may likely impair force transduction, decrease sarcomere assembly, and thus decrease myocyte stiffness. This

connection is highly plausible given the link shown here between MHC knockdown, reduced myofibrillar content (Figure 3.4), and cell softening (Figure 3.5).

It is important to note that genetic changes can be localized to cardiomyocytes with the Hand-Gal4 (II) driver (Han et al., 2006) so that comparisons of control and RNAi–treated Drosophila can occur in a cardiac-autonomous fashion in the absence of other defects. For example, Figure 3.5 confirms the ability of our method to parse individual tissue properties between the muscle layers and likely does not reflect contributions from matrix between the muscle layers as it exceedingly thin, i.e. tens of nanometers (Miller, 1950), compared to the muscle layers and the size of our indenter. Moreover, the lack of change in ventral muscle despite MHC knockdown in adjacent cardiomyocytes also suggests that there is little interdependence between the two muscle layers as observed classically in single cells (Vogel and Sheetz, 2006).

One complication of using biological specimens is the significant animal-to-animal variation in stiffness; measurement variance within a given fly for example ranged from 5 to 40 percent as a function of age (1 to 5 weeks) and up to 20 percent with MHC knockdown. Although this variance is similar to that seen with other AFM cell and tissue analyses (Collinsworth et al., 2002; Engler et al., 2004a), the relatively high temporal throughput of the Drosophila model for age-or disease- related analyses combined with the automated analysis of our method makes the number of specimens analyzed limited only by the ability of the end user to generate AFM-ready samples. We found that sufficient statistical power was achieved, i.e. significant differences in populations as determined by Wilcoxon rank-sum tests, when sample populations exceeded 20. With an average measurement time of 20 min per fly, a sufficiently large data set for a given fly genotype can be obtained in a matter of hours, making this technique suitable for the average end user to perform relatively large high throughput screens.

We believe that this linearized-Hertz method of force-indentation analysis can provide biologists with a direct biophysical understanding of how specific perturbations alter tissue function, which cannot be done with preexisting genetic tools and imaging methods (Ocorr et al., 2007; Taghli-Lamallem et al., 2008; Wolf et al., 2006). Though we present analysis in the fly heart tube here, and to our knowledge the first demonstration of direct, passive mechanical in situ measurements of cellular bilayers, applications in other layered tubes and tissues which previously could not be analyzed biophysically, such as blood vessels and skin, respectively, can be easily adapted to this method making it a useful tool to the biological community.

Acknowledgements

This chapter is adapted from a previously-published journal article: Kaushik G., Fuhrmann A., Cammarato A., and Engler A.J. (2011). In situ mechanical analysis of myofibrillar perturbation and aging on soft, bilayered Drosophila myocardium. Biophysical journal *101*, 2629-2637. The authors thanks Dr. Rolf Bodmer, Nakissa Alayari, and Joan Choi (Sanford-Burnham Medical Research Institute) for support and for assistance with Drosophila microsurgery and Ludovic Vincent (University of California, San Diego) for assistance with microfabrication.

References

A-Hassan, E., Heinz, W.F., Antonik, M.D., D'Costa, N.P., Nageswaran, S., Schoenenberger, C.A., and Hoh, J.H. (1998). Relative microelastic mapping of living cells by atomic force microscopy. Biophys J 74, 1564-1578.

Azeloglu, E.U., and Costa, K.D. (2011). 19. Atomic Force Microscopy in Mechanobiology: Measuring Microelastic Heterogeneity of Living Cells. In Atomic Force Microscopy in Biomedical Research Methods and Protocols, P.C.R. Braga, D., ed. (New York: Springer), pp. 303-329.

Cammarato, A., Dambacher, C.M., Knowles, A.F., Kronert, W.A., Bodmer, R., Ocorr, K., and Bernstein, S.I. (2008). Myosin transducer mutations differentially affect motor

function, myofibril structure, and the performance of skeletal and cardiac muscles. Molecular biology of the cell 19, 553-562.

Carl, P., and Schillers, H. (2008). Elasticity measurement of living cells with an atomic force microscope: data acquisition and processing. Pflugers Arch 457, 551-559.

Charras, G.T., and Horton, M.A. (2002). Single cell mechanotransduction and its modulation analyzed by atomic force microscope indentation. Biophys J 82, 2970-2981.

Clifford, C.A., and Seah, M.P. (2006). Modelling of nanomechanical nanoindentation measurements using an AFM or nanoindenter for compliant layers on stiffer substrates. Nanotechnology 17, 5283.

Clifford, C.A., and Seah, M.P. (2009). Nanoindentation measurement of Young's modulus for compliant layers on stiffer substrates including the effect of Poisson's ratios. Nanotechnology 20, -.

Collinsworth, A.M., Zhang, S., Kraus, W.E., and Truskey, G.A. (2002). Apparent elastic modulus and hysteresis of skeletal muscle cells throughout differentiation. Am J Physiol Cell Physiol 283, C1219-1227.

Dimitriadis, E.K., Horkay, F., Maresca, J., Kachar, B., and Chadwick, R.S. (2002). Determination of elastic moduli of thin layers of soft material using the atomic force microscope. Biophysical journal 82, 2798-2810.

Engler, A.J., Humbert, P.O., Wehrle-Haller, B., and Weaver, V.M. (2009). Multiscale modeling of form and function. Science 324, 208-212.

Engler, A.J., Rehfeldt, F., Sen, S., and Discher, D.E. (2007). Microtissue elasticity: measurements by atomic force microscopy and its influence on cell differentiation. Methods Cell Biol 83, 521-545.

Engler, A.J., Richert, L., Wong, J.Y., Picart, C., and Discher, D.E. (2004a). Surface Probe Measurements of the Elasticity of Sectioned Tissue, Thin Gels and Polyelectrolyte Multilayer Films: Correlations Between Substrate Stiffness and Cell Adhesion. Surface Science 570, 142-154.

Engler, A.J., Richert, L., Wong, J.Y., Picart, C., and Discher, D.E. (2004b). Surface probe measurements of the elasticity of sectioned tissue, thin gels and polyelectrolyte multilayer films: Correlations between substrate stiffness and cell adhesion. Surface Science 570, 142-154.

Fink, M., Callol-Massot, C., Chu, A., Ruiz-Lozano, P., Izpisua Belmonte, J.C., Giles, W., Bodmer, R., and Ocorr, K. (2009). A new method for detection and quantification of heartbeat parameters in Drosophila, zebrafish, and embryonic mouse hearts. Biotechniques 46, 101-113.

Goffin, J.M., Pittet, P., Csucs, G., Lussi, J.W., Meister, J.J., and Hinz, B. (2006). Focal adhesion size controls tension-dependent recruitment of alpha-smooth muscle actin to stress fibers. The Journal of cell biology 172, 259-268.

Guo, S., and Akhremitchev, B.B. (2006). Packing density and structural heterogeneity of insulin amyloid fibrils measured by AFM nanoindentation. Biomacromolecules 7, 1630-1636.

Haag, T.A., Haag, N.P., Lekven, A.C., and Hartenstein, V. (1999). The role of cell adhesion molecules in Drosophila heart morphogenesis: faint sausage, shotgun/DE-cadherin, and laminin A are required for discrete stages in heart development. Developmental biology 208, 56-69.

Han, Z., Yi, P., Li, X., and Olson, E.N. (2006). Hand, an evolutionarily conserved bHLH transcription factor required for Drosophila cardiogenesis and hematopoiesis. Development 133, 1175-1182.

Hertz, H. (1882). Über die Berührung fester elastischer Körper. J Reine Angew Mathematik 92, 156-171.

Kim, Y., Kim, M., Shin, J.H., and Kim, J. (2011). Characterization of cellular elastic modulus using structure based double layer model. Med Biol Eng Comput 49, 453-462.

Lieber, S.C., Aubry, N., Pain, J., Diaz, G., Kim, S.J., and Vatner, S.F. (2004). Aging increases stiffness of cardiac myocytes measured by atomic force microscopy nanoindentation. American journal of physiology Heart and circulatory physiology 287, H645-651.

Lin, D.C., Shreiber, D.I., Dimitriadis, E.K., and Horkay, F. (2009). Spherical indentation of soft matter beyond the Hertzian regime: numerical and experimental validation of hyperelastic models. Biomech Model Mechanobiol 8, 345-358.

Mahaffy, R.E., Park, S., Gerde, E., Kas, J., and Shih, C.K. (2004). Quantitative analysis of the viscoelastic properties of thin regions of fibroblasts using atomic force microscopy. Biophys J 86, 1777-1793.

Miller, A. (1950). The internal anatomy and histology of the imago of Drosophila melanogaster. In Biology of Drosophila, M. Demerec, ed. (Cold Spring Harbor, NY Cold Spring Harbor Laboratory Press), pp. 420-534.

Neely, G.G., Kuba, K., Cammarato, A., Isobe, K., Amann, S., Zhang, L., Murata, M., Elmen, L., Gupta, V., Arora, S., Sarangi, R., Dan, D., Fujisawa, S., Usami, T., Ocorr, K., Bodmer, R., Penninger, J.M. (2010). A global in vivo Drosophila RNAi screen identifies NOT3 as a conserved regulator of heart function. Cell 141, 142-153.

Ocorr, K., Reeves, N.L., Wessells, R.J., Fink, M., Chen, H.S., Akasaka, T., Yasuda, S., Metzger, J.M., Giles, W., Posakony, J.W. (2007). KCNQ potassium channel mutations cause cardiac arrhythmias in Drosophila that mimic the effects of aging. Proceedings of the National Academy of Sciences of the United States of America 104, 3943-3948.

Radmacher, M. (2002). Measuring the elastic properties of living cells by the atomic force microscope. Methods Cell Biol 68, 67-90.

Reinhart-King, C.A., Dembo, M., and Hammer, D.A. (2003). Endothelial cell traction forces on RGD-derivatized polyacrylamide substrata. Langmuir 19, 1573-1579.

Rotsch, C., Jacobson, K., and Radmacher, M. (1999). Dimensional and mechanical dynamics of active and stable edges in motile fibroblasts investigated by using atomic force microscopy. Proc Natl Acad Sci U S A 96, 921-926.

Senatore, S., Rami Reddy, V., Semeriva, M., Perrin, L., and Lalevee, N. (2010). Response to mechanical stress is mediated by the TRPA channel painless in the Drosophila heart. PLoS Genet 6.

Sun, Y., and Walker, G.C. (2005). Viscoelastic response of poly(dimethylsiloxane) in the adhesive interaction with AFM tips. Langmuir 21, 8694-8702.

Taghli-Lamallem, O., Bodmer, R., Chamberlain, J.S., and Cammarato, A. (2008). Genetics and pathogenic mechanisms of cardiomyopathies in the Drosophila model. Drug Discovery Today: Disease Models 5, 125-134.

Vogel, V., and Sheetz, M. (2006). Local force and geometry sensing regulate cell functions. Nat Rev Mol Cell Biol 7, 265-275.

Vogler, G., and Ocorr, K. (2009). Visualizing the beating heart in Drosophila. Journal of visualized experiments : JoVE.

Wasserthal, L.T. (2007). Drosophila flies combine periodic heartbeat reversal with a circulation in the anterior body mediated by a newly discovered anterior pair of ostial valves and 'venous' channels. J Exp Biol 210, 3707-3719.

Wolf, M.J., Amrein, H., Izatt, J.A., Choma, M.A., Reedy, M.C., and Rockman, H.A. (2006). Drosophila as a model for the identification of genes causing adult human heart disease. Proceedings of the National Academy of Sciences of the United States of America 103, 1394-1399.

Chapter 4

Vinculin-mediated Cytoskeletal Remodeling in Aging Myocardium

Introduction

The average age worldwide is projected to increase dramatically in the coming decades. Advanced age is a primary risk factor for cardiac dysfunction and subsequent morbidity and mortality (*1*). Treating age-related heart failure is complicated due to its heterogeneous etiologies. A multitude of events are associated with cardiac aging on multiple scales, which are thought to impair systolic and diastolic myocardial performance (*1, 2*). Remodeling fundamentally begins with molecular changes, such as altered cell growth regulation (*3*) and protein expression (*4*). Cardiomyocyte renewal in the heart is limited (*5*), suggesting that functional maintenance may depend upon molecular remodeling over time or age-related compensatory responses to minimize damage. Identifying individual, age-associated compensatory events is made difficult because of the diversity of aging processes within and between conventional models as well as an abundance of maladaptive events. However, integrated approaches that identify conserved hallmarks of cardiac aging and verify their positive or negative functional consequences can assist in the identification of therapeutic targets for treating age-related heart failure or improving outcomes during aging.

The cortical cytoskeleton in cardiomyocytes couples sarcomeres to the membrane at cell-matrix (costameric) and cell-cell (intercalated disc or ID) junctions to translate molecular movements into cell shortening. It undergoes significant remodeling during heart failure in aging patients (*6, 7*). Mechanical unloading via left-ventricular assist devices can restore baseline cytoskeletal gene expression and contractile function (*8-10*), suggesting a relationship between the cytoskeleton, ventricular contractility, and ventricular load. Aging can also alter specific cytoskeletal proteins' mechanotransduction, the signaling induced by changing physical forces. Vinculin, for example, is force-sensitive (*11, 12*) and regulates cell shape (*13, 14*) and intracellular signaling (*15, 16*). In cardiomyocytes, vinculin is localized to both costameres and IDs (*14, 17*), reinforces each during mechanical loading (*14, 18*) by crosslinking

transmembrane adhesion proteins with the cortical actin superstructure (*19, 20*), and its mutation results in cardiomyopathy (*21*). In aging patients, vinculin is overexpressed at both costameres and IDs (*7*), which suggests that it may influence myocardial performance with age. However, conservation of cardiomyocyte cytoskeletal remodeling and the structural and functional consequences of vinculin overexpression specifically have never been investigated. Therefore, we investigated the impact of cortical cytoskeletal remodeling on cardiac cell structure, mechanics, and the maintenance of function with advanced age.

To assess conservation of known aging hallmarks and identify novel molecular alterations, we analyzed the left ventricular proteomes of adult and aged simians (*Macacca mulatta*) and rats (*Rattus norvegicus*) using mass spectrometry (MS), representing the first detailed analysis of the human-like aging simian cardiac proteome. Subsequent bioinformatic investigations suggest that the cytoskeletal vinculin network has a central, conserved role during cardiac aging. Using *Drosophila melanogaster*, a rapidly aging and genetically tractable model system, we observed that cardiac overexpression of vinculin induced extensive cytoskeletal and cellular remodeling. It also correlated with preserved contractile function with age and enhanced contractility in both wildtype and myosin-deficient fly hearts. Average and maximal lifespan were significantly extended in *Drosophila* with cardiac-specific vinculin-overexpression. In contrast to a perception that remodeling is predominantly maladaptive, these data strongly suggest a beneficial role for vinculin-mediated restructuring in the aging heart. This is the first study demonstrating cytoskeletal remodeling as a compensatory mechanism in which tensional homeostasis is altered in order to preserve cardiac function during aging.

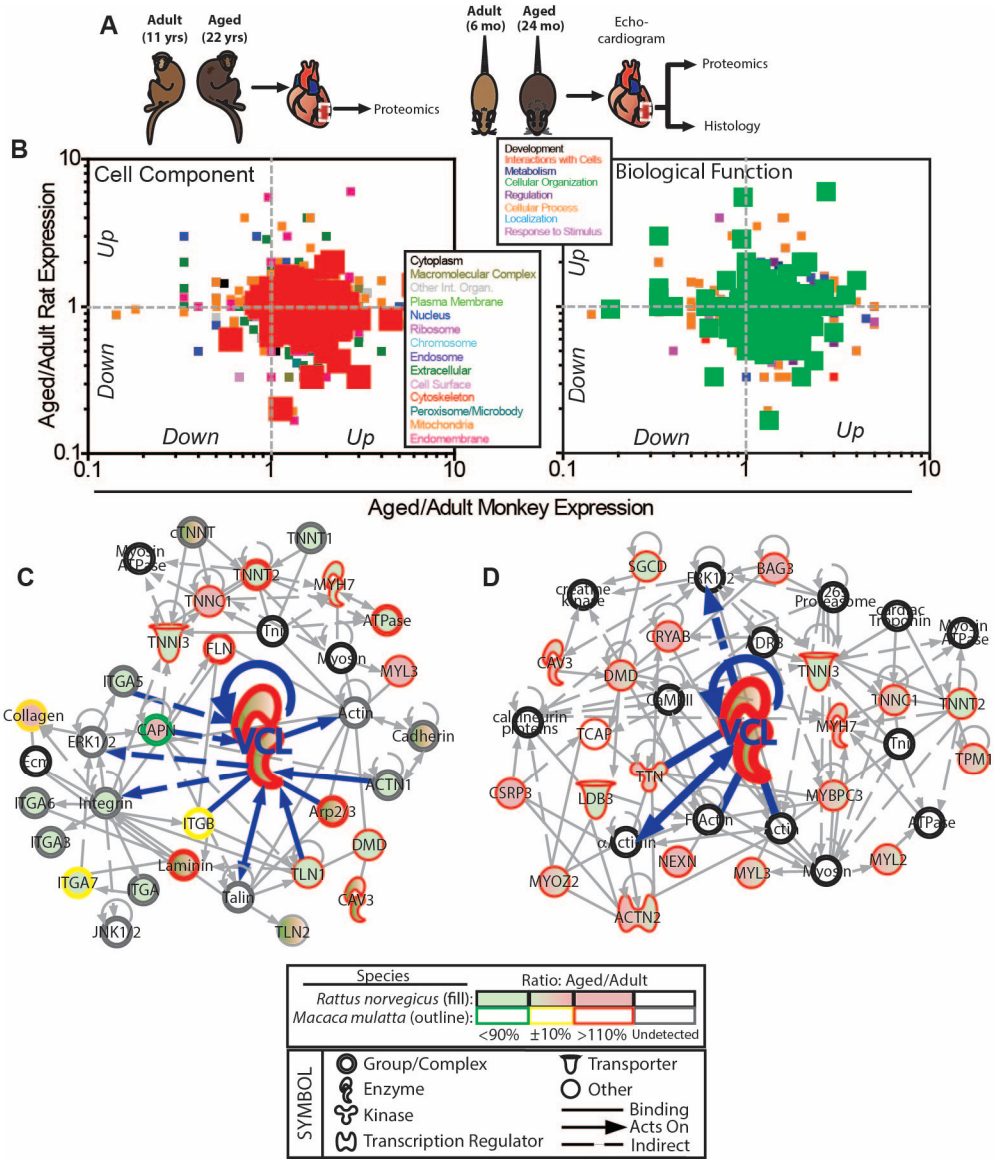

Figure 4.1: Proteomic analysis of mammalian left ventricles. A. Experimental design for protein isolation. B. Comparative changes in protein expression grouped by gene ontology. C. Changes in the vinculin (VCL) pathway. D. Biomarkers and therapeutic targets of cardiac aging.

Results

Age-related Remodeling in Simian and Murine Ventricles

Proteomic quantification of adult versus aged rhesus monkey (11 vs. 22 years) and rat (6 vs. 24 months) left ventricular free wall myocardium was performed (Figure 4.1A). Tryptic

peptides were detected using reversed-phase liquid chromatography mass spectrometry (MS) and a total of 1,206 (monkey) and 1,086 (rat) non-redundant proteins were quantified. Of these proteins, 602 were common between species (Figure 1). Categorization by ontological annotation was performed using Software Tool for Rapid Annotation of Proteins (STRAP) and Ingenuity Pathway Analysis (IPA). Cytoskeletal proteins were among the most enriched groups in monkeys and rats despite differences in gender and age ratio. Moreover, the most enriched biological functions in both species involved molecules with a role in subcellular organization, or formation and maintenance of intracellular and/or organelle structure. Based on label-free quantification via normalized spectral counts, proteins involved in the cytoskeleton and cell organization had increased quantity with age, especially in monkeys. These proteins were associated with cardiac biological functions, including cardiovascular disease, and with numerous cardiac toxicological functions, such as arrhythmia, hypertrophy, and failure. Given increased spectral counts with age for peptides associated with cytoskeletal ontologies, IPA was employed to determine interaction networks. In Figure 4.1C, expression changes for rat (filled shapes) and monkey (shape outlines) are shown for a cytoskeletal network centered on vinculin, which exhibited significant age-associated upregulation in rhesus monkeys (+32%). A significant change in vinculin abundance in rats (-9%) was not detectable by our MS-based approach. However, increases in vinculin network components (Figure 4.1C,D) and expression of vinculin itself, as measured by western blotting (+35%), were observed (Figure 4.2A). Figure 4.1D additionally depicts a vinculin-centric network of cardiac aging biomarkers whose deletion or mutation are associated with cardiomyopathy and may serve as therapeutic targets. Most of these markers are contractile- or cytoskeletal-related.

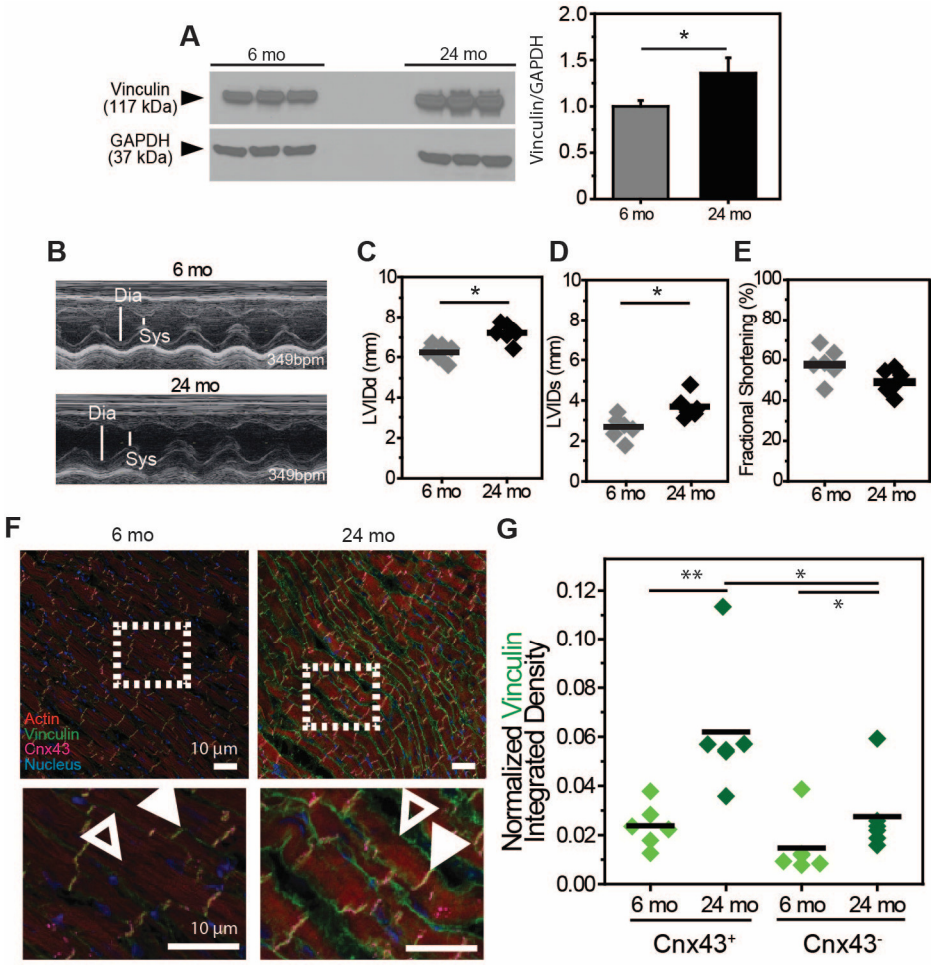

Figure 4.2: Vinculin expression and localization in aging rats. A. Cardiac vinculin expression increases with age. B. Comparison of ventricular contraction. C, D, E. Biometrics of adult and aged rats. F, G. Altered vinculin expression in aged rats.

Vinculin Localization Changes in Aging Rat Ventricles

Prior to isolation, physiological assessments were performed on rat hearts. Rats experienced increased body weight, heart weight, and total cardiac output with age, although heart weight and cardiac output normalized to body weight did not differ, suggesting that they were undergoing physiological aging and non-pathological hypertrophy at this age. Kymographs from echocardiography (Figure 4.2B) identified significant changes in diastolic and systolic left ventricular internal dimension (Figure 4.2C and D) and resulted in a modest

decrease in fractional shortening with age (Figure 4.2E) as observed previously (*22*). Histology indicated an age-related increase in ID (connexin 43-colocalizing) and non-ID localization of vinculin in aged rat myocardium (Figure 4.2F, G), reminiscent of elderly patient hearts (*23*). Vinculin is found at the cell membrane in association with transmembrane proteins with its head domain and, upon binding, bundles cortical filamentous actin with its tail domain (*19, 24, 25*). Thus, increased vinculin expression may reinforce the cortical actin superstructure during aging, leading to measurable changes in cortical stiffness. To directly evaluate this, atomic force microscopy (AFM)-based nanoindentation was performed on isolated rat cardiomyocytes to probe cortical stiffness, a reflection of cytoskeletal integrity or actin superstructure abundance and its cross-linking by actin-binding proteins such as vinculin. Stiffness measurements revealed no significant differences between age groups. However, isolated cells at these ages were poorly spread and minimally adherent, suggesting that isolation compromised cytoskeletal integrity. Thus, mechanical assessment required use of *Drosophila,* an *in situ* model system with preserved cytoskeletal structure and protein homology.

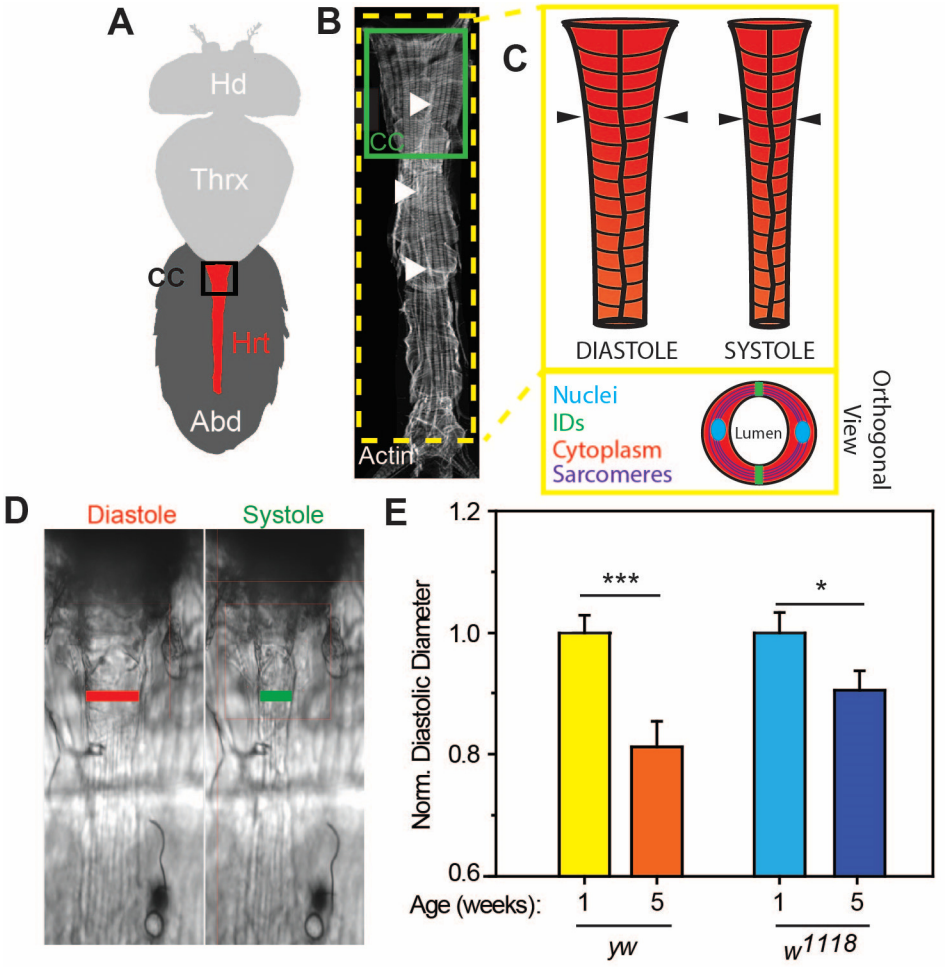

Figure 4.3: Age-related diastolic remodeling in the *Drosophila* heart. A. Ventral image of fly. B. Actin stain of heart. C. Cartoon depicting contraction of heart. D. Ventral brightfield image of heart during diastole and systole. E. Diastolic diameter declines with age.

Drosophila Heart Remodeling is Genotype-dependent

The *Drosophila* heart consists of a bilateral row of cardiomyocytes that form a contractile, tubular structure with a prominent anterior region called the conical chamber (CC; Figure 4.3A and B). β1-integrin (βPS1)-positive IDs exist at the ventral and dorsal surfaces (green; Figure 4.3C). We first quantified changes in heart size with age in various laboratory fly strains. Direct visual recording of heartbeats (Figure 4.3D) was performed as previously (*26, 27*). Altered diastolic diameters are characteristic of cardiac remodeling and we found that they

were significantly diminished at 5 weeks of age (aged) as compared to 1 week (adult) for both yellow-white (*yw*) and white (w^{1118}) strains, consistent with previous results, which have implicated diastolic restriction as the primary driver of impaired function in aged *Drosophila* (*28*). Both absolute and percent reduction in diastolic diameter was genotype-dependent, with the greatest reduction occurring in *yw* (Figure 4.3E), suggesting genotype-specific severity in chamber remodeling.

A correlation between decreased diastolic diameters and increased cortical stiffness has been previously demonstrated in aging and *Drosophila* cardiomyopathy models (*26, 29*). Thus, we examined whether genotype-dependent changes in diastolic diameters with age correlated with altered cortical stiffness in intact *Drosophila* hearts. The conical chambers (Figure 4.4A) of relaxed hearts were indented at the ventral midline, proximal to IDs (Figure 4.4B, green overlay), and at distal positions, upon the costameres (red dots, Figure 4.4B). Cardiomyocytes from 1 wk flies had relatively homogeneous stiffness independent of genotype (Fig. 4C, yellow squares). However, *yw* exhibited dramatic stiffening (Figure 4.4C, orange squares) with preference to the ventral midline, indicating that diastolic chamber remodeling correlates with increased cortical stiffness. Analogous measurements have indicated that apparent cortical stiffness is a function of the tension within the myocytes, which is proportional to the number of cross-bridges engaged, and the degree of cytoskeletal assembly and bundling by actin-binding proteins (*29-31*). To examine the underlying contributors to stiffness in our system, hearts were incubated in either blebbistatin, which inhibits actomyosin cross-bridge cycling, or cytochalasin D, which impairs cardiac contractility and additionally depolymerizes cortical, filamentous actin (*32*). 5 wk *yw* flies exhibited significant reduction in stiffness at the midline following blebbistatin treatment but reduction was more pronounced in cytochalasin D-treated flies (Figure 4.4D). These data suggest that increased resting tension in the relaxed heart does

not account for the majority of the measured stiffness increase and that the state of the actin cytoskeleton is a significant component of age-related stiffening.

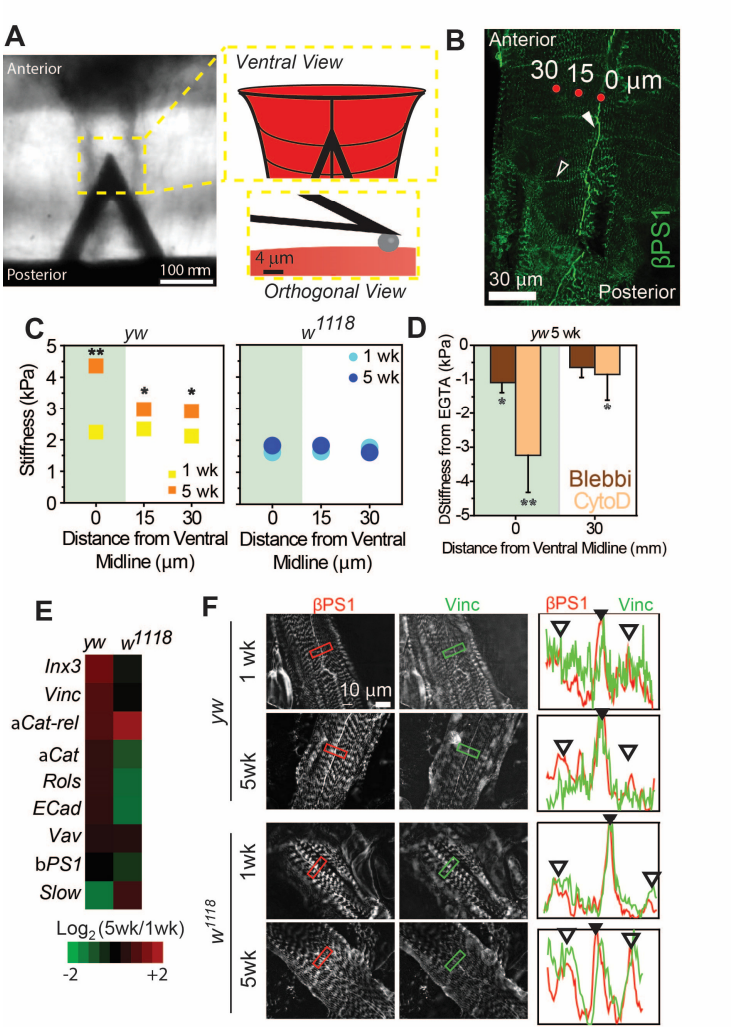

Figure 4.4: Stiffening is correlated with increased cytoskeletal gene expression. A. Indenter above heart. B. Indentation protocol. C. *yw* stiffness increases with age. D. Stiffening is associated with increased actin polymerization and E. actin-binding gene expression. F. Vinculin and integrin colocalization.

Expression changes in candidate actin-binding molecules were investigated from excised fly hearts using qPCR to determine the molecular underpinnings of these changes. A majority of cytoskeletal gene transcripts were upregulated with age in *yw*, including *Innexin 3*

(152%), *Vinculin* (103%), *αCatenin-related* (99%), and *αCatenin* (58%). However, in w^{1118} *flies*, most of these gene transcripts did not change significantly (Figure 4.4E). Since age-related vinculin localization changes were present in aged rats, we next assessed its preservation in *Drosophila*. Costameric localization was age- and genotype-dependent in flies; β*PS1* marked both the IDs (Figure 4.4F, filled arrowheads) and costameres (Figure 4.4F, open arrowheads) but exhibited a dominant peak co-localizing with *Drosophila* vinculin (*Vinc*) in aged *yw* flies. However, linescans of β*PS1* and vinculin along w^{1118} cardiomyocytes suggest that preferential localization shifts from IDs to both discs and costameres with age (Figure 4.4F). These data, in conjunction with an observed lack of cortical stiffening, indicate that minimal cytoskeletal reinforcement occurs in w^{1118} flies with age as compared to *yw*.

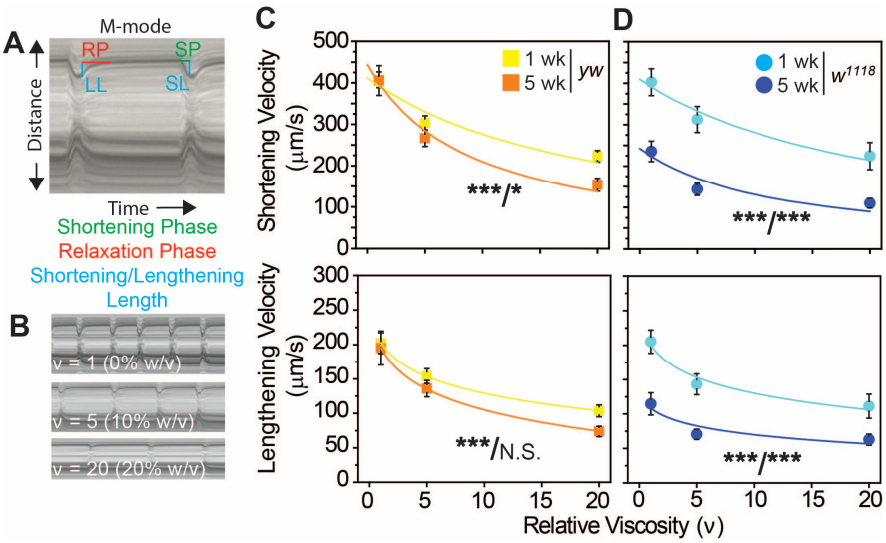

Figure 4.5: Contractile function in aging *Drosophila*. A. Motion-mode kymography of beating hearts with B. altered hemodynamic loads (viscosity). C. *yw* has preserved shortening velocity with age while D. w^{1118} does not.

Mechanical Function is Preserved in Remodeled, Aged Drosophila

To determine if increased passive stiffness is accompanied by altered active mechanics, we assessed the contractile dynamics of hearts beating against acute hemodynamic loads *in situ*. Precise measurements of shortening and lengthening intervals were made from high speed videos as indicated in representative m-mode images (Figure 4.5A) and the effect of load on contraction velocities was assessed (Figure 4.5B) (*33*). Shortening velocity is interpreted as an index of relative force production. 1 wk w^{1118} flies had indistinguishable performance relative to age-matched *yw* flies ($p = 0.94$; one-way ANOVA), but the decline in all velocities with age was more severe in w^{1118} than in *yw* ($p < 0.01$; two-way ANOVA). w^{1118} velocities declined with age at all loads (Figure 4.5D, teal vs. blue) while *yw* had preserved unloaded shortening and lengthening velocities with age ($p = 0.96$; one-way ANOVA) and only exhibited impaired shortening and relaxation at extreme loads. Relative power output during shortening, as calculated from Hill's equation (*34*) fit over the loads tested, was diminished to a greater degree with age in w^{1118}. These data indicate that mechanical performance is impaired in aged w^{1118} but is only evident under significant load for *yw* flies. Therefore, diastolic restriction and cortical stiffening was not accompanied by contractile dysfunction; rather, restriction and stiffening correlated with preservation of unloaded shortening and lengthening velocities. Correlation between age-related diastolic restriction, cortical stiffening, cardiac vinculin expression, and preserved basal contractility was further confirmed in a third genotype, *white-CantonS* (*wCS*).

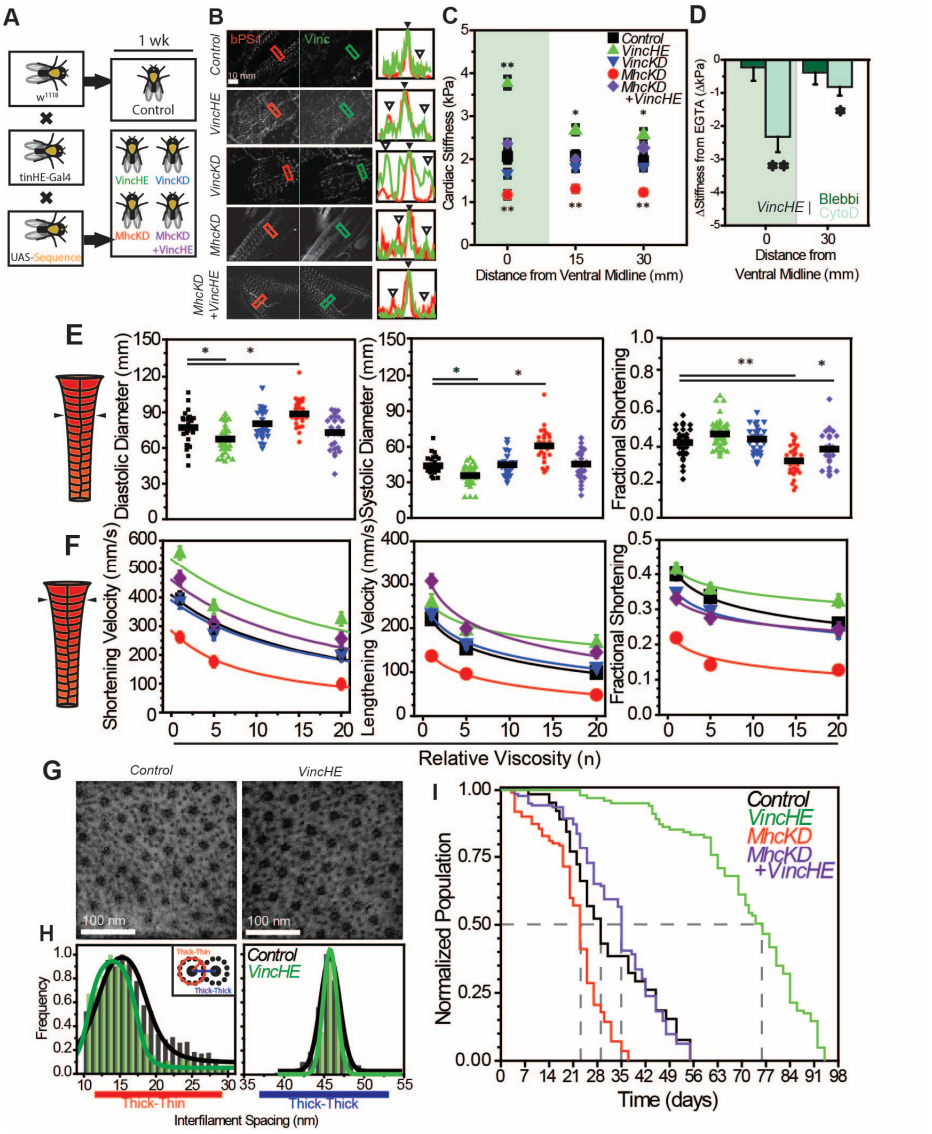

Figure 4.6: Vinculin overexpression improves cardiac performance and lifespan. A. Experimental design of cardiac-specific vinculin and MHC gene expression. B. Vinculin and integrin co-localization. C, D. Vinculin overexpression increases stiffness is an actin-dependent manner. E. Vinculin overexpression improves fractional shortening reduction under load and F. increases shortening and lengthening velocities. G, H. Transmission electron micrograph of VincHE and Control flies reveal reduced myofilament spacing variance. I. VincHE has >150% increase in median lifespan.

Vinculin-Overexpression Recapitulates Aging Phenotype

The aforementioned data suggest that *Vinculin* overexpression is a conserved aging event, though its relationship to cardiac performance and organismal outcome remain unclear.

Therefore, we examined the functional consequences of cardiac-specific *Vinculin* -overexpression. Transgenes were strategically expressed in w^{1118} hearts, which did not stiffen with age, via the cardiac-specific tinHE-Gal4 driver in conjunction with UAS-inducible Vinculin (*VincHE*) or -interfering RNA (RNAi) against Vinculin (*VincKD)* (Figure 4.6A). Since decreased myosin and increased vinculin expression were both observed in our proteomic screen (Figure 4.1C) and in heart failure patients (*4*), we also made a line co-expressing myosin heavy chain RNAi (*MhcKD)* and overexpressing vinculin (*VincHE*), dubbed *MhcKD*+*VincHE*, to determine if vinculin overexpression could rescue dysfunction resulting from impaired myosin motor expression. Cardiac-specific qPCR verified knockdown and/or overexpression for each respective gene.

Vinculin overexpression (*VincHE*) resulted in increased cardiac stiffness compared to controls, with preference at IDs, similar to 5 wk *yw* (green triangles, Figure 4.6C). *MhcKD* exhibited reduced cardiac stiffness across the heart (red circles, Figure 4.6C), consistent with results obtained using other transgene drivers (*26*). However, *MhcKD*+*VincHE* flies were indistinguishable from control (purple diamonds, Figure 4.6C), suggesting that *Vinc* overexpression could restore basal cardiac stiffness in hearts with myosin deficiency. Cytochalasin D treatment reversed the stiffening phenotype in *VincHE* (Figure 4.6D) in a pattern matching 5 wk old *yw* genotype, suggesting remodeling of the actin superstructure by increased vinculin in *VincHE*. Increased vinculin expression and costameric localization, cortical stiffening, and similar response to drug treatments occurred in both *VincHE* and 5 wk *yw* flies, suggesting a causative role for vinculin in inducing age-related changes observed in *yw* and correlate with age-associated cytoskeletal changes observed in mammals and aging patients.

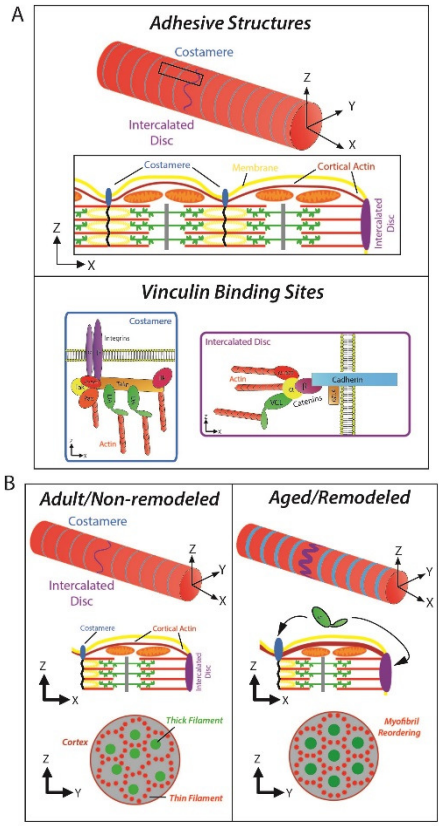

Figure 4.7: Model of cytoskeletal remodeling on filament structure. A. Vinculin expression and localization at membrane-bound attachment sites. B. Remodeling by cortical actin reinforcement and restriction of myofilament chaos.

Cardiac-specific Overexpression of Vinculin Increases Performance

Heart dimensions and contractility were evaluated to examine the effects of *Vinculin* overexpression on cell structure and function. *VincHE* had restricted diameters though fractional shortening did not differ significantly from control (Figure 4.6E). *MhcKD* hearts were significantly dilated and had impaired fractional shortening relative to control. Compensation for myosin knockdown with vinculin overexpression in *MhcKD+VincHE* renormalized heart dimensions and partially rescued fractional shortening. With regards to relative force production, *VincHE* shortening velocity was significantly higher at all loads and displayed a less-severe load-dependent decline in fractional shortening versus all other genotypes (Figure

4.6F). *MhcKD* velocities were diminished at all loads and resembled 5 wk w^{1118} flies (Figure 4.6F; p=0.19, two-way ANOVA). However, *MhcKD+VincHE* had substantially improved shortening velocity compared to *MhcKD* (Figure 4.6F) and significant extension in lifespan (Figure 4.6I). Heart rate and heart period did not differ between *VincHE* and control. Heart rate was depressed in MhcKD but renormalized in *MhcKD+VincHE*. These data suggest that *VincHE* hearts produce greater contractile forces and that increased vinculin expression can rescue the diminished force production of *MhcKD*, as observed in *MhcKD+VincHE*. Computational studies have suggested a role for vinculin in regulating sarcomere lattice spacing and contractility (Tangney et al., 2013). Therefore, electron micrographs of cross-sections through *VincHE* and control cardiac myofibrils were examined to investigate alterations in ultrastructure (Figure 4.6G). Similar average thick filament lattice spacing (45.74 and 45.79 nm) were observed in control and *VincHE*. However, a significant reduction in lattice spacing variance (3.11 nm for control vs. 0.89 nm for *VincHE;* F-test $p < 10^{-48}$) was observed for *VincHE* flies (Figure 4.6H). A similar reduction in variance was observed for thick-to-thin filament spacing. These data suggest improved myofilament arrangement contributes to the enhanced contractility observed in *VincHE*.

Discussion

Age-related heart failure is the result of chronic stressors that induce a heterogeneous array of physiological changes associated with an overall negative outcome. A role for cytoskeletal regulation of contractility has been presented recently (*16*) and post-mortem analysis of age-associated heart failure corroborated altered expression and localization of cytoskeletal proteins (*7, 8*). While a link between cytoskeletal or junctional remodeling and mechanical function has been inferred (*35*), this hypothesis has not been thoroughly vetted in aging cardiac systems. Our goal was to examine the contribution of cytoskeletal remodeling in

the maintenance of function in aged organisms with varying myocardial complexity. To accomplish this, we opted for a novel, integrated approach that utilized expansive proteomic analyses of mammals as well as in-depth mechanistic analyses in a rapidly aging, genetically tractable invertebrate system. These data establish age-associated, vinculin-mediated cytoskeletal remodeling as a regulator of myocardial structure and contractility. Additional therapeutic targets for age-related heart failure are proposed based on their expression changes and known importance to cardiac function (Figure 4.1D) as well as those specific to vinculin.

Choice of Models

A primary aim of this study was to assess conservation of age-related cytoskeletal remodeling and elucidate its impact on myocardial performance across species (simian, rat, and fly) and during normal aging (as opposed to pathological conditions). The unique but complimentary utility of each chosen model allows for cooperative approaches to study aging networks or cellular functions of interest. Monkeys have a relatively long lifespan and cardiac physiology similar to humans. Rat models are widely-used as a model for human cardiac pathophysiology. However, neither is optimally suited for mechanistic dissection of candidate proteins. *Drosophila melanogaster* is a rapidly aging, genetically-tractable organism with extensive proteomic and cellular homology to murine models (*36*), especially with vinculin. Flies also enable tissue-specific genetic manipulation and provide a unique experimental advantage because their myocytes can be interrogated *in situ* with tools that directly visualize heart structure (*27, 28, 37*) or probe mechanics (*26, 29, 38, 39*). This utility circumvents the need for isolation and *in vitro* culture, which is exceedingly difficult in adult and especially aged mammalian myocytes. Cultured neonatal or stem-cell derived myocytes are used to correlate mechanical and functional metrics (*40, 41*), but such models often lack adult morphology or mature, defined cell junctions, and can instead remodel their intracellular structure (*42*) and

contractility (*43*) based on the culture substrate's stiffness. Therefore, the cell isolation process perturbs the ordered cytoskeleton and myofilament lattice to be studied. Conversely, the *Drosophila* heart model allows us to examine the intersection of tensional and functional homeostasis as a function of age and genetic manipulation with intact structures. It is important to note that genotype-dependent differences exist within *Drosophila* (*28*), similar to what has been observed between mouse strains (*44, 45*) and thus we selected two genotypes with diverse age-associated metrics (*yw* and w^{1118}) for comparison. However, it should be noted that conservation of age-related cytoskeletal remodeling and negative correlation with contractile dysfunction was observed in a third genotype.

Choice of Approach

Heart function is dependent upon hierarchical organization of molecular and cellular components across multiple length scales. For this reason, we chose to perform an analysis that integrates complimentary information across multiple scales to investigate the hypothesis that aging is accompanied by conserved, compensatory remodeling events. Recognizing a need for a comprehensive library of myocardial proteins with altered expression with age, we performed a broad spectrum analysis of left ventricular free wall protein expression from which we identified target groups of interest (Figure 4.1). Validation of MS data with standard molecular and cellular techniques confirmed the validity of specific network hits such as vinculin (Figure 4.2), which has significant homology in *Drosophila*. However, since this work represents the first use of the fly heart as a model to study the mechanobiology of cardiac aging, it was necessary to assess the extent to which the model recapitulates mammalian cytoskeletal changes of interest (Figure 4.3, 4). As opposed to MS methods that enrich for extracellular matrix proteins (*46*), we chose to employ those that examined cellular components and especially the cytoskeleton, given that age- (*6, 7*) and mutation-associated (*21*) cytoskeletal alterations have

been associated with heart failure or cardiomyopathy. Using our MS approach, we identified biomarkers of cardiac muscle aging and, due to their requirement for appropriate muscle function, implicate them as therapeutic targets for enhancing cardiac function. Subsequent data examining changes in physiology and function following vinculin-mediated cytoskeletal remodeling (Figure 4.6) provide proof-of-principle that plasticity of the cardiac cytoskeleton allows for beneficial structural remodeling to maintain function during the aging process. More broadly, recapitulation of this aging hallmark resulted in improved myocardial performance and lifespan extension, which suggests that molecular compensation during aging is crucial for longevity.

Localization and Concentration of Vinculin in the Aging Heart

Vinculin was a promising candidate for investigation as it formed a central hub within the network of changes identified by proteomics (Figure 4.1B, C) as well as its known overexpression (*6, 7*) in aging patient myocardium. Additionally, vinculin has been implicated in integrin (*12, 47*) and cadherin-mediated (*18*) mechanotransduction and subsequent interactions with actin cytoskeleton via its tail domain (*24, 48*). Cardiac-specific vinculin deletion disrupts junctional structures and results in sudden death or cardiomyopathy in mice (*49, 50*). Thus, vinculin is an appropriate first candidate to induce cytoskeletal remodeling with age and examine its effect on myocardial performance and indicate a potential functional role in cardiac aging. Increased transcription of *Vinculin* in flies correlated with greater localization at the cortical cytoskeleton or cell cortex, subsequent cytoskeletal reinforcement, and increased contractility (Figure 4.6B-F). Partial rescue of impaired contractility and fractional shortening due to impaired myosin expression (Figure 4.6F) in *MhcKD+VincHE* also supports a role for vinculin in compensation during heart failure, as proposed previously (*6*). While it has been suggested that age-related cytoskeletal remodeling would result in stiffening and subsequent

dysfunction (*35*), these data suggest the cytoskeletal integrity positively influences contractility and lifespan, as evidenced by >50% extension in average lifespan in *MhcKD+VincHE*, compared to *MhcKD*, and >150% increase in *VincHE*, compared to control (Figure 4.6I).

Modulating Vinculin Expression in the Heart

Our data suggest that vinculin overexpression is a consistent hallmark of aging across species. Though the intrinsic molecular causes of vinculin overexpression in the aging human heart remain unclear, several exogenous, upstream regulators of vinculin and vinculin-activating reagents have been identified which could be manipulated to regulate vinculin expression in the heart (Figure 4.1D). For example, TGF-β signaling is a potent regulator of vinculin expression (Figure 4.1B-D). GDF-11, a TGF-β family protein, positively regulates vinculin expression in the brain (*51*) and its overexpression can reverse pathophysiological hypertrophy in aged mice (*3*). However, aged mice express less GDF-11 than younger counterparts while humans have an even lower concentration of GDF-11 than mice at all ages (*3*), implying that reduced GDF-11 with age is not the underlying cause of vinculin overexpression in aging patients. Antagonism of miR-34, a negative transcriptional regulator of vinculin, led to improved outcome in mice following pressure overload or myocardial infarction (*52*). While vinculin-overexpression was observed, it was not specifically implicated as an active component of protection. Post-translational modification of vinculin may also play a role in the aging process. Vinculin localization is dependent upon post-translational modification by the kinase *Abelson* (*53*) whose chronic inhibition by imatinib has been associated with contractile dysfunction (*54*). Thus, post-translational modifications of vinculin in the heart by *Abelson* or other kinases may regulate its localization and influence contractility in the heart with age. Our data suggest that vinculin overexpression alone is sufficient for improving cardiac outcome during aging, indicating that intervention with vinculin mRNA may be a viable

therapeutic. Future studies should determine the efficacy of previously-identified upstream regulators (enzymes, kinases, miRNAs, and drug compounds) on contractility, while minimizing off-target effects to maximize cardiac protection. Additionally, the effects of cardiac vinculin overexpression on heart function and organismal lifespan in larger animal models should be examined as such studies can directly elucidate how vinculin affects diastolic and systolic performance in a four-chambered heart. In broader studies that investigate cardiac performance, we posit that relative changes in vinculin expression may serve as a biomarker or predictor of outcome.

Mechanisms by which Vinculin Regulates Heart Function

Vinculin is localized to both costameres and IDs (Figure 4.7A) where it plays separate but complementary roles for cell-matrix and cell-cell adhesion, respectively. At the cell-matrix interface, our data suggest that vinculin-overexpression led to cytoskeletal reinforcement, indicated by cortical stiffness, and increased myofilament lattice order as measured by decreased interfilament spacing variance (Figure 4.6H, 7B). Since costameres directly couple Z-discs to the membrane, increased vinculin could improve costamere integrity and their ability to anchor myofilaments sufficiently and in correct orientation. These data are consistent with *in vitro* assessment of isolated vinculin-null myocytes, which had reduced cortical stiffness and increased interfilament spacing (*55*). Improved interfilament order is predicted to enhance the probability of actin-myosin cross-bridge formation, and thus the number of actively cycling motors, and potentially impact myofilament calcium-sensitivity (*56-58*). Since the number of myosin heads engaged with thin filament actin determines muscle performance (*59*), increased order predicts elevated force production. On the other hand, vinculin's role at cell-cell junctions, where it was preferentially expressed (Figures 2 and 4) and where stiffness increased most significantly with age, is less clear. We hypothesize that junctional vinculin may help anchor

myofibrils at the membrane in a more ordered fashion and facilitate longitudinal force transmission between myocytes, but additional studies are necessary to confirm this. Finally, cross-talk between the cortex and the Z-disk, which is itself mechanosensitive (*60*), may also occur during remodeling and impact force production.

Conclusions

Here we have outlined a cytoskeleton-based compensatory mechanism in the aging heart that is conserved across species (Figure 4.7). Although the vinculin network was primarily featured here, proteomic analysis suggests an even wider array of cytoskeletal proteins are differentially expressed with age (Figure 4.1) and warrant further investigation. Other vinculin/catenin-family proteins, and the signaling molecules that regulate their translation and post-translational modifications, could play complementary roles (*53*). By presenting distinct aging proteomes and providing a proof-of-concept study of vinculin in cardiac aging, we aimed to establish a resource that will facilitate a broader examination of cardiac senescence so that additional therapeutic targets and regulators of aging heart function can be identified. We believe that our findings highlight the interplay of the molecular, cellular, and ultrastructural state of the cytoskeleton in maintenance of function in the aging heart and that these studies of mechanotransduction can directly inform clinical outcomes.

Materials and Methods

Vertebrate Animals

6 adult (6mo) and 6 aged (24mo) female F344xBN F1 rats were obtained from the National Institutes on Aging (NIA). Rat colony maintenance and all experiments were performed in accordance with UC San Diego IACUC protocol S11032. 4 adult (average: 11.63 years old, range: 8.75-14.12) and 5 aged (average: 22.4 years old, range: 18.81-25.48) male

rhesus monkeys were maintained at the NIA in in accordance with NIA IACUC protocol AG000238-07 (Effects of Aging on Experimental Atherosclerosis in Nonhuman Primates). For echocardiography and ventricular rat cardiomyocyte isolation, please see the Extended Experimental Procedures section. Freshly-isolated sections of left ventricular rat myocardium were embedded in OCT and then flash-frozen in liquid nitrogen for subsequent histological analysis. Alternatively, rat and rhesus monkey samples where flash frozen for subsequent proteomic analysis.

Drosophila Husbandry and Culture

All fly lines were obtained from the Bloomington *Drosophila* Stock Center at Indiana University or the Vienna *Drosophila* RNAi Center (VDRC) and are detailed in the Extended Experimental Procedures. *yw* and w^{1118} strains served as the wild-type control lines. Flies were raised on standard agar-containing food at 25^{o}C. Cardiac-specific perturbation of gene expression was achieved via the Gal4-UAS system as described previously (*61, 62*). Virgin females expressing the transcription factor Gal4 under control of the cardiac-specific tinman (tinHE-Gal4) promoter were crossed with male flies containing the desired transgene under control of the Gal4-recognizing UAS promoter sequence. Please see Extended Experimental Procedures for stock information. The UAS-Vinculin/UAS-MHC-RNAi line (*MhcKD+VincHE*) was generated through a series of crosses between the individual UAS lines and the balancer line *Cdc42/FM6;Sco/CyO*. Female progeny of tinHE-Gal4 and w^{1118} served as control. Proper insertion of both constructs was validated via heart-specific gene expression analysis.

Statistical Analyses

Data comparison was subjected to either a non-parametric Student's t-test with unequal variance assumption, repeated measures one-way ANOVA, one-way ANOVA with post-hoc Tukey correction, or two-way ANOVA with post-hoc Tukey correction as indicated. Significance was assigned for $p < 0.05$. Scatter plots reflect the average measurement of individual animals with black bars indicating the mean. Pooled data are represented as mean +/- standard error of the mean unless otherwise indicated. All *Drosophila* experiments were performed with biological triplicates of 15-31 flies. All other experiments were performed with biological triplicates of indicated sample size.

Acknowledgements

The author would like to acknowledge his committee for support with this endeavor.

References

C. W. Yancy, M. Jessup, B. Bozkurt, J. Butler, D. E. Casey, Jr., M. H. Drazner, G. C. Fonarow, S. A. Geraci, T. Horwich, J. L. Januzzi, M. R. Johnson, E. K. Kasper, W. C. Levy, F. A. Masoudi, P. E. McBride, J. J. McMurray, J. E. Mitchell, P. N. Peterson, B. Riegel, F. Sam, L. W. Stevenson, W. H. Tang, E. J. Tsai, B. L. Wilkoff, F. American College of Cardiology, G. American Heart Association Task Force on Practice, 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Journal of the American College of Cardiology* **62**, e147-239 (2013).

K. Sharma, D. A. Kass, Heart failure with preserved ejection fraction: mechanisms, clinical features, and therapies. *Circulation research* **115**, 79-96 (2014).

F. S. Loffredo, M. L. Steinhauser, S. M. Jay, J. Gannon, J. R. Pancoast, P. Yalamanchi, M. Sinha, C. Dall'Osso, D. Khong, J. L. Shadrach, C. M. Miller, B. S. Singer, A. Stewart, N. Psychogios, R. E. Gerszten, A. J. Hartigan, M. J. Kim, T. Serwold, A. J. Wagers, R. T. Lee, Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy. *Cell* **153**, 828-839 (2013).

J. Schaper, S. Kostin, S. Hein, A. Elsasser, E. Arnon, R. Zimmermann, Structural remodelling in heart failure. *Experimental and clinical cardiology* **7**, 64-68 (2002).

J. H. van Berlo, O. Kanisicak, M. Maillet, R. J. Vagnozzi, J. Karch, S. C. Lin, R. C. Middleton, E. Marban, J. D. Molkentin, c-kit+ cells minimally contribute cardiomyocytes to the heart. *Nature* **509**, 337-341 (2014).

S. Hein, S. Kostin, A. Heling, Y. Maeno, J. Schaper, The role of the cytoskeleton in heart failure. *Cardiovascular research* **45**, 273-278 (2000).

A. Heling, R. Zimmermann, S. Kostin, Y. Maeno, S. Hein, B. Devaux, E. Bauer, W. P. Klovekorn, M. Schlepper, W. Schaper, J. Schaper, Increased expression of cytoskeletal, linkage, and extracellular proteins in failing human myocardium. *Circulation research* **86**, 846-853 (2000).

E. J. Birks, J. L. Hall, P. J. Barton, S. Grindle, N. Latif, J. P. Hardy, J. E. Rider, N. R. Banner, A. Khaghani, L. W. Miller, M. H. Yacoub, Gene profiling changes in cytoskeletal proteins during clinical recovery after left ventricular-assist device support. *Circulation* **112**, I57-64 (2005).

E. J. Birks, Molecular changes after left ventricular assist device support for heart failure. *Circulation research* **113**, 777-791 (2013).

R. A. de Weger, M. E. Schipper, E. Siera-de Koning, P. van der Weide, M. F. van Oosterhout, R. Quadir, H. Steenbergen-Nakken, J. R. Lahpor, N. de Jonge, N. Bovenschen, Proteomic profiling of the human failing heart after left ventricular assist device support. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **30**, 497-506 (2011).

C. Grashoff, B. D. Hoffman, M. D. Brenner, R. Zhou, M. Parsons, M. T. Yang, M. A. McLean, S. G. Sligar, C. S. Chen, T. Ha, M. A. Schwartz, Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature* **466**, 263-266 (2010).

A. del Rio, R. Perez-Jimenez, R. Liu, P. Roca-Cusachs, J. M. Fernandez, M. P. Sheetz, Stretching single talin rod molecules activates vinculin binding. *Science* **323**, 638-641 (2009).

A. Zemljic-Harpf, A. M. Manso, R. S. Ross, Vinculin and talin: focus on the myocardium. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research* **57**, 849-855 (2009).

J. M. Leerberg, A. S. Yap, Vinculin, cadherin mechanotransduction and homeostasis of cell-cell junctions. *Protoplasma* **250**, 817-829 (2013).

A. W. Holle, X. Tang, D. Vijayraghavan, L. G. Vincent, A. Fuhrmann, Y. S. Choi, J. C. del Alamo, A. J. Engler, In situ mechanotransduction via vinculin regulates stem cell differentiation. *Stem cells* **31**, 2467-2477 (2013).

E. Spanjaard, J. de Rooij, Mechanotransduction: vinculin provides stability when tension rises. *Current biology : CB* **23**, R159-161 (2013).

B. A. Danowski, K. Imanaka-Yoshida, J. M. Sanger, J. W. Sanger, Costameres are sites of force transmission to the substratum in adult rat cardiomyocytes. *The Journal of cell biology* **118**, 1411-1420 (1992).

Q. le Duc, Q. Shi, I. Blonk, A. Sonnenberg, N. Wang, D. Leckband, J. de Rooij, Vinculin potentiates E-cadherin mechanosensing and is recruited to actin-anchored sites within adherens junctions in a myosin II-dependent manner. *J Cell Biol* **189**, 1107-1115 (2010).

H. Hirata, H. Tatsumi, C. T. Lim, M. Sokabe, Force-dependent vinculin binding to talin in live cells: a crucial step in anchoring the actin cytoskeleton to focal adhesions. *American journal of physiology. Cell physiology* **306**, C607-620 (2014).

S. Huveneers, J. de Rooij, Mechanosensitive systems at the cadherin-F-actin interface. *Journal of cell science* **126**, 403-413 (2013).

V. C. Vasile, S. R. Ommen, W. D. Edwards, M. J. Ackerman, A missense mutation in a ubiquitously expressed protein, vinculin, confers susceptibility to hypertrophic cardiomyopathy. *Biochemical and biophysical research communications* **345**, 998-1003 (2006).

M. O. Boluyt, K. Converso, H. S. Hwang, A. Mikkor, M. W. Russell, Echocardiographic assessment of age-associated changes in systolic and diastolic function of the female F344 rat heart. *J Appl Physiol (1985)* **96**, 822-828 (2004).

S. Kostin, S. Hein, E. Arnon, D. Scholz, J. Schaper, The cytoskeleton and related proteins in the human failing heart. *Heart failure reviews* **5**, 271-280 (2000).

C. E. Tolbert, K. Burridge, S. L. Campbell, Vinculin regulation of F-actin bundle formation: what does it mean for the cell? *Cell adhesion & migration* **7**, 219-225 (2013).

R. P. Johnson, S. W. Craig, F-actin binding site masked by the intramolecular association of vinculin head and tail domains. *Nature* **373**, 261-264 (1995).

G. Kaushik, A. Fuhrmann, A. Cammarato, A. J. Engler, In situ mechanical analysis of myofibrillar perturbation and aging on soft, bilayered Drosophila myocardium. *Biophys J* **101**, 2629-2637 (2011).

K. Ocorr, N. L. Reeves, R. J. Wessells, M. Fink, H. S. Chen, T. Akasaka, S. Yasuda, J. M. Metzger, W. Giles, J. W. Posakony, R. Bodmer, KCNQ potassium channel mutations cause cardiac arrhythmias in Drosophila that mimic the effects of aging. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 3943-3948 (2007).

M. Fink, C. Callol-Massot, A. Chu, P. Ruiz-Lozano, J. C. Izpisua Belmonte, W. Giles, R. Bodmer, K. Ocorr, A new method for detection and quantification of heartbeat parameters in Drosophila, zebrafish, and embryonic mouse hearts. *Biotechniques* **46**, 101-113 (2009).

M. C. Viswanathan, G. Kaushik, A. J. Engler, W. Lehman, A. Cammarato, A Drosophila melanogaster model of diastolic dysfunction and cardiomyopathy based on impaired troponin-T function. *Circ Res* **114**, e6-17 (2014).

H. Qiu, Y. Zhu, Z. Sun, J. P. Trzeciakowski, M. Gansner, C. Depre, R. R. Resuello, F. F. Natividad, W. C. Hunter, G. M. Genin, E. L. Elson, D. E. Vatner, G. A. Meininger, S. F. Vatner, Short communication: vascular smooth muscle cell stiffness as a mechanism for increased aortic stiffness with aging. *Circulation research* **107**, 615-619 (2010).

E. U. Azeloglu, K. D. Costa, Cross-bridge cycling gives rise to spatiotemporal heterogeneity of dynamic subcellular mechanics in cardiac myocytes probed with atomic force microscopy. *American journal of physiology. Heart and circulatory physiology* **298**, H853-860 (2010).

B. M. Rothen-Rutishauser, E. Ehler, E. Perriard, J. M. Messerli, J. C. Perriard, Different behaviour of the non-sarcomeric cytoskeleton in neonatal and adult rat cardiomyocytes. *Journal of molecular and cellular cardiology* **30**, 19-31 (1998).

A. Cammarato, S. Ocorr, K. Ocorr, Enhanced assessment of contractile dynamics in Drosophila hearts. *BioTechniques*, (2015).

A. V. Hill, The heat of shortening and the dynamic constants of muscle. *Proc R Soc Ser B-Bio* **126**, 136-195 (1938).

J. C. Perriard, A. Hirschy, E. Ehler, Dilated cardiomyopathy: a disease of the intercalated disc? *Trends in cardiovascular medicine* **13**, 30-38 (2003).

A. Cammarato, C. H. Ahrens, N. N. Alayari, E. Qeli, J. Rucker, M. C. Reedy, C. M. Zmasek, M. Gucek, R. N. Cole, J. E. Van Eyk, R. Bodmer, B. O'Rourke, S. I. Bernstein, D. B. Foster, A mighty small heart: the cardiac proteome of adult Drosophila melanogaster. *PloS one* **6**, e18497 (2011).

M. J. Wolf, H. Amrein, J. A. Izatt, M. A. Choma, M. C. Reedy, H. A. Rockman, Drosophila as a model for the identification of genes causing adult human heart disease. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 1394-1399 (2006).

G. Kaushik, A. C. Zambon, A. Fuhrmann, S. I. Bernstein, R. Bodmer, A. J. Engler, A. Cammarato, Measuring passive myocardial stiffness in Drosophila melanogaster to investigate diastolic dysfunction. *Journal of cellular and molecular medicine* **16**, 1656-1662 (2012).

M. Nishimura, C. Kumsta, G. Kaushik, S. B. Diop, Y. Ding, J. Bisharat-Kernizan, H. Catan, A. Cammarato, R. S. Ross, A. J. Engler, R. Bodmer, M. Hansen, K. Ocorr, A dual role for integrin-linked kinase and beta1-integrin in modulating cardiac aging. *Aging cell* (2014).

J. Zhang, M. Klos, G. F. Wilson, A. M. Herman, X. Lian, K. K. Raval, M. R. Barron, L. Hou, A. G. Soerens, J. Yu, S. P. Palecek, G. E. Lyons, J. A. Thomson, T. J. Herron, J. Jalife,

T. J. Kamp, Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method. *Circulation research* **111**, 1125-1136 (2012).

S. P. Sheehy, A. Grosberg, K. K. Parker, The contribution of cellular mechanotransduction to cardiomyocyte form and function. *Biomechanics and modeling in mechanobiology* **11**, 1227-1239 (2012).

J. G. Jacot, A. D. McCulloch, J. H. Omens, Substrate stiffness affects the functional maturation of neonatal rat ventricular myocytes. *Biophysical journal* **95**, 3479-3487 (2008).

L. B. Hazeltine, C. S. Simmons, M. R. Salick, X. Lian, M. G. Badur, W. Han, S. M. Delgado, T. Wakatsuki, W. C. Crone, B. L. Pruitt, S. P. Palecek, Effects of substrate mechanics on contractility of cardiomyocytes generated from human pluripotent stem cells. *International journal of cell biology* **2012**, 508294 (2012).

C. J. Barrick, M. Rojas, R. Schoonhoven, S. S. Smyth, D. W. Threadgill, Cardiac response to pressure overload in 129S1/SvImJ and C57BL/6J mice: temporal- and background-dependent development of concentric left ventricular hypertrophy. *American journal of physiology. Heart and circulatory physiology* **292**, H2119-2130 (2007).

L. Garcia-Menendez, G. Karamanlidis, S. Kolwicz, R. Tian, Substrain specific response to cardiac pressure overload in C57BL/6 mice. *American journal of physiology. Heart and circulatory physiology* **305**, H397-402 (2013).

L. E. de Castro Bras, T. A. Ramirez, K. Y. DeLeon-Pennell, Y. A. Chiao, Y. Ma, Q. Dai, G. V. Halade, K. Hakala, S. T. Weintraub, M. L. Lindsey, Texas 3-step decellularization protocol: looking at the cardiac extracellular matrix. *Journal of proteomics* **86**, 43-52 (2013).

I. Thievessen, P. M. Thompson, S. Berlemont, K. M. Plevock, S. V. Plotnikov, A. Zemljic-Harpf, R. S. Ross, M. W. Davidson, G. Danuser, S. L. Campbell, C. M. Waterman, Vinculin-actin interaction couples actin retrograde flow to focal adhesions, but is dispensable for focal adhesion growth. *The Journal of cell biology* **202**, 163-177 (2013).

K. Shen, C. E. Tolbert, C. Guilluy, V. S. Swaminathan, M. E. Berginski, K. Burridge, R. Superfine, S. L. Campbell, The vinculin C-terminal hairpin mediates F-actin bundle formation, focal adhesion, and cell mechanical properties. *The Journal of biological chemistry* **286**, 45103-45115 (2011).

A. E. Zemljic-Harpf, J. C. Godoy, O. Platoshyn, E. K. Asfaw, A. R. Busija, A. A. Domenighetti, R. S. Ross, Vinculin directly binds zonula occludens-1 and is essential for stabilizing connexin-43-containing gap junctions in cardiac myocytes. *Journal of cell science* **127**, 1104-1116 (2014).

A. E. Zemljic-Harpf, J. C. Miller, S. A. Henderson, A. T. Wright, A. M. Manso, L. Elsherif, N. D. Dalton, A. K. Thor, G. A. Perkins, A. D. McCulloch, R. S. Ross, Cardiac-myocyte-specific excision of the vinculin gene disrupts cellular junctions, causing sudden death or dilated cardiomyopathy. *Molecular and cellular biology* **27**, 7522-7537 (2007).

G. Williams, M. P. Zentar, S. Gajendra, M. Sonego, P. Doherty, G. Lalli, Transcriptional basis for the inhibition of neural stem cell proliferation and migration by the TGFbeta-family member GDF11. *PloS one* **8**, e78478 (2013).

B. C. Bernardo, X. M. Gao, C. E. Winbanks, E. J. Boey, Y. K. Tham, H. Kiriazis, P. Gregorevic, S. Obad, S. Kauppinen, X. J. Du, R. C. Lin, J. R. McMullen, Therapeutic inhibition of the miR-34 family attenuates pathological cardiac remodeling and improves heart function. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 17615-17620 (2012).

J. L. Bays, X. Peng, C. E. Tolbert, C. Guilluy, A. E. Angell, Y. Pan, R. Superfine, K. Burridge, K. A. Demali, Vinculin phosphorylation differentially regulates mechanotransduction at cell-cell and cell-matrix adhesions. *J Cell Biol* **205**, 251-263 (2014).

W. Hu, S. Lu, I. McAlpine, J. D. Jamieson, D. U. Lee, L. D. Marroquin, J. R. Heyen, B. A. Jessen, Mechanistic investigation of imatinib-induced cardiac toxicity and the involvement of c-Abl kinase. *Toxicological sciences : an official journal of the Society of Toxicology* **129**, 188-199 (2012).

J. R. Tangney, J. S. Chuang, M. S. Janssen, A. Krishnamurthy, P. Liao, M. Hoshijima, X. Wu, G. A. Meininger, M. Muthuchamy, A. Zemljic-Harpf, R. S. Ross, L. R. Frank, A. D. McCulloch, J. H. Omens, Novel role for vinculin in ventricular myocyte mechanics and dysfunction. *Biophys J* **104**, 1623-1633 (2013).

M. Webb, R. Jackson del, Jr., T. J. Stewart, S. P. Dugan, M. S. Carter, C. R. Cremo, J. E. Baker, The myosin duty ratio tunes the calcium sensitivity and cooperative activation of the thin filament. *Biochemistry* **52**, 6437-6444 (2013).

G. P. Farman, J. S. Walker, P. P. de Tombe, T. C. Irving, Impact of osmotic compression on sarcomere structure and myofilament calcium sensitivity of isolated rat myocardium. *American journal of physiology. Heart and circulatory physiology* **291**, H1847-1855 (2006).

J. P. Konhilas, T. C. Irving, P. P. de Tombe, Myofilament calcium sensitivity in skinned rat cardiac trabeculae: role of interfilament spacing. *Circulation research* **90**, 59-65 (2002).

G. Piazzesi, M. Reconditi, M. Linari, L. Lucii, P. Bianco, E. Brunello, V. Decostre, A. Stewart, D. B. Gore, T. C. Irving, M. Irving, V. Lombardi, Skeletal muscle performance determined by modulation of number of myosin motors rather than motor force or stroke size. *Cell* **131**, 784-795 (2007).

M. Hoshijima, Mechanical stress-strain sensors embedded in cardiac cytoskeleton: Z disk, titin, and associated structures. *American journal of physiology. Heart and circulatory physiology* **290**, H1313-1325 (2006).

N. Staudt, A. Molitor, K. Somogyi, J. Mata, S. Curado, K. Eulenberg, M. Meise, T. Siegmund, T. Hader, A. Hilfiker, G. Bronner, A. Ephrussi, P. Rorth, S. M. Cohen, S. Fellert, H. R. Chung, O. Piepenburg, U. Schafer, H. Jackle, G. Vorbruggen, Gain-of-function screen for genes that affect Drosophila muscle pattern formation. *PLoS genetics* **1**, e55 (2005).

Y. Bidet, T. Jagla, J. P. Da Ponte, B. Dastugue, K. Jagla, Modifiers of muscle and heart cell fate specification identified by gain-of-function screen in Drosophila. *Mechanisms of development* **120**, 991-1007 (2003)

Chapter 5

The Role of the Cytoskeleton in Cardiac Function

Introduction

In the previous chapters, I have outlined molecules and mechanisms known to be involved in cardiac mechanosensation (Chapter 1), discussed differences between model cardiac systems that can affect measurements of intrinsic mechanics (Chapter 2), outlined the utility of the Drosophila heart as an complementary model for dissecting the effects of genetic perturbations on structure and mechanics (Chapter 3), and proposed that the cortical cytoskeleton plays a role in maintenance of form and function in multiple aging cardiac systems (Chapter 4). The experiments described in this dissertation strongly suggest that cytoskeletal reinforcement, while it does lead to a form of myocardial stiffening, this event does not correlate with impaired function in either chronically aged or transgenic Drosophila. In this concluding chapter, I discuss how this body of experiments fits in the context of other studies concerning the role Vinculin in cardiac function as well as discuss potential applications of this research for modeling disease and developing therapeutics for age-related cardiac dysfunction.

The Role of Vinculin in Diastole

Chapter 2 of this dissertation is adapted from a journal article written in the Fall of 2011 and published in early 2012. At that stage, we had developed the ability to measure cortical stiffness, a measure of both the abundance of localized cytoskeletal and myofibrillar content, and correlate it with measures of change in the heart's diameter with age. What appeared evident was that the greater the reduction in diastolic diameter with age, the more likely the cortex was to stiffen, and this stiffness manifested most significantly at the ventral midline, proximal to the intercalated discs. This suggested to us that 1) transverse/cortical stiffening may contribute to diastolic remodeling and that 2) this suggested the cortical cytoskeleton played a role in diastolic dysfunction. Since tractable animal models for diastolic dysfunction remain elusive, we chose

to explore the role of the cytoskeleton in maintenance of diastolic function. The potential role of the cytoskeleton in diastology was emphasized in Chapter 2 for this reason.

However, halfway through these experiments, it became clear that the assumption that cardiac stiffening meant dysfunction needed to be tested. To date, it was just that – an assumption. And so a second set of experiments, measurements of the heart's shortening and relaxation velocity with increased hemodynamic load (Chapter 4, Figure 4.5), was formulated and executed by myself and Dr. Anthony Cammarato with assistance from Meera Viswanathan (Johns Hopkins School of Medicine, Dept. of Cardiology). From these experiments, we found that genotypes with pronounced age-related stiffening had preserved basal shortening function and no deficit in relaxation velocity. Thus, the dynamics of heart function were preserved – it was only end diastolic and systolic morphology that had changed. Conversely, the non-stiffening genotypes had extreme age-related deficits in cardiac function. The hypothesis demanded to be altered – perhaps cytoskeletal remodeling was a marker of attempted compensation during age, a structural buffer against functional deterioration?

In order to explore this hypothesis, we embarked upon the study that would later become Figure 4.6 in Chapter 4, investigation of Vinculin expression via transgenic lines. By now it was 2013, and the set of experiments we performed revealed an increase in contractile function in Vinculin-overexpressing, juvenile flies. I thought perhaps that this was only possible in an intact, functional, juvenile heart. With the help of Danielle Pohl (University of Iowa), we crossed the UAS-Vinculin line with a UAS-MHC RNAi line. In this way, when crossed to a cardiac promoter line, the progeny would have a deficit in MHC but an overexpression of Vinculin. If Vinculin could actually improve function in a MHC-deficient heart, it would further suggest a role in protecting against dysfunction in aging tissues. This proved to be the case when we measured shortening velocities greater than those in wildtype, albeit with much greater beat-to-beat irregularity.

Vinculin and its Mechanism of Action

The mechanism of action remained elusive. I formed, at the time, three hypotheses by which vinculin could enhance contractile function: 1) preferential localization of vinculin to the intercalated discs reinforces those couplings, resulting in greater efficiency in the conversion of sarcomeric contraction to myocyte shortening at the membrane, 2) improved calcium-handling by alterations in t-tubule morphology, as suggested by previous studies in which dysregulation of the cortical cytoskeleton impaired intracellular calcium signaling, and 3) that cortical compression reorganized the myofilament lattice, improving longitudinal force transmission, as suggested by Dr. Andrew McCulloch (UC San Diego, Dept. of Bioengineering). The first hypothesis would require knowledge of how to deactivate vinculin's localization to the intercalated disc. In doing so, one could investigate the site-specific role of vinculin in mechanosignaling and contraction. At the time, this was unknown, though it has recently been discovered to be regulated by the receptor tyrosine kinase Abelson (Abl) (Bays et al., 2014). The second hypothesis would require introgressing another transgenic line which allows for calcium imaging in Drosophila hearts into each of our lines (Lin et al., 2011), a doable but likely cumbersome and lengthy process as well. The final hypothesis appeared to be the most straightforward to test. We would need only the flies we had on hand and the assistance of experts in the field of Drosophila muscle (Dr. Sanford I. Bernstein and Adriana Trujillo, SDSU). If reorganization or compression of the lattice occurred in vinculin-overexpressing hearts, this would suggest improved contractile performance based on comparable studies on the effects of osmotic compression on myofilament structure and dynamics. It should be noted that, regardless of what we found in the TEM imaging, the other two hypotheses also have supporting evidence in the literature and may act as cooperative mechanisms in protecting against dysfunction with age.

A link between cytoskeletal remodeling and impaired relaxation during diastole may exist in aging human hearts. Active remodeling in the form of increased cross-bridges formed during diastole is thought to contribute to acute diastolic dysfunction; passive remodeling via the cytoskeleton may have a secondary effect of stiffening the myocardium and elevating diastolic pressures. I would hypothesize that cytoskeletal remodeling is a major contributor to heart failure with preserved ejection fraction (HFpEF) in which the myocardium hypertrophies and both systolic and diastolic pressures are elevated. These studies suggests that cytoskeletal remodeling would lead to increased contractile function and therefore allow for increased systolic pressures; elevated diastolic pressure may be a secondary event. In sum, ejection fraction would be preserved and the whole event is perhaps at an attempt to compensate for damage elsewhere in the myocytes or its niche.

Regarding the Role of Vinculin in Cardiac Function

In the closing of Chapter 4, I suggested a potential mechanism by which vinculin (and perhaps other F-actin binding cortical molecules) can regulate contractile function. The effects of myofilament lattice compression on structure and function has been proposed as a potential variable in determining cross-bridge cycling kinetics but, thus far, has appeared to be physiologically-irrelevant as no in vivo mechanism has been identified. However, I believe that these experiments inadvertently shed a light on a physiological mechanism, i.e. cortical compression of the lattice. Decompression of the lattice has been shown in an in vitro murine Vcl-KO model (McCulloch et al., 2013; Tangney et al., 2013). In that case, increased interfilament spacing is thought to have contributed to a loss of contractile function and subsequent embryonic lethality, presumably as the heart would be unable to undergo appropriate mechanically-induced morphogenesis or transport blood to the growing organ system. The

possibility of vinculin playing a relevant role in enhancement of function remained an open question, which this dissertation seeks to expound upon.

Alongside such mechanistic studies of vinculin's contribution to cardiac function, a large population of studies have revealed its association with cardiac development, maturation, and aging. This work was first motivated by reports that vinculin is overexpressed in geriatric patients with dilated cardiomyopathy and end-stage heart failure (Hein et al., 2000; Heling et al., 2000). These studies also revealed other cytoskeletal molecules with age-related overexpression, such as Desmin.

Vinculin has also recently been identified as a blood-circulating biomarker in patients with atherosclerosis (Kristensen et al., 2014). Presumably, leakage of vinculin into the bloodstream is caused by stenosis of coronary arteries and subsequent cell death. If this is the case, then vinculin may be overexpressed in ischemic tissue.

A recent study investigating recent speciation and rapid evolution of divergent populations of North American bears revealed that the gene expression profile of polar bears strongly favors genes associated with cardiac function. The authors argue that this adaptation allows for improved cardiac function despite the polar bears' relatively high fat diet, which in other species would present an extreme cardiovascular disease risk. Interestingly, Vcl is among the most highly selected for adaptation. This finding further bolsters a potential protective role for vinculin in encumbered cardiac systems (in the case of the polar, an extreme high-fat diet) (Liu et al., 2014).

Mechanical unloading of a patient heart by a left ventricular assist device is associated with molecular remodeling and improved ventricular contractile function. Vinculin is among the molecules that are upregulated during this process, further implying a relationship between Vcl expression and gained mechanical function (Aquila et al., 2004; Birks et al., 2005).

Another recent study has identified the TGF-beta family protein GDF-11 (growth differentiation factor 11) as a blood-circulating factor that, when overexpressed, can reverse remodel aged, hypertrophic murine hearts (Loffredo et al., 2013). Other groups have gone on to show that GDF-11 is a positive regulator of vinculin (Williams et al., 2013) and thus the later may serve as a functional endpoint for the remodeling achieved by GDF-11. However, while GDF-11 expressed in decreased with age, vinculin expression is increased. Thus, age-associated overexpression of vinculin is likely regulated by a separate mechanism or event. One potential candidate is oxidative stress, which is known to increase vinculin expression at cellular junctions in human cells. Such a mechanism may have evolved as an adaptation to aggregated oxidative stress in aging systems.

Acknowledgements

This chapter is an original work by the author, unique to this dissertation.

References

Aquila, L.A., McCarthy, P.M., Smedira, N.G., Young, J.B., and Moravec, C.S. (2004). Cytoskeletal structure and recovery in single human cardiac myocytes. The Journal of heart and lung transplantation: the official publication of the International Society for Heart Transplantation 23, 954-963.

Bays, J.L., Peng, X., Tolbert, C.E., Guilluy, C., Angell, A.E., Pan, Y., Superfine, R., Burridge, K., and Demali, K.A. (2014). Vinculin phosphorylation differentially regulates mechanotransduction at cell-cell and cell-matrix adhesions. The Journal of cell biology 205, 251-263.

Birks, E.J., Hall, J.L., Barton, P.J., Grindle, S., Latif, N., Hardy, J.P., Rider, J.E., Banner, N.R., Khaghani, A., Miller, L.W., Yacoub, M.H. (2005). Gene profiling changes in cytoskeletal proteins during clinical recovery after left ventricular-assist device support. Circulation 112, I57-64.

Hein, S., Kostin, S., Heling, A., Maeno, Y., and Schaper, J. (2000). The role of the cytoskeleton in heart failure. Cardiovascular research 45, 273-278.

Heling, A., Zimmermann, R., Kostin, S., Maeno, Y., Hein, S., Devaux, B., Bauer, E., Klovekorn, W.P., Schlepper, M., Schaper, W., Schaper, J. (2000). Increased expression of cytoskeletal, linkage, and extracellular proteins in failing human myocardium. Circulation research 86, 846-853.

Kristensen, L.P., Larsen, M.R., Mickley, H., Saaby, L., Diederichsen, A.C., Lambrechtsen, J., Rasmussen, L.M., and Overgaard, M. (2014). Plasma proteome profiling of atherosclerotic disease manifestations reveals elevated levels of the cytoskeletal protein vinculin. Journal of proteomics 101, 141-153.

Lin, N., Badie, N., Yu, L., Abraham, D., Cheng, H., Bursac, N., Rockman, H.A., and Wolf, M.J. (2011). A method to measure myocardial calcium handling in adult Drosophila. Circulation research 108, 1306-1315.

Liu, S., Lorenzen, E.D., Fumagalli, M., Li, B., Harris, K., Xiong, Z., Zhou, L., Korneliussen, T.S., Somel, M., Babbitt, C., Wray, G., Li, J., He, W., Wang, Z., Fu, W., Xiang, X., Morgan, C.C., Doherty, A., O'Connell, M.J., McInerney, J.O., Born, E.W., Dalen, L., Dietz, R., Orlando, L., Sonne, C., Zhang, G., Nielsen, R., Willerslev, E., Wang, J. (2014). Population genomics reveal recent speciation and rapid evolutionary adaptation in polar bears. Cell 157, 785-794.

Loffredo, F.S., Steinhauser, M.L., Jay, S.M., Gannon, J., Pancoast, J.R., Yalamanchi, P., Sinha, M., Dall'Osso, C., Khong, D., Shadrach, J.L., Miller, C.M., Singer, B.S., Stewart, A., Psychogios, N., Gerszten, R.E., Hartigan, A.J., Kim, M.J., Serwold, T., Wagers, A.J., Lee, R.T. (2013). Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy. Cell 153, 828-839.

McCulloch, A.D., Pfeiffer, E., Tangney, J., and Omens, J. (2013). Biomechanics of Cardiac Electromechanical Coupling and Mechanoelectric Feedback. Journal of biomechanical engineering.

Tangney, J.R., Chuang, J.S., Janssen, M.S., Krishnamurthy, A., Liao, P., Hoshijima, M., Wu, X., Meininger, G.A., Muthuchamy, M., Zemljic-Harpf, A., Ross, R.S, Frank, L.R., McCulloch, A.D., Omens, J.H. (2013). Novel role for vinculin in ventricular myocyte mechanics and dysfunction. Biophysical journal 104, 1623-1633.

Williams, G., Zentar, M.P., Gajendra, S., Sonego, M., Doherty, P., and Lalli, G. (2013). Transcriptional basis for the inhibition of neural stem cell proliferation and migration by the TGFbeta-family member GDF11. PloS one 8, e78478.