The Connection of Composition, Structure, and Dynamic Processes to Tendon Mechanics: Structure-Function Relationships in Collagen V Deficient Tendons

Brianne Kathryn Connizzo  
University of Pennsylvania, connizzo@seas.upenn.edu

Follow this and additional works at: http://repository.upenn.edu/edissertations

Part of the Biomechanics Commons

Recommended Citation
http://repository.upenn.edu/edissertations/1666

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/edissertations/1666
For more information, please contact libraryrepository@pobox.upenn.edu.
The Connection of Composition, Structure, and Dynamic Processes to Tendon Mechanics: Structure-Function Relationships in Collagen V Deficient Tendons

Abstract
Tendons are able to withstand the broad range of stresses and strains via their finely tuned composition and structure. In addition, tendons undergo a coordinated set of dynamic responses, specifically collagen uncrimping, re-alignment, sliding and deformation, within the matrix. To date, a complete understanding of the hierarchical structure-function relationships in tendon is lacking. Therefore, the overall goal of this thesis was to measure tendon structure and function in a mouse supraspinatus model of altered structure, and to analyze links between mechanical properties, dynamic processes and composition/structure using a series of statistical analyses. In the studies presented here, we used novel and established methods to measure the multi-scale composition, structure and mechanical function of mouse supraspinatus tendons from wild type, collagen V heterozygous and collagen V null mice. Overall, we found that the experimental groups were mechanically inferior to the wild type group, with larger changes in both macroscale function and the dynamic responses (re-alignment, crimp, deformation, sliding). In addition, while fibril morphology was altered at both locations, the insertion site also exhibited alterations in cell and fiber morphology as well as extracellular matrix composition. Finally, using a novel regression approach, we found that the contribution of composition and structure as well as the contribution of dynamic processes to determining macroscale mechanical function was highly dependent on location and that the dynamic processes were significant mediators of the relationship between composition/structure and mechanical properties. Overall, we conclude that although collagen V is a quantitatively minor component in mature tendon/ligament, it is a major regulator of composition and structure during development which ultimately leads to mechanical function. Furthermore, we conclude that the dynamic responses to load are crucial factors in ultimately determining regionally-dependent mechanical function. This information will help to guide clinicians in developing preventative techniques and appropriate rehabilitation strategies, as well as help to define the appropriate and important parameters on which to base tissue engineering efforts for tendon augmentation or replacement. Finally, this work presents a strong foundation on which to develop future experimental and modeling efforts in order to fully understand the complex structure-function relationships present in tendon.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Bioengineering

First Advisor
Louis J. Soslowsky

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/1666
Keywords
collagen V, composition, dynamic, mechanics, structure, tendon

Subject Categories
Biomechanics

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/1666
THE CONNECTION OF COMPOSITION, STRUCTURE, AND DYNAMIC PROCESSES TO TENDON MECHANICS: STRUCTURE-FUNCTION RELATIONSHIPS IN COLLAGEN V DEFICIENT TENDONS

Brianne Kathryn Connizzo

A DISSERTATION

In

Bioengineering

Presented to the Faculties of the University of Pennsylvania

In

Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2015

Supervisor of Dissertation
Louis J. Soslowsky, Ph.D.
Fairhill Professor of Orthopaedic Surgery

Graduate Group Chairperson
Jason A. Burdick, Ph.D.
Professor of Bioengineering

Dissertation Committee
Robert L. Mauck, Ph.D. (Committee Chair)
Associate Professor of Orthopaedic Surgery and Bioengineering, University of Pennsylvania
Andrew F. Kuntz, M.D.
Assistant Professor of Orthopaedic Surgery, University of Pennsylvania
X. Sherry Liu, Ph.D.
Assistant Professor of Orthopaedic Surgery, University of Pennsylvania
David E. Birk, Ph.D.
Distinguished Professor, Molecular Pharmacology and Physiology, University of South Florida
Abbas F. Jawad, Ph.D.
Associate Professor of Biostatistics in Pediatrics at the Children's Hospital of Philadelphia
THE CONNECTION OF COMPOSITION, STRUCTURE, AND DYNAMIC PROCESSES TO TENDON MECHANICS: STRUCTURE-FUNCTION RELATIONSHIPS IN COLLAGEN V DEFICIENT TENDONS

COPYRIGHT

2015

Brianne Kathryn Connizzo
ACKNOWLEDGEMENTS

Without the generous assistance and counsel of my many mentors, colleagues, friends, and family, the completion of this thesis would have been immeasurably more difficult. I’d like to first thank Dr. Louis Soslowsky, my advisor and mentor, for his continued guidance, commitment and support. I’d also like to thank Dr. David Birk for his support of our many collaborative studies over the past five years, as well as his insight and knowledge in the planning and execution of this thesis. Thank you Dr. Rob Mauck, my committee chair, for continuing to support me from the first day I stepped into McKay. I’d also like to thank Dr. Sherry Liu for her professional and personal support throughout my career. I also thank Dr. Andy Kuntz for his clinical perspective and Dr. Abbas Jawad for his assistance with planning and executing my statistical regressions. Finally, I’d like to thank all of my committee members for always providing valuable commentary and suggestions throughout my studies.

I’d like to also express my gratitude to the many people who contributed directly to this thesis work. I extend my deepest gratitude to the entire laboratory staff at the University of South Florida for all of their hard work and hospitality during my several visits. I’m incredibly grateful for Mei Sun and Qingmei (Chris) Yao for all of their work breeding and caring for all of the mice necessary for this work. I’d also like to thank Thomas and Sheila Adams for their dedication and attention to detail in collecting and analyzing data in this thesis. I can’t thank these colleagues and friends enough for the many hours we worked together on my marathon day trips to Florida. I’d also like to thank Dr. Lin Han and Dr. Joseph Sarver for their mentorship, patience, and guidance in development of the fibril deformation assay. Additionally, I thank Jennica Tucker, Carrie Barnum, Benjamin Freedman, and Pankti Bhatt for always having a positive attitude while helping out on long days of assay development and execution.

Of course this work would not have been possible without a lot of hard work from members in the McKay Orthopaedic Research Laboratory as well. Thank you to all of the office staff who do all of the behind-the-scenes work for the lab. I’d especially like to thank Susan Dinella, who always lights up the lab with her smile and is one of the most dedicated and
hardworking individuals I’ve ever met. I’d also like to thank Kelly McGinnis for always being available for a chat and constantly having snacks to fuel us. I’d also like to thank Dr. Mike Hast and Dr. Snehal Shetye for their help with my several hard drive failures and frozen computers.

I would not be able to accept full credit for this work without acknowledging several of the opportunities that lead me to be able to do this work. I’d like to thank my high school volleyball coach and close friend, Beth Powell, who always encouraged and challenged me to be my best against all adversity. I’d also like to thank all of the incredible women before me at Smith College who paved the way for me in the first all women engineering program. I am truly proud to be a part of such an incredible group of alumni. Thank you to Dr. Kristin Miller who sparked my interest in this work at the very beginning of my career and to Dr. Katie Reuther for her continued friendship and support. I’m so lucky to have been part of such an incredibly talented and collaborative community. It would not have been such a fun journey without the encouragement and friendship from my fellow labmates and friends. In particular, I’d like to thank Sarah Rooney and Jennica Tucker for being with me, sometimes literally next to me, every day since the very beginning. I’d also like to express my gratitude to the Graduate Association of Bioengineering (GABE) and the Graduate Student Engineering Group (GSEG) for all of the opportunities I’ve been given and the friends I’ve made during my four years of service.

Finally, none of this work would have been possible without the love and support of my family and friends. Thank you to my mom, Barbara, who is my confidant, my number one supporter and my best friend. Thank you to my dad, Al, who challenged me to be the best I could be no matter what I was doing. Thank you to my older brother Alex for teaching me new things and always reminding me that there’s much more to life than science. Thank you also to my oldest brother Nick and his wife Kate for inspiring me to keep learning in hopes of someday surpassing them in trivia knowledge. Finally, I thank Nick Trojanowski for his endless patience, understanding and love. I am so thankful that we’ve been able to share this experience and I can’t wait to see what comes next.
ABSTRACT

THE CONNECTION OF COMPOSITION, STRUCTURE, AND DYNAMIC PROCESSES TO TENDON MECHANICS: STRUCTURE-FUNCTION RELATIONSHIPS IN COLLAGEN V DEFICIENT TENDONS

Brianne Kathryn Connizzo
Dr. Louis J. Soslowsky

Tendons are able to withstand a broad range of stresses and strains via their finely tuned composition and structure. In addition, tendons undergo a coordinated set of dynamic responses, specifically collagen uncrimping, re-alignment, sliding and deformation, within the matrix. To date, a complete understanding of the hierarchical structure-function relationships in tendon is lacking. Therefore, the overall goal of this thesis was to measure tendon structure and function in a mouse supraspinatus model of altered structure, and to analyze links between mechanical properties, dynamic processes and composition/structure using a series of statistical analyses. In the studies presented here, we used novel and established methods to measure the multi-scale composition, structure and mechanical function of mouse supraspinatus tendons from wild type, collagen V heterozygous and collagen V null mice. Overall, we found that the experimental groups were mechanically inferior to the wild type group, with larger changes in both macroscale function and the dynamic responses (re-alignment, crimp, deformation, sliding). In addition, while fibril morphology was altered at both locations, the insertion site also exhibited alterations in cell and fiber morphology as well as extracellular matrix composition. Finally, using a novel regression approach, we found that the contribution of composition and structure as well as the contribution of dynamic processes to determining macroscale mechanical function was highly dependent on location and that the dynamic processes were significant mediators of the relationship between composition/structure and mechanical properties. Overall, we conclude that although collagen V is a quantitatively minor component in mature tendon/ligament, it is a major regulator of composition and structure during development which ultimately leads to mechanical function.
Furthermore, we conclude that the dynamic responses to load are crucial factors in ultimately determining regionally-dependent mechanical function. This information will help to guide clinicians in developing preventative techniques and appropriate rehabilitation strategies, as well as help to define the appropriate and important parameters on which to base tissue engineering efforts for tendon augmentation or replacement. Finally, this work presents a strong foundation on which to develop future experimental and modeling efforts in order to fully understand the complex structure-function relationships present in tendon.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ III

ABSTRACT ............................................................................................................................... V

TABLE OF CONTENTS ........................................................................................................ VII

LIST OF TABLES ................................................................................................................... X

LIST OF FIGURES ................................................................................................................ XI

CHAPTER 1: INTRODUCTION ................................................................................................. 1
A. Introduction .......................................................................................................................... 1
B. Background ........................................................................................................................ 1
   B-1. Tendon Mechanics ....................................................................................................... 1
   B-2. Tendon Composition and Structure .......................................................................... 2
   B-3. Tendon Dynamic Parameters .................................................................................... 4
C. Significance of Studies ..................................................................................................... 8
   C-1. Multiscale Structure-Function Relationships in Tendon ................................................. 8
   C-2. Classic Ehlers-Danlos Syndrome .............................................................................. 9
D. Specific Aims ................................................................................................................... 10
E. Study Design ................................................................................................................... 12
   E-1. Animal Model .......................................................................................................... 12
   E-2. Animal Use and Sample Size Justification ................................................................ 13
F. Chapter Overview ........................................................................................................... 14
G. References ....................................................................................................................... 14

CHAPTER 2: IN SITU FIBRIL STRETCH AND SLIDING IS LOCATION-DEPENDENT IN MOUSE SUPRASPINATUS TENDONS ................................................................................. 21
A. Introduction ....................................................................................................................... 21
B. Methods ............................................................................................................................ 23
   B-1. Sample Preparation .................................................................................................... 23
   B-2. Atomic Force Microscopy (AFM) ............................................................................. 24
   B-3. Data Analysis ............................................................................................................ 25
   B-4. Statistical Analysis .................................................................................................... 26
C. Results ............................................................................................................................... 27
D. Discussion ......................................................................................................................... 28
E. References ......................................................................................................................... 32

CHAPTER 3: COLLAGEN V-DEFICIENT TENDONS EXHIBIT ALTERED DYNAMIC MECHANICAL BEHAVIOR AT MULTIPLE HIERARCHICAL SCALES ................................................................................................. 36
A. Introduction ....................................................................................................................... 36
B. Methods ............................................................................................................................ 38
   B-1. Dynamic Viscoelastic Testing .................................................................................... 39
   B-2. Fatigue Testing .......................................................................................................... 40
LIST OF TABLES

CHAPTER 2

Table 2-1: Fibril Strains.............................................................................................................28

CHAPTER 5

Table 5-1: Parameters in Statistical Analysis.................................................................91
Table 5-2: Independent Parameter Correlations...........................................................95
Table 5-3: Mechanical Properties Regressed on Dynamic Processes.........................96
Table 5-4: Dynamic Processes Regressed on Composition and Structure....................98
Table 5-5: Multiple Mediator Modeling with Single Independent Variable......................100
LIST OF FIGURES

CHAPTER 1

Figure 1-1: Tendon Composition and Structure .................................................. 2
Figure 1-2: Dynamic Re-O rganizations of Collagen Fiber Structure .................. 5
Figure 1-3: Mouse Models of Classic Ehlers-Danlos Syndrome ..................... 9
Figure 1-4: Overall Study Design ....................................................................... 13

CHAPTER 2

Figure 2-1: Testing Schematic for AFM Method .............................................. 24
Figure 2-2: AFM Analysis ................................................................................. 25
Figure 2-3: AFM Study Fibril Deformation ..................................................... 27
Figure 2-4: AFM Study Fibril Sliding ............................................................... 28

CHAPTER 3

Figure 3-1: Viscoelastic Testing and Analysis .................................................. 39
Figure 3-2: Fatigue Testing and Analysis .......................................................... 40
Figure 3-3: Re-Alignment Analysis ................................................................. 41
Figure 3-4: Fiber Uncrimping and Fibril Deformation Protocol ..................... 42
Figure 3-5: Animal and Tendon Morphology .................................................. 44
Figure 3-6: Quasi-Static Mechanical Parameters .......................................... 45
Figure 3-7: Dynamic Mechanical Parameters ................................................ 46
Figure 3-8: Fatigue Parameters ...................................................................... 47
Figure 3-9: Collagen Fiber Re-Alignment ........................................................ 49
Figure 3-10: Collagen Fiber Uncrimping ........................................................ 50
Figure 3-11: Pre-Ramp to Failure Crimp Pilot Study ....................................... 51
Figure 3-12: Fibril Deformation and Sliding ................................................... 53
CHAPTER 4

Figure 4-1: Joint Morphology.................................................................70
Figure 4-2: Crimp Morphology.................................................................71
Figure 4-3: Representative Fibril Micrographs........................................72
Figure 4-4: Fibril Diameter Distribution..................................................73
Figure 4-5: Fibril Morphology.................................................................74
Figure 4-6: Cell Morphology.................................................................75
Figure 4-7: Biochemical Quantification..................................................76
Figure 4-8: Elastin Immunofluorescence...............................................77
Figure 4-9: Humerus Histomorphometry..............................................80

CHAPTER 5

Figure 5-1: Model Schematic...............................................................93
CHAPTER 1: INTRODUCTION

A. Introduction

Tendons function primarily as mechanical, load-bearing structures to transmit forces from muscle to bone, thus allowing for motion. To perform these tasks efficiently and to withstand the broad range of stresses and strains, tendon composition and organization are finely tuned. When focusing on the initial response of tendon to load, there is not enough time to synthesize new or remodel the existing matrix and thus we consider the structure and composition to be unchanging during this early time period. However, during this initial loading, tendons do undergo a coordinated set of responses which we term ‘dynamic’, specifically fiber uncrimping and realignement and fibril sliding and deformation within the matrix. To date, a complete understanding of these unique structure-function relationships in tendon is lacking. Recent statistical models have been used to correlate some compositional/structural measures, such as fibril morphology, glycosaminoglycan (GAG) content, and collagen content with tendon mechanical properties. However, the dynamic response to loading is often overlooked. Advances in methodology have recently allowed measurement of these dynamic properties and evidence suggests that the dynamic response is critical for proper tendon function. Therefore, the overall goal of this thesis was to measure tendon structure and function in a mouse supraspinatus model of altered structure, and to analyze links between mechanical properties, dynamic processes and composition/structure using a series of statistical analyses.

B. Background

B-1. Tendon Mechanics

Tendon is a compliant, anisotropic material which has a high modulus under tension, but collapses under compression. This primary uniaxial tensile function along the longitudinal axis of its collagen fibers enables transmission of generated muscle force to bone. Furthermore, tendon
exhibits nonlinear biomechanical behavior as exhibited by a typical stress-strain curve with an initial, non-linear “toe-region” followed by the “linear-region” (Rigby et al., 1959). Clinically, the ability of tendon to demonstrate these properties allows for its ability to both guide movement (low stiffness) and provide stability (high stiffness). The toe-region is thought to be caused by gradual recruitment of crimped collagen fibers (Hansen et al., 2002; Woo et al., 2000). After the toe-region, all collagen fibers are believed to be supporting load and thus, the stress-strain curve becomes linear. In addition to their anisotropic, non-linear behavior, tendons exhibit viscoelastic properties identified as stress relaxation, hysteresis, and creep (Woo et al., 2000). Stress relaxation refers to a non-linear decrease in stress over a period of time when a tendon is held under constant tension. This process is both static and dynamic, as demonstrated by a similar decrease in peak stress over time with repetitive, cyclic tensile loading. Hysteresis represents the energy loss within the tendon with dynamic testing, accounting for a gradual change in load elongation curves with tendon loading and unloading. Creep occurs when a tendon is held under constant tension, and a measurable increase in tendon length along its longitudinal axis over time is observed. These viscoelastic properties emphasize the ability of tendon to structurally adapt to constant or cyclical loads in order to reach biomechanical equilibrium (Einhorn et al., 2007).

B-2. Tendon Composition and Structure

Figure 1-1. (A) Hierarchical structure of tendon spanning from the single collagen molecule up to fibrils, fascicles, and whole tendon. Inset image describes the structure of fibril-associated
proteoglycans (Reproduced from Voleti et al. 2012) (B) Histological (4x) section of the rat supraspinatus tendon-to-bone insertion site, highlighting the transition zone (Reproduced from Thomopoulos et al. 2002)

Tendon’s mechanical function is tuned by the unique composition and structure of the tissue, primarily by its extracellular matrix. This matrix, comprised predominantly of collagen type I, is organized in a hierarchical manner parallel to the mechanical axis of the tendon (Fig. 1-1). Collagen microfibrils organize by lateral and longitudinal stacking, thus forming a lattice-type configuration, called a fibril. Fibrils then associate together to form fibers, which then further combine to form fascicles, which then bundle to form full tendon (Birk et al., 1995; Birk et al., 1997). The process of fibril association and growth, called fibrillogenesis, is modulated mainly by proteoglycans (decorin, biglycan, fibromodulin, lumican) and minor collagens, such as collagen types V, XI, XII, and XIV. The unique structure of the collagen fibrils is suggested to play a role in the mechanical function of the tendon. This collagen orientation promotes very high strength in the direction of fiber alignment, which is dependent on the underlying organizational structure of collagen molecules. In addition, literature suggests that collagen fiber crimp is responsible for some of the nonlinearity of tendon by acting as a shock absorber (Hansen et al., 2002).

In addition to collagen I, the extracellular matrix is composed of minor collagens, elastin, proteoglycans (PGs), glycolipids, and cellular material (Woo et al., 2005). Proteoglycans attach to collagen fibrils in an orthogonal manner (Cribb and Scott, 1995; Scott et al., 1981), and extend their specialized carbohydrate chains, known as glycosaminoglycans (GAGs), into the interfibrillar space. GAGs have a negative charge, thus attracting and binding to water molecules (Woo et al., 2005). The role of PGs and GAGs in tendon’s response to load has been highly debated over the past decade (Fessel et al., 2012; Fessel and Snedeker, 2009; Lujan et al., 2007; Lujan et al., 2009; Screen et al., 2005). It has been speculated that GAGs may mechanically interconnect adjacent collagen fibrils, specifically, the GAG chains linked to decorin. Relative movements of stained GAGs during mechanical relaxation tests have been quantified, which led to the hypothesis of interfibrillar force transfer through a ratchet mechanism (Cribb and Scott, 1995). However, studies with enzymatic removal of GAGs in tendon and ligament have not
provided conclusive evidence to support this mechanism. There have been a few studies to suggest that GAGs and PGs may play a role in dynamic mechanical properties (Rigozzi et al., 2009). Most recently, the influence of decorin on the patellar tendon mechanical properties was investigated in a dose dependent manner. This study found no differences in elastic or compressive properties but a dependence on strain rate and frequency, necessitating further exploration into the dynamic behavior of decorin and biglycan knockout tendons (Dourte et al., 2012). In addition, proteoglycan removal was found to have a significant effect on collagen fiber re-alignment and aging in the mouse supraspinatus tendon, suggesting a relationship between composition and the dynamic processes (Connizzo et al., 2013).

In vivo, the structure and composition of tendon is capable of adapting to altered loading, overuse, injury and healing via changes in gene and protein expression following the event. However, when focusing on the initial response of tendon to load, there is not enough time to synthesize new or remodel the existing matrix and thus we can consider these parameters to be unchanging during this early time period. This period, the focus of these studies, is when direct relationships between structure/composition and function can be defined.

**B-3. Tendon Dynamic Parameters**

There are a number of ‘dynamic’ processes that occur during the mechanical test, specifically fiber uncrimping, fiber re-alignment, fibril deformation and fibril sliding.

**B-3-1. Fiber Uncrimping**

Crimp is a periodic waveform configuration visualized within the collagen fibers and has been implicated in the mechanical behavior of collagen (Woo et al., 2000). Particularly, the flattening or disappearance of the crimp morphology has been implicated in the non-linear behavior observed in the toe-region of the stress-strain curve (Fig. 1-2A) (Atkinson et al., 1999; Diamant et al., 1972). The disappearance of crimp in collagen fiber bundles with mechanical
testing as visualized by polarized light microscopy has been well documented (Hansen et al., 2002; Jozsa and Kannus, 1997), but few have been able to quantify crimp during the mechanical test. Using a custom freeze-spraying technique and more recently using an integrated polarized light testing setup, our lab has measured changes in crimp frequency throughout mechanical testing. A recent analysis of mouse supraspinatus tendon demonstrated that the uncrimping of collagen fibers along the entirety of the tendon was confined to the toe-region (Miller et al., 2012b).

**B-3-2. Fiber Re-Alignment**

![Figure 1-2](image)

**Figure 1-2.** Typical stress-strain curve of tendon shows an elongated low stiffness toe region transitioning to a high stiffness linear region. Inset are two mechanisms of tendon’s response to load within the toe region, (A) the uncrimping and (B) re-alignment of collagen fibers, represented by a decreased in the variance of the distribution of fiber angles from the toe to linear region.

The native collagen alignment of the tissue varies along the length of a tendon, with a more disorganized fiber matrix located at the tendon-to-bone insertion (Lake et al., 2009; Lake et al., 2010). When load or displacement is applied to tendon, collagen fibers shift their orientation
towards the axis of loading, decreasing the distribution of fiber angles (Fig. 1-2B). This mechanism is called collagen fiber re-alignment. Recent efforts in our lab have focused on the ability to quantify collagen fiber re-alignment using a novel polarized light setup that allows for simultaneous measurement and mechanical testing (Lake et al., 2009; Lake et al., 2010; Miller et al., 2012a; Miller, 2012; Miller et al., 2012b). This approach has determined that re-alignment occurs differently when measured at various points throughout the mechanical test, with the majority of re-alignment occurring during preconditioning and the toe region of the mechanical test. In addition, measurement of local re-alignment has determined that changes in collagen fiber alignment throughout loading are location-dependent (Miller, 2012; Miller et al., 2012b).

**B-3-3. Fibril Deformation and Sliding**

A number of researchers have studied the behavior of collagen fibrils individually in addition to fibril-fibril interactions (Gautieri et al., 2011, 2012; Heim et al., 2007; Svensson et al., 2010; Szczesny and Elliott, 2014; van der Rijt et al., 2006; Veres and Lee, 2012). Collagen fibrils have been likened to nanostructural biological cables and with techniques such as atomic force microscopy, the isolated study of their mechanical properties is possible. Although mechanical properties of single fibrils have been investigated by one-dimensional tensile testing using optical tweezers (Wang et al., 2005) and atomic force microscopy (AFM) (Graham et al., 2004; Yang et al., 2007), the mechanical behavior of the collagen fibrils themselves has not been able to account for full tendon mechanics. Recent studies have been directed toward understanding the behavior of single collagen fibrils in native arrangement (bundled) in response to the application of macroscopic load. Collagen fibril deformation has been measured in situ recently by measuring changes in d-period spacing with different level strains, providing evidence that d-period spacing increases with increased applied load, thus supporting stretch of individual collagen fibrils (Connizzo et al., 2014; Rigozzi et al., 2011). Macroscopic tendon extension is enabled by deformation and sliding mechanisms that simultaneously occur between fibers and between
fibrils, which is partially regulated by matrix components such as proteoglycans (Rigozzi et al., 2009; Rigozzi et al., 2011; Screen et al., 2005). Fibril sliding has been cited as one contributor to tendon viscoelastic behavior (Silver et al., 2002), and is thought to occur during the toe region of the mechanical test. Recent evidence also suggests that fibril deformation and sliding are location-dependent, alluding to structure-function relationships that are unique even along the length of the tendon (Connizzo et al., 2014).

B-3-4. Regression Modeling of Mechanical Properties

Recent studies have addressed the relationships of quasi-static mechanical behavior of tendon to composition/structure. One study measured the mechanical properties of rat tail tendon fascicles and seven structural and compositional variables, attempting to find evidence of direct structure-function relationships using a multiple regression model (Robinson et al., 2004). The most prevalent predictors of mechanical behavior in this study were GAG content and collagen fibril area fraction, suggesting that both GAGs and collagen fibril size can predict mechanical properties. Interestingly, mean collagen fibril diameter was not a significant predictor. This contrasts several studies where fibril diameter was correlated with mechanical properties (Derwin and Soslowsky, 1999; Hansen et al., 2010; Parry, 1988), but agrees with other studies observing the lack of correlation in developing tendon or self-assembled collagen fibers (Birk et al., 1991; Christiansen et al., 2000; Hansen et al., 2010). A study in human patellar tendon found differences in collagen fibril distribution between the posterior and anterior aspect of the tendon, but no correlation with fascicle mechanical properties (Hansen et al., 2010). Another study investigated fibril morphology and mechanical properties in the Achilles tendon, noting that larger fibrils may be associated with a stiffer tendon but that this could be due to fibril-fibril interactions or fibril-non-fibrillar matrix interactions (Rigozzi et al., 2010). Confusion in the literature suggests that the mechanical behavior of the tendon cannot be explained solely by composition and
structure and that there must be alternative mechanisms of load transfer such as re-alignment, uncrimping, sliding, etc.

Recent advances in technology have allowed for the measurement of dynamic parameters, but only a limited number of studies have investigated the relationship between dynamic measures and mechanical properties. In a study of collagen fiber re-alignment in the human supraspinatus tendon, initial fiber alignment was found to be significantly correlated with linear region modulus (Lake et al., 2010). This was confirmed in the mouse supraspinatus tendon, showing that increased mechanical properties were associated with increased alignment (Miller et al., 2012a). Recent evidence also suggests a dynamic relationship between collagen crimp and mechanical parameters from fatigue loading (Freedman et al., 2015). In addition, changes in dynamic properties have been noted to occur with changes in composition and structure as well (Connizzo et al., 2013; Miller et al., 2012a; Miller et al., 2012c), suggesting a complex, yet unidentified, relationship between the three hierarchical levels of properties.

C. Significance of Studies

C-1. Multiscale Structure-Function Relationships in Tendon

Tendons function in a complex environment and are subjected to a variety of multi-axial loads. The ability to respond effectively under these conditions is directly tuned by the unique structure of the tissue. Characterization of structure-function relationships in tendon has been mostly limited to comparisons between macroscopic (full tissue) mechanical properties and nanoscale composition and structure. These studies will greatly enhance the fundamental knowledge of structure-function relationships at several hierarchical levels and elucidate the relationships between them. An improved understanding of these relationships also provides insight on the mechanisms driving normal tissue adaptation, aging, and degeneration. Further, the complete nature of the strategy and approach presented in this study, which is more
comprehensive than that of any previous study of tendon, can be used as a paradigm to thoroughly evaluate tendon and other fiber-reinforced soft biological tissues.

Previous work has used linear correlations to assess the independent, univariate effects of a single organization or a single biochemical variable on tendon mechanics. Unfortunately, in vivo systems are much more complicated than can often be addressed with such an approach, particularly when considering the complex structural hierarchy in tendon. Two recent studies in our lab have utilized a method for evaluating structure-function relationships in tissue taking into consideration multiple independent variables that may be present (Ansorge et al., 2012; Robinson et al., 2004). These studies build on these previous studies by investigating if the dynamic processes (re-alignment, uncrimping, deformation, and sliding) act as mediators in the relationship between composition/structure and mechanical properties. Given that the dynamic processes take place at a larger hierarchical scale (nano/micro) than the compositional and structural parameters, this unique modeling technique is able to determine if there is a mediating relationship between these parameters and to determine which dynamic processes act as mediators for various mechanical parameters.

C-2. Classic Ehlers-Danlos Syndrome

![Figure 1-3](image)

**Figure 1-3.** (A) Classic EDS mouse model exhibits hyperelastic skin and (B) tendon/ligament-specific collagen V null mouse exhibits extreme joint hypermobility.

In addition to the advantages this work has for the basic study of tendon biomechanics, this work also provides valuable information for the clinical population of classic Ehlers-Danlos
Syndrome (EDS) patients and the physicians that treat them. The classic form of EDS, defined by haploinsufficiency for COL5A1 which is present in ~67% of affected individuals (Malfait et al., 2005; Symoens et al., 2012), is characterized by hyperextensible skin, joint hypermobility and instability, as well as abnormal wound healing and scarring (Steinmann, 2002). These functional deficiencies can often lead to recurrent joint dislocations, particularly at the knee and shoulder (Ainsworth and Aulicino, 1993; Stanitski et al., 2000), as well as to injury and disease. Our established haploinsufficient Col5a1+/− classic EDS mouse model recapitulates the human clinical phenotype (Wenstrup et al., 2006), and our tendon-specific col5a1 null mouse exhibits even more severe effects (Fig. 1-3). In addition to the mechanical studies presented here, we also examined the macroscopic tissue morphology and quantified changes in cell morphology and many key matrix proteins (total collagen, GAGs, pyridinoline, elastin). Investigation into the alterations in soft tissue structure and function, specifically in the tendon studied here, provide more insight into the functional limitations and abnormalities in this severely understudied patient population. In addition, this greatly improves the ability to treat those affected as well as help to better prevent injuries.

D. Specific Aims

The overall goal of this thesis was to measure tendon structure and function in a mouse supraspinatus model of altered structure, and to analyze links between mechanical properties dynamic processes, and composition/structure using a series of statistical analyses. To accomplish this objective, transgenic animal models with a reduction in collagen V expression were analyzed for changes in multi-scale composition, structure, and mechanical function (Aim 1). Our global hypothesis was that alterations at the fibril level due to reduced collagen V expression would cause alterations in composition and structure at increasing hierarchical levels and ultimately to decreased dynamic and quasi-static mechanical function. We then used this data to investigate relationships between structure and function a series of multiple regression
analyses followed by a causal analysis using single and multiple mediator models (Aim 2). Our global hypothesis was that dynamic processes are mediating the relationship between composition/structure and mechanical properties, mimicking the native structural hierarchy in tendon.

**Specific Aim 1:** Define the structural, compositional, and mechanical properties of mouse supraspinatus tendons from wild type, collagen V heterozygous and collagen V null mice.

*Hypothesis 1a:* Based on the reduction in fibril nucleation sites, collagen V heterozygous and null tendons will have increased fibril diameters and decreased fibril density with similar collagen content when compared to controls. Collagen V heterozygous and null tendons will also have reduced cross-linking and GAG content due to the changes in diameter and intrafibrillar spacing.

*Hypothesis 1b:* Based on previous and pilot mechanical data, collagen V heterozygous and null tendons will have inferior quasi-static, dynamic, and fatigue properties, when compared to control tendons. Since collagen V does not play a major role in fibrocartilage, these changes will be more pronounced at the midsubstance than at the insertion site.

*Hypothesis 1c:* Collagen V heterozygous and null tendons will have increased crimp but reduced alignment compared to control tendons, allowing for a longer toe region and later transition to the linear region. They will also have reduced uncrimping, later re-alignment, and increased fibril sliding. Finally, collagen V deficient tendons will have reduced fibril properties due to altered collagen fibrillogenesis.

**Specific Aim 2:** Use correlation and multiple linear regression to investigate relationships between (1) mechanical properties and dynamic processes and (2) dynamic processes and composition/structure and determine if dynamic processes are mediators of the relationship between composition/structure and mechanical properties.

*Hypothesis 2a:* As in previous studies, tendon mechanical properties will be correlated with composition/structure. Since evidence suggests that the initial response to load is a major
determinant of overall mechanics, mechanical properties will also be strongly correlated to dynamic measurements.

*Hypothesis 2b:* Dynamic processes will be strongly correlated with composition/structure, since these parameters control structure and subsequently structural responses to load.

*Hypothesis 2c:* Dynamic processes will act as mediators in the relationship between composition/structure and mechanical properties, due to the ability to mimic native tendon structure and account for relationships between hierarchical levels.

### E. Study Design

**E-1. Animal Model**

This study investigated the compositional, structural and mechanical properties in the collagen V heterozygous and null mouse model. The collagen V heterozygous mouse model (Col5a1\(^{+/−}\)) has been well characterized as a model of classic Ehlers-Danlos syndrome, exhibiting many clinical phenotypes (Wenstrup et al., 2006; Wenstrup et al., 2000; Wenstrup et al., 2011). Due to the critical role of collagen V in fibril nucleation, the traditional collagen V null mouse model exhibits embryonic lethality. Therefore, we used a tendon-specific collagen V null mouse, Col5a1\(^{Δten/Δten}\). Col5a1\(^{floxfloxFlox}\) mice were created by flanking exons 3 and 4 of the Col5a1 gene with loxP elements. The tendon/ligament-specific scleraxis-Cre (Scx-Cre) transgenic mice were gifts from Dr. Ronen Schweitzer (Shriners Hospital, Portland, Oregon). Tendon/ligament-specific expression was characterized by breeding Scx-Cre mice with Cre reporter mT/mG mice (The Jackson Laboratory). To generate tendon specific type V collagen conditional mice Scx-Cre transgenic mice were cross-bred with Col5a1floxfloxFlox mice two generations to create scx-Cre/Col51a1floxfloxFlox (Col51a1\(^{Δten/Δten}\)) mice. These mice were bred at the University of South Florida and provided by Dr. David Birk (University of South Florida, Tampa, Florida).
E-2. Animal Use and Sample Size Justification

Three hundred mice from three different groups were used in this study: control (WT), Col5a1<sup>+/−</sup> (het), and Col5a1<sup>Δten/Δten</sup> (null). All animal procedures were IACUC approved prior to the start of work. Mice were bred to 120 days of age and sacrificed humanely. The overall study design is shown in Figure 1-4. Supraspinatus (SST) tendons from shoulders designated for biological assays were dissected immediately following sacrifice and stored appropriately for the particular assay. All other limbs were frozen at -20°C until dissection for mechanical testing. Based on the correlation coefficients and R<sup>2</sup> values from previous multiple regression analyses performed in our lab, as well as knowledge of the variance in the specific parameters measured here, we determined that a sample size of 20 tendons would provide us with sufficient power (80%) to test the hypotheses presented above with significance set at p<0.05. Although not explicitly stated in the aims of the study, qualitative or semi-quantitative measurements were also performed to better characterize changes with the reduction of collagen V, specifically histology to obtain a macroscopic view of tendon structure and immunofluorescence staining of elastin. For these measurements, we used a sample size of 8-10 tendons, based on previous studies.
F. Chapter Overview

Chapter two will describe the development of an AFM-based method to measure instantaneous in situ collagen fibril deformation and sliding in mouse supraspinatus tendons. Chapter three will describe the methods, results, and discussion for the experimental studies performed to investigate the multi-scale mechanical function of mouse supraspinatus tendons from collagen V wild type, heterozygous, and null mice. Chapter four will describe the methods, results, and discussion for the experimental studies performed to investigate the multi-scale composition and structure of mouse supraspinatus tendons from collagen V wild type, heterozygous and null mice. Chapter five will describe the statistical studies investigating the relationship between composition/structure, dynamic processes, and mechanical function. Chapter six will summarize the conclusions of the previous chapters and provide future directions for this area of research.

G. References


over 90% of patients with classic EDS and allows to refine diagnostic criteria. Hum Mutat 33, 1485-1493.


CHAPTER 2: IN SITU FIBRIL STRETCH AND SLIDING IS LOCATION-DEPENDENT IN MOUSE SUPRASPINATUS TENDONS

A. Introduction

Tendon’s primary function is to transmit mechanical load and displacement from muscle to bone (Lichtwark and Barclay, 2010). It is able to perform this function due to its finely tuned hierarchical structure, composed of collagen fibrils organized into fibers or fascicles and further bundled to form tendon proper (Kastelic et al., 1978). Macroscopic structure-function studies of tendon have shown that mechanical changes occurring at lower scale levels are likely responsible for the complex non-linear and viscoelastic response of full tendon (Derwin and Soslowsky, 1999; Fessel and Snedeker, 2009; Lake et al., 2009; Lake et al., 2010; Rigozzi et al., 2009; Robinson et al., 2004). Recent evidence suggests that tendons are able to withstand high forces by employing a number of unique mechanisms occurring at many of the fibril and fiber length scales, including uncrimping, re-alignment, sliding, and deformation or stretch (Connizzo et al., 2013b; Gupta et al., 2010; Miller et al., 2012b; Screen et al., 2013). While collagen fiber uncrimping and re-alignment have been studied extensively in recent literature (Connizzo et al., 2013a; Connizzo et al., 2013b; Miller et al., 2012a; Miller et al., 2012b; Miller et al., 2012c; Miller et al., 2012d), the quantification of collagen fiber and fibril sliding and stretch has been studied less due to the experimental difficulties, particularly the inability to visualize individual collagen fibrils in vivo during mechanical loading.

Mechanical properties of single collagen fibrils have recently been investigated using several different technologies (Eppell et al., 2006; Graham et al., 2004; Tang et al., 2010; Yang et al., 2007). While these studies substantially improved our understanding of mechanics of individual collagen fibrils, they do not replicate the in vivo environment of collagen, where collagen fibrils are interacting with other collagen fibrils and with the surrounding extracellular matrix proteins. Furthermore, it has been reported that fiber-level elongation cannot be solely attributed to the deformation of the individual collagen fibrils, suggesting fibril-fibril and fibril-matrix interactions are likely responsible for this discrepancy (Fratzl et al., 1998; Puxkandl et al., 2002).
Recent investigations utilizing atomic force microscopy have successfully measured d-period length changes as a quantitative measure of collagen fibril stretch in situ (Li et al., 2013; Rigozzi et al., 2011). This work introduces a significant advancement in the literature, allowing for the ability to study fibril stretch under various types of mechanical loading as well as with cases of altered structure, such as disease, aging, or injury (Li et al., 2013). However, these studies have primarily investigated fiber sliding during or following stress relaxation or creep events (Gupta et al., 2010; Li et al., 2013; Rigozzi et al., 2011; Screen et al., 2013). The strain rate dependence of tendon identifies that the timing and rate of loading, in addition to the magnitude, is extremely important to tendon’s response. Furthermore, since tendons have been known to rupture clinically due to a single traumatic event or impact (Moller et al., 1996), the instantaneous response to load, as well as the ability to repetitively undergo that impact stress, is critical to the overall function and has not been investigated.

In addition, fibril sliding and deformation have primarily been studied during the linear region of the mechanical test. Due to the prevalence of collagen uncrimping and re-alignment during the initial toe region (Miller et al., 2012b; Miller et al., 2012c), it is likely that these other fibrillar responses are also occurring. Finally, while many studies have demonstrated that the specific transition in composition, structure and collagen organization from the midsubstance to the insertion site contributes significantly to the full tendon’s mechanical response (Lake et al., 2009; Lake et al., 2010; Shaw and Benjamin, 2007), the location-dependent response to mechanical load at the fibril level has not been studied. Therefore, the purpose of this study was to quantify the instantaneous response of collagen fibrils throughout a mechanical loading protocol both in the insertion site and midsubstance of the mouse supraspinatus tendon. We hypothesized that more fibril stretch will occur at the insertion site than the midsubstance (higher strains) and that more fibril sliding will occur at the midsubstance than at the insertion site.
B. Methods

B-1. Sample Preparation

Fifteen C57BL/6 mice at 150 days of age were used in this study (IACUC approved). Supraspinatus tendons from both shoulders of each mouse were used for this study, but no two tendons from the same animal were used in the same testing group to ensure independence of samples. All soft tissues were removed from around the tendon, leaving the supraspinatus tendon attached to the humerus. Tendon cross-sectional area was then measured using a custom laser-based device (Peltz et al., 2009). The humerus was then embedded in an acrylic tube with PMMA. A second coating of PMMA was applied to prevent failure at the growth plate. The proximal end of the tendon was glued between two pieces of sandpaper with an initial gauge length of 2.5mm and both the tendon and the acrylic pot were placed in custom grips for tensile testing (Fig. 2-1A,B), as described previously (Connizzo et al., 2013a).

All samples were kept hydrated using phosphate buffered saline (PBS) and were then loaded in a tensile testing system (Instron, Norwood, MA) for mechanical testing. A 10 N load cell was used for all tests with a resolution of 0.01 N. All tendons underwent a preload to 0.02N and ten cycles of preconditioning from 0.02N to 0.04N followed by a 60 second hold before the ramp to failure. Tendons were then divided into 6 groups and stretched to a randomly assigned grip-to-grip strain value (0, 1, 3, 5, 7, or 10%) at a rate of 0.1% strain per second. Tendons were then frozen using freezing spray (McMaster-Carr Electrical Cleaning and Maintenance Aerosol, Product #7437K43), removed from the mechanical testing setup, and placed in a specimen dish with tissue freezing medium. The sample was kept frozen during this process using freezing spray and the dish was then submerged in liquid nitrogen to complete the flash-freezing phase. Tendons were then stored at -20°C until they were sectioned in a cryostat microtome in the coronal anatomical plane at 20 microns and sections were then again kept frozen at -20°C until further processing. Frozen sections were then immersed in cold 10% neutral buffered formalin for 4 minutes for fixation and allowed to dry prior to imaging.
Figure 2-1. (A) Testing image of supraspinatus tendon prepared for mechanical testing. (B) Zoomed-in view of supraspinatus tendon. (C) Diagram depicting insertion site and midsubstance regions for d-period analysis. (D) Microscope images depicting typical scan regions for the midsubstance (top image) and insertion site (bottom images).

B-2. Atomic Force Microscopy (AFM)

Tendons were imaged in air using Peak Force Quantitative Nanomechanical Mapping mode using a Dimension Icon AFM (BrukerNano, Santa Barbara, CA). Imaging of 2 µm × 2 µm regions was performed using Bruker ScanAsyst Fluid+ probes (nominal spring constant k ≈ 0.70 N/m, radius R ≈ 2 nm). Tendons were scanned at 2-3 regions across the width of the insertion site and midsubstance of the tendon. The insertion and midsubstance locations were determined consistently by taking images within the bottom quarter (about 0.5mm) of the specimen for the insertion site and the top quarter of the specimen for the midsubstance region (a single sample region shown in Figure 2-1C). Scans were also taken from 4-6 sections throughout the depth of the tendon.
Figure 2-2. (A) Sample scan of collagen fibrils in tendon with line highlighting the measurement of d-period in a single fibril. (B) Intensity versus length plot for line in panel (A) which is used to measure d-period length using custom software.

B-3. Data Analysis

Several parameter maps were produced from imaging, including Height, Peak Force, Peak Force Error, Modulus, LogModulus, Dissipation, and Adhesion. Analysis on approximately two tendons (~20 images) showed no difference between the different parameter maps for measurement of d-period. Due to the strength of contrast from the LogModulus and Adhesion maps, these two maps were used to analyze all specimens. Custom MATLAB software (MathWorks, Natick, MA) was written to allow for the measurement of d-period length for many fibrils in a single image and for the ability to enhance the processing technique in the future. Images were first contrast enhanced by equalizing the histogram of intensities locally using a
default MATLAB function (adapthisteq) with the default parameters. A line spanning at least 10 dark bands was then drawn manually over each fibril in the image by the user (Fig. 2-2A). Image intensity over the course of the line was then plotted and the distance between peaks or valleys in the image was calculated using a standard MATLAB peak-finding function, reducing user error from the analysis (Fig. 2-2B). The median distance between all peaks was then calculated and defined as the d-period length for that particular fibril. Fibrils with d-periods below 55nm and above 80nm were considered to be noise and were excluded from the analysis based on previous studies that cite high variability in collagen fibril populations using AFM (Choi et al., 2011; Fang et al., 2012; Wallace et al., 2010). D-period length was measured for fibrils in at least 5 images per specimen and 3-5 specimens per strain level. Fibril stretch/strain was calculated by dividing the d-period length at each applied strain level by the initial d-period length. Distributions of d-period lengths for each strain level in both the insertion site and midsubstance of the tissue were also produced and the variance of the distributions was calculated. A change in fibril d-period variance from one strain to the next is indicative of strain heterogeneity between fibrils, or fibril sliding.

**B-4. Statistical Analysis**

Two tendons were excluded from this study due to failure prior to the allotted strain level and one tendon was excluded due to a tissue processing error leaving a sample size of n=3-5 tendons per strain level. It was determined via Mann-Whitney test that the variance within a single specimen was not different from the variance between specimens in the same strain level. Therefore, fibrils from all specimens in each strain level were pooled. Statistical comparisons of d-period length across different strains were then made using a non-parametric Kruskal-Wallis test at each location (insertion site and midsubstance), followed by post-hoc Dunn’s tests between the strain levels. Comparisons of variance between strain levels were performed using a Bartlett’s test for unequal variances at each location with post-hoc F tests between strain levels. A p-value of p < 0.05 was considered as statistically significant.
C. Results

**Figure 2-3.** Insertion site d-period length (left) is increased significantly from 0% to 1% tendon strain and again from 3% to 5% tendon strain before decreasing after 5% strain. Midsubstance d-period length is increased from 0 to 1% strain and then decreased after 7% strain. Data is presented as median ± interquartile range (*p < 0.05). Sample sizes are as follows: 0% (ins: n = 267, mid: n = 254), 1% (ins: n = 190, mid: n = 245), 3% (ins: n = 200, mid: n = 142), 5% (ins: n = 273, mid: n = 221), 7% (ins: n = 206, mid: n = 206), 10% (ins: n = 250, mid: n = 219).

At both the insertion and midsubstance of the tendon, Kruskal-Wallis tests determined that there was a highly significant effect of increasing tendon strain on d-period length (*p < 0.001). D-period length at the insertion site significantly increased from 0% to 1% tendon strain, increased again from 3% to 5% strain, and decreased after 5% strain (Fig. 2-3). At the midsubstance, d-period length increased from 0% to 1% strain and then decreased after 7% strain. In addition, d-period distribution data displayed heterogeneity in d-period from one strain level to the next. Variance of the insertion site d-period distribution did not significantly change with increasing tendon strain (Fig. 2-4A). At the midsubstance, there was a highly significant increase in d-period variance with a peak at 3% strain and then a sharp decrease immediately following (Fig. 2-4B).
Figure 2-4. Variance in d-period distribution changes with strain. (A) At the insertion site, d-period variance was not significantly different between any of the strain levels. (B) At the midsubstance, d-period variance is significantly and sharply increased with a peak at 3% strain. Bartlett’s test for unequal variance over the strain levels was highly significant at the midsubstance, but not significant at the insertion site. Data was calculated from fibril d-period distributions in Figure 3 and is presented as variance ± 95% confidence interval of variance.

D. Discussion

Table 2-1. Fibril strains are calculated by the change in fibril d-period from the initial (0%) d-period length at each level of full tendon strain. Data is presented as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Applied Strain</th>
<th>Insertion Fibril Strain</th>
<th>Midsubstance Fibril Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>1.83±5.06%</td>
<td>0.95±2.65%</td>
</tr>
<tr>
<td>3%</td>
<td>1.57±4.85%</td>
<td>0.94±3.06%</td>
</tr>
<tr>
<td>5%</td>
<td>3.66±4.84%</td>
<td>0.66±2.15%</td>
</tr>
<tr>
<td>7%</td>
<td>0.83±5.04%</td>
<td>1.10±2.67%</td>
</tr>
<tr>
<td>10%</td>
<td>-0.47±5.06%</td>
<td>0.24±1.10%</td>
</tr>
</tbody>
</table>

This is the first study to measure location-dependent collagen fibril stretch, or changes in collagen fibril d-period length with applied tendon strain. It has been well established that the insertion site and midsubstance of tendons often exhibit different mechanical, compositional, and structural properties, particularly in the supraspinatus tendon (Lake et al., 2010; Miller et al.,
In this study, the insertion site experienced higher fibril strains overall, reaching a peak of 3.66±4.84% fibril strain at 5% tendon strain compared to 1.10±2.67% fibril strain at 7% tendon strain in the midsubstance (Table 2-1). This is supported in the literature by full tendon studies that describe the insertion site as a high stress region (Shaw and Benjamin, 2007). Recent data have also shown that the processes of collagen fiber re-alignment and uncrimping occur differently at these two locations (Connizzo et al., 2013a; Miller et al., 2012d), so it is expected that collagen fibril stretch would also show a regional-dependence. This dependence could be due to organizational or compositional differences between the two regions such as an altered ratio or relationship between collagen fibril organization and extra-fibrillar matrix, which could lead to altered mechanical function (Thomopoulos et al., 2003; Thorpe et al., 2012). Due to the higher degree of disorganization at the insertion site, more re-alignment and uncrimping must occur here (Miller et al., 2012a; Miller et al., 2012b; Miller et al., 2012d). Given that these processes usually take place during the toe or transition region of the mechanical test, this could be one explanation for the pause in fibril stretch at the insertion site from 1 to 3% strain. Recent studies reported that the transition strain for the mouse supraspinatus at 90 and 300 days of age is at 2-3% strain (Connizzo et al., 2013a), supporting this theory.

In addition to regional dependence, this study is the first to quantify the instantaneous response of collagen fibrils to overall tendon strain during the mechanical test. The instantaneous response of collagen fibrils is an important measure of tendon biomechanical function, as many tendon injuries result from single, high impact loading trauma, particularly in athletes (Moller et al., 1996). However, previous investigations were unable to study this phenomenon due to fixation technique. Using the flash freezing method, we were able to preserve the instantaneous structural changes of tendon at varied strain levels. Our work demonstrated higher fibril strains than those previously reported (Rigozzi et al., 2011). Lower fibril strains in previous work could be attributed to the elongated holding period for chemical fixation, which would allow for creep and/or relaxation of the tissue to occur. Considering that a large amount of fiber/fibril sliding has been shown to occur during relaxation events (Gupta et al., 2010), it is possible that fibrils are relaxing
to an altered state than the instantaneous positioning of fibrils. Future studies should focus on measuring both the instantaneous and relaxation response of collagen fibrils as both the elastic and viscoelastic components significantly contribute to the overall mechanical response (Dunn and Silver, 1983; Silver et al., 2002; Woo et al., 1993).

Results in this study are consistent with those in the literature demonstrating that strains of individual fibrils were much lower when compared to overall tendon tissue strain (Fratzl et al., 1998; Puxkandl et al., 2002), once again supporting the theory that inter- and intra-fibril mechanisms must be contributing to the overall mechanical response. The variance in d-period length distributions from one strain level to the next was also calculated in this study, where differences in d-period distribution variance across strain levels would indicate that fibril sliding is occurring. This analysis showed that most of the collagen fibril sliding, noted by either a positive or negative change in d-period variance, was occurring between 1 and 5% strain, which corresponds to the toe and transition periods of the tissue-level stress-strain curves. Simultaneously, fibril stretch was highest initially (from 0 to 1% strain) and then after 5% strain until gross tissue failure. This can be explained by a cooperative relationship between fibril stretch and fibril sliding, where these mechanisms occur in coordination. Furthermore, this study found a regional dependence on fibril sliding, with a much higher amount of fibril sliding occurring in the midsubstance, as indicated by the increased d-period variance. This provides another complexity to the relationship between fibril stretch and sliding, as organization seems to play a role in how and/or when these processes can occur. While we are not aware of any studies that have directly linked collagen organization with collagen fibril sliding, recent data suggests that tail tendon, which is highly organized like the supraspinatus tendon midsubstance, does exhibit collagen fibril and fiber sliding. In addition, studies investigating differences in functionally different tendons have shown structural differences as well as differences in the relative amounts of fiber sliding and stretch, also supporting the results here (Thorpe et al., 2013; Thorpe et al., 2012).

While this work provides a novel approach to answering complicated questions, this technique is not without limitations. First, this work employs a flash freezing and processing
technique that is destructive to the tissue, requiring a large sample size to obtain a snapshot of each portion of the stress strain curve. However, with our ability to pool fibrils across several samples as described earlier, we can obtain enough fibrils to make powerful statistical comparisons in these experiments. In addition, our specific hypothesis was related to the instantaneous response of the tissue and as such, this technique does not allow for the real-time measurement of fibril stretch or sliding during loading of the tissue. Future efforts will focus on development of a technique to measure both the instantaneous and relaxation response in the same specimen since both responses contribute significantly to the overall function of the tissue. This study also did not measure the cross-sectional area of individual collagen fibrils. While evidence suggests that collagen fibril d-period length is independent of fibril diameter (Bozec et al., 2007; Reale et al., 1981), it is possible that some heterogeneity in fibril stretch could be due to fibril size and as such, future investigations will seek to normalize these measures. Finally, the heterogeneity between and within tendons is still a major area that needs to be explored since many analyses are performed on a single tendon type, making comparisons difficult to make. However, we believe the mouse supraspinatus tendon is an excellent model for this type of work due to the large changes in structure along the length of the tendon, which allow us to make generalizations about structure-function relationships that could be applicable to other tendons.

In conclusion, this work demonstrates a novel and innovative approach to measure the regional instantaneous response of collagen fibrils to overall tendon load without removing the fibrils from their native environment. Fibril stretch and sliding were found to be location-dependent and are speculated to have a strong relationship with collagen organization. This concept could lead to an explanation for differences that have been found recently in the mechanical responses of tendons with differing functional needs. Finally, this work could be a powerful tool utilized to study the altered fibrillar response to load in various animal models and could lead to a more complete visualization of the hierarchical complexity of tendon’s mechanical response.
E. References


CHAPTER 3: COLLAGEN V-DEFICIENT TENDONS EXHIBIT ALTERED DYNAMIC MECHANICAL BEHAVIOR AT MULTIPLE HIERARCHICAL SCALES

A. Introduction

Tendons function to stabilize the skeleton and allow efficient transfer of energy through their unique set of mechanical properties. The primarily uniaxial tensile function along the longitudinal axis of its collagen fibers enables transmission of generated muscle force to bone. Tendon exhibits nonlinear biomechanical behavior as exhibited by a typical stress-strain curve with an initial, non-linear “toe-region” followed by the “linear-region.” This non-linearity, in particular the low stiffness toe-region, is thought to be attributed to a number of dynamic microstructural re-arrangements (Connizzo et al., 2014b; Hansen et al., 2002; Miller et al., 2012b; Miller et al., 2012e; Rigozzi et al., 2011; Thorpe et al., 2013b; Woo et al., 2000). Particularly, the flattening or disappearance of the collagen fiber crimp morphology has been implicated in the non-linear behavior observed in the toe-region (Atkinson et al., 1999; Diamant et al., 1972). In addition, re-orientation of collagen fibers towards the axis of mechanical load also has been found to occur during this period. Macroscopic tendon extension is also enabled by deformation and sliding mechanisms that simultaneously occur between fibers and between fibrils within each fiber (Rigozzi et al., 2009; Rigozzi et al., 2011; Screen et al., 2005). In addition to their anisotropic and non-linear behavior, tendons exhibit several viscoelastic properties, such as stress relaxation, hysteresis and creep (Dourte et al., 2012; Dunkman et al., 2013; Elliott et al., 2003; Gautieri et al., 2012; Gimbel et al., 2004; Screen, 2008; Silver et al., 2002; Woo et al., 2000). This process is both static and dynamic, as demonstrated by a similar decrease in peak stress over time with repetitive, cyclic tensile loading (Freedman et al., 2014). Fibril sliding has also been cited as a contributor to dynamic viscoelastic behavior during this initial response to load (Silver et al., 2002). These viscoelastic properties emphasize the ability of tendon to structurally adapt to constant or cyclical loads in order to reach biomechanical equilibrium (Einhorn et al., 2007).
Tendon’s unique set of mechanical properties is controlled by the location-dependent and site-dependent composition and structure of the tissue, primarily by its extracellular matrix. This matrix, comprised predominantly of collagen type I, is organized in a hierarchical manner parallel to the mechanical axis of the tendon (Birk et al., 1995; Birk et al., 1989; Birk and Trelstad, 1986; Birk et al., 1997). In addition to collagen I, the extracellular matrix is composed of quantitatively minor collagens, elastin and fibrillins, proteoglycans (PGs) and their glycosaminoglycan (GAG) chains, glycolipids, and cellular material (Woo et al., 2005). The development of this unique structure is modulated mainly by proteoglycans and minor collagens, such as collagen types V, XI, XII, and XIV. Collagen V, although a quantitatively minor component (~2%) in mature tendon and ligament composition, is a major regulator of fibrillogenesis. Collagen V plays a critical role during the early process of fibril nucleation and reduction of collagen V expression during this process results in fewer collagen I fibrils with increased diameters in tendons, ligaments, dermis and cornea (Segev et al., 2006; Sun et al., 2015b; Wenstrup et al., 2004; Wenstrup et al., 2006; Wenstrup et al., 2011).

A clinical representation of collagen V reduction is the classic form of Ehlers-Danlos Syndrome (EDS), with more than 50% of patients being haploinsufficient for COL5A1 (Malfait et al., 2005; Symoens et al., 2012). These patients exhibit hyperextensible skin, joint hypermobility and instability, as well as abnormal wound healing and scarring, suggesting a crucial role for collagen V in both structure and function of tendons and ligaments. Recent studies with both an established haploinsufficient Col5a1+/− classic EDS mouse model and a tendon/ligament-specific collagen V-null model have demonstrated altered fibril structure and inadequate macroscale tensile mechanical function (Connizzo et al., 2015; Sun et al., 2015a; Wenstrup et al., 2006; Wenstrup et al., 2011). In addition, evidence suggests that the role of collagen V in establishing tendon mechanical properties is tissue-specific, alluding to a limited understanding of the mechanisms by which structure relates to mechanical function. The goal of this study was to define the relationship between hierarchical collagen structure and function by evaluating the dynamic mechanical properties of collagen V heterozygous and null tendons at a hierarchy of
length scales, including fibril, fiber and whole-tissue levels. We hypothesized that collagen V-deficiency would result in impaired dynamic mechanical function at all length scales, with a stronger phenotype in the null than heterozygous mice. We also hypothesized that changes in dynamic responses at nanoscale (fibril) and microscale (fiber) (collagen re-alignment, deformation, sliding) are more apparent than those at the tissue-level, and can be used to interpret the tissue-level changes.

B. Methods

Three hundred male mice from three different groups were used in this IACUC-approved study: C57BL/6 control (WT), Col5a1^{+/+} (HET), and a tendon/ligament specific collagen V null model, Col5a1^{Δten/Δten} (NULL) (Sun et al., 2011; Sun et al., 2015a). Mice were bred to 120 days of age and sacrificed humanely, at which point shoulders were frozen at -20°C until dissection for mechanical testing. To prepare tendons for mechanical testing, supraspinatus (SST) tendons were dissected from the shoulder and surrounding soft tissue was carefully removed (Connizzo et al., 2013; Connizzo et al., 2014b). Tendon cross-sectional area was measured using a laser-based device and Verhoeff's stain lines were applied for optical tracking (Beason et al., 2013; Carpenter et al., 1998; Connizzo et al., 2013; Derwin et al., 1994). The humerus was fixed in a polymethylmethacrylate (PMMA) pot while the tendon was positioned between a sandpaper–cyanoacrylate construct. Both sides were then placed in custom grips for testing with a gage length of 2.5mm. All analyses below were performed at both the insertion site and midsubstance of the supraspinatus tendon.
**B-1. Dynamic Viscoelastic Testing**

![Diagram of Viscoelastic TestingProtocol](image)

**Figure 3-1.** Viscoelastic mechanical testing protocol (left), depicting points at which alignment maps were captured (blue circles). Analysis of ramp to failure (middle) and dynamic data (right).

Twenty specimens from each group designated for dynamic viscoelastic testing were subjected to a testing protocol (Fig. 3-1) as described previously (Dourte et al., 2012; Dourte et al., 2013a; Dunkman et al., 2013). Tendons were first preloaded and preconditioned for 10 cycles to 1.5% grip strain at 0.25 Hz. Following a five minute hold, tendons underwent three stress relaxations (to 4%, 6%, and 8% grip strain) at 5%/s with sinusoidal frequency sweeps superimposed upon the static strain. Each frequency sweep consists of 10 cycles of 0.125% amplitude sinusoidal grip strain at frequencies of 0.1, 1, and 10 Hz. The tendons were then returned to zero displacement and then subjected to a final ramp to failure at 0.05%/s. Images were captured during the ramp to failure and analyzed for standard tensile mechanical properties using optical tracking using custom software (Matlab, Natick, MA), as described previously (Ansorge et al., 2011; Connizzo et al., 2013; Dourte et al., 2013b). A bilinear curve fit was also applied to the optical stress-strain data to quantify transition stress, transition strain and the moduli in the toe- and linear-regions (Fig. 3-1). In addition, the dynamic modulus $|E^*|$ (defined as the stress amplitude divided by the strain amplitude) and the phase angle $\delta$ (between the stress and strain) were computed at each frequency and strain level. The dynamic modulus ($|E^*|$) represents how difficult the material is to deform under dynamic loading, while the tangent of the phase angle $\Tan(\delta)$ is the ratio of loss over storage moduli (Dourte et al., 2012; Dourte et al., 2013b; Dunkman et al., 2014; Dunkman et al., 2013). Comparisons were made using one-way
ANOVA for each parameter with post-hoc Bonferroni tests to correct for family-wise error associated with multiple comparisons.

**B-2. Fatigue Testing**

![Fatigue Testing Protocol](image)

**Figure 3-2.** Fatigue testing protocol (above) and analysis (right)

An additional twenty specimens from each group were subjected to a fatigue loading protocol (Fig. 3-2), as described previously (Freedman et al., 2014; Freedman et al., 2015). Preliminary studies determined that the mean failure loads were significantly different between groups and as a result, all tendons were fatigue tested at 1Hz between 20-75% of their ultimate failure load (0.75N-2.75N for wild type and heterozygous groups, 0.25-1.0N for the null group). As described previously, several parameters were computed (Fig. 3-2): (1) maximum cyclic strain, (2) tangent stiffness (calculated as the slope between the maximum and minimum force and displacements for each cycle), (3) hysteresis (defined as the area enclosed by the stress–strain curve for a cycle), (4) damage (defined as the ratio of displacement and gauge length at a set threshold to the tissue displacement and displacement at a set threshold after the first cycle of fatigue loading, and (6) cycles to failure (defined as the number of cycles until specimen failure) (Duenwald-Kuehl et al., 2012; Freedman et al., 2015; Provenzano et al., 2002). Comparisons were made using one-way ANOVA for each parameter with post-hoc Bonferroni tests.

**B-3. Collagen Fiber Re-Alignment**

Collagen fiber re-alignment (n=20/group) was quantified during the dynamic viscoelastic testing using our established integrated cross-polarizer technique (Lake et al., 2009; Lake et al.,
At several points during the mechanical test (before and after preconditioning, before and after each stress relaxation event, and throughout the ramp to failure), sets of 13 images were acquired as the polarizers rotate through a 125° range for calculation of fiber alignment. Comparisons of circular standard deviation, a measure of the spread of the distribution of fiber angles (Lake et al., 2009; Miller et al., 2012a; Miller et al., 2012c), was used to determine if significant re-alignment occurred during each portion of the mechanical test. Statistical comparisons between regions of the mechanical test were made using non-parametric Kruskal-Wallis tests at each location.

Figure 3-3. (A) Analysis of re-alignment during stress relaxation tests. (B) Analysis of re-alignment during ramp to failure.

Re-alignment during the stress relaxation tests were split into two parameters (Fig. 3-3A). The first compared the change in circular standard deviation between 0% (after preconditioning) and 4% peak strain time point, which represent toe region strain levels (Toe Re-Alignment). The second compared the circular standard deviation between 4% peak strain and 8% peak strain, which represent linear region strain levels (Linear Re-Alignment). In addition, re-alignment during the ramp to failure exhibited bilinear behavior with a linear decrease in circular standard deviation followed by a plateau (Fig. 3-3B). The strain required to reach the plateau and the amount of re-alignment that occurred (change in circular standard deviation) was measured for each specimen using a bilinear curve fit. Statistical comparisons were made between these measures using one-way ANOVA for each parameter with post-hoc Bonferroni tests.
**B-4. Collagen Fiber Uncrimping**

![Diagram of load vs. displacement showing steps at 0%, 1%, 3%, 5%, and 7% strain.]

**Figure 3-4.** Testing protocol for fiber uncrimping and fibril deformation/sliding.

Samples for collagen fiber uncrimping and fibril deformation/sliding were prepared for mechanical testing and subjected to a standard preload and preconditioning prior to the ramp to failure. Tendons were then divided into 5 groups (n=20/group) and stretched to a randomly assigned grip-to-grip strain value (0, 1, 3, 5, or 7%) at a rate of 0.1% strain per second (Fig. 3-4). Tendons were then frozen using freezing spray, placed in a specimen dish with tissue freezing medium, and submerged in liquid nitrogen. Samples were frozen sectioned at 20 microns and immersed in cold 10% neutral buffered formalin for 4 minutes for fixation. At this point, samples were imaged using atomic force microscopy for fibril deformation/sliding analysis (described in detail below). Samples were then stained with Picosirius Red and Hematoxylin and analyzed using previously validated quantitative methods with custom software (Miller et al., 2012d; Miller et al., 2012e). Collagen fiber uncrimping was compared from one strain level to the next within each group using non-parametric Kruskal-Wallis tests.

**B-5. Fibril Deformation and Sliding**

Samples for collagen fibril deformation/sliding were prepared for mechanical testing and subjected to a standard preload and preconditioning prior to the ramp to failure. Tendons were then divided into 5 groups and stretched to a randomly assigned grip-to-grip strain value (0, 1, 3,
5, or 7%) at a rate of 0.1% strain per second (See Chapter 2). Tendons were then immediately frozen using freezing spray, placed in a specimen dish with tissue freezing medium, and submerged in liquid nitrogen (Connizzo et al., 2014b; Miller et al., 2012d). Samples were frozen sectioned at 20 microns and immersed in cold 10% neutral buffered formalin for 4 minutes for fixation. For analysis of fibril deformation and sliding, imaging of 2 \( \mu \text{m} \times 2 \ \mu \text{m} \) regions was performed by tapping mode imaging using NCHV-A probes (nominal spring constant \( k \approx 42\text{N/m} \), radius \( R \approx 10 \text{ nm} \)) and a Dimension Icon AFM (BrukerNano, Santa Barbra, CA) using a modified protocol described previously (Connizzo et al., 2014b; Rigozzi et al., 2013; Rigozzi et al., 2011). Tendons were scanned at 2-3 regions across the width of the insertion site and midsubstance of the tendon, and from 4-6 sections throughout the depth of the tendon (See Chapter 2). Custom software allowed for the measurement of d-period length for many fibrils in a single image (Connizzo et al., 2014b). Variability between and within specimens was determined to not be significantly different and therefore fibrils were pooled across approximately 5 specimens sampled per strain level per group. Median and variance were obtained from fibril d-period distributions. Fibril stretch was calculated by subtracting the d-period length at each applied strain level by the initial d-period length. A change in fibril d-period variance from one strain to the next is indicative of strain heterogeneity between fibrils, or fibril sliding (Connizzo et al., 2014b). Statistical comparisons of d-period length across different strains were made using non-parametric Kruskal-Wallis tests followed by post-hoc Dunn’s tests between strain levels. Comparisons of variance across strain levels were performed using a Bartlett’s test for unequal variances at each location with post-hoc F tests between strain levels. For all statistical comparisons in this study, a p-value of \( p < 0.05 \) was considered statistically significant.
C. Results


**Figure 3-5.** (A) Body weight, (B) Tendon cross-sectional area, (C) Maximum stress, and (D) Maximum strain. Data is reported at mean ± standard deviation.

Body weight and whole tendon cross-sectional area (Fig. 3-5A,B) were significantly reduced in the heterozygous and null groups compared to the wild type group. Maximum load (not shown) and maximum stress were also significantly reduced in the null group, but there were no differences between the heterozygous and wild type groups (Fig. 3-6C). Maximum tissue strain, determined optically, also was increased in both groups at the midsubstance, but not at the insertion site (Fig. 3-6D).
Figure 3-6. (A) Transition strain, (B) Transition stress, (C) Toe modulus, and (D) Linear modulus. Data is reported at mean ± standard deviation.

Transition strain was not different between the groups in either region (Fig. 3-6A), but transition stress was significantly less in the null group than the wild type group at the insertion site (Fig. 3-6B). The moduli in both toe and linear regions were reduced in the null group at both sites (Fig. 3-6C,D). The heterozygous group also exhibited reduced toe modulus at the midsubstance and reduced linear modulus at the insertion site (Fig. 3-6C,D).
Dynamic modulus increased and tangent delta decreased with increasing strain level as previously reported (Fig. 3-7). Dynamic modulus was significantly reduced in the null group at all frequencies compared to the wild type group at both 6% and 8% strain and compared to the heterozygous group at 8% strain (Fig. 3-7A). Tangent delta of the null group was significantly greater than both the wild type and heterozygous groups at all frequencies at both 6% and 8% strain levels (Fig. 3-7B). At 4% strain, the null and heterozygous groups were no different and the null group was higher than the wild type group at 0.1Hz and 1.0Hz. Finally, failure generally occurred at the insertion site, at the midsubstance or just below the top grip. The null tendons
failed least often at the insertion site, while the other two groups displayed failure at all three locations equally.

C-2. Tissue-level Fatigue Mechanics

![Fatigue parameters](chart)

**Figure 3-8.** Fatigue parameters (A) Cycles to Failure, (B) Damage, (C) Tangent stiffness, (D) Peak cyclic strain, and (E) Hysteresis. Data is reported at mean ± standard deviation.

The heterozygous group exhibited approximately 40% fewer cycles to failure than the wild type group (Fig. 3-8A). No statistical comparisons were made between the null group and
other groups since the loading protocols were different, but the null group was lower than both other groups. Damage was significantly decreased only in the initial phase (5%) of fatigue life in the null group (Fig. 3-8B). Peak cyclic strain and damage increased while tangent stiffness and hysteresis decreased throughout fatigue life, as previously reported (Freedman et al., 2014; Freedman et al., 2015). The null group had a significantly increased peak cyclic strain than the heterozygous group at 50% and both groups at 95% fatigue life (Fig. 3-8C). Tangent stiffness was reduced in the null group compared to the other groups at all three points in fatigue life (Fig. 3-8D). The heterozygous group also had an increased tangent stiffness when compared to the wild type group at 50% fatigue life. Hysteresis was significantly reduced in the null group when compared to the wild type and heterozygous groups at all three stages of fatigue life (Fig. 3-8E).

C-3. Collagen Fiber Re-Alignment

In both the insertion and midsubstance of the wild type groups, re-alignment occurred first following an increase in strain applied to the tissue (after the peak of stress relaxation events at 4%, 6%, and 8% strain). At the insertion site, the collagen fibers of the heterozygous and null groups re-aligned after the first two strain levels, but not with the final strain increase at 8% strain (Data not shown). At the midsubstance, the heterozygous group re-aligned initially during the preconditioning and then again during each subsequent strain level, while the null group again only re-aligned after the addition of 4% and 6% strain. This is evidenced by comparing the circular standard deviation between 0% and 4% strain (Fig. 3-9A) to denote toe region re-alignment, which occurs in all groups, and between 4 and 8% strain (Fig. 3-9B) to denote linear region alignment, which is reduced in the null and heterozygous groups than in the wild type group. All of the groups returned to a more disorganized state with the removal of strain (at the return to zero following the last stress relaxation) and then re-aligned again during the ramp to failure. However, the null group re-aligned fully at an early strain than both the heterozygous and wild type groups at the midsubstance (Fig. 3-9C) and re-aligned less over that time period than the wild type group at the insertion site (Fig. 3-9D).
Figure 3-9. (A) Toe Region Re-Alignment (B) Linear Region Re-Alignment, (C) Strain Required to Fully Re-Align, and (D) Amount of Full Re-Alignment. Data is reported at mean ± standard Deviation.
Figure 3-10. Crimp frequency over applied strain for (A) wild type, (B) heterozygous, and (C) null tendons. Data is reported at mean ± standard deviation.
None of the groups had significantly decreased crimp frequency (indicating uncrimping) during the applied strain levels from 0% to 7% (Fig. 3-10). This likely suggests that our study design was not adequate to detect uncrimping earlier in the application of load, i.e. prior to the ramp to failure. In a small pilot study, wild type tendons (n=5 per group) were tested at three additional points: (1) just before the preconditioning cycles, (2) just after the preconditioning, and at the (3) 0% applied strain. Results demonstrated that crimp frequency increases during the preconditioning cycles and decreases during the hold following preconditioning (Fig. 3-11). Therefore, we conclude that uncrimping was likely occurring during the hold and was not detected in this study.

![Crimp frequency data for additional pilot points: Before preconditioning (PC), after preconditioning, and 0% applied strain.](image)

**Figure 3-11.** Crimp frequency data for additional pilot points: Before preconditioning (PC), after preconditioning, and 0% applied strain.

**C-5. Fibril Deformation**

Fibril d-period at 0% strain in the heterozygous group was increased at the insertion site and decreased at the midsubstance compared to both other groups (data not shown). Differential
fibril deformation was observed at the insertion compared to the midsubstance. At the insertion site of the wild type tendons, collagen fibril d-period showed a bimodal response, with an initial hold to 1% applied strain, followed by another increase at 5% applied strain with a decrease in d-period between them at 3% applied strain (Fig. 3-12A). At the midsubstance of the tissue, the fibril d-period increased monotonically, peaking at 3% applied strain and decreasing following to 7% strain (Fig. 2-13A, middle). In the heterozygous group, the insertion site showed a similar trend to the wild type group, but with a larger decrease at 3% applied strain (Fig. 3-12B). At the midsubstance, the heterozygous group showed an initial increase in d-period at 1% and 3% applied strain, but this was followed by no change in d-period length rather than the typical decrease attributed to fibril failure (Fig. 3-12B, middle). In addition, initial fibril d-period in the heterozygous group was increased at the insertion site and decreased at the midsubstance when compared to the other groups. At the insertion site of the null group, there was an initial decrease of fibril d-period at 1% applied strain followed by an increase at 3% applied strain and a decrease thereafter (Fig. 3-12C, left). At the midsubstance, the null tendon d-period initially decreased at 1% and increased thereafter with no apparent decrease in fibril d-period near the end of the test (Fig. 3-12C, middle).

C-6. Fibril Sliding

Fibril sliding is indirectly measured in this study by changes in the variance of fibril d-periods, which would suggest heterogeneity in fibril stretch and therefore fibril sliding. At the insertion site of the wild type group, fibril sliding occurred in the insertion site between 1% and 3% applied strain (Fig 3-12A, right). Contrary to our previous study (Connizzo et al., 2014b), we did not find any significant fibril sliding occurring in the midsubstance of the wild type group. In the heterozygous group, fibril sliding occurred between 0 and 1% applied strain at the insertion site, between 1 and 3% applied strain at the midsubstance, between 3 and 5% applied strain in both regions and between 5 and 7% applied strain at the insertion site (Fig 3-12B, right). The null group did not exhibit any significant fibril sliding (Fig 3-12C, right).
D. Discussion

This study measured the dynamic multi-scale mechanical response of collagen V heterozygous and null supraspinatus tendons and determined the relationship between dynamic responses at multiple hierarchical scales. At the macroscale, almost every material parameter exhibited a dose-dependent decrease in properties as collagen V expression decreased from the wild type to the heterozygous and null groups, particularly at high strains and high frequency loading. This is consistent with several previous studies in multiple tendons (Connizzo et al., 2015; Sun et al., 2015a; Wenstrup et al., 2006). Interestingly, there were few differences between the groups at low levels of strain, suggesting the heterozygous and null groups perform similarly.
to the wild type group initially. Taken with larger failure strains in the heterozygous and null groups, these groups exhibit a longer, low stiffness stress-strain curve, which could support increased elasticity with low strain/low load exercise, as reported in clinically. However, both experimental groups were also unable to withstand the same repetitive cyclic loading as the wild type group, suggesting earlier damage accumulation due to inferior dynamic responses. While the null tendons were more viscoelastic initially and with low levels of strain (increased tangent delta, relaxation), the null tendons were unable to recover lost fluid during high and repetitive fatigue loading, resulting in early failure. The heterozygous group, which exhibited only small changes in many other macroscale properties, was significantly affected by the cyclic loading protocol, suggesting that the dynamic responses governing recovery from repetitive cyclic strain were diminished in this group (Freedman et al., 2014; Freedman et al., 2015; Shepherd et al., 2014; Thorpe et al., 2014a, b).

As hypothesized, a larger diminished response at the microscale than the macroscale was revealed in both the heterozygous and null groups in the measures of collagen fiber re-organization. At the microscale, collagen fiber re-alignment occurred earlier or with a smaller application of strain in both the heterozygous and null groups. While an earlier response to load could be seen as a positive trait, this also could suggest that the protection of fibrils from strain via other dynamic mechanisms are occurring earlier and subsequent damage can then accumulate in the tissue at a lower strain level. In fact, earlier re-alignment with decreased overall mechanical properties has been reported before in a mouse model of disease (Connizzo et al., 2014a). In addition, the null group re-aligned less than the wild type group. Given no significant difference in the initial tissue organization was detected between groups, this suggests that the null group also has reduced capacity to re-align overall. Reduced ability to re-align could be associated with the lack of or deterioration of inter-fiber connectivity such as in development or aging, (Connizzo et al., 2013; Miller et al., 2012e) or by alterations in the extracellular matrix.

Furthermore, the heterozygous and null groups also exhibited an altered fibril response to load as well. Early structural re-organization resulting in fibril strain reduction at the insertion site
appeared to be larger in the heterozygous group and earlier in the null group, confirming our results at the microscale. Furthermore, the fibrils also exhibited increased strain at an earlier applied strain level, implying an earlier response at the nanoscale. Unlike the wild type group which exhibited a coordinated response of fibril stretch and sliding consistent with previous studies (Connizzo et al., 2014b; Szczesny and Elliott, 2014; Thorpe et al., 2013b), the heterozygous and null groups had more unclear stretch/sliding relationships. This study revealed a significant amount of fibril sliding in the heterozygous group and no sliding in the null group. Both results (increased and decreased fibril sliding) imply that not only are the fibrils themselves altered by the reduction of collagen V expression during development, but that the interfibrillar matrix is most likely also altered (Thorpe et al., 2013a; Thorpe et al., 2012).

With all of the multi-scale results taken together, these results suggest different mechanisms by which the experimental groups attempt to withstand the same loading as the wild type group. The wild type group is able to reduce stress at the lower hierarchical scales (fibers, fibrils) through a series of coordinated dynamic responses, specifically collagen re-alignment and sliding. The heterozygous group compensates for the lack of fibril strength via earlier re-alignment and a large amount of fibril sliding. The sliding reduces strain on the collagen fibrils initially, and thus, prevents early fibril failure, but with a large amount of repeated fibril sliding, the fibrils eventually pull away from each other and fail in shear, as is evidenced by reduced cycles to failure during fatigue loading. This allows the heterozygous group to respond elastically but only at low strain levels, which is consistent with clinical observations (Castori et al., 2011; Malfait et al., 2005; Nielsen et al., 2014; Nordschow and Marsolais, 1969; Wenstrup et al., 2006). In contrast, the null group also responds early to load, but is incapable of producing significant fibril sliding, and therefore, the tendons fail earlier and with lower maximum loads.

These results provide insight into how structural alterations ultimately lead to functional deficiencies, particularly in the classic EDS patient population. Given that the heterozygous and null tendons exhibited an earlier response to load at multiple hierarchical scales, it is possible that some sub-failure damage was already present in these tendons prior to our experiments due to
normal in vivo activities. This makes determining the cause and effect of these mechanical deficiencies difficult, particularly without knowledge of in vivo tendon strain levels and alterations in activity in the collagen V deficient mice. Nevertheless, these studies suggest that repeated loading may be a mechanism for EDS-related hypermobility. While classic EDS patients may be able to perform reasonably during low impact activity, increased duration or magnitude could cause tendon damage which would ultimately lead to rotator cuff weakness, instability and injury. However, further work is necessary to confirm this hypothesis, particularly since the rotator cuff tendons act as dynamic stabilizers of the joint and EDS patients are often plagued primarily by insufficient static stabilization.

While this study provides a unique set of multi-scale mechanical parameters to understand hierarchical structure-function relationships, it is not without limitations. First and foremost, this study sought to investigate relationships between dynamic parameters but direct comparisons could not be made between these assays due to the inability to measure multiple hierarchical scales in a single measurement using the assays performed here. Additionally, this study was unable to reveal changes in the timing or quantity of collagen fiber uncrimping which could further elucidate changes at the microscale. Finally, no compositional or structural measures are reported. This is particularly important as many of the dynamic responses studied here are functions of the innate structure and composition of the tissue. For example, alterations in fibril sliding and deformation could be caused by modifications to fibril morphology, such as increased interconnectivity of fibrils due to branching or interfibrillar crosslinking, or to the alterations in the interfibrillar matrix, where a decrease in stiffness would facilitate fibril movement. Similarly, increases of viscoelastic or elastic proteins (proteoglycans or elastin, respectively) could be responsible for some of the mechanical changes found here. Ongoing studies are focused on measuring these changes in composition, organization and overall structure to investigate why these dynamic mechanical responses are altered.

Altogether, these studies highlight the relationships that exist between fibril, fiber, and tissue level mechanical function in the context of collagen V heterozygous and null tendons.
Investigation into dynamic responses at the microscale and nanoscale could provide more information to elucidate the mechanisms by which mechanical function at the macroscale is ultimately altered. Furthermore, these studies suggest that these unique set of dynamic processes provide normal tendons with a series of protective measures to prevent early failure. It is likely that these mechanisms are the initiation of large scale tissue damage, and ultimately this work could aid in the goal of improving tendon repair techniques or developing tissue-engineered constructs for tendon replacement.

E. References


CHAPTER 4: COLLAGEN V EXPRESSION IS CRUCIAL FOR PROPER DEVELOPMENT OF SUPRASPINATUS INSERTION SITE STRUCTURE AND COMPOSITION

A. Introduction

Classic Ehlers-Danlos Syndrome (EDS) is an inheritable disease that affects approximately 1 in 20-40,000 people worldwide. Clinical manifestations of the disease include joint hypermobility, skin hyperextensibility, and poor wound healing (Ainsworth and Aulicino, 1993). These functional deficiencies can often lead to recurrent joint dislocations, particularly at the knee and shoulder (Ainsworth and Aulicino, 1993; Stanitski et al., 2000), as well as to injury and disease. The major disease-causing mechanism of classic EDS is reduced availability of collagen V, with collagen V mutations identified in over 90% of patients (Malfait and De Paepe, 2014; Symoens et al., 2012) and approximately half are null-allele mutations resulting in COL5A1 haploinsufficiency (Malfait et al., 2005; Malfait and De Paepe, 2005; Symoens et al., 2012).

Collagen V is a fibril-forming collagen that can be found as heterotypic fibrils with collagens I, II, and III (Wenstrup et al., 2006; Wenstrup et al., 2011). Although it is a minor component of mature tendon composition quantitatively, collagen V plays a critical role in fibrillogenesis, specifically by nucleating fibril formation. Manipulation of these collagens both in cell culture and mouse models have demonstrated that reduction of collagen V results in decreased fibril assembly and increased fibril diameters in a number of tissues, including cornea, anterior cruciate ligament, and flexor digitorum longus tendon (Sun et al., 2011; Sun et al., 2015; Wenstrup et al., 2006). However, the role of collagen V in mature tissue is unknown. Collagen V does play a role in tendon healing after injury and classic Ehlers-Danlos syndrome patients do exhibit alterations in wound healing (Kivirikko and Risteli, 1976; Malfait and De Paepe, 2005; Wenstrup et al., 2006). Given that other regulators of fibril assembly, specifically decorin and biglycan, have recently been found to play significant roles in aging (Connizzo et al., 2013;
Dunkman et al., 2013), it is likely that collagen V also exists at a low level in mature tendon to assist in remodeling of the fibrillar matrix during these processes.

Due to the strong role of collagen structure in determining mechanical properties, alterations in fibril morphology resulting from reduced collagen V expression are thought to be responsible for the functional deficiencies in the EDS patient population. Recent mechanical evaluations have confirmed this hypothesis with collagen V deficient mouse tendons exhibiting as much as 80% reduction in tissue modulus (Connizzo et al., 2015a; Sun et al., 2015; Wenstrup et al., 2011). However, these changes were tissue-dependent, with the strongest changes found in the knee and rotator cuff (Connizzo et al., 2015a). It's still unclear whether the role of collagen V in determining tendon structure and composition is also tissue-dependent since previous tissues that have been characterized are highly organized and homogeneous. Characterization of a more complex tendon, such as the supraspinatus tendon, could provide insight into what factors contribute to the differential regulation of fibril assembly by collagen V.

Therefore, the objective of this study was to determine the effect of reduced collagen V expression in determining the multiscale structure and composition of mouse supraspinatus tendons, specifically on parameters that may contribute to mechanical function. We accomplished this using our established traditional heterozygous collagen V and tendon/ligament specific collagen V-null mouse models. We hypothesized that reduction of collagen V expression would result in altered fibril morphology (increased fibril diameters and decreased fibril density) with no additional alterations in cell morphology, fiber morphology or extracellular matrix (ECM) composition.

B. Methods

B-1. Animals and Sample Collection

A total of 144 mice from three genotypes were used in this study: C57BL/6 control (WT), Col5a1\textsuperscript{+/−} (HET), and a tendon/ligament specific collagen V-null model, Col5a1\textsuperscript{Δten/Δten} (NULL) (Sun et al., 2011; Sun et al., 2015). Animal use was approved by The University of Pennsylvania
and University of South Florida’s Institutional Animal Care and Use Committees. All mice were bred to 120 days of age by the authors at the University of South Florida and shipped to the University of Pennsylvania following sacrifice and tissue harvest.

**B-2. Collagen Fiber Crimp Morphology**

Tendons designated for assessment of crimp morphology (n=20/group) were prepared for mechanical testing as described and preconditioned for 10 cycles between 0.02 and 0.04N (Connizzo et al., 2013; Connizzo et al., 2014b). Following a 1 minute hold after preconditioning, tendons were immediately flash frozen (Connizzo et al., 2014b; Miller et al., 2012b), detached from mechanical testing setup and embedded in tissue freezing medium for frozen histology. Samples were sectioned at 20 µm and stained with Picrosirius Red and Hematoxylin. At least two images at each region (insertion, midsubstance) were taken under polarized light per sample. Images were analyzed using custom quantitative software (Matlab, Natick, MA) to calculate crimp frequency and amplitude as determined by pixel fluctuations along the length of a collagen fiber (Miller et al., 2012a; Miller et al., 2012b). Values from multiple images were averaged within a specimen.

**B-3. Fibril Morphology**

Tendons designated for fibril morphology analysis (n=20/group) were prepared as described previously (Dunkman et al., 2013). The sample was immediately fixed in "Karnovsky's Fixative," a specialized solution containing 4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate and 8 mM CaCl2, adjusted to pH 7.4 with NaOH. Samples were then placed on a rocker in a 4 °C cold-room for at least 2 h. Tendons were then rinsed with cacodylate buffer and post-fixed for 1 h with 1% osmium tetroxide. They were then dehydrated in an ethanol series followed by 100% propylene oxide, infiltrated and embedded over a 3 day period in a mixture of Embed 812, nadic methyl anhydride, dodecenylsuccinic anhydride and DMP-30 (EM Sciences, Fort Washington, PA) and polymerized over-night at 60 °C. Just prior to embedding, tendons were cut in half to separate the insertion site and midsubstance of the tissue for analysis. Ultra-
thin, approximately 90 nm, cross-sections were prepared using a Leica ultramicrotome and post-
stained with 2% aqueous uranyl acetate and 1% phosphotungstic acid, pH 3.2. The sections were
examined and imaged at 80 kV using a JEOL 1400 transmission electron microscope (JEOL Ltd.,
Tokyo, Japan) equipped with a Gatan Orius widefield side mount CC Digital camera (Gatan Inc.,
Pleasanton, CA).

Ten digital images from each tendon were taken from non-overlapping areas at ×60,000.
Images were randomized and masked before fibril diameters were measured using a RM
Biometrics-Bioquant Image Analysis System (Nashville, TN). A region of interest (ROI) of
appropriate size was determined within the image so that a minimum of 80 fibrils were measured
from each image. All fibrils in the region of interest were measured and multiple regions of
interest were used if necessary to collect at least 80 fibril diameter measurements per image.
Fibril diameters were measured along the minor axis of the fibril cross-section. Fibril density was
obtained as the fibril number per unit area. A measure of fibril roundness, fibril irregularity factor
(FIF), was defined as the ratio of the radius as determined from a circle with the fibril’s perimeter
to the radius as determined from a circle with the fibril’s area, where increasing values would
define an increasing number of folds along the surface of the fibril.

**B-4. Cell Morphology**

For assessment of cell morphology, supraspinatus tendons (n=8/group) were grossly
harvested from the shoulder, leaving the muscle and bony insertions intact. Specimens were
immediately fixed, decalcified, and processed for paraffin-embedding using standard techniques.
Coronal sections were cut to 7μm thickness and stained with hematoxylin and eosin (H&E). The
insertion site and midsubstance of each sample was then imaged at 20x and evaluated using
BioQuant software (Bioquant Image Analysis Corporation, Heidelberg, Germany) for cellularity
and cell shape (Connizzo et al., 2014c; Dunkman et al., 2013). Briefly, cellularity was calculated
as a cell density per area of region analyzed. Cell shape was measured as the average aspect
ratio of nuclei in a region of interest on a scale from 0 to 1, where 1 represented a perfect circular shape.

**B-5. Biochemistry**

Tendons designated for biochemistry (*n*=20/group) were dissected for location-dependent analysis as described previously (Connizzo et al., 2014a). All tendons were removed with muscle intact from the insertion site. The muscle was then removed with a scalpel blade to leave only the tendon portion. A consistent piece of the insertion and the midsubstance was taken from each tendon by splitting the tissue into two even 1-mm regions. Tendons were then digested in a Proteinase K and Ammonium Acetate solution overnight at 37°C. DNA content and GAG content in the digest were quantified using the PicoGreen and dimethylmethylene blue (DMMB) assays, respectively (Ansorge et al., 2011; Singer et al., 1997). The remaining digest was hydrolyzed with hydrochloric acid, resuspended in assay buffer and used to quantify total collagen (COL) using the hydroxyproline (OHP) assay (Ansorge et al., 2011; Dourte et al., 2012) and pyridinoline crosslinks (PYD) using the MicroVue PYD EIA kit (Quidel Corp., San Diego, CA). GAG and COL content were normalized to DNA content to account for differences in tissue size between samples. PYD content was normalized to total COL content.

**B-6. Elastin Immunofluorescence**

Tendons designated for immunofluorescence (*n*=10/group) were taken from extra frozen sections prepared for fiber crimp morphology as described in Section B-2. Sections were first subjected to antigen retrieval via 5mg/mL hyaluronidase for 1 hour at 37°C. Sections were then treated with 0.1% sodium borohydride for 15 minutes at room temperature followed by blocking the samples with 5% goat serum for 1 hour at room temperature. Samples were then washed with 1x PBS and incubated at 4°C overnight with mouse anti-rabbit primary antibody against elastin (Abcam, Cambridge, MA). Following a 1x PBS wash, sections were then treated for 1 hour at room temperature in the dark with goat anti-rabbit Alexa Fluor 488 secondary antibody (Abcam, Cambridge, MA). Finally, samples were washed again in 1x PBS and mounted with
prolong Gold Antifade Mountant with DAPI (Life Technologies, Carlsbad, CA). Two images per sample were taken at each region using fluorescent microscopy. All images were normalized to a negative control sample which was subjected to the same staining conditions except for the primary antibody. A positive control sample, dorsal skin from wild type mouse, was also used to compare stain intensity visually. Custom stain intensity software was used to calculate stain intensity for each specimen by converting the fluorescent image into grayscale and measures the average intensity of pixels above a threshold in the region of interest. Results were averaged between the images per region.

**B-7. Statistical Analysis**

All data was analyzed using a one-way ANOVA with post-hoc Bonferroni-corrected t-tests. Comparisons were only made between the genotypes and not between the insertion site and midsubstance in order to test the specific hypotheses of the study. Statistical significance was set at p<0.05 and a trend was noted at p<0.10.

**C. Results**

**C-1. Joint Morphology**

![Figure 4-1](image)

*Figure 4-1.* Joint morphology and whole supraspinatus tendon morphology from wild type, heterozygous, and null mice.

The shoulder joint overall in the null mice appeared to have less connective tissue between and surrounding the rotator cuff tendons (Fig. 4-1). In addition, the rotation of the
humerus when the forearm was externally rotated appeared to be increased in the null group, exhibited by the increased angle of the tendon in the dissection images. Once the tendon and humerus was dissected from the animal, the tendons appeared macroscopically to be morphologically similar. The humerus bones were visually smaller in the null group than in the wild type or heterozygous groups and the null tendons appeared to be shorter.

C-2. Fiber Morphology

The collagen fibers of null tendons exhibited altered crimp morphology at the insertion site, with decreased crimp frequency and amplitude (Fig. 4-2). The heterozygous group also displayed decreased crimp amplitude at the insertion site but crimp frequency was unchanged. No differences between groups were detected in crimp frequency or amplitude at the midsubstance.

![Figure 4-2](image)

**Figure 4-2.** (A) Crimp frequency was decreased in the null tendons compared to both other groups at the insertion site. (B) Crimp amplitude was decreased in the heterozygous and null groups at the insertion site. No differences between groups were detected in crimp frequency or amplitude at the midsubstance.
Collagen fibril assembly in wild type, heterozygous and null tendons was analyzed using transmission electron microscopy at the insertion site and midsubstance. Wild type, heterozygous and null tendon collagen fibrils at the midsubstance all exhibited normal circular cross-sectional profiles throughout the tendon extracellular matrix (Fig. 4-3). Fibrils were slightly larger in the heterozygous and null tendons than in the wild type group. In contrast, null tendons at the insertion site exhibited aberrant fibril arrangement and cross-sectional profiles. Larger and smaller diameter fibrils were present with altered fibril arrangement. In addition, null tendons were structurally abnormal, exhibiting irregular cross-sectional profiles.

Wild type tendons exhibited a normal distribution of collagen fibrils in the tendon midsubstance with the median diameter at approximately 70nm (Fig. 4-4). At the insertion site, the wild type fibril distribution was slightly shifted towards smaller diameter fibrils compared to the midsubstance. In the heterozygous tendons, there was an increase in large diameter fibrils at both the insertion and the midsubstance compared to the wild type group. At the midsubstance of
the null group, there was also a shift with increased large diameter fibrils, with diameters greater than both the wild type and heterozygous group. However, the insertion site of the null group exhibited an increase in both large and small diameter fibrils with more positive skew in the distribution than the other groups.

![Histograms showing fibril diameter distribution](image)

**Figure 4-4.** TEM analysis of fibril diameter distribution (n = 10/genotype) for (A/B) wild type, (C/D) heterozygous, and (E/F) null tendons for both insertion site (left column) and midsubstance (right column).
Quantitatively, mean fibril diameters were larger in the heterozygous group than the wild type group at the midsubstance and both groups at the insertion site (Fig. 4-5). The null group had larger fibril diameters than the wild type but only at the midsubstance. Fibril density was decreased in both groups at both the insertion site and midsubstance of the tendon. Interestingly, the null tendons also exhibited some fibrils with irregular fibril morphology (not round), which was not found in the other groups (Fig. 4-5C). When fibril irregularity was quantified, the null group had more irregularly shaped fibrils than both the wild type and heterozygous tendons at both the insertion site and midsubstance.

**Figure 4-5.** Quantitative analysis confirmed that (A) fibril diameter was larger in the heterozygous groups at both regions but only in the null group at the midsubstance. (B) Fibril density was decreased in the heterozygous and null group at the midsubstance and was slightly decreased.
(trend) at the insertion site in the null group as well. (C) Fibril irregularity was quantified and was significantly increased in the null group at both regions.

C-4. Cell Morphology

Figure 4-6. (A) Cell density in tendon proper was slight decreased (trend) at the insertion site of the null group compared to the wild type group. (B) There were no differences in cell shape between groups in either region. (C) Representative images display pockets of hypercellular endotendinous tissue present in null tendons.

Cell density was slightly decreased (trend) in the tendon proper at the insertion site of the null tendons, but there were no differences in any of the groups at the midsubstance (Fig. 4-6). Similarly, there were no differences in cell shape between groups at either the insertion site or midsubstance, although cell shape was always rounder at the insertion site than in the midsubstance. Upon visual inspection of histological sections, small pockets of hypercellular tissue were observed in between collagen fibers of the tendon proper in the null groups (Fig. 4-6C). These pockets were not present in either the heterozygous or wild type groups.
Double stranded DNA content was increased in the null group at the insertion site of the tendon, likely due to the hypercellular pockets of tissue found in histological results which were included in biochemical analysis and not in histological analysis (Fig. 4-7A, Fig. 4-6C). The null tendons also exhibited decreased glycosaminoglycan (GAG) and collagen content at the insertion site compared to both heterozygous and wild type groups (Fig. 4-7B,C). However, no differences in DNA, GAG, or total collagen content were found between groups at the midsubstance. Pyridinoline, a mature collagen cross-link, was increased in the heterozygous group at the insertion site and in the null group at both regions (Fig. 4-7D). Quantification of elastin immunofluorescence staining revealed that the null and heterozygous tendons had decreased
elastin at the insertion site compared to the wild type tendons (Fig. 4-8). Again, there were no differences found at the midsubstance of the tissue.

![Figure 4-8](image)

**Figure 4-8.** Immunofluorescence imaging of elastin and quantification displayed decreased elastin content at the insertion site in both heterozygous and null tendons. No differences were detected between groups at the midsubstance.

D. Discussion

This study was the first to investigate regional collagen structure and extracellular matrix composition at multiple hierarchical scales in mouse supraspinatus tendons. Morphology and structure at the microstructural level was disrupted in both the heterozygous and null tendons. Reduced crimp amplitude and crimp frequency in the experimental groups could be due to alterations in fibril size or distribution (Magnusson et al., 2002; Morgan et al., 2006). Alternatively, alterations in crimp structure have been attributed to changes in ECM content, particularly GAG and elastin, as well as changes in actin cytoskeleton tensioning (Buckley et al., 2013; Grant et al., 2015; Legerlotz et al., 2014; Schiele et al., 2015). Functionally, decreased crimp morphology indicates that the period of uncrimping is shorter, thus putting strain on the fibers and subsequently the fibrils earlier. This could ultimately lead to earlier damage of the tendon structure.

Reduction in collagen V expression in the supraspinatus tendon caused changes in fibril morphology at both regions. In the wild type tendons, the distribution of fibril diameters was shifted towards smaller diameters at the insertion site compared to the midsubstance. Previous
studies have shown that fibril diameter distributions can differ from the bone-tendon junction to the myotendinous junction in superficial digital flexor tendons (Watanabe et al., 2007; Watanabe et al., 2012). Small diameter fibrils provide less overall surface area on which frictional forces can act, thus possibly allowing for more dynamic motion (re-alignment, fibril deformation, sliding, etc.), which has been recently reported in these tendons (Connizzo et al., 2015b). With the reduction of collagen V expression, the midsubstance fibril diameters became larger and fibrils were less dense in both groups, which is consistent with previous literature in other tendons and ligaments (Sun et al., 2015; Wenstrup et al., 2006). A larger but less dense fibril population stems from a decrease in fibril nucleation sites due to the lack of collagen V during fibrillogenesis (Wenstrup et al., 2004b; Wenstrup et al., 2011). This is also present at the insertion site of the heterozygous tendons. Interestingly, the fibril distribution at the insertion site of the null tendons includes an increase in large diameter fibrils but also an increase in small diameter fibrils. An increase in small diameter fibrils has been observed in collagen V heterozygous tendons in the past (Wenstrup et al., 2011), and could indicate increased dynamic motion during movement as discussed above. However, with repeated or increased magnitude of movement, smaller diameter fibrils will not be able to withstand the same amount of loading and will likely suffer early damage.

Fibril irregularity was also present in the groups with reduced collagen V expression, particularly at the insertion site of the null tendons. Fibrils with abnormal shapes were present in many specimens and these fibrils also appeared to be less dense, perhaps due to their irregular shapes. ‘Cauliflower-like’ fibrils have been seen previously in approximately 5% of fibrils visualized in classic EDS patient skin (Hausser and Anton-Lamprecht, 1994), as well as in mouse skin (Wenstrup et al., 2004a) and collagen V and collagen XI heterozygous mouse tendons (Wenstrup et al., 2011). However, the irregular fibrils in our study appeared to have a large center with smaller round protrusions surrounding it, as if a large fibril had fused with several small diameter fibrils around it. However, it is clear from this study that the number of irregular fibrils increased with the reduction in collagen V expression. Irregular fibril shapes could indicate that
fibril assembly is either disrupted or unregulated due to the lack of collagen V expression. Furthermore, this irregular morphology could be associated with fusion of small and large diameter fibrils or could be associated with the interdigitation of fibrils given their presence at the insertion site of the tendon where fibrils from tendon and bone are interacting. In general, irregular fibrils could have an abnormal relationship with the interfibrillar matrix (increased friction, altered ECM production), thus disrupting important dynamic re-organizations during movement (Gupta et al., 2010; Screen et al., 2005; Szczesny and Elliott, 2014). Additionally, aberrant assembly at the fibril level likely also causes disruption of assembly at higher orders, such as the fiber and fascicle level.

Although fiber and fibril morphology was disrupted in the null group, no large differences in cell morphology were found in the tendon proper, except for a slight decrease in cell density. Since it’s likely that these tendons are experiencing the same magnitude of loading during normal cage activity, this suggests that the number or morphology of cells is not directly related to ECM composition or structure alone. An increase in DNA content overall in the tendon and sheaths as determined by biochemical assays though suggests that there were more cells present in the tendon sheaths or in between the collagen fibers. Pockets of hypercellular tissue were observed in histological sections in the null group, particularly in between the tendon fibers. An increase in cell numbers, particularly in the tendon sheaths, could be an injury response to small levels in damage or injury occurring in the tendon (Dyment et al., 2014; Dyment et al., 2013).

Despite the increase in cells in the null tendons, total collagen, elastin, and GAG content were all reduced in the null group compared to the other groups at the insertion site. This could suggest that ECM production is altered with reduced collagen V expression, alluding to possible deficiencies in the tendon healing which have been observed in collagen V heterozygous mouse skin (Wenstrup et al., 2006) and in the EDS patient population (Ainsworth and Aulicino, 1993; Malfait et al., 2010). Interestingly, pyridinoline, a mature collagen crosslink, was reduced in the heterozygous and null groups in both locations. This suggests that the molecular processing of collagen could be altered with the removal of collagen V expression. The increase in pyridinoline
crosslinks and reduction in elastin could allude to alterations in lysyl oxidase activity, which is responsible for collagen and elastin crosslinking and may play a role in fibril shape formation (Herchenhan et al., 2015; Igoucheva et al., 2015; Marturano et al., 2014). An alternative explanation is that crosslink density must be increased to handle stresses on the tendon that cannot be otherwise reduced via dynamic re-organizations during loading.

![Figure 4-9](image)

**Figure 4-9.** (A) Cortical bone, (B) epiphyseal bone, and (C) metaphyseal bone of the humerus from wild type and null mice at 60 days of age postnatal.

Large changes in many of the compositional and structural parameters measured here occurred at the insertion site of the tendon, which is the junction between tendon and bone. Although there is not much literature on the role of collagen V in bone (Roulet et al., 2007) or the incidence of bone fractures in the classic EDS patient population, a small pilot study was
performed to determine if the removal of collagen V expression in the conditionally-targeted tendon/ligament-specific null mouse model affected the humerus (Fig. 4-9). This pilot study showed that at just 60 days of age postnatally, the humerus of tendon/ligament specific collagen V null mice is significantly altered, with decreased and thinner trabeculae in the epiphyseal and metaphyseal trabecular bone above the superior growth plate as well as inferior polar moment of inertia in the humeral head cortical bone. Although there were no differences in mineral density in the cortical bone, bone mineral density in the epiphyseal and metaphyseal trabeculae was reduced in the collagen V null group (not shown), which could affect the insertion site as the attachment of the tendon on the greater tuberosity of the humeral head is adjacent to the growth plate. This suggests that there may be substantial deficiencies in the insertion site of the supraspinatus and other rotator cuff tendons due to abnormal bone development as well as abnormal tendon development. However, this study does not elucidate whether this alteration is due to reduced activity of the animal, and therefore abnormal bone remodeling, or due to dysregulation of proper bone development due to the reduction in collagen V expression. Characterization of non-load bearing bones, such as the skull, could clarify the origins of this abnormal bone structure.

Since this study was the first to rigorously evaluate and define regional changes in mouse supraspinatus composition and structure, several limitations exist. Given the small amount of tissue available, several semi-quantitative measures were made in this study which could have benefited from quantitative metrics, such as ELISA or western blotting techniques. Furthermore, semi-quantitative quantification of elastin via commercially available antibodies may not provide a complete description as specificity has not been determined. Co-localization of elastin with another associated protein, such as fibrillin, would significantly enhance our understanding of regional compositional changes present in the tissue. Although overall joint morphology was visually inspected, no quantification of joint laxity or mechanical evaluation of the auxiliary rotator cuff tendons was performed. This data would provide information on the overall state of the joint in the context of patient-reported increases in shoulder dislocations and instability. Additionally,
fibril measurements were taken from two-dimensional cross-sections to measure collagen fibril diameter, density and irregularity. However, it would be useful to determine if there were any differences in connectivity between fibrils or significant branching effects using three-dimensional fibril tracking. Alterations in fibril branching could ultimately affect dynamic re-organizations of the matrix during loading and are likely important to overall stabilization of the tissue and dynamic mechanical function (Birk et al., 1989; Provenzano and Vanderby, 2006). Future studies will address these limitations and build upon this work.

Altogether, these results demonstrate that the role of collagen V in determining the structure and composition of mouse supraspinatus tendons is location-dependent. Although there were fibril morphology changes without any other compositional change at the midsubstance, the insertion site displayed alterations in almost every parameter. This could be explained by the increased functional requirements that are present at the insertion site compared to the tendon midsubstance or by the presence of two tissues, bone and tendon, that are both affected by reduced collagen V expression during development (Beighton and Thomas, 1969; Roulet et al., 2007). However, the cascade of events that leads to the abnormal structure and composition in the null tendons is still unclear as it’s difficult to specifically separate the effects that one might have on the other and the timing of those effects. Future studies also will build upon this work by investigating the effect of reduced collagen V expression on tendon injury in both a normal and abnormal fibril structure, which could elucidate mechanisms for the development of altered structure and composition as well as further aid in developing a therapeutic target for classic EDS patients.

E. References


A. Introduction

Tendon is a compliant, anisotropic material which has a high modulus under tension, but collapses under compression. This primary uniaxial tensile function along the longitudinal axis of its collagen fibers enables transmission of generated muscle force to bone. Furthermore, tendon exhibits nonlinear biomechanical behavior as demonstrated by a typical stress-strain curve with an initial, non-linear “toe-region” followed by the “linear-region” (Connizzo et al., 2013b). In addition to their anisotropic, non-linear behavior, tendons display viscoelastic properties identified as stress relaxation, hysteresis, and creep (Woo et al., 2000). Clinically, the ability of tendon to demonstrate these properties allows for its ability to both guide movement (low stiffness) and provide stability (high stiffness). Additionally, the dynamic and viscoelastic properties emphasize the ability of tendon to structurally adapt to constant or cyclical loads in order to reach biomechanical equilibrium (Einhorn et al., 2007).

Mechanical function is modulated by a number of dynamic processes that occur at the micro- and nanoscale during mechanical loading, specifically fiber uncrimping, fiber re-alignment, fibril deformation and fibril sliding. These mechanisms are thought to reduce strain on individual fibrils during the initial response to load and ultimately to prevent irreversible damage. Collagen fiber uncrimping has been implicated in the mechanical behavior of tendon, particularly in the non-linear behavior observed in the toe-region of the stress-strain curve (Atkinson et al., 1999; Diamant et al., 1972; Miller et al., 2012b; Woo et al., 2000). In addition to collagen uncrimping, collagen fibers are also capable of shifting their orientation towards the axis of loading, called collagen fiber re-alignment (Lake et al., 2009, 2010; Miller et al., 2012c). Finally, macroscopic tendon extension is enabled by deformation and sliding mechanisms that simultaneously occur between fibers and between fibrils (Rigozzi et al., 2009; Rigozzi et al., 2011; Screen et al., 2005;
Szczesny and Elliott, 2014). Fibril sliding has been cited as a major contributor to tendon viscoelastic behavior and dynamic behavior.

The contribution of these dynamic processes to the mechanical response to load is thought to be highly dependent on region (insertion site versus midsubstance), alluding to the significance of extracellular matrix composition and structure in these processes. This matrix, comprised predominantly of collagen type I, is organized in a hierarchical manner spanning from collagen fibrils which associate together to form fibers, which then further combine to form fascicles, which then bundle to form full tendon (Birk et al., 1995; Birk et al., 1997). In addition to collagen I, the extracellular matrix is composed of minor collagens, elastin, proteoglycans (PGs) with their associated glycosaminoglycans (GAGs), glycolipids, and cellular material (Woo et al., 2005). These elements are all suggested to play a large role in the mechanical function of the tendon. Collagen orientation promotes very high strength in the direction of fiber alignment, which is dependent on the underlying organizational structure of collagen molecules. In addition, collagen fiber crimp may be responsible for some of the nonlinearity of tendon by acting as a shock absorber (Hansen et al., 2002). The extrafibrillar matrix (GAGs, elastin, cells) may also play a role in both quasi-static and dynamic mechanical function (Dourte et al., 2012; Dunkman et al., 2013; Grant et al., 2015; Henninger et al., 2015; Rigozzi et al., 2009) via their abilities to regulate fluid flow and stiffness of the interfibrillar tissue.

A number of recent studies have sought to understand the structure-function relationships present in tendon (Ansorge et al., 2012; Best et al., 1993; Chan et al., 1998; Danielsen and Andreassen, 1988; Derwin and Soslowsky, 1999; Freedman et al., 2015; Hansen et al., 2010b; Haut et al., 1992; Parry, 1988; Robinson et al., 2004). However, confusion in the literature as well as insufficient explanation of load sharing suggests that the mechanical behavior of the tendon cannot be explained solely by composition and structure and that there must be alternative mechanisms of load transfer. Recent advances in technology have allowed for the measurement of dynamic parameters, but only a limited number of studies have investigated the relationships between the dynamic processes (re-alignment, uncrimping, deformation, sliding).
and mechanical properties (Lake et al., 2010) (Miller et al., 2012a) or composition/structure (Connizzo et al., 2013a; Miller et al., 2012a; Miller et al., 2012b). Furthermore, only Pearson correlations between individual properties have been used to describe a complex, yet unidentified, relationship. Therefore, the purpose of this study was to investigate the role of composition, structure, and the dynamic response to load in predicting tendon mechanical properties in a multi-level fashion mimicking native hierarchical collagen structure. We hypothesized that dynamic responses to load would strongly predict mechanical properties and that composition and structure would predict dynamic responses. Our second hypothesis was that dynamic processes would act as a mediator of the relationship of composition/structure to mechanical properties, mimicking the native structural hierarchy in tendon.

B. Methods

B-1. Data Collection

Data analyzed in this study was gathered in the series of studies presented in Chapter 3 and 4 of this dissertation. Briefly, these studies measured the multi-scale composition, structure, and mechanical response of mature (120 days postnatal) supraspinatus tendons from wild type, collagen V heterozygous and tendon/ligament-specific collagen V null mice (Table 5-1). Statistical linear regression was performed on the data using each animal's tissue as individual data points. To determine the sample size for multiple regression analysis, an a priori power analysis was performed based on 15 predictors (the largest number of predictors for any of the regressions planned in the study) with an alpha set at 0.05, an anticipated R-squared of 0.8 and a desired statistical power of 0.8. The anticipated R² value was chosen based on an assumption that this study would better predict mechanical parameters than previous multiple regression analyses performed in our lab (Ansorge et al., 2012; Robinson et al., 2004). Based on this analysis, it was determined that a sample size of 16 tendons was sufficient, but a sample size of 20 tendons was used to account for experimental error and to remain conservative.
Table 5-1. Summary of parameters utilized in study.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Experimental Method</th>
<th>Variable Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Collagen Content</td>
<td>Hydroxyproline Assay</td>
<td>COL</td>
</tr>
<tr>
<td>Glycosaminoglycan (GAG) Content</td>
<td>Dimethylmethylen Blue Assay</td>
<td>GAG</td>
</tr>
<tr>
<td>Pyridinoline Content</td>
<td>Pyridinoline Serum ELISA</td>
<td>PYD</td>
</tr>
<tr>
<td>Cell Shape</td>
<td>Paraffin Histology</td>
<td>CellShape</td>
</tr>
<tr>
<td>Cell Density</td>
<td>Paraffin Histology</td>
<td>CellDens</td>
</tr>
<tr>
<td>Fibril Diameter</td>
<td>Electron Microscopy</td>
<td>FibDiam</td>
</tr>
<tr>
<td>Fibril Density</td>
<td>Electron Microscopy</td>
<td>FibDens</td>
</tr>
<tr>
<td>Fibril Irregularity Factor</td>
<td>Electron Microscopy</td>
<td>FIF</td>
</tr>
<tr>
<td>Fibril D-Period</td>
<td>Atomic Force Microscopy</td>
<td>Dperiod</td>
</tr>
<tr>
<td>Fibril D-Period Variance</td>
<td>Atomic Force Microscopy</td>
<td>InitSlid</td>
</tr>
<tr>
<td>Amount of Re-Alignment</td>
<td>QPLM During Mechanical Testing</td>
<td>ReAlignAmount</td>
</tr>
<tr>
<td>Strain Required to Re-Align</td>
<td>QPLM During Mechanical Testing</td>
<td>ReAlignStrain</td>
</tr>
<tr>
<td>Toe Region Re-Alignment</td>
<td>QPLM During Mechanical Testing</td>
<td>ToeReAlign</td>
</tr>
<tr>
<td>Linear Region Re-Alignment</td>
<td>QPLM During Mechanical Testing</td>
<td>LinearReAlign</td>
</tr>
<tr>
<td>Crimp Frequency</td>
<td>Histology+Polarized Light Imaging</td>
<td>CrimpFreq</td>
</tr>
<tr>
<td>Crimp Amplitude</td>
<td>Histology+Polarized Light Imaging</td>
<td>CrimpAmp</td>
</tr>
<tr>
<td>Fibril Deformation (0-1% Strain)</td>
<td>Atomic Force Microscopy</td>
<td>FibDef1</td>
</tr>
<tr>
<td>Fibril Deformation (0-3% Strain)</td>
<td>Atomic Force Microscopy</td>
<td>FibDef3</td>
</tr>
<tr>
<td>Fibril Deformation (0-5% Strain)</td>
<td>Atomic Force Microscopy</td>
<td>FibDef5</td>
</tr>
<tr>
<td>Fibril Deformation (0-7% Strain)</td>
<td>Atomic Force Microscopy</td>
<td>FibDef7</td>
</tr>
<tr>
<td>Fibril Sliding (0-1% Strain)</td>
<td>Atomic Force Microscopy</td>
<td>Slide1</td>
</tr>
<tr>
<td>Fibril Sliding (0-3% Strain)</td>
<td>Atomic Force Microscopy</td>
<td>Slide3</td>
</tr>
<tr>
<td>Fibril Sliding (0-5% Strain)</td>
<td>Atomic Force Microscopy</td>
<td>Slide5</td>
</tr>
<tr>
<td>Fibril Sliding (0-7% Strain)</td>
<td>Atomic Force Microscopy</td>
<td>Slide7</td>
</tr>
<tr>
<td>Transition Strain</td>
<td>Dynamic Viscoelastic Testing</td>
<td>TransStrain</td>
</tr>
<tr>
<td>Transition Stress</td>
<td>Dynamic Viscoelastic Testing</td>
<td>TransStress</td>
</tr>
<tr>
<td>Toe Modulus</td>
<td>Dynamic Viscoelastic Testing</td>
<td>ToeMod</td>
</tr>
<tr>
<td>Linear Modulus</td>
<td>Dynamic Viscoelastic Testing</td>
<td>LinMod</td>
</tr>
<tr>
<td>Maximum Stress</td>
<td>Dynamic Viscoelastic Testing</td>
<td>MaxStress</td>
</tr>
<tr>
<td>Dynamic Modulus (6% Strain, 1Hz)</td>
<td>Dynamic Viscoelastic Testing</td>
<td>DynMod6</td>
</tr>
<tr>
<td>Tangent Delta (6% Strain, 1Hz)</td>
<td>Dynamic Viscoelastic Testing</td>
<td>TanDelta6</td>
</tr>
<tr>
<td>Stress Relaxation (6% Strain)</td>
<td>Dynamic Viscoelastic Testing</td>
<td>Relax6</td>
</tr>
<tr>
<td>Cycles to Failure</td>
<td>Fatigue Testing</td>
<td>FailCycle</td>
</tr>
<tr>
<td>Peak Cyclic Strain (50% Fatigue Life)</td>
<td>Fatigue Testing</td>
<td>CycStrain50</td>
</tr>
<tr>
<td>Tangent Stiffness (50% Fatigue Life)</td>
<td>Fatigue Testing</td>
<td>TanStiff50</td>
</tr>
<tr>
<td>Hysteresis (50% Fatigue Life)</td>
<td>Fatigue Testing</td>
<td>Hyst50</td>
</tr>
<tr>
<td>Laxity (50% Fatigue Life)</td>
<td>Fatigue Testing</td>
<td>Lax50</td>
</tr>
</tbody>
</table>

Note: Collagen and glycosaminoglycan content were normalized to DNA content. Pyridinoline content was normalized to total collagen; QPLM = quantitative polarized light microscopy; Dynamic parameters were measured at 3 frequencies and 3 strain levels but only the values at 6% strain and 10 Hz were used in this study since results were similar across strains and across frequencies. Similarly, fatigue parameters were measured at multiple points during fatigue life but only the values at 50% fatigue life were used in the statistical studies since results were similar throughout fatigue life.

The regression analysis was “blind” to mouse genotype, allowing for the hypotheses to be tested by determining how variances in composition and structure affect dynamic re-

91
organizations and mechanical properties, regardless of how those variances were achieved. The transgenic groups ensure a distribution of properties in addition to those achieved with normal biological variation. Bilateral samples from each mouse were analyzed to maximize the number of parameters measured from a single animal. However, due to the destructive nature of many of the assays performed in this study, it was not possible to obtain values for every dependent and independent parameter from a single specimen. To overcome and investigate this limitation, we performed all analyses on two data sets as a sensitivity analysis: (1) the complete data set (87% overall missing values), and a (2) ‘compact’ data set, in which the experimental data was compressed into 20 specimens (32% overall missing values). Imputation was performed on both data sets using the fully conditional specification method with 10 iterations for five imputations (White et al., 2011). Both data sets lead to the same overall conclusions, therefore presented here are the results from the complete data set only. The results for the ‘compact’ data can be found in Appendix B for completeness and to further support the approach presented.

B-2. Multiple Regression Analysis

Summary statistics of all variables were examined and described by mean, median, standard deviation, minimum, and maximum to ensure that assumptions requisite for linear regression analysis existed (Table B-1). These included: general linearity between single predictor and dependent variables, normality, non-multicollinearity, neutrality of the dependent variables, and lack of significant outliers. This study consisted of three sets of defined variables: (1) dependent variables (mechanical properties), (2) dynamic processes (re-alignment, crimp, fibril deformation, fibril sliding), and (3) composition and structure (cell morphology, fibril morphology, initial d-period and variance, extracellular matrix composition). Pearson correlation coefficients were first used to determine univariate relationships between predictor variables with significance set at p<0.05.
Multiple linear regression models were used to quantify two primary relationships: (A) the relationship between mechanical properties and dynamic processes, (B) the relationship between composition/structure and dynamic processes (Fig. 5-1). The relationship between mechanical properties and composition/structure (C) was also determined to compare results with previous findings in the literature and this data is reported in the Supplemental Data (Table B-2). A series of stepwise regression models using least-square estimation with forward elimination were used to predict these relationships. Variables were included in the model if they significantly improved the model via an F test with significance set at p<0.05.

Figure 5-1. Model schematic depicting regression analyses. Multiple stepwise linear regressions were performed between (A) composition/structure and dynamic processes and (B) dynamic processes and mechanical properties. Regression of mechanical properties on composition/structure (C) was also performed for completeness. Mediator modeling was then used to determine if the dynamic processes mediate the relationships between composition/structure and mechanical

**B-3. Mediator Modeling**

It is well established that tendons exhibit dynamic, viscoelastic, and non-linear properties, indicating that the dynamic processes must play a role as composition/structure are unchanging during the instantaneous response to load. Multilevel or hierarchical models are often used in psychological and sociological applications to handle data that inherently has data that is categorically nested, with the most common example being individuals nested in schools nested in districts (Gelman and Hill, 2006; Krull, 2001). However, one of the major benefits of multilevel
models is to deal with cases where the assumption of independence is violated, which is not necessarily the case for the present study. In addition, these models assume that the residuals between two levels are uncorrelated. Another type of analysis useful for investigating these relationships is termed causal inference, which uses statistical methods to test specific hypotheses about relationships. This includes structural equation modeling, most notably models of mediation and moderation (Baron and Kenny, 1986; Huang and Pan, 2015). In these models, the dynamic processes would either act as to mediate, acting as a go-between, or moderate, interacting with, the relationship between composition/structure and mechanical properties. Given the hierarchical nature of tendon’s structure, we hypothesized that dynamic processes would be mediators of the relationship between composition/structure and mechanical properties.

Mediator models are causal models that have recently been used in a variety of fields to explain the relationship between correlated parameters (Huang and Pan, 2015; Tripp et al., 2015; Wright and DeKemper, 2015). Traditional mediator models make several assumptions in addition to all of the standard assumptions of the general linear model, as well as independence of measured parameters and the lack of significant correlation between the independent variable and the mediator. In addition, this analysis was performed on imputed data (predicted values), which homogenizes the variance in the data. Nevertheless, this method tests the causal hypothesis that a third parameter, called the mediator, is contributing to the relationship between the independent and dependent variables and these models are capable of measuring the strength of that mediation (Baron and Kenny, 1986; Hayes, 2013; Judd and Kenny, 1981; MacKinnon et al., 2002). For all of the regression models that displayed strong relationships ($R^2>0.5$), single mediator models were first analyzed by defining the most important contributor of regression B as the mediator and the most important contributor of regression C as the independent variable. Partial mediation was determined using previously established methods, briefly by comparing the difference between the mediated path and the direct path (Baron and Kenny, 1986). Multiple mediator models were then analyzed using the same single independent variable from the single models and all significant contributors (dynamic processes) to regression
A as the mediators (Hayes, 2013). Significance for the single and multiple mediator models was determined using the Sobel method (MacKinnon et al., 2002) with significance set at p<0.05.

C. Results

C-1. Correlations Between Independent Variables

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>INS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAG</td>
<td>COL</td>
</tr>
<tr>
<td></td>
<td>GAG</td>
<td>PYD</td>
</tr>
<tr>
<td></td>
<td>COL</td>
<td>PYD</td>
</tr>
<tr>
<td></td>
<td>COL</td>
<td>FibDens</td>
</tr>
<tr>
<td></td>
<td>PYD</td>
<td>FibDens</td>
</tr>
<tr>
<td>MID</td>
<td>FibDiam</td>
<td>FibDens</td>
</tr>
</tbody>
</table>

Note: Moderate to strong observed correlations shown. r = Pearson correlation coefficient; p = correlation significance.

Moderate to strong correlations between independent variables are displayed in Table 5-2. At the insertion site, glycosaminoglycan (GAG) content was positively correlated with total collagen (COL) content and negatively correlated with pyridinoline (PYD) content. Collagen was negatively correlated with PYD content and positively correlated with fibril density. Fibril density was also negatively correlated with PYD. At the midsubstance, fibril diameter and fibril density were strongly correlated. There were no significant correlations between any of the dynamic parameters.
### Table 5-3. Mechanical Properties Regressed on Dynamic Processes

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>$R^2$</th>
<th>Re-Alignment</th>
<th>Crimp</th>
<th>Deformation</th>
<th>Sliding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition Strain</td>
<td>0.11</td>
<td>P</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Transition Stress</td>
<td>0.32</td>
<td>N</td>
<td>P</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Toe Modulus</td>
<td>0.57</td>
<td>N</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Linear Modulus</td>
<td>0.65</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Max Stress</td>
<td>0.72</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Dynamic Modulus</td>
<td>0.55</td>
<td>N</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Tangent Delta</td>
<td>0.53</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>Stress Relaxation</td>
<td>0.59</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Cycles to Failure</td>
<td>0.49</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Peak Cyclic Strain</td>
<td>0.11</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Tangent Stiffness</td>
<td>0.82</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Hysteresis</td>
<td>0.59</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Laxity</td>
<td>0.08</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
</tbody>
</table>

**Note:** All analyses were run on imputed data as stated in Methods section; $S = \text{ReAlignStrain}$, $RA = \text{ReAlignAmount}$, $TR = \text{ToeReAlign}$, $LR = \text{LinearReAlign}$, $F = \text{CrimpFreq}$, $A = \text{CrimpAmp}$; $R^2$ = correlation coefficient of model; $P$ = positive predictor, $N$ = negative predictor; Darkness of color represents the importance of the contribution of each predictor to the overall model for each mechanical parameter.

Results from the regression of mechanical properties on dynamic parameters are presented in Table 5-3 and key findings will be discussed in the text that follows. Dynamic parameters (re-alignment, crimp, deformation, sliding) were strong predictors of mechanical
properties, with $R^2$ values reaching as high as 0.89. Tangent stiffness was the strongest predicted mechanical parameter in both regions. Transition strain and stress, as well as peak cyclic strain, cycles to failure, and laxity were the weakest predicted mechanical parameters in both regions, with all parameters having an $R^2$ value less than 0.50. At the insertion site, mechanical parameters were well predicted by linear combinations of all four dynamic parameters, with fibril sliding and deformation making the largest contributions to the models. Crimp frequency and amplitude were positive predictors of mechanical properties. The amount of re-alignment (total, toe and linear regions) was a positive predictor of mechanical properties and a negative predictor of viscoelastic properties (stress relaxation, tangent delta). Fibril deformation at 1% and 5% applied strain were strong positive predictors of mechanics while deformation at 3% and 7% applied strain did not predict any parameter strongly. Fibril sliding was a strong positive predictor at 1% applied strain and a strong negative predictor at 3% and 5% applied strain.

In contrast, the midsubstance mechanical parameters were best predicted by fibril deformation, with sliding and re-alignment playing more minor roles. Crimp amplitude and frequency were weak predictors of mechanics. The strain required to fully re-align was a positive predictor of mechanical properties while the amount of re-alignment was either not predictive or a negative predictor. Fibril deformation at all strain levels were strong predictors of mechanics with the contribution increasing with increasing strain level. Deformation from 1-5% applied strain was generally a positive predictor of mechanics while deformation at 7% applied strain was a negative predictor. Finally, fibril sliding was a positive predictor of mechanical properties at 1% applied strain and a negative predictor at 3-7% applied strain.
### Table 5-4. Dynamic Processes Regressed on Composition and Structure

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>R²</th>
<th>FDi</th>
<th>FDe</th>
<th>FIF</th>
<th>COL</th>
<th>GAG</th>
<th>PYD</th>
<th>CS</th>
<th>CD</th>
<th>DP</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ReAlignStrain</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ReAlignAmount</td>
<td>0.44</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ToeReAlign</td>
<td>0.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LinearReAlign</td>
<td>0.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrimpFreq</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrimpAmp</td>
<td>0.41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef1</td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef3</td>
<td>0.59</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef5</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef7</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide1</td>
<td>0.70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Slide3</td>
<td>0.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Slide5</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td>P</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Slide7</td>
<td>0.52</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>N</td>
</tr>
</tbody>
</table>

**Note:** All analyses were run on imputed data; FDi = fibril diameter, FDe = fibril density, FIF = fibril irregularity factor, COL = collagen, GAG = glycosaminoglycan, PYD = pyridinoline, CS = cell shape, CD = cell density, DP = initial d-period, IS = initial d-period variance; R² = correlation coefficient of model; P = positive predictor, N = negative predictor; Darkness of color represents the importance of the contribution of each predictor to the overall model for each dynamic.
Results from the regression of dynamic processes on composition and structure are presented in Table 5-4 and key findings will be discussed in the text that follows. Dynamic properties (re-alignment, crimp, deformation and sliding) were moderately predicted by parameters of composition and structure, with $R^2$ values reaching 0.70. Fibril deformation and sliding were the best predicted parameters, while re-alignment and crimp were only moderately predicted. The strain required to fully re-align and fibril deformation at 7% applied strain were the weakest predicted variables at the insertion, while the amount of re-alignment (total and toe region), crimp amplitude, and fibril sliding at 3% applied strain were weakest at the midsubstance ($R^2$ values below 0.25).

Many parameters at the insertion site were strongly predicted by pyridinoline crosslinks content, fibril density, and initial d-period variance. Pyridinoline content was a negative predictor of re-alignment, crimp and fibril deformation, but a positive predictor of fibril sliding. Fibril density was a positive predictor of all dynamic processes. Initial d-period variable was a negative predictor of re-alignment and sliding. Weaker predictors included fibril irregularity, collagen and GAG content, cell shape, and initial d-period. Fibril irregularity and collagen content were primarily positive predictors of dynamic processes while GAG content, cell shape, and initial d-period were all negative predictors.

At the midsubstance, the strongest predictors of dynamic properties were fibril morphology (diameter, density, irregularity) and initial d-period length and variance. Fibril diameter and density were negative predictors of fibril deformation and positive predictors of fibril sliding. Initial d-period variance was a positive predictor of re-alignment, crimp, and fibril deformation and a negative predictor of fibril sliding. Initial d-period was a positive predictor of fibril sliding, crimp amplitude, and the linear region re-alignment while negatively predicted fibril deformation. Weaker predictors included cell shape and pyridinoline content, which were negative predictors of fibril deformation and positive predictors of fibril sliding.
### C-4. Mediator Modeling

#### Table 5-5. Multiple Mediator Modeling with Single Independent Variable

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>Re-Alignment</th>
<th>Crimp</th>
<th>Deformation</th>
<th>Sliding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>RA</td>
<td>TR</td>
<td>LR</td>
</tr>
<tr>
<td><strong>INSERTION SITE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toe Modulus</td>
<td>PYD</td>
<td>M</td>
<td>M</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Linear Modulus</td>
<td>PYD</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Dynamic Modulus</td>
<td>PYD</td>
<td>M</td>
<td>M</td>
<td>M*</td>
<td>M</td>
</tr>
<tr>
<td>Tangent Delta</td>
<td>PYD</td>
<td>P</td>
<td>P</td>
<td>M</td>
<td>P</td>
</tr>
<tr>
<td>Max Stress</td>
<td>PYD</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>M</td>
</tr>
<tr>
<td>Stress Relaxation</td>
<td>PYD</td>
<td>M</td>
<td>P</td>
<td>M</td>
<td>P</td>
</tr>
<tr>
<td>Tangent Stiffness</td>
<td>PYD</td>
<td>M</td>
<td>P</td>
<td>P</td>
<td>M</td>
</tr>
<tr>
<td>Hysteresis</td>
<td>PYD</td>
<td>M</td>
<td>P</td>
<td>P</td>
<td>M</td>
</tr>
</tbody>
</table>

| **MIDSUBSTANCE**   |                      |    |      |    |    |    |   |    |    |    |    |    |    |    |    |
| Toe Modulus        | FibDiam              | P  | M   | M  | P  | M* | P  | M* | P  |    |    |    |    |    |
| Linear Modulus     | FibDiam              | P  | M   | M  | M  | M* | P  | M* | M* | M  |    |    |    |    |    |
| Dynamic Modulus    | FibDiam              | M  | P   | P  | M  | M* | P  | M* | P  | M  |    |    |    |    |    |
| Tangent Delta      | FibDiam              | M  | P   | M  | P  | M* | P  | M* | P  | M  |    |    |    |    |    |
| Max Stress         | FibDiam              | M  | M   | M  | M  | P  | M  | M* | P  | M  |    |    |    |    |    |
| Stress Relaxation  | FibDiam              | M  | M   | M  | M  | P  | M  | M* | P  | M  |    |    |    |    |    |
| Tangent Stiffness  | FibDiam              | P  | P   | M  | M  | P  | M* | P  | M* | M  |    |    |    |    |    |
| Hysteresis         | FibDiam              | M  | P   | M  | P  | M* | P  | M* | M  |    |    |    |    |    |    |

**Note:** Analysis performed on data from multiple imputation; PYD = pyridinoline content; FibDiam = fibril diameter; InitSlid = initial d-period variance; M* = mediator and top 3 predictor, M = mediator, P = predictor only

Results from the multiple mediator models are presented in Table 5-5. Single mediator models demonstrated that the dynamic processes are mediating the relationship between composition/structure and mechanical properties, with 78% and 67% of parameters significantly mediated by their strongest dynamic process (primarily fibril deformation and sliding) at the insertion site and midsubstance, respectively (data not shown). Multiple mediator models also showed that dynamic processes were mediating the relationship, although there was no case in which all of the significant predictors in the regression model were mediators. On average, approximately 35% and 44% of the dynamic processes were significant mediators at the insertion site and midsubstance, respectively (Table 5-5). Many of the strong predictors at the insertion site were not strong mediators of the relationship between PYD and mechanical properties. In contrast, the strongest predictors at the midsubstance (fibril deformation) were strong mediators of the relationship between fibril diameter and mechanical properties.
D. Discussion

Correlations between independent parameters revealed relationships consistent with previous studies. The strongest relationship present was that between fibril diameter and fibril density, where a larger fibril diameter correlated strongly with decreased fibril density. This relationship has been noted in several other studies investigating adaptation to loading (Michna, 1984; Sanders and Goldstein, 2001). Several relationships were discovered between extracellular matrix proteins, primarily between GAG, collagen, and PYD. These relationships are likely due to normalization measures as GAG and total COL were both normalized to DNA content, and PYD was normalized to total COL content. Finally, collagen and PYD were both correlated with fibril density, alluding to the relationship between fibril number and collagen production. Increased collagen could suggest increased fibrils and thus increased fibril density. Similarly, increased PYD could indicate smaller diameter fibrils as collagen molecules are more tightly bound together (Couppe et al., 2009; Hansen et al., 2010a) and thus would decrease fibril density. Despite these correlations, data did not exhibit multi-collinearity and met the assumptions for linear regression.

Mechanical properties were strongly predicted by dynamic processes, and the contribution of each process was location-dependent. The mechanical properties of the insertion site were predicted by all dynamic processes with fibril deformation and sliding playing major roles. Given that the insertion site experiences the highest strains (Shaw and Benjamin, 2007), it likely utilizes re-alignment, uncrimping, and sliding in order to delay the onset of direct deformation to the fibrils/fibers. Due to the increased organization and structure at the midsubstance, this region of the tissue likely responds primarily through deformation of the fibrils and fibers themselves. In both locations, elastic properties (moduli values) had opposite predictors from viscoelastic properties, alluding to the complex interplay between fluid flow and elasticity during the dynamic response to load (Ahmadzadeh et al., 2015; Buckley et al., 2013).

Re-alignment and crimp were stronger predictors of mechanical properties at the insertion site than at the midsubstance, suggesting their importance in that region. Since it is well established that the insertion site is more disorganized than the midsubstance, the importance of
re-alignment at the insertion site is perhaps not surprising. Interestingly, the insertion site was best predicted by the amount of re-alignment, while the midsubstance was best predicted by the strain required to re-align. This could allude to strain transfer occurring along the tendon from bone to muscle. Increased mechanical properties at the insertion site would require more re-alignment to occur and this may translate to an increased time or strain required to perform that re-alignment, which would delay loading at the midsubstance. Crimp frequency and amplitude also were positive predictors of many mechanical parameters. Given that initial crimp frequency and amplitude are indicative of the potential for uncrimping, this suggests that the insertion site also relies on uncrimping to delay deformation and subsequent damage. However, crimp at the midsubstance was primarily a negative predictor of elastic mechanical properties (dynamic modulus, maximum stress, cycles to failure) and a positive predictor of viscoelastic parameters (tangent delta, stress relaxation, hysteresis), insinuating that the role of crimp at the midsubstance is more related to controlling viscoelasticity and fluid flow.

Fibril deformation was a strong predictor of mechanical function in both regions, but the timing of the contribution revealed location-dependent response mechanisms. At the insertion site, fibril deformation at 1% and 5% applied strain were positive predictors of mechanical properties (and negative predictors of viscoelasticity). This agrees with our previous experimental data showing a bimodal deformation response at the insertion site (Connizzo et al., 2014) as well as with previous studies revealing structure/function-dependent deformation mechanisms (Miller et al., 2012d; Screen et al., 2013). In contrast, the midsubstance displayed an increasing importance of fibril deformation with the strongest contributions at 7% applied strain. However, deformation was a negative predictor at 7% applied strain, indicating perhaps that failure at the fibril level may be occurring at 7% applied strain.

In contrast to all of the other dynamic processes, fibril sliding displayed a similar role in prediction of mechanical properties at both regions although the contribution was stronger at the insertion site. Early sliding was a positive predictor of mechanical properties at both regions, advocating for sliding to protect fibrils/fibers from deformation. In contrast, fibril sliding later in the
test was a negative predictor of mechanics, possibly alluding to failure due to fibrils pulling away from each other in shear. This work is in contrast to several recent studies showing continuously increasing fibril sliding during macroscopic loading (Gupta et al., 2010; Szczesny and Elliott, 2014), rather than more sliding early during the test. However, these studies investigated the relaxation response rather than the instantaneous response studied in the present work which could account for the differences as the fibrils are not able to recover in the instantaneous loading response (Connizzo et al., 2014).

In contrast to the mechanical parameters, dynamic processes were only moderately predicted by structure and composition. Fibril deformation and sliding were the best predicted dynamic processes, which could be a function of the skewed measurement of fibril morphological parameters (diameter, density, irregularity, d-period, d-period variance) compared to other parameters. Since collagen re-alignment and crimp are measured at the fiber level, and not the fibril level, the reduced ability to explain these dynamic parameters via regression could also be due to the lack of appropriate parameters at that hierarchical scale or an incomplete understanding of what contributes to or controls these processes.

Pyridinoline content was a strong predictor of dynamic properties at the insertion site, as a negative predictor for re-alignment, crimp and deformation but a positive predictor of fibril sliding. While the role of collagen crosslinks in these dynamic processes has not yet been studied, increased crosslink density has been thought to increase mechanical properties (Alfredo Uquillas et al., 2012; Ng et al., 2013). Our model suggests that increasing PYD crosslink density reduces the dynamic response to load and thus ultimately would result in early strain on the fibrils. Since increased crosslink density has been reported to increase the brittleness of collagen fibrils (Buehler, 2008), this could result in early damage or injury. However, the contribution of PYD crosslinks to the dynamic processes at the midsubstance was not as strong, suggesting that in tendons that are not as structurally complex, this relationship may be less important.

Fibril diameter and density were strong predictors of dynamic function at the midsubstance, but not at the insertion site. Since the midsubstance mechanical properties were
predicted heavily by fibril deformation, which is directly related to fibril morphology, this is not surprising. Additionally, as the midsubstance is more organized and structurally homogeneous, less dynamic responses are occurring and those that are heavily depend on that fibril structure. Interestingly, fibril diameter and density were often both predictors in the same direction, which is in contrast to the correlations we found previously. While fibril diameter and fibril density are often negatively correlated, these regression models suggest that perhaps that isn’t the most efficient method for these dynamic responses. However, while fibril deformation was predicted negatively by fibril diameter and density and fibril sliding was predicted positively by fibril diameter and density, it stands to reason that perhaps these parameters are negatively correlated to balance these two dynamic responses which are extremely important for mechanical function, particularly at the midsubstance.

D-period and initial variance of the d-period were also determined to be strong predictors of dynamic function at both regions. However, while both variables were negative predictors of realignment, crimp, deformation, and sliding at the insertion site, the prediction at the midsubstance was more complicated. One would expect that in order to have increased deformation and sliding, a decreased initial d-period and decreased initial variance would be advantageous (as at the insertion site). At the midsubstance, again initial variance of the d-period and d-period length opposite predictors of sliding and deformation, alluding to the coordinate relationship between deformation and sliding in this region.

Given the hierarchical nature of tendon structure and therefore likely strain transfer during loading, we hypothesized that a model reflecting this innate structure would be able to better predict mechanical parameters than a model similar to those performed in the past, directly regressing composition and structure with mechanics. We determined that for many of the regression models, the dynamic processes (deformation, sliding, re-alignment, crimp) were mediators of the relationship between composition/structure and mechanical function, confirming our hypothesis. However, in all of our regressions, we found that dynamic processes were only partial mediators. Given that we only analyzed relationships between a single independent
parameter and each mechanical property, this could mean that some of the dynamic processes that predict mechanics may mediate relationships with other compositional/structural parameters. For example, fibril deformation at 1% was a strong predictor of mechanics at the insertion site but did not mediate the relationship between PYD and mechanical properties in almost 60% of the parameters. Fibril deformation at 1% could be a better mediator of the relationship between other compositional properties, such as fibril diameter, d-period, or collagen content, with mechanical properties, which was not analyzed in these studies. It’s likely that mediation of these relationships is shared by many of the dynamic processes, particularly at the insertion site where all of these processes contribute to overall mechanical function. In contrast, the midsubstance is highly predicted by fibril deformation which is also a strong mediator of the relationship between fibril diameter and mechanical properties. Since there are a number of independent parameters not represented in this analysis, the use of complex multi-mediator and multi-independent variable models would be necessary to fully explain these differences.

With all statistical analyses, limitations of the dataset used are present in this study which must be discussed. While all of the assumptions of linear regression were met prior to analyses, it is important to note that the complete data set was designed with a large amount of missing data. This is an inherent and unavoidable limitation in many studies which require analysis of biological tissue with limited availability. To address this limitation, the data was analyzed in two ways (‘complete’ and ‘compact’) as a sensitivity analysis of our conclusions. The data presented in the tables and text here are analyses of the ‘complete’ dataset, however all of the conclusions presented in this work were also present in the ‘compact’ dataset, instilling confidence in the analyses that were performed here. The results from our ‘compact’ data regressions can be found in Appendix B for further interpretation. Nevertheless, while the imputed datasets in this study appeared to be efficient in capturing the real distribution of the data based on summary statistics, imputation can have unintended effects on interpretation of the analysis, such as the homogenization of the data if there is a large amount of missing data, misrepresentation of the
experimental data due to selection of imputed values from a normal distribution and specific biases based on the imputation method, which must be considered.

Additionally, although a large number of parameters were measured in this study, there are still a number of important properties that were not measured here. For example, initial crimp morphology is present in this analysis but uncrimping was not measured, which would be a better measure for the dynamic process. Similarly there are a number of extracellular matrix proteins and other collagen crosslinks that were not measured and could contribute to mechanical function, most notably elastin and fibrillins (Boregowda et al., 2008; Grant et al., 2013; Grant et al., 2015; Henninger et al., 2015), as well as other non-enzymatic and enzymatic collagen crosslinks (Hanada et al., 2014; Marturano et al., 2014; Takaluoma et al., 2007). Finally, this analysis assumes that the relationships between all of the independent and dependent parameters are linear, when it is possibly and perhaps likely that many of these relationship are non-linear.

All of the parameters measured here are important assessments of the unique and complex mechanical function of tendon, with inhomogeneity, viscoelasticity, and nonlinearity all taken into account. However, it is still unclear which mechanical parameters would be the best to use when interpreting the capacity of the tendon to withstand load as loading conditions are extremely important in vivo. This remains a limitation not only of this thesis, but of the field at large, as it will be challenging to create an engineered tendon replacement or develop treatment strategies without determining what the appropriate or best outcome measure is. However, the work presented here provides a more complete picture than has ever been presented before.

We conclude that the mechanical properties at the midsubstance of the tendon are controlled primarily by fibril structure and this region responds to load via fibril deformation and sliding, which we hypothesize would be similar to organized tendons such as rat tail tendon fascicles or flexor tendons. Conversely, the mechanical function at the insertion site is more complicated, as it is controlled by many other important parameters and the region responds to load via all four dynamic mechanisms. Overall, this study presents a strong foundation on which
to design future experimental and modeling efforts in order to fully understand the complex structure-function relationships present in tendon.

E. References


110


CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

A. Introduction

The overall objective of this dissertation was to measure composition, structure, and mechanical function in a mouse model of altered structure, and to analyze links between mechanical properties, composition/structure, and dynamic processes using a unique multilevel regression and mediator modeling strategy. A novel method to measure in situ fibril deformation and sliding in mouse supraspinatus tendons was first developed using a flash-freezing mechanical testing technique combined with atomic force microscopy imaging (Chapter 2). Established animal models, specifically the collagen V traditional heterozygous mouse and a tendon/ligament-specific collagen V null mouse, combined with sophisticated and rigorous mechanical analysis at multiple hierarchical scales were then used to define the functional changes in mature mouse supraspinatus tendons due to the reduction of collagen V expression at birth (Chapter 3). Additional characterization of cell and fibril morphology as well as extracellular matrix composition was then performed to elucidate regional changes in structure and composition in these animal models (Chapter 4). Finally, this complete hierarchical characterization of composition, structure and function was then used in a novel multiple regression model to elucidate relationships between composition and structure, dynamic processes (re-alignment, uncrimping, deformation, sliding), and mechanical parameters (Chapter 5). The main findings and conclusions from these studies, as well as several potential future directions, are included in this final chapter.

B. Chapter Two Conclusions

Recent evidence suggests that tendons are able to withstand high forces by employing a number of unique mechanisms occurring at many of the fibril and fiber length scales, including uncrimping, re-alignment, sliding, and deformation or stretch. While collagen fiber uncrimping and re-alignment have been studied extensively in recent literature, the quantification of collagen fiber
and fibril sliding and stretch has been studied less due to the experimental difficulties, particularly the inability to visualize individual collagen fibrils in vivo during mechanical loading. Recent investigations utilizing atomic force microscopy have successfully measured d-period length changes as a quantitative measure of collagen fibril stretch in situ. However, these studies have primarily investigated fiber sliding during or following stress relaxation or creep events. The strain rate dependence of tendon identifies that the timing and rate of loading, in addition to the magnitude, is extremely important to tendon's response. Furthermore, since tendons have been known to rupture clinically due to a single traumatic event or impact, the instantaneous response to load, as well as the ability to repetitively undergo that impact stress, is critical to the overall function and has not been investigated.

The overall objective of this study was to develop a novel AFM-based method to quantify the instantaneous response of collagen fibrils throughout a mechanical loading protocol in the insertion site and midsubstance of the mouse supraspinatus tendon. Using a flash-freezing method to immediately arrest strain throughout the tissue, we were able to successfully capture the instantaneous response to applied macroscale strain at multiple hierarchical scales. We hypothesized that more fibril stretch will occur at the insertion site than the midsubstance (higher strains) and that more fibril sliding will occur at the midsubstance than at the insertion site. As with previous studies, fibril stains were much lower than overall tissue strain and the insertion site exhibited increased fibril strains when compared to the midsubstance. Consistent with our hypothesis, we found that fibril deformation occurred in a bimodal fashion at the insertion site and continuously throughout loading at the midsubstance. Fibril sliding also occurred primarily at the midsubstance of the tissue and not at the insertion site. These findings suggest a coordinate relationship between fibril deformation and sliding as well as with re-alignment and uncrimping at the fiber and/or fibril level. This work also confirms that the contribution of these dynamic processes to the overall mechanical response is likely dependent on collagen structure and organization as many of these processes are location-dependent.
Overall, this work demonstrated a novel and innovative approach to measure the regional instantaneous response of collagen fibrils to overall tendon load without removing the fibrils from their native environment. This method could ultimately be a powerful tool utilized to study the altered fibrillar response to load in various animal models and could lead to a more complete visualization of the hierarchical complexity of tendon’s mechanical response.

C. Chapter Three Conclusions

Tendons function to stabilize the skeleton and allow efficient transfer of energy through their unique set of mechanical properties, including anisotropy, viscoelasticity, non-linearity, and inhomogeneity. These properties are thought to be attributed to a number of dynamic microstructural re-arrangements, particularly collagen fiber uncrimping and re-alignment and fibril deformation and sliding. These mechanisms are directly controlled by the location-dependent and site-dependent composition and structure of the tissue which is highly regulated by proteoglycans and minor collagens, such as collagen V. The goal of this study was to investigate hierarchical dynamic relationships by evaluating the dynamic mechanical function of collagen V heterozygous and null tendons at a hierarchy of length scales, including fibril, fiber and whole-tissue levels. We hypothesized that collagen V-deficiency would result in impaired dynamic mechanical function at all length scales, with a stronger phenotype in the null than heterozygous mice. We also hypothesized that changes in dynamic responses at nanoscale (fibril) and microscale (fiber) (collagen re-alignment, deformation, sliding) are more apparent than those at the tissue-level, and can be used to interpret the tissue-level changes.

Consistent with our primary hypothesis, mechanical function was severely reduced in almost every mechanical parameter and these results appeared to be dose-dependent, although much larger differences were found in the null group than in the heterozygous group. Interestingly, there were few differences between the groups at low levels of strain, suggesting that the heterozygous and null groups perform similarly to the wild type group initially. However, both experimental groups were also unable to withstand the same repetitive cyclic loading as the
wild type group, suggesting earlier damage accumulation due to inferior dynamic responses. Furthermore, the heterozygous group, which exhibited only small changes in many other macroscale properties, was significantly affected by the cyclic loading protocol, suggesting that the dynamic responses governing recovery from repetitive cyclic strain were diminished.

Consistent with our secondary hypothesis, a larger diminished response at the microscale and at the nanoscale was found in both the heterozygous and null groups. Although we were unable to detect collagen fiber uncrimping, collagen fiber re-alignment occurred earlier and in response to a lower strain level in both experimental groups. This suggests that the protection of fibrils from strain via other dynamic mechanisms is completed earlier and subsequent damage can then accumulate in the tissue at a lower strain level. Furthermore, collagen fibrils exhibited increased strain at an earlier applied strain level, confirming that the tendons were responding with multiple hierarchical levels earlier than in the wild type group. However, the null tendons did not exhibit any fibril sliding while the heterozygous tendons exhibited drastically increased fibril sliding compared to the wild type group. This suggests that the heterozygous group is able to compensate for the lack of fibril strength via fibril sliding, which reduces strain on the collagen fibrils initially, but with repetitive loading would ultimately result in fibrils pulling away from each other and tendon failure via shear. These results may explain clinical observations of increased elasticity during in vivo loading, which occurs at relatively low strain levels, as well as increased joint laxity over time, as tendons would elongate with repetitive loading.

In summary, this study was the first to rigorously investigate regional mechanical function at multiple hierarchical scales. Furthermore, these studies suggest that these unique set of dynamic processes provide normal tendons with a series of protective measures to prevent early failure. It is likely that these mechanisms are the initiation of large scale tissue damage and increased understanding of the relationships between dynamic mechanisms could elucidate proper strategies for prevention of tendon damage and injury.
D. Chapter Four Conclusions

Dynamic mechanical function, such as viscoelasticity and non-linearity, is dependent on the unique extracellular matrix present in tendon. Due to the strong role of collagen structure in determining mechanical properties, alterations in fibril morphology resulting from reduced collagen V expression are thought to be responsible for the functional deficiencies in the classic Ehlers-Danlos Syndrome (EDS) patient population. However, several studies have determined that the role of minor collagens in developing collagen structure is tissue-dependent and that collagen V in particular may have a larger effect on joint-stabilizing tendons and ligaments, such as the supraspinatus tendon. The objective of this study was to determine the effect of reduced collagen V expression during tendon development in determining the structure and composition of mature mouse supraspinatus tendons. We hypothesized that reduction of collagen V expression would result in altered fibril morphology (increased fibril diameters and decreased fibril density), consistent with previous literature in mouse flexor digitorum longus tendon and anterior cruciate ligament. We also hypothesized that the tendons would exhibit no additional alterations in cell morphology, fiber morphology or extracellular matrix (ECM) composition.

Consistent with our hypotheses, fibril morphology was altered at the midsubstance of the tendon similar to other studied tendons, specifically with increased fibril diameters and decreased fibril density. This is explained by the reduction in fibril nucleation sites thus resulting in fewer produced, but larger fibrils. This altered fibril morphology was present with no changes in cell morphology or quantity, or extracellular matrix composition. Interestingly, while there were no changes in glycosaminoglycan or total collagen content at the midsubstance either, pyridinoline, a mature collagen crosslink, was increased in the null groups, suggesting that collagen processing may be disrupted.

Contrary to our hypotheses, alterations in every parameter studied were found at the insertion site. Fibril diameter distributions shifted in both directions, exhibiting an increased number of larger fibrils and an increased number of smaller fibrils. In addition, the prevalence of abnormally-shaped fibrils was increased in the null group at the insertion site. Both of these
findings suggest a disruption in the processing or assembly of collagen fibrils. Total collagen content also was reduced and pyridinoline crosslinks was increased, supporting this theory. Reduction in GAG content alludes to alterations in the interfibrillar matrix as well, which could suggest dysfunction in extracellular matrix production or regulation.

Altogether, these results demonstrate that the role of collagen V in determining the structure and composition of mouse supraspinatus tendons is location-dependent. This could be explained by the increased functional requirements present at the insertion site or by the presence of two tissues, bone and tendon, that are both affected by reduced collagen V expression during development. However, the cascade of events that leads to the abnormal structure and composition in the null tendons is still unclear as it is difficult to specifically separate the effects that one might have on the other and the timing of those effects. An understanding of the mechanism by which these alterations occur during development and/or during the injury response could further aid in developing a therapeutic target for classic EDS patients.

E. Chapter Five Conclusions

A number of recent studies have sought to understand structure-function relationships present in tendon. However, confusion in the literature as well as insufficient explanation of load sharing suggests that the mechanical behavior of the tendon cannot be explained solely by composition and structure and that there must be alternative mechanisms of load transfer. Recent advances in technology have allowed for the measurement of dynamic parameters, but only a limited number of studies have investigated the relationships between the dynamic processes (re-alignment, uncrimping, deformation, sliding) and mechanical properties. Furthermore, only Pearson correlations between individual properties have been used to describe a complex, yet unidentified, relationship. The overall objective of this study was to investigate the role of composition, structure, and the dynamic response to load in predicting tendon mechanical properties in a multi-level fashion mimicking native hierarchical collagen structure. Pearson correlations between all independent parameters as well as between dependent parameters were
used to understand direct relationships. Multiple linear regression models were then investigated to determine the primary predictors of mechanical properties and dynamic parameters in a hierarchical manner. Our primary hypothesis was that dynamic responses to load would strongly predict mechanical properties and that composition and structure would predict dynamic responses. Our second hypothesis was that the dynamic responses would be mediators of the relationship between composition and structure and mechanical properties.

Dynamic processes (re-alignment, crimp, deformation, sliding) were strong predictors of mechanical properties, consistent with our primary hypothesis. These predictions were location-dependent, with the insertion site utilizing all four dynamic responses and the midsubstance responding primarily with fibril deformation and sliding. Fibril deformation was predictive of mechanical properties with a bimodal response at the insertion site and a continuously increasing response at the midsubstance, supporting experimental results from Chapter 2. Dynamic properties were predicted moderately by composition and structure and these predictions were also location-dependent. The insertion site was primarily predicted negatively by pyridinoline crosslink content, fibril density and initial d-period variance. The midsubstance however was predicted primarily by fibril morphology, similar to past studies using regression analysis which studied more organized tendon regions.

In addition, both single and multiple mediator models demonstrated that the dynamic processes were partial mediators of the relationship between single independent composition/structure parameters and mechanical properties. This result confirms our hypothesis that strain or load may be transferred from the macroscale to the individual structural elements via these dynamic processes. However, our analyses only explored mediation of the relationship between a single independent parameter and each mechanical property, when mechanical properties are much better predicted using multiple regression as demonstrated in many studies. Some of the dynamic processes that predict mechanics may mediate relationships with other compositional/structural parameters as well, advocating for the use of complex multivariate and multi-mediator modeling in the future. Furthermore, mediation of these relationships is likely
shared by many of the dynamic processes, particularly at the insertion site where all of these processes contribute to overall mechanical function.

The relationships determined in this study represent important assessments of the unique and complex mechanical function of tendon, with inhomogeneity, viscoelasticity, and nonlinearity all taken into account. However, it is still unclear which mechanical parameters would be the best to use when interpreting the capacity of the tendon to withstand load as loading conditions are extremely important in vivo. This remains a limitation not only of this study, but of the field at large, as it will be challenging to create an engineered tendon replacement or develop treatment strategies without determining what the appropriate or best outcome measure is. Based on these studies alone, the best predicted mechanical properties based on dynamic processes and structure/composition were tangent stiffness, maximum stress, and linear modulus. This is encouraging as quasi-static mechanical function is the most commonly assessed parameters in the field and can be easily assessed for tissue engineering tendon replacements. However, these studies have also determined that the dynamic response to load is crucial in replicating the unique mechanical function of tendon, which supports the need for application-specific tissue engineering practices in the tendon field. However, the work presented here creates a more complete picture than has ever been presented before and future studies should focus on measuring the important predictors of mechanical function as designed here.

F. Overall Study Conclusions

In the studies presented here, we developed a novel method for measuring the regional deformation and sliding of collagen fibrils in situ during the instantaneous response to load. We then used this method, along with other previously established techniques, to measure the multiscale mechanical function of mouse supraspinatus tendons from wild type, collagen V heterozygous and collagen V null mice. Overall, we found that the experimental groups were mechanically inferior to the wild type group, with larger changes in the dynamic responses to load (re-alignment, crimp, deformation, sliding) than in macroscale mechanical properties, particularly
in the heterozygous group. We then investigated the regional structure and composition of supraspinatus tendons from these mouse models. While fibril morphology was altered at both locations, the insertion site also exhibited alterations in cell morphology, fiber morphology, and extracellular matrix composition, alluding to the complexity of structure in this region. Finally, we utilized the data from our two experimental studies to analyze relationships between hierarchical scales using a unique regression modeling approach. We found that the contribution of composition and structure as well as the contribution of dynamic processes to determining macroscale mechanical function was highly dependent on location.

Overall, we conclude that although collagen V is a quantitatively minor component in mature tendon/ligament, it is a major regulator of composition and structure during development which ultimately leads to mechanical function. Results indicate that repetitive or increased activity could lead to early damage or injury in the rotator cuff, consistent with what has been reported clinically. This information will help to guide clinicians in developing preventative techniques and appropriate rehabilitation strategies, as well as help to define the appropriate and important parameters on which to base tissue engineering efforts for tendon augmentation or replacement. Furthermore, we conclude that the composition and structure as well as dynamic responses to load are crucial factors in ultimately determining mechanical function and that these roles are regionally-dependent. This work presents a strong foundation on which to base future experimental and modeling efforts in order to fully understand the complex structure-function relationships present in tendon.

G. Future Directions

Based on the results presented here, several possible future directions should be considered for this avenue of research. In terms of investigating the role of collagen V in the classic EDS patient population, these include (1) investigating overall joint mechanics and their relation to patient-reported hypermobility, (2) understanding the role of collagen V in injury, healing, and aging, (3) examining the impact of gender on the role of collagen V in tendon and (4)
exploring the possibility of early detection and prevention of injury. In relation to further elucidating multi-scale structure-function relationships in tendon, future directions should focus on (1) investigating structure-function relationships in alternate models, (2) exploring alternative loading conditions, and (3) understanding the role of biological adaptation in recovery and remodeling of the mechanical response.

**G-1. Joint Mechanics**

These studies sought to investigate the role of collagen V in tendon composition, structure, and function as a means to understand the functional deficits that exist in the classic EDS patient population. Classic EDS is often characterized by joint hypermobility and recurrent joint dislocations, particularly at the knee, hip and shoulder (Ainsworth and Aulicino, 1993). This study investigated the supraspinatus tendon, which is a major dynamic joint stabilizer of the shoulder, with the added benefit of being able to investigate basic structure-function relationships in addition to clinical inquiry. However, the supraspinatus tendon is just one tendon in the rotator cuff and there are a number of other tissues present that may play a role joint hypermobility.

The objective of this study would be to investigate crucial components in joint function and their contribution to joint mobility with the hypothesis that joint hypermobility at the shoulder is primarily caused by mechanically inferior rotator cuff tendons and/or capsule with no alterations in glenoid or humeral head cartilage thickness or function. Using the established transgenic mouse model of classic EDS, the collagen V heterozygous mouse, shoulder function and passive joint mechanics would be measured using in vivo functional assays adapted for mice (Reuther et al., 2014b). Ex vivo assays would include mechanical testing of the rotator cuff tendons (infraspinatus, subscapularus), anterior and posterior capsule and the biceps tendon using dynamic mechanical testing and fatigue testing protocols as described previously (Reuther et al., 2013; Reuther et al., 2014a; Thomas et al., 2012), as well as quantification of cartilage thickness and mechanical properties as described previously (Reuther et al., 2012; Reuther et al., 2014b).
An understanding of alterations in adjacent tissues and quantification of overall joint mechanics may lead to a better comprehension of the contributors to joint hypermobility and recurrent dislocations. In addition, this work could lead to the development of potential therapeutic interventions or prevention strategies for recurrent joint dislocations.

**G-2. Injury and Healing**

While this study focused on measuring the baseline mechanical function of mature classic EDS and collagen V-null tendons as a model for understanding complex structure-function relationships in the supraspinatus tendon, classic EDS is often diagnosed primarily due to repeated injury and insufficient healing. This is especially evident in the skin, as hypertrophic scarring and bruising are major symptoms among classic EDS patients (Ogawa and Hsu, 2013; Remvig et al., 2010; Symoens et al., 2012). Reduction of collagen V expression in wounded skin results in an abnormal skin morphology and function (Wenstrup et al., 2006), but tendon injury and healing has yet to be studied. Since collagen V is also significantly upregulated in tendons and ligaments after injury (Niyibizi et al., 2000; Shimomura et al., 2003), it likely plays a crucial role in tendon healing as well.

The purpose of this study would be to investigate the role of collagen V expression during the tendon injury and healing response. This study could be performed in a number of tendons, but the simplest would be a midsubstance biopsy punch of the patellar tendon (Dunkman et al., 2014a; Dunkman et al., 2014b) as it is a reliable and repeatable injury that isolates the injury in the midsubstance of the tendon. However, injury and repair in a more clinically applicable model, such as an Achilles tendon midsubstance rupture or supraspinatus tendon insertion site detachment and repair, would be a more desirable model on which to base clinical observations for the classic EDS patient population. Injury would then be assessed at various stages of healing via biomechanical assays, gene expression arrays, and measurements of composition and structure via analysis of protein expression and fibril morphology.
A second iteration of this study would utilize novel inducible animal models, which would allow researchers to precisely control the removal of collagen V expression during the injury response via injectables. This would allow us to decouple the abnormal structure and function of the tendon prior to injury and the removal of collagen V expression from the injury process and to define the specific temporal roles of collagen V in the re-establishment of structure and function. Furthermore, the data will provide a critical foundation for developing therapeutic interventions to enhance the clinically important problem of abnormal wound healing in classic EDS.

G-3. Aging

In this thesis and several previous studies (Connizzo et al., 2013; Dunkman et al., 2013; Legerlotz et al., 2014; Swan et al., 2014), we have demonstrated that aging affects both tendon structure, composition, and mechanical function in a manner opposite to development. Since this work has shown that reduction of collagen V expression during development results in significantly inferior mature tendon properties, it is likely that reduction of collagen V expression could also affect the aging process. This is especially important as the incidence of rotator cuff tears increases dramatically with increasing age (Buckwalter et al., 2003; Thomopoulos et al., 2015). Furthermore, it has been reported clinically that hypermobility in the classic EDS patient population differentially affects younger patients (Ainsworth and Aulicino, 1993; Castori et al., 2010; Castori et al., 2011), alluding to a possible link with age in this disease. The objective of this study would be to examine changes in composition, structure and function throughout aging in the supraspinatus tendon. Animals from four ages (P60-young, P120-mature, P300-aging, P570-aged) would be investigated for changes in fibril morphology, dynamic mechanical function, and macroscale mechanical properties, as described here. The overall hypothesis for these studies is that collagen V is important for age-related declines in tendon structure and function. These studies would highlight the importance of collagen V in maintenance of the extracellular
matrix as we age, similar to recent studies with proteoglycan-deficient animals (Connizzo et al., 2013; Dunkman et al., 2013).

**G-4. Gender**

This study used primarily male mice, but the prevalence of classic EDS is heavily skewed towards the female population, with ratios as high as 12:1 for female:male incidence (Ainsworth and Aulicino, 1993; Stanitski et al., 2000). This can result in a number of problems, most notably a much greater prevalence of obstetric and gynecologic issues with EDS patients than in the general population (Hurst et al., 2014). These data indicate that there is likely a relationship between hormone and collagen V expression. More generally, the incidence of shoulder complaints in women is higher than that of men (Hart et al., 1998; van der Windt et al., 1995) and it is well established that women and men have significantly different composition and mechanical function (Onambele et al., 2007), suggesting that gender-dependent structure-function relationships exist in these tissues. The proposed studies are two-prong, designed to investigate the interaction between gender and classic EDS as well as the gender-dependence of tendon function. Control and collagen V heterozygous female mice would be subjected to the same series of studies as performed in this thesis. Comparing between male (this thesis) and female control mice would define the role of gender in structure-function relationships at baseline, while comparing the male and female classic EDS mice would illuminate the relationship between gender and classic EDS pathology. Overall, these studies would aid in elucidating the mechanism for gender-related prevalence of classic EDS and identifying therapeutics to target the disease.

**G-5. Detection and Prevention of Injury**

The primary goal of this thesis was to explore the baseline properties of classic EDS and collagen V-null tendons as a model system for understanding overall structure-function relationships across tendons. However, another important goal was to understand the effect that
these changes would have on the classic EDS patient population, with hopes of possibly aiding to
develop therapeutic strategies. In the classic EDS (heterozygous) tendons, quasi-static and
dynamic mechanical properties were only slightly altered, but there were significant alterations in
fatigue properties, alluding to changes occurring at the microscale and nanoscale. Results
suggested that tendons were responding faster to load at lower hierarchical scales and accruing
sub-rupture damage faster in the classic EDS tendons. However, we did not directly visualize or
quantify damage in these studies. In order to prevent injury, it is necessary to first be able to
accurately detect injury. Numerous studies have recently sought to quantify sub-rupture tendon
damage using a variety of animal models, imaging techniques, and statistical methods (Buckley
et al., 2013; Duenwald-Kuehl et al., 2012; Freedman et al., 2015; Fung et al., 2010; Reyes et al.,
2014; Ros et al., 2013; Spiesz et al., 2015; Sun et al., 2008; Veres et al., 2013). The ideal
proposed future studies would be designed to determine and define a strain-based or load-based
limit on which to base clinical recommendations for appropriate exercise and therapy.

First, we would want to be able to detect injury both ex vivo and in vivo in mouse
tendons. We could do this ex vivo by subjecting tendons to a series of fatigue loading profiles with
increasing cycle number and/or increasing maximum load/strain (Fung et al., 2009). In this study,
we only used a high magnitude fatigue loading protocol until failure, but it would be useful to look
at a variety of loading regimes, particularly those closer to in vivo loading levels which could give
us more insight into damage accumulation clinically. We could then detect the damage caused by
each loading profile via histology, scanning electron microscopy, or atomic force microscopy
(Freedman et al., 2015; Ros et al., 2013; Veres et al., 2013). However, ultimately we would want
to be able to detect injury in vivo, which could be performed using an imaging-based method
(Riggin et al., 2014), and then correlate that injury level to the stresses the tissue was subjected
to (Buckley et al., 2013; Fung et al., 2009). Naturally, this process would be performed in both
wild type and classic EDS tendons to determine differences in the diseased state. Finally, we
could then scale this process up to human tendons, possibly working with a clinical population of
classic EDS patients, which would ultimately allow us to develop therapeutic criteria for injury prevention and rehabilitation.

**G-6. Alternative Models of Structure-Function Relationships**

This thesis clearly demonstrated the structure-function relationships at multiple hierarchical scales are highly dependent on tendon location, thought to be attributed to changes in structure and composition. However, this is just one model system with specific changes in many of the parameters studies. By perturbing various parameters via the use of different tendons, different animal models, and different disease models, we can systematically uncover the direct contribution of each parameter. Furthermore, studying the contributions of various parameters to mechanical function in other tendons, such as Achilles tendon, flexor tendons, cruciate ligaments or patellar tendon, could help to establish if global (cross-tissue) structure-function relationships exist. This work would not only support the studies here but also inform site-specific tissue engineering strategies.

In addition, this work was an excellent platform to study disease-specific alterations in structure, function and composition. There are a number of other diseases that have been shown to create mechanically or structurally inferior tendons, including diabetes (Bermudez et al., 2011; Connizzo et al., 2014a; Thomas et al., 2014), and hypercholesterolemia (Abboud et al., 2012; Beason et al., 2013; Beason et al., 2014; Hast et al., 2014). Both of these diseases result from metabolic changes, but mechanisms by which they alter biomechanical function is still not well understood. We hypothesize that small changes in composition due to systemic metabolic changes are ultimately responsible for altering the dynamic response to load and ultimately macroscale mechanical function. For example, mechanical changes in diabetic tendons are hypothesized to be caused by changes in non-enzymatic collagen crosslinks within and between collagen fibrils, which develop with normal aging and in diabetic patients (Monnier et al., 1996; Reddy, 2004). This would likely affect the midsubstance heavily due to its dependence on fibril
structure and morphology and both regions due to their dependence on fibril sliding and
deformation. Performing rigorous evaluation of structure, composition and function at multiple
hierarchical scales could confirm this hypothesis, as well as to support the studies presented in
this thesis.

Furthermore, the Achilles tendon is one of the strongest tendons in the human body,
withstanding loads up to 6-8 times body weight, and an efficient source of energy storage during
walking, running, and jumping (Fukashiro et al., 1995; Komi et al., 1992). It is also one of the
most commonly injured tendons (Houshian et al., 1998; Jozsa et al., 1989). Interestingly, unlike
the rotator cuff tendons which often rupture due to degeneration at the junction of tendon and
bone, the Achilles tendon often ruptures at the midsubstance, suggesting alternative structure-
function relationships regionally. Given that the structure of the insertion site and midsubstance
regions of these two functionally distinct tendons are thought to be similar, a better understanding
of the hierarchical and dynamic response to load is warranted in this tendon. We would
hypothesize that the Achilles tendon midsubstance is more like the supraspinatus tendon
insertion site, relying on a number of dynamic processes that would allow for viscoelasticity and
energy storage.

Additionally, healing tendon has a severely disrupted collagen network initially that over
time repairs and then remolds (Beredjiklian et al., 2003; Galatz et al., 2006). This system relies
on coordinated processes of rebuilding and remodeling while maintaining enough stability to
withstand normal loading. We would hypothesize that early during the healing process, the
tendon scar would likely respond to load primarily through fiber re-alignment and fibril deformation
due to its disorganization and stiff, scar-like tissue. Over time, the collagen network would re-
organize and respond more like the insertion site of the tendon, with re-alignment, uncrimping
and fibril sliding as well as deformation. Since it is well established that tendons do not fully heal
(Andarawis-Puri et al., 2015; Yokota et al., 2005), the scar would never return to native properties
and we hypothesize that the hierarchical structure-function relationships would also be
permanently altered. Completion of these studies would drastically increase the knowledge of
hierarchical relationships in tendon and allow researchers to create predictive models which could inform therapeutic strategies.

Ultimately, making use of several of these ideas may prove to be the best approach towards understanding clinically relevant structure-function relationships. The methodology presented in this dissertation to define properties at the nanoscale, microscale, and macroscale could be combined with measures at the joint level, using gait analysis for measurement of joint forces and ankle function analysis to measure joint stiffness and range of motion (Hsu et al., 2011; Humphries et al., 2015; Perry et al., 2009). As these methods are in vivo measurements, they would not add to the sample size and could be measured for all samples. This would provide a complete description of clinical function from the joint level down to the protein level and yield a novel, and complex data set on which to base modeling efforts. Furthermore, we could add additional mechanical testing points in order to study alternative loading conditions or recovery (see discussion below). An ideal model system for these studies is the Achilles tendon given its clinical relevance and our interest in regional properties given the findings presented here. Finally, these baseline studies would be combined with a perturbation, such as injury, healing, aging, or addition/removal of specific extracellular matrix proteins, to further characterize the important contributors to multiscale function.

G-7. Alternative Loading Conditions

Although the primary loading profile is in tension along the longitudinal axis of the tendon, many tendons in the human body experience multi-axial loading, including the rotator cuff tendons. The rotator cuff tendons stabilize the shoulder, which is capable of extension/flexion, adduction/abduction and three-dimensional rotations (Humphries et al., 2015). Furthermore, the supraspinatus tendon passes under the coracoacromial arch during normal loading (Soslowsky et al., 1994; Soslowsky et al., 1996), which exerts compressive and shear forces on the tendon, thought to contribute to tendon degeneration over time and the development of tendinopathy...
Composition and structure are highly tuned to meet the functional requirements of each tendon, and therefore investigating the full spectrum of function for each tendon is necessary to fully understand the multi-scale relationships that may be present.

In the proposed studies, tendons will be subjected to shear and compressive loading and analyzed for changes in the dynamic processes (re-alignment, uncrimping, deformation, and sliding). Since composition and structure are not changing during the time frame of the mechanical test, these measurements are still valid for the different mechanical tests. With minor adaptations to current mechanical testing setups, shear loading could be performed via ramp to failure mechanical testing and compressive loading via incremental compressive strain steps as previously described (Fang and Lake, 2015; Henninger et al., 2015; Kondratko-Mittnacht et al., 2015). Collagen fiber re-alignment would be measured during the mechanical test, while collagen uncrimping as well as fibril deformation and sliding would be measured incrementally using a flash freezing protocol immediately following the test (Connizzo et al., 2014b; Miller et al., 2012).

We would hypothesize that shear mechanical properties would be heavily influenced by fibril sliding and deformation, rather than re-alignment and uncrimping, consistent with a few recently published studies (Fang and Lake, 2015; Henninger et al., 2015). This is likely due to the frictional forces between the interfibrillar matrix and the fibrils, which when pulled in opposite directions would result in fibril sliding. In contrast, re-alignment and uncrimping will play a larger role in compressive mechanical properties, with a very small contribution of fibril deformation. Furthermore, we hypothesize that glycosaminoglycan content will play a bigger role in shear and compressive loading than as we found in tensile loading due to the role of fluid exudation during compressive loading, as reported in the cartilage and meniscus literature (Garcia et al., 1996; Han et al., 2011). These studies will ultimately aid in the complete understanding of what role each component of tendon plays in the tissue’s overall function.
This dissertation provides a more complete understanding of multiscale mechanical function in mouse supraspinatus tendons in ex vivo loading conditions. However, the role of cytoskeletal tensioning during and the subsequent biological response following dynamic mechanical loading is relatively unstudied. To investigate the role that tenocytes may play in the passive mechanical response, the studies outlined here could be modified to be performed on live tissue, either in vivo or directly following animal sacrifice in a tendon explant culture system (Ilic et al., 2008; Leigh et al., 2008). We would hypothesize that cytoskeletal tension would increase the dynamic response during mechanical function, particularly re-alignment, uncrimping, and deformation due to the placement of cytoskeletal anchors on and cell bodies spanning between collagen fibers. The tenocyte cytoskeleton itself may also deform as well prior to transferring load to the collagen fibrils.

Furthermore, it is well established that tendon’s composition and structure is able to adapt to mechanical loading over time. Tendon’s structure-function relationships are maintained by a remodeling mechanism regulated by the tenocytes, which are situated between collagen fibers and sense and respond to mechanical load (Jelinsky et al., 2008). Mature tendon responds to load with a temporary upregulation of collagen synthesis and degradation which balances following a period of rest. Without this period of rest, microtrauma accumulates and can eventually lead to chronic disease (Thornton and Hart, 2011; Thornton et al., 2010). Investigating the mechanobiological response following load is crucial in a population hypothesized to have a poor healing response in addition to altered initial structure and function, such as in classic EDS.

Exploring this response would require the development of an in vivo loading system that could measure the mechanical function of the tissue as well as some of the real-time dynamic responses. The development of this system would allow for the measurement of mechanical properties at multiple hierarchical scales simultaneously, which could elucidate the mechanism of strain transfer between hierarchical scales in real time. As no currently available technology is
able to measure all four dynamic processes simultaneously, this would provide a significant advancement to our understanding of hierarchical structure-function relationships. Following loading and then a period of rest, remodeled composition, structure, and mechanical function can then be assessed. This would allow us to further understand the importance of the complex structure-function relationships defined in this dissertation. This could be investigated with respect to various exercise and overuse activity protocols. Furthermore, performing these studies in the classic EDS tendons would examine the effects of various levels of exercise in these patients and ultimately inform clinical therapies.

H. Final Conclusions

Overall, these studies demonstrate that the contribution of composition and structure as well as the contribution of dynamic processes to determining macroscale mechanical function is highly dependent on location. In addition, we conclude that collagen V is a major regulator of tendon during development, impacting tendon structure, composition and function at a number of hierarchical scales. A number of future avenues for research have been presented here that would highly impact basic science research of tendon function, as well as classic EDS research. Ultimately, this work will not only help to guide clinicians in developing preventative techniques and appropriate rehabilitation strategies for classic EDS patients, but also aid to define the appropriate and important parameters on which to base tissue engineering efforts for tendon augmentation or replacement. In conclusion, this dissertation is a strong foundation on which future experimental and modeling efforts can build in order to fully understand the complex structure-function relationships present in tendon.

I. References


Reuther, K.E., Thomas, S.J., Tucker, J.J., Yannascoli, S.M., Caro, A.C., Vafa, R.P., Liu, S.S.,
collagen organization in mouse achilles tendon using high-frequency ultrasound imaging. J
Biomech Eng 136, 021029.
of procollagen alpha1 (V) chain in human patellar tendon fibroblasts: potential application in
properties of the coracoacromial ligament and their relationship to rotator cuff disease. Clin
Orthop Relat Res, 10-17.
use of an animal model for investigations on rotator cuff disease. J Shoulder Elbow Surg 5, 383-
392.
Soslowsky, L.J., Thomopoulos, S., Tun, S., Flanagan, C.L., Keefer, C.C., Mastaw, J., Carpenter,
2015. Tendon extracellular matrix damage, degradation and inflammation in response to in vitro


Wenstrup, R.J., Florer, J.B., Davidson, J.M., Phillips, C.L., Pfeiffer, B.J., Menezes, D.W.,
Chervoneva, I., Birk, D.E., 2006. Murine model of the Ehlers-Danlos syndrome. col5a1
haploinsufficiency disrupts collagen fibril assembly at multiple stages. J Biol Chem 281, 12888-
12895.

composition remains altered long after tendon detachment. J Shoulder Elbow Surg 14, 72S-78S.
APPENDIX A: EXPERIMENTAL PROTOCOLS

Mouse Supraspinatus Mechanical Testing Preparation

Dissection
1. Tape the mouse down on the black mat, fixing it behind the shoulder as well as above the neck.
2. Make incision in the skin. You should be able to make a small cut and then widen with your fingers.
3. Rotate and visualize shoulder. Tape down in ~30° external rotation.
4. Locate and break the acromioclavicular (AC) joint with fine forceps.
5. Follow spine of scapula with the forceps to gently free the supraspinatus tendon-muscle unit from the scapula.
6. ‘Flip’ supraspinatus tendon and muscle to rest on humeral shaft.
7. Cut around the humeral head with scalpel blade to transect the other rotator cuff tendons and the biceps tendon.
8. Bring the scalpel blade down the shaft of the humerus and disarticulate at the elbow.
9. Place sample in PBS to hydrate.
10. Scrape the muscle away from the tendon using the fine forceps.
11. Use fine forceps and micro-scissors to remove all non-tendon material from tendon insertion site and mid-substance. This tissue should be pinkish or yellowish and somewhat transparent. It also would not move with the tendon.
12. Remove any tissue that is not well-formed enough to bear load; leave tissue that bears load.
13. Manually grip end of tendon and apply minimal amount of load.
14. Make sure to fine dissect all sides of the tendon, focusing particularly on the insertion site. You may need to hold down the humerus using small pins. **Hydrate the tendon with PBS constantly during this dissection period. It’s best to do this in an assembly line-style taking 2-3 passes on each specimen.

Stain Line Application
1. Build your custom ruler setup out of wax pieces and your ruler. You only have to do this once and it should be useable forever!
2. Dab tendon on gauze to remove excess moisture.
3. Line up tendon on ruler setup with the ruler.
4. Dab away excess moisture from tendon on a Kim Wipe. Do this by holding the tibia with one hand and dabbing with the other. This will prevent the tendon from coming out of position.
5. With suture, mark lines across the tendon based on the gage length of your tendon. For the supraspinatus tendon, the gauge length is approximately 2.5mm, so we will place stain lines at 1, 2, and 2.5mm from the insertion site. **Be careful; too much ink will obscure the tissue and not enough won’t be able to track strain!
6. Dab gently with Kim Wipe to blot excess stain.
7. Place tendon back in weigh boat with PBS to keep it hydrated.
8. Once you are finished with the specimen stain lines, cut with a fresh scalpel from the base of the insertion site to the base of the humeral head on the medial side. You are doing this to remove the extra bone that would hide the insertion site from perfect view. You want to cut enough bone off to see the insertion but not too much so that you don’t damage the growth plate. You’ll also want to do this in one fast motion to prevent extra breaks in the bone.
9. Place tendon back in weigh boat with PBS to keep it hydrated.

Area Measurement and Analysis
1. Uncover GISMO and turn on box (~10 minutes ahead of time).
3. Open ‘mogware.vi’.
4. Click ‘Capture Mode’ on. The button will turn blue.
5. Prepare tendon for measurement.
   a. Start timer for 60 seconds when tendon is removed from PBS.
   b. Dab tendon on Kim Wipe to remove excess liquid.
   c. Place tibia posterior side up with tendon laying flat on stage.
   d. Line laser up with the insertion site (first stain line).
   e. Click ‘Run (top left corner)’, ‘Start’, then ‘zero’ all 3 axes.
6. Turn the black wheel to scan across the tendon width. Watch computer screen to be sure you see the bump when crossing the tendon.
7. Turn the blue handle to move 0.5mm on the y-axis (should be approximately 2 full turns).
8. Repeat every 0.5mm until you reach the top stain line.
9. Click ‘Stop’.
10. Replace tendon in PBS.
12. Click Capture mode Off.
13. Doublecheck file in Notepad to be sure all values were zeroed at start and that measurements were taken. You should see values increase during passes in the thickness column.
14. Log off of computer, turn off Gismo box, cover machine.
15. Open MATLAB R2012a
16. Set the directory or set a path to:
    `\medfiles\ort\McKay_Lab_Folders\shared\Software_released\Gismo_area\released`
17. Type into the command line: scale_gismo;
18. Choose the file to scale and click Enter. This program will create a new file that is appropriately scaled in order to measure the passes. It should be used on all mouse data.
19. Next we’ll analyze the gismo data. Type into the command line: gismo_area;
20. A gui will appear as shown on the right.
21. Click Load. Select the .txt file you want to analyze.
22. Click Mark Gismo and use the cursor to select the sides of each peak going from left to right. When all the peaks are marked click enter. Create a folder with the name of the tendon and save all files there.
23. An area vs. length bar graph will pop up. Record the area from the MATLAB command window.
24. If you want to analyze local data, you can use the ‘Local Area’ button at this point. Choose the first 1mm for the insertion site (0-1mm) and the next 1mm for the midsubstance (1-2mm). Enter the values in the ‘Pass 1’ and ‘Pass 2’ box and click ‘Local Area’. Record the area for each region.

**Potting and Sandpapering**

1. Dry samples with Kimwipe to make sure the bone is dry.
2. To prepare PMMA, mix powder and catalyst in a separate container. This is a tricky step because there is no perfect ratio for powder:liquid. You want the mixture to be liquid enough to pour but the more liquid it is, the longer it takes to cure.
3. Pour PMMA into each acrylic pot. You can do this step with up to 10 specimens at a time. You want the PMMA level at the rim of the pot.
4. Put humerus of each sample into the pot, oriented such that the medial side (back of the tendon) is facing the center of the pot.
5. As the pot is curing, make sure the tendon is oriented vertical. You may need to tilt the humerus to make sure this happens. You'll also want to make sure that the insertion site is not obstructed by PMMA.

6. Wait ~30 minutes. Apply PBS to pot during this time to keep the tendons hydrated.

7. Prepare enough PMMA for a 1mL syringe.

8. Pull up PMMA into the syringe. Wipe the tip of the syringe with a Kim Wipe.

9. Apply PMMA around the edges of the humeral head taking care not to touch the tendon with PMMA and not to obscure the insertion site from view.

10. Wait ~30 minutes for the PMMA to completely dry. Apply PBS to tendons during this time. Also, cut pieces of sandpaper approximately 3-5mm.

11. Dry the tendon and surrounding bone.

12. Place a drop of glue on each sandpaper piece (with rough side facing up) and spread around sandpaper with glue applicator.

13. Spread tendon out with fine forceps. Then drag tendon (holding onto the top most portion) onto the first sandpaper piece making sure that the top stain line lines up with the bottom of the sandpaper piece (see right).

14. Then flip other piece of sandpaper on top of the initial piece with the tendon attached.

15. Press down for approximately 30 seconds. Rehydrate tendon with PBS.

**Viscoelastic Testing**

**Testing Protocol**

![Viscoelastic Testing Protocol](image)

- **Block 1**: Preload to 1% strain
- **Block 2**: Preconditioning at 1 Hz, 10 cycles
- **Block 3**: Hold, 60 seconds
- **Block 4**: Ramp to 4% Absolute Strain at 100% strain/second
- **Block 5**: Hold, 600 seconds
- **Block 6**: Frequency sweep, 10 cycles at 0.1 Hz
- **Block 7**: Ramp to 6% Absolute Strain at 100% strain/second
- **Block 8**: Hold, 600 seconds
- **Block 9**: Frequency sweep, 10 cycles at 1 Hz
- **Block 10**: Ramp to 6% Absolute Strain at 100% strain/second
Block 11: Hold, 600 seconds
Block 12: Frequency sweep, 10 cycles at 10 Hz
Block 13: Return to Zero
Block 14: Hold, 60 seconds
Block 15: Ramp to Failure at 0.1% strain/second

Quasi-Static Ramp to Failure Data Analysis
1. Open MATLAB.
2. Set the directory to or set a path to:
   \medfiles\ort\McKay_Lab_Folders\shared\Software_released\optikos\released
3. This protocol assumes you have the correct file format for running optical data (obtained using rawful or manually). The document should have for columns: Time, Disp, Load, Block.
4. Type into the command line: optikos;
5. A GUI will pop up on the side with Load at the top.
6. Load the images for your specimen. If you load one the rest will automatically load.
7. Change the display from Not Enhanced to Contrast + Filtered
8. Click Instron Restrict and load the file you created earlier. It should be a .txt extension.
9. Select the Image capture correction where you can adjust the time your testing began. Usually it is 0.0 and click Enter.
10. Choose the maximum displacement. Click ok and use the cursor to choose the point where the slope is maximum, before tendon failure.
11. After you select the point a warning dialog will pop up and tell you how many images there are to analyze. The software will automatically analyze a certain number of images. Keep track to make sure you are measuring the same number of images per specimen.
12. Choose ROIs (regions of interest). There are four regions of interest, PMMA, Stain line 1 (S1), Stain line 2 (S2), or Stain line 3 (S3, near the grip). Select boxes on the edges of each ROI. You want these boxes to be unique enough to track the region well. An example is shown to the right for PMMA and stain line 2.
13. Track ROIs.
14. After the tracking is done a graph will appear showing how far your regions moved. To ensure your tracking worked use the scroll feature located right above the Instron Restrict button to view your boxes across all the images.
15. Click Text Output and save the file as “Specimen Name_PMMA-LR.roip” and change PMMA to S1, S2, or S3 depending what stain line was tracked.
16. Now we will apply the displacement of the PMMA to the first stain line so that we can determine the tendon strain relative to bone. These markers should not move a lot.
17. Type into the command line: apply_disp;
18. Choose the .roip file for the PMMA. Then choose the .roip file for S1.
19. A graph will appear and another window asking you to save the output file. Label this output file: “Specimen Name_S1SYN-LR_trk.txt”.
20. Now we are going to compare the movements between the stain lines.
21. Type into the command line: trk2element;
22. Choose the file for the first stain line (S1SYN-LR_trk.txt). The choose the file for the second stain line.
23. A graph will pop up showing how the two stain lines are moving relative to one another. When the tracking is complete, save as “Specimen Name_S1-S2_trk.txt”.
24. Repeat steps for S2-S3.
25. Next we will calculate the stiffness. Type into the command line: el2d_stiffness;
26. Choose the _eld2D.txt file for the region you want to analyze.
27. Open the mechanical test data file.
28. Select the Image capture correction where you can adjust the time your testing began. Usually it is 0.0 and click Enter.
29. It will then ask you the primary loading direction. Since we stretched in the Y direction click Y.
30. A graph will appear with Load against Optical Strain and a box with the image number will appear. Each image is represented on the graph with the blue diamond and black circle around it. The black line represents the best fit to these images. In order to achieve the best fit, delete the images associated with the toe region and try and get the line as fit as possible to the point. An example is shown below. Specifically, choose the steepest region that best represents the curve.
31. Click close on the image selector when your graph looks good and save the graph as the default name.
32. Next we will obtain our image calibration. Note: this step is not necessary if using GetLength command for frequency sweep analysis
33. Navigate to the folder on the computer with the images for your specimen. Open up the images labeled _calib and preview them to make sure they look normal.
34. In the MATLAB console enter the command “calib_image;”
35. A cursor will appear and select a region on the ruler. When clicking the cursor select the bottom of the line and drag the cursor to the bottom of another line to mark the range.
36. After you draw the line double click and a box will appear that says enter calibration object length. Enter the length that your line spans and click enter.
37. In the MATLAB console copy the third number shown (L_cal (pix/mm)) into the excel file.
38. Now we will make out calculations:
   a) Stiffness (optical) = Kopt*Calib (pix/mm). We can also report grip stiffness (Kgrip).
   b) Modulus (optical) = Kstrn*100/CSA
39. Now we will run the bilinear fit program. Navigate to or set a path to the directory:
   \max\software cooker-freezer\TendonMechanics\cooker-BKC
40. Into the command line, type: close all; clear all; clc; Bilin_bri;
41. Choose the _Kel2d.txt optical strain file that was generated from optikos.
42. Choose the minimum force point you want to use (typically 0N) with the cursor.
43. Choose the maximum force point you want to use with the cursor.
44. Copy and paste data output from MATLAB.
45. If the data is not fitting properly, go into the code and adjust the initial values as necessary.

Dynamic Mechanical Analysis
1. Open MATLAB.
2. Set working directory or set a path to:
   \max\software cooker-freezer\SLRP\Cooker\v1.0.1_Matlab M files combined
3. Type into the command line: csv2txt;
4. Select the Instron .csv file. The program will create a new file in the same location with the same name but with .txt after. To streamline the process, you can type the same command for as many specimens as you have and run them all in a row.
5. Next, we’ll analyze the mechanics. Type into the command line: LoadandFitSine_BKC;
6. Navigate to the instron file for the specimen, which should be a ‘_csv.txt’ extension.
7. Dialog Box: Input the proper vector of block numbers and frequencies for this protocol. For Mouse SST (Bri Thesis), the block numbers are [11 12 13 18 19 20 25 26 27] and the frequency vectors are [0.1 1 10 0.1 1 10 0.1 1 10]. Also input the seconds to subtract from position time stamp, which is 0.002.
8. Dialog Box: Enter the starting cycle and ending cycle. As default, this is 1 and 10.
9. The program will then run the mechanical evaluation for all frequencies and strain levels (should be 27 for this protocol). The images should look like this:
10. You'll want to look out for any odd fitting of the data and note it when it occurs.
11. Copy and paste the data from the MATLAB into the viscoelastic mechanical data receptacle template.
12. Next we will get the gauge length of the specimen. Type into the command line: GetLength;
13. Type “y” to select a new calibration image (each new specimen should have its own calibration image)
14. Select the calibration image (ruler image) for that specimen. Maximize the image.
15. Click to the bottom of one tick mark on the ruler and then to the bottom of another tick mark on the ruler to span as big of a region as possible. Enter this span in mm and click OK.
16. Select the first image saved for the specimen. This image should be when the specimen is in an unloaded state prior to the ramp to failure. Maximize the image.
17. Use the cross-hairs to click to select the bottom of the tendon (in the middle of the insertion stain line) and then click to select the top of the tendon at the sandpaper.

Collagen Fiber Re-Alignment Analysis
1. Open MATLAB.
2. Set working directory or set a path to: \max\software cooker-freezer\cross-polar\beta-Ben-v5
3. Type in “close all; clear all; clc; kris_cross;”
4. Dialogue Box: Set Bundle Width (pixels) as 10 and Bundle Spacing (pixels) as 20. Note: This is the default. This setting was determined based on slight motion artifact of the image during CP rotation. Click OK.
5. Dialogue Box: Choose Sample Image File: Navigate to where your images were saved. Note: If you don’t have an output file for motor information, you will receive an error. Click open for any of the images.
6. Dialogue Box: Enter the minimum number of images/alignment maps as 15.
8. Dialogue Box: Was this a fatigue test? Click Yes/No/Cancel.
9. A box will then appear asking you to choose what alignment map series to analyze. After the specimen ID, image time stamps for the start and end of each alignment map are listed. Navigate to the excel spreadsheet that has the times at which the specimen failed and only choose images prior to the failure point. Once the desired maps are selected, click OK.
10. Select a region to crop. Move the ROI to contain the tendon and region above it. A smaller ROI is better to reduce computational time, but make sure you consider that the tendon will
elongate during the test. Once the ROI is selected, double click on it and the maps will start analyzing.

11. After the first map is processed, it will ask for a location to save. You’ll want to save them in a folder titled ‘10x20’ to define the bundle width and spacing you’re using. You’ll also want to save the tendon files and the waveplate files in separate folders.

12. Repeat for each specimen and waveplate.

13. Type “close all; clear all; clc; batch_nonintdfit;”

14. Navigate to the saved locations of the .intd files. Click one and they will all run.

15. Type “close all; clear all; clc; nnfmask;”

16. Choose a square region around your mask. You’ll be doing this for the insertion site and the midsubstance. Save the file as “_ins_NNFMSK.txt” or “_mid_NNFMSK.txt”.

17. Type “close all; clear all; clc; nnfmask_p2plogn_multi;”

18. This will apply the thresholds to all .nnf files and generate the nnf_sum.txt file.

19. Type “close all; clear all; clc; nnf_mech_slrp;”

20. This will add the load-displacement data into the re-alignment data.

21. Type “close all; clear all; clc; Bilin_Align_BKC;”

22. Choose the nnfsum file from the re-alignment data.

23. Choose the KEL2D file from the optikos data.

24. Choose the breakpoint between the two lines.

25. Save the output data, which will describe the strain required to reach the breakpoint and the amount of re-alignment occurring before the breakpoint.

**Fatigue Testing**

*Testing Protocol*

**Fatigue Testing Protocol**

![Fatigue Testing Protocol](image)

Block 1: Preload to 1% strain
Block 2: Preconditioning at 1 Hz, 10 cycles
Block 3: Hold, 60 seconds
Block 4: Cyclic Load at 1 Hz from 25%-75% maximum load

- Wild Type/Heterozygous: 0.75N – 2.75N
- Null: 0.25N – 1.00N

**Notes: Set P/I/D to -5/1.25/0 for these tests

**Data Analysis**

1. Open Matlab.
2. Set working directory or set a path to:
   \max\software cooker-freezer\Fatigue\Cooker\Version3.05-5848-Bri
3. Run program: mainFatigue.m
4. Choose the specimen fatigue data file. This file should be a ‘.csv.txt’ extension file.
5. A user input box will pop up asking if specimen dimensions exist. Inputting specimen dimensions will allow the program to calculate stress and strain from load-displacement data.
Click ‘yes’ and navigate to the specimen dimensions file, which should be a ‘.txt’ file that has the following format:

<table>
<thead>
<tr>
<th>Specimen: Col5B-LSST_004</th>
<th>Gage(mm)</th>
<th>CSA(mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Specimen: Col5B-LSST_004</td>
<td>2.727210809</td>
<td>0.178</td>
</tr>
</tbody>
</table>

6. A user input box will pop up asking what genotype this sample is. This will tell the program which protocol was used. Choose the correct genotype.

7. A box will then pop up to name the output file, which will have a ‘_FTG-raw.txt’ extension. Save the file.

8. The analysis will then run and the results of program calculations will appear. This software calculates Peak Cyclic Strain, Hysteresis, Secant Stiffness, Tangent Stiffness, Cycles to Failure, and Damage. A typical fatigue curve will look like the sample shown below.

9. Copy and paste the output data from the MATLAB command window.

Collagen Fiber Uncrimping

*Testing Protocol*

Block 1: Preload to 0.02 N
Block 2: Preconditioning from 0.02N to 0.04N, 10 cycles
Block 3: Hold, 60 seconds
Block 4: Ramp to failure at 0.1% strain/second

*Flash-Freezing Protocol*

1. Prepare tendon for testing as described in the mechanical testing protocol.
2. Fill dewar flask with liquid nitrogen.
3. Set up camera (same as mechanical testing protocol).
4. Make sure the tendon is oriented straight for tensile testing and in plane of the camera.
5. Zero the load once the tendon is slightly slack.
6. Manually preload the tendon to 0.02N.
7. Zero the displacement.
8. Begin the test.
9. Record the displacement value after the cycles of preconditioning. Use this value to calculate the stop point (i.e. 5% is 5% of the 2.5mm gauge length = 0.02+displacement after preconditioning).
10. Once the test has reached the desired stop point, press ‘Stop’ in the Bluehill software.
11. Immediately spray the tendon with flash freezing spray for approximately 15 seconds, or until some frost appears on the tendon. The further away you are from the tendon, the longer this will take.
12. Cut the tendon at the insertion site carefully with the microscissors. Try to get as close to the insertion site as possible.
13. Freeze spray the tendon again to ensure the tissue is frozen.
14. Remove the top grip and tendon from the mechanical testing setup.
15. Freeze spray the tendon again to ensure the tissue is frozen.
16. Cut the tendon from the top grip with the microscissors. Again, try to get as close to the sandpaper as possible.
17. Place tendon into the specimen dish with the appropriate orientation. (I usually place the tendon vertically in the dish with the insertion closest to the bottom and the midsubstance closest to the top). It doesn’t matter what orientation you use, as long as you write it down somewhere and know what it is.
18. Cover the tendon in OCT gently. Make sure not to turn the specimen when putting OCT into the dish.
19. Use large forceps to place plastic specimen dish with tendon into the liquid nitrogen until completely frozen.
20. Place in labeled specimen bag. Roll up the specimen bag and put into a larger biohazard bag. You want to roll the bag so that it’s more difficult for the block to come out of the plastic dish once frozen (which would cause you to lose the orientation).

**Cryosectioning**

1. To begin, the cryostat microtome should be set to approximately -22 to -25 degrees Celsius, which is optimal for sectioning tendon. Also, turn on the light.
2. Next, prepare the block containing your specimen by squeezing OCT onto one of the metal chucks, which should be sitting in one of the holes of the Peltier elements.
3. Quickly place the block on top of the button and situate it so that its top is parallel to the surface of the button. Make sure to know the orientation of your specimen (insertion site down and midsubstance up) and be consistent across specimens.
4. Squeeze a small amount of OCT on top of the block in the direction of the longitudinal axis of the tendon. This will allow you to have OCT to section through the properly line up and adjust the angle of sectioning.
5. Wait 5-10 minutes for the OCT to harden until it appears white. During this time, you can label your slides.
6. Obtain a new blade and place in the cryosection stage. Be careful as the blades are extremely sharp!
7. Grab the button and place it within the specimen clamping.
8. Twist the clamping lever to firmly hold the chuck and pull the orienting lever to situate the chuck such that its surface is parallel to the metal plate behind the clamp.
9. Turn the knob controlling thickness to 20 microns.
10. Adjust the entire position of the block with respect to the blade by using the arrow buttons to retract or pull it closer. Test the best position by removing the knife guard and turning the handwheel on the right side of the machine until you are able to section a very thin part of the block.

11. Adjust the orienting lever until sections span the entire block and the blade is slicing parallel to the button.

12. Slice away the excess OCT until you encounter your specimen. During this time, adjust the block orientation so that your blade is slicing evenly across the entire area containing your section.

13. When close to your section, cut the block into a small rectangle. This will make your sections small in area and allow more sections to be placed on a single slide.

14. Cut 3-5 sections and arrange them in a line (all in the same orientation) on the stage. You can usually cut multiple sections in a row if you’re using the plastic anti-roll plate.

15. Apply slide to the sections.

16. Leave slides in cryostat until you have finished sectioning the sample. Section all the way through the sample every time. It’s very difficult to obtain the appropriate orientation when trying to resection a sample.

17. Remove all slides from the cryostat and put directly into a slide box in the freezer. Store at -20°C until use.

**Picosirius Red Staining**

<table>
<thead>
<tr>
<th>Step</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap H₂O</td>
<td>1 minute</td>
</tr>
<tr>
<td>Picosirius Red</td>
<td>45 minutes</td>
</tr>
<tr>
<td>Acidified H₂O</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Hematoxylin</td>
<td>10-15 minutes</td>
</tr>
<tr>
<td>Tap H₂O</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Acid EtOH</td>
<td>20 seconds</td>
</tr>
<tr>
<td>Tap H₂O</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Scott’s Buffer</td>
<td>20 seconds</td>
</tr>
<tr>
<td>Tap H₂O</td>
<td>3 minutes</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>1 minute</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>1 minute</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>1 minute</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>1 minute</td>
</tr>
<tr>
<td>CitroSolv</td>
<td>1 minute</td>
</tr>
</tbody>
</table>

**Polarized Light Imaging**

1. Turn on the microscope and the camera (plug firewire cable into either input on side of camera).

2. Using the 5X objective, focus the condenser so that the edges of the hexagon are sharp. (Dial at bottom left of microscope, on the base, changes the size of the hexagon. Condenser is focused using black knobs under stage).

3. Find the region of interest (e.g., insertion site or midsubstance) and focus the specimen.

4. As you change objectives, ensure that the edges of the hexagon are still in focus and that the specimen is still in focus.

5. Upon reaching the desired magnification (10x for mature SST), move the condenser hexagon out of view.

6. On the computer connected to the microscope, load QCapture Pro software. Click on the camera icon to bring up the capture menu. Click Preview to view the image.

7. Rotate the stage until the tendon is at a ~45 degree angle (bottom left to top right) and then lightly tighten the stage.
8. Set the analyzer to 180 degrees and the polarizer to 270 degrees. This position will be considered 45 degrees when saving the images.
9. Turn the light all the way up and ensure that all light is directed toward the camera.
10. **Take the non-compensated image.** For crimp analysis, you will only need one non-compensated image at 75°. With the 10X objective in place, load the Noncomp Polarized Light profile. Adjust exposure as needed. This can be done by checking that the image is not too bright (with the analyzer at 180° and the polarizer at 270°) or too dark (with the analyzer at 135° and the polarizer rotated 45° clockwise to the blue line).
11. Rotate both the analyzer and polarizer in a clockwise direction to 75°.
12. Press Snap and save each picture as a .tiff file.
13. When you have finished, put away the compensator and analyzer and turn off the microscope lamp.

**Data Analysis**
1. Open MATLAB (v7.0 or later).
2. Set a new path to where your software is.
3. In the command window prompt, type `[dt1,dt2, magnitude, distance, amp, meanamp, MeanDistance, MeanAmplitude,RegionDistMeans, RegionAmpMeans, NUMpixs, RegionPeriod,MeanPeriod] = fft_profile_BRI_allangles3reg_mag_fig`
4. You will be prompted to navigate to the file where your crimp images are saved.
5. Choose the image file you want to analyze (should be a *.tif file extension). For this analysis, choose the noncompensated image taken at a 75 degrees angle (please see the “Polarized Light Imaging” for more details).
6. First, draw a rectangle by clicking four points around a representative section of crimp for the image. Then click ‘Enter.’
   a. It is important to draw these lines so that opposite sides of the rectangle are parallel to each other and the fibers as possible.
   b. Pressing ‘Delete’ will remove the previous click and allow you to choose a new corner instead.
7. Now you want to draw a line in the direction of the collagen fibers. Choose to follow a fiber that has visible differences in light intensity (crimp). Click once to start drawing a line and again to finish the line. You may edit this line until you click ‘Enter.’ This line should represent the fiber direction of the tissue but ideally should be oriented towards 45 degrees.
8. Next, you will be prompted to save the Excel file and the MatLab figure to your desired location.
9. This program splits the rectangle into three regions and averages the intensity across many lines within that region. The user will have to pick the peaks from those average plots for each region, and for each angle. The Intensity vs. Length plot will come up for the first angle.
10. Before beginning to click, make sure the rectangles (regions) within the blue lines on the original image are appropriate for qualitative analysis and parallel to the fiber direction.
11. In the Intensity vs. Length plot, select the **peaks** of intensity in order to obtain the coordinates of crimp peaks. Hit enter after all of the peaks have been chosen. The next sub-region in the rectangle will come up. Continue to pick peaks on each region.
   a. Only choose peaks that cross the mean intensity threshold (noted by a red dashed line at 0 degrees – This line comes from a dtrend of the data) and drop below the green line. The peaks crossing the red line should generally have an intensity of 20 or greater.
   b. Note: For type III uncrimped tissue, it is important to pay attention to there being too numerous peaks that falsely represent the tissue due to_____.
12. SAVE the following data from the command prompt into an Excel file:
   a. RegionAmpMeans
   b. MeanAmplitude
   c. RegionPeriod
d. MeanPeriod

13. Repeat for all specimens, for midsubstance and insertion.

**Collagen Fibril Deformation/Sliding**

**Fixing**
1. Follow protocol for collagen uncrimping testing, flash-freezing and sectioning prior to this protocol.
2. Place slides into slide rack for staining. Do this in the walk-in freezer so the samples do not melt.
3. Bring a slide jar or plastic container of 4% neutral buffered formalin into the cold room or walk in freezer. Cool formalin until it's cold to the touch.
4. Place slide rack with slides in formalin for 4 minutes.
5. Remove from formalin.
6. Wipe back of slide with Kim wipe and air dry slides under a fume hood. Let samples dry for approximately 1 day before imaging with AFM.

**AFM Imaging**
1. Switch on the AFM Power.
2. Attach the probe tip to the AFM.
   a. Obtain the fluid holder for the AFM tip.
   b. Place the probe holder on the loading tool (round black cylinder with 3 stations for loading tips on it) in the station that has four prongs and a small staple.
   c. Push the probe holder down on the loading station so that the staple on the probe holder is raised.
   d. With a fine forcep, carefully obtain the probe tip from the box of probes. Make sure you have selected the right probe for your application (This will vary). For Tapping mode, the best tip for our samples are NCHV-A probes. For QNM Peak Force Tapping mode, the best tip is ScanAsyst-Fluid+ probes.
   e. Place the probe underneath the staple on the probe holder and lift the holder off the loading station such that the staple comes down and secures the probe in place.
   f. Take the loaded probe holder to the AFM and attach to the head of the machine. The head of the machine can be removed by unscrewing the screw on the right side of the head and sliding the head out of the groove upwards. Be careful when manipulating the scanning head.
      i. Do NOT touch the mirror on the underside of the left surface of the head.
      ii. Do NOT pull on the scan head too hard. It is attached to the machine.
   g. Re-attach the scan head to the machine by gently sliding it back into its groove and screwing it in place. DO NOT DROP THE HEAD.
3. Open the program and choose the experiment you want to run. The software for the Bruker Dimension Icon AFM is called ‘Nanoscope’.
   a. For scanning tendon for image analysis (d-period) we will want Mechanical Properties → Tapping Mode → Tapping Mode in Air.
   b. For scanning tendon for more mechanical evaluation we will want Mechanical Properties → Quantitative Nanomechanics → Peakforce in Fluid.
4. Initialize the stage (This should be automatically prompted by the program).
5. In the ‘Setup’ panel, choose the correct probe that you are using for the experiment.
6. In the ‘Setup’ panel, align the laser up with the probe tip using the gold plated alignment tool (small black cylinder with gold plate on top).
   a. Place the alignment tool underneath the probe. This should make the image background become bright. You should turn down the brightness for this step.
b. Line up the laser with the tip of the probe in reflected view (this should be the lowest view of the tip when you are moving the optics down).

c. By using the two knobs on the top of the scan head, maximize the sum value of those knobs on the display.

d. You should also move the vertical and horizontal displacement of the laser to zero at this time by moving the knobs on the left of the scan head.

7. Perform a Thermal Tune (This is one of the drop down menus at the top of the screen). When the Thermal Tune dialog box appears, change the tune range to be the same as the range listed on the case of the probe tips. Acquire data. A good thermal tune should look like a single nice peak.

8. Back in the ‘Setup’ panel, focus on the real image by moving the optics upward. Then put the crosshead on top of the probe tip.

9. For tapping mode, you’ll also need to optimize the tip at this time using the optimization setup in the bottom left corner.

10. Switch to the ‘Navigate’ panel.

11. Load your specimen for the protocol you are using. (With a standard slide, you can put it in place and hold it with a magnet.)

12. Navigate the laser to the tendon using the navigation panel at the bottom left. You may also move the scan head down to about ½” from the specimen surface by clicking the down arrow for scan head. BE CAREFUL not to zoom down to the specimen too quickly; you could break the tip or cause a crash of the AFM and the specimen.

13. Adjust focus by moving the scan head.
   a. Use ‘tip reflection’ mode first. Focus on the specimen here; at this point, the sample will be about 2 mm from the tip. You should only move at speeds less than 20% at this step.
   b. Then use ‘sample’ mode and focus on the specimen again. At this point, the sample will be 1 mm from the tip.

14. Switch to the ‘Check Parameters’ panel. If you chose the probe correctly in Step 5, you should not have to do anything here. Make sure the Autocontrol buttons are on to begin with.

15. Switch to the ‘Engage’ panel. This will initiate the scan sequence and move the tip of the cantilever to the surface of the specimen. You should be scanning at this point.

16. To save files: In the ‘Scan’ panel, select the ‘Select Capture Directory’ button in the upper right hand corner. Browse to the directory you want to save in. Change the filename prefix by typing into the entry box to the left of the capture directory icon. You will have several options:
   a. Capture (This will capture the image files)
   b. Capture Continuous (This will capture a video of your scan)
   c. Capture Now (This will capture a snapshot of your scan)

Data Analysis

1. Open MATLAB.

2. Set working directory or set a path to:
   \max\software cooker-freezer\dbandaide\released\v2.9

3. Type into the command line: dbandaide;

4. Choose the Zply Height file. This is the file generated from applying a zply fit to the height map of a Tapping Mode image. This is specific to this protocol and can be adjusted to fit any type of map.

5. Apply Histogram equalization to the map by pushing the ‘HistEq’ button.
6. Add lines across the collagen fibrils using the 'Add Line' button. You want to make sure that each line spans at least 10 bands to get an accurate measure of d-period length. After each fibril, double click to finalize the line. Each line will produce three figures:
   a. Intensity vs. Length
   b. Intensity vs. Length Fitted
   c. Cumulative Spectral Power
7. When you've finished adding lines to the image, click 'Output Data'.
8. Save the '_AFO.txt' file.
9. A histogram of the d-periods from that image will appear. It will most likely look strange since there are only about 10 fibrils in each image.
10. Open each AFO file to obtain the d-period for each fibril chosen in the image. This software outputs the p50, which is the d-period as determined by the FFT, and the pMED, which is the d-period determined by the median d-period of all the band widths calculated from the intensity vs. length plot.

Histology

*Tissue Processing for Paraffin Histology*
1. Dissect tendon for histological processing, i.e. with the muscle and humerus intact.
2. Fold Kimwipe into a small square to fit inside of the histology cassette. The last fold should open like a book inside the cassette.
3. Place muscle-tendon-bone unit in cassette inside the Kimwipe. Be careful about orientation. You want the tendon to be straight so your section (in the coronal plane) captures all of the relevant tissue.
4. Fold Kimwipe and close cassette.
5. Place cassettes in a labeled specimen cup filled with 4% neutral buffered formalin for at least 7 days. You can leave them in formalin longer if you’re not ready to process.
6. Transfer cassettes to a labeled specimen cup filled with decalcifier for another 7 days. We use Immunocal typically. Place the specimen cup on an orbital shaker to ensure all of the tissue is infiltrated. Do not leave samples in decalcified for more than 7 days.

7. Transfer cassettes to a labeled specimen cup filled with 70% EtOH until ready for processing.

8. Once ready to process, transfer to automated processor system with the following protocol:
   a. 70% EtOH for 45 minutes at 37°C
   b. 85% EtOH for 45 minutes at 37°C
   c. 100% EtOH for 45 minutes at 37°C
   d. 100% EtOH for 45 minutes at 37°C
   e. 100% EtOH for 45 minutes at 37°C
   f. 100% EtOH for 45 minutes at 37°C
   g. Xylene for 50 minutes at 37°C
   h. Xylene for 60 minutes at 37°C
   i. Xylene for 60 minutes at 37°C
   j. Paraffin Wax for 30 minutes at 62°C
   k. Paraffin Wax for 45 minutes at 62°C
   l. Paraffin Wax for 60 minutes at 62°C
   m. Paraffin Wax for 60 minutes at 62°C

9. Remove samples from processor and embed.
   a. Place cassette on heat to melt excess wax.
   b. Orient specimen in plastic specimen dish for proper sectioning.
   c. Fill specimen dish with wax.
   d. Place cassette bottom over filled specimen dish.
   e. Add more wax so that the specimen wax fills through the cassette bottom.
   f. Place samples in cold to solidify wax.

10. Section samples at 10µm.
    a. Face samples with microtome until close to specimen.
    b. Place in cold immunocal for approximately 10 minutes.
    c. Begin taking sections. Because the mouse supraspinatus is small, we’ll take all of the sections through the thickness of the tissue.

### Hematoxylin and Eosin Staining Protocol

<table>
<thead>
<tr>
<th>Time</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 minutes</td>
<td>Xylene</td>
</tr>
<tr>
<td>3 minutes</td>
<td>Xylene</td>
</tr>
<tr>
<td>2 minutes</td>
<td>100% Ethanol</td>
</tr>
<tr>
<td>2 minutes</td>
<td>100% Ethanol</td>
</tr>
<tr>
<td>2 minutes</td>
<td>95% Ethanol</td>
</tr>
<tr>
<td>2 minutes</td>
<td>95% Ethanol</td>
</tr>
<tr>
<td>2 minutes</td>
<td>70% Ethanol</td>
</tr>
<tr>
<td>2 minutes</td>
<td>DH2O</td>
</tr>
<tr>
<td>10 minutes</td>
<td>Hematoxylin (clean off iodized surface)</td>
</tr>
<tr>
<td>Until water is clear</td>
<td>DH2O</td>
</tr>
<tr>
<td>3 Dips</td>
<td>Clarifier</td>
</tr>
<tr>
<td>2 minutes</td>
<td>DH2O</td>
</tr>
<tr>
<td>3 Dips</td>
<td>Bluing</td>
</tr>
<tr>
<td>2 minutes</td>
<td>DH2O</td>
</tr>
<tr>
<td>2 minutes</td>
<td>95% Ethanol</td>
</tr>
<tr>
<td>10 seconds</td>
<td>Eosin</td>
</tr>
<tr>
<td>2 minutes</td>
<td>95% Ethanol</td>
</tr>
<tr>
<td>2 minutes</td>
<td>95% Ethanol</td>
</tr>
</tbody>
</table>
2 minutes 100% Ethanol
2 minutes 100% Ethanol
2 minutes 100% Ethanol
3 minutes Xylene
3 minutes Xylene
3 minutes Xylene

**Elastin Immunofluorescence**

1. If the samples are currently frozen, remove from freezer and let air dry for a couple minutes. If samples are fixed (via formalin as for crimp/AFM testing), they are ready.

   a. 5 mg/mL hyaluronidase
   b. 0.1% sodium borohydride solution (0.01 g sodium borohydride + 10 mL 1X PBS)
   c. 5% goat serum (0.5 mL NGS goat serum + 9.5 mL 1X PBS). Make enough goat serum for your block and to dilute your antibodies.

3. Dry around sections with a kim wipe and circle with a pap pen. Be careful not to have sections circles touching and make the circles wide enough for easy waste collection.

4. Rehydrate in PBS for approximately 1 minute.

5. Perform antigen retrieval for fixed slides by adding 5 mg/mL hyaluronidase for 1 hour at 37°. You can do this by putting slides on the slide warmer or in the oven. Liquid will dry after approximately 30 minutes so you’ll have to reapply.

6. Treat sections with sodium borohydride for 15 minutes.

7. Wash 3 times for 5 minutes each time in 1X PBS.

8. Treat with goat serum block solution for 1 hour at room temperature.

9. During this time you can prepare your primary antibody dilution. (Anti-Elastin dilution is 1:100 with 5% goat serum, abcam21610) Spin aliquots down first and mix after pipetting.

10. Treat with primary antibody at 4°C overnight with water in troughs.
   a. Drain block from slides but do not wash.
   b. Leave negative control slide in goat serum block.
   c. Label slides with pencil not pen.

11. Wash 3 times for 5 minutes each time in 1X PBS. Be careful not to contaminate your negative controls.

12. Prepare secondary antibody (Goat Anti-Rabbit Alexa Fluor 488, abcam150077) by diluting with 1x PBS at a dilution ratio of 1:500. The secondary antibody is light sensitive so wrap in foil.

13. Treat with secondary antibody in closed black box (dark) for 1 hr at room temperature.

14. Wash 3 times for 5 minutes each time in 1X PBS. Cover between washes.

15. Mount slides with ProLong Gold Antifade Mountant with DAPI (Life Technologies, P36935)
   a. Tap off the PBS on to a paper towel.
   b. Add a small line of mounting media to the side of the slide.
   c. Flip the slide over and attach coverslip from by rocking the slide onto the coverslip from one side to the other. This will ensure mounting media on all parts of the slide.
   d. Remove any visible bubbles.

16. Leave slides to dry for 24 hours in a dark box.

17. Seal around edges with nail polish.

18. Image.

19. Open MATLAB.

20. Set working directory or set a path to:
    ```
    \max\software cooker-freezer\immuno\cooker\cooker_BKC
    ```

21. Type into the command line: IF_analysis_BKC;

22. Choose an image in the folder which holds all of the images to be analyzed. This program will run through all of the images in this folder.

23. Draw a polygon around the tissue sample.
24. Double-click at the end to complete the polygon.
25. Copy and paste the data into an Excel file for analysis. It should look like this:
   col5B-299_S1-3_MID_GFP_20x.jpg 18.5
   The image intensity (value output) is the percentage of colored pixels in the polygon chosen.
   You’ll want to normalize these values to a negative control image.

**Bioquant Data Analysis**

1. Create new data volume. You’ll want to do this at the start of each project or each part of your project. Usually, each stain or analysis is a separate data volume.
2. Create new data set. A data set should be created for each specimen and you’ll want to do this in the appropriate data volume. If you are already working in the data volume you want, you can also select ‘Quick data set’.
3. Open a file.
4. On Large Image Navigator, set it to 1:2 to show the entire picture.
5. Choose the correct calibration.
   a. Parameters>> Mag (Nikon “-N”; Leica “-L”…)>> Assign
   b. To switch cameras, Image>> Calibration>> File>> Load…>> e.g. Nikon.cal
6. Define ROI. Chose Irregular shape and click Spacebar to End. Then click Define. You’ll want to choose a region of interest that only contains tendon. For some samples, this might be the whole image. For mouse SST, this is part of the image. Push spacebar to finish the ROI and right click the screen to end ROI definition.
7. Select an array to measure. You always want to measure the total ROI area first (A2 Area
8. Double click on the array.
9. Click preview. At this point, the outline of what’s to be measured will be highlighted in yellow.
10. Click measure. A single value will be measured.
11. Next measure the cell areas by clicking the array for A1 Cell Area.
12. Adjust the threshold for each image individual. Press “Select” and click on cells. Regions with the same color will turn pink as the software is picking up these regions. Adjust until all cells are chosen.
13. Press ‘Preview’ to highlight the cells and press ‘Show’ to remove the pink inside. This will give you a good idea of how well your threshold is picking up each cell.
14. Edit your cells
   a. Draw Threshold (paintbrush): This tool will allow you to add cells. You can change the size of your drawing tool by scrolling with the wheel on the mouse.
   b. Erase Threshold (eraser): This will allow you to erase cells (if they are only partially in the ROI) and erase errors.
   c. When you are finished editing, click ‘Preview’. Then ‘Measure’.
   d. Then ‘Save’

**Biochemistry**

**Dissection**

1. Wearing gloves, get 2 tubes for each sample (1 for insertion, 1 for midsubstance).
2. Dry tubes for 24 hours at 65C, with the caps off (or 2 hrs in the lyophilizer).
3. Under dissection microscope, expose tendon. Remove overlying tissue and separate sides from surrounding tissue.
4. Use micro scissors to remove the tendon from bone. Ensure all other non-tendon tissue has been removed under dissection scope.
5. Place on top of petri dish with PBS with protease inhibitors.
6. Using a ruler, cut tendon just under the myotenodinous junction, leaving approximately a 2 mm section of tendon.
7. Cut this piece in half to separate the insertion site and mid substance of the tendon.
8. Blot sample on Kim wipe for ~3 seconds.
9. Place in eppendorf tube and close lid
10. Freeze for storage (Place in -80°C until ready to complete assay)

**Proteinase K Digestion**
1. Add 125 µL of 0.1M Ammonium Acetate and 100 µL of 5mg/mL Proteinase K solution to each sample (Make a solution for all tubes in this ratio and then add 225 uL of solution).
2. Finger flick each tube and give a brief spin on vortexer (excessive mixing is bad!).
3. Incubate samples at 65°C for 16 hours in oven, thermomixer, or water bath.
4. Add an additional 25 µL of 5mg/ml proteinase K (gives total volume of 250µL).
5. Incubate for an additional 1.5 hours.
6. Turn oven to 100°C and incubate for 10 minutes (to inactivate enzyme).
7. Take tubes out and allow to cool.
8. Freeze for storage (Place in -80°C until ready to complete assay)

**DMMB Assay**
1. Prepare standards from a 1 mg/mL chondroitin sulfate solution.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Concentration</th>
<th>Amount Stock</th>
<th>Amount ddH2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0 µg/mL</td>
<td>0 µL</td>
<td>1000 µL</td>
</tr>
<tr>
<td>S1</td>
<td>0.5 µg/mL</td>
<td>50 µL of S8</td>
<td>950 µL</td>
</tr>
<tr>
<td>S2</td>
<td>1 µg/mL</td>
<td>1 µL</td>
<td>999 µL</td>
</tr>
<tr>
<td>S3</td>
<td>2 µg/mL</td>
<td>2 µL</td>
<td>998 µL</td>
</tr>
<tr>
<td>S4</td>
<td>3 µg/mL</td>
<td>3 µL</td>
<td>997 µL</td>
</tr>
<tr>
<td>S5</td>
<td>4 µg/mL</td>
<td>4 µL</td>
<td>996 µL</td>
</tr>
<tr>
<td>S6</td>
<td>5 µg/mL</td>
<td>5 µL</td>
<td>995 µL</td>
</tr>
<tr>
<td>S7</td>
<td>8 µg/mL</td>
<td>8 µL</td>
<td>992 µL</td>
</tr>
<tr>
<td>S8</td>
<td>10 µg/mL</td>
<td>10 µL</td>
<td>990 µL</td>
</tr>
<tr>
<td>S9</td>
<td>12 µg/mL</td>
<td>12 µL</td>
<td>988 µL</td>
</tr>
<tr>
<td>S10</td>
<td>15 µg/mL</td>
<td>15 µL</td>
<td>985 µL</td>
</tr>
</tbody>
</table>

2. Add 30µL of sample or standard to each well. Always run samples in duplicate or triplicate. Do not add dye just yet because precipitate will form 5 minutes after adding.
3. Open program KC4. Use your favorite GAG protocol, borrow a labmate’s, or create your own with the protocol wizard. You’ll want to read the plate at 540nm and 595nm.
4. Using a multichannel pipetter, add 190uL of DMB dye to each well.
5. Read plate.
6. Export the data to Excel.
7. Normalize all of the data on the plate to the blank wells. Do this by averaging the blank wells and then subtracting that value from all other wells. This should be done at 540nm and 595nm.
8. Subtract 540nm-595nm.
9. Create standard curve by fitting a linear trend to the data.
10. Calculate the concentration of all of your samples using the equation determined by the linear fit (where \( x \) is the DMMB value from the assay and \( y \) is your concentration in \( \mu g/mL \)). Then average your three replicates to get a single GAG concentration value.

11. Normalize to DNA content, quantified by the PicoGreen Assay.

**PicoGreen Assay**

1. Right before use, take out the kit, and leave the reagents in a light-proof drawer to warm up to RT. Do not open the vials until they have reached equilibrium at RT.

2. Map out the standards and samples on the plate worksheet.

3. Using molecular biology grade water, make a 1X TE solution from the 20X stock in a sterile 50mL tube. You will need 100ul of TE per well. To run a full plate, you will need about 20mL

4. Make a 1:200 component A:TE solution (i.e. add 50ul to 9.950mL of TE), which contains the fluorescent dye that will bind to the nucleic acids in your samples. Use a sterile 15mL tube. This solution is light sensitive, store in foil or dark drawer until ready to dispense.

5. Prepare the standards as follows:
   a. **DNA Stock**: Dilute 18.75ul of provided DNA Stock in 750uL of 1X TE to get a 2.5ug/mL stock.
   b. Prepare Standards (250 µL is enough of each standard to run one plate):

<table>
<thead>
<tr>
<th>Standard</th>
<th>Vol 1X TE</th>
<th>Vol DNA Stock</th>
<th>Vol Reagent Sol'n</th>
<th>Final LR [ ]</th>
<th>Final [ ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>250 µL</td>
<td>0 µL</td>
<td>250 µL</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S1</td>
<td>245 µL</td>
<td>5 µL</td>
<td>250 µL</td>
<td>50 ng/mL</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>240 µL</td>
<td>10 µL</td>
<td>250 µL</td>
<td>100 ng/mL</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>235 µL</td>
<td>15 µL</td>
<td>250 µL</td>
<td>150 ng/mL</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>230 µL</td>
<td>20 µL</td>
<td>250 µL</td>
<td>200 ng/mL</td>
<td></td>
</tr>
</tbody>
</table>
6. Add 80µL of 1X TE solution to each sample well. Add 20µL of each sample. Do samples at least in duplicate.
7. Add 100µL of prepared standard where plated.
8. Using the multi-channel pipette, add 100µL of reagent solution to each well. Thoroughly mix by pipette.
9. Seal the plate and incubate in a dark drawer for 5 minutes.
11. Export results to excel file. (Plate reader takes about 15 minutes to warm up).
12. Fit a linear trend to the standards to create a standard curve (Shown below).
13. Calculate concentrations for each sample by using the equation determined by the linear fit (where x is the DMMB value from the assay and y is your concentration in ng/mL). Then average your replicates to get a single DNA concentration value.

Hydrolysis and Resuspension
1. Vortex samples
2. Remove 100µL and place in labeled Wheaton ampoules (glass)
3. Add 500µl 12N HCl to each ampoule. Do this step in the fume hood. (1:5, sample:HCl)
4. Using the propane torch, heat the stem of the ampoule while gripping the end of the step with the tongs. Rotate the vial slowly in the jet for 10 seconds until the glass becomes malleable, then begin twisting the base of the ampoule. After a full twist, continue to twist and begin to pull the stem tip and base away from each other. Notes: The hottest part of the flame is the tip of the jet. After sealing the vials, place them in a white box with a divider with a paper towel on the inside of the lid. Flip over for 10 seconds. Then check out the paper towel for wet spots. This trick will tell you if any of the vials aren't completely sealed.
5. Place the sealed ampoules on a heat block set at 110°C for 16hrs or longer.
6. Remove ampoules and place in fume hood to cool for 30min (make sure they've come to room temp before placing in vacuum so they don't boil!).
7. Using a wet paper towel, grip the stem and snap it off.
8. Dry samples in the acid concentrator (40 samples should take 2 hours at 2.5 torr).
9. Discard HCl waste when finished and clean acid concentrator.
10. Remove dried samples from vacuum.
11. Add 500µl of the assay buffer to each vial.
12. Seal with a strip of parafilm (be careful!) and vortex for 5s.
13. Store at 4°C for 16 hours.
14. After 16 hours, vortex. Then unwrap and transfer to prelabeled microcentrifuge tubes.
6. Store samples at -20C or move on to OHP assay.

**OHP Assay**

1. Prepare standards. First dilute a 1mg/mL solution of hydroxyproline in ddH$_2$O 1:10 in OHP Assay Buffer to create the stock solution. You will need at least 300 µL per standard per plate.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Concentration</th>
<th>Amount Stock</th>
<th>Amount ddH$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0 µg/mL</td>
<td>0 µL</td>
<td>1000 µL</td>
</tr>
<tr>
<td>S1</td>
<td>0.4 µg/mL</td>
<td>4 µL</td>
<td>996 µL</td>
</tr>
<tr>
<td>S2</td>
<td>0.8 µg/mL</td>
<td>8 µL</td>
<td>992 µL</td>
</tr>
<tr>
<td>S3</td>
<td>1 µg/mL</td>
<td>10 µL</td>
<td>990 µL</td>
</tr>
<tr>
<td>S4</td>
<td>2 µg/mL</td>
<td>20 µL</td>
<td>980 µL</td>
</tr>
<tr>
<td>S5</td>
<td>4 µg/mL</td>
<td>40 µL</td>
<td>960 µL</td>
</tr>
<tr>
<td>S6</td>
<td>6 µg/mL</td>
<td>60 µL</td>
<td>940 µL</td>
</tr>
<tr>
<td>S7</td>
<td>8 µg/mL</td>
<td>80 µL</td>
<td>920 µL</td>
</tr>
<tr>
<td>S8</td>
<td>10 µg/mL</td>
<td>100 µL</td>
<td>900 µL</td>
</tr>
<tr>
<td>S9</td>
<td>12 µg/mL</td>
<td>120 µL</td>
<td>880 µL</td>
</tr>
</tbody>
</table>

2. Prepare solutions.
   a. In a small glass bottle, suspend DMAB in propanol. Slowly add perchloric acid. Wrap in aluminum foil or store in a dark drawer. This solution also needs to be made fresh the day of the assay.
   b. In a 50ml tube, dissolve chloramine T in ddH$_2$O. Add propanol and the previously made stock buffer. Wrap tube with aluminum foil or store in a dark drawer. This solution needs to be made fresh the day of the assay.

**Chloramine T**

<table>
<thead>
<tr>
<th># of plates</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chloramine T (grams)</em></td>
<td>0.1175</td>
<td>0.2350</td>
<td>0.3525</td>
<td>0.4700</td>
<td>0.5875</td>
<td>0.7050</td>
</tr>
<tr>
<td>ddH2O (mL)</td>
<td>1.725</td>
<td>3.450</td>
<td>5.175</td>
<td>6.900</td>
<td>8.625</td>
<td>10.350</td>
</tr>
<tr>
<td>n-propanol (mL)</td>
<td>2.167</td>
<td>4.333</td>
<td>6.500</td>
<td>8.667</td>
<td>10.833</td>
<td>13.000</td>
</tr>
<tr>
<td>OHP Stock (mL)</td>
<td>4.442</td>
<td>8.883</td>
<td>13.325</td>
<td>17.767</td>
<td>22.208</td>
<td>26.650</td>
</tr>
<tr>
<td>Volume (mL)</td>
<td>8.333</td>
<td>16.667</td>
<td>25.000</td>
<td>33.333</td>
<td>41.667</td>
<td>50.000</td>
</tr>
</tbody>
</table>

**DMAB**

<table>
<thead>
<tr>
<th># of plates</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DMAB (grams)</strong></td>
<td>1.4535</td>
<td>2.9070</td>
<td>4.3605</td>
<td>5.8140</td>
<td>7.2674</td>
<td>8.7209</td>
</tr>
<tr>
<td>n-propanol (mL)</td>
<td>5.814</td>
<td>11.628</td>
<td>17.442</td>
<td>23.256</td>
<td>29.070</td>
<td>34.884</td>
</tr>
<tr>
<td>Perchloric (mL)</td>
<td>2.519</td>
<td>5.039</td>
<td>7.558</td>
<td>10.078</td>
<td>12.597</td>
<td>15.116</td>
</tr>
<tr>
<td>Volume (mL)</td>
<td>8.333</td>
<td>16.667</td>
<td>25.000</td>
<td>33.333</td>
<td>41.667</td>
<td>50.000</td>
</tr>
</tbody>
</table>

*Chloramine T stored at 4C
** p-dimethylaminobenzaldehyde

3. Set waterbath to 60C. Remove most of the water so that there’s only about half a centimeter of water above the metal grating.

4. Set up arrangement of plate prior to loading samples with standards on top in at least duplicate. ***Avoid using the outer rows and columns as they heat faster than the inner ones***

5. Uptake 150ul of a sample and transfer to appropriate well.
6. Put chloramine T in transfer well and use multi-channel pipetter to transfer 75µl of chloramine T reagent, mix by pipetting up and down to each well. Mix each well until solution is transparent. Put a plate cover over your plate and incubate at room temperature for 20 minutes. While waiting, turn on the microplate reader.

7. Add 75µl of the DMAB solution and mix by pipetting up and down.

8. Place the plate with a plate cover carefully in the 60°C waterbath for 7 minutes. You will likely need to balance the plate on another unused plate or hold it down with a weight.

9. Transfer to a glass casserole dish partially filled with cold tap water for 5 minutes.

10. Start the KC4 program and load the collagen assay protocol. Confirm that the plate layout matches your plate and that the read settings are set to 540nm absorbance. Remove the plate from the cold bath, dry the bottom of the plate with paper towels, and read it!

11. Export the data to Excel.

12. Normalize all of the data on the plate to the blank wells. Do this by averaging the blank wells and then subtracting that value from all other wells.

13. Create standard curve by fitting a linear trend to the standards data.

14. Calculate the concentration of all of your samples using the equation determined by the linear fit (where x is the DMMB value from the assay and y is your concentration in µg/mL). Then average your replicates to get a single concentration value.

15. Calculate the total collagen amount using the following equation, where the dilution ratio is the inverse percent of original sample (e.g., if after ProK digestion, 100µl of 250µl was removed for HCl digestion, then the dilution ratio is 1/(100/250) = 2.5)

\[ \text{Total Col} = \text{OHP Average} \times \text{Dilution ratio} \times 7.143 \]

(Reynolds and Logan 1950)

16. Normalize to DNA content, quantified by the PicoGreen Assay.

**PYD ELISA**

1. Prepare solutions.
   a. Remove Substrate Buffer from refrigerator at this time as well. For a full plate, you’ll need 2 bottles of buffer.
   b. Prepare Enzyme Conjugate within 2 hours of use. Reconstitute each required vial of Enzyme Conjugate with 7 mL of cold Assay Buffer. Store reconstituted Enzyme Conjugate at 2-8°C until use. For a full plate, you’ll need 2 vials.
   c. Dilute Standards A-F and Control 1:10 with Assay Buffer (e.g. 50µL sample + 450µL Assay Buffer). For a full plate, you’ll need 100µL of each standard. If you are working with mouse tendon, DO NOT DILUTE YOUR SAMPLES.

2. Remove Stripwell Frame and the required number of Coated Strips from the pouch. Ensure that the pouch containing unused strips is completely resealed. Label the strips you are using to prevent mix-up in case of accidental removal from the frame.
3. Add 50µL of standards, controls, and samples to each well of the strips. This step should be completed within 30 minutes.
4. Add 100µL of reconstituted Enzyme Conjugate to each well.
5. Cover plate with Tape Cover provided.
6. Incubate for 3 hours (±10 minutes) at 2-8°C IN THE DARK.
7. During incubation, prepare other solutions.
   a. Prepare Working Substrate Solution within 1 hour of use. Put one Substrate Tablet into each required bottle of 20-28°C Substrate Buffer. Allow 30-60 minutes for tablets to dissolve. Vigorously shake bottle completely to mix.
   b. Prepare required amount of 1X Wash Buffer by diluting 10X Wash Buffer 1:10 with deionized water. You should be adding approximately 250µL of wash per well per wash in a squirt bottle. For an entire plate, you will probably need 100-150mL to be safe.
8. At the end of the incubation, manually invert/empty strips.
9. Add at least 250µL of 1X Wash Buffer to each well and manually invert/empty strips.
10. Repeat two more times for a total of three washes.
11. After the last wash, vigorously blot the strips dry on paper towels. Carefully wipe the bottom of strips with a Kimwipe to ensure that the bottom of the strips are clean as well.
12. Add 150µL of Working Substrate Solution to each well.
13. Incubate for 60 minutes (±5 minutes) at 20-28°C. During this time, turn on the microplate reader and set up your protocol.
14. Add 100µL of Stop Solution to each well. Add Stop Solution in the same pattern and time intervals as the Substrate Solution was added.
15. Read the optical density at 405nm. Assure that no large bubbles are present in the wells and that the bottom of the strips are clean. Strips should be read within 15 minutes of the Stop Solution addition.
16. Use quantitation software with a 4-parameter calibration curve fitting equation to analyze the MicroVue PYD EIA results.
   a. Equation \( y = \frac{(A-D)}{(1+(x/C)^B)} + D \)
17. Determine concentration of samples and controls from the standard curve or have your software calculate the concentrations for you!
18. Double check that your method ran smoothly by checking that the calibration high and low controls are within their accepted ranges.
19. Average your replicates to get a single PYD concentration value.
20. Normalize to collagen content, quantified by the OHPAssay.
Electron Microscopy

Sample Preparation
1. Bring Karnovsky’s fixative to room temperature (Karnovsky’s can be used for 2 consecutive days but no more).
2. Prepare Eppendorf tubes by labeling and covering the labels with scotch tape (the glossy kind is best). This will ensure that none of the processing chemicals will remove the label.
3. Just after sacrifice, tape the mouse down on the black mat, fixing it behind the shoulder as well as above the neck.
4. Make incision in the skin. You should be able to make a small cut and then widen with your fingers.
5. Rotate and visualize shoulder. Tape down in ~30° external rotation.
6. Locate and break the acromioclavicular (AC) joint with fine forceps to expose the supraspinatus tendon.
7. Apply Karnovsky’s fixative (4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate and 8 mM CaCl2, adjusted to pH 7.4 with NaOH) to the shoulder. Wait approximately 5 minute, making sure that the tendon is completely bathed in fixative.
8. Follow spine of scapula with the forceps to gently free the supraspinatus tendon-muscle unit from the scapula.
9. ‘Flip’ supraspinatus tendon and muscle to rest on humeral shaft.
10. Cut the tendon and muscle off of the humerus bone at the insertion site using microscissors. Place this tissue in a petri dish, covered in Karnovsky’s.
11. Use microscissors and a #22 scalpel blade to fine dissect the tendon and to trim the muscle to fit nicely into an Eppendorf tube.
12. Place in an Eppendorf tube filled with Karnovsky’s fixative for 2-4 hours and keep cold.
13. Rinse tendons with cold cacodylate buffer 2 times for 10 minutes.
14. Add 1% osmium tetroxide to each sample for 1 hour.
15. Dehydrate samples in a series of ethanol dilutions (50%, 70%, 70%, 85%, 95%, 100% x4) at approximately 5-10 minutes per dilution.
16. Wash sample in a solution of 100% propylene oxide 3x.
17. Change sample into a solution of 2:1 Propylene oxide:EPON (Embed 812, nadic methyl anhydride, dodecenylsuccinic anhydride and DMP-30) overnight. Keep samples on a shaker at all times during this process.
18. Every 2-3 hours or so, change the sample into a solution with increasing concentrations of EPON (once in morning and once in afternoon is okay). Keep samples on a shaker at all times during this process.
19. Finally, change the samples into 100% EPON overnight.

Sample Embedding
1. Cut samples into insertion site and midsubstance pieces and embed each piece into a separate mold with 100% EPON. See figure at the right.
2. Cure samples overnight at 60-70°C.

Sectioning
1. **Block Trimming:** Prior to ultrathin sectioning, polymerized blocks must be trimmed free hand using saw and/or razor blades under a dissecting microscope. The shape of the block face will vary for each specimen but the top and bottom should be parallel. Glass knives are used for final block face. The dimensions of the block face should be approximately 0.5mm.
on the top with bottom slightly longer- no side should exceed 1mm. This is adjusted as necessary depended on the diameter of the tendon--edge of tendon should be included in final section for reference.

2. **Ultramicrotomy (Leica Ultracut- diamond knife).** Settings will vary per block:
   a. Approximately 0.8-1.0 cutting speed
   b. The specimen should be between 60-100nm, ideally around 90nm thick
   c. The following section colors relate to their thickness:
      i. 60 nm gray
      ii. 60-90 nm silver
      iii. 90-150 nm gold
      iv. 150-190 nm purple
      v. 190-240 nm blue
      vi. 240-280 nm green
   d. Isolate floating sections using the eyelash and pick up on the dull side of 150-200 mesh, naked (no support film) copper GRIDS. Number of sections will vary per grid, however, 4-6 centrally located is usually sufficient. At least 2 grids per specimen.

**Staining**
1. Secure a piece of parafilm to bench top (either by scoring or taping corners)-OR for multiple grids use grid pad.
2. Use a syringe to draw up an appropriate amount of 2% uranyl acetate (UA) solution and attach a 0.2µm syringe filter. (UA solution may be stored in the syringe for use later if covered with foil).
3. Discard the first few drops of solution and then place one drop per grid on the parafilm for staining
4. Place grids section side down onto the drops. Protect from light using a foil-covered lid while staining and stain for ~20 min
5. Rinse grids (hold grid with tweezers) with dH2O using a syringe with a 0.2µm syringe filter gently by slowly trickling over the grid -OR- rinse grids by dipping in and out of a full beaker of dH2O gently and quickly (~20 times.) -OR- for grid pad use squirt bottle.
6. Use lens paper to carefully and gently blot off excess water on grids or dry by wicking off most of the water from the grid using #42 Whatman filter paper and then drying directly down onto another piece of filter paper.
7. Repeat steps 1-6 using the 1% phosphotungstic acid solution

**Imaging**
1. Images taken at 60k scope magnification on JEOL 1400 transmission electron microscope using Gatan Orius widefield side mount digital camera
3. **Imaging Criteria.** Goal: to measure a significant number of fibrils to obtain data that represents specimen which allows for comparison (statistical significance) between experimental groups
   - fibrils in cross section
   - from center region of fiber (avoid edges)
   - not from peripheral region of tendon
     i. avoid cell and cell processes
   - avoid processing (artificial) breaks in tendon structure
   - avoid folds, holes in section and staining artifacts
     i. collect from multiple fibers, sample area should be well represented
   - measureable fibrils at least 80*
4. In abnormal specimens (young, mutant or injured specimens) where tendon morphology is not typical or mature, imaging criteria is adjusted on a project to project basis.

5. Typically 12-15 images are taken then 10 representative images are chosen to be measured using the above criteria.

6. When possible images were taken of an area of cross-sectioned of fibrils in the center region of a fiber not near the peripheral region of the tendon, away from cells, folds or holes in sections, and staining artifacts. Effort was made to not to take images close together.

**Microcomputed Tomography Imaging of Humerus Bones**

**Scanning**

1. Check to see if there is anything in the microCT.
   a. Check paper sign on front of microCT- should be flipped down to indicate availability.
   b. Check the orange light on the top of the machine. If it’s on, there is something scanning in the machine.
   c. Check the orange light on the front of the machine. If it’s on, there is something scanning in the machine.
   d. Check the queue.
      i. Go to the Session Manager → App → DEC term
      ii. Type ‘show queue’ in the command line.
      iii. Check SC2361_Batch → This is the queue for the microCT 35 and will show current scans. Queue will show “idle” status if there is nothing scanning.
   e. Check to see if there are samples in the machine. If so, find out who was last using the microCT and contact them. Place samples in the drawer below the microCT for storage.

2. Prepare your samples.
   a. Find your sample tube in the top drawer of the microCT. For mouse humeri, we will use the tall skinny tube.
   b. Cut gauze into long strips for each sample. You should be using a large square of gauze for this (not standard size).
   c. Put a small amount of fluid in the top (whatever fluid you are using).
   d. Wrap each sample in the gauze tightly by placing the sample in the middle of the gauze on the crease and rolling the humerus in the gauze.
   e. *** IF YOU ARE DOING MULTIPLE SAMPLES IN A SCAN, YOU NEED TO DRAW THE ORIENTATION OF SAMPLES IN THE TUBE TO KEEP TRACK OF WHICH SPECIMEN IS WHICH***
   f. Place the rolled sample into the tube and push to the bottom, making sure to cover the sample with fluid. You do not want to unroll the gauze or disturb the humerus, so be careful when pushing down. Your next specimen will be placed on top of this one, so add extra fluid before placing the next specimen.
   g. Once you have loaded all of your specimens into the tube, cover the top of the tube with parafilm. Typically this tube can hold approximately 4 mouse humerus bones.

3. Load samples into the machine.
   a. Place the tube into the machine by inserting the bottom piece into the hole.
   b. Twist gently until the notch in the tube lines up with that of the machine and then pushes the specimen tube down all the way.
   c. Close the door to the microCT securely.
   d. Flip paper sign up to indicate machine is in use.

4. Assign sample numbers to your specimens.
1. Click on the icon shown in the red box above.
2. Name your specimen. Naming format is as follows: PI_You_Study_specimen
3. push 'save' when you are finished inputting the sample name. A number will be generated at this time. **WRITE THIS NUMBER DOWN
4. Generate sample numbers for all of the specimens that you will scan. Generally it will be easiest to name them from the bottom up.
5. Close this window by File→Exit.

5. Set scan regions and Scan

a. Click on the icon shown above in the red box.
b. Enter the sample number you want to scan. You will start with the bottom specimen.
c. Select a control file on which to scan. For mouse humerus, you can use control file #11: XSL_bone-6um-D11.5mm
d. Click ‘Scout_view’
   i. Choose the field in which to perform your scout view. For the tube we are using, this will be from 60 to 140.
   ii. Push ‘scout_view’. This will turn the X-Ray on and take a scan of the tube. This may take a couple minutes. Make sure the X-Ray parameters read 55 kVp and 114 µA. If it doesn’t or if an error message appears, let someone know as soon as possible.
   iii. When the scan appears, push ‘Reference_line’ to set the scan region.
      1. The mouse movement will determine where the region is set vertically.
      2. Hold shift and move the mouse to increase the scan area. These are in stacks so moving from one stack to the next will increase the scan time significantly. You will want to have the same scan time and number of slices across specimens. (These parameters are shown on the bottom of the screen)
      3. Click the mouse to choose the region you want. If you click too early, you can re-do this by clicking ‘Reference_line’ again.
   iv. Push ‘Add scan’. This will add the measurement of that particular region.
e. Without closing any windows, click ‘Other’ by the sample number properties (main window). Enter your next sample number and repeat the Scout_view process for each sample.
f. Click ‘ok’ in the Scout_view window when you are finished adding all your specimens.
g. Click ‘Task List’ in the main window. A window will come up with all of your measurements that you defined. Make sure the correct protocol number and correct specimen number are there.

h. Click ‘Submit batch scans.’ Scout view windows will close and scans will begin.

i. Check that your scans are now in the queue by typing ‘show queue’ in the command line.

6. Clean-Up

a. When you come back at the end of your scans, check if your scans are finished or if there are scans occurring (See Section 1).

b. Remove the specimen tube from the microCT.

c. Remove each of your samples from the tube. Make sure you keep track of the order you had them in to keep the specimens separate.

d. Remove any excess fluid from your specimens in the tube.

e. Spray down the tube with 70% Ethanol and wipe down the surface and inside of the tube.

f. Maybe include website info w/ password and how to track scans online.

Analysis

1. Open Sample

   a. Log into program with your name or assigned number.
   
   b. Click on the button shown in the red box above.
   
   c. Open sample by finding the sample number and scan number.
   
   d. Go through the images to find the start and end of the growth plate. You will define epiphyseal bone as 10 lines above the start of the growth plate and metaphyseal bone as 10 lines below the end of the growth plate. Cortical bone will be measured in the last 50 lines of the scan (unless the end of the growth plate falls in that region).
   
   **These guidelines are loose and will vary from sample to sample.

   2. Analyzing Cortical Bone

      a. Push the button that says ‘C…’ – This will open the ‘countouring’ menu
      
      b. Define the contours
         i. Draw a loose circle counterclockwise around the outside border of the cortical bone in the region of interest in your first slice of the group.
         ii. Draw a tighter circle clockwise around the inside border of the cortical bone.
         iii. Highlight Forwards and then click ‘iterate’ and the software will iterate this through all of the remaining slices to the end.
      
      c. Fix the contours
         i. Edit specific maps by drawing the appropriate lines in the map of choice. You must do these edits in the same direction as the line was chosen (CCW for the outside border and CW for the inside border). You also must cross the line twice in order to count as an edit.
         ii. If you must edit a section, you should delete the maps following and re-iterate them.
iii. You can also play with the contouring thresholds if the software is having a difficult time keeping the correct contours. Generally the outer value will be 31 and the inner value around 500. If it is difficult, you want both values to come closer to 300. Record values to keep consistent between samples.

iv. Save the GOBJ file and record assigned number (should be 1).

d. Analyzing
   i. Click on the ‘T…’ button.
   ii. Select your analysis. Cortical bone is analyzed with #7: Bone Midshaft Evaluation.
   iii. Set your threshold values
       1. Gaussian Sigma: 1.2
       2. Gaussian Support: 2
       3. Upper Limit: 1000
       4. Lower Limit: VARIABLE. **This limit should be chosen based on a couple images and then kept constant for all samples through the study for consistency. Use “non-control” sample to set threshold.
   iv. Go back and forth between the grayscale and preview of scan in order to determine what your threshold needs to be. You want to get your scan region to match the grayscale in terms of pores that may exist in the cortical bone.
   v. Click ‘Default VOI’ before you start.
   vi. Click ‘Start Evaluation’

3. Analyzing Metaphysis or Epiphysis trabecular bone
   a. You may define the contours of a scan region while another analysis is going but you MUST wait until that one is finished before actually starting the evaluation of the second scan.
   b. Push the button that says ‘C…’ if it’s not already open from the previous analysis.
   c. Find the region of interest (where the cortical boundary starts to become clear before or after the growth region (depending on metaphysis or epiphysis)) and then add 10 lines away from the growth plate for good measure.
   d. Define your contours
      i. Draw a loose circle counterclockwise around the inside border of the cortical bone in order to capture the trabeculae in the middle of the bone. You do not want to include any cortical bone in this, but the full trabeculae.
      ii. Draw the contours on the sample every 5-10 slices for the full scan region of your sample (should be approximately 100 slices, but this will be defined by how many slices you have for the most different group in the study).
      iii. In the contour menu, highlight All and then click ‘Morph’. This will allow the software to interpolate drawings between your defined drawings. During the first few samples, you should be making sure that the morph is valid over that region, but as you continue, you’ll notice it is doing fine.
      iv. Save GOBJ file.
   e. Analyzing
      i. Click on the ‘T…’ button.
      ii. Select your analysis. Trabecular bone is task #5: Bone Trab.
      iii. Set your threshold values
          1. Gaussian Sigma: 1.2
          2. Gaussian Support: 2
          3. Upper Limit: 1000
          4. Lower Limit: VARIABLE. **Different for cortical vs. trabecular bone.
      iv. Go back and forth between the grayscale and preview of scan in order to determine what your threshold needs to be. You want to get your scan region to match the grayscale in terms of trabecular thickness and capturing the small “flecks” of trabeculae.
v. Click ‘Default VOI’ before you start.
vi. Click ‘Start Evaluation’

4. Creating 3D Images

![Image of 3D model and buttons]

a. Click on the button shown in the red box above.
b. Open a sample measurement. This file will be a _SEG.AIM;# file where the # is the number of the contour definitions you drew. You will be able to open the regions you chose for each of your measurements and create 3D images from them.
c. Use the Subdiv menu to subdivide your scan into different regions or to view cross-sectional pieces.
d. Use the formatting menus to change colors. White bone on black background is the best contrast for these types of images.
e. Choose File→Print to create images. You can change the file type to .TIFF and make sure the Directory is Measurement.
f. If you want to make a 3D image only and don’t want to measure parameters, you can use the task #1 in the ‘T…’ directory.

5. Computer Analysis

a. Go to MicroCT Website: 130.91.97.250/~microct/
b. Click on ‘Show current measurements’
c. Find the sample number that you want to analyze.
d. Click ‘Files’
e. For the cortical bone, you should be able to get the parameters from the MOI file. For the trabecular bone, you need the 3Dresults_bonemorphometry file.
f. Copy and paste the data from these files into an Excel spreadsheet. Obtain the following parameters to compare between groups:

<table>
<thead>
<tr>
<th>Cortical Bone</th>
<th>Trabecular Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMOI (Polar Moment of Inertia)</td>
<td>BV/TV (Bone Volume/Tissue Volume)</td>
</tr>
<tr>
<td>BA/TA (Bone Area/Tissue Area)</td>
<td>TV (Tissue Volume)</td>
</tr>
<tr>
<td>Porosity (1-BA/TA)</td>
<td>Connective Density</td>
</tr>
<tr>
<td>Ct.Th (Cortical Thickness)</td>
<td>SMI (Structural Model Index, Lower = Better)</td>
</tr>
<tr>
<td>Mean 1, BMD (Bone Mineral Density)</td>
<td>Tb.N (Trabecular Number)</td>
</tr>
<tr>
<td>Mean 2, TMD (Tissue Mineral Density)</td>
<td>Tb.Th (Trabecular Thickness)</td>
</tr>
<tr>
<td></td>
<td>Tb.Sp (Trabecular Spacing)</td>
</tr>
<tr>
<td></td>
<td>Mean 1, BMD (Bone Mineral Density)</td>
</tr>
<tr>
<td></td>
<td>Mean 2, TMD (Tissue Mineral Density)</td>
</tr>
<tr>
<td></td>
<td>BS/BV (Bone Surface/Volume)</td>
</tr>
<tr>
<td></td>
<td>BS (Bone Surface)</td>
</tr>
<tr>
<td></td>
<td>DA (Degree of Anisotropy)</td>
</tr>
</tbody>
</table>
### APPENDIX B: SUPPLEMENTAL REGRESSION TABLES

#### Table B-1. Descriptive Statistics for Parameters Included in Statistical Modeling

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
<th>Q1</th>
<th>Q3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INSERTION SITE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CellDens</td>
<td>24</td>
<td>0.0006</td>
<td>0.0005</td>
<td>0.0002</td>
<td>0.0002</td>
<td>0.0005</td>
<td>0.0008</td>
<td></td>
</tr>
<tr>
<td>CellShape</td>
<td>24</td>
<td>0.6</td>
<td>0.6</td>
<td>0.08</td>
<td>0.5</td>
<td>0.8</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>FibDiam</td>
<td>60</td>
<td>67.8</td>
<td>66.9</td>
<td>10.9</td>
<td>51.1</td>
<td>115.5</td>
<td>60.0</td>
<td>72.0</td>
</tr>
<tr>
<td>FibDens</td>
<td>60</td>
<td>1410.8</td>
<td>1405.5</td>
<td>297.1</td>
<td>730.2</td>
<td>2228.5</td>
<td>1257.9</td>
<td>1577.0</td>
</tr>
<tr>
<td>GAG</td>
<td>60</td>
<td>94.8</td>
<td>93.0</td>
<td>22.5</td>
<td>60.0</td>
<td>169.3</td>
<td>77.8</td>
<td>105.8</td>
</tr>
<tr>
<td>COL</td>
<td>60</td>
<td>1139.3</td>
<td>1056.3</td>
<td>307.0</td>
<td>558.4</td>
<td>1820.8</td>
<td>922.5</td>
<td>1394.1</td>
</tr>
<tr>
<td>PYD</td>
<td>60</td>
<td>2.9</td>
<td>2.6</td>
<td>1.3</td>
<td>0.6</td>
<td>6.8</td>
<td>1.9</td>
<td>3.9</td>
</tr>
<tr>
<td>FIF</td>
<td>30</td>
<td>1.5</td>
<td>1.5</td>
<td>0.2</td>
<td>1.3</td>
<td>2.2</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>DPeriod</td>
<td>17</td>
<td>61.9</td>
<td>62.3</td>
<td>1.0</td>
<td>60.2</td>
<td>63.1</td>
<td>60.9</td>
<td>62.7</td>
</tr>
<tr>
<td>InitSld</td>
<td>17</td>
<td>3.1</td>
<td>2.7</td>
<td>1.0</td>
<td>1.8</td>
<td>5.6</td>
<td>2.2</td>
<td>3.7</td>
</tr>
<tr>
<td>ReAlignStrain</td>
<td>46</td>
<td>5.3</td>
<td>5.1</td>
<td>1.6</td>
<td>2.0</td>
<td>9.1</td>
<td>3.9</td>
<td>6.8</td>
</tr>
<tr>
<td>ReAlignAmount</td>
<td>46</td>
<td>3.6</td>
<td>3.5</td>
<td>1.6</td>
<td>0.9</td>
<td>8.9</td>
<td>2.5</td>
<td>4.4</td>
</tr>
<tr>
<td>ToeReAlign</td>
<td>53</td>
<td>0.8</td>
<td>0.7</td>
<td>0.7</td>
<td>-1.2</td>
<td>2.6</td>
<td>0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>LinearReAlign</td>
<td>53</td>
<td>0.5</td>
<td>0.6</td>
<td>0.8</td>
<td>-3.8</td>
<td>1.8</td>
<td>0.2</td>
<td>0.9</td>
</tr>
<tr>
<td>CrimpFreq</td>
<td>54</td>
<td>48.1</td>
<td>46.6</td>
<td>12.6</td>
<td>19.3</td>
<td>75.0</td>
<td>37.9</td>
<td>56.4</td>
</tr>
<tr>
<td>CrimpAmp</td>
<td>54</td>
<td>33.7</td>
<td>32.4</td>
<td>7.0</td>
<td>21.7</td>
<td>54.9</td>
<td>28.1</td>
<td>39.3</td>
</tr>
<tr>
<td>FibDef1</td>
<td>15</td>
<td>-0.4</td>
<td>-0.5</td>
<td>0.8</td>
<td>-1.7</td>
<td>1.2</td>
<td>-1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>FibDef3</td>
<td>16</td>
<td>-0.3</td>
<td>-0.3</td>
<td>1.2</td>
<td>-2.1</td>
<td>2.0</td>
<td>-1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>FibDef5</td>
<td>15</td>
<td>0.2</td>
<td>0.4</td>
<td>0.7</td>
<td>-1.3</td>
<td>1.4</td>
<td>-0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>FibDef7</td>
<td>16</td>
<td>-0.2</td>
<td>-0.2</td>
<td>0.8</td>
<td>-2.0</td>
<td>1.1</td>
<td>-0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Slide1</td>
<td>15</td>
<td>-0.4</td>
<td>-0.6</td>
<td>1.1</td>
<td>-1.9</td>
<td>2.2</td>
<td>-1.1</td>
<td>-0.2</td>
</tr>
<tr>
<td>Slide3</td>
<td>16</td>
<td>-0.4</td>
<td>-0.3</td>
<td>0.9</td>
<td>-2.2</td>
<td>1.1</td>
<td>-1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Slide5</td>
<td>15</td>
<td>-0.9</td>
<td>-1.1</td>
<td>1.1</td>
<td>-2.8</td>
<td>1.1</td>
<td>-1.9</td>
<td>-0.04</td>
</tr>
<tr>
<td>Slide7</td>
<td>16</td>
<td>-0.5</td>
<td>-0.6</td>
<td>1.4</td>
<td>-2.8</td>
<td>1.6</td>
<td>-1.5</td>
<td>0.9</td>
</tr>
<tr>
<td>TransStrain</td>
<td>53</td>
<td>4.0</td>
<td>4.0</td>
<td>1.5</td>
<td>1.0</td>
<td>8.1</td>
<td>3.1</td>
<td>4.8</td>
</tr>
<tr>
<td>TransStress</td>
<td>53</td>
<td>1.1</td>
<td>0.55</td>
<td>1.3</td>
<td>0.02</td>
<td>6.4</td>
<td>0.2</td>
<td>1.9</td>
</tr>
<tr>
<td>MaxStress</td>
<td>51</td>
<td>26.6</td>
<td>26.2</td>
<td>12.4</td>
<td>8.2</td>
<td>54.3</td>
<td>15.6</td>
<td>34.7</td>
</tr>
<tr>
<td>ToeMod</td>
<td>53</td>
<td>159.8</td>
<td>122.2</td>
<td>111.4</td>
<td>23.3</td>
<td>450.1</td>
<td>79.6</td>
<td>215.3</td>
</tr>
<tr>
<td>LinMod</td>
<td>53</td>
<td>572.8</td>
<td>498.7</td>
<td>393.6</td>
<td>42.5</td>
<td>1535.3</td>
<td>279.1</td>
<td>864.4</td>
</tr>
<tr>
<td>DynMod6</td>
<td>51</td>
<td>158.5</td>
<td>153.8</td>
<td>84.1</td>
<td>36.3</td>
<td>406.8</td>
<td>96.2</td>
<td>211.0</td>
</tr>
<tr>
<td>TanDelta6</td>
<td>51</td>
<td>0.05</td>
<td>0.05</td>
<td>0.02</td>
<td>0.03</td>
<td>0.10</td>
<td>0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>Relax6</td>
<td>51</td>
<td>44.0</td>
<td>42.8</td>
<td>14.2</td>
<td>24.6</td>
<td>117.2</td>
<td>36.2</td>
<td>49.0</td>
</tr>
<tr>
<td>FailCycle</td>
<td>48</td>
<td>3709.2</td>
<td>2747.0</td>
<td>2859.6</td>
<td>236</td>
<td>12901</td>
<td>1595</td>
<td>4915.5</td>
</tr>
<tr>
<td>CycStrain50</td>
<td>48</td>
<td>1.4</td>
<td>1.4</td>
<td>0.2</td>
<td>1.3</td>
<td>2.4</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>TanStiff50</td>
<td>48</td>
<td>15.4</td>
<td>17.1</td>
<td>4.0</td>
<td>8.8</td>
<td>21.4</td>
<td>11.1</td>
<td>18.7</td>
</tr>
<tr>
<td>Hyst50</td>
<td>48</td>
<td>0.02</td>
<td>0.02</td>
<td>0.01</td>
<td>0.005</td>
<td>0.05</td>
<td>0.008</td>
<td>0.03</td>
</tr>
<tr>
<td>Lax50</td>
<td>48</td>
<td>4.3</td>
<td>4.0</td>
<td>1.5</td>
<td>2.3</td>
<td>10.5</td>
<td>3.4</td>
<td>4.5</td>
</tr>
<tr>
<td><strong>MIDSUBSTANCE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CellDens</td>
<td>23</td>
<td>0.0005</td>
<td>0.0006</td>
<td>0.0002</td>
<td>0.0002</td>
<td>0.0008</td>
<td>0.0004</td>
<td>0.0007</td>
</tr>
<tr>
<td>CellShape</td>
<td>23</td>
<td>0.4</td>
<td>0.4</td>
<td>0.07</td>
<td>0.3</td>
<td>0.6</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>FibDiam</td>
<td>56</td>
<td>79.8</td>
<td>78.3</td>
<td>12.2</td>
<td>55.1</td>
<td>109.7</td>
<td>70.9</td>
<td>88.0</td>
</tr>
<tr>
<td>FibDens</td>
<td>56</td>
<td>1082.4</td>
<td>1046.0</td>
<td>245.8</td>
<td>713.6</td>
<td>1734.3</td>
<td>888.9</td>
<td>1241.9</td>
</tr>
<tr>
<td>GAG</td>
<td>60</td>
<td>62.9</td>
<td>61.3</td>
<td>14.5</td>
<td>26.8</td>
<td>115.9</td>
<td>55.1</td>
<td>68.9</td>
</tr>
<tr>
<td>COL</td>
<td>60</td>
<td>705.9</td>
<td>700.0</td>
<td>223.4</td>
<td>269.3</td>
<td>1542.3</td>
<td>574.7</td>
<td>813.3</td>
</tr>
<tr>
<td>Variable</td>
<td>N</td>
<td>Min</td>
<td>1st Quartile</td>
<td>Median</td>
<td>3rd Quartile</td>
<td>Max</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>----</td>
<td>-----</td>
<td>--------------</td>
<td>--------</td>
<td>--------------</td>
<td>------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PYD</td>
<td>60</td>
<td>1.81</td>
<td>1.66</td>
<td>0.75</td>
<td>0.71</td>
<td>4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIF</td>
<td>30</td>
<td>1.3</td>
<td>1.3</td>
<td>0.1</td>
<td>1.2</td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPeriod</td>
<td>17</td>
<td>61.9</td>
<td>61.5</td>
<td>0.8</td>
<td>60.9</td>
<td>63.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>InitSlid</td>
<td>17</td>
<td>2.7</td>
<td>2.9</td>
<td>0.9</td>
<td>0.8</td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ReAlignStrain</td>
<td>37</td>
<td>5.2</td>
<td>5.1</td>
<td>1.9</td>
<td>1.7</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ReAlignAmount</td>
<td>37</td>
<td>2.4</td>
<td>2.2</td>
<td>1.0</td>
<td>1.0</td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ToeReAlign</td>
<td>53</td>
<td>0.7</td>
<td>0.6</td>
<td>0.8</td>
<td>-1.2</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LinearReAlign</td>
<td>53</td>
<td>0.5</td>
<td>0.4</td>
<td>0.6</td>
<td>-0.6</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrimpFreq</td>
<td>52</td>
<td>51.3</td>
<td>49.4</td>
<td>13.9</td>
<td>27.3</td>
<td>97.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrimpAmp</td>
<td>52</td>
<td>39.2</td>
<td>38.9</td>
<td>7.8</td>
<td>20.6</td>
<td>60.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef1</td>
<td>15</td>
<td>-1</td>
<td>0.3</td>
<td>1.4</td>
<td>-2.6</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef3</td>
<td>16</td>
<td>0.4</td>
<td>0.3</td>
<td>1.3</td>
<td>-1.7</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef5</td>
<td>15</td>
<td>0.2</td>
<td>-0.002</td>
<td>0.9</td>
<td>-1.5</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef7</td>
<td>16</td>
<td>-0.1</td>
<td>0.2</td>
<td>0.9</td>
<td>-1.3</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide1</td>
<td>15</td>
<td>-0.1</td>
<td>0.2</td>
<td>0.6</td>
<td>-1.1</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide3</td>
<td>16</td>
<td>-0.09</td>
<td>0.2</td>
<td>1.2</td>
<td>-2.1</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide5</td>
<td>15</td>
<td>-0.02</td>
<td>0.1</td>
<td>0.6</td>
<td>-0.9</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide7</td>
<td>16</td>
<td>0.5</td>
<td>0.5</td>
<td>0.8</td>
<td>-0.7</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TransStrain</td>
<td>54</td>
<td>3.8</td>
<td>3.4</td>
<td>2.1</td>
<td>0.4</td>
<td>13.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TransStress</td>
<td>53</td>
<td>1.5</td>
<td>0.7</td>
<td>2.5</td>
<td>0.02</td>
<td>15.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MaxStress</td>
<td>51</td>
<td>26.6</td>
<td>26.2</td>
<td>12.4</td>
<td>8.2</td>
<td>54.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ToeMod</td>
<td>54</td>
<td>126.8</td>
<td>102.4</td>
<td>89.0</td>
<td>15.8</td>
<td>342.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LinMod</td>
<td>49</td>
<td>438.2</td>
<td>348.7</td>
<td>303.6</td>
<td>64.2</td>
<td>1543.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DynMod6</td>
<td>51</td>
<td>158.5</td>
<td>153.8</td>
<td>84.1</td>
<td>36.3</td>
<td>406.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TanDelta6</td>
<td>51</td>
<td>0.05</td>
<td>0.05</td>
<td>0.02</td>
<td>0.03</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relax6</td>
<td>51</td>
<td>44.0</td>
<td>42.8</td>
<td>14.2</td>
<td>24.6</td>
<td>117.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FailCycle</td>
<td>48</td>
<td>3709.2</td>
<td>2747</td>
<td>2859.6</td>
<td>236</td>
<td>12901</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CycStrain50</td>
<td>48</td>
<td>1.4</td>
<td>1.4</td>
<td>0.2</td>
<td>1.3</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TanStiff50</td>
<td>48</td>
<td>15.4</td>
<td>17.1</td>
<td>4.0</td>
<td>8.8</td>
<td>21.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyst50</td>
<td>48</td>
<td>0.02</td>
<td>0.02</td>
<td>0.01</td>
<td>0.005</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lax50</td>
<td>48</td>
<td>4.3</td>
<td>4.0</td>
<td>1.5</td>
<td>2.3</td>
<td>10.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note:* Explanation of variable names can be found in Table 5-1 of the text; N = sample size of real data; SD = standard deviation, Min = minimum, Max = maximum, Q1 = 1st quartile or 25th percentile, Q3 = 3rd quartile of 75th percentile.
<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>R²</th>
<th>FDi</th>
<th>FDe</th>
<th>FIF</th>
<th>COL</th>
<th>GAG</th>
<th>PYD</th>
<th>CS</th>
<th>CD</th>
<th>DP</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition Strain</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>Transition Stress</td>
<td>0.27</td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>Toe Modulus</td>
<td>0.60</td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>Linear Modulus</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>Max Stress</td>
<td>0.79</td>
<td>P</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>Dynamic Modulus</td>
<td>0.58</td>
<td>P</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>P</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Tangent Delta</td>
<td>0.59</td>
<td>N</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>P</td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Stress Relaxation</td>
<td>0.67</td>
<td>N</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Cycles to Failure</td>
<td>0.51</td>
<td>P</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Peak Cyclic Strain</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>P</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Tangent Stiffness</td>
<td>0.87</td>
<td>P</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>N</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Hysteresis</td>
<td>0.63</td>
<td>P</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>N</td>
<td>P</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Laxity</td>
<td>0.07</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
</tr>
</tbody>
</table>

**INSERTION SITE**

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>R²</th>
<th>FDi</th>
<th>FDe</th>
<th>FIF</th>
<th>COL</th>
<th>GAG</th>
<th>PYD</th>
<th>CS</th>
<th>CD</th>
<th>DP</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition Strain</td>
<td>0.20</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Transition Stress</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>Toe Modulus</td>
<td>0.61</td>
<td>N</td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Linear Modulus</td>
<td>0.58</td>
<td>N</td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Max Stress</td>
<td>0.75</td>
<td>N</td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Dynamic Modulus</td>
<td>0.54</td>
<td>N</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>N</td>
<td>P</td>
<td>P</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Tangent Delta</td>
<td>0.55</td>
<td>P</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Stress Relaxation</td>
<td>0.67</td>
<td>P</td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td>P</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Cycles to Failure</td>
<td>0.51</td>
<td>N</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>P</td>
<td>P</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Peak Cyclic Strain</td>
<td>0.08</td>
<td>P</td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Tangent Stiffness</td>
<td>0.83</td>
<td>N</td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Hysteresis</td>
<td>0.58</td>
<td>N</td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Laxity</td>
<td>0.07</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
</tr>
</tbody>
</table>

**MIDSUBSTANCE**

**Note:** FDi = FibDiam, FDe = FibDens, CS = CellShape, CD = CellDens, DP = DPeriod, IS = InitSld. R² = correlation coefficient of model; Darkness of shade represents the importance of the contribution of each predictor to the overall model.
### Table B-3. Compact Data – Mechanical Properties Regressed on Dynamic Processes

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>R²</th>
<th>Re-Alignment</th>
<th>Crimp</th>
<th>Deformation</th>
<th>Sliding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>RA</td>
<td>TR</td>
<td>LR</td>
</tr>
<tr>
<td>Transition Strain</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transition Stress</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toe Modulus</td>
<td>0.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear Modulus</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max Stress</td>
<td>0.32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynamic Modulus</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tangent Delta</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stress Relaxation</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycles to Failure</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Cyclic Strain</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tangent Stiffness</td>
<td>0.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hysteresis</td>
<td>0.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laxity</td>
<td>0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.41</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: S = ReAlignStrain, RA = ReAlignAmount, TR = ToeReAlign, LR = LinearReAlign, F = CrimpFreq, CA = CrimpAmp; NM = no significant model was found; R² = correlation coefficient of model; P = positive predictor, N = negative predictor; Darkness of color represents the importance of the contribution of each predictor to the overall model.
Table B-4. Compact Data – Dynamic Processes Regressed on Composition/Structure

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>$R^2$</th>
<th>FDi</th>
<th>FDe</th>
<th>FIF</th>
<th>COL</th>
<th>GAG</th>
<th>PYD</th>
<th>CS</th>
<th>CD</th>
<th>DP</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ReAlignStrain</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ReAlignAmount</td>
<td>0.26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ToeReAlign</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LinearReAlign</td>
<td>0.34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrimpFreq</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrimpAmp</td>
<td>0.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef1</td>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef3</td>
<td>0.51</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FibDef5</td>
<td>0.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide1</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide2</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide5</td>
<td>0.54</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide7</td>
<td>0.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: FDi = FibDiam, FDe = FibDens, CS = CellShape, CD = CellDens, DP = DPeriod, IS = InitSlid; NM = no significant model was found; $R^2$ = correlation coefficient of model; Darkness of shade represents the importance of the contribution of each predictor to the overall model.
Table B-5. Compact Data – Mechanical Properties Regressed on Composition/Structure

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>R²</th>
<th>FDi</th>
<th>FDe</th>
<th>FIF</th>
<th>COL</th>
<th>GAG</th>
<th>PYD</th>
<th>CS</th>
<th>CD</th>
<th>DP</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition Strain</td>
<td>0.08</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transition Stress</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toe Modulus</td>
<td>0.23</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear Modulus</td>
<td>0.44</td>
<td>N</td>
<td>P</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max Stress</td>
<td>0.51</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynamic Modulus</td>
<td>0.27</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tangent Delta</td>
<td>0.37</td>
<td></td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stress Relaxation</td>
<td>0.34</td>
<td>N</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycles to Failure</td>
<td>0.12</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Cyclic Strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tangent Stiffness</td>
<td>0.70</td>
<td>P</td>
<td>N</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hysteresis</td>
<td>0.54</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laxity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>R²</th>
<th>FDi</th>
<th>FDe</th>
<th>FIF</th>
<th>COL</th>
<th>GAG</th>
<th>PYD</th>
<th>CS</th>
<th>CD</th>
<th>DP</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition Strain</td>
<td>0.10</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transition Stress</td>
<td>0.11</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toe Modulus</td>
<td>0.20</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear Modulus</td>
<td>0.29</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max Stress</td>
<td>0.48</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynamic Modulus</td>
<td>0.20</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tangent Delta</td>
<td>0.34</td>
<td>N</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stress Relaxation</td>
<td>0.18</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycles to Failure</td>
<td>0.10</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Cyclic Strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tangent Stiffness</td>
<td>0.52</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hysteresis</td>
<td>0.48</td>
<td>N</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laxity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: FDi = FibDiam, FDe = FibDens, CS = CellShape, CD = CellDens, DP = DPeriod, IS = InitSlid; NM = no significant model was found; R² = correlation coefficient of model; Darkness of shade represents the importance of the contribution of each predictor to the overall model.