Tendon resident macrophages are dependent on fibroblast-derived CSF1 and are essential for normal collagen fibrillogenesis

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INTRODUCTION: Resident macrophages are essential for the development of many tissues throughout the body. Most resident macrophage populations require CSF1 receptor (CSF1R) signaling for their differentiation and survival. Recent studies in our laboratory have demonstrated that Csf1rGFP^{+} resident macrophages reside adjacent to tendon fibroblasts throughout tendon development, growth, and homeostasis [1]. The proportion of resident macrophages relative to the total tendon cell population increases over the course of postnatal growth, suggesting the importance of these cells during this time. Additionally, our gene expression analyses suggest there is crosstalk between resident macrophages and neighboring fibroblasts. Therefore, in this study we investigated the impact of tendon fibroblast-derived CSF1 on the resident macrophage population as well as the effects of resident macrophage depletion on tendon development.

METHODS: All procedures were IACUC approved. Mouse models. Scleraxis-lineage CSF1 knockout mice were generated by breeding Csf1$^{flox/flox}$ mice [2] with Scx^{Cre} mice [3] to obtain Scx^{Cre};Csf1$^{flox/flox}$ knockouts ("cKO"), as well as Cre-negative Csf1$^{flox/flox}$ littermate controls ("WT"). Scx^{Cre};Csf1$^{flox/flox}$ mice were crossed with Csf1rGFP mice for cryohistology. Experimental design. Achilles tendons ("AT") and patellar tendons ("PT") from 12-week-old (12w) mice were harvested for cryohistology (n = 3-4) and transmission electron microscopy ("TEM"; n = 6) whereas P14 ATs were used for qPCR (n = 3). Cryohistology. Nuclei and mean GFP intensity within each nuclear mask were quantified using Fiji (3-4 sections/sample) from sagittal sections. TEM. Transverse sections were stained with UranyLess and 1% phosphotungstic acid and imaged. Collagen fibril diameters (12 images/sample) were measured using custom MATLAB scripts. qPCR. AT midsubstance was dissected from sagittal sections for qPCR using a Fluidigm IFC for 96 genes of interest (n = 4). Statistics. WT and cKO groups were compared by two-sample t-test for ΔC_T, and Mann Whitney U test for cell density, % GFP, and collagen fibril diameter metrics.

RESULTS: Loss of tendon fibroblast CSF1 expression resulted in Csf1rGFP^{+} resident macrophage depletion. Using the Csf1rGFP reporter, we found a depletion of Csf1rGFP^{+} resident macrophages following tendon fibroblast-derived CSF1 ablation (**Fig. 1A**), with significant decreases in Csf1rGFP^{+} cell density (**Fig. 1B**) and proportion (**Fig. 1C**) in cKO ATs ($p<0.05$). Similar trends were found in PTs. Resident macrophage depletion resulted in larger diameter collagen fibrils. We hypothesized that resident macrophages may affect collagen fibrillogenesis either directly via ECM remodeling or indirectly by signaling to tendon fibroblasts. In fact, we found that cKO tendons had a wider range of collagen fibril diameters and a higher frequency of larger diameter fibrils (**Fig. 2**). Average fibril diameter was significantly higher in cKO PTs (92.7±4.9 nm vs. 80.9±5.7 nm; p=0.004) but not significantly different in cKO ATs (88.9±6.2 nm vs. 86.0±8.7 nm; p=0.7). Gene expression changes in P14 cKO ATs. To investigate changes in gene expression in cKO tendons during early postnatal growth, we performed qPCR on P14 ATs. As expected, we found significant downregulation of genes that are prominent in macrophages, including *Csf1r*, *Il1b*, *C1qc*, *Cx3cr1*, *C1qa*, *Pf4*, and *Adgre1* in cKO tendons (**Fig. 3**). We also found significant downregulation of ECM-related genes (*Lair1*, *Ctss*, *Mrc1*, *Ctsc*) that are enriched in resident macrophages compared to tendon fibroblasts. Additionally, we found a decrease in several cytokines (*Il1b*, *Tnf*, *Ccl2*) involved in inflammatory processes. Interestingly, the cytokine *Il2*, which is associated with activated T cells, was significantly upregulated in cKO tendons.

DISCUSSION: This study demonstrated that tendon resident macrophages require fibroblast-derived CSF1 for their survival (**Fig. 1**). This macrophage depletion model was specific to the tendon body, as macrophages still existed in the peritendinous tissues. Resident macrophage depletion resulted in a wider distribution of collagen fibril diameters driven by the formation of larger diameter fibrils compared to wild-type controls (**Fig. 2**). Surprisingly, despite lacking normal numbers of resident macrophages and possessing an altered collagen fibril diameter distribution, there were no detectable changes in mechanical properties in 12w cKO tendons (data not shown). These findings suggest that resident macrophages do not have a critical role in tendon formation and maturation at the ages examined in this study. It is possible, however, that the effects of resident macrophage depletion and aberrant collagen fibrillogenesis require more time to become evident, which is a focus of future studies.

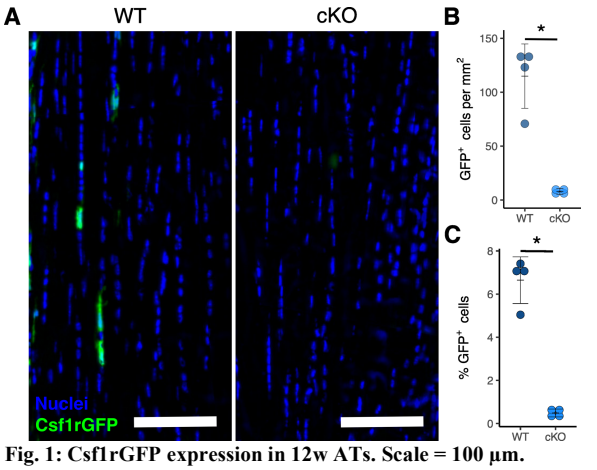

Fig. 1: Csf1rGFP expression in 12w ATs. Scale = 100 µm.

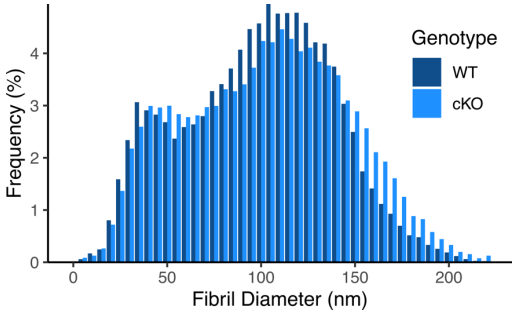

Fig. 2: Collagen fibril diameter distribution of 12w ATs.

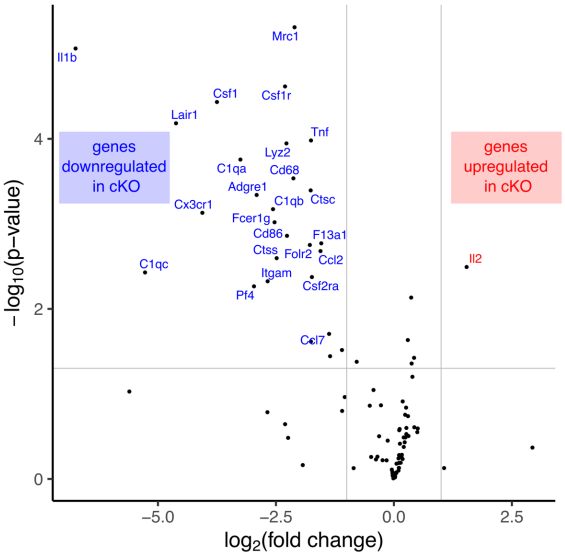

Fig. 3: Volcano plot comparing gene expression in P14 ATs.

SIGNIFICANCE: This study gives new insight into potential roles of resident macrophages during tendon development and growth and their interaction with fibroblasts and the ECM, which may inform future studies in the contexts of disease pathogenesis and tendon repair.

REFERENCES: 1) Bautista et al., 2023. 2) Harris et al., 2012. 3) Blitz et al., 2013. 4) Sasmono et al., 2003.

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Acute Knockdown of Collagen V Alters Mature Supraspinatus Tendon Regional Structure and Function

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INTRODUCTION: Collagen V is a critical fibril-forming collagen expressed during development and in mature tendons that is essential in regulating initial fibril assembly and fiber organization [1]. Clinically, classic Ehlers-Danlos syndrome (EDS) is a connective tissue disorder with greater than 50% of patients being haploinsufficient for *COL5A1* and is characterized by hyperextensible skin, joint hypermobility and instability, and abnormal wound healing [2]. Recent data from mature mouse supraspinatus tendon, which experiences a complex, region-dependent (insertion and midsubstance) loading environment within the rotator cuff of the shoulder, demonstrated that deficiency of collagen V from development resulted in severely altered collagen fibril structure, biomechanical properties, and dynamic responses to load [3]. However, the region-specific regulatory roles of collagen V during tendon homeostasis have not been differentiated from its role in development. Determining the role of collagen V during tendon homeostasis is critical for establishing the baseline effect of collagen V knockdown in both mature and aging tendons. Therefore, the objective of this study is to elucidate the effect of acute knockdown of collagen V on region-specific structure and function of mature supraspinatus tendons. Since tendon hierarchical structure is well-established by maturity, we hypothesized that acute knockdown of collagen V would result in minimal changes to regional mechanical properties, collagen fiber realignment and fibril diameter distribution.

METHODS: Animals: Male wild-type (WT; n=10) and bitransgenic *Col5a1*$^{flox/+}$ (I-HET; n=10) and *Col5a1*$^{flox/flox}$ (I-NULL; n=10) with a tamoxifen-inducible *ROSA-CreER*^{T2} were used. At 120 days old, Cre excision was induced via two consecutive IP injections of tamoxifen. Mechanics and Collagen Fiber Realignment: All mice were sacrificed at 150 days old (IACUC) and were subjected to our mechanical testing and collagen fiber realignment protocol [3]: stress relaxations at 3%, 5%, and 7% strain each with subsequent frequency sweeps at 0.1, 1, 5, and 10 Hz, followed by a quasistatic ramp-to-failure. Throughout the ramp-to-failure, dynamic collagen fiber realignment was quantified using cross-polarization imaging, and regional fiber alignment data was interpolated with a polynomial fit as a function of strain from the load-displacement data. Elastic parameters failure load and linear stiffness were quantified. Viscoelastic parameters percent relaxation, dynamic modulus and phase shift (tan δ) were also quantified for each stress relaxation and frequency sweep. Images were acquired during the ramp-to-failure for optical strain tracking of stain lines demarcating the insertion and midsubstance regions of the tendon to calculate the modulus of each region. Collagen Fibril Diameter: Tendons were fixed, processed, sectioned, stained, and imaged via transmission electron microscopy (TEM) as described [4]. Collagen fibril diameter was measured across the fibril minor axis using a custom MATLAB script. Statistics: Comparisons between genotypes were conducted using one-way ANOVAs followed by Bonferroni post-hoc tests for mechanical properties and collagen fiber realignment. Collagen fibril diameter distributions from each genotype were compared to those of the other genotypes using Kolmogorov-Smirnov tests. Significance was set at $p \leq 0.05$ and trends at $p \leq 0.1$.

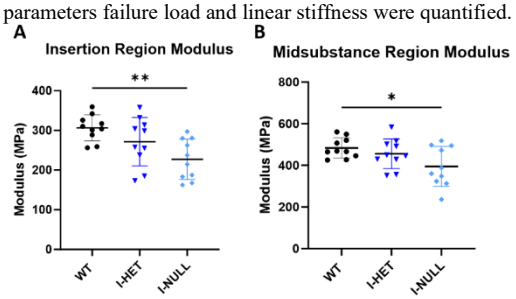

Figure 1. Acute collagen V knockdown (I-NULL) resulted in tendons with reduced insertion (A) and midsubstance (B) moduli relative to wild type (WT) controls. Data as mean ± standard deviation (--p≤0.1, *p≤0.05, **p≤0.01, ***p≤0.001).

RESULTS: As expected, no differences were observed in elastic mechanical properties, failure load and linear stiffness, and viscoelastic properties, percent relaxation, dynamic modulus, and phase shift, with acute collagen V knockdown (I-HET and I-NULL) (data not shown). Surprisingly, acute collagen V knockdown resulted in reductions in both the insertion (**Fig. 1A**) and midsubstance (**Fig. 1B**) moduli relative to wild type controls. These results are further supported by reductions in collagen fiber realignment in I-HET and I-NULL tendons across region, as demonstrated by greater normalized circular variance values for insertion (**Fig. 2A**) and midsubstance (**Fig. 2B**) regions from 3-9% strain, encompassing the toe and linear elastic regions of these tendons. Collagen fibril diameter distributions were different across genotype (**Fig. 3**, $p<0.0001$). I-NULL tendons exhibited more heterogeneous fibril diameter distributions with a greater percentage of larger diameter fibrils compared to WT and I-HET tendons in the insertion (**Fig. 3A**) and midsubstance (**Fig. 3B**) regions.

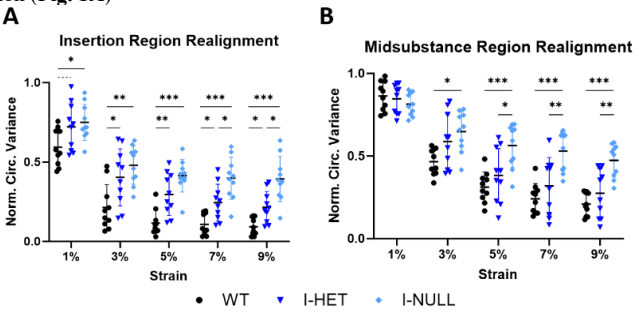

Figure 2. I-HET and I-NULL tendons demonstrated reduced collagen fiber realignment in the insertion (A) and midsubstance (B) regions. Decreased normalized circular variance is indicative of increased collagen fiber realignment. Data as mean ± standard deviation (--p≤0.1, *p≤0.05, **p≤0.01, ***p≤0.001).

DISCUSSION: In direct contrast to our hypothesis, acute reduction of collagen V expression in mature supraspinatus tendons resulted in regional structural and functional differences with reduced regional moduli and collagen fiber realignment and altered collagen fibril diameter distributions. Although mature tendons were generally believed to be quiescent tissues, there is increasing evidence that tendon collagen fibril networks are dynamic and remodel on shorter time scales than previously presumed [5]. Results of this study strongly support this notion, with collagen V playing a large role in regulating fibril properties beyond the postnatal developmental timeframe. While this study is limited by global knockdown models and potential confounding effects on neighboring tissue, the induced and short period of knockdown minimizes these effects. Future studies will evaluate the composition and gene expression of these acute collagen V-knockdown tendons to further elucidate the surprising regulatory role of collagen V in mature supraspinatus tendons.

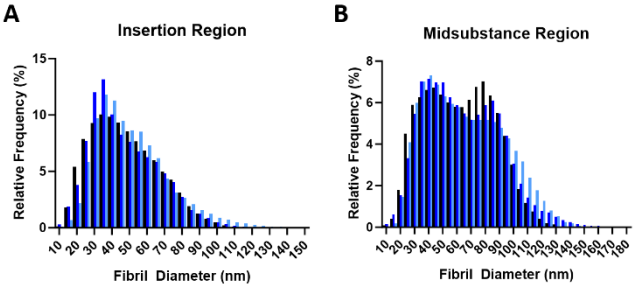

Figure 3. Fibril diameter distributions demonstrate a shift towards larger diameter fibrils in I-NULL tendons relative to the distributions of I-HET and WT tendons in the insertion (A) and midsubstance (B) regions. All distributions were significantly different from each other ($p<0.0001$).

SIGNIFICANCE/CLINICAL RELEVANCE: This study reveals that acute reduction in collagen V expression alters supraspinatus tendon regional mechanical properties, collagen fiber realignment and collagen fibril diameter distributions. These results provide further insight into the surprising role of collagen V in regulating tendon function during homeostasis and establishes a baseline for elucidating the role of collagen V in both mature and aging tendons.

ACKNOWLEDGEMENT: Study supported by NIH/NIAMS (AR070750) and Penn Center for Musculoskeletal Disorders (NIH/NIAMS, P30 AR069619).

REFERENCES: [1] Wenstrup et al. J Biol Chem. 2011. [2] Steinmann et al. Conn Tissue, Heritable Disorders. 2002. [3] Connizzo et al., Interface Focus. 2016. [4] Dunkman et al., Matrix Biol. 2014. [5] Yeung et al., Curr Top Dev Biol. 2019.

Tendon-Targeted Collagen V Deficiency and Knockout Attenuate Mature Supraspinatus Tendon Mechanics

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INTRODUCTION: Collagen V is a critical tendon matrix protein that regulates fibrillogenesis and is expressed throughout development and in mature tendons [1]. Clinical manifestation of collagen V deficiency is the classic form of Ehlers-Danlos syndrome (EDS), a connective tissue disorder with greater than 50% of patients being haploinsufficient for *COL5A1*, characterized by hyperextensible skin, joint hypermobility and instability, and abnormal wound healing [2]. Recent data from mouse supraspinatus tendon, which experiences a complex, region-dependent (insertion and midsubstance) loading environment within the rotator cuff of the shoulder, demonstrated that deficiency of collagen V during development resulted in severely altered collagen fibril structure, biomechanical properties, and dynamic responses to load [3]. However, the region-specific roles of collagen V tendon-targeted deficiency and knockout on mature supraspinatus tendons remain unknown. The objective of this study is to elucidate the regulatory role of collagen V on supraspinatus tendon whole-tissue and regional mechanics in mature mice using tendon-targeted (Scleraxis-Cre) collagen V heterozygous and knockout mice. Due to the role of collagen V in the regulation of tendon structure during development, we hypothesized that collagen V heterozygous and knockout supraspinatus tendons would have inferior whole-tissue and regional elastic mechanical properties, whole-tissue viscoelastic mechanical properties and reduced regional collagen fiber realignment compared to wild type control tendons.

METHODS: Animals: Supraspinatus tendons (n=10/genotype) from tendon-targeted collagen V heterozygous (TEN-HET) mice (ScxCre;*Col5a1*$^{f/wt}$), knockout (TEN-KO) mice (ScxCre;*Col5a1*$^{f/f}$), and wild-type (WT) control mice (Cre- littermates) were used (IACUC approved). Mechanics and Collagen Fiber Realignment: All mice were sacrificed at 150 days old and were subjected to our mechanical testing and collagen fiber realignment protocol [3]: stress relaxations at 3%, 5%, and 7% strain each with subsequent frequency sweeps at 0.1, 1, 5, and 10 Hz, followed by a quasistatic ramp-to-failure. Throughout the ramp-to-failure, dynamic collagen fiber realignment was quantified using cross-polarization imaging, and regional fiber alignment data was interpolated with a polynomial fit as a function of strain from the load-displacement data. Images were acquired during the ramp-to-failure for optical strain tracking of stain lines demarcating the insertion and midsubstance regions of the tendon. Statistics: Comparisons between genotypes were conducted using one-way ANOVAs followed by Bonferroni post-hoc tests. Significance was set at $p \leq 0.05$ and trends at $p \leq 0.1$.

RESULTS: Whole-tendon cross-sectional area was reduced in the TEN-KO group compared to the TEN-HET and WT groups (**Fig. 1A**). Consistent with our hypothesis, collagen V deficiency and knockout resulted in dose-dependent reductions in elastic mechanical properties (e.g., failure load and linear stiffness (**Figs. 1B, C**)). Viscoelastic differences were also observed. Percent relaxation was increased in TEN-KO tendons compared with TEN-HET and WT tendons at all strain levels (7% strain shown in **Fig. 2A**). Additionally, collagen V TEN-HET and TEN-KO resulted in dose-dependent reductions in dynamic modulus, while phase shift was increased in TEN-KO tendons relative to TEN-HET and WT across all strain levels and frequencies (7% strain at 1 Hz shown in **Figs. 2B and 2C**). As hypothesized, collagen V TEN-HET and TEN-KO resulted in dose-dependent reductions in insertion modulus, while midsubstance modulus was reduced in TEN-KO tendons relative to TEN-HET and WT tendons (**Figs. 3A, B**). These results are supported by reductions in collagen fiber realignment in TEN-HET and TEN-KO tendons across region, as demonstrated by greater normalized circular variance values for insertion and midsubstance regions from 3-7% strain (Figs. 3C-D), encompassing the toe and linear elastic regions of these tendons.

DISCUSSION: This study investigated the role of collagen V on supraspinatus tendon elastic and viscoelastic mechanics using TEN-HET and TEN-KO mice. Consistent with previous data [3], we demonstrated that tendon-targeted collagen V TEN-HET and TEN-KO resulted in reductions in regional and whole-tissue elastic and viscoelastic mechanical properties. Further, reductions in these properties in our collagen V TEN-HET tendons highlight the allele-dependency of collagen V on tendon elastic and viscoelastic mechanical function and collagen fiber realignment. These functional deficits could be attributed to the improper hierarchical assemblies of TEN-HET and TEN-KO tendons resulting in disorganized tendon matrices with an inferior ability to respond to load [4]. This was evidenced by marked reductions in the TEN-HET and TEN-KO tendons' responses to realign resulting in inferior whole-tissue and regional elastic and viscoelastic mechanical properties. Overall, results demonstrate that decreased collagen V expression detrimentally affects supraspinatus whole-tissue and regional elastic and viscoelastic mechanical properties and collagen fiber realignment.

SIGNIFICANCE/CLINICAL RELEVANCE: This study elucidates the critical role of collagen V in regulating supraspinatus tendon function. Future studies will evaluate the structural and compositional mechanisms that contribute to these mechanical results. Understanding the effects of collagen V in tendon can be used to develop potential treatments modalities for classic Ehlers-Danlos syndrome.

ACKNOWLEDGEMENT: This study was supported by NIH/NIAMS (AR070750) and the Penn Center for Musculoskeletal Disorders (NIH/NIAMS, P30 AR069619).

REFERENCES: [1] Wenstrup et al. J Biol Chem. 2011. [2] Steinmann et al. Conn Tissue, Heritable Disorders. 2002. [3] Connizzo et al., Interface Focus. 2016. [4] Connizzo et al., J Orthop Res. 2016.

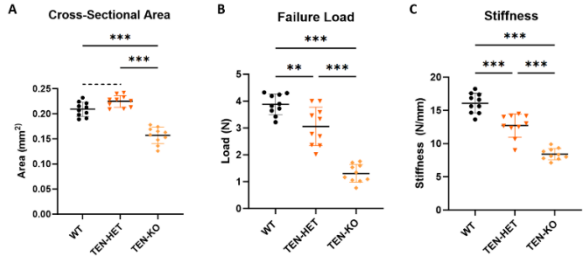

Figure 1. TEN-KO tendons demonstrated reduced cross-sectional area relative to TEN-HET and WT tendons (A). Tendon-targeted deficiency and knockout of collagen V resulted in significant reductions in elastic mechanical properties failure load and stiffness in a dose-dependent manner (B-C). Data as mean ± standard deviation (--p≤0.1, *p≤0.05, **p≤0.01, ***p≤0.001).

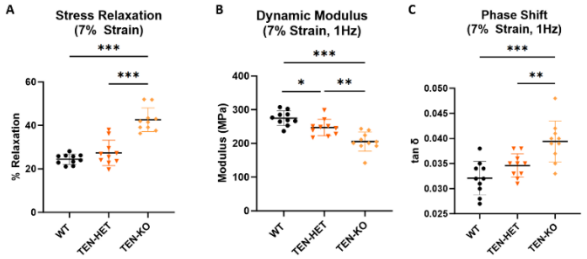

Figure 2. TEN-KO tendons had increased percent relaxation relative to TEN-HET and WT tendons (A). Tendon-targeted collagen V deficiency and knockout resulted in significant reductions in dynamic modulus in a dose-dependent manner (B), while phase shift was significantly increased in TEN-KO tendons relative to TEN-HET and WT tendons (C). Data as mean ± standard deviation (*p≤0.05, **p≤0.01, ***p≤0.001).

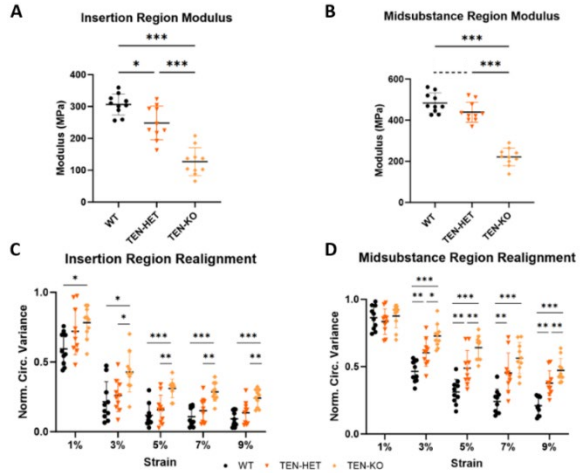

Figure 3. TEN-HET and TEN-KO tendons demonstrated reduced moduli and collagen fiber realignment in the insertion (A, C) and midsubstance (B, D) regions. Decreased normalized circular variance is indicative of increased collagen fiber realignment. Data as mean ± standard deviation (--p≤0.1, *p≤0.05, **p≤0.01, ***p≤0.001).

Strain-Induced Collagen Fibril Deformation is Diminished with Advanced Age in Mouse Supraspinatus Tendon

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Disclosures: none

INTRODUCTION: Age-related tendon degeneration increases the risk of rotator cuff injuries which can lead to significant pain and disability.[1] The supraspinatus tendon, as part of the rotator cuff, exhibits region-dependent mechanical properties that change with advanced age which are likely a contributing factor to the increased risk of rupture in the elderly population.[2,3] While these age-related changes to bulk tissue properties in the supraspinatus tendon have been demonstrated,[2,4] tendon is a complex hierarchical tissue that dynamically responds to mechanical loading though changes in structural organization at multiple length scales. Despite this, it is unclear how aging affects the relationship between bulk tissue strain and collagen fibril deformation on the nanoscale level.[5] Therefore, the objective of this study was to determine how collagen fibrils deform with applied strain in different regions of the supraspinatus tendon at two distinct ages. We hypothesized that collagen fibrils would experience deformation earlier in older tendons because of a reduction in early strain attenuation mechanisms such as uncrimping and changes in fiber alignment.

METHODS: Supraspinatus tendon-humerus complexes were harvested from p300 and p570 male wild-type C57BL/6 mice (IACUC approved). Tendon cross-sectional area was measured using a laser displacement sensor. After preparation for mechanical testing, samples were subjected to 10 cycles of preconditioning between 0.02 and 0.04 N followed by a one-minute rest and then a ramp to a randomly assigned strain (1%, 5%, or 9%; n = 5-6/group) at a rate of 0.1% strain per second. The tendon was immediately flash frozen after reaching the target strain, removed from the test fixture, and embedded in optimal cutting temperature compound while keeping the tissue frozen to maintain the applied strain.[6,7] Cryosections of the tendons were collected at 20 µm thickness and fixed in formalin. Nanoscale topographical images of the sections were acquired using tapping-mode atomic force microscopy (AFM) to visualize collagen fibrils. Five 2x2 µm images were acquired in both the insertion region (within 1 mm of humeral insertion) and midsubstance region (1-2mm away from humeral insertion) across multiple tissue sections for each sample (Fig 1). Collagen fibril d-period was measured using Fourier transform analysis.[8] The average d-period length, local variance (average variance in d-period length within individual images), and global variance (variance in d-period length across entire sample) were calculated for the insertion and midsubstance regions of each sample. Data for p300 and p570 samples were analyzed independently using two-way ANOVAs including the main effects of region, strain, and their interaction with Tukey-adjusted post-hoc testing within significant main effects.

RESULTS: The applied strains of 1%, 5%, and 9% corresponded to the early toe, early linear, and early yield portions of the stress-strain curves, respectively, in both ages (Fig 2a,b,f,g). Average d-period length increased from 67.8 nm to 68.7 nm with applied strains of 1% to 5% in p300 samples, corresponding to a fibril strain of approximately 1.3% (Fig 2c). However, d-period length was not different between applied strains of 1% and 9%. In contrast with the p300 data, fibrils from p570 samples experienced no strain-induced changes in d-period length (Fig 2h). Moreover, local and global variance in d-period length showed no effect of strain or region in both p300 and p570 ages (Fig 2d,e,i,j).

DISCUSSION: Significant deformation of collagen fibrils was observed in p300 supraspinatus tendons. Unexpectedly, this fibril deformation occurred at the lower applied strain of 5%. Therefore, some of the applied strain is transmitted from the bulk tissue level to the collagen fibrils between the early toe and early linear regions of the loading curve despite the uncrimping and reorganization that would be expected concurrently between these strains.[9] At the larger applied strain of 9%, the d-period length was no longer different than the 1% strain baseline value. Because the tissue exhibited early yield behavior (i.e., a reduction in modulus) at 9% strain, these data suggest that tissue yielding may result from early damage to the extracellular matrix that causes the collagen fibril d-period to begin to return to its initial length. Similar strain-dependent changes, with larger fibril deformations at intermediate applied strains, were found previously in supraspinatus tendons from younger p150 mice.[6,7] Contrary to our hypothesis, no fibril deformation was observed in supraspinatus tendons from p570 mice in this study. At this advanced age, the lack of strain transmission from the bulk tissue scale to the fibril scale indicates that smaller-scale mechanisms are likely dominated by structural reorganization such as uncrimping, sliding, and/or realignment rather than deformation of collagen fibrils.[9,10] Identifying the interplay between, and combination of, these mechanisms which become prominent with advanced age is a promising area for future study. In addition to measuring fibril deformation, this study also investigated the variation in d-period lengths. Even with changing d-period length in p300 samples, the variation remained similar for all strains. Therefore, the increase in d-period length was homogenous across all fibrils in the tissue, rather than the alternative where some fibrils would experience deformation while others would not. However, it should be noted that while fibril deformation was homogeneous in this controlled experiment, more complex mechanical loading of the supraspinatus tenon *in situ* could result in heterogenous fibril engagement.

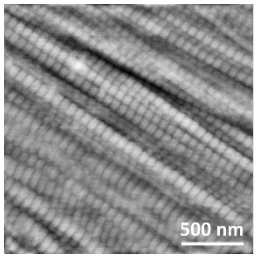

Figure 1. Representative image.

SIGNIFICANCE: Results from this study provide insights regarding nanoscale mechanisms that influence age-related degeneration and changes in mechanical properties of supraspinatus tendon.

ACKNOWLEDGEMENT: This study was supported by NIH/NIAMS (AR070750) and the Penn Center for Musculoskeletal Disorders (NIH/NIAMS, P30 AR069619).

REFERENCES: [1] Zhao+ *Orthop J Sports Med* 2021. [2] DiStefano+ *ORS* 2022. [3] Johnson+ *J Orthop Res* 2023. [4] Connizzo+ *J Biomech Eng* 2013. [5] Fang+ *J Orthop Res* 2017. [6] Connizzo+ *J Biomech* 2014. [7] Connizzo+ *Interface Focus* 2016. [8] Rigozzi+ *J Struct Biol* 2011. [9] Miller+ *J Biomech* 2012. [10] Szczesny+ *Acta Biomater* 2014.

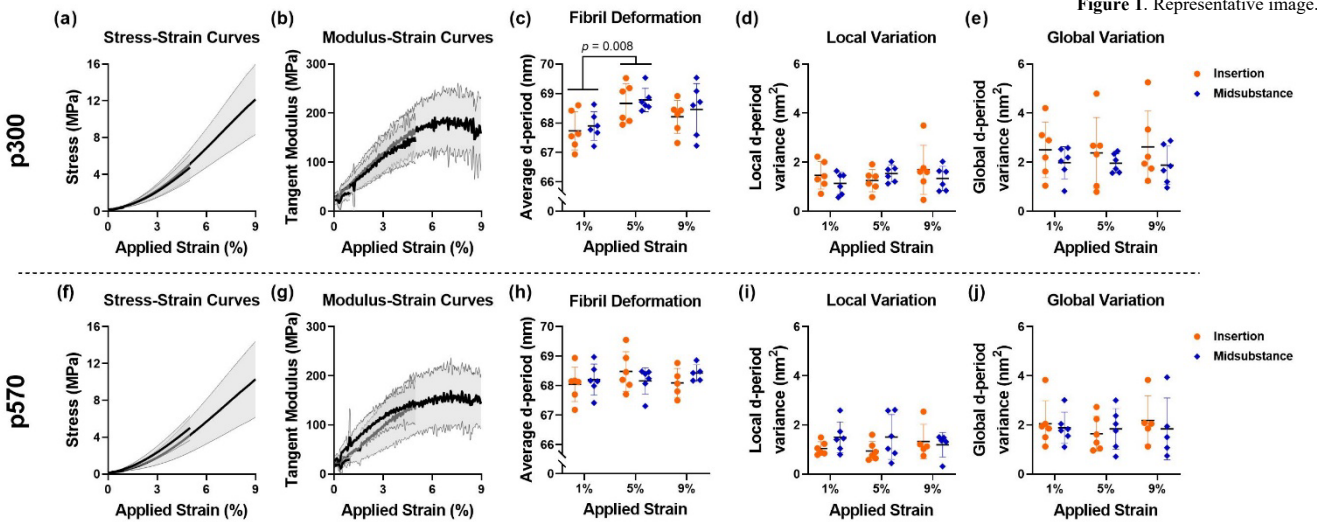

Figure 2. Average stress-strain curves (a,f) and modulus-strain curves (b,g) from p300 and p570 samples. Fibril deformation was significantly increased from 1% to 5% applied strain independently of region in p300 samples (c) but was unaffected by strain and region in p570 samples (h). Local and global variation were unaffected by strain and region in both p300 and p570 samples (d,e,i,j).

Treadmill Running Does Not Induce Mechanical Changes in the Rat Subscapularis Tendon

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DISCLOSURES: CSF (N), CAN (N), JDE (N), LJS (N), AFK (5 – FX Shoulder USA, Integra Life Sciences, Orthofix; 9 – American Board of Orthopaedic Surgery, American Shoulder and Elbow Surgeons)

INTRODUCTION: Rotator cuff tendinopathy, often caused by overuse,[1,2] results in functional deficits and pain,[3] presenting a substantial clinical problem that impacts people of all ages and levels of physical activity.[4] Previous studies in rat models have shown that exercise-induced overuse results in tendinopathy in the supraspinatus tendon.[5] However, the impact of increased activity on the remainder of the rotator cuff is unknown. Although previously believed to be an infrequent source of clinical pathology, the subscapularis is now recognized as a common cause of rotator cuff tendinopathy.[6] Therefore, the objective of this study was to evaluate the impact of treadmill running on inducing tendinopathy in the subscapularis tendon in the rat model. We hypothesized that high levels of treadmill running would lead to tendinopathy and result in decreased mechanical properties in the upper and lower bands of the subscapularis.

METHODS: Treadmill Protocol: Forty-five 16-week-old male Sprague-Dawley rats were subjected to one of three levels of exercise by treadmill running – cage activity (CA), moderate-speed running (MSR, 17 m/min at 10° decline)[5], or high-speed running (HSR, 22 m/min at 10° decline). Animals subjected to running protocols underwent an acclimation period of two weeks in the MSR group and three weeks in the HSR group, followed by four weeks of treadmill running (1 hour per day, 5 days per week). Animals were sacrificed after completion of the treadmill protocol for mechanical testing. Sample Preparation: Subscapularis and supraspinatus tendons were harvested with the left humerus and fine dissected free of non-tendon soft tissue. Subscapularis tendons were separated into upper and lower bands by cutting through the clear delineation point, in order to test the two distinct bands of the subscapularis individually (Fig 1). Tendon cross-sectional areas were measured using a laser-based device[7] and humeri were potted in polymethyl-methacrylate for testing. Mechanical Testing and Analysis: Tendons were secured in custom fixtures at a gauge length of 8mm from the insertion. Testing was performed on an Instron 5542 test frame (Instron, Norwood, MA), and consisted of preconditioning (30 cycles between 0.125% and 0.375% strain at 0.25 Hz), stress relaxation (6% strain for 10 minutes), and quasi-static ramp-to-failure (0.1 mm/s). Tendons were then analyzed for elastic properties (stiffness, modulus), viscoelastic properties (percent relaxation), and failure properties (maximum force, maximum stress) using custom MATLAB scripts. Samples that failed at the grip were excluded from failure property analysis. Data was analyzed using one-way ANOVA followed by a Tukey's post-hoc test ($\alpha = 0.05$).

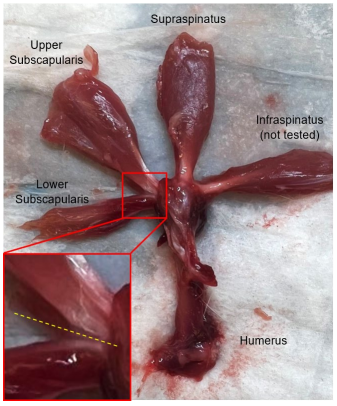

Fig. 1: Photograph of dissected humerus with rotator cuff tendons attached, with delineation point between upper and lower bands of the subscapularis highlighted in yellow.

RESULTS: Upper and lower bands of the subscapularis tendon showed no differences in stiffness (Fig. 2A), grip modulus (Fig. 2B), or percent relaxation (Fig. 2C) between activity levels. Additionally, upper and lower bands showed no differences in maximum force (Fig. 2D) or maximum stress (Fig. 2E) between activity levels. Similarly, supraspinatus tendons showed no differences in any mechanical properties between activity levels (*data not shown*).

DISCUSSION: This study demonstrated that the downhill treadmill running protocols utilized did not induce mechanical changes consistent with overuse in the subscapularis tendon. Although there were no mechanical changes observed, it is possible that treadmill running may have induced biological and/or histopathological changes that have not been evaluated to date. Prior studies using the MSR model focused on the supraspinatus tendon.[5,8,9] The lack of observed mechanical changes in the subscapularis indicates that the model may not be suitable for this tendon, perhaps due to the unique organization, composition, local loading environment, or relative location of the acromion to the supraspinatus when compared to the subscapularis. Interestingly, this study did not find mechanical changes in the supraspinatus tendon, adding to the mixed results of the treadmill model,[5,8,9] which may be affected by protocol variables such as treadmill inclination, as well as duration and time of day of running. Taken together, the results of this study in the subscapularis and supraspinatus indicate the described protocol does not induce mechanical changes consistent with tendinopathy that may lead to rotator cuff tears. While recent clinical studies have highlighted the high and previously unrecognized incidence of subscapularis tears,[6] the relationship of subscapularis tears to preexisting tendon damage, potentially due to overuse, remains unknown and further studies are needed.

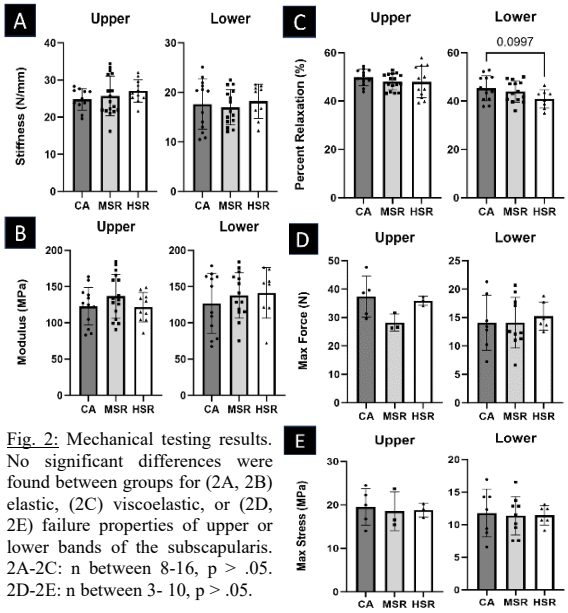

Fig. 2: Mechanical testing results. No significant differences were found between groups for (2A, 2B) elastic, (2C) viscoelastic, or (2D, 2E) failure properties of upper or lower bands of the subscapularis. 2A-2C: n between 8-16, $p > .05$. 2D-2E: n between 3- 10, $p > .05$.

CLINICAL SIGNFICANCE: Increased physical activity did not result in tendinopathy in the subscapularis, suggesting the possibility that exercise-induce overuse does not lead to subscapularis tendon damage and tears in the absence of other contributing factors.

REFERENCES: [1] Gomoll+ Arthritis Rheumatol, 2004. [2] van der Windt+ Ann Rheum Dis, 1995. [3] Millar+ Nat Rev Dis Primers, 2021. [4] Hopkins+ Asia Pac J Sports Med Arthrosc Rehabil Technol, 2016. [5] Soslowsky+, JSES, 2000. [6] Bennett+, Arthroscopy, 2001. [7] Favata PhD Thesis, 2006. [8] Parks+ J Orthop Res, 2017. [9] Attia+ J Orthop Res, 2011.

ACKNOWLEDGEMENTS: This study was supported by the Department of Veterans Affairs (RX003652) and the Penn Center for Musculoskeletal Disorders (NIH/NIAMS P30AR069619). We thank Steph Weiss, Maggie Tamburro, Rebecca Betts, Nat Thurlow, and Mitch Hallman for their contributions to rat running.

Tendon-Targeted Collagen V Knockout Influences Mechanical Properties of Aged Supraspinatus Tendon

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Disclosures: none

INTRODUCTION: Collagen V is one of the minor collagens in tendon, yet it plays a critical role in collagen fibrillogenesis by influencing the hierarchal assembly of collagen I into fibrils, fibers, and fascicles [1]. Clinical manifestations of reduced collagen V expression are present in patients with classic Ehlers-Danlos syndrome (EDS), a heritable connective tissue disorder with generalized connective tissue fragility as well as joint hypermobility and instability [2]. In addition to the altered tissue properties caused by reduced collagen V expression in EDS, the supraspinatus tendon is at high risk for injury with increased age and exhibits region-specific properties due to its complex leading environment [3-4]. Previous work has demonstrated that collagen V deficiency during development resulted in severely altered collagen fibril structure, biomechanical properties, and dynamic responses to load in the mouse supraspinatus tendon [5]. However, little remains known regarding the region-specific roles of collagen V in mouse supraspinatus tendon with more advanced age. Therefore, the objective of this study was to elucidate the region-specific role of collagen V in supraspinatus tendon mechanical properties of aged mice. We hypothesized that reduction in collagen V would result in inferior mechanical properties of the supraspinatus tendon, and that the mechanical changes would be greater in the insertion region than in the midsubstance region.

METHODS: Supraspinatus tendons (n=10/group) from male, 300 day old tendon-targeted collagen V heterozygous (Ten-Het) (ScxCre;*Col5a1*$^{f/wt}$), knockout (Ten-Null) (ScxCre;*Col5a1*$^{f/f}$), and Cre- littermate controls (control) were used in this IACUC approved study. Supraspinatus tendon-humerus complexes were finely dissected, and the cross-sectional area was measured using a laser displacement sensor. Lines were applied to the tendon using Verhoeff's stain at 0, 1, 2, and 2.5mm from the humeral insertion to demarcate the insertion region (0-1mm) and midsubstance region (1-2mm) for optical strain tracking and to establish the gauge length (2.5mm). After potting the humerus and securing the free end of the tendon between sandpaper using cyanoacrylate glue, the samples were subjected to mechanical testing: after preloading to 0.05N and performing 10 cycles of preconditioning, stress relaxations were conducted at 3%, 5%, and 7% strain each with subsequent frequency sweeps at 0.1, 1, 5, and 10 Hz, followed by a quasistatic ramp-to-failure at a rate of 0.1% strain/second. Failure load and linear stiffness were quantified from the ramp to failure. Percent relaxation was calculated for each stress relaxation, and dynamic modulus and phase shift (tan δ) were quantified for each frequency sweep. Images were acquired during the ramp-to-failure for optical strain tracking of stain lines to calculate the modulus of the insertion and midsubstance regions. Comparisons between genotypes were conducted using one-way ANOVAs followed by Bonferroni post-hoc tests.

RESULTS: Whole tendon cross-sectional area did not differ between groups (Fig. 1A). Significant differences were seen in tendon elastic and viscoelastic mechanical properties. Ten-Null tendons failed at a significantly lower loads compared to both control and Ten-Het tendons and demonstrated a lower stiffness than controls (Fig. 1B-C). Additionally, Ten-Null tendons exhibited increased percent relaxation at 7% strain compared to control tendons (Fig. 1D). There were no differences in percent relaxation between groups at 3% or 5% strain (data not shown). Ten-Null tendons demonstrated a decreased dynamic modulus and increased phase shift across all strain levels and frequencies (7% strain at 1 Hz shown in Fig. 1E-F). Ten-Null tendons demonstrated a decreased elastic modulus in the insertion region compared to control and Ten-Het tendons, while the Ten-Null tendons had a lower modulus only compared to controls in the midsubstance region (Fig.2A-B).

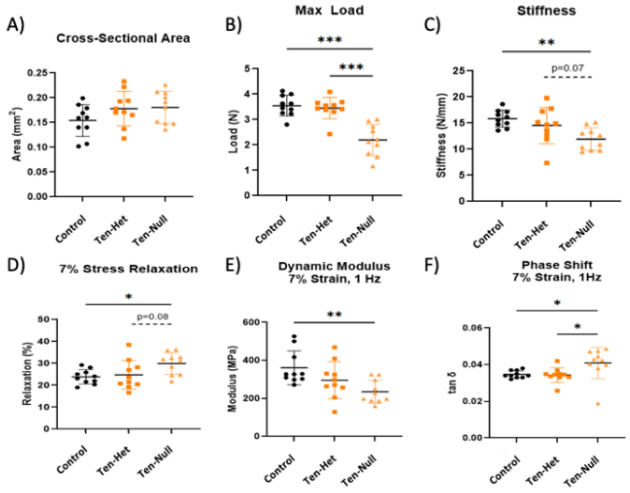

Figure 1. (A) No differences were seen in whole-tendon cross-sectional area. Ten-Null tendons demonstrated decreased in (B) max load and (C) stiffness. Within viscoelastic properties, Ten-Null tendons had (D) increased percent relaxation, (E) decreased dynamic modulus, and (F) increased phase shift. Data shown as mean ± standard deviation. (--p≤0.1, *p≤0.05, **p≤0.01, ***p≤0.001).

DISCUSSION: This study investigated the role of collagen V on aged supraspinatus tendon elastic and viscoelastic mechanical properties. Results demonstrate that collagen V plays a critical role in regulating the extracellular matrix of supraspinatus tendon that has lasting effects into advanced age. Both the insertion and midsubstance regions were affected by collagen V knockout, yet its influence caused a greater decrease in modulus in the insertion region where the supraspinatus tendon is the least organized and experiences the highest strains [6]. Previous work investigating the regional influence of collagen V on fibril morphology in the supraspinatus tendon has shown that collagen V knockout mice demonstrate a significant disruption of fibril assembly with an increase in structurally aberrant fibrils at the insertion region compared to controls [7]. This regional variation in how collagen V influences collagen fibril structure could contribute to the respective mechanical responses of the insertion and midsubstance regions. This is the first study to evaluate the role of collagen V in tendons from mice aged 300 days as previous work evaluated tendons from younger mice (60-120 days) [5-7]. Evaluating tendons at this age allows us to gain an understanding of the influence of collagen V on tendon properties after maturation into more advanced age. Future studies will investigate the underlying structural and compositional properties of the tendon extracellular matrix caused by knockout of collagen V that give rise to these mechanical findings. Moreover, continued work will investigate the varied effect of collagen V knockout at different ages to gain a better understanding of the distinct roles of collagen V in tendon properties during development, maturation, and aging.

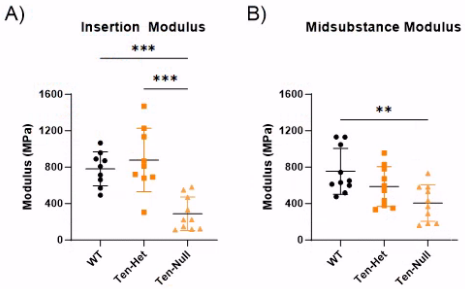

Figure 2. Ten-Null tendons demonstrated reduced modulus in the (A) insertion region, compared to control and Ten-Het samples, and in the (B) midsubstance, compared to controls. Data shown as mean ± standard deviation. (**p≤0.01, ***p≤0.001).

CLINICAL RELEVANCE: EDS is a clinical syndrome with limited treatment options. Furthering our understanding of collagen V in tendon under different conditions will aid the development of therapeutic targets for EDS.

ACKNOWLEDGEMENT: This study was supported by NIH/NIAMS (AR070750) and the Penn Center for Musculoskeletal Disorders (NIH/NIAMS, P30 AR069619).

REFERENCES: [1] Wenstrup et al. J Biol Chem. 2011. [2] Steinmann et al. Conn Tissue, Heritable Disorders. 2002. [3] Minagawa et al. J Orthop. 2013. [4] DiStefano et al. ORS Annual Meeting. 2022. [5] Connizzo et al. Interface Focus. 2016. [6] Connizzo et al. J Biomech. 2017. [7] Connizzo et al. J Orthop Res. 2017.

Focal Adhesion Kinase Regulates Physiological Tendon Development and Growth

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INTRODUCTION: Mechanical stimuli are known to impact tendon formation and homeostasis via cell mechanotransductive signaling. Focal adhesion kinase (FAK, gene: *Ptk2*) is an intracellular protein kinase that regulates cytoskeletal dynamics and transmission of mechanical strain to the cell nucleus from its surrounding extracellular matrix (ECM).[1] Pharmacological FAK inhibition alters cell morphology and tenogenic gene expression in monolayer cell culture and attenuates ECM to nuclei strain transmission and mechanotransductive gene expression in explant tendon culture.[2-7] Despite these known roles for FAK in tendon cells, the mechanism by which FAK regulates tendon physiology and the cell mechano-response throughout tendon development and postnatal growth remains unknown. Therefore, the objective of this study was to define the role of FAK in promoting cell proliferation and ECM deposition during the stage of rapid postnatal growth. We hypothesized that tendon-targeted FAK conditional knockout will reduce cell proliferation and impair matrix assembly, resulting in mechanically inferior tendons.

METHODS: To attenuate FAK expression *in vivo*, we utilized tendon-targeted FAK knockout (Scx-Cre;FAK$^{F/F}$; FAK-KO) mice,[8] in which we have previously validated reduced *Ptk2* expression.[6] Achilles tendons (ATs), flexor digitorum longus (FDL) tendons, and patellar tendons (PTs) from P10, P30, and P60 FAK-KO and WT littermate controls were used for viscoelastic mechanical testing, histology, and collagen fibril structure measures. In addition, we performed an EdU labeling experiment to quantify cell proliferation in P10 mice. Viscoelastic Mechanics: Tendon cross-sectional areas (CSAs) were measured, and tendons were subjected to a viscoelastic mechanical testing protocol (preconditioning, viscoelastic stress relaxation and dynamic frequency sweep, and a quasi-static ramp to failure). Histology: Whole knee joints from P10 mice were fixed, decalcified, paraffin embedded, and sectioned in the transverse plane to visualize the PT cross-section. Overall tissue morphology was visualized via toluidine blue staining. Cell Proliferation Analysis: Mice were injected with EdU (3μg/g bodyweight; Invitrogen A10044) at P0 and P2 and euthanized at P10. Knees joints were cut into sagittal sections and stained with Click-iT™ Cell Reaction Buffer Kit (Invitrogen C10269) to quantify EdU-positive nuclei within the PT. Collagen Fibril Structure: To quantify collagen fibril diameter distributions, PTs from all timepoints were fixed, embedded, sectioned at 85 nm, and imaged with transmission electron microscopy at 60,000x.

RESULTS: In our mechanical assessment, FAK-KO ATs and PTs exhibited reduced CSAs at P10 (**Fig. 1**). Despite this, there were few mechanical differences in structural or material properties in FAK-KO tendons at P10. This contrasts with tendons at P30 and P60 ages, in which FAK-KO tendons exhibited reduced size and structural properties (i.e., stiffness and max load) yet increased material properties (i.e., modulus and max stress) relative to WT tendons. Viscoelastic dynamic modulus values followed a similar trend to the other material properties (data not shown). Interestingly, while the reduced size of FAK-KO tendons was visible histologically at P10 (**Fig. 2A**), EdU labeling did not demonstrate a difference between proliferative cell behavior at this age (percent EdU-positive nuclei (Mean±SD); WT:7.0±2.5; FAK-KO:8.9±2.2). Finally, while the collagen fibril diameter distribution was not robustly altered between groups at P10 (**Fig. 2B**), the FAK-KO tendons demonstrated markedly smaller fibril diameters compared to WT tendons at both P30 and P60 (**Fig. 2C**-**D**).

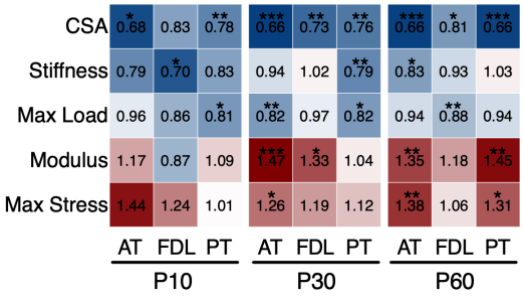

Figure 1. Viscoelastic mechanical testing datasets for all tendons evaluated in this study. Color and numbers within the cells indicate the ratio of the FAK-KO group mean relative to the WT group mean for that parameter. n=7-16/genotype/timepoint. Asterisks represent significant differences between WT and FAK-KO groups, which were compared with t-tests (*p<0.05; **p<0.01; ***p<0.001).

DISCUSSION: This study investigated the regulatory roles of FAK signaling on tendon physiology during postnatal growth. Consistent with our hypothesis, we observed reduced tissue size in FAK-KO tendons at all experimental timepoints. Interestingly, the differences in tissue mechanical properties were more drastic at the P30 and P60 timepoints compared to P10. Overall, these findings suggest that FAK regulates the generation of tendon size early in development, while altered ECM mechanical properties develop later during postnatal growth in FAK-KO mice. Given this finding, we hypothesized that FAK-KO led to altered tendon physiology by controlling cell proliferation and matrix deposition. While we did not observe a difference in EdU labeling or collagenous matrix deposition at P10, FAK ablation markedly reduced the size of collagen fibrils at P30 and P60, which suggests altered ECM deposition and remodeling behavior in FAK-KO tendons. Ongoing studies will further identify the effect of FAK-KO on the ECM structure by evaluating ECM-related gene expression and protein content. In addition, our future work will explore the effect of *in vivo* mechanical loading paradigms on FAK-dependent mechanotransduction and the tendon physiological response.

SIGNIFICANCE: Mechanical stimuli are essential for regulating tendon physiology, and defining the key signaling pathways that control tendon cell mechanotransduction will improve our understanding of disease and enable the development of improved therapies. Our results indicate that FAK signaling is important for tendon growth and the establishment of native structure/function.

ACKNOWLEDGEMENTS: We acknowledge support from NIH/NIAMS (T32AR007132 and P30AR069619).

REFERENCES: 1. Huveneers *et al.*, J Cell Sci, 2009; 2. Li *et al.*, J Orthop Res, 2019; 3. Liu *et al.*, Stem Cells Int, 2018; 4. Xu *et al.*, J Cell Physiol, 2012; 5. Leahy *et al.*, ORS Annual Meeting, 2022; 6. Leahy *et al.*, ORS Annual Meeting, 2023; 7. Leahy *et al.*, SB3C, 2023; 8. Beggs *et al.*, Neuron, 2003;

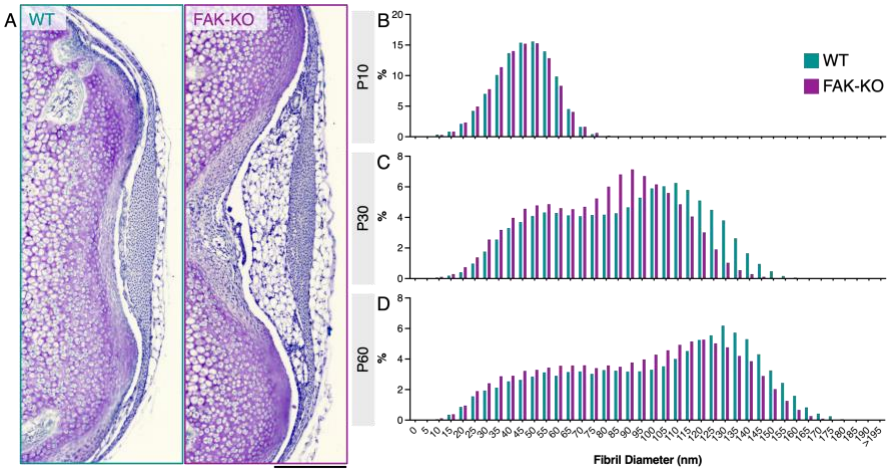

Figure 2. A) Representative images of transverse sections of P10 WT and FAK-KO tendons demonstrating reduced tendon CSA (n=4-5/genotype). B) P10, C) P30, and D) P60 collagen fibril diameter distribution quantifications (n=5-6/genotype/timepoint). Fibril diameter distributions were compared between groups using Kolmogorov-Smirnov tests, which demonstrated statistical significance (p<0.001) between groups at all experimental timepoints.

Disruption of The Anterosuperior Rotator Cuff Leads to Ambulatory and Grip Strength Deficits in a Rat Model

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INTRODUCTION: Rotator cuff tears are a highly prevalent condition, with tears involving the subscapularis tendon now more frequently recognized.[1] Subscapularis tears disrupt the anterior aspect of the rotator cuff force couple and often occur in association with supraspinatus tears.[2] However, prior studies that investigated joint damage caused by rotator cuff injuries did not include tears of the subscapularis and focused largely on post-mortem experimentation.[3] Additionally, although we previously assessed rat gait following an isolated supraspinatus injury with and without repair, longitudinal *in-vivo* diagnostics for tears involving the subscapularis tendon have not been evaluated in a rat model.[3,4] Therefore, the objective of this study was to determine the impact of a combined subscapularis and supraspinatus tear on gait and grip strength in a rat rotator cuff model. We hypothesized that there would be a loss in medial-lateral shoulder kinematics and kinetics, changes in stride length and speed, and a decrease in grip strength post-injury.

METHODS: Adult male Sprague-Dawley rats (400-450g) were used in this IACUC approved study. Animals were acclimated and trained to use our custom ambulatory[5] and grip devices[6], which assess shoulder joint function and reflexive grip strength, respectively. Baseline gait and grip measurements were obtained at 7, 4, and 1 days prior to surgery. In the right shoulder of each animal, the supraspinatus and subscapularis tendons were sharply detached at the insertion sites and biceps tenotomy was performed (n=5). Animals were administered one dose of extended-release buprenorphine at the time of injury, which provided 72 hours of analgesia. Forelimb gait and ground reaction forces as well as grip strength were recorded with our custom devices longitudinally at 2, 4, 7, 14, 20, 27, 41, 55, and 82 days after injury (n=5/timepoint). Gait kinematics were measured by tracking paw positions over time and measuring stance, stride speed, stride length, and stride width. Gait kinetics were assessed using 6 degree-of-freedom load cells to determine lateral force, propulsion force, normal force, braking force, and rate of loading[5]. Reflexive grip strength was measured using a custom grip strength device with independent grip bars and load cells for each limb, allowing for simultaneous measurements from both forelimbs.[6] Six trials were completed for each time point, with average values across all trials reported. One rat was excluded from grip strength data because of mild injury to the left forepaw. Statistical comparisons were made using two-way ANOVAs with main effects of time, limb, and their interaction. Multiple comparison post hoc tests between limbs were performed where appropriate based on significance of main effects. Significance was set at $p<0.05$ for all comparisons and trends at $p<0.10$.

RESULTS: *Gait Analysis*: A decrease in normal force in the injured limb compared to contralateral was observed after injury at 2, 4, 7, and 20 days with a trend at day 14 (Fig.1A). Braking force was also decreased in the injured limb compared to contralateral 2 and 4 days after injury (Fig.1B). Furthermore, there was a decreased rate of loading in the injured limb at 2 days after injury (Fig.1E). Propulsion force, lateral force, and stance time (Fig.1C,D,F) were not different between limbs at any time point. In contrast to several of the kinetic measures that did show differences, kinematic gait measures (i.e., speed, stride length, and stride width) showed no difference due to injury (data not shown). *Grip Strength*: The reflexive grip strength force was decreased in the injured limb compared to contralateral at 2, 4, and 7 days after injury, and this difference resolved at 14 days after injury and all subsequent time points (Fig.2).

DISCUSSION: The purpose of this study was to evaluate the utility of gait analysis and grip strength assays after a large anterosuperior rotator cuff injury in a rat model. Results demonstrated that functional deficits were detectable post-injury using these assays. We observed changes in gait kinetics after injury which gradually resolved over the course of ~20 days post-injury; however, no changes were observed in temporal gait kinematic data. This greater sensitivity of gait kinetics in comparison to kinematics is consistent with prior studies,[7] and highlights the importance of the increased sensitivity of load measures during gait analysis experiments. Similar to gait data, reflexive grip strength demonstrated functional deficits in the injured limb post-injury, while this deficit resolved between 7 and 14 days post-injury. The differences between limbs in both gait and grip strength highlight the importance of early post-injury time points to capture functional deficits post-injury and their temporal changes after a rotator cuff injury, especially with less severe injuries that would be expected to produce more subtle changes than observed here. While this study confirmed the presence of functional deficits post-injury, it remains unclear how pain, or lack of pain, contributes to the observed changes in gait and grip strength when pain relievers are introduced. Future work will investigate pain as a factor through the use of analgesics.

SIGNIFICANCE/CLINICAL RELEVANCE: The ability to quantify longitudinal functional deficits in animal models is essential for minimizing animal use and informing translation to the clinic. Results from this study illustrate changes in upper limb function that result from rotator cuff tears involving the subscapularis and supraspinatus.

REFERENCES:[1] Denard PJ and Burkhart SS, Arthrosc Tech, Oct 2013. [2] Barth J et al. Ortho and Trauma Dec 2012. [3] Reuther KE et al. J Orthop Res, May 2014. [4] Reuther KE et al. J Shoulder Elbow Surg, Mar 2015. [5] Sarver JJ et al. J Biomech, Mar 2011.[6] Reiter A et al. J Biomech Eng, Apr 2019. [7] Hsu JE et al. JOR Feb 2011.

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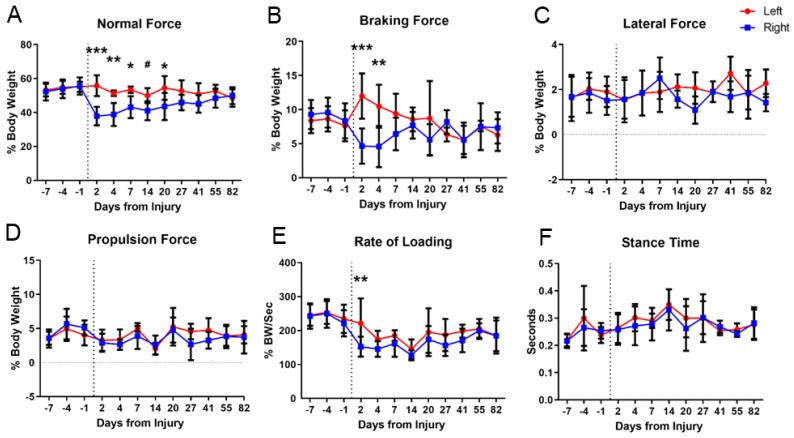

Figure 1. A decrease in normal force in the injured limb compared to contralateral was observed after injury at 2, 4, 7, and 20 days, and is trending at day 14 (Fig.1A). Braking force was decreased in the injured limb compared to contralateral 2 and 4 days after injury (Fig.2B). A decreased rate of loading in the injured limb was observed 2 days after injury (Fig.1E). Lateral force, propulsion force, and stance time (Fig.1C,D,F) were not different between limbs at any time point. (#p<0.10, *p<0.05, **p<0.01, ***p<0.001).

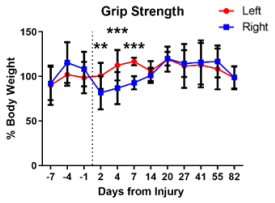

Figure 2. Reflexive grip strength force was decreased in the injured limb compared to contralateral at 2, 4, and 7 days after injury, and is resolved by 14 days after injury. (**p<0.01, ***p<0.001).

Evaluating Learning on a Limb: A high school orthopaedic research outreach workshop

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INTRODUCTION: Musculoskeletal disorders cost approximately $380.9 billion annually, making them the aggregated health category with the greatest spending in the United States.[1] Combating these high economic costs and the burden that musculoskeletal disorders have on the global population will require diverse research teams, which produce higher impact work.[2] To establish diverse research teams in orthopaedics, targeted efforts to recruit young, diverse students to the field are needed. One effective way of engaging K-12 students to study science, technology, engineering, and mathematics (STEM) is outreach activities.[3] Last year, our group designed and implemented Learning on a Limb, a half-day workshop to teach high school students about orthopaedic research.[4] We have now incorporated expanded rigorous evaluation metrics to determine how effectively this workshop taught principles of orthopaedic research and inspired high school students to consider careers in orthopaedics.

METHODS: The workshop, called Learning on a Limb, was planned and executed by the McKay Orthopaedic Research Laboratory's Diversity, Equity, and Inclusion (DEI) Committee and the Perelman School of Medicine (PSOM) Office of Outreach, Education, and Research. The Learning on a Limb Team was a diverse group of principal investigators, postdoctoral research fellows, and graduate students who all conduct orthopaedic research. High school students were recruited to participate in Learning on a Limb by the PSOM Office of Outreach, Education, and Research through flyers sent to high schools in the Greater Philadelphia area. The workshop can be divided into 3 stages: (1) Pre-activity exercises, (2) hands-on activities, and (3) post-activity exercises. During the pre-activity exercise, instructors provided students with a brief background on orthopaedic tissues and the techniques they would learn during the hands-on activities. For the hands-on activities, students were divided into four groups for circuit-style, hands-on breakout sessions. Each group consisted of 3-4 students that completed each of the following activities: (1) biomechanical testing of healthy and diseased rat tendons, (2) microcomputed tomography analysis of healthy and diseased rat bones, (3) culture of mouse bone marrow mesenchymal stem cells, and (4) histological assessment of intervertebral discs and tendons from humans, rabbits, and rats. Groups worked with Learning on a Limb instructors to complete each activity, spending approximately 25 min per activity. After completing the hands-on activities, students participated in a post-activity discussion about what they learned and a speed-networking discussion with diverse members of the Learning on a Limb Team. The workshop was evaluated using pre/post-tests to assess learning gains and a post-survey to assess interest in orthopaedic and biomedical engineering research. The pre/post-test consisted of 8 multiple-choice questions (2 per activity). Average pre/post-test results were compared using paired Student's t-tests ($\alpha = 0.05$). This study was approved by the University of Pennsylvania Institutional Review Board.

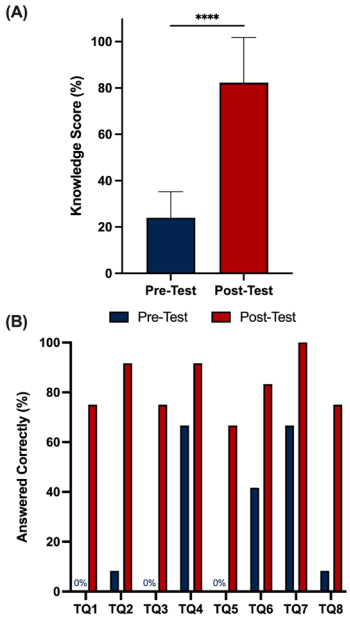

Figure 1. Students showed significant learning gains by pre/post-test. (A) *Average pre/post-test scores.* ***(B)*** *Percentage of students who answered individual test questions (TQs) correctly.* **** = $p<0.0001$.*

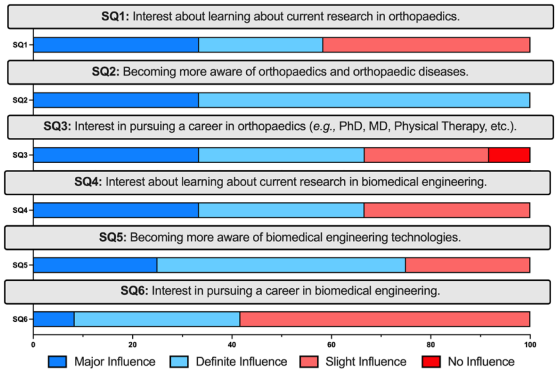

Figure 2. Post-survey data showing future interest in orthopaedics.

RESULTS: 12 high school students participated in our Learning on a Limb workshop. Most students identified as female (N=7, 58.33%). Additionally, students in our cohort identified as black (N=4, 33.33%), Latinx (N=1, 8.33%), Asian (N=6, 50%) and other (N=1, 8.33%). Thus, a large portion of the students in our cohort were considered gender and racial minorities in STEM. Results from our pre/post-test showed that students experienced significant learning gains from participating in Learning on a Limb. Specifically, the average score increased from 24.0% on the pre-test to 82.3% on the post-test (**Fig 1A**). In addition to increases in overall score, we found that the percentage of students who answered each test question (TQ) correct was greater in the post-test than the pre-test (**Fig 1B**). Our post-survey assessment demonstrated that Learning on a Limb also had a positive influence on students' interest in orthopaedic and biomedical engineering research (**Fig 2**). After completing the workshop, at least half of the students indicated that Learning on a Limb influenced their interest in learning about orthopaedics research (SQ1 & SQ2), pursuing careers in orthopaedics (SQ3), and learning about biomedical engineering (SQ4 and SQ5). Less than half of the students' felt that Learning on a Limb influenced their interest in pursuing a career in biomedical engineering (SQ6), which was plausible since the goal of the workshop was orthopaedics and not necessarily biomedical engineering. Additional comments on the post-survey indicated that students had fun participating in all aspects of Learning on a Limb (*i.e.,* hands-on activities and conversations with the Learning on a Limb Team). Overall, our results demonstrate that completing this workshop was a fun and effective way to teach students about orthopaedics and spark their interest in orthopaedic research.

DISCUSSION: Orthopaedic research is necessary to combat the high economic and societal burden that musculoskeletal diseases place on the world. To continue advancing orthopaedic research, there is a need to teach diverse students about orthopedic research at a young age. Towards this goal, we designed, implemented, and evaluated Learning on a Limb, a half-day workshop to introduce high school students to the field of orthopaedics. We found that our workshop effectively taught principles of orthopaedics and sparked student interest in orthopaedics. Given the success of this workshop, we plan to conduct Learning on a Limb annually as a means of promoting diverse participation in orthopaedic research. We will also leverage our partnership with the PSOM Office of Outreach, Education, and Research to longitudinally track the students who participated in Learning on a Limb, as has been done in the field of reproductive health[5] and for women in orthopaedics through the Perry Initiative.[6] This long-term longitudinal tracking of students' college and career choices will allow us to more rigorously evaluate the long-term benefits students received from participating in Learning on a Limb.

SIGNIFICANCE: Developing engaging workshops for high school students is important for increasing diverse participation in orthopaedics. Learning on a Limb is an effective workshop, which orthopaedics department can use as a model to inspire the next generation of orthopaedic researchers.

REFERENCES: **[1]** Dieleman+ *JAMA* 2020, **[2]** Valantine+ *CBE Life Sci Edu* 2016, **[3]** Spencer+ *BMJ* 1999, **[4]** Assi+ *Trans ORS* 2023, **[5]** Castle+ *Biol Reprod* 2016, **[6]** Buckley+ *J Am Acad Orthop Surg* 2022

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Collagen Fibril Deformation is Not Observed in Developing Mouse Patellar Tendon regardless of Collagen XI Expression

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INTRODUCTION: The development of functional tendon requires proper collagen assembly in hierarchical structures [1]. As part of this process, collagen XI co-assembles with collagens I and II during heterotypic fibril formation and its disruption leads to abnormal extracellular matrix development [2]. Deficiency of collagen XI disturbs tendon structure, causing nuclear disorganization, increased lateral growth of fibrils, and degradation of mechanical properties [1,4]. However, how nanoscale mechanisms contribute to the weakened mechanical integrity of collagen XI deficient tendon is unknown. Therefore, the goal of this study was to elucidate the role of collagen XI in the fibril deformation mechanisms in developing mouse patellar tendon. We hypothesized that tendon-targeted knockout of collagen XI would result in increased deformation of collagen fibrils.

METHODS: Male and female wild-type control (WT), *Scx-Cre;Col11a1*$^{flox/wt}$ heterozygous (HET), and *Scx-Cre;Col11a1*$^{flox/flox}$ knockout (KO) mice at 30 days of age were used (n=7-8/group, IACUC approved). Tibia-patellar tendon-patella complexes were isolated, and the cross-sectional area of all samples was measured using a custom laser-scanning device. The tibia was embedded with polymethyl methacrylate (PMMA) and the patella was gripped with sandpaper and secured in a metal fixture. Tendons were preloaded to 0.025N and subjected to a testing protocol consisting of 10 cycles of preconditioning followed by a 1-minute hold. Based on loading curves obtained from preliminary quasi-static ramp-to-failure data using the abovementioned mouse models [4], tendons were strained at a rate of 0.1% strain/second to the toe region (1% strain for all genotypes) or linear region (8.9%, 6.2%, or 4.4% strain for WT, HET, or KO, respectively) of the stress-strain curves. Then, the samples were flash-frozen at the target strain, precisely cut free from the tibia and patella, and placed in a cryo-embedding medium. Subsequently, tendons were cryo-sectioned at 20µm thickness and fixed in formalin. To observe changes at the nanoscale, a Bioscope Catalyst Atomic Force Microscope was used in tapping mode on tendon sections to obtain topographical images of the sample surfaces. A minimum of five 2.0µm x 2.0µm images at a resolution of 512x512 pixels were acquired within 1mm of the tibial insertion, where tissue strains were expected to be the greatest [5], across 3 sections per sample. The length of the d-band periodicity of collagen fibrils was measured using Fourier analysis in MATLAB. Changes in the average d-period with applied tendon strain were taken as indicative of fibril deformation, and changes in the average variance in the d-period within individual images and across the entire sample were taken as indicative of local and global variation in fibril deformation, respectively [6]. Data was analyzed using two-way ANOVAs with main effects of genotype and applied strain (i.e., toe vs. linear regions). Significance was set at $p < 0.05$.

RESULTS: Qualitative analysis of the AFM images revealed the presence of a heterogeneous population of collagen fibrils with larger diameters in the HET and KO groups compared to WT controls (Fig. 1), showing evidence of the poorly regulated lateral growth of fibrils. Despite this difference, the average d-period length was not different across genotypes and did not change across strain levels (Fig. 2A). Similarly, local and global variance in the collagen fibril d-period were not affected by genotype or applied strain (Fig 2B, C).

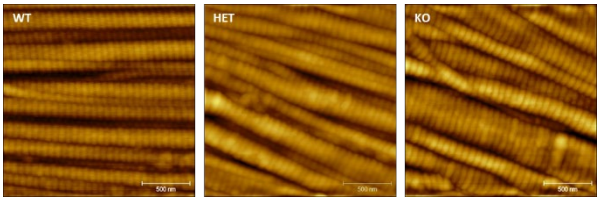

Figure 1. Representative AFM images of collagen fibrils of the three different genotype groups (Wild-type, heterozygous, and knockout group, respectively)

DISCUSSION: Tensile mechanical testing of tendons was performed to apply macroscale strains and subsequently measure the fibril deformation of collagen fibrils at the nanoscale. We found that tendon-targeted collagen XI knockout disrupted the nanoscale organization and increased the heterogeneity of fibril morphology. This abnormal fibril structure is consistent with prior work that used transmission electron microscopy to observe the collagen fibrillar matrix [1]. Based on previous studies, fibril deformation was expected when different levels of strain were applied to the tendon [6,7]. Contrary to our hypothesis, the fibril d-period did not increase over the applied strain range in the patellar tendon. This result may indicate differences in the nanoscale loading mechanism that occurs within the patellar tendon in comparison to other tendons, which could be due to the patellar tendon's unique structure with two bony attachments. Further work is needed to explain these surprising findings and investigate the role of collagen XI in the deformation mechanism of other tendons.

SIGNIFICANCE: Collagen XI regulates collagen fibrillogenesis and is essential during the development of tendons. This study shows that the deficiency or absence of collagen XI causes structural changes in collagen fibrils at the nanoscale and emphasizes its importance in the assembly of tendon hierarchical structure.

ACKNOWLEDGEMENTS: This study was supported by NIH/NIAMS (R01AR073231) and the Penn Center for Musculoskeletal Disorders (P30 AR069619)

REFERENCES: [1] Sun et al. *Matrix Biol* (2020). [2] Linsenmayer et al. *J Cell Biol* (1993). [3] Holmes et al. *Current Topics in Developmental Biology* (2018). [4] Cohen et al. *ORS* (2022). [5] Gilday et al. *J Biomech* (2013). [6] Connizzo BK et al. *J Biomech (*2014). [7] Rigozzi et al. *Journal of Structural Biology* (2011).

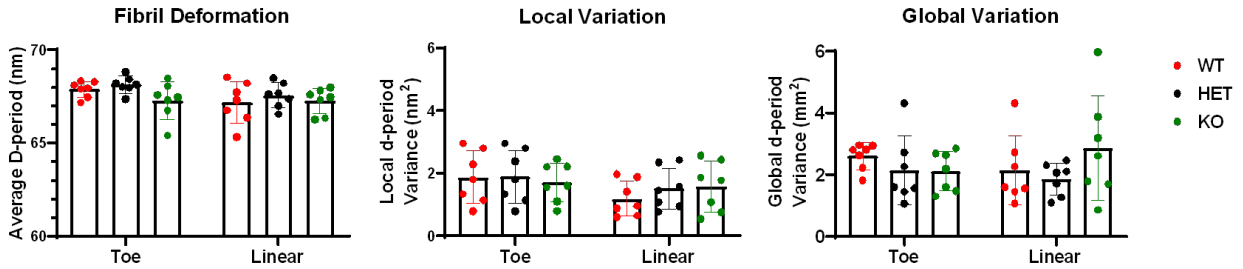

Figure 2. Fibril deformation (A), local variation (B), and global variation (C) were unaffected by genotype and applied strain. Data shown as mean ± SD.

Type III Collagen Expression Decreases During Neonatal Tendon Development and is Unchanged in Early Neonatal Tendon Healing

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INTRODUCTION: After tendon injury, fibrovascular scarring leads to inferior tendon function and high re-injury risk. Specifically, poor remodeling of the provisional, type III collagen (Col3)-rich matrix to a highly aligned, type I collagen (Col1)-rich matrix results in a disorganized and weak matrix throughout healing. Much like the early healing matrix in adult tendon, developing embryonic tendon contains high levels of Col3 [1]. However, the magnitude and timing of Col3 expression in the developing and healing neonatal tendon have not been elucidated and may provide crucial foundation for investigations of neonatal development and healing as potential mechanisms of superior tendon remodeling from a Col3- to Col1-rich matrix. Therefore, the objective of this study was to define the expression profile of the *Col3a1* gene throughout early neonatal development and healing. We hypothesized that *Col3a1* expression would be highest immediately post-partum and decrease throughout neonatal development. Additionally, we expected healing neonatal tendons to mount a quick and robust Col3 response with increased *Col3a1* expression during early healing timepoints.

METHODS: For investigations of neonatal development, thirty-five right knees from wild-type (WT) mice were harvested at postnatal days 0, 3, 7, 10, and 14 (p0, p3, p7, p10, p14; n ≥ 6/group mixed sex). For investigations of neonatal healing, twelve WT mice received right patellar tendon biopsy punch injury (0.3 mm diameter, performed under 10X magnification; Fig 1) at 7 days of age. Right knees were harvested at 3- and 7-days post-injury, corresponding to p10 and p14 of the mice, respectively (n = 6/group mixed sex). All studies were IACUC approved. For all groups, patella-patellar tendon-tibia complexes were fixed for 4 hours in 4% paraformaldehyde, dissected, and cryo-embedded. Tendons were sectioned coronally (40µm) and micro-dissected with a 25G needle to ensure proper isolation of the neonatal tendon for developmental ages (p0, p3, p7, p10, p14) or injured matrix for healing timepoints (3 days post-injury/p10, 7 days post-injury/p14). Dissected tendon tissue was digested, and RNA was isolated as described [2]. qPCR for *Col3a1* and *Abl1* (housekeeper) was performed. ΔCt values were calculated with reference to *Abl1* expression ($\Delta Ct = Ct_{Abl1} - Ct_{Col3a1}$). A one-way ANOVA was used to assess differences in *Col3a1* expression between developmental age and healing timepoints. Significance was set at $p < 0.05$.

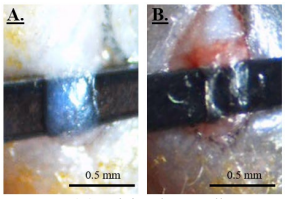

Figure 1: (A) Uninjured p7 patellar tendon. (B) p7 patellar tendon after biopsy punch (0.3 mm diameter) injury.

RESULTS: Supporting our hypothesis in the developing neonatal tendon, *Col3a1* expression was highest at p0 and decreased through p14, representing a 76% decrease in *Col3a1* expression throughout this period (Fig 2A). Interestingly, *Col3a1* expression was not increased with neonatal injury throughout early healing timepoints. *Col3a1* expression 3 and 7 days after injury was not different from the uninjured baseline at p7 (Fig 2B) or from *Col3a1* expression at corresponding, uninjured developmental timepoints (p10 and p14; Fig 2B).

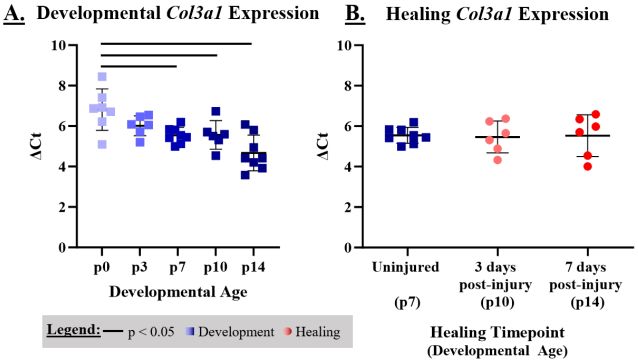

Figure 2: (A) Throughout postnatal development, *Col3a1* expression decreases. (B) After injury induced at p7, *Col3a1* expression is not increased 3 or 7 days after injury.

DISCUSSION: In this study, we defined the expression profile of the *Col3a1* gene throughout early neonatal development and healing to provide crucial foundation for investigations of neonatal development and healing as potential mechanisms of superior tendon remodeling.

Development is regarded as the ideal physiologic process for tendon matrix formation. Many regenerative approaches seek to recapitulate development, making the study of a key component of the developing tendon matrix, Col3, an important foundational step. *Col3a1* expression was previously known to be high *in utero*, and the current study is the first to measure the decrease in *Col3a1* expression in early neonatal development. Given the importance of temporally coordinated *Col3a1* expression in other developing, fibroblast-rich tissues [3], this *Col3a1* expression decrease may implicate Col3 in regulation of neonatal tendon development. Moreover, the temporal profile of *Col3a1* expression during neonatal development follows the same temporal profile of *Col3a1* expression during mature tendon healing [4] where expression is high after injury and decreases as healing progresses. Excitingly, this highlights commonalities between neonatal development and mature healing which may be leverageable in approaches which seek to improve mature healing through biomimicry of neonatal development. Further research is evaluating additional developmental timepoints to identify when homeostatic *Col3a1* expression is achieved.

Neonatal tendon healing is another model of improved tendon matrix formation as neonatal healing is superior in speed and quality [6, 7] to mature healing. Given the similarities between healing in neonatal and mature contexts, neonatal tendon healing has become a model for investigations of improved healing. Interestingly in the current study, neonatal injury did not affect overall *Col3a1* expression during early healing. This indicates a significant deviation from mechanisms of mature tendon healing where dramatically increased *Col3a1* expression is considered a hallmark of the healing response. Our previous investigations of mature mice (same C57/B6 strain) demonstrate increased *Col3a1* expression in early healing (Fig 3) [7, unpublished]. Given the improved healing observed in neonatal tendon, this finding may reveal potential for *Col3a1* modulation as a therapeutic method for improved tendon healing. Additional earlier and later healing timepoints are being explored to understand the complete temporal profile of *Col3a1* expression after neonatal tendon injury. Moreover, immunostaining for Col3a1 will be completed for all developmental and healing timepoints to evaluate protein translation to add to the gene expression data in the current study.

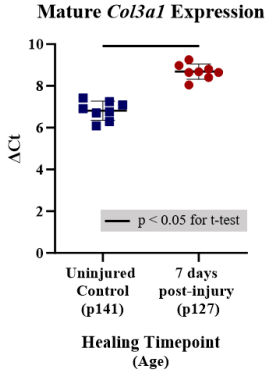

Figure 3: *Col3a1* expression during early tendon healing increases in mature mice [7, unpublished].

SIGNIFICANCE/CLINICAL RELEVANCE: Understanding temporal and mechanistic dynamics of neonatal tendon development and healing may highlight novel targets for improving tendon healing through regenerative approaches.

REFERENCES: [1] Birk et al, Eur J Cell Biol, 1997. [2] Leiphart et al, ORS, 2022. [3] Niederreither et al, Matrix Biol, 1995. [4] Dyment et al, PLoS ONE, 2013. [5] Ansorge, JOR, 2012. [6] Howell, Sci Rep, 2012. [7] Leahy et al, JOR, 2023.

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High-Speed Treadmill Running Does Not Induce a Tendinopathic Phenotype in Rat Achilles Tendon

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INTRODUCTION: Achilles tendon pathology comprises an increasing and consequential clinical burden,[1,2] but robust and reproducible preclinical animal models of Achilles tendinopathy are lacking. Overuse is a common etiology of tendon pathology, and exercise-induced overuse has been considered a promising mechanism for creating a clinically relevant tendinopathy model. In rat Achilles tendon, treadmill running at moderate speed (17-20 m/min) results in variable structural and functional outcomes,[3-7] failing to induce a consistent tendinopathy phenotype. Effects of running at higher speeds (> 25 m/min) on Achilles tendon structure and function have not been thoroughly investigated, though early results have shown potential for a tendinopathic phenotype.[7,8] Therefore, the objective of this study was to rigorously assess the structural and biomechanical impacts of high-speed treadmill running on rat Achilles tendon. We hypothesized that 16 weeks of high-speed treadmill running would induce an overuse tendinopathy phenotype characterized by matrix disorganization, rounded cell morphology, and reduced tensile mechanical properties.

METHODS: Sprague-Dawley rats (~400 g) were randomized into two groups: cage activity (n = 12) and running (n = 9). The running group underwent a 3-week acclimation protocol followed by 16 weeks of high-speed treadmill running (27 m/min, 10° incline, 1 hour/day, 5 days/week); mild electrical shock was used at the back of the treadmill to encourage running. After 16 weeks, Achilles tendons were harvested bilaterally for histological and mechanical assessment. For histology, ankles were prepared for paraffin histology with standard techniques,[9] sectioned sagittally (7 µm thickness), stained serially with DRAQ5™ (abcam, Waltham, MA, USA) then 0.1 % toluidine blue, and imaged (10X magnification). Midsubstance regions (~1.3 x 0.65 mm) from two sections per tendon were analyzed (CellProfiler™ [10]) for cell count and nuclear shape. Tendons designated for mechanical testing were first µCT imaged (10 µm resolution, µCT35, Scanco Medical, Brüttisellen, Switzerland) to identify heterotopic ossification (HO). Images were segmented and HO volume was quantified with Amira 6.7 (Thermo Fisher Scientific, Waltham, MA). After scanning, tendons were prepared[9] and tested with a viscoelastic testing protocol (preconditioning; stress relaxation at 9% strain; sinusoidal frequency sweeps at 0.1, 1, 5, and 10 Hz) followed by a quasi-static ramp (0.3% strain/s) to failure with image capture for optical strain measurement. Digital image correlation software (Vic2D, Correlated Solutions, Irmo, SC) was used to determine strain distributions along the length of the tendon at the transition point, mid-linear region (2 x transition strain), and failure. T-tests were used to compare histological and mechanical properties between cage activity and running groups, and 2-way repeated measures ANOVAs with Šídák's multiple comparison tests were used to assess differences in regional strain and modulus between activity groups. Significance was set at $p < 0.05$.

RESULTS: All histology samples demonstrated varying amounts of discrete pockets of matrix disorganization, increased staining intensity, and rounded cell morphology, demonstrating an HO phenotype (Fig. 1A). In regions of interest, chosen to exclude regions of suspected HO, cell density and nuclear shape were unaffected by treadmill running (Fig. 1B-D). In contralateral limbs, we consistently detected the presence of HO on µCT, though HO volume (Fig. 2) and mineral density (data not shown) were unaffected by activity level. While running was associated with a decrease in cross-sectional area (CSA, $p = 0.04$), when normalized to body weight, runners demonstrated increased normalized CSA ($p = 0.02$). Despite this, no differences were detected between groups in viscoelastic (relaxation at 9% strain, dynamic modulus, phase shift) or elastic (stiffness, modulus) mechanical properties (Fig. 3A-F). Local strain and modulus varied along the tendon length as expected ($p < 0.05$) but were unaffected by activity group (data not shown).

DISCUSSION: Contrary to our hypothesis, 16 weeks of high-speed treadmill running did not induce an overuse tendinopathy phenotype. While rat Achilles tendon is a well-established model for investigations of HO,[11] previous studies of impacts of treadmill running on rat Achilles tendon have not considered potential impacts of HO on tendon structure and biomechanics. We speculate that the high incidence of HO may impact the consistency of both histological and mechanical findings from previous rat Achilles tendon tendinopathy models. Future methods for inducing Achilles tendinopathy should consider alternative approaches to achieve a reproducible phenotype.

SIGNIFICANCE: High-speed treadmill running for 16 weeks did not induce tendinopathic overuse in rat Achilles tendon based on histologic, structural, and mechanical assessments.

REFERENCES: [1] Longo, Sports Med Arthrosc Rev, 2009. [2] Lanto, Scand J Med Sci Sports, 2015. [3] Huang, Ann Biomed Eng, 2004. [4] Glazebrook, JOR, 2008. [5] Abraham, BMC MSK Disord, 2011. [6] Heinemeier, J Appl Physiol, 2012. [7] Xu, Exp Ther Med, 2018. [8] Xu, Med Sci Monit, 2016. [9] Leahy, AJSM, 2022. [10] Stirling, BMC Bioinform, 2021. [11] Pierantoni, FASEB J, 2023.

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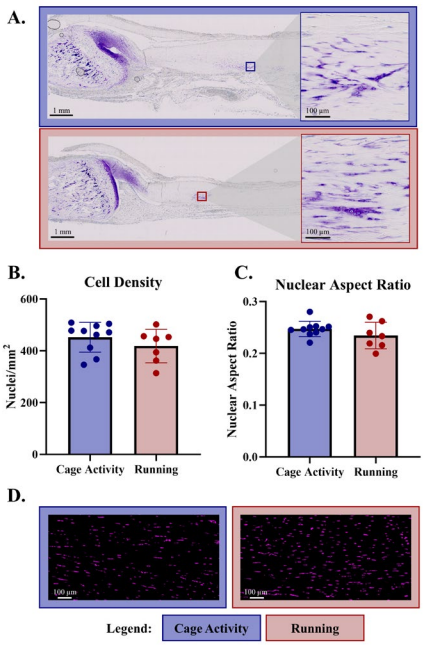

Figure 1: Both cage activity (blue) and running (red) tendons contain discrete regions of disorganization with rounded cells, indicative of HO (A). Running did not impact cell density (B) or nuclear aspect ratio (C) in the midsubstance (representative images shown in D).

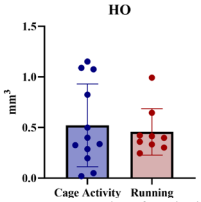

Figure 2: Tendons from both activity groups demonstrated HO by µCT. Running did not influence HO volume.

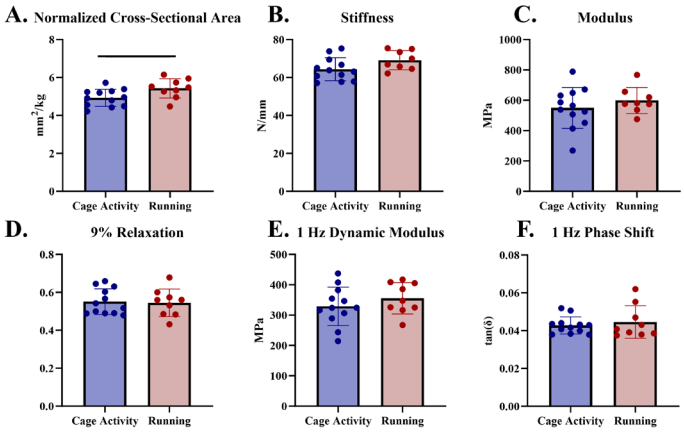

Figure 3: Running increased Achilles tendon CSA normalized to body weight (A). Neither stiffness (B) nor optical modulus (C) were influenced by treadmill running. Similarly, percent relaxation (D), dynamic modulus (E), and phase shift (F) were unaffected by treadmill running (data shown for 1 Hz, consistent across frequencies).

Collagens V and XI Jointly Regulate Fibril Assembly and Elastic Mechanical Properties during Tendon Maturation

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Disclosures: None

INTRODUCTION: Tendon hierarchical structure is established during development through the coordinated assembly of matrix proteins, including minor fibril-forming collagens such a collagens V and XI. Collagen V influences collagen fibrillogenesis through nucleating fibril formation and co-assembling with collagens I and II[1], and lack of *Col5a1* expression leads to larger fibrils, reduced fibril density, and smaller tendon cross-sectional area[2]. Collagen XI has a similar role in fibril regulation during development[3] and co-assembles with collagen V to form heterotypic fibrils[1]. The expression of genes for collagen V and XI is similar in developing tendons, but the expression of collagen XI encoding genes is decreased in mature tendons compared to collagen V genes. Moreover, in global knockdown mouse models, haploinsufficiency of both *Col5a1* and *Col11a1* in tandem yielded more irregular fibril shapes and greater heterogeneity of fibril diameters in developing tendons than *Col5a1* haploinsufficiency alone[1]. Together, these findings suggest interactive roles between collagens V and XI during development. However, the structural and functional deficits associated with coordinated knockdown of *Col5a1* and *Col11a1* remain unknown. Since the tendon-specific compound *Col5a1, Col11a1* knockout is postnatally unviable, the objective of this work was to assess the cooperative roles of collagens V and XI during fibril growth and assembly using a tendon-specific (ScxCre) compound *Col5a1* null, *Col11a1* heterozygous mouse model. Based on prior work in tendons lacking *Col5a1* expression, we hypothesized that ScxCre;$Col5a1^{flox/flox}$;$Col11a1^{flox/+}$ (VKO-XIHet) tendons would demonstrate structural changes consistent with aberrant fibril growth.

METHODS: Animals: Male and female postnatal day 30 VKO-XIHet mice (n=10) and ScxCre- littermate controls (Ctrl, n=10) were used (IACUC approved). Transmission Electron Microscopy: Immediately after sacrifice, Achilles tendons (ATs) (n=4/genotype) were isolated, fixed, embedded, sectioned, stained, and imaged as described[4]. Fibril diameters were measured using a custom MATLAB script (n=10 images/sample). Mechanics: AT-calcaneus complexes were harvested, finely dissected, and cross-sectional area was measured using a custom laser device. The free end of the tendon was secured in sandpaper with cyanoacrylate glue, and the calcaneus and sandpaper were gripped in custom fixtures. Tendons were tested in a PBS bath at 37°C using a protocol of preloading to 0.03N, preconditioning for 10 cycles, stress relaxations at 3% and 5% strain, and quasistatic ramp-to-failure at 0.1% strain/sec (Instron 5848). Each stress relaxation was followed by a frequency sweep of 10 cycles at 0.1, 1, 5, and 10 Hz. Statistics: Fibril diameter distributions were compared between genotypes using a Kolmogorov-Smirnov test. Cross-sectional area and mechanical properties were compared across genotypes using a two-sample t-test. Significance was set at $p \leq 0.05$, and all data visualization and statistics were conducted in R (v4.3.1).

RESULTS: VKO-XIHet ATs demonstrated substantial changes in fibril structure and mechanical properties. The collagen fibril distribution in VKO-XIHet tendons was different than Ctrl with a distinct population of larger (>175 nm) fibrils (**Fig 1A**). While fibrils in Ctrl tendons had circular cross-sections, many fibrils in VKO-XIHet tendons had irregularly shaped cross-sections with these irregularities most apparent and severe in the population of larger fibrils (**Fig 1B-C**). Despite larger fibril diameters, overall tendon cross-sectional area was smaller in VKO-XIHet tendons (**Fig 2A**). Maximum load, stiffness, and maximum stress were also lower in VKO-XIHet tendons compared to WT **(Fig 2B-D)**. Viscoelastic properties showed minimal differences between genotypes (data not shown).

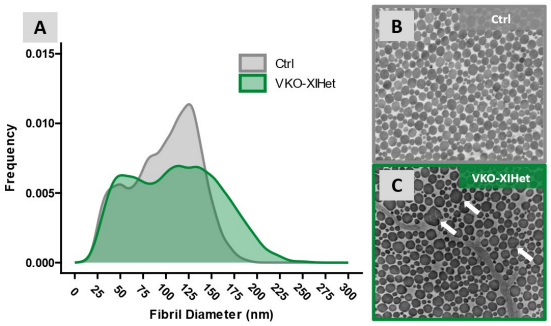

Figure 2: (A) VKO-XIHet fibril distributions demonstrate statistical differences with a population of larger diameter fibrils. Fibril distributions were compared using a Kolmogorov-Smirnov test. (B-C) Fibril boundaries are irregularly shaped in VKO-XIHet tendons (white arrows), especially in larger fibrils.

DISCUSSION: We studied the combined roles of collagens V and XI in establishing structural and mechanical properties of the AT during postnatal growth. Supporting our hypothesis, VKO-XIHet tendons showed fibril-level structural and tissue-level mechanical changes consistent with altered fibril assembly. The shift towards larger diameter fibrils and irregularity of fibril boundaries in VKO-XIHet tendons suggest that these collagen types work in concert to regulate lateral growth of fibrils. This finding is consistent with previous work where the absence of *Col5a1* expression led to larger fibril dimeters[3,5] and irregular fibril boundaries[5]. Additionally, we previously found a 39% decrease in maximum load and a 19% decrease in maximum stress[2] in post-natal day 60 ScxCre;$Col5a1^{flox/flox}$ ATs. In comparison, the post-natal day 30 ScxCre;$Col5a1^{floxflox}$;$Col11a1^{flox/+}$ tendons in this study showed 75% and 45% decreases in the same parameters, respectively. These markedly reduced mechanical properties coupled with increased lateral growth in a sizable portion of fibrils demonstrate that ablation of 1 allele of *Col11a1* in addition to both alleles of *Col5a1* further exacerbates the phenotype during tendon development. Future work will focus on delineating possible compensatory mechanisms between collagens V and XI and understanding interactions at early stages of development.

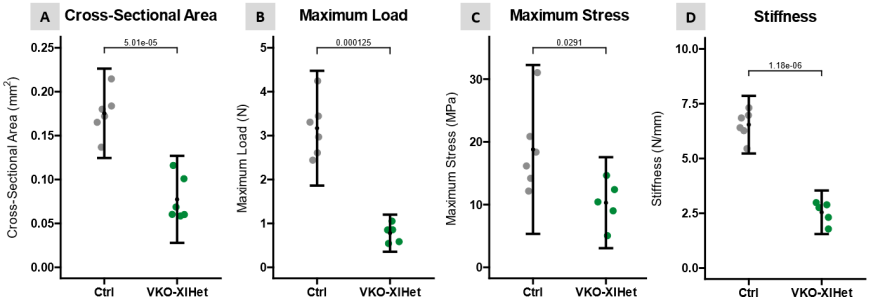

Figure 1: Cross-sectional area (A), maximum load (B), stiffness (C), and maximum stress (D) were significantly decreased in VKO-XIHet tendons. Properties were compared between genotypes using t-tests; p-values are listed above significance bars. Data shown as means ± SD.

SIGNIFICANCE: Collagens V and XI have known roles in fibrillogenesis and the acquisition of tendon structure during development. Due to their coordinated roles and structural similarities, defining the interactions between collagens V and XI in tendon is essential to understanding mechanisms underlying collagen fibril formation.

REFERENCES: 1. Wenstrup et al., J Biol Chem, 2011. 2. Connizzo et al., J Orthop Res, 2015. 3. Sun et al., Matrix Biol, 2020. 4. Dunkman et al., Matrix Biol, 2014. 5. Connizzo et al., J Orthop Res, 2016.

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