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Collagen XI regulates the acquisition of collagen fibril structure, organization and functional properties in tendon

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Abstract

Collagen XI is a fibril-forming collagen that regulates collagen fibrillogenesis. Collagen XI is normally associated with collagen II-containing tissues such as cartilage, but it also is expressed broadly during development in collagen I-containing tissues, including tendons. The goals of this study are to define the roles of collagen XI in regulation of tendon fibrillar structure and the relationship to function. A conditional *Col11a1*-null mouse model was created to permit the spatial and temporal manipulation of *Col11a1* expression. We hypothesize that collagen XI functions to regulate fibril assembly, organization and, therefore, tendon function. Previous work using *cho* mice with ablated *Col11a1* alleles supported roles for collagen XI in tendon fibril assembly. Homozygous *cho/cho* mice have a perinatal lethal phenotype that limited the studies. To circumvent this, a conditional $Col11a1^{flox/flox}$ mouse model was created where exon 3 was flanked with *loxP* sites. Breeding with Scleraxis-Cre (*Scx-Cre*) mice yielded a tendon-specific *Col11a1*-null mouse line, $Col11a1^{\Delta ten/\Delta ten}$. $Col11a1^{flox/flox}$ mice had no phenotype compared to wild type C57BL/6 mice and other control mice, e.g., $Col11a1^{flox/flox}$ and *Scx-Cre*. $Col11a1^{flox/flox}$ mice expressed *Col11a1* mRNA at levels comparable to wild type and *Scx-Cre* mice. In contrast, in $Col11a1^{\Delta ten/\Delta ten}$ mice, *Col11a1* mRNA expression decreased to baseline in flexor digitorum longus tendons (FDL). Collagen XI protein expression was absent in $Col11a1^{\Delta ten/\Delta ten}$ FDLs, and at ~50% in $Col11a1^{+/\Delta ten}$ compared to controls. Phenotypically, $Col11a1^{\Delta ten/\Delta ten}$ mice had significantly decreased body weights ($p < 0.001$), grip strengths ($p < 0.001$), and with age developed gait impairment becoming hypomobile. In the absence of *Col11a1*, the tendon collagen fibrillar matrix was abnormal when analyzed using transmission electron microscopy. Reducing *Col11a1* and, therefore collagen XI content, resulted in abnormal fibril structure, loss of normal

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Author contributions

M.S., L.J.S. and D.E.B. designed and interpreted this work. M.S. was involved in all experiments, E.L. was involved in characterization of the mouse model. S.M.A. and T.A. performed TEM analysis. Y.Y, and S.S performed biomechanics analysis. All authors contributed to the data analysis, as well as preparation of figures and the manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

Supplementary materials

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fibril diameter control with a significant shift to small diameters and disrupted parallel alignment of fibrils. These alterations in matrix structure were observed in developing (day 4), maturing (day 30) and mature (day 60) mice. Altering the time of knockdown using inducible I-*Col11a1*$^{-/-}$ mice indicated that the primary regulatory foci for collagen XI was in development. In mature *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs a significant decrease in the biomechanical properties was observed. The decrease in maximum stress and modulus suggest that fundamental differences in the material properties in the absence of *Col11a1* expression underlie the mechanical deficiencies. These data demonstrate an essential role for collagen XI in regulation of tendon fibril assembly and organization occurring primarily during development.

Keywords

Collagen XI; *Col11a1*; Tendon; Collagen fibrillogenesis; Tendon structure; Tendon biomechanics; Conditional mouse model

Introduction

The hierarchical assembly of collagen into connective tissues during development, growth and maturation determines tissue structure and function [1–3]. Disruption of this assembly results in tissue dysfunction [4] and often underlies connective tissue diseases [5,6]. In addition, mechanical influences such as tension on fibrils has been shown to influence degradation with varying effects at different hierarchal levels [7].

Tendon structure and function is dependent upon the regulated assembly of collagen I into fibrils and higher order structures. Collagen I is the major fibril-forming collagen in tendons and ligaments [1–3]. The regulatory fibril-forming collagens XI and V are essential in the regulation of initial fibril assembly and fiber organization, despite being quantitatively minor components in tendons [1,3]. Collagen XI is expressed during development, but is virtually absent in mature tendons [8] In contrast, collagen V is expressed throughout development and maturation, as well as in mature tendon [8]. Mutations in *COL11A1* result in Stickler syndrome type II [9] while mutations in collagen V define classic Ehlers Danlos Syndrome (EDS) [10]. Both diseases are generalized connective tissue disorders with broad tissue involvement and patients with these disorders have abnormal regulation of collagen fibril assembly. Both clinical phenotypes include joint laxity involving tendons and ligaments [11]. This supports roles for these collagens in establishing tendon structure and function. *COL11A1* and *COL11A2* genes have been linked to tendinopathy [12,13] supporting critical roles for this collagen in tendon biology.

Collagens V and XI are fibril-forming collagens, but they form a subclass, termed regulatory fibril-forming collagens [1,14,15]. These collagens are very homologous and can be considered different molecular forms of a single collagen type [1,16–18]. The major isoforms in tendon, are $[\alpha 1(V)]_2\alpha 2(V)$ and $\alpha 1(XI)\alpha 2(XI)\alpha 3(XI)$ [8]. Collagen V has multiple isoforms including: $[\alpha 1(V)]_2\alpha 2(V)$, $[\alpha 1(V)]_3$, and $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ while collagen XI has a single isoform $\alpha 1(XI)\alpha 2(XI)\alpha 3(XI)$ [16]. However, hybrid molecules containing both collagen V and XI gene products, such as $[\alpha 1(XI)]_2\alpha 2(V)$, and $\alpha 1(XI)\alpha 2(V)\alpha 3(XI)$ have been described [18–20]. Additional variation results from

alternative splicing. The N-terminus of the α1 (XI) chain of collagen XI undergoes alternative splicing resulting in multiple variants [18,21,22]. The alternatively spliced forms differ in the acidic or basic domains that may alter interactions with other components of the extracellular matrix resulting in tissue-specific functions [23]. For example, in the embryonic tendon a basic exon is preferentially utilized that may influence interactions with extracellular matrix proteins including small leucine rich proteoglycans [21].

These collagens are characterized by partial processing of their N-propeptide domain. The N-terminal domain is composed of a variable domain and a proline/arginine rich protein domain. Processing removes the proline/arginine rich domain, but retains a hinge region, short collagenous (COL2) domain, and variable domains [16,24]. These regulatory fibril-forming collagens co-assemble with collagens I and II as heterotypic fibrils with the N-terminal regulatory domain on the fibril surface [1,25,26]. Assembly of heterotypic fibrils is closely associated with cell surfaces, and interactions with tenocytes would permit cellular control of fibril deposition and organization [27].

Collagen XI is not generally considered a component of the tendon extracellular matrix. Collagens XI and V are generally associated with collagen II- and I-containing tissues respectively [15]. However, collagen XI is broadly localized during development in a variety of tissues including tendons, [28–30] while collagen V also is found in cartilage and other collagen II-containing tissues [20,31]. In tissues primarily containing collagen I, collagen V has been shown to be a critical regulator of fibrillogenesis; determining fibril structure, fiber organization and functional properties in a variety of tissues including tendons and ligaments [8,32–34] as well as skin [35–37] and cornea [38,39]. Collagen XI has comparable regulatory roles in regulating the assembly of collagen II-containing fibrils in cartilage [26,40]. Our previous analyses in the tendon provided unexpected results suggesting that regulation of tendon fibril nucleation and initial assembly involved coordinate interactions of both collagens V and XI. In addition, collagen XI was shown to interact with collagen I to regulate fibril assembly in vitro [17]. These studies strongly support regulatory roles for collagen XI in tendon.

Our previous work demonstrated a role for collagen XI in the tendon [8]. A transcript analysis demonstrated expression of *Col5a1, Col5a2, Col11a1, Col11a2 and Col11a3* in the tendon. In addition, the α1(V) and α1(XI) chains were present. These data suggested that the ubiquitous forms of collagen XI, α1(XI)α2(XI)α3(XI) and collagen V, $[\alpha 1(V)]_2\alpha 2(V)$ were both expressed during tendon development and maturation. However, there was a differential expression of collagens XI and V. The analyses indicated comparable expression of the collagen V and XI genes during development and early post-natal stages. In mature tendons, collagen XI expression was virtually absent while collagen V expression was decreased, but constant. Immuno-localization in postnatal tendons demonstrated that collagens V and XI were not co-localized suggesting independent regulatory functions rather than redundant functions. Hybrid collagen V/XI molecules were not were not supported with a lack of co-localization. Studies utilizing mouse models where collagen V and/or XI expression was manipulated during development suggested a key role for collagen XI in establishing tendon structure. In addition, synergistic roles are supported by the analyses [8]. However, these previous studies were limited by the perinatal lethal phenotype in the

collagen XI-null (cho/cho) mice [41]. To address this limitation, we have developed a conditional *Col11a1*-null mouse model and targeted it to the tendon using Scleraxis-Cre (*Col11a1*$^{\Delta ten/\Delta ten}$). Using this novel model where *Col11a1* was knocked out in tendons supports an essential role for collagen XI in the regulation of early fibril assembly and fiber organization in tendons processes that are critical in the acquisition of mechanical function.

Results

Generation of a tendon-targeted *Col11a1* conditional knockout mouse model

To analyze the role(s) of collagen XI in establishing and maintaining tendon structure and function, a tendon-targeted *Col11a1* conditional knockout mouse model was created. *Col11a1* was chosen since its knockout would preclude the formation of the collagen XI trimer (α1(XI)α2(XI)α3(XI)). In addition, the 2 known collagen V/XI hybrid molecules ([α1(XI)]$_2\alpha$2(V), and α1(XI)α2(V)α3(XI)) both contain the α1(XI) chain. Therefore, the knockout of *Col11a1* would preclude the assemble of these molecular forms as well. The α1(XI) chain has 3 major alternatively spliced forms and several minor ones. These alternatively spliced variants involve the N terminal domain that is retained after processing. Exon 3 is present in all the splice variants. Therefore, targeting exon 3 ensures the knockout of α1(XI) and precludes transcription/translation of an alternatively spliced α1(XI) isoform. Deletion of exon 3 results in a skip from exon 2 to exon 4 where the resulting transcript is out of frame. This transcript codes for a 102 amino acid peptide before the first of several stop codons. This peptide that includes the signal sequence coded by exons 1 and 2 is susceptible to nonsense mediated decay with no expression of the *Col11a1* transcript. This strategy does not allow for the targeting of a specific collagen XI isoform, but rather will knock out all collagen XI isoforms.

The strategy utilized was similar to that in our published work (Fig. 1) [34]. Targeted ES cells were obtained after electroporation of the *Col11a1* targeting vector into mouse embryonic stem (ES) cells. ES cells containing the targeted *Col11a1* allele were identified by the absence of the negative selection marker DTA, the presence of LacZ, and orientation was confirmed by 3' and 5' gene and vector specific sequence using short-range and long-range PCR (Fig. 2A). The successfully targeted ES cells were then injected into wild type blastocysts, resulting in chimeric mice. The chimeric mice were backcrossed with wild type mice to produce the germline transmitted mice with the targeted allele *Col11a1*$^{+/ta}$. Breeding *Col11a1*$^{+/ta}$ mice with FLPe mice resulted in the conditional knockout mice *Col11a1*$^{flox/flox}$, where the FRT flanked neo sequences were excised. Mice at each different stage were characterized using PCR that amplified specific element sequences (Fig. 2B). This demonstrated that a conditional *Col11a1*$^{flox/flox}$ knockout was obtained.

To target the knockout to tendons the *Col11a1*$^{flox/flox}$ mice were bred with *Scx-Cre* mice. Scleraxis is widely expressed in early embryonic development, but with maturation becomes specific for tendons and ligaments. The promoter region utilized to drive Cre expression has been shown to be specific for tendons and ligaments [42–44]. In our previous work using double reporter mice, it was demonstrated that Cre excision occurred only in tendons and ligaments with other limb tissue showing no excision [39]. Genotyping analysis of these mice was carried out using Cre primers and specific primers at the junction of the 3' arm and

targeting sequence. The excision of the *Col11a1* exon 3 was identified in tendon-targeted *Col11a1* conditional knockout $Col11a1^{+/\Delta ten}$ and $Col11a1^{\Delta ten/\Delta ten}$ mice, but as expected, not in parental *Scx-Cre* mice or $Col11a1^{flox/flox}$ mice (Fig. 2C). The results confirmed a knockout of *Col11a1* expression in the FDL tendon.

Col11a1 gene and protein expression is knocked out in FDLs of $Col11a1^{\Delta ten/\Delta ten}$ mice

The tendon-targeted FDLs were *Col11a1* null after *Scx-Cre*- mediated exon 3 excision. Since in tendon *Col11a1* is only highly expressed in development and perinatal periods, FDLs from day 14 mice were analyzed using qPCR (Fig. 3A). Expression of *Col11a1* mRNA in control, wild type and conditional control mice was compared to heterozygous ($Col11a1^{+/\Delta ten}$) and homozygous $Col11a1^{\Delta ten/\Delta ten}$ mice using qPCR with primers from the C terminal non-helical region. Expression of *Col11a1* was not different in the 2 control groups. In contrast, the expression was at baseline in the $Col11a1^{\Delta ten/\Delta ten}$ mice. Compared to the control group, expression of *Col11a1* mRNA was reduced by approximately 50% in the heterozygous mice, $Col11a1^{+/\Delta ten}$ mice (Fig. 3A). Primers from the helical region gave comparable results (data not shown).

The α1 chain of collagen XI in tendon-targeted *Col11a1* knockout mice was analyzed immunochemically. The α1(XI) chain was analyzed in day 4 FDL tendons using a WES simple western blotting system. The α1(XI) chain was present at comparable levels in the control group; wild type, *Scx-Cre* and $Col11a1^{flox/flox}$ mice. In contrast, the α1(XI) chain was present at lower levels in $Col11a1^{+/\Delta ten}$ mice and not detectable in $Col11a1^{\Delta ten/\Delta ten}$ mice (Fig. 3B). Taken together the gene and protein expression data show a knockout of the α1(XI) chain in the $Col11a1^{\Delta ten/\Delta ten}$ mice. Since the α1(XI) chain is present in the collagen XI trimer and collagen V/XI hybrid molecules, the data support a knockout of collagen XI and its isoforms.

Altered fibril structure and organization in $Col11a1^{\Delta ten/\Delta ten}$ FDLs

FDLs from maturing male mice at day 30 were analyzed using transmission electron microscopy in control tendons with normal *Col11a1 expression* and $Col11a1^{+/\Delta ten}$ AS as well as $Col11a1^{\Delta ten/\Delta ten}$ tendons with reduced and no expression respectively (Fig. 4). The control FDLs contained well organized uniaxial fibrils with heterogeneous fibril diameters. In longitudinal sections fibrils were linear with parallel fibril alignment. In transverse sections, the fibrils had regular, roughly circular cross-sectional profiles with a broad distribution of sizes (Fig. 4A,D). In contrast, $Col11a1^{\Delta ten/\Delta ten}$ FDLs lacked linearity and parallel fibril alignment in longitudinal sections. In cross sections the contrast with the control tendons was striking. The regular fibril packing seen in the control was lost. There was a substantially larger percentage of smaller, heterogeneous fibrils compared to the control tendons. In addition, the fibrils assembled in the absence of *Col11a1* expression displayed aberrant fibril structures with structurally abnormal fibrils with irregular fibril profiles in the cross sections (Fig. 4C,F). There was a virtual absence of larger fibrils characteristic of the mature FDL in the absence of *Col11a1* expression. In $Col11a1^{+/\Delta ten}$ FDLs, with reduced collagen *Col11a1* expression, the fibril phenotype was mild. There was some disruption of longitudinal organization and in cross section irregular fibril profiles were common (Fig. 4B,E). However, fibril structure and organization were more comparable

to the control tendons than the *Col11a1*$^{\Delta ten/\Delta ten}$ tendons. In all genotypes, cross sections of large diameter fibrils often displayed surface bumps consistent with lateral fusion of small diameter fibrils. However, *Col11a1*$^{\Delta ten/\Delta ten}$ tendons showed evidence of poorly regulated lateral fibril fusion in both longitudinal and cross sections. A quantitative analysis of fibril diameter distributions was performed on the 3 genotypes (Fig. 4G–I). Both the *Col11a1*$^{+/\Delta ten}$ and *Col11a1*$^{\Delta ten/\Delta ten}$ distributions are significantly different from the control distribution and from each other (p<0.0001, Kolmogorov-Smimov Test). In the control and *Col11a1*$^{+/\Delta ten}$ while statistically different, the fibril distributions are very comparable. Both are broad multimodal distributions with a well-defined subpopulation of small diameter fibrils. The haploinsufficient distribution has a larger representation of larger diameter fibrils. In contrast, the *Col11a1*$^{\Delta ten/\Delta ten}$ distribution is strikingly different from the other 2 genotypes. The small diameter fibril distribution is over-represented with greater than 75 percent of the fibril diameters under 100 nm compared to less than 50 percent in the other distributions. The rest of the distribution is poorly developed suggesting an aberrant regulation of lateral fibril growth in the absence of *Col11a1* expression. The data are consistent with dose dependent regulation of fibril structure and organization in the tendon. Comparable results were observed in female 30 day FDLs (Supplemental Fig. 4).

Phenotype of tendon-targeted *Col11a1*$^{\Delta ten/\Delta ten}$ mouse models

The *Col11a1*$^{\Delta ten/\Delta ten}$ mice were viable and fertile. However, they were not optimal breeders. This may be due to the stress and difficulty accessing food and water because of impaired mobility. The *Col11a1*$^{\Delta ten/\Delta ten}$ mice were reluctant to move around their cages. In addition, there was excessive joint laxity in the *Col11a1*$^{\Delta ten/\Delta ten}$ mice compared with controls. The *Col11a1*$^{\Delta ten/\Delta ten}$ mice were smaller than control mice. The average body weight of male day 60 *Col11a1*$^{\Delta ten/\Delta ten}$ mice was significantly smaller (p<0.0001) than that of the control, *Col11a1*$^{flox/flox}$ mice. The mean body weights were 27.72 ± 1.40g (n=9) and 24.22 ± 1.14 g (n=15) for control and *Col11a1*$^{\Delta ten/\Delta ten}$ mice, respectively. As the *Col11a1*$^{\Delta ten/\Delta ten}$ mice aged they developed gait impairment seen as dragging of their hind limbs and becoming hypomobile. Euthanasia had to be performed at this stage. To evaluate musculoskeletal and motor function in the *Col11a1*$^{\Delta ten/\Delta ten}$ mouse model, grip strength tests were conducted on the fore-limbs of day 60 male *Col11a1*$^{\Delta ten/\Delta ten}$ mice and *Col11a1*$^{flox/flox}$ control mice. Significant impairment of function was observed with the *Col11a1*$^{\Delta ten/\Delta ten}$ mice being weaker than the control mice (Fig. 5). The mean grip strengths were significantly different (p<0.0001). The value for control mice was 0.998 ± 0.165N, n=9 (0.224 ± 0.037 lb), and 0.664 ± 0.042N, n=9 (0.149 ± 0.009 lb) in the *Col11a1*$^{\Delta ten/\Delta ten}$ mice.

Reduction in biomechanical properties in *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs

The FDL tendons from *Col11a1*$^{\Delta ten/\Delta ten}$ day 60 male mice demonstrated significant alterations in their biomechanical properties. The *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs were smaller and weaker compared to the control *Col11a1*$^{flox/flox}$ FDLs. A significant decrease in cross-sectional area was observed compared to the control tendons (Fig. 6A). Maximum load and maximum stress were both significantly reduced in the *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs compared to controls (Fig. 6C,D) as was stiffness, and modulus (Fig. 6E, F). These data indicate that the absence of *Col11a1* expression in the FDL significantly reduces the biomechanical properties of these tendons. Decreases in maximum stress and modulus suggest that

fundamental changes in the material properties of *Col11a1*$^{\Delta ten/\Delta ten}$ and control FDL tendons underlie the observed mechanical deficiencies. In contrast, no differences were observed in tendon stress relaxation (Fig. 6B). This viscoelastic (time-dependent) response was comparable in *Col11a1*$^{\Delta ten/\Delta ten}$ and control *Col11a1*$^{flox/flox}$ tendons. This suggests that *Col11a1* expression does not play a substantial role in modulating fibril sliding or crosslinking properties, that have been previously correlated with changes in the viscoelastic response of tendons [45,46].

Col11a1$^{\Delta ten/\Delta ten}$ FDLs have abnormal fibril structure at all ages primarily due to regulatory effects in development

An analysis of fibril structure was done in control and *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs in mature (day 60) and developing (day 4) FDLs to define critical regulatory stages. In the mature FDL, the absence *Col11a1* expression resulted in a shift to smaller diameter fibrils when compared to controls (Fig. 7A–D). In addition to the shift to small diameter fibrils there was aberrant fibril structure in the absence of *Col11a1* expression. Overall, these results are a logical extension based on the day 30, maturing tendon data (Fig. 4). In the absence of *Col11a1* expression, the diameter distribution is dominated by two distinct subpopulations, small diameter fibrils and a smaller population of large diameter fibrils (Fig. 7E). Fibril density was significantly higher in *Col11a1*$^{\Delta ten/\Delta ten}$ compared to control tendons (Fig. 7F). This is consistent with the larger number of small diameter fibrils observed in the absence of *Col11a1* expression.

Collagen XI genes are expressed in early stages of tendon development while expression was minimal in mature FDLs [8], therefore, the effect of its absence was evaluated in day 4 developing FDLs (Fig. 8). *Col11a1*$^{\Delta ten/\Delta ten}$ tendons had a significant disruption in fibril packing and organization with a subpopulation of very large fibrils. This subpopulation of large diameter fibrils demonstrated evidence of lateral fibril fusion suggesting that they arose from fusion of the small diameter fibrils seen in the controls at this developmental stage. The overall disruption in fibril packing observed in the absence of *Col11a1* expression further supports abnormal lateral fibril growth. Overall, at day 4, fibril structure was severely abnormal in the absence of *Col11a1* expression (Fig. 8A,B). In the control tendons, fibrils are small with a unimodal distribution. In contrast, *Col11a1*$^{\Delta ten/\Delta ten}$ fibrils have a broader distribution with a distinct right shoulder with larger diameter fibrils. The median diameter is smaller compared with fibrils from control tendons (Fig. 8C–E). Fibril density in *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs was more heterogenous compared to controls (Fig. 8F), consistent with the fibril disorganization observed in this genotype. These data suggest a primary regulatory role in tendon development and the perinatal period.

To determine whether the regulatory dysfunction during tendon development resulted in the mature phenotype of if there was a continued regulatory influence of *Col11a1* expression during tendon maturation we utilized an inducible *Col11a1* knockdown mouse model (I-*Col11a1*$^{-/-}$). Tendons were allowed to develop normally and then knockdown of *Col11a1* was induced during maturation (day 25) and the FDLs analyzed in mature tendons at day 60 (Fig. 9). Collagen fibril structure was comparable in both I-*Col11a1*$^{-/-}$ FDLs with an induced knockdown of *Col11a1* during maturation, and control FDLs. Both had

heterogeneous populations of fibrils with regular circular profiles (Fig. 9A,B). Fibril diameter distributions were comparable with both having a bimodal distribution with a narrow subpopulation of small diameter fibrils and a subpopulation of larger diameter fibrils with a broad distribution. Fibril densities were not significantly different in the induced and control tendons (Fig. 9C–F). The larger diameter subpopulation was marginally broader with a modest increase in largest diameter fibrils in the absence of *Col11a1* expression. Overall, these data indicate a critical regulatory role for *Col11a1* expression during the development of tendon structure and function. Disruption of regulation during this period is sufficient to continue to exert an influence on tendon structure and function in the mature tendon, however, continued minor regulatory influences of *Col11a1* expression during maturation cannot be excluded.

Discussion

The overall goals of this study were to define the roles of collagen XI in regulation of tendon fibrillar structure and organization as well as the relationship to function. We hypothesize that collagen XI functions during developmental stages to regulate tendon fibril assembly, and organization. In addition, it is likely that the primary regulatory mechanism is confined to development and early perinatal stages based on the expression pattern of *Col11a1* in the FDL [8]. However, little is known about regulation involving collagen XI in the mature tendon. To address these goals a conditional *Col11a1*-null mouse model was created. The α1(XI) chain is required for assembly of the major collagen XI isoform, α1(XI)α2(XI)α3(XI). In addition, it is required for assembly of the described collagen V/XI hybrid trimers. Therefore, we anticipate that all collagen XI isoforms would be knocked out. This approach allows an analysis of the functional roles of collagen XI in the tendon but will not address roles for specific isoforms. Our previous work indicated that the major collagen XI isoform (α1(XI)α2(XI)α3(XI)) was present in the FDL.[8] However, collagen V is more prominent in tendons and the presence of collagen V/XI hybrid isoforms was not addressed. Therefore, definition of the different isoforms in tendons and their individual roles will require further analysis.

Our previous work was done using the cho/cho mouse model [8]. This mouse is described as being null (cho/cho) or haploinsufficient (+/cho) for collagen XI [40,41]. The cho mutation results in a loss of the α1(XI) chain and as a result none of the collagen XI isoforms can assemble. Similar to the data presented here, the +/cho tendons had a mild disruption in fibril structure compared to controls in both embryonic and mature tendons. In contrast, as shown here, cho/cho embryonic tendons had a more severe phenotype with aberrant fibril structure, altered diameter regulation, disorganized fibrils and a reduced number of fibrils compared to wild type controls [8]. However, the cho/cho mouse model is perinatal lethal and mature null mice are not available for these studies [41]. Our conditional *Col11a1* knockout model circumvents this limitation. The conditional mice ($Col11a1^{flox/flox}$) are comparable to wild type mice in gross phenotype and collagen XI expression. We created 2 derivative models, one that was targeted to tendons and ligaments using *Scx-Cre* and a second TM inducible model. Both of these mouse lines avoid the perinatal lethal phenotype. These *Col11a1*-null mouse models permit the spatial or temporal manipulation of collagen XI expression required for these studies. Tendon-targeted $Col11a1^{\Delta ten/\Delta ten}$ mice also allowed

the spatial targeting of the mutation to tendons. This circumvents potential secondary influences on the tendons such as those due to alterations in cartilage, bone and muscle. The inducible knockdown mice (I-*Col11a1*$^{-/-}$) allow for temporal control of *Col11a1* knockdown. This permits normal development in the presence of collagen XI with the knockdown occurring at maturity thus isolating the effect(s) to this specific time.

Collagen XI is a fibril-forming collagen found in collagen II containing tissues like cartilage and vitreous where it has been shown to regulate collagen fibril assembly [26,40]. Generally, collagen XI has not been considered to be a major factor in collagen I-containing tissues such as tendons, ligaments, cornea, and skin. However, it is widely distributed in embryonic tissues including tendons [28–30]. Also, collagen XI has been shown to interact with collagen I influencing fibril assembly in in vitro studies [17]. Using *Col11a1*-null mouse models, we demonstrate a significantly regulatory role for collagen XI in the tendon. Given that the α1(XI)α2(XI)α3(XI) isoform is expressed in FDLs, it is likely that this form has a role. However, as discussed above, independent or synergistic roles with other collagen XI isoforms cannot be excluded.

In the absence of *Col11a1* expression and therefor collagen XI, mature tendons have a significant disruption in fibril phenotype with a relative absence of large diameter fibrils compared to wild type mice. The heterozygous mice more closely resembled the wild type mice suggesting that the reduced collagen XI expression was sufficient for regulation of fibrillogenesis. This is consistent with in vitro fibrillogenesis assays that demonstrated decreased fibril diameter as collagen XI concentration was increased relative to collagen I [17]. This result implicates collagen XI as a critical regulator of tendon fibril assembly. Collagen V has also been shown to be a major regulator of the fibril assembly in collagen I containing tissues where it initiates/nucleates the fibril assembly process in association with the fibroblast surface [27,35,36,39]. It also has a key role in regulation of tendon fibril assembly [34]. Collagens V and XI are closely related and can be considered different isoforms of the same collagen type [16,17]. These data suggest that collagens XI and V both have regulatory roles in the tendon. Our work is consistent with an independent role for collagen XI. This work and previously published studies [8] suggest different, but coordinate roles for collagens XI and V in the tendon. The coordinate roles require further study to be fully elucidated.

Interestingly, consistent with *Col11a1* expression restricted to developmental and perinatal periods [8], we demonstrate that the absence of collagen XI leads to a more severe regulatory dysfunction in developing compared to mature tendons. In contrast, the situation is reversed in the absence of collagen V with developing tendons having a very mild phenotype [34]. These data suggest a restricted time frame for collagen XI regulation during the establishment of tendon extracellular matrix architecture. To address this, the FDL was allowed to develop normally in the presence of *Col11a1* expression until day 25 and then during the final stages of maturation induced a knockdown of *Col11a1* expression. When we analyzed mature tendons at day 60, we found fibril structure and organization comparable, but not identical in the knockdown and the control tendons. This indicates that the major regulatory role of collagen XI is restricted to periods when tendon structure is being established coincident with the period of collagen XI expression [8]. However, the

developing phenotype is not lost, diluted out, in the day 60 mature FDL. This suggests that the tendon matrices assembled in the presence or absence of collagen XI continue to guide assembly in maturing tendons. Collagen V regulated fibril assembly is dominant during this period and has only a minor function during development [34]. However, fibril structure, organization and tendon mechanical properties are all abnormal in mature, day 60 FDLs from the *Col11a1*$^{\Delta ten/\Delta ten}$ mice. The knockout of *Col11a1* results in the knockout of the major collagen XI isoform as well as collagen V/XI hybrid molecules. It is possible that these isoforms could have differential regulatory roles, and this requires further evaluation. However, the data clearly support a critical regulatory role for collagen XI.

Our studies also demonstrated a disorganization of fibril alignment in the absence of *Col11a1* expression. Both collagens XI and V are associated with the tenocyte surface and do not co-localize [8,27]. It is possible that the association of collagen XI with the tenocyte surface allows the tenocytes to position the assembling fibrils in the developing matrix. This control would be lost in the absence of collagen XI resulting in disorganized fibril deposition. The presence of collagen XI in the early stages of tendon development suggest a key role is establishing tendon fibrillar matrix architecture. It is possible that the collagen XI-directed deposition of fibrils provides a template for further matrix assembly. The abnormal assembly of a template may explain the continued influence of collagen XI seen in the mature tendon. In addition, the lack of co-localization of collagens XI and V at the tenocyte surface,[8] suggests separate, non-redundant functions for these collagens. One might speculate that collagen XI nucleates fibril assembly and that there is a strong affinity of collagen XI for the tenocyte surface resulting in a slow release of these fibrils into the developing matrix forming a stable template. In contrast, [2] collagen V would be rapidly released into the matrix with the collagen XI-directed template responsible for their organization. In mature tendon where collagen V is the regulator, the matrix architecture is established there would be no further need for a template. In addition, the data suggested an alteration in fibril structure consistent with dysfunctional regulation of lateral fibril fusion. This may result directly from the absence of charged α1(XI) domains on the surface of heterotypic tendon fibrils. Alternatively, the lack of specific charges domains may alter the interaction of molecules such as decorin, fibromodulin known to regulate lateral fibril growth [1,2]. In addition to altered fibril structure, changes in fibril-associated molecules can result in altered growth factor availability with resulting changes in tenocyte behavior that would influence tissue function [47]. In addition, collagen V/XI fragments can bind to specific growth factors including FGF-2 [48]. This suggest specific sites can bind these factors and sequester or present them during physiological and pathological processes. The different alternatively spliced domains vary in charge and charge density. This may provide a mechanism for differential regulation in tissues, developmental stages, and after injury.

Finally, collagen XI underlies a human congenital disorder with a broad spectrum of connective tissue defects. Stickler syndrome type II is caused by mutations in *COL11A1*. It is characterized by skeletal and joint abnormalities specifically hypermobility [11,49] among other features. Joint hypermobility involves defects in supporting tendons and ligaments. Interestingly, in affected children and young adults, joints are often loose and very flexible, however, the joints become less flexible with age. This is consistent with the structural phenotype in our mouse model. Joint hypermobility may involve contributions from both

affected cartilages and tendons. However, the reduction in structural and material tendon mechanical properties, i.e., decreased strength and stiffness supports tendon involvement. It has previously been postulated that a bimodal distribution of collagen fibril diameters is necessary for optimal tendon mechanical resilience [50]. A near complete loss of larger diameter fibrils, and therefore the bimodal distribution, was observed in the *Col11a1*$^{\Delta ten/\Delta ten}$ tendons, that might explain the substantial loss of mechanical integrity in these tendons. The tendon targeted *Col11a1* null mouse model excludes cartilage involvement. In addition, the improvement in hypermobility from childhood to adult is consistent with a critical role for collagen XI in early tendon development and lesser role in the mature tendon. This is consistent with the definition of the primary regulatory foci of collagen XI being during tendon development in the FDLs. Stickler syndrome type II is a heterozygous condition and our *Col11a1* mouse is predicted to be an excellent experimental model for this human congenital disorder. The conditional model can be utilized in its heterozygous and/or homozygous state to isolate the contributions of different tissues, define the temporal requirements for *Col11a1* expression and to probe the mechanisms underlying the pathobiology.

In conclusion, unique conditional *Col11a1* mouse models were created that permit the spatial and temporal targeting of tissues and times. Utilizing these mice, it was demonstrated that *Col11a1* expression has a critical regulatory function in tendon development but has little to no direct regulatory function in mature tendons. However, the mature *Col11a1*$^{\Delta ten/\Delta ten}$ mice had a severe disruption of fibril structure and organization associated with a severe loss of mechanical function suggesting a continued indirect role for *Col11a1 expression* in tendon matrix assembly. This work suggests that the lack of the α1(XI) chain results in the absence of α1(XI)α2(XI)α3(XI) and a collagen XI null model. However, the influence of the *Col11a1* knockout on other isoforms and hybrid collagen V/XI molecules requires investigation.

Experimental procedures

The work described was approved by the University of South Florida and the University of Pennsylvania Institutional Animal Care and Use Committees. These studies were performed using male and female mice. The tendon-targeted (*Scx-Cre*) collagen XI knockout mouse model (*Col11a1*$^{\Delta ten/\Delta ten}$) is in a C57/BL6 Charles River background. Control mice were wild type, *Col11a1-flox/flox*, and *Scx-Cre* all in a C57/BL6 Charles River background.

Development of tendon-targeted *Col11a1* knockout mouse model

The strategy for generating conditional *Col11a1* knockout *Col11a1*$^{flox/flox}$ mice is presented in Fig. 1. A promoter-driven knockout first targeting vector was obtained from the KOMP Repository (University of California at Davis, project ID: CSD80258). In this targeting vector, *Col11a1* exon 3 was flanked by loxP elements. Briefly, the targeting vector was linearized and electroporated into V6.5 129 Sv/J mouse embryonic stem (ES) cells. The ES cells were cloned and screened for appropriate targeting as previously described [39]. After selection, ES cells containing the targeted *Col11a1* allele were identified by the absence of the negative selection marker DTA, the presence of LacZ, and orientation was confirmed using 3’ and 5’ gene and vector specific sequence using short-range and long range PCR.

The successfully targeted ES cell clones were karyotyped and appropriate clones were injected into wild type C57 BL/6-Albino blastocysts, resulting in chimeric mice. The chimeric mice were backcrossed with wild type C57 BL/6 mice to produce the germline transmitted mice with the targeted allele *Col11a1*$^{+/ta}$. The *Col11a1*$^{+/ta}$ mice were bred with FLPe mice (B6; SJL-Tg(ACTFLPe) 9205Dym/J, Jackson Labs) to excise the FRT flanked neo and lacZ sequences. The resulting offspring were cross bred with C57BL/6 (Charles River) mice for 6 generations and then inter-crossed resulting in the conditional knockout mice, *Col11a1*$^{flox/flox}$. Mice at each different stage were characterized using PCR that amplified specific element sequences. To target the *Col11a1* knockout to tendons, the *Col11a1*$^{flox/flox}$ mice were bred with Scleraxis-Cre mice (*Scx-Cre*) mice [34]. The *Scx-Cre* mice were a gift from Dr. Ronen Schweitzer, Oregon Health and Science University. Genotyping analysis of tendon-targeted *Col11a1* conditional knockout mouse was carried out using Cre primers and specific primers at the junction of the 3' arm and targeting sequence. The primers for the genotyping and characterization of these mice are listed in Table 1.

A bitransgenic inducible *Col11a1*-null (*I-Col11a1*$^{-/-}$) mouse model also was created for use in these studies. A tamoxifen (TM) inducible *Col11a1*-null mouse model (I-*Col11a1*$^{-/-}$) was generated by breeding the conditional *Col11a1*$^{flox/flox}$ mice with knockin TM-inducible Cre mice (B6.129-*Gt(ROSA) 26Sor*$^{tm1(cre/ERT2)Tyj}$/J, Jackson Labs) as previously described [51]. Two male *Cre*$^{+/+}$*/Col11a1*$^{flox/flox}$ mice (I-*Col11a1*$^{-/-}$) were injected i.p with TM (Sigma, St. Louis) at 10 mg/100g body weight starting at day 25 and continued once daily for three days. Efficient Cre excision is obtained after induction with TM using this protocol [51].The mice were euthanized at day 60 and processed for electron microscopy.

RT-PCR

Samples of FDL tendons were removed from wild type, *Col11a1*$^{flox/flox}$ *Col11a1*$^{\Delta ten/\Delta ten}$, and *Col11a1*$^{+/\Delta ten}$ mice at day 14. The tissue samples were cut into small pieces and lysed in QIAzol reagent (Qiagen, Germantown, MD), the crude total RNA was cleanup with the RNeasy MinElute Cleanup Kit (Qiagen, Germantown, MD). The resulting RNA underwent reverse transcription with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). This was followed by real time PCR using SYBR Green PCR master mix (Thermo Fisher Scientific) in a StepOnePlus Real Time PCR system (Applied Biosystems). The resulting *Col11a1* mRNA expression levels were normalized with β-actin. Primers from the C terminal non-helical region were used for the real time PCR reactions. They are *Col11a1* FW: GACCAGAAGACACACTGAAAGCA, *Col11a1* RV: TCCATGCCATCTGAGTAGTCAAGA; β-actin FW: AGATGACCCAGATCATGTTTGAGA, β-actin RV: CACAGCCTGGATGGCTACGT. Primers from the helical region also were used and they are: *Col11a1* FW2: CTGGTCATCCTGGGAAAGAA; *Col11a1 RV2*: AGCCCTTGAGACCTCTGACA.

Immuno-blots

Collagen XI content was analyzed immuno-chemically using a Wes™ automated western blotting system (ProteinSimple, San Jose, CA). FDL samples were dissected from wild type, *Scx-Cre, Col11a1*$^{+/\Delta ten}$, and *Col11a1*$^{\Delta ten/\Delta ten}$ mice at day 4. Developing mice were used to

minimize the effects of crosslinking and collagen extraction. In addition, the use of young healthy mice should minimize the potential effects of non-specific proteolysis on the susceptible variable domain. Individual mice (n=3–8) were used for each genotype. Two FDLs from each mouse were cut into small pieces and protein was extracted using an extraction buffer composed of 50 mM Tris-HCl, pH 6.8, 1% SDS with proteinase inhibitor cocktail (ThermoFisher Scientific). Equal amounts (1.5 mg) of denatured protein samples were loaded into single designated wells of Wes Separation 12–230 kDa 25 Capillary Cartridges, 1:50 diluted rabbit anti-mouse Col XI antibody (300A) and the Wes anti-rabbit detection module was used for collagen XI detection. Quantification by densitometry was performed using the area of the targeted protein (α1(XI)) and normalized to total protein amount, which was analyzed by loading an equal amount of protein to a separate capillary cartridge and detected with the Wes total protein detection module. All Wes reagents (separation module and detection modules) were purchased from ProteinSimple and the Wes assay was carried out following the manufacturer's instructions. Data analyses were performed using the Compass Software (ProteinSimple).

Antibody

A Rabbit anti-mouse α1(XI) antibody was customer produced by Zymed Laboratory Inc, CA. Peptide (C)YGTMEPYQTETPRR-amide conjugated KLH was used to immunize rabbits, antisera was affinity purified against the peptide and eluted with 3M KSCN. The peptide (Ms #309–322) is coded within exon 7 and located in the α1(XI) N terminal nonhelical region.

Grip strength

Grip strength was evaluated in *Col11a1*$^{flox/flox}$ control and *Col11a1*$^{\Delta ten/\Delta ten}$ knockout male mice at day 60. A grip strength meter (San Diego Instrument, San Diego, CA) was used to record the peak force each mouse exerts in grasping a grip placed at their fore limb. The mouse was held by the tail and lowered toward the grip strength platform until it grasped the grip with its forepaws. The mouse was then pulled steadily by the tail away from the rod until the grip was broken. The force applied to the grip just before the animal loses its grip was recorded as the peak tension. In each genotype 8–9 mice were tested. Ten measurements from each mouse were recorded and the average force was used to represent the grip strength for individual mice.

Transmission electron microscopy

Samples of FDL taken from wild type, *Col11a1*$^{\Delta ten/\Delta ten}$, and *Col11a1*$^{+/\Delta ten}$ mice at day 4, day 30 and day 60 were examined using transmission electron microscopy as previously described [52–54]. The tendons were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4, with 8.0 mM $CaCl_2$ followed by post-fixation with 1% osmium tetroxide. Both transverse and longitudinal sections were stained with 2% aqueous uranyl acetate and examined at 80 kV using a JEOL 1400 transmission electron microscope. An Orius wide-field CCD camera with a resolution of 3648 × 2672 and magnification of 60,000X was used to capture the images. Images from the tendon midsubstance were masked and transferred to an RM Biometrics-Bioquant Image Analysis System for analysis. Fibril diameter analyses were done from transverse section images. All fibrils within a

predetermined region of interest (ROI) on the digitized image were measured. Non-overlapping ROIs were placed in the central tendon based on fibril orientation (i.e., cross section) and absence of cells. Diameters were measured along the minor axis. For measurement of fibril density, the total number of fibrils within the ROI was normalized for area. Statistical analysis of differences in the diameter distributions was done using the Kolmogorov–Smirnov (K-S) test.

Mechanical testing

Male mice, *Col11a1*$^{flox/flox}$ (n=12) and *Col11a1*$^{\Delta ten/\Delta ten}$, (n=15) were euthanized at 60 days. FDL tendons were removed from the ventral aspect of the mouse foot and cleaned free of excess soft tissue. Mechanical testing was done as previously described [33,34,55,56]. Verhoeff's stain lines were placed 2.5 mm apart within the mid-substance to track the strain optically. Cross-sectional area of the tendon was then measured with a custom built optical device [57]. Each end of the FDL tendon was glued to sandpaper and the gauge length is approximately 5 mm for mechanical testing. The tendon was then placed in custom made grips and a custom holding fixture was secured to the grips.

Samples were placed in phosphate buffered saline bath at room temperature and loaded in a tensile testing system (model 5542, Instron, Norwood, MA). To determine biomechanical properties, tensile testing along the long axis of the tendon was performed with the following protocol. For ACH and SST, the mechanical testing protocol involved a preload to 0.02 N; 10 cycles of preconditioning (0.02–0.04 N at 1% strain/s); rest for 300 s; stress relaxation at 5% strain (ramp rate of 5%/s) followed by a 600 s hold; a return to zero-displacement, 60 s hold, and ramp to failure at 0.1%/s [33]. For FDL, the mechanical testing protocol involved a preload to 0.01 N; 10 cycles of preconditioning (0.01–0.02 N at 1% strain/s); rest for 300 s; stress relaxation at 5% strain (ramp rate of 5%/s) followed by a 600 s hold; a return to zero-displacement, 60 s hold, and ramp to failure at 0.5%/s. A 10 N load cell was used with a resolution of 0.01 N. During the testing, images were obtained with a digital camera (Basler, Exton, PA) every 5 s for optical strain analysis. Maximum stress was calculated as the maximum force divided by cross sectional area. Maximum force and maximum stress were only calculated for samples that experienced physiological failure within the midsubstance or insertion site and failures that occurred at the grips were excluded. A custom Matlab program (Matlab R2017a, Natick, MA) was used to optically track strain lines to quantify stiffness and regional modulus in the linear region of the mechanical test [58].

Shapiro–Wilk tests were performed to verify normality of the data sets. Independent-sample t-tests (two-tailed) were used to determine statistical significance between the control and *Col11a1*$^{\Delta ten/\Delta ten}$ groups for normally distributed data sets. Mann-Whitney U tests were used for non-normal data sets. Statistical evaluation for realignment of collagen fiber organization used a two-way ANOVA. Any significant main effects were followed by one-way ANOVAs with Dunnett post-hoc tests to compare circular variance (VAR) at 1% strain to VAR at every other strain level. Significance was set at $p \leq 0.05$ and trends at $p \leq 0.1$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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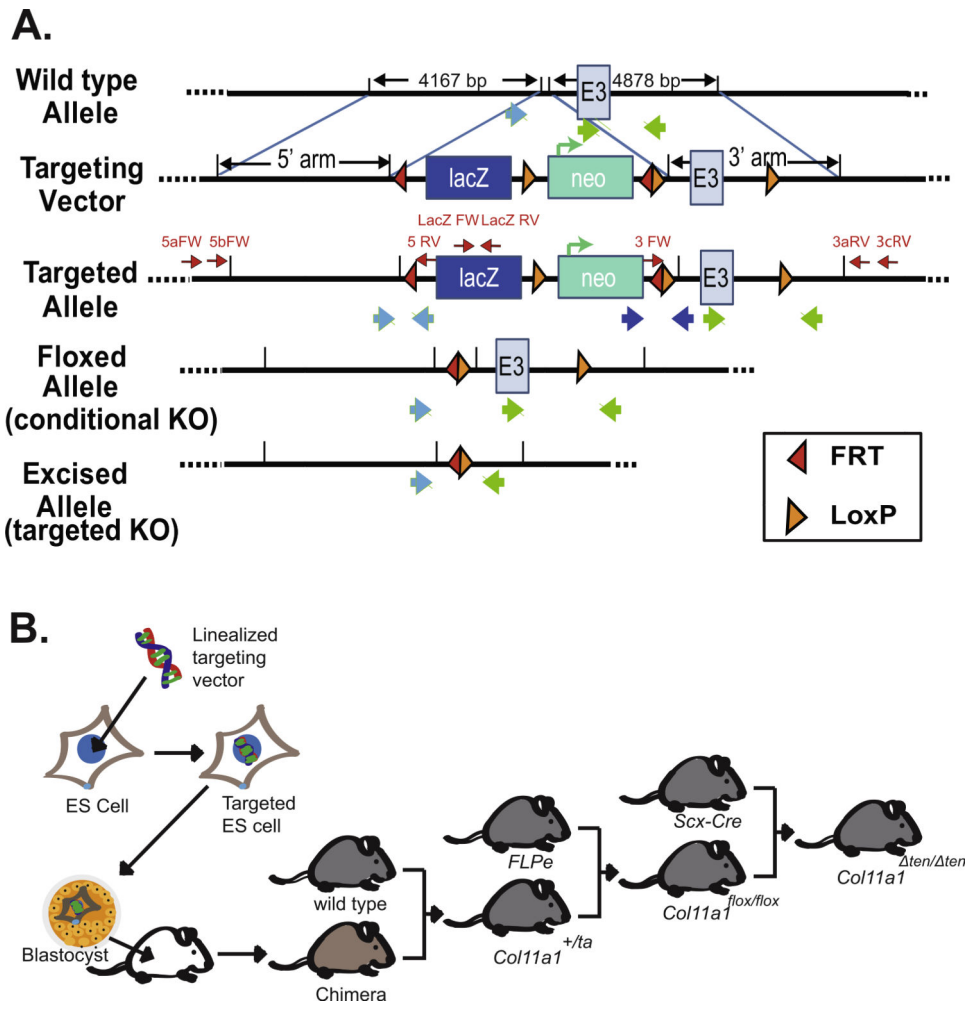

Fig. 1.

Strategy for creating tendon targeted *Col11a1* conditional knockout mice. (**A**) Schematic diagram showing the wild-type allele, targeting vector, targeted allele, and floxed allele as well as the excised allele after Cre recombination of *Col11a1* gene. The *Col11a1* targeting vector was obtained from the KOMP Repository (project ID: CSD80258). Exon 3 of *Col11a1* is flanked with LoxP sites and the Neo cassette is flanked with FRT sites. Location of primers for LacZ, as well as 3' and 5' primers used to determine insert orientation are shown (red arrows). The large blue and green arrows indicate the location of the primers used to identify specific alleles. (**B**) Strategy for gene targeting of embryonic stem cells and generation of tendon-targeted *Col11a1* conditional-knockout mice. Linearized *Col11a1* targeting vector was electroporeated into ES cells. Blastocysts were injected with targeted ES cells yielding chimeric mice. The chimeric mice were crossed with wild type mice yielding F1 progeny with the targeted allele ($Col11a1^{+/ta}$). Breeding of $Col11a1^{+/ta}$ with FLPe mice resulted in excision of FRT flanked sequences, removal of the Neo cassette and creation of conditional knockout mice ($Col11a1^{flox/flox}$). These mice were bred with mice expressing *ScxCre* to target the knockout to tendons ($Col11a1^{\Delta ten/\Delta ten}$).

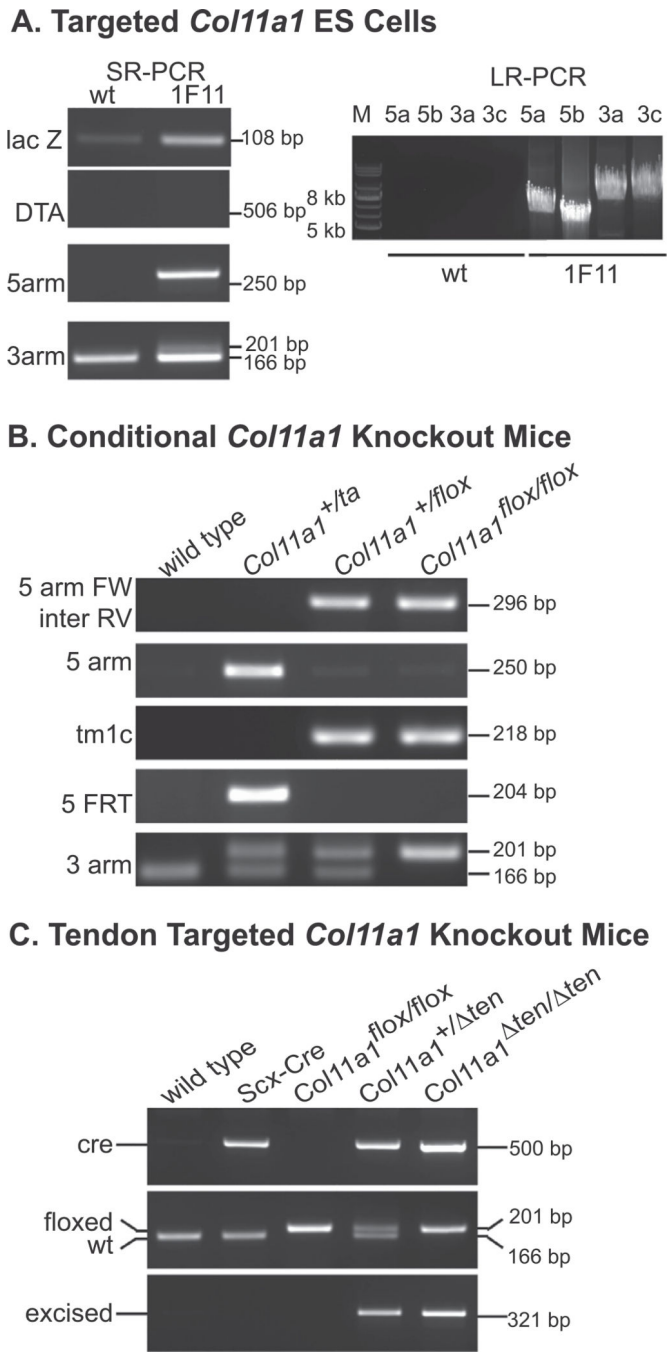

Fig. 2.

Analysis of tendon targeted *Col11a1* knockout mice. (**A**) Characterization of the targeted *Col11a1* ES cells using short-range PCR (left panel) and long-range PCR (right panel). Clone 1F11 is the targeted ES cell clone injected into blastocysts. (**B**) Genotyping of the targeted allele, and floxed alleles in different stages of creating the conditional *Col11a1* knock-out mice. (**C**) Genotyping of the Scleraxis-Cre mice (Scx-Cre), heterozygous ($Col11a1^{+/\Delta ten}$) and homozygous ($Col11a1^{\Delta ten/\Delta ten}$) tendon targeted *Col11a1* knockout mice.

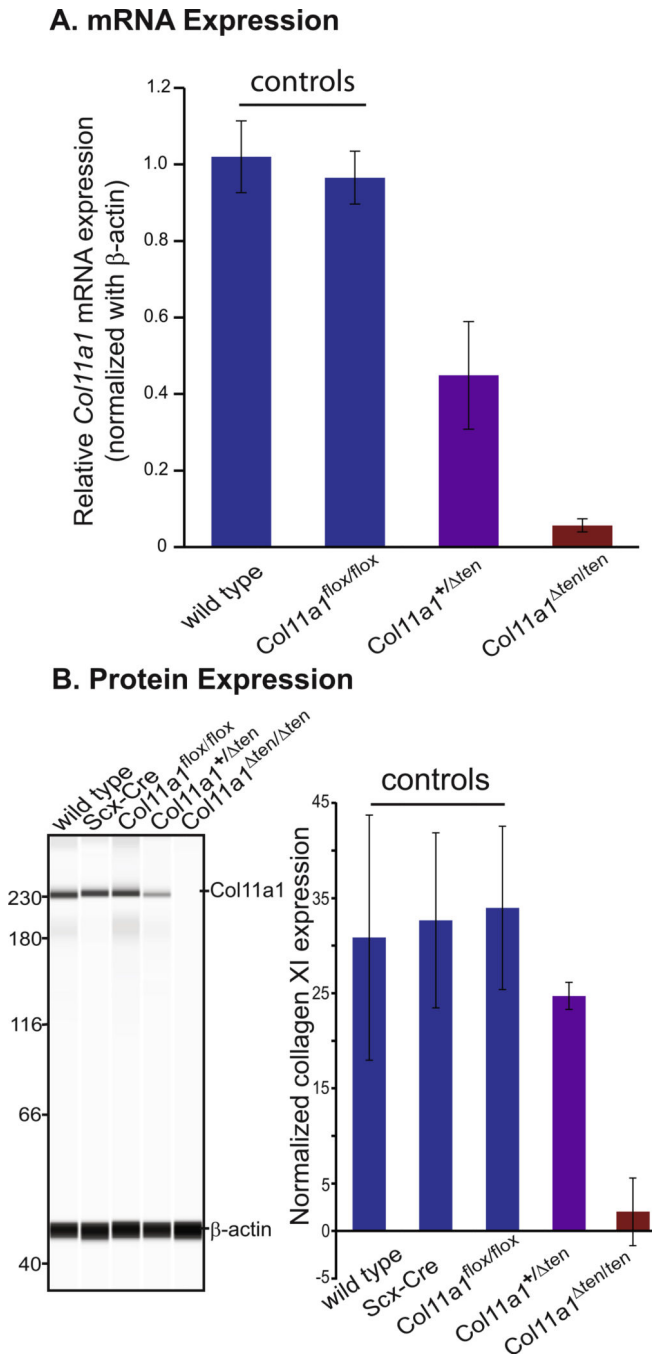

Fig. 3.

Knockout of *Col11a1* mRNA and protein expression in the *Col11a1* tendon targeted conditional mice. (**A**) Quantitative real-time PCR shows comparable expression of *Col11a1* mRNA in the wild type and $Col11a1^{flox/flox}$ controls. *Col11a1* mRNA expression in $Col11a1^{\Delta ten/\Delta ten}$ FDLs is reduced to baseline while expression in the $Col11a1^{+/\Delta ten}$ FDLs was ~50% of control values. Day 14 wild-type (n=3), $Col11a1^{flox/flox}$ (n=5), $Col11a1^{+/\Delta ten}$ (n=8), $Col11a1^{flox/flox}$ (n=7) mice for each genotype. (**B**) Western blot analysis of Col11a1 protein (α1(XI)) content in control; wild type (n=3), Scx-Cre (n=4), $Col11a1^{flox/flox}$ (n=7)

mice was done using Wes automated western blotting. The *Col11a1*$^{+/\Delta ten}$ (n=3), and *Col11a1*$^{\Delta ten/\Delta ten}$ (n=4) FDL contained reduced and virtually no α1(XI) reactivity relative to controls. All 3 control mice contained comparable amounts of α1(XI). Day 4 mice. Left panel shows a representative image and the right panel presents the quantitation results from all mice.

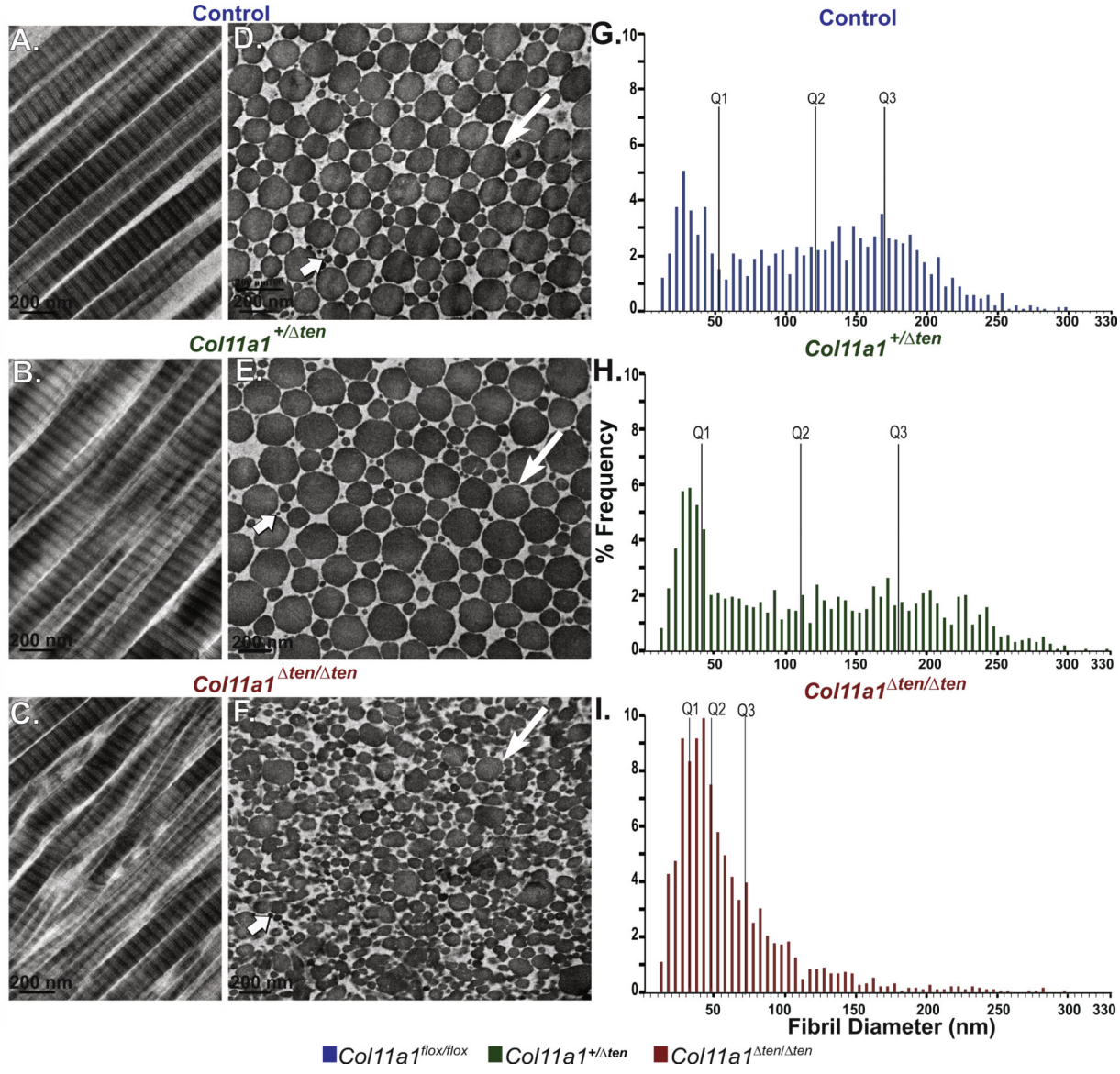

Fig. 4.

Abnormal fibril structure in FDLs from tendon targeted *Col11a1* knockout mice. **(A-C)** Transmission electron microscopy demonstrates a disruption in the parallel fibril alignment in FDLs in *Col11a1*$^{\Delta ten/\Delta ten}$ mice compared to wild type mice while fibril alignment in *Col11a1*$^{+/\Delta ten}$ FDLs is partially disrupted. **(D-F)** *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs have a larger percentage of smaller, more heterogeneous and structurally abnormal fibrils compared with wild types controls. In contrast, haploinsufficient *Col11a1*$^{+/\Delta ten}$ FDLs show only a mild disruption of fibril structure. **(G-I)** All 3 distributions are significantly different from one another ($p<0.001$ K-S test). There is a striking difference between the *Col11a1*$^{\Delta ten/\Delta ten}$ and the other 2 distributions. The fibril diameter distribution is significantly shifted to the smaller diameter fibrils with the larger diameter subpopulation reduced to a minor shoulder in the *Col11a1*$^{\Delta ten/\Delta ten}$ mice compared to the wild type mice. The wild type and *Col11a1*$^{+/\Delta ten}$ distributions are very similar with the reduction in *Col11a1* associated with a mild

phenotype with an increase in large diameter fibrils relative to the control. Day 30 males, n=2 for each genotype.

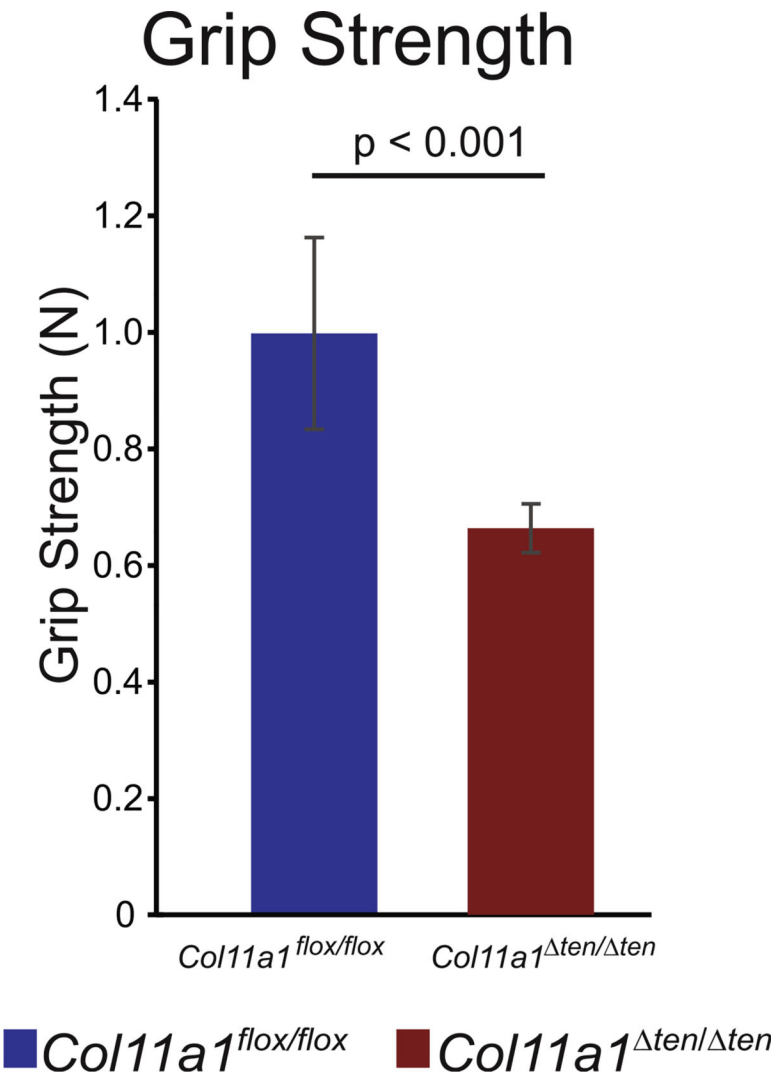

Fig. 5.

Tendon targeted *Col11a1* knockout mice are weaker. Fore-limb grip strength is significantly decreased in *Col11a1*$^{\Delta ten/\Delta ten}$ mice (n=9) compared to *Col11a1*$^{flox/flox}$ control mice (n=8). Day 60 male mice.

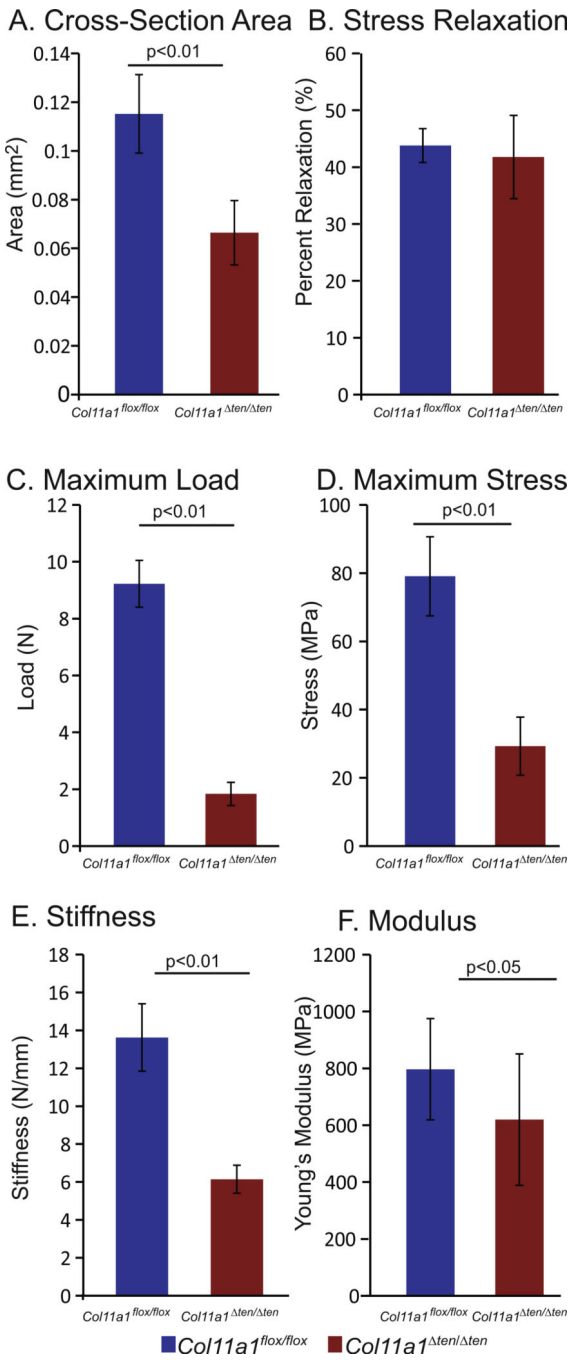

Fig. 6.

Altered biomechanical properties in FDLs in tendon targeted *Col11a1* knockout mice. FDL tendons from tendon-targeted *Col11a1*$^{\Delta ten/\Delta ten}$ null mice demonstrated significant alterations in the biomechanical properties. The *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs were smaller, weaker, and stiffer compared to the wild type controls. (**A**) A significant decrease in cross-sectional area was observed. In addition, (**B**) maximum load, (**C**) maximum stress, (**D**) stiffness, and (**E**) modulus were significantly reduced in the *Col11a1*$^{\Delta ten/\Delta ten}$ *mice compared to controls*. Decreases in maximum stress and modulus suggest that fundamental differences in the

material properties of *Col11a1*$^{\Delta ten/\Delta ten}$ and control FDL tendons underlie the mechanical deficiencies. Day 60 male *Col11a1*$^{flox/flox}$ (n=12) and *Col11a1*$^{\Delta ten/\Delta ten}$ (n=15) male mice.

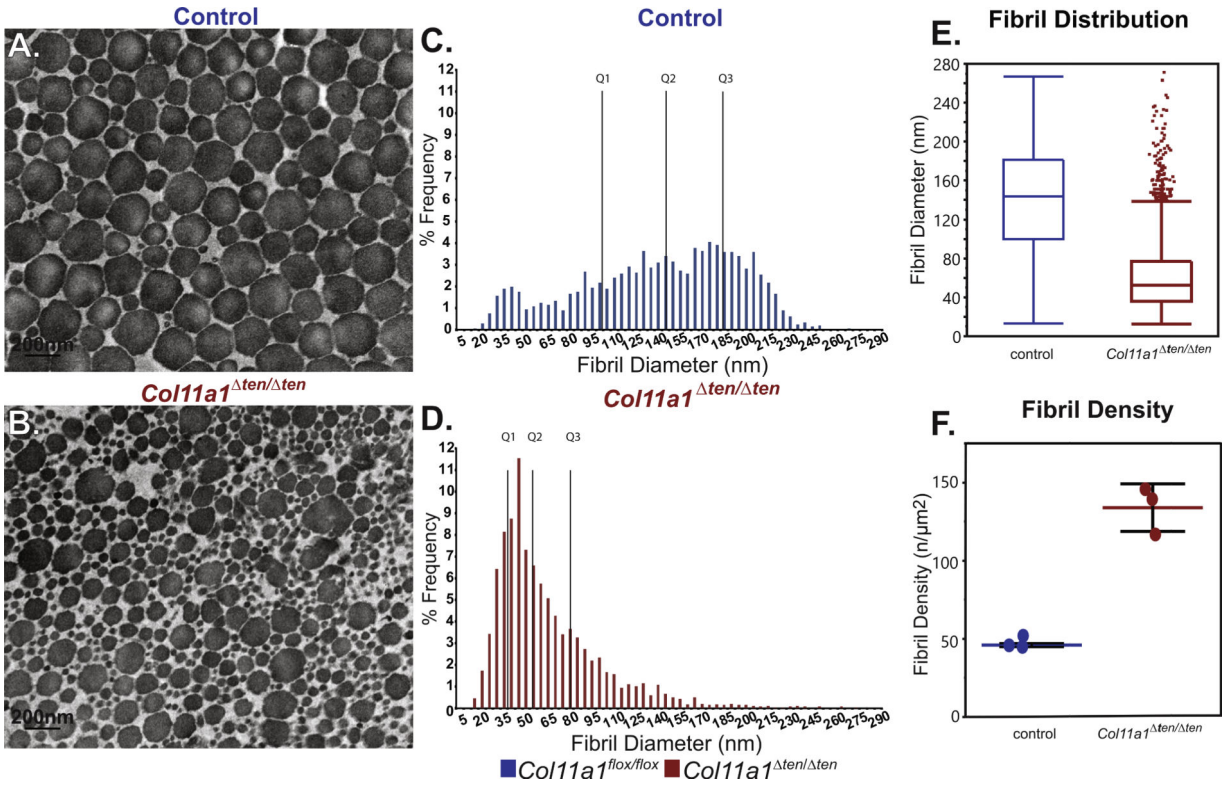

Fig. 7.

The absence of *Col11a1* expression results in abnormal FDL fibrils in mature *Col11a1*$^{\Delta ten/\Delta ten}$ mice. FDLs from mature male *Col11a1*$^{\Delta ten/\Delta ten}$ mice (day 60) have a severe disruption in collagen fibril structure and diameter distributions compared to control tendons. **(A,B)** Electron microscopic analysis demonstrates a substantial increase in small diameter fibrils as well as overall smaller diameters in the FDLs from *Col11a1*$^{\Delta ten/\Delta ten}$ compared to control FDLs. **(C-E)** The fibril distributions from wild type and *Col11a1*$^{\Delta ten/\Delta ten}$ tendons are significantly different (KS $p<0.0001$). In the *Col11a1*$^{\Delta ten/\Delta ten}$ mice there is a significant shift to small diameter fibrils and a virtual absence of large diameter fibrils characteristic of the mature FDL. The mature day 60 mice show the same trends as seen in maturing day 30 mice (Fig. 4). **(F)** Fibril density is significantly increased in the *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs compared to controls, consistent with the significant shift to small diameter fibrils observed in this genotype. (n=3 mice for each genotype).

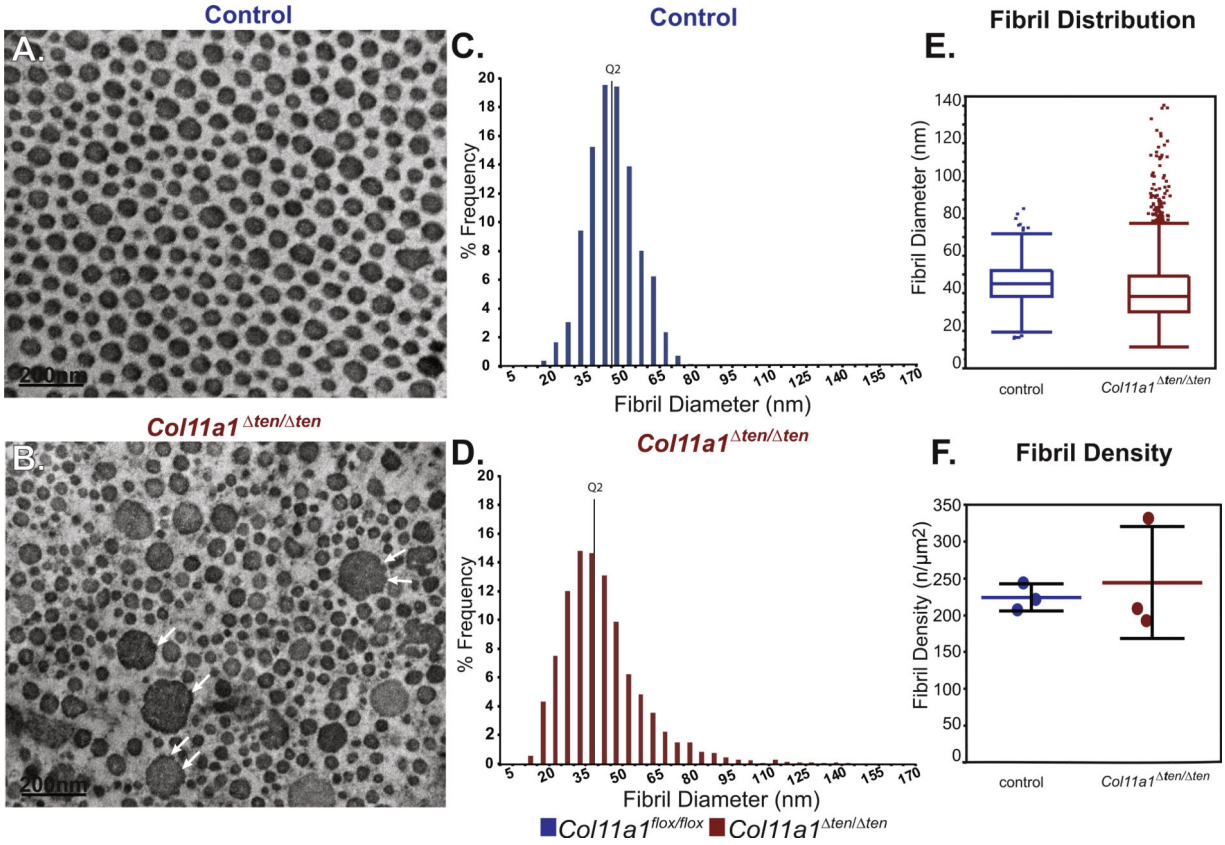

Fig. 8.

Expression of *Col11a1* regulates early steps of fibrillogenesis. **(A,B)** Electron microscopic analysis demonstrates smaller and more heterogeneous fibrils in the FDLs from developing *Col11a1*$^{\Delta ten/\Delta ten}$ mice compared with the wild type mice at day 4. The large diameter fibrils seen in *Col11a1*$^{\Delta ten/\Delta ten}$ showed evidence of fusion with small diameter fibrils (arrows) suggesting their formation was a result of unregulated lateral fibril growth. **(C-E)** The diameter distributions from wild type and *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs are significantly different (KS $p<0.0001$). The fibril diameter distributions shows a substantial increase in small diameter fibrils and the broader distribution of fibril diameters in the *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs compared to controls. **(F)** Fibril density in *Col11a1*$^{\Delta ten/\Delta ten}$ FDLs is similar to the controls, but considerably more variable consistent with the presence of a distinct subpopulation of very large diameter fibrils not seen in the control tendons. (n=3 mice for each genotype).

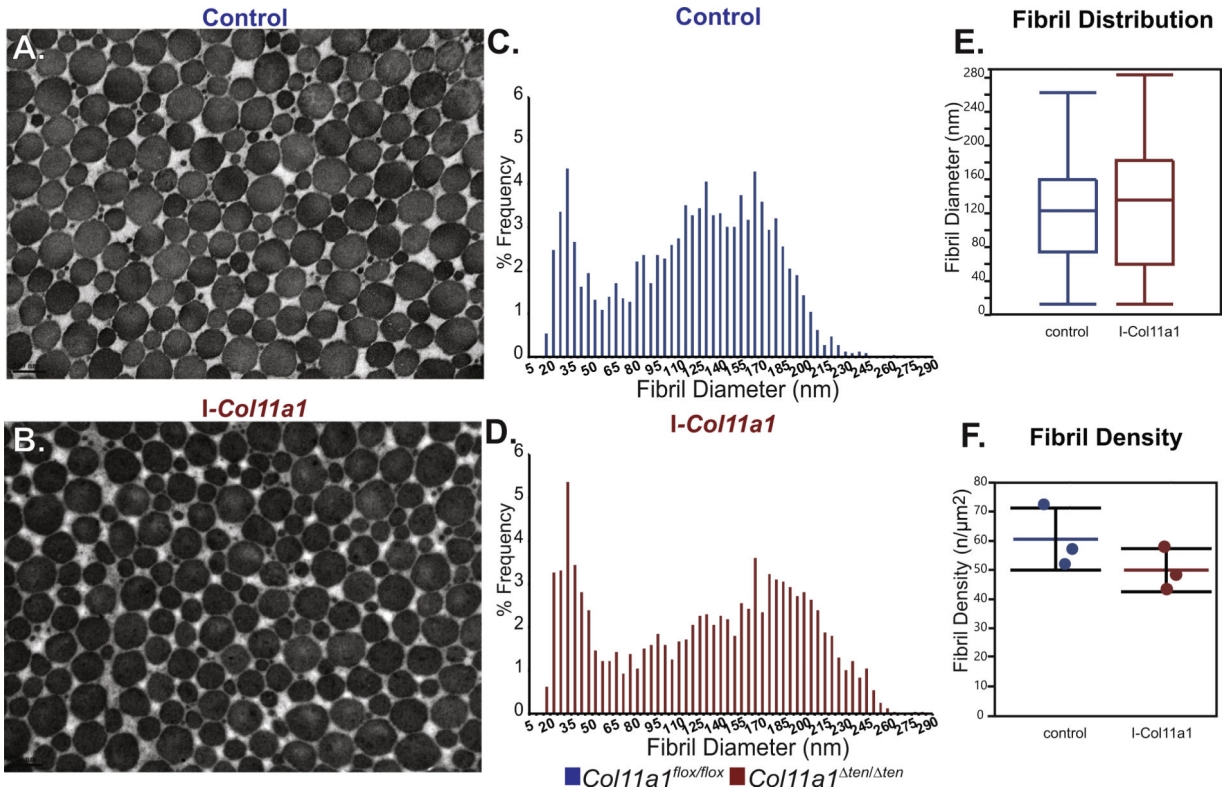

Fig. 9.

Induced knockdown *Col11a1* in the mature tendon does not affect fibril structure. Knockdown of *Col11a1* was induced using tamoxifen once daily for 3 days (i.p 10mg/100g body weight) beginning at day 25 and fibril structure analyzed at day 60. This approach allows the FDLs to develop as in wild type through development and into maturation. Knockdown is during maturation and mature FDLs are analyzed. **(A,B)** Electron microscopic analysis shows no significant effect on the fibril diameter and organization. Fibrils from both genotype have normal circular profiles. **(C-E)** The diameter distributions from wild type and I-*Col11a1*$^{-/-}$ FDLs are both bimodal and very similar. The larger diameter subpopulation is shifted modestly toward larger diameter in the I-*Col11a1*$^{-/-}$ compared to wild type FDLs. **(F)** Fibril density in I-*Col11a1*$^{-/-}$ FDLs is decreased, consistent with the increase in diameter observed. (n=3 mice for each genotype).

Table 1:

Primers for selection and characterization of *Col11a1* targeted ES cells and conditional mice.

Reaction name	Primer name	*Col11a1* primer sequence	Size	Detect	Function
LacZ Insertion	LacZ FW LacZ RV	ATCACGACGCGCTGTATC ACATCGGGCAAATAATATCG	108 bp	targeted vector targeted allele	Vector characterization ES cell selection
FRT	5FRT_F	AGGCGCATAACGATACCACGAT	204 bp	targeted vector	Vector characterization
	5FRT_R	CCACAACGGGTTCTTCTGTT		targeted allele	ES cell selection
DTA negative selection	DTA-5 DTA-3	AGGGAAGGCTGAGCACTACA CATTCTGCACGCTTCAAAAG	506 bp	targeted vector	Vector characterization ES cell selection
5 arm	5 arm FW 5 arm RV	TTCAGTGGCATGGTTTTCAA CCAACCCCTTCCTCCTACAT	250 bp	targeted vector targeted allele	ES cell selection targeted mice section
3 arm	exon1 FW 3 arm RV	TCAGCCAACTGAACAACGAC CATCCTCTTCTCCTGCCTTG	166 bp 203 bp	wild type, targeted, and floxed alleles	genotyping wild type, targeted, and floxed alleles
5’ insertion/ orientation*	5a FW 5b FW 5 RV	CCATAGGTGATCACAAGAGTTTGC GAATAATGCCAGAGTGAACACACC CACAACGGGTTCTTCTGTTAGTCC	6299 bp 5397 bp	targeted allele targeted allele	ES cell selection ES cell selection
3’ insertion/ orientation*	3 FW 3a RV 3c RV	CACACCTCCCCCTGAACCTGAAAC CTTGGAATCAGGGAGGAGAAAAAG CACCCCAGTGACCACATCTTTAGC	7736 bp 8312 bp	targeted allele targeted allele	ES cell selection ES cell selection
tm1c	tm1c_F tm1c_R	AAGGCGCATAACGATACCAC CCGCCTACTGCGACTATAGAGA	218 bp	floxed allele	floxed mice selection
Scx Cre	ra47 ra48	GCAGAACCTGAAGATGTTCGC ACACCAGAGACGGAAATCCATC	500 bp	Cre transgene	Cre mice selection
Rosa Cre	wt forw wt rev mut rev	AAAGTCGCTCTGAGTTGTTAT GGAGCGGGAGAAATGGATATG CCTGATCCTGGCAATTTCG	825 bp 8312 bp	Rosa Cre allele wild type	TM-induced Cre mice genotyping
Cre excised	5 arm FW 3 arm RV	TTCAGTGGCATGGTTTTCAA CATCCTCTTCTCCTGCCTTG	321 bp	Cre-excised allele	Cre-excision characterization

*PCR products are ~5–9kb. Long range PCR (LRPCR) will be performed (Invitrogen SequalPrep LRPCR kit) between a universal primer in the 5’ or 3’ arm of the targeting cassette and 2 different genomic specific primers in the region upstream or downstream of the construct.