Targeted conditional collagen XII deletion alters tendon function

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Abstract

Collagen XII is a fibril-associated collagen with interrupted triple helices (FACIT). This non-fibrillar collagen is a homotrimer composed of three \( \alpha \)1(XII) chains assembled into a collagenous molecule with a C terminal collagenous domain and a large N terminal non-collagenous domain. During tendon development and growth, collagen XII is broadly expressed throughout the extracellular matrix and enriched pericellularly around tenocytes. Tendons in a global \( \text{Col12a1}^{-/-} \) knockout model demonstrated disrupted fibril and fiber structure and disordered tenocyte organization, highlighting the critical regulatory roles of collagen XII in determining tendon structure and function. However, muscle and bone also are affected in the collagen XII knockout model. Therefore, secondary effects on tendon due to involvement of bone and muscle may occur in the global knockout. The global knockout does not allow the definition of intrinsic mechanisms involving collagen XII in tendon versus extrinsic roles involving muscle and bone. To address this limitation, we created and characterized a conditional \( \text{Col12a1} \)-null mouse model to permit the spatial and temporal manipulation of \( \text{Col12a1} \) expression. Collagen XII knockout was targeted to tendons by breeding conditional \( \text{Col12a1} \)-null with Scleraxis-Cre (\( \text{Scx-Cre} \)) mice to yield a tendon-specific \( \text{Col12a1} \)-null mouse line, \( \text{Col12a1}^{\text{Dten/Dten}} \). Both mRNA and protein expression in \( \text{Col12a1}^{\text{Dten/Dten}} \) mice decreased to near baseline levels in flexor digitorum longus tendons (FDL). Collagen XII immuno-localization revealed an absence of reactivity in the tendon proper, but there was reactivity in the cells of the surrounding peritenon. This supports a targeted knockout in tenocytes while peritenon cells from a non-tendon lineage were not targeted and retained collagen XII expression. The tendon-targeted, \( \text{Col12a1}^{\text{Dten/Dten}} \) mice had significantly reduced forelimb grip strength, altered gait and a significant decrease in biomechanical properties. While the observed decrease in tendon modulus suggests that differences in tendon material properties in the absence of \( \text{Col12a1} \) expression underlie the functional deficiencies. Together, these findings suggest an intrinsic role for collagen XII critical for development of a functional tendon.

Introduction

The unique hierarchical structure of tendon is critical for efficient force transmission and directly influences mechanical function [1,2]. The highly aligned tendon extracellular matrix is composed of hierarchically organized components: collagen molecules assemble into fibrils, fibrils bundle to
form fibers, and fibers together with tendon cells organize into fascicles [1–5]. However, the mechanisms underlying the development of tendon hierarchical structure and how it dictates overall tendon function are not fully understood.

Collagen XII, a member of the Fibril-Associated Collagens with Interrupted Triple Helices (FACIT) family, is a quantitatively minor component in tendons and ligaments [6–11]. It is a non-fibrillar collagen that associates with both collagen I fibrils and cell interfaces [12,13]. The molecular structure and localization to flexible bridges between neighboring collagen fibrils suggest a critical role for collagen XII in regulating collagen I fibrillogenesis [6,7,14,15], fibril organization, and interactions with other extracellular matrix components [14,16]. Additionally, collagen XII has been shown to control cell organization and assembly into communicating networks [10,11,17,18], suggesting dual regulatory roles for collagen XII.

Collagen XII is expressed in tendons, as well as bones and muscles [10]. Human mutations in COL12A1 result in myopathic Ehlers-Danlos Syndrome (mEDS), and a number of COL12A1 dominant and recessive mutations have been identified. In these patients, there is an overlapping phenotype with involvement of muscle and connective tissues [19–24]. COL12A1 mutations result in excessive weakness at birth, strikingly hypermobile distal joints, and an absence of deep tendon reflexes. These clinical manifestations indicate a critical role(s) of collagen XII in tendon and ligament as well as muscle and bone development and function.

Previous work in flexor digitorum longus (FDL) tendons from global Col12a1 conditional knockout mice supported the dual roles of collagen XII in driving tendon structure and function [11]. In addition to altered fibril packing and fiber assembly, collagen XII deficiency altered the formation of interacting cellular processes and tenocyte shape, resulting in impaired cell–cell communication possibly via connexin 43. However, these studies using global knockout models were not able to distinguish between intrinsic roles for collagen XII in the tendon versus extrinsic roles due to altered muscle, and bone or other alterations external to the tendon.

The objectives of this work were threefold. One, to create and characterize a novel conditional Col12a1 mouse model. This mouse model allows targeting of the Col12a1 deletion to specific tissues as well as temporal control of collagen XII knockdown. Second, to demonstrate that the deletion can be targeted to tendons using Scleraxis-Cre (Scx-Cre) resulting in a deletion of Col12a1 and collagen XII expression in cells of the tendon lineage. This would be critical to isolating the roles of collagen XII in tendons. Third, to evaluate the effects of a tendon targeted collagen XII knockout on gait and tendon biomechanics. Targeting the collagen XII knockout to the tendon allows an analysis of its intrinsic regulatory roles separate from extrinsic effects due to it roles in muscle and other tissues that exert indirect effects on tendons. This work provides a foundation for future studies to define the intrinsic and extrinsic regulatory mechanisms whereby collagen XII influences the development and maintenance of tendon structure and function.

Results

Generation of a tendon-targeted Col12a1 conditional knockout mouse model

The strategy presented in Fig. 1A was used to generate a novel conditional Col12a1 mouse line (Col12a1floxflox). In this line, exons 15 and 16 in the Col12a1 allele were flanked by loxP elements. Deletion of these exons results in a skip from exon 14 to 17 where the resulting transcript is out of frame. The sequence coded by exons upstream of exon 14 is susceptible to nonsense mediated decay. Therefore, there is no expression of either the long or short Col12a1 transcript. Since collagen XII is a homotrimer of Col12a1 chains, a knockout of Col12a1 results in a knockout of all possible collagen XII isoforms. The presence of the wild type, targeted and floxed alleles were followed at different stages of model development using PCR analyses (Fig. 1B).

Tendon-targeted bitransgenic collagen XII knockout models were obtained after breeding conditional mice with scleraxis Cre (Scx-Cre) mice. Using reporter mice, Cre excision was only observed in tendons and ligaments with other limb tissue showing no excision [25]. These mice were subject to genotyping analysis using Cre primers and specific primers at the junction of the 3′ arm and targeting sequence. Excision of the Col12a1 exons was identified in tendon-targeted Col12a1 conditional knockout Col12a1tm1ten and Col12a1tm1ten mice, but as expected, not in parental Scx-Cre mice or Col12a1tm1ten mice (Fig. 2). The results confirmed Cre-mediated recombination of the Col12a1 allele in the FDL tendon.

Col12a1 gene and protein expression is knocked out in FDLs of Col12a1tm1ten/ten mice

Scx-Cre-mediated excision of exons 15–16 resulted in targeting of the Col12a1 nonsense mutation to tendons. FDLs from day 10 mice were analyzed using qPCR (Fig. 3). Expression of Col12a1 mRNA in wild type control mice was compared to heterozygous (Col12a1tm1/ten) and homozygous (Col12a1tm1tm1/ten) mice. Col12a1 had a significant 76% reduction in Col12a1tm1tm1/ten mice compared to wild type control mice. In contrast, expression of Col12a1 mRNA was reduced by 24% in Col12a1tm1/ten mice compared to wild type control mice. Baseline expression was determined using traditional collagen XII knockout mice [11].
The \( \alpha_1 \) (XII) chain of collagen XII was analyzed immuno-chemically in tendon-targeted Col12a1 knockout mice. The \( \alpha_1 \) (XII) chain was analyzed in day 10 FDL tendons using a WES simple western blotting system. The \( \alpha_1 \) (XII) chain was present at comparable levels in the control group: wild type, Scx-Cre and Col12a1flox/flox mice (data not shown). In contrast, the \( \alpha_1 \) (XII) chain was present at lower levels in Col12a1+/Dten mice and just above background in Col12a1Dten/Dten mice (Fig. 4). Representative samples are shown in Fig. 4A, and the total results are quantitated in Fig. 4B. The Col12a1Dten/Dten and Col12a1+/Dten were reduced 80% and 36%, respectively relative to control.

As seen for mRNA expression, the protein expression in the Col12a1Dten/Dten mice did not reach the baseline established using traditional collagen XII knockout mice. This suggests that there are collagen XII expressing cells from a non-tendon lineage that would not be targeted in these samples. To address this,
PCR analyses of Col12a1 show a knockout of the gene and protein expression data while peritenon cells from a non-tendon narrow zone that require further study. Overall, the gene and protein expression data cannot exclude functional interactions in this narrow zone that require further study. However, cells of the surrounding peritenon show reactivity for collagen XII where the tendon is not in perfect cross section (Fig. 5). In areas the tendon proper in Col12a1+/− mice compared to wild type controls. However, cells of the surrounding peritenon show reactivity for collagen XII in both genotypes (Fig. 5). In areas the tendon proper in Col12a1+/− mice compared to wild type controls. However, cells of the surrounding peritenon show reactivity for collagen XII in both genotypes (Fig. 5). In areas where the tendon is not in perfect cross section there is some reactivity in the adjacent matrix that we believe to be artifact. However, collagen XII is enriched in a variety of interfacial matrices and can interact with collagen fibrils. Therefore, we cannot exclude functional interactions in this narrow zone that require further study. Overall, the data support a targeted knockout in tenocytes while peritenon cells from a non-tendon lineage retained collagen XII expression. Taken together, the gene and protein expression data show a knockout of the α1(XII) chain in the Col12a1+/− mice resulting in the absence of tenocyte produced collagen XII.

Gross phenotype of tendon-targeted collagen XII null mouse models

The Col12a1+/− mice were fertile. Grossly, homozygous Col12a1+/− mice were smaller than both heterozygous Col12a1+/− mice and wild type controls. The wild type and heterozygous mice were comparable in size (Fig. 6A). The mean body weights of mature male Col12a1+/− and Col12a1+/− mice were significantly less (p < 0.05) than that of wild type control male mice. The mean body weights were 26.6 ± 1.6 g (n = 5), 24.2 ± 1.7 g (n = 5) and 23.8 ± 1.8 g (n = 8) for male wild type control, Col12a1+/− and Col12a1+/− mice, respectively. Comparable to the male mice, grossly female homozygous Col12a1−/− mice were smaller than both heterozygous Col12a1+/− mice and wild type controls with the wild type and heterozygous mice comparable in size (data not shown). Female mice had decreased body weights compared to male mice. The mean body weights of female homozygous Col12a1−/− mice were significantly less than both heterozygous Col12a1+/− mice and wild type controls with no significant difference between wild type and heterozygous mice. The mean body weights were 21.8 ± 1.0 g (n = 2), 21.5 ± 1.5 g (n = 8), and 19.5 ± 0.9 g (n = 7) for female wild type control, Col12a1+/− and Col12a1+/− mice. Data from both male and female mice were comparable with wild type and heterozygous Col12a1+/− mice smaller than wild type controls without statistical significance, while Col12a1−/− mice were significantly smaller than wild type mice.

An analysis of gait was done, and mice from all genotype groups walked with regular, even steps with no obvious change in the width of toe spread irrespective of genotype. However, there was a decrease in stride length in the Col12a1−/− mice compared to wild type controls (Fig. 6B). The mean stride lengths in wild type and Col12a1+/− mice were comparable. In contrast, there was a significant decrease (p < 0.05) in the stride length of Col12a1+/− mice compared to wild type controls (Fig. 6C). The mean stride lengths for wild type, Col12a1+/− and Col12a1+/− mice were 73.7 +/- 3.6 mm (n = 2), 76.3 +/- 13.1 mm (n = 3), 59.1 +/- 4.6 mm (n = 3), respectively. Additionally, gross observation of the knee joint upon dissection indicated joint laxity in Col12a1−/− mice, with greater varus and valgus rotation and occasional patella dislocation.

To evaluate musculoskeletal and motor function in the tendon-targeted heterozygous and homozygous mice, grip strength tests were conducted on the forelimbs of mature female and male mice and compared to wild type controls.
Significant impairment of function was observed with the Col12a1\textsuperscript{Dten/Dten} male mice being weaker than the control mice. Heterozygous mice also showed reduced strength, but the results were not statistically significant. The mean grip strengths were significantly different (p < 0.01). The value for male control mice was 0.94 ± 0.10 N (n = 5) while the heterozygous and homozygous mice were 0.76 ± 0.24 N (n = 5) and 0.72 ± 0.13 N (n = 8), respectively. For females, the value for control mice was 0.73 ± 0.17 N (n = 2) while the heterozygous and homozygous mice were 0.86 ± 0.09 N (n = 5) and 0.61 ± 0.11 N (n = 8), respectively. There was no statistical significance in female heterozygous or homozygous mice vs wild type control mice due to small sample size.

Reduction in biomechanical properties of FDL tendons in Col12a1\textsuperscript{Dten/Dten} mice

FDL tendons from day 60 male and female Col12a1\textsuperscript{Dten/Dten} mice exhibited a reduction in biomechanical properties. There was no difference in cross-sectional area (Fig. 8A). However, stiffness and modulus were both significantly decreased in the Col12a1\textsuperscript{Dten/Dten} FDLs compared to controls (Fig. 8B,C). In female mice, stiffness of Col12a1\textsuperscript{Dten/Dten} FDLs was also significantly less compared to heterozygous Col12a1 Col12a1\textsuperscript{+/Dten}.
and there were no significant biomechanical changes due to partial Col12a1 knockdown in Col12a1\textsuperscript{+/ten} mice. These results suggest that an absence of Col12a1 expression significantly reduces the biomechanical properties of these FDL tendons. In contrast, there were no differences stress relaxation (Fig. 8D), indicating a comparable viscoelastic response between Col12a1\textsuperscript{+/ten}, Col12a1\textsuperscript{+/ten}, a and control Col12a1\textsuperscript{flox/flox} tendons.

**Discussion**

We created a conditional Col12a1-null mouse model and specifically targeted collagen XII knockout to tendons using a scleraxis (Sox) Cre driver. Collagen XII is a fibril-associated collagen with interrupted triple helices. Although collagen XII is considered a quantitatively minor component in collagen I-containing tissues, it is widely expressed in embryonic tissues and throughout tendon development [11]. Collagen XII localizes to the fascicular ECM, interfacial regions, and near cell–cell junctions within tendons [6]. Collagen XII also has been shown to interact with collagen I [12], influencing fibril assembly [13,26].

In humans, mutations in COL12A1 result in myopathic Ehlers-Danlos syndrome (mEDS), leading to a broad range of connective tissue defects. Patients classified with mEDS present with myopathy as well as symptoms similar to other EDS types, such as joint laxity and hypermobility, joint contractures, and abnormal wound healing. These symptoms clearly implicate alterations in tendon and ligament, and a global Col12a1\textsuperscript{-/-} knockout murine model has been shown to recapitulate many features of mEDS [21]. Our previous work using the Col12a1\textsuperscript{-/-} model demonstrated that collagen XII affects tenocyte organization, cell–cell communication, and collagen fibril interactions, thereby regulating tendon structure and function [11]. However, a broad range of musculoskeletal tissues including muscle and bone are affected in this model, and the intrinsic regulatory roles of collagen XII on tendon structure and function remain unelucidated. Our conditional Col12a1 knockout model overcomes this limitation by permitting spatial and temporal manipulation of collagen XII expression. Using this model to target the collagen XII deficiency specifically to cells of the tendon lineage allowed the analysis of the intrinsic roles of collagen XII in the tendon as opposed to extrinsic effects due to alterations in bone and muscle and their interactions with tendon. The current results support intrinsic roles for collagen XII in determining tendon function.

In FDLs of tendon-targeted Col12a1\textsuperscript{+/ten} mice, both mRNA and protein expression levels were near baseline, but did not reach the baseline established using global collagen XII knockout.
mice. This suggests that cells from a non-tendon lineage are not targeted, as expected. Immunofluorescence localization of collagen XII demonstrated efficient knockdown in the tendon proper, but not in the surrounding peritenon, indicating that this population contributes to above baseline expression in whole tendons. This finding is not unexpected as peritenon cells express significantly lower levels of scleraxis [27–29], and collagen XII also is expressed in tissue sheaths and basement membrane zones [30,31].

In the absence of Col12a1 expression and therefore collagen XII, Col12a1<sup>flox/ten</sup> mice have impaired joint and tendon function, as evidenced by reduced forelimb grip strength and FDL tendon mechanical properties. Reduced grip strength in this tendon-targeted model is consistent with joint function in the global Col12a1<sup>T</sup> knockout model [21]. This indicates that altered collagen XII interactions in the tendon proper are critical determinants of joint function. Grip strength in the Col12a1<sup>ten/ten</sup> mice is reduced, but not to the same magnitude as in the global knockout [21]. This suggests that involvement of extrinsic influences such as from muscle and bone are important. However, the deletion in tendon contributes substantially to the observed effect supporting intrinsic roles for collagen XII in the tendon. Interestingly, FDL tendon mechanical properties in the targeted mice were altered compared to control tendons, but contrasted with previous findings in the global knockout model. In the global Col12a1<sup>T</sup> knockout model, FDLs had larger cross-sectional area and greater stiffness with no difference in tendon material properties [11]. In this study, however, there were no differences in FDL cross-sectional area in Col12a1<sup>ten/ten</sup> mice, but stiffness was significantly lower, resulting in inferior tendon elastic modulus. Differences in mechanical properties suggest that collagen XII is a critical regulator of tendon structure–function, and the discrepancy from the global knockout model may be a result of extrinsic effects, such as those due to altered muscle and bone. Therefore, joint and tendon functional changes observed in the tendon-targeted Col12a1<sup>ten/ten</sup> model support significant contributions intrinsic to tendon and ligament in altered mechanical properties and in the reduction in grip strength.

Tendon-targeted Col12a1<sup>ten/ten</sup> mice allowed the spatial targeting of the mutation to tendons, circumventing any potential secondary influences such as those due to alterations in cartilage, bone, and muscle. The conditional model also can be utilized in its heterozygous and/or homozygous state to isolate dose dependent effects, the contributions of different tissues, define the temporal requirements for Col12a1 expression, and to probe the mechanisms underlying pathobiology. In conclusion, we created a conditional Col12a1 mouse model that permits the spatial and temporal targeting of the deletion. Utilizing these mice, intrinsic roles for Col12a1 in tendon function were supported.

**Experimental procedures**

This work was approved by the University of South Florida and the University of Pennsylvania Institutional Animal Care and Use Committees. These experimental studies utilized male and female mice. The tendon-targeted (Scx-Cre) collagen XII knockout mouse model (Col12a1<sup>ten/ten</sup>) is in a C57/BL6 Charles River background. Control mice were wild type, Scx-Cre, Col12a1<sup>flox/fox</sup> and Col12a1<sup>flox/flox</sup>, all in a C57/BL6 Charles River background.

**Development of tendon-targeted collagen XII knockout mouse model**

A novel conditional Col12a1 mouse line (Col12a1<sup>flox/fox</sup>) was created using a strategy previously described [41–44]. The strategy is presented in (Fig. 1A). A targeted Col12a1 ES cell line, Col12a1<sup>flox/fox</sup>/KOMP<sup>Tm2a(KOMP)Wtsi</sup> was obtained from the KOMP Repository (University of California at Davis, project ID: CSD29388). The line was validated, and the ES cell were microinjected into blastocysts and subsequently implanted. We obtained chimeric mice that were crossed with C57BL/6 mice and targeted Col12a1<sup>T/+</sup> mice were selected (Fig. 1B). The targeted mice were crossed with a germine-specific FLPe transgenic mouse (B6;SJL-Tg(CTFLPe)9205Dym/J, Jackson Labs) to remove the FRT flanked neo cassette and Lac Z sequences to yield heterozygous floxed Col12a1<sup>T/+</sup> mice (Fig. 1A).

**Fig. 6.** Smaller size and altered gait in Col12a1<sup>ten/ten</sup> mice. (A) Representative gross images showing that Col12a1<sup>ten/ten</sup> mice are smaller than control Col12a1<sup>T/+</sup> and heterozygous Col12a1<sup>T/+ten</sup> mice, with no obvious body size difference between control and heterozygous mice. All mice are male litter mates at 60 days. (B) Representative gait analysis showed shorter stride length in Col12a1<sup>ten/ten</sup> mice compared to wild type controls. However, all mice walk with regular, even steps, and there is no obvious change in the width of toe spread. The arrowed vertical lines indicate the stride lengths from the 3rd toe to adjacent 3rd toe of the same hind foot. Male 60 day mice. (C) Quantitative presentation of the stride length data from (B). There was a significant decrease in stride length in Col12a1<sup>ten/ten</sup> mice compared to heterozygous and wild type mice. There was no difference in the latter two groups. (*p < 0.05).
The resulting offspring were cross-bred with C57BL/6 (Charles River) mice for 6 generations and then inter-crossed resulting in the conditional knockout mouse model, Col12a1^flox/flox. Mice at each different stage were characterized using PCR that amplified specific element sequences.

The conditional mice were bred with Scleraxis Cre (Scx-Cre) mice to produce bitransgenic tendon-targeted collagen XII knockout models (Fig. 2) as previously described [32,33]. The Scx-Cre mice were a gift from Dr. Ronen Schweitzer, Oregon Health and Science University. Genotyping analysis of tendon-targeted Col12a1 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.
RT-PCR

FDL tendons were removed from wild type, Scx-Cre, Col12a1\textsuperscript{floxflo}, Col12a1\textsuperscript{Dten/Dten}, and Col12a1\textsuperscript{Dten/Dten} mice (gender not determined) at day 10. The tissue samples were cut into small pieces and lysed in QIAzol reagent (Qiagen, Germantown, MD), and the crude total RNA was processed using 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 Col12a1 mRNA expression levels were normalized with β-actin. The primers for the real time PCR reactions are Col12a1 FW: 5'-CCCTA CAACAGATGGGGCTAC-3', Col12a1 RV: 5'-TCT TCTCCCGCTGTTTGA-3'; β-actin FW: 5'-AGAT GACCCAGATCGTTTGA-3', β-actin RV: 5'-CACAGGCCTGGATGGTACG-3'. A one-way ANOVA with Tukey post-hoc tests was conducted to compare genotypes. Significance was set at \( p \leq 0.05 \).

ImmunobLOTS

Collagen XII content was analyzed immunologically using a Wes\textsuperscript{TM} automated western blotting system (ProteinSimple, San Jose, CA). FDL samples were dissected from wild type, Scx-Cre, Col12a1\textsuperscript{floxflo}, Col12a1\textsuperscript{Dten/Dten}, and Col12a1\textsuperscript{Dten/Dten} mice at day 10. Individual mice (\( n = 3–8 \)) were used for each genotype. The FDLs from each mouse were cut into small pieces, and protein was extracted using RIPA buffer (EMD Millipore) with proteinase inhibitor cocktail (ThermoFisher Scientific). Denatured protein samples (0.375 \( \mu g \)) were loaded into single designated wells of Wes Separation 12–230 kDa 25 Capillary Cartridges, 1:50 diluted rabbit anti-mouse Col XII antibody (KR33, gift from Dr. Manuel Koch, University of Cologne, Cologne, Germany), and the Wes anti-rabbit detection module was used for collagen XII detection. Quantification by densitometry was performed using the area of the targeted protein (collagen XII) and normalized to the area of β-actin. The loading of denatured protein samples β -actin was 1.5 \( \mu g \) and the monoclonal anti- β -actin antibody produced in mice (AC-15) was ordered from Sigma. 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). A one-way ANOVA with Tukey post-hoc tests was conducted to compare genotypes. Significance was set at \( p \leq 0.05 \).

Immunofluorescence microscopy

FDL tendons were dissected from wild type and Col12a1\textsuperscript{Dten/Dten} mice at day 14, fixed in 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) compound, and frozen at –80 °C. Frozen sections (5 \( \mu m \)) were cut using a HM 525 NX cryostat (ThermoFisher Scientific). Immunofluorescence localization was performed to analyze spatial expression of collagen XII using a rabbit anti-mouse Col XII antibody (KR33, 1:500 dilution) with a donkey anti-rabbit IgG-Alexa Fluor 568 (ThermoFisher Scientific) secondary antibody. Sections were mounted using Fluoromount-G\textsuperscript{TM} (SouthernBiotech, Birmingham, AL) with DAPI was used as a nuclear marker. Images were captured with a Leika DMi8 Microsystem (Wetzlar, Germany) and Leica DMC6200 digital camera. Antibody incubations and image acquisition were done concurrently for WT and Col12a1\textsuperscript{Dten/Dten} sections using identical procedures and settings to facilitate comparison.

Gait analysis

For gait analysis footprint recording was done for individual mice. The hind feet were dipped in
nontoxic paint, and the mouse was allowed to walk freely on a piece of white paper in a walkway consisting of two Plexiglas walls, spaced 8 cm apart. Stride length was the distance between adjacent prints made by the same hind limb from 3rd toe to 3rd toe. A mixed sample of male and female mice at day 60 was analyzed. The footprint recording was repeated at least twice for each mouse.

Grip strength

Grip strength was evaluated in mature Col12a1^fl/fox^ control, Col12a1^f/+^ten and Col12a1^f/+^ten^m^ mature male and female mice at day 65 to 90. 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 forelimb. 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. Mice were tested for each genotype (n = 5–8). Ten measurements from each mouse were recorded, and the average force was used to represent the grip strength for individual mice. For female and male mice, separate one-way ANOVAs with Tukey post-hoc tests were conducted to compare genotypes. Significance was set at p ≤ 0.05.

Biomechanical analyses

FDL tendons (n = 4–9/group) were dissected from the mouse foot, cleaned free of excess soft tissue, and mechanically evaluated as previously described [32–35]. Tendon cross-sectional area was measured using a custom laser device [36], and each end of the FDL tendon was adhered to sandpaper using cyanoacrylate glue at a gauge length of approximately 5 mm. Tendons were then secured in custom made fixtures, placed in a phosphate buffered saline bath at 37 °C, and loaded in a mechanical testing system (model 5542, Instron, Norwood, MA). To determine biomechanical properties, mechanical tensile testing was performed with the following protocol: a preload to 0.05 N; 10 cycles of preconditioning (0.1–0.2 N); 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 a rate of 0.5 %/s. A 10 N load cell was used with a resolution of 0.01 N. During testing, images were captured (Basler, Exton, PA) every 5 s for optical strain analysis. A custom Matlab program (Matlab R2019b, Natick, MA) was used to optically track strain lines to quantify elastic modulus. For female and male mice, separate one-way ANOVAs with Tukey post-hoc tests were conducted to compare genotypes. Significance was set at p ≤ 0.05.

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Author contributions

A.F. and M.S were involved in investigation, methodology, validation, visualization, and writing the original draft. All authors were involved with data curation, and formal analysis of the data. L.J.S. and D.E.B. were involved in project conceptualization, funding acquisition, supervision, administration, and review and editing of the manuscript.

DATA AVAILABILITY

Data will be made available on request.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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