Rcn3 is Involved in Postnatal Tendon Development by Regulating Collagen Modification and Fibrillogenesis

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Conflict of Interest Statement: The authors have declared that no conflict of interest exists.

Abstract

Tendon plays a critical role in the joint movement by transmitting force from muscle to bone. This transmission of force is facilitated by its structure, which consists of an aligned and organized type I collagen. Despite the importance of collagen structure, the biological mechanisms regulating fibrillogenesis are not well understood. Here we examine the function of Rcn3 in postnatal tendon development and collagen fibrillogenesis using a genetic mouse model. Loss of Rcn3 in tendon caused decreased tendon thickness, abnormal tendon cellular maturation, and decreased mechanical properties. Interestingly, Rcn3 deficient mice exhibited a relatively smaller distribution of collagen fibril and over-hydroxylation in C-telopeptide cross-linking lysine from α1(1) chain. Besides, the proline 3-hydroxylation sites in type I collagen were also over-hydroxylated in Rcn3 deficient mice. Our data collectively suggest that Rcn3 is required for proper postnatal tendon development via regulation of collagen modification and fibrillogenesis.

Introduction

Tendon is critical tissue for both structure and function of the musculoskeletal system. Tendons are primarily composed of type I collagen. Individual collagen fibrils arrange themselves into the highly organized collagen bundles that provide strong tensile strength. The tensile strength differs significantly from 50 MPa to 120 MPa, depending on the type of tendon[1]. Uniaxial biomechanical testing is most commonly used to characterize the mechanical properties of tendons, which are important indicators of tissue function[2]. This test is composed of cross-sectional area structural properties such as stiffness, failure load, and material properties such as stress relaxation, failure stress, and modulus.

Developmental stages of tendons are marked by an increased number of collagen fibrils deposited in the matrix[3]. However, postnatal stages are shown to increase the diameter of the collagen fibrils rather than the increase in collagen fibril number[3]. This transition occurs in the rough endoplasmic reticulum and involves a complex process in which small collagen fibrils undergo lateral fusion to form thicker fibrils[4]. Mutations in genes that regulate this process, including Decorin, Fibromodulin, Lumican, and Tenomudulin, can cause defects in tendon maturation[5–10].

Tendons undergo dramatic changes in tissue and cell morphology throughout postnatal development. Tendon structure becomes denser and more organized via matrix production and collagen fibrillogenesis. Cell density becomes lower as matrix production increases, and cell proliferation decreases[11]. The nuclei shape of tendon cells turned from round to a short spindle-shaped, and eventually to a long, flat spindle-shaped[12]. Previous studies showed that several signaling pathways and transcription factors are critical regulators of embryonic and postnatal tendon development, but the precise regulatory mechanisms for cell and collagen fibrillogenesis during postnatal tendon development are still not clear[13–19].

Collagens consist of repeating Gly-X-Y triplets. Procollagen biosynthesis occurs in the ER and has characteristic post-translational modifications, including hydroxylation and glycosylation. Two modifications of proline residues are known, prolyl 4-hydroxylation and prolyl 3-hydroxylation[4]. Almost all proline residues lying in the Y position are prolyl 4-hydroxylation. Thus prolyl 4-hydroxylation stabilizes the collagen triple helix because the melting temperature of the collagen triple helix is directly proportional to the prolyl 4-hydroxylation content[20,21]. Three modification sites on prolyl 3-hydroxylation are identified in type I collagen, including α1(I) Pro986, α1(I) Pro707, α2(I) Pro707, and a C-terminal (GPP)n motif, which are mainly modified by P3H2[22]. The total prolyl 3-hydroxylation content in the adult Achilles tendon collagen was higher than that in fetal Achilles tendon collagen[23]. Several studies suggested that prolyl 3-hydroxylation involved in collagen fibril assembly[22,24,25].

Reticulocalbin 3 (Rcn3) is an endoplasmic reticulum (ER) protein localized to the secretory pathway. Rcn3 was first discovered by bioinformatics analysis of cDNA from the human hypothalamus and indicated that Rcn3 functioned as a chaperone protein[26]. Moreover, Rcn3 emerged as a new potential regulator of collagen production *in vitro*[27]. Rcn3 knockout mice exhibited neonatal lethality caused by impaired synthesis and secretion of surfactant in lung tissue[28]. However, the function of Rnc3 in postnatal tendon development is not known.

In this study, we investigated the function of Rcn3 in postnatal tendon development by conditionally deleting Rcn3 in tendon cells. Loss of Rcn3 caused impaired postnatal tendon maturation with markedly decreased tendon thickness. Consistently, mechanical properties were reduced in Rcn3 deficient mice due to structural properties but not material properties. Rcn3 deficient mice also exhibited impaired fibrillogenesis and hyper-modification of type 1 collagen. These data suggest that Rcn3 is a critical regulator of postnatal tendon development via regulation of collagen modification and fibrillogenesis.

Results

Loss of Rcn3 caused decreased tendon thickness.

To understand the function of Rcn3 in tendons, we generated *Scx-Cre; Rcn3*$^{fl/fl}$ mice in which Rcn3 was specifically deleted in Scx-expressing cells that are abundant in tendons[29,30]. The growth of *Scx-Cre; Rcn3*$^{fl/fl}$ mice was normal compared with wild-type mice, which was evident with normal body weight (Figure 1A). To access the knockout efficiency of Rcn3 in tendons, we performed the immunohistochemistry analysis using an antibody against Rcn3 (Figure 1B). Rcn3 was highly expressed in patellar and Achilles tendons in wild-type mice at the early postnatal stage including P5, P21, and P30, but its expression was decreased at a later stage, P60 (Figure 1B). Immunohistochemistry analysis demonstrated that the deletion of Rcn3 in *Scx-Cre; Rcn3*$^{fl/fl}$ tendons was efficient in both patellar and Achilles tendons throughout the postnatal maturation (Figure 1B). In the patellar tendon, we found residual expression till P30, but the expression was not detected at P60. In the Achilles tendon, we observed residual Rcn3 expression at P5 and P21, but the expression was not detected at P30 and P60. These data suggest that the deletion of Rcn3 is more efficient in the patellar tendon than Achilles tendon.

We performed histological analyses on patellar and Achilles tendons to assess the tendon phenotypes in *Scx-Cre; Rcn3*$^{fl/fl}$ mice. Although *Scx-Cre; Rcn3*$^{fl/fl}$ mice had no difference in body weight compared to wild-type mice (Figure 1A), *Scx-Cre; Rcn3*$^{fl/f}$ mice clearly displayed decreased tendon thickness at P30 compared to wild-type littermate in both patellar (Figure 2A) and Achilles tendon (Figure 2C). Our quantification results confirmed the 40% reduced thickness in the patellar tendon and 20% reduced thickness in the Achilles tendon (Figure 2B and 2D). We also found that the cellularity of tendons in *Scx-Cre; Rcn3*$^{fl/fl}$ mice was higher than wild-type mice. Our quantification result verified the higher cell density in both patellar (3 fold) and Achilles (1.5 fold) tendons of *Scx-Cre; Rcn3*$^{fl/fl}$ mice (Figure 2B and 2D). The more severe phenotypes in the patellar tendon are consistent with our deletion efficiency result (Figure 1B).

Collectively, these results suggest that Rcn3 is critical for postnatal tendon development for both patellar and Achilles tendons.

Loss of Rcn3 reduced collagen fibril diameter.

The decreased tendon thickness prompted us to examine the collagen fibril diameter because collagen fibrillogenesis is critical for tendon growth. We performed transmission electron microscopy (TEM) using patellar and Achilles tendons at two months of age to measure collagen fibril diameter. *Scx-Cre; Rcn3*$^{fl/fl}$ mice exhibited decreased fibril diameter in both patellar (Figure 3A) and Achilles tendons (Figure 3C) compared with wild-type mice. Specifically, quantification results showed that the distribution of collagen fibril diameter in patellar tendon ranged from 20 to 210nm in wild-type mice, whereas *Scx-Cre; Rcn3*$^{fl/fl}$ mice showed a narrow distribution of collagen fibril diameter ranged from 10 to 170nm in the patellar tendon (Figure 3B). Consistent with the patellar tendon, the Achilles tendon also showed the same trend to the patellar tendon. The Achilles tendon of wild-type mice had the distribution of collagen fibril diameter ranging from 10 to 260nm, but *Scx-Cre; Rcn3*$^{fl/fl}$ mice showed the distribution of collagen fibril diameter ranging from 10 to 190nm (Figure 3D). These results suggest that Rcn3 is required for proper collagen fibrillogenesis, and decreased tendon thickness could be due to the smaller diameter of collagen fibril in *Scx-Cre; Rcn3*$^{fl/fl}$ mice.

Loss of Rcn3 increased collagen modification.

Collagen post-translational modifications and cross-linking analysis were investigated in *Scx-Cre; Rcn3*$^{fl/fl}$ mice at 1 month of age. The modification state of several key residues was examined using peptide mass spectrometry. Notable findings include the C-telopeptide cross-linking lysine from α1(I) chain, which was slightly over-hydroxylated in *Scx-Cre; Rcn3*$^{fl/fl}$ patellar

(20% more hydroxylated) and Achilles (9% more hydroxylated) tendons compared to wild-type mice (Table 1A). These data support that partial telopeptide lysine hydroxylation in tendon is being shifted to fuller hydroxylation in *Scx-Cre; Rcn3*$^{fl/fl}$ mice. This is a useful mark of altered collagen cross-linking and is consistent with an increase in pyridinoline cross-links. Indeed, the cross-linking analysis revealed an increase in hydroxylysylpyridinoline in both tendons of the *Scx-Cre; Rcn3*$^{fl/fl}$ mice. It should be noted that while hydroxylysylpyridinoline provides a useful measure of stable (irreversible) cross-links, it is not a measure of the total cross-links (Table 1B). The pattern of collagen over-modification was also observed across the sites of proline 3-hydroxylation in type I collagen in *Scx-Cre; Rcn3*$^{fl/fl}$ Achilles and patellar tendons (Table 1). A function has yet to be discovered for this rare modification, but it has been suggested that 3-hydrxyproline can regulate the postnatal lateral fusion of smaller fibrils in developing tendons[23].

Loss of Rcn3 caused abnormal tendon cell morphology.

To investigate the cellular phenotype caused by the deletion of *Rcn3*, we analyzed tendon cell morphology using confocal microscopy after Zo1 and phalloidin double staining (Figure 4A). Tendon cell areas became largest at P30 and decreased subsequently in wild-type mice (Figure 4B, white bar). Interestingly, tendon cells in *Scx-Cre; Rcn3*$^{fl/fl}$ mice exhibited significantly increased cell area at P21, but significantly decreased cell areas at P30 compared to wild-type mice (Figure 4B). We also found the changes in protrusion number in tendon cells of *Scx-Cre; Rcn3*$^{fl/fl}$ mice. The protrusion is projections from the cell body that is critical for tendon cell interaction. The protrusion number decreased during postnatal tendon maturation in wild-type mice (Figure 4C, white bar). Interestingly, the *Scx-Cre; Rcn3*$^{fl/fl}$ mice showed a significantly increased protrusion number compared with wild-type mice at all stages of postnatal tendon development (Figure 4C). Collectively, these results suggest that the function of Rcn3 is indispensable for morphological maturation of tendon cells.

Loss of Rcn3 enhanced cellular maturation.

To further understand the cellular phenotype in the molecular level, we performed qRT-PCR using Achilles tendon from wild-type mice and *Scx-Cre; Rcn3*$^{fl/fl}$ mice. First, we confirmed the efficient deletion of Rcn3 in the Achilles tendon of *Scx-Cre; Rcn3*$^{fl/fl}$ mice (Figure 5). The expression of tenogenic markers, such as *Scx*, *Mkx*, *Col1a1,* and *Tnmd*, were significantly increased in *Scx-Cre; Rcn3*$^{fl/fl}$ mice compared with wild-type mice (Figure 5). These results suggest that the loss of Rcn3 enhanced the tendon cell maturation.

Loss of Rcn3 decreased mechanical properties

To measure the functional outcome of Rcn3 loss in tendons, we performed the uniaxial biomechanical test on Achilles tendon at 2 months of age. Consistent with our histological analysis, cross sectional area (CSA) was significantly reduced in *Scx-Cre; Rcn3*$^{fl/fl}$ tendons (Figure 6A). Concomitantly, stiffness and failure load were also significantly lower, suggesting a loss of structural integrity in the *Scx-Cre; Rcn3*$^{fl/fl}$ mice (Figure 6B and 6C). No difference was observed in percent stress relaxation between the two groups, suggesting that Rcn3 does not perturb the viscoelastic response of Achilles tendon (Figure 6D). No differences were observed in failure stress and tissue modulus, which indicates that loss of Rcn3 did not cause an inherent material change in the tendon tissue (Figure 6E and 6F). These data suggest that reduced mechanical properties caused by loss of Rcn3 is due to decreased structural properties but not material properties.

Discussion

We investigated the function of Rcn3 in postnatal tendon development by characterizing the phenotypes of tendon-specific Rcn3 knockout mouse (*Scx-Cre; Rcn3*$^{fl/fl}$) model. The *Scx-Cre; Rcn3*$^{fl/fl}$ mouse showed impaired tendon maturation phenotypes, including decreased thickness, increased cellularity, impaired tenocyte maturation, and reduced collagen fibril diameter with altered modification. These data suggest that Rcn3 is indispensable for proper postnatal tendon development via regulation of cellular maturation and collagen fibrillogenesis.

We observed that the deletion efficiency of Rcn3 was better in patellar tendons than Achilles tendons (Figure 1B). Consistent with this result, the overall phenotypes of *Scx-Cre; Rcn3*$^{fl/fl}$ mice were more pronounced in patellar tendons. The different deletion efficiency could be due to the different expression levels of CRE recombinase in Scx-Cre line. Further studies will be required to determine specific CRE recombinase activity in the different tendons.

To measure morphological changes of tendon cells in single-cell level, we developed a new confocal imaging method (Figure 4). We found that the protrusion number was decreased throughout the postnatal tendon development in wild-type mice. Interestingly, the protrusion number is correlated with the cell density. Consistently, we found increased cell density and protrusion number in *Scx-Cre; Rcn3*$^{fl/fl}$ mice. We also observed that the cross-sectional cell area became largest at P30, which suggests that the active tenocyte population is most abundant at P30. Further thorough analyses of stage-specific tenocytes using our confocal imaging method will be necessary for a comprehensive understanding of morphological maturation of tenocytes during postnatal tendon development.

Scx-Cre; Rcn3$^{fl/fl}$ mice displayed higher post-translational modifications and cross-linking in type I collagen. *Scx-Cre; Rcn3*$^{fl/fl}$ mice showed an increased telopeptide lysine hydroxylation and increased hydroxylysylpyridinoline in both patellar and Achilles tendons. An over-

hydroxylated C-telopeptide cross-linking lysine and increased hydroxylysylpyridinoline reflect stable cross-links and are discovered in adult tendon than fetal tendon. In addition, *Scx-Cre; Rcn3*$^{fl/fl}$ mice exhibited over-hydroxylated proline 3-hydroxylation in type I collagen. The function of this rare modification is not clear, but it has been suggested that 3-hydrxyproline can regulate the postnatal lateral fusion of smaller fibrils in developing tendons[23]. Based on these collagen modification data, we carefully suggest that the reduced collagen fibril diameter in *Scx-Cre; Rcn3*$^{fl/fl}$ mice is due to the inhibition of lateral fusion caused by over-modification in type I collagen. The precise regulatory mechanism of collagen modification by Rcn3 will be the future direction of this study.

Materials and Methods

Animals. Animal care and experiments were performed in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of the University of Pennsylvania (Philadelphia, Pennsylvania, USA). The *Scx-Cre* mouse line was previously described[31]. Mouse sperms carrying Knockout-first *Rcn3* allele (*Rcn3* $^{tm1a(EUCOMM)Hmgu}$) were obtained from the Knockout Mouse Programme (KOMO), and mouse carrying *Rcn3* $^{tm1a(EUCOMM)Hmgu}$ was generated by in vitro fertilization. *Rcn3* $^{tm1a(EUCOMM)Hmgu}$ mice were crossed with *Rosa26-Flippase* (*Flp*) mice to delete β-gal and the neo cassette in order to generate the *Rcn3* conditional knockout allele (*Rcn3*$^{fl/fl}$).

Histological analyses. Mouse hindlimbs were collected from specific stages and fixed in 10% neutral buffered formalin overnight at 4°C. Samples were paraffin-embedded and sectioned at 6 µm, following decalcification in 10% (w/v) EDTA (pH 7.4) for 2 weeks with daily solution changes. The paraffin sections of the patellar and Achilles tendons were used for Hematoxylin and Eosin (H&E). Histological evaluation was blindly performed by 2 independent individuals.

Uniaxial biomechanical test. All mice assigned for mechanical testing were frozen at -20C till the day of testing. Mice were thawed at room temperature, and calcaneal bone – Achilles tendon – muscle complexes were grossly dissected. Under microscope guidance, all extraneous soft tissues and muscles were finely dissected with care to ensure no damage occurred to the tendinous tissue. Following dissection, a custom laser device was used to measure the cross-sectional area (CSA) of the Achilles tendon. The myotendinous junction was sandwiched between two sandpaper tabs with cyanoacrylate glue to prevent any slippage. The calcaneal bone was gripped with a custom fixture, and the construct was mounted onto a

material testing machine (Instron 5542, Instron Inc., Norwood, MA). All testing was conducted in phosphate buffered saline bath at room temperature. Each sample was preloaded to 0.02N followed by 10 cycles of preconditioning between 0.02 to 0.04N. After a resting period of 300 seconds at 0N, the sample underwent stress relaxation after a ramp to 5% strain (assuming a gauge length of 5mm) for 600 seconds. This was followed by a rest of 60 seconds at zero load. Finally, the sample was quasi-statically ramped to failure at a strain rate of 0.03%/s. All data were collected at 100Hz. Ensuing force-displacement curves were analyzed to obtain failure load (N) and tissue stiffness (N/mm, defined as the slope of the linear region). Cross-sectional areas (mm2) and gauge length (mm) values were used to obtain stress-strain curves for each sample. Modulus (N/mm2) was calculated as the slope of the linear region of the stress-strain curve and failure stress (N/mm2) as the maximum stress value observed. Stress relaxation (%) was defined as the percent change in stress between the peak and equilibrium stress during the stress relaxation period of 600 seconds.

Collagen EM analysis. Mouse hindlimbs were fixed in freshly prepared 1.5% glutaraldehyde/1.5% paraformaldehyde (Tousimis) with 0.05% tannic acid (Sigma) in DPBS at 4 °C with agitation overnight. The following day, dissect the relevant tendons out of the limb in PBS as follows: for the Achilles, take the mid-tendons that lies below the myotendinous junction and above the enthesis (~1-1.5 mm in length); and for the patellar tendon, take the complete mid-tendon between the patella and tibial enthesis. Following dissection, samples were post-fixed in 1% OsO4, rinsed in DMEM and dehydrated in a graded series of ethanol to 100%. Samples were then rinsed in propylene oxide, infiltrated in Spurrs epoxy, and polymerized at 70 °C overnight. TEM images were acquired using a FEI G20 TEM at multiple magnifications to visualize transverse sections of collagen fibrils. Collagen fibril diameter was measured using ImageJ.

Collagen extraction. Intact type I collagen was solubilized from mouse patellar and Achilles tendons by acid extraction in 3% acetic acid for 24h at 4°C. Acid-extracted collagen α-chains were resolved by SDS-PAGE and stained with Coomassie Blue R-250.

Collagen cross-linking analysis. Collagen pyridinoline cross-link content was determined by fluorescence monitoring with reverse-phase HPLC. Pyridinoline cross-links were analyzed in mouse tendons by HPLC after acid hydrolysis in 6M HCl for 24h at 108°C. Dried samples were dissolved in 1% (v/v) n-heptafluorobutyric acid for quantitation of hydroxylysyl pyridinoline (HP) by reverse-phase HPLC and fluorescence monitoring as previously described[32].

Mass spectrometry. Collagen α-chains were cut from SDS-PAGE gels and subjected to in-gel trypsin digestion as previously described[24]. Electrospray mass spectrometry was carried out on the trypsin-digested peptides using an LTQ XL linear quadrupole ion-trap mass spectrometer equipped with in-line Accela 1250 liquid chromatography and automated sample injection (ThermoFisher Scientific). Proteome Discoverer software (ThermoFisher Scientific) was used for peptide identification. Tryptic peptides were also identified manually by calculating the possible MS/MS ions and matching these to the actual MS/MS spectrum using Thermo Xcalibur software. Differences in post-translational modifications were determined manually by averaging the full scan MS over several LCMS minutes to include all the post-translational variations of a given peptide. Protein sequences used for MS analysis were obtained from the Ensembl genome database.

Immunofluorescent staining. For immunofluorescent staining, mouse hindlimbs were collected during postnatal tendon maturation from P10 to P60 and fixed in 4% PFA overnight at 4°C. Samples were OCT compound embedded and sectioned at 10 μm, following decalcification in 10% (w/v) EDTA (pH 7.4) for 10 days with daily solution changes. The frozen sections of the patellar tendon blocked with blocking solution (Thermo, 8120) for 1hr RT, followed by incubation at 4°C overnight with antibodies against ZO1 (Fisher, 61-7300) and then incubated for 1hr at RT with Phalloidin Alexa Fluor™ 488 conjugate. Finally, sections were mounted in antifade solution with DAPI (Invitrogen, P36935).

Primary tendon cell culture. For primary tendon cell culture, the tail was collected form P25 in PBS on ice. The skin was removed and then pull out the tendon from the tail. Tail tendons were incubated for 1 hr at 37°C in 2mg/ml collagenase (Sigma, C0130-1G). Tail tendon and cells which were collected by centrifugation were plated on culture dishes. Cells were maintained in growth media (αMEM, 20% FBS, 2mM L-glutamine).

qRT-PCR. RNA was extracted with Trizol and Direct-zol kit (Zymo Research, R2060) from primary tendon cells. And cDNA was synthesized from 1μg RNA using iScript Reverse Transcription (Bio-rad, 1708841). qRT-PCR was performed using Fast SYBR Green Master Master Mix (Pec, 4385612). The sequences of primers are listed in Table 2.

Statistical analysis. Results are expressed as mean ± SD. At least three mice per group were analyzed. Differences between values were analyzed by Student's t-test. $P<0.05$ is considered significant.

Figures and figure legends

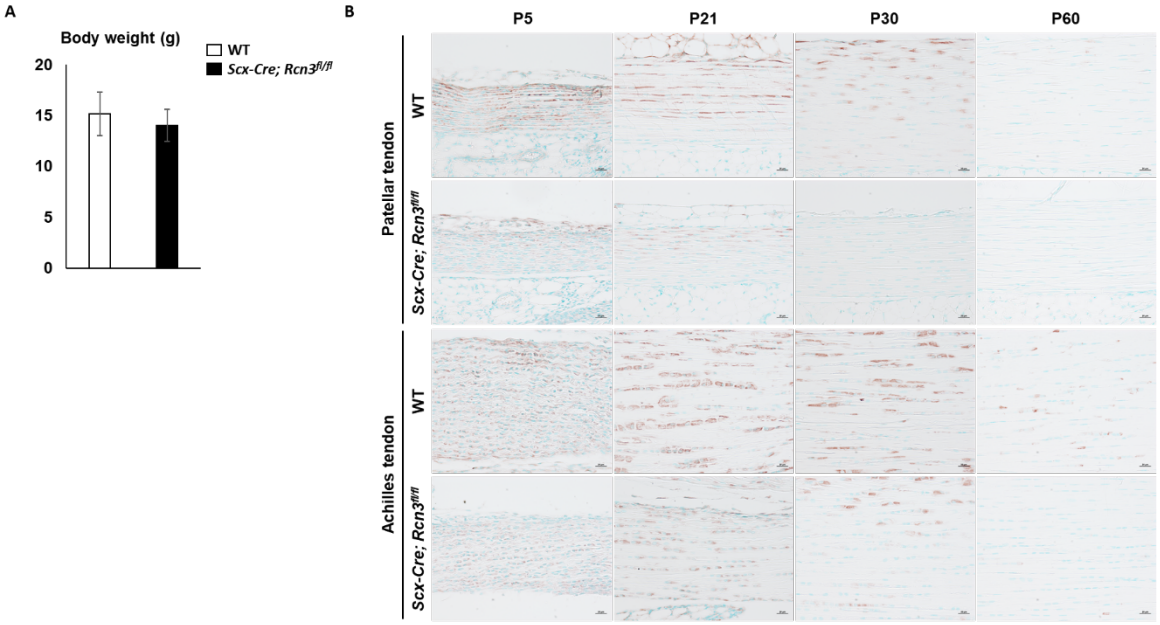

Figure 1. The weight of wild-type mice and *Scx-Cre; Rcn3*$^{fl/fl}$ mice at P30 (A). Immunohistochemical analysis of Rcn3 on patellar and Achilles tendons from wild-type mice and *Scx-Cre; Rcn3*$^{fl/fl}$ littermates during postnatal tendon maturation (B). (Brown color indicates Rcn3, Scale bar indicates 20μm (B) and n=5 (A))

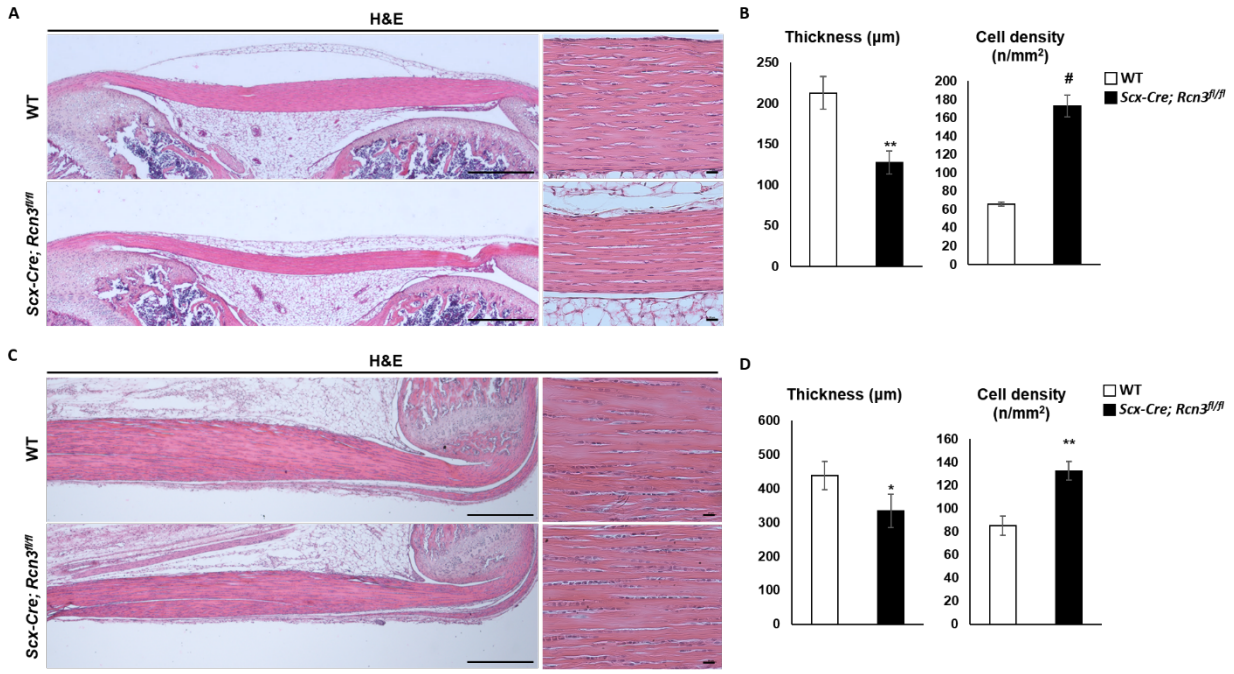

Figure 2. Histology of patellar and Achilles tendons from wild-type mice and *Scx-Cre; Rcn3*$^{fl/fl}$ littermates during postnatal tendon development (A and C). The thickness and cell density of patellar tendon and Achilles tendon at P30 (B and D). (Scale bar indicates 500μm (A) and 20μm (B), * indicates $P<0.05$, ** indicates $P<0.01$ and $^{\#}$ indicates $P<0.001$ between genotypes, n=3)

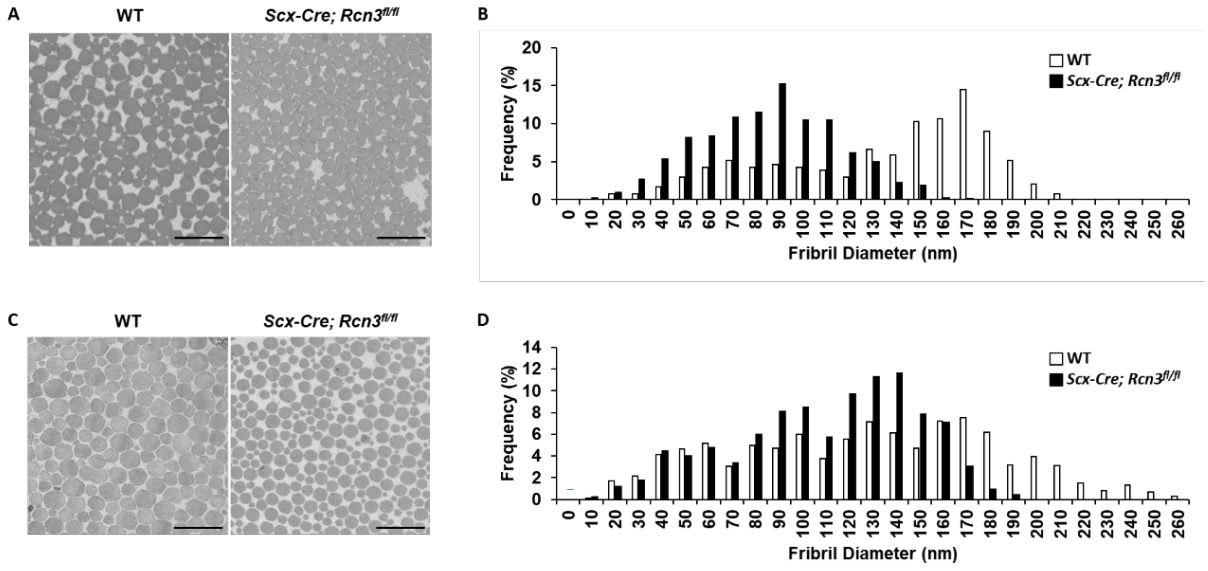

Figure 3. EM image of patellar (A) and Achilles (C) tendons and frequency graph of patellar (B) and Achilles (D) tendons of wild-type mice and *Scx-Cre; Rcn3*$^{fl/fl}$ littermates at P60. (Scale bar indicates 500nm (A and C), n=3)

A

Hydroxylation analysis		Patellar tendon		Achilles tendon	
Site		WT	*Scx-Cre; Rcn3$^{fl/fl}$*	WT	*Scx-Cre; Rcn3$^{fl/fl}$*
Hydroxylysine	α1(I)K87	100%	100%	100%	100%
	α2(I)K87	100%	100%	100%	100%
	α1(I) C-telo Hyl	45%	65%	39%	48%
3-Hydroxyproline	α1(I)P986	92%	96%	92%	94%
	α1(I)P707	60%	80%	50%	71%
	α2(I)P707	45%	65%	77%	87%

B

Cross-linking analysis	Patellar tendon		Achilles tendon	
	WT	*Scx-Cre; Rcn3$^{fl/fl}$*	WT	*Scx-Cre; Rcn3$^{fl/fl}$*
Hydroxylysylpyridinoline (mol/mol)	0.25	0.36	0.09	0.17 *

Table 1. Collagen cross-link and mass spectral analysis to investigate the hydroxylation levels (A) of lysine and proline in type 1 collagen (n=2) and the collagen cross-linking levels (B) (* indicates $P<0.05$ between genotypes, n=1 for patellar and n=3 for Achilles tendon) from wild-type mice and *Scx-Cre; Rcn3$^{fl/fl}$* (tendon-specific Rcn3 loss-of-function model) littermates at P30.

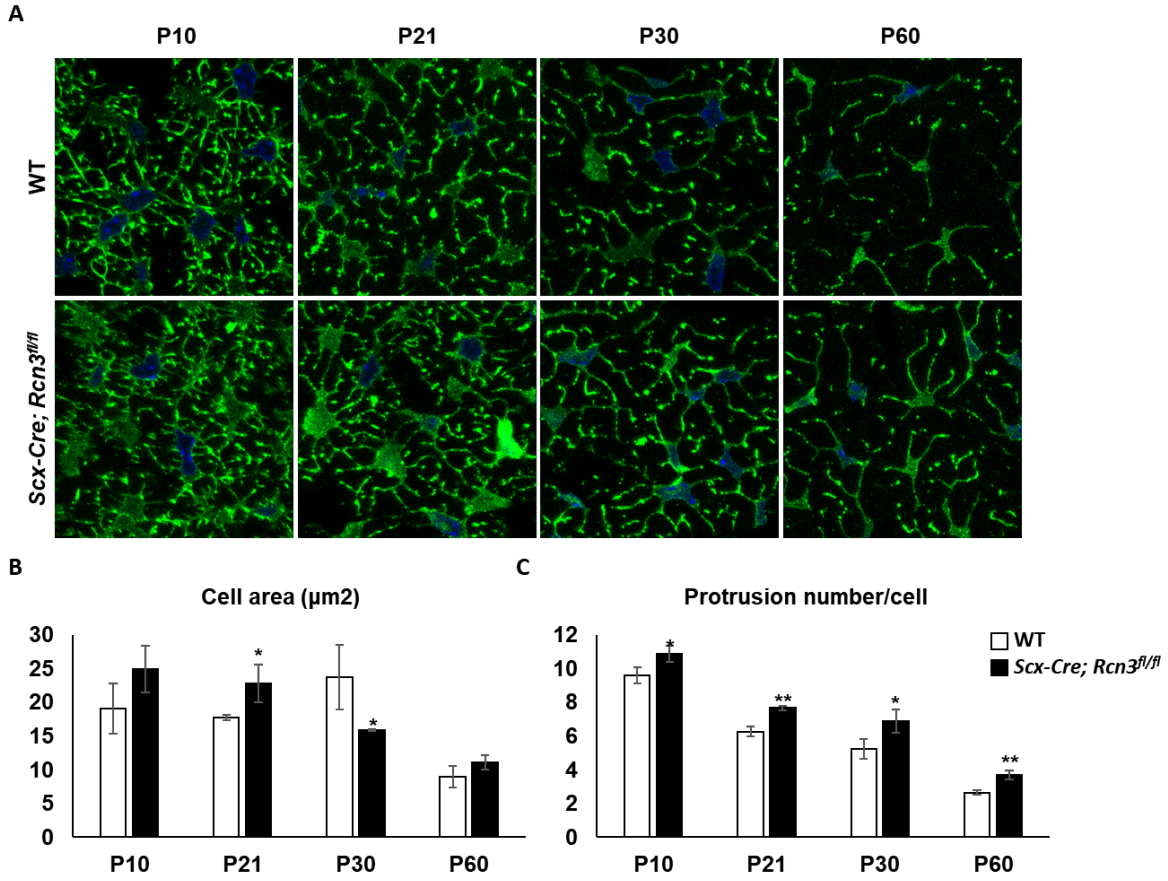

Figure 4. Immunofluorescence staining of ZO1 and phalloidin double staining on cross-sectional patellar tendon from wild-type mice and *Scx-Cre; Rcn3*$^{fl/fl}$ (tendon-specific Rcn3 loss-of-function model) littermates during postnatal tendon maturation (A). Cell area (B) and protrusion number (C) of the patellar tendon. (Scale bar indicates 5µm (A), * indicates $P<0.05$, and ** indicates $P<0.01$ between genotypes, n=3).

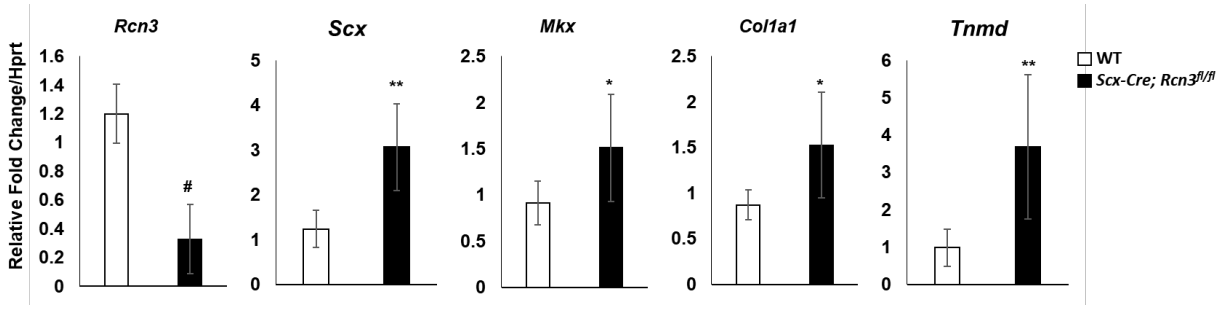

Figure 5. Quantitative real-time PCR of Achilles tendons from wild-type mice and *Scx-Cre; Rcn3$^{fl/fl}$* (tendon-specific Rcn3 loss-of-function model) littermates at P28 (C). (* indicates $P<0.05$, ** indicates $P<0.01$ and # indicates $P<0.001$ between genotypes, wild-type mice n=6, *Scx-Cre; Rcn3$^{fl/fl}$* mice n=5)

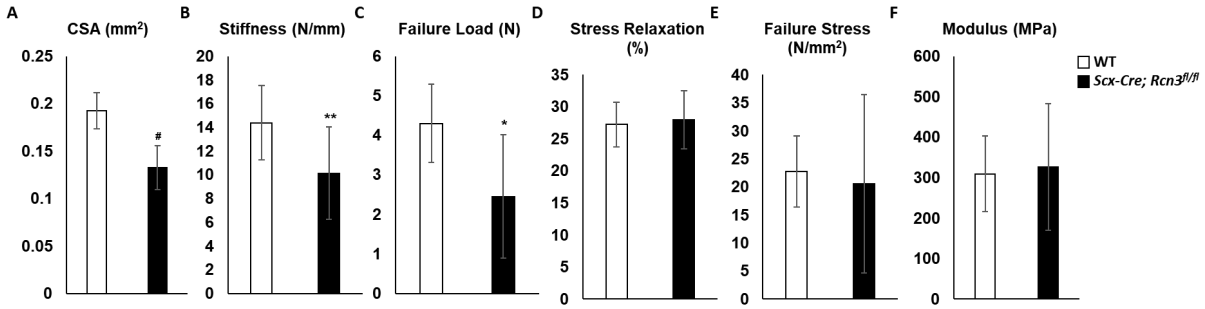

Figure 6. Mechanical property, cross-sectional area (A), stiffness (B), failure load (C), stress relaxation (D), failure stress (E), and modulus (F) of Achilles tendons from wild-type mice and *Scx-Cre; Rcn3*$^{fl/fl}$ (tendon-specific Rcn3 loss-of-function model) littermates at P60. (* indicates $P<0.05$, ** indicates $P<0.01$, and # indicates $P<0.001$ between genotypes, n=7)

Gene	Primer sequences (5'-3')
mRcn3	F: CTA CTC GGG AAG AGC TGA CG
	R: ACC TGC ACG TAG CCA TCT TT
mScx	F: AAG ACG GCG ATT CGA AGT TAG AAG
	R: TCT CTC TGT TCA TAG GCC CTG CTC ATA G
mMkx	F: GAT GGC GAC TCC TGC TCT GA
	R: CGG TCT GCC GCC AGC TTT TA
mCol1a1	F: TTG GGG CAA GAC AGT CAT CGA AT
	R: TTG GGG TGG AGG GAG TTT ACA CGA A
mTnmd	F: CTT TAC TAG GCT ACT ACC CAT ACC CCT ACT
	R: ATA TAT TGG CTA ACA GAA GGT TAA GCG TTT

Table 2. Primer sequences for the quantitative real-time PCR.

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Acknowledgments: We would like to thank Penn Center for Musculoskeletal Disorders (PCMD) Histology and Biomechanics Core for technical assistance with the histology and biomechanical testing, respectively. We also thank Cell and Developmental Biology (CDB) Microscopy Core for technical assistance with confocal microscopy. We thank Dr. Ronen Schweitzer for providing *Scx-Cre* mouse line.

Funding: This work was supported in part by NIH/ NIAMS K01 AR069002 (K. S. J.). The PCMD Cores were supported by NIH/ NIAMS P30AR069619.