

Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons

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The scleraxis (*Scx*) gene, encoding a bHLH transcription factor, is expressed in the progenitors and cells of all tendon tissues. To determine *Scx* function, we produced a mutant null allele. *Scx*^{-/-} mice were viable, but showed severe tendon defects, which manifested in a drastically limited use of all paws and back muscles and a complete inability to move the tail. Interestingly, although the differentiation of all force-transmitting and intermuscular tendons was disrupted, other categories of tendons, the function of which is mainly to anchor muscles to the skeleton, were less affected and remained functional, enabling the viability of *Scx*^{-/-} mutants. The force-transmitting tendons of the limbs and tail varied in the severity to which they were affected, ranging from dramatic failure of progenitor differentiation resulting in the loss of segments or complete tendons, to the formation of small and poorly organized tendons. Tendon progenitors appeared normal in *Scx*^{-/-} embryos and a phenotype resulting from a failure in the condensation of tendon progenitors to give rise to distinct tendons was first detected at embryonic day (E)13.5. In the tendons that persisted in *Scx*^{-/-} mutants, we found a reduced and less organized tendon matrix and disorganization at the cellular level that led to intermixing of tenocytes and endotenon cells. The phenotype of *Scx*^{-/-} mutants emphasizes the diversity of tendon tissues and represents the first molecular insight into the important process of tendon differentiation.

KEY WORDS: Scleraxis, Tendon, Connective tissue, Mouse mutant

INTRODUCTION

The musculoskeletal system consists of three major components: skeletal tissue, which provides structural support and defines the mechanical units; muscular tissue, which generates force; and tendons, which transmit to the skeleton the force generated during muscle contraction. The tendons are thus crucial for both the architecture and the biomechanics of the musculoskeletal system. To fulfill their roles throughout the musculoskeletal system, tendon tissues demonstrate considerable diversity, which has not received much attention in structural and anatomical studies. The most visible tendons are the long chord-like tendons of the limbs and tail, which serve to transmit muscle force to specific skeletal positions. To perform their biomechanical roles, these force-transmitting tendons generate a complex and remarkably organized extracellular structure in the form of tightly packed parallel collagen fibers, enabling the vectorial transmission of force (Benjamin and Ralphs, 2000). Conversely, the origin tendons at the proximal end of the same arm or shank muscles mainly serve to anchor the muscles at their skeletal origins. An extreme example of anchoring tendons can be found in the monolayer of tendon cells that surround the ribs and provide the matrix that connects the intercostal muscles to the ribs.

Tendon studies have focused mostly on force-transmitting tendons and considerable effort has been directed towards the analysis of the protein composition and organization of these

tendons and towards understanding how this organization provides the physical properties necessary for tendon function (Benjamin and Ralphs, 2000; Zhang et al., 2005). By contrast, the ontogeny of the tendons has received far less attention, and little is currently known about the molecules regulating tendon specification, differentiation, patterning or matrix deposition.

Some insight into the requirements for tendon formation has been attained via the analysis of mice harboring genetic mutations that result in discrete tendon phenotypes (reviewed in Tozer and Duprez, 2005). For example, mutations in the small leucine-rich proteoglycan genes decorin (*Dcn*) (Zhang et al., 2006), fibromodulin (*Fmod*) (Svensson et al., 1999) and biglycan (*Bgn*) (Ameye and Young, 2002) result in irregularities in the diameter of collagen fibers in the tendons. Tendon disruptions have also been described in patients with conditions affecting matrix molecules, such as the Ehler-Danlos syndrome, in which the collagen I gene is mutated (Mao and Bristow, 2001). Although these studies highlight the obvious importance of the structural elements that make up the tendon, to date, there is no description of mutations affecting tendon differentiation and patterning.

Several years ago, we found that the bHLH transcription factor scleraxis (*Scx*) is a distinct marker for tendon and ligament progenitors, and differentiated cells (Cserjesi et al., 1995; Schweitzer et al., 2001). *Scx* is a highly specific marker of the tendon/ligament lineage and appears to be induced at the earliest stage of specification of this lineage (Brent et al., 2003; Brent and Tabin, 2004; Schweitzer et al., 2001). As the first robust marker for tendon progenitors, *Scx* expression provided a unique opportunity to follow the origin and regulation of tendon cell fate. We identified a tendon progenitor pool in the limb subjacent to the ectoderm (Schweitzer et al., 2001), and a somitic compartment of tendon progenitors, the syndetome, was later identified at the

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dorsolateral edge of the early sclerotome, arising in response to Fgf signaling from the adjacent myotome (Brent et al., 2003; Brent et al., 2005).

Analysis of *Scx* expression has thus proven to be an extremely powerful tool in tendon studies, but *Scx* function in tendon formation has not been elucidated. Although *Scx* misexpression is not sufficient to alter the normal progression of tendon formation (Schweitzer et al., 2001), as a bHLH transcription factor expressed in all tendon cells from progenitor stages through development, *Scx* is none-the-less a likely candidate to be a transcription factor that is involved in regulating the tendon cell fate.

In a previous attempt to explore *Scx* function, embryos homozygous for a targeted *Scx^{KO}* allele died in the early stages of embryogenesis, thus precluding the ability to address a possible role of *Scx* in tendon differentiation (Brown et al., 1999). Surprisingly, we found that the reported *Scx^{KO}* phenotype was largely due to an effect of the neomycin-resistance (Neo) cassette on neighboring genes. We therefore generated a new loss-of-function allele of *Scx* to re-evaluate the phenotype of a simple *Scx* mutation.

Homozygous *Scx^{-/-}* mice were viable but showed a dramatic disruption of tendon differentiation, manifesting in dorsal flexure of the forelimb paw, the limited use of all paws, reduced functionality of the back muscles and the complete loss of the ability to move the tail. Surprisingly, *Scx* loss did not affect all tendon categories equally. Whereas all force-transmitting and intermuscular tendons were affected in *Scx^{-/-}* mutants (although the effects varied in severity), the muscle-anchoring tendons and the ligaments were not affected. The tendon defects were first detected at or close to E13.5 in all tendons, concurrent with the condensation and differentiation of tendon progenitors that result in emergence of discreet tendon morphologies. *Scx* however, has additional roles in tendon formation and, in tendons that persist in *Scx^{-/-}* mice, we found that the tendon matrix was reduced and disorganized, and the cellular organization of the tendons was disrupted.

This is the first report of a genetic alteration producing a tendon differentiation phenotype. While emphasizing the diversity of tendon tissues, it lays the foundation for unraveling the genes required for tendon formation, and opens the door to the analysis of the transcriptional network regulating the tendon cell fate.

MATERIALS AND METHODS

Generation of *Scx* mutant mice

The vector to target the *Scx* locus was generated using sequences from a 12 kb genomic clone (Brown et al., 1999). The 5' loxP sequence followed by a *HindIII* site was introduced into a *SacII* restriction site in the 5' UTR of *Scx* and the first exon of *Scx* was flanked by inserting the FLP recombinase target (FRT)-Neo cassette appended with a 3' loxP site (Meyers et al., 1998) into an *EcoRI* restriction site in the single intron of the *Scx* gene. Out of 180 embryonic stem cell (ES) clones assayed, approximately 60 were targeted

to the *Scx* locus and 27 retained both loxP sites, as determined by Southern blot using the *HindIII* site in the 5' loxP site. A single *Scx^{fneo}* male was generated by blastocyst injection and mated with FLPeR females (Farley et al., 2000) to produce the conditional *Scx^{fllox}* allele. *Scx^{fllox}* males, in turn, were mated with *prx1/Cre* females, taking advantage of the stochastic ubiquitous Cre activity in females from this line (Logan et al., 2002) to generate the *Scx*-null allele (see Fig. 1A). For genotyping conditions, see Table 1.

Immunohistochemistry and in situ hybridization

For whole-mount visualization of the *ScxGFP* signal, tissues were skinned and observed directly or after 2-6 hours fixation in 4% PFA. Pictures were captured on an MZFLIII dissecting microscope (Leica) with a DXM1200 camera (Nikon). For sections, 12 μ m cryosections were stained with My32 monoclonal antibody (mAb; 1:400, Sigma) to detect the myosin heavy chain, anti-collagen XII antibody (Gregory et al., 2001), anti-EphA4 antibody (1:50; R&D Systems) and the anti-tenascin C antibody (1:100; gift from Harold Erickson, Duke University Medical Center, Durham, NC). Images were captured using a Nikon Eclipse E800 compound microscope and a MicroPublisher cooled CCD camera from QImaging.

Whole-mount and section in situ hybridizations were performed using protocols found on the Tabin laboratory web pages: <http://genepath.med.harvard.edu/~cepko/protocol/ctlab/ish.ct.htm> and <http://genepath.med.harvard.edu/~cepko/protocol/insituprotocol.pdf>.

Probes included *Scx* (Brown et al., 1999) that was also used to detect the *ScxGFP* transcript (Pryce et al., 2007) and tenomodulin (Brandau et al., 2001). The probe for collagen XIV was amplified from the fourth exon of the gene using the following primers: Col14for, 5'-TCCTGATCA-GCCTGAGTTCACACCG-3'; Col14rev, 5'-CAGGAGTGTGTTTCAG-GGCTTGCT-3'.

Cell proliferation was detected using the BrdU In-Situ Detection Kit (BD Pharmingen, #550803). The *ScxGFP* signal was captured from directly alternating sections and merged with the BrdU signal in Photoshop (Adobe). For apoptosis, a standard TUNEL protocol was followed performing the terminal transferase reaction with a biotin-16-dUTP, followed by streptavidin-Cy5 for detection.

Counting tenocytes in a cross section of the FDP tendon

ScxGFP-positive cells were counted in flexor digitorum profundum (FDP) tendons in sections at the level of the digit vinculum of both wild-type and mutant embryos at E18.5 (Fig. 4B,E, white arrowhead). The sections were stained with an antibody to beta-catenin to highlight the cell membranes and with DAPI to highlight the nuclei and help identify the individual cells. Cells were counted in ten sections from five different embryos and gave an average of 97 FDP tenocytes/section in wild-type embryos and 56 FDP tenocytes/section in mutant embryos.

RESULTS

Generation of an *Scx*-null allele

Scx expression in early tendon progenitor cells suggested that it might be important for tendon formation (Schweitzer et al., 2001), but *Scx^{KO}* embryos died before E8.5, precluding the evaluation of the effects on tendon formation (Brown et al., 1999). We therefore

Table 1. Genotyping of *Scx* alleles

PCR	Forward primer (5'-3')	Reverse primer (5'-3')	PCR conditions	Product
ScxNull	Scx5' gen: CACACGGCCTGGCACAAAAGACC	Scxint1rev: GAGGGGTAGTGGCACATCAGC	40 \times (94°C, 1 min; 62°C, 1 min; 72°C, 1 min)	578 bp
Scxflox	Scx5' UTRA: TGGCGGCCCACTCCAGTCC	ScxExon1rev: AAGCCCGTGTTCACGCTGTTGG	40 \times (94°C, 1 min; 63°C, 1 min; 72°C, 1 min) add 10% DMSO	485 bp
ScxWT	Scx5' UTRA: TGGCGGCCCACTCCAGTCC	ScxExon1rev: AAGCCCGTGTTCACGCTGTTGG	40 \times (94°C, 1 min; 63°C, 1 min; 72°C, 1 min) add 10% DMSO	451 bp

The ScxNull PCR extends across the sequences of the first exon of *Scx* missing in the *Scx⁻* allele. The Scxflox and ScxWT PCRs both make use of the same primer set and generate a product that spans the 5' loxP site in the *Scx^{fllox}* allele. In a heterozygous mouse, *Scx^{fllox/+}*, this PCR reaction would therefore result in two bands representing both the wild-type and *Scx^{fllox}* alleles.

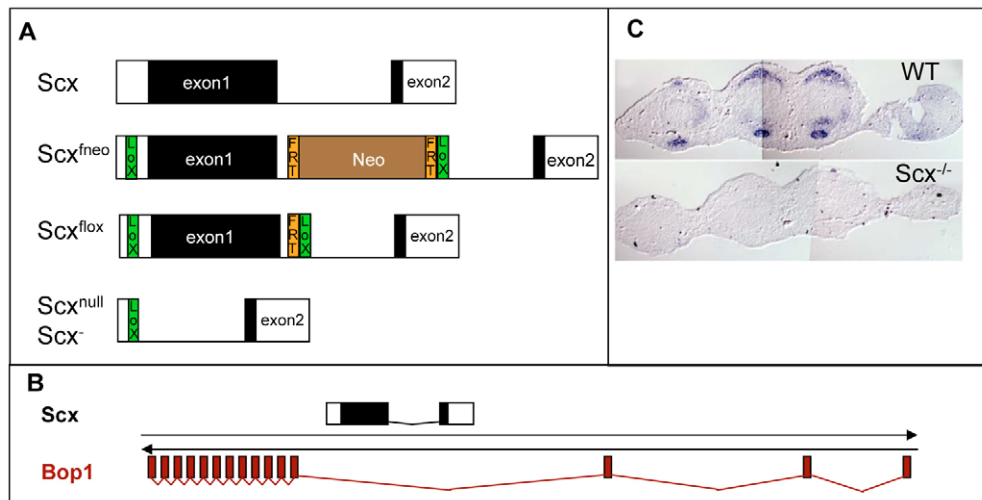


Fig. 1. Generation of an *Scx* null allele. (A) Schematic representation of alleles generated in the *Scx* locus. Black boxes represent coding regions of the *Scx* gene, and open boxes represent the 5' and 3' untranslated regions (UTRs). The recognition sequences for the Cre and FLP recombinases, the *loxP* (green boxes) and *FRT* (orange boxes) sites, respectively, are shown. The brown box represents the neomycin-resistance (Neo) cassette used for negative selection during targeting in embryonic stem cells (see Materials and methods for details). (B) Representation of the unique organization of the *Scx* locus. The two exons of the *Scx* gene (A) are located within the third intron of a second gene called block of proliferation 1 (*Bop1*), which is transcribed in the opposite orientation. (C) *Scx* in situ hybridization on cross sections through the digits of wild-type (WT) and *Scx^{-/-}* embryos at E15.5.

decided to generate a conditional allele in the *Scx* locus (by flanking the first exon, which includes most of the open reading frame, with *loxP* sites) and used the *FRT*-Neo cassette (Meyers et al., 1998) to enable selective removal of the Neo cassette (Fig. 1A). Founder *Scx^{fneo}* mice were mated with mice expressing FLP recombinase and Cre recombinase in the germline to generate a conditional allele, *Scx^{fllox}*, and a null allele, *Scx⁻* (Fig. 1A and see Materials and methods). Homozygous *Scx^{fllox/fllox}* mice were viable, but, surprisingly, homozygous *Scx^{fneo/fneo}* embryos died at approximately E10.5 (data not shown). The only difference between the two alleles is the Neo cassette embedded in the single intron of the *Scx* gene, suggesting that the Neo cassette in this locus is sufficient to cause embryonic lethality.

Effects of the Neo resistance cassette on the expression of neighboring genes have been demonstrated in numerous cases (Muller, 1999; Olson et al., 1996; Sartorelli and Caretti, 2005). The organization of the genomic locus of *Scx* provides a likely explanation for the dramatic effect of the Neo cassette in this instance. The entire *Scx* gene is located in the third intron of block of proliferation 1 (*Bop1*; Fig. 1B), a housekeeping gene required for ribosome biogenesis (Strezoska et al., 2000; Strezoska et al., 2002). It is therefore likely that the lethality observed in *Scx^{fneo/fneo}* embryos is caused by an effect on the transcription levels and/or splicing of the *Bop1* gene. Because the original *Scx^{KO}* allele (Brown et al., 1999) also introduced a Neo cassette in a similar genomic position, it is likely that the phenotype observed in that study was also affected by the presence of the Neo cassette. We therefore decided to re-evaluate the phenotype of a simple deletion of the *Scx* gene using the *Scx⁻* allele that we have generated.

***Scx^{-/-}* mice are viable and display severe disruption of force-transmitting tendons**

Contrary to the early lethality of *Scx^{KO/KO}* embryos, homozygous *Scx^{-/-}* mice survived to term. Indeed, homozygous *Scx^{-/-}* embryos could not be distinguished from their littermates (data not shown),

and hence their identity was verified by PCR (data not shown) and by in situ hybridization with an *Scx* probe (Fig. 1C). Although *Scx^{-/-}* mutant mice were viable, they displayed phenotypes that suggest a severe tendon disruption (see Movie 1 in the supplementary material). In mutants examined immediately after birth, the autopod of the forelimb was locked in a dorsal flexure, and the mice walked on their wrists (Fig. 2A). Furthermore, *Scx^{-/-}* mutants could not grip the cage railing with the paws of the fore or hind limbs and their tails were completely immobile. In addition, *Scx^{-/-}* mutants demonstrated a severe impairment in the use of their back muscles and found it very difficult to turn over when placed lying on their back (see Movie 1 in the supplementary material). In principle, some of these impairments could be caused by skeletal malformations, but we found no significant skeletal defects, with the exception of a loss of the deltoid tuberosity – a lateral outgrowth of the humerus whose formation is dependent on the activity of the attached muscles – suggesting that the deltoid tuberosity may also be missing in *Scx^{-/-}* mice because of a tendon phenotype (Fig. 2B) (E. Zelzer and R.S., unpublished results). To characterize possible tendon disruptions that cause these phenotypes, we wanted to examine tendon morphology in the mutants. Because we could not use *Scx* expression to detect the tendons in *Scx^{-/-}* mutant embryos, we took advantage of *ScxGFP*, a transgenic tendon reporter mouse line, in which the expression of GFP is regulated by *Scx* enhancers, resulting in strong GFP expression in all tendon and ligament cells (Pryce et al., 2007). Crossing this transgene into the *Scx^{-/-}* background enabled visualization of tendons and tendon progenitors in the mutants. The tendon phenotype was additionally corroborated by staining for the tendon structural protein tenascin C (Chiquet and Fambrough, 1984) and by in situ hybridization for collagen I (data not shown).

Using the *ScxGFP* reporter, we indeed found a severe disruption of tendon formation in *Scx^{-/-}* mutants at E18.5. We first observed a significant loss of limb tendons; some tendons were completely missing in *Scx^{-/-}* mutants, whereas others appeared small and

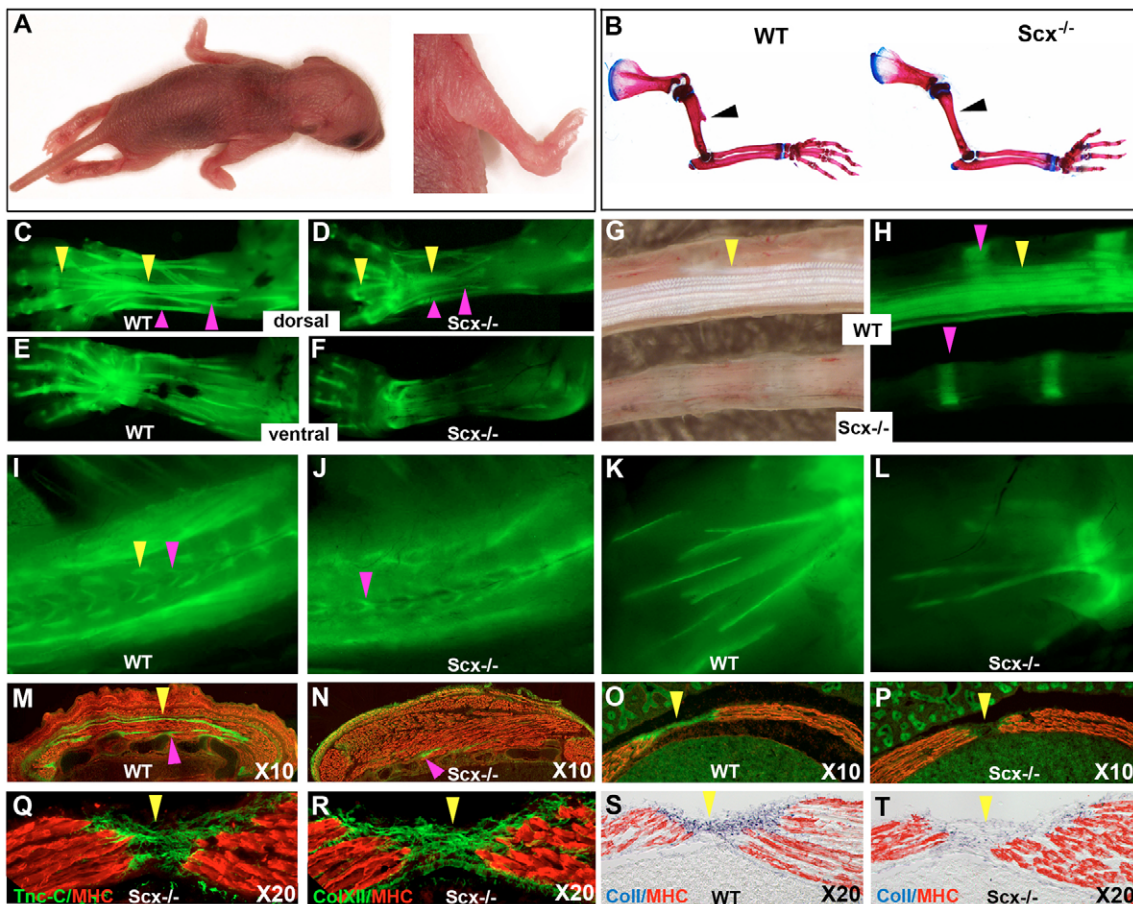


Fig. 2. The long force-transmitting tendons and intermuscular tendons are severely disrupted in *Scx*^{-/-} mice. Tendons in wild-type and mutant littermates were visualized directly in skinned tissues using the *ScxGFP* transgenic reporter (C-L). (A) The forelimbs of an *Scx*^{-/-} newborn mouse at postnatal day (P)3 are locked in a dorsal flexure. (B) Forelimbs of P14 wild-type (WT) and *Scx*^{-/-} mutant mice stained with Alcian blue and alizarin red. Black arrowheads point to the deltoid tuberosity, which is missing in the mutant. (C,D) The dorsal extensor tendons of the forelimb. Yellow arrowheads show the extensor digitorum communis (EDC). Long and short purple arrowheads show the extensor digiti quinti and extensor carpi ulnaris, respectively. (E,F) The ventral flexor tendons of the forelimb in wild type (E) and mutants (F). (G,H) Tail tendons at P14 visualized by direct illumination (G) or by fluorescence of the *ScxGFP* reporter (H). Yellow arrowheads show the tail tendons. Pink arrowheads show the annulus fibrosus. (I,J,M,N) Tendons of the trunk at E18.5. (I,J) Dorsal view of skinned trunks. (M,N) Sagittal trunk sections stained with an antibody to myosin heavy chain (MHC). Pink arrowheads show short-range tendons. Yellow arrowheads show long tendons. (K,L) The intermuscular tendons of the subscapularis muscle at E18.5 seen in a ventral view of the scapula. (O-T) Frontal sections through the trunk of E18.5 wild-type and *Scx*^{-/-} embryos at the level of the diaphragm, carrying the *ScxGFP* reporter and counterstained with an MHC antibody. Yellow arrowheads show the middle tendon of the diaphragm. (O,P,S,T) Comparison of the signal of *ScxGFP* (O,P) and in situ hybridization with a collagen I probe (S,T) in the diaphragm tendon from a wild-type and a mutant embryo. (Q,R) Expression of tenascin C and collagen XII in the diaphragm tendon of a mutant embryo.

rudimentary compared with those of wild-type littermates (Fig. 2C-F). For example, in wild-type embryos, the major extensor tendons of the forelimb, the extensor digitorum communis (EDC), originated in the EDC muscles close to the elbow and extended in close proximity up to the wrist, where they separated and continued along each digit, finally inserting at the tip of the digit (Fig. 2C, yellow arrowheads). In *Scx*^{-/-} embryos, however, the EDC could be detected in the digits, but the tendons disappeared at the wrist level (Fig. 2D, yellow arrowheads). By contrast, the extensor digiti quinti and the extensor carpi ulnaris tendons were complete in the mutant, but were still significantly reduced compared with their wild-type counterparts (Fig. 2C,D, long and short pink arrowheads, respectively). In *Scx*^{-/-} mutants, the flexor tendons were affected more severely than the extensors; not even rudimentary flexor tendons were apparent in the zeugopod and only some flexors were detected in the digits (Fig. 2E,F). Even more dramatically, the

inability of the mutants to use their tails was the result of a complete loss of all tail tendons, as visualized in 2-week-old mice, when collagen fibers of the tendons can be detected by direct illumination and the cells in these tendons could be seen by *ScxGFP* fluorescence (Fig. 2G,H, yellow arrowheads). Finally, we found a similar scenario in the trunk (Fig. 2I,J,M,N): whereas short axial tendons were possibly somewhat disrupted or reduced (Fig. 2, pink arrowhead), the long tendons were completely absent (Fig. 2, yellow arrowheads), as was seen both in whole-mount GFP images (Fig. 2I,J) and in sections counterstained with an antibody directed against myosin heavy chain (MHC) to visualize the axial musculature (Fig. 2M,N).

Interestingly, another category of tendons that was severely affected in *Scx*^{-/-} mutants was the ‘intermuscular tendons’, which are tendinous tissues interconnecting two muscle segments. Intermuscular tendons that were affected included the lateral

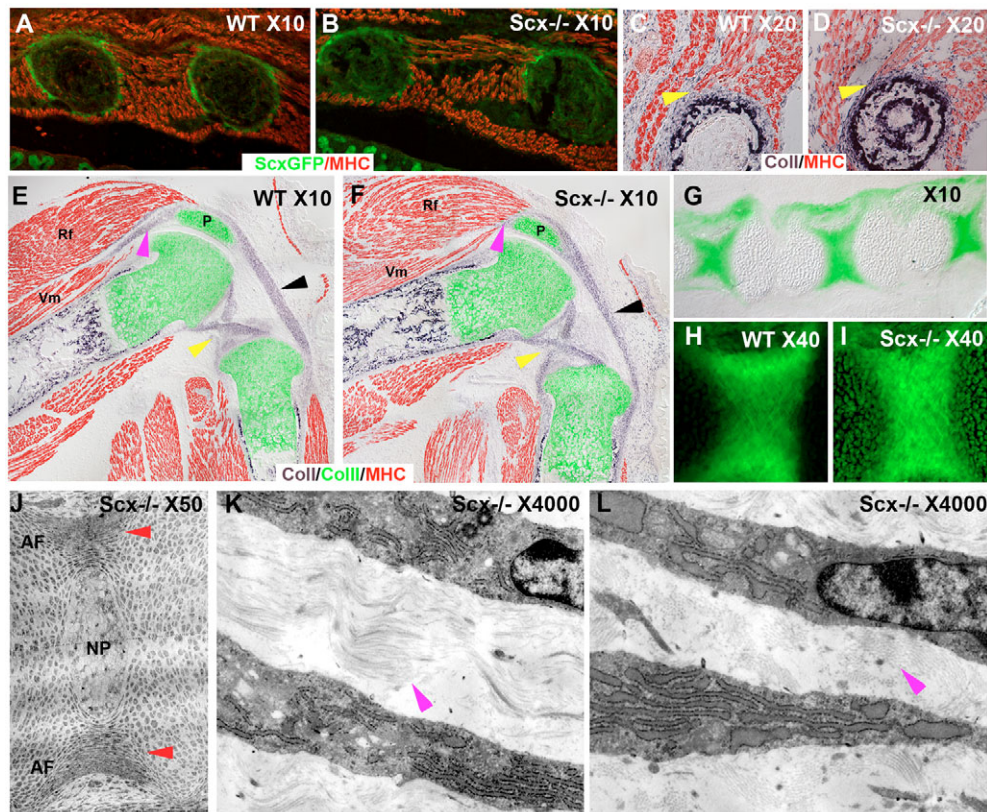


Fig. 3. Short-range anchoring tendons and ligaments are not affected in *Scx*^{-/-} mice. (A-D) Frontal sections through the rib cage of wild-type (WT) and *Scx*^{-/-} embryos at E18.5 stained with a myosin heavy chain (MHC; red) antibody to detect intercostal muscle attachments to the ribs. (A,B) *ScxGFP* signal (green) at the intercostal muscle attachments. (C,D) Section in situ hybridization (ISH) detecting collagen I expression in rib osteoblasts and in the tendinous attachment layer (yellow arrowheads) of wild-type and mutant embryos. (E,F) Sagittal sections through the knees of wild-type and *Scx*^{-/-} embryos at E18.5. Collagen I was detected by section ISH followed by antibodies to MHC (red) and collagen II (green). Fluorescent antibody signals were merged with the ISH image in Photoshop. Yellow arrowheads show the cruciate ligaments of the knee; black arrowheads show the patellar ligament; purple arrowheads show the rectus femoris tendon and vastus medialis tendon. (G-I) Superficial sagittal sections through the tail of wild-type and *Scx*^{-/-} embryos at E18.5 that are carrying the *ScxGFP* reporter. (G) The cartilage of tail bones visualized by DIC optics and overlaid with *ScxGFP* signal in the annulus fibrosus. (H,I) *ScxGFP* signal in the annulus fibrosus at high magnification. (J-L) Transmission electron microscope images of a medial sagittal section through the tail of an E18.5 *Scx*^{-/-} embryo. (J) Low magnification reveals the nucleus pulposus (NP; the intervertebral disc), which is flanked laterally by cartilage cells and by dorsal and ventral tightly stacked layers of ligament cells of the annulus fibrosus (AF; red arrowheads). (K,L) The annulus fibrosus at a higher magnification. The collagen fibers (purple arrowheads) are aligned along the section plane (K) and perpendicular to the section plane (L). AF, annulus fibrosus; NP, nucleus pulposus; P, patella; Rf, rectus femoris muscle; Vm, vastus medialis muscle.

tendinous stripes in the rectus abdominis muscle of the abdomen (data not shown), the subscapularis muscles at the ventral side of the scapula (Fig. 2K,L), and the middle tendon of the diaphragm (Fig. 2O,P, yellow arrowheads). *ScxGFP*-positive cells could not be detected in any of these tendons in *Scx*^{-/-} mutant embryos. The diaphragm is a flattened muscle bisected by a broad middle tendon, which is essential for normal breathing. The viability of mutant mice therefore indicates that a functional connective tissue was formed in the mutants. Indeed, we found that expression of matrix proteins, such as tenascin C and collagen XII, was not affected in the diaphragm tendon of mutant embryos (Fig. 2Q,R), and collagen I expression was also maintained, although at drastically reduced levels (Fig. 2S,T).

Despite the severe disruption in their force-transmitting and intermuscular tendons, *Scx*^{-/-} mice were viable and mobile, a testament to the fact that the capacity of tendons to attach muscles to bones is not disrupted in the mutants. Indeed we did not detect a significant change in the distribution of *ScxGFP*-positive cells in

anchoring tendons, such as the origin tendons of limb muscles (data not shown) or the tendinous layer that anchors the intercostal muscles to the ribs (Fig. 3A-D). A section through the ribs showed a normal distribution of tendon cells (Fig. 3A,B) and normal expression of collagen I (Fig. 3C,D) in the intercostal tendons of *Scx*^{-/-} embryos.

Ligaments, which connect bone to bone, are closely related to tendons and also express *Scx* from early stages (Schweitzer et al., 2001). There are, however, distinct differences between tendons and ligaments in the patterning, shape, composition and structure of their collagen fibers. We therefore examined the integrity of ligaments in *Scx*^{-/-} mutants. In sagittal sections through the knee of *Scx*^{-/-} embryos, we found that the cruciate ligaments of the knee and the patellar ligament that connects the patella to the tibia appeared normal in both morphology, and in expression of *ScxGFP* (data not shown) and of collagen I (Fig. 3E,F, yellow and black arrowheads, respectively). Conversely, the rectus femoris tendon, connecting the patella to the rectus femoris muscle, was almost entirely missing in

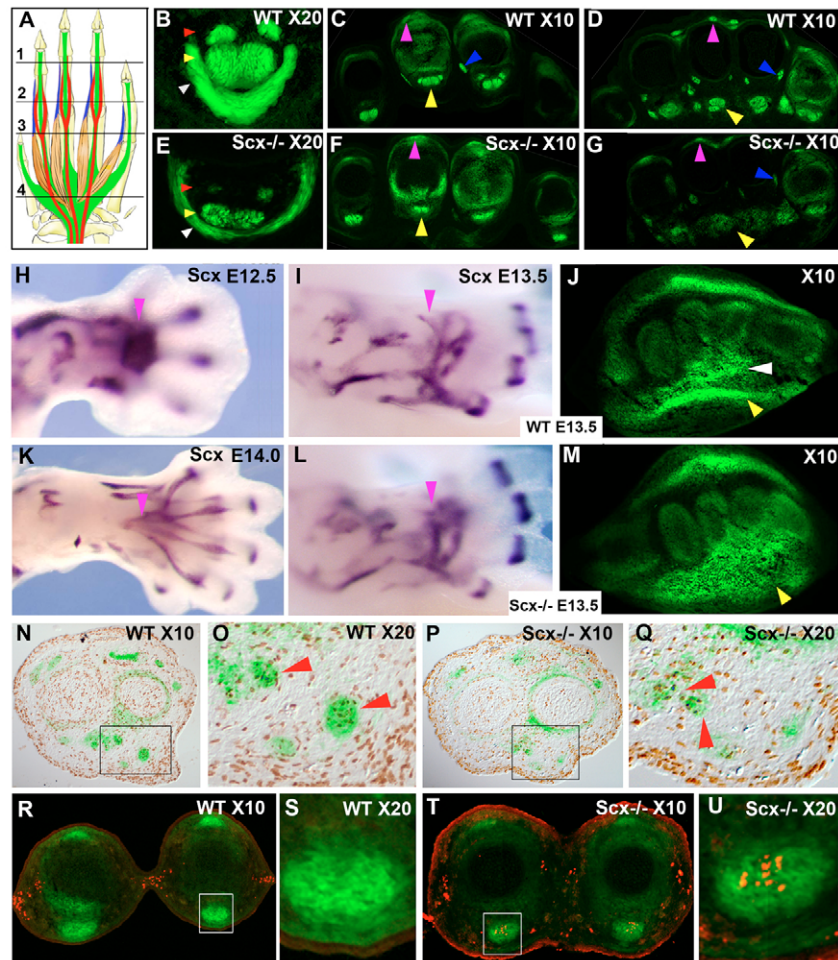


Fig. 4. The earliest tendon phenotype in $Scx^{-/-}$ embryos is detected at E13.5, concurrently with the overt formation of tendons.

(A) Schematic representation of the major flexor tendons of the forelimb: FDP tendons (green), the FDS (red), and the lumbrical muscles and tendons (blue). Section levels are marked by black lines: (1) digit level, (2) metacarpal-phalangeal joint, (3) mid metacarpal, (4) proximal metacarpal. (B-M) Arrowhead assignments are: yellow, flexor digitorum profundus (FDP); red, flexor digitorum superficialis (FDS); blue, lumbrical tendons; pink, extensor digitorum communis (EDC); white, flexor vinculum (B,E) or the palmar metacarpal ligament (J). (B-G) Sections through the autopod of wild-type (WT) and $Scx^{-/-}$ embryos at E18.5 highlighting the flexor and extensor tendons. The sections were taken at levels 1 (B,E), 2 (C,F) and 3 (D,G). (H,K) *Scx* in situ hybridization in limbs at E12.5 (H) and E14.0 (K), showing that, in wild-type mice, overt tendon formation occurs between E12.5 and E14.0. (I,L) *ScxGFP* detected by in situ hybridization in wild-type (I) and $Scx^{-/-}$ (L) forelimbs at E13.5. The EDC phenotype is first detected at E13.5 in mutant mice. (J,M) Sections through wild-type (J) and $Scx^{-/-}$ (M) forelimbs at E13.5 at a proximal metacarpal level (level 4 in A). The onset of the FDP phenotype occurs at E13.5. (N-Q) Cell proliferation in tendon cells of wild-type (N,O) and $Scx^{-/-}$ (P,Q) embryos at E14.5 in sections directly proximal to the wrist. BrdU was detected using a DAB stain and the images were overlaid with the *ScxGFP* signal from an immediate alternate section to highlight the tendons. (O,Q) Enlargements of the boxed areas in N and P, respectively. Arrowheads indicate tendons with BrdU-positive cells. (R-U) TUNEL staining in sections at digit level (level 1 in A) of wild-type (R,S) and $Scx^{-/-}$ (T,U) embryos at E14.5. (S,U) Enlargements of the boxed areas in R and T, respectively.

$Scx^{-/-}$ mutants, emphasizing the sharp contrast between the phenotypic effects on tendons and ligaments (Fig. 3E,F, purple arrowheads).

Surprised to find no phenotype in the ligaments of the knee, we examined in greater detail the structural features of another highly organized ligament, the annulus fibrosus (AF) – the ligament that connects adjacent vertebra. The AF is made up of elongated cells organized in layers in which the cells and collagen matrix have alternating perpendicular directionality to generate a criss-cross pattern. The AF has a distinct morphology in a superficial longitudinal section through the tail (Fig. 3G), and the alternating directionality of cells in the AF became distinct at a higher magnification in both wild-type and mutant embryos (Fig. 3H,I).

To examine the organization of the collagen matrix in the AF, we used medial sagittal sections through the tail of an $Scx^{-/-}$ embryo. Low-magnification transmission electron microscopy revealed the tightly packed layers of ligament cells in the AF, surrounding the inter-vertebral disc (Fig. 3J, red arrowheads). Higher magnification revealed the intact organization of the collagen matrix, with layers in which the collagen fibers were oriented along or perpendicular to the section plane (Fig. 3K,L, purple arrowhead).

In addition to its expression in the tendon and ligament lineages, the *Scx* gene is expressed in a number of other tissues, including in the sertoli cells of the testes (Muir et al., 2005), in the bronchi of the lungs and in the kidneys (Pryce et al., 2007). Major structural defects

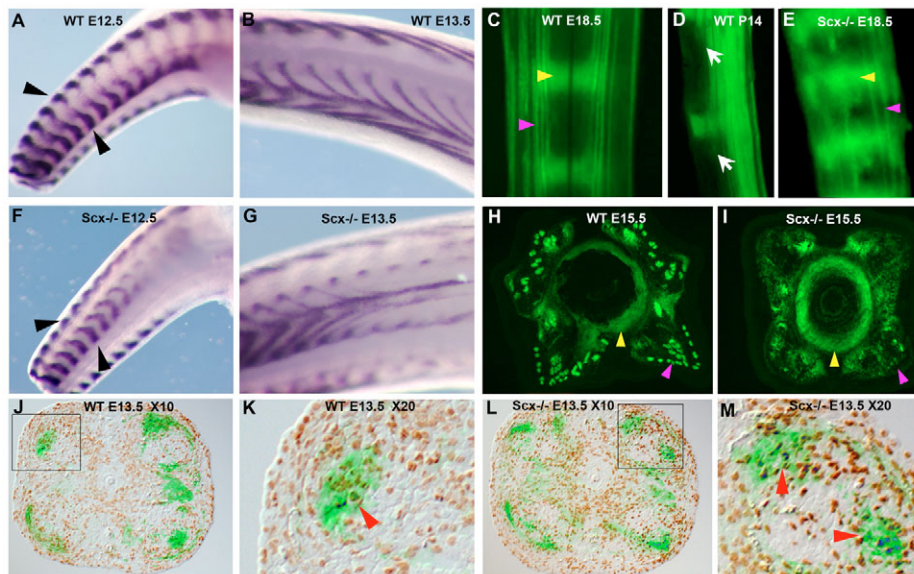


Fig. 5. The onset of the tail tendon phenotype in *Scx*^{-/-} embryos occurs during the transition from progenitors to organized tendons at E13.5. (A-I) In situ hybridization to detect the *Scx* transcript (A,B) and *ScxGFP* transcript (F,G) in tails at E12.5 (A,F) and E13.5 (B,G) and *ScxGFP* in later stages (C-E,H,I). The rostral end of the tail is pointing right in A,B,F,G and pointing up in C,D,E. (C,E,H,I) Yellow arrowheads show the annulus fibrosus, pink arrowheads show tail tendons and white arrows show the insertion of individual tendons. (A-D,H) The development of tail tendons in wild-type (WT) embryos: (A,B) *Scx* in situ hybridization of whole tails. Black arrowheads show the splitting of the syndetome at E12.5; (C,D) detection of expression of the *ScxGFP* reporter in skinned tails at E18.5 (C) and at P14 (D); (H) cross section of a tail at E15.5. (E,F,G,I) The tail tendon phenotype in *Scx*^{-/-} embryos. (F,G) In situ hybridization of tails to detect the transcript of the *ScxGFP* reporter. (E,I) The later tail phenotype, detected by *ScxGFP* fluorescence in a whole tail at E18.5 (E) and in a cross section at E15.5 (I). (J-M) Cell proliferation in tail tendons of wild-type (J,K) and *Scx*^{-/-} (L,M) embryos at E13.5. BrdU was detected using a DAB stain and the images were overlaid with the *ScxGFP* signal from an immediate alternate section to highlight the tendons. (K,M) Enlargements of the boxed areas in J and L, respectively. Arrowheads show tendons with BrdU-positive cells.

were not observed in these tissues in *Scx*^{-/-} mutants (data not shown), but potential roles for *Scx* in these tissues might be revealed upon closer examination in future studies.

The differentiation and condensation of tendon progenitors is disrupted in the limb of *Scx*^{-/-} embryos

A major feature of the *Scx*^{-/-} phenotype, the dorsal flexure of the forelimb paw, can be explained by a specific loss of flexor tendon activity, such that dorsal extension is not counteracted by flexor tendon activity. We therefore decided to analyze the flexor and extensor tendons in *Scx*^{-/-} mutants in detail. In wild type mice, there are two major flexor tendons in the autopod, shown schematically in Fig. 4A. The flexor digitorum profundus (FDP) tendons insert at the distal joint of each digit and extend along the digits and metacarpals, fusing to a seemingly continuous tendon sheath at the proximal end of the metacarpals (Fig. 4A, green). The flexor digitorum superficialis (FDS) tendons insert more proximally, at the second digital joint, and bifurcate to wrap around the FDP tendons, subsequently fusing to a single tendon ventral to the FDP at the metacarpal level (Fig. 4A, red). The morphology and relative organization of these tendons in cross sections are therefore excellent indicators for the proximal-distal position within the autopod. In *Scx*^{-/-} mutants, we found smaller FDP tendons and only rudimentary FDS tendons when compared with wild-type in cross sections through the digits (Fig. 4B,E) and at the metacarpal-phalangeal joint (Fig. 4C,F). However, at metacarpal levels, both flexors were completely absent, replaced by clouds of unorganized *ScxGFP*-positive cells (Fig. 4D,G, yellow arrowheads). Conversely,

whereas the extensors were dramatically small in *Scx*^{-/-} embryos, they persisted throughout the autopod (Fig. 4C,D,F,G, pink arrowheads). The absence of flexor activity in *Scx*^{-/-} mice may thus be an underlying cause for the dorsal flexure of the forelimb paw.

To determine which steps of tendon morphogenesis are affected in *Scx*^{-/-} mutants, we examined the limbs at earlier stages. In wild-type mice, the distribution of tendon progenitors in limb buds is highly dynamic up to E12.5 (Fig. 4H) (Schweitzer et al., 2001). A sharp transition involving the condensation of the progenitors occurs by E13.5 (Fig. 4I) and, by early E14.0 (Fig. 4K), distinct tendons can be detected. *ScxGFP* expression in *Scx*^{-/-} embryos was not different from that of wild type up until E12.5, and distinct defects were first observed at E13.5. For example, in wild type, the EDC tendons at the wrist level originated from a patch of progenitors that transformed into a triangular tendon blastema at E13.5 and into the distinct EDC tendons by E14.0 (Fig. 4H,I,K, pink arrowheads). The triangular EDC tendon blastema did not form in the mutant at E13.5 (Fig. 4L, pink arrowhead), representing the first manifestation of the EDC phenotype described above (Fig. 2C,D). The dramatic flexor phenotype at the metacarpal level was also initiated at this stage. In cross sections of E13.5 forelimbs at the proximal metacarpal level in wild-type mice (Fig. 4A, level 4), the ventral progenitors split into two distinct condensed layers (Fig. 4J) that will give rise to the FDP (Fig. 4J, yellow arrowhead) and the palmar metacarpal ligament (Fig. 4J, white arrowhead). In the mutant, these condensations failed to form, and the tendon progenitors remained as loosely organized mesenchyme (Fig. 4M). Both phenotypes indicate a specific requirement for *Scx* activity during the condensation of the tendon progenitors to form the long tendons of the limb.

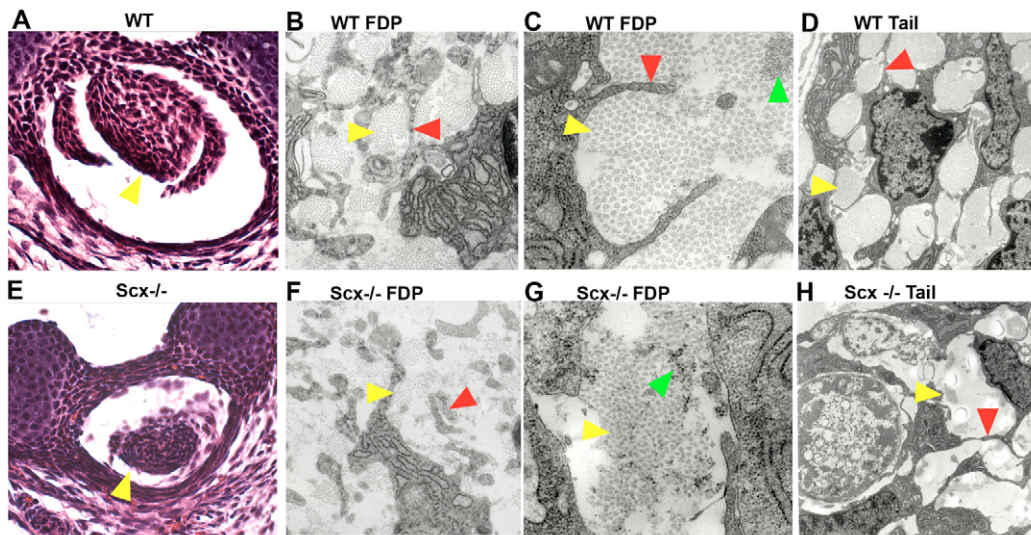


Fig. 6. The ECM of the FDP tendon in the digits of *Scx*^{-/-} embryos is also disrupted. (A-H) Yellow arrowhead, collagen fiber bundles; red arrowhead, cytoplasmic extensions of tenocytes; green arrowhead, microfilibrils. (A,E) Hematoxylin and Eosin (H&E) staining of a section of wild-type (WT) and mutant digit at E18.5. (B,C,F,G) TEM images of wild-type and *Scx*^{-/-} flexor digitorum profundus (FDP) tendons at E18.5 amplified $\times 7000$ (B,F) and $\times 38,000$ (C,G). (B,H) TEM images of sections of wild-type and *Scx*^{-/-} tail tendons at E18.5 magnified $\times 4000$.

In addition to the dramatic tendon loss discussed above, other limb tendons that formed in the mutant appeared smaller than their wild-type counterparts (Fig. 2D), and this mutant phenotype could be detected as soon as these tendons were formed at E14.5. We therefore looked for changes in cell proliferation or cell death that may cause these size differences. TUNEL staining of limb sections at digit levels of E14.5 embryos identified the expected cell death in interdigital mesenchyme in mutant mice (Fig. 4R,T). Cell death was not detected in wild-type tendons (Fig. 4R,S), but distinct cell death was observed in the FDP of mutant embryos (Fig. 4T,U). Interestingly, events of cell death were not identified in other limb tendons, ruling out the possibility that cell death is responsible for the general rudimentary nature of tendons in *Scx*^{-/-} embryos (data not shown). Next, we looked for changes in cell proliferation in limb tendons of *Scx*^{-/-} embryos. Interestingly, in all proximal-distal levels that showed positive BrdU signals in tendons, we found proliferating cells in the same tendons in limbs from both wild-type and mutant embryos (Fig. 4N-Q, red arrowheads), demonstrating that *Scx* is not essential for cell proliferation in tendons. However, because of the rudimentary nature of mutant tendons, we could not faithfully compare rates of cell proliferation and therefore cannot exclude the possibility of slower rates of proliferation in mutant tendons, but the early detection of the tendon phenotype suggests that a mere reduction in proliferation would not be sufficient to account for the observed tendon phenotype in mutants. In conclusion, these results suggest that proliferation and cell death do not play a major role in determining the size of tendons in mutant embryos. Because the tendon phenotypes emerged concomitantly with overt tendon formation, we hypothesize that, similar to the dramatic tendon phenotypes described above, the reduced tendon size in mutant embryos might also be attributable to a tendon condensation defect in which a smaller number of progenitors are incorporated into the tendons in *Scx*^{-/-} embryos.

Overt differentiation and elongation of tail tendons are also disrupted in *Scx*^{-/-} mutants

A striking feature of the *Scx*^{-/-} phenotype was the complete loss of tail tendons. Because the development of tail tendons has not been previously described, we first characterized their normal morphology and development. In wild-type mice, four massive tendon bundles were found to extend through the tail, and each was composed of individual tendons that originated at the sacral muscles

at the base of the tail and sequentially inserted at the rostral end of each tail bone, providing exquisite control of tail movement (Fig. 2G,H and Fig. 5D, white arrows).

Axial tendon progenitors are induced in the syndetome, at the intersection between two somites (Brent et al., 2003). In somites of the wild-type tail, we observed that the syndetome is subsequently separated into dorsal and ventral domains giving rise to the progenitor populations of the two dorsal and two ventral tendons (Fig. 5A, black arrowheads). Subsequently, the syndetomal progenitor populations condensed and extended rostrally (Fig. 5B). The dorsal and ventral tendons elongated to the base of the tail, thus establishing the individual tendons that connect each tail bone with the tail muscles (Fig. 5B). In cross section at E15.5, the tight individual tendon condensations could be clearly seen (Fig. 5H, pink arrowhead). By the end of embryogenesis, at E18.5, each vertebrae generated complete tendons and these tendons were organized in four radial groups (Fig. 5C,H) that coalesced after birth to give rise to the massive tendons of the tail (Fig. 5D).

In *Scx*^{-/-} mutant embryos, the induction of the syndetome and the separation of the dorsal and ventral progenitor populations were not disrupted (Fig. 5F, black arrowheads). As in the limb, the tendon phenotype emerged at E13.5 with very limited condensation of tendon progenitors (Fig. 5G), and there was a continued presence of sparsely distributed tendon cells in later stages (Fig. 5I, pink arrowhead). Consequently, tendon elongation was very limited in mutant embryos and tendon cells from multiple segments seemed to get incorporated into one or a small number of tendons (Fig. 5G). Similar to limb tendons, we did not detect cell death in tail tendons from mutant embryos at these stages (data not shown), and cell proliferation could easily be detected in all the rudimentary tail tendons of mutant embryos (Fig. 5J-M). At the end of embryogenesis, the tails of *Scx*^{-/-} embryos contained only a small number of tendon rudiments (Fig. 5E).

In conclusion, in tail as well as limb tendons, the stage that is affected most in tendons of *Scx*^{-/-} embryos appears to be the condensation and incorporation of progenitors into mature tendons. The small number of tenocytes that persist in the tail retain a capacity to organize a tendon structure, suggesting that, as in the limb, this essential feature of tenocytes is not regulated by *Scx*. However, these tenocytes subsequently undergo cell death (Fig. 6H), leading to the complete loss of tail tendons (Fig. 2H).

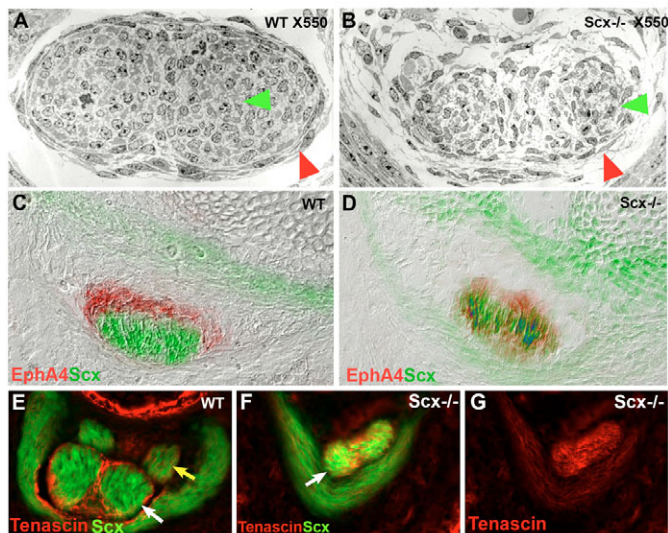


Fig. 7. In *Scx*^{-/-} mutant embryos, the endotenon cells fail to form a tendon sheath and are intermixed with the tenocytes in the FDP tendon. TEM (A,B) and antibody staining (C-G) of sections at the second digit phalange of wild-type (WT) and *Scx*^{-/-} mouse embryos at E18.5. (A,B) TEM of the flexor digitorum profundus (FDP) tendon amplified $\times 550$ to emphasize cellular aspects of the FDP. Green arrowheads, tenocytes; red arrowheads, the endotenon. (C,D) *ScxGFP* signal (green) and anti-EphA4 antibody (red) are overlaid on a DIC image of a cross section through the FDP tendon. (E-G) *ScxGFP* signal (green), and anti-tenascin C antibody (red) highlighting the endotenon in wild-type (E) and mutant (G,F) FDP tendon. Notice the collapse of the endotenon signal in the mutant tendon.

Organization of the endotenon and of the tendon matrix is disrupted in *Scx*^{-/-} mutants

We have so far shown an essential role for *Scx* in the early stages of tendon differentiation. In wild-type embryos, once the tenocytes are properly organized they produce the remarkably structured extracellular components of the tendons, the tightly packed bundles of collagen fibers (Benjamin and Ralphs, 2000). To investigate the possible involvement of *Scx* in these later aspects of tendon morphogenesis, we focused on the most robust tendon that does form in *Scx*^{-/-} mutants – the digit segment of the FDP tendon (Fig. 4E,F). The FDP tendon in the digit of *Scx*^{-/-} embryos was significantly smaller compared with a wild-type littermate (Fig. 4B,E and Fig. 6A,E). The size difference might

result from lower cell numbers caused by the cell death that we detected in these tendons (Fig. 4R-U), and, indeed, we found that mutant FDP tendons had approximately 60% of the number of cells found in the wild-type FDP tendons (see Materials and methods). Moreover, the cells in the mutant tendons appeared to be more compact in histological staining (Fig. 6A,E), indicating that matrix deposition is probably also affected. The reduced matrix content was apparent in intermediate-magnification transmission electron microscope (TEM) images, which showed the parallel collagen fibers in cross section (Fig. 6B,F, yellow arrowheads). Although bundles of collagen fibers could be detected in the mutant, the amount of matrix was greatly reduced (Fig. 6B,F), and unoccupied spaces were common in the mutant, but not in the wild-type, tendon. Moreover, in wild-type embryos, the tenocytes developed a complex network of cytoplasmic extensions (Fig. 6B-D, red arrowheads) that engulfed bundles of collagen fibers in order to regulate the localized deposition of matrix components. These cytoplasmic extensions were also severely decreased and appeared less complex in the mutant (Fig. 6F,G). Another component of the tendon matrix affected in the *Scx*^{-/-} mutants were the microfibrils (Fig. 6C, green arrowhead). Normally found in discrete bundles in the wild-type tendon, the microfibrils in mutant tendons were highly disorganized (Fig. 6G, green arrowhead). The decrease in collagen deposition and loss of cytoplasmic extensions was seen even more dramatically in the highly rudimentary tail tendons of *Scx*^{-/-} mutants at E18.5 (Fig. 5E and Fig. 6D,H). In tail sections, the tenocytes could also be seen undergoing apoptosis in the mutant, as evident by their highly enlarged nuclei (Fig. 6H). Indeed, although tendon rudiments could be seen in mutant embryos, they were completely absent in 2-week-old mutant mice.

The cellular organization of the tendons also appeared to be affected in *Scx*^{-/-} mutants. In wild type, tendons are composed of fibroblastic tenocytes, which are surrounded by flattened cells making up a tendon sheath called the endotenon and, in major tendons, of a second external layer called epitenon (Fig. 7A, red arrowhead) (Benjamin and Ralphs, 2000; Kannus, 2000). Whereas endotenon cells were organized in a sheath of stretched cells in the FDP of wild-type E18.5 embryos, in *Scx*^{-/-} embryos, the endotenon cells appeared disorganized and failed to generate a continuous layer (Fig. 7B). Staining for endotenon markers further highlighted the disrupted organization of the endotenon. In agreement with previous observations in chick embryos (D'Souza and Patel, 1999), we found that the ephrin receptor *EphA4* was expressed in the endotenon of limb tendons (Fig. 7C). In the mutant, *EphA4*-expressing cells were

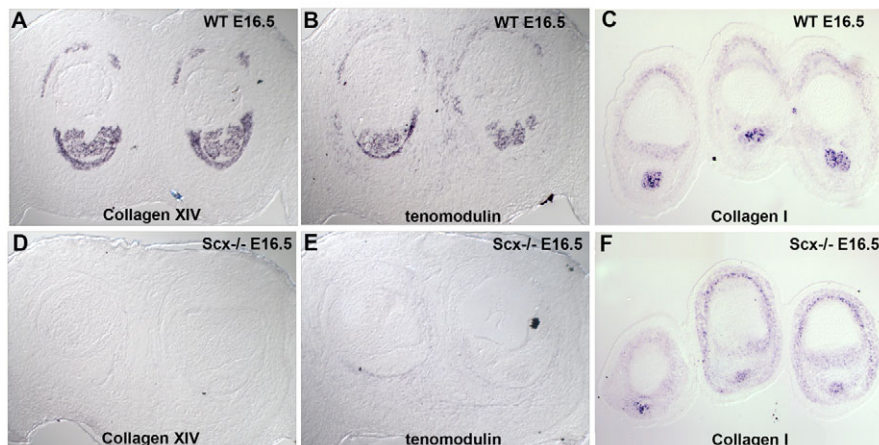


Fig. 8. Genes regulated by *Scx*. In situ hybridization on sections from the forelimb of an E16.5 *Scx*^{-/-} mutant embryo (D-F) and a wild-type (WT) littermate (A-C) using probes for the collagen XIV (A,D), tenomodulin (B,E) and collagen I (C,F) genes.

intermixed with the tenocytes, emphasizing a disruption of the regular organization of the endotenon (Fig. 7D). Similarly, in the digit flexors, the matrix protein tenascin C was exclusively organized in the endotenon (Fig. 7E), but, in *Scx*^{-/-} embryos, tenascin C protein was fully intermixed with the FDP tenocytes (Fig. 7F,G), again demonstrating that the structural integrity of the endotenon is lost in *Scx*^{-/-} mutants.

It is important to note that *Scx* is exclusively expressed in tenocytes, suggesting that the effect on the sheath is indirect. However, because the lineage of the endotenon cells has not been determined, it is also possible that these cells may express *Scx* at earlier stages of their development.

Molecular characterization of the *Scx*^{-/-} tendon phenotype

We have found that *Scx* has multiple essential roles in tendon formation, affecting both tendon differentiation and organization. To identify genes regulated by *Scx* that could be responsible for *Scx* function in tendon formation, we therefore examined the expression of genes known to be expressed specifically in all tendons [e.g. tenomodulin (Brandau et al., 2001); collagen I and collagen XIV (Walchli et al., 1994; Young et al., 2000); collagen XII (Dublet and van der Rest, 1987); tenascin C (Chiquet and Fambrough, 1984); fibromodulin, lumican and decorin (Ezura et al., 2000)] or genes described as being specifically expressed in tendons within the context of the limb bud [e.g. *Six2* (Oliver et al., 1995), *Eyal* (Xu et al., 1997) and *Fgf18* (Liu et al., 2002)]. Of these, only two showed a complete loss of expression in *Scx*^{-/-} mutants: collagen XIV and tenomodulin (Fig. 8A,B,D,E and data not shown). Collagen XIV is a fascicle-associated collagen involved in the regulation of the diameter of collagen fibers (Gelse et al., 2003; Young et al., 2002). Tenomodulin is a single transmembrane protein (Brandau et al., 2001), and a mild proliferation defect has been detected in tendons of tenomodulin mutants (Docheva et al., 2005). Interestingly, the induction of tenomodulin gene expression by *Scx* misexpression was recently demonstrated in chick (Shukunami et al., 2006). We also detected a slight but reproducible decrease in the levels of collagen I gene expression in *Scx*^{-/-} embryos (Fig. 8C,D). Based on their known properties, it is unlikely that the modulation of these three genes is sufficient to explain the phenotypic effects that we observe upon the loss of *Scx* activity.

The fact that tendon progenitors are present and many tendons still form in the mutants suggests that, despite its early expression in tendon progenitors, *Scx* is not necessary for tenocyte specification. We wanted to test whether this could be explained by partial redundancy with other, related transcription factors. *Scx* belongs to the small Twist subfamily of the bHLH transcription factors (Atchley and Fitch, 1997). Within this subfamily, paraxis (also known as Tcf15 – Mouse Genome Informatics) (Burgess et al., 1995), is the closest relative to *Scx* and the twist genes represent more-distant subfamily members. The small Hand subfamily of bHLH transcription factors is also highly related to *Scx* (Atchley and Fitch, 1997; Firulli, 2003). We did not detect a distinct tendon expression for any of these genes when examined by in situ hybridization (data not shown). Furthermore, neither paraxis nor *Hand1* or *Hand2* expression was upregulated in *Scx*^{-/-} mutants.

DISCUSSION

Scleraxis, a bHLH transcription factor, is the earliest and most persistent marker known for the tendon lineage (Schweitzer et al., 2001; Tozer and Duprez, 2005). To determine the role of *Scx* in tendon formation, we created a targeted null allele of the *Scx* gene.

Homozygous *Scx*^{-/-} mice were viable but showed a dramatic defect in tendon differentiation resulting in a severe loss of intermuscular tendons and of the tendons responsible for transmitting musculoskeletal force in the limbs, tail and trunk. Surprisingly, there was no discernible defect in other categories of tendons whose function is mostly to anchor muscles and dissipate the force generated by muscle contraction.

Tendon progenitors were induced in *Scx*^{-/-} embryos and tendon phenotypes were first detected at E13.5, concomitant with the incorporation of tendon progenitors into histologically distinct tendons. The most striking phenotype in *Scx*^{-/-} embryos was the failure of tendon progenitors to condense into morphologically distinct tendons. The variable effect on different tendon categories highlights the molecular and structural diversity within the broad definition of tissues as tendons. Moreover, we show that *Scx* plays a role in later aspects of tendon formation, including in the secretion of structural matrix components, in the production of extended cytoplasmic extensions that support matrix organization, and in the interplay between tenocytes and endotenon cells.

As a result of differential severity in the defects in tendon formation, the normal balance between extensor and flexor forces in the limbs was disrupted in mutant mice, resulting in non-physiological flexures and the complete loss of some functions of the muscular skeletal system. These phenotypic features are reminiscent of a congenital human malformation known as arthrogyposis, suggesting that mild *Scx* mutations may serve as a model for this condition.

Scx is required for the differentiation and formation of force-transmitting and intermuscular tendons

The definition of tendons as the connective tissues that connect muscle to bone accounts for a highly diverse set of tissues. Most studies focus on the robust tendons of the limbs and tail, which show a remarkable specialization to support transmission of force over considerable distances. However, the force-transmitting tendons are complemented by tendons whose physiological role is mostly to anchor muscles to respective skeletal attachments. Anchoring tendons include the origin tendons of limb muscles and many of the trunk tendons, notably including the attachments of the intercostal muscles through a monolayer of tendon cells that surround the ribs. Anchoring tendons frequently exhibit broad contact areas with the muscles or skeletal elements, reflecting the need to dissipate the force generated by the muscles. Finally, a divergent group of tendons, the intermuscular tendons, connect muscle segments such as the middle tendon of the diaphragm and probably play a role in maintaining the integrity of the muscles in question.

When we first cloned *Scx*, we noticed that it was expressed in all tendon tissues, reinforcing the notion that they all represent a single well-defined cell and tissue identity (Brent et al., 2003; Schweitzer et al., 2001). It is therefore fascinating that *Scx* function does not seem to be required for that core identity of the tendon tissues, but rather is specific to a distinct subset of tendon tissues, thus providing the first direct demonstration of differences in the molecular processes that confer the cellular identities of the different tendon categories.

Our analysis suggests that *Scx* function is required for the normal differentiation of force-transmitting tendons and of intermuscular tendons, and, noticeably, all the tendons in these categories are affected in *Scx*^{-/-} mutants. Although there is no obvious similarity between these two tendon groups, it is important to note that, in both categories, in the mutants, most of the muscles remained attached to

their targets, emphasizing that *Scx* function is crucial for the generation of the complex tendon structures, but not for the capacity of tendons to mediate tissue attachments. Moreover, despite the considerable variability observed in the phenotypes of force-transmitting tendons in mutants, in all cases, the phenotype was first detected in the transition from tendon progenitors to condensed and overtly distinct tendons, suggesting that the scleraxis function is likely to be related to the incorporation of tendon progenitors into discrete tendons. Consequently, the difference in phenotypic severity might represent the difference between the complete failure of progenitor condensation and partial condensation resulting in smaller rudimentary tendons.

Scx affects both cellular differentiation and extracellular matrix organization in tendons

The functional components of the mature tendon are highly organized bundles of collagen fibers that transmit the forces between the muscles and the skeleton. Little, however, is known about the molecular processes that enable the tenocytes to coordinate the secretion and organization of these matrix structures during tendon genesis (Benjamin and Ralphs, 2000; Kannus, 2000). The most robust tendon in *Scx*^{-/-} mutants, the digit segment of the FDP, displayed a variety of defects in its ability to produce the tendon matrix, manifesting in a dramatic decrease in the number of collagen fibers and in their organization. Concomitantly, we observed a decrease in the number of tenocytes present and a partial loss of the network of cytoplasmic processes that enable the tenocytes to regulate the local secretion and synthesis of collagen fibers. It will be interesting to determine in future studies whether the effects on the organization of the matrix and on the cellular morphology are both directly regulated by *Scx* or whether these aspects of tendon structure are mutually dependent so that a primary effect on one of them may secondarily disrupt the other.

Transcriptional regulation of tendon cell fate

The transcriptional regulation of the tendon cell fate has not been addressed previously. This study provides for the first time two pivotal observations regarding the tendon transcriptome. First, *Scx* is essential for tendon differentiation and for the incorporation of tendon progenitors in mature tendons. Future discovery of the identity and function of *Scx* targets will therefore help unravel the molecular processes involved in tendon differentiation. Second, the induction and maintenance of the tendon cell fate are not dependent on *Scx* function, nor is the expression of *Scx* itself, because expression of the *ScxGFP* reporter was maintained in tendon cells of *Scx*^{-/-} mutants. Transcriptional regulators of the *Scx* gene may therefore represent key players in tendon formation.

This study represents the first demonstration of a tendon differentiation phenotype. Moreover, the elucidation of *Scx* function and the tools that it allowed us to develop provide a powerful starting point for future studies of the transcriptional regulation of this important and often neglected tissue.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/14/2697/DC1>

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