The Scleraxis Transcription Factor Directly Regulates Multiple Distinct Molecular and Cellular Processes During Early Tendon Cell Differentiation

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Proper development of tendons is crucial for the integration and function of the musculoskeletal system. Currently little is known about the molecular mechanisms controlling tendon development and tendon cell differentiation. The transcription factor Scleraxis (Scx) is expressed throughout tendon development and plays essential roles in both embryonic tendon development and adult tendon healing, but few direct target genes of Scx in tendon development have been reported and genome-wide identification of Scx direct target genes in vivo has been lacking. In this study, we have generated a ScxFlag knockin mouse strain, which produces fully functional endogenous Scx proteins containing a 2xFLAG epitope tag at the carboxy terminus. We mapped the genome-wide Scx binding sites in the developing limb tendon tissues, identifying 12,097 high quality Scx regulatory cis-elements in ≈7,520 genes. Comparative analysis with previously reported embryonic tendon cell RNA-seq data identified 490 candidate Scx direct target genes in early tendon development. Furthermore, we characterized a new Scx gene-knockout mouse line and performed whole transcriptome RNA sequencing analysis of E15.5 forelimb tendon cells from Scx−/− embryos and control littermates, identifying 68 genes whose expression in the developing tendon tissues significantly depended on Scx function. Combined analysis of the ChIP-seq and RNA-seq data yielded 32 direct target genes that required Scx for activation and an additional 17 target genes whose expression was suppressed by Scx during early tendon development. We further analyzed and validated Scx-dependent tendon-specific expression patterns of a subset of the target genes, including Fmod, Kera, Htra3, Ssc5d, Tnmd, and Zfp185, by in situ hybridization and real-time quantitative polymerase chain reaction assays. These results provide novel insights into the molecular mechanisms mediating Scx function in tendon development and homeostasis. The ChIP-seq and RNA-seq data provide a rich resource for aiding design of further studies of the
INTRODUCTION

Tendons and ligaments are specialized connective tissues densely packed with collagen fibers, composed primarily of type I collagen fascicles with several other collagens, elastin, and various proteoglycans making up the remainder of the extracellular matrix (ECM) surrounding the resident tenocytes (Birch et al., 2013; Davis et al., 2013). Tendons connect skeletal muscles to bones and transmit mechanical forces generated from muscle contraction whereas the ligaments align bones within joints and reinforce their stability and flexibility. Many musculoskeletal diseases involve injuries and tissue degeneration in tendons and ligaments (Yang et al., 2013; Nourissat et al., 2015). Although it is expected that many genes and molecular pathways are shared during the processes of tendon development and tendon healing/regeneration, the molecular mechanisms controlling tendon development, including specification of tendon progenitor cells from mesenchymal stem cells, migration to local domains and differentiation into mature tenocytes, secretion of ECM molecules and organization of fibrils and fibers into tendon bundles, are not well understood. With the development and wide application of gene knockout technologies, thousands of mutant mouse lines carrying loss-of-function mutations in individual genes have been generated and analyzed in the last 30 years. However, only loss of function of the Scleraxis (Scx) transcription factor and TGF-β signaling led to severe disruption of tendon development whereas mice deficient in several other genes individually or in combination, including genes encoding the Mohawk (Mkx) transcription factor, the early growth response (EGR) 1 and 2 transcription factors, various proteoglycans, and tenomodulin, exhibited mild postnatal tendon defects (Docheva et al., 2005; Murchison et al., 2007; Kilts et al., 2009; Pryce et al., 2009; Ito et al., 2010; Liu et al., 2010; Lejard et al., 2011; Douste et al., 2013; Guerquin et al., 2013; Dunkman et al., 2014; Delgado Caceres et al., 2018; Shukunami et al., 2018). While these data suggest that the tendon developmental processes are well orchestrated with built-in compensatory regulatory mechanisms (Delgado Caceres et al., 2018), better understanding of the molecular mechanisms controlling tendon development and tendon cell differentiation will be instrumental for the development of effective methods for tendon repair and regeneration.

Scleraxis is a basic helix-loop-helix (bHLH) transcription factor that is expressed in early embryonic tendon progenitor cells and stays strongly expressed throughout tendon cell differentiation into mature tenocytes (Cserjesi et al., 1995; Schweitzer et al., 2001). Mice homozygous for Scx null mutation exhibit severe hypoplasia or complete absence of force-transmitting tendons, while the muscle anchoring tendons and ligaments are less affected (Murchison et al., 2007; Shukunami et al., 2018). Further studies showed that Scx function is not required for tendon progenitor cell initiation but is crucial for tendon cell differentiation (Murchison et al., 2007). Studies in mouse and chick embryos showed that TGF-β and FGF signaling pathways are essential for induction of tendon progenitor cells by activating expression of Scx and other tendon genes (Kuo et al., 2008; Pryce et al., 2009; Hasson, 2011; Havis et al., 2016). Moreover, a recent study demonstrated that Scx function is also required for adult tendons in response to mechanical loading (Gumucio et al., 2020). How Scx regulates tendon cell differentiation is still unclear. Currently very few genes, including Colla1 and Tnmd, have been identified as potential direct target genes of Scx in tenocytes primarily through in vitro functional analysis of putative Scx-binding promoter elements (Shukunami et al., 2006, 2018; Lejard et al., 2007), whereas large scale identification of Scx direct transcriptional target genes during tendon cell differentiation in vivo is still lacking.

In this study, we first generated a novel ScxFlag knock-in mouse line, which expresses the endogenous Scx protein with a 2xFLAG epitope tag at the carboxy terminus, through CRISPR/cas9-mediated genome editing. Using the ScxFlag mice, we mapped the genome-wide Scx binding sites in embryonic tendon progenitors and differentiating tendon cells using chromatin immunoprecipitation followed by high throughput DNA sequencing, which mapped high quality Scx-binding sites in the promoter and/or enhancer regions in 7,520 genes. Furthermore, we characterized a new Scx gene-knockout mouse resource that is accessible to the wide biomedical research community and performed whole transcriptome RNA sequencing analysis of early differentiating limb tendons in Scx−/− and wildtype littersmates. These ChiP-seq and RNA-seq datasets provide a rich resource for further studies of the molecular mechanisms regulating tendon formation and tendon cell differentiation. Combined analyses of the ChiP-seq and RNA-seq data provide novel insights into the molecular mechanisms mediating Scx regulation of tendon cell differentiation.

MATERIALS AND METHODS

Mice

Scx-GFP transgenic mice (Pryce et al., 2007) were generously provided by Dr. Ronen Schweitzer (Shriners Hospitals for Children, Portland, Oregon). To generate ScxFlag knock-in mice, pretested guide RNAs targeting the genomic sequence containing the endogenous Scx translational STOP codon...
were co-injected with a single-stranded oligonucleotide donor template (Figure 1A) and humanized Cas9 mRNAs into zygotes of B6D2F1 (C57BL/6 X DBA2) mice. Genome-modified founder mice were identified by using polymerase chain reaction (PCR) assay and crossed to CD-1 mice to generate Scx<sup>Flag</sup>/+ hemizygous mice. Genotypically verified G1 Scx<sup>Flag</sup>/+ hemizygous mice were intercrossed to generate Scx<sup>Flag/Flag</sup> homozygous mice. Scx+/− mutant mice were purchased from the Mutant Mouse Research and Resource Center at the University of California Davis (KOMP catalog#13478). All mouse strains were maintained by crossing to CD-1 wildtype mice (Charles River, Stock# 022), or by intercrossing between siblings. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Cincinnati Children’s Hospital Medical Center (CCHMC). The mice were housed in AAALAC accredited barrier housing conditions at CCHMC.

**Histology, in situ Hybridization, and Immunofluorescent Staining**

Mice were euthanized at predetermined stage and embryos were collected in cold PBS, fixed in 4% PFA overnight, washed in PBS for 3 times and processed to 100% methanol (for whole mount in situ hybridization), or paraffin for section (for histology staining and section in situ hybridization). Histology staining and in situ hybridization procedures were performed as previously described (Liu et al., 2013, 2015). In situ probe templates were amplified by PCR from total cDNA sample of E13.5 wildtype forelimbs. Antisense RNA probes were synthesized from templates using T7 RNA polymerase (Promega catalog# P2075). Immunofluorescent staining was performed as previously described (Xu et al., 2014). The primary antibodies used include anti-Sox9 (Santa Cruz, catalog# sc-20095, 1:25 dilution) and anti-FLAG antibody (Sigma, catalog# F1804, 1:50 dilution). The secondary antibodies were Alexa Fluor 568-conjugated goat anti-mouse IgG (H + L) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L) [Thermo Fisher Scientific, Waltham, MA, United States], catalog# A11004 and A27034, 1:500 dilution.

**Skeletal Preparations**

Mice were euthanized at postnatal day 0, heated at 65°C in distilled water for 3 min, and de-skinned manually. Whole de-skinned embryos were stained in Alcian Blue solution (15 mg Alcian Blue dissolved in 20 ml glacial acetic acid plus 80 ml 95% glacial acetic acid) for 3 min. Whole de-skinned embryos were then washed in PBS three times, and stained with 0.5% OsO<sub>4</sub> for 3 min. Embryos were dehydrated and embedded in paraffin. Sections were cut at 3 μm and stained with hematoxylin-eosin (H&E) and alcian blue. Whole mount in situ hybridization, or paraffin for section (for histology staining and section in situ hybridization). Histology staining and in situ hybridization procedures were performed as previously described (Liu et al., 2013, 2015). In situ probe templates were amplified by PCR from total cDNA sample of E13.5 wildtype forelimbs. Antisense RNA probes were synthesized from templates using T7 RNA polymerase (Promega catalog# P2075). Immunofluorescent staining was performed as previously described (Xu et al., 2014). The primary antibodies used include anti-Sox9 (Santa Cruz, catalog# sc-20095, 1:25 dilution) and anti-FLAG antibody (Sigma, catalog# F1804, 1:50 dilution). The secondary antibodies were Alexa Fluor 568-conjugated goat anti-mouse IgG (H + L) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L) [Thermo Fisher Scientific, Waltham, MA, United States], catalog# A11004 and A27034, 1:500 dilution.
motif analysis of the Scx peaks was performed within a novo than 0.05 was considered statistically significant and marked as ∗ followed by Turkey’s multiple comparison posttest. P differences among the three genotypes, we used one-way ANOVA processed for statistical analysis. To compare the body weight
normalized to that of Hprt mRNAs. Student’s t test was used for pairwise comparison. P < 0.05 was considered significantly different. P value less than 0.05 was considered statistically significant and marked as *. P value less than 0.01 was marked as ***, whereas P value less than 0.001 was marked as **** when applicable.

RESULTS

Generation of a Novel Scx<sup>Flag</sup> Mouse Line and Genome-Wide Mapping of Endogenous Scx Binding Sites in the Tendon Progenitor Cells in vivo

No specific antibody for the Scx protein that allows direct analysis of endogenous Scx-binding at target genes has been reported. To facilitate direct and specific analysis of endogenous Scx protein activity, we used the CRISPR/Cas9-mediated genome editing strategy (Cong et al., 2013; Scott and Hu, 2019) to insert a well-characterized 2xFLAG epitope tag at the carboxy terminus of the endogenous Scx protein in mice (Figure 1A). Detailed description of the procedures for generating the Scx<sup>Flag</sup> founder mice is provided in the Materials and Methods section. The Scx<sup>Flag</sup>/+ founder mice were crossed to wildtype CD1 mice and the G1 Scx<sup>Flag</sup>/+ hemizygous progenies (Figure 1B) were sequence-verified for correct integration and germ-line transmission of the Scx<sup>Flag</sup> allele as designed. The Scx<sup>Flag</sup>/+ mice were then intercrossed to generate Scx<sup>Flag/Flag</sup> homozygous mice, which were born at expected Mendelian ratio and did not display any phenotypic difference from their hemizygous or wildtype littermates (Figures 1C–F). Immunofluorescent staining of sections of mouse embryos using an anti-FLAG antibody (Sigma, catalog# F1804) showed that the Scx-FLAG fusion protein was strongly and specifically expressed in both tendons and ligaments (Figures 1G–J), consistent with previously reported patterns of expression of endogenous Scx mRNAs and of the transgenic Scx-GFP reporter activity (Murchison et al., 2007; Pryce et al., 2007; Watson et al., 2009). Since the Scx<sup>Flag/Flag</sup> homozygous mice do not have any phenotypic abnormality, the insertion of the FLAG epitope tag at the carboxy-terminus of the endogenous Scx protein did not affect Scx function, providing a valuable new tool for direct analysis of endogenous Scx function during development, tissue homeostasis, and injury repair.

To investigate Scx-mediated transcriptional regulation during early tendon development, we dissected forelimbs from E13.5 Scx<sup>Flag/Flag</sup> embryos and performed ChIP-seq experiments in triplicates using the anti-FLAG antibody (Sigma, catalog# F1804). Analysis of the ChIP-seq data identified 12,097 highly enriched Scx-associated chromatin regions (ChIP-seq peaks) (Figures 2A,B). About 77% (9,272 of 12,097) of the Scx ChIP-seq peaks were located in intronic or intergenic regions associated with one or two genes in the immediate vicinity within 500 kb from the gene’s transcription start site (TSS), whereas only about 14% (1,670 of 12,097) of the Scx-binding sites were located within gene promoter regions (Figures 2A–C). De novo motif analysis revealed that the most enriched endogenous Scx-binding domain contains a core sequence C-A-G/T-A/C-T-G (Figure 2D), which was present in >45% of the ChIP-seq peaks and regions and matches the Scx-binding core sequence previously determined by electrophoresis mobility shift assays (Shukunami et al., 2018). The second most enriched motif, which is present in 15.8% of the ChIP-seq peaks, contains a core sequence that matches the consensus binding motif of the Nfat family transcription factors, TGGAAA (Yu et al., 2015; Klein-Hessling et al., 2017; Figure 2D). It has been reported that Scx and Nfatc4, a member of the Nfat family, act cooperatively to regulate the transcription of Colla1 in tendon fibroblasts (Lejard et al., 2007). The significant enrichment of the Nfat binding motif in the Scx ChIP-seq peaks indicate that Scx and Nfat family proteins act together to regulate expression of many genes during tendon development.

Genomic Regions Enrichment of Annotations Tool analysis of the ChIP-seq data recovered 7,520 genes associated with the Scx-binding genomic regions. To gain insight into Scx-mediated transcriptional regulation during early tendon development, we compared the ChIP-seq data with the previously reported RNA-seq data analyzing transcriptome profiles of developing mouse hindlimb tendons (Liu et al., 2015). We found that high quality Scx ChIP-seq peaks were detected in 490 of the 970 genes whose expression was upregulated by more than 1.5-fold in Scx-GFP<sup>+</sup> cells during early tendon cell differentiation in the mouse hindlimb from E13.5 to E15.5 (Figure 2E). GO analysis showed that this group is highly enriched with genes playing roles in “extracellular matrix organization,” “collagen fibril organization,” and “connective tissue development” (Figure 2F), consistent with a major role of Scx in regulating tendon formation.

Characterization of a New Scx Mutant Mouse Resource

Whereas several distinct Scx mutant mouse lines have been reported (Murchison et al., 2007; Yoshimoto et al., 2017; Shukunami et al., 2018), none of these are available to the wide biomedical research community through the Mouse Resource Centers. On the other hand, the United States National Institutes of Health-funded Knock-out Mouse Project (KOMP) Consortium has generated a Scx-knockout mouse line [C57BL/6N-Scx<sup>tm1.1(KOMP)Vkg/MbpMmucd</sup>] and made available through the Mouse Biology Program at the University of California Davis, but the tendon developmental defects in mice homozygous for this particular Scx knockout allele has not been described. We obtained the C57BL/6N-Scx<sup>tm1.1(KOMP)Vkg/MbpMmucd</sup> mouse line and established a breeding colony. The Scx<sup>tm1.1(KOMP)</sup> allele (abbreviated as Scx<sup>−</sup> in the rest of the report) contains an insertion of the VelociGene ZEN-Ub1 LacZ reporter cassette replacing the entire protein-coding sequences from the translation start site in Exon-1 to 56 bp 3′ to the translation STOP codon in Exon-2 of the Scx gene (Figure 3A). We confirmed the structure and correct integration of the lacZ reporter cassette at the Scx locus through Sanger sequencing of genomic PCR products. However, no specific beta-galactosidase reporter
activity was detected in developing tendon tissues in the heterozygous and homozygous mutant mouse embryos, likely due to reporter gene silencing described previously in multiple other KOMP mouse lines (Kirov et al., 2015). To confirm that Scx function is lost in the Scx\(^{-/-}\) embryos, we carried out in situ hybridization assay using a probe specific for the Scx coding sequence and found that Scx mRNA expression was completely absent in the Scx\(^{-/-}\) embryos (Figures 3B,C). We further characterized the phenotypes of the Scx\(^{-/-}\) mutant mouse. Scx\(^{-/-}\) mice were born at Mendelian ratio, but with reduced body size and severely impaired limbs, with the autopod of both fore- and hind-limbs locked in a dorsal flexure (Supplementary Figure 1). All neonatal Scx\(^{-/-}\) mutant mice exhibited severe hypoplasia of major limb and tail tendons (Figures 3D–I). Analysis of skeletal preparations showed that Scx\(^{-/-}\) mutants lacked the deltoid tuberosity of the humerus in the forelimb and exhibited reduced size of the patella and the enthesal cartilage of the calcaneus in the hindlimb (Supplementary Figures 2A–D). In addition, the transverse processes of the lumbar vertebrae were reduced in the Scx\(^{-/-}\) mutants (Supplementary Figures 2E, F). Using a previously reported Scx-GFP transgenic reporter (Pryce et al., 2007), we found that tendon differentiation and condensation in the developing limbs were disrupted by E15.5 in the homozygous mutant embryos (Supplementary Figure 3). The tendon and skeletal defects in these Scx\(^{-/-}\) mice are similar to those reported previously in two other independent Scx mutant mouse lines (Murchison et al., 2007; Shukunami et al., 2018). Thus, this KOMP-generated Scx knockout mouse line is a valuable resource for the biomedical research community for further studies of Scx function in vivo.

**Analysis of Scx-Dependent Transcriptome Expression Profiles During Early Tendon Cell Differentiation in vivo**

We crossed the Scx-GFP transgenic reporter (Pryce et al., 2007) into the new Scx\(^{+/+}\) mouse line and then intercrossed Scx\(^{+-}\); Scx-GFP mice for analysis of transcriptomic effects of Scx during early tendon cell differentiation. Scx-GFP\(^{+}\) cells were isolated by FACS from freshly dissected forelimb tissues from E15.5 wildtype, Scx\(^{+-}\), and Scx\(^{-/-}\) mutant embryos for RNA-seq.
FIGURE 3 | Characterization of a Scx null mutant mouse strain. (A) Schematic diagram and strategy of generating Scx null mutant allele. A VelociGene ZEN-Ub1 LacZ reporter cassette was inserted after the endogenous Scx ATG codon, by homologous recombination, and replaced the entire Scx coding sequence plus a 56 bp 3’ UTR sequence. (B,C) In situ hybridization on E13.5 Scx−/− mutant and control forelimbs, with an anti-sense RNA probe against the protein coding region of the Scx mRNAs. (D–I) Whole mount view of tendons in forelimb (D,E), hindlimb (F,G), and tail (H,I) from Scx−/− mutant (E,G,I) and control littermates (D,F,H) at P21. White arrowheads point to extensor digitorium communis in panels (D,E), Achilles tendons in panels (F,G), and tail tendons in panels (H,I). Scale bar is 500 µm in panel (B) and 1000 µm in panel (D).

analysis. Analysis of the RNA-seq results from three Scx−/− embryos and three control littermates identified 68 genes that exhibited significant changes in expression levels (FDR < 0.05) in the forelimb Scx-GFP+ cells in E15.5 Scx−/− embryos compared with their control littermates (Figures 4A–D). Comparison of the Scx-dependent differentially expressed genes with the Scx ChIP-seq data revealed that 32 of the significantly down-regulated genes were associated with Scx occupancy in the tendon progenitor cells (Figure 4E and Table 1). These genes are likely critical direct target genes mediating Scx function in tendon formation and early tendon cell differentiation. Indeed, GO analysis of this gene group showed that “tendon development,” “tendon cell differentiation,” “keratan sulfate biosynthesis,” and “ECM organization” are among the most significantly enriched biological processes (Figure 4F). The most significantly down-regulated Scx target genes in the E15.5 Scx−/− forelimb tendon cells include Fmod, Tnmd, Kera, and Col11a1 (Figure 4C and Table 1), of which each plays important roles in tendon cell differentiation and/or collagen fibrillogenesis (Svensson et al., 1999; Pellegata et al., 2000; Docheva et al., 2005; Kilts et al., 2009; Wenstrup et al., 2011; Sun et al., 2020) but only Tnmd has previously been identified as a Scx transcriptional target gene.
The Scx-dependent target genes also include three genes encoding transcription factors, Mkx, Six2, and Eya1 (Table 1). Mkx function is required for tendon fibril growth and tendon homeostasis (Ito et al., 2010; Liu et al., 2010, 2019; Suzuki et al., 2016). Analysis of RNA-seq data also revealed 31 genes that were significantly up-regulated in the Scx-GFP⁺ forelimb cells of E15.5 Scx⁻/⁻ embryos compared with control littermates (Figures 4B–D). Among these, 17 genes were associated with Scx ChIP-seq peaks in the tendon progenitor cells (Figure 4E and Table 2). GO analysis of this group of genes showed that many of these, including Ccbe1, Cxcl12, Epha3, Fgf10, Igf1, Lab2, Postn, Smoc2, and Surf1, are involved in “regulation of cell migration” (Figure 4G). In addition, Surf1, Postn, Smoc2, Ehn, and Nid2 are involved in regulation of “extracellular matrix organization,” whereas Cxcl12 and Fgf10 have been implicated in “induction of chemotaxis” (Figure 4G and Table 2). Taken together, Scx regulates early tendon cell differentiation by controlling the expression of multiple ECM components as well as signaling pathways controlling cell behavior.

**Multiple Scx Target Genes Exhibited Specific Scx-Dependent Expression During Tendon Differentiation**

We further analyzed the patterns of expression of the top 10 down-regulated genes in the E15.5 Scx⁻/⁻ embryos from the RNA-seq data. Whole mount in situ hybridization analyses showed that the Fmod, Htra3, Kera, Ssc5d, Tnmd, and Zfp185 genes each exhibited highly specific patterns of expression in the developing tendon tissues in E14.5 wildtype embryos and their expression in the developing limb tendons was dramatically reduced in the Scx⁻/⁻ littermates (Figures 5A–L). Furthermore, quantitative real-time RT-PCR analysis revealed that expression of each of these genes was already significantly reduced in the Scx-GFP⁺ forelimb mesenchyme cells in Scx⁻/⁻ embryos by E13.5, in comparison with their control littermates (Figure 5M). Among
Zfp185
−
Tnmd
Tmem44
+
−
Syt7
+
St8sia1
−
Thbs4
−
Tmem44
+
−
Olfml2b
+
Naalad2
+
Htra3
−
Fmod
−
Mtcl1
+
Mkx
−
Fat3
−
Eya1
Enpp2
+
−
Dock4
Cyr61
Chst5
−
Ccdc88a
−
Ccdc85a
C1qtnf3
−
Adgrg2

in tendon development remain unknown, our finding that they
those, Fmod and Tnmd have been reported to play important
roles in tendon development whereas Kera plays crucial roles in
other connective tissues including the cornea (Svensson et al.,
1999; Pellegrata et al., 2000; Docheva et al., 2005; Kilts et al.,
2009). Htra3 and Zfp185 exhibited specific expression in the
developing tendons, similar to Tnmd, whereas Ssc5d exhibited a
similar pattern of expression as Fmod in both tendon cells and
in joint cells (Figure 5). Further in situ hybridization analysis of
serial transverse sections through the digit, metacarpal, and
zeugopod regions of the E15.5 forelimb samples confirmed the
specificity of expression of Htra3, Zfp185, and Tnmd throughout
the differentiating tendon cells (Supplementary Figure 4).
Examination of the Scx ChIP-seq profiles associated with
these genes showed high enrichment of Scx binding at the
basal promoter regions of Fmod, Ssc5d, and Zfp185
whereas Scx binding was also specifically enriched at distal
regulatory elements associated with Fmod, Kera, Htra3, and
Tnmd (Figure 6). Whereas the roles of Htra3, Ssc5d, and Zfp185
in tendon development remain unknown, our finding that they
are direct Scx targets that exhibit Scx-dependent activation
during early tendon differentiation suggests that they play crucial
roles downstream of Scx in tendon development.

**DISCUSSION**

Previous genetic studies have identified Scx as the most
crucial transcriptional regulator of tendon development as well
as tendon homeostasis and repair (Murchison et al., 2007;
Yoshimoto et al., 2017; Shukunami et al., 2018; Gumucio et al.,
2020). Mice lacking Scx exhibited loss or severe disruption
of force-transmitting tendons throughout the body as well as
defects in enthesal development (Murchison et al., 2007;
Killian and Thomopoulos, 2016; Yoshimoto et al., 2017). However,
the molecular mechanisms mediating Scx function in tendon
development is not well understood and very few Scx target
genes in tendon cells have been identified. The lack of large scale
identification of direct target genes of Scx in tendon development

| **TABLE 1** | Genes with associated Scx-binding peaks and down-regulated in Scx−/− tendon cells at E15. |
|----------------|--------------------------------------------------------------------------------------------------|
| **Gene symbol** | **Scx-binding peaks (numbers indicate distance to TSS, in bp)** |
| Agrp2           | −184004                                                                                          |
| Aqp1            | −90524*                                                                                           |
| C1qtnf3         | −229323*                                                                                          |
| Ccdc95a         | +114267                                                                                           |
| Ccdc98a         | −13517                                                                                           |
| Chat5           | +5231                                                                                             |
| Clbp2           | +9479                                                                                             |
| Cal11a1         | −170353                                                                                           |
| Cal22a1         | +105886                                                                                           |
| Cyn61           | +931                                                                                              |
| Dock4           | +210387                                                                                           |
| Enpp2           | +114774                                                                                           |
| Eya1            | −375541                                                                                           |
| Fat3            | −425380                                                                                           |
| Fmod            | −95614                                                                                           |
| Htra3           | +57656                                                                                           |
| Kera            | +5138                                                                                             |
| Mlx              | −231675                                                                                           |
| Mtd1            | +20857                                                                                           |
| Nalad2          | +4887                                                                                             |
| Olfm2b          | −38976                                                                                           |
| Pch1            | +27008                                                                                           |
| Rlnk            | −132608*                                                                                          |
| Scube2          | +14689                                                                                           |
| Sis2            | −247206                                                                                           |
| Ssc5d           | −776                                                                                              |
| Stbisa1         | +18010                                                                                           |
| Syt7            | −60962                                                                                           |
| Thbs4           | −37977                                                                                           |
| Tmem44          | +14520                                                                                           |
| Tnmd            | −90582                                                                                           |
| Zfp185          | −423                                                                                              |

*Peak located in neighboring gene.
TABLE 2 | Genes with associated Scx-binding peaks and upregulated in Scx<sup>−/−</sup> tendon cells at E15.5.

| Gene symbol | Scx-binding peaks (numbers indicate distance to TSS, in bp) | TSS (bp) |
|-------------|------------------------------------------------------------|---------|
| Adgrb3      | −746                                                        | +600918* |
| Aspn        | −37162                                                    | +1353   |
| Ccbe1       | −112895                                                   | +57507* |
| Cxcl12      | −311414                                                   | −222186 |
| Dio2        | +329510                                                   |         |
| Eln         | −153983*                                                   | −148138 |
| Epha3       | +7097                                                     | +244725 |
| Fgf10       | −237483                                                   | +19587 |
| Flb         | −50132                                                    | −48436 |
| Igf1        | −206628                                                   | −182419 |
| Ldb2        | −322438*                                                   | −123018 |
| Lingo2      | +801973*                                                   | +15799 |
| Nicd2       | +66867*                                                   |         |
| Postn       | −10601                                                   | +127772 |
| Prgd        | −339455*                                                   | −294031* |
| Smoc2       | −48448                                                   | +34969 |
| Sulf1       | −513097                                                   | −254232 |

*Peak located in neighboring gene.

is largely due to the lack of a reliable specific antibody for mapping genome-wide Scx binding in tendon cells in vivo. Scx belongs to the class II bHLH family of transcription factors, of which many have been shown to bind to an E-box (CANNTG) motif (Cserjesi et al., 1995; Massari and Murre, 2000). However, this short redundant sequence can be found throughout the

FIGURE 5 | Characterization of candidate genes from ChIP-seq and RNA-seq. (A–L) Whole mount in situ hybridization of six candidate genes in E14.5 Scx<sup>−/−</sup> mutant (B,D,F,H,J,L) and control (A,C,E,G,I,K) forelimbs. Expression of Fmod, Kera, Ssc5d, and Zfp185 was dramatically reduced in Scx<sup>−/−</sup> mutant limbs (B,F,H,L), while expression of Htra3 and Tnmd were nearly completely absent in Scx<sup>−/−</sup> mutant (D,J). Expression of Fmod and Ssc5d persisted in digit ligaments in the mutant limbs (B,H). (M) Quantitative RT-PCR analysis of expression of the six candidate genes in Scx<sup>−/−</sup> mutant and control forelimb samples at E13.5 and E15.5. All genes were significantly reduced in Scx<sup>−/−</sup> mutants at both stages, compared with control littermates. *p < 0.05, **p < 0.01, ***p < 0.001.
genome. Thus, previous studies of Scx-mediated transcriptional regulation of putative target genes primarily relied on in vitro biochemical assays including electrophoresis mobility shift assay of recombinant Scx protein binding to E-box containing promoter sequences and promoter reporter assays in cell transfection studies (Lejard et al., 2007; Shukunami et al., 2018; Paterson et al., 2020), and more recently ChIP-qPCR assays of candidate promoter regions (Paterson et al., 2020). In this study, we have generated Scx<sup>Flag</sup> mice and demonstrate that this new mouse line enables direct analysis of endogenous Scx protein function in the normal developmental and physiological context. Whereas we have used this mouse line for genome-wide mapping of Scx binding sites in early developing limb tendon tissues, this mouse line will provide a valuable tool for direct analysis of endogenous Scx protein function in many developmental and disease processes where Scx plays a role.

Our ChIP-seq analysis identified 12,097 high quality Scx binding sites in the E13.5 mouse forelimb, with the most highly enriched de novo motif identified as 5′ CAG/TA/CTG 3′. Whereas previous in vitro studies have shown Scx binding to various E-boxes including CAGTG in the Col1a1 promoter (Lejard et al., 2007), CAGGTTG in the Col2a1 promoter (Furumatsu et al., 2010), and CAAATG and CAGATG in the Tnmd promoter (Li et al., 2015), direct comparative EMSA analysis of Scx binding to five distinct E-box containing elements from the mouse Tnmd promoter region showed that Scx preferentially bound to CAGATG and CATCTG, but not the others (Shukunami et al., 2018). Thus, the consensus Scx binding motif identified from our ChIP-seq data matches perfectly with the EMSA-determined preferential Scx binding motif, which affirms our genome-wide mapped Scx binding sites. Whereas several in vitro studies have repeatedly demonstrated Scx binding to the Tnmd promoter regions and Scx activated expression of Tnmd promoter-reporter constructs in co-transfected cells (Li et al., 2015; Shukunami et al., 2018), our ChIP-seq results showed that endogenous Scx binding was primarily enriched at two upstream locations at about 20 and 90 kb, respectively, from the Tnmd TSS (Figure 6E). A minor Scx binding peak was detected at the Tnmd basal promoter region (Figure 6E), which is enriched over 4-fold over the input but the FDR value, at 0.025, was below the stringent threshold (4-fold enrichment over input and FDR < 0.01) used for identifying the genome-wide Scx binding peaks in our analysis. It is possible that Scx binding to both the distal enhancers and the promoter region to synergistically activate Tnmd gene expression in tenocytes.

Whereas previous studies have shown that activation of Tnmd expression during tendon development depends on Scx function and that Tnmd plays crucial roles in tenocyte proliferation and maturation, Tnmd null mice exhibited a much milder tendon phenotype than Scx<sup>−/−</sup> mice (Docheva et al., 2005; Murchison et al., 2007; Yoshimoto et al., 2017; Shukunami et al., 2018). Our ChIP-seq and RNA-seq results demonstrate that Scx directly regulates expression of a large number of genes during early tendon development, including activation of expression of many tendon cell-specific genes. In particular, we validated Fmod, Htra3, Ssc5d, and Zfp185 as new direct target genes that dependent on Scx for their expression in the differentiating tendon cells. Fmod-deficient mice exhibited defects in tendon collagen fibrillogenesis (Chakravarti, 2002), but the roles of Htra3, Ssc5d, and Zfp185 in tendon development are unknown. Htra3 encodes a serine peptidase that cleaves proteoglycans, thus may function in ECM remodeling (Nie et al., 2003; Glaza et al., 2015). Ssc5d (scavenger receptor cysteine rich family member with 5 domains) has been implicated in playing a role at the interface between adaptive and innate immunity and in placental functions.

![FIGURE 6 | Visualization of the Scx-binding peaks associated with the candidate Scx direct target genes. (A–F) genome browser views of genomic regions containing, Fmod (A), Htra3 (B), Kera (C), Ssc5d (D), Tnmd (E), and Zfp185 (F) genes. Numbers under each peak indicate the distance to the transcription start site (TSS) the marked gene. The red arrow in panel (E) point to a Scx-binding peak at the Tnmd gene promoter region that is recognizable upon careful examination but was below the FDR < 0.01 threshold for identifying genome-wide Scx binding sites in our ChIP-seq data analysis.](image-url)
function (Goncalves et al., 2009). Zfp185 encodes LIM domain type zinc finger protein (Heiss et al., 1997; Zhang et al., 2016). Whereas the Zfp185 protein was originally hypothesized to reside in the cell nucleus, subsequent in vitro cell biological studies suggested that Zfp185 binds to F-actin and may be involved in modulating dynamics of actin filaments (Wang et al., 2008; Zhang et al., 2016). Thus, these gene products have diverse cellular functions and loss of their expression during early tendon cell differentiation in the Scx−/− embryos likely contributed to the severe disruption of tendon condensation phenotype.

Whereas our ChIP-seq analysis identified more than 12,000 high quality Scx-binding regions associated with over 7,500 genes in the embryonic forelimb tissues, our RNA-seq analysis uncovered a relatively small number of genes that exhibited significant differential expression in the Scx-GFP+ forelimb tendon cells between E15.5 Scx−/− and control littersmates. These results suggest that Scx participates in the regulation of a large number of genes during early tendon development, but other factors likely partly compensate for Scx function in the Scx−/− mice. Nevertheless, the differential gene expression profiles uncovered by our RNA-seq data provide new insights into the molecular mechanisms underlying Scx-mediated regulation of tendon formation. It has been shown that the tendon microfilaments are highly disorganized in the Scx−/− mouse embryos (Murchison et al., 2007). We found that expression of Col11a1 was among the most down-regulated genes in the E15.5 Scx−/− forelimb tendon cells. Recent studies have shown that ColXI, the gene product of Col11a1, plays an essential role in tendon fibril assembly and organization (Wenstrup et al., 2011; Sun et al., 2020). Mice with tendon-specific disruption of Col11a1 in the Scx-expressing lineages exhibited abnormal tendon fibril structure, smaller fibril diameter and disrupted fibril alignment (Sun et al., 2020). Thus, activation of Col11a1 expression in the differentiating tenocytes is likely an important part of Scx function in tendon formation. In addition to regulating tendon ECM organization and fibrillogenesis, Scx plays a crucial role in regulating tendon cell morphology and organization (Murchison et al., 2007). Whereas wildtype tenocytes develop a complex network of cytoplasmic extensions that engulf the collagen fibril bundles during tendon fibrillogenesis, the tenocytes in Scx−/− mouse embryos exhibited much reduced and less complex cytoplasmic extensions (Murchison et al., 2007). Scx plays an important role in tendon cell migration and/or chemotaxis in the tendons of adult mice due to conditional inactivation of Scx (Gumucio et al., 2020). We found that Dock4, encoding a key regulator of filapodia and lamellipodia protrusions (Hiramoto et al., 2006; Abraham et al., 2015), was among the top downregulated genes in the E15.5 tendon cells. As tenocyte cytoplasmic extensions likely also involve spatially regulated changes of the actin cytoskeleton, our finding of significant down-regulation of Zfp185, Cdc85a and Cdc88a, which all encode actin binding proteins, in the tendon cells in E15.5 Scx−/− embryos suggests that Scx regulates expression of multiple cytoplasmic proteins to control tenocyte morphology and organization. Moreover, to maintain the stretched tenocyte morphology with a complex network of cytoplasmic extensions during tendon growth and elongation likely requires cell membrane repair mechanisms to maintain plasma membrane integrity. In Scx−/− mice tendon rudiments were detected in the embryonic tail but tail tendon was completely absent in 2-week-old mutant mice due to increased apoptosis (Murchison et al., 2007). Among the most significantly downregulated genes in the Scx−/− tendon cells in our RNA-seq data is Syt7 (Figure 4C), which encodes a transmembrane protein with a crucial role in maintenance of plasma membrane integrity and repair via regulating lysosomal exocytosis (Chakrabarti et al., 2003). Further studies will be needed to investigate whether Syt7 plays an important role in Scx-mediated tenocyte differentiation and maintenance. Furthermore, it has been shown that Scx function is required for proper organization of tendon sheath such as the endotenon and that EphA4-expressing endotenon cells were detected intermixed with tenocytes in the limb tendons in Scx−/− embryos (Murchison et al., 2007). Our RNA-seq data showed that expression of EphA3 was significantly increased in the limb tendon cell in E15.5 Scx−/− embryos. It is possible that the increased EphA3 expression in the tenocytes resulted in disruption of the Eph-Efn signaling involved in regulation of tendon sheath formation.

Whereas Scx is expressed in all tendon progenitor cells and throughout of tendon development (Schweitzer et al., 2001; Brent et al., 2003), only a subset of tendon tissues were significantly disrupted in Scx−/− mice (Murchison et al., 2007). Huang et al. (2019) demonstrated that Scx function is required for recruitment of mesenchymal progenitor cells into the initially formed tendon rudiments during the growth and elongation of the limbs and tail (Huang et al., 2019). Remarkably, lineage-specific genetic analysis and cell transplantation assays demonstrated that Scx function is exclusively required in the recruited mesenchymal cells, but not in the recruiting tendon, for the recruitment and integration of the mesenchymal progenitor cells during tendon elongation (Huang et al., 2019). The molecular mechanism acting downstream of Scx in mediating the tendon cell recruitment and elongation is currently unknown. Although our RNA-seq data show significant changes in expression of several signaling molecules involved in regulation of cell migration and/or chemotaxis in the Scx−/− Scx-GFP+ forelimb cells, it is not clear whether and how some of these molecules may mediate Scx function in tendon cell recruitment. Further analysis of the detailed spatiotemporal patterns of expression of these genes during tendon elongation and/or single-cell transcriptomic profiling combined with lineage-specific functional studies will help resolve the underlying molecular mechanism.

In a recent report, Gumucio et al. (2020) performed RNA-seq analysis to identify genes and signaling pathways that respond differently to mechanical overloads in the plantaris tendons in adult mice due to conditional inactivation of Scx (Gumucio et al., 2020). We compared our RNA-seq results from embryonic tendon cells with their RNA-seq results from the adult tenocytes and found that there were a number of overlapping genes in both the down-regulated and up-regulated groups of Scx-dependent differentially expressed genes. In particular, the Fmod, Kera, Ssc5d, Tnmd, and Zfp185 genes were significantly downregulated in the adult Scx−/− deficient mutant tendon tissues. These genes likely play important roles in tendon cell differentiation at both embryonic and adult stages. Thus, further elucidation of the molecular mechanisms of tendon development will facilitate
investigation of adult tendon cell behaviors during injuries and regeneration and contribute ultimately to improvement in strategies for tendon therapies. In this regard, our ChIP-seq and RNA-seq datasets provide a rich resource for aiding the design of new studies of the molecular and cellular mechanisms of tendon development and repair.

DATA AVAILABILITY STATEMENT

ChIP-seq and RNA-seq data was deposited in the NCBI GEO database under the accession number(s) GSE173428.

ETHICS STATEMENT

The animal study was reviewed and approved by Cincinnati Children’s Research Foundation Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

HL, YL, and RJ conceptualized and designed the research. HWL helped revise the research design and data analysis during the manuscript revision. HL and JX performed the research. HL, JX, YL, HWL, and RJ analyzed the data and critically revised the manuscript. HL and RJ wrote the manuscript. All authors approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

REFERENCES

Abraham, S., Scardia, M., Bagshaw, R. D., McMahon, K., Grant, G., Harvey, T., et al. (2015). A Rac/Cdc42 exchange factor complex promotes formation of lateral filopodia and blood vessel lumen morphogenesis. Nat. Commun. 6:7286. doi: 10.1038/ncomms8286

Birch, H. L., Thorpe, C. T., and Rumian, A. P. (2013). Specialisation of extracelluar matrix for function in tendons and ligaments. Muscles Ligaments Tendons J. 3, 12–22. doi: 10.11138/mltj/2013.3.1.012

Brent, A. E., Schweitzer, R., and Tabin, C. J. (2003). A somitic compartment of tendon progenitors. Cell 113, 235–248.

Chakrabarti, S., Kobayashi, K. S., Flavell, R. A., Marks, C. B., Miyake, K., Liston, D. R., et al. (2003). Impaired membrane resealing and autoimmune myositis in synaptotagmin VII-deficient mice. J. Cell Biol. 162, 543–549. doi: 10.1083/jcb.200305131

Chakravarti, S. (2002). Functions of lumican and fibromodulin: lessons from transgenic mouse models. Stem Cells Dev. 11, 287–293. doi: 10.1023/A:1025348417078

Chen, J., Bardes, E. E., Aronow, B. J., and Jegga, A. G. (2009). ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. Nucleic Acids Res. 37, 305–311. doi: 10.1093/nar/gkp427

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823. doi: 10.1126/science.1231143

Cserjesi, P., Brown, D., Ligon, K. L., Lyons, G. E., Copeland, N. G., Gilbert, D. J., et al. (1995). Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. Development 121, 1099–1110.

Davis, M. E., Gumucio, J. P., Sugg, K. B., Bedi, A., and Mendias, C. L. (2013). MMP inhibition as a potential method to augment the healing of skeletal muscle and tendon extracellular matrix. J. Appl. Physiol. 115, 884–891. doi: 10.1152/japplphysiol.00137.2013

Delgado Caceres, M., Pfeifer, C. G., and Docheva, D. (2018). Understanding tendons: lessons from transgenic mouse models. Stem Cells Dev. 27, 1161–1174. doi: 10.1089/scd.2018.0121

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21. doi: 10.1093/bioinformatics/bts635

Docheva, D., Hunziker, E. B., Fassler, R., and Brandau, O. (2005). Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Mol. Cell. Biol. 25, 699–705. doi: 10.1128/MCB.25.2.699-705.2005

Dourte, L. M., Pathmanathan, L., Mienaltowski, M. J., Jawad, A. F., Birk, D. E., and Soslowski, L. J. (2013). Mechanical, compositional, and structural properties of the mouse patellar tendon with changes in biglycan gene expression. J. Orthop. Res. 31, 1430–1437. doi: 10.1002/jor.22372

Dunkman, A. A., Buckley, M. R., Mienaltowski, M. J., Adams, S. M., Thomas, S. J., Kumar, A., et al. (2014). The injury response of aged tendons in the absence of biglycan and decorin. Matrix Biol. 35, 232–238. doi: 10.1016/j.matbio.2013.10.008

Furumatsu, T., Shukunami, C., Amemiya-Kudo, M., Shimano, H., and Ozaki, T. (2010). Scleraxis and E47 cooperatively regulate the Sox9-dependent transcription. Int. J. Biochem. Cell. Biol. 42, 148–156. doi: 10.1016/j.biocel.2009.10.003

Glaza, P., Osipiuk, J., Wenta, T., Zuraaws-Janicka, D., Jarzab, M., Lesner, A., et al. (2015). Structural and functional analysis of human HtrA3 protease and its subdomains. PLoS One 10:e0131142. doi: 10.1371/journal.pone.0131142

Goncalves, C. M., Castro, M. A., Henriques, T., Oliveira, M. I., Pinheiro, H. C., Sampaio, J. et al. (2015). A Rac/Cdc42 exchange factor complex promotes formation of lateral filopodia and blood vessel lumen morphogenesis. J. Biol. Chem. 287, 19569–19576. doi: 10.1074/jbc.M115.642837

Guerrquin, M. J., Charvet, B., Nourissat, G., Havis, E., Ronson, O., Bonnin, M. A., et al. (2013). Transcription factor EGR1 directs tendon differentiation and development and repair.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.654397/full#supplementary-material
promotes tendon repair. *J. Clin. Invest.* 123, 3564–3576. doi: 10.1172/JCI67521

Gumucio, J. P., Schonk, M. M., Kharaz, Y. A., Comerford, E., and Mendias, C. L. (2020). Scleraxis is required for the growth of adult tendons in response to mechanical loading. *JCI Insight* 5, e136295. doi: 10.1172/jci.insight.136295

Hasson, P. (2011). “Soft” tissue patterning: muscles and tendons of the limb take their form. *Dev. Dyn.* 240, 1100–1107. doi: 10.1002/dvdy.22608

Havis, E., Bonnin, M. A., Esteves de Lima, J., Charvet, B., Milet, C., and Duprez, D. (2016). TGFbeta and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development* 143, 3839–3851. doi: 10.1242/dev.136242

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., et al. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell.* 38, 576–589. doi: 10.1016/j.molcel.2010.05.004

Heiss, N. S., Gloeckner, G., Bachner, D., Kioschi, P., Klauck, S. M., Hinzmann, B., et al. (1997). Genomic structure of a novel LIM domain gene (ZNFL185) in Xq28 and comparisons with the orthologous murine transcript. *Genomics* 43, 329–338. doi: 10.1006/geno.1997.4810

Hiramoto, K., Negishi, M., and Katoh, H. (2006). Dock4 is regulated by RhoG.

Hasson, P. (2011). “Soft” tissue patterning: muscles and tendons of the limb take their form. *Dev. Dyn.* 240, 1100–1107. doi: 10.1002/dvdy.22608

Havis, E., Bonnin, M. A., Esteves de Lima, J., Charvet, B., Milet, C., and Duprez, D. (2016). TGFbeta and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development* 143, 3839–3851. doi: 10.1242/dev.136242

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., et al. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell.* 38, 576–589. doi: 10.1016/j.molcel.2010.05.004

Heiss, N. S., Gloeckner, G., Bachner, D., Kioschi, P., Klauck, S. M., Hinzmann, B., et al. (1997). Genomic structure of a novel LIM domain gene (ZNFL185) in Xq28 and comparisons with the orthologous murine transcript. *Genomics* 43, 329–338. doi: 10.1006/geno.1997.4810

Hiramoto, K., Negishi, M., and Katoh, H. (2006). Dock4 is regulated by RhoG.

Hasson, P. (2011). “Soft” tissue patterning: muscles and tendons of the limb take their form. *Dev. Dyn.* 240, 1100–1107. doi: 10.1002/dvdy.22608

Havis, E., Bonnin, M. A., Esteves de Lima, J., Charvet, B., Milet, C., and Duprez, D. (2016). TGFbeta and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development* 143, 3839–3851. doi: 10.1242/dev.136242

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., et al. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell.* 38, 576–589. doi: 10.1016/j.molcel.2010.05.004

Hasson, P. (2011). “Soft” tissue patterning: muscles and tendons of the limb take their form. *Dev. Dyn.* 240, 1100–1107. doi: 10.1002/dvdy.22608

Havis, E., Bonnin, M. A., Esteves de Lima, J., Charvet, B., Milet, C., and Duprez, D. (2016). TGFbeta and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development* 143, 3839–3851. doi: 10.1242/dev.136242

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., et al. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell.* 38, 576–589. doi: 10.1016/j.molcel.2010.05.004

Hasson, P. (2011). “Soft” tissue patterning: muscles and tendons of the limb take their form. *Dev. Dyn.* 240, 1100–1107. doi: 10.1002/dvdy.22608

Havis, E., Bonnin, M. A., Esteves de Lima, J., Charvet, B., Milet, C., and Duprez, D. (2016). TGFbeta and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development* 143, 3839–3851. doi: 10.1242/dev.136242

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., et al. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell.* 38, 576–589. doi: 10.1016/j.molcel.2010.05.004

Hasson, P. (2011). “Soft” tissue patterning: muscles and tendons of the limb take their form. *Dev. Dyn.* 240, 1100–1107. doi: 10.1002/dvdy.22608

Havis, E., Bonnin, M. A., Esteves de Lima, J., Charvet, B., Milet, C., and Duprez, D. (2016). TGFbeta and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development* 143, 3839–3851. doi: 10.1242/dev.136242
Suzuki, H., Ito, Y., Shinohara, S., Ichinose, S., Kishida, A., et al. (2016). Gene targeting of the transcription factor Mohawk in rats causes heterotopic ossification of Achilles tendon via failed tenogenesis. Proc. Natl. Acad. Sci. U S A 113, 7840–7845. doi: 10.1073/pnas.1522054113

Svensson, L., Aszodi, A., Reinhold, F. P., Fassler, R., Heinegard, D., and Oldberg, A. (1999). Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and altered lumican deposition in tendon. J. Biol. Chem. 274, 9636–9647. doi: 10.1047/jbc.274.14.9636

Wang, N., Zheng, Q., Zhang, J. S., and Zhao, Y. (2008). Molecular cloning and characterization of a novel mouse actin-binding protein Zlp185. J. Mol. Histol. 39, 295–302. doi: 10.1007/s10735-008-9165-2

Watson, S. S., Riordan, T. J., Pryce, B. A., and Schweitzer, R. (2009). Tendons and muscles of the mouse forelimb during embryonic development. Dev. Dyn. 238, 693–700. doi: 10.1002/dvdy.21866

Wenstrup, R. J., Smith, S. M., Florez, J. B., Zhang, G., Beason, D. P., Srengmuller, R. E., et al. (2011). Regulation of collagen fibril nucleation and initial fibril assembly involves coordinate interactions with collagens V and XI in developing tendon. J. Biol. Chem. 286, 20455–20465. doi: 10.1047/jbc.M111.223693

Xu, J., Liu, H., Lan, Y., Park, J. S., and Jiang, R. (2020). Genome-wide identification of Foxf2 target genes in palate development. J. Dent. Res. 99, 463–471. doi: 10.1177/0022034520904018

Xu, J., Liu, H., Park, J. S., Lan, Y., and Jiang, R. (2014). Osr1 acts downstream of and interacts synergistically with Six2 to maintain nephron progenitor cells during kidney organogenesis. Development 141, 1442–1452. doi: 10.1242/dev.103283

Yang, G., Rothrauff, B. B., and Tuan, R. S. (2013). Tendon and ligament regeneration and repair: clinical relevance and developmental paradigm. Birth Defects Res. C Embryo Today 99, 203–222. doi: 10.1002/bdrc.21041

Yoshimoto, Y., Takimoto, A., Watanabe, H., Hiraki, Y., Kondoh, G., and Shukunami, C. (2017). Scleraxis is required for maturation of tissue domains for proper integration of the musculoskeletal system. Sci. Rep. 7:45010. doi: 10.1038/srep45010

Yu, H. B., Yurieva, M., Balachander, A., Foo, I., Leong, X., Zelante, T., et al. (2015). NFATc2 mediates epigenetic modification of dendritic cell cytokine and chemokine responses to dectin-1 stimulation. Nucleic Acids Res. 43, 836–847. doi: 10.1093/nar/gku1369

Zhang, Y., Lan, H., Shao, Q., Wang, R., Chen, H., Tang, H., et al. (2016). An A20/AN1-type zinc finger protein modulates gibberellins and abscisic acid contents and increases sensitivity to abiotic stress in rice (Oryza sativa). J. Exp. Bot. 67, 315–326. doi: 10.1093/jxb/erv464

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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