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The Mohawk homeobox transcription factor regulates the differentiation of tendons and volar plates

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N.O., Y.I., Y.T. and H.A. designed the study; N.O., Y.I. and M.I. performed the research; N.O., Y.I., M.L., S.T., H.N. and H.A. analyzed the data; and N.O. and H.A. wrote the paper.

Conflict of interests
The authors declare no conflict of interest.

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Abstract

Background—Mohawk *(Mkx)* is a homeodomain-containing transcription factor that is expressed in various mesoderm-derived tissues, particularly in developing tendons. In this study, we investigate the exact expression pattern and functions of *Mkx* in forelimbs.

Methods—We analyzed the forelimbs of *Mkx* knockout mice (from embryonic day [E] 18.5 to postnatal day [P] 28-week) by using knocked-in Venus signals, Masson trichrome staining, and hematoxylin and eosin (H&E) staining.

Results—We detected Venus signals in forelimb tendons, pulleys, and volar plates (VPs) in P21 mice. In-depth histological analysis showed that compared to the wild-type mice, the *Mkx* knockout mice showed significant hypoplasia in the flexor digitorum profundus (FDP) tendons from E18.5. The VPs and pulleys appeared normal until P0, however, by P14, they became increasingly thicker in *Mkx*-null mice compared to wild-type mice. The fiber alignment was particularly disrupted in VPs of *Mkx*-null mice.

Conclusions—These results suggest that *Mkx* is an important regulator of the differentiation of VPs and pulleys, as well as of tendon differentiation.

Introduction

Tendons are dense connective tissues of mesodermal origin, which connect and transmit force from muscles to bones. There is one flexor tendon for the thumb and two for each of the other fingers[1]. Flexor tendon injuries such as lacerations or ruptures are common and are challenging for orthopedic surgeons [2].

The functions of tendons are supported by several peritendinous tissues, such as pulleys and volar plates (VPs). VPs are small fibrocartilaginous structures located in the proximal interphalangeal (PIP) joints (Fig. 1) [3]. VPs can be injured by hyperextension, sometimes resulting in dorsal dislocation of the phalanx or major joint instability[4], such as in PIP joint fracture-dislocations[5]. Even with adequate treatment, this injury can result in chronic pain, stiffness, and swelling [6,7]. Although tendon and VP injuries are major clinical issues for orthopedic surgeons, the molecular mechanisms underlying their development are not completely understood.

To establish new therapies for these injuries, knowledge regarding tendon biology is required. To date, two transcription factors, scleraxis *(Scx)* and mohawk (*Mkx*), have been identified as critical regulators of tendon development. Previous studies have shown that *Scx* is essential for the initiation of tendon and tendon sheath differentiation[8–10], whereas *Mkx* plays an important role in tendon maturation[11,12]. *Scx*, a basic helix–loop–helix (bHLH) transcription factor expressed in tendon progenitors[8,9], is required for the proper development of limb tendons by promoting collagen synthesis in tendons[10]. *Col1a1*, *Col1a2*, aggrecan, and tenomodulin *(Tnmd)* genes are the key targets of the *Scx*-dependent transcription[10,13–15]. The *Mkx* homeobox gene, also known as iroquois-related homeobox like 1 (*Irxl1*), is a homeodomain-containing transcription factor that belongs to the three amino acid loop extension (TALE) superclass of atypical homeodomain proteins, which have three extra amino acid residues between the first and second helices of their homeodomains[16–18]. *Mkx* encodes a 353-amino-acid protein with a putative homeodomain

motif highly similar to those of the invertebrate iroquois and vertebrate *Irx* subfamily members[16–20]. Biochemical studies have shown that *Mkx* has transcriptional repressor activity[21,22].

We previously identified *Mkx* as a transcription factor expressed in developing tendons by constructing a whole-mount in situ hybridization database, termed "EMBRYS" (http://embrys.jp/embrys/html/MainMenu.html)[23]. The initial characterization of mouse *Mkx* showed a dynamic transcription pattern in various mesoderm-derived tissues, such as the dorsomedial lip and the ventrolateral lip of somites, limb buds, endocardia, kidneys, and male gonads[11,16,18]. In the somite and limb bud, the expression of *Mkx* is observed in the progenitors of skeletal muscles, tendons, and cartilage[16–18].Our group, along with two other groups, generated *Mkx* knockout mice to investigate the functions of *Mkx* and reported the critical function of *Mkx* in the development of tendons, showing that tendons of *Mkx*-null mice were significantly hypoplastic throughout the body[11,12, 24]. It was also found that the mRNA levels of *Col1a1* and *Col1a2*, which encode type I collagen, as well as those of decorin (*Dcn*), fibromodulin (*Fmod*), and *Tnmd* were lower in *Mkx*-null mice than in their wild-type littermates[11,12].

However, the role of *Mkx* in peritendinous tissues is largely unknown. In this study, by analyzing gene-targeted mice, we found that the *Mkx* transcription factor is required for VP and pulley maturation, as well as for tendon development.

Materials and methods

Venus knock-in *Mkx*-deletion mice

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at the National Institute for Child Health and Development. Venus, an improved green fluorescent protein (GFP) gene, was inserted in mice heterozygous for exon 2 deletion of the *Mkx* gene ($Mkx^{+/-}$), as described previously[12]. The $Mkx^{+/-}$ mice were intercrossed to generate $Mkx^{-/-}$ homozygous mice.

Immunohistochemistry

For immunohistochemical analysis of Venus signals, forelimbs from postnatal day (P) 21-mice were dissected and fixed with 4% paraformaldehyde in phosphate-buffered saline PBS) at 4 °C for 1 h. The tissues were embedded in optimum cutting temperature (O.C.T.) compound (Sakura Finetek) and frozen rapidly in liquid nitrogen. The specimens were sectioned at 7 μm. Cryosections were air-dried and blocked with Blocking One (Nacalai Tesque) for 1 h. The sections were then incubated at 4 °C overnight with polyclonal rabbit anti-GFP antibody (MBL), rinsed, and incubated for 1 h with Alexa Fluor 488 conjugates of anti-rabbit IgG antibody (Molecular Probes) and DAPI (4′ 6-diamidino-2-phenylindole) for staining of the cell nuclei. High-magnification images of the fluorescent signals were captured using a BX53 compound microscope (Olympus). These experiments were performed with at least three independent samples to confirm their reproducibility.

Histological analysis

For histological analysis, forelimbs harvested from embryos (E18.5) and mice (from P0 to 7 months) were collected at predetermined stages (P0, 1-, 2-, 8-, 24- and 28-weeks) and fixed in 4% paraformaldehyde in PBS at 4°C overnight. Tissues were decalcified in 20% formic acid and 20% citric acid, dehydrated through graded methanol, embedded in paraffin, and sectioned at 7-μm thickness, followed by staining with hematoxylin and eosin (H&E).

Forelimbs from P21-mice were dissected and fixed with 4% paraformaldehyde in PBS at 4 °C for 1 h. The tissues were embedded in O.C.T. compound (Sakura Finetek) and frozen rapidly in liquid nitrogen. Specimens were sectioned at 7 μm. Cryosections were stained with Masson trichrome.

Statistical analysis

The data were processed using the StatView J-4.5 software. Values have been reported in terms of mean ± standard deviation (SD). Student's *t*-test was used to determine the level of significance. The probability level accepted for significance was $P < 0.05$.

Results

Expression of *Mkx* in the forelimbs of mice

Previous studies have shown the expression patterns of *Mkx* in embryos or adult tendons[11,12]. Here, we focused on examining *Mkx* expression in the postneonatal stages in forelimbs. Interestingly, Venus expression, which recapitulates *Mkx* expression, was strongly detected in VPs of P21-$Mkx^{-/-}$ (Fig. 1, Fig. 2 A–C) and $Mkx^{+/-}$ mice (Fig. 2 G–I) in addition to tendons (Fig. 2 A–I) and pulleys (Fig. 2 D–F). Conversely, no signals were detected in the wild-type littermates (Fig. 2 J–L). We also confirmed that the Venus signals were detected in the VPs, pulleys, and tendons of 7-month-old $Mkx^{-/-}$ and $Mkx^{+/-}$ mice, while no signals were detected in the wild-type littermates (data not shown).

Mkx$^{-/-}$ mutant mice have forelimb tendon defects

To investigate the function of *Mkx* in tendon formation, we analyzed the tendons of *Mkx*-null mice. As previously described[11,12,24], tendons throughout the body, such as Achilles, tail, back, and patellar tendons are hypoplastic in adult *Mkx*-null mice. Here, we performed detailed analysis of forelimbs tendons in *Mkx*-null mice from the postneonatal to the adult stages. This analysis showed that both the extensor and flexor digitorum profundus (FDP) tendons of P2 (Fig. 3 E–G), P21 (Fig. 3 M–P), and 7-month-old (Fig. 3 S–V) *Mkx*-null mice were dramatically hypoplastic compared with those of the wild-type littermates (Fig. 3 A–D, H–L, Q–R).

To further confirm the tendon phenotype observed in *Mkx*-null mice, we performed Masson trichrome staining of the forelimbs of P21 mice (Fig. 4) and H&E staining of the forelimbs of P0-, P14-, 8-, 24- and 28-week-old mice, and found that the FDP tendons of *Mkx*-null mice were smaller than those of the wild-type counterparts (Fig. 5–A, Fig. 6–A).

To determine whether tendon defects in *Mkx*-knockout mice are also observed in embryonic stages, we performed H&E staining of the forelimbs of *Mkx*-null embryos at E18.5. The size of the FDP tendons in *Mkx*-null embryos was also smaller than that in the wild-type littermates (Fig. 5–B, Fig. 6–B). Taken together, *Mkx* plays a critical role in hand tendon differentiation in the development stages.

Abnormality in VPs and pulleys in *Mkx*$^{-/-}$ mutant mice

Next, we observed two section levels as described by Watson et al. [25]: one was the PIP level, in which we observed the FDP and the VP; the other was the level of the center of the proximal phalanx, in which we observed the FDP, the flexor digitorum superficialis (FDS), and the A2 pulley (Fig. 5–C). The pulleys and VPs appeared normal at the embryonic stage (Fig. 5–B, Fig. 6–B) and P0 in *Mkx*-null mice (Fig. 5–A, a and b ; Fig. 6–A, a and b). However, compared to the VPs of the wild-type littermates, the VPs became thicker in *Mkx*-null mice by P14 (Fig. 5–A, c and d). We found that 8, 24 and 28-week-old *Mkx* null mice also had much thicker VPs (Fig. 5–A, e–j; Fig. 5–D). Comparison with the results for the

wild-type littermates showed that pulleys as well as VPs became thicker in *Mkx*-null mice by P14 (Fig. 6–A, c and d). Twenty eight-week-old *Mkx*-null mice also had thicker pulleys Fig. 6–A, e and f).

In wild-type mice, VPs are complex structures with three separate layers (Fig. 5–A a, c, e, g, and i; Fig. 7–A). In the palmar-most layer (Fig.7–A, c), fibers run transversely. It has been reported that the fibers of this layer are in continuity with the accessory collateral ligament[4]. The second layer (Fig. 7–A, b), which is the central core of the plate, has both longitudinal and transverse fibers that create a tight "basket-weave" pattern. The dorsal-most layer (Fig. 7–A, a) has fibers that run longitudinally, that is, the same direction as the flexor tendon. We also observed that the dorsal surface of the VP forms the floor of the PIP joint capsule (Fig. 7–A) and that the palmar surface of the VP forms the floor of the flexor tendon sheath. Interestingly, the VP of *Mkx*-null mice was composed of fibers whose orientation was quite disorganized (Fig. 5–A, b, d, f, h, and j; Fig. 7–B).

Thus, in addition to regulating tendon growth, *Mkx* plays an important role in the differentiation and homeostasis of the VPs and pulleys.

Discussion

The VPs of the PIP joints are fibrocartilaginous structures[26] with three distinct layers[27]. Because VPs have the specific orientation of the collagen bundles, they are thought to resist the tension created by hyperextension and lateral torsional strain[4].

In this study, we observed the expression of *Mkx* in VPs and pulleys as well as in tendons. Although VPs and pulleys in *Mkx*-null mice appeared grossly normal until P0, they became thicker in *Mkx*-null mice by P14 compared to those in wild-type littermates. Detailed histological analysis showed that the VPs of *Mkx*-null mice had markedly disorganized fibers and did not have three distinct layers, suggesting that the VPs in *Mkx*-null mice may be easily injured by hyperextension and lateral torsional strain, and result in dorsal dislocation of the phalanx or major joint instability. These data suggest that this morphologic difference might be due to mechanical stress or degenerative changes after birth. In this regard, we recently reported the potential role of *Mkx* in human tendon/ligament tissue homeostasis [28]. Since *Mkx* expression in VPs and pulleys was observed in adult mice, *Mkx* may also be involved in VP and pulley homeostasis and regeneration.

Pulleys are composed of two layers of fibrocartilaginous tissues. Snapping or locking of the thumb or fingers is caused by the thickening of the digit's A1 pulley [29]. Although the etiological factors of these diseases remain unclear, overload on pulleys is known to cause fibrocartilaginous metaplasia. In this regard, it would be of interest to test *Mkx* expression in such conditions. We also observed that *Mkx* was expressed in the annulus fibrosus of the intervertebral disc (data not shown), suggesting that *Mkx* is also related to the differentiation of other parts of fibrocartilaginous components.

Taken together, our results indicate that *Mkx* is a regulator not only of tendon differentiation, but also of VP and pulley development and homeostasis. Understanding how *Mkx* regulates peritendinous tissues will likely provide an important insight into the etiological factors of VPs- and pulley-related diseases and can lead to further development of the related treatment.

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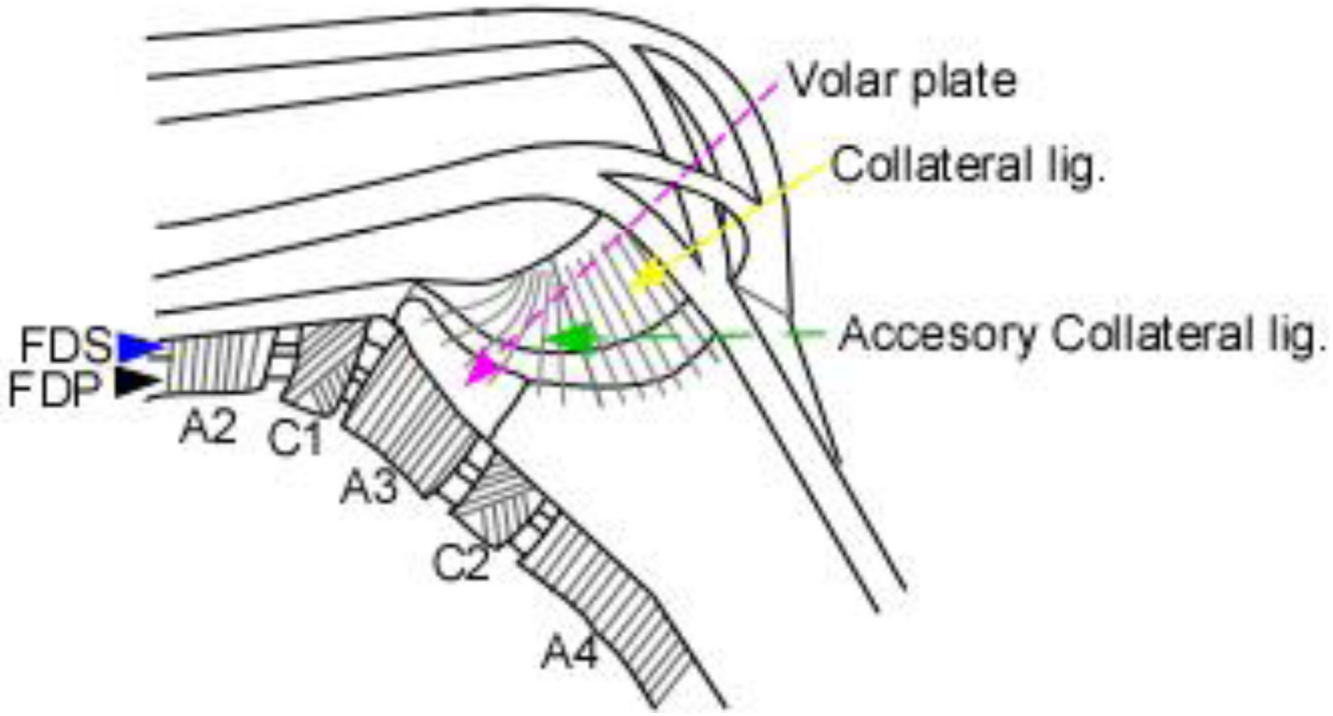

Figure 1. Schematic representation of the volar plate (VP), flexor digitorum profundus (FDP) tendons, flexor digitorum superficialis (FDS) tendons, annular pulleys (A2-A4), and cruciform pulleys (C1, C2)

Pink dashed arrow, VP. Black arrowhead, FDP. Blue arrowhead, FDS. Yellow arrow, collateral ligament. Green dashed arrow, accessory collateral ligament. (Modified from Ueba Y. Hand anatomy and its function. 2010.[3])

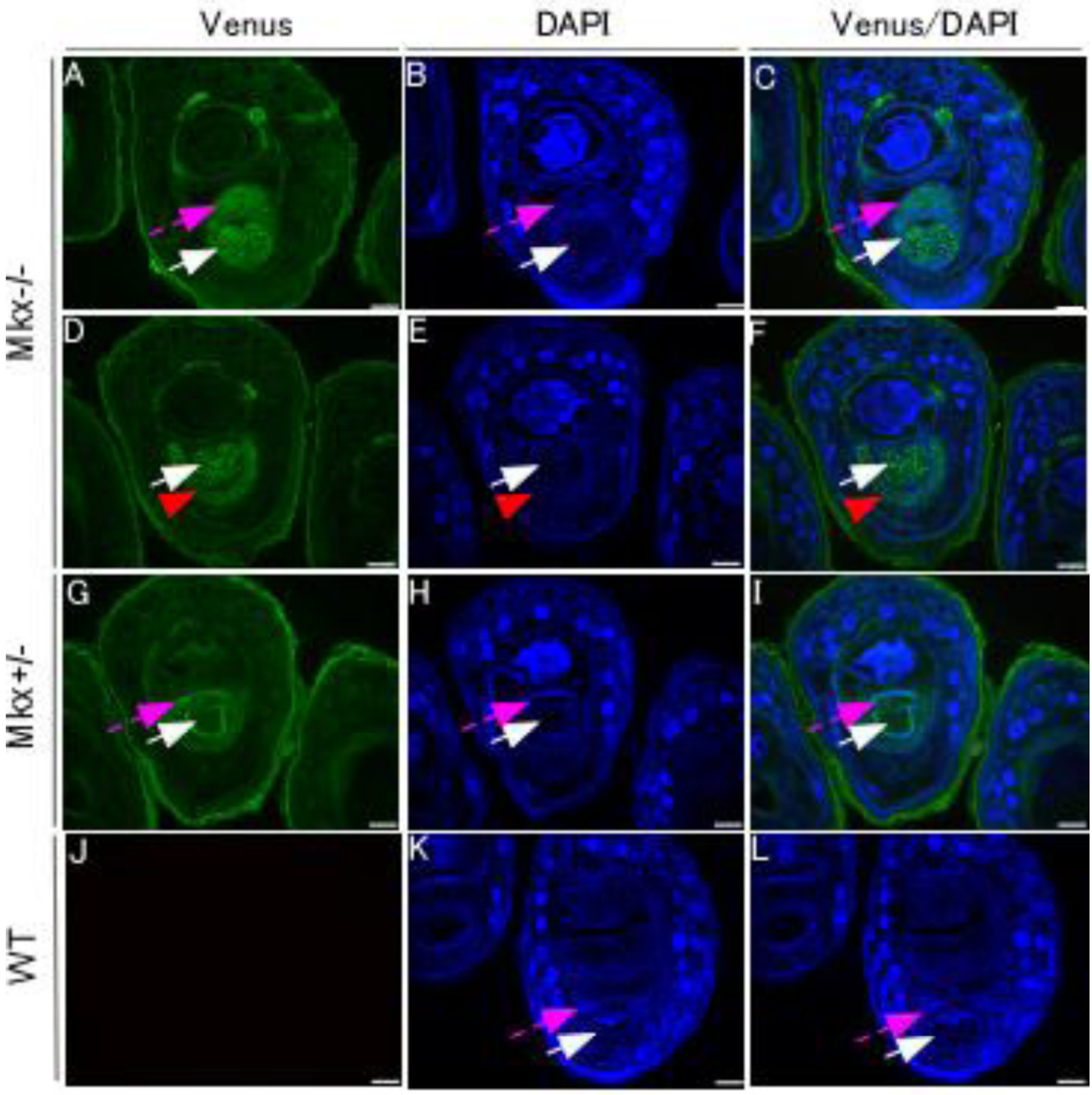

Figure 2. Venus signals were detected in flexor digitorum profundus (FDP) tendons, pulleys, and the VPs in P21 Venus knock-in *Mkx*$^{+/-}$ and $^{-/-}$ mice

(A, D, G, and J)Venus signals of *Mkx*$^{-/-}$, *Mkx*$^{+/-}$, and wild-type (WT) digits at P21. (B, E, H, and K) DAPI (4′6-diamidino-2-phenylindole) staining of a section of *Mkx*$^{-/-}$, *Mkx*$^{+/-}$, and WT digits at P21. (C, F, I, and L) Merge of a section of *Mkx*$^{-/-}$, *Mkx*$^{+/-}$, and WT digits at P21. Pink dashed arrows, VPs. White arrows, FDP tendons. Red arrowheads, pulleys. Scale bar, 100 μm.

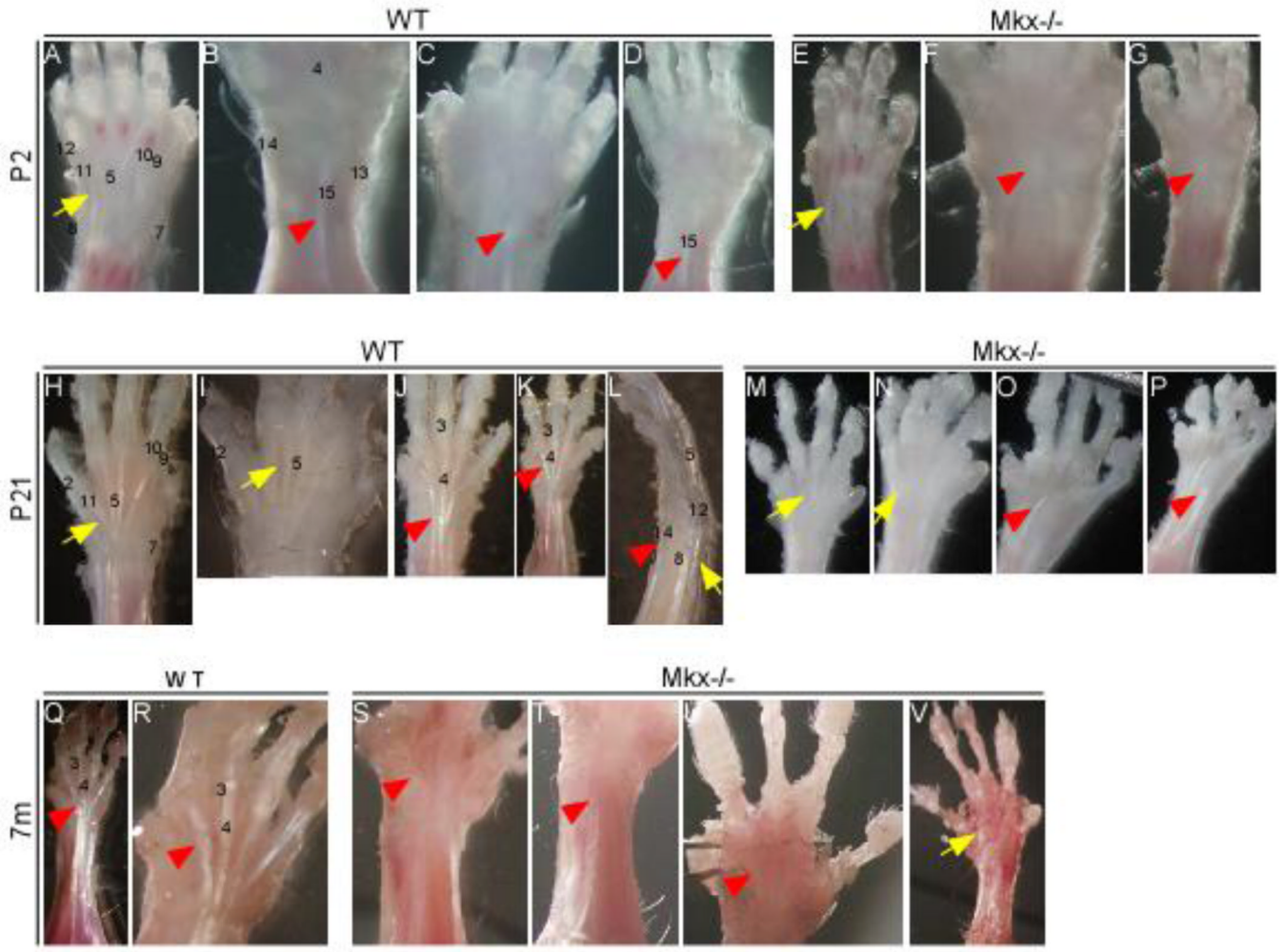

Figure 3. Significant hand tendon defects are observed in *Mkx*-null mice

The extensor tendons and flexor digitorum profundus (FDP), in P2, P21, and 7-month-old *Mkx*-null mice were dramatically reduced compared to those of the wild-type littermates. (A–G) Tendon appearance of wild-type (WT) (A–D) and mutant (E–G) digits at P2. (H–P) Tendon appearance of wild-type (WT) (H–L) and mutant (M–P) digits at P21. (Q–V) Tendon appearance of wild-type (WT) (Q, R) and mutant (S–V) digits at 7 months. Red arrowheads, FDP tendons. Yellow arrows, extensor tendons. The numbering of the tendons refers to that introduced by Watson et al. [25] as follows: 3, flexor digitorium sublimis (FDS); 4, flexor digitorium profundus (FDP); 5, extensor digitorium communis (EDC); 7, extensor pollicis; 8, extensor carpi ulnaris; 9, extensor carpi radialis longus; 10, extensor carpi radialis brevis; 11, extensor digiti quarti; 12, extensor digiti quinti; 13, flexor carpi radialis; 14, flexor carpi ulnaris; 15, palmaris longus.

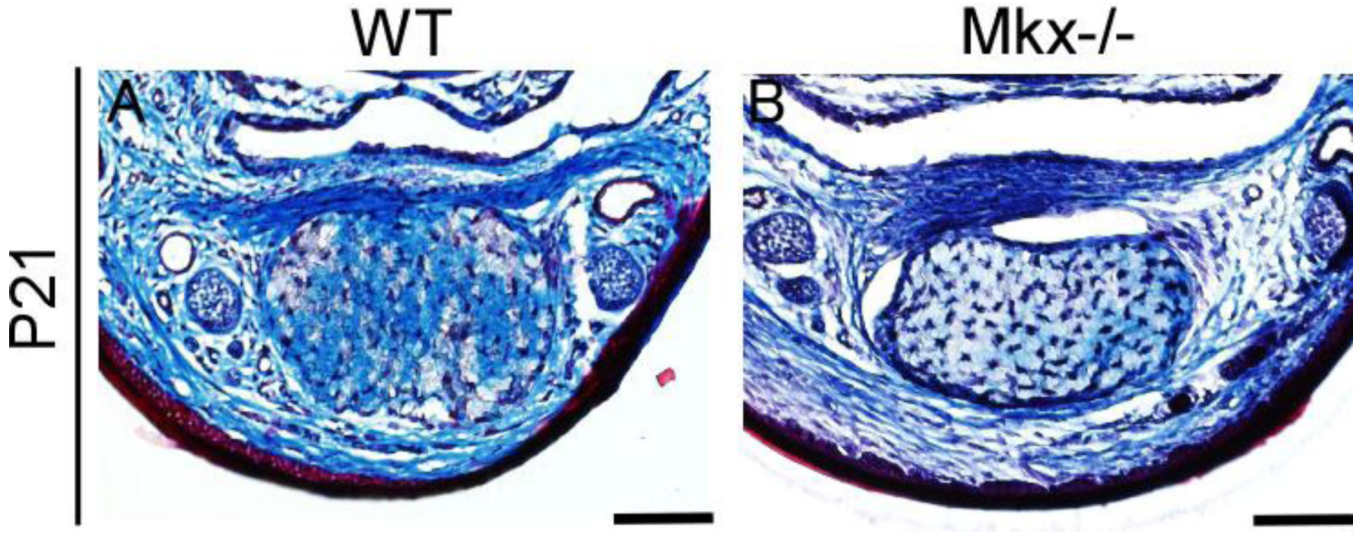

Figure 4. VP is composed of collagen

Masson trichrome staining of the flexor digitorium profundus (FDP) tendon and volar plates (VP) in wild-type (A) and *Mkx*-null mice at P21 (B). Scale bar, 100 μm.

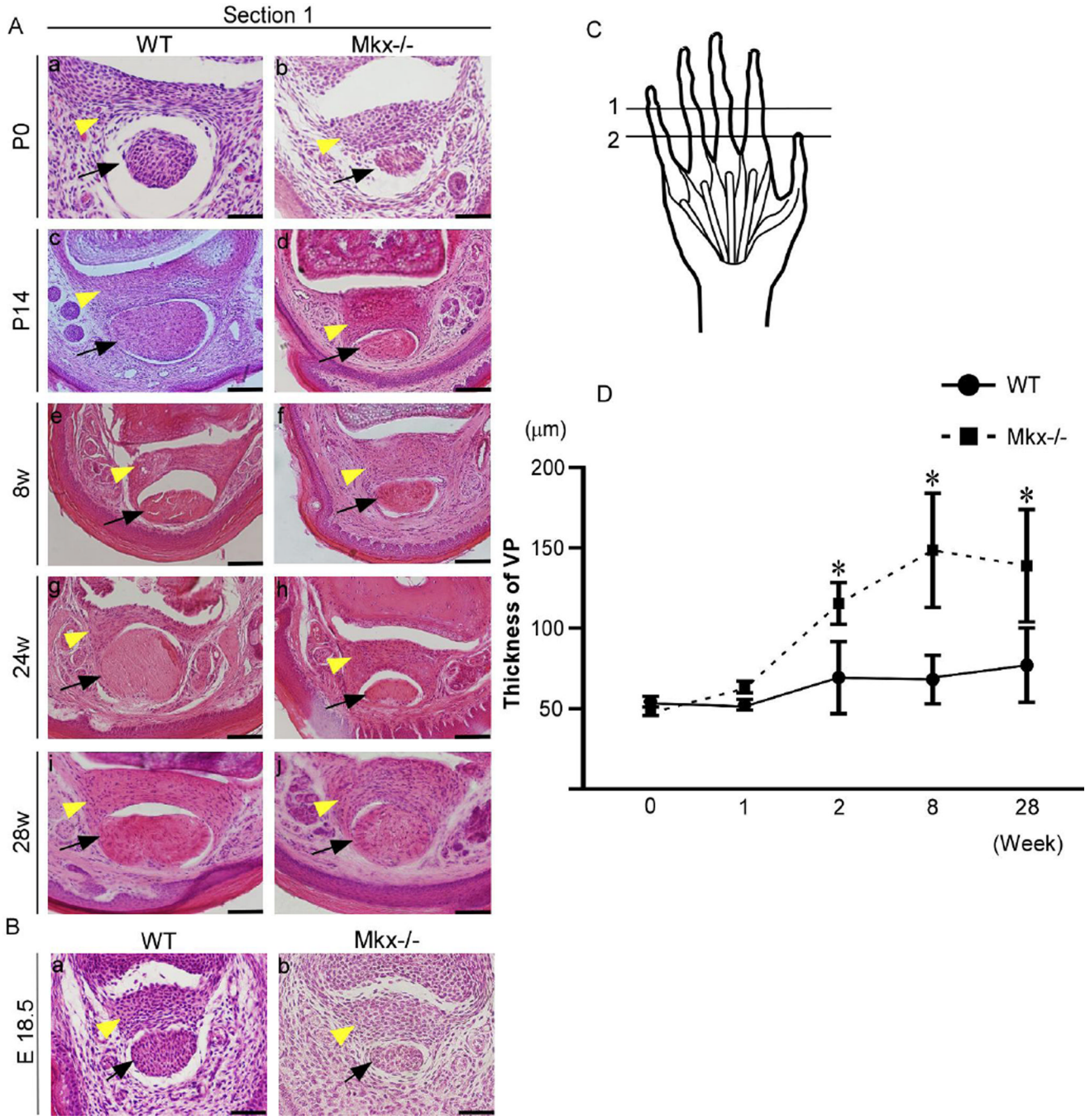

Figure 5. Tendon mass decreases in *Mkx*-null mice since E18.5. Volar plates (VPs) are normal in embryonic *Mkx*-null mice and become thick in *Mkx*-null mice after P14

(A) Hematoxylin and eosin (H&E) staining of the flexor digitorium profundus (FDP) tendon and VP in wild-type (a, c, e, g, and i) and *Mkx*-null mice (b, d, f, h, and j), in the time range from P0 to 28 weeks. Scale bars are 50 μm in a and b and 100 μm in c–j. (B) H&E staining of the FDP tendon and VP in E18.5 wild-type (a) and *Mkx*-null mice (b). Scale bar, 50 μm. Yellow arrowheads, VPs. Black arrows, FDP tendons. (C) Section levels are marked by black lines: (1) proximal interphalangeal (PIP) joint, (2) middle of the proximal phalanx (level of the A2 pulley). The sections were taken at level 1. (D) Comparison of the thickness of VPs between *Mkx*-null mice and wild-type littermates. *$P < 0.05$, $n = 5$.

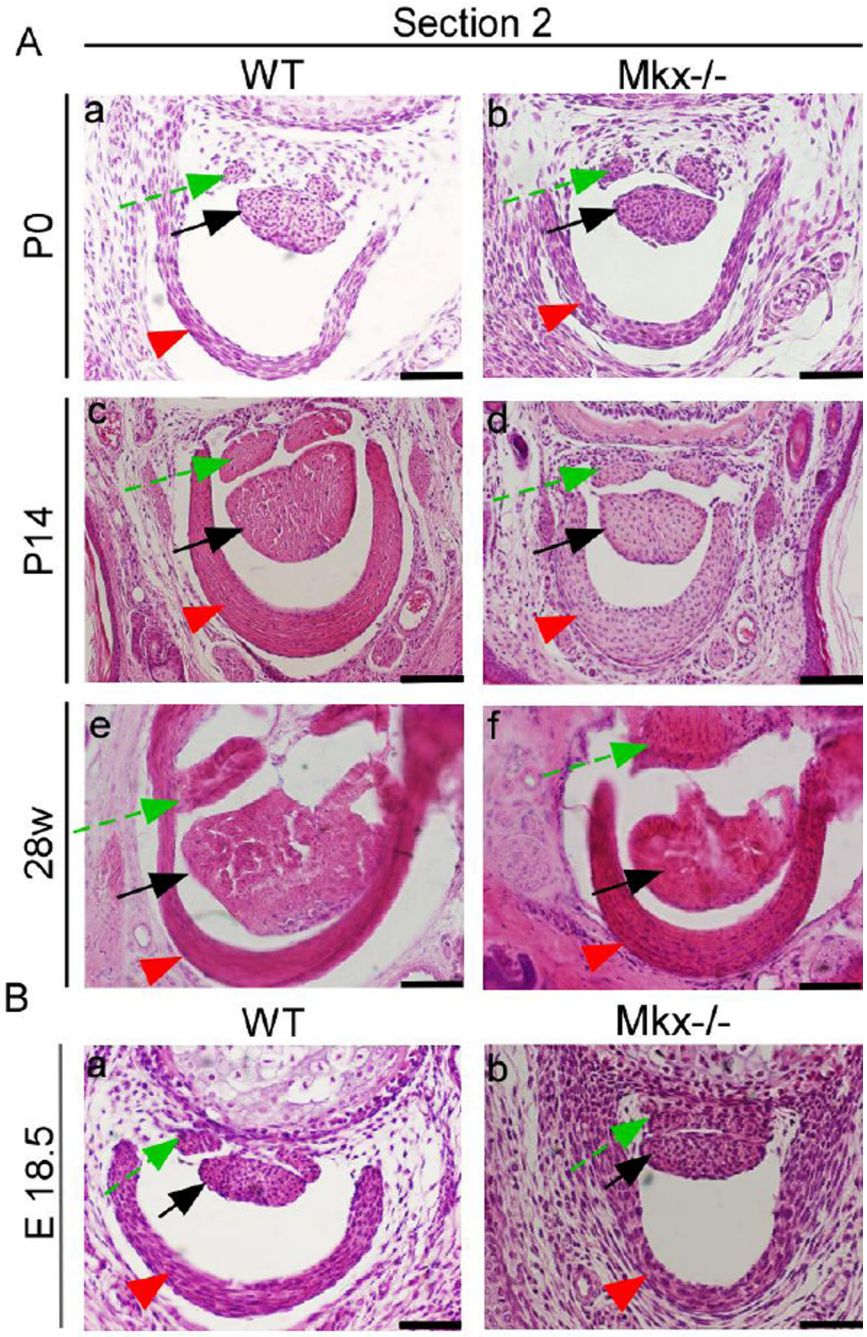

Figure 6. Pulleys are normal in embryonic *Mkx*-null mice and become thick in *Mkx*-null mice after P14

(A) Hematoxylin and eosin (H&E) staining of the flexor digitorium profundus (FDP) tendon, flexor digitorum sublimis (FDS) tendon, and pulleys in wild-type (a, c, and e) and *Mkx*-null mice (b, d, and f) in the time range from P0 to 28 weeks. Scale bars are 50 μm in a and b and 100 μm in c–f. (B) H&E staining of the FDP tendon, FDS tendon, and pulley in E18.5 wild-type (a) and *Mkx*-null mice (b). Scale bar, 50 μm. Green dashed arrows, FDS tendons. Red arrowheads, pulleys. Black arrows, FDP tendons. The sections were taken at level 2 (for details, see the caption of Fig. 5-C).

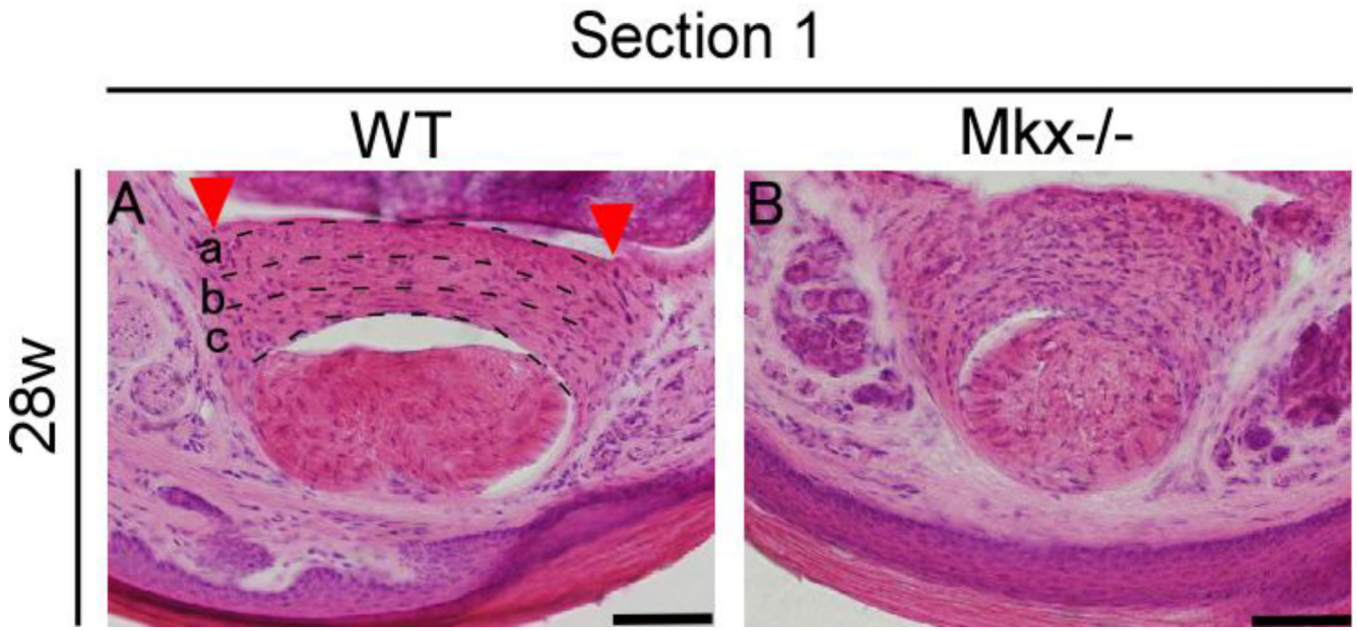

Figure 7. Enlarged view of Fig. 5-A, i and j. Volar plates (VPs) are complex structures with three separate layers in wild-type mice. In *Mkx*-null mice, the orientation of the fibers of the VPs is disorganized

(A) Hematoxylin and eosin (H&E) staining of the flexor digitorium profundus (FDP) tendon and VP in 28-week-old wild-type mice. a, The dorsal-most layer. b, The second layer. c, The palmar-most layer. (B) H&E staining 355 of the FDP tendon and VP in 28-week-old *Mkx*-null mice.