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Forelimb Contractures and Abnormal Tendon Collagen Fibrillogenesis in Fibulin-4 Null Mice

Dessislava Z. Markova[1,#,†], **Te-Cheng Pan**[1,#], **Rui-Zhu Zhang**[1], **Guiyun Zhang**[2], **Takako Sasaki**[3], **Machiko Arita**[1,†], **David E. Birk**[4], and **Mon-Li Chu**[1]

[1]Department of Dermatology and Cutaneous Biology, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA 19107, USA.

[2]Department of Pathology, Anatomy and Cell Biology, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA 19107, USA.

[3]Department of Biochemistry, Faculty of Medicine, Oita University, Oita, Japan.

[4]Department of Molecular Pharmacology & Physiology, University of South Florida, Morsani College of Medicine, Tampa, FL, 33612,USA.

Abstract

Fibulin-4 is an extracellular matrix glycoprotein essential for elastic fiber formation. Mice deficient in fibulin-4 die perinatally due to severe pulmonary and vascular defects associated with lacking intact elastic fibers. Patients with fibulin-4 mutations demonstrate similar defects and a significant number die shortly after birth or in early childhood owing to cardiopulmonary failure. The patients also demonstrated skeletal and other systemic connective tissue abnormalities, including joint laxity, and flexion contractures of the wrist. A fibulin-4 null mouse strain was generated and used to analyze the roles of fibulin-4 in tendon fibrillogenesis. This mouse model displayed bilateral forelimb contractures, in addition to pulmonary and cardiovascular defects. The forelimb and hindlimb tendons demonstrated a disruption in collagen fibrillogenesis in the absence of fibulin-4 analyzed using transmission electron microscopy. Fewer fibrils were assembled and fibrils were disorganized compared to wild type controls. The organization of developing tenocytes and compartmentalization of the extracellular space also was disrupted. Fibulin-4 was co-localized with fibrillin-1 and fibrillin-2 in limb tendons using immunofluorescence microscopy. Our studies demonstrate that fibulin-4 plays a role in regulating tendon collagen fibrillogenesis, in addition to its essential function in elastogenesis.

Keywords

Elastic fiber; cutis laxa; fibrillin; tendon; collagen fibrillogenesis

[*]Corresponding author: Mon-Li Chu, Ph.D., Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107, USA. Phone: +1 215-503-4834, Fax: +1 215-503-5788. ; Email: mon-li.chu@jefferson.edu.

[#]These two authors contributed equally.

[†]Present address: Department of Orthopaedic Surgery, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA 19107, USA.

Introduction

Fibulin-4 is a secreted glycoprotein belonging to the fibulin family, characterized by tandem repeats of calcium binding epidermal growth factor-like (cbEGF) modules and a C-terminal fibulin (FC) domain (de Vega et al. 2009; Timpl et al. 2003). Within this protein family, fibulin-3, -4 and -5 are relatively small in size (50–60 kDa) and have essentially identical modular structures (Timpl et al. 2003; Yanagisawa and Davis 2010). Studies of knockout mice have demonstrated that both fibulin-4 and -5 are indispensable for elastic fiber formation (McLaughlin et al. 2006; Nakamura et al. 2002; Yanagisawa et al. 2002). However, loss of fibulin-4 results in a more severe phenotype than absence of fibulin-5. Fibulin-4 null mice die perinatally and display pulmonary emphysema, aortic aneurysm, and artery anomalies (tortuous, dilation, narrowing, rupture). By contrast, the fibulin-5 null mice live into adulthood and show loose skin, pulmonary emphysema and cardiovascular defects.

In humans, mutations in fibulin-4 and fibulin-5 underlie autosomal recessive cutis laxa (ARCL) type 1B and 1A, respectively (Urban and Davis 2014). ARCL is a heterogeneous group of disorders characterized by loose skin with significant internal organ involvement. Like the phenotypes of the knockout mice, there are notable differences in the clinical manifestations of ARCL 1A and ARCL 1B patients. A significant proportion of the patients with fibulin-4 mutations die shortly after birth or in early childhood owing to cardiopulmonary failure (Al-Hassnan et al. 2012; Dasouki et al. 2007; Erickson et al. 2012; Hebson et al. 2014; Hoyer et al. 2009; Hucthagowder et al. 2006; Iascone et al. 2012; Kappanayil et al. 2012; Renard et al. 2010; Sawyer et al. 2013). The common pathological findings are pulmonary emphysema, arterial tortuosity and aortic aneurysm. In addition, patients show skeletal and other systemic connective tissue abnormalities, including bone fragility, joint laxity, arachnodactyly, pectus excavatum, flexion contracture of wrists, feet abnormalities, hypotonia, diaphragmatic and inguinal hernias. On the other hand, patients with fibulin-5 mutations present with cutis laxa, emphysema and supravalvular aortic stenosis, but without aortic aneurysm and skeletal connective tissue abnormalities (Callewaert et al. 2013; Loeys et al. 2002).

Previous studies of fibulin-4 global and conditional null mice have focused on the elastic fiber abnormalities in the vascular and pulmonary systems (Horiguchi et al. 2009; Huang et al. 2010; McLaughlin et al. 2006). However, in these studies it was not determined whether the loss of fibulin-4 leads to skeletal and other systemic connective tissue anomalies resembling those seen in human patients. To address this deficiency, a fibulin-4 null mouse strain that we generated was characterized. Our mouse model exhibited bilateral forelimb contractures, in addition to vascular and pulmonary defects. We found that fibulin-4 co-localized with fibrillin microfibrils in wild type tendons. In the absence of fibulin-4 collagen fibrillogenesis was disrupted. Fewer fibrils were assembled and fibrils were disorganized compared to wild type controls. The developing tenocytes and compartmentalization of the extracellular space also were disrupted in the fibulin-4 null mice. Our studies demonstrate that fibulin-4 not only is essential for elastic fiber assembly, but also plays a specific role in regulating collagen fibrillogenesis during development.

Materials and methods

Antibodies

A rabbit polyclonal antibody against full length recombinant mouse fibulin-4 was reported previously (Kobayashi et al. 2007). A guinea pig polyclonal antibody for fibulin-4 was generated using the N-terminal region of mouse fibulin-4 as the antigen by the custom antibody services of Cocalico Biologicals (Reamstown, PA). The antigen corresponded to amino acids 28 to 203 of mouse fibulin-4 and was produced in HEK293 cells by the methods described previously (Kobayashi et al. 2007).

Targeted inactivation of the *Fbln4/Efemp2* gene

A 10 kb XbaI genomic fragment containing the 5' portion of the *Fbln4/Efemp2* gene kindly provided by Dr. Günter Kostka was used to construct the gene targeting vector. Generation of the fibulin-4 null mice is described in supplementary material, Fig. S1. Genotyping of mutant mice was performed by PCR analysis of tail DNA. Forward prime CCTCTCTGCAGATGTCAACG and reverse primer GAGGCAGGCAGATTTCTGAG generated a 358 bp PCR product from the wild type allele. The same reverse primer and forward primer TAAAGCGCATGCTCCAGACTGC yielded a ~310 bp PCR product from the targeted allele. All animal experiments were performed under animal protocols approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Histology and immunostaining

Mouse embryos dissected from pregnant females and newborn mice were frozen in OCT compound or processed for paraffin embedding. Cryosections (8 μm) were fixed with methanol and used for immunostaining with a polyclonal antibody against fibulin-4 (Kobayashi et al. 2007) and Cy3-labeled secondary antibody (Jackson ImmunoResearch Laboratories). Paraffin- embedded sections (5 μm) were subject to Verhoeff von Gieson elastin staining. Samples were viewed using a Zeiss Axioskop epifluorescence microscope with a Toshiba 3CCD camera and ImagePro software (Media Cybernetics, Rockville, MD).

Flexor digitorum longus (FDL) tendons were dissected from wild type mice at postnatal day 4 and embedded in OCT compound. Cryosections (6 μm) were co-immunostained with the guinea pig polyclonal antibody for fibulin-4, and rabbit polyclonal antibodies against fibrillin-1 or fibrillin-2 (kindly provided by Dr. Lynn Sakai). Secondary antibodies used were Alexa Fluor-488 and -568 conjugated goat anti-guinea pig and anti-rabbit IgG, respectively. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured using a Leica CRT 550 fluorescence microscope and Leica DFC 340 FX digital camera.

Skeleton staining

Whole-mount skeleton staining was performed on eviscerated embryos. After skin removal, mice were fixed in 95% ethanol for 4 days at room temperature and then transferred to acetone for 1 day. Staining was carried out in 0.005% alizarin red, 0.015% alcian blue, 5% acetic acid and 70% ethanol at 37°C for 3 days. The embryos were rinsed with water and treated with 1% KOH for 1–3 days to clear muscles, followed by clearing steps of 1 day

each with 0.8% KOH in 20% glycerol, 0.6% KOH in 40% glycerol, 0.4% KOH in 60% glycerol, 0.2% KOH in 80% glycerol and 100% glycerol.

Transmission electron microscopy

Forelimb and hindlimb flexor tendons from E16, E18 and newborn mice were dissected and analyzed by transmission electron microscopy as described (Izu et al. 2011). Thin sections were examined at 80 kV using a Tecnai 12 or JEOL 1400 transmission electron microscope equipped with a Gatan Ultrascan US100 2K digital camera or Gatan Orius widefield side mount CC Digital camera (Gatan Inc., Pleasanton, CA).

Results

Generation of fibulin-4 null mice

A mutant mouse model lacking fibulin-4 was generated by gene targeting, which deleted exons 2–4 of the *EFEMP2/Fbln4* gene (Fig. S1a, exon 2 encodes the translational start site). Correct targeting of the mouse embryonic stem (ES) cells (129sv strain) and germ line transmission were identified by Southern blot analysis of genomic DNA using an external probe. The resulting heterozygous mice were intercrossed to generate $Fbln4^{+/+}$, $Fbln4^{+/-}$, $Fbln4^{-/-}$ mice as shown by Southern blot analysis (Fig. S1b). Although all three genotypes were obtained in the expected Mendelian ratio, the homozygous pups died within 1–2 days after birth. Dermal fibroblasts were established from embryonic day 18 (E18) littermates of all three genotypes. Total RNA prepared from fibroblasts was analyzed by Northern blotting. Fibulin-4 mRNA was absent in the homozygous $Fbln4^{-/-}$ fibroblasts and reduced in the $Fbln4^{+/-}$ fibroblasts (Fig. S1c). Culture media collected from the fibroblasts were analyzed by immunoblotting. Fibulin-4 protein was not produced by the $Fbln4^{-/-}$ fibroblasts and was secreted at a reduced level by the $Fbln4^{+/-}$ fibroblasts (Fig. S1d). The amount of fibulin-2 protein was similar in culture media of all three genotypes (Fig. S1d).

Bilateral forelimb contractures and diaphragmatic hernia in $Fbln4^{-/-}$ mice

The $Fbln4^{-/-}$ mice could be readily distinguished from the $Fbln4^{+/+}$ *and* $Fbln4^{+/-}$ animals morphologically, as shown by the gross appearance of eight E19 littermates obtained from crossing heterozygous male and female mice (Fig. 1a). The $Fbln4^{+/+}$, $Fbln4^{+/-}$, $Fbln4^{-/-}$ littermates were similar in size, but the homozygous mutants (Fig. 1a, #3, 4, 5) invariably exhibited bilateral forelimb contractures. One of the heterozygous mice (#7) showed unilateral forelimb contracture. The forelimbs, and to a lesser extent, the hindlimbs of the $Fbln4^{-/-}$ mice were found to be very soft and pliable, and limb joints were hypermobile. The forelimb contractures of the $Fbln4^{-/-}$ mice were consistently noted at E16 through postnatal day 1–2. The hindlimbs did not display apparent contractures. The homozygous mice had translucent skin and their rib cages were clearly visible. Moreover, their chest cavity appeared fuller and the abdomen seemed caved in. On dissection, a significant proportion of the homozygous mice had diaphragmatic hernias, showing the presence of abdominal organs (liver, intestine) in the chest cavity (Fig. 1b). We also obtained the previously reported fibulin-4 null mouse strain (McLaughlin et al. 2006) from Jackson Laboratory (B6.129P2-Efemp2tm1Dgen/J) and all fibulin-4 null mice from this strain also displayed bilateral forelimb contractures (data not shown).

To determine whether skeletal anomalies caused the forelimb contracture phenotype, skeletal preparations of newborn *Fbln4*$^{-/-}$ mice and the wild type littermates were stained with alcian blue and alizarin red. During the skeletal preparation, the distal ends of the phalanges in the forelimbs were often detached from the body (Fig. 1c). Mineralization of skeletal elements in the forelimbs and the entire body, indicated by alizarin red staining, appeared similar between *Fbln4*$^{-/-}$ and *Fbln4*$^{+/+}$ littermates (Fig. 1c and data not shown). There was no apparent difference in the number or shape of carpal bones in the wrists between the homozygous and wild type mice.

Immunostaining of ascending aortas from E18 littermates confirmed that fibulin-4 was absent in the *Fbln4*$^{-/-}$ mice (Fig. 1d). Verhoeff von Gieson elastin staining showed that the *Fbln4*$^{-/-}$ aortic wall was thick and had no well-defined elastic laminae. By contrast, elastic laminae were clearly visible in the *Fbln4*$^{+/+}$ and *Fbln4*$^{+/-}$ littermates (Fig. 1d).

Abnormal collagen fibrillogenesis in limb tendons of the *Fbln4*$^{-/-}$ mice

To assess whether the forelimb contracture phenotype resulted from soft tissue abnormality, forelimb tendons from littermates were examined by transmission electron microscopy. In the E16 *Fbln4*$^{+/+}$ forelimb tendon, tenocytes were well organized and cellular processes compartmentalized the extracellular space into organized micro-domains (Fig. 2a, c). The micro-domains containing developing collagen fibers were filled with uniformly sized and regularly packed collagen fibrils oriented parallel to the tendon axis. In contrast, the tenocytes and fiber-forming micro-domains in the *Fbln4*$^{-/-}$ forelimb tendons were disorganized (Fig. 2b, d). The collagen fibrils being organized into bundles (fibers) were less numerous than in the wild type controls. In addition, the fibrils were less regularly packed in the *Fbln4*$^{-/-}$ tendons. Similar ultrastructural alterations of collagen fibrils and fibers, and tenocytes were observed in E16 hindlimb tendons (Fig. 3). As development progressed, the number of collagen fibrils in the *Fbln4*$^{-/-}$ compartments of both forelimb and hindlimb tendons increased gradually and therefore the collagen fiber abnormality was less pronounced in E18 (data not shown) and newborn (P0) tendons (Fig. 4).

Fibulin-4 co-localizes with fibrillin-1 and fibrillin-2 in tendon

The localization of fibulin-4 in tendon is unknown. Therefore, FDL tendons from hindlimbs of wild type mice at postnatal day 4 were immunostained with fibulin-4 antibody (Fig. 5). In cross sections, fibulin-4 immunoreactivity exhibited a fine punctate pattern that localized preferentially around tenocytes and less frequently within collagen fibril bundles. In longitudinal sections, fibulin-4 positive microfibrils were often found close to groups of tenocytes organized in linear arrays along the tendon axis. Double immunostaining experiments showed that fibulin-4 co-localized with both fibrillin-1 and fibrillin-2, major structural components of connective tissue microfibrils. However, fibulin-4 was not present in the tendon sheath where fibrillin-1 and fibrillin-2 were strongly and moderately expressed, respectively.

Discussion

In this study, we show that absence of fibulin-4 results in bilateral forelimb contractures in mice from ages E16 to newborn and affects collagen fibrillogenesis during tendon development. We also show that fibulin-4 co-localizes with fibrillin-1 and fibrillin-2 around tenocytes, and between collagen fiber bundles in tendon. Fibrillin-rich microfibrils form a scaffold that guides elastin deposition during elastic fiber assembly (Wagenseil and Mecham 2007). In tendons, fibrillin-1 and fibrillin-2 have been shown to co-localize with tropoelastin around groups of tenocytes and between collagen fascicles (Grant et al. 2013). A pivotal role for fibulin-4 in elastic fiber assembly has been well-documented (Horiguchi et al. 2009; Huang et al. 2010; McLaughlin et al. 2006). However, the defect in tendon collagen fibrillogenesis in the fibulin-4 null mice cannot be explained by impaired elastic fiber formation, as absence of fibulin-5, another molecule essential for elastic fiber assembly, does not lead to skeletal connective tissue abnormalities in mice and humans (Papke and Yanagisawa 2014). The sporadic localization of fibulin-4 within collagen fibers in wild type tendons suggests that soluble factors rather than structural components may play a role in abnormal collagen fibrillogenesis. Specifically, impaired lysyl oxidase (LOX) activity and altered growth factor signaling in the absence of fibulin-4 likely contribute to the abnormal collagen fibrillogenesis in tendon for the following reasons.

Several lines of evidence suggest that fibulin-4 regulates the activity of LOX, a key enzyme responsible for cross-linking of both elastin and collagen (Lucero and Kagan 2006). In fibulin-4 null mice, the desmosine cross-links of elastin catalyzed by LOX are dramatically reduced to ~10% of the level in wild type mice (McLaughlin et al. 2006). *In vitro* binding assays show a direct interaction of fibulin-4 with the LOX precursor (Choudhury et al. 2009; Horiguchi et al. 2009). The binding interaction has been mapped to the propeptide of LOX and the amino-terminus of fibulin-4, implying that fibulin-4 might influence the activation of LOX from its precursor (Lucero and Kagan 2006). LOX null mice die perinatally and display aortic aneurysms, cardiovascular dysfunction, diaphragmatic rupture and impaired pulmonary airway development (Hornstra et al. 2003; Maki et al. 2002). Loss of LOX in mice leads to fragmented elastic fibers and abnormally organized collagen fiber bundles (Maki et al. 2005). The observations of abnormal collagen fibrillogenesis in conjunction with impaired elastogenesis in the fibulin-4 null mice suggest that the overall phenotypic abnormalities associated with fibulin-4 deficiency likely result from LOX dysfunction. Consistent with this proposition, we recently showed that crosslinking of both collagen and elastin is significantly reduced and that conversion of LOX from its precursor is less complete in fibulin-4 E57K knock-in mice, which produce a low level of abnormal fibulin-4 and no normal fibulin-4 (Igoucheva et al. 2015). In addition, the fibulin-4 E57K mutant mice display bent forelimbs and collagen fibrils in tendon and skin are abnormal. Moreover, a recent study has demonstrated abnormal collagen fibers in aortas of smooth muscle-specific fibulin-4 null mice and decreased LOX activity in fibulin-4 null cells (Papke et al. 2015).

Fibulin-4 has been localized to fibrillin-rich microfibrils by immuno electron microscopy (Kobayashi et al. 2007). It is possible that the forelimb and collagen fibril abnormalities in the absence of fibulin-4 could be related to changes in the composition of fibrillin microfibils. A key physiological function of fibrillin microfibrils is to sequester latent

transforming growth factor β (TGF-β) complex, consisting of latent TGF- β binding proteins (LTBPs) and TGF-β associated with its propeptide, in the extracellular matrix, thereby regulating TGF-β bioavailability (Ramirez and Sakai 2010). Previous studies have shown that fibulin-4 binds fibrillin-1 and that the fibulin-4 binding site is located in the N-terminal region of fibrillin-1, which also contains the binding site for LTBP-1 (El-Hallous et al. 2007; Kobayashi et al. 2007; Ono et al. 2009). Biochemical analysis indicates that LTBP-1 and fibulin-4 compete for binding to fibrillin-1 (Ono et al. 2009). Fibulin-4 has also been shown to bind LTBP-1, and can interact with fibrillin-1 and LTBP-1 simultaneously (Massam-Wu et al. 2010). It is therefore conceivable that absence of fibulin-4 would affect extracellular storage and activation of latent TGF-β complex. Indeed, increased TGF-β signaling has been found in hypomorphic fibulin-4 mice and a patient with fibulin-4 mutations, in a manner similar to fibrillin-1 mutant mice and Marfan syndrome patients with fibrillin-1 mutations (Hanada et al. 2007; Renard et al. 2010). It is noteworthy that LOX deficiency also leads to upregulation of TGF- β signaling (Kutchuk et al. 2015). TGF-β plays a major role in the formation of tendons and ligaments during development (Pryce et al. 2009) and is a master regulator of extracellular matrix proteins, including collagens and matrix metalloproteinases. Increase in TGF-β signaling thus may contribute to the abnormal tendon collagen fibrillogenesis and skeletal phenotypes associated with fibulin-4 deficiency.

Fibrillin-2 null mice display transient bilateral forelimb contractures in the first few days after birth, and their tendons contain less collagen cross-linking (Arteaga-Solis et al. 2001; Boregowda et al. 2008). Because fibulin-4 null mice die 1–2 day after birth, whether their forelimb contracture phenotype is transient like the fibrillin-2 null mice remains unknown. Our observation that both forelimbs and hindlimbs of the fibulin-4 null mice show abnormal tendon collagen fibrillogenesis suggests that the forelimb contractures may not be directly related to collagen abnormalities. Consistent with this notion, at E16 when the collagen fibrillogenesis abnormalities are most obvious, forelimb contractures are not more pronounced compared to E18 and P0 stages. Recently, the transient forelimb contractures of the fibrillin-2 null mice was attributed to decreased muscle mass resulting from activated BMP signaling (Sengle et al. 2015). Whether absence of fibulin-4 affects limb muscle development and BMP signaling remains to be investigated.

In summary, our studies are consistent with previous reports (Hanada et al. 2007; Horiguchi et al. 2009; Huang et al. 2010; McLaughlin et al. 2006) and demonstrate that mice deficient in fibulin-4 do not form intact elastic fibers. In addition, our studies report novel data demonstrating forelimb contractures and dysregulation of tendon collagen fibrillogenesis. Our fibulin-4 null mouse model thus recapitulates the full spectrum of clinical features of human ARCL type 1B (OMIM #614437).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ARCL	autosomal recessive cutis laxa
LOX	lysyl oxidase
LTBP	latent TGF-β binding protein
FDL	flexor digitorum longus

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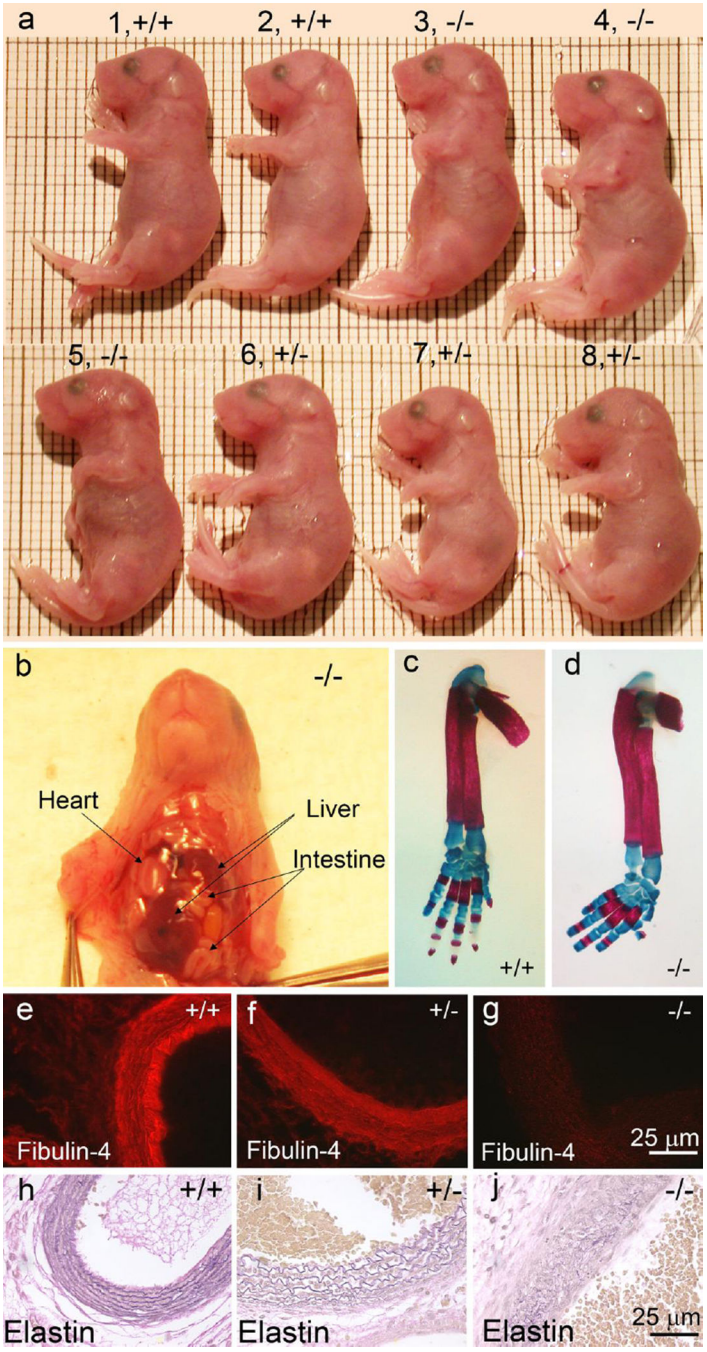

Figure 1.

Phenotypes of the fibulin-4 null mice. **a**. Photos of 8 embryos at E19 dissected from a pregnant *Fbln4*$^{+/-}$ female. All three *Fbln4*$^{-/-}$ embryos (#3, 4, 5) showed bilateral forelimb contractures. One of the three *Fbln4*$^{+/-}$ embryos (#7) showed unilateral forelimb contracture. Note that the *Fbln4*$^{-/-}$ embryos were of the same size as the wild type littermates and the skin in their chests appeared translucent and thus rib cages were visible. **b**. Diaphragmatic hernia in a representative newborn *Fbln4*$^{-/-}$ mouse. Abdominal organs, liver and intestine, were found in the chest cavity. **c, d**. Forelimb skeletons of newborn *Fbln4*$^{+/+}$ (**c**) and

Fbln4$^{-/-}$ (**d**) mice stained with Alcian blue and Alizarin red. **e, f, g**. Aortas from E18 *Fbln4*$^{+/+}$, *Fbln4*$^{+/-}$ and *Fbln4*$^{-/-}$ embryos stained with the rabbit polyclonal antibody for fibulin-4, showing absence of fibulin-4 expression in the *Fbln4*$^{-/-}$ mice. **h, i, j**. Verhoeff von Gieson elastin staining of aortas from E18 *Fbln4*$^{+/+}$, *Fbln4*$^{+/-}$ and *Fbln4*$^{-/-}$ embryos, showing thick aortic wall with an absence of well-defined elastic laminae in the *Fbln4*$^{-/-}$ aorta. Magnification bars = 25 μm.

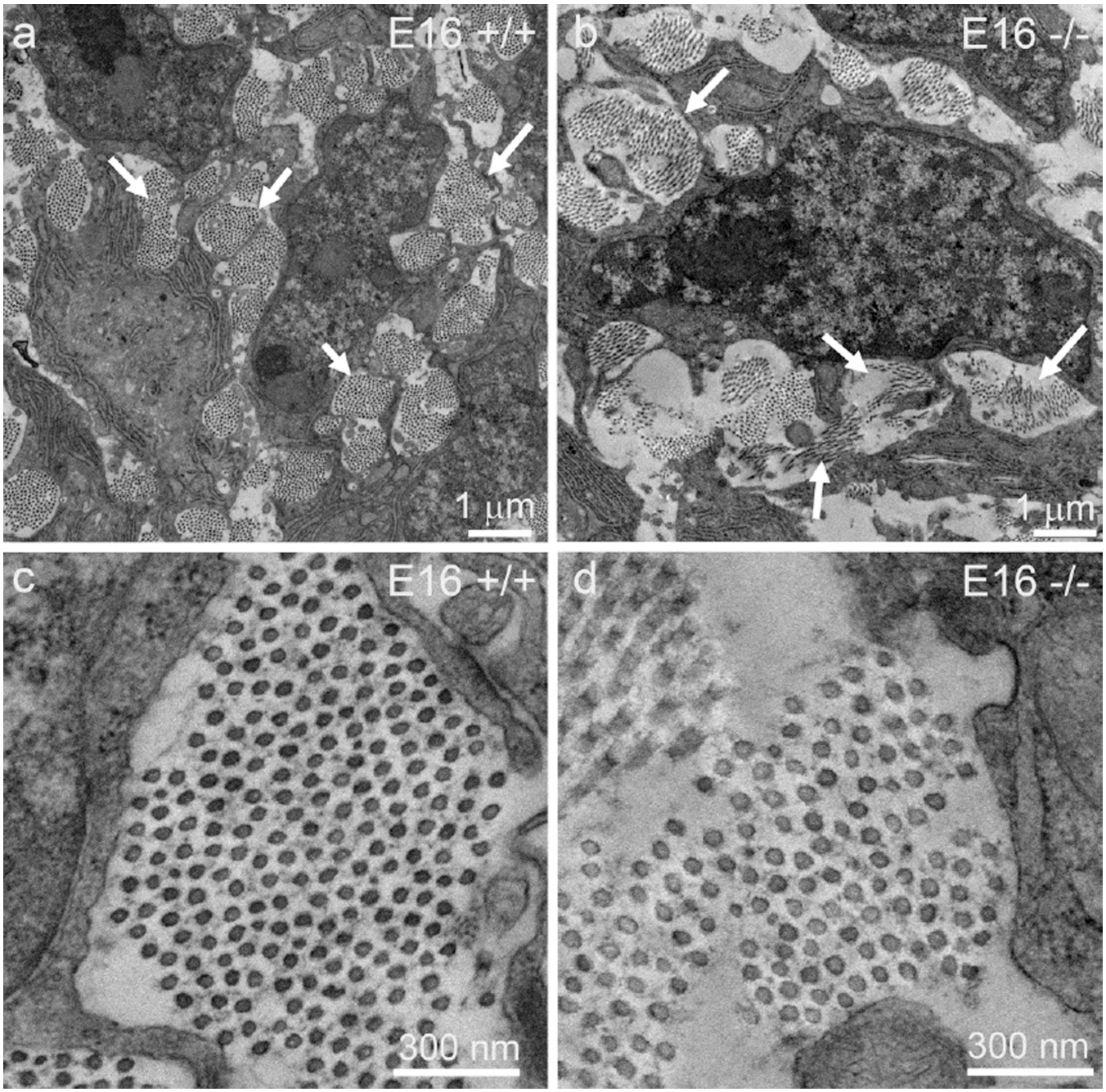

Figure 2.

Transmission electron micrographs of cross sections from flexor tendons in the forelimbs of E16 *Fbln4*$^{+/+}$ (**a, c**) and *Fbln4*$^{-/-}$ (**b, d**) embryos. In the *Fbln4*$^{+/+}$ tendons, the tenocytes were well organized and the extracellular space was compartmentalized into organized micro-domains where newly assembled collagen fibrils were organized into fibril bundles (developing fibers). In contrast, in the *Fbln4*$^{-/-}$ tendons, the tenocytes were disorganized with an associated disruption in the extracellular compartmentalization. The fibrils in the *Fbln4*$^{-/-}$ tendons were also disorganized with less regular packing and an apparent reduction in fibril number compared to the wild type controls. **a, b**: Low magnification images (scale

bar = 1 μm); **c, d**: high magnification images (scale bar = 300 nm). Arrows indicate fiber-forming spaces.

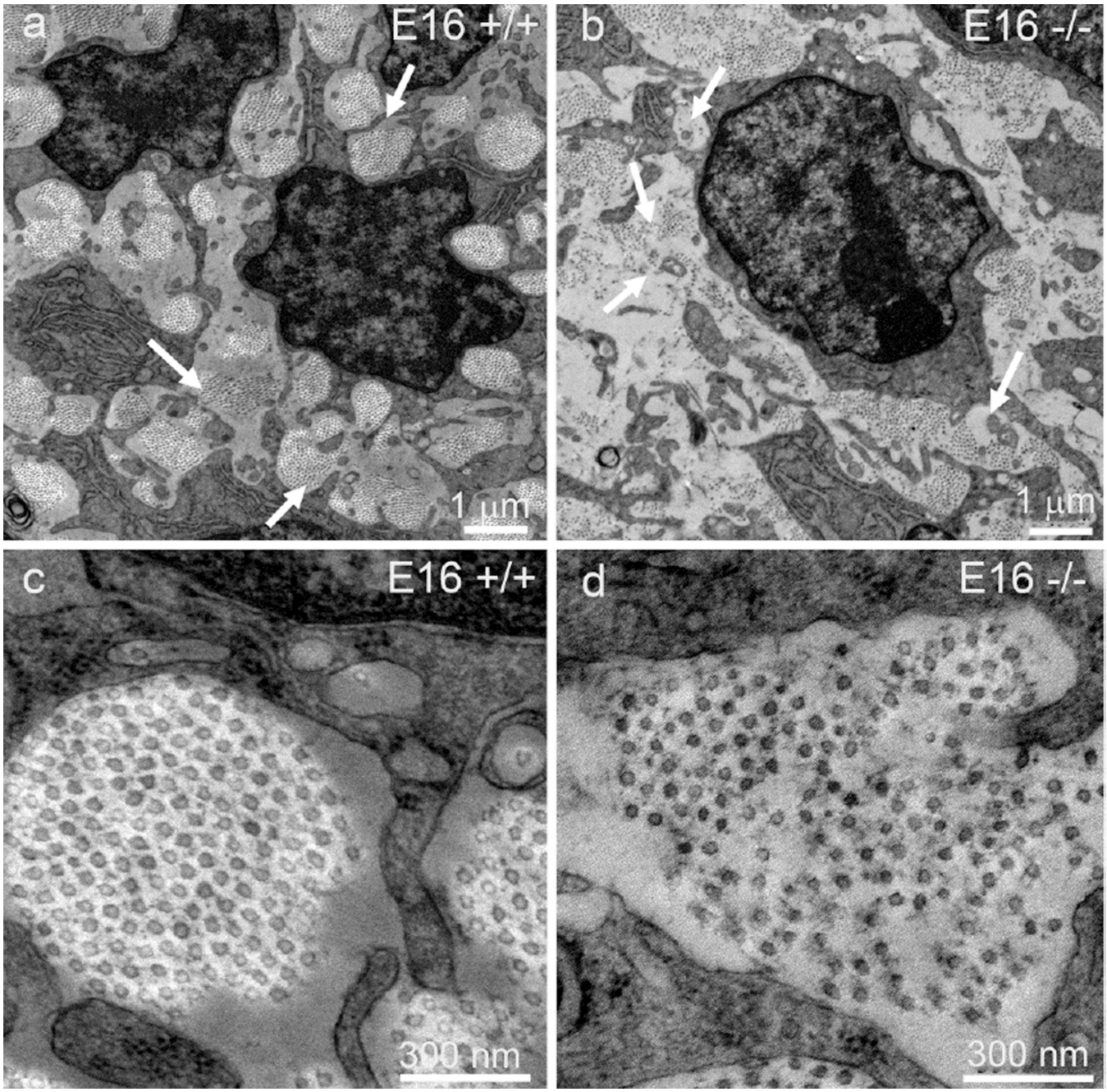

Figure 3.

Transmission electron micrographs of cross sections from flexor tendons in the hindlimbs of E16 *Fbln4*$^{+/+}$ (**a, c**) and *Fbln4*$^{-/-}$ (**b, d**) embryos. The *Fbln4*$^{-/-}$ hindlimb tendons show similar ultrastructural alterations of tenocytes, collagen fibrils and fibers as seen in the forelimb tendons described in the legend of Figure 2. **a, b**: Low magnification images (scale bar = 1 μm); **c, d**: high magnification images (scale bar = 300 nm). Arrows indicate fiber-forming spaces.

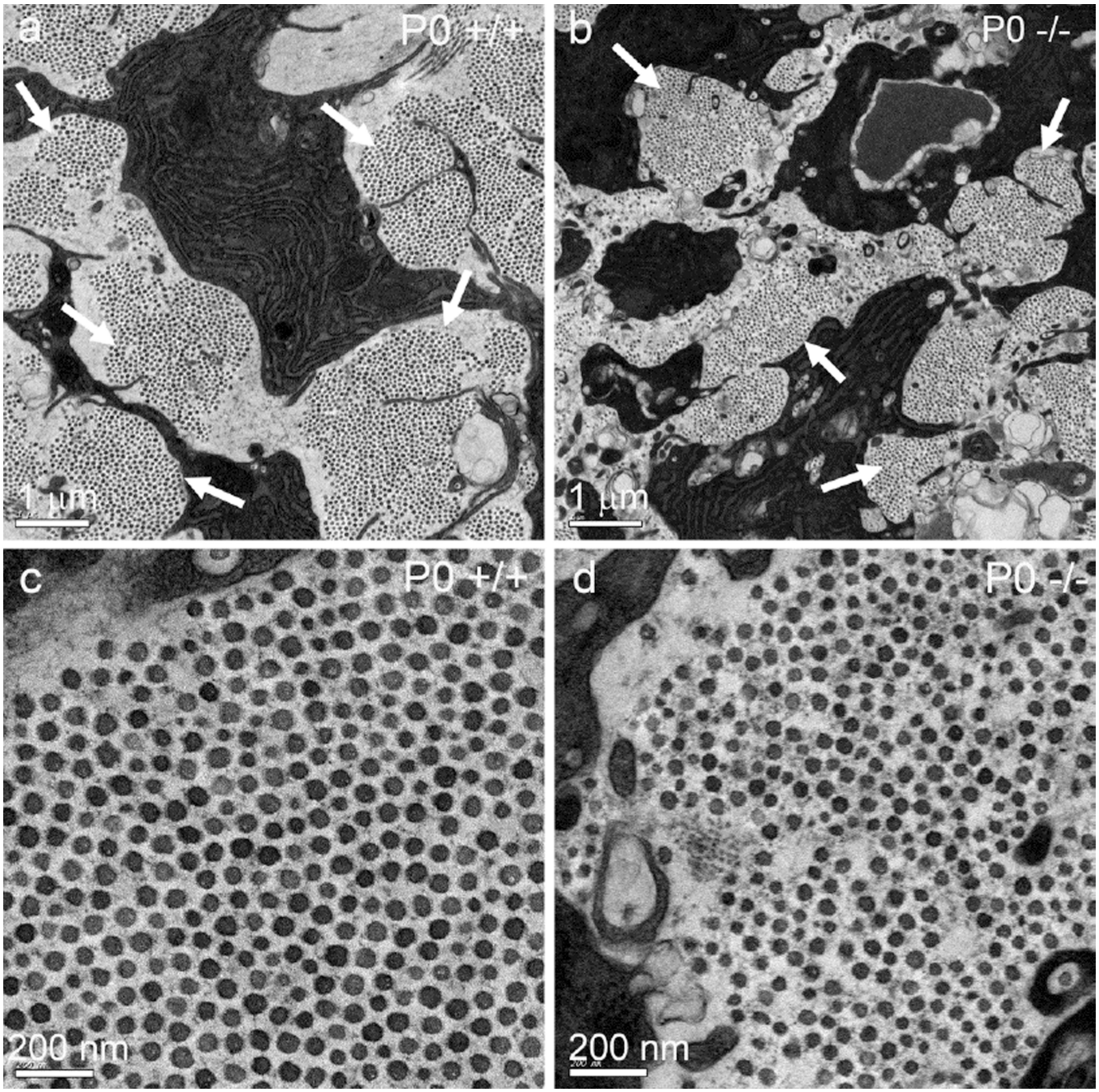

Figure 4.

Transmission electron micrographs of cross sections from flexor tendons in the forelimbs of newborn (P0) *Fbln4*$^{+/+}$ (**a, c**) and *Fbln4*$^{-/-}$ (**b, d**) mice. The P0 *Fbln4*$^{-/-}$ forelimb tendons had less pronounced collagen fibrillogenesis abnormality compared to the E16 counterpart shown in Figure 2. **a, b**: Low magnification images (scale bar = 1 μm); **c, d**: high magnification images (scale bar = 200 nm). Arrows indicate fiber-forming spaces.

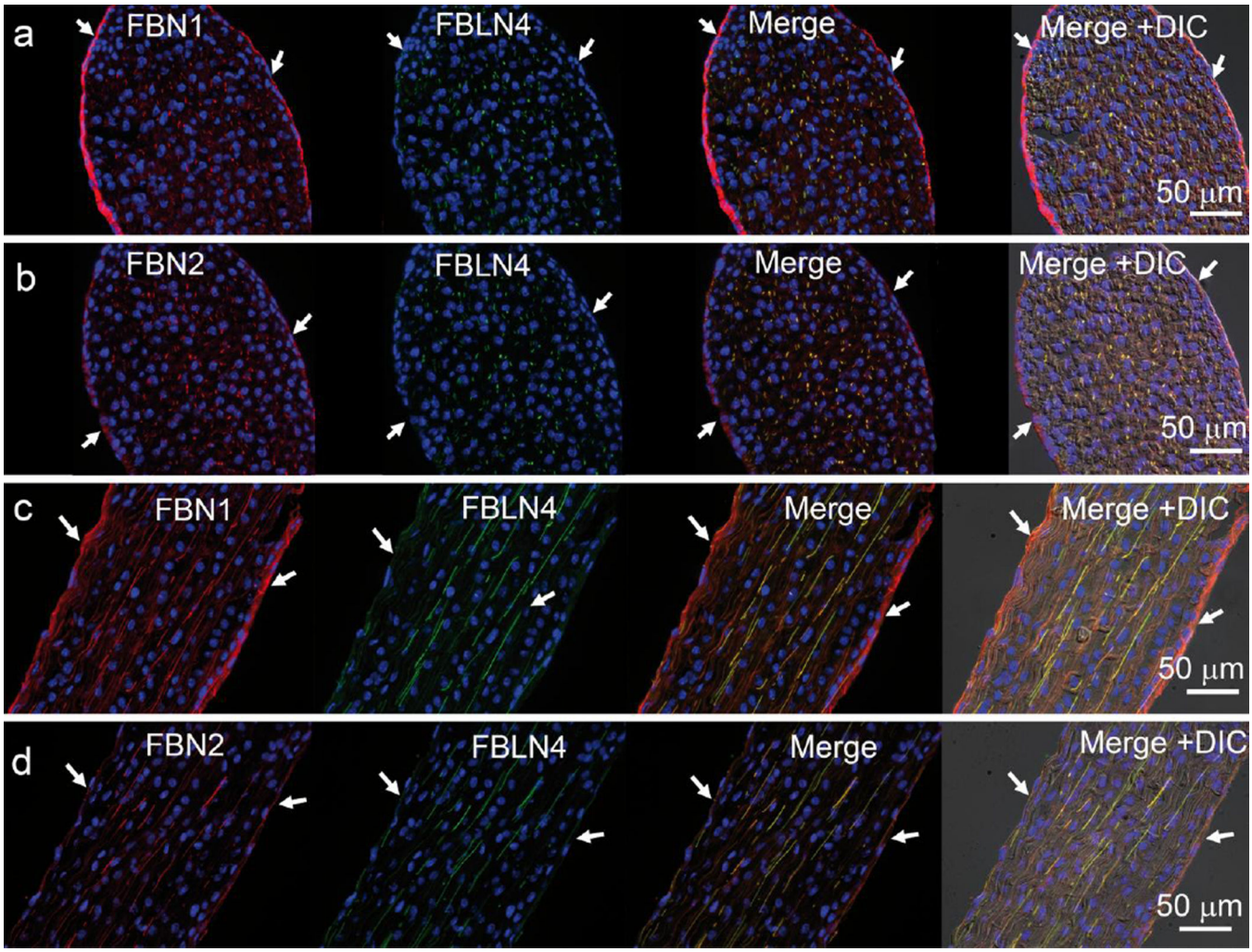

Figure 5.

Localization of fibulin-4 in tendons from wild type mice at postnatal day 4. Cross sections of FDL tendon co-immunostained with antibodies for fibulin-4 (FBLN4) and fibrillin-1 (FBN1) (**a**) and for fibulin-4 and fibrillin-2 (FBN2) (**b**). Longitudinal sections of FDL tendons co-immunostained with antibodies for fibulin-4 and fibrillin-1 (**c**) and for fibulin-4 and fibrillin-2 (**d**). Merged immunostained images are superimposed on images taken with differential interference contrast (DIC) in the far right panels. Arrows indicate tendon sheath. Scale bars = 50 μm.