Cell Tissue Res (2016) 364:637–646
DOI 10.1007/s00441-015-2346-x

REGULAR ARTICLE

Forelimb contractures and abnormal tendon collagen fibrillogenesis in fibulin-4 null mice

Dessislava Z. Markova[1,5] **· Te-Cheng Pan**[1] **· Rui-Zhu Zhang**[1] **· Guiyun Zhang**[2] **·**
Takako Sasaki[3] **· Machiko Arita**[1,5] **· David E. Birk**[4] **· Mon-Li Chu**[1]

Received: 4 August 2015 / Accepted: 10 December 2015 / Published online: 28 December 2015

Abstract Fibulin-4 is an extracellular matrix glycoprotein essential for elastic fiber formation. Mice deficient in fibulin-4 die perinatally because of severe pulmonary and vascular defects associated with the lack of intact elastic fibers. Patients with fibulin-4 mutations demonstrate similar defects, and a significant number die shortly after birth or in early childhood from cardiopulmonary failure. The patients also demonstrate 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 exhibited disruption in collagen fibrillogenesis in the absence of fibulin-4 as analyzed by transmission electron microscopy. Fewer fibrils were assembled, and fibrils were disorganized compared with wild-type controls. The organization of developing tenocytes and compartmentalization of the extracellular space was also disrupted. Fibulin-4 was co-localized with fibrillin-1 and fibrillin-2 in limb tendons by using immunofluorescence microscopy. Thus, fibulin-4 seems to play a role in regulating tendon collagen fibrillogenesis, in addition to its essential function in elastogenesis.

Keywords Elastic fiber · Cutis laxa · Fibrillin · Tendon · Collagen fibrillogenesis

Dessislava Z. Markova and Te-Cheng Pan contributed equally to this work.

Research reported in this publication was supported by the National Institutes of Health under Award Number GM55625 (MLC) and AR44745 (DEB). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Electronic supplementary material The online version of this article (doi:10.1007/s00441-015-2346-x) contains supplementary material, which is available to authorized users.

✉ Mon-Li Chu
mon-li.chu@jefferson.edu

1 Department of Dermatology and Cutaneous Biology, Sidney Kimmel Medical College, Thomas Jefferson University, 233 South 10th Street, 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, Morsani College of Medicine, University of South Florida, Tampa FL 33612, USA

5 Present address: Department of Orthopaedic Surgery, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia PA 19107, USA

Abbreviations

ARCL Autosomal recessive cutis laxa
BMP Bone morphogenetic protein
FDL Flexor digitorum longus
LOX Lysyl oxidase
LTBP Latent TGF-β-binding protein
TGF Transforming growth factor

Introduction

Fibulin-4 is a secreted glycoprotein belonging to the fibulin family, characterized by tandem repeats of calcium-binding epidermal growth factor-like modules and a C-terminal fibulin 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 the 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, fibulin-5 null mice live into adulthood and show loose skin, pulmonary emphysema, and cardiovascular defects.

In humans, mutations in fibulin-4 and -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, notable differences are found in the clinical manifestations of ARCL 1A and ARCL 1B patients. A significant proportion of patients with fibulin-4 mutations die shortly after birth or in early childhood because of 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, and 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, no determinations have been made as to 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 have generated has been characterized. Our mouse model exhibits bilateral forelimb contractures, in addition to vascular and pulmonary defects. We have found that fibulin-4 co-localizes with fibrillin microfibrils in wild-type tendons. In the absence of fibulin-4, collagen fibrillogenesis is disrupted. Fewer fibrils are assembled, and fibrils are disorganized compared with wild-type controls. The developing tenocytes and compartmentalization of the extracellular space are also 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.

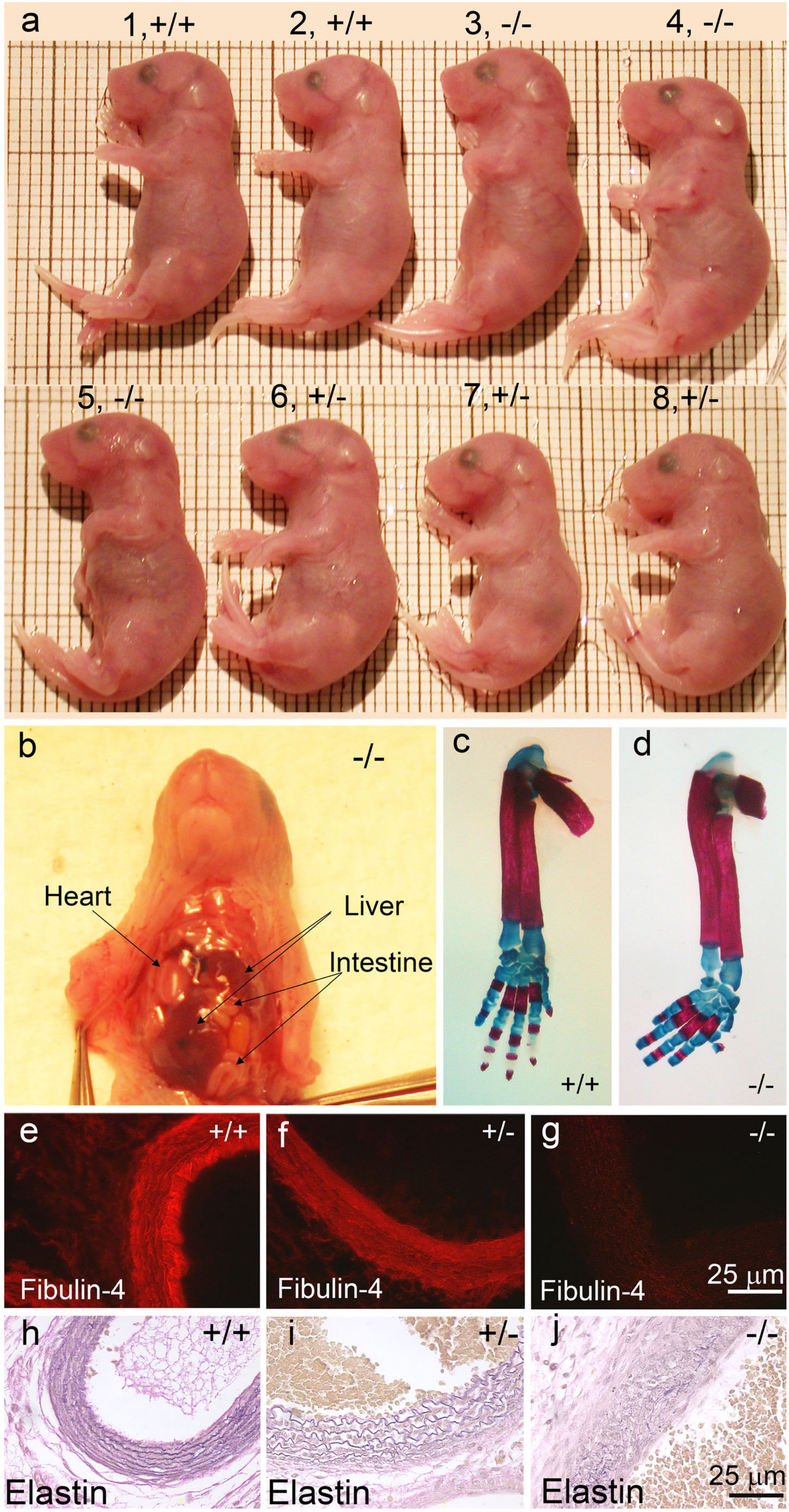
Materials and methods

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 by using the N-terminal region of mouse fibulin-4 as the antigen by the custom antibody services of Cocalico Biologicals (Reamstown, Pa., USA). The antigen corresponded to amino acids 28–203 of mouse fibulin-4 and was produced in HEK293 cells by the methods described previously (Kobayashi et al. 2007).

Targeted inactivation of *Fbln4/Efemp2* gene

A 10-kb *Xba*I 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 was as described in Supplementary Material, Fig. S1. Genotyping of mutant mice was performed by polymerase chain reaction (PCR) analysis of tail DNA. Forward primer 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


a
1,+/+
2, +/+
3, -/-
4, -/-
5, -/-
6, +/-
7,+/-
8,+/-
b
-/-
Heart
Liver
Intestine
c
+/+
d
-/-
e
+/+
Fibulin-4
f
+/-
Fibulin-4
g
-/-
Fibulin-4
25 µm
h
+/+
Elastin
i
+/-
Elastin
j
-/-
Elastin
25 µm

◀ **Fig. 1** Phenotypes of fibulin-4 null mice. **a** Photographs of eight embryos at embryonic day 19 dissected from a pregnant *Fbln4*$^{+/-}$ female. All three *Fbln4*$^{-/-}$ embryos (nos. 3, 4, 5) showed bilateral forelimb contractures. One of the three *Fbln4*$^{+/-}$ embryos (no. 7) had a 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, their 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–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 *Fbln4*$^{-/-}$ mice. **h–j** Verhoeff-Van 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 *Fbln4*$^{-/-}$ aorta. *Bars* 25 μm

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-Van Gieson elastin staining. Samples were viewed by using a Zeiss Axioskop epifluorescence microscope with a Toshiba 3CCD camera and ImagePro software (Media Cybernetics, Rockville, Md., USA).

Flexor digitorum longus (FDL) tendons were dissected from wild-type mice at postnatal day 4 (P4) and embedded in OCT compound. Cryosections (6 μm) were co-immunostained with the guinea pig polyclonal antibody for fibulin-4 and with 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. Images were captured by using a Leica CRT 550 fluorescence microscope and Leica DFC 340 FX digital camera.

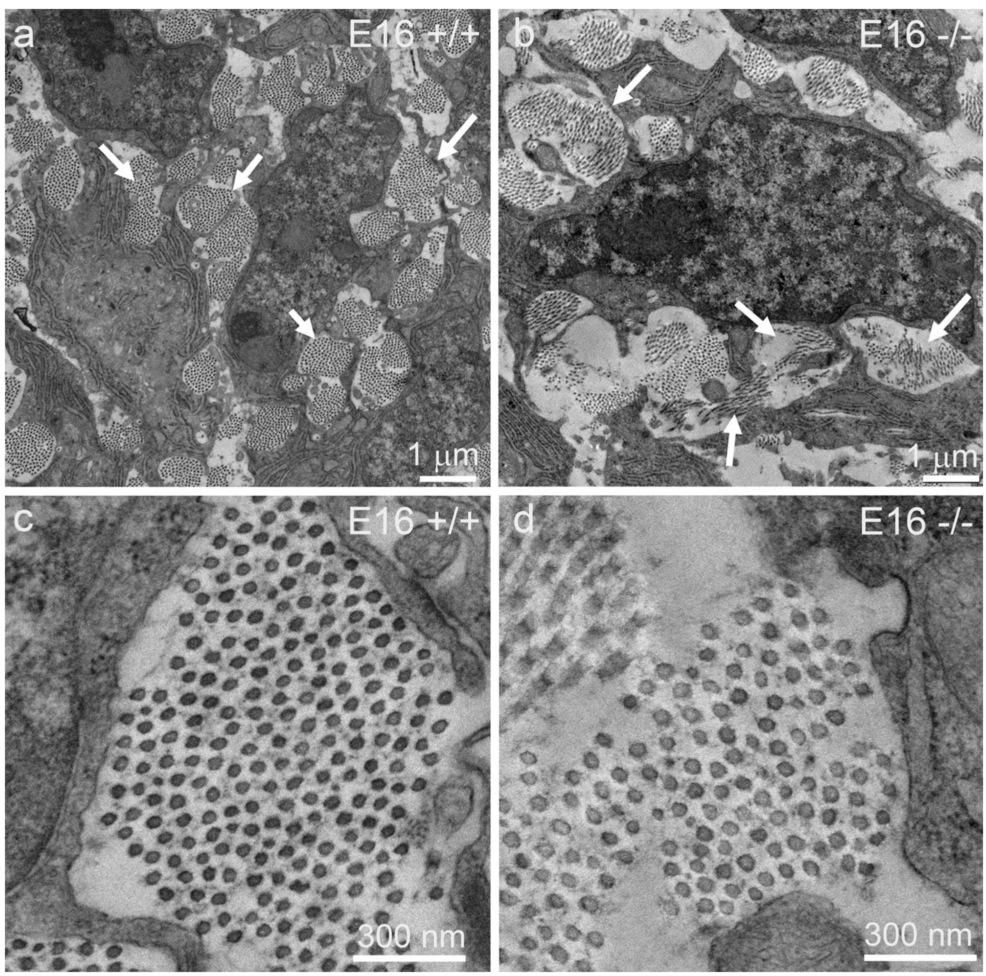


Fig. 2 Transmission electron micrographs of cross sections from flexor tendons in the forelimbs of *Fbln4*$^{+/+}$ (**a**, **c**) and *Fbln4*$^{-/-}$ (**b**, **d**) embryos at embryonic day 16 (*E16*). In *Fbln4*$^{+/+}$ tendons, the tenocytes were well organized and the extracellular space was compartmentalized into organized micro-domains in which 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 with the wild-type controls. *Arrows* indicate fiber-forming spaces. *Bars* 1 μm (**a**, **b**), 300 nm (**c**, **d**)

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 by using a Tecnai 12 or JEOL 1400 transmission electron microscope equipped with a Gatan Ultrascan US100 2 K digital camera or Gatan Orius widefield side mount CC Digital camera (Gatan, Pleasanton, Calif., USA).

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 cells (129sv strain) and germ line transmission were identified by Southern blot analysis of genomic DNA by using an external probe. The resulting heterozygous mice were intercrossed to generate *Fbln4*$^{+/+}$, *Fbln4*$^{+/-}$, and *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

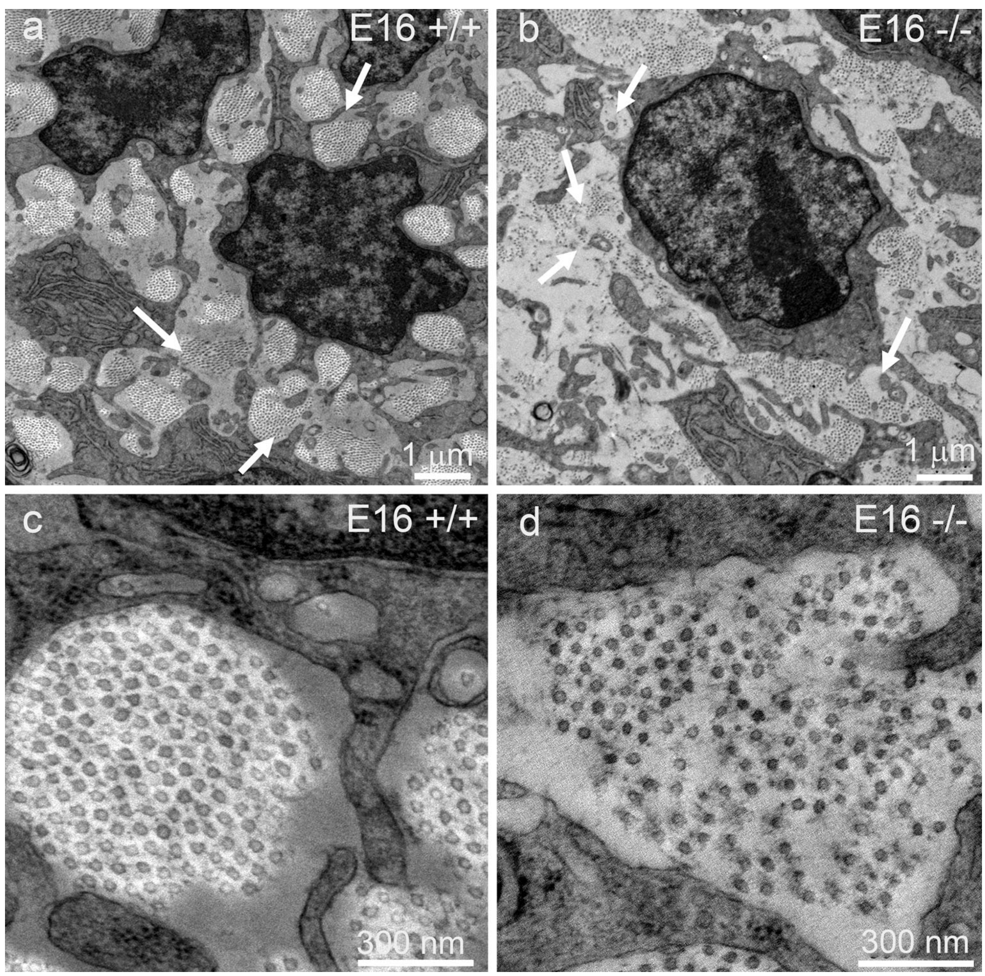


Fig. 3 Transmission electron micrographs of cross sections from flexor tendons in the hindlimbs of E16 *Fbln4*$^{+/+}$ (**a**, **c**) and *Fbln4*$^{-/-}$ (**b**, **d**) embryos. *Fbln4*$^{-/-}$ hindlimb tendons show similar ultrastructural alterations of tenocytes, collagen fibrils, and collagen fibers as seen in the forelimb tendons in Fig. 2. *Arrows* indicate fiber-forming spaces. *Bars* 1 µm (**a**, **b**), 300 nm (**c**, **d**)

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*$^{+/-}$, and *Fbln4*$^{-/-}$ littermates were similar in size, but the homozygous mutants (Fig. 1a, nos. 3, 4, 5) invariably exhibited bilateral forelimb contractures. One of the heterozygous mice (no. 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 P1-2. 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 their wild-type littermates were stained with alcian blue and alizarin red. During 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

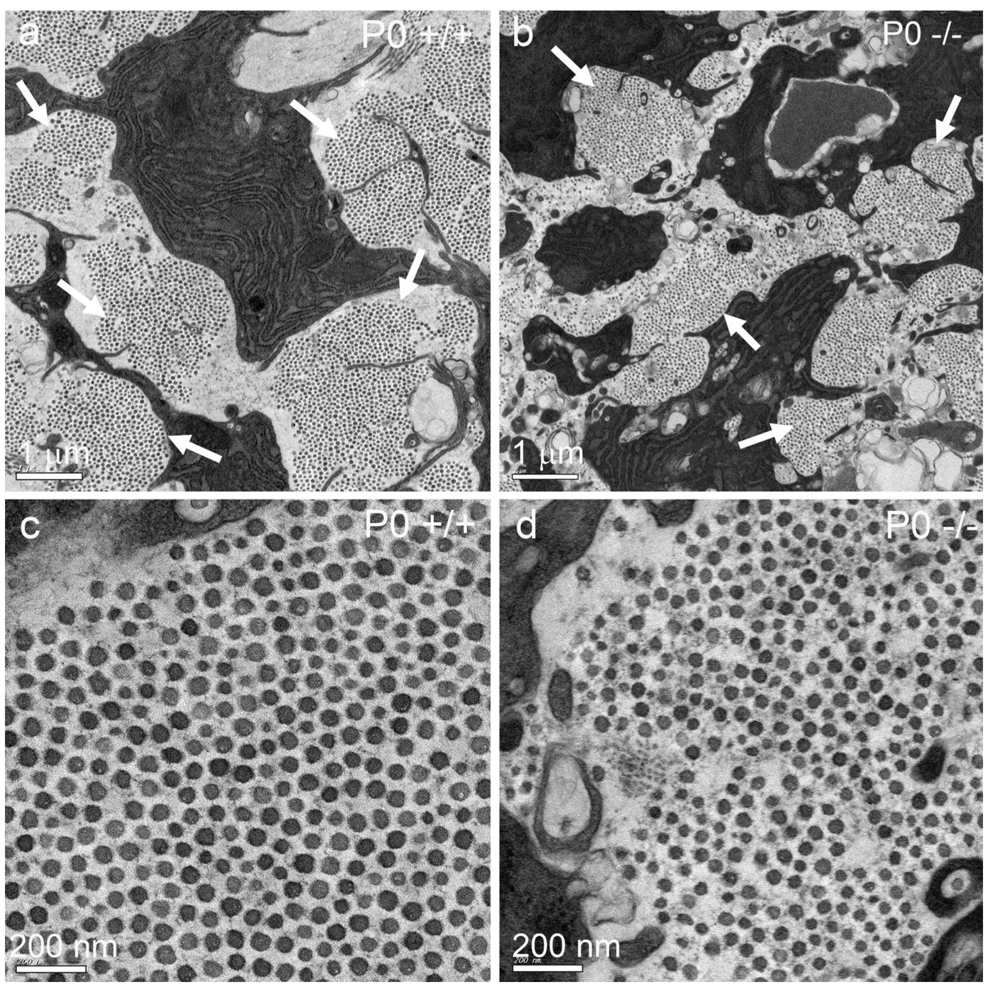


Fig. 4 Transmission electron micrographs of cross sections from flexor tendons in the forelimbs of newborn *Fbln4*$^{+/+}$ (**a, c**) and *Fbln4*$^{-/-}$ (**b, d**) mice at postnatal day 0 (*P0*). The P0 *Fbln4*$^{-/-}$ forelimb tendons had less pronounced collagen fibrillogenesis abnormality compared with the E16 counterpart shown in Fig. 2. *Arrows* indicate fiber-forming spaces. *Bars* 1 μm (**a, b**), 200 nm (**c, d**)

alizarin red staining, appeared similar between *Fbln4*$^{-/-}$ and *Fbln4*$^{+/+}$ littermates (Fig. 1c, d, data not shown). No difference was apparent 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. 1e–g). Verhoeff-Van Gieson elastin staining revealed 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. 1h–j).

Abnormal collagen fibrillogenesis in limb tendons of *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 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 of 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 P4 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 in which fibrillin-1 and fibrillin-2 were strongly and moderately expressed, respectively.

Discussion

In this study, we show that the 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 the 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 play a role in abnormal collagen fibrillogenesis. Specifically, impaired lysyl oxidase (LOX) activity and altered growth factor signaling in the absence of fibulin-4 probably 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 have revealed the 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 influences 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 probably result from LOX dysfunction. Consistent with this proposition, we have recently shown that the crosslinking of both collagen and elastin is significantly reduced, and that the conversion of LOX from its precursor is less complete in fibulin-4 E57K knock-in mice, which produce a low level of abnormal

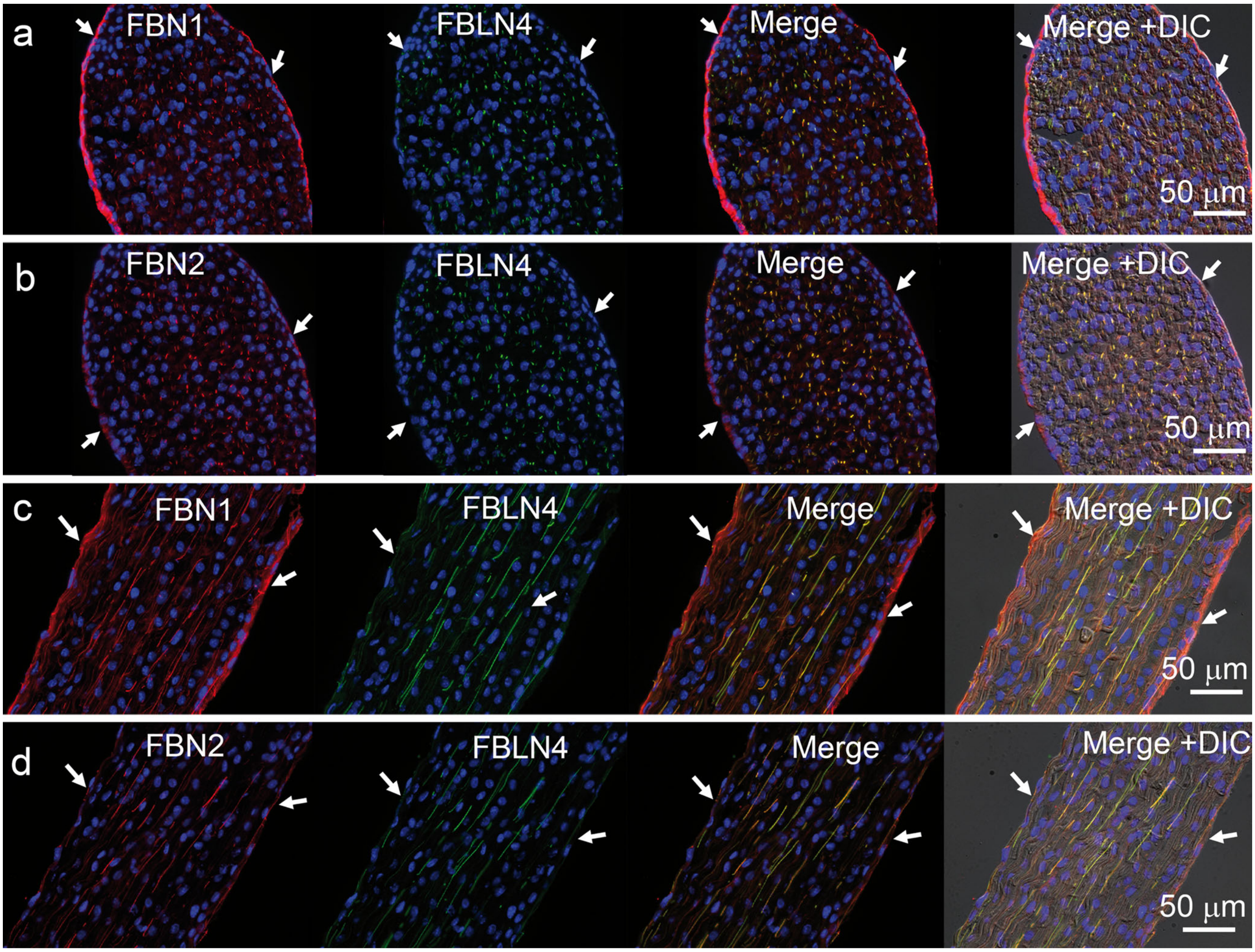


Fig. 5 Localization of fibulin-4 in tendons from wild-type mice at P4. Cross sections of flexor digitorum longus (FDL) tendon co-immunostained with antibodies for fibulin-4 (*FBLN4*) and fibrillin-1 (*FBN1*; **a**) and for fibulin-4 and fibrillin-2 (*FBN2*; **b**). Longitudinal sections of FDL tendons co-immunostained with antibodies for fibulin-4 and fibrillin-1 (**c**) and for fibulin-4 and fibrillin-2 (**d**). Merged immunostained images (*Merge*) are superimposed on images taken with differential interference contrast (*DIC*; *far right*). *Arrows* indicate tendon sheath. *Bars* 50 μm

fibulin-4 and no normal fibulin-4 (Igoucheva et al. 2015). In addition, the fibulin-4 E57K mutant mice display bent forelimbs, and the collagen fibrils in tendon and skin are abnormal. Moreover, another recent study has demonstrated abnormal collagen fibers in the 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). The forelimb and collagen fibril abnormalities in the absence of fibulin-4 might 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). Therefore, the absence of fibulin-4 might affect the extracellular storage and activation of the latent TGF-β complex. Indeed, increased TGF-β signaling has been found in hypomorphic fibulin-4 mice and in 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). Notably, LOX deficiency also leads to the 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. An increase in TGF-β signaling might thus 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 at 1-2 days after birth, whether their forelimb contracture phenotype is transient as in 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 are not directly related to collagen abnormalities. Consistent with this notion, at E16 when the collagen fibrillogenesis abnormalities are most obvious, forelimb contractures are no more pronounced than the E18 and P0 stages. Recently, the transient forelimb contractures of fibrillin-2 null mice have been attributed to decreased muscle mass resulting from activated bone morphogenetic protein (BMP) signaling (Sengle et al. 2015). Whether the 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, we 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).

Acknowledgments We thank the late Dr. Günter Kostka for graciously providing the mouse fibulin-4 genomic subclone, and Dr. Lynn Sakai for providing the antibodies for fibrillin-1 and -2.

References

Al-Hassnan ZN, Almesned AR, Tulbah S, Hakami A, Al-Omrani A, Al Sehly A, Mohammed S, Majid S, Meyer B, Al-Fayyadh M (2012) Recessively inherited severe aortic aneurysm caused by mutated EFEMP2. Am J Cardiol 109:1677–1680

Arteaga-Solis E, Gayraud B, Lee SY, Shum L, Sakai L, Ramirez F (2001) Regulation of limb patterning by extracellular microfibrils. J Cell Biol 154:275–281

Boregowda R, Paul E, White J, Ritty TM (2008) Bone and soft connective tissue alterations result from loss of fibrillin-2 expression. Matrix Biol 27:661–666

Callewaert B, Su CT, Van Damme T, Vlummens P, Malfait F, Vanakker O, Schulz B, Mac Neal M, Davis EC, Lee JG, Salhi A, Unger S, Heimdal K, De Almeida S, Kornak U, Gaspar H, Bresson JL, Prescott K, Gosendi ME, Mansour S, Pierard GE, Madan-Khetarpal S, Sciurba FC, Symoens S, Coucke PJ, Van Maldergem L, Urban Z, De Paepe A (2013) Comprehensive clinical and molecular analysis of 12 families with type 1 recessive cutis laxa. Hum Mutat 34:111–121

Choudhury R, McGovern A, Ridley C, Cain SA, Baldwin A, Wang MC, Guo C, Mironov A Jr, Drymoussi Z, Trump D, Shuttleworth A, Baldock C, Kielty CM (2009) Differential regulation of elastic fiber formation by fibulin-4 and -5. J Biol Chem 284:24553–24567

Dasouki M, Markova D, Garola R, Sasaki T, Charbonneau NL, Sakai LY, Chu ML (2007) Compound heterozygous mutations in fibulin-4 causing neonatal lethal pulmonary artery occlusion, aortic aneurysm, arachnodactyly, and mild cutis laxa. Am J Med Genet A 143A:2635–2641

de Vega S, Iwamoto T, Yamada Y (2009) Fibulins: multiple roles in matrix structures and tissue functions. Cell Mol Life Sci 66:1890–1902

El-Hallous E, Sasaki T, Hubmacher D, Getie M, Tiedemann K, Brinckmann J, Batge B, Davis EC, Reinhardt DP (2007) Fibrillin-1 interactions with fibulins depend on the first hybrid domain and provide an adaptor function to tropoelastin. J Biol Chem 282:8935–8946

Erickson LK, Opitz JM, Zhou H (2012) Lethal osteogenesis imperfecta-like condition with cutis laxa and arterial tortuosity in MZ twins due to a homozygous fibulin-4 mutation. Pediatr Dev Pathol 15:137–141

Grant TM, Thompson MS, Urban J, Yu J (2013) Elastic fibres are broadly distributed in tendon and highly localized around tenocytes. J Anat 222:573–579

Hanada K, Vermeij M, Garinis GA, de Waard MC, Kunen MG, Myers L, Maas A, Duncker DJ, Meijers C, Dietz HC, Kanaar R, Essers J (2007) Perturbations of vascular homeostasis and aortic valve abnormalities in fibulin-4 deficient mice. Circ Res 100:738–746

Hebson C, Coleman K, Clabby M, Sallee D, Shankar S, Loeys B, Van Laer L, Kogon B (2014) Severe aortopathy due to fibulin-4 deficiency: molecular insights, surgical strategy, and a review of the literature. Eur J Pediatr 173:671–675

Horiguchi M, Inoue T, Ohbayashi T, Hirai M, Noda K, Marmorstein LY, Yabe D, Takagi K, Akama TO, Kita T, Kimura T, Nakamura T (2009) Fibulin-4 conducts proper elastogenesis via interaction with cross-linking enzyme lysyl oxidase. Proc Natl Acad Sci U S A 106: 19029–19034

Hornstra IK, Birge S, Starcher B, Bailey AJ, Mecham RP, Shapiro SD (2003) Lysyl oxidase is required for vascular and diaphragmatic development in mice. J Biol Chem 278:14387–14393

Hoyer J, Kraus C, Hammersen G, Geppert JP, Rauch A (2009) Lethal cutis laxa with contractural arachnodactyly, overgrowth and soft tissue bleeding due to a novel homozygous fibulin-4 gene mutation. Clin Genet 76:276–281

Huang J, Davis EC, Chapman SL, Budatha M, Marmorstein LY, Word RA, Yanagisawa H (2010) Fibulin-4 deficiency results in ascending aortic aneurysms: a potential link between abnormal smooth muscle cell phenotype and aneurysm progression. Circ Res 106:583–592

Hucthagowder V, Sausgruber N, Kim KH, Angle B, Marmorstein LY, Urban Z (2006) Fibulin-4: a novel gene for an autosomal recessive cutis laxa syndrome. Am J Hum Genet 78:1075–1080

Iascone M, Sana ME, Pezzoli L, Bianchi P, Marchetti D, Fasolini G, Sadou Y, Locatelli A, Fabiani F, Mangili G, Ferrazzi P (2012) Extensive arterial tortuosity and severe aortic dilation in a newborn with an EFEMP2 mutation. Circulation 126:2764–2768

Igoucheva O, Alexeev V, Halabi CM, Adams SM, Stoilov I, Sasaki T, Arita M, Donahue A, Mecham RP, Birk DE, Chu ML (2015) Fibulin-4 E57K knock-in mice recapitulate cutaneous, vascular and skeletal defects of recessive cutis laxa 1B with both elastic fiber and collagen fibril abnormalities. J Biol Chem 290:21443–21459

Izu Y, Ansorge HL, Zhang G, Soslowsky LJ, Bonaldo P, Chu ML, Birk DE (2011) Dysfunctional tendon collagen fibrillogenesis in collagen VI null mice. Matrix Biol 30:53–61

Kappanayil M, Nampoothiri S, Kannan R, Renard M, Coucke P, Malfait F, Menon S, Ravindran HK, Kurup R, Faiyaz-Ul-Haque M, Kumar K, De Paepe A (2012) Characterization of a distinct lethal arteriopathy syndrome in twenty-two infants associated with an identical, novel mutation in FBLN4 gene, confirms fibulin-4 as a critical determinant of human vascular elastogenesis. Orphanet J Rare Dis 7:61

Kobayashi N, Kostka G, Garbe JH, Keene DR, Bachinger HP, Hanisch FG, Markova D, Tsuda T, Timpl R, Chu ML, Sasaki T (2007) A comparative analysis of the fibulin protein family. Biochemical characterization, binding interactions, and tissue localization. J Biol Chem 282:11805–11816

Kutchuk L, Laitala A, Soueid-Bomgarten S, Shentzer P, Rosendahl AH, Eilot S, Grossman M, Sagi I, Sormunen R, Myllyharju J, Maki JM, Hasson P (2015) Muscle composition is regulated by a Lox-TGFbeta feedback loop. Development 142:983–993

Loeys B, Van Maldergem L, Mortier G, Coucke P, Gerniers S, Naeyaert JM, De Paepe A (2002) Homozygosity for a missense mutation in fibulin-5 (FBLN5) results in a severe form of cutis laxa. Hum Mol Genet 11:2113–2118

Lucero HA, Kagan HM (2006) Lysyl oxidase: an oxidative enzyme and effector of cell function. Cell Mol Life Sci 63:2304–2316

Maki JM, Rasanen J, Tikkanen H, Sormunen R, Makikallio K, Kivirikko KI, Soininen R (2002) Inactivation of the lysyl oxidase gene Lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice. Circulation 106:2503–2509

Maki JM, Sormunen R, Lippo S, Kaarteenaho-Wiik R, Soininen R, Myllyharju J (2005) Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. Am J Pathol 167:927–936

Massam-Wu T, Chiu M, Choudhury R, Chaudhry SS, Baldwin AK, McGovern A, Baldock C, Shuttleworth CA, Kielty CM (2010) Assembly of fibrillin microfibrils governs extracellular deposition of latent TGF beta. J Cell Sci 123:3006–3018

McLaughlin PJ, Chen Q, Horiguchi M, Starcher BC, Stanton JB, Broekelmann TJ, Marmorstein AD, McKay B, Mecham R, Nakamura T, Marmorstein LY (2006) Targeted disruption of fibulin-4 abolishes elastogenesis and causes perinatal lethality in mice. Mol Cell Biol 26:1700–1709

Nakamura T, Lozano PR, Ikeda Y, Iwanaga Y, Hinek A, Minamisawa S, Cheng CF, Kobuke K, Dalton N, Takada Y, Tashiro K, Ross J Jr, Honjo T, Chien KR (2002) Fibulin-5/DANCE is essential for elastogenesis in vivo. Nature 415:171–175

Ono RN, Sengle G, Charbonneau NL, Carlberg V, Bachinger HP, Sasaki T, Lee-Arteaga S, Zilberberg L, Rifkin DB, Ramirez F, Chu ML, Sakai LY (2009) Latent transforming growth factor beta-binding proteins and fibulins compete for fibrillin-1 and exhibit exquisite specificities in binding sites. J Biol Chem 284:16872–16881

Papke CL, Yanagisawa H (2014) Fibulin-4 and fibulin-5 in elastogenesis and beyond: insights from mouse and human studies. Matrix Biol 37:142–149

Papke CL, Tsunezumi J, Ringuette LJ, Nagaoka H, Terajima M, Yamashiro Y, Urquhart G, Yamauchi M, Davis EC, Yanagisawa H (2015) Loss of fibulin-4 disrupts collagen synthesis and maturation: implications for pathology resulting from EFEMP2 mutations. Hum Mol Genet 24:5867–5879

Pryce BA, Watson SS, Murchison ND, Staverosky JA, Dunker N, Schweitzer R (2009) Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. Development 136:1351–1361

Ramirez F, Sakai LY (2010) Biogenesis and function of fibrillin assemblies. Cell Tissue Res 339:71–82

Renard M, Holm T, Veith R, Callewaert BL, Ades LC, Baspinar O, Pickart A, Dasouki M, Hoyer J, Rauch A, Trapane P, Earing MG, Coucke PJ, Sakai LY, Dietz HC, De Paepe AM, Loeys BL (2010) Altered TGFbeta signaling and cardiovascular manifestations in patients with autosomal recessive cutis laxa type I caused by fibulin-4 deficiency. Eur J Hum Genet 18:895–901

Sawyer SL, Dicke F, Kirton A, Rajapkse T, Rebeyka IM, McInnes B, Parboosingh JS, Bernier FP (2013) Longer term survival of a child with autosomal recessive cutis laxa due to a mutation in FBLN4. Am J Med Genet A 161A:1148–1153

Sengle G, Carlberg V, Tufa SF, Charbonneau NL, Smaldone S, Carlson EJ, Ramirez F, Keene DR, Sakai LY (2015) Abnormal activation of BMP signaling causes myopathy in Fbn2 null mice. PLoS Genet 11, e1005340

Timpl R, Sasaki T, Kostka G, Chu ML (2003) Fibulins: a versatile family of extracellular matrix proteins. Nat Rev Mol Cell Biol 4:479–489

Urban Z, Davis EC (2014) Cutis laxa: intersection of elastic fiber biogenesis, TGFbeta signaling, the secretory pathway and metabolism. Matrix Biol 33:16–22

Wagenseil JE, Mecham RP (2007) New insights into elastic fiber assembly. Birth Defects Res C Embryol Today 81:229–240

Yanagisawa H, Davis EC (2010) Unraveling the mechanism of elastic fiber assembly: the roles of short fibulins. Int J Biochem Cell Biol 42:1084–1093

Yanagisawa H, Davis EC, Starcher BC, Ouchi T, Yanagisawa M, Richardson JA, Olson EN (2002) Fibulin-5 is an elastin-binding protein essential for elastic fibre development in vivo. Nature 415:168–171