Loss of fibulin-4 results in abnormal collagen fibril assembly in bone, caused by impaired lysyl oxidase processing and collagen cross-linking

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Abstract

The extracellular matrix protein fibulin-4 has been shown to be indispensable for elastic fiber assembly, but there is also evidence from human mutations that it is involved in controlling skeletal development and bone stability. Fibulin-4 mutations were identified in patients suffering from vascular abnormality and/or cutis laxa, and some of these patients exhibited bone fragility, arachnodactyly and joint laxity. In order to elucidate the role of fibulin-4 in bone structure and skeletal development, we analyzed structural changes in skeletal tissues of *Fbln4*$^{-/-}$ mice. Immunostaining confirmed that fibulin-4 is highly expressed in cartilage, bone, ligaments and tendons. No morphological abnormalities were found in the skeleton of *Fbln4*$^{-/-}$ mice as compared to wild type littermates except forelimb contractures as well as unusually thick collagen fibrils. Furthermore, fibulin-4 deficiency caused enhanced susceptibility of bone collagen for acid extraction, consistent with significantly reduced lysylpyridinoline and hydroxylysylpyridinoline cross-links in bone. In accordance with that, the amount of lysyl oxidase in long bones and calvaria was strongly decreased and proteolytic activation of lysyl oxidase was reduced in fibulin-4 deficient osteoblasts, while addition of recombinant fibulin-4 rescued the activation. The finding suggested that fibulin-4 is important for the proteolytic activation of lysyl oxidase which has a pivotal role in cross-linking of collagen and elastin.

Introduction

Collagens and elastin are major extracellular matrix proteins which serve important structural functions in the animal body. Type I collagen is the most abundant protein in vertebrates and comprises 90% of the organic components of bone. Intermolecular cross-links are essential for the functions of collagen and elastic fibers, and lysyl oxidase (LOX) is the responsible enzyme which initiates the first step of cross-link formation. Lysine (Lys) ε-amino groups in elastin are oxidatively deaminated by LOX to form allysine, which subsequently condense to yield desmosine or isodesmosine [1]. In collagen I, specific Lys and hydroxylysine (Hyl) residues in the N- and C-telopeptides are oxidatively deaminated to

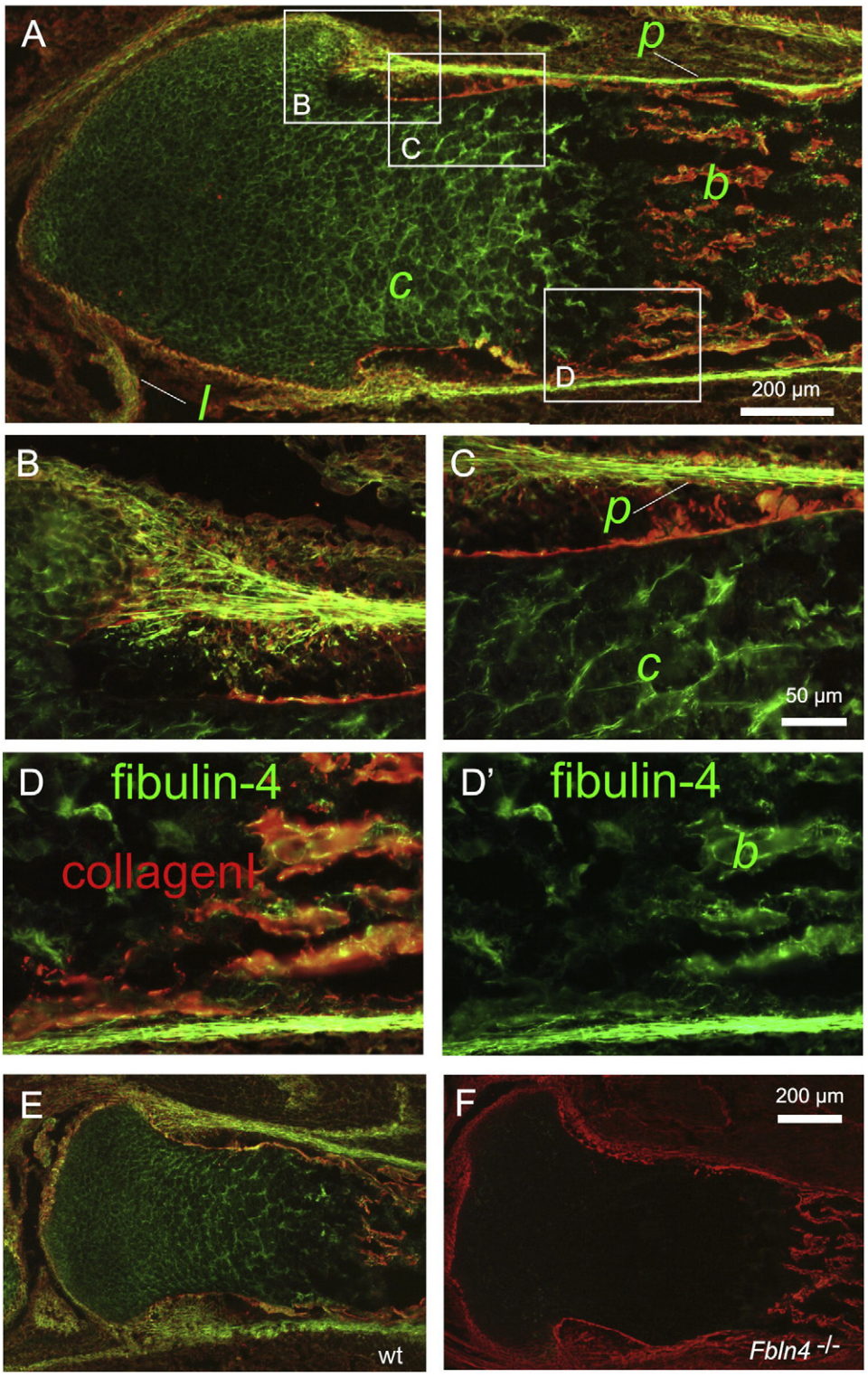


Fig. 1. Expression of fibulin-4 in mouse skeletal tissue. (A–D) Staining of the tibia and adjacent tissues from newborn mice with anti-fibulin-4 antibody (green) and anti-collagen I antibody (red) shows fibulin-4 in cartilage (*c*), ligaments (*l*), periosteum (*p*) and bone (*b*). (B–D, D') B–D labeled in A are shown with a higher magnification. D' is the same as D but only fibulin-4 staining is shown. (E, F) Staining of wild type (E) and *Fbln4*$^{-/-}$ femur (F) with the same antibodies as in A shows the absence of fibulin-4 in *Fbln4*$^{-/-}$ mice.

create reactive aldehydes that initiate non-enzymatic condensation reactions to form covalent bonds with Lys or Hyl residues on juxtaposed adjacent collagen molecules [2–4].

The LOX family consists of five members including four LOX-Like proteins LOXL1–LOXL4 [5]. All possess the catalytic domain at the C-terminus showing high homology, however the N-terminal regions are very diverse. Structurally LOX and LOXL1 are similar; they are activated by BMP-1/mammalian Tolloid and mammalian Tolloid-like proteases which release the N-terminal region [6]. The N-terminal regions of LOXL2, LOXL3 and LOXL4 consist of scavenger receptor cysteine-rich domains and need not to be cleaved off in order to convert to an active enzyme. Inactivation of the *Lox* gene in mice results in perinatal death due to aortic aneurysms, rupture of diaphragm and dysfunction of lung [7–9]. In contrast, *Loxl1* null mice are viable but develop pelvic organ prolapse, enlarged airspaces of lung, loose skin and vascular abnormality, suggesting that LOXL1 is mainly involved in cross-linking of elastin [10].

Fibulins are extracellular glycoproteins associated with elastic fibers and basement membranes (for reviews see [11–13]). The unique structural feature of this protein family consisting of 7 members (fibulin-1 to 7) is a tandem array of calcium-binding epidermal growth factor (cbEGF)-like modules and a fibulin-type C-terminal (FC) domain. Fibulin-4, 50 kD in size, is a relatively small member of the fibulin family and consists of an N-terminal modified cbEGF module, followed by five consecutive cbEGF modules and the FC domain [14]. It plays an important role in development and integrity of elastic fibers. Evidence came from human patients suffering from vascular abnormality and/or cutis laxa, arachnodactyly, pectus excavatum and joint laxity that were associated with different mutations in the fibulin-4 gene, *EFEMP2*/*FBLN4* [15–19]. The clinical phenotypes varied with the type of *FBLN4* mutation; for example, one patient diagnosed with autosomal recessive cutis laxa 1B caused by E57K missense mutation had multiple bone fractures at birth and also presented with arterial tortuosity, aneurysm, emphysema, inguinal and diaphragmatic hernia, and joint laxity [15]. A homozygous missense mutation C267Y was found in the *FBLN4* gene in another patient died perinatally and this patient was affected with cutis laxa, arachnodachtyly, microcephaly and facial dysmorphism in addition to collapsed lung and fragility of vessels [17]. A recent report by Erickson et al. [18] showed an osteogenesis imperfecta-like phenotype including arachnodactyly and multiple bone fractures. This phenotype was caused by homozygous deletion of a single nucleotide in exon 1 which introduced a premature stop codon in exon 2, most likely resulting in a condition similar to fibulin-4 null mice.

A pivotal role of fibulin-4 in the formation of elastic fibers and tissues was confirmed by targeted inactivation of the fibulin-4 gene in mice which resulted in perinatal lethality due to aneurysm, artery tortuosity and ruptures, and lung abnormalities [20]. In the aorta of fibulin-4 deficient mice, disorganized elastic fibers and perturbation of TGF-β signaling were observed, and elastin cross-links in tissues were largely diminished.

In these studies it remained open, however, whether the loss of fibulin-4 in mice also caused skeletal and other systemic connective tissue anomalies as in human patients. We recently generated a *Fbln4* deficient mouse strain ($Fbln4^{-/-}$) which exhibited bilateral forelimb contractures, in addition to vascular and pulmonary defects causing perinatal death [48]. Here we addressed the question whether fibulin-4 also affects structure and development of bone in addition to elastic tissue. Morphological and biochemical analysis of skeletal development and bone structure of these $Fbln4^{-/-}$ mice showed normal fetal bone development, but bones from newborn animals exhibited a strong disturbance in collagen fibril assembly, with a high rate of unusually large diameter collagen fibrils. Furthermore, type I collagen was more easily extractable with acid from $Fbln4^{-/-}$ bones due to strongly impaired cross-link formation. Consistent with this finding, osteoblasts from $Fbln4^{-/-}$ mice exhibited reduced amounts of the active form of LOX. Thus, here we show for the first time that fibulin-4 is not only essential for the formation of elastic fibers and for elastin cross-linking, but also for the cross-linking of type I collagen in bone by controlling the activation of LOX.

Results

Expression of fibulin-4 in mouse skeletal tissues

Guinea pig anti-mouse fibulin-4 and rabbit anti-mouse collagen I were used to co-stain cryosections of newborn mouse hindlimb (Fig. 1). Fibulin-4 was prominently expressed in cartilage, bone, perichondrium and ligaments (Fig. 1A, B, C). In bone, perichondrium and ligaments, fibulin-4 colocalized with collagen I (Fig. 1D). Fibulin-4 was also detected in bone marrow stroma (Fig. 1A), but as expected, no fibulin-4 was detected in sections of fibulin-4 null mice (Fig. 1F).

Morphological and histological analysis of the skeleton of fibulin-4 null mice

The inactivation of both alleles of the *Fbln4* gene resulted in postnatal lethality [20,48], therefore only embryos or newborn fibulin-4 null mice were

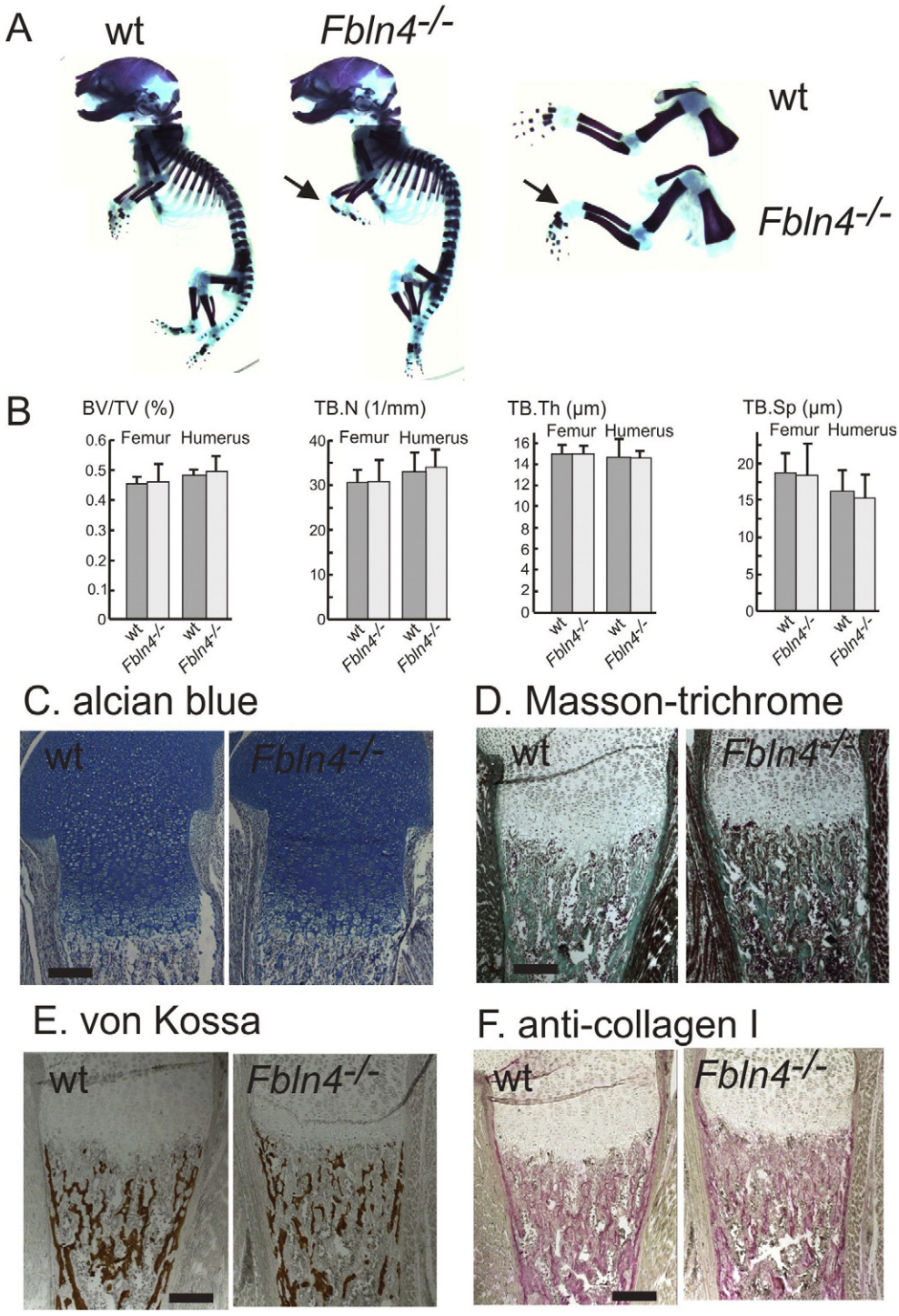
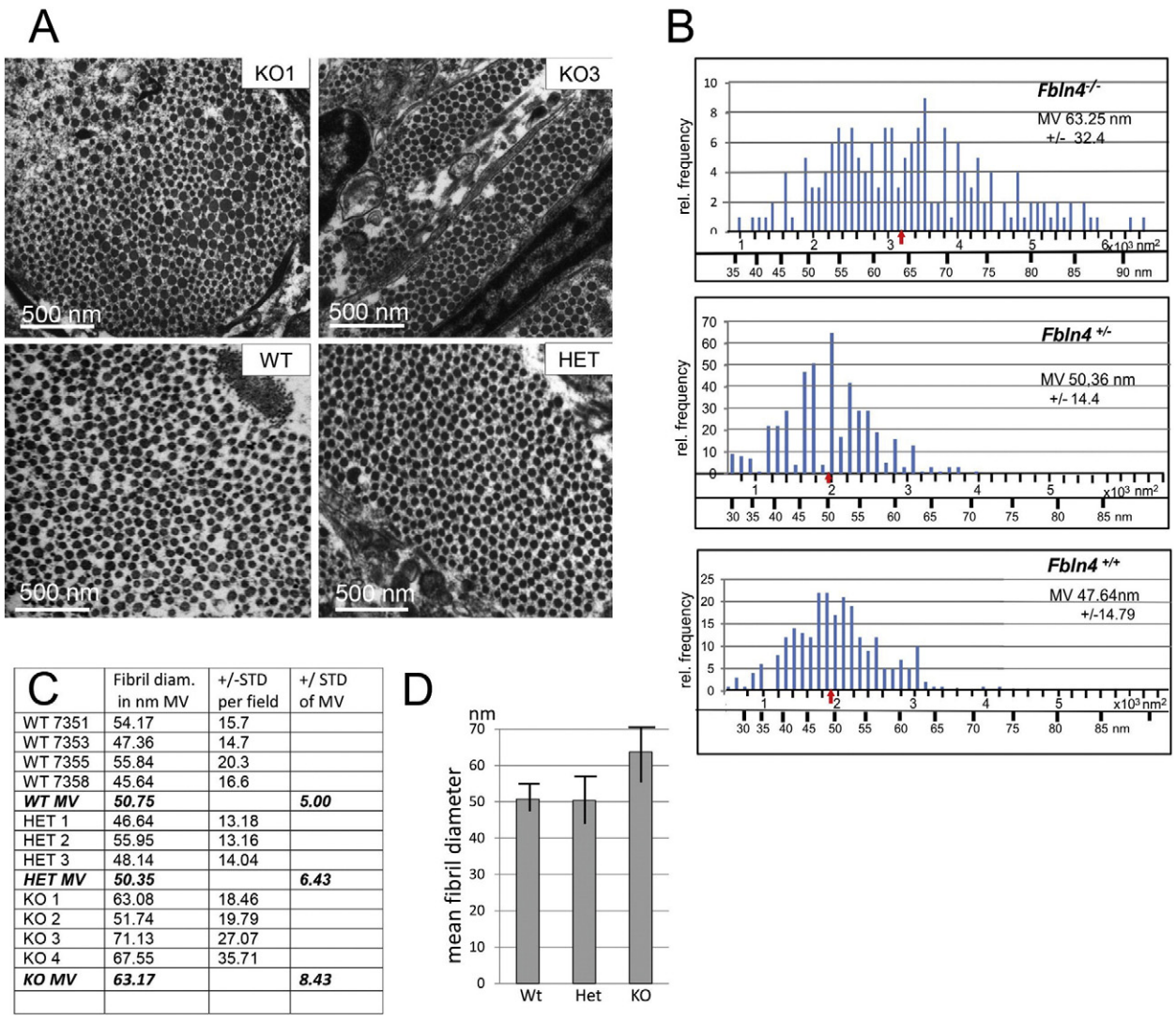


Fig. 2. Skeletal analyses of wt and *Fbln4*$^{-/-}$ mice show no major alterations in bone development but forelimb contractures (A, arrow). (A) Whole mount skeletal staining with Alcian blue and Alizarin red. (B) Histograms summarizing μCT measurements of femur and humerus. BV, bone volume; TV, total volume; TB.N, trabecular number; TB.Th, trabecular thickness; TB.Sp, trabecular separation. (C–F) Histochemical staining of femur at E18.5 with Alcian blue (C), and of femur at P1 with Masson-trichrome (D) and with von Kossa (E). Immunostaining with anti-collagen I of femur at P1 (F). Scale bars, 200 μm.

available for analysis. Alcian blue and alizarin red staining of newborn *Fbln4* null mice demonstrated normal morphology of the skeleton (Fig. 2A), with the exception of striking contractures of the forelimbs (Fig. 2A, arrow). Preparation of mice for Alcian blue and Alizarin red staining revealed that *Fbln4*$^{-/-}$ mice



C	Fibril diam. in nm MV	+/-STD per field	+/ STD of MV
WT 7351	54.17	15.7	
WT 7353	47.36	14.7	
WT 7355	55.84	20.3	
WT 7358	45.64	16.6	
WT MV	***50.75***		***5.00***
HET 1	46.64	13.18	
HET 2	55.95	13.16	
HET 3	48.14	14.04	
HET MV	***50.35***		***6.43***
KO 1	63.08	18.46	
KO 2	51.74	19.79	
KO 3	71.13	27.07	
KO 4	67.55	35.71	
KO MV	***63.17***		***8.43***

Fig. 3. Disregulated bone collagen fibril size in *Fbln4*$^{-/-}$ mice. (A) Ultrastructural analysis of bone collagen fibrils of *Fbln4*$^{-/-}$(KO), *Fbln4*$^{+/-}$ (HET) and wt femora shows a substantial proportion of unusually thick fibrils, not seen in wt or *Fbln4*$^{+/-}$ bones, and a higher variation of fibril diameters was observed in *Fbln4*$^{-/-}$ bones, (B) cross section areas of fibrils were measured in 4 different thin sections of each genotype, counting between 200 and 400 fibrils per image using the ImageJ program. Calculation of the fibril cross section areas (in nm^2) and diameters (in nm) shows that a high proportion of *Fbln4*$^{-/-}$ fibrils had large diameters above 70 nm, which were never seen in heterozygote or wt bone sections; depicted are the results of one typical experiment. (C) Owing to the higher variation in collagen fibril diameters, the standard deviation for each mean value (MV) calculated for each image is higher for the *Fbln4*$^{-/-}$ fibrils than for wt or heterozygote fibrils. (D) Comparison of mean values of each genotype shows that the average diameter of *Fbln4*$^{-/-}$ fibrils is higher than that of wt and heterozygotes.

were more sensitive to KOH maceration than wt (wild type) littermates, and especially distal parts of limbs fell into pieces without the fixation with paraformaldehyde or formalin during KOH maceration, suggesting enhanced fragility of limb ligaments. Bone analysis by microCT measurements did, however, not reveal any significant differences in bone volume or trabecular thickness between wt and *Fbln4*$^{-/-}$ mice (Fig. 2B). No abnormality of the staining for proteoglycans, calcium and collagens was detected with Alcian blue (Fig. 2C), von Kossa staining (Fig. 2E), Masson-trichrome (Fig. 2D) and anti-c-ollagen I (Fig. 2F), respectively.

Abnormal size distribution of collagen fibrils in *Fbln4*$^{-/-}$ bone

Next long bones from wt, *Fbln4*$^{+/-}$ and *Fbln4*$^{-/-}$ mice were analyzed by electron microscopy. Surprisingly, a striking variation in the thickness of

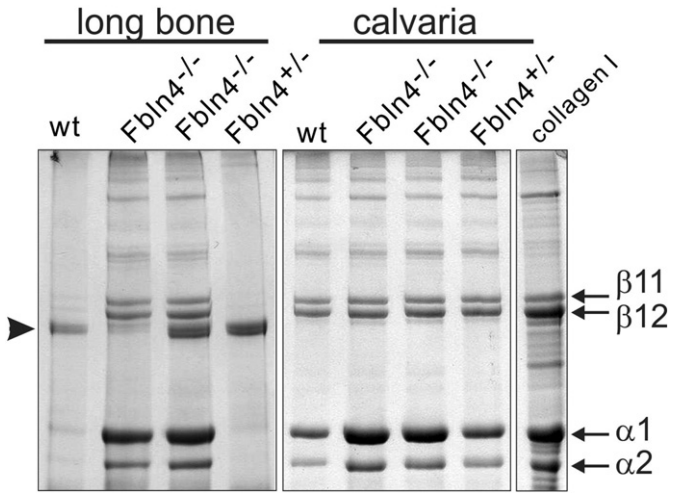


Fig. 4. SDS-PAGE of acetic acid-extracted collagen I from bones and calvaria stained with Coomassie brilliant blue. Extracts from two different *Fbln4*$^{-/-}$ mice were shown and collagen I isolated from mouse tail tendon was loaded as a reference. The band marked with an arrow head was identified as embryonal muscle myosin heavy chain by N-terminal sequencing. The figure shows that *Fbln4*$^{-/-}$ bones are susceptible to collagen extraction with acid, while wt and *Fbln4*$^{+/-}$ long bones are almost resistant to acid extraction. The differences were less striking in calvarial bones.

bone collagen fibrils was observed in *Fbln4*$^{-/-}$ bone, with numerous fibrils thicker than 70 nm which were never observed in wt or *Fbln4*$^{+/-}$ bones (Fig. 3). The cross sectional areas of collagen fibrils in 4 different sections of each genotype were measured, and the results showed confirmed a substantial amount of fibrils thicker than 70 nm in *Fbln4*$^{-/-}$ bones. Accordingly, the mean diameter of collagen fibrils in *Fbln4*$^{-/-}$ bones was 63.2 nm as compared to about 50 nm of wt or *Fbln4*$^{+/-}$ bone collagen fibrils (Fig. 3B, C, D), indicating that fibulin-4 is involved in the control of collagen I fibril assembly in bone.

Changes in collagen cross-links in *Fbln4*$^{-/-}$ bones

In order to address the question whether fibulin-4 affects the cross-linking of collagens in bones, collagen was extracted from bones from 5 to 7 newborn *Fbln4*$^{-/-}$, *Fbln4*$^{+/-}$ and wt littermates with 0.5 M acetic acid, followed by pepsin digestion. A representative result obtained from one wt, one *Fbln4*$^{+/-}$ and two *Fbln4*$^{-/-}$ bones shows that acid treatment solubilized significantly more collagen I from long bones of *Fbln4* $^{-/-}$ mice, compared with wt and *Fbln4*$^{+/-}$ long bones. The differences were less striking in calvarial bones (Fig. 4). Also pepsin extraction solubilized more monomers and dimers, but the difference was less significant than those seen in acid extracts (data not shown).

The amounts of lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP) cross-link components were determined in cartilage and bone by reverse-phase high performance liquid chromatography. Both LP and HP contents in bone but not in cartilage were reduced to close to 50% in *Fbln4*$^{-/-}$ mice compared to wt mice (Table 1). The trifunctional collagen cross-links, LP and HP, result from the hydroxyallysine route in which a Lys residue in the telopeptide is hydroxylated. Therefore we compared the amounts of Hyl and the ratio of Hyl/Hyl + Lys, but found no significant difference between wt and *Fbln4*$^{-/-}$ mice (Table 2).

mRNA expression of LOX is not changed but the protein is significantly decreased in *Fbln4* knockout bones

In order to elucidate whether the reduced amounts of LP and HP cross-links in *Fbln4*$^{-/-}$ bones were due to reduced LOX expression, LOX mRNA in *Fbln4*$^{-/-}$ and wild type mice was analyzed by in situ hybridization and real-time PCR (Fig. 5). The in situ hybridization analysis showed that pattern and

Table 1. The content of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) crosslink compounds in cartilage and bone. Tissues were hydrolyzed with 6 N HCl and crosslink compounds were quantified by HPLC.

	Hyp/pro	HP/triple helix	LP/triple helix	HP/LP	(HP + LP)/triple helix
Cartilage					
Wt (n = 4)	0.414 ± 0.008	0.0462 ± 0.00462	0.0148 ± 0.0016	3.08 ± 0.060	0.061 ± 0.0062
Fbln4$^{-/-}$ (n = 5)	0.401 ± 0.020	0.0520 ± 0.00872	0.0198 ± 0.0042	2.68 ± 0.327	0.072 ± 0.0124
Bone					
Wt (n = 4)	0.527 ± 0.045	0.0215 ± 0.0860	0.0140 ± 0.0013	1.55 ± 0.166	0.035 ± 0.132
Fbln4$^{-/-}$ (n = 5)	0.521 ± 0.030	0.0117 ± 0.00213	0.00732 ± 0.0023	1.67 ± 0.343	0.019 ± 0.004

Table 2. Amounts of total hydroxylysine in cartilage and bone. Amounts of Hyl are shown as the number of residues per collagen triple helix.

	Hyp/pro	Hyl/triple helix	Hyl/Hyl + Lys
Cartilage			
Wt (n = 3)	0.45	76.8 ± 1.01	0.14
Fbln4$^{-/-}$ (n = 3)	0.42	82.8 ± 3.12	0.14
Bone			
Wt (n = 3)	0.48	47.0 ± 1.48	0.09
Fbln4$^{-/-}$ (n = 3)	0.48	45.0 ± 1.43	0.09

intensity of LOX expression were very similar in wt and fibulin-4 null mice (Fig. 5A). Also the mRNA levels of *Col1a1*, *Lox*, *Bmp1*, *fibronectin* (*Fn*) and *periostin* (*Postn*) in long bones as measured by qRT-PCR were not significantly different (Fig. 5B). However, Western blot analyses revealed that the protein levels of active LOX were significantly reduced in bone and calvaria extracts from *Fbln4*$^{-/-}$ mice (Fig. 6). Pro LOX could not be detected in those extracts.

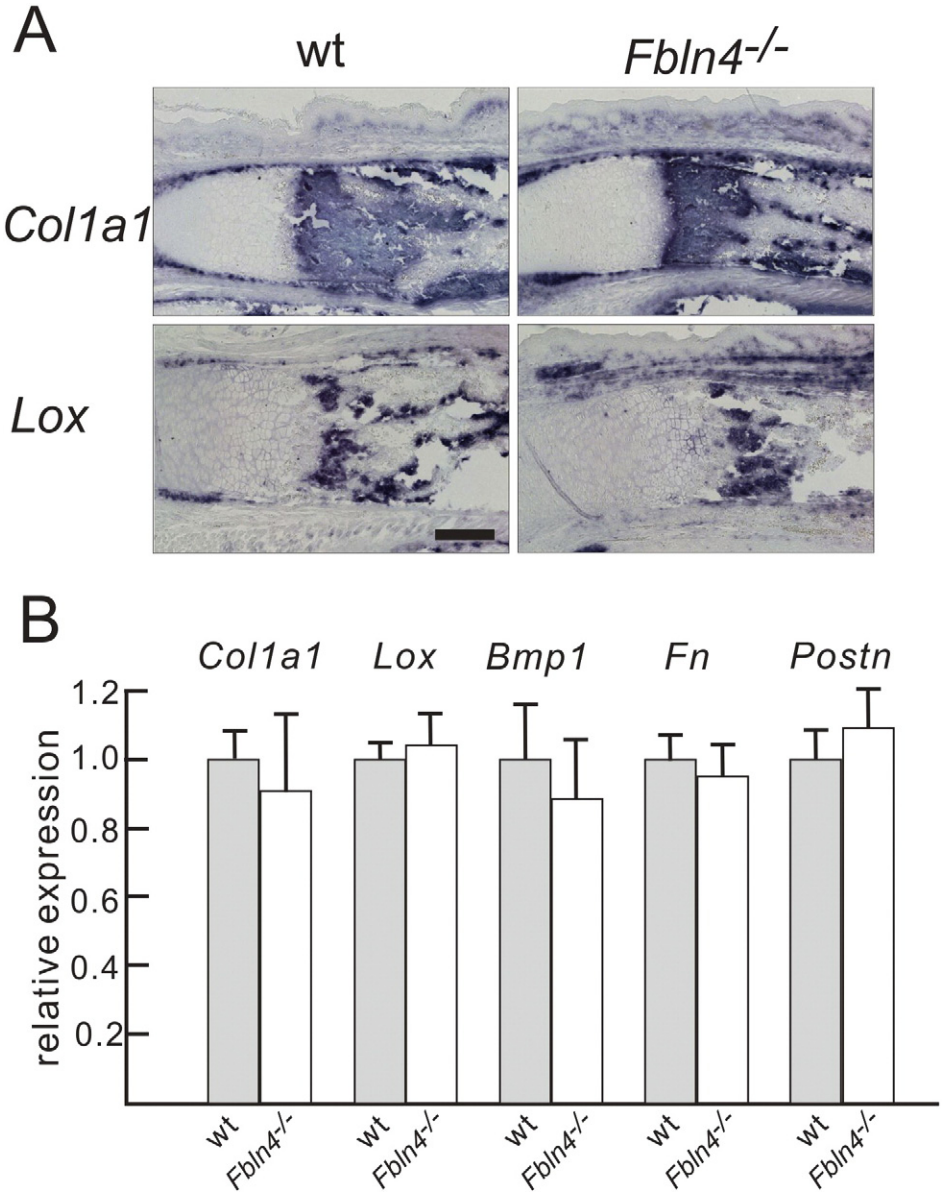


Fig. 5. mRNA expression of lysyl oxidase (LOX) is not changed in Fbln4 knockout bones. (A) mRNA expression of *Col1a1* and *Lox* in radius determined by in situ hybridization. Scale bar, 200 μm. (B) Total RNA was isolated from long bones and mRNA expression was determined by real-time PCR. Expression of BMP1, fibronectin (Fn) and periostin (Postn) which affect pro LOX activation are not different between wt and *Fbln4*$^{-/-}$ mice.

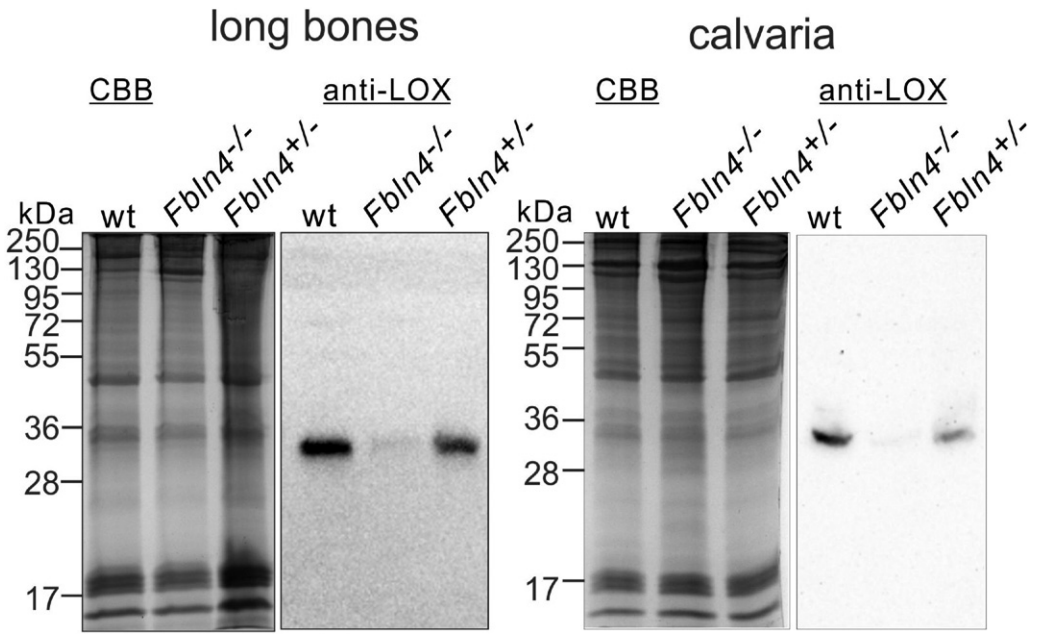


Fig. 6. Western blot analysis of LOX in bones. Whole proteins were extracted with SDS-PAGE sample buffer from long bones or calvaria of wt, *Fbln4*$^{-/-}$ and *Fbln4*$^{+/-}$ mice. Staining with Commassie brilliant blue confirms that the same amounts of proteins were loaded. Western blot analysis with anti-LOX antibody detects only the activated form of LOX which is nearly absent in the extracts from *Fbln4*$^{-/-}$ mice.

Activation of pro LOX is reduced in *Fbln4*$^{-/-}$ osteoblasts

In order to analyze whether fibulin-4 is involved in the activation and/or stability of LOX, the unprocessed pro LOX and processed form LOX were analyzed in cultures of primary calvarial osteoblasts from *Fbln4*$^{-/-}$ mice and wt littermates. Both LOX and pro LOX were detected in the conditioned media and cell lysates with an antibody prepared against the activated form of LOX (Fig. 7A), but less activated LOX was found in the media and cell lysates of *Fbln4*$^{-/-}$ osteoblasts. This antibody detected several more bands in the cell lysates but it is not clear whether those bands belong to LOX.

Addition of recombinant fibulin-4 to the medium of osteoblasts from *Fbln4*$^{-/-}$ mice enhanced processing and activation of LOX (Fig. 7B), confirming a role of fibulin-4 in LOX processing. However, fibulin-4 did not induce mRNA expression of LOX and BMP-1, which is the enzyme responsible for LOX activation (data not shown). Recombinant fibulin-4 used did not contain BMP-1 (Suppl. Fig. 2), excluding the possibility that the observed activation of LOX processing was due to contaminating BMP-1.

We also investigated the possibility that osteoblasts from *Fbln4*$^{-/-}$ mice might have a defect in their maturation, but Alizarin red staining of osteoblast cultures prepared from calvaria did not indicate a delay in differentiation and calcification in comparison to wt osteoblasts (Suppl. Fig. 3).

Discussion

Recent reports on patients with *FBLN4* mutations as well as studies on targeted disruption of the *Fbln4* gene in mice have provided ample evidence that fibulin-4 has a pivotal role in elastogenesis and stability of elastic tissues [20]. Moreover, the studies indicated that fibulin-4 is involved in the development and stability of the skeleton [15,17,18]. In order to elucidate the exact role of fibulin-4 in bone development and structure, we investigated the morphology and ultrastructure of developing bone of *Fbln4*$^{-/-}$ mice including changes in bone collagen structure in these animals. Our results did not show significant abnormalities in bone development except for the reported contracture of forelimbs, but an abnormal morphology of bone collagen fibrils in *Fbln4*$^{-/-}$ mice was seen with a substantial proportion of fibrils having diameters larger than 70 nm, which were never observed in wt or heterozygous bones. Furthermore, treatment with acetic acid extracted substantial amounts of soluble collagen monomeric α-chains from *Fbln4*$^{-/-}$ bones, but not from wild type bones, indicating impaired cross-linking. This notion was substantiated by three observations: 1) Our Western blot analyses revealed that the amount of processed LOX was strongly reduced in bones from *Fbln4*$^{-/-}$ mice. 2) Although levels of *Lox* mRNA were similar in *Fbln4*$^{-/-}$ and wild type bones, processing and activation of the pro LOX to the active form of LOX was impaired in cultured *Fbln4*$^{-/-}$ osteoblasts, while the addition of fibulin-4 restored the processing of LOX.

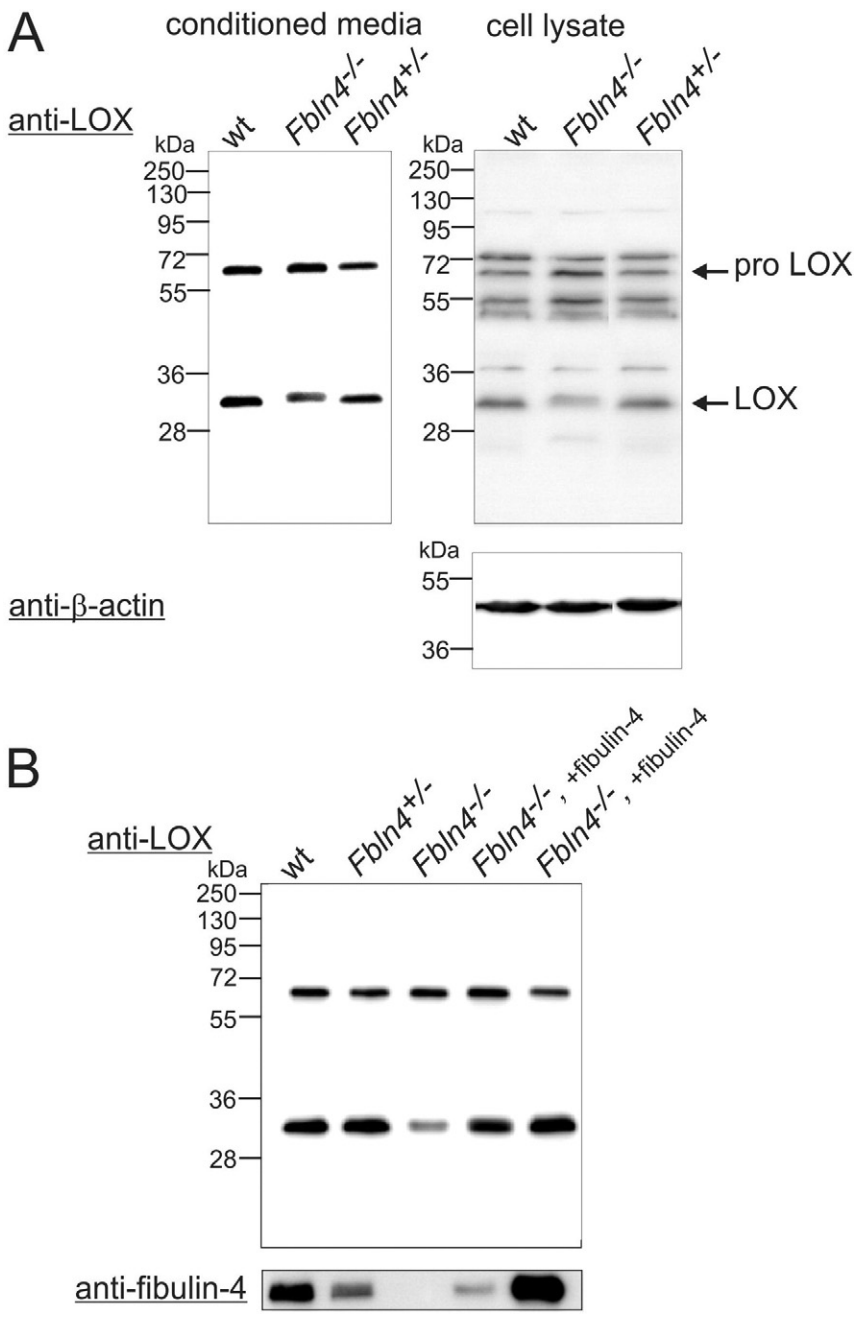


Fig. 7. Less activated LOX found in cultured calvarial osteoblasts isolated from *Fbln4*$^{-/-}$ mice. **(**A) Western blot with anti-LOX of conditioned media and cell lysates collected from 48 h cultures of calvarial osteoblasts from wt, *Fbln4*$^{+/-}$ and *Fbln4*$^{-/-}$ mice. In contrast to tissue extracts (Fig. 6), processing of pro-Lox to LOX is not complete in osteoblast cultures. The exact nature of the 4 LOX bands of about 55 kDa and 36 kDa in cell lysates is unclear yet. (B)The media of osteoblasts from wt, *Fbln4*$^{+/-}$ and *Fbln4*$^{-/-}$ mice were subjected to SDS-PAGE, and LOX (upper panel) and fibulin-4 (lower panel) were detected by specific antibodies. Addition of recombinant fibulin-4 to osteoblasts from *Fbln4*$^{-/-}$ mouse enhanced the activation of LOX.

3) HPLC analysis of heterocyclic collagen cross-link components revealed only half amount of HP and LP in *Fbln4*$^{-/-}$ bones as compared to wt bones.

These results indicate that fibulin-4 is involved in the regulation of collagen cross-linking by LOX. The importance of LOX-mediated cross-links in mechanical stability of tissues is well documented by the experimentally induced phenotype of osteolathyrism, caused by β-aminopropionitrile (BAPN), which is an irreversible inhibitor of LOX [21]. In fact,

previously it has been shown that fibulin-4 interacts with the LOX propeptide [22,23], and intriguingly, the phenotypes of *Fbln4* deficient mice are very similar to those of *Lox* deficient mice which present aortic aneurysms, cardiovascular dysfunction, and perinatal death, as well as abnormal integrity of elastic and collagen fibers [7–9].

Intermolecular cross-links are essential for the functions of collagen and elastic fibers, and LOX is the responsible enzyme which initiates the first step of cross-link formation. LOX activity is highest against nascent fibrils of native collagen and elastin, and low against the protein monomers in solution. Lysine ε-amino groups in elastin are oxidatively deaminated by LOX to the reactive aldehyde components allysine which subsequently condense to yield desmosine or isodesmosine [1]. Similarly, in collagen I, specific lysine and hydroxylysine (Hyl) residues in the N- and C-telopeptides are oxidatively deaminated to create allysine or hydroxyallysine, respectively, that initiate non-enzymatic condensation reactions to form covalent, Schiff-base type aldimine bonds with Lys or Hyl residues on juxtaposed adjacent collagen molecules [2,4,3]. The allysine route predominates in skin and the hydroxyallysine route in bone and cartilage [24,25]. The Schiff-base type aldimine crosslinks are acid-labile, while the mature aldol cross-links are stable to low pH. Therefore, the enhanced extraction of collagen α chains with acetic acid is most likely due to accumulation of acid-labile aldimine cross-links found in bones of fibulin-4 null mice, but this issue could not be finally settled due to perinatal lethality of fibulin-4 null mice and lack of sufficient bone tissue.

The dehydro-lysinonorleucine (deH-LNL) cross-links formed between lysine aldehyde (allysine) and lysine remain immature and are reducible with sodium borohydride, whereas dehydro-dihydroxy-lysinonorleucine (deH-HLNL) formed between hydroxyallysine and Lys, or between allysine and Hyl can mature to a non-reducible trivalent crosslink [2,3,26]. Also the amount of trifunctional cross-links, HP and LP, was decreased by about 50% in bones from *Fbln4*$^{-/-}$ mice as compared to wt bones. In newborn wt bones, the content of HP and LP was determined 0.0215 and 0.014 mol/mol collagen, respectively, which is about 10–20 times lower than that of adult bones, 0.427 and 0.051 for mouse femur [26], 0.35 and 0.09 for human bone [27], and 0.28 and 0.04 for bovine bone [28]. The decrease of active LOX found in tissues may also explain the reduction of desmosine level in tissues of fibulin-4 null mice [20]. The ratio of Hyl/Lys + Hyl (0.09) in *Fbln4*$^{-/-}$ bones was comparable with that of wt bones, indicating that the enhanced solubility of collagen in *Fbln4*$^{-/-}$ bones was not due to the changes in lysine hydroxylation.

Also fibronectin and periostin were shown to promote proteolytic activation of LOX, but this occurs through their interaction with BMP-1 [29,30]. BMP-1 also processes type I procollagen, chordin, and pro-biglycan, reactions which are enhanced by fibronectin. Fibulin-4 was shown to interact with the LOX propeptide [22,23] but not with BMP-1 (Sasaki, unpublished), and inactivation of *Fbln4* gene did not affect the expression of fibronectin and periostin mRNAs. Fibulin-4 is not involved in the processing of type I procollagen (Suppl. Fig.1). The exact mechanism of how fibulin-4 promotes the activation of LOX remains to be elucidated.

The question remained how the lack of fibulin-4 in bone is related to the high variability and striking increase in collagen fibril diameter up to two fold of wild type collagen fibrils, and whether there is a connection to the impaired LOX activity in *Fbln4*$^{-/-}$ mice. Important regulators of collagen I fibrillogenesis are collagen V as well as small leucine-rich proteoglycans (SLRPs) including fibromodulin, lumican, biglycan, decorin and others [31,32]. Ultrastructural analyses of bone collagen fibrils from biglycan deficient mice revealed that the average diameter and the range of fibril diameters were increased compared with wild type mice [33]. Interestingly, the analysis of tendon collagen I from fibromodulin-deficient mice revealed that fibromodulin also regulates collagen cross-links by modulating LOX action on the C-telopeptide lysines. The authors suggested that the individual SLRPs may have distinct roles regulating cross-linking during fibril growth and maturation [34]. Thus, there may be a possibility that fibulin-4 modulates collagen cross-linking through the interaction with SLRPs. Papke et al. [35] identified fibromodulin and biglycan as potential fibulin-4 binding candidates by proteomic screening. Biglycan and decorin have important role in collagen fibrillogenesis in bone, while fibromodulin is involved in this process in tendons which also exhibit anomaly in fibulin-4 null mice [48]. BMP-1 and related enzymes process probiglycan to a mature form. Removal of the N-propeptide of biglycan induces conformational changes which presumably affect the interactions with other molecules [36]. It remains to be elucidated whether biglycan is properly processed in fibulin-4 deficient mice, and whether fibulin-4 has a similar function as SLRPs on collagen fibrillogenesis.

Recently, a number of studies support the concept that LOX may be directly involved in the control of collagen fibrillogenesis. Pischon et al. reported that loss of LOX in mice resulted in a slight reduction of collagen fiber diameter in calvaria [37]. Furthermore, dispersed and shorter collagen fibers which did not form tight bundles as observed in wild type mice were found in *Lox*$^{-/-}$ mouse lung [9]. Finally, strong evidence for the notion that LOX activity is required for proper collagen fibrillogenesis by tenocytes in vitro was provided by Herchenhan et al. [38]. They

showed that inhibition of LOX by BAPN lead to structurally abnormal collagen fibrils with wider distribution of diameters and irregular shapes [38]. Collagen V, decorin, fibromodulin, and tenascin-X proteins were unaffected by the cross-link inhibition, suggesting that LOX regulates fibrillogenesis independently from these molecules.

In conclusion, we have shown that loss of fibulin-4 results in abnormality of collagen cross-links and collagen fiber size in developing bone. This phenotype is partially due to the reduction of LOX protein, which has been shown to be involved in the regulation of collagen fibrillogenesis. With these findings we provide further insight into the pathological mechanisms caused by FBLN4 mutations.

Materials and methods

Animals

Generation of *Fbln4* deficient mice is described elsewhere [48]. In brief, the 3' portion of exon 1 including the translational start site (ATG) and entire exon 2 were replaced by the gene for YFP (yellow fluorescent protein) and neomycin resistance genes. For the analysis of bone development and structure in this paper the $Fbln4^{-/-}$ mice were backcrossed into a C57BL/6 background.

Immunohistological staining

Cryosections of hindlimbs from newborn *Fbln4* null, heterozygote and wild type mice were fixed with cold acetone for 10 min and treated with hyaluronidase (1 mg/ml, Sigma H3506) at room temperature for 30 min before immunostaining. First antibodies used were guinea pig anti-mouse fibulin-4 (Markova et al., unpublished) and rabbit anti-mouse collagen I generated by immunizing with collagen I from mouse tails and both antibodies were affinity-purified. Immunization of rabbit and affinity-purification were carried out using established protocols [39]. The secondary antibodies were Alexa Fluor 488-conjugated goat anti-guinea pig IgG (Molecular Probes, Eugene, OR) and Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). Images were acquired on an Axiophot fluorescence microscope (Zeiss) using Axiovision software.

Paraffin sections were rehydrated and incubated with rabbit anti-collagen I (Abcam ab21286, 1:500) at 4 °C overnight. Bound antibody was detected using Link-Label IHC Detection system (Biogenex, San Ramon, CA) with biotinylated anti-rabbit Ig, avidin-coupled alkaline phosphatase, and Fast Red (Sigma-Aldrich) according to the manufacturer's instruction.

Histological staining

Whole mount staining of mouse skeletons with Alcian blue and Alizarin red were performed as described with a minor modification [40]. Tissues were fixed with 10% formalin overnight, and stained with 0.015% Alcian blue for 12 h. After washing with 95% ethanol tissues were treated with 2% KOH for several days, followed by staining with 0.5% alizarin red.

Limbs were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C overnight and processed for paraffin embedding. Sections were stained with Alcian blue, Masson-Trichrome and von Kossa staining.

Micro-computer tomography

MicroCT images of mouse femurs and humeruses were acquired on a laboratory cone-beam microCT scanner developed at the Institute of Medical Physics, University of Erlangen-Nuremberg, Germany, for ultra-high resolution imaging (ForBild scanner). It uses a m-Focus X-ray tube (Hamamatsu) and a 2D cooled CCD detector array (1024 × 1024 elements, 19 μm pitch; Photometrics, USA) with a dynamic range of 16 bit. The data were processed and analyzed in Amira (Mercury) and MagNan (BioCom) [41].

Extraction of collagen

Long bones from newborn mice were decalcified in 0.5 M EDTA (pH 7.4) at 4 °C for one week before sequential extractions. Decalcified bones were homogenized in PBS and stirred at 4 °C overnight and centrifuged at 16,000 × *g* for 30 min. Supernatants were removed and pellets were suspended in 0.5 M acetic acid and stirred at 4 °C overnight. Extracts (acid extract) were collected as supernatants after centrifugation at 16,000 × *g* for 30 min. The pellets were treated with pepsin (1 mg/ml) in 0.5 M acetic acid at 4 °C overnight and then centrifuged to separate the supernatant (pepsin extract). Both extracts were dialyzed against 0.7 M NaCl in 0.5 M acetic acid to precipitate collagen I. The resulting precipitates were dissolved in 0.5 M acetic acid and analyzed on SDS-PAGE. Collagens from calvaria were extracted with 0.5 M acetic acid.

Electron microscopy

Isolated Tibiae were fixed in 2.5% glutaraldehyde/2.5% paraformaldehyde/0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C overnight and then decalcified in 1.9% glutaraldehyde/0.15 M EDTA/0.06 M sodium cacodylate buffer (pH 7.4) at 4 °C for one week. Specimens were postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in graded alcohol concentrations, and embedded in epoxy resin

according to standard protocols. 1 µm semithin sections for orientation were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (LEO 906E; Carl Zeiss Microscopy, Oberkochen, Germany). Cross sectional areas of 200–400 collagen fibrils per image were measured using ImageJ software in 4–5 sections of each genotype and depicted in nm^2 (area) and recalculated in diameter (nm).

In situ hybridization

In situ hybridization was performed using antisense riboprobes for mouse *Lox* and *Col1a1* as described previously [42].

Calvarial osteoblasts

Calvarial osteoblasts were isolated according to the protocol by [43]. Cells were cultured in α-MEM containing 10% FBS. Differentiation to mature osteoblasts was induced by supplementing the medium with ascorbic acid (50 µg/ml) and 10 mM β-glycerophosphate. The medium was changed every 2 days until day 21. Mineralized matrix was stained with Alizarin red.

For analyses of LOX synthesis and maturation, osteoblasts were cultured in 6-well plate. At confluency, the cells were washed with PBS twice and the medium was replaced with serum-free medium containing ascorbic acid and cultured for another 48 h. Supernatants were harvested, and cells were lysed with RIPA buffer. The conditioned media and cell lysates were analyzed by Western blotting. For some of the experiments, total RNA was isolated from the cells using RNeasy Plus Mini kit (Qiagen) and analyzed for Lox RNA by real-time PCR.

Western blot

Long bones and calvaria were homogenized in liquid nitrogen and extracted in Laemmli's sample buffer containing 10 mM dithiothreitol at 95 °C for 5 min. Samples were separated on 10% SDS-PAGE and the blotted onto Immobilon-P membranes (Millipore). Blots were incubated with rabbit anti-LOX (Sigma L4669), rabbit anti-mouse fibulin-4 [44] and anti-C-telopeptide of α1 chain of collagen I (LF68, kindly provided by Dr. Fisher) followed by HRP-conjugated goat anti-rabbit IgG (BioRad). Bound antibodies were detected with ECL Plus Western blotting substrate (Pierce).

Determination of LP and HP, amino acid analysis and N-terminal sequencing

After freeze-drying and weighing, samples were hydrolyzed with 6 M HCl at 110 °C for 20 h. The amount of hydroxyproline and proline was determined by reverse-phase high performance liquid chromatography (HPLC) of 9-fluorenylmethyl chloroformate (Fluka, Buchs, Switzerland)-derivatized amino acids, as described by Bank et al. [45]. Collagen content was calculated assuming 300 residues hydroxyproline per triple helix and a molecular weight of 300,000 g/mol. For the determination of the collagen cross-links lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP), acid-hydrolyzed samples were diluted to 50% acetic acid and injected on an HPLC system equipped with online sample purification on CC31 cellulose using a Prospekt solid-phase extractor (Separations, Jasco Benelux BV, IJsselstein, The Netherlands). The retained cross-links were eluted from the CC31 material and online chromatographed on a cation exchange column (Whatman Partisil SCX; Fisher Scientific, Waltham, MA). Eluting cross-links were detected by a Jasco fluorometer (model FP-920; Separations). The PYD/DPD HPLC calibrator (Metra, Palo Alto, CA) was used as standard. Values are expressed as total amount of residues per collagen molecule.

For amino acid analysis the samples were loaded onto a column packed with Hitachi Custom Ion Exchange Resin equipped on an L-8900 amino acid analyzer (Hitachi, Japan) after hydrolysis with 6 M HCl at 110 °C for 24 h. N-terminal sequences were determined after transfer to Immobilon PSQ membrane (Millipore) on a Procise Protein Sequencing system (Applied Biosystems) following the manufacturer's instructions.

Real-time PCR

RNAs were isolated using RNAeasy plus mini kit (QIAGEN). Reverse transcription was performed with random hexamers (Roche) using 0.5 µg total RNA and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. Real-time PCR was performed on a CFX96 C1000 Thermal Cycler (BioRad) using HOT FIREPol EvaGreen qPCR Mix Plus (Medibena, Vienna, Austria) and the primers listed in supplemental table.

Miscellaneous materials

Mouse perlecan domain V fragment and BMP-1 were prepared as described [46,47], respectively.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.matbio.2015.12.002.

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Abbreviations used:
cbEGF, calcium-binding epidermal growth factor; FC, fibulin-type C-terminal; LOX, lysyl oxidase; wt, wild type; LP, lysylpridinoline/deoxypyridinoline; HP, hydroxylysylpridinoline/prydinoline; Lys, lysine; Hyl, hydroxylysine; BAPN, β-aminopropionitrile.

References

[1] H.A. Lucero, H.M. Kagan, Lysyl oxidase: an oxidative enzyme and effector of cell function, Cell. Mol. Life Sci. 63 (2006) 2304–2316.
[2] M. Yamauchi, M. Sricholpech, Lysine post-translational modifications of collagen, Essays Biochem. 52 (2012) 113–133.
[3] M. Saito, K. Marumo, Effects of collagen crosslinking on bone material properties in health and disease, Calcif. Tissue Int. 97 (2015) 242–261.
[4] D.R. Eyre, M.A. Weis, Bone collagen: new clues to its mineralization mechanism from recessive osteogenesis imperfecta, Calcif. Tissue Int. 93 (2013) 338–347.
[5] J. Molnar, K.S. Fong, Q.P. He, K. Hayashi, Y. Kim, S.F. Fong, et al., Structural and functional diversity of lysyl oxidase and the LOX-like proteins, Biochim. Biophys. Acta 2003 (1647) 220–224.
[6] M.I. Uzel, I.C. Scott, H. Babakhanlou-Chase, A.H. Palamakumbura, W.N. Pappano, H.H. Hong, et al., Multiple bone morphogenetic protein 1-related mammalian metalloproteinases process pro-lysyl oxidase at the correct physiological site and control lysyl oxidase activation in mouse embryo fibroblast cultures, J. Biol. Chem. 276 (2001) 22537–22543.
[7] J.M. Maki, J. Rasanen, H. Tikkanen, R. Sormunen, K. Makikallio, K.I. Kivirikko, et al., Inactivation of the lysyl oxidase gene Lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice, Circulation 106 (2002) 2503–2509.
[8] I.K. Hornstra, S. Birge, B. Starcher, A.J. Bailey, R.P. Mecham, S.D. Shapiro, Lysyl oxidase is required for vascular and diaphragmatic development in mice, J. Biol. Chem. 278 (2003) 14387–14393.
[9] J.M. Maki, R. Sormunen, S. Lippo, R. Kaarteenaho-Wiik, R. Soininen, J. Myllyharju, 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 (2005) 927–936.
[10] X. Liu, Y. Zhao, J. Gao, B. Pawlyk, B. Starcher, J.A. Spencer, et al., Elastic fiber homeostasis requires lysyl oxidase-like 1 protein, Nat. Genet. 36 (2004) 178–182.
[11] W.S. Argraves, L.M. Greene, M.A. Cooley, W.M. Gallagher, Fibulins: physiological and disease perspectives, EMBO Rep. 4 (2003) 1127–1131.
[12] R. Timpl, T. Sasaki, G. Kostka, M.L. Chu, Fibulins: a versatile family of extracellular matrix proteins, Nat. Rev. Mol. Cell Biol. 4 (2003) 479–489.
[13] S. de Vega, T. Iwamoto, Y. Yamada, Fibulins: multiple roles in matrix structures and tissue functions, Cell. Mol. Life Sci. 66 (2009) 1890–1902.
[14] R. Giltay, R. Timpl, G. Kostka, Sequence, recombinant expression and tissue localization of two novel extracellular matrix proteins, fibulin-3 and fibulin-4, Matrix Biol. 18 (1999) 469–480.
[15] V. Hucthagowder, N. Sausgruber, K.H. Kim, B. Angle, L.Y. Marmorstein, Z. Urban, Fibulin-4: a novel gene for an autosomal recessive cutis laxa syndrome, Am. J. Hum. Genet. 78 (2006) 1075–1080.
[16] M. Dasouki, D. Markova, R. Garola, T. Sasaki, N.L. Charbonneau, L.Y. Sakai, et al., 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 (2007) 2635–2641.
[17] J. Hoyer, C. Kraus, G. Hammersen, J.P. Geppert, A. Rauch, Lethal cutis laxa with contractural arachnodactyly, overgrowth and soft tissue bleeding due to a novel homozygous fibulin-4 gene mutation, Clin. Genet. 76 (2009) 276–281.
[18] L.K. Erickson, J.M. Opitz, H. Zhou, 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 (2012) 137–141.
[19] M. Renard, T. Holm, R. Veith, B.L. Callewaert, L.C. Ades, O. Baspinar, et al., 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 (2010) 895–901.
[20] P.J. McLaughlin, Q. Chen, M. Horiguchi, B.C. Starcher, J.B. Stanton, T.J. Broekelmann, et al., Targeted disruption of fibulin-4 abolishes elastogenesis and causes perinatal lethality in mice, Mol. Cell. Biol. 26 (2006) 1700–1709.
[21] C.I. Levene, J. Gross, Alterations in state of molecular aggregation of collagen induced in chick embryos by beta-aminopropionitrile (lathyrus factor), J. Exp. Med. 110 (1959) 771–790.
[22] R. Choudhury, A. McGovern, C. Ridley, S.A. Cain, A. Baldwin, M.C. Wang, et al., Differential regulation of elastic fiber formation by fibulin-4 and -5, J. Biol. Chem. 284 (2009) 24553–24567.
[23] M. Horiguchi, T. Inoue, T. Ohbayashi, M. Hirai, K. Noda, L.Y. Marmorstein, et al., Fibulin-4 conducts proper elastogenesis

via interaction with cross-linking enzyme lysyl oxidase, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 19029–19034.
[24] G. Mechanic, P.M. Gallop, M.L. Tanzer, The nature of crosslinking in collagens from mineralized tissues, Biochem. Biophys. Res. Commun. 45 (1971) 644–653.
[25] N.R. Davis, A.J. Bailey, Chemical synthesis of the reduced form of an intermolecular crosslink of collagen: a re-evaluation of the structure of syndesine, Biochem. Biophys. Res. Commun. 45 (1971) 1416–1422.
[26] K. Takaluoma, M. Hyry, J. Lantto, R. Sormunen, R.A. Bank, K.I. Kivirikko, et al., Tissue-specific changes in the hydroxylysine content and cross-links of collagens and alterations in fibril morphology in lysyl hydroxylase 1 knock-out mice, J. Biol. Chem. 282 (2007) 6588–6596.
[27] R.A. Bank, S.P. Robins, C. Wijmenga, L.J. Breslau-Siderius, A.F. Bardoel, H.A. van der Sluijs, et al., Defective collagen crosslinking in bone, but not in ligament or cartilage, in Bruck syndrome: indications for a bone-specific telopeptide lysyl hydroxylase on chromosome 17, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 1054–1058.
[28] M. Terajima, I. Perdivara, M. Sricholpech, Y. Deguchi, N. Pleshko, K.B. Tomer, et al., Glycosylation and cross-linking in bone type I collagen, J. Biol. Chem. 289 (2014) 22636–22647.
[29] G. Huang, Y. Zhang, B. Kim, G. Ge, D.S. Annis, D.F. Mosher, et al., Fibronectin binds and enhances the activity of bone morphogenetic protein 1, J. Biol. Chem. 284 (2009) 25879–25888.
[30] T. Maruhashi, I. Kii, M. Saito, A. Kudo, Interaction between periostin and BMP-1 promotes proteolytic activation of lysyl oxidase, J. Biol. Chem. 285 (2010) 13294–13303.
[31] L. Ameye, M.F. Young, Mice deficient in small leucine-rich proteoglycans: novel in vivo models for osteoporosis, osteoarthritis, Ehlers-Danlos syndrome, muscular dystrophy, and corneal diseases, Glycobiology 12 (2002) 107R–116R.
[32] S. Kalamajski, A. Oldberg, The role of small leucine-rich proteoglycans in collagen fibrillogenesis, Matrix Biol. 29 (2010) 248–253.
[33] A. Corsi, T. Xu, X.D. Chen, A. Boyde, J. Liang, M. Mankani, et al., Phenotypic effects of biglycan deficiency are linked to collagen fibril abnormalities, are synergized by decorin deficiency, and mimic Ehlers-Danlos-like changes in bone and other connective tissues, J. Bone Miner. Res. 17 (2002) 1180–1189.
[34] S. Kalamajski, C. Liu, V. Tillgren, K. Rubin, A. Oldberg, J. Rai, et al., Increased C-telopeptide cross-linking of tendon type I collagen in fibromodulin-deficient mice, J. Biol. Chem. 289 (2014) 18873–18879.
[35] C.L. Papke, J. Tsunezumi, L.J. Ringuette, H. Nagaoka, M. Terajima, Y. Yamashiro, et al., Loss of fibulin-4 disrupts collagen synthesis and maturation: implications for pathology resulting from EFEMP2 mutations, Hum. Mol. Genet. (2015).
[36] I.C. Scott, Y. Imamura, W.N. Pappano, J.M. Troedel, A.D. Recklies, P.J. Roughley, et al., Bone morphogenetic protein-1 processes probiglycan, J. Biol. Chem. 275 (2000) 30504–30511.
[37] N. Pischon, J.M. Maki, P. Weisshaupt, N. Heng, A.H. Palamakumbura, P. N'Guessan, et al., Lysyl oxidase (lox) gene deficiency affects osteoblastic phenotype, Calcif. Tissue Int. 85 (2009) 119–126.
[38] A. Herchenhan, F. Uhlenbrock, P. Eliasson, M. Weis, D. Eyre, K.E. Kadler, et al., Lysyl oxidase activity is required for ordered collagen fibrillogenesis by tendon cells, J. Biol. Chem. 290 (2015) 16440–16450.
[39] R. Timpl, Antibodies to Collagens and Procollagens, Methods Enzymol. 82 Pt A (1982) 472–498.
[40] B.B.R. Hogan, F. Constantini, E. Lacy, Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 2014.
[41] T. Hattori, C. Muller, S. Gebhard, E. Bauer, F. Pausch, B. Schlund, et al., SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification, Development 137 (2010) 901–911.
[42] N. Eitzinger, C. Surmann-Schmitt, M. Bosl, G. Schett, K. Engelke, A. Hess, et al., Ucma is not necessary for normal development of the mouse skeleton, Bone 50 (2012) 670–680.
[43] P. Ducy, M. Starbuck, M. Priemel, J. Shen, G. Pinero, V. Geoffroy, et al., A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development, Genes Dev. 13 (1999) 1025–1036.
[44] N. Kobayashi, G. Kostka, J.H. Garbe, D.R. Keene, H.P. Bachinger, F.G. Hanisch, et al., A comparative analysis of the fibulin protein family. Biochemical characterization, binding interactions, and tissue localization, J. Biol. Chem. 282 (2007) 11805–11816.
[45] R.A. Bank, B. Beekman, N. Verzijl, J.A. de Roos, A.N. Sakkee, J.M. TeKoppele, Sensitive fluorimetric quantitation of pyridinium and pentosidine crosslinks in biological samples in a single high-performance liquid chromatographic run, J. Chromatogr. B Biomed. Sci. Appl. 703 (1997) 37–44.
[46] J.C. Brown, T. Sasaki, W. Gohring, Y. Yamada, R. Timpl, The C-terminal domain V of perlecan promotes beta1 integrin-mediated cell adhesion, binds heparin, nidogen and fibulin-2 and can be modified by glycosaminoglycans, Eur. J. Biochem. 250 (1997) 39–46.
[47] T. Sasaki, W. Gohring, K. Mann, C. Brakebusch, Y. Yamada, R. Fassler, et al., Short arm region of laminin-5 gamma2 chain: structure, mechanism of processing and binding to heparin and proteins, J. Mol. Biol. 314 (2001) 751–763.
[48] D.Z. Markova, T.-C. Pan, R.-Z. Zhang, G. Zhang, T. Sasaki, M. Arita, D.E. Birk and M.-L. Chu, Forelimb contractures and abnormal tendon collagen fibrillogenesis in fibulin-4 null mice, Cell Tissue Res., in press.