The Critical Role of the TB5 domain of Fibrillin-1 in Endochondral Ossification

Author Information

**Abstract**

Mutations in the Fibrillin-1 (*FBN1*) gene are responsible for the autosomal dominant form of geleophysic dysplasia (GD), which is characterized by short stature and extremities, thick skin, and cardiovascular disease. All known *FBN1* mutations in GD patients are localized within the region encoding the TB5 (TGFβ binding protein-like) domain of this protein. Conversely, other mutations in this gene are mainly known to be associated with Marfan syndrome (MFS), which often results in tall stature, suggesting that FBN1 plays a complex role in the regulation of bone growth. Herein, we generated a knock-in mouse model, Fbn1TB5+/- by introducing the p.Tyr1696Cys mutation from a GD patient into the TB5 domain of murine *Fbn1* to elucidate the specific role of this domain in endochondral ossification. We found that both Fbn1TB5+/- and Fbn1TB5-/- mice exhibited a reduced stature reminiscent of the human GD phenotype. In contrast to murine MFS models harboring *Fbn1* mutations, our novel mouse model does not present with aortic disease. The *Fbn1* point mutation introduced in these mice affected the growth plate formation owing to abnormal chondrocyte differentiation such that mutant chondrocytes failed to establish a dense network composed of fibrillin-1 fibrils. Interestingly, the TGFβ signaling pathway was not impaired in these cells, supporting that TGFβ signaling may not be a direct pathogenic driver of GD. This original Fbn1 mutant mouse model offers new insight into the pathogenic events underlying GD. Our findings suggest that the underlying etiology of GD involves the dysregulation of fibrillin microfibril deposition, potentially due to improper interactions between the FBN1 TB5 domain and heparan sulfate residues. In GD, a protein complex composed of ADAMTSL2 and FBN1 (TB5) is essential for long bone growth.

**Keywords**: fibrillin-1, mutations, geleophysic dysplasia, growth plate, extracellular matrix

**Introduction**

The acromelic dysplasia group consists of four skeletal disorders characterized by short stature, including geleophysic dysplasia (GD, OMIM#213050), acromicric dysplasia (AD, OMIM#102370) Weill Marchesani syndrome (WMS, OMIM#608328), and Myhre syndrome (MS, OMIM#139210) [1,2]. GD is further associated with short extremities, joint stiffness, facial dysmorphism, thick skin, and cardiac defects [3]. We have previously evaluated the molecular basis of GD, revealing mutations in *ADAMTSL2* to drive the autosomal recessive form of this disease, whereas mutations in *FBN1* are responsible for autosomal dominant GD [3]. We further demonstrated that ADAMTSL2 and FBN1 are both genetically and biochemically linked [4]. The generation of an *Adamtsl2-*/- mouse model led us to highlight the impairment of chondrogenesis and microfibrillar network establishment that occur in the context of *Adamtsl2* deficiency, suggesting a role for Adamtsl2 in the maintenance of the growth plate extracellular matrix (ECM) owing to its ability to regulate the microfibrillar network [5]. Intriguingly, GD and other diseases of the acromelic dysplasia group have been described as “mirror images” of the best-known fibrillinopathy, Marfan syndrome (MFS, OMIM#154700).

MFS is a rare connective tissue disorder with an autosomal dominant mode of transmission. It is a multisystem disease with a characteristic unique combination of skeletal, cardiovascular, and ocular features including long bone overgrowth, aortic dilatation and dissection, and ectopia lentis [6]. Many *FBN1* mutations spread throughout the gene have been identified in MFS patients, all resulting in the same pathogenetic phenotype [7]. Interestingly, in GD patients, all identified *FBN1* mutations are restricted to the TGFβ binding protein-like domain 5 (TB5)-coding region of the gene [4]. This TB5 domain is not the only TB domain in FBN1 to have been linked to a specific fibrillinopathy. Indeed, TB4, which harbors the only integrin-binding RGD motif in this protein, has been linked to the development of Stiff Skin Syndrome (SSKS, OMIM#184900), which is characterized by joint contractures and thick, hard skin [8].

 The *FBN1* gene encodes fibrillin-1, which is a large and ubiquitous glycoprotein within the ECM and the major component of microfibrillar networks [9]. The FBN1 protein is primarily composed of multiple repeating calcium-binding (cb)-epidermal growth factor-like (EGF) domains and TB domains, which contain eight cysteine residues essential for disulfide bond formation within the FBN1 molecule. Functionally, FBN1 confers structural support to tissues and serves as essential scaffolding for elastin deposition. Fibrillin microfibrils are also a niche for growth factors [10]. As such, FBN1 microfibrils have been implicated in the bioavailability of TGF-β and the sequestration of bone morphogenetic proteins (BMPs). Moreover, FBN1 can interact with several ECM proteins including ADAMTS, integrins, fibronectin, and LTBPs [11], with the structures of LTBPs 1-4 being closely related to those of fibrillins [12].

At present, it remains unclear as to how mutations in the TB5 domain of *FBN1* result in short stature rather than the tall stature phenotype often associated with mutations in other domains of FBN1. Moreover, the skeletal phenotype observed in GD patients suggests that the TB5 domain of this protein may play a role in the process of endochondral ossification. To test this hypothesis, we herein generated a *Fbn1TB5+/-* knock-in (KI) mouse model that was used to explore the role of FBN1 in the pathophysiology of GD pathophysiology and the importance of the FBN1 TB5 domain in the context of skeletal development.

**Results**

**Generation of a new *Fbn1* Knock-In mouse model with skeletal abnormalities**

To investigate the role of FBN1 and its TB5 domain in short stature, we generated a *Fbn1TB5* KI mouse model by introducing the GD-associated human p.Tyr1696Cys (mouse p.Tyr1698Cys) mutation into this gene. This mutation in exon 42 is a single base substitution (A>G) resulting in a change in the amino acid sequence of the resultant protein (Tyr>Cys), as described in the Methods section and Figure 1a. This substitution was confirmed in all mice via genomic DNA sequencing (Fig. 1b). A quantitative polymerase chain reaction (qPCR) analysis of femoral head RNA samples from these mice indicated that this TB5 mutation did not impact *Fbn1* gene expression (Fig.1c).

The litters resulting from the breeding of *Fbn1TB5+/-* mice followed a normal Mendelian distribution. Heterozygous *Fbn1TB5+/-* (HT) mice were viable and fertile. Both HT and homozygous (Ho) mice exhibited a skeletal phenotype. Given that short stature, short long bones, and brachydactyly are the primary skeletal features of GD, total naso-anal and long bone length values were measured for these mice, revealing that the stature of both HT and Ho Fbn1 KI mice was reduced relative to corresponding control animals at P1 (HT: 3.2%; Ho: 4.1% p<0.05) (Fig. 1d). These differences in stature between control and mutant grew with age, reaching 8.9% in Ho animals and 4.2% in HT animals at P30 (Fig 1i). Consistently, femur length was also reduced significantly in Ho mice at P1 compared with control animals (-6%, p<0.05), and the same was true of tibia length (Fig. 1e and f). X-ray analyses conducted at P30 also confirmed the presence of skeletal abnormalities in these TB5 mutant mice, including short stature and shortened long bones (Fig.1g and h). No differences in Alcian blue or Alizarin red staining were observed in extremities of these animals when comparing controls and mutants at P1 or P30 (data not shown).

***Fbn1* KI mice do not exhibit thick skin or aortic disease**

 An *Fbn1* mouse model, WMΔ, that replicates phenotypes associated with Weill Marchesani syndrome including thick skin and shorter long bones has previously been reported [21]. As such, we evaluated skin phenotypes to confirm the specificity of our mouse model. Macroscopic inspection of the skin of our mutant and WT mice revealed no differences between these groups with respect to skin thickness. Histological evaluation of skin biopsies following Masson’s Trichrome staining revealed normal collagen deposition in the dermis of HT mice but excessive collagen in Ho mice as compared to controls (Fig. 2a), with quantification of these results further confirming the increased accumulation of collagen in the skin of Ho mice (Fig. 2b). No collagen upregulation was detected via qPCR in skin samples from Ho mice (Fig. 2c), nor were changes in *Fbn1* gene expression observed in these samples, suggesting that the introduced TB5 mutation does not affect the expression of this gene. To test whether this point mutation in TB5 causes GD rather than MFS, we analyzed aortic root morphology, which is altered in MFS. Both HT and Ho mice exhibited a normal aortic wall without any elastic fiber fragmentation (Fig. 2d). In addition, no evidence of aortic disease typical of MFS such as thoracic aortic dilation was observed in mutant mice. Taken together, these results confirmed the specificity of our mouse model as one that replicates many of the phenotypes associated with GD but not MFS. As such, our *Fbn1* mutantmouse model is distinguished by a skeletal phenotype and increased collagen deposition in the skin of Ho animals.

**Fbn1 is involved in the regulation of growth plate cytoarchitecture**

Given that our HT and Ho mutant mice presented with a shorter stature as well as shorter long bones, we next analyzed these animals to investigate the femoral growth plate, which is a cartilaginous tissue that plays critical roles in bone elongation. In the femoral head of WT animals, chondrocytes proliferated and exhibited a discoidal shape, and were aligned in a columnar conformation (Fig. 3a). Chondrocytes that had reached the hypertrophic stage of maturation were identified by their enlarged volume, spherical shape, and decreased surrounding matrix (Fig. 3a, black box). While the chondrocyte zones within growth plates of WT and mutant femurs at P1 were clearly defined, the organization and shape of the hypertrophic chondrocytes were altered in mutant mice in comparison to controls, with the size of the hypertrophic chondrocyte surface area being decreased in both *Fbn1TB5* mutant mice (Fig. 3b). Moreover, the Ho mice exhibited impaired hypertrophic chondrocyte columnar organization (Fig. 3a, lower panel), with cartilage exhibiting more severe disorganization than that observed in HT mice. At P1, the hypertrophic zone length was reduced in HT and Ho mice (Fig 3c). At P30, global reductions in growth plate length were also observed in both types of Fbn1TB5 mutant mice (Fig. 3d and e). These results led us to conclude that growth plate defects may be responsible for the skeletal abnormalities observed in both Ho and HT *Fbn1TB5*mutant animals.

***Fbn1* KI mice exhibit alterations in key chondrocyte markers**

To assess whether the changes observed in mutant growth plates are associated with temporal and/or spatial differences in the expression patterns of key growth plate marker genes, mRNA *in situ* hybridization and immunohistochemistry were performed on serial femur sections to detect markers of hypertrophic chondrocytes [type X collagen (*Col10*)] and proliferative chondrocytes [type II collagen (*Col2*) and SRY box 9 (Sox9)]. Wild-type femurs exhibited robust *Col10a1* expression restricted to hypertrophic chondrocytes (left panel). However, the level of *Col10a1* expression in HT and Ho mutants was drastically decreased near the chondro-osseous junction, especially in Ho mice (Fig. 3 bis a and b). These *in situ* hybridization results were confirmed using a specific Col10 antibody (Fig. 3 bis b). The quantification of Col10 mean fluorescence intensity revealed a significant decrease in such expression in Ho mice (Fig 3bis b). The expression pattern of *Col2a1*, a common cartilage marker expressed by all committed chondrocytes, was not significantly altered by the TB5 mutation, despite the observed variations in chondrocyte morphology (data not shown). Sox9, a critical transcription factor that controls chondrocyte differentiation, is expressed concomitantly with Col2a1 during cartilage development. Sox9 in the wild-type growth plate was detected throughout the resting zone and followed by an abrupt decline in its expression in the hypertrophic zone (Fig. 3 bis c). Conversely, mutant growth plates exhibited an increase in Sox9 expression in the prehypertrophic zone (Fig. 3 bis c). Ki-67-based analyses of proliferative activity indicated that chondrocyte proliferation rates were unaffected at P1 in the Fbn1-deficient growth plates as compared to controls (Fig. 3bis d and e).

**Mutations in the TB5 domain of fibrillin-1 impair microfibrillar network assembly**

As Fbn1 is an extracellular matrix protein and the major component within microfibrillar networks, we analyzed the impact of TB5 disruption on the structure of the ECM in the growth plate. Fibronectin was also studied given that it plays a role in regulating microfibril assembly and was affected by the absence of Adamtsl2 in a mouse model of GD [5]. Therefore, we evaluated microfibrillar network formation in cultures of primary chondrocytes isolated from the ribs of *Fbn1TB5* mutant and WT mice. Visualization of fibril networks via confocal microscopy indicated that fibronectin networks in *Fbn1TB5+/-* and *Fbn1TB5-/-*cultures were as dense and organized as in WT cultures (Fig. 4d). In contrast, fibrillin-1 fibrils were much thicker and present within a less dense and organized microfibrillar network in *Fbn1TB5+/-* and *Fbn1TB5-/-*cultures as compared to WT cultures (Fig. 4e-g). Based on this result, we investigated Fbn1 expression in the control and mutant growth plates. However, no differences in Fbn1 protein or mRNA levels were observed following TB5 mutation (Fig. 4a-c). Taken together, these results indicated that the Fbn1 TB5 of impaired fibril network assembly without affecting *Fbn1* expression levels.

**The impact of TB5 mutation on TGFβ signaling**

Given the link between TGFβ and Fbn1, we next investigated whether this mutation in the TB5 domain of Fbn1 could impair TGFβ signaling in primary chondrocytes. Immunohistochemical analysis revealed comparable levels of TGFβ expression in mutant and WT growth plates, and this was confirmed via an ELISA used to detect TGFβ-1 present in chondrocyte growth media (Fig. 5a and b). Western blotting analyses of the activation of the TGFβ signaling pathway revealed no differences in phosphorylated SMAD2 levels in mutant chondrocytes as compared to controls (Fig. 5c). Finally, mRNA levels of downstream TGFβ-1 target genes including *Ctgf*  (Connective tissue growth factor) and *Pai1* (Plasminogen activator inhibitor 1) were evaluated by real-time quantitative PCR. Their expression levels were not altered in mutant mice (Fig. 5d), confirming that this TB5 mutation had no effect on TGFβ activity in growth plates.

**Discussion**

In the present study, we reported for the first time the crucial role of Fbn1 in endochondral ossification. The skeletal anomalies observed in *Fbn1TB5+/-*and *Fbn1TB5-/-* mice highlight the importance of Fbn1 as a positive regulator of chondrogenesis. Heterozygous *Fbn1TB5+/-* micerecapitulated some characteristics of GD such as short stature and short long bones, whereas the homozygous *Fbn1TB5-/-* mice exhibited a more severe phenotype. More importantly, this novel mouse model does not exhibit the aortic disease observed in MFS mouse models harboring *Fbn1* mutations. Overall, this report offers further insight regarding the involvement of the TB5 domain of Fbn1 in growth plate abnormalities.

Fbn1 has previously been reported to be linked to stature-related phenotypes in MFS [13,14]. Multiple mouse models have been generated to decipher the pathophysiological mechanisms underlying MFS, including the hypomorphic Fbn1mgR/mgR model and the *Fbn1C1039G/+* model with a cbEGF domain substitution [15,16]. However, these prior studies focused on the role of Fbn1 in MFS-related aortic phenotypes, and its role in skeletal phenotypes has was not been fully explored.

By controlling TGFβ and BMP signaling, FBN1 is involved in bone remodeling. A study of bone phenotypes in the *Fbn1mgR/mgR* MFS mouse model revealed a decrease in bone mineral density and trabecular bone anomalies that mimicked skeletal manifestations observed in MFS patients [17]. FBN1 can thus play a negative role in osteoclastogenesis by sequestering RANKL, an osteoclastogenic factor, in the ECM[18]. Osteopenia was observed in *Fbn1Prx1-/-*and *Fbn1Osx-/-* mice, putatively due to premature osteoprogenitor differentiationsuggesting that FBN1 modulates the osteoblast/osteoclast balance [19]. The fate of mesenchymal stem cells depends upon the partition of adult bone marrow tissue into functionally distinct microenvironments called niches [20]. FBN1 can shape the fate of skeletal stem cells by modulating the bioavailability of TGF-β in these bone marrow niches. Our identification of mutations specifically within the TB5 domain of *FBN1* in GD patients with short stature also suggests a possible link between FBN1 and endochondral ossification. The skeletal anomalies exhibited by our novel *Fbn1TB5-* mouse model conclusively demonstrate that the introduced TB5 domain mutation contributes to the incidence of short stature. Reductions in long bone growth mimicking human WMS have previously been observed in WMS model mice harboring an *Fbn1* deletion, although this phenotype was transient [21]. Here, we show that Fbn1 is a key factor involved in the differentiation of chondrocytes owing to its ability to impact *Col10*, a hypertrophic chondrocyte marker that was downregulated in both *Fbn1TB5+/-*and *Fbn1TB5-/-* mice. As a result, disruption of the TB5 domain in Fbn1 profoundly altered the normal differentiation of chondrocytes. The observed decreases in the size of chondrocytes in the growth plates of mutant mice may explain or exemplify such abnormal differentiation. Sox9 also exhibits a distinct expression profile in mutant hypertrophic chondrocytes. Sox9 negatively regulates Col1014, so we posit that mutations in the TB5 domain are conducive to the upregulation of Sox9, in turn leading to the downregulation of Col10. Indeed, the ectopic expression of Sox9 in a transgenic mouse model has been reported to repress Col10 expression. Sox9 can also activate the expression of ECM components in cartilage. This may suggest the existence of a regulatory feedback loop that maintains the Fbn1 microenvironment within the cartilage as we observed previously in an *Adamtsl2*-KO mouse model of GD (Delhon et al 2019).

Our study of Fbn1, the major component of the microfibrillar network, demonstrated that defects in mutant growth plates were associated with a loss of density of Fbn1-rich network density. While fibrillins compose just 1-3% of total ECM proteins in the skeleton [20], mutations in the *Fbn1* genelead to severe skeletal anomalies. As a structural ECM glycoprotein, Fbn1 may be essential for chondrocyte column formation. Indeed, the absence of appropriate chondrocyte column formation observed in *Fbn1* mutant mice in the present study underscores the importance of ECM in this process.

Mutations in the FBN1 TB5 domain identified in GD have been shown to disrupt heparan sulfate interactions in a cellular context [22]. Indeed, similar findings have been reported in co-cultures of fibroblasts and GD mutants overexpressed in HEK293 cells. Heparan sulfate permits interaction between cells and the ECM. More importantly, heparan sulfate is involved in the regulation of the early stages of microfibril assembly [23,24]. This may explain the fact that despite the mutation of the TB5 domain, we observed Fbn1 fibrils in our mutant mice, suggesting that this mutation does not impact the expression of Fbn1 but rather alters its capacity to form an abundant fibril network. A defect in the fibril network formed by fibrillin-1 and fibronectin has also been reported in an *Adamtsl2* deficient mouse model [5]. In a separate GD mouse model with a specific deletion of Adamtsl2 in tendinous tissues, Hubmacher *et al*. observed a disrupted network and an impaired microfibril assembly in the ECM [25]. In prior studies, we determined that FBN1 deposition in GD patient-derived fibroblasts was disturbed [4]. Moreover, our previous human genetic data have established a link between ADAMTSL2 and FBN1 that was reinforced by biochemical results exhibiting a direct interaction between these two proteins [4]. ADAMTSL2 co-localizes with FBN1 fibrils in cell culture and *in vivo* [3,5,25,27]. An analysis of our CMV-Cre *Adamtsl2* KO mouse model revealed that the complex formed by a minimum of Adamtsl2 and Fbn1 creates microenvironmental conditions that are critical for appropriate chondrocyte column formation [5]. In summary, abnormal fibril assembly in distinct tissues has been observed in all human and mouse GD models, suggesting that the underlying mechanistic basis for GD involves the dysregulation of fibrillin microfibril deposition through mechanisms associated with Adamtsl2 and the Fbn1 TB5 domain.

We have previously reported abnormal TGFβ signaling in GD patient-derived fibroblasts harboring mutations in the *ADAMTSL2* or *FBN1* genes [4]. Increased TGFβ signaling activity was also detected in *Adamtsl2* deficient mice in our previous study [5]. However, we did not observe any comparable increase in TGFβ signaling activity in chondrocytes harboring a mutation in the *Fbn1* TB5 domain. These findings raise the question as to whether the observed alterations in TGFβ signaling are a cause or a consequence of GD pathogenesis. A lack of TGFβ signaling overactivation has similarly been reported in GD caused by mutations in the *LTBP3* gene [26]. Moreover, treatment with TGFβ neutralizing antibodies was not sufficient to rescue severe phenotypes in *Adamtsl2* KO lungs [27]. Altogether, these data suggest that TGFβ signaling may not be a direct pathogenic driver of GD but may instead contribute to the regulation of an abnormal ECM.

This original Fbn1 mutant mouse model provides new insight into the molecular events underlying GD. Our study confirmed that FBN1 microfibril assembly plays a major role in the pathophysiological etiology of GD and demonstrated that the FBN1 TB5 domain is a structural domain involved in the assembly of growth plate ECM scaffolds, thereby impacting column structure. Importantly, this study brought to light the pivotal role of the FBN1-associated ECM in the control of chondrogenesis. Overall, we propose the existence of a protein complex containing ADAMTSL2 and FBN1 in which the FBN1 TB5 domain may act as a sensor to gauge the state of the fibril network that is necessary for appropriate ECM regulation.

**Methods**

**Generation of *Fbn1TB5* mice**

To generate *Fbn1TB5+/-*mice, a *Fbn1TB5* targeting vector with a substitution of one base was generated as described in Figure 1 with the collaboration of the Clinique de la Souris (Strasbourg, France). The germ-line transmission of the mutated allele was achieved by crossing the chimeric mice with C57BL/6 mice. The *Fbn1TB5+/-*mice were intercrossed to generate heterozygous (*Fbn1TB5+/-*) and homozygous (*Fbn1TB5-/-*) mice carrying the mutations in the TB5 domain of fibrillin-1. Genotyping was performed using tail samples and a Direct PCR Lysis Reagent (Viagen Biotech Cat # 101-T, 102-T) followed by sequencing. All research protocols were approved by the respective Institutional Animal Care and Use committees of IMAGINE Institute/Necker Hospital and Paris Descartes University.

**Bone histology**

All mice were analyzed via X-Ray (Faxitron MX-20DC12 Edimex) and measured after euthanasia. For histological analyses, bone tissues were fixed in 4% paraformaldehyde at 4°C for 24 hours, decalcified in 0.5 M EDTA (pH7.4) for one week, and embedded in paraffin. Bone sections (5 μm) were prepared with a microtome (Thermo Scientific) and used for Safranin O staining, immunohistological analyses, and *in situ* hybridization.

For Safranin O staining, tissue sections were deparaffinized and rehydrated with ethanol. Slides were stained with Weigert’s iron hematoxylin working solution for 5 minutes then rinsed in running tap water for 4-5 minutes. Slides were then stained with 0.02% fast green (FCF) solution for 30 seconds, rinsed with 1% acetic acid for 30 seconds, and stained in 0.1% safranin O solution for 45 minutes. Slides were then mounted by using Euparal Mounting Medium.

**Skin and aorta staining**

For orcein staining, aortic tissue sections were deparaffinized and rehydrated with ethanol. Slides were stained with an orcein 1% working solution for 15 minutes then rinsed under running tap water. Slides were differentiated with lithium carbonate 1% solution for 15 minutes with constant agitation, and were then mounted in xylene-based Eukitt® medium (Sigma-Aldrich).

Hematoxylin and eosin Y staining of skin tissue sections was performed following deparaffinization and rehydration thereof using ethanol. Slides were stained with Mayer’s hematoxylin for 8 minutes, rinsed in warm running tap water for 10 minutes, then dipped briefly with 95% ethanol 10 times. Samples were then counterstained with eosin Y (Sigma-Aldrich) for 30 seconds.

For Masson’s trichrome staining, skin tissue sections were deparaffinized and rehydrated with ethanol. Slides were stained with Mayer’s hematoxylin for 10 minutes then rinsed under running tap water. Samples were then subjected to fuchsin/ponceau S staining for 5 minutes and rinsed with distilled water. Slides were differentiated in phosphomolybdic acid for 5 minutes, directly stained in aniline blue for 2 minutes, and incubated in 1% acetic acid for 5 minutes.

In all cases, aorta and skins sections were mounted in xylene-based Eukitt® medium (Sigma-Aldrich) and observed using Nanozoomer 2.0. Slide Scanner (HAMAMATSU).

***In Situ* Hybridization**

PCR products were used to generate antisense and sense cRNA probes (Table1 ) with a T7 DIG RNA Labeling kit (Roche) and digoxigenin-11-UTP (Roche) being used according to the manufacturer's specifications. Paraformaldehyde-fixed paraffin-embedded sections obtained from *Fbn1* model mice were hybridized to 1 µg /ml of DIG-11-UTP-labeled Col2a1 and Col10 *c*RNA probes as described previously. After staining with BCIP/NBT in the dark (Roche) based on provided directions, the slides were imaged with an Olympus PD70-1X2-UCB microscope and analyzed with the CellSens and ImageJ software.

|  |  |  |
| --- | --- | --- |
| Probes | Target genes | Sequences (5’ 🡪 3’) |
| Col10a1 S forward | Col10 (sense probe) | TAATACGACTCACTATAGGGAGACAAACGGCCTCTACTCCTCTGA |
| Col10a1 S reverse | Col10 (sense probe) | CGATGGAATTGGGTGGAAAG |
| Col10a1 AS forward | Col10 (antisense probe) | CAAACGGCCTCTACTCCTCTGA |
| Col10a1 AS reverse | Col10 (antisense probe) | TAATACGACTCACTATAGGGAGACGATGGAATTGGGTGGAAAG |
| Col2 S forward | Col2a1 (sense probe) | TAATACGACTCACTATAGGGAGAGAACTGGTAAGTGGGGCAAGAC |
| Col2 S reverse | Col2a1 (sense probe) | CCACACCAAATTCCTGTTCA |
| Col2 AS forward | Col2a1 (antisense probe) | ACTGGTAAGTGGGGCAAGAC |
| Col2 AS reverse | Col2a1 (antisense probe) | TAATACGACTCACTATAGGGAGAGACCACACCAAATTCCTGTTCA |
| Col2 S forward | Col2a1 (sense probe) | TAATACGACTCACTATAGGGAGAGAACTGGTAAGTGGGGCAAGAC |

Table 1. Riboprobe sequences used for *in situ* hybridization

**Immunohistochemical analysis**

Proliferation was analyzed by staining 5 µm-thick paraffin-embedded tissue sections collected from mouse hindlimbs with anti-Sox9 (1:50; Santa Cruz Biotechnology sc-20095), anti-fibrillin-1 (1:100; LS-C358981, LS-Bio), and anti-TGFβ-1 (1:100; Abcam ab66043) antibodies using an indirect (two-step) immunoenzymatic method in which the secondary goat–anti-rabbit antibody was labeled with horseradish peroxidase (HRP, Dako). HRP was then detected using 3-3′-diaminobenzidene (DAB, Abcam) as a substrate. Images were collected using an Olympus PD70-1X2-UCB microscope and analyzed with CellSens and ImageJ.

**Primary chondrocyte culture and immunofluorescent staining**

After mice were sacrificed, chondrocytes were isolated from the rib cage and cultured as previously described*.* Chondrocytes were grown in cell culture chambers in DMEM/HamF12 supplemented with 10% SVF and antibiotics until confluent, at which time they were fixed with 3% formaldehyde for 20 minutes. After treatment with 0.2 M glycine, cells were blocked with 3% BSA for 1 h and incubated overnight with primary anti-fibronectin (1:100; Abcam ab2413) and anti-fibrillin-1 (1:100; LS-Bio LS-C358981) in 3% BSA. Cells were then rinsed thrice with PBS/MgCl2/CaCl2, incubated with secondary antibodies diluted in 3% BSA for 45 min, mounted in ProLong® with DAPI (Life Technologies), and analyzed with an LSM-700 confocal microscope from the platform of Cell Imagery of Imagine Institute. Resultant images were analyzed with ImageJ.

**Western blotting**

Tissues for protein extraction were placed on dry ice during dissection and stored at -80°C until analysis. For protein extraction, whole tissues were lysed with a SCIENCEWARE® Liquid Nitrogen Cooled Mini Mortar and RIPA buffer (Thermo Scientific) containing a protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein concentrations were measured with a PierceTM BCA Protein Assay Kit (Thermo Scientific). Antibodies specific for Smad2 (1:500, Abcam ab53100) and phospho-Smad2 (Ser467) (1:500; Abcam ab188334) were utilized to probe blots, with anti-actin (1:1000; Millipore MAB1501) serving to detect actin as a loading control.

**RNA extraction, reverse-transcription, and quantitative-PCR.**

Skin tissues were samples were pulverized in liquid nitrogen, after which RNA was extracted using the NucleoSpin® RNA kit (Macherey-Nagel) according to the manufacturer’s instructions. Then, 1 μg of RNA was utilized for reverse transcription using the RNA-to-cDNA™ kit (ThermoFisher Scientific) based on provided directions. RT-qPCR was performed using a Step One Plus RT-qPCR instrument (Applied Biosystems, Thermo Scientific) according to the manufacturer’s instructions. Absolute Blue qPCR SYBR Green Supermix (Thermo Scientific) was used to quantify *Col1a1*, *Col1a2*, *Col3a1*, and *Fbn1* expression levels as per provided directions. Samples were analyzed in triplicate. The results were normalized to the expression of three housekeeping genes (*Gapdh*, *Hprt,* and *Hsp90*) by calculating the geometric means.

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| --- | --- | --- |
| Target genes | Forward sequences (5’ – 3’) | Reverse sequences (5’ – 3’) |
| *Col1a1* | GCTCCTCTTAGGGGCCACT | CCACGTCTCACCATTGGGG |
| *Col1a2* | CTGGTCCTGTTGGAAGTCGT | CAGATGCACCTGTTTCTCCA |
| *Col3a1* | CAATGTAAAGAAGTCTCTGAAG | CAAACAGGGCCAATGTCCAC |
| *Fbn1* | CCTGTGCTATGATGGGTTCA | AGGTCCCACTAAGGCAGATGT |
| *Gapdh* | TGTCCGTCGTGGATCTGAC | CCTGCTTCACCACCTTCTTG |
| *Hprt* | GTTGGGCTTACCTCACTGCT | TCATCGCTAATCACGACGCT |
| *Hsp90* | CCAGAAACCCGGATGACA | TGACCTCTACAGAGAAGTGCTTG |

Table 2. Sequences of primers used in qPCR analyses

**ELISA detection of active and total TGF-β1**.

TGF-β1 levels in 100 µl samples of culture medium from confluent primary chondrocytes from the ribs of mutant and WT mice were quantified with a Quantikine mouse TGF-β1 ELISA kit (R&D systems #MB100B). The samples were acidified for measurements of total (active plus latent) TGF-β1. TGF-β1 standard curves were generated for each assay. All experiments were performed in triplicate, and the results were analyzed using Student’s t-tests.

**Statistical analysis**

All statistical analyses were performed using PRISM 5.03 (GraphPad, La Jolla, CA, USA). All values are shown as means ± standard deviation (SD).

**Conflict of interest statement**

The authors declare no conflict of interest.

**Author contributions**

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**Figure legends**

**Figure 1**. **Skeletal abnormalities in *Fbn1TB5* mutant mice**. **(a)** The targeted *Fbn1* locus, with the introduced point mutation in exon 42 (tyrosine 1696 replacement with a cysteine) shown in red. **(b)** Sequencing electropherogram sections showing the point mutation (A>G) in *Fbn1TB5* mutant mice. Heterozygous mutants exhibit a double peak corresponding to A and G (denoted as an “R”) whereas the homozygous mutants exhibit a single peak corresponding to G. **(c)** Quantification of *Fbn1* mRNA levels in skin samples from wild-type and mutant littermates. Expression of mRNA levels was normalized to the levels of three housekeeping genes: Hsp90 (mouse heat shock protein 90 kDa), Gapdh (glyceraldehyde 3-phosphate dehydrogenase), and Hprt (hypoxanthine-guanine phosphoribosyltransferase) (n = 3). **(d)** Quantification of murine naso-anal stature and **(e)** Femur length at P1 (nWT>5, nHT>5 and nHo>5; Mann–Whitney U test; error bars represent the SEM, \*p < 0.05). (**f)** Front and **(g)** side X-ray images of the whole skeleton of WT (at left), *Fbn1TB5+/-* (HT) and *Fbn1TB5-/-* (Ho) mutant mice at P30. **(h)** Quantification of the data from f and g (nWT>5, nHT>5 and nHo>5; Mann–Whitney U test; error bars represent the SEM, \*p < 0.05)

**Figure 2.**  **Skin and aortic phenotypes in *Fbn1TB5* mutant mice. (a. upper panel)** Hematoxylin and eosin staining of skin from 1-month-old *Fbn1TB5*littermates. Scale bar = 200 µm. **(a. lower panel)** Masson’s trichrome staining of skin from 1-month-old *Fbn1TB5* littermates. *Fbn1TB5-/-* (Ho) mutant mouse skin exhibited increased collagen deposition. Scale bar: 100 µm. **(b)** Quantification of collagen (*Col1a1*, *Col1a2*, and *Col3a1*) mRNA levels in skin samples from wild-type and mutant littermates as measured by qPCR, with *Gapdh*, *Hprt*, and *Hsp90* being used to normalize gene expression (n = 3). No significant upregulation of collagen mRNA levels was observed in *Fbn1TB5+/-* (HT) and *Fbn1TB5-/-* (Ho) mutant mice**. (c)** Aortic root morphologywas assessed via the orcein staining of aorta samples. *Fbn1TB5-/-*(Ho) and *Fbn1TB5+/-* (HT) mice exhibited no elastic fiber alterations (such as fragmentation) as compared to wild-type mice (n=3). Scale bar: 100 µm

**Figure 3**. **Impact of *Fbn1* KI on chondrocyte function.** **(a)** Safranin O staining of *Fbn1TB5+/-* (HT), *Fbn1TB5-/-* (Ho), and WT newborn (P1) mouse femur and femoral proximal growth plate sections. Arrowheads indicate the lack of chondrocytes in the growth plate samples from mutant mice. Scale bar: 100 µm. **(b)** Quantification of hypertrophic chondrocyte size in WT, *Fbn1TB5+/-* (HT), and *Fbn1TB5-/-* (Ho) mouse femoral proximal growth plate samples (nWT>5, nHT>5 and nHo>5; \*p<0.05) **(c)** Safranin O staining of *Fbn1TB5+/-* (HT) and *Fbn1TB5-/-* (Ho) and WT mouse femoral proximal growth plate sections at P30. Scale bar: 200 µm. **(d)** Quantification of hypertrophic zone size in WT, HT, and Ho femoral proximal growth plate samples at P30 (nWT>5, nHT>5 and nHo>5, \*\*\*p<0.001; \*\*p<0.01). **(e)** For *in situ* hybridization experiments, WT, *Fbn1TB5+/-* (HT), and *Fbn1TB5-/-* (Ho) femoral growth plates were hybridized with an antisense riboprobe specific for *Col10*. A sense riboprobe was used as a negative control. Scale bar: 100 μm. **(f)** Immunohistochemical staining of femoral growth plate sections using anti-Sox9. PZ: proliferative zone; PHZ: prehypertrophic zone; HZ: hypertrophic zone. Scale bar: 100 µm

**Figure 4.** **Composition of the ECM in the femur growth plate**. Primary chondrocytes isolated from P1 WT and *Fbn1TB5* KI ribs and labe led with antibodies specific for fibronectin **(a)** and fibrillin-1 **(b)** revealed the impairment of the Fbn1 microfibrillar network in *Fbn1* KI chondrocytes upon fluorescent secondary antibody staining. Scale bar: 20µm. **(c)** Immunohistochemical staining of femoral growth plate sections using an antibody against fibrillin-1. Scale bar: 100 µm.

**Figure 5. Analysis of the TGFβ signaling pathway**. **(a)** Immunostaining for TGF-β1 in proximal femoral sections from WT, HT, and Ho mice at P1. HZ: hypertrophic zone. Scale bar: 100µm. **(b)** TGFβ1 levels in culture media from WT, HT, and Ho chondrocytes were measured via ELISA (n=3 per genotype). **(c)** Western blotting analysis and **(d)** quantification of phosphorylated SMAD2/SMAD3 levels in extracts from P1 WT, Ht, and Ho whole hindlimb samples, with total SMAD2 being used for normalization (n = 3).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5