Bi-allelic premature truncating variants in *LTBP1* cause autosomal recessive cutis laxa syndrome

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53
54 **Abstract**
55 Latent transforming growth factor β (TGFβ) binding proteins (LTBPs) are microfibril-associated
56 proteins essential for the anchoring of TGFβ in the extracellular matrix (ECM) as well as for correct
57 assembly of ECM components. Gene variants affecting *LTBP2*, *LTBP3*, and *LTBP4* have been
58 identified in several autosomal recessive Mendelian disorders with skeletal abnormalities with or
59 without impaired development of elastin-rich tissues. Thus far, the human phenotype associated with

60 LTBP1 deficiency has remained enigmatic. In this study, we report homozygous premature truncating
61 *LTBP1* variants in eight affected individuals from four unrelated consanguineous families. Affected
62 individuals present with connective tissue features (cutis laxa and inguinal hernia), craniofacial
63 dysmorphology, and variable heart defects and prominent skeletal features (craniosynostosis, short
64 stature, brachydactyly and syndactyly). *In vitro* studies on proband dermal fibroblasts indicate distinct
65 molecular mechanisms depending on the position of the variant in the *LTBP1* gene. C-terminal
66 variants lead to an altered LTBP1 loosely anchored in the microfibrillar network and cause increased
67 ECM deposition in cultured fibroblasts associated with excessive TGFβ growth factor activation and
68 signaling. In contrast, N-terminal truncation results in a loss of LTBP1 that does not alter TGFβ levels
69 and ECM assembly. *In vivo* validation of two independent zebrafish lines carrying mutations in *ltbp1*
70 induce abnormal collagen fibrillogenesis in skin and intervertebral ligaments and ectopic bone
71 formation on the vertebrae. In addition, one of the mutant zebrafish lines shows voluminous and hypo-
72 mineralized vertebrae. Overall, our findings in humans and zebrafish show that LTBP1 is important for
73 skin and bone ECM assembly and homeostasis.

74

75 **Introduction**

76 Latent transforming growth factor β (TGFβ) binding proteins (LTBPs) are microfibril-associated
77 multidomain proteins essential for the sequestration of TGFβ in the extracellular matrix (ECM). Mature
78 TGFβ growth factor dimers associate non-covalently with the latency associated peptide (LAP) in
79 order to form the small latent complex (SLC) which is covalently tethered via two disulfide-bridges to
80 LTBPs[1]. SLCs of the three human TGFβ isoforms were shown to bind to LTBP1 and LTBP3 while
81 TGFβ1 SLC exclusively interacts with LTBP4[2]. Most LTBPs are targeted to the extracellular (ECM)
82 via their N- and C-terminal regions. LTBP1, LTBP2, and LTBP4 interact through their carboxy-
83 terminal region with fibrillin-1 (FBN1)[3-5], while the amino-terminal region of LTBP1 and LTBP4 interact
84 with fibronectin (FN)[6; 7]. Moreover, LTBP4 facilitates the deposition of tropoelastin onto the
85 microfibrillar scaffold through interaction with fibulin-4 (EGF containing fibulin extracellular matrix
86 protein 2: EFEMP2) and fibulin-5 (FBLN5)[8-13]. Similar to LTBP4, LTBP2 facilitates the deposition of
87 tropoelastin onto the microfibrillar scaffold through interaction with fibulin-5[14]. Dysfunction of any
88 member of the LTBP superfamily has multiple consequences on the TGFβ bioavailability and elastic
89 fiber assembly in various tissues both *in vitro* and *in vivo*[9-11; 15-19].

Pathogenic variants in LTBP genes have been identified in several autosomal recessive (AR) Mendelian disorders presenting with impaired development of the skeleton and/or elastin-rich tissues. Pathogenic variants in *LTBP2* cause AR primary congenital glaucoma (MIM: 613086), AR microspherophakia and/or megalocornea, with ectopia lentis and with or without secondary glaucoma (MIM: 251750), and AR Weill-Marchesani syndrome (MIM: 614819)[20-22]. Pathogenic variants in *LTBP3* cause AR dental anomalies and short stature (MIM: 613086), and geleophysic dysplasia 3 (MIM: 617809)[23; 24]. In addition, homozygous loss-of-function (LOF) variants in *LTBP3* were reported in syndromic forms of thoracic aortic aneurysm and dissection (TAAD)[25]. Finally, pathogenic variants in *LTBP4* cause AR cutis laxa (CL) type 1C, characterized by loose redundant skin folds, emphysema and diverticula of the gastrointestinal and urinary tract[26-28]. Thus far, the human phenotype associated with *LTBP1* deficiency has remained enigmatic.

Nevertheless, the molecular consequences of *LTBP1* deficiency have been studied in mice. In most vertebrates, *LTBP1* encodes two alternatively spliced isoforms: a long (*Ltbp1L*) and a short (*Ltbp1S*) isoform. Mice lacking *Ltbp1L* only or both *Ltbp1S* and *Ltbp1L* show a persistent truncus arteriosus and an interrupted aortic arch that associates with perinatal lethality[15; 29]. At the embryonal stage, the outflow tract of $Ltbp1L^{-/-}$ mouse hearts show decreased TGFβ activity[15]. Mice lacking *Ltbp1S* while still retaining expression of an alternatively spliced form of *Ltbp1L* (Δ55 variant) are viable, show mild craniofacial and skeletal abnormalities, impaired ovarian function, and are less prone to hepatic fibrosis after bile duct ligation, which was attributed to decreased bio-availability of TGFβ[29-31]. Together, data from animal studies indicate that *Ltbp1L*, and hence intact TGFβ signaling, is required for proper embryonal cardiovascular development, while Ltbp1S could play a role in craniofacial development[15; 29; 30].

Here we report homozygous premature truncating *LTBP1* variants in eight affected individuals from four unrelated consanguineous families. Affected individuals present with cutis laxa, craniofacial dysmorphism, mild variable heart defects and altered skeletal development including short stature, craniosynostosis, brachydactyly, clinodactyly, and syndactyly, which we propose to coin as the *LTBP1*-related CL syndrome[32]. *In vitro* studies on proband dermal fibroblasts indicate distinct

120 molecular consequences and effects on TGFβ signaling depending on the position of the variant in
121 the *LTBP1* gene. For *in vivo* validation, we generated and characterized *ltbp1*$^{-/-}$ Δ29 and *ltbp1*$^{-/-}$ Δ35
122 zebrafish. We found abnormal collagen fibrillogenesis in the skin and in the intervertebral ligaments.
123 In addition, *ltbp1*$^{-/-}$ Δ29 zebrafish show hypo-mineralized vertebrae with ectopic bone formation and
124 increased vertebral volume. These observations were not yet confirmed in the majority of the affected
125 individuals. Our data indicate that LTBP1 has dual functions in humans and zebrafish affecting
126 cutaneous and skeletal development.
127
128 ***Subjects and Methods***
129 **Clinical Assessment**
130 Informed consents were obtained from all individuals or from their parents in case of minor
131 individuals, including specific consent to publish the clinical pictures in **Figure 1** with the exception of
132 F2:V-3 and F2:V-4. All individuals were evaluated at one of the collaborating referral centers and
133 clinical data were recorded using a clinical checklist (**Supplementary Table S1**). Skin biopsies were
134 obtained from several probands for dermal fibroblast culture (F1:IV-2 and F4:II-1) and transmission
135 electron microscopy (F1:IV-2). This study was conducted in accordance with the declaration of
136 Helsinki and approved by the Ghent University Hospital ethical committees (registration number
137 B670202000194). Family 2, Family 3, and Family 4 were identified through GeneMatcher[33].
138
139 **Exome Sequencing**
140 Exome sequencing (ES) was performed on genomic DNA (gDNA) extracted from blood leukocytes of
141 each person. gDNA was enriched using the SureselectXT Human All Exon v6 kit (Agilent
142 Technologies, Santa Clara, CA, USA), followed by sequencing on a HiSeq 3000 platform (Family 1)
143 (Illumina, San Diego, CA, USA). LTBP1 (RefSeq NM_206943.3) nucleotides were numbered
144 according to the Human Genome Variation Society guidelines (http://www.hgvs.org) with nucleotide
145 'A' of the ATG start codon of the long isoform of LTBP1 = c.1. The following algorithms were used to
146 predict the consequences of variants identified with ES: Polyphen-2
147 (http://genetics.bwh.harvard.edu/pph2/), PhD-SNP (http://snps.biofold.org/phd-snp/phd-
148 snp.html), SIFT (https://sift.bii.a-
149 star.edu.sg/), SNAP (https://www.rostlab.org/services/SNAP/), MAPP (http://mendel.stanford.edu/sido

150 wlab/downloads/MAPP/index.html), and REVEL (https://sites.google.com/site/revelgenomics/), and
151 allele frequencies were evaluated using the Gnomad population database. Homozygosity mapping
152 was performed prior to ES in family 2 using an Affymetrix Genome-Wide Human SNP Array 6.0
153 (ThermoFisher, Waltham, MA, USA). Segregation analysis was performed in parents of affected
154 individuals using Sanger sequencing. ES (Family 3 and family 4) was done as previously described[34;
155 35].
156

157 **Transmission Electron Microscopy**

158 For human dermal biopsies, 3 mm skin fragments from individual (F1:IV-2) and an age- and sex-
159 matched control were initially immersed in a fixative solution of 4% glutaraldehyde for transport.
160 Subsequently, samples were placed in 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M Na-
161 Cacodylate buffer in a vacuum oven for 30 min, followed by further fixation for 3 hours at room
162 temperature on a sample rotator. This solution was then replaced with fresh fixative and samples
163 were incubated overnight at 4 °C on a sample rotator. After washing in double-distilled H_2O, samples
164 were post-fixed in 1% OsO_4 with $K_3Fe(CN)_6$ in 0.1 M Na-Cacodylate buffer, pH 7.2. After washing in
165 double-distilled H_2O, samples were subsequently dehydrated through a graded ethanol series,
166 including bulk staining with 2% uranyl acetate at the 50% ethanol step, followed by embedding in
167 Spurr's resin. To select the area of interest on the block and in order to have an overview of the
168 phenotype, semi-thin sections were first cut at 0.5 µm and stained with toluidine blue. Ultrathin
169 sections were cut using an ultramicrotome (Leica EM UC6, Wetzlar, Germany), followed by post-
170 staining in a Leica EM AC20 for 40 min in uranyl acetate at 20°C and for 10 min in lead stain at 20°C.
171 Sections were collected on formvar-coated copper slot grids. Grids were viewed with a JEM 1400plus
172 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV. Results are
173 representative of three independent experiments. For zebrafish, skin biopsies and vertebrae of 4 to 6-
174 month-old male zebrafish were fixed and processed for ultrastructural analysis as previously
175 described[36]. Sections were viewed with Jeol JEM 1010 TEM (Jeol Ltd., Tokyo, Japan) equipped with
176 a CCD side mounted Veleta camera operating at 60 kV. Experiments were performed in collaboration
177 with the TEM facility of the Nematology Research Unit at Ghent University. Results are representative
178 of three independent experiments.
179

Cell Culture

Dermal fibroblasts obtained from a skin biopsy from individuals F1:IV-2 and F4:II-1, and four healthy individuals (2 control subjects, age- and gender-matched, for each individual, see **Table S2**) were cultured in Dulbecco's Modified Eagle Medium (Gibco; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (PAN-Biotech, Aidenbach, Germany), 1% non-essential amino acids (Gibco), 1% penicillin/streptomycin (Gibco), 0.1% fungizone (Gibco) and incubated at 37°C with 5% CO_2. Cells were tested for mycoplasma contamination by biochemical analysis of mycoplasmal enzymes (Lonza, Basel, Switzerland) and confirmed to be mycoplasma free.

Antibodies

The following primary antibodies and dilutions were used for immunoblot analysis: anti-Phospho-Smad2 (Ser465/467) (#3108, Cell Signaling Technologies (CST), 1/500), anti-Smad2 (#5339, CST, 1/1000), anti-Vinculin (#13901, CST, 1/1000), anti-fibronectin (ab23750, Abcam, 1/1500). Anti-rabbit IgG HRP-linked Antibody (#7074 CST, 1/2500-1/4000) was used as secondary antibody. Polyclonal rabbit anti-FBN1 antiserum (1/1000 for immunofluorescence (IF) and 1/2000 for WB) was raised against the recombinantly produced N-terminal half of human fibrillin-1 (F90)[37; 38]. Polyclonal rabbit anti-LTBP-1 antiserum (1/1000 for IF) was raised against the last 214 C-terminal residues of human LTBP-1 L1K[3; 38]. Polyclonal rabbit anti-Fbn2 antibody was raised against the C-terminally double-strep-tagged N-terminal recombinant human FBN2 polypeptide rF86 (Gln^{29}-Asp^{535})[39]. Polyclonal rabbit anti-LTBP-2 antiserum (1/1000 for IF) was raised against the last 254 C-terminal residues of human LTBP-2 (Asp^{1568}-$Glut^{1821}$) similar to as described for L1K[38]. Anti-collagen I antibody (#ab34710, Abcam, 1/1000), and polyclonal goat anti-collagen III (#1330-01, Southern Biotech, 1/1000) were used for IF. Polyclonal rabbit antibody recognizing human fibulin-4 (1/1000 for IF) was a kind gift from Dr. Takako Sasaki (Oita University). Goat anti-Rabbit IgG Alexa Fluor 555 (A32732, Life Technologies, 1/800) was used as secondary antibody.

Recombinantly produced proteins

For recombinant expression of LTBP1 proteins, cDNA encoding the wild-type (WT) human LTBP1 fragment of 541 C-terminal amino acid residues and corresponding fragments carrying c.4431T>A and c.4844del mutations were overexpressed in HEK 293 cells together with the unaffected control

210 sequence. Encoding cDNAs were cloned into a variant of the pCEP-Pu vector, and stably transfected
211 overexpressing cells were established after puromycin selection as previously described[9]. Proteins
212 were expressed with a C-terminally placed double-strep-tag and purified via affinity chromatography
213 from collected serum-free culture medium. Fresh medium was filtered with a suitable membrane filter,
214 then subjected to Strep-Tactin®XT gravity flow column (2 ml beads; IBA GmbH, Germany) at 4°C
215 overnight. LTBP1 proteins were eluted with elution buffer (100 mM Tris/HCl, pH 8.0 150 mM NaCl 1
216 mM EDTA 2.5 mM desthiobiotin). The collected fractions were concentrated, and exchanged to PBS
217 by Amicon® Ultra Centrifugal Filter, 3kDa (Merck Millipore, Massachusetts, United States). Using the
218 same protocol, the N-terminal region of human fibrillin-1 (after signal peptide cleavage site, up to the
219 amino acids coding for the fourth epidermal growth factor (EGF4) domain, encompassing the binding
220 site for LTBP1) was recombinantly produced. Production and purification of the N-terminal region of
221 human FBN2 (rF86) was as previously described[40].
222

223 **RT-qPCR**

224 Total RNA was extracted from dermal fibroblast cultures from control subjects and individuals (F1:IV-2
225 and F4:II-1) using the RNeasy® kit (Qiagen, Hilden, Germany) with DNase digestion of genomic DNA,
226 followed by cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules,
227 CA, USA). Gene expression levels of *EFEMP2*, *FBLN5*, *LTBP3*, *LTBP4*, *FBN1*, *FBN2*, *FN*, *LOX*,
228 *POSTN*, *CTGF*, *SERPIN1* were investigated between control subjects and affected individual (F1:IV-2
229 and F4:II-1) dermal fibroblast cultures. Gene expression levels of *COL1A1*, *COL1A2* and *COL3A1*
230 were investigated between control subjects and affected individuals (F1:IV-2 and F4:II-1) dermal
231 fibroblast cultures stimulated with 25 µg/ml ascorbate (Sigma-Aldrich, St. Louis, MO, USA) for 3 days.
232 All measurements were obtained from three separate dermal fibroblast culture samples originating
233 from individuals F1:IV-2 and F4:II-1, and from 2 control subjects. Average values of the two control
234 subjects were plotted as 'control' for each experiment. Total RNA was extracted from juvenile
235 zebrafish in quintuplicate, in which 10 zebrafish larvae were pooled per sample. Gene expression
236 levels of *ltbp1* was investigated between *ltbp1*$^{-/-}$ Δ29, *ltbp1*$^{-/-}$ Δ35 and wild-type zebrafish controls.
237 Assays were prepared with the addition of SsoAdvanced SYBR Green supermix (Bio-Rad
238 Laboratories) and were subsequently run on a LightCycler® 480 Instrument II (Roche, Basel,
239 Switzerland). Primers were designed using Primer-BLAST (**Table S3**). Biogazelle qBase+3.0 software

240 was used for data analysis using *YWAHZ*, *HPRT1*, and *RLP13A* for normalization of human dermal
241 fibroblasts and *loopern*, *hatn10*, and *tdr7* for normalization of zebrafish samples[41].
242
243 **NMD Analysis**
244 Dermal fibroblasts from individuals (F1:IV-2 and F4:II-1) and control subjects were incubated with 5
245 mg cycloheximide for 17 hours (h) or vehicle, followed by reverse transcription quantitative PCR (RT-
246 qPCR). All measurements were obtained from three separate dermal fibroblast culture samples
247 originating from individuals F1:IV-2 and F4:II-1, and from 2 control subjects. Average values of the
248 two control subjects were plotted as 'control' for each experiment.
249
250 **Immunoblot Analysis and determination of TGFβ levels**
251 For the investigation of extracellular proteins, conditioned serum-free medium of dermal fibroblast
252 cultures from control subjects and affected individuals (F1:IV-2 and F4:II-1) was collected at day 14 as
253 previously described[42]. Protein samples were subjected to 3-8% Tris-Acetate sodium dodecyl sulfate
254 polyacrylamide gel electrophoresis (SDS-PAGE) before blotting, either by wet or dry blotting onto a
255 polyvinylidene difluoride (PVDF) or nitrocellulose (NC) membrane. Imperial protein staining (Life
256 Technologies, Carlsbad, California, USA) was used to visualize the total protein amount. Imaging was
257 performed on an Amersham Imager 680 (GE Healthcare Life Sciences, Chicago, Illinois, USA).
258 Resulting images were processed with Fiji software[43]. For the investigation of intracellular proteins,
259 cell lysate of confluent dermal fibroblast cultures from control subjects and affected individuals (F1:IV-
260 2 and F4:II-1) was collected. Protein samples were subjected to 4-12% Bis-Tris SDS-PAGE before
261 dry blotting onto a PVDF membrane. Recombinant Human TGF beta 1 (Bio-Techne Corporation,
262 Minneapolis, MI, USA) was added at 2.5 ng/mL to one confluent control dermal fibroblast culture
263 acting as positive control for Smad2 phosphorylation. Total TGFβ protein levels were measured in
264 conditioned serum-free medium of dermal fibroblast cultures from control subjects and affected
265 individuals (F1:IV-2 and F4:II-1) collected at day 9 using the Quantikine ELISA (#MB100B, R&D
266 Systems, Minneapolis, MI, USA) according to manufacturer's instructions. All measurements were
267 obtained from three separate dermal fibroblast culture samples originating from individuals F1:IV-2
268 and F4:II-1, and from 2 control subjects. Average values of the two control subjects were plotted as
269 'control' for each experiment.

Immunofluorescence

For analysis of ECM network formation, cells were seeded on uncoated glass coverslips at a density of 8×10^4 cells/well in a 24-well plate. After culture, cells were washed with PBS, fixed at –20°C in methanol/acetone, blocked in a phosphate-buffered saline/1% bovine serum albumin solution, and subsequently incubated with primary and secondary antibodies diluted in the blocking solution. Images were obtained from three independent experiments.

Solid-phase binding assay

Multiwell plates were coated with purified LTBP1 proteins (100 nM) in 50 mM carbonate/ bicarbonate buffer, pH 9.6 at 4 °C overnight. Coated wells were blocked with 5 % nonfat dry milk in TBS at room temperature for 1 h. Recombinant fibrillin-1, and -2 were serially diluted 1:2 in 2% milk, TBS and incubated in the wells for 2 h, followed by a 1 h incubation with anti-fibrillin-1, or -2 antibody (1/5000). Color reaction of the enzyme immunoassay was achieved using the TMB (3,3′,5,5′-tetramefhyl-benzidine) substrate Kit (Thermo Fisher Scientific, Waltham, MA, USA) and stopped with 0.1 M HCl after streptavidin-HRP (biotinylated) antibody incubation. Absorbance was read at 450 nm using a Microplate Reader Sunrise (Tecan, Maennedorf, Switzerland). Curve fits to obtain affinity constants were achieved by employing Graphpad Prism 9 (La Jolla, CA, USA) selecting the nonlinear one-site model.

Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) experiments were performed as described previously[44] using a BIAcore 2000 system (BIAcore AB, Uppsala, Sweden). Recombinant human fibrillin-1 protein covering the N-terminal region including EGF4 was covalently coupled to CM5 sensor chips at 3600 resonance units (RUs) using the amine coupling kit following the manufacturer's instructions (Cytiva, Uppsala, Sweden), and 0-320 nM of recombinant LTBP1 proteins were flown over in HBS-P buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 10 mM $CaCl_2$, 0.005% (v/v) surfactant P20). Affinity constants (K_Ds) were calculated by nonlinear fitting (1:1 interaction model with mass transfer) to the association and dissociation curves according to the manufacturer's instructions (BIAevaluation version 3.0 software). Apparent equilibrium dissociation constants (K_D values) were then calculated as the ratio of k_d/k_a.

300

301 **Zebrafish lines and maintenance.**

302 Zebrafish lines were housed in a Zebtec semi-closed recirculation housing system at a constant
303 temperature (27– 28 °C), pH (~7.5), conductivity (~550 µS) and light/dark cycle (14/10). Fish were fed
304 twice a day with dry food (Gemma Micro, Skretting) and once with artemia (Ocean Nutrition, Essen,
305 Belgium). *Ltbp1*$^{-/-}$ Δ29 and *ltbp1*$^{-/-}$ Δ35 zebrafish were generated using CRISPR-Cas9 mutagenesis
306 according to the workflow previously described[45]. Zebrafish were genotyped with primers listed in
307 **Supplemental Table S4**. We adhered to the general guidelines, in agreement with EU Directive
308 2010/63/EU for laboratory animals, for zebrafish handling, mating, embryo collection and
309 maintenance[46; 47]. Approval for this study was provided by the local committee on the Ethics of Animal
310 Experiments (Ghent University Hospital, Ghent, Belgium; Permit Number: ECD 17/63K and ECD
311 18/05).

312

313 **Echocardiography**

314 Ultrasound imaging was performed on 10 to 11-month-old male zebrafish using a dedicated
315 ultrasound apparatus Vevo 2100 (Visualsonics, Toronto, Canada) equipped with a high-frequency
316 linear array transducer (MS 700, frequency 30-70 MHz). Zebrafish were placed in an anesthetic
317 chamber containing 200 mg/L tricaine (Sigma-Aldrich). Zebrafish were transferred to a 3D printed
318 imaging chamber where the zebrafish was positioned ventral side up containing 100 mg/L tricaine to
319 minimize movements. Water temperature was maintained at 28°C throughout the whole procedure.
320 Image acquisition was conducted within 5 minutes after the induction of anesthesia.
321 Echocardiographic images were obtained in two planes: long axis (LAX), enabling normalized 2D
322 ventricular dimension parameters using the body surface area (BSA) normalization factor, and
323 abdominal-cranial axis (ACX), for color Doppler and pulse-wave Doppler image acquisition, enabling
324 cardiac function measurements[48; 49]. Measurements and functional calculations were performed in
325 Vevo LAB 1.7.0. Volumes of systole and diastole are calculated in Vevo LAB 1.7.0. based on the
326 geometry of Mammalian heart. Therefore, we reported the area of the systole and diastole normalized
327 to the body surface area[50]. Measurements were performed by a researcher blinded to the genotype.

328

329 **Whole Mount Staining with Alizarin Red S**

Alizarin Red staining for mineralized bone of 4-month-old adult zebrafish was performed as previously described[51]. Stained specimens were analyzed for the presence of ectopic bone with a Leica M165 FC Fluorescent Stereo Microscope (Leica Microsystems, GmbH, Wetzlar, Germany). Ectopic bone counts started from the second caudal vertebral body (VB) (with complete neural and haemal arches and complete neural and haemal spines) (VB16 – VB27). The vertebral columns were scored by two observers blinded to the genotype of the samples.

µCT analysis

For µCT-based phenotyping and quantification, four-month-old adult zebrafish were euthanized using an overdose of tricaine, fixed in 4% PFA for 48 hours and transferred to a 70% ethanol solution for scanning. Whole-body µCT scans of *Ltbp1*$^{-/-}$ Δ29 (n=5)*, ltbp1*$^{-/-}$ Δ35 (n=5) zebrafish and corresponding controls (n=4-5) were acquired on a SkyScan 1275 (Bruker, Kontich, Belgium) using the following scan parameters: 0.25 mm aluminum filter, 50 kV, 160 µA, 65 ms integration time, 0.5° rotation step, 721 projections/360°, and 21 µm voxel size. DICOM files of individual zebrafish were generated using NRecon V1.7.3.2 (Bruker) software, which were segmented in MATLAB using custom FishCuT software, followed by data analysis in the R statistical environment, as previously described[52; 53].

Statistical analysis

Statistical calculations, including multiple testing corrections, were performed using GraphPad Prism 9. P values < 0.05 were considered significant.

Results

Biallelic premature truncating variants in *LTBP1* cause cutis laxa with impaired craniofacial, skeletal and cardiac development

Table 1, **Figure 1**, and **Table S1** summarize and illustrate the clinical findings in all eight affected individuals. Detailed case reports and pedigrees are available in the Supplemental Data. Core clinical features include cutis laxa, craniosynostosis, short stature, and discernible craniofacial characteristics. Affected individuals show facial asymmetry, coarse facial features, arched eyebrows, proptosis, downslanting palpebral fissures, long eyelashes, a prominent nose with convex nasal ridge,

360 wide nasal bridge and broad nasal tip, sagging cheeks with prominent nasolabial folds, long philtrum,
361 thick lower lip vermillion, and a highly arched palate. Skin features include mild to moderate cutis laxa,
362 deep palmar creases, and bilateral inguinal hernia (F1:II-2, F2:V-3, F2:V-8 and F2:V-9). Individual
363 F1:IV-2 further presents with a congenital diaphragmatic hernia, but this is also present in her carrier
364 mother. All affected individuals, with the exception of F1:IV-2 and F2:V-4, present with
365 craniosynostosis, involving the coronal suture (F2:V-3 and F2:V-9), coronal, sagittal and lambdoid
366 suture (F2:V-8) or right coronal and sagittal suture (F3:II-1 and F3:II-2). Individual F4:II-1 shows
367 pansynostosis, resulting in a copper beaten skull on three dimensional reconstruction of computed
368 tomography images. In addition to short stature, most individuals show other skeletal abnormalities,
369 including brachydactyly (F1:IV-2, F2:V-3, F2:V-4, F2:V-8, F2:V-9, F3:II-1 and F3:II-2), clinodactyly of
370 the fifth finger (F1:IV-2, F2:V-3, F2:V-8, F2:V-9, F3:II-1, F3:II-2 and F4:II-1), syndactyly of the 2nd ,3rd
371 and 4th toe (F2:V-8, F2:V-9), syndactyly of the 2nd and 3rd toe (F2:V-3), syndactyly of the 4th and 5th
372 toe (F3:II-1 and F3:II-2), genua vara (F1:IV-2, F3:II-2 and F4:II-1), and joint hypermobility (F1:IV-2,
373 F3:II-1, F3:II-2 and F4:II-1). Less frequent skeletal findings include camptodactyly (F3:II-1 and F3:II-
374 2), scoliosis (F1:II-2), lumbar hyperlordosis (F1:II-2), hip dislocation (F2:V-3), and a short thorax with
375 pectus excavatum (F4:II-1). X-ray images of the spine from F1:IV-2 showed 'ovoid' shaped vertebral
376 bodies at the age of 3 years. No evidence for exostoses could be observed on the X-ray images, but
377 no CT scan was made to exclude this with more certainty.
378 Variable heart defects were found in three individuals. A moderate secundum atrial septum defect of
379 congenital origin with mild right ventricular volume overload was observed in F3:II-2. F1:IV-2 shows
380 mitral and tricuspid insufficiency, and mild concentric left ventricular hypertrophy is present in F2:V-3.
381 These observations are not considered to be from congenital origin. Neurodevelopment was normal in
382 most individuals, but F2:V-3 and F2:V-4 experience learning difficulties. Severe intellectual disability
383 of unknown cause has been recorded in individual F2:V-4. Cranial nerve dysfunction occurred in
384 family 2 and 3. In family 2, optic nerve hypoplasia and associated visual impairment is present in
385 individuals F2:V-4, F2:V-8 and F2:V-9, while F2:V-3, F2:V-8 and F2:V-9 have hearing loss. Both
386 affected individuals from family 3 display ophthalmoplegia due to a 3th and 4th cranial nerve palsy.
387 Feeding problems, attributable to gastroesophageal reflux or poor appetite, were recorded in F2:V-4,
388 F3:II-1, F3:II-2 and F4:II-1. Finally, urological abnormalities are observed in two families, including a
389 low and small right kidney in F1:IV-2 and left hydroureter in F2:V-8.

390

391 Exome sequencing (ES) identified homozygous premature truncating variants in *LTBP1* (RefSeq
392 NM_206943.3) in eight affected children from four different families. Prior to ES, homozygosity
393 mapping in family 2 showed one shared 22.9 Mb homozygous region on chromosome 2 (20,605,248-
394 43,530,418), containing the *LTBP1* gene, between the three affected individuals tested and absent in
395 unaffected family members. Family 1 and 3 harbor homozygous frameshift variants in *LTBP1*
396 consisting of a 1 base-pair (bp) (c.4844del, p.(Asn1615Ilefs*)) and 5 bp deletion (c.3391del5
397 p.(Thr1331Asnfs*20)), respectively. Family 2 and 4 harbor homozygous nonsense variants (c.
398 4431T>A, p.(Cys1477*) and c.1342C>T, p.(Gln448*) respectively). All variants segregate in family
399 members according to disease – and carrier status. According to different *in silico* algorithms, the
400 identified variants are predicted to be disease causing and all variants are absent from the population
401 databases. Schematic presentation of the corresponding alterations in LTBP1 and their amino acid
402 homology in other species are shown in **Figure 2**. We used dermal fibroblasts of F1:IV-2 and F4:II-1
403 in this study. Skin biopsies and dermal fibroblasts of F2:V-3, F2:V-4, F2:V-8, F2:V-9, F3:II-1, and
404 F3:II-2 are not available. Transmission electron microscopy (TEM) analysis of the dermis from a skin
405 biopsy was done in individual F1:IV-2 (**Figure 3**). The elastic fiber shows microfibril infiltration in its
406 periphery with mild fragmentation of elastin that still formed a central core (**Figure 3C-D**). Collagen
407 fibrils appear similar to the control subject with regular fiber diameters (**Figure 3E-H**).

408

409 ***LTBP1* deficient ECM responses are variant-specific**

410 We characterized *LTBP1* transcript and protein levels in dermal fibroblasts derived from individuals
411 F1:IV-2 and F4:II-1. Both variants (c.4844del and c.1342C>T respectively) are predicted to be
412 susceptible to NMD[54]. RT-qPCR indicates that *LTBP1* mRNA expression is completely abolished in
413 dermal fibroblast cultures of F4:II-1 (c.1342C>T) compared to control fibroblasts, but partly rescued
414 upon cycloheximide treatment, indicative of NMD (**Figure 4A**). In contrast, *LTBP1* mRNA expression
415 in dermal fibroblasts of F1:IV-2 (c.4844del) is present at equal levels as in fibroblasts of control
416 subjects (**Figure 4B**). In line with the mRNA expression data, immunofluorescent analysis at 9 days
417 post confluency (dpc) shows complete absence of LTBP1 in dermal fibroblasts of F4:II-1 (**Figure 4B**),
418 but rudimentary LTBP1 fibers in dermal fibroblasts of F1:IV-2 (**Figure 4D**). The C-terminus of LTBP1
419 interacts with the N-terminus of fibrillin-1 and fibrillin-2 (**Figure 2**). To evaluate the interaction of

420 truncated LTBP1 with fibrillin-1, we used recombinantly expressed C-terminal LTBP-1 fragments
421 containing the c.4844del and c.4431T>A variants in solid phase binding studies with the N-terminal
422 region of fibrillin-1 and fibrillin-2. Binding studies using surface plasmon resonance (SPR) showed that
423 both mutant LTBP1 fragments show negligible binding to the immobilized N-terminal region of fibrillin-
424 1 when compared to the control fragment ($K_D = 12 \pm 2$) (**Figure S2A-D**). Solid phase binding studies
425 in the opposite direction (LTBP1 immobilized; fibrillin-1 and -2 proteins incubated in solution) also
426 show a significant reduction of binding affinity of the N-terminal regions of fibrillin-1 (8- to 12-fold) and
427 fibrillin-2 (16- to 40-fold) to either mutant LTBP1 fragment (**Figure 2E-F, Figure S2E**). Taken
428 together, these results suggest that LTBP1 is loosely anchored to the fibrillin microfibril network
429 assembled by dermal fibroblasts derived from F1:IV-2.
430
431 We next analyzed the mRNA and protein expression of fibronectin and fibrillin-1, the most important
432 binding partners of LTBP1. *FN* mRNA (**Figure S3A,D**) and fibronectin protein expression in the
433 conditioned media (**Figure S3G-J**) and in the ECM fraction (**Figure S3K,L**) are unaltered in cultured
434 dermal fibroblasts from both affected individuals (F1:IV-2 (c.1342C>T) and F4:II-1 (c.4844del))
435 compared to control fibroblasts. In cultured dermal fibroblasts of F1:IV-2, *FBN1* mRNA (**Figure S3B**)
436 and fibrillin-1 protein expression in the conditioned media is equal to control fibroblasts (**Figure**
437 **S3M,N**), but fibrillin-1 immunofluorescent analysis shows increased fibrillin-1 deposition in the ECM
438 fraction (**Figure S3Q**). Of note, *FBN2* mRNA expression was significantly increased in cultured
439 dermal fibroblasts of F1:IV-2 (**Figure S3C**). In contrast, cultured dermal fibroblasts of F4:II-1 show
440 significantly reduced *FBN1* mRNA but normal *FBN2* mRNA levels (**Figure S3E,F**) and, accordingly,
441 significantly decreased fibrillin-1 protein presence in the conditioned media compared to control
442 fibroblasts (**Figure S3O,P**). Fibrillin-1 immunofluorescent analysis of the ECM fraction in cultured
443 dermal fibroblasts of F4:II-1 is comparable to control fibroblasts although the fibers appear more
444 patchy (**Figure S3R**).
445
446 In cultured dermal fibroblasts of F1:IV-2, *EFEMP2* (*FBLN4*) mRNA levels are normal, but EFEMP2
447 fibers are completely abolished in the ECM fraction (**Figure S4A,E**) suggesting that the presence of
448 the c.4844del variant interferes with EFEMP2 ECM incorporation. In contrast, cultured dermal
449 fibroblasts of F4:II-1 show normal abundance of EFEMP2 fibers in the ECM fraction but significantly

450 decreased *EFEMP2* mRNA levels (**Figure S4B,I**). We addressed gene and protein expression levels
451 of other members of the LTBP protein family. Immunofluorescent analysis shows a remarkable
452 increase in LTBP2 fibers in cultured dermal fibroblasts of F1:IV-2 at 9 dpc, while no change is
453 detected for F4:II-1 fibroblasts compared to control fibroblasts (**Figure S4C,D**). *LTBP3* gene
454 expression was significantly increased in cultured dermal fibroblasts of F1:IV-2 (**Figure S4G**) but
455 significantly decreased in cultured dermal fibroblasts of F4:II-1 (**Figure S4K**). *LTBP4* gene expression
456 remained equal between cultured dermal fibroblasts of F4:II-1, cultured dermal fibroblasts of F1:IV-2
457 and control fibroblasts (**Figure S4H,L**). Finally, *FBLN5* gene expression is unchanged in both affected
458 individuals (**Figure S5F,J)**.Together, these data indicate that complete loss (c.1342C>T) of LTBP1 or
459 the presence of C-terminally aberrant LTBP1 (c.4844del) have a different effect on ECM assembly.
460
461 **TGFβ signaling response to *LTBP1* deficiency is variant-specific**
462 LTBP1 interacts with the SLC and plays an important role in regulating the bioavailability of TGFβ in
463 the ECM. Therefore, we investigated the canonical TGFβ pathway in cultured fibroblasts derived from
464 affected individuals and control subjects. Total TGFβ protein levels are significantly increased in
465 conditioned media of F1:IV-2, but not of F4:II-1 compared to control subjects (**Figure 5A,D**).
466 Accordingly, the pSmad2/Smad2 ratio is significantly increased in cultured dermal fibroblasts of F1:IV-
467 2 at 1 dpc, but unaltered in cultured dermal fibroblasts of F4:II-1 compared to controls (**Figure 5G-J**).
468 Also, gene expression levels of the canonical (SMAD2/3-dependent) TGFβ-target genes[55; 56], *CTGF*
469 and *POSTN* are significantly upregulated in cultured dermal fibroblasts of F1:IV-2 at 1 dpc compared
470 to control fibroblasts (**Figure 5B,C**), while gene expression of *POSTN* remains equal and *CTGF* gene
471 expression is significantly decreased in cultured dermal fibroblasts of F4:II-1 at 1 dpc compared to
472 control fibroblasts (**Figure 5E,F**). However, *COL1A1*, *COL1A2*, and *COL3A1* are significantly
473 upregulated in cultured dermal fibroblasts of both F1:IV-2 and F4:II-1 at 1 dpc (**Figure 5O-T**).
474 Immunofluorescent analysis shows a remarkable increase in collagen I and collagen III fibers in
475 cultured dermal fibroblasts of F1:IV-2 at 9 dpc (**Figure 5K,M**), while collagen I and collagen III fibers
476 are equal in F4:II-1 fibroblasts compared to control fibroblasts (**Figure 4L,N**). Together, these data
477 indicate that complete loss (c.1342C>T) of LTBP1 or the presence of C-terminally aberrant LTBP1
478 (c.4844del) differently impact TGFβ signaling.
479

480 **Ltbp1 deficiency causes ectopic bone and reduced tissue mineral density in the zebrafish**
481 **skeleton but does not affect cardiac function.**
482 In order to further investigate the impact of *ltbp1* deficiency in an *in vivo* setting, we generated new
483 zebrafish models. *LTBP1* is well-conserved between humans and zebrafish, and zebrafish Ltbp1
484 protein shows a (predicted) domain homology similar to human LTBP1 (**Figure S5**). However, in
485 contrast to humans, zebrafish only express a long form of the *ltbp1* gene, and no other isoforms are
486 present in the genome. Using CRISPR-Cas9 technology, we generated two *ltbp1*$^{-/-}$ zebrafish models,
487 one harboring a 1 bp deletion, c.3525delG, in exon 29 and one harboring a 10 bp deletion,
488 c.4293delTGCGGTGTGC, in exon 35 (**Figure S6**). Both deletions result in a premature stop codon
489 and cause reduced *ltbp1* gene expression at the juvenile stage (**Figure S7A**). *Ltbp1*$^{-/-}$Δ29 zebrafish
490 lack 2 TGFβ-binding domains, 3 calcium-binding EGF-like domains, and 1 EGF-like domain at the
491 Ltbp1 C-terminus. *Ltbp1*$^{-/-}$Δ35 zebrafish lack the last calcium-binding EGF-like domains, and the last
492 EGF-like domain.
493
494 *Ltbp1*$^{-/-}$Δ29 and *ltbp1*$^{-/-}$Δ35 zebrafish have similar weight and length compared to WT siblings, show
495 Mendelian inheritance, and do not show premature mortality (**Figure S7B-C**). Investigation of the
496 skeletal phenotype demonstrated that the neural and haemal arches of the vertebrae of *ltbp1*$^{-/-}$Δ29
497 and *ltbp1*$^{-/-}$Δ35 zebrafish have ectopic bone formation (of intramembranous origin) (**Figure 6A-J**). In
498 addition, the arch bases that sit on the vertebrae clearly show more intramembranous bone (white
499 dotted lines in **Figure 6C,G,D,H**). Quantitative µCT analysis of four-month-old zebrafish reveals a
500 significant decrease in tissue mineral density (TMD) of the vertebral centrum and neural and haemal
501 associated elements of the skeleton in *ltbp1*$^{-/-}$Δ29 zebrafish compared to WT siblings (**Figure 6K,M,N**
502 **and Figure S8**). The volume of these skeletal elements tends to be increased in *ltbp1*$^{-/-}$Δ29 zebrafish,
503 although statistical significance is not reached. This finding is further supported by an increased
504 volume of the vertebrae observed in alizarin red-stained *ltbp1*$^{-/-}$Δ29 zebrafish vertebral columns
505 (**Figure 6D,H**). Bone thickness also tends to be increased (P-value < 0.07) in *ltbp1*$^{-/-}$Δ29 zebrafish.
506 Interestingly, quantitative µCT parameters were not different between *ltbp1*$^{-/-}$Δ35 zebrafish and WT
507 siblings (**Figure 6, Figure S8**). *Ltbp1*$^{-/-}$Δ29 and *ltbp1*$^{-/-}$Δ35 zebrafish have normal interfrontal, coronal,
508 sagittal, and lambdoid sutures (**Figure S8D-K**) and do not show alterations in cranial morphological
509 structures, including the hyomandibula, premaxilla, and basioccipital bone (**Figure S8D-K**). Since the

510 complete knockout of *ltbp1* in mice causes a severe cardiovascular phenotype, we investigated the
511 cardiac parameters. Assessment of cardiovascular function in adult zebrafish by ultrasound imaging
512 however revealed no significant differences between *Ltbp1*$^{-/-}$Δ29 zebrafish, *ltbp1*$^{-/-}$Δ35 zebrafish, and
513 corresponding WT siblings at 10 months of age (**Figure S9**). Taken together, *ltbp1*$^{-/-}$Δ29 zebrafish
514 reveal vertebral hypo-mineralization, voluminous vertebrae, and ectopic bone formation, but normal
515 heart function.
516
517 ***Ltbp1* deficiency causes abnormal collagen fibrillogenesis in zebrafish skin and intervertebral**
518 **ligaments.**
519 TEM analysis of skin biopsies of *ltbp1*$^{-/-}$Δ29 and *ltbp1*$^{-/-}$Δ35 zebrafish demonstrated an abnormal
520 dermal collagen architecture showing a folded appearance of the typical plywood-like organization.
521 (**Figure 7A-H**). In contrast, TEM of the notochord sheet part of the intervertebral disc, shows normal
522 diameters and structural organization of collagen type II (**Figure S10E-H**). Also, the immature
523 collagen deposited by osteoblasts in the outer edges of the intervertebral ligament appears normally
524 structured (**Figure S10A-D**). However, the mature collagen structure (**Figure 7I-P**) consistently shows
525 a lack of the plywood-like organization with a chaotic assembly of the collagen fibrils in intervertebral
526 ligament samples from *ltbp1*$^{-/-}$Δ29 and *ltbp1*$^{-/-}$Δ35 zebrafish. Taken together, our experiments highlight
527 a role for *ltbp1* in collagen architecture *in vivo* in zebrafish.
528
529 **Discussion**
530 We describe a novel AR CL syndrome caused by bi-allelic truncating variants in *LTBP1*. The
531 craniofacial features, short stature, brachydactyly, variable craniosynostosis, and variable mild heart
532 defects clearly distinguish this novel AR CL syndrome from other subtypes of CL syndrome. Because
533 of the pleiotropic manifestations, we propose the name *LTBP1*-related CL. The identified premature
534 truncating variants are distributed across the *LTBP1* gene and correspond to protein alterations in the
535 second (family 4, c.1342C>T) and third EGF-like domain (family 1, c.4844del), and the 12th (family 3,
536 c.3391del5) and 13th calcium-binding EGF-like domain (family 2, c.4431T>A) of the long isoform of
537 LTBP1[1]. We demonstrate distinct molecular consequences of truncating variants in *LTBP1* depending
538 on their position within the gene. No NMD is observed for the c.4844del variant, allowing for
539 rudimentary (altered) LTBP1 fiber formation in the ECM. In contrast, NMD is observed in the

540 c.1342C>T variant, resulting in absent LTBP1 protein expression in the ECM layer. Mutant LTBP1
541 protein expressed by c.4844del or c.4431T>A *LTBP1* variants shows reduced binding affinity for the
542 N-terminal regions of fibrillin-1 and fibrillin-2 causing loss-of-function.
543
544 Reduced LTBP1 binding to fibrillin-containing microfibrils would yield in LLCs that fail to be
545 targeted correctly to the ECM resulting in their inappropriate activation. Therefore, we hypothesize
546 that the increased TGF-β levels observed in F1:IV2 fibroblast culture is the result of an unstable
547 anchorage of LTBP1 to fibrillin microfibrils. Our finding of activated TGF-β signaling in F1:IV2
548 fibroblasts which may still express a C-terminally truncated form of LTBP1 is consistent with a
549 previous study in murine skin. Transgenic overexpression of a truncated LTBP1 variant that is still
550 capable to bind TGF-β but fails to interact with the ECM due to lack of the known N- and C-terminal
551 ECM-binding regions resulted in an excess of active TGFβ (Mazieri et al. 2005; doi:
552 10.1242/jcs.02352). Moreover, strongly increased ECM production (as evidenced by mRNA and/or
553 protein expression of collagens, FBN1 and LTBP2) in cultured dermal fibroblasts expressing the
554 c.4844del variant, may be secondary to aberrant canonical TGFβ growth factor activation[55; 56]. In
555 contrast, absent LTBP1 protein expression in the ECM layer (c.1342>T variant) does not alter
556 canonical TGFβ signaling and does not induce strong alterations of collagen I and III fiber
557 incorporation in the ECM of cultured fibroblasts. Hence, functional redundancy of other LTBP family
558 members may be sufficient for TGFβ transport and sequestering in absence of LTBP1[2]. Nevertheless,
559 newly produced collagen might be degraded by other specific factors such as matrix
560 metalloproteinases (MMPs) and trigger other pathological cascades[58].
561
562 In addition, absence of LTBP1 does not alter fibulin-4 deposition in the ECM layer, while the presence
563 of altered LTBP1 protein impedes fibulin-4 incorporation into the ECM. Fibulin-4 acts as an adaptor
564 molecule to guide tropoelastin and lysyl oxidase to fibrillin-containing microfibrils[59; 60]. *Efemp2*$^{R/R}$ mice
565 have mild elastic fiber alterations[61] in line with the observation of mild elastic fiber defects upon
566 ultrastructural analysis of a skin biopsy of the individual harboring the *LTBP1* c.4844del variant.
567 Concomitantly, we observed increased deposition of LTBP2 in the ECM. LTBP2 is a known
568 interaction partner of fibulin-5 and facilitates tropoelastin deposition in human dermal fibroblasts[14]. It is
569 tempting to hypothesize that LTBP2 and fibulin-5 might compensate for the loss of fibulin-4

incorporation in the ECM in human dermal fibroblasts. Further studies should confirm the distinct molecular consequences related to a loss of LTBP1 or altered LTBP1 expression in cultured dermal fibroblast samples derived from other diagnosed individuals with *LTBP1* variants in similar regions.

However, some differences in clinical features between F1:IV-2 and F4:II-1 may be at least partly TGF-β related. For instance, F1:IV-2 shows mitral valve prolapse (MVP) which was suggested to be caused by increased TGF-beta activity also in a mouse model of Marfan syndrome (Ng et al., 2004, doi: 10.1172/JCI22715). Also recently, increased circulating levels of TGFβ-1 and -2 were detected in young adults with MVP (Malev et al., 2021, https://doi.org/10.1016/j.ppedcard.2021.101347). A homozygous premature truncation mutation after 171 amino acids in *LTBP3* also causes MVP (Dugan et al., 2015; https://doi.org/10.1002/ajmg.a.37049), while a heterozygous missense mutation in *LTBP3* resulted only in a mildly thickened mitral valve with mild mitral regurgitation (McInerney-Leo et al., 2015; doi:10.1136/jmedgenet-2015-103647). Skin fibroblast from patients with this less severe *LTBP3* missense mutation also did not show any signs of increased total or activated levels of TGFβ (McInerney-Leo et al., 2015; doi:10.1136/jmedgenet-2015-103647), suggesting that only the *LTBP3* truncation variant leads to activated TGF-β and MVP similar to our findings in F1:IV-2. In addition, the occurrence of hernias was reported to be a feature of neonates with Marfan syndrome (Parida and Kriss, 1997; doi: 10.1002/(sici)1096-8628(19971017)72:2<156::aid-ajmg6>3.0.co;2-t; Herman et al., 2013, https://doi.org/10.1038/jp.2013.15), a disorder suggested to be generally driven by aberrant TGFβ activation. F4:II-1 did not present with mitral valve prolapse or hernias, but was initially presented with deformities of the skull. Craniosynostosis, a pathology that is closely linked to dysregulated TGFβ signaling[62], was also reported to be caused by a reduced bioavailability of TGFβ within the bone matrix due to the genetic ablation of *Ltbp3* in mice (*Ltbp3* KO Dabovic et al. 2002, doi.org/10.1083/jcb.200111080; Dabovic et al. 2002, doi.org/10.1677/joe.0.1750129). Also deformities of the skull were reported in LTBP1 null mice (Drews et al., 2008; doi: 10.1016/j.bbamcr.2007.08.004). These reports in mice are consisting with the idea of reduced TGFβ bioavailability in bone of F4:II-1. However, the mechanisms controlling the tissue bioavailability of TGF-β are likely tissue-specific. Depending on the tissue-specific ECM composition and biomechanical properties LTBP deficiency may have different effects on TGFβ bioavailability. In addition, LTBP1 might have other, yet unknown functions that cannot be compensated by other

600 LTBPs and are causative for the clinical features in the reported patients. These could include
601 unknown roles in modulating the deposition of ECM components or cell - matrix interactions.
602
603 Little is known about the role of *LTBP1* in chondrogenesis. LTBP1, fibrillin-1, and FN are localized in
604 developing long bones of *R. Novergicus*. LTBP1 and fibrillin-1 are present in the longitudinal fibrillar
605 structures in the outer periosteum and in the perichondrium, and in the layer of osteoblasts adjacent
606 to the surface of newly forming osteoid[63; 64]. Many microfibrillar genes have been associated with
607 short stature including *LTBP2*, *LTBP3*, *ADAMTS10*, *ADAMTS17*, *ADAMTSL2*, *FBN1*, and *FBN2*[65].
608 *FBN1* and *FBN2* may even cause opposite phenotypes depending on the domain harboring the
609 pathogenic variant [66-69]. How these defects affect ECM interactions and microenvironment and growth
610 factor signaling pathways in chondrocytes is poorly understood[70]. Genes involved in isolated and
611 syndromic forms of craniosynostosis suggest a link between fibroblast growth factor and TGFβ
612 signaling dysregulation[62] which suggests a delicate cellular and molecular interplay between
613 osteoblastogenic and osteoclastogenic pathways[71]. Of note, most craniosynostosis syndromes do not
614 present with clear cutaneous manifestation or short stature. In this context, growth factor signaling in
615 fibroblasts may not be fully representative for the molecular consequences in osteogenic pathways. At
616 least, our study adds a novel player to the short stature and craniosynostosis phenotypes.
617
618 Our *in vivo* experiments furthermore provide evidence that LTBP1 is required for proper cutaneous
619 and skeletal homeostasis in adult zebrafish. Both homozygous mutant zebrafish models have an
620 abnormal dermal collagen architecture showing a folded plywood-like organization, indicating skin
621 redundancy, the hallmark phenotype of CL syndrome, as well as abnormal fibrillogenesis in the
622 intervertebral ligament. *Ltbp1*$^{-/-}$Δ29 zebrafish have vertebral hypo-mineralization, voluminous
623 vertebrae, and ectopic bone formation. *Ltbp1*$^{-/-}$Δ35 zebrafish show normal mineralization but still
624 display ectopic bone formed by intramembranous ossification. Increased vertebral volume in zebrafish
625 associates with ECM defects[52]. PLOD2 deficiency in zebrafish, phenotypically concordant with clinical
626 findings in individuals with Bruck Syndrome, causes loss of the typical hourglass-shape morphology in
627 zebrafish due to excessive bone formation and therefore increases vertebral body thickness, and
628 disrupts type 1 collagen fibrillar organization in the bone[52]. Collagen maturation defects which are
629 clearly observed in *ltbp1*$^{-/-}$Δ29 and *ltbp1*$^{-/-}$Δ35 zebrafish could potentially contribute to the observed

ectopic bone formation. However, neither craniofacial abnormalities nor craniosynostosis were observed in both homozygous mutant zebrafish models. A possible explanation for the lack of these features could be the induction of genetic compensation mechanisms, which could partly rescue the craniosynostosis phenotype [72]. A recent study showed that knockdown of *ltbp1* leads to abnormal craniofacial cartilage structures in zebrafish embryos[73], suggesting a role in cartilage development which we did not observe in our models (data not shown). Considering the reduction of mutant *ltbp1* mRNA levels in *ltbp1*$^{-/-}$Δ29 and *ltbp1*$^{-/-}$Δ35 zebrafish (30-10% of WT ltbp1 levels, respectively), an RNA-less *ltbp1* allele model, which would preclude activation of the genetic compensation mechanisms[74], might be informative in this context. However, we cannot exclude the possibility that mutant Ltbp1 protein might still retain some level of activity, mitigating the severity of the observed phenotype. The C-terminal TGFβ-binding domains, absent in *ltbp1*$^{-/-}$Δ29 zebrafish but present in *ltbp1*$^{-/-}$Δ35 zebrafish may play a role in the observed differences in the bone mineral density. Indeed, excessive TGFβ-signaling has been implicated in the pathogenesis of osteogenesis imperfecta (MIM: 259420)[75]. Unfortunately, this hypothesis could not be confirmed nor rejected due the lack of suitable zebrafish TGFβ antibodies. Further studies should delineate if aberrant TGFβ signaling exists in *ltbp1*$^{-/-}$Δ29 zebrafish using a luciferase reporter assay driven by a TGFβ responsive promotor as feasible read-out.

Remarkably, *Ltbp1L*$^{-/-}$ and *Ltbp1*$^{-/-}$ mice present with truncus arteriosus, interrupted aortic arch and perinatal lethality. Our observations imply differences in functional redundancy of LTBP family members or other compensatory mechanisms for *Ltbp1* deficiency in mice versus humans and teleosts.

In conclusion, we identified bi-allelic truncating variants in *LTBP1* in a novel CL syndrome with altered skeletal development. Data from *in vitro* experiments on cultured fibroblasts show that different *LTBP1* truncating variants have distinct molecular signatures on ECM development and TGFβ signaling, depending on the absence or presence of mutated protein. Moreover, *ltbp1* deficiency in zebrafish confirms a prominent role for Ltbp1 in skeletal morphogenesis *in vivo*. Taken together, our data underscores the importance of the LTBP1 LLC in matrix assembly and bone homeostasis.

660 **Description of Supplemental Data:** Supplemental Data include 11 supplemental figures and 4
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662
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664
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686
687 **Web resources**
688 Polyphen-2, http://genetics.bwh.harvard.edu/pph2/
689 PhD-SNP, http://snps.biofold.org/phd-snp/phd-snp.html

690 SIFT, https://sift.bii.a-star.edu.sg/

691 SNAP, https://www.rostlab.org/services/SNAP/

692 MAPP, http://mendel.stanford.edu/sidowlab/downloads/MAPP/index.html

693 REVEL, https://sites.google.com/site/revelgenomics/

694

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941

942 **Figure 1: Clinical Characteristics.** Clinical pictures of F1:IV-2 (at age 3 years, A), F2:V-8 (at age 17
943 years, B), F2:V-9 (at age 9 years, C), F3:II-1 (at age 1.6 years, F and G), F3:II-2 (at age 4 years, H
944 and I), F4:II-1 (at age 2 years, J-L). Brachydactyly is observed in multiple families but clinical pictures
945 (D and E) are only available from Family 2. Short stature and ovoid-shaped vertebral bodies are
946 observed in F1:IV-2 (at age 3 years, M and N). A copper beaten calvarium and a coronal suture
947 (arrow) are present in F1:IV-2 (at age 3 years, O). Craniosynostosis involving the right coronal and
948 sagittal suture is observed in F3:II-2 (at age 6 months, P and Q). A copper beaten calvarium due to
949 high intracranial pressure is present in F4:II-1 (R and S). Pedigrees of all affected families can be
950 found in **Supplemental Figure 1**.
951

952 **Figure 2: Schematic representation of the truncating variants in the LTBP1 gene and in the**
953 **LTBP1 protein in 4 unrelated consanguineous families.** (A) Schematic representation of the
954 location of the 4 distinct LTBP1 variants identified in the corresponding affected families. The genomic
955 position of each variant is indicated on the exon structure of both the short (LTBP1S) and long
956 (LTBP1L) isoforms of the LTBP1 gene. (B) Sequence alignment shows conservation of the mutated
957 residues among different species. (C) Schematic representation of the domains of the LTBP1 protein.
958 LTBP1 consists of fifteen calcium-binding (cb) EGF-like domains, three EGF-like domains, two TGFβ-
959 binding domains, a hybrid motif and a 4-cysteine domain. The position of the corresponding
960 alterations on protein level are indicated by an asterisk.
961
962 **Figure 3: Ultrastructural analysis of the ECM in dermal biopsies.** Ultrastructural analysis of the
963 elastic fibers in a skin biopsy of affected individual F1:IV-2 (C-D) and a control subject (A-B).
964 Ultrastructural analysis of collagen structures in a skin biopsy of affected individual F1:IV-2 (G-H) and
965 control subject (E-F). Scale bar: 2 µm (A,C,E,G) and scale bar: 500 nm (B,D,F,H). The elastin core
966 (dotted while line) is surrounded by a spare mantle of microfibrils in the control subject (red dotted
967 line, white arrow). Note that microfibrils infiltrate into the periphery of the elastic fiber in a skin biopsy
968 of affected individual F1:IV-2 (red dotted line, black triangle). Col: collagen; Ef: elastic fiber; Mf:
969 microfibrils.
970
971 **Figure 4: Effect of the *LTBP1* variants on the assembly of LTBP1 in the ECM.** (A-C)
972 Quantification of *LTBP1* gene expression with and without CHX treatment by RT-qPCR. (B-D)
973 Representative images of immunofluorescent analysis of LTBP1 in 9 dpc fibroblast cultures derived
974 from affected individuals and control subjects. Scale bar represents 50 µm. (E-F) Solid-phase binding
975 assay of soluble LTBP1 fragments to immobilized N-terminal region of FBN1. The negative control
976 was incubated with buffer only. Results are representative of three independently conducted
977 experiments. Data are expressed as mean ± standard deviation (SD). **** P-value <0.0001. Two-
978 tailed unpaired t-test with Welch's correction was used for statistical analysis.
979
980 **Figure 5: *LTBP1* variant-specific canonical TGFβ signaling responses**

981 (A, D) Measurement of total TGFβ in 9 dpc conditioned media obtained from fibroblast cultures
982 derived from individuals F1:IV-2 and F4:II-1 and respective gender- and age-matched control
983 subjects. (B-C, E-F) Quantification of *CTGF* and *POSTN* gene expression by RT-qPCR. (G-J)
984 Immunoblot of intracellular lysates at 1 dpc obtained from fibroblast cultures derived from individuals
985 F1:IV-2 and F4:II-1 and respective gender- and age-matched control subjects. One of the control
986 subjects was stimulated with TGFβ as positive control. (K-L) Representative images demonstrating
987 immunofluorescent analysis of collagen I and collagen III fibers in 9 dpc fibroblast cultures stimulated
988 with ascorbate. Scale bar represents 50 μm. (O-T) Quantification of *COL1A1*, *COL1A2*, and *COL3A1*
989 gene expression by RT-qPCR after ascorbate stimulation. Data are expressed as mean ± SD. * P-
990 value < 0.05, ** P-value <0.01, **** P-value <0.0001. Two-tailed unpaired t-test with Welch's
991 correction was used for statistical analysis.
992
993 **Figure 6: *Ltbp1$^{-/-}$*Δ29 in zebrafish show hypo-mineralization and voluminous vertebrae with**
994 **ectopic bone.** (A) Quantification of the amount of ectopic bone present on the neural and haemal
995 arches of the caudal vertebrae of *ltbp1$^{-/-}$*Δ29 and *ltbp1$^{-/-}$*Δ35 zebrafish and corresponding WT siblings.
996 The amount of ectopic bone is significantly increased in *ltbp1$^{-/-}$*Δ29 and *ltbp1$^{-/-}$Δ35* zebrafish. (B)
997 Graphical representation of the amount of ectopic bone on the individually scored VBs of *ltbp1$^{-/-}$*Δ29
998 and *ltbp1$^{-/-}$*Δ35 zebrafish and corresponding WT siblings. (C-J) Representative images of the neural
999 and haemal arches on the vertebrae of *ltbp1$^{-/-}$*Δ29 and *ltbp1$^{-/-}$*Δ35 zebrafish and their respective WT
1000 siblings. Note that the shape of the neural and haemal part of the vertebrae is indicated with dashed
1001 lines. *Ltbp1$^{-/-}$*Δ29 zebrafish have more erratic and voluminous vertebral shapes than their respective
1002 WT siblings. Ectopic bone is indicated with a circle. (K-L) Quantitative μCT analysis of the vertebral
1003 column in five *ltbp1$^{-/-}$*Δ29 zebrafish versus five WT siblings and in five *ltbp1$^{-/-}$*Δ35 zebrafish versus four
1004 WT siblings at the age of four months. The bone volume, tissue mineral density (TMD) and bone
1005 thickness were calculated from the vertebral centrum. The x-axis represents individual abdominal and
1006 caudal VB along the anterior-posterior axis. The TMD is significantly reduced in the vertebral centrum
1007 of *ltbp1$^{-/-}$*Δ29 zebrafish compared to WT siblings. In contrast, equal TMD is observed in the vertebral
1008 centrum of *ltbp1$^{-/-}$*Δ35 zebrafish compared to WT siblings. Data were analyzed in the R statistical
1009 environment. (M-P) Representative 2D μCT images of the skeleton of *ltbp1$^{-/-}$*Δ29 and *ltbp1$^{-/-}$*Δ35
1010 zebrafish and corresponding WT siblings. Data are expressed as mean ± standard error of the mean

1011 (SEM) and analyzed in the R statistical environment in K and L. Data are expressed as mean ± SD in
1012 A. * P-value < 0.05, ** P-value <0.01. Two-tailed unpaired t-test with Welch's correction was used for
1013 statistical analysis in A. Eb: ectopic bone; ha: haemal arch; hs: haemal spine; HA: hydroxyapatite; na:
1014 neural arch; ns: neural spine; prez: prezygapophysis; pstz: postzygapophysis; vc: vertebral column.
1015

1016 **Figure 7: LTBP1 deficiency causes abnormal collagen fibrillogenesis in skin and intervertebral**
1017 **ligaments.** (A-H) Representative images of ultrathin sections taken from the dermis of 4-months old
1018 adult *ltbp1*$^{-/-}$ Δ29, *ltbp1*$^{-/-}$ Δ35 zebrafish and corresponding WT siblings. Increased interfibrillar spaces
1019 and disorganized collagen architecture are noted in *ltbp1*$^{-/-}$Δ29 and *ltbp1*$^{-/-}$Δ35 zebrafish samples. Col:
1020 collagen; f: fibroblast; m: muscle, n: nucleus; p: pigmentation; sc: stratum compactum. Scale = 1 µm
1021 in A-D, scale = 200 nm in E-H. (I-P) Representative images of ultrathin parasagittal sections showing
1022 internal structures of zebrafish vertebral centra and intervertebral ligament of 4-months-old adult *ltbp1*$^{-}$
1023 $^{/-}$Δ29 and *ltbp1*$^{-/-}$Δ35 zebrafish and corresponding WT siblings. Note that the notochord sheet is
1024 composed of collagen type II. Collagen type II is secreted by the chordoblasts lining the notochord
1025 sheet on the inside and in-between the chordocytes and the notochord sheet. Abnormal mature
1026 collagen architecture is noted in adult *ltbp1*$^{-/-}$Δ29 and *ltbp1*$^{-/-}$Δ35 zebrafish compared to corresponding
1027 WT siblings. Ac: autocenter; b: bone; cb: chordoblasts; colII: collagen type II (notochord sheet); e:
1028 outer elastin layer; imc: immature collagen; mc: mature collagen; nc: vacuolated notochord cells
1029 (chordocytes). Scale = 200 µm in I-L, scale = 500 nm in M-P.
1030

1031 **Table 1: Overview of homozygous genotypes and clinical characteristics.**

Proband number Family	1 F1:IV-2	2 F2:V-3	3 F2:V-4	4 F2:V-8	5 F2:V-9	6 F3:II-1	7 F3:II-2	8 F4:II-1	Number and % affected individuals
Demographic features									
Age at last evaluation	3y4mo	17y5mo	16y	17y	9y	4y	1y6mo	1y9mo	
Sex	F	F	F	M	F	F	M	F	
Parental consanguinity	+	+	+	+	+	+	+	+	
Ethnicity	Turkish	Pakistani	Pakistani	Pakistani	Pakistani	Pakistani	Pakistani	Saudi Arabic	
Clinical characteristics									
Craniofacial dysmorphism									
Coarse face	+	+	-	+	+	+	+	+	7/8 (87.5%)
Arched eyebrows	-	+	+	+	+	+	+	-	6/8 (75%)
Proptosis	-	+	-	+	+	+	+	+	6/8 (75%)

Downslanted palpebral fissures	+	+	-	+	+	-	-	+	5/8 (62.5%)
Long eyelashes	+	+	+	+	+	+	+	Unknown	7/8 (87.5%)
Convex nasal ridge	-	+	+	+	+	+	+	+	7/8 (87.5%)
Wide nasal bridge and broad tip	+	+	+	+	+	+	+	+	8/8 (100%)
Sagging cheeks	+	+	-	+	+	+	+	+	7/8 (87.5%)
Prominent nasolabial folds	+	+	-	+	+	-	+	+	6/8 (75%)
Long philtrum	+	+	-	+	+	+	+	+	7/8 (87.5%)
Thick lower lip vermillion	+	+	-	+	+	+	+	+	7/8 (87.5%)
Highly arched palate	-	-	+	+	+	+	+	-	5/8 (62.5%)
Connective tissue features									
Cutis laxa	+	+	+	+	+	+	+	+	8/8 (100%)
Deep palmar creases	+	+	-	+	+	+	+	+	7/8 (87.5%)
Inguinal hernia	+	+	-	+	+	-	+	-	5/8 (62.5%)
Skeletal features									
Craniosynostosis	-	+	-	+	+	+	+	+	6/8 (75%)
Short stature	+	+	+	+	+	+	+	+	8/8 (100%)
Brachydactyly	+	+	+	+	+	+	+	-	7/8 (87.5%)
Clinodactyly	+	+	-	+	+	+	+	+	7/8 (87.5%)
Syndactyly	-	+	-	+	+	+	+	-	6/8 (75%)
Joint hyperlaxity	+	-	-	-	-	+	+	+	4/8 (50%)
Genua vara	+	-	-	-	-	-	+	+	3/8 (37.5%)
Additional features									
Learning difficulties	-	+	+	-	+	-	-	Unknown	3/8 (37.5%)
Cardiac abnormalities	+	+	-	-	-	-	+	-	3/8 (37.5%)
Hearing loss	-	+	-	+	+	-	-	-	3/8 (37.5%)
Feeding problems/ GER	-	-	-	-	-	+	+	+	3/8 (37.5%)
Urological abnormalities	+	-	-	-	+	-	-	-	2/8 (25%)
Molecular characteristics									
cDNA change	*LTBP1* c.4844del	*LTBP1* c.4431T>A	*LTBP1* c.4431T>A	*LTBP1* c.4431T>A	*LTBP1* c.4431T>A	*LTBP1* c.3391del5	*LTBP1* c.3391del5	*LTBP1* c.1342C>T	
Protein change	p.Asn1615Ilefs*	p.Cys1477*	p.Cys1477*	p.Cys1477*	p.Cys1477*	p.Thr1331Asnfs20	p.Thr1331Asnfs20	p.Gln448*	

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