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Loss of Type I Collagen Telopeptide Lysyl Hydroxylation Causes Musculoskeletal Abnormalities in a Zebrafish Model of Bruck Syndrome

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Abstract

Bruck syndrome (BS) is a disorder characterized by joint flexion contractures and skeletal dysplasia that shows strong clinical overlap with the brittle bone disease Osteogenesis Imperfecta (OI). BS is caused by bi-allelic mutations in either the *FKBP10* or the *PLOD2* gene. *PLOD2* encodes the lysyl hydroxylase 2 (LH2) enzyme, which is responsible for the hydroxylation of lysine residues in fibrillar collagen telopeptides. This hydroxylation directs cross-linking of collagen fibrils in the extracellular matrix, which is necessary to provide stability and tensile integrity to the collagen fibrils. To further elucidate the function of LH2 in vertebrate skeletal development, we created a zebrafish model harboring a homozygous *plod2* nonsense mutation resulting in reduced telopeptide hydroxylation and cross-linking of bone type I collagen. Adult *plod2* mutants present with a shortened body axis and severe skeletal abnormalities with evidence of bone fragility and fractures. The vertebral column of *plod2* mutants is short and scoliotic with compressed vertebrae that show excessive bone formation at the vertebral end plates, and increased tissue mineral density in the vertebral centra. The muscle fibers of mutant zebrafish have a reduced diameter near the horizontal myoseptum. The endomysium, a layer of connective tissue ensheathing the individual muscle fibers, is enlarged. Transmission electron microscopy of mutant vertebral bone shows type I collagen fibrils that are less organized with loss of the typical plywood-like structure. In conclusion, *plod2* mutant zebrafish show molecular and tissue abnormalities in the musculoskeletal system that are concordant with clinical findings in BS patients. Therefore, the *plod2* zebrafish mutant is a promising model for the elucidation of the underlying pathogenetic mechanisms leading to BS and the development of novel therapeutic avenues in this syndrome.

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Keywords

BRUCK SYNDROME; OSTEOGENESIS IMPERFECTA; PLOD2; LYSYL HYDROXYLASE 2; COLLAGEN

INTRODUCTION

Bruck syndrome (BS) is a rare congenital connective tissue disorder first described a century ago⁽¹⁾. The hallmark of BS is a combination of joint contractures with various skeletal anomalies. The contractures are mainly flexion deformities of the large joints (knees, elbows, ankles) with pterygia, and to a lesser extent flexion deformities of the small joints (camptodactyly, adducted thumb). The skeletal anomalies include generalized osteoporosis and bone fragility resulting in fractures starting in infancy or early childhood. The vertebrae are severely malformed with platyspondyly, leading to a severely shortened trunk, dwarfism and progressive kyphoscoliosis. The extremities show curvature deformities of the long bones that are slender and have thin cortices. Macrocephaly with Wormian bones might be present, while dentinogenesis imperfecta is uncommon^(2,3).

BS is a monogenic disorder caused by bi-allelic mutations in either the *FKBP10* or the *PLOD2* gene, resulting in respectively type I and type II BS, which are clinically indistinguishable. With regard to the skeletal anomalies and bone fragility, BS shows significant clinical overlap with Osteogenesis Imperfecta (OI). OI is usually inherited as an autosomal dominant (AD) disorder, caused by a heterozygous mutation in either *COL1A1* or *COL1A2*, encoding the $\alpha 1$ and $\alpha 2$ chain of type I (pro)collagen, respectively⁽²⁾. A small proportion of patients have an autosomal recessive form of OI due to bi-allelic mutations in genes encoding key players in the biosynthesis and processing of type I collagen, osteoblast differentiation and function or bone matrix mineralization⁽³⁾.

Type I (pro)collagen belongs to the family of the fibril-forming collagens and is the major extracellular matrix (ECM) protein of bone, dentin and skin. Type I procollagen is composed of two pro $\alpha 1$ (I) chains and one pro $\alpha 2$ (I) chain that fold into a heterotrimeric triple helix. The pro α chains contain a central core of uninterrupted repeats of the Gly-X-Y tripeptide, flanked by globular propeptide domains at the amino (N-) and carboxyl (C-) ends which are necessary for chain association and triple helix formation. These pro α chains are synthesized at the rough endoplasmic reticulum (ER), post-translationally modified in the ER lumen and subsequently assembled into a trimeric procollagen molecule. This procollagen molecule is then transported through the trans-golgi network to the extracellular matrix (ECM). The propeptides are serially removed first by C- then N-propeptidases preceding and during the process of assembly of mature triple helical collagen molecules into fibril polymers⁽⁴⁾. Collagen molecules become cross-linked covalently by lysyl oxidase during this process. The unique pattern of cross-linking of bone collagen is believed to be important for the normal mineralization and material strength of bone tissue.

The causal genes for BS (*PLOD2*, *FKBP10*) encode ER-residing proteins that play an important role in the biosynthesis of type I (pro)collagen. FKBP65, encoded by the *FKBP10* gene, functions as a molecular chaperone that aids in the folding of type I procollagen⁽⁵⁾.

PLOD2 encodes lysyl hydroxylase 2 (LH2), which is the enzyme responsible for the hydroxylation of lysine residues in collagen telopeptides. Telopeptide hydroxylysines are essential for the hydroxyallysine pathway of cross-linking, which produces mature (permanent) lysylpyridinoline and hydroxylslypyridinoline cross-links in extracellular collagen fibrils. A combination of these mature cross-links and labile cross-links formed from lysine aldehydes are believed to be required for the normal material properties of bone^(6–9). Patients suffering from BS have reduced levels of hydroxyallysine-derived cross-links in bone type I collagen^(10,11).

Despite the finding that BS is generally caused by defective type I collagen cross-linking, many questions remain to be answered regarding the pathogenesis of this disease. First, although BS and OI patients both suffer from a decrease in bone strength and an increase in bone fragility that lead to a higher susceptibility to fractures, both syndromes show a large clinical variability that is still poorly understood. Second, in BS, defects in LH2 and FKBP65 result in a phenocopy, both clinically and in terms of abnormal collagen cross-linking, while the role of FKBP65 (*FKBP10*) in collagen cross-linking and its relation to LH2 (*PLOD2*) has yet to be established. Third, the pathogenic basis of the congenital joint contractures in BS and how they are linked to defective collagen cross-linking remain unsolved. Therefore, a better understanding of the pathogenetic mechanisms in BS and OI is needed to answer these questions and to fully establish gene-phenotype relationships. Moreover, this will aid in the development of more effective therapeutics, as current therapeutic strategies for BS and OI fail to positively affect the quality of the bone.

Insights into the disease mechanisms underlying skeletal disorders such as BS and OI are strongly coupled to the tractability of model systems available to understand their pathogenesis. Growing studies suggest the small fresh water teleost *D. rerio* (zebrafish) may serve as a model for skeletal dysplasias^(12–14). As zebrafish are aquatic animals, the weight-bearing demand on the skeleton is reduced, enabling many skeletal mutant zebrafish lines to survive into adulthood far longer than their orthologous mouse counterparts, even those that may be embryonic lethal. Furthermore, the molecular basis and the key regulators of skeletogenesis appear to be highly conserved between zebrafish and mammals^(15,16). The broad availability of transgenic zebrafish lines expressing fluorescent proteins under the control of skeletal specific promoters enables *in vivo* visualization of cell and signaling dynamics that are difficult to resolve in mammalian systems. Finally, the amenability of zebrafish to genetic analysis has facilitated the rapid generation of mutant lines for cartilage and bone genes to understand their functional roles in skeletogenesis^(13,17,18). These lines include the zebrafish mutants *frilly fins*, *microwaved* and *chihuahua*, that have been reported to model aspects of human OI^(19,20).

Plod2-null mice do not survive beyond embryonic day 12⁽²¹⁾. Thus, a viable animal model of BS caused by mutations in *PLOD2* has yet to be identified. In this work we show that a zebrafish mutant carrying a nonsense mutation in *plod2* displays molecular and tissue abnormalities in the musculoskeletal system resembling those in human patients with BS. Hence, this mutant can contribute to a better understanding of the pathogenetic mechanisms in BS caused by *PLOD2* mutations, and by virtue of the unique attributes of zebrafish, accelerate the identification of new therapeutic targets.

MATERIAL AND METHODS

Animals

plod2^{sa1768} mutant zebrafish were generated by the zebrafish mutation project (ZMP)⁽²²⁾. *plod2*^{sa1768} mutant and wild-type AB fish were obtained from the Zebrafish International Resource Center (ZIRC, <http://zebrafish.org>). Heterozygote *plod2*^{sa1768} mutant zebrafish were incrossed to obtain homozygous *plod2* mutants. Fish were housed in ZebTEC semi-closed recirculation housing systems (Techniplast, Italy) and were kept at a constant temperature (27–28°C), pH (7.5) and conductivity (500 µS) on a 14/10 light/dark cycle. Fish were fed three times a day with dry food (Gemma Micro, Skretting, UK) and brine shrimps (Ocean Nutrition, Belgium). Embryos were collected by natural spawning, staged and raised according to Kimmel and colleagues⁽²³⁾. All animal studies were performed in agreement with EU Directive 2010/63/EU for animals, permit Number: ECD 15/68.

Alcian Blue and Alizarin red staining

Cartilage was stained with Alcian Blue, using a modified protocol of Neuhauss *et al.*⁽²⁴⁾ (details in supplement) and stained specimens were analyzed under a Zeiss Axio Imager Microscope and photographed using an Axiocam MRC camera. Mineralized bone was stained with Alizarin Red using a modified protocol of Spoorendonk *et al.*⁽²⁵⁾ for larvae and as described by Lewis⁽²⁶⁾ for adult fish (details in supplement), and the stained specimens were examined under a Leica M165 FC Fluorescent Stereo Microscope.

Histology and Transmission Electron Microscopy (TEM)

Fish were fixed and embedded in epon following the procedures outlined by Huysseune & Sire⁽²⁷⁾ (details in supplement). All semi-thin sections were examined using a Zeiss Axio Imager Microscope and photographed using an Axiocam MRC camera. Ultrathin sections were observed in a Jeol JEM 1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan). Pictures were digitized using a Ditabis system (Ditabis Ltd., Pforzheim, Germany).

µCT-scanning analysis

For µCT-based phenotyping and quantification, adult zebrafish were euthanized using 0.4% tricaine, stored frozen, and thawed immediately prior to scanning. Whole body µCT scans of 3 mutant and 3 control fish were acquired on a Scanco Viva CT 40 using the following scan parameters: 55kV, 145µA, 200ms integration time, 500 proj/180°, and 21µm voxel size. For each vertebral body, the haemal arch/ribs, centrum, and neural arch were manually segmented, and the mean tissue mineral density (TMD) and bone thickness computed. TMD was defined as previously described⁽²⁸⁾, i.e. the mineral density in only thresholded and segmented out bone was taken into account. A global test for association of groups of covariates/features with a response variable⁽²⁹⁾ was used to assess for phenotypic alterations across all vertebrae with genotype. For each fish, standard length was measured on maximal projections of scans. For whole-body µCT projections, adult specimens were fixed in 4 % paraformaldehyde for 48 hours and then transferred to a 70% ethanol solution for scanning. Specimens were scanned with the HECTOR µCT scanner at the Centre for X-ray Tomography of the Ghent University (UGCT, www.ugct.ugent.be)^(30,31) using a tube voltage

of 130kV, 15W target power, 1s integration time, 1801 projections, and 13.5µm voxel size. All projections were reconstructed using the Octopus-package developed at UGCT (<http://www.octopusreconstruction.com>)⁽³²⁾.

Immunoblotting

Embryos were collected at 2 dpf, chorion and yolk sac were manually removed and protein extracts were prepared from whole embryos in 1xSDS-laemmli buffer containing 5% 2-mercapto-ethanol. For adult specimens the caudal fin was amputated, tissues were collected in 1xSDS-laemmli buffer containing 5% 2-mercapto-ethanol and homogenized using a TissueLyzer (Retsch, Germany). All lysates were subjected to SDS-PAGE using a 4–12% Bis-Tris gel (Invitrogen, NuPAGE). Subsequently, proteins were transferred to a nitrocellulose membrane using the Mini Trans-Blot® Electrophoretic Transfer Cell system (Bio-rad, USA), immunolabeled with anti-LH2 primary antibody (21214-1-AP, Proteintech, USA) and horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technologies, USA). Membranes were developed using the SuperSignal® West Dura Extended Duration Substrate kit (ThermoScientific, Life Technologies Europe, Belgium) and scanned with the ChemiDoc-It® 500 Imaging System (UVP, USA). Following stripping, the membrane was reprobed with α-tubulin (Sigma-Aldrich, USA) in order to check for equal loading. Intensities were quantified using Fiji software⁽³³⁾.

Collagen cross-linking analysis

Adult zebrafish were euthanized with 0.4% tricaine and vertebral bone was dissected manually from two *plod2* mutant and two control fish, residual soft tissue was removed by incubation in Accumax solution (Sigma-Aldrich) for 1 hour. Bone was demineralized in 0.1 M HCl at 4°C, washed, and solubilized by heat denaturation in SDS-PAGE sample buffer. The method of Laemmli was used with 6% gels for the denaturant extracts. Collagen α-chains were cut from SDS-PAGE and subjected to in-gel trypsin digestion. Electrospray MS was performed on tryptic peptides using an LTQ XL ion-trap mass spectrometer (Thermo Scientific) equipped with in-line liquid chromatography using a C4 5µm capillary column (300µm × 150mm; Higgins Analytical RS-15M3-W045) eluted at 4µl min. The LC mobile phase consisted of buffer A (0.1% formic acid in MilliQ water) and buffer B (0.1% formic acid in 3:1 acetonitrile:n-propanol v/v). The LC sample stream was introduced into the mass spectrometer by electrospray ionization (ESI) with a spray voltage of 4kV. Proteome Discoverer search software (Thermo Scientific) was used for peptide identification using the NCBI protein database. Proline and lysine modifications were examined manually by scrolling or averaging the full scan over several minutes so that all of the post-translational variations of a given peptide appeared together in the full scan.

RESULTS

Zebrafish *plod2* nonsense mutation resides in highly conserved catalytic domain of lh2

The zebrafish *plod2* gene resides on chromosome 24 and spans a region of 77 kb. Its genomic structure is highly similar to that of the human *PLOD2* gene as it consist of an equal number of exons (Fig. 1A). Both orthologues encode 2 differentially spliced transcripts, which differ by the presence or absence of a 63bp exon 13a. Alignment of the

zebrafish and human lysyl hydroxylase 2 (lh2/LH2) protein sequence, which is encoded by *plod2/PLOD2*, revealed an overall 69 % amino acid (AA) similarity (Fig. 1B). The highest AA conservation (81%), can be found in the C-terminal region of the protein, encoded by the last 4 exons, and containing the catalytic domain of the protein which is vital for its lysyl hydroxylase activity, suggesting a similar enzymatic function for both orthologues. The zebrafish mutant that we report here (ZIRC, sa1768) harbors a homozygous c.2037T>A mutation in *plod2*, generating a premature stop codon in exon 18 at AA position 679 (p.Y679X), located centrally in the highly conserved catalytic domain.

Reduced telopeptide lysyl hydroxylation with aberrant cross-linking of bone type I collagen in *plod2* mutants

The presence of a nonsense mutation in *plod2* predicts nonsense-mediated decay of mutant *plod2* mRNA and consequently diminished levels of lh2 protein. In order to assess lh2 protein levels in the *plod2* mutant fish, immunoblotting was performed on protein extracts prepared from whole embryos at 2 days post fertilization (dpf) and from amputated caudal fins from adult fish. In homozygous mutant embryos and adult fins, a mean lh2 reduction of resp. 60% and 65% was observed (Fig. 2). Although a residual signal is still detected upon immunoblotting in *plod2* mutants, this signal could be explained by cross reaction of the antibody with the lh2 paralogues lysyl hydroxylase 1 (lh1) and lysyl hydroxylase 3 (lh3), which share the same approximate molecular weight (85 kDa) and have a significant sequence similarity with lh2. It is unlikely that this residual signal accounts for lh2 encoded by mutant *plod2* mRNA that escapes NMD, as the presence of the non-sense mutation would result in a 6 kDa smaller protein which would cause a band shift upon immunoblotting. To evaluate the consequences of lh2 protein reduction on type I collagen modification in bone, we examined the hydroxylation status of the C-telopeptide lysines, which are crucial for intermolecular cross-linking in collagen fibrils of bone matrix and are conserved residues in zebrafish and human type I collagen⁽³⁴⁾. For this purpose, collagen was extracted from vertebral bone dissected from *plod2* mutant and control fish and subjected to SDS-PAGE (Fig. 3A). After electrophoresis, an increase in the relative amount of extractable type I collagen was shown, consistent with a decrease in mature cross-links^(10,11) in *plod2* mutant bone when compared to control. Individual collagen chains were excised from the gel, digested with trypsin and lysine hydroxylation was assessed by mass spectrometry in targeted peptides. While 75% of the K1029 residues in the C-telopeptide of $\alpha 1(I)$ were shown to be hydroxylated in samples from control fish, a complete loss of K1029 hydroxylation was evident for *plod2* mutant fish (Fig. 3A–B). Since in zebrafish a paralogue of the $\alpha 1(I)$ encoding *colla1a* gene exists, namely *colla1b*, which encodes the $\alpha 3(I)$ chain, we also analyzed the equivalent tryptic peptide from $\alpha 3(I)$ and observed similar results as for $\alpha 1(I)$ (data not shown): The K1029 residue in the $\alpha 3(I)$ C-telopeptide was 70 % hydroxylated in samples from control fish while in *plod2* mutant fish there was a complete lack of hydroxylation at this residue. These results confirm the dysfunctionality of lh2 in *plod2* mutant fish. Interestingly, the mass spectra revealed evidence of partial additional prolyl hydroxylation at residues P1001 and P1013 in *plod2* mutant bone but not control. The significance and exact nature of this apparent overmodification is unclear but could reflect a suspected interplay between lysyl and prolyl hydroxylation mechanisms in the ER during synthesis^(9,35).

***Plod2* mutants display severe skeletal and muscular malformations**

Homozygous *plod2* mutant fish were generated at an expected Mendelian ratio and though there is higher mortality, most survive into adulthood. As adults, *plod2* mutant fish have a shorter standard length (20.1 ± 1.2 mm, age 6 months) when compared to their wild type siblings (27.9 ± 0.7 mm, age 6 months) ($p < 0.001$) and they display cranial malformations that are most apparent in the jaws, the opercular apparatus and the neuro-cranial roof (Fig. 4A–B). Within the axial skeleton μ CT reconstructions revealed that *plod2* mutant adult fish have a severely malformed vertebral column with compression of the vertebrae along the anteroposterior axis and marked kyphoscoliosis (Fig. 4B). Abundant ectopic bone formation was noted at the vertebral body centra throughout the vertebral column.

Quantitative μ CT analysis revealed multi-faceted effects on vertebral morphology and mineralization. Vertebral body (centrum) tissue mineral density (TMD) and thickness were both significantly elevated in the vertebral column of *plod2* mutant fish ($p < 0.01$) (Fig. 4C). Intervertebral variability for these quantitative characters was increased in *plod2* mutant fish compared to the control group. Analysis of the haemal arch, neural arch, and ribs revealed no significant effects of the *plod2* mutation on bone thickness or TMD. Consistent with an overall reduction in standard length, the length of vertebral body centra was significantly reduced in *plod2* mutant fish ($p < 0.001$). The length of neural arches ($p < 0.05$) and haemal arches/ribs ($p = 0.06$) were also reduced, though the latter did not reach statistical significance (Fig. S1).

Whole-mount alizarin red staining for mineralized bone further illustrated that vertebral bodies in *plod2* mutant fish are deformed through ectopic bone formation located at the vertebral endplates. Excess bone causes a loss of the typical hourglass-shape morphology of zebrafish vertebral bodies (Fig. 5A). Bowing and kinking was observed in the ribs and in the hypurals of the caudal fin endoskeleton (Fig. 5B and 5C). Bone callus formation in ribs of mutant zebrafish is indicative for fracture repair (Fig. 5D). Fractures are likely a result of increased bone fragility. Toluidine blue stained semi-thin sections (Fig. 6A–B) revealed excess bone, altering the shape of the vertebral bodies and causing the protuberances that were also observed on whole mount stains (Fig. 5A). Further, cross sections illustrated that the horizontal myoseptum, which functions as a tendon transducing force from the contracting trunk muscles towards the vertebral column⁽³⁶⁾, was also affected. The endomysium, a layer of connective tissue ensheathing the muscle fibers, was enlarged along the horizontal myoseptum of *plod2* mutant fish (Fig. 6C). In addition, muscle fibers appeared smaller in diameter and were more densely packed. Sagittal sections confirmed the compressed nature of the vertebral bodies in mutant as opposed to regularly formed vertebrae in wildtype fish (Fig. 7A). Excess bone was developed around the notochord and showed irregular remodeling (Fig. 7B). Vertebral endplates and intervertebral space were distorted in mutant fish and lacked the orderly stratification (Fig. 7C). In return, the notochord epithelium had significantly proliferated and the notochord sheath was greatly expanded in the mutant fish.

The notochord sheath of *plod2* mutant larvae displays structural defects

To determine at what stage the musculoskeletal abnormalities arise in the *plod2* mutant fish, 8 dpf, 15 dpf and 20 dpf larvae were stained with Alcian Blue and Alizarin Red to visualize cartilage and mineralized structures. No abnormalities in the cranial cartilage could be observed at these time points (data not shown). However, from 15 dpf onwards, severe structural defects, including a disturbed vertebral centra formation, protuberance and kinking of the notochord sheath, could be seen in mutant larvae (Fig. 8A). Sagittal sections at 15 dpf revealed a wavy appearance of the notochord, confirming the phenotype observed in Alcian Blue stained whole mounts, as well as a precocious thickening of the notochordal sheath (Fig. 8B), which was also detected in the adult mutants (Fig. 7C). Alizarin Red staining in control and mutant fish at 20 dpf showed a similar pattern of segmented notochord sheath mineralization in wild type and mutant fish. However, the notochord showed the same protuberances as seen upon Alcian Blue staining (Fig. 8C). This finding was expected since Alizarin Red indicates the mineralized portion of the notochord sheath, which itself was deformed at this stage.

Type I collagen exhibits a disturbed fibrillar organization in *plod2* mutant fish

In zebrafish, after initial mineralization of the notochord sheath, bone collagen is deposited by osteoblasts around the mineralized notochord sheath, especially in the vertebral body growth zone that is located at the periphery of the vertebral end plates. This collagen matures with age while developing a more pronounced banding pattern and a better organized structure, causing collagen fibril bundles to increase in density and thickness from the outer to the inner bone layer, with their orientation gradually becoming more ordered⁽³⁷⁾. To assess bone collagen fibrillar architecture in the vertebrae of *plod2* mutant fish, TEM photographs were acquired in the vertebral body growth zone (Fig. 9A). In general, bone type I collagen in *plod2* mutant fish displayed a lower degree of organization, which is especially evident in mature collagen adjoining the notochord. In wild type fish this collagen is present in layers exhibiting the typical plywood-like organization for bone, while in mutant fish this is not observed (Fig. 9B).

The notochord sheath, a cartilage-like extracellular matrix structure that surrounds the notochord, was also examined using TEM analysis. The outer portion of the sheath consists of a continuous layer of elastin (the elastica externa), which covers thick lamellar layers of mainly collagen type II. This elastica externa is covered on the outside by collagen type I rich connective tissue that surrounds the notochord⁽³⁸⁾. Interestingly, the elastica externa was of irregular thickness and fragmented in *plod2* mutant fish, pointing to a disturbed integrity of the outer layer of the notochord sheath (Fig. 9C, 7C). Furthermore, the collagen fibers of the notochord sheath, adjoining the chordoblasts, also displayed a more disorganized distribution in *plod2* mutant fish when compared to the control fish.

DISCUSSION

In this work we present a zebrafish model of BS, characterized by severe musculoskeletal malformations with evidence of bone fragility, and caused by a loss-of-function mutation in the *plod2* gene, which encodes the lh2 protein. It has been shown that human patients with

BS exhibit reduced hydroxylation of the lysine residues in bone type I collagen telopeptides^(39,40), a step which is catalyzed by the LH2 enzyme. The lack of telopeptide hydroxylysines prevents pyridinoline and pyrrole formation and results in the absence of hydroxylysine aldehyde-based cross-links in bone collagen⁽⁴¹⁾. Using SDS-PAGE and mass-spectrometry we showed a similar bone collagen cross-linking defect in *plod2* mutant fish. While 75 % of the lysine K1029 residues in the $\alpha 1(I)$ C-telopeptide were shown to be hydroxylated in wild type fish, a complete lack of hydroxylation of the same residue was observed in bone collagen from *plod2* mutant fish, consistent with a reduction of telopeptide hydroxylysine-aldehyde cross-linking as evidenced by the increased solubility⁽⁴²⁾ of type I bone collagen in these mutants. Structurally, type I collagen fibrils were shown to exhibit a lower degree of organization with loss of the typical plywood-like structure in the vertebral bone of *plod2* mutant fish when compared to control. The disturbed fibrillar architecture of collagen is most likely a consequence of the reduction of intermolecular telopeptide hydroxylysine-aldehyde cross-links within the collagen fibril, resulting in a decreased fibril stability, also affecting the supramolecular structure. In a study of human patients with recessive mutations in *FKBP10* the authors also report a reduced fibrillar architecture of type I collagen in cultured patient fibroblasts⁽⁴³⁾. Moreover, RNAi-mediated knock-down of *PLOD2* in cell culture was shown to negatively affect collagen fibril architecture and block parallel fiber arrangement⁽⁴⁴⁾. Although little is known about the relation of the ultrastructure of collagen to the mechanical integrity of mineralized bone, most likely the observed abnormalities in collagen fibril organization and ultrastructural deviations contribute to the compromised mechanical integrity of the bone in *plod2* mutant fish, comparable to observations made in mouse models for OI^(45,46).

At the tissue level, loss of lh2 function in zebrafish causes an early-onset and progressive phenotype that is strongly reminiscent of human BS. Human BS patients with biallelic *PLOD2* mutations display a variable degree of pterygia, congenital or progressive joint contractures, (kypho)scoliosis, bone fragility with osteopenia or osteoporosis, bowing of the limbs, spontaneous fractures, including rib and vertebral fractures, and short stature⁽⁴⁰⁾. Many of these features are recapitulated in the *plod2* mutant fish: they have a short body axis, kyphoscoliosis and compression of the vertebral column, and bowing and kinking of the ribs and the fin endoskeletal bones (hypurals). The *plod2* mutants also display a reduced diameter of the muscle fibers at the horizontal myoseptum, a structure that is functionally a tendon that serves as attachment site for the trunk muscles to transduce contractile force to the vertebral column during swimming⁽⁴⁷⁾. An enlargement of the endomysium, a connective tissue layer ensheating the muscle fibers, was also observed. These abnormalities may reflect a similar dysfunctionality as seen in the tendons of contracted joints in human BS patients. Contractures in BS are thought to arise due to an altered cross-linking chemistry in the tendons of large joints, however the exact etiology remains unclear⁽⁹⁾. We could demonstrate a higher TMD within the vertebral centra of *plod2* mutant fish, as well as the presence of excessive periosteal bone. Although this has not been described for humans with *PLOD2* mutations, hypermineralization has been reported as a feature in different forms of dominant and recessive OI and is believed to be related to the compromised bone properties in OI.

We also observed structural notochord abnormalities in both mutant larvae and adults. In mutant adult fish histological and TEM analysis revealed a loss of the integrity of the elastica externa, the elastin layer that forms the outer portion of the notochord sheath. During early larval development, this layer is important to restrict the expansion of the notochord sheath that results from the internal osmotic pressure generated by vacuoles in the notochord lumen⁽³⁸⁾. Although the connective tissue of the notochord sheath is believed to be mainly composed of collagen type II, also the presence of collagen type I has been reported for different vertebrates^(48–50). As the elastica externa is intimately associated with this underlying collagen network, defective cross-linking of type I collagen in the notochord sheath could negatively affect the structure of this elastin layer. Alternatively, this defect could arise due to a more direct effect of lh2 deficiency on elastin. Although cross-linking in elastin is independent of lysine hydroxylation⁽⁷⁾, interaction of elastin and FKBP65 has been reported⁽⁵¹⁾. As a result of the disturbed integrity of the elastin layer, the ability to resist internal colloid osmotic pressure may be impaired, leading to the protuberances of the notochord and possibly scoliosis in adult fish, as scoliosis in humans is also associated with elastic fiber degeneration⁽⁵²⁾. Interestingly, fragmentation of elastic fibers was reported as a feature in the dermis of the skin of OI patients⁽⁵³⁾.

Until now only 9 different mutations in *PLOD2* have been reported in patients with either OI or BS (<https://oi.gene.le.ac.uk>^(40,41,54,55)). Five of these are missense mutations and two splice site mutations that cause an in-frame exon deletion, all affecting both LH2 isoforms. The remaining two account for insertion-deletion mutations causing a frameshift, that are either in compound heterozygous state with a missense mutation or in homozygous state but only affecting the long isoform of LH2⁽⁵⁴⁾. This may suggest that a complete loss of LH2 function is not likely to be viable in humans. Similarly, Lh2-null mice do not survive beyond embryonic stage⁽²¹⁾. Conversely, most of *plod2* zebrafish mutants survive into adulthood, despite the complete loss of functional lh2 protein. This could be explained by the reduced requirement for weight bearing on the zebrafish skeleton within the aquatic environment, which causes skeletal phenotypes in fish to often present as being milder and having less impact on general survival, when compared to humans or mice enabling the study of skeletal abnormalities beyond embryonal and larval stages.

Taken together, we report a *plod2* zebrafish mutant with diminished type I collagen telopeptide lysine hydroxylation affecting the formation of intermolecular cross-links. This mutant displays musculoskeletal abnormalities resembling the clinical features seen in BS. Given the lack of viable animal models of BS caused by *PLOD2* mutations, we believe that this mutant can serve as a useful model to help unravel the underlying pathogenetic mechanisms in BS as well as to serve as a tool for the development and evaluation of possible therapeutic interventions. Finally, in this work we further illustrate the usefulness and relevance of using zebrafish as a model for heritable bone diseases such as BS and OI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Evolutionary conservation of the zebrafish and human *plod2/PLOD2* gene and *lh2/LH2* protein

A. Schematic representation of the genomic structure of the *H. Sapiens PLOD2* and *D. Rerio plod2* genes. Boxes and lines represent exons and introns of the *PLOD2/plod2* genes, respectively. Exon 13a (thick box border) is subject to differential splicing. The catalytic domain is encoded by the last four exons of the *PLOD2/plod2* genes (indicated by the dashed line). The asterisk shows the position of the mutation (c.2037T>A) in the *plod2*^{sa1768} mutant zebrafish. **B.** Amino acid sequence alignment (with Clustal Omega) between *H. Sapiens LH2* and *D. Rerio lh2* (long isoform). The catalytic domain is highlighted in light grey and the mutated residue (p.Y679X) in the *plod2*^{sa1768} mutant is indicated in dark grey. Conservation of amino acid sequences are shown below the alignment: “*” means residues identical in all sequences in the alignment; “:” means conserved substitutions; “.” means semi-conserved substitutions; space means no conservation.

Figure 2. Immunoblotting analysis of lh2 protein in *plod2* mutants

Analysis of lh2 expression in protein extracts prepared from a pool of whole embryos (whole embryo) at 2 dpf and from amputated caudal fins from three different *plod2* mutant and three different control fish at 4 months of age (adult caudal fin). Normalization was carried out by reprobing for α -tubulin (bottom lane).

J Bone Miner Res. Author manuscript; available in PMC 2017 March 24.

Figure 4. μ CT analysis of adult fish

A. *plod2* mutant fish (4 months old) have a shorter body length compared to their wild type siblings and exhibit cranial malformation. **B.** μ CT scans of adult *plod2* mutant fish (4 months old) reveal a severe malformation of the vertebral column with compression of the vertebrae along the anteroposterior axis. The animals also suffer from kyphoscoliosis (arrow, on these lateral images only kyphosis is visible) and increased bone formation. *Plod2* mutants also display malformation of the cranium, with apparent increased vertical positioning of the jaw (arrowhead) and the excess growth of the frontal and parietal bones (asterisk). **C.** Quantitative μ CT analysis of the vertebral column in three mutant versus three control fish was performed at the age of 6 months. The x-axis represents the different vertebra (centrum) from anterior to posterior. Tissue mineral density (TMD), was significantly increased in the vertebral bodies of *plod2* mutant fish ($p < 0.01$). The Centrum length, reflecting the length of each vertebral body along the anteroposterior axis, was shown to be significantly decreased in *plod2* mutant fish ($p < 0.01$), while centrum thickness, representing the diameter of each vertebral body along the left-right axis (coronal plane) was significantly increased in *plod2* mutant fish ($p < 0.01$). Error bars indicate standard errors.

Figure 5. Alizarin Red staining for mineralized bone

Adult fish were analyzed at 4 months of age by whole mount Alizarin Red staining. **A.** Most apparent defects were observed in the vertebral column, with vertebral bodies displaying an altered shape and bone protuberance occurring at the vertebral endplates (indicated at three positions by arrowheads) in *plod2* mutant fish. Neural and haemal arches also display irregularities in shape (arrows) **B.** Bowing of the parhypurals and hypurals in the caudal fin of *plod2* mutants (indicated by arrowheads). **C.** Bowing and kinking (arrowheads) of the ribs in mutant fish. **D.** Detail of a *plod2* mutant rib showing callus formation upon fracture healing. Scale bars represent 1 mm in A, 200 µm in B and C and 100 µm in D.

Figure 6. Histological analysis of the vertebral column of adult fish, cross sections

The vertebral column of 4 months old adult fish was embedded, serially sectioned and stained with toluidine blue. **A.** Schematic representation of the transverse profile through the trunk of an adult zebrafish. Light blue segments represent the individual muscle segments that are separated from each other by sheets of matrix, namely the horizontal and vertical myosepta (HM, VM). The orange and red square indicate the region investigated in panel B and C respectively. **B.** Transverse sections at a position close to the intervertebral space (location of displayed sections is also indicated in figure 7A by asterisk), showing the

presence of excessive bone (B) in *Plod2* mutants. This bone prominently envelops the notochord (Nc), which is bent in the coronal plane (along the left-right axis), and is consequently sectioned twice in the same plane. **C.** Transverse sections demonstrating a reduced muscle fiber (Mf) diameter and the presence of a thicker endomysium (Em) between the muscle fibers, at the horizontal myoseptum of *plod2* mutant fish. Other abbreviations: At, adipose tissue; Cb, caudal blood vessels; Ha, haemal arch; Mu, muscle tissue; Na, neural arch; Pc, peritoneal cavity; Sc, spinal cord; Vb, vertebral body. Scale bars represent 200 μ m in B and C.

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Figure 7. Histological analysis of the vertebral column of adult fish, sagittal sections

A. Sagittal sections, stained with toluidine blue, showing compressed and deformed vertebral bodies in *plod2* mutants, as opposed to regularly formed vertebrae in control fish. The red and black squares indicate the regions investigated in panel B and C respectively. The vertical lines marked with an asterisk indicate the location of the transverse sections displayed in Fig 6B **B.** Sagittal sections demonstrating excess bone that is heavily remodeled (asterisk) in the mutant. Note that the stratification in layers, typically surrounding the notochord (1 = notochord sheath, 2 = elastin layer, 3 = lamellar bone layer, 4 = Sharpey fiber bone), is absent or severely distorted in the mutant. Osteocytes are indicated by arrows. **C.** Sagittal sections at the level of the intervertebral space, which is dislocated in mutants. Note the proliferated notochord epithelium (Ne), expanded notochord sheath (1), and fragmented elastin layer (2) underneath a putative lamellar bone layer (3*) and Sharpey fiber bone (4). Other abbreviations: At, adipose tissue; Nc, notochord. Scale bars represent 200 μ m in A, 20 μ m in B, and 30 μ m in C.

Figure 8. Phenotypic analysis of *plod2* mutant larvae

A. Whole mount Alcian Blue staining at 15 dpf marking the notochord sheath. Three different *Plod2* mutant larvae are shown (mutant 1–3), each displaying a similar, but structurally different abnormality (arrowheads) in the notochord sheath. **B.** Toluidine blue stained sagittal sections of 15 dpf control fish show a smooth and rather straight notochord, while the notochord in the mutant has a wavy appearance, consistent with the phenotypes shown in panel A. Note early thickening of the notochord sheath (arrowheads), produced by a thickened notochord epithelium (arrows). **C.** Whole mount Alizarin Red staining for mineralized bone at 20 dpf in a control and representative *plod2* mutant larva indicates no obvious abnormalities in *plod2* mutants. Detail on the right shows that upon detailed examination subtle abnormalities are noted, which are similar to the structural defects observed after Alcian Blue staining. Scale bars represent 100 µm in A, 30 µm in B and 500 µm and 100 µm (detail) in C.

Figure 9. Analysis of bone type I collagen in adult fish using transmission electron microscopy (TEM)

A. Toluidine blue stained longitudinal sections of the vertebral column to illustrate where TEM images were acquired (black rectangles) both in control and *plod2* mutant fish. **B.** TEM images of bone type I collagen in the vertebral end plates (Vep), which represents the growth zone of the vertebral bodies. Due to the presence of excessive periosteal bone (B) in mutant fish a larger image is shown for the *plod2* mutant, compared to the control. Collagen is deposited by Osteoblasts (Ob) on the outer edges of the Vep as immature collagen (IC)

and gradually matures towards the inside, adjoining the notochord (Nc), while developing a more organized structure. Hence mature collagen (MC) displays a typical plywood-like organization (indicated by asterisk), which is clearly visible in control fish but not in comparable regions (asterisk) for *plod2* mutant fish. **C.** TEM images of the notochord sheath showing a region where the elastin layer delineating the notochord sheath (elastica externa, arrowhead), is clearly visible in control fish but interrupted in *plod2* mutants. Also the collagen of the notochord sheath, which is mainly type II collagen (cII), has a disorganized appearance. Other abbreviations: cI, type I collagen; B, bone; Ne, notochord epithelium; Ovs, outer vertebral space; Vep, vertebral endplate. Scale bars represent 500 μm in A, 2 μm in B and 4 μm in C.