Mutations in the latent TGF-beta binding protein 3 (LTBP3) gene cause brachyolmia with amelogenesis imperfecta

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Received: November 15, 2014. Revised and Accepted: February 6, 2015
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Inherited dental malformations constitute a clinically and genetically heterogeneous group of disorders. Here, we report on four families, three of them consanguineous, with an identical phenotype, characterized by significant short stature with brachyolmia and hypoplastic amelogenesis imperfecta (AI) with almost absent enamel. This phenotype was first described in 1996 by Verloes et al. as an autosomal recessive form of brachyolmia associated with AI. Whole-exome sequencing resulted in the identification of recessive hypomorphic mutations including deletion, nonsense and splice mutations, in the LTBP3 gene, which is involved in the TGF-beta signaling pathway. We further investigated gene expression during mouse development and tooth formation. Differentiated ameloblasts synthesizing enamel matrix proteins and odontoblasts expressed the gene. Study of an available knockout mouse model showed that the mutant mice displayed very thin to absent enamel in both incisors and molars, hereby recapitulating the AI phenotype in the human disorder.

Abstract

Inherited dental malformations constitute a clinically and genetically heterogeneous group of disorders. Here, we report on four families, three of them consanguineous, with an identical phenotype, characterized by significant short stature with brachyolmia and hypoplastic amelogenesis imperfecta (AI) with almost absent enamel. This phenotype was first described in 1996 by Verloes et al. as an autosomal recessive form of brachyolmia associated with AI. Whole-exome sequencing resulted in the identification of recessive hypomorphic mutations including deletion, nonsense and splice mutations, in the LTBP3 gene, which is involved in the TGF-beta signaling pathway. We further investigated gene expression during mouse development and tooth formation. Differentiated ameloblasts synthesizing enamel matrix proteins and odontoblasts expressed the gene. Study of an available knockout mouse model showed that the mutant mice displayed very thin to absent enamel in both incisors and molars, hereby recapitulating the AI phenotype in the human disorder.

Introduction

Brachyolmia (from the Greek ‘short trunk’) refers to a heterogeneous group of skeletal dysplasias with as major clinical feature a disproportionate short stature with short trunk. Radiographic abnormalities are predominantly present in the axial skeleton and include generalized platyspondyly (i.e. flattened vertebral bodies). Amelogenesis imperfecta (AI) is a defect in enamel formation and mineralization (1). AI can be an isolated finding or occur in association with other anomalies (syndromic AI) (2). In 1996, Verloes et al. (3) described an autosomal recessive form of platyspondyly with AI [MIM 601216]. Absence of enamel and oligodontia were the major dental findings. Bertola et al. (4) subsequently published two other families, one of them with abnormal yellow coloration of primary and permanent teeth, as well as retarded dental eruption compatible with a diagnosis of AI. Here we report on four families, three of them being consanguineous, with an identical phenotype, characterized by platyspondyly (brachyolmia) and AI. Affected individuals have very thin or almost absent enamel. By using a combined strategy of homozygosity mapping and whole-exome sequencing, recessive mutations in the latent TGF-beta binding protein 3 (LTBP3) gene were identified. Analysis of Ltbp3 expression during mouse development and the study of dental anomalies observed in the Ltbp3−/− knockout mouse model underscored the key role of the latent TGF-beta binding protein 3 in amelogenesis and skeletal development.

Results

Patients’ phenotype

The index family 1 presented with AI and short stature. The almost complete absence of enamel in both primary and permanent dentitions (Fig. 1A and B) led to the diagnosis of hypoplastic AI, thereby explaining the yellow, small and spaced appearance of the teeth. The panoramic radiographs confirmed the absence of enamel associated with large pulp chambers and taurodontic molars (Fig. 1C). Class III mandibular prognathism encountered in family 1 was due to maxillary underdevelopment (Fig. 1D). Radiographs of the skeleton revealed brachyolmia (Fig. 1E and F).

Subsequently, three additional families with a similar phenotype were identified. Additional bone anomalies such as osteopenia and scoliosis were present in family 4. Missing teeth (family 4) and retarded teeth eruption (family 3) were also reported.

Enamel shows quantitative and qualitative defects

The enamel structure of a permanent tooth, the left upper second premolar (25) extracted within the course of treatment of patient IV-1 of family 1 was further analyzed by scanning electron microscopy (Fig. 1G and H). With this evaluation the enamel hypoplasia was confirmed and very thin or absent enamel was noted. Dentin was normal. The initial aprismatic enamel layer was absent. A very thin shell of irregular prismatic enamel (PE), with a comparatively at the same site on a control tooth), was deposited covering the dentin scaffold. In this layer, a Hunter-Schreger band pattern, featuring the arrangement of enamel prisms, was present. In these areas, no aprismatic outer layer was deposited. However in some areas, amelogenesis continued and some ‘bubbling’ of non-prismatic enamel (NPE) occurred on top of this basal first enamel layer. Waves of aprismatic and prismatic enamel alternated. The outermost layer was always aprismatic in areas where enamel formation continued.
Mutations in LTBP3 underlie syndromic AI with brachyolmia

Whole-exome sequencing was performed independently (in two different labs) in families 1 and 2 and in families 3 and 4. Coverage and variant calling data for families 1 and 2 are provided in Supplementary Material, Table S1.

A single gene, LTBP3, was found to carry bi-allelic mutations in all affected individuals from the four families (Fig. 2A and B). In family 1, we identified a homozygous 14 bp deletion, c.[2071_2084delTACCGGCTCAAAGC] (Table 1 and Fig. 2A). This mutation lies within a zone of homozygosity that is shared between the two affected individuals, but that is absent in the unaffected sibling and parents (Supplementary Material, Fig. S1).

In family 2 we identified compound heterozygosity for a nonsense and a splice donor site mutation c.[421C>T];[1531+1G>T]; in families 3 and 4 homozygosity for a single nucleotide deletion was found [c.[2216_2217delG] in family 3 and c.[2356_2357delG]
Figure 2. Detected LTBP3 mutations and corresponding protein domains. (A) Pedigrees and sequences analysis of families 1–4 (na: not available). Simplified pedigree of family 1 demonstrates consanguinity between the parents III.3 and III.4. Electropherograms reveal a 14-bp homozygous deletion in the affected individuals IV.1 and IV.2. The parents III.3 and III.4 are heterozygous for the deletion. (B) The LTBP3 human gene is located on chr11q13.1, extends over 20 kb and contains 28 exons (vertical gray hatches); the position of the start codon (ATG) and the stop codon (TGA) are indicated. The mutations detected in this study are bold and underlined, vertical lines indicate positions relative to exons (only the p’Tyr774* was previously described in Noor et al. [5]). (C) Arrows indicate positions relative to the cDNA and the protein domain according to EMBL-EBI Interpro.
in family 4). All mutations segregated with the disease phenotype in each family and were confirmed by Sanger sequencing (Fig. 2). In addition, they were absent in the Exome Variant Server (EVS) and the Thousand Genomes Project Catalog. Interestingly a single nucleotide insertion (c.2216_2217insG; p.Gly740Argfs*51) was tabulated in EVS in the homozygous state in 7 out of more than 6000 individuals. However, coverage information was not available for these individuals to verify if this is a false or true positive variant.

The Ltbp3 mutations identified in our families are most likely hypomorphic. The 14-bp deletion found in family 1 does not seem to result in nonsense-mediated decay because it was present in RNA extracted from a gingival biopsy of patient IV.1 (Supplementary Material, Fig. S2). The mutation most likely gives rise to a truncated protein that lacks the terminal 612 amino acids, which encode essential functional domains (epidermal growth factor like-domain, TB domain, EGF-like calcium-amino acids, which encode essential functional domains (epidermal growth factor like-domain, TB domain, EGF-like calcium-amino acids, which encode essential functional domains (epidermal growth factor like-domain, TB domain, EGF-like calcium-amino acids.

**Expression pattern of Ltbp3 in developing mouse bone and tooth**

To gain insight into Ltbp3 expression during bone and tooth development in mice, we performed an in situ hybridization analysis, using a digoxigenin-labeled antisense riboprobe generated from the same DNA template as previously used by the Eurexpress consortium (www.eurexpress.org). Mouse embryos were analyzed at E14.5, E16.5 and E18.5. Using a combined strategy of homozygosity mapping and whole-exome sequencing in four unrelated families with the association of brachyolmia and AI, we identified the LTBP3 (latent TGF-beta binding protein 3) gene as the underlying causal gene responsible for this rare autosomal recessive disorder [MIM 601216]. Mutations in the LTBP3 gene have already been reported in individuals with oligodontia (5), a dental developmental disorder defined by the absence of more than six permanent teeth. The question therefore arises if mutations in LTBP3 can cause different dental phenotypes and how we can explain the presence of extra-dental manifestations such as brachyolmia in our families.

### Table 1. Mutations described in LTBP3 (NM_001130144.2)

| Family | Affected | Ethnic origin | #Exon | Chromosomic position (hg19) | cDNA change | Protein change |
|--------|----------|---------------|-------|-----------------------------|-------------|---------------|
| 1      | Two siblings | Turkey         | Exon 14 | g.65314933 | c.[2071_2084delTACCGCTCAAAACC]; [2071_2084delTACCGCTCAAAACC] | p.[Tyr691Leufs*95]; [Tyr691Leufs*95] |
| 2      | Two siblings | Caucasian French | Exon 2 and 8 | g.65321762 | c.[421C>T]; [1531+1G>T] | p.[Gln141*]; [Tyr691Leufs*95] |
| 3      | One boy | Brazil            | Exon 15 | g.65314283 | c.[2216_2217delG]; [2216_2217delG] | p.[Gly739Alafs*7]; [Gly739Alafs*7] |
| 4      | Three siblings | Pakistan        | Exon 17 | g.65311018 | c.[2356_2357delG]; [2356_2357delG] | p.[Val786Trps*82]; [Val786Trps*82] |

Mutation affecting splice are in italics.
Based on the available data in the report, we believe that the oligodontia phenotype in this family was either related to early loss of teeth due to enamel defects or caused by defective tooth development leading to absent teeth. Close examination of the skull X-ray of an affected patient in their published Figure 2 clearly shows absent enamel and short roots in the remaining teeth, which suggests the diagnosis of an AI. Verloes et al also mentioned oligodontia as a feature in his family with brachyolmia and AI (3). Missing teeth were also found in our family. Oligodontia may therefore be part of the clinical spectrum of this condition. The affected individuals in the consanguineous Pakistani family with oligodontia also presented with short stature. Varying degrees of scoliosis were apparently present but no other skeletal anomalies were observed. The authors do not report flattening of the vertebral bodies in their family and no lateral views of the spine are shown in their article. They only describe a higher bone mineral density in the spine found by dual-energy X-ray absorptiometry. Although we cannot judge on the presence or absence of brachyolmia as cause of the short stature in the affected individuals, we believe that this reported family with oligodontia may be affected by the same disorder given the presence of a recessive mutation in the LTBP3 gene.

**LTBP3 and TGF-beta acting as partners**

The LTBP3 gene (NM_001130144.2, 11q13.1) spans 28 exons, has 20,372 base pairs and generates 22 different transcripts (only two major transcripts with CCDS number, eight without a protein and two resulting in nonsense-mediated decay).

**LTBP3 and TGF-beta coding for a protein of the extracellular matrix that is involved in regulation of TGF-beta secretion, trapping and activation** (11-13). LTBP3 (1139 amino acids, NCBI RefSeq: NP_001157738.1; 1303 amino acids NM_001130144.2) is one of the four members of the LTBP/fibrillin family and can form a large complex with latent TGF-beta 1, 2 and 3 (14). The LTBP protein domain structure consists of eight TGF-beta-binding protein-like (TB) domains also named eight cysteine repeats as well as insulin-like growth factor binding protein domains (Fig. 2). This domain structure is also found in TGF-binding protein and fibrillins.

**LTBP3 and AI**

TGF-beta 1, 2 and 3 are growth factors involved in various biological functions such as cell growth and differentiation, extracellular matrix secretion and remodeling. TGF-beta signaling is essential for hair and tooth growth especially enamel formation. Enamel is a complex structure secreted by ameloblasts presenting different successive stages during their life cycle. In mammals it is possible to recognize an initial aprismatic enamel layer at the dentin–enamel junction deposited by secreting ameloblasts without Tomes’ process, then the bulk of enamel with rod and interrod compartments forms with secreting ameloblasts with Tomes’ process and finally the last-formed surface apismatic layer of enamel with the retraction of ameloblasts’ Tomes’ process (15). Ltbp3 expression pattern in differentiating ameloblasts and odontoblasts is highly similar to that of TGF-beta1 (16-18). Blockade of TGF-beta signaling results in a failure of ameloblasts to produce an enamel layer in incisor teeth (11). Overexpression of TGF-beta1 in teeth results in detachment of ameloblasts and enamel defects (1,19). TGF-beta1 and TGF-beta receptor 1 (TGFBR1) genes are strongly expressed in secreting...
ameloblasts where they promote the expression of MMP20, an enamel matrix protease (20). TGF-beta1 is also expressed later in the maturation-stage ameloblasts and seems to play an important role in ameloblast apoptosis (21). In addition, TGF-β1 is able to induce KLK4 (a protease degrading enamel proteins to increase mineralization) expression (22,23). TGF-beta1 may also control cell layer integrity for odontoblasts (24).

The enamel defects, observed in our families, at the ultrastructural level (Fig. 1) with both the absence of the initial prism-matic enamel layer and the abnormal secretion of non-prismatic bulk enamel, strongly suggest a role of LTBP3 during the life cycle of ameloblasts especially at the secretory stages with Tomes process formation/modulation. The molecular mechanisms that control the formation, function and retraction of Tomes’ process...
are not well understood. However clear molecular differences exist between NPE and PE (25) and this may involve TGF-beta signaling. Transgenic mice presenting with enamel defects such as the amelotin (AMTN) overexpression model also exhibit enamel defects in relation with the disappearance of Tomes’ process (26). We therefore formulate the hypothesis that TGF-beta signaling is involved in the modulation of Tomes’ process and the deposition of a decussating prisms enamel pattern. Interestingly, the Kik4 ablated mice display rod/interod anomalies besides enamel maturation defects (27).

**LTBP3 and brachyolmia**

LTBP3 is also involved in bone formation and remodeling. Inactivation of LTBP3 reduces TGF-beta activation and therefore diminishes associated cell proliferation and osteogenic differentiation (28). TGF-beta is the most abundant growth factor in bone playing a major role in bone development and skeletogenesis, especially during endochondral ossification but also later in adult bone homeostasis and remodeling (29). Deregulation of the TGF-beta signaling pathway is likely to interfere with axial skeleton patterning (30).

Ltbp3 is expressed in cartilage primordia especially in developing vertebrae (7). Recently, genes such as TRPV4 (transient receptor potential cation channel, subfamily V, member 4 gene) have been identified in autosomal dominant brachyolmia (31) and PAPSS2 (β-phosphoadenosine 5′-phosphosulfate synthase 2) in autosomal recessive brachyolmia (32). A direct relationship may exist between the TGF-beta signaling pathway and the TRPV4 and PAPSS2 genes. TRPV4 regulates cardiac fibroblast differentiation to myofibroblasts by integrating signals from TGF-beta1 and mechanical factors (33). Altered responsiveness to TGF-beta results in reduced Papss2 expression and alterations in the biomechanical properties of mouse articular cartilage (34).

The role of LTBP3 in bone development is also exemplified by the Ltbp3−/− knockout mouse phenotype (7,8,35,36). These animals show growth retardation, obliteration of the skull synchondrosis within 2 weeks of birth, osteoporosis of long bones and vertebrae and osteoarthritis. Thoracic kyphosis (curvature of the cervical and thoracic vertebrae) and distal ribcage were also observed in the mutant mice. The high bone mass was related to a defect in bone resorption with compromised osteoclast function and decreased bone turnover (9). Platyspondyly however was not observed in the Ltbp3−/− knockout mice.

**A wider phenotype**

A previous report has described expression of LTBP3 in various human tissues and cell lines, predominantly in heart, skeletal muscle, prostate and ovaries, as well as testis and small intestine (14). Ltbp3 was also found to be expressed in the rat brain, with a partially overlapping expression with several other TGF-beta family members (37). Basiocipital-basiphenoid synchondrosis of P1 mouse expressed Ltbp3 in prehypertrophic chondrocytes. Ltbp3 transcripts were also present in adult mouse tissues in heart, lung and kidney (7,8). We also report the transcription of ltbp3 in brain vesicles, in cells surrounding the lumen of the neural tube, in the developing inner ear, in the small and large intestine, in the differentiating lung, in heart outflow tract and in the wall of the large blood vessels (Fig. 3).

It is surprising that the mutations of LTBP3 in human seem to affect only two organ systems, the skeleton and the teeth. It will be very important to follow over time the cohort of patients to assess the clinical relevance of this in situ hybridization data and to record any additional occurring illness that may extent the phenotype spectrum.

**Ltbp3−/− knockout mouse model display an enamel phenotype**

Analyzing further the Ltbp3−/− knockout mouse with respect to the craniofacial and odontal phenotypes was of importance to convince that LTBP3 is indeed the causative gene behind brachyolmia associated with Al.

As described in refs. (7–9) craniofacial malformations were identified in Ltbp3−/− mice at postnatal day 12. By 3 months of age mutant mice displayed a pronounced rounding of the cranial vault, extension of the mandible beyond the maxilla and kyphosis. The altered skull shape with occipital bossing was believed to be caused by the premature ossification of the cranial base synchondroses. Shortened skull base and disproportionately short upper jaw were evident. The external facial appearance was characterized by a rounded head and a shortened snout. Our analysis indicated that almost all craniofacial structures were affected in the Ltbp3-null mice. Alterations were observed in intramembranous and endochondral bones, along with in teeth. Compared with wild-type (WT) mice, Ltbp3-null mutants presented with an overall reduction in craniofacial size and modifications of the shape of various parts of the craniofacial skeleton. Modifications of viscerocranium, neurocranium and mandible were prominent changes.

Some of the skeletal and growth anomalies observed in the Ltbp3−/− mice were also present in our patients (short maxilla, mandibular prognathism) but others (such as the increased bone density) were not present, underscoring the fact that the mouse phenotype is not always recapitulating the human phenotype. We do know secreting ameloblasts express Ltbp3. The enamel was clearly defective in the mouse mutant. It was thinner, less mineralized and exhibited an unusual wavy pattern with alternating area of presence/absence. Both incisors and molars were affected. Ltbp3−/− ameloblasts as seen on histological sections of continuously growing incisor enamel organ were, in spotted area, able to synthesize an extruding NPE matrix confirming at the cellular level the impaired amelogenesis and the wavy pattern appearance.

This knockout-mice is now to be inscribed in the list of animal models presenting with enamel defects (38) and will likely serve as an important model to study further brachyolmia with Al (39).

In conclusion, the study of four unrelated families with the association of Al and brachyolmia has led to the identification of LTBP3 as causal gene. Our study not only confirms the existence of this rare, autosomal recessive disorder but also highlights the role of LTBP3 and TGF-beta signaling in amelogenesis, both in humans and mice. It also adds another member to the growing list of genes causing isolated and syndromic forms of brachyolmia. Study of additional families is warranted for better understanding of the phenotypic spectrum of this disorder given the wide expression of the LTBP3 gene.

**Materials and Methods**

**Patients**

In Family 1, two sisters born from a consanguineous family of Turkish origin were referred to the Reference Center for Rare Or- donental Diseases at the Strasbourg University Hospital because of pain and enamel defects (Fig. 1). Besides short stature (140 cm adult height, -4.2 SD), they also showed facial dysmorphism with large forehead, thick eyebrows, almond shaped eyes,
myopia and mild learning difficulties. Radiographs of the skeleton revealed brachyolmia (Fig. 1E and F) and there were no signs of a generalized skeletal dysplasia. Bone age was considered as normal.

Their orodental findings were documented using the D4/phenodent Diagnosing Dental Defects Database registry (www.phenodent.org).

Enamel was almost absent (hypoplastic AI) in both primary and permanent dentitions (Fig. 1A and B). The teeth were yellow, small and spaced. Several teeth were surgically extracted because of recurrent infections. On orthopantomogram, no enamel was observed, pulp chambers were large and molars were taurodontic (Fig. 1C). Lateral cephalogram showed somewhat thickened cortical plates of the frontal bone and an absence of pneumatization of the frontal and sphenoid sinuses. Posterior clinoid apophyses of the sphenoid were abnormally shaped. Class III mandibular prognathism was due to maxillary underdevelopment (Fig. 1D).

Family 2 consisted of two sibs born to non-consanguineous, unrelated Caucasian parents. The female proband (III.6) is the sixth child. Birth weight was 2.760 g, birth length 46 cm and head circumference was 33 cm at 39 weeks of gestation. She had normal psychomotor development and no general health problems. Primary teeth were small and yellowish with a poor square morphology and rounded cusps without pronounced fissures. The enamel was smooth and showed external crown resorption before molars erupted. She suffered of repeated dental abscesses, leading to the extraction of several teeth at ages 2½ and 5 years. When seen at age 8, she had clearly hypoplastic AI, microdontia and taurodontism. At age 13, she was 149 cm tall (−1.5 SD), weighed 42 kg and had an occipital-frontal circumference of 51 cm. She had a triangular face with retraction of the midface. She had short hands with stubby interphalangeal joints. Prominent sternum and hypermobile small joints. The family had a triangular face, converging squint, a narrow thorax with anteroposterior flattening with posterior scalloping and superior and inferior indentations in the posterior third of the lumbar vertebrae. The oldest girl underwent surgery for an S-shaped scoliosis. The scoliosis in the two other children was rather mild. Recurrent dental abscesses were noted and the dental practitioner confirmed a diagnosis of AI with missing teeth.

None of the family members had intellectual disability. The father’s height was 165.2 cm (3rd–10th centile), and the mother’s height was 149.4 cm (>3rd centile). None of the unaffected members of the family have skeletal nor dental anomalies.

**Genetic analyses**

This study was approved by the ethics committee of the Strasbourg University Hospital (ClinicalTrials.gov Identifier: NCT01746121). Informed consent and DNA samples were obtained from all participating individuals. Genomic DNA was isolated either from blood using the Flexigene DNA kit (Qiagen, Courtaboeuf, France) or from saliva using the prepIT-L2P OG-250 Oragene®DNA kit (DNA Genotek Inc., ON, Canada). Whole-exome sequencing was performed by IntegraGen (Evry, France). The coding parts of the genome were captured using the SureSelect Human All Exon Kits V5+UTR 70 Mb (Agilent, Massy, France) and the resulting libraries were sequenced as paired-end 75 base pair reads on a HiSeq 2000 (Illumina, San Diego, USA). Image analysis and base calling were performed using the Real Time Analysis (RTA) Pipeline version 1.9 with default parameters (Illumina). The bioinformatic analysis of sequencing data was based on the CASAVA1.8 pipeline (Illumina). CASAVA performs alignment and detects variants (SNPs and indels) based on the allele calls and read depth. The variants were annotated and prioritized using an in-house pipeline VaRank (http://lbgi.fr/VaRank/).

Sanger sequencing (GATC Biotech, Applied Biosystems ABI 3730xl™, Konstanz, Germany) was used to validate the mutations and verify segregation using the primers shown in Supplementary Material, Table S2.

Homozygosity mapping via GeneChip Human All Exon Kits V5+UTR 70 Mb (Agilent, Massy, France) and the resulting libraries were sequenced as paired-end 75 base pair reads on a HiSeq 2000 (Illumina, San Diego, USA). Image analysis and base calling were performed using the Real Time Analysis (RTA) Pipeline version 1.9 with default parameters (Illumina). The bioinformatic analysis of sequencing data was based on the CASAVA1.8 pipeline (Illumina). CASAVA performs alignment and detects variants (SNPs and indels) based on the allele calls and read depth. The variants were annotated and prioritized using an in-house pipeline VaRank (http://lbgi.fr/VaRank/).

**cDNA analysis**

RNA was extracted from fibroblasts of patient IV.1 (gingival biopsy) and one unrelated control by using a RiboPure™ Kit, followed by a DNAse treatment with the TURBO DNA-free™ Kit (Life Technologies, Carlsbad, CA). RNA integrity was assessed by gel electrophoresis and RNA concentration was measured with the Eppendorf Biophotometer Plus™ with the Hellma® TrayCell™ (Eppendorf, Hamburg, Germany). Reverse transcription of 1 µg total RNA to cDNA was performed using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA). Reverse transcription polymerase chain reaction was performed to determine the exon content of the cDNA from the patient. Primers used for reverse transcription polymerase chain reaction are shown in Supplementary Material, Table S2.
Scanning electron microscopy
The permanent left upper second premolar (25) of patient IV2, family 1, as well as an identical control tooth, were available for the comparative study of enamel AI/control ultrastructure. After extraction, the teeth were rinsed with tap water and immersed in a sodium hypochlorite solution (1.2 chlorometric degree) for 24 h. After a rinsing with distilled water the tooth was dehydrated in a graded series of ethanol, transferred in a solution of propylene oxide/epon resin (v/v) for 24 h, then embedded in Epon 812 (Euromedex, Souffelweyersheim, France). The tooth was sectioned into two halves along its vertical axis using a water-cooled diamond circular saw (Buronwil, NY, USA) and both surfaces were polished with diamond paste (Escil, Chassieu, France). One half was then etched with a 20% (v/v) citric acid solution for 2 min, rinsed with distilled water, dehydrated in a graded series of ethanol solutions and finally left to dry at room temperature. The samples were coated with a gold–palladium alloy using a HUMMER JR sputtering device (Technics, CA, USA). Scanning electron microscope assessments and microanalysis (Energy dispersive X-ray) were performed with an XL SIRION 200 FEG SEM (FEI company, Eindhoven, The Netherlands) operating with an electron accelerating voltage of 5 kV.

In situ hybridization

Sample preparation
Mouse embryos/fetuses were collected at E12.5, E14.5, E16.5 and E18.5, after natural mating between C57BL6 mice. All experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC). The project was approved by the ICS/IGBMC animal experimentation ethics committee (2012-097). For E14.5 and older samples, the whole body was embedded in OCT 4583 medium (Tissue-Tek, Sakura) and frozen on the surface of dry ice. E12.5 embryos were fixed overnight in 4% paraformaldehyde (pH 7.5, w/v) in phosphate buffered saline (PBS), cryoprotected by overnight incubation in 20% sucrose (w/v) in PBS and cryoembedded as described above. Cryosections (Leica CM3050S cryostat) at 10 µm were collected on Superfrost plus slides and stored at −80°C until hybridization. E12.5 and E14.5 samples were sectioned in a frontal plane, whereas other stages were sectioned sagitally.

Probe synthesis
The Ltbp3 probe was synthesized from PCR-generated DNA templates kindly provided by the EURExpress consortium (http://www.eurexpress.org). The template sequence is given in Supplementary Material, Fig. S3. DIG-labeled antisense riboprobe was transcribed in vitro by incubation for 2 h at 37°C using 1 µg of the PCR product, 20 U SP6 RNA polymerase, 5× transcription buffer (Promega), 10× DIG RNA labeling Mix (Roche), 0.5 M 1,4-Dithiothreitol, 20 U RNase inhibitor (Roche) in a 20-µl volume. The PCR product, 20 U SP6 RNA polymerase, 5× transcription buffer (Promega), 10× DIG RNA labeling Mix (Roche), 0.5 M 1,4-Dithiothreitol, 20 U RNase inhibitor (Roche) in a 20-µl volume. The reaction was stopped with 2 µ1 EDTA (0.2 M, pH 8), and RNA was precipitated with 1 µl yeast tRNA (10 mg/ml), 2.5 µl LiCl (4 M) and 75 µl absolute ethanol, followed by an incubation for 30 min at −80°C and centrifugation at 12 000 rpm (30 min at 4°C). The pellet was washed with 0.5 ml ethanol (70%) and re-centrifuged. The supernatant was discarded and the pellet was allowed to dry. The probe was then diluted in 20 µl sterile H2O. The quality of the probe was verified by electrophoresis in a 1% agarose gel. If no smear was observed and the size was as expected, the probe was considered to be ready for use. The quantity of RNA was evaluated by Nanodrop (ND-1000 Spectrophotometer, Labtech) and adjusted to 150 ng/µl in hybridization buffer, then stored at −20°C until use.

In situ hybridization
In situ hybridization was performed as previously described (43).

MicroCT imaging
Fixed in ethanol heads of 7 (5 × 3.5 months-old and 2 × 5.5 months-old) mutant Ltbp3−/− mice and 7 WT littermates were analyzed through X-ray micro-CT using Quantum FX® microCT Pre-clinical In Vivo Imaging System (Caliper Life Sciences, Inc., Hopkinton, MA, USA), which operates at an energy of 80 kV and current intensity of 160 µA, with high-resolution detection at 10–295 µm pixel size. 3D data reconstructions were performed using Analyze software (v 11.0; Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). Animals necropsy material was kindly provided by B. Dabovic and D.B. Rifkin, Department of Cell Biology, NYU Langone Medical Center, New York, NY.

Histological analysis
Fixed in 10% formalin and then transferred in 70% ethanol, heads of 3.5 months-old WT and Ltbp3−/− mice were washed in water and then demineralized in EDTA 10% in H2O at 37°C for 10 days (the demineralizing solution was changed every day for the first 3 days and then every other day). After thorough water washes, the heads were dehydrated in graded ethanol, cleared in histosol and embedded in paraffin at 60°C. 10 µm transversal sections were stained with hematoxylin/eosin. A detailed histology protocol can be found at http://www.empress.har.mrc.ac.uk.

Supplementary Material
Supplementary Material is available at HMG online.

Acknowledgements
We would like to thank the patients and families for their participation. We are grateful to Nadia Messaddeq from the IGBMC Imaging centre for her help and support and to Yann Hérault and Isabelle Goncalves da Cruz from the Mouse Clinical Institute for facilitating access to the Micro-CT imaging. We thank Vanessa Stoehr for her efficient management of the INTERREG project. All contributors have read and approved the submitted manuscript.

Conflict of Interest statement. None declared.

Funding
This work was supported by grants from the University of Strasbourg, the French Ministry of Health (National Program for Clinical Research, PHRC 2008 No. 4266 Amelogenesis imperfecta), the Hôpitaux Universitaires de Strasbourg (API, 2009–2012, ‘Development of the oral cavity: from gene to clinical phenotype in Human’), IFRO (Institut Français pour la Recherche Odontologique) and the EU-funded project (ERDF) A27 “Oro-dental manifestations of rare diseases”, supported by the RMT-TMO Offensive Sciences initiative, INTERREG IV Upper Rhine program www.genomsile.eu. Work performed at IGBMC was supported by the grant ANR-10-LABX-0030-INRRT, a French State fund managed by the Agence Nationale de la Recherche under the frame programme Investissements d’Avenir labelled ANR-10-IDEX-0002-02. Funding was also received under the European Commission Seventh...
Framework Programme (SYBIL project; grant number 602300). E.B. holds a post-doctoral fellowship of the FWO (Fund for Scientific Research—Flanders). B.B.D. and D.B.R. are funded by National Institutes of Health: NIH R01 CA034282. Funding to pay the Open Access publication charges for this article was provided by the EU-funded project (ERDF) A27 "Oro-dental manifestations of rare diseases", supported by the RMT-TMO Offensive Sciences initiative, INTERREG IV Upper Rhine program www.genosmile.eu.

Web Resources
The URLs for data presented herein are as follows:
1000 Genome Browser, http://browser.1000genomes.org
BDGP, http://www.fruitfly.org
D4[phenodont Diagnosing Dental Defects Database, www. phenodont.org
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP
Ensembl Genome Browser, http://www.ensembl.org
Eurexpress, http://www.eurexpress.org/eex
Exome Variant Server (EVS) http://evs.gs.washington.edu/EVS/
GenBank, http://www.ncbi.nlm.nih.gov/Genbank
GeneHub-GEPI3, http://www.cgl.ucsf.edu/research/genentech/ genehub-gepis/genehubgepis-search.html
Gene expression in tooth, http://bite-it.helsinki.fi
GenePaint, http://www.genepaint.org
HSF2.4.1, http://www.umd.be/HSF
NCBI, http://www.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway
UniGene, http://www.ncbi.nlm.nih.gov/unigene
VaRank, http://lab3.ualberta.ca/VaRank/
EMBL—EBI Interpro, http://www.ebi.ac.uk/interpro
EMPReSS, http://www.empress.har.mrc.ac.uk

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