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Prolyl 3-hydroxylase 1 deficiency causes a recessive metabolic bone disorder resembling lethal/severe osteogenesis imperfecta

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Abstract

A recessive form of severe osteogenesis imperfecta that is not caused by mutations in type I collagen has long been suspected. Mutations in human *CRTAP* (cartilage-associated protein) causing recessive bone disease have been reported. CRTAP forms a complex with cyclophilin B and prolyl 3-hydroxylase 1, which is encoded by *LEPRE1* and hydroxylates one residue in type I collagen, $\alpha 1(I)Pro986$. We present the first five cases of a new recessive bone disorder resulting from null *LEPRE1* alleles; its phenotype overlaps with lethal/severe osteogenesis imperfecta but has distinctive features. Furthermore, a mutant allele from West Africa, also found in African

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AUTHOR CONTRIBUTIONS

W.A.C., W.C., A.M.B. were responsible for the design and execution of all experiments except for the differential scanning calorimetry (E.M. and N.V.K., under the guidance of S.L.) and mass spectrometry (M.A.W. and M.A.S., under the guidance of D.R.E.). K.N.R., C.J.T., D.I.B., C.K. and P.A.S. referred the probands to J.C.M. and contributed syndrome description and clinical insights. The manuscript was primarily written by J.C.M., with contributions from W.A.C. and critical revisions from W.C., A.M.B., D.R.E. and S.L. J.C.M. was responsible for overall study strategy and design, and clinical insight in selection of cases to be screened.

GenBank accession numbers. *Homo sapiens* *LEPRE1*: gene, NC_000001.9; mRNA, NM_022356.2; protein, NP_071751.2.

Note: Supplementary information is available on the Nature Genetics website.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Americans, occurs in four of five cases. All proband *LEPRE1* mutations led to premature termination codons and minimal mRNA and protein. Proband collagen had minimal 3-hydroxylation of $\alpha 1(I)Pro986$ but excess lysyl hydroxylation and glycosylation along the collagen helix. Proband collagen secretion was moderately delayed, but total collagen secretion was increased. Prolyl 3-hydroxylase 1 is therefore crucial for bone development and collagen helix formation.

Classical osteogenesis imperfecta is a well-known skeletal disorder with dominant inheritance that is caused by mutations in *COL1A1* or *COL1A2*, the genes that encode type I collagen^{1,2}. Recessive forms of lethal/severe osteogenesis imperfecta have been suspected since the original Sillence classification in 1979 (ref. 3). Although some familial recurrences are due to parental mosaicism for a dominant collagen mutation, others have no linkage to type I collagen^{4,5} and/or no detectable collagen mutation by gene sequencing. Some of these individuals have abnormal collagen biochemistry⁶.

Prolyl 3-hydroxylase 1 (P3H1) has been independently isolated for three distinct functions. Initially, it was isolated from a rat cDNA library as leprecan ('leucine proline-enriched proteoglycan'), a previously undescribed matrix proteoglycan⁷. Leprecan is a chondroitin sulfate proteoglycan with a core protein of 804 residues (90 kDa) and is detected in the basement membrane of blood vessels in kidney and skeletal muscle and in pericellular material in hyaline cartilage⁷. It contains both an RGD cell attachment site, suggesting that it mediates cell-matrix interactions, and a KDEL endoplasmic reticulum retention signal, implying that it has a function in intracellular secretory pathways. Subsequently, the human homolog of leprecan, also known as Gros1, was isolated as a growth suppressor⁸ and assigned to chromosome 1. Finally, the intracellular function of P3H1 in collagen hydroxylation was recognized when P3H1 was isolated from chick embryos⁹, confirming its assignment to a family of 2-oxoglutarate dioxygenases that are required for modifications that are essential to the synthesis, folding and assembly of collagen. The purification of P3H1 on gelatin-Sepharose showed that it bound to denatured collagen. Two other proteins, cartilage-associated protein (CRTAP) and cyclophilin B, have been shown to form an intracellular collagen-modification complex with P3H1 (refs. 9,10).

Intracellular modifications of type I collagen facilitate its folding and stability. Lysyl hydroxylase and prolyl 4-hydroxylase modify multiple lysine (Xaa-Lys-Gly) and proline (Xaa-Pro-Gly) residues along the helical portion of collagen chains¹¹. Certain hydroxylysine residues are subsequently glycosylated. Folding of procollagen proceeds from the C to N terminus; the constituent chains are accessible for modification during translation, but are inaccessible after completion of helix folding. The collagen structural defects that cause classical osteogenesis imperfecta delay helix folding and expose the constituent chains to the modifying enzymes for a longer time, resulting in chain 'overmodification' and delayed electrophoretic migration¹².

Collagen is also modified by P3H1, which hydroxylates a single proline, Pro-4Hyp-Gly, at $\alpha 1(I)Pro986$ ¹³, which is of unknown function. Because proline 3-hydroxylation is more abundant on other collagens, particularly types IV (basement membrane) and V collagen, its role in bone formation was unappreciated¹⁴. The relationship of 3-hydroxylation to recessive

osteogenesis imperfecta was suggested by the *Crtap*^{-/-} mouse¹⁰, which develops severe bone dysplasia and has abnormal type I collagen biochemistry. *CRTAP* has the same chromosomal location as moderately severe recessive type VII osteogenesis imperfecta^{15,16}, previously delineated by distinctive bone histology¹⁷. Type VII osteogenesis imperfecta is caused by a hypomorphic *CRTAP* defect¹⁰, whereas null *CRTAP* mutations result in lethal osteogenesis imperfecta-like bone dysplasia¹⁸. Because the enzymatic activity in the 3-hydroxylation complex resides in P3H1, we postulated that its absence would result in severe bone dysplasia.

We screened individuals with lethal/severe osteogenesis imperfecta-like bone dysplasia and overmodified collagen but without a type I collagen mutation detected by complete cDNA sequencing of both chains. Real-time RT-PCR of total fibroblast RNA identified five individuals, three with lethal and two with severe bone dysplasia (Fig. 1 and Supplementary Note online), whose *LEPRE1* mRNA was 5%–21% of normal control (Fig. 2a). Four probands, including the three lethal cases, were African, African American or Afro-Caribbean. The fifth proband was Pakistani. The parents of proband 1 denied consanguinity and had a previous affected child, who died in Africa; the parents of proband 2 had a second affected child. The phenotype of the probands overlapped Sillence lethal type II/severe type III osteogenesis imperfecta (severe osteoporosis, shortened long bones and a soft skull with wide open fontanelle^{2,3}). However, in contrast to the classical blue sclerae, triangular face and narrow thorax of severe and lethal osteogenesis imperfecta, our probands have white sclerae, a round face and a short barrel-shaped chest. Prenatal radiographs demonstrate gracile, undermineralized ribs and long bones. Multiple fractures were present at birth. Long bone radiographs of surviving probands showed bulbous metaphyses and apparent matrix disorganization, which awaits future histomorphometry. Their hands appear relatively long compared to their forearms, with long phalanges, short metacarpals and disorganized matrix. Bone age was normal. Vertebral compression fractures occurred in surviving probands 3 and 5 by 14 months and 5 years of age, respectively. Their bone density was lower than almost all individuals with severe osteogenesis imperfecta, with an L1–L4 vertebral DEXA z-score of –7.

The decreased expression of *LEPRE1* transcripts in proband fibroblasts was partially rescued by emetine treatment (Fig. 2a), suggesting rapid transcript degradation by nonsense-mediated decay¹⁹. Parental *LEPRE1* mRNA levels were comparable to controls (Fig. 2b). Sequencing of the 14 exons of *LEPRE1* and adjacent intronic sequences yielded mutations in both alleles of each proband (Table 1 and Supplementary Fig. 1 online). A common mutant allele was found in the African and African American probands. This mutation, IVS5+1G>T, occurred in both alleles of proband 1 and one allele of proband 3, with parents from Ghana and Nigeria, respectively, as well as in both alleles of proband 2 (both of whose parents are African American) and one allele of proband 4, whose parents are African American and Afro-Caribbean. Both Ghanaian parents and the Nigerian mother are heterozygous carriers, as are both parents of proband 2 and the mother of proband 4. These data point to a mutant allele originating and persisting in West African populations and transported to North America. The common African allele has five alternatively spliced isoforms (Supplementary Fig. 1) that lead to premature translational termination. These *LEPRE1* mutations were not found in 100 alleles from clinically normal controls.

Most *LEPRE1* mutations, like the mutant West African allele, result in altered splicing of the transcript, leading to premature translational termination and severely decreased levels of *LEPRE1* mRNA in probands. The exon 9 splice donor site mutation (IVS9+1G>T) found in the Nigerian father of proband 3 activates an intronic donor site, resulting in the retention of five intronic nucleotides and predicting the translational termination in exon 11 (Supplementary Fig. 1). The 1-nt deletion (c.747delC in exon 3) in the allele from the Afro-Caribbean father of proband 4 leads to premature termination in exon 5 in the majority of transcripts (Supplementary Fig. 1). Some transcripts from this allele are alternatively spliced, restoring the frame at P3H1 residue Val336, after translating 64 missense residues and deleting 65 nt. The putative protein product is missing two CXXXC motifs²⁰ that are highly conserved and are important for dimerization and two TPR domains²⁰, which function in protein-protein interactions. Only proband 5, whose parents are Pakistani, is homozygous for a point mutation (c.1656C>A) that directly generates a stop codon in exon 11 at residue 552 of 804.

The absence of P3H1 protein was demonstrated by protein blot (Fig. 2c) in four probands; a residual amount was present in proband 4. Because homozygotes for the mutant African allele have no detectable protein, we attributed proband 4's residual P3H1 to the paternal transcripts that have a restored reading frame. We also confirmed that null *LEPRE1* mutations virtually abolished type I collagen 3-hydroxylation. Tandem mass spectrometry of collagen tryptic peptides (Fig. 3 and Table 1) showed an absence of 3-hydroxylation of $\alpha 1(I)Pro986$ in four probands, and 10% of control hydroxylation in proband 4, consistent with residual P3H1. Parental $\alpha 1(I)Pro986$ hydroxylation was only slightly reduced (Table 1).

Absence of P3H1 affects folding of the type I collagen helix (Fig. 4a and Table 1). All probands have abnormal electrophoretic migration of the collagen chains, consistent with overmodification. In addition, amino acid chromatography showed an increase in the percentage of hydroxylated lysine residues along the collagen helix, comparable to the increased modification caused by a delay in helix folding from a collagen structural mutation near the carboxyl end of the $\alpha 1(I)$ chain in a case of type III osteogenesis imperfecta. Lack of $\alpha 1(I)Pro986$ hydroxylation and/or a direct chaperone effect of P3H1 evidently results in a delay of helix folding and longer exposure of the constituent chains to lysyl hydroxylases and prolyl 4-hydroxylases. The full overmodification of type I collagen is most likely the major contributor to the ~ 1 °C increase in proband collagen melting temperature, T_m , seen by calorimetry, as overmodified collagen with a normal primary structure from cells grown at 40 °C (ref. 21) has a comparable increase in T_m (Fig. 4b). We cannot totally exclude that lack of Pro986 hydroxylation contributes to the increased T_m . Prolyl 3-hydroxylation destabilizes host-guest peptides, especially in the Y-position^{22,23}, but peptides with 3Hyp in the X-position still form a typical collagen superhelical structure, with the 3Hyp residue pointed away from the helix²⁴. It is therefore likely that neither the presence nor absence of 3Hyp contributes substantially to the global stability of the type I collagen helix. Furthermore, in all probands, we detected overmodification of type V collagen, a fibrillar collagen found with type I collagen in skin and bone, which contains several sites for prolyl 3-hydroxylation. We verified that overmodification of collagen was not due to a secondary increase in transcription of lysyl hydroxylase isoforms (*PLOD1-3*).

Transcripts coding for LH1 (0.84 ± 0.15) and LH3 (0.99 ± 0.22), which hydroxylate the helical region of collagen chains^{25,26}, were normal for the *LEPRE1* probands, compared with a pooled control of five normal individuals. Transcripts coding for LH2 were increased (2.28 ± 1.69) in *LEPRE1* probands; however, LH2 hydroxylates the collagen telopeptide and has not been shown to hydroxylate the collagen helical region²⁷.

Overmodified collagen with a primary structural abnormality is often secreted from the cell into the matrix slower than normal collagen¹. Pulse-chase experiments showed that this was also true for fibroblasts with P3H1 deficiency (Fig. 5), which have moderately slower secretion kinetics than are seen in two control lines, and is consistent with a possible chaperone role for the 3-hydroxylation complex. Notably, the total collagen secreted by *LEPRE1* null cells was 20%–55% higher than that secreted by the same number of control cells.

These data delineate a new recessive metabolic bone disorder caused by deficiency of P3H1. Our cases have extreme skeletal under-mineralization and a disorganized radiographic appearance of bone matrix. This bone dysplasia may result from a disruption of the intracellular functions of P3H1, presented here, and also its putative function(s) as the matrix proteoglycan leprecan, as it can be extracted from normal bone (Supplementary Fig. 2 online). Absence of P3H1 may affect basement membrane function; P3H1 was first detected in renal basement membrane⁷ and type IV collagen has multiple 3-hydroxylation sites²⁸. Immunohistochemical staining⁹, however, showed that P3H1 localized specifically to tissues expressing fibrillar collagen and there was minimal expression in kidney and liver, which are rich in basement membrane.

Homozygosity for the West African mutant allele was lethal in the first month of life, whereas compound heterozygosity for this allele permits survival beyond 1 year, with severe bone disease and growth deficiency. Thus, the P3H1 deficiency phenotype, which modifies a single residue of type I collagen, overlaps that of osteogenesis imperfecta, which is caused by structural defects of type I collagen. We propose that the nosology of the new recessive forms of osteogenesis imperfecta extends recent osteogenesis imperfecta types defined by bone histology. Defects in *CRTAP*, which encodes the helper protein for P3H1, cause lethal¹⁸ to severe^{10,17} bone dysplasia, and are logically grouped together as Type VII osteogenesis imperfecta. Defects in *LEPRE1*, resulting in a lethal to severe recessive bone dysplasia that is characterized by white sclerae, severe growth deficiency, extreme skeletal under-mineralization and bulbous metaphyses, should be classified as Type VIII osteogenesis imperfecta.

Overmodification of types I and V collagen in individuals with *LEPRE1* mutations suggests that P3H1 may directly function as a collagen chaperone. The presence of P3H1 (known as leprecan in matrix) in normal bone matrix (Supplementary Fig. 2) indicates that its absence may have direct consequences for matrix structure. The relative contributions of 3-hydroxylation deficiency and putative chaperone and matrix glycoprotein functions of P3H1 to the clinical manifestations of this syndrome remain to be delineated. These investigations will be aided in the future by generation of mice that are deficient for *Lepre1* and *Ppib* (encoding cyclophilin B). Furthermore, because this severe disorder is caused by a

deficiency of an essential protein, it may be more amenable to gene or cell therapy than classical osteogenesis imperfecta, which has a dominant negative mechanism.

METHODS

Clinical information.

Detailed descriptions of proband phenotypes are presented in the Supplementary Note. Collection of proband and parental samples and information was approved by the National Institute of Child Health and Human Development institutional review board. Written informed consent was received for probands 1, 3 and 5. Samples from probands 2 and 4 were referred to the US National Institutes of Health for CLIA-certified diagnostic testing.

Mutation analysis.

Genomic DNA was extracted from leukocytes or primary fibroblast cultures using the Puregene DNA Isolation kit (Gentra Systems). We designed primers (Supplementary Table 1 online) that corresponded to intronic sequences flanking the exons of *LEPRE1* on chromosome 1. Primers for exons 3 (3F/3R), 5 (5F/5R), 9–10 (9F/10R) and 11 (11F/11R) were used to obtain 544, 471, 692 and 312-bp products, respectively. PCR was performed with Platinum Taq High Fidelity (Invitrogen) using standard conditions. Amplification products were sequenced using primers internal to the PCR fragments, and analyzed on a Beckman Coulter CEQ 2000 DNA analysis system. Sequence variants were confirmed by digestion of independent PCR products using appropriate restriction endonucleases (*NlaIV*, *BsaI*, *MseI* and *Tsp509I*, New England Biolabs) followed by electrophoresis on 6% or 10% acrylamide gels (Invitrogen). Leukocyte genomic DNA from 50 control samples was amplified by PCR using the same primers described above. PCR products were digested with the appropriate restriction enzymes to test for the four distinct mutations identified in probands.

RNA analysis.

Total RNA was extracted from primary fibroblasts cultured in the presence or absence of emetine (100 µmg/ml) using TriReagent (Molecular Research Center); RNA integrity was verified by analysis on an Agilent 2100 Bioanalyzer. For real-time RT-PCR analysis, 5 µg of RNA were reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems) with random primers. A TaqMan *LEPRE1* expression assay specific for the mRNA sequence was used for quantification of transcripts (Applied Biosystems, Hs00223565_m1). *LEPRE1* expression was calculated using a control fibroblast mRNA standard curve, then normalized to *GAPDH*. Levels of transcripts for lysyl hydroxylase isoforms were determined by real-time RT-PCR using Applied Biosystems Assays-on-Demand (*PLOD1*, Hs00609372_m1; *PLOD2*, Hs00168688_m1; *PLOD3*, Hs00153670_m1).

To identify alternatively spliced transcripts, RNA (1 µg) was reverse transcribed using antisense primers, e7as or e11as, specific for the *LEPRE1* transcript (Supplementary Table 1), and murine leukemia virus (MuLV) reverse transcriptase (Applied Biosystems). Subsequent PCR used the same antisense primers and a sense primer corresponding to exon 4 sequence, e4s. To specifically amplify the paternal allele in proband 4, we reverse

transcribed RNA with the e7as primer, and amplified it by PCR using e2s and e5/6as. PCR used Platinum Taq High Fidelity (Invitrogen) under standard conditions. PCR products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. Alternatively spliced transcripts were characterized by cloning of RT-PCR products into plasmids (TOPO TA cloning kit, Invitrogen) and sequencing using vector-specific primers.

Protein blot analysis.

Primary fibroblast cultures were lysed in radioimmunoprecipitation buffer containing a protease inhibitor cocktail (Sigma); protein concentrations of the lysates were quantitated using the BCA Protein Assay (Pierce). Samples (15 μ g of protein) were resolved by 10% SDS-PAGE, transferred to PVDF membranes (Invitrogen) and probed with a monoclonal antibody raised against a peptide fragment corresponding to residues 335–429 of leprecan protein (Abnova Corporation) or monoclonal antibody to β -actin (Sigma). Reactive bands were visualized with a secondary antibody conjugated with HRP (Santa Cruz Biotech, Inc.) and chemiluminescence (Western Lightning, Perkin Elmer Life Sciences).

Collagen modification analysis.

Confluent primary fibroblasts were labeled overnight in serum-free DMEM containing 260 μ Ci/ml [3 H]-labeled proline. Collagen was precipitated from medium and cell lysates, pepsin digested and electrophoresed on SDS-urea polyacrylamide gels under nonreducing conditions. Unlabeled procollagens were harvested from the medium of cultures stimulated in serum-free conditions containing ascorbate²⁹. Differential scanning calorimetry was performed in 0.2 M sodium phosphate and 0.5 M glycerol (pH 7.4)²⁹ from 10–50 $^{\circ}$ C at 0.125 $^{\circ}$ C min⁻¹ heating rates in a NanoII DSC instrument (Calorimetry Sciences Corporation). To determine prolyl 3-hydroxylation, procollagen samples were acidified and digested with pepsin at 4 $^{\circ}$ C for 24 h. Collagens were precipitated at 1.0 M NaCl and resolved by SDS-PAGE. The α 1(I) band was excised from the gel and subjected to in-gel trypsin digestion. Electrospray mass spectrometry was performed on the tryptic peptides using an LCQ Deca XP ion-trap mass spectrometer equipped with inline liquid chromatography (Thermo Finnigan) using a C8 capillary column (300 μ m \times 150 mm; Grace Vydac 208MS5.315) eluted at 4.5 μ l per min. To determine lysyl hydroxylation, collagens were purified twice by selective salt precipitation in 0.9 M NaCl. Purified collagen was redissolved in 0.5 M acetic acid, dialyzed against 5 mM acetic acid, analyzed by SDS-PAGE and circular dichroism and lyophilized. Amino acid composition was analyzed by HPLC (Commonwealth Biotechnologies, Inc).

Collagen synthesis and secretion kinetics.

For pulse-chase assays, performed as previously described³⁰, normal control and proband fibroblasts were plated in 35-mm wells and grown to confluence in DMEM and 10% FBS. For each cell line, two wells were used for cell counts before labeling. Cells were labeled for 4 h with 1.75 μ Ci [14 C]-proline and then chased with fresh medium containing 2 mM cold proline. Medium and cell layer procollagens were harvested at the indicated times and digested with pepsin. Sample loading for gel electrophoresis was normalized to cell number. Collagen was quantitated by densitometry of autoradiograms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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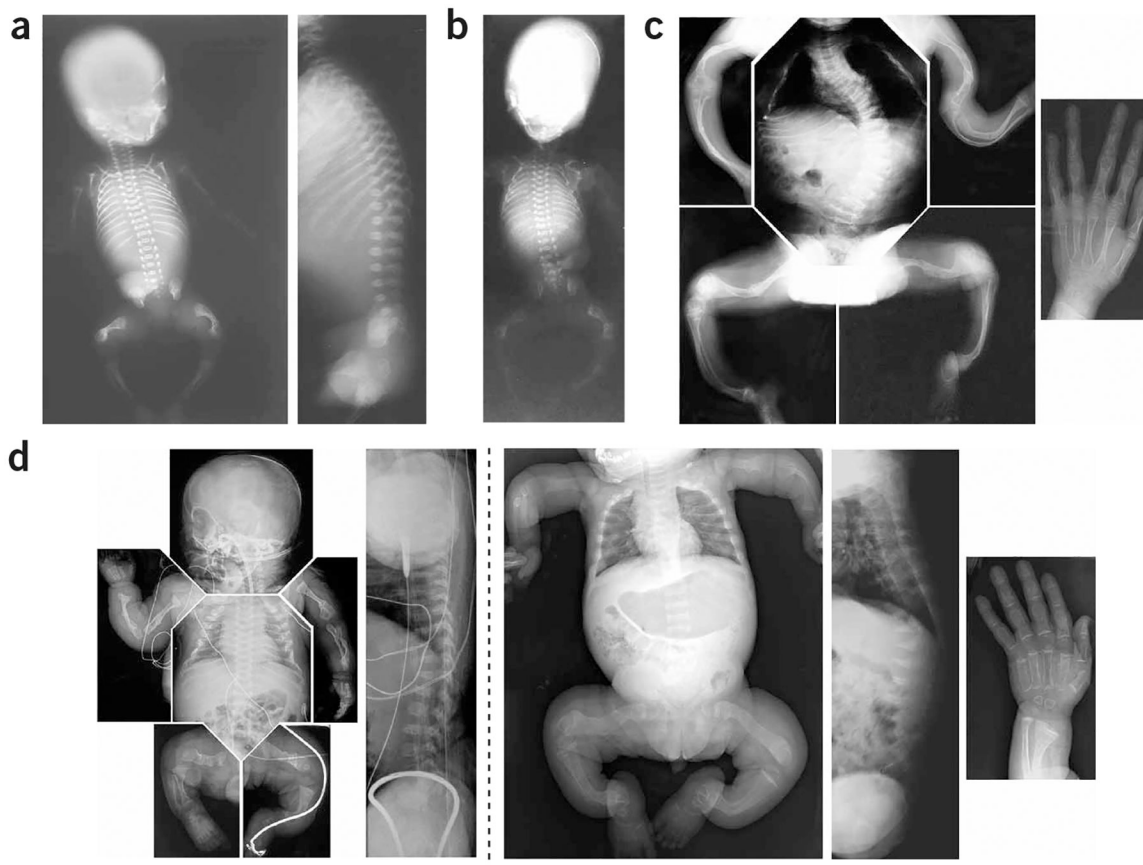


Figure 1.

Clinical and radiological manifestations of severe recessive osteogenesis imperfecta. **(a)** Fetal anteroposterior and lateral radiographs of proband 2–2 at 19-weeks gestation demonstrate irregular fractures with periosteal reaction and bowing of the femurs. The skull is markedly thin. The metaphyses are irregularly ossified. The vertebrae and ribs are not fractured. **(b)** Fetal anteroposterior radiograph of proband 4 at 18 weeks gestation. Bones are severely osteopenic with irregular fractures of the long bones. Femurs and tibiae are bowed. Ribs and skull are markedly thin. **(c)** Proband 5 at 6 years of age. The bones are severely osteopenic. Long bones are thin and gracile with prominent epiphyses and multiple fractures resulting in severe bowing. The ribs are markedly thin. There is significant platyspondyly with thoracolumbar scoliosis. Anteroposterior view of the hand at 9 years of age demonstrates severe osteopenia with disorganized matrix. The phalanges are unusually long and metacarpals are short. Bone age is normal. **(d)** Proband 3 newborn films (left of dotted line). The skull is thin. Long bones have numerous fractures with irregular callus formation, resulting in ‘accordion’ shaped femurs. There is bowing of the tibia, fibula and radius. Asymmetric platyspondyly is most marked at the lower thoracic vertebrae. Proband radiographs at 16 months of age (right of dotted line) demonstrate healing of fractures with resultant irregular bowing of the long bones, particularly the tibiae. Severe osteopenia has progressed. Vertebra plana has worsened with resultant kyphosis. Anteroposterior view of the hand demonstrates long phalanges with abnormal matrix; bone age is normal.

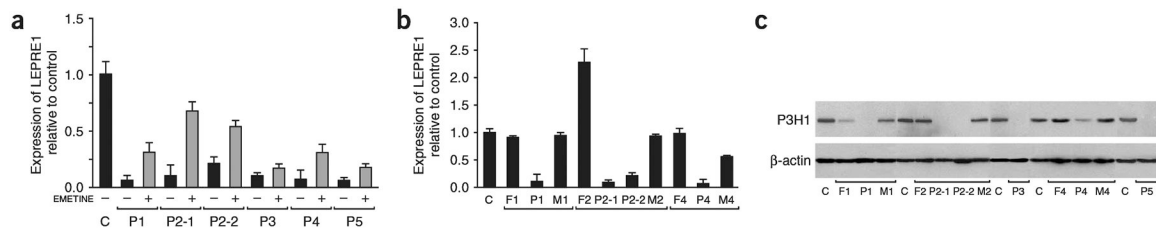
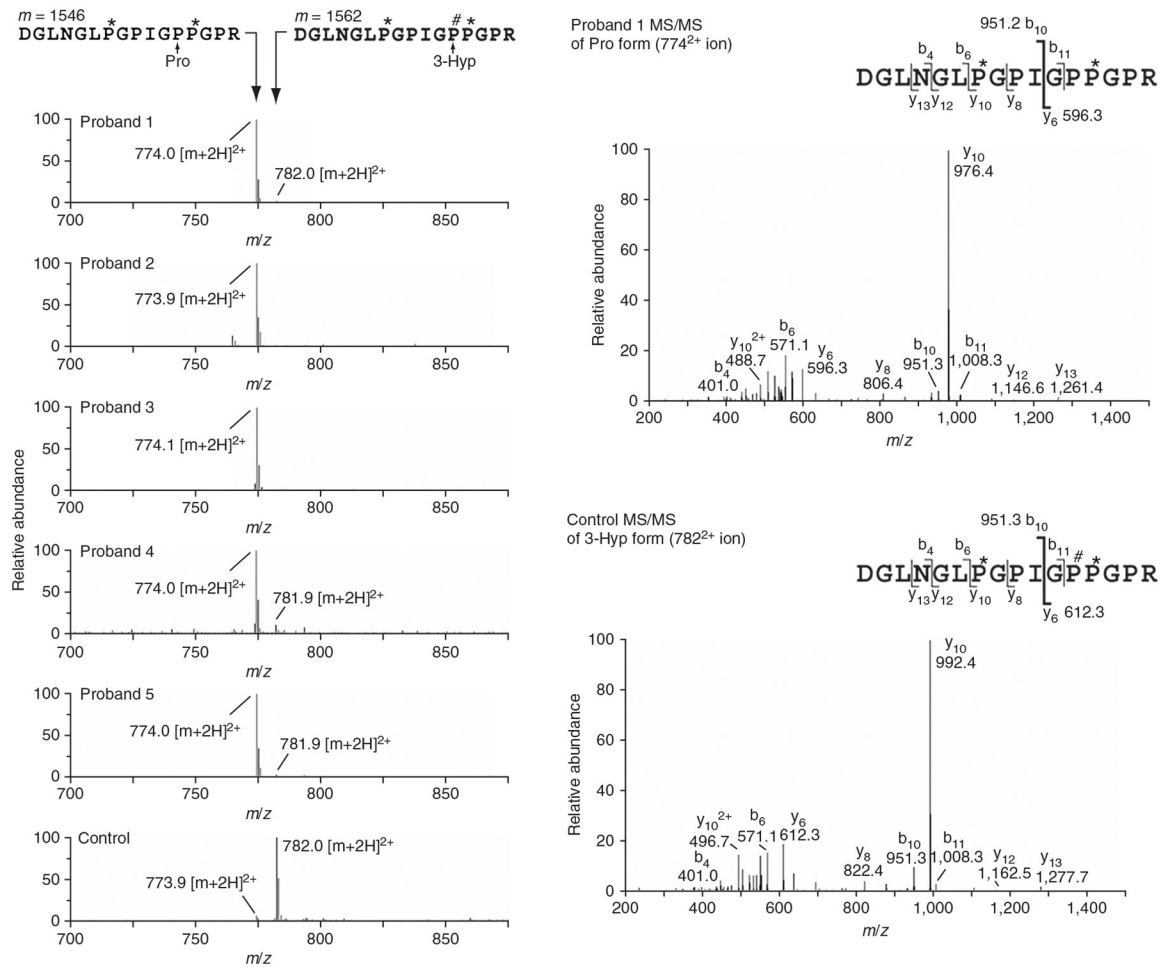


Figure 2.

LEPRE1 mRNA and P3H1 protein expression in probands and parents. **(a)** Proband fibroblast total RNA was screened for *LEPRE1* mRNA transcripts by real-time RT-PCR. Decreased *LEPRE1* mRNA was identified in primary fibroblasts from five probands with possible quantitative defects in *LEPRE1*. Partial rescue of *LEPRE1* mRNA expression in fibroblasts incubated with the translational inhibitor emetine (+) suggests that transcripts are degraded by nonsense-mediated decay because of the presence of premature termination codons. **(b)** Decreased expression of *LEPRE1* transcripts was evident only in affected probands by real-time RT-PCR. Parental fibroblasts (F, father; M, mother) showed relatively normal expression of *LEPRE1* mRNA compared with normal control fibroblasts. **(c)** Protein blots of total cell lysates probed with antibody to P3H1. Complete absence of P3H1 protein is seen in fibroblasts of proband 1 (P1), both siblings from family 2 (P2–1, P2–2), proband 3 (P3) and proband 5 (P5). A residual amount of P3H1, about 25% of control, was detected in proband 4 (P4). β -actin was probed as a loading control.

**Figure 3.**

Tandem mass spectrometry of proband and normal control secreted type I collagen. Proband 1 was observed to have absent or decreased 3-hydroxylation at $\alpha 1(I)Pro986$. Left, ions are displayed by their relative abundance and mass/charge (m/z) ratio. Doubly charged ions shown for probands differ from control by 16 a.m.u., because of a lack of an oxygen atom. In the presence of normal 3-hydroxylation, the tryptic peptide ions containing $\alpha 1(I)Pro986$ yield a mass of 782, whereas the peptide ions without 3-hydroxylation have a mass of 774. Right, fragmentation spectra of tryptic peptide ions containing the $\alpha 1(I)Pro986$ residue. N-terminal (b) and C-terminal (y) daughter ions are labeled for control and proband 1 samples. The y_6 ion localizes the extra 16 a.m.u. to the C-terminal six amino acid residues containing the $\alpha 1(I)Pro986$ site of 3-hydroxylation.

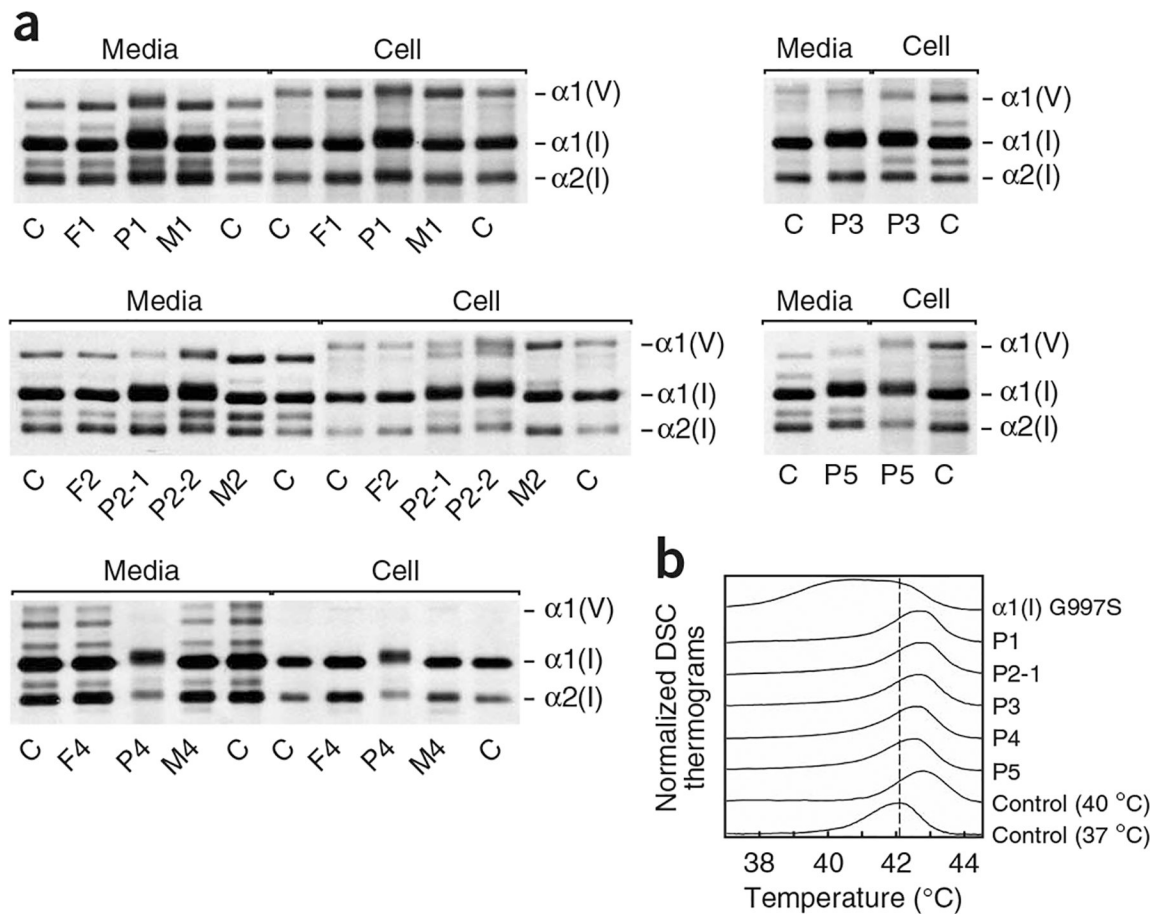


Figure 4.

Collagen chains with normal primary structure are overmodified in the helical region. (a) SDS-urea-PAGE analysis of type I collagens synthesized by proband and available parental fibroblasts. Overmodification, detected as delayed electrophoretic migration of collagen alpha chains, is present in all proband samples, but is absent in all parental samples. (b) Differential scanning calorimetry thermograms of proband type I collagens. The T_m of the proband samples are increased approximately 1 $^{\circ}C$ compared with normal control collagen, but are comparable to collagen synthesized at 40 $^{\circ}C$, consistent with increased modification in the absence of a primary structural defect. A thermogram of overmodified type I collagen containing an $\alpha 1(I)$ Gly997Ser substitution (top tracing) shows the decreased T_m characteristic of collagen structural defects.

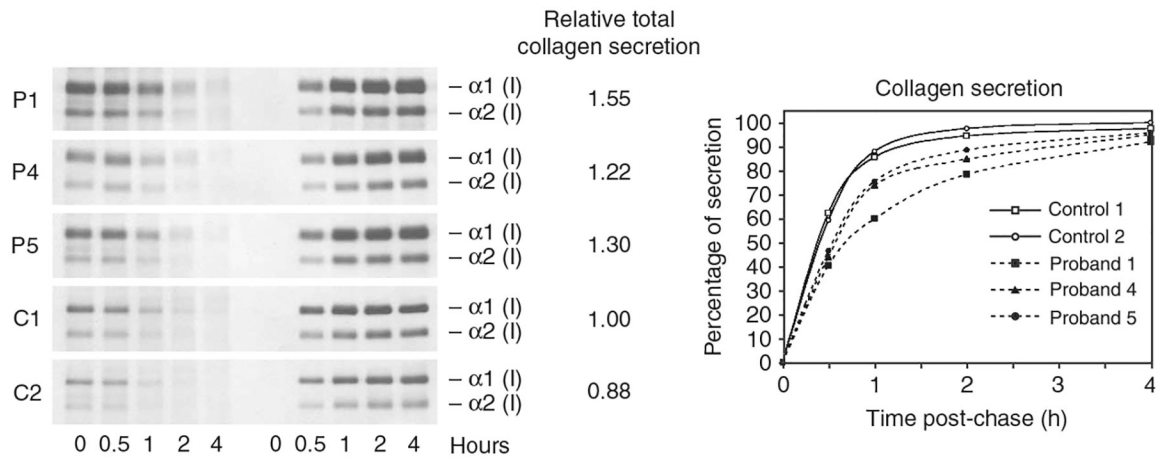


Figure 5. The effect of P3H1 absence on type I collagen secretion. **(a)** Pulse-chase studies of collagen synthesis by proband and control cells were conducted as described in Methods. Each scan shows intracellular and secreted collagen from the indicated cell line from $t = 0$ to 4 h after chase was initiated; sample loading was normalized to cell number. **(b)** Densitometry of radiographs of pulse-chase gels was used to calculate relative total collagen secretion (ratio of $\alpha 1(I)$ band in proband versus control in medium at 4 h), and time course of secretion (proportion of $\alpha 1(I)$ band in media versus media + cell at a given time for each cell line).

Table 1

Proband mutations and hydroxylation of type I collagen

Proband	Total Hyl (%) ^a	Hyl proband / Hyl control	3-Hydroxylation (%) ^b
Normal (nl) control ^c	24.3	–	97
Osteogenesis imperfecta III control (α1(I) G997S) ^d / nl	34.5	1.42	98
Father 1 (IVS5+1G>T) / nl			87
Proband 1 (IVS5+1G>T) / (IVS5+1G>T)	32.4	1.33	3
Mother 1 (IVS5+1G>T) / nl			85
Father 2 (IVS5+1G>T) / nl			89
Proband 2-1 (IVS5+1G>T) / (IVS5+1G>T)	32.9	1.35	0
Mother 2 (IVS5+1G>T) / nl			76
Father 3 (IVS9+1G>T) / nl			ND ^e
Proband 3 (IVS5+1G>T) / (IVS9+1G>T)	34.3	1.41	0
Mother 3 (IVS5+1G>T) / nl			ND
Father 4 (c.747delC) / nl			87
Proband 4 (c.747delC) / (IVS5+1G>T)	35.3	1.45	10
Mother 4 (IVS5+1G>T) / nl			87
Father 5 (c.1656C>A) / nl			ND
Proband 5 (c.1656C>A) / (c.1656C>A, Y552X)	33.6	1.38	4
Mother 5 (c.1656C>A) / nl			ND

^aTotal Hyl: the percentage of lysine residues in type I collagen modified by lysyl hydroxylase.

^b3-Hydroxylation: the percentage of α1(I)P986 that is 3-hydroxylated, as determined by mass spectrometry of tryptic peptides.

^cnl: normal allele.

^dG997S is a case of progressive deforming osteogenesis imperfecta (type III in the Sillence classification) with a heterozygous collagen structural mutation near the carboxyl terminus of the α1(I) chain.

^eND: not determined due to lack of sample availability.