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REVIEW

Null mutations in *LEPRE1* and *CRTAP* cause severe recessive osteogenesis imperfecta

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Abstract Classical osteogenesis imperfecta (OI) is a dominant genetic disorder of connective tissue caused by mutations in either of the two genes encoding type I collagen, *COL1A1* and *COL1A2*. Recent investigations, however, have generated a new paradigm for OI incorporating many of the prototypical features that distinguish dominant and recessive conditions, within a type I collagen framework. We and others have shown that the long-sought cause of the recessive form of OI, first postulated in the Sillence classification, lies in defects in the genes encoding cartilage-associated protein (*CRTAP*) or prolyl 3-hydroxylase 1 (P3H1/*LEPRE1*). Together with cyclophilin B (*PPIB*), CRTAP and P3H1 comprise the collagen prolyl 3-hydroxylation complex, which catalyzes a specific post-translational modification of types I, II, and V collagen, and may act as a general chaperone. Patients with mutations in *CRTAP* or *LEPRE1* have a lethal to severe osteochondrodystrophy that overlaps with Sillence types II and III OI but has distinctive features. Infants with recessive OI have white sclerae, undertubulation of the long bones, gracile ribs without beading, and a small to normal head circumference. Those who survive to childhood or the teen years have severe growth deficiency and extreme bone fragility. Most causative mutations result in null alleles, with the absence or severe reduction of gene transcripts and proteins. As expected, 3-hydroxylation of the Pro986 residue is absent or severely reduced, but bone severity and survival length do not correlate with the extent of residual hydroxylation. Surprisingly, the collagen produced by cells with an absence of Pro986 hydroxylation has helical overmodification by lysyl hydroxylase and prolyl 4-hydroxylase, indicating that the folding of the collagen helix has been substantially delayed.

Keywords Recessive osteogenesis imperfecta · CRTAP · P3H1/LEPRE1 · Prolyl 3-hydroxylation · Osteochondrodysplasia

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Introduction

Osteogenesis imperfecta (OI), or "brittle bone disease", is a well-known heritable disorder of connective tissue whose hallmark feature is bone fragility. Most cases of OI are caused by autosomal dominant defects in either of the genes (*COL1A1* or *COL1A2*) that code for type I collagen, the major structural protein of the extracellular matrix of bone and skin. Mutations in the type I collagen genes account for the full range of the OI clinical phenotypes described by the Sillence classification (Sillence et al. 1979), from perinatal lethal to barely detectable. Genotype-phenotype correlations based on over 800 collagen mutations have shown different localization patterns for lethal mutations in *COL1A1* and *COL1A2*, implying distinct functions for each chain (Marini et al. 2007b).

About 75%–80% of collagen structural defects are point mutations in codons for glycine and result in substitutions by other residues. Glycine is required in the first position of the repeating triplet (Gly-Xaa-Yaa) comprising the collagen helical region and faces the sterically restricted inner aspect of the collagen triple helix. Most of the remaining collagen structural mutations involve splice donor or acceptor sites; many of these result in splicing out of a discrete collagen exon, although a surprising proportion involve the use of a

cryptic splice site and out-of-frame transcripts, leading to premature termination codons (PTCs). Mechanistically, insufficiency of structurally normal collagen is known to lead to clinically mild OI, whereas the presence of structurally abnormal collagen leads to the more clinically serious cases involving abnormal extracellular matrix structure and cell-matrix interactions.

A new paradigm for OI at the junction of four different approaches

A new paradigm for OI has generated great interest because it incorporates many of the prototypical features that distinguish dominant and recessive conditions, within the type I collagen-related framework. Classical OI is caused by dominant negative structural mutations in either of the type I collagen genes. Recessive OI is caused by a deficiency of an enzyme or its helper protein involved in the post-translational modification of collagen. How did this expanded view of OI emerge?

Following the complete sequencing of the collagen coding regions, it became increasingly clear that a subset of patients with lethal and severe OI did not have structural defects in type I collagen. Four distinct and independent approaches came together over the space of a few years to generate a new understanding of OI. The first approach involved clinical genetics and histomorphometry (Ward et al. 2002). An autosomal recessive form of OI was identified in an extensive First Nations pedigree in northern Quebec Province. The distinctive features of these individuals were rhizomelia, coxa vera, minimally bluish sclerae, and a high turnover of bone tissue in the context of normal type I collagen sequence. Although they had fractures at birth, many affected individuals had moderate bone fragility, resulting in several dozen fractures through young adulthood, and were ambulatory without assistive devices. This index pedigree, in a new moderate-to-severe OI category, was designated type VII OI. Because of the moderate severity of OI in this pedigree and their genetic isolation, the connection to other severe and lethal cases of OI without collagen defects was not made until new data on collagen biochemistry and bone genetics became available several years later.

The second approach leading to the new OI paradigm was collagen biochemistry. The isolation of the protein responsible for collagen prolyl 3-hydroxylation, prolyl 3-hydroxylase 1 (P3H1), had eluded investigators for over 20 years despite its existence having been inferred. Bachinger and colleagues successfully purified P3H1 from chick embryo rER extracts by using a protocol involving affinity chromatography for gelatin (denatured collagen) and a monoclonal antibody affinity column for P3H1 (Vranka et al. 2004). Co-elution of P3H1, cartilage-associated protein (CRTAP), and cyclophilin B (CyPB) from the P3H1 affinity column suggested the presence of a collagen-interacting complex in the endoplasmic reticulum (ER).

The third approach involved generation of a genetic mouse model. In approximately the same timeframe as P3H1 isolation, an investigation of CRTAP by Morello and colleagues connected CRTAP to a recessive bone phenotype through the knock-out *Crtap* mouse. *CRTAP* had first been identified in a subtracted cDNA library during screening for uniquely expressed genes in hypertrophic chick chondrocytes in culture (Castagnola et al. 1997). In the skeletal system, *CRTAP* expression was identified in tibial and femur epiphyses, articular cartilage, and lower hypertrophic zone cartilage in the chick (Vranka et al. 2004) and in the growth plate, bone collar, and calcified cartilage of the chondro-osseous junction in the mouse (Morello et al. 2006). Furthermore, analysis by reverse transcription with the polymerase chain reaction (RT-PCR) confirmed the expression of *Crtap* in both osteoblasts and osteoclasts. The *Crtap* knock-out mouse had a recessive osteochondrodysplasia (Morello et al. 2006), including growth deficiency, osteopenia, shortening of the femurs (rhizomelia), and progressive kyphosis, which revealed the bone function of CRTAP. Consistent with the localization of *Crtap* to the growth plate, the proliferating chondrocytes of the *Crtap*$^{-/-}$ mouse were disorganized. An absence of CRTAP resulted in the loss of 3-hydroxylation of the P986 residue in α1(I) and α1(II) collagen chains, confirming the in vivo role of CRTAP as a crucial component of the collagen 3-hydroxylation complex.

The results from the skeletal and biochemical studies of the *Crtap* knock-out mouse were combined with those from the chick biochemical studies of the collagen prolyl 3-hydroxylation complex and depicted an ER-resident collagen-modification complex that interacted with types I and II collagen and was important for bone structure and function.

Finally, the clinical molecular genetics approach identified the appropriate patients to complete the paradigm. The interaction of P3H1 and CRTAP with collagen, together with the role of CRTAP in bone structure, were precisely the features that we were seeking in candidate gene(s) for cases of OI that did not have defects in the type I collagen genes. About 10%–15% of the children with clinical OI who are referred to the NIH do not have demonstrable collagen mutations. The absence of a collagen mutation was particularly perplexing in a subgroup of these cases, including families with more than one affected child, in whom the type I collagen synthesized by dermal fibroblasts was biochemically abnormal, with unequivocal overmodification of the helical regions of the collagen chains. Given

their positive biochemical test for abnormal collagen, we postulated that the genetic defect in this group of patients lay in a protein(s) that interacted with collagen. The P3H1 and CRTAP data combined to elevate these proteins from distant possibilities to prime candidates. Recessive deficiency of P3H1 or CRTAP was found to cause essentially all OI cases with positive collagen biochemistry and negative collagen sequencing. Furthermore, the connection to the original type VII OI pedigree, with rhizomelia and moderately severe OI and a chromosomal localization at 3p22–24.1 (Labuda et al. 2002), fell into place when a hypomorphic mutation of *CRTAP* mapping to chromosome 3p22, was identified in these individuals (Morello et al. 2006; Tonachini et al. 1999).

Type I collagen structure and post-translational modification

In the new paradigm for collagen-related bone disorders, post-translational modification of collagen plays an important role. Each of the alpha chains of heterotrimeric type I collagen [$\alpha1(I)_2\alpha2(I)$] contains 338 repeats of the Gly-Xaa-Yaa amino acid triplet. The X and Y residues of the collagenous Gly-X-Y triplet are often proline and 4-hydroxyproline, which comprise approximately 25% of the amino acid content of collagen. During chain synthesis and folding into a heterotrimeric helix, each of the collagen alpha chains is subject to several post-translational modifications (Myllyharju and Kivirikko 2004). These modifications include *cis* to *trans* prolyl isomerization by CyPB, 5-hydroxylation of approximately one quarter of the lysyl residues (Xaa-Lys-Gly) by lysyl hydroxylase (LH1), and 4-hydroxylation of about one-half of the prolyl residues (Xaa-Pro-Gly) by prolyl 4-hydroxylase (P4H; Lehmann et al. 1995). The post-translational modifications are essential for helix formation and stability at the molecular and tissue level; 4-hydroxyproline residues provide stability to the trimer, whereas hydroxylysine is required for the formation of intermolecular crosslinks and attachment sites for glycosylation (Kivirikko and Myllyla 1979).

An additional post-translational modification of unknown function occurs and results in 3-hydroxylation of a single X position residue (Pro986) in each of the α1(I) chains by using the recognition sequence Pro-4Hyp-Gly (Fietzek et al. 1972). Tryggvason and colleagues first demonstrated that 3-hydroxyproline and 4-hydroxyproline were synthesized by unique enzymes (Risteli et al. 1977). A partially purified protein fraction with prolyl 3-hydroxylase activity was subsequently isolated from chick embryo extracts (Tryggvason et al. 1979). It is this enzymatic activity that is lacking in the cases of recessive OI, with genetic defects in the collagen prolyl 3-hydroxylation complex components. The role of this modification presumably involves an important general function of fibrillar collagens, since this complex hydroxylates the same site in α1(I), α1(II), several sites in type V collagen, and, possibly, types III and IV collagen.

CRTAP and P3H1 are multifunctional proteins

Demonstration that *CRTAP* is important for the development of connective tissues has been provided by the characterization of its expression in several chick, murine, and human tissues (Castagnola et al. 1997; Morello et al. 1999; Tonachini et al. 1999). Although CRTAP was first identified in cultured chick hypertrophic chondrocytes (Castagnola et al. 1997), *Crtap* transcripts were also detected by Northern analysis in chick embryonic skin, heart, and lungs. In human tissues, the expression of *CRTAP* was identified in heart, lung, and small intestine, in addition to skeletal tissue (Tonachini et al. 1999). In mouse, the majority of CRTAP was intracellular and co-localized with markers for the ER, but light staining for CRTAP was identified in cartilage matrix by immunohistochemistry and indicated matrix, and cellular, functions.

Prolyl 3-hydroxylase 1 is a multifunctional protein that has now been independently identified three times, suggesting that the hydroxylation of collagen is not the only role of the P3H1 protein. It was first isolated from a rat yolk sac tumor cell line as a novel matrix chondroitin sulfate proteoglycan by Wassenhove-McCarthy and McCarthy (1999); the authors isolated, from a rat cDNA library, a single clone found to contain a fragment coding for the COOH terminal region of a novel cDNA. The sequence was used to isolate a full-length transcript by a cDNA capture assay, producing a 3.1-kb transcript predicted to encode a 728-amino-acid leucine-proline enriched proteoglycan. Termed "leprecan", the proteoglycan was detected in the ER and Golgi of CHO cells transfected with the full-length cDNA. Furthermore, despite containing a carboxyl terminal KDEL ER retrieval signal, leprecan was shown to be secreted by rat L2 cells and was localized to the basement membrane of several rat tissues by immunostaining. The polyclonal antibody recognizing the C-terminus of leprecan gave strong staining in the basement membranes of the vasculature and smooth muscle of several organs, including the glomerulus, mesangial matrix, and tubules of kidney, the liver, and skeletal muscle, and the pericellular matrix surrounding chondrocytes in cartilage.

The human homolog of leprecan, Gros1, was isolated as a potential growth suppressor located at chromosome 1p31 (Kaul et al. 2000). In this study, two alternatively spliced transcripts encoding 41- and 84-kDa proteins denoted as Gros1-S and Gros1-L, respectively, were identified. The

first 360 residues of the 736-amino-acid 84-kDa isoform are identical to the predicted translated sequence of the Gros1-S variant. Gros1-S, however, utilizes a cryptic exon (exon 5b) in intron 5 while also retaining the entire intron 7 sequence, leading to a premature stop codon. The alternative transcript would generate a truncated 363-amino-acid peptide lacking the carboxyl leucine repeat and P4H domain; this protein product has yet to be demonstrated. Semiquantitative PCR analysis has demonstrated that Gros1-L is the dominantly expressed form in adult human tissues, such as heart, lung, and kidney (Tiainen et al. 2008). In human fetal tissues, Gros1-S predominates in brain and thymus, whereas both forms of the transcript are seen in equimolar ratios in liver, skeletal muscle, spleen, and pancreas.

The third independent isolation of the *LEPRE1* gene product was as P3H1, the enzymatic component of the collagen prolyl 3-hydroxylation complex (see A new paradigm for OI at the junction of four different approaches).

Collagen prolyl 3-hydroxylation complex

Recent investigations by a small number of laboratories have demonstrated that the 3-hydroxylation modification of the major fibrillar collagens is performed by a protein complex consisting of P3H1, CRTAP, and CyPB, in a 1:1:1 ratio (Morello et al. 2006; Vranka et al. 2004). Since P3H1 is able to 3-hydroxylate collagen substrate independently in vitro (Vranka et al. 2004), P3H1 is now believed to serve in vivo as the enzymatic component, whereas CRTAP functions as a "helper" protein for the complex.

Comparison of the human protein sequences of the components of the 3-hydroxylation complex show several striking features (Marini et al. 2007a). CRTAP has a high degree of homology (55%) with the amino half of the larger P3H1 protein. Putative transmembrane sequences can be found in both CRTAP (Gly_4–Ser_{32}) and P3H1 (Ala_5–Val_{33} and Glu_{372}–Phe_{387}), although these proteins have not been demonstrated to associate with or to form a complex on the ER membrane. Interestingly, the amino terminal location of the putative transmembrane sequences in P3H1 and CRTAP would be consistent with the topology of type II membrane proteins, allowing for several functional motifs to be situated within the ER lumen where they could interact with collagens. P3H1 contains four tetratricopeptide repeats (TPR), which are important for protein-protein interactions. These motifs are also found in the mouse, rat, cow, and chicken homologs of P3H1. CRTAP, however, contains only a single TPR domain, which corresponds to the second TPR of human P3H1, and can be identified in the human, mouse, rat, chicken, and zebrafish proteins.

Both proteins have four CX_3C motifs, which also occur in the small proteins of the mitochondrial inner membrane translocase complex TIM and in the chemokine fractalkine (Koehler 2004; Stievano et al. 2004). Interestingly, a similar CX_2C motif is conserved among thiol/disulfide oxidoreductases and participates in redox reactions and the isomerization of incorrectly formed disulfide bonds (Chivers et al. 1998; Horibe et al. 2004; Quan et al. 2007) and in the formation of metal-binding sites (Bruschi and Guerlesquin 1988; Coruzzi and Tzagoloff 1979; Glerum et al. 1996). Furthermore, the occurrence of the CX_3C sequence in both P3H1 and CRTAP can be identified across species, including the human, mouse, and cow proteins, each of which contain four of these motifs. Chicken CRTAP has three CX_3C motifs, whereas rat and zebrafish CRTAP contain only two.

P3H1 also contains a prolyl 4-hydroxylase (P4H) domain, plus four catalytic residues that are highly conserved among the 2-oxoglutarate dioxygenase family (His_{587}, Asp_{589}, His_{659} and Arg_{669}). This group of proteins includes P4H, the P3H isoenzymes (P3H1-3), and the lysyl hydroxylases (LH1-3), all of which require Fe^{2+}, 2-oxoglutarate, O_2, and ascorbic acid for their activity. Other features of P3H1 include a putative integrin association site ($RGD_{48–50}$), three potential glycosaminoglycan attachment sites ($Ser_{190,\ 496,\ 653}$), and three putative N-glycosylation sites ($Asn_{316,\ 467,\ 540}$). P3H1 contains a leucine zipper (L_{445}TREGGPLLYE-GISLTMNSKLL_{466}), which is known to be involved in protein dimerization, and two potential myristoylation sequences (G_{53}VVLSM_{58} and G_{667}QRCAI_{672}). There are also 20 potential recognition sites for phosphorylation, although no evidence yet exists that these sites are used. Piltti and coworkers (2008), however, have suggested that phosphorylation of P3H1/leprecan is increased in human HCS 2/8 chondrosarcoma cells in response to mechanical stimulation and is presumably related to altered intracellular collagen synthesis and assembly.

The third member of the 3-hydroxylation complex, CyPB, is a peptidyl-prolyl isomerase and is known to play a central role in the in vivo folding of procollagens in the ER. CyPB does not have any structural similarities to CRTAP or P3H1-3. Procollagen alpha chain association occurs at the carboxyl-terminal propeptides, which are the last portions of the polypeptide to be translated. Although the collagen triple helix can only accommodate peptide bonds that are in the *trans* conformation, there is sufficient time for the formation of an equilibrium state of unfolded alpha chains in which as much as 12% of X-Pro bonds in type I procollagen are in the *cis* conformation, as determined by nuclear magnetic resonance (Fischer et al. 1984). Hence, the peptidyl-prolyl *cis-trans* isomerase function of CyPB catalyzes the efficient folding of procollagen alpha chains into the triple helical structure. Evidence in support of this role has been presented by Bachinger and colleagues, who used partially purified

enzyme to study the in vitro rate of folding of type III procollagen (Bachinger 1987); they concluded that CyPB increased the rate of folding three-fold, and that *cis-trans* isomerization of the X-Pro bonds in collagen alpha chains was the rate-limiting step of helical folding (Bachinger et al. 1980). Complementary findings by Steinmann and colleagues (1991) support an in vivo role of CyPB in collagen folding. Treatment of fibroblasts with cyclosporin A (CsA) induces the overmodification of type I collagen along the full length of the alpha chains. Since CsA binds to and inhibits the isomerase activity of CyPB by an incompletely characterized mechanism (Fischer et al. 1989), the loss of *cis-trans* prolyl isomerization and the subsequent delayed folding is believed to result in collagen overmodification. This is supported by amino acid analysis, which has demonstrated an increase in the proportion of hydroxylysine residues from 21% of lysyl residues in normal control collagen to 28% of lysyl residues in CsA-treated cells.

Recent investigations into the functioning of the collagen 3-hydroxylation complex have suggested that the P3H1/CRTAP/CyPB complex functions as a chaperone in classical chaperone assays involving citrate synthase aggregation and rhodanese refolding (Ishikawa et al. 2009). Furthermore, although CyPB has greater PPIase activity on a peptide substrate as part of the complex than as free CyPB, free CyPB is a better catalyst of pN-collagen III refolding than is the full complex. In addition, the collagen 3-hydroxylation complex has been shown to inhibit fibril formation of type I collagen in vitro, suggesting that it may prevent premature aggregation of collagen molecules within the secretory pathway of cells (Ishikawa et al. 2009).

Mutations in *CRTAP* and *LEPRE1* cause recessive types VII and VIII OI

Recent developments in bone biology have demonstrated that deficiency of the components of the collagen 3-hydroxylation complex is the elusive cause of recessive OI, first postulated to exist in the 1979 Sillence classification (Sillence et al. 1979). In our original screening cohort of 10 children with lethal to severe OI, overmodified collagen protein, and normal *COL1A1* and *COL1A2* sequences, all 10 were demonstrated to have deficiency of CRTAP (3 cases; Barnes et al. 2006) or P3H1 (7 cases including 2 unpublished; Cabral et al. 2007). To date, defects in *COL1A1*, *COL1A2*, *CRTAP*, or *LEPRE1* account for all cases of severe OI with abnormal collagen biochemistry.

Identification of the first human cases of *CRTAP* deficiency (Barnes et al. 2006) paralleled the characterization of the *Crtap* knock-out mouse (Morello et al. 2006) and fulfilled our hypothesis that recessive OI would be caused by a molecule that interacted with type I collagen. To date, 13 independent probands with 15 distinct *CRTAP* mutant alleles have been published (Table 1). When parental analysis has been possible, each parent has been shown to be the heterozygous carrier of a mutant allele. The occurrence of confirmed parental consanguinity in six of the probands has also been consistent with recessive inheritance.

Most of the mutations are of a type that is expected to cause loss of *CRTAP* transcripts by nonsense-mediated decay (NMD), consistent with the absent or severely reduced transcript levels that have been reported in eight cases. Two mutant alleles directly generate a stop codon; one alters the initiating Met residue, whereas 10 mutant alleles contain small deletions, duplications, or splice site defects that shift the frame of the transcript directly or through alternative splicing, leading to PTCs and NMD. In contrast, two mutant alleles contain missense mutations: (1) a homozygous Leu67Pro substitution of unknown mechanism, and (2) a mutant allele that contains two non-conservative changes, one in the signal peptide and the second mid-chain altering a residue charge; this allele occurs in compound with a null mutation. Notably, almost all the identified *CRTAP* mutations occur in exons 1 and 4 and their adjacent intronic sequences.

In humans, the absence of CRTAP protein or the loss of its functional role in the 3-hydroxylation complex causes severe to lethal osteochondrodysplasia, now designated as type VII OI (OMIM 610682). Interestingly, *CRTAP* deficiency in humans results in a more severe phenotype than in the knock-out mouse. The human phenotype overlaps Sillence types II and III (Barnes et al. 2006; Van Dijk et al. 2009). Patients have severe osteoporosis with rhizomelia, neonatal fractures, broad undertubulated long bones, gracile ribs (several born without rib fractures), and a relatively normal head circumference. In almost all cases, their sclerae have been described as white or light grayish. Those who survive into childhood manifest extreme symptoms of chondrodystrophy, with disorganized “popcorn” epiphyses similar to those seen in about half of the children with type III OI, and extreme growth deficiency (Baldridge et al. 2008; Obafemi et al. 2008).

The data available concerning levels of Pro986 hydroxylation in some of these probands do not suggest that the collagen modification is related to clinical outcome in a simple way (Table 2). Three infants with 0%–5% Pro986 hydroxylation died before 1 year of age. However, a child with 24% Pro986 hydroxylation also died at 10 months after multiple pneumonias. The level of Pro986 hydroxylation was not determined in either a 4-year-old child homozygous for a splice site defect in intron 1 or a 2-year-old homozygous for a small frame-shifting duplication in exon 1, and so the relationship of greater longevity to

Table 1 Mutations in genes encoding collagen 3-hydroxylation components (*ND* not determined, PTC premature termination codon)

Mutation	Location of exon/intron mutation	Number of alleles	PTC cDNA location	Effect of mutation on protein	Reference
CRTAP					
c.3G>A	Exon 1	1	Missense	p.M1I	Barnes et al. 2006
c.21_22dupGG	Exon 1	2	c.35_37 (exon 1)	p.Ala8fsX6	Van Dijk et al. 2009
c.24_31del	Exon 1	2	c.122_124	p.Ala8fsX31	Baldridge et al. 2008
c.38C>A, c.469A>G	Exon 1	1	Missense	p.Ala13Glu, p. Lys157Glu	Van Dijk et al. 2009
c.198C>A	Exon 1	1	c.198 (exon 1)	p.Tyr66X	Van Dijk et al. 2009
c.200 T>C	Exon 1	2	Missense	p.Leu67Pro	Baldridge et al. 2008
c.278_293dupAGCCCGAG CCCGCCGC	Exon 1	2	c.477_479 (exon 1)	p.Ala98fsX68	Barnes et al. 2006; Baldridge et al. 2008
c.404delG	Exon 1	2	c.518_520 (exon 2)	p.Ser135fsX39	Van Dijk et al. 2009
c.471+1G>C	Intron 1	2	c.471+61_+63 (intron 1)	p.Lys157fsX21	Barnes et al. 2006
c.471+2C>A	Intron 1	5	ND	ND	Van Dijk et al. 2009; Bodian et al. 2009
c.472-1021C>G	Intron 1	First Nations	c.472–1057_−1055 (intron 1)	p.Lys157fsX13	Morello et al. 2006
c.822_826delAATACinsT	Exon 4	1	c.831_833 (exon 4)	p.Lys274fsX10	Baldridge et al. 2008
c.826C>T	Exon 4	2	c.826_828 (exon 4)	p.Gln276X	Barnes et al. 2006
c.879delT	Exon 4	2	c.923_925 (exon 5)	p.Phe293fsX15	Morello et al. 2006
c.923–2A>G (cryptic accept. c.936)	Intron 4	1	c.972_974 (exon 5)	p.Leu308fsX13	Van Dijk et al. 2009
LEPRE1					
c.232delC	Exon 1	Irish Travelers	c.320_322 (exon 1)	p.Gln78fsX30	Baldridge et al. 2008
c.392C>A	Exon 1	2	c.391_393 (exon 1)	p.Ser131X	Baldridge et al. 2008
c.628C>T	Exon 3	2	c.628_630 (exon 3)	p.Arg210X	Willaert et al. 2008
c.747delC	Exon 3	1	c.1007_1009 (exon 5)	p.249fsX87	Cabral et al. 2007
c.765C>A	Exon 3	1	c.763_765 (exon 3)	p.Tyr255X	Baldridge et al. 2008
c.933C>G	Exon 4	2	c.931_933 (exon 4)	p.Tyr311X	Baldridge et al. 2008
c.1080+1G>T	Intron 5	22	5 splice forms (exons 6, 7, 9)	p.Arg359fsX-multiple	Cabral et al. 2007; Bodian et al. 2009; Baldridge et al. 2008
c.1102C>T	Exon 6	1	c.1102_1104 (exon 6)	p.Arg368X	Willaert et al. 2008
c.1170+2 T>A	Intron 6	2	In-frame exon 6 splice-out	p.Ser361_390del	Baldridge et al. 2008
c.1170+5G>C	Intron 6	2	In-frame exon 6 splice-out	p.Ser361_390del	Baldridge et al. 2008
c.1346–340_1473+36del	Intron 8–9	1	ND	ND	Baldridge et al. 2008
c.1365_1366delAGinsC	Exon 9	2	c.1382_1384 (exon 9)	p.Glu455fsX6	Willaert et al. 2008
c.1383_1389dupGAACTCC	Exon 9	2	c.1437_1439 (exon 9)	p.464fsX19	Baldridge et al. 2008
c.1473+1G>T	Intron 9	1	c.1622_1624 (exon 11)	p.492fsX52	Cabral et al. 2007
c.1656C>A	Exon 11	2	c.1654_1656 (exon 11)	p.Tyr552X	Cabral et al. 2007
c.2041C>T	Exon 14	2	c.2041_2043 (exon 14)	p.Arg681X	Baldridge et al. 2008
c.2055+18G>A	Intron 14	3	c.2055+104_+106 (intron 15) c.2073+2075 (exon 15)	p.Arg685fsX34 p.Arg685fsX13	Willaert et al. 2008

Table 2 Functional effects of defects in collagen 3-hydroxylation components (*NA* not applicable, *ND* not determined)

Proband	Transcript levels [a] (% versus normal control)	3Hyp/Pro [a] (% 3-hydroxylated Pro986)	5Hyl/Lys (% hydroxylated lysines)	Western analysis	Reference
Normal control	NA	97	24.3	+	Barnes et al. 2006; Cabral et al. 2007
α1(I) p.Gly1175Ser (Gly997Ser helix)	ND	98	34.5	ND	Barnes et al. 2006; Cabral et al. 2007
CRTAP					
c.3G>A/c.278_293dup	21	21	31.3	−	Barnes et al. 2006
c.24_31del/c.24_31del	19	4 (fb), 2 (bone)	ND	ND	Baldridge et al. 2008
c.200 T>C/c.200 T>C (p.Leu67Pro)	128	79	ND	ND	Baldridge et al. 2008
c.278_293dup/ c.822_826delAATACinsT	4	5	ND	ND	Baldridge et al. 2008
c.471+1G>C/c.471+1G>C	0	4	35.1	−	Barnes et al. 2006
c.472–1021C>G/c.472–1021C>G	10	Variable	ND	−/ residual	Morello et al. 2006
c.826C>T/c.826C>T	16	0	34.7	−	Barnes et al. 2006
c.879delT/c.879delT	1	40	ND	−	Morello et al. 2006
LEPRE1					
c.232delC/c.232delC	0.8	15	ND	ND	Baldridge et al. 2008
c.628C>T/c.628C>T	10	Severely reduced	ND	−	Willaert et al. 2008
c.933C>G/c.933C>G (p.Tyr311X)	6	5	ND	ND	Baldridge et al. 2008
c.747delC/c.1080+1G>T	7	10	35.3	ND	Cabral et al. 2007
c.1080+1G>T/c.1080+1G>T	5 (skin), 0.4 (bone)	4 (fb), 55 (bone)	ND	ND	Baldridge et al. 2008
	11	3	32.4	−	Cabral et al. 2007
	12	0	32.9	−	Cabral et al. 2007
c.1080+1G>T/c.1473+1G>T	10	0	34.3	−	Cabral et al. 2007
c.1102C>T/c.2055+18G>A	37	Severely reduced	ND	−	Willaert et al. 2008
c.1170+2 T>A/c.1170+2 T>A (exon 6 spliceout)	87	7	ND	ND	Baldridge et al. 2008
c.1383_1389dup/c.1383_1389dup	8	7	ND	ND	Baldridge et al. 2008
c.1656C>A/c.1656C>A (p.Tyr552X)	8	4	33.6	−	Cabral et al. 2007
c.2041C>T/c.2041C>T (p.Arg681X)	57	4	ND	ND	Baldridge et al. 2008
c.2055+18G>A/c.2055+18G>A	70	Severely reduced	ND	−	Willaert et al. 2008

[a] Fibroblast collagen, unless otherwise stated

residual CRTAP function is unknown in these cases. Individuals in the index pedigree for type VII OI, a genetic isolate in northern Quebec with a hypomorphic *CRTAP* mutation, have variable levels of transcript and Pro986 hydroxylation, with about 10% residual transcripts and protein. These individuals have a moderately severe phenotype, living at least through middle-age, with fractures declining after puberty, moderate short stature, and DEXA L1–L4 z-scores ranging from −1.3 to −4.8. Given the tissue and cellular functions of CRTAP, these genotype-phenotype variations are likely to reflect more than the level of type I collagen modification.

Furthermore, in the case of the one well-studied *CRTAP* missense mutation, near-normal levels of collagen 3-hydroxylation do not prevent a severe phenotype. The proband who is homozygous for the Leu67Pro substitution is 9 years old, with fractures, extremely severe growth deficiency (size of a 10.5-month-old) and wheel-chair

mobility (Baldridge et al. 2008). The fibroblasts of this child have normal transcript levels and 79% Pro986 hydroxylation, compared with 94%–98% in normal controls and 88%–91% in clinically normal carrier parents (Barnes et al. 2006). Factors in addition to a 10% decrease in type I collagen 3-hydroxylation, compared with carriers, must be contributing to phenotypic severity in this case.

Similarly, the mutations that have been identified in the 15 exons and surrounding intronic regions of *LEPRE1* gDNA cause an absence or loss of function of P3H1 and result in the lethal to severe type VIII OI (OMIM 610915). There are now 17 known mutant *LEPRE1* alleles in about two dozen independent families (Table 1). The mutations occur throughout the *LEPRE1* gene. Most can be detected by screening for the loss of expression of *LEPRE1* by real-time RT-PCR with RNA isolated from patient fibroblasts (Cabral et al. 2007). Almost all of the mutations are consistent with the reduction of *LEPRE1* transcripts, as the resulting stop codons or frame-shifts lead to introduction of a PTC. We found that the severely reduced or absent *LEPRE1* mRNA could be partially rescued by treatment of the fibroblasts with emetine, supporting a mechanism in which the PTCs trigger NMD of the transcripts. *LEPRE1* transcripts were less than 15% of control fibroblasts in the eight of 12 cases with unique mutations in which it was measured, 40%–60% in two cases, and 70%–87% in two others. The lowest levels of transcripts were associated with single nucleotide deletions, stop codons, and splice site defects, and all led to less than 15% of the normal Pro986 hydroxylation level. Moderate and near-normal levels of transcripts were associated with mutations in the final exon or with in-frame splice-out of exon 6. These transcripts apparently did not trigger NMD; in these cases, the level of type I collagen Pro986 hydroxylation was also less than 10% or “severely reduced”.

Three *LEPRE1* mutations deserve special mention. The first mutation is a splice site defect that is the most commonly identified *LEPRE1* mutation; it has now been identified in 22 mutant alleles (Baldridge et al. 2008; Bodian et al. 2009; Cabral et al. 2007), almost as frequently as all other *LEPRE1* mutations combined. We identified this recurring mutation, IVS5+1G>T, (c.1080+1G>T), among six NIH OI patients with severely reduced or absent *LEPRE1* mRNA, each of whom was of African-American or contemporary West African descent (Cabral et al. 2007). This splice donor site mutation results in five alternatively spliced forms of the transcript, each of which contains a PTC. To determine the incidence of carriers, we screened three contemporary African-American cohorts. The data yielded a carrier frequency for the recurring mutation of 1 in 200–300 (0.32%–0.50%) Mid-Atlantic African-Americans and predicted an estimated incidence for type VIII OI attributable to homozygosity for this mutation of 1 in 160–380,000 African-American births (Cabral et al. 2008). Homozygosity for this mutation was uniformly lethal in the first few months after birth, whereas compound heterozygotes lived into their teens, although they did not have a significant difference in collagen 3-hydroxylation. The occurence of this mutation in both contemporary African-Americans and West Africans implies that the mutation is a founder mutation of African origin and was brought to the Americas with the Atlantic slave trade (1450–1860 C.E.).

The second notable mutation occurs in the genetic isolate known as the Irish Travelers. This frame-shift mutation in exon 1 has a variable phenotype, ranging from perinatal lethal to survival to school age with severe OI, despite minimal levels of *LEPRE1* transcripts and collagen 3-hydroxylation (Baldridge et al. 2008; Williams et al. 1989).

The third distinctive *LEPRE1* mutation affects the KDEL sequence of P3H1 via a frame-shift in the last exon. P3H1 is the only component of the collagen prolyl 3-hydroxylation complex, which contains an ER-retrieval KDEL sequence. The P3H1 isoform containing the KDEL motif was not present in a proband with severely reduced hydroxylation of Pro986, suggesting that P3H1 catalytic function is restricted to this form (Willaert et al. 2008). Secreted P3H1 was not identified in media of proband cultured fibroblasts, although the transcripts were demonstrated to escape NMD, as predicted by the location of the PTC. Since leprecan was originally identified in the extracellular matrix of mouse tissues (Wassenhove-McCarthy and McCarthy 1999), and since a protein reactive to P3H1 antibody has been extracted from murine femurs (Cabral et al. 2007), the isoform of P3H1 that is secreted into the extracellular matrix has yet to be identified.

Clinically, type VIII OI, like type VII, overlaps with the presentation of Sillence types II and III OI, although there are distinctive features. As infants, type VIII OI presents with rhizomelia and severe undertubulation of the long bones, especially the femurs; long bones are crumpled from *in utero* fractures. The rib cage does not have the beaded appearance common for severe/lethal OI infants, but rather the ribs are gracile, with no or few fractures. The head circumference is normal, although the skull is poorly mineralized with wormian bones and enlarged fontanelles, and the sclerae are white or light grayish as found in normal infants.

Type VIII OI mutations, which do not represent founder mutations or genetic isolates, most often occur in consanguineous marriages. Many of these cases were terminated. Among affected individuals who were live-born, survival ranges from hours to weeks to school age. Several teenagers with type VIII OI have been reported, the oldest of whom are now 17 (P3H1 p.Tyr552X) or 17–23 (p. Arg685fsX) years old. The skeletal features of non-lethal

type VIII OI include enlarged head circumference, scoliosis, extreme bone fragility, and undermineralization at the most severe end of the OI spectrum (Baldridge et al. 2008; Cabral et al. 2007). Their lower extremity long bones become gracile with flared metaphyses containing the exuberant "popcorn" formation, which often also occurs in upper extremity long bones (Baldridge et al. 2008). Their hands and fingers have a long gracile appearance.

Types VII and VIII OI are difficult, if not impossible, to distinguish from each other clinically. The level of suspicion for type VIII OI should be high in African-American infants, although mutations in *CRTAP* have also been identified in this group. However, in African-Americans, de novo classical dominant OI will have a higher incidence than recessive OI in North America.

Expected consequences of null mutations in *CRTAP* and *LEPRE1*

Most of the known *CRTAP* and *LEPRE1* mutant alleles are expected to reduce the level of transcripts by NMD. This expectation has been confirmed in all cases where transcript levels have been assayed by real-time RT-PCR and is further corroborated by the normal level of *CRTAP* transcripts associated with the p.Leu67Pro missense mutation (Baldridge et al. 2008). Consistent with the mutations and resulting degradation of transcripts, CRTAP and P3H1, respectively, were absent from fibroblasts of four probands with *CRTAP*-null mutations and of seven probands with *LEPRE1*-null mutations whose protein levels were assayed on Western blots. One of the NIH *LEPRE1*-null probands had a residual level of protein from the paternal alelle, presumably translated from the small amount of in-frame transcripts that were detected (Cabral et al. 2007), and a residual amount of CRTAP was detected in the type VII OI index pedigree from Quebec, consistent with their milder phenotype (Morello et al. 2006). Consequently, in the absence of either of the components of the collagen-modification complex, the 3-hydroxylation of the Pro986 residue of α1(I) collagen is abolished or reduced. Although not demonstrated, 3-hydroxylation of types II, III and V collagen is expected also to be reduced or abolished in these cases. Only 0%–21% (in six type VII OI probands) or 0%–15% (in 11 type VIII OI probands) of α1(I) Pro986 residues were 3-hydroxylated in fibroblast collagen, as demonstrated by tandem mass spectrometry of trypsin-digested collagen peptides (Table 2). This is in contrast to 94%–98% Pro986 hydroxylation in normal controls, 85%–91% hydroxylation in carrier parents, and 79% hydroxylation in collagen synthesized by cells with a *CRTAP* missense mutation (Baldridge et al. 2008; Barnes et al. 2006; Cabral et al. 2007).

Unexpected consequences

In collagen synthesized by cells with a deficiency of either CRTAP or P3H1, collagen alpha chains exhibited delayed electrophoretic migration on analysis by polyacrymaide gel electrophoresis. Amino acid analysis of type I collagen from *CRTAP*-null and *LEPRE1*-null fibroblasts revealed an increase in lysyl hydroxylation compared with normal control collagen (31%–35% versus 24% respectively; Barnes et al. 2006; Cabral et al. 2007). This is comparable with the level of overmodification by the prolyl 4-hydroxylase and lysyl hydroxylase modification system found in collagen with a structural defect near the carboxyl end of the helix (Barnes et al. 2006). Differential scanning calorimetry of type I collagen from our probands was consistent with the modification data. In classical OI, the presence of a glycine substitution causes a less stable collagen structure with decreased thermal stability. The scans from our proband collagens, however, show an increased melting temperature of 1°C compared with the normal control, the expected consequence of collagen overmodification in the absence of a primary structural defect (Torre-Blanco et al. 1992). Since these data are consistent with longer exposure of the collagen chains to lysyl hydroxylase, we have concluded that the loss of P3H1 protein or its role in the 3-hydroxylation complex results in the delayed folding of the collagen helix, leading to the overmodification. Of note, no discernable collagen-modification abnormalities have been detected in the parents who are heterozygous carriers for the *LEPRE1* mutations; gel migration of the parental collagen is normal, as are the levels of *LEPRE1* mRNA on real-time RT-PCR, consistent with near normal (85%–90%) hydroxylation of Pro986 residues (Cabral et al. 2007).

A second unexpected consequence of deficiency of P3H1 is the increased secretion of type I collagen by proband fibroblasts. We performed pulse-chase experiments comparing fibroblast collagen synthesis from our cases with P3H1 deficiency and two independent normal controls to observe collagen secretion kinetics (Cabral et al. 2007). Similar to classical OI (Byers and Cole 2002), we saw a 15– to 20-min delay in the rate of type I collagen secretion from *LEPRE1*-null fibroblasts, presumably attributable to the overmodification of the collagen helix. Surprisingly, when we normalized the amount of secreted collagen to the number of cells, we found that total collagen secretion was increased 20%–50% compared with controls.

This result is especially surprising because slower secretion of overmodified collagens is expected to cause ER stress with subsequent upregulation of the unfolded protein response (UPR) pathway (Kojima et al. 1998; Lisse et al. 2008; Wilson et al. 2005). In classical OI, retention of abnormal procollagen has been linked to increases in

several ER-resident proteins, including the collagen-specific chaperone HSP47, protein disulfide isomerase (PDI/P4Hβ), BiP, and prolyl 4-hydroxylase (Chessler and Byers 1993; Ko and Kay 2004; Kojima et al. 1998; Walmsley et al. 1999). Conversely, the absence of a collagen chaperone or post-translational modifying enzyme would be expected to increase intracellular retention and degradation with a corresponding decrease in total collagen secretion, as has been demonstrated in HSP47, P4Hα, and LH3 knock-out mice (Holster et al. 2007; Marutani et al. 2004; Rautavuoma et al. 2004). Since the UPR response results in the downregulation of translation, the occurrence of increased collagen synthesis in P3H1-deficient fibroblasts is counterintuitive but may hint at a direct or indirect role for P3H1 in regulating collagen synthesis and secretion.

Speculations on mechanisms

Types VII and VIII OI are recessive osteochondrodystrophies about whose mechanisms we have more questions than answers. Given the multifunctional nature of CRTAP and P3H1, the respective contributions of the loss of the ER-collagen-modification functions versus the loss of the matrix function of the secreted proteins remains to be sorted out. Furthermore, the collagen-modification function involves not just type I collagen, on which we focus because of its complementarity to collagen structural defects in dominant OI, but also types II, V, and possibly type IV collagen. Some of the most severe findings in children with recessive OI, including the extreme growth deficiency, may have as much or more of a relationship to cartilage and type II collagen, than to type I collagen. Many of these issues will require conditional animal models for answers; however, since knock-out mice for *Crtap* and *Lepre1* have much milder phenotypes than their human counterparts, these answers may be elusive (Bachinger et al. 2009; Morello et al. 2006).

The possibility that P3H1 modifies type IV collagen remains unverified. Tiainen and coworkers (2008) have shown that P3H2 preferentially hydroxylates peptides with a sequence corresponding to type IV collagen versus type I collagen; these findings are supported by data confirming P3H2 as the dominant P3H isoform expressed in kidney (Vranka et al. 2009). Nevertheless, the expression of P3H1/leprecan at low levels in podocytes and mesangial cells in renal glomeruli in adult rat suggests a possible function in this tissue (Lauer et al. 2007). Since the kidney normally has little type I or type III collagen, a role for P3H1 in renal development awaits either the verification of type IV collagen as a substrate for this enzyme or the identification of a matrix function for the secreted form of the protein. The expression of several isoforms of P3H1 in the kidney also complicates the determination of its role; whether these isoforms include the carboxyl terminal KDEL sequence or whether they represent transcripts with an alternatively spliced carboxyl terminus that encodes a secreted extracellular matrix proteoglycan remains unclear (Lauer et al. 2007; Wassenhove-McCarthy and McCarthy 1999). Whereas elevated urine calcium levels and increased incidence of kidney stones have been reported, no evidence of compromised kidney function has been detected in patients with dominant or recessive OI (Chines et al. 1995; Vetter et al. 1989). Indeed, the only published reports of glomerulopathy occur in the *oim* mouse, which is a model for an extremely rare form of recessive OI characterized by synthesis of α1(I) homotrimers because of a null *Col1a2* allele (Brodeur et al. 2007; Phillips et al. 2002). Investigation into kidney functioning in the few surviving patients with P3H1 deficiency should help to determine its role in renal development.

Another major issue concerns the role of prolyl 3-hydroxylation itself. In types I and II collagen, it occurs at a single residue of many potential such X-position prolines. From the standpoint of collagen structure, hydroxylation of Pro986 seems unnecessary for the initiation of the correct folding of the collagen helix. If the 3-hydroxylation of Pro986 is not crucial to the configuration or stability of the collagen substrate *per se*, it might alternatively be important for the function of the complex. Completion of the modification reaction might change the secondary structure of one or more complex components or the tertiary structure of the complex. It might also serve as a binding site and initiation point for CyPB or other PPIases important for prolyl isomerization, which is the rate-limiting step in collagen folding. Hydroxylation of Pro986 may be necessary, if not sufficient, as the trigger for the proper folding of the collagen helix. These questions await the results of experiments involving the substitution of the Pro986 residue or the re-positioning of the triplets surrounding the modified residue in order to eliminate or reposition the 3-hydroxylation site despite the presence of a functional collagen-modification complex.

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