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# Prolyl 3-hydroxylase 1 and CRTAP are mutually stabilizing in the endoplasmic reticulum collagen prolyl 3-hydroxylation complex

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**Null mutations in cartilage-associated protein (*CRTAP*) and prolyl 3-hydroxylase 1 (P3H1/*LEPRE1)* cause types VII and VIII OI, respectively, two novel recessive forms of osteogenesis imperfecta (OI) with severe to lethal bone dysplasia and overmodification of the type I collagen helical region. CRTAP and P3H1 form a complex with cyclophilin B (CyPB) in the endoplasmic reticulum (ER) which 3-hydroxylates the Pro986 residue of α1(I) and α1(II) collagen chains. We investigated the interaction of complex components in fibroblasts from types VII and VIII OI patients. Both CRTAP and P3H1 are absent or reduced on western blots and by immunofluorescence microscopy in cells containing null mutations in either gene. Levels of *LEPRE1* or *CRTAP* transcripts, however, are normal in *CRTAP*- or *LEPRE1*-null cells, respectively. Stable transfection of a *CRTAP* or *LEPRE1* expression construct into cells with null mutations for the transfected cDNA restored both CRTAP and P3H1 protein levels. Normalization of collagen helical modification in transfected *CRTAP*-null cells demonstrated that the restored proteins functioned effectively as a complex. These data indicate that CRTAP and P3H1 are mutually stabilized in the collagen prolyl 3-hydroxylation complex. CyPB levels were unaffected by mutations in either *CRTAP* or *LEPRE1*. Proteasomal inhibitors partially rescue P3H1 protein in *CRTAP*-null cells. In *LEPRE1*-null cells, secretion of CRTAP is increased compared with control cells and accounts for 15–20% of the decreased CRTAP detected in cells. Thus, mutual stabilization of P3H1 and CRTAP in the ER collagen modification complex is an underlying mechanism for the overlapping phenotype of types VII and VIII OI.**

## INTRODUCTION

Osteogenesis imperfecta (OI), or 'brittle bone' disease, is a genetic disorder of connective tissue. Affected individuals are susceptible to fracture from mild trauma or even acts of daily living (1,2). Classical dominant OI (types I–IV) ranges in severity from minimally detectable to perinatal lethal (3) and is caused by mutations in *COL1A1* or *COL1A2*, the genes encoding the two type I collagen alpha chains (4,5). Type I collagen is a heterotrimer [$\alpha1(I)_2\alpha2(I)$] which is subject to post-translational modification (6,7). Prolyl 4-hydroxylase (P4H) and lysyl hydroxylase (LOH) modify multiple proline (Xaa-Pro-Gly) (8) and lysine residues (Xaa-Lys-Gly) (9) along the length of the collagen helical region, with subsequent glycosylation of some hydroxylated lysine residues (6,10–12). Mutations causing structural changes in the collagen alpha chains delay folding of the collagen helix and expose the chains to post-translational modification for a longer time (13). Excess modification, or 'overmodification' of the collagen helix can be detected as delayed electrophoretic migration of collagen alpha chains (14,15).

Recently, recessive forms of OI have been attributed to null mutations in the genes encoding cartilage-associated protein (CRTAP) and prolyl 3-hydroxylase 1 (P3H1/*LEPRE1*) in a small percentage (5–7%) of patients with severe to lethal

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**Table 1.** Proband cell lines

| Proband OI type | Proband | Mutations | Location of PTCs | Parental allele | Reference |
|---|---|---|---|---|---|
| VII (*CRTAP*-null) | 1[a] | c.3G > A, p.M1 | | F | (18) |
| | | c.278_293Dup | e2 (c.477–479) | M | |
| | 2[a] | c.826C > T, p.Q276X | e4 (c.826–828) | F | (18) |
| | | c.826C > T, p.Q276X | e4 (c.826–828) | M | |
| | 3[a] | c.471 + 1G > C | i1(g.532–534) | F | (18) |
| | | c.471 + 1G > C | i1(g.532–534) | M | |
| | 4 | c.826C > T, p.Q276X | e4 (c.826–828) | ND | This report |
| | | c.634C > T, p.R212X | e3 (c.634–636) | | |
| VIII (*LEPRE1*-null) | 5 | c.1383_1389dupGAACTCC | e9 (c.1437–1439) | F | This report |
| | | c.1924_1934delCAGCCTCAGTG | e14 (c.1982–1984) | M | |
| | 6[b] | c.1656C > A, p.Y552X | e11 (c.1654–1656) | F | (19) |
| | | c.1656C > A, p.Y552X | e11 (c.1654–1656) | M | |
| | 7[b] | c.1080 + 1G > T | Multiple PTCs[c] | F | (19) |
| | | c.1080 + 1G > T | Multiple PTCs[c] | M | |
| | 8[b] | c.747delC | e5 (c.1007–1009) | F | (19) |
| | | c.1080 + 1G > T | Multiple PTCs[c] | M | |

[a]Probands 1, 2 and 3 correspond to cases 3, 2 and 1, respectively, from our previous publication (18).
[b]Probands 6, 7 and 8 correspond to Probands 5, 2–2 and 4, respectively, from our previous publication (19).
[c]Multiple PTCs: e6 (c.1140–1142), e7 (c.1193–1195), e9 (c.1362–1364).

OI, now classified as types VII (OMIM # 610682) and VIII OI (OMIM # 610915), respectively (16–22). CRTAP, P3H1 and cyclophilin B (CyPB) form the collagen prolyl 3-hydroxylation complex in the endoplasmic reticulum (ER) (23). This complex is responsible for the modification of a single proline residue, Pro986, on the α1(I) and α1(II) collagen chains (24). P3H1, originally isolated as the matrix proteoglycan leprecan (25), contains the enzymatic activity of the complex and is the only complex component with a KDEL ER-retrieval signal (23,25). CRTAP was first identified in cultured chondrocytes and embryonic cartilage in chick (26,27) and mouse (26,27), and acts as the helper protein of the complex (17,20,23). *Crtap* knock-out mice have a recessive osteochondrodystrophy associated with lack of the prolyl 3-hydroxylation modification in cartilage and bone (17).

Phenotypically, types VII and VIII OI probands have lethal or extremely severe OI that overlaps with the clinical features of dominant types II and III OI. However, the recessive cases have the distinctive features of rhizomelia, white or light blue sclerae and a small to normal head circumference as infants (16,18,19,21,22). Biochemically, recessive and dominant OI also overlap in that both groups have overmodification of the helical region of type I collagen, detected as delayed collagen chain migration on electrophoresis (18,19). Collagen overmodification in recessive OI involves the full length of the collagen helix, comparable to dominant OI with a collagen structural defect at the carboxyl end of the helix (18,19). These data indicate that absence of the components of the collagen prolyl 3-hydroxylation complex delays collagen folding by an as yet undetermined mechanism.

To explore the interactions of the components in the complex and the mechanism by which their absence causes an overlapping phenotype, we undertook an examination of each protein in fibroblasts from individuals with types VII and VIII OI. We focused on the fate of the normal complex components in *CRTAP*- or *LEPRE1*-null cells, including their stability, site of degradation and/or secretion from the cell. We found that CRTAP and P3H1 are mutually protective in the complex. Each protein is absent or substantially decreased in cells in which either gene has a null mutation. Both proteins are restored when an expression construct for a normal cDNA copy of each gene is transfected into cells with a null mutation in that gene. Correct functioning of the restored complex for collagen folding was shown in transfected *CRTAP*-null cells. These data demonstrate that mutual stabilization of P3H1 and CRTAP in the ER-resident collagen modification complex is the underlying mechanism for the overlapping phenotype of types VII and VIII OI.

## RESULTS

### Probands and their mutations

Probands 1–4 are homozygous or compound heterozygous for null mutations in *CRTAP* (Table 1). P4 has not been previously published and has compound heterozygosity for two *CRTAP*-null mutations, p.Q276X and p.R212X (Fig. 1A; Case Report in Supplementary Material, Fig. S1). Probands 5–8 are homozygous or compound heterozygous for null mutations in *LEPRE1*. P5 is also a novel case and is compound heterozygous for paternal c.1383_1389dupGAACTCC and maternal c.1924_1934delCAGCCTCAGTG mutations, each leading to frameshifts and premature termination codons in exons 9 and 14, respectively (Fig. 1B, Case Report in preparation for publication).

### Both P3H1 and CRTAP proteins are absent or reduced in cell lysates from *CRTAP*-null or *LEPRE1*-null fibroblasts

P3H1, CRTAP and CyPB form the collagen prolyl 3-hydroxylation protein complex in the ER. To examine the stability of CRTAP and P3H1 in the complex, we inhibited protein translation in control cells with 100 μg/ml cycloheximide for increasing times up to 24 h. Both CRTAP and P3H1 levels were unchanged for the full duration of the treatment (Fig. 2), demonstrating that both of these proteins

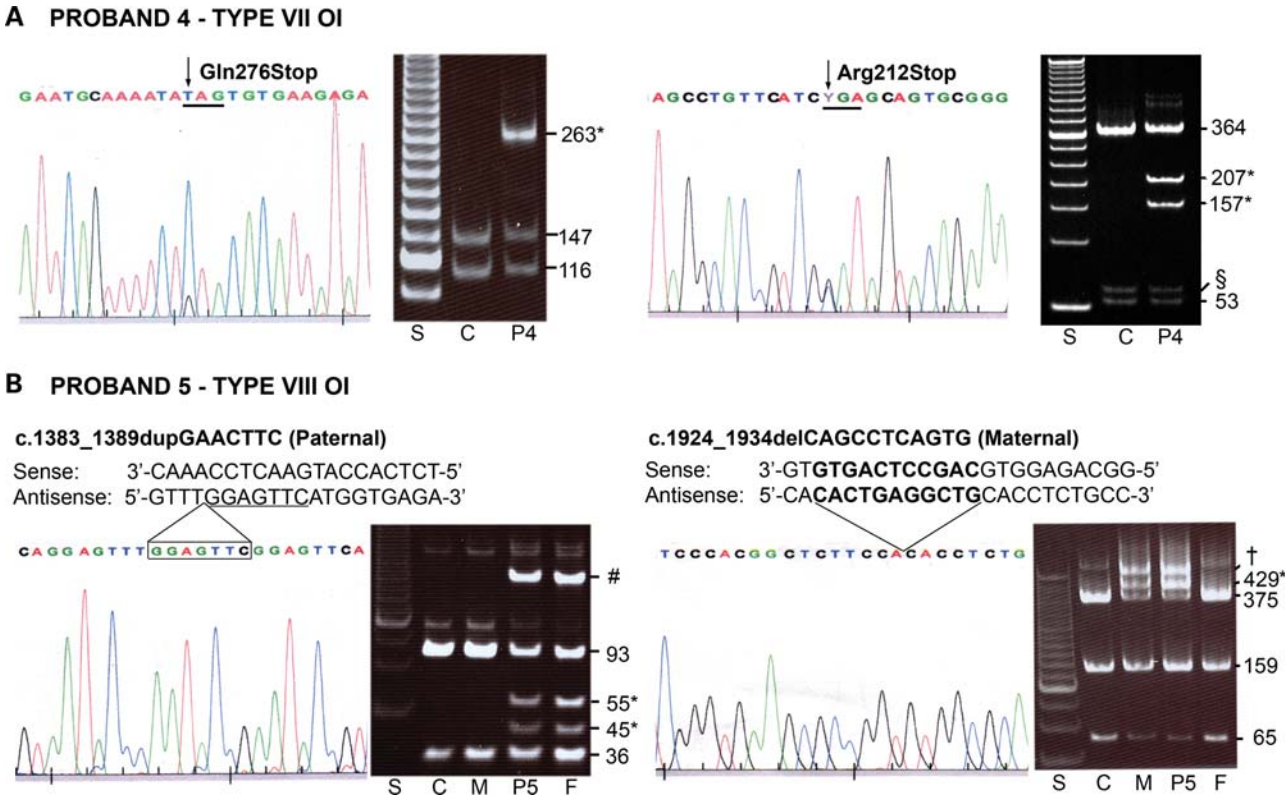


**Figure 1.** Two novel cases of recessive osteogenesis imperfecta. (**A**) Type VII OI Proband 4 has compound heterozygosity for two point mutations in *CRTAP*. *Left*, the proband is heterozygous for a point mutation in exon 4 (p.Q276X, c.826C > T), causing a Gln276Stop substitution. The mutation identified by DNA sequencing eliminates an HpyCH4 III restriction site. Digestion of a gDNA PCR product with HpyCH4 III yields an undigested 263 bp fragment from the mutant allele, and 147 and 116 bp fragments from the normal allele of the proband. *Right*, the proband is also heterozygous for a mutation in exon 3 (p.R212X, c.634C > T), causing an Arg212Stop substitution. The mutation identified by DNA sequencing generates a novel DdeI restriction site. The 207 and 157 bp fragments generated by DdeI digestion of a gDNA PCR product confirm the mutation. (§) is a partial digestion product (53 + 9 bp) from 3′-end of fragment. (**B**) Type VIII OI Proband 5 has compound heterozygosity for two mutations in *LEPRE1*. *Left*, sequencing of cDNA subclones identified a 7 nt duplication (boxed) in exon 9 (c.1383_1389 dupGAACTCC), which generates a novel Hpy188I restriction site. Digestion of a gDNA PCR product yields a 93 bp product from the normal allele and 55 and 45 bp fragments from the mutant allele in the proband and his father. The slowest migrating band (#) is a heteroduplex of normal and mutant PCR products, which was indigestable. *Right*, exon 14 gDNA subclone sequences reveal an 11 nt deletion (bold) (c.1924_1934delCAGCCTCAGTG), which eliminates a BslI restriction site. Digestion of a gDNA PCR product with BslI confirms the presence of the 429 bp product from the mutant allele in the proband and his mother, as well as the normal 375 and 65 bp digestion products. The slowest migrating DNA fragment (†) is a heteroduplex of normal and mutant PCR products and was not BslI digestable. S, molecular size standard; C, normal control; P, Proband; F, father; M, mother.

are very stable when all complex components are present in the cell.

We examined the interaction of the complex components by investigating their fate in cells with null mutations in *CRTAP* or *LEPRE1*. Both P3H1 and CRTAP proteins are absent in western blots of *CRTAP*-null fibroblasts (Fig. 3A). Conversely, in *LEPRE1*-null fibroblasts, P3H1 protein is absent, while CRTAP is only minimally detectable (Fig. 3B). When cells were extracted under denaturing conditions (28), no increase in the amount of P3H1 detected in *CRTAP*-null cells or CRTAP detected in *LEPRE1*-null cells was seen, suggesting that substantial amounts of aggregated insoluble P3H1 or CRTAP were not present in cells (Fig. 3C and D). Levels of CRTAP (M1:64 ± 6%; F1:73 ± 10%) and P3H1 (M1:46 ± 6%; F1:85 ± 6%) were consistently reduced in the heterozygous carrier parents of Proband 1, with more normal values in F1, who has a mutation in the Met start codon which allows partial re-initiation at Met 42. In the heterozygous carrier parents for *LEPRE1*-null Proband 5, both P3H1 (M5: 56 ± 15%; F5: 57 ± 6%) and CRTAP (M5:39 ± 10%; F5:33 ± 9%) protein levels were also reduced compared with normal control. The third component of the complex, CyPB, was as abundant in both sets of mutant cell lines as in control.

The absence of P3H1 or severe reduction of CRTAP in the *CRTAP*- and *LEPRE1*-null cell lines, respectively, is not associated with reduced levels of the normal gene transcripts. Probands with *CRTAP*- and *LEPRE1*-null mutations have been previously demonstrated to have nonsense-mediated decay of the mutant transcripts (18,19). We found normal or increased transcript levels of *LEPRE1* in *CRTAP*-null cells (Fig. 3E) and of *CRTAP* in most *LEPRE1*-null cells (Fig. 3F). P8 had about half the normal *CRTAP* transcript level, but the same residual level of CRTAP protein as the other cases. These data suggest that P3H1 or CRTAP protein is produced in cells with null mutations in the other gene, but that both proteins are required for their mutual stabilization in the collagen prolyl 3-hydroxylation complex.

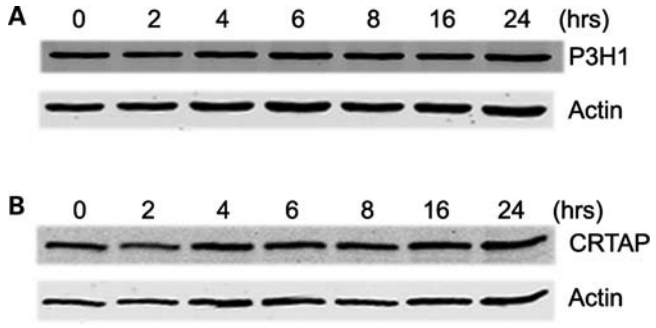


**Figure 2.** Stability of P3H1 and CRTAP in normal fibroblasts. Dermal fibroblasts from a 5-year-old boy were treated with 100 μg/ml cycloheximide for the indicated times to inhibit protein translation. P3H1 (**A**) and CRTAP (**B**) protein levels were assessed by western blot, with actin as a loading control. Both proteins are very stable when all components of the collagen prolyl 3-hydroxylation complex are present in cells.

### Both CRTAP and P3H1 proteins are undetectable in *CRTAP*-null and *LEPRE1*-null fibroblasts *in vivo*

To examine CRTAP in *LEPRE1*-null and P3H1 in *CRTAP*-null fibroblasts *in vivo*, we used immunofluorescence microscopy of *CRTAP*-null and *LEPRE1*-null fibroblasts from type VII (Proband 2) and type VIII OI (Proband 7) probands, respectively (Fig. 4). The cells were stained with antibodies to CRTAP (Fig. 4A) or P3H1 (Fig. 4B) and the ER luminal protein GRP94 (29). The overlap of CRTAP or P3H1 signals with GRP94 in the control cells demonstrates that CRTAP and P3H1 are localized in the ER. In agreement with the western blot data, both CRTAP and P3H1 signals are absent from the *CRTAP*-null (Fig. 4A) and *LEPRE1*-null (Fig. 4B) cells. In *LEPRE1*-null cells, immunocytochemistry does not detect the residual level of CRTAP detected by the more sensitive LI-COR Odyssey system in western blots (Fig. 3B). Control staining with only the secondary antibody yielded no signal (data not shown).

### Restoration of *CRTAP* expression in *CRTAP*-null cells or P3H1 in *LEPRE1*-null cells rescues both CRTAP and P3H1 protein levels

To demonstrate that CRTAP can stabilize P3H1 *in vivo*, we transfected a full-length *CRTAP* expression construct and a series of deletion constructs into immortalized *CRTAP*-null fibroblasts from Proband 2 (Fig. 5A and B). Restoration of full-length CRTAP substantially rescued P3H1, supporting the interpretation of CRTAP/P3H1 mutual protection in the ER *in vivo* (Fig. 5B, left). Cells transfected with *CRTAP* deletion constructs did not rescue P3H1 efficiently. This may be explained by the existence of multiple essential CRTAP and P3H1 interaction regions along the CRTAP peptide chain. Alternatively, the sizable deletions may have directly or indirectly altered the folding of a single interaction region in the CRTAP peptide chain.

Conversely, we transfected Proband 7 primary fibroblasts with a full-length *LEPRE1* expression construct. Restoration of P3H1 protein in *LEPRE1*-null cells also restored intracellular CRTAP levels (Fig. 5B, right).

### Restoration of *CRTAP* expression in *CRTAP*-null cells partially corrects excess collagen modification

Type I collagen produced by *CRTAP*-null cells is overmodified, resulting in delayed electrophoretic migration. Although collagen chain electrophoresis is not completely normalized, transfection of full-length *CRTAP* substantially reduced the collagen overmodification in *CRTAP*-null cells in cell layer and media (Fig. 5C), demonstrating that complex function has been restored.

### P3H1 is partially degraded by the proteasome pathway in *CRTAP*-null fibroblasts

To investigate the possible role of the two major intracellular protein degradation pathways (30,31) in the degradation of P3H1 in *CRTAP*-null fibroblasts, we treated cells with the proteasomal inhibitors ALLN and MG132 or lysosomal inhibitors E64 and Pepstatin A (Fig. 6). We used the MG132 treatment duration and concentration shown to be most effective in P3H1 rescue in dose response and time course experiments (Supplementary Material, Fig. S2). Both proteasomal inhibitors partially rescued P3H1 in two independent lines of *CRTAP*-null fibroblasts (Fig. 6A and C). Neither lysosomal inhibitor affected P3H1 levels, although the inhibition was sufficient to rescue BACE from the lysosomal pathway (Fig. 6B and D).

### Proteasomal inhibition of *LEPRE1*-null cells rescues a short form of CRTAP

*LEPRE1*-null fibroblast lines from Probands 6 and 7 were treated with proteasomal or lysosomal inhibitors (Fig. 7A and C). The level of full-length CRTAP was not increased by any inhibitor treatment, although the effectiveness of proteasomal and lysosomal inhibition was demonstrated by significant increases in P53 and BACE (Fig. 7B and D), proteins known to be degraded by proteasomes (32) and lysosomes (33). However, a smaller protein reactive with CRTAP antibody was rescued by both proteasomal inhibitors in *LEPRE1*-null and in control fibroblasts.

### Secretion of CRTAP from *LEPRE1*-null fibroblasts is increased

Although P3H1 was first isolated from matrix as the proteoglycan leprecan, it is the only component of the collagen prolyl 3-hydroxylation complex which contains a KDEL ER-retention signal. CRTAP was previously detected as a secreted protein (17,26,27). Therefore, we hypothesized that CRTAP is not retained in the ER in the absence of P3H1, and that, consequently, more CRTAP would be secreted into the extracellular space from *LEPRE1*-null cells.

To determine the level of CRTAP secreted from control human fibroblasts, conditioned media were compared with cell lysates on western blots (Fig. 8A). CRTAP accumulated in media, with secreted CRTAP constituting 1.2, 2.6, 6.0 and 12.0% of total CRTAP (cell layer + media) at 4, 8, 16 and 24 h of collection, respectively. The approximately linear increase in CRTAP accumulation in media suggests

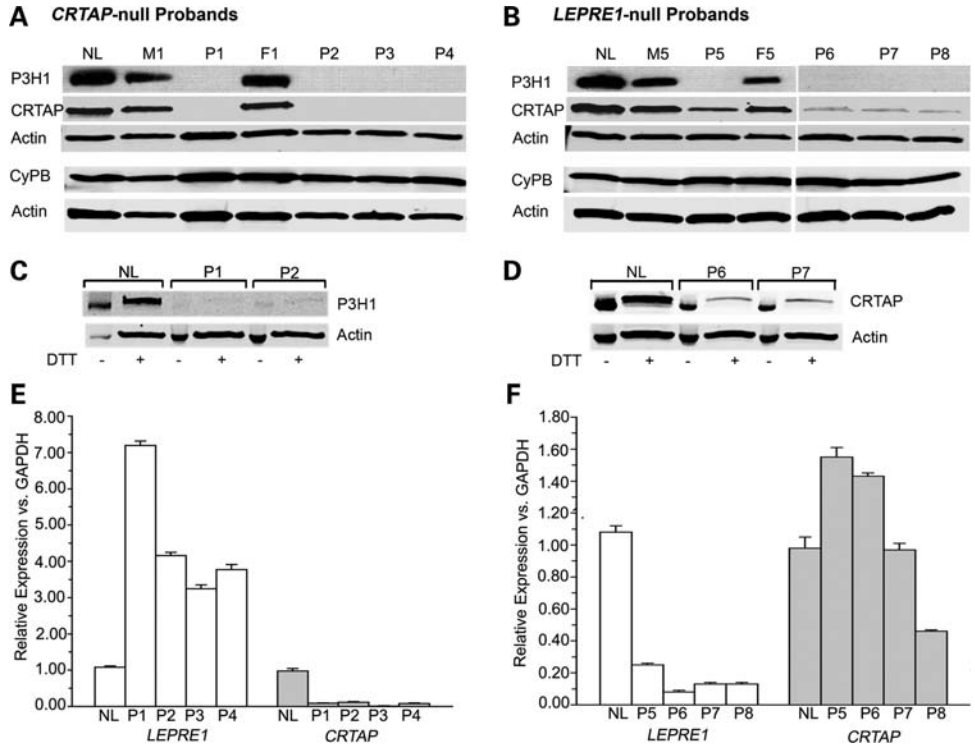


**Figure 3.** P3H1 and CRTAP are mutually stabilized in the ER-resident collagen prolyl 3-hydroxylation protein complex. P3H1, CRTAP and CyPB levels in *CRTAP*-null and *LEPRE1*-null fibroblasts were assayed by western blot and real-time RT-PCR. (**A**) In *CRTAP*-null cells, both CRTAP and P3H1 proteins are undetectable by western blot; cell extraction under denaturing conditions did not detect aggregates of misfolded protein (**C**). Although P3H1 protein is undetectable, *LEPRE1* transcript levels are normal by real-time RT-PCR (**E**). Levels of P3H1 and CRTAP proteins in the carrier parents (M1 and F1) of Proband 1 are reduced (A). (**B**) In *LEPRE1*-null cells, P3H1 is absent on western blot, while CRTAP is barely detectable. CRTAP levels are not increased by cell extraction under denaturing conditions (**D**), although *CRTAP* transcripts levels are normal by real-time RT-PCR (**F**). The carrier parents (M5 and F5) of Proband 5 have approximately half the levels of P3H1 and CRTAP proteins found in control (B). Note the transcript levels shown in E and F show relative expression versus *GAPDH*. In accordance with the MIQE Guidelines to validate results by comparison with a second housekeeping gene (49), transcript levels were determined versus *18s rRNA and/or beta-2-microglobulin* (data not shown). Normalized to *18s rRNA* transcripts, Proband *LEPRE1* transcripts in *CRTAP*-null cells were 75–177% of normal control and *CRTAP* transcripts in *LEPRE1*-null cells were 30–40% of normal because *18s rRNA* was consistently elevated in proband cells. However, when the level of *CRTAP* transcripts in *LEPRE1*-null cells was normalized to *beta-2-microglobulin*, results similar to those shown in (F) with *GAPDH* were obtained, in which proband levels were normal except Proband 8 (30% of normal). Taken together, these data confirm that adequate CRTAP transcript levels were present for protein synthesis. In both *CRTAP*- and *LEPRE1*-null cells (A, B), levels of CyPB are stable in all proband cells. NL, control cells; P1–P8, Probands 1–8; M1 and F1, mother and father of Proband 1; M5 and F5, mother and father of Proband 5.

that the secreted protein is stable in the culture system over this time frame.

We compared CRTAP secretion in two *LEPRE1*-null cell lines with secretion from control cells at 24 h. The proportion of total CRTAP that was secreted from *LEPRE1*-null cells was more than triple that of control cells (Fig. 8B). The increase of secreted CRTAP does not completely account for the decrease of intracellular CRTAP in *LEPRE1*-null cells, but it does represent a significant proportion (15–20%). These data show that most CRTAP is not retained in the ER in the absence of P3H1 and the collagen prolyl 3-hydroxyation complex; instead, most CRTAP is degraded and an increased proportion is secreted. Interestingly, Proband 6 consistently had a doublet in CRTAP media samples, but normal gel migration of intracellular samples. The CRTAP media doublet occurred despite DTT in gel loading solutions and did not occur in samples from normal cells or other probands. The slower migrating form may be altered in folding or post-translational modification.

## DISCUSSION

Both classical dominant OI and recessive OI are collagen-related dysplasias (1,2). Dominant OI is caused by structural defects in the type I collagen alpha chains, due to mutations in *COL1A1* and *COL1A2* (4,5). Recessive OI is caused by the deficiency of components of the collagen prolyl 3-hydroxylation complex, P3H1 or CRTAP, due to null mutations in *LEPRE1* or *CRTAP*, respectively (18,19,21,22). The prolyl 3-hydroxylation complex catalyzes one of the post-translational modifications of type I collagen, hydroxylating the Pro986 residue on $\alpha$1(I) chains in the ER (23,24). Recessive and dominant OI also share abnormal collagen biochemistry. Collagen structural defects and deficiency of the P3H1 complex both delay collagen folding (13), causing overmodification of the helical region of collagen chains (15).

In the present study, we demonstrated that CRTAP and P3H1 are mutually stabilized in the ER prolyl 3-hydroxylation complex. In control cells, P3H1 and CRTAP are stable

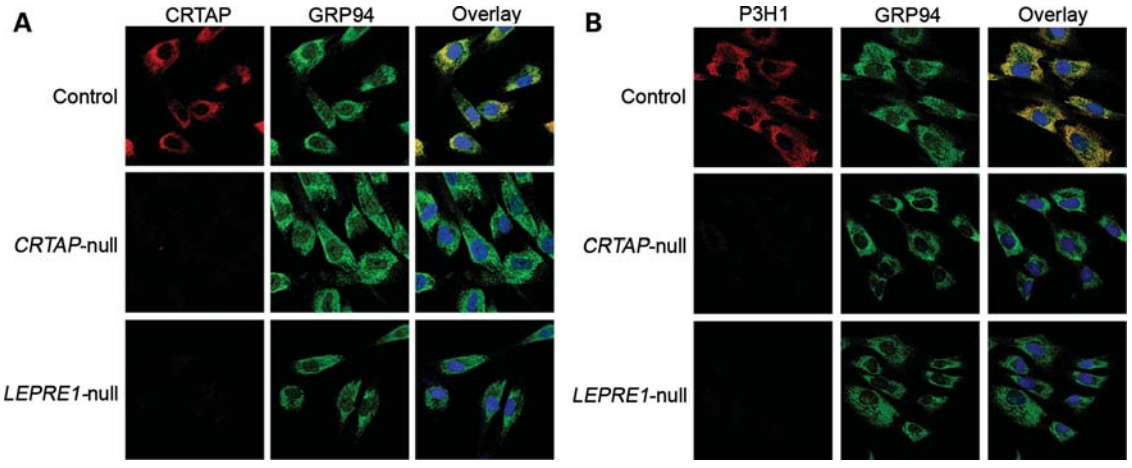


**Figure 4.** Immunofluorescence microscopy of fibroblasts from type VII and VIII OI probands. Fibroblasts were stained with antibodies to CRTAP and the ER luminal protein GRP94 (**A**) or with antibodies to P3H1 and GRP94 (**B**). Proband 2 (*CRTAP*-null) and Proband 7 (*LEPRE1*-null) fibroblasts were compared with control cells. CRTAP and P3H1 colocalized with GRP94 in control cells. Both P3H1 and CRTAP proteins are absent in fibroblasts with null mutations in *CRTAP* or *LEPRE1*.

intracellularly for at least 24 h. However, a null mutation for one of these proteins results in absence or substantial decrease of both proteins on western blots and immunofluorescence microscopy, despite normal levels of transcript from the non-mutated gene. Re-introduction of CRTAP or P3H1 into *CRTAP*- or *LEPRE1*-null cells restores the intracellular levels of both proteins. In the *CRTAP*-null cells with restored *CRTAP* expression, the rescued proteins function effectively as the ER-resident modification complex, since collagen helical modification is substantially normalized in transfected cells. Although we have not obtained sufficient stably transfected *LEPRE1*-null cells to conduct the collagen assay, we would expect that these cells would also have normalized collagen modification, since the complex has been restored.

The mutual stabilization of P3H1 and CRTAP differs from the situation for P4Hα, the enzyme located in the rough ER that catalyzes the 4-hydroxylation of X-Pro-Gly residues in the helical regions of collagens and proteins with collagen-like sequences. P4Hα is solubilized by the β-subunit of the complex (8,34), protein disulfide isomerase (PDI) (35), which acts to prevent P4Hα aggregation and to retain it in the ER via the PDI KDEL retention signal (36). However, PDI is independently stable. Thus, the P3H1/CRTAP association is the first example of a eukaryotic complex with mutual stabilization of components in the ER. Other mutually protective complexes have been described in the nucleus, the cytoplasm and the secretory pathway. The nuclear transcription factor STRA13, which plays important roles in neurogenesis, chondrogenesis and adipogenesis, forms a mutually protective nuclear complex with the cell cycle-dependent transcriptional regulator MSP58 (37), which is also a member of several different transcriptional repression complexes. In the melanosome, an organelle of lysosomal origin, the β3A and μ3 subunits of adaptor complex-3 are mutually protective (38); deficiency of the complex causes the severe autosomal recessive oculocutaneous albinism, Hermansky–Pudlak syndrome. Finally, the cytokine IL-15 is mutually stabilized by its receptor IL-15Rα, leading to both increased intracellular levels and increased secretion of the complex into serum (39).

The levels of the third component of the complex CyPB/*PPIB* are not affected by null mutations in *CRTAP* and *LEPRE1*. CyPB is an abundant protein in the ER and secretory pathways (40,41). The *cis*/*trans* isomerization of prolines in collagen by CyPB is thought to be rate limiting for collagen folding (42,43). While the normal levels of CyPB in types VII and VIII OI indicate that the complex is not essential for CyPB stabilization in the ER, they do not formally demonstrate whether CyPB is important for the integrity of the complex. The reverse assertion is supported indirectly, however, by several facts. First, no CyPB defects have been reported in individuals with OI, although a severe OI phenotype would be expected from all causes of complex destabilization. Second, the only reported genetic defect in *PPIB*, a missense mutation in American quarter horses (44), is associated with the skin condition hyperelastosis cutis, rather than bone dysplasia, although the collagen biochemistry associated with the *PPIB* missense mutation has not been reported.

We also examined the fate of normal complex components in cells in which null mutations in *CRTAP* or *LEPRE1* prevent complex formation. Proteasomal inhibition partially rescues P3H1 and a shortened form of CRTAP in *CRTAP*- and *LEPRE1*-null cells, respectively. The smaller CRTAP-reactive band is most likely a partially degraded form of the protein, as it occurs also in control cells, and does not specifically reflect the fate of CRTAP in *LEPRE1*-null cells. It is also possible that the short form corresponds to the naturally occurring *CRTAP* transcripts with a splicing isoform missing exon 4 (45), although this is less likely because these are rare isoforms on RT-PCR. More importantly, CRTAP secretion is increased. In conditioned media from control cells, P3H1 is not detectable by western blot, while about 10–12% of total CRTAP is secreted. Apparently P3H1 is very efficiently retained in the ER by its KDEL sequence (23,25) and retains the majority of CRTAP along with it. However, in *LEPRE1*-null cells, P3H1 is not present to retain CRTAP in the ER, and the proportion of CRTAP secreted from cells into media is more than tripled, accounting for 15–20% of

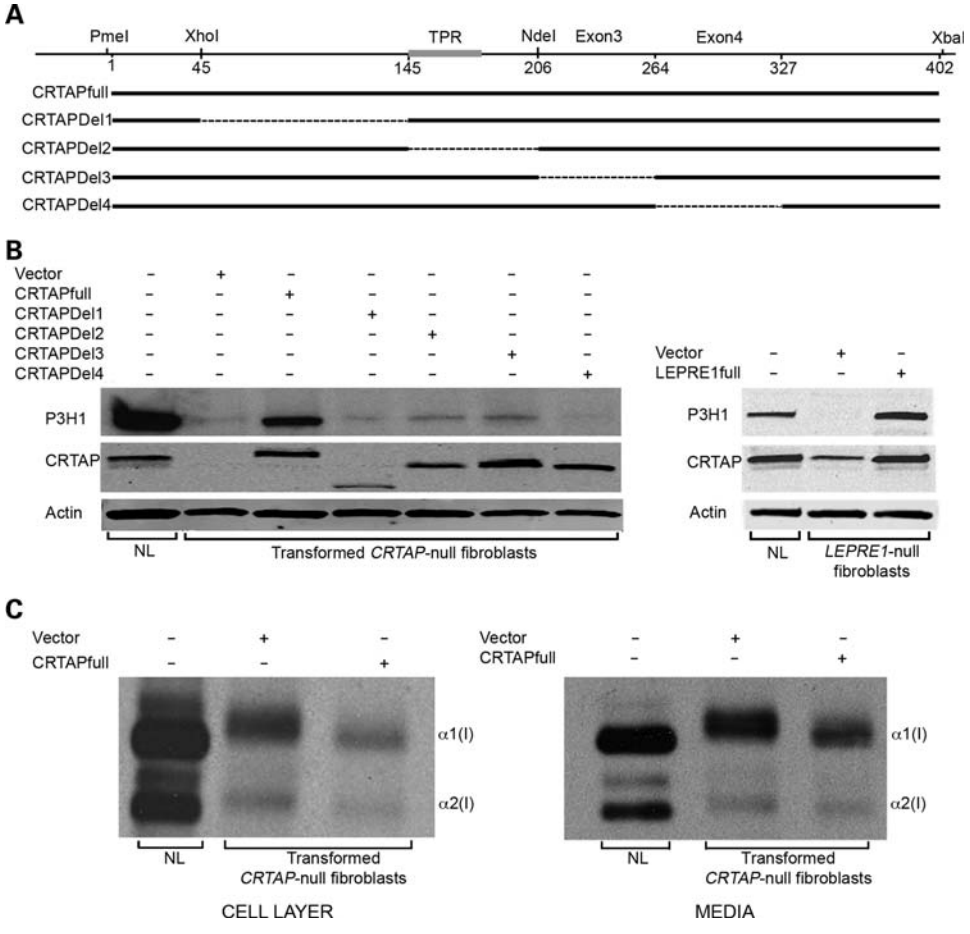


**Figure 5.** Transfection of *CRTAP* or *LEPRE1* expression constructs into cells with corresponding null mutations restores levels and functions of both proteins. (**A**) Diagram of full-length *CRTAP* expression construct and four deletion constructs. Thick black lines represent *CRTAP* coding sequences. Dashed lines indicate deleted regions of CRTAP. Restriction sites used to assemble constructs, the TPR domain and exons 3 and 4 are indicated above the CRTAP map line; amino acid residues at boundaries of deleted regions are indicated below the line. (**B**) P3H1 and CRTAP levels in transformed *CRTAP*-null fibroblasts (left) or primary *LEPRE1*-null fibroblasts (right) stably transfected with *CRTAP* or *LEPRE1* expression constructs were determined by western blot. Gel migration of full-length transfected CRTAP is slightly delayed compared with control because of the presence of an HA-tag in the construct. P3H1 was substantially rescued by expression of full-length *CRTAP* but not by constructs with *CRTAP* deletions. Also, the CRTAP protein level was restored in *LEPRE1*-null fibroblasts transfected with a full-length *LEPRE1*expression construct (right). (**C**) Steady state type I collagen synthesized by *CRTAP*-null fibroblasts stably transfected with the full-length *CRTAP* expression construct. Collagen electrophoretic migration in transfected cells was substantially normalized in cell layer (left) and media (right) compared with *CRTAP*-null cells transfected with vector. NL, untransfected normal control fibroblasts.

the decrease in intracellular CRTAP in these cells compared with control. CRTAP may not require stabilization by P3H1 in the extracellular compartment, where it is not digested by proteasomes. Finally, denaturing extracts of proband cells yielded no evidence for the presence of CRTAP or P3H1 aggregates in the absence of proper folding and mutual stabilization, as is the outcome for unstabilized components of other complexes. P4Hα forms aggregates which associate with the immunoglobulin heavy chain binding protein in the absence of PDI (36). STRA13 and MSP58 are subject to proteasome-mediated degradation in the absence of complex formation (37), while the degradation pathways of the other complex components have not been reported.

The prolyl 3-hydroxylation complex has recently been reported by the Bachinger laboratory to function as a chaperone in both classical chaperone assays inhibiting citrate synthase aggregation and rhodanese refolding and aggregation, as well as in a fibril formation assay of type I collagen (46). Although these assays do not directly address the delayed folding of the collagen helix that occurs in recessive OI, these experiments raise interesting questions relating to collagen prolyl 3-hydroxylation. The unique hydroxylation of Pro986 (24), out of several dozen Pro-4Hyp-Gly triplets in the α1(I) chain, requires a selective binding that is not apparent *in vitro*, while the complex also has a general function to assist protein folding.

Finally, the mutual protection of P3H1 and CRTAP is an underlying mechanism for the overlapping phenotype of types VII and VIII OI. On a clinical and radiographic basis, types VII and VIII OI are indistinguishable at birth and early childhood. Both are lethal to severe osteochondrodysplasias, reflecting the fact that the prolyl 3-hydroxylation complex also modifies type II collagen in cartilage. Both have rhizomelia, undertubulation of long bones, white sclerae, normal to small head circumference and popcorn metaphyses reflecting growth plate disorganization. While

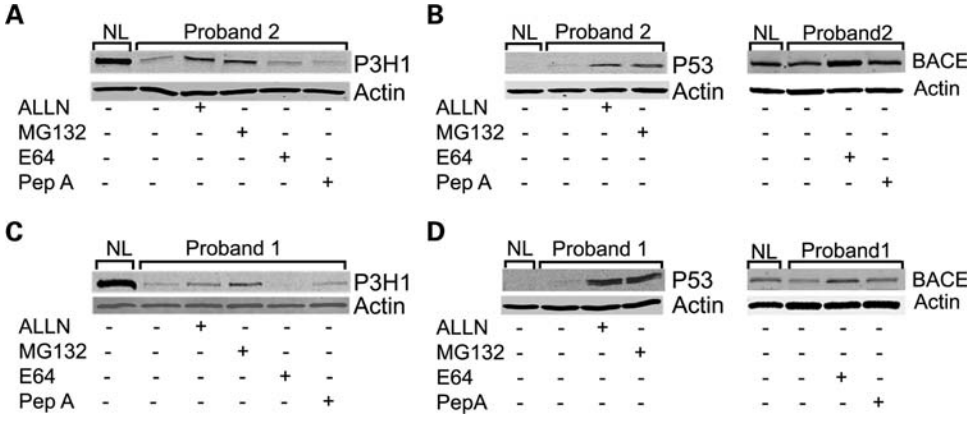


**Figure 6.** Proteasomal inhibition rescues P3H1 in *CRTAP*-null fibroblasts. Two independent *CRTAP*-null fibroblast cell lines (Proband 2: **A**, **B** and Proband 1: **C**, **D**) were treated with proteasomal (ALLN or MG132) or lysosomal (E64 or Pep A) inhibitors. Cell lysates were probed with P3H1 (A, C) or P53 and BACE antibodies (B, D). Actin was probed as a loading control. Proteasomal inhibitors MG132 and ALLN partially rescued P3H1 in *CRTAP*-null fibroblasts of both probands. Lysosomal inhibitors E64 and pepstatin A did not affect P3H1 levels (A, C). P53 and BACE were probed as controls for effective proteasomal and lysosomal inhibition, respectively (B, D).

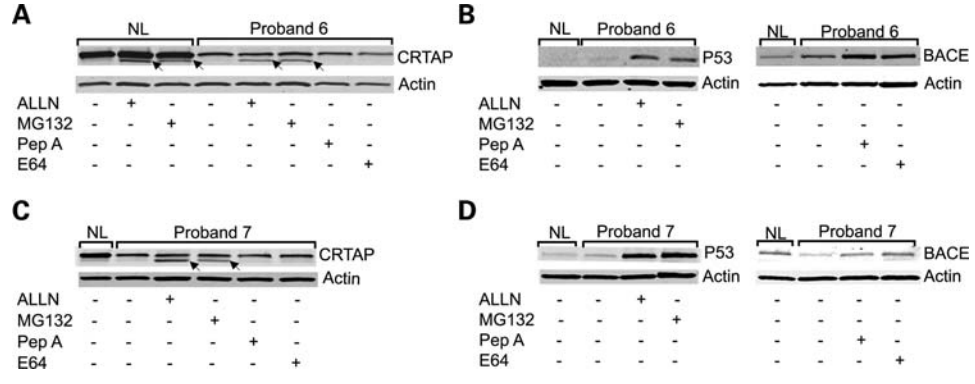


**Figure 7.** Proteasomal inhibition rescues short form of CRTAP in *LEPRE1*-null and normal fibroblasts. Two independent *LEPRE1*-null fibroblast cell lines (Proband 6: **A**, **B** and Proband 7: **C**, **D**) were treated with proteasomal (ALLN or MG132) or lysosomal (E64 or Pep A) inhibitors. Cell lysates were probed with CRTAP (A, C) or P53 and BACE antibodies (B, D). Actin was probed as a loading control. None of the inhibitors increased the level of full-length CRTAP protein in *LEPRE1*-null fibroblast cell lines. However, both proteasomal inhibitors rescued a short form of the CRTAP protein (arrow, A, C). P53 and BACE were probed as controls for effective proteasomal and lysosomal inhibition, respectively (B, D).

P3H1 and CRTAP share a joint function in the ER complex, both proteins are multifunctional and can be secreted from cells into matrix (25,27). Absence of the specific matrix functions of each protein and/or increased matrix CRTAP in type VIII OI may well provide distinguishing features between types VII and VIII OI. Studies of serum chemistries or bone histomorphometry in lethal and surviving cases of both types can be expected to reveal these distinctions.

## MATERIALS AND METHODS

### Ethics statement

All patient skin biopsies were obtained with informed consent under a protocol approved by the NICHD IRB.

### Mutation identification

The Proband 4 and Proband 5 mutations in *CRTAP* and *LEPRE1*, respectively, were detected as previously described (18,19) except for the paternal mutation of Proband 5 in *LEPRE1* exon 9, which was not detected by sequencing gDNA PCR products. To identify this mutation, full-length *LEPRE1* cDNA was amplified from proband fibroblast total RNA using standard RT-PCR protocols (47) with Oligo$(dT)_{16}$ (Applied Biosystems) and *LEPRE1*-specific primers LEPRE1–5 and LEPRE1–3 (All primers are listed in Supplementary Material, Table S1). The PCR product was cloned into PCR4-TOPO (Invitrogen) and subclones were sequenced. To confirm this mutation, exon 9 gDNA was amplified for restriction digestion.

### Cell lines and cell culture

Fibroblasts from Type VII OI probands (*CRTAP*-null; Probands 1–4) and type VIII OI probands (*LEPRE1*-null; Probands 5–8) were from the BEMB cell repository (Table 1), obtained with informed consent. Cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% $CO_2$.

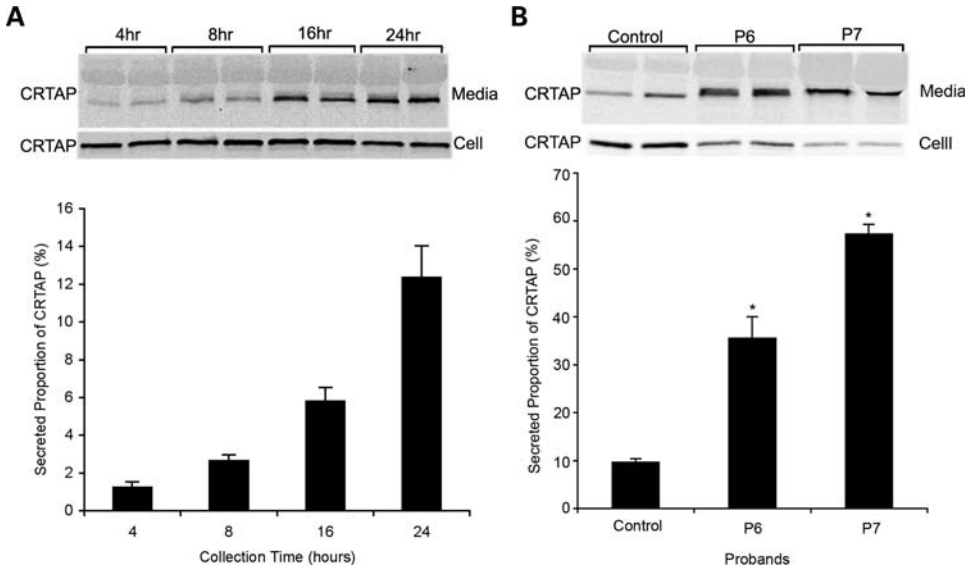


**Figure 8.** CRTAP secretion from control and *LEPRE1*-null fibroblasts. (**A**) CRTAP is secreted by normal fibroblasts. Conditioned media and cell lysates collected at indicated time points over 24 h were probed by western blot with CRTAP antibody (top). A minor percentage of CRTAP is secreted; approximately 10–12% of total CRTAP is detected in conditioned media after 24 h (bottom). (**B**) CRTAP secretion is increased in *LEPRE1*-null fibroblasts from Probands 6 and 7. Top panel is a representative western blot of CRTAP in 24 h conditioned medium and cell lysate collected from Probands 6 and 7, compared with normal control fibroblasts. The bottom panel is a plot of the proportion of secreted CRTAP in western blots from four independent samples of each proband, compared with control cells.

For treatment of cells with proteasomal or lysosomal inhibitors, unless otherwise specified, cells were treated for 8 h with the concentrations indicated: MG132 50 μM (Sigma), ALLN 50 μM (Sigma), Pepstatin A 20 μM (Sigma), E64 10 μM (Sigma).

### Western analysis

Cultured fibroblasts were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4) supplemented with protease inhibitor cocktail (Sigma). Protein from each cell lysate was quantitated using the BCA Protein Assay Kit (Thermo Scientific); 15 μg total protein was added to loading buffer containing 100 mM DTT. For cell extractions under denaturing conditions, a protocol utilized to detect insoluble protein in dermis (28) was adapted to our cells. Briefly, cultured fibroblasts were lysed in a buffer containing 4 M guanidine HCl, 0.05 M Tris-Cl and 10 mM DTT, supplemented with protease inhibitor cocktail. Samples were dialyzed against water to remove guanidine, dried and resuspended in loading buffer, with and without DTT.

Protein extracts were separated on 4–15% Tris–HCl Ready Gels in Tris-Glycine-SDS buffer and then electroblotted onto nitrocellulose membranes (Bio-Rad). The membranes were treated with Blocking Buffer (LI-COR) for 1 h, then primary antibody and 0.5% Tween-20 (Bio-Rad) were added prior to incubation overnight at 4°C. After washing in 1X TBST, the membrane was incubated with the corresponding IRDye infrared secondary antibody (LI-COR), according to the manufacturer's protocol. Finally, the membranes were scanned to visualize the specific protein bands using an Odyssey Infrared Imaging System (LI-COR). The primary antibodies used in this study are as follows: mouse anti-P3H1 (MaxPab, Abnova), mouse anti-CRTAP (Abnova), rabbit anti-actin and mouse anti-p53 (Santa Cruz), mouse anti-actin (Sigma), rabbit anti-CyPB and rabbit anti-BACE (Novus).

### Real-time RT–PCR

*CRTAP* and *LEPRE1* transcript levels were determined by real-time RT-PCR, as described previously (18,19). Briefly, total RNA was extracted from primary fibroblast cultures using TriReagent (48) (Molecular Research Center) and RNA integrity was verified on an Agilent 2100 Bioanalyzer. Total RNA (5 μg) was reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems) with random primers. TaqMan *LEPRE1* and *CRTAP* expression assays (Applied Biosystems) specific for mRNA sequence were used for quantification of transcripts. *LEPRE1* and *CRTAP* expression was calculated using a control fibroblast mRNA standard curve, then normalized to *GAPDH*, *18s rRNA* or *beta-2-microglobulin* (49). Technical replicates of a single assay versus *GAPDH* are shown in Figure 3, although multiple biological replicates have confirmed the results on these cells.

### Immunocytochemistry

Fibroblasts ($1.6 \times 10^4$ cells/cm$^2$) were plated on 2-well chamber slides (Nunc) and grown for 48 h. For CRTAP/GRP94 co-staining, cells were washed with PBS, then fixed for 15 min in 4% paraformaldehyde. Fixed cells were washed with PBS, permeabilized with 0.1% TritonX-100 for 15 min on ice, washed again with PBS and incubated for 1 h in blocking buffer containing 1% BSA in PBS. Cells were

then incubated in blocking buffer containing primary antibody overnight in a humidified chamber at 4°C. After washing, cells were incubated with 1:200 Alexa Fluor-555 anti-mouse (CRTAP) or 1:200 Alexa Fluor-488 anti-rabbit (GRP94) conjugated secondary antibody in blocking buffer for 1 h in a humidified chamber. Slides were mounted using mounting media with DAPI (Vectashield) and coverslips. Finally, the cells were imaged using a Zeiss LSM 510 Inverted Meta microscope and LSM510 software. P3H1/GRP94 staining was done following the protocol of Willaert *et al*. (22). Briefly, cells were washed in TBS, then fixed and permeabilized in cold acetone. Cells were then blocked in 10% goat serum in TBS and incubated with primary antibody in blocking buffer for 2.5 h in a humidified chamber at room temperature. Secondary staining and imaging was done by the same protocol as for CRTAP.

### *CRTAP* and *LEPRE1* expression constructs

Full-length *CRTAP* cDNA was amplified from total RNA of human control fibroblasts using standard RT-PCR protocols (47) with Oligo(dT)$_{16}$ and *CRTAP*-specific primers CRTAP5 and CRTAP3HA. The PCR product was initially cloned into the PCR4-TOPO vector (Invitrogen), then released by EcoRI (subsequently filled with T4 DNA polymerase) and XbaI digestion, for subsequent subcloning into the EcoRV and XbaI sites of pcDNA3Hyg(+) (Invitrogen). This construct was designated pcDNA3HygCRTAPfull.

The strategy for assembly of each *CRTAP* deletion construct involved ligation of two fragments into pcDNA3Hyg(+). One segment was a restriction digestion fragment from the pcDNA3HygCRTAPfull construct, generated using unique restriction sites in the *CRTAP* cDNA. The second segment was a PCR amplification fragment, generated with one primer containing a restriction site matching the prepared vector and one primer containing a restriction site matching the digested cDNA fragment and maintaining the reading frame.

The pcDNA3HygCRTAPDel1 construct was assembled by ligation of (a) the pcDNA3CRTAPfull PmeI-XhoI fragment and, (b) the XhoI-XbaI digested CRTAP PCR fragment generated using primers CRTAPXHOI and CRTAP3HA, into PmeI-XbaI digested pcDNA3Hyg(+). This construct contains an in-frame deletion of aa 46–144 within exon 1 of *CRTAP*. The pcDNA3HygCRTAPDel2 construct required ligation of (a) the EcoRI(filled)-NdeI digested CRTAP PCR fragment generated using primers CRTAP5 and CRTAPNDEI and (b) the pcDNA3HygCRTAPfull NdeI-XbaI digestion fragment, into EcoRV-XbaI digested pcDNA3Hyg(+). This construct contains an in-frame deletion of aa 145–204 within exon 2, including the CRTAP TPR-like domain. The pcDNA3HygCRTAPDel3 construct was assembled by ligation of (a) the pcDNA3CRTAPfull PmeI-NdeI digestion fragment and (b) the NdeI-XbaI digested CRTAP PCR fragment generated using primers CRTAPNDEIF and CRTAP3HA, into PmeI-XbaI digested pcDNA3Hyg(+). This construct contained an in-frame deletion of aa 207–264 in exon 3. The pcDNA3HygCRTAPDel4 construct is a subclone of a rare RT-PCR product from control human fibroblasts. This isoform with deletion of exon 4 has been previously described (45). All final constructs were completely resequenced.

The full-length *LEPRE1* expression construct was generated in the same manner as the full-length *CRTAP* construct. The *LEPRE1*-specific primers LEPRE1–5, with a NheI restriction site, and LEPRE1–3, with an Xba1 restriction site, were used. The full-length *LEPRE1* cDNA was cloned into NheI-XbaI digested pcDNA3Hyg(+).

### Stable transfections

Proband 2 fibroblasts were immortalized by transfection with pcDNA3 expressing SV40T, following the 3T3 protocol (50). Transformed *CRTAP*-null fibroblasts from Proband 2 or primary *LEPRE1*-null fibroblasts from Proband 7 were transfected with full-length and deletion constructs using LT1 (Mirus). Stably transfected clones were selected and maintained using hygromycin (300 and 150 μg/ml, respectively).

### Steady-state collagen preparation

The steady-state collagen synthesized by stably transfected *CRTAP*-null fibroblasts was radiolabeled by overnight incubation of confluent fibroblasts in serum-free media containing 260 μCi/ml [$^3$H]-Proline (14). Procollagen was precipitated from both media and cell lysates with $(NH_4)_2SO_4$, pepsin digested for 4 h on ice and electrophoresed on 6% SDS-Urea-PAGE. Collagen bands were visualized on the dried gel by autoradiography.

### Conditioned media

To collect secreted protein, fibroblasts were cultured in 100 mm Petri dishes. Serum-free DMEM was applied to confluent cells and the conditioned media were collected after 24 h, unless stated otherwise. After addition of a protease inhibitor cocktail, the conditioned media were concentrated 10-fold and washed three times with PBS using an Amicon Ultra-4 centrifugal filter device (Millipore). The cell count from each dish was used to normalize loading. Conditioned media from 100 000 cells and cell lysate from 50 000 cells were loaded on each lane for western blot analysis and bands were quantified with a LI-COR Odyssey Infrared Imaging System. Proportion of CRTAP secreted from a cell line was calculated as (Media)/[(Cell × 2) + (Media)]. Compensation of secreted media sample in proband for decreased CRTAP in cells was calculated as $(Media_{mut}\text{-}Media_{nl})/[(Cell_{nl} - Cell_{mut}) \times 2]$.

### GenBank accession numbers

Homo sapiens LEPRE1: gene, NC_000001.9; mRNA, NM_022356.2; protein, NP_071751.2.

Homo sapiens CRTAP: gene, NC_000003.10; mRNA,NM_006371.3; protein, NP_006362.1.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

*Conflict of Interest statement*. None declared.

## FUNDING

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