

## Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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## APPENDIX

### Cell Culture

Dermal fibroblasts were grown from punch biopsies in Dulbecco's Modified Eagle's Media (DMEM) containing Glutamax (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. For prolyl 3-hydroxylation analysis and amino acid chromatography, secreted type I procollagen was collected with protease inhibitors from confluent fibroblasts that had been incubated for 24 hours in DMEM containing Glutamax supplemented with 50 µg/ml ascorbic acid, 0.1% fetal bovine serum, penicillin and streptomycin.

### *PPIB* Sequencing and mRNA Expression Levels

PCR of *PPIB* Exon 1 gDNA was performed with the following primers - Forward: 5'-GCAATA ACGTGGCAACCACCCA-3' and Reverse: 5'-CGGGTCGTTCCCTGGACT GAAT-3', and sequenced using the Forward primer. Total RNA was collected using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) from untreated proband and control fibroblasts (ATCC2127) or cells treated with 100 µg/ml emetine for eight hours. RNA integrity was confirmed using an Agilent 2100 Bioanalyzer. Samples and standard curves for real-time reverse-transcriptase PCR were performed in triplicate with three independent samples. Relative expression of each gene of interest was compared to expression of *GAPDH*. Expression levels were normalized to the untreated control.

### ***In vitro* Biochemistry**

To examine collagen modification, confluent fibroblasts were labeled overnight with serum-free DMEM containing 280  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-proline. Radiolabeled collagens were precipitated with ammonium sulfate, pepsin-digested and separated by electrophoresis. Collagen bands were visualized using autoradiography. For Western blots, cell lysates were collected in radioimmunoprecipitation buffer (RIPA: 50 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 0.25% Na-deoxycholate) supplemented with a protease inhibitor cocktail (Sigma). Conditioned media was collected with protease inhibitors and concentrated  $\sim 50\text{X}$  using a spin column. Proteins were separated on pre-cast 4-15 % gradient Ready Gels (Bio-Rad, Hercules, CA), transferred to 0.45  $\mu\text{M}$  or 0.22  $\mu\text{M}$  nitrocellulose membranes and blocked with 5% bovine serum albumin plus 1X casein before probing with antibody. Cyclophilin B was probed with three separate antibodies targeted against different regions of the protein: full-length, C-terminal half, and the C-terminal 12 amino acids.

Amino acid chromatography was performed by high pressure liquid chromatography (Commonwealth Biotechnologies, Inc., Richmond, VA). Prolyl 3-hydroxylation determination was performed as previously described.<sup>1</sup> In brief, pepsinized collagen was resolved by SDS-PAGE, subjected to in-gel trypsin digestion and analyzed by electrospray mass spectrometry. Helical 4-proline and lysyl hydroxylation were determined by mass spectrometry of tryptic peptides as previously described.<sup>2</sup> Differential scanning calorimetry was performed on a Nano III DSC instrument from 10-55  $^{\circ}\text{C}$  at 0.125  $^{\circ}\text{C}/\text{minute}$  heating rate.<sup>3</sup>

## **Immunofluorescence Microscopy**

Cells for immunofluorescence microscopy were fixed in 4% paraformaldehyde in PBS. Permeabilization was performed on ice with 0.1% TritonX-100 in PBS. Blocking and antibody dilutions were made in 5% goat serum (P3H1/GRP94) or 1% BSA in PBS (CyPB/PDI, CRTAP/GRP94). Alexa-Fluor secondary antibodies (Molecular Probes) specific for mouse or rabbit antibodies were used at a 1:200 dilution. A Zeiss LSM 510 Inverted Meta confocal microscope with LSM software was used to collect and analyze the data.

## Detailed Clinical Case Reports

The probands are siblings born to healthy Senegalese parents of normal stature (F, 182.9 cm (75%); M, 160 cm (25%)), whose mothers were sisters (Fig. 1A). The mother was age 25 and 33 years old and the father was age 32 and 40 years old when probands III-1 and III-4, respectively, were born. The unaffected offspring of this couple are of normal stature: III-2 is age 10 years with height 126.5 cm (3% for age; 50% for 8 years), and III-3 is age 8 years with height 128 cm (70% for age).

Proband III-1 is a 12 year old male with osteogenesis imperfecta and sickle cell anemia who was born at term after an uncomplicated pregnancy, via spontaneous vaginal delivery. His birth weight was 5 lbs (< 5%). No further details of the newborn exam in Senegal are available. He crawled at 9 months, stood at 18 mos, and first walked at 2 years of age; he currently ambulates with weight bearing as tolerated. Intellectual development is normal.

His first fracture occurred at age 3 months, with 2 right femoral re-fractures by age 3 years resulting in a 5 cm leg length discrepancy. Between ages 4 and 10 years, he fractured his left tibia multiple (> 6) times, in a presumed acute-on-chronic fracture cycle. He has undergone right femoral osteotomy and realignments at ages 4 and 11 with intramedullary rodding (Rush rod) at 4 years and exchange-nailing with custom Smith & Nephew trochanteric entry nail at age 11 years. Left tibial osteotomy were performed at ages 11 and 12 with external fixation (Ilizarov spacial frame) at 11 years and placement of intramedullary titanium elastic nails at 12 years. At age 11, he was treated with oral alendronate (35 mg/wk) for one month, but discontinued for fractures and surgeries.

His growth has been moderately delayed, with height of 93 cm (50% for 2.75 yrs) and 125 cm (50% for 7.5 yrs) at ages 4 and 11 years respectively. His weight was proportional to height. Head circumference of 49 cm at age 11 years was normocephalic (25% for age). Hearing, vision and echocardiogram are normal. Pulmonary history is significant for two episodes of respiratory distress secondary to anesthesia, and pneumonia requiring hospitalization for oxygen and antibiotics at age 11 years. He has had no SCA crises and does not bruise easily.

On physical exam, his skull is asymmetric with a flat occiput and parietal bossing. Eyes are proptotic with epicanthal folds; the nasal bridge is flat with antiverted nares. His sclerae are white. The palate is mildly arched; dentition is normal. His thorax is somewhat narrow but is not deformed; no pectus is present. He has an innocent murmur. Musculoskeletal exam remarkable only for deformity secondary to malunion of chronic fracture sites. He is not rhizomelic. The proportion of span to height is normal. He has bilateral pes planus and moderate ligamentous laxity with 20-30 degrees dorsiflexion, positive thumb to forearm, hyperextension of 5<sup>th</sup> digit and elbow recurvatum.

His skeletal radiographs are characteristic of moderately severe OI, with generalized osteopenia but not rhizomelia (Fig. 1B). The long bones have thin cortices and normal diaphyseal modeling. The femoral metaphyses are mildly undertubulated and the R femur is shorter than the L by about 5 cm. Vertebrae are not compressed. Hand films reveal normal proportion for age of metacarpals to phalanges. L1-L4 DEXA z-score was -1.3 at age 11 years.

Proband III-4 is a 4 year old girl with osteogenesis imperfecta who was born at term via caesarean section due to breech position. At birth, she weighed 6 lbs (10% for age)

and had apgar scores of 4/8. She was noted to have bilateral fractures of humeri, radius and ulna, femora, and tibiae although an ultrasound at 37 weeks showed no fractures. She was also noted to have a wide anterior fontanelle, two natal teeth and low muscle tone and weakness. She sat independently at age 2.75 years and first walked at age 3.5 years wearing HKAOs and using a walker. Currently she ambulates with locked KFOs. Intellectual development is normal.

Post-natal fractures began at 6 months; she has sustained 4 femoral, 2 tibial, 1 clavicular and several rib fractures. All 4 lower extremity long bones have undergone osteotomy procedures with Fassier-Duval nailing. She was treated with intravenous pamidronate (3 mg/kg/cycle) q3mos for 1 year beginning at age 2 years, and for a single cycle at age 4 years.

Proband III-4 has more severe growth delay than her brother, with length falling below the normal curve by 6 months of age and plateauing after a year; weight gain has been proportionate. At 28 months of age, her length is 70 cm and weight 8.5 kg (both 50% for 9 months). Hearing, vision and echocardiogram are normal. Cardiac and respiratory systems are within normal limits. She does not bruise easily.

On physical exam, she has a triangular face with a high bossed forehead, proptotic eyes, white sclerae, and a flat nasal bridge. There is no dentinogenesis imperfecta. The thorax is symmetric without deformity. Her musculoskeletal exam at 5 months was notable for ligamentous laxity, umbilical hernia, and mild anterior tibial bowing. She is not rhizomelic. Both hands are 12.5 cm long (50% for age). She has bilateral pes planus with moderate ligamentous laxity, including elbow recurvatum, positive thumb to forearm and 5<sup>th</sup> finger hyperextension.

Radiographs shortly after birth reveal osteoporotic lower extremity long bones, with undertubulation and mid-shaft bowing of femora and tibiae (Fig. 1B). By age 15 months, T11-L1 vertebral bodies had significant anterior compressions. At 33 months, long bone tubulation has improved but is still impaired in femoral metaphyses. A series of 3 sharp pamidronate lines are visualized. Hand films reveal proportionate segments. L1-L4 DEXA z-score is -3.9 at age 3.5 years.

## References

1. Barnes AM, Chang W, Morello R, et al. Deficiency of cartilage-associated protein in recessive lethal osteogenesis imperfecta. *N Engl J Med* 2006;355(26):2757-64.
2. Giunta C, Elcioglu NH, Albrecht B, et al. Spondylocheiro dysplastic form of the Ehlers-Danlos syndrome--an autosomal-recessive entity caused by mutations in the zinc transporter gene SLC39A13. *Am J Hum Genet* 2008;82(6):1290-305.
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Supplementary Table 1: Updated Sillence Classification

|                        | <b>Type</b> | <b>Inheritance</b> * | <b>Phenotype</b>   | <b>Defect</b>                                  |
|------------------------|-------------|----------------------|--|--|
| <b>Sillence Types:</b> | I           | AD                   | Mild   | Null (1I) allele<br>Some Glycine substitutions |
|                        | II          | AD                   | Lethal   | Structural defects in Type I Collagen          |
|                        | III         | AD                   | Progressive Deforming  | Structural defects in Type I Collagen          |
|                        | IV          | AD                   | Moderate   | Structural defects in Type I Collagen          |
| <b>New Types:</b>      | V           | AD                   | Hypertrophic Callus<br>Dense Metaph. Band<br>Distinctive Histology | Unknown  |
|                        | VI          | ?                    | Mineralization Defect<br>"Fish-scale" Lamellae                     | Unknown  |
|                        | VII         | AR                   | Severe to Lethal   | Mutations in <i>CRTAP</i>                      |
|                        | VIII        | AR                   | Severe to Lethal   | Mutations in <i>LEPRE1</i>                     |
|                        | IX          | AR                   | Moderately Severe  | Mutations in <i>PPIB</i>                       |

\* AD, autosomal dominant; AR, autosomal recessive

## Figure Legends

### **Supplementary Figure 1. Radiographs of probands.**

Lateral spine film of Proband III-1 shows no vertebral compressions. Hand films of Probands III-1 and III-4 show normal proportions of metacarpals to phalanges for age and gender.

### **Supplementary Figure 2. Type I Collagen 3-Hydroxylation and Thermal Stability.**

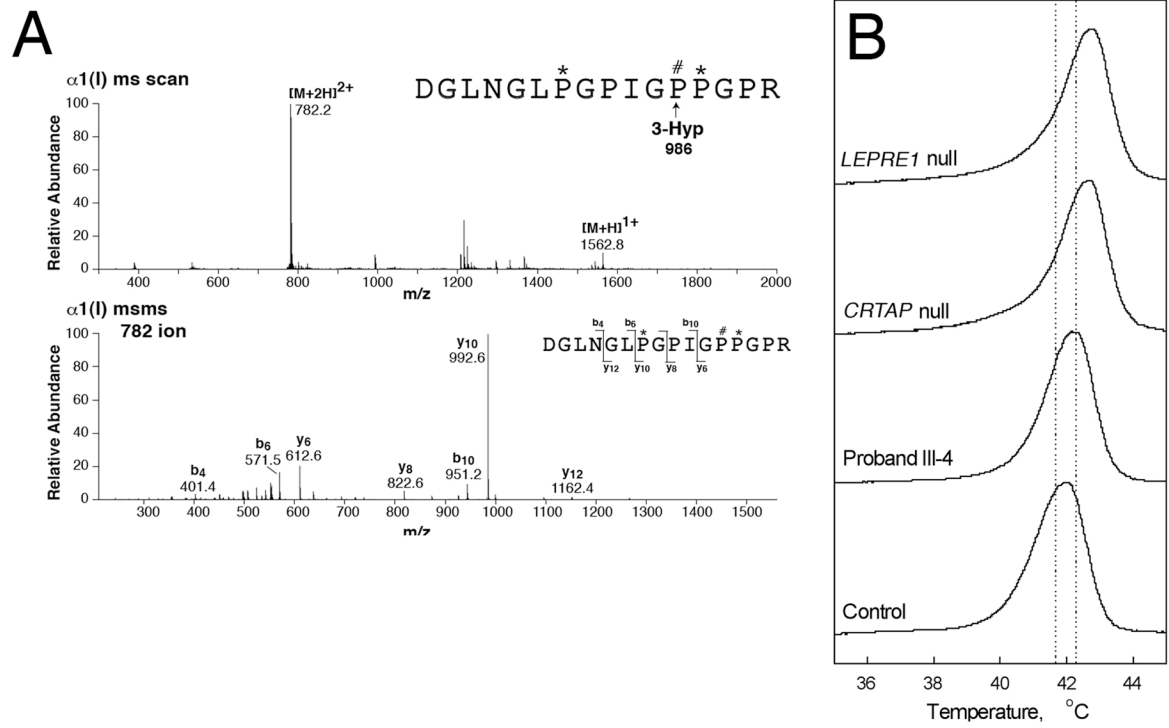
Panel A shows the fragmentation spectra of the peptide containing  $\alpha 1(I)$ Pro986. The top spectrum shows that virtually all Pro986 residues are 3-hydroxylated. In the bottom spectrum, the b and y ions correspond to the amino-terminal (b) and carboxyl-terminal (y) daughter ions. Daughter ion y6 is increased by 16 mass units reflecting the Pro986 3-hydroxylation. Panel B shows thermograms of collagen stability performed by differential scanning calorimetry. The dotted lines show the range of normal collagen thermal stability. The  $T_m$  of Proband III-4 is within the normal range, in contrast to collagen from *CRTAP*- or *LEPRE1*-null fibroblasts which are increased by 1°C.

III-1



III-4





Supplementary Figure 2