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## Kuskokwim syndrome, a recessive congenital contracture disorder, extends the phenotype of *FKBP10* mutations

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### Abstract

Recessive mutations in *FKBP10* at 17q21.2, encoding FKBP65, cause both osteogenesis imperfecta (OI) and Bruck syndrome (OI plus congenital contractures). Contractures are a variable manifestation of null/missense *FKBP10* mutations. Kuskokwim syndrome (KS) is an autosomal recessive congenital contracture disorder found among Yup'ik Eskimos. Linkage mapping of KS to chromosome 17q21, together with contractures as a feature of *FKBP10* mutations, made *FKBP10* a candidate gene. We identified a homozygous 3-nucleotide deletion in *FKBP10* (c. 877\_879delTAC) in multiple Kuskokwim pedigrees; 3% of regional controls are carriers. The mutation deletes the highly conserved p.Tyr293 residue in FKBP65's 3<sup>rd</sup> PPIase domain. *FKBP10* transcripts are normal, but mutant FKBP65 is destabilized to a residual 5%. Collagen synthesized by KS fibroblasts has substantially decreased hydroxylation of the telopeptide lysine crucial for collagen cross-linking, with 2–10% hydroxylation in probands vs 60% in controls. Matrix deposited by KS fibroblasts has marked reduction in maturely cross-linked collagen. KS collagen is disorganized in matrix, and fibrils formed *in vitro* had subtle loosening of monomer packing. Our results imply that *FKBP10* mutations affect collagen indirectly, by ablating FKBP65 support for collagen telopeptide hydroxylation by LH2, thus decreasing collagen crosslinks in tendon and bone matrix. *FKBP10* mutations may also underlie other arthrogyposis syndromes.

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## Keywords

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## INTRODUCTION

Kuskokwim syndrome (KS; MIM# 208200) is an autosomal recessive congenital contracture disorder that occurs solely among Yup'ik Eskimos in and around the Kuskokwim River delta region of southwest Alaska. Affected individuals usually have congenital contractures of large joints, especially knees and/or elbows, and spinal, pelvic, and foot deformities; they sometimes have proximally or distally displaced patellae, due to tendon attenuation (Petajan, et al., 1969). Minor skeletal features have been reported, including mild vertebral wedging, mild to moderate scoliosis, pedicle elongation, and clubbing of the clavicle (Petajan, et al., 1969; Wright, 1970). Individuals with KS have white sclerae, normal teeth and hearing.

The first individual identified with KS was born in 1922. The syndrome was recognized as a new disorder in 1969, and 17 patients from seven families living near Bethel and Togiak were described (Wright, 1970). The sudden emergence of this syndrome can be explained by the history of the region and of the Yup'ik Eskimo people (Fienup-Riordan, 1991). From the 1840s to 1880s, Bethel, a small (population about 40) centrally located village, was important for periodic tribal social gatherings among villages along the river, and also for interaction with Russian traders. At the native celebrations of religious festivals, social interactions led to genetic admixture in offspring conceived in Bethel but born in their own villages. In 1884, Moravian missionaries arrived in Bethel. As Christianity took hold under the Moravians, these gatherings were discontinued, requiring the Yup'ik to find mates within their own small villages, thus greatly increasing the frequency of consanguineous relationships.

Mating selection was further reduced by the 1918–1919 “Spanish flu” epidemic, which cost the lives of fully half of the Yup'ik population. Some epidemic survivors who had resided in Bethel then migrated to Togiak and other distant villages. Two years later the first child known to have Kuskokwim syndrome was born. Thus, KS emerged due to the effective small population size, the shift in marriage patterns before and after the arrival of Moravian missionaries, and the population bottleneck caused by the influenza epidemic.

Under the hypothesis that the mutation causing KS arose on a single founder chromosome, we used both homozygosity mapping and linkage analysis to localize the gene to chromosome 17q12–21. Two candidate genes, *COL1A1* (MIM# 120150, NM\_000088.3) and *NOG* (MIM# 602991, NM\_005450.4), were excluded in the 1990's. More recently, *FKBP10* (MIM# 607063, NM\_021939.3) was identified as a candidate gene for KS based on gene location at 17q21.2 and the variable phenotype of previously identified *FKBP10* mutations (MIM# 610968) (Kelley, et al., 2011; Setijowati, et al., 2012; Shaheen, et al., 2011; Shaheen, et al., 2010).

*FKBP10* encodes FKBP65, an ER-localized peptidyl-prolyl *cis-trans* isomerase (PPIase) and chaperone molecule (Ishikawa, et al., 2008). FKBP65 is the largest member of the immunophilin subfamily that binds FK506 and has 4 PPIase domains. FKBP65 has multiple ligands, including type I collagen and elastin (Davis, et al., 1998; Zeng, et al., 1998). Mutations in *FKBP10* were first identified as causing autosomal recessive type XI osteogenesis imperfecta (OI) in 2010 (Alanay, et al., 2010). Affected individuals had severe progressive deforming or moderately severe OI. The *FKBP10* phenotype rapidly expanded

to include Bruck syndrome 1, which is severe OI with congenital contractures (Kelley, et al., 2011; Schwarze, et al., 2013; Setijowati, et al., 2012; Shaheen, et al., 2011; Shaheen, et al., 2010).

We have identified homozygosity for a small deletion in exon 5 of *FKBP10* as the cause of Kuskokwim syndrome, extending the phenotypic spectrum of *FKBP10* mutations from OI alone, and OI with contractures (Bruck syndrome), to a predominantly congenital contracture syndrome. We demonstrate that the collagen-related mechanism of KS involves decreased hydroxylation of the collagen telopeptide lysine crucial for cross-linking of collagen monomers into fibrils, resulting in decreased deposition of mature collagen in matrix, and a looser appearance of fibrils formed *in vitro*.

## MATERIALS AND METHODS

### Patient and control samples

Eight families with KS, including 11 affected individuals from whom DNA was attained, were initially ascertained by a search of the Indian Health Service database in Anchorage, AK (Supp. Figure S1). An additional four families were ascertained by WP, to a total of 16 affected individuals, 6 probably/mildly-affected individuals, and 47 unaffected first-degree relatives of patients. Patients were examined either in the villages or Anchorage by SJB, GD and WP (under a protocol approved by the Indian Health Service, the Yukon-Kuskokwim Health Corporation, and the Yup'ik Tribal Council), or at the National Institutes of Health (NIH), under IRB-approved protocol 97-HD-0089. Buccal swabs were also collected from individuals in Alaska, and EDTA blood from patients seen at NIH. Buccal swabs were collected from 48 unaffected, unrelated Yup'ik individuals to serve as ethnically-matched controls.

### Clinical Examinations

Patients seen in Alaska had detailed reviews of medical records, brief physical examinations and buccal swab collection. Three families with five probands (Fig. 1A) underwent more extensive examinations. The five patients seen at the NIH underwent physical examinations including dermatology, physical medicine and rehabilitation, physical and occupational therapy, as well as ECG, audiometry, and skin biopsy. Laboratory studies included CBC and serum chemistry, radiographic skeletal survey, MRI of knees and elbows, and bone mineral density (DXA). Ultrasound examination of patellar and elbow tendons was performed on several patients.

### Linkage Mapping

DNA was extracted from buccal swabs and blood, per standard protocols. Three DNA pools were prepared as follows: Pool 1: 11 affected individuals; Pool 2: 17 obligate heterozygotes (parents and offspring of affected individuals); Pool 3: 20 unaffected, unrelated Yup'ik controls. The pooled samples were screened using 387 polymorphic markers, with average heterozygosity of 76% and average spacing of 10 cM (Vers 8, CHLC Human Screening Set, Research Genetics, Huntsville, AL), and examined by incorporating <sup>32</sup>P in amplification reactions. The markers that showed a reduction in the number of alleles in the affected pool, as compared to the control pool, were verified by typing of individual family members and by analysis of other nearby markers.

### PCR and Sequencing

Genomic DNA (gDNA) was prepared from fibroblasts or leukocytes using a Gentra Puregene DNA extraction kit (Qiagen, Valencia, CA). Proband fibroblast gDNA was used to amplify the ten exons of *FKBP10* (NM\_021939.3) by PCR. PCR products were then

sequenced (MacroGen Corp, Rockville, MD). The homozygous mutation was confirmed by the loss of an *MspI* restriction enzyme site. The KS mutation was submitted to the OI Variant Database (<http://www.LOVD.nl/FKBP10>).

### Real-time PCR

Total RNA was extracted from primary fibroblasts using TriReagent (Molecular Research Center, Cincinnati, OH); RNA integrity was verified on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Complementary DNA was reverse transcribed from 5 µg RNA using a High Capacity cDNA Archive Kit (Applied Biosystems/Life Technologies Corporation, Carlsbad, CA). The relative expression of *FKBP10* was assessed by comparing it to the expression of *GAPDH*, using Taqman Gene Expression Assays. Expression levels were normalized to control primary fibroblasts.

### Western Blot

Cell lysates from proband fibroblasts were collected in RIPA buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Proteins were separated on precast 4–15% Ready Gels (Bio-Rad, Hercules, CA), transferred to 0.2 µM nitrocellulose membranes and blocked with 5% bovine serum albumin plus 1X casein before probing with FKBP65 (Abnova, Taipei, Taiwan) and actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies overnight. Blots were washed, incubated with secondary IR-conjugated antibodies for 1 hr, washed, and visualized and quantified on a LI-COR Odyssey infrared imager (LI-COR, Lincoln, NE).

### Steady-state Collagen

For type I collagen studies, control and proband fibroblasts were grown to confluence in 6-well culture dishes. Steady-state collagen analysis was conducted as previously described (Cabral, et al., 2005). Cells were incubated with 437.5 µCi/ml L-[2,3,4,5-<sup>3</sup>H] proline for 16–18 hours, prior to collection and ammonium sulfate precipitation, then pepsin-digested and analyzed by 6% SDS-urea-PAGE.

### Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) of secreted collagen solutions in 0.2 M sodium phosphate and 0.5 M glycerol at pH 7.4 from proband K5-II.8 and null cells was performed from 10 to 55 °C in a Nano III DSC instrument (Calorimetry Sciences Corporation, Lindon, UT), as previously described (Makareeva, et al., 2008).

### Collagen Secretion

Collagen secretion rate was measured in the presence of ascorbic phosphate and 0.5% serum as described before (Makareeva, et al., 2010). Absolute collagen concentration was quantified by adding AlexaFluor488-labeled MMP-1 fragments of type I mouse tail tendon collagen as an internal standard followed by Cy5-labeling of the mixture and separated on 3–8% gradient Tris-Acetate mini gels (Invitrogen/Life Technologies, Grand Island, NY). The collagen to internal standard ratio was measured by densitometry of gel fluorescence on an FLA5000 scanner (Fuji Medical Systems, Tokyo, Japan) which was analyzed with Multi-Gauge software supplied with the scanner. The secretion rate was normalized to the number of cells in the well.

### Matrix Deposition

For extracellular matrix deposition assays, post-confluent cells were stimulated with 100 µg/ml ascorbic acid three times a week for 14 days. After fourteen days, fibroblasts were either labeled for 24 hours with 406.25 µCi/ml L-[2,3,4,5-<sup>3</sup>H] proline and the media and cell layer

were harvested, as described previously (Bateman and Golub, 1994), or used for Raman micro-spectroscopy. The  $^3\text{H}$ -proline-labeled collagens were sequentially extracted from the cell-layer fraction with neutral salt, acetic acid and pepsin. These matrix extracts were run on 6% SDS-urea-PAGE. Collagen content of each fraction was measured by densitometry and normalized to sample volume.

### Immunofluorescence Microscopy

For FKBP65 and type I collagen immunofluorescence staining, fibroblasts were plated on 2-well chamber slides (Nalge Nunc International, Rochester, NY), grown for 48 hr and stained. Briefly, for co-staining of FKBP65 (BD, Franklin Lakes, NJ) and LF-68 (  $\alpha$ (I) C-telopeptide; a generous gift from Dr. Larry Fisher, NIH) cells were washed with PBS, then fixed in 4% paraformaldehyde. Fixed cells were washed, permeabilized on ice in 1% goat serum plus 0.2% TritonX-100 for 5 minutes, washed again and blocked with 1% BSA in PBS. Cells were incubated with primary antibody for 1 hr, washed in PBS, and incubated with Alexa Fluor-555 and Alexa Fluor-488 conjugated secondary antibodies for 1 hr then washed extensively. Slides were mounted using mounting media with DAPI (Vector Laboratories, Burlingame, CA) and coverslips. Stained cells were imaged using a Zeiss LSM 510 Inverted Meta microscope and LSM510 software.

Staining of the extracellular matrix was performed essentially as described (Valli, et al., 2012). Cells were grown to confluence on chamber slides, then treated with DMEM + 10% FBS containing 100  $\mu\text{g}/\text{ml}$  ascorbic acid three times a week for 2 weeks. Matrices were washed in PBS, then fixed in 4% paraformaldehyde. The matrix was blocked in 1% BSA in PBS plus 0.02% Tween-20, washed with PBS and incubated with primary antibody (LF-68) in 1% BSA/PBS for 1 hr. After washing, the matrix was incubated with secondary antibody for 1–1.5 hr, washed, and examined as above.

### Raman Micro-Spectroscopy

Confluent cell cultures fixed in 1% formaldehyde in PBS for 4 hr at 37°C were hydrated with PBS supplemented with 1 ng/ml DAPI stain for nuclei and examined in a Senterra confocal Raman microscope (Bruker Optics) as described before (Barnes, et al., 2012). (Auto)fluorescence images of the pieces were taken under 365 nm light to locate cell cytoplasm. The excitation laser was focused to  $2 \times 2 \mu\text{m}$  areas pointed at the cytoplasm (between nuclear edge and cell periphery). Raman-scattered light was collected from a 7  $\mu\text{m}$  high focal volume, which encapsulated both cells and their matrix. Raman scattering spectra with 10–15  $\text{cm}^{-1}$  resolution were collected from different  $140 \times 170 \mu\text{m}$  regions within each culture, averaged over 10 points within each region, and corrected for water and quartz contributions as well as for 1–6  $\text{cm}^{-1}$  instrumental variability in frequency calibration. Matrix collagen to cell organics ratios were evaluated from decomposition of the corrected spectra on spectra of collagen-free cell cytoplasm and of purified collagen, normalized to have similar integral intensities of stretching vibrations of all organic CH groups. Decomposition was in the regions of the CH stretching (2,780–3,040  $\text{cm}^{-1}$ ) or in the amide III region (1,180–1,380  $\text{cm}^{-1}$ ) where collagen and cells have very distinct spectra. Alternatively, the spectra were decomposed from ratios of integral intensities of baseline-corrected, spectral bands dominated by (hydroxy)proline (832–893  $\text{cm}^{-1}$ ) to those of CH stretching (2,810–3,028  $\text{cm}^{-1}$ ) vibrations dominated by proteins and lipids. Content of intracellular collagen in these cultures was negligible. Standard errors and *P* values (two-tail *t*-test) were calculated for variations between different regions within the control ( $n = 14$ ) and mutant ( $n = 6$ –8) cultures.



## Amino Acid Analysis

Amino acid analysis to quantify 4-hydroxyproline, proline, hydroxylysine and lysine was performed by high pressure liquid chromatography (AIBiotech, Richmond, VA). Telopeptide lysyl hydroxylation of COL1A1 p.Lys1208 was assessed by ion-trap mass spectrometry. Secreted procollagen was precipitated by 1M (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, from which pro- $\alpha$  chains were resolved by SDS-PAGE and subjected to in-gel trypsin digestion for analysis of targeted peptides by electrospray mass spectrometry.

*In vitro Fibrillogenesis:* To analyze the morphology of collagen fibrils formed from collagen molecules with aberrant hydroxylation of telopeptides we employed *de novo* fibril formation assays with the use of procollagen I isolated from the cultures of probands' dermal fibroblasts, as described (Cabral, et al., 2007; Stepkowski, et al., 2004). In brief, fibril formation was initiated by processing procollagen I at 150  $\mu$ g/ml with endopeptidase Lys-C (EC.3.4.21.50, endoproteinase Lys-C; Roche, Basel, Switzerland) at 37°C. Note: As Lys-C cleaves proteins at the C-terminal side of the lysine residues, employing this enzyme to process procollagen I propeptides conserves a critical lysine residue of the C-terminal telopeptide of the  $\alpha$ 1(I) chain (p.Lys1208) but not the lysine residues present within the N-terminal telopeptides.

To preserve the structure of newly formed fibrils and to minimize their aggregation, the fibril formation process was carried out in a hanging drop according to a described method (Fertala, et al., 1996). In brief, samples of 5  $\mu$ l of mixture contain procollagen I and Lys-C were transferred to a Teflon-insulated wire to form a hanging drop, the wire was mounted inside a screw cap, and then it was placed above a buffer in a tightly closed tube. The tube was incubated at 37°C for 24 hr and then the whole drop was placed on a carbon-coated copper grid (Ted Pella Inc., Redding, CA) and left to adsorb for 2 min. Subsequently, collagen fibrils were negatively stained with 1% phosphotungstic acid (Ted Pella, Inc., Redding, CA). The negatively stained samples were analyzed using a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan).

## RESULTS

### Clinical phenotype

Kuskokwim syndrome patients usually have congenital contractures, especially of lower extremities, which progress during childhood and persist for the lifetime of the individual. Not all patients with KS have contractures at birth, reflecting the variability of contractures with *FKBP10* mutations. The severity of contractures can be strikingly asymmetrical in any given homozygous individual. The knees and elbows are frequently involved. Bony deformities of the spine, pelvis, and feet are frequently seen. Occasional patients with full bilateral contractures of the knees ambulate either by "duck walking" (hunkering), where the individuals sit with buttocks on their heels, or by "knee walking", where they ambulate on their knees with their lower legs drawn up behind them to their buttocks (Petajan, et al., 1969), although most are treated with leg braces and/or surgery in childhood and walk upright. Hip flexion contractures can be masked by lumbar hyperlordosis. Foot and ankle involvement included internal tibial torsion and planovalgus or equinovarus deformities. There may be associated muscle atrophy of limbs with contractures, and proximal or distal displacement of patellae due to attenuation of patellar or quadriceps tendons. Proximal patella displacement, when it occurs, may be progressive in childhood, but only is present in some severe individuals. Distal displacement is a subtle, but more common, finding and is a good indicator of clinically significant contractures.

Mild skeletal features are common, with osteopenic or osteoporotic vertebrae (L1-L4 z-score from -0.48 to -2.29) and mild vertebral wedging. X-rays reveal severe lumbar

lordosis with associated horizontal sacrum, severe sacral kyphosis (hooked sacrum), superior angulation of the transverse processes of the lower lumbar vertebrae, and elongation of the lumbar pedicles. Spondylolisthesis and mild to moderate scoliosis are occasionally seen (Fig. 1B–C, Supp. Figures S2–S3). Lordosis is usually much less apparent on physical exam than on X-ray, being masked by pedicle elongation. Affected individuals have marked lordosis with the sacrum position being nearly horizontal (Supp. Figure S3). They tend to have barrel chests. Many probands have incurred several low energy fractures, including femora. In more severe cases, protrusio acetabulae occurs. Affected individuals may also have hallux valgus, plano valgus feet or talipes equinovarus. A very consistent x-ray finding is clubbing of the distal clavicle.

Individuals with KS have white sclerae and normal dentition and hearing. Probands have relative short stature, with height below the 5<sup>th</sup> percentile for age (50<sup>th</sup> percentile for 9.5 to 15 year olds) when compared to the standard U.S. growth tables, but are less short compared with other Yup'ik people. Head circumference is macrocephalic.

The detailed descriptions of the probands in the Supporting Information show the variability of symptoms among affected individuals, even within sibships. Among probands examined in detail, probands 4 and 5 (K3-III.6 and K5-II.8) have more severe symptoms and findings, while proband 1 (K2-II.1) is intermediate and probands 2 and 3 (K2-II.2 and K3-III.5) are relatively mild. Probands 1 and 2 are siblings, as are probands 3 and 4, showing variability even with sibships.

### Mapping of Kuskokwim syndrome to 17q12–21

Twelve families, residing in eight Yup'ik villages and two towns, were ascertained. Homozygosity mapping using pooled DNA from affected individuals showed only a single allele for three markers on chromosome 17q21 (*D17S800*, *KRT9-CA repeat*, *D17S846*), while control and relative pools showed multiple alleles (Supp. Table S1). On radiation hybrid mapping, these three markers gave LOD scores ranging from 8.58 to 12.8, confirming the likelihood of the mutation in this region. Patients and family members were all typed individually for the three conserved markers, revealing homozygosity in all affected for a “4-4-1” haplotype. Of six “probably/mildly” affected individuals included in the analysis, five were homozygous 4-4-1, and one carried one 4-4-1 haplotype and one 4-4-2 haplotype. Flanking markers on the centromeric side showed the presence of multiple alleles in the affected pool, originally identifying a conserved haplotype of approximately 0.83 Mb on chromosome 17q21. When the conserved region was first defined, the *D17S1793* marker qter to this region was not examined in all individuals. In retrospect, the available data on *D17S1793* indicates that this marker should be included in the conserved haplotype. In 1999, *COL1A1* (17q21.33) was explored as a candidate gene, however, radiation hybrid mapping placed *COL1A1* distal to the conserved haplotype and the *COL1A1* sequence was normal in affected individuals.

### *FKBP10* mutation in Kuskokwim pedigrees

Because the updated KS homozygous region included *FKBP10* at 17q21.2, in which congenital contractures had been shown to be a variable feature of recessive type XI OI (Shaheen, et al., 2010), we sequenced the 10 exons of *FKBP10* in K3-III.6 fibroblast DNA. We identified a homozygous 3 nucleotide deletion in *FKBP10* exon 5 (c.877\_879delTAC, Fig. 2A), which causes deletion of a single residue, p.Tyr293. This deletion occurs in the 3<sup>rd</sup> PPIase domain of FKBP65, at a highly conserved residue (Fig. 2B). The mutation eliminates an *MspI* restriction site, which was used to confirm presence of the homozygous deletion in probands with varying clinical severity from three Kuskokwim pedigrees (Fig. 2C). Furthermore, DNA samples from 48 unaffected controls from the Kuskokwim delta region

were examined; about 3% (3/96 alleles) were found to be heterozygous carriers of the *FKBP10* in-frame deletion. This *FKBP10* mutation was not present in the Exome Variant Server (<http://evs.gs.washington.edu/EVS/>). This mutation extends the phenotype of *FKBP10* mutations to a predominantly congenital contracture condition (Fig. 2D).

### ***FKBP10* expression and FKBP65 stability in Kuskokwim syndrome**

Transcript levels of *FKBP10* in fibroblasts from five Kuskokwim probands who were homozygous for the in-frame deletion were in the normal range (79–167% of control) (Table 1). Expression levels varied as much between siblings as between pedigrees, but did not correlate with severity.

The in-frame deletion destabilizes the FKBP65 protein. Only residual FKBP65 was detected on Western blots of proband fibroblast lysates (2.68 – 6.44% of control, Fig. 2E).

### **Effect of KS *FKBP10* mutation on collagen synthesis, secretion and stability**

We examined type I collagen biochemistry to determine the effect of decreased FKBP65 on collagen post-translational modification and metabolism. Steady-state collagen synthesized by probands K3-III.5 and K3-III.6 fibroblasts showed normal electrophoretic migration with a slight band broadening (Fig. 3A), consistent with a minimal increase in collagen melting temperature on DSC (Fig. 3B). Amino acid analysis of the collagen helix by liquid chromatography revealed a 30% increase in number of hydroxylated lysines (Table 1). On average, this is equivalent to hydroxylation of an additional 2 or 3 lysines in a collagen monomer vs control samples.

The absolute amount of collagen secreted into media by proband fibroblasts in culture was determined with an assay using internal collagen standards. Collagen secretion (pg/hr/cell) was normal by t-test, ranging from 119–141% of control cell values (Table 1). Intracellular collagen showed no evidence of aggregation on immunofluorescence microscopy (Fig. 3C).

### **Effect of KS *FKBP10* mutation on collagen deposition**

Collagen deposition into matrix was examined biochemically, as well as by confocal microscopy and Raman micro-spectroscopy. In the biochemical assay, fractions were successively extracted from the deposited matrix by neutral salt, which contains newly incorporated collagen without cross-links; acetic acid, which contains collagen with immature cross-links; and pepsin, which contains collagen with mature cross-links. Fibroblasts from individuals with relatively milder (K3-III.5) and more severe (K5-II.8) KS were utilized. There was a consistent decrease of collagen deposited into the maturely cross-linked fraction, ranging from half to one-sixth of control. In addition, the  $\alpha$ -forms in the cross-linked fractions, were clearly reduced, with  $\alpha$ <sub>1</sub> and  $\alpha$ <sub>2</sub> nearly absent and only  $\alpha$ <sub>1</sub>,2 well-detected (Fig. 4A).

On confocal microscopy, the collagen deposited by fibroblasts homozygous for the Kuskokwim *FKBP10* mutation was generally more sparse and disorganized than control and contained spindly structures with bright edges (Fig. 4B). The amount of collagen in matrix was intermediate between the control and *FKBP10-null* cells. Further examination of deposited matrix by Raman micro-spectroscopy revealed variable collagen deposition in KS matrix, ranging from 1/3 to normal levels, with no correlation with disease severity (Fig. 4C). Raman micro-spectroscopy confirmed the decreased deposition in K3-III.5 and K5-II.8 in culture, but two other KS probands had normal matrix deposition by Raman.



### Collagen telopeptide lysine crucial for cross-linking has reduced hydroxylation

The reduced deposition of mature collagen into matrix in culture led us to examine the hydroxylation status of the type I collagen C-telopeptide lysine, which is crucial for cross-linking of collagen into matrix, using mass spectroscopy of tryptic peptides (Table 1). Although telopeptide residues are not normally hydroxylated in skin tissue, this residue is 60% hydroxylated in cultured normal fibroblasts and has been shown to be <1% hydroxylated in *FKBP10*-null cells (Barnes, et al., 2012). In KS samples, hydroxylation of COL1A1 p.Lys1208 was found to be significantly reduced, although not absent, ranging from 2–10%. More mildly affected individuals had slightly greater levels of telopeptide hydroxylation than more severely affected individuals, 8–10% vs 2–3%.

### *In vitro* fibrillogenesis of KS collagen yields looser collagen fibrils

*In vitro* fibrillogenesis of probands K3-III.5 and K5-II.8 secreted collagen yielded fibrils with a normal D-periodicity. The presence of collagen molecules with decreased content of hydroxylated p.Lys1208 was associated with less tightly bundled fibrils, with evidence of splitting and splaying of the fibers (Fig. 5A). Transmission electron microscopy of collagen fibrils from K3-III.6 skin had cross-sections that were less rounded than control while longitudinal fibrils had a normal D-periodicity (Fig. 5B).

## DISCUSSION

In this report, we have identified the causative mutation of Kuskokwim syndrome, a congenital contracture disorder occurring among Yup'ik Eskimos, as a 3-nucleotide deletion in *FKBP10*, resulting in loss of the highly conserved p.Tyr293 residue of FKBP65. Linkage mapping localized KS to chromosome 17q12–21 in the 1990's and several potential candidate genes, including *COL1A1*, were ruled out at that time. Subsequently, null and missense mutations in *FKBP10* were shown to be the cause of moderate to severe recessive OI, as well as Bruck syndrome 1, which is severe OI with congenital contractures (Alanay, et al., 2010; Cabral, et al., 2005; Kelley, et al., 2011; Puig-Hervas, et al., ; Setijowati, et al., 2012; Shaheen, et al., 2011; Shaheen, et al., 2010; Steinlein, et al., 2011; Venturi, et al., 2012). Because the same *FKBP10* mutation may cause OI alone or OI with contractures in unrelated individuals or even in siblings, contractures are now understood to be a variable manifestation of *FKBP10* mutations. The combination of variable contractures in the *FKBP10* phenotype and the location of *FKBP10* at 17q21.2 made *FKBP10* a prime KS candidate gene. All tested probands with KS are homozygous for the p.Tyr293 deletion, and ~3% of ethnically matched normal controls are heterozygous carriers. This discovery will provide carrier and prenatal testing to the Kuskokwim community, via a simple and inexpensive blood test. Should the Yup'ik community so choose, they now have the potential to allow the elimination of this syndrome in a generation or two. In addition, this mutation extends the phenotypes associated with recessive *FKBP10* mutations in a manner that complements the existing phenotypes, so that an individual homozygous for a null or missense mutation will have either OI or Bruck syndrome, while an individual homozygous for deletion of the p.Tyr293 residue will have a congenital contracture condition with milder skeletal findings (Fig. 2D).

As in our previously published report of a null *FKBP10* mutation (Barnes, et al., 2012), and in a subsequent report which included examination of a bone sample from a Bruck syndrome patient (Schwarze, et al., 2013), hydroxylation of the type I collagen telopeptide lysine is impaired by the KS mutation. While null mutations totally eliminate *FKBP10* transcripts and FKBP65, leading to essentially undetectable hydroxylation of p.Lys1208, the Kuskokwim mutation destabilizes FKBP65 but leaves a residual 5% of control level of the PPIase in fibroblasts, leading to 2–10% hydroxylation of the telopeptide lysine. Affected

individuals with KS have variable and minor skeletal manifestations, including normal to osteopenic bone density, scoliosis and lordosis, short stature and low energy fractures. However, these findings are likely due in part to abnormal and restricted mobility from their contractures and did not attain a severity that previously merited an OI designation. Apparently, residual FKBP65 and COL1A1 p.Lys1208 hydroxylation do not significantly alter the ability of collagen molecules to self-assemble into fibrils and is sufficient to surpass a threshold crucial for osteoblasts to deposit and cross-link collagen well enough to support bone structure. In fact, KS fibroblasts deposit an intermediate level of collagen into matrix *in vitro*, between normal and *FKBP10*-null cells. Furthermore, this correlation is supported by the clinical findings of the KS probands examined extensively at the NIH, in which probands K3-III.6 and K5-II.8, who have 2–3% residual telopeptide hydroxylation, have more significant skeletal symptoms than do probands K2-II.2 and K3-III.5, with 8–10% hydroxylation, notwithstanding the sibling relationship between the K3 probands.

Interestingly, the collagen-related function that is ablated by *FKBP10* deficiency is not a function performed directly by FKBP65. Lysyl hydroxylase 2 (LH2), encoded by *PLOD2* (NM\_182943.2), hydroxylates the collagen telopeptide lysine. It is possible that the speculation applied to tropoelastin (Davis, et al., 1998) concerning steric hindrance applies to the type I collagen telopeptide, that is, that prolines which have not been isomerized and are located close to a lysine will block access of LH2. In the primary sequence of collagen the nearest prolines are amino to the telopeptide lysine and are located in the triple helical region. Steric hindrance of LH2 would then need to involve transient altered folding of the helical region, which is not supported by evidence. The more likely alternative is that FKBP65 is crucial in some way for the stability or activity of LH2, as previously proposed (Barnes, et al., 2012; Schwarze, et al., 2013). This might involve complex formation, or simply LH2 as a substrate ligand of FKBP65.

Decreased collagen cross-links in bone likely underlie the bone fragility component of the *FKBP10* phenotype (Barnes, et al., 2012; Schwarze, et al., 2013). Cross-links of collagen in bone are decreased in post-menopausal osteoporosis (Bailey, et al., 1993; Oxlund, et al., 1996). Treatment of animal models with ̢-aminopropionitrile (BAPN) to totally inhibit cross-link formation results in weakened bone (Oxlund, et al., 1995; Oxlund, et al., 1996). Additionally, the phenotype is related to that found in Bruck syndrome 2, a recessive condition caused by absence of the enzyme LH2 (Ha-Vinh, et al., 2004).

The variable occurrence of contractures with null and missense *FKBP10* mutations and their highly prevalent occurrence in KS are more difficult to explain. Even in KS, some homozygous individuals lack contractures into adulthood. The descriptive clinical term for the occurrence of multiple congenital joint contractures that limit ambulation is arthrogryposis; Kuskokwim syndrome was originally called “arthrogryposis of the Eskimo”. Several dozen arthrogryposis entities have been classified with a total population prevalence estimated between 1/3300–5100. The contractures are found in the upper and lower extremities and are often symmetric. A common mechanism for the contractures is absence or decrease in the movement normally required *in utero* for correct formation of tissue around the joints. Large joints develop in a flexion position, so if *in utero* movement is decreased by neurological (central or non-central), muscular or vascular conditions, then extra tissue will form around the joint (the Swinyard phenomenon) and fix the flexion position. Presuming that the immediate cause of contractures in *FKBP10* mutations is decreased *in utero* movement, then the originating cause could be an abnormality in the strength or organization of the connective tissue. In mice, *FKBP10* was shown to be developmentally regulated, as 12-day-old mice had higher expression in aorta, brain, kidney and lung when compared with near absent expression in adult mice (Patterson, et al., 2000). Additionally, *FKBP10* is upregulated at sites of active matrix production (Patterson, et al.,

2005) and therefore its mutation may affect the quality of extracellular matrix that is produced, both during development and throughout life.

Since congenital contractures are not seen in other types of OI, we are led to hypothesize that lack of collagen telopeptide hydroxylation must have a special consequence in tendon collagen. In the absence of tendon tissue for examination, we reflect on the distinctive pathway for collagen secretion into tendon matrix, involving linear secretion and deposition through fibripositors (Canty, et al., 2004). Disturbance of this very directional process by lack of telopeptide hydroxylation could account for this special feature of *FKBP10* mutations. It is also possible that the level of modifying enzymes such as LH2 might be different in tendon than in dermis or bone, adding to the susceptibility of this tissue. Finally, one wonders whether the phenotypes of *FKBP10* mutations might not be further extended by targeted examination of this gene in other congenital contracture conditions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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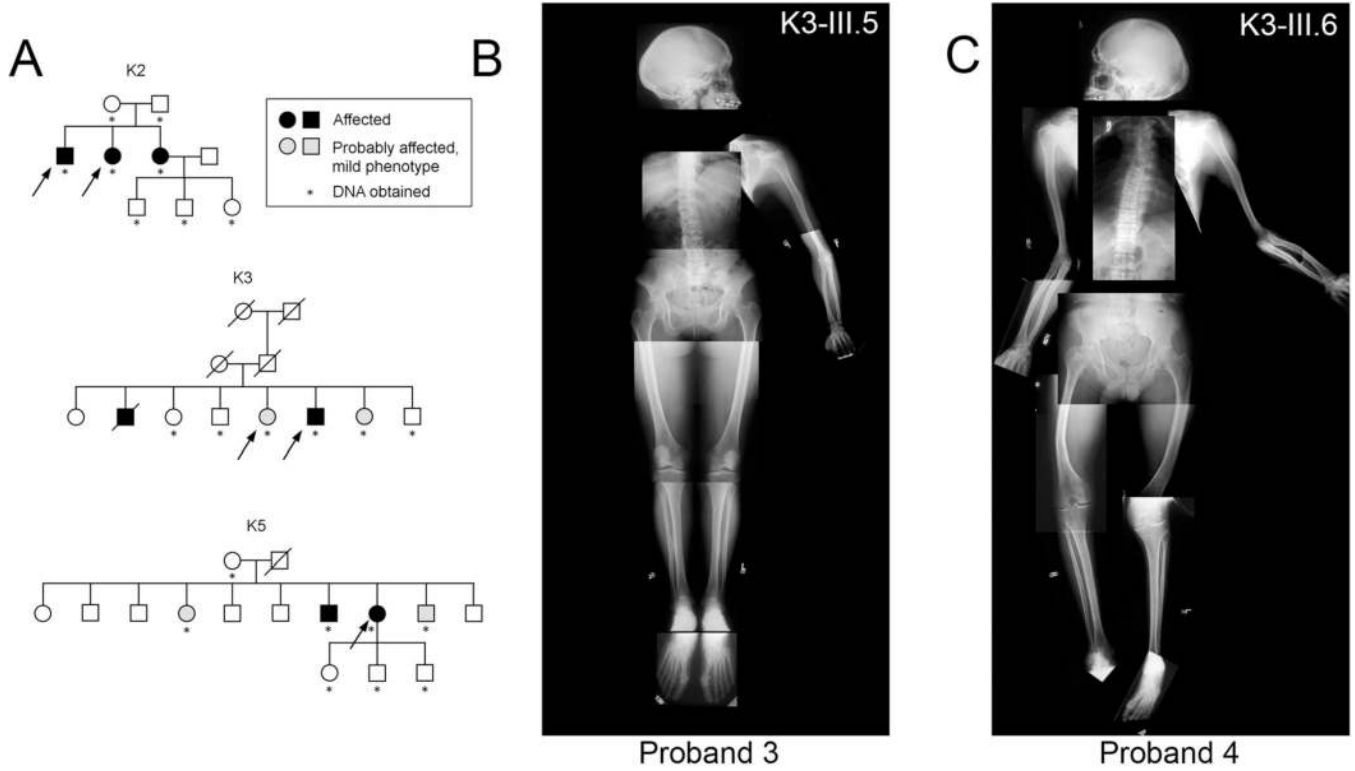
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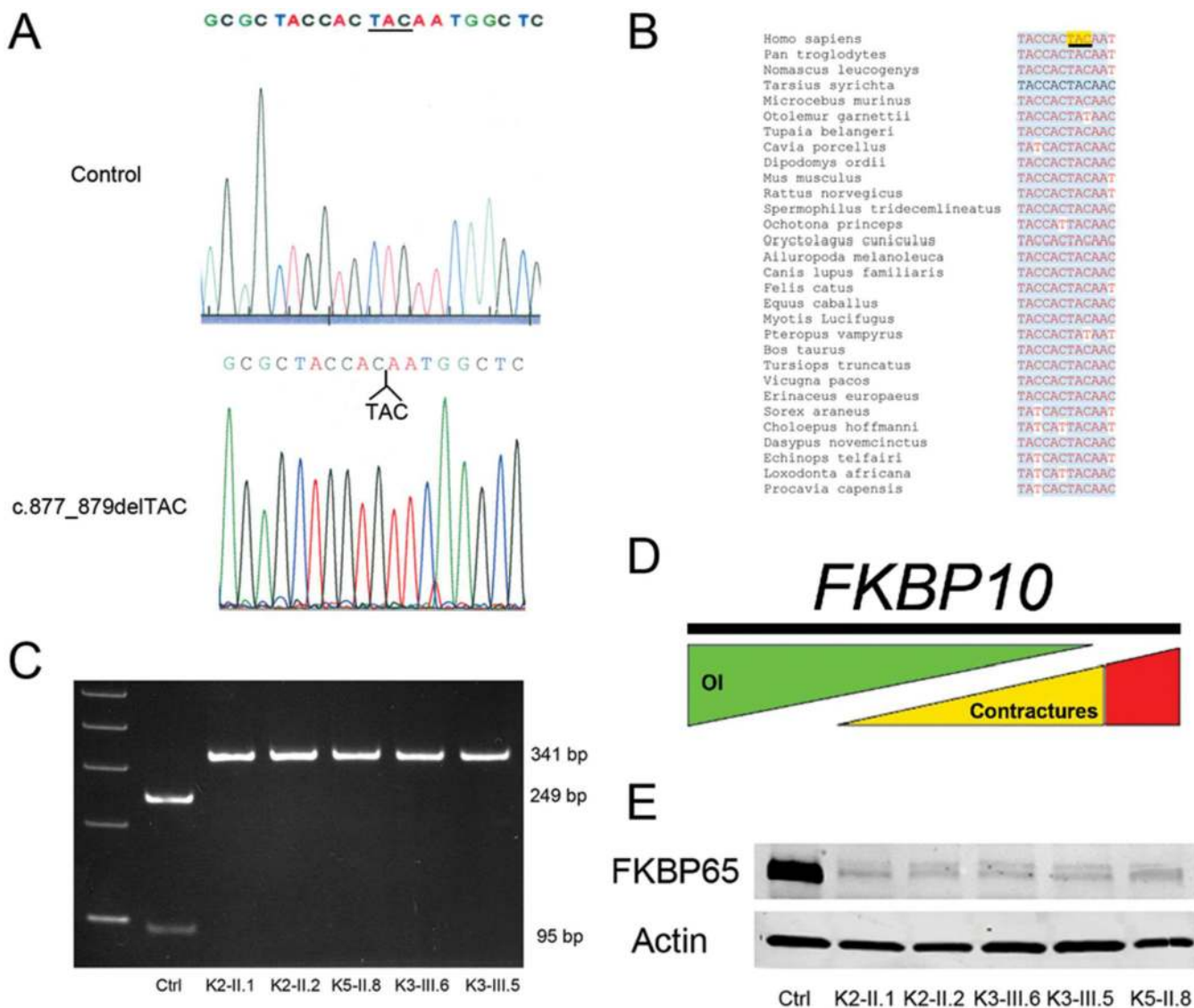
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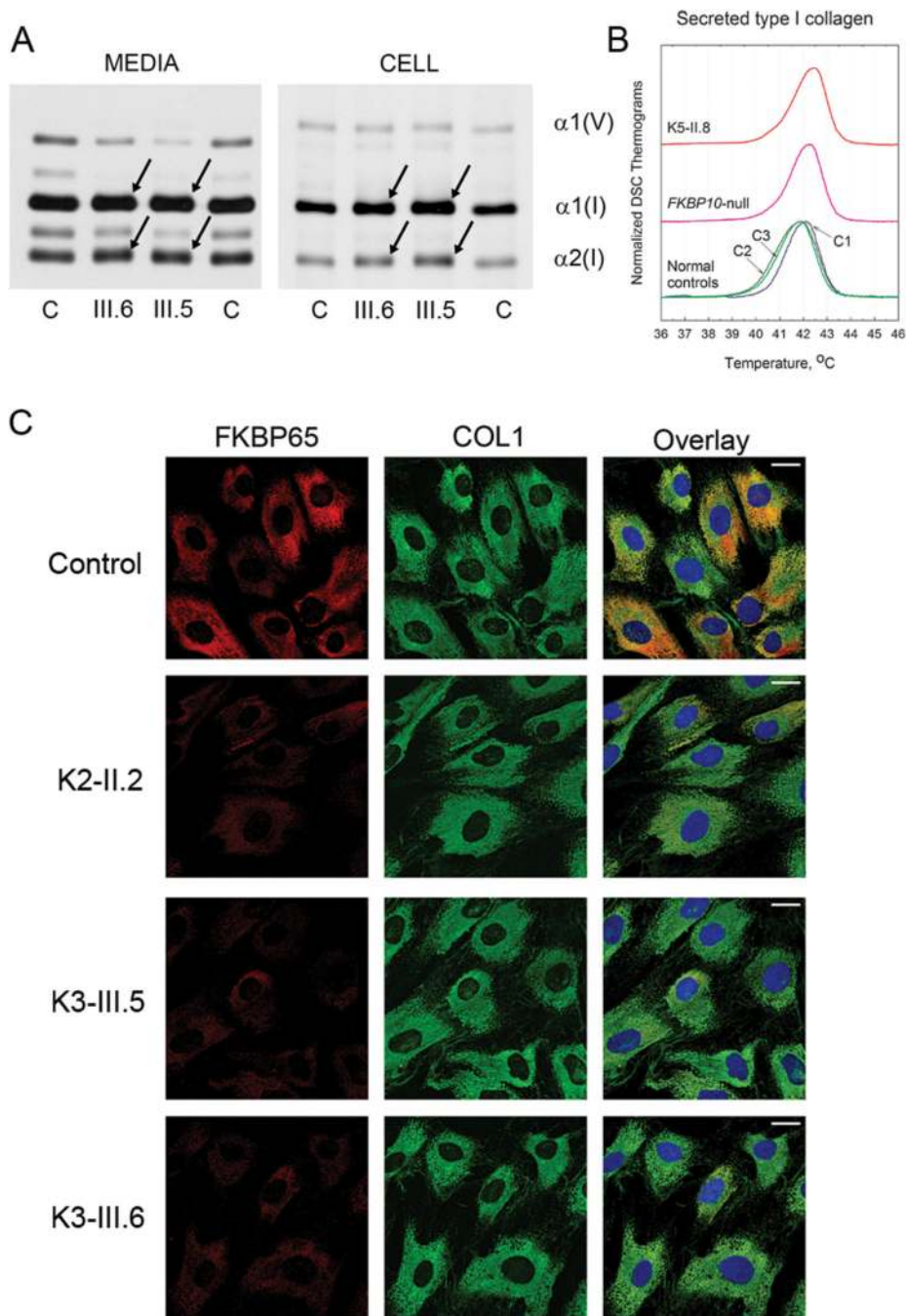
**Figure 1. Pedigrees and Radiographs of KS Probands**

A) Pedigrees of three Alaskan families with KS (arrows denote probands). Black symbols indicate individuals with KS; gray symbols represent probable KS/mild phenotype. B, C) Radiographs of sibling probands 3 and 4 showing phenotype variability. B) Proband 3 (K3-III.5) at 46 years shows clubbing of the clavicles, mild femoral bowing and shortening, and no patellar displacement. She has minor contractures of the elbows. C) Proband 4 (K3-III.6) at 41 years demonstrates mild thoracic scoliosis, mild R acetabular protrusion, lateral bowing of femora, proximal migration of patellae, and a L triple arthrodesis. He has knee, hip and elbow contractures.



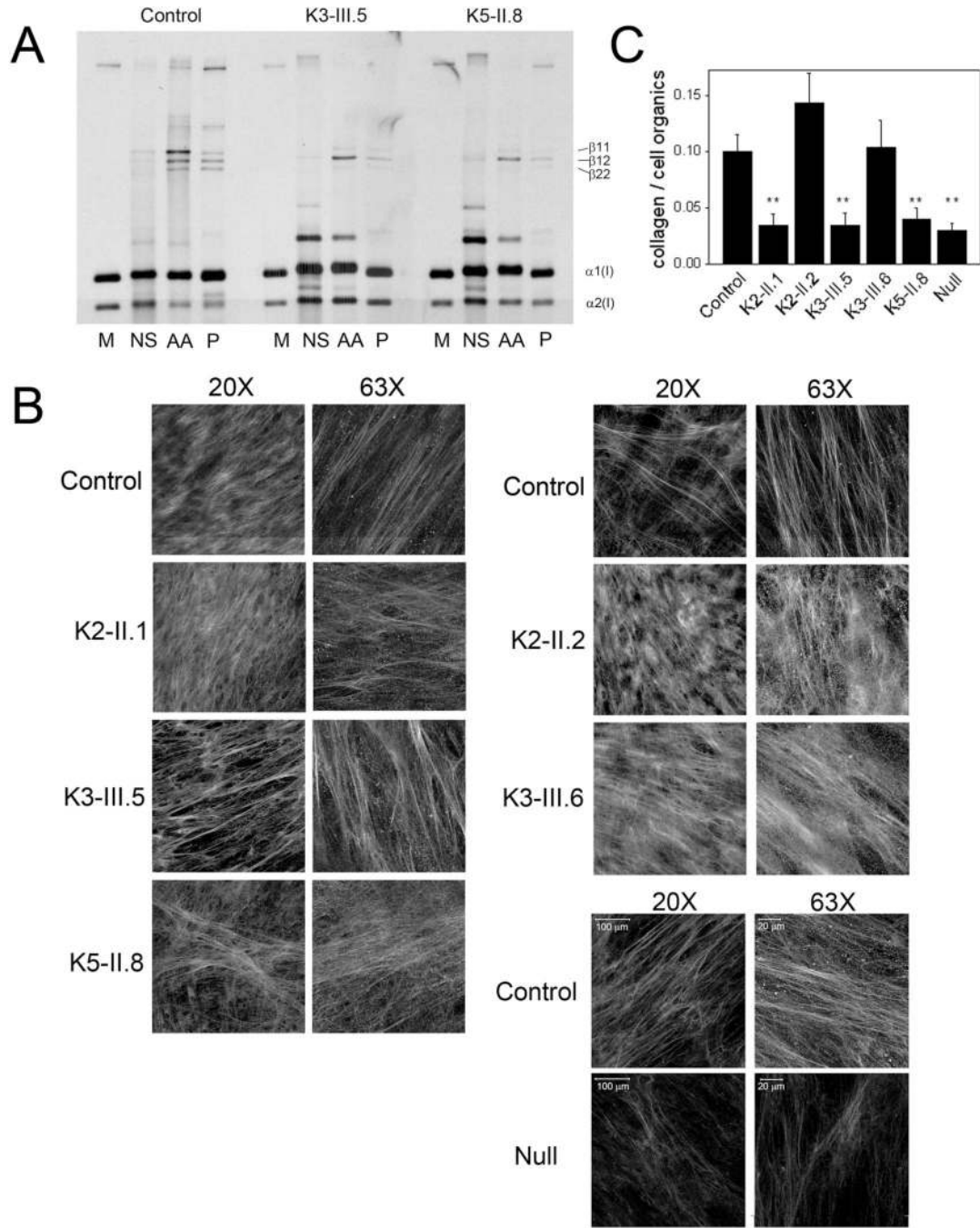
**Figure 2. Kuskokwim mutation in *FKBP10***

A) Sequencing tracing of *FKBP10* c.877\_879delTAC in KS proband K3-III.6 compared to normal sequence. B) Conservation of p.Tyr293 across various species. The 3 nucleotide deletion (underline) results in the deletion of p.Tyr293 (highlighted). C) Confirmation of homozygous *FKBP10* c.877\_879delTAC in all probands by elimination of the *MspI* restriction site. D) Schematic diagram of the phenotype of *FKBP10* mutations. Recessive null or missense *FKBP10* mutations lead to OI with or without contractures while the KS in-frame *FKBP10* deletion leads to a contracture phenotype. E) Western blot of proband RIPA cell lysates showing residual FKBP65 protein in all probands (for quantitation, see Table 1). □Actin is used as a loading control.



**Figure 3. Kuskokwim mutation has minimal effect on type I collagen biochemistry**

A) Steady-state collagen gel electrophoresis of siblings K3-III.5 and K3-III.6. Collagen has normal migration with a very slight broadening (arrows), consistent with the increase in helical hydroxylysines. B) Differential scanning calorimetry of proband K5-II.8, *FKBP10*-null and control secreted collagen from normal newborn (C1), 5 year-old (C2) and 9 year-old (C3) fibroblasts, showing a 0.5°C increase in thermal stability in KS collagen. C) Immunofluorescence microscopy of cultured proband fibroblasts shows residual FKBP65 staining and lack of intracellular collagen aggregation. Scale bar = 20  $\mu$ m.

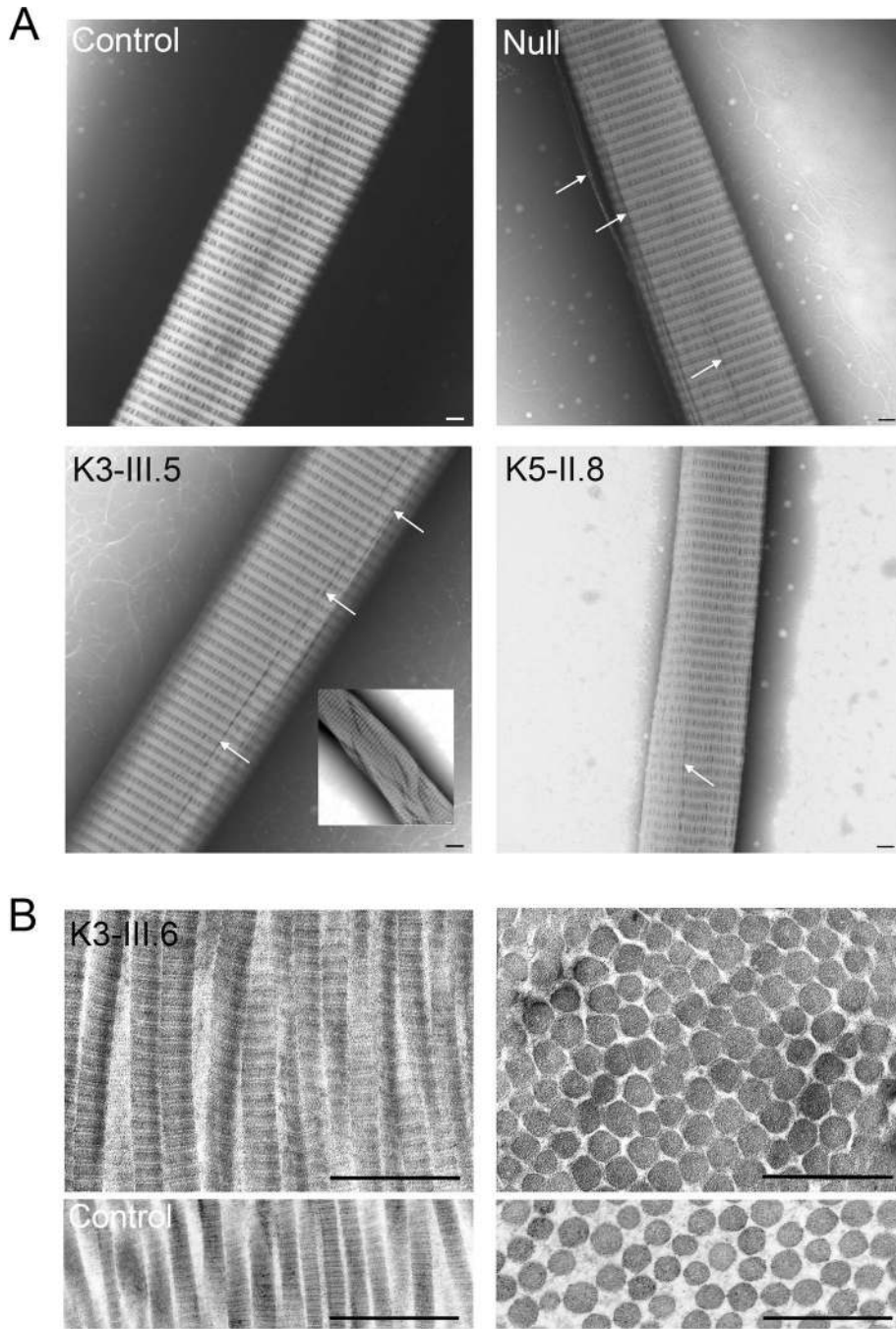


**Figure 4. Diminished collagen in extracellular matrix with fewer cross-links**

A) Matrix deposited by cultured fibroblasts was extracted in three fractions Neutral Salt (NS, newly incorporated collagen without cross-links), Acetic Acid (AA, immaturely cross-linked collagen) and Pepsin-digested (P, mature cross-linked collagen) and compared to collagen secreted in the media (M). KS probands K3-III.5 and K5-II.8 show a decrease in beta cross-linking forms  $\beta 1,1$  and  $\beta 2,2$  as well as a marked reduction in mature collagen matrix, when normalized for loading to control. B) Immunofluorescence microscopy of extracellular matrix deposited by control, KS or *FKBP10*-null fibroblasts. Matrix stained for type I collagen shows a modest decrease in total collagen matrix from KS probands, intermediate between control and null matrix. Fibrils appear disorganized and less tightly

bundled than control. C) Matrix collagen to cell organics ratio in proband cultures measured by Raman micro-spectroscopy at locations where matrix and cell cytoplasm overlap. The plotted ratio is the arithmetic average of such ratios measured in the spectral regions of amide III, CH-stretching or 853  $\text{cm}^{-1}$  proline bands (Supp. Figure S4). Consistent differences between collagen contents of these cultures were found using each ratio. The error bars represent the mean square root of the mean standard errors of the ratios. \*\*  $P < 0.005$ .





**Figure 5. *In vitro* and dermal collagen fibrils from KS probands**

A) Fibrils formed *in vitro* from secreted collagen have a normal D-periodicity, but appear less tightly bundled in both null and KS probands. Areas of fibril splaying and splitting are denoted by arrows. Inset shows unraveling of the collagen fibril in proband K3-III.5. Scale bar = 100 nm. B) Transmission electron microscopy of dermal collagen fibrils of proband K3-III.6 (top panels) and an age-matched control (bottom panels). Proband fibrils have a normal D-periodicity and a fairly normal longitudinal (left panel) appearance. Cross-sectional fibrils (right panel) appear less rounded than control. Scale bar = 500 nm.

Table 1

Effect of the *FKBP10* mutation on collagen

	K2-IL1	K2-IL2	K3-IL5	K3-IL6	K5-IL8	Null	Control
mRNA expression <i>FKBP10</i>	1.14 ± 0.12	1.67 ± 0.18	0.79 ± 0.09	1.07 ± 0.20	1.59 ± 0.42	0.06 ± 0.01	1
Protein expression (%) FKBP65	5.58 ± 2.38	3.46 ± 1.14	2.68 ± 0.37	3.13 ± 1.46	6.49 ± 2.88	<0.01	100
Hydroxylation (%) Hyl/total Lys (% difference from control)			+ 35	+ 37	+ 22	+ 12.75	---
Hyp/total Pro (% difference from control)			- 5	- 7	- 1	+ 7	---
Telopeptide lysyl hydroxylation (%)	5	8	10	3	2	<1	58-59
Collagen Secretion pg collagen/ (hr cell)	1.35 ± 0.33	1.51 ± 0.32	1.48 ± 0.09	1.54 ± 0.11	1.59 ± 0.24	0.87	1.13 ± 0.36

Values are means ± SD, n = 2-3. The relative levels of mRNA expression were normalized to *GAPDH* expression, then compared to the level of expression in control fibroblasts (arbitrarily set to 1). The relative levels of protein were normalized to actin, then compared to normal protein levels from a control cell line (arbitrarily set to 100).