CLINICAL REPORT

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Severe Osteogenesis Imperfecta Caused by a Small In-Frame Deletion in *CRTAP*

I.M. Ben Amor,[1] F. Rauch,[1]* K. Gruenwald,[2] M. Weis,[3] D.R. Eyre,[3] P. Roughley,[1] F.H. Glorieux,[1] and R. Morello[2,4]

[1]Genetics Unit, Shriners Hospital for Children and McGill University, Montreal, Canada

[2]Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas

[3]Department of Orthopedics and Sports Medicine, University of Washington, Seattle, Washington

[4]Division of Genetics, University of Arkansas for Medical Sciences, Little Rock, Arkansas

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Mutations of proteins involved in posttranslational modification of collagen type I can cause osteogenesis imperfecta (OI) inherited in a recessive pattern. The cartilage-associated protein (CRTAP) is part of a heterotrimeric complex (together with prolyl-3-hydroxylase-1 [P3H1] and cyclophilin B) that 3-hydroxylates the alpha 1 chain of collagen type I at proline residue 986 and plays a collagen chaperon role. *CRTAP* mutations usually cause severe OI. We report on a patient with OI and a homozygous in-frame deletion in *CRTAP* and a severe form of OI. The girl was born with markedly deformed long bones. Despite intravenous bisphosphonate treatment, she developed multiple vertebral compression fractures and severe scoliosis and at 4 years of age was able to sit only with support. Although *CRTAP* transcript levels were normal in the patient's fibroblasts, protein levels of both CRTAP and P3H1 were severely reduced. The degree of 3-hydroxylation at proline residue 986 was also decreased. This report characterizes a patient with a *CRTAP* small in-frame deletion. We are unaware of prior reports of this finding. We suggest that this deletion affects crucial amino acids that are important for the interaction and/or stabilization of CRTAP and P3H1. © 2011 Wiley Periodicals, Inc.

Key words: recessive osteogenesis imperfecta; *CRTAP*; 3-hydroxylation complex; *P3H1*

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INTRODUCTION

Osteogenesis imperfecta (OI) is the most common heritable bone fragility disorder and in the majority of cases is inherited in an autosomal dominant pattern and caused by mutations in *COL1A1* or *COL1A2*, the two genes that code for collagen type I alpha chains [Dalgleish, 1998]. These chains undergo extensive posttranslational modification in the endoplasmic reticulum (ER). Defects in a number of proteins that participate in posttranslational modification, chaperoning, and intracellular trafficking of type I procollagen, cause OI that is inherited in an autosomal recessive pattern [Morello et al., 2006; Cabral et al., 2007; van Dijk et al., 2009a; van Dijk et al., 2009b; Alanay et al., 2010; Christiansen et al., 2010].

Cartilage-associated protein (CRTAP) is an ER protein that, when missing or severely reduced, causes recessive OI. Together with prolyl-3-hydroxylase-1 (P3H1, encoded by the *LEPRE1* gene) and the prolyl cis-trans isomerase cyclophilin-B (encoded by the *PPIB* gene), CRTAP forms a trimeric complex that not only has collagen chaperon function but also 3-hydroxylates a single proline residue at position 986 of the alpha 1 chain of collagen type I [Morello et al., 2006; Morello and Rauch, 2010]. The CRTAP protein does not have enzymatic activity itself, but appears to stabilize P3H1, the enzymatically active protein responsible for the proline modification. This is suggested by the observation that P3H1 protein is absent or reduced in fibroblasts from patients with *CRTAP* null mutations even though *LEPRE1* transcript levels are normal [van Dijk et al., 2009b; Baldridge et al., 2010; Chang et al., 2010].

The database of *CRTAP* mutations currently contains 19 distinct mutations (http://www.le.ac.uk/ge/collagen/). These mutations affect mostly exon/intron 1 or exon/intron 4 of the *CRTAP* gene, are thought to completely inactivate *CRTAP*, and result in very severe or lethal OI (type II or III according to the Sillence

*Correspondence to:
F. Rauch, Genetics Unit, Shriners Hospital for Children, 1529 Cedar Avenue, Montréal, Québec H3G 1A6, Canada.
E-mail: frauch@shriners.mcgill.ca
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classification) [Barnes et al., 2006; Baldridge et al., 2008; Bodian et al., 2009; van Dijk et al., 2009a; Chang et al., 2010]. The only known exception is a single base-pair substitution in intron 1 that introduces a cryptic splice site, causes abnormal splicing, leads to a 90% reduction in intracellular CRTAP protein, and causes a moderately severe phenotype [Morello et al., 2006; Baldridge et al., 2008]. This will be referred to in the present manuscript as a hypomorphic mutation since it is causing a significant reduction in the protein product. In-frame deletions in the *CRTAP* gene have not yet been identified.

Here, we describe a girl with severe OI due to a small in-frame deletion in exon 4 of *CRTAP*. The molecular consequences of this mutation are characterized.

CLINICAL REPORT

The girl was born after 38 weeks gestation by elective cesarean for a suspected prenatal diagnosis of severe OI. Shortened, bowed limbs, beaded ribs, and bilateral club feet were visible on third trimester ultrasound. Birth weight was 2,810 g (50th centile for gestational age), head circumference was 34 cm (~75th centile). She was the sixth child of consanguineous parents of Sudani origin. One older brother had been diagnosed with severe OI in infancy and had died at 20 months of age due to respiratory complications. The other four siblings were healthy (Fig. 1A).

At birth, the girl presented with bluish sclerae, more than 10 fractures of both upper and lower extremities at different stages of healing, posterior rib fractures, and multiple vertebral compression fractures. There was diffuse osteopenia with thin long-bone cortices. Numerous Wormian bones were seen on skull X-ray. A tentative diagnosis of OI type III was made, but testing for *COL1A1* and *COL1A2* mutations was negative.

When first evaluated at our institution at the age of 15 months, she had apparently normal cognitive functions, but delayed motor milestones with no rolling and a poor head control. Her bone mineral density at the lumbar spine (QDR Discovery, Hologic Inc., Waltham, MA) corresponded to an age-matched z-score of −7.1 [Salle et al., 1992]. Intravenous treatment with zoledronic acid was initiated at a dose of 0.05 mg per kg body weight and repeated every 6 months. Despite treatment, the girl continued to have fractures of long bones and significant motor delay.

At the most recent follow-up visit at the age of 4.2 years, she was able to sit only with support. Her long bones were deformed and shortened, but the shortening was not rhizomelic, as had been described in patients with hypomorphic *CRTAP* mutations [Ward et al., 2002; Morello et al., 2006]. There were multiple vertebral compression fractures and severe scoliosis (Fig. 1B). She had no dentinogenesis imperfecta. Height was 65.8 cm (z-score: −9.2, average for a 6 month old female), weight was 8.6 kg (z-score: −4.5, average for an 8-month-old female). After nine cycles of zoledronic acid, the lumbar spine areal bone mineral density z-score was −6.3.

Sequencing

Total genomic DNA was isolated from peripheral blood using the QIAamp DNA Blood Midi Kit (Qiagen Inc., Mississauga, ON, Canada). The coding sequences and the flanking sequences of the seven exons of the *CRTAP* gene were amplified by polymerase chain reaction using primers described before [Barnes et al., 2006]. The sequencing reaction was performed using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The nucleotide sequence was determined using an Applied Biosystems 3100 DNA sequencer.). Sequence traces were aligned with the GenBank reference sequences of the *CRTAP* genomic DNA (NG_008122.1), cDNA (NM_006371.4), and amino acid numbering (NP_006362.1).

Cell Culture

Human primary skin fibroblasts from controls and patients with OI were grown in DMEM with 4,500 mg/L glucose and 110 mg/L sodium pyruvate (HyClone) and supplemented with 10% FBS, L-Glutamine (2 mM final), 100 units/ml penicillin, and 100 µm/ml streptomycin. These cells were utilized for protein analysis by western and immunofluorescence, or to prepare RNA for real-time PCR analysis as described below. Ascorbic acid (100 µg/ml) was added to the cell culture medium and supernatants from confluent 100 mm dishes were harvested for the 3-hydroxyproline analysis of type I collagen.

Immunofluorescence Staining

Control and OI cells were plated onto glass LAB-TEK 2-well chamber slides and grown to 70–90% confluence. Then they were fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized in 0.1% Triton in PBS, and washed in PBS. Cells were incubated in a blocking solution made of 10% normal goat serum and 1% BSA in PBS at room temperature for 30 min and then sequentially incubated with 1:500 dilution of CRTAP antisera or P3H1 mouse MaxPab polyclonal antibody (Abnova Inc., Walnut, CA), 1:400 goat anti-rabbit or goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 or 594 (Invitrogen Inc., Carlsbad, CA), respectively. The chamber dividers were removed and the slides were mounted with Dapi Fluoromount-G (Southern Biotech Inc., Birmingham, AL). Images were captured using a Zeiss Axio Imager M1.

Western Blotting

Cells were lysed into RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 0.5% Sodium Deoxycholate, and 1% Triton x-100) containing a cocktail of protease inhibitors including EDTA (AMRESCO Inc., Solon, OH). Lysates were spun down and supernatants collected and quantified using the Bio-Rad protein assay dye reagent. About 4 µg of protein lysates were loaded in each well, run on SDS–PAGE and then blotted onto a nitrocellulose membrane using a Mini Protein Tetra System (Bio-Rad Inc., Hercules, CA). Membranes were blocked using 5% non-fat milk in TBS (50 mM Tris-HCl and 150 mM NaCl, pH 7.6) and then incubated with CRTAP antisera (1:500), P3H1 mouse MaxPab polyclonal antibody (1:500) or β-tubulin monoclonal antibody (1:1,000) (Clone TBN06 from Fisher Scientific, Pittsburgh, PA) in

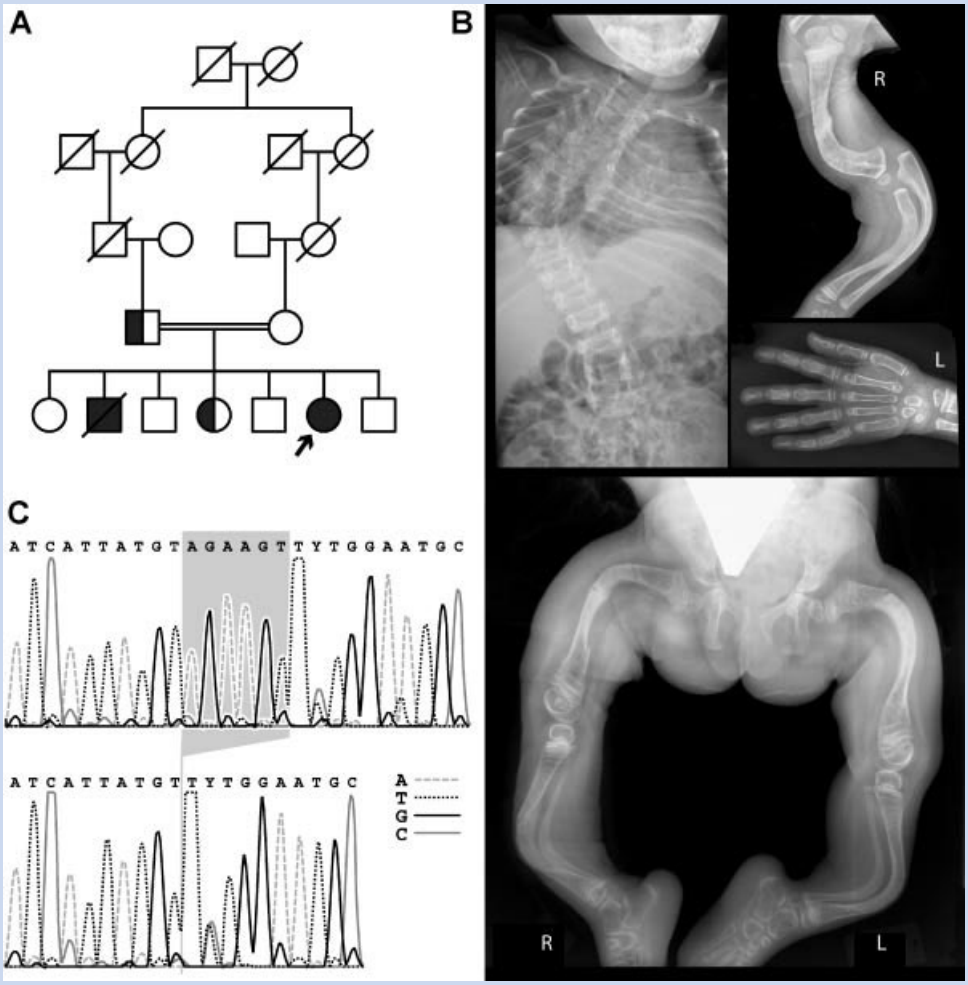

FIG. 1. A: Family pedigree. B: Severe thoracolumbar right convex scoliosis in proband at 3 years of age. Severe bowing deformities of the right humerus, ulna, and radius with healing fracture at the apex of the humerus at same age. The hand radiograph shows thin cortices with osteopenia. Severe anterior convex bowing of femora, tibiae, and fibulae with a fracture at the proximal shaft of the left femur, and an oblique fracture at the distal shaft of the right femur at the same age. C: Control sequence: Exon 4 in the *CRTAP* gene (upper panel). In-frame deletion in proband: c.804_809delAGAAGT in exon 4 in the *CRTAP* gene (lower panel).

5% milk in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.6). The membranes were washed with PBS and then incubated with goat anti-rabbit or goat anti-mouse IRDye 680LT secondary antibodies (LI-COR Biosciences, Lincoln, NE), which were diluted to 1:20,000 in 5% milk in TBST. Finally membranes were washed with PBS and then scanned using a LI-COR Odyssey instrument. Protein quantification was performed by densitometry using the LI-COR Odyssey instrument and after normalization to β-tubulin levels. For the secretion experiment, serum-free DMEM was added to confluent six-well plates and conditioned media and cells were collected after 24 h. A protease inhibitor cocktail was added to the media that were then concentrated about 10-fold and then washed with PBS using an Amicon Ultra (30 K) centrifugal filter unit (Millipore Inc., Billerica, MA). Cells were harvested, counted for the normalization of loading and lysed into RIPA buffer. Conditioned media from 80,000 cells and cell lysates from 40,000 cells were loaded on each lane of a western blot and bands were quantified with the LI-COR Odyssey instrument. The percentage of CRTAP secreted into the medium was calculated as described by Chang et al. [2010].

Real-Time PCR

The TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN) was used to extract RNA from cells. cDNA was obtained using the Transcriptor First Strand cDNA Synthesis kit from Roche according to the manufacturer recommendations. cDNA yields were quantified at the nanodrop and appropriate dilutions were used as templates in a PCR reaction using the Light Cycler FastStart Master^{PLUS} Sybr Green I Kit; PCR reactions were run in glass capillaries on a LightCycler 1.5 Instrument as described before [Morello et al., 2006]. Primers used were: *CRTAP* For: 5′- ATCCGGTTGAGAAATTTGTGGCTACCAT; *CRTAP* Rev: 5′- GAGAGGCCCCAAGTGTCCCTGTG; *P3H1*

For: 5′-ACTTCCTCCCATCGCATTAT; *P3H1* Rev: 5′-CAGTTCTTTTTCCAGTAGGCTT.

Mass Spectrometry

For quantification of 3-Hyp in collagen, type I collagen was prepared from primary skin fibroblast culture media. Collagen α-chains were cut from SDS–PAGE gels and subjected to in-gel trypsin digestion. Electrospray MS was performed on the tryptic peptides using an LCQ Deca XP ion-trap mass spectrometer equipped with in-line liquid chromatography (Thermo Finnigan, West Palm Beach, FL) using a C8 capillary column (300 μm × 150 mm; Grace Vydac 208MS5.315) eluted at 4.5 μl per min. Sequest search software (Thermo Finnigan) was used for peptide identification using the NCBI protein database.

RESULTS

Sequence analysis of the exons and flanking intronic sequences of the *CRTAP* gene showed a homozygous six base pair in-frame deletion c.804_809delAGAAGT in exon 4, compared to control sequence (Fig. 1C). This deletion eliminates two amino acids from the CRTAP protein sequence, i.e., p.Glu269_Val270del. Both parents of the proband were heterozygous for this mutation.

Real-time PCR on cDNA from the patient's skin fibroblasts showed slightly decreased levels of CRTAP transcript although these were within the normal range as compared to three unrelated healthy controls (Fig. 2A). This was in contrast to the marked decrease in CRTAP mRNA observed in fibroblasts from a patient with the hypomorphic mutation in intron 1 [Morello et al., 2006]. Fibroblasts from patients with either the in-frame deletion or the hypomorphic mutation expressed similar levels of *P3H1* transcript compared to healthy controls (Fig. 2A). To evaluate CRTAP and P3H1 protein expression, western blot analyses were conducted on lysates of patient fibroblasts. Fibroblasts carrying the in-frame deletion had about 65% reduction of CRTAP protein compared to controls, whereas fibroblasts with the hypomorphic mutation had over 90% reduction (Fig. 2B). In contrast, P3H1 protein was similarly reduced in the two fibroblast lysates (21% vs. 13% of control levels, respectively) (Fig. 2B). Immunofluorescence staining confirmed that CRTAP protein expression in fibroblasts from the patient with the in-frame deletion was about intermediate between that seen in controls and that of patients with the hypomorphic *CRTAP* mutation in intron 1 (Fig. 2C). Immunofluorescence with the P3H1 antibody showed that the proposita had severely reduced levels of P3H1 in the ER of skin fibroblasts, similar to those of the patient with the hypomorphic mutation (Fig. 2C).

Others have shown that in P3H1-deficient fibroblasts CRTAP is mostly degraded but also increasingly secreted into the extracellular matrix, compared to controls [Chang et al., 2010]. Therefore, the amount of secreted and intracellular CRTAP protein in a 24 hr period was analyzed by western blot. Fibroblasts harboring the in-frame *CRTAP* deletion showed a slightly increased percentage of CRTAP in the medium relative to intracellular CRTAP compared to control fibroblasts, 63% versus 50%, respectively (Fig. 2D).

Finally, to analyze the impact of the CRTAP in-frame mutation on the collagen type I 3-hydroxyproline level, mass spectrometry on type I collagen isolated from fibroblast culture medium was performed [Morello et al., 2006]. The spectra of the tryptic peptide containing proline residue 986 demonstrated that 48% of proline residues at that position were 3-hydroxylated in fibroblasts with the in-frame deletion. In contrast, the fraction of 3-hydroxylated proline 986 residues was close to 100% in the identical peptide derived from control fibroblasts (Fig. 2E).

DISCUSSION

Here we describe a patient with OI and an in-frame deletion in the *CRTAP* gene. This patient has a more severe phenotype than the previously described patients with OI type VII, who have a hypomorphic *CRTAP* mutation [Ward et al., 2002; Morello et al., 2006]. We are unaware of prior reports of patients with OI and an in-frame deletion. In fact, the patient reported here presented at birth with severe shortened and deformed long bones, multiple vertebral compression fractures, and rib fractures with a significant low bone mineral density. The response to intravenous bisphosphonate treatment was poor, as the girl continued to have fractures of her long bones and vertebrae, which were associated with progressive scoliosis and significant motor delay. Overall, the clinical presentation was similar to patients presenting with severe OI type III. Consistent with previously reported patients with *CRTAP* mutations, dentinogenesis imperfecta was absent [Barnes et al., 2006; Morello et al., 2006; Baldridge et al., 2008; van Dijk et al., 2009a].

Our mechanistic studies yielded somewhat conflicting results on the mRNA and on the protein level. As expected for an in-frame deletion, fibroblasts from the present patient had normal *CRTAP* transcript levels. In contrast, CRTAP protein levels were significantly reduced compared to normal controls, but were higher than in patients carrying the hypomorphic mutation (35% vs. 7% of control levels) [Ward et al., 2002; Morello et al., 2006]. Nevertheless, the clinical features of the present patient were more severe than those of patients with hypomorphic mutations [Ward et al., 2002]. This suggests that the deletion of Glu269 and Val270 renders the CRTAP protein dysfunctional.

Further investigations into this hypothesis showed that the in-frame deletion was associated with a severe reduction in the amount of P3H1 in the ER. This indicates that the mutated CRTAP does not stabilize P3H1 and points to a critical functional role of Glu269 and Val270 for the interaction between CRTAP and P3H1 [Chang et al., 2010]. This is supported by earlier evidence that amino acids encoded by *CRTAP* exon 4 are critical for the co-stabilization between CRTAP and P3H1 [Chang et al., 2010].

We conclude that the phenotype in this patient is one of a severe OI similar to type III but with no DI as is typical in OI type VII. The association with an in-frame deletion has not been previously reported to our knowledge. The severe shortening of long bones did not follow a rhizomelic pattern but was proportionate. The two deleted amino acids (Glu269 and Val270) reduced the amount of the CRTAP protein and altered its function in particular its interaction with P3H1 that was found to be drastically reduced. These two amino acids seem to play a critical role in the interaction and co-stabilization between the CRTAP and the P3H1 proteins and therefore affecting the 3-hydroxylation of the alpha 1 chain of collagen type I at proline residue 986.

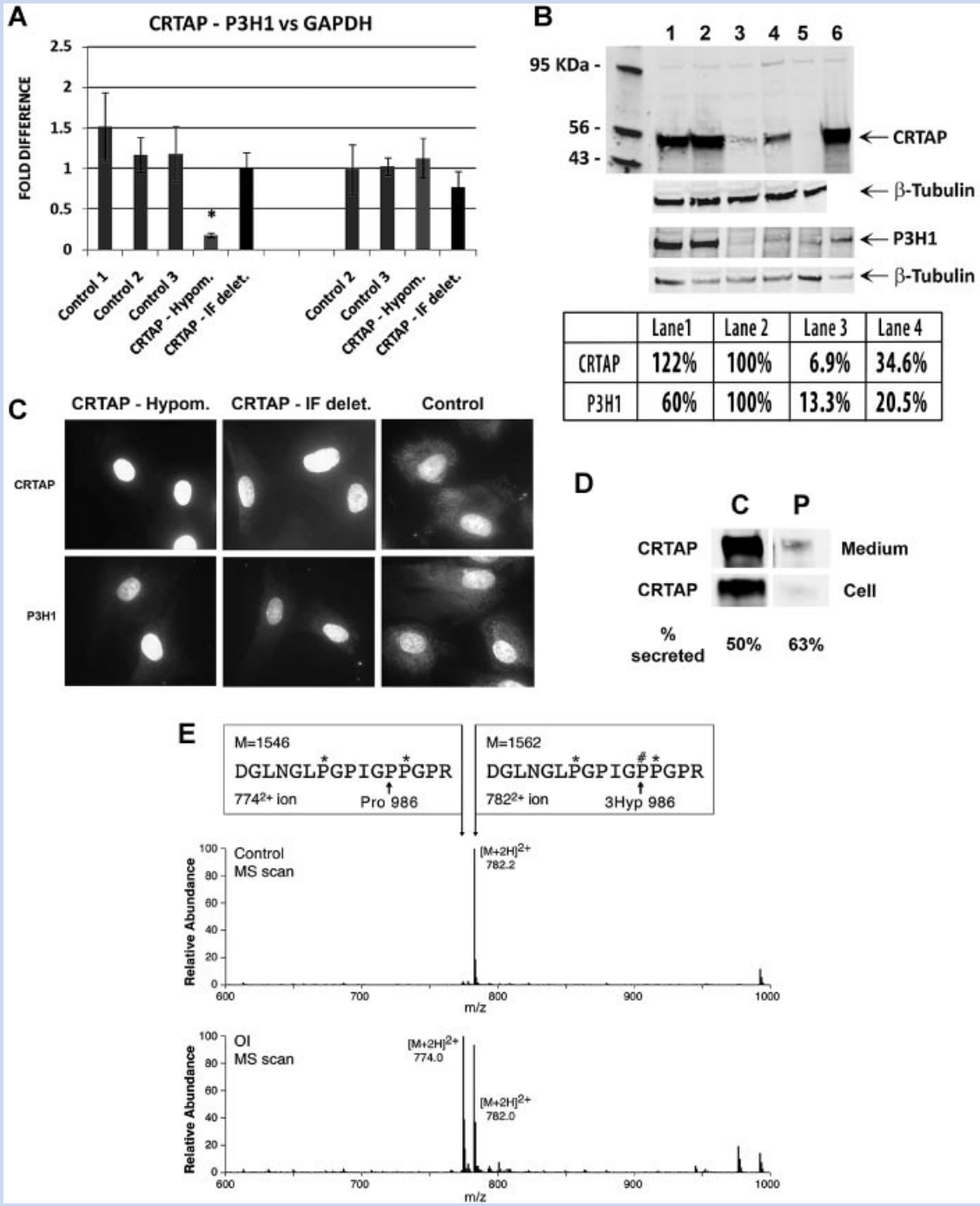

FIG. 2. A: Real time PCR quantification of *CRTAP* and *P3H1* transcripts normalized against GAPDH. B: Western blot with CRTAP antibody: Lane 1: Human control fibroblasts; lane 2: Human control fibroblasts; lane 3: *CRTAP* hypomorphic mutation; lane 4: New in-frame deletion; lane 5: *Crtap*$^{-/-}$ murine osteoblasts used as negative control; lane 6: Wild type murine osteoblasts used as positive control. C: CRTAP and P3H1 immunofluorescence on skin fibroblasts from a control, the patient with the in-frame *CRTAP* deletion presented here and a patient with a hypomorphic *CRTAP* mutation. D: Representative western blot of CRTAP in 24 hr conditioned medium and cell layer showing increased CRTAP secretion in the studied patient compared to a control. E: Mass spectrometry of a tryptic peptide of the collagen type I alpha 1 chain containing Pro986 and showing a significant amount of non 3-hydroxylated proline in the patient fibroblasts compared to a control.

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