# Abnormal type III collagen produced by an exon-17-skipping mutation of the *COL3A1* gene in Ehlers–Danlos syndrome type IV is not incorporated into the extracellular matrix

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A novel heterozygous mutation of the *COL3A1* gene that encodes the α1(III) chains of type III collagen was identified in a family with the acrogeric form of Ehlers–Danlos syndrome type IV (EDS-IV). Cultured dermal fibroblasts produced normal and shortened α1(III) chains. The triple helix of the latter chain was shortened owing to a 33 amino acid deletion of Gly-184 to Pro-216. The corresponding region of cDNA lacked 99 base pairs from nucleotides 1051 to 1149. The deletions corresponded exactly to the normal sequence encoded by exon 17 of the *COL3A1* gene. The proband was heterozygous for a T to G transversion at position +2 of intron 17, which resulted in skipping of exon 17. The splicing defect was not corrected by growing the fibroblasts at 33 °C and no other splicing variants were identified at 33 or 37 °C. The affected brother had the same mutation but his unaffected mother did not. Heterotrimeric type III collagen molecules containing normal and mutant chains were retained within the cell. The mutant homotrimeric molecules were modified and secreted normally and were thermally stable. These normal characteristics of the mutant homotrimers suggested that the loss of ten Gly-Xaa-Yaa triplets (where Gly-Xaa-Yaa is a repetitive amino acid triplet structure in which Xaa and Yaa are other amino acids, proline and hydroxyproline being more common in the Yaa position) did not adversely affect the formation and stability of the triple helix or the structural requirements for secretion. However, the mutant homotrimers were not incorporated into the extracellular matrix of an *in vitro* model of EDS-IV dermis. The EDS-IV phenotype in this family was probably due to a deficiency in the amount of normal type III collagen available for formation of the heterotypic collagen fibrils of the extracellular matrix. Intracellular and extracellular quality-control mechanisms prevented the incorporation of heterotrimeric and homotrimeric mutant type III collagen molecules into the cross-linked extracellular matrix.

## INTRODUCTION

The type IV form of Ehlers–Danlos syndrome (EDS-IV) is a heritable connective-tissue disease characterized by fragility of the skin, blood vessels and viscera [1]. Dominant-negative mutations of the *COL3A1* gene, which encodes the pro-α1(III) chains of type III procollagen, have been identified in EDS-IV. The reported mutations alter the primary structure of the helical domain of type III collagen. They include large and small deletions, point mutations that produce abnormal pre-mRNA splicing and point mutations that result in the substitution of glycine residues in Gly-Xaa-Yaa triplets, which are the essential repetitive elements required for normal formation of the triple helix [1].

In this paper we describe a family with the acrogeric form of EDS-IV that is due to a novel skipping mutation of the *COL3A1* gene. The proband had a heterozygous T to G transversion at position +2 of intron 17, which resulted in complete skipping of exon 17 and the deletion of 33 amino acids, Gly-184 to Pro-216, from the triple-helical domain of mutant α1(III) chains. The splicing defect was not corrected by growing the fibroblasts at a low temperature, nor was there evidence at normal or low temperatures of other splicing products. The mutant homotrimeric molecules were secreted normally and were thermally stable and modified normally. However, they were not incorporated into the extracellular matrix.

## EXPERIMENTAL

### The patient

The proband and his affected brother and father had the typical features of the acrogeric form of Ehlers–Danlos syndrome [1]. The father died suddenly at the age of 31 years from a spontaneous rupture of the abdominal aorta.

### Collagen production by short-term fibroblast cultures

EDS-IV and control fibroblast cultures were grown to confluency and sodium ascorbate was then added daily to the culture medium at a final concentration of 0.25 mM for 3 days. Procollagens synthesized on the fourth day were labelled with 10 Ci/ml L-[5-$^3$H]proline (30 Ci/mmol, Amersham Corporation) for 18 h in medium containing 10% (v/v) dialysed foetal calf serum (Flow Laboratories), 0.25 mM sodium ascorbate and 0.1 mM β-aminopropionitrile fumarate (Sigma). The cell-layer and medium fractions were separated for analysis and the procollagens were precipitated with 25% saturated $(NH_4)_2SO_4$. Procollagens were converted to collagen by limited pepsin digestion and the collagen chains were resolved by SDS/5%-PAGE and detected by fluorography [2,3]. Cyanogen bromide peptides were resolved by two-dimensional gel electrophoresis [4].

Abbreviations used: CB, cyanogen bromide peptide; *COL3A1*, gene locus for the pro-α1(III) chains of type III procollagen; EDS-IV, type IV form of the Ehlers–Danlos syndrome; Gly-Xaa-Yaa, repetitive amino acid triplet structure of the triple-helical domain of α1(III) chains in which Xaa and Yaa are other amino acids, proline and hydroxyproline being more common in the Yaa position.

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### Collagen production by long-term fibroblast cultures

EDS-IV and control fibroblast cultures were grown to confluency, after which sodium ascorbate was added daily to the medium, at a final concentration of 0.25 mM, for 8 weeks [5]. The medium was collected every 3 days and pooled. After 8 weeks, the cell matrix was recovered and extracted in 10 mM Tris/HCl buffer, pH 7.5, containing 150 mM NaCl, 5 mM EDTA and protease inhibitors, at 4 °C for 18 h. The extract was added to the pooled medium fraction. The collagens, prepared from the insoluble matrix and medium by limited pepsin digestion, were analysed by SDS/5%-PAGE followed by staining with Coomassie Brilliant Blue. The thermal stability ($T_m$) of collagen molecules in the medium was also determined [6].

### Amplification and sequencing of α1(III) cDNA from EDS-IV fibroblasts

First-strand cDNA was synthesized from fibroblast RNA by using a cDNA synthesis kit (Amersham Corporation) and an internal oligonucleotide primer 5′-ATCCCAGCAATGGCA-GCGGC-3′, corresponding to nucleotides 3604–3623 of the α1(III) cDNA. The nucleotides are numbered from the start site of translation of the pro-α1(III) mRNA [7]. A 2086-nucleotide region of the cDNA, extending from Arg-135 to Met-797, was amplified as three overlapping fragments by PCR [7]. The amino acid residues are numbered from the start of the triple-helical domain of the α1(III) chains [7]. The PCR products were analysed and sequenced by previously described methods [8].

### Amplification and sequencing of genomic DNA

A 1.1 kb genomic DNA product was amplified by PCR by using the forward oligonucleotide primer 5′-ACTGCCGGATTCC-CTGGATC-3′ from exon 16 and the reverse oligonucleotide primer 5′-CCTTTACCACCAGGACTACC-3′ from exon 18 [7]. The products were purified, cloned and sequenced as described for the cDNA-PCR products except that a forward primer 5′-GGTTCAAATGGTGCCCCTGG-3′ that is specific for exon 17 was used for sequencing. Genomic DNA extracted from lymphocytes from the proband's brother and mother was amplified and sequenced as described above [9].

## RESULTS

### Analysis of type III collagen chains and peptides

The EDS-IV fibroblasts produced and secreted normal and faster-migrating mutant forms of disulphide-linked type III collagen molecules (Figure 1). Quantification by scintillation counting showed that ~ 32% of the type III collagen molecules in the medium were in the mutant form. The chain composition of the mutant molecules was not determined. However, as the bands were sharp and distinct from the normal molecules it is likely that they were mutant homotrimers. In contrast, the cell layer contained a broad type III collagen band that migrated slightly faster than the control type III collagen but more slowly than the abnormal type III collagen in the medium (Figure 1). The composition of these molecules was also not determined, but they were likely to be heterotrimers containing normal and mutant α1(III) chains.

The abnormally fast migration of the mutant type III collagen molecules in the medium suggested that the mutant α1(III) chains were shorter than normal. These molecules migrated as a sharp band and did not show the tailing that is characteristic of molecules containing mutations that impair helix formation,

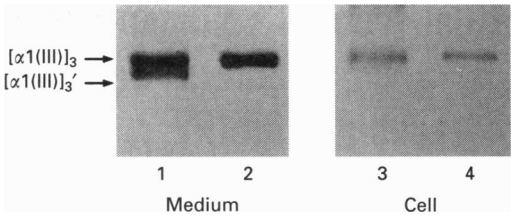


**Figure 1 Electrophoresis of type III collagen produced by cultured fibroblasts**

Radiolabelled procollagens were prepared from the cell layer and medium of short-term cultures of control and EDS-IV fibroblasts. They were converted to collagen by limited pepsin digestion and resolved by SDS/5%-PAGE. Collagen bands were detected by fluorography and only the section of the gel containing the type III collagens is shown. Lanes 1 and 3, EDS-IV; lanes 2 and 4, controls. The normal type III collagen is labelled as $[\alpha1(III)]_3$ and the faster-migrating mutant type III collagen is labelled as $[\alpha1(III)]_3'$.

with consequent overmodification of the more amino-terminal lysine residues [2,3,8]. This finding suggested that the mutant α1(III) chains contained a deletion that maintained the normal Gly-Xaa-Yaa triplet structure and enabled the triple helix to form normally. Electrophoresis of cyanogen bromide peptides showed that the carboxy-terminal α1(III) CB5 and 9 peptides migrated normally (results not shown). As a result, it was likely that the mutation was amino-terminal to Pro-561, the first amino acid residue of the CB5 peptide [7]. The mutation could not be localized more accurately as the amino-terminal peptides were too small to be analysed by the two-dimensional electrophoresis system. Western blotting did not show any abnormal peptides.

### Characterization of the mutant α1(III) cDNA

Amplification of nucleotides 903–1269 yielded a normal 367 bp product, as observed in control reactions, and an abnormal 267 bp product (Figure 2). Quantification by image analysis

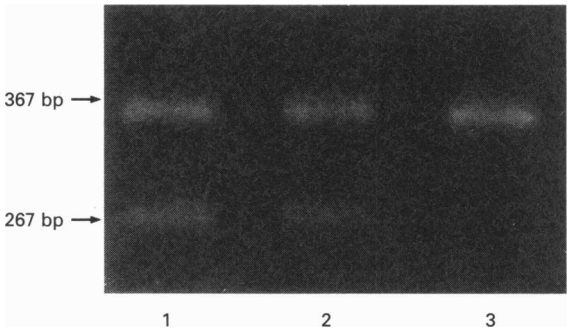


**Figure 2 Amplification of α1(III) cDNA from EDS-IV fibroblasts grown at 31 and 37 °C**

Total RNA was prepared from EDS-IV fibroblasts grown at 31 and 37 °C. Approx. 10 ng of cDNA was amplified by PCR with the forward oligonucleotide primer 5′-AAGAGGGGCTC-CTGGTGAGC-3′ and the reverse oligonucleotide primer 5′-ACCATTAGCACCGGCTGGTC-3′ corresponding to nucleotides 903–922 and 1250–1269 of the α1(III) cDNA, respectively [7]. One-tenth of the reaction mixture was resolved by 2.5% (w/v) Nusieve agarose gel electrophoresis and stained with ethidium bromide. Lane 1, amplification products from EDS-IV fibroblasts grown at 31 °C; lane 2, amplification products from EDS-IV fibroblasts grown at 37 °C; lane 3, amplification products from control fibroblasts grown at 37 °C. The mutant 267 bp product, lacking exon-17-encoded sequences, and the normal 367 bp product are shown.

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A AGA GGG GCT CCT GGT GAG CGA GGA CGG CCA GGA CTT CCT GGG GCT GCA GGT GCT CGG GGT  963
  Arg Gly Ala Pro Gly Glu Arg Gly Arg Pro Gly Leu Pro Gly Ala Ala Gly Ala Arg Gly  154

  AAT GAC GGT GCT CGA GGC AGT GAT GGT CAA CCA GGC CCT CCT GGT CCT CCT GGA ACT GCC 1023
  Asn Asp Gly Ala Arg Gly Ser Asp Gly Gln Pro Gly Pro Pro Gly Pro Pro Gly Thr Ala  174

  GGA TTC CCT GGA TCC CCT GGT GCT AAG GGT GAA GTT GGA CCT GCA GGG TCT CCT GGT TCA 1083
  Gly Phe Pro Gly Ser Pro Gly Ala Lys Gly Glu Val Gly Pro Ala Gly Ser Pro Gly Ser  194

  AAT GGT GCC CCT GGA CAA AGA GGA GAA CCT GGA CCT CAG GGA CAC GCT GGT GCT CAA GGT 1143
  Asn Gly Ala Pro Gly Gln Arg Gly Glu Pro Gly Pro Gln Gly His Ala Gly Ala Gln Gly  214

  CCT CCT GGC CCT CCT GGG ATT AAT GGT AGT CCT GGT GGT AAA GGC GAA ATG GGT CCC GCT 1203
  Pro Pro Gly Pro Pro Gly Ile Asn Gly Ser Pro Gly Gly Lys Gly Glu Met Gly Pro Ala  234

  GGC ATT CCT GGA GCT CCT GGA CTG ATG GGA GCC CGG GGT CCT CCA GGA CCA GCC GGT GCT 1263
  Gly Ile Pro Gly Ala Pro Gly Leu Met Gly Ala Arg Gly Pro Pro Gly Pro Ala Gly Ala  254

  AAT GGT 1269
  Asn Gly  256
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**Figure 3 Normal and mutant cDNA and deduced amino acid sequences from EDS-IV**

The PCR products shown in Figure 2 were purified, cloned and sequenced. The nucleotides are numbered from the start site of translation and the amino acids are numbered from the start of the triple helix. The 5′ and 3′ oligonucleotide primers are underlined and the deleted exon-17-encoded sequences from the mutant PCR product are shown within the box.

showed that the two products in the EDS-IV reaction were present in equal amounts. The normal α1(III) sequence was observed in clones of the 367 bp product from the control and EDS-IV reactions. A deletion of 99 nucleotides from the normal sequence was observed in clones of the abnormal 267 bp product (Figure 3). The deleted nucleotides 1051–1149, and the corresponding 33 amino acids Gly-184 to Pro-216, are normally encoded by exon 17 [10]. The sequences encoded by exons 16 and 18 were contiguous with maintenance of the repetitive Gly-Xaa-Yaa triplet structure.

### Characterization of mutant *COL3A1* genomic DNA

Amplification of EDS-IV and control genomic DNA, encompassing exons 17 and 18, generated a single product of 1.1 kb. Sequencing revealed normal and abnormal EDS-IV clones. The abnormal clones contained a T to A substitution at position −1 and a T to G substitution at position +2 of the 5′ splice site of intron 17. The T to A change in exon 17 did not alter the amino acid sequence. In addition, it did not alter the splice donor-site score of 86 calculated for the normal intron 17 sequence by using the formula of Shapiro and Senapathy [11]. In contrast, the T to G substitution at position +2 reduced the score to 68, which is below the average mammalian splice donor-site score of > 70.

Sequencing of the same region of genomic DNA from lymphocytes showed that the proband's clinically affected brother was also heterozygous for these substitutions, whereas his clinically unaffected mother was normal. Samples from the proband's clinically affected father were not available for analysis.

### Analysis of pre-mRNA splicing products

EDS-IV fibroblasts were grown at 33 and 37 °C to determine whether splicing of the mutant allele could be corrected at a lower temperature [12]. Normal splicing was not restored, as the same proportions of the normal 367 bp and the mutant 267 bp cDNA-PCR products were observed at both temperatures (Figure 2). The absence of other PCR products indicated that alternative splicing sites were not activated at either temperature.

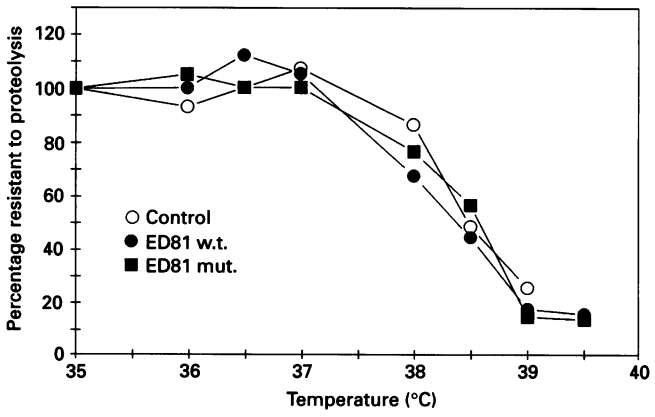


**Figure 4 Thermal stability of EDS-IV collagen**

Collagen was prepared by limited pepsin digestion of procollagen from long-term EDS-IV fibroblast cultures. Samples were incubated from 35 to 39 °C and aliquots were digested with a mixture of trypsin and chymotrypsin [6]. The triple-helical molecules that were resistant to enzymic digestion were resolved by SDS/5%-PAGE, stained with Coomassie Brilliant Blue and quantified by densitometry. The results are expressed as the relative proportion of helical molecules resistant to proteolysis at each of the temperatures tested. Key to symbols: ○, control; ●, ED81 wild-type; ■, ED81 mutant.

### Thermal stability of mutant type III collagen

The thermal stability of the normal and mutant type III collagen molecules in the medium from EDS-IV fibroblasts was determined. Deletion of the 33 amino acids encoded by exon 17 did not alter the thermal stability of the mutant collagen (Figure 4). The $T_m$ of the normal and mutant type III collagen molecules was 38.5 °C.

### Formation of an extracellular matrix by EDS-IV fibroblasts

The incorporation of type III collagen into the extracellular matrix was examined after EDS-IV fibroblasts had been cultured for 8 weeks. A dense extracellular matrix formed during this time. It contained cross-linked type I and III collagens but the

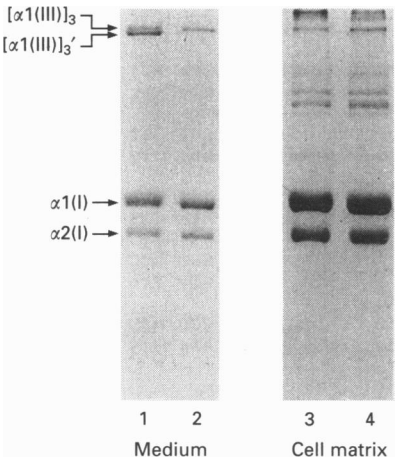


**Figure 5 Extracellular matrix formation by long-term EDS-IV fibroblast cultures**

EDS-IV and control fibroblasts were grown for 8 weeks. The pooled medium and cell-matrix layer were subjected to limited pepsin digestion. The collagens were resolved by SDS/5%-PAGE and stained with Coomassie Brilliant Blue. Lanes 1 and 3, EDS-IV; lanes 2 and 4, controls. The type I and III collagen bands are shown and the mutant type III collagen band is designated $[\alpha 1(III)]_3'$.

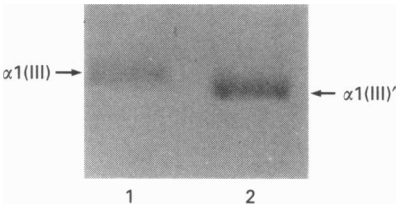


**Figure 6 Chain composition of normal and mutant type III collagen from the medium of long-term fibroblast cultures**

The pooled medium from EDS-IV fibroblasts grown over an 8 week period was subjected to limited pepsin digestion. Samples were separated by SDS/5%-PAGE and stained with Coomassie Brilliant Blue. The normal and mutant type III collagen-containing bands were excised from the gel and their disulphide bonds were reduced with $\beta$-mercaptoethanol. The reduced $\alpha 1$(III) chains were resolved by SDS/5%-PAGE and stained with Coomassie Brilliant Blue. Lane 1, normal type III collagen from EDS-IV fibroblast medium; lane 2, mutant type III collagen from EDS-IV fibroblast medium. The normal and mutant $\alpha 1$(III) chains are labelled.

shortened mutant form of type III collagen was not detected (Figure 5). This finding was confirmed by Western blot analysis of normally loaded and overloaded gels with antibodies that were specific for human type III collagen (results not shown). Mutant chains were also not detected by silver staining of overloaded gels. The densitometry ratio of disulphide-linked $\alpha 1$(III) chains to the $\alpha 1$(I) chains in the EDS-IV cell matrix was approximately equal to control values. However, the amount of type I and III collagens in the EDS-IV cell matrix was reduced to $\sim 50\%$ of that present in the normal cell matrices. These differences were determined from the densitometry results adjusted for DNA content and sample size.

In contrast, the pooled medium contained normal and mutant type III collagen molecules. Densitometry of the bands stained with Coomassie Brilliant Blue showed that 61% of the type III collagen in the pooled medium was in the mutant form. The mutant, disulphide-linked type III collagen molecules were shown to contain only the short mutant $\alpha 1$(III) chains (Figure 6).

## DISCUSSION

The EDS-IV phenotype in this family was shown to be due to heterozygous deletions of 99 nucleotides from the $\alpha 1$(III) mRNA and 33 amino acids from the $\alpha 1$(III) chains of type III collagen. These deletions corresponded to sequences normally encoded by exon 17 of the *COL3A1* gene [10]. We concluded that the T to G transversion in the highly conserved +2 position of intron 17 was responsible for the abnormal splicing of pre-mRNA with loss of the exon-17-encoded sequences from the $\alpha 1$(III) mRNA and chains.

The abnormal skipping of exon 17 was not altered when the fibroblasts were grown at a low temperature [8]. This finding is in accordance with the lack of temperature modulation of exon skipping in fibroblasts from EDS-IV patients with substitutions of G in the +1 position of other introns of the *COL3A1* gene [8]. However, exon skipping due to substitutions further along the introns, such as substitutions of G in the +5 position of introns 25 and 37, can be corrected at low temperatures [12].

From our analyses of the products generated from cDNA-PCR we concluded that about half of the $\alpha 1$(III) mRNA was normal and half lacked the exon-17-encoded sequence. We did not find any evidence of alternatively spliced mRNA species that retained part or all of intron 17. As the steady-state levels of normal and mutant 1(III) mRNAs were approximately equal, it is likely that $\sim 12.5\%$ of the type III collagen molecules were normal homotrimers, 12.5% were mutant homotrimers and 75% were heterotrimers containing a mixture of normal and mutant chains [13]. The latter molecules would be unstable owing to the differing lengths of the triple-helical domains of the normal and mutant chains, as observed in other skipping mutations and deletions of the *COL3A1* gene [8,14]. This proposal is supported by our observation that normal homotrimers and mutant homotrimers were the major forms of type III collagen obtained after the conversion of type III procollagen to collagen by limited pepsin digestion. The residual heterotrimers were retained and probably degraded within the cells as observed with other skipping mutations [8,14].

The mutant homotrimers were secreted normally by cultured dermal fibroblasts and had the same thermal stability as the normal homotrimers. The level of enzymic modification of their lysine residues was also probably normal, as the mutant homotrimers and mutant $\alpha 1$(III)′ chains did not show the electrophoretic tailing characteristic of overmodification of lysine residues. These normal characteristics of the mutant homotrimers indicated that the loss of ten Gly-Xaa-Yaa triplets did not adversely affect helix formation and stability or the structural requirements for secretion.

The incorporation of the mutant homotrimers into the extracellular matrix was studied with an *in vitro* model of the dermis. In this model, which used long-term ascorbate-supplemented dermal fibroblast cultures, the normal extracellular matrix contained cross-linked type I and III collagens that were almost identical to those extracted from normal human dermis [5,15]. In the EDS-IV dermal model, the mutant homotrimers accumulated in the medium but were not detectable in the extracellular matrix. We did not determine whether this abnormality was due to deficient processing of mutant homotrimeric procollagen to collagen or to impaired incorporation of the processed collagen into the extracellular matrix.

The amounts of type I and III collagen were halved in the extracellular matrix of the long-term EDS-IV cultures. As the collagen fibrils contain type I and III collagens, it is likely that the deficiency of normal type III collagen also impaired the incorporation of normal type I collagen into the EDS-IV fibrils

[15]. This proposal is in accordance with the thin collagen fibrils reported in EDS-IV dermis [13].

The clinical phenotype of the proband was similar to that of a child that we studied with skipping of exon 41 due to a $G^{+1}$ to A substitution in intron 41 of the *COL3A1* gene [8]. In the latter case, the mutant homotrimers, which lacked the mammalian collagenase cleavage site, were the major pepsin-resistant form of type III collagen produced by cultured dermal fibroblasts. However, the amount of type III collagen in the proband's dermis was reduced to 11% of normal, close to the 12.5% level that would be expected if only the normal homotrimers were incorporated into the extracellular matrix. This finding and our observations in the present case suggest that substitutions in the $G^{+1}$ or $T^{+2}$ position of any intron within the region of the *COL3A1* gene that encodes the triple-helical domain of the $\alpha$1(III) chains are likely to produce a severe deficiency in the amount of normal type III collagen in the tissues and a severe clinical phenotype. Intracellular and extracellular quality-control mechanisms prevented the incorporation of heterotrimeric and homotrimeric mutant type III collagen molecules into the cross-linked extracellular matrix.

Intracellular quality control resulting in the retention and degradation of mutant heterotrimers has also been reported in osteogenesis imperfecta as a result of dominant-negative mutations of the *COL1A1* and *COL1A2* genes of type I collagen, and in chondrodysplasias as a result of dominant-negative mutations of the *COL2A1* gene of type II collagen [2,3,16,17]. Extracellular quality control resulting in the partitioning of normal molecules into the cross-linked extracellular matrix and mutant molecules into a more soluble collagen pool has also been observed in bone and dermis from patients with osteogenesis imperfecta [3,15].

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