A COL17A1 Splice-Altering Mutation Is Prevalent in Inherited Recurrent Corneal Erosions

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Purpose: Corneal dystrophies are a genetically heterogeneous group of disorders. We previously described a family with an autosomal dominant epithelial recurrent erosion dystrophy (ERED). We aimed to identify the underlying genetic cause of ERED in this family and 3 additional ERED families. We sought to characterize the potential function of the candidate genes using the human and zebrafish cornea.

Design: Case series study of 4 white families with a similar ERED. An experimental study was performed on human and zebrafish tissue to examine the putative biological function of candidate genes.

Participants: Four ERED families, including 28 affected and 17 unaffected individuals.

Methods: HumanLinkage-12 arrays (Illumina, San Diego, CA) were used to genotype 17 family members. Next-generation exome sequencing was performed on an uncle—niece pair. Segregation of potential causative mutations was confirmed using Sanger sequencing. Protein expression was determined using immunohistochemistry in human and zebrafish cornea. Gene expression in zebrafish was assessed using whole-mount in situ hybridization. Morpholino-induced transient gene knockdown was performed in zebrafish embryos.

Main Outcome Measures: Linkage microarray, exome analysis, DNA sequence analysis, immunohistochemistry, in situ hybridization, and morpholino-induced genetic knockdown results.

Results: Linkage microarray analysis identified a candidate region on chromosome chr10:12,576,562–112,763,135, and exploration of exome sequencing data identified 8 putative pathogenic variants in this linkage region. Two variants segregated in 06NZ–TRB1 with ERED: COL17A1 c.3156C→T and DNAJC9 c.334G→A. The COL17A1 c.3156C→T variant segregated in all 4 ERED families. We showed biologically relevant expression of these proteins in human cornea. Both proteins are expressed in the cornea of zebrafish embryos and adults. Zebrafish lacking Col17a1a and Dnajc9 during development show no gross corneal phenotype.

Conclusions: The COL17A1 c.3156C→T variant is the likely causative mutation in our recurrent corneal erosion families, and its presence in 4 independent families suggests that it is prevalent in ERED. This same COL17A1 c.3156C→T variant recently was identified in a separate pedigree with ERED. Our study expands the phenotypic spectrum of COL17A1 disease from autosomal recessive epidermolysis bullosa to autosomal dominant ERED and identifies COL17A1 as a key protein in maintaining integrity of the corneal epithelium. Ophthalmology 2016;121:14 © 2016 by the American Academy of Ophthalmology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Supplemental material is available at www.aaojournal.org.

Technological advances in the clinic and laboratory have allowed us a greater appreciation of the phenotypic and genetic diversity of corneal dystrophies. The International Committee for Classification of Corneal Dystrophies1,2 has responded to this newfound diversity by developing a clear classification system, considering both clinical features and the underlying genetic cause in its categorical designations. We previously described a 3-generation New Zealand family (06NZ–TRB1) with a unique autosomal dominant corneal dystrophy. Members of the 06NZ–TRB1 family experienced frequent, painful, recurrent corneal erosions from the age of 5 years.3 The corneal features include small, fine grey anterior stromal flecks, with more unique larger grey-white opacities at the level of the Bowman layer and the immediately subjacent anterior stroma, with increased prominence of the corneal nerves.3 This corneal appearance is distinct from classic Fleck dystrophy (Online Mendelian Inheritance in Man identifier, 121850), and patients with Fleck dystrophy typically are relatively asymptomatic. Painful recurrent corneal erosions in this family ceased in the patients’ second decade, although the resulting accumulation of corneal scarring impaired vision in some
clinical changes. A validated IVCM grading scale for the quantification of anterior corneal stromal haze and fibrosis was applied.

### Clinical Examination of Patients

All family members underwent a comprehensive clinical examination to determine the presence or absence of disease (described previously). This included visual acuity, slit-lamp examination, intraocular pressure (Goldmann applanation tonometry), and, in selected affected individuals, clinical photography and in vivo confocal microscopy (IVCM) with the Heidelberg Retina Tomograph 2 Rostock Cornea Module (Heidelberg Engineering GMBH, Heidelberg, Germany). Representative IVCM images from all corneal layers were selected for the United Kingdom family (UKOGA) by 2 experienced examiners (B.S., V.R.) for analysis of pathologic changes. A validated IVCM grading scale for the quantification of anterior corneal stromal haze and fibrosis was applied.

### Linkage Analysis Using HumanLinkage-12 Arrays

HumanLinkage-12 arrays (Illumina, San Diego, CA) were used to genotype 17 06NZ-TRB1 family members (7 affected and 8 unaffected members and 2 members with unknown status subsequently confirmed as unaffected). Genotypes were imported into ALOHOMORA for subsequent manipulation. Mendelian errors were identified with PedCheck, and unlikely close-recombinant genotypes were identified with Merlin and were removed before multipoint parametric linkage analysis with Allegro version 2 using the deCODE genetic map.

### Screening of Candidate Genes

Protein–protein interactions were analyzed with Search Tool for the Retrieval of Interacting Genes/Proteins (STRING). This created functional protein association networks among known corneal dystrophy genes located within the reduced interval of the linkage region, identifying 8 genes of interest. Primer pairs for amplicons spanning at least 1 full exon, and its 5’ and 3’ intron—exon boundaries, were designed with the National Center for Biotechnology Information’s Primer-BLAST (available at [www.ncbi.nlm.nih.gov/tools/primer-blast/](http://www.ncbi.nlm.nih.gov/tools/primer-blast/)) for all coding exons within the identified genes of interest, as shown in Supplemental Table 1 (available at [www.aaojournal.org](http://www.aaojournal.org)). Sanger sequencing was performed on an ABI 3700 sequencer (Applied Biosystems, Waltham, MA) as previously described. Sequence chromatograms were compared with reference sequences in CodonCode Aligner version 4.2.3 (CodonCode Corporation, Centerville, MA).

### Exome Sequencing and Bioinformatic Analysis

DNA from 2 affected individuals (II.5 and III.3, an uncle–niece pair; Fig 1A) was sent to the Otago Genomics Facility (New Zealand Genomics Ltd.) for library preparation, exome enrichment, and Illumina HiSeq sequencing (Illumina, Inc., San Diego, CA). Illumina TruSeq DNA sequencing libraries and TruSeq 64Mb exome enrichment were carried out according to the manufacturer’s instructions. Exomes were subjected to 100-bp paired-end sequencing using the Illumina HiSeq2000 instrument.

Reads were aligned to the human reference GRCh37 with Burrows-Wheeler Alignment tool (BWA). Aligned reads then were marked for duplicates with Picard and base quality score recalibration and realignment of local insertions and deletions

### Methods

#### Collection of DNA Samples

This study adhered to the principles of the Declaration of Helsinki and received institutional ethics approval (Northern A Health and Disability Ethics Committee, NTX/06/12/161; Northern Ireland Office for Research Ethics Committee, 350/03). After obtaining informed consent, genomic DNA was extracted from peripheral venous blood or saliva as described previously. Samples were collected from 17 members of the 06NZ-TRB1 family (7 affected and 10 unaffected members) and from 4 members of the 15NZ-LED1 family (3 affected members and 1 member with unknown status). Samples from the additional families were received as genomic DNA from Tasmania (11 affected and 4 unaffected members) and the United Kingdom (7 affected and 2 unaffected members).

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were performed with the Genome Analysis Toolkit. Recalibrated reads were genotyped with the Genome Analysis Toolkit unified genotyper, and variant quality scores were readjusted to standard hard filtering parameters.

Variants were annotated with Snpeff to identify genes, locations within genes, and possibly impact on genes, and then were filtered by the critical region (as defined by linkage), by heterozygosity in both affected family members, and finally by population allele frequency in ESP6500 and the 1000 Genomes data sets. MutationTaster, MutationTaster2, and Human Splicing Factor were used to predict the effect of variants on splicing. MutationTaster and PolyPhen-2 were used to predict pathogenicity of missense variants.

**Sanger Sequencing Confirmation**

Primer pairs flanking the variants of interest were designed with Primer3Plus (available at: primer3plus.com/) and the National Center for Biotechnology Information’s BLAST (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov/BLAST). Primers for COL17A1 c.3156C→T were 5’-CGTGGGAGAACATGTCC-3’ (forward) and 5’-AAAGTCTCGCCTGTGATGGT-3’ (reverse) and those for DNAJC9 c.334 G→A were 5’-CCCTTCCCTGGTCCCTAGTT-3’ (forward) and 5’-CTTGATGACTGTATGCCCA-3’ (reverse).

Polymerase chain reaction (PCR) analysis was performed using the AmpliTaq Gold DNA polymerase (Life Technologies, Waltham, MA), with a final MgCl2 concentration of 1.5 mmol/L. Polymerase chain reaction amplicons were purified using the DNA Clean & Concentrator-5 PCR Puriﬁcation Kit (Zymo Research, Irvine, CA) according to the manufacturer’s instructions. Sanger sequencing was performed on an ABI 3700 sequencer as described previously. Sequence data were compared with the National Center for Biotechnology Information reference sequence NM_000494.3 with CodonCode Aligner Software version 4.2.3 (CodonCode Corporation).

**Microsatellite Marker Analysis**

Microsatellite markers were identiﬁed adjacent to COL17A1 with the UCSC Genome Browser microsatellite track. Microsatellite repeats were identiﬁed within COL17A1 (19×CA repeat; chr10:105815203→105815240, GRCh37) and OBFC1 (20×AT repeat; chr10:105674094→105674133), as shown in Supplemental Figure 1 (available at: www.aaajournal.org). Primers were designed with Primer3Plus. Primers for COL17A1 (19×CA) were 5’-HEX-CCAAAGACTGTGTCCTTCCAC-3’ (forward) and 5’-ACTCACTTCAACTGCGGAGA-3’ (reverse; 60°C annealing temperature). Primers for OBFC1 (20×AT) were 5’-FAM-TGATTGTACCACCTGCTCTCA-3’ (forward) and 5’-TCCACA...
ACCACAGCAAGACA-3′ (reverse; 63°C annealing temperature). Polymerase chain reaction was performed on 250 ng DNA in a 50-μl reaction with AmpliTaq Gold DNA Polymerase (Life Technologies) according to the manufacturer’s guidelines. Polymerase chain reaction products were cleaned with the DNA Clean & Concentrator-5 PCR Purification Kit (Zymo Research) according to the manufacturer’s instructions. The purified samples were run alongside a 500 LIZ Size Standard (Applied Biosystems) on an ABI 3730 capillary sequencer (Applied Biosystems). The microsatellite results were allele binned with the STRand analysis freeware (available at: www.vgl.ucdavis.edu/STRand).

Immunohistochemistry

Immunohistochemistry was performed to show expression of COL17A1 and DNAJC9 in both human and zebrafish cornea. Cryosections of 16 to 20 μm were prepared from fresh keratoconic human corneal buttons (collected during corneal transplant surgeries; New Zealand Eye Bank, www.eyebank.org.nz/) and 4% paraformaldehyde–treated zebrafish tissue (isolated adult eyes and whole embryos) as follows. Tissues were processed through a sucrose gradient, embedded in 2:1 sucrose–O.C.T. compound media (Tissue-Tek; Sakura Finetek U.S.A., Inc., Torrance, CA) and snap-frozen on dry ice, then stored at −80°C until use. Cryosections were mounted on Superfrost Plus Microscope Slides (Fisher Scientific, Hampton, NH) and dried overnight. For immunochemistry, sections were rehydrated in 0.1 M phosphate-buffered saline (PBS), then digested with 2 mg/ml testicular hyaluronidase for 1 hour at 37°C, followed by 2 mg/ml tyrode solution (Earle’s) and then stored in PBS. For immunohistochemistry, sections were rehydrated in 0.1 M phosphate-buffered saline (PBS), then rinsed in PBS before applying an antibody to the tissue. Antifadent-mounted media (CitiFlour, Sigma-Aldrich, St. Louis, MO) was used at 1:60 (8.3 ng/μl) to label the basement membrane. For the visualization of basement membrane, a 1:150 dilution of an antibody to laminin (Cat. No. L-9393; Sigma-Aldrich, St. Louis, MO) was used at 1:60 (8.3 ng/μl) to label the basement membrane. The rehydrated sections were then treated in 20 mmol/L glycine for 30 minutes. Sections then were blocked with 2% normal goat serum in PBS (plus 0.1% Triton X-100) for 30 minutes at room temperature before labelling. A mouse monoclonal antibody raised against the NC16a-domain of human COL17A1 (catalog no. ab79878; Abcam, Cambridge, UK) was used at a dilution of 1:40 for both species. For DNAJC9, a rabbit monoclonal antibody raised against a synthetic peptide corresponding to residues in human DNAJC9 (catalog no. EPR9856; Abcam) was used at a dilution of 1:100 for both species. A polyclonal rabbit antimouse laminin (laminin, α 1) primary antibody (catalog no. L-9393; Sigma-Aldrich, St. Louis, MO) was used at 1:60 (8.3 ng/μl) to label the basement membrane. The primary antibody was incubated overnight at 4°C in a humidifying chamber. Sections were washed with 0.1 M PBS before secondary antibody application. For COL17A1 detection, goat antimouse immunoglobulin G Alexa-546 (catalog no. A11003; Molecular Probes, Eugene, OR) was used at 1:1000 for both human tissue and 1:400 for zebrafish tissue were used. Goat antirabbit immunoglobulin G Alexa-488 (catalog no. A11031; Life Technologies) was used at 1:300 (6.7 ng/μl) for laminin and DNAJC9 detection in both human and zebrafish tissue. Secondary antibody labelling was performed for 2 hours at room temperature in the dark to prevent quenching of the conjugated fluorophores. Unbound secondary antibody was removed by washing sections with 0.1 M PBS. Sections then were stained with 4′,6-diamidino-2-phenylindole to show cell nuclei and were mounted in CitiFlour Antifadent-mounted media (CitiFlour Ltd, London, UK). Labelling was visualized using confocal microscopy.

Zebrafish Whole-Mount In Situ Hybridization

Zebrafish were used with institutional ethics approval from the University of Auckland Animal Ethics Committee (reference no. 001343). Hybridization of antisense RNA probes specific to a transcript of interest were used to visualize expression via antibody-conjugated alkaline phosphatase staining.35 Digoxigenin (Roche, Penzberg, Germany)—labelled antisense RNA probes were produced against zebrafish orthologs of COL17A1 (Col17a1a and Col17a1b, as previously published36) and Dnae9 (Ensembl transcript ENSDART00000137683, drDNAJC9_WISH_F2: AGAAAGCTCCAGACTCGAGA and drDNAJC9_WISH_R2: GCCCATCTTCTCCTGATTT). Reverse-transcriptase PCR was performed on cDNA from embryos 8 days after fertilization to amplify the region of interest. The PCR products were then ligated into the pCR II-TOPO vector (Life Technologies), linearized with SpeI, NotI, or BamHI (NEB; New England Biolabs, Ipswich, MA), and transcribed with T7 or Sp6 polymerase. Embryos were prepared by fixation in 4% paraformaldehyde (ProSciTech, Thuringowa Central, Australia) overnight and stored in 100% methanol. An established whole-mount in situ hybridization protocol was followed as previously described.35 Posthybridization staining was performed using an alkaline phosphatase–conjugated anti-digoxigenin (DIG) antibody. Samples were imaged under a Leica MZ16FA stereomicroscope with a Leica DC490 camera and software (Leica, Wetzlar, Germany).

Morpholino Microinjections

Morpholinos were designed and synthesized by GeneTools LLC (Philomath, OR): Col17a1a_TBB:TTGGTTTGTGTTAGCTTGTCATTCC to target NM_001145565.1 and DNAJC9_SB Disease:GAATTCTGCGGCAAGATGTGCACAA to target exon 2 of ENSDART00000044150. The Standard Control Morpholino from GeneTools LLC was used as a control. Morpholinos were resuspended in 5-mmol/L stock solutions, from which 0.25-mmol/L (Col17a1a) and 0.5-mmol/L (Dnae9 and control) injection mixes were made, including 2X Phenol Red dye. One nanoliter of morpholino was injected into single-cell embryos. Embryos were grown in the dark at 28°C and examined using a Zeiss Discover V20 stereo microscope and camera (Zeiss, Oberkochen, Germany).

Results

Phenotype Description

Four white families with suspected autosomal dominant ERED were identified independently (Fig 1). These families are not knowingly related to each other, nor to an ERED family previously described in New Zealand.37 The phenotype of the index New Zealand family (06NZ-TRB1; Fig 1A), as previously described,3 was remarkably similar to the other 3 families (Table 1). In all families, affected individuals presented between 5 and 7 years of age for recurrent significant corneal epithelial erosions. As the individuals aged, these episodes tended to decrease in frequency and eventually burnt out, variably from the third to fourth decade. Symptoms continuing beyond the cessation of erosions predominantly were foreign body sensation, photophobia, and a variable reduction in vision.

All families had features on slit-lamp biomicroscopy similar to those described in the index family, additional examples of which are shown in Supplemental Figure 2 (available at www.aaojournal.org).3 The key features of this 06NZ-TRB1 New Zealand corneal phenotype are a small number of focal, disc-shaped, circular, or wreath-like grey-white opacities (typically 0.2–1.5 mm in diameter) involving the Bowman layer and adjacent anterior stroma on a relatively subtle background of numerous smaller (<100 μm) grey flecks limited to the anterior 20% of the stroma (Fig 2). The earliest lesions are similar in general appearance to the minor Bowman layer scarring that follows small, superficial corneal foreign bodies. Symptoms of recurrent corneal erosions typically commence at approximately
Table 1. Clinical Features of the 4 Epithelial Recurrent Erosion Dystrophy Families: 06NZ-TRB1, UKOGA, 15NZ-LED1, and CDTAS1

<table>
<thead>
<tr>
<th>Family Identification</th>
<th>Patient Identification</th>
<th>Affected Status</th>
<th>Genotype</th>
<th>COL17A1 DNAJC9</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Visual Acuity</th>
<th>Corneal Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>06NZ-TRB1</td>
<td>I.2</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>64</td>
<td>“Childhood”</td>
<td>Right Eye: 6/7.5−1, Left Eye: 6/7.5−</td>
</tr>
<tr>
<td></td>
<td>II.2</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>41</td>
<td>10</td>
<td>Right Eye: 6/12, Left Eye: 6/7.5</td>
</tr>
<tr>
<td></td>
<td>II.5</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>38</td>
<td>5</td>
<td>Right Eye: 6/6, Left Eye: 6/6+1</td>
</tr>
<tr>
<td></td>
<td>III.3</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>12</td>
<td>5</td>
<td>Right Eye: 6/5−1, Left Eye: 6/5−</td>
</tr>
<tr>
<td></td>
<td>II.6</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>10</td>
<td>5</td>
<td>Right Eye: 6/6, Left Eye: 6/6</td>
</tr>
<tr>
<td></td>
<td>III.6</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>75</td>
<td>7</td>
<td>Right Eye: 6/9, Left Eye: 6/12</td>
</tr>
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<td>UKOGA</td>
<td>I.1</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>75</td>
<td>7</td>
<td>Right Eye: 6/9, Left Eye: 6/12</td>
</tr>
<tr>
<td></td>
<td>II.2</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>42</td>
<td>7</td>
<td>Right Eye: 6/9, Left Eye: 6/5</td>
</tr>
<tr>
<td></td>
<td>III.3</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>55</td>
<td>7</td>
<td>Right Eye: 6/6, Left Eye: 6/9</td>
</tr>
<tr>
<td></td>
<td>II.7</td>
<td>Putative affected</td>
<td>—</td>
<td>—</td>
<td>M</td>
<td>30</td>
<td>(4−6)</td>
<td>—</td>
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<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>25</td>
<td>7</td>
<td>Right Eye: 6/5, Left Eye: 6/4</td>
</tr>
<tr>
<td></td>
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<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>21</td>
<td>7</td>
<td>Right Eye: 6/5, Left Eye: 6/4</td>
</tr>
<tr>
<td></td>
<td>III.3</td>
<td>Affected</td>
<td>—</td>
<td>—</td>
<td>F</td>
<td>10</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>III.4</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>9</td>
<td>8.5</td>
<td>Right Eye: 6/6, Left Eye: 6/6</td>
</tr>
<tr>
<td>15NZ-LED1</td>
<td>II.3</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>30</td>
<td>(&lt;8)</td>
<td>—</td>
</tr>
<tr>
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<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>25</td>
<td>7</td>
<td>Continuing</td>
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<tr>
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<td>III.2</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>10</td>
<td>8.3</td>
<td>Continuing</td>
</tr>
<tr>
<td>CDTAS1</td>
<td>III.4</td>
<td>Predicted affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>II.2</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
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<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>61</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>IV.2</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>41</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td></td>
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<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>40</td>
<td>28</td>
<td>—</td>
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<tr>
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<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td>IV.11</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
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<td>44</td>
<td>6</td>
<td>—</td>
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<tr>
<td></td>
<td>IV.14</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>42</td>
<td>6</td>
<td>—</td>
</tr>
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<td></td>
<td>V.1</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>4</td>
<td>12</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>V.2</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>7</td>
<td>19</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>V.3</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>F</td>
<td>6</td>
<td>16</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>V.14</td>
<td>Affected</td>
<td>C/T</td>
<td>DNAJC9</td>
<td>M</td>
<td>5</td>
<td>18</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>V.13</td>
<td>Affected</td>
<td>—</td>
<td>—</td>
<td>F</td>
<td>6</td>
<td>12</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>V.14</td>
<td>Affected</td>
<td>—</td>
<td>—</td>
<td>F</td>
<td>6</td>
<td>9</td>
<td>—</td>
</tr>
</tbody>
</table>

BCL = B-cell lymphoma; F = female; L = left; M = male; MDF = map-dot-fingerprint dystrophy; ph = pinhole; PTK = protein tyrosine kinase; R = right; — = no information available; ? = unsure.
6 years of age and seem to precede these corneal clinical signs. Although there was a tendency for stromal opacities to appear and gradually increase throughout life, variable expression was present within family members. The phenotypic variability observed in the United Kingdom family in 4 individuals spanning 3 generations is demonstrated in Figure 2.

In vivo confocal microscopy examination in the UKOGA family showed features similar to those in the index 06NZ-TRB1 New Zealand family: brightly hyperreflective polymorphous intraepithelial opacities were present in the epithelium, although invisible on biomicroscopic examination (Fig 3A). Areas with clinically visible disc-like opacities showed bowl-like epithelial thickening extending into the anterior stroma, with complete destruction of Bowman layer and the subepithelial nerve plexus (Fig 3B). The adjacent anterior stroma was remarkable for diffuse accumulation of anterior stromal extracellular matrix (grade 2; Fig 3C). Although anterior stromal extracellular matrix accumulation was limited to focal clinical lesions in younger patients, it was not limited to these lesions in older individuals (Fig 3D). Also, the corneal stroma in younger patients did not yield any abnormal findings, whereas patients older than 60 years were found to show needle-like stromal opacities affecting the anterior more than the posterior stroma (Fig 3E), resembling findings previously described in granular and Reis-Bückler corneal dystrophy. The corneal endothelium was not affected. Only single endothelial guttae were found in 1 female patient who was 74 years of age (Fig 3F).

**Candidate Gene Screen in 06NZ-TRB1**

The 100-Mb linkage region identified on chr10:12,576,562–112,763,135 contains approximately 996 genes. To identify candidate disease-causing genes, we created functional protein association networks between known corneal genes and those within our chr10:12,576,562–112,763,135 linkage region. This identified 8 strong candidate genes of interest that are located within the reduced interval, expressed in the cornea, make biological sense, and interact with other known corneal proteins, as shown in Supplemental Table 2 (available at www.aaojournal.org). Sanger sequencing of all coding exons of these 8 candidate genes was performed on 2 brothers discordant for disease from the 06NZ-TRB1 family. No sequence variants were found in the genomic DNA sequences examined between these 2 individuals.

**Exome Sequencing Results in 06NZ-TRB1**

Given the size of the linkage region and the number of genes it contained, exome sequencing was performed on 2 affected individuals (Fig 1A, II.5 and III.3, an uncle–niece pair). Bioinformatic filtering identified 46 heterozygous variants with
allele frequencies of less than 0.001 in the National Heart, Lung, and Blood Institute ESP Exome Variant Server and 1000 Genomes databases, as shown in Supplemental Table 3 (available at www.aaojournal.org). MutationTaster was used to predict pathogenicity of all heterozygous variants, as provided in Supplemental Table 3 (available at www.aaojournal.org). The evidence supporting 4 of the variants was poor because of low read depth; therefore, these variants were given a low priority and were not investigated further. Of the remaining 42 variants, 10 were homozygous in more than 4 individuals and a further 8 were heterozygous in more than 20 individuals in the 1000 Genomes Project data. In total, we identified 8 potential disease-causing variants in our whole-genome exome sequencing data, as shown in Table 2 and Supplemental Table 4 (available at www.aaojournal.org).

Previously, Sullivan et al identified an ERED phenotype—associated linkage region between markers D10S677 (chr10:95,964,310) and D10S1671 (chr10:106,852,499) in a family originally described as having Thiel-Behnke corneal dystrophy. Four of our potential disease-causing variants lie in genes found within this region: ARHGAP19, COL17A1, GBF1, and SLIT1. COL17A1 previously was excluded as the causative gene in this previously described corneal dystrophy family. However, the COL17A1 variant c.3156C>T, which superficially seems to produce a synonymous substitution in codon 1052 encoding glycine (chr10:105,797,446, GRCh37; ENST00000353479; NM_000494), segregated with disease in our original New Zealand family (06NZ-TRB1; 7 affected and 10 unaffected individuals), as confirmed by Sanger sequencing (Fig 1; Table 1). This variant is present only once in the Exome Aggregation Consortium database (available at exac.broadinstitute.org), with an allele frequency of 8.249e-06. Jonsson et al predicted (using a minigene splicing assay and in vitro mutagenesis) that the c.3156C>T variant leads to the introduction of a splice donor site and causes truncation of exon 46, resulting in the hypothesized insertion of 1 amino acid and deletion of 17 amino acids; (p.Gly1052_Thr1070delinsAla).

The only in silico—predicted missense variant detected by whole-exome sequencing was in DNAJC9 (c.334G>A, p.D112N, chr10:75,005,922, GRCh37; NM_015190, exon 3), later confirmed by Sanger sequencing. This aspartic acid is conserved among all species (MutationTaster), and the change to asparagine is predicted to be probably damaging, with a PolyPhen-2 score of 0.999. We confirmed segregation of this variant with disease in all 17 of the 06NZ-TRB1 family members analyzed (7 affected and 10 unaffected individuals). This variant was allocated the dbSNP identifier rs200630658 and was reported at a heterozygote (C/T) frequency of 0.002 in exome sequencing data from 662 individuals (The ClinSeq Project). It is present twice in the Exome Aggregation Consortium database with an allele frequency of 1.65e-05.

**COL17A1 and DNAJC9 Variant Screening in Additional Pedigrees**

Members from 3 subsequently identified ERED families underwent genetic screening at COL17A1 c.3156C>T and DNAJC9
c.334G→A, because segregation with disease status was confirmed in the 06NZ-TRB1 family at these 2 loci. A second affected family was identified in Tasmania, Australia (identifier, CDTAS1; Fig 1; Table 1). Clinically affected individuals in this pedigree showed recurrent ERED with a progressive increase in anterior stromal haze, similar to the 06NZ-TRB1 and UKOGA families (described above). The allelic status of 15 members of this family (10 affected and 5 unaffected) was determined for the COL17A1 variant c.3156C→T (p.Gly1052Gly, chr10:105,797,446, GRCh37; ENST00000353479; NM_000494) and DNAJC9 variant c.334G→A (p.D112N, chr10:75,005,922, GRCh37; NM_015190; Fig 1; Table 1) was determined with Sanger sequencing. All affected family members were heterozygous for the aberrant variant for the COL17A1 variant C/T), whereas unaffected family members were homozygous (C/C). However, at the DNAJC9 variant c.334G→A, all individuals examined (10 affected and 5 unaffected) were homozygous for the wild-type c.334G allele. These results suggest that COL17A1 is causative of disease in this family.

A third affected family was identified in Hamilton, New Zealand (identifier, 15NZ-LED1; Fig 1; Table 1). This family demonstrated clinical features consistent with ERED. Of the 4 family members tested, 3 were affected and 1 was considered typically too young, at 6 years of age, to demonstrate symptoms. All 4 family members tested were heterozygous for the aberrant COL17A1 variant c.3156C→T. The DNAJC9 variant c.334G→A was not present in the 15NZ-LED1 family. Our results suggest that the youngest family member, although currently asymptomatic, likely will demonstrate signs of the disease in the near future.

The fourth family with ERED was identified in the United Kingdom (identifier, UKOGA; Fig 1; Table 1). Analysis of the variant loci confirmed segregation of COL17A1 c.3156C→T with disease (present in 7 affected members and absent in 2 unaffected members), but not DNAJC9 c.334G→A, which was not present in any family members.

Microsatellite Screening across Epithelial Recurrent Erosion Dystrophy Families

Two microsatellite markers flanking the COL17A1 c.3156C→T variant—COL17A1 (20×AT) and OBFC1 (19×CA), as shown in Supplemental Figure 1 (available at www.aaojournal.org)—indicate segregation of the haplotype COL17A1 number 7 allele (C7; 7 repeats) and the OBFC1 number 12 allele (012; 12 repeats) in affected individuals in all 4 families (provided in Supplemental Table 5, available at www.aaojournal.org). In the 06NZ-TRB1 family, 5 of 5 affected family members and 2 of 4 unaffected family members carry the C7/012 haplotype. All UKOGA family members tested carry the C7/012 haplotype (3 affected and 2 unaffected individuals), as with the 15NZ-LED1 family (3 affected individuals). In the CDTAS1 family, 9 of 9 affected family members carry the C7/012 haplotype, compared with only 1 of 4 unaffected family members.

Table 2. Candidate Heterozygous Variants at COL17A1 and DNAJC9 Observed in 2 Affected Individuals within the chr10: 12,576,562-112,763,135 Linkage Region by Exome Sequencing (Positive DNA Strand)

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Chr10 Position (GRCh37)</th>
<th>Observed Allele</th>
<th>Reference Allele</th>
<th>Alteration Type</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL17A1</td>
<td>Collagen, type XVII, alpha 1</td>
<td>105797446</td>
<td>A</td>
<td>G</td>
<td>Single base substitution in CDS; gain of splice donor site</td>
<td>27</td>
</tr>
<tr>
<td>DNAJC9</td>
<td>Dnaj (Hsp40) homolog, subfamily C, member 9</td>
<td>75005922</td>
<td>T</td>
<td>C</td>
<td>Nonsynonymous missense mutation causing substitution D112N; this amino acid is highly conserved in all species</td>
<td>33</td>
</tr>
</tbody>
</table>

Expression of COL17A1 and DNAJC9 in Human Corneal Tissue

Immunohistochemistry analysis was used to examine expression of COL17A1 and DNAJC9 in a fresh keratoconic cornea (Fig 4). COL17A1 is expressed in both corneal epithelial cells and the Bowman layer (basement membrane; Fig 4, red), whereas DNAJC9 is expressed in the Bowman layer of the corneal epithelium (Fig 4, green). The pattern of DNAJC9 localization is distinct from that of COL17A1.

Expression of Col17a1 and Dnajc9 Proteins in Zebrafish

The Dnaj9 and Col17a1 staining in our adult (13 months after fertilization) zebrafish samples suggests protein presence in the external surface membranes of cells in the superficial squamous layer (Fig 5). In embryonic fish 3 days after fertilization, Dnaj9 and Col17a1 are present throughout the 2-cell layer of the developing cornea (Fig 5). Dnaj9 staining also appears to be present in the migrating endothelial cells, which at 3 days after fertilization are moving from the limbis to the cornea. Both Col17a1 and Dnaj9 are expressed in different corneal layers in the adult zebrafish compared with human keratoconic tissue.

Expression of Col17a1 and Dnajc9 Transcripts in Zebrafish

At 50 hours after fertilization, the Col17a1a transcript is present in a punctate pattern on epithelial cells across the embryo, including the cornea (Fig 6). The Col17a1b transcript is expressed within neurornast cells, consistent with previous observations by Kim et al. and is absent in the cornea.

At 50 hours after fertilization, Dnaj9 is expressed throughout the head region of the embryo, including in the retinal proliferative zone, as described previously (Fig 6). There is no clear evidence to support corneal expression of Dnajc9 in embryos 50 hours after fertilization. However, under stress conditions induced by ethanol exposure, we observe upregulation of Dnajc9 in the head of embryos 2 days after fertilization, including the cornea, as shown in Supplemental Figure 5 (available at www.aaojournal.org).

Transient Knockdown of Col17a1a and Dnajc9 with Morpholinos

We transiently knocked down Col17a1a using a translation-blocking morpholin. At 3 days after fertilization, we observed morphant embryos with atypical tail morphologic features, where the tip of the tail is distended compared with embryos injected with the standard morpholin control (Fig 7). Several morphants demonstrated heart edema and spinal curvature (Fig 7, arrow). No obvious changes were present in the morphant eye, although phenotypic characterization is limited by the sensitivity of this tissue to disruption during microdissection.
When we transiently knocked down Dnajc9 with a splice-blocking morpholino, embryos 3 days after fertilization demonstrated a bent spine phenotype, which was not observed in control-injected morphants (Fig 7). No obvious ocular phenotype was observed in the Dnajc9 morphants.

Discussion

In this study, we used genetic linkage to identify a haplotype on chr10:12,576,562–112,763,135 associated with autosomal dominant corneal ERED. Further characterization of the area with exome sequencing allowed us to show independently that the COL17A1 genomic variant c.3156C>T segregates with affected individuals and is likely to be causative of disease in the 06NZ-TRB1 family. The presence of this same variant in a further 3 white ERED families (15NZ-LED1, CDTAS1, and UKOGA) suggests that it is highly likely to be the causative mutation and is prevalent in autosomal dominant ERED. Jonsson et al recently identified a missense mutation in COL17A1 (c.2816C→T, p.T939I) in a Northern Swedish family with ERED. A sequence variant in COL17A1 (c.3156C→T) identified in the misclassified Thiel-Benhke dystrophy was reported as a nonpathogenic synonymous variant; however, Jonsson et al predicted that this variant leads to the introduction of a splice donor site and causes truncation of exon 46 (p.(Gly1052_Thr1070delinsAla)), and therefore is highly likely to be pathogenic. Combined with the findings in our 4 families described here, the c.3156C→T COL17A1 variant is causative and COL17A1 is an integral player in the pathogenesis of corneal ERED.
In the index 06NZ-TRB1 New Zealand family, although numerous fine, small (25–100 μm), grey stromal flecks are present, it is the larger grey-white opacities at the level of the Bowman layer and immediately subjacent anterior stroma that distinguish the corneal phenotype from other corneal fleck dystrophies. Subtle corneal flecks are common yet asymptomatic and can be associated with contact lens wear, drugs, and inherited corneal dystrophies. Although on clinical biomicroscopy such flecks may appear to have a predominantly pre–Descemet membrane stromal location (e.g., Maeder Danos) or anterior stromal location (e.g., Francois Neetans), more typically, and especially on assessment by in vivo confocal microscopy, these flecks are variably distributed throughout the stroma.45,46 Because these flecks typically are asymptomatic, there are few histologic data available, but they may represent enlarged or metabolically altered keratocytes or stromal deposits.45–47

In the index 06NZ-TRB1 New Zealand family, the small grey flecks are limited to the very anterior 20% of stroma (clinically and by IVCM) and extend from the central to peripheral cornea. But these flecks are not in isolation unique to, or diagnostic of, the corneal phenotype. However, the larger, focal grey-white, predominantly disk-shaped, circular, or wreath-like lesions (with central clarity), which vary from 0.2 to 1.5 mm in diameter, in conjunction with these aforementioned small, grey anterior stromal flecks, seem to be clinically diagnostic of this variant fleck and anterior membrane dystrophy (see Supplemental Fig 2, available at www.aaojournal.org). The larger grey-white lesions typically are few (range, 5–10). Prominent corneal nerves are present variably, but this is not a feature unique to this dystrophy.

Based on the available genealogic information, the 4 ERED families we have described are not knowingly related to each other. Our haplotype analysis with flanking microsatellite markers suggests that a C7/012 haplotype cosegregates with the c.3156C→T COL17A1 variant in affected individuals. The discovery of this haplotype is consistent with a founder effect. Because the white population in Australia and New Zealand is derived largely from United Kingdom emigrants, it is possible that these families share a common ancestor.

There is biological evidence supporting an essential role of COL17A1 in the cornea. COL17A1, a member of the collagen family, is an integral part of the hemidesmosome structure. Our observed expression of COL17A1 in the corneal epithelium is consistent with its reported role as a hemidesmosome protein,48,49 suggesting a function biologically relevant to the ERED phenotype. Furthermore, COL17A1 is associated with other diseases characterized by compromised epithelial attachment. Autoimmunity against COL17A1 produces the skin-blistering disease bullous pemphigoid,50 whereas mutations in COL17A1 cause the recessive, mechanically induced skin-blistering disease junctional epidermolysis bullosa,51 which manifests as corneal erosion in many patients.52 COL17A1 is linked to keratinocyte mobility,53 and increased levels of COL17A1 have been observed during corneal wound healing,54 suggesting it plays a crucial role not only in maintaining epithelial attachment, but also in recovering from injury.55

Our expression analysis of human keratoconic corneal samples indicates that COL17A1 is present in the Bowman layer and around the epithelial cells, which is comparable with previous corneal findings.55,56 Although our expression analysis was performed on keratoconic cornea, no difference in COL17A1 expression is found between keratoconic and normal corneal tissue.55,56

We identified a second potentially pathogenic variant in our original New Zealand family (06NZ-TRB1) in the DNAJC9 gene (c.334G→A, p.D112N); this is the only variant identified in our exome sequencing data set (after filtering) that is a clear missense change. DNAJC9 is a member of the heat shock (HSP40) family of proteins and is purported to have a role in the molecular chaperoning of
HSP70 family members. In human tissue, DNAJC9 is expressed ubiquitously and is upregulated in response to stress.57 Because the nonsynonymous DNAJC9 variant is found in only 1 of the 4 ERED families (06NZ-TRB1), we conclude that it is not necessary for ERED corneal disease, but it cannot be excluded as a modifier of disease severity. Of note, disease presentation in the 06NZ-TRB1 family trended toward an earlier age at onset than in the

**Figure 7.** Photographs of zebrafish injected with 0.25 pmol Col17a1 TB morpholino (MO), 0.5 pmol Dnajc9 SB (disease exon) MO, or 0.5 pmol control MO obtained 3 days after fertilization (dpf). Col17a1a TB morphants present with morphologic changes in the shape of the tail tip and occasional heart edema (arrow). Although most Dnajc9 SB (disease exon) morphants appear unaffected, several demonstrated spine curvature (arrow).
additional 3 families (Table 1). DNAJC9 expression has not been characterized previously in the human cornea. The expression pattern within the human keratoconic cornea is consistent with a potential role in corneal adhesion. Colocalization of DNAJC9 and COL17A1 in both human and zebrafish cornea is suggestive of a potential interaction between the 2 proteins, possibly with regard to response to injury and tissue regeneration. We hypothesize that under stress conditions, such as mild trauma, mutant DNAJC9 protein may not adequately perform its roles in regulating cell proliferation, survival, and apoptosis by chaperoning HSP70s.

In addition, we examined the expression pattern of COL17A1 and DNAJC9 orthologs (protein and transcript) in the zebrafish. The zebrafish cornea contains all 5 of the major corneal layers (including the Bowman layer) and is readily visible early in development.36 The mature zebrafish corneal epithelium is 4 to 6 cells deep, constituting approximately 60% of the corneal thickness, whereas the stroma comprises a further 30%.43 Although we did observe Col17a1 and Dnajc9 in the zebrafish cornea, these proteins appeared to be localized to the squamous epithelial cells on the surface of the cornea. The difference in protein localization between the zebrafish and human corneas may be attributed to the differences in cornea between these 2 species; the zebrafish cornea presumably is under less mechanical stress than the human cornea (absence of eyelids in the fish). Therefore, zebrafish epithelial cells may not require the same tight anchoring to the Bowman layer. The zebrafish has undergone historical genome duplication,36 resulting in 2 copies of many genes, some of which have undergone functional divergence. Whole-mount in situ hybridization analysis of gene expression was used to identify the zebrafish corneal ortholog of human COL17A1 in zebrafish embryos. The expression of the COL17A1 homologs has been assessed previously in zebrafish with regard to junctional epidermolysis bullosa mutations.36 We confirmed the previously published expression patterns of the zebrafish orthologs of COL17A1: Col17a1a and Col17a1b,36,44 and established that Col17a1a is likely to be the functional ortholog in zebrafish cornea. We did not observe high expression of Dnajc9 in embryos; however, as a member of the heat shock protein family, high expression is not necessarily expected from embryos in the absence of stress. Under stress conditions induced by ethanol exposure,59 we noted increased Dnajc9 expression in the zebrafish cornea. When Col17a1a was knocked down transiently during early development, we observed changes to tail morphologic features, but no obvious alteration to the gross corneal or ocular phenotype. A similar tail distension phenotype was characterized by Kim et al,36 who identified the phenotype as resulting from vacuolization in the epidermis and attributed it to compromised hemidesmosomes. Transient knockdown of Dnajc9 caused spinal curvature in some morphants. Accurate characterization of the cornea of the developing zebrafish is challenging because morphologic artefacts are introduced readily during the microdissection process. As a further limitation, the zebrafish cornea is not developed fully until adulthood,43 challenging the feasibility of transient, morpholino-induced gene knockdowns.

Until recently, morpholinos were considered the gold standard approach; however, phenotypic discrepancies now are known to be common between morphant and knockout zebrafish.60 The CRISPR/Cas9 genome-editing technique opens up exciting opportunities to introduce (via homology-directed repair) our identified COL17A1 and DNAJC9 variants into zebrafish,61 establishing lines suitable for examining the role of these genes in adult zebrafish cornea using both ophthalmic (e.g., optical coherence tomography)26 and molecular diagnostic tools. We have found that although the zebrafish is an ideal model for genetic engineering of the Col17a1a variant c.3156C→T, it has limitations for use in examining corneal dystrophy onset during early development.

The rare COL17A1 disease-causing variant (c.3156C→T) occurring in our 4 families with phenotypically similar disease replicates the association of a COL17A1 missense mutation (c.2816C→T) in the Swedish ERED family.13 It also suggests that the phenotype described as Thiel-Behnke by Yee et al12 is the result of the same COL17A1 disease-causing variant detected in our families. This study expands the phenotypic spectrum of COL17A1 disease from autosomal recessive epidermolysis bullosa to autosomal dominant ERED and suggests that COL17A1 is a key protein in maintaining corneal epithelial integrity.

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Abbreviations and Acronyms:
ERED = epithelial recurrent erosion dystrophy; IVCM = in vivo confocal microscopy; PBS = phosphate-buffered saline; PCR = polymerase chain reaction.

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