The Role of *crumbs* Genes in the Vertebrate Cornea

Jill Beyer,¹ Xinping C. Zhao,² Richard Yee,² Shagufta Khaliq,³ Timothy T. McMahon,⁴ Hongyu Ying,⁴ Beatrice Y. J. T. Yue,⁴ and Jarema J. Malicki¹

PURPOSE. To evaluate the role of *crumbs* genes and related epithelial polarity loci in the vertebrate cornea.

METHODS. The authors used histologic analysis and electron microscopy to evaluate the corneas of zebrafish mutant for a *crumbs* locus *oko meduzy* (*ome*) and in mutants of four other loci, *nagie oko* (*nok*), *heart and soul* (*bas*), mosaic eyes (*moe*), and *ncad* (formerly *glass onion*), that function in the same or related genetic pathways. In parallel, they performed an evaluation of corneas in human carriers of a *crumbs* gene, *CRB1*, and mutations using topography and biomicroscopy. The expression of the *CRB1* gene in the normal human cornea was examined by polymerase chain reaction (PCR) and immunohistochemical staining.

RESULTS. The corneas of zebrafish mutants display severe abnormalities of the epithelial and stromal layers. The epithelial cells do not properly adhere to each other, and fluid-filled spaces form between them. In addition, the layering of the corneal stroma is poorly formed or absent. The corneas of human carriers of *CRB1* mutations display shape deviations compared with what has been observed in normal individuals. A PCR product of the correct size was obtained from normal human corneal samples. Sequence analyses confirmed its identity to be the human *CRB1* gene. Immunohistochemical staining using anti-*CRB1* yielded positive brown deposits in the human cornea.

CONCLUSIONS. *crumbs* genes play a role in the differentiation of the vertebrate cornea. Corneal defects associated with *crumbs* gene mutations are very severe in the zebrafish model and, in comparison, appear clinically less pronounced in the human eye. (*Invest Ophthalmol Vis Sci.* 2010;51:4549–4556) DOI:10.1167/iov.09-4549

From the ¹Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts; the ²Department of Ophthalmology and Visual Science, University of Texas Health Science Center at Houston, Houston, Texas; the ³Centre for Human Genetics and Molecular Medicine, Sindh Institute of Urology and Transplantation, Karachi, Pakistan; and the ⁴Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, Illinois.

Supported by National Eye Institute awards RO1 EY011882 and EY016859 (JJM), EY017571 (TTM), EY018728 (XCZ), EY03890 (BYJTY), EY01792 (Core Grant UIC), and EY10608; Research to Prevent Blindness (Department of Ophthalmology and Visual Science, The University of Texas Health Science Center at Houston); Hermann Eye Fund Awards (XCZ, RY); and Wellcome Trust Grant 063406/Z/2000/Z.

Submitted for publication August 27, 2009; revised February 12, 2010; accepted March 8, 2010.

Disclosure: J. Beyer, None; X.C. Zhao, None; R. Yee, None; S. Khaliq, None; T.T. McMahon, None; H. Ying, None; B.Y.J.T. Yue, None; J.J. Malicki, None

Corresponding author: Xinping C. Zhao, Department of Ophthalmology and Visual Science, University of Texas Health Science Center at Houston, 6431 Fannin Street, MSB 7.024, Houston, TX 77030; xinping.zhao@uth.tmc.edu.

Optimal vision relies on the clarity and optical characteristics of the cornea. Its smooth optical surface and transparent nature allow the cornea to act as the eye's primary refractive surface. Any breakdown in this function presents a risk for a loss of visual acuity. Given the many variations of corneal abnormalities and the estimated number of affected patients, corneal diseases continue to be a valuable area of research.^{1–3} Corneal pathology such as trachoma, ocular trauma, and corneal ulceration make up a large portion of severe corneal morbidity, yet corneal dystrophies and degenerations also contribute a noteworthy portion to overall corneal pathologies. For example, in keratoconus—a type of corneal degeneration—prevalence has been estimated as 0.05% by one group of investigators, whereas another study reported the frequency of definite keratoconus cases of 0.12%.^{4,5} Although the exact prevalence of corneal dystrophies and degenerations is not always known, their clinical relevance is evident. It is worth noting that a portion of corneal dystrophies and degenerations have been suspected as genetically based, but in other cases the role of genetic factors remains unknown.^{2,6–9}

The cornea of the vertebrate eye is a stratified structure. Each of the five primary layers that form the cornea must maintain its proper function to sustain optimal vision. The most superficial layer is the corneal epithelium, which, in the adult human eye, consists of three major components: the squamous cell layer, wing cell layer, and basal cell layer.¹⁰ Cells of the corneal epithelium are continuously added by mitotic divisions in the basal layer of the cornea and shed at the corneal surface.¹¹ Postmitotic basal cells continuously move anteriorly, becoming wing cells and, with time, superficial epithelial cells. All corneal epithelial cells adhere to one another through desmosomes and gap junctions. In addition, the superficial squamous cells also adhere to each other through tight junctions.^{12,13} Beneath the corneal epithelium lies Bowman's layer, followed by a thick layer known as the stroma; both layers are rich in collagen fibrils. Bowman's layer consists of a thin, randomly structured meshwork, whereas the corneal stroma contains many lamellae of collagen fibrils, which provide thickness and strength to the cornea. In the fully developed human eye, the stroma contributes approximately 90% of the corneal thickness.¹³ Layers (lamellae) of the corneal stroma maintain an organized structure; collagen fiber bundles run parallel to each other within each layer, and each layer of bundles lies at an angle to adjacent layers.^{11,13–15} Collagen fibrils are maintained by keratocytes, which lie between stromal lamellae.¹³ Beneath the corneal stroma sits Descemet's layer and finally the most posterior corneal cell layer, the endothelium. Descemet's layer is a basal lamina secreted by the endothelium; with age, it increases in thickness.¹³ The endothelium performs an important function by acting as a pump to maintain corneal transparency and as a barrier to prevent the ingress of aqueous fluid. It removes fluid from the cornea, thus maintaining proper hydration and lamellar organization that preserve the clarity of the cornea.¹⁰ Endothelial cell membranes interdigitate with one another and are tightly bound by

junctional complexes.¹² The overall structure of the cornea is similar in different vertebrate species, including higher vertebrates and teleost fish.¹⁶ This provides the basis for the use of animal models in the studies of human corneal differentiation and function.

Both corneal epithelium and endothelium display apico-basal polarity. The most obvious manifestation of this polarity is the subdivision of their cell surface into two distinct domains by belts of cell junctions.^{13,16–20} These junctions confer mechanical strength and provide a barrier that limits the exchange of molecules between the outer environment and the extracellular spaces inside tissues. Given these characteristics, we hypothesized that mutations that cause a breakdown of epithelial polarity would also cause corneal defects. A number of genes have been shown to play essential roles in the formation of epithelial polarity in vertebrate and invertebrate genetic model organisms.^{21,22} One class of the major regulators of epithelial polarity is *crumbs* genes. Originally discovered in *Drosophila*,²³ mutations in *crumbs* genes have been shown to produce apicobasal polarity defects in fly embryonic epithelia, the zebrafish retinal neuroepithelium, and in cultured mammalian epithelial sheets.^{24–28} To test their possible role in the cornea, we inspected zebrafish mutants at a *crumbs* locus, *oko meduzy* (*ome*), and found obvious abnormalities in the architecture of the corneal epithelium. Similar defects of the cornea were present in mutants of other loci that function in the *ome* pathway: *nagie oko* (*nok*), *heart and soul* (*bas*), and *mosaic eyes* (*moe*), as well as in a cadherin mutant *ncad*^{m117}.

Based on studies conducted in animal models, human carriers of *crumbs* gene mutations are also likely to display defects of epithelial structures. In fact, defects in one of the three human *crumbs* genes, *CRB1*, have been shown to cause several forms of photoreceptor degeneration in the human population.^{29–32} As photoreceptor cells display several epithelial characteristics,³³ this phenotype is consistent with *crumbs* function in epithelial polarity. To investigate the possibility that *crumbs* genes function in the human cornea, we examined the expression of the *CRB1* gene in the cornea and examined carriers of *CRB1* mutations for corneal abnormalities. Although we found that these individuals do not display striking aberrations in the appearance of their corneas, compared with their genotypically normal siblings, their corneas do display a tendency to develop aberrant curvature.

METHODS

Electron microscopy was performed as described previously.¹⁶ To quantify defects in the cornea of mutant zebrafish, we counted epithelial desmosomes at 3 days post fertilization (dpf). For each mutation, two mutant individuals and one wild-type sibling were used for measurements. Desmosomes were counted in five separate adjacent cells in the central cornea for each mutant and wild-type individual and the average number of desmosomes per cell was calculated. The length of the interface between two cell layers of the central corneal epithelium and the length of gaps that separate them were also measured and compared between mutant and wild-type siblings. All studies were performed in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and were approved by the animal welfare committee at each institution involved in this study.

Informed consent was obtained from all participants in this study according to the tenets of the Declaration of Helsinki. Clinical evaluations were conducted on a subset of patients from families carrying *CRB1* mutations.³² Affected persons and their genetically normal siblings were examined. Corneal topographies were performed with a keratograph (CTK 922; Haag-Strait, Koeniz, Switzerland). Biomicroscopy was performed using standard clinical slit lamp approaches. Statistical analysis of data was performed using Wilcoxon, Mann-Whitney *U*, and Student's *t*-tests.

Polymerase chain reaction (PCR) and immunohistochemical staining were carried out to determine whether the *CRB1* gene is expressed in the human cornea. Institutional review board approval was obtained for use of human eye tissues. Total RNA was extracted from normal human corneal and retinal tissues (RNeasy Mini Kit; Qiagen, Valencia, CA) according to the manufacturer's protocol. Tissues were dissected from eye bank eyes (Illinois Eye Bank, Chicago, IL) from donors 49 and 61 years of age. At least 1 μ g RNA was reverse transcribed into cDNA using random hexamers (First-Strand cDNA Synthesis Kit; Fermentas, Glen Burnie, MD). PCR was performed using 500 ng corneal or retinal (positive control),³⁴ cDNA template, and human *CRB1*-specific primer sets (forward, 5'-AACAACACCAGGTGCTCTC-3'; reverse, 5'-GGCATGTAGCCTCGTTCTTG-3'). Water was used as a negative control. PCR conditions were 94°C denaturation for 1 minute, followed by 11 cycles of touchdown (94°C, 30 seconds; 65°C–55°C, decrease 1°C per cycle, 30 seconds; 72°C, 1 minute); 30 cycles of 94°C, 30 seconds; 55°C, 30 seconds, 72°C, 1 minute; and one cycle of 72°C, 7 minutes. The expected PCR product measures 516 bp. PCR products, along with DNA molecular weight marker (Marker VI; Roche Laboratories, Nutley, NJ) or 100-bp ladder markers (GeneRuler; Fermentas), were resolved on 1% agarose gels. They were subsequently column purified (QIAquick PCR Purification kit; Qiagen). Sequencing analyses were conducted to verify the identity of PCR products. The experiments were repeated three times.

For immunohistochemistry, corneas from eye bank donors 24, 25, 49, 61, 61, and 61 years of age were fixed in formalin and embedded in paraffin. Sections (5 μ m) were deparaffinized and treated with boiling citrate buffer, pH 6.0, for antigen retrieval. Tissue sections were then blocked in blocking buffer (3% bovine serum albumin in phosphate-buffered saline) and incubated with goat anti-*CRB1* antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The primary antibody was omitted in negative controls. The slides were further incubated with biotin-conjugated rabbit anti-goat secondary antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA), and the immunoreactive products were visualized (Vectastain ABC kit; Vector Laboratories, Burlingame, CA).

Zebrafish mutant alleles that were used in this analysis harbor the following defects. *ome*^{m289} contains a termination codon at position 764, which eliminates approximately half the extracellular domain and the entire cytoplasmic region.^{26,35} The *nok*^{m227} allele contains a substitution of a conserved amino acid in the PDZ domain at position 329.^{35,36} The strength of this allele is indistinguishable from *nok*^{m520}, which involves a truncation of 150 C-terminal amino acids. The *glo*^{m117} features a substitution of a tryptophane at position 2.^{35,37} This amino acid is very well conserved in evolution and is deemed essential for cadherin function. The *bas*^{m567} allele causes a C-terminal truncation of approximately 70 amino acids.^{38,39} Finally, *moe*^{b781} contains a premature termination codon within the first 20% of the polypeptide. All these alleles are predicted to produce either null or near-null phenotypes.

RESULTS

The Role of *crumbs* and Related Genes in the Zebrafish Cornea

We previously showed that zebrafish and human corneas share many structural similarities, such as five major layers, keratocytes in the stroma, and a multilayered organization of the stromal matrix.¹⁶ To investigate the genetic bases of corneal differentiation, we took advantage of a large collection of zebrafish eye mutants available for analysis.⁴⁰ Regulators of apicobasal polarity are one group of loci potentially important for corneal morphogenesis and maintenance. At least five such genes were found in past mutagenesis screens: *oko meduzy* (*ome*), *nagie oko* (*nok*), *heart and soul* (*bas*), *mosaic eyes* (*moe*), and *ncad* (previously *glass onion/parachute*).^{35,41} They encode, respectively, a transmembrane protein related to

the fly *crumbs* gene, a MAGUK scaffolding factor, an atypical protein kinase C, a FERM protein, and an adhesion molecule.^{26,36–39,42,43} Mutations in these loci affect the polarity of retinal neuroepithelium. Given that the cornea contains two cell layers that display apicobasal polarity, we hypothesized that these regulators are likely to play a role in corneal differentiation.

In the wild-type zebrafish cornea at 3 dpf, the two layers of epithelial cells adhere tightly to one another and are connected by cell junction complexes, most likely desmosomes¹⁶ (Figs. 1C; 2, top). By contrast, the *ome*^{m289} mutation produces extensive extracellular spaces between the inner and the outer layer of the corneal epithelium (Fig. 1D). These spaces indicate a defect in adhesive properties of corneal epithelial cells. Given that *ome* mutations produce a defect of apical cell junctions in both the zebrafish retinal neuroepithelium and in fly embryonic epithelia,^{44,45} it was unexpected that the apical junctions of the corneal epithelium would be grossly intact (Fig. 1F; compare to wild-type in Fig. 1E). Some junctions also persist at the interface of the two epithelial cell layers of mutant corneas (Fig. 1H).

To extend this analysis, we investigated the corneas of mutants in four loci related to *ome*: *nagie oko* (*nok*), *heart*

FIGURE 1. Electron micrographs of transverse sections through eyes of wild-type and *oko meduzy* (*ome*) mutant zebrafish at 3 dpf. (A, B) Low-magnification view of the lens and the overlying cornea. Note that the surface of the mutant cornea features more protrusions than the wild-type cornea. Additionally, fluid-filled spaces are found between epithelial cells in the mutant. *Arrowheads*: corneal surface. (C, D) Higher-magnification images of the cornea. (D, *arrows*) Fluid-filled spaces between epithelial cells. (E, F) Higher magnifications of the corneal surface. Wild-type and mutant apical junctions (*arrows*) do not display any obvious differences. (G, H) Junctions are present on the interface between two layers of corneal epithelial cells. At least some of them persist in the mutant (*arrows*). L, lens.

FIGURE 2. Electron micrographs of transverse sections through eyes of wild-type and mutant zebrafish at 3 dpf. *Left*: lens and overlying cornea (*arrows*). *Right*: higher magnifications of the corneal epithelium and stromal layer. Each row of images corresponds to a different genotype. *Top to bottom*: corneas of the wild-type and the following mutant strains: *glo*, *bas*, *nok*, and *moe*. *Right, arrows*: fluid-filled spaces between epithelial cells of mutant corneas. L, lens.

and *soul* (*bas*), *mosaic eyes* (*moe*), and *ncad*. All mutants display defects similar to these seen in *ome*, although the magnitude of epithelial abnormalities varies (Fig. 2). To quantitatively analyze these defects, we measured the portion of the interface between the two epithelial layers occupied by intercellular spaces and recorded the number of desmosomes (Table 1). Mutations in the *ncad* (*glo*^{m117}) and *bas* (*bas*^{m567}) genes produce the strongest loss of adhesion between the outer and the inner epithelial layer. In these mutants, approx-

TABLE 1. Quantitative Evaluation of Corneal Defects in Zebrafish Epithelial Polarity Mutants				% of Cell Surface in Direct Contact with Other Cells	
Fish	Desmosomes (n)				
	Wt	Mt	Mt/Wt (%)	Wt	Mt
<i>glo</i> ^{m117}	20.8	5.1	24.6	97	24
<i>bas</i> ^{m567}	21.0	5.3	25.0	96.2	20.2
<i>nok</i> ^{m227}	21.5	10.9	50.7	97.8	66.9
<i>ome</i> ^{m289}	20.3	10.7	52.7	95.7	66.9
<i>moe</i> ^{b781}	21.0	14.1	67.2	93.6	75.1

Wt, phenotypically wild-type siblings of each strain; Mt, phenotypically mutant siblings of each strain.

imately 75% of adhesion interface is occupied by extracellular spaces, and only approximately 25% of the desmosomes are present, compared with their wild-type siblings (Table 1). Corneal epithelial defects in *nok* (*nok*^{m227}) are similar to those in *ome* (*ome*^{m289}): approximately 30% of adhesion interface is affected, and about half the desmosomes are lost. Finally, the *moe* (*moe*^{b781}) epithelial defect is the weakest. Approximately 20% of adhesive interface is defective, primarily at the borders between adjacent cells of the posterior layer of the corneal epithelium (Fig. 2). Similar to *ome*^{m289}, the apical junctions are intact in all four mutants.

In *ome*^{m289} and all other mutants, the stroma appears to be less organized. In wild-type fish, the stroma contains 8 to 10 layers of fibrils, arranged at angles to each other.¹⁶ In contrast, this regularity of layering of the stromal fibrils is compromised in all epithelial polarity mutants (data not shown).

In addition to the cornea, mutations in epithelial polarity loci also affect the lens. The *glo* defect is again the most severe. *glo*^{m117} lens is much smaller in the mutant compared with that in its wild-type siblings. There is no defined layer of the lens epithelium; instead, the lens appears to be composed of largely undifferentiated cells (Fig. 2). In the *nok*^{m227} mutants, fluid-filled spaces are present in the central region of the lens (Fig. 2). Lens differentiation appears to be delayed in this mutant, as

evidenced by the presence of cell nuclei in the central region of the lens (data not shown). Similar defects are also seen in some of the more severely affected *ome*^{m289} mutant individuals (not shown). The lens in *bas*^{m567} and *moe*^{b781} mutants is largely normal, though intercellular spaces are present under the lens epithelium (Fig. 2). These are absent in their wild-type siblings.

Abnormalities in Human *crumbs* Mutant Corneas

Mutations in one of the human *crumbs* genes, *CRB1*, are known to cause several forms of retinal abnormalities that lead to blindness, including Leber congenital amaurosis, and several forms of retinitis pigmentosa.^{29,30,32,46,47} These human *CRB1*-associated diseases are autosomal recessive in inheritance. Studies of these disorders identified numerous human carriers of defects in the *CRB1* gene⁴⁸ (example in Fig. 3). Given the presence of corneal abnormalities in zebrafish *crumbs* mutants, we began to search for corneal defects in human carriers of *crumbs* mutations. To accomplish that, we performed corneal topography and biomicroscopy measurements in individuals homozygous for *CRB1* genetic defects (example shown in Fig. 4).

Corneal evaluations were performed on the members of two Pakistani families who were previously shown to carry

FIGURE 3. Pedigrees of two Pakistani families that carry defects in the *CRB1* gene based on previously published data.³² Shaded boxes: family members who were tested for corneal defects. Asterisks: patients who show signs of corneal abnormalities based on KSS analysis. S, severe corneal scarring.

FIGURE 4. Normal and *CRB1* mutant corneas. (A, B) Corneal topography data from a 3330RP family control (A) and from a 010LCA family homozygous carrier of a *CRB1* mutation (B). (A, B) *Top*: graphic representations of corneal curvature. Steeper areas are represented by warmer colors, and flatter regions are represented by cooler colors, as color-coded on the scale to the right. *Bottom*: raw numerical data of corneal curvature from the same two patients. The cornea of a *crumbs* mutation carrier is characterized by steeper curvatures and irregular astigmatism, most pronounced in the inferior temporal region. The control features much more homogeneous corneal curvatures. (C) Cornea of patient V-4 (010LCA pedigree; Fig. 3A). Severe corneal scarring, possibly a result of corneal ectasia, prevented topography measurement in this patient.

CRB1 mutations³² (Fig. 3). In the 3330RP pedigree, we inspected corneal phenotypes in five homozygous carriers of the *CRB1* mutation and in five control members either heterozygous or wild type at the *CRB1* locus. In the 010LCA family, we inspected four homozygous carriers and four control members. We were unable to collect topography data from two family members because of severe corneal defects (labeled “S” in Fig. 3). On slit lamp examination, both persons displayed severe corneal scarring, combined in at least one case with Munson’s sign (Fig. 4C). Such defects were not seen in relatives of these two persons; thus, they may represent severe examples of the *CRB1* mutant phenotype. We also removed several topography measurements from the data pool because of patients’ inability to focus on the target during data collection. All the instances of poor fixation were found among topography measurements of homozygous carriers of *CRB1* mutations. This was not unexpected because they had poor vision and, in the most extreme cases, were blind.^{31,49}

To quantitate differences between clinically normal persons and homozygous carriers of *CRB1* mutations, we examined three parameters based on topographic measurements: the steepest point on the cornea, the flattest point on the cornea, and total corneal toricity. The steepest point on the cornea was steeper on average in carriers of *CRB1* mutations compared with the steepest point in noncarriers, but this difference was

not statistically significant (Fig. 5; 44.67 vs. 45.11 D in normal and affected persons, respectively). Similarly, the flattest point on the cornea was flatter on average in carriers of *crumbs* mutations. However, the difference again was not statistically significant given our sample size (41.67 vs. 40.78 D in normal and affected persons). Consistent with these findings, strong differences were present in the total corneal toricity, measured as the difference between the steepest and the flattest point (2.99 vs. 4.40 D). This difference was significant based on the Student’s *t*-test and the Mann-Whitney *U* test ($P < 0.05$). Additionally, the first two measurements displayed dramatically higher variability in carriers of *CRB1* mutations (Fig. 5).

In addition, to evaluate differences between persons homozygous for the *CRB1* mutation and their normal relatives, we performed a modified version of the keratoconus severity score (KSS) analysis.¹ This assessment revealed that two homozygous carriers of *CRB1* mutations feature defective corneas (Fig. 3, asterisks). None of the control persons displayed this defect. Finally, specular microscopy did not reveal any obvious abnormalities in the morphology of endothelial cells (data not shown). Taken together, these observations reveal deviations in the shape of *CRB1* homozygous mutant corneas compared with the corneas of control persons, either wild-type homozygous or heterozygous carriers of the *CRB1* muta-

FIGURE 5. Measurements of corneal curvature in controls (WT) and in homozygous carriers of *CRB1* mutations (CRB). Measurements of the steepest point on the cornea (*left*), the flattest point (*middle*), and the difference between the two (*right*) are provided. The *vertical axis* shows dioptric values, and the *horizontal axis* shows genotypes. Averages and standard deviations are provided below each graph.

tion. Our results suggest that *crumbs* gene defects create a predisposition to corneal abnormalities.

Expression of Human *CRB1* Gene in Normal Human Cornea

To investigate whether *CRB1* is expressed in the cornea, we performed two experiments. First, a 516-bp product was obtained from the normal human corneal cDNA samples by PCR (Fig. 6A). Sequencing analyses confirmed the identity of this PCR product to be the human *CRB1* gene. An identical PCR

product was also detected in human retinal samples that served as the positive control but not from the negative control (Fig. 6A). In agreement with these results, immunohistochemical staining using anti-*CRB1* yielded positive brown deposits in the human cornea (Figs. 6Ba, 6Bb). The most prominent staining was seen in the epithelial layer. Staining of *CRB1* appeared enriched in nuclei.

DISCUSSION

Analysis of the zebrafish *crumbs* (*oko meduzy*) mutant cornea revealed a loss of integrity in the corneal epithelial layer, as evidenced by the appearance of fluid-filled spaces between epithelial cells. Additionally, the layering of the corneal stroma was poorly defined or absent. Such structural defects are not obviously reminiscent of those seen in human corneal dystrophies. In a keratoconic human cornea, epithelial cells display irregular or elongated shape, yet the desmosomes and interfaces between epithelial cells appear to be normal.⁵⁰⁻⁵² In contrast to our observations in the zebrafish, fluid-filled spaces were reported inside, but not in between, corneal epithelial cells in keratoconus patients.^{50,52} Additionally, in affected humans, stromal lamellae decrease in number and are frequently irregular or wavy.^{14,52} These abnormalities differ from defects observed in zebrafish *ome* mutants. Similarly, a number of other dystrophies affect corneal structure, but their characteristics differ considerably from what we found in zebrafish *ome* mutants. For example, though Reis-Buckler dystrophy affects the epithelium, Bowman's layer and the superficial stroma, and epithelial basement membrane dystrophy primarily affects the epithelial basement membrane, neither produces the magnitude of stromal defects we observed in zebrafish mutants.⁵³ On the other hand, many stromal dystrophies are characterized by the presence of stromal deposits or opacities, which frequently are visible as linear or spotted forms in the cornea.⁵³ We did not observe these in human carriers of *crumbs* mutations or in the zebrafish model. Additionally, such dystrophies as posterior polymorphous dystrophy, and iridocorneal endothelial syndrome (ICE) feature corneal abnormalities originating primarily in the posterior aspect of the cornea.⁵⁴ Again, their characteristics differ from those seen in zebrafish mutants. Thus, we do not see a clear similarity between zebrafish *ome* defects and those in well-known human corneal dystrophies.⁵⁴

Given that *crumbs* genes function in the formation of adherens junctions,^{26,44} abnormal adhesion between epithelial cells in *ome* mutants is not surprising. It is important to note, however, that obvious differences exist between the phenotype of the corneal epithelium and those of other epithelia that were studied in *crumbs* mutants. Although the apical surface is disrupted in fly embryonic epithelia and in the retinal epithelium of the zebrafish *ome* mutant,^{26,44,45} the integrity of the apical surface in the corneal epithelium of *crb2a* (*ome*) mutants is intact. Our observations also show that the same is true for the corneas of mutants that carry defects in several other epithelial polarity loci related to *ome*. In particular, apically located cell junctions appear grossly intact in this group of mutants (Figs. 1, 2). This suggests that *crumbs* genes function differently in the corneal epithelium than in other epithelia. Perhaps their function is limited to regulating some aspects of protein trafficking and is not relevant to the formation of junctional structures. Mistargeting of apical polypeptides to the basolateral surface could change adhesive properties of epithelial cells and result in the appearance of fluid-filled spaces between them, as observed in *ome* mutants. The origins of stromal defects in zebrafish *crumbs* mutants are also unclear. In one scenario, they may be secondary to epithelial abnormalities, as discussed previously by others.⁵⁵⁻⁵⁸ Alternatively, a

FIGURE 6. Expression of *CRB1* gene in the human cornea. (A) PCR amplification of the human *CRB1* gene. A 516-bp *CRB1* PCR product was amplified from normal human corneal and retinal cDNA templates but not from the water control. (B) Immunohistochemical staining for *CRB1* in the normal human cornea (a, b). Immunoreactive products (brown) were noted in the epithelial layer. Negative control (c) in which primary antibody was omitted showed minimal staining. Brackets: the corneal epithelium.

keratocyte defect could be responsible for changes in stromal architecture. This does not appear to be the case, however, because corneal keratocytes are not found in the zebrafish cornea until after 2 weeks of age.¹⁶ Finally, we cannot exclude the possibility that stromal defects are, at least in part, secondary to endothelial abnormalities.

As in zebrafish corneas, human corneas display defects in homozygous carriers of *crumbs* mutations. The human defects appear, however, considerably milder. Differences between animal models and human models are not without a precedent. The mouse mutants of some Usher syndrome genes, for example, do not display photoreceptor degeneration.⁵⁹ Alternatively, the differences between human and zebrafish phenotypes may be caused by different strengths of mutant alleles. The zebrafish *ome*^{m289} mutant allele used in this study truncates most of the protein sequence and is most likely null,²⁶ whereas *CRB1* mutations in the human pedigrees we studied involve single amino acid substitutions and thus may result in a partial loss of gene function.³² Another likely explanation for differences between zebrafish and human phenotypes could lie in the fact that the zebrafish *ome* locus encodes a homolog of the human *CRB2* gene, whereas the affected family members we studied carry *CRB1* defects. It is thus possible that human carriers of *CRB2* mutations display more severe corneal defects. To our knowledge, however, human abnormalities associated with *CRB2* mutations have not been reported thus far.⁶⁰ Two of our patients (Fig. 4C and data not shown) displayed severe corneal scarring. These two patients may represent a more severe form of *crumbs*-associated corneal defects that eventually produced scarring, possibly as a result of recurrent erosion. If this is the case, their corneas may, in fact, display abnormalities reminiscent of those seen in the zebrafish model. Ultrastructural analysis of tissue in nonscarred areas of the corneas of such patients will be necessary to determine whether this is the case. We also note that in a related effort, McMahon et al.,⁶¹ while investigating a cohort of patients with Leber congenital amaurosis, determined that carriers homozygous for the *CRB1* gene, and possibly the *CRX* gene, seem to have higher propensity of concurrent signs of keratoconus compared with other genotypes studied (*AIPL1*, *RetGC*, *RPE65*).⁶¹

Our analysis has identified a tendency toward corneal defects in human carriers of *CRB1* gene mutations. This observation is supported by our studies that detected both *CRB1* transcript and protein in the human cornea, indicating that the *CRB1* gene is expressed therein. *CRB1* mutations are likely to be overlooked in most patients because they display severe retinal degeneration. Our findings suggest that *CRB1* patients should also be routinely examined for corneal defects. Although human carriers of homozygous *CRB1* mutations have vision loss primarily caused by photoreceptor degeneration, benefit derived from the diagnosis and treatment of corneal defects may enhance their overall functional vision and may improve their quality of life.

References

- McMahon TT, Szcotka-Flynn L, Barr JT, et al. A new method for grading the severity of keratoconus: the Keratoconus Severity Score (KSS). *Cornea*. 2006;25:794–800.
- Poulaki V, Colby K. Genetics of anterior and stromal corneal dystrophies. *Semin Ophthalmol*. 2008;23:9–17.
- Javadi MA, Rafee'i AB, Kamalian N, Karimian F, Ja'farinasab MR, Yazdani S. Concomitant keratoconus and macular corneal dystrophy. *Cornea*. 2004;23:508–512.
- Kennedy RH, Bourne WM, Dyer JA. A 48-year clinical and epidemiologic study of keratoconus. *Am J Ophthalmol*. 1986;101:267–273.
- Hofstetter HW. A keratoscopic survey of 13,395 eyes. *Am J Optom Arch Am Acad Optom*. 1959;36:3–11.
- Rabinowitz YS. The genetics of keratoconus. *Ophthalmol Clin North Am*. 2003;16:607–620, vii.
- Edwards M, McGhee CN, Dean S. The genetics of keratoconus. *Clin Exp Ophthalmol*. 2001;29:345–351.
- Bechara SJ, Waring GO 3rd, Insler MS. Keratoconus in two pairs of identical twins. *Cornea*. 1996;15:90–93.
- Rabinowitz YS, Maumenee IH, Lundergan MK, et al. Molecular genetic analysis in autosomal dominant keratoconus. *Cornea*. 1992;11:302–308.
- Kaufman P, Alm A. *Adler's Physiology of the Eye*. St. Louis: Mosby; 2003.
- Hanna C, Bicknell DS, O'Brien JE. Cell turnover in the adult human eye. *Arch Ophthalmol*. 1961;65:695–698.
- McLaughlin BJ, Caldwell RB, Sasaki Y, Wood TO. Freeze-fracture quantitative comparison of rabbit corneal epithelial and endothelial membranes. *Curr Eye Res*. 1985;4:951–961.
- Kaufman H, Barron B, McDonald MB. *The Cornea*. Boston: Butterworth-Heinemann; 1998.
- Polack FM. Morphology of the cornea, I: study with silver stains. *Am J Ophthalmol*. 1961;51:1051–1056.
- McCally RL, Farrell RA. Structural implications of small-angle light scattering from cornea. *Exp Eye Res*. 1982;34:99–113.
- Zhao XC, Yee RW, Norcom E, et al. The zebrafish cornea: structure and development. *Invest Ophthalmol Vis Sci*. 2006;47:4341–4348.
- Anderson SC, Stone C, Tkach L, SundarRaj N. Rho and Rho-kinase (ROCK) signaling in adherens and gap junction assembly in corneal epithelium. *Invest Ophthalmol Vis Sci*. 2002;43:978–986.
- Barry PA, Petroll WM, Andrews PM, Cavanagh HD, Jester JV. The spatial organization of corneal endothelial cytoskeletal proteins and their relationship to the apical junctional complex. *Invest Ophthalmol Vis Sci*. 1995;36:1115–1124.
- Mandell KJ, Holley GP, Parkos CA, Edelhauser HF. Antibody blockade of junctional adhesion molecule-A in rabbit corneal endothelial tight junctions produces corneal swelling. *Invest Ophthalmol Vis Sci*. 2006;47:2408–2416.
- Mandell KJ, Berglin L, Severson EA, Edelhauser HF, Parkos CA. Expression of JAM-A in the human corneal endothelium and retinal pigment epithelium: localization and evidence for role in barrier function. *Invest Ophthalmol Vis Sci*. 2007;48:3928–3936.
- Knust E, Bossinger O. Composition and formation of intercellular junctions in epithelial cells. *Science*. 2002;298:1955–1959.
- Shin K, Fogg VC, Margolis B. Tight junctions and cell polarity. *Annu Rev Cell Dev Biol*. 2006;22:207–235.
- Jurgens G, Wieschaus E, Nusslein-Volhard C, Kluding C. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. *Roux Archiv of Dev Bio*. 1984;193:283–295.
- Roh MH, Fan S, Liu CJ, Margolis B. The Crumbs3-Pals1 complex participates in the establishment of polarity in mammalian epithelial cells. *J Cell Sci*. 2003;116:2895–2906.
- Fogg VC, Liu CJ, Margolis B. Multiple regions of Crumbs3 are required for tight junction formation in MCF10A cells. *J Cell Sci*. 2005;118:2859–2869.
- Omori Y, Malicki J. *oko* meduzy and Related crumbs genes are determinants of apical cell features in the vertebrate embryo. *Curr Biol*. 2006;16:945–957.
- Tepass U, Theres C, Knust E. crumbs encodes an EGF-like protein expressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia. *Cell*. 1990;61:787–799.
- Campbell K, Knust E, Skaer H. Crumbs stabilises epithelial polarity during tissue remodelling. *J Cell Sci*. 2009;122:2604–2612.
- den Hollander AI, ten Brink JB, de Kok YJ, et al. Mutations in a human homologue of *Drosophila* crumbs cause retinitis pigmentosa (RP12). *Nat Genet*. 1999;23:217–221.
- den Hollander AI, Heckenlively JR, van den Born LI, et al. Leber congenital amaurosis and retinitis pigmentosa with Coats-like exudative vasculopathy are associated with mutations in the crumbs homologue 1 (*CRB1*) gene. *Am J Hum Genet*. 2001;69:198–203.

31. den Hollander AI, Davis J, van der Velde-Visser SD, et al. CRB1 mutation spectrum in inherited retinal dystrophies. *Hum Mutat.* 2004;24:355-369.
32. Khaliq S, Abid A, Hameed A, et al. Mutation screening of Pakistani families with congenital eye disorders. *Exp Eye Res.* 2003;76:343-348.
33. Kennedy B, Malicki J. What drives cell morphogenesis: a look inside the vertebrate photoreceptor. *Dev Dyn.* 2009;238:2115-2138.
34. Gosens I, den Hollander AI, Cremers FP, Roepman R. Composition and function of the Crumbs protein complex in the mammalian retina. *Exp Eye Res.* 2008;86:713-726.
35. Malicki J, Neuhauss SC, Schier AF, et al. Mutations affecting development of the zebrafish retina. *Development.* 1996;123:263-273.
36. Wei X, Malicki J. *nagie oko*, encoding a MAGUK-family protein, is essential for cellular patterning of the retina. *Nat Genet.* 2002;31:150-157.
37. Malicki J, Jo H, Pujic Z. Zebrafish N-cadherin, encoded by the glass onion locus, plays an essential role in retinal patterning. *Dev Biol.* 2003;259:95-108.
38. Horne-Badovinac S, Lin D, Waldron S, et al. Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. *Curr Biol.* 2001;11:1492-1502.
39. Peterson RT, Mably JD, Chen JN, Fishman MC. Convergence of distinct pathways to heart patterning revealed by the small molecule concentrinamide and the mutation heart-and-soul. *Curr Biol.* 2001;11:1481-1491.
40. Avanesov A, Malicki J. Approaches to study neurogenesis in the zebrafish retina. *Methods Cell Biol.* 2004;76:333-384.
41. Jensen AM, Walker C, Westerfield M. *mosaic eyes*: a zebrafish gene required in pigmented epithelium for apical localization of retinal cell division and lamination. *Development.* 2001;128:95-105.
42. Jensen AM, Westerfield M. Zebrafish *mosaic eyes* is a novel FERM protein required for retinal lamination and retinal pigmented epithelial tight junction formation. *Curr Biol.* 2004;14:711-717.
43. Masai I, Lele Z, Yamaguchi M, et al. N-cadherin mediates retinal lamination, maintenance of forebrain compartments and patterning of retinal neurites. *Development.* 2003;130:2479-2494.
44. Grawe F, Wodarz A, Lee B, Knust E, Skaer H. The *Drosophila* genes *crumbs* and *stardust* are involved in the biogenesis of adherens junctions. *Development.* 1996;122:951-959.
45. Malicki J, Driever W. *oko meduzy* mutations affect neuronal patterning in the zebrafish retina and reveal cell-cell interactions of the retinal neuroepithelial sheet. *Development.* 1999;126:1235-1246.
46. den Hollander AI, Ghiani M, de Kok YJ, et al. Isolation of *Crb1*, a mouse homologue of *Drosophila* *crumbs*, and analysis of its expression pattern in eye and brain. *Mech Dev.* 2002;110:203-207.
47. Lotery AJ, Jacobson SG, Fishman GA, et al. Mutations in the CRB1 gene cause Leber congenital amaurosis. *Arch Ophthalmol.* 2001;119:415-420.
48. Easter SS Jr, Malicki JJ. The zebrafish eye: developmental and genetic analysis. *Results Probl Cell Differ.* 2002;346-370.
49. Richard M, Roepman R, Aartsen WM, et al. Towards understanding CRUMBS function in retinal dystrophies. *Hum Mol Genet.* 2006;15:R235-R243.
50. Aktekin M, Sargon MF, Cakar P, Celik HH, Firat E. Ultrastructure of the cornea epithelium in keratoconus. *Okajimas Folia Anat Jpn.* 1998;75:45-53.
51. Lohman LE, Rao GN, Aquavella JV. In vivo microscopic observations of human corneal epithelial abnormalities. *Am J Ophthalmol.* 1982;93:210-217.
52. Wong S, Rodrigues MM, Blackman HJ, Guimaraes R, Lemp MA. Color specular microscopy of disorders involving the corneal epithelium. *Ophthalmology.* 1984;91:1176-1183.
53. Miller CA, Krachmer JH. Epithelial and stromal dystrophies. In: Kaufman H, Barron B, McDonald MB, eds. *The Cornea*. Boston: Butterworth-Heinemann; 1998:411-452.
54. Miller CA, Krachmer JH. Endothelial dystrophies. In: Kaufman H, Barron B, McDonald MB, eds. *The Cornea*. Boston: Butterworth-Heinemann; 1998:453-475.
55. Wilson SE, He YG, Weng J, et al. Epithelial injury induces keratocyte apoptosis: hypothesized role for the interleukin-1 system in the modulation of corneal tissue organization and wound healing. *Exp Eye Res.* 1996;62:325-327.
56. Kim WJ, Rabinowitz YS, Meisler DM, Wilson SE. Keratocyte apoptosis associated with keratoconus. *Exp Eye Res.* 1999;69:475-481.
57. Sawaguchi S, Fukuchi T, Abe H, Kaiya T, Sugar J, Yue BY. Three-dimensional scanning electron microscopic study of keratoconus corneas. *Arch Ophthalmol.* 1998;116:62-68.
58. Teng CC. Electron microscope study of the pathology of keratoconus, I. *Am J Ophthalmol.* 1963;55:18-47.
59. Saihan Z, Webster AR, Luxon L, Bitner-Glindzicz M. Update on Usher syndrome. *Curr Opin Neurol.* 2009;22:19-27.
60. van den Hurk JA, Rashbass P, Roepman R, et al. Characterization of the Crumbs homolog 2 (CRB2) gene and analysis of its role in retinitis pigmentosa and Leber congenital amaurosis. *Mol Vis.* 2005;11:263-273.
61. McMahon TT, Kim LS, Fishman GA, et al. CRB1 gene mutations are associated with keratoconus in patients with Leber congenital amaurosis. *Invest Ophthalmol Vis Sci.* 2009;50:3185-3187.