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Drosophila Crumbs Is Required to Inhibit Light-Induced Photoreceptor Degeneration

Kevin Johnson, Ferdi Grawe, Nicola Grzeschik, and Elisabeth Knust[1]
Institut für Genetik
Heinrich-Heine-Universität Düsseldorf
Universitätsstrasse 1
40225 Düsseldorf
Germany

Summary

Mutations in the human transmembrane protein CRB1 are associated with severe forms of retinal dystrophy, retinitis pigmentosa 12 (RP12), and Leber's congenital amaurosis (LCA) [1–3]. The *Drosophila* homolog, *crumbs*, is required for polarity and adhesion in embryonic epithelia [4–6] and for correct formation of adherens junctions and proper morphogenesis of photoreceptor cells [7, 8]. Here, we show that mutations in *Drosophila crumbs* result in progressive, light-induced retinal degeneration. Degeneration is prevented by expression of p35, an inhibitor of apoptosis, or by reduction of rhodopsin levels through a vitamin A-deficient diet. In the dark, rhabdomeres survive but exhibit morphogenetic defects. We demonstrate that it is the extracellular portion of the Crumbs protein that is essential to suppress light-induced programmed cell death, while proper morphogenesis depends on the intracellular part. We conclude that human and *Drosophila* Crumbs proteins are functionally conserved to prevent light-dependent photoreceptor degeneration. This experimental system is now ideally suited to study the genetic and molecular basis of RP12- and LCA-related retinal degeneration.

Results and Discussion

Drosophila embryos mutant for *crumbs* (*crb*) fail to maintain a proper zonula adherens (ZA), a belt-like adhesive structure encircling the apex of epithelial cells [9, 10]. As a consequence, cells lose contact with each other and undergo programmed cell death (PCD) in several epithelia. The human homolog CRB1 displays a similar overall organization to the *Drosophila* protein [1], and its cytoplasmic tail can functionally substitute for the fly protein in the *Drosophila* embryo [11]. Mutations in CRB1 are associated with progressive degeneration of the retina in patients suffering from RP12 or LCA [1–3]. RP12 is characterized by an early onset of retinal degeneration and the loss of vision in patients before the age of 20 years, while LCA, the earliest and most severe form of retinal dystrophy, causes blindness at birth or during the first months of life. This connection of structural and functional preservation prompted us to assay *crumbs* mosaic eyes for any phenotype relating to retinal degeneration.

[1]Correspondence: knust@uni-duesseldorf.de

Crumbs Is Expressed in the Stalk Membrane of the Adult Eye

In a first series of experiments, we analyzed the pattern of Crumbs expression in the adult eye. Immunohistochemistry revealed that Crumbs protein is found in a circumferential stripe at the apical sides of the photoreceptor cells, bordering the ZA, which is visualized as a circumferential belt by anti-Armadillo staining (Figures 1A–1C). Within the apical domain, Crumbs is localized to the apical stalk membrane, which connects the ZA with the rhabdomeres (Figure 1E; also see Figure 2G for the demonstration of the stalk membrane). The rhabdomeres are also apical derivatives containing highly pleated stacks of microvilli, rich in F-actin, that carry the photosensitive pigment rhodopsin and the phototransduction complexes.

Loss of *crb* in the Eye Leads to Gradual, Light-Induced Retinal Degeneration

Since, in *Drosophila*, hereditary retinal degenerations are light dependent in several cases [12], we analyzed mosaic eyes containing large *crb* mutant clones of flies kept under constant illumination. The *Drosophila* eye is composed of about 800 ommatidia, cylindrical, barrel-like structures, containing eight photoreceptor cells in their center that are arranged in a stereotypic manner (Figures 2A, 2E, and 2G). When flies carrying crb^{11A22} mosaic eyes are kept in constant light for 7 days, the retina shows massive degeneration (Figure 2B). This phenotype strictly depends on continous exposure to light, since, in flies kept under standard laboratory conditions, i.e., in artificial low light, no degeneration of photoreceptors occurred. *crb* mosaic flies raised under these conditions show a slightly variable, mutant phenotype. Their rhabdomeres are thicker and shorter compared to wild-type and are often found in close contact with other rhabdomeres of the same ommatidium (Figures 2C, 2F, and 2H). Serial cross-sections (Figures 2C and 2D) and horizontal sections stained with FITC-phalloidin, which highlights the F-actin bundles of the rhabdomeres (Figure 1D), reveal that the rhabdomeres fail to reach the basal lamina and extend from the distal pole near the lens to only about one third of the normal length. In addition, the stalk membrane is reduced in length (Figures 2F and 2H). However, the tightly stacked "semicristalline" internal structure of the rhabdomere is unaffected. The catacomb-like rhabdomere base [13] is also unaffected in mutant photoreceptor cells. In many ommatidia, ZAs are visible in the distal regions of the cells (Figures 2F and 2H). Control of morphogenesis seems to be cell autonomous, since ommatidia composed of wild-type and mutant cells only exhibit defects in the mutant cells (Figures 1F and 2C).

To analyze the temporal course of degeneration, eyes carrying mutant clones were sectioned at different time points after light exposure. Eyes kept for only 1 day in constant illumination do not show any sign of photoreceptor degeneration but only exhibit the morphoge-

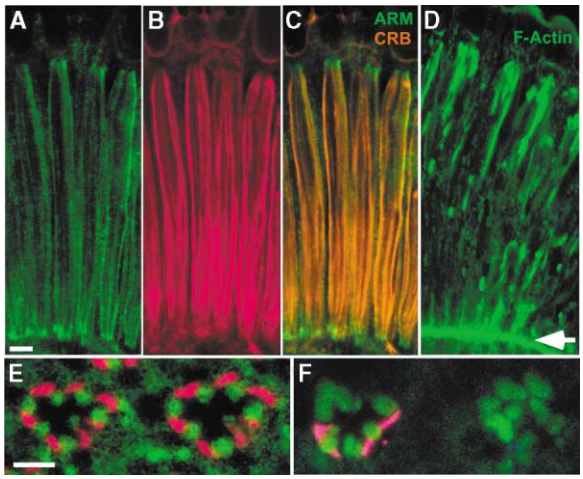

Figure 1. Expression of Crumbs in Adult Eyes and Morphogenetic Defects in *crb* Mutant Ommatidia

(A–D) (A–C) Horizontal optical sections of wild-type adult retinae stained with anti-Crumbs (red) and anti-Armadillo (green) and (D) crb^{11A22} eyes stained with FITC-phalloidin. Crumbs and Armadillo extend along the entire length of the photoreceptor apex in nonoverlapping belts. The distal and proximal ZA contacts between cone cells and photoreceptors are enriched for Armadillo. The highest F-actin concentration is seen in rhabdomeres and the proximal feet of cone and pigment cells (arrow). Rhabdomeres of mutant cells are short and thick and do not reach the basal lamina.

(E and F) Optical cross-sections through (E) wild-type and (F) crb^{11A22} mosaic ommatidia of adult eyes, stained with anti-Crumbs (red) and FITC-phalloidin (green). Crumbs is localized in the stalk membrane adjacent to the rhabdomeres. In (F), note that the mosaic ommatidia (left) is composed of mutant and wild-type cells.

The scale bars represent 10 μm in (A)–(D) and 5 μm in (E) and (F). (A–D) The distal pole points to the top and contains the lens.

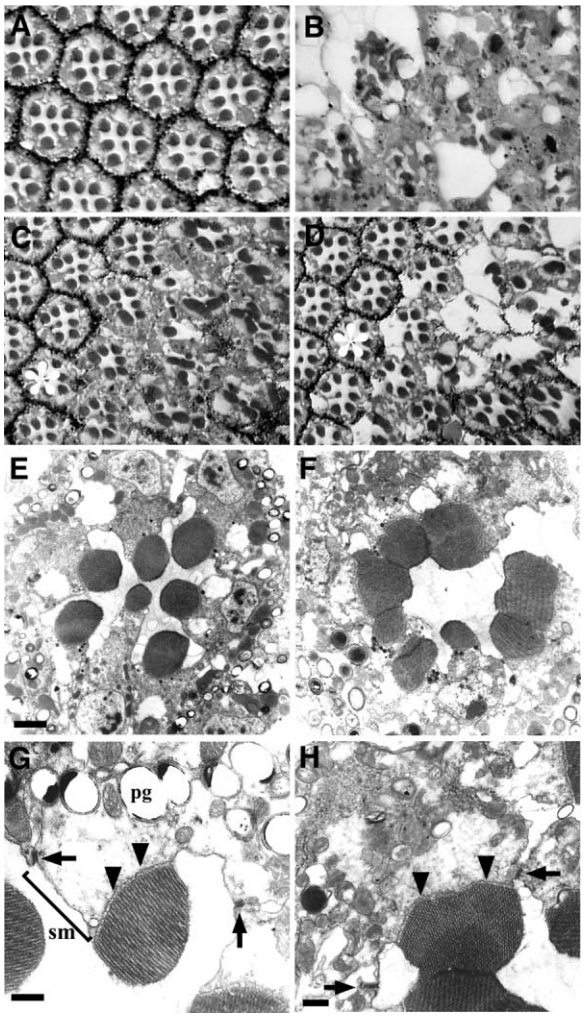

Figure 2. Mutant Phenotypes in adult crb^{11A22} Ommatidia

(A and B) Cross-sections through (A) wild-type and (B) crb^{11A22} mutant eyes kept in constant illumination for 7 days. Most mutant photoreceptor cells have degenerated.

(C and D) (C) Distal and (D) more proximal cross-sections (15 μm apart) through the same crb^{11A22} mosaic eye kept under low-light conditions (5 weeks old). Wild-type ommatidia are identifiable by the presence of pigment granules in the surrounding pigment cells. Note the absence of most mutant rhabdomeres in (D) at a sectional level of less than 50% (the asterisk in [C] and [D] indicates the same ommatidium).

(E–H) Electron micrographs of distal sections of (E and G) wild-type and (F and H) crb^{11A22} mutant ommatidia. Mutant rhabdomeres are thicker and often make contact with each other. (H) The stalk membrane (sm; bracket) is reduced in size in mutant photoreceptor cells. The ZAs (arrow) between photoreceptor cells are present in the distal region of most mutant ommatidia. The tightly packed microvilli in the rhabdomere and the rhabdomere base (arrowhead) appear to be unaffected.

The scale bars represent 2 μm in (E) and (F) and 1 μm in (G) and (H); pg, pigment granule.

netical defects described above (compare Figures 2F, 2H, and 3A). After 5 days in constant light, each ommatidium contains one or two photoreceptor cells with signs of degeneration (Figure 3B). In ommatidia exposed to constant light for 7 days, most of the photoreceptor cells are degenerating (Figures 2B and 3C). Signs of degeneration include the devolution of the highly pleated microvillar rhabdomere structure, concomitant with the loss of the catacomb-like rhabdomere base. Affected nuclei round up, and the nucleolus, nucleoplasm, and cytoplasm appear condensed (Figures 3B–3D). These features are indicative of PCD. In order to support PCD as the mechanism involved, we assayed whether overexpression of the baculovirus-derived p35 survival protein can inhibit light-induced photoreceptor cell death in crb^{11A22} mutant ommatidia. It is well established that PCD involves a conserved cascade of cysteine proteases, the caspases, many of which can be inhibited by the ectopic expression of p35 protein. During *Drosophila* eye development, all stages of endogenous, pattern-forming PCD can be inhibited by using a transgene expressing p35 [14]. Similarly, age-related and light-induced retinal degeneration in flies mutant for $ninaE^{RH27}$ (rhodopsin1), $rdgC^{306}$ (rhodopsin phosphatase), $norpA^{EE5}$ (phospholipase C), or *arr2(P261S)* (arrestin) can be prevented by overexpressing p35 survival protein [15, 16]. We found that overexpression of p35 protein rescues light-induced degeneration of crb^{11A22} photoreceptor cells, prevents devolution of the rhabdomeres, and preserves the rhabdomere bases (Figures 3E and 3F). Even when exposed to constant illumination for 14 days, the rescue by p35 expression is similar (not shown), implicating PCD as the downstream conse-

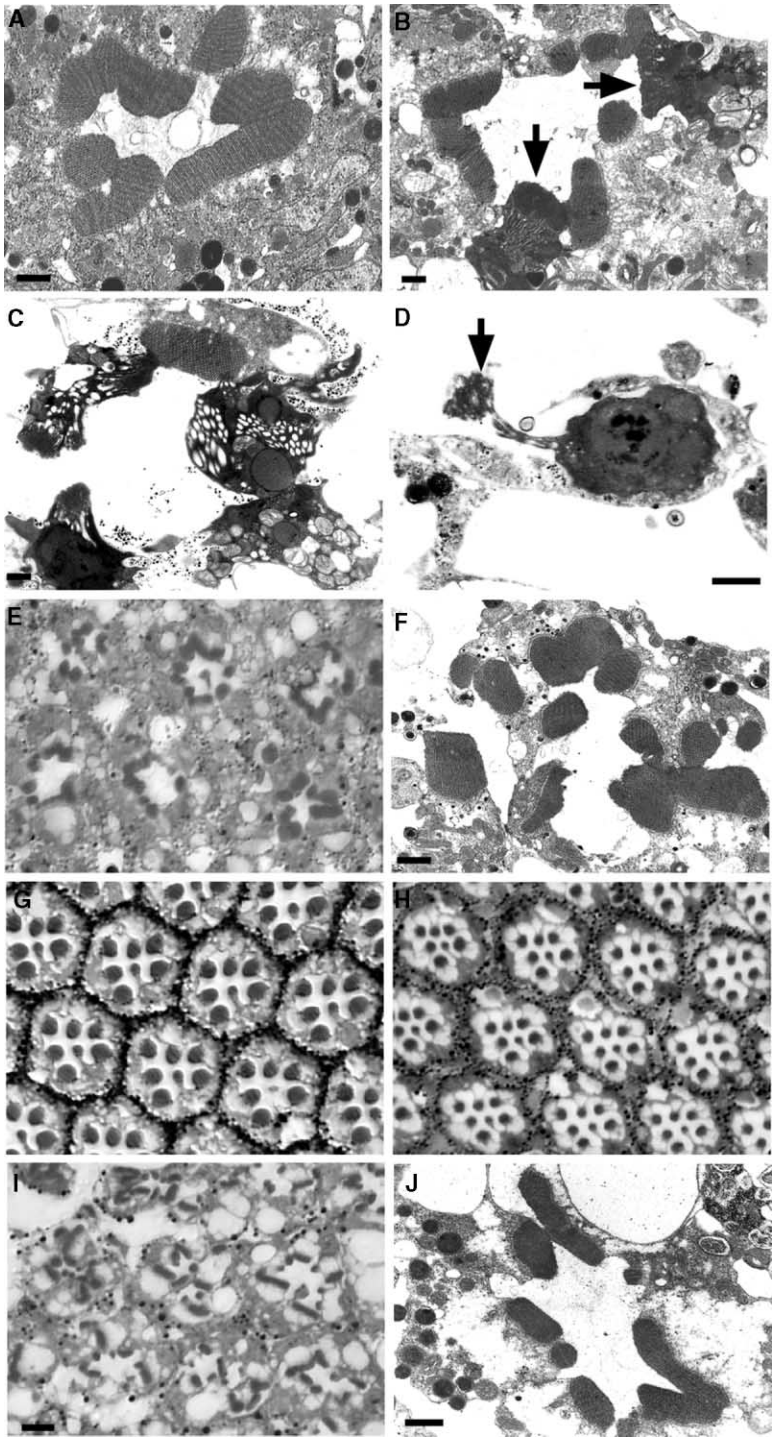

Figure 3. Light-Induced Retinal Degeneration in *crb*^{11A22} Photoreceptor Cells and Its Rescue by Overexpression of p35 and through a Vitamin A-Deficient Diet

(A–D) Electron micrographs of distal cross-sections through *crb*^{11A22} adult ommatidia exposed to constant light for (A) 1 day, (B) 5 days, or (C and D) 7 days. While photoreceptor cells show morphogenetic defects, no degeneration is initially observed. However, in time, progressively more photoreceptors show signs of PCD. (D) This panel illustrates higher magnification of a single photoreceptor cell to demonstrate a round nucleus with condensed nucleoplasm, a condensed cytoplasm, and remnants of the rhabdomere in devolution (arrow). It seems to be engulfed by a cytoplasmic finger of a neighboring cell. (E and F) Distal cross-sections through *crb*^{11A22} ; GMR-p35 eyes exposed to constant light for 7 days. Many photoreceptors survive but show similar morphogenetic defects to *crb*^{11A22} eyes kept in low light (compare with Figures 2C and 2F).

(G–J) Sections of (G and H) wild-type and (I and J) *crb*^{11A22} eyes of flies raised on (G) standard or on (H–J) vitamin A-deficient medium [18], after 7 days under constant illumination. Note that rhabdomere size is significantly reduced in (H)–(J). Photoreceptor cells of *crb*^{11A22} ommatidia survive, and rhabdomeres are present. They exhibit the *crb*-specific morphogenetic defects (compare with Figures 2C and 2F).

The scale bars represent 5 μm in (E) and (G)–(I); 2 μm in (A)–(C), (F), and (J); and 1 μm in (D).

quence of *crb*-induced retinal degeneration. Together these results suggest that *crb* mediates two functions in photoreceptor cells. One function controls the proper morphogenesis of the rhabdomeres and allows the formation of highly elongated (>100 μm) cells [7, 8] (this study). The other function is required after eclosion for survival of photoreceptor cells when exposed to light.

Since Crumbs is not expressed in the rhabdomere, it cannot directly participate in the signal transduction process, which raises the question of how Crumbs controls cell survival. Recently, a mechanism has been put forward that explains retinal degeneration in a subset of retinal degeneration mutations (*arr2*, *norpA*, *rdgB*, and *rdgC*) as a result of abnormally stable, light-induced meta-rhodopsin/arrestin complexes. In mutant eyes, these stable complexes are internalized by clathrin-mediated endocytosis [16, 17]. The accumulation of internal meta-rhodopsin/arrestin complexes triggers PCD

through an unknown mechanism. In these mutants, PCD is prevented if internalization is inhibited in a *shibire* (D-dynamin) mutant background or when larvae are raised on a vitamin A-deficient medium. The depletion of vitamin A reduces the amount of rhodopsin to about 3% of its normal amount [18], which leads to the development of normal, though smaller, rhabdomeres in wild-type flies (Figure 3H).

To test whether light-induced degeneration in *crb* mutant eyes is based on a similar mechanism, we raised crb^{11A22} mosaic flies on this vitamin A-deficient medium and exposed them to continuous room light. Mutant photoreceptor cells show smaller, thinner rhabdomeres due to the reduced amount of rhodopsin. They also show morphogenetic defects similar to those of crb^{11A22} ommatidia raised on standard medium and kept in the dark (compare Figures 3I and 3J with Figures 2C and 2F). However, we found only minor signs of photoreceptor degeneration (compare Figures 3I and 2B). This finding is consistent with the possibility that PCD in illuminated crb^{11A22} photoreceptor cells is due to the increased internalization of meta-rhodopsin/arrestin complexes. Proof of this will require a direct determination of the number of complexes formed, their localization, and the possible involvement of endocytosis in *crb*-mediated degeneration.

The Extracellular Domain of Crumbs Is Essential for Survival of Photoreceptor Cells

For the embryo, it is well documented that the short intracellular domain of Crumbs is crucial for proper function since its truncation leads to a complete loss of function phenotype [19]. The cytoplasmic domain recruits the MAGUK protein Stardust and the four PDZ-domain protein Discs Lost [20] into a subapical protein scaffold (SAC) [6, 21, 22]. Overexpression of the membrane-bound cytoplasmic domain ($Crumbs_{intra}$) is sufficient to achieve a partial rescue of *crb* embryos, and the degree of rescue is comparable to that obtained by overexpression of the full-length Crumbs protein [5, 6]. In order to determine which part of the Crumbs protein is necessary to prevent retinal degeneration, we induced clones that are homozygous mutant for the crb^{8F105} allele. The crb^{8F105} allele encodes a protein that lacks the C-terminal 23 amino acids of the cytoplasmic domain and is completely nonfunctional in the embryo [19]. The morphogenetic phenotype observed in mosaic eyes of crb^{8F105} is comparable to that of the crb^{11A22} null allele kept in the dark (compare Figures 4A and 2C). In contrast to crb^{11A22}, however, crb^{8F105} mutant ommatidia do not show major signs of degeneration, even after 14 days of exposure to light (Figure 4B). This indicates that either the extracellular domain or the N-terminal portion of the cytoplasmic tail of Crumbs prevents light-induced degeneration. To discriminate between these two possibilities, crb^{8F105} and crb^{11A22} mutant ommatidia were analyzed in the presence of a transgene expressing the membrane-bound cytoplasmic domain of Crumbs. This transgene largely rescues the morphogenetic defects in mosaic eyes of both alleles under low-light conditions. This is manifested by the fact that many rhabdomeres are elongated to reach the basal lamina (Figures 4C and 4E and data not shown). In contrast, crb^{11A22} photoreceptor cells exposed to light still undergo retinal degeneration despite the expression of the cytoplasmic domain (Figures 4D and 4F). We conclude that the intracellular domain of Crumbs is not sufficient to prevent light-induced photoreceptor degeneration and suggest a function for the extracellular domain in the prevention of PCD. Interestingly, all mutations mapped to the *CRB1* gene in RP12 or LCA patients have been localized to the extracellular portion of the protein and lead either to amino acid exchanges, frame shifts, or stop codons [1–3]. While this may simply reflect the fact that the intracellular domain is a small target for mutagenesis, the large number of RP12 cases showing exclusive amino acid exchanges extracellularly indicate a requirement of an intact extracellular CRB1 domain.

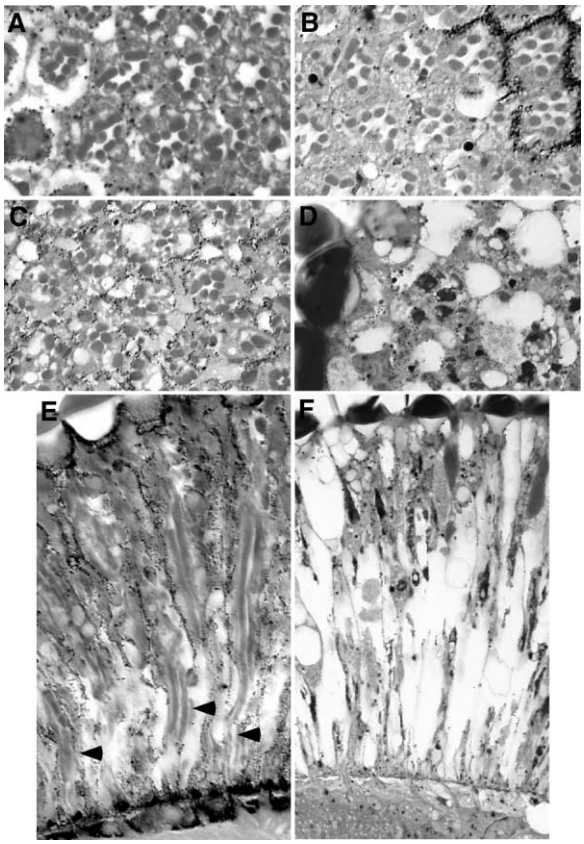

Figure 4. Different Requirements of Crumbs Protein Domains in the Eye

(A and B) Cross-section of crb^{8F105} mosaic eyes kept in the (A) dark and (B) exposed to constant light for 14 days. Mutant ommatidia are recognized by the absence of pigment granules. Morphogenetic defects are observed in both cases, but no light-induced degeneration occurs (compare to Figures 2B and 2C).

(C–F) (C and D) Cross-section and (E and F) transverse section of (C and E) crb^{8F105} ; GMR-crb_{intra} eyes kept in the dark and (D and F) crb^{11A22} ; GMR-crb_{intra} eyes exposed to constant light for 7 days. While morphogenetic defects in crb^{8F105} ommatidia are largely rescued by the expression of the intracellular domain (note that many rhabdomeres reach the basal lamina [arrowheads]), crb^{11A22}-associated retinal degeneration is not rescued.

The scale bar represents 5 μm in (A)–(D) and 10 μm in (E) and (F). Note that (C) is a proximal section, while (A), (B), and (D) are distal sections close to the lens.

The data shown here provide striking support for a functional conservation between human *CRB1* and *Drosophila crumbs*. It has recently been reported that the cytoplasmic domain of CRB1 can substitute for the lack of *crb* function in the *Drosophila* embryo. Expression of $CRB1_{intra}$ rescues epidermal cell polarity and PCD of a *crb* mutant embryo to the same degree as the corresponding region of the *Drosophila* protein [11]. Similarly, overexpression of either the *Drosophila* or the human cytoplasmic domain in the embryo or the eye provokes the same dominant phenotype [8, 11]. In the eye, the cytoplasmic tail of Crumbs is required for proper morphogenesis of photoreceptor cells, suggesting that this domain may be involved in the organization of an apical protein scaffold; this is similar to what occurs in embryonic epithelia. Strikingly, the stalk membrane of photoreceptor cells and the localization of the SAC in epithelial cells correspond spatially, apical to the ZA, and by the enrichment of Crumbs protein. The recently shown presence of Crumbs and β_{heavy}-spectrin in the same protein complex suggests that both proteins cooperate in the formation of the stalk membrane [7]. Our data now indicate that the functional conservation also extends to the extracellular domains of human CRB1 and *Drosophila* Crumbs, which are both required for the survival of their respective photoreceptor cells. We can only speculate whether it is the interaction with an extracellular ligand in the interrhabdomeral space or a transmembrane protein on the photoreceptor cell that is responsible to prevent PCD under visual stress.

It is particularly evident that retinal degeneration in RP12 patients is a continuously progressing process, leading from night blindness to a restricted field of view to the subsequent loss of vision. It is tempting to speculate that daylight might have a deleterious effect on disease progression. In mosaic eyes lacking Crumbs protein in *Drosophila*, we observe a progressive degeneration of photoreceptor cells under continuous visual stress above a minimal intensity (K.J. and E.K., unpublished data). Thus, an intriguing speculation would be that reduced amounts and/or intensities of daylight might be beneficial to RP12 patients.

Our results in *Drosophila* further indicate that the depletion of rhodopsin through a vitamin A-deficient diet prevents the light-induced crb^{11A22} degeneration of the retina. A randomized study of ungenotyped RP patients, given high-dose oral vitamin A supplementation, suggested a modest slowing of the disease progression [23], although the interpretation is controversial [24]. Our finding may indicate that, at least for *CRB1*-induced RP12 and LCA cases, a high-dose vitamin A supplementation might be counterproductive rather than beneficial in slowing disease progression.

Both assumptions on the progression of the disease should be tested in a vertebrate animal model, i.e., a knockout mouse. The *Drosophila* system, on the other hand, can now be exploited to unravel the underlying mechanism of *crb*-dependent retinal degeneration.

Experimental Procedures

Fly Strains and Clonal Analysis

crumbs mosaic eyes were generated by crossing *y w* eyFLP2 glass-lacZ; FRT82B w^+ cl3R3/TM6B females [25] to FRT82B crb^{11A22}/TM6B or FRT82B crb^{8F105}/TM6B males. The P(GMRp35)-X1 chromosome (donated by B. Hay) was crossed into the FRT82B crb^{11A22}/TM6B background to block caspase activity and suppress PCD [14–16]. GMR-crb_{intra} transgenic lines were generated by subcloning crb_{intra} [5] into pGMR [14]. Germline transformation was done according to standard protocols [26]. The mild GMR-crb_{intra}-X35.2 insertion on the second chromosome was used in Figures 4C–4F. Light-induced retinal degeneration was analyzed after exposure of flies to constant room light (neon and incandescent light combined). The total intensity was 17 $\mu mol/m^2\ s^1$ of photosynthetically active radiation (380–710 nm) measured by a quantum sensor. Flies were kept at 25°C. For vitamin A depletion experiments, larvae were raised and flies were maintained on vitamin A-deprived food [18].

Electron Microscopy

Sections were done according to [27], with modifications. In brief, 0.1 M phosphate buffer (pH 7.4) was used to fix bisected heads in 25% glutaraldehyde, followed by simultanous fixation in 1% osmium/2% glutaraldehyde, followed by 2% osmium fixation. After dehydration, eyes were embedded in araldite, and 2.5 μm semi-thin sections were taken with a Reichert OM U2 microtome, followed by toluidine staining. Ultrathin sections were 0.1 μm thin and were contrasted and analyzed with a Zeiss EM9 S2.

Immunohistochemistry and Confocal Microscopy

For immunolabeling, the following method was used (adapted from [28]): adult eyes (1–4 days posteclosion) were dissected and fixed in 8% formaldehyde/75 mM PIPES/15% picric acid. Subsequent antibody staining with mouse anti-Crb-Cq4 (1:5) and rabbit anti-$ArmN_2$ (1:100) was performed as described in [6, 28]. Combinatory F-actin staining was achieved by initial stabilization with phalloidin, followed by FITC-phalloidin staining. Whole-mount confocal images are z-series of 5 μm depth projected into a single image taken with a Leica TCS NT confocal microscope.

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