Mutant prominin 1 found in patients with macular degeneration disrupts photoreceptor disk morphogenesis in mice

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Familial macular degeneration is a clinically and genetically heterogeneous group of disorders characterized by progressive central vision loss. Here we show that an R373C missense mutation in the prominin 1 gene (PROM1) causes 3 forms of autosomal-dominant macular degeneration. In transgenic mice expressing R373C mutant human PROM1, both mutant and endogenous PROM1 were found throughout the layers of the photoreceptors, rather than at the base of the photoreceptor outer segments, where PROM1 is normally localized. Moreover, the outer segment disk membranes were greatly overgrown and misoriented, indicating defective disk morphogenesis. Immunoprecipitation studies showed that PROM1 interacted with protocadherin 21 (PCDH21), a photoreceptor-specific cadherin, and with actin filaments, both of which play critical roles in disk membrane morphogenesis. Collectively, our results identify what we believe to be a novel complex involved in photoreceptor disk morphogenesis and indicate a possible role for PROM1 and PCDH21 in macular degeneration.

Introduction

Retinal photoreceptor cells are highly specialized. One compartment, organized as an outer segment (OS) in vertebrates, contains a stack of over 1,000 disks harboring rhodopsin and the phototransduction machinery. The entire OS is renewed by the continuous formation of new disks at its base and the shedding of older disks from its distal end (1). In humans and most other primates, the region comprising about 20° in the center of the retina is known as the macula. Both rod and cone photoreceptor cells have regions of highest density in the macula, so that the macula provides high-acuity vision.

While genetically diverse and rare in prevalence, focus on familial macular degeneration has most often identified disease-causing gene mutations affecting photoreceptor and retinal pigment epithelial (RPE) cell function (2–7). Previously, we mapped 2 inherited autosomal-dominant macular degeneration phenotypes to an overlapping region on chromosome 4p, a Stargardt-like macular dystrophy (Stargardt disease 4 [STGD4]; OMIM 603786; ref. 8) and bull’s-eye macular dystrophy (MCDR2; OMIM 608051; ref. 9). STGD4 is characterized by bilateral, symmetric, atrophic lesions in the macula and by the presence of yellow fundus flecks, whereas MCDR2 is characterized by bilateral annular RPE atrophy at the macula (9).

In the present study, genetic screening and mutation analysis of the 12 genes residing within this overlapping disease interval revealed an R373C missense mutations in the prominin 1 gene (PROM1; GenBank accession no. AF027208, also known as CD133). PROM1 encodes a 5-transmembrane domain protein containing 2 large, highly glycosylated extracellular loops and a cytoplasmic tail (10). It is specifically associated with plasma membrane protrusions (11, 12), but its function and specific molecular interactions remain largely unknown. It is present in many tissues. In the retina, PROM1 is found at the base of the photoreceptor OSs, where the new disk membranes are formed, and has previously been linked to autosomal-recessive retinal degeneration (13, 14).

In order to study the cellular basis for macular degeneration caused by mutant PROM1, as well as the molecular function of PROM1 in photoreceptor cells, we generated transgenic mice carrying the R373C mutant PROM1 and studied the ensuing pathogenesis. Additionally, we carried out molecular interac-

Nonstandard abbreviations used: CNGCA1, rod cyclic nucleotide gated channel alpha subunit coo; ERG, electroretinography; IS, inner segment; LOD, logarithm of odds; MCDR2, bull’s-eye macular dystrophy; ONL, outer nuclear layer; OS, outer segment; PCDH21, protocadherin 21; PROM1, prominin 1; RPE, retinal pigment epithelial/epithelium; STGD4, Stargardt disease 4.

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Results

Identification of the PROM1 mutation in STGD4, MCDR2, and cone-rod dystrophy pedigrees. Previously, we identified family pedigrees showing STGD4, clinically characterized by macular dystrophy and yellow fundus flecks (8), and an autosomal-dominant MCDR2, characterized by RPE atrophy (9) (Figure 1A and Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI35891DS1). In this study we narrowed the disease interval to a 12-cM region between markers D4S1582 and GATA1582G03. We screened 12 candidate genes and identified a R373C missense mutation in PROM1 in both phenotypes (Figure 1B). We then identified the same R373C mutation in a third pedigree with an autosomal-dominant cone-rod dystrophy characterized by both cone and rod photoreceptor degeneration (Supplemental Figure 1C). Comparison of disease haplotypes revealed that affected members in the STGD4, MCDR2, and cone-rod dystrophy families had distinct disease haplotypes, indicating that the PROM1 mutation in each family arose independently (Supplemental Figure 1C). The log of odds (LOD) score in each family was 7.7, 2.1, and 4.4, respectively, and the combined LOD score of the 3 families was 14.2 at \( \theta \) of 0 with marker D4S403. The mutation segregated with affected individuals in each family (LOD score 5.8, 1.7, 4.3; combined LOD, 11.8 at \( \theta \) of 0), and was absent in 400 normal matched controls. PROM1 was expressed in both cone and rod photoreceptors (Supplemental Figure 2).

Generation of transgenic mice expressing WT PROM1 and the R373C human PROM1 mutation. To gain insight into the molecular mechanism of retinal degeneration caused by mutant PROM1, we generated transgenic mice carrying the R373C mutant human PROM1 mutation under the control of the 4.4-kb rhodopsin promoter (15). Expression of the human PROM1 transgene in the retina was verified by Western blotting (Supplemental Figure 3A). As a control for overexpression of PROM1 in mice, we also generated transgenic mice expressing human WT PROM1. We characterized 2 human WT (PWT20 and PWT31) and 2 mutant (PMT3 and PMT14) PROM1 transgenic mouse lines. By Western blot we found that the relative expression levels of WT (PWT20 and PWT31) and mutant (PMT3 and PMT14) human PROM1 transgenes were 1-, 2-, 8-, and 2-fold, respectively, that of endogenous mouse Prom1 expression (Supplemental Figure 3B).

WT and mutant PROM1 transgenic mouse phenotypes. Fundus images and light microscopy of retinal sections from transgenic mice expressing mutant PROM1 revealed progressive retinal abnormalities visible as subretinal deposits and photoreceptor atrophy (Figure 1C and Supplemental Figure 4). Compared with PWT20 mice, PMT14 mice showed fundus changes characteristic of changes seen in humans with Stargardt disease. Yellow deposits and small lesions (Figure 1C, yellow arrows) were scattered throughout the
4 months of age, the remaining photoreceptors contained either membranes were evident in PMT3 retinas (Figure 2A, arrows). By after birth, during photoreceptor development, overgrown disk membranes were evident in the RPE of PMT3 mice at 4 months of age. Accumulation of lipofuscin-like material (arrows) was evident. (C) Electron micrograph showing abnormalities in the RPE of PMT3 mice at 4 months of age. Accumulation of lipofuscin-like material (arrows) was evident. (D) Electron micrograph of a WT retina, showing normal OS disk membranes and adjacent RPE. Scale bars: 1 μm (A, B, and D); 300 nm (C); 30 nm (B, inset).

Figure 2
Electron microscopy of PROM1 transgenic mouse rod photoreceptors. (A and B) Overgrown and abnormally oriented disk membranes (arrows) in PMT3 retinas were visible at 16 days (A) and 4 months (B) of age. Arrowheads indicate vesiculated membrane. Higher-magnification view of the boxed region in B is shown in the inset. (C) Electron micrograph showing abnormalities in the RPE of PMT3 mice at 4 months of age. Accumulation of lipofuscin-like material (arrows) was evident. (D) Electron micrograph of a WT retina, showing normal OS disk membranes and adjacent RPE. Scale bars: 1 μm (A, B, and D); 300 nm (C); 30 nm (B, inset).

central fundus in 4-month-old PMT14 mice. By 13 months of age, large coalescing deposits and atrophic lesions (Figure 1C, black arrows) covered much of the fundus. With fluorescein angiography, the atrophic lesions showed hyperfluorescence at the earlier phase and hypofluorescence in the late phase, indicating a window defect and RPE atrophy (data not shown).

Mutant PROM1 transgenic mice exhibited progressive photoreceptor degeneration, measured by loss of photoreceptor nuclei in the outer nuclear layer (ONL; Figure 1D and Supplemental Figure 4). The rate of photoreceptor loss correlated with expression levels in the outer nuclear layer (ONL; Figure 1D and Supplemental Figure 4). The rate of photoreceptor loss correlated with expression levels in the mutant PROM1 mouse lines, with 50% of photoreceptors lost from the regions located 200–300 μm dorsal and ventral to the optic nerve head at 4 weeks in PMT14 mice and 7 weeks in PMT3 mice (Figure 1D). In contrast, higher levels of WT PROM1 transgene expression did not cause retinal abnormalities or degeneration (Figure 1D and Supplemental Figures 4 and 5).

Electron microscopy revealed that most of the rod photoreceptor OSs of mutant PROM1 retinas were disorganized. By 16 days after birth, during photoreceptor development, overgrown disk membranes were evident in PMT3 retinas (Figure 2A, arrows). By 4 months of age, the remaining photoreceptors contained either vesiculated OS membranes (Figure 2B, arrowheads) or stacks of disk membranes that were of excessive size and aligned perpendicular to normal disk orientation (Figure 2B, arrows and inset). The OSs of a WT retina are shown in Figure 2D. In the RPE, there were abnormal lipofuscin-like deposits (Figure 2C, arrows), indicating that this cell type was also compromised. Such deposits were not present in 4-month-old control (i.e., WT or PWT20) mice.

Electroretinography (ERG) recordings were performed to assess retinal function in PM14 mice (Supplemental Figure 5A). The rod and cone ERG b-waves were both significantly reduced relative to normal C57BL/6 controls (t test, 2.9; P < 0.01) and PWT20 mice (t test, 2.1; P < 0.05) at 3 months of age, indicating that both rod and cone photoreceptor cells are affected by mutant PROM1. B-wave responses were not detectable in the PM14 line at 9 months of age. Correspondingly, maximum high-intensity rod a-wave responses, which are proportional to the total number of functioning photoreceptor disks, were reduced by an average of 50% at 3 months and were not detectable at 9 months of age (Supplemental Figure 5B).

WT and mutant PROM1 localization was examined by immunohistochemistry in transgenic and control mouse retinas. PROM1 was previously observed by immunoelectron microscopy to be localized to the nascent disk membranes at the base of rod OSs in control retinas (13). Our immunofluorescence images were consistent with this finding; label was restricted to a narrow region that corresponded to base of the photoreceptor OSs (Figure 3, A–C). In PWT20 mice, PROM1 showed a similar localization pattern (Figure 3, D–F). Conversely, in PMT3 and PMT14 mice, mutant PROM1 was found most strongly in the myoid region of photoreceptors, a site containing predominantly ER, and throughout photoreceptors extending from OSs to the synaptic termini (Figure 3, G and J). The endogenous mouse Prom1 in these mutant retinas appeared to show mislocalization throughout the photoreceptor layers (Figure 3, H and K). Nevertheless, PROM1 immunofluorescence should be interpreted with caution, because Western blot analysis showed low levels of PROM1 expression in PMT3 and PMT14 retinas (Supplemental Figure 3C).

Protocadherin 21 localization in PROM1 transgenic mice. Protocadherin 21 (PCDH21; GenBank accession no. AB053448), previously shown to also localize to the nascent disks at the base of the OSs, is thought to play a critical role in disk membrane morphogenesis (16). Based on its location similar to that of PROM1, we immunolabeled PCDH21 in WT and mutant PROM1 transgenic mice. In PWT20 mice, PCDH21 colocalized with WT PROM1 at the base of OSs (Figure 3, M–O). In mutant PROM1 retinas, PCDH21 was mislocalized in a pattern similar to that of PRO1 throughout photoreceptors (Figure 3, P–R). In contrast, immunolocalization of retinal OS membrane protein 1 (ROM1; found in the rims of the mature disk membranes; refs. 17, 18), rod cyclic nucleotide gated channel alpha subunit cook (CNGCA1; an OS marker; ref. 19), and Na+/K+-ATPase (an inner segment [IS] marker; ref. 20) proteins were not disturbed in mutant PROM1 mice (Supplemental Figure 6), indicating that mutant PROM1 does not have a generalized effect on intracellular vectorial or compartmental transport.

Based on these results, we also examined immunolocalization of the murine Prom1 in Pcdh21+/− mice. Pcdh21+/− mice were generated previously and were shown to form disorganized OSs and undergo photoreceptor degeneration (16). Using the mouse-specific Prom1 antibody, we found WT Prom1 mislocalized throughout OSs in the absence of Pcdh21 (Figure 3, S–U). Similar to the mutant PROM1 transgenic mice, the Pcdh21+/− mice
did not show perturbations in CNGCA1 (Figure 3T) or Na+/K-ATPase (Figure 3U) locations in the OS or IS photoreceptor regions, respectively. These results suggest that the interaction of these proteins is required for normal Prom1 localization to the base of OSs and that this interaction plays a role in normal disk morphogenesis.

**Physical interaction of PROM1 and PCDH21.** Migration of WT PROM1 on SDS gel reflected the predominance of the glycosylated form (~114 kDa) when HEK293 cells were transfected with PROM1 constructs (Figure 4A, lane 1). Infrequently, an additional faster-moving PROM1 polypeptide (~105 kDa) was detected (Figure 4A, lane 3).

Because PCDH21 was mislocalized with mutant PROM1 in transgenic mice, we carried out coimmunoprecipitation studies to determine whether PROM1 and PCDH21 directly interact. HEK293 cells were cotransfected with either WT or mutant PROM1 and a PCDH21-Myc fusion construct. Interestingly, we found that WT and mutant PROM1 coimmunoprecipitated with PCDH21 (Figure 4A, lanes 2 and 4). Reciprocally, PCDH21 coimmunoprecipitated with the human PROM1 antibody (Figure 4B, lanes 2 and 3). To confirm their in vivo interaction, the PCDH21 antibody was used in immunoprecipitation reactions with WT and mutant PROM1 transgenic mouse retinal lysates. Western blot analysis showed the interaction of PCDH21 protein with WT and mutant PROM1 in vivo (Figure 4C).
PCDH21 undergoes proteolytic processing releasing the ectodomain and retaining the transmembrane and intracellular domains during disk morphogenesis (21). We observed an increase in levels of unprocessed, full-length PCDH21 (~125 kDa) and decreased levels of the cleaved fragment (~25 kDa) in cell lysates from mutant POM1 mouse retinas compared with WT POM1 transgenic and C57BL/6 retinas (Figure 4D). These results suggested that the R373C mutation may also affect proteolytic processing of PCDH21 in mutant POM1 mice.

Physical interaction of POM1 with actin. The overgrown and misoriented disk membranes in R373C POM1 transgenic mice (Figure 2, A and B) are strikingly similar to those previously observed in retinas treated with cytochalasin D in order to depolymerize actin filaments (22, 23). Hence, we explored whether there is an interaction between POM1 and actin filaments and whether any interaction would be affected by the R373C mutation. Cells transfected with WT POM1 showed protrusions and membrane processes, and some of the POM1 colocalized with actin, especially in the protrusions (Supplemental Figure 7). In contrast, cells transfected with mutant POM1 did not possess protrusions, and the mutant POM1 did not colocalize with actin (Supplemental Figure 7). We found a physical interaction between WT POM1 and actin by immunoprecipitation in transfected cells (Figure 5) and in transgenic retinas (Supplemental Figure 8), and this interaction was markedly reduced by the R373C mutation (Figure 5). Furthermore, polymerization of actin filaments in photoreceptors was affected in mutant POM1 transgenic mice compared with that in WT transgenic mice (Supplemental Figure 9). Taken together, these results indicate that POM1 can interact with actin filaments and that this interaction is decreased by the R373C mutation.

Discussion

We report that a missense mutation in POM1 causes 3 forms of autosomal-dominant retinal dystrophy. In transgenic mice expressing mutant POM1, the mutant protein was mislocalized, and defects were evident in both rod and cone photoreceptor cells. ERG responses were reduced, and photoreceptor cell loss occurred at a rate that correlated with the expression level of the mutant POM1. Most notably, the mutation in POM1 interfered with its interaction with PCDH21 and actin, and prior to degeneration, the disks of the photoreceptor OSs were aberrant. Both these observations support a role for POM1 in the morphogenesis of new disk membranes.

POM1 and retinal degeneration. Mutations in the POM1 gene have been previously linked to pedigrees with autosomal-recessive retinitis pigmentosa (13, 14), where they mainly affect rod photoreceptors and peripheral vision. These mutations were frameshift mutations, resulting in premature stop codons and truncation of the encoded protein, most likely representing null mutations. In transfection studies, the truncated protein was found to be degraded quickly; a minor amount was detected in the endoplasmic reticulum and it was absent from the cell surface, so that the degeneration in these patients appeared to have resulted from loss of POM1 function (13). In contrast, the R373C mutation results in a stable mutant protein, and, as we show here, causes dominant macular degeneration that mainly affects cone photoreceptors and central vision. The R373C mutant POM1 was mislocalized and caused the mislocalization of WT Prom1 as well as that of at least one other critical protein, PCDH21.

Interaction with PCDH21. Rattner et al. (16, 21) described a photoreceptor cadherin, PCDH21; like POM1, PCDH21 localizes to the nascent disks of rod and cone OSs. Moreover, like the POM1 R373C mutant mice, Pdb21−/− mice exhibit disorganized photoreceptor membrane disks in OSs with corresponding photoreceptor degeneration (16). The present results support a functional interaction between POM1 and PCDH21. POM1 and PCDH21 were colocalized at the base of OSs, and POM1 was immunoprecipitated with full-length PCDH21. Further evidence of this interaction was obtained upon analysis of molecular abnormalities in R373C POM1 transgenic mice. PCDH21 showed an overlapping mislocalized immunolabeling pattern with the R373C mutant protein in photoreceptors. With immunoblots, we found a marked reduction in cleaved PCDH21 levels in R373C mutant retinal lysates. Given that loss of PCDH21 results in retinal degeneration (16), the dominant effect of the R373C POM1 mutation on retinal degeneration could stem from impairment of PCDH21 function.

Because POM1 and PCDH21 are both present on the nascent disks, their interaction appears likely to be within the same membrane or between adjacent membranes of 2 nascent disks. In the latter case, they may play a role in maintaining the spacing between
DNA was extracted from blood samples and have high curvature (10–12). Recently, PROM1 was detected at the port to the evagination/rim growth model. PROM1 is best characterized as a component of membrane domains that are protruding and have high curvature (10–12). Recently, PROM1 was detected at the tips of primary cilia on the neuroepithelium of the neocortex (34). Membrane was observed to bud off from these cilia (34), in a manner reminiscent of that from photoreceptor cilia in rds mice (35), which lack peripherin/rds (2) and are thus unable to form the rims of the disk membranes.

In the present study, we observed overgrown disk membranes, oriented abnormally along the OS plane, in R373C PROM1 transgenic mice (Figure 2, A and B). A strikingly similar phenotype has previously been described following the depolymerization of actin filaments with cytochalasin D in eyecup cultures and in retinas in vivo (22, 23). In these studies, observations of especially the cone disk membranes provided strong evidence that the overgrowth was caused by continued membrane evagination (23). The overgrown disks incorporated newly synthesized protein, and their formation appeared to result from a failure in the initiation of new disk membranes—the initial protrusion of the ciliary plasma membrane was inhibited, so that the new membrane was channeled into nascent disks that were already growing (23). In addition to actin filaments, it seems likely that PROM1 is also required for the normal, regular initiation of new disks. This suggestion is consistent with the general association of PROM1 on membrane protrusions (refs. 10–12 and Supplemental Figure 7), including those from primary cilia (34), and our present finding that PROM1 bound actin (Figure 5). Mice lacking RGRGIRP, which interacts with the retinitis pigmentosa GTPase regulator (RPGR) in the photoreceptor connecting cilium, also produce overgrown disk membranes, and it has been suggested that RPGRIP may regulate actin cytoskeletal dynamics (36).

In summary, our results identify what we believe to be a novel molecular complex involved in photoreceptor disk membrane morphogenesis and support the model whereby new disk membranes form by an evagination of the ciliary plasma membrane followed by rim growth.

Methods

Patients. Approval from the institutional review boards of the University of Utah, Moorfields Eye Hospital, and Institut de Recherche en Ophtalmologie was obtained for this study, and informed consent was obtained from all patients. Participating in the study were 27 individuals at risk for inheriting STGD4 in the first kindred, from the United States, a black family; 9 individuals at risk for inheriting MCDR2 in the second kindred, from England, a family of mixed European descent; and 15 individuals at risk for inheriting cone-rod dystrophy in the third kindred, from Switzerland.

Genetic linkage and mutation screening. DNA was extracted from blood samples, and genetic linkage was assessed using microsatellite markers D4S1582, D4S2906, D4S403, D4S2362, GGA18G02, D4S1525, D4S2946, D4S2633, D4S2926, and GATA158G03 (tightly linked to the STGD4 and MCDR2 loci) using previously established methods (6). We performed 2-point linkage analysis using the FASTLINK (37) version of MLINK from the LINKAGE Program Package (38). An autosomal-dominant mode of inheritance with full penetrance was used for LOD score computation. Disease allele frequency was set at 0.0001 (39). PROM1 mutation screening was performed by denaturing HPLC (dHPLC) analysis followed by direct sequencing of PCR-amplified DNA fragments for all 23 PROM1 exons using previously established methods (6). PCR primers were designed to include flanking intronic sequences of each exon according to published protocols (6). Amplified products were purified using the QiAQuick Gel Extraction Kit (Qiagen) and sequenced with forward and reverse primers by the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Beckman-Coulter) according to the manufacturer’s instructions. dHPLC analysis was used according to the manufacturer’s instructions (Transgenomics Inc.)

Figure 5

Communoprecipitation of PROM1 and β-actin. (A) HEK293 cells were transfected with either WT or mutant (MT) PROM1. Whole cell lysates with equal amounts of input WT or mutant PROM1 were immunoprecipitated with PROM1 polyclonal antibody, followed by anti–β-actin immunodetection on Western blots (WB). (B) Quantification of PROM1 and β-actin interaction by densitometry. Values (mean ± SD) denote the amount of β-actin, immunoprecipitated with PROM1 antibodies from cells, transfected with WT or mutant PROM1. Each experiment was done in triplicate, and 3 independent transfection experiments were performed for each PROM1 construct. Significance was examined using independent sample t test.
to screen for changes in PROM1 sequence and to determine the presence or absence of the mutation in the controls.

*Generation of expression constructs.* Forward and reverse primers (5'-CCGCTTGACGGTGTTCAATGAAGACCCTC-3' and 5'-ATAGTTTACGGGCGGACATTCTTATTCAATGGTTGATGGGCTTGTC-3', respectively) containing XhoI and NotI restriction sites were designed for amplification of WT human PROM1 cDNA. Following XhoI and NotI restriction digestion, the amplified cDNA was cloned into the XhoI and NotI sites of the pcDNA3.1(-) vector (Invitrogen). The 1117 C>T (R373C) mutation was introduced into the WT PROM1 pcDNA3 construct by PCR-based site-directed mutagenesis. The recombinant plasmids were purified using a Qiagen plasmid isolation kit (Qiagen). Mouse PCDH21 expression constructs were generously provided by A. Rattner and J. Nathans (Johns Hopkins University School of Medicine, Baltimore, Maryland, USA; ref. 21). All constructs were verified by restriction digestion and DNA sequencing.

*Generation of PROM1 transgenic mice.* WT or mutant human PROM1 cDNAs, generated as described above, were subcloned into the pHRO4.4 plasmid, containing the 4.4-kb human rhodopsin promoter — directing expression to rod and cone photoreceptors (15) — and a bovine poly A site. All constructs were verified by restriction enzyme digestion and direct DNA sequencing. Inserts were isolated by NotI and KpnI digestion, microinjected into C57BL/6 mouse embryos, and implanted into pseudopregnant foster female mice. Founder mice were identified by PCR. Human PROM1 primers (forward, 5'-CCGCTTGACGGTGTTCAATGAAGACCCTC-3'; reverse, 5'-CCGGATCCCCTGTATCTTATTCAATGGTTGATGGGCTTGTC-3') were used for PCR analysis of genomic DNA extracted from mouse tail biopsies. WT and mutant transgenic lines were established and propagated in the C57BL/6 mouse strain. All procedures were approved by the IACUC of the University of Utah and carried out according to NIH guidelines.

**PROM1 expression in transgenic mice.** After removal of the lens, retinas from 1-month-old mice were manually separated from the RPE and lysed in 150 mM NaCl; 50 mM Tris, pH 7.5; 1 mM EDTA; 1% Triton X-100; 0.5% SDS; and protease inhibitor mixture (Roche Applied Science). Protein from each retinal lysate (10 μg) was separated by SDS-PAGE, transferred to PVDF filters, and probed with either human PROM1 or mouse Prom1-specific antibodies, followed by incubation with HRP-conjugated secondary antibodies and standard ECL detection.

**Fundus photography and fluorescein angiography.** Fundus photographs and fluorescein angiographs for C57BL/6 and WT mutant PROM1 transgenic mice were recorded at 6 and 9 months of age using a Kowa RC-2 handheld fundus camera (Kowa Genesis). The eyes were held with scopolamine (0.25%; Isopto Hyoscin; Alcon) 1 h prior to photography. Fluorescein angiographs were recorded with negative black and white film after intraperitoneal injection of 0.2 ml 25% sodium fluorescein diluted 1:1 with sterile PBS.

**Histology and light microscopy.** Mice were maintained in a continuous 12-h light/12-h dark cycle and sacrificed 8–12 h after the onset of the light phase. Anesthetized mice were perfused with 0.1 M PBS and then with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) by intracardiac injection. The superior scera of each eye was marked for orientation. Eyecups were processed for embedding in Epon. Sections of 0.5 μm, oriented along the dorsoventral axis of the retina and containing the optic nerve head, were used for measuring photoreceptor ONL width. Photoreceptor nuclear counts were measured within 200–300 μm dorsal and ventral regions flanking the optic nerve head. Five separate counts per side (total 10 counts) were averaged, and ONL width was expressed as average number of nuclei.

**Dark-adapted ERG.** Electrotretinograms were obtained from 42 mice between the ages of 3 and 15 months: 19 PMT14, 8 PWT20, and 15 C57BL/6. Several mice were tested at more than one age. Mice were diluted with 0.25% scopolamine, dark-adapted overnight, and prepared for recording under a dim red light while the dilation was reinforced with scopolamine. Mice were anesthetized with intraperitoneal injection of 0.008 ml/g of a mixture of ketamine (20%), xylazine (0.5%), and sodium chloride solution (79.5%). The corneal electrode was a Burian-Allen mouse electrode referenced to a needle electrode in the scalp. A second needle electrode in the tail served as ground. Mice were placed between 2 heating pads to stabilize body temperature, which was continuously monitored with a digital probe. The eye was numbed with proparacaine hydrochloride (Alcaine; Alcon) and Refresh Celluvise (Allergan Inc.) was added to the contact lens electrode to protect the eye. Signals were amplified 10,000x and filtered (8-pole Butterworth 60-Hz notch filter) to remove line noise before averaging (n = 20–200) by computer. A ganzfeld dome and the incorporated Grass photostimulator, similar to systems used in human testing, was used to produce flashes comparable to the International Society for the Clinical Electrophysiology of Vision standard (40). Rod a-waves were elicited by a high-intensity Xenon flash (Novatron). The rod phototransduction model was fit to the leading edges of a-waves generated in response to high-intensity stimuli, and the dashed curves were the best fitting curves (41). Cone b-wave responses were obtained in the presence of a rod-saturating background (3.2 log photopic trolands).

**Electron microscopy.** Eyes from WT and mutant PROM1 transgenic mice were fixed in 2% paraformaldehyde plus 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and embedded in Epon 812 resin. Ultrathin sections were mounted on copper grids and stained with uranyl acetate and lead citrate and examined with a Philips electron microscope (model 208).

**Immunohistochemistry.** Antibodies used in these studies included rabbit polyclonal anti-human PROM1 (42); rat monoclonal anti-mouse Prom1 (11); rabbit polyclonal anti-mouse PCDH21 (diluted 1:500; gift from A. Rattner and J. Nathans; ref. 16); mouse monoclonal anti-mouse CNGCA1 (diluted 1:100; gift from R. Molday, University of British Columbia, Vancouver, British Columbia, Canada; ref. 19); mouse monoclonal anti-mouse ROM1 (diluted 1:100; gift from R. Molday; ref. 43); and rabbit polyclonal anti-mouse Na+/K+-ATPase (diluted 1:100; obtained from the Developmental Studies Hybridoma Bank, University of Iowa).

**Cryostat sections (12 μm) were washed with 1x PBS, blocked in immunohistochemical buffer (0.2% Triton X-100 in PBS) containing 10% goat serum at room temperature for 1 h. Sections were incubated in primary antibody overnight at 4°C. After washing, sections were incubated in FITC-conjugated goat anti-rabbit or Texas Red-conjugated goat anti-mouse secondary antibodies (diluted 1:100; Invitrogen) at room temperature for 1 h. Immunofluorescence was examined, and images were captured on a Zeiss 510 confocal microscope.

**Cell transfection and immunoprecipitation reactions of POM1 and PCDH21.** Cultured cells were propagated in DMEM (Gibco; Invitrogen) supplemented with 10% FBS (Gibco; Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with recombinant human WT or mutant PROM1 and mouse recombinant PCDH21 (containing a C-terminal Myc epitope tag) with FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer’s recommended protocol. At 36 h after transfection, cells were lysed in 150 mM NaCl; 50 mM Tris, pH 7.5; 1 mM EDTA; 1% Triton X-100; 0.5% SDS; and a protease inhibitor mixture (Roche Applied Science). Cell lysates were centrifuged at 1,600 g for 5 min at 4°C to remove nuclei and insoluble material. The anti-PROM1 antibody was used to immunoprecipitate PROM1-binding proteins. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF filters, and probed with the Myc antibody on Western blot. The reciprocal experiment was performed using the PCDH21 antibody for immunoprecipitations followed by Western blot with anti-PROM1. For retinal coimmunoprecipitation studies, retinas were isolated and pooled from 10 WT or mutant PROM1 transgenic mice, and protein was extracted as described above.
Western blot analysis of PRO1M and PCDH21 in transgenic mouse retinas. Dissected retinas were briefly homogenized in buffer containing 150 mM NaCl, 50 mM Tris, pH 7.5; 1 mM EDTA; 1% Triton X-100; 0.5% SDS; and complete protease inhibitor mixture (Roche Applied Science). Following a 20-min incubation on ice, lysate was cleared of nuclei and insoluble material by centrifugation at 1,600 g for 5 min at 4°C. Soluble proteins were separated by SDS-PAGE, after which Western blot analysis was performed. The polyclonal PCDH21 C-terminal antibody was used to detect full-length PCDH21 and the C-terminal protolytic fragment in C57BL/6 and PRO1M transgenic mice. A polyclonal antibody specific for human PRO1M without cross-reactivity to the mouse PRO1M was used to detect WT and mutant PRO1M transgene expression. An anti-mouse β-actin antibody (Sigma-Aldrich) was used as a loading control. For visualization, blots were probed with a HRP-conjugated anti-rabbit or anti-mouse secondary antibody (diluted 1:4,000; GE Healthcare) and developed with an ECL kit according to the manufacturers’ protocol (GE Healthcare).

Immunoprecipitation of PRO1M and actin. HEK293 cells transfected with WT or mutant PRO1M were lysed in the wash buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 1% NP-40; and 0.5% sodium deoxycholate) supplemented with complete protease inhibitors (Roche Applied Science), centrifuged at 10,000 g for 5 min to obtain postnuclear supernatant, and immunoprecipitated using affinity-purified polyclonal PRO1M antibodies. Immunocomplexes were precipitated by Protein A-Agarose (Pierce Biotechnologies Inc.) and washed 4 times with wash buffer. Immunoprecipitated protein samples were eluted with Laemmli buffer with 100 mM DTT, resolved by 10% SDS-PAGE, and immunoblotted with the β-actin monoclonal antibody (Sigma-Aldrich).

Dissected retinas from 1-month-old PWT20 mice were homogenized in buffer containing 150 mM NaCl; 50 mM Tris, pH 7.5; 1 mM EDTA; 1% Triton X-100; 0.5% SDS; and complete protease inhibitor mixture (Roche Applied Science). Lysate was centrifuged at 10,000 g for 5 min to obtain postnuclear supernatant and then immunoprecipitated using affinity-purified polyclonal PRO1M antibody. Immunocomplexes were precipitated by Protein A-Agarose, resolved by 10% SDS-PAGE, and immunoblotted with a β-actin monoclonal antibody.

Immunostaining and fluorescence microscopy of PRO1M and actin. HEK293 cells were transfected with WT or mutant PRO1M. Immunostaining was performed essentially as described previously (44). Slides were incubated for 1 h at room temperature in anti-PROM1 (diluted 1:500) or rhodamine-conjugated phallolidin for F-actin (diluted 1:2,000). Laser-scanning confocal microscopy was performed on a Zeiss LSM 510 microscope with krypton-argon and helium-neon lasers.

Cryosections of PWT20 and PM3 retinas were labeled with FITC-conjugated phallolidin (Invitrogen) to visualize actin filaments and with tubulin antibody. Immunofluorescence was examined, and images were captured on a Zeiss 510 confocal microscope.