Deletion of the transmembrane protein Prom1b in zebrafish disrupts outer-segment morphogenesis and causes photoreceptor degeneration

Received for publication, March 28, 2019, and in revised form, July 24, 2019. Published, Papers in Press, July 30, 2019, DOI 10.1074/jbc.RA119.008618

Zhaojing Lu,† Xuebin Hu,‡§ James Reilly,‡ Danna Jia,‡ © Fei Liu,§ Shanshan Yu,‡ Xiliang Liu,‡ Shanglun Xie,‡ Zhen Qu,‡ Yayan Qin,‡ Yuwen Huang,‡ Yuexia Lv,‡ Jingzhen Li,‡ Pan Gao,‡ Fulton Wong,‡ Xinhua Shu,‡ Zhaohui Tang,‡, and Mugen Liu,‡

From the †Key Laboratory of Molecular Biophysics of Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, Hubei 430074, China, ‡State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, Guangdong 510600, China, §Department of Life Sciences, Glasgow Caledonian University, Glasgow G4 OBA, Scotland, United Kingdom, and ‡Department of Ophthalmology, Duke University School of Medicine, Durham, North Carolina 27710

Edited by Xiao-Fan Wang

Mutations in human prominin 1 (PROM1), encoding a transmembrane glycoprotein localized mainly to plasma membrane protrusions, have been reported to cause retinitis pigmentosa, macular degeneration, and cone–rod dystrophy. Although the structural role of PROM1 in outer-segment (OS) morphogenesis has been demonstrated in Prom1-knockout mouse, the mechanisms underlying these complex disease phenotypes remain unclear. Here, we utilized a zebrafish model to further investigate PROM1’s role in the retina. The Prom1 orthologs in zebrafish include prom1a and prom1b, and our results showed that prom1b, rather than prom1a, plays an important role in zebrafish photoreceptors. Loss of prom1b disrupted OS morphogenesis, with rods and cones exhibiting differences in impairment: cones degenerated at an early age, whereas rods remained viable but with an abnormal OS, even at 9 months postfertilization. Immunofluorescence experiments with WT zebrafish revealed that Prph2, an ortholog of the human transmembrane protein peripherin 2 and also associated with OS formation, is localized to the edge of OS and is more highly expressed in the cone OS than in the rod OS. Moreover, we found that Prom1b deletion causes mislocalization of Prph2 and disrupts its oligomerization. We conclude that the variation in Prph2 levels between cones and rods was one of the reasons for the different PROM1 mutation–induced phenotypes of these retinal structures. These findings expand our understanding of the phenotypes caused by PROM1 mutations and provide critical insights into its function.

Human PROM1 (also known as CD133, RP41, MCDR2, STGD4, and CORD12) encodes a five-transmembrane glycoprotein localized mainly to plasma membrane protrusions (1). It was identified originally as a surface antigen in the hematopoietic stem and progenitor cells (2) and later also detected in various differentiated epithelial and nonepithelial cells (3–6). Although PROM1 is expressed in various tissues throughout embryogenesis and adulthood, mutations in the PROM1 gene cause mainly retinal diseases. Human PROM1 gene mutations lead to extremely variable retinal clinical phenotypes, including cone photoreceptor– or macula-dominated disorders such as macular degeneration (MD), autosomal dominant Stargardt-like macular dystrophy (7), autosomal dominant bull’s-eye macular dystrophy (7), autosomal dominant cone–rod dystrophy (CRD) (7, 8), autosomal recessive CRD (8–15), and the rod photoreceptor–dominated disorder autosomal recessive retinitis pigmentosa (RP) (6, 16–20). So far, 24 PROM1 mutations, predominantly truncation mutations along with some missense mutations, have been reported: 22 of them are associated with an autosomal recessive mode of inheritance, and 16 of them have been identified in patients with CRD. The mechanisms underlying this phenotypic heterogeneity are largely unknown.

Vertebrate photoreceptors are neural cells specialized in light detection. The phototransduction apparatus is housed in a cellular compartment known as the outer segment (OS). The OS, which contains a stack of membranous disks, is renewed daily by the formation of new disks at its base and the shedding of older disks from its distal tip. The shed disks are phagocyto-
Prom1b deletion causes photoreceptor degeneration

sed by retinal pigment epithelium (RPE) cells. Several studies have shown that mouse PROM1 is localized to the base of the photoreceptor OS where the new disk membranes are formed (1, 7), whereas, in Xenopus, Prom1 is distributed to the outer rims of open disk lamellae of cone OSs and basal disks of rod OSs (21). In human and murine retina, the PROM1 protein is purported to have a crucial role in the formation of the OS. Deletion of PROM1 in mouse resulted in abnormal photoreceptor OS morphogenesis of both rods and cones. However, the function of PROM1 in photoreceptor OS formation is still unclear.

PROM1 is evolutionarily conserved from Caenorhabditis elegans to Drosophila, zebrafish, Xenopus, mouse, and human. Zebrafish prom1 orthologues include prom1a and prom1b (22). In the present study, we constructed prom1a- and prom1b-knockout zebrafish lines. Our results show that prom1a deletion in zebrafish has no deleterious effect on photoreceptors. In contrast, prom1b-knockout zebrafish show serious retinal degeneration at an early age. It is notable that cones and rods are affected differently, although the primary defect in both photoreceptor subtypes is disruption of disk morphogenesis. Cones degenerate with the onset of OS formation, whereas the rods are still present at 9 months postfertilization (mpf) although with partly disorganized and longer OSs.

Another gene that is known to be involved in OS formation is PRPH2 (also known as retinal degeneration slow (RDS)). This protein forms oligomers and localizes to the rim of OS. Like PROM1, mutations in PRPH2 also cause phenotypic heterogeneity, resulting in RP, CRD, or MD (23–25). Here, we report that deletion of Prom1b causes mislocalization and disrupts oligomerization of Prph2. Additionally, our immunofluorescence results indicate that cones possess a higher protein content of Prph2 than rods, which may be one of the reasons for the different phenotypes in rods and cones in prom1b−/− zebrafish. Accordingly, our results provide mechanistic insights into the function of Prom1b and the nature of the phenotype caused by its deletion.

Results

Generation of prom1a−/− and prom1b−/− zebrafish lines

In this study, we constructed prom1a- and prom1b-knockout zebrafish lines by the transcription activator–like effector nuclease (TALEN) technology. Both of their TALEN-binding sites were separated by a 16-bp DNA spacer (Fig. 1, A and B). By mutant screening over three generations of zebrafish, we selected prom1a mutation (prom1a−/−) (c.138_141delTACT, p.Asp46Glu fs*15) and prom1b mutation (prom1b−/−) (c.174_177delACCA, p.Pro59Val fs*62) zebrafish lines for subsequent experiments; the genomic DNA sequencing results are shown in Fig. 1, C and D. Additionally, we detected Prom1b protein expression by Western blotting (antibody against Prom1b was prepared by Dia-An, Inc., Wuhan, China), and the result confirmed that no Prom1b protein existed in the mutant zebrafish, which further demonstrated the successful construction of the prom1b−/− zebrafish line (Fig. 1G). Unfortunately, we failed to generate a usable antibody against Prom1a. Compared with wildtype (WT) zebrafish, the mRNA expression levels of prom1a showed no significant change at 2 mpf, whereas that of prom1b exhibited a decrease at 7 days postfertilization (dpf) (Fig. 1, E and F). The mutated cDNA sequences of prom1a and prom1b, including a section of the region encompassing exon 1 and exon 2, are shown in Fig. S1. This finding could also indicate the knockout design was effective.

prom1b−/− zebrafish displayed retinal degeneration phenotypes

To assess the effects of prom1a and prom1b knockout on the zebrafish retina, we first examined their retinal morphology. Retinal sections were obtained at the age of 1 mpf and stained with hematoxylin/eosin. The results showed that there was no obvious difference in cell layer organization between the mutant and WT zebrafish. However, cells in the outer nuclear layer (ONL) of prom1b−/− zebrafish were sparsely distributed compared with WT zebrafish (Fig. 2A). Furthermore, an increased level of apoptotic cells (about 3-fold) was clearly observed in prom1b−/− compared with WT zebrafish at 1 mpf (Fig. 2, B and C). The thicknesses of the ONL and OSs in prom1a−/− was similar to those in WT zebrafish even at 11 mpf (Fig. S2), which suggested that knockout of prom1a in zebrafish might cause no impairment to photoreceptors. To investigate the reason for the different phenotypes of prom1a−/− and prom1b−/− zebrafish, we generated GFP-hPROM1, GFP-zfProm1a, and GFP-zfProm1b and expressed the plasmids in ARPE-19 cells. We observed that zfProm1b was mainly localized on the protuberances of the cell membrane, which was similar to hPROM1, whereas zfProm1a was localized in the cytoplasm. We also observed a similar distribution of zfProm1b and hPROM1 using HeLa cells (Fig. S3). These results indicated that the function of Prom1a is different from that of hPROM1 in photoreceptors.

Additionally, we examined the expression of some proteins involved in the phototransduction cascade, including rod-specific proteins (GNB1 and GNAT1) and cone-specific proteins (GNB3 and GNAT2) (26). These proteins are essential for converting light signals into electrical signals, and any impairment would affect normal visual function. Results from Western blotting showed a significant decrease in expression of Gnb3 and Gnat2 in prom1b−/− compared with WT zebrafish at 1 and 3 mpf, whereas an increased expression of Gnb1 and Gnat1 was observed at the same ages (Fig. 3, A, B, and C). However, there was no significant differences in the expression of the four proteins between prom1a−/− and WT zebrafish even at 11 mpf (Fig. 3, A and D).

The results obtained above indicate that prom1b, rather than prom1a, plays an important role in zebrafish photoreceptors and that loss of Prom1b in zebrafish would lead to severe retinal degeneration phenotypes. Therefore, in the subsequent research, we mainly study the pathogenic mechanism of retinal degeneration caused by PROM1 mutation in the prom1b−/− zebrafish line.

Prom1b deletion in zebrafish caused different impairments to different photoreceptors

As obvious characteristics of retinal degeneration were observed in the prom1b−/− zebrafish retina, we wanted to fur-
Prom1b deletion causes photoreceptor degeneration

Other explore possible differences in different photoreceptors. With the exception of their ultraviolet (UV) cones, zebrafish possess photoreceptor types similar to those of human. We labeled rods and cones (red, green, blue, and UV cones) with specific antibodies (Rhodopsin, Opn1lw, Opn1mw, Opn1sw2, and Opn1sw1, respectively) in retinal sections for WT and prom1b−/− zebrafish at different ages. Our results showed that the OSs of all five types of photoreceptor exhibited a neat and compact arrangement in the WT zebrafish but that prom1b−/− zebrafish OSs displayed different impairments with respect to number and morphology (Fig. 4, A and B). The number of red and green cone OSs in prom1b−/− zebrafish decreased from the age of 7 dpf, and with time this decrease became more obvious (Fig. 5, A and B). Compared with the WT zebrafish, the number of blue and UV cone OSs showed only a slight decrease at 7 dpf but had decreased noticeably at 1 mpf, with only a few OSs remaining by 3 mpf (Figs. 5C and D, S4, and S5). The number of rod OSs in prom1b−/− zebrafish showed no difference from the WT zebrafish at 7 dpf; however, the length of rod OSs showed a clear increase at 1 mpf, whereas rods still existed even at 9 mpf (Figs. 5E and Fig. S6). In short, our results indicate that Prom1b deletion in zebrafish had different effects on cones and rods: the number of cones was reduced, whereas the length of rod OSs became longer.

Outer-segment morphogenesis was disrupted in prom1b−/− zebrafish

Next, we carried out a transmission EM (TEM) assay to assess ultrastructural changes of the photoreceptors in prom1b−/− zebrafish. At 3 dpf, the normal stacked disk morphology of photoreceptor OSs could be clearly observed in WT zebrafish; by contrast, there were few, or occasionally several, quite small whorl-like disk structures detected in prom1b−/− zebrafish at 1 mpf. The zebrafish pcDNA3.1-Prom1b expressed in human lymphatic endothelial cells was used as a positive control, and the mock was used as a negative control. Tuba served as a loading control. The Prom1b band marked by the black arrowhead was undetectable in prom1b−/− zebrafish.

Figure 1. Generation of the prom1a+/− and prom1b+/− zebrafish lines. A and B, zebrafish prom1a and prom1b genes are shown with the left and right arms of the TALEN-binding sequences underlined and the spacer sequences highlighted in red. C, sequencing of the c.138_141delTACT prom1a mutation in homozygous zebrafish. The 4-bp deletion is indicated by the red line. D, sequencing of the c.174_177delACCA prom1b mutation in homozygous zebrafish. The 4-bp deletion is indicated by the red line. E and F, quantitative real-time PCR analysis of prom1a at 2 mpf and prom1b at 7 dpf. Glyceraldehyde-3-phosphate dehydrogenase served as an endogenous control. Error bars represent S.D. (n = 3). G, Western blot analysis of Prom1b in retinal extracts from WT and prom1b−/− zebrafish at 1 mpf. The zebrafish pcDNA3.1-Prom1b expressed in human lymphatic endothelial cells was used as a positive control, and the mock was used as a negative control. Tuba served as a loading control. The Prom1b band marked by the black arrowhead is undetectable in prom1b−/− zebrafish.
Prom1b deletion causes photoreceptor degeneration

Figure 2. prom1b<sup>−/−</sup> zebrafish displayed retinal degeneration phenotypes. A, retinal histology analysis of WT and prom1a/1b-knockout zebrafish at 1 mpf. IS, inner segment; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars, 20 μm. B, TUNEL staining of WT and prom1b<sup>−/−</sup> zebrafish at 1 mpf. White arrows indicate the TUNEL-positive signals (red). Scale bars, 50 μm. C, quantification of TUNEL-positive cells in ONL of whole-retina sections at 1 mpf (n = 3). Error bars represent S.D.

Prom1b deletion affects Prph2 protein distribution and oligomerization

PRPH2 is a key structural protein of the OS. It is localized to the disk rim of the rods and cones and is necessary for the formation of the OS (23, 31). Prph2<sup>−/−</sup> mice failed to develop OS structures, whereas Prph2<sup>+/−</sup> mice formed disorganized OSs with whorl-like structures (32, 33). It is notable that PRPH2 mutations in human manifest as rod and/or cone dystrophies with varying levels of severity (34) and that previous studies have demonstrated distinct functions of PRPH2 in rods and cones (35–37). In our current studies, Prom1b deletion in zebrafish exerted different effects on the disk formation of rod and cone OSs, with the pattern of disks being similar to that seen in Prph2<sup>+/−</sup> mice, all of which led us to consider whether there was a relationship between prom1b and prph2. Therefore, we focused on the Prph2 protein in the subsequent research.

To examine the cellular localization of Prph2, we labeled photoreceptors with specific antibodies (Prph2, Rhodopsin, and Opn1lw) in retinal sections from WT and prom1b<sup>−/−</sup> zebrafish at 2 mpf (rods and a few red cones were still present in the retina at this period). In the WT zebrafish, Prph2 was mainly localized to the edge (most likely the disk rim) of the rod and cone OSs (Fig. 8, A–F, a–f), with signal intensity in cone OSs being stronger than that in rod OSs (Fig. S8), indicating a higher protein content of Prph2 in cone OSs. In the prom1b<sup>−/−</sup> zebrafish, we found Prph2 signals almost completely filling the red cone OS rather than just on its edge (Fig. 8, F–I, f–i), whereas occasional mislocalization of Prph2 appeared in the partial OSs of rods (Fig. 8, G–I, g–i). Moreover, we also found mislocalization of Prph2 in the OSs of prom1b<sup>−/−</sup> zebrafish at an early age (14 dpf; Fig. S9).

In photoreceptor OSs, Prph2 forms higher-order oligomers, which localize to the disk rim and play a crucial role in disk rim formation (24). A number of previous studies have confirmed that oligomerization of Prph2 is essential for OS formation (24, 38). Because mislocalization of Prph2 and malformation of OSs were observed in the current study, we asked whether the Prom1b deletion would affect the oligomerization of Prph2. By nonreducing Western blot analysis, we found that the ratio of Prph2 dimer to monomer was reduced in zebrafish at an earlier age (7 dpf) (Fig. 9, A and a). These results further demonstrated that the localization of Prph2 in the disk was affected in the mutant zebrafish.

Furthermore, to evaluate the influence on protein levels of Prph2, Western blot analysis was performed on retinal extracts from WT and prom1b<sup>−/−</sup> zebrafish at different ages. The results showed that the protein expression of Prph2 also significantly decreased at 7 dpf and 2 mpf (Fig. 9, B and b). In addition, we found that the mRNA expression of prph2 (there are two isoforms, prph2a and prph2b, in zebrafish) also decreased in prom1b<sup>−/−</sup> zebrafish at the same period (Fig. 9, C and D). Based on the fact that photoreceptor number was reduced at an early age, the decreased level of Prph2 likely resulted from a consequence of the OS phenotypes following Prom1b deletion.

Discussion

PROM1 mutations lead to more complex clinical phenotypes of retinal degeneration. Patients with mutations in PROM1 were initially diagnosed as having autosomal recessive retinal degeneration. The affected individuals reported night blindness and loss of peripheral vision from childhood with progression to profound visual impairment and extinguished electroretinograms by their third decade (6). Subsequently, a
A homozygous mutation resulting in a truncated PROM1 protein was identified in a family with severe RP, the affected patients reporting night blindness and decreased visual acuity in early childhood, with all of them having typical and advanced RP fundus changes accompanied by macular changes (20). Recently, many mutations of PROM1 have been implicated in patients with CRD: these patients had central visual dysfunction from early childhood, manifesting as nystagmus, mild photophobia, and color vision deficiency, whereas night vision deteriorated in adolescence (11, 12, 19). Additionally, there is another autosomal dominant mutation, PROM1/R373C, that results in stable mutant protein and interferes with the action of the normal protein, causing Stargardt-like MD, bull’s-eye MD, and CRD (7). Due to complex and variable clinical phenotypes caused by PROM1 mutations, it is not possible to use one animal model to reflect all clinical phenotypes. In this study, we generated a new PROM1-knockout animal model to provide a new perspective for research into the function of PROM1. The major findings of this study are the following. 1) Prom1b, rather than Prom1a, plays an important role in the zebrafish photoreceptors. 2) Prom1b deletion disrupts OS morphogenesis and causes photoreceptor degeneration, but the effect on cones is more acute than in rods. 3) Mislocalization of Prph2 and decreased ratio of Prph2 dimer to monomer are part of the mechanisms for the phenotypes caused by Prom1b deletion. The retinal phenotype of prom1b-knockout zebrafish is not identical to that of the Prom1-knockout mouse. In both animal models, photoreceptors degenerate at an early age, and the disks are disorganized. However, there are still clear differences. In Prom1-knockout mouse, both rod and cone disks appear overgrown and misoriented throughout the entire OS and degenerate at the same time. In prom1b−/− zebrafish, cone disks exhibit serious disorganization, whereas rod OSs exhibit only mild misorientation; cones degenerated at an early age, whereas rods were alive, albeit with abnormal OS, even at 9 mpf (1). We speculate that the distinct phenotypes between mouse and zebrafish models may be caused by different photoreceptor structure and by a different distribution of PROM1. In some species, the OSs of the photoreceptors are surrounded by structures called calyceal processes, which are composed of actin filaments and which play a supporting role for OS structure. Calyceal processes surround the proximal outer segment in rods and are paraxially aligned with the outer segment in cones (39, 40). These calyceal processes exist in human, rhesus monkey, and zebrafish but not in mouse (41). PROM1 might have an essential role in maintaining the calyceal processes through two of its interaction proteins: actin and eyes shut homolog. Due to the function of PROM1 in calyceal processes, deletion of
Prom1b deletion causes photoreceptor degeneration

Prom1b in zebrafish might have different effects on rods and cones. In humans, the PROM1 protein has been reported to be distributed throughout the entire cone OS and the basal disk of the rod OS (42). A similar distribution of Prom1 has been observed in *Xenopus laevis*, whereas in mouse Prom1 is localized exclusively to the base disk of both rods and cones. Based...
on the phenotype of prom1b<sup>-/-</sup> zebrafish, we speculate that zebrafish is likely to have a Prom1 distribution similar to that of X. laevis and human. However, we failed to confirm this speculation as our antibody against zebrafish Prom1b did not work in immunofluorescence analysis.

PRPH2 is another crucial protein for disk morphogenesis. Mutations that affected PRPH2 oligomerization caused more severe defects in cones than in rods (24). In our study, we detected that Prph2 signal intensity in cones was notably stronger than that in rods, which is an important finding, indicating that cones and rods possess different Prph2 protein levels. We consider this different protein content of Prph2 as one of the reasons for the different phenotypes of cones and rods. Furthermore, our results showed that loss of Prom1b disrupted oligomerization and caused mislocalization of Prph2. Han et al. (21) report that Prom1 and Prph2 are mutually exclusively distributed in X. laevis cone OSs and suggest the two proteins must be tightly coordinated (possibly through a counterbalance relationship) to mediate disk morphogenesis. Here, we detected that there is no direct protein interaction between Prom1b and Prph2, which suggests that the relationship between the two proteins in zebrafish may be similar to that in X. laevis. Interestingly, our results show that Prph2 does not localize specifically to the disk rims, as it also appeared to localize to disk membranes in prom1b<sup>-/-</sup> zebrafish. Another independent study by Conley et al. (43) showed that Prph2 deletion in amphibian leads to Prom1 localization in both disk rim and membrane but does not affect distribution of other disk membrane–specific proteins (such as S-opsin). The results of Conley et al. (43) and our experimental results together support the suggestion provided by Han et al. (21). Therefore, we consider that the abnormal distribution of Prph2 in prom1b<sup>-/-</sup> zebrafish may result from disruption of a counterbalance relationship between Prom1 and Prph2. Moreover, we found that the ratio of Prph2 dimer to monomer was significantly reduced following Prom1b deletion. It is not clear what caused the ratio to decrease, but we speculate that this is associated with the mislocalized Prph2. Given that impairment of Prph2 oligomerization would affect disk formation, the changes in the Prph2 oligomer and distribution are likely to be part of the mechanism for OS defects in prom1b<sup>-/-</sup> zebrafish. Our findings provide critical clues for understanding the relationship between PROM1 and PRPH2 in disk formation.

However, it is still difficult to account for the fact that rod OS length becomes longer in prom1b<sup>-/-</sup> zebrafish. Typically, disks in rods are detached from the plasma membrane, whereas cone disks are continuous with the membrane; it could be that loss of Prom1b may affect structures or mechanisms that are responsible for shedding of the distal rod OSs, thus accounting for the longer OS. A recent study showed that PROM1 is a regulator of autophagy in the human RPE (44). Autophagy is important to

**Figure 7.** Outer-segment morphogenesis was disrupted in prom1b<sup>-/-</sup> zebrafish. A–C, ultrastructural analysis of WT and mutant (MT) (prom1b<sup>-/-</sup>) zebrafish photoreceptors at 10 dpf. Scale bars, 5 μm. D–H, outer-segment disks of mutant zebrafish exhibited different morphological characteristics compared with WT zebrafish. Scale bars, 2 μm. I and J, retinal ultrastructural analysis of mutant zebrafish photoreceptors at 2 mfp. White arrows indicate the shedding OSs. The areas within the dashed rectangles are shown in the higher-magnification images (a and b). Scale bars, 10 μm in I and J, and 1 μm in a and b.

**Figure 8.** Prph2 was mislocalized in prom1b<sup>-/-</sup> zebrafish. A–C and G–I, retinal cryosections from WT and prom1b<sup>-/-</sup> zebrafish were immunostained with anti-Rhodopsin (green) and anti-Prph2 (red) antibodies at 2 mfp. Occasionally, mislocalization of Prph2 appeared in the partial OSs of rods. The areas within the dashed rectangles are shown in the higher-magnification images (a–c and g–i). Scale bars, 10 μm in A–C and G–I and 2 μm in a–c and g–i. D–F and J–L, retinal cryosections from WT and prom1b<sup>-/-</sup> zebrafish were immunostained with anti-Opn1lw (green) and anti-Prph2 (red) antibodies at 2 mfp. The OS of red cones in prom1b<sup>-/-</sup> zebrafish showed mislocalization of Prph2. The areas within the dashed rectangles are shown in the higher-magnification images (d–f and j–l). Scale bars, 10 μm in D–F and J–L and 2 μm in d–f and j–l.
the health and function of the RPE and retina (45), and alterations in autophagy flux have been reported in some retinal degeneration diseases like MD and RP (46–48). The effects of Prom1b deletion on retinal autophagy activity and its relationship with photoreceptor degeneration require further study to elucidate the precise role of PROM1.

In summary, we generated a new PROM1-knockout animal model in this study. Using prom1b−/− zebrafish, we found that loss of Prom1b caused more severe impairments in cone photoreceptors and that Prom1b plays a vital role in regulating the distribution of Prph2 in disks. These findings contribute to the understanding of PROM1-related retinal degeneration diseases and provide key clues for further understanding of the molecular function of PROM1.

**Experimental procedures**

**Zebrafish maintenance**

Zebrafish larvae and adults were maintained at 26–28.5 °C under a 14/10-h light/dark cycle. Fertilized eggs were collected and maintained in E3 medium in an incubator (at ~28.5 °C) for 72 h until the larvae hatched (49). All procedures involving zebrafish were approved by the Ethics Committee of Huazhong University of Science and Technology and were in accordance with Animal Research: Reporting of in Vivo Experiments (ARRIVE) guidelines.

**Cell culture and transfection**

Cells were cultured in Dulbecco’s modified Eagle’s medium (human lymphatic endothelial cells and HeLa) or Dulbecco’s modified Eagle’s medium/F-12 medium (ARPE-19) containing 10% fetal bovine serum and incubated at 37 °C in 5% CO2 in a humidified incubator. When grown to 80–90% confluence, cells were replated at a split ratio of 1:3–1:5. Cell transfection was performed with Lipofectamine™ 3000 (Invitrogen).

**Antibodies**

The list of antibodies used in the present study is provided in Table S1.

**Generation of prom1a−/− and prom1b−/− zebrafish lines**

Both prom1a−/− and prom1b−/− zebrafish lines were constructed utilizing the Golden Gate TALEN kit as described pre-
Prom1b deletion causes photoreceptor degeneration

TUNEL staining

TUNEL staining was performed using the TUNEL BrightRed Apoptosis Detection kit (Vazyme Biotech) according to the manufacturer’s instructions. Generally, cryosections were air-dried at room temperature and fixed with 4% paraformaldehyde in PBS for 30 min. The slides were washed two times with PBS for 15 min and incubated with proteinase K buffer for 10 min. After that, slides were washed two to three times with PBS and incubated with equilibration buffer for 10–30 min. The retinal sections were then incubated in TdT buffer at 4 °C overnight. The next day, after DAPI labeling, the slides were mounted under glass coverslips.

RNA extraction and RT-qPCR

Total RNA of zebrafish was extracted using TRIzol (Takara) and quantitated by NanoDrop spectrometry (Thermo Scientific). Real-time PCR was performed using AceQ® qPCR SYBR® Green Master Mix (Vazyme) according to the manufacturer’s instructions, and relative gene expression was quantified using the StepOnePlus™ Real-Time PCR System (Life Technologies). Gene primers are listed in Table S2.

Western blotting

Cells and zebrafish eyes were collected and lysed in SDS lysis buffer with protease inhibitor mixture. Protein concentration was determined using the BCA protein assay kit (Beyotime, China). Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The blots were incubated with primary antibodies (1:500–5000) followed by horseradish peroxidase–labeled secondary antibodies (1:20,000; Thermo Scientific). Super Signal ECL substrate (Pierce) was used for the detection of signals.

Plasmid constructs

The full-length PROM1, Prom1a, and Prom1b cDNAs were cloned into the pEGFP-N1 and pcDNA3.1 vectors.

Statistical analysis

All data are presented as mean ± S.D. Data groups were compared by Student’s t test (Prism 6.0 software, GraphPad Software, Inc., La Jolla, CA). Differences between groups were considered statistically significant if the p value was <0.05.

Author contributions—Z. L. and X. H. data curation; Z. L., S. Y., Z. T., and M. L. funding acquisition; Z. L., X. H., D. J., F. L., S. Y., X. L., S. X., Z. Q., Y. Q., Y. H., Y. L., J. L., P. G, and P. G. investigation; Z. L. writing-original draft; J. R., F. W., and X. S. writing-review and editing; Z. T. and M. L. conceptualization.

References

1. Zacchigna, S., Oh, H., Wilsch-Bräuninger, M., Missol-Kolka, E., Jászai, J., Jansen, S., Tanimoto, N., Tonagel, F., Seeliger, M., Huttner, W. B., Corbeil, D., Dewerchin, M., Vinckier, S., Moons, L., and Carmeliet, P. (2009) Loss of the cholesterol-binding protein prominin-1/CD133 causes disk dys-
36. Chakraborty, D., Conley, S. M., Stuck, M. W., and Naash, M. I. (2010) Differences in RDS trafficking, assembly and function in cones versus rods: insights from studies of C150S-RDS. *Hum. Mol. Genet.* **19**, 4799–4812 CrossRef Medline

37. Stuck, M. W., Conley, S. M., and Naash, M. I. (2015) Retinal degeneration slow (RDS) glycosylation plays a role in cone function and in the regulation of RDS-ROM-1 protein complex formation. *J. Biol. Chem.* **290**, 27901–27913 CrossRef Medline

38. Chakraborty, D., Ding, X. Q., Fliesler, S. J., and Naash, M. I. (2008) Outer segment oligomerization of Rds: evidence from mouse models and subcellular fractionation. *Biochemistry* **47**, 1144–1156 CrossRef Medline

39. Lin-Jones, J., Parker, E., Wu, M., Dosé, A., and Burnside, B. (2004) Myosin 3A transgene expression produces abnormal actin filament bundles in transgenic *Xenopus laevis* rod photoreceptors. *J. Cell Sci.* **117**, 5825–5834 CrossRef Medline

40. Nagle, B. W., Okamoto, C., Taggart, B., and Burnside, B. (1986) The teleost cone cytoskeleton. Localization of actin, microtubules, and intermediate filaments. *Invest. Ophthalmol. Vis. Sci.* **27**, 689–701 CrossRef Medline

41. Sahly, I., Dufour, E., Schietroma, C., Michel, V., Bahloul, A., Perfettini, I., Pepermans, E., Estivalet, A., Carette, D., Aghaie, A., Ebermann, I., Lelli, A., Irbarne, M., Hardelin, J. P., Weil, D., et al. (2012) Localization of Usher 1 proteins to the photoreceptor calyceal processes, which are absent from mice. *J. Cell Biol.* **199**, 381–399 CrossRef Medline

42. Jászai, J., Fargeas, C. A., Florek, M., Huttner, W. B., and Corbeil, D. (2007) Focus on molecules: prominin-1 (CD133). *Exp. Eye Res.* **85**, 585–586 CrossRef Medline

43. Conley, S. M., Al-Ubaidi, M. R., Han, Z., and Naash, M. I. (2014) Rim formation is not a prerequisite for distribution of cone photoreceptor outer segment proteins. *FASEB J.* **28**, 3468–3479 CrossRef Medline

44. Bhattacharyya, S., Yin, J., Winborn, C. S., Zhang, Q., Yue, J., and Chaum, E. (2017) Prominin-1 is a novel regulator of autophagy in the human retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* **58**, 2366–2387 CrossRef Medline

45. Zhou, Z., Vinberg, F., Schottler, F., Doggett, T. A., Kefalov, V. J., and Ferguson, T. A. (2015) Autophagy supports color vision. *Autophagy* **11**, 1821–1832 CrossRef Medline

46. Bergmann, M., Schätt, F., Holz, F. G., and Kopitz, J. (2004) Inhibition of the ATP-driven proton pump in RPE lysosomes by the major lipofuscin fluorophore A2-E may contribute to the pathogenesis of age-related macular degeneration. *FASEB J.* **18**, 562–564 CrossRef Medline

47. Hu, X., Lu, Z., Yu, S., Reilly, J., Liu, F., Jia, D., Qin, Y., Han, S., Liu, X., Qu, Z., Lv, Y., Li, J., Huang, Y., Jiang, T., Jia, H., et al. (2019) CERKL regulates autophagy via the NAD-dependent deacetylase SIRT1. *Autophagy* **15**, 453–465 CrossRef Medline

48. Yu, S., Li, C., Biswas, L., Hu, X., Liu, F., Reilly, J., Liu, X., Liu, Y., Huang, Y., Lu, Z., Han, S., Wang, L., Yu Liu, J., Jiang, T., Shu, X., et al. (2017) CERKL gene knockout disturbs photoreceptor outer segment phagocytosis and causes rod-cone dystrophy in zebrafish. *Hum. Mol. Genet.* **26**, 2335–2345 CrossRef Medline

49. Liu, F., Qin, Y., Yu, S., Soares, D. C., Yang, L., Weng, J., Li, C., Gao, M., Lu, Z., Hu, X., Liu, X., Jiang, T., Liu, J. Y., Shu, X., Tang, Z., et al. (2017) Pathogenic mutations in retinitis pigmentosa 2 predominantly result in loss of RP2 protein stability in humans and zebrafish. *J. Biol. Chem.* **292**, 6225–6239 CrossRef Medline

50. Lu, Z., Hu, X., Liu, F., Soares, D. C., Liu, X., Yu, S., Gao, M., Han, S., Qin, Y., Li, C., Jiang, T., Luo, D., Guo, A. Y., Tang, Z., and Liu, M. (2017) Ablation of EYS in zebrafish causes mislocalisation of outer segment proteins, F-actin disruption and cone-rod dystrophy. *Sci. Rep.* **7**, 46098 CrossRef Medline