Disruption of the Retinitis Pigmentosa 28 gene Fam161a in mice affects photoreceptor ciliary structure and leads to progressive retinal degeneration

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Abstract

Mutations in the FAM161A gene were previously identified as the cause for autosomal-recessive retinitis pigmentosa 28 (RP28). To study the effects of Fam161a dysfunction in vivo, we generated gene trapped Fam161a\textsuperscript{GT/GT} mice with a disruption of its C-terminal domain essential for protein-protein interactions. We confirmed the absence of the full-length Fam161a protein in the retina of Fam161a\textsuperscript{GT/GT} mice using Western blots and showed weak expression of a truncated Fam161a protein by immunohistochemistry. Histological analyses demonstrated that photoreceptor segments were disorganized in young Fam161a\textsuperscript{GT/GT} mice and that the outer retina was completely lost at six months of age. Reactive microglia appeared in the outer retina and electroretinography showed an early loss of photoreceptor function in four month old Fam161a\textsuperscript{GT/GT} animals. Light and electron microscopy revealed a remarkable phenotype of a significantly shortened connecting cilium, spread ciliary microtubule doublets and disturbed disk organization in Fam161a\textsuperscript{GT/GT} photoreceptor cells. Co-immunolabeling experiments demonstrated reduced expression and mislocalization of centrin 3 and disturbed targeting of the Fam161a interactors lebercilin and Cep290, which were restricted to the basal body and proximal connecting cilium in Fam161a\textsuperscript{GT/GT} retinas. Moreover, we identified misrouting of the outer segment cargo proteins opsin and rds/peripherin 2 in Fam161a\textsuperscript{GT/GT} mice. In conclusion, our results suggest a critical role for the C-terminal domain of Fam161a for molecular interactions and integrity of the connecting cilium. Fam161a is required for the molecular delivery into the outer segment cilium, a function which is essential for outer segment disk formation and ultimately visual function.
Introduction

Retinitis pigmentosa [RP (MIM 268000)] is the most prevalent hereditary degeneration of the human retina with an incidence of 1:4000 worldwide. Initial symptoms include night blindness and a gradual constriction of peripheral vision caused by rod photoreceptor death (1, 2). Secondary loss of cone photoreceptors may subsequently lead to impairment of central vision and ultimately legal blindness (3). RP can be transmitted in an X-linked, autosomal-dominant or autosomal-recessive inheritance pattern. So far, mutations in at least 50 different genes have been identified as genetic causes for non-syndromic RP (RetNet, https://sph.uth.edu/retnet/). Genetic heterogeneity of RP is also reflected in the broad clinical variability in course and severity of the disease (1).

On the molecular level, proteins encoded by RP genes are involved in a multitude of cellular processes including the phototransduction cascade, cytoskeletal dynamics, regulation of gene transcription and intracellular trafficking of proteins (1, 3). Due to the complex organization and high extent of specialization of photoreceptors, these cells are very vulnerable to stress. As a consequence, genetically-induced dysfunctions of single photoreceptor gene products often lead to a loss of photoreceptor function and programmed cell death, a common hallmark in the pathogenesis of inherited retinal degenerations (4, 5).

We and others have previously identified mutations in the FAM161A gene as cause for autosomal-recessive RP28 (6, 7). Homozygosity for a 685C-T transition in FAM161A exon 3 was identified in affected members from an Indian family, converting the codon for arginine 229 into a termination codon in FAM161A exon 3 (p.Arg229X). Other mutations causing frameshifts or premature termination have been identified in RP cohorts of German, Jewish, Palestinian, and North American decent (6-9). Notably, except for one mutation in exon 4 (p.Arg596X) all of the identified mutations are located in the large exon 3 of FAM161A.
The orthologous human and mouse FAM161A genes consist of 7 exons and the Fam161A protein is present in several tissues with high expression level in the retina (6). Fam161A protein is found in inner photoreceptor segments and co-localizes with ciliary marker proteins in the connecting cilium and the adjacent centriole of photoreceptor cells (10, 11). FAM161A has been shown to interact with microtubules in vitro via its C-terminal part and a putative role in microtubule stabilization has been proposed (10, 11). The FAM161A protein contains a highly conserved UPF0564 domain which is encoded by exon 3, and the N-terminal part of this domain is crucial for both homo- and heterotypic protein-protein interactions with the ciliopathy proteins Cep290 and lebercilin (Lca5) (11). In vitro data suggest that FAM161A is involved in transport processes across the photoreceptor cilium, however, it is currently unknown whether loss of FAM161A function affects ciliary structure in vivo.

To study the consequences of Fam161a disruption in the retina and to determine its role in photoreceptor cells in vivo, we generated and analyzed homozygous gene trapped Fam161a (Fam161a\textsuperscript{GT/GT}) mice. We found that Fam161a\textsuperscript{GT/GT} mice displayed progressive photoreceptor degeneration, early impairment of retinal signal processing and microglial activation. Further investigations of the histopathology revealed that Fam161a\textsuperscript{GT/GT} retinas displayed a spread and shortened photoreceptor connecting cilium, mislocalization of Fam161a interacting proteins and disturbed outer segment formation. Our findings suggest that Fam161a plays a crucial role in the assembly and function of photoreceptor cilia and highlight the Fam161a\textsuperscript{GT/GT} mouse line as a novel retinal ciliopathy model.

Results

Generation of a mouse line with gene trap disruption of Fam161a

We generated a mouse line with targeted disruption of the Fam161a gene using a CMMR ES cell clone GT\textunderscore462E7\textunderscore5S with the UPA-vector (12) gene trap allele Fam161a\textsuperscript{GT(462E7)Chmd}.
inserted into exon 3 of the gene (Fig. 1A). The insertion of the gene trap cassette disrupts the highly conserved UPF0564 domain after amino acid position 262 (Fig. 1B, Figure S1). Mice heterozygous and homozygous for the targeted allele were identified by PCR genotyping (Fig. 1C). Mice homozygous for the Fam161a<sup>GT</sup>-allele and hereafter referred to as Fam161a<sup>GT/GT</sup> mice showed no behavioral abnormalities compared to their heterozygous littermates. RT-PCR to amplify the 5’ and 3’ parts of the Fam161a mRNA did not show major expression differences in wild-type, Fam161a<sup>+/GT</sup> and Fam161a<sup>GT/GT</sup> retinas (Fig. 1D). This suggests that it is unlikely that Fam161a<sup>GT</sup> mRNA is subjected to complete nonsense mediated decay.

**Weak expression of a truncated Fam161a protein in the Fam161a<sup>GT/GT</sup> retina**

Next, we studied Fam161a protein expression. First, we over-expressed a corresponding Fam161a (aa 1-363) variant with an EGFP tag in Hek293 cells (Fig. 2A). This truncated EGFP-tagged Fam161 was readily detected by staining with an antibody against an N-terminal epitope of Fam161a and retained its ability to localize to the microtubule cytoskeleton (Fig. 2B). The specificity of this antibody was further confirmed by immunoblotting, as it detected the truncated version as well as the full length form of Fam161a at the expected sizes of 70 and 80 kDa, respectively (Fig. 2C). Next, we analyzed Fam161a protein expression in the retina by immunoblotting with two different anti-Fam161a antibodies (Fig. 2D, Fig. S1). We detected a specific band of 80 kDa in lysates from wild-type retinas which was absent in one month old Fam161a<sup>GT/GT</sup> animals. However, we did not detect the truncated Fam161a protein in retinal lysates of Fam161a<sup>GT/GT</sup> mice, probably due to the low expression level of this polypeptide. Nevertheless, we studied Fam161a protein expression by immunohistochemistry in longitudinal retinal sections from one month old wild-type, Fam161a<sup>+/GT</sup> and Fam161a<sup>GT/GT</sup> mice. Fam161a immunoreactivity was detected in inner segment of photoreceptors, the outer plexiform layer and the inner plexiform layer of wild-type mice confirming previous findings (10) (Fig. 2E). Retinas of heterozygous
Fam161a-mutants showed similar immunoreactivity in photoreceptor inner segments, but lower levels in the plexiform layers (Fig. 2E). Fam161a\textsuperscript{GT/GT} mice showed strongly reduced levels of immunoreactivity in all retinal layers. Based on these observations, Fam161a\textsuperscript{GT/GT} mice can be considered to weakly express a truncated form of Fam161a in the inner segment of retinal photoreceptor cells.

\textit{Fam161a} gene trapped mice show a severe and progressive degeneration of the outer retina

We then analyzed the morphology of retinal layers in Fam161a\textsuperscript{GT/GT} mice at different ages using histology and spectral domain optical coherence tomography (SD-OCT) imaging. Longitudinal retinal sections of Fam161a\textsuperscript{GT/GT} mice displayed a severe and progressive retinal degeneration (Fig. 3A-G), involving a gradual loss of the outer nuclear layer which was almost absent in five month old Fam161a\textsuperscript{GT/GT} mice (Fig. 3F). Higher magnification microscopy further revealed that Fam161a\textsuperscript{GT/GT} retinas lacked clear photoreceptor inner/outer segment junctions and displayed disorganized photoreceptor outer segments already at one month of age (Fig. 3H, I). Thinning of the outer retina was accompanied by disturbance of the structural order and the appearance of pyknotic photoreceptor nuclei (Fig. 3J-N). In later stages, the outer retina had completely disappeared, whereas the inner retina remained largely unaffected (Fig. 3G, N). Progressive retinal degeneration of Fam161a\textsuperscript{GT/GT} mice was also observed using SD-OCT imaging with early absence of the defined layer between inner and outer segments (Fig. S2A). Further quantitative morphometric analyses along the nasal/temporal axis revealed that Fam161a\textsuperscript{GT/GT} animals have a significantly thinner outer nuclear layer already at one month compared to wild-type littermates (Fig. S2B). At two months of age, retinal thickness had declined to 50% until more than 90% of the outer nuclear layer were lost after six months in Fam161a\textsuperscript{GT/GT} retinas (Fig. S2B).
Degeneration on the Fam161a<sup>GT/GT</sup> retina is related to microglial reactivity

As microglial activation is a hallmark of inherited retinal degeneration, the morphology and spatial distribution of retinal microglial cells were examined in Fam161a<sup>GT/GT</sup> mice. The morphology of Iba1-positive microglial cells in wild-type retinas is characterized by small cell bodies and long ramified processes (Fig. 4A), with the cells being distributed in a highly ordered array throughout the plexiform layers (Fig. 4B). In two month old Fam161a<sup>GT/GT</sup> mice, microglia became bloated with shortened protrusions and a significant number of cells migrated to the outer retina (Fig. 4A, B). Reactive microglia then increasingly accumulated in the subretinal space of older Fam161a<sup>GT/GT</sup> mice until the territorial microglial network was completely disrupted in five month-old animals (Fig. 4A, B). To specify this glial reactivity in relation to cell death, we performed expression profiling using qRT-PCR. The retinal apoptosis marker Caspase 8 (Casp8) was significantly elevated in Fam161a<sup>GT/GT</sup> retinas from four weeks and showed a peak at two months (Fig. S3A). The microglia markers activated microglia whey acidic protein (Amwap) and Cd68 indicated initiation of microglial reactivity between two and three weeks of age with maximum levels in two month old Fam161a<sup>GT/GT</sup> retinas (Fig. S3B, C). The Müller glia cell and astrocyte marker Gfap was also significantly up-regulated at all time points and showed a maximum of expression between six and eight weeks after birth (Fig. S3D). These data suggest that initiation of immune activation is tightly associated with the onset of photoreceptor degeneration in Fam161a<sup>GT/GT</sup> retinas.

Fam161a-associated retinal degeneration leads to an early loss of visual function

To test the impact of the observed photoreceptor degeneration on retinal function, we recorded scotopic and photopic electroretinograms (ERGs) from Fam161a<sup>GT/GT</sup> mice and age matched controls. Fam161a<sup>GT/GT</sup> mice exhibited abnormal scotopic ERG responses already at one month of age, and at four months, ERG responses were almost undetectable (Fig. 5A). We then analyzed the residual visual function in one month old Fam161a<sup>GT/GT</sup> mice in detail
The dark-adapted ERG response waveforms (Fig. 5B) of Fam161a<sup>GT/GT</sup> animals showed lower a- and b-wave amplitudes (Fig. 5C) and prolonged implicit times (Fig. 5D) for all flash intensities. However, the elevated b/a-wave amplitude ratio (Fig. 5E) indicates a photoreceptor dominated degeneration. Light-adapted ERGs showed a less profound amplitude reduction (Fig. 5F, G) and prolonged implicit times (Fig. 5H) in Fam161a<sup>GT/GT</sup> animals compared to control mice.

**Spreading of the connecting cilium and aberrant disk organization in Fam161a<sup>GT/GT</sup> photoreceptor cells**

Fam161a has been previously localized to the cilium of rod photoreceptor cells (10, 11). Here, co-staining of FAM161A in the human retina with the cone marker peanut agglutinin (PNA) revealed FAM161A localization not only in cilia of rods, but also of cone photoreceptor cells (Fig. S4). We therefore focused on the analysis of photoreceptor cilia in Fam161a<sup>GT/GT</sup> mice applying transmission electron microscopy (TEM). Comparative TEM analysis of Fam161a<sup>GT/GT</sup> and wild-type photoreceptor cells revealed the following striking morphological changes in Fam161a<sup>GT/GT</sup> mice (Fig. 6): i) the connecting cilium was significantly reduced in length (WT: ~1.5 µm; Fam161a<sup>GT/GT</sup>: ~1.1 µm), ii) the characteristic microtubule doublets of the connecting cilium were spread in the proximal half thereby, iii) the outer segment base was markedly dilated (Fig. 6B), and iv) the membrane disk stacks in the outer segment were distorted, often arranged perpendicular to the disk stack orientation in wild-type photoreceptors (Fig. 6A). In contrast, the structural arrangements of the basal body and the adjacent centriole of the cilium were not altered and no ultrastructural changes were obvious in the inner segment and at the synapses of Fam161a<sup>GT/GT</sup> photoreceptor cells (Fig. 6 and data not shown). These structural Fam161a<sup>GT/GT</sup> phenotypes were identified as early as four weeks of age. At that time point alterations in ciliary structures were observed in 95% of all photoreceptors while the disk phenotype was present only in 60% of outer segments. Later
at 10 weeks, all photoreceptor cells in Fam161a\textsuperscript{GT/GT} mice showed defective cilia and almost all photoreceptor outer segments were altered.

**Altered distribution of ciliary molecules in spread photoreceptor cilia of Fam161a\textsuperscript{GT/GT} mice**

Next, we studied whether spreading of microtubule doublets in the connecting cilium and structural disruptions of the outer segments were associated with molecular changes in the ciliary compartment of Fam161a\textsuperscript{GT/GT} photoreceptor cells. We first analyzed the localization of the truncated Fam161a protein in the ciliary compartment. In wild-type mice, Fam161a was present in the connecting cilium, the basal body and the adjacent centriole (Fig. 7A, B) confirming our previous studies (6, 10), a report from rat retina (11), and our data for human rod and cone photoreceptor cells (Fig. S4). Fam161a co-localized with centrin3, a marker for the adjacent centriole, the basal body and the connecting cilium and with acetylated-tubulin, a marker for stabilized microtubules of cilia and centrioles (Fig. 7A, B, left panels). All ciliary marker stains confirmed the shortening of the connecting cilium in Fam161a\textsuperscript{GT/GT} photoreceptor cells (Fig. 7). Co-labelling for Fam161a and centrin3 or acetylated-tubulin revealed that the truncated Fam161a protein was restricted to the basal body and the adjacent centriole of the photoreceptor cilium (Fig. 7A, B, right panels). However, Fam161a staining was absent in the connecting cilium of Fam161a\textsuperscript{GT/GT} photoreceptors cells. In contrast, acetylated-tubulin remained in all compartments of the cilium, namely the axoneme, the shortened connecting cilium, the basal body and the adjacent centriole of the Fam161a\textsuperscript{GT/GT} photoreceptor cilium (Fig. 7C, D, E). Centrin3 also remained at the two centrioles of the cilium but was significantly reduced in the connecting cilium of Fam161a\textsuperscript{GT/GT} photoreceptors (Fig. 7A, B, D, E). The retraction of centrin3 to the proximal part of the connecting cilium was more evident in double labeling experiments for centrin3 and glutamylated-tubulin, both markers for the adjacent centriole, the basal body and the
connecting cilium in wild-type mice (Fig. 7F). In contrast to glutamylated-tubulin, which was stained all along the shortened connecting cilium, centrin3 was found only at its base in Fam161a\textsuperscript{GT/GT} photoreceptor cells (Fig. 7F).

Next, we tested whether the molecular composition of the photoreceptor axoneme, which projects from the tip of the connecting cilium into the photoreceptor outer segment, was altered in Fam161a\textsuperscript{GT/GT} photoreceptor cells. We performed immunolabeling of Rp1 and BBS5, two molecules which have been previously described as molecular components of the axoneme in photoreceptor outer segments (13, 14). Immunofluorescence analysis revealed that both proteins accumulated at the tip of the connecting cilium of Fam161a\textsuperscript{GT/GT} photoreceptors (Fig. 7G, H, I).

To specify our results on the disruption of the connecting cilium at higher resolution and by molecular means, we applied immunoelectron microscopy. We labeled centrin3 and acetylated-tubulin on ultrathin LR White sections through wild-type and Fam161a\textsuperscript{GT/GT} photoreceptor cells (Fig. S5). Our analysis confirmed that centrin3, which is normally present along the entire connecting cilium of photoreceptor cells (Fig. S5A) (15), was restricted to the lower part of the connecting cilium in Fam161a\textsuperscript{GT/GT} photoreceptor cells (Fig. S5B, C). In the apical region, where the microtubule doublets spread, no centrin3 was detected (Fig. S5B, C). In contrast, acetylated-tubulin was also present in the spread microtubules projecting into the remains of the outer segments of Fam161a\textsuperscript{GT/GT} photoreceptors (Fig. S5D-F). Quantification of the silver-enhanced immunogold labeling of centrin3 revealed that the total density of centrin3 per cilium was significantly reduced (Fig. S5G). Furthermore, our quantitative analysis demonstrates that the spatial distribution of centrin3 over the ciliary compartments was severely altered in Fam161a\textsuperscript{GT/GT} cilia (Fig S5H), confirming our immunofluorescence data.
**Fam161a-interacting partners are mislocalized in Fam161a\(^{GT/GT}\) photoreceptor cells**

A recent study demonstrated the interaction of Fam161a with the ciliopathy proteins lebercilin (Lca5) and Cep290 (11). The interaction of both proteins is mediated by the C-terminal domain mostly deleted in Fam161a\(^{GT/GT}\) mice. To analyze the consequences of Fam161a deletion/truncation for the distribution of these disease proteins, we double-labeled specimens for the ciliary marker centrin3 and lebercilin or Cep290, respectively. In wild-type photoreceptors, centrin3 and lebercilin co-localized in the basal body and the connecting cilium (Fig. 8A, left panel). Lebercilin was also present in the axoneme of the outer segment (Fig. 8A, left panel). In contrast, lebercilin was absent from the axoneme and the connecting cilium in Fam161a\(^{GT/GT}\) photoreceptors (Fig. 8A, right panel). Its co-localization with centrin3 was restricted to the basal body of Fam161a\(^{GT/GT}\) cilia (Fig. 8A, right panel). Cep290 also co-localized with centrin3 in the connecting cilium of wild-type mice (Fig. 8B, left panel). In Fam161a\(^{GT/GT}\) photoreceptor cells, Cep290 exclusively localized to the proximal connecting cilium and the basal body (Fig. 8B, right panel). The fact that both interacting partners of Fam161a mislocalized in the photoreceptor cilia of Fam161a\(^{GT/GT}\) mice (Fig. 8C) indicates that interaction with Fam161a is essential for their correct position in the connecting cilium of photoreceptor cells.

**The ciliary localization of the IFT complex B transport module is not altered in Fam161a\(^{GT/GT}\) photoreceptor cells**

The assembly and maintenance of cilia requires intraflagellar transport (IFT), a process mediated by molecular motors and IFT proteins organized into supramolecular protein complexes (16). Previous studies showed that IFT proteins are associated with photoreceptor cilia (17, 18). This prompted us to also examine whether IFT proteins were affected in the cilium of Fam161a\(^{GT/GT}\) photoreceptor cells. However, immunostainings of IFT20, IFT57 and
IFT88 revealed that none of these IFT molecules was mislocalized in Fam161a<sup>GT/GT</sup> retinal photoreceptor cilia (Fig. 8D-G).

**Spreading of Fam161a<sup>GT/GT</sup> photoreceptor cilia impairs transport to outer segment proteins**

We next investigated the functional consequences of the ciliary phenotype observed in Fam161a<sup>GT/GT</sup> photoreceptor cells. Therefore, we stained cryosections through the retina for the outer segment proteins opsin and rds/peripherin2. Most opsin is normally localized within photoreceptor outer segments of wild-type mice (Fig. 9A). In contrast, Fam161a<sup>GT/GT</sup> retinal opsin was found in the inner segments, the outer nuclear layer and at photoreceptor synapses (Fig. 9B). The higher resolution of anti-opsin immunoelectron microscopy then revealed the presence of opsin in the cytoplasmic and internal membranes of the inner segment of photoreceptor cells (Fig. 9C), the cytoplasmic membrane facing the perinuclear cytoplasm of rod nuclei (Fig. 9D), and in the synaptic terminals of rod cells (Fig. 9E). At the same developmental stage the amount of rds/peripherin2 was reduced to ~48% in photoreceptor outer segments (Fig. 9F, G). Furthermore, a mislocalization of rds/peripherin2 to synapses of the outer plexiform layer was obvious in Fam161a<sup>GT/GT</sup> retinas (Fig. 9E, G).

**Discussion**

We and others have earlier reported mutations in the FAM161A gene as one genetic cause for retinitis pigmentosa (6-9). *In vitro* biochemical studies have indicated that Fam161a may contribute to the function of the photoreceptor cilium (10, 11). To gain insights into the molecular pathomechanism underlying RP28 we continued with the functional characterization of Fam161a in vivo using a novel Fam161a gene trapped mouse line.
Photoreceptor cell death and microglial activation represent common pathological features in several retinal degeneration models (4, 19, 20). Fam161a<sup>GT/GT</sup> retinas showed a progressive loss of photoreceptor cells and had severely impaired visual function. In the Fam161a<sup>GT/GT</sup> retina, microglia also changed their morphology and migrated to the outer retina. Reactive microglial cells can secrete neurotoxic substances which then accelerate photoreceptor death and influence the function of retinal pigment epithelial cells (21-23). Transcript profiling showed that cell death in Fam161a<sup>GT/GT</sup> retinas was coincident with the induction of microglial activation. Notably, the peak of gliosis was reached between one and two months of age, which corresponds to the period with the steepest rate of cell loss and the time with most decline of retinal function. We therefore conclude that glial reactivity parallels retinal degeneration in the Fam161a<sup>GT/GT</sup> retina.

The overt accumulation of opsin in photoreceptor inner segments, around photoreceptor nuclei and at photoreceptor synapses in Fam161a<sup>GT/GT</sup> mice may trigger photoreceptor cell death. The same phenomenon was observed in Rp1-deficient or Lca5<sup>Gt/Gt</sup> gene trapped mice, two ciliopathy models in which defective ciliary transport also leads to the accumulation of opsin in the outer nuclear layer (13, 24). Moreover, rd16 mice, which express a truncated version of the ciliary protein Cep290 in the retina, display abnormal outer segment structure, retention of outer segment proteins, and rapid degeneration (25, 26). Photoreceptors may tolerate this to some extent but mislocalization of truncated rhodopsin to membranes of the inner segment favors cell death (27). A recent study raised some concerns that photoreceptor death in several RP mouse models may be caused by proteasomal insufficiency due to misfolded proteins (28). In this study, we did not observe accumulation of ubiquitinylated proteins in Fam161a<sup>GT/GT</sup> retinas (data not shown) and thus exclude proteasomal dysfunction as a trigger of cell death.
In $Fam161a^{GT/GT}$ mice, the layered retinal architecture is preserved similarly to other retinal ciliopathy models (13, 24, 29). However, our analyses revealed a significant reduction of connecting cilium length and spreading of microtubule doublets and thereby distortion of outer segment disks at early stages of degeneration. These results provide in vivo evidence for an essential role of Fam161a in the structural composition, maintenance and function of the connecting cilium of photoreceptor cells. Our analyses showed that the gene trapped N-terminal part of Fam161a targeted to microtubules and to the basal body, but not to the connecting cilium of photoreceptor cells. Interestingly, acetylation and glutamylation of microtubules in the cilium were not reduced, indicating that microtubules were not destabilized in $Fam161a^{GT/GT}$ mice. This is in line with in vitro studies linking the N-terminal part of Fam161a with microtubule binding and stabilization (10, 11).

Recent cell culture data indicated that Fam161a is integrated into a network of ciliary disease proteins present in the transition zone of cilia (11). The transition zone is localized between the basal body and the axoneme and its overlapping protein modules play a crucial role in the regulation of ciliary transport (30, 31). This strategic zone is homolog to the connecting cilium of photoreceptor cells (32, 33). The ciliary phenotype of $Fam161a^{GT/GT}$ mice could therefore result from the disruption of these protein modules. The truncated version of Fam161a lacks the C-terminal interaction sites for Cep290 and lebercilin and thereby their connection to Fam161a is probably disturbed. Indeed, both proteins do not correctly localize to the connecting cilium in $Fam161a^{GT/GT}$ mice, indicating that their interaction with Fam161a is crucial for their correct ciliary position. Cep290 normally tethers the transition zone microtubule pairs to the membrane (34) and interacts with the Bardet-Biedl Syndrome protein MKKS (35). The disruption of this connection may thus underly the spreading of microtubule doublets in the connecting cilium in $Fam161a^{GT/GT}$ mice. $Fam161a^{GT/GT}$ photoreceptors were also devoid of lebercilin in the connecting cilium. Lebercilin and
Fam161a are associated with the intraflagellar transport (IFT) module, which is essential for assembly and maintenance of cilia and flagella (24, 36). However, like the absence of lebercilin (24), Fam161a inactivation did not affect the ciliary localization of IFT proteins, indicating that both proteins do not serve as core components of IFT particles. Nevertheless, the molecular interplay between Fam161a, Cep290, and lebercilin may provide a structural framework for correct organization of the connecting cilium and delivery of cargo molecules to outer segments.

Our data from Fam161a$^{GT/GT}$ photoreceptor cells also indicate a widening of the outer segment basis, where novel membrane disks containing all components of the visual cycle are generated (37, 38). These outer segment alterations suggest that Fam161a may participate in disk neogenesis. Since we did not find Fam161a at the rims of the outer segments, we exclude its direct involvement in disk stacking. A comparable phenotype has been described in the Prph2$^{Rd2}$ mouse, where the disruption of the disk rim protein peripherin-2 causes defective disk organization (39). In accordance, our data show a reduction of Prph2 in Fam161a$^{GT/GT}$ photoreceptor outer segments, indicating that Fam161a may participate in the delivery of peripherin-2 to the site of disk formation.

In conclusion, we have established the Fam161a$^{GT/GT}$ mouse as a novel retinal degeneration model with a remarkable phenotype of a shortened connecting cilium and spread ciliary microtubule doublets. Mislocalization of essential transition zone components, misrouting of outer segment cargo and disturbed disk organization in Fam161a$^{GT/GT}$ mice implicates that Fam161a is crucial in the structural composition, maintenance and function of the connecting cilium. The Fam161a$^{GT/GT}$ mouse line may be very useful to reveal further insights into Fam161a containing protein modules and into photoreceptor ciliary biology in general.
Materials and Methods

*Animals.* Mice were kept in an air-conditioned environment on a 12-hour light-dark schedule at 22°C, and had free access to food and water. All procedures complied with the German Law on Animal Protection and the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals, 2011. When necessary, mice were anesthetized by intraperitoneal or subcutaneous injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). Pupils were dilated using 1% tropicamide and 2.5% phenylephrine.

*Human material.* Human donor eyes were obtained from the Department of Ophthalmology, University Medical Center Mainz, Germany. The guidelines to the declaration of Helsinki were followed.

*Fam161a gene trapped mouse.* The stem cell gene trap clone CMHD-GT_462E7_5S was obtained from the Canadian Mouse Mutant Repository. ES cell DNA was screened for correct gene trap insertion at the *Fam161a* locus by Sanger sequencing. ES cells were microinjected into C57BL/6J blastocysts and transferred to pseudopregnant female CD1 mice to generate chimeras. Germline transmission of the *Fam161a* gene trap allele was checked by genotyping and gene-trap positive males were bred with C57BL/6J females. The subsequent progeny was intercrossed to obtain homozygous *Fam161a*\(^{GT/GT}\) mice. Genotyping was performed by PCR with DNA isolated from tail tips using the primers *GT1-F* (5’-TCCCTGAAGCCCTTTAAGTTC-3’), *GT2-R* (5’-AAAGCATTGGGCGATTCACA-3’), *GT5-F* (5’-CGGACAGACACAGATAAGTTGC-3’), *GT6-R* (5’-AAAGCATTGGGCGATTCACA-3’). Primer pairs *GT1-F/GT2-R* (485 bp fragment) and *GT5-F/GT6-R* (929 bp fragment) were used to detect the gene trapped sequence. The wild-type sequence was detected using the primers *GT1-F/GT6-R* (385 bp fragment). Prior to
breeding, founder animals were tested negative for the *rd1*, *rd8* and *rd10* allele as described earlier (40-42).

RNA isolation and RT-PCR. Total RNA was extracted from murine retinas using the RNeasy Micro Kit (Qiagen, Hilden, Germany). cDNA synthesis was performed with the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, St-Leon-Roth, Germany). RT-PCR was performed with 50 ng cDNA to amplify intron-spanning fragments between *Fam161a* exon 2-3 (329 bp) and exon 4-5 (267 bp) using primers *Ex2-3_F* (5’-TGGCATTCGAGAGCACTATG-3’), *Ex2-3_R* (5’-CCCGGGAGTCTTTTCCTCTTA-3’), *Ex4-5_F* (5’-AGGAGTGAAAGGGCCAGGAT-3’) and *Ex4-5_R* (5’-TTTCTCCAGTGCGAGCCTCTTCT-3’). A 292 bp fragment of β-actin was amplified as the reference transcript with primers β-actin_F (5’-ACCCACACTGTGCCCATCTA-3’) and β-actin_R (5’-CGGAACCGCTCATTGCC-3’). PCRs were carried out using the Qiagen Taq Core kit (Qiagen, Hilden, Germany) and standard PCR conditions.

Quantitative real-time RT-PCR. Amplifications of 50 ng cDNA were performed on the ABI7900HT system (Applied Biosystems) in 10 µl reactions containing 1xTaqMan Universal PCR Master Mix (Applied Biosystems), 200 nM of primers, and 0.25 µl of dual-labeled probe (Roche ProbeLibrary, Roche Applied Science). Measurements were performed in triplicates and results were analyzed with an ABI sequence detector software version 2.3 using the ΔΔCt method for relative quantification.

Cloning and recombinant protein expression. For expression in Hek293 cells, EGFP-mFAM161A aa 1-363 was subcloned from full length EGFP-mFAM161A plasmid (10) using primer pair 5’-AAG CTT TGA TGG CTG CGC CCACC-3’ and 5’-GTC GAC TTA CGC AGC TGG CCT GTA AAT AAA-3’; the sequence was verified by direct sequencing.
**Western blotting.** Mouse retinal tissue or Hek293 cells were homogenized in cold RIPA buffer using a TissueLyser LT (Qiagen, Hilden, Germany) and protein concentrations were determined by Bradford assay (Roti-quant, Roth, Karlsruhe, Germany). 50 µg of total proteins were separated by SDS-PAGE on 10% gels and subjected to Western blotting. Membranes were incubated with a primary antibody against Fam161a as described previously (10) (Fam161a ab1), a commercial anti-Fam161a antibody (ab115810, Abcam, Cambridge, UK) (Fam161a ab2), and anti-Actin antibody (sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were visualized with chemiluminescence signals using secondary IgG-HRP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Spectral-domain optical coherence tomography (SD-OCT).** SD-OCT was performed on a Spectralis HRA + OCT device (Heidelberg Engineering GmbH, Dossenheim, Germany). Mice were placed on a custom made mounting platform for SD-OCT measurements (λ=870 nm; acquisition speed, 40,000 A-scans per second; average images per scan, 24). SD-OCT volume scans of 61 B-scans with 70 µm distance between B-scans (human dimension) were performed on the upper quadrant of the globe for all eyes; this corresponds to 23.33 µm distance between B-scans for murine eyes.

**Electroretinography.** Mouse ERGs were performed as described previously (43). Statistical significant difference between groups was calculated by One-way ANOVA, followed by multiple t-test using the Holm-Sidak method. All analyses and plotting were processed with R 2.15.2 and ggplot 0.9.2.

**Histology.** Semi-thin (1 µm) sections for light microscopy were prepared along the nasal-temporal plane of the retina and stained with Richardson’s stain as described previously (44).
Immunohistochemistry was performed on 10 µm thick horizontal retinal cryo-sections prepared from eyes embedded in optimal cutting temperature (OCT) compound (Hartenstein, Wuerzburg, Germany). For subcellular analysis, eyes were cryofixed in melting isopentane and cryosectioned as described elsewhere (45). The following antibodies were used: rabbit anti-Iba1 (Wako Chemicals, Neuss, Germany), rabbit anti-Fam161a ab1, mouse monoclonal anti-Rhodopsin (Invitrogen), mouse anti-Prph2 (5H2; provided by M. Biehl, Munich), rabbit anti-IFT20 and rabbit anti-IFT57 (provided by G. Pazour, Worcester, MA), rabbit anti-IFT88 and rabbit anti-lebercilin (Proteintech), mouse anti-Cep290 (3G4, provided by Chen and Shou), rabbit anti-BBS5 (provided by M. Nachury, Stanford, CA), chicken anti-Rp1 (provided by E. Pierce, Boston, MA), rabbit anti-glial fibrillary acidic protein (Sigma), mouse anti-acetylated α-tubulin (Sigma), mouse monoclonal anti-centrin3 (15), rabbit polyclonal anti-centrin3 antibodies, and fluorescein labelled lectin peanut agglutinin (FITC-PNA).

Samples were analyzed with a Leica DM 6000 B microscope (Leica microsystems, Bensheim, Germany) and images were processed with Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA).

For retinal whole mounts, eyes were enucleated and fixed in 4% formaldehyde for 4h before dissection. Isolated retinas were permeabilized with a solution of 25% Triton X-100 and 25% Tween 20 in 1x PBS followed by blocking in BLOTTO solution for 1h (1% milk powder and 0.01% in Triton X-100 in 1x PBS). Retinas were incubated overnight with rabbit anti-Iba1 (1:500) in antibody solution (2% BSA, 0.02% NaN₃ 0.1% Triton X-100 in 1x PBS) at 4°C followed by 1h secondary antibody incubation. Whole mounts and cross-sections were mounted in Dako fluorescent mounting medium (DakoCytomation GmbH, Hamburg, Germany) and imaged on an Axioskop2 MOT Plus Apotome microscope (Carl Zeiss).
Fixation for conventional electron microscopy. Enucleated eyes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 0.1 M sucrose for 1.5 h at 4°C. After 30 min fixation the cornea and lens were removed and the eye cups were fixed for additional 1 h. Eye cups were then washed with 0.1 M cacodylate buffer containing 0.1 M sucrose for 30 min. Subsequently, eye cups were fixed with 2% osmium tetroxide (OsO4) in 0.1M cacodylate buffer containing 0.1 M sucrose for 1 h at room temperature, followed by dehydration in ethanol (30–100%) and embedding in Renlam® M-1 resin (Serva Electrophoresis, Heidelberg, Germany).

Fixation and postembedding labelling for immunoelectron microscopy. Mouse retinas were fixed 0.1% glutaraldehyde and 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Fixed tissue was dehydrated stepwise in 30-98% ethanol, embedded in LR White hard (Science Services, Munich, Germany), polymerized at 4°C under ultraviolet (UV) light for 48–60 h, and postembedding immunolabelling was performed as described previously (46). In brief, ultrathin LR White sections were incubated with mouse anti-acetylated α-tubulin (Sigma), mouse monoclonal anti-centrin3 (15), or mouse monoclonal anti-opsin (clone K16-155) antibodies. A secondary goat anti-mouse Fab conjugated to nanogold™ (Nanoprobes, Stony Brook, NY) was applied in IgG-gold buffer. After postfixation in 2% glutaraldehyde for 10 min the nanogold™ labeling was silver-enhanced for 25 min.

Ultrathin sectioning and transmission electron microscopy. Ultrathin sections were made using a Reichert Ultracut S ultramicrotome (Leica), collected on Formvar-coated copper or nickel grids and counterstained with 2% uranyl acetate in 50% ethanol aund aq. 2% lead citrate. Ultrathin sections were analysed in a Tecnai 12 BioTwin transmission electron microscope (FEI, Eindhoven, The Netherlands). Images were obtained with a CCD camera (charge-coupled-device camera; SIS MegaView3; Surface Imaging Systems, Herzogenrath,
Germany) and processed with Adobe Photoshop CS (Adobe Systems).

Supplementary material

Supplementary Figures S1-S5.

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Conflict of Interest statement: non declared
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Figure legends

Figure 1. Disruption of the murine *Fam161a* locus and molecular characterization. (A) Schematic presentation of the *Fam161a* locus. The UPA vector disrupts the UPF0564 domain encoded by exon 3. Features of the UPA vector were described earlier (12, 41). SA, splicing acceptor, GFP, green fluorescent protein gene; Neo, neomycin resistance gene, SD, splicing donor. (B) Electropherograms showing sequencing results of genomic DNA isolated from wild-type and *Fam161a* gene trapped (*Fam161a*<sup>GT/GT</sup>) murine embryonic stem cells. Letters above the electropherograms indicate the nucleotides and amino acid sequences. (C) PCR-genotyping for detection of the mutant *Fam161a*<sup>GT/GT</sup> (GT) allele in genomic DNA from wild type (+/+), heterozygous (GT/+) and homozygous (GT/GT) mice. The location of genotyping primers is given as black arrows in (A). (D) RT-PCR analysis with retinal cDNA from wild-type (+/+), heterozygous (+/GT) and one month-old homozygous (GT/GT) *Fam161a* gene trapped mice. Truncation of the *Fam161a* locus by gene trapping did not affect transcript expression or induce mRNA decay. β-actin gene expression was used as positive control.

Figure 2. *Fam161a* protein expression in vitro and in the *Fam161a*<sup>GT/GT</sup> retina. (A) Schematic diagram of the gene trap within the Fam161a protein and the EGFP-Fam161a truncation construct (Fam161aΔ) used for expression in Hek293 cells. (B) Fluorescence images show Hek293 cells overexpressing the Fam161aΔ construct. EGFP fluorescence (green) and the anti-Fam161a antibody (red) both detect the truncated Fam161a protein in a cytoskeletal localization. (C) Immunoblot analysis of lysates isolated from Hek293 cells overexpressing Fam161aΔ or GST-tagged wild type Fam161a (Fam161a) using antibody Ab1. (D) Western blot analysis with two different anti-Fam161a antibodies (Ab1 and Ab2)
shows the absence of full-length Fam161a protein expression in $Fam161a^{GT/GT}$ animals. (E) Representative fluorescent images of retinal sections from one month-old $Fam161a^{+/+}$, $Fam161a^{+/GT}$ and $Fam161a^{GT/GT}$ mice, stained with anti-Fam161a antibody Ab1 recognizing the N-terminus. The retina was counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to define nuclear layers. Fam161a immunostaining was detected in inner segments and the OPL of both $Fam161a^{+/+}$ and $Fam161a^{+/GT}$ mice. Strongly reduced Fam161a immunoreactivity was seen in $Fam161a^{GT/GT}$ retinas. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar, 20µm.

**Figure 3. Disruption of the Fam161a locus leads to progressive retinal degeneration.**

Light-microscopy of horizontal retinal cross-sections from one to six month-old animals (A-G) reveal massive photoreceptor loss in $Fam161a^{GT/GT}$ retinas (C-G), which is preceded by the appearance of abnormal outer and inner segment morphology (B). High-magnification histological images of the outer retina illustrate disorganization of photoreceptor segments (I), distorted disk stacks (J-N) and pyknotic photoreceptor nuclei (J-N) in $Fam161a^{GT/GT}$ retinas. After six months, $Fam161a^{GT/GT}$ retinas were almost devoid of photoreceptors. RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 20µm.

**Figure 4. Microglial reactivity in the Fam161a$^{GT/GT}$ retina.** (A) Retinal whole mount images of Iba1-positive microglial cells in retinas from one to six months-old wild-type and $Fam161a^{GT/GT}$ mice reveal progressive changes of microglial morphology, including bloated
cell somata and loss of ramification. (B) Fluorescence images of Iba1-immunolabeling and DAPI counterstaining in retinal sections show microglial migration to the damaged outer retina in Fam161a<sup>GT/GT</sup> mice compared to wild-type controls. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars, 50µm.

Figure 5. Disruption of the Fam161a gene causes early ERG defects. (A) Representative scotopic ERG recordings are shown for two different stimulus intensities in one, three, four, and six month-old Fam161a<sup>GT/GT</sup> mice (colored lines) and age-matched wild-type mice (Fam161a<sup>+/+</sup>, black lines). (B) Average of dark-adapted ERG waveforms for all stimulus intensities (green lines, one month-old Fam161a<sup>GT/GT</sup>; black lines, age-matched wild-type). (C, D and E) Comparison of corresponding a- and b-wave amplitudes, implicit times and b/a-wave ratios of Fam161a<sup>GT/GT</sup> (green) mice versus age-matched controls (black). (F) Average light-adapted (photopic) ERG recordings at three different stimulus intensities of one month-old Fam161a<sup>GT/GT</sup> animals (green lines) vs. age-matched wild-type (black lines).

Fam161a<sup>GT/GT</sup> animals (green) showed reduced waveform amplitudes (G) and prolonged implicit times (H) in comparison to wild type (black) at the age of one month. *p<0.05; **p<0.01; ***p<0.001. One-way ANOVA, followed by multiple t-test using the Holm-Sidak method.

Figure 6. Ultrastructural analysis of Fam161a<sup>GT/GT</sup> photoreceptor cilia. (A) Electron micrographs of longitudinal sections through the ciliary region of photoreceptor cells of one month-old Fam161a<sup>GT/GT</sup> and wild-type mice. Photoreceptor outer segment (OS) disks of Fam161a<sup>GT/GT</sup> mice were disorganized in a perpendicular orientation and the outer segment
base was widened (double headed arrow). (B) High magnification view of the connecting cilium (CC). Fam161a<sup>GT/GT</sup> photoreceptors had shortened connecting cilia with their microtubules spread (arrowheads) in their upper part (above arrow). The structure of the ciliary base, namely the basal body (BB) and the adjacent daughter centriole (Ce), or other organelles of inner segment (IS) were not altered in Fam161a<sup>GT/GT</sup> mice. Scale bars, 500 nm.

**Figure 7.** Ciliary marker analysis in Fam161a<sup>GT/GT</sup> photoreceptor cells. (A) Indirect immunofluorescence co-labeling of Fam161 and centrin3 of inner- (IS) and outer segments (OS) of photoreceptors revealed strongly reduced Fam161a levels in a disrupted ciliary region (CR) of Fam161a<sup>GT/GT</sup> mice compared to wild-type animals. (B-D) High magnifications of the connecting cilium (CC) in wild-type retinas show the co-localization of Fam161a with the ciliary proteins centrin3 (B), acetylated α-tubulin (C), and co-localization of centrin3 with acetylated α-tubulin (D). (E) The cartoon describes the organization of the photoreceptor connecting cilium and the (mis)localization of the respective proteins examined in wild-type and Fam161a<sup>GT/GT</sup> mice. (F-H) High magnifications of the connecting cilium in wild-type and Fam161a<sup>GT/GT</sup> retinas show the co-localization of glutamylated α-tubulin with the ciliary protein centrin3 (F) and the outer segment marker proteins BBS5 (G) and RP1(H). (I) The cartoon describes the organization of the photoreceptor connecting cilium and the localization of the respective proteins examined in wild-type and Fam161a<sup>GT/GT</sup> mice. Yellow color in merged pictures denotes co-localization, arrowheads point to missing protein labels. The truncated Fam161a protein is restricted to the basal body (BB) and the adjacent centriole (Ce) in Fam161a<sup>GT/GT</sup> mice (B, C). Centrin3 resembles Fam161a mislocalization but remains slightly at the base of the connecting cilium (B, D). Tubulin acetylation was mainly detected adjacent to the proximity of Fam161a/centrin3 staining in both, wild-type and Fam161a<sup>GT/GT</sup> mice (C, D), while labelling of glutamylated tubulin was shortened in Fam161a<sup>GT/GT</sup> mice (F-
Both axoneme (Ax) markers, BBS5 and RP1 are linearly located in wild-type OS, but aggregate at the OS base in Fam161a<sup>GT/GT</sup> mice (G-H). OS, outer segment; IS, inner segment; Ax, axoneme; CC, connecting cilium; BB, basal body; Ce, centriole. Scale bar, 2 µm.

**Figure 8. Analysis of Fam161a interaction partners and IFT components in Fam161a<sup>GT/GT</sup> photoreceptor cells.** (A-F) High magnifications of the connecting cilium co-labeled with the ciliary marker centrin3 and lebercilin (Lca5) (A), Cep290 (B), or the intraflagellar transport components IFT20 (D), IFT57 (E), and IFT88 (F). (C, G) Cartoons describe the organization of the photoreceptor connecting cilia and the localization of the respective proteins examined in wild-type and Fam161a<sup>GT/GT</sup> mice. Yellow color in merged pictures denotes co-localization, arrowheads point to missing protein labels. In wild-type photoreceptors lebercilin (A) was located in all ciliary compartments and Cep290 perfectly co-localized with centrin3 (B). In Fam161a<sup>GT/GT</sup> mice only the lebercilin localization at the ciliary base remained (A), whereas Cep290 partially co-localized with centrin3 (B). IFT20 (D) was localized at the ciliary base, whereas IFT57 (E) and IFT88 (F) exhibited additional localization and at the tip of the connecting cilium (D-F). This localization pattern was not changed in Fam161a<sup>GT/GT</sup> mice. OS, outer segment; IS, inner segment; Ax, axoneme; CC, connecting cilium; BB, basal body; Ce, centriole. Scale bar, 2 µm.

**Figure 9. Mislocalization of opsin and peripherin2 in Fam161a<sup>GT/GT</sup> retinas.** (A, B) Merged immunofluorescence images of opsin-immunoreactivity (red) with DAPI-counterstain (blue) in retinal sections from (A) wild-type mice and (B) one month-old Fam161a<sup>GT/GT</sup> mice. (B) Images of retinas from one month-old Fam161a<sup>GT/GT</sup> mice reveal opsin immunoreactivity lining photoreceptor nuclei in the outer nuclear layer (ONL), as well as scattered opsin immunoreactivity along the inner segments of photoreceptors. (C-E) Silver
enhanced immunogold labeling of opsin in rod cells of one month-old $Fam161a^{GT/GT}$ mice. Opsin was mislocalized to the cytoplasmic membrane (arrowheads) and internal membranes (arrows) of the IS (C), in the perinuclear cytoplasmic membrane (arrowheads) in the ONL (D) and in the synaptic terminals of rod ribbon synapses (E, asterisks) of $Fam161a^{GT/GT}$ mice. (F, G) Merged immunofluorescence images of peripherin2 immunoreactivity (red) with DAPI-counterstain (blue) in retinal sections from (F) wild-type mice and (G) one month-old $Fam161a^{GT/GT}$ mice. (G) Images of retinas from one month-old $Fam161a^{GT/GT}$ mice reveal decreased peripherin2 immunoreactivity in photoreceptor outer segments (~50% compared to wild-type) and mislocalized peripherin2 in photoreceptor synaptic endings (white arrows OPL). OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; OLM, outer limiting membrane. Scale bars: A, B, F, G. 50 µm, C-E, 0.5 µm.
## Abbreviations

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<td>Amwap</td>
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