MPP3 Regulates Levels of PALS1 and Adhesion Between Photoreceptors and Müller Cells

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MPP3 and CRB1 both interact directly with PALS1/MPP5 and through this scaffold protein may form a large protein complex. To investigate the role of MPP3 in the retina we have analyzed conditional mutant Mpp3 knockout mice. Ultrastructural localization studies revealed that MPP3 is predominantly localized in apical villi of Müller glia cells. Retinas lacking MPP3 developed late onset retinal degeneration, with sporadic foci of rosette formation in the central part of the retina. Retinal degeneration in Mpp3 cKO mice was accelerated by exposure to moderate levels of white light. Electoretinography recordings in aging mice under both scotopic and photopic conditions ranged from normal to mildly subnormal, while the magnitude correlated with the strength and extent of morphological alterations. Loss of MPP3 resulted in significant loss of PALS1 at the subapical region adjacent to adherens junctions, and loss of MPP3 in Pals1 conditional knockdown retinas significantly accelerated the onset of retinal degeneration. These data suggest that MPP3 is required for maintaining proper levels of PALS1 at the subapical region, and indicate that the MPP3 gene is a candidate modulator of the Crumbs complex.

Key Words: retinal degeneration, Müller glia, Crumbs complex, aging, adherens junctions

Introduction

The mammalian retina is a polarized tissue consisting of seven different major cell types in three different cell layers and two plexiform layers, where synaptic contacts between different cell types are established (Agathocleous and Harris, 2009). At the outer limiting membrane adherens junctions are formed between photoreceptor cells and Müller glia cells (Lee et al., 2011; Reichenbach and Bringmann, 2013). The integrity of these adherens junctions between photoreceptors and Müller glia cells is vital to maintain proper organization and function of the retina. Besides typical adherens junction complex proteins, such as Nectins, Cadherins, and Catenins, there is also another complex of proteins essential for proper Müller glia-photoreceptor interaction. This protein complex is called the Crumbs complex and consists of CRB1, CRB2 and CRB3, MUPP1, PATJ, and PALS1/MPP5 (Bazellieres et al., 2009). All of these proteins are present in both photoreceptors and Müller glia cells at the subapical region adjacent to adherens junctions, except for CRB1, which is only present at the subapical region in apical villi of mouse Müller glia cells (van Rossum et al., 2006).

Mutations in CRB1 are associated with retinitis pigmentosa or Leber congenital amaurosis (den Hollander et al., 2001; den Hollander et al., 1999; Khalig et al., 2003; Lotery et al., 2001). Loss of mouse CRB1 function results in retinal disorganization followed by degeneration (Mehalow et al., 2003; van de Pavert et al., 2004; van de Pavert et al., 2007a,b). CRB1 is required for the maintenance of adherens junctions during light exposure (van de Pavert et al., 2004; van de Pavert et al., 2007b). Also loss of other members of the
Crumbs complex leads to retinal degeneration in mice. Loss of CRB2 in the retina results in retinal degeneration and these mice lose vision from one month of age (Alves et al., 2013).

Likewise, reduced levels of PALS1/MPP5 in the retina resulted in progressive retinal degeneration (Cho et al., 2012; Park et al., 2011). Additionally, a naturally occurring avian model of retinal dystrophy with a nonsense mutation in the Mupp1 (Mpdz) gene was identified (Ali et al., 2011).

Membrane Associated Guanylate Kinase (MAGUK) proteins have a number of functions, including roles in cell signaling, synaptogenesis and synaptic maintenance and scaffolding cell-cell adhesion proteins and receptors (Funke et al., 2005). One group of MAGUK proteins is the Membrane Palmitoylated Protein (MPP) family that contains a GuK, a PDZ, and a SH3 domain. In addition, all but MPP1 contain a tandem L27 domain. MAGUK proteins have a number of functions, including roles in cell signaling, synaptogenesis and synaptic maintenance and scaffolding cell-cell adhesion proteins and receptors (Funke et al., 2005).

In the retina at least four members of this family are expressed, MPP1, MPP3, MPP4 and MPP5 (Gosens et al., 2007; Kantardzhieva et al., 2006; Pavert et al., 2004). MPP1 is a potential link between the Usher syndrome modulator of the Crumbs complex. As such, we hypothesize that the PALS1/MPP5 in the retina resulted in progressive retinal degeneration (Cho et al., 2012; Yang et al., 2007). PALS1 is a core protein of the Crumbs complex, as it interacts with CRB1, CRB2 and CRB3 via its PDZ domain and with PATJ and MUPP1 via its N-terminal L27 domain.

The human MPP3 gene consists of 18 exons and there exist two isoforms due to splicing of exon 11. Full-length MPP3 protein consists of 585 amino acids, whereas the truncated isoform contains 315 amino acids and lacks the GUK domain (MPP3 GuK) (Kantardzhieva et al., 2006). In vitro experiments showed that MPP3 can interact with the adherens junction proteins Nectin1 and Nectin3, where association of MPP3 with Nectin1 increased cell surface expression of Nectin1 (Dudak et al., 2011) and with the serotonin 5-hydroxytryptamine2C receptor (Gavarini et al., 2006). In the retina, MPP3 has a direct interaction with PALS1/MPP5 at the outer limiting membrane and with DLG1 at the outer plexiform layer (Kantardzhieva et al., 2006).

To investigate the physiological role of MPP3 in the retina, we have analyzed Mpp3 conditional knockout (cKO) mice with Mpp3 expression specifically ablated in the retina using RxCre and Chx10Cre mice. Retinal degeneration developed between 3 and 12 months of age in retinas lacking MPP3 and we demonstrate that MPP3, like CRB1, is required for maintenance of adherens junctions during light exposure. Furthermore, we show that MPP3 is required for maintenance of proper levels of PALS1 at the subapical region, and Pals1 knockout in Mpp3 cKO mice prominently increased retinal disorganization and accelerated the onset of retinal degeneration. As such, we hypothesize that the MPP3 gene is a candidate modulator of the Crumbs complex.

**Abbreviations**

AF = Autofluorescence  
cKD = conditional Knockdown  
cKO = conditional Knockout  
cSLO = confocal Scanning Laser Ophthalmoscopy  
ERG = Electoretinography  
FA = Fluorescence Angiography  
GCL = Ganglion Cell Layer  
HRA = Heidelberg Retina Angiograph  
INL = Inner Nuclear Layer  
IPL = Inner Plexiform Layer  
IS = Inner Segment  
M = Months  
MAGUK = Membrane Associated Guanylate Kinase  
MG = Müller Glia  
MPP = Membrane Palmitoylated Protein  
OLM = Outer Limiting Membrane  
ONL = Outer Nuclear Layer  
OPL = Outer Plexiform Layer  
OS = Outer Segment  
PFA = Parafomaldehyde  
PRC = Photoreceptor Cell  
RF = Red-free  
RPE = retinal pigmented epithelium  
SAR = Subapical Region  
SD-OCT = spectral domain optical coherence tomography

**Materials and Methods**

**Mpp3 Conditional Knockout Mice**

Generation of Mpp3 conditional knockout mice has been described previously (Dudak et al., 2011). All mice were maintained as a cross of C57BL/6J/OlaHsd and 129/Ola (50%/50%). C57BL/6J/OlaHsd mice do not contain the Crb1 rd8 mutation. To obtain Mpp3 cKO mice, RxCre (Swindell et al., 2006) and Chx10Cre mice (Rowan and Cepko, 2004), maintained as a cross of C57BL/6J/OlaHsd and 129/Ola (50%/50%), with less than 1% FVB background and backcrossed onto C57BL/6J/OlaHsd and 129/Ola (50%/50%), were used as Cre mediated recombination. RxCre mice were obtained on FVB background, and backcrossed onto C57BL/6J/OlaHsd and 129/Ola (50%/50%), with less than 1% FVB background, and had no mutations in the phosphodiesterase 6b (pde6b0) gene. Genotyping of the mice was done by PCR on genomic DNA. For genotyping the following primers were used: RxCre 5′-GTGGGAGAATGTCTCGTGA-3′ and 5′-GTATCCCAACTTCTCT TGCG-3′, Chx10Cre 5′-GGGCGGGCCTGGGGGCACCTTCAAGA-3′ and 5′-CGGCGGGCGGCTCGAATCTCC-3′, Mpp3 5′-ATGCACGG CGAGGATCTGCTGTC-3′ and 5′-AATGTCTCAGCCTGTCGCT GG-3′. Mpp3F Chx10CreTg/ + and Mpp3F RxCreTg/ + were used as experimental animals, and Mpp3F Chx10CreTg/ − and Mpp3F RxCreTg/ − were used as controls.
RxGr^{Tg/+} were used as control animals. Generation and genotyping of Paltd cKD mice has been described previously (Park et al., 2011). All animals were kept at a 12 hour dark light cycle (100 lux) with food and water provided ad libitum. All animal experiments carried out were approved by the experimental animal committee of the Royal Netherlands Academy of Arts and Sciences (KNAW).

Morphological Analysis and Analysis of Retinal Degeneration

For morphological analysis of retinas, mice were euthanized with CO₂/O₂, eyes were enucleated and incubated in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 20 min. After incubation in PFA, eyes were shortly washed in PBS and taken through a series of ethanol dehydration of 30 min 50% ethanol, 30 min 70% ethanol, two times 30 min 90% ethanol and two times 100% ethanol. After the ethanol dehydration series, the eyes were incubated for 30 min in 100% ethanol and Technovit 7100 (1:1). Subsequently, eyes were incubated overnight in Technovit 7100 supplemented with 1 g hardener/100 mL. To embed eyes Technovit 7100 supplemented with 1 g hardener/100 mL was mixed with hardener 2 (15:1).

Thin 3 μm sections were cut on a microtome. After cutting, sections were briefly incubated in toluidine blue, short washed in ddH₂O and allowed to dry. Images from the Technovit sections were taken on a Leica DMRE light microscope and subsequent image analysis was performed using Adobe Photoshop 7.0. All procedures were carried out at room temperature unless otherwise stated. In order to quantify retinal degeneration, one retinal section of every 30 μm was used to count degenerative spots. The thickness of the outer nuclear layer and photoreceptor segment length was measured at 250, 500, 1,000, 1,500, 2,000, and 2,500 μm. Measurements were taken on a Leica DMRE light microscope and subsequent image analysis was performed with Zeiss LSM Image Browser 3.5 and Adobe Photoshop 7.0 software.

Immunohistochemistry

Mice were killed with CO₂/O₂, eyes were enucleated and incubated in 4% PFA in PBS (pH 7.4) for 20 min. Eyes were shortly washed in PBS and incubated for 30 min in PBS (pH 7.4) 15% sucrose and subsequently for 2 h in PBS (pH 7.4) 30% sucrose at 4°C and frozen in Tissuetek (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands). On a cryostate 8 μm sections were cut, and stored at −20°C until use for immunohistochemistry. First, sections were incubated in PBS (pH 7.4) supplemented with 0.3% Triton-X100 (PBS-TX100) and 10% normal serum for one hour. Next, sections were incubated in the primary antibodies in PBS TX100 for 2 h at room temperature or overnight at 4°C. After incubation in primary antibodies, sections were washed with PBS and incubated in secondary antibodies diluted in PBS for one hour. Finally, sections were washed in PBS and embedded in Dabco-Mowiol 4-88 (Sigma Aldrich). All reactions were carried out at room temperature unless otherwise stated.

The following primary antibodies were used: MPP3 CPH8 (1:100) (Kantardzhieva et al., 2006), MPP4 AK4 (1:300) (van de Pavert et al., 2004), CRB2 SKII (1:700; obtained from Dr. Rashbash), MUPP1 (1:200; BD Biosciences), PATJ (1:400; obtained from Dr. Le Bivic), DLG1 (1:200 BD Biosciences), PSD-95 (1:200; Cell Signaling), anti-Nectrin1 (1:500; MBL), β-catenin (1:100; BD Biosciences), Catenin p120 (1:100; BD Biosciences), GFAP (1:200; Dako). Fluorescent-labeled secondary antibodies were rabbit anti-chicken, goat antiserum, goat anti-rabbit or goat anti-rat IgGs conjugated to Cy3, Alexa 488 or Alexa 555 (1:500; Jackson Immunoresearch, Stanford, USA and Invitrogen). Nuclei were counterstained with TO-PRO-3 iodine (Invitrogen) at 1 μM. Sections were analyzed using a Zeiss CLSM 510 confocal microscope. Image analysis was performed with Zeiss LSM Image Browser 3.5 and Adobe Photoshop 7.0 software.

Immuo-Electron Microscopy

To perform immuno-electron microscopy, mice were euthanized by ketamine (100 mg/kg)/xylazine (10 mg/kg) intravenous injection and transiently perfused with PBS (pH 7.4) followed by 4% PFA in PBS (pH 7.4). After perfusion, eyes were removed, enucleated and incubated in PBS 15% and PBS 30% sucrose as described above. Eyes were frozen in Tissuetek and stored at −80°C until use. For immunoelectron microscopy, retinas were sectioned in 20 μm sections and incubated overnight at room temperature with rabbit anti-MPP3 CPH8 (1:50). After washing, the sections were incubated with a PowerVisionPoly-HRP-Goat Anti-rabbit IgG (ImmunoVision Technologies Co., Daly City, CA). To visualize the peroxidase, the sections were incubated in a Tris–HCl diaminobenzidine (DAB) solution containing 0.03% H₂O₂. The DAB reaction product was then intensified by a gold-substituted silver peroxidase method (van den Pol and Gorcs 1986). Sections were rinsed in sodium cacodylate buffer 0.1 M (pH 7.4) and postfixed for 20 min in 1% OsO₄ supplemented with 1% potassium ferrocyanide in sodium cacodylate buffer 0.1 M (pH 7.4). After washing in the sodium cacodylate buffer, the material was dehydrated and embedded in epoxy resin. Ultrathin sections were observed and photographed in FEI Tecnai 12 electron microscope. Electron micrographs were acquired as TIFF files with IMAGE II cameras.

Light Exposure Experiment

For the light exposure experiments, mice were continuously exposed to 3000 lux of white light for 72 hours without pupillary dilatation. In order to check for the Rpe65 Leu450Met polymorphism (Wenzel et al., 2001) a 545 bp fragment containing Rpe65 codon 450 sequence was PCR amplified using the following primers: Forward 5'-ACCAG AAATTTGGAGGGAAAC-3' and reverse 5'-CCCTTCATTTACCAGA GCTTCA-3' and used for a sequence reaction. A correlation between Rpe65 Leu450Met polymorphism and light-sensitivity in control or Mpp3 cKO mice was excluded. Food pellets and the water bottle were removed from the lid of the cage to avoid shade. Food pellets and solid drink was provided ad libitum in the cage. The mice were euthanized immediately after the 72 hours light exposure by CO₂/O₂. Morphological and immunohistochemical analysis and quantification of degenerative spots in these retinas was performed as described above.

In Vivo Analysis

Animal groups of 6 and 12 months of age were subjected to cSLO, SD-OCT and ERG measurements. The groups consisted of 4 to 6
animals per genotype. In the 6M old group Mpp3\(^{F+/−}\) RxCre\(^{Te/+}\) and Mpp3\(^{F+/−}\) RxCre\(^{Te/+}\) mice were analyzed, whereas in the 12M group Mpp3\(^{F+/−}\) RxCre\(^{Te/+}\), Mpp3\(^{F+/−}\) RxCre\(^{Te/+}\) and Mpp3\(^{F+/−}\) mice were analyzed.

**Electroretinography**

Ganzfeld electroretinograms (ERGs) were recorded according to the procedures described previously (Seeliger et al., 2001; Tanimoto et al., 2009). Briefly, after overnight dark adaptation, mice were anesthetized with ketamine (66.7 mg/kg body weight) and xylazine (11.7 mg/kg body weight), and the pupils were dilated with tropicamide eye drops (Mydriaticum Stulln, Pharma Stulln, Stulln, Germany). Single-flash responses were obtained under dark-adapted (scotopic) and light-adapted (photopic) conditions. Light adaptation was achieved with a background illumination of 30 cd/m\(^2\) starting 10 min before photographic recordings. Single white-flash stimuli ranged from −4 to 1.5 log cd/s/m\(^2\) under scotopic and from −2 to 1.5 log cd/s/m\(^2\) under photopic conditions, divided into ten and eight steps, respectively. Ten responses were averaged with interstimulus interval of 5 s (for −4 to −0.5 log cd/s/m\(^2\)) or 17 s (for 0 to 1.5 log cd/s/m\(^2\)).

**Confocal Scanning-Laser Ophthalmoscopy (cSLO)**

After ERG recording, the retinal structures of the still anesthetized animals were visualized via SLO imaging with a Heidelberg Retina Angiograph (HRA I) and a confocal scanning-laser ophthalmoscope, according to previously described procedures (Seeliger et al., 2005). The HRA features two argon wavelengths (488 nm and 514 nm) in the short wavelength range and two infrared diode lasers (795 nm and 830 nm) in the long wavelength range. Laser wavelengths used for fundus visualization were: 514 nm (RF, red-free channel), and 488 nm for autofluorescent images (AF), with a barrier filter at 500 nm.

**Spectral Domain Optical Coherence Tomography (SD-OCT)**

Spectral domain optical coherence tomography (SD-OCT) imaging was done with a commercially available Spectralis™ HRA+OCT device from Heidelberg Engineering featuring a broadband superluminescent diode at 870 nm as low coherent light source (Fischer et al., 2009). Each two-dimensional B-scan recorded at 30° field of view consists of 1536 A-scans, which are acquired at a speed of 40,000 scans per second. Optical depth resolution is ~7 μm with digital resolution reaching 3.5 μm. Imaging was performed using the proprietary software package Eye Explorer (version 3.2.1.0, Heidelberg Engineering). Retinal thickness was quantified using horizontal slices, located 1,500 μm distant from the optic nerve head in the temporal hemisphere.

**Protein Chemistry**

Retinas from 1- to 3-month-old mice were dissected and homogenized in lysis buffer containing 10 mM Hepes pH 7.9, 10 mM NaCl, 3 mM MgCl\(_2\), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na\(_3\)VO\(_4\), protease inhibitors (Roche) and the membrane fraction obtained as previously described (Kantardzhieva et al., 2006). After overnight incubation at 4°C, samples were centrifuged and protein concentration in the supernatant was measured by Bradford’s assay. Retina lysate was loaded on a gradient SDS-PAGE gel (NuPAGE gels, Invitrogen) and subsequently transferred onto PVDF membranes. After transfer, membranes were blocked by TBS (50 mM Tris, pH 7.5, 150 mM NaCl) supplemented with 1% Casein, and incubated with primary antibodies (anti-MPP3 1/500, anti-PALS1, 1/500 anti-Acin, 1/1,000) for one hour in T-TBS (TBS and 0.05% Tween 20) 0.3% Casein. Blots were washed with T-TBS and incubated in secondary antibodies (Streptavidin IRDye conjugated, Rockland Immunochemicals) in T-TBS 0.3% Casein for 1 h. After washing, bands were visualized using the Odyssey Infrared Imaging system (LI-COR Biosciences, Nebraska USA) and quantified by ImageJ (National Institutes of Health, USA) or Adobe Photoshop 7.0.

**Results**

**Mpp3 Conditional Knockout Mice**

In order to conditionally delete Mpp3 in the retina, the Mpp3 cKO mice were crossed with either RxCre or Chx10Cre mice. RxCre mice express Cre recombinase in the eye-determining field of the forebrain from embryonic Day 9 onwards, whereas Chx10Cre mice express Cre in the outer neuroblastic layer of the retina from embryonic Day 11.5 onwards (Rowan and Cepko, 2004; Swindell et al., 2006). Both Mpp3 floxed mice and Mpp3 floxed mice crossed with Cre mice were born in the expected Mendelian ratio.

In Mpp3\(^{F+/−}\) Chx10Cre\(^{Ts/+}\) and Mpp3\(^{F+/−}\) RxCre\(^{Ts/+}\) control retinas (from now on referred to as Mpp3 Chx10Cre and Mpp3 RxCre control, respectively), MPP3 is localized at the subapical region adjacent to the outer limiting membrane and in the outer plexiform layer (Fig. 1A) (Kantardzhieva et al., 2006). However, both in the Mpp3\(^{F+/−}\) Chx10Cre\(^{Ts/+}\) and in the Mpp3\(^{F+/−}\) RxCre\(^{Ts/+}\) (from now on referred to as Mpp3 Chx10Cre cKO and Mpp3 RxCre cKO, respectively) levels of MPP3 at the outer limiting membrane and in the outer plexiform layer were clearly reduced (Fig. 1A). Quantification of MPP3 levels revealed a 42.2 ± 4.1% (n = 3) reduction of MPP3 levels in Mpp3 cKO retinas compared with Mpp3 control retinas (Fig. 1B). The remnant MPP3 is most likely due to the mosaic expression of both the Chx10Cre and RxCre transgene (arrowheads in cKO in Fig. 1A) (Cho et al., 2012; Rowan and Cepko, 2004).

**MPP3 Is Localized to the Subapical Region of Müller Glia Cells**

Previous experiments showed that the Crumbs complex members CRB2, CRB3, PALS1, MUPP1, and PAT1 are present both in Müller glia apical villi and in photoreceptors at the subapical region adjacent to the outer limiting membrane, whereas CRB1 localizes at the subapical region of Müller glia but not of photoreceptors (van Rossum et al., 2006). For MPP3, however, the cellular localization at the outer limiting
membrane is still unknown. In order to investigate the cellular localization of MPP3, we performed immuno-electron microscopy for MPP3 using 1 month (M) old Mpp3 RxCre control and Mpp3 RxCre cKO retinas. No staining was detected in cKO retinas, whereas in Mpp3 control retinas we observed, at the level of the outer limiting membrane, MPP3 localization in apical villi of Müller glia cells, but no significant staining in photoreceptors, suggesting that, at the level of the outer limiting membrane, MPP3 is almost exclusively localized in Müller glia cells and not in photoreceptors (Fig. 1C,D).

**In Vivo Morphological and Functional Analysis**

The morphological and functional impact of MPP3 deficiency was assessed in vivo in 6M and 12M old Mpp3 RxCre cKO, Mpp3 RxCre control, and Mpp3F/F control mice (n = 4–6 mice/group) using confocal scanning-laser ophthalmoscopy (cSLO), spectral domain optical coherence tomography (SD-OCT) and electroretinography (ERG). cSLO imaging at 6M of age (Fig. 2) revealed signs of degeneration, as indicated by the white spots, in the central part of the retina of Mpp3 cKO mice, but not in heterozygous littermates (Fig. 2, left column). This finding correlated with areas of accumulation of fluorescent material found in the autofluorescence analysis (Fig. 2, second column from left). In the homozygous group, the disease effects were variable (Fig. 2, lower three rows). The fundus image in native (Fig. 2E,I,M) and autofluorescence mode (Fig. 2F,J,N) showed alterations in each Mpp3 RxCre cKO. Interestingly, where we observed detectable morphological damage such as disruption of OLM integrity, we also observed vascular

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**FIGURE 1:** Mpp3 cKO mice and subcellular localization of MPP3. A: Immunohistochemistry analysis for MPP3 in the retina in control mice showed that MPP3 is distributed at the subapical region and the outer plexiform layer. In Mpp3 cKO, MPP3 levels at the subapical region are markedly reduced, with some remaining MPP3 (arrowheads) likely due to mosaic expression of Cre in the Cre lines used. Also at the outer plexiform layer the level of MPP3 is clearly reduced, with arrowheads pointing to some remaining MPP3. B: Western blotting for MPP3 in retina lysate shows a 42.2 ± 4.1% reduction of MPP3 levels in Mpp3 cKO retinas compared to Mpp3 control retinas. C: Immuno electron microscopy showed that in Mpp3 RxCre control retina MPP3 is localized predominantly at the subapical region at the outer limiting membrane in apical villi of Müller glia cells (indicated by asterisks). Arrowheads point to MPP3 labeling. Inset is a magnification of the black box showing Müller glia apical villi. Black line marks the border of the photoreceptor inner segment, whereas the grey line delineates the Müller glia apical villi. Several grains of MPP3 are localized in the Müller glia apical villi. D: No staining for MPP3 in Mpp3 RxCre cKO retina. Scale bars: A: 20 μm, D: 500 nm.
abnormalities (Fig. 2G,H,K,L,O,P). At 12M of age, the morphological findings became more distinct (Supp. Info. Fig. S1). In general, the area of visible degenerations in native (Supp. Info. Fig. S1, left column) and autofluorescence mode (Supp. Info. Fig. S1, second column from left) appeared expanded around the central part of the retina. Furthermore, some hyperfluorescent areas were found in heterozygous mice (Supp. Info. Fig. S1F). Again, sites of retinal disorganization in the OCT analysis were found only in the animals with the highest degree of degeneration based on native and autofluorescence imaging (Supp. Info. Fig. S1S). The morphological findings correlated well with the functional data obtained with ERG (Fig. 3 and Supp. Info. S2). Depending on the extent of morphological alterations at 6M and 12M of age (see Fig. 3 and Supp. Info. S2), ERG recordings under both scotopic and photopic conditions ranged from normal to mildly subnormal but were not significantly different between control and Mpp3 cKO mice. Generally, the Mpp3 cKO mice with the highest degree of damage were associated with subnormal recordings in either scotopic or photopic ERG or both.
Loss of MPP3 Results in Late Onset Retinal Degeneration

In order to investigate whether loss of MPP3 resulted in retinal degeneration and to confirm the in vivo data obtained by cSLO and SD-OCT, we performed a histological analysis on 2, 3, 6 and 12M retinas from Mpp3 RxCre cKO and Mpp3 RxCre KO mice (n = 3–6 mice/group). This showed that histologically 2M Mpp3 cKO retinas were largely unaffected and indistinguishable from control retinas, suggesting that MPP3 is dispensable for proper morphological development of the retina (Fig. 4A,B). However, occasionally we found ingression of photoreceptors in the outer plexiform layer and ectopic photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium (subretinal space), suggesting that the integrity of the outer limiting membrane might be compromised locally (Fig. 4C–E). Also retinas from 3M old Mpp3 cKO mice were largely unaffected with occasionally ectopic photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium and ingression of photoreceptors in the outer plexiform layer (Fig. 4F,G). However, occasionally we found a clear region of retinal degeneration with rosettes, ingression of photoreceptors in the outer plexiform layer and protrusion of photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium (Fig. 4H). Additionally, in 6M and 12M Mpp3 cKO retinas we also found occasionally photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium, ingression of photoreceptors in the outer plexiform layer, disruption of the integrity of the outer limiting membrane and thinning of the outer nuclear layer (Fig. 4I–M). Retinas with Chx10Cre mediated Mpp3 deletion showed a comparable phenotype (Fig. 4N–U). There is some variability in the onset and extent of retinal degeneration, as in one 12M Mpp3 cKO animal there was a more prominent and widespread retinal degeneration. In this retina, there was a clearly disorganized outer

FIGURE 3: In vivo retinal function in Mpp3 RxCre cKO mice at 6M of age. Electroretinographic (ERG) single-flash intensity series data obtained from 6M old Mpp3 RxCre control and Mpp3 RxCre cKO mice. Shown are ERG responses of Mpp3 RxCre cKO mice (red traces) with different degrees of fundus alterations based on SLO and OCT data in comparison to a Mpp3 RxCre control mouse (black traces). A: Dark-adapted (scotopic) ERG responses. In each panel, the vertical line indicates the time of the stimulus onset. B: Light-adapted (photopic) ERG responses. C: Quantification of scotopic ERG b-wave amplitudes of each Mpp3 RxCre cKO mouse (red traces) as a function of the logarithm of the flash intensity (Vlogl function). For comparison, the Vlogl range of Mpp3 RxCre control mice (n = 3) is shown. The shaded area marks the range between 5% (lower black trace) and 95% quantiles (upper black trace) of amplitude data. D: Photopic b-wave amplitude quantification (panel format as in C). In A and B traces 1, 2 and 3 are from E-H, I-L and M-P mice respectively in Fig. 2. In summary, a variable functional deficit was found in 6M and 12M (see Supp. Info. Fig. S2) Mpp3 RxCre cKO mice under both scotopic and photopic conditions. This deficit correlated with the degree of morphological alteration.
nuclear layer and loss of photoreceptors (Fig. 4N). The degeneration was mostly confined to the outer nuclear layer, as the inner nuclear layer and ganglion cell layer seemed largely unaffected as judged from gross morphology. Finally, retinas from 18M old Mpp3 cKO mice showed sporadic loss of photoreceptors from the outer nuclear layer (Fig. 4O,P) with areas of disruption of the outer limiting membrane and ectopic photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium (Fig. 4Q) and at areas a more prominent loss of photoreceptors from the outer nuclear layer (Fig. 4R). Quantification of photoreceptor layer thickness in 6M, 12M (data not shown) and 18M mice revealed that in Mpp3 cKO retinas the outer nuclear layer is slightly thinner, albeit not significant (Fig. 4V). Also, analysis of photoreceptor outer segment length revealed no difference between control and Mpp3 cKO in 6M, 12M and 18M mice (data not shown).

In conclusion, retinas lacking MPP3 develop normally, but in aging retinas there are areas of disorganization starting at two months of age. A more prominent retinal degeneration phenotype developed between 3 and 12 months, with protrusion of photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium.
membrane and retinal pigment epithelium, ingestion of photoreceptors in the outer plexiform layer and occasionally rosette formation.

**Loss of MPP3 Does Not Affect DLG1 and MPP4 in the Outer Plexiform Layer**

In addition to the subapical region, MPP3 is localized at the outer plexiform layer (Fig. 1A) (Kantardzhieva et al., 2006). Like MPP3, MPP4 is also localized at the outer plexiform layer where it is required for correct localization of PSD95, Veli3, TMEM16B, and PMCA at the photoreceptor synapse (Aartsen et al., 2006; Sohr et al., 2009; Yang et al., 2007). Although in the retina MPP3 and MPP4 do not interact, both proteins form a complex with DLG1 at the photoreceptor synapse (Kantardzhieva et al., 2006). We performed immunohistochemistry for DLG1 and MPP4 in retinas from 6M old mice to assess whether loss of MPP3 affects levels or localization of DLG1 and MPP4 in photoreceptor synapses. This showed that localization and levels of DLG1 and MPP4 were indistinguishable between Mpp3 RxCre cKO and control retinas, suggesting that loss of MPP3 does not result in a loss of DLG1 or MPP4 from photoreceptor synapses (Fig. 5A–H). To investigate whether there are gross structural changes in photoreceptor synapses upon MPP3 removal, we performed immunohistochemistry for PSD-95. This showed that levels and localization of PSD-95 were indistinguishable between Mpp3 RxCre cKO and control retinas, suggesting that MPP3 is not required for assembly or maintenance of photoreceptor ribbon synapses (Fig. 5I–L).

**Loss of MPP3 Results in Disrupted Localization of PALS1 and CRB2**

In the retina, it has been shown that MPP3 does not directly bind CRB1, but MPP3 and CRB1 both interact with PALS1 at the subapical region. Hence, these may form a protein complex or form mutually exclusive complexes (Kantardzhieva et al., 2006; van de Pavert et al., 2004). To elucidate whether reduced levels of MPP3 resulted in a loss or delocalization of Crumbs complex member proteins, we performed

![Diagram](image)

**FIGURE 5** Reduced levels of PALS1 at the subapical region of RxCre cKO retinas, whereas localization of DLG1 and MPP4 at the outer plexiform layer is not affected. A,B: Immunohistochemistry on retinas from 6M old mice showing no difference in distribution and levels of DLG1 between Mpp3 RxCre control (A) and Mpp3 RxCre cKO (B) retina. C,D: Magnification of the red boxes in A and B respectively. E,F: No difference in localization of MPP4 between Mpp3 RxCre control and Mpp3 RxCre cKO retina. G,H: Magnification of the red boxes in E and F respectively. I,J: Immunohistochemistry for PSD-95 showed that gross morphology of ribbon synapses was unaffected in Mpp3 RxCre cKO retina (J) compared with control (I). K,L: Magnification of the red boxes in I and J respectively. M: In Mpp3 RxCre control retinas, PALS1 is distributed in patches at the outer limiting membrane. N: In Mpp3 RxCre cKO retinas, PALS1 is still distributed in patches at the outer limiting membrane, but the staining intensity is reduced, suggesting reduced levels of PALS1 at the outer limiting membrane. O,P: Magnification of the red boxes in M and N respectively. Q: Western blot on retina lysate showed reduced levels of PALS1 in Mpp3 RxCre cKO retinas. R,S: Patchy pattern of PALS1 at the subapical region in Mpp3 RxCre control retina (R), in Mpp3 RxCre cKO retina (S) at areas with protrusions of photoreceptors (asterisks in S) there is an interruption in patches of PALS1 at the outer limiting membrane. T,U: CRB1 is localized at the outer limiting membrane, just apical of β-catenin. Localization and protein level is not different between Mpp3 RxCre control and Mpp3 RxCre cKO retina. (V,W) Like CRB1, CRB2 is also localized at the outer limiting membrane, apically of β-catenin. At areas without retinal degeneration CRB2 levels and localization is not affected in Mpp3 RxCre cKO retinas. (X-AA) Levels and localization of Crumbs complex proteins MUPP1 (X,Y) and PATJ (Z,AA) are not affected upon MPP3 removal. (AB-AG) At areas with protrusion of photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium (asterisks in AE), gaps in CRB2 punctate pattern at the outer limiting membrane are observed (arrows in AC). (AB,AC) Immunohistochemistry for CRB2, (AD,AE) TO-PRO-3 nuclear staining. (AF,AG) Merged images of AB and AD (AF) or AC and AE (AG). Scale bars: J, S, AG: 20 μm, P: 10 μm L: 5 μm, M,N. 50 μm.
immunohistochemistry for the Crumbs complex members CRB1, CRB2, MUPP1, PALS1, and PATJ on 3M old retinas.

Interestingly, immunohistochemistry for PALS1 revealed a clear decrease in PALS1 immunostaining intensity at the subapical region in Mpp3 RxCre cKO retinas, suggesting that there is a partial loss of PALS1 upon MPP3 removal (Fig. 5M,N). These data were confirmed by the use of a second independent antibody against PALS1 (SN47; Supp. Info. Fig. S3A,B). In addition, we have previously shown that the anti-PALS1 antibodies do not cross react with MPP3 (Kantardzhieva et al., 2006). Immunohistochemical analysis of PALS1 in Mpp3 Chx10Cre mice also revealed a comparable reduction in PALS1 intensity at the outer limiting membrane in cKO retinas compared to control retinas (Supp. Info. Fig. S3C–F). In Mpp3 RxCre cKO retinas PALS1 was distributed in patches along the subapical region just like in control retinas, but with a clear reduction in staining intensity in the Mpp3 cKO (Fig. 5O,P). Western blotting to quantify levels of PALS1, which was detected as a doublet (Hurd et al., 2003; Makarova et al., 2003; van de Pavert et al., 2004), showed a 32 ± 7.3% (n = 6) decrease in PALS1 levels in Mpp3 RxCre cKO retinas compared to Mpp3 RxCre control (Fig. 5Q). At areas with protrusions of photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium there was a more prominent loss of patches of PALS1 in Mpp3 RxCre cKO retinas, suggesting that the integrity of the outer limiting membrane is compromised (Fig. 5R,S). As PALS1 is required for the correct localization of CRB1 and Crumbs family members at the subapical region (van Rossum et al., 2006), we asked whether partial loss of PALS1 in Mpp3 RxCre cKO retinas affected Crumbs complex members at the subapical region (van Rossum et al., 2006), we asked whether partial loss of PALS1 in Mpp3 RxCre cKO retinas affected Crumbs complex members at the subapical region. Immunohistochemistry for Crumbs complex members revealed that levels and localization of CRB1, CRB2, MUPP1 and PATJ were not affected upon MPP3 loss (Fig. 5T-AA). However, in the few regions where ectopic cells were found in the space between the outer limiting membrane and retinal pigment epithelium, there was clearly an interruption in the punctate pattern of the Crumbs complex member CRB2 (Fig. 5AB–AG). However, staining for adherens junction markers (e.g., β-catenin, catenin p120, see Fig. 7A–H) did not show a significant change in localization under these conditions. In conclusion, MPP3 is required for maintaining proper levels of PALS1 at the subapical region.

**Exposure to White Light Accelerates Retinal Degeneration in Mice Lacking MPP3**

Previously, we showed that Crb1 KO mice developed retinal degeneration between P14 and 9M of age. Exposure to light prominently accelerated the retinal degeneration in the Crb1 KO mice (van de Pavert et al., 2004; van de Pavert et al., 2007b). Since the Mpp3 cKO retinal phenotype we observed is comparable to the Crb1 KO retinal phenotype, we set out to investigate whether exposure to light accelerated and/or increased the extent of retinal degeneration in Mpp3 RxCre...
FIGURE 7: MPP3 is required for maintenance of adherens junctions during light exposure. A,B: Levels and localization of β-catenin is not affected in 6M of age Mpp3 RxCre cKO retinas. Note the reduced level of PALS1 in Mpp3 RxCre cKO retina (B). C,D: CRB1 expression at the outer limiting membrane is not affected upon MPP3 loss in 6M old retinas. E–H: No difference in localization and levels of adherens junction markers catenin p120 (E,F) and Nectin1 (G,H) upon MPP3 loss. I–L: At the outer limiting membrane Mpp3 RxCre control retinas, Nectin1 (I) co-localizes with the adherens junction marker β-catenin (J). (L) is a magnification of the red box in (K). (M–P) PALS1 (N), which is localized apically of adherens junctions, localized in Mpp3 RxCre control retinas just apical of Nectin1 (M). (P) is a magnification of the red box in (O). Q–T: Retinas from 12M old Mpp3 RxCre cKO mice (R) show reduced levels of PALS1 at the subapical region compared to control (Q,S) and where retinal degeneration is observed a clear loss in integrity of PALS1 localization was noticeable (T). U–W: Loss of Nectin1 at the subapical region in Mpp3 RxCre cKO retinas (V,W): compared with Mpp3 RxCre control retina (U). X–Z: Immunohistochemistry for PSD-95 showed ectopic photoreceptor ribbon synapses in outer nuclear layer of Mpp3 RxCre cKO retinas (arrowheads in Y), some of which almost reach the outer limiting membrane (arrow in Z) compared with Mpp3 RxCre control retina (X). (AA,AB) Immunohistochemistry for GFAP shows glial reactivity in 12M old Mpp3 RxCre cKO retina (AB) compared to Mpp3 RxCre control (AA). (AC,AD) Immunohistochemistry for β-catenin and PALS1 in 6M old Mpp3 RxCre control retina (AC) and Mpp3 RxCre cKO retina (AD) exposed to white light showing that in Mpp3 RxCre cKO retina at foci there is a loss of PALS1 and β-catenin at the outer limiting membrane, which is in some areas more prominent than in others. (AE) Example of interruption of outer limiting membrane integrity (arrowhead). (AF,AG) Loss of adherens junction markers Nectin1 and catenin p120 at foci of retinal degeneration in Mpp3 RxCre cKO retinas exposed to light. (AH) Magnification of the red box in (AG). Note the interruptions in outer limiting membrane integrity (indicated by the arrowheads). (AI,AJ) Immunohistochemistry for PSD-95 revealed ectopic PSD-95 labeling in the outer nuclear layer in Mpp3 RxCre cKO retinas at areas with ingression of photoreceptors in the outer plexiform layer. (AK) Magnification of the red box in (AJ), arrow indicates ingestion of photoreceptors, whereas the arrowhead indicates ectopic PSD-95 labeling in the outer nuclear layer. (AL,AM) Immunohistochemistry for GFAP shows that in Mpp3 RxCre cKO retinas there is prominent and widespread gliosis (AM), compared to Mpp3 RxCre control retinas where there is only mild gliosis (AL). Scale bars: H,R,T,Y,AB,AD,AG,AJ and AM: 20 μm, O,W, Z and AH: 10 μm, P, AE and AK: 5 μm.
cKO mice. To this end 6M Mpp3 RxCre control and cKO mice \((n = 4 \text{ mice/group})\) were continuously exposed to 3,000 lux of white light for 72 h, after which a histological analysis of retinal morphology was performed. Analysis of retinal morphology of these mice showed that in retinas from Mpp3 RxCre cKO mice exposed to light there were foci of ectopic cells in the space between the outer limiting membrane and retinal pigment epithelium, disruption of the integrity of the outer limiting membrane and sporadic rosette formation (Fig. 6A–C). Quantification of degenerative spots showed that light exposure increased retinal degeneration in Mpp3 RxCre cKO mice \((32.6 \pm 4.4 \text{ degenerative spots/retina})\) compared to Mpp3 RxCre cKO mice exposed to cycled light \((12 \text{ h dark/12 h 100 lux}) (13.9 \pm 1.3 \text{ degenerative spots/retina}, P = 0.05, \text{Fig. 6D})\). Exposure to white light did not increase retinal degeneration in Mpp3 RxCre control mice \((5.1 \pm 1.9 \text{ degenerative spots/retina})\) compared to Mpp3 RxCre control mice exposed to cycled light \((12 \text{ h dark/12 h 100 lux}) (2.7 \pm 1.8 \text{ degenerative spots/retina}, P = 0.25, \text{Fig. 6D})\). Interestingly, albino mice are naturally more prone to retinal degeneration and more sensitive to light. Therefore we analyzed Mpp3 RxCre cKO and control albino mice which were occasionally present in the litters from Mpp3 breedings and investigated the extent of retinal degeneration in these albino mice. In 6M old control Mpp3 RxCre albino mice retinas were grossly normal, although occasionally an interruption at the outer limiting membrane and protrusion of photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium was observed (Fig. 6E). In contrast, in 6M old Mpp3 RxCre cKO albino mice we observed several areas with groups of photoreceptor nuclei in the space between the outer limiting membrane and retinal pigment epithelium, where clearly the integrity of the outer limiting membrane was compromised (Fig. 6F). In these degenerative foci we observed rows of two or more photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium (Fig. 6G). These data show that loss of MPP3, similar as CRB1, results in mild retinal degeneration, which is accelerated by exposure to moderate levels of light.

**MPP3 Is Required for Maintenance of Adherens Junctions During Exposure to White Light**

As described above, the adherens junctions and expression of Crumbs complex proteins were hardly affected and the morphology of the retina only mildly affected in 6M old mice deficient for MPP3 (Fig. 7A–F). Only at sporadic foci where the integrity of the outer limiting membrane was disrupted, adherens junctions were disrupted as well. Recently it was shown that MPP3 has a direct interaction with Nectin1 and -3 and that MPP3 is important for trafficking and processing of Nectin1 (Dudak et al., 2011). Nectins are cell-cell adhesion proteins that form both homotypic and heterotypic interactions at adherens junctions between cells. We therefore tested the localization of Nectins in the retina. Nectin1 is localized at the outer limiting membrane, just basally of PALS1, and colocalized with the adherens junction marker \(\beta\)-catenin, showing that also at the outer limiting membrane Nectin1 is a marker for adherens junctions (Fig. 7I–P).

Distribution of Nectin1 was indistinguishable between 6M old Mpp3 RxCre cKO and control retinas (Fig. 7G,H). As histological analysis of 12M old Mpp3 RxCre cKO retinas revealed a somewhat more prominent degeneration phenotype we investigated whether in these retinas we could observe a loss of Crumbs complex and adherens junction proteins at more foci. Immunohistochemistry analysis for PALS1 in 12M old retinas showed that levels of PALS1 are reduced at the subapical region in Mpp3 cKO retinas compared to control (Fig. 7Q,R). At foci with ectopic photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium there was a clear loss of integrity in patches of PALS1 (Fig. 7S,T). Notably, in 12M old Mpp3 cKO retinas there was loss and mislocalization of Nectin1 at the subapical region (Fig. 7U–W). Immunohistochemistry for the photoreceptor synapse marker PSD-95 showed that in 12M old retinas we observed ectopic photoreceptor synapses in the outer plexiform layer (Fig. 7X,Y), with some ribbon synapses ectopically localized at the level of the subapical region (Fig. 7Z). Finally, immunohistochemistry for GFAP revealed that in 12M old Mpp3 cKO retinas there was glial reactivity compared with control (Fig. 7AA,AB).

As exposure to white light prominently accelerated the retinal degeneration phenotype, we were interested to investigate whether exposure to white light induced loss of adherens junctions and Crumbs complex proteins at the subapical region. PALS1 and \(\beta\)-catenin staining showed that in 6M old Mpp3 control retinas exposed to light there were no areas with loss of these markers (Fig. 7AC). However, in Mpp3 cKO retinas exposed to light at foci with ectopic photoreceptors there was a loss of PALS1 and \(\beta\)-catenin (Fig. 7AD,AE). In addition, we also observed a loss of the adherens junction markers Nectin1 and catenin p120 (Fig. 7AF–AH). Since we also observed ingression of photoreceptors in the outer plexiform layer in Mpp3 cKO retinas exposed to light at these foci, we wondered whether ribbon synapses might be affected in these areas. Staining for PSD-95 revealed that at these areas there was ectopic PSD-95 labeling, indicative of ectopic photoreceptor synapses in the outer nuclear layer (Fig. 7AI–AK). Finally, we performed GFAP staining to assess whether and to which extent there might be gliosis in Mpp3 cKO retinas exposed to light. This showed that, in contrast to Mpp3 control retinas, which only showed mild gliosis, there was...
massive and prominent gliosis in Mpp3 cKO retinas exposed to light (Fig. 7AL,AM).

Thus, retinas from 12M old Mpp3 cKO mice develop retinal degeneration due to loss of adherens junctions at the outer limiting membrane and retinal pigment epithelium and loss of groups of photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium and protrusion of photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium. Exposure of 6M old Mpp3 cKO animals to light resulted in a comparable phenotype as in 12M old mice not exposed to light, showing that exposure to moderate levels of light accelerated the phenotype and that Mpp3 is required for maintaining proper levels of PALS1 at the subapical region and for maintenance of adherens junctions during light exposure.

Knockdown of PALS1 in Mpp3 cKO Mice Prominently Accelerates and Increases Retinal Degeneration

Finally, we investigated whether conditional knockdown (cKD) of Pals1 in Mpp3 cKO retinas would result in increased and more widespread retinal degeneration as compared with that seen in the individual cKO/cKD mice. To achieve this we crossed Pals1 Chx10Cre cKD mice with Mpp3 Chx10Cre cKO mice. Our previous analysis of Pals1 cKD mice showed that RxCre mediated cKD of Pals1 in the retina resulted in widespread retinal degeneration due to an important function for PALS1 in the retinal pigment epithelium, whereas Chx10Cre mediated Pals1 cKD only resulted in mild and late-onset retinal degeneration (Park et al., 2011).

In order to investigate whether knockdown of Pals1 in Mpp3 cKO mice would result in increased retinal degeneration, we analyzed the extent of retinal degeneration in 1M old Mpp3 Chx10Cre control, Mpp3 Chx10Cre cKO, Mpp3+/+ Pals1Te/+ Chx10CreTe/+ (Pals1 cKD), Mpp3+/+ Pals1Te/+ Chx10CreTe/+ (Mpp3/Phal1 cKD), and Mpp3+/+ Pals1Te/+ Chx10CreTe/+ (Mpp3/Phal1 double cKO/KD) mice (n=2-4 mice/group). Quantification of degenerative spots in 1M old Mpp3 Chx10Cre control (5.3 ± 4.3 degenerative spots/retina) and Mpp3 Chx10Cre cKO (4.7 ± 3.1 degenerative spots/retina) showed that there were only very few degenerative spots in Mpp3 Chx10Cre cKO retinas. As we have recently shown (Park et al., 2011), histological analysis of retinas from Pals1 cKD mice showed that only occasionally a spot of retinal degeneration was observed, with protrusion of groups of photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium and loss of photoreceptors. Quantification of degenerative spots in 1M old Pals1 cKD (29.0 ± 48.4 degenerative spots/retina) and in Mpp3/Phal1 Chx10Cre cKD (40.3 ± 7.0 degenerative spots/retina) showed an increased number of degenerative spots in the retina compared to Mpp3/Phal1 cKO mice (p<0.05). Interestingly, quantification of degenerative spots in retinas from 1M old Mpp3/Phal1 Chx10Cre double cKO/KD mice (178.8 ± 20.7 degenerative spots/retina) showed that there is a ~4-fold increase in number of degenerative spots in Mpp3/Phal1 double cKO/KD mice (p<0.05) compared to Mpp3/Phal1 Chx10Cre cKD mice (Fig. 8).

These data suggest that MPP3 is required to maintain PALS1 at the subapical region, and that knockdown of Pals1 in Mpp3 cKO mice, which already have reduced levels of PALS1 at the subapical region, clearly accelerated the onset and increased the extent of retinal degeneration. Finally, these data suggest that there is a direct relation between the level of PALS1 and the extent of retinal degeneration.

Discussion

In this study we showed that loss of MPP3 in the retina results in retinal degeneration which developed between 3 and 12 months of age and which is accelerated upon exposure to moderate levels of white light. Additionally, we showed that MPP3 is essential to maintain levels of PALS1 at the subapical region near the outer limiting membrane. At areas with protruding photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium there is a loss of Crumbs complex proteins. Also, we showed that loss of MPP3 in Pals1 knockout retinas further decreased PALS1 levels and significantly accelerated the onset of retinal degeneration.

To some extent the phenotype in the Mpp3 cKO retinas is comparable to that of Crib1 knockout retinas, since the retinal degeneration shows slow progression, retinal development...
is not affected, retinal disorganization and degeneration is confined to a specific part of the retina and retinal degeneration is accelerated upon exposure to moderate levels of white light in both knockouts (van de Pavert et al., 2004; van de Pavert et al., 2007b). However, it seems that the Mpp3 cKO phenotype is somewhat more subtle than the Crb1 phenotype in terms of extent of retinal degeneration. One explanation for this could be that the Crb1 mutant mouse is a complete knockout, whereas the Mpp3 mutant mouse is a conditional knockout, since Chx10Cre and -RxCre lines show a mosaic Cre expression in the retina (Cho et al., 2012; Rowan and Cepko, 2004; Swindell et al., 2006). Thus, in the Crb1 KO there is a complete removal of the CRB1 protein, while in the Mpp3 cKO the removal is not complete and remnant MPP3 protein is present, explaining the more subtle phenotype.

In contrast to the Crb2 cKO, Pals1 RxCre cKD and Pals1 RxCre cKO phenotypes (Alves et al., 2013; Cho et al., 2012; Park et al., 2011), the Mpp3 and Crb1 retinal phenotypes are relatively mild. An explanation for this could be that CRB2 and PALS1 are present in the Crumbs complex at the subapical region both in Müller glia apical villi and in photoreceptor inner segments, whereas CRB1 and MPP3 are predominantly localized in Müller glia apical villi (van Rossum et al., 2006). Therefore, in Crb2 Chx10Cre cKO, Pals1 RxCre cKD or Pals1 RxCre cKO retinas there is a deficiency or reduction of CRB2 or PALS1 in both photoreceptor inner segments and Müller glia apical villi, resulting in disturbed Crumbs complex function in both photoreceptors and Müller glia cells at the subapical region. In contrast, loss of CRB1 or MPP3 only disturbs Crumbs complex function in Müller glia apical villi, while the Crumbs complex is still intact in the photoreceptors. Additionally, there might be functional redundancy between CRB1 and CRB2, and between MPP3 and MPP4, so that loss of CRB1 in Müller glia apical villi might be compensated for by CRB2 in the apical villi, whereas loss of MPP3 from the subapical region in Müller glia apical villi might be compensated for by other MPP family members.

In retinas lacking MPP3 we found that the localization and levels of the Crumbs complex members at the subapical region is not affected, although at foci with protrusions of photoreceptors in the space between the outer limiting membrane and retinal pigment epithelium the integrity of the outer limiting membrane and the Crumbs complex is compromised. Surprisingly, levels of PALS1 at the subapical region were reduced in the Mpp3 cKO. Since PALS1 is a key protein in the Crumbs complex, one could expect a concomitant loss of Crumbs complex proteins. Indeed, loss of the PALS1 orthologue magie oko in zebrafish and knockdown of PALS1 in shPals1-RxCre mouse retina resulted in progressive retinal degeneration and clear defects on retinal stratification (Park et al., 2011; Wei and Malicki, 2002), but knockdown of PALS1 in shPals1-Chx10Cre mouse retina resulted in mild retinal degeneration, yet somewhat more severe than observed in Mpp3-RxCre or Mpp3-Chx10Cre cKO retinas. Despite reduced levels of PALS1 at the subapical region in Mpp3 cKO, retinal degeneration is relatively mild, and retinal stratification is moderately affected. One explanation is that levels of PALS1 are not sufficiently reduced in Mpp3 cKO retinas. Indeed, Chx10Cre driven PALS1 cKD resulted in only mild retinal degeneration, demonstrating that ~34% loss of PALS1 in the neural retina is not sufficient to induce retinal degeneration (Park et al., 2011). As shown here, removal of MPP3 resulted in a comparable reduction in levels of PALS1 as in Pals1 Chx10Cre cKD retinas, strongly suggesting that a ~30% loss of PALS1 is not sufficient to induce severe retinal degeneration defects. Additionally, in Mpp3 cKO retinas levels of PALS1 at the subapical region are likely still sufficient to maintain the Crumbs complex. Indeed, knockdown of Pals1 in Mpp3 cKO mice resulted in a ~4-fold increase in degenerative spots, suggesting that extent and onset of retinal degeneration is related to the level of PALS1 at the subapical region. Another explanation might be that MPP3 is predominantly present in Müller glia cell apical villi at the subapical region, explaining the partial loss of PALS1, as in photoreceptor inner segments other MPP proteins might be responsible for PALS1 modulation, likely MPP4 and MPP1. Indeed, MPP4 and MPP1 can interact with PALS1, and are expressed at the subapical region in photoreceptors (Gosens et al., 2007; Kantardzhieva et al., 2005). Alternatively, the MPP3-PALS1 complex might represent a reserve pool of PALS1. MPP3 might be a modulator required for stabilization of PALS1 at this reserve pool, and hence loss of MPP3 might result in partial depletion of PALS1. As there is likely only a partial depletion of PALS1 from this pool in Mpp3 cKO retinas, there is still sufficient to replenish PALS1 at the Crumbs complex. In contrast, further reducing or depleting PALS1 from this reserve pool might eventually compromise PALS1 replenishment in the Crumbs complex, resulting in prominent and widespread retinal degeneration. MPP3 and PALS1 interact directly with the adherens junction proteins Nectin1 and -3, where association of MPP3 with Nectins is important for their trafficking and processing (Dudak et al., 2011). As we have shown here, Nectin1 is expressed at the outer limiting membrane, and localized at the adherens junctions where it co-localized with other adherens junction markers. Loss of MPP3 did not result in loss of Nectin1, suggesting that there is functional redundancy, presumably with PALS1. However, at areas where the integrity of the outer limiting membrane was compromised, there was a loss of adherens junction proteins, including Nectin1. Also, in aging retinas lacking MPP3 there was a more prominent loss of integrity of the outer limiting membrane at foci, loss of Crumbs complex proteins and...
loss of adherens junction proteins including Nectin1. It is unclear whether loss of MPP3 eventually results in a loss of Nectin1 and therefore leads to loss of adherens junctions, or whether there is first a loss of adherens junctions and therefore as a result loss of Nectin1.

In humans, several mutations in CRB1 are associated with inherited retinopathies such as retinitis pigmentosa and Leber congenital amaurosis (den Hollander et al., 2001; den Hollander et al., 1999; Khalig et al., 2003; Lottery et al., 2001). Retinitis pigmentosa model mice rdi8 were shown to have a mutation in the Crb1 gene, resulting in truncated CRB1 lacking the transmembrane and the cytoplasmic domain due to a premature stop codon (Mehalow et al., 2003). Likewise, the Crb1 KO and knock-in mice showed a similar retinal degeneration phenotype (van de Pavert et al., 2004; van de Pavert et al., 2007a). So far, no mutations in MPP3 have been found which are associated with inherited retinopathies. However, the fact that mice lacking Mpp3 in the retina develop mild retinal degeneration that is accelerated upon exposure to light suggests that MPP3 is a new candidate gene for inherited retinopathies.

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