Neurobiology of Disease

PALS1 Is Essential for Retinal Pigment Epithelium Structure and Neural Retina Stratification

Bokyung Park,1 Celso Henrique Alves,1 Ditte M. Lundvig,1 Naoyuki Tanimoto,3 Susanne C. Beck,3 Gesine Huber,3 Fabrice Richard,1 Jan Klooster,1 Till F. M. Andlauer,1 Eric C. Swindell,3 Milan Jamrich,4 André Le Bivic,1 Mathias W. Seeliger,3 and Jan Wijnholds1

Departments of 1Neuromedical Genetics and 2Retinal Signal Processing, The Netherlands Institute for Neuroscience, Royal Netherlands Academy of Arts and Sciences, 1105 BA Amsterdam, The Netherlands, 3Division of Ocular Neurodegeneration, Centre for Ophthalmology, Institute for Ophthalmic Research, University of Tübingen, 72076 Tübingen, Germany, 4IBDML, CNRS UMR 6216, Université de la Méditerranée, Campus de Luminy, Case 907, 13288 Marseille Cedex 09, France, 5Department of Pediatrics, The University of Texas Medical School at Houston, Houston, Texas 77030, and 6Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030

The membrane-associated palmitoylated protein 5 (MPP5 or PALS1) is thought to organize intracellular PALS1-CRB-MUPP1 protein scaffolds in the retina that are involved in maintenance of photoreceptor–Müller glia cell adhesion. In humans, the Crumbs homolog 1 (CRB1) gene is mutated in progressive types of autosomal recessive retinitis pigmentosa and Leber congenital amaurosis. However, there is no clear genotype–phenotype correlation for CRB1 mutations, which suggests that other components of the CRB complex may influence the severity of retinal disease. Therefore, to understand the physiological role of the Crumbs complex proteins, especially PALS1, we generated and analyzed conditional knockdown mice for Pals1. Small irregularly shaped spots were detected throughout the PALS1 deficient retina by confocal scanning laser ophthalmoscopy and spectral domain optical coherence tomography. The electroretinography a- and b-wave was severely attenuated in the aged mutant retinas, suggesting progressive degeneration of photoreceptors. The histological analysis showed abnormal retinal pigment epithelium structure, ectopic photoreceptor nuclei in the subretinal space, an irregular outer limiting membrane, half rosettes of photoreceptors in the outer plexiform layer, and a thinner photoreceptor synaptic layer suggesting improper photoreceptor cell layering during retinal development. The PALS1 deficient retinas showed reduced levels of Crumbs complex proteins adjacent to adherens junctions, upregulation of glial fibrillary acidic protein indicative of gliosis, and persisting programmed cell death after retinal maturation. The phenotype suggests important functions of PALS1 in the retinal pigment epithelium in addition to the neural retina.

Introduction

Polarization of cells depends on the asymmetric distribution of multiprotein complexes, and these complexes provide the basis for formation of cell-type-specific junctions that are crucial for their function (Bulgakova et al., 2008). Central components of many protein complexes are scaffolding proteins, and membrane-associated guanylate kinases (MAGUKs) form a family of scaffolding proteins involved in cell polarity at tight junctions (Funke et al., 2005). PALS1 (Protein associated with Lin7) belongs to the MAGUK family of proteins, which are scaffolding factors containing one PDZ, one SH3, and two L27 domains (Kamberov et al., 2000). The PDZ domain of PALS1 links it to CRB1, 2, and 3, whereas the N-terminal L27 domain links it to PALS1-associated tight junction protein (PATJ) or multi-PDZ domain protein 1 (MUPP1). CRB-PALS1-PATJ and -MUPP1 are the core proteins in the Crumbs complex, and the complex is conserved in mammalian cells and localizes at tight junctions or subapical regions (SARs) immediately adjacent to adherens junctions in polarized epithelia (Kamberov et al., 2000; Roh et al., 2002; Makarova et al., 2003).

In humans, mutations have been identified in the CRB1 gene in individuals with Leber congenital amaurosis (LCA), and retinitis pigmentosa (RP) type 12 characterized by preservation of para-arteriolar retinal pigment epithelium (PPRPE), RP with Coats-like exudative vasculopathy, and early-onset RP without PPRPE (den Hollander et al., 1999; den Hollander et al., 2004; Gosens et al., 2008; Richard et al., 2006). Various animal models have been used to study the function of Crumbs. In Drosophila, loss of Crumbs results in the reduction of the stalk membrane,
and the photoreceptor cells undergo light-dependent degeneration (Johnson et al., 2002). Loss of CRB1 function in the mouse impairs the integrity of the outer limiting membrane (OLM), resulting in the delamination of parts of the photoreceptor layer and neuronal cell death (Mehalow et al., 2003; van de Pavert et al., 2004). However, the severity of the phenotype is strongly dependent on the genetic background as different mutations in CRB1/Crb1 cause various retinal phenotypes in human and mice. The lack of a clear genotype–phenotype correlation for CRB1/Crb1 mutations may suggest that other components of the Crumbs complex have a function influencing the severity of the retinal disease. For instance, as orthologs to Pals1, Nagie oko zebrafish lacking Pals1 display severe defects in the organization of the retinal cell layers (Wei and Malicki, 2002; Wei et al., 2006), and Drosophila flies lacking the PALS1 ortholog Stardust develop an eye phenotype characterized by a shortened stalk membrane and altered rhabdomere morphogenesis (Hong et al., 2003; Nam and Choi, 2003; Berger et al., 2007). Previously, we demonstrated the function of PALS1 by in vitro study using Pals1 shRNA in cultured retinas, which resulted in the loss of localization of CRB1, CRB2, MUPP1, VEL13, and partially of CRB3 (van Rossum et al., 2006). Here, to address the involvement of PALS1 as a component of the Crumbs complex in retinal dystrophy, we generated conditional shPals1 knockdown mice and analyzed the consequence of lowered PALS1 levels in the retina.

Materials and Methods

Generation of conditional shPals1 knockdown mice. Animal care and experiments were performed in accordance with guidelines established at the institutions in which the experiments were performed and approved by the Animal Care and Use Committee. The shPals1 oligonucleotides, TGTTCAATGGAATGCTGAGATCACGGATTTCTGAAGAATTCAAGAGATTCACCATGTTCAAT and CGACGGAGAGTTGCAAACTCTTCCTACACG, were annealed and subcloned into the HpaI/Xhol site of pSico-pgk-puro (Ventura et al., 2004) behind the second loxP site. A 2.9 kb PstI/KpnI fragment containing the U6 promoter, the two TATA-loxP sites, the intervening pgk-puro sequence, the shPals1 sequence, and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequences was isolated from pSico-U6-loxP-pgk-puro-loxP-shPals1 and electroporated into mouse E14 embryonic ES cells, and verified by long-template PCR of a construct-specific 2.3 kb fragment using specific primers in the U6 promoter (5′-AGACAAGATGGAGGAATGCAGATGGATGCATACCCTGACAAGGC-3′ and WPRE (5′-CCACATTGCTGTAACAAAGGAC AACATG-3′) of the targeting vector. After verifying correctly targeted shPals1 ES clones, 10 different Pals1 shRNA cell clones were selected and 3 clones were used for the blastocyst injection, of which 2 gave germ line transmission (clones 7 and 13). In the studies described here, we used transgenic shPals1 mice derived from ES clone 13. ChimERIC conditional floxed shPals1 mRNA mice were generated and heterozygous shPals1 (shPals1fllox/+), from here on referred to as shPals1) and control mice were maintained as a cross of C57BL/6 and 129/Ola (50%/50%). To obtain shPals1 conditional knockdown mice, RxCre (Swindell et al., 2006) and Chx10Cre (Rowan and Cepko, 2004) mice, maintained as a cross of C57BL/6 and 129/Ola (50%/50%), were used for Cre-mediated recombination. RxCre mice were obtained on FVB background, and thereafter backcrossed onto C57BL/6 and 129/Ola (50%/50%), with the shPals1 mice. A 4.8 kb fragment was used, for the Chx10Cre/+/H11001 primers were used, for the Chx10Cre gene. Genotyping of the mice was done by PCR on genomic DNA. For Chx10Cre-mediated recombination was verified by PCR using the following primer pair: 5′-GGGCACGAGGAGATCCAGGT-3′ and 5′-TTCTCGAGGTCGACCGTGATATC-3′. The PCR product of 581 b was excised from agarose gel and sequenced. For all analyses, wild-type, shPals1, RxCre, Chx10Cre, shPals1fllox/+; RxCre/+ (from here on referred to as shPals1–RxCre) or shPals1fllox/+; Chx10Cre (as shPals1–Chx10Cre) mouse litters of either sex were used. Cre-mediated recombination was confirmed by PCR using the following primer pair: 5′-GGGCACGAGGAGATCCAGGT-3′ and 5′-TTCTCGAGGTCGACCGTGATATC-3′.

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). 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 photopic 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 10 and 8 steps, respectively. Ten responses were averaged with an interstimulus interval of 5 or 17 s (for 0–1.5 log cd · s/m^2).

Electron microscopy analysis. For immuno–electron microscopy (immuno–EM) analysis, eyes were processed according to the procedures described previously (Klooster et al., 2009). Briefly, eyes were fixed for 1 h at room temperature in 0.1 M phosphate-buffered (pH 6.5) 4% formaldehyde. After fixation, the eyes were cryoprotected and embedded in a Tris–HCl dianinobenzidine (DAB) solution containing 0.03% H2O2. The DAB reaction product was then intensified by a gold-substituted silver peroxidase method. Sections were immersed in 0.1 M sodium cacodylate buffer (pH 7.4), and washed in TBST (TBS and 0.05% Tween 20). Bands were visualized using the Odyssey Infrared Imaging system (LI-Cor Biosciences) and quantified by Image (National Institutes of Health).

Confocal scanning laser ophthalmoscopy and spectral domain optical coherence tomography. Confocal scanning laser ophthalmoscopy (cSLO) imaging was performed with HRA 1 and HRA 2 (Heidelberg Engineering) featuring up to two Argon wavelengths (488/514 nm) in the short wavelength range and two infrared diode lasers (HRA 1: 795/850 nm, HRA 2: 785/815 nm) in the long wavelength range as previously described (Seeliger et al., 2005). For spectral domain optical coherence tomography (SD-OCT) imaging, Spectralis HRA+OCT device (Heidelberg Engineering) was used as described previously (Fischer et al., 2009). Imaging was performed using the proprietary software package Eye Explorer version 3.2.1.0 (Heidelberg Engineering). Resulting data were exported and processed in Corel DrawX3.
glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, and postfixed in 1% osmium supplemented with sodium cacodylate buffer (0.1 M, pH 7.4). After fixation, eyes were dehydrated in a graded series of ethanol and embedded in epon 812 (Polysciences). Ultrathin sections (80 nm) were made with an Ultramicrotome UCT (Leica). Then, the sections were examined with a Zeiss EM 912 electron microscope (Zeiss), and images were made with an Ultramicrotome UCT (Leica). After fixation, eyes were dehydrated in a graded series of ethanol and embedded in Tissue-Tek O.C.T. compound. Immunostainings were performed on cryosections using the following primary and secondary antibodies: rabbit anti-PALS1 (1/200, Millipore Bioscience Research Reagents, ProteinTech), mouse anti-β-catenin (1/300, BD), rabbit anti-CRB1 (1/100, AK2) (van de Pavert et al., 2004), rabbit anti-gal glutamate kinase acidic protein (GPAP) (1/200, DAKO), mouse anti-MUPP1 (1/300, BD), mouse anti-glutamine synthetase (1/300, BD), rabbit anti-MPP3 (1/100, ProteinTech), rabbit anti-Recoverin (1/200, Millipore Bioscience Research Reagents), mouse anti-β-catenin (1/300, BD), and rabbit anti-Caspase-3 (cleaved form, 1/200, Cell Signaling). Cone outer segments and pedicles were stained with fluorescein-labeled peanut agglutinin lectin (PNA, 1/200, Vector Laboratories). Fluorescent-labeled secondary antibodies were donkey anti-chicken, goat anti-mouse, or goat anti-rabbit IgGs conjugated to Cy3, Alexa488, or FITC (Jackson ImmunoResearch Laboratories and Invitrogen). For retinal flat-mount analysis, eyes were fixed in 4% PFA in 0.1 M PB, dehydrated in 30% sucrose, and embedded in Tissue-Tek O.C.T. compound. Immunostainings were performed on cryosections using the following primary and secondary antibodies: rabbit anti-PALS1 (1/200, Millipore Bioscience Research Reagents, ProteinTech), mouse anti-β-catenin (1/300, BD), rabbit anti-CRB1 (1/100, AK2) (van de Pavert et al., 2004), rabbit anti-gal glutamate kinase acidic protein (GPAP) (1/200, DAKO), mouse anti-MUPP1 (1/300, BD), mouse anti-glutamine synthetase (1/300, BD), rabbit anti-MPP3 (1/100, ProteinTech), rabbit anti-Recoverin (1/200, Millipore Bioscience Research Reagents), mouse anti-β-catenin (1/300, BD), and rabbit anti-Caspase-3 (cleaved form, 1/200, Cell Signaling). Cone outer segments and pedicles were stained with fluorescein-labeled peanut agglutinin lectin (PNA, 1/200, Vector Laboratories). 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**Quantification and measurement.** To quantify the apoptotic cells, retina sections (P6 and P18) were stained with cleaved Caspase-3 (c-CAS3) antibody and counterstained with TO-PRO-3 for nuclei. Total numbers of apoptotic cells were determined by manually counting c-CAS3 antibody-positive cells in the unit area (100 μm²) on digital images generated by Zeiss LSM browser v3.2 image software. For the measurement of cone outer segments, retina sections (P10, P18, and P30) were stained with fluorescein-labeled PNA lectin and the lengths of the stainings were measured by Zeiss LSM browser v3.2 image software. For ONL thickness, the thickness was measured at 0.5, 1, 1.5, 2, and 2.5 mm distance from the optic nerve head using Zeiss LSM browser v3.2 image software. For statistical analysis, Student’s t test was applied for calculating significance and significance levels were set at <0.05.

**Results**

**Generation of shPals1 conditional knockdown mice**

The effectiveness of the shPals1 sequences in cultured retinas and in utero brain ventricle electroporation has previously been shown (van Rossum et al., 2006; Kim et al., 2010). The conditional targeting construct (Fig. 1A) was electroporated into mouse E14 ES cells, and cells harboring the complete expression cassette were used for blastocyst injection and generation of shPals1 mice. Cre-mediated recombination was verified by PCR using retinal chromosomal DNA from wild-type, RxCre, shPals1, and shPals1-RxCre. PCR analysis confirmed the recombinated DNA product (lower band, 466 bp) in shPals1-RxCre (Fig. 1B), but also indicated the presence of cells that escaped from recombination (upper band, 1979 bp). Next, we performed Western blot analysis to evaluate the reduction of PALS1 levels using total retina lysates (n = 6–8 eyes; Fig. 1C) at postnatal day 6 (P6). Upon quantification of the blots, PALS1 levels were decreased to 52 ± 3% (n = 10, ± SD) in shPals1-RxCre retinas compared to RxCre control retinas.

**Reduced retinal PALS1 levels impairs vision in adult mice**

To determine the phenotype, we analyzed the retinas of 1-, 3-, 6-, and 12-month-old shPals1-RxCre mice by cSLO, optical coherence tomography (OCT), and electroretinography (ERG). In cSLO and OCT analysis, all control littermate animals showed normal retinal morphology and retinal vasculature (Fig. 2A). However, the shPals1-RxCre mice showed changes in fundus appearance as well as vascular alterations especially in aged shPals1-RxCre mice. In 1- and 3-month-old shPals1-RxCre mice, an almost normal fundus appearance by cSLO examination was observed, but sites of cellular mislocalization as well as roundish structures in the outer plexiform layer (OPL) were found with OCT imaging (Fig. 2A) corresponding to the half rosettes observed in histological sections (Fig. 3A). In 6-month-old shPals1-RxCre mice, spotty and patchy areas as well as vascular leakage were observed with cSLO imaging (Fig. 2A, arrows). In 12-month-old shPals1-RxCre mice, the spotty and patchy areas and

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**Figure 1.** Decreased levels of PALS1 in the retina after conditional gene silencing. **A.** Schematic representation of shPals1-RxCre retinas after Cre-mediated recombination. After Cre recombination, the U6 promoter drives the expression of shRNA against the Pals1 gene as illustrated. The small arrow indicates the insertion of the short hairpin Pals1 sequence behind the second loxp PGK, Phosphoglycerate kinase promoter; PURO, puromycin gene; U6, U6 promoter. **B.** Genotyping of the mice (RxCre, shPals1, and shPals1-RxCre) was done by long-distance PCR on genomic DNA isolated from E15 or 3-month-old retinas. The upper PCR bands (1979 bp) are from non-recombined and the lower bands (466 bp) are from recombinated DNA products. 3M, 3 Months of age; E15, embryonic day 15. C, PALS1 protein levels were decreased to 52 ± 3% in P6.shPals1-RxCre retinas. Total retina cell lysates were used for SDS-PAGE and Western blot analysis. PALS1 antibody was used to detect PALS1 protein in the retinas and actin antibody was used for loading control. Expression levels were normalized to actin.
Figure 2. Decreased levels of PALS1 in shPals1-RxCre retinas caused early onset of loss of vision. A, cSLO and SD-OCT analysis from 1-, 3-, 6-, and 12-month-old shPals1-RxCre and control mice (RxCre). Spotty and patchy areas as well as vascular abnormalities (arrows in angiography) were found in shPals1-RxCre mouse retinas with cSLO fundus imaging. OCT (Figure legend continues.)
vascular leakage were more obvious and spread to the entire retina in cSLO imaging (Fig. 2A, arrows). OCT imaging revealed further sites of cellular mislocalization, as well as roundish structures in the OPL, and a reduction of the retinal thickness of the outer retina (red lines), as well as cellular mislocalization. Examinations of the retinal function in shPals1-RxCre mice by ERG showed reduced scotopic and photopic responses, with a- and b-wave amplitudes (reflecting activities of photoreceptors and ON-bipolar cells, respectively) lower than littermate controls (shPals1 and RxCre), indicating alterations of both rod and cone system components (Fig. 2B). Upon aging of shPals1-RxCre retinas, the ERG signals were severely attenuated, suggesting progressive degeneration of photoreceptors and reduced vision compared to controls (Fig. 2C).

Reduced PALS1 levels cause retinal disorganization and degeneration
To investigate the retinal shPals1-RxCre phenotype, we collected eyes from P6 to 1 year and analyzed the morphology of the retinas (Fig. 3A). In control retina at P10, the three nuclear layers, outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL) are well laminated, but photoreceptor segments are still short and the OPL and inner plexiform layer (IPL) synapses are not yet fully matured (Cepko et al., 1996; Reese, 2011). At P18, the retina is almost matured and the retina morphology is similar to adult retinas. However, the shPals1-RxCre retinas showed abnormal retina structure. At P6, the GCL and IPL are well laminated, but regions of the developing INL, ONL, and OPL are disorganized. From P10 to P30, the three layers were relatively well separated, but showed retinal folds and half rosettes of photoreceptors in the ONL and OPL, and ectopic photoreceptor nuclei in the subretinal space. In addition, the OLM showed gaps and irregularities. In aged shPals1-RxCre retinas, the ONL and OPL became progressively thinner and the retina showed gradual degeneration of the ONL as well as INL. Overall, the phenotype in shPals1-RxCre retinas started with sporadically detectable disorganizations at the peripheral part of the retina at the early postnatal days (P6 till P30) and progressed throughout the entire retina following retinal maturation.

Tangential imaging of wild-type retina (P10) shows the localization of PALS1 in the apical cell membrane of retinal pigment epithelium (RPE) cells and in the apical microvilli (Fig. 3Ba). To get more detail, we examined the retina at EM level. Immuno-EM images of wild-type retinas (P10) display the localization of PALS1 at the tight junctions of RPE cells (arrows in Fig. 3Bb) without detectable staining at the basolateral membrane (Fig. 3Bb, arrowheads). PALS1 staining was also detected in RPE apical microvilli (Fig. 3Bc, arrows). In EM examination of shPals1-RxCre retinas at P10, basal infoldings (Fig. 3Cc, arrows) were disorganized and vacuoles (asterisk) were increased in number and size. The apical microvilli of the mutant RPE were either absent or shorter (Fig. 3Cd, double-headed arrows) and did not interdigitate with the outer segments of photoreceptor cells (Fig. 3Cb, double-headed arrows). From the EM results, we suggest that PALS1 is essential for the RPE structure, and especially their apical microvilli that have important roles in phagocytosis of shed photoreceptor outer segments and removal of waste products from photoreceptor cells (Bok, 1993; Bonilha et al., 2004).

The retinal phenotype depends on dosage and location of the decrease of PALS1
In RxCre mice, Cre starts to be expressed as early as embryonic day 9 in the forebrain in the eye determining field (Swindell et al., 2006). The region expressing Cre encompasses the future neural retina and the RPE. In Chx10Cre mice, Cre starts to be expressed a little later at E10.5 in the neural retina but not in the RPE (Rowan and Cepko, 2004). To examine the function of PALS1 in the early neural retina, we examined shPals1-Chx10Cre mice. In shPals1-Chx10Cre retinas, conditional recombination occurred as in shPals1-RxCre retinas (Fig. 4A), and PALS1 levels were decreased to 66 ± 4% in shPals1-Chx10Cre retinas compared to Chx10Cre control retinas (n = 6–8 eyes; n = 8 blots, ±SD; Fig. 4B). The shPals1-Chx10Cre retinas showed a significant milder phenotype than shPals1-RxCre retinas. Retinas of shPals1-Chx10Cre at 12 months of age showed normal vascular pattern in cSLO fundus imaging, but OCT imaging revealed lamination defects at the OPL (Fig. 4C). In ERG analysis, the mutant mice showed only slightly reduced scotopic and photopic responses compared to control littermates (Fig. 4D). Sections of plastic embedded retinas confirmed the morphological abnormalities observed by OCT imaging (Fig. 4E). At 3 months of age, we detected a morphological phenotype in shPals1-Chx10Cre retinas much milder than in shPals1-RxCre retinas. In shPals1-Chx10Cre retinas, we only sporadically detected gaps at the OLM, ectopic photoreceptor nuclei in the subretinal space and OPL, and upregulated GFAP (Fig. 4E and data not shown). The shPals1-Chx10Cre retinas of 12 months of age showed a thinner photoreceptor layer and OPL with shortened photoreceptor segments, and at foci disorganization of the OLM, ONL, and INL. EM analysis at P10 did not show differences between shPals1 and shPals1-Chx10Cre retinas (Fig. 4F). The milder phenotype in shPals1-Chx10Cre retinas may be the result of less efficient gene silencing of Pals1, and an intact RPE required for maintenance of the outer retina (Longbottom et al., 2009). In summary, the data from shPals1-RxCre and shPals1-Chx10Cre retinas suggests an important function for PALS1 in the RPE in addition to the neural retina.

Reduced PALS1 levels disturbs the Crumbs complex and cause gliosis
The phenotype in shPals1-RxCre and shPals1-Chx10Cre mice resembles in part the phenotype observed in Crb1 mutant mice (Mehalow et al., 2003; van de Pavert et al., 2004, 2007a,b). A striking difference is that in Crb1 mutant mice the phenotype is limited to one quadrant of the inferior retina, whereas in shPals1 conditional knockdown retinas, the phenotype is not restricted to one quadrant, resulting in significant loss of retinal activity. We therefore examined by immunohistochemistry the localization of Crumbs complex members at the SAR immediately adjacent to...
adherens junctions at the OLM. Whereas, at 3 month of age, in control retinas PALS1, CRB1, and MUPP1 stained the SAR adjacent to the OLM, in shPals1-RxCre retinas the staining for SAR proteins is reduced or lost especially at regions of protrusions of photoreceptor nuclei into the subretinal space and ingression of photoreceptor nuclei into the OPL (Fig. 5). Staining for markers of the adherens junctions at the OLM (cadherins, β-catenin, p120) gave similar results (data not shown). In Crb1 mutant mice, upregulation of markers for intermediate filaments in Müller glia cells such as GFAP was detected at foci of retinal disorganization (van de Pavert et al., 2004, 2007a,b). In shPals1-RxCre retinas, we detect upregulation of GFAP throughout the retina suggestive of gliosis (Fig. 5). Previous coimmunoprecipitation studies showed a complex of retinal PALS1 with CRB1 and MUPP1, immunohistochemistry showed their colocalization, and shPals1 gene silencing studies in cultured retinas showed that PALS1 is required for the localization of these proteins (van de Pavert et al., 2004; van Rossum et al., 2006). From these, we suggest that reduced levels of PALS1 in the retina result in disruptions at the OLM and apical protein complex.

Figure 3. Decreased levels of PALS1 in shPals1-RxCre retinas caused retinal degeneration and abnormal RPE structure. A, Technovit retinal sections of control RxCre mice at postnatal day 6 (P6), P10, P18, and P30 and 12 months of age (12M), and shPals1-RxCre mice at P6, P10, P18, and P30 and 3, 6, and 12 months of age (3M, 6M, 12M). The phenotype in shPals1-RxCre retinas is detectable initially at the peripheral part of the retina at P6, and it spreads to the entire retina following retinal maturation. Even though the retinal layers (ONL, INL, GCL) are separated, retinal folds and half rosettes of the ONL are detected, and photoreceptor nuclei protruded into the subretinal space and ingress into the OPL from P10 onwards. In aged mutant retinas (3–12M), the ONL and OPL become thinner due to gradual retinal degeneration. The ONL is also severely damaged and abnormal large vacuoles are detected in the RPE layer. Scale bar, 50 μm. B, Representative PALS1 staining images from immuno-EM and IHC analysis are shown from wild-type at P10. a, IHC flat mount of RPE cells stained with anti-PALS1. At immuno-EM level, the RPE monolayer has apical microvilli toward the photoreceptor outer segments and the cells are interconnected by tight junctions (b, c). Arrowheads indicate the basolateral membrane. b, c, Arrows indicate PALS1 at the tight junction and in apical microvilli surrounding a photoreceptor outer segment (c). Scale bars: 20 μm (a), 0.5 μm (b, c). C, EM analysis of RxCre and shPals1-RxCre mice at P10. RPE cells appeared to be disorganized with short microvilli (double-headed arrows in d), reduced/disorganized basal infoldings (arrow in e), and in addition, numerous vacuoles were present within the cells (e, asterisk). N, RPE cell nucleus; OS, photoreceptor outer segments; Mv, microvilli; BM, Bruch’s membrane; BI, basal infoldings. Scale bars: 5 μm (a, c), 1 μm (b, d).
Retinal cell specification is not affected, whereas retina laminar positioning is aberrant.

Retinal progenitor cells initiate a cell-type-specific differentiation program, controlling their migratory behavior and their morphogenesis. In this way, the different cell types achieve their laminar positioning, adult morphologies, and synaptic connections (Cepko et al., 1996; Reese, 2011). Lack of functional PALS1 in zebrafish nagie oko mutant retinas results in retinas that do not
show the normal nuclear layering, the cells occupy highly abnormal positions, but cell-specification mechanisms are not affected (Wei and Malicki, 2002). To verify the cell specification and patterning in \textit{shPals1-RxCre} retinas, we stained for markers of photoreceptors and bipolar and Müller glia cells. Control retinas at P10 show lamination of the retina, and normal location of bipolar cells and photoreceptors (Fig. 6). In \textit{shPals1-RxCre} retinas, all cell types were generated and most but not all retinal cells were properly located in the retina. The mutant retina showed folds, some photoreceptor cells ectopically localized in the subretinal space or OPL, and some bipolar cells ectopically localized in the ONL, suggesting incorrect patterning of retinal cells. We suggest that reduced levels of PALS1 do not affect retinal cell-specification mechanisms, but affect correct patterning of newly born retinal cells, especially photoreceptors, and that may have negative consequences for establishing and maintenance of stable neuronal connections at the OPL.

Lack of the PALS1 ortholog Stardust in the fruit fly \textit{Drosophila} leads to loss of Crumbs and Patj from the stalk membrane, and to shortened stalk membranes, and increased sensitivity to light, although different \textit{Stardust} mutant alleles give different phenotypes (Hong et al., 2003; Nam and Choi, 2003; Berger et al., 2007). Lack of Crumbs in \textit{Stardust} photoreceptors leads to loss of Stardust and Patj from the stalk membrane, and to shortened stalk membranes, and increased sensitivity to light (Johnson et al., 2002). Similarly, lack of CRB ortholog Crb2b in zebrafish \textit{Danio rerio} leads to reduced photoreceptor segments length (Omori and Malicki, 2006). We therefore studied the length of cone outer segments in \textit{shPals1-RxCre} retinas by measuring peanut agglutinin (PNA) stains of P10, P18, and P30 retinas, but detected no significant effect on outer segment length upon decreased levels of PALS1 except for foci of severely disorganized retina (Fig. 7A, B). The reduced length of cone outer segments at P30 is likely to be the consequence of the increased retinal degeneration throughout the retina (Fig. 7B). The data suggest that moderately reduced levels of PALS1 do not directly affect cone photoreceptor outer segment length.

**Figure 5.** PALS1 localizes with CRB1 and MUPP1 to the subapical region adjacent to adherens junctions at the outer limiting membrane. Immunohistochemistry on 3-month-old retinas showing confocal images of staining for proteins associated with the Crumbs complex, PALS1, CRB1, and MUPP1, and for GFAP, a Müller glial cell marker. Staining for the Crumbs complex shows fragmented staining (arrows) of PALS1, CRB1, and MUPP1 in the \textit{shPals1-RxCre} retinas, which indicate the dislocation of the Crumbs complex proteins at the SAR adjacent to adherens junctions at the OLM. GFAP staining shows increased reactive gliosis in the mutant retinas. Scale bar, 50 μm.

**Retinas with reduced levels of PALS1 undergo persistent programmed cell death**

\textit{shPals1-RxCre} retinas showed disorganization during late retinal development followed by progressive degeneration. Cell death is a normal process during retinal development (Young, 1984). It takes place in two waves concomitant with the processes of neurogenesis, cell migration, and cell differentiation at an early phase and when connections are established and synapses are formed, resulting in selective elimination of inappropriate connections at a later phase (Vecino et al., 2004). To study the incidence of apoptosis in \textit{shPals1-RxCre} retinas, we used cleaved-Caspase-3 (c-CAS3) antibody as a marker for apoptosis. The late phase of apoptosis normally occurs around P6, and apoptosis rates are similar as in the control retinas (Fig. 8A, B). The late phase of apoptosis normally ends after P10, but \textit{shPals1-RxCre} retinas showed persistent apoptosis, suggesting continuous clearance of inappropriate connections. Untimely apoptosis is detected in the photoreceptor layer as well as INL, whereas the ganglion layer is spared. In aged retinas of 3, 6, and 12 months of age, we detected loss of photoreceptors and bipolar cells (Fig. 8C, D). Our data on \textit{shPals1-RxCre} retinas suggest that reduced levels of PALS1 cause inappropriate localization of photoreceptor and INL neurons that subsequently undergo programmed cell death.

**Discussion**

In the current study, we have generated and analyzed \textit{shPals1-RxCre} and \textit{shPals1-Chx10Cre} mice. The phenotype in \textit{shPals1-RxCre} is more severe than in \textit{shPals1-Chx10Cre} retinas, suggesting a role for PALS1 in the RPE and neural retina during retinal development.

We have crossed the \textit{shPals1} mice with two different \textit{Cre} lines, RxCre and Chx10Cre, both expressing \textit{Cre} in retinal progenitor cells; but \textit{shPals1-RxCre} and \textit{shPals1-Chx10Cre} retinas show a difference in onset and severity of the retinal phenotype. We have also generated \textit{shPals1-NestinCre} mice that gave as severe a retinal phenotype, in ERG, SLO, OCT, histology, and IHC analysis, as \textit{shPals1-RxCre} (data not shown). The difference in severity of
the phenotype might be explained by (1) difference in cell type-specific expression of Cre (expression in neural retina, or neural retina and RPE), (2) difference in time of expression of Cre and subsequently of Pals1 shRNA (from E9 in RxCre, or from E10.5 in Chx10Cre), or (3) difference in mosaic expression of Cre and silencing of PALS1 expression. The Chx10Cre mouse line used is known to express Cre in most but not all retinal progenitor cells, resulting in a mosaic retina of wild-type and mutant cells (Rowan and Cepko, 2004).

The RPE performs highly specialized, unique functions essential for homeostasis of the retina, and loss/malfunction of RPE causes loss of homeostasis between the retina and RPE (Ashburn et al., 1980), and photoreceptor degeneration and atrophy of the choriocapillaris (Korte et al., 1984). Using EM analysis, we detected disturbances in mutant RPE cells such as shortened microvilli, disorganized basal infoldings, and increased number and size of vacuoles. Therefore, our data suggest that reduced levels of PALS1 in the RPE and neural retina cause the phenotype observed in shPals1-RxCre retinas. In zebrafish, PALS1 is expressed in the early RPE, and nagie oko zebrafish that completely lack PALS1 showed disturbances in RPE cells, and the phenotype could be rescued in part by expression of a nagie oko transgene in the RPE showing proper patterning of ganglion cells and cells of the INL (Zolesi et al., 2006; Zou et al., 2008; Randlett et al., 2011). Transgenic mouse lines expressing Cre in the RPE are available, but are unfortunately not useful to address the hypothesis because they either express Cre recombinase in the RPE as well as in the neural retina, or in a small subset of RPE cells, or they express postnatally (Mori et al., 2002; Delmas et al., 2003; Longbottom et al., 2009; Zhao et al., 2011).

In mouse neural retina, PALS1 localizes to the SAR adjacent to adherens junctions between photoreceptors and Müller glia cells and forming complexes with CRB1 and CRB2 in Müller glia cells, and with CRB2 in rod and cone photoreceptors (van Rossum et al., 2006). The phenotype observed in Figure 3 (e.g., gaps in the OLM, retinal folds, half-rosettes, ectopic photoreceptors in the subretinal space and OPL, progressive photoreceptor death) is in part similar to the phenotype observed in Crb1 knock-out mice.
As shown in Figure 5, the Crumbs complex proteins were reduced or lost at the SAR. Therefore, PALS1 may be required for the recruitment of the Crumbs complex components, and reduced levels of PALS1 are likely to give a complex phenotype due to the reduced levels of PALS1-CRB1 and PALS1-CRB2 complexes in Müller glia cells and photoreceptors (van Rossum et al., 2006).

During retinal development, newborn neurons, which make up six of the seven major classes of cells in the retina, must be produced in proper ratios, migrate to the proper layer, and form synaptic connections with each other (Cepko et al., 1996; Wei and Malicki, 2002). Previous studies indicated that all retinal cell classes were present in nagie oko mutant retinas; the mutant showed, however, a severe disruption of retinal architecture, which indicated that zebrafish PALS1 did not have a role in cell-class specification (Wei et al., 2006). To verify the involvement of mouse PALS1 in cell specification, we used specific markers for retinal cells as shown in Figure 6. However, unlike the nagie oko mutant, most cells in the mutant retinas were well separated and migrated properly, even though the retina layers are folded and some photoreceptor cells are dislocated into the subretinal space. The data suggest that PALS1 levels are not critical for cell class specification, but are critical for proper stratification of the retina.

Figure 8. Retinas lacking PALS1 undergo persistent programmed cell death at maturation of the retina. A, Immunohistochemistry for c-CAS3 on representative sections of shPals1-RxCre and RxCre retinas at P6 and P18. Apoptotic cells were stained with c-CAS3 antibodies (red). Nuclear DNA was counterstained with TO-PRO-3 (blue). Scale bar, 50 μm. B, Histogram depicting the number of c-CAS3-positive cells in shPals1-RxCre and control retinas at P6 and P18. Asterisk (*) indicates significant difference compared to the control (Student’s t test, p < 0.0017 for P18 OLM, p < 0.0029 for P18 INL). Error bars indicate ± SEM. C, Immunohistochemistry for apoptotic cells and bipolar cells on representative sections of shPals1-RxCre and control RxCre retinas at 12 months of age. Apoptotic cells were stained with c-CAS3 antibodies (red, arrowheads), and bipolar cells with anti-PKCα (green). Nuclear DNA was counterstained with TO-PRO-3 (blue). Dashed quadrants in the upper panels indicate examples of a c-CAS3-positive cell, and are shown enlarged in the panel down on the left (scale bar, 10 μm). The number of photoreceptor and bipolar cells decreased over time. Scale bar, 50 μm. D, Quantification of the outer nuclear layer thickness. ONL thickness measured every 500 μm from the optic nerve to an area near the peripheral edge of retinal sections in control (12M) and shPals1-RxCre (3, 6, 12M). Error bars indicate ± SEM.
pocampus (Kim et al., 2010). Even heterozygote mice with reduced levels of PALS1 in the cortex showed a significant phenotype, suggesting dosage sensitivity of PALS1. We analyzed the number of mitotic cells between E12 and E16 to examine the involvement of PALS1 in progenitor cell proliferation in the retina, but could not detect significant differences (data not shown). In shPals1-RxCre retinas, a reduction of up to 52% of PALS1 may not be sufficient to affect cell proliferation, but we cannot exclude the possibility that cell proliferation is inappropriately timed. The time course of neurogenesis in the retina is longer than in the cortex and hippocampus (Götz and Huttner, 2005), and levels of PALS1 might not be as critical in retinal progenitor cell proliferation as in cortical progenitor cell proliferation.

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