Development/Plasticity/Repair

Function of Atypical Protein Kinase C λ in Differentiating Photoreceptors Is Required for Proper Lamination of Mouse Retina

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The photoreceptor is a highly polarized neuron and also has epithelial characteristics such as adherens junctions. To investigate the mechanisms of polarity formation of the photoreceptor cells, we conditionally knocked out atypical protein kinase C λ (*aPKCλ*), which has been proposed to play a critical role in the establishment of epithelial and neuronal polarity, in differentiating photoreceptor cells using the Cre-loxP system. In *aPKCλ* conditional knock-out (CKO) mice, the photoreceptor cells displayed morphological defects and failed to form ribbon synapses. Intriguingly, lack of *aPKCλ* in differentiating photoreceptors led to severe laminar disorganization not only in the photoreceptor layer but also in the entire retina. Cell fate determination was not affected by total laminar disorganization. After Cre recombinase began to be expressed in the developing photoreceptors at embryonic day 12.5, both the immature photoreceptors and mitotic progenitors were dispersed throughout the CKO retina. We detected that adherens junction formation between the immature photoreceptors and the progenitors was lost in the CKO retina, whereas it was maintained between the progenitors themselves. These results indicate that the expression of aPKCλ in differentiating photoreceptors is required for total retinal lamination. Our data suggest that properly polarized photoreceptors anchor progenitors at the apical edge of the neural retina, which may be essential for building correct laminar organization of the retina.

Key words: **aPKC; retina; photoreceptor; lamina; polarity; adherens junction; cadherin; catenin**

Introduction

The vertebrate retina is a well described and accessible structure that provides an excellent model system for studies of patterning and cell fate determination within the CNS. In vertebrates, the neural retina develops from a single-layered neuroepithelium. With maturation, it develops into a highly ordered laminated tissue with seven classes of cells: rod and cone photoreceptors, horizontal, bipolar, amacrine, ganglion cells, and Müller glia. During retinal development, all mitosis occurs at the ventricular edge of the neural retina. Postmitotic progenitor cells, therefore, migrate some distance to occupy positions characteristic of their class within the retina. Each cell then extends its neurites or processes leading to laminar organization of the retina.

Retinal photoreceptor cells, composed of rods and cones, work as light detectors through phototransduction. The mammalian photoreceptor is a highly polarized neuron consisting of an outer segment (OS), an inner segment (IS), a cell body (CB), and a synaptic terminus (ST). In addition, at the apical side of the photoreceptors, adherens junctions (AJs) between the photoreceptors and the Müller glia are formed (Willbold and Layer, 1998). These AJs are considered to be important for photoreceptor cell shape and retinal tissue integrity. Thus, the photoreceptors have at the same time neuronal and epithelial characteristics, and the establishment and maintenance of apico-basal polarization in the photoreceptors are very crucial. However, the molecular mechanisms that control cell polarity formation in the retinal photoreceptors are poorly understood.

The Par3/Par6/atypical protein kinase C (aPKC) complex is required for the regulation of polarity in a variety of cells in multicellular organisms (Kemphues, 2000; Doe, 2001; Ohno, 2001; Wodarz, 2002). aPKC forms a complex with Par6 and Par3 and contributes to the cell polarity in various biological contexts. In *Drosophila*, *Drosophila* aPKC (DaPKC) colocalizes with DmPAR6 (*Drosophila melanogaster* PAR6) and Bazooka (PAR3) at the apical cell cortex of epithelial cells and neuroblasts. In mammals, these three proteins colocalize to the tight junction (TJ) in epithelium and to the AJ in neuroepithelial cells (Izumi et al., 1998; Manabe et al., 2002). The overexpression of a dominant-negative mutant of aPKC severely affects the biogenesis of the TJ structure and epithelial cell surface polarity, indicating that the Par3/Par6/aPKC complex plays critical roles in the development of the junctional structures and apico-basal polar-

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ization of mammalian epithelial cells (Suzuki et al., 2001). In addition, Par3, Par6, and aPKC have been proposed as axon determinants in cultured mammalian hippocampal neurons (Shi et al., 2003; Nishimura et al., 2004).

To study molecular mechanisms of cell polarity formation in the retinal photoreceptors, we generated an *aPKCλ* conditional knock-out (CKO) mouse line in which the *aPKCλ* gene is inactivated in postmitotic photoreceptors under the control of the *Crx* promoter. We report here that lack of *aPKCλ* in differentiating photoreceptors led to severe laminar disorganization not only in the photoreceptor layer but also in the entire retina. In the *aPKCλ* CKO mice, the photoreceptors failed to develop proper morphology and establish neuronal connections. These findings indicate that aPKCλ is required for polarization in postmitotic photoreceptors and the proper laminar formation of the entire retina.

Materials and Methods

Generation of conditional knock-out mouse. Mice harboring a floxed *aPKCλ* gene in which exon 5 was flanked by loxP sequences ($aPKC\lambda^{wild/flox}$ mice) were generated by homologous recombination (K. Akimoto, T. Noda, and S. Ohno, unpublished observations). The *Crx-cre* transgenic mouse line was described previously (Nishida et al., 2003). All procedures conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the procedures were approved by the Institutional Safety Committee on Recombinant DNA Experiments and Animal Research Committee of Osaka Bioscience Institute.

Immunostaining and in situ *hybridization.* Mouse eyeballs were fixed with 4% formaldehyde in PBS for immunostaining and *in situ* hybridization. Cryosections were subjected to immunostaining and analyzed using a confocal microscope LSM 510 (Carl Zeiss, Oberkochen, Germany). We acquired the following primary antibodies: monoclonal antibodies specific against rhodopsin (RET-P1; Sigma, St. Louis, MO), HPC-1 (Sigma), Cre recombinase (Covance, Berkeley, CA), N-cadherin (TDL, Lexington, KY), β-catenin (TDL), γ-tubulin (Sigma), calbindin (Sigma), vimentin (Zymed, South San Francisco, CA), Rom1 and cGMP-gated channel (CNCG) (both provided by Dr. R. Molday, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada) (Molday, 1998), afadin (provided by Dr. Y. Takai, Department of Molecular Biology and Biochemistry, Faculty of Medicine, Osaka University School of Medicine, Osaka, Japan) and Ki-67 (BD Pharmingen, San Diego, CA), rat monoclonal antibodies against nectin-1, -2, -3 (provided by Dr. Y. Takai); rabbit polyclonal antibodies against Pax6 (Zymed), phosphohistone H3 (Upstate, Lake Placid, NY), cyclin D3 (Santa Cruz Biotechnology, Santa Cruz, CA), S-opsin (Chemicon, Temecula, CA), ZO-1 (Zymed), aPKCλ (Akimoto et al., 1994), Par-3 (Izumi et al., 1998), Par-6 (Suzuki et al., 2001), aPKCζ (C-20; Santa Cruz), Cre recombinase (Novagen, Madison, WI); a goat polyclonal antibody against Brn3b (Santa Cruz Biotechnology). We raised polyclonal antibodies against Chx10 in rabbits (MBL, Nagoya, Japan) (Nishida et al., 2003). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma) or TOTO-3 iodide (642/660) (Invitrogen, Eugene, OR). Full-length *Crx* cDNA and 891 bp of N terminus of *aPKCλ* cDNA were used as probes for *in situ* hybridization (Freund et al., 1997; Furukawa et al., 1997b). The procedure for *in situ* hybridization was described previously (Furukawa et al., 1997a).

Retinal cell count. For quantitative analysis of retinal cells, postnatal day 8 (P8) retina were dissociated with trypsin and immunostained with respective retinal marker antibodies for 1 h and secondary antibodies for 30 min. Three independent experiments were performed to count each type of retinal cells.

Processing of tissues for electron microscopy. Killed animals were perfused with 1× PBS followed by fixation with 2% glutaraldehyde in 0.1 M Na-cacodylate for 3 h in an ice bath. Retinas were osmicated and then dehydrated with graded series of ethanol, followed by propylene oxide, and finally embedded in Epon for morphology. Ultrathin sections (70 nm) were cut by an LKB-8800 ultramicrotome (LKB-Produkter, Stockholm, Sweden) and mounted on copper grids before staining with 2% uranyl acetate.

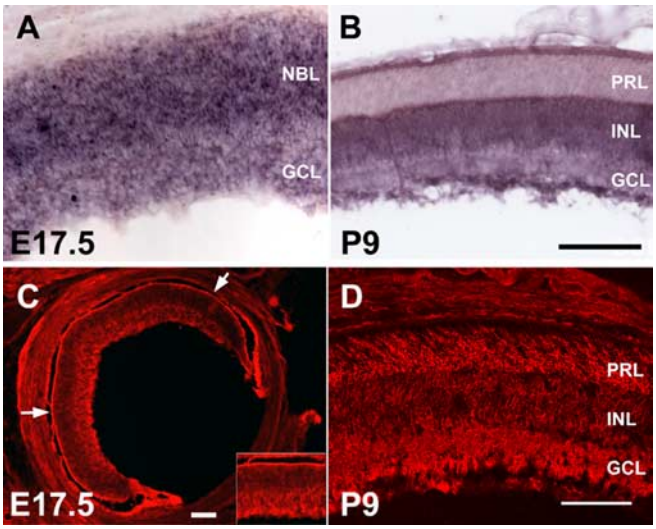

Figure 1. aPKCλ expression during mouse retinal development. ***A***, ***B***, *In situ* hybridization of mouse *aPKCλ* on retinal sections of E17.5 (***A***) and P9 (***B***). ***C***, ***D***, aPKCλ immunostaining of the retina at E17.5 (***C***) and P9 (***D***). Arrows indicate aPKCλ expression at the apical edge. Insets are higher-magnification images of retina. NBL, Neuroblastic layer; GCL, ganglion cell layer; PRL, photoreceptor layer; INL, inner nuclear layer. Scale bars, 100 μm.

Results

Temporal and spatial expression of aPKCλ in developing mouse retina

We first analyzed the expression pattern of aPKCλ in the developing mouse retina. *aPKCλ* mRNA was expressed broadly, relatively stronger in the outer neuroblastic layer, during embryogenesis of the mouse retina (Fig. 1*A*). After birth, the expression of *aPKCλ* mRNA was observed strongly in the inner nuclear layer and the ganglion cell layer, and weakly in the photoreceptor layer (Fig. 1*B*). The expression pattern of aPKCλ protein was not consistent with that of *aPKCλ* mRNA. Immunohistochemical analysis using an anti-aPKCλ antibody showed that aPKCλ protein was detected intensely at the apical edge of the embryonic retina during embryogenesis (Fig. 1*C*). Strong immunoreactivity was observed in the photoreceptor layer and the ganglion cell layer at P9 (Fig. 1*D*). These expression patterns of aPKCλ during retinogenesis suggest that aPKCλ plays important roles in polarity formation of retinal cells.

Expression of aPKCλ in differentiating photoreceptors is required for proper retinal lamination

To ablate *aPKCλ* in the photoreceptors, we used a transgenic mouse line expressing Cre under the control of the mouse *Crx* promoter (*Crx-cre*) (Nishida et al., 2003). In this transgenic mouse line, we showed previously that Cre-mediated recombination occurred in the postmitotic differentiating photoreceptors and pinealocytes of the pineal gland using CAG-CAT-Z mouse line in which *lacZ* expression is directed by Cre-mediated recombination (Sakai and Miyazaki, 1997; Nishida et al., 2003). In addition, we confirmed that the recombination in *Crx-cre*/CAG-CAT-Z mice occurred in photoreceptor-committed cells also at the embryonic stages (supplemental Fig. 1, available at www.

jneurosci.org as supplemental material). We further confirmed by immunostaining that Cre recombinase is not significantly expressed in progenitors. The Ki-67 antigen is a nuclear protein expressed in proliferating cells during all active parts of the cell cycle (Schluter et al., 1993). Immunostaining results showed that Cre recombinase was expressed at the outermost layer in the retina after embryonic day 12.5 (E12.5) and was not colocalized with Ki-67-positive progenitors (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Furthermore, β-galactosidase was not colocalized with Ki-67 in the *Crx-cre*/CAG-CAT-Z retina at E12.5, indicating that the recombination does not occur in the progenitors (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Next, to examine whether or not Cre recombinase is expressed in ganglion cells, we immunostained E13.5 CKO retina with anti-Cre and anti-Brn3b antibodies and confirmed that these signals do not overlap each other (data not shown). We also found that early-differentiated ganglion cells were properly located and laminar structure is properly organized in the innermost layer of the E15.5 retina; thus, it is unlikely that aPKCλ is ablated in ganglion cells (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). We mated the *Crx-cre* mouse line with an $aPKC\lambda^{flox}$ mouse line in which the *aPKCλ* allele is flanked by *loxP* sites (Fig. 2*A*) (Akimoto, Noda, and Ohno, unpublished observation). We obtained $aPKC\lambda^{flox/flox}$/*Crx-cre* (*aPKCλ* CKO) and analyzed phenotypes by comparing them with those of control mice with the genotype $aPKC\lambda^{flox/wild}$/*Crx-cre*, which showed no abnormal phenotype. The *aPKCλ* CKO mouse was viable but showed moderate microphthalmia and hydrocephalus. Although the cause of hydrocephalus is not clear, hydrocephalus is considered very unlikely to cause and affect retinal phenotypes. We analyzed details of retinal sections from the CKO and the control mice at various developmental stages. Mouse retinas begin to differentiate at E11.5, and Cre expression begins ~E12.5 in postmitotic differentiating photoreceptors (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) (Nishida et al., 2003). No obvious difference between the CKO and the control retina was observed at E11.5 (Fig. 2*B*,*C*), but slight disorganization of the retina was observed in the CKO retina at E13.5 (Fig. 2*D*,*E*), E14.5, P3, and P8 (data not shown). At P14, the retinal laminar structure was fully developed in the control retina, but the lamina of the CKO retina was remarkably disrupted and plexiform patches were observed (Fig. 2*F*,*G*). These results show that ablation of aPKCλ function in postmitotic photoreceptors led to lamination defects not only in the photoreceptor layer but also in the total retina.

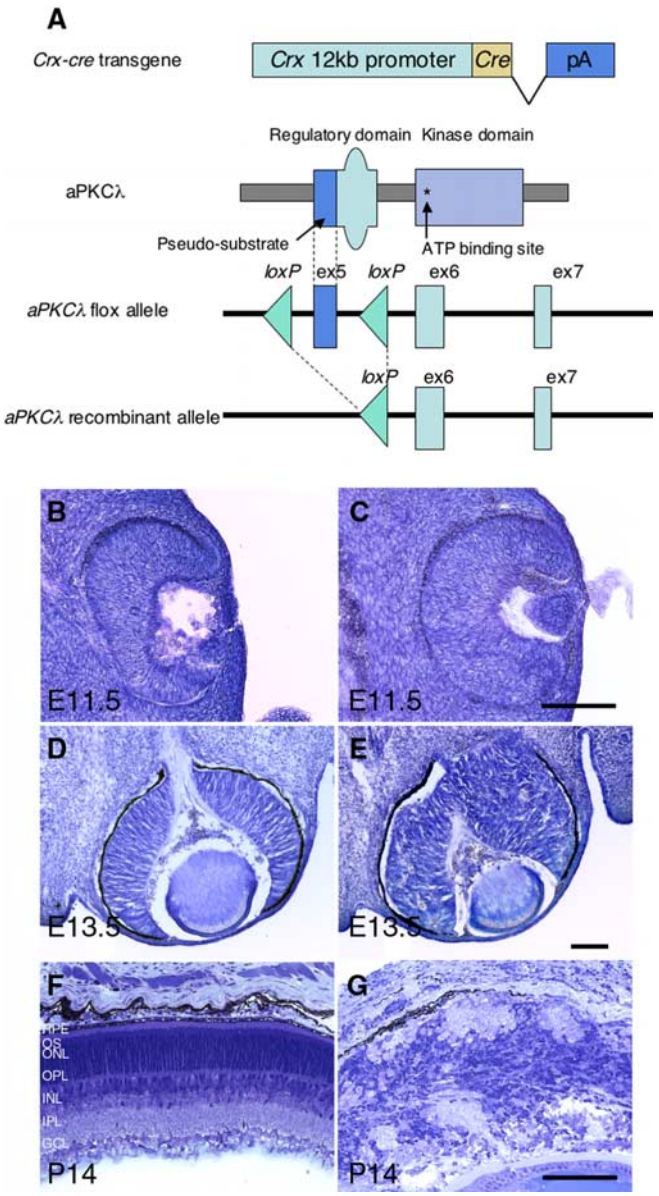

Figure 2. aPKCλ ablation in differentiating photoreceptor cells causes severe retinal lamination defect. ***A***, The *Crx-cre* transgene construct (top) and the *aPKCλ* genomic allele flanked with *loxP* before (middle bottom) and after (bottom) the recombination by Cre recombinase. Exon 5 is designed to be deleted, which corresponds to the pseudosubstrate region of aPKCλ (middle top). ***B–G***, Laminar formation of retina during development in control (***B***, ***D***, ***F***) and *aPKCλ* CKO retina (***C***, ***E***, ***G***). By P14, retinal lamination is complete and distinguishable as RPE, photoreceptor layer composed of OS and outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) in control (***F***). Note that laminar disorganization of the neural retina is observed even at E13.5, whereas Cre expression in differentiating photoreceptors begins at ~E12.5 (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Scale bars, 100 μm.

Lamination defect does not affect cell fate determination

To determine the distribution of various retinal cell types, we performed immunohistochemical analysis of retinal sections from the CKO and the control mice at P14, using antibodies against retinal cell type markers (Fig. 3*A–J*). All types of retinal cells were dispersed in the CKO retina (Fig. 3*A–J*) (data not shown); ganglion (anti-Brn3b antibody), photoreceptor (anti-rhodopsin antibody), amacrine (anti-Pax6 antibody for a nuclear marker; anti-HPC-1 antibody for a cytoplasmic marker), bipolar (anti-Chx10 antibody), and horizontal cells (anti-calbindin antibody), and Müller glia (anti-cyclin D3 antibody for a nuclear marker; anti-S-100β antibody for a cytoplasmic marker). Amacrine (anti-HPC-1 antibody) and Müller glia (anti-S-100β antibody) processes were also detected in patches (Fig. 3*A*,*B*,*G*,*H*). To examine whether the absence of photoreceptor cell polarity affects cell fate, we harvested and dissociated retinas from both the CKO and the control mice followed by immunostaining with the cell type-specific markers. There was no significant difference in cell numbers of each retinal cell type including the photoreceptor between the *aPKCλ* CKO and the control retinas (Fig. 3*K*). Therefore, this indicates that neither defects of photoreceptor cell polarity nor disorganized lamination affect retinal cell fate determination. Furthermore, our observation suggests that a cell-intrinsic program may play a major role in retinal cell fate deter-

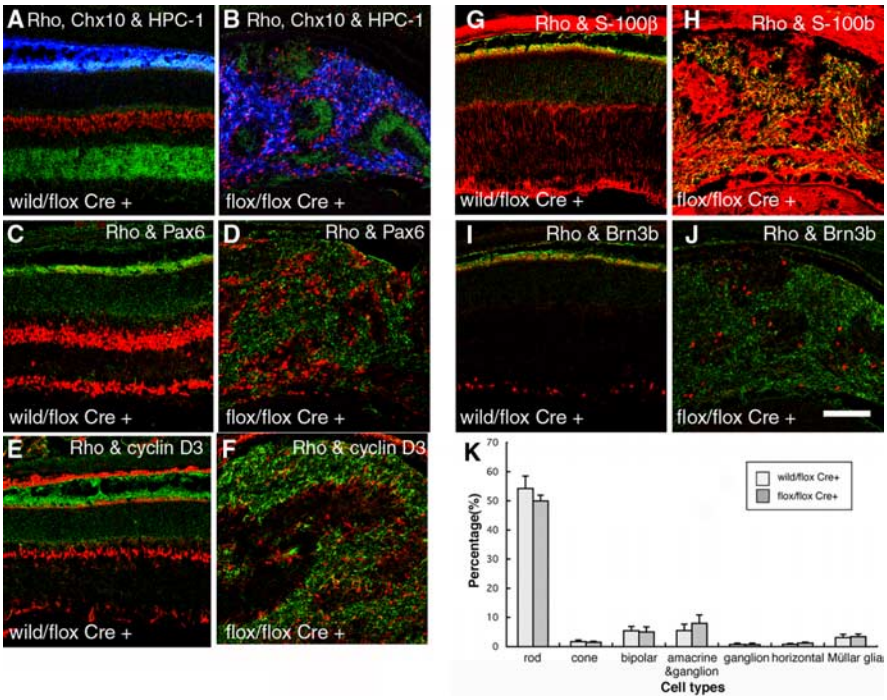

Figure 3. Lamination defect does not affect on cell fate determination. ***A***, ***B***, Distribution of rod photoreceptor cells (Rho, rhodopsin; blue), bipolar cells (Chx10, red) and amacrine processes (HPC-1, green) was detected by immunohistochemistry in control (***A***) and in *aPKC*λ CKO retina (***B***) at P14. ***C–J***, Distribution of rod photoreceptors (green) (***C–J***) and amacrine and ganglion cells (Pax6, red) (***C***, ***D***), Müller glial cells (cyclin D3, red) (***E***, ***F***), Müller glial processes (S100-β, red) (***G***, ***H***), and ganglion cells (Brn3b, red) (***I***, ***J***) was detected by immunohistochemistry in control (***C***, ***E***, ***G***, ***I***) and *aPKC*λ CKO retina (***D***, ***F***, ***H***, ***J***) at P14. Scale bar, 100 μm. ***K***, The percentage of cell types in control or *aPKC*λ CKO retina. Note that lamination defect did not affect retinal cell fate determination. Scale bars indicate SD.

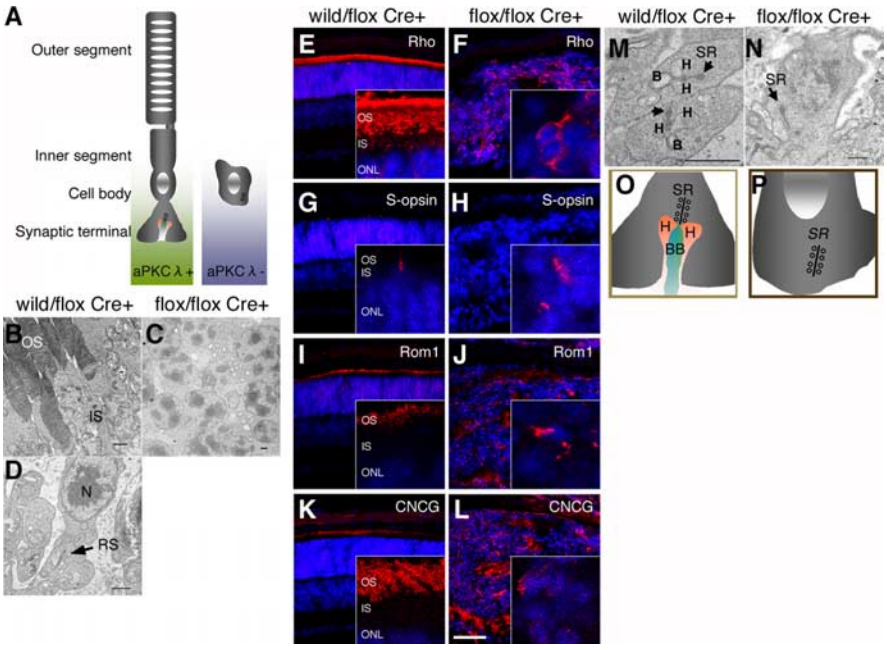

Figure 4. aPKCλ is essential for photoreceptor polarization but not for differentiation. ***A***, Model illustration of control and *aPKC*λ-null photoreceptors. ***B–D***, Ultrastructure of control (***B***, ***D***) and *aPKC*λ-null photoreceptors (***C***). Arrow indicates ribbon synapse (RS). Scale bar, 1 μm. ***E–L***, Failure of morphogenesis but existence of components of photoreceptors. Expression and distribution of rhodopsin (red) (***E***, ***F***), S-opsin (red) (***G***, ***H***), Rom1 (red) (***I***, ***J***), and CNCG (red) (***K***, ***L***) in control (***E***, ***G***, ***I***, ***K***) and *aPKC*λ-null photoreceptors (***F***, ***H***, ***J***, ***L***). Insets are higher-magnification images of retina. Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm. ***M–P***, Ultrastructure of synaptic terminals in control (***M***) and *aPKC*λ-null photoreceptors (***N***). Model illustration of synaptic terminal (***O***, ***P***). Note SR is formed, but there is no penetration of horizontal (H) and bipolar (B) cells into *aPKC*λ-null photoreceptor (***N***). N, Nucleus; SR, synaptic ribbon; ONL, outer nuclear layer. Scale bars, 1 μm.

mination. A similar observation implicating the importance of a cell-intrinsic property in cell fate determination was recently reported using the retinal clonal-density culture system and ganglion cell deprivation (Cayouette et al., 2003; Mu et al., 2005).

*aPKC*λ is required for proper polarization of photoreceptors *in vivo*

To investigate the effect of *aPKC*λ ablation on photoreceptor polarization, we used electron microscopy to examine the morphology of P14 CKO mutant photoreceptors. The mammalian photoreceptor is a highly polarized cell composed of OS, IS, CB, and ST (Fig. 4*A*). Each of them shows distinguishable morphology. The OS, which is thought to evolve from the ciliated dendrites (Goldstein and Yang, 2000), contains stacks of flattened double lamellas in the form of discs, and the IS contains the large number of long mitochondria (Fig. 4*B*,*D*). In the *aPKC*λ CKO retina, the photoreceptors, which can be distinguished by their cell nuclei with condensed heterochromatin, were randomly distributed in the retina, and no evident OS, IS, or ST structures were observed (Fig. 4*C*). Although these structures were not formed, the components of the OS, including rhodopsin (Fig. 4*F*), S-opsin (Fig. 4*H*), Rom1 (Fig. 4*J*), and rod photoreceptor CNCG (Fig. 4*L*) were detected in the *aPKC*λ CKO retina (Fig. 4*E–L*) by immunohistochemical analysis. The normal photoreceptor forms a complex synaptic arrangement, designated as a triad. The horizontal and bipolar cell processes invaginate into a photoreceptor terminal at the site of the synaptic ribbon structure (Missotten, 1962) (Fig. 4*M*,*O*). At the terminal of *aPKC*λ CKO photoreceptors, we could not detect any typical triad although synaptic ribbons were observed (Fig. 4*N*,*P*). In contrast, many synaptic termini of the retinal neurons other than the photoreceptors were found in the CKO retina by electron microscopy (data not shown). Together, all of these findings suggest that aPKCλ is necessary for proper photoreceptor polarization *in vivo*, although loss of cell polarity does not affect differentiation and production of photoreceptor components.

Proper polarizations of differentiating photoreceptors are required for apical junction formation

Ablation of *aPKC*λ function in postmitotic photoreceptors led to lamination defects not only in the photoreceptor layer but also in the entire retina. To understand

the mechanism of severe retinal lamination defects, we analyzed the distribution of photoreceptor-committed cells during retinal development. In the control retina, photoreceptor-committed cells that expressed *Crx* were observed at the outer neuroblastic layer at E14.5 (Fig. 5*A*). In contrast, in the E14.5 *aPKCλ* CKO retina, *Crx*-expressing cells were already separated from the outermost layer and located randomly in the inner layer (Fig. 5*B*). aPKCλ, Par3, and Par6 were located at the apical edge of the embryonic control retina, but expression of these molecules was mislocalized in the CKO retina (supplemental Fig. 5*A–F*, available at www.jneurosci.org as supplemental material). We confirmed by Western blotting that the amount of aPKCλ protein was substantially reduced in the CKO retina, whereas the amounts of Par3 and Par6 were not affected (supplemental Fig. 5*G*, available at www.jneurosci.org as supplemental material). These data suggest that aPKCλ is required for proper localization of photoreceptor-committed cells at the apical edge of the retina. The aPKC complex is known to localize at the TJs in epithelial cells as well as the AJs in neuroepithelial cells (Manabe et al., 2002). We examined whether or not aPKCλ localizes to the AJ systems in the mouse retina. aPKCλ colocalized with N-cadherin, β-catenin, and afadin in E15.5 retina (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). Zona occludens-1 (ZO-1), a PDZ [PSD-95 (postsynaptic density 95)/discs large/ZO-1] domain protein, localizes both at the TJs and the AJs (Itoh et al., 1993; Izumi et al., 1998; Suzuki et al., 2001). N-cadherin colocalizes with ZO-1 in the apical surface of neuroepithelial cells (Aaku-Saraste et al., 1996). We found that continuous expression of ZO-1, N-cadherin, and β-catenin, which are located at the apical edge of E13.5 control retina, were dispersed in the CKO retina (Fig. 5*C–H*). We confirmed by Western blotting that the protein amount of N-cadherin and β-catenin was not changed in the CKO retina. We also observed the dispersion of other AJ-associated proteins, nectin and afadin, from the apical edge of the CKO retina (Fig. 5*I,J*) (data not shown). We then examined the formation of apical junction complex (AJC) in E15.5 retina by electron microscopy. In the control retina, we observed that the AJs were formed between immature photoreceptors harboring distinct segment extension beyond the AJC (Fig. 5*K,M*). The AJC will become the outer limiting membrane (OLM), and the photoreceptors form AJs at the OLM with microvilli of Müller glial cells, which are not yet differentiated at this stage. All retinal cells other than the photoreceptors locate inner than the OLM; therefore retinal cells that extend their segments beyond the AJC are considered to be immature photoreceptors. In addition, photoreceptors are the only retinal cells that harbor segments. It is interesting that the AJ formation between the photoreceptors are detected, because only AJs between the photoreceptor and the Müller glia have been reported so far. In contrast, in the *aPKCλ* CKO retina, we observed the AJs neither at the retinal apical edge nor around scattered photoreceptors (Fig. 5*L,N*) (data not shown). These data therefore suggest that ablation of *aPKCλ* leads to disruption of the AJs and resulted in the dispersion of the photoreceptors. Thus, our results indicate that the expression of aPKCλ is required for formation of the AJs between the differentiating photoreceptor cells.

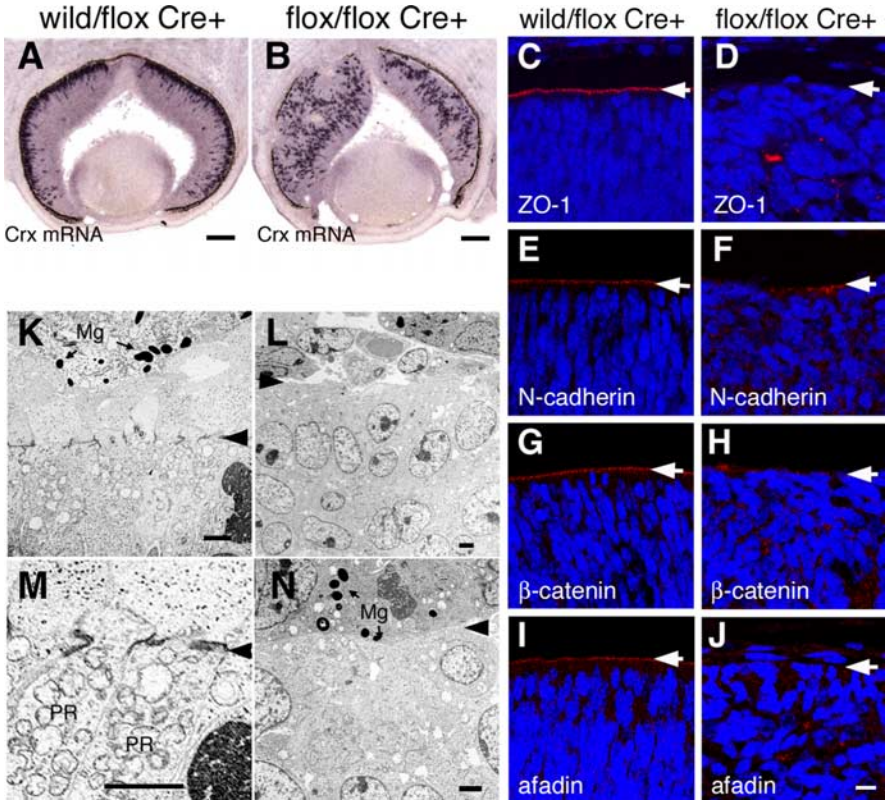

Figure 5. Differentiating photoreceptors in *aPKCλ* CKO retina failed to form adherens junctions. ***A***, ***B***, *In situ* hybridization of *Crx* on retinal sections from control (***A***) and *aPKCλ* CKO (***B***) at E14.5. Scale bar, 100 μm. ***C–J***, Immunostaining of adherens junction-related molecules in control (***C***, ***E***, ***G***, ***I***) and *aPKCλ* CKO retina (***D***, ***F***, ***H***, ***J***). ZO-1 (***C***, ***D***), N-cadherin (***E***, ***F***), β-catenin (***G***, ***H***), and afadin (***I***, ***J***) were detected using respective antibodies (red). Arrows indicate apical edge. Nuclei were counterstained with TOTO-3 iodide (642/660) (blue). Scale bars, 10 μm. ***K–N***, Ultrastructure of apical edge in control and *aPKCλ* CKO retina. Adherens junction was detected at the apical edge in control (***K***, ***M***, arrowhead) but not in *aPKCλ* CKO retina (***L***, ***N***). PR, Photoreceptor; Mg, melanin granule in retinal pigment epithelium indicated by arrow. Scale bars, 2 μm.

Polarized photoreceptor cells anchor progenitors at the apical edge of neural retina

The absence of the AJs in the *aPKCλ* CKO photoreceptors may explain why the photoreceptors scatter; however, it does not explain why all types of the retinal cells are dispersed. During retinal development, proliferating progenitor cells are bipolar in shape with a process extending toward the apical surface of the epithelium and another process that terminates at the basal surface (Saito et al., 2003). The somal position of the progenitors varies with cell cycle stage: mitotic progenitors are found near the apical surface adjacent to the developing pigment epithelium. Newly postmitotic cells leave the apical surface, migrate into the proper layers, and form synaptic connections. We investigated the possibility that loss of the AJC leads to abnormal localization of the progenitors followed by differentiation in the CKO retina. At E15.5, mitotic progenitors were localized at the apical edge in the control retina (Fig. 6*A*). In contrast, the CKO retina showed scattered distribution of mitotic progenitors (Fig. 6*B*). We also examined the distribution of S-phase retinal progenitors in E15.5

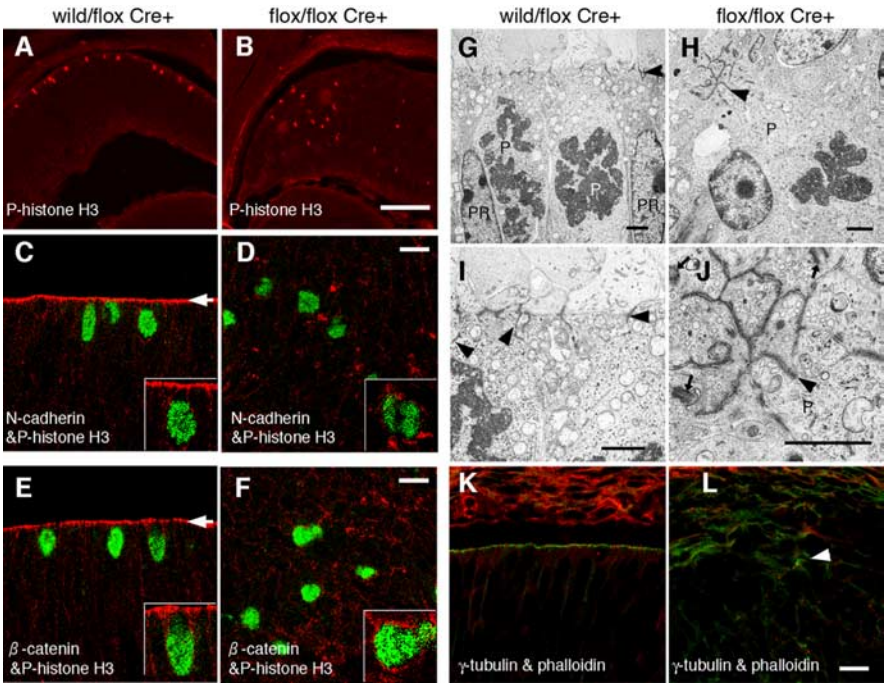

Figure 6. Progenitors failed to form adherens junctions during mitosis and were distributed randomly throughout retina. ***A***, ***B***, Distribution of progenitors during mitosis in control (***A***) and *aPKC*λ CKO retina (***B***) immunostained with phospho-histone H3 antibody at E15.5. Scale bar, 100 μm. ***C–F***, N-cadherin (red) (***C***, ***D***) and β-catenin (red) (***E***, ***F***) were expressed around progenitors (phospho-histone H3, green) both in control (***C***, ***E***) and *aPKC*λ CKO retina (***D***, ***F***). Arrows indicate the apical edge. Scale bars, 10 μm. Insets are higher-magnification images of progenitors. ***G–J***, AJC at electron-microscopic level of E15.5 retina. Immature photoreceptor (PR) and dividing progenitor (P) containing distinct nuclear alignment at the apical edge (***G***, arrowhead). AJs were formed between progenitors in mitosis and differentiating photoreceptors (***I***, arrowheads). Apical junction formed at abnormal position with a dividing progenitor (***H***, ***J***, arrowheads). Centrosomes were directed to AJC (arrow) (***J***). Scale bars, 2 μm. ***K***, ***L***, AJ-associated actin bundles (phalloidin, green) and centrosomes (γ-tubulin, red) localize apically in the control retina at E15.5 (***K***). This polarized distribution was disrupted in *aPKC*λ CKO retina (***L***). The arrowhead indicates gathering of centrosomes and AJ-associated actin bundles. Scale bar, 10 μm.

retinas. S-phase progenitors were scattered all over the retina including the apical edge in the mutant retina, whereas they are located in the inner layer in the control retina (supplemental Fig. 7, available at www.jneurosci.org as supplemental material). Thus, in the mutant retina, the abnormal distribution of mitotic progenitors is unlikely to be caused by the disruption of interkinetic nuclear migration by abnormal photoreceptors. We therefore consider that dispersion of the progenitors and after differentiation at the inappropriate location may lead to laminar disorganization.

To understand the mechanisms of progenitor dispersion in the CKO retina, we examined junction formation in the progenitors. In the control retina, N-cadherin was distributed mainly at the apical surface of the mitotic progenitors as was β-catenin (Fig. 6*C*,*E*). In the *aPKC*λ CKO retina, randomly distributed mitotic progenitors also expressed N-cadherin and β-catenin in characteristic sites (Fig. 6*D*,*F*). At the electron-microscopic level, we found that the AJs were formed between the mitotic progenitors, which have distinct nuclear morphologies, and the photoreceptors (Fig. 6*G*,*I*). The AJs were not formed at the apical surface of the *aPKC*λ CKO retina (Fig. 5*L*,*N*) but were observed at dislocated progenitors (Fig. 6*H*,*J*). Abnormal formation of the AJC between mislocalized progenitors was detected by gathering of centrosomes in the aPKCλ CKO retina by electron microscopy (Fig. 6*J*). Immunohistochemical staining also showed that ectopic distribution of centrosomes and AJ-associated actin bundles in the CKO retina (Fig. 6*L*). The centrosomes and the AJ-associated actin bundles serve as reliable indicators of apical polarity of the control retina (Chenn et al., 1998; Wei and Malicki, 2002) (Fig. 6*K*).

Thus, the progenitors seem to maintain AJ formation ability even in the *aPKC*λ CKO retina. Therefore, these progenitors may disperse because of lack of the AJ formation ability in unpolarized differentiating photoreceptor cells in the *aPKC*λ CKO retina. In other words, photoreceptors may anchor progenitors at the apical edge of the embryonic retina.

Discussion

In vivo role of *aPKC*λ in photoreceptor polarization

The photoreceptor cells have both the neuronal and the epithelial characteristics. They are generated and differentiated from the neuroepithelium and have axon–dendrite polarity as well as the other neurons in the CNS (Goldstein and Yang, 2000). At the same time, they also have epithelial properties, such as linear arrangement of AJs and apico-basal polarity. In the *aPKC*λ CKO retina, the OS and IS of the photoreceptors were absent while they expressed components of OS, including rhodopsin, cone opsin, rom1, and CNCG (Fig. 4*C*,*F*,*H*,*J*,*L*). Similarly, in the CKO retina, ST of the photoreceptors were also absent while they expressed components of ST such as synaptic ribbons (Fig. 4*N*). These results indicate that the photoreceptors cannot form OS and ST without aPKCλ function even if they express OS and ST components. Then, is the loss of photoreceptor polarity in the CKO retinas primarily caused by loss of aPKCλ or not? We cannot eliminate both possibilities, but the result that the synaptic termini of retinal neurons other than photoreceptors were formed in the CKO retinas (data not shown) supports the idea that aPKCλ is primarily required at least for synapse formation of the photoreceptors. The failure of formation of OS and IS in the CKO retina is supposed to be primarily caused by lack of aPKCλ as well; however, it awaits future analysis to draw a clear conclusion.

Several downstream targets of aPKCλ have been suggested. The evolutionally conserved Crumbs (Crb) complex is localized to the apical side and required to organize apico-basal polarity in the epithelial cells of the *Drosophila* embryo (Muller, 2000; Ohno, 2001; Tepass et al., 2001; Knust and Bossinger, 2002; Henrique and Schweisguth, 2003; Roh and Margolis, 2003). Bazooka, a Par3 homolog, interacts with Crb directly, and phosphorylation of Crb by DaPKC is required for epithelial polarity in *Drosophila* (Sotillos et al., 2004). The mouse *Crumbs* homolog, Crumbs1 (Crb1), actually localizes to the IS of photoreceptors, and mutations in the human *CRB1* are reported to cause retinitis pigmentosa and Leber congenital amaurosis (van Soest et al., 1999; Cremers et al., 2002; den Hollander et al., 2002). However, the mouse *Crb1* mutants show only mild retinal pheno-

types, discontinuous OLM, and spotty rosettes of photoreceptors (Mehalow et al., 2003; van de Pavert et al., 2004). Therefore, another or unknown Crumbs homolog other than CRB1 are more likely to be involved in inducing photoreceptor polarization as a target of aPKC.

One of the other possible targets of aPKC may be the microtubule-based dynein/kinesin motor complex (Goldstein and Yang, 2000; Nishimura et al., 2004). So far, a kinesin II subunit, KIF3A, is known to be required for normal transportation in photoreceptors (Marszalek et al., 2000). Other kinesins and/or related proteins may contribute to structural establishment of photoreceptors, because significant morphological change of photoreceptors was not observed in the *KIF3A* mutants (Marszalek et al., 2000).

Interestingly, a recent study shows that glycogen synthase kinase-3β (GSK-3β) regulates neuronal polarity through the phosphorylation of collapsing response mediator protein-2 (CRMP-2) (Yoshimura et al., 2005). aPKC can phosphorylate GSK-3β and inactivate its kinase activity (Etienne-Manneville and Hall, 2003). We detected the expression of CRMP-2 in developing photoreceptors (data not shown). Thus, aPKC may also regulate photoreceptor polarity through the regulation of GSK-3β and CRMP-2.

Role of photoreceptor in retinal lamination

In vertebrates, the neural retina develops from a single-layered neuroepithelium. With maturation, the retina develops to form three major laminas: the photoreceptor layer, the inner nuclear layer, and the ganglion cell layer. In the *aPKC*λ CKO retina in this study, severe laminar disorganization was observed not only in the photoreceptor layer but also in the entire retina. What is the mechanism that leads to the entire retinal lamination defect although *aPKC*λ is ablated only in the photoreceptors?

There have been several reports of mouse mutants showing laminar disorganization in mammalian retina, which suggest important roles of retinal pigment epithelium (RPE) and Müller glia for retinal lamination. In the RPE-ablated mice, the retinal lamina is weakly disorganized, but each retinal layer is still distinguishable (Raymond and Jackson, 1995). *shh* (*sonic hedgehog*) signaling from retinal ganglion cells plays a role in laminar organization in the mouse retina through the Müller glia, which express *Patched-1* (Wang et al., 2002). In the *aPKC*λ CKO retina, the boundary between the retina and the RPE is partially missing (Fig. 2*E*). However, we consider that abnormality of the RPE or Müller glia is unlikely to be a primary cause of the lamination defect in the CKO retina because of the following reasons. First, Cre recombinase is expressed in neither the RPE nor Müller glia (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) (Nishida et al., 2003). Second, differentiating photoreceptor cells have already been detached from the apical edge at E14.5, when most of the RPE seems intact (Fig. 5*B*). Third, Müller glia have not yet developed until E17 at which the lamination defect has already been observed in the CKO retina.

Genetic studies of zebrafish show several alleles that play important roles in epithelial polarity of the retinal neuroepithelium. The retinal phenotypes of several zebrafish mutants, including *nok* (Wei and Malicki, 2002), *glo* (Malicki et al., 2003), *pac* (Masai et al., 2003), *ncad* (Masai et al., 2003), *moe* (Jensen et al., 2001; Jensen and Westerfield, 2004), and *ome* (Malicki et al., 1996; Malicki and Driever, 1999), are similar with those of *aPKC*λ CKO retina to some extent; laminar arrangement is disorganized and patches of the plexiform matter are scattered. *nok* and *moe* are considered to be necessary for TJ formation of RPE and also for the polarity formation of RPE and the neuroepithelial sheet. *Glo*, *pac*, and *ncad* alleles encode N-cadherin and are thought to play a role in neuroepithelial integrity. They also function in the integrity of the inner plexiform layer. We showed in this report that the expression of N-cadherin was dispersed in the *aPKC*λ CKO retina (Fig. 5*F*). Therefore, the retinal laminar disorganization of the zebrafish mutants described above may be at least partly attributable to loss of N-cadherin in postmitotic photoreceptor cells. Intriguingly, only the *heart and soul* (*has*) mutant, which encodes aPKC, shows a different phenotype from that of mouse *aPKC*λ CKO retina; plexiform matter is not scattered in patches (Malicki et al., 1996; Horne-Badovinac et al., 2001). The loss of plexiform matter in zebrafish *has* mutant retina indicate that aPKCλ contributes to process formation of all types of the retinal cells. The complete laminar disorganization observed in these zebrafish mutants has been considered to be attributable to polarity defects in retinal progenitor cells, but we speculate that polarity defects in the photoreceptors may also contribute to these phenotypes.

At the apical side of the photoreceptors, OLM, containing AJs, is formed between the photoreceptors and Müller glia cells. We reported here that AJ formation was also detected between the photoreceptors and the progenitors, in addition to AJ formation between the photoreceptors themselves (Figs. 5, 6). Because the photoreceptors have AJs and the Par3/Par6/aPKC complex locates at the apical edge, we considered that the similar mechanisms proposed for epithelial cells could apply for the retinal integrity. AJ as well as cell adhesion molecules such as N-cadherin and nectin were not detected at the apical edge in the CKO retina (Fig. 5*F*,*L*,*N*) (data not shown). Based on these findings, we propose the following mechanism for the striking lamination defect of the *aPKC*λ CKO retina (supplemental Fig. 8*B*, available at www.jneurosci.org as supplemental material). First, disruption of polarity and AJ formation in postmitotic differentiating photoreceptor cells leads to the absence of AJC during retinogenesis. Second, mitotic progenitors then fail to anchor to the apical surface and scatter. Third, progenitors exiting the cell cycle undergo normal differentiation even in the abnormal position. Fourth, irregular AJC formation of progenitors in abnormal positions may lead to rosette formation. Together, we consider that formation and maintenance of AJC between photoreceptor-committed cells and progenitors are required for proper lamination during development. Other retinal cells are unlikely to be involved in anchoring progenitors, because photoreceptors are the only retinal cells that deposit at the outermost layer and are supposed to maintain AJC until E17 when OLM is formed with the terminal process of Müller glia, which functions as a supporting cell.

In this study, we have shown that normal photoreceptor polarization is required for retinal lamination by deprivation of aPKCλ in postmitotic photoreceptors. How is retinal laminar properly organized during retinal development? We have shown that aPKCλ is expressed at the apical edge of the embryonic retina (Fig. 1; supplemental Fig. 9, available at www.jneurosci.org as supplemental material). During development, neural retina is generated from single neuroepithelium sheet, and neurogenesis begins at ~E11.5. Morphological defects of the retina were detected after Cre recombinase began to be expressed in photoreceptor-committed cells. Because gliogenesis begins at ~E17, our observations suggest that correct polarization in postmitotic photoreceptors is required for the proper laminar formation of the mammalian retina before gliogenesis begins (supple-

mental Fig. 8*A*, available at www.jneurosci.org as supplemental material).

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