Development/Plasticity/Repair

Blimp1 Suppresses *Chx10* Expression in Differentiating Retinal Photoreceptor Precursors to Ensure Proper Photoreceptor Development

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The zinc finger transcription factor Blimp1 plays fundamentally important roles in many cell lineages and in the early development of several cell types, including B and T lymphocytes and germ cells. Although *Blimp1* expression in developing retinal photoreceptor cells has been reported, its function remains unclear. We identified *Blimp1* as a downstream factor of Otx2, which plays an essential role in photoreceptor cell fate determination. To investigate Blimp1 function in the mouse retina, we ablated *Blimp1* in the developing retina by conditional gene targeting. In the *Blimp1* conditional knockout (CKO) retina, the number of photoreceptor cells was markedly reduced in the differentiated retina. We found that the numbers of both bipolar-like cells and proliferating retinal cells increased noticeably, with ectopic localizations in the postnatal developing retina. In contrast, a reduction of the number of photoreceptor precursors was observed during development. Forced expression of *Blimp1* by *in vivo* electroporation suppressed bipolar cell genesis in the developing retina. Multiple genes involved in bipolar development, including *Chx10*, were upregulated in the *Blimp1* CKO retina. Furthermore, we showed that Blimp1 can bind to the *Chx10* enhancer and repress *Chx10* enhancer activity. These results suggest that Blimp1 plays a crucial role in photoreceptor development by repressing genes involved in bipolar cell fate specification and retinal cell proliferation in differentiating photoreceptor precursors.

Introduction

The vertebrate retina is comprised of five types of neurons: photoreceptor, horizontal, bipolar, amacrine, and ganglion cells, and one type of glial cell, Müller glia. These cell types arise from a multipotent progenitor cell population in a stage-specific manner (Cepko et al., 1996). We showed previously that the Otx2 transcription factor is essential for the cell fate determination of photoreceptor cells (Nishida et al., 2003; Sato et al., 2007). The *Otx2* conditional knockout (CKO) mouse retina showed a total absence of photoreceptors and a marked increase of amacrine-like cells, suggesting that the cell fate of photoreceptor precursors was converted to that of amacrine-like cells in the absence of *Otx2*. To reveal the transcriptional network in developing photoreceptor cells, we performed a genomewide expression profiling of the *Otx2* CKO retina (Y. Omori, K. Katoh, S. Sato, and T. Furukawa, unpublished data). We identified the *B lymphocyte-induced maturation protein 1* [*Blimp1*, also known as *PR domain containing 1* (*Prdm1*)] gene as one of the downstream targets of Otx2. Blimp1 is a Prdm family transcription factor and was originally identified as a repressor of *β-interferon* gene expression (Keller and Maniatis, 1991). Blimp1 has been shown to function mainly as a transcriptional repressor. Blimp1 orchestrates development and homeostasis in a diverse variety of tissues through repression of cell-cycle progression and/or global change of gene expression (Shaffer et al., 2002; Ohinata et al., 2005; Shapiro-Shelef and Calame, 2005; Kallies and Nutt, 2007). Blimp1 functions as a master regulator of B lymphocyte terminal differentiation by repressing both cell proliferation and expression of a large number of genes critical for B lymphocyte identity (Turner et al., 1994). Blimp1 also specifies germ cell fate through silencing of the default somatic gene program and promotion of germ cell-related gene expression (Ohinata et al., 2005; Vincent et al., 2005). In T lymphocytes, Blimp1 governs T lymphocyte homeostasis by attenuating both proliferation and survival (Kallies et al., 2006; Martins et al., 2006). It was reported that Blimp1 is involved in specification of both neural crest cells and a subset of sensory neurons in zebrafish (Roy and Ng, 2004). In the mouse retina, several studies reported that *Blimp1* is expressed in the photoreceptor cell layer (Chang et al., 2002; Wilm and Solnica-

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Krezel, 2005; Hsiau et al., 2007). However, Blimp1 function in the developing retina remains elusive.

In the present study, to assess *in vivo* Blimp1 function in the retina, we generated *Blimp1* CKO mice by crossing *Blimp1*$^{flox/flox}$ mice (Shapiro-Shelef et al., 2003) with *Dkk3-Cre* mice (Sato et al., 2007). We report here that *Blimp1* CKO mice showed a significant decrease of photoreceptor precursors and, in contrast, a marked increase of bipolar-like cells and proliferating retinal cells during development. Forced expression of Blimp1 leads to a specific reduction of developing bipolar cells. In addition, we demonstrated that Blimp1 interacts with *Chx10* enhancer regions and represses *Chx10* transcription. These results suggest that Blimp1 ensures the proper development of photoreceptor cells through suppressing genes involved in bipolar cell fate specification and retinal cell proliferation in differentiating photoreceptor precursors.

Materials and Methods

Generation of Blimp1 CKO mouse. We mated a *Blimp1*^{flox} (Shapiro-Shelef et al., 2003) mouse line with a *Dkk3-Cre-BAC* transgenic mouse line (Sato et al., 2007), which expresses Cre recombinase under control of the *Dkk3* promoter. The *Blimp1-mVenus* (Ohinata et al., 2008), *Crx-Cre* (Nishida et al., 2003), and *Crx-LacZ* (Furukawa et al., 2002) transgenic mouse lines were described previously. All procedures conformed to the Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology, and these procedures were approved by the Institutional Safety Committee on Recombinant DNA Experiments and the Animal Research Committee of Osaka Bioscience Institute. Mice were housed in a temperature-controlled room with a 12 h light/dark cycle. Fresh water and rodent diet were available at all times.

Histology and immunohistochemistry. Embryonic ages were determined as the time since the appearance of the vaginal plug [the first day was designated as embryonic day 0.5 (E0.5)]. Mouse embryos and eye cups were fixed in 4% paraformaldehyde in PBS for either 30 min to 3 h (for immunostaining) or overnight (for *in situ* hybridization). The samples were cryoprotected, embedded, frozen, and sectioned (20 μm thick). Slides were incubated with blocking solution (4% normal donkey serum and 0.5% Triton X-100 in PBS) for 1 h, and then with the primary antibodies for 4 h at room temperature. Slides were washed with PBS three times for 10 min each time and incubated with the secondary antibodies for 2 h at room temperature. The specimens were observed under a laser confocal microscope (LSM510; Carl Zeiss). We used the following primary antibodies: mouse monoclonal antibodies specific to β-galactosidase (1:500; Developmental Studies Hybridoma Bank), Crx (1:3000; Medical & Biological Laboratories), calbindin D-28K (1:500; Sigma), Islet1 (1:100; Developmental Studies Hybridoma Bank), Pax6 (1:200; Developmental Studies Hybridoma Bank), proliferating cell nuclear antigen (PCNA; 1:500; Dako Cytomation), rhodopsin RET-P1 (1:5000; Sigma), and S-100β (1:1000; Sigma); rabbit polyclonal antibodies to active caspase3 (1:100; Promega), Chx10 (1:100) (Koike et al., 2007), Ki-67 (1:100; Neomarkers), M-opsin (1:300; Millipore), phospho-histone H3 (1:100; Millipore), PKCα (1:10,000; Sigma), recoverin (1:1000; Millipore), rhodopsin (1:5000; LSL), S-opsin (1:1000; Millipore), and Sox9 (1:750; Millipore); rat monoclonal antibodies against Blimp1 (1:200; Santa Cruz Biotechnology) and green fluorescent protein (GFP; 1:1000; Nakalai); goat polyclonal antibodies to Bhlhb5 (1:200; Santa Cruz Biotechnology), Brn3b (1:100; Santa Cruz Biotechnology), and Otx2 (1:500; R&D Systems); a guinea pig polyclonal antibody to Trb2 (1:50; MBL); and a sheep polyclonal antibody against bromodeoxyuridine (BrdU; 1:300; Fitzgerald). We used rhodamine-labeled peanut agglutinin (1:300; Vector Laboratories) and Alexa Fluor 488 phalloidin (1:3000; Invitrogen). For BrdU experiments, mice were injected intraperitoneally with 50 μg/g body weight of BrdU for 3 h (see Fig. 3*K*,*L*) or two days (supplemental Fig. S4*A*,*B*, available at www.jneurosci.org as supplemental material) (retinal tissues) before being killed. Terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assays were performed using the *in situ* Apoptosis Detection Kit (Takara) according to the manufacturer's instructions. The full-length cDNAs of *Otx2* and *Crx* were used as templates to generate probes for *in situ* hybridization as described previously (Furukawa et al., 1997; Nishida et al., 2003). A *Blimp1* cDNA fragment for an *in situ* probe was obtained by reverse transcriptase PCR.

Transfection and luciferase assay. Luciferase reporter plasmids (*pGL3-Chx10-A*, *pGL3-Chx10-B*, and *pGL3-Chx10-C*) were generated by subcloning an ~2.5 kb fragment from around 20 kb upstream (Chx10 region A; −20102 to −17585 bp) (Kim et al., 2008), an ~0.7 kb fragment from around 7 kb upstream (Chx10 region B; −7851 to −7183 bp), or an ~1.0 kb fragment from around 13 kb downstream (Chx10 region C; +12470 to +13358 bp intron3) of the mouse *Chx10* gene into the *pGL3-promoter* luciferase reporter vector (Promega). The mouse *Blimp1/pcDNA3.1* expression vector was a gift from Dr. Kathryn Calame (Columbia University Medical Center, New York, NY). The Neuro2A neuroblastoma cell line was used for the luciferase assay. Transient transfection and measurement of luciferase activity were as described previously (Furukawa et al., 1997; Omori et al., 2001).

Microarray analysis. Mouse retinas were dissected at postnatal day 6 (P6). Total retinal RNA (5 μg) was isolated using TRIzol reagent (Invitrogen) and converted to cDNA using the One-Cycle cDNA synthesis kit (Affymetrix) according to the manufacturer's instructions. Biotin-labeled cRNA was prepared using the *in vitro* transcription labeling kit and hybridized to the GeneChip mouse genome 430 2.0 array (Affymetrix). Signal intensity was determined using GeneChip operating software version 1.4.

Quantitative real-time PCR and Northern blot analysis. Quantitative real-time PCR (Q-PCR) was performed using SYBR GreenER Q-PCR Super Mix (Invitrogen) and Thermal Cycler Dice Real Time System Single MRQ TP870 (Takara) according to the manufacturer's instructions. Quantification was performed by Thermal Cycler Dice Real Time System software version 2.0 (Takara). The primer sequences are listed in supplemental Table S3 (available at www.jneurosci.org as supplemental material). For the Northern blot analysis, a 364 bp (574–937 bp) fragment of *Blimp1* cDNA was used as a radiolabeled probe.

Electroretinogram. Electroretinographic recordings were performed as described in detail previously (Koyasu et al., 2008). In brief, mice were dark adapted overnight, and then anesthetized with an intraperitoneal injection of ketamine and xylazine. Electroretinograms (ERGs) were picked up with a gold-wire loop electrode placed on the cornea. The mice were placed in a Ganzfeld dome and stimulated with stroboscopic stimuli ranging from −7.0 to 1.0 log cd-s/m^2 to elicit the scotopic ERGs, and with five levels of stimuli ranging from −1.0 to 1.0 log cd-s/m^2 for the photopic ERGs. The photopic ERGs were recorded on a rod-suppressing white background of 1.3 log cd-s/m^2.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as described previously (Peng and Chen, 2005) using an anti-Blimp1 antibody (Santa Cruz Biotechnology). The immunoprecipitated chromatin DNA was analyzed by PCR using gene-specific primers (supplemental Table S3, available at www.jneurosci.org as supplemental material).

In vivo *electroporation into the mouse retina.* The *in vivo* electroporation into the mouse retinas was performed as described previously (Matsuda and Cepko, 2004). P0 mice were electroporated *in vivo* with *pCAG-GFP* or *pCAG-Blimp1*. P6 retinas were dissected and examined by immunohistochemical analysis using the anti-Chx10 antibody.

Statistical analysis. Statistical significance was calculated with a Student's *t* test. A value of $p < 0.03$ was taken to be statistically significant. Data are presented as means ± SD.

Results

Blimp1 is expressed in photoreceptor precursors during development

We showed previously that Otx2 is essential for the cell fate determination of photoreceptors (Nishida et al., 2003). To identify factors that are critical for retinal development, we performed a microarray analysis of the control and the *Otx2*

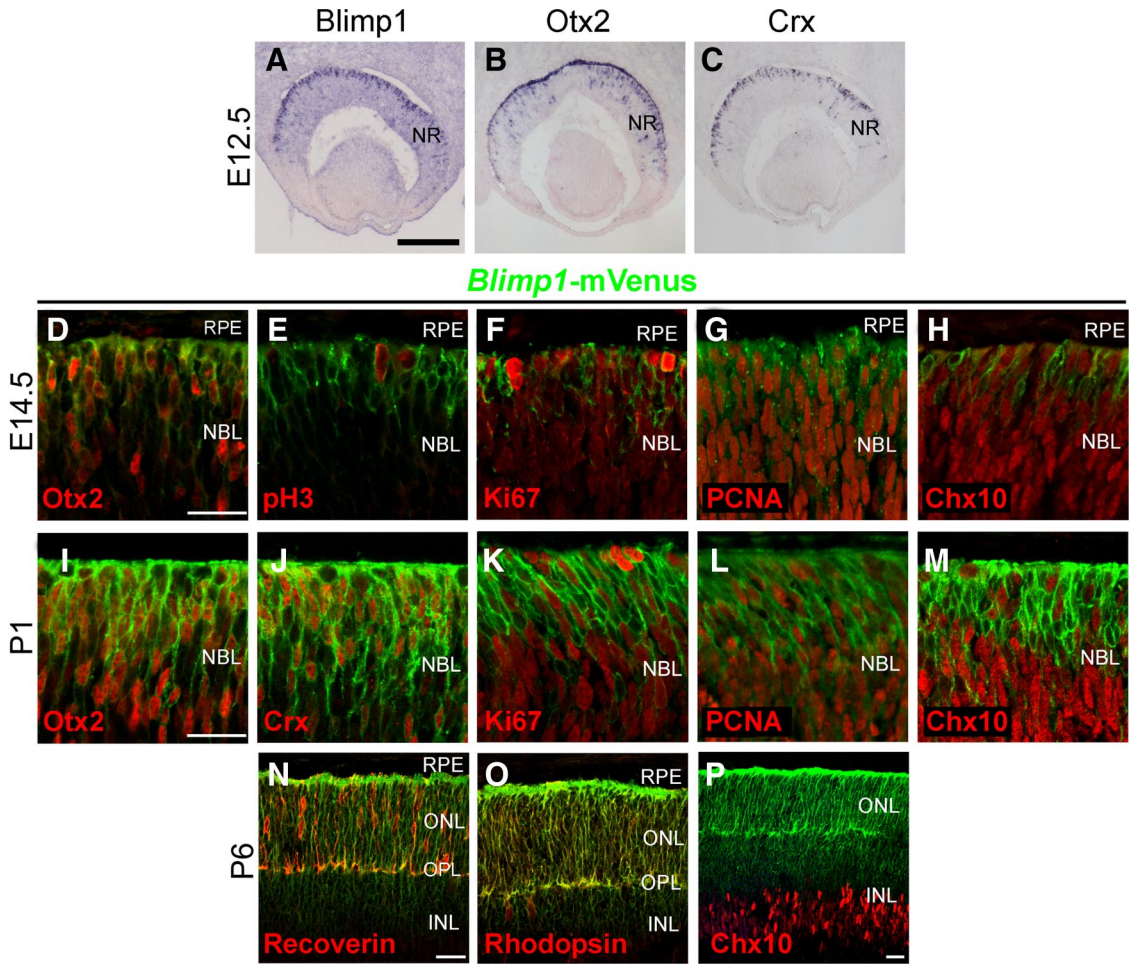

Figure 1. Blimp1 is expressed in photoreceptor precursor cells in the developing retina. ***A–C***, Expression of *Blimp1* (***A***), *Otx2* (***B***), and *Crx* (***C***) mRNA in the E12.5 mouse retina were detected by *in situ* hybridization. ***D–P***, Immunostaining of the retina from a transgenic reporter mouse strain that expresses mVenus under the control of *Blimp1* regulatory elements (*Blimp1-mVenus*). Retinal sections were stained with anti-Otx2 (***D***, ***I***), anti-pH3 (***E***), anti-Ki67 (***F***, ***K***), anti-PCNA (***G***, ***L***), anti-Chx10 (***H***, ***M***, ***P***), anti-Crx (***J***), anti-Recoverin (***N***), and anti-Rhodopsin (***O***) antibodies at E14.5 (***D–H***), P1 (***I–M***) and P6 (***N–P***). Scale bars: (in ***A***) ***A–C***, 200 μm; (in ***D***, ***I***, ***N***, ***P***) ***D–P***, 20 μm. NR, Neural retina; RPE, retinal pigment epithelium; NBL, neuroblastic layer.

CKO retinas (Omori, Katoh, Sato, and Furukawa, unpublished data). In this analysis, we identified that *Blimp1* is almost absent in the *Otx2* CKO retina at P1 and P12. We confirmed that *Blimp1* was markedly downregulated in the *Otx2* CKO retina by Q-PCR analysis (1.000 ± 0.060 in control; 0.012 ± 0.003 in *Otx2* CKO; $p < 0.03$).

It was reported previously that *Blimp1* is expressed in the developing retina (Chang et al., 2002; Wilm and Solnica-Krezel, 2005; Hsiau et al., 2007). To determine the detailed expression pattern of *Blimp1* in the developing retina, we performed *in situ* hybridization analysis of *Blimp1* at several developmental stages. *Blimp1* mRNA was first detected in the E11.5 retina (supplemental Fig. S1*A*, available at www.jneurosci.org as supplemental material). Crx is an Otx2 family transcription factor that is positively regulated by Otx2 and is essential for the terminal differentiation of photoreceptor cells (Furukawa et al., 1999). Similar to the *Otx2* and *Crx* transcripts, a significant *Blimp1* transcript signal was observed in the outer aspect of the neural retina corresponding to the prospective photoreceptor layer at E12.5 (Fig. 1*A–C*). During embryonic and postnatal retinal development, *Blimp1* expression was observed in the prospective photoreceptor layer from E15.5 to P3 (supplemental Fig. S1*B–D*, available at www.jneurosci.org as supplemental material). After P6, the expression of *Blimp1* decreased and became almost undetectable at the adult stage (supplemental Fig. S1*E*,*F*, available at www.jneurosci.org as supplemental material). Consistent with the result by *in situ* hybridization, Northern blot analysis showed the expression of *Blimp1* reaches its peak between P3 and P6 (supplemental Fig. S1*G*, available at www.jneurosci.org as supplemental material).

We then immunostained the developing retina from the transgenic reporter mouse strain which expresses membrane-targeted Venus (mVenus) under control of the *Blimp1* regulatory elements (*Blimp1-mVenus* mouse) (Ohinata et al., 2008). In the E14.5 retina, mVenus-positive cells overlapped with the Otx2 signal, a marker for photoreceptor precursors at this stage (Fig. 1*D*). On the other hand, mVenus-positive cells rarely overlapped with the phospho-histone H3 (pH3) signal (mitosis marker), the Ki67 signal (marker of proliferating cells), the PCNA signal (marker of proliferating cells), or the Chx10 signal (marker of progenitor in this stage) at E14.5 (Fig. 1*E–H*). At P1, mVenus-positive cells overlapped with Otx2 and Crx signals, which are photoreceptor precursor markers (Fig. 1*I*,*J*). Conversely, the mVenus signal rarely colocalized with Ki67-, PCNA-, or Chx10-positive cells at P1 (Fig. 1*K–M*). In the P6 retina, the cells expressing mVenus were co-stained with an anti-recoverin antibody or an anti-rhodopsin antibody, both of which are rod photoreceptor markers (Fig. 1*N*,*O*). In contrast, mVenus-expressing cells rarely overlap with the Chx10 signal, which is a marker for immature and mature bipolar cells and proliferating progenitors (Fig. 1*H*,*M*,*P*). These results suggest that *Blimp1* is predomi-

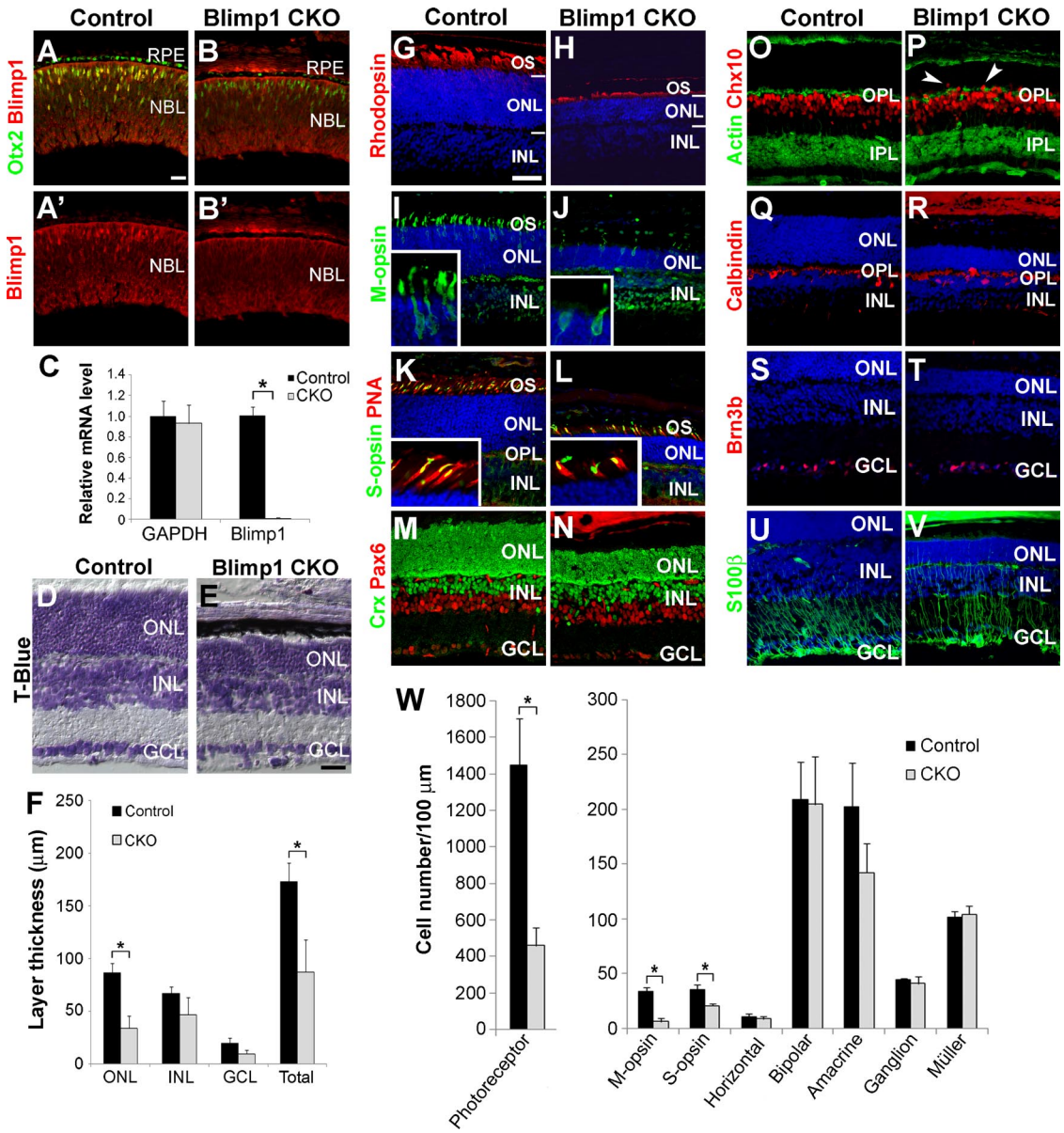

Figure 2. Targeted disruption of *Blimp1* caused a specific reduction of photoreceptor cells in the retina. ***A–B′***, Retinal sections from the control (***A***, ***A′***) and *Blimp1* CKO (***B***, ***B′***) mice at E15.5 were stained with the anti-Otx2 (green) and anti-Blimp1 (red) antibodies. ***C***, Expression levels of *Blimp1* and *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) were quantified by Q-PCR analysis in the control and *Blimp1* CKO retinas at P6 ($n = 4$). *$p < 0.03$. ***D***, ***E***, Toluidine blue (T-Blue) staining of the control (***D***) and the *Blimp1* CKO (***E***) retina at P14. ***F***, Thicknesses of GCL, INL, ONL, and total retina (total) were measured ($n = 15$ from 3 retinas). *$p < 0.03$. ***G–V***, P14 retinal sections from the control (left) and *Blimp1* CKO (right) mice were immunostained with cell-type-specific antibodies for rod photoreceptors (Rhodopsin; ***G***, ***H***), cone photoreceptors (M-opsin, ***I***, ***J***; S-opsin and PNA, ***K***, ***L***), photoreceptor and amacrine cells (Crx and Pax6; ***M***, ***N***), plexiform layers and bipolar cells (phalloidin, which stains actin in the OPL, and Chx10; ***O***, ***P***), horizontal cells (calbindin; ***Q***, ***R***), ganglion cells (Brn3b; ***S***, ***T***), and Müller glia (S100β; ***U***, ***V***). The OPL stained with phalloidin was slightly irregular in the *Blimp1* CKO retina (***P***, arrowheads). Small boxes in ***I–L*** show higher-magnification images. ***W***, Retinal cell type composition evaluated from section immunohistochemistry with various retina cell-specific markers. All data were represented as mean ± SD ($n = 15$ from 3 retinas). *$p < 0.03$. Scale bars: 50 μm. NR, Neural retina; RPE, retinal pigment epithelium; NBL, neuroblastic layer; OS, outer segment; IPL, inner plexiform layer.

nantly expressed in postmitotic photoreceptor precursors in the developing retina.

Reduction of the number of photoreceptors in the *Blimp1* CKO retina

To investigate the *in vivo* function of *Blimp1* in the developing retina, we ablated *Blimp1* from the developing retina by conditional gene targeting. To accomplish this, we mated the $Blimp1^{flox}$ mouse line with the *Dkk3-Cre* transgenic mouse line in which Cre recombinase-mediated recombination occurs in almost all retinal progenitors (Sato et al., 2007). We obtained $Blimp1^{flox/flox}$; $Dkk3\text{-}Cre^{+}$ (*Blimp1* CKO) mice by mating $Blimp1^{flox/flox}$ mice with $Blimp1^{flox/+}$; $Dkk3\text{-}Cre^{+}$ mice. We analyzed the phenotypes and compared them to those of control mice with the genotype $Blimp1^{flox/flox}$; $Dkk3\text{-}Cre^{-}$, which show no abnormal phenotype. *Blimp1* CKO mice were viable and fertile. We confirmed a total loss of Blimp1 protein in the *Blimp1* CKO retina at E15.5 by coimmunostaining with anti-Blimp1 and anti-Otx2 antibodies (Fig. 2*A–B′*). We also observed an almost complete loss of *Blimp1* mRNA expression in the *Blimp1* CKO retina by Q-PCR analysis (Fig. 2*C*).

First, we examined the retinal tissue of the *Blimp1* CKO and control mouse retinas at P14 by toluidine blue staining (Fig. 2*D*,*E*). At this stage, retinogenesis is almost complete, and all

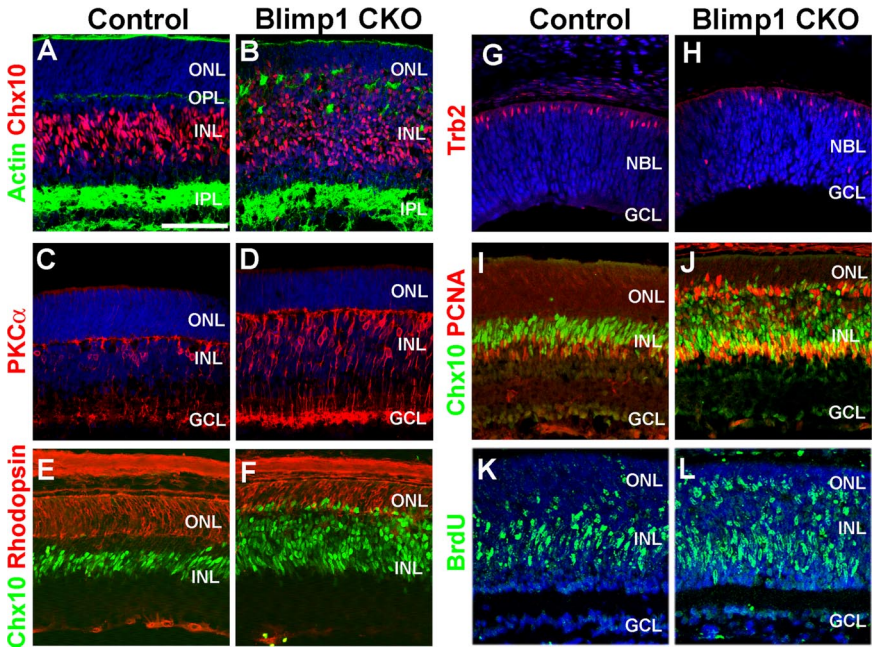

Figure 3. Cell composition is impaired in the developing *Blimp1* CKO retina. ***A–D***, The number of bipolar-like cells increased in the *Blimp1* CKO retina (***B***, ***D***) at P6 compared to the control (***A***, ***C***) stained with the anti-Chx10 (***A***, ***B***, red) and anti-PKCα (***C***, ***D***, red) antibodies. The OPL is visualized by phalloidin staining (***A***, ***B***, green), which stains actin filaments in the plexiform layers. ***E***, ***F***, Decreased number of photoreceptors in the *Blimp1* CKO retina (***F***) compared to the control (***E***) at P6. Maturing photoreceptor precursors are stained with anti-rhodopsin antibody (red). ***G***, ***H***, Cone photoreceptor precursors decreased in the *Blimp1* CKO at E14.5. Retinal sections from control (***G***) and *Blimp1* CKO (***H***) mice were stained with the anti-Trb2 antibody (red), a marker for cone precursor in the embryonic retina. ***I–L***, Increased numbers of proliferating cells were observed in the *Blimp1* CKO (***J***, ***L***) compared to the control (***I***, ***K***). Proliferating cells are stained with the anti-PCNA antibody (***I***, ***J***, red) and BrdU (***K***, ***L***, green). Bipolar cells and proliferating retinal cells are visualized by the anti-Chx10 antibody (***I***, ***J***, green). Scale bars: 50 μm. NBL, Neuroblastic layer.

three nuclear layers and the two plexiform layers are fully formed in both the control and *Blimp1* CKO retinas (Fig. 2*D–F*). However, the thickness of the outer nuclear layer (ONL) is significantly thinner in the *Blimp1* CKO retinas compared with that of the control retinas (Fig. 2*D–F*). In the mouse retina, ~97% of photoreceptors are rod (Carter-Dawson and LaVail, 1979). The number of rod photoreceptors, determined by counting the number of 4′,6′-diamidino-2-phenylindole (DAPI)-positive nuclei in the ONL of the *Blimp1* CKO retina at P14, was markedly decreased (Fig. 2*W*). Cone photoreceptors stained with the anti-S-opsin and M-opsin antibodies also decreased (Fig. 2*I–L*,*W*). The length of outer segments of both rod and cone photoreceptors in the *Blimp1* CKO retinas were significantly shorter than those of the control retinas (Fig. 2*G–L*). In contrast to the decrease of the number of photoreceptors, the numbers of the Chx10-positive bipolar cells, calbindin-positive horizontal cells, Pax6-positive amacrine cells, Brn3b-positive ganglion cells, and S100β-positive Müller glial cells in the *Blimp1* CKOs were unaffected at this stage, compared to those in the control retinas (Fig. 2*M–W*). The outer plexiform layer (OPL) stained with phalloidin was slightly irregular in the *Blimp1* CKO retinas (Fig. 2*O*,*P*).

Next, to evaluate the retinal physiological function *in vivo*, ERGs were recorded from 8-week-old *Blimp1* CKO and control mice (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). The scotopic ERG amplitudes of *Blimp1* CKO mice were significantly smaller than those of the control mice, and the degree of amplitude reduction was similar for the a- and b-waves (supplemental Fig. S2*A*,*B*, available at www.jneurosci.org as supplemental material). We found that the photopic ERG amplitudes of the *Blimp1* CKO mice were significantly smaller than those of the control mice (supplemental Fig. S2*C*,*D*, available at www.jneurosci.org as supplemental material). These results suggest that the functional abnormality of the *Blimp1* CKO retina lies mainly in the rod and cone photoreceptors.

Abnormal cell composition in the developing *Blimp1* CKO retina

We then examined retinal development of *Blimp1* CKO retinas at E14.5, E15.5, P3, P6, and P9 and compared them to those of control retinas. We observed the most severe abnormality at P6 in the *Bilmp1* CKO retina (Fig. 3*A–F*,*I–L*). In the normal retina, the production of bipolar cells reaches its peak at around P3 (Cepko et al., 1996). We found that the thickness from the inner nuclear layer (INL) up to the ONL was similar between the control (112.6 $\pm$ 8.2 μm) and the *Blimp1* CKO retinas (104.1 $\pm$ 11.1 μm) at P6 (Fig. 3*A*,*B*). However, ONL thickness of the *Blimp1* CKO retina was significantly reduced (47.4 $\pm$ 5.8 μm in control; 29.4 $\pm$ 4.5 μm in CKO; $p < 0.03$) (Fig. 3*A*,*B*). Notably, we found that the number of Chx10-positive cells increased in the *Blimp1* CKO retina, as did the dispersion of those Chx10-positive cells (number of Chx10$^+$ cells/100 μm, 146.2 $\pm$ 31.8 in control, 373.0 $\pm$ 64.3 in CKO; $p < 0.03$) (Fig. 3*A*,*B*). We also observed an increased number of PKCα-positive cells, which are rod bipolar cells (Fig. 3*C*,*D*). Chx10 is known to be expressed in both all bipolar cells and proliferating progenitor cells; therefore, we next coimmunostained with Chx10 and a proliferating cell marker, PCNA. Most of the ectopically localized Chx10-positive cells did not overlap with PCNA in the *Blimp1* CKO retina (Fig. 3*I*,*J*), suggesting that bipolar cells are overproduced in the developing *Blimp1* CKO retina at P6. Similar to the P14 *Blimp1* CKO retina, the number of differentiating rod photoreceptor precursors detected by the rhodopsin or recoverin signal was markedly decreased in the P6 *Blimp1* CKO retina (number of recoverin$^+$ cells/100 μm, 84.8 $\pm$ 15.6 in control, 47.0 $\pm$ 3.8 in CKO; $p < 0.03$) (Fig. 3*E*,*F*).

Furthermore, we also found a remarkable increase of PCNA-positive proliferating cells in the *Blimp1* CKO at P6 (number of PCNA$^+$ cells/100 μm, 48.0 $\pm$ 5.7 in control, 103.6 $\pm$ 7.9 in CKO; $p < 0.03$) (Fig. 3*I*,*J*). Consistent with this, after BrdU pulse labeling for 3 h in the P6 retina, we found increased numbers of BrdU-positive cells in the *Blimp1* CKO retina (Fig. 3*K*,*L*). All these observations suggest that lack of *Blimp1* in the developing retina causes an abnormal increase of Chx10-positive cells and proliferating cells.

To confirm that the loss of Blimp1 in photoreceptor precursors causes these abnormal retinal cell compositions, we generated *Blimp1*$^{flox/flox}$; *Crx-Cre*$^+$ (*Blimp1 Crx-Cre* CKO) mice. *Crx-Cre* mice specifically express Cre in photoreceptor precursors under the control of the *Crx* promoter (Furukawa et al., 2002). We observed reduced numbers of photoreceptor precursors and excess bipolar-like and proliferating cells in the *Blimp1 Crx-Cre* CKO retina at P6 (supplemental Fig. S3*A–H*, available at www.jneurosci.org as sup-

plemental material), similar to the *Blimp1* CKO retina. The retinal phenotype of *Blimp1 Crx-Cre* CKO is similar to but less severe than that of *Blimp1 Dkk3-Cre* CKO. This is likely because of *Blimp1* expression beginning as early as *Crx* expression and inactivation of *Blimp1* in *Crx-Cre* mice occurring slightly later than in *Dkk3-Cre* mice.

What cells are these excess bipolar-like cells and proliferating cells in the *Blimp1* CKO retina derived from? One possibility is that these cells are autonomously derived from photoreceptor precursors in which Bimp1 is inactivated. The other possibility is that the deficiency of Blimp1 in photoreceptor precursors secondarily causes abnormal production of bipolar-like cells and/or proliferating cells from cells other than photoreceptor precursor cells.

To test these possibilities, we used the *Crx-LacZ* transgenic mouse line in which β-gal, under the control of the *2 kb-Crx* promoter, specifically labels photoreceptor precursor linage (Fig. 4*A*) (Furukawa et al., 2002). We generated *Blimp1*$^{flox/flox}$; *Dkk3-Cre*$^{+}$; *Crx-LacZ*$^{+}$ mice and analyzed their retinas by immunostaining at P1, P4, and P6. In the control retina (*Blimp1*$^{flox/flox}$; *Dkk3-Cre*$^{-}$; *Crx-LacZ*$^{+}$), most of the β-gal-positive cells are negative for the Chx10 signal (Fig. 4*B*,*F*,*J*). We found that β-gal and Chx10 double-positive cells increased in the *Blimp1* CKO retina (number of β-gal^{+} Chx10^{+} cells/100 μm at P4, 2.6 ± 1.7 in control, 40.6 ± 10.1 in CKO; $p < 0.03$) (Fig. 4*B*,*C*,*F*,*G*,*J*,*K*). Furthermore, β-gal and Ki67 double-positive cells also increased in the *Blimp1* CKO retina (number of β-gal^{+} Ki67^{+} cells/100 μm at P4, 9.6 ± 4.7 in control, 47.1 ± 18.3 in CKO; $p < 0.03$) (Fig. 4*D*,*E*,*H*,*I*,*L*,*M*). These results suggest that *Blimp1*-deficient photoreceptor precursors were converted to bipolar-like cells or proliferation marker-positive cells autonomously in the *Blimp1 Dkk3-Cre* CKO retina. In contrast, most rhodopsin-positive cells, which correspond to more mature photoreceptors than Crx-LacZ-positive cells, rarely overlap with the Chx10 signal either in the *Blimp1* CKO or control retinas (Fig. 3*E*,*F*).

It should be noted that we observed a significant number of Chx10-positive cells in the *Blimp1 CKO* retina at P6; however, we found no significant change in mature bipolar cell numbers between the control and *Blimp1 CKO* retinas at P14. One possible mechanism underlying this phenotype is the elimination of bipolar cells by apoptotic cell death after P6 in the *Blimp1* CKO retina. To test this possibility, we examined apoptotic cell death by active caspase3 staining and TUNEL assay in the *Blimp1* CKO and the control retinas (Fig. 5*A*–*M*). We stained apoptotic cells using the anti-active caspase3 antibody and counted the number of apoptotic cells in the retina at E15.5, E17.5, P1, P3, P6, P9, and P14. At all stages examined, we found the number of active caspase3-positive cells significantly increased (Fig. 5*K*). Consistent with this, TUNEL-positive cells also significantly increased in the *Blimp1* CKO retina (Fig. 5*M*). In addition, we separately counted the apoptotic cell numbers in the ONL, INL, and ganglion cell layer (GCL) at P6 and P9. We found that apoptotic cells increased in the ONL as well as in the INL of the *Blimp1* CKO retina (Fig. 5*L*). These results suggest that a subset of aberrant photoreceptors died of apoptosis in early development, and that overproduced bipolar-like cells and proliferating cells in the *Blimp1* CKO retina were eliminated by apoptotic cell death before maturation of bipolar cells. We also observed a significant increase of apoptotic cells in the early retina (Fig, 5*K*). Since rod photoreceptor production reaches its peak around P0 (Cepko et al., 1996), the increase of apoptotic cell death in the early *Blimp1* CKO retina is likely caused by the death of aberrant rod precursors.

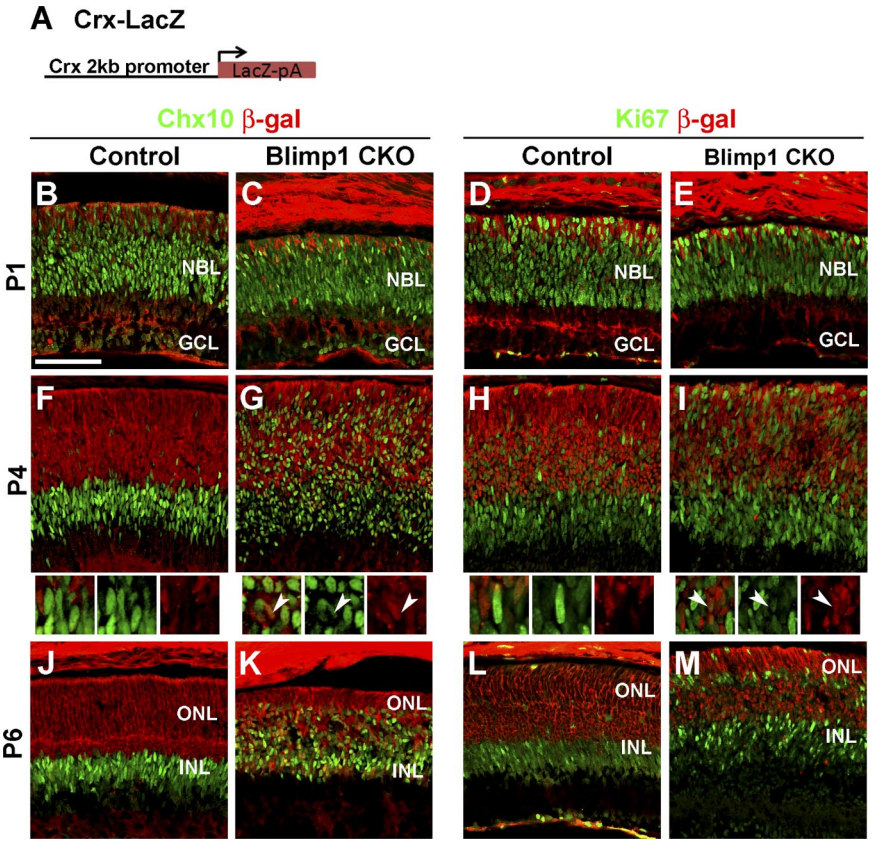

Figure 4. Linage-tracing analysis of *Blimp1*-deficient photoreceptor precursors using the *Crx-LacZ* mouse line. ***A***, The *Crx-LacZ* transgenic mouse line specifically expresses the β-gal reporter in the photoreceptor precursor linage under the control of the *2 kb-Crx* promoter. We generated *Blimp1*$^{flox/flox}$; *Dkk3-Cre*$^{+}$; *Crx-LacZ*$^{+}$ mice, and performed the linage-tracing analysis. ***B–M***, The retinal sections at P1 (***B–E***), P4 (***F–I***), and P6 (***J–M***) of control (***B***, ***D***, ***F***, ***H***, ***J***, ***L***) and *Blimp1* CKO mice (***C***, ***E***, ***G***, ***I***, ***K***, ***M***) were stained with anti-β-gal (red signal) and anti-Chx10 (green signal in ***B***, ***C***, ***F***, ***G***, ***J***, ***K***) or anti-Ki67 antibodies (***D***, ***E***, ***H***, ***I***, ***L***, ***M***, green signal). Arrowheads indicate Chx10 and β-gal double-positive cells (***G***, bottom), or Ki67 and β-gal double-positive cells (***I***, bottom). Scale bar, 50 μm. NBL, Neuroblastic layer.

Decrease of cone photoreceptors in the early *Blimp1* CKO retina

We observed a marked reduction of photoreceptor precursors in the *Blimp1* CKO retina at P6. Most photoreceptors generated at this stage are rods. In contrast, the majority of cone photoreceptors are generated during the first wave of retinogenesis during embryonic stages (Cepko, 1996). To investigate whether Blimp1 function affects cone development, we examined the *Blimp1* CKO retina at E14.5. We stained cone precursors in the embryonic retina using the anti-Trb2 antibody. We found that the number of Trb2-positive cells significantly decreased in the *Blimp1* CKO retina at E14.5 (number of Trb2^{+} cells/100 μm, 14.7 ± 2.6 in control, 7.4 ± 0.4 in CKO; $p < 0.03$) (Fig. 3*G*,*H*).

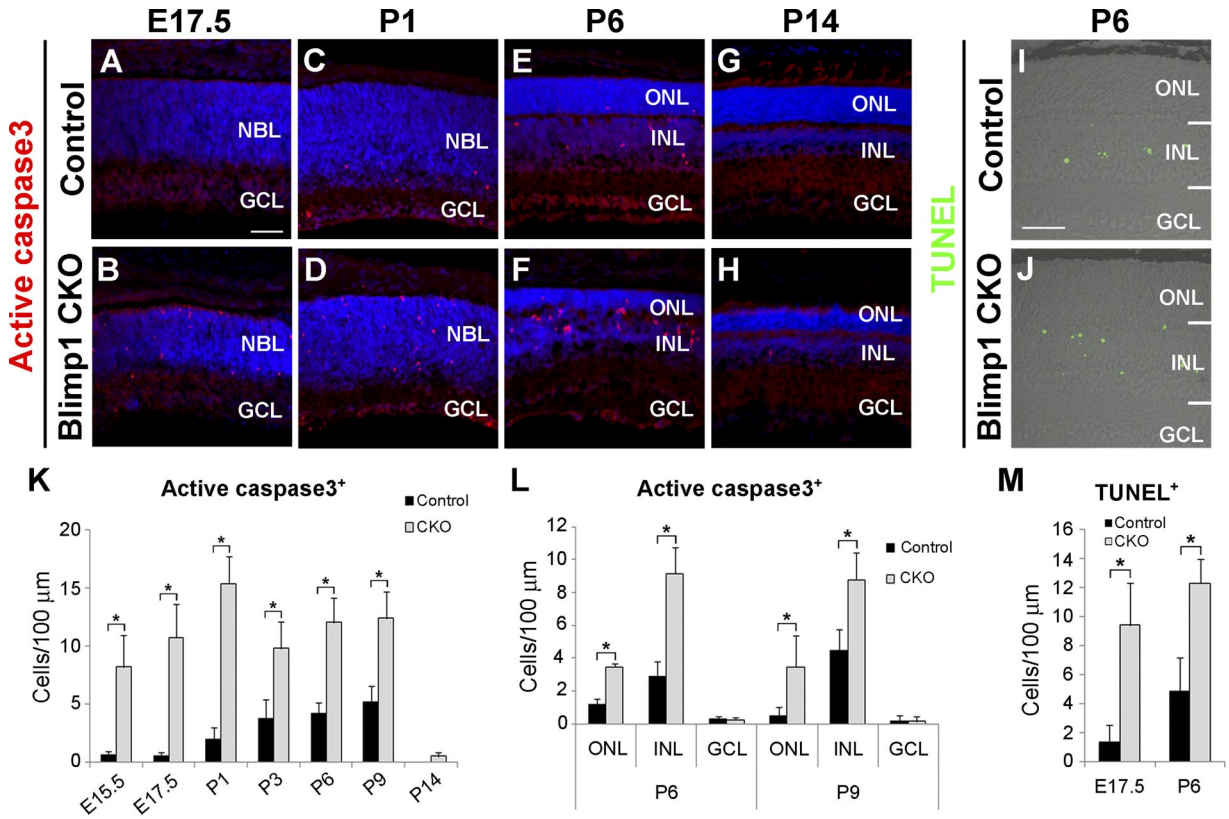

Figure 5. Significant increase of retinal cell death in the developing *Blimp1* CKO retina. ***A–H***, Apoptotic cells were stained with anti-active caspase3 (red signal) in the control (***A***, ***C***, ***E***, ***G***) and *Blimp1* CKO (***B***, ***D***, ***F***, ***H***) retinas at E17.5 (***A***, ***B***), P1 (***C***, ***D***), P6 (***E***, ***F***), and P14 (***G***, ***H***). Nuclei were stained by DAPI (blue signal). ***I***, ***J***, TUNEL staining of P6 retinas from control (***I***) and *Blimp1* CKO (***J***) mice. ***K–M***, Cell numbers of active caspase3-positive cells (***K***, ***L***) and TUNEL-positive cells (***M***) in the control and *Blimp1* CKO retinas were counted. $*p < 0.03$. Scale bars: 50 μm. NBL, neuroblastic layer.

This result in the embryonic retina is consistent with a reduction of mature cones at later stages (Fig. 2*I–L*,*W*). In addition, we examined whether Blimp1 deficiency affects embryonic retinal cell fate choice by immunostaining using cell proliferation and cell type-specific markers. We observed that the numbers of BrdU- and pH3-positive proliferating cells, Brn3b-positive ganglion precursors, Bhlhb5-positive amacrine precursors, and Islet1-positive cells (horizontal, amacrine, ganglion precursors) were unaltered between the wild-type and *Blimp1* CKO retinas (supplemental Fig. S4*A–J*,*M–Q*, available at www.jneurosci.org as supplemental material). In contrast, the apoptotic cells were significantly increased in the *Blimp1* CKO retina at E14.5 (supplemental Fig. S4*K*,*L*,*R*, available at www.jneurosci.org as supplemental material). We also observed that Trb2 and Chx10 double-positive cells significantly increased in the *Blimp1* CKO retina at E15.5 (supplemental Fig. S5*A*,*B*, available at www.jneurosci.org as supplemental material). These results show that Blimp1 deficiency affects cone photoreceptor development. The aberrant cone precursors in the *Blimp1* CKO retina appear to express Chx10 and die at this stage.

Gene expression profiles of the *Blimp1* CKO retina

To investigate the molecular mechanisms underlying the histological phenotype of the *Blimp1* CKO retina at P6, we analyzed a gene expression profile of the *Blimp1* CKO retina using a microarray containing 45,101 probe sets covering >34,000 genes. We identified 222 downregulated probe sets (signal log ratio, less than or equal to −1.0; signal intensity, ≥100) and 218 upregulated probe sets (signal log ratio, ≥1.0; signal intensity, ≥100) in the *Blimp1* CKO retina, compared with the control retina (the lists of top 20 probes in each group are shown in supplemental Tables S1, S2, available at www.jneurosci.org as supplemental material). Consistent with the results of immunohistochemical analysis, we observed several photoreceptor-specific genes (e.g., *Arr3*, *Pde6h*, and *Rhbdf2*) in these downregulated genes in the *Blimp1* CKO retina. We also identified several bipolar cell-related genes (e.g., *Scgn*, *Vsx1*, and *Cabp5*) in the upregulated genes in the *Blimp1* CKO retina. To confirm the differential expression of genes involved in bipolar and photoreceptor development in the *Blimp1* CKO retina, we selected 11 genes involved in bipolar cell development and 10 genes involved in photoreceptor cell development, and assayed the expression levels of these genes in the *Blimp1* CKO retina at P6 by Q-PCR analysis. Similar to the result of the microarray analysis, we found that the expression of several genes involved in bipolar development (*Chx10*, *Vsx1*, *Lhx3*, *Lhx4*, *Prox1*, *Islet1*, and *Scgn*) were notably upregulated (twofold to fivefold) in the *Blimp1* CKO retina at P6 (Fig. 6*A*). In contrast, several genes involved in photoreceptor development [*Crx*, *Nrl* (*neural retina leucine zipper*), *Rbp3*, *NeuroD1*, *Thrb*, *Arr3*, and *Sag*] were significantly downregulated (by 50 to 80% from control) in the *Blimp1* CKO retina (Fig. 6*B*). These results suggest that loss of *Blimp1* in the developing photoreceptors leads to a downregulation of photoreceptor-related gene expression and to an upregulation of bipolar cell-related gene expression.

Blimp1 can directly downregulate *Chx10* transcription

What is the molecular mechanism underlying the overproduction of Chx10-positive cells and proliferating cells in the *Blimp1* CKO retina? Ectopic expression of Chx10 has been shown to increase the number of bipolar-like cells and reduce the number of photoreceptors (Livne-Bar et al., 2006). This phenotype is par-

tially similar to our observed phenotype in the developing *Blimp1* CKO retina. In fact, we observed an increased number of Chx10-positive cells in the *Blimp1* CKO retina. Blimp1 has been known to direct gene regulation during cell differentiation through the recruitment of interacting factors to specific sites (Gyory et al., 2004). Based on these observations, we focused on *Chx10* as one of the putative targets of the Blimp1 transcription factor in the developing retina. We hypothesized that Blimp1 might suppress *Chx10* expression in the photoreceptor precursors and prevent their cell fate change toward the bipolar cells.

To examine whether or not Blimp1 can directly suppress *Chx10* enhancer activity, we performed a luciferase reporter assay using the *Chx10* enhancer and the *Blimp1* expression vector (Fig. 6*C*,*D*). A previous study showed that a 2.4 kb region upstream of the mouse *Chx10* locus was sufficient to drive reporter expression in bipolar cells and proliferating progenitor cells in transgenic mouse lines (Rowan and Cepko, 2005) (Fig. 6*C*, region A). We identified at least two Blimp1-binding consensus sequences AGNGAAAG (Kuo and Calame, 2004; Ancelin et al., 2006) in this region, called region A. In addition, we searched other possible Blimp1-binding consensus sequences in the *Chx10* locus between −10 kb and +26 kb, and identified two more regions (regions B and C) containing at least two (region B) and four (region C) Blimp1-binding consensus sequences GGGAAAG or AGNGAAAG, respectively. Region B is located ~7 kb upstream of the *Chx10* gene, and region C is localized ~12 kb downstream of the transcriptional starting site of the *Chx10* gene. All of these regions are relatively well conserved among mammalian *Chx10* genes.

To test whether or not Blimp1 is able to regulate transcription through these regions, we performed a luciferase reporter assay by transfecting both a *Blimp1* expression plasmid and a luciferase reporter vector containing each of the three candidate regulatory regions of the *Chx10* gene, separately. We observed a significant dose-dependent repression of the reporter gene by Blimp1 in all three constructs that we tested (Fig. 6*D*). To confirm the binding of Blimp1 to these regions *in vivo*, we performed a ChIP assay using an anti-Blimp1 antibody on P3 mouse retinal tissues. We found that Blimp1 specifically immunoprecipitated selected sequences in regions A and B; however, we did not detect a significantly amplified band in the ChIP assay for region C (Fig. 6*E*). These results suggest that Blimp1 is a direct upstream regulator of *Chx10* via binding to the consensus sequences in the *Chx10* upstream region.

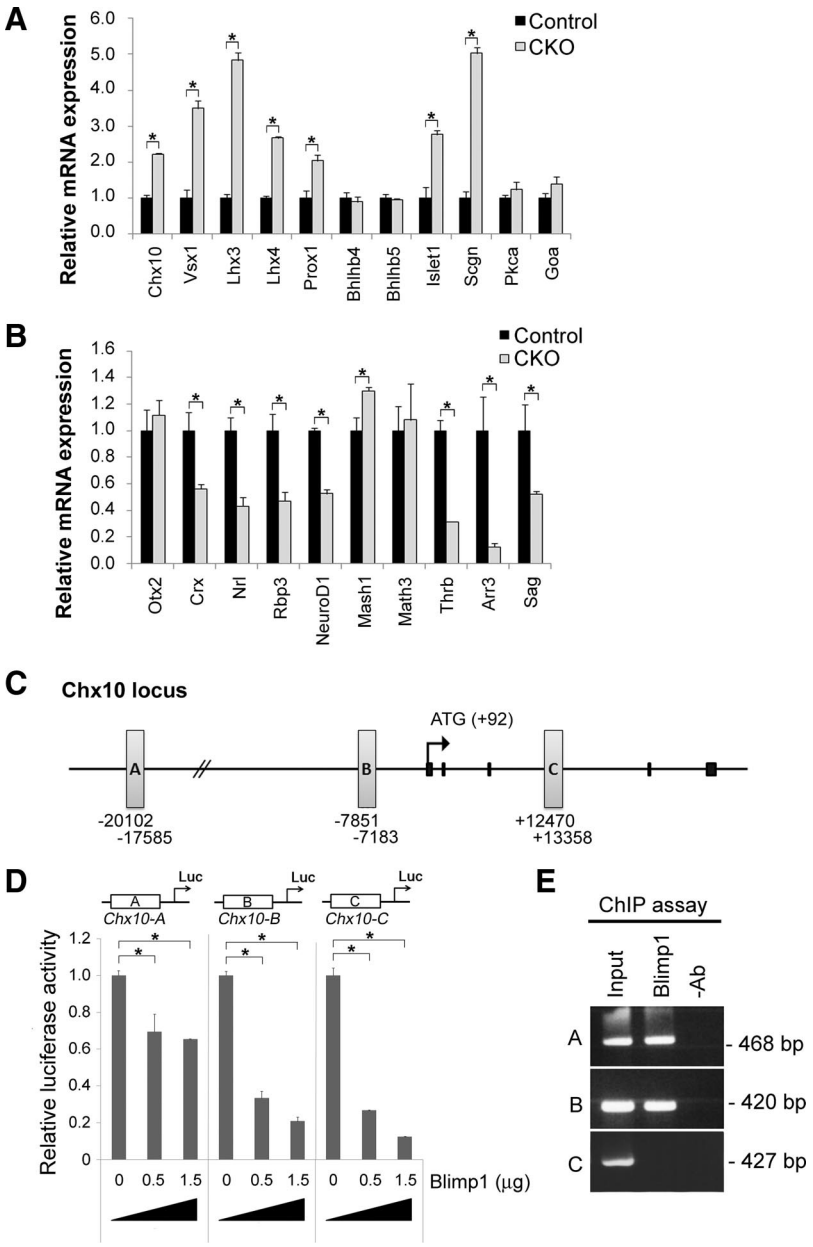

Figure 6. Gene expression profiling of the *Blimp1* CKO retina by Q-PCR and direct regulation of *Chx10* promoter by Blimp1. ***A***, ***B***, Expression levels of selected genes involved in bipolar (***A***) and photoreceptor (***B***) development were analyzed by Q-PCR. Expression levels of genes were normalized to the expression level of a housekeeping gene, *GAPDH*. Several genes involved in bipolar cell development were upregulated (***A***) in the *Blimp1* CKO retina. Several genes involved in photoreceptor development were downregulated in the *Blimp1* CKO retina (***B***). The mean value of each control was set as 1.0. Error bars show the SD ($n = 4$ from 7 retinas). *$p < 0.03$. ***C***, Schematic diagram of three potential Blimp1-binding regions (shown as A, B, and C) found on 5′ upstream (regions A and B) and downstream (region C) of the transcriptional start site (arrow) of the mouse *Chx10* gene. ***D***, Dose-dependent repression by Blimp1 of three possible *Chx10* regulatory regions. Neuro2A cells were transfected with reporter plasmids containing region A, B, or C along with various amounts of the *Blimp1* expression plasmid. The results were normalized against the *Renilla* luciferase activities obtained from the *pRL-TK* plasmid as an internal control. Error bars show the SD ($n \geq 3$). *$p < 0.03$. ***E***, Interaction of endogenous Blimp1 with genomic DNA of the *Chx10* locus by ChIP assay. Cell extracts (Input) from isolated P3 retinas were immunoprecipitated with (Blimp1) or without (−Ab) the anti-Blimp1 antibody. Three putative Blimp1-binding regions (regions A–C) were amplified by PCR.

Overexpression of Blimp1 by electroporation inhibits bipolar genesis in the developing retina

To test whether Blimp1 is sufficient to suppress bipolar cell development, likely by suppressing *Chx10* expression, we forced the expression of Blimp1 in the developing mouse retina by *in vivo* electroporation (Fig. 7). We electroporated both the *Blimp1* ex-

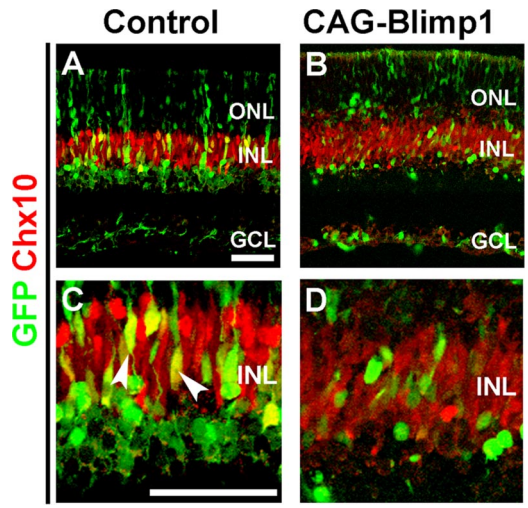

Figure 7. Overexpression of *Blimp1* suppresses bipolar cell genesis in the developing mouse retina. ***A–D***, Immunohistochemical analysis of P6 retinas electroporated *in vivo* at P0 with *CAG-GFP* (***A***, ***C***) and *CAG-Blimp1* (***B***, ***D***) vectors. Bipolar cells were stained with the anti-Chx10 antibody (red). ***C***, ***D***, Higher-magnification images focused on the INL. White arrowheads point to Chx10-expressing electroporated cells. Scale bars: 50 μm.

pression vector and a *GFP* expression vector together, or a *GFP* expression vector alone as a control into P0 mouse retinas. At P6, when the most significant phenotype was observed in the *Blimp1* CKO retina, transfected retinas were immunostained with the anti-Chx10 antibody and analyzed by confocal microscopy. Identification of cell types was determined by the characteristic morphologies and locations of cells. The majority of electroporated cells at P0 are rod precursors, which natively express Blimp1. We actually observed that the number of GFP-positive cells in the ONL was not significantly altered between the *Blimp1* expression vector-electroporated (50.5 ± 9.2%) and control retinas (53.5 ± 5.0%) (Fig. 7*A*,*B*). In contrast, the Chx10 and GFP double-positive cells were markedly decreased in the *Blimp1*-transfected cells (20.7 ± 3.8% in control, 6.1 ± 5.2% in CAG-Blimp1; $p < 0.03$) (Fig. 7*A–D*). These results suggest that Blimp1 repressed bipolar cell development but not photoreceptor cell development. Consistent with the results from the loss of function experiments of Blimp1, forced expression of Blimp1 inhibited bipolar cell development in the developing retina.

Discussion

Regulation of *Blimp1* expression in differentiating photoreceptor precursor cells

In the present study, we investigated the expression and function of the Blimp1 transcription factor in the developing retina. To explore *Blimp1* expression in detail, the *Blimp1-mVenus* transgenic mouse line has been used (Ohinata et al., 2008). By taking advantage of the *Blimp1-mVenus* transgenic line, we found that *Blimp1* is expressed mostly in postmitotic photoreceptor precursors, suggesting that Blimp1 may play a significant role downstream of the Otx2 transcription factor in photoreceptor precursor differentiation. In the current study, we showed *Blimp1* expression in the embryonic retina begins as early as E11.5, when *Otx2* begins to be expressed as well (Nishida et al., 2003); however, *Otx2* expression was unaltered in the *Blimp1* CKO retina. These results suggest that *Blimp1* expression is regulated by Otx2, but *Otx2* expression is not regulated by Blimp1. Unlike *Otx2*, whose expression is known to begin in the late S to early G2 phase of the last cell cycle in retinal progenitor cells (Trimarchi et al., 2008a,b), our study showed that *Blimp1* expression was mainly observed in postmitotic photoreceptor precursor cells. Only a very limited number of Blimp1-mVenus-positive cells overlap with Ki67, suggesting that even if *Blimp1* begins expression in mitotic cells, it is likely to be expressed from the last step of the cell cycle.

Functional role of Blimp1 in photoreceptor development

We demonstrated that lack of *Blimp1* results in decreased numbers of both cone and rod photoreceptor cells. Moreover, Q-PCR and microarray analysis of the control and the *Blimp1* CKO retinas also showed that expression of many photoreceptor-related genes except *Otx2* were downregulated in the *Blimp1* CKO retina. These observations suggest that although initial photoreceptor cell fate is determined by Otx2, approximately half of photoreceptor precursors did not develop normally in the *Blimp1* CKO retina. On the other hand, we found that both Chx10-positive bipolar-like cells and proliferation marker-positive cells were notably increased around at P6, and at the same time, cell death was enhanced in the *Blimp1* CKO retina.

How does the lack of *Blimp1* in photoreceptor precursors induce the overproduction of bipolar-like cells? Several lines of evidence suggest that Blimp1 ablation results in a direct cell fate change from photoreceptor precursors to bipolar-like cells. We demonstrated by reporter assay that Blimp1 can bind to *Chx10* enhancer regions and repress *Chx10* expression. Moreover, *in vivo* electroporation of *Blimp1* also presented the possibility that Blimp1 blocks bipolar cell development. Intriguingly, the *Crx-LacZ* tracer *Blimp1* CKO mouse (*Blimp1*$^{flox/flox}$; *Dkk3-Cre*$^{+}$; *Crx-LacZ*$^{+}$) showed that the β-gal signal partially merged with Chx10, suggesting that Crx-positive postmitotic photoreceptor precursors aberrantly expressed Chx10. We showed previously that strong Otx2 expression is shifted from photoreceptors to bipolar cells after birth (Koike et al., 2007). It was speculated that Otx2 expressed in bipolar cells could promote bipolar cell fate determination by increasing Chx10 expression (Kim et al., 2008). In normal retinal development, Chx10 expression is lost in postmitotic cells, except for bipolar cells and a subset of Müller glia (Rowan and Cepko, 2004). However, in the *Blimp1* CKO retina, some photoreceptor precursors that originally express *Otx2* may aberrantly upregulate *Chx10* through escaping suppression by Blimp1, and therefore these cells are driven to become bipolar-like cells. Since these cells have already executed a program toward becoming photoreceptors, many of these cells may differentiate into neither photoreceptors nor bipolar cells, and subsequently they are eliminated by apoptosis. How does the loss of Blimp1 in photoreceptor precursors cause an aberrant increase of proliferating cells? We found that PCNA-positive proliferating cells increased in the *Blimp1* CKO retina. We also observed that proliferating cells were ectopically localized in the OPL region in the *Blimp1* CKO retina at P6. Indeed, Blimp1 is known to regulate proliferation in several tissues by repressing expression of cell-cycle regulators (Lin et al., 1997, 2000; Horsley et al., 2006). Blimp1 might function to ensure cell cycle exit and/or prevent newly generated photoreceptor precursor cells from reentering the cell cycle. It has been reported previously that ectopic expression of cyclin D1 or E2F1 in rods results in the ectopic increase of proliferating cells and cell death (Howes et al., 1994; Lin et al., 2001). Furthermore, in the *Rb*$^{-/-}$; *p107*$^{+/-}$; *p130*$^{-/-}$ retina, even mature horizontal cells were shown to reenter the cell cycle (Ajioka et al., 2007). Some of these cell cycle reentry cells exit the cell cycle again later in development and differentiate either into photoreceptors or bipolar cells, both of which express *Otx2*. At

this time point, differentiating progenitors tend to become bipolar cells at a higher rate than they become photoreceptors, since later progenitors produce more bipolar cells than photoreceptors during postnatal retinal development. Given that *Otx2* expression begins in the late S to early G2 phase of the last cell cycle in retinal progenitor cell (Trimarchi et al., 2008a,b), and that a small population of retinal progenitor cells may also express *Blimp1* just after *Otx2* expression in mitotic progenitors, there is a possibility that *Blimp1* deficiency lead to an overproliferation of progenitor cells rather than reentry into the cell cycle. In addition, we observed an increase of β-gal and Ki67 double-positive cells in the *Crx-LacZ* tracer *Blimp1* CKO mice. This result also supports the idea that excess proliferating cells in the *Blimp1* CKO are derived from aberrant photoreceptor precursors.

Again, the possible role of Blimp1 in the repression of Chx10 may account for the mechanism of overproduction of proliferating cells in the *Blimp1* CKO retina. In the embryonic retina, Chx10 is essential for progenitor cell proliferation, and a defect of Chx10 in the retinal progenitor cells causes microphthalmia in both mice and humans (Burmeister et al., 1996; Ferda Percin et al., 2000). It is possible that loss of Blimp1 in the photoreceptor precursors causes a failure to repress Chx10 expression, and that aberrant Chx10 expression in the photoreceptor precursors might prevent proper cell cycle exit or cause abnormal cell cycle reentry of photoreceptor precursors.

In the adult CNS, it has been reported that failure of cell-cycle arrest results in the reexpression of various cell cycle proteins such as cyclins, cyclin-dependent kinases, and PCNA in neurons from patients with various neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and stroke (Vincent et al., 1996; Nagy et al., 1997; Jordan-Sciutto et al., 2003; Love, 2003). These studies suggest that mature neurons need to continuously suppress their cell cycle. Together with these studies, the phenotypes observed in the current study might contribute to understanding of the mechanism by which postmitotic neurons regulate the cell cycle machinery.

Thus, our results in this study support the hypothesis that in the *Blimp1* CKO retina cell fate-committed photoreceptor precursors autonomously change their cell fate to bipolar-like cells in the majority of the cell population, or to proliferating cells in a small number of cells, and

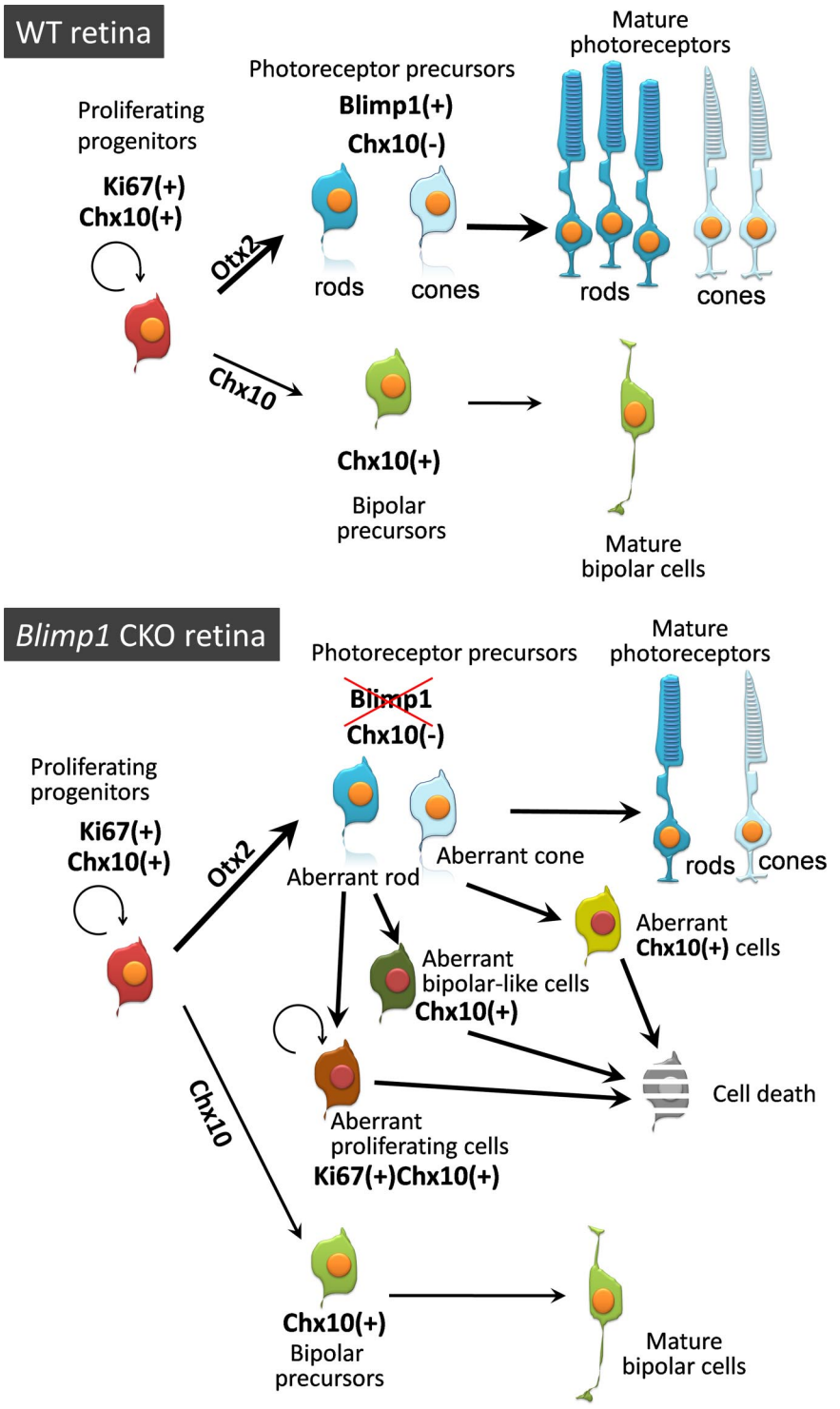

Figure 8. A hypothetical model of the functional mechanism of Blimp1. In this hypothetical model, Blimp1 is expressed in the both rod and cone photoreceptor precursors and ensures photoreceptor cell fate in the wild-type (WT) developing retina (top). In the *Blimp1* CKO retina (bottom), loss of *Blimp1* produces some proportion of rod photoreceptor precursors aberrantly expressing Chx10, resulting in overproduction of bipolar-like cells and proliferating retinal cells. Some proportion of aberrant rod photoreceptors appear to undergo cell fate switch to become bipolar-like cells. The improper cell cycle exit and/or reentry of aberrant photoreceptor precursors into the cell cycle might contribute to the increase of proliferating cells in the *Blimp1* CKO retina. The excess bipolar-like cells and proliferating cells derived from abnormal photoreceptor precursors are eliminated by apoptosis. Some proportion of cone precursors in the *Blimp1*-null retina aberrantly express Chx10 and die at embryonic stages.

then these abnormal cells die by apoptosis. Blimp1 seems to ensure the cell fate determination of photoreceptor precursors and prohibit aberrant cell fate choice or abnormal cell cycle regulation in the developing retina (Fig. 8).

In the present study, we showed that Blimp1 plays an essential role in photoreceptor development and identified *Chx10* as one of the possible targets of Blimp1. However, approximately half of the normal number photoreceptor cells were generated in the *Blimp1* CKO retina. One possibility is that another family member or unknown factor compensated for the loss of *Blimp1*, although we did not detect any significant upregulation of other Prdm family members in the *Blimp1* CKO retina in our microarray analysis of the control and the *Blimp1* CKO retinas. It should be noted that remaining photoreceptors expressed rhodopsin or cone opsins; however, their outer segments were significantly shorter than those of the control photoreceptors.

Very recently, Brzezinski et al. (2010) independently reported retinal phenotypes in *Blimp1* CKO mice using an *αPax6-Cre* line, which expresses Cre in retinal progenitors in the peripheral part of the retina. Consistent with our results, they showed an increase of bipolar-marker positive cells instead of a decrease of photoreceptor precursors in the *Blimp1* CKO retina. In contrast to our result, they did not mention the increase of proliferating cells in the developing *Blimp1* CKO retina. They mentioned excess Sox9-positive Müller glial cells in the *Blimp1* CKO retina. We also found increased Sox9-positive cells in the *Blimp1* CKO; however, these cells at least partially colocalized with the cell proliferation marker, suggesting that Sox9-positive cells are progenitor-like cells rather than Müller glial cells (supplemental Fig. S6, available at www.jneurosci.org as supplemental material). This may require additional analysis in future.

It has been reported that Blimp1 functions with histone methyltransferases (G9a, Prmt5), histone demethylase (LSD1), histone deacetylases, and transcriptional cofactors (Groucho family protein) by recruiting its multiple domains in a context-dependent manner (Ren et al., 1999; Yu et al., 2000; Gyory et al., 2004; Ancelin et al., 2006; Su et al., 2009). Whether or not epigenetical regulation by Blimp1 plays a role in retinal photoreceptor development awaits future analysis.

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