Blimp1 controls photoreceptor versus bipolar cell fate choice during retinal development

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SUMMARY
Photoreceptors, rods and cones are the most abundant cell type in the mammalian retina. However, the molecules that control their development are not fully understood. In studies of photoreceptor fate determination, we found that Blimp1 (Prdm1) is expressed transiently in developing photoreceptors. We analyzed the function of Blimp1 in the mouse retina using a conditional deletion approach. Developmental analysis of mutants showed that Otx2+ photoreceptor precursors ectopically express the bipolar cell markers Chx10 (Vsx2) and Vsx1, adopting bipolar instead of photoreceptor fate. However, this fate shift did not occur until the time when bipolar cells are normally specified during development. Most of the excess bipolar cells died around the time of bipolar cell maturation. Our results suggest that Blimp1 expression stabilizes immature photoreceptors by preventing bipolar cell induction. We conclude that Blimp1 regulates the decision between photoreceptor and bipolar cell fates in the Otx2+ cell population during retinal development.

KEY WORDS: Blimp1 (Prdm1), Retina, Cell fate determination, Photoreceptor, Rod, Cone, Bipolar cell, Mouse

INTRODUCTION
The retina comprises seven primary cell types: rod and cone photoreceptors, amacrine cells, retinal ganglion cells (RGCs), horizontal cells, bipolar cells and Müller glia. These cells are formed from a common pool of retinal progenitor cells during development and are born (exit the cell cycle) in a characteristic, but overlapping, order (Livesey and Cepko, 2001; Rapaport et al., 2004). The molecular mechanisms that control retinal cell fate determination involve both cell-intrinsic and extrinsic factors (Livesey and Cepko, 2001).

Photoreceptors are the most numerous cells in the mammalian retina and are responsible for detecting light stimuli. Cones are born early in mouse retinal development, starting around embryonic day (E) 12 and finishing by parturition (Carter-Dawson and LaVail, 1979). Rods are born starting at ~E13.5 in mice, with the bulk formed postnatally, finishing around postnatal day (P) 7 (Carter-Dawson and LaVail, 1979). Previous studies have identified several key transcription factors required for photoreceptor fate specification and differentiation. Otx2 is a homeodomain transcription factor that is expressed in early and mature photoreceptors and bipolar cells (Fossat et al., 2007; Nishida et al., 2003). Loss of Otx2 from the retina prevents the development of these cell types, the cells adopting amacrine fate instead (Nishida et al., 2003; Sato et al., 2007). These data show that Otx2 is required for bipolar cell genesis, but for differentiation (Chow et al., 2001; Livne-Bar et al., 2006).

Bipolar cells (Burmeister et al., 1996) and is necessary for bipolar generation as Chx10-null retinas have few, if any, bipolar cells (Burmeister et al., 1996; Green et al., 2003; Livne-Bar et al., 2006). Vsx1 is expressed in a subset of cone bipolar cells and is not required for bipolar cell genesis, but for differentiation (Chow et al., 2001; Chow et al., 2004; Clark et al., 2008; Ohtoshi et al., 2001; Ohtoshi et al., 2004).

Photoreceptors and bipolar cells are similar in that they both express Otx2. This raises the question of how the Otx2+ population chooses between these cell fates during development. This is likely to be mediated through differential expression of transcription factors within the Otx2+ population. We examined the role of the PR-SET domain zinc-finger transcription factor Blimp1 (Prdm1) in more detail, based on preliminary expression characterization in the retina (Chang and Calame, 2002; Hsiau et al., 2007; Wang et al., 2008). Blimp1 can recruit Groucho family co-repressors and...
histone-modifying machinery to repress transcription (Anclin et al., 2006; Hayashi et al., 2007; Ren et al., 1999; Yu et al., 2000). Blimp1 (B-lymphocyte-induced maturation protein) is required for B-cell maturation into plasma cells (Shapiro-Shelef et al., 2003; Turner et al., 1994) and is also important for T-cell, skin, vibrissae, pharyngeal arch, muscle, neural crest, limb, heart and primordial germ cell development (John and Garrett-Sinha, 2009). Here, we determined that Blimp1 is transiently expressed in developing photoreceptors. We generated conditional loss-of-function mice and show that Blimp1 mutants have fewer photoreceptors and more bipolar cells than wild-type mice. Blimp1 mutants have normal numbers of Otx2+ cells during development, but most of them develop as bipolar cells instead of photoreceptors, providing evidence for a one-to-one fate shift. After the first postnatal week, many of these excess bipolar cells die, yielding an adult retina with ~50% more bipolar cells than normal. Our results show that Blimp1 controls the choice between photoreceptor and bipolar cell fates during development by repressing bipolar cell-specific gene expression in Otx2+ cells.

**MATERIALS AND METHODS**

**Mice**

For wild-type Blimp1 characterization, tissues were collected from C57BL/6 mice. For conditional knockout mice (CKO), two different Cre lines were used: Foxg1-Cre (Hebert and McConnell, 2000) and αPax6-Cre-GFP (Marquardt et al., 2001). These lines were crossed to Cre::Blimp1Flox/+. These lines were crossed to Blimp1Flox/+. These lines were crossed to Cre::Blimp1Flox/+. The populations used for analysis: Cre::Blimp1Flox/+ controls and Cre::Blimp1Flox/+. Mice were used in accordance with University of Washington IRB approved protocols.

**Human fetal tissue**

Tissues from 74, 96, 115 and 163 day post-conception fetuses, without identifiers, were obtained from the University of Washington Birth Defects Laboratory. The donated fetal tissue was collected from non-diseased populations used for analysis: Cre::Blimp1Flox/+ controls and Cre::Blimp1Flox/+. Mice were used in accordance with University of Washington IACUC approved protocols.

**Immunohistochemistry and microscopy**

Tissues of various stages were fixed in 2% paraformaldehyde for 15 minutes to 2 hours at room temperature. Prior to fixation, the lens was removed from postnatal eyes. Tissues were cryopreserved through 30% sucrose, frozen in OCT (Sakura, Torrance, CA, USA) and sectioned at ~10 μm. We found that Blimp1, Trβ2 and NeuroD1 immunostaining was fixation sensitive. A shorter fixation time resulted in more robust staining. Sections were blocked with milk-block (the supernatant from a solution of 5% dried milk powder and 0.5% Triton X-100 in PBS spun for 15 minutes at 11,750 g in a microcentrifuge) for 2-4 hours at room temperature. Primary antibodies were diluted in milk-block solution and incubated with the sections overnight. The sections were washed in PBS and incubated with appropriate fluorescent conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) or streptavidin conjugate (Invitrogen) diluted in milk-block solution for 2 hours along with DAPI to counterstain nuclei. The sections were washed and mounted for microscopy.

Primary antibodies were as follows: mouse anti-Blimp1 (1:100) (NB600-235, Novus, Littleton, CO, USA), rat anti-Blimp1 (1:100) (sc47732, Santa Cruz, Santa Cruz, CA, USA), goat anti-Bmi1 (1:50) (sc6029, Santa Cruz, rabbit anti-calbindin D-28K (Calb1) (1:500) (ab1778, Millipore, Billerica, MA, USA), rabbit anti-cleaved caspase 3 (1:200) (555955, BD Biosciences, San Jose, CA, USA), sheep anti-Chx11 (1:200) (X1179P, Exalpa, Shirley, MA, USA), mouse anti-CLALBP (1:250) (ab15051, Abcam, Cambridge, MA, USA), rabbit anti-CrX (1:100) (sc30150, Santa Cruz), chicken anti-GFP (1:500) (ab13970, Abcam), goat anti-NeuroD1 (1:50) (sc1084, Santa Cruz), rabbit anti-Olig2 (1:250) (ab33427, Abcam), goat anti-Otx2-biotin (2.5 μg/ml) (BAF1797, R&D Systems, Minneapolis, MN, USA), rabbit anti-Otx2/CrX (1:1000) (a gift of Cheryl Craft, University of California, Los Angeles) (Zhu and Craft, 2000), rabbit anti-m-opsin (Opn1mw) (1:250) (ab5405, Millipore), goat anti-s-opsin (Opn1sw) (1:150) (sc14363, Santa Cruz), rabbit anti-Pax6 (1:500) (PRB-278P, Covance, Princeton, NJ, USA), mouse anti-PKC (1:750) (P5704, Sigma, St Louis, MO, USA), rabbit anti-recoverin (1:1000) (ab5585, Millipore), mouse anti-rhodopsin (1:500) (a gift of Robert Molday, University of British Columbia) (Laird and Molday, 1988), goat anti-Sox2 (1:1000) (sc17320, Santa Cruz), rabbit anti-Sox9 (1:750) (ab5535, Millipore), rabbit anti-Trβ2 (1:500) (a gift of Douglas Forrest, NIDDK) (Ng et al., 2001) and rabbit anti-Vsx1 (1:250) (a gift of Ed Levine, University of Utah) (Clark et al., 2008). The rat Blimp1 antibody worked much better than the previously published mouse antiserum (Chang and Calame, 2002) and was used for all the images shown. For Otx2 immunostaining we used fluorescent streptavidin conjugates instead of secondary antibodies because this resulted in a cleaner signal.

Images were captured using a Zeiss (Thornwood, NY, USA) LSM 510 confocal microscope or a Nikon (Melville, NY, USA) A1 confocal microscope. Images were used as single planes or as z-stack projections. Images were processed in Adobe Photoshop (San Jose, CA, USA).

**RT-PCR**

For relative Blimp1 expression, we collected retinas in Trizol (Invitrogen) from BL/6 mice from several time-points and made three pools of RNA. RNA was prepared following the Trizol protocol and cleaned up using the RNeasy Kit (Qiagen, Germantown, MD, USA) according to the manufacturer’s instructions. Each pool of RNA was treated with DNase, reverse transcribed with Superscript II (Invitrogen), and then subjected to RT-PCR twice using a DNA Engine Opticon and Opticon Monitor software (BioRad, Hercules, CA, USA) with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Primer sequences: for Blimp1, 5′-GAACCTGCTTTTAAGTATGCTG-3′ and 5′-TCCATAGGCGGTA-AATCAA-3′. Mouse Blimp1 was normalized to Gapdh values and relative expression was determined by normalizing each point to the average E12.5 value. Data were plotted using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Blimp1-GFP plasmid construction and in vivo electroporation

Human BLIMP1 cDNA was acquired from Tom Maniatis (Ren et al., 1999) and subcloned into the pMES expression vector (Swartz et al., 2001) to generate pMES-BLIMP1 (Blimp1-GFP). The pMES backbone contains chicken β-actin and CMV-1E promoter and enhancer sequences followed by a multiple cloning site and IRES2-EGFP sequences. For control electroporations, unmodified pMES (GFP) was used. For electroporations, these plasmids were mixed (2 to 1) with pCAGGS (Niwa et al., 1991), a vector that robustly drives GFP expression, in order to better visualize transfected cells over time, as the pMES and pMES-BLIMP1 vectors were not as abundantly expressed after 4 days in neurons (data not shown). For electroporations, P1 mice were anesthetized and a small cut was made to pull the eyelids apart. A 30-gauge needle was used to make a hole in the vitreous near the limbus of one eye. A pulled-glass micropipette containing the plasmid mix was inserted through the hole and ~1 μl (~6 μg) of DNA injected. Tweezertrodes (BTX, Holliston, MA, USA) were placed on either side of the head such that the positive electrode was opposite the injected eye. The eyes were electroporated with a BTX T-820 electroporator with the following parameters: 90 V olts, five pulses, 50 milliseconds per pulse. Eyes were collected 4 days later at P5 for histology. Approximately 80% of the electroporated eyes had transfected cells in the retina and ~25% had large patches of transfected cells, which were used for counting.
RESULTS

Blimp1 is expressed in developing photoreceptors

Previous experiments have shown that Blimp1 is expressed in the embryonic and early postnatal retina of rodents (Chang and Calame, 2002; Hsiau et al., 2007; Wang et al., 2008). Although its expression pattern was consistent with developing photoreceptors, this was not explicitly characterized. To more fully describe the Blimp1 expression domain during retinal development, we immunostained sections from multiple developmental time-points.

We first observed Blimp1 protein in the nucleus of cells in the central retina at E12. These Blimp1+ cells always co-expressed Otx2, a marker of developing photoreceptors and retinal pigmented epithelium (RPE) (Fossat et al., 2007; Nishida et al., 2003) (Fig. 1A). However, not all Otx2+ cells in the retina expressed Blimp1, nor did any Blimp1+ cells co-express Brn3 (Pou4F2 – Mouse Genome Informatics), an RGC marker (Xiang et al., 1995). We also observed that Blimp1 labeled vascular endothelial cells in the vitreous (Fig. 1A,E,F). At E14.5, the Blimp1 expression domain spanned from the central to peripheral retina, similar to what has been observed previously (Chang and Calame, 2002). The intensity of Blimp1 labeling was variable, but again, every Blimp1+ cell co-expressed Otx2 (Fig. 1B, Table 1). Crx is another photoreceptor-specific marker at E14.5 (Chen et al., 1997; Furukawa et al., 1997; Nishida et al., 2003). Every Crx+ cell co-expressed Blimp1 (Fig. 1C) and Otx2 (data not shown). However, there were more Blimp1+ cells than Crx+ cells and more Otx2+ cells than Blimp1+ cells (Table 1). Together, this showed that Blimp1 is expressed in developing photoreceptors and implied that Otx2 is expressed before Blimp1, which is expressed before Crx. Blimp1+ cells rarely co-expressed the progenitor marker Sox9 (Poche et al., 2008) (Fig. 1D, Table 1) and always co-expressed NeuroD1, which is expressed in postmitotic, developing photoreceptors and amacrine cells (Liu et al., 2008; Morrow et al., 1999) (Fig. 1E). Developing cones, labeled by Trp2 (Ng et al., 2001), always co-expressed Blimp1, but accounted for only ~60% of Blimp1+ cells at this stage (Fig. 1F, Table 1). This indicated that Blimp1 is expressed in both nascent postmitotic rods and cones at E14.5. We then examined the expression of Pax6 and Olig2, transcription factors that are expressed in both progenitors and postmitotic cells (de Melo et al., 2003; Nakamura et al., 2006). Pax6 labels progenitors weakly and postmitotic amacrine and RGCs intensely (de Melo et al., 2003). Although we observed no intensely labeled Blimp1+/Pax6+ cells, a small number of Blimp1+ cells expressed low levels of Pax6 (see Fig. S1 in the supplementary material; Table 1). Olig2 was expressed by ~30% of Blimp1+ cells at E14.5 (see Fig. S1 in the supplementary material; Table 1). This implied that Olig2+ progenitors can give rise to photoreceptors and/or that Olig2 is transiently expressed in newly born photoreceptors. Transient retention of some progenitor markers is expected in newly postmitotic neurons. The small amount of progenitor marker overlap with Blimp1 implies that Blimp1 is an early marker for photoreceptor development.

Blimp1 is needed for normal photoreceptor development

The transient expression of Blimp1 suggested that it plays a role in the development of photoreceptors. To test this, we examined Blimp1 loss-of-function mutants. Blimp1 knockout mice die in utero (Vincent et al., 2005), so we made conditional knockout (CKO) mice by crossing the previously generated Blimp1Flox strain (Shapiro-Shelef et al., 2003) with one of two strains that express Cre recombinase in retinal progenitors. The Foxg1-Cre strain expresses Cre recombinase in ~50% of retinal progenitors during development, starting before the onset of Blimp1 expression (Hebert and McConnell, 2000). The αPax6-Cre-GFP strain expresses Cre in retinal progenitors in the peripheral part of the retina, starting before the onset of Blimp1 expression (Marquardt et al., 2001). This transgene is expressed in amacrine cells throughout the life of the animal (Marquardt et al., 2001). We crossed both Cre strains to Blimp1Flox mice to generate heterozygous controls (Cre::Blimp1Flox/+ and CKO mice (Cre::Blimp1Flox/−). Since Foxg1-Cre CKO mice did not survive to birth, we focused on αPax6-Cre-GFP CKO mice, which were viable.

We first examined 3- to 4-week-old CKO mice, an age when all the cells in the retina have differentiated. The peripheral retinas of heterozygous controls and CKO mice were compared using various cell type-specific markers. It was immediately clear from nuclear counterstaining that the outer nuclear layer (ONL) of CKO mice contained fewer cells than control retinas (Fig. 2A-F). Moreover, the inner nuclear layer (INL) was thicker in CKO mice (Fig. 2A-F). This was consistent with a loss of photoreceptors and a gain of interneurons or glia. We then immunostained retinas for Otx2 to measure photoreceptors and bipolar cells. Mature photoreceptors have small nuclei that are stained near the edges, where euchromatin is preferentially packaged (Fossat et al., 2007; Solovei et al., 2009). Bipolar cells have larger, intensely stained nuclei located in the INL. Controls had normal Otx2 staining patterns, but CKO mice had far fewer Otx2+ photoreceptors and ~50% more Otx2+ bipolar cells (Fig. 2A-C, Table 2). This reduction in photoreceptors affected rods and cones (see Fig. S3 in the supplementary material). The regular laminar organization of bipolar cells was lost in CKO mice and the outer
plexiform layer (OPL) was disrupted. By contrast, the localization and number of Pax6+ cells in the INL did not differ between the strains (Fig. 2A–C, Table 2), nor were any differences seen in the number of RGCs or horizontal cells (data not shown). We stained retinas for Chx10 to label most bipolars (Burmeister et al., 1996) and observed ~50% more Chx10+ bipolars in CKO mice (Fig. 2D–F, Table 2). Again, their laminar organization was disrupted and bipolar cell nuclei were often observed in the ONL (Fig. 2D–F). The increase in bipolar cells was confirmed with other markers, including protein kinase C (PKC), which labels rod bipolar cells (Greferath et al., 1990). Similar to the Otx2 and Chx10 staining results, there were more PKC+ rod bipolars in CKO mice and these bipolars showed disrupted lamination (Fig. 2G,H). Cone bipolars labeled for Vsx1 (Chow et al., 2001; Ohtoshi et al., 2001) were increased in CKO mice and showed disrupted laminar organization (Fig. 2I,J). Vsx1+ cells in CKO mice were more likely to co-express Chx10 than Vsx1+ cells in controls (Fig. 2I,J), but the relevance of this co-expression is unknown.

In addition to the increase in bipolar cells that we observed in CKO peripheral retina, there were also ~50% more Sox9+ Müller glia (Poche et al., 2008) (Fig. 2D–F, Table 2). Sox9+ glial nuclei were not organized into a single row and were occasionally observed in the ONL of CKO retinas (Fig. 2D–F). We also observed an increase

Table 1. Blimp1 expression at E14.5

| Marker (M) | Blimp1+ M+ (%) ± s.d. | M+=Blimp1+ (%) ± s.d. |
|------------|-----------------------|-----------------------|
| Otx2       | 100 (ND)              | 82.36 (4.30)          |
| NeuroD1    | 98.86 (2.27)          | 79.07 (1.16)          |
| Crx        | 64.40 (2.21)          | 100 (ND)              |
| Trβ2       | 62.83 (4.64)          | 100 (ND)              |
| Olig2      | 32.31 (6.01)          | 35.43 (4.92)          |
| Pax6*      | 16.27 (2.49)          | 2.30 (0.33)           |
| Sox9       | 2.06 (1.44)           | 0.44 (0.30)           |

ND, standard deviation not determined.

*Only faintly labeled Pax6* cells ever co-expressed Blimp1.
We first examined E15.5 earlier developmental stages for changes in photoreceptor markers. Regulating photoreceptor fate choice. We examined CKO mice at photoreceptors in CKO mice suggested a role for Blimp1 in the presence of extra bipolar and Müller glia at the expense of photoreceptors. Blimp1 represses bipolar fate in nascent development. During development.

The loss of photoreceptors in adult CKO mice was about four times greater than the gains seen in bipolars and Müller glia (Table 2). The Müller glia-specific marker CRALBP (Hatakeyama et al., 2001; Rowan and Cepko, 2004) compared with Sox9 in the number of glia that co-expressed Sox9 and Chx10 (Hatakeyama et al., 2001; Rowan and Cepko, 2004) with Sox9 in CKO mice (Fig. 2K,L).

The loss of photoreceptors in adult CKO mice was about four times greater than the gains seen in bipolars and Müller glia (Table 2), raising the possibility of excess bipolar and glial genesis or photoreceptor cell death. We did not observe any proliferating cells or an increase in apoptotic cells in adult CKO retinas (data not shown), suggesting that all the cell population changes occurred during development.

Blimp1 represses bipolar fate in nascent photoreceptors

The presence of extra bipolar and Müller glia at the expense of photoreceptors in CKO mice suggested a role for Blimp1 in regulating photoreceptor fate choice. We examined CKO mice at earlier developmental stages for changes in photoreceptor markers. We first examined E15.5 Blimp1 CKO mice using the Foxg1-Cre line (for this age only). These mice had a broad loss of Blimp1 from the retina. Other than the loss of Blimp1, we did not observe any changes in Otx2, Sox9, Pax6, Trf2, Crx or NeuroD1 (see Fig. S4 in the supplementary material). When we examined E18 CKO mice for the same markers, they appeared the same as in the controls (Fig. 3A-C). However, at this stage we observed a small number of Vsx1+ cells in Blimp1-deleted regions (Fig. 3D,E), which suggested precocious bipolar cell specification. The number of precocious Vxs1+ cells was even greater at P0 and P1 (Fig. 3D-G). At these stages, we detected cells that were intensely labeled for Chx10 (Fig. 3F,G,N), also indicative of precocious bipolar cell specification (Burmeister et al., 1996). The Vsx1+ and Chx10+ cells always co-expressed Otx2, consistent with bipolar fate choice (Fig. 3J and data not shown). At P1 and P3, we saw fewer recoverin- and S-opsin-labeled cells (Fig. 3I,J) in CKO than in control mice, consistent with a reduction in rod and cone differentiation. At P3, Blimp1 CKO mice had a large number of brightly labeled Chx10+ and Vxs1+ cells, whereas control mice still did not express these markers (Fig. 3Q,R). This suggested that Otx2+ cells were adopting bipolar fate at the expense of photoreceptor fate in CKO mice. At P1 and P3, expression of Otx2 and Sox9 was similar in CKO and control retinas (Fig. 3H,I,L,O,P). Together, these data show that Blimp1 is not required for the initial expression of photoreceptor markers (Otx2, Crx, Trf2 and NeuroD1), but that it negatively regulates Chx10 and Vsx1 in the Otx2+ population.

Next, we examined time-points at the end of retinogenesis. At P7, heterozygous controls had an Otx2+ ONL and brightly stained Otx2+ bipolar cells in the INL (Fig. 4A). These layers were noticeably separated by the OPL, with a few photoreceptors present on the wrong (inner) side of this border. By contrast, P7 CKO mice had numerous Otx2+ bipolar cells and few.

Table 2. Cell density differences in adult Blimp1 CKO mice

|                     | Otx2+ photoreceptors | Otx2+ bipolars | Chx10+ | Sox9+ | Chx10+/Sox9+ | Pax6* |
|---------------------|----------------------|----------------|--------|-------|--------------|-------|
| Blimp1+/+           | 126.31 (20.42)       | 22.20 (4.43)   | 19.39 (2.75) | 10.70 (1.52) | 1.20 (0.67) | 23.29 (5.29) |
| Blimp1−/−           | 48.66 (9.82)         | 34.52 (6.28)   | 29.57 (5.66) | 15.76 (3.24) | 3.38 (1.93) | 26.86 (4.49) |
| p                   | 0.000                | 0.000          | 0.000    | 0.000    | 0.000        | 0.07  |

Cell density is given in cells per 100 μm (± s.d.) retinal length.
*Pax6-labeled cells in the inner nuclear layer.
†Unpaired, two-tailed t-test. n≥5 600× fields from four retinas for controls and n≥5 600× fields from eight retinas for CKO mice.
photoreceptors (Fig. 4B,C). The OPL was hard to discern in CKO retinas, but when observed, many Otx2+ bipolar cells were misplaced into the ONL. Blimp1 CKO retinas had increased numbers of Chx10+ and Vsx1+ bipolar cells compared with controls at P7 (Fig. 4D-G). When we counted the number of Otx2+ photoreceptors and bipolars, there were more bipolars and fewer photoreceptors in CKO than in control mice (Fig. 4O, Table 3). The decrease in photoreceptors was proportional to the increase in bipolar cells in CKO mice, yet there were no differences in the total number of Otx2+ cells between CKO mice and controls (Fig. 4O, Table 3). This implied that there was an approximately one-to-one fate shift between photoreceptors and bipolars. By P10, heterozygous control mice had laminated retinas with Otx2+ photoreceptors in the ONL and Otx2+ bipolars in the INL (Fig. 4H). Blimp1 CKO mice had a much thinner retina than controls, reminiscent of adult CKO mice (Fig. 4I). At P10, there were few photoreceptors, but also fewer bipolar cells than seen at P7 (Fig. 4I,P, Table 3). In contrast to P7 retinas, there were significantly fewer total Otx2+ cells in P10 CKO than control retinas (Fig. 4P, Table 3). At P10, there was excess PKC staining in CKO mice, consistent with the increase in bipolar cells (Fig. 4J,K). By contrast, PKC staining was not seen in the most

Table 3. Cell density differences in juvenile Blimp1 CKO mice

|                | Otx2+ photoreceptors | Otx2+ bipolars | Total Otx2+ | Sox9* | Otx2+/Sox9* | Caspase 3** |
|----------------|----------------------|----------------|-------------|-------|-------------|-------------|
| P7 Blimp1+/–   | 133.42 (22.39)       | 46.66 (9.96)   | 180.08 (31.45) | 18.06 (3.22) | 2.47 (1.21) | 1.93 (0.76) |
| P7 Blimp1–/–   | 65.10 (12.23)        | 130.96 (14.79) | 195.27 (12.85) | 30.34 (5.61) | 5.45 (1.41) | 5.56 (2.20) |
| P10 Blimp1+/–  | 129.48 (18.97)       | 35.49 (4.62)   | 164.98 (22.73) | 14.32 (2.21) | 0.52 (0.36) | 1.09 (0.43) |
| P10 Blimp1–/–  | 55.63 (13.42)        | 60.80 (18.79)  | 116.43 (26.57) | 18.95 (3.26) | 1.23 (0.93) | 1.01 (0.59) |

*Cell density is given in cells per 100 μm (± s.d.) retinal length.
**Cleaved caspase 3+ apoptotic cells.
†Unpaired, two-tailed t-test. n=10 400× fields from four retinas for P7 and n=5 400× fields from four retinas for P10s.
peripheral parts of P7 control or CKO retinas, suggesting that the excess bipolar cells do not fully differentiate ahead of schedule (data not shown).

The majority of Otx2+ cells in CKO mice adopted bipolar cell fate by P7. However, we also saw a smaller increase in the Sox9+ population at this stage (Table 3). At P7, in the control most of the Sox9+ cells were Müller glia, which formed a single layer in the INL (Fig. 4A). Blimp1 CKO mice had ~2-fold more Sox9+ cells, which were spread throughout the area where bipolar cells are observed (Fig. 4B,C). Interestingly, both Blimp1 CKO and control retinas had a sizeable population of Otx2+/Sox9+ cells at P7 (Fig. 4A-C, Table 3). The ~2-fold increase in Otx2+/Sox9+ cells paralleled the increase in Sox9+ cells in CKO mice (Table 3). What these double-labeled cells represent is unclear, but they might be newly formed Müller glia, raising the possibility that the excess glia seen in CKO retinas derived from Otx2+ cells. At P10, there are few, if any, progenitors left in the retina (Carter-Dawson and LaVail, 1979; Young, 1985). However, the presence of a small population of Otx2+/Sox9+ cells in both genotypes at this stage (Table 3) further suggested that these double-labeled cells were Müller glia.

Fig. 4. Otx2+ cells in Blimp1 CKO mice switch from photoreceptor to bipolar fate. All panels show peripheral retina. (A-C) P7 control (A) and CKO (B,C) retinas stained for Otx2 (red) and Sox9 (green). The small, fainter Otx2+ nuclei are photoreceptors and the large, bright nuclei are bipolar cells. The photoreceptor layer (bracketed) is much thinner in CKO mice and there is a proportional increase in the number of bipolar cells. In both control and CKO sections, several Sox9+ cells co-express Otx2 (arrows). CKO mice also have more Sox9+ cells (mostly glia), which are scattered throughout the retina. (D,E) P7 control (D) and CKO (E) sections stained for Chx10 (red) and Sox9 (green). CKO mice have more Chx10+ bipolars and Sox9+ cells. The arrow shows a double-labeled Müller cell. (F,G) P7 control (F) and CKO (G) retinas stained for Vsx1. There are many more Vsx1+ bipolars in CKO mice and their distribution is broader. (H,I) P10 control (H) and CKO (I) sections stained for Otx2 (red) and Sox9 (green). At this stage, the ONL (bracketed) is much thinner in CKO mice and there are fewer excess bipolars and glia than seen at P7. Otx2+/Sox9+ cells were detected (arrow). (J,K) P10 control (J) and CKO (K) mice stained for Chx10 (red) and PKC (green) to label bipolars. (J) All rod bipolars express PKC and Chx10 (arrow), forming a row in the INL. In CKO mice (K), there are more PKC- and Chx10-labeled bipolars (arrow). (L-N) P7 control (L) and CKO (M,N) retinas stained for Otx2 (red) and activated caspase 3 (AC3) (green). Most of the dying cells in controls and CKO mice are in the bipolar cell area rather than in the photoreceptor area (bracketed). CKO mice have ~3-fold more dying cells than controls, some of which are still Otx2+ (arrows). (O,P) Otx2+ photoreceptor (black) and bipolar (red) cell density measured at P7 (O) and P10 (P). At P7 there is an approximately one-to-one fate shift in the CKO Otx2+ population. The error bars indicate the s.d. of the total Otx2+ cells counted. Scale bars: 50 μm, except 100 μm in F,G and 5 μm in insets.
The difference in the number of Otx2+ cells between P7 and P10 suggested that many of the fate-shifted bipolar cells were dying between these stages. We examined cell death at several time-points. At P1 and P3, no differences in activated caspase 3 labeling, a marker of apoptotic cells (Thornberry and Lazebnik, 1998), were seen between control and CKO mice (data not shown). However, at P7, we observed ~3-fold more caspase 3+ cells in CKO mice as compared with control mice (Fig. 4L-N, Table 3). Most of the cell death was in the INL (Fig. 4M,N), consistent with the loss of bipolar cells and Müller glia. By P10, the amount of cell death was equivalent between genotypes (Table 3). Thus, many of the excess bipolar cells generated in the Blimp1 CKO mice died between P7 and P10, which correlated with the timing of bipolar maturation and the peak of normal cell death in this layer (Bramblett et al., 2004; Young, 1984).

**Blimp1 overexpression inhibits bipolar cell fate choice**

Blimp1 CKO mice have fewer photoreceptors and excess bipolar cells, suggesting that Blimp1 inhibits bipolar cell development. To determine whether Blimp1 is sufficient to inhibit bipolar cell genesis, we engineered expression plasmids to drive GFP or Blimp1-GFP in the retina at a time when bipolar versus photoreceptor fate decisions are being made. These constructs were injected into the vitreous of P1 eyes and electroretinoplotared into the retina. We examined the fate of transfected (GFP+) cells at P5, allowing 4 days for effects on bipolar versus photoreceptor fate to become apparent. At P5, most transfected cells co-expressed Otx2 in control (GFP) and Blimp1-GFP retinas (Fig. 5A,C) (see Table S1 in the supplementary material). Photoreceptors had smaller, faintly labeled nuclei, whereas bipolars and undifferentiated cells (nascent bipolars and photoreceptors) had bright, elongated Otx2 staining. Chx10 co-labeling was used to more definitively mark transfected bipolar cells (Fig. 5B,D). Transfected cells were scored for faint Otx2 (Otx2+), strong Otx2 (Otx2++), and Chx10 (Chx10+) expression (Fig. 5E). Blimp1-GFP-transfected cells adopted photoreceptor fate more readily (26% more) and bipolar (and undifferentiated) fate less frequently (24% fewer) than the GFP control (Fig. 5E). Thus, Blimp1 overexpression caused a shift in the fate distribution of the Otx2+ population, but in the opposite direction to that observed in Blimp1 CKO retinas. The inhibitory effect of Blimp1 was more pronounced on definitive, Chx10+ bipolar cells, which were reduced by 59% compared with GFP-transfected controls (Fig. 5E).

**DISCUSSION**

We observed that the PR-SET domain zinc-finger transcription factor Blimp1 is expressed in developing photoreceptors in mammals. Genetic deletion of this transcription factor in the retina led to conversion of most of the photoreceptors into bipolar cells, although some may also have adopted Müller glial fate. As the Blimp1 CKO retinas matured, many of the fate-shifted cells were lost; however, there were still ~50% more bipolars and glia than normal. Overexpression of Blimp1 promoted photoreceptor fate at the expense of bipolar cells. We conclude that during retinal development, Blimp1 regulates cell fate choice by inhibiting the ‘bipolar cell program’ in nascent Otx2+ photoreceptors.

Previous experiments have shown that Blimp1 is likely to be expressed in developing photoreceptors (Chang and Calame, 2002; Hsiau et al., 2007; Wang et al., 2008). Our data are consistent with Blimp1 marking developing photoreceptors for the following reasons. First, Blimp1 expression starts at E12, the time when the first photoreceptors exit the cell cycle (Carter-Dawson and LaVail, 1979). Second, at all stages, Blimp1+ cells co-express Otx2, a marker of photoreceptors. Third, all Crx+ photoreceptors and Trpβ2+ cones co-expressed Blimp1. Fourth, all Blimp1+ cells co-expressed NeuroD1, which is a marker of postmitotic photoreceptors and amacrine cells. Fifth, many recoverin+ photoreceptors co-express Blimp1 neonatally. Sixth, Blimp1 expression rarely coincided with markers of non-photoreceptor cell types. These criteria establish that Blimp1 is expressed in photoreceptors, although it remains possible that Blimp1 is produced transiently in all Otx2+ cells, including those that give rise to bipolar cells. A lineage-tracing study is needed to examine this possibility. Previous experiments have suggested that Otx2 expression precedes that of Crx (Nishida et al., 2003). At E14.5, all Crx+ cells expressed Blimp1 and both these populations...
co-expressed Otx2. This nested pattern suggests that the order of gene expression in nascent photoreceptors is Otx2, then Blimp1, and lastly Crx.

Conditional Blimp1 deletion resulted in retinas with fewer photoreceptors in the affected areas. A similar result has been reported in Blimp1 mutant fish, which have reduced expression of opsin genes (Wilm and Solnica-Krezel, 2005). It is unclear why some photoreceptors remain in Blimp1 CKO mice. It is possible that some cells in the affected regions did not lose Blimp1. Furthermore, although Otx2 expression is expressed before Blimp1, it is possible that some cells did not lose Blimp1 until after it had been briefly expressed, stabilizing photoreceptor identity in this subset of cells. If only the latest born Otx2+ cells retained photoreceptor identity in Blimp1 CKO mice, the retina should contain only rods, as cones are not generated postnatally. However, both rods and cones were observed in CKO mice. Another possibility is that only postnatally generated Otx2+ cells convert to bipolar. This is unlikely because cones (early born) were also reduced in Blimp1 CKO mice. The cone reduction is seen early on (P1-P3), which argues against the possibility of selective cone death in adult CKO mice. Lastly, it is possible that another factor partially compensates for Blimp1 activity. In support of this, there are many Prdm family members in mice, most of which are expressed in the developing retina (J.A.B. and T.A.R., unpublished).

Blimp1 CKO mice had increased bipolar and Müller glial cell numbers and, conversely, overexpression of Blimp1 resulted in fewer bipolar cells and increased photoreceptors. These experiments suggest that Blimp1 regulates cell fate during retinogenesis. Blimp1 has been shown to regulate fate choice in other systems. In B-cells, Blimp1 directly represses key transcription factors to allow plasma cell differentiation (Calame et al., 2003). In fish, Blimp1 acts as a switch between slow and fast twitch muscle types (Elworthy et al., 2008; von Hofsten et al., 2008). In primordial germ cells, Blimp1 is required to repress the somatic program, allowing germ cell specification in cells otherwise competent to adopt somatic fate (Ohnata et al., 2005; Vincent et al., 2005). In Blimp1 CKO mice, we observed a direct fate shift in the Otx2+ population, which is competent for photoreceptor and bipolar fates. Prior to birth, rod and cone fate specification was unaffected in Blimp1 CKO mice. By P1, there were fewer rods and cones, but no changes in Otx2 expression. In Blimp1 CKO mice, Otx2 cells prematurely co-express the bipolar-specific markers Vsx1 and Chx10 (Fig. 6A,B) such that many previously generated rods and cones lose their photoreceptor identity in the absence of Blimp1. This indicates that Blimp1 is needed to stabilize, but not specify, photoreceptor fate during development (Fig. 6C). This also implies that photoreceptor identity is not permanently established at the time of cell cycle exit, but might require continued stabilization from the ‘bipolar cell program’ until late in retinogenesis. Consistent with this hypothesis, Chx10 overexpression can directly repress the photoreceptor program (Dorval et al., 2006; Livne-Bar et al., 2006). It might be that Chx10 is needed to repress photoreceptor fate (Livne-Bar et al., 2006), whereas Blimp1 represses bipolar fate, allowing fate diversification in the postmitotic Otx2+ population. Our data suggest that Blimp1 represses Chx10 and Vsx1 expression, blocking bipolar development. Cells with high-level Chx10 and Vsx1 expression are not normally seen until P4 or later (Fig. 6A,B), well after many bipolar cells have exited the cell cycle (Morrow et al., 2008; Young, 1985). This raises the possibility that postmitotic Otx2+ cells remain plastic and do not commit to bipolar fate for up to several days after exiting the cell cycle.

Blimp1 regulates the choice between photoreceptor and bipolar fates. Two other mouse mutants exhibit similarly extensive direct fate shifts in the retina. In Nrl-null mice, all rods become cones (Mears et al., 2001). In conditional Otx2 mutants, photoreceptors and bipolars are lost and these cells adopt amacrine cell fate (Nishida et al., 2003; Sato et al., 2007). In both of these mutants, many of the excess (fate shifted) cells are lost by apoptosis (Mears et al., 2001; Nishida et al., 2003; Sato et al., 2007). Similarly, many excess bipolar cells generated in Blimp1 CKO mice are lost around the time of bipolar cell maturation. It is unclear why these excess bipolar cells are lost. One possibility is that bipolar cells need trophic support from other neurons to mature and survive. The most likely sources
of this support are photoreceptors (presynaptic) and RGCs (postsynaptic for most). In photoreceptor-degeneration mouse lines, bipolar cells change molecularly and physically, but survive (Punzo and Cepko, 2007). In mutants lacking RGCs, there are fewer bipolar cells, but most remain, suggesting that RGCs are not required for bipolar cell survival either (Brzezinski et al., 2005). Another possibility is that Müller glia are required to maintain bipolar cell numbers. In support of this, both bipolar and Müller glial numbers are increased by ~50% in adult Blimp1 CKO mice. Thus, a 50% increase in glia might support a 50% increase in bipolar cells, but this remains to be demonstrated directly.

Although not as extensive as for bipolar cells, we also saw an increase in the number of Müller glia generated in CKO mice. This increase could also be the result of a direct fate shift of Otx2+ cells into glia. In support of this, we observed a ~2-fold increase in both Sox9 and Otx2/Sox9+ cells in Blimp1 CKO mice at P7, suggesting that at least some glia derive from Otx2+ cells. We also observed a population of Otx2+/Sox9+ cells in adult CKO retinas (see Fig. S5 in the supplementary material). Nonetheless, it remains possible that the change in glial numbers is non-cell-autonomous. A lineage-tracing experiment is needed to distinguish these possibilities.

During retinal development a cohort of cells competent for cone, rod, bipolar and possibly glial fates exits the cell cycle and expresses Otx2 (Fig. 5C.) To achieve cell diversity in the Otx2+ population, a switch that regulates photoreceptor versus bipolar fate choice during retinal development.

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Competing interests statement
The authors declare no competing financial interests.

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References
Akimoto, M., Cheng, H., Zhu, D., Brzezinski, J. A., Khanna, R., Filippova, E., Oh, E. C., Jing, Y., Linares, J. L., Brooks, M. et al. (2006). Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. Proc. Natl. Acad. Sci. USA 103, 3890-3895.
Ancelin, K., Lange, U. C., Hajkova, P., Schneider, R., Bannister, A. J., Kozariodes, T. and Surani, M. A. (2006). Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. Nat. Cell Biol. 8, 623-630.
Bramblett, D. E., Pennesi, M. E., Wu, S. M. and Tsai, M. J. (2004). The transcription factor Bhlhb4 is required for rod bipolar cell maturation. Neuron 39, 433-442.
Chow, R. L., Snow, B., Novak, J., Looser, J., Freund, C., Vidgen, D., Ploder, L. and McInnes, R. R. (2001). Vsx1, a rapidly evolving paired-like homeobox gene expressed in cone bipolar cells. Mech. Dev. 109, 315-322.
Chow, R. L., Volgyi, B., Szillard, R. K., Ng, D., McKerlie, C., Bloomfield, S. A., Birch, D. G. and McConnell, S. K. (2004). Control of late off-center cone bipolar cell differentiation and visual signaling by the homeobox gene Vsx1. Proc. Natl. Acad. Sci. USA 101, 1754-1759.
Clark, A. M., Yun, S., Veien, E. S., Wu, Y. Y., Chow, R. L., Dorsky, R. I. and Levine, E. M. (2000). Negative regulation of Vsx1 by its paralog Chx10/Vsx2 is conserved in the vertebrate retina. Brain Res. 929, 199-213.
de Melo, J., Qiu, X., Du, G., Cristante, L. and Eisenstat, D. D. (2003). Dlx1/Dlx2, Pax6, Bm3b, and Chx10 homeobox gene expression defines the retinal ganglion and inner nuclear layers of the developing and adult mouse retina. J. Comp. Neurol. 461, 187-201.
Dorval, K. M., Bobechko, B. P., Fujieda, H., Chen, S., Zack, D. J. and Brenner, R. (2006). CHX10 targets a subset of photoreceptor genes. J. Biol. Chem. 281, 744-751.
Eisenfeld, A. J., Bunt-Milam, A. H. and Saari, J. C. (1985). Localization of retinoic acid-binding proteins in developing rat retina. Exp. Eye Res. 41, 299-304.
Elworthy, S., Harragwe, M., Knight, R., Mebus, K. and Ingham, P. W. (2008). Expression of multiple slow myosin heavy chain genes reveals a diversity of zebrafish slow twitch muscle fibres with differing requirements for Hedgehog and Pdm1 activity. Development 135, 2115-2126.
Fossat, N., Le Greneur, C., Beck, V., Vincent, S., Godement, P., Chatelain, G. and Lamonerie, T. (2007). A new GFP-tagged line reveals unexpected Otx2 protein localization in retinal photoreceptors. BMC Dev. Biol. 7, 122.
Furukawa, T., Morrow, E. M. and Cepko, C. L. (1997). Chx10, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Neuron 19, 641-651.
Furukawa, T., Morrow, E. M., Li, T., Davis, F. C. and Cepko, C. L. (1999). Retinopathy and attenuated circadian entrainment in Chx10-deficient mice. Nat. Genet. 23, 466-470.
Green, E. S., Stubbs, J. L. and Levine, E. M. (2003). Genetic rescue of cell number and photoreceptor function in a mouse phototransduction mutant: interactions between Chx10 and G1-phase cell cycle regulators. Development 130, 539-552.
Grefrath, U., Grunert, U. and Wassef, H. (1990). Rod bipolar cells in the mammalian retina show protein kinase C-like immunoreactivity. J. Comp. Neurol. 301, 435-442.
Hayatake, J., Tomita, K., Inoue, T. and Kageyama, R. (2005). Roles of homeobox and bHLH genes in specification of a retinal cell type. Development 132, 1313-1322.
Hayashi, K., de Sousa Lopes, S. M. C. and Surani, M. A. (2007). Germ cell specification in mice. Science 316, 394-398.
Hebert, J. M. and McConnell, S. K. (2000). Among the lines of crosstalk between Chx10 and Vsx1, one that mediates loxP recombination in the telencephalon and other developing head structures. Dev. Biol. 222, 296-306.
Hsiau, T. H., Dacoucos, C., Myers, C. A., Lee, J., Cepko, C. L. and Corbo, J. C. (2007). The cis-regulatory logic of the mammalian photoreceptor transcriptional network. PLoS ONE 2, e443.
John, S. A. and Garrett-Sinha, L. A. (2009). Blimp1: a conserved transcriptional repressor critical for differentiation of many tissues. Exp. Cell Res. 315, 1077-1084.
Laird, D. W. and Molday, R. S. (1988). Evidence against the role of rhodopsin in rod outer segment binding to RPE cells. Invest. Ophthal. Vis. Sci. 29, 419-428.
Levit, H., Etter, P., Hayes, S., Jones, I., Nelson, B., Hartman, B., Forrest, D. and Reh, T. A. (2008). NeuroD regulates expression of thyroid hormone receptor 2 and cone opsin in the developing mouse retina. J. Neurosci. 28, 749-756.
Livesey, F. J. and Cepko, C. L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. Nat. Rev. Neurosci. 2, 109-118.
Livne-Bar, I., Pacal, M., Cheung, M. C., Hankin, M., Trogadis, J., Chen, D., Dorval, K. M. and Brenner, R. (2006). CHX10 is required to block photoreceptor differentiation but is dispensable for progenitor proliferation in the postnatal retina. Proc. Natl. Acad. Sci. USA 103, 4988-4993.
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Nakamura, K., Harada, C., Namekata, K. and Harada, T. (2007). Cellular responses to photoreceptor death in the retina using a novel multifunctional BAC expression transfectants with a novel eukaryotic vector. Nat. Genet. 37, 345-349.

Young, R. W. (2001). Pax6 is required for the multipotent state of retinal progenitor cells. Nat. Genet. 27, 94-98.

Ohinata, Y., Payer, B., O’Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S. J., Kreysing, M., Lanctot, C., Sciammas, R., Shapiro-Shalef, M., Davis, M. M., and Gruss, P. (2008). Blimp-1 is required for the formation of primordial germ cells in the mouse. Development 135, 1315-1325.

Vincent, S. D., Dunn, N. R., Sciammas, R., Shapiro-Shalef, M., Davis, M. M., Calame, K., Bikooff, E. K. and Robertson, E. J. (2005). The zinc finger transcriptional repressor Blimp1/Pdml is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. Development 132, 1315-1325.

Wang, D., Zhuang, L., Gao, B., Shi, C. X., Cheung, J., Liu, M., Jin, T. and Wen, X. (2006). The Blimp-1 gene regulatory region directs EGFP expression in multiple hematopoietic lineages and tests in mice. Transgenic Res. 17, 193-202.

Wassle, H., Puller, C., Muller, F. and Haverkamp, S. (2009). Cone contacts, mosaics, and territories of bipolar cells in the mouse retina. J. Neurosci. 29, 106-117.

Wilm, T. P. and Solника-Krezel, L. (2005). Essential roles of a zebrafish prdm 1/blimp 1 homolog in embryo patterning and organogenesis. Development 132, 393-404.

Xiang, M., Zhou, Z., Macke, J. P., Yoshioka, T., Hendry, S. H., Eddy, R. L., Show, T. B. and Nathans, J. (1995). The Brn-3 family of POU-domain factors: primary structure, binding specificity, and expression in subsets of retinal ganglion cells and somatosensory neurons. J. Neurosci. 15, 4762-4785.

Young, R. W. (1984). Cell death during differentiation of the retina in the mouse. J. Comp. Neurol. 229, 362-373.

Young, R. W. (1985). Cell differentiation in the retina of the mouse. Anat. Rec. 212, 199-205.