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Blimp1 (Prdm1) prevents re-specification of photoreceptors into retinal bipolar cells by restricting competence

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Abstract

During retinal development, photoreceptors and bipolar cells express the transcription factor Otx2. Blimp1 is transiently expressed in Otx2+ cells. *Blimp1* deletion results in excess bipolar cell formation at the expense of photoreceptors. In principle, Blimp1 could be expressed only in Otx2+ cells that are committed to photoreceptor fate. Alternatively, Blimp1 could be expressed broadly in Otx2+ cells and silenced to allow bipolar cell development. To distinguish between these alternatives, we followed the fate of Blimp1 expressing cells using *Blimp1-Cre* mice and *Lox-Stop-Lox* reporter strains. We observed that Blimp1+ cells gave rise to all photoreceptors, but also to one third of bipolar cells, consistent with the latter alternative: that Blimp1 inhibits bipolar competence in Otx2+ cells and must be silenced to allow bipolar cell generation. To further test this hypothesis, we looked for transitioning rod photoreceptors in *Blimp1* conditional knock-out (CKO) mice carrying the *NRL-GFP* transgene, which specifically labels rods. Control animals lacked NRL-GFP+ bipolar cells. In contrast, about half of the precociously generated bipolar cells in *Blimp1* CKO mice co-expressed GFP, suggesting that rods become re-specified as bipolar cells. Birthdating analyses in control and *Blimp1* CKO mice showed that bipolar cells were birthdated as early as E13.5 in *Blimp1* CKO mice, five days before this cell type was generated in the wild-type retina. Taken together, our data suggest that early Otx2+ cells upregulate photoreceptor and bipolar genes, existing in a bistable state. Blimp1 likely forms a cross-repressive network with pro-bipolar factors such that the winner of this interaction stabilizes the photoreceptor or bipolar state, respectively.

Keywords

Retina; Photoreceptor; Bipolar Cell; Cell Fate Specification; Transdifferentiation; Competence

Introduction

The retina is responsible for detecting and relaying light stimuli to the brain. The retina comprises seven cell types; rod and cone photoreceptors, retinal ganglion cells (RGCs), Müller glia, and horizontal, amacrine and bipolar interneurons. These derive from a common

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pool of multipotent progenitor cells (Turner and Cepko, 1987; Turner et al., 1990). Lineage tracing studies have shown that retinal fate determination is a stochastic process; nonetheless, birthdating studies have shown that fate choice correlates with the time of permanent cell cycle exit (Carter-Dawson and LaVail, 1979; Gomes et al., 2011; La Vail et al., 1991; Livesey and Cepko, 2001; Rapaport et al., 2004; Sidman, 1961; Turner and Cepko, 1987; Turner et al., 1990; Young, 1985). The numbers of each retinal cell type are the result of precise temporal regulation of competence acquisition and restriction. *Foxn4* and *Math5* (*Atoh7*) act to establish competence for horizontal and amacrine, and RGC fates, respectively (Brzezinski et al., 2012; Feng et al., 2010; Li et al., 2004; Yang et al., 2003). Conversely, *Ascl1* and *Olig2* expression defines progenitors that have lost competence (Brzezinski et al., 2011; Hafler et al., 2012). The dynamic regulation of competence acquisition and restriction during retinogenesis requires the action of miRNAs (Georgi and Reh, 2010).

Otx2 is expressed by nascent and mature rods, cones, and bipolar cells in the retina. *Otx2* conditional knock-out (CKO) mice do not form photoreceptors or bipolar cells, but generate amacrine cells instead (Nishida et al., 2003; Sato et al., 2007). The transcriptional repressor Blimp1 (*Prdm1*) is expressed solely by Otx2+ cells in the retina during the period of photoreceptor genesis (Brzezinski et al., 2010; Katoh et al., 2010). *Blimp1* CKO mice generate the same number of Otx2+ cells, but have an approximately 1-to-1 fate shift of photoreceptors (rod and cone) into bipolar cells. Interestingly, *Blimp1* CKO mice generate photoreceptors normally until around birth, when bipolar-specific markers are upregulated and photoreceptor markers reduced (Brzezinski et al., 2010). The transcription factor *Chx10* (*Vsx2*) is required for the formation of bipolar cells (Burmeister et al., 1996; Green et al., 2003). Chx10 has been shown to repress photoreceptor genes and biases postnatal progenitors to bipolar fate when overexpressed (Dorval et al., 2006; Livne-Bar et al., 2006). Blimp1 can directly repress *Chx10* expression (Katoh et al., 2010). Several predictions can be made from these observations: (1) Blimp1 must be silenced to allow bipolar fate, (2) Blimp1 restricts bipolar competence, (3) photoreceptors become re-specified to bipolar cell fate without *Blimp1*, and (4) instructive factors that promote bipolar cell fate are not present in the embryonic retina.

To test these predictions, we first conducted Blimp1 expression fate mapping. Blimp1+ cells gave rise to all photoreceptors and about one third of bipolar cells, suggesting that bipolars are generated from Otx2+ cells that quickly silenced *Blimp1*. We reasoned that without *Blimp1*, photoreceptors might retain bipolar cell competence and become susceptible to bipolar reprogramming. To test this, we examined *Blimp1* CKO mice carrying the *NRL-GFP* transgene (Akimoto et al., 2006). NRL-GFP+ rods that transition to bipolar fate would transiently retain GFP and co-express bipolar-specific markers. Transitioning cells were not seen in controls but were common in *Blimp1* CKO mice, suggesting that photoreceptors are re-specified as bipolar cells in the absence of *Blimp1*. To further test whether Blimp1 restricts bipolar competence, we conducted birthdating experiments at stages well before bipolar cells are normally born. In contrast to controls, we found birthdated bipolars as early as embryonic day (E) 13.5 in *Blimp1* CKO mice. Nonetheless, bipolar markers were not seen before birth in *Blimp1* CKO mice. These data indicate that Blimp1 restricts bipolar competence and that factors instructive for the bipolar cell fate are not present in the embryonic retina. Together, our data suggest that Otx2 initiates a cross-repressive program that stabilizes either photoreceptor or bipolar fate.

Materials and Methods

Animals

Wild-type *C57Bl6/J* mice (The Jackson Laboratory, Bar Harbor, ME, USA) (strain #000664) were used for chromatin immunoprecipitation (ChIP) experiments. *Blimp1*^{Flox} (*Prdm1*) floxed mice (*B6.129-Prdm*^{1tm1Clme}*/J*) (Jackson #008100) (Shapiro-Shelef et al., 2003) were crossed to α*Pax6-Cre-GFP* (*Tg(Pax6-cre,GFP)2Pgr*) transgenic mice (Marquardt et al., 2001) (from Ruth Ashery-Padan, Tel Aviv University) to generate CKO (α*Pax6-Cre-GFP*;*Blimp1*$^{Flox/Flox}$) and heterozygous controls (α*Pax6-Cre-GFP*;*Blimp1*$^{Flox/+}$) as described previously (Brzezinski et al., 2010). *Blimp1-Cre* BAC transgenic mice (*B6.Cg-Tg(Prdm1-Cre)1Masu/J*) were acquired from Michel Nussenzweig (The Rockefeller University) (Ohinata et al., 2005) and The Jackson Laboratory (#008827). *Lox-Stop-Lox* Cre reporter strains used were *ROSA-nGFP* (Stoller et al., 2008) (*B6;129-Gt(ROSA)26Sor*^{tm1Joe}*/J*) (Jackson #008516), *ZEG* (Novak et al., 2000) (*B6.129(Cg)-Tg(CAG-Bgeo/GFP)21Lbe/J*) (Jackson #004178), and *mTmG* (Muzumdar et al., 2007) (*B6.129(Cg)-Gt(ROSA)26Sor*$^{tm4(ACTB-tdtomato,-EGFP)Luo}$*/J*)(Jackson #007576). To specifically label rods, we acquired *NRL-GFP* (*B6.Cg-Tg(Nrl-EGFP)1Asw/J*) mice from Anand Swaroop (NEI) (Akimoto et al., 2006). The morning a vaginal plug was observed was considered E0.5. All animals were used with approval from the University of Washington and the University of Colorado Denver institutional animal care and use committees.

Histology

Eyes were collected, cryosectioned, immunostained, and imaged as described previously (Brzezinski et al., 2011; Brzezinski et al., 2010). Primary antibodies used were mouse anti-AP2α (0.5 μg/mL; clone 5E4, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), rat anti-Blimp1 (1:100; sc47732, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rat anti-BrdU (1:100; OBT0030, Accurate Chemical & Scientific, Westbury, NY, USA), goat anti- Brn3a/b/c (pan- specific) (1:50; sc6026, Santa Cruz), rabbit anti-calbindin D-28K (1:500; ab1778, Millipore, Billerica, MA, USA), rabbit anti-calretinin (1:1000; 7699/4, SWANT, Marly, Switzerland), sheep anti-Chx10 (Vsx2) (1:200; X1179P, Exalpha, Shirley, MA, USA), mouse anti-Cre recombinase (1:250; mab3120, Millipore), chicken anti-GFP (1:750; ab13970, Abcam, Cambridge, MA, USA), goat anti-Otx2-biotin (1.5 μg/mL; BAF1979, R&D Systems, Minneapolis, MN, USA), rabbit anti-Pax6 (1:500; PRB-278P, Covance, Princeton, NJ, USA), mouse anti-PKC (1:250; P5704, Sigma, St. Louis, MO, USA), goat anti-Prox1 (1:500; AF2727, R&D Systems), rabbit anti-Prox1 (1:500; ab11941, Abcam), rabbit anti-Scgn (0.2μg/mL; BioVendor, Asheville, NC, USA), goat anti-Sox2 (1:100; sc17320, Santa Cruz), rabbit anti-Sox9 (1:500; ab5535, Millipore), rabbit anti-Sox9 (1:100, sc20095, SCBT), rabbit anti-Thrb2 (1:500, a gift from Douglas Forrest, NIDDK) (Ng et al., 2001), and rabbit anti-Vsx1 (1:250; a gift from Ed Levine, University of Utah)

Expression fate mapping

We crossed *Blimp1-Cre* mice to several *Lox-Stop-Lox* reporter lines to indelibly mark cells that expressed *Blimp1* during development. *Blimp1-Cre* and *Blimp1-Cre;mTmG* mice were used to characterize the specificity of the transgene and the fate of Blimp1+ cells at E14.5, postnatal day (P) 0, P10, and adult ages. Recombined cells expressed membrane localized GFP. To more readily quantify the labeling frequency, we used *Blimp1-Cre;ROSA-nGFP* mice, which discretely labeled nuclei with GFP/β-galactosidase fusion protein expression (Stoller et al., 2008). Three week old *Blimp1-Cre;ROSA-nGFP* mice (N=3) were immunostained with GFP and cell type-specific markers (Otx2, Chx10, AP2α, Brn3, Calbindin, Pax6, and Sox2). At least nine 400× fields were imaged for each marker and the percentage of GFP labeling in each was calculated and averaged. We immunostained P11

Blimp1-Cre;ZEG retinas with additional bipolar- and amacrine-specific markers (Vsx1, Scgn, PKC, Prox1, and calretinin) to determine whether Blimp1+ cells adopted specific interneuron subtype identities. No labeled cells were seen in any reporter animal lacking Cre recombinase (not shown).

Chromatin immunoprecipitation

ChIP was adapted from a prior protocol (Dahl and Collas, 2008). We dissected and pooled retinas from E18.5, P1, or 6 week-old *C57Bl6/J* mice in PBS. Retinas were quickly dissociated with trypsin to single cell density and 10 million cells were fixed for 7.0 minutes in 0.5% formaldehyde/PBS. Chromatin was sheared to approximately 500bp with a probe tip sonicator (Dismembrator-150E, Fisher, Pittsburgh, PA, USA) or a Bioruptor sonicator (Diagenode, Denville, NJ, USA). For immunoprecipitation (IP), 2–4 μg of goat anti-Otx2 antibodies (BAF1979, R&D Systems) or goat IgG fraction (AB-108-C, R&D Systems) were bound to 10 μL of Protein G dynabeads (Diagenode or Life Technologies, Grand Island, NY, USA). Approximately 10% of the sheared chromatin (one million cells) was used per IP. For input control, non-precipitated chromatin was used. PCR was run in triplicate from 2–5 independent immunoprecipitations. Percent input was calculated as $2^{((\text{Ct-input}) - (\text{Ct-IP}))} \times 100\%$; where Ct is the threshold cycle. Statistical differences were evaluated by Welch's t-test. Primer sequences and PCR conditions are listed in Supplemental Table 1.

Rod re-specification assay

To determine whether photoreceptors adopt bipolar or glial identities, we crossed *NRL-GFP* (Akimoto et al., 2006) and *Blimp1 CKO* animals to generate heterozygous control and *Blimp*1 CKO mice carrying the *NRL-GFP* transgene. Eyes were collected at P1, P4, P7, and P14 for immunohistochemistry. We examined 3 animals (6 eyes) for both control and experimental conditions at P1 and P7. We also examined 1–2 animals (2–4 eyes) for each condition at P4 and P14. Sections were immunostained for GFP and bipolar- and glial-specific markers. Since α*Pax6-Cre-GFP* mice express GFP in some amacrine cells (Marquardt et al., 2001; Yaron et al., 2006), we also examined P3 and P7 *Blimp1 CKO* mice that lacked the *NRL-GFP* transgene to rule out an amacrine source for GFP labeled bipolar cells and glia.

Birthdating

For EdU (5-ethynyl-2'-deoxyuridine) birthdating, *Blimp1 CKO* mice were bred to heterozygous controls and pregnant dams were administered a single pulse of EdU (200 μg) intraperitoneally at E13.5, E15.5, or E17.5. For BrdU (5-bromo-2'-deoxyuridine) studies, pregnant dams were given a single pulse of BrdU (100 μg/g body mass) at E15.5 and E17.5. We also administered a single subcutaneous dose of BrdU (100 μg/g) to P4 control and *Blimp1* CKO pups. In all cases, animals were allowed to develop to three weeks of age and their eyes were harvested for histology. For EdU detection, slides were immunostained and then incubated with Click-iT fluorescent reagents according to the manufacturer's instructions (Life Technologies). BrdU was detected as previously described (Brzezinski et al., 2012). We quantified 3–6 EdU injected *Blimp1* heterozygous control and *Blimp1 CKO* experimental animals at each time-point (N>11 400× fields each). A Mann-Whitney test was used to evaluate statistical differences between control and experimental animals. One to two BrdU injected animals were quantified from each time-point and genotype.

Results

Blimp1+ cells contribute to multiple cell fates in the retina

Previous results demonstrate that Blimp1 inhibits bipolar cell development in Otx2+ cells (Brzezinski et al., 2010; Katoh et al., 2010), however, it remains unclear how the choice between photoreceptors and bipolar cells is initially made. In principle, Blimp1 could be expressed in Otx2+ cells that are already committed to photoreceptor fate, stabilizing photoreceptor identity. Blimp1 expressing cells should exclusively adopt photoreceptor fate in this model. Alternatively, Blimp1 may be expressed by all Otx2+ cells and then silenced within a subset to allow bipolar fate commitment. If so, Blimp1+ cells should adopt photoreceptor and bipolar identities. To distinguish between these possibilities, we conducted an expression fate mapping experiment using *Blimp1-Cre* mice (Ohinata et al., 2005) and multiple *Lox-Stop-Lox* reporter mouse lines to indelibly mark cells that express *Blimp1* during development.

Blimp1-Cre transgenic mice were previously shown to recapitulate Blimp1 expression in primordial germ cells and in several other cell types (Harper et al., 2011; Horsley et al., 2006; Ohinata et al., 2005; Robertson et al., 2007). To demonstrate specificity of this line in the retina, we examined transgenic mice with antibodies to Cre and Blimp1 at two developmental timepoints (Fig. 1). At E14.5, during photoreceptor development but before bipolar cell genesis, Cre+ cells always co-expressed Blimp1 (Figs 1A–C). At P0, when both photoreceptors and bipolar cells are being generated, Cre+ cells also co-expressed Blimp1 (Figs 1D–F). The Blimp1+ population tended to be larger than the Cre+ population (Fig. 1), which could be caused by incomplete transgene expression or by differences in antibody sensitivity or protein stability. Together, these results demonstrate that *Blimp1-Cre* largely recapitulates the Blimp1 spatial and temporal expression pattern during retinal development.

Next, we crossed *Blimp1-Cre* mice to *Lox-Stop-Lox* reporter strains to permanently label cells that expressed *Blimp1* (Fig. 2). We first examined *mTmG* mice, which express membrane-GFP upon Cre-mediated recombination. We examined mice at P10, shortly after the completion of cell fate choice in the retina. We observed staining of the retina and the retinal vasculature (Fig. 2A), as predicted from prior experiments (Brzezinski et al., 2010; Katoh et al., 2010; Robertson et al., 2007; Vincent et al., 2005). GFP staining was evident in all photoreceptors and in subsets of cells of the inner retina (Fig. 2A). To determine the frequency of each cell type that expressed Blimp1 during development, we crossed *Blimp1-Cre* mice to *ROSA-nGFP* mice to discretely mark nuclei with GFP. *Blimp1-Cre;ROSA-nGFP* mice were examined at three weeks of age with multiple cell type-specific markers and the percentage of each cell type quantified (Figs 2B–I). We observed GFP-labeled cells throughout the retina and the staining intensity and subcellular localization of GFP was variable. Nuclear staining was often punctate in the inner retina (Figs 2B–H), as seen in other tissues from *ROSA-nGFP* mice (Stoller et al., 2008). The reason for these staining differences remains unclear, nonetheless, the overall labeling pattern matches the *Blimp1-Cre;mTmG* lineage traced mice (Fig. 2A). Otx2 was used to identify all photoreceptors (faint) and bipolar cells (intense) (Nishida et al., 2003). As expected, nearly every Otx2+ photoreceptor was GFP+ (Figs 2B,I). We also observed that about a third of intensely Otx2+ + bipolar cells co-expressed GFP+ (Figs 2B,I). Chx10 intensely marks rod bipolars and subset of cone bipolars in the mature retina (Clark et al., 2008; Shi et al., 2011). About one third of Chx10+ bipolar cells co-expressed GFP (Figs 2C,I). Blimp1+ cells generated the same percentage of Otx2++ (pan) and Chx10+ (subsets) bipolars, suggesting that bipolar labeling is stochastic instead of subtype-specific. To further test this, we examined P11 *Blimp1-Cre;ZEG* mice with additional bipolar-subtype markers (Vsx1, Scgn, and PKC) (Supplemental Fig. 1). Blimp1+ cells gave rise to subsets of Vsx1+ cone bipolars (types 1, 2, and 7) (Shi et al., 2011), Scgn+ cone bipolars (all but types 1 and 7) (Puthussery et al.,

2010), and PKC+ rod bipolars (Greferath et al., 1990) (Supplemental Fig. 1). These data show that Blimp1+ cells do not solely give rise to particular bipolar subtypes. Next, we examined Müller glia with Sox2 antibodies (Taranova et al., 2006). GFP staining was especially intense in glia (Figs 2D, F). Nonetheless, GFP+ glia accounted for less than 1% of the total Müller population (Figs 2D,F,I). We also observed many GFP+ horizontal and amacrine cells (Figs 2E–G). Roughly half of the calbindin+ horizontal cells (Peichl and Gonzalez-Soriano, 1994) were intensely GFP+ (Figs 2E,I). Like bipolar cells, about one third of the inner nuclear layer (INL) amacrine cells, marked with Pax6 or AP2α (Bassett et al., 2007; de Melo et al., 2003), were GFP+ (Figs 2F,G,I). The same percentage of displaced AP2α+ amacrine cells in the ganglion cell layer (GCL) were GFP+ (Figs 2G,I). Amacrine cells also appeared to be labeled stochastically, since calbindin+, Sox2+, and AP2α+ amacrine subpopulations (Bassett et al., 2007; Cherry et al., 2009; Haverkamp and Wassle, 2000; Surzenko et al., 2013; Taranova et al., 2006) were each partially GFP labeled (Figs 2D, E, G). Similar results were seen with Prox1 and calretinin labeled amacrine subpopulations (Cid et al., 2010; Dyer et al., 2003; Haverkamp and Wassle, 2000) (Supplemental Fig. 1). Labeling of horizontals and amacrines must have occurred in the embryonic retina since both of these cell types were observed in newborn *Blimp1-Cre;mTmG* mice (Supplemental Fig. 2). The lack of Cre expression in amacrine cells suggests that they derive from cells that transiently express Cre (Fig. 1, Supplemental Fig. 2). Lastly, we examined RGCs with pan-Brn3 antibodies (Xiang et al., 1995). RGCs, like glia, were essentially absent from *Blimp1-Cre;ROSA-nGFP* mice (Figs 2H–I, Supplemental Fig. 2).

These Blimp1 expression fate mapping experiments suggest the existence of three populations of cells. One population consists of cells where Blimp1 (Cre) expression is high and/or sustained, resulting in ~100% reporter labeling. This accounts for rod and cone photoreceptors. The second population consists of cells that weakly and/or transiently express Blimp1, which generate incomplete and stochastic labeling patterns. Bipolar, horizontal, and amacrine interneurons fall into this group. Lastly, RGCs and Müller glia derive from a population of cells that do not express Blimp1.

Otx2 binding of the *Chx10* bipolar-specific enhancer precedes Chx10 expression

The Blimp1 expression fate mapping experiments suggest that Otx2+ cells are competent to form photoreceptors and bipolar cells simultaneously. If Otx2 establishes photoreceptor and bipolar cell competence, it may “prime” genes required for both cell types. These primed loci would require Otx2 and the action of transcriptional co-activators and co-repressors to ensure proper gene regulation and cell fate outcome. A bipolar-specific enhancer was recently characterized for *Chx10* (Kim et al., 2008). This element is 17.7kb upstream of the transcription start site and contains an evolutionarily conserved Otx2 binding site that when mutated, largely prevents bipolar-specific expression (Kim et al., 2008). Chx10 is not expressed in bipolar cells until P4, but is seen as early as P0 in *Blimp1* CKO mice (Brzezinski et al., 2010), suggesting that Otx2 primes the bipolar-specific enhancer of *Chx10*. To test this, we conducted Otx2 ChIP on wild-type retinas.

We first optimized Otx2 ChIP on the *Irbp* (*Rbp3*) proximal promoter, which was previously shown to bind Otx2 in the retina (Peng and Chen, 2005). We observed strong immunoprecipitation of this site using Otx2 antibodies in P1 retina, but saw no enrichment with control goat IgG (Fig. 3A). We then examined the *Chx10* bipolar-specific enhancer. Goat IgG was unable to pull-down the *Chx10* enhancer, but Otx2 immunoprecipitated the site well (Fig. 3A). To control for specificity of Otx2 immunoprecipitation, we also examined two evolutionarily conserved non-coding genomic regions upstream of *Otx2* and *Id3*, which lack consensus Otx2 binding sites (Bunt et al., 2011). As expected, Otx2

antibodies failed to pull-down these genomic sites (Fig. 3A). With the Otx2 ChIP validated, we then compared Otx2 pull-downs of the *Chx10* bipolar-specific enhancer from different aged retinas by quantitative PCR. Otx2 ChIP showed significant (t-test, $P<0.05$) enrichment over goat IgG ChIP at E18.5, P1, and in adult retinas (Fig. 3B). Due to the paucity of Otx2+ cells, we were unable to determine whether binding occurred earlier than E18.5. Regardless, these data show that a bipolar-specific enhancer is bound by Otx2 several days in advance of its activation.

Photoreceptors are re-specified into bipolars and glia in the absence of *Blimp1*

In the absence of *Blimp1*, photoreceptors form normally until about birth (Supplemental Fig. 3) (Brzezinski et al., 2010). Definitive photoreceptor markers, such as opsins, then disappear as bipolar markers increase (Brzezinski et al., 2010). In our previous analysis of *Blimp1* CKO mice, we did not observe significant changes in cell death or the number of Otx2+ cells between P0 and P7 (Brzezinski et al., 2010). Blimp1 may act by preventing bipolar competence from persisting in recently specified photoreceptors. If so, previously specified photoreceptors that lack *Blimp1* may be re-specified as bipolar cells in the early postnatal period. This would manifest as cells that expresses photoreceptor- and bipolar-specific markers simultaneously. As fate-transition events are likely brief, we used *NRL-GFP* mice to specifically label rods with GFP upon their specification (Akimoto et al., 2006). GFP has a relatively long half-life, which provides a much longer time window to identify transitioning cells (GFP+/Bipolar marker+).

We first examined the peripheral retinas of *Blimp1* CKO and heterozygous control mice carrying the *NRL-GFP* transgene at P1 (Figs 4A–D). Control mice had GFP+ rods near the scleral surface and a separate population of GFP labeled amacrine cells near the GCL (Figs 4A,C). These GFP+ amacrines are presumably the result of α*Pax6-Cre-GFP* transgene expression (Marquardt et al., 2001; Yaron et al., 2006). At P1, control mice lacked Chx10+ bipolars and Vsx1+ cells (Figs 4A,C). In contrast, *Blimp1* CKO mice had many Chx10 and Vsx1 labeled cells (Figs 4B,D) at this age. Cells co-expressing NRL-GFP and Chx10 or Vsx1 were abundant in all mutant animals examined (Figs 4B,D) (Supplemental Table 2). Similar results were observed in P4 mice (not shown). We also examined P1 mice with the bipolar subtype marker Scgn (Secretagogin), which is expressed in most cone bipolars (Puthussery et al., 2010). Scgn was not expressed in control retinas, but was present in *Blimp1* CKO mice, where it overlapped with a subset of NRL-GFP+ cells (Supplemental Fig. 4). We next examined mice at the end of bipolar cell genesis (Figs 4E–G). By P7, control mice had many Chx10+ bipolar cells and NRL-GFP+ rods, but the Chx10+ bipolar cells did not co-express NRL-GFP (Fig. 4E) (Supplemental Table 2). In contrast, *Blimp1* CKO mice had far fewer NRL-GFP+ rods and more abundant and widespread Chx10 labeling (Fig. 4F). Cells that co-expressed Chx10 and GFP were frequently observed in all *Blimp1 CKO* animals examined (Fig. 4F) (Supplemental Table 2). To rule out the possibility that amacrine cells labeled by the α*Pax6-Cre-GFP* transgene express Chx10, we examined *Blimp1* CKO mice that did not carry the *NRL-GFP* transgene. None of the GFP+ amacrine cells co-expressed Chx10, demonstrating that double labeled cells express NRL-GFP and not αPax6-Cre-GFP (Fig. 4G). We also examined P14 mice, several days after neurogenesis is complete. NRL-GFP+ cells only co-expressed Chx10 in *Blimp1* CKO mice (Supplemental Fig. 4). These double labeled cells were much less abundant (~20%) at P14 compared to P1 and P7 *Blimp1 CKO* retinas (Supplemental Table 2), consistent with our previous observations on *Blimp1* mutants (Brzezinski et al., 2010). Together, these new data suggest that existing rods are re-specified as bipolar cells in the absence of *Blimp1*.

We previously observed excess Müller glia in *Blimp1* CKO mice (Brzezinski et al., 2010). These cells had glial morphology and expressed definitive markers; nonetheless, they often

co-expressed Chx10 and/or Otx2 (Brzezinski et al., 2010). Thus, these glia may inappropriately derive from previously specified photoreceptors or bipolar cells. To test this, we examined glia in heterozygous control and *Blimp1* CKO mice carrying the *NRL-GFP* transgene (Figs 4H–J). At P7, control mice did not have any NRL-GFP+ rods that co-expressed the glial marker, Sox9 (Poche et al., 2008) (Fig. 4H). In contrast, we observed a small number of NRL-GFP+/Sox9+ cells in every *Blimp1* CKO retina we examined (Fig. 4I) (Supplemental Table 3). The αPax6-GFP+ amacrine cells in *Blimp1* CKO mice never co-expressed glial markers (Fig. 4J). These data suggest that without *Blimp1*, photoreceptors can also be re-specified as Müller glia.

Bipolars and Müller glia are born precociously in Blimp1 mutants

Rods are generated throughout retinal development while bipolars and glia are generated postnatally. The *NRL-GFP* experiments suggest that *Blimp1* CKO rods are re-specified as bipolar cells and glia. If true, this implies that these cell types would be born during the period of rod genesis in *Blimp1* CKO mice. To test this, we conducted EdU birthdating of control heterozygous and *Blimp1* CKO mice at three embryonic time-points (E13.5, E15.5, and E17.5) that precede bipolar genesis (Fig. 5). Adult retinas were co-labeled with Chx10 and Sox9 antibodies to specifically mark bipolar (Chx10+/Sox9−) and glial (Sox9+) cells. We examined the peripheral retinas, the affected region of *Blimp1* CKO mice. We first examined mice given EdU at E13.5, which birthdates the earliest photoreceptors of the peripheral retina. In control animals, E13.5 birthdated cells (EdU+) rarely, if ever, contributed to bipolar or glial fates (Figs 5A,C). In stark contrast, E13.5 birthdated cells in *Blimp1* CKO mice gave rise to both bipolar cells and Müller glia (Figs 5B–C). While modest in absolute number, these cells were seen in every retina we examined and were significantly more abundant compared to controls (Mann-Whitney test, $P<0.01$). E15.5 birthdated control cells rarely, if ever, formed bipolars or glia (Figs 5D,F). Birthdated bipolars and glia were again seen in all *Blimp1* CKO mice (Figs 5E–F). By E17.5, birthdated bipolars and glia were seen in some control animals, but remained rare overall (Figs 5G,I). There were significantly more E17.5 birthdated bipolars and glia (Mann-Whitney test, $P<0.05$) in *Blimp1* CKO mice (Figs 5H–I). Birthdated glia in the mutants always expressed Chx10. To complement the EdU experiments, we also conducted BrdU birthdating and examined adult retinas with Otx2 or Sox9 antibodies (Supplemental Fig. 5). As before, there were E15.5 and E17.5 birthdated bipolars (Otx2++) in *Blimp1* CKO mice (Supplemental Fig 5). We also examined mice that received BrdU injections at P4, during normal bipolar and glial genesis. We saw no differences in the number of birthdated Otx2++ or Sox9+ cells at this age between *Blimp1* CKO and control mice (Supplemental Fig. 5). This suggests that excess bipolar and glial cells seen in adult *Blimp1* mutants derive from cells that would not have normally adopted these fates.

Together, these data show that bipolars and glia in *Blimp1* CKO mice can be generated from cells born as early as E13.5. This is several days in advance of precocious bipolar marker expression and corresponds to the earliest peripheral photoreceptor birthdates.

Discussion

We investigated the mechanisms of photoreceptor and bipolar cell fate determination by examining Blimp1, a transcription factor expressed by Otx2+ cells that inhibits bipolar development (Brzezinski et al., 2010; Katoh et al., 2010). Blimp1+ cells could give rise to both photoreceptors and bipolars, arguing that Blimp1 is normally silenced to allow Otx2+ cells to adopt bipolar cell fate. Consistent with this hypothesis, we found that; (1) Otx2 binding to at least one bipolar-specific enhancer preceded bipolar genesis, (2) bipolar cells could be generated well in advance of their normal genesis period in *Blimp1* CKO mice, and

(3) that rods could become re-specified as bipolar cells in postnatal *Blimp1 CKO* mice. Together, these data suggest that a cross-repressive network is established in Otx2+ cells, which results in the stabilization of either photoreceptor or bipolar cell identity. In this cross-repressive network, Blimp1 acts by restricting bipolar cell competence.

A cross-repression model for photoreceptor versus bipolar fate choice

Expression fate mapping experiments showed that Blimp1+ cells gave rise to all photoreceptors and a subset of bipolar cells. Bipolar labeling appeared stochastic, as there was no gross bipolar subtype preference or exclusion. These observations argue that bipolar cell labeling is the result of weak and/or transient Cre activity (see below). Otx2 likely activates a gene regulatory network containing factors that antagonize one another to establish a bistable state. The winner of these cross-repressive interactions would stabilize either photoreceptor or bipolar cell identity (Fig. 6A). An unknown factor "X" represses Blimp1 and photoreceptor formation while Blimp1 does the opposite (Figs 6A,B). Chx10 (Vsx2) is a strong candidate for factor "X" because: (1) Chx10 expression is positively regulated by Otx2 (Kim et al., 2008), (2) Chx10 is required for bipolar cell formation (Burmeister et al., 1996; Green et al., 2003), (3) Chx10 is precociously upregulated in the absence of *Blimp1* (Brzezinski et al., 2010), (4) *Chx10* appears to be a direct regulatory target of Blimp1 (Katoh et al., 2010), and (5) because Chx10 can directly repress photoreceptor-specific genes (Dorval et al., 2006; Livne-Bar et al., 2006). However, Chx10 is not normally expressed in bipolar cells until P4, which is several days after bipolar cells are first born (~P0). This delay suggests that Chx10 may act to prevent photoreceptor gene networks from superseding bipolar programs (Dorval et al., 2006), while not directly instructing bipolar cell fate. Thus, Blimp1 and Chx10 would restrict bipolar and photoreceptor competence, respectively.

Fate mapping also revealed that Blimp1 expressing cells give rise to one third to one half of horizontal and amacrine interneurons, but few RGCs or Müller glia. This suggests that horizontals and amacrine cells weakly and/or transiently express Blimp1 during development. Alternatively, this could be interpreted as inappropriate spatial and/or temporal *Blimp1-Cre* transgene expression. This does not appear to be the case for several reasons. First, Blimp1-Cre was only observed in Blimp1+ cells. Second, radial clones were not observed at any time-point, arguing against stochastic Cre activation in proliferative progenitor cells. Third, transient Otx2 expression by developing amacrine cells has been suggested to occur in rodents (Baas et al., 2000; Das et al., 2009). Fourth, the paucity of labeled RGCs and Müller glia argues against random recombination. Since RGCs and horizontal cells are generated at the same time and in similar numbers (Jeon et al., 1998; Rapaport et al., 2004), they would have the same labeling frequency if progenitors were to inappropriately express *Blimp1-Cre*. The same situation applies to glia and bipolar cells. Lastly, horizontal and amacrine neurons were labeled during development, indicating temporal accuracy of the transgene. Taken together, these data strongly argue that *Blimp1-Cre* accurately reflects *Blimp1* expression during development.

Without *Blimp1*, instructive factors cause photoreceptors to transdifferentiate into bipolar cells

Blimp1 CKO mice have no visible changes in photoreceptor development until around birth, when bipolar genes are precociously activated. In our previous analysis, we did not observe excess apoptosis of early generated photoreceptors (Brzezinski et al., 2010), raising the possibility that photoreceptors partially differentiate and then directly become re-specified (transdifferentiate) as bipolar cells. To test this, we examined the fate of NRL-GFP+ rods. Strikingly, we observed that NRL-GFP+ cells co-expressed multiple bipolar markers in *Blimp1 CKO* mice. These overlapping cells were abundant during bipolar formation (P1, P4,

P7), but much less common after neurogenesis was complete (P14). These data have several implications. First, the lack of NRL-GFP overlap in control mice indicates that bipolar cells do not normally express Nrl, even transiently. Though, if the period of Nrl expression was sufficiently brief, NRL-GFP might not be detectable. Second, the presence of NRL-GFP+ cells that co-express bipolar markers in *Blimp1* CKO mice suggests that photoreceptors become re-specified as bipolar cells. Third, the presence of NRL-GFP+ bipolar cells after P4 suggests that many (perhaps all) rods are specified normally in *Blimp1* CKO mice, but their identity is rapidly superseded by pro-bipolar signals present in the postnatal retina. Together, these results suggest that bipolar cell competence persists for several days in *Blimp1* CKO photoreceptors.

We observed that the *Chx10* bipolar enhancer (Kim et al., 2008) was bound by Otx2 at E18.5, about 5 days in advance of bipolar-specific Chx10 expression. Thus, additional activators (instructive factors) and/or the loss of inhibitors (competence restrictors) are needed for *Chx10* activation and bipolar cell identity. Competence restriction could be due to Blimp1, which can repress Chx10 directly (Katoh et al., 2010). This implies that embryonic Otx2+ cells would become bipolar-competent in the absence of *Blimp1*. We observed that bipolar cells in the peripheral retinas of *Blimp1 CKO* mice were born as early as E13.5, demonstrating an embryonic shift in bipolar competence. Nonetheless, bipolar markers were not present until ~P0, indicating that bipolar instructive factors are not present in the embryonic retina. Without *Blimp1*, early born photoreceptors retain bipolar competence until instructive factors are present, causing transdifferentiation to bipolar fate.

It is unclear how the fate balance is shifted in postnatal Otx2+ cells to allow bipolar genesis. One possibility is that an unknown factor is required to drive *Blimp1* expression and this activator becomes down-regulated in the postnatal retina, allowing bipolar genesis (Fig. 6B). Another possibility is that the combination of bipolar instructive factors and Otx2 is sufficient to overwhelm Blimp1-mediated repression of *Chx10* in some Otx2+ cells (Fig. 6B). Higher *Chx10* expression levels would then shift the equilibrium toward bipolar fate. The nature of bipolar instructive factors is unclear. These factors could reflect the intrinsic properties of late-stage progenitors (Cayouette et al., 2003; Reh and Kljavin, 1989) or be the result of environmental signaling events present only in the postnatal retina.

Blimp1 CKO mice have a modest increase in the number of Müller glia, which often co-expressed Chx10 and/or Otx2 (Brzezinski et al., 2010). We speculated that some of these glia may be derived from photoreceptors. Accordingly, some Müller glia were NRL-GFP labeled in *Blimp1 CKO* mice. Glia in *Blimp1 CKO* mice were also birthdated as early as E13.5. These data suggest that excess Müller glia in *Blimp1* mutants are derived from Otx2+ cells, some of which were previously specified as photoreceptors. This is contrary to our expression fate mapping experiments, which showed that Blimp1+ (and thus Otx2+) cells rarely adopted glial fate. Photoreceptors may be susceptible to glial reprogramming, such that Blimp1 has an additional role in maintaining glial restriction or making it permanent.

Fate diversification in the retina

While the seven major retinal cell types are generated in a stereotypical sequence, there is considerable overlap such that multiple cell types form concurrently throughout development (La Vail et al., 1991; Rapaport et al., 2004; Sidman, 1961; Young, 1985). Given this condition, progressive competence restriction within molecularly distinct subpopulations of retinal progenitors is a mechanism that could profoundly influence cell fate choice. Two such populations of competence-restricted progenitors have been recently characterized by expression fate mapping techniques. Progenitors that express Ascl1 do not give rise to RGCs, but can adopt the remaining six cell fates (Brzezinski et al., 2011). Olig2 expressing progenitors are able to form all cell types except for RGCs and Müller glia

(Hafler et al., 2012). Notably, this is the same pattern that we observe in the Blimp1 expression fate mapping experiments. Since all Blimp1+ cells express Otx2+, it is possible that Otx2+ cells derive from progenitors that have become sequentially competence-restricted, having passed through Ascl1 and Olig2 positive states (Fig. 6C). This suggests that Otx2 is expressed broadly and becomes; (1) stabilized in the subset of cells that will adopt photoreceptor and bipolar fates or (2), repressed quickly in the subset of cells that will adopt horizontal and amacrine fates (Fig. 6C). Prior histological studies show that Blimp1 expression rapidly follows Otx2 (Brzezinski et al., 2010; Katoh et al., 2010). Blimp1 expression would thus be weak and/or short-lived in those cells fated to become interneurons (Fig. 6C), consistent with the stochastic labeling we observed in bipolars, horizontals, and amacrines.

It is unclear how the decision to stabilize Otx2 expression is made. Otx2 is activated during or shortly after the terminal cell cycle (Koike et al., 2007). It is possible that Otx2 is inherited asymmetrically during the terminal division. This would allow for diversification, but most divisions would have to be symmetric due to the superior numbers of photoreceptors and bipolars compared to horizontals and amacrines (Jeon et al., 1998). Another possibility is that Otx2 promotes its own expression via an autoregulatory feedback loop. Thus, stochastic differences in the initial levels of Otx2 could determine whether it becomes stabilized or not. Lastly, it is possible that a bistable state exists between Otx2 and another transcription factor (Fig. 6C). One intriguing candidate is Foxn4, which is expressed by a subset of retinal progenitors and is required for horizontal and amacrine cell competence (Li et al., 2004). Foxn4 can promote Notch signaling via Dll4 regulation, which has been shown to inhibit photoreceptor formation (Luo et al., 2012). While *Foxn4* mutants have more photoreceptors, *Otx2* and *Blimp1* expression are lower at E14.5, suggesting that Foxn4 does not repress *Otx2* directly (Li et al., 2004; Luo et al., 2012). Moreover, though *Otx2* mutants have more amacrine cells (Nishida et al., 2003; Sato et al., 2007), it is unclear whether *Otx2* silences *Foxn4* or its critical target, *Ptf1a* (Fujitani et al., 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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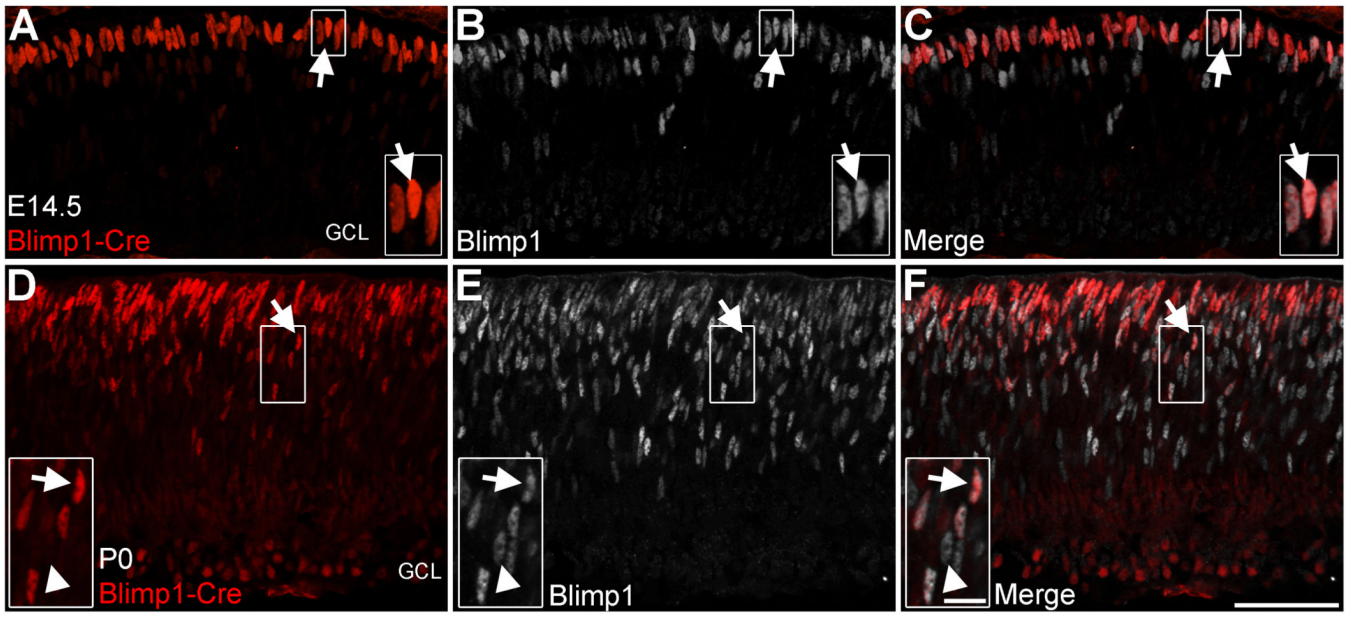

Figure 1.

Blimp1-Cre recapitulates Blimp1 expression in the retina. **(A–C)** Retinal sections from E14.5 *Blimp1-Cre* mice immunostained with antibodies to Cre (red) and Blimp1 (grey). All Cre+ cells are Blimp1+ (arrows). The Cre immunostaining appears less sensitive than Blimp1. **(D–F)** *Blimp1-Cre* mice examined at P0. Cre+ cells are Blimp1+ (arrows). A small number of Blimp1+ cells are Cre-negative (arrowheads). Non-specific staining is seen in the GCL. GCL, ganglion cell layer. Scale bars are 50 μm for all panels and 10 μm for insets.

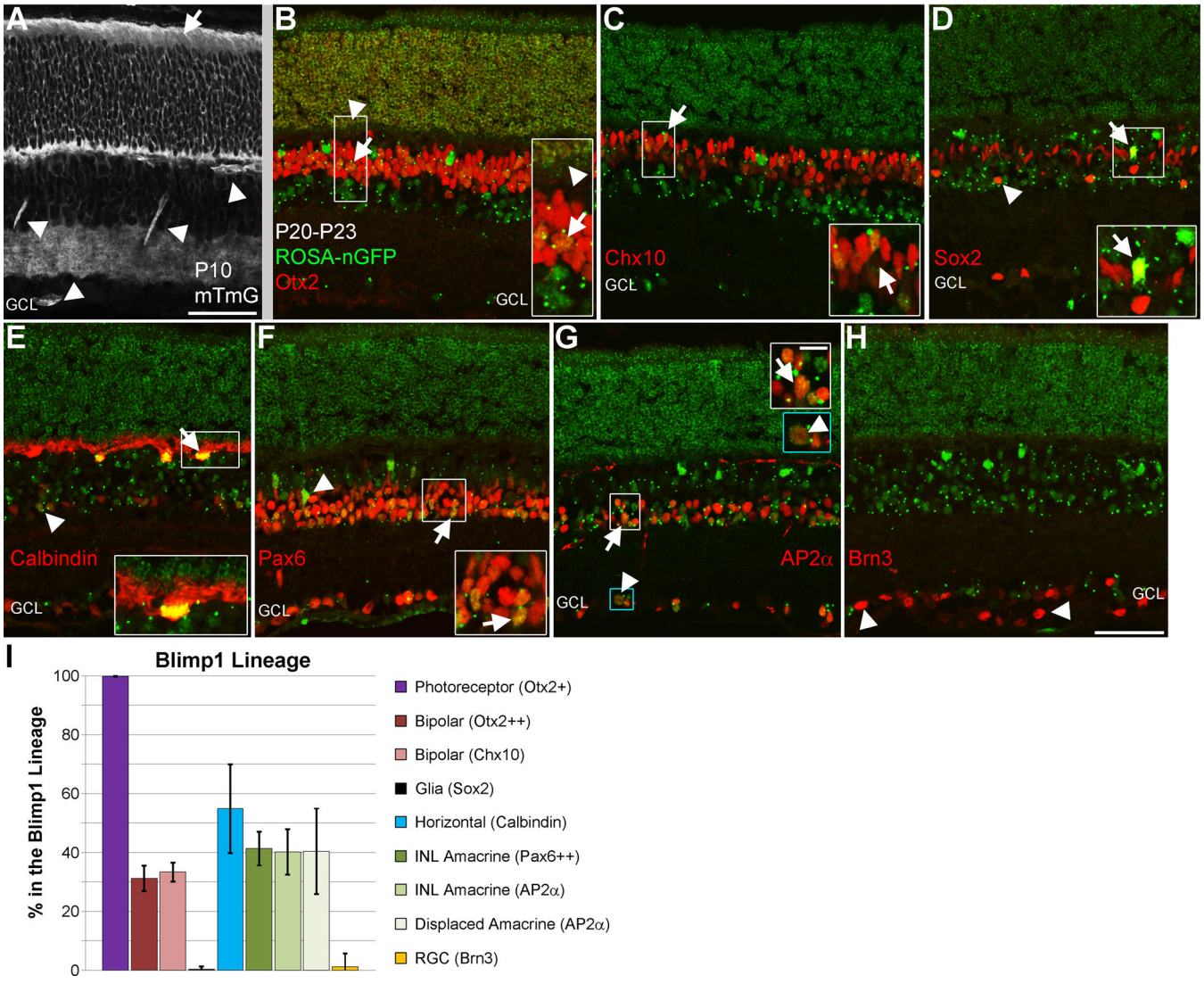

Figure 2.

Blimp1+ cells generate multiple cell types. *Blimp1-Cre* mice were crossed with *mTmG* (A) or *ROSA-nGFP* (B–H) reporter mice to permanently mark cells that expressed *Blimp1* during development. **(A)** Retinal sections from P10 *Blimp1-Cre;mTmG* mice immunostained for GFP. Photoreceptor inner and outer segments (arrow) and blood vessels (arrowheads) are conspicuous. **(B–H)** Retinal sections from approximately 3 week old *Blimp1-Cre;ROSA-nGFP* mice immunostained with GFP (green) and cell type-specific markers (red). GFP staining is overwhelmingly nuclear, but varies from intense to punctate. Occasionally, GFP+ puncta are located outside the nucleus. **(B)** Nearly all photoreceptors (Otx2+) are GFP+ (arrowhead) and about one third of bipolars (Otx2++) express GFP (arrows). **(C)** About one third of Chx10+ bipolar cells are GFP+ (arrows). **(D)** Few Sox2+ glia (elongated nuclei, middle-INL) co-express GFP (arrows). Glia are intensely GFP labeled and signal is seen outside the nucleus. A subset of Sox2+ amacrine cells are GFP+ (arrowhead). **(E)** About half of the calbindin+ horizontal cells strongly co-express GFP (arrows). A subset of calbindin+ amacrines is GFP+ (arrowheads). **(F)** About one third of Pax6+ amacrine cells (bright round nuclei, inner-INL) are GFP+ (arrows). An arrowhead marks a Pax6+/GFP+ Müller cell. **(G)** A large subset of amacrines is marked with AP2α. About one third of INL

(arrows) and GCL (arrowheads) AP2α+ amacrines are GFP+. **(H)** Brn3+ RGCs (arrowheads) are nearly always GFP-negative. INL, inner nuclear layer. Scale bars are 50 μm for A, B–H, and 10 μm for insets. **(I)** Quantification of the *Blimp1-Cre;ROSA-nGFP* expression fate mapping data plotted as percentage of each cell type that co-expresses GFP. Error bars are S.D.

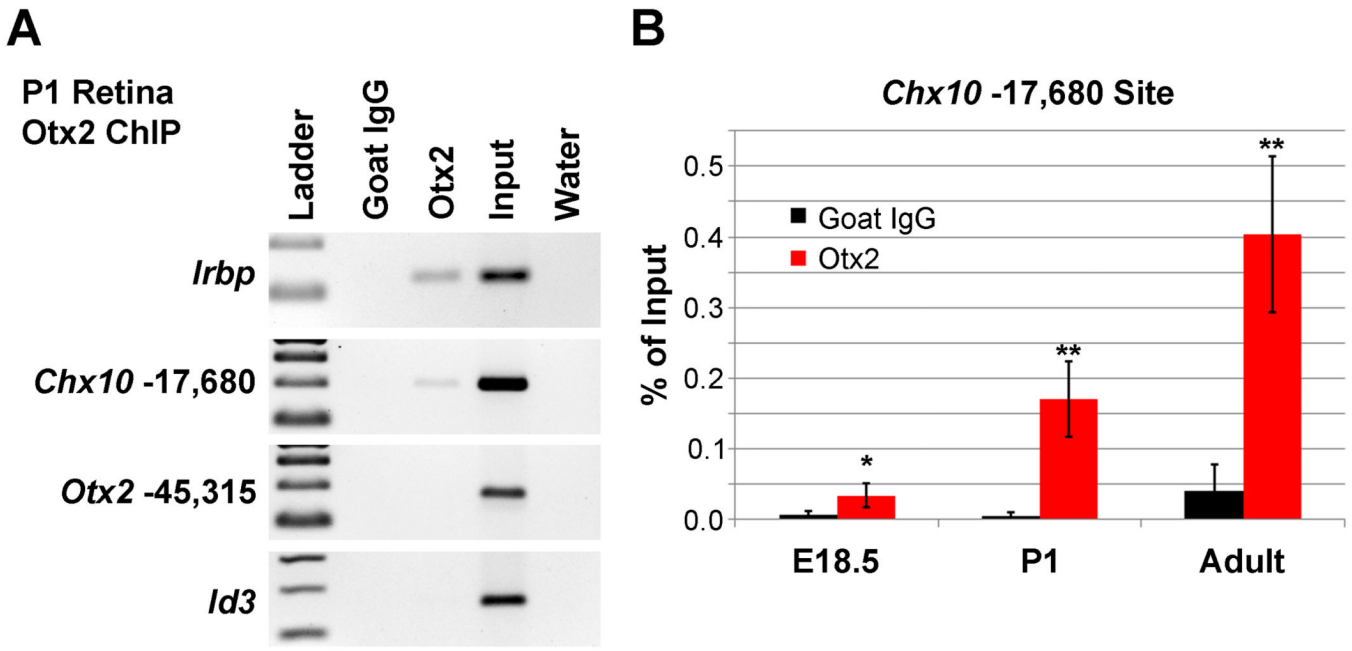

Figure 3.

The *Chx10* bipolar-specific enhancer is primed by Otx2. **(A)** Otx2 and goat IgG ChIP from wild-type P1 retina. Gel images following 36 cycles of PCR. Primers flanking the *Irbp* transcription start site (TSS) serve as a positive control for Otx2 ChIP (top panel). The second panel shows primers that amplify the bipolar-specific enhancer of *Chx10* (17,680bp upstream of the TSS). Primers to conserved non-coding sequences 45kb upstream of the *Otx2* start codon and within the *Id3* promoter are used as specificity controls (bottom two panels). **(B)** Otx2 and goat IgG ChIP from E18.5, P1, and adult wild-type retinas. Quantitative PCR for the *Chx10* enhancer normalized to input levels shows significant enrichment (t-test, * = P<0.05, ** = P<0.001) at each time-point compared to goat IgG control. Bipolar-specific Chx10 expression starts at P4. Error bars are S.D.

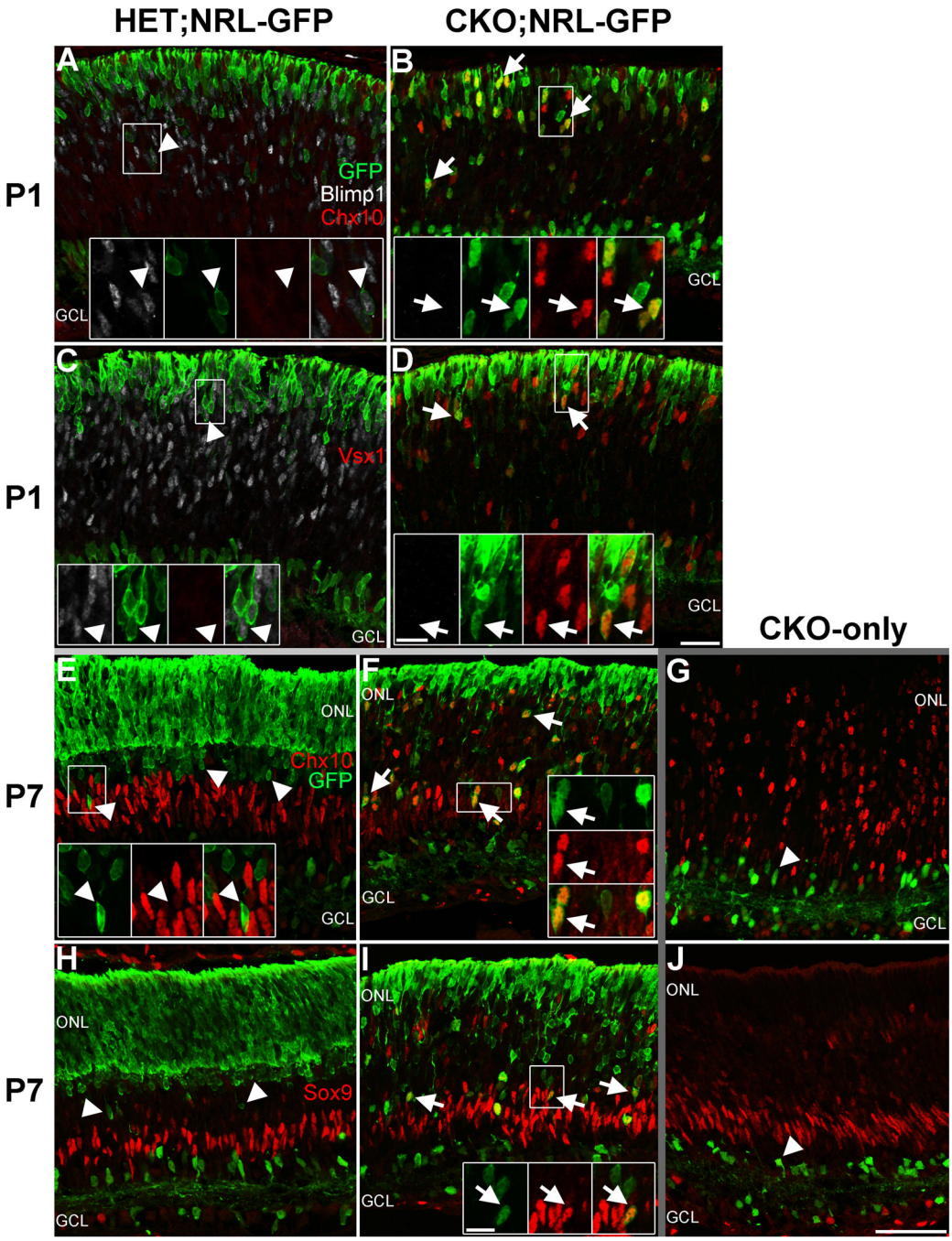

Figure 4.

Photoreceptors are re-specified as bipolar cells and glia. Control and *Blimp1* CKO mice carrying the *NRL-GFP* transgene to specifically label rods. **(A–D)** P1 retinal sections from *Blimp1* heterozygous (A, C) and CKO mice (B, D) immunostained for Blimp1 (grey), Chx10 (red) (A, B) or Vsx1 (red) (C, D), and GFP (green). **(A)** No intensely Chx10 labeled cells (bipolars) are seen in controls. NRL-GFP+ cells that co-express Blimp1 are seen (arrowheads in A, C) as are Blimp1+ cells that do not express GFP. **(B)** In *Blimp1* CKO mice, intensely labeled Chx10 cells are present and frequently (53% ± 5.1% S.D.) co-express GFP (arrows) (Supplemental Table 2). **(C)** Vsx1 is never seen in P1 control animals.

(D) In *Blimp1* CKO mice, a subset of Vsx1+ cells (23.8% ± 5.7% S.D.) co-expresses GFP (arrows). **(E–J)** P7 *Blimp1* heterozygous control (E, H) and CKO mice (F, G, I, J) immunostained for GFP (green) and Chx10 (red) (E–G) or Sox9 (red) (H–J). **(E)** P7 control mice have many NRL-GFP+ rods, including those that have failed to migrate into the ONL. These GFP+ cells do not express Chx10 (arrowheads). **(F)** *Blimp1* CKO mice have fewer NRL-GFP+ rods and more Chx10+ cells. Many Chx10+ cells express GFP (arrows) (9.4% ± 4.2% S.D.). **(G)** *Blimp1* CKO mice without the *NRL-GFP* transgene have many extra Chx10+ cells, none of which overlap with GFP+ amacrine cells marked by the α*Pax6-Cre-GFP* transgene (arrowhead). **(H)** Sox9 labeled Müller glia do not co-express GFP in control animals (arrowheads). **(I)** *Blimp1* CKO mice have Sox9+ cells that co-express GFP (arrows), though these are less frequent than Chx10+/GFP+ cells (F) (Supplemental Tables 2, 3). **(J)** GFP+ amacrine cells (arrowhead) in *Blimp1* CKO mice not carrying the *NRL-GFP* transgene do not co-express Sox9. ONL, outer nuclear layer. Scale bars are 25 μm for panels A–D, 50 μm for E–J, and 10 μm for insets A–D, E–I.

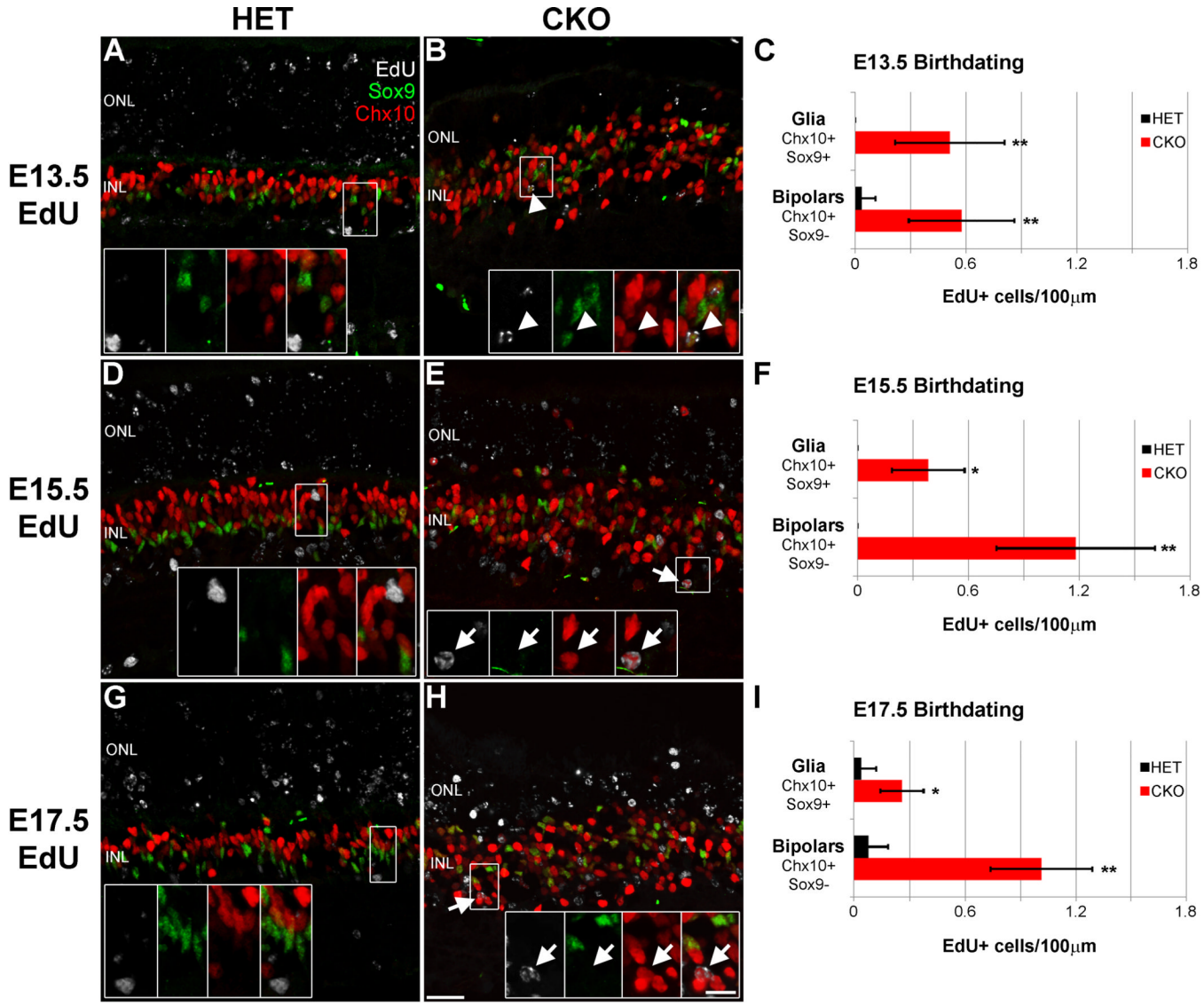

Figure 5.

Bipolars and glia are born precociously in *Blimp1* CKO mice. A single pulse of EdU was administered at E13.5. (A–C), E15.5 (D–F), or E17.5 (G–I) and mice examined around 3 weeks of age. Retinal sections from heterozygous control (A, D, G) and *Blimp1* CKO mice (B, E, H) immunostained for Chx10 (red), Sox9 (green) and developed for EdU (grey). **(A)** E13.5 birthdated control mice lack birthdated bipolar cells (Chx10+/Sox9-) and glia (Sox9+). **(B)** *Blimp1* CKO mice have more Chx10 and Sox9 labeled cells. EdU+ bipolars and glia (arrowheads) are present. **(D, E)** E15.5 birthdated bipolars (arrows) and glia are present in *Blimp1* CKO retinas, but are not seen in controls. **(G)** E17.5 birthdated control retinas rarely and variably contain birthdated bipolars or glia. **(H)** In contrast, EdU+ bipolars (arrows) and glia are seen in every *Blimp1* CKO retina. Scale bars are 25 μm for panels and 10 μm for insets. **(C, F, I)** Quantification of EdU+ bipolars (Chx10+/Sox9−) and glia (Sox9+) in control (black) and *Blimp1* CKO mice (red). Cells are counted in each section and normalized by the horizontal length of the retina imaged (cells/100 μm). **(C, F)** Control values are too low to be visible in the plots. Error bars are S.E.M. (N=3 to 6 mice). Significant differences are evaluated by Mann-Whitney test, * = P<0.05, ** = P<0.005.

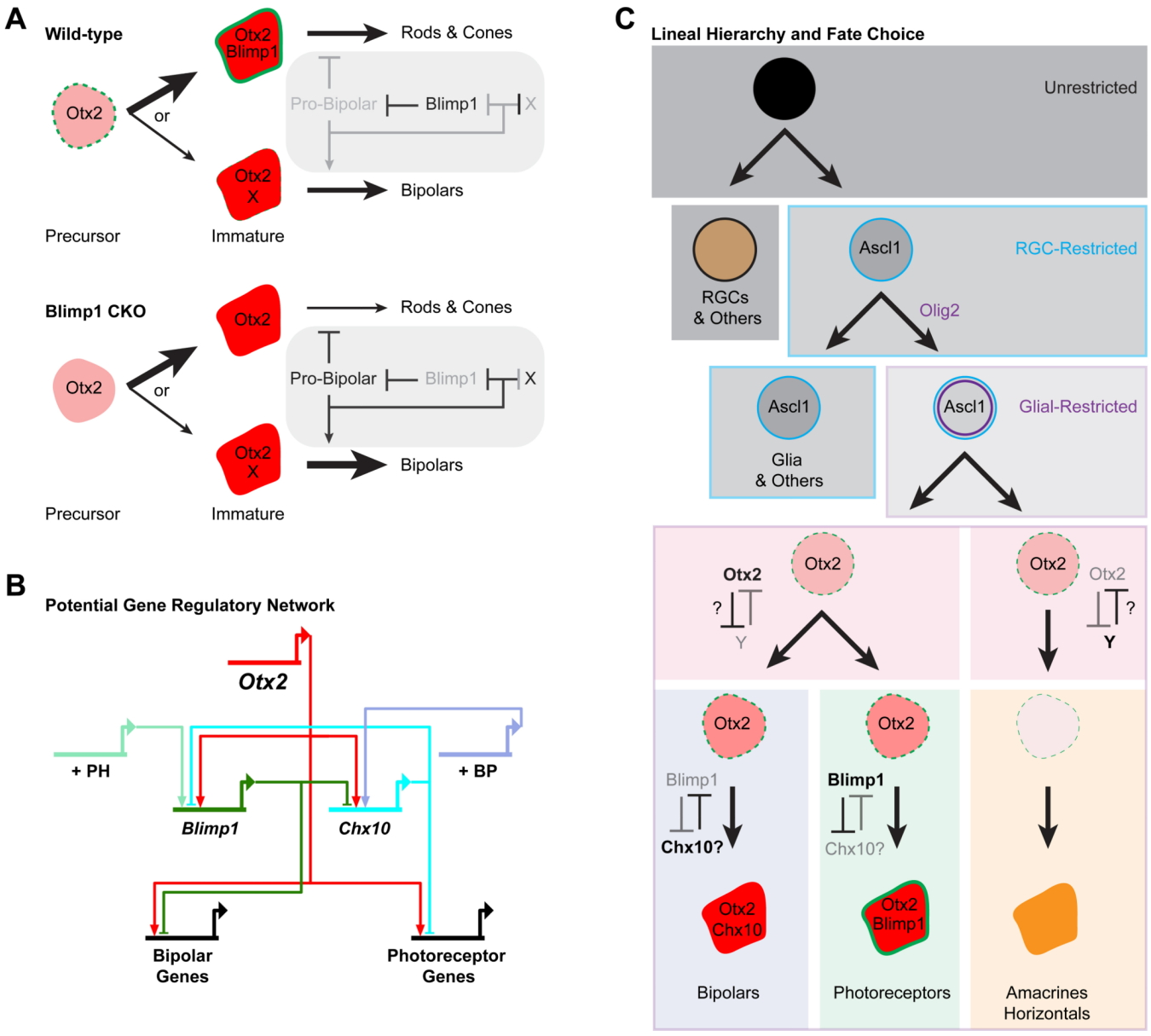

Figure 6.

Retinal cell fate determination models. **(A)** An Otx2+ cell (pink/red) rapidly upregulates Blimp1 (green ring) as it exits the cell cycle. Blimp1 restricts competence to respond to pro-bipolar instructive factors (grey box). A small population of Otx2+ cells silences Blimp1 via unknown factor X (perhaps Chx10) and adopts bipolar fate. Blimp1 and factor X likely form a cross-repressive interaction, the winner stabilizing photoreceptor or bipolar fate, respectively. In *Blimp1* CKO mice (bottom), Otx2+ cells remain competent to respond to bipolar instructive factors leading to a fate shift and/or re-specification (transdifferentiation) of photoreceptors as bipolar cells. **(B)** A potential gene regulatory network for photoreceptor and bipolar cell development. Drawn with BioTapestry software (http://www.biotapestry.org/). Otx2 (red) is required for the activation of Blimp1 (green) and Chx10 (blue). Blimp1 represses Chx10 and bipolar-specific genes. Chx10 represses Blimp1 and photoreceptor genes. This forms a cross-inhibitory circuit where the winner decides the cell fate outcome. Other factors must influence the expression of Blimp1 and Chx10 besides

Otx2. Putative bipolar instructive factors (+ BP, purple) likely co-activate Chx10 along with Otx2. As not all Otx2+ cells in the eye or the embryo express Blimp1, a pro-photoreceptor factor (+ PH, lime) likely co-activates Blimp1. Otx2 may autoregulate its own expression during development (not drawn). **(C)** Competence restriction and retinal fate diversification. A multipotent progenitor (black circle) can give rise to all seven retinal cell fates. Most progenitors lose ganglion cell competence, as marked by Ascl1 expression (blue ring, box). Ascl1-negative progenitors (brown) give rise to RGCs and possibly other cell fates. The Ascl1+ population likely becomes restricted again, losing glial competence, as marked by Olig2 expression (purple ring, box). Blimp1+ cells, and thus Otx2+ cells, are limited to the same fates as Olig2+ cells. It is possible that indirect cross-repressive interactions between Otx2 and other transcription factors (Y) act to further restrict competence.