Ezh2 maintains retinal progenitor proliferation, transcriptional integrity, and the timing of late differentiation

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ABSTRACT

Epigenetic regulation, including histone modification, is a critical component of gene regulation, although precisely how this contributes to the development of complex tissues such as the neural retina is still being explored. We show that during retinal development in mouse, there are dynamic patterns of expression of the polycomb repressive complex 2 (PRC2) catalytic subunit EZH2 in retinal progenitors and some differentiated cells, as well as dynamic changes in the histone modification H3K27me3. Using conditional knockout of Ezh2 using either Pax6-αCre or Six3-Cre, we find selective reduction in postnatal retinal progenitor proliferation, disruption of retinal lamination, and enhanced differentiation of several late born cell types in the early postnatal retina, including photoreceptors and Müller glia, which are ultimately increased in number and become reactive. RNA-seq identifies many non-retinal genes upregulated with loss of Ezh2, including multiple Hox genes and the cell cycle regulator Cdkn2a, which are established targets of EZH2-mediated repression. ChIP analysis confirms loss of the H3K27me3 modification at these loci. Similar gene upregulation is observed in retinal explants treated with an EZH2 chemical inhibitor. There is considerable overlap with EZH2-regulated genes reported in non-neural tissues, suggesting that EZH2 can regulate similar genes in multiple lineages. Our findings reveal a conserved role for EZH2 in constraining the expression of potent developmental regulators to maintain lineage integrity and retinal progenitor proliferation, as well as regulating the timing of late differentiation.

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Introduction

During the development of complex tissues, the balance between proliferation and differentiation of progenitors, and the timing of differentiation is critical to ensure that various cell types are generated in appropriate numbers. The dramatic changes in gene expression driving these events are regulated primarily by transcription factors that are expressed in stage and cell-type dependent manner. This has been well defined in the developing neural retina, where specific sets of transcription factors define retinal progenitor identity and regulate the ordered genesis of distinct retinal cell types (Bassett and Wallace, 2012). But there is increasing evidence that in many systems epigenetic regulation, such as histone modification, also plays an important role. These modifications can serve as gatekeepers for the activity of transcription factors, and thus regulate gene activation or repression (Laugesen and Helin, 2014). While the molecular machinery involved in histone modifications have been well studied, their precise roles during in vivo development are still being explored.

One of the key repressive modifications is the trimethylation of lysine 27 on histone H3 (H3K27me3). The di- and tri-methylation of H3K27 is primarily catalyzed by polycomb repressive complex 2 (PRC2), with the enzymatic activity of the complex mediated by the subunit Enhancer of zeste 2 (EZH2), and in some instances by EZH1 (Margueron and Reinberg, 2011). Polycomb group proteins were originally identified in Drosophila for their role in silencing Hox genes (Schuettengruber et al., 2007), but are now recognized to have broader roles in both invertebrate and vertebrate development, including neural development (Aldiri and Vetter, 2012; Margueron and Reinberg, 2011). In embryonic stem cells (ESCs) PRC2 is required for neural differentiation (Pasini et al., 2007; Shen

et al., 2008), and PRC2 targets many of the genes involved in neurogenesis for H3K27me3 modification (Boyer et al., 2006; Lee et al., 2006).

The in vivo role *of Ezh2* during neural development is complex. During the late phase of neocortical development PRC2 cooperates with PRC1 to restrict the generation of neurons to allow astrocyte production, with *Ezh2* conditional knockouts showing a prolonged neurogenic phase (Hirabayashi and Gotoh, 2010; Hirabayashi et al., 2009). In contrast, early deletion *of Ezh2* in the developing cerebral cortex causes enhanced early neurogenesis, accompanied by earlier astrocyte production and a premature termination of cell proliferation (Pereira et al., 2010). Furthermore, knockdown *of Ezh2* in neural stem cell cultures also leads to a reduction in their proliferation and self-renewal potential and enhanced production of astrocytes rather than oligodendrocytes upon differentiation (Sher et al., 2008). Interestingly, in the neural crest, *Ezh2* is not required for neural development (Schwarz et al., 2014). Thus, *Ezh2* appears to play complex roles in the developing nervous system, and can promote or block differentiation, maintain proliferation, and/or regulate the timing of cell fate acquisition. These diverse developmental roles have also been ascribed to *Ezh2* function in other tissues, including skin, skeletal muscle, heart and lung (Ezhkova et al., 2009; He et al., 2012; Juan et al., 2011; Snitow et al., 2015; Woodhouse et al., 2013). However, it is unclear whether core molecular mechanisms are responsible for these functions in multiple tissues.

The developing neural retina is an excellent system to investigate this question, since progenitor properties and the transcriptional regulation of cell fate determination are well described (Bassett and Wallace, 2012). Furthermore, genome-wide H3K27me3 histone modification has been characterized at several key stages of mouse retinal development (Popova et al., 2012). In *Xenopus*, we previously found that knocking down or blocking the PRC2 core component *Ezh2* reduces retinal neurogenesis and promotes Müller glial cell differentiation (Aldiri et al., 2013). We also observed reduced retinal progenitor proliferation, in part due to upregulation of the Cdk inhibitor p15 (*Ink4b; Cdkn2b*), which is a direct target of EZH2-mediated repression in multiple tissues (Popov and Gil, 2010). Conditional disruption *of Ezh2* in the mouse retina using Dkk-Cre showed that *Ezh2* is also required to repress *cdkn2a* and maintain progenitor proliferation and the normal timing of retinal differentiation (Aldiri et al., 2013; Iida et al., 2014).

However, we lack a detailed understanding of the molecular changes associated with loss of PRC2-mediated repression in the developing retina and how this relates to PRC2 function in other tissues. We performed conditional disruption *of Ezh2* in the mouse retina using Pax6-αCre, and assessed effects on retinal development as well as gene expression changes by RNAseq. Our analysis shows that *Ezh2* and its repressive histone modifications are necessary to maintain the transcriptional identity as well as proliferation state of retinal progenitors, to regulate proper timing of differentiation during early postnatal retinal development, and to constrain the generation of Müller glia.

Materials and methods

Ezh2$^{fl/fl}$, Pax6-αCre and *R26EYFP* mice and their genotyping have been described previously (Marquardt et al., 2001; Shen et al., 2008; Srinivas et al., 2001; Su et al., 2003). Mouse breeding details are provided in SI Materials and methods. For immunostaining, retinal sections were generated as previously described (La Torre et al., 2013). Nuclear counterstain was done using either DAPI or Hoechst. Detailed methods and antibodies used are listed in SI Materials and methods. In situ hybridization was performed on whole embryos and retinal sections using digoxigenin (DIG)-labeled riboprobe for mouse *Ezh2*, as previously described (Zhang et al., 2008). Hematoxylin and eosin

staining of retinal sections was performed as previously described (Zhang et al., 2008). For explant culture, retinas from E14 mouse embryos were dissected and cultured as previously described (La Torre et al., 2013) with GSK126 (Xcess Biosciences) added at 2 μM or 10 μM, and corresponding concentrations of vehicle (DMSO) added to the control samples.

For RNAseq analysis the peripheral one-third of E16.5 retinas was dissected, left and right eyes combined, and total RNA isolated using RNeasy Plus Mini Kit according to manufacturer's protocol (Qiagen). RNA integrity was confirmed using a Bioanalyzer RNA 6000 Nano Chip. Four biological replicates *of Ezh2*$^{fl/-}$ CKO animals and *Ezh2*$^{fl/+}$ controls from the same litter were used for RNA sequencing on a 50 bp single-end run on Illumina HiSeq 2000. See SI Materials and methods for details. RNAseq data have been deposited to the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) under series accession number GSE65082.

Primers for PCR and ChIP are listed in Supplemental Table 7. Detailed methods for RNA isolation, RT-PCR and quantitative RT-PCR are described in SI Materials and methods. For chromatin immunoprecipitation (ChIP) analysis, the peripheral one-third of postnatal day 0 retinas were dissected and processed, as described in SI Materials and methods. Values were averaged from 3 biologically independent experiments and normalized for ChIP efficiency across biological replicates.

Results

Ezh2 is dynamically expressed during retinal development

In the developing *Xenopus* retina, *Ezh2* is enriched in retinal progenitors and downregulated in differentiated cells (Aldiri et al., 2013). To determine where *Ezh2* is expressed during mouse retinal development we performed *in situ* hybridization and found expression in progenitor cells throughout the optic cup at e9.5 (Fig. 1A), and in retinal progenitors at e12.5 (not shown). Expression was reduced in the emerging retinal ganglion cell layer (GCL) at e14.5, but maintained in retinal progenitors (Fig. 1B). By P0, Ezh2 expression was evident in the GCL, as well as in the neuroblastic layer (Fig. 1C), and by P10 Ezh2 expression was largely restricted to the GCL and inner nuclear layer (INL). Thus *Ezh2* is enriched in both retinal progenitors and subsets of differentiating cells, with dynamic changes in expression over the course of development.

Loss of Ezh2 results in impaired retinal development

To assess the function *of Ezh2* during retinal development, we conditionally inactivated *Ezh2* in retinal progenitors by crossing Pax6-αCre mice with either *Ezh2*$^{fl/fl}$ or *Ezh2*$^{fl/-}$ mice (Shen et al., 2008; Su et al., 2003). The Pax6-αCre retinal driver promotes recombination in the peripheral retina from E10.5 onwards, which is at the onset of retinal neurogenesis (Marquardt et al., 2001). By immunostaining retinal sections at P3, EZH2 protein was detected at high levels through the neuroblastic layer and at lower levels in the GCL in peripheral retina of control *Ezh2*$^{fl/+}$::Pax6-αCre (*Ezh2*$^{fl/+}$) mice, with mosaic loss of EZH2 protein in *Ezh2*$^{fl/fl}$::Pax6-αCre (*Ezh2*$^{fl/fl}$ CKO) retina, that was more extensive in *Ezh2*$^{fl/-}$::Pax6-αCre (*Ezh2*$^{fl/-}$ CKO) retina (Fig. 1E–G). In addition, H3K27me3 labeling was enriched in the GCL and in the inner part of the neuroblastic layer at P3 in *Ezh2*$^{fl/+}$ control retina (Popova et al., 2012), but showed mosaic loss in *Ezh2*$^{fl/fl}$ CKO retina, that was more extensive in *Ezh2*$^{fl/-}$ CKO retina (Fig. 1H–J). When crossed with *R26EYFP* mice, the reporter showed mosaic expression that corresponded to the areas with loss of H3K27me3 (Supplemental Fig. 1).
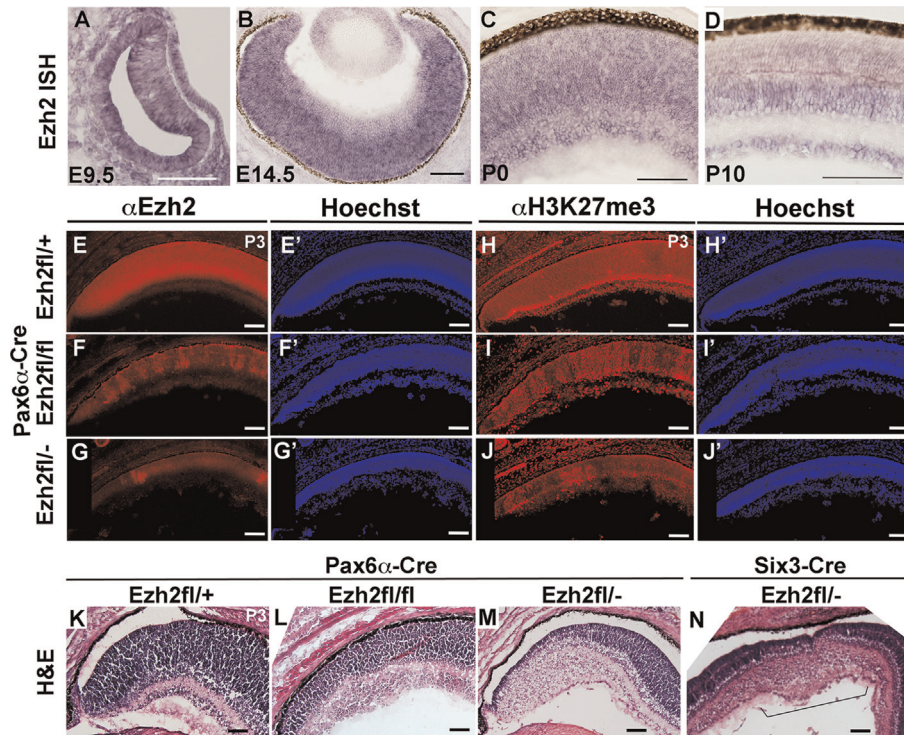
Fig. 1. Ezh2 expression during retinal development and after *Ezh2* conditional deletion. (A–D) In situ hybridization (ISH) analysis. (E–G) EZH2 immunostaining shows increasing loss of protein in the peripheral retina with conditional deletion using Pax6-αCre in *Ezh2$^{fl/fl}$* and *Ezh2$^{fl/-}$* mice relative to control *Ezh2$^{fl/+}$* mice at P3. (E'-G') Hoechst staining of the same sections. (H–J) Similarly H3K27me3 immunostaining shows increasing loss of this histone mark. (H'-J') Hoechst staining of the same sections. (K–M) Hematoxylin and eosin staining shows increasing disruption to retinal lamination with conditional deletion using Pax6-αCre in *Ezh2$^{fl/fl}$* and *Ezh2$^{fl/-}$* mice relative to control *Ezh2$^{fl/+}$* mice at P3. (N) Similar disruption of retinal lamination in the central retina is observed after conditional deletion *of Ezh2* using Six3-Cre in *Ezh2$^{fl/-}$* mice. Scale bar=100 μm.
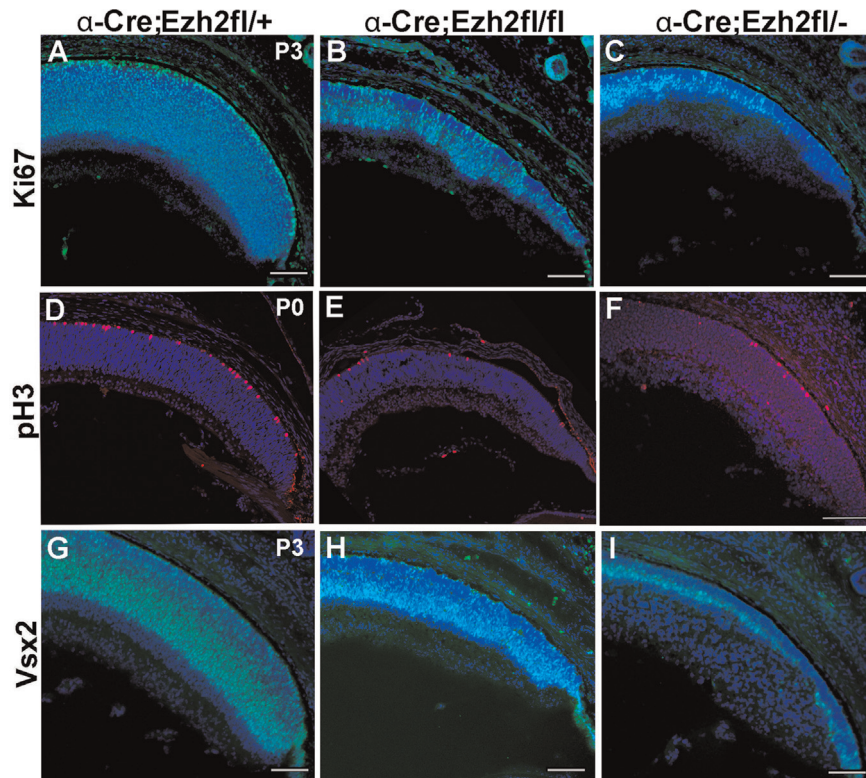

Fig. 2. Ezh2 deletion results in reduced retinal progenitor proliferation. (A–C) Increasing loss of Ki67 labeling (green) in the peripheral retina in *Ezh2$^{fl/fl}$* and *Ezh2$^{fl/-}$* CKO mice relative to control *Ezh2$^{fl/+}$* mice at P3. (D–F) Similarly, there is increasing reduction of pH3 labeling (red), and (G–I) reduced labeling for the progenitor marker Vsx2 (green). Scale bar=100 μm.

Since there was evidence for increasing penetrance of the phenotype with $Ezh2^{fl/-}$ versus $Ezh2^{fl/fl}$ CKO, we analyzed the phenotype in both to assess effects on retinal development. Hematoxylin and eosin staining of P3 retinal sections revealed disruption of retinal lamination and reduced thickness of the neuroblastic layer in $Ezh2^{fl/fl}$ CKO mice, that was more extensive in $Ezh2^{fl/-}$ CKO mice (Fig. 1K–M). For $Ezh2^{fl/fl}$ CKO mice, the $R26EYFP$ reporter confirmed that the phenotype corresponded to the areas of Cre-mediated recombination (Supplemental Fig. 1). We also confirmed disruption of retinal lamination that was restricted to the central retina region in $Ezh2^{fl/-}$ CKO animals using Six3-Cre (Fig. 1N), which drives recombination in retinal progenitors (Furuta et al., 2000). Thus, $Ezh2$ is required for normal retinal development.

Ezh2 is required to maintain late retinal progenitor proliferation

Reduced thickness of the neuroblastic layer suggested reduced retinal progenitor number in $Ezh2$ CKO retina. Labeling of progenitors with the proliferation marker Ki67 was reduced at P3 in $Ezh2^{fl/fl}$ CKO mice and to a greater extent in $Ezh2^{fl/-}$ CKO mice (Fig. 2A–C). The reduction in proliferation was confirmed by a similar reduction of pH3 labeling, which marks progenitor cells at the G2-M transition (Fig. 2D–F). We also found significant reduction at P3 in the numbers of cells labeled for the progenitor marker

Vsx2 in $Ezh2^{fl/fl}$ CKO mice that was more pronounced in $Ezh2^{fl/-}$ CKO mice (Fig. 2G–I).

There was no effect on EdU labeling at e15 in $Ezh2^{fl/fl}$ CKO mice, suggesting that the proportion of cells in S-phase was unaltered (Supplemental Fig. 1). In addition, the proportion of cycling cells appeared unchanged since there was no effect on PCNA labeling or Ki67 labeling at e15.5 in $Ezh2^{fl/-}$ CKO mice, although we cannot rule out subtle effects on cell cycle length (Supplemental Fig. 1 and data not shown). Although there was no apparent effect on retinal progenitor proliferation, there was loss of EZH2 and H3K27me3 labeling of progenitors in the CKO region of the peripheral retina at this stage (Supplemental Fig. 1). By P0 there was modest reduction in Ki67 labeling in $Ezh2^{fl/-}$ CKO mice, suggesting that premature exit of progenitors has begun by this stage (Supplemental Fig. 1). Thus, we conclude that $Ezh2$ is required to maintain proliferating progenitors during postnatal development, but not embryonic retinal development.

Ezh2 deletion alters retinal ganglion cell and amacrine cell numbers.

Since disruption of $Ezh2$ has an effect on retinal progenitor maintenance, we predicted that this would impact retinal cell fate specification. Retinal neurons in mouse are generated in a defined sequence beginning at e10.5 with retinal histogenesis largely complete by postnatal day 14. An early wave of differentiation during the embryonic period generates the majority of retinal
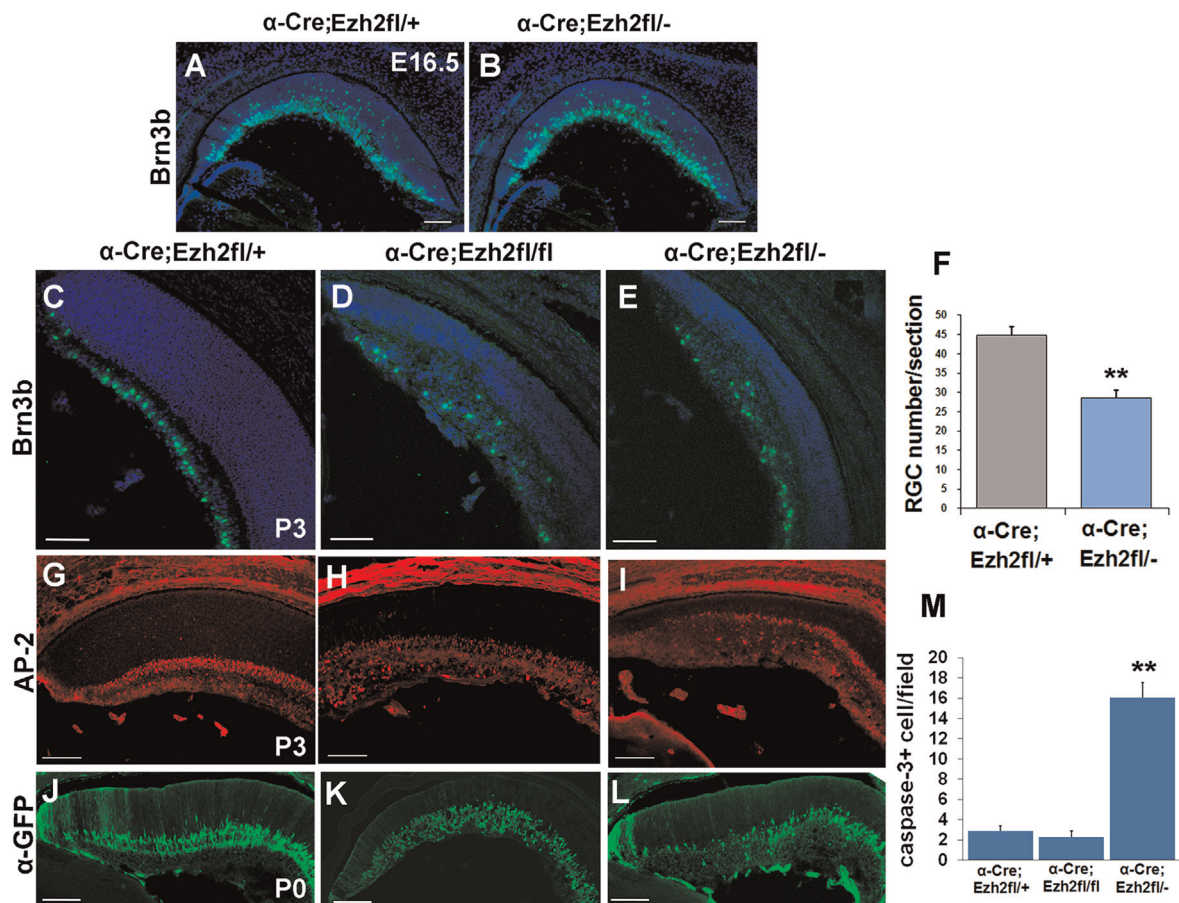

Fig. 3. Ezh2 deletion alters retinal ganglion cell and amacrine cell numbers. (A,B) No change in Brn3b labeling at e16.5 (green). (C–E) Brn3b immunostaining (green) shows disruption of the RGC layer and reduced RGC number in $Ezh2^{fl/fl}$ and $Ezh2^{fl/-}$ CKO retina relative to control $Ezh2^{fl/+}$ at P3. (F) There is a 36% reduction in Brn3b+ cells at P3 in $Ezh2^{fl/-}$ CKO retina. N = 45 sections from 6 retinas. Error bars represent the standard error, ** = $P < 0.001$ Student's t-test. (G–I) Ap2+ amacrine cells are increased relative to control $Ezh2^{fl/+}$ retina at P3 in $Ezh2^{fl/fl}$ CKO, but reduced in $Ezh2^{fl/-}$ CKO. (J–L) Similarly, at P0 the GFP reporter that is expressed from the Pax6-αCre transgene in amacrine cells is expanded in $Ezh2^{fl/fl}$ CKO, but reduced in $Ezh2^{fl/-}$ CKO retina relative to control $Ezh2^{fl/+}$. (M) The number of cleaved caspase 3-positive cells is increased at P0 in $Ezh2^{fl/-}$ CKO but not $Ezh2^{fl/fl}$ CKO retina relative to control $Ezh2^{fl/+}$. N = 24, 24 and 27 sections from 6 retinas each, respectively for $Ezh2^{fl/+}$, $Ezh2^{fl/fl}$ and $Ezh2^{fl/-}$. Error bars represent the standard error, ** = $P < 0.001$ Student's t-test. Scale bar = 100 μm.

ganglion cells, horizontal cells, cones and amacrine cells, while a later postnatal wave generates the bulk of rod photoreceptors, bipolar cells and Müller glia (Bassett and Wallace, 2012). We first assessed early born retinal neurons since we did not observe an effect on proliferation during the embryonic period of retinal neurogenesis. Consistent with this, there was no major effect on Brn3b-positive RGCs at e15.5 in $Ezh2^{fl/-}$ CKO mice (Fig. 3A and B). But by P3 the numbers of Brn3b-labeled RGCs was significantly reduced in both $Ezh2^{fl/-}$ and $Ezh2^{fl/fl}$ CKO retina (Fig. 3C–F). These results suggest that although the ganglion cells are generated in approximately normal numbers, these cells are reduced post-natally (see below).

Amacrine cells are also largely born during the embryonic period, so we investigated whether loss of Ezh2 affected this retinal cell population. We observed an increase in Ap2-labeled amacrine cells in $Ezh2^{fl/fl}$ CKO mice relative to $Ezh2^{fl/+}$ controls at P3 (Fig. 3G and H). This was confirmed by an increase in GFP-labeled cells in $Ezh2^{fl/fl}$ CKO mice, since at postnatal stages the GFP reporter from the Pax6-αCre transgene is expressed in amacrine cells (Fig. 3J and K) (Marquardt et al., 2001). In contrast, there was a decrease in Ap2-labeled amacrine cells in $Ezh2^{fl/-}$ CKO mice, as

well as a decrease in GFP-labeled cells at P3 (Fig. 3I and L). By P10, Ap2-labeled amacrine cells were significantly reduced in Cre+ regions in $Ezh2^{fl/fl}$ CKO retina, largely due to reduced number of labeled cells in the INL ($N=3$ retinas per genotype, 3 sections/retina, $P<0.01$ by Student's t-test), and were also clearly reduced at P14 in $Ezh2^{fl/-}$ CKO retina (data not shown). Thus we observe distinct effects on amacrine cell number in $Ezh2^{fl/-}$ versus $Ezh2^{fl/fl}$ CKO mice, but in both cases the amacrine and ganglion cell layers were disorganized and intermixed.

To determine whether the survival of the neurons was compromised from the loss in Ezh2, we labeled the retinas for cleaved caspase 3. We observed an increase in the numbers of cells labeled for cleaved caspase 3 at P0 in $Ezh2^{fl/-}$ CKO mice (Fig. 3M). A small subset of cleaved caspase 3 positive cells colabeled for Brn3b (data not shown), indicating that an increase in cell death that may partially account for the reduced number of early born cell types in $Ezh2^{fl/-}$ CKO animals. This increase in cell death was not evident at P3 and was not significant at either stage in $Ezh2^{fl/fl}$ CKO mice. Thus, we conclude that $Ezh2$ is not required for the genesis of early born neurons during the embryonic period but is ultimately required for the normal complement of early retinal cell types.
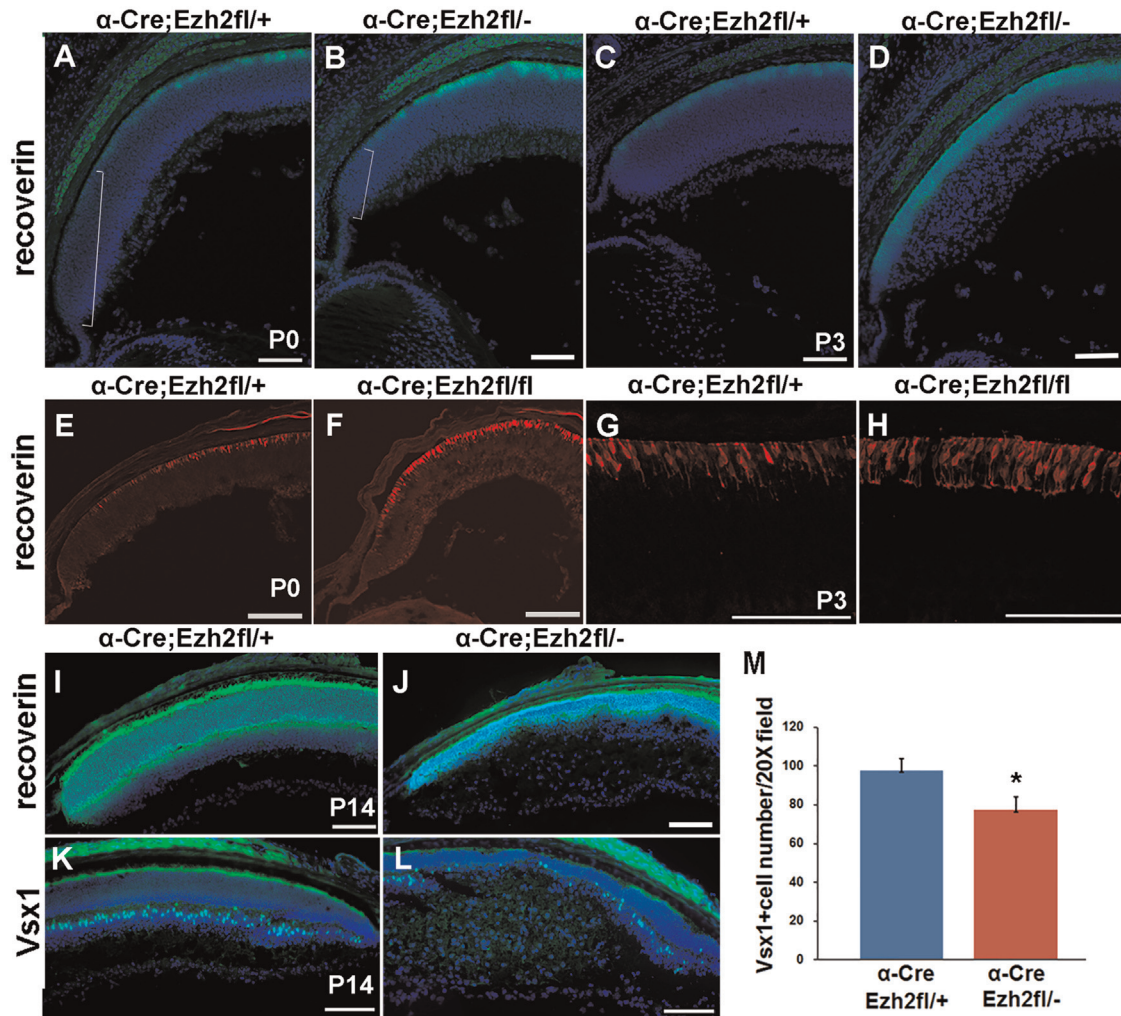

Fig. 4. Ezh2 deletion increases rod photoreceptor differentiation in the early postnatal retina, and affects overall numbers of later born retinal cell types. (A,B) Conditional deletion in $Ezh2^{fl/-}$ retina results in increased photoreceptor differentiation relative to control $Ezh2^{fl/+}$ at P0 as detected by recoverin labeling (green). Bracket indicates the extent of the undifferentiated region at the peripheral margin of the retina, which is reduced in CKO animals. (C,D) Increased photoreceptor differentiation is also evident at P3 as evident by the thickened ONL. (E,F) This is similarly observed in $Ezh2^{fl/fl}$ CKO retina at P0, and (G,H) at P3 (recoverin, red). (I,J) By P14 recoverin-positive photoreceptors are reduced in $Ezh2^{fl/-}$ CKO. (K,L) Bipolar cells are reduced in the $Ezh2$ CKO region at P14 as detected by Vsx1 labeling (green). (M) Cell counts show a significant reduction in Vsx1-positive cells at P14 in $Ezh2^{fl/-}$ CKO retina. $N=10$, 12 sections, 4 retinas each respectively for $Ezh2^{fl/+}$ and $Ezh2^{fl/-}$. Error bars represent the standard error. $^*=P<0.01$ Student's t-test. Scale bar$=100$ μm.

Ezh2 disruption results in increased photoreceptor differentiation in the early postnatal retina:

Since loss *of Ezh2* results in reduced proliferation and progenitor depletion at early postnatal ages, we assessed whether this would affect the differentiation of later born cell types. We found that there was increased expression of photoreceptor differentiation markers in the peripheral retina of both $Ezh2^{fl/-}$ and $Ezh2^{fl/fl}$ CKO mice at early postnatal ages. At P0, recoverin labeling in the outer nuclear layer (ONL) extended more peripherally in both $Ezh2^{fl/-}$ and $Ezh2^{fl/fl}$ CKO retina, suggesting that more progenitors in this region were differentiating at this timepoint than normal (Fig. 4A, B, E and F), with the average distance from the ciliary edge to the first recoverin labeling decreased by 40% in $Ezh2^{fl/fl}$ CKO retina (76 μM) compared to $Ezh2^{fl/+}$ controls (126 μM) ($N=3$, $P < 0.01$). Consistent with this, by P3 there was a thickened layer of recoverin-labeled cells in both $Ezh2^{fl/-}$ and $Ezh2^{fl/fl}$ CKO retina (Fig. 4C, D, G and H). This was not simply due to derepression of recoverin expression, since we also observed increased labeling for

the synaptic marker SV2 in the outer plexiform layer in $Ezh2^{fl/fl}$ CKO retina compared to $Ezh2^{fl/+}$ controls, indicating that more functional photoreceptors are present in the P3 *Ezh2* CKO retina (Supplemental Fig. 2). This is likely due to more cells exiting and differentiating as photoreceptors at the earlier timepoint, but it is also possible that the process of photoreceptor differentiation is accelerated, or that retinal cell birthorder is altered.

However, by P14 the recoverin-labeled ONL was noticeably thinner in $Ezh2^{fl/-}$ CKO retina (Fig. 4I and J). The reduction in proliferation in the postnatal retina, coupled with the reduced ONL thickness suggests that the progenitor depletion ultimately leads to an overall reduction in photoreceptor production. Consistent with this, the number of cells labeled with the photoreceptor progenitor marker Otx2 was reduced by P3 in $Ezh2^{fl/fl}$ CKO retina (data not shown).

Although the loss in Ezh2 leads to an acceleration in the timing of differentiation of photoreceptors in the postnatal retina, we did not observe a similar premature expression of the bipolar cell marker Vsx1 in $Ezh2^{fl/-}$ CKO retina at P3, prior to the onset of
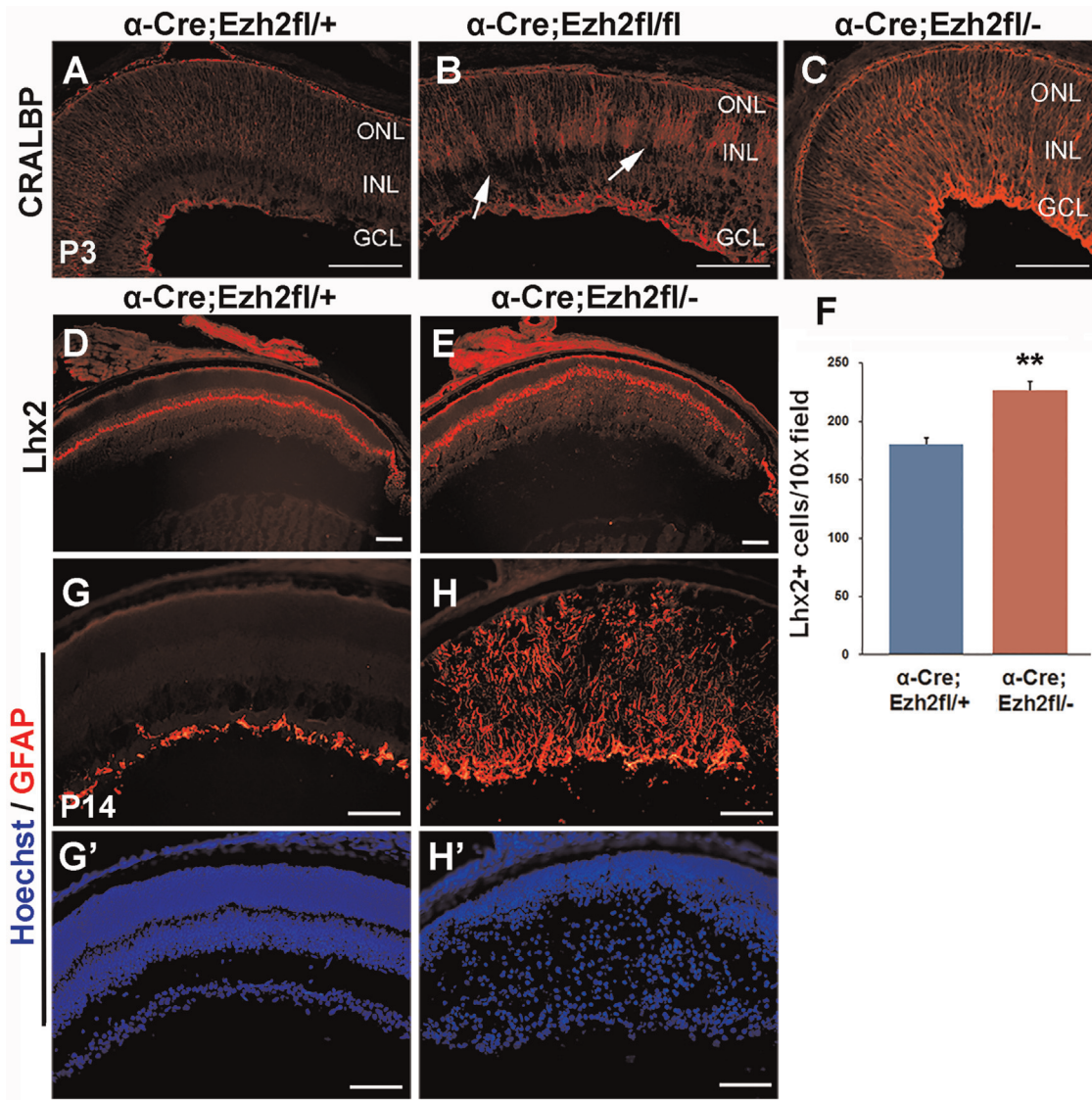

Fig. 5. Ezh2 deletion enhances Müller glia differentiation and leads to reactivity. (A–C) Conditional deletion in $Ezh2^{fl/fl}$ and $Ezh2^{fl/-}$ retina results in increased Müller glia differentiation at P3 relative to control $Ezh2^{fl/+}$ retina as detected by Cralbp immunstaining. (D,E) Lhx2 immunostaining at P14 shows an increase in Müller glia in $Ezh2^{fl/-}$ CKO retina. (F) Counts of Lhx2–labeled cells show a 25% increase in Müller glia in $Ezh2^{fl/-}$ CKO retina relative to $Ezh2^{fl/+}$ controls. N=20, 24 sections, 6 retinas each respectively for $Ezh2^{fl/+}$ and $Ezh2^{fl/-}$. **=$P < 0.001$, Student's t-test. (G,H) Müller glia at P14 in the $Ezh2^{fl/-}$ CKO region become reactive and show increased GFAP expression. (G',H') Hoechst staining of the same sections. Scale bar=100 μm. GCL=ganglion cell layer, INL=inner nuclear layer, ONL=outer nuclear layer.
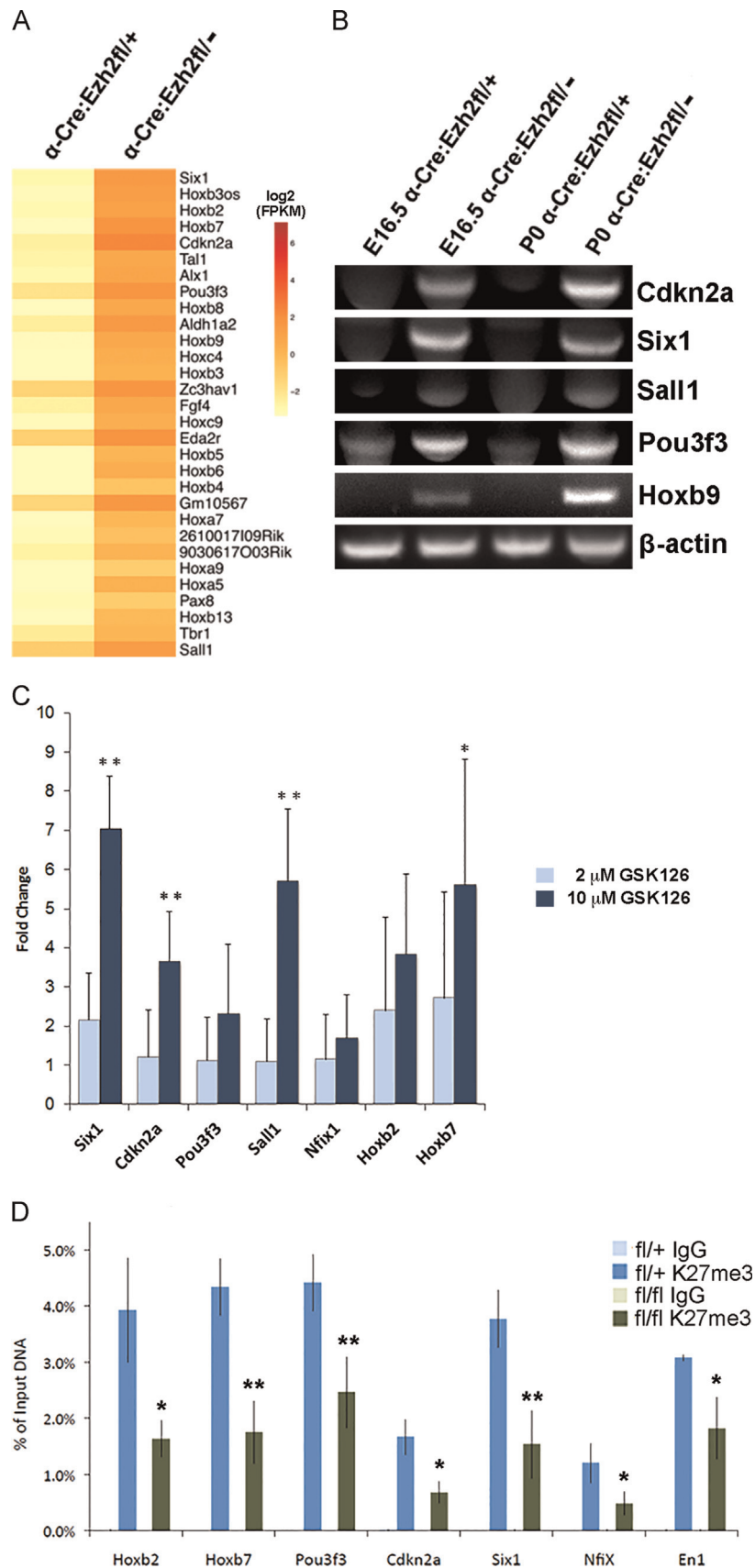
Fig. 6. RNA-Seq reveals significant gene upregulation following conditional deletion *of Ezh2*. (A) Heat map representation of log 2 (avg FPKM) for the top thirty genes differentially expressed by at least twofold in peripheral retina at e16.5 in *Ezh2$^{fl/-}$* CKO mice relative to control *Ezh2$^{fl/+}$* mice. Genes with no expression are assigned an FPKM value of 0.1. A color-coded log 2 scale is shown at the side of the heat map. (B) RT-PCR for a subset of these genes confirms their upregulation following conditional deletion *of Ezh2* at both e16.5 and P0. (C) Quantitative RT-PCR analysis following treatment of retinal explants with GSK126, a small molecule inhibitor of EZH2, also shows upregulation of many genes that increase in the *Ezh2* CKO. (D) Chromatin immunoprecipitation (ChIP)-qPCR for H3K27me3 on a subgroup of genes that are upregulated after Ezh2 deletion confirms depletion of H3K27me3. The *Engrailed1* promoter was also assayed as a gene that is not upregulated after Ezh2 deletion. $^{*}=p < 0.05$, $^{**}=p < 0.01$, Students' *t*-test.

bipolar cell differentiation, or at P6, which is at the early stages of bipolar cell differentiation (data not shown). By P14, the number of Vsx1 labeled bipolar cells was significantly reduced in *Ezh2*^{fl/−} CKO retina (Fig. 4K and L). Thus, the requirement for *Ezh2* in the normal timing of photoreceptor differentiation does not extend to all late generated cell types.

Ezh2 disruption results in increased early Müller glia differentiation

Since blocking *Ezh2* in the *Xenopus* retina results in increased Müller glial cell differentiation (Aldiri et al., 2013), we assessed whether these cells would be affected with loss *of Ezh2* in the mouse retina. We found that at P3 there was increased Müller glial differentiation in the peripheral retina of both *Ezh2*^{fl/−} and *Ezh2*^{fl/fl} CKO mice as detected by CRALBP expression (Fig. 5A–C). This increase was already evident at P0 (data not shown). By P14 there was a 25% increase in Lhx2 positive Müller glia in the CKO region in *Ezh2*^{fl/−} CKO retina as compared to *Ezh2*^{fl/+} retina (Fig. 5D–F). Consistent with this, at P10 there was also an expansion of Sox2-labeled cells in the INL *of Ezh2*^{fl/fl} CKO retina, suggesting an increase in Müller glia (data not shown). However, at P14 in *Ezh2*^{fl/−} CKO retina Müller glia were reactive, and showed significant disorganization and upregulation of GFAP (Fig. 5G and H). Glutamine synthase, was significantly downregulated, consistent with Müller glia reactivity (data not shown). At P10 we found no coexpression of NeuN, which labels neurons, and Id1, which labels Müller glia, in the CKO region *of Ezh2*^{fl/fl} retina, indicating that the distinction between neuron versus glial identity is maintained (data not shown). Thus, we conclude that *Ezh2* is required to constrain Müller glial cell differentiation, and to regulate the timing of their genesis.

Ezh2 is required to inhibit the expression of many developmental regulators and non-retinal genes

To understand the mechanisms underlying this retinal phenotype we sought to determine which genes are deregulated by loss *of Ezh2*. We dissected peripheral retina tissue at e16.5 to enrich for regions with loss *of Ezh2*, and then performed RNA-seq. This age is prior to apparent changes in retinal development, since we sought to identify gene expression changes that are directly due to loss *of Ezh2*. A total of 232 genes were differentially expressed in *Ezh2*^{fl/−} CKO versus *Ezh2*^{fl/+} control animals with a fold change cutoff of 2 (Abs log 2 > 1, $p < 0.05$), with the majority of genes upregulated and only four significantly downregulated, which is consistent with the canonical function of EZH2 in promoting gene repression (Supplemental Table 1).

Examination of the top 30 most highly upregulated genes showed that most of them had either no or very low expression in the control retina, but were significantly upregulated in *Ezh2*^{fl/−} CKO retina (Fig. 6A). This included genes encoding many developmental regulators such as multiple *Hox* transcription factors (15/30 top upregulated genes), transcription factors (e.g. *Six1*, *Pou3f3*), signaling factors (e.g. *Fgf4*) and the cell cycle inhibitor *Cdkn2a*. *Cdkn2b* was also upregulated (Supplemental Table 1). To confirm the results of our RNA-seq analysis we performed RT-PCR for a subset of the most highly upregulated genes, *Cdkn2a*, *Six1*, *Sall1*, *Pou3f3*, and *Hoxb9* using RNA isolated from peripheral retina tissue and found that these candidate genes were upregulated at e16.5, consistent with our RNA-seq timepoint, as well as at P0, when the retinal phenotype is apparent (Fig. 6B). To further confirm these findings, we sought to independently reduce EZH2 activity. E14 retinal explants were treated with the EZH2 chemical inhibitor GSK126 at 2 μM or 10 μM for four days, then RNA isolated and quantitative RT-PCR performed to assess fold change in expression for *Six1*, *Cdkn2a*, *Pou3f3*, *Sall1, Nfix, Hoxb2* and *Hoxb7*.

There was significant upregulation of *Six1, Cdkn2a*, *Sall1,* and *Hoxb7*, particularly at the 10 μM concentration, although *Pou3f3*, *Nfix,* and *Hoxb2* were upregulated but not significantly (Fig. 6C).

Many of these most highly upregulated genes are repressed via EZH2 in other tissues, suggesting that EZH2 may target common genes regardless of cell lineage. To further investigate this, we compared our gene expression profiling data for e16.5 retina (GSE65082), which is ectodermal in origin, to data available in the GEO data repository for gene expression changes in other lineages after loss *of Ezh2* (http://www.ncbi.nlm.nih.gov/geo/). For example, out of the 232 genes differentially expressed in our study, 40 were also significantly increased in e12.5 cardiomyocytes, which is mesodermal in origin (GSE29992; abs log 2 > 0.585, $p < 0.05$; (He et al., 2012)). Similarly, 29 were also significantly increased in e14.5 lung, which is endodermal in origin (GSE66060; abs log 2 > 0.585, p < 0.05; (Snitow et al., 2015)). Multiple genes or gene families were upregulated in all three datasets, including *Cdkn2a, Hox* genes and *Pou3f3* (Supplemental Table 2). While some genes are upregulated in specific lineages, the fact that there is such overlap suggests that *Ezh2* is required to repress similar classes of genes in multiple lineages, regardless of embryonic germ layer origin.

We also compared the gene expression changes we found in embryonic retina to those reported for adult tissues. An analysis of gene expression from right ventricles of adult wild type and *Ezh2*-deficient hearts showed up to 60 genes common with our dataset (GSE34274; Abs log 2 > 0.585, $p < 0.05$; (Delgado-Olguin et al., 2012)). Of these, 18 genes were also upregulated in either e12.5 cardiomyocytes or e14.5 lung (Supplemental Table 3). This includes the skeletal muscle gene *Six1*, which was among the most highly upregulated in both lineages. This analysis strikingly shows that overlapping genes are repressed in an *Ezh2*-dependent manner regardless of lineage or developmental stage.

Many, but not all Ezh2 regulated genes have high levels of H3K27me3

To assess the link between H3K27me3 and retinal gene expression changes in the *Ezh2* CKO, we examined the genes that are upregulated in the *Ezh2* CKO and their levels of H3K27 trimethylation, as reported for the postnatal day 1 (P1) mouse retina (Popova et al., 2012). Among the genes that are upregulated in the *Ezh2* CKO are those with the highest levels of H3K27me3 (Supplemental Table 4). In order to confirm that the conditional knockout *of Ezh2* results in depletion of H3K27me3, we performed chromatin immunoprecipitation (ChIP) for this histone modification on a subgroup of these genes. The promoter regions (within 1 kb upstream of the transcription start site) of the upregulated genes *Hoxb2*, *Hoxb7*, *Pou3f3*, *Cdkn2a*, *Six1*, *Nfix* were assayed for H3K27me3 enrichment by ChIP-qPCR from *Ezh2*^{fl/fl} CKO *and Ezh2*^{fl/+} littermates. The *Engrailed1* promoter was also assayed as a gene that is not upregulated after Ezh2 deletion. At all assayed gene promoters, H3K27me3 levels were reduced, confirming the predicted effect of Ezh2 deletion (Fig. 6D). H3K27me3 levels were reduced, but not absent, at least in part due to the incomplete nature of the conditional deletion throughout the retina.

We found that H3K27me3 levels were reduced at the *Engrailed1* promoter, although this gene is not upregulated following Ezh2 deletion. We identified additional genes that are most highly marked with H3K27me3 in the P1 mouse retina, but that are not significantly increased in the *Ezh2* CKO (Supplemental Table 5), suggesting additional repressive mechanisms, or developmental gain of H3K27me3 at P1 as compared to e16.5. Conversely, many genes that increase in the *Ezh2* CKO do not appear to have significant levels of H3K27me3 (Supplemental Table 6), suggesting indirect regulation, or developmental loss of H3K27me3 by P1. An important caveat is that different retinal cell types likely have

different levels of these modifications (Popova et al., 2014, 2012), which may not be detected by bulk analysis. Overall, we conclude that EZH2 maintains repression of many potent developmental regulators, including genes not normally expressed in the developing retina, although clearly other repressive mechanisms are also involved in maintaining appropriate levels of gene expression in retinal progenitors.

Discussion

Development of the retina involves the ordered genesis of distinct retinal cell types from proliferating retinal progenitors, and by conditional deletion *of Ezh2* in developing retina, we show that *Ezh2* plays multiple roles in the development of this tissue. *Ezh2* is required in postnatal retina to maintain progenitor proliferation, coordinate the timing of differentiation and generate the appropriate complement of late retinal cell types. In addition, RNAseq analysis revealed that loss *of Ezh2* results in upregulation of cell cycle inhibitors and many non-retinal genes, indicating that *Ezh2* also maintains transcriptional integrity of cells in this lineage. Genes upregulated after loss *of Ezh2* in retina overlap significantly with those repressed by *Ezh2* in other tissues of distinct developmental origin, suggesting that *Ezh2* may have a core function in repressing the expression of potent developmental regulators and cell cycle genes regardless of lineage.

Loss of Ezh2 disrupts H3K27me3 in the developing retina

We observed that *Ezh2* is highly expressed in retinal progenitors and weakly in postmitotic cells of the inner retina, consistent with previous studies showing significant expression in proliferating cells and embryonic tissues of multiple lineages, including retina (Aldiri et al., 2013; Aldiri and Vetter, 2012; Ezhkova et al., 2009; Iida et al., 2014; Margueron et al., 2008). Iida et al. (2014) showed EZH2 protein localized to Pax6-positive retinal progenitors and RGCs at embryonic stages, while postnatally EZH2 localized to glutamine synthase-positive Müller glia. In our study, conditional deletion *of Ezh2* in retinal progenitors resulted in a large decrease in the level of H3K27me3 in the deleted region, particularly in progenitors. The presence of residual H3K27me3, particularly in some postmitotic cells, suggests potential contribution by *Ezh1*, which is generally more highly enriched in postmitotic cells and adult tissues than *Ezh2* (Ezhkova et al., 2009; Margueron et al., 2008). However, the expression and function of *Ezh1* in the developing retina remains to be determined.

We also noted increasing penetrance of phenotype, as well as loss *of Ezh2* and H3K27me3, when comparing *Ezh2fl/fl* versus *Ezh2fl/−* CKO retina using the Pax6-αCre driver (Marquardt et al., 2001). Thus, the recombination efficiency and/or differences in the timing of deletion seem to impact the phenotype. We confirmed that the *Ezh2fl/fl* CKO phenotype was identical when generated by either lab. In addition, the *Ezh2* CKO phenotype was also apparent using the Six3-Cre driver (Furuta et al., 2000), and has also recently been reported using Dkk-Cre (Iida et al., 2014). Together these findings demonstrate a consistent role for *Ezh2* in the regulation of retinal development.

Ezh2 maintains retinal progenitor proliferation

One of the most highly conserved functions *of Ezh2* in developing tissues is the maintenance of progenitor proliferation (Laugesen and Helin, 2014). Similar to the findings of Iida et al., 2014, we observed that loss *of Ezh2* in retinal progenitors results in reduced proliferation at early postnatal ages, as well as upregulation of *Cdkn2a* (*Arf/Ink4a*) and *Cdkn2b* (*Ink4b*), which regulate the

G1/S transition of the cell cycle and control of apoptosis. Both loci are not normally expressed in the developing retina, and have high levels of H3K27me3 histone modification in newborn mouse retina (Popova et al., 2012). It is likely that the proliferation defect is due to premature cell cycle exit of retinal progenitors, as demonstrated in the *Xenopus* retina (Aldiri et al., 2013), although changes in cell cycle kinetics or progenitor cell death may also contribute to progenitor depletion. In both *Xenopus* and mouse, loss or inhibition *of Ezh2* leads to an increase in *Arf/Ink4a* or *Ink4b*, with ectopic expression of these genes causing reduced proliferation (Aldiri et al., 2013; Iida et al., 2014). Cell cycle genes are dysregulated in other regions of the CNS upon loss *of Ezh2* (Pereira et al., 2010). Deficiency of *Ink4a/Arf* was sufficient to restore proliferation in *Ezh2*-deficient neural stem cells, demonstrating that it is the principal factor resulting in loss of proliferation (Hwang et al., 2014). *Cdkn2a* is a well-documented direct target for repression by *Ezh2* in many other tissues, and is also associated with cancer (Popov and Gil, 2010). Thus maintaining proliferation by preventing the expression of cell cycle inhibitors is a core function *of Ezh2*.

Progenitor proliferation was unaffected by loss *of Ezh2* in embryonic retina, suggesting that additional mechanisms regulate cell cycle gene expression. Consistent with this possibility, retinal progenitor proliferation and cell cycle genes are also regulated by G9a methyltransferase via H3K9 methylation (Katoh et al., 2012). It is also possible that distinct mechanisms function during embryonic versus postnatal development to regulate proliferation, since conditional knockout of the DNA methyltransferase gene *Dnmt1* also alters cell cycle progression in postnatal but not embryonic retina (Rhee et al., 2012).

Ezh2 maintains the timing of late retinal cell differentiation

Ezh2 deletion and the concomitant loss of H3K27me3 in the developing retina caused striking alterations in histogenesis and retinal lamination. Differentiation of retinal ganglion cells, an early born cell type, was normal during the embryonic period, similar to the effect on proliferation, although numbers were reduced by early postnatal stages, potentially due to increased apoptosis. Although we confirmed loss of EZH2 and H3K27me3 embryonically, it is possible that other mechanisms act redundantly with *Ezh2* to ensure normal differentiation during the embryonic period. We observed increased differentiation for several late born cell types at early postnatal ages, particularly rod photoreceptors and Müller glia, similar to Iida et al. (2014). Although generated early, by P14 rod photoreceptors were reduced in number, potentially due to early cell cycle exit and progenitor depletion. However, overall numbers of Müller glia were increased, suggesting a shift from neuron to glial fate. In *Xenopus*, *Ezh2* knockdown also caused an increase in Müller glia differentiation at the expense of neurons (Aldiri et al., 2013), possibly due to reduced proneural gene expression, which we do not observe in our RNAseq data. Instead we find increased expression of the glial gene *Nfix*, and *Ink4a/Arf*, both of which have been shown to regulate glial fate acquisition (Price et al., 2014; Wilczynska et al., 2009).

Ezh2 regulates the timing of differentiation and the proportion of cell types generated in multiple developmental contexts. In the cerebral cortex after conditional Ezh2 deletion using Emx1-Cre, there is an acceleration of developmental timing, with early onset of gliogenesis, and a shift from self-renewal to differentiation in cortical progenitors (Pereira et al., 2010). In contrast, conditional deletion *of Ezh2* during late corticogenesis results in an extended period of neurogenesis and failure to switch to astrocyte production (Hirabayashi et al., 2009). Thus the effects *of Ezh2* loss on developmental timing are highly context-dependent (Aldiri and

Vetter, 2012; Testa, 2011). Accelerated developmental timing has also been reported for non-neural tissues; basal cells in the developing lung epithelium appear earlier and in increased numbers upon loss of *Ezh2* (Snitow et al., 2015). Thus *Ezh2* may act to regulate the timing of differentiation and the proportion of cell types generated in multiple developmental contexts.

Not all late born cell types were affected, since there was no evidence for increased early bipolar cell differentiation (see also Iida et al. (2014)). In addition, in *Ezh2*$^{fl/fl}$ CKO retina there was an increase in amacrine cell number, while in *Ezh2*$^{fl/-}$ CKO retina, amacrine cells were decreased, potentially due to increased penetrance of the phenotype, resulting in distinct effects on timing of differentiation or survival of these neurons. Overall, the gene expression changes induced by loss of *Ezh2* may selectively affect the differentiation of a subset of retinal neurons, perhaps by mimicking the effects of related endogenous developmental regulators with shared binding specificity.

Upregulation of non-retinal genes with loss of Ezh2

Several classes of genes appear to be particularly susceptible to silencing by the H3K27me3 histone modification. In addition to cell cycle inhibitors, 24 *Hox* genes were upregulated in *Ezh2*$^{fl/-}$ CKO retina, including multiple genes from the *Hoxa*, *Hoxb* and *Hoxc* clusters, consistent with the *Hox* loci having some of the highest levels of H3K27me3 in the P1 retina (Popova et al., 2012). *Hox* genes are upregulated in many tissues upon loss of *Ezh2*, including tissues with very different embryonic origin such as cardiac tissue or lung endoderm (He et al., 2012; Snitow et al., 2015). Such deregulation can have significant developmental consequences. For example, derepression of *Hox* genes in neural crest cells, where they are not normally expressed, prevents craniofacial bone and cartilage formation (Schwarz et al., 2014). Polycomb genes were first identified for their role in the regulation of *Hox* genes in Drosophila, suggesting an ancient and strongly conserved relationship between *Hox* gene regulation and H3K27 trimethylation (Sparmann and van Lohuizen, 2006).

We confirmed that other potent developmental regulators are upregulated in the *Ezh2* CKO retina, including genes with high levels of H3K27 trimethylation, such as *Six1*, *Sall1*, and *Pou3f3*, which showed reduced H3K27me3 by ChIP analysis in the *Ezh2* CKO retina. For all these genes the H3K27me3 modification levels are much higher than the levels of the permissive mark H3K4me2 in the P1 retina (Popova et al., 2012). However, unlike Iida et al. (2014), we did not observe changes in the expression of *bHLHb4*, *Vsx1* or *Isl1* (Iida et al., 2014).

Most of the upregulated genes are not significantly expressed during normal retinal development, consistent with a role for *Ezh2* in repressing non-relevant gene programs to preserve lineage integrity, as proposed for skeletal muscle stem cells (Conerly et al., 2011; Juan et al., 2011). Comparing RNAseq and previous gene profiling studies from embryonic and adult tissues of diverse developmental origin, we find that loss of *Ezh2* in retina leads to changes in expression of many of the same genes. While there are lineage-specific changes in gene expression after loss of *Ezh2*, many of these genes represent families of developmental regulators originally identified as PRC2 targets in embryonic stem cells (Lee et al., 2006). Promoters of many developmental control genes are poised for expression (Zeitlinger and Stark, 2010), and bivalent domains in their promoter regions containing both permissive H3K4me3 and repressive H3K27me3 modifications have been proposed to silence expression while keeping the genes poised for activation, which can be triggered by loss of H3K27me3 (Voigt et al., 2013). While bivalent domains can be dynamically modified, it is intriguing to consider that for a core set of genes this poised state may be maintained across lineages and into adult

tissues (Weishaupt et al., 2010). Since H3K4me3 was not assessed here, it remains to be determined whether such bivalent domains are present in the developing mouse retina, and whether there is variation among the heterogeneous cell populations that are present in this tissue.

H3K27me3 levels do not strictly correspond to gene expression changes after loss of Ezh2

Not all genes with high levels of H3K27me3 were upregulated in the *Ezh2* CKO retina. For example, RNAseq analysis showed that no *Hoxd* genes were upregulated, although *Hoxd* genes have high levels of H3K27me3 in the newborn mouse retina (Popova et al., 2012), and are known targets for PRC2-mediated repression. For example, in human cells the *Hoxd* locus is specifically targeted for PRC2-mediated repression via the long non-coding RNA HOTAIR (Rinn et al., 2007). Other genes, including developmental regulators such as *Pax3*, *En1* and *Olig2*, are also highly marked with H3K27me3 in the P1 mouse retina (Popova et al., 2012), but were not upregulated with loss of *Ezh2* in the retina. These data suggest that either other repressive histone modifications are present at these loci, or that the transcription factors necessary for their activation are not present in the retina. In contrast to retina, after loss of *Ezh2* from adult mouse neural stem cells, *Olig2* is upregulated and prevents neuronal differentiation (Hwang et al., 2014), suggesting context-dependent regulation of gene repression at certain loci.

Some genes that increase in the *Ezh2* CKO do not appear to have significant levels of H3K27me3, although we cannot rule out cell type-specific epigenetic regulation (Popova et al., 2014), which can result in different levels of these modifications. Alternatively, these genes may not be directly regulated by *Ezh2*, but may be regulated by one of the many transcription factors that are derepressed directly.

Chromatin modifications and regulation of retina development

Together with previous studies, our results show that different sets of developmental genes are repressed by distinct complementary epigenetic regulators. Two other well-established repressive chromatin modifications, H3K9me2 and DNA methylation, are also required for retinal progenitor proliferation and/or differentiation, involving genes that are different from those regulated by *Ezh2* (Katoh et al., 2012). Many long non-coding RNAs (lncRNAs) interact with PRC2 to coordinate gene silencing (Brockdorff, 2013), and several lncRNAs are expressed in retina and regulate retinal neurogenesis (Meola et al., 2012; Rapicavoli et al., 2010; Rapicavoli et al., 2011). In addition, we found significant upregulation of the PRC1 component *Bmi1* in *Ezh2* CKO retina. *Bmi1* has also been implicated in the regulation of proliferation and differentiation in the developing nervous system (Fasano, 2009; Zencak et al., 2005). Thus, multiple repressive complexes are required to coordinate retinal neurogenesis, and there is the potential for cross-talk and complementary interactions in the regulation of different repressive complexes during development.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.05.010.

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