

Development 140, 2867–2878 (2013) doi:10.1242/dev.088096
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Polycomb repressive complex PRC2 regulates *Xenopus* retina development downstream of Wnt/ β -catenin signaling

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SUMMARY

The histone methyltransferase complex PRC2 controls key steps in developmental transitions and cell fate choices; however, its roles in vertebrate eye development remain unknown. Here, we report that in *Xenopus*, PRC2 regulates the progression of retinal progenitors from proliferation to differentiation. We show that the PRC2 core components are enriched in retinal progenitors and downregulated in differentiated cells. Knockdown of the PRC2 core component Ezh2 leads to reduced retinal progenitor proliferation, in part due to upregulation of the Cdk inhibitor p15^{Ink4b}. In addition, although PRC2 knockdown does not alter eye patterning, retinal progenitor gene expression or expression of the neural competence factor Sox2, it does cause suppression of proneural bHLH gene expression, indicating that PRC2 is crucial for the initiation of neural differentiation in the retina. Consistent with this, knocking down or blocking PRC2 function constrains the generation of most retinal neural cell types and promotes a Müller glial cell fate decision. We also show that Wnt/ β -catenin signaling acting through the receptor Frizzled 5, but independent of Sox2, regulates expression of key PRC2 subunits in the developing retina. This is consistent with a role for this pathway in coordinating proliferation and the transition to neurogenesis in the *Xenopus* retina. Our data establish PRC2 as a regulator of proliferation and differentiation during eye development.

KEY WORDS: Ezh2, Histone methylation, Retina development, Neurogenesis, Epigenetics, Wnt signaling

INTRODUCTION

In the developing vertebrate retina, the transition of progenitors from proliferation to differentiation is highly coordinated, and involves downregulation of cell cycle genes and activation of the retinal cell fate specification machinery (Agathocleous and Harris, 2009; Agathocleous et al., 2009). This switch is regulated by crosstalk between transcription factors and signaling pathways, and requires broad chromatin restructuring and extensive changes in gene expression (Hsieh and Gage, 2004; Ohsawa and Kageyama, 2008). Mounting evidence suggests that mechanisms that regulate chromatin structure and global gene expression orchestrate crucial transitional steps during neural development, including retinal differentiation (Yamaguchi et al., 2005; Lessard and Crabtree, 2010).

The highly conserved Polycomb group proteins repress gene expression through the formation of distinct chromatin remodeling complexes, termed polycomb repressive complexes (PRCs) (Martinez and Cavalli, 2006; Margueron and Reinberg, 2011). PRC2 methyltransferase activity catalyzes the addition of histone H3 lysine 27 trimethylation (H3K27me3) to specific genomic loci, which act as docking sites for recruiting additional repressive complexes (Kuzmichev et al., 2002; Fischle et al., 2003; Rajasekhar and Begemann, 2007). PRC2 consists of four core subunits: EZH2 (the catalytic subunit), SUZ12, EED and RbBP4/7 (Pietersen and van Lohuizen, 2008; Margueron and Reinberg, 2011). SUZ12 and EED are indispensable for the EZH2 enzymatic function, whereas RbBP4/7 is required for nucleosome binding (Nekrasov et al., 2005; Pasini et al., 2007).

PRC2 plays crucial roles in governing the balance between proliferation and differentiation (Yu et al., 2007; Margueron and Reinberg, 2011; Aldiri and Vetter, 2012). Ezh2 is required for cell proliferation during tissue formation and is a known marker for metastatic cells, mainly owing to repression of the tumor suppressor locus Ink4b-ARF-Ink4a (Popov and Gil, 2010; Aguilo et al., 2011).

Furthermore, PRC2 is an important regulator of cellular differentiation (Testa, 2011). Embryonic stem cells (ESCs) with mutated Suz12 fail to differentiate into neurons in culture, suggesting that PRC2 is essential for neural differentiation (Boyer et al., 2006; Lee et al., 2006; Pietersen and van Lohuizen, 2008). In the developing cortex, PRC2 modulates the developmental timing of neuron and glia production (Hirabayashi and Gotoh, 2010; Pereira et al., 2010). PRC2 also regulates the cell fate choices between oligodendrocytes and astrocytes of cultured neural stem cells (Sher et al., 2008). Thus, PRC2 function is context dependent during development.

Whether PRC2 regulates eye development and underlying cell fate decisions is unknown. In ESCs, PRC2 and its repressive mark H3K27me3 occupy the promoters of many genes that are involved in retinal development, and PRC2 components are expressed during both *Xenopus* and mouse eye development (Lee et al., 2006; Aldiri and Vetter, 2009; Rao et al., 2010). Here, we show that expression of the PRC2 core components is enriched in retinal progenitors and diminishes with the onset of retinal differentiation. Blocking Ezh2 impairs retinal proliferation but does not alter retinal progenitor specification. Notably, PRC2 is required for proneural gene expression in retinal progenitors, and for normal retinal neuron differentiation, as blocking PRC2 function biases cell fate towards the Müller glia fate. We find that canonical Wnt signaling, which coordinates proliferation and differentiation in the *Xenopus* retina (Van Raay et al., 2005; Agathocleous et al., 2009), regulates the expression of several PRC2 subunits in the developing eye in a Sox2-independent manner, supporting a role for PRC2 as an important component of this developmental

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Accepted 7 May 2013

transition. Collectively, our data reveal that in *Xenopus*, PRC2 acts at a crucial step in the progression of retinal progenitors towards a differentiated fate.

MATERIALS AND METHODS

Construction of Δ SET-Ezh2, microinjections of mRNAs and transgenesis

Dominant-negative Ezh2 (Δ SET-Ezh2) lacks the C-terminal 135 amino acids containing the SET domain, and was subcloned by PCR into pCS2+. Capped mRNAs were synthesized using Message Machine kit (Ambion). mRNAs for GFP (300 pg), β -galactosidase (β -gal; 200 pg) or *Xenopus* p15^{Ink4b} (1 ng; Open Biosystems) were injected in one dorsal animal blastomere at the eight-cell stage (Huang and Moody, 1993). Embryos were collected and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967), and X-gal staining performed on β -gal-injected embryos as previously described (Turner and Weintraub, 1994). Transgenic embryos were generated as previously described (Kroll and Amaya, 1996; Hutcheson and Vetter, 2002), using the *Rx*: Δ Ntcf3-GFP construct (Van Raay et al., 2005) or the *Rx*:Sox2-BD construct, in which Sox2-BD (Kishi et al., 2000) was cloned under control of the *Rx* promoter (see Van Raay et al., 2005).

In situ hybridization analysis

In situ hybridization was performed on whole embryos and retinal sections as previously described (Hutcheson and Vetter, 2001). The following digoxigenin (DIG)-labeled riboprobes were used for the analysis: Ezh2, Rbbp4/7, Eed, Suz12 (Aldiri and Vetter, 2009), Rx (Casarosa et al., 1997), Xash1 (Ferreiro et al., 1993), Xath5 (Kanekekar et al., 1997), *Xenopus* Fz5 (Sumanas and Ekker, 2001), cyclin D1 (Vernon and Philpott, 2003), Xngnr-1 (Ma et al., 1996), Xash3 (Zimmerman et al., 1993), Vsx1 (D'Autilia et al., 2006), Sox2 (Mizuseki et al., 1998), Pax6 (Hirsch and Harris, 1997), NeuroD (Lee et al., 1995), Six3 (Zhou et al., 2000), Hermes (Patterson et al., 2000), Sbt1 (Logan et al., 2005a), Delta (Dorsky et al., 1997), Notch (Coffman et al., 1990), Esr-1 (Wettstein et al., 1997) and Nrarp (Lamar et al., 2001).

Morpholinos and pan-caspase inhibitor

The following translation blocking morpholinos (Gene Tools; Philomath, OR) were used in the eight-cell injections: Ezh2 ATG MO, 5'-CAGATTTCTTCCCGTCTGGCCCAT-3' (5 ng); Ezh2 UTR MO, 5'-TATCCAAAGGATGAATGGTCGCTCA-3' (20-25 ng); control MO (scrambled sequence of Ezh2 ATG MO), 5'-CGAATTCTTCTCCGCTTCGCGCACT-3' (5 ng); Rbbp4/7 MO, 5'-CGAACGCAGCTTCTT-ATCAGCCAT-3' (10 ng).

Fz5 MO (15 ng) and Suz12 MO (15 ng) have been previously described (Van Raay et al., 2005; Peng et al., 2009). The efficacy of the Ezh2 UTR MO was confirmed by its ability to block *in vitro* protein translation using Ezh2 cDNA as a template (data not shown). A pan-caspase inhibitor [Z-VAD (OMe)-FMK; Calbiochem] (5 ng) was used in injections.

Immunohistochemistry, TUNEL analysis and BrdU labeling

Immunostaining on retinal cryosections was performed as previously described (Moore et al., 2002). Antibodies used were: rabbit anti-H3K27me3 (Millipore, 1:100), rabbit anti-HP3 (Upstate, 1:300), rabbit anti-Ezh2 (1:4000, Active Motif; sections treated with 50 mM NH₄Cl for 10 minutes after fixation) and Alexa Fluor 568-conjugated goat anti-rabbit antibody (Molecular Probes, 1:2000). TUNEL labeling was performed as previously described (Hensley and Gautier, 1998; Agathocleous et al., 2009). BrdU labeling for 30 minutes was followed by detection on stage 41 retinal sections as previously described (Perron et al., 1998).

For cumulative BrdU labeling (Nowakowski et al., 1989), 5 ng of control MO or Ezh2 ATG MO plus 300 pg of GFP mRNA was injected into one dorsal blastomere at the eight-cell stage, then embryos selected at stages 17-18, sorted for GFP expression in the optic vesicle then injected with BrdU as described previously (Perron et al., 1998), except that embryos were then allowed to recover for 30, 60 or 90 minutes, or 4 hours prior to fixation. During this time, BrdU is continuously available, and is incorporated by cells entering S-phase. Embryos were embedded in paraffin wax, sectioned at 14 μ m and anti-BrdU antibody staining was performed as described previously (Agathocleous et al., 2009). Cell counts were made

using NIS Elements AR4 and statistical analysis using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). The labeling index (LI) was quantified as the number of BrdU-labeled nuclei over total Hoechst-positive nuclei in the optic vesicle. The LI increases over time until maximal labeling is attained, which defines the growth fraction or the proportion of cells in the optic vesicle that are actively cycling. The slope of the linear region of the curve measures the rate of BrdU incorporation, which is a reflection of total cell cycle length (Nowakowski et al., 1989).

Retinal cell fate analysis

Morpholinos against Ezh2 (ATG, 0.25 ng; UTR, 2.5 ng) or mRNA encoding Δ SET-Ezh2 (1 ng) were injected in one dorsal animal blastomere of 32-cell stage embryos along with 300 pg of GFP mRNA (Huang and Moody, 1993). GFP mRNA alone (300 pg) or control MO (0.5 ng) were injected as controls. GFP-positive cells in stage 41 retinal sections were scored for cell type based on cell position and morphology, as previously described (Moore et al., 2002).

Semi-quantitative RT-PCR

Embryos were injected with 300 pg GFP mRNA with either Ezh2 ATG or control MOs at the eight-cell stage. Embryos were grown to stage 27, GFP-positive eyes dissected and total RNA isolated (TRIzol Reagent, Invitrogen). First-strand cDNA was synthesized using SuperScript First-Strand (Invitrogen). Semi-quantitative polymerase chain reaction (PCR) was performed with Phusion DNA Polymerase (New England Biolabs). Initial experiments validated exponential amplification at different cycle numbers (20-33 cycles). Amplified products for histone H4 (25 cycles) (Logan et al., 2005b) and p15^{Ink4b} (30 cycles) were visualized in agarose gel with ethidium bromide and a UV transilluminator (Gel Doc 2000; Bio-Rad), and relative band intensity (Image lab software 4.0; Bio-Rad) in relation to histone H4 was measured. The following primers for p15^{Ink4b} were used: forward, 5'-GCCCCAATTCATGGATTTCAATGCC-3'; reverse, 5'-GCCCTCTGAGTCAGCGATATCCTAGC-3'.

Microarray analysis

Embryos were injected at the eight-cell stage with 5 ng Ezh2 ATG MO or control MO together with 400 pg mRNA for GFP, and at stage 27 GFP-positive eyes were isolated. Pools of 20-25 eyes were used to prepare total RNA for two-color microarray analysis on the *Xenopus* Agilent microarray by the University of Utah Microarray core facility, with subsequent statistical analysis as described previously (Green and Vetter, 2011). Four biological replicates for each condition were performed. Microarray data have been deposited at GEO with accession number GSE47456.

RESULTS

PRC2 components are expressed in retinal progenitors

PRC2 genes are dynamically expressed in the developing central nervous of *Xenopus* embryos and at all stages of eye development, including in retinal progenitors throughout the optic vesicle (Reijnen et al., 1995; Yoshitake et al., 1999; Showell and Cunliffe, 2002; Aldiri and Vetter, 2009) (supplementary material Fig. S1A-D). To assess expression in specific retinal cell populations, we characterized the expression patterns of Ezh2 and other PRC2 core components in the retina at stage 41 (tadpole stage; Fig. 1A). At this stage, differentiated retinal cells occupy the central part of the retina, while undifferentiated proliferating cells reside in the peripheral region, termed the ciliary marginal zone (CMZ) (Fig. 1B) (Perron et al., 1998; Agathocleous et al., 2009). Transcripts of all PRC2 core subunits, Ezh2, Suz12, Eed and Rbbp4, were expressed in the CMZ region, and not detected in terminally differentiated cells (Fig. 1C-F). The expression of these genes was also excluded from the most peripheral retinal stem cell compartment (Fig. 1C-F). Thus, the PRC2 genes appear to be expressed in retinal progenitors in both the embryonic retina and in the CMZ.

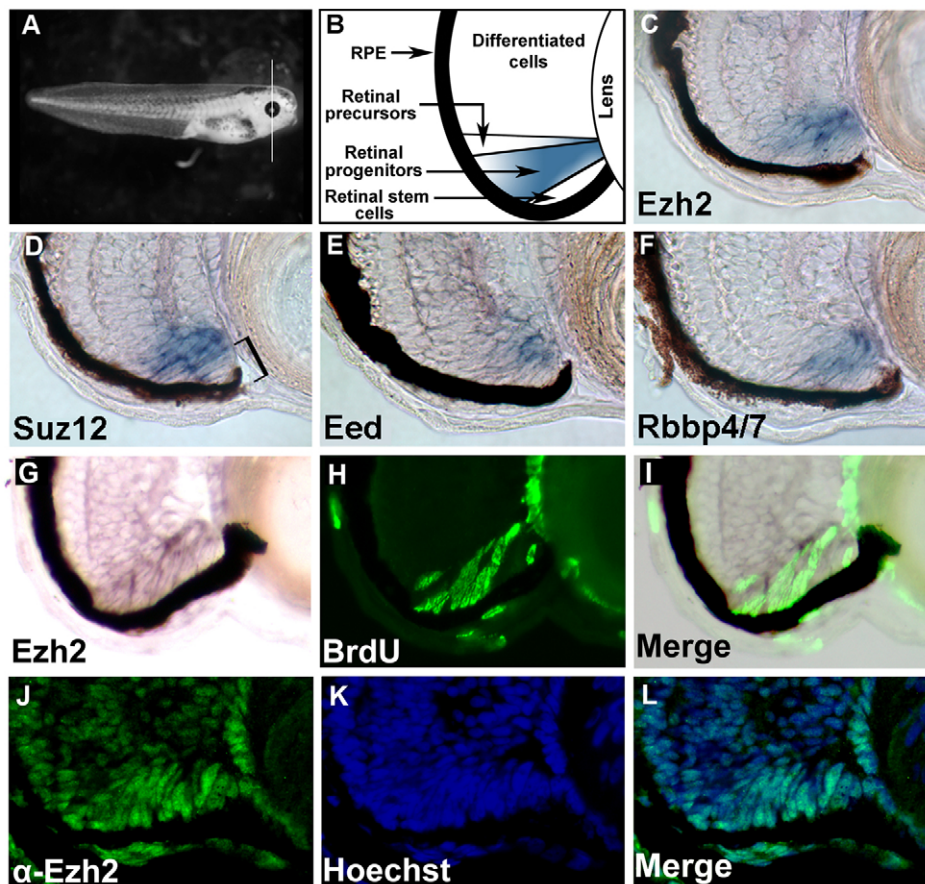


Fig. 1. The PRC2 core components are enriched in the CMZ region of *Xenopus* retina. (A) Stage 41 frog embryo. (B) Domains in the ciliary marginal zone (CMZ) of the *Xenopus* retina at stage 41. (C-F) Expression of Ezh2, Suz12, Eed and Rbbp4 by *in situ* hybridization on retinal sections. Retinal stem cell domain in the distal tip of the CMZ is negative for staining (bracket in D provides an example). (G-I) Ezh2 expression coincides with BrdU labeling. (J-L) EZH2 protein is enriched in the CMZ. RPE, retinal pigment epithelium.

To confirm that PRC2 components are expressed in actively dividing cells, we performed BrdU labeling with *in situ* hybridization and found that most BrdU-labeled cells were also positive for PRC2 transcripts (Fig. 1G-I; supplementary material Fig. S1E-J). Furthermore, by immunostaining, we found that EZH2 protein is enriched in the CMZ region (Fig. 1J-L). EZH2 immunostaining was reduced by injections of 5 ng of translation blocking morpholino for EZH2 (hereafter termed EZH2 ATG MO) together with GFP mRNA into one cell of eight-cell stage embryos, whereas control MO (a scrambled sequence of Ezh2 ATG MO) had no effect (supplementary material Fig. S1K-P). Thus, the expression of the PRC2 components is transient, and highest in proliferating retinal progenitor cells.

PRC2 regulates H3K27me3 deposition in *Xenopus* retina

As PRC2 catalyzes the addition of H3K27me3, we examined the distribution pattern of global H3K27me3 by immunostaining the retina at stage 41. We found that H3K27me3 levels were clearly enriched in postmitotic retinal cells (Fig. 2A-C). To address whether Ezh2 regulates H3K27 trimethylation in the retina, we injected EZH2 ATG MO together with GFP mRNA into one cell of 32-cell stage embryos and immunostained retinal sections at stage 41 for H3K27me3. GFP-positive cells exhibited a marked reduction in the levels of H3K27me3 (Fig. 2D-F), whereas control MO did not affect H3K27me3 (Fig. 2G-I). To confirm this, we generated a construct encoding a dominant-negative form of Ezh2 that lacks the catalytic SET domain (Δ SET-Ezh2) (Akizu et al., 2010). Injection of mRNA encoding Δ SET-Ezh2 caused a similar reduction in HEK27me3 levels (supplementary material Fig. S2). Finally, targeting another

core component of the PRC2 complex (Suz12) by injecting Suz12 MO (Peng et al., 2009) had a similar effect (supplementary material Fig. S3). We conclude that PRC2 regulates H3K27me3 deposition in *Xenopus* retina.

PRC2 is required for retinal proliferation

We have shown that PRC2 subunits are expressed in retinal progenitors, indicating a possible role in retinal proliferation and/or differentiation (Fig. 1; supplementary material Fig. S1A-D) (Aldiri and Vetter, 2009). To assess the function of PRC2 in eye development, we injected Ezh2 ATG MO into one dorsal cell of eight-cell stage embryos along with GFP mRNA as a tracer, and observed a reduction in eye size on the injected side at stage 41 (Fig. 3B), while control MO had a minimal effect (Fig. 3C). Eye diameter was on average smaller by 15% in embryos injected with Ezh2 MO compared with the ones injected with control MO (Ezh2 ATG MO, $n=18$ embryos; control MO, $n=11$ embryos; t -test, $P<0.001$). Furthermore, we injected a morpholino that targets the 5' UTR region of Ezh2 (Ezh2 UTR MO), and obtained weaker but similar results (Fig. 3E). Finally, targeting other components of the complex by injecting translation blocking morpholino against Rbbp4/7 or a Suz12 (Peng et al., 2009) phenocopied the effect of the Ezh2 MOs (Fig. 3F,D).

We assessed whether reduced eye size upon knockdown of Ezh2 is due to decreased proliferation. We injected Ezh2 ATG MO or control MO at the eight-cell stage, then stained retinal sections at stage 27 with an antibody against phosphorylated histone H3 (HP3), a marker for mitotic cells. Knockdown of Ezh2 function resulted in reduced fraction of HP3-positive cells when compared with control MO (Fig. 3G-K).

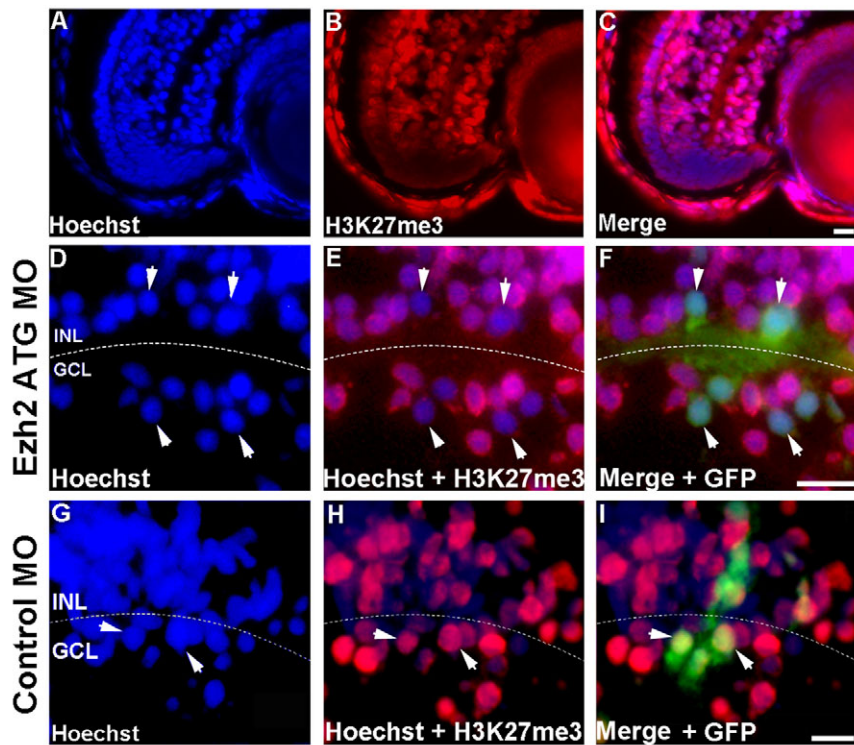


Fig. 2. Ezh2 is required for H3K27me3 deposition in *Xenopus* retina.

(A-C) Immunostaining of H3K27me3 on a stage 41 retinal section showing increased labeling in differentiated cells. (D-I) Immunostaining of H3K27me3 after co-injection of GFP mRNA with Ezh2 ATG MO (D-F) or control MO (G-I). Arrowheads in D-F show GFP-labeled cells with reduced H3K27me3 levels, whereas in G-I the arrowheads indicate GFP-labeled cells with normal H3K27me3 staining. Hoechst labels nuclei (blue). INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars: 10 μ m.

To determine how cell cycle kinetics were altered with knockdown of PRC2 function, we used a BrdU cumulative labeling method (Nowakowski et al., 1989; Siegenthaler and Miller, 2005) to compare the rate of proliferation in the optic vesicle and the proportion of cells that were actively cycling. This was performed over a 4 hour period, as we previously found that this was sufficient to achieve maximal BrdU labeling in the optic vesicle (Van Raay et al., 2005). We found that the initial linear increase in labeling index (LI) in the optic vesicle was similar with either Ezh2 MO or control MO injection, with no statistical difference in slope, indicating that the rate of proliferation, and thus cell cycle length, was not altered (Fig. 3L) (Nowakowski et al., 1989). However, there was a significant decrease in the maximum BrdU labeling attained with Ezh2 ATG MO when compared with control MO (Fig. 3L). Thus, Ezh2 ATG MO reduced the growth fraction, which is the proportion of cells in the optic vesicle that are cycling, indicating that PRC2 activity appears to be required for cell cycle progression.

We ruled out apoptosis as a potential cause of reduced eye size. First, we found that embryos injected with Ezh2 ATG MO or control MO showed no difference in TUNEL-positive cells in the eye at stage 24 ($n=43$ and $n=21$ embryos respectively; t -test, $P=0.33$, data not shown). Second, co-injection of pan-caspase inhibitor (Z-VAD (OMe)-FMK, Calbiochem) (Walker and Harland, 2009; Bonev et al., 2011) along with Ezh2 ATG MO did not rescue the microphthalmia (supplementary material Fig. S4A).

We next asked whether the reduced proliferation was due to alteration in the expression of cell cycle regulators. A known target of PRC2 regulation in other systems is the Ink4b-ARF-INK4a locus, which encodes the cyclin-dependent kinase inhibitors p15^{Ink4b} and p16^{Ink4a}, as well as an unrelated protein, p14^{ARF}. In *Xenopus laevis*, we identified transcripts in GenBank for p15^{Ink4b} (CDKN2B; NM_001096861), but not for other PRC2 targets that are part of the Ink4b-ARF-INK4a locus p16^{Ink4a} or p14^{ARF}. Thus, we dissected the optic vesicle at stage 27 from embryos injected at the eight-cell stage with Ezh2 ATG MO or control MO together with GFP mRNA

to mark the injected side. We then compared the levels of p15^{Ink4b} expression relative to histone H4 by semi-quantitative RT-PCR. We found a significant upregulation in p15^{Ink4b} levels upon knocking down of Ezh2 (Fig. 4A). To examine whether the effect of Ezh2 MO on retinal proliferation can be attributed to increased levels of p15^{Ink4b}, we first overexpressed p15^{Ink4b} in retinal progenitors by injecting *Xenopus* p15^{Ink4b} mRNA at the eight-cell stage and found that eye size was reduced, similar to what was observed with Ezh2 MO injection (Fig. 4B,C). Furthermore, overexpression of p15^{Ink4b} led to a significant reduction in HP3 staining (Fig. 4D). Thus, PRC2 activity may maintain retinal proliferation in part by inhibiting the tumor suppressor gene p15^{Ink4b}.

However, co-injection of Ezh2 MO and p15^{Ink4b} MO did not lead to rescue of eye size (supplementary material Fig. S4B). We considered the possibility that other genes, including other cell cycle inhibitors, may also be upregulated upon blocking Ezh2 function. To assess, this we performed microarray analysis on optic vesicles isolated at stage 27 from Ezh2 MO-injected embryos or control MO-injected embryos. We found that at least two other known cell cycle inhibitors were upregulated upon Ezh2 inhibition (supplementary material Fig. S5): Fizzy-related (Fzr1), which encodes Cdh1, an important activator of the anaphase-promoting complex (Skaar and Pagano, 2008); and p16Xic3, which encodes a cyclin-dependent kinase (cdk) inhibitor of the Cip/Kip family. Other members of this family, p27Xic1 or p16Xic2, as well as the cdk inhibitor p19Ink4d were not upregulated (supplementary material Fig. S5). Collectively, our data suggest that PRC2 activity regulates retinal proliferation in part by inhibiting, either directly or indirectly, cell cycle inhibitors, including the tumor suppressor gene p15^{Ink4b}.

Knockdown of PRC2 does not affect retinal progenitor specification

In some cases, PRC2 function is required for progenitors to properly acquire or maintain their tissue identity (Wyngaarden et al., 2011; Aldiri and Vetter, 2012), so we assessed whether PRC2 function is

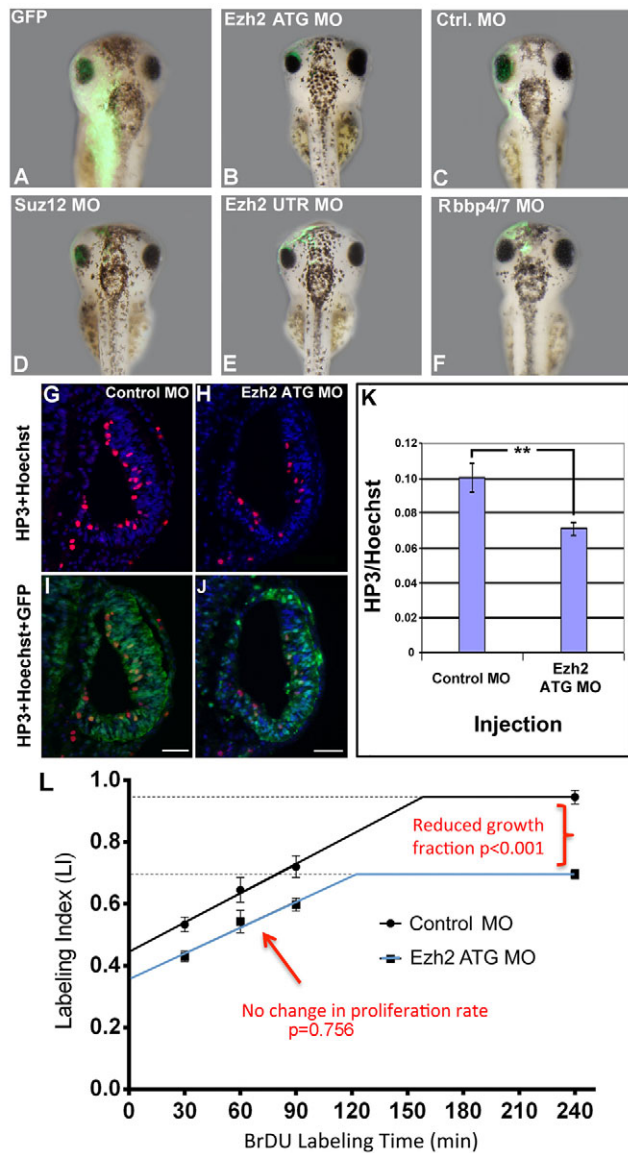


Fig. 3. Inhibition of PRC2 function negatively affects retinal proliferation. (A-F) Knockdown of PRC2 core components causes reduced eye size ($n=103/117$ for Ezh2 ATG MO; $6/36$ for Ezh2 UTR MO; $19/24$ for Rbbp4/7 MO; $14/34$ for Suz12 MO), whereas control MO does not (C; $n=44$). (G-J) Injection of Ezh2 ATG MO results in reduced fraction of HP3-labeled cells within the optic vesicle (H,J) when compared with control MO (G,I). Scale bars: $50\ \mu\text{m}$. (K) Quantification of data represented in G-J ($n=12$ embryos for control MO, $n=10$ embryos for Ezh2 MO embryos). Data are mean \pm s.e.m., Student's t -test, $**P<0.01$. (L) Cumulative BrdU labeling in the optic vesicle with Ezh2 ATG MO or control MO injection together with GFP mRNA. The labeling index (LI) is the number of BrdU-labeled nuclei over total Hoechst-positive nuclei in the optic vesicle. The slope of the initial linear increase in BrdU labeling in both conditions was similar ($P=0.756$), which reflects no change in proliferation rate and cell cycle length. There was a significant decrease in the maximum BrdU labeling attained with Ezh2 ATG MO (LI=0.695) when compared with control MO (LI=0.945, $P<0.001$); thus, the growth fraction (the proportion of cells in the optic vesicle that are cycling) is reduced. Data are mean \pm s.e.m.

required for progenitor specification or retinal identity. We injected one dorsal blastomere of eight-cell stage embryos with Ezh2 ATG MO plus β -galactosidase (β -gal) mRNA as a tracer, and investigated

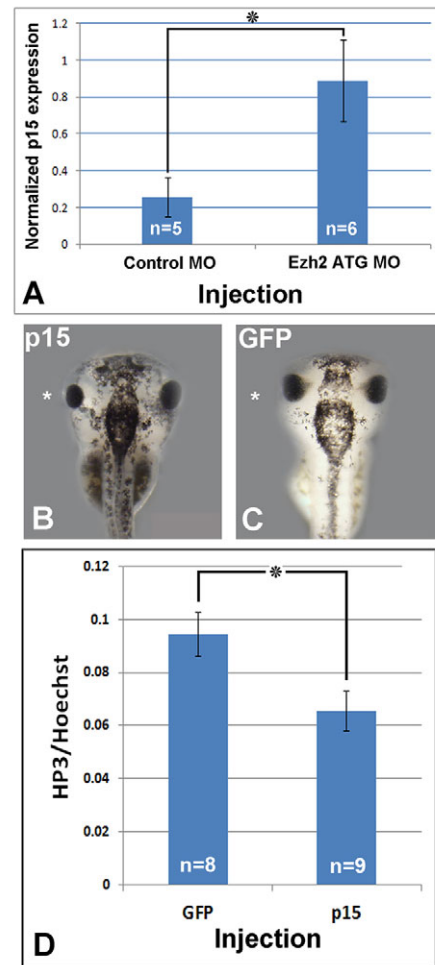


Fig. 4. Ezh2 knockdown increases p15^{Ink4b} expression and p15^{Ink4b} causes reduced proliferation. (A) Quantification of p15^{Ink4b} expression by semi-quantitative PCR analysis of isolated optic vesicle tissue after either control MO or Ezh2 ATG MO injection, normalized to the internal standard histone H4. $n=5$ for control MO, $n=6$ for Ezh2 ATG MO. (B,C) p15^{Ink4b} overexpression by mRNA injection at the eight-cell stage causes a small eye phenotype ($n=35/39$) when compared with injection of GFP mRNA alone. Asterisk marks injected side. (D) p15^{Ink4b} overexpression results in reduced HP3 labeling in the optic vesicle when compared with GFP alone. In A,D, data are mean \pm s.e.m.; Student's t -test, $*P<0.05$.

the expression of the retinal progenitor markers Rx, Pax6, Six3 and Vsx1 by *in situ* hybridization analysis at stage 20 (optic vesicle stage). There was no change in the expression of the tested genes, although there was a clear reduction in the size of the expression domain on the injected side, consistent with reduced eye size (Fig. 5A-D). Similarly, we saw no effect on the expression intensity of Fz5, which regulates neural competence in the *Xenopus* retina, or its downstream target Sox2 (Van Raay et al., 2005), although the expression domain was smaller (Fig. 5E,F). Similar results were obtained for the progenitor gene cyclin D1 (Fig. 5G). Embryos injected with control MO showed no or minimal effect on the expression of retinal progenitor genes (Fig. 5H,I; supplementary material Fig. S6).

We also injected the Ezh2 UTR MO or Suz12 MO, and assayed for expression of Rx, Vsx1 and Fz5. Although eye size was reduced, we observed no change in the expression of these progenitor genes

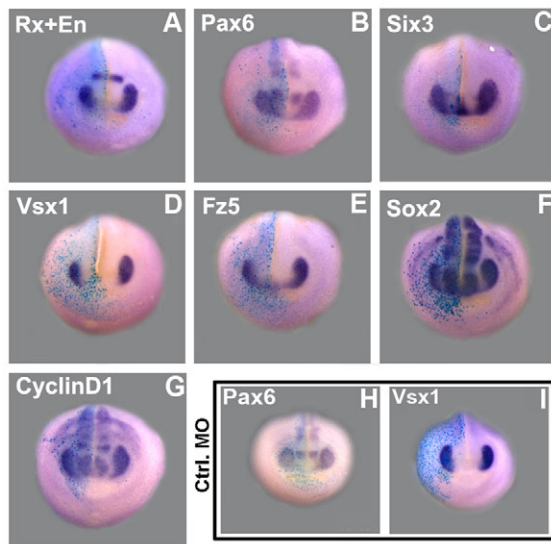


Fig. 5. Knockdown of Ezh2 does not affect retinal progenitor specification. (A-G) Anterior view of stage 20 embryos after injection of Ezh2 ATG MO and mRNA encoding β -galactosidase to label the injected side. X-gal staining is light blue. Progenitor genes show normal levels of expression, although the eye domain is smaller (embryos with a reduced expression domain: 70%, $n=90$ for Rx; 85%, $n=47$ for Pax6; 81%, $n=43$ for Six3; 82%, $n=74$ for Vsx1; 82%, $n=76$ for Fz5; 81%, $n=62$ for Sox2; 81%, $n=16$ for cyclin D1). (H,I) Control MO-injected embryos.

(supplementary material Fig. S7). Taken together, we conclude that PRC2 function is not required for retinal progenitors to be specified or to become Sox2-positive neural-competent retinoblasts.

Knockdown of PRC2 blocks expression of retinal differentiation genes

In cortical progenitors, PRC2 can inhibit neural differentiation during the gliogenic period by repressing the proneural bHLH factor Ngn1 (Hirabayashi et al., 2009). To assess the effects on proneural gene expression in the developing retina, we injected Ezh2 ATG MO, along with β -gal or GFP mRNA as a tracer in one dorsal blastomere of eight-cell stage embryos and analyzed the expression of several retinal differentiation factors by *in situ* hybridization analysis. Surprisingly, we found that the expression of the proneural bHLH genes, Xath5, Xash1, Xash3, NgnR1 and NeuroD was lost or dramatically reduced on the Ezh2 ATG MO-injected side (Fig. 6A-J). Consistent with the loss of proneural function, the expression of Sbt1, a downstream target of Xath5, NeuroD and Ngn2 (Logan et al., 2005a; Seo et al., 2007), as well as Hermes, a differentiated retinal ganglion cell marker (Patterson et al., 2000) was suppressed (Fig. 6K-N). Again at this stage, the expression levels of the progenitor genes Rx, Vsx1, Fz5 and Sox2 were not affected (Fig. 6O-P; supplementary material Fig. S8). Embryos injected with control MO showed minimal effect on the expression of proneural genes or their targets (Fig. 6Q-X). We confirmed that injection of the Ezh2 UTR MO had a similar effect to the Ezh2 ATG MO in blocking the expression of Xash1 and Xath5 (supplementary material Fig. S7). In addition, targeting two other core PRC2 components by injecting either Suz12 MO or Rbbp4 MO mirrored the effect of Ezh2 knockdown by inhibiting Xath5 and Xash1 expression (supplementary material Fig. S7; data not shown).

As Ezh2 causes an increase in p15^{Ink4b} expression and reduction in retinal progenitor proliferation, we tested whether this would

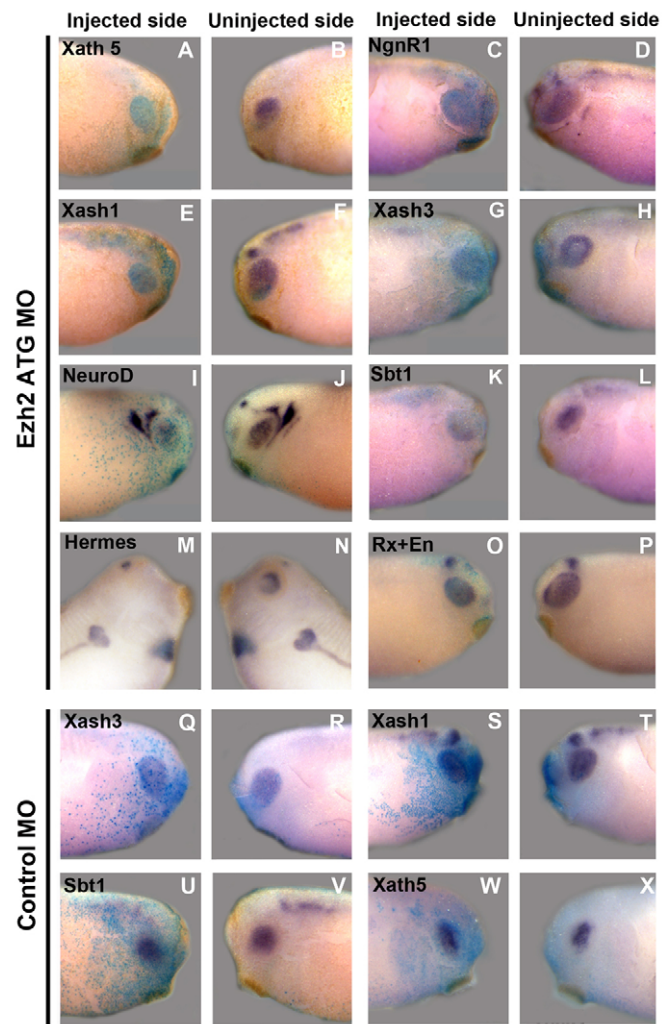


Fig. 6. Initiation of retinal differentiation genes is blocked by Ezh2 inhibition. Lateral view of embryos injected with Ezh2 ATG or control MOs along with β -galactosidase or GFP mRNAs to mark injected side. X-gal staining is light blue. (A-J) Proneural bHLH gene expression is reduced or absent after Ezh2 ATG MO injection (55%, $n=53$ for Xath5; 57%, $n=52$ for Xash1; 47%, $n=19$ for NeuroD; 40%, $n=16$ for Xash3; 43%, $n=30$ for NgnR1). (K-P) The bHLH target gene Sbt1 (K,L; 67%, $n=14$) and the ganglion cell marker Hermes (M,N; 84%, $n=31$) are reduced or absent, whereas Rx expression level is normal (O,P). (Q-X) Control MO has minimal effect ($n=28$ for Xash1; $n=21$ for Xash3; $n=15$ for Xath5; $n=14$ for Sbt1).

be sufficient to block proneural gene expression. Although overexpression p15^{Ink4b} by mRNA injection at the eight-cell stage caused reduced eye size (Fig. 4B), there was no effect on levels of proneural gene expression (supplementary material Fig. S9), suggesting that inhibiting cell cycle progression is not sufficient to prevent the onset of differentiation. In addition, as activation of Notch signaling blocks retinal differentiation, we reasoned that loss of PRC2 might increase Notch signaling. However, we found that the Notch pathway components Delta, Notch and its downstream targets Esr1 and Nrarp at this stage were not increased (supplementary material Fig. S8). These findings were also supported by our microarray analysis, which indicates that although proneural gene expression was reduced, eye-specifying factors and Notch signaling genes were unaffected (supplementary

material Fig. S5). Thus, although PRC2 does not disrupt the specification of retinal progenitors, it is essential for proneural gene expression in the *Xenopus* retina, in addition to regulating progenitor proliferation.

Knockdown of PRC2 causes an increase in Müller glial cell differentiation

As both cell cycle regulators and proneural bHLH factors are major determinants of neural cell fate decisions (Agathocleous and Harris, 2009), we reasoned that blocking PRC2 may affect retinal neuron differentiation. To address this, we injected one dorsal blastomere at the 32-cell stage with Ezh2 ATG MO along with GFP mRNA, and collected embryos at stage 41. Individual GFP-labeled cells in retinal sections were counted and scored for cell type based upon morphology and laminar position, as previously described (Van Raay et al., 2005). Blocking Ezh2 function caused a sevenfold increase in non-neural Müller glia when compared with GFP mRNA alone (Fig. 7A). The increase in Müller glia with the Ezh2 ATG MO was coupled to a decrease in the percentage of some retinal neurons, specifically retinal ganglion cells and bipolar cells, although amacrine cell numbers were significantly increased (supplementary material Fig. S10). Injection of control MO did not significantly alter the proportion of Müller glia or other retinal cell types relative to GFP mRNA alone (Fig. 7A).

To confirm that the effect is due to blocking Ezh2 function, we next injected Ezh2 UTR MO and observed a sixfold increase in Müller glia (Fig. 7A). Similarly, injection of mRNA encoding a dominant-negative Δ SET-Ezh2 caused a 10-fold increase in Müller glia (Fig. 7A), confirming that Ezh2 function is indeed required for retinal neuron differentiation. Fig. 7B shows an example of GFP-labeled cells co-injected with Ezh2 UTR MO, many showing the

hallmark morphology of Müller glia. Taken together, we conclude that PRC2 is essential for generating the normal complement of retinal cell types in *Xenopus*.

Expression of PRC2 subunits depends upon Wnt/ β -catenin signaling

The PRC2 core components are dynamically expressed during *Xenopus* eye development, but little is known about whether their expression is regulated by developmental signaling pathways. We have previously shown that Wnt/ β -catenin signaling, acting through the Fz5 receptor, regulates the differentiation of retinal progenitors in *Xenopus*, similar to PRC2 (Van Raay et al., 2005). Furthermore, Fz5 and its downstream target Sox2 are expressed in progenitors, similar to the PRC2 core components (Van Raay et al., 2005). We therefore investigated whether PRC2 gene expression is regulated by Wnt/ β -catenin signaling in the *Xenopus* retina and whether it inhibited Fz5 activity by injection at the eight-cell stage of Fz5 MO or mRNA encoding a dominant-negative form of Fz5 (dnFz5) (Van Raay et al., 2005). We confirmed that Wnt/ β -catenin signaling was disrupted due to loss of Sox2 and Delta expression on the injected side of stage 24 embryos, as previously reported (Van Raay et al., 2005). We found that injection of Fz5 MO or mRNA for dnFz5 results in a significant loss in the expression of Ezh2 and Suz12 (Fig. 8A-D; data not shown). We note that there is no reciprocal regulation, as we had already found that inhibition of Ezh2 does not affect the expression of Fz5 or the downstream target Sox2 (Fig. 5; supplementary material Fig. S8).

To confirm that PRC2 subunit expression depends on Wnt/ β -catenin signaling, we generated transgenic embryos and used the *Xenopus* Rx promoter to drive expression in the eye of a truncated version of Tcf3 that functions as a constitutive repressor and blocks Wnt/ β -catenin signaling (Rx: Δ NTcf3-GFP) (Molenaar et al., 1996). We then examined gene expression by *in situ* hybridization analysis at stage 28. In the *Xenopus* retina, this phenocopies the effects of the Fz5 MO (Van Raay et al., 2005). As expected, only a minor proportion of embryos (13% of $n=48$) showed reduced expression of Rx, as this gene is not dependent upon Wnt/ β -catenin signaling (Van Raay et al., 2005). The minor effect was generally due to altered development observed in a subset of embryos with the nuclear transfer procedure. Both Sox2 and Xath5 showed reduced or absent expression in the eye (Fig. 8F,I; data not shown; 33%, $n=34$ for Sox2). This is consistent with their dependence upon Wnt/ β -catenin signaling (Van Raay et al., 2005), and in accordance with typical rates of transgene expression of 25-50% (Kroll and Amaya, 1996; Hutcheson and Vetter, 2002). Furthermore, we found that the expression of Ezh2 and Suz12 was also reduced or missing (Fig. 8E,I; data not shown), in agreement with the Fz5 MO knockdown experiments.

Given that Sox2 is a downstream target for Wnt signaling in the *Xenopus* retina (Van Raay et al., 2005), we asked whether PRC2 expression is also dependent on Sox2 function. We generated transgenic embryos using the Rx promoter to drive expression of dominant-negative Sox2 that lacks DNA-binding domain (Kishi et al., 2000). We examined the expression of Suz12 and Ezh2 in transgenic embryos by *in situ* analysis and compared this with Xath5 expression. Our data show that although Xath5 expression was reduced or lost in transgenic animals (Fig. 8H,I), consistent with Sox2 being required for Xath5 expression (Van Raay et al., 2005), Suz12 and Ezh2 expression was minimally affected (Fig. 8G,I; data not shown). Thus, we conclude that Sox2 is not required for the expression of Suz12 or Ezh2 in the developing *Xenopus* retina.

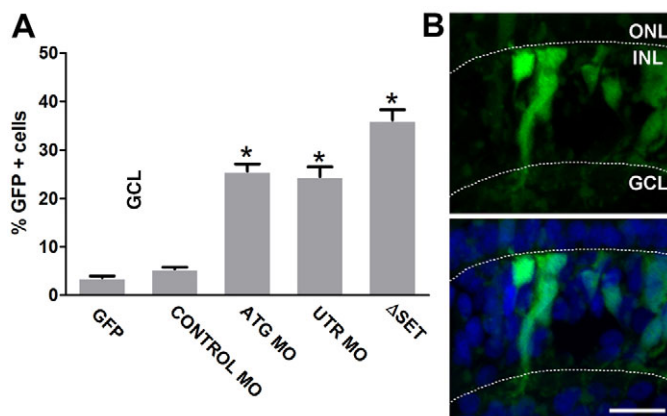


Fig. 7. Inhibiting PRC2 function in retinal progenitors biases cells to adopt the Müller glial cell fate. (A) Injection of Ezh2 MO (ATG MO) caused a sevenfold increase in the proportion of cells that become Müller glia when compared with GFP alone or with control MO [25.6 \pm 1.5% (s.e.m.), $n=2065$ cells total, 11 retinas for Ezh2 ATG MO; 3.6 \pm 0.37%, $n=3574$ cells total, 11 retinas for GFP mRNA alone, $P<0.001$; 5.6 \pm 0.51%, $n=2699$ cells total, 10 retinas, for control MO, no significant difference, $P=0.06$ compared with GFP mRNA alone]. Injection of Ezh2 UTR MO (UTR MO) had a similar effect [24.5 \pm 2.04% (s.e.m.), $n=2125$ cells, 9 retinas, $P<0.001$] as did injection of Δ SET-Ezh2 mRNA [Δ SET; 36.2 \pm 2.2% (s.e.m.), $n=3782$ cells, 17 retinas, $P<0.001$]. The percent representation of each cell type is a weighted average, and error bars represent s.e.m.; * $P<0.001$, by Student's *t*-test. (B) Confocal image of a retinal section (stage 41) showing Hoechst-labeled retinal cells (blue) and GFP-labeled cells (green) from an embryo injected with Ezh2 UTR MO plus GFP mRNA. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 20 μ m.

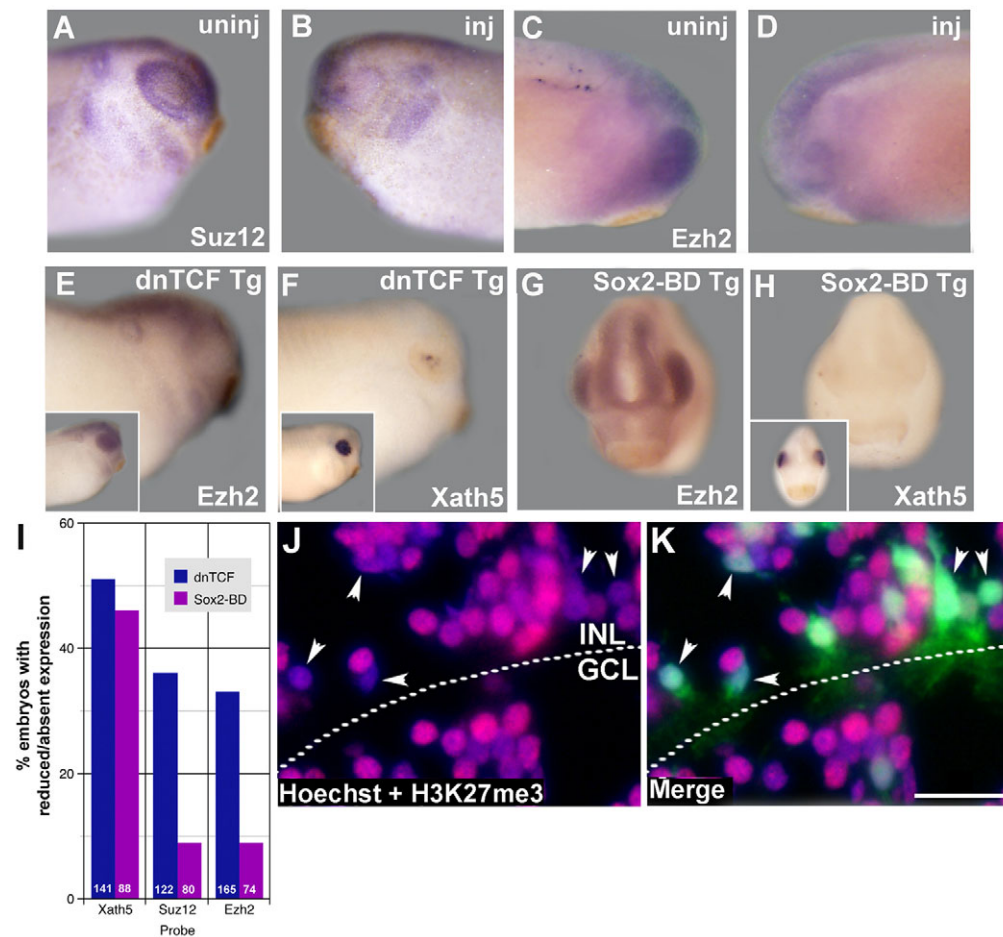


Fig. 8. Wnt signaling is required for PRC2 subunit expression and H3K27me3 in the retina. (A–D) Fz5 MO-injected embryos showing reduced expression of Suz12 (A,B; 51%, $n=55$) and Ezh2 (C,D; 44%, $n=41$) within the eye on the injected side. (E,F) *Rx:ΔNTcf3*-GFP transgenic embryos showing loss of Ezh2 and Xath5 expression in the eye. Embryos with normal expression are shown in the inset. (G,H) Anterior view of *Rx:Sox2-BD* transgenic embryos showing normal Ezh2 expression while Xath5 expression is lost. Inset in H shows an embryo with normal Xath5 expression. (I) A high proportion of dnTCF transgenic embryos have reduced or absent retinal expression of Xath5, Suz12 or Ezh2, whereas for Sox2-BD transgenic embryos, only Xath5 is affected. Total numbers of embryos analyzed for each are indicated on the bars of the graph. (J,K) Immunostaining with antibody against H3K27me3 on a retinal section from a stage 41 embryo injected with Fz5 MO plus GFP mRNA. Hoechst labels nuclei (blue). Arrowheads indicate GFP-labeled cells with reduced H3K27me3 levels. INL, inner nuclear layer; GCL, ganglion cell layer; Tg, transgenic. Scale bar: 20 μ m.

Finally, we reasoned that if Wnt/ β -catenin signaling is necessary for the expression of PRC2 core subunits, then blocking Wnt/ β -catenin signaling should reduce global H3K27me3 levels. Indeed, injection of Fz5 MO results in reduced H3K27me3 labeling in the *Xenopus* retina (Fig. 8J,K). Taken together, our data suggest that active Wnt/ β -catenin signaling, independent of the downstream target Sox2, governs the expression of PRC2 core subunits during retinal development in *Xenopus*.

DISCUSSION

We have defined a role for PRC2 in orchestrating the transition from proliferation to differentiation and subsequent lineage determination during retinal development. We found that PRC2 acts in retinal progenitors to maintain proliferation potential, and to promote the initiation of neural differentiation gene expression and consequently the generation of retinal neuron cell types. We show that Wnt/ β -catenin signaling, by acting through the Fz5 receptor but independently of Sox2, governs the expression of PRC2 subunits in the developing eye. Our findings support a model in which PRC2 functions to prime progenitors for the establishment of differentiation programs and reveal a crosstalk between signaling pathways and epigenetic mechanisms to promote retinal development.

Expression of PRC2 core subunits and H3K27me3 in *Xenopus* retina

We found that although the mRNAs of PRC2 core subunits are transiently enriched in retinal progenitors and downregulated in

differentiated cells, global H3K27me3 tends to increase upon differentiation. This observation is consistent with previously reported patterns in other tissues such as mouse retina and chick spinal cord, where PRC2 mRNAs are enriched in progenitors, whereas the H3K27me3 is highest in postmitotic differentiated cells (Akizu et al., 2010; Rao et al., 2010). We found that EZH2 protein expression in the *Xenopus* retina was also enriched in progenitors in the CMZ, although lower levels of protein may also be present in postmitotic cells of the central retina. In other tissues, the related protein EZH1 has been shown to persist in postmitotic cells where it contributes to H3K27 trimethylation (Akizu et al., 2010; Ezhkova et al., 2011), yet we could not identify any Ezh1-like sequences in the *Xenopus laevis* genome database, suggesting that Ezh2 may be primarily responsible. Consistent with this, we found that knocking down Ezh2 function in retinal progenitors was sufficient to reduce H3K27me3 labeling in postmitotic retinal neurons. However, we cannot rule out the possibility that other factors may contribute to histone methylation in retinal postmitotic cells.

Although the bulk of the H3K27me3 mark is found in postmitotic neurons, it does not rule out the possibility of lower levels being present on selected genes in progenitors. ChIP-seq analysis (Akkers et al., 2009) and mass spectrometry analysis (Schneider et al., 2011) suggest that H3K27me3 deposition is low during early stages of *Xenopus* development and increases as differentiation proceeds. Furthermore, genome-wide studies of H3K27me3 distribution in *Xenopus tropicalis* embryos suggest a dynamic pattern of H3K27me3 occupancy on genes involved in neurogenesis (Veenstra, G. J., personal communication) (Akkers et al., 2009;

Bogdanović et al., 2012). In addition, inhibiting PRC2 activity affects progenitors, by slowing their proliferation and preventing proneural gene expression, indicating that PRC2 has a function in this cell population. We hypothesize that PRC2 may have multiple distinct functions in the developing eye, regulating developmental progression early then potentially stabilizing differentiated cell fate later. Stage-specific roles for PRC2 have been described in the development of other tissues, including muscle and skin (Ezhkova et al., 2011; Stojic et al., 2011).

Regulation of retinal proliferation by PRC2

We found a reduction in eye size upon inhibition of PRC2 function, and observed that the proportion cells in the optic vesicle that were cycling was reduced. PRC2 has been shown to control cell proliferation during development, in part due to repression of the tumor suppressor locus $p16^{\text{Ink4a}}$ -ARF- $p15^{\text{Ink4b}}$ (Popov and Gil, 2010). Consistent with this, we detected a significant upregulation of $p15^{\text{Ink4b}}$ upon loss of *Ezh2*. We could not find a homologue for $p16^{\text{Ink4a}}$ /ARF in the *Xenopus* genome database, so we conclude that $p15^{\text{Ink4b}}$ is the most likely target for regulation by PRC2 in *Xenopus* retina. We found that misexpression of $p15^{\text{Ink4b}}$ was sufficient to cause reduced retinal progenitor proliferation and a small eye phenotype. Little is known about $p15^{\text{Ink4b}}$ function in the developing retina. We found that although $p15^{\text{Ink4b}}$ is expressed in the neural tube and optic vesicle as it first forms (stage 19-20; data not shown), it is downregulated and not significantly expressed during embryonic eye development, nor is it detectable in the retina at stage 41, including the CMZ (data not shown). Similarly, during cortical development, genes from the *Ink4/Arf* locus are not expressed but are upregulated with loss of PRC2 (Pereira et al., 2010). We also found evidence for upregulation of other cell cycle inhibitors, including *Fzr1* and *p16Xic3*, but observed no significant increase in *p27Xic1*, *p16Xic2* or *p19Ink4d*. Thus, the mechanisms by which PRC2 maintains retinal progenitor proliferation are likely to be complex. Indeed, loss of the PRC1 gene *Bmi1* leads to derepression *p16Ink4a*, as well as to activation of the DNA damage response pathway, leading to S-phase arrest (Liu et al., 2009). So we cannot rule out additional effects due to loss of PRC2. Nevertheless it is clear that normal PRC2 function is important for cell cycle progression of retinal progenitors in *Xenopus*.

Tight regulation of proliferation is also crucial for retinal histogenesis (Bilitou and Ohnuma, 2010). Cyclin-dependent kinase inhibitors have been shown to act in a context-dependent manner to influence not only proliferation but retinal cell differentiation. For example, in the *Xenopus* retina *p27Xic1* can cooperate with proneural genes to promote neurogenesis, whereas on its own *p27Xic1* promotes gliogenesis, independent of its cell cycle inhibitory activity (Ohnuma et al., 1999; Ohnuma et al., 2002; Bilitou and Ohnuma, 2010). Whether $p15^{\text{Ink4b}}$ can influence retinal cell fate decisions in a similar manner is unknown.

Regulation of progenitor progression to differentiation

It is widely established that PRC2 regulates embryonic patterning through regulation of Hox gene expression during development (Sparmann and van Lohuizen, 2006; Alexander et al., 2009). However, we saw no effect on eye specification or the expression of retinal progenitor genes when PRC2 components were knocked down. We did observe a failure to initiate expression of proneural bHLH factors and downstream differentiation genes. There is precedence for PRC2 activity being required for neuronal differentiation. ESCs lacking *Suz12* fail to form neurons under

differentiation conditions, suggesting that H3K27me3 deposition is essential for the proper execution of the neural differentiation program (Pasini et al., 2007). By contrast, during mouse cortical development PRC2 acts to constrain differentiation. Conditional inactivation of *Ezh2* in cortical progenitors before the onset of neurogenesis accelerates the timing for both neurogenesis and gliogenesis (Pereira et al., 2010), whereas conditional disruption of *Ezh2* later during the neurogenic period prevents the normal shift from neuron to astrocyte production due to a failure to repress expression of the proneural bHLH gene *Ngn1* (Hirabayashi et al., 2009). We found no evidence for premature expression of proneural or retinal differentiation genes upon inhibition of PRC2 function in retinal progenitors, but rather a failure to initiate their expression. However, we cannot rule out that PRC2 may also have a later function to control developmental timing or to repress proneural genes at the end of neurogenic period. It is also possible that there are species-specific functions for PRC2 during neural development.

In ESCs, the H3K27me3-specific demethylase *Jmjd3* is also required for ESC neural differentiation, suggesting that dynamic regulation of H3K27me3 is essential for the execution of the neural differentiation program (Burgold et al., 2008; Sen et al., 2008). *Jmjd3* and *Utx* are expressed in the developing *Xenopus* eye but their roles during retinogenesis have not been explored (Kawaguchi et al., 2012).

How PRC2 regulates the differentiation program are unclear. We showed that increased expression of the cell cycle regulator $p15^{\text{Ink4b}}$, although sufficient to cause reduced progenitor proliferation, did not suppress the expression of proneural and differentiation genes. We cannot rule out an effect from the other upregulated cell cycle genes, such as *Fzr1* or *p16Xic3*. *Fzr1* has been implicated in cellular differentiation in the lens and muscle (Hu et al., 2011), and overexpression of *p16Xic3* affects retinal cell fate in *Xenopus* (Daniels et al., 2004). The failure to initiate the proper differentiation program in the *Xenopus* retina upon loss of PRC2 may be due to global or selective derepression of non-neural genes, which are normally silenced. Alternatively, PRC2 might be required to silence repressors of proneural factors in progenitors to allow neurogenesis to initiate. No clear candidates have emerged from the microarray analysis, but future work will explore these possibilities in detail.

Regulation of cell fate choices by PRC2

We found that blocking the function of PRC2 in retinal progenitors biases cell fate decisions toward the generation of Mueller glia, largely at the expense of ganglion cells and bipolars cells. Loss of PRC2 function leads to inhibition of proneural gene expression, which may account for the increase in retinal gliogenesis (Ohnuma et al., 1999; Vetter and Moore, 2001). Other manipulations that prevent proneural gene expression in retinal progenitors in *Xenopus*, including inhibition of *Fz5* or *Sox2*, lead to increased Mueller glia differentiation at the expense of retinal neurons (Van Raay et al., 2005). Alternatively, retinal gliogenesis can be increased by factors that instructively promote glial fate, such as Notch signaling, although we observed no change in the expression of Notch pathway components with loss of PRC2 function. Overexpression of the cell cycle inhibitor *p27Xic1* in *Xenopus* retinal progenitors can promote Mueller glia formation (Ohnuma et al., 1999), but whether $p15^{\text{Ink4b}}$ overexpression has a similar effect is unknown. Additionally, the interaction between PRC2 and long non-coding RNAs, which have been implicated in the recruitment of PRC2 to its targets, may also be involved in retinal cell fate choices (Margueron and Reinberg, 2011). *Ezh2*

interacts with the long non-coding RNA Six3OS, and knockdown of the latter promotes Mueller glia formation in the mouse retina (Rapicavoli et al., 2011).

We observed that although most other neuronal cell types are suppressed, amacrine cell numbers increased upon loss of PRC2. This occurred despite inhibition of NeuroD, which promotes amacrine cell genesis (Morrow et al., 1999), suggesting the involvement of other regulators of amacrine cell differentiation. Overall, our analysis suggests a complex role for PRC2 in retinal subtype specification.

Regulation of PRC2 expression by Wnt signaling

Although the expression of PRC2 genes is dynamically modulated, the mechanisms that regulate their expression during development remain largely unknown. We show that Wnt/ β -catenin signaling regulates the expression of PRC2 core subunits. Given that Wnt signaling is important in multiple developmental contexts (van Amerongen and Nusse, 2009), it is possible that this mode of regulation is used in other tissues. However, as Wnt/ β -catenin signaling has distinct functions in the developing mammalian and chick eye to promote ciliary body/iris formation rather than retinal neurogenesis (Cho and Cepko, 2006; Liu et al., 2007), it remains to be determined how PRC2 gene expression is regulated in this context. Interestingly, during adipogenesis PRC2 is required to repress several Wnt ligands to prevent the activation of Wnt signaling during adipocyte differentiation, suggesting potential for cross-regulation (Wang et al., 2010).

How Wnt signaling regulates PRC2 expression remains unknown. We have shown that Sox2 is regulated by Wnt/ β -catenin signaling in *Xenopus* (Van Raay et al., 2005), but find that the expression of Ezh2 and Suz12 is independent of Sox2. Thus, PRC2 and Sox2 may be co-regulated by Wnt/ β -catenin signaling and their activities coordinated. It has recently been proposed that PRC2 and Sox2 are recruited to shared targets through long non-coding RNAs (Ng et al., 2012). Our data open the window for future investigations into the functional link(s) between β -catenin signaling, Sox2 and PRC2.

Acknowledgements

We are grateful for technical support from Joy Corley. We thank Brett Milash for help with the microarray data analysis and Gert Veenstra for sharing unpublished data and for helpful discussions.

Funding

This work was supported by the National Institutes of Health [EY012274 to M.L.V.]. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Author contributions

I.A. conceived the project with M.L.V., performed PRC2 expression analysis, morpholino experiments, microarray and PCR analysis and wrote the manuscript; K.B.M. performed cumulative BrdU labeling, retinal cell fate analysis and Wnt pathway analysis, including morpholino and transgenic experiments; D.A.H. helped with transgenic experiments; J.Z. performed Ezh2 immunostaining; M.L.V. supervised the experiments and analysis, edited and submitted the manuscript. All authors discussed the results and commented on the manuscript.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.088096/-DC1>

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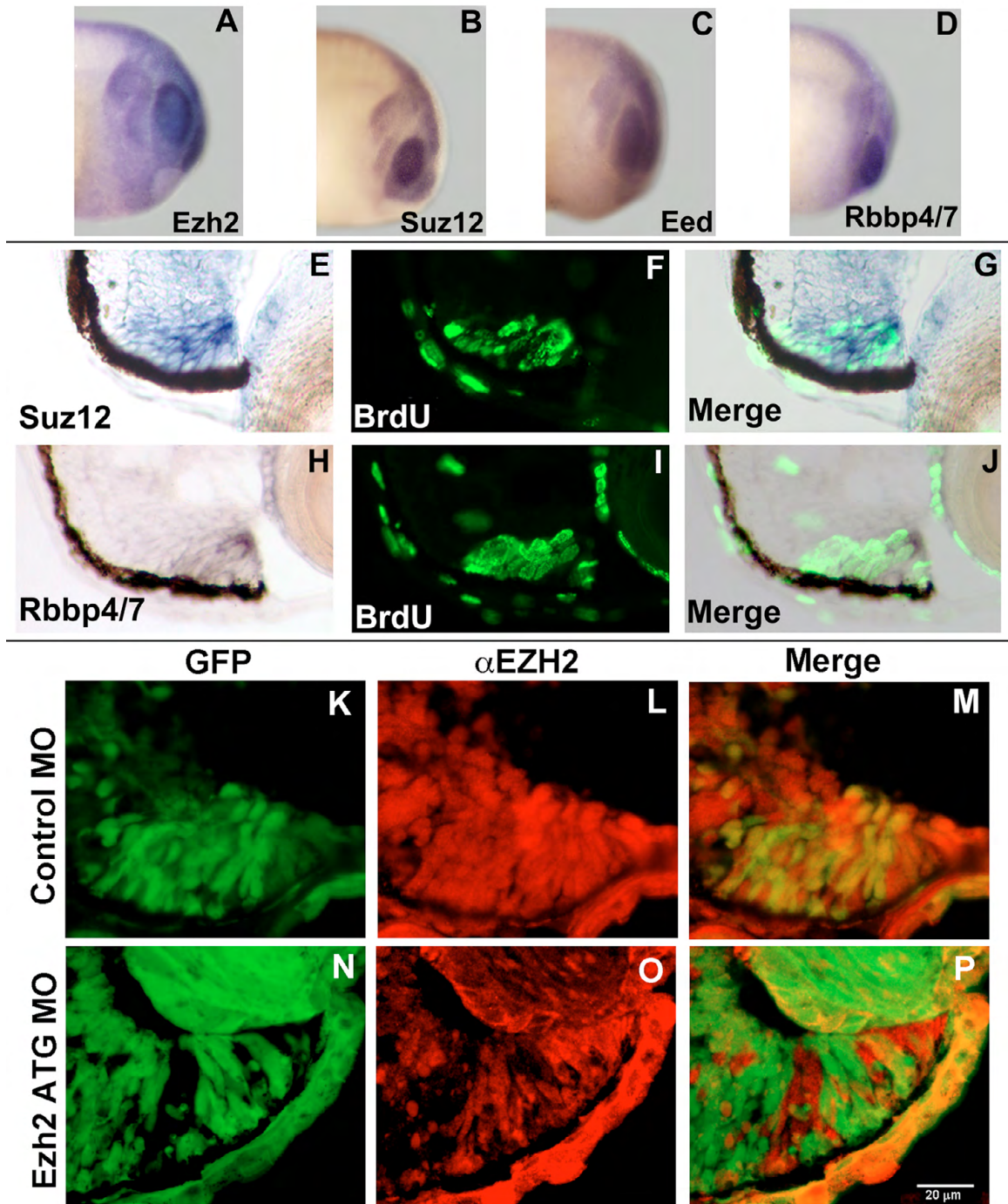


Fig. S1. The PRC2 core components are expressed in dividing retinal progenitors. (A-D) Expression of the PRC2 core subunits Ezh2, Suz12, Eed and Rbbp4 in the developing eye by whole-mount *in situ* hybridization of stage 22/23 embryos. (E-J) Expression of Suz12 and Rbbp4/7 (by *in situ* hybridization) largely coincides with BrdU immunostaining in the CMZ of the *Xenopus* retina at stage 41. (K-P) Injection of GFP mRNA together with 5 ng control MO or Ezh2 ATG MO at the eight-cell stage, followed by immunostaining for EZH2 on stage 41 retinal sections. Control MO does not reduce the level of EZH2 immunolabeling in the GFP-marked clones (see yellow cells in M), whereas EZH2 immunostaining is significantly reduced by Ezh2 ATG MO (GFP-positive cells do not significantly co-label with EZH2 immunostain in P).

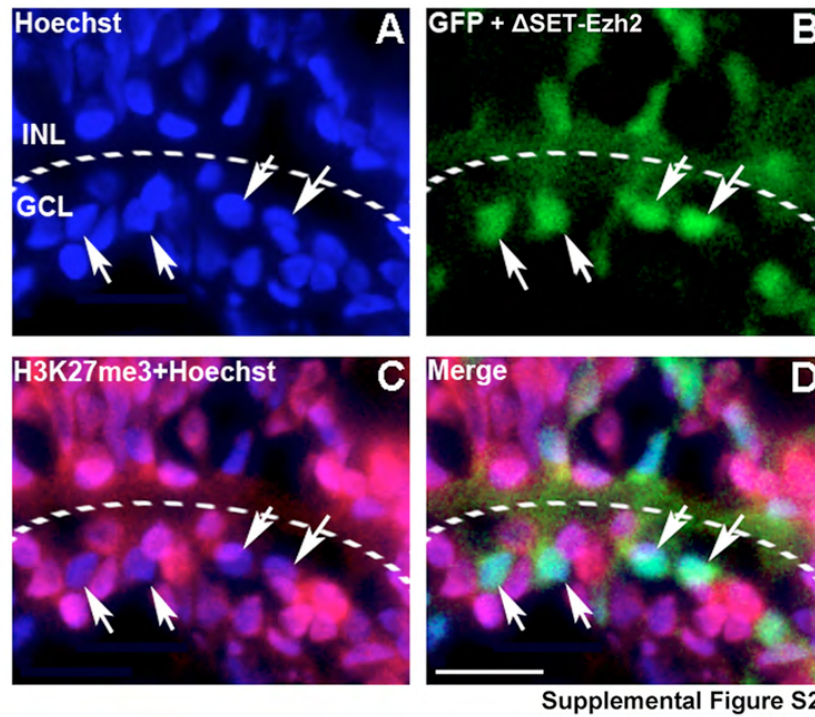


Fig. S2. Ezh2 lacking the SET domain functions as a dominant negative and prevents H3K27me3 labeling in *Xenopus* retina. (A-D) Immunostaining of a retinal section of stage 41 embryo with antibody against H3K27me3 (red) after co-injection of GFP mRNA with Ezh2 Δ SET mRNA (green) at the eight-cell stage. Hoechst labels nuclei (blue). Arrows show GFP-labeled cells with reduced H3K27me3 levels. INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 20 μ m.

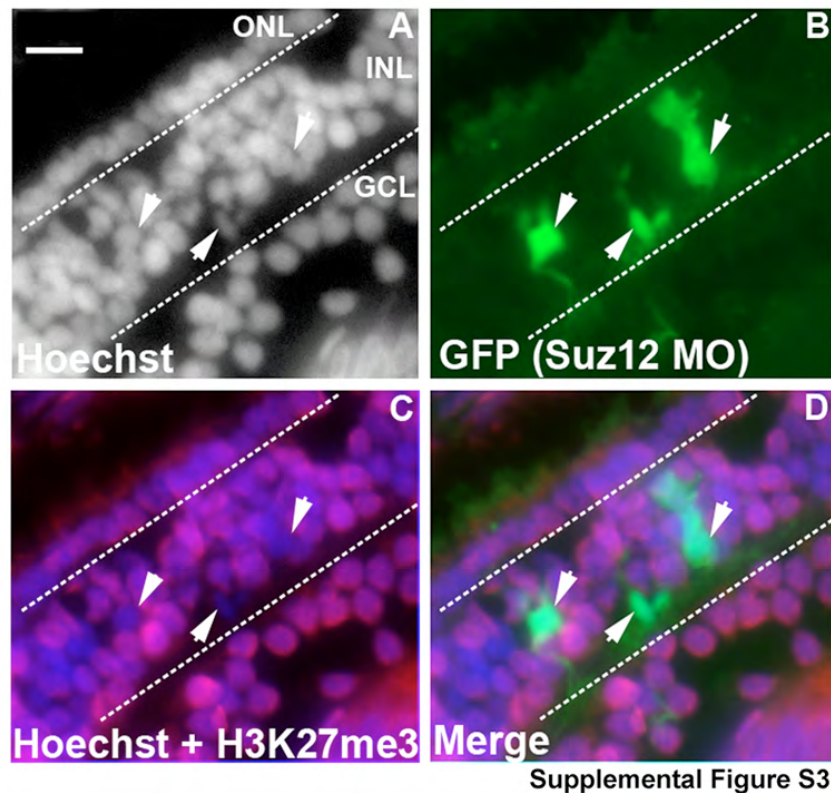
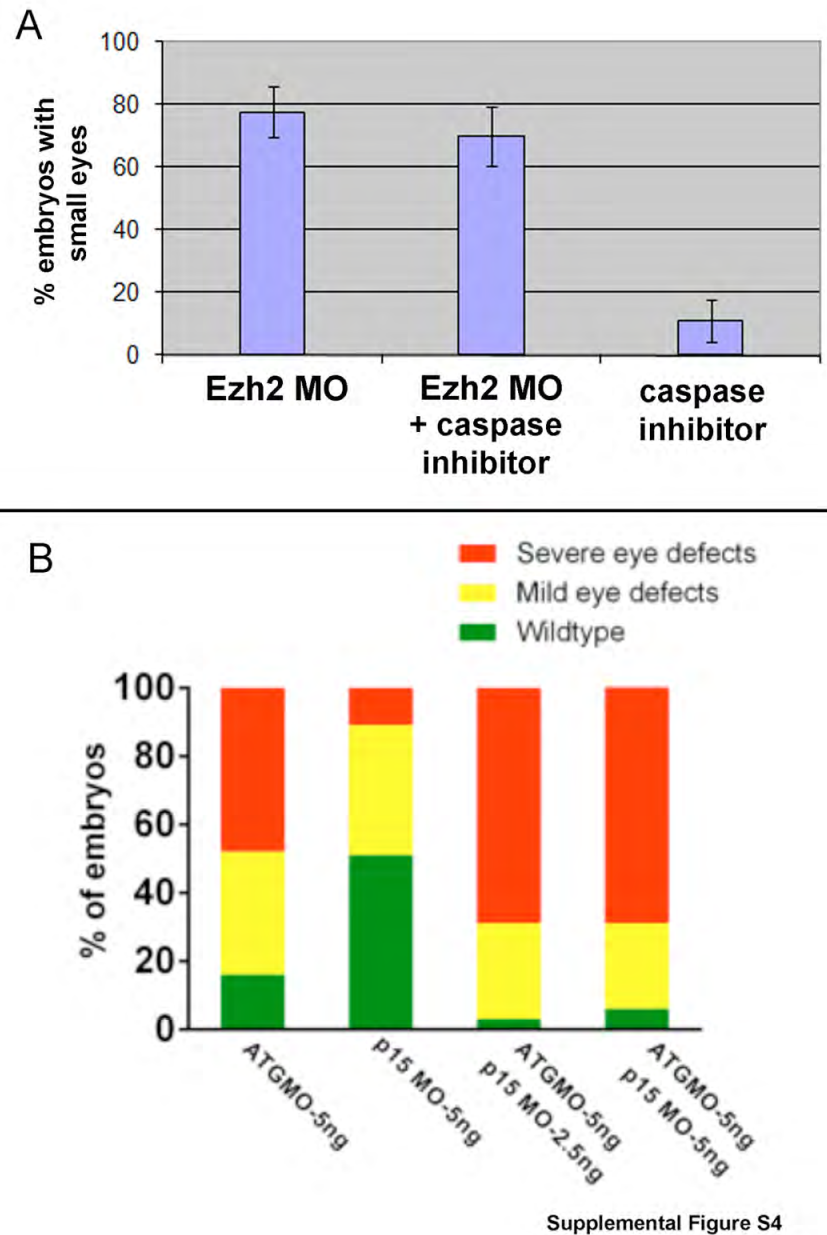
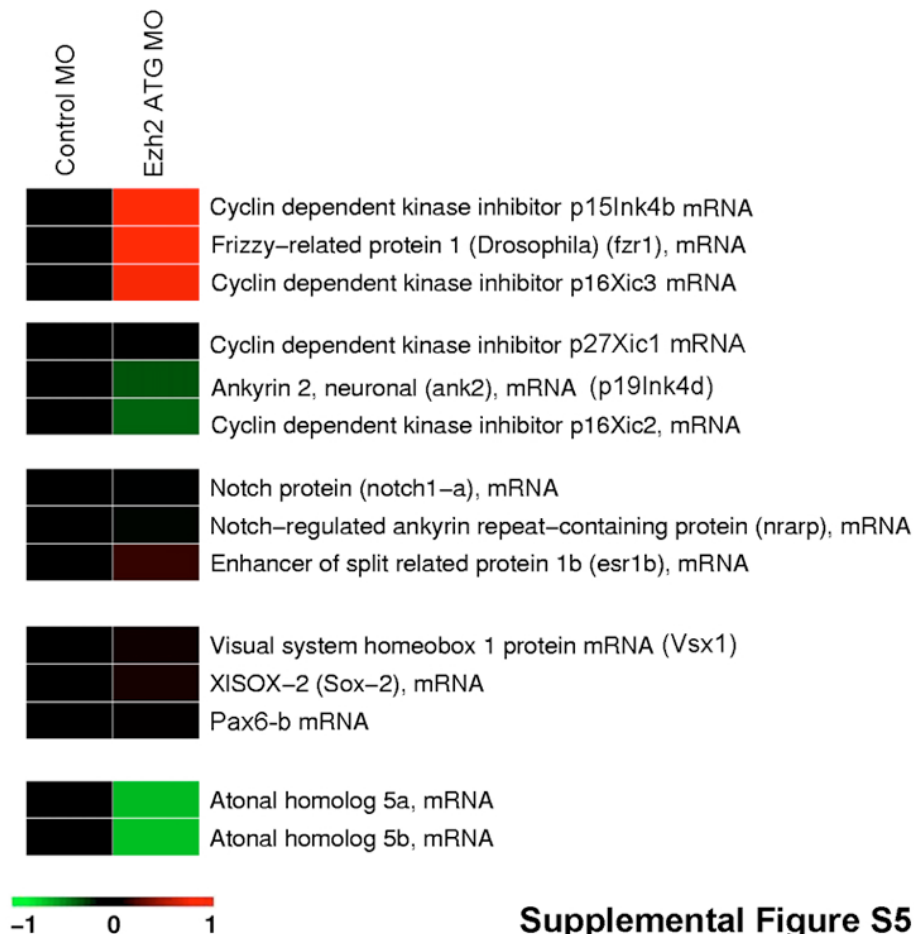


Fig. S3. Suz12 is required for H3K27me3 in *Xenopus* retina. (A-D) Knockdown of Suz12 by injection of translation blocking morpholino (Suz12 MO) together with GFP mRNA results in decreased H3K27me3 staining in the retina in GFP-labeled cells (arrowheads). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 10 μ m.



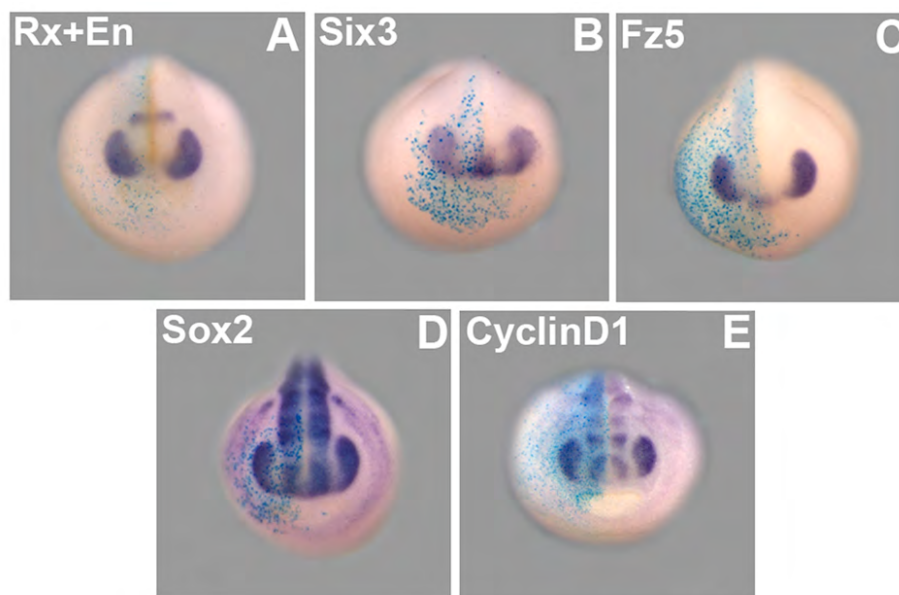
Supplemental Figure S4

Fig. S4. Blocking cell death or knockdown of $p15^{Ink4b}$ does not rescue eye size after knockdown of Ezh2. (A) Ezh2 ATG MO or pan-caspase inhibitor, or both, were injected into eight-cell embryos and number of embryos that showed reduced eye size was counted at stage 41. No significant difference in the percent of embryos with microphthalmia was observed between injection of Ezh2 ATG MO alone ($n=55$) or in combination with pan-caspase inhibitor ($n=46$; Student's t -test $P=0.55$). Injection of pan-caspase inhibitor alone has minimal effect on eye size ($n=28$). (B) Co-injection of Ezh2 ATG MO with two different doses of p15 MO failed to rescue the eye size defects caused by injection of Ezh2 ATG MO alone. Injection of p15 MO alone also causes reduced eye size, potentially owing to expression in the neural tube and very early optic vesicle (not shown).



Supplemental Figure S5

Fig. S5. Changes in optic vesicle gene expression after injection of control MO versus Ezh2 ATG MO by microarray analysis. Relative changes in gene expression shown by heatmap of \log_2 -transformed microarray signals for the indicated genes, with the color scale shown at the bottom in log base 2 units. Data represent an average of four biological replicates.



Supplemental Figure S6

Fig. S6. Control MO has no effect on eye specification genes. (A-E) Frontal view of stage 20 embryos injected with control MO plus mRNA encoding β -galactosidase to mark injected side. X-gal stain is in light blue. The expression domain as well as expression levels of all tested genes appear normal in the injected side. Embryos with normal expression: 100%, $n=13$ for Rx; 91%, $n=11$ for Six3; 94%, $n=16$ for Fz5; 100%, $n=15$ for Sox2; 93%, $n=15$ for Cyclin D1.

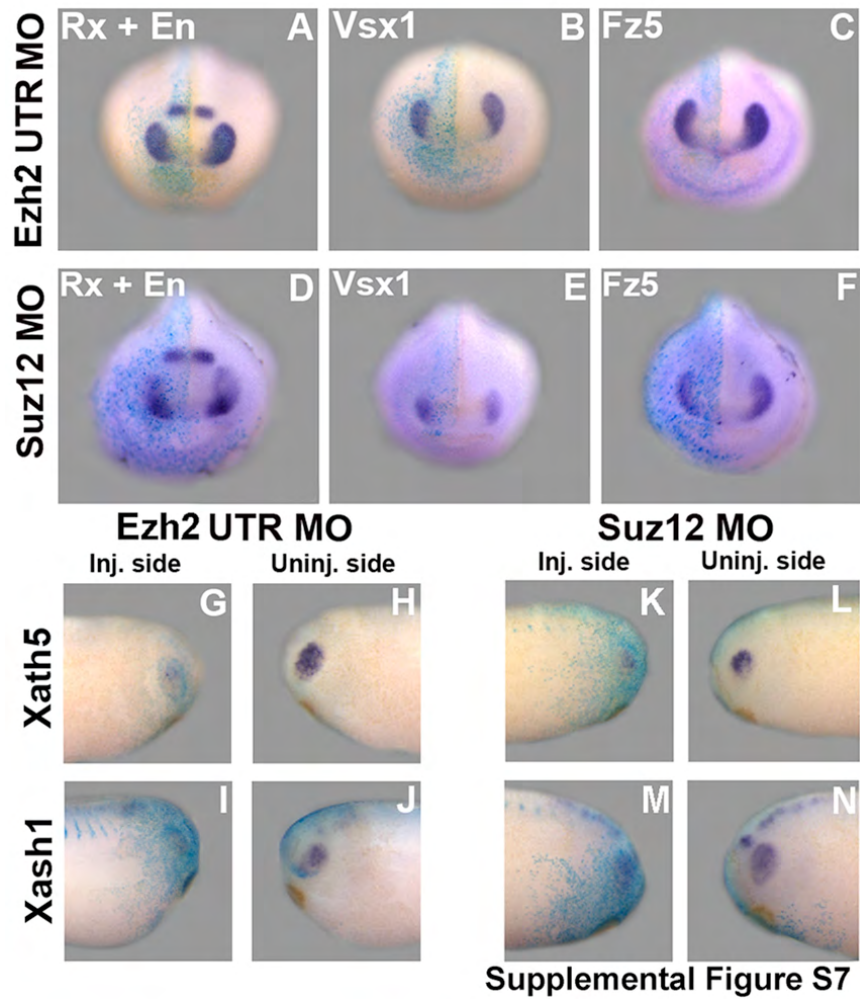
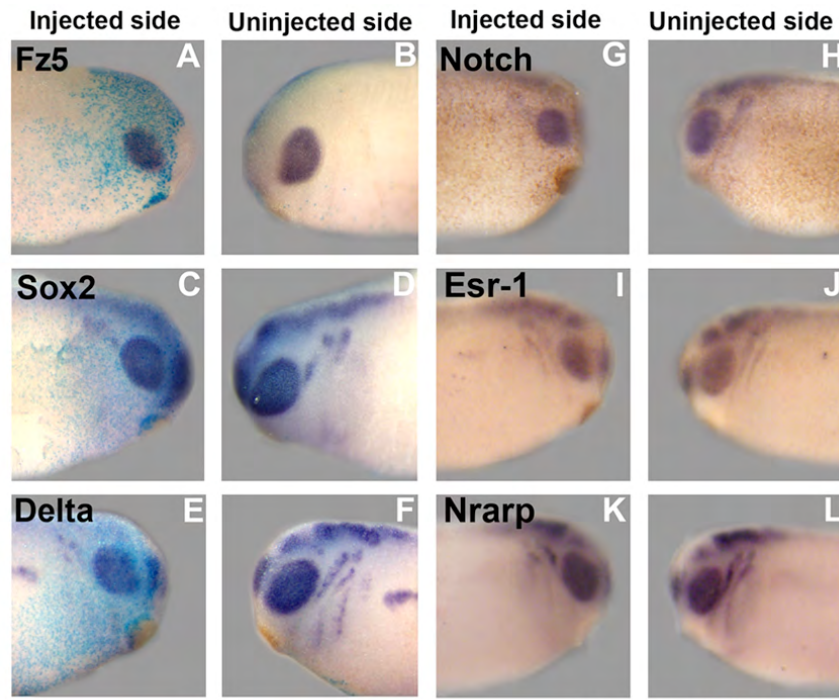
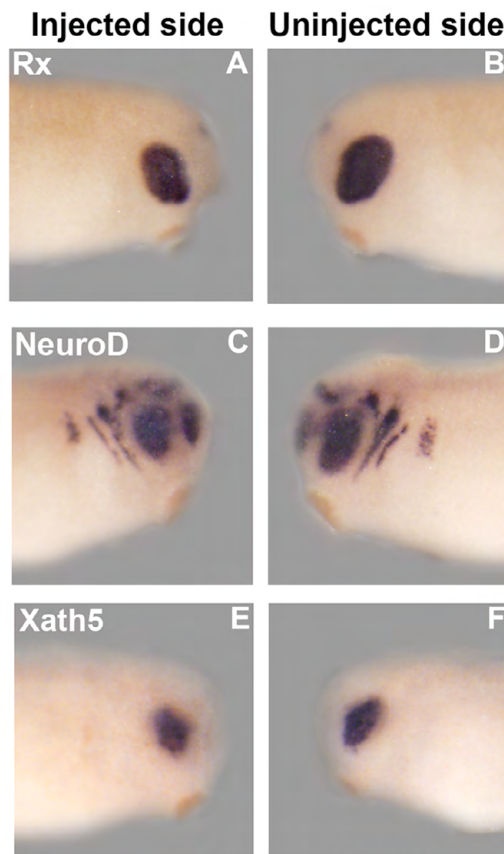


Fig. S7. Ezh2 UTR MO and Suz12 MO phenocopy the Ezh2 ATG MO effect. (A-F) Frontal view of embryos injected with Ezh2 UTR MO (A-C) or Suz12 MO (D-F) showing reduction in expression domain of the progenitor genes Rx, Vsx1 and Fz5 on the injected side. (G-N) Lateral view of embryos injected with Ezh2 UTR MO (G-J) or Suz12 MO (K-N) exhibiting reduced levels of the bHLH factors Xath5 and Xash1 on the injected side.



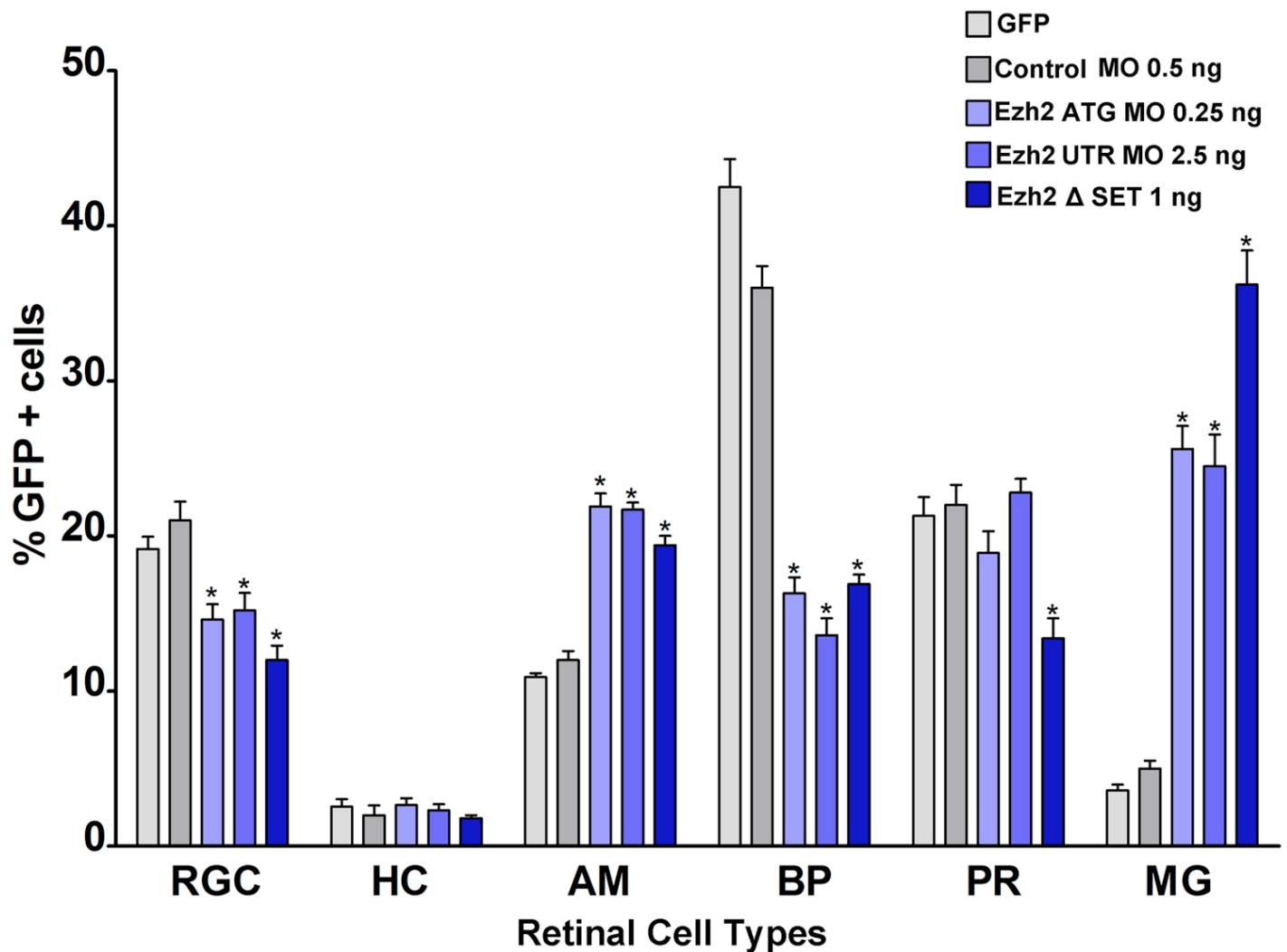
Supplemental Figure S8

Fig. S8. Ezh2 MO injection does not affect Wnt or Notch signaling. Lateral view of stage 27 embryos injected with Ezh2 ATG MO. (A-D) There are relatively normal levels of expression of Fz5 and the downstream target Sox2 on the injected side, although the eye domain is smaller. (E-L) Likewise, there is normal expression of Notch signaling components Delta, Notch, Esr-1 and Nrarp.



Supplemental Figure S9

Fig. S9. Overexpression of p15 does not affect retinal progenitor specification or neural differentiation. (A-F) Lateral view of stage 27 embryos after injection of p15 mRNA. The progenitor gene Rx and neural differentiation genes NeuroD and Xath5 show normal levels of expression.



Supplemental Figure S10

Fig. S10. Blocking PRC2 in retinal progenitors alters the normal complement of retinal cell types. Analysis of GFP-labeled retinal cell types in stage 41 retinal sections after injection of GFP mRNA plus MO into a dorsal animal blastomere at the 32-cell stage. Injection of either the Ezh2 ATG MO or the Ezh2 UTR MO caused an increase in the representation of Müller glia when compared with GFP mRNA alone or control MO, and a corresponding decrease in several major retinal cell types (ganglion cells and bipolar cells). Amacrine cells are significantly increased whereas horizontal cells and photoreceptor cells are unchanged, although this analysis does not distinguish rods and cones. Injection of mRNA for dominant-negative Δ SET-Ezh2 had a similar effect, confirming that the effect is due to loss of PRC2 function. RG, ganglion cells; C, horizontal cells; AM, amacrine cells; BP, bipolar cells; PR, photoreceptor cells; MG, Müller glia. The percent representation of each cell type was calculated as a weighted average; error bars represent s.e.m; * $P < 0.001$ (Student's t -test).