

# Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina

Yaping Wang<sup>1</sup>, Gabriel D. Dakubo<sup>1</sup>, Sherry Thurig<sup>1</sup>, Chantal J. Mazerolle<sup>1</sup> and Valerie A. Wallace<sup>1,2,\*</sup>

<sup>1</sup>Molecular Medicine Program, Ottawa Health Research Institute and University of Ottawa Eye Institute, 501 Smyth Road, Ottawa, Ontario K1H 8L6, Canada

<sup>2</sup>Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, Ontario K1H 8M5, Canada

\*Author for correspondence (e-mail: vwallace@ohri.ca)

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## Summary

The timing of cell cycle exit and temporal changes in the developmental competence of precursor cells are key components for the establishment of the normal complement of cell types in the mammalian retina. The identity of cell extrinsic cues that control these processes is largely unknown. We showed previously in mouse retina that sonic hedgehog (Shh) signalling from retinal ganglion cells (RGCs) to retinal precursor cells (RPC) is required for the establishment of normal retinal organization. Here, we show that conditional ablation of *Shh* expression in the peripheral mouse results in a depletion of the RPC pool, owing to precocious cell-cycle exit and neuronal

differentiation. These changes were correlated with the downregulation of cyclin D1 and *Hes1* gene expression. *Shh* inactivation also results in an increase in RGC number owing to a bias of RPC towards RGC production. In contrast to zebrafish, where Shh signalling drives cell cycle exit and RGC development, our findings indicate that in the mouse retina Shh signalling is required to maintain RPC proliferation and to control the timing of RGC development.

Key words: Retina, Proliferation, Differentiation, CyclinD1, Hedgehog, Mouse

## Introduction

The mammalian retina is an excellent model in which to investigate the contributions of cell intrinsic and extrinsic processes on precursor cell proliferation and diversification. It is one of the most accessible regions of the CNS, and the different cell types are well characterized and can be identified by their position within the retinal laminae and by their expression of cell-type-specific markers. The lineage of the cells in the retina is known: all of the cells, except for astrocytes, are derived from multipotential retinal precursor cells (RPC) (Holt et al., 1988; Turner and Cepko, 1987; Wetts and Fraser, 1988). The different cell types are generated in an invariant sequence, with retinal ganglion cells (RGCs), cone photoreceptors, horizontal cells and the majority of the amacrine cells generated first, followed by bipolar cells, Müller glia and the remaining amacrine cells generated in a second wave of histogenesis, which extends into the postnatal period (Young, 1985). Rod photoreceptors are generated throughout retinal development.

A prevailing model of retinal development that reconciles the evidence that RPC are multipotential with the temporal control of cell type production is the competence model, whereby RPC progress irreversibly through a series of competence states where their developmental potential is restricted (Livesey and Cepko, 2001). Cell mixing experiments

and the comparison of RPC development in different environments indicate that cell-cycle exit and cell diversification are intrinsically determined, at least for late born retinal cell types (Belliveau et al., 2000; Cayouette et al., 2003; Watanabe and Raff, 1990). Cell ablation experiments, however, indicate a role for neuron-derived signals in the regulation of these processes (Mu et al., 2005; Negishi et al., 1982; Reh, 1987; Waid and McLoon, 1998). RGC development and RPC proliferation, for example, appear to be controlled by signalling from RGCs (Mu et al., 2005; Waid and McLoon, 1998). Two candidate RGC-derived signalling molecules that may mediate these effects are Shh and Gdf11 (Kim et al., 2005; Mu et al., 2005; Zhang and Yang, 2001).

Hedgehog (Hh) proteins are extracellular signalling molecules that control patterning and growth of a number of tissues in the developing embryo (reviewed by Ingham and McMahon, 2001). Hh signalling has been implicated in retinal development in several vertebrate species (reviewed by Amato et al., 2004); however, there is considerable controversy regarding its role in this context. In the zebrafish retina, Shh signalling from RGCs is required for RGC development (Neumann and Nüsslein-Volhard, 2000), whereas in the chick retina Shh inhibits RGC development (Zhang and Yang, 2001). Although Shh is a mitogen for perinatal RPC in rodent and chick (Jensen and Wallace, 1997; Levine et al., 1997; Moshiri

et al., 2005), it is not clear whether it functions as a mitogen in the embryonic retina. Differences in the source of Hh signals (extra-retinal versus intra-retinal) or temporal aspects of Hh signalling, could account for some of these discrepancies or they could reflect species-specific functions for this pathway in retinal development.

*Shh* is expressed in RGCs in the murine retina and we showed that it is required for the maintenance of Hh target gene expression in RPC in vivo and in retinal explants, and that treatment with recombinant Shh protein could promote normal lamination in retina explants from perinatal mice (Jensen and Wallace, 1997; Wang et al., 2002). To examine the requirement for intra-retinal *Shh* expression on RPC proliferation and cell diversification in the mouse embryonic retina in vivo, we used the Cre-loxp system to target inactivation of the *Shh* gene to the peripheral retina. Reduced Shh signalling is associated with precocious cell-cycle exit during embryogenesis and a depletion of the RPC pool. These changes are associated with a reduction in the expression of cyclin D1 and *Hes1*, a negative regulator of differentiation, in RPC. The failure to maintain RPC in cycle has profound consequences on histogenesis, as differentiation of photoreceptors is accelerated and the production of late born cell types, such as bipolar and Müller glia is reduced. In addition, RGCs are overproduced in the peripheral retinas of the mutant mice because of a sustained RPC bias towards RGC development. Thus, RGC-Shh signalling plays a pivotal role in regulation of RPC proliferation, as well as the timing of RGC development during embryonic retinal development.

## Materials and methods

### Transgenic mice and in situ hybridization

The  $\alpha$ -Cre transgenic mice were obtained from P. Gruss (Marquardt et al., 2001) and were maintained on an FVBN background. Rosa26R mice were obtained from the Jackson Laboratories (Soriano, 1999). Mice heterozygous for a *Shh*-null allele (*Shh*<sup>+/−</sup>) and homozygous for the conditional *Shh* allele (*Shh*<sup>cre</sup>) were maintained on a C57Bl/6 background (Lewis et al., 2001).  $\alpha$ -Cre;*Shh*<sup>+/−</sup> mice were crossed to *Shh*<sup>cre</sup> mice to generate  $\alpha$ -Cre;*Shh*<sup>−/−</sup> mice and were compared with  $\alpha$ -Cre;*Shh*<sup>+/−</sup> littermates (referred to as wild type). Genotyping for the  $\alpha$ -Cre transgene, *Shh* null and conditional alleles was performed by the PCR. To label cells in S phase in vivo, time-mated females were given two intraperitoneal injections 2 hours apart of BrdU at 0.1 mg/g body weight. Tissues were processed for immunohistochemistry (see below) or in situ hybridization with digoxigenin-labelled antisense riboprobes, as previously described (Dakubo et al., 2003; Wallace and Raff, 1999). For more reliable comparisons of gene expression patterns, wild-type and mutant tissues were processed on the same slides.

### Cell culture and immunohistochemistry

Embryonic retinal tissues were obtained from time mated C57Bl/6 mice, the day of the vaginal plug was taken as day 0 of the pregnancy. Neural retinas were dissected in MEM-HEPES (ICN) and cultured on 13 mm polycarbonate filters (0.8  $\mu$ m pore size, Nucleopore) (perinatal retinal explants) or submerged (E12 explants) in 24-well plates in 1 ml of serum-free culture medium [1:1 DMEM-F12, supplemented with insulin (10  $\mu$ g/ml), transferrin (100 mg/ml), bovine serum albumin (BSA Fraction V: 100 mg/ml), progesterone (60 ng/ml), putrescine (16  $\mu$ g/ml), sodium selenite (40 ng/ml) and gentamycin (25  $\mu$ g/ml)]. The cultures were supplemented with recombinant myristoylated N-terminal fragment of Shh (Shh-N) at 2  $\mu$ g/ml or Smoothed agonist (Hh agonist) at 10 nM (Frank-Kamenetsky et al.,

2002) (kind gifts from Curis), bFGF (50 ng/ml, Sigma), EGF (100 ng/ml, Sigma) or purified anti-Hh [5E1 (Ericson et al., 1996)] and anti-LFA-3 (1E6) monoclonal antibodies at 30  $\mu$ g/ml. The dose of growth factors was determined to be optimal for RPC proliferation in vitro (data not shown) and the medium and growth factors were refreshed every 3 days. To label progenitor cells, explants were incubated in [<sup>3</sup>H]thymidine for 4 hours and autoradiographic analysis was performed as previously described (Jensen and Wallace, 1997). In some experiments, BrdU (10  $\mu$ M) was added for the last 6 hours of the culture. At the end of the culture period, explants were dissociated into single cells with trypsin or fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, cryoprotected in 30% sucrose/PBS, cut in half and sectioned from the middle of the explant at 8  $\mu$ m. Immunohistochemistry was performed, as described previously (Jensen and Wallace, 1997), with the following antibodies: goat polyclonal anti-Brn3b (Pou4f2 – Mouse Genome Informatics) (Santa Cruz Biotechnology, Santa Cruz, California, USA), rabbit polyclonal anti-CRALBP (a gift from J. Saari), anti-cone arrestin [LUMIj (Zhu et al., 2002), a gift from C. Craft and X. Zhu] and mouse monoclonal anti-rhodopsin [B630 (Rohlich et al., 1989)], anti-syntaxin (Sigma), anti-PKC (Pharmingen) and anti-BrdU (Becton Dickinson). Sections were analyzed on a Zeiss Axioplan microscope and digital images were captured using an AxioVision 2.05 (Zeiss) camera and processed with Adobe® Photoshop. To quantify cell types in mutant and wild-type retinas, the number of marker<sup>+</sup> cells in 300  $\mu$ m (100  $\mu$ m for E14) regions adjacent to the optic nerve and in the peripheral retina was counted. The cell counts in the peripheral region of the  $\alpha$ -Cre;*Shh*<sup>−/−</sup> retina corresponded to the region where *Gli1* expression was downregulated and the RGC layer was thicker. After P6, the cell counts from the peripheral  $\alpha$ -Cre;*Shh*<sup>−/−</sup> retina were based on counting cells in a 300  $\mu$ m region proximal to the site of degenerative changes in the outer nuclear layer. Cell counts were performed on at least three sections at the level of the optic nerve head/mouse and, for most analyses, from three mice/genotype. Data are presented as mean $\pm$ s.d. and data sets were compared with the Student's *t*-test. All *P*-values are based on two-sided hypothesis testing.

### RT-PCR analysis

RNA from pools of three explants per condition was isolated using Trizol reagent (Sigma) and 2  $\mu$ g of total RNA was reverse transcribed in a total volume of 40  $\mu$ l. Equal amounts of first strand cDNA were then amplified using primer pairs specific for the following genes: *Gli1F*, ggcgtctcaggaaggatgag; *Gli1R*, ggcgtctcaggaaggatgag; *Hes1F*, cagccagtgtcaacacgacac; *Hes1R*, tcgttcactcactcgtctgag; *Hes5F*, cgcatacaacagcagcatag; *Hes5R*, tggagtggtaagcagcttc; *GAPDHf*, caagacccttcattgacctc; *GAPDHR*, atccagcagggacacattgg; *Ptc2F*, ggtctccgagtggctgtaat; *Ptc2R*, ccagttgtgccactggata. The cycling parameters used were as follows: 94°C for 30 seconds; 58°C for 30 seconds; 72°C 45 seconds (*Gapdh*, *Gli1*, *Hes5*); 94°C for 30 seconds; 55°C for 30 seconds; 72°C for 45 seconds (*Ptc2*); 94°C for 30 seconds; 53°C for 30 seconds; 72°C for 45 seconds (*Hes1*) for 20–31 cycles. For semi-quantitative RT-PCR, 0.3  $\mu$ l of <sup>32</sup>P-dATP was added to each reaction and PCR products were run on a native polyacrylamide gel electrophoresis gel, which was then dried and exposed to film. The number of cycles required to achieve linear amplification of PCR products was determined for each primer pair.

## Results

### RGC-derived Shh acts locally to pattern the neuroblast layer

RGCs differentiate in a wave that spreads from the centre to the periphery of the retina, and RGC-derived signals, including Shh, have been implicated in the propagation of this wave in the chick and fish retina (Neumann and Nüsslein-Volhard,

2000; Shkumatava et al., 2004; Zhang and Yang, 2001). To address whether activation of the Hh signalling pathway follows a similar pattern in the mouse retina, we compared the pattern of RGC differentiation with the expression of *Shh* and *Gli1*, a Hh target gene (Goodrich and Scott, 1998), in the embryonic retina. At E12, *Shh* was expressed in the RGC layer in the central retina, behind the leading edge of RGC differentiation, which was marked by Brn3b staining (Fig. 1A,B). *Gli1* expression was upregulated in the neuroblast layer adjacent to the *Shh*-expressing cells (Fig. 1C). The *Gli1* expression domain at E12 did not extend past the domain of *Shh* expression in the RGC layer, indicating that Shh functions as a short-range signal at this stage (Fig. 1, compare B with C). By E13, *Shh* and *Gli1* expression expanded towards the periphery, but still lagged behind the wave of RGC differentiation (Fig. 1D-F). Although the induction of *Shh* expression occurred after the onset of Brn3b expression, it mirrored the central-to-peripheral wave of RGC development and induced a wave of Hh response across the developing retina, as suggested by the induction of *Gli1* expression.

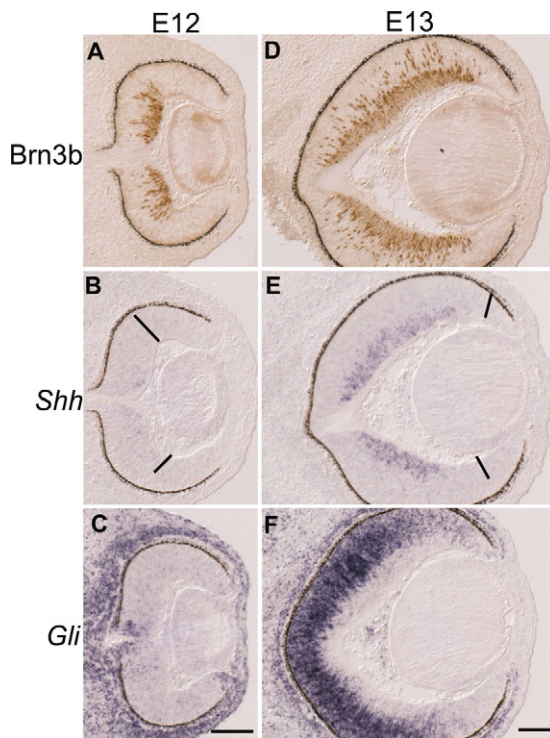
The local induction of *Gli1* expression in the neuroblast layer by *Shh*-expressing RGCs indicated that these first-born neurons are imparting patterning information to the cells in the adjacent neuroblast layer. To address the functional

significance of this cell-cell interaction, we generated mice with a retina-restricted conditional ablation of the *Shh* gene by crossing  $\alpha$ -Cre transgenic mice with *Shh* conditional mutant mice to generate  $\alpha$ -Cre;*Shh*<sup>-/-</sup> mice (see Materials and methods). Beginning at E10, Cre activity in  $\alpha$ -Cre mice is restricted primarily to RPC, RGCs and amacrine cells in the peripheral retina, although there is a low level of Cre activity in the central retina (Marquardt et al., 2001) (see Fig. S1A in the supplementary material). Inactivation of the *Shh* gene in the peripheral retina of  $\alpha$ -Cre;*Shh*<sup>-/-</sup> mice would, therefore, be expected to result in a loss of Hh target gene expression in this region. Consistent with this prediction, *Gli1* expression was downregulated in the peripheral retina of  $\alpha$ -Cre;*Shh*<sup>-/-</sup> mice at E14 and P0 (Fig. 2A-D). Thus, based on the absence of *Gli1* expression, Hh pathway activation is reduced in the peripheral retina of  $\alpha$ -Cre;*Shh*<sup>-/-</sup> mice from E10 onwards. For simplicity, we will refer to the *Gli1*<sup>-</sup> region of the retina as the region where the *Shh* gene has been inactivated.

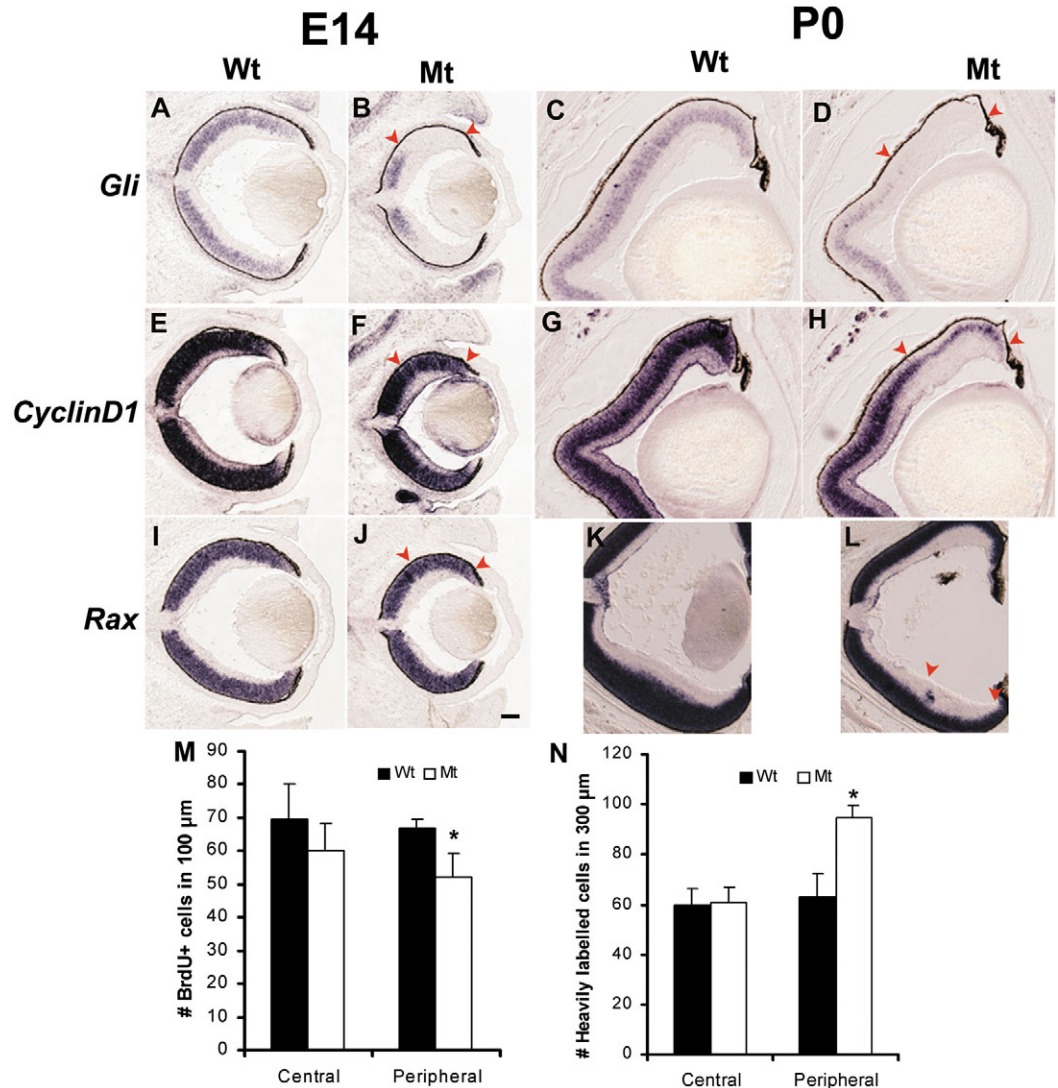
### Loss of Hh signalling is associated with precocious cell-cycle exit

As Shh signalling has been shown to promote RPC proliferation in the perinatal rodent retina, we sought to determine whether *Shh* inactivation would affect RPC proliferation at embryonic stages (Jensen and Wallace, 1997; Levine et al., 1997; Wallace and Raff, 1999; Wang et al., 2002). RPC were labelled at E14 with a short pulse of BrdU and the number of cells in S phase was quantified. We found that BrdU incorporation was reduced in the peripheral retinas of  $\alpha$ -Cre;*Shh*<sup>-/-</sup> mutants at E14 (Fig. 2M, see Fig. S1B,C in the supplementary material). TUNEL staining at several stages during embryogenesis did not reveal any appreciable differences in cell death in the  $\alpha$ -Cre;*Shh*<sup>-/-</sup> retina (data not shown), indicating that the reduction in proliferation was not secondary to changes in cell survival. Several lines of evidence indicated that the reduction of proliferation in the  $\alpha$ -Cre;*Shh*<sup>-/-</sup> mice was due to precocious cell-cycle exit. We performed birth-dating analyses at E13 by injecting BrdU and quantifying the number of heavily labelled cells in the peripheral retina at P0. The heavily labelled cells were those that exited the cell-cycle shortly after the BrdU pulse; cells that continued to divide following the BrdU pulse would be expected to be lightly labelled or unlabelled. We observed a greater than 1.5-fold increase in the number of heavily labelled cells in the peripheral retinas of  $\alpha$ -Cre;*Shh*<sup>-/-</sup> mice compared with wild-type mice (Fig. 2N). Tuj1 staining of dissociated cells from the entire retina revealed that the proportion of differentiated neurons was increased in the retinas of  $\alpha$ -Cre;*Shh*<sup>-/-</sup> mice at E14 compared with wild-type (% Tuj1<sup>+</sup> cells: wild type, 32.0±2.0; mutant 38.6±2.0;  $P<0.005$ ). By P0, the RPC pool was reduced in size in the  $\alpha$ -Cre;*Shh*<sup>-/-</sup> retina, as indicated by a thinner domain of *Rax* expression, a RPC marker, in the peripheral retina (Fig. 2, compare K with L), a reduction in the proportion of S-phase cells (% BrdU<sup>+</sup> cells: wild type, 22.5±2.3; mutant, 15.4±0.7;  $P<0.005$ ) (see Fig. S1D,E in the supplementary material) and in total cell number (wild type  $3.4 \times 10^6 \pm 0.7$ ; mutant  $2.3 \times 10^6 \pm 0.1$ ;  $P<0.05$ ).

Hh signalling upregulates the expression of Mycn and D-type cyclins in a number of tissues, and cyclin D1 expression is required for RPC proliferation (Duman-Scheel et al., 2002; Kenney et al., 2003; Sicinski et al., 1995). To investigate the



**Fig. 1.** Spatial and temporal expression pattern of *Shh*, *Gli1* and RGC differentiation. In situ hybridization for *Shh* and *Gli1* mRNA and immunohistochemistry for Brn3b staining on serial sections of the mouse eye at E12 (A-C) and E13 (D-F). (A-C) *Shh* expression is initiated behind the leading edge of RGC differentiation, as marked by Brn3b staining (lines in B) at E12. (C) *Gli1* expression is localized to the neuroblast layer overlying the *Shh*-expression domain. (D-F) By E13, the *Shh* and *Gli1* expression domains have expanded across the retina, but still lag behind the leading edge of RGC differentiation (lines in E). Scale bar: 200  $\mu$ m.



**Fig. 2.** *Shh* inactivation in the peripheral retina is associated with increased cell cycle exit and depletion of the RPC pool. (A-L) In situ hybridization on wild-type and  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retinas at E14 and P0. (A-D) *Gli* and (E-H) cyclin D1 expression are downregulated in the peripheral retina of mutant mice at both ages (between arrowheads in B,D,F,H). (I-K) In situ hybridization for *Rax* expression to mark the neuroblast layer, which by P0 is reduced in thickness in the peripheral retina of the mutant mice (between the arrowheads in L). (M) The number of S-phase cells is reduced in the peripheral retina of the mutant mice at E14. (N) The number of birthdated cells is increased in the peripheral retinas of mutant mice. RPC were labelled at E13 with BrdU and the number of heavily labelled nuclei was counted in the central versus peripheral retina at P0. \* $P < 0.005$ . Scale bar: 100  $\mu$ m.

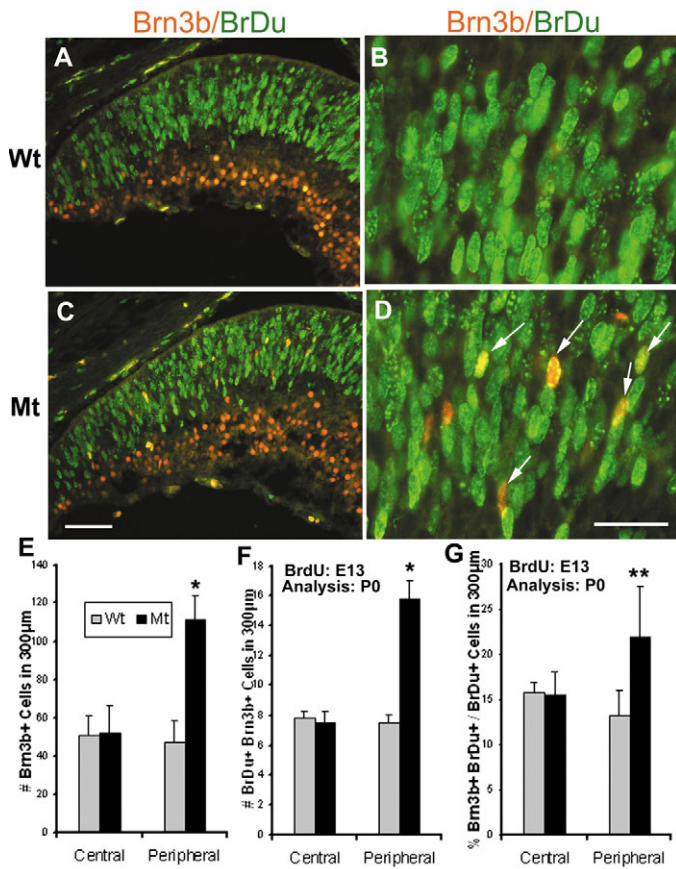
mechanism of the proliferative defect caused by the lack of Shh signalling, we examined the expression of these genes in the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina. Cyclin D1 expression was markedly downregulated in the peripheral retina of  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice at E14 and P0 compared with wild-type retinas (Fig. 2E-H). *Mycn* mRNA levels, however, were not different in the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retinas compared with controls and the levels of *Mycn*, *Rlf* (previously L-Myc) and *Myc* were not changed in Shh-treated retinal explants compared with controls (see Fig. S4 in the supplementary material; data not shown).

### Loss of Shh signalling results in overproduction of RGCs

As Shh is required for RGC development in zebrafish (Neumann and Nusslein-Volhard, 2000; Zhang and Yang, 2001), we therefore examined RGC development in  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice. RGCs differentiated in the peripheral region of the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina, as indicated by the presence of Brn3b<sup>+</sup> cells and the induction of *Shh* expression (from the mutant allele) in the RGC layer (Fig. 3A,C; data not shown). However, the RGC layer in peripheral region of the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina was thicker, disorganized and contained more

RGCs (Fig. 3A,C,E). These RGC abnormalities were restricted to the peripheral region of the retina where *Gli1* expression is downregulated, as RGC number and organization were normal in the central retina of  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice (Fig. 3E; data not shown).

In the chick, increased Shh signalling was associated with reduced cell survival in the RGC layer (Spence et al., 2004). We did not observe a change in cell survival in the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina or in Shh-treated retinal explants and RGC development was initiated at the normal time in mutant and wild-type retinas, as judged by the pattern of Brn3b staining at E12 (data not shown). Thus, the increase in RGC number in the mutant retina was probably due to an overproduction of these cells during the period when RGCs are normally produced. To further test this conclusion, we examined the development of RGCs in vivo. RPC undergo mitosis and cytokinesis at the apical side of the retina neuroepithelium, and postmitotic RGCs differentiate immediately and express Brn3b as they migrate away from the apical surface towards the RGC layer (Xiang, 1998). To identify newly generated RGCs, we labelled RPC at E16 with BrdU, harvested the retinas 24 hours later, and stained frozen sections for Brn3b and BrdU. Cells in the neuroblast layer that stain for



**Fig. 3.** *Shh* inactivation in the peripheral retina is associated with increased RGC development. (A–D) Retinas from wild-type and  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice at E17 stained for BrdU and Brn3b. RPC were labelled in vivo with BrdU at E16 and harvested 24 hours later. B and D are high magnification views of the neuroblast layers in A and B, respectively. Arrows in D indicate examples of double-labelled cells. The layer of Brn3b<sup>+</sup> RGCs is thickened and there is an increase in double-labelled cells in the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina (C,D). (E) Quantification of the number of Brn3b<sup>+</sup> cells in a 300  $\mu$ m region in the central and peripheral regions of wild-type and mutant retinas at P0. (F,G) Birthdating analysis in the embryonic  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina. RPC were labelled in vivo with BrdU at E13 and the retinas were harvested at P0. The number of Brn3b<sup>+</sup> cells with intense BrdU-labelling (F) and the proportion of Brn3b<sup>+</sup> cells among the heavily BrdU-labelled cohort (G) were quantified in 300  $\mu$ m regions of the central and peripheral retinas in wild-type and mutant mice. \**P* < 0.005. Scale bar: 100  $\mu$ m in A,C; 50  $\mu$ m in B,D.

both markers must have exited the cell-cycle after incorporating BrdU and initiated RGC differentiation. We found that there was an increase in newly generated RGCs in the neuroblast layer in the peripheral retinas of the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice compared with wild-type littermates (Fig. 3A–D).

We also quantified the change in RGC development by injecting mice with BrdU at E13 and E16 and then counting the number of cells at P0 that exhibited heavy nuclear staining for BrdU and that co-stained with Brn3b. The number of such cells was increased by more than twofold in the peripheral retinas of  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice compared with wild-type retinas and this increase was not observed in the central retina, indicating the localized effect of *Shh* inactivation on RGC

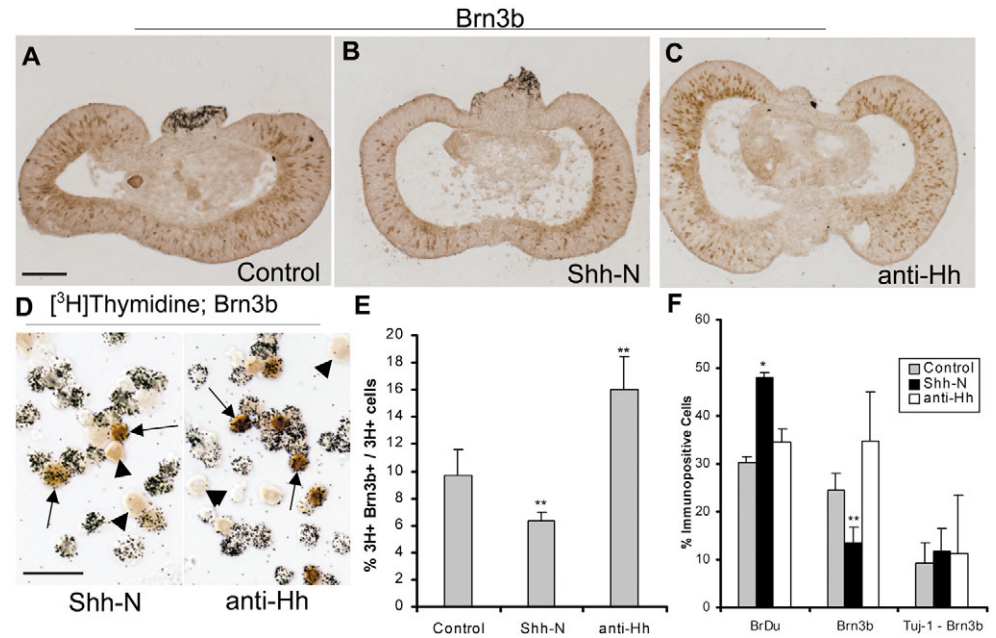
development (Fig. 3F, see Fig. S2A in the supplementary material). The lack of *Shh* signalling did not extend the period of RGC development, as we did not observe a persistence of Brn3b<sup>+</sup> cells in the neuroblast layer of  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice at P0, which corresponds to the end of the normal period of RGC development (data not shown).

To determine whether *Shh* inactivation affected the development of other early-born cell types, such as cones, amacrine cells and horizontal cells, we injected BrdU at E13 and compared the distribution of heavily labelled cells in the retina of P0 mice, assigning the cells to one of three layers: the outer region of the outer nuclear layer (ONL) (presumptive cone photoreceptors); the inner region of the inner nuclear layer (INL) (presumptive amacrine cells); or the RGC layer (RGCs and displaced amacrine cells). In the peripheral retina, the proportion of heavily labelled cells was increased in the RGC layer, reduced in the inner neuroblast layer and unchanged in the outer neuroblast layer of  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice compared with wild-type mice (see Fig. S2B in the supplementary material). Moreover, the proportion of RGCs among the heavily labelled cohort at E13 was increased in the peripheral retina of  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice compared with wild-type mice (Fig. 3G). Taken together, these data suggest that, in the absence of *Shh* signalling, there is a bias towards the production of RGCs at the expense of cells destined for the inner nuclear layer, presumably mostly amacrine cells.

### **Shh signalling inhibits RGC development in explants**

The disproportionate increase in RGC development at E13 in the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mutant suggested that *Shh* might also antagonize RGC development. To test this suggestion, we asked whether short exposure to *Shh* signalling in retinal explants would inhibit RGC development. We treated explants from E12 wild-type mice, a stage in development when RGC development is maximal, with either recombinant N-terminal fragment of *Shh* (*Shh*-N) or anti-Hh antibodies, for 48 hours. RGCs normally die by apoptosis within 24–48 hours of culture in explants, but, because they differentiate rapidly following terminal mitosis, we could follow the development of newly formed RGCs by limiting the culture time to 48 hours. To ensure that we were following newly differentiated RGCs, we labelled RPC with [<sup>3</sup>H]thymidine at the beginning of the culture and quantified the proportion of RGCs among the labelled cohort. *Shh*-N treatment reduced RGC development in the explant cultures, whereas anti-Hh treatment had the opposite effect (Fig. 4A–E). *Shh*-N-treatment also increased the proportion of dividing cells in the explants, which raised the possibility that it decreased RGC development by delaying RPC cell-cycle exit (Fig. 4F). We reasoned that, if this were the case, then the differentiation of other cell types would also be reduced in *Shh*-N-treated explants. To test this possibility, we stained dissociated cells from explants with Tuj1, an anti- $\beta$ III-tubulin antibody, which detects both RGCs and amacrine cells, another cell type that is generated at this stage of retinal development. The proportion of amacrine cells (Tuj1<sup>+</sup>-Brn3b<sup>+</sup>) was not significantly different in the *Shh*-N or anti-Hh treated explants (Fig. 4F), indicating that the effect of increased *Shh* signalling was specific to RGCs, at least at this stage. Finally, treatment with other RPC mitogens, Egf and basic Fgf, did not reduce RGC development in explants (data not shown).

**Fig. 4.** Hh signalling inhibits RGC development in vitro. (A-C) Brn3b staining in E12 retinal explants cultured for 48 hours under control conditions (A) or in the presence of recombinant Shh-N (B) or anti-Hh (C). The most dramatic change in Brn3b staining is observed in the peripheral region (closer to the lens) of the retinal explants. (D) Co-localization of silver grains marking [<sup>3</sup>H]thymidine labelled cells and Brn3b staining. RPC were labelled with [<sup>3</sup>H]thymidine for 4 hours, washed and cultured for an additional 48 hours. Arrows indicate double-labelled cells, arrowheads indicate Brn3b<sup>+</sup> [<sup>3</sup>H]thymidine<sup>-</sup> cells. (E) Dissociated cell scoring to quantify the proportion of RGCs among the [<sup>3</sup>H]thymidine<sup>+</sup> cohort in E12 retinal explants cultured for 48 hours under the indicated treatment conditions. Treatment with isotype-matched anti-LFA antibodies (1E6) was no different than control and is not shown.  $n \geq 3$  for each condition. (F) Proportion of S-phase, Brn3b<sup>+</sup> and the non-RGC component of the Tuj1<sup>+</sup> population (determined by subtracting the number of Brn3b<sup>+</sup> from the number of Tuj1<sup>+</sup> cells) in E12 explants cultured for 48 hours under the indicated conditions and analyzed by dissociated cell scoring. \* $P < 0.005$ , \*\* $P < 0.005$ . Scale bars: 100  $\mu\text{m}$  in A-C; 25  $\mu\text{m}$  in D.



### Loss of Shh signalling results in accelerated differentiation

Although our birth-dating analyses indicated that the production of some early cell types was either not changed (cones) or reduced (amacrine cells), the differentiation of these and other retinal cell types was accelerated. For example, horizontal cell differentiation, as revealed by a row of syntaxin<sup>+</sup> and calbindin<sup>+</sup> cells in the outer layer of the retina, was apparent in the peripheral region of the  $\alpha\text{-Cre};\text{Shh}^{-/c}$  retina at P3, but not in wild-type retinas (Fig. 5A,B; data not shown). The number of photoreceptors was also increased in the peripheral retina of  $\alpha\text{-Cre};\text{Shh}^{-/c}$  mice at P1 (rods) and P6 (cones), and these cells appeared to be more differentiated in the mutant compared with the control retinas, based on the increased intensity of cone-arrestin and rhodopsin staining (Fig. 5C-E; data not shown). As we did not observe an increase in the number of birth-dated photoreceptors at E13 (Fig. S2B), the increase in photoreceptors in the perinatal  $\alpha\text{-Cre};\text{Shh}^{-/c}$  retina probably reflects an acceleration of their differentiation program, rather than an increase in their production.

We encountered two problems when we attempted to quantify late born cell types, such as Müller glia and bipolar cells, in the  $\alpha\text{-Cre};\text{Shh}^{-/c}$  retina. First, the markers for these cells are not expressed until late in the postnatal period, and, at this stage, we could not rely on the absence of *Gli1* expression to delineate the mutant region of the retina, as *Gli1* expression is downregulated at this stage. Second, at late stages of development, there were considerable degenerative changes in the peripheral retina of  $\alpha\text{-Cre};\text{Shh}^{-/c}$  mice, especially in the nasal quadrant, which made quantification unreliable (Fig. 5H,L; data not shown). For these reasons, we adopted a different counting strategy for the late peripheral retina by comparing cell counts in a region 300  $\mu\text{m}$  proximal to the start of degeneration. The numbers of Müller cells (CRALBP<sup>+</sup>) and bipolar cells (PKC<sup>+</sup>) were reduced in the

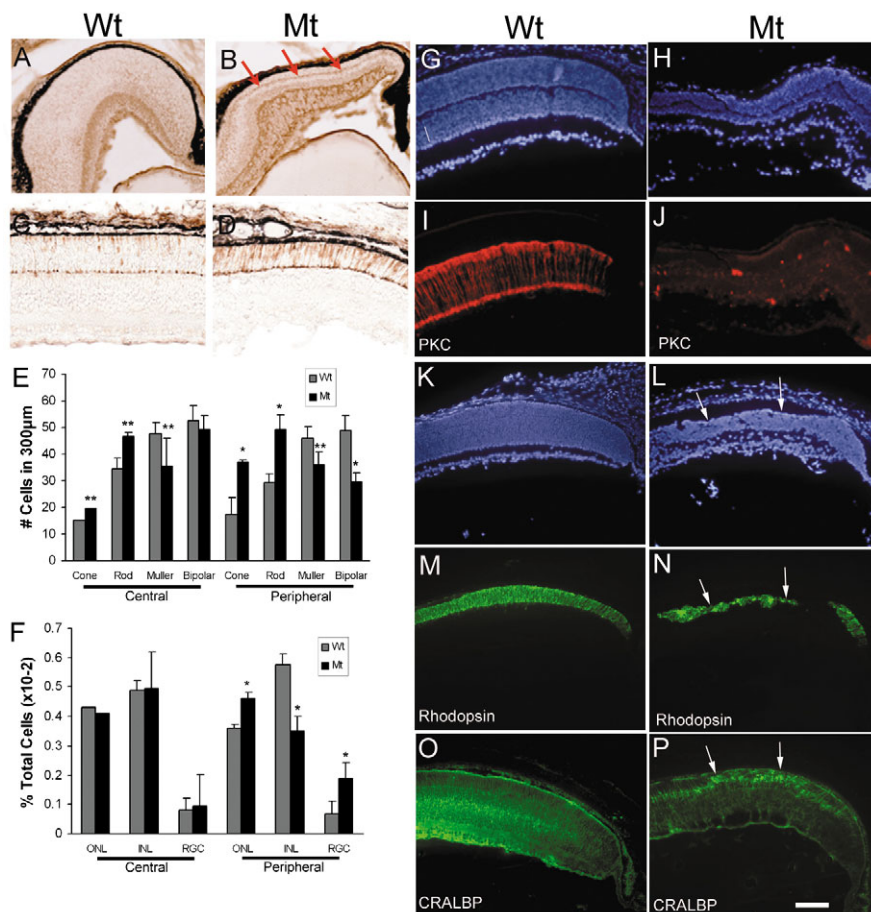
peripheral region of the  $\alpha\text{-Cre};\text{Shh}^{-/c}$  retina compared with wild-type mice (Fig. 5E). We also counted the total cell number in a 100  $\mu\text{m}$  region of the central and peripheral retina and calculated the proportion of cells in the different layers (Fig. 5F). The proportion of INL cells was reduced in the peripheral retina of the  $\alpha\text{-Cre};\text{Shh}^{-/c}$  mice (Fig. 5F), indicating that Müller and bipolar cells are also reduced as a proportion. The reduction in Müller cells in the central retina probably reflects a partial reduction in Shh signalling in this region, as we have noted a low level of Cre activity in the central region of the  $\alpha\text{-Cre}$  retina and a reduction in the intensity of *Gli1* expression in the central region of some  $\alpha\text{-Cre};\text{Shh}^{-/c}$  mice (data not shown). As it is possible that our counting strategy for the peripheral retina at these late stages could have included Shh<sup>+</sup> regions of the retina, we also examined the development of these cell types in the peripheral-most degenerating regions of the retina. Whereas Müller cells were present in these regions, bipolar cells were almost completely absent; the few that were present were severely disorganized (Fig. 5, compare I with J).

As early as P1, the peripheral nasal retina of  $\alpha\text{-Cre};\text{Shh}^{-/c}$  mice exhibited abnormalities in the outer nuclear layer, which included photoreceptor rosettes and gaps in rhodopsin staining that were readily apparent by P6 (Fig. 5K-P; data not shown). Staining with anti-CRALBP antibodies revealed that these gaps contained cells from the INL, including Müller glia (Fig. 5P). Ultimately, the degeneration progressed to the point where the entire outer nuclear layer was lost and the remaining inner and RGC layers were markedly disorganized (data not shown).

### Ectopic Hh pathway activation restores bipolar cell development in $\alpha\text{-Cre};\text{Shh}^{-/c}$ retinas

The defects in late cell development and degeneration that we observed in the  $\alpha\text{-Cre};\text{Shh}^{-/c}$  perinatal stages could be indirect

**Fig. 5.** *Shh* inactivation affects differentiation and the development of late-born cell types. Retinal sections from wild-type (A,C,G,I,K,M,O) and  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice (B,D,H,J,L,N,P) were stained at P0 for syntaxin (A,B) to identify amacrine and horizontal cells; at P6 for cone-arrestin (C,D) to identify cone photoreceptors; at P9 for Hoechst (G,H) to mark nuclei and for PKC (I,J) to identify rod bipolar cells; and at P6 for Hoechst (K,L), rhodopsin (M,N) to identify rod photoreceptor cells and CRALBP (O,P) to identify Müller glia. Red arrows in B indicate the line of differentiating horizontal cells in the outer plexiform layer in the periphery of the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina. Horizontal cells are not differentiated in the control retina at this stage (A). The intensity of cone arrestin staining is greater in the periphery of the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina compared with wild-type littermates (C,D). (E) Quantification of retinal cell types in the central and peripheral regions of the wild-type and mutant retinas at P1 (rods), P6 (cones, Müller glia) and P9 (bipolar cells). (F) The distribution of cells between the different retinal layers in a 100  $\mu$ m region of the central and peripheral retinas of wild-type and mutant mice at P6. (G-J) Bipolar cells are reduced in number and disorganized in degenerating peripheral retinas of  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice. \*\**P*<0.05, \**P*<0.005. (K-P) Degenerative changes take place in the periphery of the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina, as indicated by the rosettes (arrows in L), gaps in rhodopsin staining (arrows in N) and infiltration of Müller glial cell bodies into the ONL (arrows in P). Scale bar: 100  $\mu$ m.



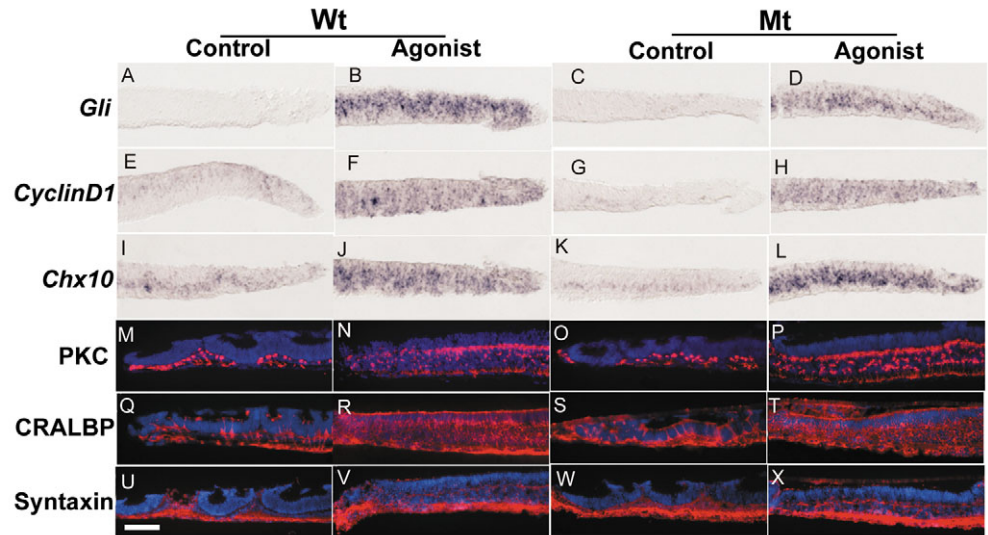
consequences of *Shh* inactivation during embryogenesis. We found, however, that blockade of Hh signalling in perinatal retinal explants and reaggregate cultures reduced BrdU incorporation and the proportions of Müller glia and bipolar cells (see Fig. S3 in the supplementary material), suggesting that sustained Hh pathway activation is required at late stages for RPC proliferation and the development of late born cell types. To explore this possibility further, we determined whether Hh pathway activation could restore proliferation and the development of late born cell types in peripheral regions of the postnatal  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina. We treated retinal explants from P1 mutant and wild-type mice with a Hh agonist and examined the expression of cell-cycle genes, Hh target genes and RPC markers, after 2 days, and late cell type markers after 9 days. Treatment with the Hh agonist increased the expression of *Gli1*, cyclin D1 and *Chx10* in peripheral regions of retinal explants from  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> and wild-type mice (Fig. 6A-L). Treatment with the Hh agonist also increased the development of Müller glia and bipolar cells in the peripheral regions of explants from  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> and control mice (Fig. 6M-T). Hh agonist treatment at this stage appeared to be more specific for promoting Müller and bipolar cell development, as it had little effect amacrine cell development (Fig. 6U-X). The restoration of proliferation and late cell type development appeared to be specific to Hh

pathway activation, as EGF and bFGF, two other RPC mitogens, had no effect on cyclin D1 expression and bipolar cell development (see Fig. S4 in the supplementary material).

### Accelerated cellular differentiation is associated with down-regulation of *Hes* gene expression

The acceleration of photoreceptor differentiation in the peripheral region of the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina was similar to the retinal phenotype of *Hes1*<sup>-/-</sup> mice (Takatsuka et al., 2004; Tomita et al., 1996). We examined the expression of *Hes* genes in the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> retina at P0 and found that *Hes1* expression was reduced in the peripheral retina at these ages compared with control retinas (Fig. 7A,B). To determine whether altering the level of Hh signalling in retinal explants could affect *Hes* gene expression, we performed semi-quantitative RT-PCR analysis on E18 retinal explants that were cultured in the presence of Shh-N, anti-Hh antibodies or control conditions (Fig. 7C). Consistent with our previous published observations, the expression of *Gli1* and *Ptch2*, Hh target genes, is downregulated in control explants because of the rapid loss of RGC in these cultures (Fig. 7C). *Hes1* and *Hes5* expression was reduced in control and anti-Hh-treated explants at 1 and 3 days compared with the levels in the E18 retina (Fig. 7C), and treatment with Shh-N restored *Hes* gene expression in explants. This effect appeared to be specific to Hh signalling, as EGF

**Fig. 6.** Ectopic Hh pathway activation restores cell cycle gene expression and bipolar cell development in retinal explants from  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice. Retinal explants from wild-type and  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mice at P1 were cultured for 2 (A-L) or 9 (M-X) days and processed for in situ hybridization to detect mRNA for *Gli* (A-D), cyclin D1 (E-H) and *Chx10* (I-L) or stained for Hoechst to reveal nuclei and anti-PKC (M-P), anti-CRALBP (Q-T) and anti-syntaxin (U-X) antibodies. The peripheral region of the explants is shown in these panels. Scale bar: 100  $\mu$ m.



treatment had little effect on *Hes1* and *Hes5* expression (Fig. 7C).

## Discussion

### Species specific functions of Hh signalling in retinal development

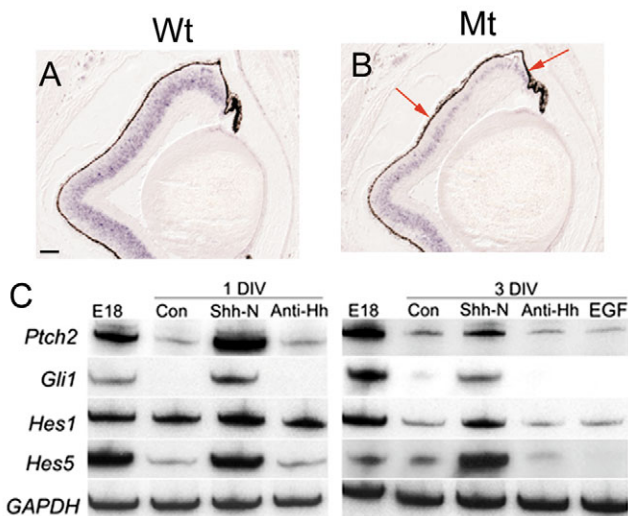
There is a striking interspecies conservation in the cell-cell interactions that mediate Hh signalling in the retina. In mouse and fish, Hh expression is linked to RGC differentiation and is thus propagated across the retina in a wave-like fashion where it signals at short range to overlying cells in the neuroblast layer (this study) (Shkumatava et al., 2004). Despite these similarities, there are notable differences in the function of this

pathway in mouse and zebrafish. In the mouse, *Shh* is the only Hh homologue expressed in the RGC layer (Jensen and Wallace, 1997), whereas in zebrafish, at least two Hh homologues, *Shh* and *tiggywinkle* hedgehog (*twhh*), are expressed in RGCs, and *Shh* is also expressed in amacrine neurons (Neumann and Nusslein-Volhard, 2000; Shkumatava et al., 2004). In mouse, *Shh* is required to maintain the proliferation of RPC and negatively regulates RGC production. In the zebrafish retina, *Shh* exerts the opposite effect, being required to induce cell cycle exit and RGC differentiation, in part via induction of *p57<sup>Kip</sup>* expression (Neumann and Nusslein-Volhard, 2000; Shkumatava and Neumann, 2005; Stenkamp and Frey, 2003). One explanation for these differences in Hh function could be related to differences in the length of the histogenic processes, which is rapid (3 days) in fish, but significantly longer (~3 weeks) in mouse. Thus, in the zebrafish retina, the need to induce cell cycle exit from a pool of rapidly dividing progenitors might take precedence over the need to maintain those cells in cycle, whereas in the mouse retina, the opposite might be the case, where maintenance of proliferation takes precedence over cell cycle exit.

### *Shh* controls RPC proliferation in the embryonic retina

Control of RPC proliferation and cell cycle exit is required to achieve the correct proportions of retinal cell types (Dyer and Cepko, 2001). Thus, the characterization of the growth control signals in the developing retina is an important step towards understanding the link between cell cycle control and neurogenesis. We have used a conditional mutagenesis approach to show that *Shh* signalling from RGCs is required for Hh target gene induction and normal RPC proliferation in the embryonic retina. Together with the evidence that Hh pathway activation is also required for RPC proliferation in the perinatal retina (Wallace and Raff, 1999) (this study), we now establish that *Shh* functions as a mitogen throughout the period of retinal development.

The effects of *Shh* on proliferation are probably mediated, in part, through cyclin D1 and *Hes1*. The retinas of cyclin D1, *Hes1* and  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> mutant mice exhibit a remarkable degree of phenotypic similarity that includes reduced



**Fig. 7.** *Shh* signalling modulates *Hes* gene expression in RPC. (A,B) In situ hybridization for *Hes1* mRNA in the retina of wild-type (A) and  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> (B) mice at P0. *Hes1* expression is reduced in the peripheral retina of the  $\alpha$ -*Cre*;*Shh*<sup>-/-</sup> (between the red arrows). (C) Semi-quantitative RT-PCR analysis of gene expression in control E18 retinas and in E18 retinal explants cultured for 1 and 3 days under the indicated conditions. Scale bar: 100  $\mu$ m in A,B.



proliferation and retinal degeneration (cyclin D1) (Ma et al., 1998; Sicinski et al., 1995), RGC overproduction and accelerated photoreceptor differentiation (*Hes1*) (Takatsuka et al., 2004; Tomita et al., 1996) and we show here that Hh signalling modulates the expression of these genes in RPC. Shh-induced proliferation in the hair follicle and cerebellum is driven by Gli1-dependent transcription control of cyclin D1 and *Mycn* and stabilization of Mycn protein (Kenney et al., 2004; Mill et al., 2005). By contrast, Myc genes do not appear to be transcriptional targets of Hh signalling in the retina; however, it remains to be seen whether Hh signalling is required for stabilization of Myc protein in RPC. Hh signalling promotes *Hes1* expression in cerebellar granule neuron precursors (Solecki et al., 2001) and this has been shown to be Notch dependent in granule neuron-derived tumours (Hallahan et al., 2004). The reduction in *Hes1* expression in the  $\alpha$ -*Cre;Shh*<sup>-/-</sup> retina is, however, not associated with a change in the expression of Notch receptors, Notch ligands or the Notch effector protein RBPJK (data not shown).

### Shh signalling and effects on cell diversification and retinal degeneration

The loss of Shh signalling *in vivo* is associated with increased RGC production and decreased late-born cell type production, such as Müller glia and bipolar cells. Given the proliferation defects in the  $\alpha$ -*Cre;Shh*<sup>-/-</sup> retina, the simplest interpretation of these findings is that as more RPC exit the cell cycle precociously, they adopt the fate that is appropriate for their stage of retinal development, in this case, RGCs; precocious cell-cycle exit also depletes the RPC pool so that too few RPC remain to produce appropriate numbers of late-born cell types. This interpretation is consistent with other studies in which forced cell-cycle exit and premature differentiation of RPC results in an increase in RGC development (Austin et al., 1995; Ohnuma et al., 2002; Takatsuka et al., 2004) and with our evidence that ectopic Hh signalling can restore cell-cycle gene expression and Müller, and bipolar cell development in explants from  $\alpha$ -*Cre;Shh*<sup>-/-</sup> mice. Alternatively, these late cell types may develop from a Hh-dependent RPC pool, which would be consistent with our observation that EGF and bFGF do not elicit a significant proliferative response or promote bipolar cell development in perinatal retinal explants.

Previously, we have shown that maintenance of Shh signalling in retinal explants promoted the development of normal lamination (Wang et al., 2002). In this study, we were able to follow the mutant retinas until adulthood and showed that *Shh* inactivation is associated with retinal degeneration. Because of the timing of *Shh* inactivation in this system it is possible that these degenerative changes could be indirect. However, blockade of Hh signalling in perinatal retinal explants results in severe lamination defects (Wang et al., 2002) (data not shown), consistent with a role for Shh signalling at late stages of retinal development for normal retinal organization.

### Shh and timing of RPC competence to generate RGCs

Several lines of evidence indicate that some of the effect of Shh on RGC development is cell cycle independent. The birthdating analyses presented in this study indicate that RPC in the peripheral region of the  $\alpha$ -*Cre;Shh*<sup>-/-</sup> retina are biased

towards RGC production at the expense of amacrine cells, which are normally generated at the same time. If all of the effects of Shh signalling were mediated through the cell cycle, we would have expected that the proportions of early born cell types would remain the same among the birthdated cohort. Hh-mediated inhibition of RGC development in the chick retina is not associated with increased RPC proliferation (Zhang and Yang, 2001), and we have found that as little as 4 hours of Shh treatment inhibits RGC development in mouse retinal explants (data not shown). Given that RGC production remains increased in the peripheral retina of  $\alpha$ -*Cre;Shh*<sup>-/-</sup> mice at E16, we suggest that Shh signalling is required to control the timing of RPC competence to generate RGCs. Gdf11 is also expressed in RGCs and is a negative regulator of RGC development, as evidenced by an overproduction of RGC at the expense of amacrine cells and photoreceptors in the retinas of *Gdf11*<sup>-/-</sup> mice (Kim et al., 2005). In contrast to our findings in the  $\alpha$ -*Cre;Shh*<sup>-/-</sup> mouse, loss of Gdf11 does not affect RPC proliferation and increases RGC development at later stages (Kim et al., 2005). Thus, Shh signalling might act at an earlier stage to control progenitor competence to generate RGCs, which is consistent with our observation that its expression is delayed until after Brn3b<sup>+</sup> cells have completed their migration to the RGC layer; earlier induction of *Shh* expression might result in insufficient RGC production. The alterations in RPC competence in the *Gdf11*<sup>-/-</sup> retina were associated with prolonged expression of *Math5*, a bHLH transcription factor that is required for RGC production (Brown et al., 2001; Wang et al., 2001), and changes in the timing of expression of other bHLH genes, such as *Mash1* (*Ascl1* – Mouse Genome Informatics) and *Neurod1* (Kim et al., 2005). Consistent with these observations, *Math5* expression is marginally increased in the  $\alpha$ -*Cre;Shh*<sup>-/-</sup> retina (data not shown).

### Similarity with mutants lacking RGCs

This study highlights the important role for a RGC-derived signal on retinal histogenesis and several aspects of  $\alpha$ -*Cre;Shh*<sup>-/-</sup> retinal phenotype are similar to other RGC-deficient mouse mutants. In *Math5* mutant mice, for example, the retina and, in particular, the INL are reduced in thickness and bipolar cells are reduced in number (Brown et al., 2001; Brzezinski et al., 2005; Wang et al., 2001). In another mouse model in which RGCs are selectively ablated by targeted expression of diphtheria toxin (*Brn3b-DTA*), RPC proliferation is reduced and this effect was associated with a reduction in *Shh*, *Gli1* and cyclin D1 expression (Mu et al., 2005), which is in good agreement with the findings presented here. Despite the reduction in Shh signalling in the *Brn3b-DTA* mouse model, however, the proportion of late-born cell types is normal and the retina does not degenerate (Mu et al., 2005). A major difference between the  $\alpha$ -*Cre;Shh*<sup>-/-</sup> and *Brn3b-DTA* models is the presence of RGCs in the former, which raises the possibility that some of the retinal phenotype in the  $\alpha$ -*Cre;Shh*<sup>-/-</sup> mouse could be due to unmasking the effects of other RGC-derived signals, such as myostatin/Gdf8 (Mu et al., 2004). Alternatively, differences in these retinal phenotypes could be due to imbalances created by the juxtaposition of mutant and wild-type tissue in the  $\alpha$ -*Cre;Shh*<sup>-/-</sup> retina.

Our results highlight a crucial role for Shh signalling in the local control of RPC proliferation and differentiation by RGCs. RGC-derived Shh signalling acts to maintain RPC

proliferation, and thereby helps to establish the proper balance between the production of early and late retinal cell types. In this way, RGCs help control the synaptic input they receive.

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/22/5103/DC1>

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