## DIRECT AND INDIRECT EFFECTS OF HEDGEHOG PATHWAY ACTIVATION IN THE MAMMALIAN RETINA.

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Running Title: Cell autonomous Hedgehog signaling in the retina

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The morphogen Sonic hedgehog (Shh) is expressed by the projection neurons of the retina, retinal ganglion cells (RGCs), and promotes retinal precursor cell (RPC) proliferation. To distinguish between direct and indirect effects of Hedgehog (Hh) pathway activation in the perinatal mouse retina, we followed the fate of cells that expressed a constitutively active allele of Smoothened (SMO-M2), the signal transduction component of the Hh pathway. SMO-M2 expression promoted a cell-autonomous increase in *CyclinD1* expression and RPC proliferation and biased RPC towards the production of cells with an inner nuclear layer fate at the expense of rod photoreceptors. SMO-M2 expression also inhibited rhodopsin expression in uninfected cells, thus highlighting an unexpected non-cell autonomous effect of Hh pathway activation on photoreceptor development.

#### INTRODUCTION

A fundamental question in central nervous system development is how the enormous cellular diversity in the adult brain is achieved. The retina is an excellent model system in which to address this question because 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 layers and by their expression of cell-type-specific markers. The adult retina contains six neuronal and one major glial cell type that are generated in a sequential, but overlapping, order where retinal ganglion cells (RGCs) are specified first and Müller glia and bipolar cells are specified last (Young, 1985). Lineage tracing analyses in a number of vertebrate species has revealed that cell diversification in the retina is lineage independent: all of the retinal cell types can be generated from multipotential precursor cells (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988). The question then becomes to what extent the cell diversification process is influenced by cell intrinsic versus cell extrinsic processes. Comparison of the developmental potential of retinal precursor cells in vivo versus in vitro indicated that diversification, as well as the timing of cell cycle exit, are determined cell intrinsically, at least for cell types that are generated postnatally (Cayouette et al., 2003). There is, however, evidence that the generation of some classes of retinal cells, in particular RGCs and amacrine cells, is regulated by negative feedback inhibition by signals from differentiated neurons of the same subclass (Belliveau and Cepko, 1999; Reh and Tully, 1986; Waid and McLoon, 1998). In the case of RGCs, these effects are mediated by RGC-derived Sonic hedgehog (Shh) and GDF11 signaling (Kim et al., 2005; Wang et al., 2005; Zhang and Yang, 2001).

The sequential development of retinal cell types is postulated to be due, in part, to temporal changes in the developmental competence of RPCs (Competence model; reviewed in

Livesey and Cepko, 2001). Thus, the timing of cell cycle exit is likely to be an important factor in determining the final complement of retinal cell types. Precocious cell cycle exit is associated with an increase in early born cell types, such as RGCs (Ohnuma et al., 2002), and delayed cell cycle exit is associated with a reduction in early born cell types and an increase in late born cell types (Dyer et al., 2003; Li et al., 2002). In addition to intercellular signaling mediated by the Notch pathway, neuron-derived signals also play an important role in the timing of cell cycle exit and differentiation (Austin et al., 1995; Dorsky et al., 1997; Dorsky et al., 1995; Henrique et al., 1997; Mu et al., 2005). For example, RGCs are required for normal RPC proliferation, which is mediated, in part, via Sonic hedgehog signaling (Mu et al., 2005; Wang et al., 2005; Wang et al., 2002).

Hedgehog (Hh) proteins are extracellular signaling molecules that control patterning and growth of a number of tissues in the developing embryo (reviewed in Ingham and McMahon, 2001). Hh proteins control target gene expression in responsive cells by regulating Smoothened (Smo), a seven-transmembrane domain protein that transduces the signal via cytoplasmic effectors to the nucleus. Normally, Smo activity is antagonized by the Patched (Ptc) tumor suppressor gene, however, Ptc insensitive activating SMO alleles that contain single point mutations have been identified in sporadic basal cell carcinomas and have been shown to drive constitutive Hh pathway activation in cells (Xie et al., 1998). *Ptc* and *Gli* are two universal target genes of the pathway, which are upregulated in Hh responding cells, and thus serve as a convenient readouts for the status of Hh pathway activation in tissues (reviewed in Hooper and Scott, 2005). In many instances, Hh proteins mediate their effects directly on target cells. However, there are instances where Hh acts indirectly to pattern tissues, in part via the induction of secreted factors (Pola et al., 2001; Strutt and Mlodzik, 1997; Tanimoto et al., 2000).

Hh signaling has been implicated in the control of eye field specification and retinal development in several vertebrate species (reviewed in Amato et al., 2004). In the mouse retina, Shh is expressed by RGCs and induces Hh target gene expression in RPC (Wang et al., 2002). The analysis of mice with a retina specific conditional Shh inactivation reveals that Shh is required for the maintenance of RPC proliferation, in part via the induction of CyclinD1 and Hesl expression (Wang et al., 2005). In addition, Shh inactivation also resulted in the accelerated differentiation of several cell types, including rod photoreceptors, which normally do not express Hh target genes, indicating a possible indirect effect of this pathway on retinal development (Wang et al., 2005). To distinguish between direct and indirect effects of Hh pathway activation in the retina we examined the effects of ectopic expression of activated Smoothened (SMO-M2) in RPC. We find that SMO-M2 expression promoted proliferation, *CyclinD1* expression and the development of cells with an inner nuclear layer identity in a cell autonomous fashion. We also found that rod differentiation was inhibited in cells that did not express SMO-M2 indicating a role for non-cell autonomous effects of Hh pathway activation in the retina.

RESULTS

To confirm that retroviral expression of SMO-M2 induces ligand independent Hh pathway activation, we infected C3H10T1/2 cells, a Hh-responsive osteoblast cell line, with SMO-M2 or eGFP (control) retroviruses (Fig. 1A) or treated them with recombinant N-terminal fragment of Shh (Shh-N) and examined Gli expression by RT-PCR analysis 2 days later. Gli mRNA was upregulated in SMO-M2-infected and Shh-N-treated cultures compared with eGFPinfected or untreated cultures (Fig. 1B), confirming that the Hh pathway is activated in SMO-M2 infected cells. To investigate whether SMO-M2 expression was sufficient to induce Hh target gene expression in primary retinal cells, we co-electroporated mouse E18.5 retina explants with expression vectors containing SMO-M2 under the control of the CMV promoter (pCLE-SMO-M2) and a GFP reporter vector (pUB-GFP) and examined gene expression by ISH after 48 hours. We have shown previously that endogenous Hh target gene expression in retinal explants is downregulated because RGCs, the source of Shh, die by apoptosis in these cultures (Wang et al., 2002). Transfected cells in the pCLE-SMO-M2-electroporated explants were identified by GFP expression and a marked increased in the intensity of the ISH signal for Smo (Fig. 1C). Glil mRNA was upregulated in GFP<sup>+</sup> regions of pCLE-SMO-M2 transfected explants, but not in the control explants transfected with the eGFP reporter alone (Fig. 1C), thus confirming the cellautonomous effect of SMO-M2 on Hh target gene expression in RPC.

To address whether cell autonomous Hh pathway activation was sufficient to promote RPC proliferation we examined the effect of ectopic SMO-M2 expression on cell cycle gene expression and proliferation. Electroporation of pCLE-SMO-M2 in E18.5 retinal explants induced high levels of *CyclinD1* expression that was restricted to the SMO-M2-expressing cells (Fig. 1C). We also compared BrdU incorporation in cells infected in the SMO-M2- or control

retroviruses. E18.5 retinal explants were infected with retroviruses, cultured for 3 days in serumfree medium and pulsed with BrdU for the last 20 hours of the culture period to label cells in Sphase. The explants were dissociated into single cells and the proportion of BrdU<sup>+</sup> cells amongst the GFP<sup>+</sup> cohort was quantified. The proportion of cells in S-phase was increased by over 2 fold in the SMO-M2-infected compared with eGFP-infected cells (Fig. 2A). The proportion  $BrdU^+$ cells in the uninfected, GFP<sup>-</sup>, cohort from SMO-M2 or eGFP-infected explants was not changed, indicating that the effects of SMO-M2 on RPC proliferation were cell-autonomous (Fig. 2A). Mutant SMO-induced signaling in granule neuron precursors has been reported to promote cell survival (Hallahan et al., 2004), however, it was difficult to compare survival of SMO-M2 versus eGFP-infected cells in retinal explants. TUNEL staining of control and Hh-treated retinal explants at daily intervals over a 7 day culture period did not reveal any differences in cell survival and conditional ablation of Shh in the retina is not associated with changes in cell survival during embryonic retinal development (Wang et al., 2005; data not shown). Therefore, it is unlikely that the effect of SMO-M2 expression on retinal progenitor proliferation is secondary to changes in cell survival.

We also addressed whether SMO-M2 expression would affect clone size in the retina. E18.5 retina explants were infected with a low concentration (10<sup>6</sup> CFU/ml) of SMO-M2- or eGFP-retroviruses and cultured for 7 days in medium containing 10% FCS. Serum was added to these cultures because it promoted better explant morphology in long-term cultures. For *in vivo* infections, the retrovirus solution was injected subretinally at P0 and the mice were sacrificed at P14. After 7 days *in vitro* or 14 days *in vivo*, the GFP-labeled cells were arranged in radial clusters (Fig. 2B and data not shown). A radial cluster of cells was considered a clone (i.e. derived from a single infected RPC) if it was separated from the nearest GFP<sup>+</sup> radial cluster by at

least 4 cell diameters. The majority of clones, however, were spaced further apart than 4 cell diameters. SMO-M2-derived clones were larger compared with eGFP-derived clones *in vitro* and *in vivo* (Fig. 2C, D). For example, there was a two-fold reduction in the proportion of single cell clones and an increase in the proportion of clones containing 3 or more cells in the SMO-M2-infected explants compared with eGFP-infected explants (Fig. 2C). A similar trend was observed after *in vivo* infection, although the reduction in single cell clones in the SMO-M2 infected cells was not as dramatic as that observed *in vitro* and the clones were generally smaller than the clones in the explants (Fig. 2D). The overall reduction in the clone size *in vivo* could be due to the later age of infection (P0 *in vivo* versus E18.5 *in vitro*), to potential growth promoting effects of serum in the culture medium, or the presence of growth inhibitory factors *in vivo*.

To address whether cell-autonomous Hh pathway activation had an effect on retinal cell type development, we analyzed the cellular composition of the clones by comparing the distribution of cells within clones between the nuclear layers. We quantified the proportion of clones that were comprised of cells located only in the outer nuclear layer (ONL), the inner nuclear layer (INL) or both layers (INL+ONL). SMO-M2 expression was associated with a reduction in the proportion of ONL-only clones and an increase in the proportion of ONL+INL containing clones *in vitro* (Fig. 3A). A similar trend was observed *in vivo*, where SMO-M2 expression resulted in a reduction in ONL-only clones, but this effect was not associated with a change in the proportion of INL-containing clones *in vivo* (Fig. 3B). Thus, cell autonomous Hh pathway activation in the perinatal retina reduced photoreceptor development, and also promoted INL cell development in vitro. Since SMO-M2 expression promoted proliferation and increased clone size, it is possible that this could account for the increase in the proportion of INL+ONL clones in SMO-M2-infected explants. The lack of an increase in the proportion of INL-containing

clones in vivo could be because of reduced growth of SMO-M2 cells in that environment, which is consistent with the overall smaller clone size that we observed *in vivo*.

Hh signaling has been reported to promote Müller glial cell development and to reduce rod photoreceptor development in rodent retinal cultures (Jensen and Wallace, 1997; Levine et al., 1997; Wang et al., 2005). To examine the effect of Hh pathway activation on the development of other retinal cell types, we treated E18.5 retinal explants with a Hh agonist (Frank-Kamenetsky et al., 2002) under serum-free conditions for 10 days and determined the proportion of cells that stained with markers for Müller glia (CRALBP), bipolar cells (Chx10, PKC), amacrine (HPC1, calretinin) and rods (rhodopsin). We confirmed that Hh-agonist treatment reduced the proportion of rods but increased the proportions of Müller glia and bipolar cells compared with control explants (Fig. 4A). Similar results were obtained when explants were cultured in the presence of serum (data not shown). Hh-agonist treatment also increased the proportion of cells expressing amacrine markers, although the increase in the proportion of HPC1<sup>+</sup> cells could reflect an increase in horizontal cells and RPCs, since those cell types also express these markers (Fig. 4A).

To address the extent to which these changes in cell type development were cell autonomous, we compared cell type development in SMO-M2 infected cells. E18.5 retinal explants were infected with SMO-M2 or eGFP retroviruses and cultured for 10 days under serum-free conditions. Explants were dissociated into single cells and the proportion of marker<sup>+</sup> cells among the GFP<sup>+</sup> and GFP<sup>-</sup> cohort was determined. Consistent with the effects of Hh agonist treatment in retinal explants, the proportion of Müller glia, bipolar and amacrine cells was increased in the SMO-M2 compared with the eGFP-infected cohort (Fig. 4B). The increase in INL-type cells in the SMO-M2-infected cohort was at the expense of rods, as there was a two-

fold reduction in the proportion of rods in SMO-M2 compared with eGFP-infected cells (Fig. 4B). The increased production of INL cells was restricted to SMO-M2-infected cells, as the proportion of Müller, amacrine and bipolar cells was not changed in the uninfected cells in SMO-M2-infected explants (Fig. 4C). There was, however, a 2-fold reduction in rhodopsin<sup>+</sup> cells amongst the uninfected cells in SMO-M2 infected retinal explants compared with explants infected with the eGFP retrovirus (Fig. 4B). Thus, SMO-M2 expression induces a cell-autonomous increase in cells with an INL-identity at the expense of rods and also had a non-cell autonomous inhibitory effect on rod development in explants.

To address the basis for the inhibitory effect of SMO-M2 expression on rod development we compared the kinetics of rhodopsin expression in E18.5 explants infected with SMO-M2 and control virus and cultured for 7 and 10 days under serum-free conditions. At 7 days, the proportion of rods in the SMO-M2-infected cohort of cells was reduced compared with eGFP-infected cells and this difference was more pronounced by 10 days (Fig. 5A). When we compared rod development among the uninfected cells we found that at 7 days the proportion of rods was not different in SMO-M2 compared with eGFP-infected explants; however, by 10 days there was a two-fold reduction in rods in the uninfected cells from the SMO-M2-infected retinal explants (Fig. 5B).

We also compared rhodopsin staining in frozen sections of retrovirus-infected explants at 7 and 10 days of culture. At 7 days, there was no difference in the pattern of rhodopsin staining in the SMO-M2- or eGFP-infected retinal explants; in both cases, staining was continuous throughout the ONL and followed a central (highest) to peripheral (lowest) gradient, which is consistent with the normal central to peripheral gradient of photoreceptor differentiation *in vivo* (Fig. 5C). By 10 days, however, the rhodopsin-staining pattern in the ONL in the SMO-M2-

infected explants was discontinuous, with areas of rhodopsin<sup>+</sup> cells interspersed with areas that exhibited reduced rhodopsin expression, even in the central region of the retinal explants; while the eGFP-infected explants exhibited a normal pattern of rhodopsin staining (Fig. 5D). The reduction of rhodopsin expression in the SMO-M2-infected explants was not secondary to a change in cell survival, as there was no difference in TUNEL staining in SMO-M2-and eGFPinfected retinal explants (data not shown). The regions of reduced rhodopsin staining in the explant were not associated with a change in the density or distribution of retrovirally infected cells compared with regions of the explant with normal rhodopsin expression.

To investigate whether the reduction in rhodopsin expression in SMO-M2-infected retinal explants was due to an effect on rod specification or differentiation, we scored rods in explants on the basis of nuclear morphology. Prior to the onset of rhodopsin expression, rods can be identified by nuclear morphology: small nuclei with one or more large clumps of heterochromatin (Cayouette et al., 2003; Neophytou et al., 1997) (Fig. 6A). The proportion of cells with a characteristic rod nuclear morphology was reduced by 30% in the SMO-M2-infected compared with the eGFP-infected cohort (Fig. 6B), indicating that specification of cells with a rod nuclear morphology was not different amongst the uninfected cohort of cells in SMO-M2-infected compared with eGFP-infected explants (Fig. 6C), indicating that SMO-M2 does not affect specification to the rod lineage of uninfected cells.

Our findings suggested that rather than blocking specification to the rod lineage, expression of SMO-M2 inhibited rod differentiation at or before the rhodopsin<sup>+</sup> stage in bystander cells. To address at what stage of rod development this occurred we examined the expression of *Crx* and recoverin. Crx is a homeodomain transcription factor that is expressed

soon after terminal mitosis in rod and cone photoreceptors, as well as in a subset of bipolar cells (Furukawa et al., 1997). There was no significant difference in the intensity of *Crx* expression in the ONL in SMO-M2-infected explants at 7 and 10 days of culture compared with eGFP-infected explants (data not shown). Recoverin is a calcium binding protein that is expressed prior to the onset of rhodopsin expression in rods (Kelley et al., 1995). The proportion of recoverin<sup>+</sup> cells was reduced in the SMO-M2-infected compared with the eGFP-infected cohort after 10 days (Fig. 6B). The proportion of recoverin<sup>+</sup> cells was also reduced in the uninfected cohort of cells from SMO-M2-infected compared with eGFP-infected explants (Fig. 6C). Thus, SMO-M2 expression appears to exert a non-cell autonomous inhibitory effect on late stage rod differentiation.

DISCUSSION

Expression of the SMO-M2 allele has been shown to drive Hh-dependent cell type differentiation in the neural tube (Hynes et al., 1995) and proliferation in a variety of tissues, including the cerebellum, skin and developing bone (Hallahan et al., 2004; Long et al., 2001; Xie et al., 1998). RPC proliferation is dependent on the expression of high levels of CyclinD1, which is dependent, in part, on Shh signaling from RGCs (Wang et al., 2005; Wang et al., 2002). Our results indicate that the mitogenic effect of Hh pathway activation on RPC is direct, as SMO-M2 expression induces a cell-autonomous increase in *CyclinD1* expression and proliferation in RPCs, which is in good agreement with the cell autonomous requirement for Hh pathway activation for proliferation in other tissues (Long et al., 2001). Our results do not, however, preclude the possibility that Hh-induced RPC proliferation is mediated by a paracrine mechanism, as Hh mediates cellular responses in other systems by regulating growth factor responsiveness in target populations (Huang et al., 1998).

Our results indicate that sustained Hh pathway activation in RPCs also promotes the development of cells with an INL identity at the expense of rod photoreceptor development. We postulate that this occurs in two ways (discussed separately below): 1) by biasing RPCs towards an INL fate and 2) by inhibiting differentiation of committed rod precursor cells. Given its role on controlling the timing of cell cycle exit in the retina (Black et al., 2003; Moshiri and Reh, 2004; Wang et al., 2005), Shh could be acting permissively to promote INL fate by delaying cell cycle exit until RPC acquire the competence to generate late born cell types. However, this explanation is inconsistent with our previous observations that driving RPC proliferation with other mitogens does not promote INL fate (Wang et al., 2005) and our findings in this study that SMO-M2 expression increases amacrine cell development, a cell type that is generated before

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Müller glia and bipolar cells. SMO-M2 expression could promote the proliferation of committed amacrine cell precursors, as amacrine cells exhibit extended proliferative capacity, especially in the absence of pRb and p107 (Chen et al., 2004). However, the majority of cells that localized to the INL in the SMO-M2 clones were located in the upper half of the INL, a region that does not normally contain amacrine cells. We suggest that some of the increase in HPC1<sup>+</sup> cells in the SMO-M2-infected cells reflects an increase in RPC, which is consistent with an increase in the proportion nestin<sup>+</sup> cells amongst the SMO-M2-infected cohort (data not shown). Taken together, our findings support a model where Hh signaling acts instructively to bias RPCs towards an INL fate.

The reduction in rod development in the presence of increased Hh pathway activation reflects a reduction in specification of cells to the rod lineage (as discussed above), but also an inhibitory effect of this pathway on rod differentiation. In the rod lineage there is a considerable lag (5.5-6.5 days) between terminal cell cycle exit and rhodopsin expression (Morrow et al., 1998). Prior to the expression of these late markers post mitotic pre-rod cells express the homeobox transcription factor Crx and exhibit a unique heterochromatin pattern (Cayouette et al., 2003; Neophytou et al., 1997). The proportion of cells with a rod nuclear morphology that co-express rhodopsin was reduced in the SMO-M2 infected cohort compared with the eGFP-infected cells (50% in SMO-M2 versus 70% in eGFP), indicating that rod differentiation in the SMO-M2 infected cohort is delayed at a stage prior to rhodopsin and recoverin expression.

This inhibition of rod differentiation was also observed in uninfected cells in SMO-M2infected explants, indicating that Hh pathway activation can exert non-cell autonomous effects on rod development. This effect is not due to changes in cell survival or to a reduction in the commitment to the rod lineage. The most likely explanation for our results is that SMO-M2 expression inhibits rhodopsin expression in committed pre-rods via a paracrine effect. Rod development is influenced by extrinsic factors, such as CNTF and LIF, which are produced by RPC and Müller glia, and have been shown to inhibit rhodopsin and recoverin expression in post-mitotic pre-rods (Ezzeddine et al., 1997; Kirsch et al., 1998; Neophytou et al., 1997; Schulz-Key et al., 2002). Thus, the inhibition of rod differentiation in SMO-M2-infected explants could be secondary to an increase in Müller glia-derived LIF or CNTF production. This explanation is consistent with the lack of correlation between the location of retrovirally-infected cells and rhodopsin<sup>-</sup> regions of the ONL, as these growth factors can act at long range. The discontinuous nature of the reduction in rhodopsin staining in the ONL of the SMO-M2-infected explants is also consistent with a role for CNTF in this process. Rods exhibit differences in susceptibility to CNTF signaling, where newly differentiated rods are more sensitive to the effects of CNTF signaling than rods that have been expressing rhodopsin for longer periods (Kirsch et al., 1998; Schulz-Key et al., 2002).

Inactivation of *Shh* in the developing mouse retina is associated with accelerated photoreceptor differentiation (Wang et al., 2005). However, rods are not direct targets of Hh signaling, as the expression of Hh target genes is excluded from the apical  $Crx^+$  region of the retina and the ONL. The data presented here supports a model whereby Hh exerts direct effects on RPC, but also acts indirectly to control rod photoreceptor differentiation, possibly via a paracrine mechanism.

#### EXPERIMENTAL METHODS

#### Retroviral generation

To generate the SMO-M2 retrovirus a 2.5 kb *EcoRI* fragment encoding the entire coding region of the human SMO-M2 cDNA was excised from the parent vector, pCLE-SMO-M2, and subcloned into the *EcoRI* site of the pMXIE-retroviral construct (Fig. 1A). The resulting retroviral vector, SMO-M2, and the control retroviral vector, eGFP, were transfected in phoenix-Eco packaging cells by calcium phosphate precipitation based on protocols published online from the Nolan laboratory (http://www.stanford.edu/group/nolan/protocols/pro\_helper\_free.html). Supernatants from the transfected cells were concentrated by centrifugation at 21,000 rpm for 2 hrs at 4 °C in a Beckman JA25.50 rotor, aliquoted and stored at -80 °C. The retrovirus stocks were titred on NIH 3T3 cells. Typical titres for the eGFP and SMO-M2 retroviruses were  $10^8$  cfu/ml and  $10^7$ - $10^8$  cfu/ml, respectively.

#### Cell culture

NIH 3T3, C3H 10T  $\frac{1}{2}$  and Phoenix-Eco cells were cultured at 37°C, in 5% CO<sub>2</sub> in DMEM, containing 10% heat-inactivated fetal calf serum (FCS) and 100 U/ml penicillin/streptomycin. Retinal explants were obtained from timed mated C57/BL6 mice; the morning of the vaginal plug was considered day 0 of the pregnancy. Retinas from E18.5 C57/BL6 mice were dissected free of RPE and lens, flattened with the RGC facing up onto a filter in 500 µl culture medium (50% DMEM, 50% HAMS F-12, Insulin (10 µg/ml), transferrin (100 mg/ml), bovine serum albumin (BSA Fraction V: 100 mg/ml), progesterone (60 ng/ml), putrescine (16 µg/ml), sodium selenite (40 ng/ml) and NAC (60 µg/ml), and Gentamycin (25 µg/ml)) and cultured at 37°C, in

8% CO<sub>2</sub>. For clonal analysis of retrovirally-infected explants the culture medium was supplemented with 10% heat inactivated fetal calf serum. The cell culture medium was refreshed every three to four days by replacing half of the culture medium with fresh medium. The cultures were supplemented with recombinant myristoylated Shh-N protein at 2  $\mu$ g/ml or Hh agonist 1.4 at 10 nM (Frank-Kamenetsky et al., 2002) (a kind gift of Curis). This agonist binds to and activates the Smoothened receptor and the agonist concentration used in these experiments was determined in dose response experiments to be optimal at stimulating retinal progenitor cell proliferation in explant cultures.

#### Retroviral infection

Within one hour of dissection and transfer to filters the explants were infected by adding 6  $\mu$ l of  $10^8$  CFU/ml SMO-M2- or eGFP-retroviruses on top of the explant. After one hour, an additional 6  $\mu$ l of virus was added to the top of the explant. For clonal analysis, the virus stock was diluted to  $10^6$  CFU/ml and the retina was infected only once. For retroviral infection *in vivo*, 1  $\mu$ l  $10^6$  CFU/ml SMO-M2 or eGFP retrovirus solution was injected subretinally into the right eye of postnatal day one F1 pups from CD-1 x C57Bl/6 matings and the animals were sacrificed at P14. For clonal analysis, tissue was sectioned at 8  $\mu$ m (*in vitro* infection) and 16  $\mu$ m (*in vivo* infection) and stained with anti-GFP antibodies. A total of 529 SMO-M2 and 779 eGFP-infected clones were scored from 3 explants per retrovirus and a total of 360 SMO-M2 and 404 eGFP-infected clones were scored from 3 injected eyes per retrovirus.

#### In vitro electroporation

Electroporation was performed as according to Matsuda and Cepko (Matsuda and Cepko, 2004). E18.5 retinae were dissected and placed into a microelectroporation chamber containing DNA solution (0.8  $\mu$ g/ $\mu$ l in HBSS). Explants were electroporated with a 4:1 (w:w) mixture of SMO-M2-pCLE and pUB-GFP (Schorpp et al., 1996) or pUB-GFP alone and cultured on filters in 500  $\mu$ l of explant culture medium at 5% CO2 for 2 days.

#### Immunohistrochemistry

Cryosections (8 µm thickness) or dissociated cells were permeabilized with 70% ethanol, washed 3 times with PBS, and incubated in the primary antibody diluted in 10% goat serum in PBS for two hours at room temperature. The primary antibodies used in this study are anti-rhodopsin (B630 mouse monoclonal, (Rohlich et al., 1989)), anti-Syntaxin (HPC1 monoclonal antibody; Sigma), anti-recoverin (rabbit polyclonal; 1:1000 Chemicon International), anti-CRALBP (a kind gift of J. Saari, rabbit polyclonal, 1:2000), anti-PKC (mouse monoclonal, 1:100; BD Biosciences), anti-BrdU (mouse monoclonal, 1:100; Becton Dickinson), and anti-GFP (mouse monoclonal 1:100, rabbit polyclonal, 1:100; Molecular Probes). After 2 hours, the sections were washed three times with PBS, and then incubated with fluorescent conjugated secondary antibodies, diluted in 2% goat serum in PBS, for 1 hour at room temperature. To visualize nuclear morphology, cells were counterstained with fluorescent DNA-binding dye bisbenzimide (Hoechst) for 5 minutes and mounted with fluorescent mounting medium. Staining was visualized on a Zeiss fluorescent upright microscope.

#### In situ hybridization

In situ hybridization was performed as described previously (Wallace and Raff, 1999). Briefly, the sections were air-dried for at least 4 hours before overnight hybridization at 65°C in a moist chamber with digoxigenin-labelled antisense specific riboprobes (diluted 1:1000 in hybridization solution). Following the usual stringency washes and an alkaline phosphatase-conjugated anti-digoxigenin antibody treatment, staining in nitro blue tetrazolium/5-bromo-4-chloro-3-indoylphosphate revealed the blue color indicative of regions of specific *in situ* gene expression.

#### **ACKNOWLEDGMENTS**

We thank Genentech for the pCLE-SMO-M2 vector and Dr. C. Lois for the pUB-GFP plasmid and Curis Inc for recombinant Shh-N and the Hh agonist. This work was supported by operating grants to Dr. V. Wallace from the Stem Cell Network of Canada and the National Cancer Institute of Canada. V. Wallace is the recipient of a Canadian Institutes of Health New Investigator Award and Chuan Yu is a recipient of a studentship award form the Stem Cell Network of Canada and M. Pacal is a recipient of a fellowship from the Vision Science Research Program, University of Toronto.

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FIGURE LEGENDS

<u>Fig. 1</u>. SMO-M2 expression induces cell-autonomous Hh pathway activation in retinal cells. *A*, Schematic of the SMO-M2 and eGFP retroviral vectors. *B*, RT-PCR analysis for *Gli* and *HPRT* gene expression in C3H 10T1/2 cells 2 days after treatment with recombinant Shh-N protein or infection with the indicated retroviruses. P0 retina refers to RNA isolated from acutely dissected P0 mouse retina and serves as a positive control for *Gli* expression; control is a PCR reaction run without template. *C*, GFP immunofluoresence and *in situ* hybridization for *Smo*, *Gli* and *CyclinD1* mRNA in frozen sections of E18.5 mouse retinal explants 2 days following electroporation with pCLE-SMO-M2 + pUB-GFP or pUB-GFP. Note that signal for *Smo*, *Gli* and *CyclinD1* is upregulated in the GFP<sup>+</sup> regions of pCLE-SMO-M2 transfected explants.

Fig. 2. Cell autonomous activation of the Hh signaling pathway promotes RPC proliferation and increases clone size in the retina. *A*, The proportion of BrdU<sup>+</sup> cells among infected (GFP<sup>+</sup>) and uninfected (GFP<sup>-</sup>) cells from mouse E18.5 retinal explants 3 days after SMO-M2 or eGFP infection. BrdU was added for the last 20 hours of culture and the explants were dissociated and stained with anti-BrdU and anti-GFP antibodies. *B*, A representative clone from a SMO-M2-infected explant. E18.5 mouse retinal explants were infected with retrovirus, cultured for 7 days in serum-containing medium and processed for anti-GFP and Hoechst staining. Note that three cellular layers are well defined at this stage and that the radially-oriented clone consists of two cells (arrows) in the outer nuclear layer. Cell processes are indicated by arrowheads. GCL, ganglion cell layer; INL, inner nuclear layer, ONL, outer nuclear layer. *C*,*D*, Comparison of clone size in SMO-M2 and eGFP-infected retinas *in vitro* (C) and *in vivo* (D). For *in vitro* analyses, E18.5 mouse retinal explants were treated as described for *B*. For *in vivo* analysis, PO

mice were injected subretinally with retrovirus and analyzed at P14. Virally infected clones were identified by immunohistochemistry with anti-GFP antibodies. Data are presented at the mean +/- SD \* p < 0.05, \*\* p <0.005, two sided hypothesis testing.

<u>Fig. 3</u>. SMO-M2 expression promotes the development of cells with an INL identity. *A*,*B*, Distribution of clonal progeny between the INL and ONL in SMO-M2 and eGFP-infected retinas *in vitro* (A) and *in vivo* (B). Retroviral infections were performed, as described Fig. 2. At 7 days (*in vitro*) and 14 days (*in vivo*), the retinas were processed for immunohistochemistry with anti-GFP antibodies and clones were categorized according the position of cell bodies within the INL and ONL. Single GFP<sup>+</sup> cells were observed occasionally in the GCL; however, these cells were very rare and were not included in the analysis. Data are presented at the mean +/- SD \* p < 0.05, two sided hypothesis testing.

<u>Fig. 4</u>. Hh signaling in the perinatal retina promotes the development of cells with an INL identity at the expense of rods. *A*, Dissociated cell scoring of marker<sup>+</sup> cells in retinal explants. Mouse E18.5 retinal explants were untreated (control) or treated with Hh agonist for 10 days and the proportions of cells staining with the indicated markers was quantified. *B*, Dissociated cell scoring of marker<sup>+</sup> cells amongst the infected (GFP<sup>+</sup>) and uninfected (GFP<sup>-</sup>) cohort in retinal explants. Mouse E18.5 retinal explants were infected with SMO-M2 or eGFP retroviruses and 10 days later were processed for immunohistochemistry with anti-GFP and cell type specific markers, as described above. Data are presented at the mean +/- SD \* p < 0.05, two sided hypothesis testing.

<u>Fig. 5</u>. Effects of the cell-autonomous activation of the Hh signaling pathway on the rod photoreceptors. *A*,*B*, Dissociated cell scoring for rhodopsin<sup>+</sup> cells in retrovirus-infected (GFP<sup>+</sup>) (A) and uninfected (GFP<sup>-</sup>) (B) cells in mouse E18.5 retinal explants 7 and 10 days after infection. *C*,*D*, Immunohistochemistry for GFP and rhodopsin in retinal explants infected with the indicated viruses and cultured under serum-free conditions for 7 (C) and 10 (D) days. Central, optic nerve head region of the explant; Peripheral, edge of the explant. GCL, ganglion cell layer; INL, inner nuclear layer, ONL, outer nuclear layer, DIV, days *in vitro*. \*indicates regions where rhodopsin expression is reduced. Data are presented at the mean +/- SD \* p < 0.05, \*\* p <0.005, two sided hypothesis testing.

<u>Fig. 6</u>. SMO-M2 expression induces a cell non-autonomous inhibition of rod differentiation. *A*, Identification of rods on the basis of nuclear morphology. Mouse E18.5 retinal explants were cultured for 10 days, dissociated into single cells and stained with anti-rhodopsin antibodies and Hoechst to reveal the pattern of heterochromatin in nuclei. Rhodopsin<sup>+</sup> cells have small nuclei with distinct clumps of heterochromatin (arrowheads). Also present in the photo are cells that exhibit the identical heterochromatin pattern, but that are rhodopsin<sup>-</sup> (arrows). Cells that exhibited this characteristic rod nuclear morphology belonged to the rhodopsin<sup>+</sup> and rhodopsin<sup>-</sup> subsets and were scored as rods for this analysis. Non-rod cells are easily distinguished by the larger nuclei and paler staining heterochromatin (\*). *B*,*C*, Dissociated cell scoring to quantify rods among infected (GFP<sup>+</sup>) and uninfected (GFP<sup>-</sup>) cells from mouse E18.5 retinal explants 10 days after infected with SMO-M2- or eGFP-retroviruses. Rods were identified by nuclear

morphology and by staining with anti-recoverin and anti-rhodopsin antibodies. Data are presented at the mean +/- SD \* p < 0.05, \*\* p <0.005, two sided hypothesis testing.













## Figure 4







D



# Figure 6

