The role of signaling pathway integration in Neurogenesis

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Authorization

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Abstract

Proper central nervous system development is critical for survival and depends on complex intracellular and extracellular signaling to regulate neural progenitor cell growth and differentiation; however, the mechanisms that mediate molecular crosstalk between pathways during neurogenesis are not fully understood. Here, we explored the integration of the Hedgehog (Hh) signaling pathway with the two critical developmental pathways, Receptor Tyrosine Kinase (RTK) and Notch signaling, in the growth and maintenance of neural progenitors in the developing neuroretina. We found combined and sustained RTK and Hh signaling was sufficient to establish long-term retinal progenitor cell (RPC) cultures and these cells maintained neurogenic and gliogenic, but not retinogenic, competence *in vitro* and *in vivo*. In addition, we identified crosstalk between Notch and Hh signaling, where Notch is required for Hh-mediated proliferation and Gli protein accumulation, and gain-of-function of Notch is sufficient to extend the window of Hh responsiveness in a subset of Müller glia. Both Hh-RPC monolayer establishment and Notch mediated Hh-responsiveness required *Gli2*. Taken together, we identified molecular cross-communication between the Hh pathway and two major pathways, Notch and RTK, during retinogenesis, advancing our understanding of mechanisms that influence Hh to control neural progenitor growth.

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List of Abbreviations

3D	3-dimensional
ADAM	A disintegrin and metalloprotease protein
AKT	V-Akt murine thymoma viral oncogene homolog
AR	Amphiregulin
Atoh	Atonal homolog
Ascl	Achaete-scute homolog
bHLH	Basic helix loop helix protein
bp	Base pair
BMP	Bone morphogenetic protein
Boc	Brother of CDO
BrdU	5-bromo-2-deoxyuridine
Brn	Pou class homeobox protein
CDO	Cell adhesion associated, oncogene regulated

c-Jun	Jun proto-oncogene
C-terminus	Carboxy-terminus
CB	Ciliary body
CcnD	Cyclin D
CcnE	Cyclin E
Cdc25	Cell division cycle 25
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
Chx	Ceh-10 Homeodomain-containing Homolog
ci	cubitus interruptus
ci^{Act}	Cubitus interruptus activator
ci^{Rep}	Cubitus interruptus repressor
CK	Case kinase
CKO	Conditional knockout
CNS	Central nervous system
COS	CV-1 in origin and carrying SV40 cell

	line
Cos2	Costal2
Ct	Cycle threshold
CRALBP	Cellular retinaldehyde binding protein
CRE	Cre recombinase
Crx	Cone-rod homoebox
CXCR	Chemokine receptor protein
DAPI	4’,6-diamidino-2-phenylindole
DAPT	N-[N-(3.5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DCL	Differentiated cell layer
Dhh	Desert hedgehog
DIV	Days *in vitro*
Dll	Delta-like ligand
DN-Maml	Dominant negative Master-mind like
dsRed	*Discosoma sp.* Red fluorescent protein
EdU	5-ethynyl-2’-deoxyuridine

EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FACS	Fluorescent activated cell sorting
FGF	Fibroblast growth factor
FBS	Fetal bovine serum
Fl	Full-length
Fu	Fused
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gas	Growth-arrest specific
GBS	Gli Binding Site
GC	Ganglion cell
GFAP	Glial fibrillary acidic protein
GFP	Green Fluorescent Protein
GRK	G protein-coupled receptor kinase
GS	Glutamate synthase
GSK	Glycogen synthase kinase

Glast	Glutamate aspartate transporter protein
Gli	Glioma-associated oncogene homolog
HB-EGF	Heparin-binding EGF-like growth factor
Hes	Homologues of Drosophila hairy and enhancer of splits
Hh	Hedgehog
Hh-Ag	Hedgehog Agonist
Hh-C	Hedgehog catalytic domain
Hh-N	Hedgehog signaling domain
Hh-RPC	Monolayer cultured RPCs
Hh-RPC^{GFP+}	GFP transfected cultured RPCs
HMG	High mobility group
HRP	Horseradish peroxidase protein
Id	Inhibitor of DNA binding protein
ICD	Intracellular domain
IHC	Immunohistochemistry

Ihh	Indian hedgehog
Ihog	Inhibitor of Hedgehog
IPL	Inner plexiform layer
INL	Inner nuclear layer
ISH	*In situ* hybridization
JNK	c-Jun N-terminal kinase
Kif	Kinesin family member
KO	Knockout
Luc	Luciferase
MAPK	Mitogen activated protein kinase
MDM2	Mouse double minute 2 homolog
MEK	MAPK/ERK kinase
Math	Mouse atonal homolog
MF	Morphogenetic Furrow
MMP	Matrix metalloprotease
Maml	Mastermind-like protein
miRNA	Micro RNA

Myc	V-Myc avian myelocytomatosis viral oncogene homology
NBL	Neuroblast layer
NCBI	National Center for Biotechnology Information
NeuroD	Neurogenic differentiation protein
Nestin	Neuroectodermal stem cell marker
NFL	Nerve fiber layer
NICD	Notch Intracellular Domain
Nrl	Neural Retinal Leucine Zipper
ONL	Outer nuclear layer
OPL	Outer plexiform layer
Or	Ocular retardation
OS	Outer segment layer
Otx	Orthodenticle homeobox
PACAP	Pituitary adenylate cyclase activate peptide

PDL	Poly D-lysine
pHH3	Phospho-histone 3
PC	Primary cilium
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phospholipase C γ
PN	Postnatal day
Ptch/Ptc	Patched
Pax	Paired box gene
qRT-PCR	Quantitative real-time PCR
RA	Retinoic acid
Rax	Retina and anterior neural fold homoebox
Rb	Retinoblastoma

RBPJκ	Recombination signal binding protein for immunoglobulin Kappa J region
Rep	Repressor
RGL	Retinal ganglion cell layer
RLU	Relative light units
RPC	Retinal Progenitor Cell
RPE	retinal pigment epithelium
RSK2	Ribosomal protein S6 kinase 2
RT	Real time
RTK	Receptor Tyrosine Kinase
SEM	Standard error of the mean
Shh	Sonic Hedgehog
Sufu	Suppressor of Fused
Six	Sine Oculis homeobox
Smo	Smoothened
SMOM2	Constitutively active mutant Smoothened

Smo-Ag	Smoothened Agonist
Sox	Sex determining region Y-box
STAT	Signal transducer and activator of transcription
TBST	Tris buffered saline with tween
TGF	Transforming growth factor
Tp53	Tumour suppressor p53
Tuj1	Neuron-specific class III beta-tubulin
UV	Ultraviolet ray
Vsx	Visual system homeobox
WNT	Wingless-type MMTV integration site family
WT	Wild type
Zf	Zinc finger

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Chapter 1:

Introduction

1.0 Introduction

The central nervous system (CNS), comprised of the brain, spinal cord and retina, is a critical organ system that integrates information from the environment to coordinate and influence the activity of all aspects of an organism. Proper early CNS development is critical for later function and survival, and a greater understanding of how this vital organ system develops could lead to cell based regenerative therapies and more effective disease treatments.

CNS development begins early during gastrulation, as the neuroepithelium is induced to differentiate from dorsal midline ectoderm by the earlier born notochord (Bertrand and Dahmane, 2006)(Figure 1-1). Mediated by key signaling molecules, a neural plate is formed that undergoes folding, cellular migration, convergent extension at critical hinge points, and fusion events to create a characteristic tube shaped structure, the neural tube, lined with a layer of rapidly dividing, pseudostratified neural stem cells, termed the neuroepithelium (Copp et al., 2003). Surrounding the neural tube is overlying surface ectoderm, which will give rise to the epidermis, and adjacent neural crest cells, formerly the cells that connected the neural plate and epidermal destined ectoderm tissue (Copp et al., 2003). The neural tube forms the primordia of the CNS and the anterior segment of the neural tube can be divided into 3 main parts - the forebrain, midbrain and hindbrain. The forebrain undergoes rapid division immediately after induction to create secondary vesicles that can be subdivided into the telecephalon and the diencephalon; these tissues will form the cerebral cortex, striatum, hypothalamus and thalamus of the adult brain, as well as the neural retina (Bertrand and Dahmane, 2006; Chow and Lang, 2001).

There are several critical cellular processes that occur during the induction and subsequent growth during CNS development that must be precisely controlled, and disruption of these delicate processes can have devastating consequences on the health of an organism (Copp et al., 2003). For example, failure of neural tube closure from the disruption of inductive signals results in insufficient brain tissue

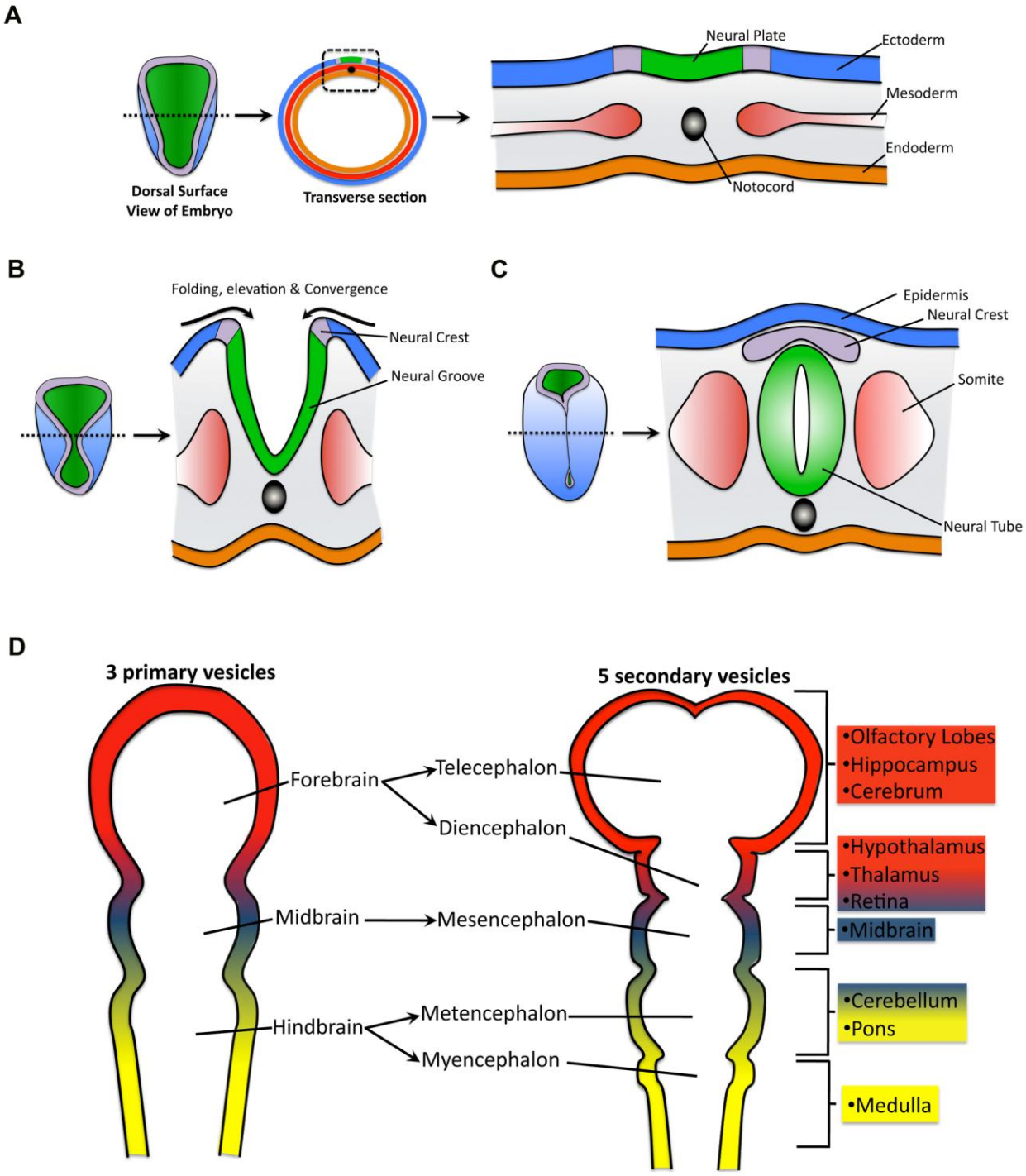
A
Dorsal Surface
View of Embryo
Transverse section
Neural Plate
Ectoderm
Mesoderm
Endoderm
Notocord
B
Folding, elevation & Convergence
Neural Crest
Neural Groove
C
Epidermis
Neural Crest
Somite
Neural Tube
D
3 primary vesicles
5 secondary vesicles
Forebrain
Telecephalon
Diencephalon
Midbrain
Mesencephalon
Hindbrain
Metencephalon
Myencephalon
•Olfactory Lobes
•Hippocampus
•Cerebrum
•Hypothalamus
•Thalamus
•Retina
•Midbrain
•Cerebellum
•Pons
•Medulla

Figure 1-1 Early development of the Central Nervous System (CNS). A) At the dorsal midline of the developing embryo, the notochord (black) signals to the overlying ectoderm layer (blue), forming the neural plate (green). B) A neural groove (green) is created by inductive signals, folding, elevation and covergent migration. C) Continued tissue remodeling and fusion creates the characteristic neural tube structure (green) and surrounding neural crest (purple), mesoderm derived somites (red) and surface ectoderm, which will give rise to the epidermis (blue). D) The anterior segment of the neural tube gives rise to the forebrain (red), midbrain (blue) and hindbrain (yellow), and following rapid expansion, 5 secondary vesicles are created that will give rise to the structures of the adult brain, including the neural retina. Adapted from (Gilbert, 2000).

at birth (Copp et al., 2003). One such example is the process of neurogenesis, whereby neurons are generated from multipotent neural progenitor cells, to produce all neurons within the CNS. Neurogenesis is complex and highly regulated, involving contributions from cell intrinsic regulators and environmental inputs and it is the co-ordination of these two inputs that control progenitor behaviour, but the intricacies of pathway integration during neurogenesis are still largely unknown.

1.1 The neural retina - a model for CNS development

The vertebrate eye is derived from surface ectoderm and neuroectoderm that evaginates bilaterally from the forming diencephalon during the onset of gastrulation (Chow and Lang, 2001; Sinn and Wittbrodt, 2014) (Figure 1-2). Mesenchymal and neural crest cells also make contributions to the eye, producing the extra ocular muscles, blood vessels, cornea stroma and connective tissues (Shyamala et al., 2015). Initially, neuroectoderm from the diencephalon moves toward the overlying, non-neural surface ectoderm, forming bilateral optic vesicles. The surface ectoderm tissue thickens, first creating the lens placode, followed by formation of a lens pit as it contacts the neuroectoderm-derived optic cup and lastly remodeling to create the lens vesicle that will eventually become the lens (Chow and Lang, 2001). At the same time the optic vesicle folds to create a double-layered optic cup – with the inner layer forming the neural retina and the outer layer producing the retinal pigment epithelium (Chow and Lang, 2001).

The retina is a highly specialized bilateral, contiguous extension of the CNS derived from the neural tube. Thus, similar to the brain, the embryonic neural retina is comprised of pseudostratified, polarized neuroepithelial tissue with progenitor cells that undergo interkinetic nuclear migration (Willardsen and Link, 2011), and many of the same inductive signaling molecules that influence progenitors of the retina play analogous roles during brain development (Guillemot, 2007). Because of its simple composition and ease of genetic manipulation *in vivo* and *in vitro*, the neural retina is a tractable model system to investigate the process of neurogenesis. Manipulations that severely disrupt the

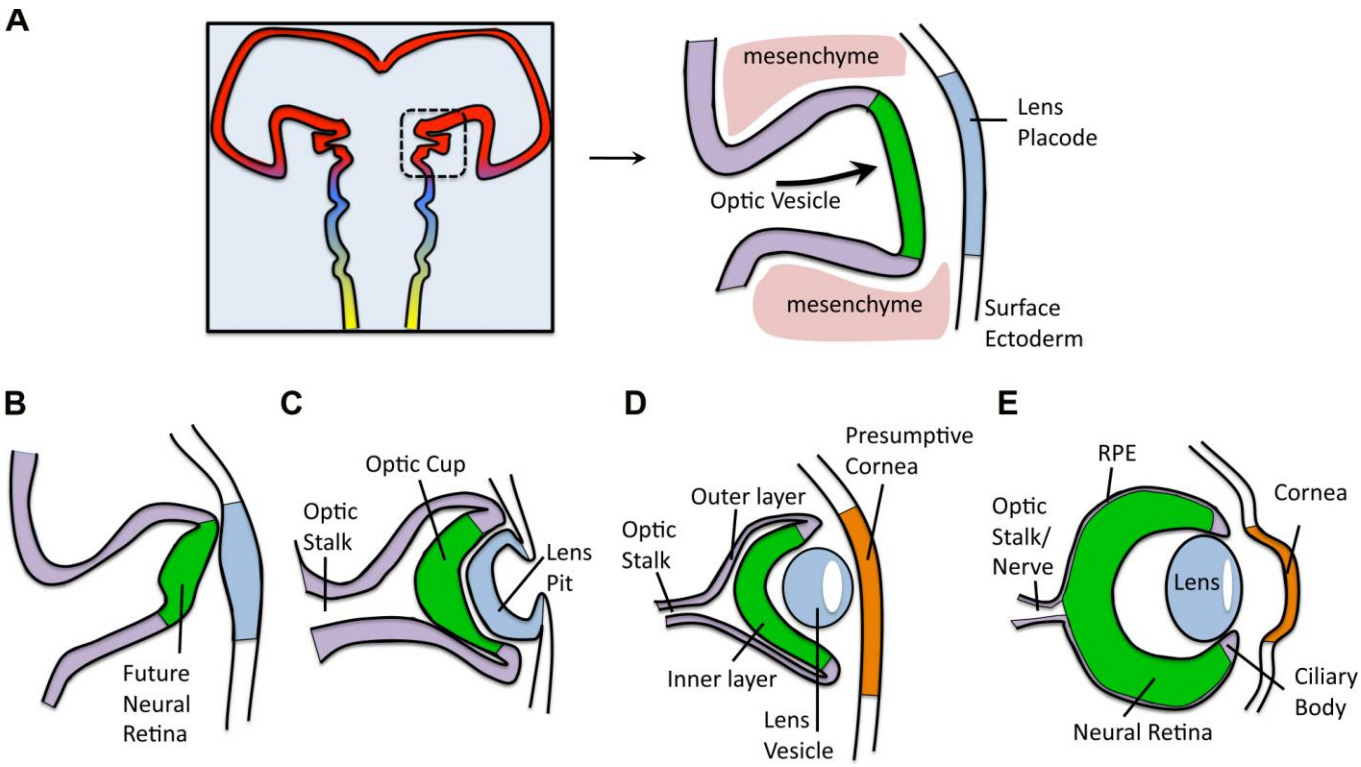
A
mesenchyme
Lens
Placode
Optic Vesicle
mesenchyme
Surface
Ectoderm
B
Future
Neural
Retina
C
Optic Cup
Optic
Stalk
Lens
Pit
D
Presumptive
Cornea
Outer layer
Optic
Stalk
Inner layer
Lens
Vesicle
E
RPE
Cornea
Optic
Stalk/
Nerve
Lens
Ciliary
Body
Neural Retina

Figure 1-2 Early eye development. A) Around embryonic day 8.5, the optic vesicle evaginates from the neuroectoderm (boxed area), extending toward the surface ectoderm. B) The presumptive neural retina (green) contacts the lens placode (light blue). C) The neural retina invaginates, forming the optic cup, while the lens placode also invagines to form the lens pit. D) As development continues, further tissue remodeling occurs - the lens vesicle separates from the surface ectoderm. The inner layer of the optic cup will give rise to the neural retina, while the outer portion will form the retinal pigment epithelium (RPE). (D). The fully formed eye containing an optic stalk region, with retinal ganglion cells projecting out of the eye, forming the optic nerve, a single cell layer of RPE surrounding the neural retina. Adapted from (Chow and Lang, 2001).

function of the retina are not lethal to the organism, where death may preclude the analysis of certain phenotypes in the brain. Similarly, the retina is an ideal model to investigate neurodegenerative disease because many of the mechanisms that adversely affect the brain are also shared by the retina, such as deposition of protein aggregates in the case of age-related degenerative disease (Sivak, 2013). In contrast to brain, which contains several neuronal and glial cell types, the retina has a limited number of mature cell types. Moreover, cellular migration and death do not contribute significantly to the total size of the cell population in the retina during development, both occurring late and involving few cells, compared to the cerebral cortex, where expansion is complicated by intricate patterning, migration and cell death (Green et al., 2003).

The light sensing mature retina is comprised of six neuronal and one glial cell type that are derived during development from a common pool of multipotent retinal progenitor cells (RPCs) (Figure 1-3)(Young, 1985). In rodents, retinal cells are born in two waves: an embryonic wave producing ganglion cells (GCs), cone photoreceptors, horizontal cells and some amacrine cells and a postnatal wave producing bipolar cells, Müller glia and the remaining amacrine cell pool. Rod photoreceptors are generated throughout retinal histogenesis (Young, 1985) and these different cell types are organized into three distinct laminated layers (Figure 1-3).

Throughout development, RPCs must make the decision to stay in cycle and continue to divide or differentiate into a mature neuron, and without proper control and balance of these two basic, binary choices the neural retina will fail to properly function or improperly regenerate. For example, early cell cycle exit would result in an insufficient progenitor pool and result in loss of later born cell types (Riesenberg et al., 2009), while failure to exit the cell cycle within the appropriate temporal window would result in loss of earlier cell types (Ohnuma et al., 1999). These seemingly simple choices made by an RPC involve a complex network of intrinsic regulators that are influenced by extrinsic inputs that elegantly come together to form the unique neural retina.

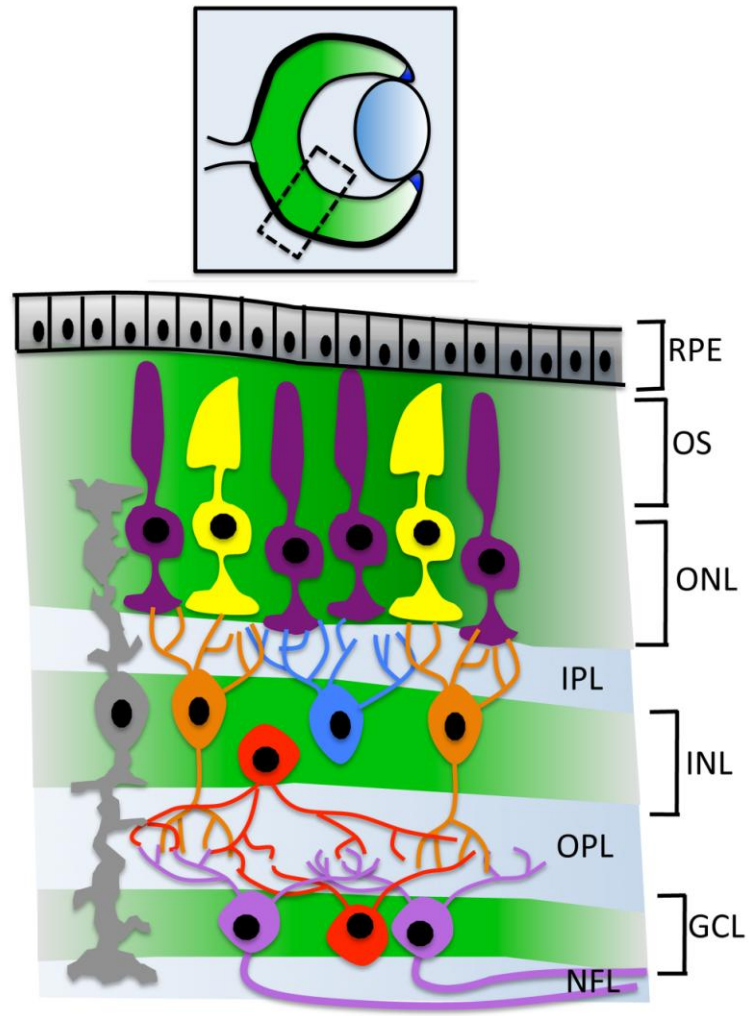
RPE
OS
ONL
IPL
INL
OPL
GCL
NFL
– Rod Photoreceptor
– Cone Photoreceptor
– Bipolar Cell
– Horizontal Cell
– Amacrine Cell
– Ganglion Cell
– Muller Glia

Figure 1-3 The mature neural retina. The adult vertebrate retina is comprised of 6 neuronal and 1 glial cell type arranged in an organized and layered composition. The photoreceptors make up the outer nuclear layer, Müller glia, amacrine, horizontal and bipolar cells comprise the inner nuclear layer and displaced amacrines and ganglion cells are found within the ganglion cell layer. RPE = retinal pigment epithelium, OS = outer segments, ONL = outer nuclear layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, GCL = ganglion cell layer, NFL = nerve fiber layer.

1.2 The invertebrate eye – a valuable model for retinal development

Vertebrate and invertebrate eye structures appear very different, but there are a number of interesting and important parallels between the development of flies, fish, frog, mouse and human species that have been exploited in the advancement of our knowledge of retinal development. One key model system is the invertebrate *Drosophila* retina, where it serves as a simple, yet elegant system to ask questions about how intrinsic and extrinsic cues regulate the growth and patterning of the retina. By integrating our understanding of retinal development across species, the field has been able to ask a number of questions that might not otherwise be explored, thus contributing to a deeper understanding of the mechanisms of retinal development in vertebrates.

In the developing *Drosophila* eye disc, mitogenic inputs in the surrounding microenvironment influence neural progenitor maintenance, growth and cell fate, not unlike vertebrate development. Retinal development begins at the third instar larval stage within the developing eye disc, when a furrow forms, known as the morphogenetic furrow (MF), which sweeps from posterior to anterior, marking the onset of photoreceptor differentiation, clustering and eye patterning (Cagan and Ready, 1989). Ahead of the advancing MF, cells remain in an undifferentiated, unorganized state, proliferating asynchronously (Cagan and Ready, 1989; Wolff and Ready, 1991). As the MF progresses, cells enter the furrow and arrest in G_1 phase of the cell cycle (Ready et al., 1976; Thomas et al., 1994). A few hours later, cells emerge posterior to the MF and organize into 2 groups: ommatidial precursor cells that will eventually form 5 of the 8 photoreceptors of the mature ommatidia, and undifferentiated surrounding cells (Baonza and Freeman, 2005; Ready et al., 1976). Undifferentiated surrounding cells not part of the ommatidial precursor cells undergo a final, synchronized, terminal round of division called the second mitotic wave before differentiating into the remaining photoreceptors, accessory cones and pigment cells (Cagan and Ready, 1989). Many general principles of intracellular signaling and the molecular signals that mediate them, including Hedgehog, Receptor Tyrosine Kinase and Notch signaling, have first been described

through genetic analysis of the *Drosophila* eye development and these signals are conserved during vertebrate retinal histogenesis.

1.3 Key Regulators of RPC fate and competence

As the vertebrate retina develops, RPCs proceed irreversibly through different competency states that are intrinsically defined by a number of key determinants of RPC fate, including multiple classes of transcription factors (Figure 1-4) (Bassett and Wallace, 2012; Cepko et al., 1996; Livesey and Cepko, 2001). The mechanism by which these different transcription factors regulate cell fate is varied. For instance, some intrinsic determinants act instructively, such as the gene neural retina leucine zipper *(Nrl)*, which is necessary and sufficient to direct the binary decision of rod over cone photoreceptor fates (Mears et al., 2001), while other genes do not directly confer specific cell types, but rather act permissively to regulate the competence of RPCs to produce specific lineages. *Atoh7* is a key competence factor that is required for GC determination (Brown et al., 2001; Wang et al., 2001), but *Atoh7* expression does not instruct cells to adopt a GC fate (Feng et al., 2010), suggesting other factors contribute to GC fates as well. Indeed, retinal cell fate determination genes function in complex genetic networks and have multiple roles in cellular subtype specification, depending on which combinations of transcription factors are present. These particular combinations, or 'transcriptional code', of transcription factors underlie the timing and type of neuronal cell produced from the common pool of RPCs. Forced co-expression of *NeuroD1* or *NeuroD4* with *Pax6* or *Six3* promotes amacrine cell genesis, while expression of each of these factors alone is insufficient for amacrine production (Inoue et al., 2002). Temporal RPC competence, the timing of cell type production, also is dependent upon specific factors within the retina. The zinc finger proteins *Ikaros* and *Casz1*, for example, are required for the generation of early and late retinal cell types, respectively (Elliott et al., 2008; Mattar et al., 2015). The network of genetic factors critical for cell fate specification and progenitor competence are becoming clearer; however, an

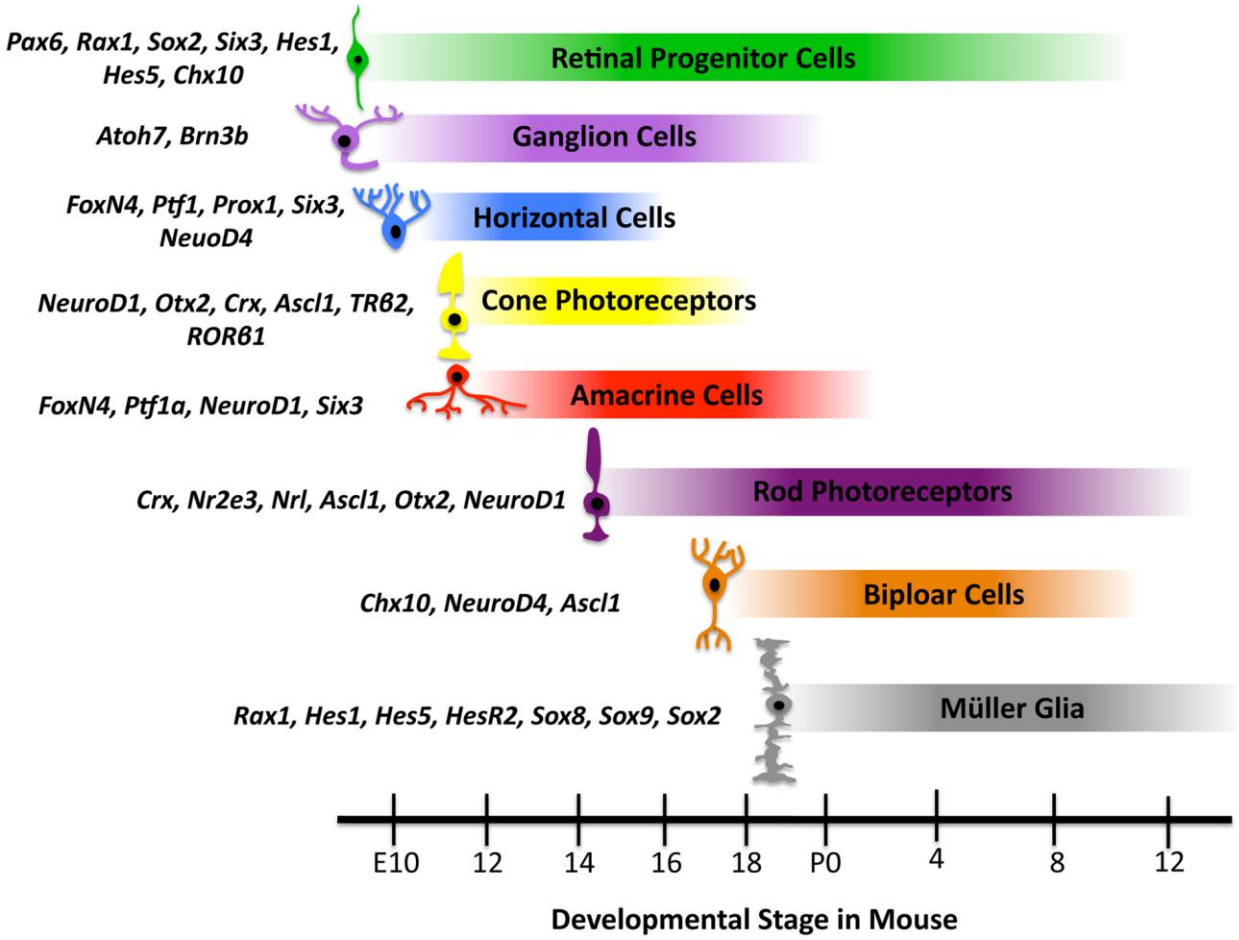
Pax6, Rax1, Sox2, Six3, Hes1, Hes5, Chx10
Retinal Progenitor Cells
Atoh7, Brn3b
Ganglion Cells
FoxN4, Ptf1, Prox1, Six3, NeuoD4
Horizontal Cells
NeuroD1, Otx2, Crx, Ascl1, TRß2, RORß1
Cone Photoreceptors
FoxN4, Ptf1a, NeuroD1, Six3
Amacrine Cells
Crx, Nr2e3, Nrl, Ascl1, Otx2, NeuroD1
Rod Photoreceptors
Chx10, NeuroD4, Ascl1
Biploar Cells
Rax1, Hes1, Hes5, HesR2, Sox8, Sox9, Sox2
Müller Glia
E10
12
14
16
18
P0
4
8
12
Developmental Stage in Mouse

Figure 1-4 Retinal cell types are generated through a conserved birth order. Retinal progenitor (RPC) birth order is conserved, where the competence to produce a particular retinal cell type is regulated by intrinsic transcription factors that are influenced by extrinsic cues and RPC competence shifts over the course of retinal development, only producing certain cells at a particular developmental time. Adapted from (Bassett and Wallace, 2012).

equally important question that precedes cell fate choices made by progenitor cells is: how is the progenitor pool size regulated?

1.4 Critical Regulators of RPC proliferation, maintenance and differentiation

The size of the eye and the timing of differentiation are important for normal histogenesis in the retina and genes involved in the regulation of cell cycle kinetics and temporal differentiation of RPCs are critical; however, only a few key players have been shown to be integral in these processes and impact the cell cycle in various ways throughout retinohistogenesis.

CcnD1 is the critical and predominant D-type cyclin expressed in RPCs (Sicinski et al., 1995). *CcnD1* couples with cyclin-dependent kinases (CDKs) 4 and 6 to promote G_1 cell cycle progression resulting in CDK2 activity, inactivation of retinoblastoma proteins, and DNA synthesis (Kozar and Sicinski, 2005; Musgrove, 2006). Germline inactivation of *Ccnd1* promotes cell cycle exit and severely impairs RPC proliferation leading to hypocellular retinas in mice (Das et al., 2009; Fantl et al., 1995; Sicinski et al., 1995) and zebrafish embryos injected with *Ccnd1* morpholinos similarly have small eye phenotype (Duffy et al., 2005). In addition to promoting proliferation by activating CDK4/6, another role of *CcnD1* is to inhibit accumulation of the primary CDK-inhibitor (CDKI) in the vertebrate retina, $p27^{kip1}$ (Geng et al., 2001). $p27^{kip1}$ is critical in the maintenance of RPCs, where it acts to restrict progenitor growth by inhibiting CDK2 (Mitsuhashi et al., 2001). Consistently, $p27^{Kip1}$-null mice display retinal dysplasia (Nakayama et al., 1996) and persistent proliferation of late RPCs (Dyer and Cepko, 2001; Levine et al., 2000). A second CDKI member in the Kip class of proteins, $p57^{Kip2}$, also modulates RPC maintenance and proliferation, such that it is necessary and sufficient for normal cell cycle exit of early, embryonic RPCs, but not later born, postnatal progenitors (Dyer and Cepko, 2000b; Dyer and Cepko, 2001). Similarly, loss of a second CDKI in the retina, $p19^{Ink4d}$, results in ectopic RPC proliferation, and $p27^{kip1}$; $p19^{Ink4d}$ double null retinas display a synergistic increase in this ectopic RPC

proliferation, suggesting a cooperative role between both CDKIs to control RPC growth and cell cycle exit (Cunningham et al., 2002). Together, these cell cycle genes ultimately regulate the size of the retina, but have modest effects upon cellular fates.

The Retinoblastoma (*Rb*) gene family includes three pocket protein members, *Rb/p105*, *p107* and *Rb2/p130*, and collectively these genes represent another important regulator of cell cycle in the retina. However, unlike the previously mentioned cell cycle genes, pocket proteins are required in differentiating precursor cells of the retina, where they negatively regulate the cell cycle re-entry and allow cells to exit the cell cycle (Chen et al., 2004). In G_1 phase of the cell cycle, hypophosphorylated Rb functions to suppress the activity of E2F transcription factors, thus inhibiting cell cycle progression and promoting exit (Weinberg, 1995). Upon phosphorylation by CDKs, E2Fs are released from Rb and cells are allowed to progress past a restriction point into the remaining stages of G_1, S, and G_2 phases of the cell cycle (Weinberg, 1995). In humans, hemizygosity for the RB gene predisposes individuals to retinoblastoma, a childhood tumour of the eye, as well as a multitude of cancers in other tissues (Giacinti and Giordano, 2006; Robanus-Maandag et al., 1998). In contrast to humans, mice heterozygous for Rb do not develop retinoblastoma type tumours (Classon and Harlow, 2002). However, loss of *p107* or *p130* in combination with *Rb* in mice results in retinal tumour growth, indicating that *p107/p130* and *Rb* act redundantly as tumour suppressors in the mouse (Chen et al., 2004; MacPherson et al., 2004; Robanus-Maandag et al., 1998). Although all retinal cell types are affected by mutations in the RB gene family, the cell of tumour origin is thought to arise from death resistant precursor cells in the inner nuclear layer (i.e. amacrine, horizontal and Müller glia cells) that is able to escape terminal differentiation in the absence of Rb and p107 or p130 (Chen et al., 2004; MacPherson et al., 2004; Robanus-Maandag et al., 1998). In humans, early cone photoreceptor precursor cells have been shown to acquire tumorigenic growth capacity in the absence of RB (Xu et al., 2014).

In addition to G_1 cell cycle components controlling RPC maintenance, a number of non-classical cell cycle regulators have also been implicated in the regulation of progenitor proliferation and

maintenance. For example, early loss of *Pax6, Rax1, Hes1, Sox2* or *Chx10* results in gross eye abnormalities due to severe growth restrictions, including microphthalmia (small eye) and anopthalmia (no eye) (Burmeister et al., 1996; Danno et al., 2008; Hill et al., 1991; Mathers et al., 1997; Tomita et al., 1996). As well as playing an early role in eye field specification, many of these transcription factors have a dual function later in development that is context dependent, where they function to regulate neuronal cell fate and play a critical role in maintaining the pool of multipotent RPCs (Agathocleous and Harris, 2009).

The homeodomain transcription factor *Pax6* is one such factor that is critical for maintaining RPCs following eyecup formation during development, and conditional inactivation of *Pax6* from RPCs causes hypocellularity as a result of decreased progenitor proliferation (Farhy et al., 2013; Marquardt et al., 2001; Oron-Karni et al., 2008). Persisting *Pax6*-null progenitors lose their multipotency and adopt an amacrine cell fate (Farhy et al., 2013; Marquardt et al., 2001; Oron-Karni et al., 2008).

Another critical group of transcription factors in the maintenance of RPCs are the basic helix-loop-helix homologues of *Drosophila* hairy and enhancer of split (Hes) gene family. These genes are essential effectors of Notch signaling that antagonize proneural genes by repressing transcription through DNA binding and recruitment of co-repressors (Kageyama et al., 2008; Kageyama et al., 2009). Hes factors can also act to passively repress transcription by forming non-DNA binding heterodimers with bHLH activators, thus inhibiting their transcriptional activity (Kageyama et al., 2008). In the retina, *Hes1* negatively regulates neurogenesis and maintains RPCs by repressing the activation of pro-neural genes, such as *Atoh7* and *Ascl1* (Chen et al., 1997). Deletion of *Hes1* in RPCs results in premature cell cycle exit (Lee et al., 2005; Tomita et al., 1996) and enhanced neurogenesis of early cell types in the retina (Tomita et al., 1996), while forced activation of *Hes1* inhibits neural differentiation in RPCs (Furukawa et al., 2000). Postnatally, *Hes1* continues to inhibit neural differentiation, biasing RPCs toward a Müller glial fate (Furukawa et al., 2000).

The SOXB1-high mobility group (HMG) box transcription factor *Sox2* is expressed throughout the developing neural progenitor populations of the CNS, where it is necessary to maintain cellular identity (Guillemot, 2007). Similarly in the retina, *Sox2* is required to maintain RPCs such that conditional deletion results in precocious RPC cell cycle exit and neural differentiation (Taranova et al., 2006). Hypomorphic mutations demonstrate that the concentrations of SOX2 are critical in neuronal progenitor cells (Cavallaro et al., 2008; Ferri et al., 2004; Taranova et al., 2006) and haplo-insufficiency for SOX2 accounts for approximately 10% of human anophthalmia and severe microphthalmia (Fantes et al., 2003; Hagstrom et al., 2005). Later during retinal development, *Sox2* expression is essential in Müller glia to maintain their progenitor-like characteristics through a Notch-dependent mechanism (Surzenko et al., 2013). These data point to a dose-dependent role for *Sox2* in maintaining RPC competence, similar its general role in maintaining multipotent potential throughout the CNS.

The paired-like homeobox transcription factor *Rax1* is another critically important factor for eye development and RPC proliferation (Bailey et al., 2004; Mathers et al., 1997; Zhang et al., 2000). Early *Rax1* ablation in vertebrates leads to loss of eye field specification (Bailey et al., 2004; Zhang et al., 2000) and misexpression of *Rax1* in *Xenopus* embryos causes formation of ectopic RPE and extra retinal tissue (Mathers et al., 1997) as a result of hyperproliferation due to delayed cell cycle exit and increased mitotic activity of RPCs (Casarosa et al., 2003; Terada et al., 2006). *Rax1*-mediated RPC hyperproliferation is attributed to activation of cell cycle genes *CcnD1* and *Myc,* and nucleosome binding protein *Xhmgb3*, as well as repression of *p27Xic* expression (Andreazzoli et al., 2003; Casarosa et al., 2003; Terada et al., 2006). In late stage development, *Rax1* functions in the determination of Müller glia and photoreceptors via regulation of *Notch1/Hes1* and *Otx2*, respectively (Furukawa et al., 2000; Nelson et al., 2009; Pan et al., 2010).

Chx10, another homeobox gene, is expressed in all RPCs throughout vertebrate retinogenesis (Chen and Cepko, 2000; Levine et al., 1997a; Liu et al., 1994; Passini et al., 1997) and later becomes restricted to bipolar cells in the mature retina (Burmeister et al., 1996). The ocular retardation (Or)

mutant mouse is a spontaneous, naturally occurring null mutation in the *Chx10* gene and these mice have severely thin, hypocellular perinatal retina from profound defects in RPC proliferation (Burmeister et al., 1996), implicating *Chx10* as an integral regulator of RPC maintenance, as well as highlighting a dual role in bipolar interneuron specification and maintenance. This function is conserved among fish and mice, since morpholino mediated knockdown of the zebrafish homolog of *Chx10* also results in a small eye phenotype (Barabino et al., 1997). RPCs lacking *Chx10* displayed longer cell cycle times compared to wildtype, which correlated with an increase in G_1 phase of the cell cycle (Green et al., 2003). Using double mutant mouse models, Green *et. al.* elegantly demonstrated that *Chx10* influences RPC proliferation by regulating post-transcriptional levels of $p27^{kip1}$ through a *CcnD1*-dependent mechanism (Green et al., 2003). Interestingly, bipolar development was unaffected by modulation of *p27kip1* in combination with *Chx10* deletion (Green et al., 2003), suggesting *Chx10* mediates bipolar specification and RPC proliferation via two distinct mechanisms. Not only does *Chx10* regulate RPC proliferation by directly influencing G_1 type cell cycle proteins, but *Chx10* also has been shown to impact the timing and magnitude of Hedgehog signaling (Hh) (Sigulinsky et al., 2008). An alteration in Hh signaling in Chx10-null retinas was shown to be a consequence of ligand availability by delayed embryonic GC differentiation (Sigulinsky et al., 2008), revealing multiple intrinsic and extrinsic roles for *Chx10* in the regulation of RPC proliferation and maintenance.

1.5 Extrinsic control of RPCs

As well as intrinsic regulators, extrinsic signals in the surrounding environment play a critical role in regulating neural progenitor behaviour during CNS and retinal development. Environmental inputs are not instructive for specific cellular lineages in developing RPCs and all cell lineages develop when cultured clonally outside of the retina (Cayouette et al., 2003; Gomes et al., 2011); however, extrinsic cues can influence cell fate choices through feedback inhibition mechanisms (Ma et al., 2007; Waid and

McLoon, 1998). For example, ligands secreted from mature neurons, such as Shh in the case of RGCs, negatively influences the development of additional neurons of the same type from the RPC pool (Belliveau and Cepko, 1999; Waid and McLoon, 1998).

On the other hand, extrinsic cues are necessary for the regulation of RPC proliferation, where dividing cells require mitogenic signals to progress through the cell cycle (Pardee, 1974; Pardee, 1989; Planas-Silva and Weinberg, 1997). Through this mechanism, extrinsic cues may also influence cell fate indirectly by impacting the timing of cell cycle exit (Locker et al., 2006; Sakagami et al., 2009; Wang et al., 2005). Thus, extrinsic cues are key to understanding proliferative and cell fate choices progenitors make during neurogenesis and in the case of the vertebrate retina these pathways include: Transforming Growth Factor ß (TGF-ß) signaling, Bone Morphogenic Proteins (BMPs), Retinoic acid, WNT signaling, Receptor Tyrosine Kinase signaling (RTK), Notch pathway and Hedgehog pathways (Urban and Guillemot, 2014).

2.0 Hedgehog Pathway - Overview

The evolutionarily conserved secreted morphogens Hedgehog (Hh) play diverse and context specific roles in multiple, critical developmental processes and disease (Ingham and Placzek, 2006). Historically, hh was first discovered in *Drosophila* as an embryonic patterning molecule, where hh mutations result in a spikey cuticle phenotype and thus inspired the 'hedgehog' name (Nusslein-Volhard and Wieschaus, 1980). In vertebrates because of genome duplication events, there are multiple Hh subgroups: Sonic *(Shh)*, Indian *(Ihh)* and Desert *(Dhh)* (Varjosalo and Taipale, 2008). Zebrafish and frog have a total of five and four *Hh* genes, respectively, due to additional gene duplication events (Takabatake et al., 1997). Birds and mammals, on the other hand, have only one *Hh* gene in each subgroup (Ingham and Placzek, 2006). Hh has several well-established roles during development, including: control of tissue patterning and cell proliferation, differentiation, survival and migration

(Ingham and McMahon, 2001), and while all Hh proteins have a similar physiological effect, the differences and versatility of Hh function are a result of diverse patterns of expression (Varjosalo and Taipale, 2008). *Dhh* regulates Sertolli and Leydig cell development of the gonads, influencing spermatogenesis and androgen secretion (Bitgood et al., 1996; Yao et al., 2002), as well as normal myelination, survival and function of the peripheral nervous system (Sharghi-Namini et al., 2006). *Ihh,* in combination with *Shh*, plays a key role in intestinal development by controlling epithelial-mesenchymal cross-talk that patterns and maintains the developing gut (van den Brink, 2007), and *Ihh* is essential for osteoblast and chondrocyte proliferation and differentiation during vertebrate endochondral bone formation (Long et al., 2001; Maeda et al., 2007; St-Jacques et al., 1999; Vortkamp et al., 1996). *Shh* is the most widely expressed of the three Hh proteins in vertebrates and is involved in the development of a multitude of tissues and organs, including the gut, limb, lung and in the notochord, neural tube and retina of the developing CNS (Briscoe and Therond, 2013).

2.1 Hh signaling and Disease

Mutations in the Hh pathway are implicated in a number of congenital abnormalities, such as pure gonadal dysgenesis, brachydactyly, holoprosencephaly, and Gorlin syndrome (Nieuwenhuis and Hui, 2005), and aberrant Hh signaling is causative for various forms of human cancer (Stecca and Ruiz, 2010). For example, medulloblastoma is a cancerous growth that originates from the cerebellum and approximately 35% of these tumours are a result of gain or loss-of-function mutations in the pathway that increase Hh activity (Guessous et al., 2008; Taylor et al., 2002; Zurawel et al., 2000). The exact mechanisms leading to medulloblastoma formation and other types of cancer remain elusive, but uncontrolled, cancerous growth is consistent with Hhs function as a mitogen in various contexts (Agathocleous et al., 2007).

2.2 Hh ligand

All Hh proteins undergo post-translational modification that ultimately regulates the activity and spread of mature Hh ligand from producing cells. During Hh biosynthesis, full-length Hh proteins are autoproteolytically cleaved, catalyzed by the C-terminal domain, to generate an N-terminal peptide that is then dually modified by the addition of cholesterol and palmitate to the C-terminus and N-terminus regions, respectively (Mann and Beachy, 2004; Steinhauer and Treisman, 2009)(Figure 1-5). This mature, modified N-terminal portion of Hh mediates all Hh signaling activity (Briscoe and Therond, 2013; Chamoun et al., 2001; Lee et al., 2001). Inhibition of the addition of the palmitate group results in loss of ligand signaling potential in vertebrates and *Drosophila* (Callejo et al., 2006; Dawber et al., 2005; Gallet et al., 2003; Pepinsky et al., 1998), and blocking SHH cleavage in humans results in holoprosencephally from loss of pathway function (Maity et al., 2005), highlighting the essential role for these post-translation modifications in the reception and downstream activation of the Hh pathway.

2.3 Hh Signal Transduction

The critical signaling components of the Hh pathway are well conserved from flies to humans and pathway activation is initiated by Hh ligand binding to the 12-pass transmembrane receptor Patched (Ptch) (Chen and Struhl, 1996; Fuse et al., 1999)(Figure 1-6). Functionally redundant co-receptors modulate the response of Hh signaling by binding ligand, and at least one of these co-receptors is required for Hh pathway activation but these proteins either lack intracellular regions or do not appear to transmit Hh signaling (McCabe and Leahy, 2014; Robbins et al., 2012). In the absence of ligand, Ptch constitutively represses the 7-pass transmembrane, G protein-coupled receptor Smoothened (Smo), which is responsible for intracellular transmission of Hh signal (Chen and Struhl, 1998; Zheng et al., 2010). Upon ligand binding, Ptch mediated Smo repression is alleviated through phosphorylation of the

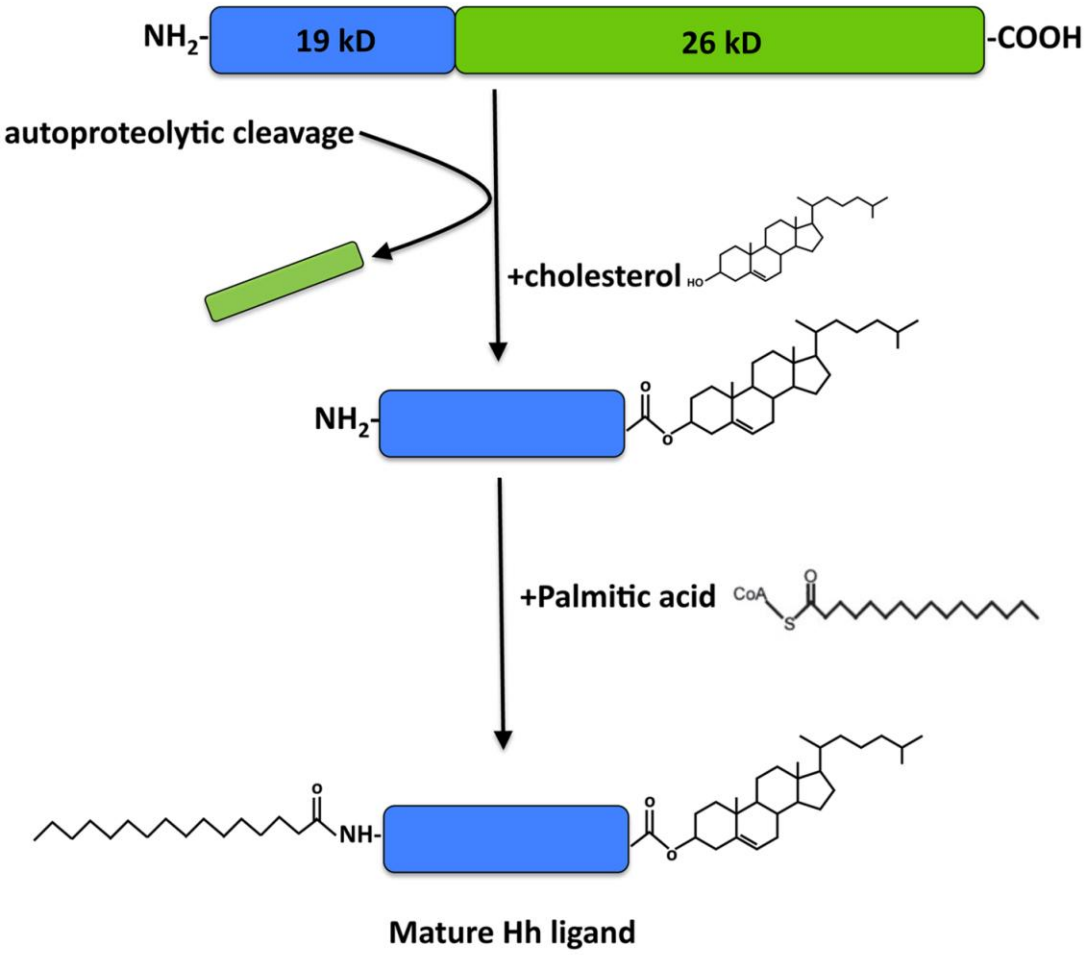
NH2-
19 kD
26 kD
-COOH
autoproteolytic cleavage
+cholesterol
HO
NH2-
O
O
+Palmitic acid
CoA
O
S
NH-
O
O
Mature Hh ligand

Figure 1-5 Hedgehog protein is posttranslationally processed and modified to form mature ligand. Full-length Hh precursor protein is autoproteolytically cleaved to generate an Hh-N fragment that is further modified by the addition of cholesterol and palmitic acid to the C- and N-terminus, respectively, forming the mature Hh ligand. Adapted from (Varjosalo and Taipale, 2008).

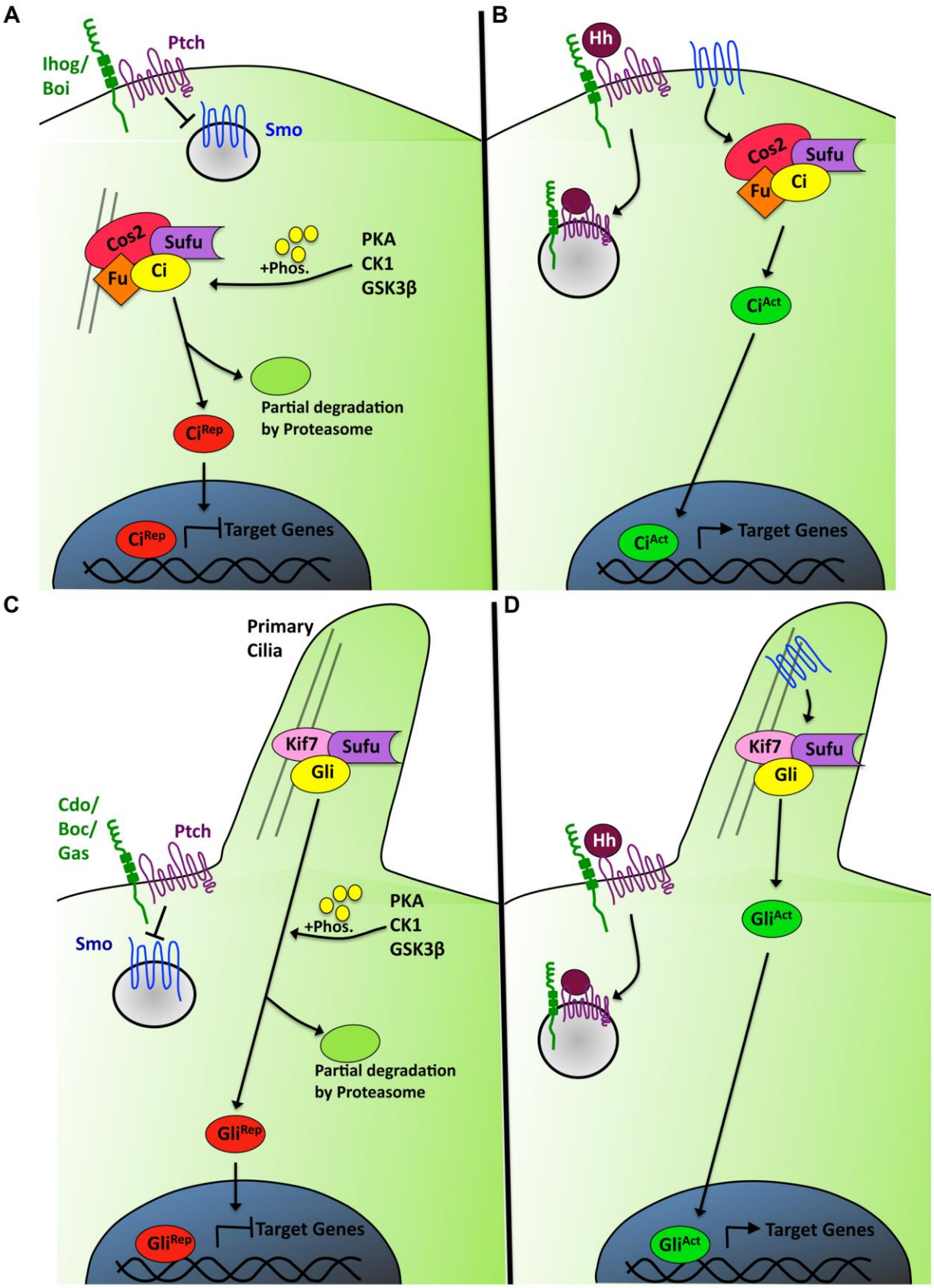
A
Ihog/
Boi
Ptch
Smo
Cos2
Sufu
Fu
Ci
+Phos.
PKA
CK1
GSK3β
Partial degradation
by Proteasome
CiRep
CiRep
Target Genes
B
Hh
Cos2
Sufu
Fu
Ci
CiAct
CiAct
Target Genes
C
Primary
Cilia
Kif7
Sufu
Gli
Cdo/
Boc/
Gas
Ptch
Smo
+Phos.
PKA
CK1
GSK3β
Partial degradation
by Proteasome
GliRep
GliRep
Target Genes
D
Hh
Kif7
Sufu
Gli
GliAct
GliAct
Target Genes

Figure 1-6 Hedgehog signal transduction in *Drosophila* and vertebrates. A) In the absence of Hh ligand, Ptch inhibits Smo activation and membrane localization, retaining Smo in intracellular vesicles. Full-length Ci is bound by Cos2, Fu and Sufu in a microtubule-associated complex, which promotes the phosphorylation of Ci by CK1, GSK3 and PKA and subsequent proteasomal processing of Ci into a transcriptional repressor (Ci^{Rep} , red). B) Upon binding of Hh to Ptch and an Ihog co-receptor (Ihog or Boi), the receptor-ligand complex is internalized and Smo is activated and translocated to the plasma membrane, interacting with Cos2 and resulting in activation of the full-length transcriptional activator form of Ci (Ci^{Act} , green). Unlike the invertebrate system, Hh signal transduction in vertebrates involves the non-motile microtubule based primary cilia. C) In the absence of ligand, Ptch inhibits Smo, similar to the Drosophila model. Kif7 and Sufu function to sequester Gli proteins, allowing their phosphorylation by PKA, GSK3 and CK1 and mediate their cleavage into truncated transcriptional repressors that actively inhibit Hh target genes. D) In response to ligand binding to Ptch and a co-receptor (Cdo, boc or Gas1), Smo is activated and translocates to the primary cilia to regulate Gli processing into full-length activator forms that enter the nucleus and upregulate Hh target genes.

cytoplasmic tail of Smo (Jia et al., 2004; Zhang et al., 2004), inducing an activating conformational change (Zhao et al., 2007). Actvated Smo translocates from cytoplasmic vesicles to the primary cilium (PC) in vertebrates (Rohatgi et al., 2007) or the cell surface in *Drosophila* (Zhang et al., 2004), initiating a signaling cascade that ultimately converges on the family of Glioma-associated Oncogene (Gli*)* zinc finger transcription factors (Nozawa et al., 2013).

2.4 Gli Family Transcription Factors

Gli proteins are key, bifunctional DNA binding transcription factors that can both repress and activate the transcription of target genes downstream of an active Hh signaling cascade (Figure 1-6) (Hui and Angers, 2011). *Drosophila* have a single Gli family gene, cubitus interruptos (*ci*), that mediates downstream Hh signaling (Methot and Basler, 2001; Orenic et al., 1990). Ci protein contains 3 distinct domains: a zinc finger DNA binding region, N-terminal repressor domain, and a C-terminal activation domain (Dai et al., 1999; Sasaki et al., 1999) (Figure 1-7) and in the absence of Hh ligand, ci is proteolytically processed into a truncated form lacking the activator domain (ci^{Rep}) that functions as a transcriptional repressor (Aza-Blanc et al., 1997; Sasaki et al., 1999). Proteolytic processing of ci is regulated by the ubiquitin proteasome, which requires a series of enzymatic reactions mediated by sequential phosphorylation by protein kinase A (PKA), glycogen synthase kinase-3 (GSK3) and casein kinase 1 (CK1), ultimately resulting in partial degradation of the C-terminal half of ci (Robbins et al., 2012). Conversely, when the Hh pathway is stimulated, ci^{Rep} processing is inhibited, allowing accumulation of the full-length transcriptional activator form of ci (ci^{Act})(Figure 1-6)(Aza-Blanc et al., 1997). It is the balance between ci^{Act} and ci^{Rep} forms that dictate transcriptional activation or repression of Hh target genes through the binding of the same promoter sites, maintaining a steady state of gene regulation even in the absence of Hh (Robbins et al., 2012; Ruiz i Altaba, 1999).

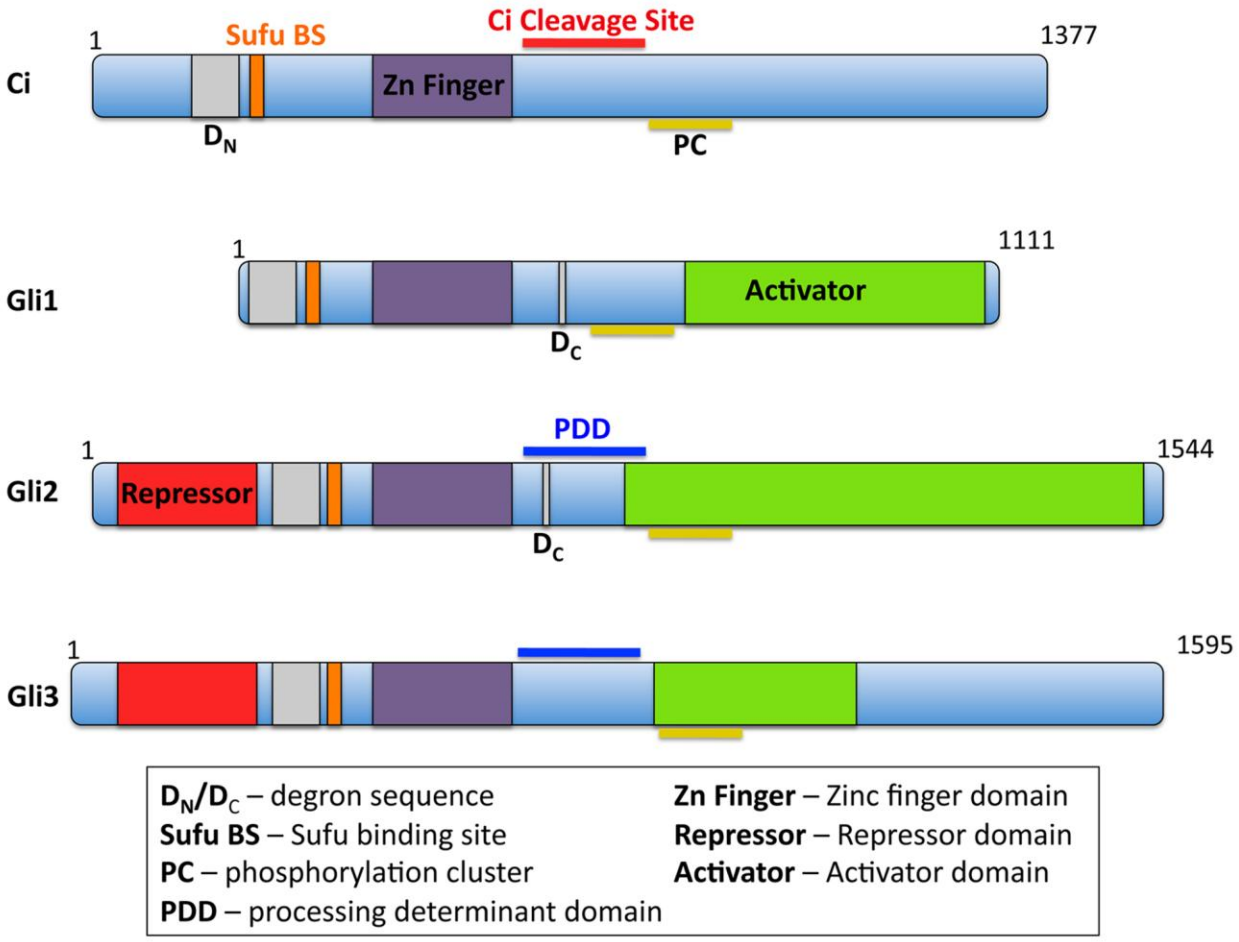
Ci
1
Sufu BS
Ci Cleavage Site
1377
Zn Finger
D_N
PC
Gli1
1
1111
Activator
D_C
Gli2
1
PDD
1544
Repressor
D_C
Gli3
1
1595
D_N/D_C – degron sequence
Sufu BS – Sufu binding site
PC – phosphorylation cluster
PDD – processing determinant domain
Zn Finger – Zinc finger domain
Repressor – Repressor domain
Activator – Activator domain

Figure 1-7 The family of Gli transcription factors. Schematic representation of *Drosophila* Cubitus interruptus (ci) and the 3 vertebrate Gli proteins (Gli1, Gli2 and Gli3) showing the various protein regulatory domains and motifs. Approximate amino acid number is shown above each protein. Adapted from (Hui and Angers, 2011).

In the mammalian system, the functions of Gli proteins are diversified amongst three *Gli* genes, *Gli1*, *Gli2* and *Gli3* (Figure 1-7) (Hui and Angers, 2011) and similar to *Drosophila* ci, Gli activity is regulated by numerous mechanisms, including protein stability, regulated expression and posttranslational modifications (Hui and Angers, 2011; Robbins et al., 2012; Ruiz i Altaba, 1999). Gli2 is the primary Hh-regulated transcriptional activator (Gli^{Act}) and Gli3 is the primary Hh-regulated repressor (Gli^{Rep})(Ding et al., 1998; Mo et al., 1997), while Gli1 is strictly a transcriptional activator (Figure 1-7)(Bai et al., 2002; Park et al., 2000). In the absence of Hh signaling, full-length Gli2 and Gli3 are partially processed by the proteasome, resulting in truncated Gli^{Rep} proteins that actively suppress Hh target gene transcription (Pan et al., 2006). Proteolytic processing of Gli2 and Gli3 is blocked in the presence of Hh, which allows the accumulation of full-length Gli^{Act} proteins that act as potent transcriptional activators in the nucleus and drive Hh target gene expression (Kolpak et al., 2005; Pan et al., 2006). Gli1 protein is not present in the absence of Hh signaling and thus does not transduce the initial Hh signal, but in response to Hh stimulation, *Gli1* transcription increases, potentiating the transcriptional output of Hh signaling (Bai et al., 2002).

Gli2 and *Gli3* are essential for mammalian development such that loss of *Gli2* function results in defective Hh signaling in the neural tube (Ding et al., 1998; Mo et al., 1997) and *Gli3* null mice display dorsal brain defects consistent with ectopic Hh pathway activation (Hui and Angers, 2011; Hui and Joyner, 1993). *Gli1*, on the other hand, is dispensable for normal development and its function is compensated by the remaining Gli proteins (Bai et al., 2002; Park et al., 2000). While a comprehensive analysis of Gli target genes has no been performed, Gli-dependent transcriptional binding has been studied in tissues of the limb, cerebellum and retina, revealing an extensive network of co-regulated transcription factors, signaling molecules and cell cycle regulators that ultimately control the diverse functions of Hh signaling (McNeill et al., 2012; Vokes et al., 2007; Vokes et al., 2008; Zuniga et al., 2012).

2.5 Hedgehog signaling in the retina

Hh signal is essential for eye development, both in invertebrates and vertebrates, where it is a critical factor in eye patterning and growth, and influences cell fates. During early vertebrate forebrain development, secreted Shh from the ventral midline of the diencephalon regulates patterning of the bilateral eye fields (Ericson et al., 1995) and promotes proximal over distal identity in the developing optic vesicle tissue (Ekker et al., 1995; Perron et al., 2003). Vertebrates lacking *Shh* display cyclopia, or a fused eye field phenotype, because of the failure of ventral neural tube specification (Chiang et al., 1996; Ekker et al., 1995; Macdonald et al., 1994), not unlike humans with loss-of-function mutations in *SHH* (Belloni et al., 1996; Roessler et al., 1996). Vertebrate Hh proteins also have a described role in the development of extraocular tissues later in eye development, where *Ihh* secreted from the developing choroid signals the peri-ocular mesenchyme and RPE to regulate differentiation (Dakubo et al., 2008). Similarly, *Xenopus* Hh homologs are also required for proper RPE differentiation in tadpole embryos (Perron et al., 2003). In addition, Hh proteins are also actively transported along retinal axons and induce neural differentiation and patterning in the brain in *Drosophila* (Huang and Kunes, 1996; Huang and Kunes, 1998), similar to Shh transported within RGC axons. Here, RGC derived Shh regulates astrocyte proliferation in the rodent optic nerve (Beug et al., 2011; Dakubo et al., 2008a; Wallace and Raff, 1999) and functions as an axon guidance molecule during neuronal GC pathway finding at the optic disc and optic chiasm (Bovolenta, 2005; Kolpak et al., 2005).

In the vertebrate retina, Shh is the main mediator of Hh signaling during development and it is secreted from the first-born neurons, the RGCs (Jensen and Wallace, 1997; Wang et al., 2005; Wang et al., 2002). Loss of the RGCs, either by conditional endotoxin expression or by culturing retinal tissue, results in loss of Hh signaling, similar to conditional Shh deletion in the retina (Mu et al., 2005; Wang et al., 2005; Wang et al., 2002). The function of Shh in the retina is two fold: it is a potent mitogen influencing the pool of multipotent RPCs (Jensen and Wallace, 1997; Moshiri et al., 2005; Wang et al.,

2005; Wang et al., 2002; Zhang and Yang, 2001) and biasing individual cell fates (Levine et al., 1997b; Sakagami et al., 2009; Wang et al., 2005; Zhang and Yang, 2001). These functions are well conserved among vertebrate and invertebrate species. For example, Hh in *Drosophila* eye disc is secreted from posterior neurons and regulates proliferation in the second mitotic wave during eye development by inducing cell cycle genes *Cyclin D* and *Cyclin E* (Duman-Scheel et al., 2002). Functioning as a feedback inhibitory cue, Hh secreted from differentiating photoreceptor cells in the *Drosophila* eye disc drives sequential induction of the morphogenetic furrow as it sweeps anteriorly across the eye disc (Heberlein and Moses, 1995; Heberlein et al., 1993; Ma et al., 1993). Similarly, in the mouse and chick retina, Hh is secreted is by the first-born neurons, the RGCs, and functions to maintain the RPC pool (Moshiri et al., 2005; Wang et al., 2005; Wang et al., 2002; Zhang and Yang, 2001) by driving expression of the cell cycle regulator *CcnD1* and other genes critical for the maintenance of RPCs, such as *Hes1* (Wall et al., 2009; Wang et al., 2005), *Sox2* (Cwinn et al., 2011) and *Ndp* (McNeill et al., 2013). Conditional deletion of *Shh* from the retina causes decreased progenitor proliferation and precocious cell cycle exit (Mu et al., 2005; Wang et al., 2005), while forced activation of the pathway increases proliferation (Yu et al., 2006). In the zebrafish and *Xenopus*, Hh has been shown to regulate cell cycle kinetics within progenitor cells, where increased Hh activity accelerates G_1/S and G_2/M transitions (Locker et al., 2006), while also promoting cell cycle exit, in part through $p57^{Kip2}$ (Locker et al., 2006; Shkumatava and Neumann, 2005). *Shh* also influences cell fate and pattern development in the retina, negatively regulating the formation of RGCs (Sakagami et al., 2009; Wang et al., 2005; Zhang and Yang, 2001). Conditional deletion of *Shh* causes gross lamination defects, increased RGC and later born photoreceptors at the expense of Müller glia and bipolar cells (Wang et al., 2005; Zhang and Yang, 2001). Postnatal gain-of-function for the *Shh* results in the opposite: increased inner retinal cell types at the expense of photoreceptors (Jensen and Wallace, 1997; Wall et al., 2009; Yu et al., 2006).

The Gli transcription factors have described roles consistent with mediating downstream effects of Hh signaling during retinohistogenesis. In the mammalian retina, Gli2 is a primary mediator of Hh-

dependent induction of target gene expression, proliferation and the development of late cell types (McNeill et al., 2012; Wall et al., 2009), and while Gli1 is dispensable for retinal development, it can partially compensate in the absence of Gli2 in RPCs to drive target gene expression and proliferation (Wall et al., 2009). In the absence of Gli3, null mice exhibit a range of eye defects ranging from microphthalmia to the complete absence of eye tissue (Franz and Besecke, 1991; Furimsky and Wallace, 2006). Moreover, gene induction in response to Hh stimulation and baseline expression of Hh target genes are increased in Gli3-null retinal explant cultures (McNeill et al., 2012), which is consistent with a role where Gli3 antagonizes Hh target gene transcription.

3.0 Receptor Tyrosine Kinase pathway - Overview

The receptor Tyrosine kinase (RTK) signaling superfamily is made up of a large and diverse set of cell surface receptors that are key regulators of a multitude of critical cellular processes, including cell migration, metabolism, survival, differentiation and proliferation (Blume-Jensen and Hunter, 2001; Ullrich and Schlessinger, 1990). The mechanisms of action and critical signaling components of RTKs are evolutionarily conserved from *Drosophila* to humans and not surprisingly, aberrant RTK signaling is associated with multiple diseases (Hausott et al., 2009).

3.1 RTK Signal Transduction

Broadly speaking, RTK receptors share a similar molecular structure consisting of an extracellular ligand-binding region, a single transmembrane helix and a cytoplasmic region containing a protein tyrosine kinase domain with additional carboxyl terminal and juxtamembrane regulatory regions (Lemmon and Schlessinger, 2010). Ligand-receptor specificity is established through different binding capacities, alternative splicing events to generate unique receptors and tissue specific expression of both

ligands and receptors (Wu et al., 1991; Zhang et al., 2006). Upon ligand binding, a cascade of events resulting in pathway activation is initiated: first beginning with receptor dimerization, followed by conformational changes in receptor structure and activation of the kinase domain by phosphorylation of tyrosine residues (Figure 1-8). The phosphorylated tyrosine residues on the dimerized receptor act as docking sites for adaptor proteins and canonically lead to activation of four key downstream pathways: RAS-RAF-MAPK, PI3K-AKT, STAT and PLCγ (Dudka et al., 2010; Eswarakumar et al., 2005; Kanazawa et al., 2010; Tsang and Dawid, 2004). Cellular context largely determines the functional outcome of RTK activation during development and this is highlighted by the diverse cellular and molecular functions RTK signaling has been implicated in, including: proliferation and migration, but also cell cycle arrest and differentiation (De Moerlooze et al., 2000). Heparan sulphate proteoglycans (HSPG) also serve a critical function during RTK signaling, where they function to sequester secreted RTK ligands at the cell surface and mediate ligand-receptor interaction by forming a ternary complex with RTK receptors (Harmer et al., 2004; Mohammadi et al., 2005).

3.2 The Epidermal Growth Factor Pathway

Epidermal growth factor (EGF) signaling, like FGF signaling, is a member of the RTK signaling superfamily and this pathway has been the focus of decades of work that has highlighted its complex and important role in regulating growth, survival, proliferation and differentiation in mammalian cells (Oda et al., 2005). There are seven known ligands (summarized in Table 1) (Schneider and Wolf, 2009) that bind to four EGFR family members (Figure 1-9) (Harris et al., 2003) that play pivotal roles during organ development and tumorigenesis (Sibilia et al., 2007). Targeted loss of EGFR family members results in neural defects as a result of progressive loss of neurons and glial cells and neurodegeneration (Kornblum et al., 1998; Sibilia et al., 1998), while gain-of-function for the EGFR, either through genetic mutation in humans or transgenic mouse models, results in overgrowth and glioblastoma-like tumor formation

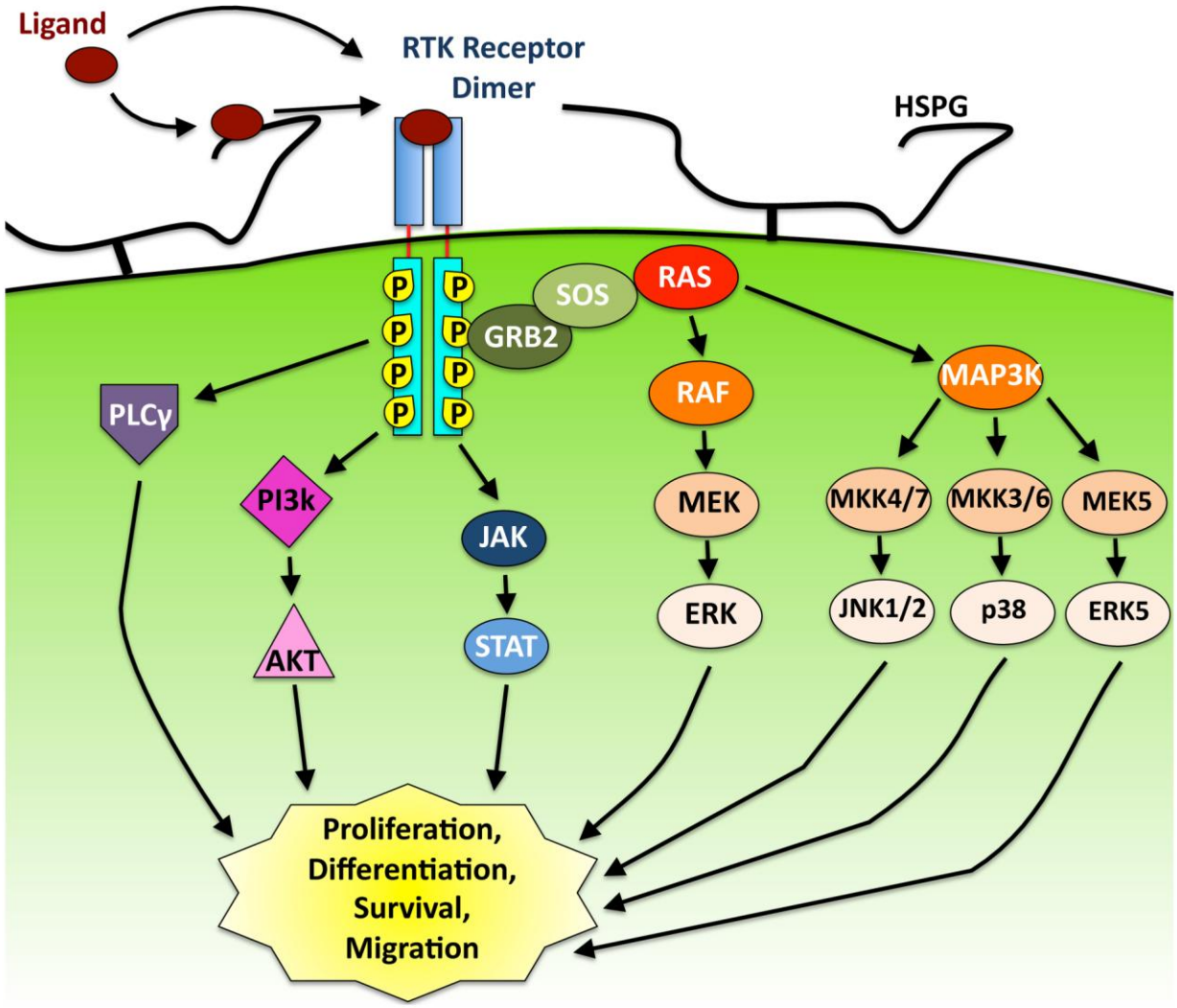
Ligand
RTK Receptor
Dimer
HSPG
P
SOS
RAS
GRB2
PLCγ
RAF
MAP3K
PI3k
MEK
MKK4/7
MKK3/6
MEK5
JAK
ERK
JNK1/2
p38
ERK5
AKT
STAT
Proliferation,
Differentiation,
Survival,
Migration

Figure 1-8 Receptor Tyrosine Kinase (RTK) signal transduction pathway. Ligand binding, mediated by heparin sulphate proteoglycans (HSPG) resulting in receptor autophosphorylation, activating Ras, which can activate several signaling cascades through consecutive phosphorylation events. RTK autophosphorylation can also activate PI3K, which in turn activates Akt, PLCγ and the JAK-STAT signalling proteins. All of these downstream RTK signaling events play an essential role in cellular differentiation, proliferation, survival and migration. Adapted from (Wang, 2016).

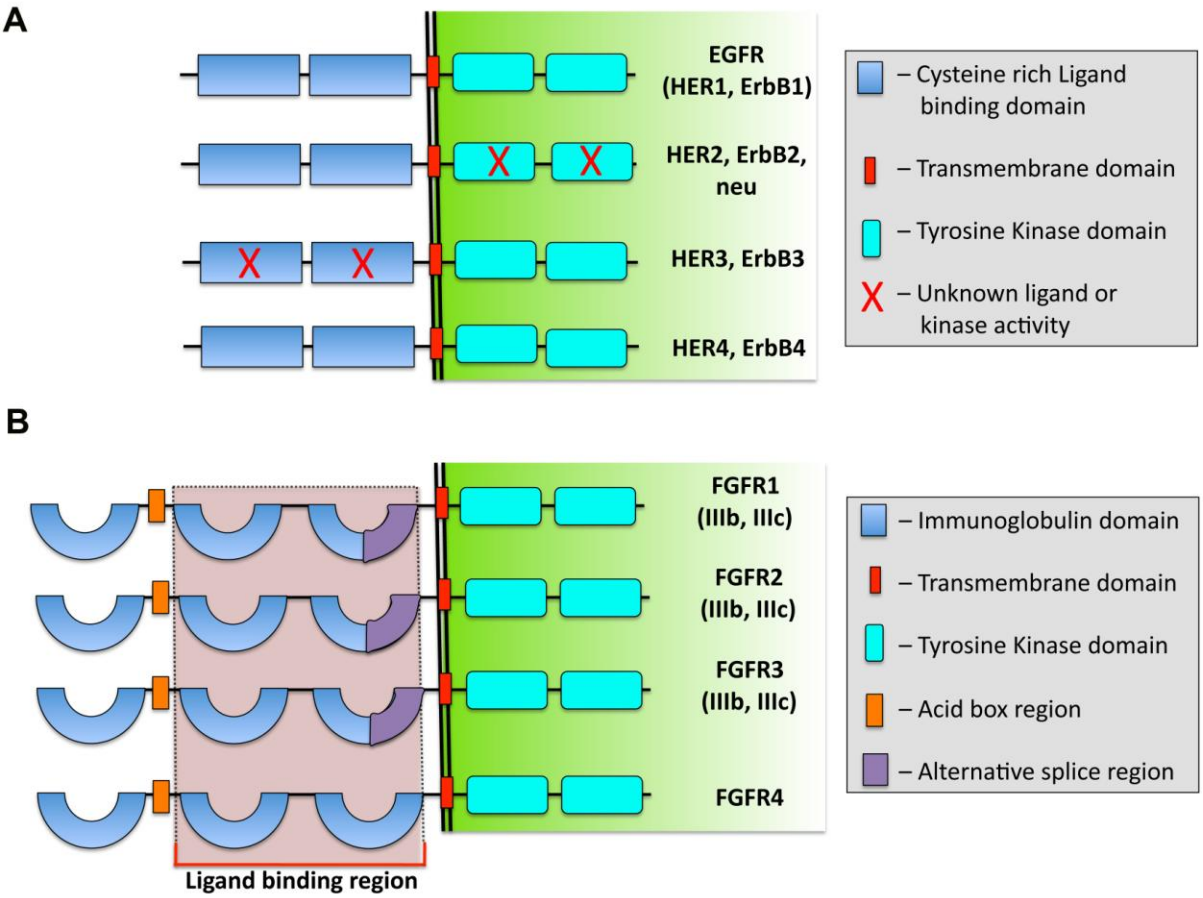
A
EGFR
(HER1, ErbB1)
HER2, ErbB2,
neu
HER3, ErbB3
HER4, ErbB4
– Cysteine rich Ligand binding domain
– Transmembrane domain
– Tyrosine Kinase domain
– Unknown ligand or kinase activity
B
FGFR1
(IIIb, IIIc)
FGFR2
(IIIb, IIIc)
FGFR3
(IIIb, IIIc)
FGFR4
Ligand binding region
– Immunoglobulin domain
– Transmembrane domain
– Tyrosine Kinase domain
– Acid box region
– Alternative splice region

Figure 1-9 Epidermal growth factor (EGF) and Fibroblast growth factor (FGF) receptor structure. Schematic representation of A) EGF and B) FGF family of RTK receptors, depicting critical protein domains and motifs. Adapted from (Harris et al., 2003).

Table 1-1 A summary of EGF ligands and respective receptor binding partners

Ligands	Receptor Preference
Epidermal Growth Factor (EGF)	EGFR/HER1/ErbB1
Transforming Growth Factor –α (TGF-α)	EGFR/HER1/ErbB1
Amphiregulin (AR)	EGFR/HER1/ErbB1
Epigen	EGFR/HER1/ErbB1
Heparin-binding EGF-like growth factor (HB-EGF)	EGFR/HER1/ErbB1, HER4/ErbB4
Epiregulin	EGFR/HER1/ErbB1, HER4/ErbB4
Betacellulin	EGFR/HER1/ErbB1, HER4/ErbB4

Adapted from (Schneider and Wolf, 2009) and (Harris et al., 2003)

(Holland et al., 1998; Ling et al., 2005; Weiss et al., 2003). In *Drosophila*, EGF, signaling through a single EGFR/ErbB family member, is implicated in multiple developmental roles, such as cell fate induction, proliferation and cell migration within the fly CNS (Baker and Yu, 2001; Baonza et al., 2001; Freeman, 1996; Huang et al., 1998).

3.3 Fibroblast Growth Factor Signaling

The secreted glycoprotein fibroblast growth factor (FGF) and its respective receptors (FGFRs) are a subclass of the RTK signaling family and in vertebrates, are made up of 18 different ligands (Summarized in Table 2) that bind and signal through 4 highly conserved FGFRs (Figure 1-9)(Iwata and Hevner, 2009; Turner and Grose, 2010) to control progenitor cell growth, differentiation, survival and patterning during embryonic development (Lanner and Rossant, 2010) and organogenesis, including the brain (Blak et al., 2007; Saarimaki-Vire et al., 2007; Trokovic et al., 2005). By recapitulating early developmental mechanisms, FGFs have critical roles in adult tissues, mediating metabolic homeostasis (Hu et al., 2013; Long and Kharitonenkov, 2011), tissue repair and regeneration (Guzy et al., 2015; Meyer et al., 2012).

3.4 EGF and FGF signaling and progenitor proliferation in the retina

RTK signaling via the EGF and FGF pathways play critical roles during CNS development and disease and, consistently, RTK signaling regulates RPC growth and patterns the retina during development in invertebrate and vertebrate species.

In the second instar eye disc prior to *Drosophila* eye patterning, EGFR signaling is required for proliferation of unpatterned cells (Xu and Rubin, 1993). At the third instar larval stage, EGFR signaling

Table 2-2 Summary of FGF ligand Family members and corresponding receptor binding partners

FGF Subfamily	Ligands	Receptor Preference
FGF1	FGF1, FGF2	FGF1 activates all FGFRs, FGF2 prefers FGFR1c and FGFR2c
FGF4	FGF4, FGF5, FGF6	FGFR1c, FGFR2c
FGF7	FGF3, FGF7, FGF10, FGF22	FGFR2b, FGFR1b
FGF8	FGF8, FGF17, FGF18	FGFR3c, FGFR4, FGFR1c
FGF9	FGF9, FGF16, FGF20	FGFR3c, FGFR2c
FGF15/19	FGF15/19, FGF21, FGF23	Hormone class, very weak activation of FGFR1c, FGFR2c
FGF11	FGF11, FGF12, FGF13, FGF14	No activation of FGFRs

Adapted from (Pownall, 2010).

plays two roles. It defines early born photoreceptor cells in the morphogenetic furrow (Baker and Yu, 2001; Baonza et al., 2001; Xu and Rubin, 1993). Later EGFR signaling is required for survival (Dominguez et al., 1998) and progression from G_2 to M phase during the second mitotic wave via activation of the *Drosophila* homolog of *cdc25*, providing sufficient uncommitted cells to produce accessory cone and pigment cells of ommatidium required for proper compound eye patterning (Baker and Yu, 2001; Baonza et al., 2002). Thus, EGF signaling plays an integral role in regulating retina progenitor growth and pattern formation in the fly compound eye.

Multiple ligands can activate the EGFR and in the vertebrate retina, mature neurons (RGCs, Müller glia, horizontal and amacrine cells) express different combinations of ligands, including EGF, TGF-α, heparin-binding EGF like ligand (HB-EGF), and amphiregulin (AR) (Chen et al., 2007; Close et al., 2006). Similarly, *Fgf12* and *Fgf13* expression has been shown in mature RGCs and amacrine cells (Trimarchi et al., 2007). EGFR expression is also evident in postmitotic, terminally differentiated adult retinal neurons, suggesting that EGF signaling has additional, non-mitogenic functions in the adult retina (Close et al., 2006).

RPCs also express a number of RTK ligands (*Fgf-15*, *fgf-3*) (Trimarchi et al., 2008) and receptors (*Egfr*, *Fgfr1*) (Close et al., 2006; James et al., 2004; Koso et al., 2007) and this expression varies as a function of developmental time, where early and late RPCs displaying distinct proliferative responses to RTK ligands (James et al., 2004; Lillien and Cepko, 1992). Activation of RTK signaling by exogenous EGF or TGF-α exposure promotes RPC proliferation *in vitro* (Anchan et al., 1991; Close et al., 2006; Lillien and Cepko, 1992) and consistently, mice carrying a homozygous deletion of the EGFR gene have reduced numbers of proliferating late RPCs (Close et al., 2006).

Not only does RTK signaling promote RPC growth, but EGF is a potent mitogen for Müller glia *in vivo* and upon retinal damage, EGFR expression is upregulated allowing Müller glia to proliferate (Close et al., 2006), in part, through a bone morphogenic protein (BMP)-dependent mechanism (Ueki and Reh, 2013). In the chicken retina, FGF2 in cooperation with other growth factors mediate Müller glia

derived progenitor proliferation in a MAPK-dependent mechanism in both uninjured and damaged retinas (Fischer et al., 2002; Fischer and Reh, 2002; Fischer et al., 2009). HB-EGF mediates dedifferentiation and formation of cycling progenitors cells from mature Muller glia to repair the injured zebrafish retina by activating MAPK and WNT/beta-catenin signalling (Wan et al., 2012) and blocking FGF signaling after a light induced lesion in fish inhibits proliferation and regeneration, likely by inhibiting Müller glial growth (Hochmann et al., 2012).

4.0 Notch signaling pathway – Overview

Notch is another evolutionarily conserved molecular pathway that regulates a diverse set of developmental processes in vertebrates and invertebrates, where it is integral for tissue homeostasis and development (Gordon et al., 2008). Thus, it is not surprising that defects in the Notch pathway are implicated in a number of human diseases, such as: Alagille syndrome (McDaniell et al., 2006), spondylocostal dysostosis (Turnpenny et al., 2007), congenital heart disease (Zhou and Liu, 2014), Alzheimer's disease (Woo et al., 2009) and several cancers (Koch and Radtke, 2007). In the context of CNS development, Notch signaling is a major regulator of neural progenitor cells, where it serves to maintain the pool of progenitor cells and regulate glial cell fate in postmitotic precursor cells (Henrique et al., 1997; Jadhav et al., 2006a; Kechad et al., 2012; Nelson et al., 2007; Riesenberg et al., 2009; Takatsuka et al., 2004; Yaron et al., 2006; Zheng et al., 2009).

4.1 Notch Signal Transduction

In mammals, there are four Notch receptors, NOTCH1-4, comprised of a single-pass heterodimer transmembrane proteins with a large extracellular domain and a small intracellular region (Brou et al., 2000) that are activated by binding of five canonical Delta-Serrate-lag (DSL) type ligands: Jagged1,

Jagged2, Delta-like 1 (Dll1), Dll3 and Dll4 (Figure 1-10) (Lindsell et al., 1995). Upon ligand binding from an adjacent cell, a disintegrin and metalloproteases (ADAM10) and γ-secretase complex proteolytically cleave the transdomain portion of the Notch receptor, which releases the intracellular domain (NICD) into the cytosol of the receiving cell (Figure 1-11). NICD then translocates to the nucleus and complexes with the DNA binding protein RBPJκ (also called CSL (CBF1, Su(H), LAG1) and members of the mastermind-like (MAML) coactivator family (Kageyama et al., 2009; Pierfelice et al., 2011). The RBPJκ-NICD-MAML1 complex acts as a transcriptional activator of target genes, most notably the Hes gene family, who antagonize proneural genes downstream of Notch signaling (Kageyama et al., 2008; Kageyama et al., 2009).

4.2 Two-way Notch signaling

There is also evidence to support bidirectional Notch signaling, where Notch ligands are able to transmit signals within ligand-expressing cells in addition to activating Notch receptors of neighbouring cells (Pintar et al., 2007)(Figure 1-11). Processing of ligands is similar to that of the receptors and γ-secretase, together with ADAM, mediate cleavage events that ultimately release a ligand intracellular domain (ligand-ICD) (Klueg et al., 1998; LaVoie and Selkoe, 2003; Six et al., 2003) and ligand cleavage is dependent upon Notch receptor binding (Ascano et al., 2003). The exact role of this bidirectional signaling is not fully known, but cleaved ligand-ICD can partially locate to the nucleus of ligand expressing cells (Bland et al., 2003; Chitnis et al., 1995; Ikeuchi and Sisodia, 2003), Consistent with this nuclear localization, Jagged1-ICD has been shown to activate gene expression *in vitro* (Ascano et al., 2003; Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003), including SMAD transcription factors in neural stem cells (Hiratochi et al., 2007). Forced expression of *Xenopus* Serrate-ICD in embryos inhibits primary neurogenesis (Kiyota and Kinoshita, 2004), while Dll1-ICD induced neuron formation in P19 embryonic carcinoma cells (Hiratochi et al., 2007), which suggests ligand-ICD signaling may be

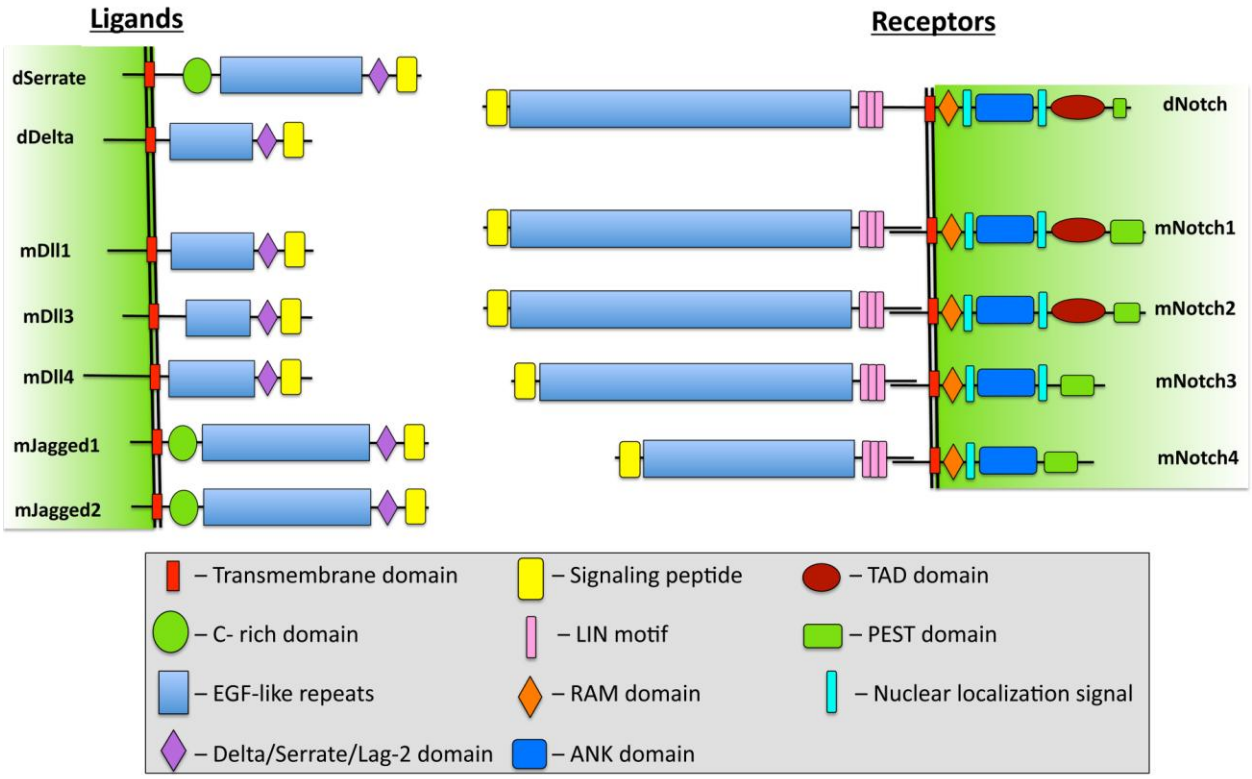
Ligands
Receptors
dSerrate
dDelta
mDll1
mDll3
mDll4
mJagged1
mJagged2
dNotch
mNotch1
mNotch2
mNotch3
mNotch4
– Transmembrane domain
– Signaling peptide
– TAD domain
– C- rich domain
– LIN motif
– PEST domain
– EGF-like repeats
– RAM domain
– Nuclear localization signal
– Delta/Serrate/Lag-2 domain
– ANK domain

Figure 1-10 ***Drosophila*** **and mammalian Notch pathway ligands and receptors.** Schematic representation of Notch ligands and receptors depicting important protein domain and motifs. Adapted from (Gordon et al., 2008).

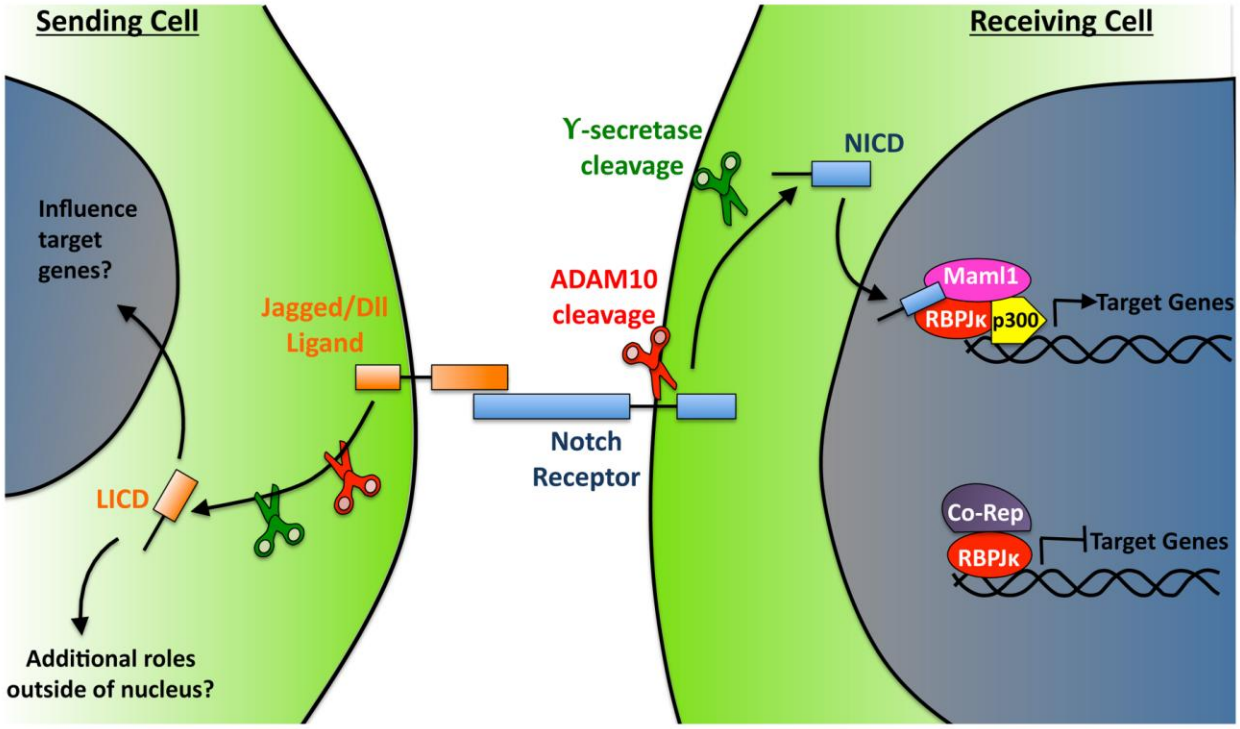
Sending Cell
Receiving Cell
Influence target genes?
Jagged/Dll Ligand
LICD
Additional roles outside of nucleus?
Y-secretase cleavage
NICD
ADAM10 cleavage
Notch Receptor
Maml1
RBPJκ
p300
Target Genes
Co-Rep
RBPJκ
Target Genes

Figure 1-11 Simplified view of Notch pathway signal transduction. Upon extracellular binding of Ligand to the Notch receptor on the receiving cell, sequential cleavage by ADAM10 and γ-secretase complex is initiated, which liberates the intracellular domain of the Notch receptor (NICD). NICD then enters the nucleus and binds DNA in a complex with RBPJκ , Maml1 and p300, driving canonical Notch pathway target gene transcription. In the absence of activated Notch signaling, RPBJκ in a co-repressor complex, inhibits target gene activation. Bidirectional Notch signaling in the sending cell, mediated by ADAM10 and γ-secretase complex cleavage of intracellular domain of the ligand can also activate target genes as well as repress signalling pathways in sending cells. Adapted from (Gordon et al., 2008).

important during neurogenesis. In addition to affecting neighbouring cells via receptor-ligand *trans*-interaction, ligand-ICD signaling has been shown to disrupt the formation of NICD-RBPJ-MAML complex formation by regulating protein stability within the same cell (Kim et al., 2011) and this raises the possibility that ligand-ICD, acting in the same cell, may function to negatively regulate receptor-ICD mediated Notch signaling and act as a mechanism to fine tune Notch output (Kim et al., 2011; Miller et al., 2009).

4.3 Notch signaling in the retina

Notch is a key regulator of retinal neurogenesis (Perron and Harris, 2000). Multiple *Notch* receptors (*Notch1, Notch 3*) and ligands (*Dll1, Dll4* and *Jagged1)* are expressed in large overlapping patterns in undifferentiated, proliferating RPCs in vertebrates (Ahmad et al., 1997; Bao and Cepko, 1997; Dorsky et al., 1995; Lindsell et al., 1996). In frogs and chick, *Notch* signaling controls the temporal development of multiple cell lineages from the RPC pool through lateral inhibition (Henrique et al., 1997). In mice, loss of Notch signaling embryonically results in early lethality, precluding the analysis of retinal phenotypes (Oka et al., 1995; Swiatek et al., 1994). Exploiting retinal specific conditional mutants, it has been shown that *Notch1* and the integral downstream Notch mediator, *Rbpj*, play a critical role in maintaining RPCs in cell cycle and preventing neuronal differentiation (Jadhav et al., 2006b; Riesenberg et al., 2009; Yaron et al., 2006). Consistent with this function, pharmacological inhibition or genetic loss of Notch function forces RPCs out of cell cycle and initiates coordinated neuronal differentiation (Mizeracka et al., 2013; Nelson et al., 2007). Conversely, ectopic Notch activation by overexpression of NICD1 or forced expression of ligand inhibits neurogenesis (Austin et al., 1995; Bao and Cepko, 1997; Dorsky et al., 1995; Scheer et al., 2001) and promotes expression of progenitor genes in the rodent and frog retina (Furukawa et al., 2000; Jadhav et al., 2006a; Lindsell et al., 1996; Nelson et al., 2011). These

effects are in part due to Notch mediated activation of repressive bHLHs Hes1 and Hes5 (Ohtsuka et al., 1999) and suppression of pro-neurogeneic bHLHs *Atoh7* and *Neurog2* within RPCs (Maurer et al., 2014).

Notch also influences cell fate decisions: inhibition early in development results in the differentiation of early born RGC and cone photoreceptor cells (Jadhav et al., 2006b; Riesenberg et al., 2009; Yaron et al., 2006), while ectopic Notch activation in postnatal RPCs influences cells to differentiate into late-born Müller glia (Furukawa et al., 2000; Nelson et al., 2011), in part by regulation of downstream inhibitors of DNA binding factors *Id1* and *Id3* (Mizeracka et al., 2013a). Mechanistically, in post-mitotic daughter cells of the retina, Notch activity is antagonized by asymmetrically inherited Numb, an endocytic protein, which creates differential pathway signaling between each daughter cell and induces distinct cellular fates (Kechad et al., 2012). Thus, Notch signaling in the retina serves to maintain the pool of RPCs by preventing neuronal differentiation and cell cycle exit, and also patterns the retina by repressing early and promoting later born glial cell fates during development.

Within mature Müller glia, Notch is essential for maintaining a quiescent state in mouse (Surzenko et al., 2013), but also plays an integral role in Müller glia derived progenitor maintenance and proliferation in the chick retina (Ghai et al., 2010; Hayes et al., 2007). Indeed, inhibiting Notch pharmacologically impairs glial cell-cycle re-entry after mitogen stimulation (Todd and Fischer, 2015).

In the invertebrate retina of *Drosophila*, Notch is required in uncommitted cells at the time of cell fate adoption as the morphogenetic furrow sweeps posterior to anterior during eye disc formation (Cagan and Ready, 1989). Without Notch, neural photoreceptor and non-neural bristle and pigment cell development was affected, suggesting Notch signaling permits interactions by other, more specific mechanisms to guide cell fate decisions (Cagan and Ready, 1989). In addition to regulating pattern formation of the *Drosophila* retina, Notch is also required to clear a critical G1-S checkpoint that triggers the initiation of the second mitotic wave behind the morphogenetic furrow (Baonza and Freeman, 2005). Notch mediates synchronized proliferation during second mitoitic wave by relieving RBF1-dependent inhibition of dE2F1 and activation of *cyclin A* expression (Baonza and Freeman, 2005), which is

consistent with the pattern of *Notch* transcripts roughly corresponding to that of mitoses in the *Drosophila* embryo (Artavanis-Tsakonas, 1988). Thus, the invertebrate system also relies on Notch to regulate pattern formation and proliferation in the developing retina.

5.0 Integration of Hh signaling with the RTK and Notch pathways

5.1 Synergistic Hh and RTK signaling

The *Drosophila* model system has revealed multiple developmental processes that rely on integrated, context dependent Hh and RTK signaling. Hh activates EGF signaling by inducing tissue-specific expression of *Drosophila* EGFR ligand during head formation (Amin et al., 1999), but EGFR signaling can also limit Hh signaling by promoting degradation of Ci during Hh-mediate morphogenetic furrow progression (Baker et al., 2009).

There is evidence to support the crosstalk between Hh and RTK signaling to regulate aberrant cellular growth during cancer development (Bigelow et al., 2005; Kasper et al., 2006; Schnidar et al., 2009; Stecca et al., 2007; Zhou et al., 2016). For example, PI3K/AKT signaling in renal cell carcinoma can activate expression of Gli (Zhou et al., 2016) and oncogenic RAS-induced melanoma in mice is prevented by inhibition of Gli function (Stecca et al., 2007). Hh and RTK signaling have been shown to integrate at the level of the Gli transcription factors, where RAS-MEK and AKT mediate the nuclear translocation of Gli1 protein (Stecca et al., 2007), and Hh and EGF-MEK signaling together can synergistically activate a set of genes during hair growth and cancer development (Kasper et al., 2006).

Cross talk between Hh and EGFR signaling is also evident in the developing and adult vertebrate brain, where these signals act cooperatively as mitogens to stimulate neural stem cell growth (Heo et al., 2007; Palma et al., 2005; Palma and Ruiz i Altaba, 2004; Reinchisi et al., 2013). In the mouse neocortex, Shh is required for stem and precursor cell proliferation in a concentration-dependent manner with EGF

(Palma and Ruiz i Altaba, 2004). Similar to embryonic and postnatal neocortical stem cells, adult stem and progenitor cells of the subventricular zone in the forebrain require synergistic Hh-RTK signaling to promote self-renewal (Palma et al., 2005). Interestingly, Shh alone could not alter neurosphere size or number and synergism was only seen over a limited, lower concentration range of EGF, suggesting combined Shh and EGF secretion may define a neurogenic niche where stem cells exist in the brain (Palma et al., 2005; Palma and Ruiz i Altaba, 2004). In follow up studies it was shown Hh transactivates the EGFR via receptor phosphorylation in a metalloprotease-dependent manner to promote neural stem cell growth in the late neocortex, thus providing a potential mechanism for such synergism (Heo et al., 2007; Reinchisi et al., 2013).

Multiple lines of evidence suggest RTK and Hh signaling can integrate to regulate growth of neural stem and progenitor cells as well as promote cancer growth and the invertebrate retina has demonstrated cross talk between these pathways during development. However, the extent of synergistic Hh-RTK signaling within the context of progenitor proliferation in the vertebrate retina remains unclear. This leaves several important questions unanswered; can synergistic mitogenic RTK and Hh signaling promote RPC growth and maintenance similar to the embryonic and adult cortex? If RTK and Hh can co-operate in RPCs, can these signaling pathways be exploited to generate an unlimited supply of multipotent cells for regenerative therapies and what are the potential mechanisms that govern pathway synergism?

5.2 Co-operative Notch and Hh signaling during neurogenesis

Notch and Hh interactions have been described in invertebrates and vertebrates, where they affect numerous developmental processes (Dave et al., 2011; Huang et al., 2012; Kong et al., 2015; Stasiulewicz et al., 2015; Todd and Fischer, 2015). For example, integrated Hh and Notch signaling in the developing neocortex control proliferative versus neurogenic divisions in Nestin+ neural progenitors (Dave et al.,

2011). Similarly, Hh and Notch together are required for Müller glial derived progenitor cell proliferation following injury in chick retina (Todd and Fischer, 2015).

Co-operative Hh and Notch signaling is also important for pattern formation. During Zebrafish spinal cord development, lateral floor plate progenitors depend on a permissive Notch signal to maintain Hh responsiveness and specify interneuron fates prior to differentiation (Huang et al., 2012). This is not unlike the patterning in mouse and chick spinal cord, where Notch, acting upstream of Hh, is required for neural progenitors to respond to high levels of Shh activity and adopt ventral-most fates in the neural tube (Kong et al., 2015; Stasiulewicz et al., 2015).

5.3 Mechanisms of Hh-Notch integration

The actual mechanism of the Hh-Notch interactions are diverse and context dependent. In the developing spinal cord, dorso-ventral Shh patterning is dependent on Notch activity to regulate subcellular localization of Ptch and Smo within the PC (Kong et al., 2015; Stasiulewicz et al., 2015) as well as to modulate the length of the PC (Kong et al., 2015; Stasiulewicz et al., 2015). Activated Notch also leads to elevated levels of full-length Gli3 protein (Kong et al., 2015). Moreover, Rbpj directly binds the Gli2, Gli3 and Smo promoter regions in neural stem cells of the mouse cortex, providing direct evidence of Notch-Hh pathway integration during neurogenesis (Li et al., 2012). Thus, several lines of evidence point to Notch integration at multiple points during Hh signal transduction.

5.4 Phenotypic Hh-Notch Overlap in the Retina

Interestingly in the context of retinal development, there is considerable overlap in the gain- and loss-of-function phenotypes associated with Notch and Hh pathway manipulation in RPCs, particularly with respect to effects on RPC maintenance and late born cell type development (Furukawa et al., 2000;

Jadhav et al., 2006b; Wang et al., 2005; Yu et al., 2006). Ectopic Notch and Hh activation results in maintenance of the RPC pool (Jadhav et al., 2006a; Wang et al., 2005) and loss of either pathway results in precocious RPC cell cycle exit (Riesenberg et al., 2009). Conversely, early loss of Notch or Hh results in inappropriate RGC and cone photoreceptor production (Riesenberg et al., 2009; Wang et al., 2005) and ectopic expression of either pathway postnatally biases late RPCs toward a Müller glia fate (Bao and Cepko, 1997; Furukawa et al., 2000; Jadhav et al., 2006a). As well, secreted Hh regulates *Delta* expression in the *Drosophila* retina (Baonza and Freeman, 2005) and both pathways activate a number of common target genes in the vertebrate retina (McNeill et al., 2012; Muto et al., 2009; Nelson et al., 2011; Wall et al., 2009; Wang et al., 2002). Consistent with this notion, our lab has shown that Hh signaling can activate the canonical Notch target *Hes1* by direct binding of *Gli2* to drive RPC proliferation and bias progenitor cell fates (Wall et al., 2009). Considering the multiple lines of evidence that point to Notch influencing Hh signaling during neurodevelopment and the striking similarities between Notch and Hh retinal mutant phenotypes, it remains to be determined how Notch can influence Hh-dependent proliferation and cell fate induction in the retina.

6.0 Objectives

It has been well established that Hh, RTK and Notch signaling are critical for proper CNS development (Artavanis-Tsakonas et al., 1999; Hausott et al., 2009; Ingham and McMahon, 2001), including the neural retina, where Hh and RTK pathways act as mitogens (Close et al., 2006; Jensen and Wallace, 1997), controlling RPC growth, both Hh and Notch signaling maintain RPC identity (Jadhav et al., 2006a) and similarly bias cell fates during development (Furukawa et al., 2000; Jadhav et al., 2006b; Wang et al., 2005). Furthermore, the integration of the RTK and Notch signaling with the Hh pathway has been demonstrated during brain (Kong et al., 2015; Palma et al., 2005; Stasiulewicz et al., 2015) and cancer development (Brechbiel et al., 2014; Stecca and Ruiz, 2010), and Hh can directly activate the

canonical Notch target *Hes1* in postnatal RPCs (Wall et al., 2009). However, cross talk between these pathways has not been extensively explored in the growth and maintenance of RPCs in the developing retina. Based on these observations, I **hypothesize** that RTK signaling will stabilize Gli proteins, maintain Hh signaling and sustain RPC growth. I also **hypothesize** that Notch signaling is required to maintain Hh-dependent RPC proliferation through regulation of Gli2 protein during development of the retina.

Therefore, the objectives of my project are to:

1) Examine the effects of synergistic RTK and Hh pathway activation in retinal progenitor cell growth and competence *in vitro*
2) Explore the relationship of Notch and Hh signaling during RPC proliferation and maintenance

Chapter 2:

Combinatorial hedgehog and mitogen signaling promotes the in vitro expansion but not retinal differentiation potential of retinal progenitor cells

2.0 Combinatorial hedgehog and mitogen signaling promotes the in vitro expansion but not retinal differentiation potential of retinal progenitor cells

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2.1 Author Contribution Statement

R.R. designed experiments, collected, analyzed and interpreted data (Figures 2-3, 2-4, 2-6 & 2-7, Supplemental Figures 2-1 & 2-2, Table 2-1), and wrote the manuscript. Y.P. designed experiments and collected and analyzed data (Figures 2-1, 2-2 & 2-5, Supplemental Figure 2-2, Table 2-1). M.A. assisted with the collection and analysis of data. A.J.M. and K.Y collected and analyzed microarray data (Supplemental Table 2-1). V.A.W. provided financial support, designed experiments, analyzed and interpreted data and wrote the manuscript.

2.2 Abstract

Purpose: The *in vitro* expansion of multilineage competent primary neural progenitor cells is typically limited. Hedgehog (Hh) signaling is required *in vivo* for the maintenance of stem cell (SC) and progenitor populations in the central nervous system, including the retina. Here we investigated the impact of Hh signaling on *in vitro* expansion of perinatal mouse retinal progenitor cells (RPCs).

Methods: Perinatal mouse retinal cells were treated with combinations of Hh-agonist, EGF/FGF2 and the cultures were assayed for long term growth, gene expression and dependence on Gli2. Differentiation was assessed in monolayer cultures, following *in vivo* transplantation and in cellular re-aggregates.

Results: Using a combination of Hh agonist, EGF and FGF2 we were able to establish long term RPC cultures (termed Hh-RPCs). The ability of this combinatorial signaling approach to block quiescence of these cells was not associated with altered Tp53/Mdm2 levels or Hh-EGF cooperativity gene expression. Efficient Hh-RPC expansion and monolayer culture establishment requires Gli2, as Hh-RPCs derived from Gli2 knock-out retinal tissue fail to generate cultures that can be passaged long term *in vitro*. Hh-RPCs retain competence for neurogenic and gliogenic differentiation *in vitro*. However, they fail to engraft and differentiate into retinal cell types following *in vivo* transplantation to the eye or *in vitro* when, mixed with acutely dissociated perinatal retinal cells.

Conclusions: Our data show that combining Hh and mitogen signaling is sufficient to promote the expansion of RPCs *in vitro*, but it is insufficient to maintain competence of these cells for retinal differentiation.

2.3 Introduction

The neural retina is an especially tractable system for investigating the regulation of cell diversification in the central nervous system. The six neuronal and one major glial cell type in the adult retina are generated in an invariant, but overlapping, sequence by multipotential retinal progenitor cells (RPCs)(Livesey and Cepko, 2001). The temporal competence of RPCs to generate the different retinal lineages is regulated largely cell intrinsically, but the final cell repertoire is also impacted by cell extrinsic cues (Cayouette et al., 2006). Because it contains a mixture of progenitors and differentiating post-mitotic cells, the heterogeneity of the retina presents a challenge for isolating the effects of environment on the developmental progression of progenitors. Moreover, the typically small sample sizes of primary tissue makes larger scale biochemical analysis on purified cell populations difficult. Furthermore, there is a need for efficient generation of large pools of clinically relevant multipotent RPCs for transplantation based replacement therapies to treat retinal degenerative diseases. Thus, *in vitro* methods to generate a large pool of purified multipotential RPCs for studies of cell diversification and therapeutics would represent a significant advance in the field.

RPCs from a number of cell sources, including the fetal retina, adult ciliary margin, and embryonic stem cells can be induced and/or expanded in culture (Lamba et al., 2008). Typically, epidermal growth factor (Egf) and fibroblast growth factor 2 (Fgf2) are used as the primary mitogens in these culture media, as they have been shown to be mitogenic for rodent RPCs (Anchan et al., 1991; Lillien and Cepko, 1992). However, RPC expansion is highly variable (Ahmad et al., 1999; Czekaj et al., 2012; Engelhardt et al., 2004; Qiu et al., 2004; Yang et al., 2002). We reasoned that activation of physiologically-relevant anti-differentiation pathways would be an effective strategy to improve RPC proliferation *in vitro*. Sonic hedgehog (Shh) signaling from ganglion cells (GCs), the projection neurons of the retina, is essential for maintenance of the RPC pool *in vivo* (Wang et al., 2005). Here we show that the combination of Hh agonist (Hh-Ag), Egf and Fgf2 promotes the long term expansion of

undifferentiated mouse RPCs from the perinatal retina. Moreover, efficient Hh-RPC cultures depend upon a Hh-Gli2 signaling axis, the major Gli transcription factor required for Hh target gene induction and proliferation in the retina (Wall et al., 2009). Expanded Hh-RPCs retain competence to differentiate into pan-neurons and glia, however, they fail to engraft and differentiate into retinal neurons when transplanted *in vivo* and when co-cultured with perinatal retinal tissue *in vitro*. Thus, while supplementation with Hh agonist and mitogens represents a facile method of long term expansion of multipotent RPCs *in vitro*, it is not sufficient to maintain competence for retinal lineage differentiation of these cells.

2.4 Materials and Methods

2.4.1 Mice and retinal cell culture

Neonatal C57BL/6 mice were used for *in vivo* transplantation and as the source of retinal tissue for cell culture. $Gli2^{+/-}$ transgenic mice (obtained from A. Joyner, Sloan-Kettering Institute, New York, NY (Mo et al., 1997)) were maintained on a CD1 background and littermates were used as controls in analyses involving mutant mice. Transgenic mice were coupled in the late afternoon and the presence of a vaginal plug the next morning was considered as embryonic day 0.5 (E0.5). Animals were cared for and handled according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. For retinal explants, eyes from postnatal day 0 (PN0) or E18.5 mice were dissected in CO_2-independent DMEM medium (GiBCo), the RPE, sclera and lens were removed, the neural retinas were transferred to a 13 mm polycarbonate filter (0.8 mm pore size, Nucleopore), flattened and cultured with the ganglion cell layer facing up in 24 well plates at 8% CO_2 and 100% humidity in wells containing 0.5 ml of serum-free retinal cell culture medium (SFRCM) (DMEM/F12 (1:1), 10μg/ml insulin, 100 μg/ml transferrin, 100 mg/ml bovine serum albumin (BSA Fraction V), 60 ng/ml progesterone, 16 μg/ml putrescine, 40 ng/ml sodium selenite, 25 μg/ml Gentamycin) supplemented with either 20 nM Smoothened agonist (Hh agonist, Hh-Ag, Frank-Kamenetsky et al., 2002; a kind gift from Curis), 25 ng/ml human EGF (Sigma) or 10 ng/ml human FGF2 (Sigma). The concentration of Hh-Ag used in the explant cultures was determined in previous dose response experiments of Hh-Ag-induced proliferation in E18 retinal explants. After 2 days, the retinal explants were digested in 1 ml of trypsin solution (0.75 μg/ml, Sigma) at 37 ^{0}C for 10 minutes. The digestion was stopped by the addition of 1 ml trypsin inhibitor (1 mg/ml in SFSCM) and triturated to single cells. The cell suspension was centrifuged at 1500rpm for 5 minutes and the pellet was re-suspended in serum-free stem cell culture medium (SFSCM) (DMEM/F12 (1:1), 6 ng/ml progesterone, 5

ng/ml selenium, 100 mg/ml transferrin, 9.5 mg/ml putrescine, 250 mg/ml insulin, 25 ng/ml human EGF, 10 ng/ml FGF2, 2 mg/ml Heparin (Sigma)(Tropepe et al., 2000)) supplemented with 5 nM Hh agonist. KAAD Cyclopamine (Calbiochem) was dissolved in DMSO or Ethanol. The cells were cultured in 6-well plates at the density of 5-10 $X10^5$ cells per well in 2 ml of SFSCM or in 24-well plates at a density of 5-$10X10^4$ cells per well in 0.5 ml of SFSCM. The medium was refreshed every 2 or 3 days. After two weeks, a monolayer formed and the cultures were passaged every 2-3 days by mechanical trituration to obtain single cells that were then diluted 1:3 with fresh culture medium. Differentiated neurons did not survive under these culture conditions and were lost upon serial passaging of the cultures. To label Hh-RPCs in S-phase for transplantation assays, BrdU (200 μM) was added to the culture medium for 48 hours prior to harvesting for transplantation assays. Typically, >80% of cells were labeled. Hh-RPCs were cloned by plating 100 cells per well in laminin and PDL-coated wells of 24 well plates. After 15 days, isolated single spheres were selected and transferred to uncoated wells in 24 well plates, which were then passaged as monolayers.

2.4.2 In situ hybridization and immunohistochemistry

In situ hybridization (*ISH*) was performed as previously described (Wang et al., 2005) using digoxigenin-labeled antisense RNA probes corresponding to mouse *Jagged 1*, *CyclinD1* and *Math5*. Hh-RPCs were grown on glass coverslips and the *ISH* reactions were performed in 24 well plates. A detailed description of the immunostaining conditions for cells and tissue sections is provided in the Supplementary Methods. The following primary antibodies were used in this study: mouse anti-Nestin (1:200, RDI); mouse anti-CyclinD1 (1:300, Santa Cruz); mouse anti-Ki67 (1:100, BD Pharmingen); rabbit anti-PCNA (1: 500, Santa Cruz); rabbit anti-CyclinD3 (1:100, Santa Cruz); sheep anti-Vsx2/Chx10 (1:2000, a kind gift from Rod Bremner, University Health Network, Toronto Ontario); mouse anti-Pax6 (1:6 of tissue culture supernatant, Developmental Studies Hybridoma Bank); rabbit anti-GFAP (1:2000,

Sigma); mouse anti-Map2 (1:200, Synaptic Systems); rabbit anti-Sox2 (1:1000 Millipore); mouse anti-β3-tubulin/Tuj1 (1:200, Stem cell Technology), mouse anti-GS (1:400, BD Biosciences); mouse anti-BrdU (1:100, Becton Dickinson); sheep anti-BrdU (1:400, Gene Tex); rabbit anti-recoverin (1:1000, Chemicon). The following secondary antibodies were used: Alexa488 or Alexa568-conjugated goat anti-rabbit or goat anti-mouse antibodies and Alexa 647-conjugated donkey anti-rabbit or donkey anti-mouse IgG (all purchased from Jackson Immunolabs). Nuclei were stained with Hoechst 33342 or 4',6'-diamidino-2-phenylindole (DAPI). All sections were viewed under a Zeiss Axioplan microscope and digital images were captured using an Axio Vision 2.05 (Zeiss) camera and processed with Adobe Photoshop®.

2.4.3 Western blot

Protein was extracted from treated retinal explants or Hh-RPCs using 1x RIPA buffer (Millipore) with cOmplete Mini EDTA-Free protease inhibitor (Roche) and total protein was estimated using a Bradford assay (BioRad). 20μg of total protein was run on a 4-12% SDS-reducing gradient gel (BioRad), transferred to a nitrocellulose membrane and probed with mouse anti-MDM2 (1:500, Millipore), mouse anti-p53 (1:3000, Leica Biosystems) or mouse anti-GAPDH (1:20 000, Millipore). Donkey anti-mouse IgG horseradish peroxidase (HRP, 1:5000, Millipore) was used as a secondary antibody and protein signals were detected using Luminata Crescendo Western HRP substrate (Millipore). GAPDH was used as a control to confirm equal loading.

2.4.4 In vivo transplantation

BrdU labelled Hh-RPCs were washed with Hank's Balanced Salt Solution (HBSS, Sigma), centrifuged at 300 x g for 5 minutes and re-suspended in HBSS at a density of 10^8 cells/ml. Cells were

stored on ice and transplanted within 2 hours of harvesting. C57BL/6 recipient mice, aged P2 to P5, were anaesthetized with isofluorane/O_2 and injected intravitreally with Hh-RPCs according to the method described by Mizumoto et al. (Mizumoto et al., 2003). Briefly, the conjunctiva was cut with small curved scissors to expose the eyeball, a hole was made with a 25 gauge needle at the cornea-sclera junction and 1 µl of the cell solution (containing 10^5cells) was injected into vitreous using a 10 ml Hamilton micro syringe with a 32-G blunt needle. After the procedure the eyelid was closed with a drop of wound adhesive. Only one eye was injected in each mouse and control mice were injected with 1 µl of HBSS. The integration of donor cells was assessed 7 to 15 days after transplantation. The data are presented as the mean ± SEM from several independent experiments ($n \geq 3$). Statistical significance was evaluated by Student's t-test. $p < 0.05$ was considered statistically significant.

2.5 Results

To investigate whether sustained Hh signaling mediates similar effects on RPCs *in vitro* as it does *in vivo*, we compared neuronal differentiation and RPC marker expression in control and Hh-Ag treated retinal explants from postnatal day 1 (PN1) mice. Untreated retinal explants serve as a loss of function model, as they rapidly become Shh deficient because of the death of Shh-expressing GCs, the main source of Hh signaling in the retina (Wang et al., 2002). Exogenous Hh signaling had two major effects on cells in retinal explants. First, compared with control explants, treatment with Hh-Ag for 2 days inhibited neuronal differentiation, as assessed by immunohistochemistry with anti-Tuj1 antibodies (Fig. 2-1A). Second, Hh-Ag treatment altered the pattern of Pax6 and CyclinD3 expression, two RPC markers. The intensity of Pax6 and CyclinD3 immunoreactivity was increased in Hh-Ag-treated explants compared with the controls (Fig. 2-1A). Moreover, $Pax6^+$ and $CyclinD3^+$ cells were distributed throughout the Hh-Ag-treated explant, compared to the equivalent stage *in vivo* (PN1), where cells expressing high levels of CyclinD3 are located in the ciliary body at the periphery of the eyecup and the intensity of Pax6 immunoreactivity in the neuroblast layer is low (Fig. 2-1A). These short-term effects of Hh-Ag on Pax6 and CyclinD3 expression are not a general consequence of mitogenic stimulation, as they were not observed in explants treated with Egf or Fgf2 (Fig. 2-1A).

Quantitative analysis of single cell dissociates from Hh-Ag-treated retinal explants and acutely dissected retina, which represents cells exposed to endogenous Shh signaling, revealed that Hh-Ag treatment *in vitro* increased the proportions of $Pax6^+$ and $CyclinD3^+$ cells by 8 and 35-fold, respectively compared with the PN1 retina (Fig. 2-1B). Although Pax6 is also expressed in a subset of retina neurons, we determined that the majority of the $Pax6^+$ cells in Hh-Ag-treated

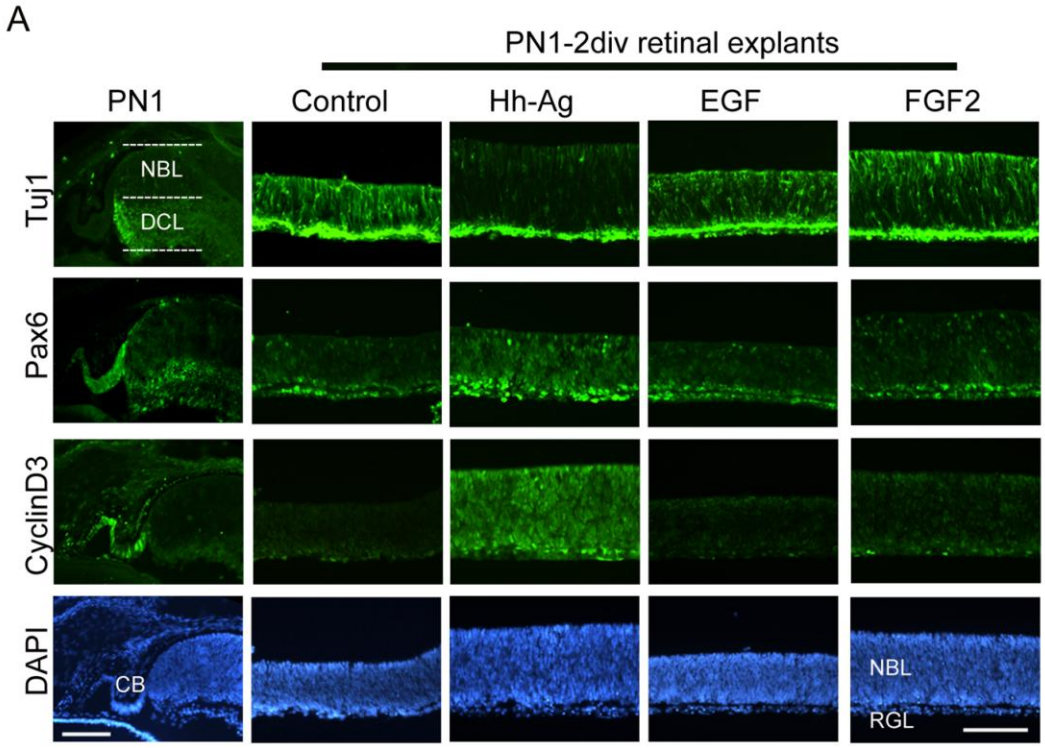
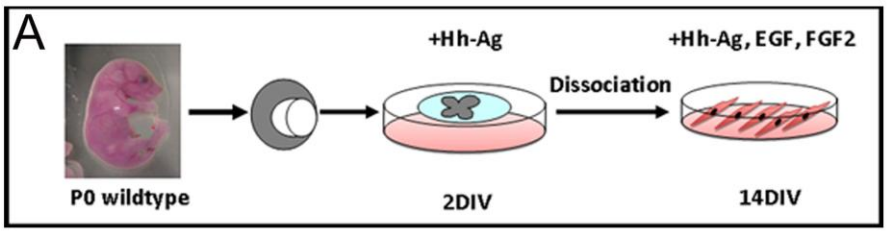
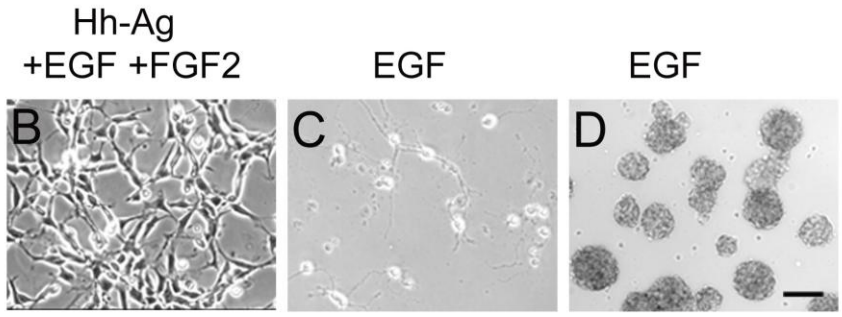

A
PN1-2div retinal explants
PN1
Control
Hh-Ag
EGF
FGF2
Tuj1
Pax6
CyclinD3
DAPI
NBL
DCL
CB
NBL
RGL
B
PN1
PN1-2DIV
% of marker positive cells
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
*
*
Chx10
Pax6
CyclinD1
Ki67
CyclinD3
Nestin

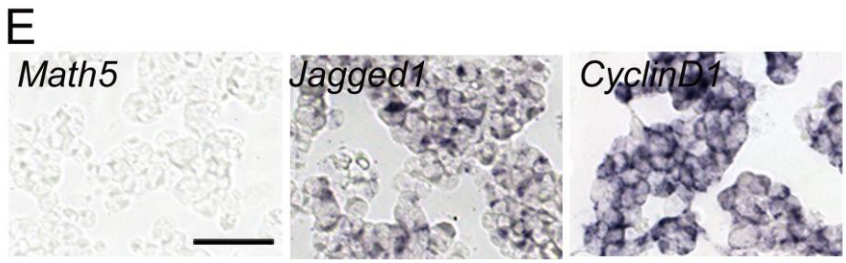
Figure 2-1. Inhibition of neuronal differentiation and rapid induction of progenitor marker expression in Hh-agonist-treated retinal explant cultures. (A) Immunohistochemistry of serial explant sections with antibodies specific for b-tubulin (Tuj1, top), Pax6 (second row), CyclinD3 (third row) and Dapi (bottom row) in the PN1 retina and PN1 retinal explants cultured for two days *in vitro* (DIV) in serum free medium under control conditions or in the presence Hh-Ag, EGF or FGF2. Note the reduced b-tubulin and the increased Pax6 and CyclinD3 immunoreactivity in Hh-Ag-treated explants compared with control, EGF or FGF2-treated explants. Scale bar, 100 mm. NBL, neuroblast layer; DCL, differentiated cell layer; RGL, retinal ganglion cell layer; CB, ciliary body. Scale bar, 100 mm. (B) Quantification of marker^{+} cells in single cell dissociates from the PN1 retina (n=3) and PN1 retinal explants cultured for 2 DIV with Hh-Ag (n=3). * $p < 0.001$.

explants were progenitor cells, as they co-stained with PCNA, a marker of cycling cells (%$PCNA^+Pax6^+/Pax6^+$ 88.0±2.7 n= 3). There was no significant difference in the proportion of cells expressing cell cycle markers and Vsx2 (Chx10) in the Hh-Ag-treated explants and the PN1 retina, (Fig. 2-1B). Thus, the enrichment for CyclinD3 and Pax6 expression in explants following *in vitro* exposure to Hh-Ag likely reflects the induction of these markers in the explants as opposed to the expansion or altered survival of a distinct subset of cells in the explants.

The enrichment of $Pax6^+$ and $CyclinD3^+$ cells in Hh-Ag treated explants prompted us to investigate the effect of sustained Hh pathway activation on the growth of RPCs outside of the explant environment. Explants were primed for 2 days with Hh-Ag, dissociated into single cells and cultured in serum free medium supplemented with Hh-Ag, Egf and Fgf2 or combinations of single and double growth factors (Fig. 2-2A). Only cultures supplemented with the combination of Hh-Ag, Egf and Fgf2 generated monolayer cultures that could be passaged routinely after 6 weeks (Fig. 2-2A, B and Table 2-1) and we refer to these monolayer cultures as Hh-RPCs. Consistent with previous reports (Angenieux et al., 2006; Young, 2005), we could establish monolayer cultures of RPCs by growing acutely dissociated retinal cells in medium supplemented with Egf/Fgf2 (referred to as 'mitogens'), however, this approach was not as reliable as the Hh-RPC method (Table 2-1), the cultures had a high tendency to quiesce (Fig. 2-2C) or to develop aggregates (Fig. 2-2C) by 14 days and could rarely be passaged after 6 weeks (Table 2-1). Treatment with the Hh pathway antagonist, cyclopamine, prevented monolayer formation and inhibited proliferation in retinal explants, confirming the specificity of the Hh-Ag in this context (Supplementary. Fig. 2-1).


A
+Hh-Ag
+Hh-Ag, EGF, FGF2
Dissociation
P0 wildtype
2DIV
14DIV


Hh-Ag
+EGF +FGF2
EGF
EGF
B
C
D


E
Math5
Jagged1
CyclinD1

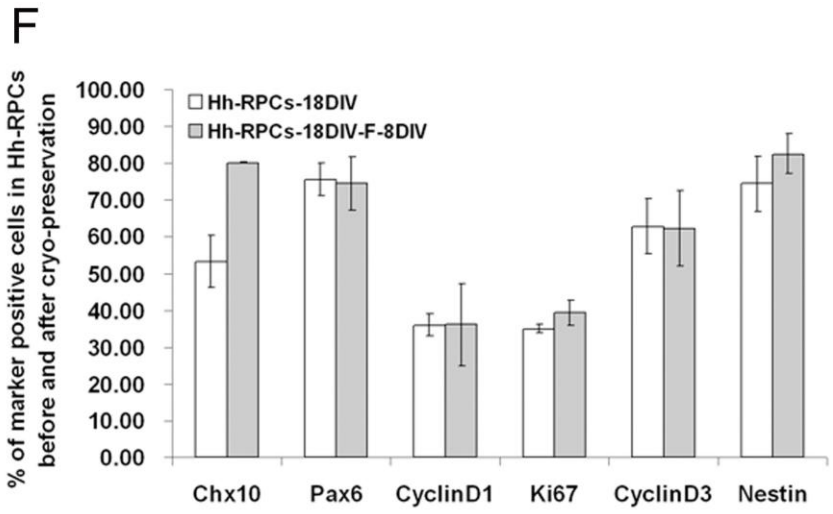

F
% of marker positive cells in Hh-RPCs before and after cryo-preservation
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Hh-RPCs-18DIV
Hh-RPCs-18DIV-F-8DIV
Chx10
Pax6
CyclinD1
Ki67
CyclinD3
Nestin

Figure 2-2. Propagation of RPCs in the presence of Hh-Ag and mitogens. (A) Schematic of methods to generate Hh-RPCs. P0 eyes are removed, the lens and retinal pigment epithelium dissected away from the retina and the tissue is placed on a polycarbonate filter in the presence of Hh-Ag for 2DIV followed by dissociation into single cells and further culturing for 14DIV in the presence of EGF, FGF2 and Hh-Ag. (B,C) Phase contrast images of dissociated cell cultures from mouse retinal explants treated for 2 days with Hh-Ag followed by dissociation and culture for 11 days with Hh-Ag, EGF and FGF2 (B) or EGF (C). (D) An example of the cellular aggregates that form in RPC cultures generated by direct dissociation of retina followed by monolayer culture in EGF and FGF2. (E) *in situ* hybridization for *Math5,Jagged-1* and *CyclinD1* mRNA in Hh-RPCs. (F) Comparison of marker expression in Hh-RPCs after culture for 18 days and following cryopreservation and culture for an additional 8 days. Scale bar, 50 mm.

Hh-RPCs exhibited a neuroepithelial morphology and based on immunohistochemistry, RT-PCR and microarray analysis expressed markers characteristic of undifferentiated neural progenitor and stem cells, including *Jagged1, CyclinD1* (Fig. 2-2E), CyclinD3, Nestin, Pax6, Vsx2, Sox2, Glutamine synthetase (GS), *Hes1*, *Notch1,* retinal progenitor markers *Vsx2/Chx10*, *Lhx2*, *Six3*, *Six6* (Fig. 2-2F, Supplementary Fig. 2-2A, B and Supplementary Table 2-1), proneural genes, including NeuroD1 and Mash1 (Supplementary Fig 2-2B), but not neurogenic genes or terminal differentiation markers (Fig. 2-2F, Supplementary Fig. 2-2B and data not shown). Since they were derived from perinatal retinal tissue, Hh-RPCs could also be Müller glia, which is consistent with expression of *ApoE* (Roesch et al., 2008) and *S100b* (Ueki et al., 2012) (Supplementary Table 2-1), and a pattern of Hh target gene expression ($Ptch1^{+}Ptch2^{+}Gli1^{-}$) that is typical of adult Müller glia (Black et al., 2003) (Supplementary Fig. 2B). However, they did not express the full complement of adult Müller glial markers, notably CRALBP/Rlbp1, suggesting that if they are Müller glia they are incompletely differentiated (data not shown). We could maintain Hh-RPCs in culture for over 1.5 years (the longest continuous culture that we attempted), and recover them following cryopreservation without significant changes in marker expression (Fig. 2-2F). Hh-RPCs were not transformed cells, as they required continuous growth factor supplementation for survival and they did not form tumours after *in vivo* injection to the eye (n = 41 *in vivo* transplantations to PN2-PN4 host analyzed 1 to 4 weeks post transplant). Thus, Hh-Ag priming in explants followed by dissociated cell culture in the presence of Hh-Ag, EGF and FGF2 is a reliable method for establishing cultures of neuroepithelial-like cells that are stable for long term passage *in vitro*.

Next, we sought to determine the molecular characteristics that distinguish Hh-RPCs from monolayers that were exposed to mitogens alone or mitogens and Hh-Ag. In the

Table 2-1. Comparison of the effects of growth factor and culture conditions in the establishment of RPC monolayer cultures.

Culture Condition	Number of Trials	Number of trials that resulted in the successful establishment of RPC monolayers[†]
[*]Direct dissociation		
Supplementation with EGF+FGF2	9	2
Supplementation with Hh-Ag+EGF+FGF2	4	3[‡]
2DIV explant step followed by dissociated cell culture		
Explant: Hh-Ag Dissociated culture: Hh-Ag+EGF+FGF2	20	20
Explant: Hh-Ag Dissociated culture: EGF + FGF2	10	0
Explant: Hh-Ag Dissociated culture: Hh-Ag+EGF	2	0
Explant: Hh-Ag Dissociated culture: Hh-Ag+FGF2	2	0
Explant: Hh-Ag Dissociated culture: Hh-Ag	2	0
Explant: Hh-Ag Dissociated culture: EGF	2	0
Explant: Hh-Ag Dissociated culture: FGF2	2	0
Explant: Hh-Ag Dissociated culture: No growth factors	2	0
Explant: No Hh-Ag Dissociated culture: Hh-Ag + EGF + FGF2	2	0
Explant: No Hh-Ag Dissociated culture: EGF + FGF2	2	0

[*] Retinas were dissected, enzymatically dissociated to single cells and plated on tissue culture plastic in the presence of the indicated growth factors.

[†]Success was determined by the establishment of a monolayer of cells with neuroepithelial morphology after 2 weeks in culture. A trial was considered a failure if there was no cell growth after 2 weeks or if cellular aggregates (spheres) formed in the cultures; in our experience the formation of aggregates was always associated with growth cessation a few weeks later.

[‡]one of these cultures could not be propagated after the 3 month mark, the other two were not passaged past 14 days.

cerebellum, Hh mediates granule neuron progenitor proliferation, in part, through Mdm2 dependent inhibition of Tp53 (Malek et al., 2011). Thus we investigated whether a similar Hh-dependent Mdm2-Tp53 axis was responsible for the long term culture of Hh-RPCs *in vitro*. RPC cultures were established, as described above and supplemented with Hh-Ag and mitogens or mitogens only at the monolayer stage of the protocol. After 7 days *in vitro*, Tp53 and Mdm2 levels were compared via western blot. Since cultures supplemented with mitogens alone are frequently quiescent by 14 days, we reasoned that molecular differences between the different growth factor supplementations would be apparent at the midway point of the culture period. Surprisingly, growth factor supplementation did not affect Mdm2 levels and Tp53 was undetectable in both conditions (Fig. 2-3A and data not shown). We next addressed whether the efficient propagation of Hh-RPCs was associated with differences in Tp53/Mdm2 levels at the explant priming stage. PN0 explants were stimulated with Hh-Ag, Egf or a combination of Hh-Ag and Egf for 2 days *in vitro* and analyzed for Tp53 and Mdm2 by western blot. Similar to Hh-RPC cultures, Tp53 protein was undetectable in retinal explants (Fig. 2-3B). Interestingly, Mdm2 levels in explants were increased to a similar extent by Hh-Ag or mitogen treatment (Fig. 2-3B), suggesting that mitogen stimulation, but not specifically Hh activation, promotes Mdm2 expression in explants.

Recently, synergistic Hh-EGF signaling has been shown to determine the oncogenic potential of basal carcinoma and pancreatic cancer initiation, in part through enhanced expression of *Sox2, Sox9, Jun, Cxcr4* and *Fgf19*, termed 'cooperation response genes' (Eberl et al., 2012). We hypothesized that synergistic Hh-mitogenic signaling may promote long term propagation of Hh-RPC *in vitro* through enhanced cooperation response gene expression. To test this hypothesis, we compared cooperation gene expression by qRT-PCR in RPC monolayer cultures and in retinal explants. We observed no significant difference in Hh-EGF cooperation response gene expression between RPC cultures treated with Hh-Ag

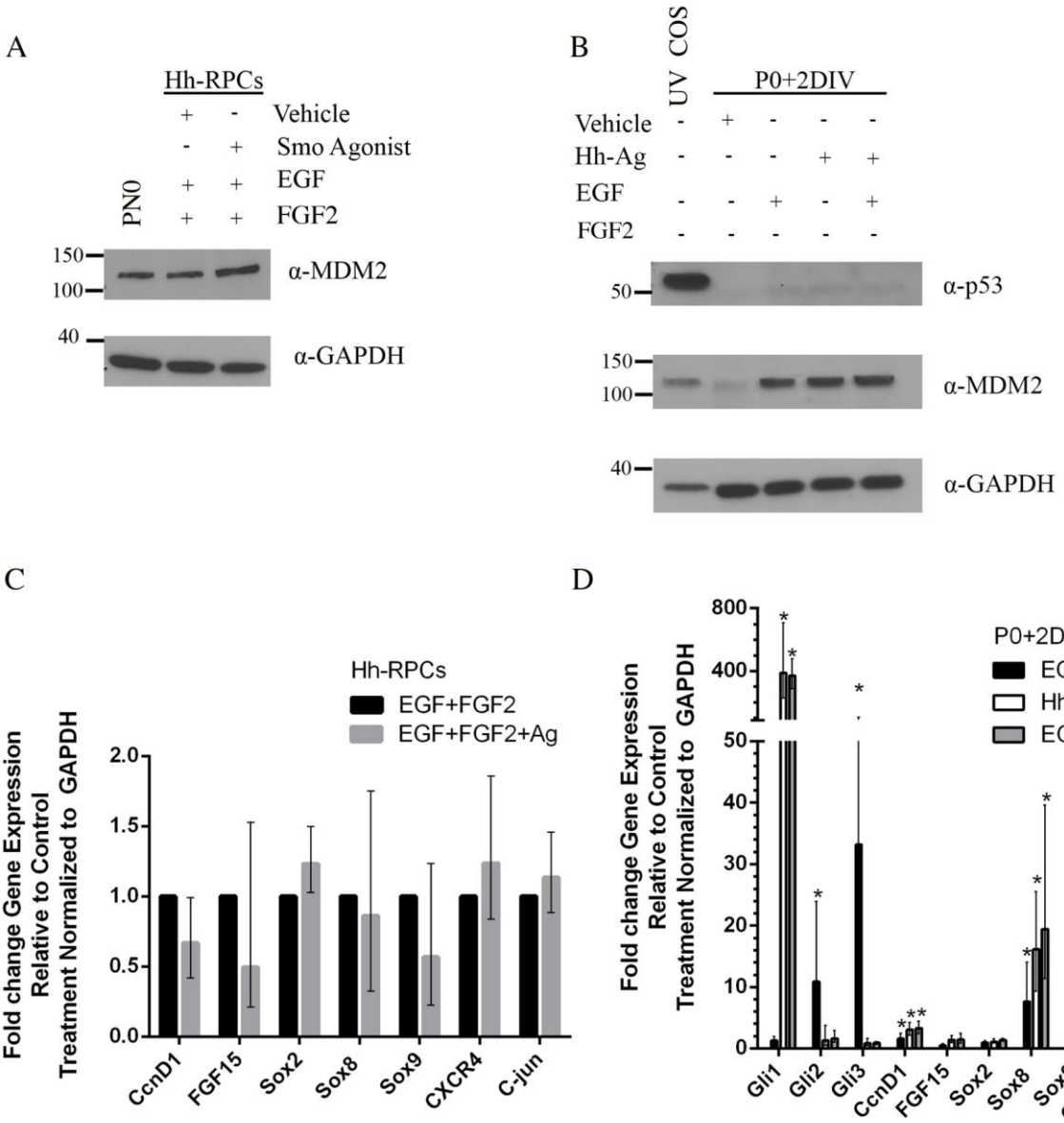
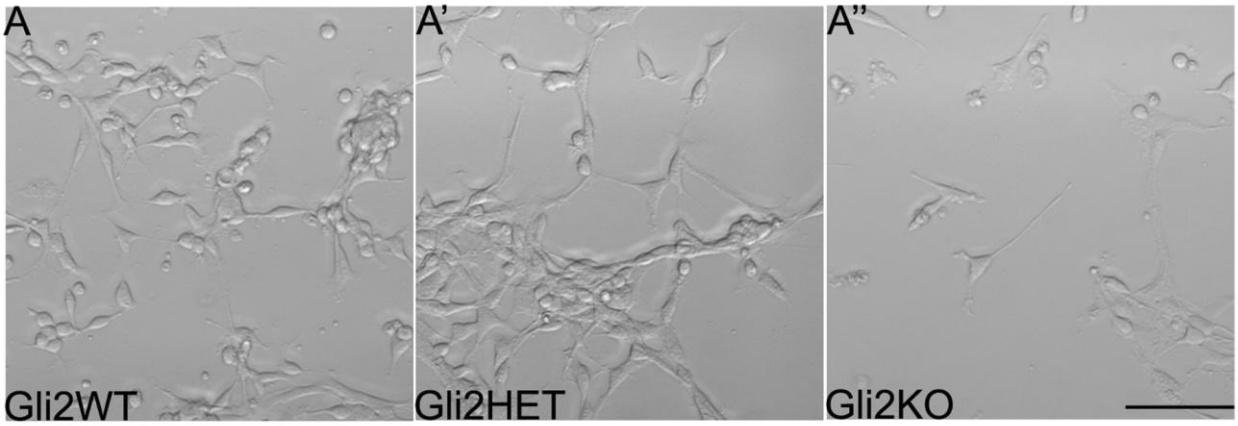

A
Hh-RPCs
+ - Vehicle
- + Smo Agonist
+ + EGF
+ + FGF2
PN0
150
100
α-MDM2
40
α-GAPDH
B
UV COS
P0+2DIV
Vehicle - + - - -
Hh-Ag - - - + +
EGF - - + - +
FGF2 - - - - -
50
α-p53
150
100
α-MDM2
40
α-GAPDH
C
Hh-RPCs
EGF+FGF2
EGF+FGF2+Ag
Fold change Gene Expression Relative to Control Treatment Normalized to GAPDH
2.0
1.5
1.0
0.5
0.0
CcnD1
FGF15
Sox2
Sox8
Sox9
CXCR4
C-jun
D
P0+2DIV
EGF
Hh-Ag
EGF + Hh-Ag
Fold change Gene Expression Relative to Control Treatment Normalized to GAPDH
800
400
50
40
30
20
10
0
Gli1
Gli2
Gli3
CcnD1
FGF15
Sox2
Sox8
Sox9
CXCR4
C-jun

Figure 2-3. The long term propagation property of Hh-RPCs does not correlate with p53/MDM2 protein levels or differences in Hh-EGF cooperative response genes at monolayer and explant stages of culture. (A) Western blot with lysate from Hh-RPCs treated with the indicated miogens for 7 days post dissociation or acutely dissected P0 retina. 25µg of total protein was run on a 4-15% SDS gel and probed with the indicated antibodies. (B) Western blot with lysate from acutely dissected PN0 retinal explants were cultured for 2 DIV in the presence of the indicated mitogen and/or Hh-Ag. 25µg of total protein was run on a 4-15% SDS gel and probed with the indicated antibodies. 5µg of UV exposed COS cell lysate was used as a positive control for p53. (C) Hh-EGF signaling cooperative response gene expression in Hh-RPCs 7 days post dissociation measured using qRT-PCR. * indicated $p<0.05$ (D) Hh-EGF signaling cooperative response gene expression in 2DIV treated retinal explants measured using qRT-PCR. * indicated $p<0.05$

and mitogens or mitogens alone after 7 days or in explants cultured for 2 days in the presence of Hh-Ag or Egf (Fig. 2-3C, D). Taken together, the long term-propagation property of Hh-RPCs is not correlated with altered expression of Hh-EGF cooperation response genes in explants or differences in Tp53/Mdm2 protein levels at the monolayer or explant stages.

The transcriptional output of the Hh pathway is mediated by the combinatorial activity of Gli1, Gli2 and Gli3 Zinc finger transcription factors (Robbins et al., 2012). Maximal Hh-mediated progenitor proliferation and target gene induction in the retina is dependent upon Gli2 (Wall et al., 2009) and therefore, we reasoned that Gli2 may also have a similar role in the *in vitro* expansion of Hh-RPCs. We generated RPC cultures from *Gli2* knock-out (*Gli2*KO), heterozygote (*Gli2*HET) or wild type (*Gli2*WT) littermates and observed that *Gli2*KO cultures consistently failed to establish a monolayer by 14 days *in vitro* (Fig. 2-4A-A''). Cell quantification at 14 days confirmed that there was a significant decrease in the total cell number in cultures established from *Gli2*KO retinae, compared with *Gli2*WT or *Gli2*HET (Fig. 2-4B), suggesting that Gli2 is required for the efficient establishment of Hh-RPCs *in vitro*.

To investigate the competence of Hh-RPCs to differentiate into retinal neurons and glia, we exposed Hh-RPCs to standard monolayer-based differentiation conditions that lack mitogens and contain low levels of serum (Tropepe et al., 2000) and stained the cells with cell-type specific markers. Hh-RPCs differentiated into cells that expressed pan neuron and astrocytic cell markers and this neurogenic potential required continuous exposure to Hh-Ag under growth conditions (Fig. 2-5). However, these conditions did not promote the differentiation of Hh-RPCs to retinal neurons or adult Müller glia, as we were unable to detect the expression of retinal cell type specific markers, including Rhodopsin (rods), Recoverin (photoreceptors, subset of bipolars), Syntaxin (amacrine), PKC (rod bipolars) and Calbindin (subset of amacrines, horizontal cells), CRALBP/Rlbp1 (Müller glia) (data not shown). RPC differentiation is context dependent (Ikeda et al., 2005); therefore, we monitored the differentiation of Hh-RPCs following exposure to a retinal environment. BrdU-labelled Hh-RPCs were transplanted into


A
A'
A''
Gli2WT
Gli2HET
Gli2KO

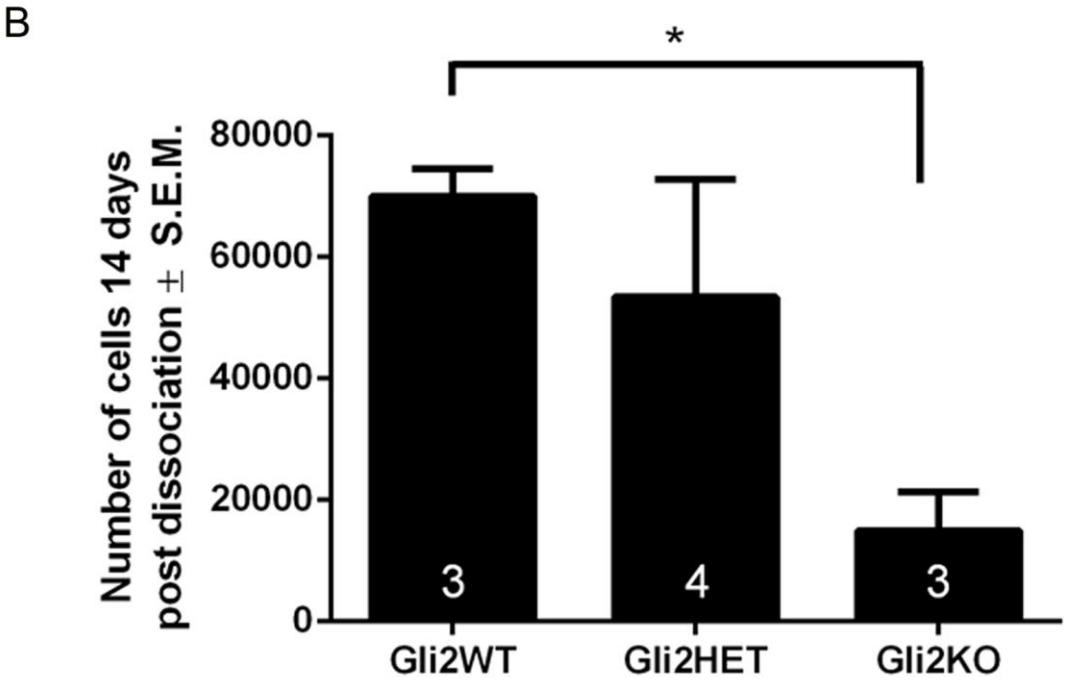

B
*
Number of cells 14 days post dissociation ± S.E.M.
80000
60000
40000
20000
0
3
4
3
Gli2WT
Gli2HET
Gli2KO

Figure 2-4. Gli2 is required for the propagation and outgrowth of Hh-RPCs. Hh-RPC cultures were established from the retinas of E18.5 mice of the indicated genotypes. Acutely dissected E18.5 Gli2 null retinae were individually dissected and cultured for 2DIV in the presence of Hh-Ag. After 2 days *in vitro*, retinal explants were individually dissociated, counted and plated at $5x10^4$cells per well and treated with Hh-Ag, EGF and FGF2. After 14 days, total live cell numbers were counted in duplicate. (A) Representative pictures of Gli2WT, Gli2HET and Gli2KO Hh-RPC cultures at 14 days post dissociation. (B) Quantification of total live cell numbers. Sample size indicated on bars in graph. * denotes $P<0.05$.

the vitreous of PN3 mouse eyes and after 14 days *in vivo* the tissues were harvested and processed for IHC for BrdU and cell type specific markers. In contrast with previous *in vivo* transplantation studies of mitogen expanded rodent RPCs or Müller glia to the perinatal retina (Canola and Arsenijevic, 2007; Das et al., 2006), we observed extensive BrdU staining in the inner and outer nuclear layers of the retina that co-labelled with every retinal cell type specific marker, with the exception of ganglion cell (GC) markers, in the correct location and morphology for that lineage (data not shown).

Since a similar degree of cell integration of transplanted cultured RPC marked with other lineage tracers has not been reported previously (Canola and Arsenijevic, 2007; Chacko et al., 2000; Das et al., 2006; MacLaren et al., 2006), we were prompted to consider the possibility that the BrdU labelling in Hh-RPC transplanted retina represents BrdU incorporation of host cells from unincorporated BrdU in the injection solution or transfer from donor cells. BrdU transfer from dead transplanted cells has been reported (Burns et al., 2006; Coyne et al., 2006) and the PN3 retina contains dividing progenitors, which could incorporate the label. To determine if BrdU from dead Hh-RPCs can be incorporated into host retinal tissue *in vivo*, BrdU labelled Hh-RPCs were repeated freeze-thawed prior to injection and loss of viability was confirmed by trypan blue staining (trypan blue+ = dead Hh-RPCs). Live, dead Hh-RPCs and a cell free preparation, used as a control for residual BrdU carry over from the cell media, were injected into PN3 mouse eyes and processed for IHC 7 days post surgery. BrdU labeling was not observed in retinae injected with the cell free preparation, ruling out any residual carry over of BrdU from labelling *in vitro*. Surprisingly, retinas transplanted with dead or live Hh-RPCs exhibited a similar high degree of BrdU labelling (Fig. 2-6), demonstrating that host tissue can incorporate BrdU from dying RPCs and that BrdU is not an adequate label to track transplanted Hh-RPCs.

As an alternative cell tracing method to track Hh-RPCs, we attempted to transfect Hh-RPCs with replication incompetent retrovirus expressing GFP, but Hh-RPCs could not be infected efficiently (data not shown). Therefore, we used nucleofection (Gartner et al., 2006), to transiently transfect Hh-RPCs with an expression vector encoding GFP under the control of the ubiquitin C promoter (33.0%

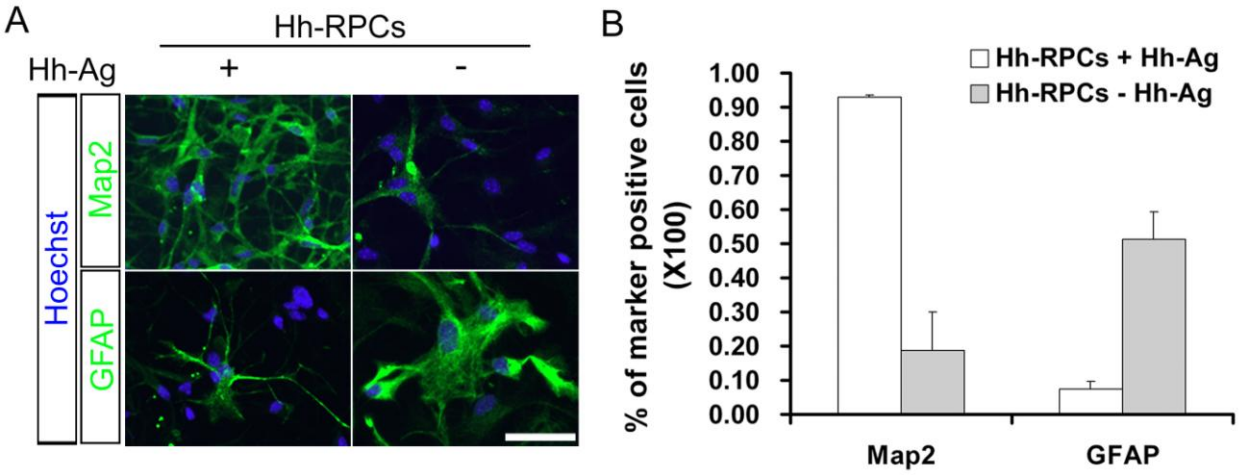

A
Hh-RPCs
Hh-Ag
+
-
Hoechst
Map2
GFAP
B
Hh-RPCs + Hh-Ag
Hh-RPCs - Hh-Ag
% of marker positive cells (X100)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Map2
GFAP

Figure 2-5. Continuous Hh pathway stimulation promotes the neurogenic competence of Hh-RPCs. (A) Analysis of Hh-RPCs grown in differentiation conditions and immunostained for MAP-2, a neuronal marker, and GFAP, an astrocyte marker. Hh-RPCs that were cultured for 31 days were cultured for an additional 2 weeks with or without Hh-Ag in medium supplemented with EGF and FGF2. To assay for differentiation, the cells were transferred to wells coated with PDL+ laminin and cultured in medium containing 1% FCS in the absence of mitogens or Hh-Ag for 7 days. Scale Bar: 50μm. (B) Quantification of neurons and glia follow differentiation of Hh-RPPs that were grown for two weeks prior to differentiation in medium with and without Hh-Ag.

GFP+/DAPI+ cells 24hr post-nucleofection, Hh-RPC^{GFP+}). Following subretinal transplantation into PN3 mouse eyes, injected Hh-RPC^{GFP+} cells were observed at the site of injection but not within the host retina (data not shown). To circumvent the limited engraftment of these cells, we adopted a pellet culture method where Hh-RPCs^{GFP+} were mixed with dissociated PN0 retinal cells, centrifuged into pellets and cultured on filters for 7 days. Pellet cultures are an established method for monitoring retinal differentiation *in vitro* (Watanabe et al., 1997), and the direct mixing of Hh-RPCs and unlabelled retinal cells bypasses the integration step associated with transplantation, thereby allowing us to assay the differentiation potential of Hh-RPCs exposed to a retinal environment. After 7 days post mixing, the pellets were harvested and processed for IHC for GFP and cell type specific markers. Hh-RPCs^{GFP+} were present throughout the pellet tissue and exhibited complex neuron-like morphology, but did not express markers of photoreceptors or glia (Fig. 2-7A and data not shown). Although pellet cultures of retinal cells are not laminated, the cells are arranged into distinct rosettes that consist of inner (amacrine, bipolar, Müller) and outer (photoreceptor) nuclear layer retinal cells (Watanabe et al., 1997). Hh-RPCs^{GFP+} did not appear to be incorporated into rosettes (Fig. 7A) or to express photoreceptor (recoverin) (Fig. 2-7B) or glial (GFAP) (Fig. 2-7C) markers and instead were more commonly located in more acellular regions of the pellet (lumens of rosettes) or in close proximity to the polycarbonate culture filter (Fig. 2-7). The absence of GFAP expression in Hh-RPC^{GFP+} cells in pellets could reflect an inhibitory effect of this environment on GFAP induction and/or poor survival of GFAP+ derivatives of these cells. Taken together, although *in vitro* expanded Hh-RPCs do not express markers of differentiated retinal neurons or glia, they retain competence for pan neuron and glia differentiation *in vitro* that does not depend on signaling from differentiating retinal cells.

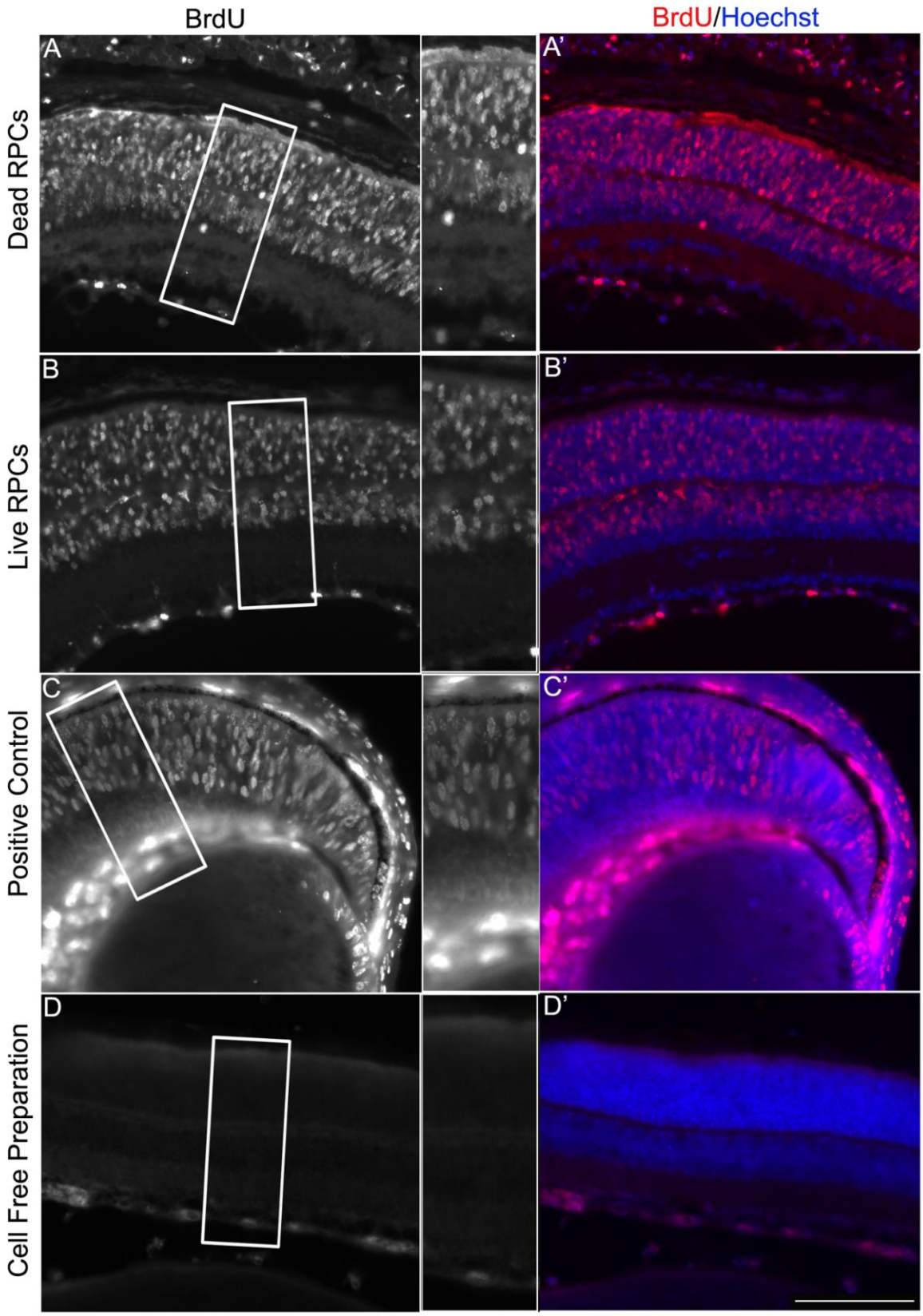

BrdU
BrdU/Hoechst
A
A'
Dead RPCs
B
B'
Live RPCs
C
C'
Positive Control
D
D'
Cell Free Preparation

Figure 2-6. Dead Hh-RPCs transplanted *in vivo* transfer the BrdU label to the host retinal tissue. 10^5 BrdU-labeled dead Hh-RPCs (A, A') or Live RPCs (B, B') were injected into the vitreous of P3 mice. After 7 days, the retinae were isolated, processed and stained with antibodies specific for BrdU (A,B) and a nuclear marker (Hoechst) (A', B'). BrdU labeled E14.5 wild type retina (C) was used as a positive control. BrdU staining in eyes injected with a cell free preparation (D) as a control for carry over of unincorporated BrdU. Boxed areas represent 1.5x zoom of panels A, B, C and D, Scale bar, 100 μm.

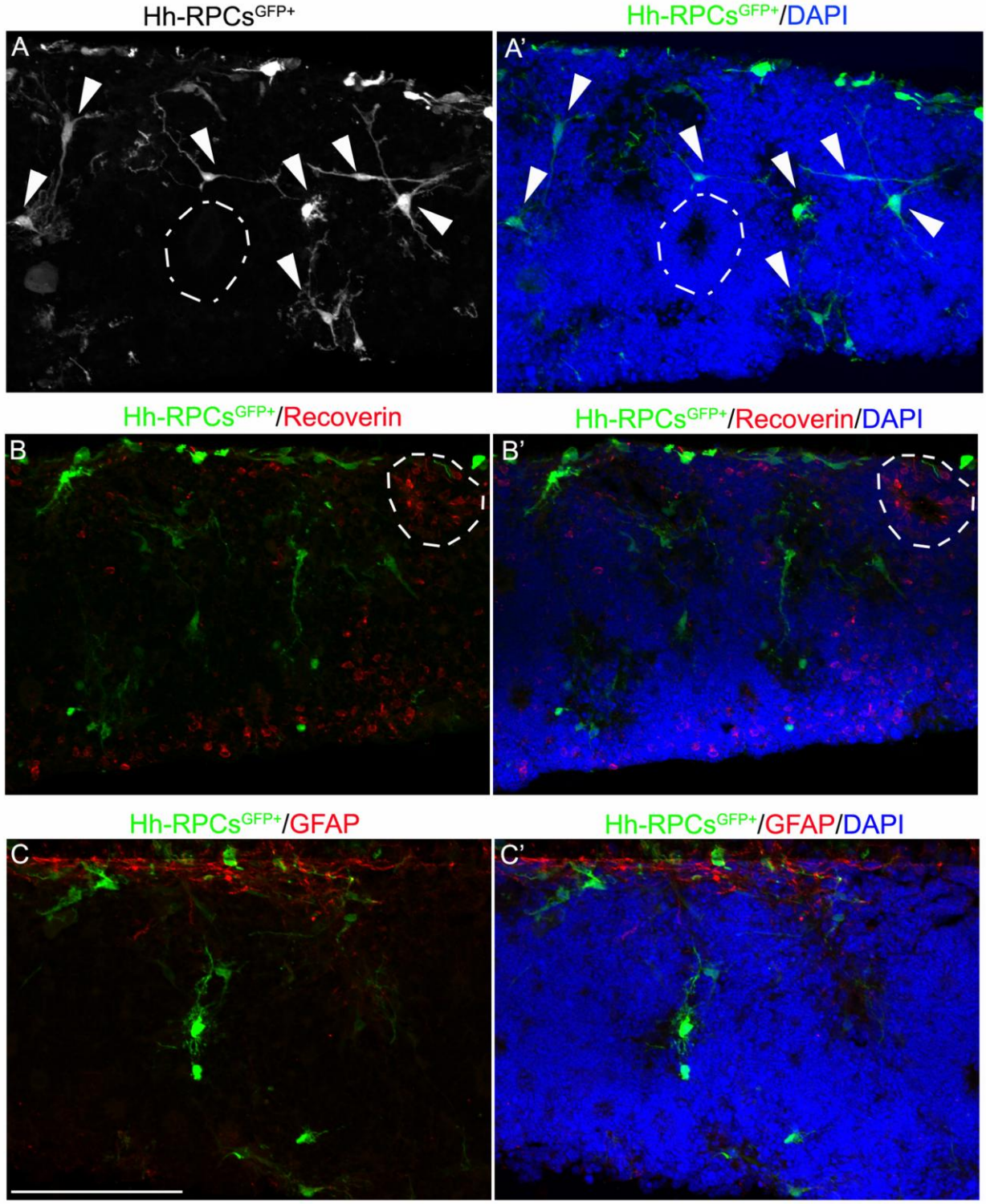

Hh-RPCsGFP+
Hh-RPCsGFP+/DAPI
A
A'
Hh-RPCsGFP+/Recoverin
Hh-RPCsGFP+/Recoverin/DAPI
B
B'
Hh-RPCsGFP+/GFAP
Hh-RPCsGFP+/GFAP/DAPI
C
C'

Figure 2-7. Hh-RPCs cultured in aggregates with neonatal wild type retinal cells adopt neuron-like morphology but do not express retinal cell markers. $2x10^4$ Gfp-transfected Hh-RPCs were mixed with $2x10^6$ acutely dissociated P0 wild type retinal cells and aggregated by gentle centrifugation. After 7 days *in vitro* pellets were fixed, cryosectioned and stained with a nuclear marker (DAPI) (A'- C') or antibodies specific for GFP, marking transfected Hh-RPCs (A-C, A'-C'), photoreceptors (anti-recoverin) (B, B') and activated Müller glia and astrocytes (anti-GFAP) (C, C'). Aggregated Hh-RPCs show variable branching patterns (indicated by arrow heads) (A, A') similar to neurons, but do not participate in rosette structures comprised of photoreceptors (indicated by dotted circles) (A,A', B, B'). Scale bar, 100 μm.

2.6 Discussion

Here we show that exposing retinal explants to Hh-Ag followed by dissociated cell culture in the presence of Hh-Ag and mitogens is a reliable approach for the establishment of monolayer cultures of RPCs that can be passaged long term and this effect appears to be dependent upon Gli2 levels. Hh-RPCs can give rise to generic neurons and glia in low serum differentiation conditions, but fail to give rise to retinal specific cell types, including photoreceptors, even when cultured in close proximity to retinal cells in a pellet culture. Our work also highlights the pitfalls of using BrdU to label transplanted cells into the retina, as we found that host tissue readily uptakes BrdU from injected cells, likely as a result of transfer from dead cells.

Synergy between Hh and EGF signaling has been reported in neural progenitor development in other brain regions (Palma et al., 2005) and is mediated through the p53 pathway (Malek et al., 2011), expression of Hh-EGF co-operation genes (Kasper et al., 2006), and stabilization of Gli2 (Riobo et al., 2006a). While the expansion of Hh-RPCs was not associated with changes in Tp53 or cooperation gene expression, it did require Gli2. In RPCs, Gli2 is required for induction of *CyclinD1* and *Hes1*, two key effector genes of Hh-induced proliferation in the mouse retina (Wall et al., 2009), an effect that is not mimicked by Egf or Fgf2 treatment in retinal explants (Wall and Wallace, unpublished observations)(Wall et al., 2009; Wang et al., 2005). The facile expansion of RPCs in response to combinatorial Hh-Ag and mitogen stimulation could, therefore, reflect a Gli2-dependent requirement for simultaneous activation of self renewal and cell cycle target genes for the maintenance of RPC self renewal. Sustained Notch signaling is also associated with persistence of progenitor cells with stem cell-like characteristics in the murine retina, however, the neurogenic competence of these cells has not been reported (Jadhav et al., 2006a).

While we show Hh-RPCs with neurogenic and gliogenic potential can be maintained *in vitro*, they do not differentiate into photoreceptors. This observation is consistent with a recent report describing the

lack of retinal differentiation capacity of Egf/Fgf expanded mouse RPCs (Czekaj et al., 2012), which was associated with the downregulation of expression of retinal identity genes. In contrast, the expression of several retinal identity genes was maintained in Hh-RPCs, including Vsx2, Pax6, Lhx2, Six3 and Six6. Although Hh-RPCs express several RPC and retinal identity markers, they could represent Müller glia, a cell type that exhibits neurogenic potential (Jadhav et al., 2009) and expresses several RPC markers (Fischer and Bongini, 2010). A Müller glial cell origin for Hh-RPCs is consistent with the observation that Hh pathway activation promotes Müller glial cell development in retinal cultures (Wan et al., 2007; Yu et al., 2006), and upregulates expression of markers of activated Müller glia, including CyclinD3, Pax6 and Vsx2 (Fischer and Reh, 2001). Moreover, Hh-RPCs express Müller markers, and exhibit an adult Müller-cell specific pattern of Hh target gene expression (Black et al., 2003). Hh-RPCs are, however, distinct from mature Müller glia because they do not express the full complement of Müller glia markers and they express *Jagged1*, which is normally restricted to non-neurogenic cells in the periphery of the retina. Irrespective of their classification as RPC or Müller glia, our findings together with those of Czekaj *et. al*. (2012), suggest that culture conditions affect the ability of these cells to differentiate into photoreceptors. One possibility is that the monolayer culture conditions could promote the re-specification of Hh-RPCs to cell types, such as hypothalamic progenitors, which are induced by higher levels of Hh signaling at the midline at the same rostral level as the eye field (Szabo et al., 2009). However, we failed to detect the expression of several hypothalamic markers in cultured Hh-RPCs.

Studies in animal models support the feasibility of cell-based transplantation strategies to restore vision in the diseased retina (Lamba et al., 2009; MacLaren et al., 2006); however, the development of efficient and reliable methods for the *in vitro* expansion of purified RPCs from a variety of stem cell sources is a significant barrier to clinical applications of this technology for the treatment of retinal degenerative diseases, such as age-related macular degeneration. Mimicking physiologically-relevant growth and cell fate signaling is a useful strategy for directing the differentiation of region specific neural progenitors and neuron subtypes from stem cells *in vitro* (Li et al., 2005; Yan et al., 2005). We reasoned

that combining physiologically relevant mitogenic and anti-differentiation signals would promote the *ex vivo* expansion of multipotential RPCs. While this strategy supports the expansion of a "generic' type of neural progenitor from the retina, it is not sufficient to maintain retina differentiation potential and suggests that to be successful, additional factors and intercellular interactions need to be considered in these *in vitro* manipulations.

2.7 Acknowledgements

We are grateful to Drs. Rod Bremner and Jack Saari for antibodies; Curis Inc. for Smoothened agonist; Drs. Rashmi Kothary, Dave Picketts and Rod Bremner for comments on the manuscript. We thank Chantal Mazerolle, Sherry Thurig and Andrew Ha for assistance with the transplantation experiments. This work was supported by operating grants to V. Wallace from the Canadian Institute of Health Research, Foundation Fighting Blindness Canada and Stem Cell Network of Canada.

2.8 Supplemental Material

2.8.1 Supplemental Methods

2.8.1.1 Immunohistochemistry

Cells and tissues (Hh-RPCs grown on glass coverslips, retinal explants, retinal tissue harvested from transplanted hosts) were fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PBS, pH7.4) for 15-30 minutes at room temperature or overnight at 4 ^{0}C, washed with PBS for 3 times (5 minutes each), and cryoprotected in 30% sucrose/PBS overnight. Tissues were embedded in OCT: 30% sucrose (1:1) and cryosectioned at 8 - 10 μm. Cells or tissue sections were blocked in 5% fetal calf serum (FCS) in TBLS with 0.1% Triton-X100 for 30 minutes and incubated with primary antibodies at room temperature for 2 hours. For BrdU immunostaining, the cells or sections were exposed to 2 N *HCl* for 20 minutes at 37 ^{0}C to denature DNA before the blocking step. Primary and secondary antibodies were diluted in blocking solution.

2.8.1.2 RNA extraction and RT-PCR analysis

Total RNA from Hh-RPCs and PN1 retinas was extracted using Trizol (Tri-reagent, Sigma) and 3μg RNA was used in first strand cDNA synthesis using the Superscript First-Strand Synthesis System according to the manufacturer's directions (Invitrogen). PCR amplification was performed using a standard procedure with Taq Polymerase (Invitrogen). Briefly, the cDNA, equivalent amount of 50 ng RNA was used in a 20-μl reaction. The cDNA was first denatured in 94 ^{0}C for 5 minutes and then following with 35 cycles of denatured at 94 ^{0}C for 30 seconds, annealing at 50 ^{0}C to 60 ^{0}C for 30 seconds

and elongation at 72 ^{0}C for 45 seconds. The primers and the size of PCR products used in this study are shown in Table 1.

2.8.1.3 In vitro Hh-RPC differentiation

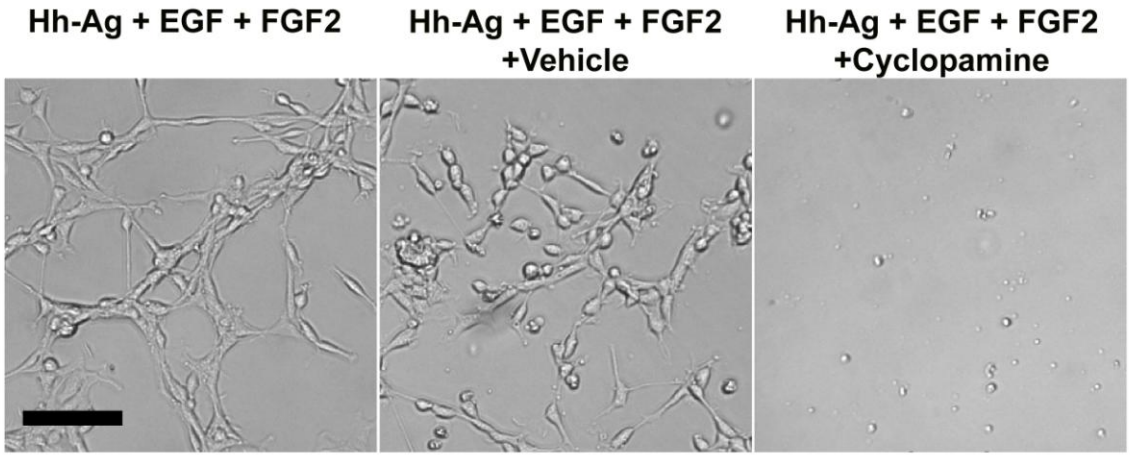
To test the competence of Hh-RPCs *in vitro*, 10^4 Hh-RPCs were plated in 24-well tissue culture plates coated by poly-D-lysine (PDL) (1 μg/ml) and laminin (50ng/ml) in stem cell culture medium supplemented with 1% FCS, but no EGF or FGF2 for 7 or 14 days, followed by IHC, as described above. For co-culture experiments, retinal explants from wildtype E18 or P0 mice were prepared as described above in SFRCM, with the GC layer oriented upwards and a suspension of BrdU-labeled Hh-RPCs (5-10 x10^4 cells) was pipetted on top of the explant. The culture medium was refreshed every 3 days and the co-cultures were harvested for IHC after 8 days.

2.8.1.4 Microarray Analysis

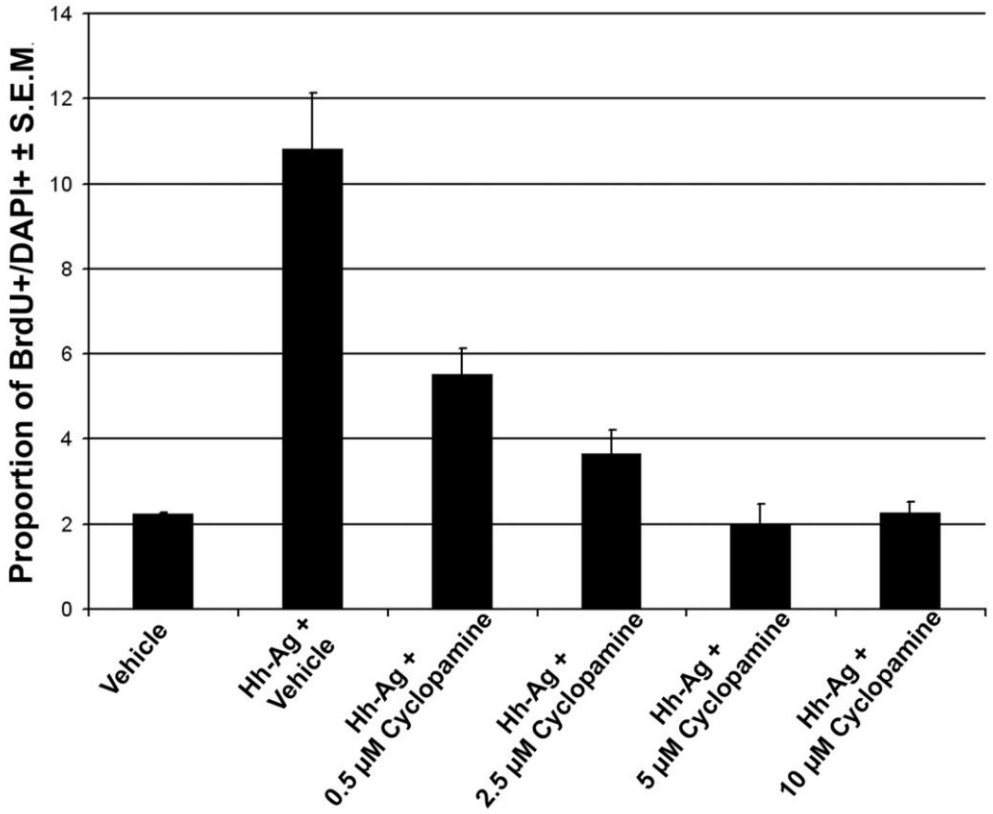
Gene expression profiling was performed on established monolayer Hh-RPCs cultured as described above. For each biologic replicate (n=2), RNA was isolated from Hh-RPCs using the RNeasy mini kit (Qiagen, Valencia, CA). 5 ug of total RNA (targets) were labeled with Cy5 or Cy3 using 3DNA Array 900 kits (Genisphere, Hatfield, PA) following the manufacturer's instructions. Samples were then hybridized to MEEBO 38.5K arrays (Microarrays Inc., Huntsville, AL). A dye-flip was performed (reversing which samples were labeled with Cy5 or Cy3) to correct for dye bias. Signals were quantified using the ScanArray express (Perkin Elmer, Waltham, MA) and ProScan Array Express software package (Perkin Elmer). A values ($\log_2$ average signal strength) were then determined for all probes. Probes (genes) with an average A above 9 ($\log_2$) were considered to have a significant signal relative to background to be scored as expressed.

2.8.2 Supplemental Figures

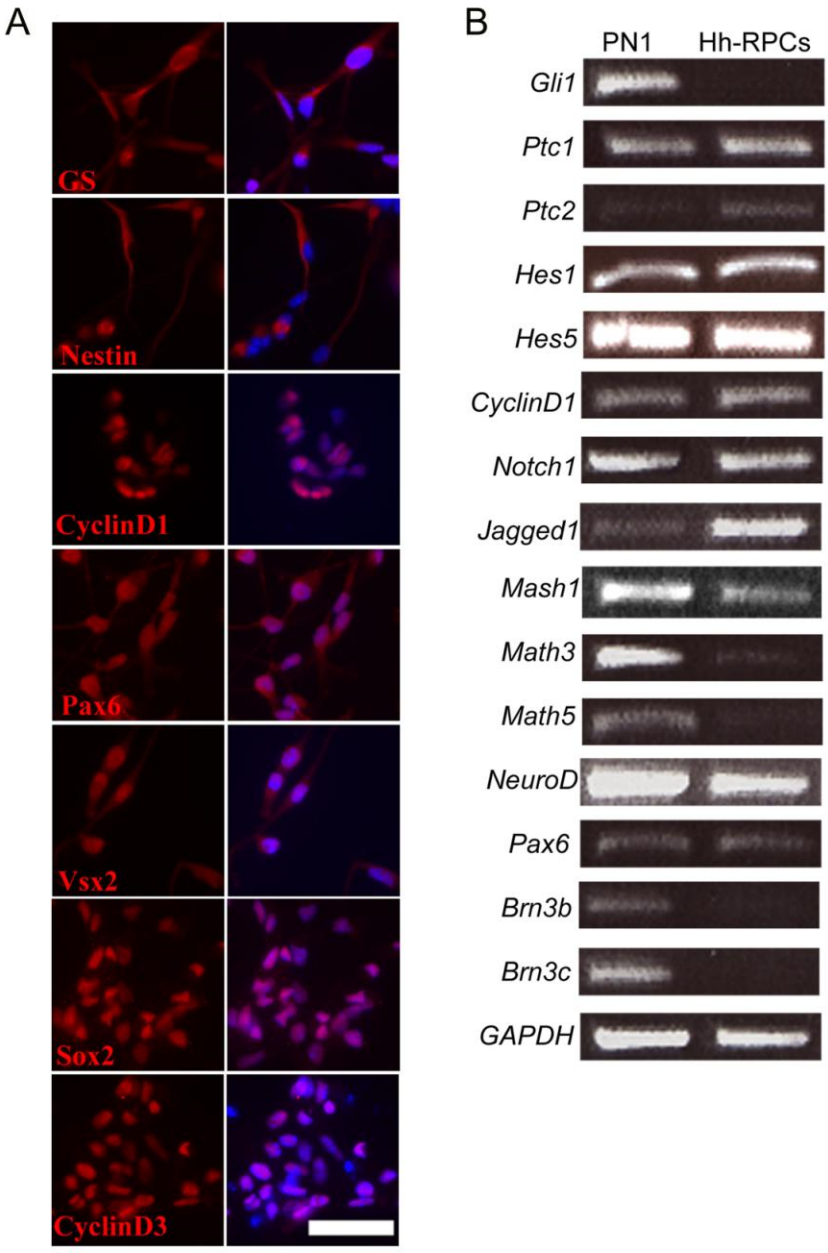
A


Hh-Ag + EGF + FGF2
Hh-Ag + EGF + FGF2 +Vehicle
Hh-Ag + EGF + FGF2 +Cyclopamine

B


Proportion of BrdU+/DAPI+ ± S.E.M.
0
2
4
6
8
10
12
14
Vehicle
Hh-Ag + Vehicle
Hh-Ag + 0.5 µM Cyclopamine
Hh-Ag + 2.5 µM Cyclopamine
Hh-Ag + 5 µM Cyclopamine
Hh-Ag + 10 µM Cyclopamine

Supplemental Figure 2-1. Treatment with SMO inhibitor, Cyclopamine, blocks monolayer formation and Hh-Ag dependent proliferation. (A) PN0 retinal explants were pre-treated with Hh-Ag, Hh-Ag + Vehicle or Hh-Ag + 5μM Cyclopamine for 2 days, dissociated and cultured for 14 days in presents of Hh-Ag + FGF2 + EGF, Hh-Ag + FGF2 + EGF + Vehicle or Hh-Ag + FGF2 + EGF + 5μM Cyclopamine. Scale Bar: 50μm. (B) PN0 retinal explants were cultured for 2 days in the presence of Vehicle, Hh-Ag + Vehicle or Hh-Ag + increasing concentrations of Cyclopamine. The proportion of BrdU+ cells were quantified in single cell dissociates from retinal explants.


A
GS
Nestin
CyclinD1
Pax6
Vsx2
Sox2
CyclinD3
B
PN1
Hh-RPCs
Gli1
Ptc1
Ptc2
Hes1
Hes5
CyclinD1
Notch1
Jagged1
Mash1
Math3
Math5
NeuroD
Pax6
Brn3b
Brn3c
GAPDH

Supplementary Figure 2-2. Marker and Gene expression pattern in Hh-RPCs. (A) IHC for the indicated markers on Hh-RPCs that were cultured for 14 days following recovery from cyropreservation. (B) RT-PCR analysis for the indicated genes in RNA isolated from the PN1 retina (lane 1) and Hh-RPCs (Lane 2). Primer sequences are reported in Supplementary table 1. Control reactions performed on samples untreated with reverse transcriptase did not give a signal.

2.8.3 Supplemental Tables

Supplementary Table 2-1. Raw gene expression profile data generated by microarray from established monolayer Hh-RPCs.

Retinal genes A>9

Accession	Symbol	Probe Sequence	A(Signal)
NM_010710	Lhx2	GAGCCCCACAGCCCTTCACAAACGACTCTTACCAACCTTTTCTAATGACTCGCCACCCCCTTCTCCCCGA	10.9
NM_016801	Stx1a	GTGCTGCCTTCTCACGGCCACCTCCCTTTACCCCTTCACCAAAGGTCTTGGTACAACCAGCTGCCCATTT	9.4
NM_020599	Rlbp1	AGGGCAGTGAGGCAGGGGAATGCCTTTCATCGGGAGATATGTGGGGCAGTTAAATTCCCACCTGCCTCTG	9.6
NM_011384	Six6	GACCCTAGGTTGGAAATTAGCCCAGATCTTCAAACAGCAATTTACTCAATCACCCGAATCAGCCCATAAA	12.12
NM_011381	Six3*	TCGGGCCGAGCGGGATGCGCTCGCTGGCCGAGCCCGGCTGCCCCACGCACGGCTCAAGCAGAGTCACCGT	10.47

Retinal genes A<9

Accession	Symbol	Probe Sequence	A(Signal)
NM_013833	Rax	AAGTCTGGCTTAGCTCGGGAAGTTTGCGTTTGAAGAAGAACCTGTTAGGGTTGAAGGTGCCATTCAGGGT	6.8
NM_008736	Nrl	GCCTCTCAGAGCTGAAGTGAGACCTCACAGGATAACAGGGAGCCGAGAGACTGTGACTTTTTGTGAATTA	5.9
NM_007770	Crx	CTTACTAGCCTATCAGGTCTCCCTTCCCTGGCTACCCAGAGTGAAACTGATTAAAAATGTGGATCCCACT	5.5
NM_013708	Nr2e3	ACTGGAATGAAAAGAAAAGGAAATGAGCAAAGAAAACCATAATAGACTGATAGAGAGAAAGCCAGAAGTC	6.2
NM_013708	Nr2e3	ACTGGAATGAAAAGAAAAGGAAATGAGCAAAGAAAACCATAATAGACTGATAGAGAGAAAGCCAGAAGTC	5.7
NM_138944	Pou4f2	ATCCAGCCAAGGCCCTCCTCGGAGAAGATCGCGGCCATCGCCGAAAAGCTGGATCTCAAGAAAAATGTGG	6.2
NM_138944	Pou4f2	TTGAGTGTTGTTTCATTGTCTTTCATTGAAGAGATGATTTTAATGTTTTACTGGCAAAGTATGCTGCTTT	5.2
NM_007586	Calb2	TCCTGCCGACCGAAGAGAATTTCCTTTTGTGCTTCAGGCAGCACGTGGGCTCCAGCGCTGAGTTTATGGA	8.8
NM_007586	Calb2	AGCTTGCTTAATGATCTAGCTGTCTTCTCAGAGCTACTGTGGAGGGTGACTGCCCTCTCCTTGTGTTCTT	6.1
NM_009788	Calb1	GCACTAAATGTCGATCCCAAAGAGGATAGTCCCAAGGAGGAAGTAGCAGTTCCTGATGAGGAATAAGGGT	6.0
NM_009788	Calb1	GTCATGTAATCTGTTATCAATTAGGGTAGAAAAGTAGATATCGTGAATCATTATAGCCGAAGGACTCTAA	5.7
NM_144841	Otx2	CAGCTCCAACTCAGCCTCCACTGTTACTAAAAAATAAAAATCGCTAGAGGAGCTCAGTCGCCACCTCTAC	6.7
NM_009107	Rxrg	AGCTTCCTCATGGAGATGTTGGAGACCCCACTGCAGATCACCTGAACCTCCTCAGCTGCAGCTTCCCCAC	7.95
BC029667	Otx2	CAGCTCCAACTCAGCCTCCACTGTTACTAAAAAATAAAAATCGCTAGAGGAGCTCAGTCGCCACCTCTAC	7.78
NM_144841	Otx2	GGGGCCTTAGAAGGGTCCATCAACCAGCAACCTGAAATGGACAAACCAATCTACTTAAGATTCTGTTATA	6.66

*second probe with low signal is likely due to poor binding kinetcs based on a BLAST alignment

Chapter 3:

A Notch-Gli2 axis sustains Hedgehog responsiveness of neural progenitors and Müller glia

3.0 A Notch-Gli2 axis sustains Hedgehog responsiveness of neural progenitors and Müller glia

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3.1 Author Contribution Statement

R.R. and V.A.W. designed the research and wrote the manuscript. R.R. performed the experiments and interpreted and analyzed data. M.A. provided critical assistance in the development of western blot techniques, performed qRT-PCR experiments and analyzed data. P.S.L. and D.J.P. assisted with electroporations. E.A.B. assisted with diagrams, provided helpful discussions and troubleshooting advice. C.C. provided technical assistance with cell culture techniques and troubleshooting advice. C.M. assisted with *in situ* hybridization and supported the performed experiments with critical advice and reagents. A.J.M. performed plasmid vector validation experiments and provided technical assistance. V.A.W. provided financial support for the project, critical conceptual design and interpretated of data.

3.2 Abstract

Neurogenesis is regulated by the dynamic and coordinated activity of several extracellular signaling pathways, but the basis for crosstalk between these pathways remains poorly understood. Here we investigated regulatory interactions between two pathways that are each required for neural progenitor cell maintenance in the postnatal retina; Hedgehog (Hh) and Notch signaling. Both pathways are activated in progenitor cells in the postnatal retina based on the co-expression of fluorescent pathway reporter transgenes at the single cell level. Disrupting Notch signaling, genetically or pharmacologically, induces a rapid downregulation of all three Gli proteins and inhibits Hh-induced proliferation. Ectopic Notch activation, while not sufficient to promote Hh signaling or proliferation, increases Gli2 protein. We show that Notch regulation of Gli2 in Müller glia renders these cells competent to proliferate in response to Hh. These data suggest that Notch signaling converges on Gli2 to prime postnatal retinal progenitor cells and Müller glia to proliferate in response to Hh.

3.3 Introduction

Neurogenesis is a complex and highly regulated process that involves a fine balance between cell cycle exit and cell cycle re-entry to ensure the proper rate of neuronal differentiation while maintaining a sufficient neural progenitor cell pool. The retina represents a tractable model system to investigate neurogenesis because of its simple composition and ease of genetic manipulation *in vivo* and *in vitro*. The retina is made up of six neuronal and one glial cell type that are derived from a common pool of multipotent retinal progenitor cells (RPCs) (Young, 1985). In the rodent retina, cells are born in two waves: an embryonic wave producing ganglion cells (GCs), cone photoreceptors, horizontal cells and some amacrine cells and a postnatal wave producing bipolar cells, Müller glia and the remaining amacrine cell pool. Rod photoreceptors are generated throughout retinal histogenesis (Young, 1985). Intracellular effectors, including basic helix-loop-helix transcription factors as well as extrinsic cues, such as Hedgehog (Hh), Notch and other mitogens, regulate RPC proliferation and differentiation (Livesey and Cepko, 2001). The factors that regulate the responsiveness of RPCs to extrinsic signals are, however, not well understood.

The secreted morphogen Sonic hedgehog (Shh) is an essential regulator of neural progenitor cell proliferation and differentiation in the CNS (Jiang and Hui, 2008). Hh pathway activation is initiated by Hh ligand binding to the Patched (Ptch) receptor, which de-represses the activity of Smoothened (Smo), allowing Smo to accumulate in the primary cilium (Goetz and Anderson, 2010). Smo activation triggers a downstream signaling cascade that converges on the activity of the family of Gli family zinc finger transcription factors, which control Hh target gene expression (Nozawa et al., 2013). In mammals there are three Gli proteins, Gli1, Gli2 and Gli3.

Gli2 is the primary transcriptional activator in the Hh pathway, whereas Gli3 primarily functions as a repressor of Hh target gene transcription when the pathway is inactive (Hui and Angers, 2011). Gli1 is a general target of the pathway and, while it is not essential in mammalian development, induction of Gli1 transcription is an accepted biomarker of Hh pathway activity (Hui and Angers, 2011; Sasaki et al., 1999).

In the developing retina, Shh is secreted from GCs and signals to RPCs to promote cell cycle progression and to maintain the RPC pool (Agathocleous et al., 2007; Wallace, 2008). Shh also mediates distinct effects on cell fate, functioning as a feedback inhibitory cue for GC development and modulating the development of late born cell types (Levine et al., 1997b; Sakagami et al., 2009; Wang et al., 2005; Yu et al., 2006; Zhang and Yang, 2001). Gli2 is a primary mediator of Hh-dependent induction of target gene expression, proliferation and the development of late cell types (McNeill et al., 2012; Wall et al., 2009). The Notch pathway also plays an essential role in retinal development, where it functions in RPCs and postmitotic precursor cells to maintain the progenitor pool and to regulate cell fate (Henrique et al., 1997; Jadhav et al., 2006a; Kechad et al., 2012; Nelson et al., 2007; Riesenberg et al., 2009; Takatsuka et al., 2004; Yaron et al., 2006; Zheng et al., 2009). Interestingly, there is considerable overlap in the gain- and loss-of-function phenotypes associated with Notch and Hh pathway manipulation in RPCs, particularly with respect to effects on RPC maintenance and late born cell type development (Furukawa et al., 2000; Jadhav et al., 2006b; Wang et al., 2005; Yu et al., 2006). Notch and Hh interactions in development have been described in species ranging from flies to mammals, and affect numerous processes, including neural progenitor proliferation (Dave et al., 2011) and cell fate specification (Huang et al., 2012). The actual mechanism of the interactions are context dependent and include: Hh regulation of Notch ligand expression (Baonza and

Freeman, 2005), overlap of target gene activation (Li et al., 2012; McNeill et al., 2012; Muto et al., 2009; Nelson et al., 2011; Wall et al., 2009; Wang et al., 2002) and regulation of Hh signal transduction (Kong et al., 2015; Stasiulewicz et al., 2015).

Here we explored the relationship between Hh and Notch in the proliferation of postnatal RPCs. Similar to the reported spatial and temporal pattern of Notch pathway effectors (Bao and Cepko, 1997; Lindsell et al., 1996; Roesch et al., 2008), we show that *Gli* expression also correlates with the presence of proliferating RPCs and that Notch and Hh can be active in the same cells. Gain and loss of function studies show that Notch is necessary but not sufficient for Hh signaling to promote RPC proliferation. Interestingly, Notch inhibition was associated with a rapid loss of Gli proteins but did not prevent Gli1 mRNA induction in response to Hh. Conversely, ectopic Notch activation increased the level of Gli2 protein in retinal explants and Notch activation in quiescent Müller glia maintains their competence to proliferate in response to Hh. Taken together, our data are consistent with a model whereby Notch signaling functions upstream of Hh, in part by regulating Gli2 availability, to maintain cellular competence to proliferate in response to Hh.

3.4 Materials and Methods

3.4.1 Experimental animals

All experiments were approved by the University of Ottawa Animal Care Ethics Committee and adhered to the guidelines of the Canadian Council on Animal Care. Male and female mice were selected randomly for all genotypes for analysis. Wildtype C57BL/6 and CD1 mice were obtained from the Charles River Laboratory and transgenic mice were obtained from Jackson Laboratory (Bar Harbor, Main). *Gli2*$^{flox/flox}$ mice (Corrales et al., 2006) were maintained on a C57BL/6 background and *Rosa26*^{LacZ} CRE reporter mice (Soriano, 1999) were maintained on a CD1 background. All transgenic mice were genotyped using PCR amplification of tail snip DNA with specific primers as per supplier's instructions.

3.4.2 Retinal explant culture, cell dissociation and EdU/BrdU labeling

Retinal explants were generated, as previously described (Wang et al., 2005). Briefly, retinal explants were prepared by removing the lens and retinal pigment epithelium from the eye and flattening the retina onto polycarbonate filters (0.8 μm pore size, Nucleopore). The explants were cultured in 0.5 ml of serum free culture medium [1:1 DMEM-F12, supplemented with 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 gentamycin (25 μg/ml)] for indicated number of days *in vitro* (DIV). As indicated, explants were supplemented with Smoothened agonist (Smo-Ag, 20 nM) (kind gift from Curis Inc. (Frank-

Kamenetsky et al., 2002)), epidermal growth factor (EGF; Sigma), N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT; 10μM, Millipore) or the appropriate vehicle control. To label cells in S-phase, P0 explants were pulsed with BrdU (10 μM) or EdU (0.25 μM) for 4 hours (or 30 min for DAPT treated samples) on the final day of culture. Single-cell dissociates were obtained using 10 mg/ml trypsin (Sigma) treatment in sterile calcium-free phosphate-buffered saline (PBS) (Life Technologies) for 5 min at 37°C. Trypsinization was inhibited with 10% FBS/CO2-independent media/DNAse I (0.2 mg/ml, Sigma), and the tissue was triturated to obtain a single-cell suspension. Cells were plated on glass chamber slides (Thermo Scientific) for 5 min at room temperature followed by 30 min at 37°C before a 5 min 4% paraformaldehyde fix.

3.4.3 RNA extraction and qRT-PCR

Total RNA was extracted from freshly dissected retinas or retinal explants using RNAeasy Mini Kit (Qiagen) as per the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from 2 μg of DNAse1-treated (DNA-Free Kit, Life Technologies) RNA using M-MLV reverse transcriptase (RT) (Life Technologies) and random hexamer primers (Fermentas). Target gene mRNA levels were assessed by qRT-PCR using SYBR® Green Jumpstart™ Taq Readymix™ (Sigma) and a MX3000P multiplex QPCR system (Agilent Technologies). Primer pairs (Supplementary Material, Table S1) were designed using PRIMER-blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were optimized by using a 5-point standard curve of 2-fold diluted composite cDNA from adult and postnatal brain and retinal tissue and acceptable primers for downstream use were those with an R^2>0.95, a

percent efficiency between 90-110%, sharp single point melt curve and positive controls with Ct values >10 cycle difference compared to no RT control samples. The specificity of all primers was verified by direct sequencing of amplified products and by running the resulting amplicons on an agarose DNA gel. The relative changes in target gene mRNA expression for unknown samples versus a control were determined using Relative Expression Software Tool (REST) and were normalized to *Gapdh* or *Gapdh* and *Gfp* in transfected material (Pfaffl, 2001; Pfaffl et al., 2002). Statistical significance was calculated using a pair wise fixed reallocation randomization test provided by the REST software (Pfaffl, 2001; Pfaffl et al., 2002).

3.4.4 Tissue preparation

For processing of postnatal retinae, whole heads were fixed in 4% PFA in PBS overnight at 4°C and adult retinas were dissected after cardiac PFA perfusion followed by overnight fixation in 4% PFA. Whole retina explants were fixed at room temperature for 30min in 4% PFA. Tissue was then washed 3 × 10 min in sterile PBS (0.14 M NaCl, 2.5 mM KCl, 0.2 M Na_2HPO_4, 0.2 M KH_2HPO_4) and cyroprotected by immersion in 30% sucrose in PBS at 4°C overnight, embedded in 50:50 30% sucrose in PBS:OCT (Tissue-Tek) and snap frozen with liquid nitrogen. Retinas were cryosectioned in the coronal plane at 12 µm using a Leica 1850 cryostat, transferred onto Superfrost Plus coated slides (Fischer Scientific), air dried for ~4 h at room temperature, and if not used immediately, were stored with desiccant at −20°C. Control and experimental tissues were compared on the same slides and anatomical landmarks were used to ensure similar regions were analyzed in postnatal experiments.

3.4.5 Immunocytochemistry, in situ Hybridization, β-galactosidase and EdU Detection

Immunohistochemistry (IHC), *in situ* hybridization (ISH) and generation of DIG-labelled antisense riboprobes for *Gli1*, *Gli2* and *Gli3* (gift from A. Joyner) were performed as described previously (Wang et al., 2005). ß-galactosidase activity in retinal explants was detected by 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal.) staining essentially as described previously (Dufort et al., 1998). EdU labeled cells were visualized using the Click-iT® EdU Alexa Fluor® 555 Imaging Kit (Life Technologies) as per manufacturer's instructions. Antibodies used in this study include mouse anti-KI67 (1:500, BD Bioscience), mouse anti-BrdU (BD Bioscience), mouse anti-GFP (1:500, Sigma), rabbit anti-GFP (1:1000, Life Technologies), rabbit anti-pHH3 (1:500, EMD Millipore), goat anti-BRN3b (1:200, Santa Cruz), goat anti-GFP (1:500, Rockland) and anti-dsRed (1;1000, Clontech). Secondary antibodies include, donkey anti-goat, donkey anti-mouse and donkey anti-rabbit conjugated to Alexa Fluor 488 or Alexa Fluor 555 (All from Life Technologies). Nuclei were counterstained with Hoechst (Sigma) and mounted with fluorescent mounting medium (DAKO). Bright field images were analyzed using an Axioplan microscope and captured with an Axiovision camera (2.05; Carl Zeiss). Fluorescent images were analyzed using an Olympus FV1000 (Olympus) or Zeiss LSM 510 (Carl Zeiss) confocal, captured as a stack with a 1µm slice distance and displayed as maximum intensity projections. Images were processed using Photoshop CS4 (Adobe). Quantification of immunolabeled cells was performed by counting 100-150 marker+ cells in single-cell dissociates of retinal explants visualized on Zeiss fluorescent upright microscope (Carl Zeiss).

3.4.6 Electroporation and Expression vectors

In vitro electroporation was performed on retinal explants as previously described (Matsuda and Cepko, 2004). Briefly, retinas were electroporated using a 10:1 molar ratio of expression vector (or empty control vector) (~0.5-1 µg/µl) and pUb-GFP or mCherry2-N1 in a 2 mm gap cuvettes (VWR) using five square 30 V pulses of 50 ms duration and 950 ms intervals using an ECM830 pulse generator (BTX Harvard Apparatus). The DNA plasmids used in this study include: SMOM2 (gift from G. Fishell, New York University Langone Medical Center, New York, NY), pUb-GFP (gift from T. Matsuda, Harvard Medical School, Boston, MA), Gli2GFP, Gli2$^{\Delta 784}$GFP (both gifts from A. Liu, Huck Institute of Life Sciences, Pennsylvania State University, PA), Gli1 (gift from Alex Joyner, Sloan Kettering Institute, New York, NY), Notch Intracellular Domain 1 (NICD1; gift from G. Weinmaster, David Geffen School of Medicine, University of California, Los Angeles, CA), Hes5p-dsRed (gift from N. Gaiano, Johns Hopkins University School of Medicine, Baltimore, MD; Addgene plasmid #26868 (Mizutani et al., 2007; Ohtsuka et al., 2006)), pCRALBP-dsRed (gift from C. Cepko, Harvard Medical School, Boston, MA; Addgene plasmid #11158 (Matsuda and Cepko, 2007)), *Gli* binding site (GBS)-GFP (gift from J. Briscoe, Francis Crick Institute, London, UK (Balaskas et al., 2012)), mCherry2-N1 (gift from M. Davidson, Department of Biological Science, Florida State University, FL; AddGene plasmid #54517), CRE recombinase (Cwinn et al., 2011), DN-Maml1 (gift from J.C. Aster, Brigham and Women's Hospital and Harvard Medical School, Boston, MA), GBS-luciferase (gift from H. Kondoh, Institute for Molecular and Cellular Biology, Osaka University, Japan) and Renilla (Promega).

3.4.7 Luciferase Assay

Luciferase activity was assayed using the Dual Luciferase Kit (Promega) according to manufacturer's instructions and quantified using a Lumat LB 9507 luminometer (Eg&G Berthhold). Briefly, explants were immersed in 150 µl of 1× passive lysis buffer solution, homogenized, and incubated at room temperature for 5 min. 90 µl of lysate was added to 150 µl of luciferin, luciferase activity was measured, and then 150 µl of 'Stop and Glo' solution was added to measure *Renilla* activity. Data is represented as arbitrary light units relative (R.L.U) to the appropriate control sample or condition.

3.4.8 Western Blot

Protein was extracted from 4 acutely dissected retinae or *in vitro* cultured primary retinal explants using 1x RIPA buffer (Millipore) with cOmplete Mini EDTA-Free protease inhibitor (Roche). Following a 2-4 second sonication at frequency of 30%, total protein was determined using a Bradford assay (BioRad) and 50 µg of total protein was run on a 4-12% SDS-reducing gradient gel (BioRad), transferred (Wet transfer, 1hr, 100v, 4^0C) in Tris-Glycine solution containing 0.05% SDS and 10% MeOH to a nitrocellulose membrane and blocked with 5% milk diluted in Tris-buffered saline solution containing 0.1% Tween (TBST) for 30 minutes. Membranes were incubated overnight at 4^0C with mouse anti-Gli1 (1:3000, Cell Signaling Technologies), goat anti-Gli2 (1:500, R&D Systems), goat anti-Gli3 (1:2000, R&D Systems), rabbit anti-phosphorylated ERK1/2 (1:2000, Cell Signaling Technologies), rabbit anti-total ERK1/2 (1:5000, Cell Signaling Technologies), rabbit anti-cleaved Notch Intracellular domain 1

(1:2000, Cell Signaling Technologies), goat anti-GFP (1:1000, Rockland) or mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:20 000, Millipore) diluted in blocking solution. Membranes were washed 3x5min in TBST prior to incubating in donkey anti-mouse IgG horseradish peroxidase (HRP), donkey anti-rabbit HRP and donkey anti-goat HRP (all at 1:5000, Millipore). Protein signals were detected using Luminata Crescendo Western HRP substrate (Millipore) as per manufacturer's instructions. GAPDH or GFP, in the case of transfected retinal explants, was used as a control to confirm equal loading.

3.4.9 Fluorescent activated cell sorting

Single cell dissociates of trypsinized retinal explants were re-suspended in 2 ml of 10% FBS/CO2-independent media and passed through a 50 μm mesh (Millipore). Cells were then placed on ice and sorted using a Dako Cytomation MOFLO (Dako) by StemCore. Live cells were gated using forward and side scatter and sorted for GFP+ cells. The apparatus was cleaned beforehand using 10% bleach and DEPC water. Cells were collected on ice in 2 ml of 10% FBS/CO2-independent media. Purity of GFP+ FACS cells was initially verified using wide-field fluorescent microscopy.

3.4.10 Statistical Analyses

All data are presented as mean ± standard error of the mean (S.E.M.). Statistical significance was evaluated between experimental and control conditions using an ANOVA followed by Dunnett's *post hoc* test for figures 3-1C, 3-3D, 3-3E, 3-3F (left panel), 3-4E, 3-5B

and 3-5D. Data shown in figures 3-5E, 3-7D, 3-7E, 3-7F and 3-7G was analyzed using a two-way ANOVA followed by Tukey's *post hoc* test to determine statistically significant differences between groups. An unpaired, two-tailed Student's t-test was performed to determine statistical significance in figure 3-3F (right panel, Control vs. Smo-Ag condition) and 3-6B. All statistical analyses were calculated using GraphPad Prism 6 software. Significance was assumed when $p<0.05$ and significance levels in histograms is indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ alpha levels.

3.5 Results

3.5.1 Gli transcription factor expression is associated with RPCs

Because the availability of Gli factors contributes to cellular competence to respond to Hh, we monitored the spatial and temporal expression of *Gli* and Hh-pathway genes as a function of differentiation in the postnatal retina from postnatal day 0 (P0) until P21, when retinal histogenesis is complete. At P0 cells expressing *Gli* transcripts were located in a wide layer corresponding to the region of proliferating RPCs (Figure 3-1A, KI67+, arrows) and excluded from the differentiated ganglion cell layer. (Figure 3-1A, BRN3B+, arrowheads). The domain of *Gli* gene expression and proliferation narrowed progressively over time to a thin band of cells located in the inner nuclear layer (INL) by P7 (Figure 3-1A, arrows). By P21, *Gli* expression and proliferation were undetectable (Figure 3-1A). The only exception to the association between *Gli* expression and proliferation was in the central retina at P7, where there were few proliferating cells yet *Gli* transcripts were still detectable (Figure 3-1A, insets). We suggest that these cells are differentiating Müller glia, based on the reported expression of *Ptch1*, an Hh target gene, in this cell type (Wang et al., 2002). Using gene profiling and western blot analysis we confirmed the progressive decline of *Gli* transcripts and protein, as well as a decline in expression of *Smo* and Hh target genes *Fgf-15, CcnD1* and *Hes1* at late stages of retinal development (Figure 3-1B, C). Interestingly, the expression of *Shh*, as well as pathway antagonists, *Ptch1* and *Sufu*, was still detectable by P21 (Figure 3-1B). Thus, Hh activity declines as a function of differentiation in the postnatal retina and is associated with a downregulation of positive mediators of the Hh signal transduction and transcriptional regulators.

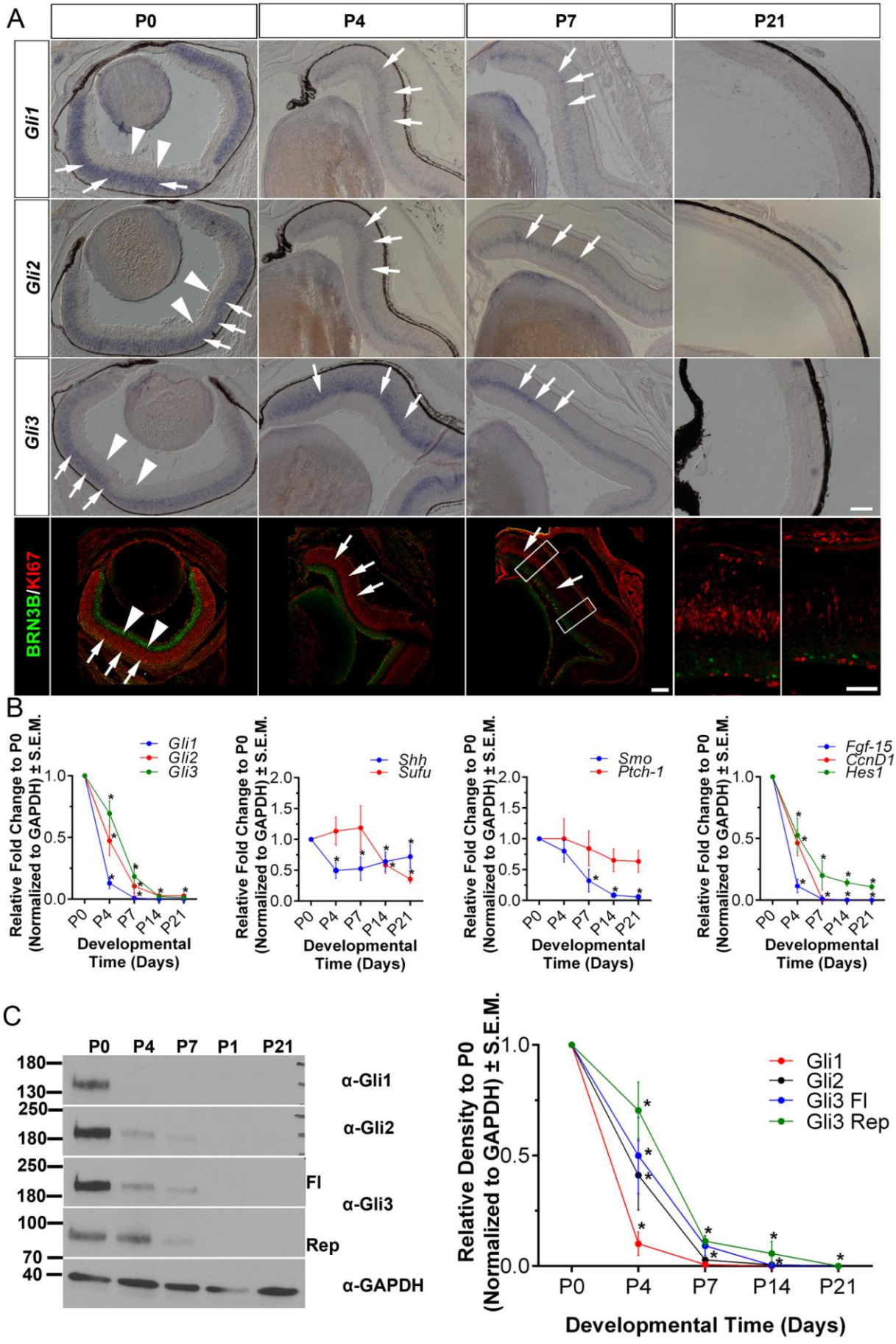

A
P0
P4
P7
P21
Gli1
Gli2
Gli3
BRN3B/KI67
B
Relative Fold Change to P0 (Normalized to GAPDH) ± S.E.M.
Gli1
Gli2
Gli3
Shh
Sufu
Smo
Ptch-1
Fgf-15
CcnD1
Hes1
Developmental Time (Days)
C
P0 P4 P7 P1 P21
180
130
250
180
250
180
100
70
40
α-Gli1
α-Gli2
Fl
α-Gli3
Rep
α-GAPDH
Relative Density to P0 (Normalized to GAPDH) ± S.E.M.
Gli1
Gli2
Gli3 Fl
Gli3 Rep
Developmental Time (Days)

Figure 3-1. Spatial and temporal pattern of proliferation and Hh pathway component expression in the postnatal retina. A) *In situ* hybridization ($\underline{n} \geq 4$ per condition) for the indicated transcripts and immunohistochemistry (IHC) for BRN3B (ganglion cells, arrowheads, green+) and KI67 (proliferating progenitor cells in the neuroblast layer, arrows, red+) in wildtype retinas at the indicated stages. Inset panels represent boxed areas. Scale bars =100μm**.** B) qRT-PCR analysis of gene expression in the postnatal retina relative to acutely dissected P0 retinal tissue (n ≥5 per condition). C) Western blot analysis for the indicated proteins from acutely dissected eyes at the indicated stages (n≥4 per condition). Fl = Full length Gli3 protein, Rep = cleaved repressor Gli3 protein.

To examine the acute effects of differentiation on *Gli* expression, we used retinal explant cultures, an *in vitro* model where it is possible to induce rapid changes in differentiation and proliferation as a function of Hh pathway activation (Wang et al., 2002). Explanting P0 retinas in culture results in a rapid reduction of Hh activity and proliferation because of the death of GCs, the only source of Shh in these cultures, and is an effect that can be reversed by supplementation with Smoothened agonist (Smo-Ag), which binds to and activates Smo (Figure 3-2A) (Wang et al., 2005). Loss of Hh activation in explants is associated with a rapid decline in the levels of Gli1 protein and transcript and an increase in cleaved Gli3 repressor protein (Gli3Rep) after one day (Figure 3-2B, C), which is consistent with what has been reported in other systems (Bai et al., 2002; Humke et al., 2010; Wang et al., 2002). We also observed a reduction in the level of Gli2 protein and transcript and *Gli3* transcripts over time, although the change at the transcript level was not as rapid as Gli1 (Figure 3-2B, C). Treatment with Smo-Ag restored Gli protein and transcripts to levels comparable to the P0 retina (Figure 2B, C, which has active endogenous Hh signaling (Frank-Kamenetsky et al., 2002). However, even in Hh-activated explants the levels of all *Gli* transcripts declined by 7 days, which follows the decline in proliferation, marked by a reduction in *CcnD1* transcript levels (Figure 3-2C). These data define an Hh-dependent Gli protein signature in retinal explants and show that the onset of differentiation is associated with rapid loss of Gli1 and Gli2 proteins and enhanced production of Gli3Rep.

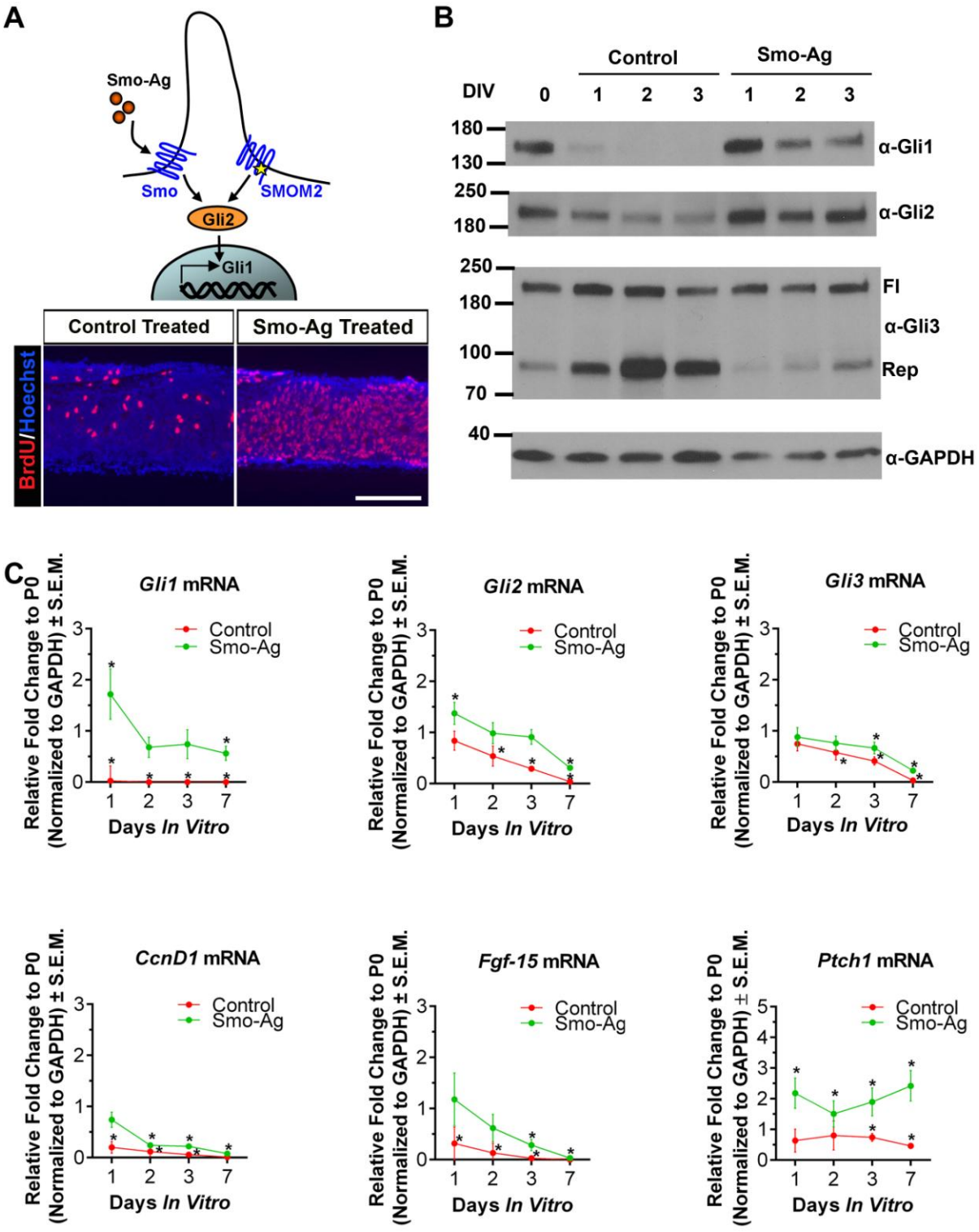

A
Smo-Ag
Smo
SMOM2
Gli2
Gli1
Control Treated
Smo-Ag Treated
BrdU/Hoechst
B
Control
Smo-Ag
DIV
0
1
2
3
1
2
3
180
130
250
180
250
180
100
70
40
α-Gli1
α-Gli2
Fl
α-Gli3
Rep
α-GAPDH
C
Gli1 mRNA
Gli2 mRNA
Gli3 mRNA
CcnD1 mRNA
Fgf-15 mRNA
Ptch1 mRNA
Relative Fold Change to P0 (Normalized to GAPDH) ± S.E.M.
Control
Smo-Ag
Days In Vitro

Figure 3-2. *Gli* expression and protein is maintained in the presence of activated Hh signaling. A) Schematic of Hh pathway activation in response to SMOM2 over-expression or Smo-Ag treatment in wildtype P0 retinal explants. Hh pathway activation results in RPC maintenance, as shown by the enhanced incorporation of the S-phase marker BrdU (red+) in Smo-Ag supplemented cultures compared to controls at 3 days *in vitro* (DIV). B) Western blot for Gli1, 2 and 3 in acutely dissected P0 wildtype tissue and retinal explants cultured for 1, 2 or 3 DIV in control or Smo-Ag conditions. C) qRT-PCR analysis for the indicated genes in control or Smo-Ag treated retinal explants (n=6 per condition). Fold changes are relative to P0 retina, which has active Shh signaling.

3.5.2 Gli2 is sufficient to promote RPC proliferation

We showed previously that *Gli2* was required for Hh responses in embryonic and postnatal RPCs (Cwinn et al., 2011; Wall et al., 2009). To test whether ectopic Gli2 expression can rescue aspects of Hh loss-of-function in RPCs, we electroporated expression vectors encoding Gli2GFP into P0 explants and cultured them in the absence of an Hh stimulus. Because Gli2 stability is regulated by phosphorylation and proteosomal degradation (Pan et al., 2006), we also tested the effect of a degradation deficient Gli2 variant, Gli2$^{\Delta 784}$GFP (Pan et al., 2006; Zeng et al., 2010)(Figure 3-3A). Acute expression of wildtype Gli2GFP or Gli2$^{\Delta 784}$GFP was sufficient to promote proliferation in RPCs (Figure 3-3B, C, D & E) and activation of an Hh reporter construct, GBS-luciferase, at levels comparable to SMOM2, a constitutively active mutant form of the Smo receptor that drives ligand-independent pathway activation upstream of Gli2 (Figure 3-3F)(Xie et al., 1998; Yu et al., 2006). This result was surprising, given that this stabilized form of Gli2 is typically associated with higher activity (Pan et al., 2006) and suggests that transfected Gli2 is not subject to a significant degree of inhibition under these conditions. Thus, Gli2 is sufficient to drive Hh reporter gene activation and to maintain proliferation in RPCs, however, the regulation of Gli2 levels in RPCs is not known.

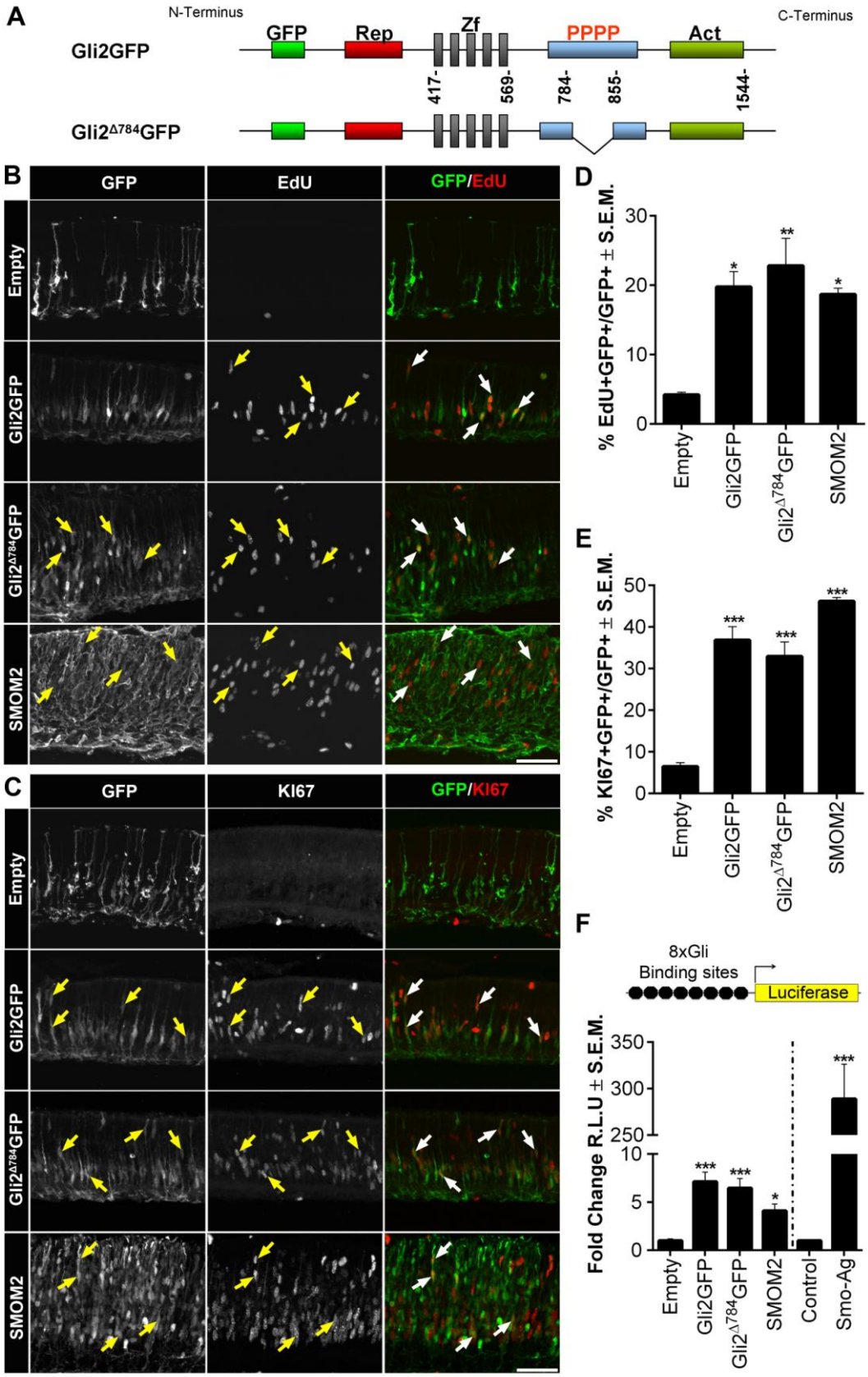

A
N-Terminus
C-Terminus
GFP
Rep
Zf
PPPP
Act
Gli2GFP
417-
569-
784-
855-
1544-
Gli2Δ784GFP
B
GFP
EdU
GFP/EdU
Empty
Gli2GFP
Gli2Δ784GFP
SMOM2
C
GFP
KI67
GFP/KI67
Empty
Gli2GFP
Gli2Δ784GFP
SMOM2
D
% EdU+GFP+/GFP+ ± S.E.M.
30
20
10
0
*
**
*
Empty
Gli2GFP
Gli2Δ784GFP
SMOM2
E
% KI67+GFP+/GFP+ ± S.E.M.
50
40
30
20
10
0
***
***
***
Empty
Gli2GFP
Gli2Δ784GFP
SMOM2
F
8xGli
Binding sites
Luciferase
Fold Change R.L.U ± S.E.M.
350
300
250
10
5
0
***
***
*
***
Empty
Gli2GFP
Gli2Δ784GFP
SMOM2
Control
Smo-Ag

Figure 3-3. Exogenous Gli2 expression promotes RPC proliferation and Hh reporter activation. A) Schematic of Gli2 constructs used in this study. Rep = Repressor domain, Zf = zinc finger domains, PPPP= critical phosphorylation sites for protein turn over located between amino acids 784-855, Act = activator domain. (See Zeng et al., 2010). Retinal explants (n=4) were electroporated with empty, Gli2GFP, Gli2$^{\Delta 784}$GFP or SMOM2 expression vectors. IHC was used to both mark proliferating progenitors with B) EdU (red+) or C) KI67 (red+) and transfected cells with GFP (green+). Arrows indicate double marker+GFP+ cells. Scale bar = 50μm. Quantification of D) EdU+ and E) KI67+ cells relative to the GFP+ transfected population in dissociated retinal explants electroporated with the indicated vector. F) Luciferase assay (schematic of GBS-luciferase construct in inset) measuring Gli activity in retinal explants (n=6 per condition) electroporated with a constitutively active Renilla vector, acting as a transfection control, GBS-luciferase and the indicated vector. Luciferase assay in retinal explants (n=3 per condition) measuring Gli reporter activity following Smo-Ag treatment relative to Control treatment conducted in a separate assay.

3.5.3 Notch regulation of Gli protein and Hh responsiveness.

Based on prevailing models, Hh synergizes with other pathways, notably receptor tyrosine kinase (RTK) signaling, to stabilize Gli proteins (Kasper et al., 2006; Stecca et al., 2007). Moreover, we have reported synergy between Hh and RTK mitogen signaling in the long-term propagation of RPCs *in vitro* (Ringuette et al., 2014). However, EGF treatment was not sufficient to maintain the levels of Gli1 or Gli2 protein in explants, and had a modest effect on proliferation compared with Hh signaling induced by Smo-Ag treatment (Figure S3-1). Notch is another key regulator of neural progenitor differentiation and similar to loss of Hh signaling, Notch inactivation in the retina induces rapid cell cycle exit and differentiation *in vivo* and *in vitro* (Imayoshi et al., 2010; Jadhav et al., 2006b; Nelson et al., 2007) and has also been reported to regulate Gli2 and Gli3 expression (Li et al., 2012). Short-term (20 hour) pharmacological inhibition of Notch with DAPT, a γ-secretase inhibitor, in retinal explants reduced both proliferation (Figure 3-4A) and the levels of all three Gli proteins (Figure 3-4C). These effects were not reversed by treatment with Smo-Ag (Figure 3-4A, C). This result was surprising given our previous observation that SMOM2 induction of *Gli1* and *Hes1*, as well as proliferation, were intact in DAPT-treated explants (Wall et al., 2009). In addition to using different approaches to activate Hh signaling (SMOM2 versus Smo-Ag), there are several technical differences between the two studies, including length of time in culture, explant preparation and mouse strain (Wall et al., 2009). Our attempts to reconcile the effects of DAPT treatment on the outcome of Hh activation in explants cultured for 3 days were unsuccessful because of toxicity (data not shown). Interestingly, we noted that in DAPT-treated explants, proliferation was more preserved in folded compared with flattened regions of the tissue (Figure S3-2), raising the possibility that

local differences in tissue architecture affected the response to these treatments. In contrast, there was less variability in proliferation across the explant if it was flattened. Therefore, to reduce this source of variability and generate a more homogenous response, we standardized our explant preparation to ensure that the tissue was flat.

Because of the level of toxicity associated with 3 day exposure to DAPT, we used a 20 hour culture period and compared the effect of DAPT treatment on induction of Hh target genes by Smo-Ag and SMOM2. In the presence of DAPT, both SMOM2 and Smo-Ag upregulated *Gli1* mRNA (albeit a blunted response), but only SMOM2 transfected explants exhibited increased *Hes1* mRNA (Figure S3-3A, B). This result is consistent with the Wall *et. al.* (2009) study showing that SMOM2 expression increased *Gli1* and *Hes1* mRNA detected by ISH in DAPT treated explants and suggests that the two approaches to activate Hh are differentially sensitive to DAPT treatment in terms of *Hes1* induction. This data also suggests that DAPT treatment can uncouple the induction of *Gli1* mRNA from stabilization of Gli1 protein.

To circumvent the toxicity and differential sensitivity of Hh activators to DAPT treatment, we blocked Notch genetically by transfecting cells with dominant negative Mastermind1 (DN-Maml1), which inhibits the pathway downstream of the Notch receptor (Wu et al., 2000). Cell autonomous Notch inhibition blocked SMOM2 (Figure 3-4B, E) and Smo-Ag (Figure S3-4) induced proliferation in retinal explants indicating that both methods of Hh pathway activation were equally sensitive to Notch inhibition downstream of γ-secretase activity. The inhibitory effect of DN-Maml1 on SMOM2 signaling was also associated with a reduction in the levels of Gli1 and Gli2 protein (Figure 3-4D). However, co-expression of SMOM2 with wildtype Gli2, degradation-deficient Gli2 transgenes (Zeng et al., 2010) or Gli1 was unable to restore proliferation in DN-Maml1-expressing cells (Figure 3-4E), suggesting that Notch

inhibition mediates additional effects that are independent of Gli1/Gli2 to block Hh-dependent proliferation. Taken together, the impact of short term and cell autonomous Notch inhibition suggests that Notch function is upstream of and is required for Hh-induced Gli protein accumulation and proliferation.

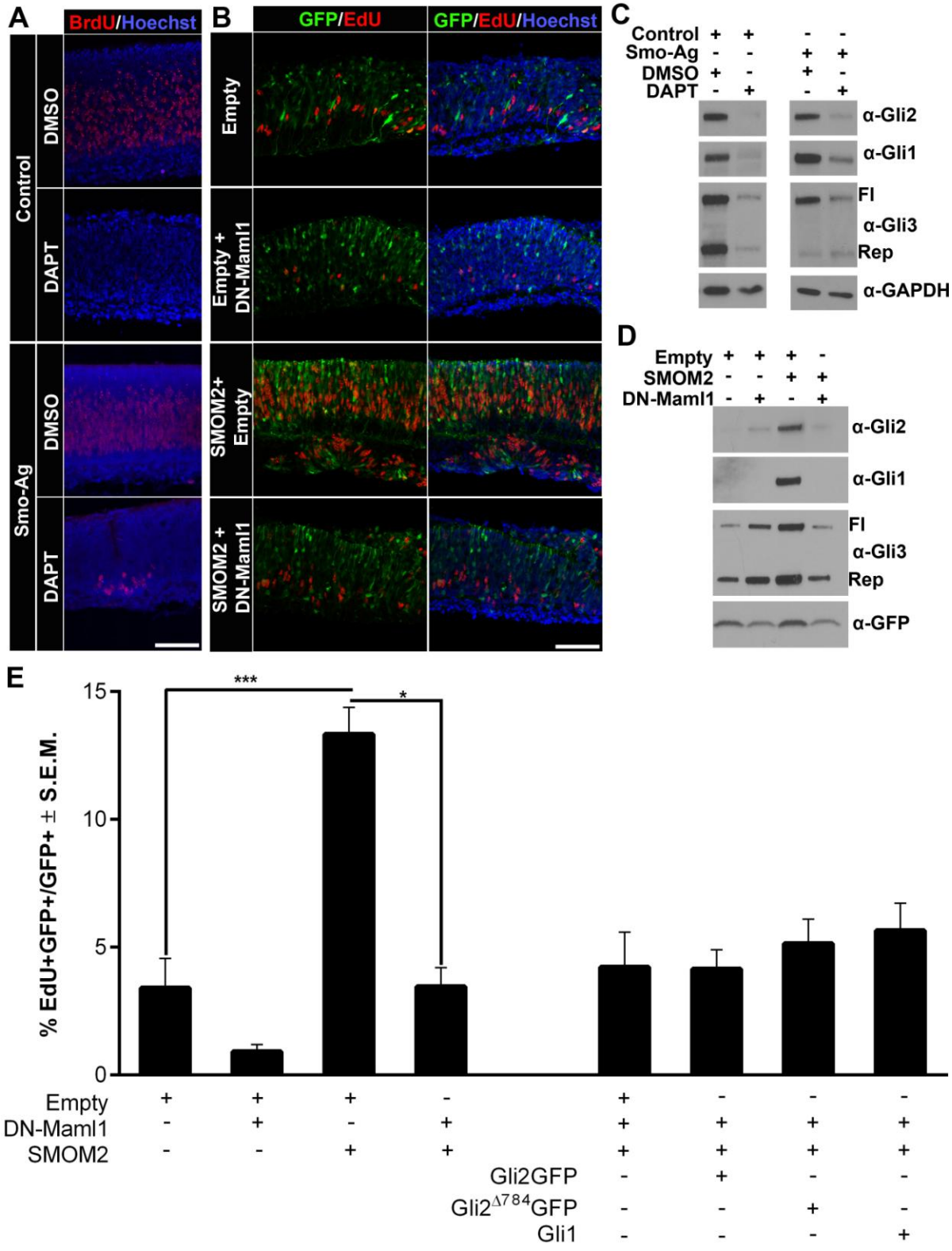

A
BrdU/Hoechst
Control
DMSO
DAPT
Smo-Ag
DMSO
DAPT
B
GFP/EdU
GFP/EdU/Hoechst
Empty
Empty + DN-Maml1
SMOM2+ Empty
SMOM2 + DN-Maml1
C
Control + + - -
Smo-Ag - - + +
DMSO + - + -
DAPT - + - +
α-Gli2
α-Gli1
Fl
α-Gli3
Rep
α-GAPDH
D
Empty + + + -
SMOM2 - - + +
DN-Maml1 - + - +
α-Gli2
α-Gli1
Fl
α-Gli3
Rep
α-GFP
E
% EdU+GFP+/GFP+ ± S.E.M.
15
10
5
0
***
*
Empty + + + - + - - -
DN-Maml1 - + - + + + + +
SMOM2 - - + + + + + +
Gli2GFP - + - -
Gli2Δ784GFP - - + -
Gli1 - - - +

Figure 3-4. The Notch pathway is required for Hh-dependent Gli protein accumulation and RPC proliferation *in vitro*. A) Retinal explants were treated with DAPT, Smo-Ag or the relevant vehicle control, cultured for 20hrs and pulsed with BrdU for the last 30 minutes of culture time. S-phase cells (BrdU, red+) were analyzed in tissue sections. All Scale bars = 50μm. B) Retinal explants were electroporated with combinations of DN-Maml1, a dominant negative downstream component of the Notch pathway, SMOM2 or corresponding empty expression vectors, and GFP, cultured for 3DIV before being pulsed with EdU (Red+). S-phase cells (EdU, red+) were analyzed in tissue sections. C) Western blot analysis for Gli proteins P0 retinal explants treated with DAPT, Smo-Ag or vehicle controls for 20hrs. D) Western blot analysis for Gli2, Gli1, Gli3 and GFP from P0 retinal explants electroporated with the indicated plasmids and cultured for 3DIV. E) Quantification of EdU+ cells relative to the GFP+ transfected population in dissociated retinal explants ($n \geq 5$ per condition) electroporated with indicated plasmids.

3.5.4 Notch signaling biases cells to a Müller glial fate and maintains Gli2 protein levels, but does not fully activate the Hh pathway

To further investigate how Notch and Hh signaling interact, we compared the outcome of acute Notch and Hh activation on proliferation and Gli protein levels in retinal explants. P0 explants were electroporated with Notch intracellular domain 1 (NICD1), a strong Notch pathway activator, or SMOM2, an Hh activator, and analyzed after 3 days. Notch and Hh activation induced markedly different outcomes on proliferation and cell fate. In contrast to the SMOM2+ cohort that contained a significant population of proliferating cells (Figure 3-5A, B) and few differentiating Müller glia (marked by pCRALBP-dsRed reporter expression) (Figure 3-5C, D), the majority of NICD1+ cells were post mitotic, based on EdU and Ki67 staining (Figure 3-5A, B and data not shown), and approximately half of them were Müller glia based on pCRALBP-dsRed expression (Figure 3-5C, D). Despite these differences in biological outcome, activation of either pathway was sufficient to promote Gli2 protein accumulation (Figure 3-5F), however, only SMOM2 expression was sufficient to activate Hh signaling, based on the presence of Gli1 protein and transcripts (Figure 3-5F, G)(Sasaki et al., 1999). Because of the requirement for Gli2 for Hh induction of Müller glia development (Wall et al., 2009), we asked whether Gli2 mediated the same effect downstream of Notch activation. To inactivate *Gli2*, we transfected explants from mice that were homozygous for a conditional *Gli2* allele ($Gli2^{flox/flox}$;$Rosa26^{LacZ}$) with NICD1 along with CRE recombinase and assessed expression of the Müller reporter transgene at 3 days. Here we found that NICD1 induction of Müller reporter+ cells did not require *Gli2* (Figure 3-5E), suggesting that Müller cell induction by Notch and Hh occur via distinct mechanisms. Moreover, the lack of change in Müller reporter induction could not be

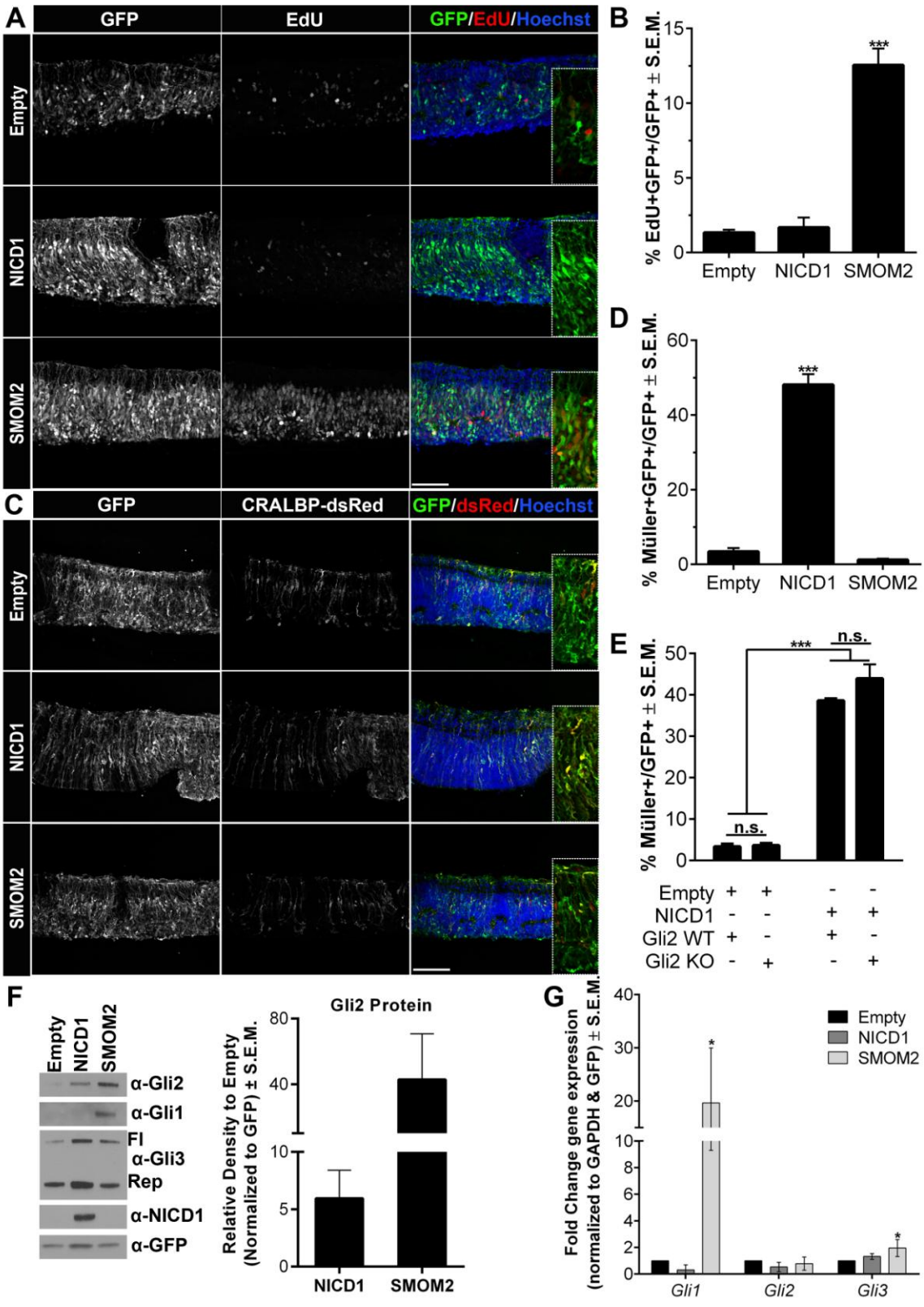

A
GFP
EdU
GFP/EdU/Hoechst
Empty
NICD1
SMOM2
B
% EdU+GFP+/GFP+ ± S.E.M.
15
10
5
0
***
Empty
NICD1
SMOM2
C
GFP
CRALBP-dsRed
GFP/dsRed/Hoechst
Empty
NICD1
SMOM2
D
% Müller+GFP+/GFP+ ± S.E.M.
60
40
20
0
***
Empty
NICD1
SMOM2
E
% Müller+/GFP+ ± S.E.M.
50
40
30
20
10
0
***
n.s.
n.s.
Empty + + - -
NICD1 - - + +
Gli2 WT + - + -
Gli2 KO - + - +
F
Empty
NICD1
SMOM2
α-Gli2
α-Gli1
Fl
α-Gli3
Rep
α-NICD1
α-GFP
Gli2 Protein
Relative Density to Empty (Normalized to GFP) ± S.E.M.
80
40
10
5
0
NICD1
SMOM2
G
Fold Change gene expression (normalized to GAPDH & GFP) ± S.E.M.
40
30
20
10
8
6
4
2
0
*
*
Empty
NICD1
SMOM2
Gli1
Gli2
Gli3

Figure 3-5. Gain of function for the Notch pathway maintains Gli2 protein in Müller glia *in vitro*. A) Retinal explants ($\underline{n} \geq 5$ per condition) were transfected with empty, NICD1 or SMOM2 and GFP expression vectors and processed for IHC to identify transfected GFP+ cells (green) and proliferating EdU+ (red) cells. All Scale bars = 50µm. B) Quantification of EdU+ relative to the GFP+ transfected population in dissociated retinal explants electroporated with expression vectors. C) Retinal explants from wildtype P0 animals were electroporated with the indicated plasmids and a Müller glial reporter gene, pCRALBP-dsRed. Explants were cultured for 3DIV, processed and stained with antibodies GFP (green+) and dsRed (red+) to mark CRALBP+ cells. D) Quantification of Müller reporter+ (CRALBP-dsRed+) cells relative to the GFP+ transfected population in dissociated retinal explants ($\underline{n} \geq 5$ per condition). E) Müller reporter+ (CRALBP-dsRed+) cells relative to the GFP+ transfected population in dissociated *Gli2*$^{flox/flox}$ explants electroporated (n=4 explants per condition) with Empty or NICD1 and CRE (Gli2 KO) or relevant control vector (Gli2 WT). F) Western blot analysis for the indicated proteins from retinal explants electroporated with control, NICD1 or SMOM2 and GFP expression vectors and cultured for 3DIV. Protein amounts ($\underline{n} \geq 4$ per condition) are quantified relative to empty vector control electroporated samples. G) qRT-PCR analysis of *Gli* transcript in retinal explants (n = 6 per condition) electroporated with the indicated plasmids and cultured for 3DIV. Fold changes are relative to mRNA levels from 3DIV Empty electroporated explants (n=6 biological replicates per condition).

simply explained by inefficient CRE-mediated recombination because we detected robust activation of the Rosa26 CRE-reporter locus (based on induction of X-gal staining) in CRE-transfected retinal explants (Figure 3-S5). While Notch is not sufficient to induce Hh signaling (based on Gli1 transcription and protein induction) or proliferation (a functional readout of Hh signaling in this context), it does promote Gli2 protein and thus may function upstream to prime cells to respond to Hh.

3.5.5 Notch and Hh pathways can be active simultaneously in the same cell.

If Notch primes cells to respond to Hh, then we predicted that these pathways should be active simultaneously or in rapid succession in retinal cells. To address this possibility, we transfected vectors encoding fluorescent Notch (Hes5-dsRed) and Hh (GBS-GFP) pathway reporters in retinal explants and monitored their expression after 24 hours. In addition to validating the pathway responsiveness of these reporters, we also confirmed that they were not cross-activated (e.g. the Hes5 reporter not activated by Hh stimulation and vice versa) (Figure S3-6, S3-7). In untreated, dissociated explants we detected single Hh+ and Notch+ as well as double Hh+Notch+ cells (Figure 3-6A, B), reflecting the presence of endogenous Hh and Notch signaling in explants at this stage (Detection of Hh+ cells likely reflects residual SHH, which would be present in the first 24 hours of the cultures (Muto et al., 2009; Wall et al., 2009; Wang et al., 2002)). Smo-Ag treatment increased the proportions of single Hh+ and double Hh+Notch+ cells at the expense of Notch+ cells (Figure 3-6B). Because the half-life of these reporters is 24 hours, we cannot say unequivocally that the pathways are active simultaneously at

the single cell level, however, these observations do support the interpretation that there is close temporal activation of both pathways in at least a subset of progenitor cells.

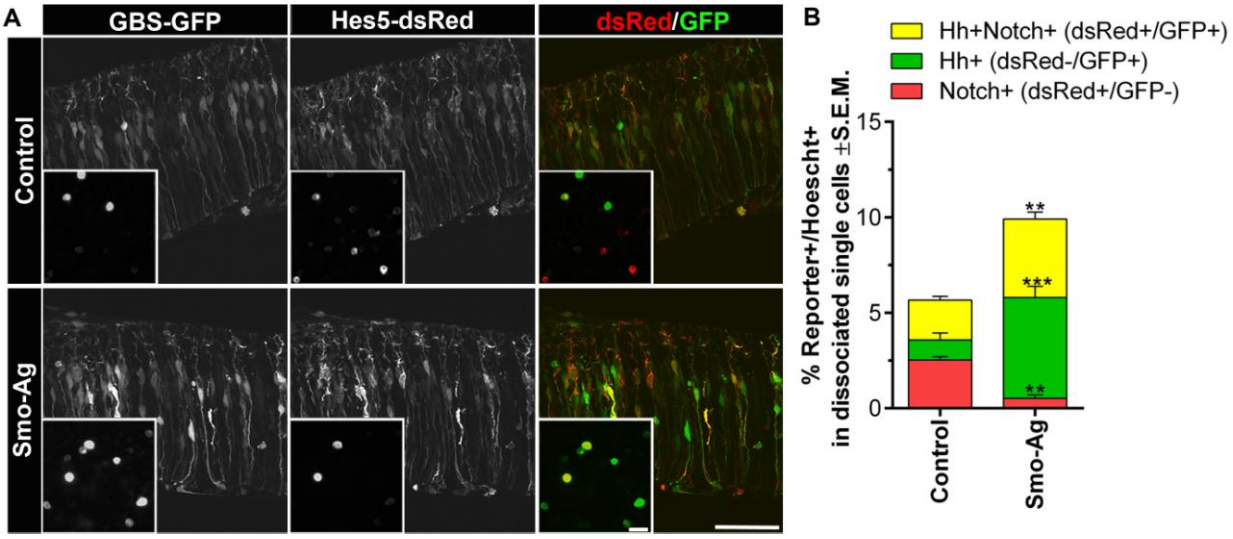

A
GBS-GFP
Hes5-dsRed
dsRed/GFP
Control
Smo-Ag
B
Hh+Notch+ (dsRed+/GFP+)
Hh+ (dsRed-/GFP+)
Notch+ (dsRed+/GFP-)
% Reporter+/Hoescht+
in dissociated single cells ±S.E.M.
15
10
5
0
**
***
**
Control
Smo-Ag

Figure 3-6. Co-activation of Notch and Hh in RPCs. A) Wildtype P0 retinal retinas were co-transfected with a Hh reporter consisting of 8 Gli binding sites upstream of GFP (GBS-GFP) and a Notch reporter containing a minimal Hes5 promoter upstream of dsRed (Hes5p-dsRed), cultured in control or Smo-Ag treated conditions for 24hrs, dissociated and processed for IHC with GFP and dsRed specific antibodies. Scale bar = 50μm. Example of dissociated cells shown in inset. Scale bar = 20μm. B) Transfected retinal explant cultures (n=4) were dissociated and counts were performed quantifying the number of Notch-responsive/Hh-negative (dsRed+/GFP-, red bars), Hh-responsive/Notch-negative (dsRed-/GFP+, green bars) and double Notch/Hh-responsive (dsRed+/GFP+, yellow bars) retinal cells as function of total Hoechst+ cells.

3.5.6 Notch extends the competence of Müller glia to respond to an Hh signal via Gli2

Because Notch activation was sufficient to increase the level of Gli2 protein in retinal explants, we next tested whether persistent Notch activation could extend the window of Hh responsiveness in retinal explants (Figure 3-7A). We took advantage of previous observations that neural progenitors become refractory to Hh stimulation after a period of Hh deprivation (Kenney and Rowitch, 2000). Using multiple proliferative markers spanning different phases of the cell cycle (Figure 3-7B), we observed that explants grown in control conditions for 3 days have very low rates of proliferation in response to re-stimulation of the Hh pathway with Smo-Ag, which is consistent with the cells adopting a state of insensitivity to Hh signaling (Figure 3-7C, D). However, when explants were transfected with NICD1 at the start of the culture and stimulated with Smo-Ag 3 days later, NICD1+ cells exhibited a robust proliferative response, based on the increase in cells staining for general (KI67+), S-phase (EdU+) and mitotic (pHH3+) cell cycle markers (Figure 3-7C, D). Since ectopic NICD1 expression promoted Müller glial development (Figure 3-5B, D), we asked if Müller glia were the source of proliferating cells upon Hh stimulation. We found that Smo-Ag induced proliferation of NICD1+ cells was restricted primarily to Müller glia, based on EdU incorporation, with little effect on non-Müller cells (Figure 3-7E, F). To test the requirement for *Gli2* in this response, we repeated the analysis using explants from $Gli2^{flox/flox};Rosa26^{LacZ}$ mice that were transfected with NICD1 and CRE recombinase or an empty vector. Here *Gli2* inactivation significantly attenuated the proliferative response of NICD1 expressing cells to Hh stimulation (Figure 3-7G). Residual proliferation after *Gli2* deletion could be due to *Gli1*, which has been shown to partially compensate for *Gli2* loss in the context of RPC proliferation (Wall et al., 2009).

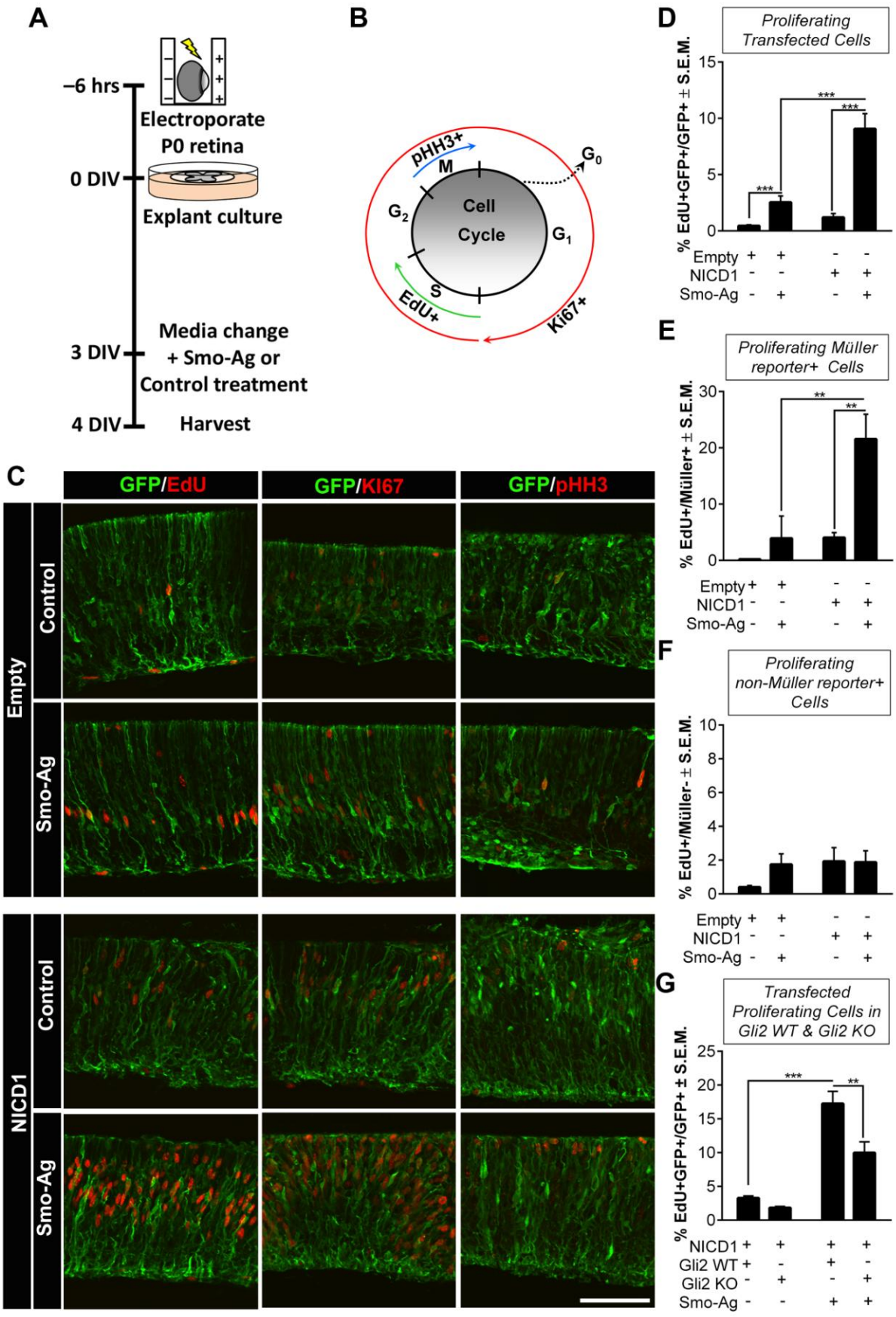

A
–6 hrs
Electroporate
P0 retina
0 DIV
Explant culture
3 DIV
Media change
+ Smo-Ag or
Control treatment
4 DIV
Harvest
B
pHH3+
M
G0
G2
Cell
Cycle
G1
S
EdU+
Ki67+
C
GFP/EdU
GFP/KI67
GFP/pHH3
Empty
Control
Smo-Ag
NICD1
Control
Smo-Ag
D
Proliferating
Transfected Cells
% EdU+GFP+/GFP+ ± S.E.M.
15
10
5
0
***
***
***
Empty + + - -
NICD1 - - + +
Smo-Ag - + - +
E
Proliferating Müller
reporter+ Cells
% EdU+/Müller+ ± S.E.M.
30
20
10
0
**
**
Empty + + - -
NICD1 - - + +
Smo-Ag - + - +
F
Proliferating
non-Müller reporter+
Cells
% EdU+/Müller- ± S.E.M.
10
8
6
4
2
0
Empty + + - -
NICD1 - - + +
Smo-Ag - + - +
G
Transfected
Proliferating Cells in
Gli2 WT & Gli2 KO
% EdU+GFP+/GFP+ ± S.E.M.
25
20
15
10
5
0
***
**
NICD1 + + + +
Gli2 WT + - + -
Gli2 KO - + - +
Smo-Ag - - + +

Figure 3-7. Gli2-dependent Notch activation preserves RPC competence to respond to Hh. A) Schematic depicting experimental design. Wildtype P0 primary retinal explants were electroporated with GFP and NICD1 or empty expressing plasmids, cultured in control treatment conditions for 3DIV followed by 1DIV Smo-Ag or vehicle treatment. B) Simplified cell cycle diagram indicating the stage that each proliferative stain marks. C) Retinal explants ($\underline{n} \geq 3$ per condition) were transfected with GFP and NICD1 or Empty and treated with Smo-Ag or Control as shown in A before being processed for IHC to identify transfected GFP+ cells (green+) and proliferating (red+) cells. All Scale bars = 50μm. D) Percentage of EdU+ cells among the GFP+ transfected cohort expressing empty or NICD1 plasmid ($n \geq 6$ explants per condition). Note: compared to explants with continuous exposure to Smo-Ag from the start of culture (see Figure 4), delaying Smo-Ag supplementation significantly attenuates proliferation. E) Percentage of the Müller glia (pCRALBP-dsRed+) in S-phase (EdU+) in response to Smo-Ag in Empty or NICD1 transfected explants (n=4 explants per condition). F) Percentage of non-Müller glia (pCRALBP-dsRed-), S-phase (EdU+) cells following Smo-Ag treatment in NICD1 or Empty transfected explants (n=4 explants per condition). G) Percentage EdU+ cells among the GFP+ transfected cohort in $Gli2^{flox/flox};Rosa26^{LacZ}$ explants electroporated (n=4 explants per condition) with a combination of Empty or NICD1 and CRE (Gli2 KO) or relevant control (Gli2 WT) expression vectors.

To characterize the Hh response of Notch-activated cells and how it compares with the Hh response of RPCs, we performed qRT-PCR analysis of Hh activated RPCs and NICD1-expressing cells. To standardize the analysis between groups, all explants were transfected with a GFP reporter plasmid and GFP+ cells that were enriched by fluorescent activated cell sorting (FACS) were used for the analysis. Using this approach we were able to compare the response of NICD1+ cells after 24 hours exposure to Smo-Ag with that of RPCs treated for 3 DIV with Smo-Ag, our standard conditions. Smo-Ag treatment increased the expression of Hh target genes (*Gli1, Ptch* and *Sox8*) in RPCs and NICD1+ cells, confirming the activation of the Hh pathway in both cell types (Figure 3-8A). *CcnE1* expression was increased in Hh stimulated NICD1+ cells (Figure 3-8A), consistent with progression to S-phase in these cells (Figure 3-7C, D). Interestingly, several genes that were modulated by Hh in RPCs, including Notch targets (*Hes1, Hes5, Nrarp)*, progenitor/ Müller glia markers (*Sox2, Sox9*), the neurogenic gene *Id1,* lineage markers (*Glast*, *Crx*) and the G_1 cell cycle regulator *CcnD1* were not induced in Hh-treated NICD1+ cells (Figure 3-8A). The failure to increase G_1 regulators is particularly curious because these are well-established targets associated with Hh-induced proliferation (Kenney and Rowitch, 2000). While this result could imply that Hh stimulation of NICD1+ cells induces a different program of gene expression, it is also possible that NICD1 expression alone is sufficient to regulate expression of this cohort of genes. Consistent with the latter possibility, NICD1 transfected cells were enriched for the expression of Notch targets, progenitor/Müller glia markers, G1 regulators and showed a reduction in photoreceptor (*Crx*) transcripts (Figure 3-8B), consistent with the increased Müller glial cell differentiation in NICD1 transfected cells. In addition, we did not see enrichment for the gliotic marker *Gfap*, suggesting that NICD1+ Müller

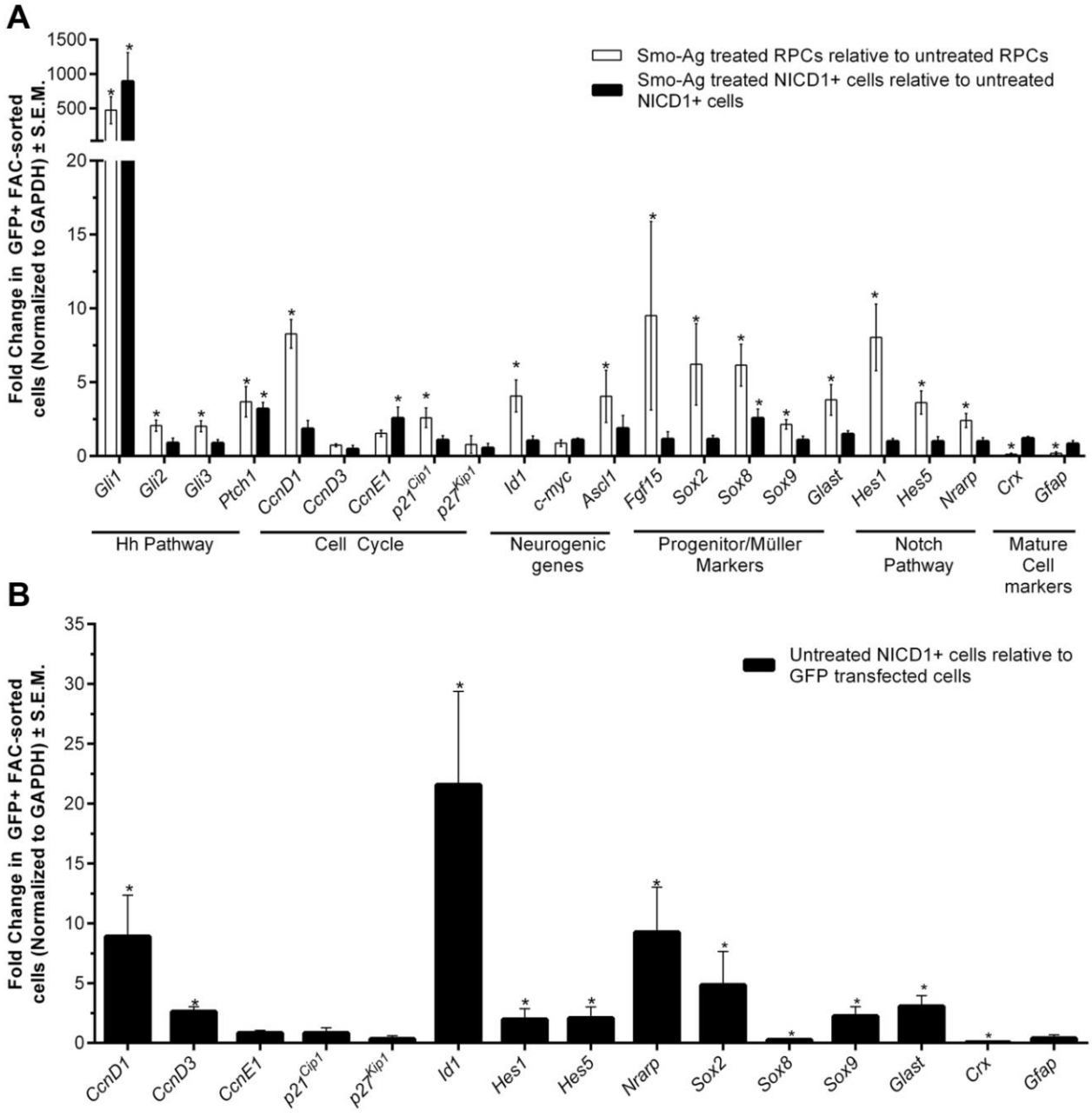

A
Fold Change in GFP+ FAC-sorted cells (Normalized to GAPDH) ± S.E.M.
Smo-Ag treated RPCs relative to untreated RPCs
Smo-Ag treated NICD1+ cells relative to untreated NICD1+ cells
Gli1
Gli2
Gli3
Ptch1
CcnD1
CcnD3
CcnE1
p21Cip1
p27Kip1
Id1
c-myc
Ascl1
Fgf15
Sox2
Sox8
Sox9
Glast
Hes1
Hes5
Nrarp
Crx
Gfap
Hh Pathway
Cell Cycle
Neurogenic genes
Progenitor/Müller Markers
Notch Pathway
Mature Cell markers
B
Fold Change in GFP+ FAC-sorted cells (Normalized to GAPDH) ± S.E.M.
Untreated NICD1+ cells relative to GFP transfected cells
CcnD1
CcnD3
CcnE1
p21Cip1
p27Kip1
Id1
Hes1
Hes5
Nrarp
Sox2
Sox8
Sox9
Glast
Crx
Gfap

Figure 3-8. Gene expression signature of Hh responding NICD1 expressing cells. A) qRT-PCR analysis of gene expression in FACS-enriched GFP+ cells from P0 wildtype primary retinal explants receiving Smo-Ag for 3DIV (Smo-Ag treated RPCs) relative their untreated controls (untreated RPCs) versus GFP+ cells from NICD1 transfected explants receiving Smo-Ag for the last 24 hours of a 4 day culture (Smo-Ag treated NICD1+ cells) relative untreated controls (untreated NICD1+ cells). Fold changes are relative mRNA levels from the respective untreated control explants (n=3 biological replicates per condition). B) qRT-PCR analysis of gene expression in FACS-enriched GFP+ cells from untreated NICD1+ cells explants relative to control untreated GFP transfected cells. Note: these samples were not Hh treated and thus are a comparison of NICD1-induced gene expression. Fold changes are relative mRNA levels control untreated GFP transfected explants (n=3 biological replicates per condition).

glia have not become reactive under these conditions (Figure 3-8B) (Dyer and Cepko, 2000). Taken together, these results show that Notch stimulation is sufficient to promote the expression of a cohort of genes that are typically upregulated in Hh-stimulated RPCs, suggesting that part of the Hh response in RPCs is coordinated with Notch signaling. While Notch activated gene expression is not sufficient to promote proliferation, it is associated with competence to respond to Hh and proliferate in a Gli2-dependent manner. Thus, Notch signaling in this context appears to maintain cells in a primed status able to respond to mitogenic signals.

3.6 Discussion

In this study, we investigated the relationship between the timing of *Gli* expression and postnatal retinal histogenesis, and crosstalk between Notch and Hh signaling in postnatal RPCs. We show that there is an inverse relationship between the expression of Hh pathway activators and neuronal differentiation. *Gli* transcripts are not detected in differentiated layers of the retina and rapid cell cycle exit of RPCs induced by loss of Shh signaling is associated with downregulation of Gli protein and transcripts. Since Gli2 is required for normal RPC responses to Hh (McNeill et al., 2013; McNeill et al., 2012; Wall et al., 2009), this rapid loss of Gli proteins would render cells non-responsive to the Shh ligand in their environment, which could represent a mechanism by which neurogenic precursors escape Hh activation. This decline in *Gli* expression could reflect a build-up of inhibitors and/or the loss of positive regulators. Consistent with the former, it has been shown that neuron-derived signals, notably PACAP, can antagonize Gli2 activity and Shh-induced proliferation in the cerebellum, as well as cell cycle gene expression and proliferation in the retina (Niewiadomski et al., 2013; Njaine et al., 2010).

Because Notch and Hh signaling overlap temporally in the retina, the relationship between these pathways has been challenging to dissect. Our findings are consistent with a model whereby Notch functions upstream to promote Hh signaling and proliferation of postnatal RPCs (Figure 3-9). This is based on the observations that Notch activation potentiates the response of progenitors to exogenous Hh and that Notch is required for full Hh signaling, which in this context is characterized by *Gli1* mRNA induction, accumulation of Gli protein and proliferation in retinal explants. Our findings are in agreement with the evidence for a similar

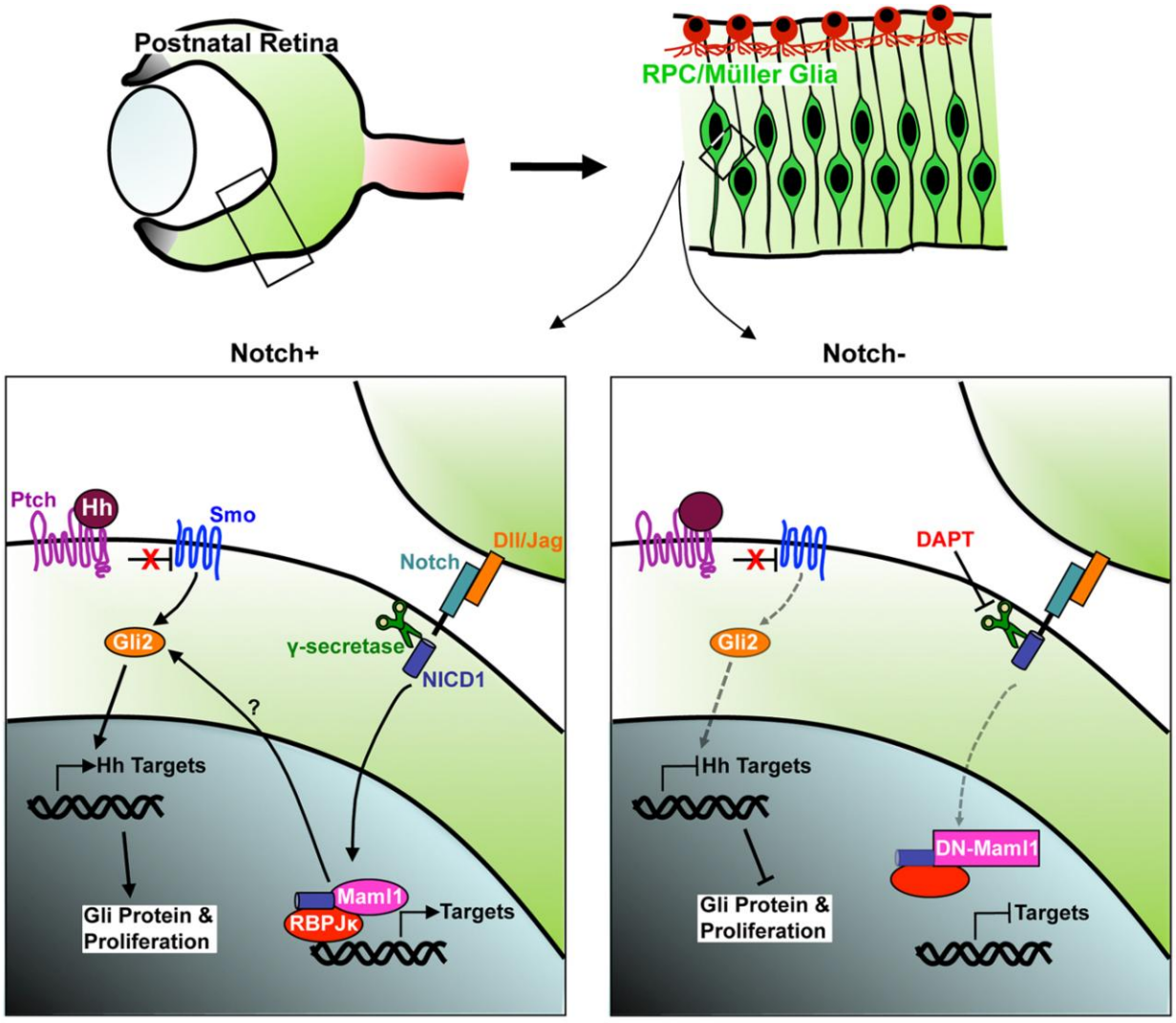

Postnatal Retina
RPC/Müller Glia
Notch+
Notch-
Ptch
Hh
Smo
Dll/Jag
Notch
γ-secretase
NICD1
Gli2
?
Hh Targets
Gli Protein &
Proliferation
Maml1
RBPJκ
Targets
DAPT
DN-Maml1

Figure 3-9. Proposed interactions between Notch and Hh signaling in RPCs and Müller glia. In RPCs and Müller glia of the developing postnatal retina, Notch signal is required to maintain Gli protein accumulation and proliferation downstream of an activated Hh signal through a Gli2-dependent mechanism (left panel). Genetic or pharmacological inhibition of Notch blocks Gli protein accumulation and proliferation (right panel).

relationship between Notch and Hh in the developing neural tube, where Notch signaling modulates Shh-dependent patterning (Kong et al., 2015; Stasiulewicz et al., 2015). In those studies, Notch functions upstream of Smo activation to control Ptch1 distribution and primary cilia length, as well as regulating Smo and full-length Gli3 protein accumulation (Kong et al., 2015; Stasiulewicz et al., 2015). In this context, this upstream requirement for Notch in Hh activation can be overcome by activating the pathway downstream of Smo (Kong et al., 2015), which contrasts with our findings in RPCs where direct activation of Smo cannot overcome the loss of Notch activity and promote proliferation. While we cannot rule out the possibility that Notch signaling also plays a similar role controlling cilia length in the retina, our data suggest that there are additional points where these pathways intersect, in this instance with Notch modulating Hh signaling downstream of Smo at the level of Gli2. Notch activity may be maintaining Gli2 protein by direct NICD1/RBPJκ transcriptional regulation of *Gli2*, as has been shown in neural progenitors of the hippocampus (Li et al.), or by controlling the translation and/or stability of Gli2 protein (Pan et al., 2006). We favour the latter mechanism, as manipulating Notch signaling did not alter the levels of Gli2 and Gli3 transcripts. Finally, we acknowledge that our evidence of Notch regulation of Gli2 protein is in the context of Müller glia, since NICD1 expression in explants promoted Müller glia differentiation rather than RPC proliferation. Whether this Notch/Gli2 relationship can be extrapolated to RPCs requires further investigation with more refined approaches to manipulate Notch levels in neural progenitors without promoting gliogenesis.

Notch signaling is maintained in retina explants, as demonstrated by persistent Hes5 reporter activity and the outcome of Notch inhibition, which could account for why ectopic Gli2 is sufficient to drive proliferation and Hh reporter induction in this context. However, Gli2 is not

sufficient to rescue proliferation in Notch inhibited cells, suggesting Notch mediated potentiation of Hh signaling in RPCs is more complex and involves additional effectors, most likely transcriptional targets of the Notch pathway. Consistent with this idea, we show that Notch activation promotes the expression of several genes known to play a role in Hh responses, including SoxB1 family member *Sox2*, which are known co-regulators of Hh target genes (Peterson et al., 2012), as well as *CcnD1* (Kenney and Rowitch, 2000) and *Hes1* (Ingram et al., 2008; Wall et al., 2009). Thus, Notch signaling could potentiate Hh signaling in RPCs through a general permissive effect involving regulation of genes that control the progenitor state and more directly by regulating components of the Hh pathway.

We found a differential ability of DAPT to modulate the effects of Hh activation in SMOM2 transfected versus Smo-Ag treated conditions where DAPT prevented the induction of *Hes1* downstream of Smo-Ag but not SMOM2. Consistent with our previous observations (Wall et al., 2009), DAPT treatment did not block the early transcriptional response, marked by increased *Gli1* mRNA, to SMOM2 and Smo-Ag, but interestingly did inhibit the accumulation of Gli1 and Gli2 protein. These observations suggest that SMOM2 and Smo-Ag act through different mechanisms to control Hh target gene expression downstream of Notch and that Notch signaling regulates the level of Gli protein. While both Hh activators function at the level of the primary cilium, it is possible that they result in different levels of Smo availability over time, resulting in different levels of Hh activation. Indeed, Notch is required for neural progenitor cells to experience high but not low levels of Shh signaling in the developing neural tube (Kong et al., 2015) and Smo-Ag potentiates a much stronger Gli transcriptional response, as measured by *Gli1* expression levels and GBS-luciferase activity in retinal explants. Alternatively, we

cannot rule out the possibility for a differential requirement for γ-secretase activity in Smo-Ag stimulated cells.

We show that Hh is sufficient to induce Notch expressing quiescent Müller glia to re-enter the cell cycle in retinal explants, which raises the issue of whether Hh modulates Müller cell proliferation *in vivo*. The potential for Notch and Hh crosstalk in Müller glia *in vivo* is consistent with the expression of *Gli* genes in the INL in non-dividing cells at P7, the persistence of Notch and Hh target gene expression in adult Müller glia (Blackshaw et al., 2004; Nelson et al., 2011; Wang et al., 2002) and with the evidence that adult Müller glia can respond to Hh *in vivo* and *in vitro* (Ferraro et al., 2015; Todd and Fischer, 2015; Wan et al., 2007). In future studies it will be important to examine the requirement for Gli2 in the regulation of Müller glial cell proliferation and response to injury *in vivo*. We suggest that Notch priming functions by maintaining high levels of *CcnD1* and Gli2 protein, thus allowing quiescent Müller glia to respond to Hh and enter S-phase, which is consistent with elevated levels of *CcnE1* and EdU uptake observed in Hh-treated NICD1+ cells. This mechanism raises an interesting evolutionary parallel with *Drosophila* eye development, where progenitor proliferation posterior to the morphogenetic furrow is dependent on a Notch signal to maintain G1 followed by downstream signaling by Hh to promote *CcnE* expression and S-phase entry (Baonza and Freeman, 2005; Duman-Scheel et al., 2002; Firth and Baker, 2005). Interestingly, Hh-stimulated NICD1+ cells did not upregulate *Ascl1*, a gene shown to induce Müller glia to form progenitor cells in the mouse (Pollak et al., 2013; Ueki et al., 2015) and fish retina (Ramachandran et al., 2010). Similarly, Hh induced proliferation of Müller glia-derived progenitor cells in the injured chick retina is not associated with *Ascl1* upregulation (Todd and Fischer, 2015), suggesting that Hh stimulation induces Müller cell cycle re-entry independent of *Ascl1* and may represent an

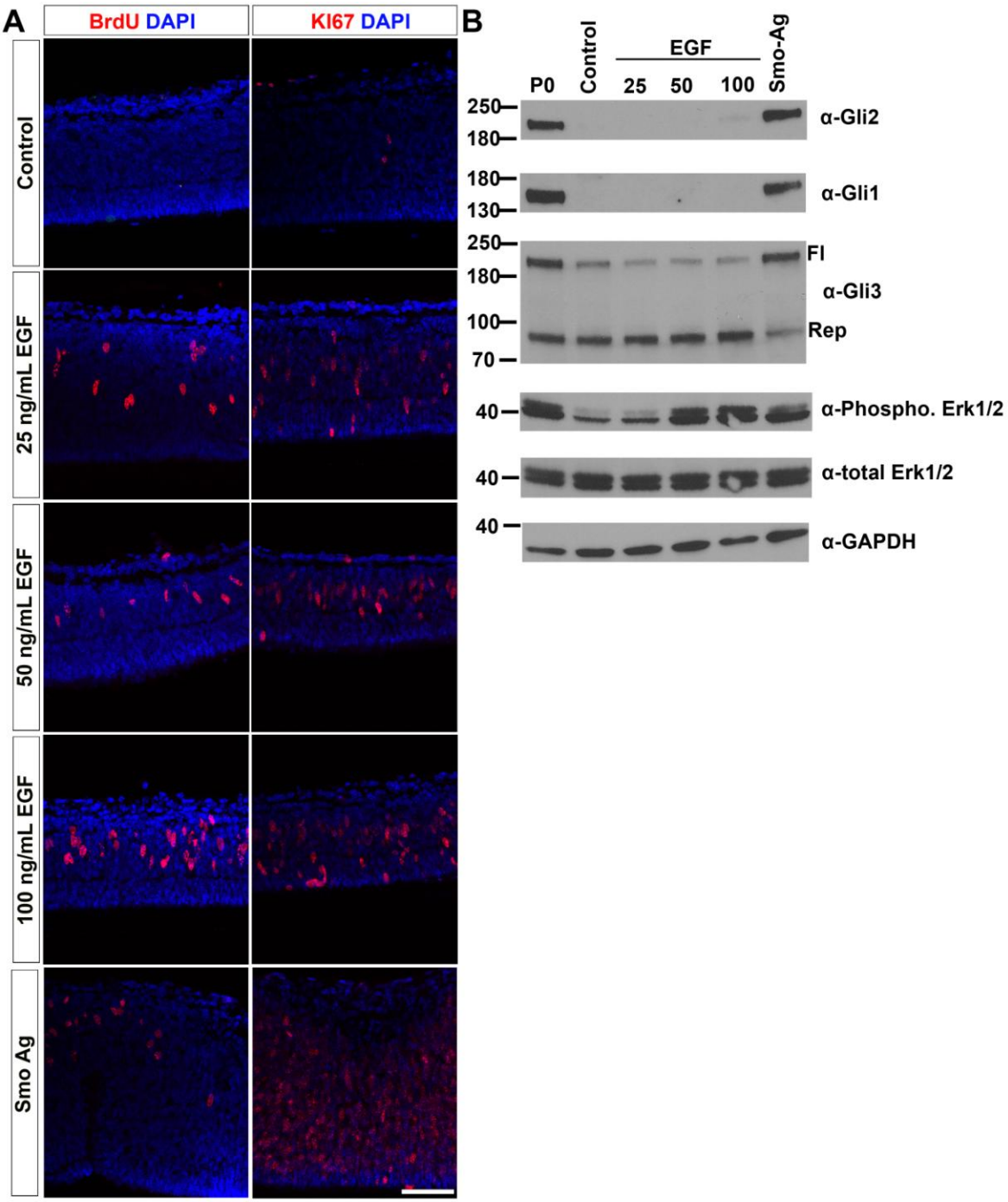
alternative approach to stimulate Müller cells. It would be interesting to explore if co-operative *Ascl1* gain-of-function with Hh stimulation could further potentiate Müller–dependent retinal regeneration in the mouse retina.

3.7 Acknowledgements

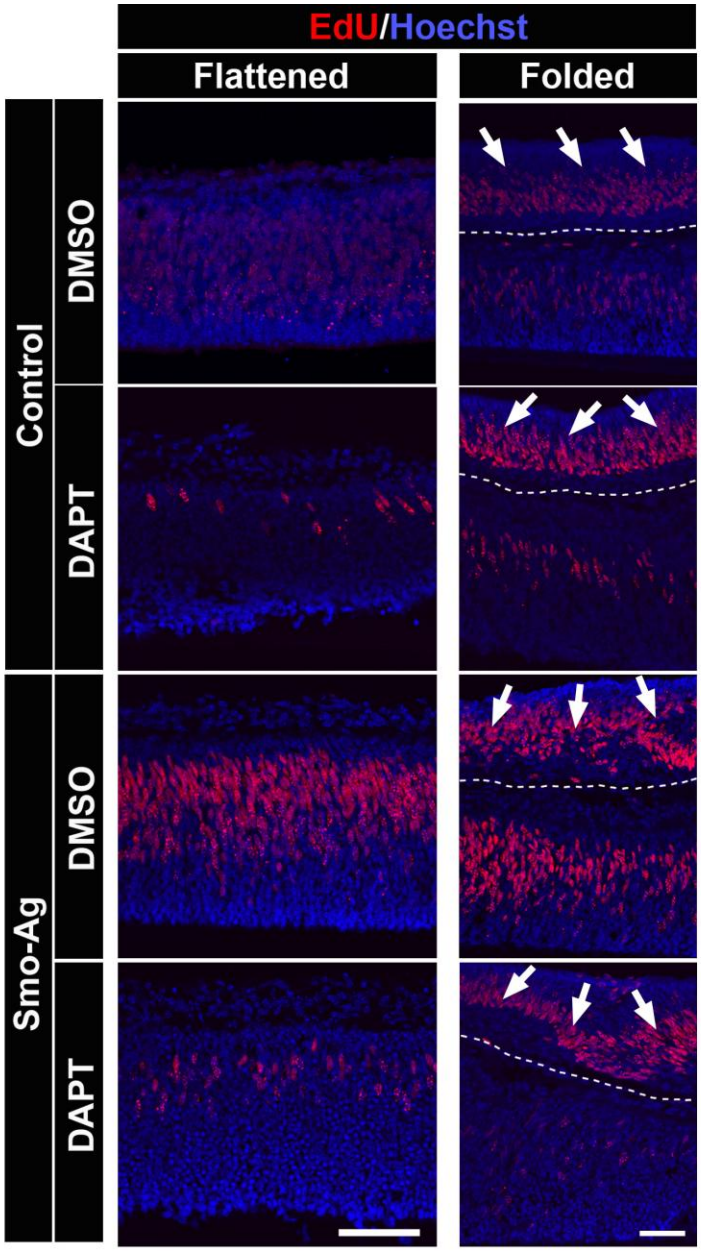
We are grateful to the Wallace laboratory members, past and present, and other OHRI members for helpful discussions and Drs. Carol Schuurmans and Phil Nickerson for critical reading of the manuscript. We thank Curis, Inc., and Drs. Alex Joyner, Gordon Fishell, Connie Cepko, Gerry Weinmaster, Michael Davidson, Nicholas Gaiano, Takahiko Matsuda, Jon C. Aster, Aimin Liu, Hisato Kondoh and James Briscoe for reagents, Dr. Jason Fernandes and StemCore laboratories for assistance with FACS, Katy Morin, Sheila Smiley and Sherry Thurig for their technical assistance and animal husbandry. We also thank University of Ottawa Animal Care for assistance with animal husbandry and surgical procedures. This research was supported by operating grants to Dr. Valerie A. Wallace from the Canadian Institute of Health Research and the Canadian Cancer Society.

3.8 Supplemental Material

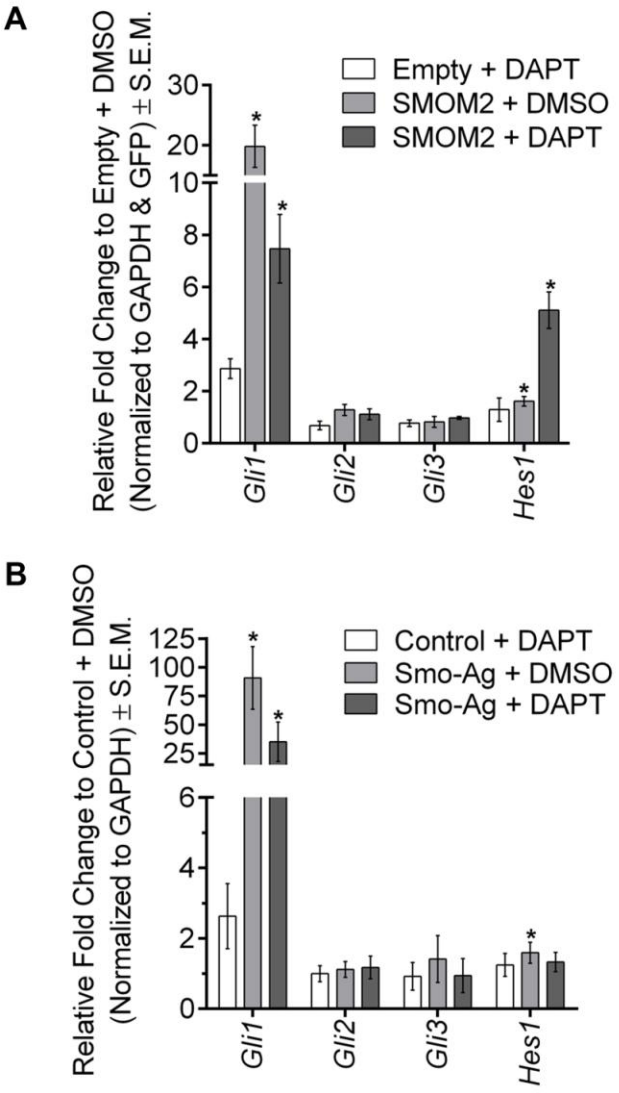
3.8.1 Supplemental Figures


A
BrdU DAPI
KI67 DAPI
Control
25 ng/mL EGF
50 ng/mL EGF
100 ng/mL EGF
Smo Ag
B
P0
Control
EGF
25
50
100
Smo-Ag
250
180
α-Gli2
180
130
α-Gli1
250
180
100
70
Fl
α-Gli3
Rep
40
α-Phospho. Erk1/2
40
α-total Erk1/2
40
α-GAPDH

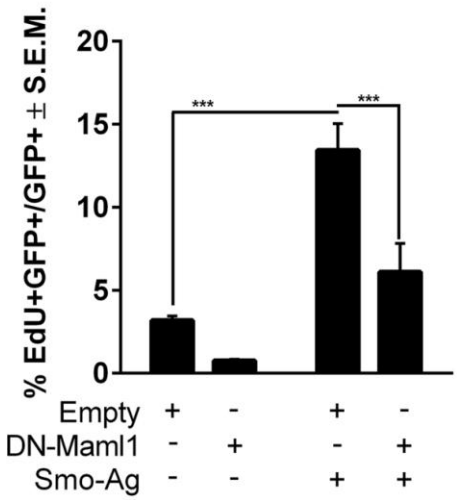
Supplemental Figure 3-1. EGF treatment promotes proliferation but is not sufficient to maintain Gli proteins *in vitro.* A) IHC measuring progenitor cells (BrdU and KI67, red+) in retinal explants as function of treatment for 3DIV. Scale bars = 50μm. B) Western blot analysis for Gli2, Gli1, Gli3, total ERK1/2 and activate phosphorylated ERK1/2, acting as a positive control for EGF stimulation, from P0 wildtype primary retinal explants treated with vehicle control, increasing concentrations of recombinant EGF or Smo-Ag for 3DIV.


EdU/Hoechst
Flattened
Folded
Control
DMSO
DAPT
Smo-Ag
DMSO
DAPT

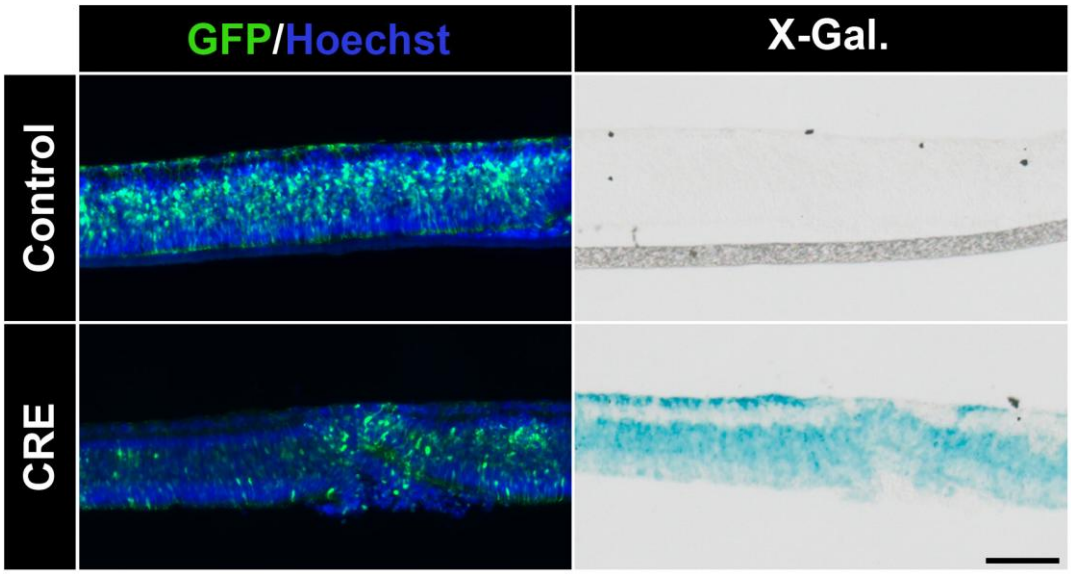
Supplemental Figure 3-2. Extensively folded regions of retinal explant cultures contain more proliferation compared to flattened regions. Retinal explants were flattened or allowed to collapse and fold (arrows, dotted line separates tissue layers) during tissue processing prior to *in vitro* culture in the presence of DAPT and Smo-Ag or the respective vehicle control conditions for 20hrs. Proliferating cells are marked by EdU (red+). All Scale bars = 50μm.


A
Relative Fold Change to Empty + DMSO
(Normalized to GAPDH & GFP) ± S.E.M.
Empty + DAPT
SMOM2 + DMSO
SMOM2 + DAPT
30
20
10
8
6
4
2
0
*
Gli1
Gli2
Gli3
Hes1
B
Relative Fold Change to Control + DMSO
(Normalized to GAPDH) ± S.E.M.
Control + DAPT
Smo-Ag + DMSO
Smo-Ag + DAPT
125
100
75
50
25
6
4
2
0
Gli1
Gli2
Gli3
Hes1

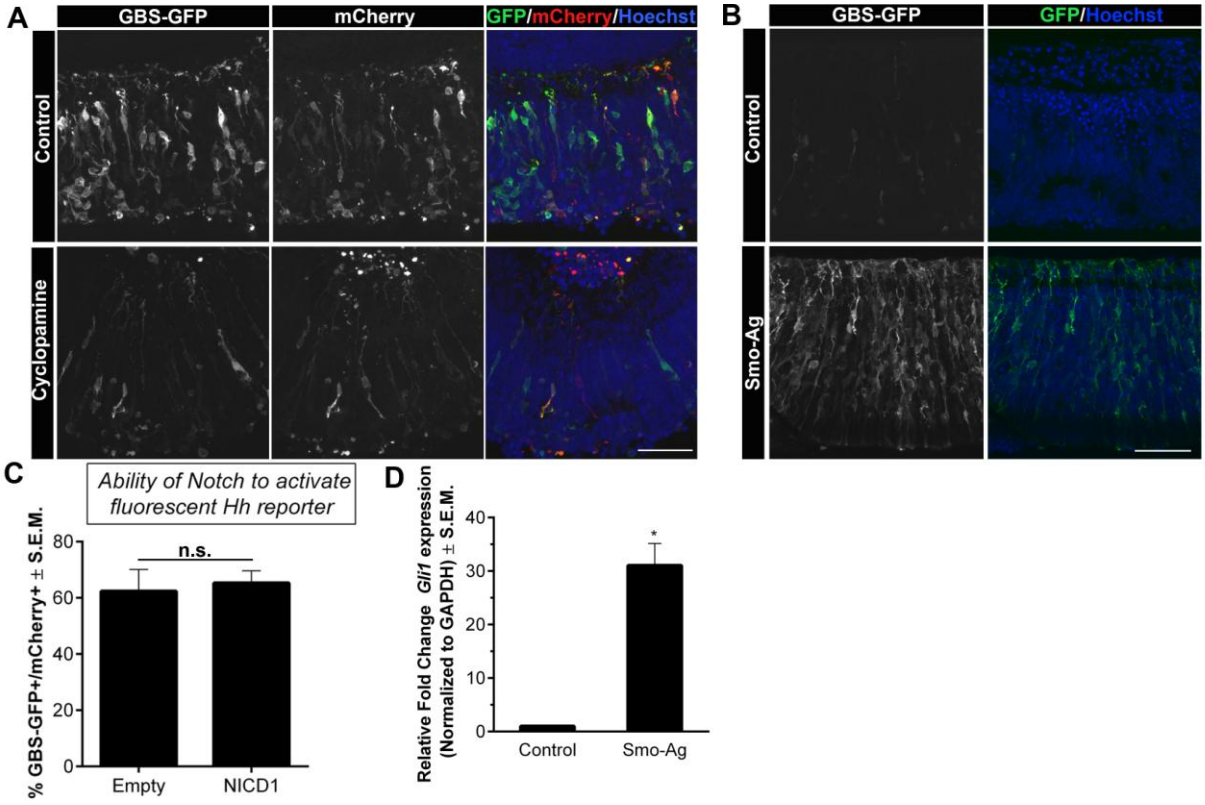
Supplemental Figure 3-3. Pharmacological Notch inhibition differentially affects SMOM2 and Smo-Ag Hh pathway activation. A) qRT-PCR analysis of gene expression in retinal explants transfected with GFP and SMOM2 or Empty and treated with DAPT or DMSO for 20hrs (n = 3 per condition). Fold changes are relative mRNA levels from Empty transfected, DMSO treated explants (n=3 biological replicates per condition). B) qRT-PCR analysis of gene expression in retinal explants treated with combinations of DAPT or DMSO and Smo-Ag or control for 20hrs (n = 3 per condition). Fold changes are relative mRNA levels from Control and DMSO treated explants (n=3 biological replicates per condition).


% EdU+GFP+/GFP+ ± S.E.M.
20
15
10
5
0
***
***
Empty + - + -
DN-Maml1 - + - +
Smo-Ag - - + +

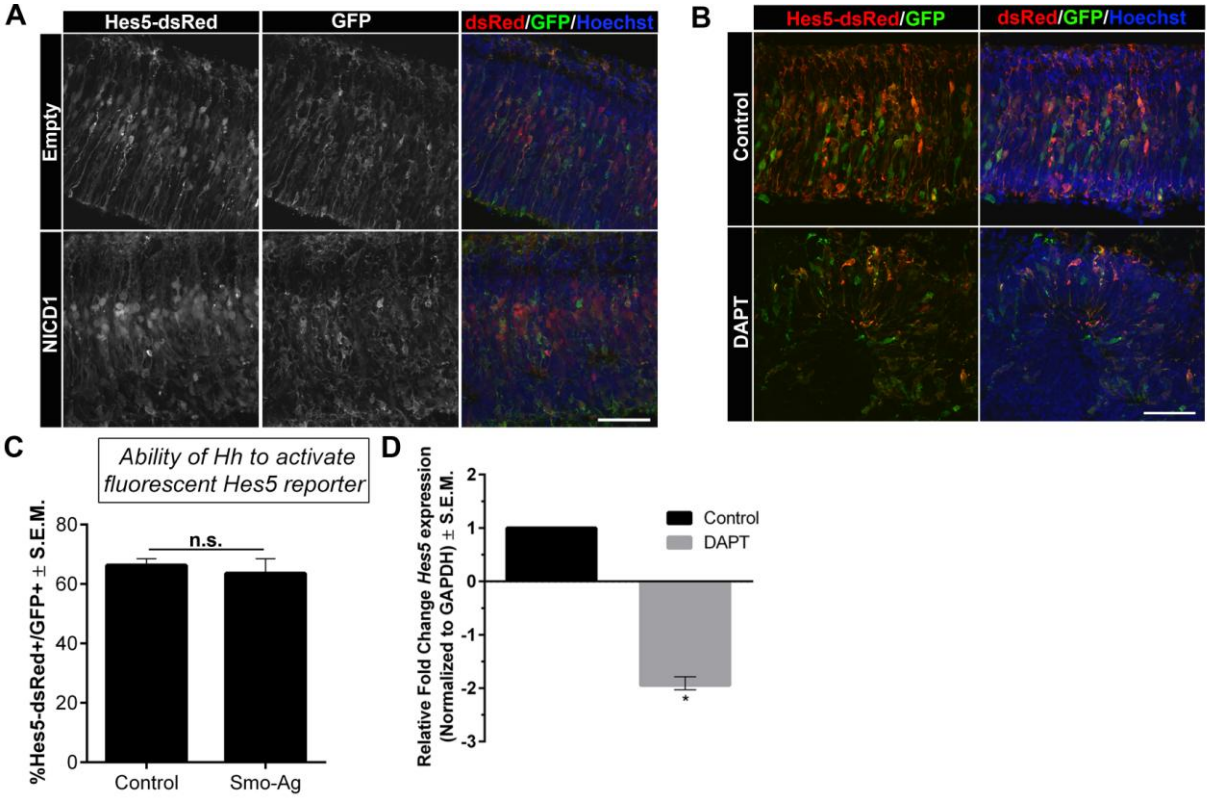
Supplemental Figure 3-4. Genetic Notch inhibition abrogates Smo-Ag dependent proliferation in retinal explant tissue. Quantification of EdU+ cells relative to the GFP+ transfected population in dissociated retinal explants (n≥3 per condition) electroporated with DN-Maml1 or Empty control vectors and treated with Smo-Ag or vehicle control after 3DIV.


GFP/Hoechst
X-Gal.
Control
CRE

Supplemental Figure 3-5. Exogenous CRE expression recombines the Rosa26 locus, expressing *LacZ,* in transfected Gli2$^{flox/flox}$;Rosa26^{LacZ} retinal explants. IHC for GFP (green+), marking transfected cells, and β-galactosidase (X-Gal., blue) staining in serial sections Gli2$^{flox/flox}$;Rosa26^{LacZ} retinal explants transfected with CRE recombinase or control vectors. Scale bar =50μm.


A
GBS-GFP
mCherry
GFP/mCherry/Hoechst
Control
Cyclopamine
B
GBS-GFP
GFP/Hoechst
Control
Smo-Ag
C
Ability of Notch to activate fluorescent Hh reporter
% GBS-GFP+/mCherry+ ± S.E.M.
n.s.
Empty
NICD1
D
Relative Fold Change Gli1 expression (Normalized to GAPDH) ± S.E.M.
*
Control
Smo-Ag

Supplemental Figure 3-6. GBS-GFP fluorescent activity reflects the activation status of Hh signaling and is not influenced by acute NICD1 expression in retinal tissue explant cultures. Fluorescent Hh reporter, GBS-GFP (green+), activity in retinal explants treated with A) 5μM Cyclopamine, a Hh pathway inhibitor, or B) Smo-Ag, an Hh pathway agonist for 1DIV or 3DIV, respectively. mCherry (red+) was included as a transfection control. Scale bars =50μm. C) In the absence of Hh signaling, exogenous NICD1 expression does not alter the fraction of Hh Reporter positive cells in retinal explants (n=4 per condition) cultured for 1DIV and dissociated into singles cells. No statistical difference was found using an unpaired, two tailed Student's *t*-test. D) qRT-PCR verifying upregulation of *Gli1* expression in Smo-Ag treated P0 wildtype retinal explants (n=6 per condition) cultured for 3DIV, relative vehicle control treatment.


A
Hes5-dsRed
GFP
dsRed/GFP/Hoechst
Empty
NICD1
B
Hes5-dsRed/GFP
dsRed/GFP/Hoechst
Control
DAPT
C
Ability of Hh to activate fluorescent Hes5 reporter
%Hes5-dsRed+/GFP+ ± S.E.M.
n.s.
Control
Smo-Ag
D
Relative Fold Change Hes5 expression (Normalized to GAPDH) ± S.E.M.
Control
DAPT
*

Supplemental Figure 3-7. The Hes5-dsRed regulatory element responds to Notch pathway gain and loss of function and is not acutely influenced by Smo-Ag treatment in retinal explant cultures. Fluorescent Notch reporter, Hes5-dsRed (red+), in retinal explants treated electroporated with A) a NICD1 expression vector and B) 10μM DAPT, a chemical inhibitor of the Notch pathway for 3DIV or 20hr in culture, respectively. GFP (green+) was included as a transfection control. Scale bars =50μm. C) Smo-Ag treatment does not alter the fraction of Hes5 Reporter positive (red+) cells in retinal explants (n=3 per condition) cultured for 1DIV and dissociated into single cells, indicating the regulatory element No statistical difference using unpaired, two tailed Student's *t*-test. D) qRT-PCR verifying decreased *Hes5* expression in DAPT treated P0 wildtype retinal explants (n=3 per condition) treated for 20hrs, relative to vehicle control treatment.

Chapter 4:

Discussion

4.0 Discussion

The overall goal of my thesis was to investigate crosstalk between Hh signaling and other developmentally critical pathways during neurogenesis. We focused on two major pathways, RTK and Notch signaling, and asked how these pathways integrate during RPC growth and maintenance. We found combined and sustained Hh and RTK signaling was sufficient to establish long-term RPC cultures, and these cells maintained neurogenic and gliogenic competence *in vitro,* but failed to engraft and differentiate into retinal cell types following *in vivo* transplantation or *in vitro* mix co-cultures with acutely dissociated postnatal retinal cells. We also identified crosstalk between Notch and Hh signaling, where Notch is required for Hh-mediated proliferation, as pharmacological or genetic inhibition of Notch signaling blocked Hh-induced proliferation and Gli protein accumulation, and forced expression of Notch was sufficient to extend the window of Hh responsiveness in a subset of Müller glia. Both Hh-RPC monolayer establishment and Notch mediated Hh-responsiveness was dependent upon *Gli2*. Taken together, this work identifies crosstalk between the Hh pathway and two major pathways, Notch and RTK, during retinal development and furthers our understanding of the molecular cross-communication mechanisms between diverse signaling networks that influence Hh to regulate progenitor growth during neurogenesis.

Although we show that Hh-RPCs with neurogenic and gliogenic potential can be maintained *in vitro*, these cultured cells did not differentiate into photoreceptors. Our findings, together with those of Czekaj et al., describing a lack of retinal differentiation capacity in mitogen-expanded mouse RPCs (Czekaj et al., 2012), suggest that long-term propagation or culture conditions affect the ability of these cells to differentiate into photoreceptors. Unlike in the study performed by Czekaj *et. al.* (2012), Hh-RPCs maintained the expression of several,

critical retinal competence factors. While we did confirm expression of these factors at the protein level, it is still possible that additional critical retinal competence factors, which were only examined at the level of mRNA,, may not have been translated.. Consistent with this suggestion, regulated translation of retinal fate determinants plays an important role in cell fate induction and progenitor competency in the retina (Cremisi, 2013; Moore et al., 2002; Okano et al., 2002). Alternatively, the lack of Hh-RPC differentiation could be due to altered activity of temporal competence factors, such as *Ikaros* or *Casz1*. *Ikaros* is needed for the generation of early cell types, while *Casz1* is required for late cell types and misexpression of these factors results in temporally inappropriate production of retinal cell types (Elliott et al., 2008; Mattar et al., 2015). If Hh-RPCs are able to track time, it is possible long-term propagation beyond the normal RPC developmental time scale may inhibit their ability give rise to later born photoreceptors and glia in our differentiation assays. To address this possibility, *Ikaros* and *Casz1* mRNA and protein levels could be measured over the course of Hh-RPC establishment and maintenance, followed by addressing if ectopic expression of either gene in Hh-RPCs could restore their retinal differentiation capacity.

Photoreceptor differentiation from Hh-RPCs may also be affected by monolayer culture conditions, where additional extrinsic signals may be required for expanded Hh-RPCs to adopt a photoreceptor fate, similar to embryonic stem cells and retinal explants, where exposure to retinoic acid can promote photoreceptor differentiation (Osakada et al., 2008; Qiu et al., 2005; Soderpalm et al., 2000). More recently, using 3-dimensional (3D) culture methods, mouse embryonic stem cells have been shown to efficiently generate neuroretina (Eiraku et al., 2011), providing a source of photoreceptors for retinal transplantation (Gonzalez-Cordero et al., 2013). It is hypothesized that 3D culture methods more faithfully recapitulate the developmental processes required for efficient photoreceptor differentiation compared to 2-dimensional,

monolayer adherent culture methods (Gonzalez-Cordero et al., 2013), as we have tried here. It may therefore be useful to investigate long-term Hh-RPC propagation using 3D culture methods as a means to maintain retinal competence within these cells.

4.2 Pitfalls of DNA incorporating S-phase markers as cell tracers

Bromodeoxyuridine (BrdU) is a thymidine analog that incorporates into the DNA of dividing cells during S-phase of the cell cycle (Taupin, 2007). Recently, additional analogs, such as IdU, CldU and EdU, have been developed, increasing the number of unique S-phase labels and allowing for more precision when asking questions related to proliferation, cell cycle dynamics and birth dating cell types (Breunig et al., 2007). Although these tools have been critical in the study of neurogenesis (Altman, 1969; Sidman et al., 1959), there exists pitfalls and limitations to thymidine analog-type S-phase markers that are often overlooked (Taupin, 2007). Our work highlights one such pitfall of using BrdU to label transplanted cells into the retina, as we found that host tissue readily uptakes BrdU from injected cells, likely as a result of transfer from dead cells. To confirm this, we subjected BrdU labeled Hh-RPCs to repeated freeze-thaw cycles prior to transplantation and observed similar robust signal to our standard Hh-RPC transplantation method, consistent with label transfer rather than integration of Hh-RPCs into the host retina. Indeed, dying cells transplanted into the brain have also been shown to release and transfer BrdU that persisted for up to 12 weeks in the surrounding host tissue (Burns et al., 2006). However, inconsistent with a model of simple BrdU transfer from dying Hh-RPCs to proliferating host cells, double BrdU+/ Arrestin+ cone photoreceptors (Zhu et al., 2002) were detected in transplanted postnatal day 3 host retinas. Cone photoreceptors are normally born during early embryonic stages (~E11) of retinal development (Swaroop et al., 2010) and presumably would

not be in S-phase at the time of injection. DNA synthesis can be initiated independent of mitosis, such as during apoptosis or DNA repair (Kuan et al., 2004; Taupin, 2007; Yang et al., 2001). Thus, it is possible that damage as a result of surgery in our transplantation model could have caused 'abortive' cell cycle re-entry in cones that subsequently picked up excessive BrdU from dying Hh-RPCs (Kuan et al., 2004), persisting for several weeks following transplantation. Alternatively, newly born rod photoreceptors would also be present in the same nuclear layer as cones and could be mistaken for double Arrestin+/BrdU+ cells without analyzing a sample at multiple focal distances, also known as focus stacking. We attempted to circumvent graft to host label transfer by transiently transfecting Hh-RPCs prior to transplantation with a fluorescent marker, but found limited integration of GFP+ cells into the host retina *in vivo*.

4.3 The mechanism of RTK and Hh synergy in cultured Hh-RPCs

Synergy between Hh and EGF signaling has been reported in neural progenitors from other brain regions during the development of multiple organisms (Palma et al., 2005; Palma and Ruiz i Altaba, 2004). Hh-RTK crosstalk has been show to be mediated by the p53 pathway (Malek et al., 2011), co-regulation of a common set of target genes (Kasper et al., 2006) and by RTK-mediated stabilization of GLI proteins (Liu et al., 2015; Stecca et al., 2007). The expansion of Hh-RPCs was not associated with changes in total TP53, the TP53 modulating protein MDM2 or the expression of cooperative Hh-RTK genes. However, we did find that efficient Hh-RPC monolayer establishment required *Gli2*. In RPCs, *Gli2* is required for induction of *CcnD1*, a key effector gene of Hh-induced proliferation in the mouse retina and *CcnD1* induction is not mimicked by EGF or FGF2 treatment in retinal explants (Wang et al., 2005). Therefore, the facile expansion of RPCs in response to combinatorial Hh-Ag and mitogen stimulation could

reflect a *Gli2*-dependent requirement for simultaneous activation of self-renewal and cell cycle target genes for the maintenance RPC self-renewal. In a transgenic glioma tumour mouse model for example, constitutively active mutant EGFR expression requires additional mutations in genes encoding cell-cycle proteins, such as CDK4, to promote lesion formation (Holland et al., 1998); thus, a similar dual mechanism, where RTK signaling requires *Hh-Gli2-CcnD1* mediated modulation of cell-cycle proteins, may be required to promote facile Hh-RPC expansion.

The requirement for *Gli2* in Hh-RPC cultures is consistent with a mechanism where stabilized GLI proteins by RTK signaling mediate growth. Several lines of evidence suggest Gli proteins, acting as a signaling nexus for the convergence of different pathway inputs downstream of Hh signaling, synergize with aberrant RTK signaling during oncogenesis and these effects are mediated by a diverse array of context specific signal transduction cascades during oncogenesis (Kasper et al., 2006; Liu et al., 2015; Riobo et al., 2006a; Riobo et al., 2006b; Schnidar et al., 2009; Stecca et al., 2007). For example, mitogen-actived protein kinase MEK1 regulates Gli2 at the transcript and protein level, where MEK1 prolongs the half-life of Gli2, resulting in increased nuclear accumulation (Liu et al., 2015). Stabilization in this context was mediated by MEK1, in cooperation with a second downstream protein kinase, RSK2, by antagonizing Gli2 phosphorylation and ubiquitination through GSK-3ß in cellular models of multiple myeloma (Liu et al., 2015). Alternatively, EGFR and MEK/ERK, but not PI3K/AKT, is important for synergistic GLI/EGF target gene induction in transformed keratinocytes (Kasper et al., 2006); however, a number of reports point to a PI3K-dependent mechanism in the potentiation of GLI and RTK signaling in the context of fibroblasts, neural explants and during chondrogenic differentiation (Riobo et al., 2006a; Riobo et al., 2006b). Thus, the downstream RTK signaling mechanisms that regulate Gli proteins display high levels of context-dependent cross-talk and to further understand the mechanism by which synergistic RTK and Hh signaling promote long-term

Hh-RPC culture, it would be important to identify the downstream RTK signaling kinases that mediate these effects in this context. By treating cultures with specific chemical inhibitors *in vitro*, you could begin to tease apart which major RTK signaling components are important in the context of Hh-RPC culture establishment and growth.

4.4 Can Hh transactive EGFRs in RPCs during normal development?

Our results suggest synergistic Hh and EGFR signaling is important in the maintenance of RPCs during retinal development, raising the question as to what mechanisms may be mediating this combined signaling. Cross-communication between G-protein coupled receptors (GPCR) and RTK can occur by a transactivation mechanism, where stimulation of a GCPR results in rapid EGFR phosphorylation and subsequent downstream signaling (Daub et al., 1996). Two major models have been proposed in the transactivation of EGFRs downstream of GPCR activation: a ligand-independent mechanism, where EGFRs are transactivated by GPCR stimulation through intracellular signaling pathways, and a second, ligand-dependent mechanism that requires GPCR-activation of matrix metalloproteases (MMPs) to proteolytically cleave and release extracellular EGF ligand, stimulating EGFRs (Prenzel et al., 1999; Wang, 2016).

Recent studies using cancerous cell lines and neural progenitors isolated from the neocortex have demonstrated that Hh signaling can transactivate the EGFR to drive proliferation in these models by an MMP-dependent mechanism (Heo et al., 2007; Reinchisi et al., 2013), presumably mediated by the GPCR, Smo. This evidence, coupled with our findings outlining a synergistic role of Hh and RTK signaling in the long-term maintenance of Hh-RPCs, raises the possibility that Hh signaling in the retina may also transactivate the EGFR to promote progenitor growth. Interestingly, several MMPs were highly expressed (average A value >9) in our

microarray data generated from Hh-RPCs, and in a separate study, we found Hh-RPCs could degrade fibrin gels when encapsulated *in vitro* in the absence of protease inhibitors (Ahmed et al., 2014), suggesting these cells actively secrete MMPs (Bini et al., 1996). Moreover, the possibility that Hh could promote RTK signaling in RPCs is also consistent with the increase in phospho-ERK1/2, a key downstream effector of RTK signaling, in Smo-Ag treated retinal explants. This is not unlike the regenerating chick retina, where Shh can induce robust Erk phosphorylation at a level comparable to FGF2 treatment and stimulate progenitor cells in the ciliary body to proliferate (Spence et al., 2007). Because Hh mediated activation of ERK1/2 through EGFR transactivation accounts for almost 50% of the EGFR-dependent mitogenic response in late neocortical neural stem cells (Reinchisi et al., 2013), it would be of interest to investigate if Hh potentiates RTK signaling by a similar mechanism during normal, mitogenic progenitor proliferation in the retina.

4.5 Retinal Regeneration and Müller Glia

Not only does our work further our understanding of neurogenesis, but a deeper comprehension of the molecular mechanisms that regulate normal RPC development are also relevant for regenerative therapies in the treatment of retinal degenerative conditions. Age-related macular degeneration (AMD) is the most common cause of blindness in developed countries (Wong et al., 2014), while retinitis pigmentosa (RP) occurs less frequently, effecting 1 in ~4000 individuals worldwide (Hartong et al., 2006). Although both diseases differ in terms of mechanism, they both result in an irreversible loss of vision due to the death of the photoreceptors in the retina and currently there is no effective treatment once photoreceptors have died, but only methods to slow disease progression (Aronow and Chew, 2014; Berson et al., 2010; Chew et al.,

2013; Clemson et al., 2011). Two potential methods to replace lost photoreceptors are cell-based replacement therapy, whereby new photoreceptors are re-introduced into the retina (Zarbin, 2016), and enhancing endogenous regenerative mechanisms in the eye; however, both therapies require a thorough understanding of the mechanisms that guide RPC growth and differentiation.

The regenerative capacity of the vertebrate retina is varied across species, where lower vertebrates such as zebrafish and frog display a remarkable ability to regenerate all mature cell types in the retina after damage (Fausett and Goldman, 2006; Raymond et al., 2006; Thummel et al., 2008b), while chick has a more limited capacity (Fischer and Reh, 2001). In contrast, regeneration is extremely limited in the mouse retina (Karl et al., 2008; Ooto et al., 2004). The regenerative ability of the retina has been linked to the capacity of late-born Müller glia to re-enter the cell cycle, differentiate and integrate into the damaged retina (Bernardos et al., 2007; Fischer and Reh, 2001). Interestingly, these glial cells are unique to the eye and share many similarities to RPCs, including exhibiting neurogenic potential (Bernardos et al., 2007; Fischer et al., 2002; Jadhav et al., 2009; Thummel et al., 2008b), maintaining the expression of several RPC markers (Blackshaw et al., 2004; Nelson et al., 2011; Roesch et al., 2008) and proliferating in response to mitogenic stimulation *in vivo* (Close et al., 2006; Fischer and Reh, 2001; Fischer et al., 2009; Karl et al., 2008; Todd and Fischer, 2015; Todd et al., 2015; Wan et al., 2012) and *in vitro* (Ferraro et al., 2015; Lawrence et al., 2007; Lillien and Cepko, 1992; Wan et al., 2007). Thus, a clear understanding of the mechanisms that control RPC proliferation and maintenance, as well as the signals that control their differentiation, are not only critical in advancing our understanding of retinohistogenesis, but could be extended to Müller glia and exploited to improve retinal regeneration and treat retinal disease.

4.6 Hh-RPCs – are they Müller glia?

There are several lines of evidence, outlined in the previous chapter, that argue for and against a Müller glial cell origin for Hh-RPCs. Another point to consider is Müller glia from adult *Tp53*$^{+/-}$ *or Tp53*$^{-/-}$ mice have an enhanced capacity to proliferate in response to EGF stimulation *in vitro*, where as wildtype Müller glia growth is restricted by the second postnatal week in dissociated or explant cultures (Ueki et al., 2012). Using our dissociated protocol method, we did not observe any changes in the expression of TP53 protein or MDM2, an upstream TP53 inhibitor whose activity is influenced by Hh signaling in the developing cerebellum (Malek et al., 2011), which suggests Hh-RPCs are distinct from Müller glia. Another notable difference between Hh-RPCs and Müller glia is the inability of Hh-RPCs to generate retinal neurons, where as proliferating Müller glia can differentiate into photoreceptors in many different contexts (Osakada et al., 2007; Wan et al., 2007). Thus, if Hh-RPCs are a form of immature Müller glia, for example, it is clear that further work will be required to understand how Hh-RPC are distinct and if regenerative mechanisms similar to Müller glia can be exploited to enhance Hh-RPCs reparative utility.

4.7 The mechanism of Hh-Notch signaling in the retina

Early, embryonic genetic manipulations of the Hh or Notch pathway preclude later analyses and both overlap temporally in the retina, which poses a challenge when trying to dissect the relationship between these critical developmental pathways (Jadhav et al., 2006a; Jadhav et al., 2006b; Riesenberg et al., 2009; Yaron et al., 2006; Zheng et al., 2009; Zhu et al., 2013). Using acute gain- and loss-of-function models of Hh and Notch pathways, we have built a model

whereby Notch, functioning upstream, promotes Hh signaling and proliferation of postnatal RPCs. This is supported by the observations that Notch activation potentiates the response of progenitors to exogenous Hh and that Notch is required for full Hh signaling, defined by *Gli1* mRNA induction, accumulation of Gli protein and proliferation in retinal explants. Consistent with a hypothesis that Notch and Hh cooperatively signal in RPCs, both pathways were active, as indicated by fluorescent reporters, in dissociated, single retinal cells together or in quick succession over a period of 24hrs. Moreover, we discovered that exogenous Gli1 or Gli2 was insufficient to restore RPC proliferation following genetic Notch inhibition in retinal explants, suggesting Notch-dependent regulation of Hh in RPCS is complex and requires other effectors in addition to Gli1 or Gli2. Consistent with this idea, we show that Notch activation promotes the expression of several genes known to play a role in Hh responses, including SoxB1 family member *Sox2*, which are known co-regulators of Hh target genes (Peterson et al., 2012), *CcnD1* (Kenney and Rowitch, 2000) and *Hes1* (Ingram et al., 2008; Wall et al., 2009). Therefore, simply bolstering Gli1 or Gli2 levels downstream of Notch inhibition may be insufficient to permit Hh signaling because Notch may be required permissively, acting through Notch transcriptional targets, to control progenitor state in addition to regulating Hh pathway components. Alternatively, robust inhibition of Notch using transgenic DN-Maml1 forces RPCs out of cycle and/or promotes differentiation and this could inhibit Gli expression, similar to the decline of Gli we observe in retina as a function of differentiation *in vitro* and *in vivo*. It would therefore be useful to confirm transgenic Gli1 and Gli2 protein expression when Notch is genetically inhibited. We did show that $Gli2^{\Delta 784}$, lacking several critical residues required for protein turnover by the ubiquitin-proteasome system (Pan et al., 2006; Zeng et al., 2010), also failed to rescue Hh proliferation downstream of genetic Notch inhibition, suggesting protein

inhibition may be mediated by alternative mechanisms independent of the proteasome or inhibitory mechanisms may act further upstream at the level of the transcript.

Our findings agree with recent evidence for a similar cooperative relationship between Notch and Hh in the developing mouse and chick neural tube demonstrating Notch signaling modulates Shh-dependent patterning (Kong et al., 2015; Stasiulewicz et al., 2015) and in the zebrafish spinal cord, where progenitor maintenance is mediated by Notch signaling, enabling later born Hh-induced cell type specification (Huang et al., 2012). In the neural tube, Notch functions upstream of Smo activation to control Ptch1 trafficking and distribution at the primary cilium through a transcriptional mechanism (Kong et al., 2015). In this context, the requirement for Notch in Hh activation could be overcome by activating the pathway at the level of Smo (Kong et al., 2015) and this is unlike our study in RPCs where direct activation of Smo cannot overcome the loss of Notch activity to promote proliferation. Unlike the first study, Stasiulewicz *et. al.* found Notch could promote full-length Gli3 protein accumulation in addition to regulating Ptch and Smo accumulation at the PC (Stasiulewicz et al., 2015). Because our protein analysis in retinal explants also includes non-transfected cells that also express Gli3 protein in addition to our transfected population, we cannot reliably draw conclusions regarding Gli3 protein ratios in Notch gain-of-function conditions. It is conceivable that full-length Gli3 could have a role in potentiating Hh signaling downstream of Notch and perhaps could explain the residual population of proliferating NICD1+ Müller glia in Gli2 conditional null explants following Hh stimulation. In the future, it would be necessary to measure Gli3 protein levels in a purified, FACS-purified population of NICD1+ retinal explants to unequivocally ask if Notch can influence Gli3 levels, which may be difficult due to technical limitation of western blotting of retinal explant tissues from limited sorted material. Similarly, Gli1 has been shown to compensate in the absence of Gli2 and could also account for the remaining dividing population in this context. Interestingly,

both studies revealed an ability of Notch to positively modulate the length of the PC (Kong et al., 2015; Stasiulewicz et al., 2015). A recent study demonstrated mature Müller glia contain PC and that these microtubule based structures are required for Shh-induced Müller glial proliferation (Ferraro et al., 2015), raising the interesting possibility that Notch could be potentiating Hh signaling in these cells through modulation of the PC.

4.8 Notch extends Hh responsiveness of Müller glia

In our second study, we show that Hh is sufficient to induce Notch expressing quiescent Müller glia to re-enter the cell cycle in retinal explants. We did not explore the neurogenic potential of Hh treated NICD1+ cells, but this question would be an interesting next step toward understanding integrated Notch and Hh signaling. In fish following injury, Müller glia activate the expression of the proneural gene *Ascl1*, dedifferentiate and re-enter the cell cycle to produce progenitor cells that regenerate the retina by replacing lost neurons (Fausett et al., 2008; Ramachandran et al., 2010; Raymond et al., 2006; Thummel et al., 2008a). By contrast, *Ascl1* fails to be upregulated in the mouse retina following injury (Karl et al., 2008) and gain-of-function for *Ascl1* causes upregulation of neuronal genes and downregulation of glial specific genes, which was mediated by ASCL1-dependent chromatin remodeling from a repressive to activated state at neuronal targets (Pollak et al., 2013). Forced Ascl1 expression reprogrammed Müller glia to adopt a neuronal fate, expressing retinal-specific markers, and exhibit a neuron-like response to neurotransmitters (Pollak et al., 2013) and *Ascl1*-expressing glia can initiate an *in vivo* regenerative response in mice similar to fish (Ueki et al., 2015). Interestingly, Hh-stimulated NICD1+ cells did not upregulate *Ascl1*, similar to Hh induced proliferation of Müller glia-derived progenitor cells in the injured chick retina (Todd and Fischer, 2015), suggesting that Hh

stimulation appears to induce Müller cell cycle re-entry independent of *Ascl1* and may represent an alternative approach to stimulate Müller cells. It would be interesting to explore if co-operative *Ascl1* gain-of-function in combination with Hh stimulation could further potentiate Müller –dependent retinal regeneration in the mouse retina.

4.9 Constitutively active versus oscillatory Notch signaling

The downstream Notch signaling effector Hes1 has been shown to oscillate in neural progenitors (Shimojo et al., 2008) and Hes1 labeling in the retina shows a 'salt and pepper' pattern, consistent with oscillating signaling and/or cellular heterogeneity (Zhu et al., 2013). Similarly, NICD1 is heterogeneously expressed in subsets of RPCs (Nelson et al., 2007). Moreover, *Hes1*+/- and *Hes1*-/- mutant retinas have differing phenotypes (Takatsuka et al., 2004), indicating RPCs are sensitive to *Hes1* dosage. Thus, it is plausible that endogenous Notch signaling may oscillate over the course of retinal development in subsets of RPCs to control their behaviour. Because we manipulated the Notch pathway using gain and loss-of-function approaches under the control of constitutively active promoters, it is possible that such strong manipulations of the Notch pathway may not accurately reflect the endogenous, dynamic levels of Notch signaling in retina and the observed Notch-dependent extension of Hh responsiveness may be an artifact as a result of unphysiological levels of Notch signaling in NICD1+ cells. However, we did observe that Hh and Notch pathways are active in close succession or within the same cell, as indicated by fluorescent reporters. In this context, we can only conclude that they are active within a 24hr time window because of the half-life of fluorescent proteins (Verkhusha et al., 2003). A critical next step in exploring our working hypothesis that Notch stabilizes Gli2 protein and allows progenitors to respond to an Hh signal would be to establish the temporal window in

which Notch and Hh are active within RPCs. The retina is an ideal model to investigate this question using fluorescent reporters because transgenes can easily be introduced *in vivo* and *in vitro* into the tissue (Matsuda and Cepko, 2004), providing a unique and powerful opportunity to determine the temporal signaling dynamics between the Hh and Notch pathway in progenitors and also test the effects of differing dosages of Notch on Hh signaling during retinal development.

4.10 Gli expression and protein – The gatekeeper of Hh signaling in an environment of persistent ligand?

We show that there is an inverse relationship between the expression of Hh pathway activators and neuronal differentiation. *Gli* transcripts are not detected in differentiated layers of the retina and rapid cell cycle exit of RPCs induced by loss of Shh signaling is associated with downregulation of Gli protein and transcripts. Since Gli2 is required for normal RPC responses to Hh, this rapid loss of Gli proteins would render cells non-responsive to the persistent Shh ligand in their environment and this observation could represent a mechanism by which neurogenic precursors escape Hh activation. Based on our expression analysis over the course of retinal development, the rapid loss of *Gli* expression cannot fully be explained by loss of key upstream Hh signaling components, such as *Smo* or *Ptch1*, since expression of these genes decays at a slower rate over retinal development *in vivo*. This raises the question: what are the mechanisms that regulate *Gli* expression in differentiating neurons? The decline in *Gli* expression could be a result of neuron specific inhibitors and/or the loss of positive *Gli* regulators in post-mitotic neurons.

Epigenetic changes, where the chromatin landscape at the *Gli* locus changes from an open, activated state to a repressive, silenced one mediated by post-translational modifications of

histones, could be a potential mechanism to rapidly extinguish *Gli* expression as a progenitor differentiates. In the retina, epigenetic regulation is important for normal retinal function and development (Rao et al., 2011). For example, histone lysine methylation marks show dynamic expression patterns over the course of retinal maturation, where marks associated with active gene transcription are detected in the proliferative layers of the neuroblast during development and later shift to a signature associated with transcriptional repression that is restricted largely to newly born RGCs (Rao et al., 2010), and accordingly, a number of histone lysine methyl-transferase proteins that catalyze the formation of repressive histone marks are also temporally expressed in RGCs (Komai et al., 2009; Rao et al., 2010). To test the idea of epigenetic regulation in the control of temporal *Gli* expression in the retina, it would be necessary to first map the key post-translational histone modifications at each *Gli* loci in proliferating RPCs and compare this histone signature with that found in post-mitotic precursor cells and mature neurons. If there were significant differences in histone acetylation and/or methylation status at the *Gli* loci, the next obvious question would be which epigenetic enzymes are important for this modulation over the course of retinal development? Because *Gli* expression declined as a function of development, we would predict activating histone modifications in progenitors that would shift to repressive marks in precursor cells and differentiated neurons. Recently, epigenetic targeting of Hh pathway transcriptional output has been explored as a strategy to control growth of cancers resistant to Smo inhibitors (Long et al., 2014; Tang et al., 2015). In these studies, the bromo and extra C-terminal (BET) bromodomain protein, BRD4, has emerged as a key regulater of *Gli* transcription downstream of Smo and Sufu, where it has been shown to directly bind *Gli1* and *Gli2* promoter regions, modulate Hh target gene expression and promote medulloblastoma growth (Long et al., 2014; Tang et al., 2015). Bromodomain proteins recognize and bind acetylated motifs on open chromatin found at lysine residues on H3 histones and

participate in large, DNA binding regulatory complexes with other proteins to control transcriptional activity (Dhalluin et al., 1999; Jacobson et al., 2000) and it is possible similar epigenetic regulation may occur in the retina to positively control *Gli* expression in progenitors that is downregulated in neurons.

Alternatively, another strong candidate mechanism in the temporal regulation of *Gli* expression in the retina is the neuropeptide pituitary adenylate cyclase-activated peptide (PACAP). Consistent with a build up of negative regulators of *Gli* expression as cells shift from a proliferative progenitor to mature neuron, PACAP expression in the neural tube is restricted to newly differentiated cells (Waschek et al., 1998). In the cerebellum, PACAP can antagonize Gli2 activity through a PKA mediated mechanism, inhibiting Shh-driven progenitor proliferation (Niewiadomski et al., 2013), and PACAP can also inhibit Hh-driven medulloblastoma derived growth in culture (Cohen et al., 2010). Consistent with a proposed role in negatively regulating mitogenic Hh signaling, PACAP modulates RPC proliferation through the downregulation of *CcnD1* expression in the retina (Njaine et al., 2010). Given the evidence for PACAP to negatively regulate Hh-driven proliferation in normal and cancerous cerebellar growth and the proposed role of Hh in the retina, it is plausible that PACAP could modulate RPC proliferation by antagonizing Gli activity during retinal development.

4.11 Notch-Hh signaling in a disease context

The requirement for Notch to sustain Hh signaling raises the possibility that Notch may be involved in potentiating Hh signaling in a disease context and inhibiting Notch could represent a novel therapeutic target. The dual requirement for Notch and Hh in the formation and growth of medulloblastoma, a tumour derived from the cerebellum as a result of activating mutations in the

Hh pathway (Goodrich et al., 1997), has been explored. A requirement for Notch in Hh-dependent tumours has been shown to be context dependent, where growth and formation of tumours are unaffected by genetic inhibition of RBPJκ *in vivo* (Julian et al., 2010a; Julian et al., 2010b), while pharmacological Notch inhibition decreased growth and cellular survival in human medulloblastoma cell lines, mouse ex vivo cultured tumours and human tumour xenografts transplanted into immunocompromised mice (Hallahan et al., 2004). These differing effects of Notch on Hh-dependent tumour growth may be related to the mechanism of Hh activation used in either study, with the former inactivating one allele of *Ptch* (Julian et al., 2010a; Julian et al., 2010b) and the latter upregulating *Smo* activity (Hallahan et al., 2004). Context dependent differences are also evident between different brain regions, because attenuation of Notch in neocortical progenitors reverses the effect of loss of *Ptch1*, restoring the balance of symmetric proliferative versus neurogenic divisions (Dave et al., 2011).

Despite the conflicting evidence for Notch playing a role in Hh-initiated tumours of the cerebellum and region specific requirements for both pathways throughout the brain, there may be other diseases in which therapeutically targeting Notch may improve disease outcomes. For example, Basal Cell Naevus (BCNS) or Gorlin syndrome patients exhibit a range of abnormalities, including birth defects, predisposition to tumour development and vitreoretinal defects in ~15-25% of affected individuals (Evans et al., 1993; Gorlin, 1987), as a result of an autosomal dominant mutations of the *PTCH* gene (Hahn et al., 1996; Johnson et al., 1996). *PtchLacZ*$^{+/-}$ mice display ectopic retinal proliferation, photoreceptor dysplasia, and Müller glia gliosis (Black et al., 2003). Glia activation in BCNS patients (McLeod et al., 1987) and gliosis in *PtchLacZ*$^{+}$ lesions (Black et al., 2003) are a hallmark of epiretinal membrane formation (ERM) and are postulated to be a result of maladapted retinal wound repair processes that ultimately lead to glia hypertrophy, upregulation of intermediate filaments and transient proliferation (Bringmann

et al., 2006), forming scar-like tissue that distorts the retina and impairs vision (Bringmann and Wiedemann, 2009; Pastor, 1998). Considering the model we propose, where Notch is required for Hh-mediated proliferation in the postnatal retina, it is tempting to speculate that inhibiting Notch may be beneficial in the treatment of BCNS by blocking overactive Hh signaling downstream of the *PTCH* mutation in Müller glia to prevent ERM formation. Future studies exploring this possibility could expand the scope of this work and may reveal a novel therapeutic target in the treatment of ERM and other proliferative vitreoretinopathies.

5.0 Conclusions

In conclusion, we have demonstrated a role for integrated signaling between Hh and two other critical pathways, Notch and RTK, to regulate retinal progenitor growth during retinal development, acting through the signaling nexus Gli2. Notch, RTK and Hh pathways have been extensively studied, but the intricate molecular crosstalk mechanisms that mediate diverse cellular functions during development are still largely unknown. This work serves to advance our understanding of cross-communication during neurogenesis and may also reveal novel therapeutic targets or unique tissue regeneration strategies to treat human disease in the future.

6.0 References

Agathocleous, M., Harris, W. A., 2009. From progenitors to differentiated cells in the vertebrate retina. Annu Rev Cell Dev Biol. 25**,** 45-69.

Agathocleous, M., Locker, M., Harris, W. A., Perron, M., 2007. A general role of hedgehog in the regulation of proliferation. Cell Cycle. 6**,** 156-9.

Ahmad, I., Dooley, C. M., Polk, D. L., 1997. Delta-1 is a regulator of neurogenesis in the vertebrate retina. Dev Biol. 185**,** 92-103.

Ahmad, I., Dooley, C. M., Thoreson, W. B., Rogers, J. A., Afiat, S., 1999. In vitro analysis of a mammalian retinal progenitor that gives rise to neurons and glia. Brain Res. 831**,** 1-10.

Ahmed, T. A., Ringuette, R., Wallace, V. A., Griffith, M., 2014. Autologous fibrin glue as an encapsulating scaffold for delivery of retinal progenitor cells. Front Bioeng Biotechnol. 2**,** 85.

Altman, J., 1969. Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J Comp Neurol. 137**,** 433-57.

Amin, A., Li, Y., Finkelstein, R., 1999. Hedgehog activates the EGF receptor pathway during Drosophila head development. Development. 126**,** 2623-30.

Anchan, R. M., Reh, T. A., Angello, J., Balliet, A., Walker, M., 1991. EGF and TGF-alpha stimulate retinal neuroepithelial cell proliferation in vitro. Neuron. 6**,** 923-36.

Andreazzoli, M., Gestri, G., Cremisi, F., Casarosa, S., Dawid, I. B., Barsacchi, G., 2003. Xrx1 controls proliferation and neurogenesis in Xenopus anterior neural plate. Development. 130**,** 5143-54.

Angenieux, B., Schorderet, D. F., Arsenijevic, Y., 2006. Epidermal growth factor is a neuronal differentiation factor for retinal stem cells in vitro. Stem Cells. 24**,** 696-706.

Artavanis-Tsakonas, S., 1988. The molecular biology of the Notch locus and the fine tuning of differentiation in Drosophila. Trends Genet. 4**,** 95-100.

Artavanis-Tsakonas, S., Rand, M. D., Lake, R. J., 1999. Notch signaling: cell fate control and signal integration in development. Science. 284**,** 770-6.

Ascano, J. M., Beverly, L. J., Capobianco, A. J., 2003. The C-terminal PDZ-ligand of JAGGED1 is essential for cellular transformation. J Biol Chem. 278**,** 8771-9.

Austin, C. P., Feldman, D. E., Ida, J. A., Jr., Cepko, C. L., 1995. Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. Development. 121**,** 3637-50.

Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C., Kornberg, T. B., 1997. Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. Cell. 89**,** 1043-53.

Bai, C. B., Auerbach, W., Lee, J. S., Stephen, D., Joyner, A. L., 2002. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. Development. 129**,** 4753-61.

Baker, N. E., Bhattacharya, A., Firth, L. C., 2009. Regulation of Hh signal transduction as Drosophila eye differentiation progresses. Dev Biol. 335**,** 356-66.

Baker, N. E., Yu, S. Y., 2001. The EGF receptor defines domains of cell cycle progression and survival to regulate cell number in the developing Drosophila eye. Cell. 104**,** 699-708.

Balaskas, N., Ribeiro, A., Panovska, J., Dessaud, E., Sasai, N., Page, K. M., Briscoe, J., Ribes, V., 2012. Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. Cell. 148**,** 273-84.

Bao, Z. Z., Cepko, C. L., 1997. The expression and function of Notch pathway genes in the developing rat eye. J Neurosci. 17**,** 1425-34.

Baonza, A., Casci, T., Freeman, M., 2001. A primary role for the epidermal growth factor receptor in ommatidial spacing in the Drosophila eye. Curr Biol. 11**,** 396-404.

Baonza, A., Freeman, M., 2005. Control of cell proliferation in the Drosophila eye by Notch signaling. Dev Cell. 8**,** 529-39.

Baonza, A., Murawsky, C. M., Travers, A. A., Freeman, M., 2002. Pointed and Tramtrack69 establish an EGFR-dependent transcriptional switch to regulate mitosis. Nat Cell Biol. 4**,** 976-80.

Barabino, S. M., Spada, F., Cotelli, F., Boncinelli, E., 1997. Inactivation of the zebrafish homologue of Chx10 by antisense oligonucleotides causes eye malformations similar to the ocular retardation phenotype. Mech Dev. 63**,** 133-43.

Bassett, E. A., Wallace, V. A., 2012. Cell fate determination in the vertebrate retina. Trends Neurosci. 35**,** 565-73.

Belliveau, M. J., Cepko, C. L., 1999. Extrinsic and intrinsic factors control the genesis of amacrine and cone cells in the rat retina. Development. 126**,** 555-66.

Belloni, E., Muenke, M., Roessler, E., Traverso, G., Siegel-Bartelt, J., Frumkin, A., Mitchell, H. F., Donis-Keller, H., Helms, C., Hing, A. V., Heng, H. H., Koop, B., Martindale, D., Rommens, J. M., Tsui, L. C., Scherer, S. W., 1996. Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. Nat Genet. 14**,** 353-6.

Bernardos, R. L., Barthel, L. K., Meyers, J. R., Raymond, P. A., 2007. Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci. 27**,** 7028-40.

Bertrand, N., Dahmane, N., 2006. Sonic hedgehog signaling in forebrain development and its interactions with pathways that modify its effects. Trends Cell Biol. 16**,** 597-605.

Bigelow, R. L., Jen, E. Y., Delehedde, M., Chari, N. S., McDonnell, T. J., 2005. Sonic hedgehog induces epidermal growth factor dependent matrix infiltration in HaCaT keratinocytes. J Invest Dermatol. 124**,** 457-65.

Bini, A., Itoh, Y., Kudryk, B. J., Nagase, H., 1996. Degradation of cross-linked fibrin by matrix metalloproteinase 3 (stromelysin 1): hydrolysis of the gamma Gly 404-Ala 405 peptide bond. Biochemistry. 35**,** 13056-63.

Bitgood, M. J., Shen, L., McMahon, A. P., 1996. Sertoli cell signaling by Desert hedgehog regulates the male germline. Curr Biol. 6**,** 298-304.

Black, G. C., Mazerolle, C. J., Wang, Y., Campsall, K. D., Petrin, D., Leonard, B. C., Damji, K. F., Evans, D. G., McLeod, D., Wallace, V. A., 2003. Abnormalities of the vitreoretinal interface caused by dysregulated Hedgehog signaling during retinal development. Hum Mol Genet. 12**,** 3269-76.

Blackshaw, S., Harpavat, S., Trimarchi, J., Cai, L., Huang, H., Kuo, W. P., Weber, G., Lee, K., Fraioli, R. E., Cho, S. H., Yung, R., Asch, E., Ohno-Machado, L., Wong, W. H., Cepko, C. L., 2004. Genomic analysis of mouse retinal development. PLoS Biol. 2**,** E247.

Bland, C. E., Kimberly, P., Rand, M. D., 2003. Notch-induced proteolysis and nuclear localization of the Delta ligand. J Biol Chem. 278**,** 13607-10.

Blume-Jensen, P., Hunter, T., 2001. Oncogenic kinase signalling. Nature. 411**,** 355-65.

Brechbiel, J., Miller-Moslin, K., Adjei, A. A., 2014. Crosstalk between hedgehog and other signaling pathways as a basis for combination therapies in cancer. Cancer Treat Rev. 40**,** 750-9.

Breunig, J. J., Arellano, J. I., Macklis, J. D., Rakic, P., 2007. Everything that glitters isn't gold: a critical review of postnatal neural precursor analyses. Cell Stem Cell. 1**,** 612-27.

Bringmann, A., Pannicke, T., Grosche, J., Francke, M., Wiedemann, P., Skatchkov, S. N., Osborne, N. N., Reichenbach, A., 2006. Muller cells in the healthy and diseased retina. Prog Retin Eye Res. 25**,** 397-424.

Bringmann, A., Wiedemann, P., 2009. Involvement of Muller glial cells in epiretinal membrane formation. Graefes Arch Clin Exp Ophthalmol. 247**,** 865-83.

Briscoe, J., Therond, P. P., 2013. The mechanisms of Hedgehog signalling and its roles in development and disease. Nat Rev Mol Cell Biol. 14**,** 416-29.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A., Israel, A., 2000. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. Mol Cell. 5**,** 207-16.

Brown, N. L., Patel, S., Brzezinski, J., Glaser, T., 2001. Math5 is required for retinal ganglion cell and optic nerve formation. Development. 128**,** 2497-508.

Burmeister, M., Novak, J., Liang, M. Y., Basu, S., Ploder, L., Hawes, N. L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V. I., Roderick, T. H., Taylor, B. A., Hankin, M. H., McInnes, R. R., 1996. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat Genet. 12**,** 376-84.

Burns, T. C., Ortiz-Gonzalez, X. R., Gutierrez-Perez, M., Keene, C. D., Sharda, R., Demorest, Z. L., Jiang, Y., Nelson-Holte, M., Soriano, M., Nakagawa, Y., Luquin, M. R., Garcia-Verdugo, J. M., Prosper, F., Low, W. C., Verfaillie, C. M., 2006. Thymidine analogs are transferred from prelabeled donor to host cells in the central nervous system after transplantation: a word of caution. Stem Cells. 24**,** 1121-7.

Cagan, R. L., Ready, D. F., 1989. Notch is required for successive cell decisions in the developing Drosophila retina. Genes Dev. 3**,** 1099-112.

Callejo, A., Torroja, C., Quijada, L., Guerrero, I., 2006. Hedgehog lipid modifications are required for Hedgehog stabilization in the extracellular matrix. Development. 133**,** 471-83.

Canola, K., Arsenijevic, Y., 2007. Generation of cells committed towards the photoreceptor fate for retinal transplantation. Neuroreport. 18**,** 851-5.

Casarosa, S., Amato, M. A., Andreazzoli, M., Gestri, G., Barsacchi, G., Cremisi, F., 2003. Xrx1 controls proliferation and multipotency of retinal progenitors. Mol Cell Neurosci. 22**,** 25-36.

Cavallaro, M., Mariani, J., Lancini, C., Latorre, E., Caccia, R., Gullo, F., Valotta, M., DeBiasi, S., Spinardi, L., Ronchi, A., Wanke, E., Brunelli, S., Favaro, R., Ottolenghi, S., Nicolis, S. K., 2008. Impaired generation of mature neurons by neural stem cells from hypomorphic Sox2 mutants. Development. 135**,** 541-57.

Cayouette, M., Barres, B. A., Raff, M., 2003. Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. Neuron. 40**,** 897-904.

Cayouette, M., Poggi, L., Harris, W. A., 2006. Lineage in the vertebrate retina. Trends Neurosci. 29**,** 563-70.

Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M., Ezzeddine, D., 1996. Cell fate determination in the vertebrate retina. Proc Natl Acad Sci U S A. 93**,** 589-95.

Chacko, D. M., Rogers, J. A., Turner, J. E., Ahmad, I., 2000. Survival and differentiation of cultured retinal progenitors transplanted in the subretinal space of the rat. Biochem Biophys Res Commun. 268**,** 842-6.

Chamoun, Z., Mann, R. K., Nellen, D., von Kessler, D. P., Bellotto, M., Beachy, P. A., Basler, K., 2001. Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. Science. 293**,** 2080-4.

Chen, C. M., Cepko, C. L., 2000. Expression of Chx10 and Chx10-1 in the developing chicken retina. Mech Dev. 90**,** 293-7.

Chen, D., Livne-bar, I., Vanderluit, J. L., Slack, R. S., Agochiya, M., Bremner, R., 2004. Cell-specific effects of RB or RB/p107 loss on retinal development implicate an intrinsically death-resistant cell-of-origin in retinoblastoma. Cancer Cell. 5**,** 539-51.

Chen, Y., Struhl, G., 1996. Dual roles for patched in sequestering and transducing Hedgehog. Cell. 87**,** 553-63.

Chen, Y., Struhl, G., 1998. In vivo evidence that Patched and Smoothened constitute distinct binding and transducing components of a Hedgehog receptor complex. Development. 125**,** 4943-8.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., Beachy, P. A., 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 383**,** 407-13.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., Kintner, C., 1995. Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature. 375**,** 761-6.

Chow, R. L., Lang, R. A., 2001. Early eye development in vertebrates. Annu Rev Cell Dev Biol. 17**,** 255-96.

Classon, M., Harlow, E., 2002. The retinoblastoma tumour suppressor in development and cancer. Nat Rev Cancer. 2**,** 910-7.

Close, J. L., Liu, J., Gumuscu, B., Reh, T. A., 2006. Epidermal growth factor receptor expression regulates proliferation in the postnatal rat retina. Glia. 54**,** 94-104.

Cohen, J. R., Resnick, D. Z., Niewiadomski, P., Dong, H., Liau, L. M., Waschek, J. A., 2010. Pituitary adenylyl cyclase activating polypeptide inhibits gli1 gene expression and proliferation in primary medulloblastoma derived tumorsphere cultures. BMC Cancer. 10**,** 676.

Copp, A. J., Greene, N. D., Murdoch, J. N., 2003. The genetic basis of mammalian neurulation. Nat Rev Genet. 4**,** 784-93.

Corrales, J. D., Blaess, S., Mahoney, E. M., Joyner, A. L., 2006. The level of sonic hedgehog signaling regulates the complexity of cerebellar foliation. Development. 133**,** 1811-21.

Coyne, T. M., Marcus, A. J., Woodbury, D., Black, I. B., 2006. Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. Stem Cells. 24**,** 2483-92.

Cremisi, F., 2013. MicroRNAs and cell fate in cortical and retinal development. Front Cell Neurosci. 7**,** 141.

Cunningham, J. J., Levine, E. M., Zindy, F., Goloubeva, O., Roussel, M. F., Smeyne, R. J., 2002. The cyclin-dependent kinase inhibitors p19(Ink4d) and p27(Kip1) are coexpressed in select retinal cells and act cooperatively to control cell cycle exit. Mol Cell Neurosci. 19**,** 359-74.

Cwinn, M. A., Mazerolle, C., McNeill, B., Ringuette, R., Thurig, S., Hui, C. C., Wallace, V. A., 2011. Suppressor of fused is required to maintain the multipotency of neural progenitor cells in the retina. J Neurosci. 31**,** 5169-80.

Czekaj, M., Haas, J., Gebhardt, M., Muller-Reichert, T., Humphries, P., Farrar, J., Bartsch, U., Ader, M., 2012. In vitro expanded stem cells from the developing retina fail to generate photoreceptors but differentiate into myelinating oligodendrocytes. PLoS One. 7**,** e41798.

Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M., Ishii, S., 1999. Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. J Biol Chem. 274**,** 8143-52.

Dakubo, G. D., Mazerolle, C., Furimsky, M., Yu, C., St-Jacques, B., McMahon, A. P., Wallace, V. A., 2008. Indian hedgehog signaling from endothelial cells is required for sclera and retinal pigment epithelium development in the mouse eye. Dev Biol. 320**,** 242-55.

Danno, H., Michiue, T., Hitachi, K., Yukita, A., Ishiura, S., Asashima, M., 2008. Molecular links among the causative genes for ocular malformation: Otx2 and Sox2 coregulate Rax expression. Proc Natl Acad Sci U S A. 105**,** 5408-13.

Das, A. V., Mallya, K. B., Zhao, X., Ahmad, F., Bhattacharya, S., Thoreson, W. B., Hegde, G. V., Ahmad, I., 2006. Neural stem cell properties of Muller glia in the mammalian retina: regulation by Notch and Wnt signaling. Dev Biol. 299**,** 283-302.

Das, G., Choi, Y., Sicinski, P., Levine, E. M., 2009. Cyclin D1 fine-tunes the neurogenic output of embryonic retinal progenitor cells. Neural Dev. 4**,** 15.

Dave, R. K., Ellis, T., Toumpas, M. C., Robson, J. P., Julian, E., Adolphe, C., Bartlett, P. F., Cooper, H. M., Reynolds, B. A., Wainwright, B. J., 2011. Sonic hedgehog and notch signaling can cooperate to regulate neurogenic divisions of neocortical progenitors. PLoS One. 6**,** e14680.

Dawber, R. J., Hebbes, S., Herpers, B., Docquier, F., van den Heuvel, M., 2005. Differential range and activity of various forms of the Hedgehog protein. BMC Dev Biol. 5**,** 21.

De Moerlooze, L., Spencer-Dene, B., Revest, J. M., Hajihosseini, M., Rosewell, I., Dickson, C., 2000. An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. Development. 127**,** 483-92.

Dhalluin, C., Carlson, J. E., Zeng, L., He, C., Aggarwal, A. K., Zhou, M. M., 1999. Structure and ligand of a histone acetyltransferase bromodomain. Nature. 399**,** 491-6.

Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J., Hui, C. C., 1998. Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. Development. 125**,** 2533-43.

Dominguez, M., Wasserman, J. D., Freeman, M., 1998. Multiple functions of the EGF receptor in Drosophila eye development. Curr Biol. 8**,** 1039-48.

Dorsky, R. I., Rapaport, D. H., Harris, W. A., 1995. Xotch inhibits cell differentiation in the Xenopus retina. Neuron. 14**,** 487-96.

Dudka, A. A., Sweet, S. M., Heath, J. K., 2010. Signal transducers and activators of transcription-3 binding to the fibroblast growth factor receptor is activated by receptor amplification. Cancer Res. 70**,** 3391-401.

Dufort, D., Schwartz, L., Harpal, K., Rossant, J., 1998. The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. Development. 125**,** 3015-25.

Duman-Scheel, M., Weng, L., Xin, S., Du, W., 2002. Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. Nature. 417**,** 299-304.

Dyer, M. A., Cepko, C. L., 2000. Control of Muller glial cell proliferation and activation following retinal injury. Nat Neurosci. 3**,** 873-80.

Dyer, M. A., Cepko, C. L., 2001. p27Kip1 and p57Kip2 regulate proliferation in distinct retinal progenitor cell populations. J Neurosci. 21**,** 4259-71.

Eiraku, M., Takata, N., Ishibashi, H., Kawada, M., Sakakura, E., Okuda, S., Sekiguchi, K., Adachi, T., Sasai, Y., 2011. Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature. 472**,** 51-6.

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T., Beachy, P. A., 1995. Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. Curr Biol. 5**,** 944-55.

Elliott, J., Jolicoeur, C., Ramamurthy, V., Cayouette, M., 2008. Ikaros confers early temporal competence to mouse retinal progenitor cells. Neuron. 60**,** 26-39.

Engelhardt, M., Wachs, F. P., Couillard-Despres, S., Aigner, L., 2004. The neurogenic competence of progenitors from the postnatal rat retina in vitro. Exp Eye Res. 78**,** 1025-36.

Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M., Edlund, T., 1995. Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. Cell. 81**,** 747-56.

Eswarakumar, V. P., Lax, I., Schlessinger, J., 2005. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev. 16**,** 139-49.

Evans, D. G., Ladusans, E. J., Rimmer, S., Burnell, L. D., Thakker, N., Farndon, P. A., 1993. Complications of the naevoid basal cell carcinoma syndrome: results of a population based study. J Med Genet. 30**,** 460-4.

Fantes, J., Ragge, N. K., Lynch, S. A., McGill, N. I., Collin, J. R., Howard-Peebles, P. N., Hayward, C., Vivian, A. J., Williamson, K., van Heyningen, V., FitzPatrick, D. R., 2003. Mutations in SOX2 cause anophthalmia. Nat Genet. 33**,** 461-3.

Fantl, V., Stamp, G., Andrews, A., Rosewell, I., Dickson, C., 1995. Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev. 9**,** 2364-72.

Farhy, C., Elgart, M., Shapira, Z., Oron-Karni, V., Yaron, O., Menuchin, Y., Rechavi, G., Ashery-Padan, R., 2013. Pax6 is required for normal cell-cycle exit and the differentiation kinetics of retinal progenitor cells. PLoS One. 8**,** e76489.

Fausett, B. V., Goldman, D., 2006. A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. J Neurosci. 26**,** 6303-13.

Fausett, B. V., Gumerson, J. D., Goldman, D., 2008. The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. J Neurosci. 28**,** 1109-17.

Feng, L., Xie, Z. H., Ding, Q., Xie, X., Libby, R. T., Gan, L., 2010. MATH5 controls the acquisition of multiple retinal cell fates. Mol Brain. 3**,** 36.

Ferraro, S., Gomez-Montalvo, A. I., Olmos, R., Ramirez, M., Lamas, M., 2015. Primary cilia in rat mature Muller glia: downregulation of IFT20 expression reduces sonic hedgehog-mediated proliferation and dedifferentiation potential of Muller glia primary cultures. Cell Mol Neurobiol. 35**,** 533-42.

Ferri, A. L., Cavallaro, M., Braida, D., Di Cristofano, A., Canta, A., Vezzani, A., Ottolenghi, S., Pandolfi, P. P., Sala, M., DeBiasi, S., Nicolis, S. K., 2004. Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain. Development. 131**,** 3805-19.

Firth, L. C., Baker, N. E., 2005. Extracellular signals responsible for spatially regulated proliferation in the differentiating Drosophila eye. Dev Cell. 8**,** 541-51.

Fischer, A. J., Bongini, R., 2010. Turning Muller glia into neural progenitors in the retina. Mol Neurobiol. 42**,** 199-209.

Fischer, A. J., McGuire, C. R., Dierks, B. D., Reh, T. A., 2002. Insulin and fibroblast growth factor 2 activate a neurogenic program in Muller glia of the chicken retina. J Neurosci. 22**,** 9387-98.

Fischer, A. J., Reh, T. A., 2001. Muller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat Neurosci. 4**,** 247-52.

Fischer, A. J., Reh, T. A., 2002. Exogenous growth factors stimulate the regeneration of ganglion cells in the chicken retina. Dev Biol. 251**,** 367-79.

Fischer, A. J., Scott, M. A., Tuten, W., 2009. Mitogen-activated protein kinase-signaling stimulates Muller glia to proliferate in acutely damaged chicken retina. Glia. 57**,** 166-81.

Frank-Kamenetsky, M., Zhang, X. M., Bottega, S., Guicherit, O., Wichterle, H., Dudek, H., Bumcrot, D., Wang, F. Y., Jones, S., Shulok, J., Rubin, L. L., Porter, J. A., 2002. Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. J Biol. 1**,** 10.

Franz, T., Besecke, A., 1991. The development of the eye in homozygotes of the mouse mutant Extra-toes. Anat Embryol (Berl). 184**,** 355-61.

Freeman, M., 1996. Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. Cell. 87**,** 651-60.

Furimsky, M., Wallace, V. A., 2006. Complementary Gli activity mediates early patterning of the mouse visual system. Dev Dyn. 235**,** 594-605.

Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M., Cepko, C. L., 2000. rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. Neuron. 26**,** 383-94.

Fuse, N., Maiti, T., Wang, B., Porter, J. A., Hall, T. M., Leahy, D. J., Beachy, P. A., 1999. Sonic hedgehog protein signals not as a hydrolytic enzyme but as an apparent ligand for patched. Proc Natl Acad Sci U S A. 96**,** 10992-9.

Gallet, A., Rodriguez, R., Ruel, L., Therond, P. P., 2003. Cholesterol modification of hedgehog is required for trafficking and movement, revealing an asymmetric cellular response to hedgehog. Dev Cell. 4**,** 191-204.

Gartner, A., Collin, L., Lalli, G., 2006. Nucleofection of primary neurons. Methods Enzymol. 406**,** 374-88.

Geng, Y., Yu, Q., Sicinska, E., Das, M., Bronson, R. T., Sicinski, P., 2001. Deletion of the p27Kip1 gene restores normal development in cyclin D1-deficient mice. Proc Natl Acad Sci U S A. 98**,** 194-9.

Ghai, K., Zelinka, C., Fischer, A. J., 2010. Notch signaling influences neuroprotective and proliferative properties of mature Muller glia. J Neurosci. 30**,** 3101-12.

Giacinti, C., Giordano, A., 2006. RB and cell cycle progression. Oncogene. 25**,** 5220-7.

Gilbert, S. F., Developmental Biology. Formation of the Neural Tube. Sinauer Associates, Sunderland (MA), 2000.

Goetz, S. C., Anderson, K. V., 2010. The primary cilium: a signalling centre during vertebrate development. Nat Rev Genet. 11**,** 331-44.

Gomes, F. L., Zhang, G., Carbonell, F., Correa, J. A., Harris, W. A., Simons, B. D., Cayouette, M., 2011. Reconstruction of rat retinal progenitor cell lineages in vitro reveals a surprising degree of stochasticity in cell fate decisions. Development. 138**,** 227-35.

Gonzalez-Cordero, A., West, E. L., Pearson, R. A., Duran, Y., Carvalho, L. S., Chu, C. J., Naeem, A., Blackford, S. J., Georgiadis, A., Lakowski, J., Hubank, M., Smith, A. J., Bainbridge, J. W., Sowden, J. C., Ali, R. R., 2013. Photoreceptor precursors derived from three-dimensional embryonic stem cell cultures integrate and mature within adult degenerate retina. Nat Biotechnol. 31**,** 741-7.

Goodrich, L. V., Milenkovic, L., Higgins, K. M., Scott, M. P., 1997. Altered neural cell fates and medulloblastoma in mouse patched mutants. Science. 277**,** 1109-13.

Gordon, W. R., Arnett, K. L., Blacklow, S. C., 2008. The molecular logic of Notch signaling--a structural and biochemical perspective. J Cell Sci. 121**,** 3109-19.

Gorlin, R. J., 1987. Nevoid basal-cell carcinoma syndrome. Medicine (Baltimore). 66**,** 98-113.

Green, E. S., Stubbs, J. L., Levine, E. M., 2003. Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. Development. 130**,** 539-52.

Guessous, F., Li, Y., Abounader, R., 2008. Signaling pathways in medulloblastoma. J Cell Physiol. 217**,** 577-83.

Guillemot, F., 2007. Cell fate specification in the mammalian telencephalon. Prog Neurobiol. 83**,** 37-52.

Guzy, R. D., Stoilov, I., Elton, T. J., Mecham, R. P., Ornitz, D. M., 2015. Fibroblast growth factor 2 is required for epithelial recovery, but not for pulmonary fibrosis, in response to bleomycin. Am J Respir Cell Mol Biol. 52**,** 116-28.

Hagstrom, S. A., Pauer, G. J., Reid, J., Simpson, E., Crowe, S., Maumenee, I. H., Traboulsi, E. I., 2005. SOX2 mutation causes anophthalmia, hearing loss, and brain anomalies. Am J Med Genet A. 138A**,** 95-8.

Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell, D. J., Gerrard, B., Goldstein, A. M., Dean, M., Toftgard, R., Chenevix-Trench, G., Wainwright, B., Bale, A. E., 1996. Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell. 85**,** 841-51.

Hallahan, A. R., Pritchard, J. I., Hansen, S., Benson, M., Stoeck, J., Hatton, B. A., Russell, T. L., Ellenbogen, R. G., Bernstein, I. D., Beachy, P. A., Olson, J. M., 2004. The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. Cancer Res. 64**,** 7794-800.

Harmer, N. J., Ilag, L. L., Mulloy, B., Pellegrini, L., Robinson, C. V., Blundell, T. L., 2004. Towards a resolution of the stoichiometry of the fibroblast growth factor (FGF)-FGF receptor-heparin complex. J Mol Biol. 339**,** 821-34.

Harris, R. C., Chung, E., Coffey, R. J., 2003. EGF receptor ligands. Exp Cell Res. 284**,** 2-13.

Hartong, D. T., Berson, E. L., Dryja, T. P., 2006. Retinitis pigmentosa. Lancet. 368**,** 1795-809.

Hausott, B., Kurnaz, I., Gajovic, S., Klimaschewski, L., 2009. Signaling by neuronal tyrosine kinase receptors: relevance for development and regeneration. Anat Rec (Hoboken). 292**,** 1976-85.

Hayes, S., Nelson, B. R., Buckingham, B., Reh, T. A., 2007. Notch signaling regulates regeneration in the avian retina. Dev Biol. 312**,** 300-11.

Heberlein, U., Moses, K., 1995. Mechanisms of Drosophila retinal morphogenesis: the virtues of being progressive. Cell. 81**,** 987-90.

Heberlein, U., Wolff, T., Rubin, G. M., 1993. The TGF beta homolog dpp and the segment polarity gene hedgehog are required for propagation of a morphogenetic wave in the Drosophila retina. Cell. 75**,** 913-26.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D., Lewis, J., 1997. Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. Curr Biol. 7**,** 661-70.

Heo, J. S., Lee, M. Y., Han, H. J., 2007. Sonic hedgehog stimulates mouse embryonic stem cell proliferation by cooperation of Ca2+/protein kinase C and epidermal growth factor receptor as well as Gli1 activation. Stem Cells. 25**,** 3069-80.

Hill, R. E., Favor, J., Hogan, B. L., Ton, C. C., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D., van Heyningen, V., 1991. Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature. 354**,** 522-5.

Hiratochi, M., Nagase, H., Kuramochi, Y., Koh, C. S., Ohkawara, T., Nakayama, K., 2007. The Delta intracellular domain mediates TGF-beta/Activin signaling through binding to Smads and has an important bi-directional function in the Notch-Delta signaling pathway. Nucleic Acids Res. 35**,** 912-22.

Hochmann, S., Kaslin, J., Hans, S., Weber, A., Machate, A., Geffarth, M., Funk, R. H., Brand, M., 2012. Fgf signaling is required for photoreceptor maintenance in the adult zebrafish retina. PLoS One. 7**,** e30365.

Holland, E. C., Hively, W. P., DePinho, R. A., Varmus, H. E., 1998. A constitutively active epidermal growth factor receptor cooperates with disruption of G1 cell-cycle arrest pathways to induce glioma-like lesions in mice. Genes Dev. 12**,** 3675-85.

Hu, M. C., Shiizaki, K., Kuro-o, M., Moe, O. W., 2013. Fibroblast growth factor 23 and Klotho: physiology and pathophysiology of an endocrine network of mineral metabolism. Annu Rev Physiol. 75**,** 503-33.

Huang, P., Xiong, F., Megason, S. G., Schier, A. F., 2012. Attenuation of Notch and Hedgehog signaling is required for fate specification in the spinal cord. PLoS Genet. 8**,** e1002762.

Huang, Z., Shilo, B. Z., Kunes, S., 1998. A retinal axon fascicle uses spitz, an EGF receptor ligand, to construct a synaptic cartridge in the brain of Drosophila. Cell. 95**,** 693-703.

Hui, C. C., Angers, S., 2011. Gli proteins in development and disease. Annu Rev Cell Dev Biol. 27**,** 513-37.

Hui, C. C., Joyner, A. L., 1993. A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. Nat Genet. 3**,** 241-6.

Humke, E. W., Dorn, K. V., Milenkovic, L., Scott, M. P., Rohatgi, R., 2010. The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. Genes Dev. 24**,** 670-82.

Ikeda, H., Osakada, F., Watanabe, K., Mizuseki, K., Haraguchi, T., Miyoshi, H., Kamiya, D., Honda, Y., Sasai, N., Yoshimura, N., Takahashi, M., Sasai, Y., 2005. Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. Proc Natl Acad Sci U S A. 102**,** 11331-6.

Ikeuchi, T., Sisodia, S. S., 2003. The Notch ligands, Delta1 and Jagged2, are substrates for presenilin-dependent "gamma-secretase" cleavage. J Biol Chem. 278**,** 7751-4.

Imayoshi, I., Sakamoto, M., Yamaguchi, M., Mori, K., Kageyama, R., 2010. Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. J Neurosci. 30**,** 3489-98.

Ingham, P. W., McMahon, A. P., 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15**,** 3059-87.

Ingham, P. W., Placzek, M., 2006. Orchestrating ontogenesis: variations on a theme by sonic hedgehog. Nat Rev Genet. 7**,** 841-50.

Ingram, W. J., McCue, K. I., Tran, T. H., Hallahan, A. R., Wainwright, B. J., 2008. Sonic Hedgehog regulates Hes1 through a novel mechanism that is independent of canonical Notch pathway signalling. Oncogene. 27**,** 1489-500.

Iwata, T., Hevner, R. F., 2009. Fibroblast growth factor signaling in development of the cerebral cortex. Dev Growth Differ. 51**,** 299-323.

Jacobson, R. H., Ladurner, A. G., King, D. S., Tjian, R., 2000. Structure and function of a human TAFII250 double bromodomain module. Science. 288**,** 1422-5.

Jadhav, A. P., Cho, S. H., Cepko, C. L., 2006a. Notch activity permits retinal cells to progress through multiple progenitor states and acquire a stem cell property. Proc Natl Acad Sci U S A. 103**,** 18998-9003.

Jadhav, A. P., Mason, H. A., Cepko, C. L., 2006b. Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development. 133**,** 913-23.

Jadhav, A. P., Roesch, K., Cepko, C. L., 2009. Development and neurogenic potential of Muller glial cells in the vertebrate retina. Prog Retin Eye Res. 28**,** 249-62.

James, J., Das, A. V., Rahnenfuhrer, J., Ahmad, I., 2004. Cellular and molecular characterization of early and late retinal stem cells/progenitors: differential regulation of proliferation and context dependent role of Notch signaling. J Neurobiol. 61**,** 359-76.

Jensen, A. M., Wallace, V. A., 1997. Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. Development. 124**,** 363-71.

Jiang, J., Hui, C. C., 2008. Hedgehog signaling in development and cancer. Dev Cell. 15**,** 801-12.

Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epstein, E. H., Jr., Scott, M. P., 1996. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science. 272**,** 1668-71.

Julian, E., Dave, R. K., Robson, J. P., Hallahan, A. R., Wainwright, B. J., 2010a. Canonical Notch signaling is not required for the growth of Hedgehog pathway-induced medulloblastoma. Oncogene. 29**,** 3465-76.

Julian, E., Hallahan, A. R., Wainwright, B. J., 2010b. RBP-J is not required for granule neuron progenitor development and medulloblastoma initiated by Hedgehog pathway activation in the external germinal layer. Neural Dev. 5**,** 27.

Kageyama, R., Ohtsuka, T., Kobayashi, T., 2008. Roles of Hes genes in neural development. Dev Growth Differ. 50 Suppl 1**,** S97-103.

Kageyama, R., Ohtsuka, T., Shimojo, H., Imayoshi, I., 2009. Dynamic regulation of Notch signaling in neural progenitor cells. Curr Opin Cell Biol. 21**,** 733-40.

Kanazawa, S., Fujiwara, T., Matsuzaki, S., Shingaki, K., Taniguchi, M., Miyata, S., Tohyama, M., Sakai, Y., Yano, K., Hosokawa, K., Kubo, T., 2010. bFGF regulates PI3-kinase-Rac1-JNK pathway and promotes fibroblast migration in wound healing. PLoS One. 5**,** e12228.

Karl, M. O., Hayes, S., Nelson, B. R., Tan, K., Buckingham, B., Reh, T. A., 2008. Stimulation of neural regeneration in the mouse retina. Proc Natl Acad Sci U S A. 105**,** 19508-13.

Kasper, M., Schnidar, H., Neill, G. W., Hanneder, M., Klingler, S., Blaas, L., Schmid, C., Hauser-Kronberger, C., Regl, G., Philpott, M. P., Aberger, F., 2006. Selective modulation of Hedgehog/GLI target gene expression by epidermal growth factor signaling in human keratinocytes. Mol Cell Biol. 26**,** 6283-98.

Kechad, A., Jolicoeur, C., Tufford, A., Mattar, P., Chow, R. W., Harris, W. A., Cayouette, M., 2012. Numb is required for the production of terminal asymmetric cell divisions in the developing mouse retina. J Neurosci. 32**,** 17197-210.

Kenney, A. M., Rowitch, D. H., 2000. Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol Cell Biol. 20**,** 9055-67.

Kim, M. Y., Jung, J., Mo, J. S., Ann, E. J., Ahn, J. S., Yoon, J. H., Park, H. S., 2011. The intracellular domain of Jagged-1 interacts with Notch1 intracellular domain and promotes its degradation through Fbw7 E3 ligase. Exp Cell Res. 317**,** 2438-46.

Kiyota, T., Kinoshita, T., 2004. The intracellular domain of X-Serrate-1 is cleaved and suppresses primary neurogenesis in Xenopus laevis. Mech Dev. 121**,** 573-85.

Klueg, K. M., Parody, T. R., Muskavitch, M. A., 1998. Complex proteolytic processing acts on Delta, a transmembrane ligand for Notch, during Drosophila development. Mol Biol Cell. 9**,** 1709-23.

Koch, U., Radtke, F., 2007. Notch and cancer: a double-edged sword. Cell Mol Life Sci. 64**,** 2746-62.

Kolpak, A., Zhang, J., Bao, Z. Z., 2005. Sonic hedgehog has a dual effect on the growth of retinal ganglion axons depending on its concentration. J Neurosci. 25**,** 3432-41.

Komai, T., Iwanari, H., Mochizuki, Y., Hamakubo, T., Shinkai, Y., 2009. Expression of the mouse PR domain protein Prdm8 in the developing central nervous system. Gene Expr Patterns. 9**,** 503-14.

Kong, J. H., Yang, L., Dessaud, E., Chuang, K., Moore, D. M., Rohatgi, R., Briscoe, J., Novitch, B. G., 2015. Notch Activity Modulates the Responsiveness of Neural Progenitors to Sonic Hedgehog Signaling. Dev Cell.

Kornblum, H. I., Hussain, R., Wiesen, J., Miettinen, P., Zurcher, S. D., Chow, K., Derynck, R., Werb, Z., 1998. Abnormal astrocyte development and neuronal death in mice lacking the epidermal growth factor receptor. J Neurosci Res. 53**,** 697-717.

Kozar, K., Sicinski, P., 2005. Cell cycle progression without cyclin D-CDK4 and cyclin D-CDK6 complexes. Cell Cycle. 4**,** 388-91.

Kuan, C. Y., Schloemer, A. J., Lu, A., Burns, K. A., Weng, W. L., Williams, M. T., Strauss, K. I., Vorhees, C. V., Flavell, R. A., Davis, R. J., Sharp, F. R., Rakic, P., 2004. Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain. J Neurosci. 24**,** 10763-72.

Lamba, D., Karl, M., Reh, T., 2008. Neural regeneration and cell replacement: a view from the eye. Cell Stem Cell. 2**,** 538-49.

Lanner, F., Rossant, J., 2010. The role of FGF/Erk signaling in pluripotent cells. Development. 137**,** 3351-60.

LaVoie, M. J., Selkoe, D. J., 2003. The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. J Biol Chem. 278**,** 34427-37.

Lawrence, J. M., Singhal, S., Bhatia, B., Keegan, D. J., Reh, T. A., Luthert, P. J., Khaw, P. T., Limb, G. A., 2007. MIO-M1 cells and similar muller glial cell lines derived from adult human retina exhibit neural stem cell characteristics. Stem Cells. 25**,** 2033-43.

Lee, J. D., Kraus, P., Gaiano, N., Nery, S., Kohtz, J., Fishell, G., Loomis, C. A., Treisman, J. E., 2001. An acylatable residue of Hedgehog is differentially required in Drosophila and mouse limb development. Dev Biol. 233**,** 122-36.

Lemmon, M. A., Schlessinger, J., 2010. Cell signaling by receptor tyrosine kinases. Cell. 141**,** 1117-34.

Levine, E. M., Close, J., Fero, M., Ostrovsky, A., Reh, T. A., 2000. p27(Kip1) regulates cell cycle withdrawal of late multipotent progenitor cells in the mammalian retina. Dev Biol. 219**,** 299-314.

Levine, E. M., Passini, M., Hitchcock, P. F., Glasgow, E., Schechter, N., 1997a. Vsx-1 and Vsx-2: two Chx10-like homeobox genes expressed in overlapping domains in the adult goldfish retina. J Comp Neurol. 387**,** 439-48.

Levine, E. M., Roelink, H., Turner, J., Reh, T. A., 1997b. Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells in vitro. J Neurosci. 17**,** 6277-88.

Li, X. J., Du, Z. W., Zarnowska, E. D., Pankratz, M., Hansen, L. O., Pearce, R. A., Zhang, S. C., 2005. Specification of motoneurons from human embryonic stem cells. Nat Biotechnol. 23**,** 215-21.

Li, Y., Hibbs, M. A., Gard, A. L., Shylo, N. A., Yun, K., 2012. Genome-wide analysis of N1ICD/RBPJ targets in vivo reveals direct transcriptional regulation of Wnt, SHH, and hippo pathway effectors by Notch1. Stem Cells. 30**,** 741-52.

Lillien, L., Cepko, C., 1992. Control of proliferation in the retina: temporal changes in responsiveness to FGF and TGF alpha. Development. 115**,** 253-66.

Lindsell, C. E., Boulter, J., diSibio, G., Gossler, A., Weinmaster, G., 1996. Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. Mol Cell Neurosci. 8**,** 14-27.

Lindsell, C. E., Shawber, C. J., Boulter, J., Weinmaster, G., 1995. Jagged: a mammalian ligand that activates Notch1. Cell. 80**,** 909-17.

Ling, B. C., Wu, J., Miller, S. J., Monk, K. R., Shamekh, R., Rizvi, T. A., Decourten-Myers, G., Vogel, K. S., DeClue, J. E., Ratner, N., 2005. Role for the epidermal growth factor receptor in neurofibromatosis-related peripheral nerve tumorigenesis. Cancer Cell. 7**,** 65-75.

Liu, I. S., Chen, J. D., Ploder, L., Vidgen, D., van der Kooy, D., Kalnins, V. I., McInnes, R. R., 1994. Developmental expression of a novel murine homeobox gene (Chx10): evidence for roles in determination of the neuroretina and inner nuclear layer. Neuron. 13**,** 377-93.

Liu, Z., Li, T., Reinhold, M. I., Naski, M. C., 2015. MEK1-RSK2 contributes to Hedgehog signaling by stabilizing GLI2 transcription factor and inhibiting ubiquitination. Oncogene. 33**,** 65-73.

Livesey, F. J., Cepko, C. L., 2001. Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci. 2**,** 109-18.

Locker, M., Agathocleous, M., Amato, M. A., Parain, K., Harris, W. A., Perron, M., 2006. Hedgehog signaling and the retina: insights into the mechanisms controlling the proliferative properties of neural precursors. Genes Dev. 20**,** 3036-48.

Long, F., Zhang, X. M., Karp, S., Yang, Y., McMahon, A. P., 2001. Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. Development. 128**,** 5099-108.

Long, J., Li, B., Rodriguez-Blanco, J., Pastori, C., Volmar, C. H., Wahlestedt, C., Capobianco, A., Bai, F., Pei, X. H., Ayad, N. G., Robbins, D. J., 2014. The BET bromodomain inhibitor I-BET151 acts downstream of smoothened protein to abrogate the growth of hedgehog protein-driven cancers. J Biol Chem. 289**,** 35494-502.

Long, Y. C., Kharitonenkov, A., 2011. Hormone-like fibroblast growth factors and metabolic regulation. Biochim Biophys Acta. 1812**,** 791-5.

Ma, C., Zhou, Y., Beachy, P. A., Moses, K., 1993. The segment polarity gene hedgehog is required for progression of the morphogenetic furrow in the developing Drosophila eye. Cell. 75**,** 927-38.

Ma, L., Cantrup, R., Varrault, A., Colak, D., Klenin, N., Gotz, M., McFarlane, S., Journot, L., Schuurmans, C., 2007. Zac1 functions through TGFbetaII to negatively regulate cell number in the developing retina. Neural Dev. 2**,** 11.

Macdonald, R., Xu, Q., Barth, K. A., Mikkola, I., Holder, N., Fjose, A., Krauss, S., Wilson, S. W., 1994. Regulatory gene expression boundaries demarcate sites of neuronal differentiation in the embryonic zebrafish forebrain. Neuron. 13**,** 1039-53.

MacLaren, R. E., Pearson, R. A., MacNeil, A., Douglas, R. H., Salt, T. E., Akimoto, M., Swaroop, A., Sowden, J. C., Ali, R. R., 2006. Retinal repair by transplantation of photoreceptor precursors. Nature. 444**,** 203-7.

MacPherson, D., Sage, J., Kim, T., Ho, D., McLaughlin, M. E., Jacks, T., 2004. Cell type-specific effects of Rb deletion in the murine retina. Genes Dev. 18**,** 1681-94.

Maeda, Y., Nakamura, E., Nguyen, M. T., Suva, L. J., Swain, F. L., Razzaque, M. S., Mackem, S., Lanske, B., 2007. Indian Hedgehog produced by postnatal chondrocytes is essential for maintaining a growth plate and trabecular bone. Proc Natl Acad Sci U S A. 104**,** 6382-7.

Maity, T., Fuse, N., Beachy, P. A., 2005. Molecular mechanisms of Sonic hedgehog mutant effects in holoprosencephaly. Proc Natl Acad Sci U S A. 102**,** 17026-31.

Malek, R., Matta, J., Taylor, N., Perry, M. E., Mendrysa, S. M., 2011. The p53 inhibitor MDM2 facilitates Sonic Hedgehog-mediated tumorigenesis and influences cerebellar foliation. PLoS One. 6**,** e17884.

Mann, R. K., Beachy, P. A., 2004. Novel lipid modifications of secreted protein signals. Annu Rev Biochem. 73**,** 891-923.

Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F., Gruss, P., 2001. Pax6 is required for the multipotent state of retinal progenitor cells. Cell. 105**,** 43-55.

Mathers, P. H., Grinberg, A., Mahon, K. A., Jamrich, M., 1997. The Rx homeobox gene is essential for vertebrate eye development. Nature. 387**,** 603-7.

Matsuda, T., Cepko, C. L., 2004. Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc Natl Acad Sci U S A. 101**,** 16-22.

Matsuda, T., Cepko, C. L., 2007. Controlled expression of transgenes introduced by in vivo electroporation. Proc Natl Acad Sci U S A. 104**,** 1027-32.

Mattar, P., Ericson, J., Blackshaw, S., Cayouette, M., 2015. A conserved regulatory logic controls temporal identity in mouse neural progenitors. Neuron. 85**,** 497-504.

Maurer, K. A., Riesenberg, A. N., Brown, N. L., 2014. Notch signaling differentially regulates Atoh7 and Neurog2 in the distal mouse retina. Development. 141**,** 3243-54.

McCabe, J. M., Leahy, D. J., 2014. Smoothened goes molecular: new pieces in the hedgehog signaling puzzle. J Biol Chem. 290**,** 3500-7.

McDaniell, R., Warthen, D. M., Sanchez-Lara, P. A., Pai, A., Krantz, I. D., Piccoli, D. A., Spinner, N. B., 2006. NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. Am J Hum Genet. 79**,** 169-73.

McLeod, D., Hiscott, P. S., Grierson, I., 1987. Age-related cellular proliferation at the vitreoretinal juncture. Eye (Lond). 1 (Pt 2)**,** 263-81.

McNeill, B., Mazerolle, C., Bassett, E. A., Mears, A. J., Ringuette, R., Lagali, P., Picketts, D. J., Paes, K., Rice, D., Wallace, V. A., 2013. Hedgehog regulates Norrie disease protein to drive neural progenitor self-renewal. Hum Mol Genet. 22**,** 1005-16.

McNeill, B., Perez-Iratxeta, C., Mazerolle, C., Furimsky, M., Mishina, Y., Andrade-Navarro, M. A., Wallace, V. A., 2012. Comparative genomics identification of a novel set of temporally regulated hedgehog target genes in the retina. Mol Cell Neurosci. 49**,** 333-40.

Mears, A. J., Kondo, M., Swain, P. K., Takada, Y., Bush, R. A., Saunders, T. L., Sieving, P. A., Swaroop, A., 2001. Nrl is required for rod photoreceptor development. Nat Genet. 29**,** 447-52.

Methot, N., Basler, K., 2001. An absolute requirement for Cubitus interruptus in Hedgehog signaling. Development. 128**,** 733-42.

Meyer, M., Muller, A. K., Yang, J., Moik, D., Ponzio, G., Ornitz, D. M., Grose, R., Werner, S., 2012. FGF receptors 1 and 2 are key regulators of keratinocyte migration in vitro and in wounded skin. J Cell Sci. 125**,** 5690-701.

Miller, A. C., Lyons, E. L., Herman, T. G., 2009. cis-Inhibition of Notch by endogenous Delta biases the outcome of lateral inhibition. Curr Biol. 19**,** 1378-83.

Mitsuhashi, T., Aoki, Y., Eksioglu, Y. Z., Takahashi, T., Bhide, P. G., Reeves, S. A., Caviness, V. S., Jr., 2001. Overexpression of p27Kip1 lengthens the G1 phase in a mouse model that targets inducible gene expression to central nervous system progenitor cells. Proc Natl Acad Sci U S A. 98**,** 6435-40.

Mizeracka, K., Trimarchi, J. M., Stadler, M. B., Cepko, C. L., 2013. Analysis of gene expression in wild-type and Notch1 mutant retinal cells by single cell profiling. Dev Dyn. 242**,** 1147-59.

Mizumoto, H., Mizumoto, K., Shatos, M. A., Klassen, H., Young, M. J., 2003. Retinal transplantation of neural progenitor cells derived from the brain of GFP transgenic mice. Vision Res. 43**,** 1699-708.

Mizutani, K., Yoon, K., Dang, L., Tokunaga, A., Gaiano, N., 2007. Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. Nature. 449**,** 351-5.

Mo, R., Freer, A. M., Zinyk, D. L., Crackower, M. A., Michaud, J., Heng, H. H., Chik, K. W., Shi, X. M., Tsui, L. C., Cheng, S. H., Joyner, A. L., Hui, C., 1997. Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. Development. 124**,** 113-23.

Mohammadi, M., Olsen, S. K., Ibrahimi, O. A., 2005. Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev. 16**,** 107-37.

Moore, K. B., Schneider, M. L., Vetter, M. L., 2002. Posttranslational mechanisms control the timing of bHLH function and regulate retinal cell fate. Neuron. 34**,** 183-95.

Moshiri, A., McGuire, C. R., Reh, T. A., 2005. Sonic hedgehog regulates proliferation of the retinal ciliary marginal zone in posthatch chicks. Dev Dyn. 233**,** 66-75.

Mu, X., Fu, X., Sun, H., Liang, S., Maeda, H., Frishman, L. J., Klein, W. H., 2005. Ganglion cells are required for normal progenitor- cell proliferation but not cell-fate determination or patterning in the developing mouse retina. Curr Biol. 15**,** 525-30.

Musgrove, E. A., 2006. Cyclins: roles in mitogenic signaling and oncogenic transformation. Growth Factors. 24**,** 13-9.

Muto, A., Iida, A., Satoh, S., Watanabe, S., 2009. The group E Sox genes Sox8 and Sox9 are regulated by Notch signaling and are required for Muller glial cell development in mouse retina. Exp Eye Res. 89**,** 549-58.

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D. Y., 1996. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell. 85**,** 707-20.

Nelson, B. R., Hartman, B. H., Georgi, S. A., Lan, M. S., Reh, T. A., 2007. Transient inactivation of Notch signaling synchronizes differentiation of neural progenitor cells. Dev Biol. 304**,** 479-98.

Nelson, B. R., Ueki, Y., Reardon, S., Karl, M. O., Georgi, S., Hartman, B. H., Lamba, D. A., Reh, T. A., 2011. Genome-wide analysis of Muller glial differentiation reveals a requirement for Notch signaling in postmitotic cells to maintain the glial fate. PLoS One. 6**,** e22817.

Nelson, S. M., Park, L., Stenkamp, D. L., 2009. Retinal homeobox 1 is required for retinal neurogenesis and photoreceptor differentiation in embryonic zebrafish. Dev Biol. 328**,** 24-39.

Nieuwenhuis, E., Hui, C. C., 2005. Hedgehog signaling and congenital malformations. Clin Genet. 67**,** 193-208.

Niewiadomski, P., Zhujiang, A., Youssef, M., Waschek, J. A., 2013. Interaction of PACAP with Sonic hedgehog reveals complex regulation of the hedgehog pathway by PKA. Cell Signal. 25**,** 2222-30.

Njaine, B., Martins, R. A., Santiago, M. F., Linden, R., Silveira, M. S., 2010. Pituitary adenylyl cyclase-activating polypeptide controls the proliferation of retinal progenitor cells through downregulation of cyclin D1. Eur J Neurosci. 32**,** 311-21.

Nozawa, Y. I., Lin, C., Chuang, P. T., 2013. Hedgehog signaling from the primary cilium to the nucleus: an emerging picture of ciliary localization, trafficking and transduction. Curr Opin Genet Dev. 23**,** 429-37.

Nusslein-Volhard, C., Wieschaus, E., 1980. Mutations affecting segment number and polarity in Drosophila. Nature. 287**,** 795-801.

Oda, K., Matsuoka, Y., Funahashi, A., Kitano, H., 2005. A comprehensive pathway map of epidermal growth factor receptor signaling. Mol Syst Biol. 1**,** 2005 0010.

Ohnuma, S., Philpott, A., Wang, K., Holt, C. E., Harris, W. A., 1999. p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina. Cell. 99**,** 499-510.

Ohtsuka, T., Imayoshi, I., Shimojo, H., Nishi, E., Kageyama, R., McConnell, S. K., 2006. Visualization of embryonic neural stem cells using Hes promoters in transgenic mice. Mol Cell Neurosci. 31**,** 109-22.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., Kageyama, R., 1999. Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J. 18**,** 2196-207.

Okano, H., Imai, T., Okabe, M., 2002. Musashi: a translational regulator of cell fate. J Cell Sci. 115**,** 1355-9.

Ooto, S., Akagi, T., Kageyama, R., Akita, J., Mandai, M., Honda, Y., Takahashi, M., 2004. Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. Proc Natl Acad Sci U S A. 101**,** 13654-9.

Orenic, T. V., Slusarski, D. C., Kroll, K. L., Holmgren, R. A., 1990. Cloning and characterization of the segment polarity gene cubitus interruptus Dominant of Drosophila. Genes Dev. 4**,** 1053-67.

Oron-Karni, V., Farhy, C., Elgart, M., Marquardt, T., Remizova, L., Yaron, O., Xie, Q., Cvekl, A., Ashery-Padan, R., 2008. Dual requirement for Pax6 in retinal progenitor cells. Development. 135**,** 4037-47.

Osakada, F., Ikeda, H., Mandai, M., Wataya, T., Watanabe, K., Yoshimura, N., Akaike, A., Sasai, Y., Takahashi, M., 2008. Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nat Biotechnol. 26**,** 215-24.

Osakada, F., Ooto, S., Akagi, T., Mandai, M., Akaike, A., Takahashi, M., 2007. Wnt signaling promotes regeneration in the retina of adult mammals. J Neurosci. 27**,** 4210-9.

Palma, V., Lim, D. A., Dahmane, N., Sanchez, P., Brionne, T. C., Herzberg, C. D., Gitton, Y., Carleton, A., Alvarez-Buylla, A., Ruiz i Altaba, A., 2005. Sonic hedgehog controls stem cell behavior in the postnatal and adult brain. Development. 132**,** 335-44.

Palma, V., Ruiz i Altaba, A., 2004. Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex. Development. 131**,** 337-45.

Pan, Y., Bai, C. B., Joyner, A. L., Wang, B., 2006. Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Mol Cell Biol. 26**,** 3365-77.

Pan, Y., Martinez-De Luna, R. I., Lou, C. H., Nekkalapudi, S., Kelly, L. E., Sater, A. K., El-Hodiri, H. M., 2010. Regulation of photoreceptor gene expression by the retinal homeobox (Rx) gene product. Dev Biol. 339**,** 494-506.

Pardee, A. B., 1974. A restriction point for control of normal animal cell proliferation. Proc Natl Acad Sci U S A. 71**,** 1286-90.

Pardee, A. B., 1989. G1 events and regulation of cell proliferation. Science. 246**,** 603-8.

Passini, M. A., Levine, E. M., Canger, A. K., Raymond, P. A., Schechter, N., 1997. Vsx-1 and Vsx-2: differential expression of two paired-like homeobox genes during zebrafish and goldfish retinogenesis. J Comp Neurol. 388**,** 495-505.

Pastor, J. C., 1998. Proliferative vitreoretinopathy: an overview. Surv Ophthalmol. 43**,** 3-18.

Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K., Taylor, F. R., Wang, E. A., Galdes, A., 1998. Identification of a palmitic acid-modified form of human Sonic hedgehog. J Biol Chem. 273**,** 14037-45.

Perron, M., Boy, S., Amato, M. A., Viczian, A., Koebernick, K., Pieler, T., Harris, W. A., 2003. A novel function for Hedgehog signalling in retinal pigment epithelium differentiation. Development. 130**,** 1565-77.

Perron, M., Harris, W. A., 2000. Retinal stem cells in vertebrates. Bioessays. 22**,** 685-8.

Peterson, K. A., Nishi, Y., Ma, W., Vedenko, A., Shokri, L., Zhang, X., McFarlane, M., Baizabal, J. M., Junker, J. P., van Oudenaarden, A., Mikkelsen, T., Bernstein, B. E., Bailey, T. L., Bulyk, M. L., Wong, W. H., McMahon, A. P., 2012. Neural-specific Sox2 input and differential Gli-binding affinity provide context and positional information in Shh-directed neural patterning. Genes Dev. 26**,** 2802-16.

Pfaffl, M. W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29**,** e45.

Pfaffl, M. W., Horgan, G. W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30**,** e36.

Pierfelice, T., Alberi, L., Gaiano, N., 2011. Notch in the vertebrate nervous system: an old dog with new tricks. Neuron. 69**,** 840-55.

Pintar, A., De Biasio, A., Popovic, M., Ivanova, N., Pongor, S., 2007. The intracellular region of Notch ligands: does the tail make the difference? Biol Direct. 2**,** 19.

Planas-Silva, M. D., Weinberg, R. A., 1997. The restriction point and control of cell proliferation. Curr Opin Cell Biol. 9**,** 768-72.

Pollak, J., Wilken, M. S., Ueki, Y., Cox, K. E., Sullivan, J. M., Taylor, R. J., Levine, E. M., Reh, T. A., 2013. ASCL1 reprograms mouse Muller glia into neurogenic retinal progenitors. Development. 140**,** 2619-31.

Pownall, M. E., Isaacs, H.V., FGF Signalling in Vertebrate Development. FGF Ligands. Morgan & Claypool Life Sciences, San Rafael (CA), 2010.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., Ullrich, A., 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature. 402**,** 884-8.

Qiu, G., Seiler, M. J., Arai, S., Aramant, R. B., Sadda, S. R., 2004. Alternative culture conditions for isolation and expansion of retinal progenitor cells. Curr Eye Res. 28**,** 327-36.

Qiu, G., Seiler, M. J., Mui, C., Arai, S., Aramant, R. B., de Juan, E., Jr., Sadda, S., 2005. Photoreceptor differentiation and integration of retinal progenitor cells transplanted into transgenic rats. Exp Eye Res. 80**,** 515-25.

Ramachandran, R., Fausett, B. V., Goldman, D., 2010. Ascl1a regulates Muller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. Nat Cell Biol. 17**,** 532.

Rao, R. C., Hennig, A. K., Malik, M. T., Chen, D. F., Chen, S., 2011. Epigenetic regulation of retinal development and disease. J Ocul Biol Dis Infor. 4**,** 121-36.

Rao, R. C., Tchedre, K. T., Malik, M. T., Coleman, N., Fang, Y., Marquez, V. E., Chen, D. F., 2010. Dynamic patterns of histone lysine methylation in the developing retina. Invest Ophthalmol Vis Sci. 51**,** 6784-92.

Raymond, P. A., Barthel, L. K., Bernardos, R. L., Perkowski, J. J., 2006. Molecular characterization of retinal stem cells and their niches in adult zebrafish. BMC Dev Biol. 6**,** 36.

Ready, D. F., Hanson, T. E., Benzer, S., 1976. Development of the Drosophila retina, a neurocrystalline lattice. Dev Biol. 53**,** 217-40.

Reinchisi, G., Parada, M., Lois, P., Oyanadel, C., Shaughnessy, R., Gonzalez, A., Palma, V., 2013. Sonic Hedgehog modulates EGFR dependent proliferation of neural stem cells during late mouse embryogenesis through EGFR transactivation. Front Cell Neurosci. 7**,** 166.

Riesenberg, A. N., Liu, Z., Kopan, R., Brown, N. L., 2009. Rbpj cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. J Neurosci. 29**,** 12865-77.

Ringuette, R., Wang, Y., Atkins, M., Mears, A. J., Yan, K., Wallace, V. A., 2014. Combinatorial hedgehog and mitogen signaling promotes the in vitro expansion but not retinal differentiation potential of retinal progenitor cells. Invest Ophthalmol Vis Sci. 55**,** 43-54.

Riobo, N. A., Haines, G. M., Emerson, C. P., Jr., 2006a. Protein kinase C-delta and mitogen-activated protein/extracellular signal-regulated kinase-1 control GLI activation in hedgehog signaling. Cancer Res. 66**,** 839-45.

Riobo, N. A., Lu, K., Ai, X., Haines, G. M., Emerson, C. P., Jr., 2006b. Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. Proc Natl Acad Sci U S A. 103**,** 4505-10.

Robanus-Maandag, E., Dekker, M., van der Valk, M., Carrozza, M. L., Jeanny, J. C., Dannenberg, J. H., Berns, A., te Riele, H., 1998. p107 is a suppressor of retinoblastoma development in pRb-deficient mice. Genes Dev. 12**,** 1599-609.

Robbins, D. J., Fei, D. L., Riobo, N. A., 2012. The Hedgehog signal transduction network. Sci Signal. 5**,** re6.

Roesch, K., Jadhav, A. P., Trimarchi, J. M., Stadler, M. B., Roska, B., Sun, B. B., Cepko, C. L., 2008. The transcriptome of retinal Muller glial cells. J Comp Neurol. 509**,** 225-38.

Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S. W., Tsui, L. C., Muenke, M., 1996. Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. Nat Genet. 14**,** 357-60.

Ruiz i Altaba, A., 1999. Gli proteins encode context-dependent positive and negative functions: implications for development and disease. Development. 126**,** 3205-16.

Sakagami, K., Gan, L., Yang, X. J., 2009. Distinct effects of Hedgehog signaling on neuronal fate specification and cell cycle progression in the embryonic mouse retina. J Neurosci. 29**,** 6932-44.

Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., Kondoh, H., 1999. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development. 126**,** 3915-24.

Scheer, N., Groth, A., Hans, S., Campos-Ortega, J. A., 2001. An instructive function for Notch in promoting gliogenesis in the zebrafish retina. Development. 128**,** 1099-107.

Schneider, M. R., Wolf, E., 2009. The epidermal growth factor receptor ligands at a glance. J Cell Physiol. 218**,** 460-6.

Schnidar, H., Eberl, M., Klingler, S., Mangelberger, D., Kasper, M., Hauser-Kronberger, C., Regl, G., Kroismayr, R., Moriggl, R., Sibilia, M., Aberger, F., 2009. Epidermal growth factor receptor signaling synergizes with Hedgehog/GLI in oncogenic transformation via activation of the MEK/ERK/JUN pathway. Cancer Res. 69**,** 1284-92.

Sharghi-Namini, S., Turmaine, M., Meier, C., Sahni, V., Umehara, F., Jessen, K. R., Mirsky, R., 2006. The structural and functional integrity of peripheral nerves depends on the glial-derived signal desert hedgehog. J Neurosci. 26**,** 6364-76.

Shimojo, H., Ohtsuka, T., Kageyama, R., 2008. Oscillations in notch signaling regulate maintenance of neural progenitors. Neuron. 58**,** 52-64.

Shkumatava, A., Neumann, C. J., 2005. Shh directs cell-cycle exit by activating p57Kip2 in the zebrafish retina. EMBO Rep. 6**,** 563-9.

Shyamala, K., Yanduri, S., Girish, H. C., Murgod, S., 2015. Neural crest: The fourth germ layer. J Oral Maxillofac Pathol. 19**,** 221-9.

Sibilia, M., Kroismayr, R., Lichtenberger, B. M., Natarajan, A., Hecking, M., Holcmann, M., 2007. The epidermal growth factor receptor: from development to tumorigenesis. Differentiation. 75**,** 770-87.

Sibilia, M., Steinbach, J. P., Stingl, L., Aguzzi, A., Wagner, E. F., 1998. A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. EMBO J. 17**,** 719-31.

Sicinski, P., Donaher, J. L., Parker, S. B., Li, T., Fazeli, A., Gardner, H., Haslam, S. Z., Bronson, R. T., Elledge, S. J., Weinberg, R. A., 1995. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell. 82**,** 621-30.

Sidman, R. L., Miale, I. L., Feder, N., 1959. Cell proliferation and migration in the primitive ependymal zone: an autoradiographic study of histogenesis in the nervous system. Exp Neurol. 1**,** 322-33.

Sigulinsky, C. L., Green, E. S., Clark, A. M., Levine, E. M., 2008. Vsx2/Chx10 ensures the correct timing and magnitude of Hedgehog signaling in the mouse retina. Dev Biol. 317**,** 560-75.

Sinn, R., Wittbrodt, J., 2014. An eye on eye development. Mech Dev. 130**,** 347-58.

Sivak, J. M., 2013. The aging eye: common degenerative mechanisms between the Alzheimer's brain and retinal disease. Invest Ophthalmol Vis Sci. 54**,** 871-80.

Six, E., Ndiaye, D., Laabi, Y., Brou, C., Gupta-Rossi, N., Israel, A., Logeat, F., 2003. The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. Proc Natl Acad Sci U S A. 100**,** 7638-43.

Soderpalm, A. K., Fox, D. A., Karlsson, J. O., van Veen, T., 2000. Retinoic acid produces rod photoreceptor selective apoptosis in developing mammalian retina. Invest Ophthalmol Vis Sci. 41**,** 937-47.

Soriano, P., 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 21**,** 70-1.

Spence, J. R., Aycinena, J. C., Del Rio-Tsonis, K., 2007. Fibroblast growth factor-hedgehog interdependence during retina regeneration. Dev Dyn. 236**,** 1161-74.

St-Jacques, B., Hammerschmidt, M., McMahon, A. P., 1999. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev. 13**,** 2072-86.

Stasiulewicz, M., Gray, S., Mastromina, I., Silva, J. C., Bjorklund, M., Seymour, P. A., Booth, D., Thompson, C., Green, R., Hall, E. A., Serup, P., Dale, J. K., 2015. A conserved role for Notch in priming the cellular response to Shh through ciliary localisation of the key Shh transducer, Smoothened. Development.

Stecca, B., Mas, C., Clement, V., Zbinden, M., Correa, R., Piguet, V., Beermann, F., Ruiz, I. A. A., 2007. Melanomas require HEDGEHOG-GLI signaling regulated by interactions between GLI1 and the RAS-MEK/AKT pathways. Proc Natl Acad Sci U S A. 104**,** 5895-900.

Stecca, B., Ruiz, I. A. A., 2010. Context-dependent regulation of the GLI code in cancer by HEDGEHOG and non-HEDGEHOG signals. J Mol Cell Biol. 2**,** 84-95.

Steinhauer, J., Treisman, J. E., 2009. Lipid-modified morphogens: functions of fats. Curr Opin Genet Dev. 19**,** 308-14.

Surzenko, N., Crowl, T., Bachleda, A., Langer, L., Pevny, L., 2013. SOX2 maintains the quiescent progenitor cell state of postnatal retinal Muller glia. Development. 140**,** 1445-56.

Swaroop, A., Kim, D., Forrest, D., 2010. Transcriptional regulation of photoreceptor development and homeostasis in the mammalian retina. Nat Rev Neurosci. 11**,** 563-76.

Szabo, N. E., Zhao, T., Cankaya, M., Theil, T., Zhou, X., Alvarez-Bolado, G., 2009. Role of neuroepithelial Sonic hedgehog in hypothalamic patterning. J Neurosci. 29**,** 6989-7002.

Takabatake, T., Ogawa, M., Takahashi, T. C., Mizuno, M., Okamoto, M., Takeshima, K., 1997. Hedgehog and patched gene expression in adult ocular tissues. FEBS Lett. 410**,** 485-9.

Takatsuka, K., Hatakeyama, J., Bessho, Y., Kageyama, R., 2004. Roles of the bHLH gene Hes1 in retinal morphogenesis. Brain Res. 1004**,** 148-55.

Tang, Y., Gholamin, S., Schubert, S., Willardson, M. I., Lee, A., Bandopadhayay, P., Bergthold, G., Masoud, S., Nguyen, B., Vue, N., Balansay, B., Yu, F., Oh, S., Woo, P., Chen, S., Ponnuswami, A., Monje, M., Atwood, S. X., Whitson, R. J., Mitra, S., Cheshier, S. H., Qi, J., Beroukhim, R., Tang, J. Y., Wechsler-Reya, R., Oro, A. E., Link, B. A., Bradner, J. E., Cho, Y. J., 2015. Epigenetic targeting of Hedgehog pathway transcriptional output through BET bromodomain inhibition. Nat Med. 20**,** 732-40.

Taranova, O. V., Magness, S. T., Fagan, B. M., Wu, Y., Surzenko, N., Hutton, S. R., Pevny, L. H., 2006. SOX2 is a dose-dependent regulator of retinal neural progenitor competence. Genes Dev. 20**,** 1187-202.

Taupin, P., 2007. BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev. 53**,** 198-214.

Taylor, M. D., Liu, L., Raffel, C., Hui, C. C., Mainprize, T. G., Zhang, X., Agatep, R., Chiappa, S., Gao, L., Lowrance, A., Hao, A., Goldstein, A. M., Stavrou, T., Scherer, S. W., Dura, W. T., Wainwright, B., Squire, J. A., Rutka, J. T., Hogg, D., 2002. Mutations in SUFU predispose to medulloblastoma. Nat Genet. 31**,** 306-10.

Terada, K., Kitayama, A., Kanamoto, T., Ueno, N., Furukawa, T., 2006. Nucleosome regulator Xhmgb3 is required for cell proliferation of the eye and brain as a downstream target of Xenopus rax/Rx1. Dev Biol. 291**,** 398-412.

Thomas, B. J., Gunning, D. A., Cho, J., Zipursky, L., 1994. Cell cycle progression in the developing Drosophila eye: roughex encodes a novel protein required for the establishment of G1. Cell. 77**,** 1003-14.

Thummel, R., Kassen, S. C., Enright, J. M., Nelson, C. M., Montgomery, J. E., Hyde, D. R., 2008a. Characterization of Muller glia and neuronal progenitors during adult zebrafish retinal regeneration. Exp Eye Res. 87**,** 433-44.

Thummel, R., Kassen, S. C., Montgomery, J. E., Enright, J. M., Hyde, D. R., 2008b. Inhibition of Muller glial cell division blocks regeneration of the light-damaged zebrafish retina. Dev Neurobiol. 68**,** 392-408.

Todd, L., Fischer, A. J., 2015. Hedgehog signaling stimulates the formation of proliferating Muller glia-derived progenitor cells in the chick retina. Development. 142**,** 2610-22.

Todd, L., Volkov, L. I., Zelinka, C., Squires, N., Fischer, A. J., 2015. Heparin-binding EGF-like growth factor (HB-EGF) stimulates the proliferation of Muller glia-derived progenitor cells in avian and murine retinas. Mol Cell Neurosci. 69**,** 54-64.

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S. L., Nakanishi, S., Guillemot, F., Kageyama, R., 1996. Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron. 16**,** 723-34.

Trimarchi, J. M., Stadler, M. B., Cepko, C. L., 2008. Individual retinal progenitor cells display extensive heterogeneity of gene expression. PLoS One. 3**,** e1588.

Trimarchi, J. M., Stadler, M. B., Roska, B., Billings, N., Sun, B., Bartch, B., Cepko, C. L., 2007. Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. J Comp Neurol. 502**,** 1047-65.

Tropepe, V., Coles, B. L., Chiasson, B. J., Horsford, D. J., Elia, A. J., McInnes, R. R., van der Kooy, D., 2000. Retinal stem cells in the adult mammalian eye. Science. 287**,** 2032-6.

Tsang, M., Dawid, I. B., 2004. Promotion and attenuation of FGF signaling through the Ras-MAPK pathway. Sci STKE. 2004**,** pe17.

Turner, N., Grose, R., 2010. Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer. 10**,** 116-29.

Turnpenny, P. D., Alman, B., Cornier, A. S., Giampietro, P. F., Offiah, A., Tassy, O., Pourquie, O., Kusumi, K., Dunwoodie, S., 2007. Abnormal vertebral segmentation and the notch signaling pathway in man. Dev Dyn. 236**,** 1456-74.

Ueki, Y., Karl, M. O., Sudar, S., Pollak, J., Taylor, R. J., Loeffler, K., Wilken, M. S., Reardon, S., Reh, T. A., 2012. P53 is required for the developmental restriction in Muller glial proliferation in mouse retina. Glia. 60**,** 1579-89.

Ueki, Y., Reh, T. A., 2013. EGF stimulates Muller glial proliferation via a BMP-dependent mechanism. Glia. 61**,** 778-89.

Ueki, Y., Wilken, M. S., Cox, K. E., Chipman, L., Jorstad, N., Sternhagen, K., Simic, M., Ullom, K., Nakafuku, M., Reh, T. A., 2015. Transgenic expression of the proneural transcription factor Ascl1 in Muller glia stimulates retinal regeneration in young mice. Proc Natl Acad Sci U S A. 112**,** 13717-22.

Ullrich, A., Schlessinger, J., 1990. Signal transduction by receptors with tyrosine kinase activity. Cell. 61**,** 203-12.

Urban, N., Guillemot, F., 2014. Neurogenesis in the embryonic and adult brain: same regulators, different roles. Front Cell Neurosci. 8**,** 396.

van den Brink, G. R., 2007. Hedgehog signaling in development and homeostasis of the gastrointestinal tract. Physiol Rev. 87**,** 1343-75.

Varjosalo, M., Taipale, J., 2008. Hedgehog: functions and mechanisms. Genes Dev. 22**,** 2454-72.

Verkhusha, V. V., Kuznetsova, I. M., Stepanenko, O. V., Zaraisky, A. G., Shavlovsky, M. M., Turoverov, K. K., Uversky, V. N., 2003. High stability of Discosoma DsRed as compared to Aequorea EGFP. Biochemistry. 42**,** 7879-84.

Vokes, S. A., Ji, H., McCuine, S., Tenzen, T., Giles, S., Zhong, S., Longabaugh, W. J., Davidson, E. H., Wong, W. H., McMahon, A. P., 2007. Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning. Development. 134**,** 1977-89.

Vokes, S. A., Ji, H., Wong, W. H., McMahon, A. P., 2008. A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. Genes Dev. 22**,** 2651-63.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M., Tabin, C. J., 1996. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science. 273**,** 613-22.

Waid, D. K., McLoon, S. C., 1998. Ganglion cells influence the fate of dividing retinal cells in culture. Development. 125**,** 1059-66.

Wall, D. S., Mears, A. J., McNeill, B., Mazerolle, C., Thurig, S., Wang, Y., Kageyama, R., Wallace, V. A., 2009. Progenitor cell proliferation in the retina is dependent on Notch-independent Sonic hedgehog/Hes1 activity. J Cell Biol. 184**,** 101-12.

Wallace, V. A., 2008. Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina. Brain Res. 1192**,** 61-75.

Wan, J., Ramachandran, R., Goldman, D., 2012. HB-EGF is necessary and sufficient for Muller glia dedifferentiation and retina regeneration. Dev Cell. 22**,** 334-47.

Wan, J., Zheng, H., Xiao, H. L., She, Z. J., Zhou, G. M., 2007. Sonic hedgehog promotes stem-cell potential of Muller glia in the mammalian retina. Biochem Biophys Res Commun. 363**,** 347-54.

Wang, S. W., Kim, B. S., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H., Gan, L., 2001. Requirement for math5 in the development of retinal ganglion cells. Genes Dev. 15**,** 24-9.

Wang, Y., Dakubo, G. D., Thurig, S., Mazerolle, C. J., Wallace, V. A., 2005. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. Development. 132**,** 5103-13.

Wang, Y. P., Dakubo, G., Howley, P., Campsall, K. D., Mazarolle, C. J., Shiga, S. A., Lewis, P. M., McMahon, A. P., Wallace, V. A., 2002. Development of normal retinal organization depends on Sonic hedgehog signaling from ganglion cells. Nat Neurosci. 5**,** 831-2.

Wang, Z., 2016. Transactivation of Epidermal Growth Factor Receptor by G Protein-Coupled Receptors: Recent Progress, Challenges and Future Research. Int J Mol Sci. 17.

Waschek, J. A., Casillas, R. A., Nguyen, T. B., DiCicco-Bloom, E. M., Carpenter, E. M., Rodriguez, W. I., 1998. Neural tube expression of pituitary adenylate cyclase-activating peptide (PACAP) and receptor: potential role in patterning and neurogenesis. Proc Natl Acad Sci U S A. 95**,** 9602-7.

Watanabe, T., Voyvodic, J. T., Chan-Ling, T., Sagara, H., Hirosawa, K., Mio, Y., Matsushima, S., Uchimura, H., Nakahara, K., Raff, M. C., 1997. Differentiation and morphogenesis in pellet cultures of developing rat retinal cells. J Comp Neurol. 377**,** 341-50.

Weinberg, R. A., 1995. The retinoblastoma protein and cell cycle control. Cell. 81**,** 323-30.

Weiss, W. A., Burns, M. J., Hackett, C., Aldape, K., Hill, J. R., Kuriyama, H., Kuriyama, N., Milshteyn, N., Roberts, T., Wendland, M. F., DePinho, R., Israel, M. A., 2003. Genetic determinants of malignancy in a mouse model for oligodendroglioma. Cancer Res. 63**,** 1589-95.

Willardsen, M. I., Link, B. A., 2011. Cell biological regulation of division fate in vertebrate neuroepithelial cells. Dev Dyn. 240**,** 1865-79.

Wolff, T., Ready, D. F., 1991. The beginning of pattern formation in the Drosophila compound eye: the morphogenetic furrow and the second mitotic wave. Development. 113**,** 841-50.

Woo, H. N., Park, J. S., Gwon, A. R., Arumugam, T. V., Jo, D. G., 2009. Alzheimer's disease and Notch signaling. Biochem Biophys Res Commun. 390**,** 1093-7.

Wu, D. Q., Kan, M. K., Sato, G. H., Okamoto, T., Sato, J. D., 1991. Characterization and molecular cloning of a putative binding protein for heparin-binding growth factors. J Biol Chem. 266**,** 16778-85.

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., Griffin, J. D., 2000. MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. Nat Genet. 26**,** 484-9.

Xu, T., Rubin, G. M., 1993. Analysis of genetic mosaics in developing and adult Drosophila tissues. Development. 117**,** 1223-37.

Xu, X. L., Singh, H. P., Wang, L., Qi, D. L., Poulos, B. K., Abramson, D. H., Jhanwar, S. C., Cobrinik, D., 2014. Rb suppresses human cone-precursor-derived retinoblastoma tumours. Nature. 514**,** 385-8.

Yan, Y., Yang, D., Zarnowska, E. D., Du, Z., Werbel, B., Valliere, C., Pearce, R. A., Thomson, J. A., Zhang, S. C., 2005. Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. Stem Cells. 23**,** 781-90.

Yang, P., Seiler, M. J., Aramant, R. B., Whittemore, S. R., 2002. Differential lineage restriction of rat retinal progenitor cells in vitro and in vivo. J Neurosci Res. 69**,** 466-76.

Yang, Y., Geldmacher, D. S., Herrup, K., 2001. DNA replication precedes neuronal cell death in Alzheimer's disease. J Neurosci. 21**,** 2661-8.

Yao, H. H., Whoriskey, W., Capel, B., 2002. Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. Genes Dev. 16**,** 1433-40.

Yaron, O., Farhy, C., Marquardt, T., Applebury, M., Ashery-Padan, R., 2006. Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. Development. 133**,** 1367-78.

Young, M. J., 2005. Stem cells in the mammalian eye: a tool for retinal repair. Apmis. 113**,** 845-57.

Young, R. W., 1985. Cell proliferation during postnatal development of the retina in the mouse. Brain Res. 353**,** 229-39.

Yu, C., Mazerolle, C. J., Thurig, S., Wang, Y., Pacal, M., Bremner, R., Wallace, V. A., 2006. Direct and indirect effects of hedgehog pathway activation in the mammalian retina. Mol Cell Neurosci. 32**,** 274-82.

Zarbin, M., 2016. Cell-Based Therapy for Degenerative Retinal Disease. Trends Mol Med. 22**,** 115-34.

Zeng, H., Jia, J., Liu, A., 2010. Coordinated translocation of mammalian Gli proteins and suppressor of fused to the primary cilium. PLoS One. 5**,** e15900.

Zhang, C., Williams, E. H., Guo, Y., Lum, L., Beachy, P. A., 2004. Extensive phosphorylation of Smoothened in Hedgehog pathway activation. Proc Natl Acad Sci U S A. 101**,** 17900-7.

Zhang, X., Ibrahimi, O. A., Olsen, S. K., Umemori, H., Mohammadi, M., Ornitz, D. M., 2006. Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. J Biol Chem. 281**,** 15694-700.

Zhang, X. M., Yang, X. J., 2001. Regulation of retinal ganglion cell production by Sonic hedgehog. Development. 128**,** 943-57.

Zhao, Y., Tong, C., Jiang, J., 2007. Hedgehog regulates smoothened activity by inducing a conformational switch. Nature. 450**,** 252-8.

Zheng, M. H., Shi, M., Pei, Z., Gao, F., Han, H., Ding, Y. Q., 2009. The transcription factor RBP-J is essential for retinal cell differentiation and lamination. Mol Brain. 2**,** 38.

Zheng, X., Mann, R. K., Sever, N., Beachy, P. A., 2010. Genetic and biochemical definition of the Hedgehog receptor. Genes Dev. 24**,** 57-71.

Zhou, J., Zhu, G., Huang, J., Li, L., Du, Y., Gao, Y., Wu, D., Wang, X., Hsieh, J. T., He, D., Wu, K., 2016. Non-canonical GLI1/2 activation by PI3K/AKT signaling in renal cell carcinoma: A novel potential therapeutic target. Cancer Lett. 370**,** 313-23.

Zhou, X. L., Liu, J. C., 2014. Role of Notch signaling in the mammalian heart. Braz J Med Biol Res. 47**,** 1-10.

Zhu, M. Y., Gasperowicz, M., Chow, R. L., 2013. The expression of NOTCH2, HES1 and SOX9 during mouse retinal development. Gene Expr Patterns. 13**,** 78-83.

Zhu, X., Li, A., Brown, B., Weiss, E. R., Osawa, S., Craft, C. M., 2002. Mouse cone arrestin expression pattern: light induced translocation in cone photoreceptors. Mol Vis. 8**,** 462-71.

Zuniga, A., Laurent, F., Lopez-Rios, J., Klasen, C., Matt, N., Zeller, R., 2012. Conserved cis-regulatory regions in a large genomic landscape control SHH and BMP-regulated Gremlin1 expression in mouse limb buds. BMC Dev Biol. 12**,** 23.

Zurawel, R. H., Allen, C., Chiappa, S., Cato, W., Biegel, J., Cogen, P., de Sauvage, F., Raffel, C., 2000. Analysis of PTCH/SMO/SHH pathway genes in medulloblastoma. Genes Chromosomes Cancer. 27**,** 44-51.

7.0 Appendix

Appendix Table 1. qRT-PCR Primer sequences used for gene expression analysis in this study

Gene	Primer Sequence	Amplicon Size (bp)	GenBank Accession
Notch1	Forward 5'- AGGTGGATGCAGGCAATAAGG -3' Reverse 5'- CAGTGAAGAGGTGGCCCAACCC -3'	563	NM_008714
Hes1	Forward 5'- AAAGACGGCCTCTGAGCACA -3' Reverse 5'- TCATGGCGTTGATCTGGGTCA -3'	376	NM_008235.2
Hes5	Forward 5'- AAGTACCGTGGCGGTGGAGATGC -3' Reverse 5'- CGCTGGAAGTGGTAAAGCAG -3'	355	NM_010419.2
CyclinD1	Forward 5'- CCGGATAGAGTTGTCAGTGT -3' Reverse 5'- CTGACACCAATCTCCTCAAC -3'	347	NM_007631
Pax6	Forward 5'- GGGGGTCTGTACCAAGGATA -3' Reverse 5'- CAGCTGAGTCGCATCTGAG -3'	292	NM_013627
NeuroD	Forward 5'- GCTCCAGGGTTATGAGATCG -3' Reverse 5'- GGCTTTTGATCCTCCTCCTC -3'	300	NM_010894
Brn3b	Forward 5'- TCTGGAAGCCTACTTCGCCA -3' Reverse 5'- CCGGTTCACAATCTCTCTGA -3'	339	S68377
Brn3c	Forward 5'- TCTTCAACGGCAGTGAGCGT -3' Reverse 5'- ACACCCTGGAGTGTCCCGTA -3'	306	S69352.1
Math5	Forward 5'- CCTCCTATCTCCACTTCTCTTGTG -3' Reverse 5'- TTCTCCACCTCCTGAATGACGC -3'	219	AF418923.1
Jagged1	Forward 5'- TGCTTGGTGACAGCCTTCTACTGG -3' Reverse 5'- CTCTGGGCACTTTCCAAGTC -3'	380	NM_013822.1
Gli1	Forward 5'- CAGGGAAGAGAGCAGACTGA -3' Reverse 5'- AGCTGATGCAGCTGATCCAG -3'	251	AF026305
Nrarp	Forward 5'- AGGGCCAGACAGCACTACAC -3' Reverse 5'- CTTGGCCTTGGTGATGAGAT -3'	176	NM_025980.2
Ptc1	Forward 5'- AACAAAAATTCAACCAAACCTC -3' Reverse 5'- TGTCTTCATTCCAGTTGATGTG -3'	244	NM_008957.1
Ptc2	Forward 5'- GGTCTCCGAGTGGCTGTAAT -3' Reverse 5'- CCAGGTTGGTCCACTGGATA -3'	436	NM_008958.1
Mash1	Forward 5'- GGCTCAACTTCAGCGGCTTC -3' Reverse 5'- TGGAGTAGTTGGGGGAGATG -3'	291	M95603
Math3	Forward 5'- AGACCGGGCTCTTATGGAAT -3' Reverse 5'- AGTTCCTTGCCAGTCGAAGA -3'	295	AF036257
GAPDH	Forward 5'- ACCACAGTCCATGCCATCAC -3' Reverse 5'- TCCACCACCCTGTTGCTGTA -3'	451	XR_007679.1

Appendix Table 2. qRT-PCR Primer sequences used for gene expression analysis in this study

Gene	Primer Sequence	Amplicon Size(bp)	GenBank Accession
Gli1	Forward 5'-TCCACGGGGAGCGGAAGGAA-3' Reverse 5'-TCTCACCCGTGTGCGACCGA-3'	204	NM_010296.2
Gli2	Forward 5'-TCCACGCGCCTTTGCCGATT-3' Reverse 5'-GGTGGGCGCTGAAGGGTGAC-3'	183	NM_001081125.1
Gli3	Forward 5'-ACCACATCTGGCGGAGCCCT-3' Reverse 5'-CGGCCCTCATGATGTCTGGCA-3'	153	NM_008130.2
CcnD1	Forward 5'-GAGACCTGTGCGCCCTCCGT-3' Reverse 5'-TCATGGCCAGCGGGAAGACCT-3'	143	NM_007631.2
Fgf15	Forward 5'-ACCTCTGCATGAGCGCGGAC-3' Reverse 5'-AGGAGCGGTGAAACACGGGG-3'	210	NM_008003.2
Gapdh	Forward 5'-GGCCGGTGCTGAGTATGTCG-3' Reverse 5'-TTCAAGTGGGCCCCGGCCTT-3'	72	NM_001289726.1
Hes5	Forward 5'- CCGCTCGCTAATCGCCTCCA -3' Reverse 5'- CGGCTTCCGCAGTCGGTTTT -3'	97	NM_010419.4
Ptch1	Forward 5'- GCAGACTGGCAGCCGAGACA -3' Reverse 5'- TCGTTGCTGACCCAAGCGGT -3'	126	NM_008957.2
CcnD3	Forward 5'- GGACACTCGCTTTGTTTGGGT-3' Reverse 5'- GGAAGGTCTTGCTGGTCCATA-3'	185	NM_001081635.1
Id1	Forward 5'- GAGTCTGAAGTCGGGACCAC -3' Reverse 5'- TCAGCGACACAAGATGCGAT -3'	135	NM_010495.3
Ascl1	Forward 5'- GACTTTGGAAGCAGGATGGC -3' Reverse 5'- CTCCCCATTTGACGTCGTTG -3'	70	NM_008553.4
Nrarp	Forward 5'- AGGGCCAGACAGCACTACAC -3' Reverse 5'- CTTGGCCTTGGTGATGAGAT -3'	176	NM_025980.2
Hes1	Forward 5'- GACCCGAGCGTGTTGGGGAA -3' Reverse 5'- ACACGTGGACAGGAAGCGGG -3'	78	NM_008235.2
Sox9	Forward 5'- GAAGGCGAGTCGGAGCGGAG-3' Reverse 5'- GGAAGTCGGAGAGCCGAGAG-3'	171	NM_011448.4
Sox8	Forward 5'- CGAAGGAAGAGTGTGAAGACT-3' Reverse 5'- CTGTGTGGTGGTCACTGTGAT-3'	133	NM_011447.3
Sox2	Forward 5'- GACTTTGGAAGCAGGATGGC -3' Reverse 5'- CTCCCCATTTGACGTCGTTG -3'	132	NM_011443.3
c-myc	Forward 5'- ATGCCCCTCAAGGTGAACTTC -3' Reverse 5'- CGGAGTCGTAGTCGAGGTCATA -3'	55	NM_010849.4
$p21^{Cip1}$	Forward 5'- CCTGGTGATGTCCGACCTG -3' Reverse 5'- CGGGACCGAAGAGACAAC -3'	61	NM_007669.4
$p27^{Kip1}$	Forward 5'- AGGCAGATGGTTTAAGAGTGC-3' Reverse 5'- TCGACGCCAGACGTAAACA -3'	200	NM_009875.4
CcnE1	Forward 5'- ACAAGACTGTGAAAAGCGAGG -3' Reverse 5'- TTTGTTAGGGGTGGGGATGAA -3'	92	NM_007633.2
Crx	Forward 5'- CCAGGCTTAAAGTCGCCCCGT -3' Reverse 5'- AGGTCCACATTGGGGCCACTCA -3'	220	NM_007770.4
Glast	Forward 5'- ACCAAAAGCAACGGAGAAGAG -3' Reverse 5'- GGCATTCCGAAACAGGTAACTC -3'	144	NM_148938.3
Gfap	Forward 5'- CCACCAAACTGGCTGATGTCTAC -3' Reverse 5'- TTCTCTCCAAATCCACACGAGC -3'	240	NM_001131020.1

Cralbp	Forward 5'- ACGGGTGAGGGAGCTACAG-3' Reverse 5'- GCACGACCCACATCGAACTT -3'	147	NM_020599.2
Cxcr4	Forward 5'- GACTGGCATAGTCGGCAATG -3' Reverse 5'- AGAAGGGGAGTGTGATGACAAA -3'	131	NM_009911.3
C-jun	Forward 5'- CCTTCTACGACGATGCCCTC -3' Reverse 5'- GGTTCAAGGTCATGCTCTGTTT -3'	102	NM_010591.2
GFP	Forward 5'- CAACAGCCACAACGTCTATAT-3' Reverse 5'- ATGTTGTGGCGGATCTTGAAG -3'	78	L29345.1