Identification of *Sox8* and *Ndp* as novel targets of the Hedgehog signaling pathway in the retina

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ABSTRACT

During embryonic development, the Hedgehog (Hh) signaling pathway plays an important role in the growth and patterning of numerous tissues and organs. In the developing retina, Hh signaling regulates the proliferation and differentiation of retinal progenitor cells (RPC) through mechanisms that are not completely understood. The principal downstream mediators of the Hh pathway are the Gli transcription factors (Gli1-3), which regulate the expression of target genes responsible for the effects of the Hh pathway on RPC. The network of genes targeted by this pathway in neural progenitor cells however, remains unknown. The objective of this thesis was to identify and characterize novel targets of Hh/Gli during retinal development. Using a computation approach, 390 genes were identified as having at least one conserved Gli binding motif within the vicinity of the coding sequence between humans and mice. During validation, I demonstrate that 30 of 46 selected targets were modulated in response to Hh pathway activation in either E14.5 and/or P0.5 retinal explants and that the induction of 25 of these were significantly different between the two developmental stages. Included in this list of Hh-modulated genes were *Sox8* and *Ndp*, two highly inducible genes that are direct targets of Gli2. Functionally, I was unable to determine a role for *Sox8* during retinal development which could reflect compensation by the closely related *Sox9* and *Sox10* genes. *Ndp* on the other hand was found to be sufficient and required for Hh mediated induction in progenitor cell proliferation and cell fate determination. Therefore, in this thesis Hh target genes have been identified which could provide some insight into the mechanisms that are responsible for the cellular outcome of a response to the pathway.

RÉSUMÉ

Pendant le développement embryonnaire, la voie de signalisation Hedgehog (Hh) joue un rôle important dans la croissance et la structuration de nombreux tissus et organes, y compris la rétine. Pendant le développement de la rétine, la voie de signalisation Hh est impliquée dans la régulation de la prolifération et la différenciation des cellules progénitrices de la rétine (CPR) à travers des mécanismes moléculaires qui ne sont pas complètement compris. Les médiateurs principaux en aval de la voie Hh sont les facteurs de transcription Gli (Gli1-3), qui régulent l'expression de gènes qui contribuent à la spécification des CPR; mais le réseau de gènes ciblés par cette voie reste inconnu. Par conséquent, l'objectif de cette thèse est d'identifier et de caractériser de nouvelles cibles de Hh / Gli pendant le développement de la rétine. En utilisant une approche bioinformatique, 390 gènes ont été identifiés comme ayant au moins un motif Gli conservé entre les humains et les souris. Pendant le processus de validation, je démontre que 30 des 46 cibles sélectionnées étaient modulées en réponse de l'activation de la voie Hh dans des explants de rétine soit de E14.5 ou P0.5 et que 25 de ceux ont démontré une réponse temporelle. Inclus dans ces objectifs ont été *Sox8* et *Ndp*, deux gènes qui démontre une haut induction de transcription et qui sont des cibles directes de Gli2. Je n'ai pu déterminer un rôle fonctionelle pour *Sox8* pendant le développement de la rétine qui pourrait refléter une compensation fonctionnelle par *Sox9* et *Sox10*. Pour *Ndp*, j`ai demontré que ce gène est suffisant et nécessaire pour la prolifération et la determination des cellules progénitrices causée par la voie the Hh. Par conséquent, cette thèse met en lumière de nouvelles cibles de la voie Hh pendant le développement de la rétine et peut donner un aperçu mécaniste des processus généraux Hh dépendantes.

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ABREVIATIONS

18S	18S ribosomal RNA
Ag	Agonist
AP	Alkaline phosphatase
bHLH	Basic helix-loop-helix
BCC	Basal cell carcinoma
BIO	6-bromoindirubin-3'-oxime
BMP	Bone morphogenic protein
Boc	Brother of CDO
Bok	Bcl2-related ovarian killer
bp	Base pair
BrdU	5-bromo-2-deoxyuridine
CcnD	Cyclin D
CcnE	Cyclin E
Ccr	C-C chemokine receptor
Cdk	Cyclin-dependent kinase
Cdc	Cell Division Cycle related
Cdon	Cell adhesion molecule-related/downregulated by oncogenes
cGMP	cyclic guanosine monophosphate
ChIP	Chromatin immounprecipitation
Chx	ceh-10 homeodomain containing homolog

Ci	Cubitus Interruptus
Ci^{Act}	Cubitus Interruptus activator
Ci^{Rep}	Cubitus Interruptus Repressor
CK	Casein kinase
CKO	Conditional knockout
CNS	Central nervous system
Cos2	Costal2
CSC	Cancer stem cell
Ct	Cycle threshold
C-terminus	Carboxy-terminus
Cys	Cystine
Dhh	Desert Hedgehog
Dkk	Dickkopf
Dsh	Disheveled
DIV	Days in vitro
Dlp	Dally-like protein
Ehh	Echidna hedgehog
ER	Endoplasmic reticulum
EST	Expressed sequence tag
FBS	Fetal bovine serum
FEVR	X-linked familial exudative vitreoretinopathy
Fox	Forkhead box
Fu	Fused

Fzd	Frizzled
Gas	growth-arrest-specific
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GC	Ganglion cells
GFP	Green fluorescent protein
Gli	Glioma-associated oncogene
GPC	Glypican
GPI	Glycosylphosphatidylinositol
GRK2	G protein-coupled receptor kinase 2
GSK	Glycogen Synthase Kinase
Hes	Hairy and enhancer of split
HeyL	Hairy/enhancer-of-split related with YRPW motif-like protein
Hh	Hedgehog
Hh-C	Catalytic domain
Hh-N	Hedgehog signaling domain
Hip	Hh-interacting protein
HMG	High mobility group box
Hpcal	Hippocalcin like
HPE	Holoprosencephaly
HSC	Hedgehog signaling complex
HSPGs	Heparan-sulfate proteoglycans
Ihh	Indian Hedgehog
IHC	Immunohistochemistry

INL	Inner layer nuclear
IP	Immunoprecipitation
ISH	*In situ* hybridization
Kb	Kilobases
Kif	Kinesin family member
KO	Knockout
Lef	Lymphoid enhancer factor
LiCl	Lithium chloride
Lrp	Low-density lipoprotein receptor-related protein
MIM	Missing in Metastasis
Mmp	Matrix metallopeptidase
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
ND	Norrie disease
Ndp	Norrie disease protein
N-Myc	v-myc Myelocytomatosis viral related oncogene
NrCAM	Neuronal cell adhesion molecule
N-terminus	Amino terminus
ONL	Outer nuclear layer
qPCR	Quantitative polymerase chain reaction
Pax6	Paired box gene 6
PC	Primary cilium
PDG	Pure gonadal dysgenedid

PCP	Planar cell polarity
PFA	Paraformaldehyde
PKA	Protein kinase A
Ptc	Patched
Pvrl	Poliovirus receptor-related
RGC	Retinal ganglion cell
ROP	Retinopathy of prematurity
RPC	Retinal progenitor cell
RPE	Retinal pigment epithelium
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAG	Smoothened agonist
SEM	Standard error of the mean
Shh	Sonic hedgehog
SHP	Small hetrodimer partner
Smo	Smoothened
Sox8	SRY (sex determining region Y)-box 2
SRR	Sterol-recognition region
Stmn	Stathmin
Su(fu)	Suppressor of Fused
Syn	Synapsin
Tcf	T-cell factor
Tef	Thyrotroph embryonic factor

Terf	Telomeric repeat binding factor
TF	Transcription factor
TGF-β	Transforming growth factor β
TSS	Transcription start site
TSPAN	Tetraspanin
Twhh	Tiggywinkle hedgehog
Wnt	Wingless-type MMTV integration site family

Chapter 1

Introduction

1.1 Hedgehog proteins

1.1.1 Hedgehog isoforms

Hedgehog (Hh) proteins are secreted morphogens that activate an evolutionary conserved signaling pathway. This pathway is an important regulator of growth and patterning in numerous tissues and organs (reviewed in Ingham and McMahon, 2001; Varjosalo and Taipale, 2008). First discovered as a segment polarity gene in *Drosophila*, the Hh protein received its name based on the disorganized denticle phenotype of the hh mutant flies (Nusslein-Volhard and Wieschaus, 1980). Since then numerous Hh homologues have been identified in a number of organisms including leech, sea urchin, zebrafish, *Xenopus*, chick, mouse and humans (reviewed in Hooper and Scott, 2005; Ingham and McMahon, 2001). In vertebrates, as a result of a genome duplication, the Hh genes have expanded into three subgroups: *Desert* (*Dhh*), *Indian* (*Ihh*) and *Sonic* (*Shh*) (reviewed in Varjosalo and Taipale, 2008). Birds and mammals have one *Hh* gene in each subgroup while zebrafish, as a result of another genome duplication, have an additional member in the Shh subgroup, *tiggywinkle* (*Twhh*) (Ekker et al., 1995), and two others in the Ihh subgroup, *echidna* (*Ehh*) (Currie and Ingham, 1996) and *Indian hedgehog homologue a* (*Ihha*) (Avaron et al., 2006).

1.1.2 Hh ligand processing

Hh proteins are synthesized as full-length precursors that undergo several post-translational modifications within the secretory pathway that contribute to the biological activity of the protein and its ability to act as a long range morphogen (Fig 1-1)

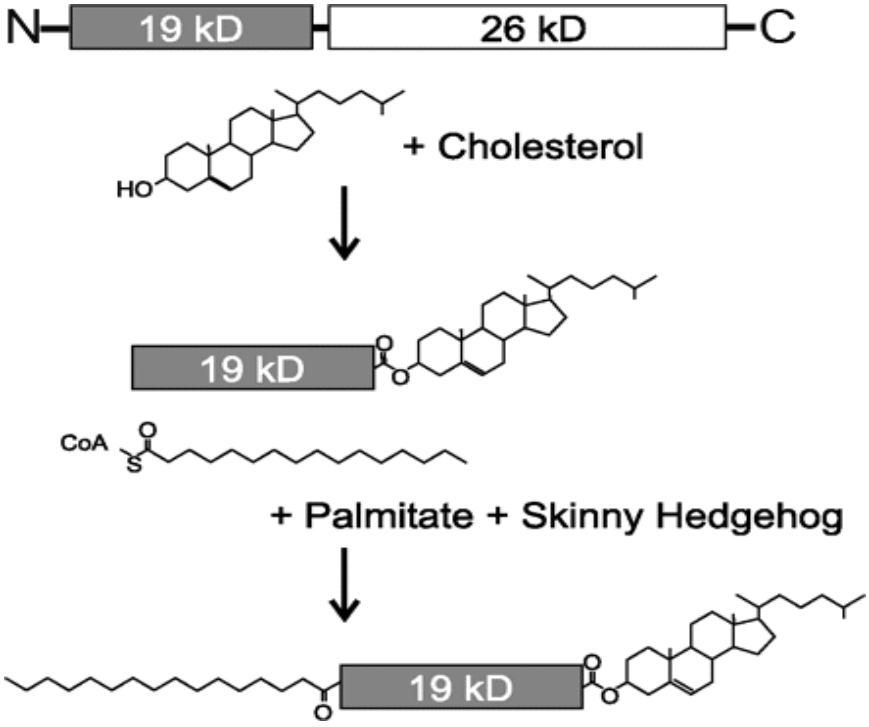

N
19 kD
26 kD
C
HO
+ Cholesterol
19 kD
CoA
S
+ Palmitate + Skinny Hedgehog
19 kD

Figure 1-1. Posttranslational Hedgehog processing. The full-length Hh precursor protein undergoes an autocatalytic cleavage event to generate a Hh-N and a Hh-C terminal proteins. During the cleavage process, a cholesterol will be added to the C-terminal end of Hh-N followed by the addition of a palmitate to the N-terminal end by Skinny hedgehog. Reproduced from van den Brink (2007).

(Chen et al., 2011). Targeted to the secretory pathway by an N-terminal signal peptide, the full-length protein undergoes a cleavage event catalyzed by the carboxy-terminus of the immature protein resulting in the generation of two Hh proteins, an amino (Hh-N) terminal fragment that mediates all Hh signaling activity and a carboxy (Hh-C) terminal fragment (Lee et al., 1994; Porter et al., 1995). During the cleavage process, a cholesterol moiety bound to a sterol-recognition region (SRR) in Hh-C is transferred to the carboxy end of the Hh-N (Fig 1-1) (Porter et al., 1996a; Porter et al., 1996b). A second lipid modification occurs at the amino terminal of Hh-N where a palmitate is added by the transmembrane acetyltransferase Skinny Hedgehog (Ski) on a Cys residue (Chamoun et al., 2001; Pepinsky et al., 1998). The addition of the palmitoyl group is important for protein function, as inhibiting this modification significantly reduces the signaling potential of this protein in *Drosophila* and vertebrates (Chamoun et al., 2001; Chen et al., 2004a; Lee et al., 2001b; Lewis and Eisen, 2001). The function of the cholesterol modification on the other hand, is controversial. Some reports describe this modification as important in the long range transport of the Hh ligand (Lewis et al., 2001; Li et al., 2006; Miura and Treisman, 2006), while others have shown that this modification restricts the movement of the ligand by promoting insertion into the lipid bilayer and preferential enrichment in membrane lipid rafts (Callejo et al., 2006; Rietveld et al., 1999).

1.1.3 Hh signaling pathway

The key components of the Hh signaling pathway are well conserved from flies to humans. Hh pathway activation is initiated following the interaction between Hh ligand and its 12-pass transmembrane receptor Patched (Ptc) (Forbes et al., 1993). In its unliganded

state, Ptc acts as a negative regulator of the pathway by repressing the activity of the seven-span G-protein coupled receptor-like transmembrane protein Smoothened (Smo) (Fig 1-2) (Apionishev et al., 2005; Jia et al., 2004) and by sequestering free Hh protein (Briscoe et al., 2001; Chen and Struhl, 1996; Torroja et al., 2004). Upon ligand binding, Ptc becomes internalized to the endosome resulting in the mobilization of Smo to the cell surface (reviewed in Teglund and Toftgard, 2010). The translocation of Smo to the plasma membrane is required but not sufficient to activate the Hh pathway (Corbit et al., 2005; Rohatgi et al., 2007). In both *Drosophila* (Denef et al., 2000; Jia et al., 2004) and mammals (Chen et al., 2004b; Meloni et al., 2006; Philipp et al., 2008) phosphorylation of the cytoplasmic tail is necessary for Smo activity. In *Drosophila*, Smo becomes hyper-phosphorylated by a series of kinases including, Protein Kinase A (PKA), Glycogen Synthase Kinase 3 (GSK3) and PKA-primed Casein Kinase 1 (CK1) (reviewed in Aikin et al., 2008). In mammals, as the cytoplasmic tail of Smo is truncated and lacks the PKA and CK1 phosphorylation sites, the G protein-coupled receptor kinase 2 (GRK2) is currently the only known Smo phosphorylating kinase (Chen et al., 2004b). While these Smo phosphorylation events are well characterized, especially in *Drosophila*, it remains unclear how phosphorylation affects the activity of Smo. It has been shown that the phosphorylation of Smo promotes its association with non-visual β-arrestins, which are involved in intracellular protein transport and receptor recycling (Kovacs et al., 2009; Kovacs et al., 2008; Rohatgi and Scott, 2008). Therefore this phosphorylation-induced Smo interaction with beta-arrestin may serve to recruit signaling components essential for the activation of the signaling cascade or to transport Smo to its obligate subcellular location (Kovacs et al., 2008; Meloni et al., 2006).

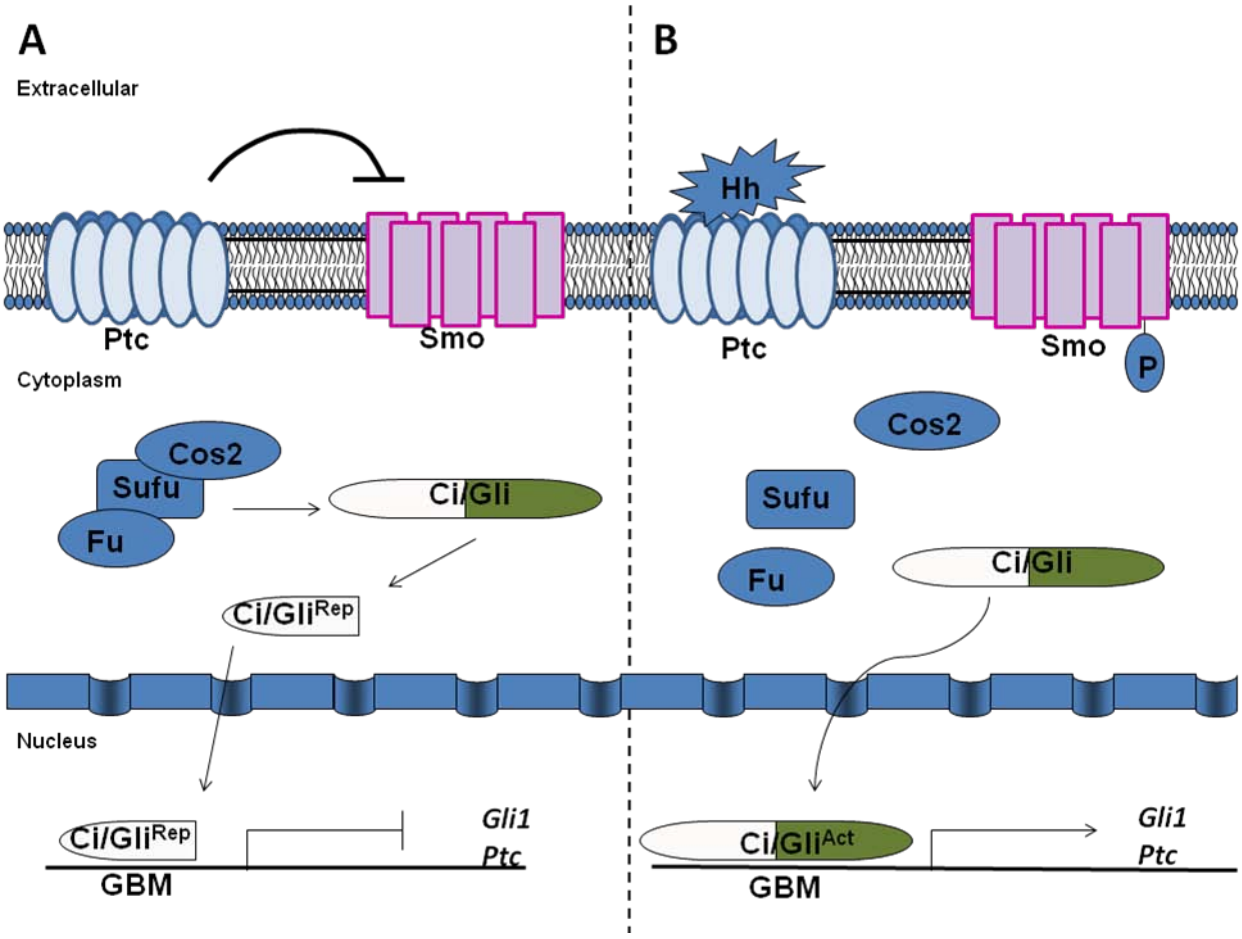

A
Extracellular
Ptc
Smo
Cytoplasm
Cos2
Sufu
Fu
Ci/Gli
Ci/Gli^Rep
Nucleus
Ci/Gli^Rep
Gli1
Ptc
GBM
B
Hh
Ptc
Smo
P
Cos2
Sufu
Fu
Ci/Gli
Ci/Gli^Act
Gli1
Ptc
GBM

Figure 1-2: Hedgehog signaling pathway. (A) In the absence of Hh ligand, Ptc antagonizes the activity of Smo. Ci/Gli associates with the HSC complex consisting of Fu, Sufu and Cos2 resulting in the cleavage of full-length Ci/Gli into repressor forms. Ci/Gli repressor translocates into the nucleus and leads to transcriptional repression of target genes such as *Gli1* and *Ptc*. (B) In the presence of ligand, the inhibition of Ptc on Smo is alleviated resulting in the dissociation of Ci/Gli from the HSC complex. Full-length Ci/Gli activator enters the nucleus and activates the transcription of target genes.

A novel feature of vertebrate Hh signaling is that it occurs in the primary cilium (PC) (Huangfu and Anderson, 2005), an evolutionary conserved structure found in all vertebrate cells (Michaud and Yoder, 2006) and only in sensory neurons and sperm cells in *Drosophila* (Corbit et al., 2005). The primary function of these immotile, cell-surface projections are to serve as sensory centers that detect both mechanical and chemical signals from the extracellular environment (Berbari et al., 2009; Michaud and Yoder, 2006). When unbound, Ptc is localized within and at the base of the PC where it prevents the accumulation of Smo to the PC (Berbari et al., 2009; Eggenschwiler and Anderson, 2007). Once bound to Hh, Ptc is removed from the PC allowing for the recruitment of Smo to the PC from either the bordering plasma membrane or directly from the Golgi (Milenkovic et al., 2009; Wang et al., 2009). When proteins involved in PC formation and maintenance are mutated, the embryonic phenotype resembles those of other Hh pathway mutations (Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005; May et al., 2005), highlighting the importance of this structure for Hh signaling. In *Drosophila*, Hh signaling in non-ciliated cells occurs at the level of the cell surface (Denef et al., 2000; Quirk et al., 1997). Furthermore, mutations in key cilium maintenance proteins in flies does not affect the activity of the Hh signaling pathway (Han et al., 2003; Sarpal et al., 2003).

The activation of Smo ultimately regulates the processing of the zinc finger transcription factors, Cubitus Interruptus (Ci) in flies (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998) and Glioma -associated oncogene (Gli) in mammals (Chen et al., 2004b), which control the transcriptional output of the Hh pathway (Fig 1-2). Ci exists in two forms, a full-length transcriptional activator (Ci^{Act}) and a truncated N-terminal fragment that functions as a repressor (Ci^{Rep}) (Zhang et al., 2005). The processing of full-length Ci to a

truncated repressor is achieved through a cytoplasmic complex known as the Hedgehog Signaling Complex (HSC) that includes the kinesin-like protein Costal-2 (Cos2), the serine-threonine kinase Fused (FU), Ci and Suppressor of Fused [Su(fu)] (Fig 1-2) (Robbins et al., 1997; Sisson et al., 2006; Smelkinson et al., 2007; Stegman et al., 2000). In the absence of Hh signalling, HSC anchors Ci to the microtubules where Cos2 recruits multiples kinases, including PKA, CK1 and GSK3 to promote Ci phosphorylation that results in a proteolytic processing of Ci into its repressive form (Fig 1-2) (Aza-Blanc et al., 1997; Chen et al., 1998; Jia et al., 2002; Methot and Basler, 1999; Smelkinson et al., 2007). Following Hh pathway activation, Fu and Cos2 are hyperphosphorylated (Nybakken et al., 2002; Therond et al., 1996) resulting in the dissociation of the HSC from microtubules and the translocation of Ci^{Act} into the nucleus (Ohlmeyer and Kalderon, 1998; Wang and Holmgren, 2000; Zhang et al., 2005) (Fig 1-2). In addition to being a component of the HSC, SuFu also negatively regulates the pathway by sequestering full-length Ci^{Act} in the cytoplasm preventing its entry into the nucleus and transcriptional activation of target genes (Methot and Basler, 2000; Wang et al., 2000).

In mammals, the functions of Ci are distributed among three Gli proteins (Gli1-3). With the exception of Gli1 (Park et al., 2000), Gli2 and Gli3 can exist in both full-length activator and truncated repressor forms like Ci (Matise et al., 1998; Sasaki et al., 1997). In the absence of Hh signaling, Gli3, and to a lesser extent Gli2, undergo proteasome-mediated carboxyl cleavage to repressor forms that silence Hh target genes (Ruiz i Altaba, 1999; Wang et al., 2006). Hh pathway activation prevents this cleavage resulting in the accumulation of full length activator forms of Gli2 and to a lesser extent Gli3 (Buttitta et al., 2003; Ding et al., 1998; McDermott et al., 2005; Pan et al., 2006; Pan et al., 2009). As the loss of Gli1

does not result in developmental defects or a decrease in Hh signaling, unless one copy of Gli2 is removed, it is believed to primarily function to enhance the activation ability of Gli2 (Bai et al., 2002; Park et al., 2000). All three Glis recognize and interact with the Gli consensus motif GACCACCCA (Agren et al., 2004; Kinzler and Vogelstein, 1990) and the combined activity of Gli repressor and activator forms in the nucleus will mediate the effects of the Hh pathway. Interestingly, in mouse neural crest stem cells treated with the chlorobenzothiophene-containing Hh pathway agonist SAG, several sites within the *Nanog* promoter were quickly occupied by Gli2 but were later replaced by Gli1 (Po et al., 2010). Therefore, for some genes Gli2 could be important for the initial upregulation in gene expression following Hh pathway activation while Gli1 is required to maintain this level of expression.

While many of the core components involved in regulating the processing of Gli2 and Gli3 into repressors in mammals are similar to *Drosophila*, there is considerable divergence in the involvement and importance of some of these components. In *Drosophila*, *fu* is a critical component of the Hh pathway and essential for strong Hh signaling (Alves et al., 1998; Ohlmeyer and Kalderon, 1998), while in mouse the loss of *Fu* does not appear to affect Hh signaling (Wilson and Chuang, 2010). In contrast with the *sufu* KO flies, which do not display any overt phenotype (Pham et al., 1995), *Sufu*-deficient mice die around embryonic day 9.5 and share a high degree of phenotypic similarity with *Ptc* mutants (Cooper et al., 2005; Svard et al., 2006). *Sufu* has also been shown to be required to achieve maximal vertebrate Hh pathway activity potentially through the stabilization of full-length Gli2 and Gli3 (Chen et al., 2009).

Cos2 is another component of the HSC where divergence exists between flies and mammals. As mentioned above, Cos2 is a key component of the HSC that recruits kinases important for the phosphorylation and truncation of Ci into its repressor form. In mammals, *Kif7* and *Kif27* are the *Cos2* orthologues and *in vitro* analysis of their function led to the conclusion that neither protein was involved in Hh signaling (Varjosalo et al., 2006). However, in a recent study using a *Kif7* knockout mouse, it was demonstrated that Kif7 acts as a negative regulator of the pathway in the neural tube, as the loss of this protein was associated with a reduction in ventral cells types in the spinal cord (Endoh-Yamagami et al., 2009). It was also shown that Kif7 accumulates at the distal tip of the PC in a Hh-dependent manner and is required for the efficient processing of Gli3 to its repressor form (Endoh-Yamagami et al., 2009). Therefore the roles of Cos2 and Kif7 between flies and mammals may not be as divergent as once believed.

The activity of the Gli transcription factors has also been shown to be either enhanced or restricted through interaction with other proteins. A number of proteins to date have been identified as Gli binding partners. For example, Small heterodimer partner (SHP) of the nuclear receptor superfamily inhibits the transcriptional activity and nuclear translocation of Gli1 via protein-protein interactions. SHP overexpression decreases the expression of Gli target genes, whereas SHP-knockdown increases the expression of these target genes (Kim et al., 2010). Parafibromin/Hyrax (Hyx) can also form a complex with each of the three Glis and regulates the transcriptional activity of Gli1 and Gli2 (Mosimann et al., 2009). Through yeast two-hybrid, Ski has been identified as Gli2 and Gli3 binding partners and is important in the repressive function of Gli3 (Dai et al., 2002). Zinc finger proteins Zic have also been shown to interact with Glis and promote Hh signaling by

promoting the retention of Gli1 (Chan et al., 2011; Koyabu et al., 2001). Dyrk1 also enhances Gli-dependent transcription by retaining Gli1 in the nucleus as well as enhancing the transcription activity of this TF (Mao et al., 2002). Itch (Di Marcotullio et al., 2010), Slimb (Smelkinson et al., 2007) and Missing in Metastasis (MIM) (Bershteyn et al., 2010; Callahan et al., 2004) are other proteins that can bind to Ci/Gli and either regulate activity or stability.

1.1.4 Hh binding proteins

Once released into the extracellular environment, Hh-N can interact with a number of proteins other than Ptc, including, Hh-interacting protein (Hip) (Jeong and McMahon, 2005; Tada et al., 2008), Cell adhesion molecule-related/downregulated by oncogenes (Cdo) (Tenzen et al., 2006), Brother of Cdon (Boc) (Okada et al., 2006), growth-arrest-specific 1 (Gas1) (Allen et al., 2007; Seppala et al., 2007) and several heparan-sulfate proteoglycans (HSPGs) (Carrasco et al., 2005; Gallet et al., 2008). As the interaction with these proteins can either enhance or inhibit Hh signaling activity, it is believed that these interactions serve to fine tune the activity of Hh. Hip, for example, is a glycoprotein that can bind all three Hh proteins with a similar affinity as Ptc1 and functions as a negative regulator of the pathway (Chuang et al., 2003; Chuang and McMahon, 1999; Marigo and Tabin, 1996). The immunoglobulin (Ig)/fibronectin (Fn)-repeat-containing proteins Cdon and Boc, as well as the glycosylphosphatidylinositol (GPI)-anchored membrane bound Gas1, have been shown to enhance Hh-Ptc interaction and are thought to function as co-receptors (Allen et al., 2011; Allen et al., 2007; Lee et al., 2001a; Seppala et al., 2007; Tenzen et al., 2006; Zhang et al., 2006). HSPGs can either enhance or inhibit the Hh pathway. In *Drosophila*, Dally-like

protein (Dlp) has been reported to be required for optimal Hh signaling by enhancing the interaction of Hh with Ptc (Desbordes and Sanson, 2003). In mammals, the Glypican-5 HSPG protein also positively affects Hh signaling by stabilizing the interaction of Hh and Ptc (Li et al., 2011) while Glypican-3 negatively affects Hh signaling by interacting with Hh leading to the subsequent degradation of the Hh-Glypican-3 complex (Capurro et al., 2008).

1.2 Function of Hh signaling

1.2.1 Embryonic development

The importance of the Hh pathway during invertebrate and vertebrate embryonic development is well established. Its activity includes the regulation of proliferation and differentiation, cell survival, self-renewal, cell migration and tissue patterning (reviewed in Ingham and McMahon, 2001). While all Hh proteins activate the pathway similarly, the temporal and tissue specific expression of these proteins emphasizes the unique set of functions in regulating different developmental processes (reviewed in Varjosalo and Taipale, 2008). In mammals, Dhh is a positive regulator of the differentiation of steroid-producing Leydig cells (Yao et al., 2002), involved in spermatogenesis (Bitgood et al., 1996) and is required for normal conductance and function of peripheral nerves (Bajestan et al., 2006; Lai et al., 2005). Ihh is a key regulator of endochondral (McMahon et al., 2003) and intramembranous (Abzhanov et al., 2007; Lenton et al., 2011) bone development and is important in epithelial differentiation in the gut (Ramalho-Santos et al., 2000). Shh is the most widely expressed homologue and is involved in the development of numerous tissues and organs including the CNS, gut, lung, limb and retina (reviewed in Varjosalo and Taipale,

2008). One key function of Shh during the development of these tissues is to act as a strong mitogen and promote proliferation to ensure proper cell numbers are present to form a functional system. In neuronal cells, this is achieved partly through the regulation of cell cycle components *CcnD1* (Lobjois et al., 2004), *CcnA2*, *CcnB1* (Locker et al., 2006; Sakagami et al., 2009), *N-Myc*, *E2f1*, *E2f2* (Oliver et al., 2003) by the Shh pathway. Furthermore, Hh signaling in the retina has been shown to be important for the G_1/S transition as the length of G_1 is significantly increased in *Smo* KO animals (Locker et al., 2006; Sakagami et al., 2009)

1.2.2 Homeostasis and regeneration

Much less is known about the function of Hh signaling in adults. It has been shown that this pathway plays an important role in the maintenance of stem and progenitor cells in a growing list of adult tissues including the epithelia of many internal organs and brain (Adolphe et al., 2004; Machold et al., 2003; Miura et al., 2001; van den Brink, 2007). Hh signaling has also been shown to be an important regulator of adult homeostasis in bone (Mak et al., 2008; Ohba et al., 2008) and small intestine (Zacharias et al., 2011). Recent work suggests that the Hh pathway also plays a critical role in tissue repair and regeneration in response to injury (Akazawa and Kohsaka, 2007; Asai et al., 2006; Bambakidis et al., 2003; Luo et al., 2009).

1.2.3 Hh signaling, disease and cancer

Aberrant Hh signaling leads to a variety of congenital abnormalities, such as Holoprosencephaly (HPE), Brachydactyly, Pure gonadal dysgenesis (PGD) and Gorlin

syndrome (reviewed in Nieuwenhuis and Hui, 2005). HPE is characterized by incomplete separation of the bilateral hemispheres, mental retardation and abnormal craniofacial development, including cyclopia, presence of a proboscis and cleft lip and palate all resulting from the loss of Shh signaling (Belloni et al., 1996; Roessler et al., 1996). Brachydactyly represents a group of inherited hand anomalies generally characterized by shortened phalanges or metacarpals caused by mutations in the Ihh (Gao et al., 2001). PGD is characterized by underdeveloped and dysfunctional gonads with an increased risk of developing gonadal tumors as a result of mutations in Dhh (Canto et al., 2004). Gorlin syndrome, a result of a Ptc mutation, is an autosomal dominant disorder characterized by a predisposition to develop basal cell carcinomas (BCC) of the skin and a variety of other tumor types, as well as congenital anomalies of the brain and skeletal system (Boutet et al., 2003).

Abnormal Hh pathway activation is also known to contribute to the onset and progression of various forms of cancers. Activation can be achieved through a loss-of-function mutation in inhibitory proteins or gain-of-function mutation in positive regulators as well as overexpression of the Hh ligands leading to autocrine and paracrine activation of the pathway (Barakat et al., 2010). For example, in medulloblastomas originating from the cerebellum, approximately 35% of these tumors arise from mutations in Hh pathway components that lead to an overactive pathway (Guessous et al., 2008). The most common mutation is the heterozygous mutation in the tumor suppressor gene *Ptc* (Zurawel et al., 2000) while mutations in *Sufu* and activating mutations in *Smo* have also been attributed to medulloblastoma development (Guessous et al., 2008; Hatton et al., 2008; Taylor et al., 2002). The mechanisms leading to medulloblastomas is unclear. In cultured cerebellar

granule neuron progenitor cells, the predicted origin of medulloblastomas, Hh pathway activation upregulates the expression of *Cyclin* (*Ccn*) *D1*, *CcnD2*, *CcnE* (Kenney and Rowitch, 2000), *N-Myc* (Hatton et al., 2006; Thomas et al., 2009) and *Bmi1* (Leung et al., 2004; Michael et al., 2008) which results in an increase in proliferation of these cells.

There is also compelling evidence that tumors originate from long-lived stem cells, termed cancer stem cells (CSC), through the misregulation of the self-renewal process (Marquardt et al., 2010; Reya et al., 2001). CSCs isolated from several types of cancers exhibit an elevated level of Hh signaling which is believed to promote their self-renewal (Bar et al., 2007; Huang et al., 2010; Mao et al., 2009; Quint et al., 2009; Song et al., 2011; Yang et al., 2008).

To address the effects of Hh signaling on neural progenitor proliferation, self-renewal and differentiation the neural retina has emerged as a valuable model system. Derived from an outgrowth of the developing brain (Hilfer, 1983), the retina is a relatively simple, accessible and experimentally tractable system that follows a well characterized developmental process. The retina can be manipulated genetically *in vivo* without compromising viability and *in vitro* in retinal tissue cultures that can be generated easily and rapidly.

1.3 The Retina

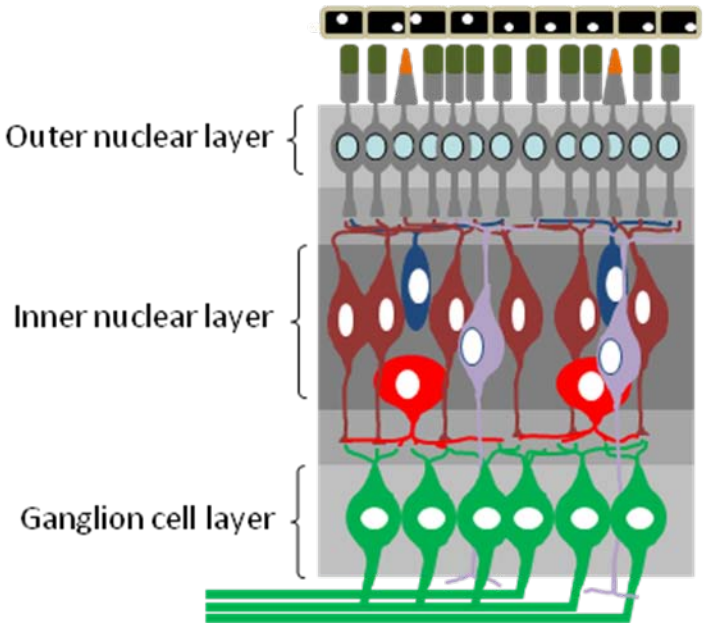
1.3.1 Structure

The retina is a sensory system that converts light signals into electrical impulses that are sent to the visual cortex within the brain. In the mature vertebrate retina there are six

neuronal and one glia cell type arranged into 3 histologically distinct nuclear layers that are separated by two plexiform layers (Fig 1-3). The outer nuclear layer is made up of rod and cone photoreceptors, the inner nuclear layer houses the horizontal, amacrine, bipolar and Müller glia, while retinal ganglion cells (RGC) and amacrine cells make up the ganglion cell layer. The visual process begins as light entering the eye is refracted and focused on the center of the retina, the fovea, by both the cornea and the lens where it will be detected by the photoreceptor cells, rods and cones. From the photoreceptors, the signal is relayed by interneurons to the RGCs then onto the brain (Marquardt, 2003; Poche and Reese, 2009)..

1.3.2 Retinal development

The developing neural retina serves as an excellent model for CNS development due to its simple organization, cellular composition and accessibility. The first morphological sign of the vertebrate eye is following the bilateral evagination of the diencephalon resulting in the formation of the optic vesicles (reviewed in Chow and Lang, 2001). The optic vesicle will extend outward until it reaches and makes contact with the overlying surface ectoderm where communication between the two tissues will result in the formation of the lens placode through ectodermal cell thickening (Fig 1-4) (reviewed in Fuhrmann, 2010). Coordinated invagination of the lens placode and optic vesicle results in the formation of the lens vesicle, which will ultimately give rise to the lens, and a double-layered optic cup. The inner layer of the optic cup will form the neural retina while the outer layer will form the retinal pigmented epithelium (RPE) (Fig 1-4) (reviewed in Chow and Lang, 2001). Within the retina the generation of the various cell types follows a highly conserved order across most vertebrate species. Typically, cell differentiation begins in the central region of the optic cup with the



RPE

Cone photoreceptors

Rod photoreceptors

Horizontal cells

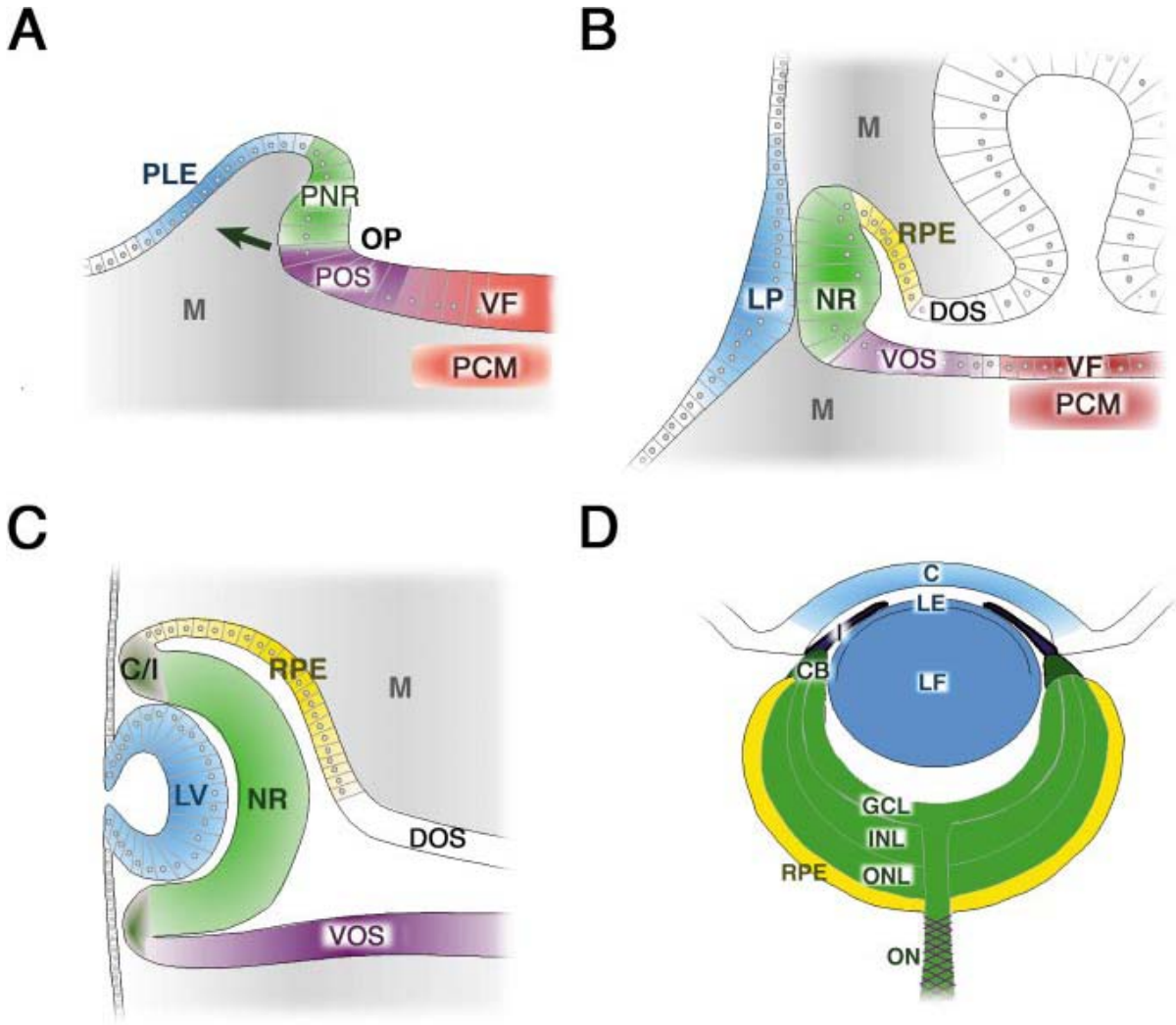
Bipolar cells

Muller Glia

Amacrine cells

Ganglion cells

1-3: Mature mouse retina. The mature retina consists of 6 neural and 1 glial cell type arranged into 3 distinct cellular layers. Neurons consist of ganglion, amacrine, bipolar, horizontal, rod photoreceptor and cone photoreceptor cells . Muller cells are a glial cell type. The ganglion cell layer consists of ganglion cells and displaced amacrine cells (not shown), the inner nuclear layer consists of amacrine, bipolar and Müllers cells while rod and cone photoreceptors are found in the outer nuclear layer.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RPE
M
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NR
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VOS
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I
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INL
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ON

Figure 1-4: Retinal Development. (A-D) , presumptive or differentiated eye tissues are color-coded in the following manner; blue, lens/cornea; green, neural retina; yellow, retinal pigmented epithelium (RPE); purple, optic stalk; red, ventral forebrain/prechordal mesenchyme; grey, mesenchyme. (A) Evagination of the presumptive forebrain results in the formation of the optic pit (OP) which contain the presumptive neural retina (PNR), presumptive ventral stalk (POS), presumptive lens ectoderm (PLE) and RPE (not shown), and the proximo-ventral region, which gives rise to the presumptive ventral optic stalk (POS); PLE, presumptive lens ectoderm; M, mesenchyme; VF, ventral forebrain; PCM, prechordal mesoderm. (B) Continued growth of the optic vesicle culminates with a period of close contact between the lens placode (LP) and the presumptive neural retina (NR) during which important inductive signal likely exchange :RPE, presumptive retinal pigmented epithelium; VOS, ventral optic stalk; DOS, dorsal optic stalk. (C) Invagination of the optic vesicle results in the formation of the lens vesicle (LV) and neural retina (NR) and establishes the overall structure of the eye. The point at which the neural retina and RPE meet gives rise to components of the ciliary body and iris (C/I). (D) Organization of the mature eye: C, cornea; LE, lens epithelium; LF, lens fiber cells; I, iris; CB, ciliary body; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; ON, optic nerve. Reproduced with the permission of Copyright Clearance Center on behalf of Annual Reviews.

appearance of the retinal ganglion cells (RGCs) followed in overlapping phases by horizontal, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells and ending with Müller glia cells (Young, 1985). From the central retina, differentiation will progress concentrically in a wave-like fashion until reaching the peripheral edges of the retina (McCabe et al., 1999; Young, 1985).

Cell lineage tracing studies have revealed that each retinal cell type arises from a common multipotent retinal progenitor cell (RPC) (Cepko et al., 1996; Marquardt and Gruss, 2002). The competence model of retinal development has been proposed by Cepko and colleagues to describe how the temporal sequence in cell type specification is achieved by the multipotent progenitor population (Cepko et al., 1996; Livesey and Cepko, 2001). This model dictates that RPCs progress irreversibly through a series of competence states characterized by their ability to only produce a restricted number of cell types at each stage of retinogenesis and that RPCs cannot be made to move precociously to a later competence state or to revert to an earlier competence state (Cepko et al., 1996; Livesey and Cepko, 2001). This model is consistent with the data from heterochronic cell mixing experiments that revealed stage-specific restrictions of RPC potential (Belliveau and Cepko, 1999; Belliveau et al., 2000). The exact mechanisms that control the progression of RPC through the different competence states remains unknown but likely reflects changes in intrinsic properties (Belliveau et al., 2000; Cherry et al., 2009; Cherry et al., 2011; Watanabe and Raff, 1990). A large body of work also exists demonstrating the importance of cell extrinsic mechanisms on the regulation of RPC cell proliferation and diversification. These include the Delta-Notch (Austin et al., 1995; Jadhav et al., 2006; Muto et al., 2009; Yaron et al., 2006), BMP (Du et al., 2010; Dudley et al., 1995), retinoic acid (Kelley et al., 1995; Levine

et al., 2000) and the Hh (Jensen and Wallace, 1997; Shkumatava et al., 2004; Wang et al., 2005; Wang et al., 2002; Zhang and Yang, 2001) signaling pathways. While both intrinsic and extrinsic factors have been shown to influence the process of cell fate determination during retinal development (reviewed in Belliveau and Cepko, 1999; Cayouette et al., 2006; Livesey and Cepko, 2001; Wallace, 2008; Yang, 2004), which factors involved in these processes remains unclear.

1. 3.3 Hh signaling during mouse retinal development

Of the three Hh isoforms, only Shh has been identified as functionally important during mammalian retinal development. First expressed in RGCs around E12.5 in mice, the downstream target of the pathway, *Gli1*, can be detected by E13.5 by *in situ* hybridization (ISH) in the underlying RPCs (Fig 1-5) (Jensen and Wallace, 1997). *In vitro* studies have shown that ectopic Hh pathway activation in these cells results in an increase in proliferation (Jensen and Wallace, 1997; Wall et al., 2009; Wang et al., 2002; Yu et al., 2006), while *in vivo* ablation of Shh results in a decrease in proliferating progenitor cells, an accelerated rate of cell cycle exit and a decrease in retinal thickness (Wang et al., 2005). The Shh pathway is also involved in regulating RPC differentiation. Loss of Shh during retinal development results in an overproduction of RGC and rod photoreceptors while bipolar and Müller cell numbers are decreased (Wang et al., 2005). These observations were consistent with those demonstrating ectopic activation of the Hh pathway *in vitro* resulted in an increased production of Müller and bipolar cells at the expense of photoreceptor development (Wall et al., 2009; Wang et al., 2005; Yu et al., 2006).

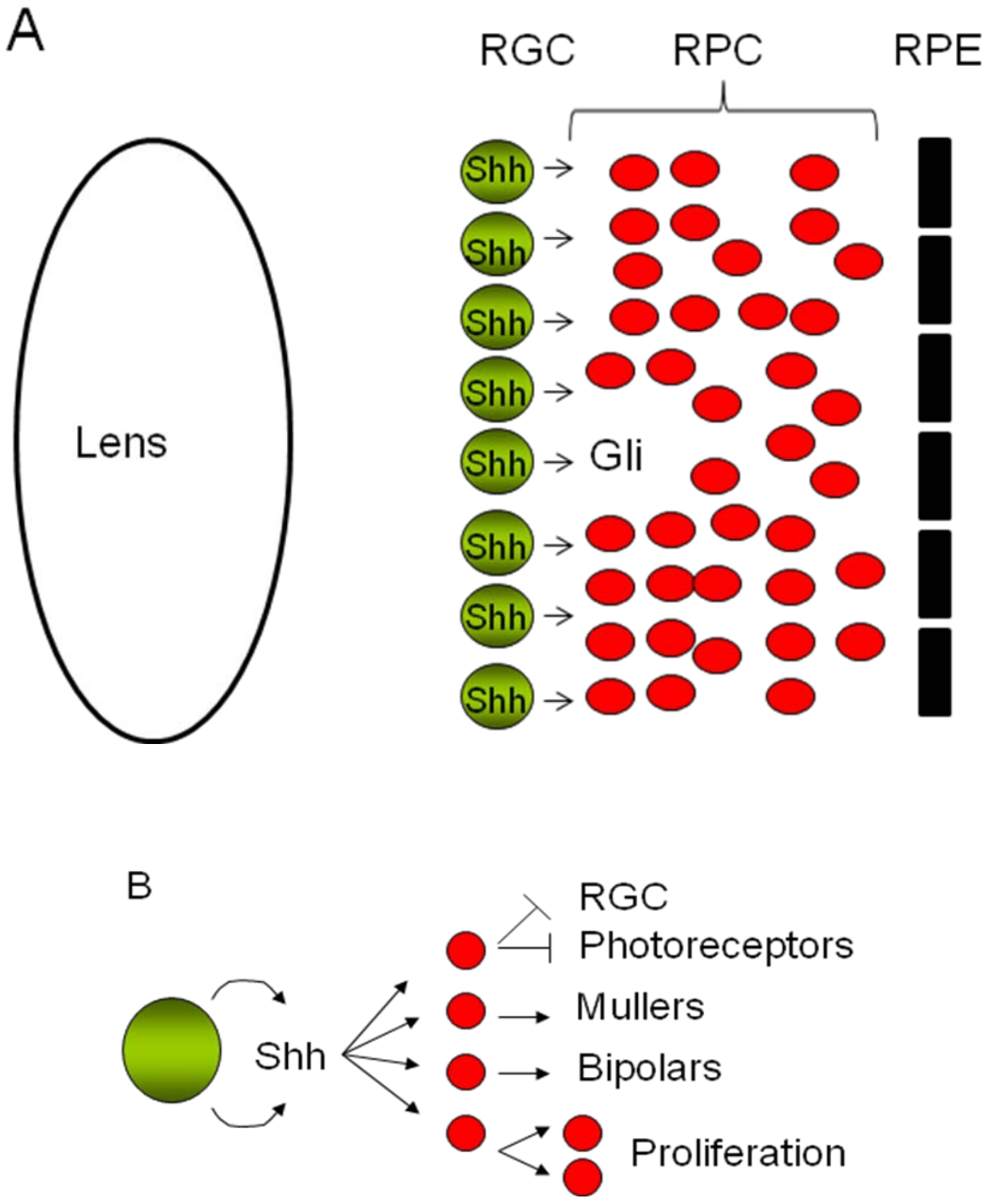

A
RGC
RPC
RPE
Lens
Shh
Shh
Shh
Shh
Shh
Gli
Shh
Shh
Shh
B
Shh
RGC
Photoreceptors
Mullers
Bipolars
Proliferation

Figure 1-5. Summary of the function of Shh in retinal development. (A) Shh secreted from the RGCs (green) targets the RPC (red) and regulates the expression of target genes through the activity of the Gli transcription factors. (B) Effects of Shh on RPC include inhibition of RGC and photoreceptor differentiation, promotion of Muller and bipolar cell production, and promoting RPC proliferation. RGC, retinal ganglion cell; RPE, retinal pigmented epithelium; RPC, retinal progenitor cell.

Other processes involving Shh in the retina include retinal lamination and axon guidance (Dakubo et al., 2003; Kolpak et al., 2005; Shkumatava et al., 2004; Wang et al., 2002). The retina is organized into 3 distinct cellular layers separated by two plexiform layers (Fig 1-3). *In vitro*, loss of Hh signaling results in rossetting in the photoreceptor layer that can be significantly reduced following media supplementation with a Hh agonist (Wang et al., 2002). *In vivo*, conditional inactivation of Shh results in an accelerated photoreceptor differentiation, rossetting and disorganization of the outer nuclear layer (Shkumatava et al., 2004; Wang et al., 2005; Wang et al., 2002). In axon guidance, both *in vivo* and *in vitro* data demonstrates that Shh can influence the growth and trajectory of RGC axons to the optic disc to the center of the retina, in which they exit the eye to join the optic nerve (Dakubo et al., 2003; Gordon et al., 2010; Kolpak et al., 2005; Sanchez-Camacho and Bovolenta, 2008).

RT-PCR analysis has detected the expression of Dhh in the retina (Takabatake et al., 1997), but no role has been identified. ISH demonstrated the Ihh expression in the periocular mesenchyme and retinal pigmented epithelium (RPE) surrounding the retina (Dakubo et al., 2008). Loss of Ihh results in a decrease in RPC proliferation and a defect in photoreceptors, but as Ihh does not appear to signal directly to the neural retina, these effects are believed to be indirect (Dakubo et al., 2008).

1.3.5 Hh target genes in the retina

The response of a cell following Hh pathway activation is mainly the result of genetic regulation by the Gli TF. The number and identity of functionally relevant target genes in discreet Hh-responsive tissues is an area of active investigation. High-throughput methods, such as ChIP-chip, have identified thousands of potential Gli targets in systems

such as the developing limb (Vokes et al., 2008) and cerebellum (Lee et al., 2010). In the retina, several Hh target genes have been identified, including *Gli1*, *Ptc*, *CcnD1*, *CcnA2*, *CcnB1*, *Cdc25B*, *Hes1* and $p57^{Kip2}$ (Locker et al., 2006; Mu et al., 2005; Shkumatava and Neumann, 2005; Wall et al., 2009; Wang et al., 2005; Yu et al., 2006). As all the effects of Hh on RPCs cannot be explained by these genes, an objective of my PhD project was to identify novel targets of the pathway to provide insight into how Hh mediates these effects on RPCs. Using a computational approach (discussed in Chapter 2), of the 390 targets identified, *Sox8* and *Ndp* were selected for characterization during retinal development. These two genes were considered good candidates based on preliminary data demonstrating a strong transcriptional response following Hh pathway activation and their reported effects during retinal development.

1.4 *Sox8* and *Ndp*, novel targets of the Hh pathway

1.4.1 *Sox8* (*Sry-like high-mobility-group box 8*)

Sox proteins form a large family of transcription factors that are related by a DNA-binding domain known as the HMG box. In mouse and humans this family consists of 20 members (Schepers et al., 2002) that are predominantly expressed during embryonic development and have been implicated in many developmental processes and human congenital diseases (Akiyama et al., 2007; Chan et al., 2003; Hargrave et al., 2000; Schlierf et al., 2007). The Sox proteins are organized into eight groups from A-H (Bowles et al., 2000; Schepers et al., 2002; Wegner, 1999). The homology within a group tends to be high (~70-95%) both within and outside the HMG box, whereas Sox proteins from different

groups tend to only share ≤ 46% identity (Lefebvre et al., 2007). Like other HMG domain proteins, Sox factors contain a 79-amino-acid, DNA-binding HMG domain that enables them to regulate gene transcription (reviewed in Wegner, 1999). However, in contrast to most other HMG domain proteins which bind DNA with little or no sequence specificity, Sox proteins are able to bind DNA with some specificity for the consensus motif, 5`-WWCAAWG-3' (Denny et al., 1992; Harley et al., 1992; van de Wetering et al., 1991). Sox proteins bind the minor groove of the DNA and induce a DNA bend that ranges from as little as 30° up to as much as 110° (Connor et al., 1994; Kiefer, 2007; Weiss, 2001; Werner et al., 1995). As with other specific DNA binding HMG proteins like Tcf/Lef-1, this bend could allow transcription factors bound to non-adjacent sites on either sides of the HMG protein to interact with each other and thereby form an active transcriptional complex (Lefebvre et al., 2007). Therefore, it is likely that the role of Sox proteins is at least partly architectural. A number of Sox proteins contain transactivation and repressor domains C-terminal to the HMG box (Lefebvre et al., 2007). It is believed that these domains, activator or repressor, serve as an interface for protein-protein interaction and recruit the co-factors necessary to achieve transcriptional regulation (Bernard and Harley, 2010; Wissmuller et al., 2006).

Sox8 falls within the Sox E group that also contains *Sox9* and *Sox10*. During embryonic development the SoxE proteins typically share overlapping expression patterns and some degree of functional compensation (Barrionuevo and Scherer, 2010; Hong and Saint-Jeannet, 2005; Stolt et al., 2004). *Sox8* KO mice exhibit idiopathic weight loss, reduced bone density and progressive loss of Sertoli cell function, resulting in compromised fertility in aging males (O'Bryan et al., 2008; Sock et al., 2001). Recently, *Sox8* has been implicated in retinal development. Muto et al., (2009) have demonstrated, using an explant

system, that knocking down *Sox8* resulted in a reduction in the number of Müller glial cells and an increase in photoreceptors generation after 14 days in culture, but had no effect on cell proliferation or survival. As both *in vivo* and *in vitro* loss of Shh signaling studies have shown to result in an overproduction of photoreceptors and a decrease in Müller cells (Shkumatava and Neumann, 2005; Wang et al., 2005; Yu et al., 2006), it is possible that *Sox8* could be a downstream target of the Shh pathway involved in regulating these processes.

1.4.2 *Ndp* (*Norrie disease pseudoglioma*) gene

The *NDP* gene is located on the short arm of the X chromosome (position Xp11.4), spans 28kb and contains 3 exons (Berger et al., 1992a; Chen et al., 1992; Sims et al., 1992). The open reading frame begins in exon 2, ends in exon 3 and encodes for the protein Norrin. Over 80 mutations have been reported in this gene that cause a variety of human eye diseases all characterized by varying degrees of vascular defects that lead to visual impairment (Aponte et al., 2009; Riveiro-Alvarez et al., 2005; Schuback et al., 1995; Wu et al., 2007). These diseases include Coat's disease (OMIM 300216) (Black et al., 1999), Retinopathy of prematurity (ROP; OMIM 133780) (Allen et al., 2006; Dickinson et al., 2006; Haider et al., 2002; Shastry et al., 1997), Familial Exudative Vitreoretinopathy (FEVR; OMIM 133780) (Pelcastre et al., 2010; Shastry, 1998; Torrente et al., 1997) and Norrie disease (ND; OMIM 310600) (Berger et al., 1992a; Berger et al., 1996; Fuchs et al., 1996). The pathology of these diseases include an aberration of retinal development associated with varying degrees of peripheral retina avascularity, abnormal vascularization, subretinal exudation, abnormal vitreous composition and vitreoretinal interface, and retinal detachments (Wu et al., 2007).

Norrie disease patients can also suffer from progressive hearing loss, delayed motor skills, mental retardation and behaviour abnormalities (Lev et al., 2007)

In mice, the expression of *Ndp* by ISH has been reported as low and evenly distributed from E12.5 to E18.5 throughout the entire embryo, except for the olfactory bulb where the expression is markedly higher (Berger et al., 1996). Within the retina, high levels were also reported in postnatal animals in the inner nuclear layer and the ganglion cell layer (Berger et al., 1996). Interestingly, *Ndp* expression during mouse development analyzed from a knock-in mouse that carries the coding sequence of human placental alkaline phosphatase (AP) inserted at the *Ndp* locus offers a different retinal expression pattern (Ye et al., 2010; Ye et al., 2009). In the knock-in mice, no signal is detected in the retina during early embryonic (E) development, but by E15.5 staining is observed at the optic disc which persists until postnatal day (P) 0 (Ye et al., 2010). By P0 and P3, AP staining can be observed throughout the retina which the authors describe as Müller cells (Ye et al., 2010; Ye et al., 2009). From P4 into adulthood, AP staining spanned the full thickness of the retina which is indicative of the expression in Müller glia (Ye et al., 2009).

1.4.3 Norrin, a cystine-knot protein

Norrin is a secreted, 133 amino acid protein with an N-terminal signal sequence and a C-terminal cystine-knot motif (Meitinger et al., 1993). While Norrin does not show strong sequence homology with any other known protein, the hydrophobic residues and spacing of the cystine residues resemble those at the C-terminus of the von Willerbrand factor and many secretory mucins (Meindl et al., 1992; Meitinger et al., 1993). As with other cystine-knot motif protein, which have a characteristic of forming disulfide-linked dimers, it has been

shown that Norrin forms oligomers that bind to the extracellular matrix (Perez-Vilar and Hill, 1997). The dimerization of these types of proteins is usually required to carry out specialized functions, such as binding to specific receptors (Bell et al., 2001; Isaacs, 1995). While mutations have been reported throughout the *NDP* gene leading to multiple diseases, only protein truncation mutations and mutations within the cystine-knot motif have been linked to ND; no other genotype:phenotype correlation has been made (Berger et al., 1992b; Schroeder et al., 1997; Wu et al., 2007).

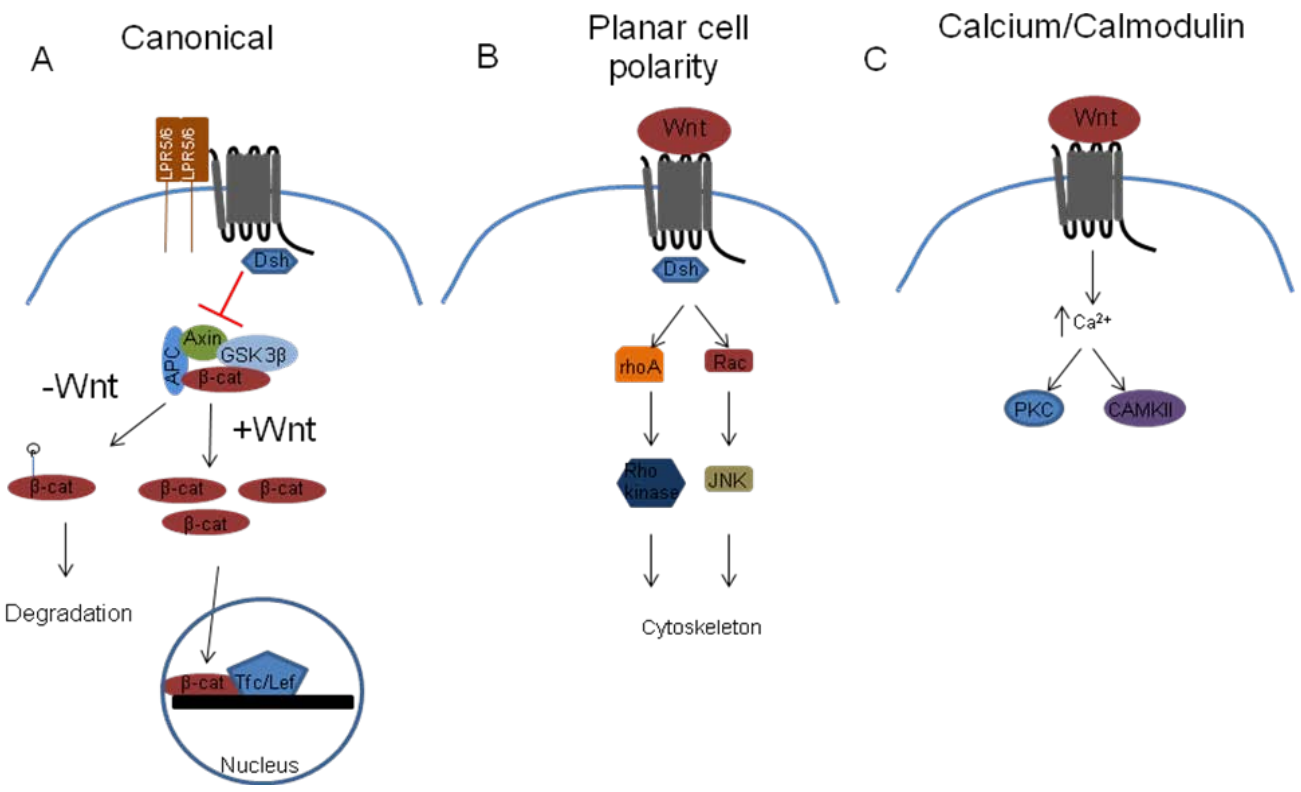
1.4.4 Norrin; vascular defects

In humans and mice, primary vascular development in the eye begins when vessels originate from the optic nerve head and spread over the surface of the retina to form a dense network (Fruttiger, 2002; Provis, 2001). From the primary vessels along the surface of the developing retina, new vessels form through angiogenesis, the process in which proliferating endothelial cells form new vessel sprouts and extend the vascular network from pre-existing vessels (Fruttiger, 2007). The new vessels will penetrate into the deeper layers of the retina to form two capillary beds that ramify in the inner and outer plexiform layers of the retina (Fruttiger, 2002, 2007; Provis, 2001). In the *Ndp* KO retinas, the vascular abnormalities have been linked to defects in angiogenesis resulting in a dramatic decrease in the number of blood vessels in the inner and outer plexiform layers (Ohlmann et al., 2005; Richter et al., 1998). *Ndp* KO retinas also exhibit defects in the process of hyaloid regression. Normally the hyaloid vessels supply nutrients to the developing eye and regress during early postnatal life as the new vascular network becomes established (Browning et al., 2001; Ito and Yoshioka, 1999). In the absence of *Ndp*, the hyaloid vessels fail to regress and often invade

the avascular retina (Luhmann et al., 2005). Morphological analysis of the *Ndp* KO mice revealed additional non-vascular defects, including fibrous masses within the vitreous body, loss of cells within the ganglion cell layer and an overall disorganization of the three layers of the retina (Berger et al., 1996; Richter et al., 1998) which is consistent with the retinal phenotype of a human with ND (Schroeder et al., 1997).

1.4.5 Norrin and the canonical Wnt signalling pathway

The vascular abnormalities in the ND mouse retina and the striking similarities in the Frizzled (Fzd)- 4 KO retinas has lead to the discovery that Fzd4 is a high affinity receptor for Norrin (Xu et al., 2004). Fzd are normally recognized as receptors for the lipid-modified, cystein-rich glycoproteins called Wnts (Mason et al., 1992). In mammals, there are 19 Wnt and 10 Fzds that through their interaction can activate three main signaling cascades; the planar cell polarity pathway (PCP), the Ca^{2+} pathway and the canonical Wnt/β-catenin pathway (Fig 1-6). For Norrin, the interaction with Fzd4 in conjunction with a co-receptor Lrp5 or Lrp6 and the integral membrane protein Tspan12, is able to activate the canonical Wnt/β-catenin pathway (Junge et al., 2009; Smallwood et al., 2007; Xu et al., 2004). In the absence of ligand, β-catenin is phosphorylated, ubiquitinated and targeted for proteasome mediated degradation by the Axin/APC/GSK3β destruction complex (reviewed in Clevers, 2006). Following ligand interaction with Fzd and the co-receptors, the cytoplasmic phosphoprotein Disheveled (Dsh) is activated, which will inhibit GSK3β activity leading to a stabilization and accumulation of β-catenin in the cytoplasm. Upon reaching a certain cytoplasmic concentration, β-catenin translocates into the nucleus and associates with the Tcf/Lef family of transcription factors to regulate the expression of target genes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Canonical
LPR5/6
LPR5/6
Dsh
Axin
APC
GSK3β
β-cat
-Wnt
+Wnt
β-cat
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β-cat
β-cat
Degradation
β-cat
Tfc/Lef
Nucleus
B
Planar cell polarity
Wnt
Dsh
rhoA
Rac
Rho kinase
JNK
Cytoskeleton
C
Calcium/Calmodulin
Wnt
Ca2+
PKC
CAMKII

Figure 1-6: Wnt signaling pathways. (A) The canonical Wnt/β-catenin signaling pathway. In the absence of Fzd ligand, β-catenin associates with a cytoplasmic complex consisting of APC, CK1, Axin and GSK3β. This association results in the phosphorylation, ubiquitination and subsequent degradation of β-catenin. Ligand interaction with Fzd and Lrp5/6 coreceptors results in the phosphorylation of GSK3β following the activation of Dishevelled (Dsh) that leads to a stabilization of β-catenin. β-catenin enters the nucleus and associates with Tcf/Lef and initiates transcription of target genes. (B) The Planar cell polarity pathway is initiated by ligand-Fzd interaction leading to the activation of rhoA and Rac through Dsh that will induce cytoskeletal rearrangements and gene expression. (C) The Calcium/Calmodulin pathway. Ligand-fzd interaction leads to the intracellular release of Ca2+, decreased cGMP and activation of CamKII (Ca^{2+}-Calmodulin-dependent Protein Kinase-II) and PKC (Protein Kinase-C).

(reviewed in Akiyama, 2000) (Fig 1-6). The role of the canonical Wnt/β-catenin signaling pathway during retina development remains unclear and somewhat controversial between different species. In addition to being involved in vascular development (see above), this pathway has also been shown to be important in the development of the ciliary margin and its non-neural derivatives in both mouse and chick (Cho and Cepko, 2006; Liu et al., 2007).

1.4.6 Potential for non-vascular effects in the retina

While the involvement of *Ndp* in retinal angiogenesis has been clearly established, there is evidence that *Ndp* may also have a non-vascular role during retinal development. Firstly, the onset of *Ndp* expression far precedes the commencement of vascular development in the retina. Berger et al., (1996) demonstrated an evenly distributed *Ndp* expression in E15.5 retinas, while vascular development begins ~P3 (Fruttiger, 2007). Secondly, the expression pattern of *Ndp* is evenly distributed during embryonic development and becomes restricted to cells within the ganglion and inner nuclear cell (Berger et al., 1996), suggesting a specific function for this gene within these cells. Thirdly, the retinas of *Ndp* KO mice appear thinner and disorganized and fibrous masses can be located within the vitreous body (Berger et al., 1996; Schroeder et al., 1997). And finally, when Ohlmann et al., (2005) attempted to rescue the vascular defects in the *Ndp* KO animals by ectopically overexpressing *Ndp* under the control of a lens-specific promoter, not only was the vascular network restored but they also noticed an increase in retinal proliferation and thickness both in the *Ndp* KO and wildtype littermates.

1.5 Statement of Hypothesis and Objectives

The Hh signaling pathway plays a key role in the growth and patterning of numerous tissues, is involved in stem cell maintenance and the progression of various cancers. The exact downstream mechanisms governing these various Hh-dependent processes remain largely unknown. As the activity of the Hh pathway is primarily mediated through the Gli transcription factors, the objective of this thesis is to identify and characterize novel Gli targets during retinal development. Through a bioinformatics approach, *Ndp* and *Sox8* were identified as having a Gli binding motif within the promoter region in both human and mouse genomes. As ectopic *Ndp* and *Sox8* knockdown have previously been linked to cell proliferation and Müller cell development in the retina respectively, I hypothesize that these genes function downstream of Gli in regulating these processes.

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Chapter 2

Comparative genomics identification of a novel set of temporally regulated Hedgehog target genes in the retina

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2.1 Co-authorship statement:

This project was carried out under the supervision of Dr. Valerie Wallace. Brian McNeill generated the data for Figures 2-1, 2-2, 2-3, 2-4, Table 1 and Supplemental Figures 1 and 2. The computation analysis, Supplemental Table 2-2, was initiated by Dr. Marosh Furimsky and performed by Dr. Carol Perez-Iratxeta and Dr. Miguel A. Andrade-Navarro. *Sox8* KO mice were generated and kindly provided by Dr. Yuji Mishina.

2.2 Abstract

The Hedgehog (Hh) signaling pathway is involved in numerous developmental and adult processes with many links to cancer. In vertebrates, the activity of the Hh pathway is mediated primarily through three Gli transcription factors (Gli1, 2 and 3) that can serve as transcriptional activators or repressors. The identification of Gli target genes is essential for the understanding of the Hh-mediated processes. We used a comparative genomics approach using the mouse and human genomes to identify 390 genes that contained conserved Gli binding sites. Q-PCR validation of 46 target genes in E14.5 and P0.5 retinal explants revealed that Hh pathway activation resulted in the modulation of 30 of these targets, 25 of which demonstrated a temporal regulation. Further validation revealed that the expression of *Bok*, *FoxA1*, *Sox8* and *Wnt7a* was dependent upon Sonic Hh (Shh) signaling in the retina and their regulation is under positive and negative control by Gli2 and Gli3, respectively. We also show using chromatin immunoprecipitation that Gli2 binds to the *Sox8* promoter, suggesting that *Sox8* is an Hh-dependent direct target of Gli2. Finally, we demonstrate that the Hh pathway also modulates the expression of *Sox9* and *Sox10*, which together with *Sox8* make up the SoxE group. Previously, it has been shown that Hh and SoxE group genes promote Müller glial cell development in the retina. Our data are consistent with the possibility for a role of SoxE group genes downstream of Hh signaling on Müller cell development.

2.3 Introduction

The Hh signaling pathway plays an essential role in growth and patterning of numerous tissues and organs during embryonic development. This pathway is also highly relevant to human health, as aberrant Hh signaling is associated with congenital abnormalities and cancer in humans (Stecca and Ruiz, 2010). One major function of this pathway is to control progenitor proliferation and fate determination in several regions of the CNS, including the retina (Marti and Bovolenta, 2002). During vertebrate retinal development, the secreted Sonic hedgehog (Shh) ligand is produced by the ganglion cells (GCs), the first neurons to differentiate in the retina, and signals to the pool of multipotential retinal progenitor cells (RPC) throughout retinal neurogenesis, which in mouse spans the period between embryonic day 11 to postnatal day 7 (Amato et al., 2004). The major functions of Shh signaling in the rodent retina include the maintenance of RPC proliferation and self renewal (Sakagami et al., 2009; Wang et al., 2005) and the regulation of cell fate by inhibiting GC development at embryonic stages and promoting interneuron and glial cell development postnatally (Wang et al., 2005; Zhang and Yang, 2001). A number of Hh targets in RPCs have been identified in several vertebrate species, including *p57* (Shkumatava and Neumann, 2005), *CyclinD1* (Locker et al., 2006; Wang et al., 2005) and *Hes1* (Wall et al., 2009); however, the latter two genes are not sufficient, at least in the mouse retina, to explain all of the stage-dependent effects of Hh on cell fate and proliferation. Since Hh function in RPCs requires Gli2 (Wall et al., 2009), a primary mediator of the transcriptional output of the Hh pathway (see below), the identification of additional Gli target genes is one avenue for characterizing the Hh response in RPCs.

Initiation of the Hh signaling cascade begins with ligand binding to the transmembrane receptor Patched (Ptch) which relieves suppression of the seven-transmembrane protein Smoothened (Smo) promoting its translocation to the plasma membrane and the primary cilium (Goetz et al., 2009). This results in the recruitment of the Gli transcription factors (TF) to this structure, followed by their nuclear translocation and transcriptional activation (Jiang and Hui, 2008). In vertebrates, there are three Gli TF proteins (Gli1, 2 and 3) that serve as the main mediators of the transcriptional output of the Hh pathway (Hui et al., 1994). In the absence of Hh signaling, Gli3, and to a lesser extent Gli2, undergo proteasome-mediated carboxyl cleavage to repressor forms that silence Hh target genes (Jiang and Hui, 2008; Stecca and Ruiz, 2010). Hh pathway activation prevents this cleavage resulting in the accumulation of full length activator forms of Gli2 and to a lesser extent Gli3 (Jiang and Hui, 2008; Stecca and Ruiz, 2010). Gli1 is not cleaved and therefore acts only as a transcriptional activator, but is believed to mainly serve to enhance the activity of Gli2 (Park et al., 2000). All three mammalian Gli TF bind to a Gli consensus motif GACCACCCA (Kinzler and Vogelstein, 1990) and it is thought that the levels of activators/repressors and their DNA binding is what determines the transcriptional output of the pathway. Notably, Gli1 is a transcriptional target of Hh signaling and provides a sensitive readout of Hh pathway activity (Goodrich and Scott, 1998).

Discovering the regulatory targets of these transcription factors could help understand the involvement of this pathway in the numerous processes that they participate in from tissue developmental to cancer progression. We used a comparative genomics approach to identify direct targets of the Gli TFs. Because conservation of noncoding DNA between genomes is a good predictor of regulatory elements and has been used to discover transcription factor

binding sites (Meireles-Filho and Stark, 2009) we used this approach to identify genes with conserved Gli binding sites in the human and mouse genomes. Our study identified 390 candidate Gli target genes including genes previously linked to Hh signaling and, more importantly, unlinked genes that could potentially play important roles in Hh signaling. Validation was performed on 46 genes in Hh gain and loss of function assays in RPCs. We demonstrate that the expression of 30 of these genes is significantly altered in response to Hh pathway activation. Interestingly, 25 of these 30 modulated genes exhibit a temporal dependence for Hh induction in RPC. Consistent with Gli binding site criteria for identification, we show that both Gli2 and Gli3 are involved in regulating the expression of *Bok, FoxA1, Sox8* and *Wnt7a*. Finally, we demonstrate that the Hh pathway activates three members of the SoxE group, *Sox8*, *Sox9* and *Sox10*, which links this pathway and *Sox* genes in the regulation of Müller glial cell development.

2.4 Materials and Methods

Computational identification of Gli target genes

To identify potential Gli target genes we searched the human genome (version hg15) for the presence of the Gli-binding motif sequence (GACCACCCA; Kinzler and Vogelstein, 1990) or a sequence with a single mismatch within an interval that spanned 2kb up and downstream of the transcription start and stop sites. The resulting gene hits were filtered by searching the mouse genome for orthologues containing a conserved Gli binding site 4kb up and downstream of the transcript. The list was further filtered by excluding all genes that did not have an EST expressed in the CNS in NCBI's database.

Experimental Animals

All animal procedures were approved by the University of Ottawa Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines. The *α-Cre* transgenic mice were obtained from P. Gruss (Marquardt et al., 2001) and were maintained on a C57BL/6 background. Mice heterozygous for a *Shh* null allele ($Shh^{+/-}$) (St-Jacques et al., 1998) and homozygous for the conditional *Shh* allele ($Shh^{c/c}$) (Lewis et al., 2004) were maintained on a C57BL/6 background. α-*Cre*;$Shh^{+/-}$ mice were crossed to $Shh^{c/c}$ mice to generate α-*Cre*;$Shh^{-/c}$ mice (referred to as Shh^{CKO}) and were compared with α-*Cre*;$Shh^{+/c}$ littermates (referred to as wildtype). *Gli1* and *Gli2* alleles contain a deletion of the zinc finger domain (Mo et al., 1997) and the $Gli3^{xt}$ allele contains a large deletion of the 3'end of the gene (Hui and Joyner, 1993). The *Gli1* and *Gli2* KO mice were maintained on CD1 background and *Gli3* KO on a C57BL/6. Explants derived from *Gli* KO animals were compared to wildtype littermates. Unless otherwise stated, wildtype retinal explants were derived from C57BL/6 mice (Jackson Laboratory). The embryos were genotyped using tail tissue and a polymerase chain reaction protocol with gene specific primers. Mice were coupled in the late afternoon and allowed to mate overnight. The next morning, males were removed from the cage and females were checked for vaginal plugs. The presence of the vaginal plug was considered as embryonic day 0.5 (E0.5)

Retinal explants

Mice were continuously mated or time-mated to generate tissue of the appropriate age. Retinal explants were generated and cultured, as previously described (Wang et al., 2005). Briefly, eyes were dissected from E14.5 and postnatal day 0.5 (P0.5) mice, the lens

and RPE removed, the retinas were flattened onto polycarbonate filters (0.8 μm pore size, Nucleopore) and placed in culture medium for 3 days *in vitro* (DIV) at 8% CO_2 and 37^{o}C. Explants were cultured in 24-well plates 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 (25μ g/ml)]. The cultures were supplemented with a Smoothened agonist (Hh-Ag) (kind gift from Curis Inc) at 20nM (Frank-Kamenetsky et al., 2002). Explanted retinas that were kept in culture in the absence of Hh-Ag were used as controls. Cell autonomous Hh pathway activation in explants was accomplished using an electroporation technique that efficiently transfect RPC (Matsuda and Cepko, 2004). Briefly, retinas electroporated at a 10:1 ratio with expression vectors (or empty vector) (1 μg/μl) and pUb-GFP in 2-mm gap cuvettes (VWR) using 5 square 30V pulses of 50-ms duration and 950-ms intervals using a ECM830 pulse generator (BTX Harvard Apparatus). The DNA plasmids used in this study were *SMO-M2* (a gift from G. Fishell, New York University Langone Medical Center, New York, NY), *Sox8* (Open Biosystems), shRNA targeting *Sox8* (Open Biosystems), double-stranded shcontrol (Invitrogen) and pUb-GFP (a gift from T. Matsuda, Harvard Medical School, Boston, MA) to visualize the transfected region.

RNA extraction and RT-qPCR

Total RNA was extracted from freshly dissected retinas or from retinal explants cultured for 3 days *in vitro* (DIV) using Tri ® reagent (Sigma-Aldrich) following the manufacturer's instructions. First strand cDNA was synthesized from 2 μg total DNase I

(Sigma-Aldrich) treated RNA using M-MLV reverse transcriptase (Invitrogen) and random hexamer primers (Fermentas). Target gene mRNA levels were assessed by RT-qPCR using Brilliant® SYBR® Green mastermix (Agilent Technologies) and a MX3000P multiplex QPCR system (Agilent Technologies). PCR conditions were carried out as outlined by the manufacturer, except that the reaction volume was scaled down from 50 µl to 20 µl. The primer pairs (Supplemental Table 2-1) were designed using DNAMAN software (Lynnon Biosoft) and primer3 (v. 0.4.0) (Rozen and Skaletsky, 2000). Relative changes in mRNA expression of target genes were determined using the Δ-ΔCt method normalized against *18S* and *Gapdh*. Statistical significance was determined using a two-tailed Student's t-test.

Chromatin Immunoprecipitation

Retinal explants treated with or without Hh-Ag for 3 DIV were fixed in cold 4% paraformaldehyde (PFA) solution for 30 min. The DNA was sheared to less than 1 kb by sonication. Chromatin Immunoprecipitation (ChIP) was performed using the EZ ChIP kit (Millipore) on E14.5 and P0.5 retinal explants according to the manufacturer's instructions. Immunoprecipitations were performed using a goat anti-Gli2 polyclonal antibody (Santa Cruz Biotechnology Inc) or a goat anti-Brn3b (Santa Cruz Biotechnology Inc) as control. DNA was analyzed by qPCR with Brilliant® SYBR® Green mastermix (Agilent Technologies) using primer sets that span the Gli consensus sequence in the *Sox8* promoter or primers targeting the β-actin promoter (control) (Supplemental Table 2-1). To calculate the enrichment of Gli2 at a binding site, the ΔCt values obtained (using the standard curve method) for each target were divided by the amount of the corresponding target in the input fraction. Values were normalized against control IPs performed in parallel using goat IgG

and values are expressed as fold enrichment of Hh-Ag-treated compared to untreated explants. Standard deviation were estimated from three independent experiments, and statistical significance was assessed in each case with a two tailed Student's t-test.

In situ hybridization (ISH)

Tissue preparation for ISH was performed as previously described (Wallace and Raff, 1999). Briefly, explants were fixed in 4% PFA in PBS for 1h at RT while eyes were fixed overnight at 4^{o}C. Following fixation, samples were washed with PBS and incubated in 30% sucrose overnight at 4^{o}C. Samples were equilibrated in a 30% sucrose OCT mix (1:1 ratio) overnight prior to embedding, freezing and sectioning using a Leica 1850 cryostat. 12µm sections from explants through the center of the tissue, transverse sections from the eye, were collected onto Superfrost plus slides (VWR) and dried overnight at RT. ISH was performed as previously described (Wang et al., 2005) using Digoxigenin (DIG)-labeled antisense RNA riboprobes prepared by *in vitro* transcription from linearized plasmids containing complete or partial cDNA sequences for *Sox8*, *Sox9* and *Gli1*. Briefly, sections were hybridized overnight at 65^{o}C in a humidified chamber, washed stringently and incubated with an alkaline phosphatase-conjugated anti-DIG antibody. Staining was performed using Nitro blue tetrazolium (NBT; Roche) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche) and analyzed on an Axioplan microscope and digital images were captured using an AxioVision camera 2.05 (Zeiss).

2.5 Results

Computational prediction of Gli target genes

To identify candidate Gli target genes computationally we searched the human and mouse genomes sequentially for genes containing a conserved 8/9 or 9/9 match to the Gli binding sequence (GACCACCCA; Kinzler and Vogelstein, 1990) in defined intervals flanking the transcript (see Methods for details). The search identified 390 genes (Supplemental Table 2-2) and included known Gli targets, such as *Ptch*, *Rab34* (Vokes et al., 2007), *Stmn1* (Chung et al., 2010) and *Nkx2* (Briscoe et al., 1999). The list of genes was prioritized for validation studies on the basis of function (proliferation, differentiation, survival) and role in eye development. Based on these criteria, we selected 41 genes and an additional 5 genes (*p57, Cdc25, Ccnna2, Pvrl1, Pvrl3*) for which binding sites were only identified in the human orthologue, for further analysis (Table 2-1).

To investigate the Hh regulation of these candidate genes, we used the retinal explant culture model. Several aspects of retinal neurogenesis, including cell fate specification and neuronal differentiation, are recapitulated in explants with the exception that Shh and Hh target gene expression are not maintained because the GCs, the source of Shh, undergo apoptosis in these cultures (Wang et al., 2002). However, Hh signaling, and target gene expression can be restored in explants by *in vitro* treatment with a Hh agonist (Hh-Ag) (Frank-Kamenetsky et al., 2002; Wang et al., 2002). Therefore, by comparing gene expression in Hh-Ag treated versus untreated (control) explants, we can determine the response of candidate genes to Hh pathway activation in primary RPCs. We cultured retinal explants from E14.5 and P0.5 mice for 3 days in the presence or absence of Hh-Ag and

analyzed gene expression by RT-qPCR. Hh pathway activation was confirmed by the upregulation of *Gli1* (data not shown) and *Ptch* in the Hh-Ag treated explants (Table 2-1). Of the 46 genes analyzed, the expression of 30 genes was modulated by Hh-Ag treatment in at least one temporal condition (asterisk marks in Table 2-1).

One of the most interesting trends we noted was that the expression in 25 of these genes was temporally regulated by Hh signaling (Table 2-1); where the change in gene expression was only significant at one age or where the change in expression between the two stages was significantly (≥ 2 fold) different (shaded rows in Table 2-1). For example, the expression of *Sox8* and *HeyL* were significantly increased by Hh-pathway activation only at P0.5, whereas the expression of *Hpcal4* and *Tef* was increased only at E14.5 (Table 2-1). The expression of *Bok* and *Terf1* was increased by Hh-Ag treatment at both stages but not to the same degree (Table 2-1). It is unlikely that these Hh-mediated temporal effects on gene expression are secondary to differences in cellular composition in the explants, as the proportion of RPCs, the primary Hh target population, was not significantly different in Hh-Ag-treated explants at E14.5 or P0.5 (Ki67 at 3DIV, E14 73.2% +/- 3.1 SEM, n=6; P0 67.1% +/- 2.5 SEM, n=6). Another possibility to account for temporal changes in gene expression is differences in the baseline level of gene expression in the control (untreated) explants at the two different ages. However, we could observe some genes with very stable temporal expression (differences in the Δ-Ct values, normalized gene expression to *18S* and *Gapdh*, between control samples at each age ≤ 1.5) and large temporal difference in induction (≥ 3 fold), such as *Bok*, *HeyL*, *Sox8* and *Wnt10b*.

Table 2-1. Change in gene expression following Hh pathway activation

		E14.5			P0.5		
Symbol	**Accession**	**Fold change**	**SEM**		**Fold change**	**SEM**	
Amn	NM_033603	4.5	1.4	*	-3.8	0.1	*
Bok	NM_016778	12.9	1.0	*	2.4	0.8	
Ccr10	NM_007721	2.0	0.7		-1.1	0.2	
Cdc25	NM_007658	1.8	0.1	*	1.5	0.5	
Csnk1g2	NM_134002	1.7	0.4		1.5	0.4	
Ccnna2	NM_145732	-1.8	0.1		-1.1	0.2	
Dscam	NM_031174	-2.4	0.1	*	-3.3	0.1	*
E2F2	NM_177733	1.1	0.4		2.6	0.7	
Egr2	NM_010118	1.1	0.5		4.2	1.1	*
FoxA1	NM_008259	4.4	1.5	*	6.8	1.3	*
Hap1	NM_010404	-1.4	0.1		1.1	0.2	
Heyl	NM_010423	-1.0	0.3		13.2	2.7	*
Hpcal4	NM_174998	4.5	1.8	*	1.4	0.5	
Inha	NM_010564	-1.1	0.3		-1.2	0.3	
Ldb2	NM_010698	1.6	0.6		5.4	0.9	*
Lifr	NM_013584	-2.1	0.0	*	2.2	0.8	
Mmp11	NM_008606	1.1	0.1		3.5	0.7	*
Mmp24	NM_0108018	-1.5	0.2		-1.3	0.2	
Nrcam	NM_176930	-1.1	0.1		4.1	0.9	*
Ogfr	NM_031373	-1.1	0.1		1.9	0.3	
Opn1lw	NM_013887	3.1	1.8		1.5	0.6	
P57	NM_009876	-3.3	0.1	*	2.3	0.2	*
Phb	NM_008831	1.3	0.2		5.1	1.4	*
Plek2	NM_013738	7.2	1.7	*	8.4	1.1	*
Plgbn	NM_010593	-1.6	0.2		2.8	0.6	*
Pou4F2	NM_138944	1.0	0.4		2.9	0.3	*
Ptch1	NM_008957	15.8	2.4	*	12.6	1.2	*
Ptger3	NM_011196	1.5	0.8		2.0	0.8	
Pvrl1	NM_021424	-2.0	0.1	*	2.4	0.4	*
Pvrl3	NM_021495	1.9	0.4		5.2	0.8	*
Rab34	NM_033475	1.6	0.2		4.1	0.1	*
Rax1	NM_013833	4.5	1.3	*	3.5	1.2	*
Rxra	NM_011305	1.1	0.1		1.1	0.1	
Rxrb	NM_011306	-1.4	0.1		1.7	0.4	
Sox18	NM_009236	2.3	0.6	*	5.6	0.4	*
Sox8	NM_011447	1.3	0.2		28.2	3.8	*
Stat5A	NM_011488	2.4	0.5	*	5.0	0.4	*
Stmn1	NM_019641	1.1	0.4		-1.4	0.4	
Susd2	NM_027890	-1.7	0.3		-1.7	0.2	
Syn1	NM_013680	-1.2	0.4		-1.0	0.3	
Tef	NM_07376	3.4	0.4	*	1.1	0.3	
Terf1	NM_009352	2.4	0.4	*	5.6	0.4	*
Uhrf1	NM_001111078	1.1	0.4		1.0	0.5	
Wnt10b	NM_011718	9.2	2.5	*	-2.6	0.2	*
Wnt11	NM_009519	-1.5	0.2		-2.4	0.1	*
Wnt7a	NM_009527	6.2	0.9	*	1.8	0.7	

Table 2-1. Target gene expression in response to Hh pathway activation in retinal explants. E14.5 and P0.5 retinal explants were cultured with or without Hh-Ag for 3 days and gene expression was analyzed by RT-qPCR. Values represent average fold changes in gene expression in Hh agonist treated explants (n=6 per age) relative to untreated explants (n=6 per age) and are normalized to *Gapdh* and *18S* +/- SEM. Genes that exhibited temporal differences in expression in response to Hh activation are highlighted in gray. Significant differences (* $p \leq 0.05$) between Hh-agonist treated and untreated explants was assessed using a two tailed Student's t-test.

To investigate the Hh-dependence of these target genes we analyzed their expression in retinas of mice with a conditional knockout of *Shh* (*Shh*^{CKO}) (Wang et al., 2005). *Shh* inactivation in the peripheral retina in this strain results in the downregulation of target genes, including *Gli1, Ccnd1 and Hes1,* in this region (Wang et al., 2005). RT-qPCR analysis of whole retinas of *Shh*^{CKO} mice revealed that the expression of known Hh target genes, *Gli1* and *Ccnd1* was reduced in the mutants compared with wildtype mice, demonstrating the sensitivity of this approach to detect specific changes in Hh-dependent gene expression (Fig. 2-1). Based on our findings in retinal explants we compared the expression of 14 Hh-responsive and 9 non-responsive genes (Table 2-1) in control and *Shh*^{CKO} retinas at P0.5. Twelve of fourteen genes that were increased by Hh activation in explants, were downregulated in the *Shh*^{CKO} mutant retina (Fig. 2-1). Unexpectedly, of the group of genes that were characterized as non-Hh modulated in retinal explants, the expression of *Ccr10, Mmp24* and *Syn1* was downregulated and the expression of *E2F2* and *Stmn1* was upregulated in the *Shh*^{CKO} retina (Fig. 2-1). One explanation for the discrepancy between the *in vitro* and *in vivo* regulation of these genes by Hh signaling could be related to compensatory changes *in vivo* that would not be present following the acute loss of Hh function in explants. In summary, we have identified 30 genes whose expression is modulated by acute Hh pathway activation and of this cohort, 12 genes that require Hh signaling for normal levels of expression *in vivo*. In addition, the expression of 25 of these genes was temporally regulated by Hh.

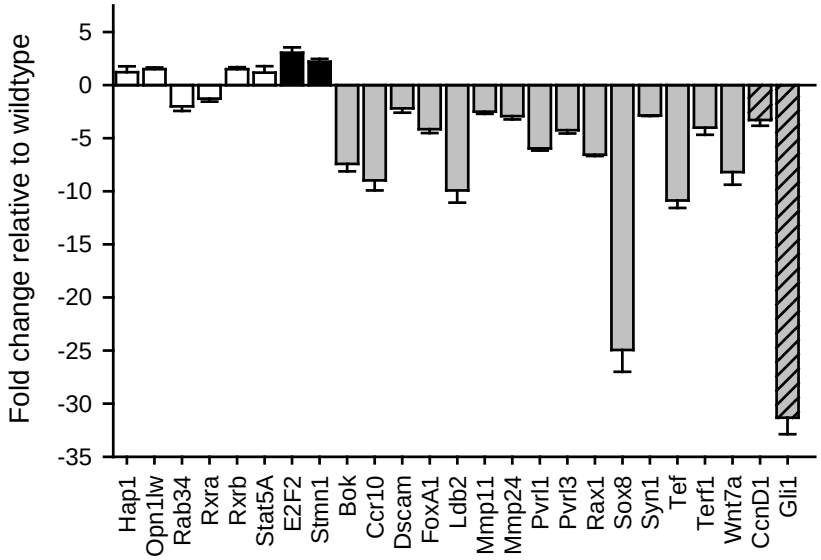

Fold change relative to wildtype
5
0
-5
-10
-15
-20
-25
-30
-35
Hap1
Opn1lw
Rab34
Rxra
Rxrb
Stat5A
E2F2
Stmn1
Bok
Ccr10
Dscam
FoxA1
Ldb2
Mmp11
Mmp24
Pvrl1
Pvrl3
Rax1
Sox8
Syn1
Tef
Terf1
Wnt7a
CcnD1
Gli1

Figure 2-1. Hh-dependence of target gene expression in the mouse retina *in vivo*. RT-qPCR analysis of whole P0.5 retinas comparing Shh^{cko} (n=6) to wt littermates (n=6) and the expression of genes that were unaffected (white bars), significantly increased (black bars) and significantly reduced (gray bars) in Shh^{cko} retinas compared to wt is shown. Known Hh target genes *CcnD1* and *Gli1* (gray hatched bars) are reduced in the mutants. Values represent average fold change in gene expression normalized to *Gapdh* and *18S* +/- SEM. Significance ($p \leq 0.05$) was assessed using Student's t-test.

Gene induction in Gli knockout tissues

We next investigated the requirement for Gli TF in the regulation of these candidate Hh target genes by comparing gene expression in control and Hh-Ag treated explants from *Gli* knockout (KO) mice. We restricted our analysis to 10 genes that were significantly upregulated by Hh treatment in wildtype explants and significantly downregulated in the Shh^{CKO} retinas. The Hh inducibility of all 10 genes was normal in *Gli1* KO explants (Fig. 2-2 a), which is consistent with a previous study documenting the non-essential role for Gli1 in the Hh response of RPCs (Wall et al., 2009). Conversely, the Hh-dependent induction of five genes was significantly reduced in *Gli2* KO explants compared with wildtype controls (Fig. 2-2 a), which suggests a role for Gli2 as a transcriptional activator downstream of Hh for the induction of these genes. With the exception of one gene (*Mmp11*), all of the genes were induced by Hh in *Gli3* KO explants (Fig. 2-2 b) with three genes (*Bok*, *FoxA1* and *Sox8*) expressed at a higher level in the Hh-Ag-treated KO explants compared with wildtype. The expression of four genes (*Bok, FoxA1, Sox8 and Wnt7a*) was de-repressed in untreated *Gli3* KO explants, consistent with Gli3 functioning as an antagonist of these genes. Interestingly, these four genes were also Gli2 dependent, suggesting that they are under positive and negative regulation by Gli2 and Gli3, respectively. In contrast to the data reported in Table 2-1, the expression of *Mmp11*, *Pvrl1*, *Tef* and *Terf1* was not modulated by Hh activation in wildtype or *Gli* KO explants in this experimental series. To circumvent the embryonic lethality associated with germline *Gli2* and *Gli3* inactivation we performed this analysis using E16.5 retinal tissue, which based on the temporal regulation of these genes (Table 2-1), is not the optimal stage at which to observe Hh induction of these genes.

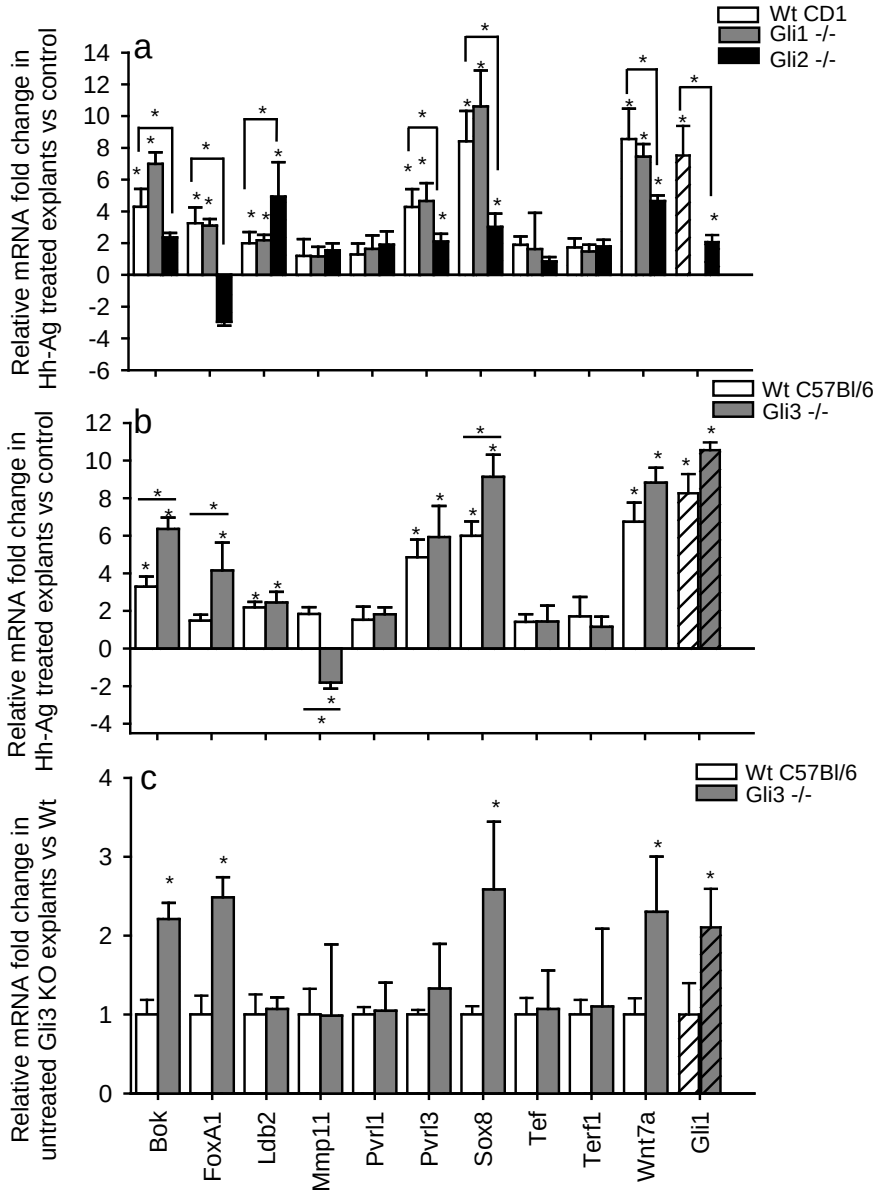

a
Wt CD1
Gli1 -/-
Gli2 -/-
Relative mRNA fold change in Hh-Ag treated explants vs control
b
Wt C57Bl/6
Gli3 -/-
Relative mRNA fold change in Hh-Ag treated explants vs control
c
Wt C57Bl/6
Gli3 -/-
Relative mRNA fold change in untreated Gli3 KO explants vs Wt
Bok
FoxA1
Ldb2
Mmp11
Pvrl1
Pvrl3
Sox8
Tef
Terf1
Wnt7a
Gli1

Figure 2-2. Hh mediates effects on target gene expression through Gli2 and Gli3. (a,b) Hh induction of target gene expression in wildtype and *Gli* KO explants. Explants from E16.5 *Gli1* KO and *Gli2* KO (a) and *Gli3* KO (b) mice and wildtype littermates were cultured with Hh-Ag or under control conditions for 3 days and gene expression was analyzed by RT-qPCR. n=6 for each genotype and littermate controls. (c) Baseline levels of gene expression in untreated Wt vs *Gli3* KO explants. *Gli1* expression serves as a readout for Hh pathway activation (hatched bars). Values represent average fold change in gene expression in Hh-Ag compared with untreated conditions (a,b) and untreated *Gli3* KO compared with wildtype (c) normalized to *Gapdh* and *18S* +/- SEM. An asterisk denotes significance ($p \leq 0.05$) as assessed using Student's t-test.

Therefore, of the six Hh-responsive genes at E16.5, we found that five required Gli2 and that four may be also regulated by Gli3.

Hh-pathway dependent enrichment of Gli2 at the *Sox8* promoter

Our analyses identified *Sox8* as a temporally regulated Hh and Gli2-dependent target gene (Table 2-1, Fig. 2-1 and 2-2). To further characterize the relationship between Hh and *Sox8* we performed chromatin immunoprecipitation (ChIP) for Gli2 on DNA isolated from Hh-Ag and untreated E14.5 and P0.5 retinal explants cultured for 3DIV. Sequence analysis of the *Sox8* promoter region revealed two potential Gli interacting elements located -606 bp and -1934 bp upstream from the transcription start site (TSS) (Fig 2-3 a). Q-PCR analysis of genomic DNA from Gli2 ChIP revealed a significant enrichment of Gli2 at both the -606 and -1934 sites in Hh-Ag treated E14.5 and P0.5 retinal explants (Fig 2-3 b). Thus, the Hh-dependent induction of *Sox8* expression correlates with an increase in Gli2 binding at two sites in the *Sox8* promoter. Interestingly, differences in Gli2 enrichment at these sites did not correlate with the temporal induction of *Sox8* by Hh activation, which is significantly lower at E14.5 compared with P0.5. This suggests that other mechanisms are involved in the regulation of *Sox8* that either prevent Hh mediated induction of gene expression at E14.5 or that promote the response at P0.5.

S*ox8* along with *Sox9* and *Sox10* comprise the group E of the Sry-box protein family and these genes exhibit overlapping expression patterns and some degree of functional compensation *in vivo* (Stolt and Wegner, 2010). In the developing retina, Sox8 and Sox9 are

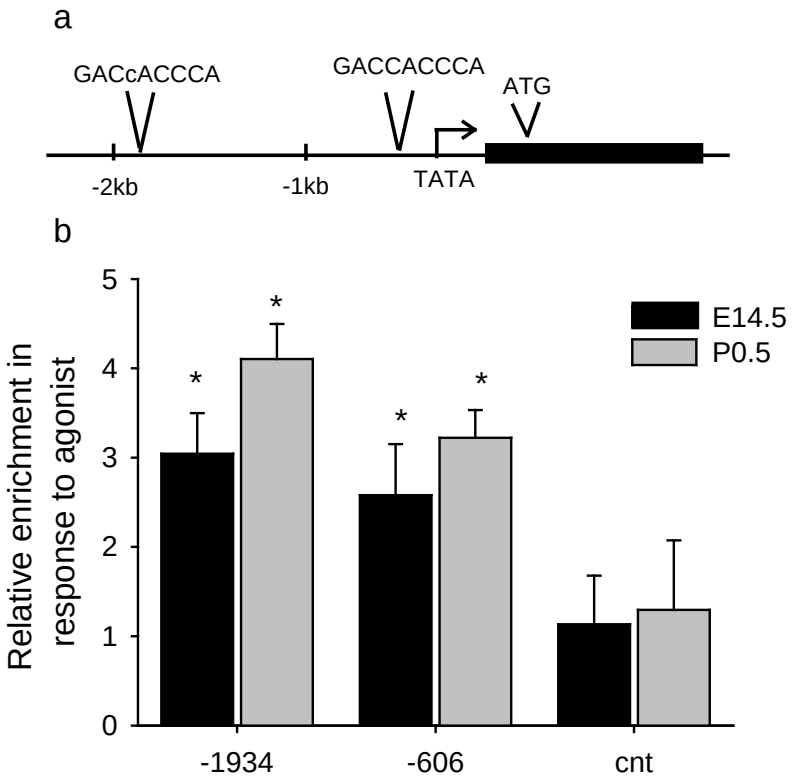

a
GACcACCCA
GACCACCCA
ATG
-2kb
-1kb
TATA
b
Relative enrichment in response to agonist
5
4
3
2
1
0
E14.5
P0.5
*
*
*
*
-1934
-606
cnt

Figure 2-3. Hh pathway activation promotes Gli2 association with the *Sox8* promoter. (a) Schematic of the 4-kb region of the *Sox8* promoter highlighting potential Gli binding sites at position -1934 and -606 bp upstream of the TSS. Mismatched nucleotides relative to the ideal Gli consensus sequence are indicated by lowercase letters. (b) qPCR amplification of genomic DNA using primers that flank identified Gli binding sites or control actin primers on Gli2-ChIPed DNA from Hh-Ag and control explants after 3 days. Values represent the fold enrichment of the amplified product in the Hh-Ag over control samples. An asterisk denotes significance ($p \leq 0.05$) over untreated as assessed using a two tailed Student's t-test.

required non-redundantly to promote Müller glial cell development (Muto et al., 2009; Poche et al., 2008). The demonstration of a direct relationship between Shh and *Sox8* in RPCs together with the evidence that Shh promotes Müller glial cell development in the vertebrate retina (Shkumatava et al., 2004; Wang et al., 2005) prompted us to investigate the temporal and spatial relationship between Hh signaling and the SoxE genes. Similar to *Sox8*, and consistent with the response in neuroepithelial cells (Scott et al., 2010), the expression of *Sox9* and *Sox10* was induced by Hh pathway activation in retinal explants (Fig. 2-4 a) and was downregulated in the retinas of Shh^{CKO} mice (Fig. 2-4 b), demonstrating the co-regulation of SoxE group genes by Hh. ISH analysis of P0.5 Shh^{CKO} retinas confirmed loss of Gli1 expression in the peripheral retina where *α-Cre* is expressed (Fig. 2-4 c, f) (Wang et al., 2005). Expression analyses of *Sox8* and *Sox9* in the adjacent sections revealed a reduction in expression of these genes, in a pattern that overlapped with the downregulation of Hh signaling (Fig. 2-4 d-e, g-h), suggesting that the expression of these genes is dependent on local Shh signaling from GCs to RPCs. The residual expression of *Sox8* and *Sox9* in the mutant region of the Shh^{CKO} retina (Fig. 2-4 f-h, brackets) could be due to Notch activity, which has also been shown to regulate the expression of these genes in RPCs (Muto et al., 2009).

To address whether Hh activation is sufficient to promote SoxE group gene expression cell autonomously, we examined gene expression by ISH in retinal explants that were transfected with Smo-M2, which drives ligand-independent constitutive activation of the Hh pathway in RPCs (Wall et al., 2009; Xie et al., 1998). As shown in Fig 2-4 i-n, *Sox8* and *Sox9* expression was increased in Smo-M2 electroporated (marked by GFP) regions of retinal explants compared with control (empty vector) transfected explants. These data

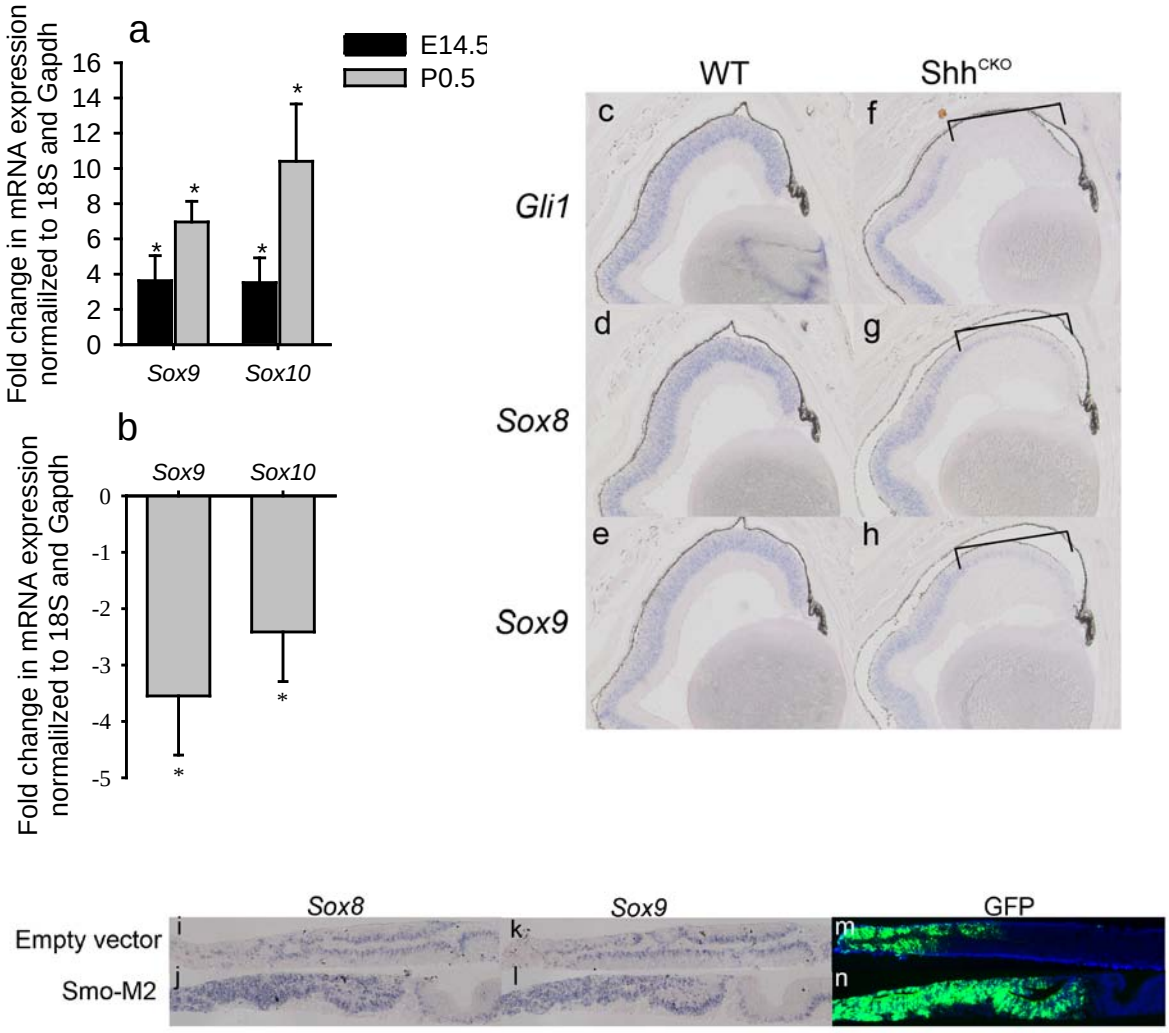

a
E14.5
P0.5
Fold change in mRNA expression normalized to 18S and Gapdh
16
14
12
10
8
6
4
2
0
Sox9
Sox10
b
Fold change in mRNA expression normalized to 18S and Gapdh
Sox9
Sox10
0
-1
-2
-3
-4
-5
WT
Shh^CKO
Gli1
Sox8
Sox9
c
f
d
g
e
h
Sox8
Sox9
GFP
Empty vector
Smo-M2
i
k
m
j
l
n

Figure 2-4. Hh regulation of SoxE group gene expression. (a) Retinal explants at E14.5 (n=6) and P0.5 (n=6), were treated with and without Hh agonist for 3 days in culture and gene expression was analyzed by RT-qPCR. (b) Change in gene expression in P0.5 Shh^{CKO} retinas compared to Wt (N=4). Values represent average fold change in gene expression normalized to *Gapdh* and *18S* +/- SEM. An asterisk denotes significance ($p \leq 0.05$) as assessed using a two tailed Student's t-test. (c-h) ISH for *Gli1* (c,f), *Sox8* (d,g) and *Sox9* (e,h) in the retina of Wt and Shh^{CKO} mice at P0.5. The reduction in the *Gli1* signal in the peripheral retina of the Shh^{CKO} mouse (between brackets), marks the region of *Shh* inactivation and overlaps with the diminished *Sox8* and *Sox9* signal. ISH for *Sox8* (i,j), *Sox9* (k,l) and Hoechst-GFP staining (m,n) on serial sections of P0.5 retinal explants that were transfected with GFP (control) or Smo-M2 and GFP expression vectors. GFP marks the transfected region of the explant.

provide strong evidence that *Sox8* is a target of Hh-Gli2 signaling and that the expression of the other members of the SoxE group are Hh regulated in the retina.

Given the positive and direct relationship between Hh signaling and *Sox8* expression in the retina, we investigated whether *Sox8* mediates the effect of Hh signaling on RPC proliferation and differentiation. RNAi-mediated knockdown of *Sox8* had no effect on *Smo-M2* induced proliferation in E14.5 or P0.5 explants after 3DIV (data not shown) nor did it alter rod, bipolar cell, Müller or amacrine cell development in P0.5 explants after 7DIV compared to the scrambled shRNA control (Supplemental Fig. 2-1 a). In addition, ectopic expression of *Sox8* in retinal explants at P0.5 did not affect neural or glial cell development (Supplemental Fig. 2-1 b), which is consistent with the findings of Muto et al. (2009). Sox8 also does not appear to be required for retinal differentiation *in vivo*, as we did not detect any significant differences in the proportions of different cell types in adult *Sox8* KO retinas compared to wildtype littermates (Supplemental Fig. 2-2, Appendix fig. 1). The absence of a retinal phenotype in the *Sox8* KO mouse is unexpected as Muto et al., (2009) reported that *Sox8* knockdown in retinal explants reduces Müller cell development. However, it is possible that this discrepancy is due to differences in compensation for germline *Sox8* loss *in vivo* compared with acute *Sox8* knockdown *in vitro*. Taken together, our data show that while *Sox8* is a Hh target gene, it is not required to mediate the effects of Hh in RPCs possibly because of redundancy with the co-regulated SoxE group genes.

2.6 Discussion

Using a comparative genomics approach we identified 390 genes that contain conserved Gli binding motifs in the human and mouse genomes. Of the 46 genes that were

tested for Hh-responsiveness, we found that the expression of 30 was altered in response to Hh activation in primary retinal explants, and of this group 12 exhibited Hh-dependent expression in the retina *in vivo*. Maximal Hh induction of four of these genes requires Gli2. Unexpectedly, we found that Hh regulation of 25 of these genes is temporally regulated. Finally, we identified a direct positive relationship between Hh signaling and the expression of SoxE group genes, thus linking this pathway with SoxE genes and Müller cell development.

This study used a bioinformatics-based approach for Hh target gene identification. The high percentage of validated genes (65%) is unanticipated because the only tissue specific inclusion criteria were hits in the brain and eye EST database. From our list of 390 candidate genes, 33 have been identified as Gli3 targets in the developing limb bud (Vokes et al., 2008), 17 as Gli1 targets in cerebellar granule neuron precursor cells and 39 as Gli1 targets in a Ptch+/- derived medulloblastoma (Lee et al., 2010). Thus, there is considerable overlap (73 genes) between our bioinformatics-based gene list and genes identified in tissue-restricted Gli1 and Gli3 ChIP-chip studies, which validates our approach and suggests that it can identify genes that are regulated by multiple Gli family members. Caveats to this bioinformatics approach for target gene identification include missing target genes because of the narrow genomic interval for the search and the high sequence conservation criteria. Functional Gli elements have been identified up to 27 kb away from the transcript of some target genes (Vokes et al., 2008) and Hh-regulation can be mediated by Gli elements that are less than 8/9 matches (Komada et al., 2008). Finally, since we restricted our validation experiments to neural progenitors it is possible that we could have missed genes that are Hh targets in other tissues.

Although there are some exceptions, the current body of evidence suggest that Gli1 and Gli2 serve as transcriptional activators and Gli3 as a transcriptional repressor (Stecca and Ruiz, 2010). The results from our study support this type of regulation in the retina as the loss of *Gli2* had a negative impact on the induction of *Bok*, *FoxA1*, *Sox8*, and *Wnt7a* in response to Hh-Ag. Because the induction of *Bok*, *Sox8* and *Wnt7a* was not completely abolished in the *Gli2* KO explants, this could suggest compensation by Gli1, which is consistent with previous studies (Bai and Joyner, 2001; Wall et al., 2009). Consistent with a repressive role for Gli3, the expression of a subset of the genes we tested was de-repressed in untreated *Gli3* KO explants and increased to a greater extent in Hh-Ag treated *Gli3* KO explants. For genes like *Bok, FoxA1, Sox8 and Wnt7a* these results implicate both Gli2 and Gli3 in their regulation, while genes like *Ldb2* and *Pvrl3* are primarily Gli2-dependent.

RPCs undergo intrinsically regulated temporal changes in developmental competence to generate different types of neurons (Livesey and Cepko, 2001). These changes in competence have been postulated to influence, in part, the response of RPCs to environmental cues (Cepko, 1999), which is consistent with the evidence for stage-specific RPC responses to mitogens (Lillien and Cepko, 1992). We identified 25 genes whose expression was temporally regulated by Hh signaling. Similar temporally regulated transcriptional output of the Hh pathway has been reported previously in the retina (Wall et al., 2009), developing limb (Ahn and Joyner, 2004) and neural tube (Lee et al., 1997). Thus, we speculate that the developmental progression of RPCs is associated with alterations in the transcriptional output of the Hh pathway. Future studies should address the extent to which the temporal difference in the outcome of Hh signaling is an intrinsic property of RPCs, whether it is functionally relevant and the molecular basis for these

changes. The temporal regulation of *Sox8* by Hh was not associated with significant differences in Gli2 association with the *Sox8* promoter, suggesting that other mechanisms are involved. While we cannot rule out the possibility of temporally-regulated recruitment of Gli factors at other regulatory elements of this gene, it is also possible that alterations in co-factor recruitment and interaction with transcriptional regulators of other signaling pathways could also play a role in this process.

The temporal regulation of Hh target genes could also reflect a stage-specific requirement for these genes in RPCs. *NrCAM* is expressed in the inner retina and Müller cells in chick (Grumet, 1997) and has been reported to antagonize Hh signaling in the developing cerebellum (Sakurai et al., 2001; Xenaki et al., 2011). The induction of *NrCAM* by Hh in the postnatal retina suggests that it may function similarly to regulate Hh responsiveness at late stages of retinal development. Similarly, the timing of Hh induction of *Sox8* is similar to the endogenous expression pattern of this gene in RPCs, namely low at E14.5 and high at P0.5 (Muto et al., 2009) and coincides with the timing of Müller cell development (Young, 1985). Hh signaling and *Sox8* and *Sox9* are required for Müller cell development (Muto et al., 2009) and *Sox9* is a direct target of the Hh pathway in other systems (Bien-Willner et al., 2007; Park et al., 2010). Here we show that Hh pathway activation is sufficient and required for the expression of all three of SoxE group genes and that *Sox8* is a direct target of Gli2. Thus, the combined of *Sox8* and *Sox9* by the Hh pathway could be one mechanism through which Hh signaling promotes Müller cell development in the retina.

2.7 Acknowledgements

We thank Alan Mears for comments on the manuscript. Brian McNeill is a recipient of a Natural Sciences and Engineering Research Council of Canada (NSERC) Doctoral Studentship. This work was supported by an operating grant to V. Wallace from the Canadian Cancer Society (National Cancer Institute of Canada Grant 016435).

2.8 Supplemental

Materials and methods

Dissociation of electroporated retinal explants

Retinal explants generated from P0.5 pups were electroporated and cultured for 7DIV. Briefly, retinas electroporated at a 10:1 ratio with expression vectors (or empty vector) (1 µg/ul) and pUb-GFP in 2-mm gap cuvettes (VWR) using 5 square 30V pulses of 50-ms duration and 950-ms intervals using a ECM830 pulse generator (BTX Harvard Apparatus). The DNA plasmids used were *SMO-M2* (a gift from G. Fishell, New York University Langone Medical Center, New York, NY), *Sox8* (Open Biosystems), shRNA targeting *Sox8* (Open Biosystems), double-stranded shcontrol (Invitrogen) and pUb-GFP (a gift from T. Matsuda, Harvard Medical School, Boston, MA) to visualize the transfected region. Single cell dissociates were obtained with 0.1mg/ml trypsin (Sigma) treatment in sterile calcium-free PBS (Invitrogen) for 15min at 37^{o}C. Trypsinization was inhibited with 10% FBS/DMEM/DNAseI (0.2mg/ml, Sigma) and tissue was triturated to obtain a single cell suspension. Cell were plated on glass slides for 15 min at room temperature followed by 40 min at 37^{o}C before a 5 min 4% PFA fix. Cells were processed immediately for immunocytochemistry or stored at -20^{o}C.

Adult tissue preparation for immunohistochemistry.

Sox8 KO animals were generated by knocking in a lacZ expression cassette to replace exon 1 and exon 2 (O'Bryan et al., 2008). Adult eyes were isolated from *Sox8* KO and wildtype animals, fixed in 4% PFA overnight at 4^{o}C and cryoprotected in 30% sucrose in PBS overnight. Tissues were embedded in 1:1 mix of 30% sucrose in PBS:OCT (Tissue-Tek) and snap frozen in liquid nitrogen. 12µM retinas cross-sections were obtained using a Leica 1850 cryostat and transferred onto Superfrost Plus coated slides (Fisher Scientific). Slides were air dried for 2-6h at room temperature and stored with desiccant at -20^{o}C.

Immunohistochemistry/ Immunocytochemistry

Slides were hydrated in PBS for 15 min prior to a 1h block in 10% FBS/PBS. The primary antibodies used in this study were as follows: αHPC1 (Sigma-Aldrich), αRhodopsin (B630; Developmental Studies Hybridoma Bank), αCralbp (gift from Dr. Xuemei Zhu and Cheryl Craft from the Mary D. Allen laboratory, Doheny Eye Institute, USC), αPKC (BD Pharmingen). Secondary antibodies used were as follows: αMouse Alexa 488 (Invitrogen), αRabbit Alexa 546 (Invitrogen). Nuclei were counterstained with Hoechst and slides were mounted with fluorescent mounting medium (DAKO). Staining was visualized using a Zeiss fluorescent upright microscope and images were captured using an AxioVision 6.05 camera (Zeiss).

2.8.2 Supplemental tables

Supplemental Table 2-1. Primer sequences

Symbol	Forward	Temp	Reverse	Temp
18S	CGGCTACCACATCCAAGG	64.2	CTGGAATTACCGCGGCT	63.7
Amn	ACTGCCTCAAGGACAATGCT	63.8	AGGTCAAATGTGGGGTCGT	64.3
Ccnna2	GTGTCACAGCCGATAGCAGA	64.1	GTAGAGGATGTGCGGAAGCA	65.3
Ccr10	CCGACTGAAAGCCTGGAC	63.5	GACCAAGCCCACAGAGCA	65.3
Cdc25	AGCGTGTCATTGTCGTGTTC	63.9	CATTGCCGAGCCTATCTCTC	63.7
ChIP b-actin	CCCTGAAGTACCCCATTGA	62.3	GGTGTGGTGCCAGATCTTC	63.2
CsnK1g2	CGGCTACGTGTTTGACTACG	63.2	CTGAGCTTTGTCGCGATGT	64.2
Dscam	ATGTCCAAGCCATAGCAACC	63.7	CCCAGCTCTCCGTCTATGAG	63.8
E2F2	AGTTGCTCCCTGAGCTTCAA	64	GCTCCTTGAAGTTGCCTACG	63.7
Egr2	GCGGGCATCTTGCAAC	64.3	TGAGTCTGGGACATGGTACACA	65.4
FoxA1	CTCCGAGCAACAGCACAAG	64.9	CTCCTCGTGGCCACTGA	64
Gapdh	TGAAGGGGTCGTTGATGG	64.7	AAAATGGTGAAGGTCGGTGT	63.1
Gli1	CACTGAGGACTTGTCCAGCTTG	66.2	AGCTGGGCAGTTTGAGACC	64.4
Hap1	TTCCGATAGCTCTGCCATCT	63.7	GACAACCTGGAGGACGAAGA	64.2
HeyL	GCTTGCCTTCCCTGAACAGT	65.5	GCTCTCTGGGTGGAAGTCAC	63.9
Hpcal4	GCCTGATAGCCAGCCTTGT	64.3	ACCCGGCTTGAAGTGAAAGT	64.8
Inha	TGAACCAGAGGAGGAAGATG	61.8	CCGGAATACATAAGTGAAGAGG	61.3
Lifr	GCCTCATTTCTCCGGTTACA	63.8	CGAGCACCACTTTGTCTTGA	64.1
Mmp11	TCACCTTCACGGGATCAAAC	64.8	AGATTGATGCTGCCTTCCAG	64.2
Mmp24	AACCCTGGACGCTAGGAAAT	63.5	TAGAGTGCTCCAAGCCCAGT	63.9
Nrcam	CGTCCATCACACCATTTCTG	64	ATGAGGGTCCCATTCTCTCC	64.1
Ogfr	CCCTCACACTGAAGGAGGTT	63.1	CTGTGTTCCGGTCCTCAAGT	64.1
Opn1lw	CTTCCTCATGTCAGTCACTCAGG	65	AGGGTGATCATGGAAGTGATG	63.7
p57	CAGATGCCCAGCAAGTTCTC	64.9	CTCTCCAAACGTGGCTCCT	64.4
Plek2	AGCAGACACAAGGGAACCAC	64.1	AAGGTGCGCAGATTTGTTCT	63.6
Plgbn	GCACAGGTGAGGACGTTGAC	65.9	GCAATCTCTCGGATGTAGCC	63.6
Pou4F2	ATGGTGGTGGTGGCTCTTAC	63.8	CGGAGAGCTTGTCTTCCAAC	63.8
Ppil5	AGCCACTGTCCGGCTAAAG	64.2	TCCACAGACACAGGTTTTGG	63.7
Ptch1	TAGCCCTGTGGTTCTTGTCC	64	TGTGGTCATCCTGATTGCAT	64.1
Ptger3	GGATCATGTGTGTGCTGTCC	64.3	GTGTCTTGCATTGCTCAACC	63.31
Pvrl1	CTGGCCTGCATTGTCAACTA	63.8	AAAGCCCTCAATGGTCACCT	63.6
Pvrl3	GAATCCGGCCCTTTTATCA	63.55	AGCCGTTACATTCCCACTTG	63.7
Rax1	TTCATGGACGACACTTCCAG	63.8	GTTAACCTACCCGAGGTTCG	62.2
Rxra	CGAGCCCAAGACTGAGACAT	64.4	GCTTGTCTGCTGCTTGACAG	64
Rxrb	GCACAGAAACTCAGCCCATT	64.1	TGTCACGCATTTTGGACACT	64.2
Sox10	AGGGGCTGCTGCTATTCAG	64.4	TCTGGGTTCCCATCTGACAT	64.4
Sox18	GACGAGTTGCGCATTCG	64.4	ACGCTTTGCCCAGCATC	65.2
Sox8	TTCTCGCTTTCACTCAGCAA	63.8	GTACCCGCATCTCCATAACG	64.1
S8 ChIP 770	TCTCTCTAGGCGCTGAGGA	62.2	CGGGTATGACCTGTGGTG	62

S8ChIP 2095	CGTGTCAGTGCTTGTACAGAG	63.6	GGTGTAGTCAGCCCTTGTTATACAG	62.9
Sox9	GTGGACATCGGTGAACTGA	62.3	GTGGGTGGCCGGAAC	64
Stat5a	GGAAGATGGGTTTTTGCTGA	63.8	AACCAGCCTCTGTTCGTTGT	63.6
Stmn1	AAGCTGACCCACAAAATGGA	64.3	TTTGTTCTTCCGCACCTCTT	63.6
susd2	CAACAATAACCCCAGGGATG	63.8	AAACAGGGAAGACGTGTTGG	63.9
Syn1	CAGCACAAAGTCTGGCTTCA	64.2	GCCAATGGTGGATTCTCTGT	63.8
Tef	GAGTCTGCCAGCTCTTCCAC	64.2	GGTCTCCCTCTCCTTTTCCA	64.4
Terf1	ACACAGACGTGCTCCATCAG	64.1	TTCCACTGGTTCTTCGGTTC	64
Uhrf1	ACGGTGCCTACTCATTGGTC	63.9	GCTTCTGGTCAGAGGACTGG	64
Wnt10b	CACTTCCGCTTCAGGTTTTC	63.6	TCTCTCGGGATTTCTTGGATT	63.6
Wnt11	ACCTGCTTGACCTGGAGAGA	64	CGATGGTGTGACTGATGGTG	65.3
Wnt7a	GGCCTGGGATCTTGTTACAG	63.4	TTTCTCAGCCTGGGCATAGT	63.6

Supplemental Table 2-2. Genes identified by computational analysis as having Gli binding sites

Accession	# Gli binding sites	Gene Title	Gene symbol
NM_001319	10	Casein Kinase1 gamma 2	CSNK1G2
NM_014571	7	Hairy/enhacer-of-split related with YRPW motif-like	HEYL
NM_030943	6	Amnionless homologue	AMN
NM_017904	5	Tetratricopeptide repeat domain 22	TTC22
NM_005563	5	Stathmin 1	STMN1
NM_003689	5	Aldo-keto reductase family 7, member A2	AKR7A2
NM_001856	5	Collagen type, XVI, alpha 1	COL16A1
NM_000403	5	UDP-Galactose-4-epimerase	GALE
NM_031934	4	RAB34, member RAS oncogene family	RAB34
NM_019601	4	Sushi domain containing 2	SUSD2
NM_148972	3	Tumor necrosis factor receptor superfamily, member 25	TNFRSF25
NM_053286	3	Aquaporin 6	AQP6
NM_025258	3	Chromosome 6 open reading frame 27	C6orf27
NM_024029	3	Yip1 domain family, member 2	YIPF2
NM_021257	3	Neuroglobin	NGB
NM_019066	3	MAGE-like 2	MAGEL2
NM_018982	3	Yip1 domain family, member 1	YIPF1
NM_016257	3	Hippocalcin like 4	HPCAL4
NM_015886	3	Protease inhibitor 15	PI15
NM_014443	3	Interleukin 17B	IL17B
NM_007346	3	Opiod growth factor receptor	OGFR
NM_007284	3	Twinfilin, actin-binding protein	TWF2
NM_004753	3	Dehydrogenase/reductase (SDR family) member 3	DHRS3
NM_004388	3	Chitobiase, di-N-acetyl-	CTBS
NM_003245	3	Transglutaminase 3	TGM3
NM_002353	3	Tumor-associated calsium signal transducer 2	TACSTD2
NM_001397	3	Endothelin converting enzyme 1	ECE1
NM_000957	3	Prostaglandin E receptor 3 (subtype EP3)	PTGER3
NM_153374	3	LysM, putative peptidoglycan-binding domain containing 2	LYSMd2

NM_152911	2	Polyamine oxidase (exo-N4-amino)	PAOX
NM_152559	2	Williams Beuren syndrome chromosome region 27	WBSCR27
NM_152545	2	RasGEF domain family, member 1B	RASGEF1B
NM_152447	2	Leucine rich repeat and fibronectin type II domain containing 5	LRFN5
NM_138790	2	Phospholipase D family, member 4	PLD4
NM_134426	2	Solute carrier family 26, member 6	SLC26A6
NM_080820	2	D-tyrosyl-tRNA deacylase 1 homolog	DTD1
NM_080431	2	Actin-related protein T2	ARPT1
NM_057157	2	Cytochrome P450, family 26, subfamily A, polypeptide 1	CYP26A1
NM_054114	2	T-cell activation RhoGTPase activating protein	TAGAP
NM_052951	2	Deocynucleotidyltransferase, terminal, interacting protein1	DNTTIP1
NM_032947	2	Chromosome 5 open reading frame 62	C5orf62
NM_032515	2	BCL2-related ovarian killer	BOK
NM_032192	2	Protein phosphatase 1, regulatory (inhibitor) subunit 1B	PPP1R1B
NM_030883	2	Olfactory receptor, family 2, subfamily H, member 1	OR2H1
NM_025141	2	TM2 domain containing 3	TM2D3
NM_025115	2	Chromosome 8 open reading frame 41	C8orf41
NM_025027	2	Zinc finger protein 606	ZNF606
NM_023937	2	Mitochondrial ribosomal protein L24, nuclear gene encoding mitochondrial protein	MRPL34
NM_023080	2	Chromosome 8 open reading frame 33	C8orf33
NM_021798	2	Interleukin 21 receptor	IL21R
NM_021160	2	HLA-B associated transcript 5	BAT5
NM_021134	2	Mitochondrial ribosomal protein L23, nuclear gene encoding mitochondrial protein	MRPL23
NM_020653	2	Zinc finger protein 287	ZNF287
NM_018957	2	SH3-domain binding protein	SH3BP1
NM_018438	2	F-box protein 6	FBXO6
NM_018419	2	SRY (sex determining region Y)-box 18	Sox18
NM_017991	2	KIAA1310	KIAA1310
NM_017911	2	Family with sequence similarity 118, member A	FAM118A
NM_017449	2	EPH receptor B2	EPHB2
NM_016734	2	Paired box 5	PAX5

NM_016546	2	Complement component 1, r subcomponent-like	C1RL
NM_016456	2	Transmembrane protein 9	TMEM9
NM_016350	2	Ninein (GSK3B interacting protein)	NIN
NM_016053	2	Coiled-coil domain containing 53	CCDC53
NM_014365	2	Heat shock 22kDa protein 8	HSPB8
NM_014320	2	Heme binding protein 2	HEBP2
NM_013388	2	Prolacting regulatory element binding	PREB
NM_012417	2	Phosphatidylinositol tranfer protein, cytoplasmic 1	PITPNC1
NM_012113	2	Carbonic anhydrase XIV	CA14
NM_007238	2	Peroxisomal membrane protein 4, 24kDa	PXMP4
NM_007104	2	Ribosomal protein L10a	RPL10A
NM_007009	2	Zona pellucida binding protein	ZPBP
NM_006803	2	Adapter-related protein complex3, mu 2 subunit	AP3M2
NM_006782	2	Zinc finger protein-like 1	ZFPL1
NM_005940	2	Matrix metalloproteinase 11 (stromelysin 3)	MMP11
NM_005720	2	Actin related protein 2/3 compex, subunit 1B, 41kDa	ARPC1B
NM_005010	2	Neuronal cell adhesion molecule	NRCAM
NM_004626	2	Wingless-type MMTV integration site family, member 11	WNT11
NM_004387	2	NK2 transcription factor related, locus 5	NKX2-5
NM_004162	2	RAB5A, member RAS oncogene family	RAB5A
NM_003405	2	Tyrosine 3-monooxygenase/trytophan 5-monooxygenase activation protein, eta polypeptide	YWHAH
NM_003394	2	Wingless-type MMTV integration site family, member 10B	WNT10B
NM_003152	2	Signal transducer and activator of transcription 5A	STAT5A
NM_002924	2	Regulator of G-protein signaling 7	RGS7
NM_002911	2	UPF1 regulator of nonsense transcripts homolog	UPF1
NM_002760	2	Protein kinase, Y-linked	PRKY
NM_002689	2	Polymerase (DNA directed), alpha 2 (70kD subunit)	POLA2
NM_002338	2	Limbic system-associated membrane protein	LSAMP
NM_002286	2	Lymphocyte-activation gene 3	LAG3
NM_002278	2	Keratin hair, acidic 2	KRTHA2
NM_002257	2	Kallikrein 1	KLK1

NM_001885	2	Crystallin, alpha B	CRYAB
NM_001613	2	Actin, alpha 2, smooth muscle, aorta	ACTA2
NM_000744	2	Cholinergic receptor, nicotinic, alpha 4	CHRNA4
NM_000691	2	Aldehyde dehydrogenase 3 family, member A1	ALDH3A1
NM_000354	2	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	SERPINA7
NM_000334	2	Sodium channel, voltage-gated, type IV, alpha subunit	SCN4A
NM_177541	1	Taste receptor, type 1, member 1	TAS1R1
NM_177533	1	Nudix (nucleoside diphosphate linked moiety X)-type motif 14	NUDT14
NM_175913	1	Junctophilin 2	JPH2
NM_175068	1	Keratin 73	KRT73
NM_175064	1	Speedy homolog E1	SPDYE1
NM_173794	1	FUN14 domain containing 1	FUNDC1
NM_173626	1	Solute carrier family 26, member 11	SLC26A11
NM_173575	1	Serine/threonine kinase 32C	STK32C
NM_173557	1	Ring finger protein 152	RNF152
NM_173527	1	RAS (RAD and GEM)-like GTP binding 2	REM2
NM_173491	1	LSM11, U7 small nuclear RNA associated	LSM11
NM_172220	1	Colony stimulating factor 3 (granulocyte)	CSF3
NM_170754	1	Tensin like C1 domain containing phosphatase (tensin 2)	TENC1
NM_153281	1	Hyaluronoglucosaminidase 1	HYAL1
NM_153271	1	Sorting nexin 33	SNX33
NM_153214	1	Fibulin 7	FBLN7
NM_152899	1	Interleukin 4 induced 1	IL4I1
NM_152870	1	Abhydrolase domain containing 1	ABHD1
NM_152603	1	Zinc finger protein 567	ZNF567
NM_152550	1	SH3 domain containing ring finger 2	SH3RF2
NM_152476	1	Zinc finger protein 560	ZNF560
NM_152328	1	Adenylosuccinate synthase like 1	ADSSL1
NM_152267	1	Ring finger protein 185	RNF185
NM_145918	1	Cathepsin L1	CTSL1
NM_145738	1	Synaptogyrin 1	SYNGR1

NM_145690	1	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase protein, zeta polypeptide	YWHAZ
NM_145260	1	Odd-skipped related 1 (Drosophila)	OSR1
NM_145200	1	Calcium binding protein 4	CABP4
NM_145053	1	Ubiquilin-like	UBQLNL
NM_145032	1	F-box and leucine-rich repeat protein 13	FBXL13
NM_145019	1	Family with sequence similarity 124A	FAM124A
NM_145008	1	Yippee-like 4 (Drosophila)	YPEL4
NM_144999	1	Leucine rich repeat containing 45	LRRC45
NM_144997	1	Folliculin	FLCN
NM_144769	1	Forkhead box I1	FOXI1
NM_144716	1	Coiled-coil domain containing 12	CCDC12
NM_144578	1	Mitogen-activated protein kinase 1 interacting protein 1-like	MAPK1IP1L
NM_139177	1	Solute carrier family 39 (metal ion transporter), member 11	SLC39A11
NM_139160	1	DEP domain containing 7	DEPDC7
NM_139053	1	EPS8-like 3	EPS8L3
NM_138993	1	Mitogen-activated protein kinase 11	MAPK11
NM_138805	1	Family with sequence similarity 3, member D	FAM3D
NM_138462	1	Zinc finger, MYND-type containing 19	ZMYND19
NM_138347	1	Zinc finger protein 551	ZNF551
NM_138334	1	Josephin domain containing 2	JOSD2
NM_134268	1	Cytoglobin	CYGB
NM_133499	1	Synapsin I	SYN1
NM_133373	1	Phospholipase C, delta 3	PLCD3
NM_080836	1	Serine/threonine kinase 35	STK35
NM_080385	1	Carboxypeptidase A5	CPA5
NM_058192	1	RNA pseudouridylate synthase domain containing 1	RPUSD1
NM_058163	1	TSR2, 20S rRNA accumulation, homolog (S. cerevisiae)	TSR2
NM_052945	1	Tumor necrosis factor receptor superfamily, member 13C	TNFRSF13C
NM_033543	1	Carcinoembryonic antigen-related cell adhesion molecule 21	CEACAM21
NM_033467	1	Membrane metallo-endopeptidase-like 1	MMEL1
NM_033413	1	Leucine rich repeat containing 46	LRRC46

NM_033200	1	Lipase maturation factor 2	LMF2
NM_033139	1	Caldesmon 1	CALD1
NM_033112	1	Ribosomal RNA processing 36 homolog (S. cerevisiae)	RRP36
NM_032872	1	Synaptotagmin-like 1	SYTL1
NM_032799	1	Zinc finger, DHHC-type containing 12	ZDHHC12
NM_032470	1	Tenascin XB , transcript variant XB-S	TNXB
NM_032342	1	Chromosome 9 open reading frame 125	C9orf125
NM_032285	1	Methylthioribose-1-phosphate isomerase homolog (S.cerevisiae)	MRI1
NM_032259	1	WD repeat domain 24	WDR24
NM_031945	1	Tetraspanin 10	TSPAN10
NM_031895	1	Calcium channel, voltage-dependent, gamma subunit 8	CACNG8
NM_031485	1	Glutamate-rich WD repeat containing 1	GRWD1
NM_031472	1	tRNA phosphotransferase 1	TRPT1
NM_031310	1	Plasmalemma vesicle associated protein (PLVAP),	PLVAP
NM_031232	1	N-terminal EF-hand calcium binding protein 3	NECAB3
NM_031208	1	Fumarylacetoacetate hydrolase domain containing 1	FAHD1
NM_030753	1	Wingless-type MMTV integration site family, member 3	Wnt3
NM_030634	1	Zinc finger protein 436 (ZNF436),	ZNF436
NM_024922	1	Carboxylesterase 3	CES3
NM_024861	1	Chromosome 2 open reading frame 54	C2orf54
NM_024815	1	Nudix (nucleoside diphosphate linked moiety X)-type motif 18	NUDT18
NM_024674	1	Lin-28 homolog A (C. elegans) (LIN28),	LIN28A
NM_024598	1	Chromosome 16 open reading frame 57	C16orf57
NM_024552	1	Homo sapiens LAG1 longevity assurance homolog 4 (S. cerevisiae) (LASS4), mRNA	LASS4
NM_024407	1	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase) (NDUFS7)	NDUFS7
NM_024313	1	Nucleolar protein 12	NOL12
NM_024106	1	Zinc finger protein 426	ZNF426
NM_024101	1	Melanophilin	MLPH
NM_023032	1	Methyltransferase-like 1	METTL1
NM_023004	1	Reticulon 4 receptor	RTN4R

NM_022831	1	Axin interactor, dorsalization associated	AIDA
NM_022740	1	Homeodomain interacting protein kinase 2	HIPK2
NM_022144	1	Tenomodulin	TNMD
NM_022119	1	Protease, serine, 22	PRSS22
NM_022096	1	Ankyrin repeat domain 5	ANKRD5
NM_022089	1	ATPase type 13A2	ATP13A2
NM_021976	1	Retinoid X receptor, beta	RXRB
NM_021910	1	FXYD domain containing ion transport regulator 3	FXYD3
NM_021642	1	Fc fragment of IgG, low affinity IIa, receptor (CD32)	FCGF2A
NM_021602	1	CD79B antigen (immunoglobulin-associated beta)	CD79B
NM_021181	1	SLAM family member 7	SLAMF7
NM_020470	1	Yip1 interacting factor homolog A (S. cerevisiae)	YIF1A
NM_020433	1	Junctophilin 2	JPH2
NM_020427	1	Secreted LY6/PLAUR domain containing 1	SLURP1
NM_020167	1	Neuromedin U receptor 2	NMUR2
NM_020061	1	Opsin 1 (cone pigments), long-wave-sensitive	OPN1LW
NM_019844	1	Solute carrier organic anion transporter family, member 1B3	SLCO1B3
NM_019596	1	Chromosome 21 open reading frame 62	C21orf62
NM_019032	1	ADAMTS-like 4	ADAMTSL4
NM_018990	1	SAM and SH3 domain containing 3	SASH3
NM_018970	1	G protein-coupled receptor 85	GPR85
NM_018849	1	ATP-binding cassette, sub-family B (MDR/TAP), member 4	ABCB4
NM_018690	1	Apolipoprotein B48 receptor	APOB48R
NM_018290	1	Phosphoglucomutase 2	PGM2
NM_018231	1	Solute carrier family 38, member 7	SLC38A7
NM_018197	1	Zinc finger protein 64 homolog (mouse)	ZFP64
NM_018097	1	HAUS augmin-like complex, subunit 2	HAUS2
NM_017979	1	Unc-45 homolog A (C. elegans)	UNC45A
NM_017870	1	Transmembrane protein 132A	TMEM132A
NM_017839	1	Lysophosphatidylcholine acyltransferase 2	LPCAT2
NM_017578	1	Ropporin, rhophilin associated protein 1	ROPN1

NM_017522	1	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	LRP8
NM_017489	1	Telomeric repeat binding factor (NIMA-interacting) 1	TERF1
NM_016657	1	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	KDELR3
NM_016613	1	Family with sequence similarity 198, member B	FAM198B
NM_016602	1	Chemokine (C-C motif) receptor 10	CCR10
NM_016564	1	Cell cycle exit and neuronal differentiation 1	CEND1
NM_016445	1	Pleckstrin 2	PLEK2
NM_016368	1	Inositol-3-phosphate synthase 1	ISYNA1
NM_016321	1	Rh family, C glycoprotein	RHCG
NM_016243	1	Cytochrome b5 reductase 1	CYB5R1
NM_016222	1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41	DDX41
NM_016180	1	Solute carrier family 45, member 2	SLC45A2
NM_016150	1	Ankyrin repeat and SOCS box-containing 2	ASB2
NM_015991	1	Complement component 1, q subcomponent, A chain	C1QA
NM_015973	1	Galanin prepropeptide	GAL
NM_015897	1	Protein inhibitor of activated STAT, 4	PIAS4
NM_015649	1	Interferon regulatory factor 2 binding protein 1	IRF2BP1
NM_015537	1	Nasal embryonic LHRH factor	NELF
NM_015488	1	Paroxysmal nonkinesigenic dyskinesia	PNKD
NM_015484	1	SYF2 homolog, RNA splicing factor (S. cerevisiae)	SYF2
NM_015322	1	Fem-1 homolog b (C. elegans)	FEM1B
NM_015292	1	Extended synaptotagmin-like protein 1	ESYT1
NM_015256	1	Acyl-CoA synthetase long-chain family member 6	ACSL6
NM_015162	1	Acyl-CoA synthetase bubblegum family member 1	ACSBG1
NM_014587	1	SRY (sex determining region Y)-box 8	SOX8
NM_014564	1	Homo sapiens LIM homeobox 3	LHX3
NM_014312	1	V-set and immunoglobulin domain containing 2	VSIG2
NM_014157	1	Coiled-coil domain containing 113	CCDC113
NM_013993	1	Discoidin domain receptor tyrosine kinase 1	DDR1
NM_013313	1	Yippee-like 1 (Drosophila)	YPEL1
NM_013282	1	Ubiquitin-like with PHD and ring finger domains 1	UHRF1

NM_012471	1	Transient receptor potential cation channel, subfamily C, member 5	TRPC5
NM_012281	1	Potassium voltage-gated channel, Shal-related subfamily, member 2	KCND2
NM_012259	1	Homo sapiens hairy/enhancer-of-split related with YRPW motif 2	HEY2
NM_012249	1	Ras homolog gene family, member Q	RHOQ
NM_012123	1	Mitochondrial translation optimization 1 homolog (S. cerevisiae)	MTO1
NM_009587	1	Lectin, galactoside-binding, soluble, 9	LGALS9
NM_007185	1	CUGBP, Elav-like family member 3	CELF3
NM_007177	1	Family with sequence similarity 107, member A	FAM107A
NM_007101	1	Sarcosine dehydrogenase	SARDH
NM_007024	1	Transmembrane protein 115	TMEM115
NM_006958	1	Zinc finger protein 16	ZFP16
NM_006890	1	Carcinoembryonic antigen-related cell adhesion molecule 7	CEACAM7
NM_006760	1	Uroplakin 2	UPK2
NM_006690	1	Matrix metallopeptidase 24	MMP24
NM_006636	1	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	MTHFD2
NM_006612	1	Kinesin family member 1C	KIF1C
NM_006546	1	Insulin-like growth factor 2 mRNA binding protein 1	IGF2BP1
NM_006541	1	Glutaredoxin 3	GLRX3
NM_006374	1	Serine/threonine kinase 25	STK25
NM_006337	1	Microspherule protein 1	MCRS1
NM_006181	1	NETRIN 3	NTN3
NM_006149	1	LECTIN, galactoside-binding, soluble, 4	LGALS4
NM_006145	1	DnaJ (Hsp40) homolog, subfamily B, member 1	DNAJB1
NM_006041	1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	HS3ST3B1
NM_005984	1	Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1	SLC25A1
NM_005764	1	PDZK1 interacting protein 1	PDZK1IP1
NM_005714	1	Potassium channel, subfamily K, member 7	KCNK7
NM_005699	1	Interleukin 18 binding protein	IL18BP
NM_005583	1	Lymphoblastic leukemia derived sequence 1	LYL1
NM_005515	1	Motor neuron and pancreas homeobox 1	MNX1

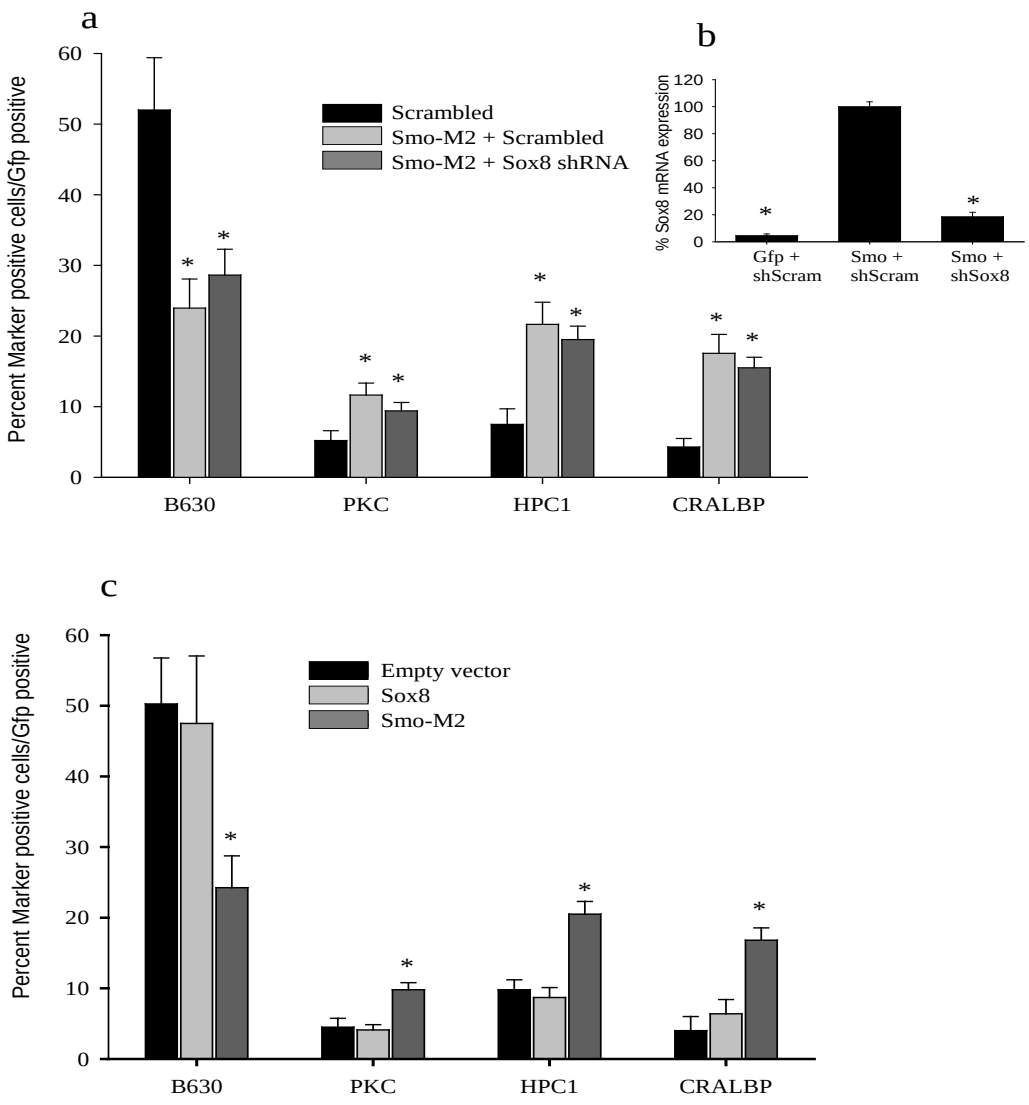
NM_005438	1	FOS-like antigen 1	FOSL1
NM_005230	1	ELK3, ETS-domain protein (SRF accessory protein 2)	ELK3
NM_005216	1	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase	DDOST
NM_005187	1	Core-binding factor, runt domain, alpha subunit 2; translocated to, 3	CBFA2T3
NM_005182	1	Carbonic anhydrase VII	CA7
NM_005154	1	Ubiquitin specific peptidase 8	USP8
NM_005044	1	Protein kinase, X-linked	PRKX
NM_004955	1	Solute carrier family 29 (nucleoside transporters), member 1	SLC29A1
NM_004887	1	Chemokine (C-X-C motif) ligand 14	CXCL14
NM_004838	1	Homer homolog 3 (Drosophila)	HOMER3
NM_004822	1	Netrin 1	NTN1
NM_004744	1	Lecithin retinol acyltransferase (phosphatidylcholine--retinol O-acyltransferase)	LRAT
NM_004625	1	Wingless-type MMTV integration site family, member 7A	WNT7A
NM_004583	1	RAB5C, member RAS oncogene family	RAB5C
NM_004575	1	POU class 4 homeobox 2	POU4F2
NM_004561	1	Homo sapiens ovo-like 1(Drosophila)	OVOL1
NM_004496	1	Homo sapiens forkhead box A1	FOXA1
NM_004427	1	Polyhomeotic homolog 2 (Drosophila)	PHC2
NM_004404	1	Septin 2	(SEPT2)
NM_004176	1	Sterol regulatory element binding transcription factor 1	SREBF1
NM_004170	1	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	SLC1A1
NM_004091	1	E2F transcription factor 2	E2F2
NM_004056	1	Carbonic anhydrase VIII	CA8
NM_003949	1	Huntingtin-associated protein 1 (neuroan 1) (HAP1),	HAP1
NM_003867	1	Fibroblast growth factor 17	FGF17
NM_003820	1	Tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	TNFRSF14
NM_003755	1	Eukaryotic translation initiation factor 3, subunit G	EIF3G
NM_003663	1	CGG triplet repeat binding protein 1	CGGBP1
NM_003649	1	D-aspartate oxidase	DDO
NM_003636	1	Potassium voltage-gated channel, shaker-related subfamily, beta member 2	KCNAB2

NM_003604	1	Insulin receptor substrate 4	IRS4
NM_003585	1	Double C2-like domains, beta	DOC2B
NM_003514	1	Histone cluster 1, H2am	HIST1H2AM
NM_003485	1	G protein-coupled receptor 68	GPR68
NM_003479	1	Protein tyrosine phosphatase type IVA, member 2	PTP4A2
NM_003436	1	Zinc finger protein 135	ZNF135
NM_003435	1	Zinc finger protein 134 (clone pHZ-15)	ZNF134
NM_003332	1	TYRO protein tyrosine kinase binding protein	TYROBP
NM_003216	1	Thyrotrophic embryonic factor (TEF),	TEF
NM_003133	1	Signal recognition particle 9kDa	SRP9
NM_003047	1	Solute carrier family 9 (sodium/hydrogen exchanger), member 1	SLC9A1
NM_002957	1	Retinoid X receptor, alpha	RXRA
NM_002946	1	Replication protein A2, 32kDa	RPA2
NM_002789	1	Proteasome (prosome, macropain) subunit, alpha type, 4	PSMA4
NM_002774	1	Kallikrein-related peptidase 6	KLK6
NM_002634	1	Prohibitin	PHB
NM_002561	1	Purinergic receptor P2X, ligand-gated ion channel, 5	P2RX5
NM_002509	1	NK2 homeobox 2	NKX2-2
NM_002428	1	Matrix metallopeptidase 15	MMP15
NM_002364	1	Melanoma antigen family B, 2	MAGEB2
NM_002349	1	Lymphocyte antigen 75	LY75
NM_002310	1	Leukemia inhibitory factor receptor alpha	LIFR
NM_002298	1	Lymphocyte cytosolic protein 1 (L-plastin)	LCP1
NM_002215	1	Inter-alpha (globulin) inhibitor H1	ITIH1
NM_002201	1	Interferon stimulated exonuclease gene 20kDa	ISG20
NM_002191	1	Inhibin, alpha	INHA
NM_002151	1	Hepsin	HPN
NM_002135	1	Nuclear receptor subfamily 4, group A, member 1	NR4A1
NM_032638	1	GATA binding protein 2	GATA2
NM_001997	1	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	FAU
NM_001964	1	Early growth response 1	EGR1

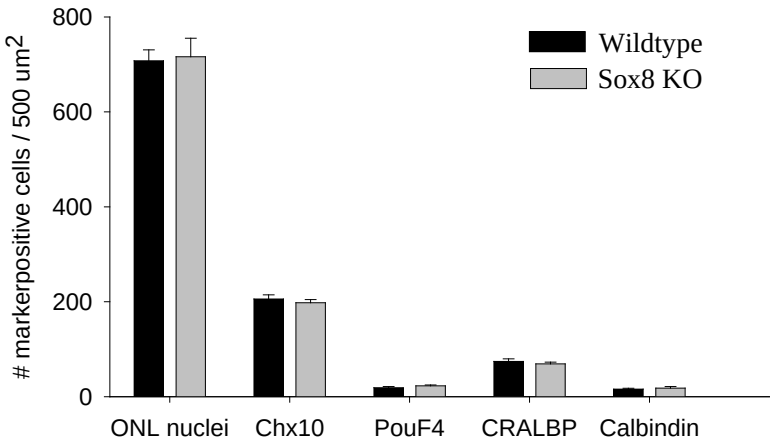
NM_001956	1	Endothelin 2	EDN2
NM_001940	1	Atrophin 1	ATN1
NM_001917	1	D-amino-acid oxidase	DAO
NM_001906	1	Chymotrypsinogen B1	CTRB1
NM_001863	1	Cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous)	COX6B1
NM_001853	1	Collagen, type IX, alpha 3	COL9A3
NM_001852	1	Collagen, type IX, alpha 2	COL9A2
NM_001837	1	Chemokine (C-C motif) receptor 3	CCR3
NM_001824	1	Creatine kinase, muscle	CKM
NM_001745	1	Calcium modulating ligand	CAMLG
NM_001677	1	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1
NM_001674	1	Activating transcription factor 3	ATF3
NM_001638	1	Apolipoprotein F	APOF
NM_001631	1	Alkaline phosphatase, intestinal	ALPI
NM_001625	1	Adenylate kinase 2	AK2
NM_001533	1	Heterogeneous nuclear ribonucleoprotein L	HNRNPL
NM_001528	1	HGF activator	HGFAC
NM_001504	1	Chemokine (C-X-C motif) receptor 3	CXCR3
NM_001428	1	Enolase 1, (alpha)	ENO1
NM_001400	1	Sphingosine-1-phosphate receptor 1	S1PR1
NM_001311	1	Cysteine-rich protein 1 (intestinal)	CRIP1
NM_001308	1	Carboxypeptidase N, polypeptide 1	CPN1
NM_001292	1	CDC-like kinase 3, transcript variant phclk3/152	CLK3
NM_001252	1	CD70 molecule	CD70
NM_001154	1	Annexin A5	ANXA5
NM_001120	1	Major facilitator superfamily domain containing 10	MFSD10
NM_001069	1	Tubulin, beta 2A	TUBB2A
NM_001051	1	Somatostatin receptor 3	SSTR3
NM_000976	1	Ribosomal protein L12	RPL12
NM_000906	1	Natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)	NPR1
NM_000878	1	Interleukin 2 receptor, beta	IL2RB

NM_000864	1	5-hydroxytryptamine (serotonin) receptor 1D	HTR1D
NM_000853	1	Glutathione S-transferase theta 1	GSTT1
NM_181657	1	Leukotriene B4 receptor	LTB4R
NM_000738	1	Cholinergic receptor, muscarinic 1	CHRM1
NM_000666	1	Aminoacylase 1	ACY1
NM_000641	1	Interleukin 11	IL11
NM_000576	1	Interleukin 1, beta	IL1B
NM_000543	1	Sphingomyelin phosphodiesterase 1, acid lysosomal	SMPD1
NM_000525	1	Potassium inwardly-rectifying channel, subfamily J, member 11	KCNJ11
NM_000512	1	Galactosamine (N-acetyl)-6-sulfate sulfatase	GALNS
NM_000424	1	Keratin 5	KRT5
NM_000399	1	Early growth response 2 (Krox-20 homolog, Drosophila)	EGR2
NM_000394	1	Crystallin, alpha A	CRYAA
NM_000391	1	Tripeptidyl peptidase I	TPP1
NM_000387	1	Solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	SLC25A20
NM_000356	1	Treacher Collins-Franceschetti syndrome 1	TCOF1
NM_000266	1	Norrie disease (pseudoglioma)	NDP
NM_000264	1	Homo sapiens patched homolog (Drosophila)	PTCH
NM_000196	1	Hydroxysteroid (11-beta) dehydrogenase 2	HSD11B2
NM_000172	1	Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	GNAT1
NM_000094	1	Collagen, type VII, alpha 1	COL7A1
NM_000093	1	Collagen, type V, alpha 1	COL5A1
NM_000066	1	Complement component 8, beta polypeptide	C8B

2.8.3 Supplemental figures



Supplemental figure 2-1. Gain and loss of function for Sox8 does not affect differentiation *in vitro*. (a) Dissociated cell scoring for marker positive cells from 7DIV P0.5 retinal explants electroporated with pUB-GFP and scrambled shRNA (n=4), Smo-M2 +scrambled shRNA(n=4) or with Smo-M2 + shRNA targeting Sox8 (n=4). An asterisk denotes significance ($p<0.05$) over scrambled electroporated explants. (b) Knockdown effects of shSox8 on the Smo-M2 induced Sox8 expression in electroporated P0.5 retinal explants (n=3 for each). Values represent *Sox8* expression as a percentage compared to Smo-M2 +shScram normalized to GFP. An asterisk denotes significance ($p<0.05$) compared to Smo-M2+ shScram electroporated explants. (c) Dissociated cell scoring of marker positive cells from P0.5 retinal explants electroporated with pUB-GFP and empty vector or Sox8 (n=5) or Smo-M2 (n=4) and cultured for 7 DIV. An asterisk denotes significance ($p<0.05$) over empty vector control plasmid electroporation. Markers included B630 to identify rod photoreceptors, PKC for bipolars, HPC1 for amacrine and CRALBP for Muller glia. Error represent SEM and significance was calculated using a student's t-test.


800
600
400
200
0
markerpositive cells / 500 um2
Wildtype
Sox8 KO
ONL nuclei
Chx10
PouF4
CRALBP
Calbindin

Supplemental figure 2-2. Adult retinas of *Sox8* KO animals do not have a defect in cell types. Quantification of cell types in adult *Sox8* KO (n=3) and wildtype (Sox8 +/-)(n=3) retina revealed no significant differences in the various cell types. Markers included were B630 to identify photoreceptors, Chx10 for amacrine, PouF4 for ganglion cells, CRALBP for Muller glia and Calbindin to identify horizontal cells. Photoreceptors were quantified as the number of nuclei in the outer nuclear layer. Counts were performed on 3 sections per retina at the level of the optic nerve head.

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Chapter 3

Norrie disease protein is a direct target of Hedgehog/Gli and mediates Hedgehog-driven neural progenitor cell cycle re-entry and survival

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3.1 Co-authorship statement:

This project was carried out under the supervision of Dr. Valerie Wallace. Brian McNeill generated the data for all Figures with the exception of Figure 3-1 a-c which was generated by Chantal Mazerolle. *Ndp* KO animals were generated at Lexicon pharmaceuticals and kindly provided by Dr. Denis Rice and Kim Peas who also provided prepared BrdU pulsed embryonic and postnatal fixed tissue samples for analysis.

3.2 Abstract

The Hedgehog (Hh) signaling pathway is a key regulator of growth and cell fate determination in the developing brain and aberrant Hh pathway activity is implicated in the development of brain tumors. In the developing eye, retinal progenitor cell (RPC) proliferation and cell fate is dependent on Shh but the downstream regulation of these processes is poorly understood. Here we identify *Norrie disease protein* (*Ndp*) as a novel mediator of Hh-dependent RPC development. *Ndp*, which encodes for the secreted Norrin protein, regulates retinal vasculogenesis and is mutated in Norrie Disease, an X-linked condition associated with severe retinal hypovascularization and blindness. We show that Hh is necessary and sufficient for *Ndp* expression in RPCs and it promotes Gli2 binding to the *Ndp* promoter, suggesting that the interaction is direct. *Ndp* is required for RPC cell cycle re-entry *in vivo* and for Hh-induced proliferation and cell fate in the perinatal retina. The requirement for *Ndp* is autonomous to RPCs suggesting that Norrin functions in a paracrine loop to promote proliferation. These data identify a novel mechanism of Hh-mediated growth control and a function for *Ndp* that is separate from its role in vasculogenesis. These observations could lead to an improved understanding of the extraocular defects seen in ND patients.

3.3 Introduction

The hedgehog signaling pathway is essential for neural progenitor proliferation in the developing brain and gain of function for this pathway is causally implicated in the development of brain tumors in humans. In the developing retina, a tractable model for the molecular analysis of neural progenitor proliferation, Sonic Hedgehog (Shh) is the ligand of the pathway. Produced by ganglion cells (GC), Shh signals to the pool of multipotent retinal progenitor cells (RPCs) to regulate proliferation (Sakagami et al., 2009), self renewal (Wang et al., 2005) and cell fate determination (Wang et al., 2005; Zhang and Yang, 2001). The effects of Hh signaling are mediated primarily through Gli-dependent regulation of target genes. Of the three mammalian Gli genes (Gli1-3), Gli2 generally functions as a positive regulator of the transcriptional output of the pathway with Gli1 serving an amplification role while Gli3 functions primarily to repress target gene expression when the pathway is inactive (Bai and Joyner, 2001). In RPCs, Gli2 is required for the transcription of *Gli1*, a universal Hh target gene, as well as *CyclinD1* and *Hes1* (Wall et al., 2009). However, the full mechanism of Hh-dependent effects on RPCs is not known.

In a screen for genes with conserved Gli binding sequences (see Chapter 2) we identified *Norrie Disease Protein* (*NDP*). *NDP* encodes Norrin, a 133 amino acid protein with an N-terminal signal sequence and a cysteine-knot motif at its carboxyl-terminal (Meitinger et al., 1993). Norrin is known primarily for its role in retinal angiogenesis, as mutations in *NDP* cause several human eye diseases, including Norrie disease (ND), X-linked familial exudative vitreoretinopathy (FEVR), Coat's disease and severe retinopathy of prematurity (reviewed by Warden et al., 2007), which all feature severe vascular defects in the eye resulting in visual impairment. ND patients also exhibit progressive hearing loss and

mental retardation (Berger et al., 1996; Halpin et al., 2005) and *Ndp* knockout (KO) mice exhibit retinal and cerebellar degeneration (Berger et al., 1996; Luhmann et al., 2008), suggesting that Norrin has additional non-retinal targets and functions. Despite the importance of Norrin function in angiogenesis and sensorineural development, the regulation of *Ndp* expression is unknown.

In the present study we explored the regulation of *Ndp* by the Shh pathway. We show that Hh signaling via Gli2 is required for normal levels of *Ndp* expression in the retina and that *Ndp* is sufficient to promote RPC proliferation and the development of inner layer retinal neurons. Importantly, we show that *Ndp* is required downstream of Hh signaling for RPC proliferation and neuronal differentiation. *Ndp* mediates its effects by enhancing RPC cell cycle re-entry rather than cell cycle kinetics. Finally, we show that Norrin signaling in RPCs does not require the canonical Wnt signaling pathway, distinguishing Norrin signaling in RPC development from its role in angiogenesis.

3.4 Materials and Methods

Experimental animals

The following transgenic mouse strains were used this in study: *TCF/Lef-LacZ* reporter mice (Mohamed et al., 2004), $Ndp^{-/Y}$ (Ndp KO) (Junge et al., 2009), α-Cre (Marquardt et al., 2001), $Shh^{+/-}$, $Shh^{c/c}$ (St-Jacques et al., 1998), $Gli1^{+/-}$ and $Gli2^{+/-}$ (Mo et al., 1997) and $Gli3^{xt}$ (Hui and Joyner, 1993). *Shh* conditional KO mice (Shh^{CKO}) were generated as described in Chapter 2. *Ndp* KO mice were generated by crossing $Ndp^{-/Y}$ males with $Ndp^{+/-}$ females and only $Ndp^{-/Y}$ and $Ndp^{+/Y}$ were used for all analyses. 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)

Retinal explants.

Retinal explants were generated by removing the lens and RPE from the eye and flattening the retina onto polycarbonate filters (0.8 μm pore size, Nucleopore). Explants were cultured in medium for 2 to 7 days *in vitro* (DIV) at 8% CO_2 and 37^{o}C in 0.5ml of 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 (25μg/ml)] supplemented with Smo agonist (Smo-Ag; 20 nM; a kind gift from Curis Inc), recombinant Norrin (; 200 ng/ml; R & D systems), recombinant Dkk1 (1 μg/ml; R & D systems); Norrin peptide (40 μg/ml; ProSci); Sox8 peptide (40μg/ml; Santa Cruz Biotechnology), Lithium Chloride (LiCl; 20mM), Sodium Chloride (NaCl; 20mM), or GSK-3β inhibitor 6-bromoindirubin-3'-oxime (BIO) (2.5μM; Tocris). 7DIV explants were also cultured in the presence of 10% fetal bovine serum (FBS). The concentration of recombinant Norrin and Norrin peptide were determined based on a dose response curve measuring BrdU incorporation in P0.5 explants and Topflash reporter activity, respectively (Supplemental Fig. 3-1 and 3-3). *In vivo* BrdU labeling was performed on pregnant gestation day E14.5 females and P0.5 pups and consisted of two BrdU injections over a 4 hour period. BrdU incorporation in explants was performed with a 2 (E14.5) or 4 (P0.5) hour BrdU pulse on the final day of culture. To analyze cell cycle re-entry in the *Ndp* KO animals, pups received a single BrdU pulse and were harvested two days later for dissociation and IHC. To label most RPCs in a P0.5

retina, gestation day 19 pregnant females were BrdU pulsed every 4hours over a 24hour period prior to retina isolation from pups for *in vitro* culture. *In vitro* BrdU incorporation for E14.5 retinal explants included a 2h pulse on the final day of culture and P0.5 explants received a 5h pulse. Single cell dissociates were obtained with 0.1mg/ml trypsin (Sigma) treatment in sterile calcium-free PBS (Invitrogen) for 15min at 37^{o}C. Trypsinization was inhibited with 10% FBS/ DMEM/ DNAseI (0.2mg/ml, Sigma) and tissue was triturated to obtain a single cell suspension. Cells were plated on glass slides for 15 min at room temperature followed by 40 min at 37^{o}C before a 5 min 4% paraformaldehyde (PFA) fix. Cells were processed immediately for immunocytochemistry (IHC) or stored at -20^{o}C.

RNA extraction and RT-qPCR.

Total RNA was extracted from freshly extracted retinas or from retinal explants using Tri ® reagent (Sigma-Aldrich) following the manufacturer's instructions and DNase treated (Sigma-Aldrich). First strand cDNA was synthesized from 2μg total RNA using M-MLV reverse transcriptase (Invitrogen) and random hexamer primers (Fermentas). Target gene mRNA levels were assessed by RT-qPCR using SYBR® Green Jumpstart™ Taq Readymix™ (Sigma-Aldrich) and a MX3000P multiplex QPCR system (Agilent Technologies). Primer pairs (Table 3-1) were designed using DNAMAN software (Lynnon Biosoft) and primer3 (v. 0.4.0) (Rozen and Skaletsky, 2000). The specificity of the *Ndp*, *Ptch1*, *Gli1*, *18S* and *Gapdh* primers were verified by direct sequencing of amplified products. Relative changes in mRNA expression of target genes were determined using the Δ-ΔCt method (Pfaffl, 2001) normalized against *18S* and *Gapdh*.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed on E14.5 and P0.5 retinal explants treated with and without Smo-Ag for 3DIV using the EZ ChIP kit (Millipore) according to the manufacturer's instructions. Briefly, explants were fixed in 4% PFA for 30 min at RT and sonicated to generate DNA fragments between ~200-1000 base pairs in length. Immunoprecipitation was performed using a goat anti-Gli2 polyclonal antibody (Santa Cruz Biotechnology) and protein G agarose beads. Goat anti-Brn3b (Santa Cruz Biotechnology) was used as a control antibody. DNA was analyzed by qPCR using Brilliant® SYBR® Green mastermix (Agilent Technologies) and primer sets (Table 3-1) that span regions within the *Ndp* promoter that contained at least a 7 bp match to the Gli consensus GACCACCCA or TGGGTGGTC sequence (Kinzler and Vogelstein, 1990). To calculate the enrichment of Gli2 to a particular binding site within the *Ndp* promoter, the values obtained for each target was divided by the amount of the corresponding target in the input fraction. Values were normalized against mock IPs performed in parallel using normal goat IgG and values are expressed as fold enrichment of Smo-Ag treated compared to untreated explants.

Luciferase assays

P0.5 retinal explants were electroporated with 0.4 µg/µl of *Ndp* plasmid (Origene), 0.2 µg/µl of *Fzd4* plasmid (Open Biosystems), 0.2 µg/µl of *Lrp5* plasmid (Open Biosystems), 0.15 µg/µl of Topflash reporter plasmid (Millipore) and 0.05 µg/µl of the transfection control renilla luciferase plasmid (Promega). For samples in which one or more components were omitted, the DNA was adjusted to the appropriate µg with an empty Sport 6.1 vector. Luciferase activities were measured 48 hr post transfection with a Dual-

Table 3-1. Summary of qPCR primers

Symbol	Forward	Temp	Reverse	Temp
18S	CGGCTACCACATCCAAGG	64.1	CTGGAATTACCGCGGCT	63.8
Bmi1 F	TGTCCAGGTTCACAAAACCA	64	CGGGTGAGCTGCATAAAAAT	63.7
Braca2	ACCAGTCGCCTTTCAGAGAA	63.9	CACAGGGTCCACTTTGGTCT	64
Ccna1	TTCTGGAAGCTGACCATTC	64.1	GGCAAGGCACAATCTCATTT	63.9
Ccna2	GTGTCACAGCCGATAGCAGA	64.1	GTAGAGGATGTGCGGAAGCA	65.3
Ccnd1	AGGAAGCGGTCCAGGTAGTT	63.8	AGTGCGTGCAGAAGGAGATT	63.9
Ccnd2	TCCCGCAGTGTTCCTATTTC	63.8	GGTAATTCATGGCCAGAGGA	63.7
Ccne1	TTTGCCTTCCTTTTTCTGGA	63.4	CTGAGTTCCAAGCCCAAGTC	63.7
Ccne2	CCCAGATAATTCAGGCCAAG	63.2	TGATTCCTCCAGACAGTACAGGT	63.7
Cdh11	CTGAAGCCTTCGACATAGCC	63.7	TCGTCCACATCCACACTGTT	64.2
Chek1	AATGTTGGCTGGAGAATTGC	63.9	AGCCAGAGGAGCAGAATCAA	64
ChIP 10	TAAGATCCTTCCCCAATCTG	60.4	AAAGACACCGTCAATAAGACAAA	61.3
ChIP 10.7	CACAAGCACCAAATCAATTG	61.5	GCTCTTTCCATGAGGCC	61
ChIP 16.6	AATCTGAATAAACCCTCCTGCC	64	TGGTTCTGGCCAGAGGATAA	64
ChIP 18	ATTCTTGGAGGCTGTTCACAC	63	CTTCCTCCCTGTGTCACACT	61.8
ChIP 18.2	AACATACTAACGGACCACCAAG	61.6	CTTCCATTGTGCCTCCCT	62.7
Egfp	TATATCATGGCCGACAAGCA	63.9	GAACTCCAGCAGGACCATGT	64.2
Gapdh	TGAAGGGGTCGTTGATGG	64.7	AAAATGGTGAAGGTCGGTGT	63.1
Gli1	CACTACCTGGCCTCACACCT	66.2	GTACTCGGTTCGGCTTCTCC	64.4
Hes1	AAGACGGCCTCTGAGCACA	66.3	TCATGGCGTTGATCTGGGTCA	71
Mcm2	AATGACCAGGACAGGACCAG	64	AAATGATGGGCTCTGTGAGG	64
Mdm2	CTTCGTGAGAACTGGCTTCC	63.9	TGTCAGCTTTTTGCCATCAG	63.9
Nanog	ACCCAACTTGGAACAACCAG	63.7	CGTAAGGCTGCAGAAAGTCC	63.7
Ndp	GCTGGCCATAATGGGAGATA	63.7	AGTGCTGAAGGACACCAAGG	63.7
Oct4	AGGCCTGGAAGAAAAAGAGC	63.5	CGAACCATGTCCTTCTCCAT	63.8
Ptch1	TAGCCCTGTGGTTCTTGTCC	64	TGTGGTCATCCTGATTGCAT	64.1
Sens2	CCTTCTCCACACCCAGACAT	64	TATAATCCTGGGCACGGAAG	63.6
Skp2	CTCCAACACCTCTCGCTCA	64.3	GTTCCCTCTGGCACGATTC	64.6

Luciferase Assay Kit (Promega). Topflash activity was corrected for Renilla activity and the results are expressed as a ratio of Topflash/Renilla.

Immunohistochemistry (IHC) and *In situ* hybridization (ISH)

Sample preparation consisted of a 1 hour 4% PFA in PBS fix for explants or overnight for entire eyes. Samples were transferred to a 30% sucrose/PBS solution overnight and embedded in a 1:1 30% sucrose OCT mixture. 12 μm sections were obtained using a Leica 1850 cryostat. IHC antibodies used in this study include rabbit anti-Ki67 (Abcam), mouse anti-Ki67 (BD Bioscience), goat anti-Brn3b (Santa Cruz Biotechnology), mouse anti-Pax6 (Abcam), mouse anti-BrdU (BD Pharmigen), rabbit anti-CRALBP (Abcam), mouse anti-rhodopsin (Rohlich et al., 1989), mouse anti-syntaxin (HPC1, Sigma), mouse anti-PKC (BD Bioscience), rabbit anti-recoverin (Millipore), sheep anti-Chx10 (Abcam), rabbit anti-phosphohistone H3 (pH3Millipore), and rabbit anti-GFP (Invitrogen), FITC-conjugated anti-GFP (Santa Cruz Biotechnology), mouse anti-calbindin (Sigma). Secondary antibodies include donkey anti-goat Alexa Fluor 488 (Invitrogen), goat anti-rabbit Alexa Fluor 488 (Invitrogen), donkey anti-rabbit Alexa Fluor 647, goat anti-mouse IgG Cy3 (Jackson ImmunoResearch Laboratories), and goat anti-rabbit Alexa Fluor 546 (Invitrogen). For IHC staining, samples were rehydrated in PBS, blocked in 10% FBS/PBS for 1 h and incubated in primary antibody overnight at 4^{o}C. Samples were washed in PBS and incubated in secondary antibody for 1 h, washed in PBS and treated with Hoechst to stain the nuclei. An antigen retrieval step consisting of a 10 min microwave in a Sodium Citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) followed by a PBS wash was performed prior to blocking for BrdU, Ki67, Chx10, pH3 and Brn3b staining. For ISH,

samples were hybridized overnight at 65^{o}C in a humidified box with riboprobes diluted in 50% Formamide, 10% dextran sulfate, 1mg/ml yeast RNA, 1X Denhardt's and 1X salt. Samples were washed 3X with 50% Formamide, 1X SSC, 0.1% Tween-20 at 65^{o}C for 30 min followed by 2X washes in MABT (100 mM Maleic acid, 150 mM NaCl, 0.1 % Tween-20) for 30 min at room temperature. Samples were blocked 2 h with MABT containing 20 % sheep serum and 2% blocking reagent (Roche). Following blocking, samples were incubated with alkaline-phosphatase-conjugated Fab fragments of sheep anti-DIG antibodies (Roche) diluted in blocking solution overnight at 4^{o}C. Samples were washed 4X in MABT solution for 20 min and 2X in staining buffer (100 mM NaCl, 50mM $MgCl_2$, 100mM Tris pH 9.5 and 0.1% tween-20) and incubated in staining buffer containing 4.5 µl NBT and 3.5 µl BCIP/ml (Roche). The antisense riboprobes used for ISH include *Gli1* (a gift from A. Joyner) and *Ndp*. Bright field images were analyzed using an Axioplan microscope and captured with an Axiovision camera (2.05; both from Carl Zeiss, Inc.). Fluorescent images were analyzed using an Axiocam microscope (HRm) and captured with an Axioimager camera (M1; both from Carl Zeiss, Inc). All images were processed using Photoshop CS4 (Adobe).

Electroporation

In vitro electroporation was performed on retinal explants as previously described (Matsuda and Cepko, 2004). Briefly, retinas were electroporated at a 10:1 ratio with expression vectors (or empty vector) (1 µg/µl) and pUb-GFP in 2-mm gap cuvettes (VWR) using 5 square 30V pulses of 50-ms duration and 950-ms intervals using a ECM830 pulse generator (BTX Harvard Apparatus). *In vivo* electroporation was performed on

isoflurane/O2 anesthetised newborn pups as previously described (Matsuda and Cepko, 2007). Approximately 0.5 µl of DNA solution (3 to 6 µg/ul) in PBS containing 0.1% fast green as a tracer was injected into the sub-retinal space of the right eye and electroporated using 80 square 30V pulses of 50-ms duration and 950-ms intervals. Electroporation was accomplished using the ECM830 pulse generator (BTX Harvard Apparatus). The DNA plasmids used in this study include: pCMV *Smo-M2* (a gift from G. Fishell, New York University Langone Medical Center, New York, NY), pCMV6 *Ndp* (Origene), *pUb-GFP* (a gift from T. Matsuda, Harvard Medical School, Boston, MA), Sport6.1 *Fzd4* (Open Biosystems), pKLO.1 *Ndp shRNA* (TRCN0000104966 and TRCN000010468; Open Biosystems), pKLO.1 *Fzd4 shRNA* (TRNCN0000071678; Open Biosystems), Sport6.1 *Lrp5* (Open Biosystems), *shRNA pLKO.1-puro* control vector (Scrambled; Sigma), *Topflash* (Millipore) and *Renilla* (Promega).

Western blotting

Proteins were extracted from 3DIV Smo-Ag treated and non-treated retinal explant using RIPA buffer [125 mM Tris-HCL, 2% SDS + protease inhibitor cocktail (Roche)] and quantified using Bradford assay (BioRad). Protein samples were run on a 12% SDS-reducing gel and transferred to a nitrocellulose membrane. Protein samples were probed with goat polyclonal anti-Norrin (1:250; R & D systems), or 1:50 dilution of mouse monoclonal antibody anti-tubulin (E7 ascites; Developmental Studies Hybridoma Bank). Secondary antibodies used include donkey anti-goat HRP (1:15,000; Santa Cruz Biotechnology, Inc.) and sheep anti-mouse HRP (1:15,000; Sigma-Aldrich). The signal was

developed using enhanced luminescence (GE Healthcare) and quantified by densitometry and normalized to tubulin using Image J.

Statistics

Statistical analysis between groups was calculated using a two-tailed Student's t-test. Values of $p < 0.05$ were considered to be significant and error bars represent standard error of the mean (SEM).

3.5 Results

Ndp expression is regulated by the Hh pathway

Ndp is required for development of the retinal vasculature postnatally, yet it has been reported to be expressed in the embryonic retina prior to the onset of vasculogenesis (Berger et al., 1996). We examined the pattern of *Ndp* expression in the embryonic and perinatal retina by *in situ* hybridization. At embryonic day 14.5 (E14.5) the retina is comprised of two cellular layers, the postmitotic GC layer and the neuroblast layer which contains dividing RPCs. At this stage *Ndp* is expressed at low levels in both retinal layers and by postnatal day 0.5 (P0.5), *Ndp* expression appears to be increased within the GC and the inner aspect of the neuroblast layers (Fig. 3-1 a-c). Thus, *Ndp* is expressed in the neuroblast layer and in postmitotic neurons prior to the onset of retinal angiogenesis in the central retina at P0.

To investigate the relationship between Hh signaling and *Ndp* we first examined *Ndp* expression in retinas of newborn mice with a conditional inactivation of *Shh* in the peripheral retina (Shh^{CKO}) (Wang et al., 2005). Compared to wildtype littermates, the

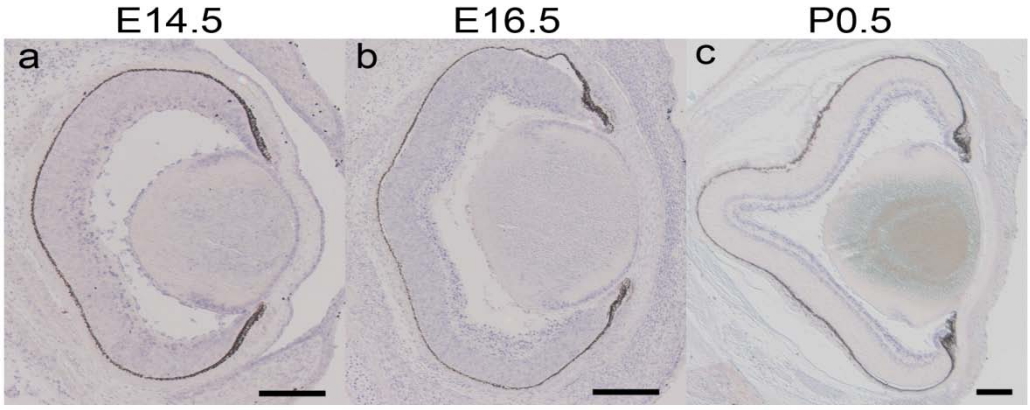

E14.5
E16.5
P0.5
a
b
c

Figure 3-1. Expression of *Ndp* in the developing retina. *In situ* hybridization showing localization expression of *Ndp* in (a) E14.5, (b) E16.5 and (c) P0.5 retinas. Scale bar equals 200μm.

expression of *Ndp* at P0.5 in the Shh^{CKO} retinas was reduced by 4-fold (Fig. 3-2 a), indicating that Shh signaling is required for *Ndp* expression in the perinatal mouse retina. To investigate the effect of acute Hh pathway activation on *Ndp* expression, an *in vitro* retinal explant model system was used. Several features of normal retina development are recapitulated in retinal explants (Zhang et al., 2002) however, because of the rapid death of GCs, the primary source of Shh, they represent an acute loss of function model for Hh signaling (Wang et al., 2002). Hh signaling can be restored in these cultures by treatment with a Hh- Agonist (Smo-Ag) that binds to and activates Smo, the signaling moiety of the pathway (Frank-Kamenetsky et al., 2002; Wang et al., 2002). In explants derived from E14.5 and P0.5 mice, Smo-Ag treatment increased *Ndp* expression by ~16 and ~5 fold, respectively (Fig. 3-2 b) and increased Norrin protein levels ~ 3-fold in P0 explants (Fig. 3-2 c). To determine whether Hh activation in dividing progenitors is sufficient to induce *Ndp* expression, we electroporated retinal explants with an expression vector encoding *Smo-M2*, which drives cell autonomous, ligand independent, constitutive Hh pathway activation (Xie et al., 1998). Because electroporation targets dividing cells (Matsuda and Cepko, 2004), this approach allowed us to monitor the effects of increased Hh pathway activation in RPCs. The expression of *Ndp* and *Gli1*, a positive control for Hh activation, were specifically increased in *Smo-M2* expressing regions of the explants (Fig. 3-3 a-d'), demonstrating that the effects of Hh on *Ndp* upregulation are local to, if not autonomous, to Hh-responsive cells. Taken together, these results show that Shh and Hh pathway activation are necessary and sufficient to promote *Ndp* expression in the embryonic and perinatal retina at a stage prior to the onset of *Ndp*-dependent vascular development.

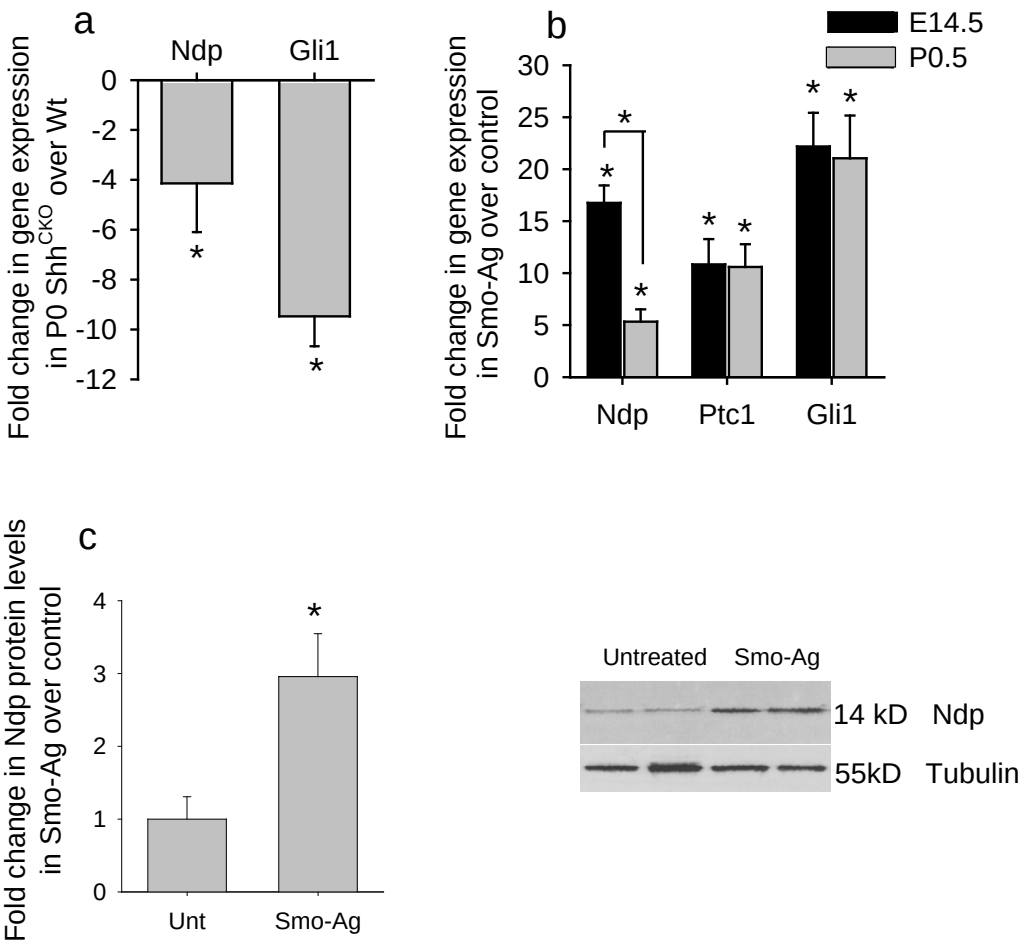

a
Ndp
Gli1
0
-2
-4
-6
-8
-10
-12
Fold change in gene expression in P0 Shh^CKO over Wt
b
E14.5
P0.5
30
25
20
15
10
5
0
Fold change in gene expression in Smo-Ag over control
Ndp
Ptc1
Gli1
c
4
3
2
1
0
Fold change in Ndp protein levels in Smo-Ag over control
Unt
Smo-Ag
Untreated
Smo-Ag
14 kD Ndp
55kD Tubulin

Figure 3-2. Shh regulation of *Ndp* expression. . (a) RT-qPCR analysis of *Ndp* and Hh target gene expression in whole retinas of Shh^{CKO} (n=6) and wildtype (n=6) mice at P0.5. (b) RT-qPCR analysis of *Ndp, Ptch* and *Gli* mRNA in E14.5 and P0.5 retinal explants cultured for 3 DIV with and without Smo-Ag (E14.5 n=6 untreated, n= 6 Smo-Ag ; P0.5 n=6 untreated, n=6 Smo-Ag) . Values represent fold change in gene expression relative to wildtype or control explants and the data are normalized using *Gapdh* and *18S*. (c) Western blot for Norrin protein in untreated and Smo-Ag treated P0.5 retinal explants cultured for 3 DIV (n=4 for untreated and Smo-Ag treated). β-tubulin was used as a loading control. Protein densitometry quantification was performed with Image J. Error bars represent s.e.m., *$p < 0.05$ Student's t-test.

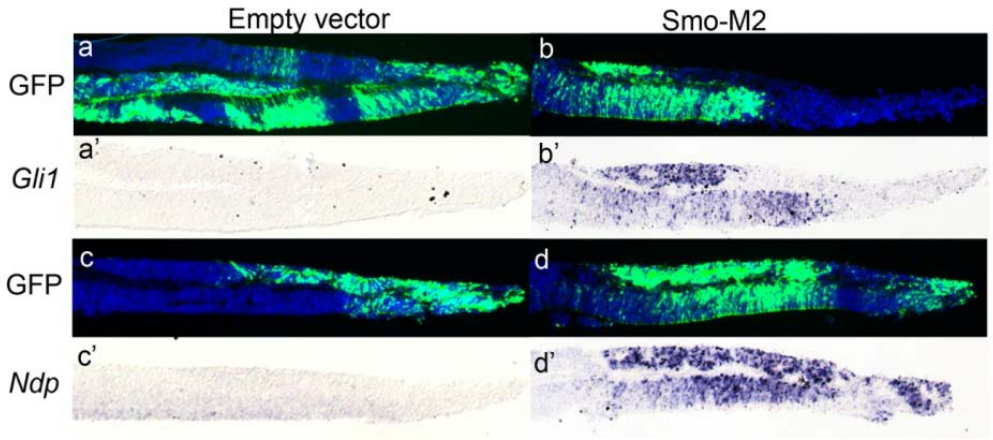

Empty vector
Smo-M2
GFP
a
b
Gli1
a'
b'
GFP
c
d
Ndp
c'
d'

Figure 3-3. Hh pathway activation increases *Ndp* expression. GFP immunoflourescence and Hoechst nuclear labelling (a,b,c,d) and *in situ* hybridization for *Gli1* (a', b') and *Ndp* (c', d') in serial sections of P0.5 retinal explants that were co-transfected with GFP and empty vector or *Smo-M2* and cultured for 3 DIV. The GFP fluorescence indicates the location of transfected cells in the tissue.

Ndp is a direct target of Gli2

Next we compared the expression of *Ndp* in Hh stimulated retina explants from *Gli* KO mice. Smo-Ag induction of *Ndp* expression was reduced ~ 50% in *Gli2* KO explants compared to wildtype controls, whereas it was unaffected in *Gli1* and *Gli3* KO explants (Fig. 3-4 a and b). Gli2 is also required for *Ndp* expression *in vivo*, as the level of endogenous *Ndp* expression was significantly reduced in *Gli2* KO retinas compared with wildtype littermates (fold change in *Gli2* KO relative to control 2.9-fold ±0.4 SEM, $p < 0.05$). Gli3 is likely to antagonize *Ndp* expression, as *Ndp* expression is de-repressed in unstimulated *Gli3* KO explants (Fig. 3-4 b). Thus Gli2 and Gli3 function as positive and negative regulators, respectively, of *Ndp* expression downstream of Hh activation.

Given the positive relationship between Gli2 and *Ndp* expression we addressed whether Gli2 interacts with the promoter of *Ndp* by performing ChIP on E14.5 and P0.5 retinal explants. We identified candidate Gli interacting elements at -366bp +9972, 10737, 16597, 18005 and 18226 bp relative to the transcriptional start side (TSS) (Fig. 3-4 c). qPCR analysis on anti-Gli2 ChIP samples revealed Smo-Ag treatment resulted in an enrichment of Gli2 at positions -366, 9972, 16597 and 18005/18226 compared with control explants at E14.5 (Fig. 3-4 d) and -366, 9972 and 18005/18226 at P0.5 (Fig. 3-4 e). Because the sites located at position 18005 and 18226bp are too close to one another to be separated efficiently by sonication, we cannot rule out the possibility that Gli2 is interacting with only one of these sites. Therefore these sites are represented together. These data show that Hh regulation of *Ndp* requires Gli2 and the ChIP studies suggest that the relationship between Gli2 and *Ndp* is direct.

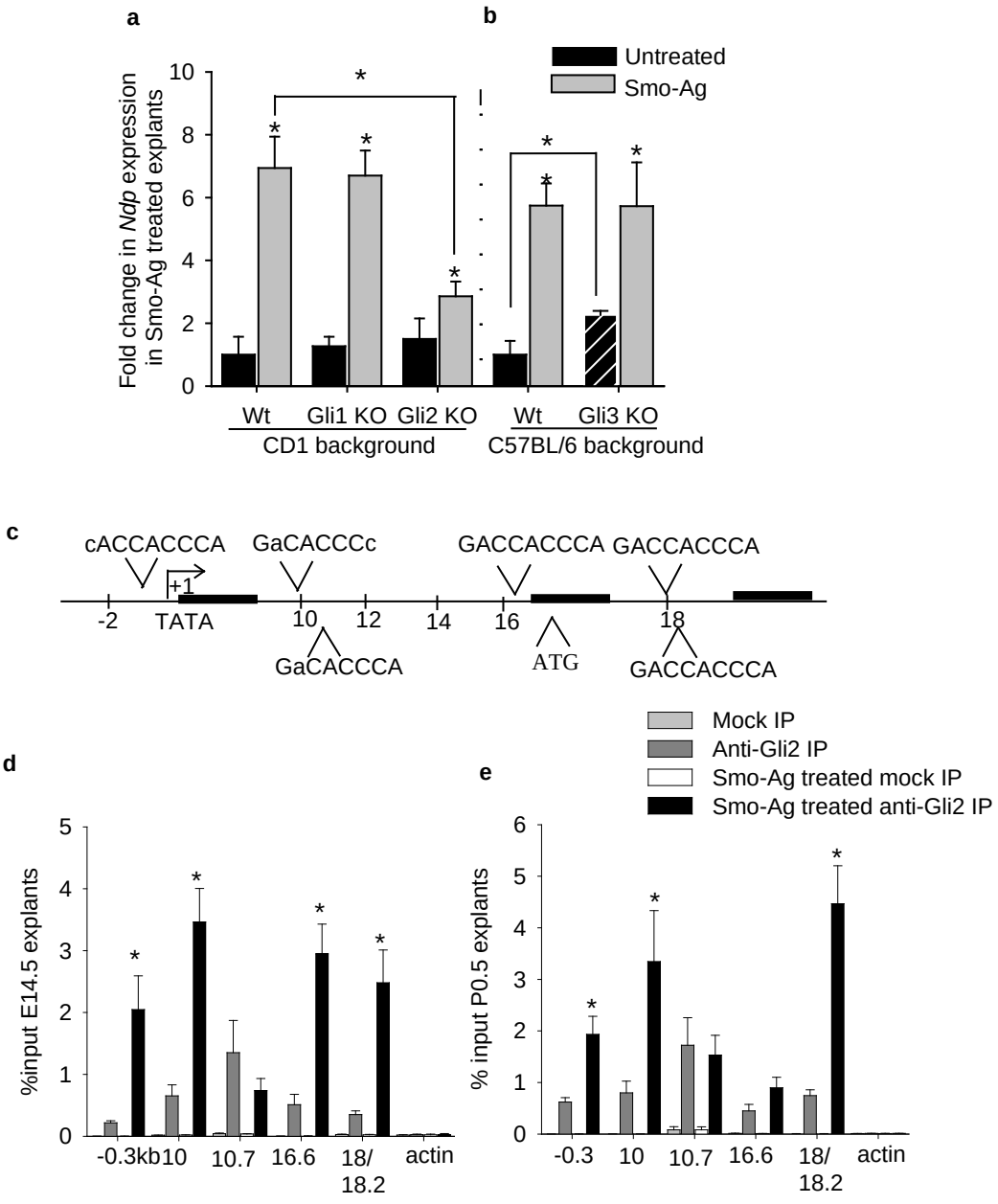
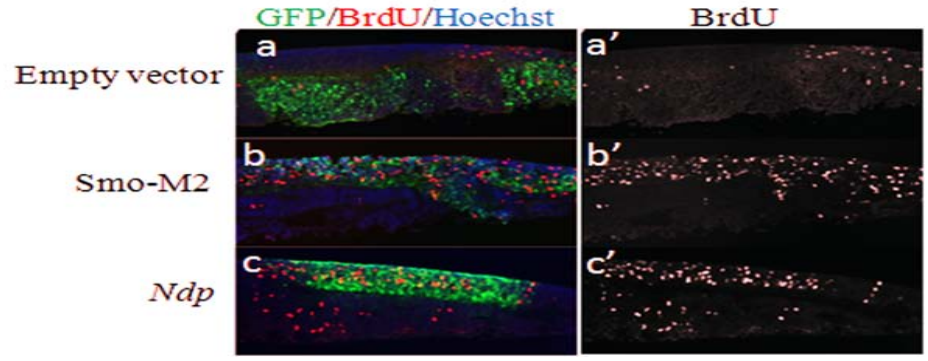

a
b
Untreated
Smo-Ag
Fold change in Ndp expression in Smo-Ag treated explants
Wt
Gli1 KO
Gli2 KO
Wt
Gli3 KO
CD1 background
C57BL/6 background
c
cACCACCCA
GaCACCCc
GACCACCCA
GACCACCCA
+1
-2
TATA
10
12
14
16
18
GaCACCCA
ATG
GACCACCCA
d
e
Mock IP
Anti-Gli2 IP
Smo-Ag treated mock IP
Smo-Ag treated anti-Gli2 IP
%input E14.5 explants
% input P0.5 explants
-0.3kb
10
10.7
16.6
18/ 18.2
actin
-0.3
10
10.7
16.6
18/ 18.2
actin

Figure 3-4. Shh induction of *Ndp* directly through Gli2. (a,b) RT-qPCR analysis of *Ndp* expression in Wt and *Gli* KO explants. E16.5 explants from Wt (n=6), *Gli1* KO (n=6) and *Gli2* KO (n=6) (a) and *Gli3* KO (n=6) mice (b) were cultured with or without Smo-Ag for 3 days. Values represent average fold change in *Ndp* expression in Smo-Ag treated compared with untreated and the data are normalized to *Gapdh* and *18S*. (c) Schematic of the *Ndp* gene. The Gli2 binding sites (-0.3, +10.0, +10.7, +16.5, +18.0 and +18.2kb) relative to the TSS are indicated with the mismatched nucleotides relative to the ideal Gli consensus sequences in lowercase letter. (d,e) ChIP analysis of E14.5 (d) and P0 (e) retinal explants cultured for 3 DIV under control or Smo-Ag conditions and immunoprecipitated with anti-Gli2 or control goat polyclonal antibodies. Note that since +18.0 and +18.2kb sites are captured by a single primer set they are grouped together. *β-actin* promoter that does not contain a Gli consensus sequence was used as a negative control . Error bars represent s.e.m., *$p < 0.05$

Ndp is required for RPC proliferation and differentiation

Hh regulation of *Ndp* expression in RPCs coincides with the stage at which Hh promotes RPC proliferation (Wang et al., 2005; Wang et al., 2002). To address whether *Ndp* mediates a similar effect on RPCs, retinal explants were electroporated with expression vectors coding for *Ndp* and GFP or an empty vector and GFP and the proportion of transfected (GFP +) cells that were in S-phase (BrdU+) was quantified after 3 DIV. Under these conditions RPCs respond to *Smo-M2*-induced Hh activation, as shown by the increase in the proportion of BrdU positive cells amongst the transfected cohort (Fig. 3-5 a-b' and d). When ectopic *Ndp* was introduced, explants responded similarly, as seen by the increase in the proportion of electroporated cells that incorporated BrdU (Fig. 3-5 c-d). E14.5 explants responded with a ~2 fold increase in proliferation while P0.5 had a ~4 fold increase in BrdU positive cells compared to the empty vector control (Fig. 3-5 d). Similar results were obtained when the media was supplemented with recombinant Norrin protein; a ~1.7 and ~3 fold increase in BrdU incorporation in E14.5 and P0.5 explants respectively (Fig. 3-5 e). Therefore, *Ndp* could be an important component in Shh mediated regulation of RPC proliferation. To address whether *Ndp* is required downstream of Shh to regulate proliferation, shRNA mediated knockdown of *Ndp* was performed in conjunction with a gain-of-function for the Hh pathway. Using two efficient shRNA (Supplemental Fig. 3-2), we observed a 50% reduction in the ability of *Smo-M2* to induce proliferation (Fig. 3-6 a). Similarly, using a Norrin-blocking peptide (Supplemental Fig. 3-3), the increase in proportion of BrdU positive cells by Smo-Ag was significantly reduced in P0.5 explants whereas treatment with the Sox8 peptide had no effect (Fig. 3-6 b). Consistent with the requirement for *Ndp* downstream of Hh to regulate RPC proliferation, *Ndp* KO explants


GFP/BrdU/Hoechst
BrdU
Empty vector
Smo-M2
Ndp
a
a′
b
b′
c
c′

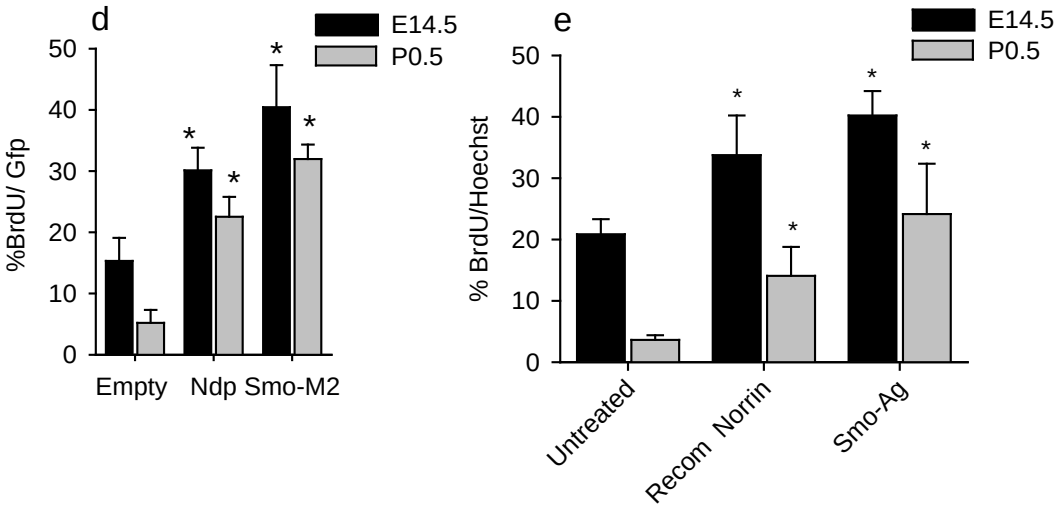

d
E14.5
P0.5
%BrdU/ Gfp
50
40
30
20
10
0
Empty
Ndp
Smo-M2
e
E14.5
P0.5
% BrdU/Hoechst
50
40
30
20
10
0
Untreated
Recom Norrin
Smo-Ag

Figure 3-5. Ectopic *Ndp* increases RPC proliferation. BrdU staining on retinal explants (P0.5 + 3DIV) electroporated with (a, a') empty vector, (b, b') *Smo-M2* and (c, c') *Ndp*. Electroporated regions are indicated by GFP fluorescence. (d) Quantification of BrdU incorporation within the GFP cohort of *Ndp* (E14.5 n=6; P0.5 n=6) or Smo-M2 (E14.5 n=6; P0.5 n=6) electroporated retinal explants (+3DIV). Cell counts were scored in dissociated explants. (e) Retinal explants treated with a recombinant Norrin protein or Smo-Ag (positive control) for 2 DIV (n=6 for each treatment and age). Explants were dissociated and scored for the proportion of BrdU/ Hoechst. Asterisk denotes significance ($p < 0.05$), as calculated using a Student's t-test, between (d) empty vector and *Ndp* or Smo-M2 and (e) between untreated and recombinant Norrin or Smo-Ag. Error bars represent SEM.

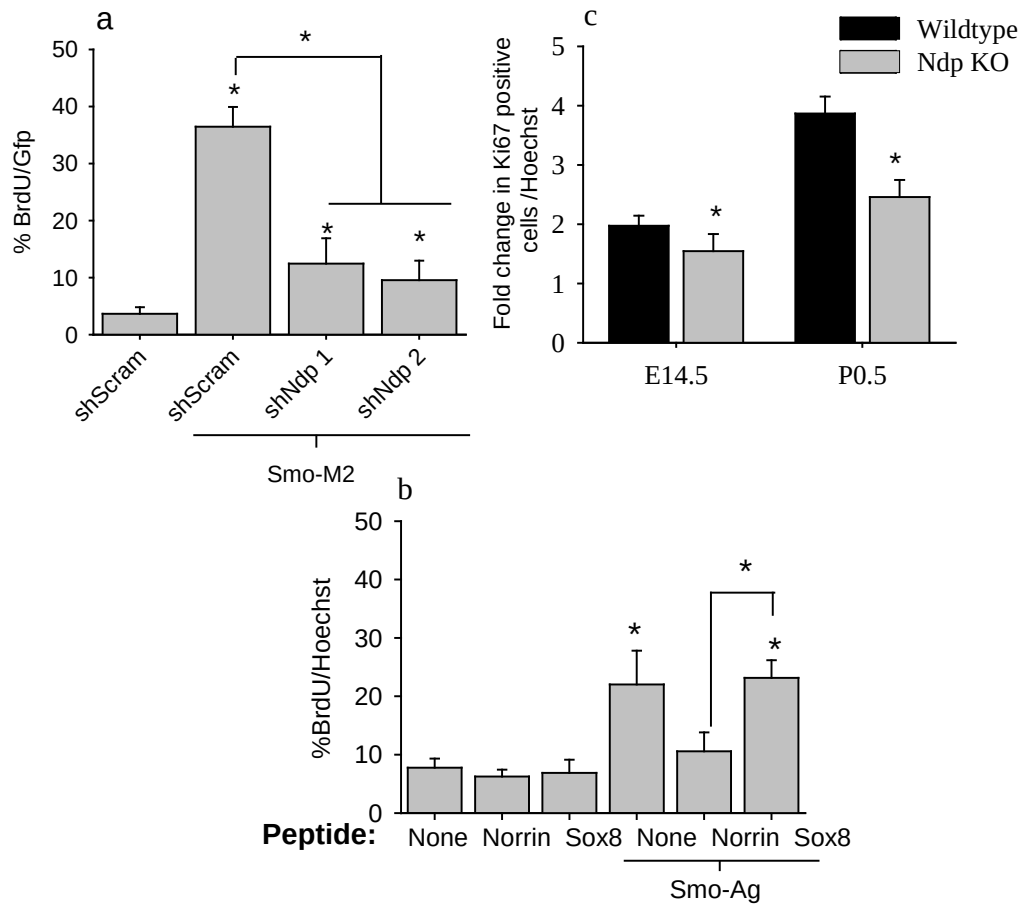

a
% BrdU/Gfp
50
40
30
20
10
0
*
*
*
*
shScram
shScram
shNdp 1
shNdp 2
Smo-M2
c
Fold change in Ki67 positive cells /Hoechst
5
4
3
2
1
0
Wildtype
Ndp KO
*
*
E14.5
P0.5
b
%BrdU/Hoechst
50
40
30
20
10
0
*
*
*
Peptide:
None
Norrin
Sox8
None
Norrin
Sox8
Smo-Ag

Figure 3-6. *Ndp* is required downstream of Hh signaling to promote proliferation. (a) shRNA mediated knockdown of *Ndp* in Smo-M2 electroporated explants (P0.5 + 3DIV). Explants were dissociated and scored for the proportion of BrdU+ GFP+/GFP+ cells. n=6 for eachcondition. (b) Retinal explants (P0.5 + 2DIV) were cultured with a peptide in the presence and absence of Smo-Ag. Explants (n=6 for each treatment) were dissociated and scored for the proportion of BrdU+/ Hoechst. (c) Hh induced proliferation in *Ndp* KO retinal explants. P0.5 explants from wildtype and *Ndp* KO mice were cultured for 3 DIV with (n=6 wildtype; n=6 *Ndp* KO) or without Smo-Ag (n=6 wildtype; n=6 *Ndp* KO) and the proportion of Ki67+ cells was determined. Error bars represent s.e.m., *$p < 0.05$ Student's t-test.

cultured with Smo-Ag also exhibited a reduced number of proliferating cells following 3 days in culture (Fig. 3-6 c).

To investigate the requirement for *Ndp* in RPC proliferation *in vivo* we analyzed the retinas of embryonic and postnatal *Ndp* KO mice. While there was no significant change in RPC proliferation in the embryonic *Ndp* KO retina, we found a significant reduction in the proportion of BrdU positive cells at P0.5 and onwards (Fig. 3-7 a) as well as a reduction in the proportion of Ki67 (data not shown) and pH3 positive cells (Fig. 3-7 b). In addition, there was also a significant increase in the number of apoptotic (TUNEL+) cells in the *Ndp* KO retina beginning at P0.5 (Fig. 3-7 c) and a reduction in total cell number in the postnatal *Ndp* KO retinas (Fig. 3-7 d). Examining the cell cycle rate by scoring the proportion of BrdU positive cells within the Ki67 cohort, we did not detect any significant difference between the *Ndp* KO and wildtype littermate retinas (Fig. 3-7 e). While these data show that *Ndp* is required for normal levels of RPC proliferation and for cell survival in the postnatal retina they do not identify the cellular source of *Ndp* that can mediate these effects. To address whether *Ndp* expression in RPCs is required for proliferation we induced *Ndp* knockdown in these cells by *in vivo* electroporation of short hairpin expression vectors targeting *Ndp* (Supplemental Fig. 3-2). As shown in Fig. 3-7 f, proliferation was significantly reduced at 2 and 4 days in cells transfected with sh*Ndp* compared with shScrambled control vector. Taken together these data show that *Ndp* is required for normal levels of RPC proliferation *in vivo* and for cell survival in the perinatal retina and that *Ndp* likely acts cell autonomously to promote RPC proliferation.

Hh promotes neural progenitor cell proliferation in at least three ways: it promotes

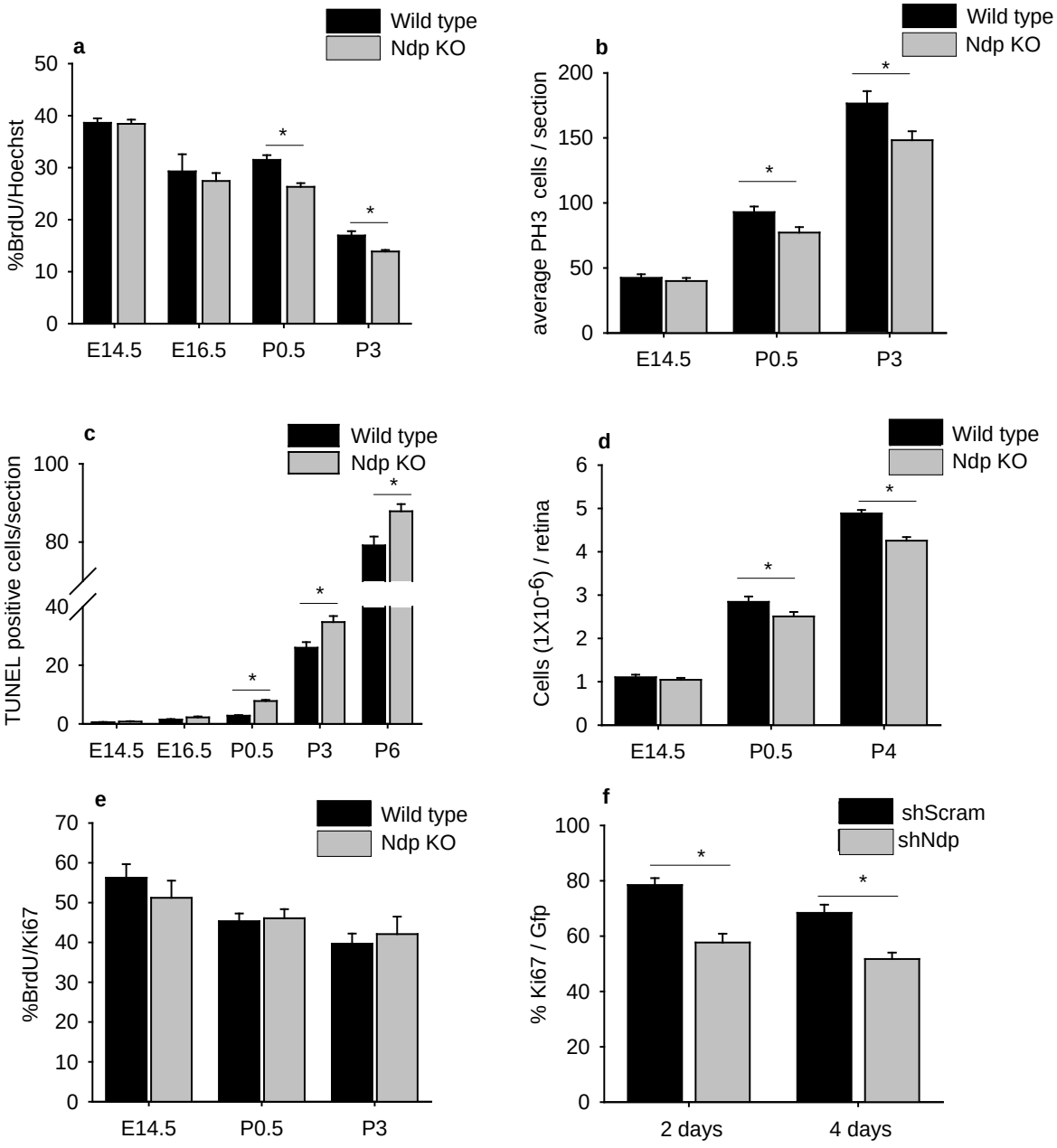

a
Wild type
Ndp KO
%BrdU/Hoechst
50
40
30
20
10
0
E14.5
E16.5
P0.5
P3
*
*
b
Wild type
Ndp KO
average PH3 cells / section
200
150
100
50
0
E14.5
P0.5
P3
*
*
c
Wild type
Ndp KO
TUNEL positive cells/section
100
80
40
20
0
E14.5
E16.5
P0.5
P3
P6
*
*
*
d
Wild type
Ndp KO
Cells (1X10-6) / retina
6
5
4
3
2
1
0
E14.5
P0.5
P4
*
*
e
Wild type
Ndp KO
%BrdU/Ki67
70
60
50
40
30
20
10
0
E14.5
P0.5
P3
f
shScram
shNdp
% Ki67 / Gfp
100
80
60
40
20
0
2 days
4 days
*
*

Figure 3-7. Analysis of *Ndp* KO retinas. (a-c)*In vivo* quantitative analysis of (a) BrdU incorporation (b) pH3 and (c) TUNEL positive cells in the retinas of *Ndp* KO and wildtype littermates (E14.5 n=5; E16.5 n=4; P0.5 n=6; P3 n=6 for each marker). Values represent the average proportion of marker+ cells as a function of Hoechst-stained nuclei counted in a retinal section at the level of the optic nerve head (1012 µm length; 3-4 sections/animal). (d) Total cell number from dissociated E14.5 (n=6), P0.5 (n=8) and P4 (n=6) Wt and *Ndp* KO retinas counted using a hemocytometer. (e) *Ndp* loss is not associated with significant changes to the BrdU labeling index. E14.5 BrdU pulsed retinas (n=5) were obtained by BrdU pulsing pregnant females for 4h whereas P0.5 (n=6) and P3 (n=6) retinas were generated by BrdU pulsing pups for 4h prior to retina fixation. The proportion of BrdU+Ki67+/Ki67+ cells was determined by counting cells in a fixed length in sections taken at the level of the optic nerve head. Data are from 3 sections per/retina. (f) Proportion of Ki67+ GFP+ /GFP + cells in retinas of newborn pups electroporated *in vivo* with GFP and sh*Ndp* (n=3) or shScrambled (n=3). Error bars represent s.e.m., *$p < 0.05$ Student's t-test.

cell cycle progression (Kenney and Rowitch, 2000; Locker et al., 2006; Sakagami et al., 2009) , cell cycle re-entry (Kenney and Rowitch, 2000; Sakagami et al., 2009; Wang et al., 2005) and cell survival (Bragina et al., 2010; Locker et al., 2006). To investigate the effects on cell cycle progression, electroporated retinal explants were BrdU pulsed on day 3 of culture and the proportion of BrdU positive cells out of the proliferating Ki67 positive cohort was quantified. Hh signaling accelerates the rate of cell cycle by shortening the length of G1/G2 (Locker et al., 2006; Sakagami et al., 2009), which is shown by the 2-fold increase in the BrdU labeling index (%BrdU+/Ki67+ cells) in *Smo-M2* expressing cells (Fig. 3-8 a). Ectopic *Ndp* expression on the other hand, did not significantly affect the labeling index of RPCs (Fig. 3-8 a), suggesting that it does not affect cell cycle kinetics. To monitor cell cycle re-entry, we marked RPCs *in vivo* with BrdU (see methods), transfected the dissected retinas with *Smo-M2* or *Ndp* and examined cell cycle re-entry by quantifying the proportion of BrdU+ electroporated (GFP +) cells that express the cell cycle marker Ki67+ at various timepoints following the BrdU pulse. BrdU+ cells that exited the cell cycle would be Ki67- and those that re-entered the cell cycle would be Ki67+. Immediately following the BrdU pulse (time= 0), the majority (≥99) of the BrdU+ cells co-expressed Ki67 (Fig. 3-8 b). After 48h and 72h the proportion of BrdU+ cells that co-stained with Ki67 was greater in the *Smo-M2* and *Ndp* expressing populations compared with the control (Fig. 3-8 b), demonstrating that both treatments promote cell cycle re-entry of RPCs. To determine whether *Ndp* expression mediates similar effects on RPCs *in vivo*, we monitored cell cycle re-entry in the retinas of *Ndp* KO mice using a similar BrdU commitment assay as described above. As shown in Fig. 3-8 c, there was a significant reduction in the proportion of BrdU labeled cells that remained in cycle after 48 hours in the *Ndp* KO compared with wildtype retinas. Thus

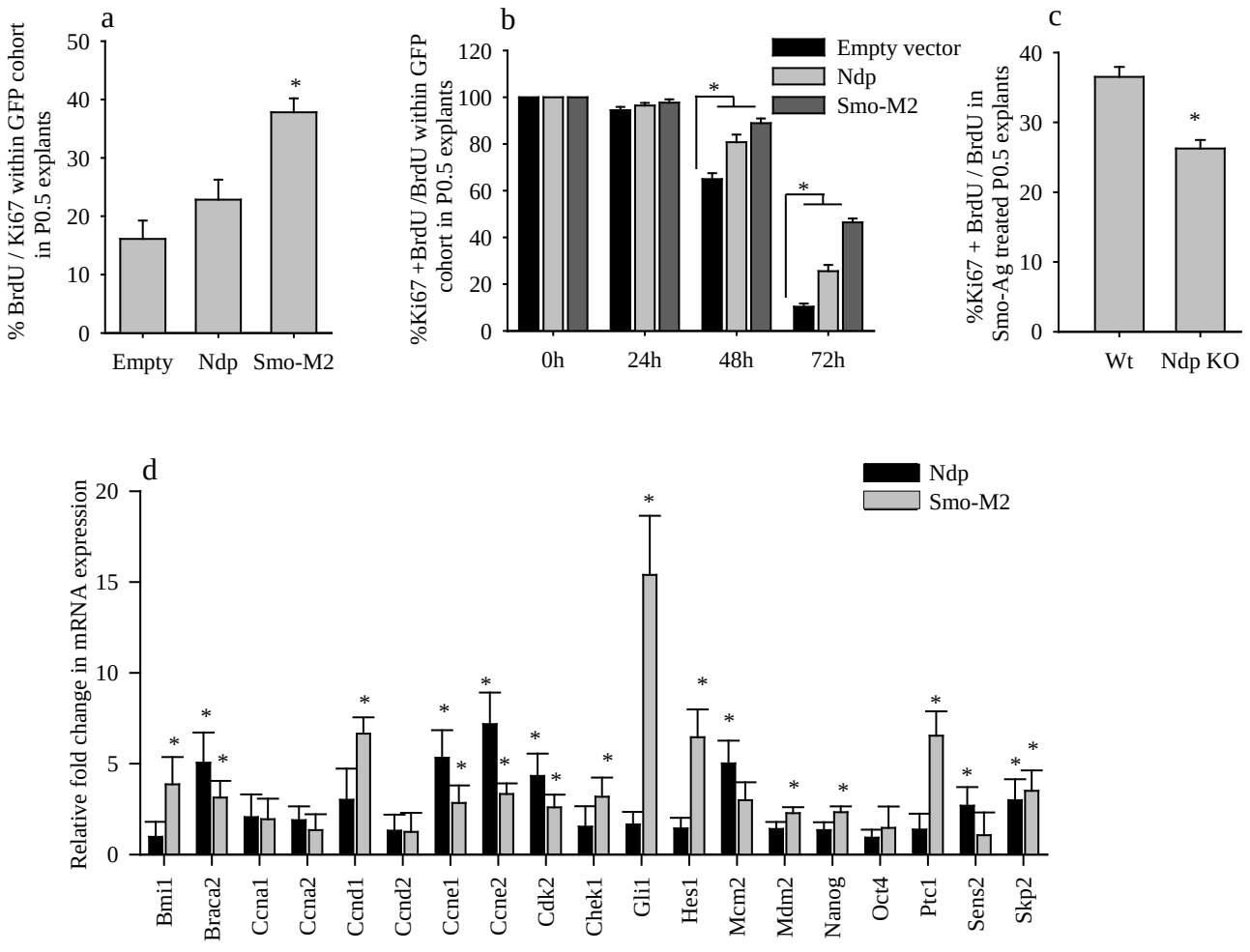

a
% BrdU / Ki67 within GFP cohort in P0.5 explants
50
40
30
20
10
0
Empty
Ndp
Smo-M2
b
%Ki67 +BrdU /BrdU within GFP cohort in P0.5 explants
120
100
80
60
40
20
0
0h
24h
48h
72h
Empty vector
Ndp
Smo-M2
c
%Ki67 + BrdU / BrdU in Smo-Ag treated P0.5 explants
40
30
20
10
0
Wt
Ndp KO
d
Relative fold change in mRNA expression
20
15
10
5
0
Ndp
Smo-M2
Bmi1
Braca2
Ccna1
Ccna2
Ccnd1
Ccnd2
Ccne1
Ccne2
Cdk2
Chek1
Gli1
Hes1
Mcm2
Mdm2
Nanog
Oct4
Ptc1
Sens2
Skp2

Figure 3-8. *Ndp* promotes RPC cell cycle re-entry. Retinal explants (P0.5 + 3DIV) were electroporated with Gfp and empty vector (n=5), *Ndp* (n=5) or *Smo-M2* (n=5) and pulsed with BrdU for 4 hours on day 3 of culture. Single cell dissociates were scored to determine the proportion of BrdU+/ Ki67+ in the Gfp+ cohort. (b) Retinas from regnant dams that had been injected at E18 with BrdU 6 times over a 24hr period were electroporated with Gfp and empty vector, *Ndp* or *Smo-M2*. Following culture, the proportion of BrdU+Ki67+/BrdU+ in the Gfp+ cohort was quantified in single cell dissociated of the explants (0h n=4; 24h n=4; 48h n=6; 72h n=6 for each condition). (c) P0.5 wildtype and *Ndp* KO pups were given a single pulse with BrdU and after 48 hours the retinas were dissected, dissociated and the proportion of BrdU+Ki67+/BrdU+ cells was quantified (n=4 for wt; n=6 for *Ndp* KO). (d) RT-qPCR analysis of Hh target and cell cycle genes in *Ndp* (n=6) and Smo-M2 (n=6) electroporated retinas (P0.5 + 3DIV). (e) Cyclin D1 protein levels in response to Smo-Ag and recombinant Norrin. Protein densitometry quantification was performed with Image J and β-tubulin protein level was used as a loading control. Error bars represent s.e.m., *$p < 0.05$ Student's t-test.

our results show that Hh signaling is associated with an increase in the rate of cycling and cell cycle re-entry in RPCs, whereas *Ndp* is necessary and sufficient to promote RPC cell cycle re-entry. The *Smo-M2* results are consistent with studies demonstrating the involvement of the Shh pathway in cell cycle re-entry in other regions of the CNS (Ahn and Joyner, 2005; Jensen and Wallace, 1997; Palma and Ruiz i Altaba, 2004), while this is the first study to identify *Ndp* as a regulator of cell cycle re-entry.

To explore the mechanism of *Ndp*-driven proliferation, we analyzed gene expression following gain and loss of function for *Ndp* in retinal explants. Ectopic *Ndp* expression had no effect on the expression of known Hh target genes including, *Gli1*, *Ptch* and *Hes1*, suggesting that *Ndp* does not mediate its effects in RPCs by regulating Hh pathway activation (Fig. 3-8 d). However, ectopic *Ndp* and Hh signaling induced the expression of overlapping sets of cell cycle genes (Fig. 3-8 d), including *CcnE1*, *CcnE2* and *Cdk2*, which are essential for entry into S-phase (Moroy and Geisen, 2004) and *Cdk2*, which is essential for neural stem cell self-renewal (Jablonska et al., 2007). One notable exception was *CcnD1*, an essential regulator of G1 progression in RPCs (Das et al., 2009), which was induced by Hh but not *Ndp* (Fig. 3-8 d). The association of *Ndp* with the expression of cell cycle genes characteristic of late but not early G1 is consistent with the observation that *Ndp* preferentially increases RPC cell cycle re-entry.

The cell cycle re-entry aspects of Hh are associated with cell diversification in that Shh promotes the production of Müller glia and bipolar cells at the expense of RGC and photoreceptors (Wang et al., 2005; Yu et al., 2006; Zhang and Yang, 2001). To address the effects of ectopic *Ndp* expression on cell fate determination, we electroporated explants with

GFP and *Smo-M2* or *Ndp* and quantified the proportion of transfected cells that stained with cell type specific markers after 7DIV. Interestingly, *Smo-M2* and *Ndp* increased the proportion of Müller glia, bipolar and amacrine marker positive cells (Fig. 3-9 a) demonstrating that *Ndp* and Hh mediate similar effects on retinal neuron differentiation *in vitro*. However, unlike *Smo-M2*, *Ndp* did not alter the proportion of rhodopsin positive cells indicating that the negative effect of the Hh pathway on the generation of these cell types is independent of *Ndp* (Fig. 3-9 a). *Ndp* mediates the INL-promoting effects of Hh signaling, as the differentiation of these cell types was inhibited in Hh-treated retinal explants from *Ndp* KO mice (Fig. 3-9 b). The requirement for *Ndp* in this context appears to be cell autonomous as the INL-promoting effects of *Smo-M2* were inhibited by treatment with sh*Ndp* (Fig. 3-9 c). Thus similar to its requirement for proliferation, these data show that *Ndp* also mediates the effects of Hh signaling on neuron and glial cell development. Furthermore, i*n vivo* cell marker analysis of *Ndp* KO animals at P6 revealed a reduction in the number of Pax6 and Chx10 positive cells (Fig 3-9 d; Appendix fig. 2). Pax6 and Chx10 are TF that are both involved in regulating RPC multipotency and proliferation (Marquardt et al., 2001; Oron-Karni et al., 2008; Rowan and Cepko, 2004) which is consistent with the reduced number of proliferating cells in the retina measured at P0.5 and P3. During later development, the expression of Chx10 becomes restricted to the INL and is required for the production of bipolar cells (Burmeister et al., 1996; Liu et al., 1994) which is also consistent with the *in vitro* gain- and loss-of- function for *Ndp* on bipolar cell development.

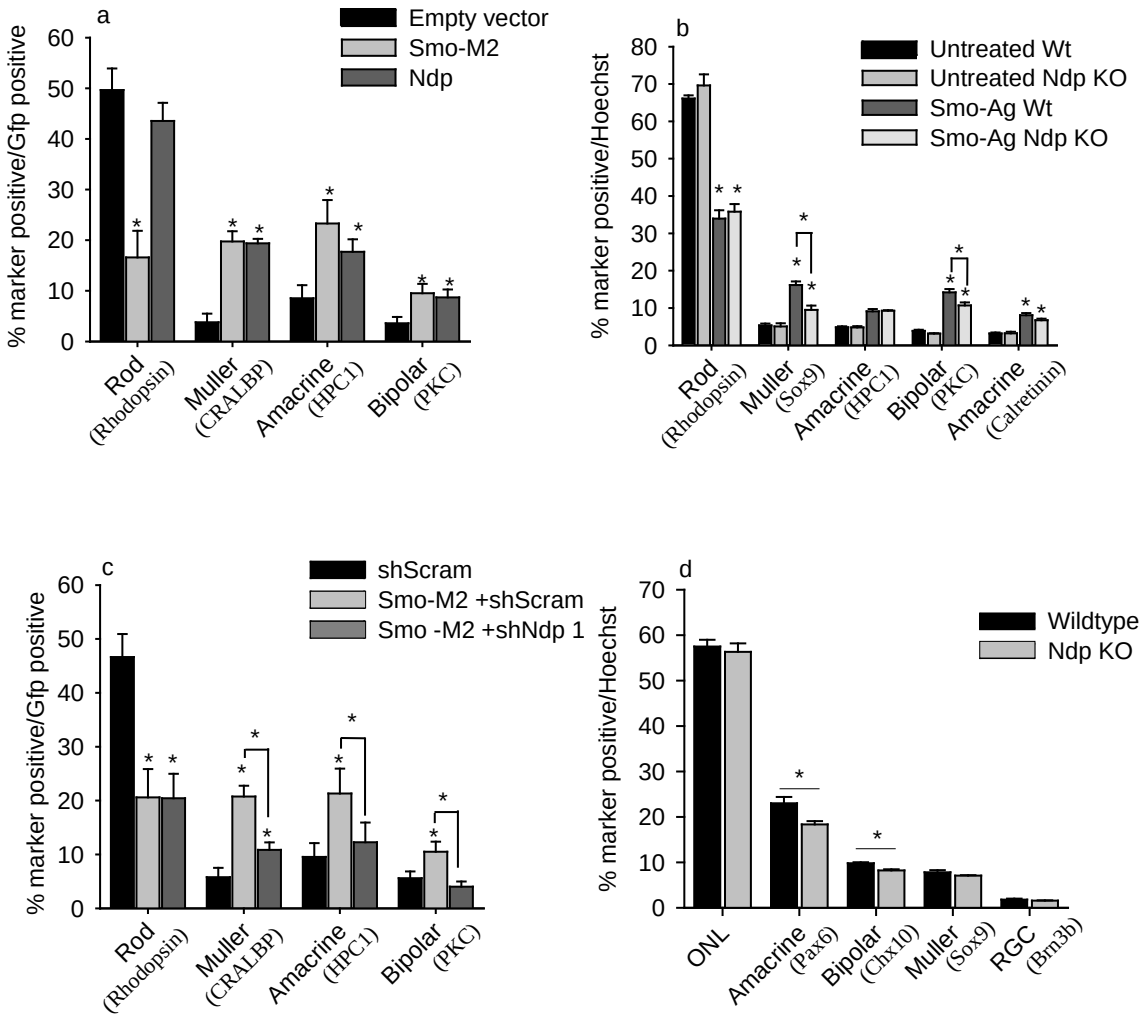

a
Empty vector
Smo-M2
Ndp
% marker positive/Gfp positive
Rod (Rhodopsin)
Muller (CRALBP)
Amacrine (HPC1)
Bipolar (PKC)
b
Untreated Wt
Untreated Ndp KO
Smo-Ag Wt
Smo-Ag Ndp KO
% marker positive/Hoechst
Rod (Rhodopsin)
Muller (Sox9)
Amacrine (HPC1)
Bipolar (PKC)
Amacrine (Calretinin)
c
shScram
Smo-M2 +shScram
Smo -M2 +shNdp 1
% marker positive/Gfp positive
Rod (Rhodopsin)
Muller (CRALBP)
Amacrine (HPC1)
Bipolar (PKC)
d
Wildtype
Ndp KO
% marker positive/Hoechst
ONL
Amacrine (Pax6)
Bipolar (Chx10)
Muller (Sox9)
RGC (Brn3b)

Figure 3-9. Ectopic *Ndp* expression promotes the development of cells with an INL identity. (a) Dissociated cell scoring of marker +cells in retinal explants (P0.5 +7DIV) electroporated with GFP and Empty vector (n=6), *Ndp* (n=6) or Smo-M2 (n=6). Counting was restricted to the GFP cohort. (b) Retinal explants (P0.5 + 7DIV) from *Ndp* KO (n=5) and wt littermates (n=4) were cultured with and without Smo-Ag. Dissociated retinas were scored for marker +cells as a function of Hoechst stained nuclei. (c) Explants (P0.5 + 7DIV) were electroporated with GFP and sh*Ndp* (n=4), Smo-M2 + shScrambled (n=6) or with Smo-M2 + shRNA targeting *Ndp* (n=6). The proportion of marker+ cells amongst the GFP cohort was determined by dissociated cell scoring. (d) Quantification of cell types in a P6 *Ndp* KO (n=4) and wt (n=4) retina. Asterisk denotes significance ($p<0.05$), as calculated using a Student's t-test, between empty vector and *Ndp* or Smo-M2 and between shScrambled and Smo-M2 or as indicated. Error bars represent SEM. The following cell type specific antibodies were used: rhodopsin (rod photoreceptors), CRALBP (Müller glia), HPC1 (amacrine), PKC (rod bipolar cells), calretinin (horizontal and amacrine), Sox9 (Müller), Brn3b (RGC), Chx10 (bipolar) and Pax6 (amacrine and ganglion cells).

***Ndp*-mediated effects on RPC proliferation are independent of the canonical Wnt signaling pathway.**

During angiogenesis Norrin binds with high affinity to the Fzd4 receptor and in cooperation with the Lrp5 and TSPAN12 co-receptors, signals through the canonical Wnt/β-catenin signaling pathway (Junge et al., 2009; Xu et al., 2004). It is unlikely that Norrin uses a similar signaling pathway in RPCs as TSPAN12 is not expressed in the developing retina (Junge et al., 2009) and activation of the canonical Wnt pathway inhibits proliferation in the rodent retina (Liu et al., 2007). Indeed, *Ndp* expression was not sufficient to activate the TCF-LacZ (Mohamed et al., 2004) (Fig. 3-10 a-c) or the Topflash luciferase reporters in retinal explants (Fig. 3-10 d). However, co-expression of *Ndp* with *Fzd4* and *Lrp5* could activate Topflash in explants (Fig. 3-10 d), suggesting that these signaling components are normally limiting in the retina, which is consistent with the low levels of *Fzd4* expression reported in the developing retina (Liu et al., 2003). Consistent with the inability of *Ndp* to activate canonical Wnt signaling in retinal explants, knockdown of *Fzd4* (sh*Fzd*; Supplemental Fig. 3-4) or inhibition of Fzd/Lrp5/6 signaling with soluble Dkk1 had no effect on *Ndp*-induced proliferation in retinal explants (Fig. 3-11 a, b), demonstrating that Fzd4 and Lrp5/6 activity are not required for *Ndp* signaling in RPCs. Moreover, pharmacological activation of the canonical Wnt pathway with Gsk-3 antagonists (BIO and LiCl), not only failed to stimulate proliferation in retinal explants but also inhibited proliferation in response to *Ndp* (Fig. 3-11 c) and Hh (Fig. 3-11 d). Taken together these results demonstrate that *Ndp* does not signal through Fzd4/Lrp5/6 and β-catenin to promote RPC proliferation and implicate a positive role for Gsk3 activation in RPC proliferation.

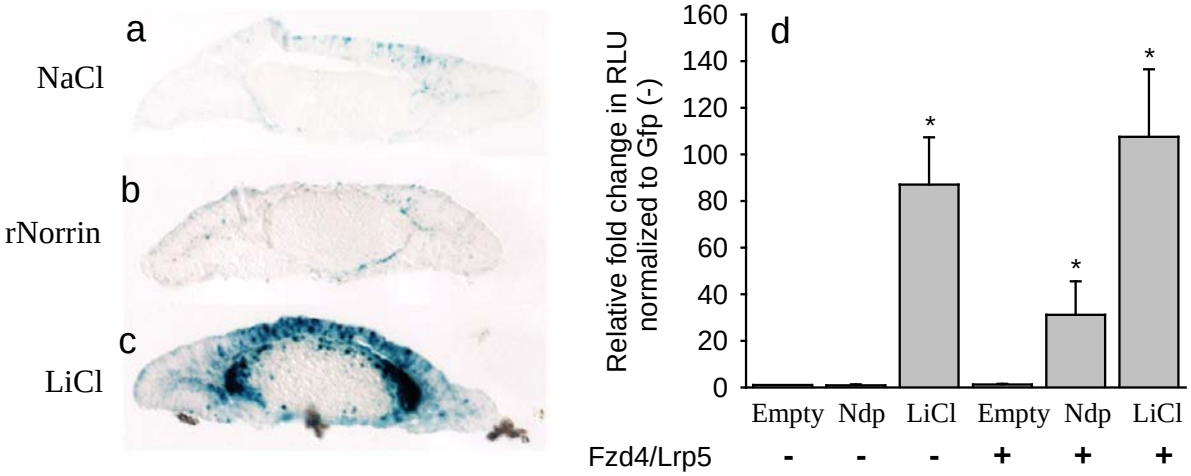

a
NaCl
b
rNorrin
c
LiCl
d
Relative fold change in RLU normalized to Gfp (-)
160
140
120
100
80
60
40
20
0
*
*
*
Empty
Ndp
LiCl
Empty
Ndp
LiCl
Fzd4/Lrp5
-
-
-
+
+
+

Figure 3-10. *Ndp* not sufficient to activate Wnt/β-catenin signaling in the retina. X-gal staining to monitor activation of the TCF/Lef-LacZ reporter transgene in response to (a) NaCl, (b) recombinant Norrin (rNorrin) and (c) LiCl in retinal explants (E16.5 + 24h). . (d) Topflash luciferase assays on retinal explants (P0.5 + 2DIV) electroporated with GFP and empty vector (n=4) or *Ndp* (n=4) with or without *Lrp5* and *Fzd4*. Asterisk denotes significance ($p<0.05$) in reporter activity between empty vector and *Ndp* or LiCl (positive control) as calculated using a Student's t-test. Error bars represent SEM.

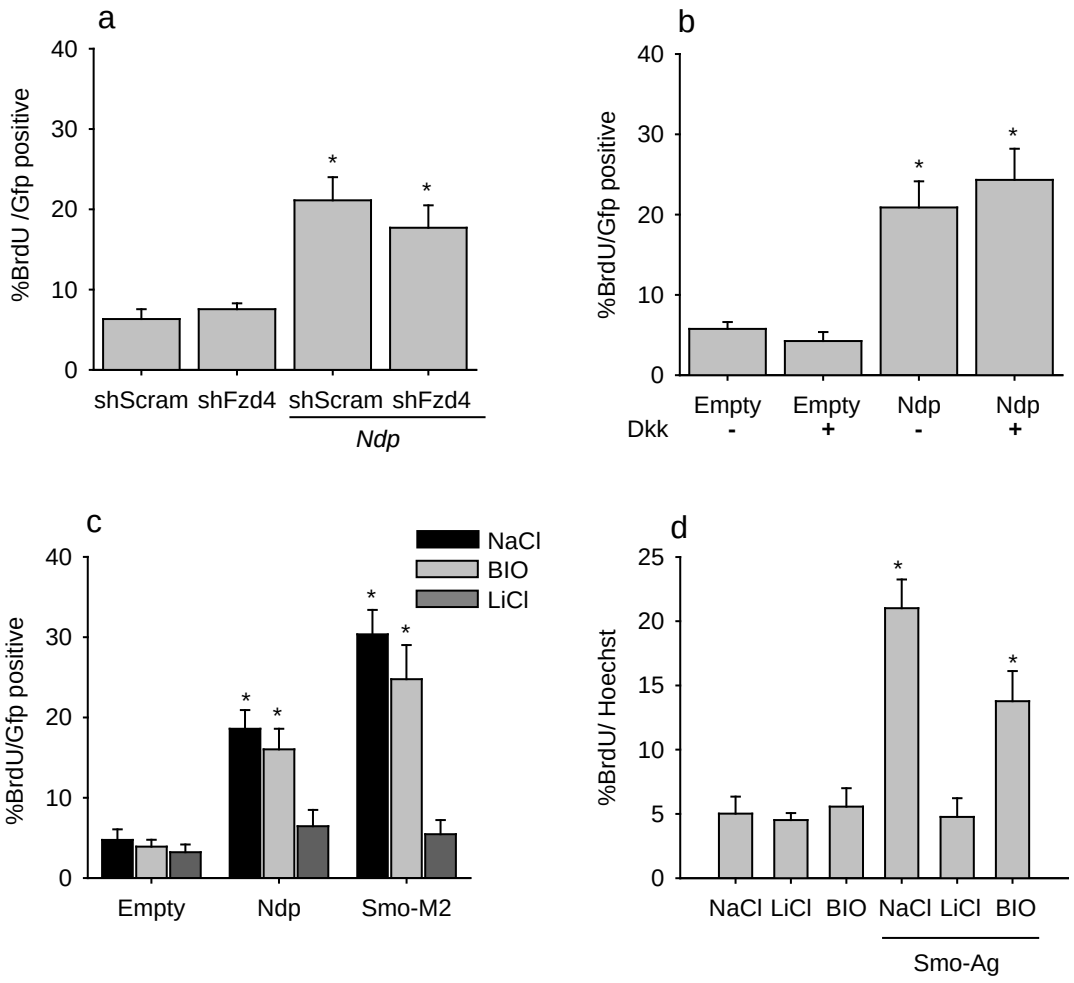

a
%BrdU /Gfp positive
40
30
20
10
0
shScram
shFzd4
shScram
shFzd4
Ndp
b
%BrdU/Gfp positive
Empty
Empty
Ndp
Ndp
Dkk
-
+
-
+
c
%BrdU/Gfp positive
NaCl
BIO
LiCl
Empty
Ndp
Smo-M2
d
%BrdU/ Hoechst
25
20
15
10
5
0
NaCl
LiCl
BIO
NaCl
LiCl
BIO
Smo-Ag

Figure 3-11. *Ndp* mediated effects on RPC are not mediated by Fzd4/Lrp5. (a) shRNA mediated knockdown of Fzd4 in retina explants (P0.5 + 3DIV) co-electroporated with GFP + shScram (n=3), GFP + sh*Fzd4* (n=3), GFP + shScram + *Ndp* (n=3) or GFP+ sh*Fzd4* + *Ndp* (n=3). Explants were dissociated and scored for BrdU/ Hoechst. (b) Explants (P0.5 + 2DIV) electroporated with GFP and empty vector (n=4) or *Ndp* (n=4) were cultured in the presence or absence of the Lrp5/6 inhibitor Dkk1. Cell scoring was performed on dissociated explants for proportion of BrdU+ GFP+/GFP+. Effect of β-catenin stabilization with LiCl (n=6) or BIO (n=6) on proliferation in (c) *Ndp* and Smo-M2 electroporated P0.5 retinas and (d) Smo-Ag treated explants. Asterisk denotes significance ($p<0.05$), as calculated using a Student's t-test, between shScrambled and *Ndp* or Smo-M2, between empty vector and *Ndp* electroporated and between Smo-Ag treated and untreated. Error bars represent SEM.

3.6 Discussion

Here we describe a novel role for *Ndp* as a downstream mediator of the effects of Hh signaling on neural progenitor proliferation and cell fate. Hh is necessary and sufficient for *Ndp* expression in the retina, as *Ndp* expression is reduced in mice with a conditional inactivation of *Shh* in the retina and Hh pathway activation increases *Ndp* expression. Hh signaling promotes the interaction of Gli2 with the *Ndp* promoter and Gli2 is required for maximal *Ndp* expression downstream of Hh activation, suggesting that *Ndp* is a direct target of Hh/Gli2. *Ndp* is necessary and sufficient to promote RPC proliferation and differentiation *in vitro* and *in vivo*. We show that it mediates the effects of Hh signaling on these processes and does so by promoting cell cycle re-entry rather than affecting cell cycle kinetics. Because acute *Ndp* knockdown alone or in conjunction with Hh activation inhibits proliferation it suggests that the secreted norrin protein is a short range extracellular signal in this context. Hh is the first signaling pathway to be implicated in *Ndp* regulation and our study identifies neural progenitors as novel cellular targets of this signaling molecule and a novel mechanism of Hh-induced neural progenitor proliferation.

Previous studies investigating the role of *Ndp* in the eye have focused almost exclusively on its role in angiogenesis (reviewed in Warden et al., 2007) where *Ndp* is required for the invasion of blood vessels from the vitreal surface into the retina to form the deeper vascular plexus. *Ndp* KO mice also exhibit retinal abnormalities, including reduced cell numbers and rosetting (Schroeder et al., 1997), however the extent to which these defects reflect a primary or secondary requirement for *Ndp* function in the retina has not been resolved. Here we show that *Ndp* expression is sufficient to promote RPC proliferation and that it is required for normal RPC proliferation and for cell survival. These data are

consistent with previous reports demonstrating the neuroprotective properties of Norrin (Lin et al., 2009; Seitz et al., 2010) and its ability to induce proliferation in the retina (Ohlmann et al., 2005) and cultured endothelial cells (Ohlmann et al., 2010). The proliferative effects of *Ndp* are observed in the absence of angiogenesis in explants and prior to the onset of angiogenesis *in vivo*, which provides strong support for our conclusion that *Ndp* has a novel role in neural progenitor proliferation.

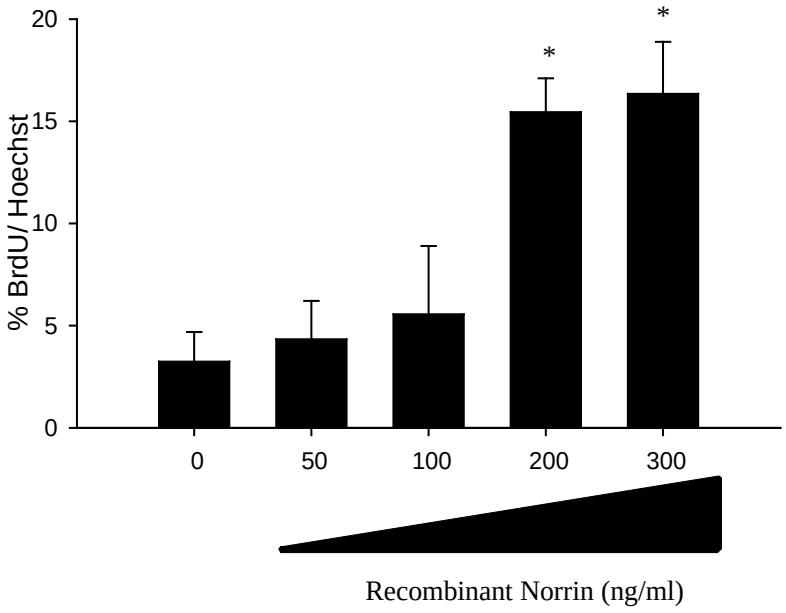
The Hh-dependent association of Gli2 with the *Ndp* promoter and the cell autonomy of the effects of *Ndp* gain and loss of function *in vitro* and *in vivo* are consistent with the evidence that *Ndp* is normally expressed in immature RPCs and that Hh-regulated *Ndp* acts at the level of RPCs to control their proliferation. This conclusion is supported by the ISH data showing *Ndp* expression in the neuroblast layer of the embryonic and perinatal retina and with analysis of an AP knock-in reporter line showing that *Ndp* is expressed in cells with a radial morphology in the retina (Ye et al., 2009). While this expression pattern was attributed to Müller glia, it very likely is also RPCs, which are also radial and are more abundant than Müller cells in the P0 and P4 retina.

Ndp knockdown in RPCs on its own or in conjunction with cell autonomous Hh pathway activation inhibits proliferation and the development of late cell types. Since Norrin is a secreted protein these observations suggest that Hh-induced Norrin signals at short range as a paracrine or possibly autocrine signal to regulate RPC development. This conclusion is consistent with our evidence that Hh effects on RPC proliferation are cell autonomous (Yu et al., 2006) and with the short range activity of Norrin *in vitro* (Xu et al., 2004). Norrin is strongly associated with the ECM (Perez-Vilar and Hill, 1997; Xu et al., 2004), which could account for its restricted range of action in retinal tissue.

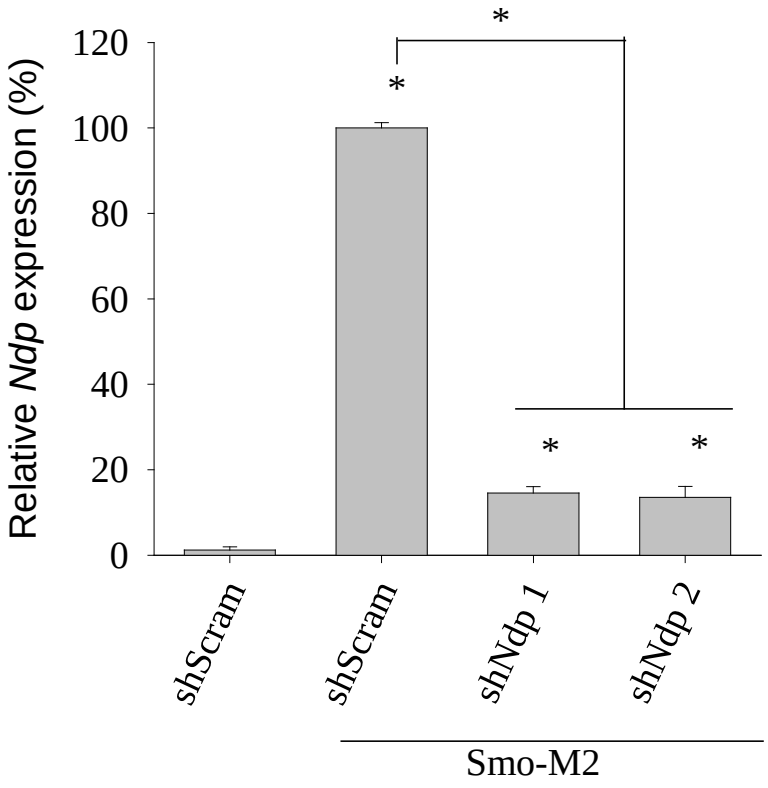
Interestingly, *Ndp* required co-expression of Fzd4/Lrp5 co-receptors to activate the canonical Wnt pathway in retinal explants and its effect on proliferation was insensitive to inhibition of the Wnt signaling pathway. Moreover, Fzd4 appears to be dispensable for retinal development, as conditional Fzd4 inactivation in the retina does not affect retinal function (Ye et al., 2009). Thus while we show that given the appropriate co-receptors Norrin can activate canonical Wnt signaling, we believe that this signaling mechanism is restricted to the developing vascular system. Norrin shares homology with CCN1, a matricellular protein that regulates growth and differentiation of multiple subtypes. CCN1 interaction with integrins and HSPG co-receptors mediates GSK3-dependent growth in fibroblasts (Leu et al., 2004; Si et al., 2006). Interestingly, Gsk3 inhibitors inhibited Norrin and Hh function in RPCs and therefore the integrin-dependence of Norrin signaling in the retina warrants further study.

Understanding the regulatory mechanisms of the Shh pathway not only has implications in retinal development but in other systems as well, such as the skin, lung and various parts of the CNS. Mutations in this pathway have deleterious consequences as they have been linked to several human disorders and linked to various types of cancers. Here we demonstrate a novel target of the Shh pathway in the retina that is sufficient and required for RPC proliferation and differentiation. The activity of *Ndp* appears to be local and signals through an alternative mechanism then the canonical Wnt/β-catenin that has been shown for the vascular system.

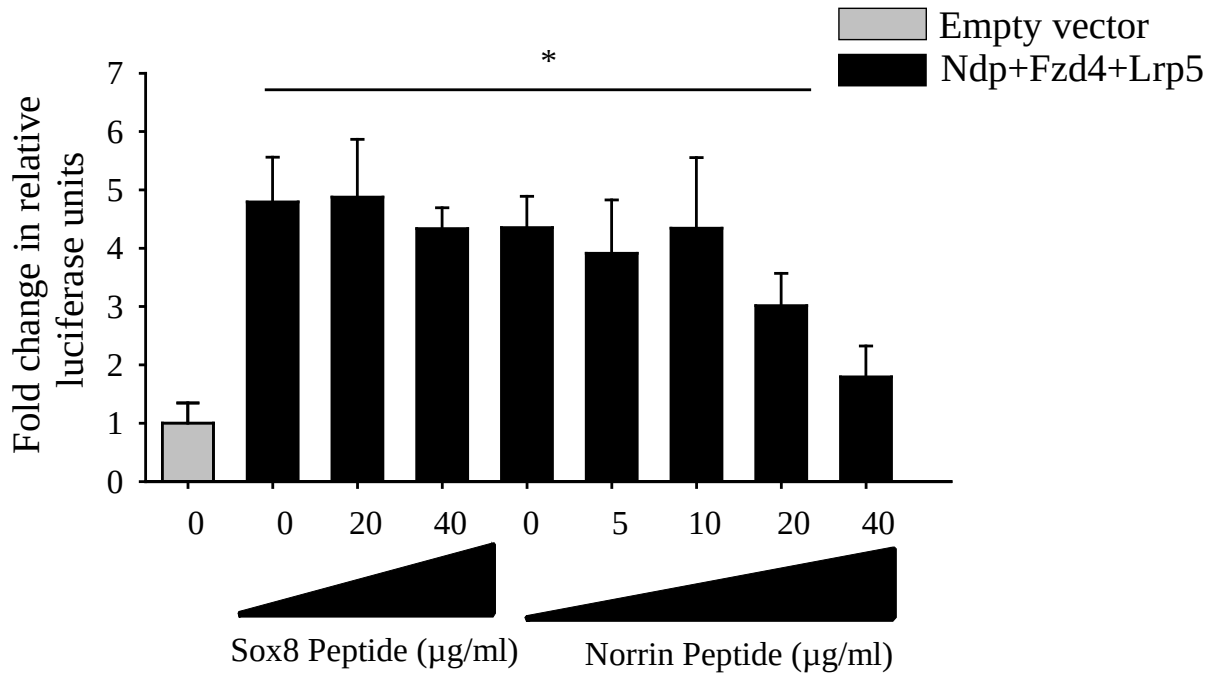
3.7 Supplementary Data



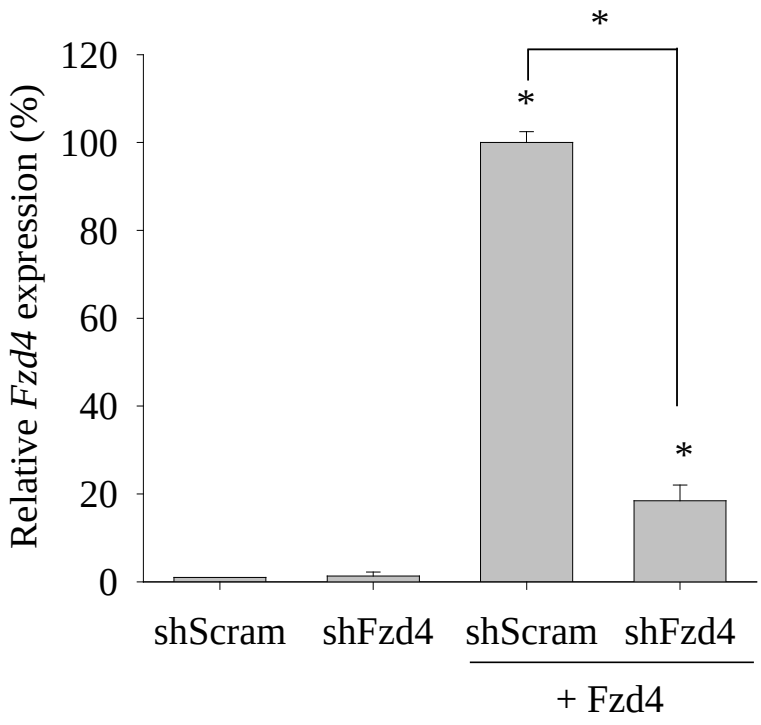
Supplemental figure 1. Recombinant Norrin dose response in retinal explants. Explants (P0.5 + 3DIV) were cultured with recombinant Norrin (rNorrin) at the indicated concentrations (n=3 explants/condition), pulsed with BrdU (4 hr) on the final day of the culture, dissociated and scored for the proportion of BrdU+ Hoechst stained nuclei. Asterisk denotes significance ($p<0.05$) between rNorrin treated and untreated as calculated using a Student's t-test. Error bars represent SEM.


Relative Ndp expression (%)
120
100
80
60
40
20
0
*
*
*
*
shScram
shScram
shNdp 1
shNdp 2
Smo-M2

Supplemental figure 2. Knockdown efficiency of shRNA targeting *Ndp*. Explants (P0.5 + 3DIV) were electroporated with GFP + shScram (n=3), GFP+ shScram +*Smo-M2* (n=3) or GFP+ sh*Ndp* +*Smo-M2* (n=3 for each shRNA) and RT-qPCR analysis of *Ndp* expression was performed. Asterisk denotes significance ($p<0.05$) between scrambled and Smo-M2 with scrambled or sh*Ndp* as indicated. Error bars represent SEM.


Empty vector
Ndp+Fzd4+Lrp5
*
Fold change in relative luciferase units
0
1
2
3
4
5
6
7
0
0
20
40
0
5
10
20
40
Sox8 Peptide (µg/ml)
Norrin Peptide (µg/ml)

Supplemental figure 3. Norrin blocking peptide dose curve measured by Topflash reporter. Explants (P0.5 + 2DIV) electroporated with *Lrp5*, *Fzd4* and *Ndp* or empty vector and cultured with Sox8 or Norrin peptides. Topflash reporter assay was performed on explants measuring RLU. Values represent fold change compared to empty vector untreated explants. Asterisk denotes significance ($p<0.05$) between empty vector and *Ndp+Fzd4+Lrp5*. Error bars represent SEM.


Relative Fzd4 expression (%)
120
100
80
60
40
20
0
*
*
*
shScram
shFzd4
shScram
shFzd4
+ Fzd4

Supplemental figure 4. Knockdown efficiency of shRNA targeting Fzd4. COS cells transfected with GFP + shScram (n=3), GFP + shFzd4 (n=3), GFP + shScram + Fzd4 (n=3) or GFP + shFzd4+ Fzd4 (n=3) and analysed by RT-qPCR for *Fzd4* expression. Asterisk denotes significance ($p<0.05$) in *Fzd4* expression between as calculated using a Student's t-test. Error bars represent SEM.

3.8 References

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Chapter 4

Discussion

The goal of this thesis was to identify novel targets of the Gli TF in the retina that could provide insight and broaden our understanding of the Hh pathway and its functions. Using a computational method, 390 genes were identified as having conserved Gli binding motifs between the human and mouse genomes. Using an *in vitro* explant model to validate the genes identified with this method we found the expression of 31 of the 47 selected targets was significantly altered following Hh pathway activation. Furthermore, 26 of these genes demonstrated a temporal response between explants derived from E14.5 and P0.5 retinas. Following subsequent validation, two genes (*Sox8* and *Ndp*) were selected for characterization during retinal development. While we demonstrate the expression of *Sox8* is highly inducible following Hh pathway activation in a Gli2 dependent manner, we did not identify a role for this gene during retinal development. *Ndp* was also shown to be highly inducible in a Gli2 dependent manner. Functional characterization revealed a role for this gene in promoting RPC cell cycle re-entry, cell survival and potential involvement in cell fate determination for cells that make up the INL. We also show that, unlike in vascular development, the effects of *Ndp* on are not through the activation of the canonical Wnt signaling pathway.

The effects of the Hh pathway are mediated primarily through the activity of the Gli TF. Understanding the network and function of these TF targets would help understand how the Hh pathway mediates the multitude of processes throughout development into adulthood. Previous studies have successfully used high-throughput ChIP-chip to identify Gli targets in cells (Vokes et al., 2007), the developing limb (Vokes et al., 2008), cerebellum and *Ptc* +/- related medulloblastomas (Lee et al., 2010). For this thesis an alternative approach was used to identify novel Gli targets. Used successfully in other systems (Bagheri-Fam et al., 2010;

Doniger et al., 2005; Dutton et al., 2008; Loots et al., 2000), we chose a bioinformatics approach to search for conserved Gli binding motifs between human and mouse. As with ChIP-chip, this type of approach is genome wide and can result in the identification of thousands of potential targets with only a small fraction actually being relevant for Gli mediated regulation. Advantages with using this type of approach when this project started was that it was more cost-friendly than the conventional experimental approaches typically used to identify TF targets and we also had access to bioinformatics expertise. Furthermore, combining bioinformatics with temporal analysis of gene expression of candidate targets removes the need for more expensive temporal ChIP-chip datasets. A disadvantage with the bioinformatics approach we used is that targets were missed. As several studies have shown TF binding is most prevalent within the first 2kb upstream of the TSS (Bieda et al., 2006; Del Bene et al., 2007; Vokes et al., 2008; Xu et al., 2007), we used this knowledge to limit the number of targets identified and to increase the probability that the identified genes are true Gli targets. In doing so, genes with Gli binding motifs greater than -2kb from the TSS in humans would be missed. Also targets with Gli binding motifs more divergent then 8/9 from the sequence we used would be missed. *CcnD1* and *N-Myc* for example, both Gli targets (Hu et al., 2006), have binding motifs within 2kb of the TSS in humans however they were not identified in our search as these motifs have a 7/9 match to the sequence we used. Nevertheless, the goal of this thesis was to identify novel targets of the pathway, not necessarily all the targets of the pathway during retinal development.

Comparing the results obtained from our computational analysis to those obtained through ChIP-chip, we found 77 of our 390 genes were also identified in these other studies. These include the known targets *Ptc*, *Nkx2.2*, *Stmn1*, cell cycle genes such as *E2F2* and

tubulin beta 2A (*Tubb2a*), *ion transport genes solute carrier* (*Slc*) *25A20* and *Slc 26A11*, and differentiation genes like *Acta1* and *Gata2*. Many of the common genes between the data sets however, have little known function such as *Grp85*, *Dhrs3*, *Stk35*, *Tm2d3* and *Ptk9I*. Interestingly, *Sox18* is a common target identified in all of these studies and has yet to be extensively studied.

From a subset of target genes selected for validation, we have found several of these genes responded differently to Hh pathway activation in explants derived from E14.5 compared to P0.5 mice. This temporal response could indicate specific roles for these genes during retinal development or that additional components are required for the Hh pathway to induce the expression of these genes, such as enhancers and Gli co-factors (discussed in Chapter 2). The temporal regulation could also be mediated epigenetically through DNA and chromatin modifications that determine the accessibility of genes to be transcribed (reviewed in Allen, 2008). DNA methylation, for example, has been shown to regulate the expression of two known Gli targets *Ptc* (Agren et al., 2004) and *CcnD2* (Yoon et al., 2002). In the *Ptc* promoter, methylation within a CpG island near the Gli binding motif prevents the induction of this gene and results in hyperactive Hh signaling (Cretnik et al., 2007). *CcnD2* promoter methylation prevents the induction of this gene by Gli1 in astrocytoma tumors but not in medulloblastomas which do not contain a highly methylated *CcnD2* promoter (Shahi et al., 2010).

Following the target validation of the computational search, we focused on characterizing two genes in particular, *Sox8* and *Ndp*. When the genes were prioritized for functional analysis there was evidence that *Sox8* was expressed in the eye (Sock et al., 2001), however, no function for this gene had been described in retinal development. While we

clearly demonstrate that expression of *Sox8* is modulated by the Hh pathway in a Gli2-dependent manner, we were unable to demonstrate the functional relevance of Hh-mediated Sox8 regulation in the developing retina. These results are consistent with our *in vivo* results of the *Sox8* KO retinas, which did not exhibit any significant differences in the proportion of cell types compared to wildtype controls. The lack of *Sox8* function from our study is contradictory to the study by Muto et al., (2009) demonstrating *Sox8* knockdown resulted in a reduction in Müller glia and an increase in photoreceptor development. This discrepancy could reflect differences in experimental design. Muto et al., (2009) examined the loss of *Sox8* in retinal explants that were cultured for 14 days whereas we only cultured for 7 days. It is possible that the consequences of knocking down *Sox8* would not be obvious by 7 days as it would be by 14 days taking into account the time required for mRNA knockdown by the shRNA and for the decay of the Sox8 protein. Also, we looked at the effects of *Sox8* knockdown in the context of Hh pathway activation. Because the other SoxE members are also upregulated by Hh signalling, it is therefore possible that these closely related proteins are able to compensate for the loss of *Sox8*. Furthermore, other genes may also have the ability to compensate or mask the effects associated with a loss of *Sox8*. *Hes1*, for example, has been shown to be involved in Müller cell development (Furukawa et al., 2000) and is a direct Hh target in the retina (Wall et al., 2009). Similar to *Sox9* and *Sox10*, the expression of this bHLH is downregulated in cultured explants (Wall et al., 2009). Therefore it is possible that in our experiments, the induction of *Hes1, Sox9* or *Sox10* following Hh pathway activation is able to compensate for loss of *Sox8* and ensure Müller cell production occurs normally. It would be interesting to knockdown *Sox9* and *Sox10* in *Sox8* KO explants and see if a decrease in Müller cells would be detected under conditions of Hh pathway

activation. As loss of *Hes1* results in a decrease in Müller cells, it would also be interesting to see if there would be a greater decrease in Müller cells when *Hes1* and *Sox8* were lost.

The other gene we selected for further investigation was *Ndp*. This gene was selected primarily based on its implication in a variety of visual diseases. While the cause of these diseases is believed to be primarily due to vascular defects, there is evidence for a non-vascular function for *Ndp* during retinal development. Here we clearly demonstrate the ability of the Hh pathway to regulate the expression of *Ndp* likely through Gli2 and possibly Gli3. Ectopic expression of *Ndp in vitro* increases RPC proliferation by promoting cell cycle re-entry. Consistent with this role in RPC proliferation, *in vivo* analysis of *Ndp* KO retinas revealed a reduction in proliferating RPCs. However this effect was restricted to retinas from postnatal animals, no effects were detected in the retinas during embryonic development. This suggests that *Ndp* does not have any function during embryonic retinal development, which is not consistent with our *in vitro* results demonstrating an increase in RPC proliferation when explants were cultured with Norrin or when *Ndp* was overexpressed. One possible explanation for the inconsistency between the *in vivo* and *in vitro* results is that during early embryonic development many cell cycle and re-entry factors are present and are able to compensate for the loss of *Ndp*. As development progresses it is possible that the function of some of these re-entry factors diminish as the rate of differentiation increases resulting in the need for *Ndp*. Another possibility is that, due to the loss of signals that would be present *in vivo*, the explants become a sensitized system that are able to respond to ectopic *Ndp* during early development.

How *Ndp* mediates its effects on the RPC during development remains unclear. Consistent with an increase in proliferation, we detected an increased expression of many genes associated with the cell cycle but did not detect any change in expression in genes associated with self renewal such as *Nanog*, *Bmi1*, *Hes1* and *Oct4*. It is possible that the expression of other self-renewal factors, such as *Sox2* (Catena et al., 2004; Ferri et al., 2004), are affected by *Ndp* or that the regulation is at the level of the protein rather than at the transcriptional level. Growing evidence also suggests that cell polarity plays a role in regulating self-renewal in both fly and mammalian neural stem cells (reviewed in Doe, 2008). During early mouse cortical neurogenesis, for example, highly polarized cells within the ventricular zone typically give rise to progenitors following cell division while non-polarized cells generally give rise to two postmitotic neurons (Haubensak et al., 2004; Noctor et al., 2004). Disrupting the polarity within the polarized cells results in precocious cell cycle exit and neuronal differentiation (Cappello et al., 2006; Costa et al., 2008). Overexpression of the polarity genes, *Par3* or *Par6*, in progenitor cells caused these cells to proliferate and remain in an undifferentiated state, an indication of enhanced self-renewal (Costa et al., 2008). It would be interesting to investigate the polarity of RPC following ectopic *Ndp* expression and to determine the fates of the progeny of the transfected progenitor cell; would two progenitor cells, one progenitor and one postmitotic or two postmitotic cells be produced?

While we and others (Lin et al., 2009; Seitz et al., 2010) have demonstrated a survival role for *Ndp* in the retina, our observed effects of *Ndp* on RPC are not likely due exclusively to survival for several reasons. Firstly, gain- and loss-of-function for *Ndp* in 7DIV explants significantly affected the production of specific cell types (Fig 3-9), a result I

would not predict if simply a survival effect. Secondly, enhanced cell survival would not result in an increase in cell cycle re-entry observed in explants overexpressing *Ndp* (Fig 3-8 b). To clearly distinguish between survival and self-renewal, it would be important to first demonstrate that Ndp affects PRC survival. While we have detected an increase in TUNEL positive cells within the neuroblast layer (Fig 3-7 c ;data not shown), these cells were not identified as progenitor or postmitotic cells.

Previous reports have demonstrated the ability of this protein to activate the canonical Wnt signaling pathway through the exclusive interaction with Fzd4 (Luhmann et al., 2008) with a requirement for Lrp5/6 and TSPAN12 (Junge et al., 2009; Xu et al., 2004; Ye et al., 2009). While we confirmed the ability of *Ndp* to induce this pathway in the retina, the addition of *Fzd4* and *Lrp5* were required to elicit a response. Furthermore, not only did stabilization of GSK-3β with LiCl or the BIO inhibitor not recapitulate the proliferative effects of ectopic *Ndp* but LiCl treatment actually inhibited both *Ndp* and Hh-Ag induced proliferation. The inability of *Ndp* to activate the Topflash reporter assay in explants without the addition of *Fzd4/Lrp5* coupled with the inability of BIO and LiCl treatment to increase RPC proliferation, suggests the effects of *Ndp* on RPC proliferation are not mediated by the Wnt/β-catenin pathway. Consistent with *Ndp* signaling through an alternative pathway other than Wnt/β-catenin in the neural retina is the weak expression of *Fzd4* in the developing retina (Liu et al., 2003 ; data not shown) and the restricted expression of TSAPN12 to the blood vessels (Junge et al., 2009). In an attempt to identify the mechanism of *Ndp* signaling in RPCs, we performed microarrays on retinas isolated from P0.5 *Ndp* null animals and wildtype littermates (Appendix Table 1) and validated a subset of these targets by RT-qPCR (Appendix fig. 3). While the expression of several genes were altered between the *Ndp* KO

and wildtype retinas, no clear pathway emerged. It would be interesting to perform immunoprecipitation for Norrin (protein product of *Ndp*) to identify proteins that associate with this molecule in RPC. This is one potential way the mechanism could be elucidated.

In conclusion, we have identified novel targets of the Gli TF that could help understand the mechanism of the Hh pathway not only in the retina but in other Hh dependent tissues and cancers. While we did not find a role of *Sox8* in the retina, we did show that the regulation of this gene is highly dependent on the Hh pathway and could play an important role in other tissues. Understanding the regulatory mechanisms and functions of *Ndp* during retinal development could help understand the hearing impairment and mental retardation that are often associated with Norrie Disease patients. Could the effects of *Ndp* in the brain be a result of a cell deficiency associated with a decrease in self-renewal? Or could *Ndp* have a more pronounced effect on neuronal differentiation in the brain? Understanding *Ndp* could also be significant in other tissues including cancer. We have detected an increase in *Ndp* expression in medulloblastomas from heterozygous *Ptc* animals (Appendix fig. 4). While we did not pursue this further, it would be interesting to see if *Ndp* is involved in the progression of this type of tumor or any other tumor and what would happen if we disrupted *Ndp* signaling.

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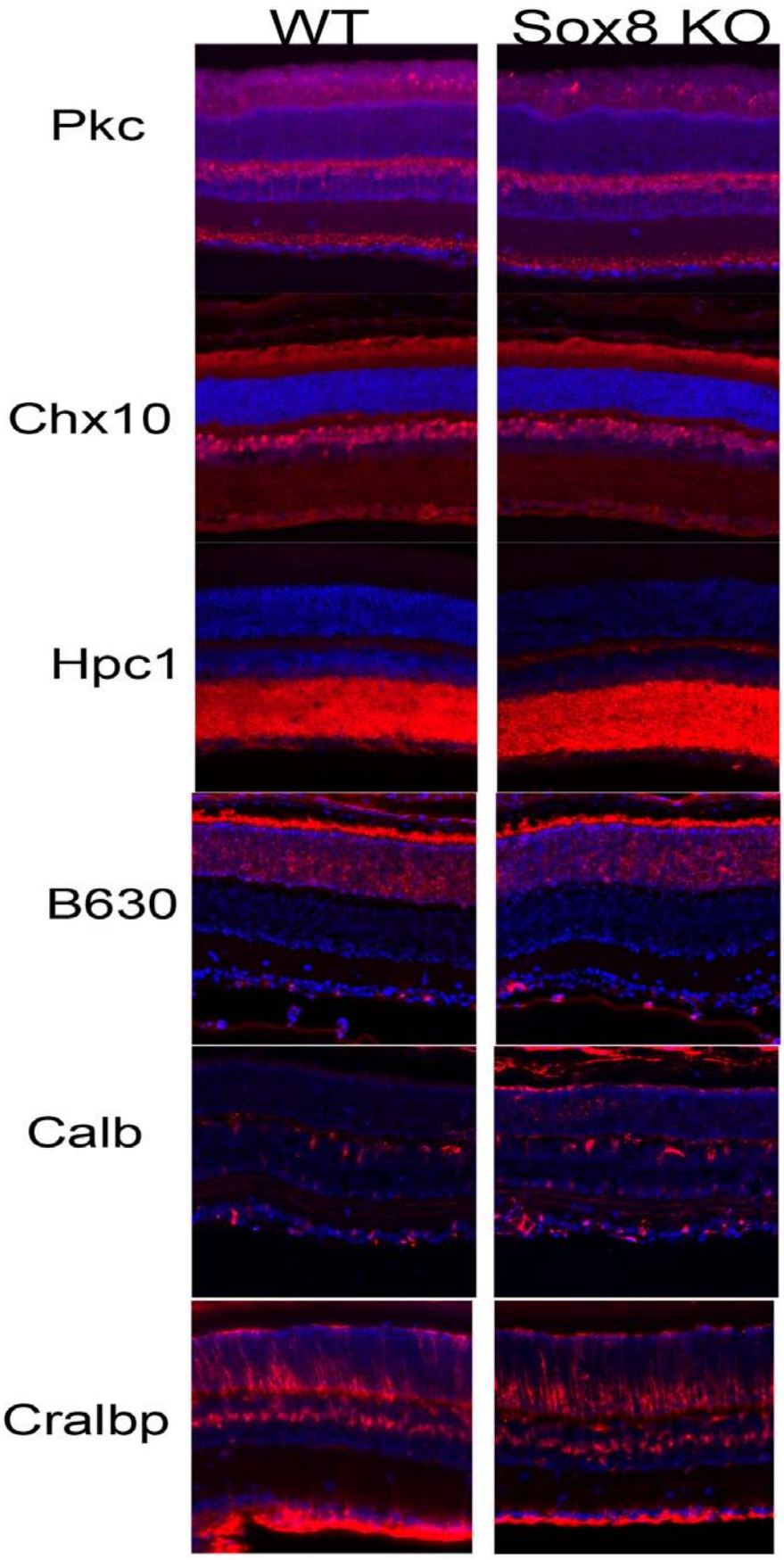
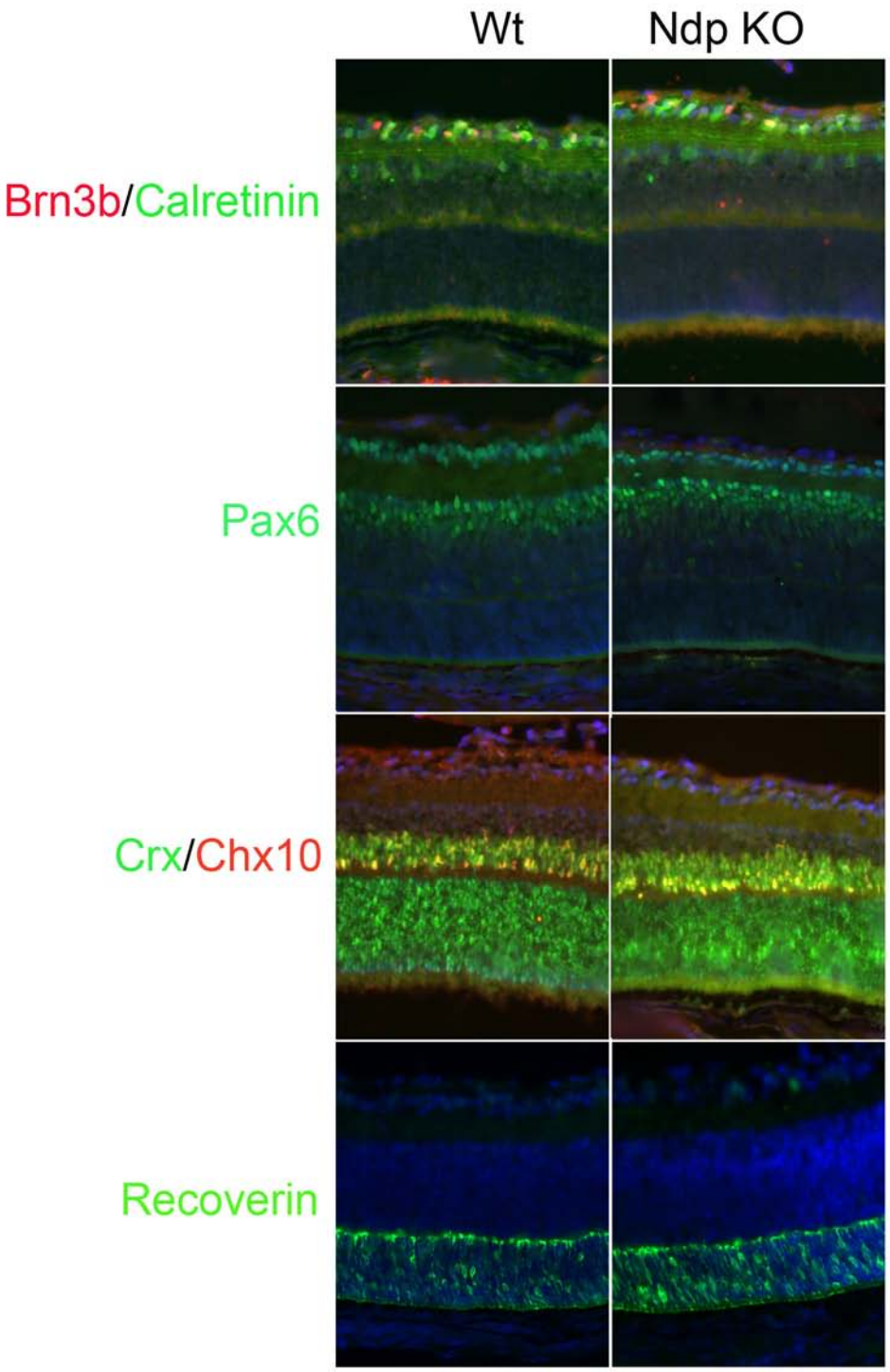
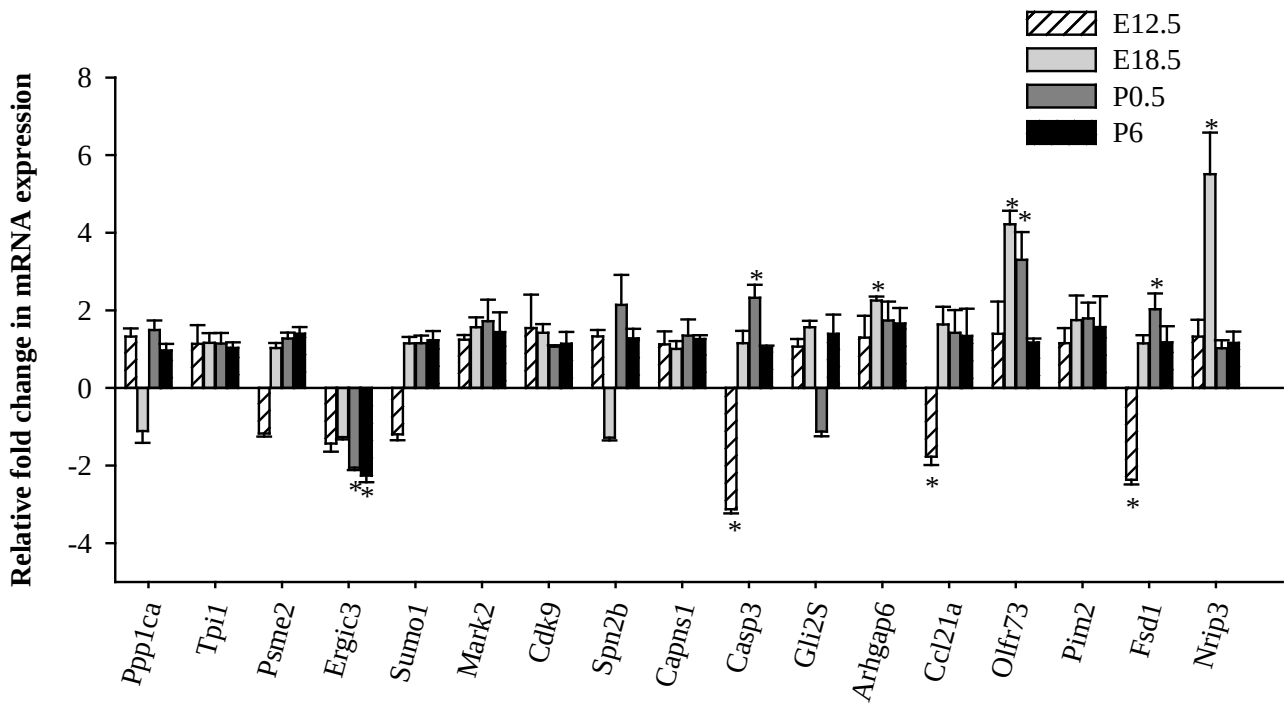
APPENDIX


WT
Sox8 KO
Pkc
Chx10
Hpc1
B630
Calb
Cralbp

Appendix figure 1. Immunohistochemistry on *Sox8* KO adult retina. Staining performed on adult (6-8 weeks) retinas from Wt and *Sox8* KO retinas using markers for bipolars (Pkc, Chx10), amacrine (Hpc1), rod photoreceptors (B630), horizontal (Calbindin) and Müller (Cralbp) cells.


Wt
Ndp KO
Brn3b/Calretinin
Pax6
Crx/Chx10
Recoverin

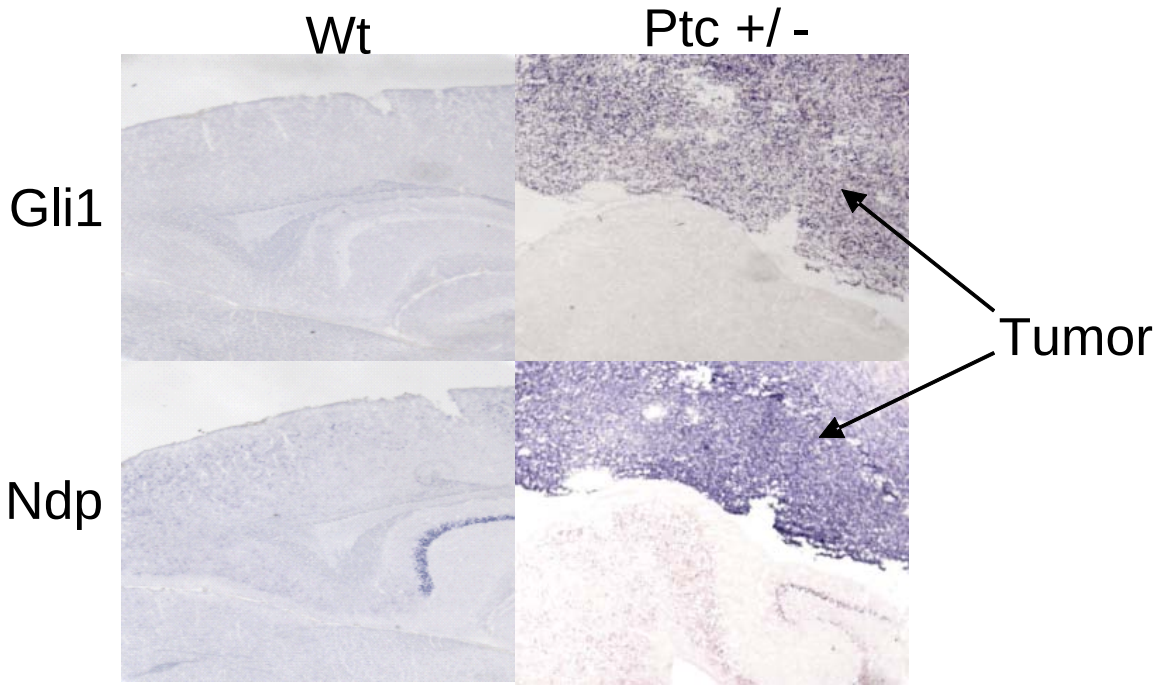
Appendix figure 2. Immunohistochemistry on *Ndp* KO p6 retinas. Staining performed on postnatal day 6 retinas from Wt and Ndp KO retinas using markers for bipolars (Chx10), amacrine (Pax6, Hpc1), rod photoreceptors (Crx, recoverin), horizontal (Calbindin) and ganglion (Brn3b) cells.


E12.5
E18.5
P0.5
P6
Relative fold change in mRNA expression
8
6
4
2
0
-2
-4
Ppp1ca
Tpi1
Psme2
Ergic3
Sumo1
Mark2
Cdk9
Spn2b
Capns1
Casp3
Gli2S
Arhgap6
Ccl21a
Olfr73
Pim2
Fsd1
Nrip3

Appendix figure 3. Microarray validation. RT-qPCR analysis performed on retinas isolated from wildtype and *Ndp* KO mice at various ages. As asterisk denotes significant (p 0.05) in *Ndp* KO retinas compared to wildtype as calculated using a Student's t-test. n=3 for each group. Bars represent SEM.

APPENDIX TABLE 1. Largest differentially expressed genes identified by microarray analysis in *Ndp* mutant retinas compared to wt littermates

				Gene Ontology (GO)	
Fold change	Accession	Symbol	Name	Biological process	Cellular component
-8.60	NM_031868	Ppp1ca	Protein phosphatase 1	Cell cycle, Metabolic process	Cytosol, Nucleus, Cytoplasm
-6.07	NM_009415	Tpi1	Triosephoshate isomerase1	Metabolic process, Glycolysis	Cytosol, Nucleus
-6.04	NM_011190	Psme2	Proteasome 28 subunit, beta	Positive regulation of endopeptidase activity	
-5.54	NM_025516	Ergic3	ERGIC and golgi 3	Vesicle-mediated transport	Membrane, Golgi apparatus
-5.06	NM_009460	Sumo1	SMT3 suppressor of mif two 3 homolog 1	Regulation of transcription, Sumoylation	Nucleus, Cytoplasm, membrane
-4.67	NM_007928	Mark2	MAP/microtubule affinity-regulating kinase2	Cell differentiation, Phosphorylation	Membrane, Nucleus
-4.08	NM_130860	Cdk9	Cyclin-dependent kinase 9	Transcription, Phosphorylation	Nucleus, Nucleoplasm
-4.00	NM_175836	Spnb2	Spectrin beta 2	Intracellular protein transport	Cytoplasm, Membrane, Nucleus
-3.57	NM_009795	Capns1	Calpain, small subunit 1	Metabolic process	Cytoplasm, Membrane
-2.68	NM_009810	Casp3	Caspase 3	Apoptosis	Cytoplasm, Nucleus, Cytosol
-2.12	NM_031184	Glis2	GLIS family zinc finger 2	Transcription	Cytoplasm, Nucleus
8.96	NM_009707	Arhgap6	Rho GTPase activating protein 6	Cytoskeleton organization, Signal transduction	Cytoplasm, Intracellular
5.64	NM_011335	Ccl21a	Chemokine (C-C motif) ligand 21A	Leukocyte chemotaxis	External side of plasma mebrane
5.17	NM_054090	Olfr73	Olfactory receptor 73	Sensory perception of smell	Intragal to membrane
4.60	NM_138606	Pim2	Proviral integration site 2	Apoptosis, Phosphorylation	
3.98	NM_183178	Fsd1	Fibronectin type 3 and SPRY domain-containing protein	Cell cycle, Cell division, Mitosis	Cytoplasm, Cyctoskeleton
3.78	NM_020610	Nrip3	Nuclear receptor interacting protein 3	Proteolysis, Biologicprocess	


Wt
Ptc +/ -
Gli1
Ndp
Tumor

Appendix figure 4. Ndp expression increased in medulloblastoma. *In situ* hybridization for *Gli1* and *Ndp* on sections from wildtype and Ptc+/- related medulloblastomas.

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