FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

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FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

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'Suppressor of Fused' Antagonizes Hedgehog Signaling and Is Required to Maintain Retinal Progenitor Cell Identify and Multipotency

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'Suppressor of Fused' antagonizes hedgehog signaling and is required to maintain retinal progenitor cell identity and multipotency.

Matthew Cwinn

THESIS

Submitted to the School of Graduate Studies in partial fulfillment
of the requirements for the degree of
Master of Science

University of Ottawa
Ottawa, Ontario, Canada
April 2009

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Your file Votre référence
ISBN: 978-0-494-59467-4
Our file Notre référence
ISBN: 978-0-494-59467-4

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Abstract

The mature retina consists of six neuronal and one glial cell type that are derived from a pool of mulipotent progenitor cells (RPC). The decision to remain as a multipotent progenitor or to specify a particular retinal cell lineage and differentiate are governed by cell intrinsic and extrinsic factors. Sonic hedgehog (Shh) is a secreted lipoprotein that is motigenic for RPCs and influences cell fate decisions. *Suppressor of fused* (*Sufu*) is an intracellular antagonist of the pathway; however, its role in regulating *Shh* signaling and influencing cell fate decisions in RPCs are unknown. Here, I demonstrate that *Sufu* antagonizes the Hh pathway in RPCs both *in vitro* and *in vivo*. Surprisingly, *Sufu* was required to maintain early RPC identity and multipotency. Conditional deletion of *Sufu* in early RPCs resulted in the down-regulation of transcription factors required to maintain RPC identity and multipotency as well as transcription factors required to specify all seven retinal cell types. *Sufu*-null RPCs were incapable of differentiating into the normal complement of retinal cell types and instead differentiated into restricted subsets of interneurons. These data demonstrate that *Sufu* antagonizes the Hh pathway in RPCs and provides novel evidence that *Sufu* is required for proper progenitor cell behavior.

Acknowledgements

First and foremost I would like to give many thanks to my supervisor, Dr. Valerie Wallace, for the great opportunity to study in her laboratory and for the guidance and support that she provided along the way. I am also grateful to my committee members, Dr. Dave Pickets and Dr. Ruth Slack for their helpful suggestions.

Of course, I must also thank everyone in the Wallace lab (past, present or honorary): Sean Beug, Andrew Ha, Rina Lall, Michael Le, Heather McGee, Brian McNeill, Chantal Mazerolle, Chantal Medina, Randy Ringuette, Sherry Thurig, Dana Wall and Dr. Yaping Wang. You all provided me with tons of technical help and know how to have an awesome time. I am especially grateful to Chantal for getting the project started, Dana, Chantal and Brian for answering millions of technical questions, Sherry and Michael for technical help, Chantal FM for answering questions about amacrines and Andrew for defining the BLOB.

Finally, I would like to thank my parents for their support and encouragement and Caryn for listening to my ideas and putting up with the numerous experiment-related complaints and surprise stop-ins to the lab to stop *in situ* reactions or change culture medium.

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Abbreviations

bp	Base pair
BrdU	Bromodeoxyuridine
C57/Bl6	C57 Black 6

Cdc2l1	Cell division cycle 2-like 1
ChAT	Choline acetyltransferase
Chx10	Ceh-10 homeodomain containing homolog
CKO	Conditional knock-out
CM	Ciliary margin
CNS	Central Nervous System
CRALBP	Cellular retinaldehyde binding protein
Crx	Cone rod homeobox
DIG	Digoxigenin
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyriboneuceic acid
dNTP	Deoxyribonucleotide
DTT	Dithiothreitol
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediaminetetraacetic acid
En	Embryonic day n
ES cells	Embryonic stem cells
EtOH	Ethanol
FBS	Fetal bovine serum
FoxG1	Forkhead/winged helix box G1
FoxN4	Forkhead/winged helix box N 4
GABA	Gamma aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
Gli1	Glioma associated oncogene homolog 1

Gli2	Glioma associated oncogene homolog 1
Gli3	Glioma associated oncogene homolog 1
Gli-Luc	Gli luciferase
Gly-T1	Glycine transporter 1
GNP	Granule neuron precursor
GSK3-β	Glycogen synthase kinase 3 beta
H&E	Hematoxylin and eosin
Hes1	Hairy and enhancer of split 1
HPLC	High performance liquid chromatography
Hh	Hedgehog
ICC	Immunocytochemistry
IHC	Immunohistochemistry
LB broth	Luria-Bertani broth
LOH	Loss of heterozygosity
Math 3	Mouse atonal homolog 3
Math5	Mouse atonal homolog 5
MMLV	Muloney murine leukemia virus
mRNA	Messenger ribonucleic acid
NeuroD1	Neurogenic differentiation 1
Nkx2.1	NK homeobox 2.1
Nrl	Neural retina leucine zipper
OCT	Optimal cutting temperature
Otx1	Orthodenticle homeobox 1
Otx2	Orthodenticle homeobox 1
P*N*	Post natal day N
Pax2	Paired box 2

Pax6	Paired box 6
Pax6 CKO	Paired box 6 conditional knock-out
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Ptc	Patched
Prox1	Prospero homeobox1
qPCR	Quantitative polymerase chain reaction
Rax/Rx1	Retinal and anterior neural fold homeoxbox 1
RGC	Retinal ganglion cell
RPC	Retinal progenitor cell
RPE	Retinal pigmented epithelium
SAP18	mSin3 associated protein, 18kDa
SEM	Standard error of the mean
Shh	Sonic hedgehog
Sox2	SRY (sex determining region-Y) box 2
Smo	Smoothened
Sufu	Suppressor of fused
Sufu CKO	Suppressor of fused conditional knock-out
TH2	Tyrosine hydroxylase 2
TUNEL	Terminal uridine deoxynucleotidyl transferase
WT	Wild type

1. Introduction

Proper central nervous system (CNS) development is dependent upon the maintenance of a pool of progenitor cells, cell fate specification and differentiation into the correct complement of neuronal and glial subtypes. The co-ordinate regulation of these processes is mediated by a complex interplay between intrinsic and extrinsic regulatory processes that are only beginning to be elucidated. Understanding these processes is not only important from a scientific perspective, but is crucial for the development of cell based regenerative therapies as well as advancing our understanding of cancer biology. The retina is an accessible and relevant model of CNS development, as many of the genetic and signal transduction pathways that regulate development in other regions of the brain are conserved in the neural retina. Other advantages of the retina as a model for CNS development are that the development of this structure is well characterized, it is tractable for *ex vivo* experimental manipulation and that targeted perturbations of developmentally relevant genes and signaling pathways to the retina are, in general, not lethal.

1.1 Development of the Retina

Eye development can be subdivided into two broad stages: 1) specification and patterning of the eye field and presumptive ocular structures, and 2) the development of these early structures into anatomically correct components of the adult eye, such as the retina. Since the focus of this work is to elucidate mechanisms that are important for the latter process, early eye development will only be touched upon briefly.

Formation of the mouse eye begins around embryonic day 8.5 (E8.5), when bilateral evaginations from the ventral diencephalon, which later form the optic stalk, extend towards the surface ectoderm (17). Upon contact with the surface ectoderm, these two structures invaginate, and the surface ectoderm becomes the presumptive lens, while the invaginated portion of the optic stalk forms the optic cup. The inner layer of the optic cup will later give rise to the neural retina, while the outer layer will give rise to the retinal pigmented epithelium (RPE) (17) (Figure 1).

The immature neural retina is organized as a pseudostratified neuroepithelium composed of a pool of multipotent retinal progenitor cells (RPC). Lineage tracing experiments have demonstrated that a single retinal progenitor is capable of giving rise to the six neural and one glial cell types found in the mature retina: ganglion, amacrine, horizontal, bipolar, rod and cone photoreceptors, Müller glia (46, 124). In the adult retina, these seven cells are arranged in three distinct nuclear layers: the ganglion cell layer, the inner nuclear layer and the outer nuclear layer (Figure 2). The ganglion cell layer contains the nuclei of RGCs and displaced amacrine cells, the inner nuclear layer contains the nuclei of amacine cells, horizontal cells, bipolar cells and Müller glia, and the outer nuclear layer contains the nuclei of the rod and cone photoreceptors. The mature retinal cell types are born in a conserved temporal order (Figure 3) beginning during embryonic development and finishing within the first ten days of post-natal life (137). Retinal ganglion cells (RGC) are born first, beginning at E11.5, followed by horizontal cells, cone photoreceptors and roughly half of the amacrine cells. The remaining amacrine cells as well as bipolar cells and Müller glia are generated postnatally, while rod photoreceptors are generated throughout retinal development (137).

Superficially, the stereotypical birth order of retinal cell types from a pool of multipotent RPCs seems counter-intuitive. If RPCs have the capacity to give rise to all seven cell types, then why are cell types specified sequentially rather than simultaneously? This phenomenon could most easily occur through a variation of the competence model proposed by Cepko and colleagues (71), whereby RPCs become restricted in their competence in a temporal manner such that a 'late' RPC will no longer have the competence to generate an 'early' cell type.

Figure 1: Early eye development. A) At ~E8.5 the optic vesicle evagninates from the neuroectoderm and extends towards the surface ectoderm. B) The presumptive retina (blue squares) comes in to contact with the lens placode (green squares). C) The presumptive retina invaginates, forming the optic cup. The lens placode also invaginates, and this will give rise to the lens. D) The inner portion of the optic cup gives rise to the neuro-retina while the outer portion forms the retinal pigmented epithelium (RPE)

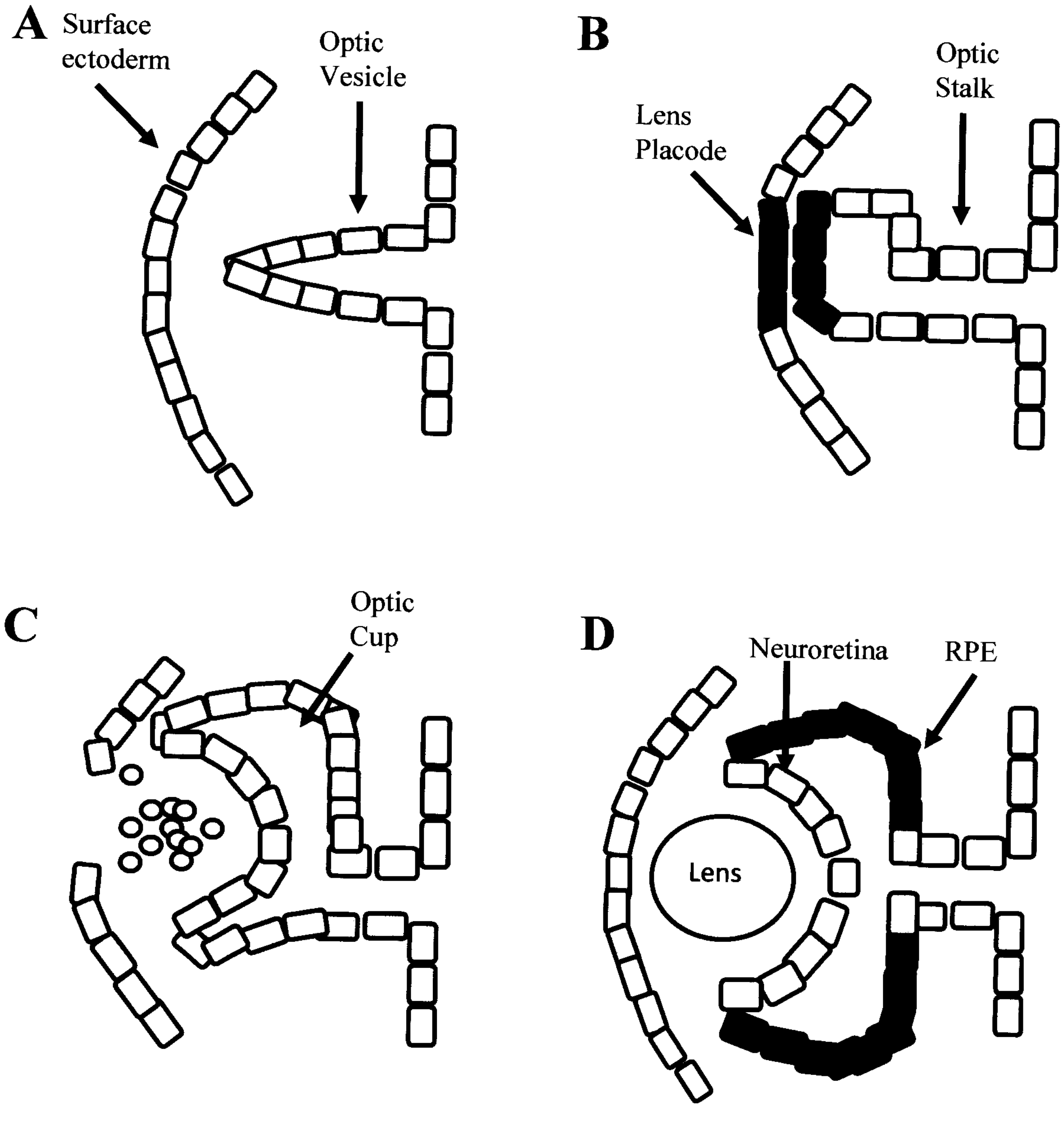

A
Surface ectoderm
Optic Vesicle
B
Lens Placode
Optic Stalk
C
Optic Cup
D
Neuroretina
RPE
Lens

Figure 2: Morphology of the developing and adult retina. A) Immunohistochemistry for Ki67 in an E12.5 retina. The developing retina consists of a neuroblast layer containing proliferating, multipotent progenitor cells that express Ki67 and a layer of differentiated cells. B/C) Morphology of a mature retina. B) A cartoon illustrating the morphology of the mature retina and C) an H&E stain of a P18 retina. The adult retina contains six neural and one glial cell types that are organized into three nuclear layers. The ganglion cell layer contains nuclei of the ganglion cells and displaced amacrine cells, the inner nuclear layer contains nuclei of the amacrine, bipolar, horizontal and Müller cells and the outer nuclear layer contains nuclei of the rod and cone photoreceptors. The cartoon in (B) was adapted (31).

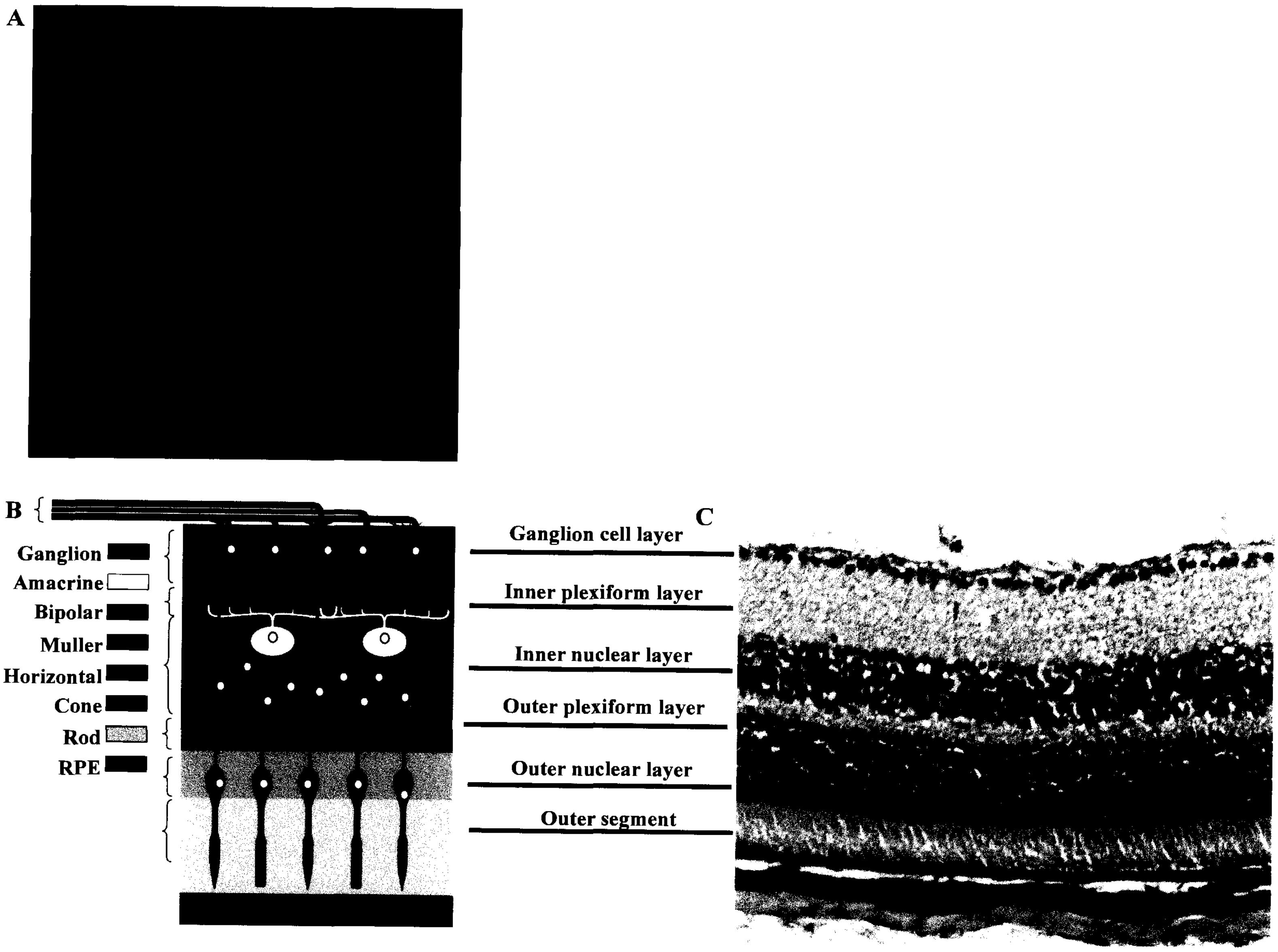
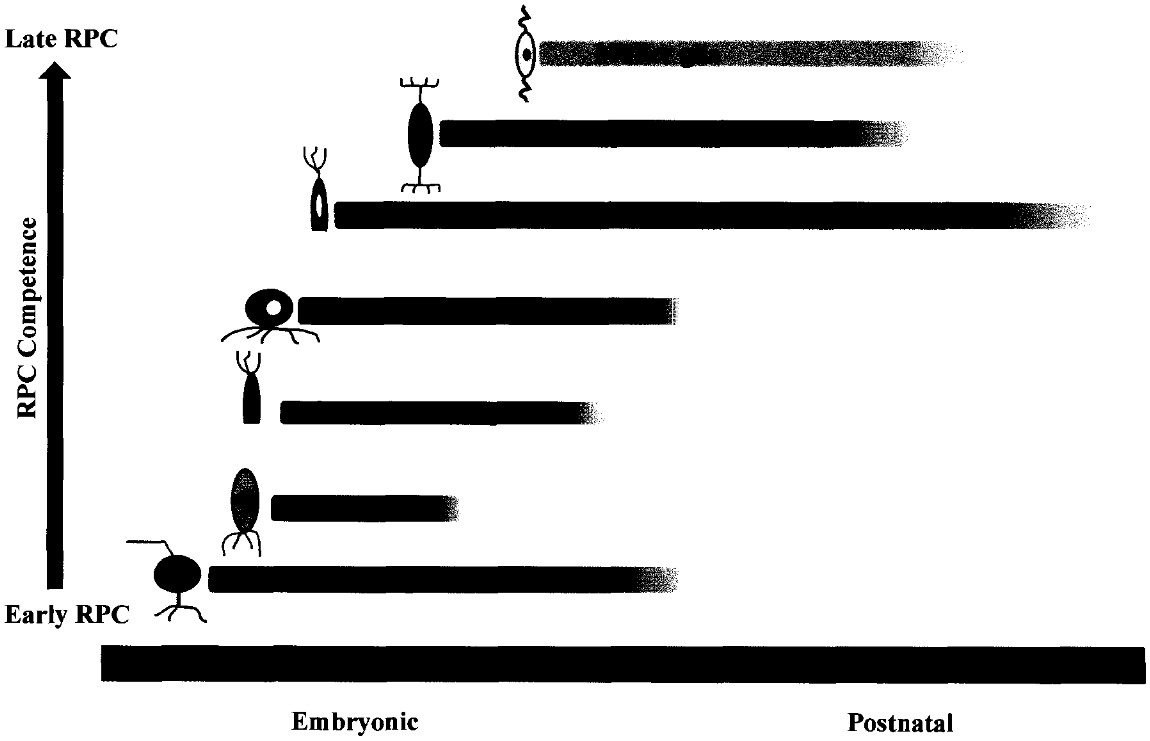

A
B
Ganglion
Amacrine
Bipolar
Muller
Horizontal
Cone
Rod
RPE
C
Ganglion cell layer
Inner plexiform layer
Inner nuclear layer
Outer plexiform layer
Outer nuclear layer
Outer segment

Figure 3: Retinal cell types have a conserved birth order. The competence of a multipotent retinal progenitor cell (RPC) to specify a particular retinal cell type is dictated by intrinsic transcription factors that are influenced by extrinsic cues. RPC competence changes during the course or retinal development so that an RPC is capable to specify a limited number of cell types at any given time. This results in the conserved temporal birth order of the seven retinal cell types.


Late RPC
RPC Competence
Early RPC
Embryonic
Postnatal

The competence of a particular RPC to maintain multipotency, continue to divide or to specify a particular cell fate is dictated by cell-intrinsic transcription factors that are influenced by cell-extrinsic cues (71).

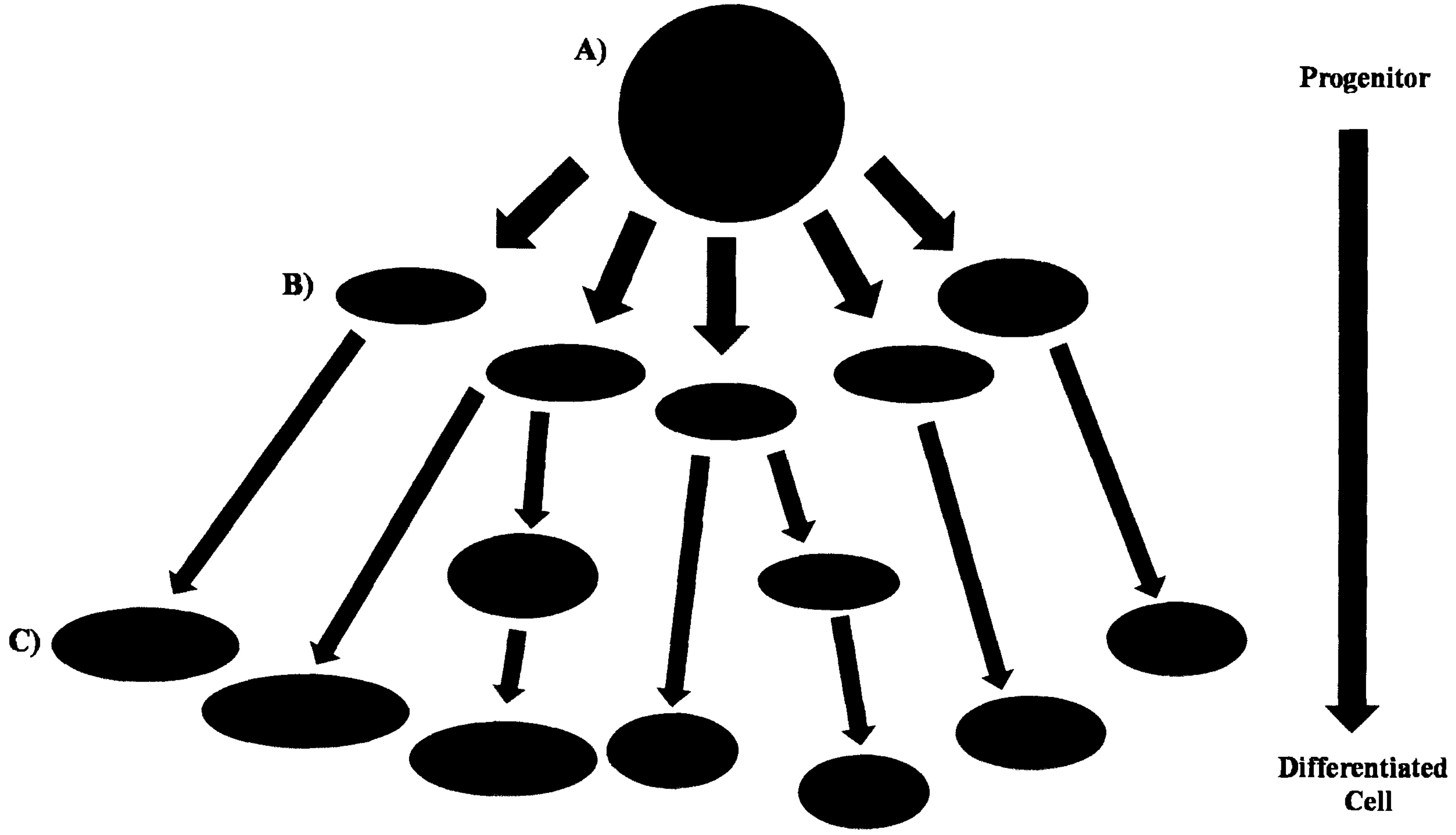
1.2 Retinal Progenitor Cell Function: Multipotency, cell cycle and cell type specification.

Retinal progenitor cell identity, which I will define as having both proliferative and multipotent capacity, is maintained by both intrinsic and extrinsic signals. Intrinsically, these two processes are influenced by the expression of multiple transcription factors (97) and the expression or function of these transcription factors is, in part, regulated by extrinsic signaling molecules (136). The complex balance and interactions of extrinsic and intrinsic factors is essential for the generation of the correct cellular complement of the retina: premature cell cycle exit would result in a deficit in the number of progenitors and could result in an increase in early born cell types at the expense of late cell types (122, 130, 131) while delayed cell cycle exit could result in an increase in later born at the expense of early born cell types (122, 127) or, if cells fail to exit the cell cycle entirely, tumorigenesis (2, 14). Similarly, alterations in the expression of transcription factors required for cell type specification could result in an incorrect proportion of the retinal cell types (36, 70, 72).

1.3 Intrinsic Regulation of RPC identity.

Maintaining RPCs in a proliferative and multipotent state is intrinsically regulated by a complex process that is influenced by several different transcription factors including *Pax6* (80, 140), *Rax* (82, 140), *Hes1* (63, 122, 127), *Chx10* (12, 47) and *Sox2* (119). All of these transcription factors are expressed in RPCs, can be used to define the progenitor population and play crucial roles in maintaining retinal progenitor cell identity (Figure 4). Loss-of-function studies have

Figure 4: RPC identity and cell fate specification are influenced by particular subsets of transcription factors. A) Maintenance of RPC identity is dependent upon the expression of a subset of transcription factors that are required for multipotency, to maintain of neuro-progenitor identity, to prevent differentiation and to promote or enable proliferation. B) Lineage commitment is dependent upon the expression of transcription factors that may be either permissive or instructive for the specification of a particular cell fate and the subsequent differentiation into that fate (C). Progenitors are shown in red and precursors or differentiated cells are shown in blue.


A)
B)
C)
Progenitor
Differentiated
Cell

demonstrated that mice deficient in *Hes1*, *Chx10* or *Sox2* exhibit microopthalmia (small eye) (12, 30, 122) and mice deficient in *Pax6* or *Rax* exhibit microopthalmia and/or anopthalmia (no eye) (45, 82). In humans, mutations in *Pax6, Rax, Chx10* or *Sox2* result in severe ocular malformations, including micropthalmia, anopthalmia and aniridia (no iris) (22, 29, 30, 49, 56)

Once the eye cup has formed, *Pax6* is crucial for the maintenance of both RPC multipotency (80,98) and proliferation (80, 140). Conditional deletion of *Pax6* results in precocious cell cycle exit and the exclusive generation of amacrine cells at the expense of all other retinal cell types (80) and therefore is one of the most important genes involved in maintaining RPC identity that has been identified to date.

The homeobox domain protein *Rax* is another transcription factor that is essential for retinogenesis. *Rax* is crucial for specifying the eye field (82). Since *Rax*-null embryos fail to specify the eye field, and thus do not form a retina (82), and a conditional *Rax* allele has not been generated, little is known about the specific functions of this transcription factor in RPCs during early and mid retinal development in mammals. However, an accumulating body of evidence suggests that *Rax* is involved in the specification of RPCs. Over-expressing *Rax* in embryonic stem cells results in the capacity to develop into an RPC-like cell and to integrate into retinal explants and generate ganglion and bipolar neurons, while ES cells that did not over-express *Rax* were not capable of integration or differentiation into retinal cell types (116). Other studies have demonstrated that the *Xenopus* homolog, *Rx1,* is involved in the proliferation and maintenance of retinal stem cells and that *Rx1* over-expression biases cell fate in a temporal fashion (140). In the rat, over-expression of *Rax* in post-natal progenitor cells results in the generation of Müller glia (36), suggesting that *Rax* plays a role in RPC maintenance during early retinal development, and specifies Müller glia during later stages of retinal development. However, it is not known if this specification is instructive or permissive.

Hes1 acts as a negative regulator of neurogenesis in RPCs (36, 122). It is thought to function in this context by repressing the transcriptional activation of pro-neural genes such as Math5 (84) and is therefore crucial for maintaining the RPC pool. In mice deficient in *Hes1*, RPCs prematurely exit the cell cycle (63, 122), resulting in the precocious generation of early retinal subtypes (63) while over-expression of *Hes1* prevents neural differentiation (36, 122). *Hes1* is also required during later stages of retinal development to prevent neural differentiation and direct RPCs towards a Müller glia cell fate (36). *Hes1* expression in RPCs is dependent, in part, upon the extrinsic signaling mediated by the Notch (55, 94) and Sonic hedgehog pathways (127), and therefore serves as an excellent example to illustrate the interactions between extrinsic and intrinsic pathways.

In RPCs, *Chx10* plays a variety of roles including maintaining neural retina identity, influencing proliferation and specifying particular cell fates. The retinas of *Chx10*-deficient mice display a proliferative defect (12, 42, 112), although the proliferation deficiency observed in these mice is, in part, non cell-autonomous due to the delayed production of ganglion cells and secretion of the mitogen *Sonic Hedgehog* (*Shh*, see below) (112). These mice also exhibit aberrant RPE differentiation at the expense of neural retina (47), and fail to form bipolar cells (12, 72). Thus, like *Rax* and *Hes1, Chx10* is implicated in both RPC maintenance as well as cell fate specification.

The SRY-box transcription factor, *Sox2* has also recently been implicated in the function and maintenance of *RPCs* (119), which is not suprising, given the role of *Sox2* in the maintenance of neural progenitors from other regions of the CNS (41). Conditionally ablating *Sox2* in RPCs inhibits their ability to both continue to cycle and to differentiate (119). Reducing, but not completely ablating, *Sox2* levels in RPCs results in aberrant cell cycle exit and differentiation, leading to microopthalmia (119). In the mature retina, *Sox2* is expressed in amacrine interneurons and Müller glia (68, 119) and over-expression of *Sox2* in RPCs at E17.5 promotes the generation of amacrine cells (68).

1.4 Intrinsic factors in cell type specification

The expression of transcription factors that specify particular cell fates is crucial for driving RPCs or post-mitotic precursors towards particular cell fates. Not surprisingly, this process is extremely complex and the hierarchal transcription factor cascades that act to specify a particular cell fate are only beginning to be elucidated. However, transcription factors required to specify each of the seven retinal cell types have been identified, mostly through gain and loss of function studies (97). For example, *Math5* is a permissive factor for the acquisition of the ganglion cell fate (135). Forced expression of *Math5* promotes ganglion cell fate (70), while mice deficient in *Math5* fail to form ganglion cells (129). As mentioned above, several transcription factors that are required during early retinal development for maintaining RPC identity later play a role in cell type specification (36, 72, 122). A summary of the pertinent transcription factors that have been found to specify specific retinal cell types is presented in Table 1.

Table 1: A summary of transcription factors required to specify or maintain the seven retinal cell types.

Cell Type	Transcription Factor	Class	Citation
Ganglion Cells	Math5 Brn3b	bHLH* POU	(70, 129) (37)
Amacrine Cells	FoxN4 Math3 and NeuroD1	Forkhead bHLH	(67) (53)
Horizontal Cells	FoxN4	Forkhead	(67)
Bipolar Cells	Chx10	Homeobox	(12, 72)
Rods and Cones	Crx	Paired Homeobox	(34, 35)
Rods	Nrl	Leucine Zipper	(85)
Müller Glia	Rax Hes1	Homeobox bHLH	(36)

*bHLH - basic Helix-Loop-Helix.

Although most of the studies to date have focused on vertical transcription factor hierarchies that lead to the production of a particular cell fate, it is becoming increasingly clear that cell fate specification in RPCs is also dependent upon the activities of transcription factors that function in parallel, rather than sequentially (90), further adding to the complexity of this process. Deletion of both *NeuroD1* and *Math3* prohibits amacrine cell genesis, while deletion of only one of these does not (53). However, combinatorial over-expression of both *NeuroD1* and *Math3* will not lead to the generation of amacrine cells, unless *Pax6* is also co-expressed (53). Additionally, some transcription factors are involved in the specification of multiple different cell types. For example, *Math3,* when co-expressed with *Six3*, drives horizontal cell genesis (53) and *NeuroD1,* but not *Math3*, is capable of restoring ganglion cell genesis when it is knocked in to the *Math5* locus on a *Math5* KO background (78). Therefore, interpreting how particular cell fates are specified is relatively simple when one modulates the activity of a transcription factor that is positioned at the apex of a regulatory cascade but becomes increasingly more complex when one examines the roles of factors further down-stream.

1.5 Extrinsic signaling factors in the retina

In the retina, numerous extrinsic signaling pathways are required to modulate the behavior of RPCs and to promote proper retinal development, including Notch, transforming growth factor β (Tgf-β), retinoic acid (RA) and sonic hedgehog (Shh).

Activation of the Notch pathway in RPCs prevents cell cycle exit and neurogenic differentiation (101) and therefore plays a role in the maintenance of the progenitor pool. In the rodent, chick and *Xenopus*, inhibition of Notch signaling in RPCs results in neural differentiation (5, 22, 44). Notch also functions to influence cell fate decisions. During early retinal development, inhibition of Notch signaling results in the preferential production of early cell types including RGCs (5, 126)and cone photoreceptors (55). Conversely, ectopic Notch pathway activation in in late RPCs biases these cells towards adopting a Müller cell fate (36, 55), which is the last retinal cell type to be specified during development.

The transforming growth factor β (Tgf-β) super-family of signaling molecules is comprised of a number of subgroups, including the Tgf-β, bone morphogenic protein (Bmp) and growth and differentiation factor (Gdf) subgroups(109).

Current evidence suggests that, in the retina, Tgf-β1,2 and 3 induce programmed cell death in RPCs and therefore help control the total cell number of the retina (24, 25, 75). In the early post-natal retina, Tgf-β2 is the most abundantly expressed Tgf-β family subtype, and it has been implicated in the induction of mitotic senescence of retinal progenitor cells and Müller glia (18). In addition to controlling PCD and proliferation, Tgf-β2 plays a role in cell fate determination, as it negatively regulates amacrine cell genesis (75).

The role of Bmps has been more substantially characterized in the retina. Bmp4 controls the expression of transcription factors required to specify dorsal retinal identity(9). Haploinsufficiency for Bmp4 in mice is associated with eye defects, such as a reduction in the number of ganglion and inner nuclear cells (13). Bmp7 dosage is also important for eye development, as gain and loss of function for Bmp7 is associated with apoptosis in the retina and microphthalmia and anophthalmia (23, 56, 74). Bmp signaling is also helps specify peripheral eye fates, such as the ciliary body. Bmp4 and Bmp7 are expressed in the ciliary margin, the distal part of the eyecup, and Bmp signaling is required to maintain this structure, as it differentiates into neurons in the presence of ectopically expressed noggin, a soluble Bmp antagonist(143).

Members of the Gdf sub-family have also recently been shown to be important for RPC cell fate specification and retinal patterning. In the mouse, Gdf-11 negatively regulates ganglion cell development by controlling the competence of RPCs to specify and/or produce this cell type

(61). In the absence of Gdf-11, an increase in RGCs is observed at the expense of amacrine cells and photoreceptors (61). In the zebrafish, another Gdf family member, Gdf-6a, is required to pattern the dorsal retina (40).

Although the functions of Tgf-β family members in retinal development is only beginning to become uncovered, it is becoming evident that members of this super-family have similar roles in modulating retinal development, such as controlling cell death, promoting dorsalization of the retina and, in some cases, influencing cell fate decisions.

Retinoic acid (RA) is a vitamin A derivative that influences many aspects of retinal development. During early retinogenesis, RA is involved in regulating the growth of the ventral optic cup (81, 83). Later, RA plays a role in rod photoreceptor development (66). However, whether RA affects commitment towards a rod photoreceptor fate or whether it affects rod differentiation (or both) is under debate (66).

1.6 The sonic hedgehog signaling pathway

During embryogenesis, *Shh* signaling is essential for proper CNS development, including development of the retina. In general, *Shh* plays a role in establishing the ventral midline, anterior/posterior patterning as well as dorsal/ventral patterning. Additionally, S*hh* can act as a mitogen to promote the proliferation of progenitor and stem cells both in the embryo and in the adult (32). It is important to note, however, that the effects of *Shh* are context dependent (32, 52), both in terms of its action as a classical morphogen, as in the neural tube (32, 54, 103, 104), or not, as in the retina (130) as well spatial and temporal differences in mitogenic activity (32). In the neural tube, *Shh* is secreted by the notochord and the floor plate and acts as a classical morphogen such that a ventral-high, dorsal-low gradient is formed (54). Neural tube progenitors are influenced to differentiate into particular neural subtypes depending on how they interpret the *Shh* signal, which in turn is dependent on where they are situated within the *Shh* gradient (54) over-activation of the Hh pathway in the neural tube causes the majority of the progenitors to specify ventrally located neuronal subtypes (ventralization) (39, 50, 115). *Shh* is also involved in the establishment of ventral structures in other regions of the CNS, including the forebrain (telencephalon and diencephalon) and the cerebellum (106) although it may not act as a classical morphogen in this context (32). In the retina, *Shh* is thought to act predominantly over very small distances, as conditional deletion of *Shh* in the peripheral retina is not rescued by *Shh* secreted by ganglion cells in the central retina (130).

Shh is also mitogenic for progenitors in various regions of the brain (32, 106) as well as the retina (11, 73, 89, 127, 130, 138) and controls the proliferation of adult neural stem cells (1, 76, 100). Thus, *Shh* not only patterns the CNS, but also plays a role in regulating total cell numbers of progenitor/stem cells in both the developing, and adult, CNS.

Given the importance of the *Shh* pathway in development, it is not surprising that both loss and gain of function for *Shh* has severe consequences for development (52, 58). Gain of function of *Shh* is frequently oncogenic (52, 58), while loss of function for hedgehog results in a congenital malformations including holoprosencephaly (10, 16, 105) and, in the most severe cases, cyclopia (16, 105), as well as ocular problems (105, 108, 130, 131). Consequently, gaining a thorough understanding of the roles of *Shh* signaling has direct implications in our understanding of human disease.

1.7 Sonic Hedgehog in the Retina

Retinal expression of *Hh* pathway components has been described in a number of vertebrate species (4). In the mammalian retina, Shh produced and secreted by RGCs (57, 130, 131) is the primary mediator of Hh signaling during retinal development (91, 130, 131). The timing of Shh and *Shh* target gene induction in RGCs and RPCs, respectively, mirrors the central to peripheral wave of RGC differentiation (130). RGC ablation in explant culture (91, 130, 131) or through conditional endotoxin expression (91) abrogates Hh pathway activation in RPCs, as does conditional inactivation of Shh in the retina (91, 130, 131). In the adult murine retina, Shh is also expressed in the inner nuclear layer, most likely in amacrine neurons (57) and Ptc is expressed in Muller glia (11, 57), however, the functional significance of Hh pathway expression in the adult retina is not known.

1.8 Shh acts as a mitogen in RPCs

Shh is mitogenic for RPCs (11, 73, 89, 127, 130, 131, 138) and therefore contributes to the regulation of cell number in the retina. Accordingly, conditional loss of Shh expression in the retina results in a decrease in the proliferative zone of the immature retina and precocious cell cycle exit (91, 130), while forced activation of the pathway leads to an increase in proliferation (138). *Shh* drives the cell cycle in RPCs, in part, by inducing the transcription of target genes involved in cell cycle progression, such as Cyclin D1 (130) as well as genes involved in progenitor cell maintenance, such as Hes1 (127). In addition to promoting proliferation, *Shh* signaling also affects cell cycle kinetics in lower vertebrates. In both *Xenopus* and zebrafish, increasing *Shh* activity in early retinal progenitor cells accelerates the cell cycle by shortening G1 and G2/M resulting in an increase in the number of cell divisions compared to control retinas (73) but also promotes cell cycle exit (73, 111), by up-regulating the cell cycle inhibitor *p57(Kip2)* (111). Therefore, although the latter effect has not been investigated in a mammalian system, it appears that *Shh* controls several aspects of the cell cycle by promoting proliferation while playing a role in the regulation of cell cycle kinetics and cell cycle exit.

1.9 Shh Patterns the Retina

In the retina *Shh* signaling also affects cell type development. *Shh* acts as a negative regulator of retinal ganglion cell development (130, 142); conditional deletion of *Shh* results in patterning defects, such as an increase in RGC and photoreceptor cell numbers and a decrease in Müller and bipolar cell number (130) as well as lamination defects (130, 142). Conversely, increased *Shh* signaling inhibits RGC development (130, 142) and ectopic *Shh* pathway activation in P0 explants results in an increase in most inner retinal cell types at the expense of photoreceptors (57, 127, 138).

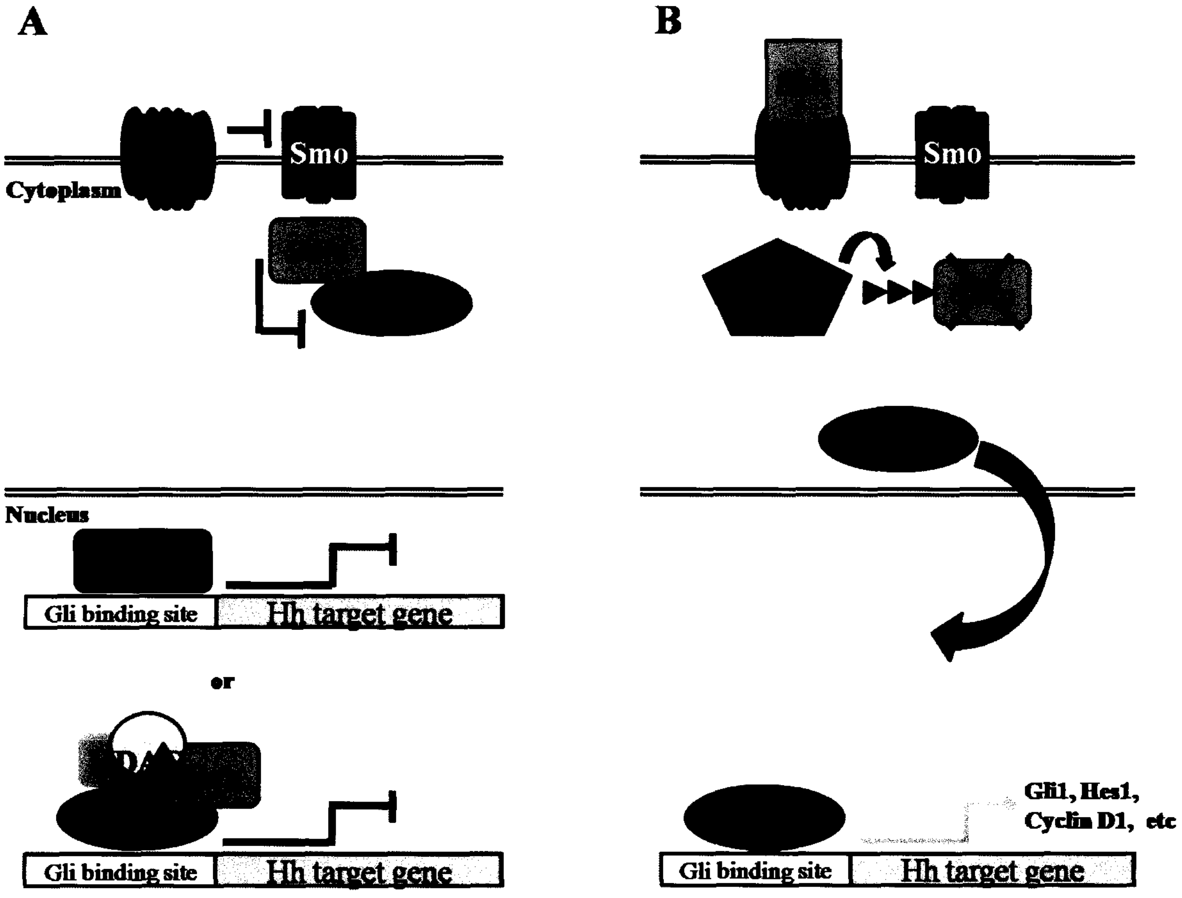
However, despite the accumulating body of knowledge regarding the roles of *Shh* in the retina, many important questions remain unanswered. First, the roles of *Sonic Hedgehog* as a mitogen and in cell fate determination have yet to be successfully uncoupled at the mechanistic level. It has not yet been determined if *Shh* directly promotes cell fate specification or if *Shh* signaling influences cell fate induction through proliferative effects that provide a permissive, rather than instructive cue. Does gain-of-function for *Shh* in late retinal progenitors bias the cells towards a Müller glia fate directly by up-regulating transcription factors involved in cell-fate specification or does the mitogenic effect on RPCs keep the progenitors in cycle longer, such that their competence becomes increasingly restricted towards the generation of the latest born cell type in the retina, the Müller cell? Uncoupling these effects would provide greater insight and understanding into the relative roles of the extrinsic versus the intrinsic environment in neural progenitor proliferation and cell fate specification. Another important question that has not been properly addressed is the potential for temporal differences in the response of RPCs to *Shh* signaling and the potential consequences of these effects. Evidence from our lab suggests that the transcriptional activation of *Shh* target genes is temporally dependent ((127), our lab, unpublished results); however, the direct implications of these observations remain to be determined.

1.10 The mechanism of Shh signaling

The overall framework of *Hh* signaling is evolutionarily conserved from the fly to mammals. However, there has been divergence throughout evolution in the specifics governing both positive and negative activation of the pathway (48).

In mammals, *Shh* exerts its effect (Figure 5) by binding to its receptor, the transmembrane protein Patched (*Ptc*) (79), which alleviates Ptc-mediated repression of another transmembrane protein, Smoothened (*Smo*). Smo activation initiates the Shh signal cascade (3, 93) that converges upon the activity of *Gli* transcription factors *(Gli1, Gli2, Gli3)* and, ultimately, the transcription of general target genes, including *Gli1* (64) , *Ptc1* (38), and tissue/context specific target genes, including *CyclinD1* (130) and *Hes1* in the retina (127). *In vivo,* Gli2 is the predominant activator of the pathway, and Gli1 acts as a secondary activator (6, 7), while Gli3 is proteolytically cleaved into a repressor form in the absence of Shh signaling (128). Since aberrant activity of the *Shh* pathway results in congenital defects, including eye malformations, and tumors in humans (43), it is not surprising that numerous regulatory mechanisms are in place to maintain tight control over the pathway. At the cell surface, the *Shh* receptor, *Ptc,* has been well characterized as a potent negative regulator of the pathway (39). In mammals, the downstream, intracellular regulators have been less well characterized. One component of the pathway that has recently been shown to act as a potent intracellular antagonist of the pathway is the atypical protein *Suppressor of fused* (*Sufu*).

Figure 5: A schematic of the Hh signaling pathway. A) In the absence of Hh ligand, Ptc represses Smo and the pathway is inactivated. Downstream of Smo, the pathway is further repressed by Sufu and Gli3 repressor. Sufu prevents pathway activation by tethering Gli1/2 in the cytoplasm and possibly by recruiting chromatin remodeling complexes in the nucleus. Gli3 repressor binds to conserved Gli binding sites in the promoter region of Hh responsive target genes to further prevent transcriptional activation by Gli1 or Gli2. B) When Shh binds Ptc, Smo is derepressed. Sufu mediated repression of Gli1 and Gli2 is abrogated, possibly through the ubiquination and subsequent proteolysis of Sufu. Gli1 and Gli2 can then translocate in to the nucleus where they induce the transcription of Hh responsive target genes, such as *Gli1*, *Hes1* and *Cyclin D1*.


A
Smo
Cytoplasm
Nucleus
Gli binding site
Hh target gene
or
Gli binding site
Hh target gene
B
Smo
Gli1, Hes1,
Cyclin D1, etc
Gli binding site
Hh target gene

1.11 Sufu is a negative regulator of the Hh pathway

Sufu is a highly conserved protein that does not share homology to any other known proteins (102), (113, 114) that acts as a negative regulator of *Hh* signaling in both flies and vertebrates (48). Sufu was originally discovered in a *Drosophila* screen as a factor that could suppress the activities of *Fused* kinase (102), a positive regulator of the *Hh* pathway in *Drosophila*. However, because *Sufu*-null flies are viable and exhibit only minor developmental anomalies (102), not much attention was paid to the functions of vertebrate *Sufu* until recently.

Recent evidence suggests that there has been a divergence in the functional regulation of the *Shh* signaling cascade in mammals. Unlike in *Drosophila*, *Sufu* functions as a principal intracellular negative regulator of mammalian *Hh* signaling *in vitro* (62, 120, 125), and *in vivo* (19, 115). The *in vivo* functions of mammalian *Sufu* during early embryonic development have recently been addressed through the generation and characterization of *Sufu* knockout mice (19, 115). These mice die *in utero* at ~E9.5 and exhibit a Shh gain-of-function phenotype similar to that observed in $Ptc^{-/-}$ mice, such as ventralization and failure to close the neural tube (19, 115). However, although the knockout mouse is informative for *Sufu* function at early developmental stages, its usefulness is limited because it does not permit one to address the roles of *Sufu* as a regulator of the *Hh* pathway during later stages of development or during adulthood.

In contrast to the heightened requirement for *Sufu* in mammals, the mammalian homologues of *Fused* and *Costal2*, which are potent activators and repressors of *Hh* signaling in *Drosophila*, respectively (96), appear to be dispensable for *Hh* signaling in mammals. Although *in vitro* evidence has suggested that *Fused* is capable of antagonizing *Sufu* and restoring *Hh* activity in the context of *Sufu* over-expression (92), *Fused* knockout mice do not display a *Shh* gain-of-function phenotype (87) indicating that *Fused* does not play an integral role in mammalian *Shh* signal transduction *in vivo*. Similarly, the mammalian homologues of *Costal2*, *Kif7* and *Kif27*, do not appear to act as negative regulators of the *Shh* pathway in mammalian cells (125), although this has not yet been demonstrated *in vivo* and thus remains a controversial issue.

1.12 Mechanism of Action- Sufu regulates of Gli function

The current consensus in the field of *Hh* signal transduction is that *Sufu* likely exerts its effects by directly interacting with Gli1 and Gli2 proteins and tethering them in the cytoplasm, thus preventing nuclear translocation and transcriptional activation of *Gli* target genes (8, 62, 88, 120, 125). Although domains in the amino and the carboxy domains of *Sufu* are capable of binding *Gli1/2* (88), the carboxy-terminal domain is sufficient to promote cytoplasmic localization of both *Gli1* and *Gli2* (8). *In vitro* experiments have also shown that the N-terminus of *Sufu* can inhibit *Gli1/2* transcriptional activity independent of cytoplasmic tethering (8, 15). This inhibition may be through the recruitment of chromatin remodeling complexes, as the Sufu-Gli1 complex is capable of translocating to the nucleus where Sufu can recruit SAP18, a component of the mSin-3 histone deacetylase complex (15, 99), which in turn represses Gli1-mediated transcriptional activity (15).

The mechanism(s) through which the effects of *Sufu* are abrogated to permit active *Gli1/2* activity are only beginning to be elucidated. Recent evidence suggests that *Sufu* activity is regulated through ubiquitination and that active *Shh* signaling leads to *Sufu* degradation by the proteosome (139). Studies also suggest that *Sufu* can be phosphorylated (99) by several kinases, including GSK3-β (118), and potentially by Cdc2l1 (28) and that both of these phosphorylation events can activate the *Hh* pathway (28, 118). However, the direct consequences of *Sufu* phosphorylation on protein function or stability have not been determined.

1.13 Hedgehog and Sufu in Cancer

Aberrant activation of the *Shh* pathway has been implicated in numerous malignancies in both humans and mice including medulloblastoma (39, 136), nevoid basal cell carcinoma (43), small-cell lung tumors (132) and pancreatic cancer (121). Based on the purported role of *Sufu* as a key negative regulator of *Shh* signaling, it is not surprising that mutations or repression of *Sufu* have also recently been implicated in cancer. Although, for the most part, heterozygosity for *Sufu* does not result in tumorigenesis (19, 65, 120), loss of heterozygosity (LOH) for *Sufu* has been implicated in the development of medulloblastoma (120) and rhabdomyosarcoma (123) in humans. In mice, *Sufu* LOH on a p53-/- background was sufficient to induce both medulloblastoma and rhabdomyosarcoma (65). Moreover, cell lines derived from human lung, breast and prostate tumors that display *Shh* pathway activation have low levels of *Sufu* expression (139) and over-expressing *Sufu* is sufficient to reduce *Hh* activity and attenuate proliferation in NCI-H322M lung cancer cells (139). Reduced *Sufu* activity has also been reported in pancreatic ductal adenocarcinoma cells (59), further underscoring the role of *Sufu* as a tumor suppressor. Thus, there is an increasing body of evidence to suggest that *Sufu* functions as a tumor suppressor in mice and humans both *in vivo* and *in vitro* and improved understanding of *Sufu* function and regulation could lead to the development of putative therapies.

1. 14 Objective

It has been well established that Shh signaling is crucial for proper development of the CNS (52, 58), including the neural retina, where *Shh* acts as a mitogen to control RPC proliferation and acts to influence cell fate decisions (127, 130, 138, 142). In mammals, *Sufu* appears to function as a key intracellular antagonist of *Shh* signaling (19, 115, 120, 125). By mediating pathway activity downstream of *Ptc/Smo* and upstream of *Gli* transcription, *Sufu* occupies a key junction where the cell-extrinsic signal converges upon intracellular machinery to influence cell-intrinsic functions. Based on these observations, I **hypothesize** that *Sufu* plays a pivotal role in the regulation of Shh hedgehog signaling in RPCs and cell fate specification in the developing retina.

The objectives of my project are to:

1) Examine the effects of *Sufu* on *Shh* pathway activation.

2) Characterize the role of *Sufu* in retinal progenitor cell behavior *in vivo.*

2. Materials and Methods

2.1 Mice

All experiments were approved by the University of Ottawa's Animal Care Ethics Committee and adhered to the guidelines of the Canadian Council on Animal Care. Wild type (WT) C57/BL6 mice were obtained from the Charles River Laboratory. The *aP0-Cre* transgenic mice, which express *Cre-recombinase* under the control of the αP0 element of the *Pax6* promoter, were obtained from Dr. P Gruss (Max-Planck Institute, Gottingen, Germany) (80) and maintained on a C57/BL6 background. $Sufu^{Fl/Fl}$ mice, which harbor loxP sites flanking *Sufu* exons 4-8, were obtained from Dr. C.C Hui (Sickkids, Toronto) and maintained on a C57/BL6 background. Retina-restricted conditional *Sufu* knockout mice were generated by crossing *aP0-Cre;* $Sufu^{+/Fl}$ mice with $Sufu^{Fl/Fl}$ mice to generate Cre^{+}; $Sufu^{Fl/Fl}$ mice.

Mice were genotyped by PCR. DNA was isolated by incubating a small tail fragment in 75 μl alkaline lysis buffer (25mM NaOH, 0.2mM EDTA) for 60 minutes at 95°C. Samples were then cooled and neutralized with 75 μl neutralization reagent (40mM Tris-HCl). Genotyping for the α-Cre transgene was done as described previously(130). Genotyping for wildtype and floxed sufu alleles was performed as follows: 2μl 10xPCR buffer (Invitrogen), 0.6μl 50mM $MgCl_2$, 2 μl 1mM dNTP (Invitrogen), 1μl each of 10mM primers: F- 5'CTGTTTGTACTCATGGTC3', R-5' CCTACCCTTTCCAGTAAG3', Del- 5'GCTGAATTCTTGACTCACTG3', Neo-5'GTGTCAGTTTCATCGCCTG3'. The thermal profile for the PCR reaction was 10 minutes at 95°C followed by 32 cycles of 95°C for one minute, 55°C for thirty seconds, 72°C for forty seconds. PCR products were run on a 1% agarose gel, products were stained with ethidium bromide and visualized using a UV transilluminator. Primers directed against the wild type allele yielded a band size of 300bp, those directed against the floxed allele yielded a band size of 350bp.

2. 2 Retinal explant culture, electroporation BrdU incorporation.

Explants were prepared and electroporated as described previously (127). Briefly, eyes from P0 mice were dissected free of the optic nerve and RPE and retinas were then electroporated (ECM 830 BTX Harvard apparatus) in a 2mm gap cuvette (VWR). The DNA plamids used in this study were *Smo-M2* (CMV promoter) (a gift from G.Fishell, New York University, New York), *Sufu*-Flag (CMV promoter) (a gift from Dr. C.C Hui, Sickkids, Toronto), *pUB-GFP*, *Gli-Luciferase* (a gift from Dr. H Sasaki, Osaka), *Renilla Luciferase* (a gift from Dr. Alan Mears, University of Ottawa) and Cre (β-actin promoter) (pML78, Mount Sinai). Plasmids were prepared using a Qiagen Maxiprep kit according to manufacturer's instructions (Qiagen). Plasmids were electroporated at a concentration of ~1μg/μl. After electroporation, the lens was removed and explants were flattened on micropore filters (Millipore) and cultured in serum-free retinal explant medium (130). For BrdU incorporation experiments, explants were pulsed with BrdU (Sigma) for 5 hours prior to cell dissociation.

2. 3 Tissue preparation for in situ hybridization or immunohistochemistry

For timed matings, the morning of vaginal plug detection was considered embryonic day 0.5 (E0.5). Animals were euthanized and tissue was harvested for experimental analyses. For fixation of embryonic retinae, whole heads were fixed in 4% PFA overnight at 4°C. For fixation of adult retinae, mice were anesthetized using CO_2 and a cardiac perfusion was performed using ~10 ml of 4% PFA. Eyes were then enucleated and fixed in 4% PFA overnight. Tissue was then washed 3 x 10 minutes in sterile phosphate buffer solution (PBS) (0.14M NaCl, 2.5mM KCl, 0.2M Na_2HPO_4, 0.2M KH_2HPO_4) and immersed in 30% Sucrose in PBS at 4°C overnight or up to 3 days. Before embedding, tissue was equilibrated for 1 hour in 50:50 30%Sucrose in PBS:OCT (Tissue-Tek) and then embedded in the equilibration solution in liquid nitrogen. All cryosections were cut in the coronal plane at 10μm using a Leica 1850 cryostat. Sections were transferred onto Superfrost Plus coated slides (Fischer Scientific), air dried for ~4 hours at room temperature and used immediately for immunohistochemistry or *in situ* hybridization or stored with desiccant at -20°C. Wild-type and mutant tissues were always compared on the same slides.

2.4 Cell Dissociation for Immunocytochemistry

Retinal explants were dissociated using 0.1mg/ml trypsin (Sigma) in sterile calcium-free PBS (Invitrogen) at 37°C for 15 minutes. Trypsinization was inhibited with 10% FBS/ DMEM/DNAseI (0.2mg/ml, Sigma) and tissue was triturated to obtain a single cell suspension. Cells were pelleted at 1000xG for 5 minutes and re-suspended in 350μl 10%FBS/DMEM/Insulin(0.01mg/ml, Sigma). 15μl of the cell suspension was plated on a glass slide, which were then incubated in a humidified chamber of 15 minutes at room temperature followed by 40 minutes at 37°C to allow the cells to adhere to the slides. Cells were then fixed with 4%PFA for 5 minutes, washed 3 times with sterile PBS and air dried. Cells were either processed immediately for immunocytochemistry or stored at -20°C.

2.5 Hematoxylin and eosin (H&E) staining

For H&E staining, slides were re-hydrated for 30 minutes in 1xPBS and then stained with hematoxylin (Fischer) for ~7 seconds. Tissue was then dehydrated through consecutive 2 minute incubations in 50% Ethanol (EtOH), 70% EtOH, 80%EtOH, 90%EtOH x2, 100% EtOH x2. Tissue was then stained with eosin (Fischer) for 1 minute and dehydrated using the same protocol as for hematoxylin. Slides were washed 1x 2 minutes in Xylene (Fischer) and mounted using PerMount (Fischer). Slides were visualized using an Axioplan microscope and images were captured using an Axiovision camera 2.05 (Zeiss). Images were processed using Adobe Photoshop CS2.

2.6 Immunohistochemistry (IHC)/ immunocytochemistry (ICC) and nuclear labeling

IHC and ICC were performed as previously described (127, 130). Sections or dissociated cells were rehydrated for 30 minutes in PBS, blocked for 1 hour in 10% FBS in PBS and incubated with primary antibodies diluted in 10%FBS/PBS overnight at 4°C . The antibodies used in this study as well as antibody-specific alterations to the staining protocol can be found in Appendix 1. Slides were then washed 3x-10 minutes in PBS and incubated for 1 hour with fluorescent secondary antibodies (Appendix 1) diluted in 10%FBS/PBS. Nuclei were counterstained with bisbenzimide (Hoechst) (Invitrogen) for 5 minutes and then mounted with fluorescent mounting medium (Dako). Staining was visualized using a Zeiss fluorescent upright microscope and images were captured using an AxioVision 6.05 (Zeiss) camera and processed with Adobe Photoshop CS2.

2.7 In situ hybridization

In situ hybridization was performed as described previously (57). Briefly, DIG-labeled RNA probes were diluted 1/1000 in hybridization buffer (50% formamide, 10% dextran sulfate, 1mg/ml yeast RNA, 1x Denhardt's and 1x salt) that had been pre-warmed to 70°C and denatured for 15 minutes at 70°C. Probes were allowed to hybridize to the tissue overnight at 65°C in a humidified box. Slides were then washed 1x30 minutes and 2x20 minutes with wash buffer solution (50% formamide, 1xSSC, 0.1% Tween-20) at 65°c. Slides were then washed 3x 20 minutes with MABT (100 mM Maleic acid, 150 mM NaCl, 0.1% Tween-20; pH 7.5). Sections were blocked 1-2 hours with blocking solution (20% Sheep serum (Sigma) and 2% blocking solution in MABT). Alkaline-phosphatase conjugated Fab fragments of sheep anti-DIG was diluted 1/1500 in blocking solution and allowed to bind overnight at 4°C in a humidified box. The following day, slides were washed 5x20 minutes with MABT at room temperature followed by 1x15 minutes in pre-stain buffer (100 mM NaCl, 50mM $MGCl_2$, 100mM Tris pH 9.5 ad 0.1% Tween-20) before being placed in the dark in staining buffer (100 mM NaCl, 50mM $MGCl_2$, 100mM Tris pH 9.5 ad 0.1% Tween-20, tetrazolium chloride (Roche) and 3.5µl/ml 5-bromo-4-choloro-3-indolylphosphate (Roche)). Slides were washed in 1x PBS to stop the color reaction and mounted using a 1:1 mixture of 1xPBS:30% glycerol. Sections were analyzed on an Axioplan microscope and digital images were captured using an AxioVision camera 2.05 (Zeiss) camera and processed with Adobe Photoshop CS2. To generate *in situ* probes, ~10µg of plasmid containing the cDNA for the gene of interest were linearized overnight using restriction digestion. Probes were then synthesized using 5µl 5x transcription buffer, 1µl RNase OUT, 2.5µl 10x DIG-UTP, 1µl RNA polymerase (T7, SP6) and incubated at 37□C for one hour. 1µl of T7 or SP6 enzyme was added, and the solution was incubated for one more hour at 37□C. DIG-labeled probes were then precipitated for 1 hour at -80°C in 2x volume 100% EtOH, 1/10 vol. 3M NaOAc and cetrifugated for 10 minutes at 12,000 x g at 4°C . The pellet was resuspended in 1 volume 70% EtOH, respun for 10 minutes at 12,000 x g, the EtOH was decanted and resuspended in 100µl HPLC grade water.

2.8 TUNEL assay

Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using the In Situ Death Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Sections were re-hydrated in 1x PBS for 20-30 minutes and then permeabilized using 0.01% Triton X in 0.1% Sodium Citrate (Sigma) at room temperature for 2 minutes. Slides were washed for 10 minutes with PBS and incubated in a mixture of 10µl enzyme solution and 90 µL labeling solution for 1 hour at 37°C in a dark, humidified box. Slides were then washed 3x 10 minutes with PBS, nuclei were counterstained with DAPI as above and mounted with fluorescent mounting medium (DAKO).

2.9 Cell dissociation for Fluorescent Activated Cell Sorting (FACS)

Cells were dissociated and pelleted as described above and then re-suspended in 2ml of 10%FBS/DMEM and filtered using 70µm filters (Millipore). Cells were then placed on ice and sorted using a Dako Cytomation MOFLO (Dako) by StemCore Inc (Ottawa), which was cleaned beforehand using 10% bleach and DEPC water. Cells were collected on ice in 2ml 10%FBS/DMEM.

2.10 mRNA isolation and cDNA synthesis from FACS sorted cells

RPCs sorted by FACS were pelleted at 1000xG for 5 minutes, re-suspended in 1ml tri-reagent (Invitrogen) and sonicated for 6 seconds at 30% amplitude. 200µl chloroform was then added, the mixture was mixed by inversion for 30 seconds and incubated at room temperature for five minutes. Phases were separated by centrifugation at 4°C, 13,000xG for 10 minutes, the aqueous phase was removed, added to 500µl isopropanol and the mRNA was precipitated at -20°C for 48 hours. Following this step, mRNA was pelleted at 4°C, 13,000 xG for 15 minutes and the isopropanol was removed. The pellet was re-suspended in 70% EtOH and then re-spun at

4°C, 13,000xG for 10 minutes. EtOH was decanted and the pellet was air-dried at room temperature for ~5 minutes and then resuspended in 20μL of HPLC grade water. mRNA purity and concentration was then determined using spectroscopy (Ependorph BioPhotometer).

cDNA was then prepared as follows: 5μl mRNA, 1μl 10mM dNTP (Invitrogen), 1.5μl 10 mM random hexamers (Invitrogen) and 6μl HPLC grade water. Solutions were incubated at 65°C for 5 minutes, cooled to 22°C over 1.5 minutes and incubated at 22°C for 8.5 minutes. 4μl 5x first strand buffer (Invitrogen), 2μl DTT (invitrogen) and 0.75μl MMLV were added to each reaction and the mixture incubated at 40°C for 50 minutes and then heated to 70°C for 10 minutes. cDNA was then diluted 1:2 in HPLC grade water.

2.11 Real-time quantitative PCR (qPCR)

For qPCR, 2μl cDNA, 1μl 10μM forward/10μM reverse primers, and 10μl HPLC grade water were added to 15μl of SYBR-Green qPCR master-mix reagent (Sigma). Reactions were carried out using an Mx3000P thermocycler (Stratagene). The thermal program used was 10minutes at 95°C followed by 40 cycles of 2 minutes at 95°C, 25 seconds at 59°C, 25 seconds at 72°C. Data was collected at the end of the 72°C extension time. Primers for GAPDH were F-5'TGAAGGGGTCGTTGATGG3' and R- 5'AAAATGGTGAAGGTCGGTGT3'. *Sufu* primers were F- 5'ATTCAGCCCAACAGTGGAAC3' R- 5'CCGTCTGTCTAATGCCTTT3'. *Sufu* transcript levels were normalized to GAPDH and fold change was calculated using 2^{ddCt}. To ensure primers directed against *Sufu* were specific, the presence of a PCR product was verified using a dissociation curve and PCR products were run on a gel to confirm that an appropriately sized amplicon was being generated. PCR products were then ligated into pGEM-T (Promega) as per manufacturers instructions and transformed into DH5-α *E.coli*. Tansformants were screened by PCR and positive colonies were grown overnight at 37°C in 100mL LB broth. The plasmid was then isolated (Qiagen midi kit or mini kit) and the insert was sequenced by Stem Core (Ottawa).

2.12 Luciferase activity

Luciferase activity was assayed using the Dual Luciferase Kit (Promega) according to manufacturer's instructions and quantified using a Lumat LB 9507 luminometer (Eg&G Berthhold). Briefly, explants were immersed in 500μL 1x passive lysis buffer solution (Promega), homogenized briefly and rocked at room temperature for 30 minutes. The lysate was added to 200μL luciferin (Promega), luciferase activity was measured and then 200μL Stop and Glo solution (Promega) was added to measure *Renilla* activity.

2.13 Image processing, data analysis and statistics.

Images were processed using Adobe Photoshop. Images of control and mutant retinae were taken at the same exposure for each magnification. In the event that contrast/levels were adjusted, they were adjusted for the entire image, and images of both control and mutant retinae were adjusted equally.

For the *in vivo* analysis, at least 3 mutant mice from each age group were examined and compared to wild type littermates. When counting cells, at least 150 transfected, GFP+, cells were scored per retina.

All data are presented as mean +/- standard error of the mean (SEM). Statistical significance was evaluated using a two-tailed, unpaired Studen't *t*-test and $p<0.05$ was considered statistically significant.

3. Results

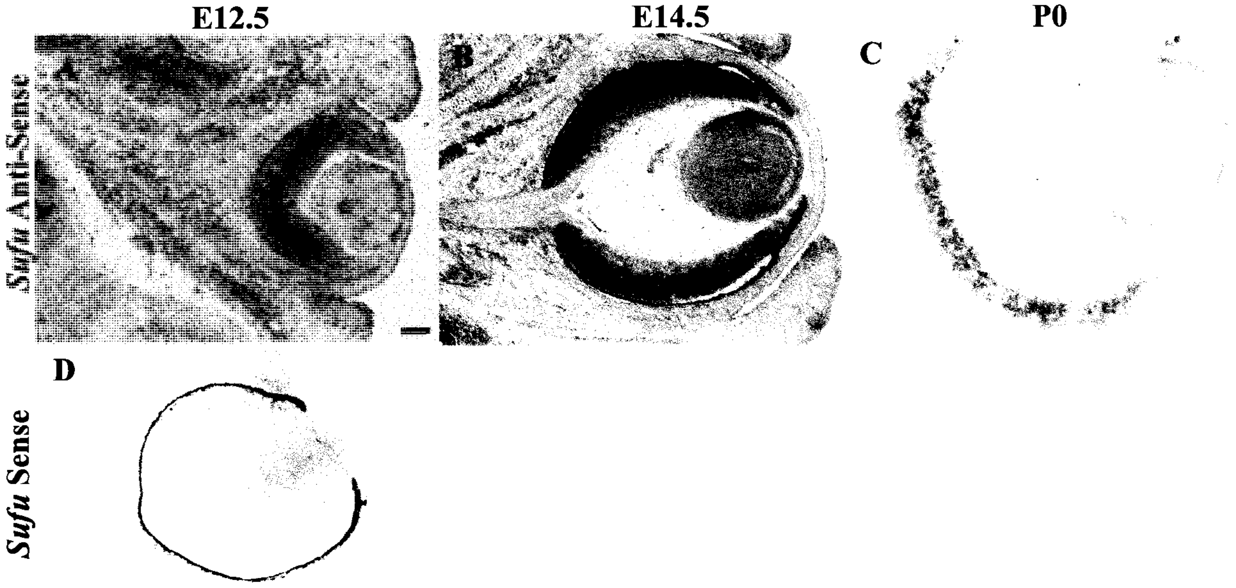
3.1 Sufu is expressed in Retinal Progenitor Cells

Sufu expression in the developing retina was characterized by *in situ* hybridization on cryosections of embryonic (E) day 12.5 and 14.5 and postnatal day 0 (P0) mouse retinas. At all stages, *Sufu* mRNA was restricted to the neuroblast layer, which contains RPCs (Figure 6) and was undetectable in GC layer, optic nerve, periocular mesenchyme, and cornea. Thus, in the developing retina, Sufu expression is a feature of undifferentiated neuroepithelial cells.

3.2 Sufu functions as a negative regulator of Shh signaling in RPCs:

The ability of *Sufu* to negatively regulate *Shh* signaling was first assayed in retinal explant culture. In retinal explants, the ganglion cells that secrete Shh protein die after ~24hrs and the Shh pathway becomes inactive (131), thus creating an ideal model to study the function of components of the Hh pathway. The Hedgehog (Hh) pathway can be activated experimentally by electroporating explants with an expressing vector containing a Smo-M2 cDNA, a constitutively active form of *Smo* that is capable of activating the *Shh* pathway in a cell-autonomous manner (134) in RPCs (138). SMO-M2-dependent Hh pathway activation can then be quantified using a *Gli-luciferase* reporter (*Gli-Luc*), which contains 8 *Gli* binding motifs up-stream of *luciferase* (107) and has been used previously to report Gli-dependent transcriptional activation (107). To determine if *Sufu* is capable of attenuating Gli-dependent transcriptional activation in RPCs, wild-type P0 retinas were co-electroporated with expression vectors for *Smo-M2*, *Gli-luc* and *Renilla luciferase* with or without a *Sufu* expression vector and luciferase

Figure 6: Sufu is expressed in the neuroblast layer during retinal development. In situ hybridization using an anti-sense probe targeting Sufu mRNA at A) E12.5, B) E14.5 and C) P0. D) Shows an E12.5 retina hybridized with the sense probe control. Unless specified, all sections are coronal sections with the dorsal region of the retina oriented upwards. Scale bar for all images is 100μM.


E12.5
E14.5
P0
A
B
C
D
Sufu Anti-Sense
Sufu Sense

activity in these explants was compared to explants transfected with an empty vector instead of Smo-M2 or Sufu. After 2.5 days *in vitro* (DIV) Smo-M2-transfected explants exhibited a ~500-fold activation of *Gli-Luc*, compared with control explants transfected with the empty vector. This induction of *Gli-Luc* activity was significantly attenuated nearly 3 fold when explants were co-transfected with *Sufu* and Smo-M2 (Figure 7), demonstrating that *Sufu* can inhibit *Gli*-dependent transcriptional activation downstream of *Smo* in RPCs.

The antagonistic effect of ectopic *Sufu* expression on Hh pathway activation in RPCs raised the possibility that inhibition of endogenous Sufu would be sufficient to activate this pathway in the absence of Hh ligand. To address this possibility, I next investigated whether acute *Sufu* deletion in RPCs is sufficient to activate the Hh pathway in RPC. To inactivate *Sufu*, P0 retinal explants from $Sufu^{Fl/Fl}$ mice (Figure 8) were co-electroporated with expression vectors for *Cre recombinase* and *pUB-GFP (GFP).*

I first verified that the *Sufu* allele was recombined in $Sufu^{Fl/Fl}$ RPCs after co-electroporation with expression vectors for *Cre recombinase* and *GFP*. P0 explants from $Sufu^{Fl/Fl}$ mice were co-electroporated with expression vectors for *Cre recombinase* and *GFP* or empty vector and *GFP* and cultured for 2.5 and 4 days. Six to eight retinas per treatment group were pooled, the transfected GFP$^+$ cells were isolated by fluorescent activated cell sorting (FACS) and mRNA was extracted for gene expression analysis by real-time PCR. To determine if recombination of *Sufu* had occurred after electroporation with *Cre*, primers were designed to target a region of the *Sufu* transcript that would be present in wild-type, but not recombined cells.

Figure 7: ***Sufu*** **functions down-stream of** ***Smo*** **to antagonize** ***Gli*****-dependent transcriptional activation in RPCs.** Wild-type P0 retinas were co-electroporated with expression vectors for *Gli-luc, Renilla luciferase, Smo-M2* +/- *Sufu* or empty vectors and cultured as explants for 2.5 days. Luciferase fold-activation was calculated as fold-change of treated/control explants after normalizing to *Renilla luciferase* activity. Data are shown as mean fold-change +/- SEM. n= 6 control explants, n=5 *Smo*-treated explants, n=6 *Smo*+ *Sufu* treated explants.

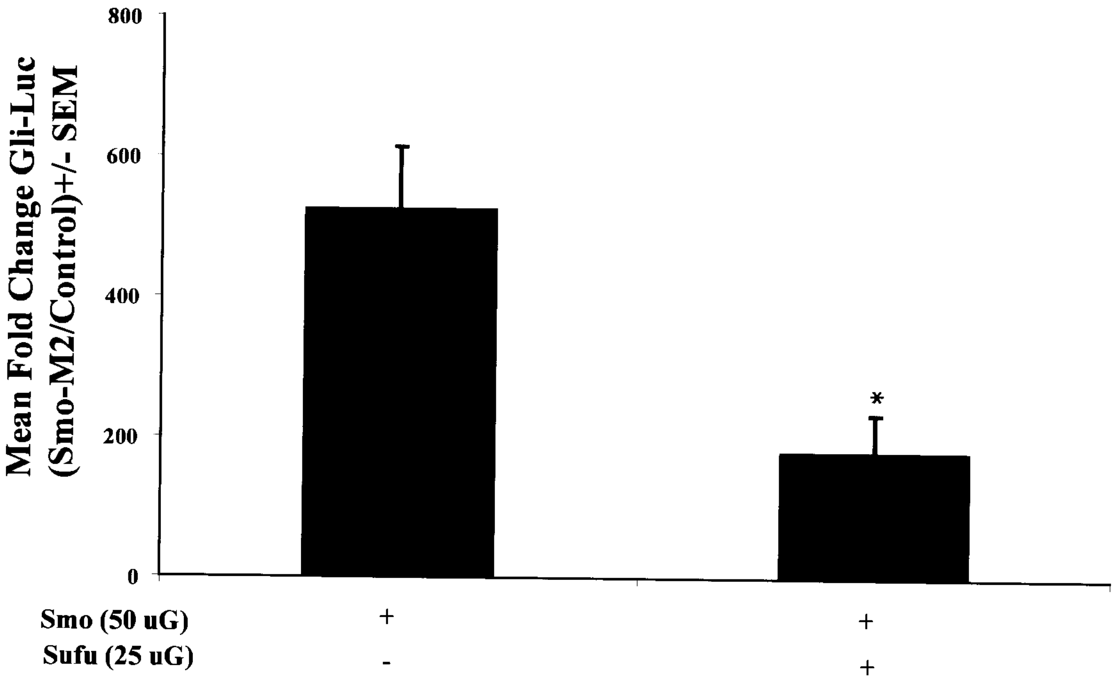
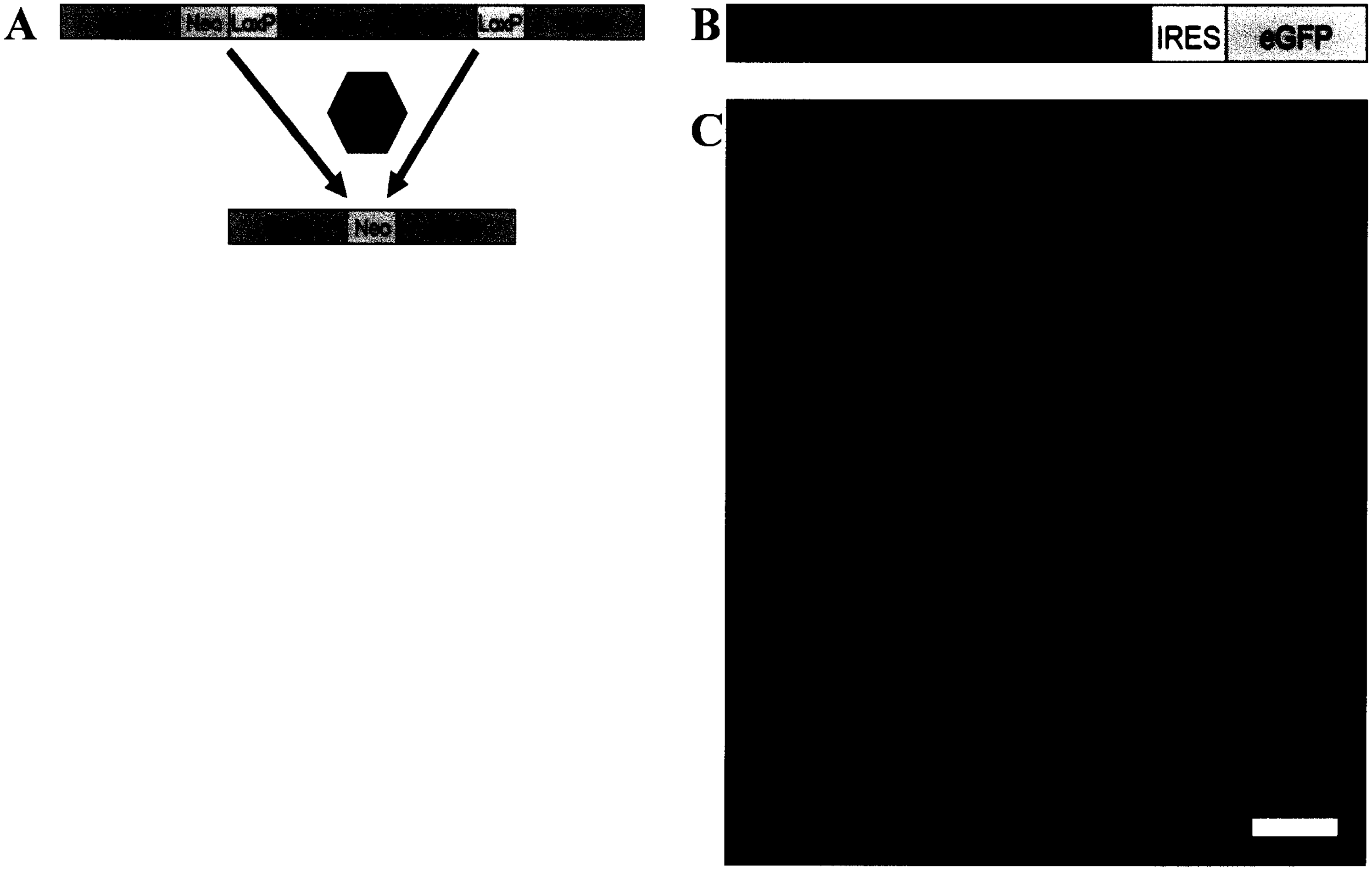

Mean Fold Change Gli-Luc
(Smo-M2/Control)+/- SEM
800
600
400
200
0
*
Smo (50 uG)
+
+
Sufu (25 uG)
-
+

Figure 8: **Transgenic alleles used in this study.** A) In *SufuFl/Fl* mice, exons 4-8 are floxed by loxP sites. Cre-mediated recombination at the lox P sites removes the intervening sequences and generates a null allele. B) The αP0-Cre locus. *Cre-recombinase* is driven by the αP0 element located within the *Pax6* promoter. The transcript contains an internal ribosome entry site (IRES) followed by the coding region for eGFP, thus serving as a surrogate reporter for the location of *Cre* expression in transgenic tissue. C) αP0-Cre is only expressed in the peripheral retina. Immunohistochemistry for GFP showing the representative expression of Cre/GFP in RPCs. Note that Cre is expressed in a much broader region ventrally than dorsally.


A
Neo
LoxP
LoxP
Neo
B
IRES
eGFP
C

WT *Sufu* transcript was reduced by ~50% and ~94% after 2.5 and 4 days in vitro (DIV), respectively in the *Cre* transfected population compared to controls (Figure 9). These results confirm that co-electroporating *Sufu*$^{Fl/Fl}$ explants with expression vectors for *Cre-recombinase* was sufficient to reduce the levels of WT transcript.

To determine if acute *Sufu* deletion resulted in *Gli*-dependent transcriptional activation, *Sufu*$^{Fl/F}$ retinas were co-electroporated with expression vectors for *Cre recombinase* or an empty vector, *Gli-luc* and *Renilla luciferase* and cultured as explants for 2.5 and 5 days. Compared with the control, Cre-electroporated explants exhibited a 5.4 and 16-fold increase in *Gli-luciferase* activity at 2.5 and 5 DIV, respectively (Figure 10) indicating that acute deletion of *Sufu* is sufficient to induce *Gli*-dependent transcription in the absence of active *Smo.*

3.3 Sufu deletion is mitogenic in RPCs.

Hh pathway activation in RPCs is mitogenic (11, 73, 127, 130, 138) and in the absence of *Shh* RPCs undergo precocious cell cycle exit (130). Since retinal explants do not have an endogenous source of *Shh,* RPC proliferation is reduced considerably in the absence of *Hh* pathway stimulation (127, 130, 138). To determine if *Sufu* deletion in RPCs is sufficient to induce progenitor cell proliferation, P0 *Sufu*$^{Fl/F}$ retinas were co-electroporated with expression vectors for *Cre recombinase* and *GFP* or an empty vector and *GFP*, cultured as explants for 2.5 days and pulsed with BrdU for five hours to label cells in S-phase of the cell cycle. Single cell dissociates obtained from these retinal explants were processed for immunohistochemistry for BrdU and Ki67, a proliferation marker. After 2.5 DIV, there was a significant increase in the proportion BrdU+ and Ki67+ cells amongst the *Cre* transfected cohort of cells, compared with

Figure 9: Cre-recombinase mediates recombination of the *Sufu* allele in the RPCs of *Sufu*$^{Fl/Fl}$ mice. qPCR analysis of wild-type *Sufu* transcripts in retinal explants from *Sufu*$^{Fl/Fl}$ mice co-electroporated with expression vectors for *Cre-recombinase* and *eGFP* or empty vector and *eGFP* and cultured as explants for 2.5 or 4 DIV. GFP^{+} cells were isolated by FACS and mRNA was extracted from the transfected cells and used to generate cDNA for gene expression analysis. Data is expressed as mean percent expression +/- SEM. *Sufu* expression was normalized to *GAPDH* and calculated using $2^{\wedge -ddCt}$. n=2 independent experiments/treatment with 6-8 retinae pooled per group.

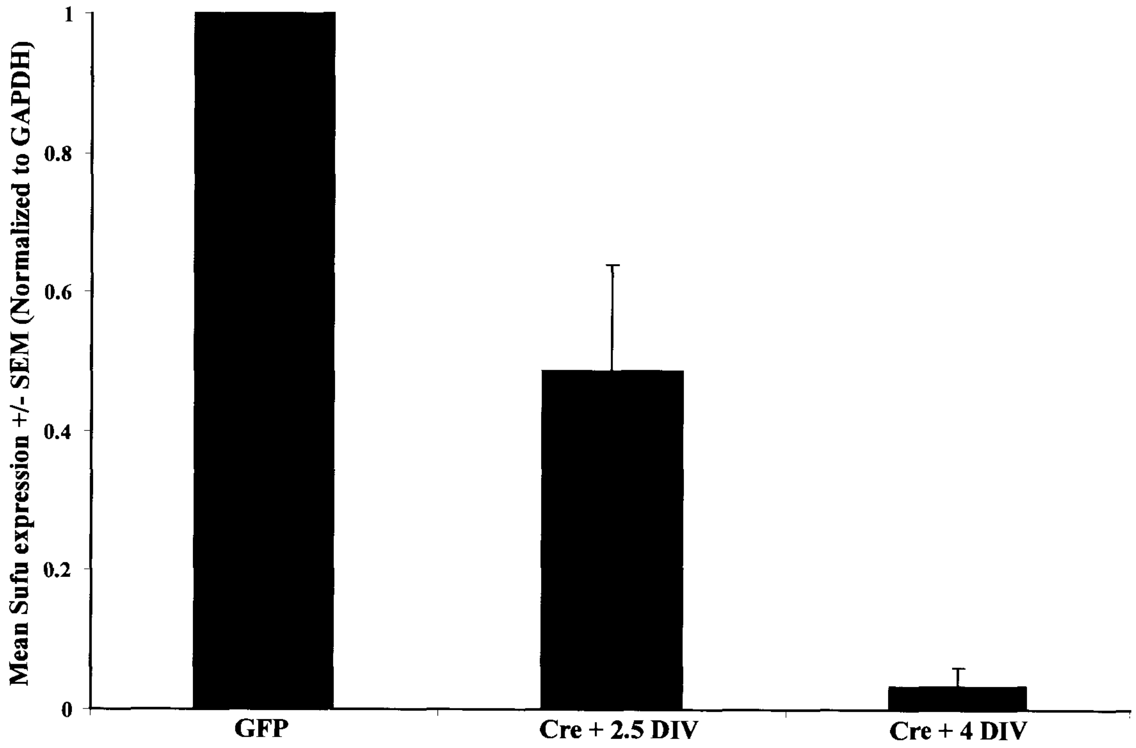
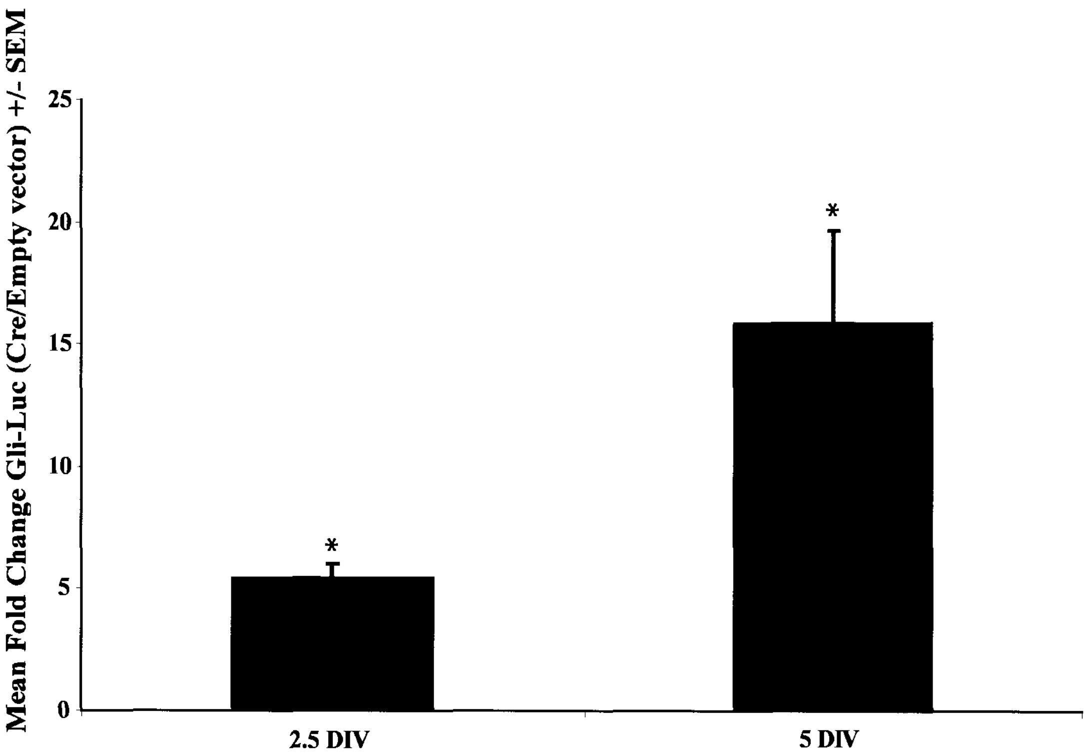

Mean Sufu expression +/- SEM (Normalized to GAPDH)
1
0.8
0.6
0.4
0.2
0
GFP
Cre + 2.5 DIV
Cre + 4 DIV

Figure 10: Acute deletion of *Sufu* in RPCs is sufficient to induce Gli-dependent transcriptional activation. Retinas from P0 *Sufu*$^{Fl/Fl}$ mice were co-electroporated with expression vectors for *Gli-luc*, *Renilla luiferase* and *Cre* or *Gli-luc*, *Renilla* and an empty vector and cultured as explants for 2.5 DIV or 5 DIV. Luciferase fold-activation was calculated as fold-change of treated/control explants after normalizing to *Renilla luciferase* activity. Data are shown as mean fold-change +/- SEM. n= 9 explants/treatment at 2.5 DIV; n=8 explants/treatment at 5 DIV. *p<0.005


Mean Fold Change Gli-Luc (Cre/Empty vector) +/- SEM
25
20
15
10
5
0
*
*
2.5 DIV
5 DIV

the controls (Figure 11), indicating that acute deletion of *Sufu* is sufficient to maintain a proportion of the RPCs in cycle. Taken together, the observed *Gli*-dependent transcriptional activation and sustained proliferation in RPCs after acute deletion of *Sufu* indicates that, in RPCs, *Sufu* functions as a negative regulator of the Hh signaling pathway.

3.4 Sufu functions in vivo to regulate retinal development.

To determine if *Sufu* influences RPC proliferation and cell-fate specification *in vivo,* we generated a conditional knockout (CKO) for sufu by crossing *Sufu*$^{Fl/Fl}$ mice with a transgenic line that expresses *Cre recombinase* under the control of the αP0 element of the Pax6 promoter (See Figure 8) (80) to generate *Cre*$^{+}$; *Sufu*$^{Fl/Fl}$. For simplicity, *Cre*$^{+}$; *Sufu*$^{Fl/Fl}$ mice will be referred to as *Sufu* CKO mice, and control *Cre*$^{-}$; *Sufu*$^{Fl/Fl}$ mice will be referred to as wild type (WT). The Pax6 αP0 element induces *Cre* expression exclusively in the peripheral retina (80) (See Figure 8) beginning at ~E10.5 (80), although the domain of expression is frequently much broader in the ventral retina compared to the dorsal retina (See Figure 8). *Sufu* expression could not be detected in the peripheral retina of *Sufu* CKO mice at E12.5 indicating that successful recombination occurs in these mutants (Figure 12). However, the domain of deletion was frequently smaller in the dorsal retina compared to the ventral retina (Figure 12).

Figure 11: Acute deletion of *Sufu* induces proliferation in RPCs. P0 *Sufu*$^{Fl/Fl}$ retinas were co-electroporated with expression vectors for *eGFP* and *Cre* or *eGFP* and an empty vector and cultured as explants for 2.5 DIV. Explants were then pulsed with BrdU for 5 hours, dissociated, and GFP+ cells were scored for **A)** the expression of Ki67 (n=7 control explants; n=8 *Cre*-treated explants) and **B)** BrdU incorporation (n=7 control explants and n=9 *Cre*-treated explants) as determined by fluorescent immunocytochemistry. Data are shown as the mean proportion of double positive cells +/- SEM.

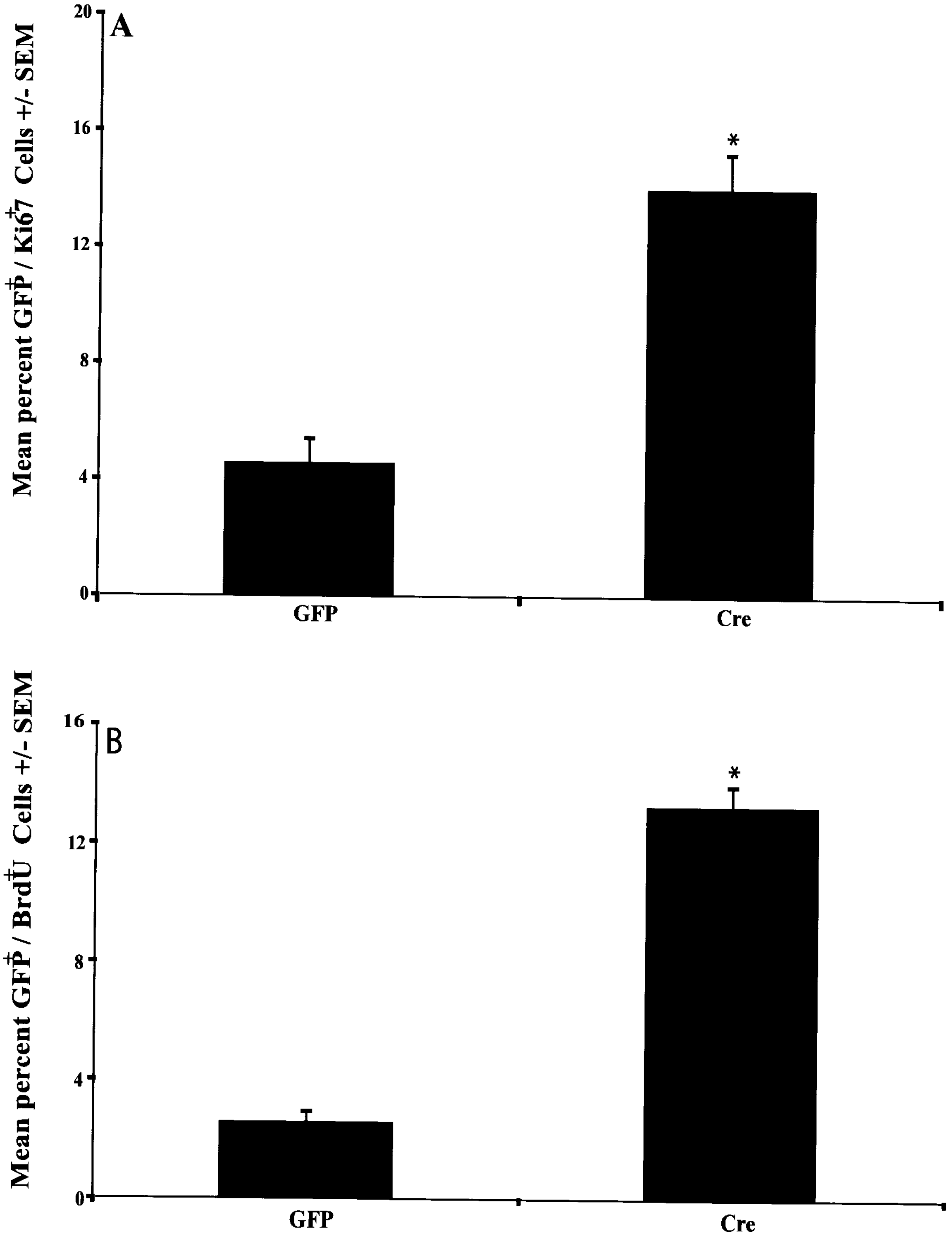
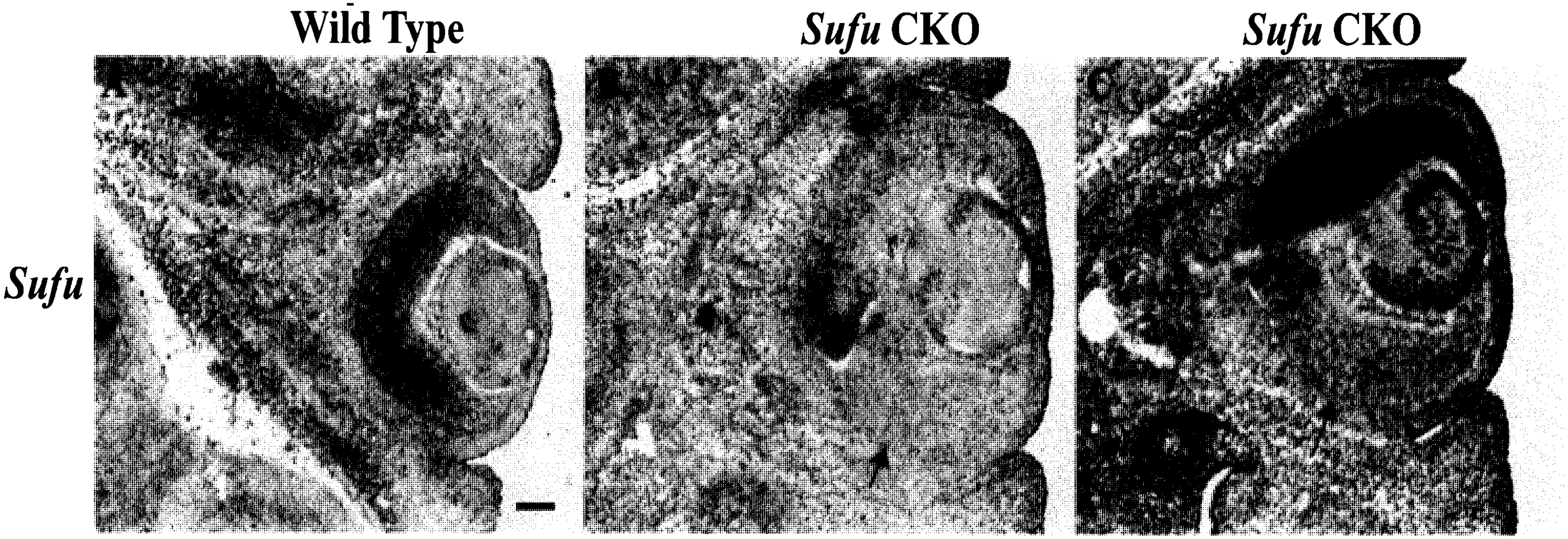

A
Mean percent GFP+ / Ki67+ Cells +/- SEM
20
16
12
8
4
0
*
GFP
Cre
B
Mean percent GFP+ / BrdU+ Cells +/- SEM
16
12
8
4
0
*
GFP
Cre

Figure 12: *Sufu* is deleted in the peripheral retinas of *Sufu* CKO mice. *In situ* hybridization of *Sufu* mRNA in A) WT or B,C) *Sufu* CKO mice at E12.5. Note the absence of *Sufu* expression in the dorsal and ventral retina in B, compared to ventrally in C (arrows). Since *Sufu* was more consistently deleted in the ventral region of the retina, this is where we focused our analysis.


Wild Type
Sufu CKO
Sufu CKO
Sufu

3.5 Sufu deletion results in disrupted retinal morphology and lamination

Histological analysis of mutant retinas was conducted at various developmental stages to determine if *Sufu* deletion had any effect on retinal morphology or lamination. Analysis of *Sufu* CKO retinae revealed that a large mass, characterized by a thickening of the retina into a globular-like shape, formed in the ventral retina by E12.5 (Figure 13 A,B), roughly two days after the onset of *Cre* expression (80). By P0, a small mass had also formed in the dorso-nasal peripheral retina (Figure 13 C,D). Histological examination of P18 retinas revealed a cellular mass, characterized by H&E as a region of the retina that was significantly wider than controls, that had an absence of normal retinal lamination and aberrant spacing of cell nuclei (Figure 13 E,F). The eyes of P18 *Sufu* CKO mice were frequently larger than that of wild-type littermates and had a notably thinner optic nerve (Figure 13 G).

3.6 The hedgehog pathway is transiently activated in the peripheral retina of Sufu CKO *mice.*

I then asked if conditional deletion of *Sufu* in early RPCs activated the *Hh* pathway. *Sufu* deletion *in vivo* resulted in activation of the Shh pathway. At E12.5, *Shh* target genes *Gli1*, and *Hes1* were expressed in the ventral mass at E12.5 and the domain of *Gli1* expression in mutant retinae was expanded compared to WT (Figure 14). By E14.5, the expression of *Gli1* and *Hes1* was reduced in the ventral mass in comparison to what was observed at E12.5; however, the domain of *Gli1* expression was still greater in the mutant compared to wild-type retinas (Figure 15 A,B). Therefore, consistent with the effect observed *in vitro*, *Sufu* appears to be required *in vivo* to antagonize the *Hh* pathway in RPCs.

Figure 13: The retinae of *Sufu* CKO mice display severe morphological defects. (A-F) Hematoxylin and eosin (H&E) staining of wild type and mutant retinas at E12.5 (A,B), P0 (C,D) and P18 (E,F). Note the formation of a cellular mass in ventral peripheral retina at E12.5 (arrow), and the ventral and dorsal regions of the mutant retina at P0 (arrows). The ventral region of the WT and mutant retina is shown in (E) and (F), respectively. (G) Dissected eyes and optic nerves from a WT and mutant mouse at P18. Note that the mutant eye is larger than the WT eye, but the optic nerve of the mutant eye is notably thinner (arrow).


Wild Type
Sufu CKO
B
E12.5
P0
E
F
P18
P18

Figure 14: Expression of Hh target genes *Gli1* and *Hes1* in the retinas of *Sufu* CKO mice. *In situ* hybridization for *Gli1* (A,B) and *Hes1* (C,D) mRNA in control and mutant retinae at E12.5. The domain of *Gli1* expression is expanded in the retinas of mutant mice (arrow,inside the brackets). *Hes1* is expressed throughout the peripheral mass (arrow) in the mutant retina.

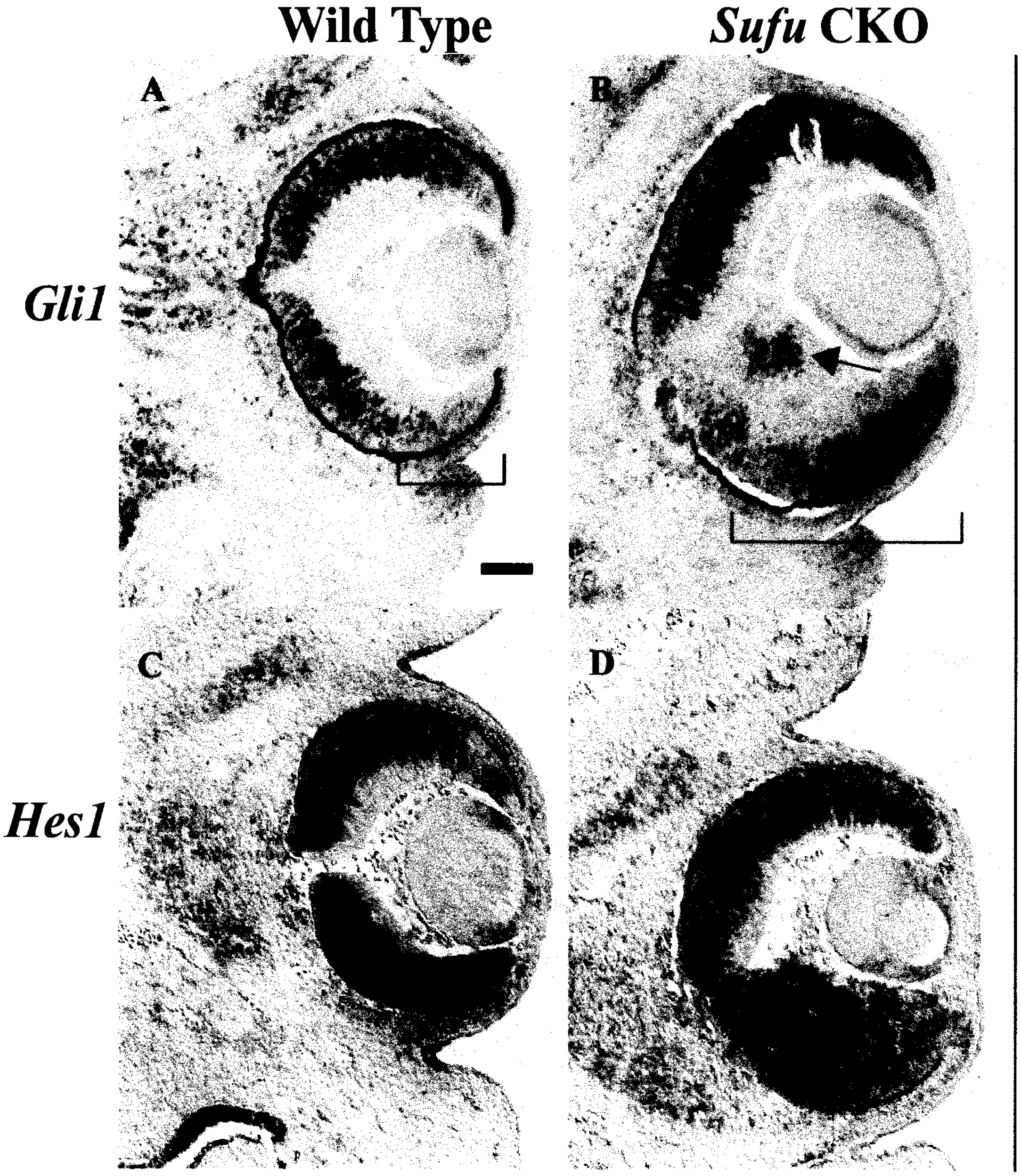
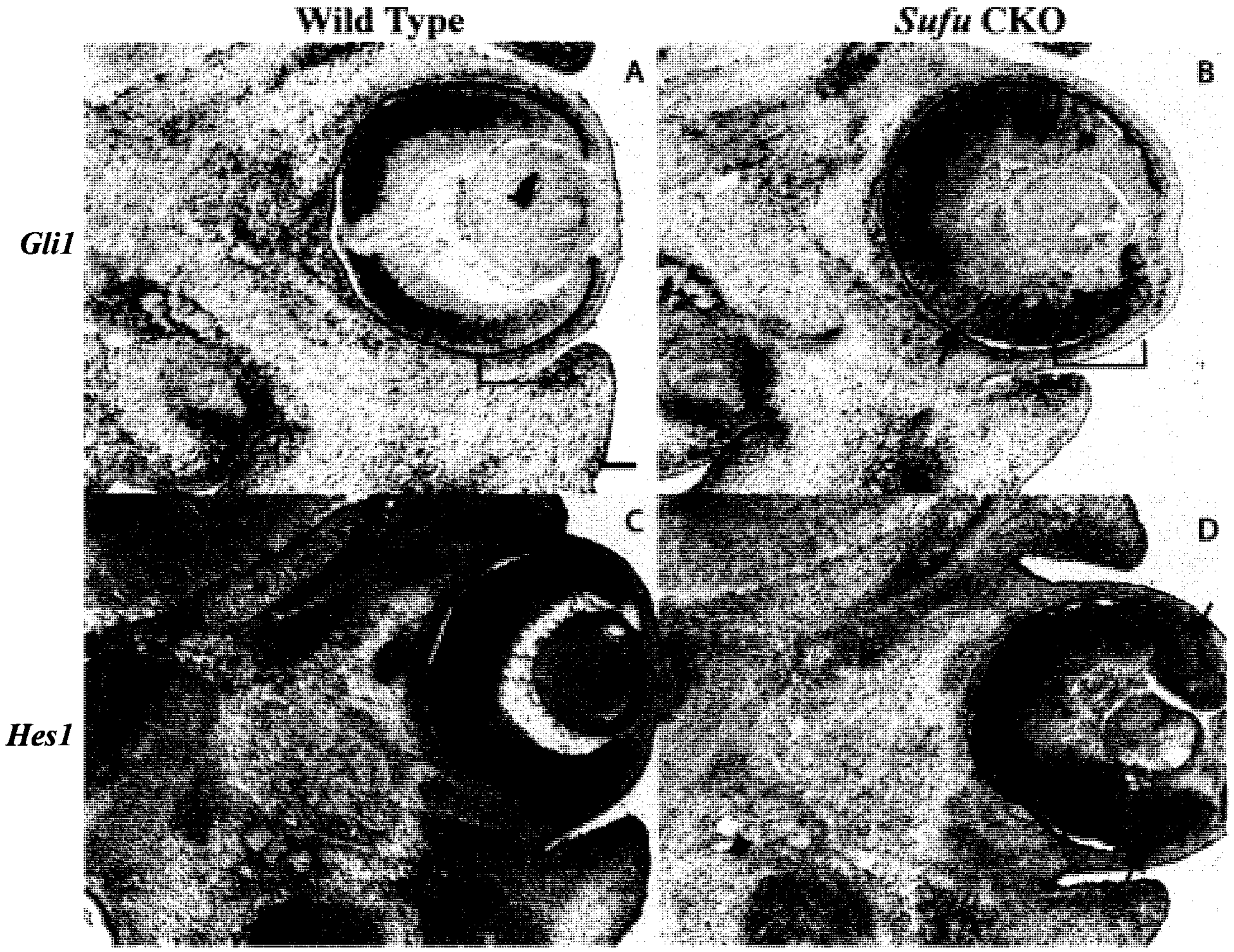

Wild Type
Sufu CKO
A
B
Gli1
C
D
Hes1

Figure 15: Expression of Hh target genes *Gli1* and *Hes1* in the retinas of *Sufu* CKO mice. *In situ* hybridization for *Gli1* (A,B) and *Hes1* (C,D) mRNA in control and mutant retinae at E14.5. The domain of *Gli1* expression is expanded in the retinas of mutant mice (within brackets). *Hes1* is expressed throughout the peripheral mass.


Wild Type
Sufu CKO
Gli1
Hes1
A
B
C
D

3.7 Sufu-null cells prematurely exit the cell cycle.

Consistent with the pattern of Hh pathway activity observed in mutant retinas, cells in the ventral retina robustly expressed the proliferation marker Ki67 at E12.5 (Figure 16 A,B) but this expression was reduced at E14.5 (Figure 16 C,D), and only sporadic Ki67 positive cells could be detected in the *Sufu*-null region at P0 (Figure 16 E,F) suggesting that, *in vivo, Sufu*-null cells prematurely exit the cell cycle. The cell cycle inhibitor *p57(Kip2)*is expressed in the murine retina between E14.5-E16.5 by a subset of progenitors just prior to cell cycle exit (26) and *p57(Kip2)* is transcriptionally up-regulated by *Shh* signaling in zebrafish (111). At both E12.5 and E14.5, *p57(Kip2)* was up-regulated in the ventral peripheral retina of *Sufu* CKO mice compared to wild-type littermates (Figure 17, A-D). This up-regulation correlates with the down-regulation of proliferation markers observed in the peripheral retinae of *Sufu* CKO mice and suggests that *Shh* signaling may also regulate *p57(Kip2)* expression in the mammalian retina.

3.8 Sufu is required to maintain RPC identity.

I next investigated whether alterations in Hh signaling and cell cycle were associated with perturbations in the expression of transcription factors that are required to specify RPCs. Unexpectedly, the expression of RPC-specific genes was extensively altered in sufu CKO retina. *Sufu* deletion was associated with altered expression of transcription factors required to maintain RPC identity and function. *Pax6, Rax* and *Chx10* were all down-regulated in the ventral peripheral retina at E12.5 and E14.5 (Figure 18 and Figure 19, respectively). In the developing retina, *Pax6* is required to maintain the multipotent state of retinal progenitors as conditional deletion of *Pax6* results in the exclusive formation of amacrine interneurons (80, 98). The down-regulation of *Pax6* expression observed in *Sufu* CKO retinas suggested that the *Sufu*-null RPCs may no longer be multipotent.

Figure 16: Cells in the peripheral mass of *Sufu* CKO mice prematurely down-regulate the proliferation marker Ki67. Fluorescent immunohistochemistry for Ki67 in WT and mutant retinae at E12.5 (A-B), E14.5 (C-D) and P0 (E-F). Note that the majority of the cells in the ventral mass appear to be Ki67+ at E12.5 (arrows). The expression of Ki67 is down-regulated in the peripheral retina of mutant mice at E14.5 and only a few Ki67+ cells could be detected in this region at P0 (arrows indicate Ki67+ positive cells).

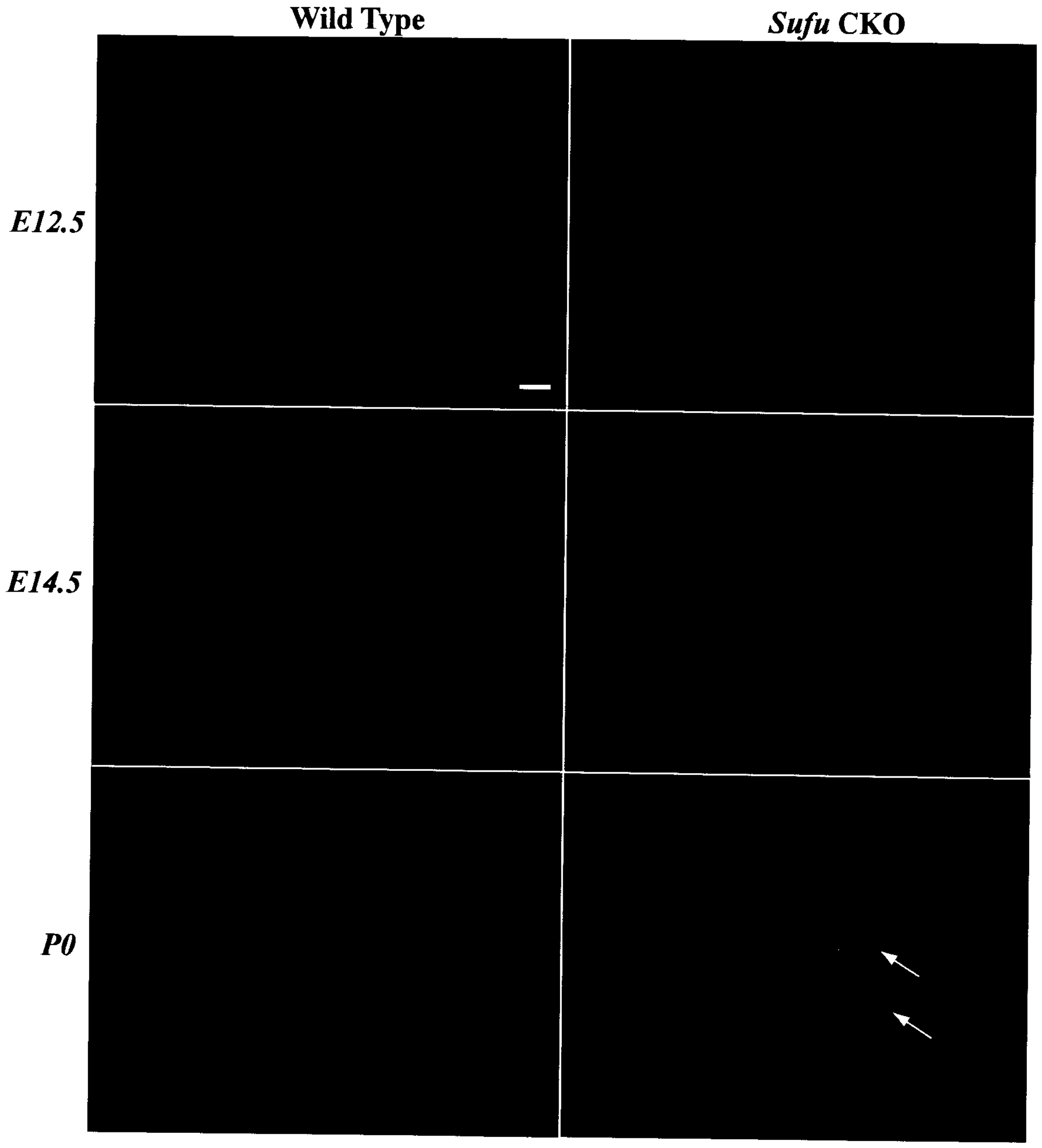

Wild Type
Sufu CKO
E12.5
E14.5
P0

Figure 17: The cell cycle inhibitor *p57(Kip2)* is up-regulated in the ventral retina of *Sufu* CKO mice. *In situ* hybridization for *p57(Kip2)* mRNA at E12.5 (A-B) and E14.5 (C-D). Ectopic expression (arrows) of *p57(Kip2)* in the ventral retina of mutant mice at E12.5 (A,B) and E14.5 (C,D).

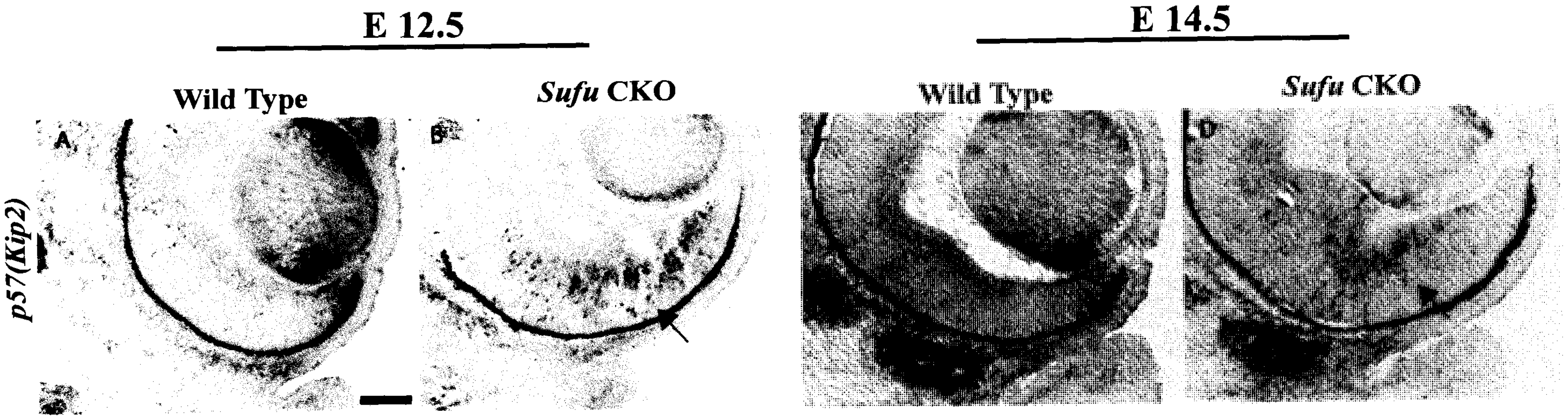

E 12.5
E 14.5
Wild Type
Sufu CKO
Wild Type
Sufu CKO
p57(Kip2)
A
B
D

Figure 18: Transcription factors that maintain RPC identity are down-regulated in the ventral retina of *Sufu* CKO mice. *In situ* hybridization for *Pax6* (A,B); *Rax* (C,D) and *Chx10* (E,F) in the retinae of E12.5 wild-type and mutant mice. Note the down-regulation of *Pax6* and the absence of *Rax* and *Chx10* expression in the ventral retina. Arrows indicate the region of mutant retinae with abnormal gene expression.

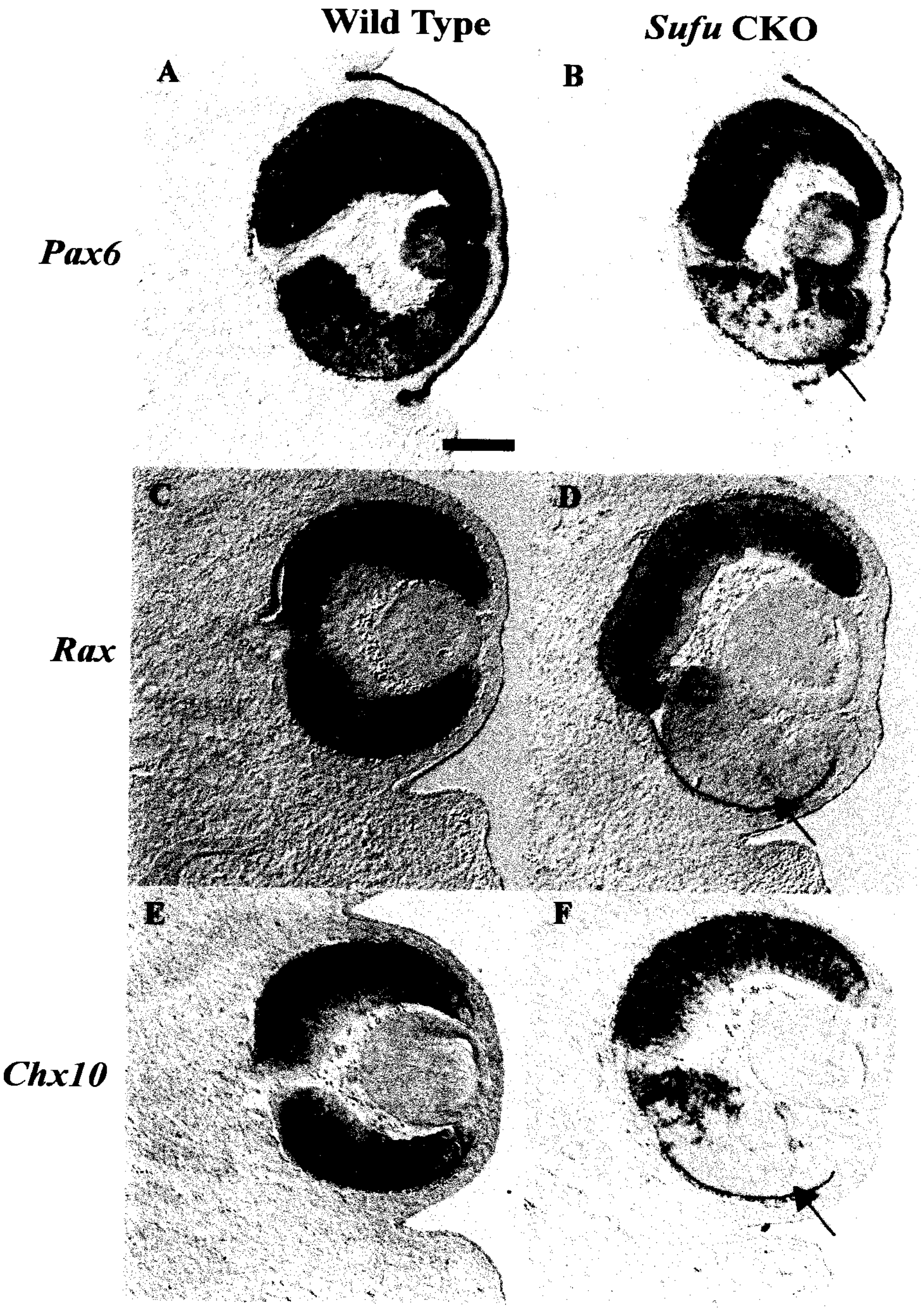
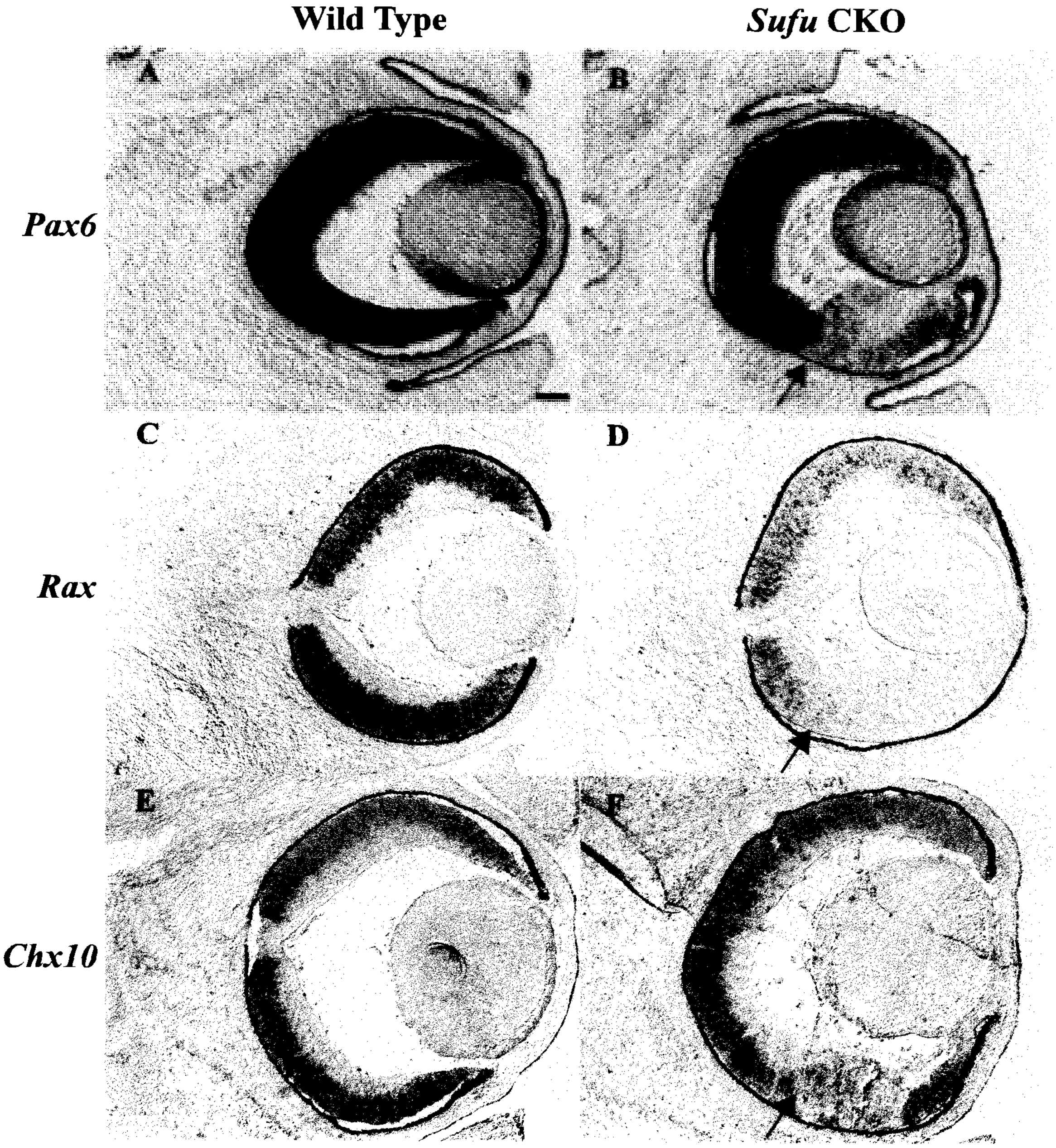

Wild Type
Sufu CKO
A
B
Pax6
C
D
Rax
E
F
Chx10

Figure 19: Transcription factors that maintain RPC identity are down-regulated in the ventral retina of *Sufu* CKO mice. *In situ* hybridization for *Pax6* (A,B); *Rax* (C,D) and *Chx10* (E,F) in the retinae of E14.5 wild-type and mutant mice. Note the down-regulation of *Pax6* and the absence of *Rax* and *Chx10* expression in the ventral retina. Arrows indicate the region of the mutant retina with abnormal gene expression.



Wild Type
Sufu CKO
Pax6
Rax
Chx10
A
B
C
D
E
F

3.9 Sufu is required to maintain multipotency.

To determine if *Sufu*-null RPCs were multipotent, I examined the expression of transcription factors required to specify each of seven the retinal cell types. In general, the expression of transcription factors required for cell fate determination of all retinal cell types was absent in the ventral and down-regulated in the dorsal retinas of the *Sufu* CKO mouse. For example, the expression of *Math5,* which is required to specify ganglion cell fate (129), was down-regulated in mutant retina at E12.5 (Figure 20 A,B) and E14.5 (Figure 20, C,D). Ganglion cells are the earliest born cell type and mature, post-mitotic, ganglion cells can be detected as early as E11.5 based on the expression of Brn3B. Brn3b is a direct target of *Math5* (70) and, consistent with the loss of Math5 expression in mutant retinae, Brn3a/b was undetectable in the mutant region of *Sufu* CKO retinae at E12.5 (Figure 20 E,F) and E14.5 (Figure 20 G,H). *FoxN4* is required for amacrine/horizontal cell development (67) and its expression was down-regulated in mutant retinas at E12.5 (Figure 21 A,B) and at E14.5 (Figure 22 A,B). *NeuroD1,* which functions downstream of *FoxN*4, was down-regulated in a similar pattern to that of *FoxN4* at E14.5 (Figure 22 C,D). *Crx* is required for the generation of both rod and cone photoreceptors (34) and its expression was also down-regulated in the mutant retina at E12.5 (Figure 21 A,B) and E14.5 (Figure 22 E,F). As mentioned previously, *Rax* and *Chx10* were also strongly down-regulated in the ventral peripheral retina and these transcription factors are involved for the generation of Müller glia (36) and bipolar cells (12, 72), respectively.

Figure 20: Reduced ganglion cell development in the retinas of *Sufu* CKO mice. *In situ* hybridization for *Math5* mRNA at E12.5 (A,B) and E14.5 (C,D) and fluorescent immunohistochemistry for Brb3a/b at E12.5 (E,F) and E14.5 (G,H) in WT and *Sufu* CKO retinae. The expression of *Math5* is abolished ventrally and down-regulated dorsally in mutant retinas at E12.5 and E14.5. The pattern of Brn3A/B expression mimics that of *Math5* at both E12.5 and E14.5. Arrows indicate regions of the mutant retina with perturbed expression of either *Math5* or Brn3A/B.

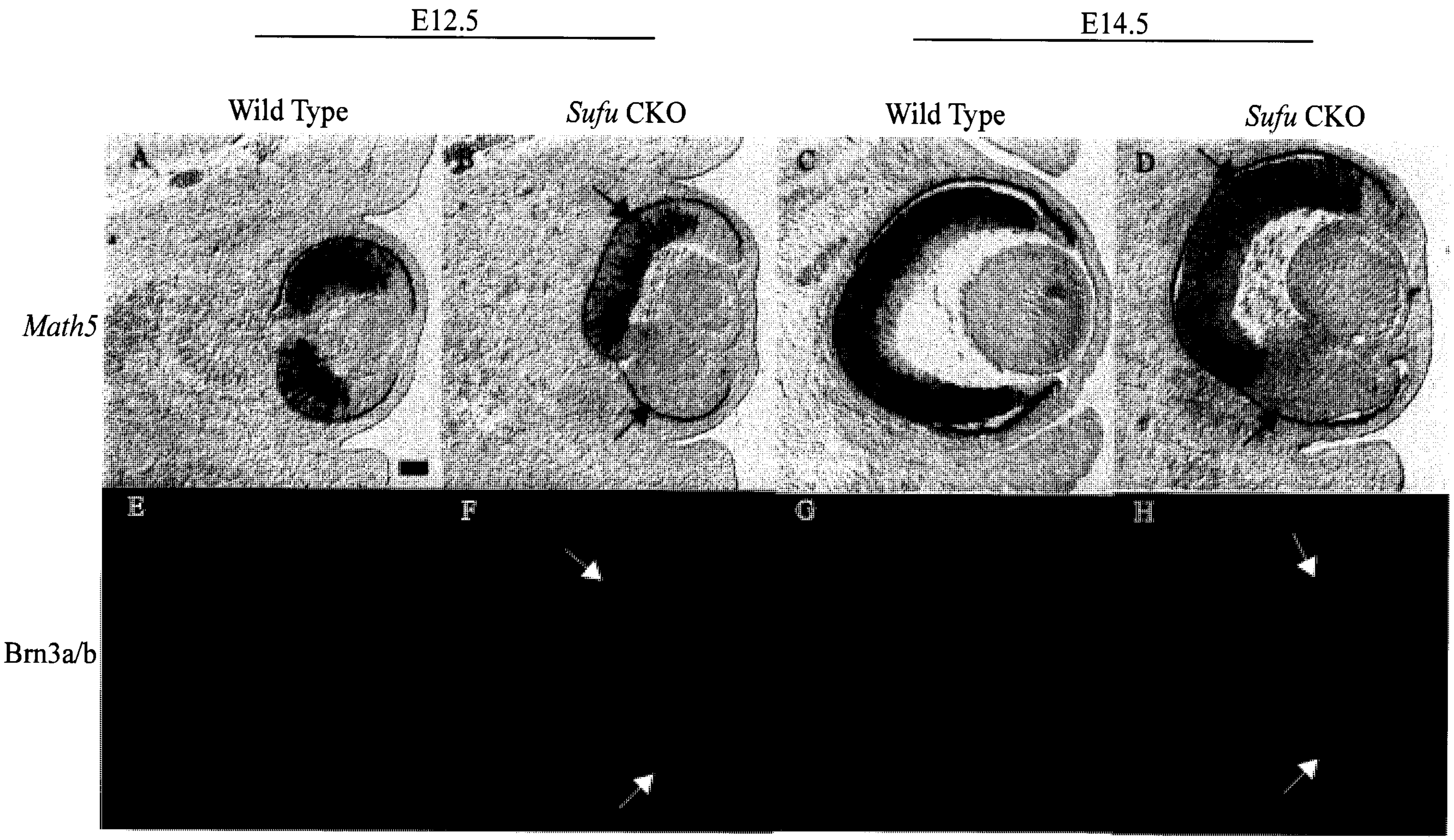

E12.5
E14.5
Wild Type
Sufu CKO
Wild Type
Sufu CKO
Math5
Brn3a/b
A
B
C
D
E
F
G
H

Figure 21: Transcription factors required to specify amacrine/horizontal interneurons and rod/cone photoreceptors are down-regulated in *Sufu* CKO retinae. *In situ* hybridization for *FoxN4* (A,B) and *Crx* (C,D) mRNA in WT and *Sufu* CKO retinae at E12.5. The expression of *FoxN4* is abolished ventrally and down-regulated dorsally in *Sufu* CKO retinas. *Crx* is weakly expressed in the WT retina at E12.5 and completely down-regulated in the peripheral retina of *Sufu* CKO mice. Arrows indicate regions of the mutant retina with perturbed expression of *FoxN4* or *Crx*.

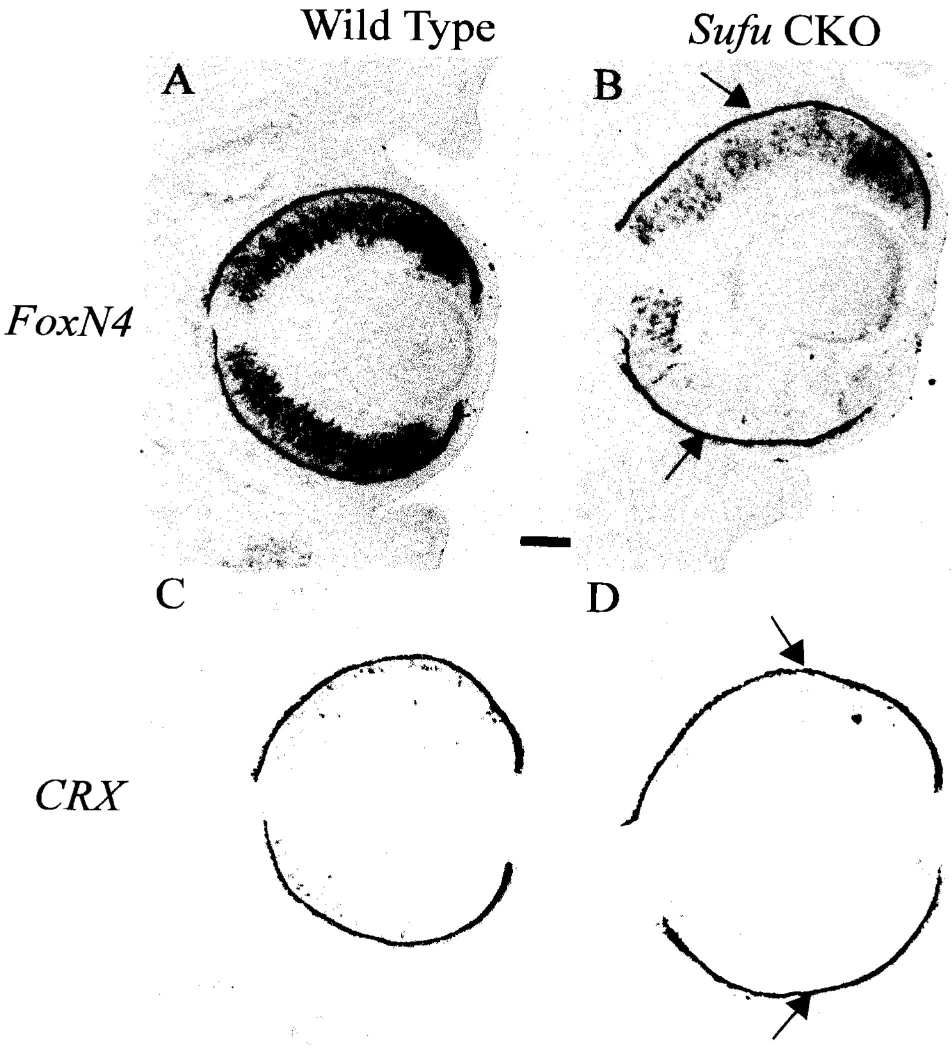
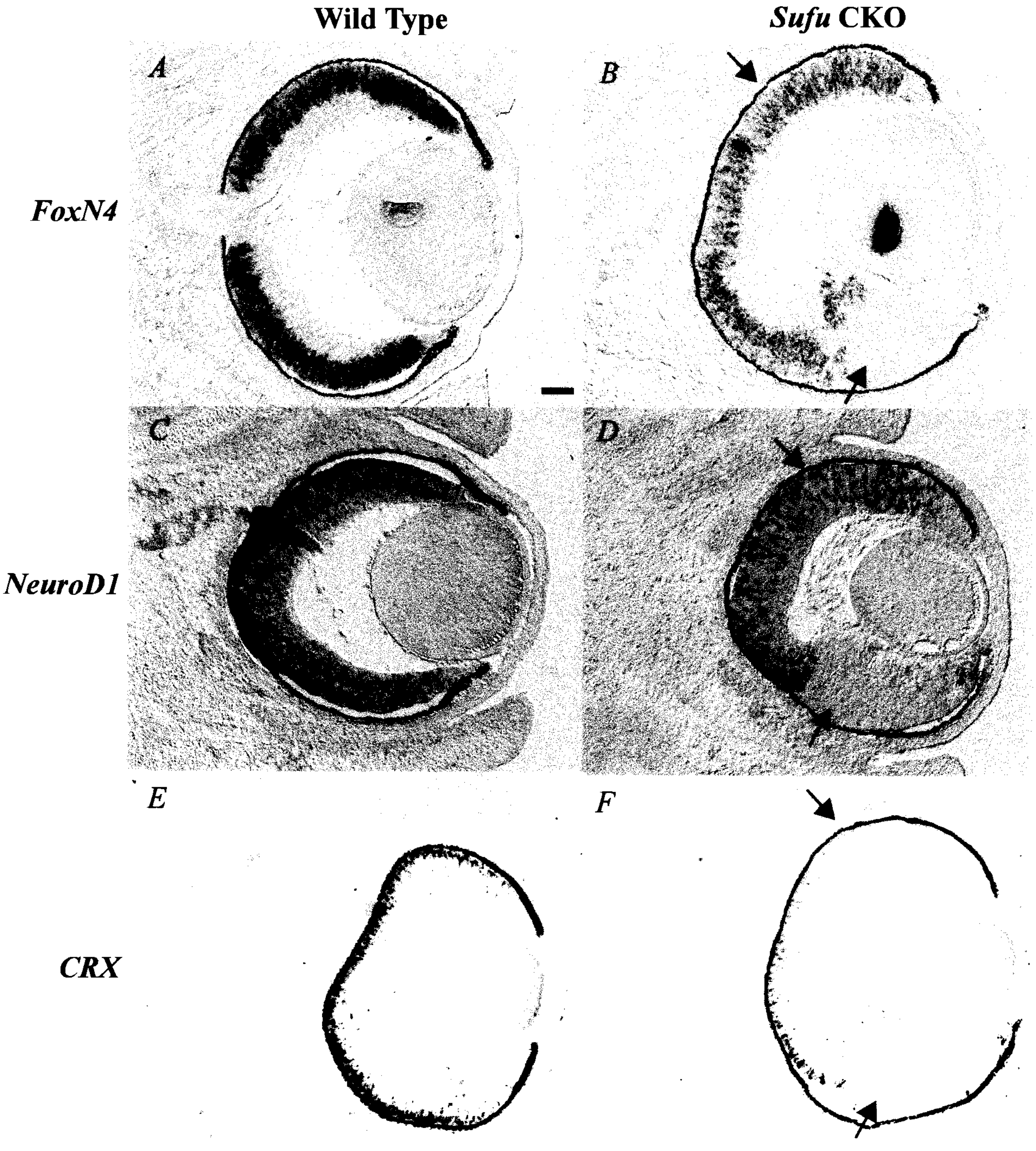

Wild Type
Sufu CKO
A
B
FoxN4
C
D
CRX

Figure 22: Transcription factors required to specify amacrine/horizontal interneurons and rod/cone photoreceptors are down-regulated in *Sufu* CKO retinae. *In situ* hybridization for *FoxN4* (A,B), *NeuroD1* (C,D) and *Crx* (E,F) mRNA expression in WT and *Sufu* CKO retinae at E14.5. The expression of *FoxN4* is abolished ventrally and down-regulated dorsally in *Sufu* CKO retinas. *Crx* is weakly expressed in the WT retina at E12.5 and completely down-regulated in the peripheral *Sufu* CKO retina. Arrows indicate regions of the mutant retina with perturbed expression of either *FoxN4* or *Crx*.


Wild Type
Sufu CKO
A
B
FoxN4
C
D
NeuroD1
E
F
CRX

3.10 Loss of RPC identity is not due to cell death

The down-regulation of transcription factors required to maintain RPC identity or to specify retinal cell types could potentially occur due to increased cell death. However, the altered expression pattern of these transcription factors was not associated with an increase in apoptosis as there was no marked increase in TUNEL positive cells in mutant retinas compared to the retinas of wild-type littermates (Figure 23).

3.11 The peripheral retina does not transdifferentiate into other ocular tissue

If *Sufu* deletion resulted in the transdifferentiation of RPCs into other tissue types, it could potentially explain the observed loss of RPC identity and down-regulation of pro-neural transcription factors. *Sufu* has been suggested to negatively regulate β-catenin (86) and our laboratory has previously demonstrated that aberrant β-catenin activity in the retina results in an expansion of the ciliary margin (CM), a non-neural portion of the eye located at the extreme periphery of the retina (69). However, the expression of *Otx1*, which defines the CM (69) and is likely a direct target of β-catenin signaling in the CM (our lab, unpublished results), was either normal or reduced in *Sufu* CKO eyes (Figure 24 C,D). It was also possible that *Sufu*-deletion resulted in transdifferentiation of the retina into optic stalk. However, this possibility was ruled out as the retinas of Sufu CKO mice had normal expression of the optic stalk marker, *Pax2* (110) (Figure 24 A,B).

Thus, *Sufu* deletion in RPCs results in a down-regulation of transcription factors required to specify RPC identity and these cells exhibit a remarkable loss of mulipotency. However, this effect is not associated with increased cell death or conversion to other optic vesicle cell fates.

Figure 23: No change in cell survival in the retinae of *Sufu* CKO mice. TUNEL staining of wild-type and mutant retinae at E12.5 (A, B) and E14.5 (C, D) does not indicate the presence apoptotic nuclei in WT or *Sufu* CKO retinae compared to the positive control (E) which is a P0 retina electroporated with GFP. The Cy3 filter (TUNEL) was taken at the same exposure for all images.

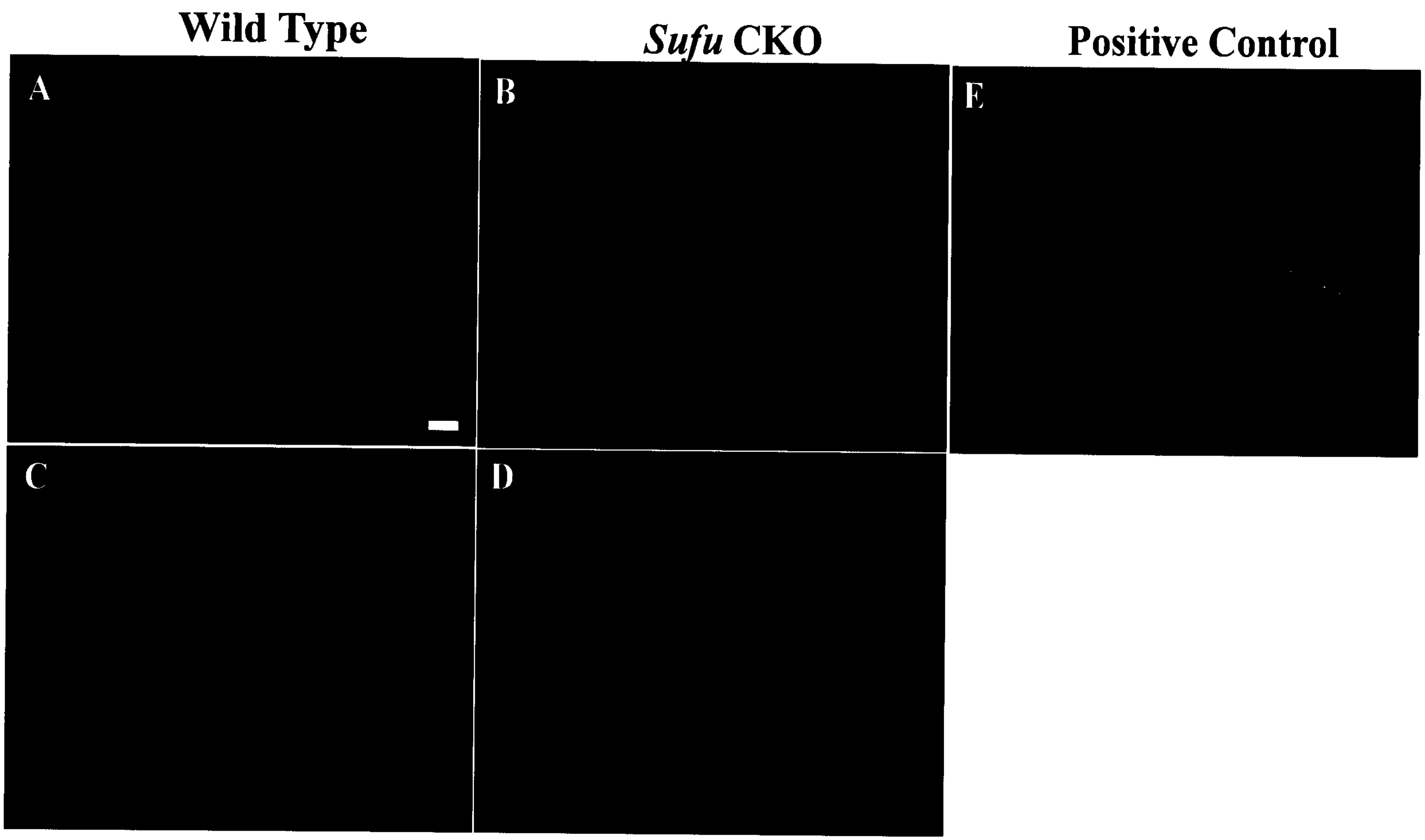
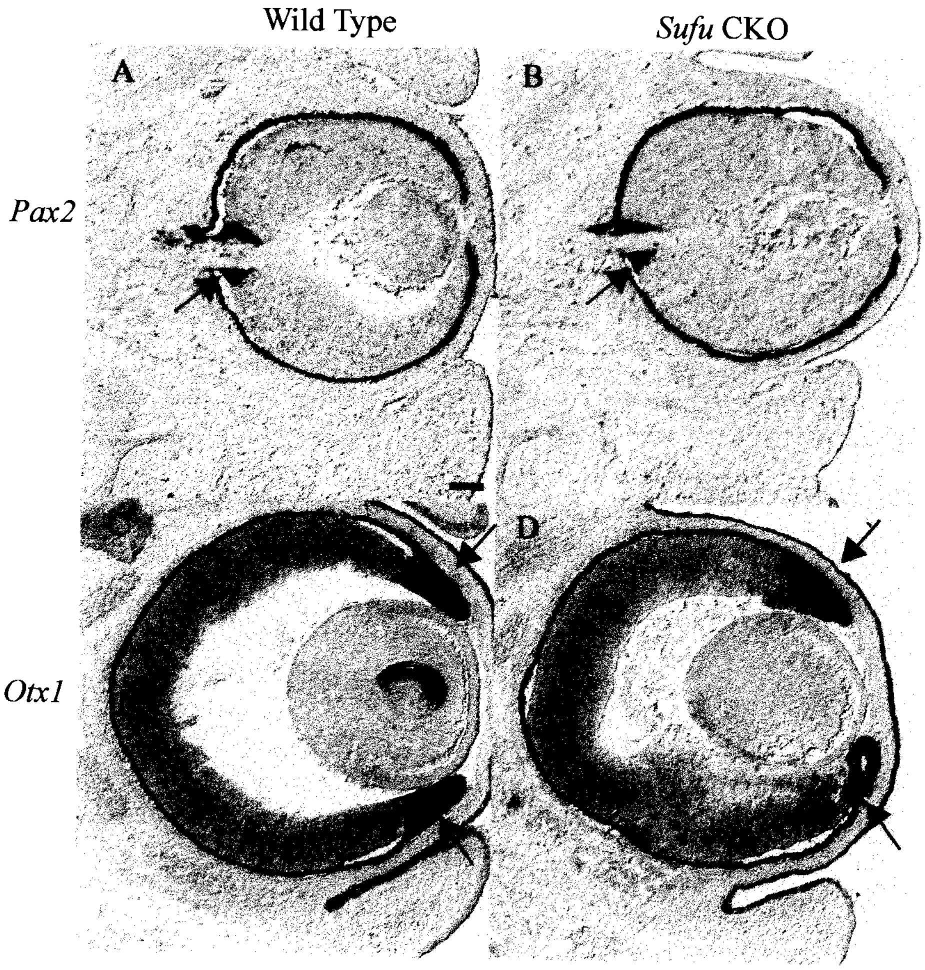

Wild Type
Sufu CKO
Positive Control
A
B
E
C
D

Figure 24: There is no expansion of the optic stalk (OS) or the ciliary margin (CM) in the retinae of *Sufu* CKO mice. *In situ* hybridization for *Pax2* (A,B) and *Otx1* (C,D) mRNA in the retinae of MT and *Sufu* CKO retinae at E12.5 and E14.5, respectively. Note the normal expression of the OS marker *Pax2* in the mutant mice (arrows) and the normal expression pattern of *Otx1*, which defines the CM (arrows).


Wild Type
Sufu CKO
A
B
Pax2
D
Otx1

3.12 Sufu-null RPCs predominantly adopt an interneuron cell fate.

To determine the fate adopted by *Sufu*-null RPCs, mature retinas from P18 mice were analyzed for the expression of retinal cell-type specific markers (Appendix 2). The mutant region of the adult retina was identified morphologically, based on the increase in thickness and abnormal spacing of the cells that was a consistent feature of the peripheral retina of mutant mice. Consistent with the loss of multipotency that I observed at E12.5 and E14.5, the cells in the peripheral retina of *Sufu* CKO mice displayed loss of retinal cell type diversity. Cells in the ventro-nasal mass exclusively expressed markers associated with mature amacrine/horizontal interneurons and did not express any other cell-type specific markers. The vast majority of the cells expressed the pan-amacrine/horizontal marker, Syntaxin (Figure 25 A-C), many of the cells stained positively for Calretinin (Figure 25 D-F), which is expressed by a sub-set of amacrine cells, although the intensity was not as strong as in Calretinin positive cells in the wild-type retina (Figure 25 D). A subset of cells also expressed the amacrine/horizontal/bipolar marker prox1 (Figure 25 G-I). In contrast, very few, if any, of the cells in the ventro-nasal mass expressed markers of other differentiated cell types, including bipolar cells, photoreceptors or Müller glia (Figure 26). Although a sub-set of cells in the peripheral Sufu CKO retina were Prox1$^+$, these cells were likely not bipolar neurons as none of the cells in the mutant-region of the retina expressed Chx10 (Figure 26 B), which is expressed by all bipolar neurons, or Recoverin (Figure 26 D) which is expressed by cone-bipolar cells as well as by rod and cone photoreceptors. Consistent with an absence of Recoverin expression, none of the cells in the peripheral mass expressed the rod photoreceptor marker, Rhodopsin (Figure 26 F). Finally, cells in the peripheral region of *Sufu*-null retina failed to form Müller glia, based on an absence of CRALBP-expressing cells (Figure 26 H). Thus, the absence of retinal diversity in the mature retina suggests that the loss of multipotency observed embryonically has functional consequences on cell fate specification.

Figure 25: Cells in the peripheral retina of *Sufu* CKO mice differentiate into interneurons. Fluorescent immunohistochemistry for Syntaxin (A-C), Calretinin (D-F) and Prox1 (G-I) in the retinae of control (A,D,G) and mutant (B,C,E,F,H,I) mice at P18. C, F and I are higher magnification views of the boxed region in B, E and H, respectively and the hatched line delineates the border between WT and mutant tissue. Cells in the peripheral retina of *Sufu* CKO mice express the pan-amacrine/horizontal maker Syntaxin. A subset of these cells also expresses Calretinin or Prox1.

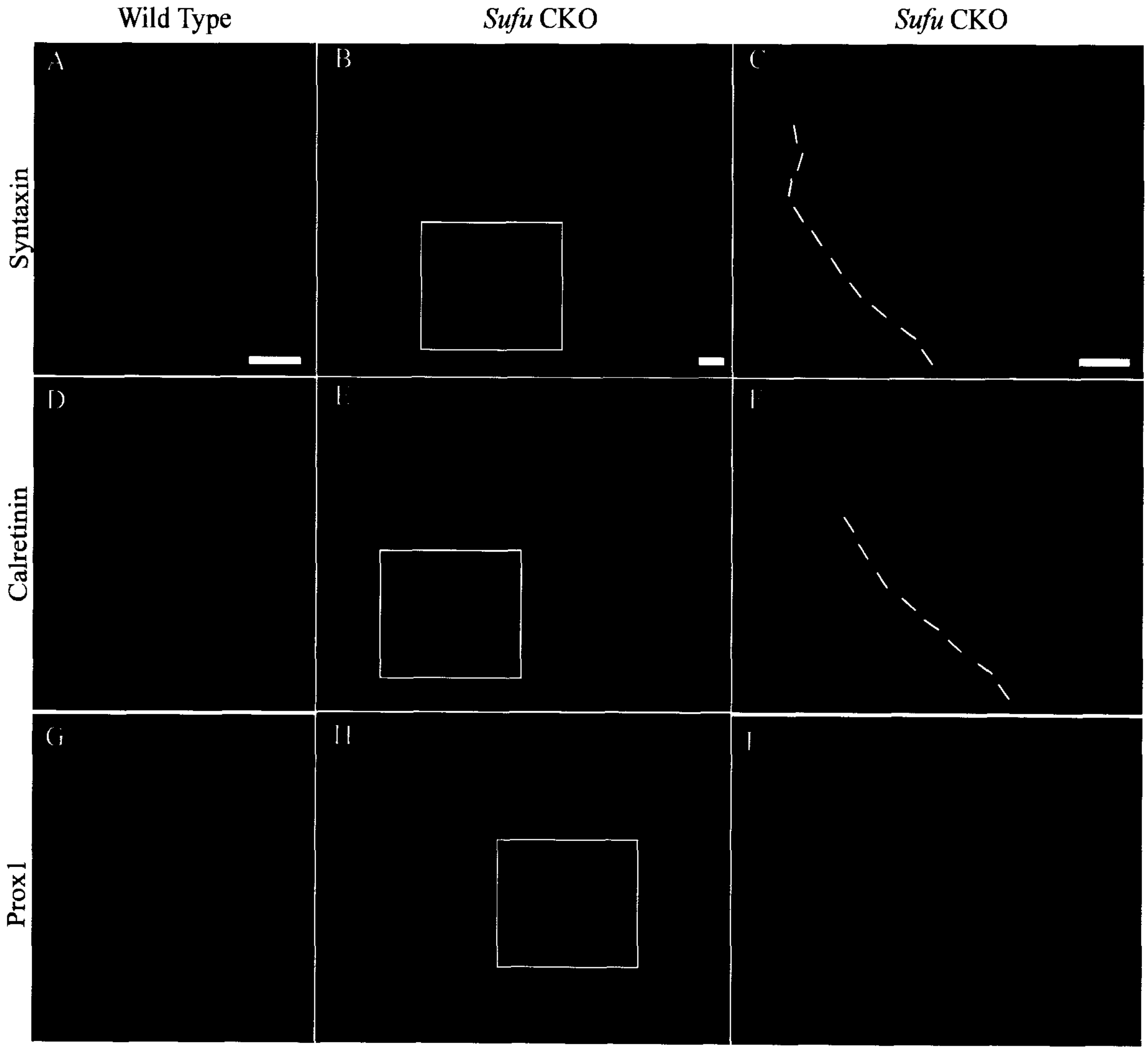
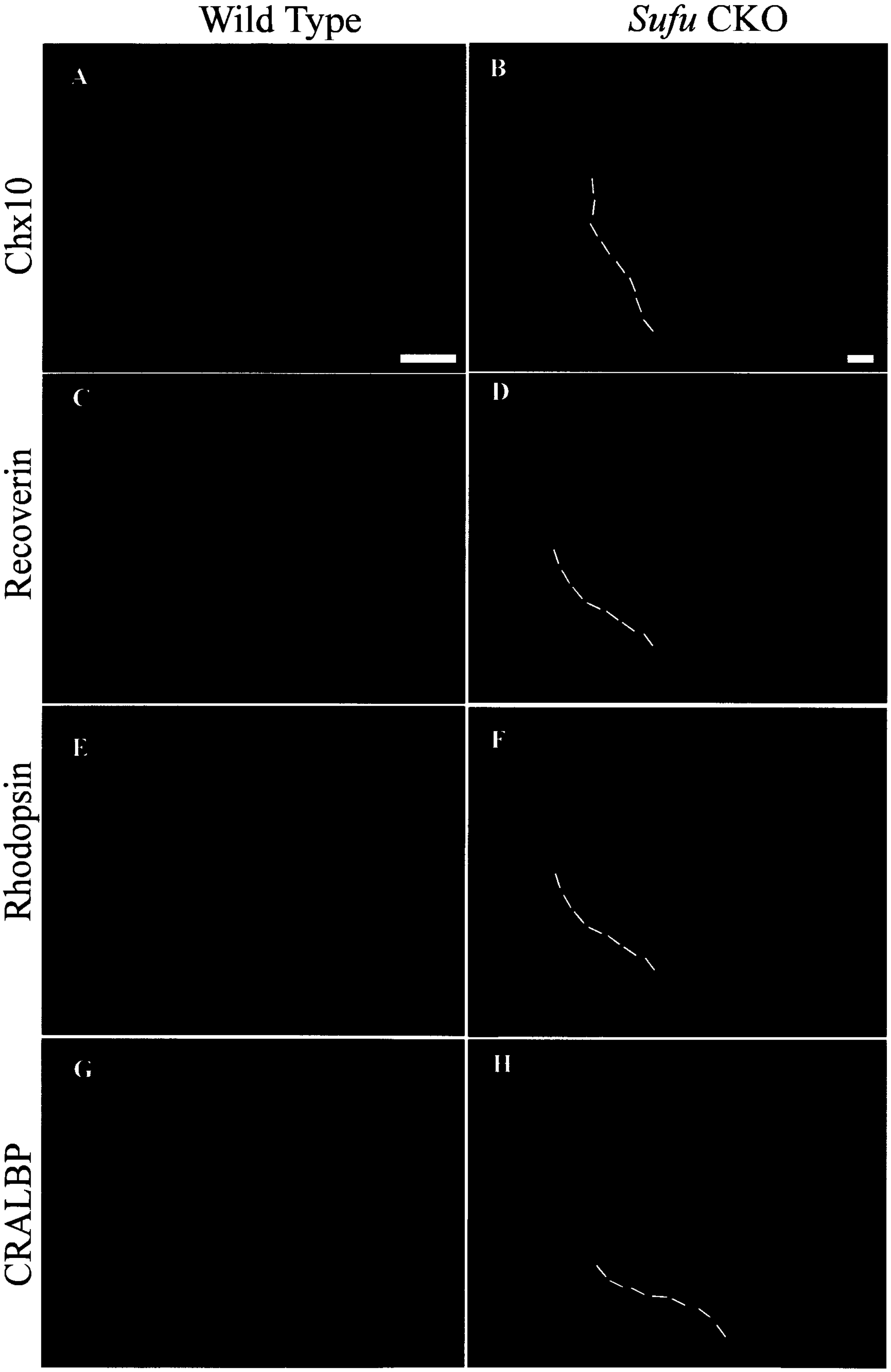
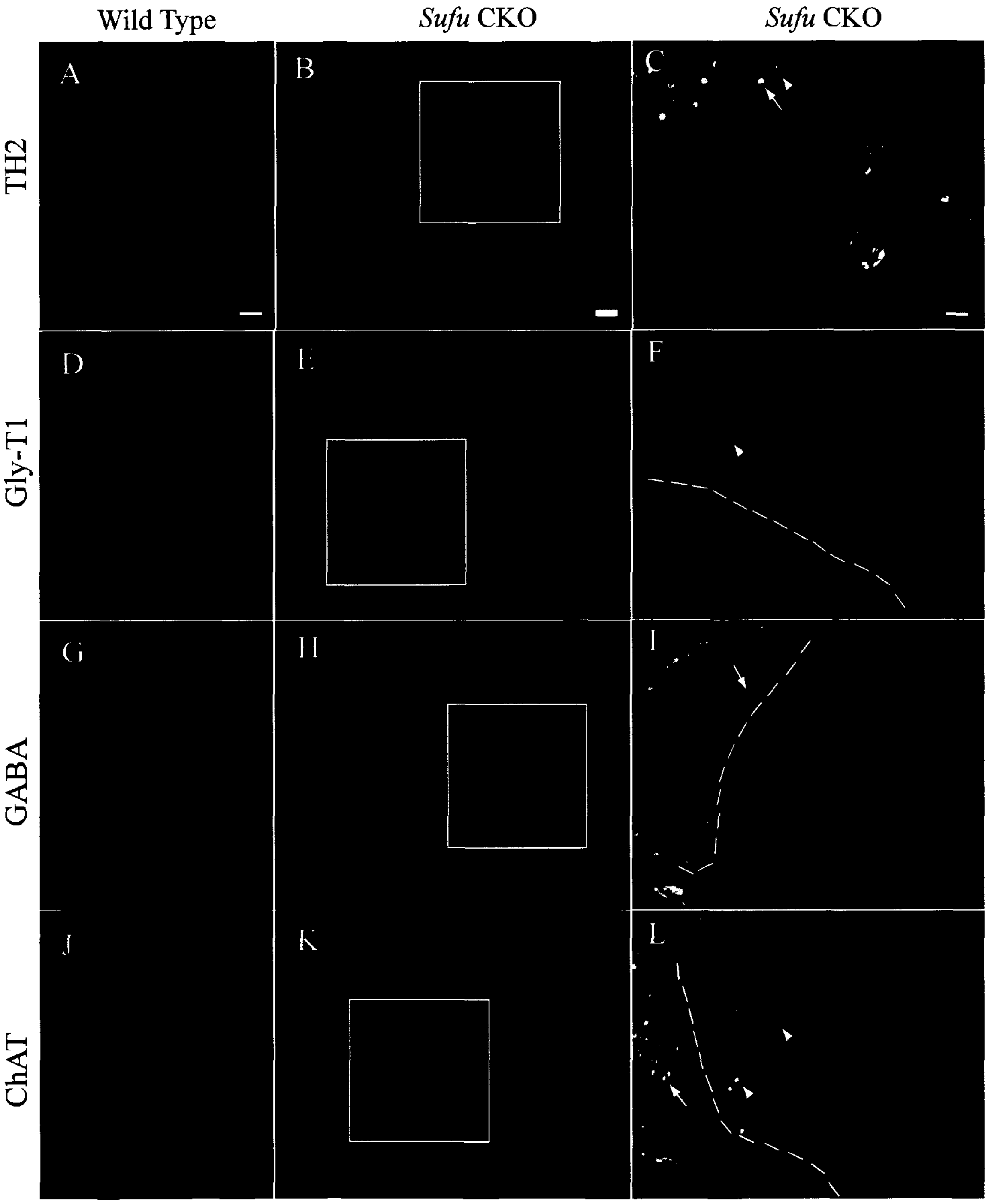

Wild Type
Sufu CKO
Sufu CKO
Syntaxin
Calretinin
Prox1
A
B
C
D
E
F
G
H
I

Figure 26: Cells in the peripheral retina of *Sufu* CKO mice do not differentiate into mature bipolar neurons, photoreceptors or Müller glia. Fluorescent immunohistochemistry for Chx10 (A,B), Recoverin (C,D) Rhodopsin (G,H) and CRALBP (I,J) in the retinae of WT and *Sufu* CKO mice at P18. In B, D, G and J, the hatched line delineates the border between WT and mutant tissue. Chx10 is expressed by mature bipolar cells, Recoverin is expressed by cone-bipolar cells as well as rod and cone photoreceptors. Rhodopsin is expressed by rod photoreceptors and CRALBP is expressed by Müller glia. Note the absence of any of these markers in the mutant region of the retina.


Wild Type
Sufu CKO
Chx10
Recoverin
Rhodopsin
CRALBP
A
B
C
D
E
F
G
H

Amacrine cells are extremely diverse, with over 20 amacrine subtypes identified to date in the mammalian retina (77), many of which can be identified by the expression of particular neurotransmitters (21, 95) or other markers, such those involved in neurotransmitter metabolism, or neural function (95). To determine if the appropriate diversity of interneuron sub-types was generated, I next investigated whether the interneurons in the sufu CKO region of the retina expressed the normal compliment of neurotransmitters. Interneurons in the cell mass were dopaminergic, based on the expression of tyrosine hydroxylase (TH2) (Figure 27 B,C), which could be seen as intense staining within cell bodies (Figure 27 C, arrow), as well as diffuse, punctate staining (Figure 27 C arrowhead). Very few, if any, of the cells within the mass were Glycinergic, GABAnergic or Cholinergic, based on an absence of expression of Glycine Transporter 1 (Gly-T1) (Figure 27 E,F), GABA (Figure 27 H,I), or Choline acetyltransferase (ChAT) (Figure 27 K,L). Therefore, *Sufu* deletion in RPCs leads not only to the exclusive generation of interneurons, but results in alterations in the types of interneurons that are generated.

Figure 27: Amacrine interneuron diversity is aberrant in the peripheral retina of *Sufu* CKO mice. Fluorescent immunohistochemistry for TH2(A-C), Gly-T1 (D-F) , GABA (G-I) and ChAT (J-L) in the retinae of control (A,D,G,J) and mutant (B,C,E,F,H,I,K,L) mice at P18. C,F, I and L are higher magnification views of the boxed regions in B, E , H and K, respectively. The hatched lines in C, F, I and L delineates the border between WT and mutant tissue. Some cells in the peripheral retina of mutant mice express the dopaminergic marker TH2. Note the bright staining in some cell bodies (arrow) and weaker, punctate staining in the neurites (arrowhead). In contrast, cells in the peripheral retina of mutant mice do not express the glycinergic marker, Gly-T1, the GABAnergic marker, GABA, or the cholinergic marker, CHaT compared to the WT region of the retina (arrows) in *Sufu* CKO mice or the retinae of WT littermates.



Wild Type
Sufu CKO
Sufu CKO
TH2
Gly-T1
GABA
ChAT
A
B
C
D
E
F
G
H
I
J
K
L

4. Discussion

The objectives of this study were to elucidate the role of *Sufu* as a regulator of Hh signaling in RPCs and to characterize the role of *Sufu* in RPC behavior *in vivo.* Gain and loss-of-function studies demonstrated that *Sufu* over-expression is sufficient to inhibit Hh signaling down-stream of *Smo,* while deletion of *Sufu* in RPCs is sufficient to activate the Hh pathway and promote proliferation. Thus, *Sufu* is an important negative regulator of the Hh pathway in RPCs. Importantly, conditional inactivation of *Sufu* in early RPCs identified a novel, and surprising, requirement for *Sufu* in the maintenance of RPCs identity and multipotency. The retinae of *Sufu* CKO mice displayed a down-regulation of transcription factors required to maintain RPC identity and specify the seven retinal cell types. This resulted in the exclusive generation of amacrine-like interneurons in the peripheral retina of *Sufu* CKO mice. These findings demonstrate that *Sufu* is required during early retinal development to specify and/or maintain progenitor cell identity and suggests that hedgehog signaling may antagonize RPC identity during early retinal development.

4.1 Sufu antagonizes the Hh pathway in RPCs.

Using both *in vitro* and *in vivo* approaches, this study has identified *Sufu* as a novel regulator of the *Hh* pathway in RPCs and further supports the theory that *Sufu* function has diverged in mammals to act as a potent antagonist of the *Hh* pathway (19, 115, 125). Over-expression of *Sufu* in retinal explant culture in the context of gain of function for Hh signaling was sufficient to abrogate Gli-dependent transcriptional activity, whereas acute deletion of *Sufu* in RPCs had the opposite effect. Deleting *Sufu* in RPCs *in vivo* also resulted in a gain-of-function for the *Hh* pathway, although this effect was transient. At E12.5, *Sufu* deletion resulted in an expansion of the *Gli1* expression domain and the formation of a cellular mass that contained predominantly cycling cells. However, these effects were not sustained, as *Gli1* down-regulation was observed in the mutant region of the retina at E14.5. Cells in this region also began to down-regulate the proliferation marker Ki67 at E14.5 and Ki67$^+$ cells were scarce by P0. Taken together, these data indicate that *Sufu* antagonizes the *Hh* pathway in RPCs, and that abrogation of *Sufu* expression results in a gain-of-function for the *Hh* pathway.

The inability of *Sufu*-null cells to remain in cycle indefinitely is perhaps not surprising. In this study, as well as others, activating the *Hh* pathway in retinal explants derived from P0 mice is sufficient to maintain only about 20% of the affected population in cycle after 2.5 or 3 days in culture (127, 138). Thus, while *Hh* pathway activation is able to promote proliferation in RPCs, it is not sufficient to completely prevent differentiation. This observation is not exclusive to retinal progenitors, and also applies to granule neuron precursors (GNP) in the cerebellum (60, 136). A key difference between RPCs and GNPs is that, in GNPs with aberrant *Hh* pathway activity, the majority of the cells differentiate, while a minor population of the GNPs remain in cycle indefinitely and develop into medulloblastoma (60, 136). In contrast, the retina appears to be resistant to tumorigenesis in the context of gain-of-function for *Hh* (This study, (11, 89)).

Conditional deletion of *Sufu in vivo* resulted in an up-regulation of the cell cycle inhibitor *p57(Kip2)*. The up-regulation of *p57(Kip2)* could potentially contribute to the premature cell cycle exit observed in retinae of *Sufu* CKO mice, as it occurred just prior to, and during, the time that I observed a down-regulation of Ki67 expression. Although this observation was not explored further, it does have two potentially important implications that should be investigated in future studies. First, *Hh* activity in the RPCs of lower vertebrates influences both proliferation (73) and cell cycle exit (73, 111), the latter function mediated through the up-regulation of *p57(Kip2)* (111). While sufficient evidence supports the mitogenic role for *Shh* in mammalian RPCs (11, 57, 127, 130, 138), there is little evidence to suggest a role for *Shh* in driving cell cycle exit in mammalian RPCs. The data presented here suggests that, under certain gain-of-function conditions, *Hh* signaling may also control cell cycle exit by modulating *p57(Kip2)* expression in mammalian RPCs, thus providing further evidence for the evolutionary conservation in *Shh* signaling. More importantly, if aberrant *Hh* activity also stimulates cell cycle exit in a proportion of mammalian RPCs, then this could provide insight into why RPCs are resistant to oncogenic transformation in a *Hh* gain-of-function context, while GNPs are not.

4.2 Sufu is required to maintain RPC identity

In early RPCs, *Sufu* was required to maintain the expression of transcription factors *Pax6, Rax, Chx10* and *Hes1*, which all play roles in specifying or maintaining RPC identity. *Rax* is required early during development to specify progenitor cells and/or the expression of progenitor cell markers (116, 141), *Pax6* is required to maintain multipotency (80) and *Chx10* and *Hes1* are required to maintain neuro- progenitor identity or prevent neural differentiation, respectively (36, 47, 122). Additionally, all four of these transcription factors are required to maintain the proliferative capacity of RPCs (42, 80, 112, 122, 140) which could also explain, in part, the premature down-regulation of Ki67 in the retinae of mutant mice.

Although the down-regulation of *Pax6, Rax, Chx10* and *Hes1* in *Sufu*-null progenitors suggests that RPC identity has been lost, it is possible that *Sufu*-null progenitors also have alterations in the expression of other transcription factors required for RPC maintenance or specification. Two potential candidates include *Sox2* and *Otx2*. *Sox2* expression is directly regulated by *Hh* signaling in neural stem cells (117). *Sox2* and *Pax6* regulation and function is tightly linked, with evidence suggesting that *Sox2* and *Pax6* reciprocally regulate their own expression (68, 133). Additionally, *Sox2* and *Otx2* co-regulate *Rx1* expression in *Xenopus* RPCs (20). Therefore, determining how *Sufu*-mediated control of the *Hh* pathway affects the expression of these transcription factors will advance our understanding of the relationships that exist between extrinsic signaling factors and the intrinsic establishment of retinal progenitor cell identity.

The down-regulation of transcription factors associated with RPC identity suggested that *Sufu*-null progenitors could be initiating apoptosis or adopting a non-RPC cell fate. However, I did not detect any appreciable increase in cell death in *Sufu* CKO retinae at either E12.5 or E14.5. Moreover, these cells did not adopt the fate of other ocular cell types, such as ciliary margin or optic stalk, based on the relatively normal expression of *Otx1* and *Pax2*, which are CM and OS markers, respectively(69, 110). I also addressed the possibility that *Sufu*-null RPCs were adopting the fate of ventral neural progenitors found in a more rostral region of the CNS. However, preliminary experiments indicated that this was not the case, as neither *Nkx2.1* nor *FoxG1* were expressed in *Sufu* CKO retinae, despite normal expression in the ventral telencephalon (data not shown). Therefore, although I have ruled out the possibility that *Sufu*-null progenitors are adopting a fate associated with the CM, OS or non-retinal neural progenitors, I have also not been able to provide evidence that *Sufu*-null progenitors have maintained a retinal identity and the identity of these cells requires further investigation.

4.3 Sufu is required to maintain RPC multipotency.

The down-regulation of *Pax6*, which is required to maintain RPC multipotency (80, 98), suggested that the expression of transcription factors required to specify retinal cell fates may be affected. Conditional inactivation of *Sufu in vivo* resulted in the down-regulation of transcription factors required to specify all seven retinal cell types. Analysis of mature mutant retinae at P18 revealed that *Sufu*-null progenitors did not differentiate into the diverse compliment of retinal cell types and were instead restricted towards an amacrine-like interneuron lineage, based on the exclusive expression of interneuron markers, and an absence of markers expressed by other differentiated retinal cell types. The absence of cellular diversity is likely not a function of selective interneuron survival, based on the absence of observed cell death and the down-regulation of transcription factors required to specify the other cell types that occurred early during retinal development.

Moreover, *Sufu*-null progenitors did not differentiate into the normal complement of amacrine interneuron subtypes normally found in the retina. Some cells within the mass expressed calretinin or prox1, both of which are expressed by amacrine cell subtypes (27, 80) . Many of the interneurons within the mass were dopaminergic, which is consistent with the association between Hh pathway activation and induction of dopaminergic neurons in other regions of the CNS (49, 51). In contrast, progenitors in the mutant region of *Sufu* CKO retinae did not differentiate into glycinergic, GABAnergic or cholinergic neuronal subtypes. However, it is important to note that not all of the cells within the mutant region of the retina were dopaminergic, indicating that they either expressed components of a neurotransmitter pathway not examined here, or failed to differentiate into mature neurons.

The phenotype observed here is somewhat consistent with the phenotype observed in the retinae of mice with a conditional deletion of *Pax6 (Pax6 CKO)* (80)– namely a loss of multipotency and the failure to form any cell types except amacrine or amacrine-like interneurons. However, three important differences suggest that the phenotype observed in the retinae of *Sufu* CKO mice is not exclusively the result of altered *Pax6* expression.

First, *Pax6* CKO RPCs continue to express transcription factors required to maintain RPC identity, such as *Rax* and *Hes1* (80) while *Sufu* CKO progenitors do not. Secondly, in the retinae of *Sufu* CKO mice, the expression of transcription factors required to specify all seven retinal cell types, including amacrine cells (*FoxN4* and *NeuroD1*), was affected. This contrasts with *Pax6* CKO retinae, which continue to express *NeuroD1* (80) but display a down-regulation of transcription factors required to specify the six other cell types (80). Finally, although progenitors from both *Sufu* CKO and *Pax6* CKO mice adopt an interneuron fate, the diversity of neural-subtypes observed differs substantially. The diversity of amacrine cell subtypes generated in the retinae of *Pax6*-null mice is relatively normal, as all neurotransmitter subtypes are made with the exception of GABAnergic amacrine interneurons (80). In comparison, *Sufu*-null progenitors are incapable of differentiating into the normal compliment of amacrine-subtypes. Therefore, although it is likely that the down-regulation of *Pax6* that is observed in the retinae of *Sufu* CKO mice contributes to the overall *Sufu*-null phenotype, it is insufficient to completely explain the behavior of *Sufu*-null progenitors and suggests that additional factors are being affected.

4.4 Shh and RPC identity

The loss of RPC identity and failure to specify retinal cell types has not been described previously in other retinal Shh gain-of-function models. For example, the RPCs of *Ptc*$^{+/-}$ mice, which have a partial Hh gain-of-function, develop all seven retinal cell types and have relatively normal retinal lamination (11, 89). Additionally, the RPCs of *Gli3*$^{-/-}$ mice, which also exhibit a Hh gain-of-function (Our lab, unpublished results), presumably due to a loss of Gli3-repressor activity (128), maintain expression *Pax6* and *Chx10* (33), indicating that Gli3 repressor is not required for the maintenance of RPC identity. The unique phenotype observed in *Sufu* CKO retinae may be due to the context and magnitude of *Hh* pathway activation or it could be the result of an unidentified *Sufu* function. Other genetic approaches to aberrantly activate the *Hh* pathway, such as complete inactivation of *Ptc* in RPCs through the use of a conditional *Ptc* allele will help distinguish between these two possibilities.

4.5 Future Directions

Although this study has answered some important questions about the role of *Sufu* in regulating the Hh pathway in RPCs and the importance of *Sufu* in the maintenance of RPC identity, it raises important issues related to the function of this pathway in multipotential neural progenitor populations.

This study did not elucidate the mechanism by which *Sufu* promotes the maintenance of PRC identity. Our findings suggest that there is an inverse relationship between the strength of Hh signaling and the expression of RPC-specific transcription factors. A key question in this context would be to dissect the relative roles of Gli2 activator and Gli3 repressor in the transcriptional regulation of these genes. This issue could be addressed through genetic epistasis experiments by generating *Sufu* CKO; *Gli2*$^{-/-}$ mice or generating *Sufu* CKO mice that over-express *Gli3* repressor. Importantly, this study also demonstrated that ectopic activation of the *Hh* pathway in RPCs *in vivo* was not sufficient to maintain RPCs in cycle indefinitely. Gaining an ind-epth understanding of the mechanism that makes the retina refractory to *Hh*-mediated tumorigenesis could potentially contribute towards the development of a treatment for other Hh-induced malignancies that occur in other regions of the CNS and in other organ systems.

5. Conclusion

The data presented here both implicates *Sufu* as a novel regulator of the Hh pathway in RPCs and further confirms that *Sufu* is a critical antagonist of the *Hh* pathway in mammalian systems. Moreover, I have also characterized the role of *Sufu* in modulating retinal progenitor cell behavior *in vivo* (Figure 28). The unexpected requirement for *Sufu* in the maintenance of early RPC identity and multipotency suggests that *Sufu* may have a critical function in mediating the intrinsic behavior of RPCs in response to extrinsic cues. Future studies should focus on elucidating the mechanism through which this mediation occurs.

Figure 28: ***Sufu*** **antagonizes the Hh pathway in RPCs and is required to maintain RPC identity and multipotency.** A) In the presence of *Sufu*, RPC identity and multipotency is established and/or preserved. These RPCs can express the transcription factors required to specify all seven retinal cell types and will give rise to the appropriate proportions of mature retinal neurons and glia. B) In the absence of *Sufu*, the Hh signaling pathway is activated and RPC identity and multipotency is lost. Transcription factors required to specify all seven retinal cell types are down-regulated prohibiting the specification and differentiation of the appropriate compliment of mature retinal neurons and glia. Through an unknown mechanism, these *Sufu*-null progenitors differentiate into amacrine-like interneurons.

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Appendix 1: Antibodies used for immunohistochemistry.

Antibody	Dilution	Species	Source	Special Treatment
Primary Antibodies				
Anti-Brn3b	1/150	Goat polyclonal	Santa Cruz Biotech	Citrate antigen retrieval – boil for 6 minutes in 5x Sodium Citrate. Biotin/Streptavidin.
Anti-Chx10	1/2000	Sheep polyclonal	Gift from Dr. Rod Bremner (University of Toronto).	Citrate antigen retrieval. Biotin/Streptavidin.
Anti-Gly-T1	1/1000	Goat polyclonal	Chemicon International	
Anti-calretinin	1/2000	Rabbit polyclonal	Swant	
Anti-CRALBP	1/500	Rabbit polyclonal	Gift from Dr. Greg Garwin (Univserity of Washington)	
Anti-Tyrosine Hydroxylase	1/500	Rabbit polyclonal	Chemicon International	
Anti-Prox1	1/3000	Rabbit polyclonal	Chemicon International	
Anti-GABA	1/3000	Rabbit polyclonal	Sigma	
Anti-ChAT	1/500	Rabbit polyclonal	Chemicon international	30 hour incubation at 4°C
Anti-GFP	1/1000	Rabbit polyclonal	Invitrogen	
Anti-B630	1/3	Mouse monoclonal	Developmental Studies Hybridoma Bank	
Anti-BrdU	1/1000	Mouse monoclonal	BD Bioscience	Prior to blocking, slides were incubated for 6 minutes in 2N HCl at 37°C and then 10 minutes in 0.1-Tris pH8.8 with 0.01% Tween-20.
Anti-Ki67	1/500	Mouse monoclonal	BD Pharmingen	Citrate antigen retrieval
Anti-Syntaxin	1/1000	Mouse monoclonal	Sigma	

Secondary Antibodies				
Anti-Rabbit FITC	1/450	Goat or Donkey	Molecular Probes	
Anti-Rabbit Cy3	1/1000	Goat	Jackson	
Anti-Mouse Cy3	1/1000	Goat	Jackson	
Biotinylated Anti-Goat	1/200	Donkey	Amersham Biosciences	
Cy3-Streptavidin	1/3000	N/A	Amersham Biosciences	

Appendix 2: Markers of retinal cell types.

Cell Type	**Marker**
Ganglion	Brn3b
Amacrine	Calretinin
	ChAT
	GABA
	Gly-T1
	Prox1
	Syntaxin
	TH2
Horizontal	Prox1
	Syntaxin
Bipolar	Chx10
	Prox1
	Recoverin
Cone Photoreceptor	Recoverin
Rod Photoreceptor	Recoverin
	Rhodopsin
Müller Glia	CRALBP

Appendix 3: Contributions of Colaborators.

Chantal Mazerolle: Research Technitian, Lab of Dr. Valerie Wallace, Vision Program, Ottawa Health Research Institute. Chantal conducted some initial analysis on adult and P0 mutant retinae and provided a framework from which to start the studies presented here.

Paul Oleynik: Technitian, StemCore Laboratories, Ottawa Health Research Insitute. Paul performed the FACS analysis and isolated the transfected RPCs that were eventually used for gene expression analysis presented in Figure 9.

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Curriculum Vitae

Education

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M.Sc – Biochemistry with a specialization in human molecular genetics.

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Honors B.Sc *Cum Laude*. Biology with a specialization in cellular and molecular biology.

Academic Awards and Scholarships

2008-2009: National Science and Engineering Research Council (NSERC) PGS Master's Fellowship.

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2007-2008: University of Ottawa Graduate Entrance Scholarship.

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Publications

Original Research

Matthew A. Cwinn, Stephanie P. Jones, Sean W. Kennedy,
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