The role of Hh signaling in mouse retinal bipolar cell subtype development

by

Di Wu
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Supervisory Committee

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Abstract

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In the vertebrate retina, bipolar interneurons consist of at least 13 distinct subtypes, which are classified based on their morphology, behavior and gene expression. The mechanisms underlying the formation of these subtypes is poorly understood. Our previous unpublished work has implicated Sonic Hedgehog (Shh) in the formation of cone and rod bipolar cell subtypes. In this thesis, I characterized the relationship between Hh signaling and bipolar subtype cell development in greater detail. Using an in vivo plasmid-based reporter approach, I show that Hh signaling is active in both retinal progenitor cells (RPCs) and bipolar cells of the postnatal retina. Next, to address function, I used a conditional gene targeting approach to show that activation of *Smoothened* (Smo), a downstream Hh signaling component, is both necessary and sufficient in postnatal RPCs to promote the formation of cone but not rod bipolar cells. In contrast, activation of *Smo* in postmitotic bipolar cells that are greater than 24 hours old from cell birth, does not affect bipolar subtype formation. Together, these results suggest that Hh signaling functions in postnatal RPCs (and potentially in early bipolar cell precursors) to promote cone bipolar cell formation.

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List of Abbreviation

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BC	Bipolar cell
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
CK1	Casein kinase 1
CNS	Central nerbous system
CNTF	Ciliary neurotrophic factor
Cos2	Costal2
Dhh	Desert hedgehog
Disp1	Dispatched 1
DMSO	Dimethyl sulfoxide
E	Embryonic
ECM	Extracellular matrix
EGF	Epidermal growth factor
EM	Electron microscopy
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GBS	Gli-binding site
GCL	Ganglion cell layer
GCL	Ganglion cell layer
GDF11	Growth differentiation factor 11
GSK3	Glycogen synthase kinase 3
HBSS	Hanks' balanced salt solution
Hh	Hedgehog
Hes1	Hairy and enhancer of split-1
HSPG	Heparin sulfate proteoglycan
Ihh	Indian hedgehog
INL	Inner nuclear layer
IPL	Inner plexiform layer

Kif7	kinesin family member 7
Mash1	Atonal homolog 1
Math5	Atonal homolog 5
mGluR6	Metabotropic glutamate receptor 6
miRNA	microRNA
NBL	Neuroblast layer
NGN2	Neurogenin 2
NPY	Neuropeptide y
ONL	Outer nuclear layer
OPL	Outer plexiform layer
Otx2	Orthodenticle homeobox 2
P	Postnatal
Pax6	Paired box protein
PB	Phosphate buffer
PKA	Protein kinase a
PKCα	Protein kinase c alpha
Ptch1	Patched1
RPC	Retinal progenitor cell
RPE	Retinal pigment epithelium
SAH	Subarachnoid hemorrhage
SGZ	Subgranular zone
Shh	Sonic hedgehog
Smo	Smoothened
SUFU	Suppressor of fused homolog
SVZ	Subventricular zone
TF	Transcription factor
Vsx1	Visual system homeobox 1
Vsx2	Visual system homeobox 2
WT	Wild-type

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Chapter 1 Introduction

In vertebrates, the Hedgehog (Hh) signaling pathway is essential for controlling retinal progenitor cell proliferation. However, little is known about the role of Hh signaling in regulating retinal cell type formation. Recent unpublished studies from our lab have implicated a role for Hh signaling in regulating bipolar cell subtype formation. This thesis investigates the function of Hh signaling in postnatal retinal progenitor cells (RPCs) generating cone and rod bipolar cells.

The first chapter of this thesis provides background about the basic anatomy and cell type composition of the vertebrate retina and the regulatory mechanism in which different retinal cell types are generated. The following section then introduces the Hh signaling pathway and subsequently, the role of Hh signaling in the central nervous system (CNS) and retinal development. Finally, I present my objectives and the hypothesis of my thesis.

1.1 Anatomy and Physiology of the Vertebrate Retina

1.1.1 Structure and visual pathways in retina

The vertebrate retina is a sensory tissue located at the back of the eye that converts light into chemical signals that are sent to the visual cortex. In the mature retina, the visual neurons in the retina are organized into five distinct layers. (Fig. 1A). Cone and rod photoreceptors are light detectors located in the outer nuclear layer (ONL), the outer-most layer of the retina. Amacrine cells, horizontal cells, and bipolar cells are interneurons located in the inner nuclear layer (INL). These cells integrate input from multiple photoreceptors and transmit the signal to retinal ganglion cells in the inner most ganglion cell layers (GCL). The axons of retinal ganglion cells are formed into the optic nerve that connects to the lateral geniculate nucleus and superior colliculus in the brain (Wohrer 2008). The Müller cells are the only glial cell type in the retina and function primarily to maintain the stability of the retinal extracellular environment. In mammals, Müller glia cells contribute to gliosis in the context of retinal injury by re-entering the cell cycle to proliferate (Bringmann et al 2006, Dyer & Cepko 2000).

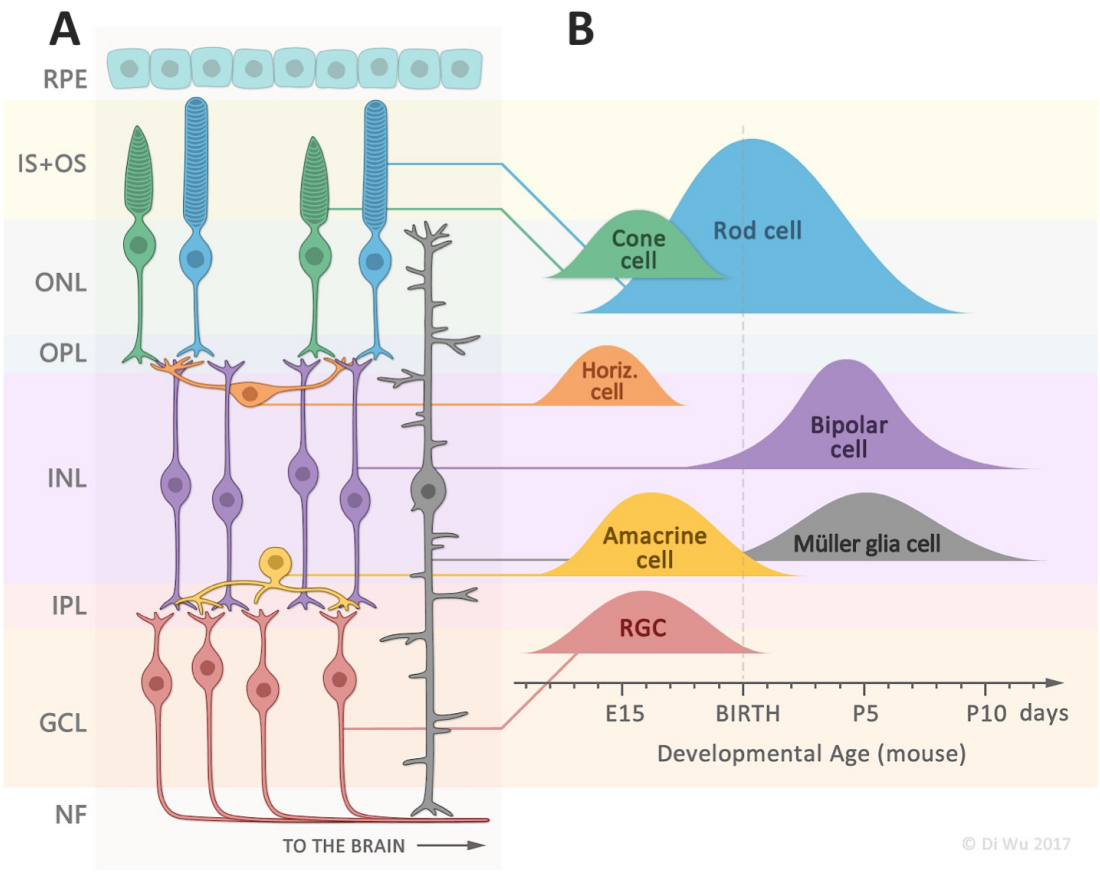

Figure 1 Organization of the mature retinal structure and the temporal ordering of cell birth in mouse retinas. (Legend on next page)

Figure 1 Organization of the mature retinal structure and the temporal ordering of cell birth in mouse retinas.

A) Mature mouse retinas consist of three nuclear layers (GCL, INL and ONL) and two synaptic layers (IPL and OPL). Five classes of neurons are present in the retina; these classes include retinal ganglion cells in the GCL, amacrine cells, bipolar cells and horizontal cells in the INL, and cone and rod photoreceptors in the ONL. The retina also consists of one glial cell type, the Müller cell. Light enters the eyes and passes through the entire retinal tissue until it reaches the RPE, where scattered light is absorbed. Light information is transformed into electrical signals by photoreceptors, and the signals are passed down to and processed by INL neurons. The signals are integrated by ganglion cells and sent to the brain. The axons derived from ganglion cells projecting to the brain form the NF layer. **B)** The temporal birth order of cells in the retina. Cell birth begins at E9.5 and ends at P10. Cell types are born in an orderly yet overlapping manner over time. Retinal ganglion cells, horizontal cells, cones, and most of the amacrine cells are born embryonically. Rods, bipolar cells, and Müller glia are born postnatally. Bipolar cells, the focus of this study, arise P0; birthing peaks at around P3 and ends at around P10. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer; IPL: inner plexiform layer; OPL: outer plexiform layer; RPE: retinal pigment epithelium; NF: neural fibre; E: embryonic; P: postnatal.

1.1.2 The diversity of retinal cell types

The vertebrate retina has been a favorite model for studying neuronal diversity. In the mouse retina, around 100 retinal cell types exist that are distinct in morphology, molecular characteristics and physiological functions (Sanes & Zipursky 2010). This cell type diversity is essential for the establishment of the many complex and functionally specialized retinal circuits that process various types of visual information (Gollisch & Meister 2010). Retinal ganglion cells, the signal output of the visual system, account for a large number of cell types in the retina. At least 32 different types of ganglion cells have been identified based on their light responses and anatomical properties (Baden et

al 2016). Amacrine cells play roles in shaping of ganglion cells responses and have at least 28 subtypes identified by Golgi staining and photofilling techniques (Macneil et al 1999). Bipolar cells are interneurons that transmit signals from photoreceptors to ganglion cells. Bipolar cells also contact horizontal cells and amacrine cells to mediate specialized visual signalling processing (Euler et al 2014, Kaneko 1983). Bipolar cells are divided into different subtypes (further discussed in section 1.1.3). Photoreceptors have two basic types: cones and rods. Cone photoreceptors are further divided into several subtypes, based on the expression of opsin proteins (Jacobs et al 2004, Ortin-Martinez et al 2014). Most mammals have around two or three horizontal cell subtypes, whereas mice and rats have only one (Kolb et al 1994, Masland 2001).

1.1.3 Classification of retinal bipolar cells

Retinal bipolar cells make up 40% of neurons in the INL (Jeon et al 1998). At least 13 bipolar subtypes have been distinguished in mice so far (Seung & Sumbul 2014), and these subtypes are grouped as ON or OFF types and cone or rod types (Fig. 2). All rod bipolar cells are ON types while cone bipolar cells are ON and OFF types of cells (Dacheux & Raviola 1986). Upon receiving the neural transmitter glutamate released by photoreceptors, ON type bipolar cells (types 5a to 9 of cone bipolar cells, and rod bipolar cells) hyperporarize and OFF type bipolar cells (types 1 to 4 of cone bipolar cells) depolarize (Werblin & Dowling 1969). The underlying mechanism for these opposing responses is that ON and OFF bipolar cells express different glutamate receptors. ON bipolar cells express the metabotropic glutamate receptor 6 (mGluR6) that couples to a G-protein cascade (Shiells & Falk 1990). Mice lacking the mGluR6 gene have complete ablation of ON bipolar cell activity (Masu et al 1995). OFF bipolar cells respond to glutamate mainly through NMDA receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, and kainate receptors; unlike mGluR6, these receptors are ionotropic (Saito & Kaneko 1983, Thoreson & Witkovsky 1999) .

Morphologically, bipolar cells are further classified into 12 different subtypes by their axonal stratification patterns in mice. In mammals, the IPL strata is divided into five equal levels of thickness (S1-S5), and each stratum is defined by the horizontal bands of neuronal processes (Ramon y Cajal 1893). Bipolar cells have their axon terminals stratified at one or multiple distinct IPL stratum to allow connectivity to certain

sets of retinal ganglion cells and amacrine cells (Kolb et al 1992, Pignatelli & Strettoi 2004). The axons of OFF bipolar cells terminate at between IPL S1 and S2 (also known as sublamina a) while the axons of ON bipolar cells terminates at between IPL S3 to S5 (Nelson et al 1978).

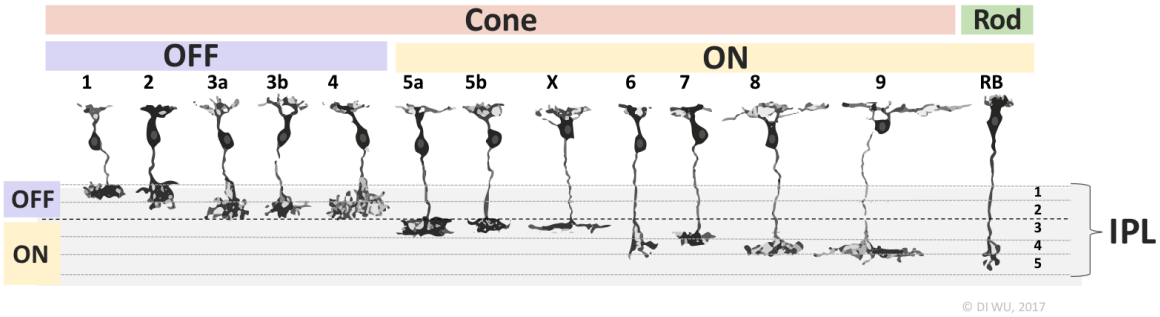

Figure 2 Classification of bipolar cells in mouse retinas.

Bipolar cells are classified into 13 subtypes based on their morphologies and physiology and are arranged based on their stratification pattern in the sublamina (1-5) of the IPL (Ghosh et al 2004, Helmstaedter et al 2013). Depending on their responses to light stimuli, bipolar cells are also grouped into ON types and OFF types (second top panel). ON-type bipolar cells depolarize in response to increased light intensity and terminate at the innermost portion of the INL (left panel), while OFF-type bipolar cells hyperpolarize to such stimuli and terminate at the outer portion of the IPL (left panel). Bipolar cells are also grouped into cone and rod types (top panel) based on their connectivity to cone or rod photoreceptors.

Several techniques have allowed the identification of different bipolar cell subtypes. A summary of bipolar cell subtype markers is given in Table.1. Immunohistochemistry is the most commonly used method to distinguish bipolar cell populations that express certain cell markers. For instance, rod bipolar cells are immunoreactive for protein kinase C alpha (PKCα) in mammals (Negishi et al 1988, Nelson et al 1978). In mice, the calcium-binding protein caldendrin is a marker for OFF-cone bipolar cells (Haverkamp & Wassle 2000, Seidenbecher et al 1998). Some protein markers are expressed in both ON cone and OFF cone bipolar cells. For example, the visual system homeobox 1 (Vsx1) protein is specifically expressed by a subset of cone bipolar cells (OFF type 2 and ON type 7) (Chow et al 2004, Shi et al 2011).

In addition to immunohistochemistry, expressions of reporter proteins driven by cell-specific promoters have been used to identify specific types of bipolar cells *in vivo*. For example, type 7 cone bipolar cells are labelled with GFP driven by the alpha-gustducin promoter (Huang et al 2003a). ON-cone bipolar cells express the nuclear-localized βgal driven by the mGluR6 promoter (Ueda et al 1997).

Both immunohistochemistry and reporter mouse models are used for identifying a certain population of bipolar cell subtypes. In order to pinpoint to specific kind of bipolar cell, techniques such as intracellular injection or gene gun targeting of bipolar cells with dyes have been used to morphologically distinguish bipolar cell at the single cell level (Ghosh et al 2004, Pignatelli & Strettoi 2004). Other advanced techniques such as serial electron microscopy (EM) have been used to classified bipolar cells into types based on their morphological criteria and neural circuits (Helmstaedter et al 2013).

Table 1 Summary of immunohistochemistry markers and transgene reporters of bipolar cell subtypes.

		Cone											Rod	References
		OFF					ON							
		1	**2**	**3**		**4**	**5**	**X**	**6**	**7**	**8**	**9**	**RB**	**References**
				3a	**3b**		**5a**	**5b**						
Cellular marker	Vsx1		+							+				(Chow et al 2001, Chow et al 2004, Shi et al 2011)
	CaB5			+	+		+	+					+	(Ghosh et al 2004, Haverkamp et al 2003)
	HCN4			+										(Mataruga et al 2007)
	PKARIIβ				+									(Mataruga et al 2007)
	NK3R	+	+											(Ghosh et al 2004, Haverkamp et al 2003)
	Recoverin		+											(Haverkamp et al 2003)
	PKCα												+	(Negishi et al 1988)
	Syt2		+						+					(Wassle et al 2009)
	Irx5		+	+	+									(Cheng et al 2005)
	Chx10	+	+	+	+	+	+	+	+	+	+	+	+	(Burmeister et al 1996a)
Transgene reporter	Gustusin:GFP									+			**(+)**	(Huang et al 2003b)
	mGluR6:NLS-βgal						+	+	+	+	+	+	+	(Shi et al 2012, Ueda et al 1997)
	Clm1	+												(Feng et al 2000, Wassle et al 2009)
	Clm12		+											(Berglund et al 2006)
	5ht3R-EGFP						+	+						(Haverkamp et al 2009, Wassle et al 2009)

1.2 Retinal cell determination and differentiation

1.2.1 The temporal order of retinal neurogenesis in vertebrates

In mice, all retinal cells are generated from a pool of multipotent RPCs between embryonic day (E) 11 and postnatal day (P) 10 in an evolutionally conserved order (Morrow et al 2008, Young 1985). Birth dating experiments in rodents have shown that retinal ganglion cells are the first types of neurons to be born, followed by horizontal cells and cone photoreceptors that are all born embryonically. Amacrine cells and rod photoreceptors are continuously born at the postnatal stage, while bipolar cells and Müller glia cells are born mainly postnatally (Fig. 1B) (Rapaport et al 2004, Young 1985). It is also believed that some retinal cell subtypes are also born in order. For instance, glycinergic amacrine cells are born postnatally while other types (Starbust types, GABAergic types, neuropeptide Y (NPY)-expressing types and tyrosine hydroxylase-expressing types) are born embryonically (Cherry et al 2009, Voinescu et al 2010). Retroviral linage analysis in mice has revealed that cone bipolar cells are born earlier than rod bipolar cells (Morrow et al 2008). This contrasts somewhat, our unpublished findings that rod bipolar cell birth tends to be completed before that of cone bipolar cells (Star et al., unpublished data, Chow lab).

1.2.2 The regulation of RPC fate determination

In developmental biology, cell fate commitment is described by three basic modes (reviewed in (Bedzhov et al 2014)). The first is called cell specification, in which cells have made a level of commitment beyond cell fates and are capable to develop cell-autonomously when placed in a neuter environment. However, cell fates at this stage can still be influenced by different environments. Beyond cell specification is cell determination. At this stage, cells have made an irreversible developmental choice. Finally, the process of cell committing their specification is called cell differentiation, in which cells have gained detectable differences. The ordering of cell birth in the retina suggests that temporal regulation is required for cell fate determination of RPCs. Although RPCs are in general multipotent, a number of studies have found that in vertebrates, RPCs progressively obtain heterogeneous competence over the course of retinal development (Harris 2008, Marquardt 2003, Ohsawa & Kageyama 2008,

Trimarchi et al 2008, Turner & Cepko 1987a, Turner et al 1990, Wetts & Fraser 1988, Wong & Rapaport 2009). Competence is defined by the ability of neuron progenitors to generate a particular types of daughter cell in a short temporal period (Cepko 2014). It is believed that the competence of RPCs is largely defined by intrinsic factors such as transcription factors (TFs) and microRNAs (miRNAs), while the temporal progression of RPC competence may be regulated by extrinsic cues coming from the environment (Cepko 2014).

1.2.2.1 Intrinsic regulation

The intrinsic competence of RPCs has been demonstrated in rodents; RPC clones from the embryonic stage generated early born cell types while those from the postnatal stage largely gave rise to late born cell types (Turner & Cepko 1987b). The competence of RPCs may be regulated primarily by a cell-autonomous mechanism as illustrated by studies in which isolated RPCs from the E16-17 stage generate a similar composition of cell types *in vitro* in serum free culture conditions (Cayouette et al 2003). Similarly, *in vivo* studies of frogs have found that young retinal tissues failed to express older cell markers at an earlier time than is usual when placed with older developing retinal cells (Rapaport et al 2001).

The expression level of certain TFs influences the intrinsic programming of RPCs; the basic helix-loop-helix (bHLH) and homeodomain (HD) TFs protein families have been studied the most extensively. For instance, it was found that that the vast majority of ganglion cells were lost in mice that were mutant for the bHLH *atonal homolog 5* (Math5) gene, and the loss of ganglion cells resulted in an increased differentiation of amacrine cells (Cepko 1999, Yang et al 2003). Some bHLH TFs such as hairy and enhancer of split-1 (Hes1) are, unlike Math5, negative regulators of early retinal development. In *Hes1*-null mice, precocious differentiation occurred throughout the retina and was thought to be a result of the up-regulated *atonal homolog 1* (Mash1) expression caused by the absence of *Hes1*(Ishibashi et al 1995, Tomita et al 1996b).

The HD proteins family is the second largest transcription protein family; the roles of these proteins in vertebrate neurogenesis have been extensively studied (Anderson et al 1997, Arber et al 1999, Hodge & Hevner 2011, Marquardt & Gruss 2002). Paired box protein (Pax6) is a well-known HD TF in the retina that regulates

RPCs differentiation. In mammalian retinas, *Pax6* deficiency led to the ablation of all retinal cell types except for non-glycinergic amacrine cells, suggesting that Pax6 is required for the maintenance of RPCs mutipotency (Marquardt et al 2001). Pax6 may also intrinsically influence retinal cell subtype formation at a later stage, as the *Pax6*-deficient amercrine cells subtype did not develop into the glycinergic subtypes (Marquardt et al 2001). Other HD TFs drive RPCs to adopt specific cell fates; for example, the *Orthodenticle homeobox 2* (*Otx2*) gene is required and sufficient to promote photoreceptor and bipolar cell fates. Additionally, the *Visual system homeobox* 2 (*Vsx2*, also known as *Chx10*) gene promotes bipolar cell determination (Burmeister et al 1996b, Nishida et al 2003, Viczian et al 2003).

Though the roles of TFs in programming RPCs fates have been well studied, the mechanisms that drive the temporal progression of RPCs cell fate have remained elusive. Potential regulators include miRNAs and the interplay of series of TFs. In mice, miRNAs appears to be required by RPCs to shift from early cell fate competence to late cell fate competence. Let-7, miR-125, and miR-9 have been found to be sufficient to accelerate the development timing of RPCs and thus increase the production of late born cells such as rod photoreceptors (La Torre et al 2013). Transcriptional networks also regulate the cell cycle progression of RPCs. In the chick retina, the progression of the last cell cycle of RPCs committed to become retinal ganglion cell fate is tightly regulated by the downregulation of HES1 and the upregulation of neurogenin 2 (NGN2) and ATH5 (Matter-Sadzinski et al 2005). Similarly, the loss of *Math5* in mice caused the deficient cell cycle progression of RPCs and *Math5*$^{-/-}$ RPCs cells were unable to adopt the fate of retinal ganglion cell (Le et al 2006).

1.2.2.2 Extrinsic regulation

Previous studies have found that the cell fate determination of RPCs is restricted by their stages of competence. However, the contribution of extrinsic cues should not be underestimated. First, the roles of extrinsic cues are evident in a classic study in which E16 RPCs from rat formed more rod photoreceptors (the main postnatally born cell types) than control when cultured in the presence of 20-fold more postnatal (P) 0 cells *in vitro*

(Belliveau & Cepko 1999) . These results may not necessarily contradict previous experiments conducted on frogs (Rapaport et al 2001) because both the animals and the culturing systems are very different. Additionally, extrinsic factors are ideal candidates for feedback regulation during retinal development. For instance, both retinal ganglion cells and amacrine cells have inhibitory regulation of their own productions (Belliveau & Cepko 1999, Waid & McLoon 1998). The stage of RPC competence is mediated by the timing of RPC exit from the cell cycle, and thus it will not be surprising that extrinsic factors can regulate cell type determination by influencing RPC cell cycle. Sonic hedgehog (Shh) is one of the extrinsic factors that play a mitogenic role in the developing retina. Inactivation of *Shh* in mouse retinas is associated with early cell cycle exit and the overproduction of retinal ganglion cells (Wang et al 2005); an opposite effect was found in the zebrafish, in which cell cycle exit is perturbed when *Shh* is absent (Shkumatava & Neumann 2005). The roles of Shh in mediating cell cycle and regulating retinal development will be further discussed in section 1.4 and 1.5.

The roles of extrinsic cues, other than Shh, such as Notch signaling and growth factors, have also been studied extensively. Notch signaling, which is mediated by cell-cell communication, has been known to play roles in preserving the pool of undifferentiated progenitor cells in vertebrate nervous system (Artavanis-Tsakonas et al 1999). In the retina, the signaling receptor Notch 1 is expressed by undifferentiated RPCs and mature Müller glia cells (Dorsky et al 1995, Furukawa et al 2000). In mice, constitutive activation of Notch signaling in embryonic RPCs leads to the prolonged progenitor cell state and the acquisition of glial fates, while the downregulation of Notch signaling is required for the production of postmitotic neurons (Jadhav et al 2006a, Tomita et al 1996a). Notch signaling not only affects the progenitor cell stage of RPCs; it is also required for inhibiting photoreceptor formation, as inactivation of Notch signaling leads to enhanced photoreceptor production in both embryonic and postnatal stages. In addition, these phenotypes are coupled with the loss of bipolar cells, amacrine cells and Müller glia cells (Jadhav et al 2006b, Yaron et al 2006) The role of Notch signaling in bipolar cell development is further discussed in section 1.2.3.3.

Fibroblast growth factor receptors (FGFR) are the receptors for the fibroblast growth factor (FGF) protein family and some non-FGF ligands such as cell-adhesion

molecules (Szebenyi & Fallon 1999, Walsh & Doherty 1997). The role of FGF signaling in retinal development has been established by introducing the mutant forms of FGFR into *Xenopus* embryo; this approach led to a reduced production of rod photoreceptors and amacrine cells and an increased production of cone photoreceptors, Müller glia cells and horizontal cells (McFarlane et al 1998). In contrast, overexpression of FGF2, one ligand of FGFR, resulted in the formation of more retinal ganglion cells and fewer Müller glia cells. Overexpression of FGF2 also led to the formation of more rod photoreceptors at the expense of cone photoreceptors (Patel & McFarlane 2000). Additionally, the role of Growth differentiation factor 11 (GDF11) is another example of growth factor-mediated regulation in RPCs.

GDF11 is a secreted molecule and is a member of the transforming growth factor beta superfamily. It is suggested that GDF11 controls the competence of RPCs and negatively regulates retinal ganglion cell genesis at the expense of photoreceptors and amacrine cells (Kim et al 2005). *GDF11* deficiency did not affect the proliferation of RPCs. Instead, it promoted retinal ganglion cell genesis at the expense of photoreceptors and amacrine cells, possibly by stabilizing *Math5* expression (Kim et al 2005). These findings suggest that regulation of cell division and cell-type determination may be independent in the retina.

1.2.3 The regulation of bipolar cell development

Nearly all bipolar cells are born postnatally in vertebrates (Bassett & Wallace 2012). A single RPC is capable to produce a bipolar cell and rod photoreceptor in its terminal division. Therefore, the generation of bipolar cells largely depends on the regulation networks that impact this binary cell fate decision. HD and bHLH TFs, as intrinsic factors, regulate the generations of pan-bipolar cells and the individual subtypes. The contribution of extrinsic cues has not been studied extensively. However, the roles of a few candidates, such as Shh and CNTF, in influencing bipolar cell development, have been studied. Figure 3 presents the developmental timeline of bipolar cells, and summarizes the intrinsic and extrinsic factors that regulate the development of bipolar cells.

1.2.3.1 The gene regulatory network in pan-bipolar cell development

The HD TF Chx10 is essential for the genesis of bipolar cells. Chx10 is expressed in RPCs and in the mature retina remains expressed in bipolar cells (Burmeister et al 1996a). *Chx10*-null retinas have impaired RPCs proliferation and a loss of bipolar cells (Burmeister et al 1996a, Livne-Bar et al 2006). Even when RPC proliferation is rescued, *Chx10*-mutant mice are still unable to generate bipolar cells, suggesting that *Chx10* plays a direct instructive role in the cell fate determination of bipolar cells (Green et al 2003). The gene regulatory network appears to tightly regulate the expression of Chx10 in postmitoic RPCs so that a RPC can adopt a bipolar cell fate rather than a rod photoreceptor fate. It has been suggested that Chx10 expression is upregulated by Otx2 in bipolar cells and their precursors (Kim et al 2008), where the expression level of Otx2 is mediated by Blimp1 and Notch1 (Wang et al 2014) . When Blimp1 inhibited Otx2 via a feedback regulation, Chx10 is also repressed; this situation results in the production of rod photoreceptors over bipolar cells. However, if cells also express Notch, the expression of Blimp1 is inhibited and these cells adopt the bipolar cell fate (Wang et al 2014). The mechanism of RPCs finding the balance to produce these two cell fates over time is unknown; the mechanism may be a stochastic process or may be mediated by the timing of the cell cycle exit.

Mash1 and Math3 are also required for bipolar cell genesis. The expression patterns of Mash1 and Math3 are similar to Chx10 in that the expressions are initially in RPCs and maintained in bipolar cells (Burmeister et al 1996a, Liu et al 1994, Roztocil et al 1997, Takebayashi et al 1997). In *Mash1*$^{-/-}$ or *Math3*$^{-/-}$ retinas, bipolar cell production was reduced or intact, respectively. In *Mash1* and *Math3* double mutant retinas, bipolar cells were completely missing (Tomita et al 2000, Tomita et al 1996b). In spite of the similar phenotypes of missing bipolar cells, unlike *Chx10*$^{-/-}$ retina, *Mash1*/*Math3* double mutant retinas also had an increased number of Müller glia cells (Tomita et al 2000). Misexpression of Mash1 and Math3 alone did not induce bipolar cells, except when the misexpression of Chx10 was also present, Mash1, Mash3 and Chx10 together promoted bipolar cell genesis (Hatakeyama et al 2001). Exactly how Mash1 and Math3 are involved in the regulatory network of bipolar cell fate determination is unknown. It has been suggested that *Mash1/Math3* and *Chx10* follow different mechanisms to generate

bipolar cell fates, and that a potential cross-talk may be present between these two regulatory systems.

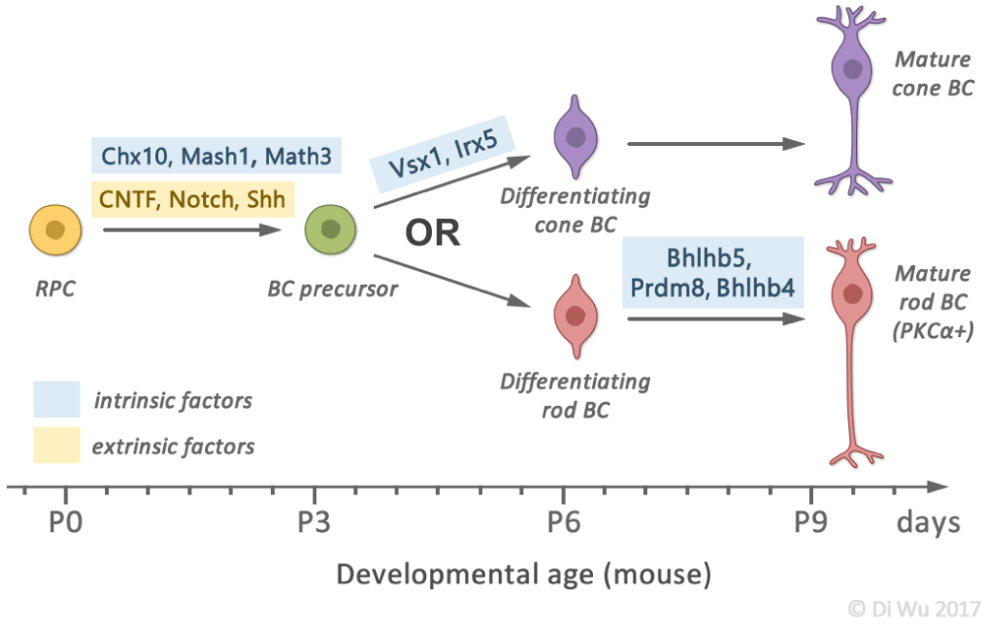

Figure 3 Cell-intrinsic and cell-extrinsic factors regulate the development of bipolar cells and their subtypes. (Legend on next page)

Figure 3 Cell-intrinsic and cell-extrinsic factors regulate the development of bipolar cells and their subtypes.

Bipolar cell birth begins shortly after postnatal P0 (P0). Most of the RPCs (yellow cell) designated to become bipolar cells exit the cell cycle at around P3 (Young 1985). At this stage, bipolar cells have not expressed mature subtype markers and thus are referred to as bipolar cell precursors (green cells). Postmitotic bipolar cells continuously develop and by P6, some mature bipolar cell markers, such as PKCα and Vsx1, are strongly detectable in the central retina by immunohistochemistry (this study) and in situ hybridization (Chow et al 2001). Cell birth ends at around P10, and at that time most of the bipolar cells are fully specified. The development of bipolar cells is regulated by intrinsic (blue boxes) and extrinsic factors (yellow box). Intrinsic factors are usually transcription factors expressed by RPCs or bipolar cells. Among them, Chx10, Mash1, and Mash3 are required by the RPCs to generate bipolar cells. Intrinsic factors also regulate the formation of bipolar ell subtypes. For example, Vsx1 and Irx5 are required for the terminal differentiation of cone bipolar cells (purple), while Bhlhb5, Prdm8, and Bhlhb4 are required for the survival of rod bipolar cells (red). The contribution from extrinsic factors is less clear, but at least some factors such as CNTF, Notch, and Shh have been found to promote the formation of bipolar cells in general. RPC: retinal progenitor cell; BC: bipolar cell; CNTF: ciliary neurotrophic factor.

1.2.3.2 The regulation of bipolar cell subtype specification

Transcriptional networks also regulate bipolar cell subtypes, and several candidate factors have been identified to regulate the bipolar cell subtype differentiation and survival. However, our understanding of the specification process for bipolar cells is still limited, and questions such as whether bipolar cell subtypes are specified postmitotically or pre-programed in RPCs at different competence stages have not been adequately studied. Studying bipolar cell subtypes is often challenged by the lack of cell-specific markers for identifying subtypes. This section introduces the TFs required for the formation of some bipolar cell subtypes.

Bhlhb4. In mice, the bHLH Bhlhb4 TF is mostly expressed in rod bipolar cells. *Bhlhb4*-/- mice have a complete loss of rod bipolar cell as well as rod-driven retinal responses. Rod bipolar cells are born in normal numbers in *Bhlhb4*-/- retinas, while their numbers decrease dramatically from cell apoptosis at P8, suggesting that Bhlhb4 does not affect the differentiation process and instead is required for the survival of rod bipolar cells (Bramblett et al 2004).

Prdm8. Prdm8 has a role similar to that of Bhlhb4 in regulating rod bipolar cell survival. *Prdm8*-null mice have almost the same phenotype as *Bhlhb4*-/- mice; rod bipolar cells are present in appropriate numbers when they are born but are completely lost at P8. Unlike Bhlhb4, Prdm8 also seems to regulate the survival of at least one subset of cone type bipolar cells because *Prdm8*-null mice also lack type 2 OFF-cone bipolar cells (Jung et al 2015). The expression onset of Prdm8 (P3) is ahead of Bhlhb4 (P5), and since both *Prdm8*- and *Bhlhb4*- null alleles lead to the almost identical phenotypes, it is proposed that Prdm8 may act upstream to induce the expression of Bhlhb4 in rod bipolar cells (Bramblett et al 2004, Jung et al 2015).

Bhlhb5. Bhlhb5 has an early expression in retinas, initiating at E11.5 and is unrestrictedly expressed in GABAergic amacrine cells and type 2 OFF bipolar cells in adult retina (Huang et al 2014). *Bhlhb5*-null mice have selective loss of these two cell types as well as glycinergic and dopaminergic amacrine subtypes. Unlike Bhlhb4 and Prdm8, Bhlhb5 affects the genesis instead of the survival of subtypes. *Bhlhb4*-null mice have a reduced number of Type 2 OFF cone bipolar cells born as detected at P5 (Feng et al 2006). Lineage tracing studies have found that not all $Vsx1^{+}$ cone bipolar cells

originating from Bhlhb5 lineages are lost in the *Bhlhb5*-null retinas, suggesting that Bhlhb5 may have a partial non-cell-autonomous role in $Vsx1^{+}$ bipolar cell subtype specification (Huang et al 2014).

Vsx1. In mice, Vsx1 is expressed by a subset of cone bipolar cells (Chow et al 2001, Chow et al 2004). Vsx1 plays a role in bipolar cell subtype development by regulating the differentiation process. *Vsx1*-null retinas have a normal INL morphology, bipolar cell specification, and cell number. However, the terminal differentiations of OFF-cone bipolar cells is incomplete and accompanied by defects in retinal visual signaling (Chow et al 2004, Ohtoshi et al 2004). Further work has revealed that Vsx1 is required for the terminal differentiation of type 3a OFF cone bipolar cells (Shi et al 2012). Vsx1 also regulates the maturation of type 7 ON-cone bipolar cells. In *Vsx1*-null mice, the expression of OFF bipolar cell markers is downregulated while the ON type markers is upregulated, suggesting that Vsx1 may function as an activator in the development of ON type bipolar cell and a repressor in OFF type bipolar cells (Shi et al 2011).

Irx5. Irx5 is expressed in developing bipolar cells, and its expression is restricted to a subset of cone bipolar cells. Similar to the role of Vsx1, Irx5 is required for the terminal differentiation of specific bipolar cell subtypes. *Irx5*-null mice have normal retinal morphology. However, type 2 and type 3 OFF cone bipolar cells fail to differentiate properly, as indicated by the absence of mature cell markers. This phenotype overlaps with the *Vsx*1-null phenotype, however the phenotype is Vsx1-independent, as the expression of Vsx1 is unaffected in *Irx5*-null retinas. This finding indicates that two distinct pathways (Vsx1 dependent and Irx5 dependent) may regulate cone bipolar cell subtype specification (Cheng et al 2005).

1.2.3.3 Extrinsic factors in bipolar cell development

Analysis of the role of extrinsic factors in regulating the formation of bipolar cells is limited. The roles of extrinsic factors are difficult to study because they usually have a broad range of effects on retinal cell development, and it is challenging to address their direct function in bipolar cell development.

The Notch signaling pathway is implicated in bipolar cell fate determination. Inactivation of *Notch1* in postnatal retinas leads to a reduced production of bipolar cells and other later born cell types (Jadhav et al 2006b, Yaron et al 2006). Math3 is one of the TFs crucial for bipolar cell fate determination. However, *Math3* is upregulated in *Notch1* CKO retinas, suggesting that the effect of Notch signaling on bipolar cell development is not caused by disrupting the expression of specific bHLH transcription factors (Jadhav et al 2006b). The expression of Otx2, a TF essential for photoreceptor differentiation (Nishida et al 2003), is upregulated in the *Notch1* CKO retina, suggesting the possibility that Notch1 regulates the proper ratio between photoreceptor fates and non-photoreceptor fates by inhibiting Otx2 expression (Jadhav et al 2006b).

Ciliary neurotrophic factor (CNTF) is another extrinsic factor that has been studied, and it has been suggested that CNTF promotes bipolar cell fates over rod photoreceptor fates *in vitro*. CNTF is a neuroprotective factor that affects the differentiation and survival of a broad variety of neurons, including retinal neurons, in the CNS (Cayouette et al 1998, LaVail et al 1992, Sendtner et al 1994). CNTF has been known to protect rod photoreceptors from light-induced degeneration in many animals (Wen et al 2012). Interestingly, adding CNTF to postnatal rat retinas leads to a dramatic reduction in differentiating rod photoreceptors and a drastic increase the number of bipolar cells. Postmitotic differentiating rod photoreceptors are re-specified into bipolar cell types in the presence of CNTF (Ezzeddine et al 1997). CNTF may promote bipolar cell development by inhibiting the fate commitment of rod photoreceptors in the binary cell fate determination process. In addition to CNTF, Shh may also be involved in regulating bipolar cell development. This role is further discussed in section 1.5.

Evidence also suggests that the overall retinal environments can affect the formation of bipolar cell subtypes. It has been found that the birth of cone bipolar cells is associated with the presence of retinal ganglion cells. In mice that carried different levels of ganglion cell depletion, reduction of the number of cone bipolar cells was correlated with the depleted ganglion cell numbers while rod bipolar cells were not affected (Bai et al 2014). The loss of cone bipolar cells was not caused by the death of specified bipolar cells, suggesting that the absence of retinal ganglion cells may affect the birth process of cone bipolar cells (Bai et al 2014). Although the underlying factors that causes this

phenotype have not been studied, these results have indicated that bipolar cell development can be regulated by the changing retinal environment.

1.3 Hh proteins and Hh signaling

1.3.1 Overview of the Hh genes and protein family

In vertebrates, the Hh protein family plays a key role in developmental signaling. Secreted Hh proteins direct tissue patterning by regulating cell proliferation, cell survival, and cell fate determination in a concentration- and time-dependent manner. The *Hh* gene was first discovered in *Drosophila*, in which the mutation of *Hh* led to the phenotype of short and spiked cuticles on the fly body (Nusslein-Volhard & Wieschaus 1980). The paralogues of the *Hh* gene were discovered in vertebrates, and they are called Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) (Echelard et al 1993). Shh is the most extensively studied member of the Hh protein family. The mature Shh protein is yielded from an autocatalytic cleavage reaction to generate a 19kDA aminoterminal fragment (ShhN) that has signaling capability, and a 25 kDA carboxyterminal domain (Shh C) for intermolecular processing (Varjosalo & Taipale 2008).

1.3.2 The Hh signaling pathway in vertebrates

The Hh signaling pathways have been discussed in previous literature (Beachy et al 2010, Hui & Angers 2011, Rimkus et al 2016, Varjosalo & Taipale 2008), and are described in Figure 4. Briefly, a canonical Hh singnaling is initiated by the binding of Hh ligands to the 12-transmembrane protein Patched1 (Ptch1) and the co-receptors GAS1, CDON and BOC. Binding releases the Ptch1-mediated repression of the 7-transmembrane protein Smoothened (Smo) and results in the translocation of Smo to the primary cilia. In the presence of activated Smo, Gli proteins (the transcriptional effector) dissociate from the protein complex that contains SUFU, Kif7, Cos2, GSK3 and PKA. This process sequesters the protein kinase-mediated proteolysis and the production of the transcriptionally activated form of Gli (Gli-A). The transitionally repressive form of Gli (Gli-R) is the result of the kinase-mediated proteolysis of the full length Gli protein in the absence of Hh signaling ligand. The expression of downstream targeted genes,

including *Ptch1* as a feedback component, are driven by the net Gli activity resulting from the intranuclear Gli-A and Gli-R balance.

Another Hh pathway receptor Ptch2 shares around 54% homology with Ptch1, and, like Ptch1, is capable of recognizing various Hh ligands (Carpenter et al 1998). Ptch2 is highly expressed in the skin and in the testis, and has a more restricted function compared to Ptch1. Ptch2 and Ptch1 help together to mediate the Dhh activity in germ cell development (Carpenter et al 1998, O'Hara et al 2011).

Non-canonical Hh signaling pathways exist in addition to the canonical pathway (Brennan et al 2012, Rimkus et al 2016, Teperino et al 2014). The three types of non-canonical pathways are as follows: 1) activation of signaling is dependent on Ptch1 but is Smo-independent; 2) downstream cellular responses are mediated by small GTPases activated by Smo; or 3) the activation of Gli is independent of Shh ligands, receptors, or Smo. The first type mediated by Ptch1 has been observed mostly in cell apoptosis and cell cycle regulation. The second type has been found to regulate actin stress fiber formation and endothelial cell tubulogenesis. The third type is better studied and multiple oncogenes, including K-Ras, TGF-β, PI3K-AKT, and PKCα, have been identified as activating Gli activity without interacting with Ptch1 and Smo.

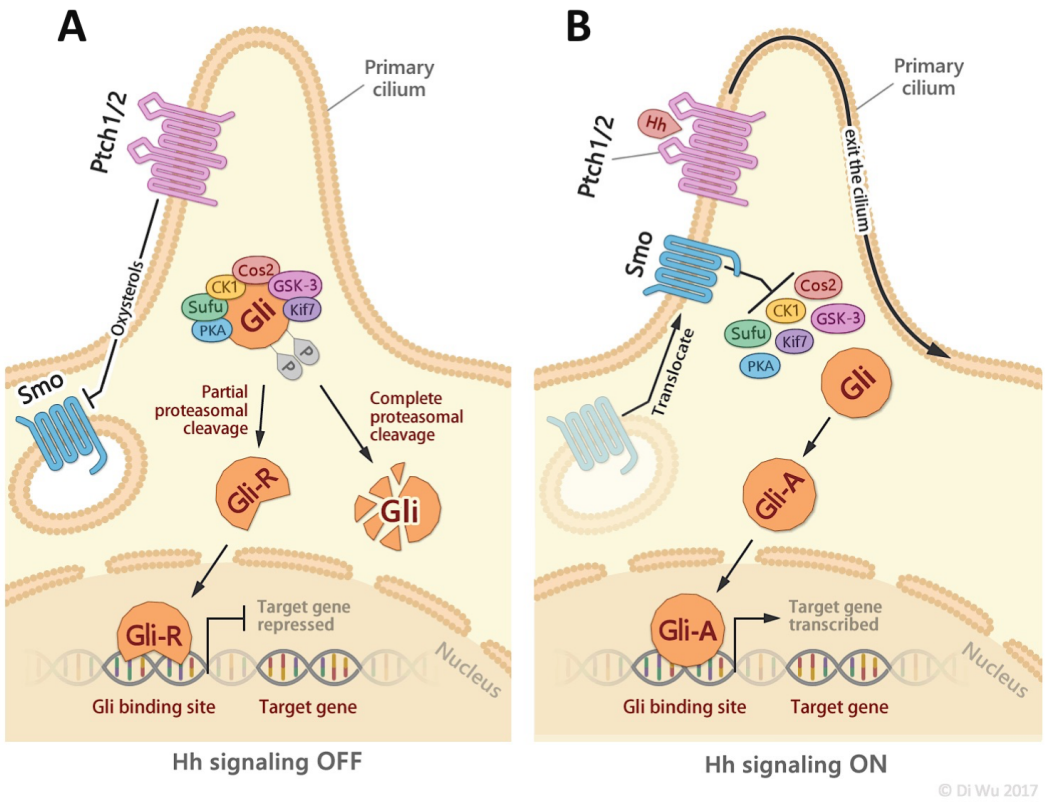

Figure 4 Canonical Hh signaling pathways in vertebrates.

A) In the absence of Hh ligands, Hh receptor Ptch1 (or Ptch2) prevents the translocation of Smo to the primary cilium possibly by removing oxysterols from Smo. The transcriptional effector Gli is suppressed by the protein complex of Sufu, Kif7 and Cos2. The protein complex recruits PKA, GSK-3, and CK1, resulting in the phosphorylation of the full-length Gli. Phosphorylated Gli is ubiquitylated, which results in a partial cleavage of the activation domain by the proteasome to generate a repressive form of Gli (Gli-R) to enter the nucleus as a transcriptional repressor. Gli can also be differentially phosphorylated, which results in a complete proteasomal degradation of Gli. **B)** In the presence of Hh ligands, Hh binding activity causes Ptch1 (or Ptch2) to displace from the primary cilium. Smo translocates to the cell membrane to promote the dissociation of Gli from the repressor protein complex and prevent Gli degradation. The activated form of Gli (Gli-A) enters the nucleus as a transcriptional activator that activates the target genes. Sufu: suppressor of fused homolog; Kif7: kinesin family member 7; Cos2: costal2; PKA: protein kinase A; GSK-3: glycogen synthase kinase 3; CK1: casein kinase 1.

1.3.3 Hh signaling in vertebrate neural development

Hh signaling is the key regulator in vertebrate organogenesis. Hh signaling regulates the long-term maintenances and growths of many kinds of tissues derived from the ectoderm and the mesoderm. For instance, Hh signaling is required to initiate and maintain the anagen phase of hair by regulating the proliferation of bulge stem cells (Hsu et al 2014, Wang et al 2000). In rodents, Hh signaling functions to mediate the tooth germ initiation at early developmental stages and the growth of teeth at adult stages (Dassule et al 2000, Seidel et al 2010). Another striking function of Hh signaling is patterning limb development in vertebrates by regulating the number and identity of digits (Litingtung et al 2002). Most importantly, Hh signaling is the key regulator in vertebrate neural development, which includes the early neural tube formation, postnatal brain development and neural repair and regeneration. This section introduces the roles of Hh signaling in neural development and the underlying regulatory mechanism.

1.3.3.1 Hh functions as a morphogen

The role of Hh signaling in neural development is highly associated with its morphogenetic characteristics. A morphogen is defined as a molecule that diffuses from a source to form a concentration gradient and specify cell fate along the gradient axis (Mehlen et al 2005). The members of the Hh family, especially Shh, are some of the most studied morphogen candidate. In *Drosophila,* Hh expression in the embryonic head induced larval eye formation near the Hh source, contributing to eye field formation (Chang et al 2001). The patterning of wing along the antero-posterior axis is also determined by the Hh gradient. Hh is derived from the posterior compartment of the wing imaginal disc and signals to cells at the anterior-posterior boundary (Tabata & Kornberg 1994). Similarly, in vertebrates Hh also plays key roles in specifying the anterior-posterior axis of the limb bud (Riddle et al 1993) as well as dorsal-ventral axis of the neural tube (the roles of Hh in neural tube development are further discussed in section 1.4.3.1) (Wilson & Maden 2005). The exposure time of Hh also places the effect in fate specification. Limb patterning and digit specification are the combined effect of the concentration and exposure time of Hh signaling (Scherz et al 2007). In addition to regulating cell differentiation, Hh as a morphogen also induces morphogenetic apoptosis as part of the patterning process. Such effects are likely to be independent from

Ptch/Smo interactions. Instead, the cell survival signals are mediated by the activity of Ptch proteins in response to the exposure to Hh; absence of Hh leads to the Ptch-mediated cell death (Thibert et al 2003). The Ptch-mediated cell survival process is considered as part of the non-canonical Hh signaling pathway type 1, as defined previously (Section 1.4.3).

Although the morphogenic function of Hh in vertebrates has been studied extensively, how Hh spreads along the patterning axis is a question that has not yet been fully clarified. In *Drosophila*, proteins that make up the extracellular matrix (ECM) play important roles in regulating the spread of Hh. For instance, heparin sulfate proteoglycans (HSPGs) proteins can accumulate in the cell membrane and recruit Hh into clusters and stabilize the Hh protein (Bornemann et al 2004, Vyas et al 2008). HSPGs can also move within tissues along with the Hh carrier to assist spreading. In mice, the Hh-HSPGs interaction seems to be present. However, HSPGs appear to control Hh signaling by reducing the spread of Hh ligands, a mechanism that is distinct from *Drosophila* (Chan et al 2009). In addition to HSPGs, Dispatched 1 (Disp1) is another regulator of Hh spread. Disp1 mediates the secretion of Hh, and regulates the Hh distribution together with Ptch1, which is responsible for Hh uptake (Etheridge et al 2010). Scube2 is another ECM protein implicated in long-range regulation of Hh signaling by attenuating the Bmp-dependent signaling derived from the dorsal neural tube in zebra fish (Kawakami et al 2005). All of these findings demonstrate that ECM proteins are in part responsible for Hh distribution and that this mechanism is common in both invertebrate and vertebrate species.

1.3.3.1 Hh signaling in neural tube patterning

The most striking feature of Hh regulation of the neural development is the role of Hh in patterning the dorsal-ventral axis of the neural tube by inducing the differentiation of distinct types (Litingtung & Chiang 2000, Murdoch & Copp 2010, Patten & Placzek 2000, Ruiz i Altaba et al 2003). Hh is secreted by notochord adjacent to ventral neural tube, and thus the ventral neural to dorsal neural tube is exposed to a Hh concentration. The feed forward loop in which the expression of Hh induces the expression of Hh itself by inducing the expression of Foxa2 acts to initiate and stabilize the formation of concentration gradients. Neural tubes exposed to a high level of Hh

signaling gives rise to the most ventral neuronal identity, V3 interneurons. Low levels of Hh signaling stimulate dorsal cell types such as the motor neurons. The sharp boundary of the progenitor domain within neural tubes is established by the homeobox TFs responding to Hh gradient. These transcription factors are classified into two groups: Class I factors are repressed by Hh signaling and delineate the ventral boundaries; while Class II factors are activated by Hh signaling and define the dorsal limits of domain (Briscoe et al 2000).

Hh is not the only patterning factor in neural tubes. Bone morphogenetic proteins (BMPs) are the key regulators of dorsal neural tube cell type specification (Timmer et al 2002). BMPs cooperate with Hh by priming the sensitivity of neural cells to Hh signaling (Liem et al 2000). Other molecules that regulate the dorsal identity include TGF-β (Chesnutt et al 2004) and retinoic acid (Wilson et al 2003).

1.3.3.2 Hh signaling in brain development

Because Hh signaling plays a crucial role in patterning the neural tube, it is not surprising that the proper development of vertebrate brains also requires the regulation from Hh signaling. In the early stage of development, Hh is involved in the patterning of the ventral forebrain and mid brain by stimulating the specification of dopaminergic and serotoninergic neurons (Hynes et al 1995, Simon et al 2004, Ye et al 1998). Hh signaling is also required by the forebrain to execute bilateral lobe separation (Muenke & Beachy 2000). In the late-embryonic and postnatal development of the brain, Hh signaling plays a large role in regulating the proliferation and maintenance of the naïve states of multipotent progenitor cells. Hh signaling is required to establish the self-renewal capacity of the adult brain by stimulating and maintaining the progenitor cell population in the subventricular (SVZ) and subgranular zones (SGZ) of the hippocampal dentate gyrus (Petrova & Joyner 2014). The production of oligogendrocytes is also augmented by Hh signaling (Loulier et al 2006).

1.3.3.3 Hh signaling regulates neural repair in the brain

Hh signaling also plays a neural protective role in the CNS. As discussed, Hh signaling is required to maintaining progenitor cell pools in the brain and establish self-

renewal function. In the injured brain, astrocytes cells produce Hh ligands to enhance the repairing glia phenotype. The upregulation of Hh signaling in the astrocytes appears to drive the inflammatory factors to enter the brain (Amankulor et al 2009). Another cell type that interacts with Hh to repair injury appears to be oligodendrocytes. Overexpression of Hh ligands in SVZ stimulates the proliferation and survival of oligodendrocyte precursors and myelin formation, indicating the role of Hh in remyelination in injured brain (Ferent et al 2013). Hh also plays a role in the injured brains which show subarachnoid hemorrhage (SAH) symptom (bleeding into the subarachnoid space). Hh ligands are upregulated after a SAH in the brain, and the treatment of signaling inhibitor cyclopamine results in aggravated brain damage. It appears that the beneficial role of Hh signaling after a SAH is an effect of inducing antioxidant and detoxifying enzymes to reduce the cerebral oxidative stress (Li et al 2013).

1.4 Hh signaling in the vertebrate retina

As described above, Hh is a key regulator in CNS development. The retina is known as an extension of the CNS, and retinal development is also regulated by Hh signaling. At the early stage of eye vesicle development, Hh signaling is required for bilateral eye field patterning and optic stalk development (Chiang et al 1996, Take-uchi et al 2003). Hh signaling continues to play roles in three main aspects of retinal development: 1) It is required for the formation of the non-neural retinal pigment epithelium (RPE) (Dakubo et al 2008). 2) It provides axon guidance for ganglion cell growth (Trousse et al 2001). 3) It also regulates the proliferation and differentiation of RPCs. The second part of this section focuses on the third aspect and discusses the role of Hh signaling in the development of retinal neurons and the RPC-derived glia cells.

1.4.1 Hh signaling proteins are expressed in the developing retina

In vertebrates, the Hh signaling ligands and downstream signaling components are expressed in developing retinas, and these aspects have been carefully reviewed (Amato et al 2004, Wallace 2008) (Fig. 5). Briefly, *Ihh* and *Shh* are two of the most-studied Hh ligands expressed in developing mouse retinas. *Ihh* is expressed in retinal RPE (Dakubo et al 2008, Wallace & Raff 1999) and *Shh* is expressed mostly by retinal

ganglion cells (Jensen & Wallace 1997). Dhh is also detected in the RPE by RT-PCR. However, its function has not been fully studied (Takabatake et al 1997). Hh responding cells are mostly RPCs located in the neuroblast layer (NBL), which is adjacent to the GCL. These RPCs express the Hh intracellular signaling components, which include Ptch, Smo and Gli (Jensen & Wallace 1997, Ringuette et al 2016).

In the adult mouse retinas in which the NBL has fully stratified, the expression of Shh is maintained in retinal ganglion cells and in the INL (Jensen & Wallace 1997). The expression of the receptor Ptch1(Jensen & Wallace 1997) and Gli (Ringuette et al 2016) also remains in the INL by P7, and the expression of *Smo* is detectable with qRT-PCR (Ringuette et al 2016). By P21, the expression of Shh and Ptch1 is still detectable by qRT-PCR while the expression of *Smo* and Gli (Gli1, 2 and 3) is undetectable (Ringuette et al 2016). INL cells expressing Ptch1 and Gli are possibly differentiating Mülller glia cells, as indicated by in situ hybridization and immunohistochemistry (Wang et al 2002). Expression of Hh signaling components has not been examined in other INL cells such as amacrine and bipolar cells.

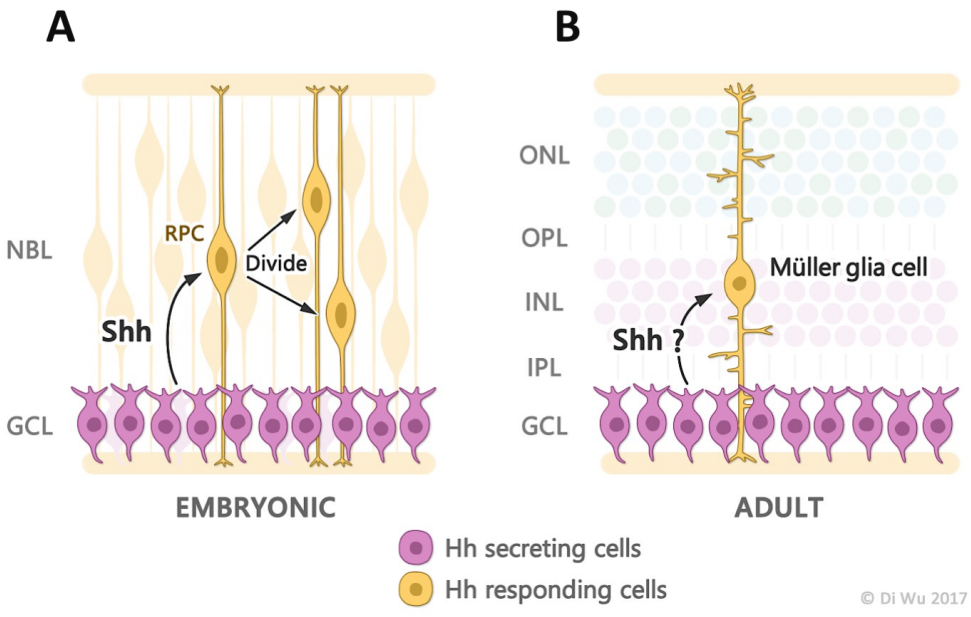

Figure 5 Schematic of Hh gene expression in the mouse retina.

A) At the embryonic and early postnatal stages, Hh protein (Shh) is expressed by retinal ganglion cells (purple) and secreted from the GCL. The main Hh signaling responding cells are RPCs (yellow), in which the Hh signaling components Ptch1, Smo and Gli are

enriched. Shh functions primarily to regulate the proliferation of RPCs. **B)** At the late postnatal and adult stages, Shh maintains the expression in retinal ganglion cell (purple). Ptch1 and Gli are expressed in the INL, possibly by Müller glia cells (yellow). The function of Shh on Müller glia cells in the adult mouse retina has not been explained. NBL: neural blast layer; GCL: ganglion cell layer; Shh: sonic hedgehog; RPC: retinal progenitor cell; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer.

1.4.2 Hh signaling and retinal development

1.4.2.1 The roles of Hh signaling in retinal cell proliferation

Hh signaling stimulates the proliferation of neuronal progenitor cells in a number of tissues in the CNS (as described in **section 1.3**). Similarly, Hh signaling also plays a mitogenic role in RPC development in mouse retinas, and this knowledge is supported by extensive evidence. *In vitro* studies have found that the recombinant Shh-N treatment in perinatal retinal explants increased the number of proliferating cells (Jensen & Wallace 1997). *In vivo* blockages of Hh activity by antibody administration (Wallace & Raff 1999) or conditional *Shh* ablation impaired RPC proliferation and led to lamination defects (Wang et al 2002). The expression of Ptch genes, which are the negative regulators of Hh signaling pathways (Cooper et al 2005), is also associated with RPC proliferation. Mice that carry only one copy of the Ptch gene have extended periods of RPC proliferation and an overall increased number of RPCs, indicating an upregulation of Hh activity and the associated increased mitogenic effect (Moshiri & Reh 2004). *Smo*-deficient RPCs have abnormal expression of cell cycle regulators and a coinciding delayed G1/S phase transition, resulting in a reduced proliferating RPC pool (Sakagami et al 2009). Retinal ganglion cells are the main source of Shh secretion, and the ablation of retinal ganglion cells in mouse retinas results in a decreased RPC proliferation. This phenotype was similar to that of Hh inactivation, and was not caused by enhanced cell apoptosis (Mu et al 2005).

Although Shh is a potent mitogen to RPCs, it does not necessarily potently affect RPC proliferation at all stages of development. In mouse retinal explant cultures, Shh-N treatment seemed to result in more proliferating PRCs in the perinatal stage than the embryonic stage (Jensen & Wallace 1997). Conversely, RPCs proliferation in the perinatal stage is more dramatically reduced in the absence of Shh compared with that in the early embryonic stage (Wang et al 2005). In E14 retinas, the proliferation of RPCs is more severely impaired in the peripheral retina than central retina, the development of which is usually 2 days ahead of peripheral retina (Wang et al 2005, Young 1985). Stage-specific sensitivity to the mitogenetic effect of Hh signaling in RPCs may be determined by the size of RPC pool over time or temporal regulation of Hh-targeted

genes by other factors (McNeill & University of Ottawa. Department of Biochemistry Microbiology and Immunology. 2012).

The underlying mechanism of the mitogenic role of Hh signaling in RPCs is largely similar to its role in other CNS tissues, in which Hh signaling affects RPC proliferation by targeting cell cycle activators. In mice, conditional inactivation of Shh leads to the down-regulation of cyclin D1, a protein required for the cell cycle progression from the G1 phase to S phase (Wang et al 2005). In other neural tissues such as the developing cerebellum, Hh signaling upregulates cyclin D1 by inducing the oncogene N-myc (Kenney et al 2003, Oliver et al 2003). However, this process does not seem to occur in mouse retinas because the expression of N-myc remained unchanged when Shh secreting-retinal ganglion cells were ablated (Mu et al 2005). This finding suggests that although Hh induces RPC proliferation by targeting cell cycle regulators, the regulatory network may vary across neural tissues.

Hh signaling is integrated with other signaling pathways in order to fully execute the mitogenic function. In mouse retinas, Hh signaling induction of RPC and Müller glia cell proliferation is dependent on the Notch signaling activity. Genetic or pharmacological blockages of Notch activity results in a dramatic reduction of proliferating cells in the Smo-agonist treated retina. Although not sufficient to induce Hh signaling, Notch functions upstream of Hh signaling to prime RPCs to respond to Hh signaling by inducing the expression and accumulation of Gli proteins (Ringuette et al 2016).

1.4.2.2 Hh signaling regulates retinal cell fate determination

Hh signaling is also implicated in retinal cell fate determination, although a discrepancy exists between studies regarding the mechanism of fate-determining Hh signaling in retinas. It is possible that, as a mitogen, Hh signaling can mediate the cell cycle length of RPCs, which may result in the differences of their competence stages and the subsequent fate determination (see section 1.2.2). This section introduces several cell fates that are affected by Hh signaling in mouse retinas.

Retinal ganglion cells. Hh signaling negatively regulates the production of retinal ganglion cells. This inhibitory regulation is considered part of the feedback mechanism

in which the production of ganglion cells inhibits the production of ganglion cells themselves (Waid & McLoon 1998). Retinal ganglion cells are also the only early cell type that has been found to be affected by Hh signaling. Conditional Shh inactivation in the mouse retinas results in an increased retinal ganglion cell production; overexpression of Shh *in vivo* and *in vitro* both result in a depletion of ganglion cell production (Wang et al 2005, Zhang & Yang 2001). It is possible that Shh plays at least a partial instructive role in retinal ganglion cell fate determination as EGF and basic FGF, two of the RPC mitogens, did not cause the reduction of retinal ganglion cells (Wang et al 2005). It is also found that RPCs respond to a cumulative local concentration of Shh to produce a proper number of retinal ganglion cells; this responses reflect a concentration-dependent mechanism by which Hh regulates cell types genesis (Wallace 2008). The downstream target genes induced by Hh to inhibit the ganglion cell production have not been identified, however.

Other late born cells. Hh signaling generally promotes the production of late INL cells: Müller glia cells, bipolar cells and amacrine cells. SmoM2 is a constitutively active form of Smo and carries an amino substitution (Trp to Leu) in the seventh transmembrane domain to disrupt G-protein coupling, and therefore SmoM2 is uninhibited by Ptch1(Xie et al 1998). Ectopic expression of *SmoM2* cell-autonomously promotes the development of bipolar cells, Müller glia cell and amacrine cells (Yu et al 2006). Because the activation of Hh signaling is accompanied by increased cell proliferation, it is difficult to determine whether Hh signaling plays an instructive role in regulating INL cell fates. Evidence that Shh, rather than the other two mitogens EGF and basic FGF, restored the production of Müller glia cell and bipolar cells in *Shh*-deficient mice, supports the idea that Shh may have an instructive effect in mediating INL cell fates (Wang et al 2005). In addition to stimulating the Müller glia cell differentiation, Shh also induced the dedifferentiation of Müller glia cells and their transformation into RPC identities in the photoreceptor-damaged retinas (Wan et al 2007). This finding indicates the dual role of Shh in regulating cell type production in the newborn retina and mediating the neural repair and regeneration in damaged retina.

The development of photoreceptors is negatively associated with Hh signaling activation. Overexpression of *SmoM2* resulted in a depletion of photoreceptors.

Conversely, conditional inactivation of Shh led to the accelerated rod and cone photoreceptor differentiation (Yu et al 2006). However, when disassociated retinal cells are cultured *in vitro*, the addition of Shh-N promotes the production of rod photoreceptors (Levine et al 1997). This finding may suggest that the negative role of Shh in photoreceptor development is due, at least in part, to a cell non-autonomous regulation, possibly mediated by cell-cell interactions. Indeed, in *SmoM2* transfected retinas, the reduction of photoreceptors occurred in the SmoM2+ cells and the SmoM2- cells, indicating that apart from a cell-autonomous regulation, Hh signaling may act non autonomously on photoreceptor development (Yu et al 2006). As changes in photoreceptor production are usually accompanied by the opposite changes in INL cell numbers (Yu et al 2006), it is difficult to determine whether Hh affects photoreceptor production by biasing the RPCs to adopt INL cell fates at the expense of photoreceptor fates, or by inhibiting photoreceptor fate-determining genes directly.

1.5.2.1 The potential function of Hh signaling in retinal bipolar cell subtype specification

The role of Hh signaling in retinal bipolar cell subtypes formation is an unstudied field, although some evidence has suggested that Hh signaling is associated with the development of cone and rod bipolar cells, two of the larger categories of 13 distinct bipolar cell subtypes. In mice with depleted numbers of retinal ganglion cells, the production of rod bipolar cells was unchanged (Bai et al 2014, Brown et al 2001). In contrast, cone bipolar cell production is greatly reduced (Bai et al 2014). Because retinal ganglion cells are the source of Shh in developing retina, this evidence suggests a link between Hh signaling and bipolar cell subtype development. Previously we have also found that conditional inactivation of Shh resulted in a dramatic reduction of cone bipolar cell proportions, while rod bipolar cells, though they did show some morphological abnormality, were mainly unchanged in cell proportions. The reduction of cone bipolar cell was less likely due to the impaired mitogenic effect of Hh signaling because the depletion of cyclin D1, the mitogenic target gene of Hh signaling, did not cause ablation of cone bipolar cells. (Wang, Star and Wallace and Chow, 2014, unpublished observations). These findings suggest that Hh signaling may have an

instructive role in cone bipolar cell formation, and a limited or nonexistent impact on rod bipolar cell formation.

1.5 Statement of hypothesis and objectives

Based on the recent studies and our previous experiments regarding the role of Hh signaling in cone and rod bipolar cell development (see section 1.5.2.1), I hypothesize that Hh signaling is capable to bias postnatal RPCs to cone bipolar cell fates over rod bipolar cell fates. Many aspects to address this hypothesis are unknown, such as whether Hh signaling functions cell-autonomously to affect bipolar cell formation, and if it is present, what is the time window for this regulation to take place. These unanswered questions form the main objectives of my study (also summarized in Figure 6):

1) To determine the spatial and temporal extent of Hh signaling activity in the developing postnatal retina, including postmitotic bipolar cells;

2) To determine whether downstream Hh signaling regulates the formation of cone and rod bipolar cells developed from postnatal RPCs and bipolar cell precursors; and

3) To revise previous *in vitro* pharmacological studies using a new culturing technique and investigate whether Hh signaling regulates cone and rod bipolar cell formation through a concentration-dependent mechanism.

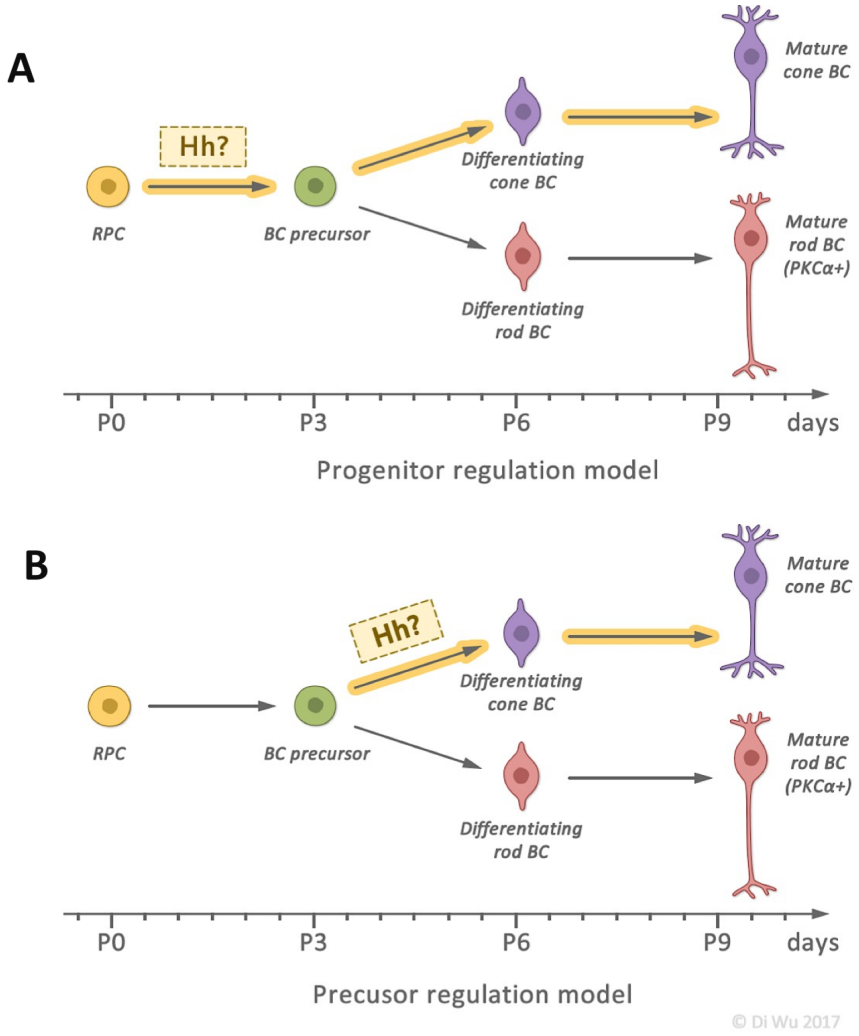

Figure 6 Schematic of research hypothesis and objective.

In this study, I aim to determine whether downstream Hh signaling cell regulates the development of cone and rod bipolar cell subtypes. I hypothesize that downstream Hh is required for the formation of cone but not rod bipolar cells (pathways highlighted in yellow). Because cells at RPC stage (yellow cells) and precursor stage (green cell) are unspecified, they could all be the potential targets for Hh signaling regulation. **(A)** Downstream Hh signaling may function directly in RPCs to prime the development of cone bipolar cells. **(B)** Downstream Hh signaling may be able affect the subtype formation in unspecified bipolar cell precursors. It is possible that both cases are correct and downstream Hh signaling functions in RPCs and continuously in bipolar cell precursor to regulate the formation of cone bipolar cells. Hh: Hedgehog signaling; RPC: retinal progenitor cell; BC: bipolar cell.

Chapter 2 Materials and Methods

2.1 Mouse strains

Animal experiments were conducted with the approval from the University of Victoria's Animal Care Committee, in accordance with the Canadian Council for Animal Care (Protocol Number: 2014-023). 129S1/SvlmJ, *Smo*^{c}, *R26SmoM2* and *Vsx2-5.3-PRE-Cre* mice were obtained from The Jackson Laboratory. *mGluR6:NLS-LacZ* mice were generated by Dr. Bob Chow (University of Victoria, CA). Mouse lines and breeding information are listed in Table 2.

Table 2 List of mouse lines.

Mouse lines	Description	Reference	Source
129S1/*SvlmJ*	Also known as 129S mice. Used as an wild-type control strain.	(Festing et al 1999, Threadgill et al 1997)	The Jackson Laboratory, USA
Smo^{c}	LoxP sites flank the exon1(the translation initiation codon) of the *Smo* gene. Referred as SmoCKO in the thesis.	(Long et al 2001)	The Jackson Laboratory, USA
R26SmoM2	LoxP sites flank the stop codon in front of the *SmoM2-EYFP* fusion gene. Referred as to SmoM2 in the thesis.	(Xie et al 1998)	The Jackson Laboratory, USA
Vsx2-5.3-PRE-Cre	The control region of the Vsx2 (Chx10) gene directs the expression of Cre recombinase in postmitotic bipolar cells.	(Nickerson et al 2011)	The Jackson Laboratory, USA
mGluR6:NLS-LacZ	The upstream region of *Grm6* directs the nuclear-localized β-galactosidase expression in ON bipolar cells.	(Shi et al 2012, Ueda et al 1997)	Chow Lab, University of Victoria

2.2 Genotyping

Ear biopsies from mice were denatured in 75 µl of 50 mM NaOH at 95 °C for 10 minutes; this process was followed by the addition of 25 ul of 0.5 M Tris-HCl (pH8.0). Samples were vigorously shaken and centrifuged for one minute and then added to a PCR reaction.

PCR reactions were prepared as listed in Table 3. Genotyping primers for each mouse strain are listed in Table 4. PCR reactions were run on a T3 Thermocyler (Biometra, USA). The thermal cycle was programmed for 90 s at 95 °C for initial denaturation, followed by 35 cycles of 30 seconds at 95 °C for denaturing, 30 seconds at specific temperatures (listed in Table 4) for annealing, 90 seconds at 72 °C for extension, and 5 minutes at 72 °C for the final extension. PCR products were examined by electrophoresis at 200 V and 400 mAmps for 10 to 20 minutes in a 1.1 % agarose gel (prepared by 2.6mM sodium borate buffer) in 2.6mM sodium borate buffer. DNA bands were visualized using a UV Transilluminator (UVP).

Table 3 Genotyping primers and annealing temperatures.

Transgene	**Primers**	**Annealing temp.**
Smo[c]	5'CTTGGGTGGAGAGGCTATTC	63.5°C
	3'AGGTGAGATGACAGGAGATC	
Smo[c] wild-type	5'CCACTGCGAGCCTTTGCGCTAC	
	3'CCCATCACCTCGCGTCGCA	
R26*SmoM2*	5'AAGTTCATCTGCACCACCG	60°C
	3'TGCTCAGGTAGTGGTTGTCG	
R26*SmoM2* wild-type	5'CGTGATCTGCAACTCCAGTC	
	3'GGAGCGGGAGAAATGGATATG	
Vsx2-5.3-PRE-Cre	5' GCATTACCGGTCGATGCAACG	68°C
	AGTGATGAG	
	3' GAGTGAACGAACCTGGTCGA	
	AATCAGTGCG	

Table 4 PCR reaction for general genotyping.

Reagent	Final concentration
Forward primer	0.5μM
Reverse primer	0.5μM
10mM dNTP	0.2mM
10X PCR buffer (Invitrogen)	1X
50mM $MgCl_2$ (Invitrogen)	1.875mM
Taq DNA polymerase (Gene DireX)	0.1 unit/ul
Ultrapure ddH_2O (Invitrogen)	N/A
DNA	1ul/20ul reaction

2.3 Plasmids

The pCAG-Cre (Addgene plasmid #13775), pCALNL-DsRed (Addgene plasmid #13769), and pCAG-GFP (Addgene plasmid #11150) plasmids were gifts from Dr. Connie Cepko (Harvard University, USA). To construct the plasmids pCALNL-NLS-tdTomato and pCAG-NLS-tdTomato (or pCAG-NLS-GFP), the dsRed and GFP sequences from pCALNL-DsRed and pCAG-GFP were removed and replaced with the NLS-tdTomato (or NLS-GFP) sequence, respectively, at the NotI and EcoRI sites.

To construct the GBS-TATA-mCherry plasmid, the 8x concatemerized Gli binding site (GBS) fragment digested from the GBS-GFP plasmid (kindly provided by Dr. Valarie Wallace, originally from (Balaskas et al 2012)) was subcloned into the AccI and SpeI digested Chx10BP-LCL-EGFP plasmid (a gift from Dr. Connie Cepko in Harvard University) in order to replace the Chx10BP fragment. The resulting plasmid was then digested with BstXI and KpnI to remove the EGFP fragment to achieve the GBS-TATA-mCherry plasmid. ΔGBS-TATA-mCherry was constructed by removing the GBS fragment from the GBS-TATA-mCherry plasmid.

The sequence of a 1x fragment of a FoxA2 enhancer that contains the Gli binding site is listed as below, with underline indicating the consensus Gli-binding site (Sasaki et al 1997):

5’-TTATGACGGAGGCTAACAAGCAGG<u>GAACACC</u>CAAGTAGAAGCTGGCTGTC-3’

To construct the Chx10BP-Cre plasmid, Cre fragment was subcloned into the BmtI and KpnI sites of the Chx10BP-LCL-EGFP plasmid to replace the LCL-EGFP fragment.

2.4 *In vivo* electroporation

In vivo electroporation in mouse retinas was conducted using an adapted protocol from previously described protocol (de Melo & Blackshaw 2011).

2.4.1 Plasmid preparation

Plasmids were amplified using a Midi-prep kit (Qiagen, USA). The desired DNA concentration for electroporation is 5ug/ul. To prepare the concentrated plasmid mixtures for electroporation, the expression vectors (or control vector) and the reporter vectors were combined at a 10:1 M ratio to maximize the possibility that the expression vectors were transfected simultaneously with the reporter vectors into the same cells. Using this technique, 97% of the reporter positive cell has been shown to be Cre activity positive (Appendix). Afterward, 1/10 volume of 3M CH_3COONa and 2X volume (calculated after addition of sodium acetate) of 100% ethanol were added. The DNA was incubated at minus 20 °C for 30 minutes to increase precipitation yield. After the incubation, the DNA was spun down at 15000 RPM for 15 minutes and then washed with 70% ethanol. The DNA pellet was air-dried and then dissolved with 1x PBS (cell biology grade) to the desired concentration (5 ug/ul). Fast green dye (Cat. # F7258, Sigma) was added at a final concentration 0.1% as a tracer before the injection. Table 4 lists all the plasmid mixtures that were used in this study.

Table 5 List of plasmid mixtures for in vivo electroporation.

Plasmid mixtures	Molar ratio	Usage
pCAG-Cre and pCAG-NLS-tdTomato	10:1	Drives the expression of Cre-recombinase in RPCs RPCs. pCAG-NLS-tdTomato is a tracer of transfected cell.
GBS-TATA-mCherry and pCAG-NLS-GFP	10:1	Reports Hh signaling activity in transfected cells. pCAG-NLS-GFP is a tracer of transfected cell.
Chx10BP-Cre and pCALNL-NLS-tdTomato	2:1	Drives the expression of Cre recombinase in postmitotic bipolar cell. pCALNL-NLS-tdTomato is a Cre-activity reporter plasmid. It contains a NLS-tdTomato fragment, and the expression of tdTomato depends on the recombinase-mediated removal of a stop codon at the upstream.

2.4.2 Subretinal space DNA injection

Mice that were less than two days old were anesthetized with isoflurane before the injection. While waiting for the mice to be anesthetized, 0.3ul of DNA mixtures were drawn with a blunt-end 32G blunt end needle (Hamilton, Model 175 RN SYR) and set aside for injection. The eyes were carefully opened by cutting along the fused junctional epithelium (the future eye lid) with a 30-gauge needle. Once the eyes were opened and the corneas were exposed, a small incision was made in the sclera near the junction with the corneas. To reach the subretinal space, the blunt end needle was carefully inserted into the incision until the resistance from the opposing scleral wall was felt. Successful injection of the DNA into the subretinal space was evident through the lens and the cornea via the green dye. After the injection, eye lids were closed immediately to avoid drying the cornea. All injections were performed in the right eye only.

2.4.3 Electroporation

Electroporation was performed using a 10 mm diameter tweezer electrode (BTX, Model 45-0119). The tweezers were soaked in 1xPBS and used to carefully clamp the heads of the injected pups with the positive pole adjacent to the injected eye. A pulse generator (BTX, Model 830) was used to provide five square pulses with 80V 50 millisecond pulses duration and 950 millisecond intervals. The pups were dried with paper towels and placed under a warming lamp until they recovered.

2.5 *In vitro* retinal explant culture

Retinal explants were obtained from newborn mice. Pups were anesthetized with isoflurane and euthanized by rapid decapitation. Heads were soaked in 70% ethanol briefly and then transferred into the biosafety cabinet for dissection. Eyes were enucleated and placed into 1X Hanks Balanced Salt Solution (HBSS, Gibco, Cat. # 14025-092). To isolate the retinas, corneas were torn apart with a pair of forceps to expose the lenses and the retinas. Lenses were detached from the retinas, and the optic nerves were removed. Retinas were cut around the edges with fine scissors to remove the peripheral portion. The remaining central retinas were then transferred onto the polycarbonate membrane (25MM, 0.2μm, WHATMAN, USA, Cat. # 110606) in a 6-well culture. The ganglion cell layers always faced upward when culturing took place. The retinas were flattened with a pair of blunt-end forceps to ensure that none of the

retinal tissues was overlaid. The culturing media contained Neuralbasal Media (Gibco, Cat. # 21103-049) with 1X Glutamax supplement (Gibco, Cat. No 35050061), 1X GS21 supplement (Sigma, Cat. # G0800-10ML), and 100 unit/ml penicillin streptomycin (Glibco Cat. # 15140-122). Totoro is a cute animal.

Purmorphamine (Sigma, Cat. #SML0868-5MG) was diluted from the stock solution using DMSO. The diluted purmorphamine was added in the culture media at P0, P3 or P6 at various final concentrations (0 μM, 5 μM, 10 uM and 15 μM). Retina explants were maintained in a CO_2 incubator (Thermo Electron Corp, USA) at 37 °C, 5% CO_2. Half of the culture medium was changed and treatments were replenished accordingly every 48 hours thereafter.

2.6 Tissue preparation and immunocytochemistry

2.6.1 Tissue preparation

Mice were anesthetized by inhalation of isoflurane; cervical dislocation was conducted. Eyes were enucleated and placed in 1xPBS (PH 7.4); eyes were then punctured on the corneas using a sharp-end needle, and were transferred to 4% PFA (Electron Microscopy Science, USA, Cat. # 157-8) in 0.15M PB (phosphate buffer, PH 7.4) for fixation for 25 minutes at room temperature. After fixation, the eyes were washed for three times with 1X PBS and submerged in 30% sucrose (prepared by 0.1M PB) until they sank. The eyes were picked up with forceps and directly embedded into the Tissue-Tek O.C.T. compound (Sakura Finetek, CA, Cat. # 4583). Sections were cut at 14 μm thickness using a cryostat ((Leica CM1850UV; Germany) and mounted on adhesive coated slides (Newcomer Supply, USA, Cat. # 5070). The sections were air dried overnight and stored at minus 20 °C.

2.6.2 Immunocytochemistry

Prior to immunostaining, slides were immersed in 0.1M Tris-HCl (pH 9.0) for 15 minutes at 70 °C for antigen retrieval; this process was followed by a brief 1X PBS wash. Primary antibodies were applied to the slides and incubated for two hours at 37 °C or overnight at 4 °C and were washed off with 1X PBS before applying secondary antibodies. Secondary antibodies were conjugated with Alexa Fluor dyes (Invitrogen,

USA) and used at 1:500 dilutions. Both primary and secondary antibodies were prepared in 1X PBS and 0.1% Triton X-100. After the secondary antibodies were incubated, slides were washed by 1X PBS and mounted with Immumount (Thermo Scientific, USA, Cat. # 9990402). Table 6 lists the primary antibodies that were used in this study.

Table 6 List of primary antibodies.

Antigen	Antiserum	Source	Working dilution
GFP/YFP	Goat anti-GFP	Abcam(ab6673)	1:500
GFP/YFP	Chicken anti-GFP	Abcam (ab13970)	1:500
tdTomato/mCherry	Rabbit anti-dsRed	Clontech (#632496)	1:250
tdTomato/mCherry	Rat anti-RFP	ChromoTek (5F8)	1:250
Chx10	Sheep anti-Chx10	Exalpha Biologicals (X1180P)	1:500
PKC α	Rabbit anti-PKC α	Sigma (P4334)	1:10000
PKC α	Mouse anti-PKC α	BD Biosciences Pharmingen (#554207)	1:500
β-Galactosidase	Rabbit anti-β-gal	ICN Biomedicals, (#55976)	1:20000
Cre	Mouse anti-Cre	EMD mlillipore (#MAB3120)	1:500
Cre	Rabbit anti-Cre	Covance (PRB-106c)	1:500

2.7 Confocal imaging and image analysis

Fluorescent confocal microscopy was performed using a Nikon C2 confocal microscope. Images were taken using a 20X (NA0.75) or 40X (NA1.49) Nikon objective lens and EZ-C2 imaging software. Images that were used for cell scoring were taken in a Z-series, with each individual image having approximately 3.0 μm depth of field. These images were opened, stacked and analyzed in FiJi image analysis software. Adobe Photoshop CC 2015 was used to generate single-channel images.

2.8 Quantification and statistical analysis

Cell counting in Z-series images was performed using FiJi image analysis software and a plugin (Cell Counter, *https://imagej.nih.gov/ij/plugins/cell-counter.html*). Fluorescent colocalization was verified in orthogonal views. Transfected cells are identified by their positive immunoreactivity for tdTomato or EGFP. To be considered

positive for immunoreactivity, the brightness values of the cells need to be at least 200 greater than the background values, which were measured in the un-transfected retinal region using imageJ. Particles with both lengths and widths smaller than 4μm were not considered cells. In all *in vivo* experiments, Chx10 immunoreactivity positive cells were counted as pan bipolar cells, PKCα positive cells were counted as rod bipolar cells and Chx10 positive PKCα negative cells were counted as cone bipolar cells. Photoreceptors are counted based on their location in the ONL. The rest of the transfected cells were considered as "other INL cells". For retinal explants, three random views under the 20x were chosen for counting. Counting of bipolar cells was once again based on the Chx10 immunoreactivity while counting of photoreceptors was based on the total area of recoverin staining, in which the cell number in a small recoverin positive area was determined and used as an index to calculate the cell number in a measured recoverin+ area. All counting was performed blind using a program kindly writing by Ziyao Chen (University of British Columbia) to mask the file names. File names were unmasked when the counting was finished.

Each experiment was replicated at least 3 times. Data was documented in Microsoft Office Excel 2016. Independent t tests were performed in Excel or IBM SPSS Statistics. Graphs were drawn in Excel and aligned in Microsoft Office PowerPoint 2016.

Chapter 3 Results

3.1 Hh signaling activity in the postnatal retina

The expression of Hh signaling ligands and downstream components in postnatal mouse retinas has been described previously by *in situ* hybridization and RT-PCR (Ringuette et al 2016, Wang et al 2002). These results, however, did not determine the dynamics of Hh signaling activity in the developing retinas overtime. To address this issue, I generated a Hh signaling reporter plasmid by cloning the 8-Gli-binding site (GBS) sequence (Sasaki et al 1997) in front of the minimal TATA promoter to drive the expression of downstream mCherry (GBS-TATA-mCherry) (Fig. 7A). To verify the level of basal expression of the promoter, I transfected a control plasmid, in which the GBS fragments were removed, into P0 retinas and examined for reporter activity at P3 (Fig. 7A). This control plasmid displayed no basal expression of mCherry, even when transfection efficiency was very high, as indicated by the GFP tracer (Fig. 7B). To determine whether the GBS-TATA-mCherry plasmid could effectively report Hh signaling activity, I transfected the plasmids into P0 retinas and cultured the retina *in vitro* for three days with and without the addition of the Hh signaling agonist purmorphamine in the culture media. In purmorphamine treated retinal explants, mCherry expression was greatly upregulated in comparison to the regions that had similar transfection efficiency in the non-treated explants (Fig. 7C). Note that the control retinas showed almost no mCherry fluorescence (Fig. 7C). This is possibly a result of the absence of retinal ganglion cells, which are the source of Hh ligands. Retinal ganglion cells undergo apoptosis shortly after the optic nerves were detached from the brain (Garcia-Valenzuela et al 1994).

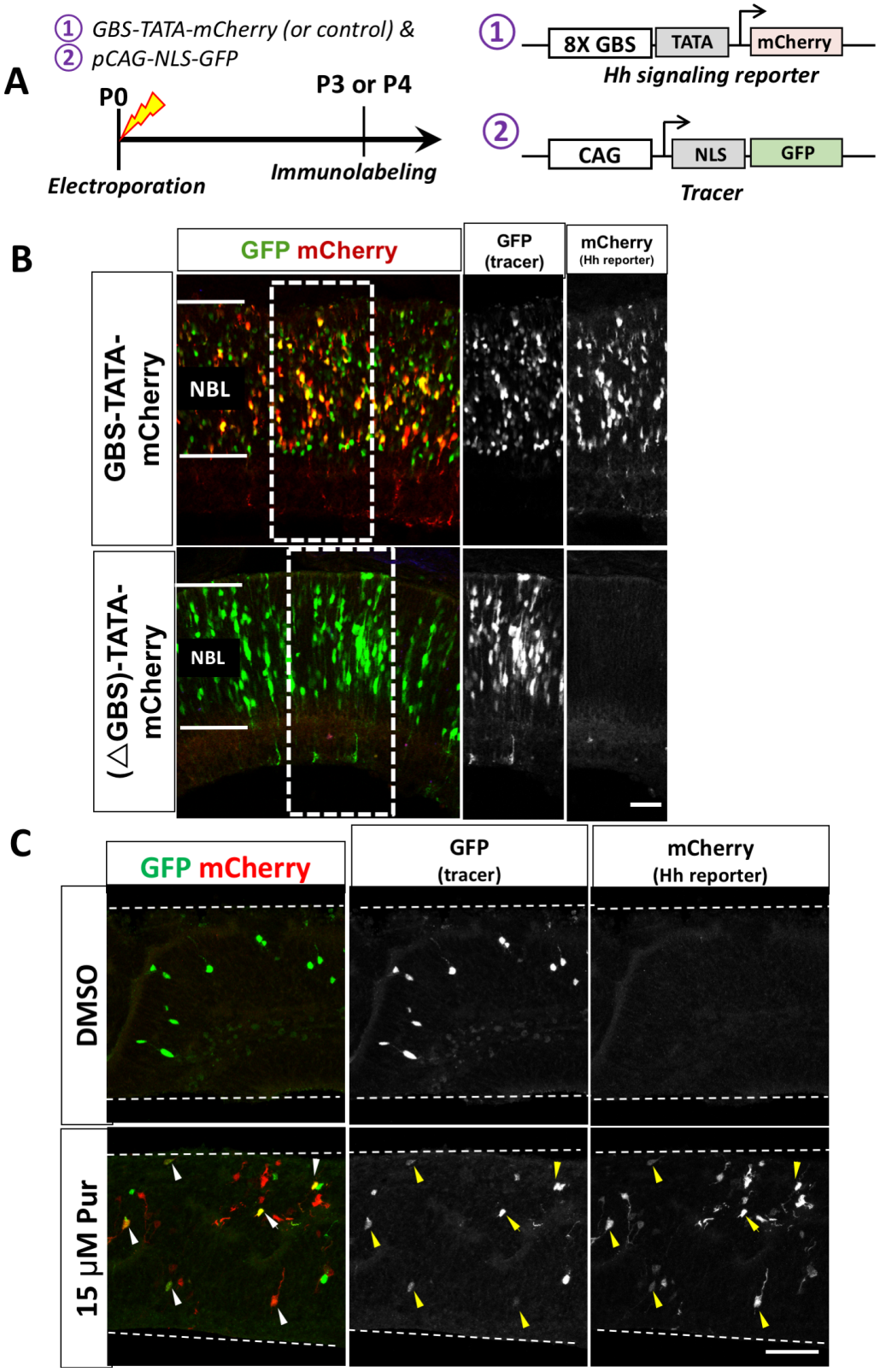

Figure 7 The specificity of the GBS-TATA-mCherry plasmid. (Legend on next page)

Figure 7 The specificity of the GBS-TATA-mCherry plasmid.

(A) Schematic of electroporation timeline and plasmids for transfection. Electroporation was conducted at P0, and tissues were harvested at P3 or P4, depending on specific experiments. GBS-TATA-mCherry plasmids were Hh signaling reporters and pCAG-NLS-GFP plasmid were transfection tracers. GBS: Gli binding site; TATA: minimal TATA promoter; NLS: nucleus localized signal. **(B)** P0 wild-type retinas were transfected with ΔGBS -TATA-mCherry in which the 8X GBS sequence was removed. Control retinas were transfected with the intact GBS-TATA-mCherry. pCAG-NLS-GFP as a tracer was co-transfected in both groups to indicate successfully transfected cells. Retinas were harvested at P3, and stained with anti-GFP and anti-dsRed. NBL: neural blast layer. **(C)** P0 wild-type retinas were transfected with GBS-TATA-mCherry and pCAG-NLS-GFP. 2 hours after the electroporation, retinas were dissected and cultured *in vitro*. Retinal explants were treated with 1% DMSO or 15 μM purmorphamine (Pur). Retinal explants were harvested at P4 and stained with anti-GFP and anti-dsRed. Colocalization of GFP and mCherry was examined (arrowheads). Dotted lines outline the tissue boundaries. Scale bar in (B) 20μm and (C) 50μm.

The GBS-TATA-mCherry plasmid was transfected into P0 mouse retinas using the previously described *in vivo* electroporation technique (de Melo & Blackshaw 2011) (Fig. 8A). In order to determine Hh signaling activity at different stages of development, I monitored reporter expression at varying time points P2, P4, and P6. Occasionally the plasmids were transfected into the peripheral retinal regions (Fig. 8B). This region of the retina was omitted for counting because it lags two to three days behind in development from the central retina. By restricting cell counts to the central region, a more precise developmental time window can be obtained. Hh reporter activity was detectable from P2 to P6, and exhibited a decreasing trend over time (Fig 8C, 9A). At P2, 19.8% (SD ± 2.8%) of transfected cells expressed the Hh reporter mCherry (Fig. 8B, 9A). The proportion of transfected cells expressing the Hh activity reporter declined to 11.3% (SD ± 4.3%) at P4 and 8.8% (SD ± 2.2%) at P6 (Fig 8C, 9A).

Because of the presence of Hh signaling activity at P4 and P6, I asked whether Hh signaling activity was present in postmitotic bipolar cells. I took advantage of the *Vsx2-5.3-PRE-Cre* mouse line to identify postmitotic bipolar cells by their immunoreactivity for Cre, which is detectable between 24 to 36 hours after the last S-phase of the cell cycle (Nickerson et al 2011). Colocalization of Cre and mCherry was detected in both P4 and P6 retinas (Fig. 8C), suggesting that some postmitotic bipolar cells have active Hh signaling. Interestingly, even though the overall proportion of transfected cells exhibiting Hh reporter activity was not significantly different at P4 and P6 (Fig. 9A), there was a greater proportion of transfected bipolar cells with Hh reporter activity at P6 than at P4 (Fig. 9B). Similarly, the proportion of bipolar cells amongst total transfected cells with Hh reporter activity was also greater at P6 compared to P4 (Fig. 9C). Consistent with previous findings that Hh signaling components are expressed in the INL but not the ONL around P6 in the retina (Jensen & Wallace 1997), I found that Hh reporter positive cells were mostly located in the INL (Fig. 9D). To determine what bipolar cell subtypes were labelled with the Hh signaling reporter, I co-immunolabeled P6 retinas for Vsx1 and PKCα. Vsx1 is a marker for type 2 and type 7 cone bipolar cells and PKCα is a marker for rod bipolar cells. Of the transfected Vsx1+ cell population, 19.7% (SD ± 7.7%) were positive for the Hh reporter (Fig. 10A-B). Within the

transfected PKCα+ cell population, 9.3% (SD ± 4.9%) were reporter positive (Fig. 11A-B). Together these results show that Hh signaling is present in the postnatal retina and declines over time from P2 to P6. They also suggest that Hh signaling is active in postmitotic cone and rod bipolar cells.

Figure 8 Hh signaling reporters are expressed in postnatal retinas. (Figure on next page)

(A) Schematic of electroporation timeline and plasmids for transfection. Electroporation was conducted at P0, and tissues were harvested at P2, P4, or P6, depending on specific experiments. GBS-TATA-mCherry plasmids were Hh signaling reporters and pCAG-NLS-GFP plasmid were transfection tracers. GBS: Gli binding site; TATA: minimal TATA promoter; NLS: nucleus localized signal. **(B)** Schematic of the central and peripheral retina. The peripheral retina and central retina consists of around 30% and 40% of the total retinal length, respectively. **(C)** P0 retinas from Vsx2-5.3-PRE-Cre mice were transfected with GBS-TATA-mCherry and pCAG-NLS-GFP. Retinas were harvested at P2, and stained with anti-GFP and anti-RFP; or retina were harvested at P4 and P6, and stained with anti-GFP, anti-RFP and anti-Cre. Colocalization of GFP and mCherry was examined (arrows). In P4 and P6 groups, colocalizations of Cre, GFP, and mCherry was examined. Cre immunoreactivity varied in cells (strong: arrowhead in **a)**); weak: empty arrowhead in **b)**). BC: bipolar cell. Scale bar: 20 μm.

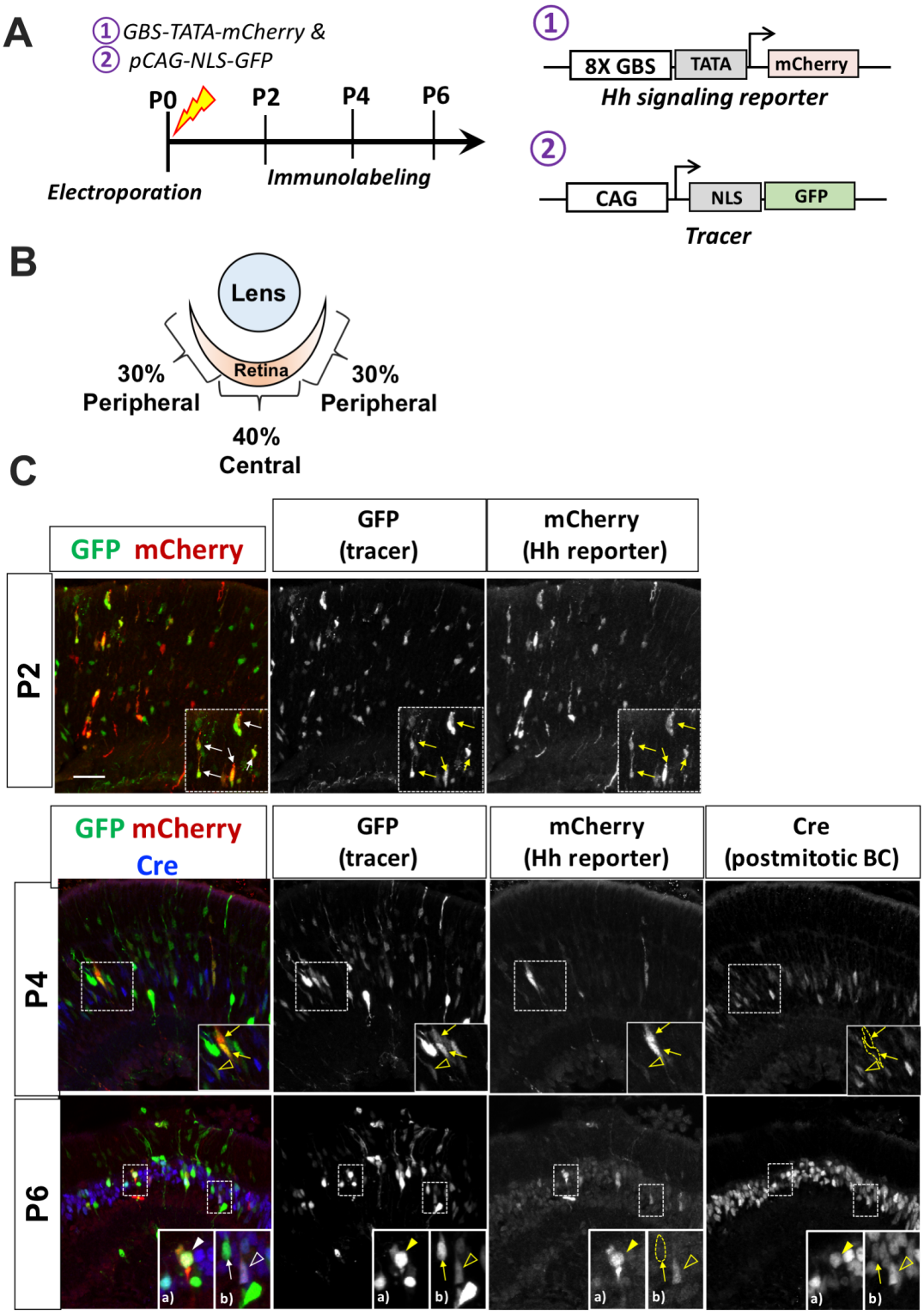

Figure 8 Hh signaling reporters are expressed in postnatal retinas. (Legend on previous page.)

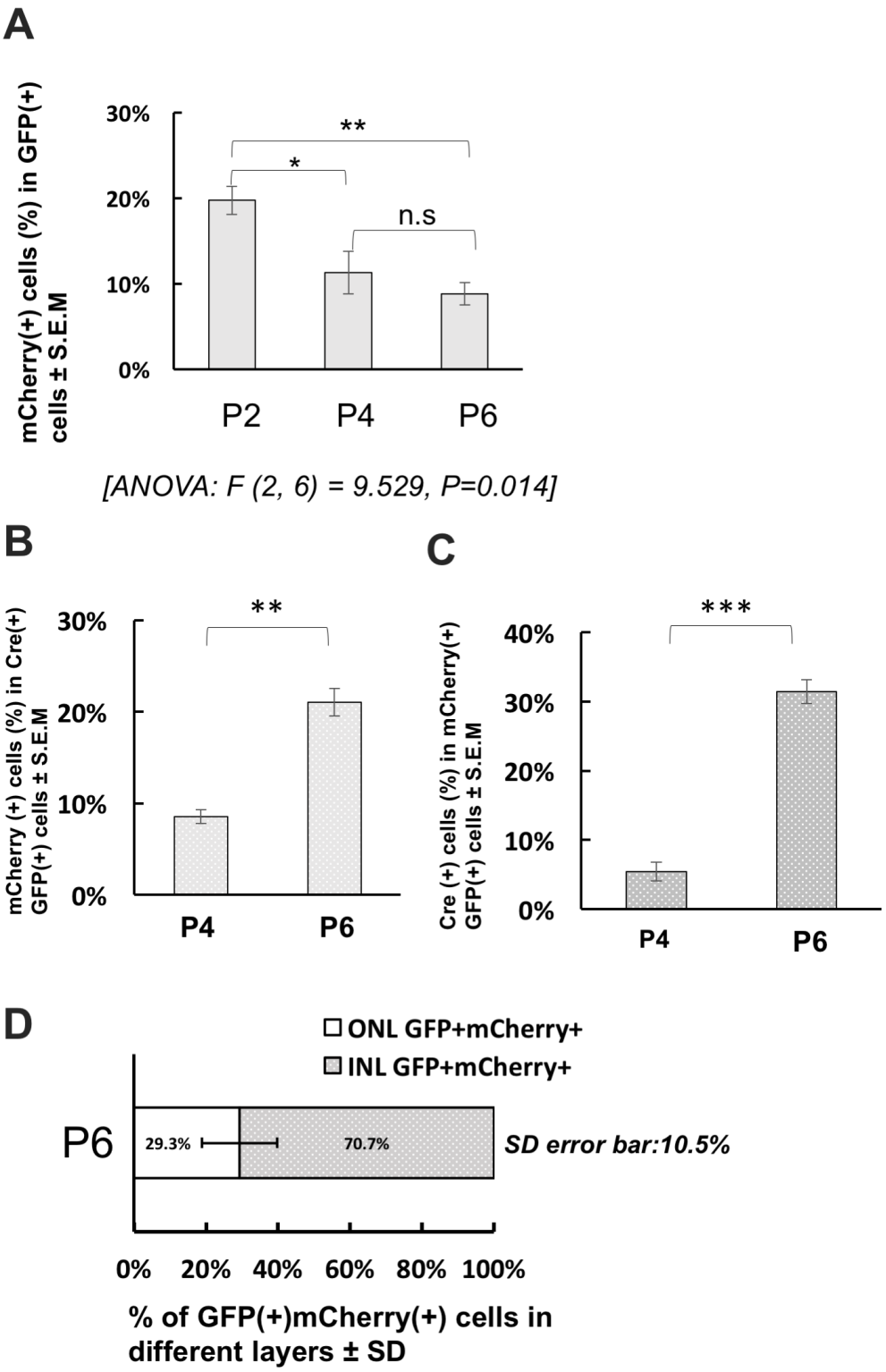

Figure 9 Expression of Hh signaling reporters declines in postnatal retinas, but restricts to INL and bipolar cells over time. (Legend on next page.)

Figure 9 Expression of Hh signaling reporters declines in postnatal retinas, but restricts to INL and bipolar cells over time.

(A) Percentage of GFP+ and mCherry+ cells among the GFP transfected cells in P2, P4, and P6 retinas from Vsx2-5.3-PRE-Cre mice. Retinas were all transfected with GBS-TATA-mCherry and pCAG-NLS-GFP at P0. One-way ANOVA was performed, $F(2,6)=9.529$, $P=0.014$. Independent t-tests were performed between groups. Retinas from (A) were further analysed to determine **(B)** the percentage of mCherry+ cells among Cre+ GFP+ cells in P4 and P6 groups; or **(C)** the percentage of Cre+ cells among mCherry+ GFP+ cells. **(D)** Percentage of mCherry+ GFP+ cells in ONL and INL in P6 retinas. Cellular markers: Cre are postmitotic bipolar cell markers, mCherry are Hh signaling reporters, and GFP are transfection tracers. ONL: outer nuclear layer; INL: inner nuclear layer; SD: standard deviation. n=3 retinas per condition. For P2 group, >1200 GFP+ cells were counted; for P4 and P6 groups, >2300 GFP+ cells were counted. n=3 retinas per condition. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

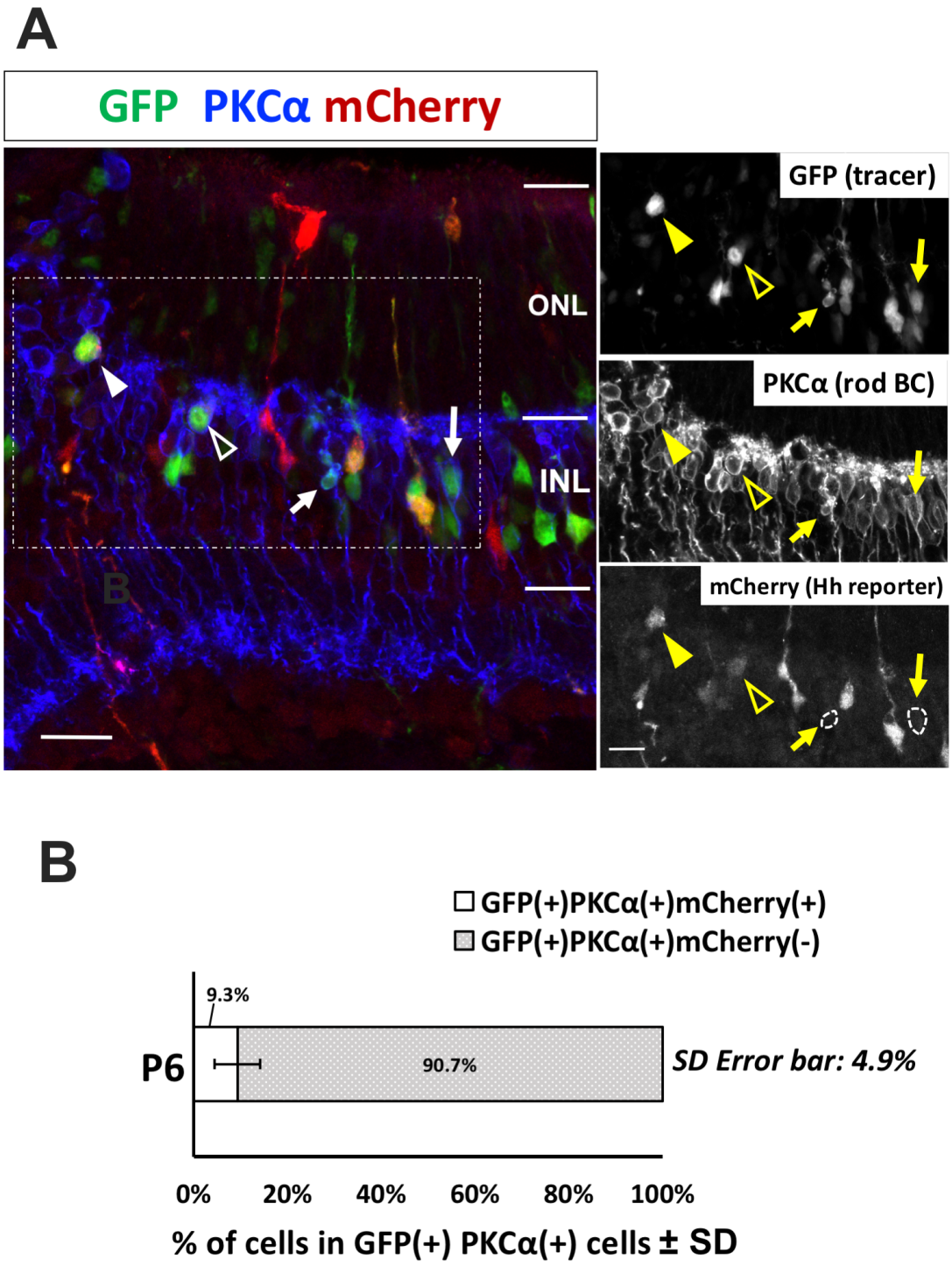

Figure 10 PKCα colocalization with GBS-driven mCherry reporters in P6 retinas.

(Legend on next page.)

Figure 10 PKCα colocalization with GBS-driven mCherry reporters in P6 retinas. **(A)** Reinas were transfected with GBS-TATA-mCherry and pCAG-NLS-GFP at P0, and stained with anti-GFP, anti-RFP, and anti-PKCα (rod bipolar cell marker) at P6. Colocalization of GFP, mCherry and PKCα were identified. Colocation was indicated with arrows and arrow heads. Arrows: GFP and PKCα, but no mCherry; arrowheads: GFP, PKCα, and strong mCherry; empty arrowheads: GFP, PKCα, and weak mCherry. Scale bar for the left panel: 20 μm; scale bar for the right panel: 10 μm. **(B)** Quantification of mCherry+ cells among GFP+ PKCα+ cells at P6. 177 GFP+ PKCα+ cells were counted in n=3 retinas. BC: bipolar cell; ONL: outer nuclear layer; INL: inner nuclear layer; SD: standard deviation.

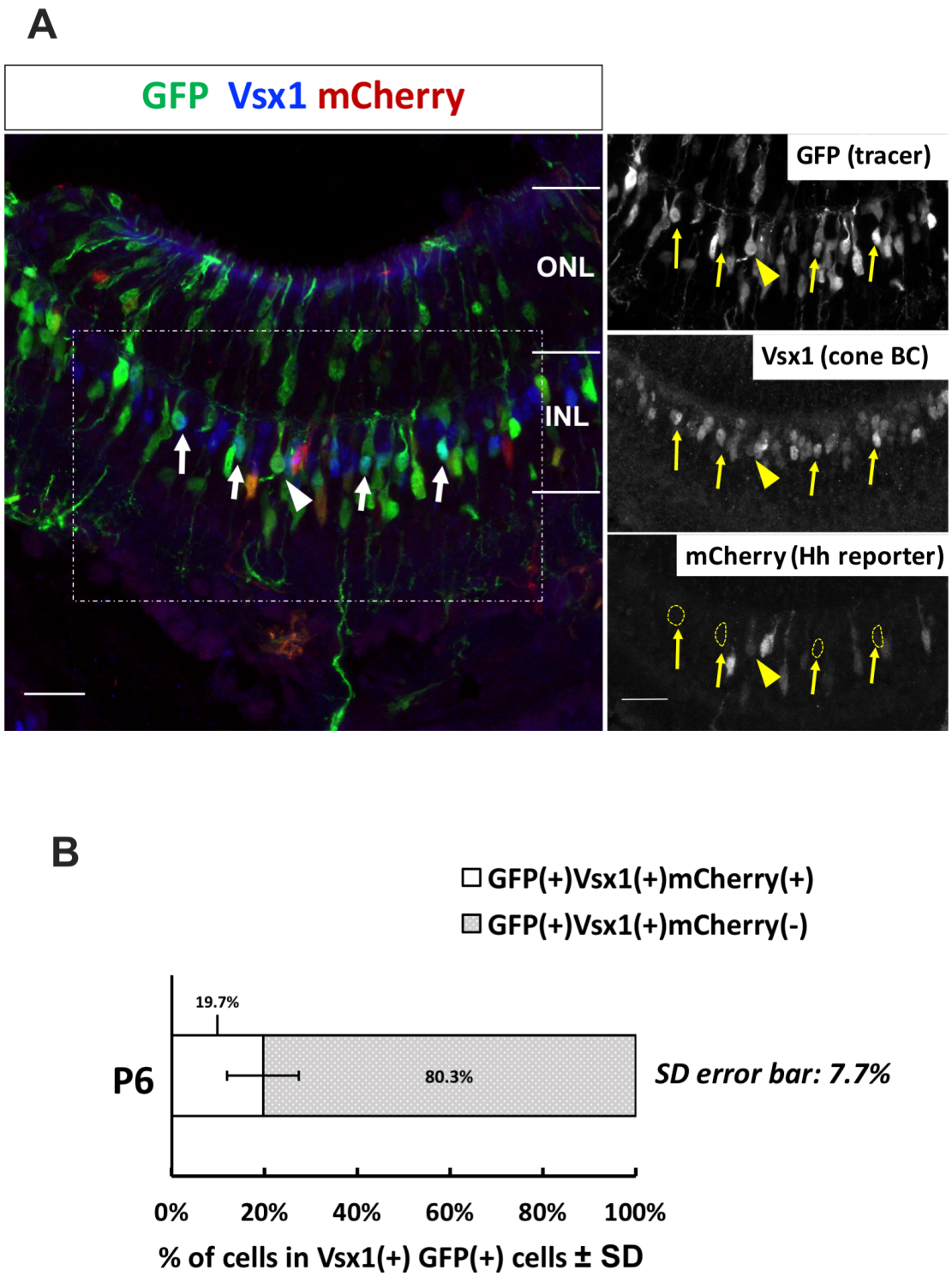

Figure 11 Vsx1 colocalization with GBS-driven mCherry reporters in P6 retinas.

(Legend on next page.)

Figure 11 Vsx1 colocalization with GBS-driven mCherry reporters in P6 retinas. **(A)** Retinas were transfected with GBS-TATA-mCherry and pCAG-NLS-GFP at P0, and stained with anti-GFP, anti-RFP and anti-Vsx1 (type 2 and type7 cone bipolar cell marker) at P6. Colocalization of GFP, mCherry and Vsx1 was identified and indicated with arrows and arrowheads. Arrows: GFP and Vsx1, but no mCherry; arrowheads: GFP, Vsx1, and mCherry. Scale bar for the left and right panel: 20 μm. **(B)** Quantification of mCherry+ cells among GFP+ Vsx1+ cells at P6. 127 GFP+ Vsx1+ cells were counted in n=3 retinas. BC: bipolar cell; ONL: outer nuclear layer; INL: inner nuclear layer; SD: standard deviation.

3.2 The postnatal requirement of Smo for regulating the formation of bipolar cells and their cone and rod subtypes

It has been previously observed that the number of cone bipolar cells in *Shh*-CKO retinas was significantly reduced while the number of rod bipolar cells was not affected (Wang, Star and Wallace and Chow, 2014, unpublished observations). Interestingly, a similar phenotype of selective cone bipolar cell reductions was observed in retinas with a deficiency of retinal ganglion cells, which are the only source of Shh in the retinas (Bai et al 2014). These results suggest a role for Shh in cone and rod bipolar cell development. However, these results did not address whether the phenotypes were the direct outcome of regulation in RPCs by Hh signaling or a secondary effect caused by the global environmental change when *Shh* was deficient. To investigate the direct role of Hh signaling in regulating the formation of cone and rod bipolar cells from RPCs, I transfected the Cre-expressing plasmid pCAG-Cre into P0 retinas of mice carrying the *Smo* floxed alleles (Long et al 2001) to conditionally delete *Smo*, the key downstream component of Hh signaling pathways, in postnatal RPCs (Fig. 12A). Even though the promoter for Cre is ubiquitous, RPCs are specifically transfected using this in vivo electroporation approach (Matsuda & Cepko 2004). By P10, *Smo* deletion in P0 RPCs led to an almost complete loss (an approximate tenfold reduction) of bipolar cells in the INL (Fig. 12B, 13A). Other INL cells were also greatly reduced, although some INL cells remained. Judging from the larger cell body and the inner location in the INL, these

were likely amacrine cells (Fig. 12B). The proportion of photoreceptors was increased (Fig. 13A). It is less likely that the cell loss in SmoCKO retinas was caused by the inhibited cell survival at a later stage since these retinas were negative for Caspase3 immunoreactivity (Fig. 13B). The loss of bipolar cells in SmoCKO retinas made bipolar cell subtype analysis challenging because not enough bipolar cells were available for sampling.

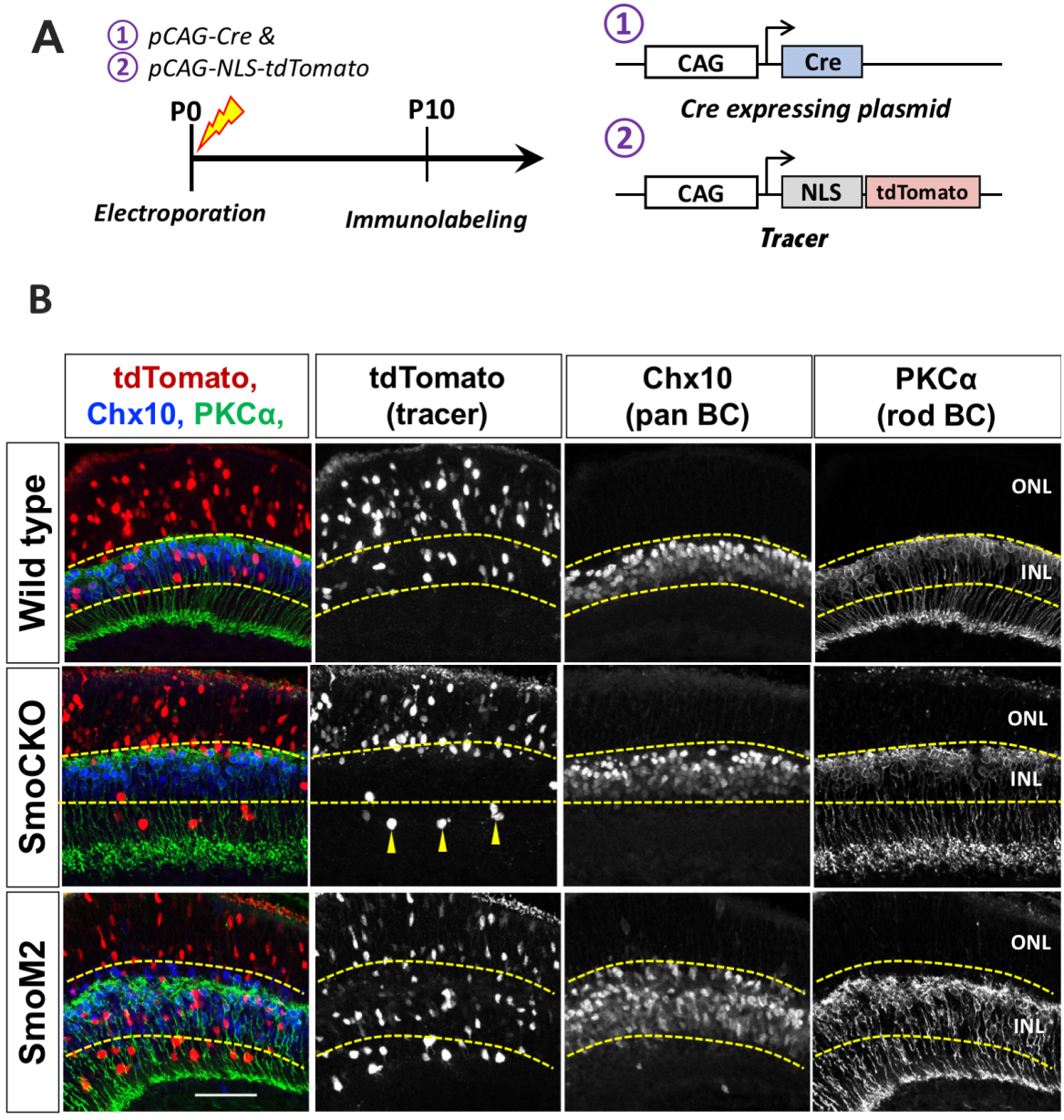

Figure 12 Deletion or activation of *Smo* in P0 retinas (Legend on next page.)

Figure 12 Deletion or activation of *Smo* in P0 retinas

(A) Schematic of electroporation timeline and plasmids for transfection. Electroporation was conducted at P0, and tissues were harvested at P10. pCAG-Cre were Cre expressing plasmids and pCAG-NLS-tdTomato were transfection tracers. NLS: nucleus localized signal. **(B)** Retinas from wild-type (WT), SmoCKO and SmoM2 mice were transfected with the above plasmids at P0 and harvested at P10. Retinas were stained with anti-dsRed, anti-Chx10 (bipolar cell marker) and anti-PKCα (rod bipolar cell marker). Note that in SmoCKO retinas, nearly all of the INL cells were lost, except for some larger cells located at the inner boundary of the INL (arrowheads). Those cells were likely amacrine cells. BC: bipolar cell; ONL: outer nuclear layer; INL: inner nuclear layer. Scale bar: 50 μm.

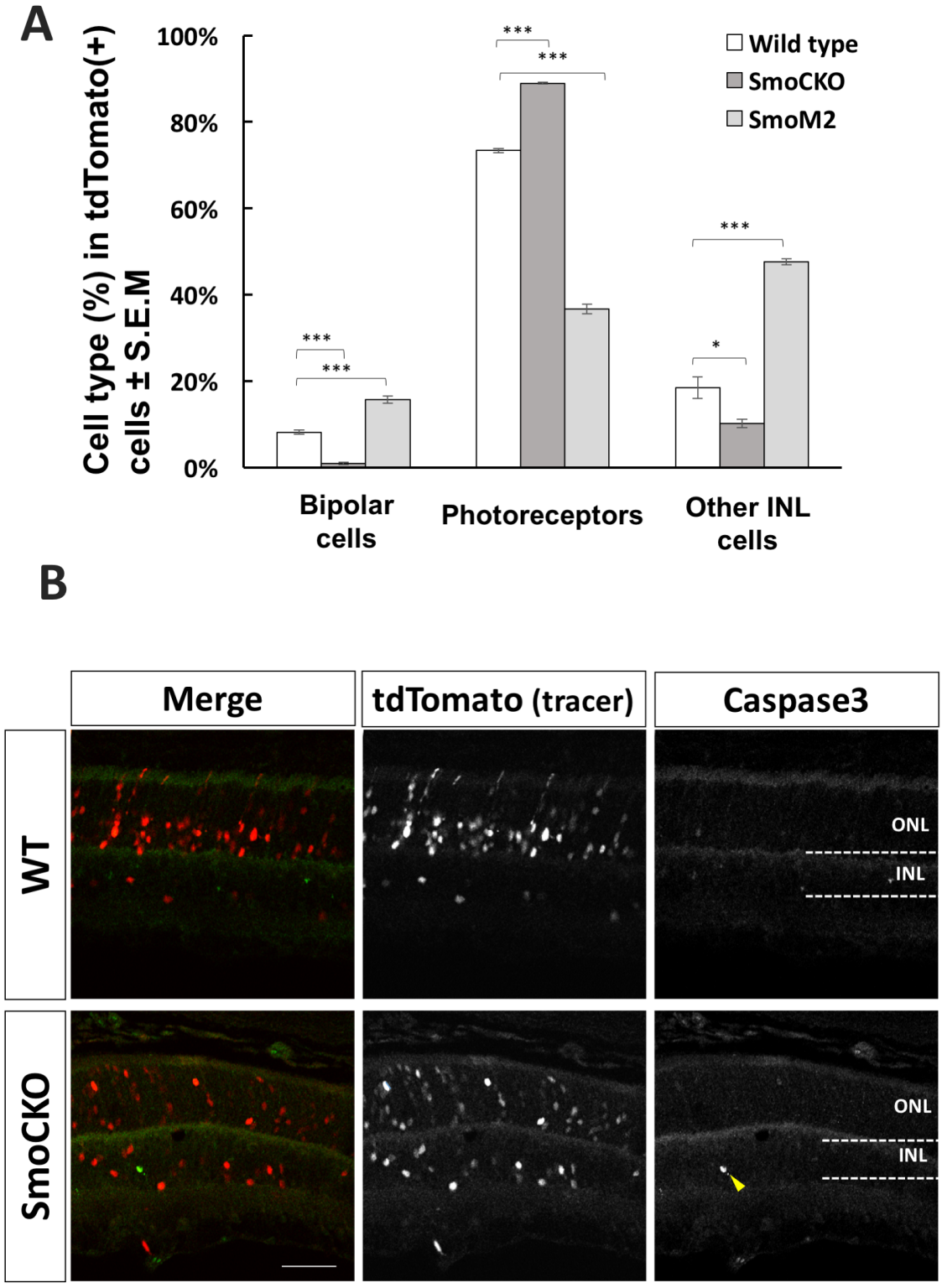

Figure 13 *Smo* activation in P0 RPCs is required and sufficient to induce bipolar cell formation. (Legend on next page.)

Figure 13 Smo activation in P0 RPCs is required and sufficient to induce bipolar cell formation.

A) Retinas from wild-type (WT), SmoCKO and SmoM2 mice were transfected with pCAG-Cre and pCAG-NLS-tdTomato at P0 and harvested at P10. Bipolar cells were identified by Chx10+ labeling, photoreceptors were identified based on their location in the ONL, and the rest of the cells were identified as other INL cells. The mean percentages of these three cell types among tdTomato+ cells were calculated in each group. INL: inner nuclear layer. >2900 tdTomato+ cells were counted within n=5 retinas per condition. * P<0.05, ***P<0.001. **(B)** P10 wild-type and SmoCKO retinas transfected with the above plasmids were stained with anti-RFP and anti-Caspase3 (apoptosis cell marker). Note that a very few amount of cell deaths occur naturally in both condition (arrowhead). ONL: outer nuclear layer; INL: inner nuclear layer. Scale bar: 50 μm. * P<0.05, **P<0.01, ***P<0.001.

Given that the competency of RPCs to generate different cell types changes over time , I transfected pCAG-Cre to delete *Smo* in P1 and P2 retinal RPCs to investigate whether the requirement of *Smo* by RPCs to generate bipolar cells is affected (Fig. 14A). Interestingly, *Smo*-deficiency in P1 and P2 RPCs did not result in a complete ablation of bipolar cells as in P0. Although, compared to the wild-type controls, fewer bipolar cells were present (Fig. 14B, 15A-B) *Smo*-deficiency in P1 and P2 RPCs did not cause significant changes in the proportions of photoreceptors and other INL cells. Wild-type retinas that were transfected at P0, P1, and P2 gave rise to similar proportion of bipolar cells (Fig. 15C) while transfected SmoCKO retinas displayed increasing bipolar cell formation overtime (Fig. 15D). Similarly, the increase of photoreceptor production and the inhibitory effect on other INL cell types in SmoCKO retinas also diminished progressively when pCAG-Cre was transfected at P1 and P2 (Fig. 15D). These results suggest that RPCs at different stages have different requirement of Smo for generating bipolar cells. Alternatively, the presence of bipolar cells in these experiments may

indicate that some bipolar cells had already been born (i.e. were postmitotic) and irreversibly determined before Smo deletion had occurred.

For both P1 and P2 transfected SmoCKO retinas, enough bipolar cells were present for subtype analysis. I performed PKCα immunohistochemistry to identify rod bipolar cells and determine whether the development of cone and rod bipolar cell subtypes was affected by cell-autonomous *Sm*o deletion in RPCs. Deleting *Smo* in both P1 and P2 RPCs led to a significant reduction of cone bipolar cell proportions within transfected cells; rod bipolar cell proportions were not affected in either group (Fig. 16A, B). The reduction in cone bipolar cells was more pronounced in P1 group (2.7 fold) than P2 group (1.8 fold) (Fig. 16A, B). These results were consistent with our unpublished data showing that cone bipolar cells are selectively reduced in the *Shh* knocked-out retinas (Wang, Star and Wallace and Chow, 2014, unpublished observations). Notably, P1-transfected wild-type retinas gave rise to more cone bipolar cells in comparison to P0-transfected and P2-transfected retinas (Fig. 16C), raising the possibility that RPCs transfected at P1 are more competent to give rise to cone bipolar subtypes. Together these results suggest that *Smo* is cell-autonomously required by RPCs to generate bipolar cells at early postnatal stages and that the development of cone bipolar cells, but not rod bipolar cells is dependent on the cell-autonomous *Smo* activation in P1 and P2 postnatal RPCs.

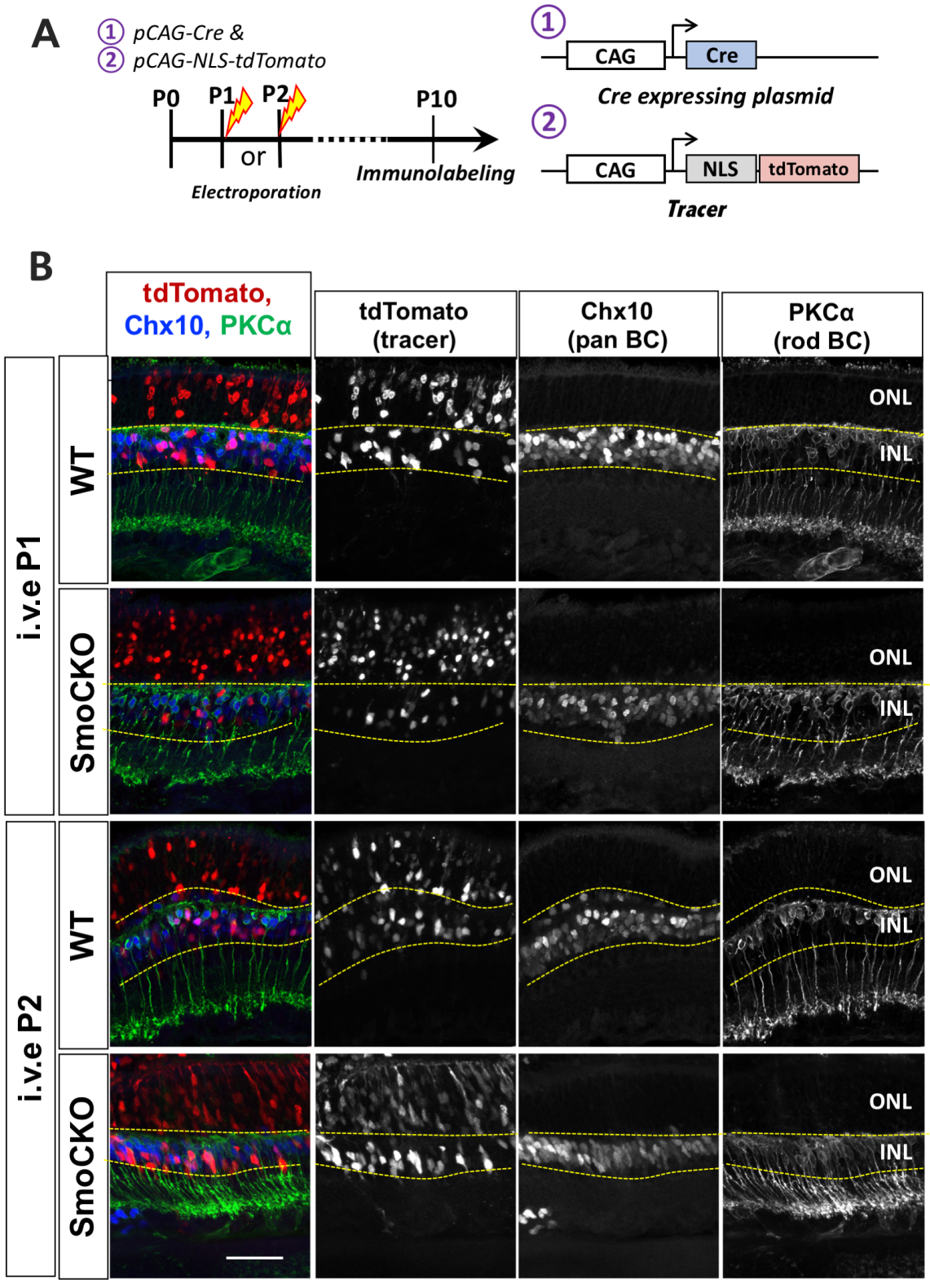

Figure 14 Deletion of *Smo* in P1 and P2 retinas.

Figure 14 Deletion of *Smo* in P1 and P2 retinas.

(A) Schematic of electroporation timeline and plasmids for transfection. Electroporation was conducted at P1 or P2, and tissues were harvested at P10. pCAG-Cre were Cre-expressing plasmids and pCAG-NLS-tdTomato were transfection tracers. NLS: nucleus localized signal. **(B)** Retinas from wild-type (WT) and SmoCKO mice were transfected with pCAG-Cre and pCAG-NLS-tdTomato at P1 or P2 and were harvested at P10. Retinas were stained with anti-RFP, anti-Chx10 (bipolar cell marker), and anti-PKCα (rod bipolar cell marker). BC: bipolar cell; ONL: outer nuclear layer; INL: inner nuclear layer. Scale bar: 50 μm.

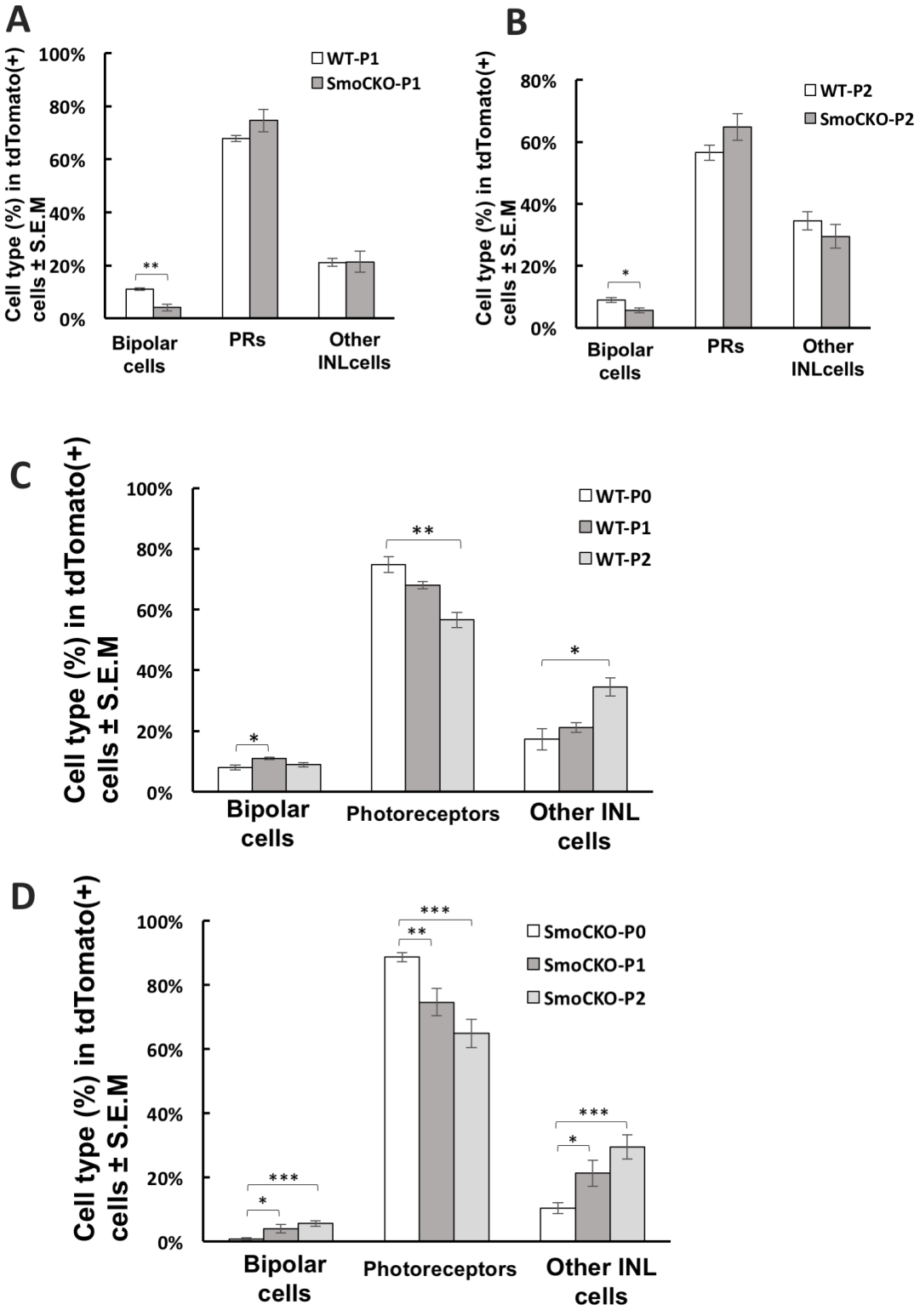

Figure 15 *Smo* inactivation in P1 and P2 retinas reduces bipolar cell formation.

Figure 15 Smo inactivation in P1 and P2 retinas reduces bipolar cell formation. Cell type percentages among tdTomato+ cells were determined in P10 retinas from wild-type (WT) and SmoCKO mice. These retinas were transfected with pCAG-Cre and pCAG-NLS-tdTomato at either **(A)** P1 or **(B)** P2. Retinas transfected with the above plasmids at P0 were included to plot the cell type composites among **(C)** wild-type group and **(D)** SmoCKO group. Bipolar cells were identified by Chx10+ labeling, photoreceptors were identified based on their location in the ONL, and the rest of the cells were identified as other INL cells. INL: inner nuclear layer. 3000~7000 tdTomato+ cells were counted in n= at least 3 retinas per condition. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

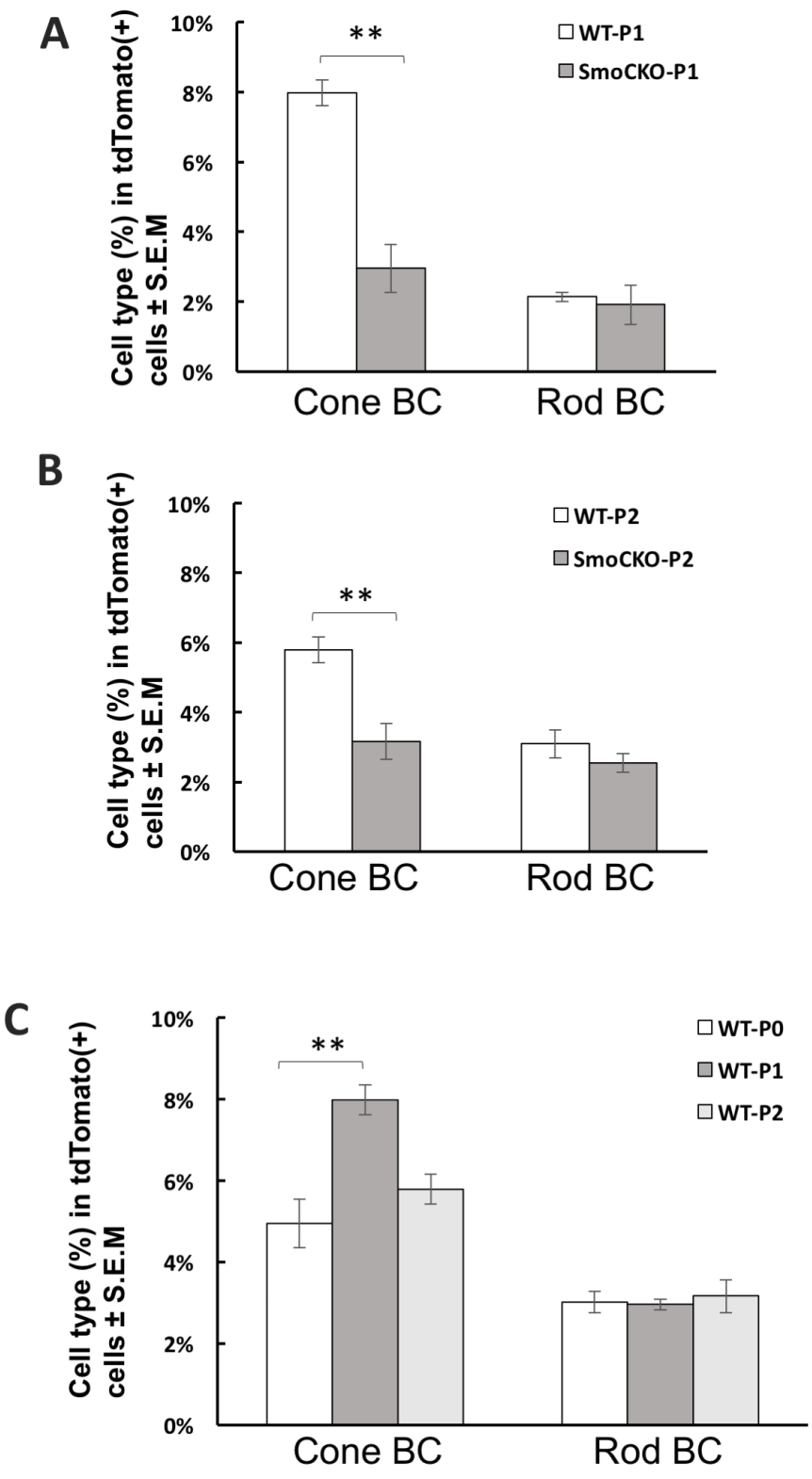

Figure 16 *Smo* inactivation in P1 and P2 retinas selectively reduces cone bipolar cell formation.

Figure 16 Smo inactivation in P1 and P2 retinas selectively reduces cone bipolar cell formation.

Percentage of cone and rod bipolar cells among tdTomato+ cells were determined in P10 retinas from wild-type (WT) and SmoCKO mice. These retinas were transfected with pCAG-Cre and pCAG-NLS-tdTomato at either **(A)** P1 or **(B)** P2. **(C)** Distribution of cone and rod bipolar cell composites among P10 wild-type retinas transfected with the above plasmid at P0, P1, and P2. Cone bipolar cells were identified by PKCα-, Chx10+ labeling; rod bipolar cells were identified by PKCα+, Chx10+ labeling. 3000~7000 tdTomato+ cells were counted in n= at least 3 retinas per condition. **P<0.01.

3.3 Postnatal activation of *Smo* is sufficient to promote cone bipolar cell development

Because the results from the previous section suggest that the development of bipolar cells requires cell-autonomous activation of *Smo*, I subsequently asked whether activation of *Smo* in postnatal RPCs is sufficient to promote bipolar cell development and to affect cone and rod subtype formation. To conditionally activate *Smo* in postnatal RPCs, pCAG-Cre plasmids were transfected into the *R26SmoM2* (hereafter referred as SmoM2) mouse line. Cre recombinase activity would activate the expression of *SmoM2*, a mutated form of *Smo*. SmoM2 is constitutively activated in the cell because its G-protein binding domain is disrupted and therefore uninhibited by Ptch (Xie et al 1998). Electroporation of pCAG-Cre at P0 in SmoM2 retinas induced the production of bipolar cells by P10 (Fig. 12B). Other INL cells were also significantly induced; however, photoreceptors were reduced drastically (Fig. 13A). Overall, these results are consistent with previous work that the expression of *SmoM2* in P0 retinas promotes the development of cells with INL identities and inhibits ONL cells (Yu et al 2006). Note that the INL in the *SmoM2* retinas appeared thicker and had more Chx10+ cells, many of which were negative for the GFP transfection control reporter (Fig. 12B). This occurrence may be due to the fact that pCAG-Cre was overloaded and therefore not all Cre+ cells had GFP reporter present (Appendix). I also can not rule out the possibility

that this was a paracrine mechanism by which Smo+ cells induced other RPCs non-cell autonomously to form into bipolar cells.

To determine whether cell-autonomous activation of *Smo* in RPCs regulates the development of cone and rod bipolar cell subtypes, I performed co-immunolabeling for Chx10 and PKCα to analyze subtype composition. Activated *Smo* expression was associated with an approximate twofold increase of cone bipolar cell proportion while the proportion of rod bipolar cells was unaffected (Fig. 17A-B)) Thus, cell-autonomous *Smo* activation in RPCs selectively promotes the development of cone bipolar cells over rod bipolar cells.

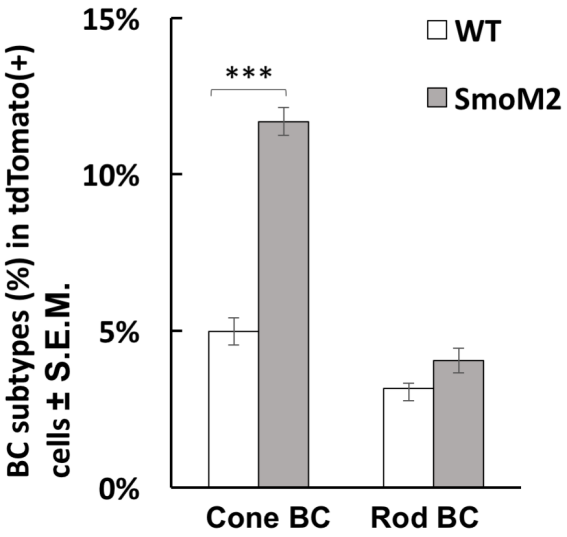

Figure 17 *Smo* activation in P0 retinas selectively induces cone bipolar cell formation.

Percentages of cone and rod bipolar cells among tdTomato+ cells were determined in P10 retinas from wild-type (WT) and SmoM2 mice. These retinas were transfected with pCAG-Cre and pCAG-NLS-tdTomato at P0. Cone bipolar cells were identified by PKCα-, Chx10+ labeling; rod bipolar cells were identified by PKCα+, Chx10+ labeling. ~3000 tdTomato+ cells were counted in n=5 retinas per condition. BC: bipolar cell. ***P<0.001.

3.4 Activation of *Smo* in postmitotic bipolar cells has no influence on bipolar cell subtype formation

I have demonstrated in the previous sections that cell-autonomous activation of *Smo* in postnatal RPCs was selectively required and sufficient to promote cone bipolar cell development, suggesting that Hh signaling in RPCs is important for regulating the bipolar cell subtype formation. However, whether Smo functions in postmitotic bipolar cells to regulate subtype formation is not known. To address this question, I transfected the Chx10BP-Cre plasmid with a Cre-activity reporter into the SmoCKO retinas to conditionally delete *Smo* in postmitotic bipolar cells. Surprisingly, I observed varying levels of leaky Cre activity in wild-type and SmoCKO groups (Fig. 18 and Table 7). The reporters were found not only in bipolar cells but also in photoreceptors and some other INL cells (Table 7). The leakiness prevented me from analyzing the composites of bipolar cell subtypes because *Smo* deletion may not be specific in postmitotic bipolar cells.

To determine whether *Smo* activation in postmitotic bipolar cells is sufficient to affect the subtype formation, I crossed the SmoM2 mice to the Vsx2-5.3-PRE-Cre (Chx10-Cre) mice (Nickerson et al 2011) to induce bipolar cell specific *Cre* expression driven by the upstream element of *Vsx2*. This element is activated 24-36 hours after RPCs exiting the cell cycle and become bipolar cells. *SmoM2* expression in bipolar cells was confirmed by the expression of *YFP*, which is fused to the *SmoM2* (Fig. 19A). By P21, activation of *SmoM2* in postmitotic bipolar cell did not affect the formation of cone and rod subtypes (Fig. 19B). This result suggests that *Smo* activation in postmitotic bipolar cells is not sufficient to induce cone bipolar cell type formation, although due to nature of the experimental approach, I cannot rule out the possibility that *Smo* functions within the first 24-36 hours after bipolar cell birth.

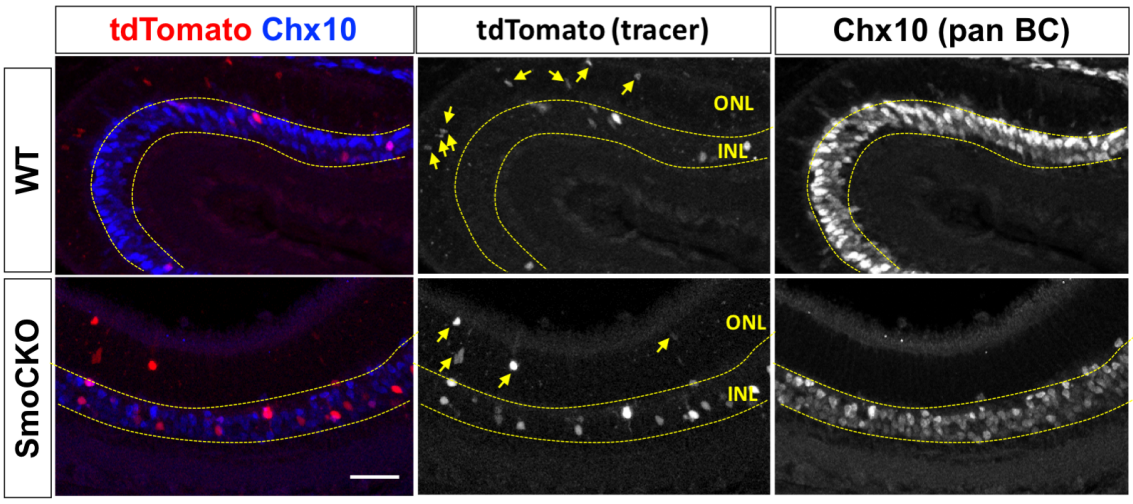

Figure 18 Leaky expression of the Chx10-BP-Cre plasmid.

Retinas from wild-type and SmoCKO mice were transfected with Chx10-BP-Cre and pCALNL-NLS-tdTomato (reporter of Cre activity) at P2 and were harvested at P10. Retinas were stained with anti-Chx10 and anti-RFP. tdTomato expression was seen in the INL cells. The expression also leaked to the ONL cells (arrows). ONL: outer nuclear layer; INL: inner nuclear layer. BC: bipolar cell. Scale bar: 40 μm.

Table 7 Percentages of tdTomato+ cell types in WT and SmoCKO animals injected with Chx10BP-Cre and pCALNL-NLS-tdTomato plasmid.

	Total cell counts*	%of PRs ± SD(%)	% of BCs ± SD	% of other INLcs ± SD
WT	878	53.84 ± 11.71	23.08 ± 1.46	23.08 ± 13.16
SmoCKO	454	23.33 ± 21.04	40.46 ± 8.44	36.03 ± 13.87

** Counting were performed in n=3 animals*

Abbreviation: PRs: photoreceptors; BCs: bipolar cells; INLcs: INL cells

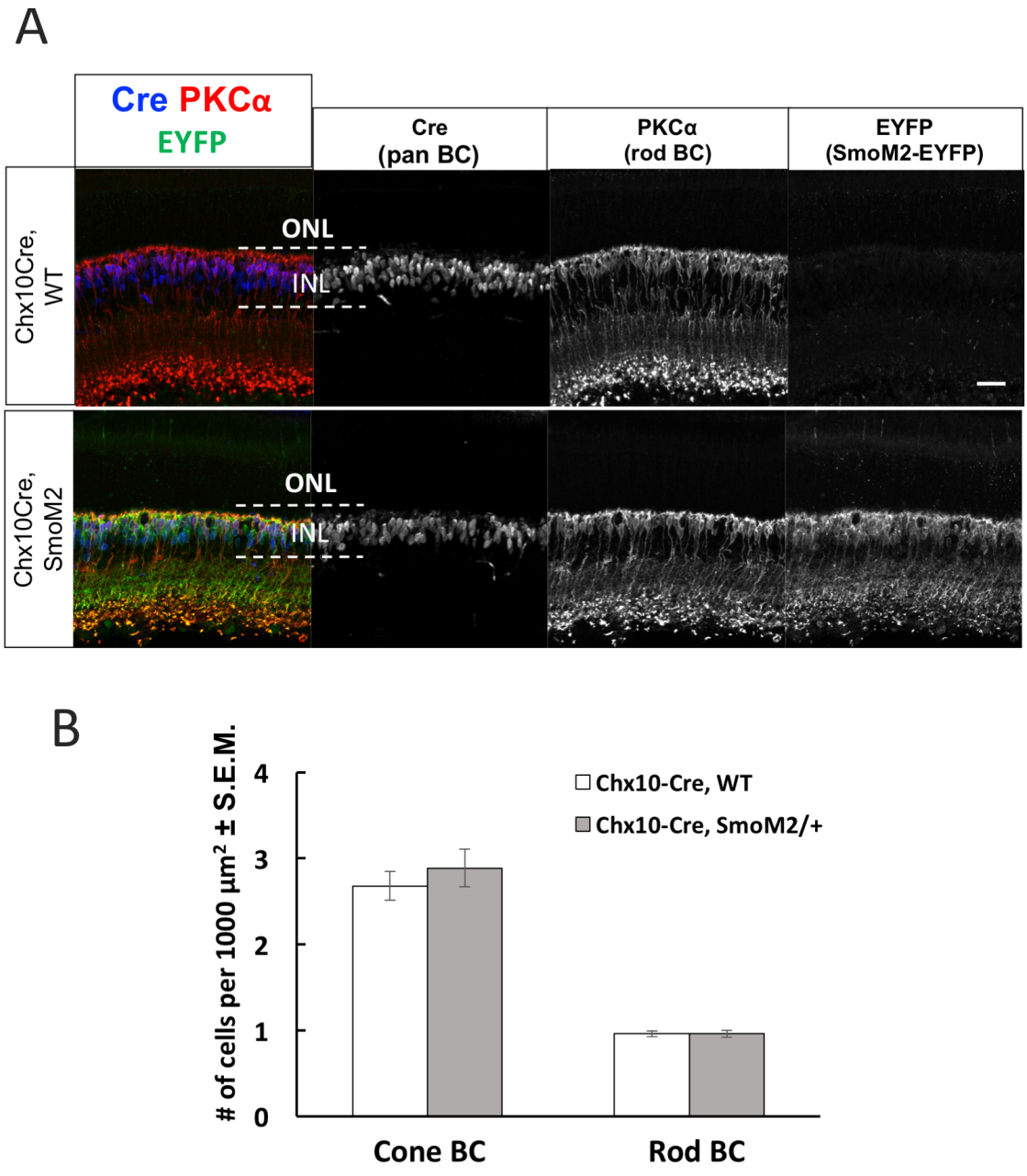

Figure 19 Activation of *Smo* in postmitotic bipolar cells is not sufficient to affect cone and rod bipolar cell subtype formation. (Legend on next page.)

Figure 19 Activation of *Smo* in postmitotic bipolar cells is not sufficient to affect cone and rod bipolar cell subtype formation.

(A) SmoM2 or wild-type mice were crossed to Vsx2-5.3-PRE-Cre (Chx10-Cre) mice. At P21, retinas were harvested from pups with the genotypes of SmoM2(+) Chx10-Cre(+) and SmoM2(-) Chx10-Cre(+). Retinas were stained with anti-Cre (bipolar cell marker), anti-PKCα (rod bipolar cell marker), and anti-GFP (labeled YFP fused with SmoM2). BC: bipolar cell; ONL: outer nuclear layer; INL: inner nuclear layer. Scale bar: 20 μm. **(B)** The number of cone and rod bipolar cells was counted in the mentioned genotypes as above. In each condition, the number of cone (or rod) bipolar cells was divided by the total retinal area in counting views. The number of cells per 1000μm^2 retinal area was thendetermined. Cone bipolar cells were identified by PKCα-, Cre+ labeling; rod bipolar cells were identified by PKCα+, Cre+ labeling. >2500 Cre+ cells were counted in n=3 retinas per condition.

3.5 Activation of Hh signaling stimulates bipolar cell production *in vitro*

Hh signaling is well known for its morphogenic effect in neural development. To investigate whether graded Hh signaling differentially regulates the development of bipolar cells and their subtypes, I cultured P0 retinal explants *in vitro* with different levels of purmorphamine to activate the Hh signaling pathway. Purmorphamine is an agonist of Hh signaling and functions by directly binding and activating Smo (Sinha & Chen 2006). A recent study has found that flattening the retinas when culturing greatly improved the morphology and reduced variation (Ringuette et al 2016). In the present study, all retinal explants were similarly cultured as a flattened single layer of tissue (as described in the Methods sections). By P10, the number of Chx10 + bipolar cells increased by more than twofold in explants treated with purmorphamine and plateaued at 10 μM purmorphamine (Fig. 20, 22A). In contrast, the number of photoreceptors decreased dramatically with increased purmorphamine concentration (Fig. 22A). These results are generally consistent with the previous finding that Hh agonist treatment in explants induces Chx10+ cells and represses rhodopsin + cells (Yu et al 2006). Bipolar cell production was also examined in explants with 15 μM purmorphamine added at later stages (P3 and P6). These treatments did not affect the production of bipolar cells and photoreceptors (Fig. 23A-C).

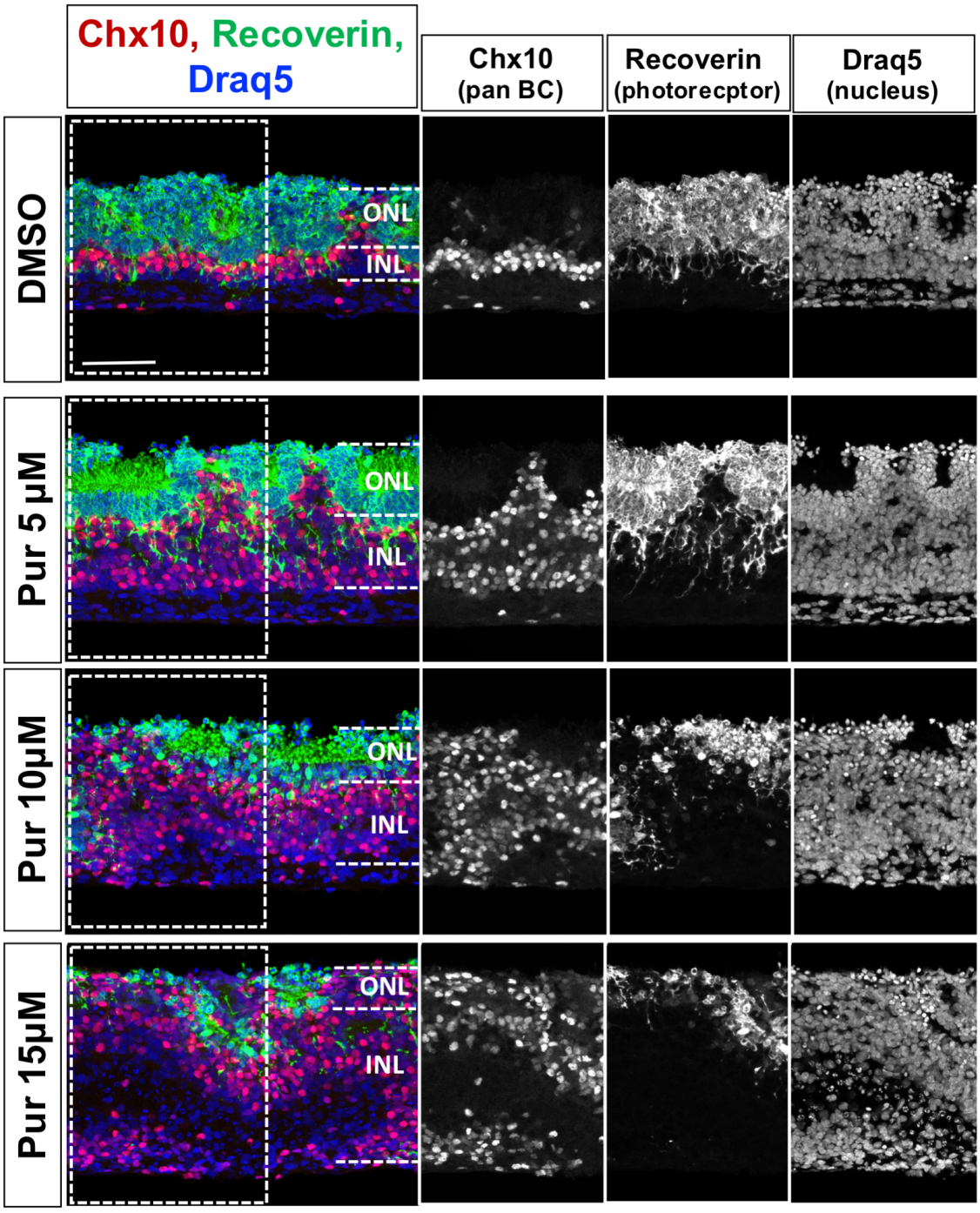

Figure 20 Purmorphamine induces bipolar cell formation and inhibits photoreceptor formation *in vitro*.(Legend on next page.)

Figure 20 Purmorphamine induces bipolar cell formation and inhibits photoreceptor formation *in vitro*.

Retinas from *mGluR6:NLS-LacZ* mice were dissected and cultured *in vitro* since P0 with 1% DMSO or graded purmorphamine treatment (5μM to 15μM) and were harvested at P10. Retinas were stained with anti-Chx10 (bipolar cell marker), Recoverin (photoreceptor marker), and Draq5 (nuclei marker). BC: bipolar cell; ONL: outer nuclear layer; INL: inner nuclear layer. Scale bar: 50 μm.

To determine the effect of purmorphamine in bipolar cell subtype formation, I analyzed the composition of rod and cone bipolar cells in each group. Adding purmorphamine at 5 μM and 10 μM similarly promoted the production of both cone and rod bipolar cells (Fig. 21A, Fig. 22B). Notably, in the highest concentration of purmorphamine (15 μM) treatment, changes were not observed in the number of cone bipolar cells, while rod bipolar cells were continuously stimulated (Fig. 22B). Since the explants were derived from the *mGluR6:NLS-βgal* mice that express βgal specifically in ON bipolar cells, I analyzed the ON and OFF subtype composition in purmorphamine treated explants and controls. Interestingly, the inducing effect of purmorphamine was limited in OFF type bipolar cells (Fig. 21A, Fig. 22C). In contrast, the production of ON type bipolar cells was greatly increased in the presence of purmorphamine, although the inducing effect displayed no relationship to the concentration of purmorphamine (Fig. 22C). I also examined ON and OFF type composition within the cone type population. Purmorphamine had mild stimulating effect on both ON cone and OFF cone bipolar cells at low to medium concentration (5-10 μM) and had no effect at high concentration (15 μM) (Fig. 22D). Adding purmorphamine at P3 and P6 did not cause an obvious change in the number of bipolar cell subtypes (Fig.21B, Fig, 24A-C). Together these results demonstrate that activation of Hh signaling by purmorphamine *in vitro* induces the production of bipolar cells at the expense of photoreceptors. These results also suggest that ON bipolar cells may be selectively regulated by Hh signaling *in vitro*.

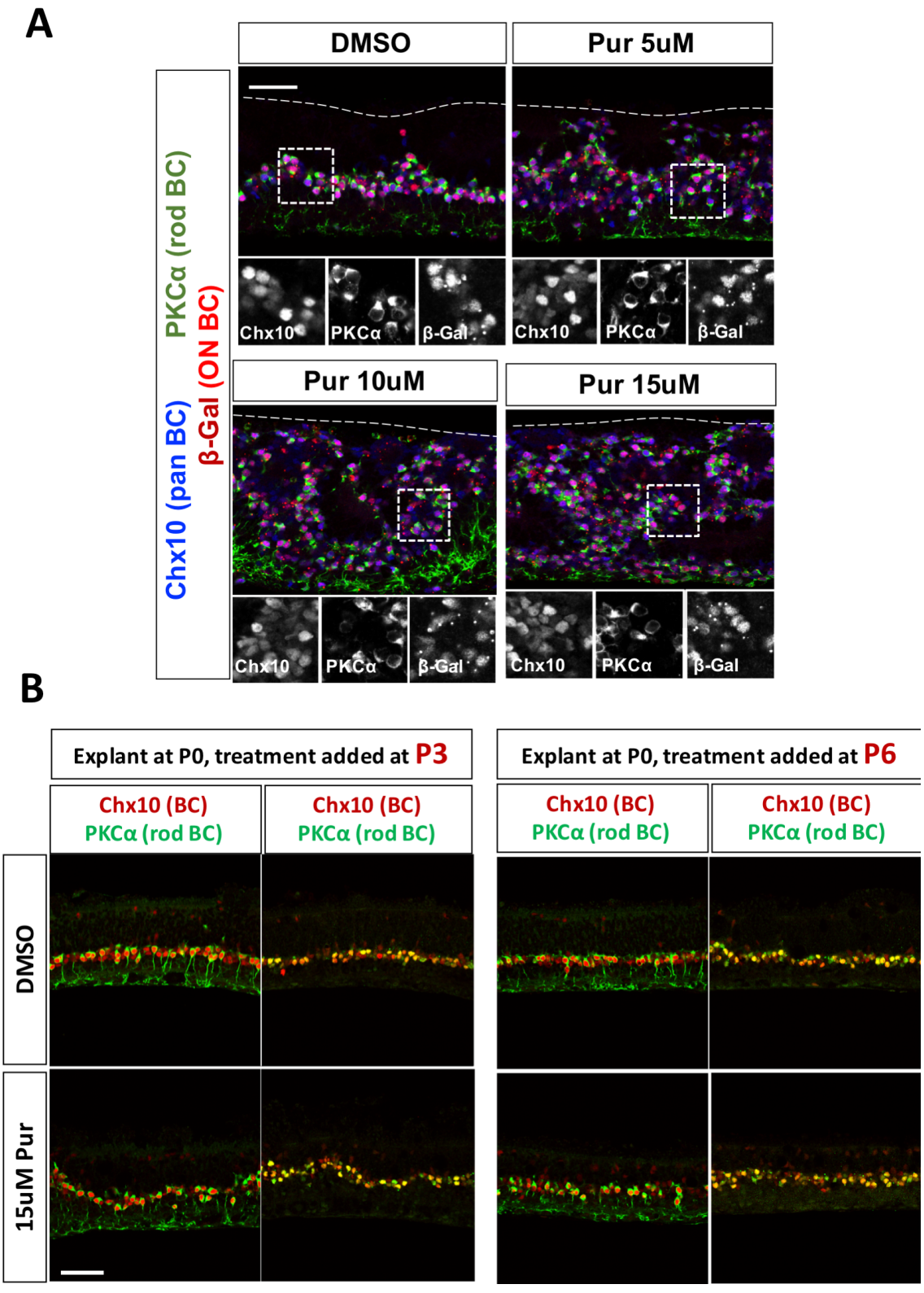

Figure 21 The effect of purmorphamine in bipolar cell subtype formation *in vitro*.

(Legend on next page)

Figure 21 The effect of purmorphamine in bipolar cell subtype formation *in vitro*. **A)** Retinas from *mGluR6:NLS-LacZ* mice were dissected and cultured *in vitro* since P0 with 1% DMSO or graded purmorphamine treatment (5μM to 15μM) and were harvested at P10. Retinas were stained with anti-Chx10 (bipolar cell marker), anti-βgal (ON bipolar cell marker) and PKCα (rod bipolar cell marker). Dotted lines outline the tissue boundary. **B)** Retinas from *mGluR6:NLS-LacZ* mice were dissected and cultured *in vitro* since P0. Treatments of 1% DMSO or 15 μM purmorphamine were applied at P3 (left panel) or P6 (right panel). Retinas were harvested at P10. Retinas were stained with the same antibodies as listed in A). BC: bipolar cell; Pur: purmorphamine; ONL: outer nuclear layer; INL: inner nuclear layer. Scale bar: 50 μm.

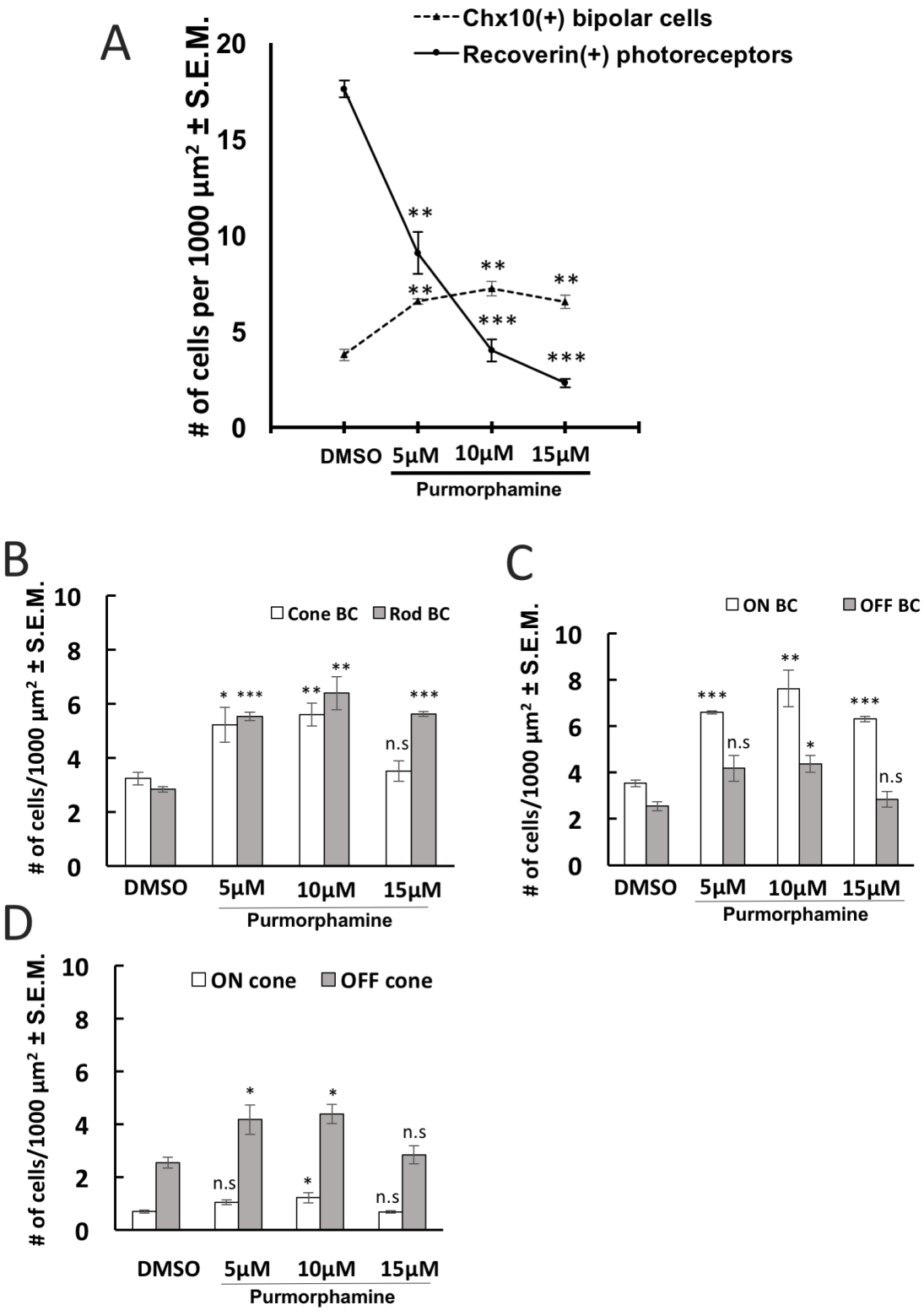

Figure 22 Purmorphamine treatment affects bipolar cell subtype formation *in vitro.* (Legend on next page.)

Figure 22 Purmorphamine treatment affects bipolar cell subtype formation *in vitro*. **(A)** Numbers of Chx10+ cells or Recoverin+ cells per 1000μm^2 retinal area were calculated in P10 retinal explants from *mGluR6:NLS-LacZ* mice; the retinas were treated with 1% DMSO or graded purmorphamine since P0. The number of **(B)** cone or rod bipolar subtypes; **(C)** ON or OFF bipolar cell subtypes; and **(D)** ON cone and OFF cone bipolar cell subtypes per 1000μm^2 were determined by cell marker analysis. Cone bipolar cells were identified by PKCα-, Ch10+ labeling; rod bipolar cells were identified by PKCα+, Chx10+ labeling; ON bipolar cells were identified by β-gal+, Chx10+ labelling; OFF bipolar cells were identified by β-gal-, Chx10+ labeling; OFF-cone bipolar cells were identified by Chx10+, PKCα-, β-gal- labeling; ON-cone bipolar cells were identified by Chx10+, PKCα-, β-gal+ labeling. The mean number of cell types per 1000μm^2 in individual purmorphamine treated group was compared to the DMSO group. >1500 Chx10+cells, and 1200~7000 Recoverin+ cells were counted in n=3 retinas per condition. Pur: purmorphamine; BC: bipolar cell. PR: photoreceptor. * P<0.05, **P<0.01, ***P<0.001.

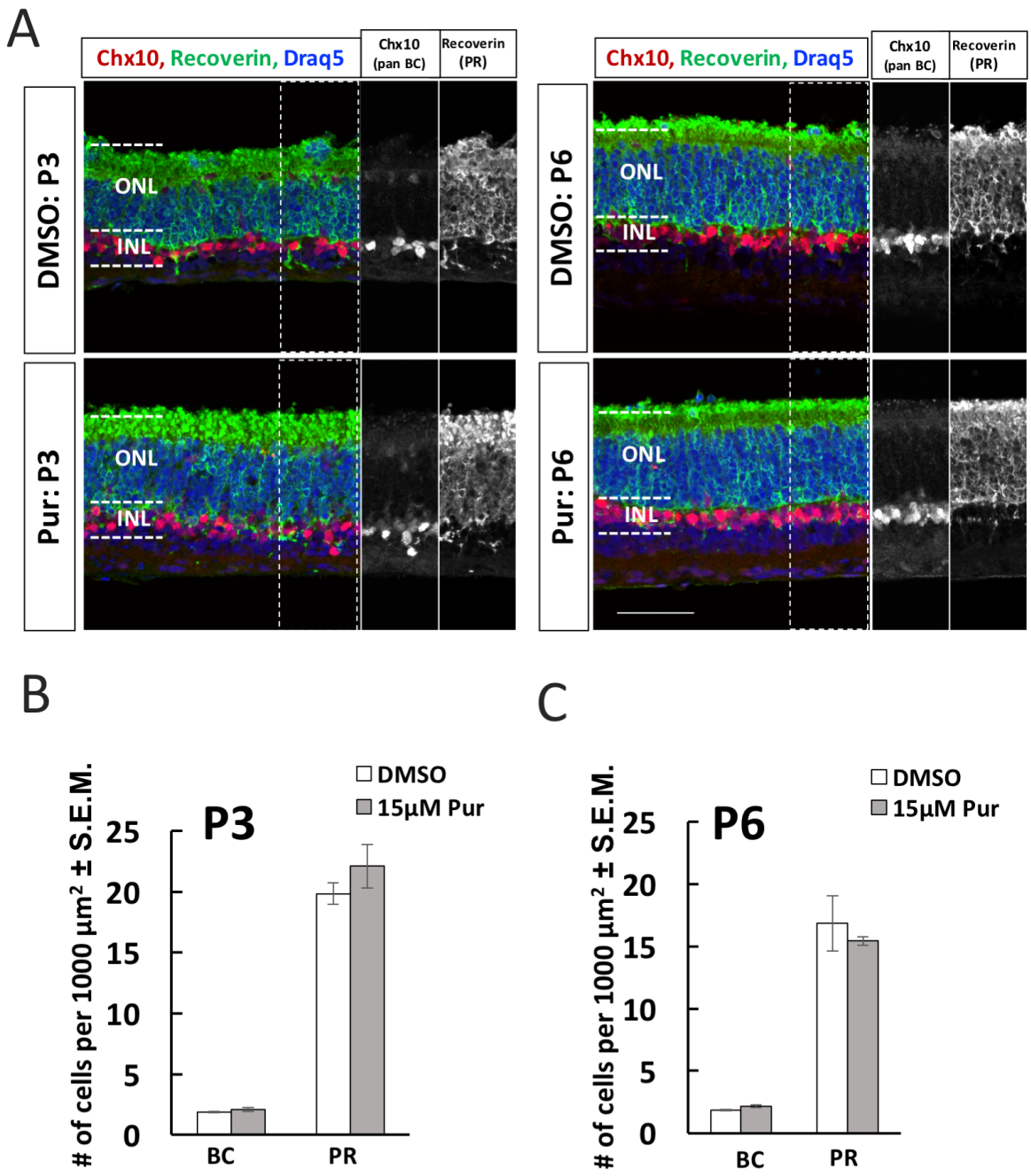

Figure 23 Purmorphamine treatment at P3 and P6 did not affect net bipolar cell production *in vitro*. (Legend on next page.)

Figure 23 Purmorphamine treatment at P3 and P6 did not affect net bipolar cell production *in vitro*.

(A) Retinas from *mGluR6:NLS-LacZ* mice were dissected and cultured *in vitro* since P0. Treatments of 1% DMSO or 15 μM purmorphamine were applied at P3 (left panel) or P6 (right panel). Retinas were harvested at P10. Retinas were stained with anti-Chx10 (bipolar cell marker), Recoverin (photoreceptor marker), and Draq5 (nuclei marker). Pur: purmorphamine; BC: bipolar cell; PR: photoreceptor; ONL: outer nuclear layer; INL: inner nuclear layer. Scale bar: 50 μm. **(B)** P3- and **(C)** P6- treated retinas as described in (A) were counted for the number of bipolar cells or photoreceptors per 1000μm^2. Bipolar cells were identified by Chx10+ labeling; photoreceptors were identified by Recoverin+ labeling. >1200 Chx10+cells and >12000 Recoverin+ cells were counted in n=3 retinas per condition. * $P<0.05$, ** $P<0.01$, n.s: non-significant.

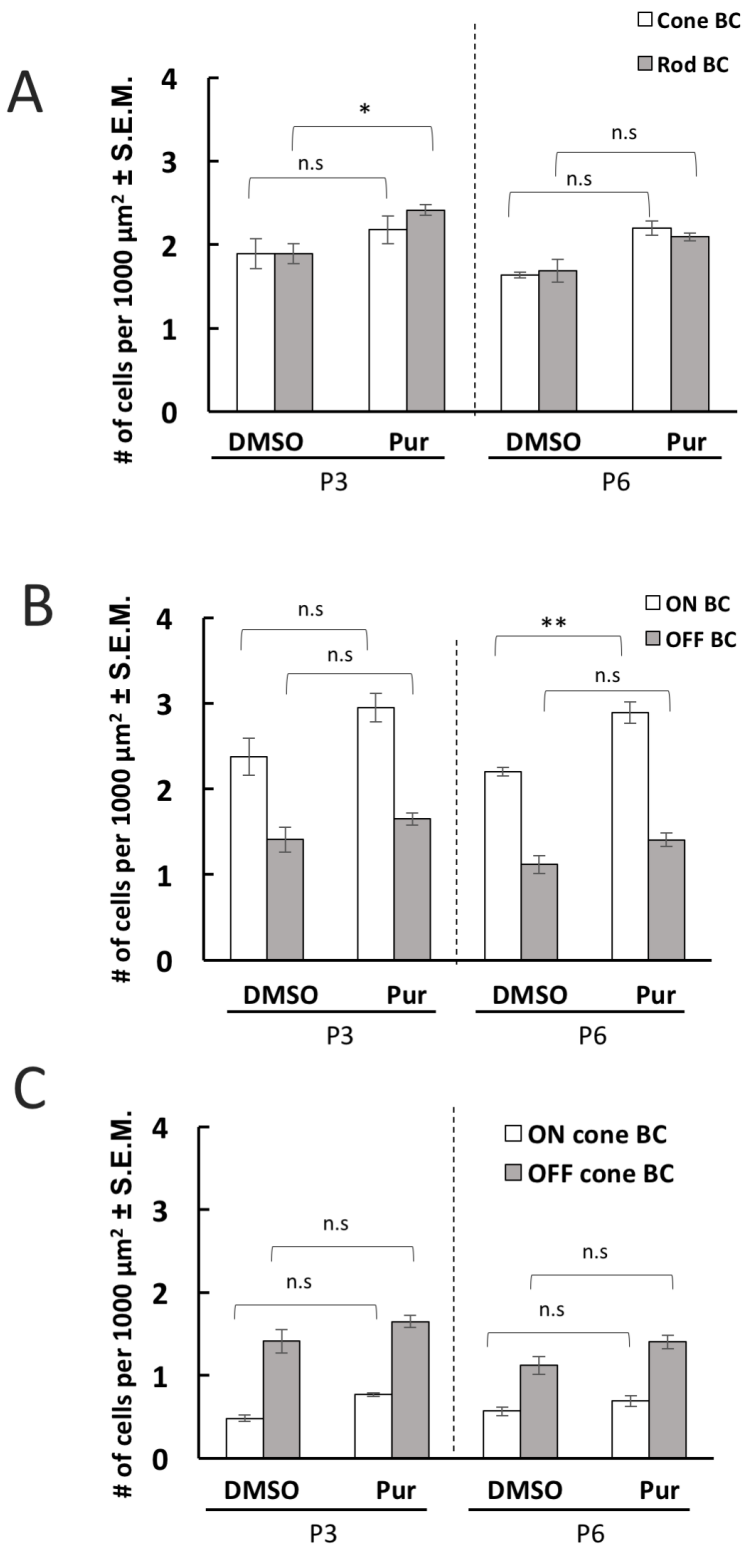

Figure 24 Purmorphamine treatment at P3 and P6 did not affect bipolar cell subtype formation *in vitro*. (Legend on next page.)

Figure 24 Purmorphamine treatment at P3 and P6 did not affect bipolar cell subtype formation *in vitro*.

Retinas from *mGluR6:NLS-LacZ* mice were dissected and cultured *in vitro* since P0. Treatments of 1% DMSO or 15 μM purmorphamine were applied at P3 or P6. Retinas were harvested at P10 for bipolar cell subtype analysis. The number of **(A)** cone or rod bipolar subtypes; **(B)** ON or OFF bipolar cell subtypes; and **(C)** ON cone and OFF cone bipolar cell subtypes per 1000μm^2 were determined by cell marker analysis. Cone bipolar cells were identified by PKCα-, Ch10+ labeling; rod bipolar cells were identified by PKCα+, Chx10+ labeling; ON bipolar cells were identified by β-gal+, Chx10+ labelling; OFF bipolar cells were identified by β-gal-, Chx10+ labeling; OFF-cone bipolar cells were identified by Chx10+, PKCα-, β-gal- labeling; ON-cone bipolar cells were identified by Chx10+, PKCα-, β-gal+ labeling >1200 Chx10+ cells were counted in n=3 retinas per condition. * $P<0.05$, ** $P<0.01$, n.s: non-significant. BC: bipolar cell; Pur: purmorphamine.

Chapter 4 Discussion

The first objective of my study was to visualize the dynamics of Hh signaling in the postnatal retina and to determine the existence of Hh activity in bipolar cells by transfecting the Hh signaling reporter plasmid into newborn retinas. The second objective was to investigate the role of *Smo* in directing the formation of pan bipolar cells and cone and rod bipolar subtypes from postnatal RPCs. This objective was carried out by delivering Cre-expressing plasmids into postnatal retinas to achieve RPC-specific *Smo* targeting. Finally, this study sought to investigate the potential morphogen effect of Hh signaling *in vitro* by applying graded Hh signaling agonist to retinal explants and monitoring the development of bipolar cells and their subtypes. The discussion below summarizes the main findings and presents an interpretation of the results. A summary of my conclusions is presented in **Figure 25**.

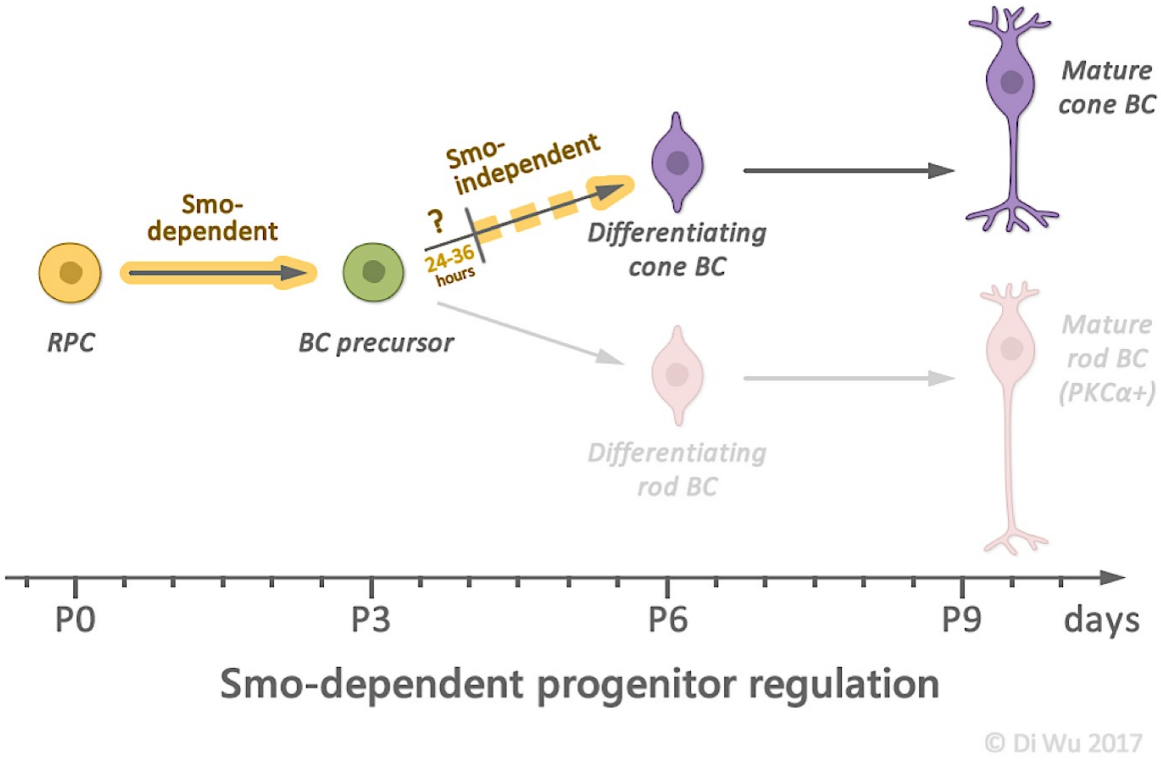

Figure 25 Summary of cone bipolar cell development mediated by Smo.

Figure 25 Summary of cone bipolar cell development mediated by Smo.

This study has revealed a Smo-mediated regulation on cone bipolar cell development. In postnatal retinas, pan-bipolar cell formation and the subsequent cone type development require the expression of *Smo* in RPCs (the yellow line). The function of Smo in newly born postmitotic bipolar cells (within 24-36 hours after cell cycle exiting) is unknown, while expression of Smo in postmitotic bipolar cells at later stage (beyond 36 hours) is not sufficient to affect the formation of cone subtype (the dotted yellow line). Smo: Smoothened; RPC: retinal progenitor cell; BC: bipolar cell.

4.1 Hh signaling activity in the postnatal retina and developing bipolar cells.

The expression of Hh signaling components (*Ptch* and *Gli*) in postnatal retinas has been described previously (Jensen & Wallace 1997, Ringuette et al 2016, Wang et al 2002), however, Hh signaling activity in postnatal retinas has not been visualized. The current study is the first to investigate the dynamics of Hh signaling activity in postnatal retinas by transfecting Hh signaling reporter plasmids into newborn retinas. The key components of the Hh signaling reporters are the 8x Gli-binding-sites, which have been successfully used as a transgenic tool to detecting Hh signaling activity in developing neural tubes (Balaskas et al 2012, Stamataki et al 2005). The 8x Gli-binding-sites were also used in a plasmid form to read out Hh activity in retinas (Ringuette et al 2016). I modified the minimal promoter of this plasmid to minimize the basal reporter expression. My data revealed that net Hh signaling activities in retinas decreased over time from P2 to P6. This result is consistent with the finding that the expression of *Ptch* and *Gli* is downregulated with the progression of neuronal differentiation (Ringuette et al 2016). My results also revealed that even though the overall expression of Hh signaling reporters declined overtime, the reporter was gradually restricted to the INL and the proportion of reporter+ bipolar cells increased and peaked at P6. This finding is interesting because the expression of Hh signaling components in bipolar cells has not been described in postnatal retinas. However, it is known that the expression of *Ptch* and *Gli* remained postnatally in the INL where bipolar cells are enriched (Wang et al 2002).

Notably, not all bipolar cells were Hh reporter positive, suggesting that Hh signaling may only be active in particular bipolar cell subtypes, or that the signaling may be transiently present. The ON-type cone bipolar cell marker Vsx1 and rod bipolar cell marker PKCα were both detected in Hh reporter+ bipolar cells, suggesting the presence of Hh signaling in both cone and rod bipolar cells. Notably, by P6 fewer Hh signaling reporter positive cells were observed in the ONL layer. This phenotype may coincide with the fact that photoreceptors are negatively regulated by Hh signaling (Yu et al 2006); thus, down-regulated Hh signaling in the ONL may be required for the development of photoreceptors. Another explanation for this phenotype is that the majority of photoreceptors have been already born by P6 and they no longer require the regulation of Hh signaling; therefore, the absence of Hh reporter in ONL may reflect the differentiation of photoreceptors.

Since the half-life of fluorescent protein is ~24 hours (Subach et al 2009), I cannot confirm that the reporter was reflecting the real-time Hh signaling activity. It is possible that some fluorescence detected in bipolar cells was the result of un-degraded fluorescent protein from the previous progenitor cell stage, in which Hh signaling was required for the cell cycle progression (Sakagami et al 2009). If the reporters were not degraded and carried over to the postmitotic stage, I expect they may appear weaker as the postmitotic cell markers begin to be expressed. The fact that some reporter positive bipolar cells had strong expression of reporters and the postmitotic bipolar cell marker Chx10-Cre suggests that in these cells, Hh signaling reporter activity was more likely derived from bipolar cells instead of their previous progenitor cell stages. An ideal approach to address this issue is using a reporter system in which the expression of reporter is dependent on both the activity of Gli-binding-sites and bipolar cell-specific Cre recombinase.

4.2 *Smo* activation in postnatal RPCs is required and sufficient to promote bipolar cell formation

The development of bipolar cells likely involves a combination of both intrinsic and extrinsic factors that primes RPCs to acquire bipolar cell fates (Cepko 2014, Mizeracka et al 2013b, Xiang 2013). The contribution of extrinsic factors to bipolar cell development has been studied less extensively, although factors such as Notch1 (Mizeracka et al 2013a) and CNTF (Ezzeddine et al 1997) have been implicated in the formation of bipolar cells. Shh is also an extrinsic stimulator of bipolar cell production. Activation of Hh signaling *in vivo* or *in vitro* promotes pan-bipolar cell formation (Yu et al 2006). My results also indicate that *Smo* activation in P0 RPCs is sufficient to promote the formation of bipolar cells and other INL cell types at the expense of photoreceptors. More importantly, my results for the first time indicate the requirement of *Smo* for postnatal RPCs to develop into bipolar cells. Given the role of Hh signaling in controlling the timing of cell cycle exit (Moshiri & Reh 2004, Wang et al 2005), I postulate that the P0 requirement of *Smo* in bipolar cell development could result from precocious cell cycle exit and the failure to acquire the competence for adopting bipolar cell fates. In addition, deleting *Smo* in P1 and P2 RPCs progressively attenuated the loss of bipolar cells. The stimulating effect of Smo on other INL cells and inhibiting effect on photoreceptors was also diminished over time. It is possible that RPCs at later stages may have already reached a stage where they are competent to generate particular cell types and, therefore, were less affected by the deficiency of *Smo* compared to P0 RPCs. Another possibility is that some RPCs have already exited the cell cycle and become bipolar cells during the time period of LoxP site recombination and Smo protein clearance; this possibility may be supported by the fact that bipolar cell birthing peaks at around P3, and therefore at P1 and P2, many transfected RPCs are likely at their last cell cycle before they become bipolar cells. If these RPCs exit the cell cycle before Smo protein is fully depleted, it might suggest that determination of bipolar cell fate in postmitotic cells is not greatly influenced by *Smo*.

Smo may also regulate bipolar cell formation by biasing the dual fate choice of RPCs towards bipolar cell fate rather than photoreceptor fate, given the observation that bipolar cells and photoreceptors were inversely affected by either the deletion or

activation of *Smo*. Future studies could investigate the expression level of Blimp1 in Smo-modulated cells; because Blimp1 is the key regulator that controls fate choice between bipolar cell fate and photoreceptor fate (Brzezinski et al 2013, Wang et al 2014). The role of Smo in promoting bipolar cell development may be instructive because other mitogens such as EGF and FGF2 did not promote bipolar cell development specifically (Yu et al 2006); even though both of these factors have been found to promote the progression of cell cycle of RPCs (Liang et al 2010, Spence et al 2007).

Notably, the phenotype of *Smo* knock-out in P0 RPCs in this study shared a remarkable similarity with the phenotype of *Notch1* knock-out in P0 RPCs (Mizeracka et al 2013b) in the RPCs. Both mutants resulted in complete ablation of INL cells except for amacrine cells and a significant increase in the number of photoreceptors. Overlapping phenotypes were also seen in retinas with clonal inactivation of Notch1 in postnatal RPCs (Jadhav et al 2006b). These similar phenotypes could be a result of the overlap of target genes in Notch and Hh pathways (Li et al 2012, McNeill et al 2012, Wall et al 2009) as well as the Notch-mediated Hh signaling transduction in which Notch signaling primes the RPCs to respond to Hh (Kong et al 2015, Ringuette et al 2016, Stasiulewicz et al 2015). The increased production of photoreceptors in *Notch1* knock-out retinas was associated with the upregulation of Blimp1 (Mizeracka et al 2013b), the photoreceptor fate determining factor. Given the overlap of target genes between Notch and Hh signaling pathways, it is reasonable to postulate that Blimp1 may also be a target gene of Hh signaling. Blimp1 may be upregulated in *Smo* deficient RPCs to facilitate the fate choice biasing of photoreceptors. Future studies could examine the phenotype of *Notch1* knock-out in a genomic context in which *Smo* is activated or deleted.

4.3 Cone bipolar cell subtype formation is dependent on *Smo* activation in postnatal RPCs

The specification of cone and rod bipolar cell subtypes is regulated by intrinsic factors (summarized in Figure 25). The contribution of extrinsic factor has not been well studied. However, some evidence suggests that Shh may be implicated in the formation of bipolar cell subtypes. For example, the development of cone bipolar cells depends on the presence of retinal ganglion cells (Bai et al 2014), the only source of Shh in developing retinas. In addition, conditional *Shh* deletion in the retina from embryonic stages resulted in a severe reduction of cone bipolar cell rather than rod bipolar cells (Wang, Star and Wallace and Chow, 2014, unpublished observations), suggesting that the development of cone bipolar cells requires the presence of Shh. However, since Hh signaling also affects RPC proliferation (Sakagami et al 2009) and early born cell type development (Zhang & Yang 2001), the phenotype of selective cone bipolar cell loss may be a confounding cumulative mutational effect. My study was designed to directly target the Hh signaling downstream component Smo only within target cells (RPCs) and restrict the experimental timing to postnatal stage; this approach would eliminate the cumulative mutational effect caused by *Shh* deficiency. I found that the development of cone bipolar cells was associated with the activation of *Smo* in RPCs, while the development of rod bipolar cells was *Smo*-independent (Figure 25). Interestingly, transfection carried in different stages resulted in different proportions of cone and rod bipolar cells within the transfected cells by P10, with P1 being the stage when most of the transfected bipolar cells became cone types. This result suggested that RPC competency may be implicated in the formation of cone and rod bipolar cell subtypes. P1 RPCs may have greater competence to generate cone bipolar cells than P0 and P2 RPCs. Thus, it may seem less surprising that knocking-out *Smo* in P1 RPCs caused a greater reduction in overall cone bipolar cell production, as the cone-type competence of the RPCs may be disrupted by the lack of *Smo*. However, I also acknowledged that the complete clearance of Smo protein in the cell likely lagged several hours behind the timing of transfection, judging from the 2-hour turnover time of Smo fused to a fluorescent protein in HIH/3T3 fibroblasts cell lines (Kim et al 2014). This situation may

interfere my interpretation of timing-mediated Smo regulation in cone bipolar cell production. Our preliminary experiments have shown that rod bipolar cells are born slightly earlier than cone bipolar cells (Star et al., unpublished data, Chow lab); therefore, rod bipolar cells are more likely to be born before Smo protein clearance is completed than cone bipolar cells. This may explain why rod bipolar cells are less affected by *Smo* deletion. It is worth mentioning that the birth order of bipolar cells is still equivocal. While evidence from our lab suggests the rod bipolar cell birth peaks before cone bipolar cells, a published study suggests that cone bipolar cells are born earlier than rod bipolar cells (Morrow et al 2008). The discrepancy may be attributed to different birth dating techniques for determining bipolar cell birth order. The published study used tritiated thymidine to "pulse" label retinal cells in different developing stages (Morrow et al 2008); while our study applied a "window labeling" technique in which two distinct thymidine analogues CldU and IdU were administrated sequentially in developing retinas. It has been demonstrated that, compared to pulse labeling technique, window labelling technique allows a greater refinement of the temporal analysis of cell cycle exit (Repka & Adler 1992). Overall, my result indicates that *Smo* activation in postnatal RPCs was necessary and sufficient to promote the formation of cone bipolar cells.

How does Smo regulate the development of cone bipolar cells? I postulate two possible explanations. First, Smo may bias the postnatal RPCs to adopt a cone bipolar cell fate over a rod bipolar cell fate, producing more cone bipolar cells as a result. Secondly, Smo may facilitate the differentiation and cell survival of cone bipolar cells. I favoured the first explanation because caspase 3 immunoreactivity was not up-regulated in *Smo* knock-out retinas by P10, suggesting that the loss of cone bipolar cells was less likely caused by increased apoptosis. However, I could not rule out the possibility that cell death may happen quickly after the transfection, and therefore may not be detectable by P10. Future studies can examine cell death within 48 hours of *Smo* deletion. In fact, apoptosis assessment has been done in retinas to address the mechanism by which the development cone bipolar cells was selectively dependent on the presence of ganglion cells. This assessment concluded that the reduced number of cone bipolar cells in retinal ganglion cell-depleted retinas was not a result of induced cell apoptosis (Bai et al 2014). Because ganglion cells are the only source of Shh in developing retinas, the fact that no

induced cell death was observed when *Smo* was deleted in RPCs again supports my hypothesis of the fate-determining model rather than the cell surviving model. It remains unclear, though, whether or not the role of Smo is dependent on its mitogenic effect in the fate-determining model. Given that the number of cone bipolar cells was not restored in *Shh*-/- mice when crossed to a *p27kip* (a cell cycle inhibitor) null background (Wang, Star and Wallace and Chow, 2014, unpublished observations), it is reasonable to hypothesize that the regulation of Smo in cone bipolar cell production is independent on its mitogenic effect.

My results also indicated that *Smo* activation in postmitotic bipolar cells was not sufficient to induce cone bipolar cell subtypes. However, because the onset of Cre-mediated *Smo* activation in *Vsx2-5.3-PRE-Cre* transgene occurs between 24 and 36 hours after the bipolar cells are born (Nickerson et al 2011), the function of *Smo* before this period of time is therefore unknown. It is possible that Smo is functional in newly born postmitotic cells but has no effect in later stages. On the other hand, it remains unknown whether Smo is required by postmitotic bipolar cells to form into cone subtypes because the attempt of deleting *Smo* in postmitotic bipolar cells was not successful. This attempt aimed to deliver Chx10BP-Cre plasmid into retinas to drive the expression of bipolar cell-specific Cre recombinase. Unexpectedly, Cre cleavage activity spread widely across bipolar cells, photoreceptors and other INL cells, suggesting a leaky activation of the Chx10BP element. This element has been used to successfully drive the expression of downstream mCherry specifically in bipolar cells (Wang et al 2014). This success, however, does not necessarily guarantee that activation of this element is specific. For example, the leakiness may happen transiently and subtly, however, sufficiently enough to drive traces of Cre expression to turn on the expression of the flox-based reporter plasmid. It is more likely that the leakiness occurs in postmitotic cells rather than RPCs because I did not observe loss of bipolar cells or other INL cells in transfected SmoCKO retinas; if Cre leaked into RPCs and deleted *Smo*, according to the results from the previous section, bipolar cell formation would be impaired.

Fortunately, in mice with *Vsx2-5.3-PRE-Cre* background (Nickerson et al 2011), Cre cleavage activity was specific in bipolar cells, as shown in this study. In fact, I have

attempted to cross SmoCKO mice to *Vsx2-5.3-PRE-Cre* mice in the hope of deleting *Smo* in bipolar cells specifically. However, I was not able to get *Cre*+ litters that were homozygous for *SmoCKO* alleles. We found in some strains, such as Ai3, that *Vsx2-5.3-PRE-Cre* displayed leaky expression (data not shown); therefore, it is possible that in SmoCKO mice, *Cre* expression is leaky. If the leakiness occurs embryonically, it may explain why I was not getting *Cre*+, *SmoCKO*/*SmoCKO* litters because embryonic deletion of *Smo* is lethal. Future studies can focus on creating a SmoCKO line in a conditional reporter background so that the Cre activity can be detected; the modified SmoCKO-reporter mice can then be used to cross with *Vsx2-5.3-PRE-Cre* mice to acquire specific *Smo* deletion in bipolar cells.

4.4 The regulatory logic of Hh signaling *in vitro*

Hh is a well-known morphogen in neural development (Varjosalo & Taipale 2008). However, it is difficult to address the morphogenic effect *in vivo* because it is challenging to manipulate the Hh dosage *in vivo*. Instead, *in vitro* pharmacological experiments provide easy access for modulating the Hh signaling dosage by applying graded signaling agonist. However, in my attempts of treating retinas with graded Hh, I found that the production of both cone and rod bipolar cells was induced. This result may seem somewhat contradictory to the *in vivo Smo* activation experiment. However, considering that the context of the *in vitro* and *in vivo* experiments were very different, the results may seem less surprising. The addition of Hh signaling agonist in explant cultures could exert a much broader effect on the overall retinal environment compared to specific targeting of *Smo* in RPCs. Despite the inducing effect on both cone and rod bipolar cells, agonist at the highest concentration did not induce the production of cone bipolar cells. Given that the condition is too extreme with the dosage of agonist, it is difficult to draw a conclusion at this point. However, it can be speculated that over-threshold Hh signaling could inhibit the development of cone bipolar cells but not rod bipolar cells. In addition, the results also suggested that the development of ON bipolar cells may favor the presence of Hh signaling because the addition of graded agonist significantly induced the production of ON type bipolar cells rather than OFF type bipolar cells. Future studies can examine ON-type and OFF-type bipolar cell production

in vivo using the same electroporation approach as in *Smo* conditional mice crossed to an mGluR6:NLS-βgal background described in the thesis.

Despites conflicting results regarding bipolar cell subtype formation, Hh agonist consistently induced bipolar cells while inhibiting photoreceptors. The inhibiting effect of Hh agonist on photoreceptors appeared to be more profound than the stimulating effect on bipolar cells. In addition, agonist treatments carried out at later stages (P3 and P6) did not cause obvious change regarding cell types. These results may have been caused by committed RPCs not responding to Hh signaling.

4.5 Conclusions and future study

My work has presented the dynamics of Hh signaling in the postnatal retina, and found evidence that some bipolar cells are Hh signaling reporter active. Furthermore, my work has demonstrated that the development of bipolar cells requires the activation of *Smo* in postnatal RPCs. More importantly, this is the first study to address the contribution of Smo, the downstream component of Hh signaling, in selectively regulating the development of cone but not rod bipolar cell subtypes. The results also revealed that Smo activation in postmitotic bipolar cells is not sufficient to affect bipolar cell subtype formation.

Future studies should be conducted to accomplish the following goals:

1) Confirm that Hh signaling reporter turns on after bipolar cell exit the cell cycle, and to explore whether or not Hh signaling components are expressed in postmitotic bipolar cells. This goal can be accomplished by using a new Hh signaling reporter. In this reporter, the expression of fluorescents relies on both the binding activity of Gli-binding-site and Cre-mediated cleavage of the upstream stop codon. For example, this reporter will be useful when introducing into the Vsx2-5.3-PRE-Cre mice, in which Cre only expresses in bipolar cells.

2) Explore the mechanism through which the development of pan bipolar cells is dependent on the postnatal activation of *Smo*, and how does the competence stage of RPCs mediates the effect of *Smo* deficiency. This could be accomplished using the previously described single cell profiling technique in which transfected cells are

isolated and used for running microarray to determine the fold change of selective target genes (Mizeracka et al 2013b). Candidate genes could be bipolar cell fate determinants Chx10, Math1, and Math3, as well as markers for photoreceptors and other INL cells. Cone and rod bipolar cell makers and regulators such as Vsx1 (cone regulator), bhlhb4, bhlhb5, and prdm8 (rod regulators) should also be included to investigate the mechanism through which Smo selectively regulates cone bipolar cell formation.

3) Visualize Hh signaling activity when deleting or activating *Smo* in order to confirm whether the Smo-mediated regulation of bipolar cell development relies on the canonical Hh signaling pathways.

4) Since the phenotypes of gain or loss of function of Smo and Notch1 greatly overlaps, it would be interesting to study the bipolar cell development in a *Notch1* CKO lines that have a conditional *Smo* genetic background.

To summarize, this study has filled in the gap in understanding about extrinsic regulation of bipolar cell subtype formation. It suggests a novel downstream role for Hh signaling in directing neuronal subtype formation in the postnatal retina.

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Appendix

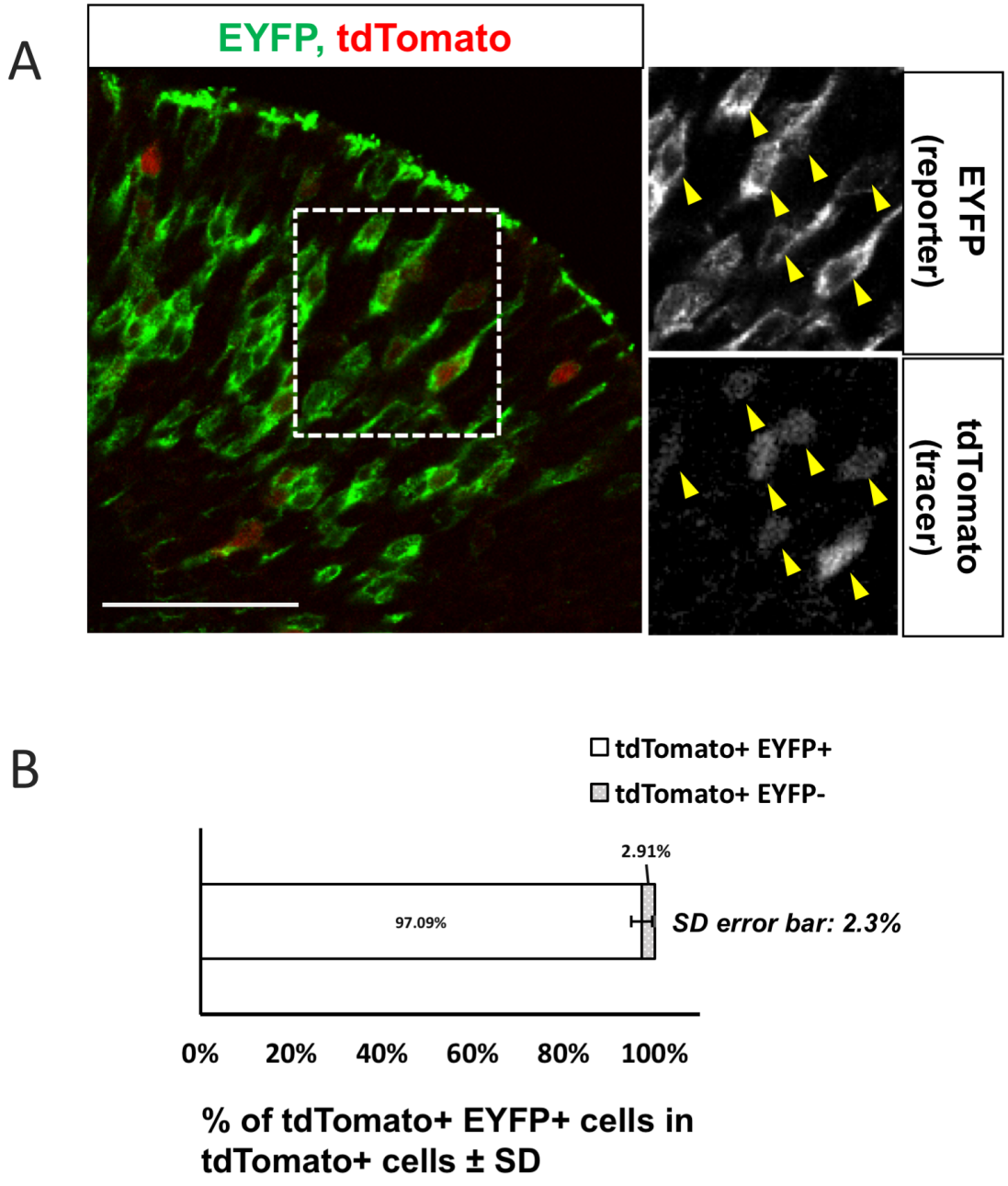

Figure A1 Use of CAG-NLS-tdTomato as a tracer to indicate Cre activity + cells. **(A)** Retinas from SmoM2 mice were transfected with pCAG-Cre and pCAG-NLS-tdTomato at 10:1 molar ratio at P0. Retinas were harvested at P4 and stained with anti-GFP and anti-dsRed. Cells that were successfully transfected with pCAG-Cre would

express fusion protein SmoM2-YFP, and therefore YFP served as the reporter of Cre activity. Nearly all of the tdTomato+ cells were YFP+ (arrowheads). Note that not all YFP+ cells were tdTomato+. Scale bar: 50 μm. **(B)** Retinas mentioned above were counted to determine the percentage of YFP+ cells within tdTomato+ cells. This percentage represents the efficiency of CAG-NLS-tdTomato as a tracer to indicate Cre+ cells. 534 cells were counted in n=4 retinas. SD: standard deviation.