RBPJ-DEPENDENT AND -INDEPENDENT NOTCH2 SIGNALING REGULATES CILIARY BODY DEVELOPMENT IN THE MOUSE EYE

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ABSTRACT

The ciliary body (CB) is a two-layered structure in the anterior eye, which is composed of the pigmented outer ciliary epithelium (OCE) and the non-pigmented inner ciliary epithelium (ICE). It is responsible for aqueous humor secretion and lens accommodation. Despite the important roles in maintaining normal eye functions, its development still remains poorly understood. The Notch signaling pathway is an evolutionarily conserved pathway that has diverse functions during tissue development and homeostasis. Canonical Notch signaling is mediated through the recombination signal binding protein for immunoglobulin kappa J region (RBPJ)-dependent transcription activation and repression. In this study, I have demonstrated that Notch2 and RBPJ are important regulators of CB development by conditionally deleting them in the developing CB. Conditional knockout of either Notch2 or RBPJ causes severe dysgenesis of the CB, although both of them are dispensable for cell fate determination of the ciliary margin zone (CMZ). RBPJ-dependent Notch2 signaling regulates CB morphogenesis partially through the promotion of cell proliferation and the maintenance of active bone morphogenetic protein (BMP) signaling in the OCE of the CB. Surprisingly, RBPJ-independent Notch2 modulates BMP signaling in the ciliary stroma cells via repressing the expression of two secreted BMP inhibitors, chordin-like 1 (Chrdl1) and neuroblastoma suppression of tumorigenicity 1 (Nbl1). In addition, Notch2-independent RBPJ also controls cell adhesion mediated by neural cadherin (N-cadherin) in the OCE to hold together two CB layers. Finally, I have shown that RBPJ in the ICE regulates Opticin (Optc) expression and secretion. Therefore, this study has revealed important roles of RBPJ-dependent canonical Notch2 signaling in regulating CB morphogenesis and development, and has also uncovered RBPJ-dependent and -independent regulation of BMP signaling by Notch2 in the developing CB.

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ABBREVIATIONS

Genes:

aPKC: atypical protein kinase C

Aqp1: aquaporin 1

AR: α2-adrenergic receptor

BMP: bone morphogenetic protein

Chrdl1: chordin-like 1

Col IX: collagen IX

Cx43: connexin 43

CYP1B1: Cytochrome P450, Family 1, Subfamily B, Polypeptide 1

Dll: Delta-like

FGF: fibroblast growth factor

Hey: hairy/enhancer-of-split related with YRPW motif

Lef1: lymphoid enhancer binding factor 1

Lmx1b: LIM homeobox transcription factor 1 beta

Mart1: melan-A, also called Mlana

Msx1: msh homeobox 1

MYOC: myocilin

Nbl1: neuroblastoma, suppression of tumorigenicity 1

Ncad: cadherin 2, neural cadherin

NICD: notch intracellular domain

Optc: opticin

Otx1: orthodenticle homolog 1

Par3: par-3 family cell polarity regulator

Par6: Partitioning defective protein 6

Pax6: paired box 6

Pitx2: paired-like homeodomain transcription factor 2

Pou4f3: POU domain, class 4, transcription factor 3

pSMAD: phospho-SMAD

Ptf1a: pancreas specific transcription factor, 1a

RBPJ: recombination signal binding protein for immunoglobulin kappa J region

Trp1: tyrosinase-related protein

Tyrp2: tyrosinase-related protein 2, also called dopachrome tautomerase (Dct)

Wnt: wingless-type MMTV integration site family

ZO-1: zonula occludens 1

Terms:

AH: aqueous humor

AJ: adherens junction

CB: ciliary body

CKO: conditional knockout

CMZ: ciliary marginal zone

EGFP: enhanced green fluorescent protein

F-actin: filamentous actin

ICE: inner ciliary epithelium

IOP: intraocular pressure

IRES: internal ribosome entry site

miRNA: microRNA

OCE: outer ciliary epithelium

POAG: primary open angle glaucoma

RGC: retina ganglion cell

RPE: retinal pigment epithelium

shRNA: short hairpin RNA

SLRP: small leucine rich repeats proteins

TM: trabecular meshwork

Z/EG: lacZ/EGFP

Reagents:

BrdU: 5-bromo-2'-deoxyuridine

CAG: CMV early enhancer/chicken β-actin (CAG) promoter

CAI: carbonic anhydrase inhibitor

CMV: Cytomegalovirus

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco's modified Eagle's minimal essential medium

DTA: diphtheria toxin A

H&E: Hematoxylin and eosin

HRP: horseradish peroxidase

IgG: immunoglobulin G

IP: immunoprecipitation

NBT/BCIP: Nitro blue tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate

NSS: normal sheep serum

PBS: phosphate-buffered saline

PFA: paraformaldehyde

SDS: sodium dodecyl sulfate

TdT: Terminal deoxynucleotidyl transferase

TUNEL: Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling assay

CHAPTER ONE: BACKGROUND

CILIARY BODY OVERVIEW

Introduction

The mammalian eye is mainly composed of three parts: the anterior segment, the vitreous body and the posterior retina. The anterior segment contains ciliary body (CB), iris, lens, cornea and aqueous drainage structures, whereas the posterior retina is organized into three distinct cell layers with six types of retinal neurons and Müller glial cells (Figure 1.1 A). In order for the eye to precept the light, the inside of the eye must remain avascular to keep the lens and cornea clear. Thus the aqueous humor secreted from the ciliary body plays the role of blood replacement to nourish anterior avascular structures. After the production from the ciliary body, the aqueous humor enters the anterior chamber through the pupil and exits the eye through the drainage system at the angle between iris and cornea. Intraocular pressure (IOP) is maintained through the balance between the inflow resulting from the production of aqueous humor from the CB and the outflow by the drainage system. High level of IOP is often a risk factor of glaucoma (Stamer & Acott 2012). Additionally, zonule fibers attached to the lens from the ciliary muscle control the accommodation of the lens for far versus near sight vision. Despite the important roles the CB plays in maintaining normal eye function, how it forms and develops is still poorly understood.

Ciliary body anatomy

The CB consists of two apically adherent epithelial sheets: the inner ciliary epithelium (ICE) derived from the neural retina and the outer ciliary epithelium (OCE) from the retinal pigment epithelium (RPE), and the underlying stroma (Figure 1.1 B). Apical sides of the non-pigmented

ICE and the pigmented OCE are apposed to each other, where cell-cell junctions are formed. The basal side of the ICE faces the vitreous, whereas the basal side of the OCE faces the ciliary vasculature. The ciliary epithelium usually forms three to four folds to expand the cell surface area of the CB. Both layers extend anteriorly to form the iris epithelium, and the ICE of the CB gains large number of melanosomes as approaching to the iris side. The stroma of the CB contains fenestrated capillaries in close contact with the OCE, allowing rapid material communication between the CB and the systemic circulation system. The ciliary muscle also exists in the stroma to control the accommodation of the lens via zonule fiber contraction. The ciliary muscle in mice is less complex and smaller than the human counterpart, which is consistent with the weaker ability of lens accommodation in mice.

Ciliary body and glaucoma

Glaucoma is a group of ocular neuropathy conditions caused by degeneration of retinal ganglion cells (RGCs), resulting in progressive and irreversible loss of vision (Zhang et al 2012). And it is the second leading cause of blindness worldwide, and expected to affect nearly 80 million people by 2020 (Quigley & Broman 2006, Resnikoff et al 2004). Despite the fact that ganglion cell death is the direct cause for vision loss, the major approach for glaucoma therapy is to lower IOP, the only known treatable risk factor for glaucoma (McLaren & Moroi 2003).

There are two major types of glaucoma: open-angle glaucoma and closed-angle glaucoma, and more than 80% of glaucoma patients in the United States are open-angle form (Weinreb et al 2014). In patients of open-angle glaucoma, they experience increased aqueous outflow resistance by the trabecular meshwork (TM), although the structure of iridocorneal angle remains largely

normal. Pathogenic mutations of several TM and CB expressed genes have been identified to be associated with primary open-angle glaucoma (POAG), including myocilin (MYOC) and cytochrome P450 family 1 subfamily B polypeptide 1 (CYP1B1) (Coca-Prados & Escribano 2007, Weinreb et al 2014), indicating the CB can also regulate TM functionality to control IOP. The closed-angle glaucoma differs from the open-angle form in the obstruction of the iridocorneal angle by the apposition of part of the iris. Occlusion of the access to the outflow pathways leads to the increase of IOP. Acute angle-closure may need immediate treatment to prevent permanent vision loss.

Pharmacological interventions of production of the aqueous humor from the ciliary body or enhancement of outflow from trabecular meshwork and uveoscleral pathway are common medications for early stage glaucoma, but surgery will be required for certain types of glaucoma. Current glaucoma therapy includes five major classes of medications: β-blockers, α2-adrenergic receptor (AR) agonists, and carbonic anhydrase inhibitors (CAIs) can decrease the production of aqueous humor production by the CB, whereas muscarinic agonists and prostaglandin agonists lower IOP by enhancing trabecular and uveoscleral outflow (McLaren & Moroi 2003, Weinreb et al 2014). Taken together, the CB plays an important role in regulating the IOP and is often a major target site for glaucoma therapy.

Ciliary body and presbyopia

Presbyopia, literally meaning “old eye”, is characterized by age-related loss of the ability of lens accommodation, resulting in farsightedness. The CB controls lens accommodation via the anchorage to the lens of zonule fibers, the microfibrillar framework composed of collagen and

elastic fibers (Schachar 2006). Age-related ciliary muscle atrophy may be an important factor causing presbyopia (Croft & Kaufman 2006). Morphological changes in the ciliary muscle including the increase of connective tissue, shortening and widening of ciliary muscle may also explain the loss of ciliary muscle contraction in aged humans (Charman 2008).

CILIARY BODY DEVELOPMENT

Ciliary body development overview

The development of the eye is a complex and tightly controlled process (Chow & Lang 2001). The formation of optic primordia is initiated with bilateral evagination of the developing forebrain. Continued evagination of the optic primordia leads to the formation of optic vesicles. As optic vesicles approaching the surface ectoderm, signals from the surface ectoderm induce the morphogenesis and formation of the optic cup, whereas the lens placode induced reciprocally from the surface ectoderm also undergoes invagination to form the lens vesicle and the presumptive lens. The optic cup contains two layers of cells: the layer facing the presumptive lens develops into the neural retina, while the layer in contact with surrounding mesenchymal cells develops into the retinal pigment epithelium (RPE). The distal tip where these two layers meet develops into the ciliary body and iris.

In the mouse eye, the ciliary body/iris region (also known as the ciliary marginal zone) starts to be visible at the rim of the optic cup around embryonic day 13.5 (E13.5) (Figure 1.2). Continued extension of the optic cup margin delineates a distinguished boundary between the CB/iris and the retina. A single layer of cells extended from the retina forms the inner layer of the CB/iris, whereas the rest of the retina remains laminated with multiple layers of cells. The RPE layer

extends anteriorly to become the outer layer of the CB/iris. Neural crest-derived mesenchymal cells from surrounding tissue migrate underneath the tip of the optic cup to form the stroma cells of the ciliary body and the iris. Around postnatal day 0 (P0), the inner ciliary epithelium (ICE) and the outer ciliary epithelium (OCE) tend to form the first fold of the ciliary processes. The first two folds are usually formed around P3, and three to four folds are formed by P7. These folds continue to grow, extend and elaborate until the adult stage. Recent studies have elucidated important genes and signaling pathways that regulate the formation and function of the CB intrinsically and extrinsically.

Inductive role of the lens

The lens has long been believed to induce the formation of the ciliary body (Beebe 1986). Early studies on ocular congenital malformations show that the ciliary body and iris fail to develop in the absence of normal lens formation, suggesting an essential role of the lens in the induction of the ciliary body (Beebe 1986). An intentional removal of the lens from the embryonic rat eye abolish the formation of the ciliary body and iris (Stroeva 1967). A complimentary experiment done in chicken embryos shows that transplantation of a supernumerary lens into the optic cup can induce additional presumptive anterior structures (Genis-Galvez 1966). However, a recent repetition of lens removal experiments in chicken embryos only provided evidence for the role of the lens in the differentiation of the cornea (Beebe & Coats 2000). The inconsistency among these experiments could be explained by the coincidence of physical disruption of the eye structure, different experimental designs and species differences (Napier & Kidson 2007).

Additional doubts on the inductive role of the lens were raised when the lens was genetically ablated with *diphtheria toxin A* (*DTA*) under the control of a lens specific promoter. Transgenic mice carrying DTA under *αA-crystallin* (Harrington et al 1991) or *γF-crystallin* (Klein et al 1992) with lens degeneration around embryonic day 12 show the failure of normal ciliary body development. However, transgenic mice carrying an alpha-crystallin-diphtheria toxin hybrid gene (*lnl* mice) display normal ciliary body development despite of the microphthalmia phenotype in the transgenic mice (Key et al 1992). A more recent study using a modified crystallin promoter driven expression of an attenuated version of DTA (*Tox176*, a point mutation of wildtype DTA leading to 30 folds less cytotoxic) shows normal specification of the ciliary body, whereas the iris differentiation was blocked (Zhang et al 2007). However, crystallin starts its expression in differentiating lens fiber cells when the ciliary body differentiation program has already initiated. To further assess the necessity of the lens in the initiation of ciliary body specification and development, the same group expressed *Tox176* under a modified Pax6 promoter, which is expressed as early as in the surface ectoderm specifically (Zhang et al 2008). In the *Pax6-Tox176* transgenic mice, the ciliary body is properly specified shown by the expression of ciliary body markers Otx1 and Opticin, though later differentiation and morphogenesis of the ciliary body is severely disrupted. Taken together, these experimental results provide strong evidence for the important role of the lens in the ciliary body differentiation and morphogenesis, but possibly not specification.

Supporting role of the periocular mesenchyme

The developing eye is surrounded by the periocular mesenchyme, which has many important roles in the regulation of normal eye development. The periocular mesenchyme not only provides multiple cell types that become part of the eye, but also secretes signaling molecules to

pattern the ocular structure. The ciliary body also benefits from the periocular mesenchyme by the contributions of formation of neural crest-derived ciliary stroma and mesoderm-derived ciliary vasculature (Gage et al 2005).

Genetic manipulation in the periocular mesenchyme can affect the development of the ciliary body. Lmx1b, a LIM homodomain class transcription factor is highly expressed in the periocular mesenchyme. In the *Lmx1b* mutant eyes, there is an absence of the ciliary folds even though the ciliary marginal zone is clearly segregated from the rest of the retina (Pressman et al 2000). However, the postnatal development of the ciliary body was not characterized well due to perinatal lethality of *Lmx1b* mutant mice. By crossing *Lmx1b* flox allele with a neural crest specific cre line, *Pitx2-Cre*, Liu & Johnson 2010 was able to confirm that Lmx1b is indeed required for the formation of ciliary folds and trabecular meshwork (Liu & Johnson 2010). Opticin was detected in the neural crest specific *Lmx1b* conditional mutant ciliary body, suggesting the ciliary body is properly specified but further morphogenesis depends on the periocular mesenchyme.

A more recent study involving knockout of p120-catenin in the neural crest cells provides another piece of evidence of the ciliary body development regulated by the neural crest derived periocular mesenchyme (Tian et al 2012). *Wnt1-Cre*-mediated depletion of p120-catenin leads to ciliary body hypoplasia and loss of trabecular meshwork structure. However, further examination of molecular markers of the ciliary body is needed to determine whether the ciliary body is correctly specified. Additionally, radial capillaries in the mesenchyme in close contact with the outer layer of the ciliary body are also believed to control the regularity of the folds (Beebe

1986). Taken together, current evidence suggests a supporting role of the periocular mesenchyme in the morphogenesis of the ciliary body.

CB specification

FGF signaling has been shown to be an important regulator of the differentiation of the retina and RPE. Exposure to FGF molecules or activated FGF signaling cascade can convert RPE into neural retina-like structure (Dias da Silva et al 2007, Galy et al 2002, Hyer et al 1998, Vogel-Hopker et al 2000, Zhao et al 2001). Interestingly, Dias da Silva *et al.* noticed that ciliary body tissues appeared in the transitioning zone between the RPE and the RPE-transformed neural retina where ectopic FGF4 was introduced to the RPE cells (Dias da Silva et al 2007). The non-neurogenic, non-pigmented tissue expresses high levels of ciliary body markers including Collagen IX, laminin, nidogen, thymyosin β, Connexin43 and tenascin C, but not Wnt2b. The authors proposed a model for the CB specification: in the chicken eye, the overlapping gradients of FGF and BMP synergistically produce an environment favorable for CB specification. Zhao *et al.* support this model by showing the expansion of the ciliary marginal zone in *FGF9* null mouse eyes (Zhao et al 2001). Together, these results show that FGF signaling is important for the patterning of the CB region.

Wnt2b and downstream Wnt signaling components are highly expressed in the peripheral CMZ in avian eyes (Cho & Cepko 2006, Kubo et al 2003). Activation of Wnt signaling by overexpressing Wnt2b or constitutive active β-catenin in the central retina leads to ectopic expression of CB markers, whereas inhibition of Wnt signaling by overexpressing a dominant negative Lef1 reduces the expression of CMZ genes and CB/iris hypoplasia. Similar

experiments have been done in the mouse eye. In the mouse eye, the expression of active β-catenin in the peripheral retina sufficiently induces, whereas its inactivation reduces, the expression of known CB markers (Liu et al 2007). Together, these results indicate that canonical Wnt signaling is sufficient and necessary for CB specification.

miRNAs are small non-coding RNAs that can regulate gene expression levels by controlling the stability or translation efficiency of the mRNA. Therefore, they are often important regulators of tissue development. Expressions of multiple miRNAs are found in the mouse eye during the ocular development (Hackler et al 2010, Xu et al 2007). The role of miRNAs in CB development was investigated by genetic ablation of *Dicer1* (Davis et al 2011). Inactivation of *Dicer1*, the key enzyme involved in miRNA biogenesis leads to abrogation of all miRNAs. Upon *α-Cre* or *Tyrp2-Cre* mediated depletion of Dicer1 in either layer of the CB at progenitor stage, the presumptive CB region failed to develop normally but developed into a cryptic population of cells with a mixed phenotype of neuronal and CB progenitors instead. Interestingly, postnatal deletion of Dicer1 using *Pou4f3-Cre* line leads to hyperplastic and disorganized CB. These results suggest that miRNAs are essential for the specification and differentiation of the CB, and are continuously required for proper morphogenesis and organization of the CB in postnatal stages.

CB morphogenesis

Following the specification, the CB undergoes folding formation during the first week after birth. Intraocular pressure has been suggested to be a physical drive force for ciliary fold formation in the avian eye (Bard & Ross 1982b, Coulombre 1957). Intubation of the chicken eye by inserting

microcapillary tubes eliminates ciliary processing, while applying physical constraints around the anterior of the eye can induce folding (Napier & Kidson 2007). Changes in cell densities, heights and volumes also correlate with morphological changes (Napier & Kidson 2005, Reichman & Beebe 1992). In the mouse, Napier *et al.* observed a surge of proliferation in the OCE of the CB around P0, with significant lower levels of proliferation rate in the ICE. The differential proliferation may induce a bulging of the OCE and promotes CB morphogenesis (Napier & Kidson 2005). Genetic studies have identified several genes and pathways controlling CB morphogenesis.

BMP proteins have been shown expressed in the ciliary margin of the optic cup, and are essential for CB morphogenesis (Chang et al 2001, Zhao et al 2002). A heterozygous deficiency of BMP4 causes severe malformation of the CB and the drainage structure, and the mutant mice develop glaucomatous phenotype similar to that of human patients with congenital glaucoma (Chang et al 2001). A lens specific overexpression of Noggin, a secreted potent BMP antagonist that binds to BMP proteins and prevents their association with BMP receptors, results in loss of the ciliary body (Zhao et al 2002). Interestingly, the ciliary body phenotype could be rescued with ectopic overexpression of BMP7, demonstrating that defective BMP signaling is the cause of CB morphogenesis defects.

Transcription factor Pax6 is a highly conserved master regulator of the eye development (Chow et al 1999, Grindley et al 1995, Quiring et al 1994). It is expressed throughout the development, differentiation and maturation stages of the CB and iris. In humans, Pax6 mutations have been associated with aniridia (no iris formation) and Peters' anomaly (congenital corneal opacity)

(Graw 1996, Napier & Kidson 2007). In mice, the haploinsufficiency of Pax6 induces iris hypoplasia but with normal development of the CB (Davis-Silberman et al 2005). Complete knockout of Pax6 using *Tyrp2-Cre* in both layers of the CB leads to dysgenesis of the CB, whereas overexpression of additional copy of Pax6 results in severe structural aberrant of the CB, indicating that the gene dosage of Pax6 is important for normal development of the CB (Davis et al 2009).

Otx1 is a homobox gene that plays important roles in normal morphogenesis and differentiation of neural tissues. Otx1 is expressed early in the dorsal optic vesicle and gradually restricted to the optic stalk, presumptive RPE and CMZ (Martinez-Morales et al 2001). *Otx1* null eyes exhibit a grossly normal phenotype except the absence of the ciliary processes and reduction of the iris size (Acampora et al 1996, Martinez-Morales et al 2001). Otx2 may compensate with Otx1 in the development of other ocular structure due to the overlapping expression patterns (Martinez-Morales et al 2001). However, no further analysis was performed to characterize the defects of the CB in *Otx1* null eyes. Further experiments are needed to investigate the exact role of Otx1 in ciliary body morphogenesis.

CELL ADHESION AND CB MORPHOGENESIS

Cell adhesion plays important roles in epithelial morphogenesis. Nectin1 and Nectin3, Ca^{2+} independent immunoglobulin like cell adhesion molecules are expressed in the cell junctions between ICE and OCE and apicolateral junctions between OCE cells of the CB. Knockout of either Nectin1 or Nectin3 leads to separation of apex junctions and abrogation of CB fold formation, but not CB specification and survival (Inagaki et al 2005). In addition to nectins,

multiple gap junction proteins are also detected in the junctions of the CB (Coffey et al 2002). Cx43 and Cx40 are enriched at the apical junctions between the ICE and OCE, whereas Cx26 is expressed at the apicolateral side of the ICE and Cx31 expressed at the basal side of the ICE. Inactivation of Cx43 in either layer of the CB does not cause CB morphogenesis defects, indicating the dispensable role of Cx43 in regulating the morphogenesis of the CB (Calera et al 2006, Calera et al 2009). However, when Cx43 is depleted in the OCE by *Nestin-Cre*-mediated gene knockout, there is a complete loss of the vitreous body and pathological changes consistent with the loss of intraocular pressure (Calera et al 2006). Similarly, knockout of Cx43 in the ICE with *Pax6α-Cre* leads to a significant reduction of the IOP although no obvious structural difference was observed (Calera et al 2009). These results indicate that gap junctions are important for the secretory function but not the morphogenesis of the CB.

Classical cadherin proteins, a family of Ca^{2+} dependent adhesion molecules are single-pass transmembrane proteins of particular importance in the dynamic regulation of cell adhesion during morphogenetic processes (Gumbiner 2005). The extracellular domain of classical cadherin contains five cadherin-type repeats bound together by Ca^{2+} ions. Homophilic binding of the extracellular domain mediates adhesive function between adjacent cells, whereas the intracellular domain interacts with β-catenin that binds to α-catenin and connects to the actin cytoskeleton. Cadherin-catenin complexes form adherens junction. The CB, as an extension of the neural epithelium, expresses N-cadherin (neural cadherin) instead of E-cadherin (Xu et al 2002). This study reveals important role of N-cadherin in the regulation of CB morphogenesis.

NOTCH SIGNALING

Notch signaling is an evolutionary conserved signaling pathway that has diverse functions during tissue development and homeostasis (Andersson et al 2011). Canonical Notch signaling involves the interaction between Notch receptors and Notch ligands. In mammals, there are four Notch receptors (Notch 1-4) and five Notch ligands (Delta-like 1, 3 and 4; Jagged 1 and 2). Upon engagement of Notch receptor with the ligand from the neighboring cell, the Notch intracellular domain (NICD) is then cleaved off the receptor and translocated into the nucleus where it binds with RBPJ to initiate transcription of Notch downstream target genes to control cell proliferation, cell death, cell fate specification and differentiation, tissue patterning and homeostasis in a context dependent manner (Figure 1.3) (Kopan & Ilagan 2009). Disruption of Notch signaling transduction has been suggested to be associated with multiple human disorders, ranging from heritable genetic diseases like Alagille syndrome and Hajdu-Cheney syndrome to adult onset diseases including cancer and Alzheimer's disease (Koch & Radtke 2007, Kopan & Ilagan 2009, Turnpenny & Ellard 2012).

Notch signaling is also involved in the ocular development. All four Notch receptors have been reported to express in the developing eye (Bao & Cepko 1997, Claxton & Fruttiger 2004, Lindsell et al 1996). Notch1 is mainly expressed in the retinal progenitors, where it promotes proliferation and prevents premature differentiation of progenitor pools (Jadhav et al 2006, Yaron et al 2006). Notch1 is continued to be required at later differentiation stage to ensure proper differentiation of rod and cone photoreceptors (Jadhav et al 2006, Mizeracka et al 2013). The expression pattern of Notch3 includes the lens epithelium, central retina and the ciliary marginal zone, whereas Notch4 is detected in the retinal vasculature (Claxton & Fruttiger 2004,

Kitamoto et al 2005, Lindsell et al 1996). However, no CB defects was reported in *Notch3* null or *Notch4* mutant eyes, suggesting the redundancy of Notch gene family in the CB development (James et al 2014, Kitamoto et al 2005, Krebs et al 2000, Krebs et al 2003). Notch2 is highly expressed in the developing lens epithelium and the RPE layer including the OCE layer of the CB (Bao & Cepko 1997, Lindsell et al 1996, Zhou et al 2013). Conditional ablation of Notch2 in the lens blocks lens differentiation and morphogenesis, indicating the important role of Notch2 in lens development (Saravanamuthu et al 2012). This study provides the evidence that Notch2 is also required for the morphogenesis of the CB by conditional inactivation of *Notch2* in the OCE using *Trp1-Cre*-mediated gene knockout approach, which will be discussed further in this study (Zhou et al 2013). An independent study from Sarode *et al.* confirmed my results with a different Cre line: *Mart1-Cre* (Sarode et al 2014).

RBPJ is the common effector required for all four Notch receptors to exert their function via transcriptional regulation. In the absence of NICD, RBPJ can recruit transcription co-repressors to inhibit transcription of downstream targets. The binding of NICD to RBPJ acts as the transcriptional switch to turn on gene expression (Kopan & Ilagan 2009). Besides its vital role in canonical Notch signaling, evidences of Notch independent RBPJ functions have emerged recently. In flies, loss of *Su(H)*, the *Drosophila* homolog of RBPJ has a weaker phenotype than *Notch* mutants in many context including bristle formation, wing development and dorsal-ventral boundary formation (Castro et al 2005, Koelzer & Klein 2003, Koelzer & Klein 2006, Morel & Schweisguth 2000). This phenomena is possibly explained by the gene expression repression role of Su(H) to at least partially compensate the loss of Notch signaling (Johnson & Macdonald 2011). In addition, transcription factor Ptf1a has been shown to interact with RBPJ and initiate

transcription independent of Notch signaling in the pancreas and spinal cord cell fate determination (Hori et al 2008, Masui et al 2007). Together, these results extend our knowledge of roles of RBPJ in both canonical Notch and Notch-independent signaling pathway.

In summary, the CB presents an attractive model to study epithelial tissue development that involves various signaling pathways. As an important structure of maintaining normal eye functions, the regulation of the function of the CB is directly related to the IOP management, whose elevation is a high risk factor for glaucoma. Taking advantage of powerful mouse genetic tools, I identified Notch2 and RBPJ as important regulators for the development and function of the CB, which could provide novel insight into pathogenesis of glaucoma and possible new treatments.

Figure 1.1 Anatomy of the mouse eye

(A) H&E staining of a cross section of the adult mouse eye. The eye can be separated into three compartments: the anterior segments, the vitreous body, and the posterior retina. The anterior segments is composed of the cornea, the iris, the lens, and the ciliary body. The space between cornea and the pupil is the anterior chamber filled with aqueous humor, and the vitreous body occupies the space between the retina and the lens. The retina is organized into three distinct layers of retinal neurons, and the optic nerve is formed of axons of ganglion cells exiting the eye through the optic disc in the center of the retina.

(B) A closer view of the ciliary body (CB). The ciliary body extends from the retina, and continues with the iris. Two layers of epithelium (ICE and OCE) and underneath stroma constitute the CB.

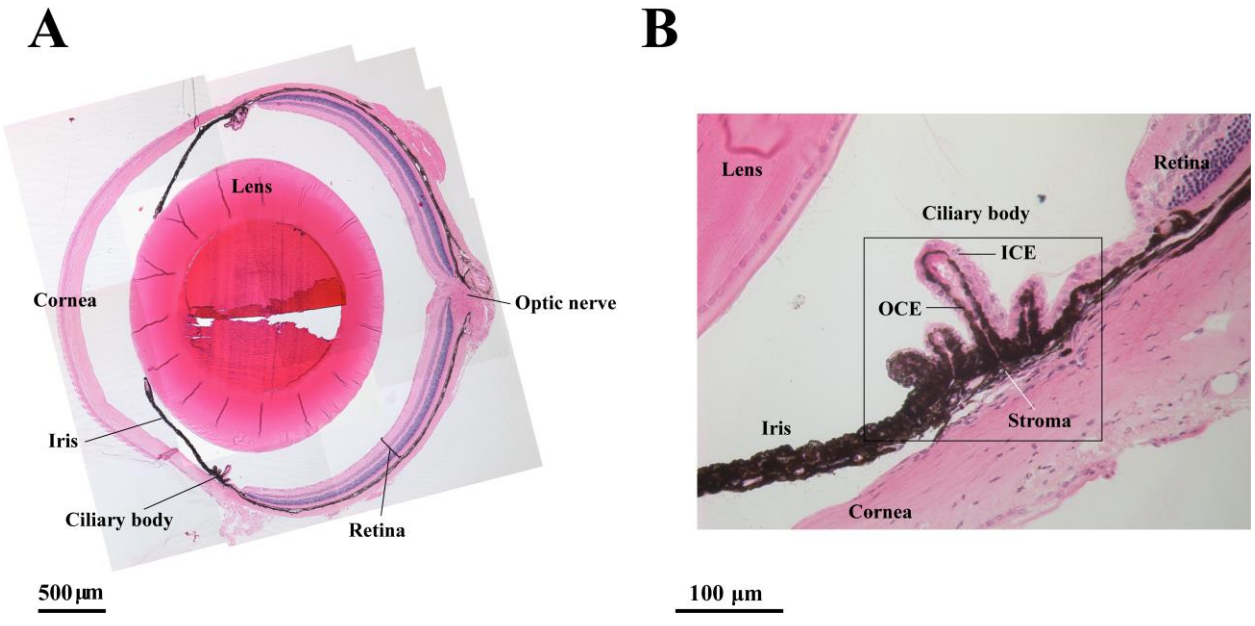

A
Lens
Cornea
Optic nerve
Iris
Ciliary body
Retina
500 µm
B
Lens
Retina
Ciliary body
ICE
OCE
Iris
Stroma
Cornea
100 µm

Figure 1.2 Development of the ciliary body

(A) Schematic illustration of the structure of the CB. The CB contains the ICE derived from the neural retina, the OCE from the retina pigment epithelium (RPE) and underlying stroma migrated from the periocular mesenchyme.

(B-D) Prenatal development of the CB at various stages. The presumptive CB/iris starts to develop from the rim of the optic cup at E13.5 (**B**). The ciliary marginal zone continues to extend anteriorly. Around E16.5 (**C**), a clear boundary between the retina and the CB/iris is formed. The CMZ grows extensively, and a bulge forms at the CB region to segregate from the iris around E17.5 (**D**).

(E-H) Postnatal development of the CB at different time points. Two epithelial layers of the CB start to fold together around P0 (**E**), and drastic morphogenetic changes occur during the first week after birth. One or two folds are usually formed around P3 (**F**), and around P7 the ciliary processes normally contain three to four folds (**G**). These folds continue to elaborate till adulthood (**H**). The CB regions are highlighted by boxes, in which the yellow arrows show the ICE, whereas the green arrowheads indicate the OCE.

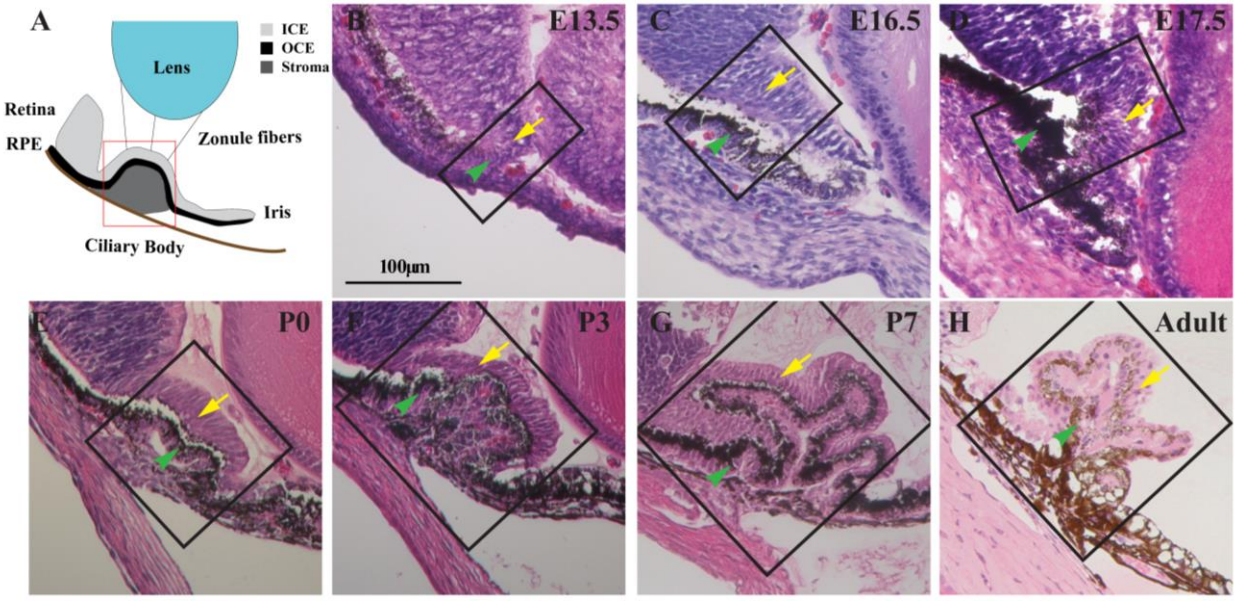
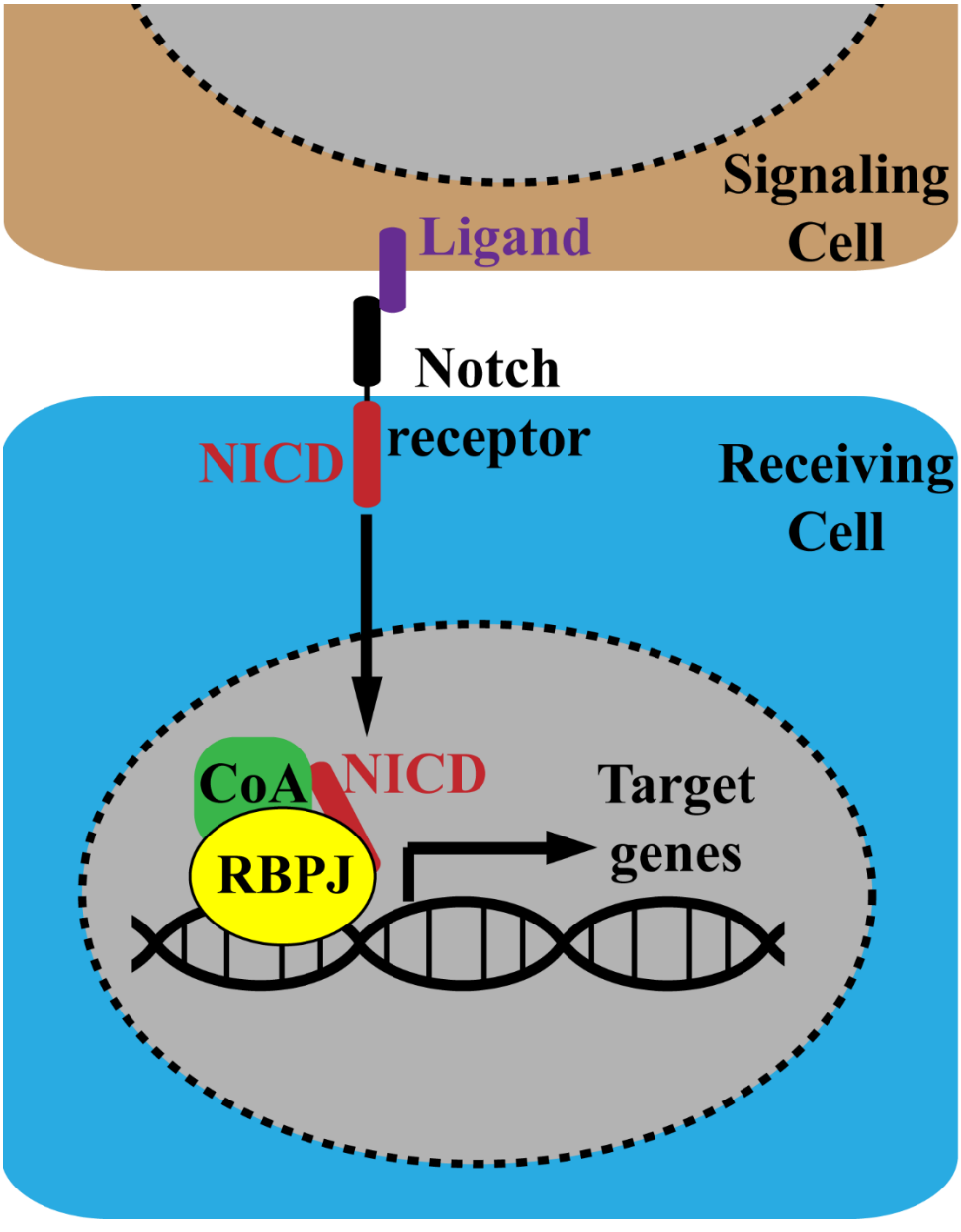

A
ICE
OCE
Stroma
Lens
Retina
RPE
Zonule fibers
Iris
Ciliary Body
B
E13.5
100μm
C
E16.5
D
E17.5
E
P0
F
P3
G
P7
H
Adult

Figure 1.3 Notch signaling pathway overview

Notch receptor is expressed at the surface of Notch responsive cells. Binding of the ligand from the signaling cell leads to the cleavage of the Notch receptor, and therefore the Notch intracellular domain (NICD) is released and translocated into the nucleus of the receiving cell. Association of NICD with effector RBPJ and transcription co-activator (CoA) initiates the expression of target genes.


Signaling
Cell
Ligand
Notch
receptor
NICD
Receiving
Cell
CoA
NICD
RBPJ
Target
genes

CHAPTER TWO: MATERIALS AND METHODS

MOUSE STRAINS

Trp1-Cre, *Notch2*$^{fx/fx}$, *RBPJ*$^{fx/fx}$ and *Ncad*$^{fx/fx}$ strains were previously described (Kostetskii et al 2005, McCright et al 2006, Mori et al 2002, Tanigaki et al 2002). To visualize *Cre* expression pattern, *Trp1-Cre* strain was crossed with a *Z/EG* reporter strain to generate *Trp1-Cre;Z/EG* mice (Novak et al 2000). All mice were housed and cared according to the Institutional Animal Care and Use Committees at the Stowers Institute for Medical Research. Tail clips were lysed by boiling in a 1x base solution followed by neutralization in a 1x neutralization buffer. Genotyping was done with standard PCR with tail lysate.

HISTOLOGY

Mice were euthanized by decapitation for pups and cervical dislocation for adults. For cyro-sectioning, eyes were fixed in 4% formaldehyde in PBS at 4°C overnight and infiltrated with 15% sucrose in PBS until the samples sank and then transferred to 30% sucrose in PBS at 4°C overnight. Phosphatase inhibitor cocktail (Calbiochem) was added during tissue fixation to prevent dephosphorylation. For paraffin or plastic sectioning, samples were fixed in Davidson's fixative at room temperature overnight, and dehydrated in 70% ethanol at room temperature overnight. Sucrose balanced or dehydrated tissues were then sent to the Stowers Histology Core Facility for further processing and sectioning. Cyrosections were cut with 12µm, dried in a 25°C oven and stored in -20°C for use of immunostaining or mRNA *in situ* hybridization. Paraffin and plastic sections were prepared at 5µm and stained with hematoxylin and eosin (H&E) for the visualization of the morphology.

IMMUNOHISTOCHEMISTRY AND MICROSCOPY

Slides with cryosections were taken out of the -20°C freezer and warm up at room temperature for at least 30mins. Then antigen retrieval was performed in 1x citrate buffer at 95°C for 10mins, followed by three washes with TBST for 10mins each. Samples were blocked with Powerblock (Biogenex) for 10mins, and incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used in this study: rabbit anti-N-cadherin, rabbit anti-aPKC, rabbit anti-Aqp1 and goat anti-Par6 (Santa Cruz Biotechnology); rat anti-N-cadherin, mouse anti-Otx1, mouse anti-Collagen IX and rat anti-Notch2 (Developmental Studies Hybridoma Bank); rabbit anti-Pax6 and rabbit anti-α-catenin (Zymed); goat anti-Opticin (R&D Systems); rabbit anti-pSMAD1/5/8 and rabbit anti-Connexin43 (Cell Signaling); rabbit anti-ZO-1, rabbit anti- β-catenin and chicken anti-GFP (Invitrogen); mouse anti-Ncadherin (BD Biosciences); rabbit anti-Par3 (Millipore); mouse anti-β-actin (Abcam); rabbit anti-BrdU (Megabase Research Products). Three washes in TBST were performed prior to secondary antibody incubation. All secondary antibodies (Alexa 488 and Alexa 568) were from Invitrogen. Slides were then counterstained with DAPI (Invitrogen) and mounted with Vectashield Mounting Media (Vector Laboratories). For pSMAD1/5/8 staining, tyramide signal amplification was performed to increase the signal intensity with TSA™ Cyanine 3 kit (PerkinElmer). All fluorescent images were taken on a Leica SP5 confocal microscope, and white field images on Leica DM550 microscope.

BRDU LABELING ASSAY

BrdU labeling was done by injecting BrdU intraperitoneally (IP) in P3 pups at the dosage of 0.1mg/g body weight 2 hours before the eyes were enucleated. Standard histology was

performed and slides were immunostained with BrdU antibody, followed by imaging with confocal microscope. Cell counting was performed with Leica LAS AF software from at least 10 CBs. A two-tailed t-test was performed to calculate the statistical significance between groups.

TUNEL ASSAY

Cell apoptosis was detected by tunel assay with the ApopTag Fluorescein In-Situ Apoptosis Detection Kit (Chemicon). Briefly, cyrosections were fixed in 1% PFA in PBS for 10mins at room temperature, washed with PBS and permeabilized with ethanol: acetic acid 2:1 for 5mins at -20°C. After two washes with PBS, samples were equilibrated with equilibration buffer for 10 seconds at room temperature and working strength TdT enzyme was applied. Slides were then incubated in a humidified chamber at 37°C for 1hr. The slides were then submerged in stop/wash buffer for 10mins at room temperature and rhodamine-conjugated anti-digoxigenin was added and incubated in a dark and humidified chamber at room temperature for 30mins after three washes with PBS. DAPI was used to counterstain the slides. Then the slides were mounted and imaged with a confocal microscope.

MRNA IN SITU HYBRIDIZATION

Otx1 and Msx1 probes were gifts from Dr. Richard Libby (University of Rochester Medical Center). Digoxigenin-labeled RNA probes were synthesized according to the manufacturer's instructions (Roche). Slides were air dried for 30mins at room temperature, and post-fixed with 4% PFA in PBS for 10mins, followed by three washes with PBT for 5mins each. Proteinase K (Roche) was then applied at a concentration of 1μg/ml in PBS for 10mins. After washing with PBT twice, the slides were treated with an additional post-fix with 4% PFA in PBS for 10mins,

and then washed again with PBT. Acetylation was done with acetylation solution (625µl acetic anhydride in 250ml 0.1M triethanolamine) for 10mins and prehybridized in hybridization buffer at 62°C for 1-4 hours. Probes were then added and incubated at 62°C overnight. On the second day, slides were washed three times with hybridization wash buffer at 62°C for 20mins each time, and blocked with 20% normal sheep serum (NSS) for 1hour at room temperature. Primary anti-digoxygenin antibody was applied, and the slides were incubated in a humid dark chamber for another overnight at 4°C. On the third day, slides were washed with TBST three times and rinsed in Alkaline Phosphatase buffer. Then NBT-BCIP substrate was added for color development. Slides were mounted in glycerol and imaged under Leica DM550 microscope.

IMMUNOPRECIPITATION AND WESTERNBLOT

For co-IP experiments *in vivo*, protein lysates from dissected CB tissues from 90 eyes of P3 B6 pups were incubated with protein G beads pre-conjugated with either mouse control IgG or mouse anti-N-cadherin antibody. The beads were washed six times with IP wash buffer and eluted in SDS sampling buffer with heat. Samples were loaded into an 8% to 12% SDS gel and transferred to a nitrocellulose membrane using iBlot2 machine (Life technologies). Primary antibodies used are as the same as in immunostaining experiments. The secondary HRP conjugated antibodies were purchased from Promega, and signal was developed with Western Lightning Plus-ECL reagent (PerkinElmer). For quantitative analysis of western blots, band intensity was measured with Adobe Photoshop CS4. Average band intensities were subtracted by background intensities and normalized to β-actin controls. Three biological replicates were quantified for statistical analysis using Student's t-test.

LENTIVIRAL VECTOR CONSTRUCTION, LENTIVIRUSES PRODUCTION AND INTRAOCULAR INJECTION

cDNAs for full-length coding sequences of *Chrdl1* and *Nbl1* were cloned by PCR and cloned into the vector pIRES2-EGFP (Addgene). Coding sequences of *Chrdl1* and *Nbl1* together with IRES-EGFP was subcloned into the pSicoR lentivirus vector (Addgene). Short hairpin RNA (shRNA) sequences are cloned under the U6 promoter of pSicoR vector. shRNA sequences targeting the all three isoforms of mouse Par3 are 5'-ACAAGCGTGGCATGATCCA-3' (Par3-i1) (McCaffrey & Macara 2009) and 5'-GGCATGGAGACCTTGGAAG-3' (Par3-i2) (Gerard et al 2007). Their knockdown efficiencies were confirmed in NIH3T3 cells by transient transfection with lipofectamine reagent (Life Technologies). High-titer lentiviruses were produced by co-transfecting the construct and packaging plasmids psPAX2 and pMD2.G (Addgene) into 293T cells and purified via ultracentrifuge. Concentrated lentiviruses were then injected into the CB region of around birth (P0) CD1 pups. Eyeballs were collected at P3 for further analysis. For the *in vitro* BMP assays, after lentiviruses had been added to cultured 293T cells for 4 h, culture medium was replaced by fresh medium supplemented with either DMEM only or DMEM containing 2 ng/mL, 5 ng/mL, and 10 ng/mL recombinant human BMP4 (PeproTech). Cells were harvested 12 h later for Western blotting analysis.

INTRAOCULAR PRESSURE MEASUREMENTS

Intraocular pressure was measured with a handheld rebound tonometer from TonoLab according to the procedure as previously described (Wang et al 2005). For each eye, six measurements were performed and averaged to the mean. Student's t-test was performed to analyze the statistical difference.

TRANSMISSION ELECTRON MICROSCOPY

Mouse eyes were dissected and immersion fixed in 2.5% glutaraldehyde for 2 hours at room temperature. Following fixation, tissues were washed three times in PBS then post-fixed in aqueous 1% OsO_4, 1% $K_3Fe(CN)_6$ overnight at 4^oC. Following three PBS washes, tissues were dehydrated through a graded series of 30-100% ethanol, 100% propylene oxide then infiltrated and embedded in Epon resin. Thick sections of 1-2 µm were cut and stained with Toluidine Blue O (TBO) on slide. Ultrathin (60-70nm) sections were collected on copper grids, stained with 2% uranyl acetate and 1% lead citrate. Sections were photographed using a FEI transmission electron microscope at 80 kV.

LASER CAPTURE MICRODISSECTION

Laser capture microdissection experiments were carried out as previously described (Morrison et al 2012). Briefly, eyes were enucleated and immediately frozen in Tissue Freezing Medium (TFM, VWR). Sixteen to twenty 10 µm thick cryosections were collected onto Zeiss MembraneSlide NF 1.0 PEN (Carl Zeiss Microscopy) and dried on a heating block. Membrane slides were either processed for laser capture on the Zeiss PALM MicroBeam laser capture microscope or stored at -80 ^oC for future experiments. Settings for Zeiss microbeam microscope were used as follows: laser energy for cut was set to 36 and focus was 52, whereas laser for LPC was used at an energy delta of 25 and a focus delta of -5. Cutting speed was set at 14 percent.

CHAPTER THREE: NOTCH2 REGULATES BMP SIGNALING AND EPITHELIAL MORPHOGENESIS IN THE CILIARY BODY OF THE MOUSE EYE

SUMMARY

The ciliary body (CB) of the mammalian eye is a structure that is responsible for the secretion of aqueous humor to maintain the intraocular pressure and the accommodation of the lens for far sight versus near sight vision. Dysfunction of CB might be associated with multiple retinal diseases including glaucoma and myopia. The CB contains two-layered apical adherent epithelial sheets composed of the non-pigmented inner ciliary epithelium (ICE) derived from the retina and the pigmented outer ciliary epithelium (OCE) derived from the retinal pigment epithelium (RPE), and the stroma underneath. Despite the important functions, how CB forms and develops remains poorly understood. In this chapter, I show that disruption of Notch2 signaling by conditional knockout of *Notch2* in the OCE can abolish CB morphogenesis, while the specification and secretory function of CB remain unaffected. Notch2 signaling is required to maintain BMP signaling, which has been shown to be essential for CB morphogenesis. Notch signaling has been show to crosstalk with BMP signaling via the interaction of downstream transcriptional factors, however this study identified novel links between Notch2 and BMP signaling that Notch2 maintains BMP signaling by repressing the expression of secreted BMP inhibitors. Our work has established an attractive model to study how Notch signaling regulates tissue morphogenesis and also provided novel insight into the cross-talk between Notch and BMP signaling pathways.

INTRODUCTION

The mammalian eye is composed of the anterior segment, the vitreous body and the posterior retina. The anterior segment contains the cornea, the lens, the iris and the ciliary body (CB), whereas the posterior retina is laminated into three distinct cell layers with six different types of retina neurons and Müller glia cells. In order for the eye to precept the light, the eye should remain inflated and no blood vessel should reside inside the eye to block light transmission. The aqueous humor (AH) secreted from the CB provides the solution to both issues. Intraocular pressure (IOP) is produced through the dynamic flow of the AH and maintains the sphere shape of the eye. Meanwhile, the AH plays the role of blood replacement to nourish the lens and cornea. IOP is maintained through the balance between the inflow resulting from the production of aqueous humor from CB and the outflow by the drainage system. High levels of IOP is often a risk factor for glaucoma (Zhang et al 2012). Consequently, the CB is often a major target site for glaucoma therapy (McLaren & Moroi 2003). Additionally, the zonule fibers attached to the lens from the ciliary muscle controls the accommodation of the lens for far versus near sight vision. Despite the important functions and medical implications of the CB, how it forms and develops is still poorly understood.

The CB consists of two apically adherent epithelial sheets: the inner ciliary epithelium (ICE) derived from neural retina and the outer ciliary epithelium (OCE) from retinal pigment epithelium (RPE), and the underlying stroma. In mice, the two epithlial layers of the CB start to fold together to undergo morphogenesis around P0 and this process persits till around P7 when 3~4 folds form. Studies have shown that the formation and morphogenesis of CB is regulated both intrincically and extrincically. Blood vessels and neural crest derived cells underneath the

CB have been suggested to be important for the formation of the folds (Beebe 1986, Pressman et al 2000, Tian et al 2012). Additionaly, normal IOP is also required for the fold formation (Reichman & Beebe 1992). Moreover, changes in cell number and cell volume in the CB may also contribute to the morphogenesis (Bard & Ross 1982a, Bard & Ross 1982b).

Genetic studies have identified many important signaling pathways that control CB development. FGF and Wnt signaling have been found to be important in controlling the fate determination for the CB at an early embronic stage (Cho & Cepko 2006, Dias da Silva et al 2007, Kubo et al 2003, Liu et al 2007). microRNA pathway is also involved in the specification and morphogenesis of the CB since deletion of Dicer1, the key enzyme in miRNA biogenesis leads to CB defects (Davis et al 2011). Transcription factor Otx1 and Pax6 are essential for the morphogenesis process (Acampora et al 1996, Davis et al 2009). Cell adhesion mediated by Nectin 1 and Nectin 3 are also important for CB morphogenesis (Inagaki et al 2005). Disruption of BMP signaling by either transgenic overexpression of Noggin, a BMP antagonist, or BMP4 halpoinsufficiency leads to the abolishment of foldings, suggesting BMP signaling is also very critical for the CB morphogenesis (Chang et al 2001, Zhao et al 2002).

Notch signaling is an evolutionary conserved signaling pathway that plays important roles in various biological processes including tissue patterning and homeostasis. Binding of Notch receptor with the ligand from the neighboring cell leads to the cleavage of Notch receptor and the Notch intracellular domain (NICD) is released from the transmembrane domain and translocated into the nucleus where it binds with RBPJ to initiate transcription of Notch downstream target genes (Kopan & Ilagan 2009). Defective Notch signaling is associated with multiple human

disorders, from congenital diseases such as Alagille syndrome and Hajdu-Cheney syndrome to adult-onset diseases including cancer (Koch & Radtke 2007, Kopan & Ilagan 2009, Turnpenny & Ellard 2012). There are four Notch receptors (Notch 1-4) and five ligands (Dll 1, 3, 4 and Jag 1, 2) expressed in mammals. In the eye, Notch1 has been shown to be expressed in the neural retina where it regulates the proliferation and differentiation of early retinal progenitor cells (Bao & Cepko 1997, Jadhav et al 2006, Yaron et al 2006). Notch2 is expressed in the lens epithelium and RPE during different developmental stages (Bao & Cepko 1997, Saravanamuthu et al 2012). It has been shown that Notch2 is essential for the lens formation (Saravanamuthu et al 2012). However, the role of Notch2 in RPE and CB remains undiscovered. By using the Cre-loxp system to conditionally knockout *Notch2* in the CB, this chapter shows that Notch2 signaling is required for the morphogenesis of the developing CB (Zhou et al 2013).

RESULTS

Notch2 is required in the OCE to control CB morphogenesis in the mouse eye

Notch2 is strongly expressed in the pigmented epithelium of the developing eye, including the CB region, but its role in eye development has not been investigated (Bao & Cepko 1997, Lindsell et al 1996). In order to investigate the role of *Notch2* in the regulation of RPE development in the developing eye, I used a floxed allele of *Notch2*, *Notch2*$^{fx/fx}$ (McCright et al 2006), and a RPE-specific Cre line, *Trp1-Cre* (the *Cre* gene under the control of the *Trp1* promoter) (Mori et al 2002), to conditionally inactivate *Notch2* function in the developing pigmented epithelium. Surprisingly, RPE-specific *Notch2* conditional knockout (*Notch2 CKO*) mutant adult eyes show no other discernible phenotype except lack of CB morphogenesis in comparison with the control (Figure 3.2). The *Trp1-Cre* line exhibits some degree of RPE

degeneration in the eye as recently reported (Thanos et al 2012), but its CB region is normal. The mutant CB phenotype is very consistent on the ventral side of the eye, but is more variable on the dorsal side ranging from no morphogenesis to certain degree of morphogenesis (Figure 3.2). This is likely caused by the uniform expression of *Trp1-Cre* in the CB on the ventral side of the developing eye and the highly mosaic expression on the dorsal side (Figure 3.1). Consequently, the analysis of the *Notch2* mutant CB phenotype in this study is focused on the ventral side. These results indicate that *Notch2* is required for driving CB morphogenesis.

Notch2 signaling is dispensable for the specification of the CB fate and secretion function of the CB

Consistent with their mRNA expression patterns, Notch2 protein is expressed in the OCE of the CB at P3 (Figure 3.3 A and A'). In contrast, Notch2 protein is not detectable in the OCE of the CB but still present in the lens epithelium in the *Notch2 CKO* mutant, further supporting that *Notch2* is specifically deleted in the OCE (Figure 3.3 B and B').

One potential explanation for the failure of the morphogenesis of the *Notch2 CKO* mutant CB is that the CB cell fate fails to be specified in the mutant. Notch signaling is known for its role in the regulation of cell fate specification (Artavanis-Tsakonas et al 1999, Bray 2006, Fortini 2009, Sprinzak et al 2010). The wild-type control CB has been previously shown to express *Msx1* (Figure 3.3 C) (Zhao et al 2002). My mRNA *in situ* results indicate that the *Notch2 CKO* mutant CB still expresses *Msx1* at normal levels (Figure 3.3). Although *Otx1* and *Pax6* have been shown to be important for CB morphogenesis (Acampora et al 1996, Davis et al 2009), their protein expression in the P0 *Notch2* mutant OCE remains unchanged in comparison with the P0 control

OCE, suggesting that Notch2 signaling must regulate other pathways to control CB morphogenesis.

In addition, I also examined Collagen IX (Col IX) and Opticin (Optc) protein expression for CB secretion function in the control and *Notch2 CKO* mutant CBs. Collagen IX and Opticin are extracellular matrix protein secreted by the CB and accumulate on the surface of the CB and the retina (Dhawan & Beebe 1994, Le Goff & Bishop 2007, Linsenmayer et al 1990). Both control and *Notch2 CKO* mutant CBs exhibit similar levels of Optc expression in the secreting ICE cells and secreted Col IX and Optc protein on the surface of the CB (Figure 3.4 A-D). These results suggest that Notch2 is dispensable for the specification of the CB region and protein secretion function.

Notch2 maintains BMP signaling in the CB

Because BMP4 haploinsufficiency and Noggin overexpression cause similar CB morphogenesis defects to that of the *Notch2 CKO* mutant (Chang et al 2001, Zhao et al 2002), I then determined if Notch2 signaling affects BMP signaling in the developing CB by examining the expression of the phosphorylated form of SMAD proteins 1, 5 and 8 (pSMAD1/5/8). Secreted BMP proteins can bind to receptor complexes composed of at least one of type I receptors (BMPR1a and BMPR1b) and a type II receptor (BMPR2), leading to the phosphorylation of SMAD1, 5 and 8 proteins (Miyazono et al 2005, Sieber et al 2009). In developing retinal progenitors, OCE cells and differentiating lens fiber cells, BMP signaling also results in pSMAD1/5/8 production (Belecky-Adams et al 2002, Haynes et al 2007, Zhao et al 2002). In the control P3 CB, cells in the OCE, but not those in the ICE, show strong pSMAD1/5/8 expression, indicating that the

BMP pathway is active in the OCE (Figure 3.5 A-A'). However, in the *Notch2 CKO* mutant CB, pSMAD1/5/8 expression diminishes in the OCE (Figure 3.5 B-B'). pSMAD1/5/8 expression in the lens remains normal, suggesting that BMP signaling reduction is restricted to the CB region (Figure 3.5 A and B). These results show that Notch2 is required for maintaining active BMP signaling in the OCE.

Notch2 represses the expression of two BMP signaling inhibitor genes, Chrdl1 and Nbl1, in the OCE of the CB

Inactivation of *Notch2* function in the OCE results in reduced BMP signaling activity not only in the OCE but also in the underlying stromal cells, suggesting that Notch2 can regulate BMP signaling in both cell-autonomous and non-cell-autonomous manners (Figure 3.5 A and B). One of the possibilities is that Notch signaling normally represses the expression of a gene(s) encoding a secreted BMP inhibitor in the OCE. In collaboration with Dr. Christopher Tanzie, I identified *Chrdl1* (*Chordin-like 1*) and *Nbl1* (*neuroblastoma, suppression of tumorigenicity 1*) upregulated in the *Notch2* mutant OCE from microarray results done by Dr. Tanzie. I have used qRT-PCRs to confirm that *Chrdl1* and *Nbl1* are upregulated in the *Notch2* mutant OCE 2.1 fold and 1.7 fold, respectively (Figure 3.6 A). I have further shown that they indeed repress BMP signaling activities in human 293T cells when overexpressed, which is consistent with published results (Balemans & Van Hul 2002, Kane et al 2008, Pearce et al 1999, Sakuta et al 2001) (Figure 3.6 B-G). To directly test if *Chrdl1* and *Nbl1* are capable of inhibiting BMP signaling in the developing OCE and prevent CB fold formation, I injected lentiviruses carrying *CMV-Chrdl1-IRES-gfp* (the *CMV* promoter controlling the expression of *Chrdl1* and *GFP* genes linked by IRES) and *CMV-Nbl1-IRES-gfp* in the CBs of the P0 eyes. Following *Chrdl1* overexpression,

pSMAD1/5/8 expression is severely reduced in both the OCE and the underlying mesenchymal cells of the P3 CBs, and normal fold formation is also disrupted, indicating that *Chrdl1* overexpression is capable of repressing BMP signaling and disrupting CB morphogenesis (Figure 3.7 A-B). Although *Nbl1* overexpression can also decrease pSMAD1/5/8 expression, it is less effective in repressing BMP signaling and disrupting CB morphogenesis than *Chrdl1* in the CB, which is consistent with the results in cultured human 293 cells (Figure 3.7 C). Therefore, I propose that Notch2 controls BMP signaling possibly by repressing *Chrdl1* and *Nbl1* expression in the OCE.

DISCUSSION

Although BMP signaling, Pax6 and Otx1 have recently been shown to be required for CB morphogenesis (Acampora et al 1996, Davis et al 2009, Zhao et al 2002), it remains unclear how they work together to control CB morphogenesis at the molecular and cellular level. In this chapter, I show that the active Notch2 signaling in the OCE drives CB morphogenesis at least in part by maintaining BMP signaling. Defective Notch2 signaling in the OCE decreases BMP signaling. Mechanistically, Notch2 signaling controls BMP signaling at least in part by repressing the expression of *Chrdl1* and *Nbl1*, encoding two secreted BMP inhibitors. In contrast with the previous findings that Notch and BMP signaling cooperate with each other by targeting their downstream transcription factors to the promoters of common target genes (Larrivee et al 2012, Moya et al 2012), this study has revealed a novel strategy for cross-talk between Notch and BMP pathway, which is critical for CB morphogenesis (Chang et al 2001, Zhao et al 2002). Although Notch signaling has been demonstrated to play a critical role in cell fate specification (Koch & Radtke 2007, Miyazono et al 2005), both *Notch2* and *Rbpj* are dispensable for the

specification of the CB fate based on the expression of multiple CB markers. Therefore, I propose that Notch2 signaling regulates BMP signaling in the CB, and consequently CB morphogenesis. However, my study does not rule out the possibility that Notch2 signaling also controls CB morphogenesis independent of BMP signaling.

The cross-talk between BMP and Notch signaling pathways has been shown to exist in different cellular contexts. In endothelial cells, the Alk1/BMPR-mediated BMP signaling pathway and the Dll4-activated Notch signaling pathway work together to transcriptionally activate the expression of *Hey1* and *Hey2* genes (Larrivee et al 2012, Moya et al 2012). In addition, Notch and BMP signaling pathways can block myogenic differentiation of C1C12 cells by regulating the expression of Hey1 through a direct interaction between Smad1 and NICD (Dahlqvist et al 2003). In the zebrafish pineal gland, BMP signaling is a competence factor for Notch signaling to efficiently activate its target gene expression (Quillien et al 2011), whereas in the regulation of the initial formation of the olfactory nerve BMP signaling negatively affects Notch signaling to achieve the balance between the two pathways (Maier et al 2011). In these two cases, it remains unclear how the two pathways are integrated. In this chapter, I have shown that Notch signaling controls BMP signaling activity in the developing CB possibly by repressing *Chrdl1* and *Nbl1* expression in the OCE. In addition, I show that *Chrdl1* overexpression in the OCE can inhibit BMP signaling in both the OCE and the underlying mesenchymal cells of the developing CB, which is similar to *Notch2* inactivation specifically in the OCE. Although *Nbl1* is also capable of repressing BMP signaling in the CB, it is less effective than *Chrdl1* and its overexpression is not sufficient to disrupt CB morphogenesis. The important unanswered questions include: whether *Chrdl1* and *Nbl1* are also expressed in the ICE to contribute to BMP regulation in the CB, how

Notch2 signaling represses *Chrdl1* and *Nbl1* expression in the OCE at the molecular level, and whether OCE-specific inactivation of *Chrdl1, Nbl1* or both sufficiently restore BMP signaling and morphogenesis in the *Notch2* mutant CB. In summary, I have identified the Notch2 signaling pathway as a key signaling pathway to control CB morphogenesis at least in part by regulating BMP signaling.

Figure 3.1 Trp1-Cre efficiently catalyzes LoxP-mediated recombination in the OCE of the ventral but not dorsal CB.

(A) Schematic illustration of the *Z/EG* reporter. In the Z/EG reporter, ubiquitously expressed CAG promoter drives the expression of the EGFP transgene. A stop cassette with two loxp sites is inserted upstream of the EGFP coding sequence to block the expression of EGFP. In the presence of Cre, Cre can mediate gene recombination between two loxp sites and remove the stop cassette. Thus, the expression of EGFP represents the expression patterns of Cre.

(**B-D**) Expression patterns of the *Trp1-Cre* as shown by the EGFP expression from the *Trp1-Cre;Z/EG* reporter at different developmental stages. *Trp1-Cre* is expressed highly in the RPE and the CB at E17.5 (**B**), P0 (**C**) and P3 (**D**). Dorsal expression of the Cre shows mosaic patterns (**B', C'** and **D**'), whereas the ventral side is more consistent (**B'', C''** and **D''**). Dashed lines highlight the OCE of the CB.

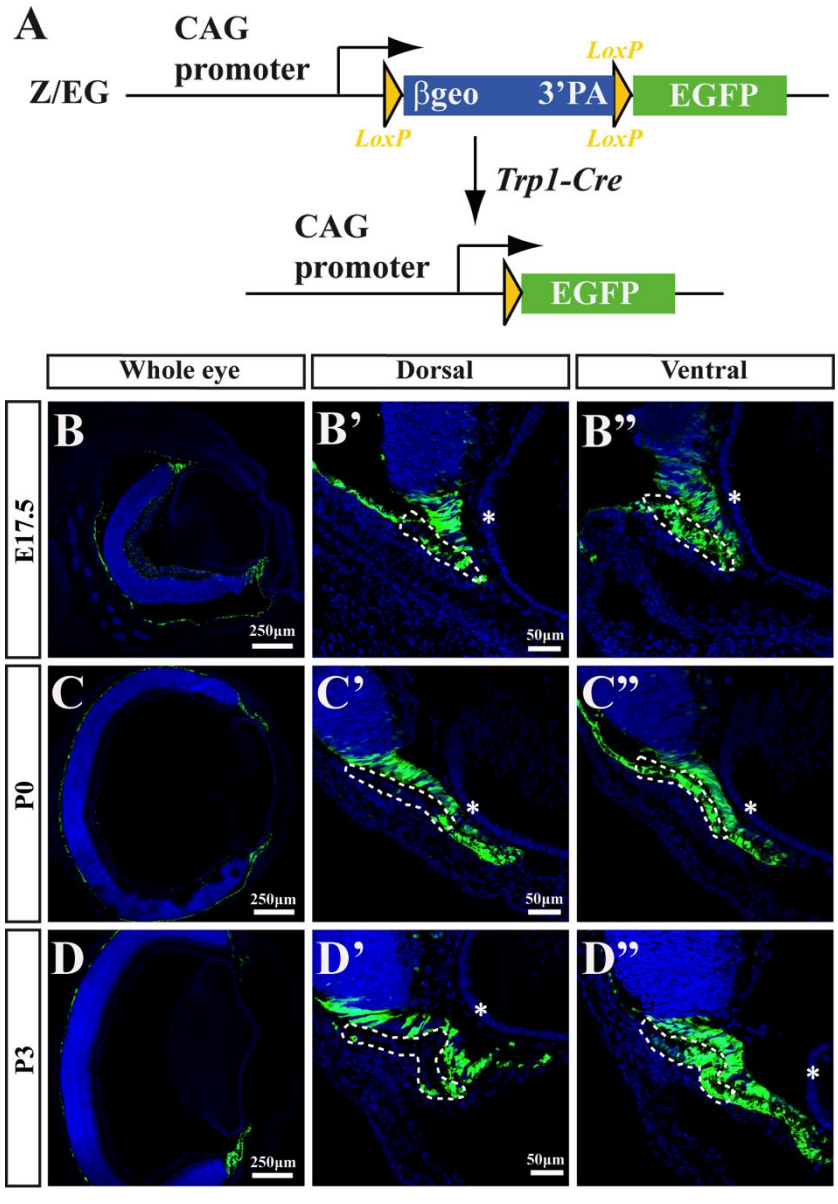

A
CAG
promoter
Z/EG
LoxP
βgeo
3'PA
EGFP
LoxP
LoxP
Trp1-Cre
CAG
promoter
EGFP
Whole eye
Dorsal
Ventral
E17.5
P0
P3
B
B'
B''
C
C'
C''
D
D'
D''
250μm
50μm

Figure 3.2 Notch2 is required for CB morphogenesis.

(A-B) H&E images of WT adult CBs from dorsal (**A**) and ventral (**B**) side show the presence of multiple ciliary processes.

(**C-F**) Conditional inactivation of Notch2 in the CB leads to CB morphogenesis defects. Phenotype of *Notch2* mutant CB varies more on dorsal side (**C, E**), but is more consistent on the ventral side (**D, F**). Boxes highlight the CB regions. The blue arrows denote the ICE, and the green arrowheads show the OCE.

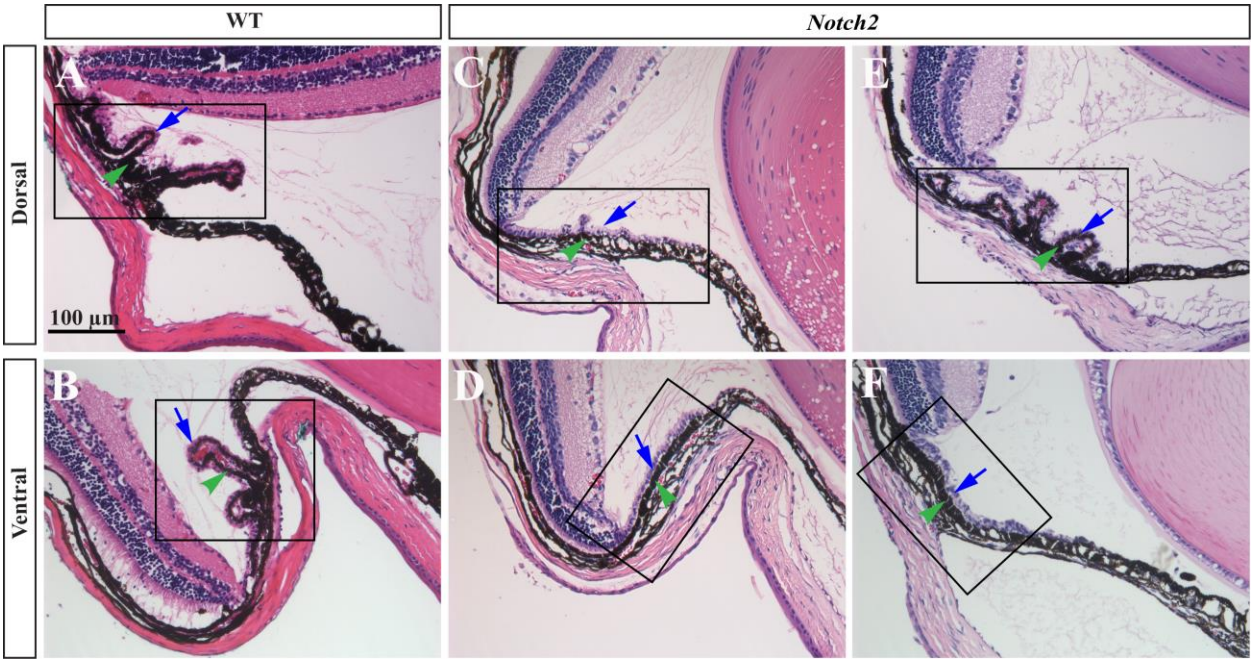

WT
Notch2
Dorsal
Ventral
A
B
C
D
E
F
100 µm

Figure 3.3 Notch2 is dispensable for CB cell fate specification.

(**A-B'**) Notch2 is highly expressed in the OCE cells of the CB in the WT control (**A-A'**), while its expression in *Notch2* mutant CB is depleted in the OCE but not the lens epithelium (**B-B'**). **A**' and **B'** are higher magnification photos of the boxed area of **A** and **B,** respectively.

(**C-D**) mRNA *in situ* hybridization results show similar expression level of *Msx1* in *Notch2* mutant CB (**D)** to control CB (**C**).

(**E-F''**) Immunohistochemistry of Otx1 and Pax6 demonstrate protein levels of Otx1 and Pax6 remain unchanged in *Notch2* mutant CB (**F-F''**) compared to control CB (**E-E''**). * denotes the lens epithelium, and dashed lines highlight the OCE of the CB.

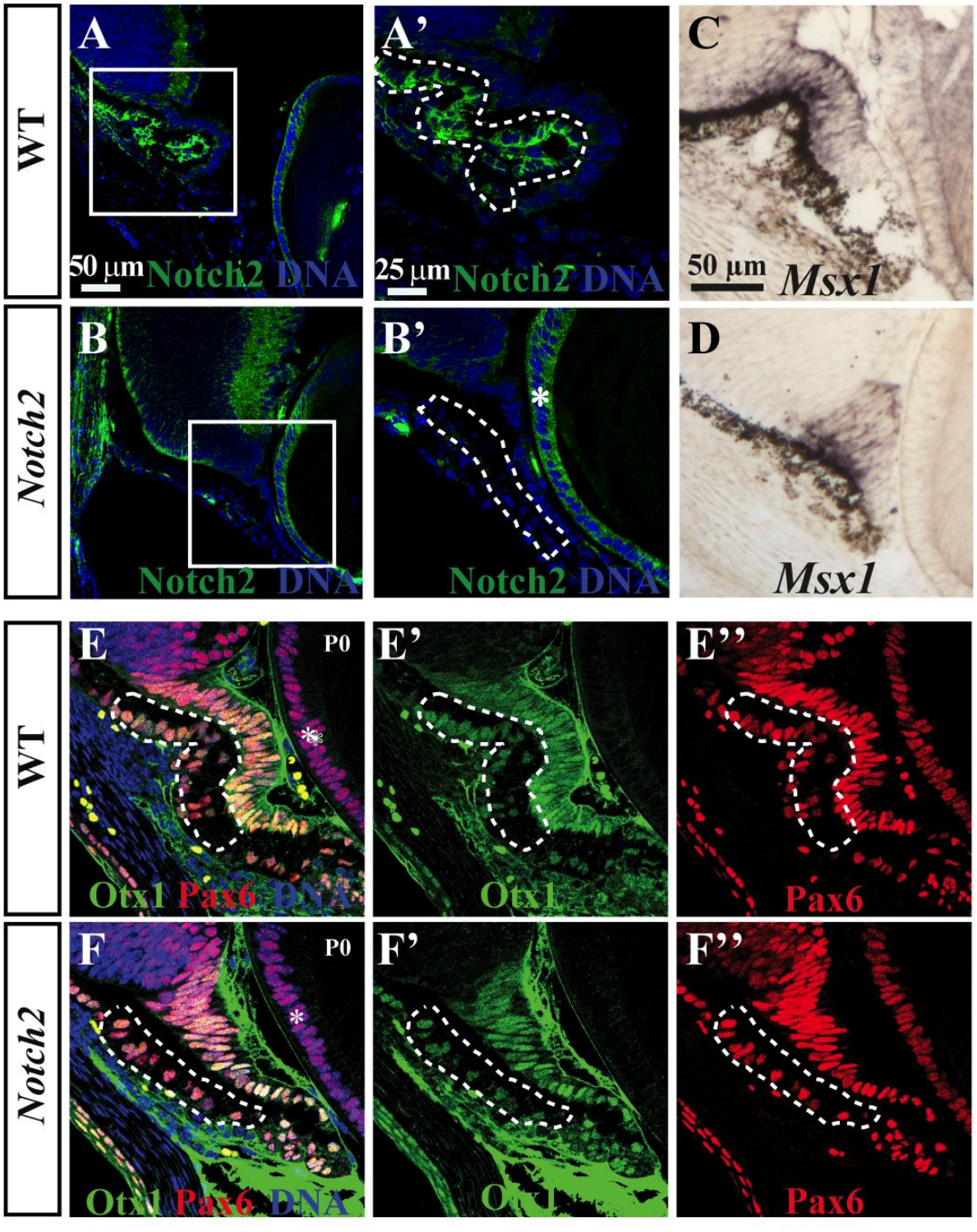

WT
Notch2
A
A'
C
B
B'
D
50 μm
Notch2 DNA
25 μm
Notch2 DNA
50 μm
Msx1
Notch2 DNA
Notch2 DNA
Msx1
E
E'
E''
F
F'
F''
P0
P0
Otx1 Pax6 DNA
Otx1
Pax6
Otx1 Pax6 DNA
Pax6

Figure 3.4 Defective CB morphogenesis does not affect CB secretion.

Col IX (**A**) is secreted from the CB and deposit at the surface of basal ICE and retina, while Optc (**B**) is expressed in secreting cells and accumulates at the CB surface. *Notch2* mutant CBs show similar expression levels of Col IX (**C**) and Optc (**D**).

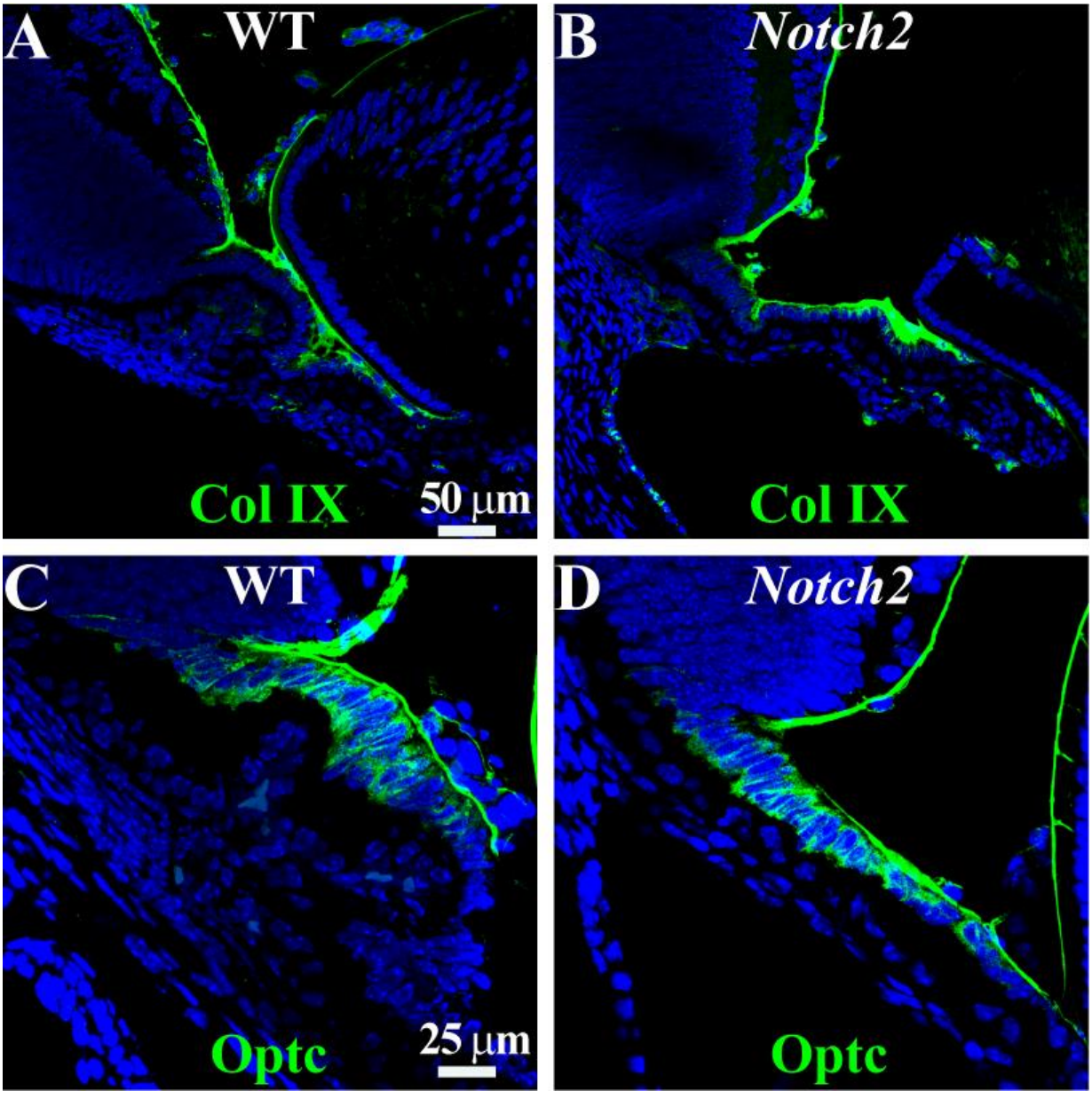

A
WT
Col IX
50 μm
B
Notch2
Col IX
C
WT
Optc
25 μm
D
Notch2
Optc

Figure 3.5 Notch2 regulates BMP signaling in the OCE of the CB.

(**A-A'**) BMP signaling is active in the OCE and stroma of the CB and differentiating lens fiber cells.

(**B-B'**) Expression levels of active BMP signaling marker pSMAD1/5/8 are drastically reduced in the *Notch2* mutant CB, but the lens still maintain high levels of pSMAD1/5/8. Arrow indicates the differentiating lens fiber cells.

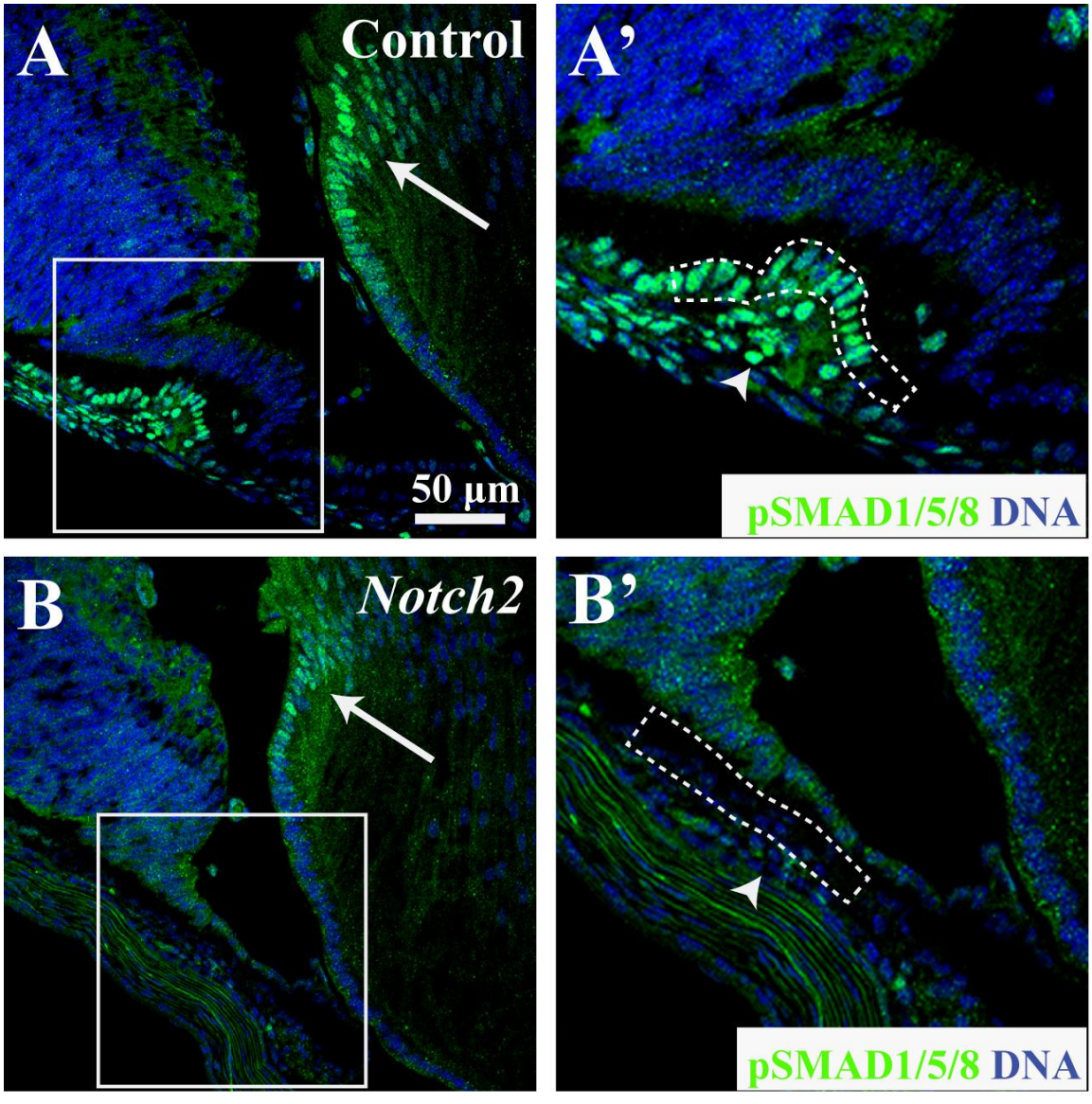

A
Control
50 µm
A'
pSMAD1/5/8 DNA
B
Notch2
B'
pSMAD1/5/8 DNA

Figure 3.6 Notch2 represses the expression of Chrdl1 and Nbl1.

(A) Quantitative PCR results show the up-regulation of *Chrdl1* and *Nbl1* in *Notch2* mutant.

(**B-C**) Chrdl1 and Nbl1 can inhibit BMP signaling *in vitro*. 293T cells were treated with different dosage of BMP4 and immunoblotted for the expression levels of pSMAD1/5/8. (**C)** is the quantitative results of three biological replicates of the western blot normalized to β-actin control. A student's t-test was performed to determine the statistical significance. * indicates P-value less than 0.05.

(**D**) RT-PCR of 293T cells infected with *gfp*, *Chrdl1* and *Nbl1* lentiviruses confirms overexpression of Chrdl1 and Nbl1.

(**E-G**) Immunofluorescent images showing GFP expression in lentiviruses infected 293T cells.

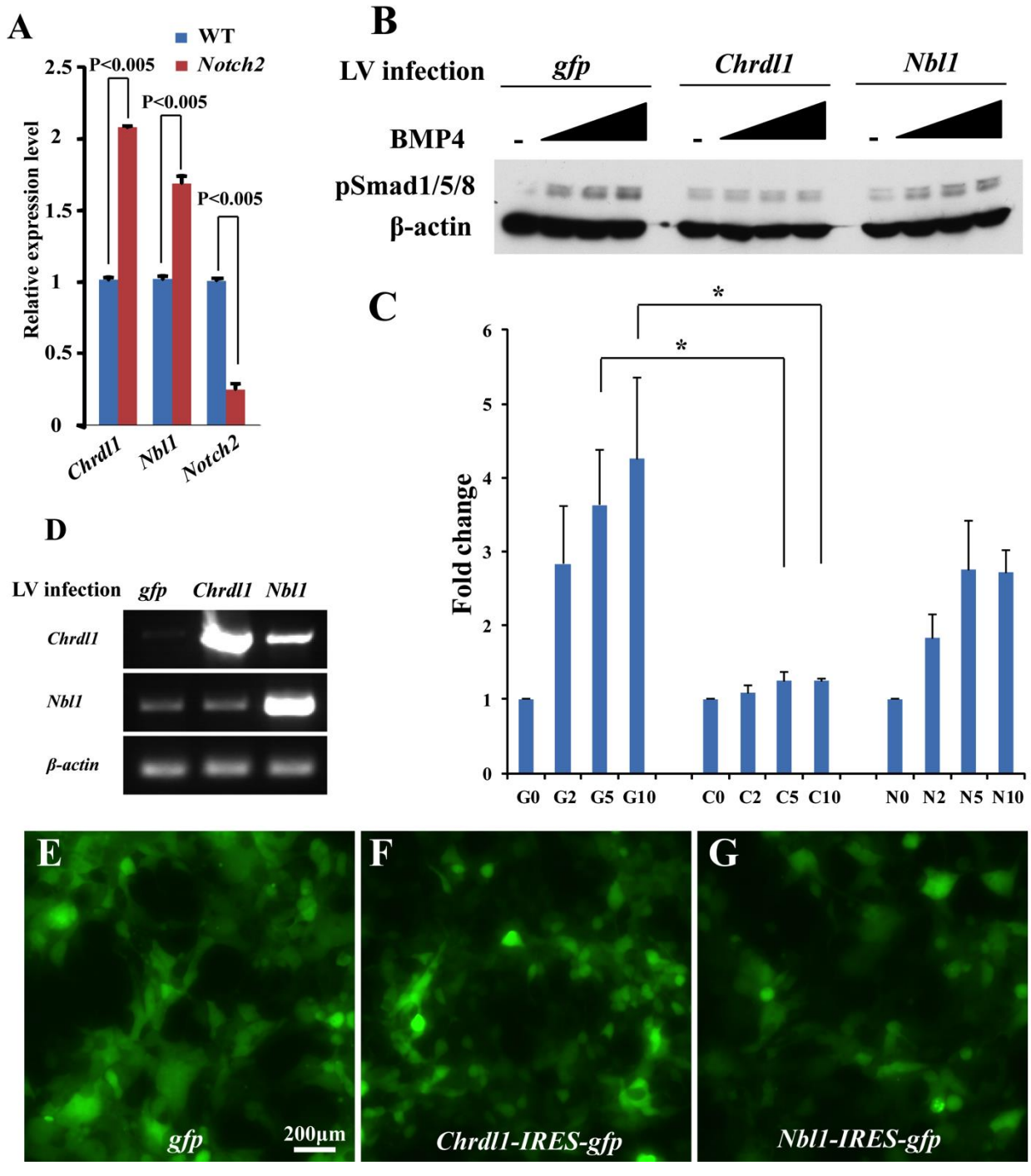
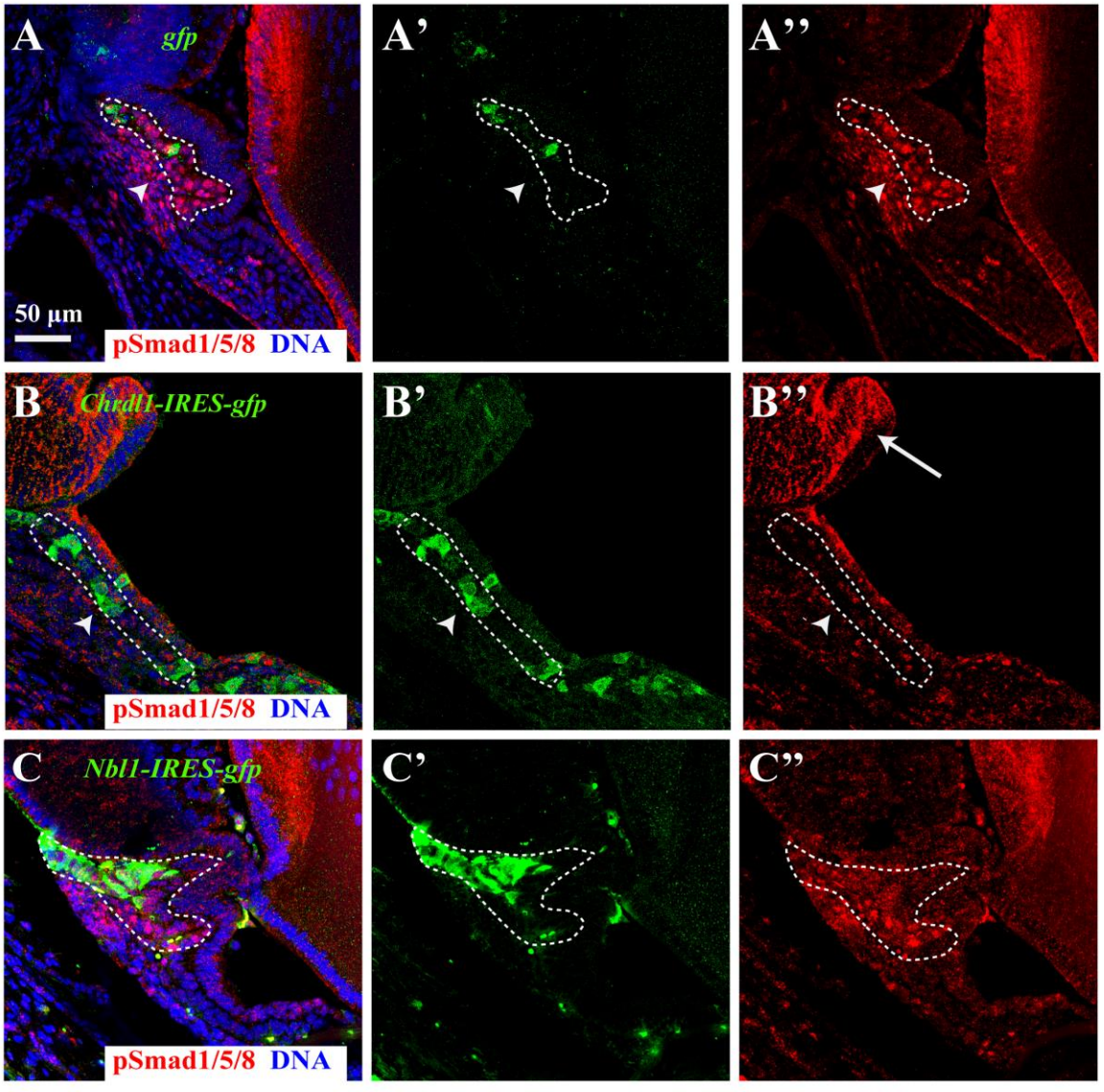

A
WT
Notch2
P<0.005
P<0.005
P<0.005
Relative expression level
2.5
2
1.5
1
0.5
0
Chrdl1
Nbl1
Notch2
B
LV infection
gfp
Chrdl1
Nbl1
BMP4
-
-
-
pSmad1/5/8
β-actin
C
*
*
Fold change
6
5
4
3
2
1
0
G0 G2 G5 G10 C0 C2 C5 C10 N0 N2 N5 N10
D
LV infection
gfp
Chrdl1
Nbl1
Chrdl1
Nbl1
β-actin
E
gfp
200μm
F
Chrdl1-IRES-gfp
G
Nbl1-IRES-gfp

Figure 3.7 Inhibition of BMP signaling by Chrdl1 can induce CB morphogenesis defects.

(A) The injection of *gfp* control lentiviruses does not affect BMP signaling and CB morphogenesis.

(B) The injection of *Chrdl1-IRES-gfp* lentiviruses reduces BMP signaling and also disrupts CB morphogenesis.

(C) The injection of *Nbl1-IRES-gfp* lentiviruses leads to partial decrease of BMP signaling, but the CB morphogenesis is normal.


A
gfp
A'
A''
50 µm
pSmad1/5/8 DNA
B
Chrdl1-IRES-gfp
B'
B''
pSmad1/5/8 DNA
C
Nbl1-IRES-gfp
C'
C''
pSmad1/5/8 DNA

CHAPTER FOUR: RBPJ-DEPENDENT AND -INDEPENDENT REGULATION OF BMP SIGNALING BY NOTCH2 DURING CILIARY BODY DEVELOPMENT

SUMMARRY

The ciliary body (CB) is composed of the inner ciliary epithelium (ICE) and the outer pigmented ciliary epithelium (OCE), and is responsible for the secretion of aqueous humor and lens accommodation. Our previous work shows that Notch2 signaling controls CB morphogenesis via the regulation of BMP signaling and cell proliferation. Canonical Notch signaling is mediated through RBPJ-dependent gene regulation. In this study, I conditionally inactivated *Rbpj* and *Notch2* in the developing CB by crossing *Trp1-Cre* with floxed conditional mutations. The ablation of *Rbpj* in the CB leads to its morphogenesis and secretion defects. Like *Notch2* mutant CBs, *Rbpj* mutant CBs exhibit normal cell fate specification and Col IX secretion, and decrease cell proliferation and BMP signaling in the OCE. Unlike *Notch2* mutant CBs, *Rbpj* mutant CBs frequently separate the ICE from the OCE possibly due to decreased N-cadherin accumulation in the ICE-OCE junction, and decrease Opticin expression and secretion in the ICE. Surprisingly, Notch2, but not RBPJ, is required in the developing CB to maintain active BMP signaling in the underlying stromal cells. This study has revealed important roles of RBPJ in regulating CB development and secretion, and has also uncovered RBPJ-independent regulation of BMP signaling by Notch2 as well as Notch2-independent RBPJ functions in the developing CB.

INTRODUCTION

As a part of the anterior segment, the ciliary body (CB) is a folded structure critical for maintaining normal eye functions (Beebe 1986). The aqueous humor (AH) secreted from the CB nourishes avascular anterior structures and generates the intraocular pressure (IOP), whose elevation is often a risk factor for glaucoma (Stamer & Acott 2012, Zhang et al 2012). In addition, zonule fibers extended from the CB controls the lens accommodation. Despite these important functions, the development of the CB remains poorly understood.

The CB consists of three major components: nonpigmented inner ciliary epithelium (ICE) extending from the retina, pigmented outer ciliary epithelium (OCE) from the retinal pigment epithelium (RPE), and the underlying stroma layer formed by neural crest and mesoderm derivatives (Beebe 1986, Gage et al 2005). It develops from the ciliary marginal zone (CMZ) at the rim of the optic cup before birth. Genetic studies have identified several important regulators that control the specification of the CB. FGF signaling controls the patterning of the presumptive CB regions, while Wnt signaling determines the specification of the CB (Cho & Cepko 2006, Dias da Silva et al 2007, Kubo et al 2003, Liu et al 2007). Abrogation of all miRNAs production via conditional inactivation of Dicer1 in the CB, the key enzyme involved in miRNA biogenesis, leads to the development of a mixed phenotype of neuronal and CB progenitors, suggesting that miRNAs are required for CB specification (Davis et al 2011).

Following the specification, ciliary folds are formed during the first week after birth (Beebe 1986, Zhou et al 2013). Bone morphogenetic protein (BMP) signaling has been shown to be

crucial for this process (Chang et al 2001, Zhao et al 2002). BMP4 haploinsufficiency (Chang et al 2001) or ectopic overexpression of BMP antagonist Noggin (Zhao et al 2002) leads to severe CB morphogenesis deficits. Our recent work and others have shown that Notch signaling is also essential to control CB morphogenesis (Aydin & Beermann 2011, Sarode et al 2014, Schouwey et al 2011, Zhou et al 2013). Our results show that Notch2 regulates ciliary fold formation by maintaining cell proliferation and BMP signaling in the OCE of the CB (Zhou et al 2013). These studies have implicated that Notch and BMP signaling pathways work cooperatively to control CB morphogenesis.

Canonical Notch signaling is mediated by the transcriptional regulation of the protein complex between the Notch intracellular domain (NICD) and the effector recombination signal binding protein for immunoglobulin kappa J region (RBPJ) (Kopan & Ilagan 2009). Although RBPJ has been shown to be required for normal CB development (Aydin & Beermann 2011, Sarode et al 2014), it remains unclear whether Notch2 signaling regulates CB morphogenesis through RBPJ. In this study, I investigated the role of RBPJ in the CB development by conditionally removing RBPJ from the developing CB. Consistent with Notch2, RBPJ regulates cell proliferation and BMP signaling to control the CB morphogenesis. Surprisingly, I have revealed the differences between Notch2 and RBPJ in the regulation of CB secretion and BMP signaling. Therefore, I hypothesize RBPJ-dependent and independent Notch2 signaling plays important roles in regulating CB development.

RESULTS

RBPJ is essential for CB and iris development and for maintaining normal ocular structure

To investigate the role of RBPJ in CB morphogenesis, I generated conditional knockout mutants of *RBPJ* by crossing a floxed allele of *Rbpj* (*Rbpj*$^{fx/fx}$) (Tanigaki et al 2002) with the *Trp1-Cre* line, which has been shown to be able to mediate gene recombination in the RPE, the CB and the iris (Mori et al 2002, Zhou et al 2013). Consistent with previous reports (Aydin & Beermann 2011, Sarode et al 2014), depletion of RBPJ in the RPE and the CB leads to CB hypoplasia without any obvious RPE defects. In WT CBs, the ciliary folds normally form during the first week after birth (Figure 4.1 A, C and E). *Rbpj* mutant CBs also show a severe disruption of CB morphogenesis (Figure 4.1 B, D and F), which is consistent with *Notch2* mutant CBs (Zhou et al 2013). In contrast with *Notch2* mutant CBs, in which the ICE and OCE remain adhered together (Zhou et al 2013), the ICE is often separated from the OCE in the absence of RBPJ (Figure 4.1 B, D and F). The separated ICE in the *Rbpj* mutant CB is often folded back on itself to form a stem lying on the top of the retina (Figure 4.1 D and F). In addition, those *Rbpj* mutant irises show severe dysgenesis and are disorganized in comparison with the control ones at P0, P3 and P7 (Figure 4.1 A-F). Consistent with the previous finding that *Rbpj* deletion results in the rosette formation in the retina (Riesenberg et al 2009, Zheng et al 2009), the retinal rosettes are often observed at the peripheral retina due to the leaked expression of the *Cre* in the neural retina (Mori et al 2002). These results show that RBPJ is required for normal CB and iris development.

In addition to the defects in the CB, the iris and the peripheral retina, one ocular defect also becomes apparent in the *Rbpj* mutant eyes in comparison with the control eyes at P7: they lack the vitreous body between the lens and the retina (Figure 4.1 G and H). In addition, adult *Rbpj*

mutant mice display a severe microphthalmia phenotype. Further histological analysis in the *Rbpj* mutant eyes shows the severe degeneration of the retina and the lens, loss of the anterior chamber and the vitreous body, leading to the shrinkage of overall eye structure (Figure 4.1 I and J). These results suggest that RBPJ is also required for maintaining the overall ocular structure.

RBPJ is dispensable for the specification of the ciliary marginal zone (CMZ)

RBPJ is known to be capable of regulating cell fate decisions of retinal progenitor cells (Riesenberg et al 2009, Zheng et al 2009). To this end, I examined the expression of the known molecular markers for the CMZ, Pax6, Otx1 and Msx1 (Acampora et al 1996, Davis et al 2009, Martinez-Morales et al 2001, Zhao et al 2002, Zhou et al 2013). They are also known to be important for the development of the CB and the iris in the mouse eye (Acampora et al 1996, Davis et al 2009, Zhao et al 2002). Our immunohistochemistry results show that Pax6 protein expression pattern and levels are normal in the *Rbpj* mutant eyes compared to the control eyes (Figure 4.2 A and B). mRNA *in situ* hybridization results show that the expression patterns and levels of *Otx1* and *Msx1* mRNAs are also normal in the *Rbpj* mutant eyes in comparison with control eyes (Figure 4.2 C-F). Along with our previous study that Notch2 is required for CB morphogenesis but is also dispensable for the specification of the CMZ (Zhou et al 2013), these results indicate that CMZ specification does not depend on Notch2/RBPJ-mediated signaling.

RBPJ is required to hold ICE and OCE together in the developing CB possibly by maintaining N-cadherin expression

The frequent separation of the ICE and OCE layers in the *Rbpj* mutant CB suggests some defect in the cell adhesion between the two layers. N-cadherin is expressed in both the epithelial layers

of the CB, and particularly accumulates in the ICE-OCE junction of the CB, raising the possibility that N-cadherin-mediated cell adhesion keeps the two layers together (Figure 4.3 A and A'). In addition, N-cadherin also accumulates at the basal and lateral sides of the ICE as well as at the basal sides of the OCE (Figure 4.3 A and A'). In contrast, N-cadherin protein levels are severely reduced in the ICE-OCE junction and the OCE of the *Rbpj* mutant CB (Figure 4.3 B and B'). However, its expression appears to be largely normal in the ICE of the *Rbpj* mutant CB (Figure 4.3 B'). Interestingly, N-cadherin expression also seems to be largely normal in the ICE-OCE junction, the ICE and the OCE of the *Notch2* mutant CB, also explaining why its two layers remain together (Figure 4.3 C and C'). Since N-cadherin is a classical cadherin member mediating hemophilic interaction, our results suggest that the diminished N-cadherin accumulation at the apical ICE-OCE junction is likely responsible for the ICE and OCE separation in the *Rbpj* mutant CB.

N-cadherin is required for CB morphogenesis in the mouse eye

To investigate the role of N-cadherin in regulating CB morphogenesis, I conditionally deleted N-cadherin from both CB epithelial layers by crossing a floxed allele of *N-cadherin* (*Ncad*), $Ncad^{fx/fx}$, with *Trp1-Cre*. The $Ncad^{fxfx}$ conditional mutant has been shown to efficiently delete N-cadherin in combination with a tissue-specific *Cre* line, whereas *Trp1-Cre* is expressed in both epithelial layers of the CB (Figure 3.1) (Kostetskii et al 2005, Mori et al 2002). N-cadherin is efficiently depleted from both layers of the CB (Figure 4.4 G-G'). As reported previously (Zhou et al 2013), the CB folding process occurs in the first week after birth (P0-P7) (Figure 4.4 A, C and E). In contrast, conditional *Ncad* mutant knockout CBs (thereafter referred to as *Ncad* mutant CBs) show a complete absence of fold formation (Figure 4.4 B, D and F) or a significant

reduction in fold number and length (Figure 4.4 B', D' and F'). It is worth noting that in the absence of N-cadherin the proximal part of the ICE tends to separate from the OCE, and folds back on itself to form a stem-like structure, suggesting that N-cadherin is important to keep the two CB epithelial layers together during morphogenesis (Figure 4.4 B, D and F). Because of the existence of some phenotypic variations, I quantified the CB mutant phenotypes according to their severity. At P3, P7 and P30, about 50% of the CBs show no fold formation, indicating that N-cadherin is required for the robust initiation of fold formation (Figure 4.4 H). Interestingly, at P7 and P30, additional 40% of the CBs showing fold formation at P3 fail to form additional folds or fail to grow to normal length ("limited morphogenesis"), indicating that N-cadherin is also required for continuous fold formation and growth (Figure 4.4 H). Taken together, our results indicate that N-cadherin-mediated cell adhesion helps keep two CB layers together during CB morphogenesis, and continues to be required for CB morphogenesis.

RBPJ promotes cell proliferation in the OCE layer of the CB

Cell proliferation has been proposed to be one of the important factors driving CB morphogenesis (Napier & Kidson 2005, Reichman & Beebe 1992, Zhou et al 2013). Our previous results showed that Notch2 signaling regulates cell proliferation during CB morphogenesis (Zhou et al 2013). To investigate whether RBPJ also controls cell proliferation like Notch2, I performed BrdU incorporation assay and quantified BrdU positive cells in the P3 developing control and *Rbpj* mutant CBs, in which N-cadherin expression was used to highlight the ICE and OCE cells. Consistent with previous studies (Napier & Kidson 2005, Reichman & Beebe 1992, Zhou et al 2013), both ICE and OCE cells proliferate fast at P3, and the proliferation rates in the OCE are significantly higher than that of the ICE (Figure 4.5 A-A'' and

C). In contrast, the cells in the OCE layer of the *Rbpj* mutant CBs proliferate significantly and drastically slower than the counterparts in the WT control CBs (Figure 4.5 B-B' and C). Interestingly, the proliferation of the cells in the ICE layer of the *Rbpj* mutant CBs is only slightly affected in comparison with that for the counterpart of the control CB (Figure 4.5 B-B'' and C). These results indicate that the *Rbpj* mutant CB behave like the *Notch2* mutant CB in terms of cell proliferation, further suggesting that RBPJ functions as the downstream of Notch2 to regulate cell proliferation in the CB.

RBPJ maintains active BMP signaling in the OCE of the CB

As reported previously (Zhou et al 2013), Notch2 regulates BMP signaling in the OCE of the CB and in the underlying stromal cells based on the expression of phosphorylated SMAD1, 5 and 8 proteins (pSMAD1/5/8). pSMAD1/5/8 is an indicator of BMP signaling activity because its production is dependent on BMP-mediated activation of BMP receptor complexes (Sieber et al 2009). Another study has recently reported that pSMAD1/5/8 expression is not changed in the adult *Rbpj* knockout CB (Sarode et al 2014). However, it remains unclear if pSMAD1/5/8 expression is changed in the developing *Rbpj* knockout CB. To test this idea, I then examined the expression of pSMAD1/5/8 in the *Rbpj* mutant P0 and P3 CBs. Consistent with our previously published findings, pSMAD1/5/8 is highly expressed in the OCE of the control CBs and the underlying stromal cells at P0 and P3, but its expression in both the OCE and the stromal cells is severely reduced in the *Notch2* mutant CBs (Figure 4.6 A-D). Interestingly, its expression is also severely reduced in the OCE of the *Rbpj* mutant P0 and P3 CBs just as in the *Notch2* mutant P0 and P3 CBs (Figure 4.6 C-F). Surprisingly, its expression is only slightly affected in the stromal cells underlying the *Rbpj* mutant P0 and P3 CBs unlike in those underlying the *Notch2* mutant P0

and P3 CBs (Figure 4.6 C-F). Our previous results suggest that Notch2 signaling represses the expression of secreted BMP inhibitors Chrdl1 and Nbl1 to modulate BMP signaling in the stroma cells (Figure 3.6 A). Our results show these genes do not change their expression in *Rbpj* mutant CB by qPCR (Figure 4.6 G). Interestingly, expression of *Notch2* decreased by half in the absence of RBPJ, suggesting a positive feedback loop of Notch2-RBPJ signaling axis. Together, these results indicate that RBPJ-mediated Notch2 signaling is required to maintain active BMP signaling in the OCE of the CB in a cell-autonomous manner, and RBPJ-independent Notch2 signaling controls BMP signaling in the underlying stromal cells in a non-cell-autonomous manner.

RBPJ regulates the secretion function of the CB

One of the major functions of the CB is to secrete aqueous humor. To determine if RBPJ is required for the secretion function of the CB, I examined the expression of Collagen IX (Col IX) and Opticin (Optc) proteins in the *Rbpj* mutant CBs as well as in the control and *Notch2* mutant CBs. Col IX is an extracellular matrix protein secreted by the CB and accumulate on the surface of the CB and the retina (Dhawan & Beebe 1994, Linsenmayer et al 1990). Optc belongs to the extracellular matrix protein family of the small leucine rich repeat proteins (SLRPs) and regulates the fibrillogenesis of collagen fibrils in the vitreous (Takanosu et al 2001). Consistent with our previous report (Zhou et al 2013), Col IX is expressed in the control and *Notch2* mutant P0 and P3 CBs at the levels comparable to those in the control CBs (Figure 4.7 A-D). *Rbpj* mutant P0 and P3 CBs also secrete comparable levels of Col IX protein as the control and *Notch2* mutant CBs (Figure 4.7 E and F). These results suggest that RBPJ and Notch2 are dispensable for Col IX secretion in the CB.

In the control P0 and P3 eyes, Optc protein is highly expressed in the ICE of the CB and is also secreted to the surface of the CB, the retina and the lens (Figure 4.7 H and I). There are no discernible differences between the control CBs and the *Notch2* mutant CBs (Figure 4.7 H-K). In contrast, in the *Rbpj* mutant P0 and P3 eyes, Optc protein levels in the ICE of the CB as well as in the surface of the CB, the retina and the lens are severely reduced in comparison with those in the control and *Notch2* mutant eyes (Figure 4.7 L and M). These results indicate that RBPJ, but not Notch2, is required to regulate Optc expression and secretion in the ICE of the CB.

DISCUSSION

Although BMP and Notch2 signaling have been shown to be essential for CB morphogenesis (Chang et al 2001, Sarode et al 2014, Zhao et al 2002, Zhou et al 2013), it remains poorly understood how they cooperate with each other to regulate CB morphogenesis at the molecular level. Our previous study has shown that Notch2 drives CB morphogenesis partially by regulating cell proliferation and BMP signaling (Zhou et al 2013). Another independent study suggests that RBPJ is dispensable for BMP signaling in the adult CB (Sarode et al 2014), but it remains unclear if RBPJ is also dispensable for BMP signaling in the developing CB. This study has further confirmed the essential roles of both Notch2 and RBPJ in controlling CB morphogenesis, and has further established the role of RBPJ-dependent Notch2 signaling in maintaining active BMP signaling in the OCE of the CB (Figure 4.8). In addition, I have revealed the role of RBPJ-independent Notch2 signaling in maintaining active BMP signaling in the stromal cells in a non-cell-autonomous manner. Finally, I have also uncovered Notch2-independent RBPJ functions for controlling Optc expression and/or secretion in the ICE and

maintaining the apical N-cadherin accumulation in the ICE-OCE junction (Figure 4.8). Therefore, this study has advanced our understanding of how Notch2 and RBPJ control BMP signaling, CB morphogenesis and the secretion function of the developing CB.

Although RBPJ-mediated Notch signaling can regulate differentiation of retinal gangion cells and photoreceptors (Riesenberg et al 2009, Zheng et al 2009), the deletion of either Notch2 or RBPJ does not affect specification of the CMZ, suggesting that Notch signaling is dispensable for specification of the CB fate. Similarly, both Notch2 and RBPJ are dispensable for normal secretion of Col IX protein. Interestingly, another aqueous humor protein Optc decreases its expression in the *Rbpj* mutant CB, but not in the *Notch2* mutant CB. This finding can help explain why the *Rbpj* mutant eye, but not the *Notch2* mutant eye, loses the vitreous body and shows the lens degeneration because Optc has been suggested to maintain vitreous gel stability and structure (Bishop 2000, Hindson et al 2005, Takanosu et al 2001) and the vitreous body also contains many growth factors (Forrester 2004, Sanders et al 2003). The reason why Notch2 is dispensable for Optc expression in the ICE is that Notch2 expression is restricted to the OCE (Zhou et al 2013). Finally, the requirement of RBPJ in the ICE for maintaining Optc expression is likely mediated by other Notch receptors. One of the likely candidates is Notch3 because it is known to be expressed in the ICE (Kitamoto et al 2005, Lindsell et al 1996). Since no obvious eye defects was reported in *Notch3* null mice (Kitamoto et al 2005, Krebs et al 2003), it is possible that another Notch receptor is also expressed in the ICE to work with Notch3 to control Optc expression. Alternatively, RBPJ works in the ICE to control Optc expression independently of Notch signaling. These possibilities await future investigation.

The RBPJ-dependent canonical Notch2 signaling likely operates in the OCE of the developing CB to control cell proliferation and BMP signaling, thereby driving CB morphogenesis. First, this study and our previous study have shown that both RBPJ and Notch2 are required for cell proliferation only in the OCE but not the ICE (Zhou et al 2013). This is also consistent with the recent finding that the RBPJ inactivation can suppress abnormal cell proliferation induced by an activated Notch receptor in the CB (Sarode et al 2014). Second, this study and our previous study have also demonstrated that both RBPJ and Notch2 are required for maintaining active BMP signaling in the developing CB (Zhou et al 2013). Interestingly, a recent study reported that BMP signaling becomes normal in the adult *Rbpj* mutant CB (Sarode et al 2014), suggesting that an RBPJ-independent mechanism exists in the adult CB to maintain BMP signaling. It will be important to identify the RBPJ-independent mechanism for maintaining active BMP signaling in the adult CB in the future.

One of the most exciting and surprising findings in this study is the revelation of the RBPJ-independent Notch2 signaling mechanism in the OCE to maintain active BMP signaling in the underlying stromal cells. Our previous study has shown that Notch2 is required in the OCE to maintain active BMP signaling in the stromal cells in a non-cell-autonomous manner by repressing the expression of two secreted BMP inhibitors, Chrdl1 and Nbl1(Zhou et al 2013). Because Chrdl1 and Nbl1 block BMP signaling activation by preventing BMPs from binding to receptors (Walsh et al 2010), the loss of Notch2 function results in the increased expression of these secreted BMP inhibitors and subsequent inhibition of BMP signaling in both OCE cells and underlying stromal cells (Figure 4.8). This study shows that RBPJ is largely dispensable for the BMP signaling in the underlying stromal cells, suggesting that RBPJ is dispensable for Notch2-

mediated repression of Chrdl1 and Nbl1. Such RBPJ-independent Notch signaling has already established in other systems. For example, Notch1 can directly bind with YY1 to activate c-myc expression independently of RBPJ in human myelogenous leukemia cell lines (Liao et al 2007), and Notch3 activates NF-kappaB signaling in T-cell development and leukemia independently of RBPJ (Vacca et al 2006). By comparing gene expression profiles between *Notch2* and *Rbpj* mutant CBs, I might be able to reveal how RBPJ-dependent and RBPJ-independent Notch2 signaling branches maintain active BMP signaling in the OCE of the CB and in the underlying stromal cells, respectively. In summary, this study has identified important roles of RBPJ-dependent and –independent Notch2 signaling in regulating BMP signaling and cell proliferation during CB morphogenesis, and also has uncovered a novel function of RBPJ in the regulation of CB secretion.

Figure 4.1 RBPJ is required for CB morphogenesis and the maintenance of normal ocular structures.

(**A, C** and **E**) WT CB undergoes morphogenesis at P0 (**A**), P3 (**C**) and P7 (**E**). (**B, D** and **F**) The morphogenesis process is disrupted in the *Rbpj* mutant CB at P0 (**B**), P3 (**D**) and P7 (**F**). In addition, the iris stroma (green arrows) is disorganized in the *Rbpj* mutant eyes. CB regions are highlighted by rectangles. Blue and yellow arrowheads indicate the ICE and the OCE, respectively. (**G**, **H**) H&E staining of cross sections of P7 eyes shows severe ocular defects in the *Rbpj* mutant eye (**H**) compared to the WT control (**G**). Besides the disruption of ciliary fold formation, the vitreous body is absent in the *Rbpj* mutant eye. (**I**) A 3 month-old control eye shows the CB, the iris, the retina, the lens and the vitreous body. (**J**) A 3 month-old *Rbpj* mutant eye shows micropthalmia, the degenerated retina and lens, the severely disorganized CB and iris and the absence of the vitreous body. Yellow and green arrows indicate the CB and the iris, respectively. Black and green asterisks mark the lens and the vitreous body, respectively. Scale bar: **A**-**F**, 100 μm; **G**-**H,** 200 μm; **I**-**J,** 500 μm.

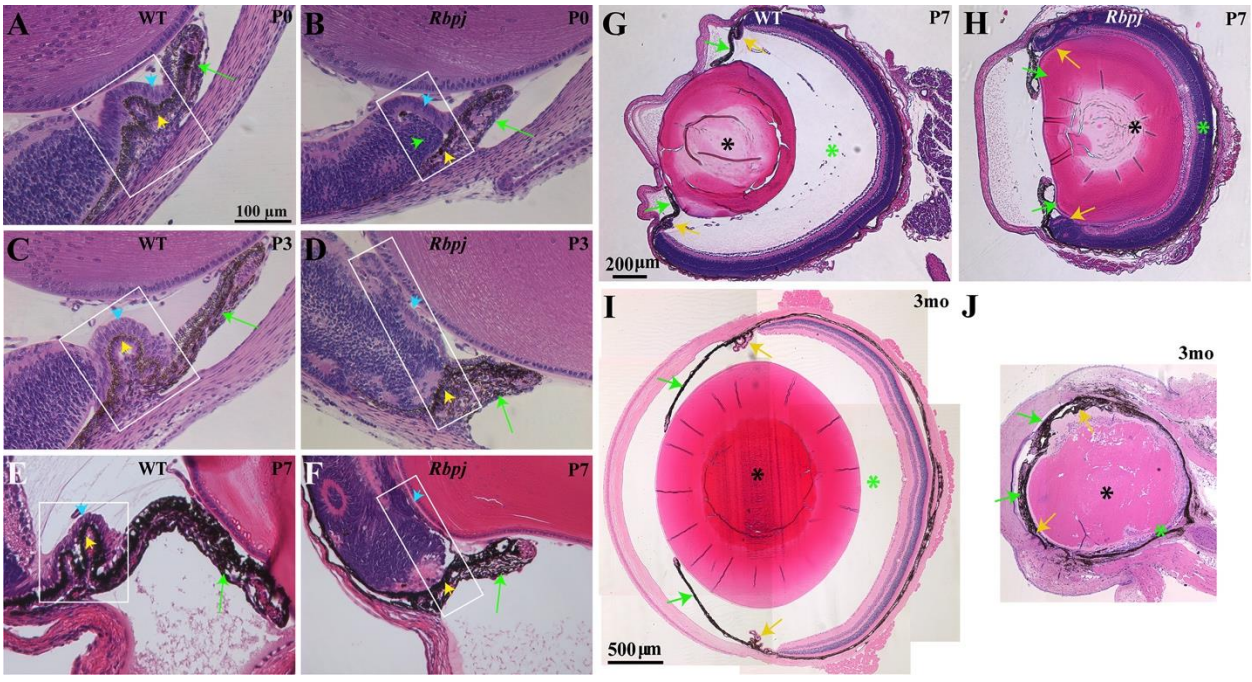

A
WT
P0
100 µm
B
Rbpj
P0
C
WT
P3
D
Rbpj
P3
E
WT
P7
F
Rbpj
P7
G
WT
P7
200µm
H
Rbpj
P7
I
3mo
500µm
J
3mo

Figure 4.2 RBPJ is dispensable for specification of the CMZ.

(**A**, **B**) Immunofluorescent images show that Pax6 protein is expressed at similar levels in *Rbpj* mutant (**B**) and WT CBs (**A**). (**C-F**) mRNA *in situ* hybridization experiments show comparable mRNA levels for *Otx1* (**C**, **D**) and *Msx1* (**E**, **F**) in *Rbpj* mutant (**D**, **F**) and WT control (**C**, **E**) CBs. White arrows denote the ICE and red arrows indicate the OCE. Scale bar: **A-B** 50 µm; **C-F** 100 µm.

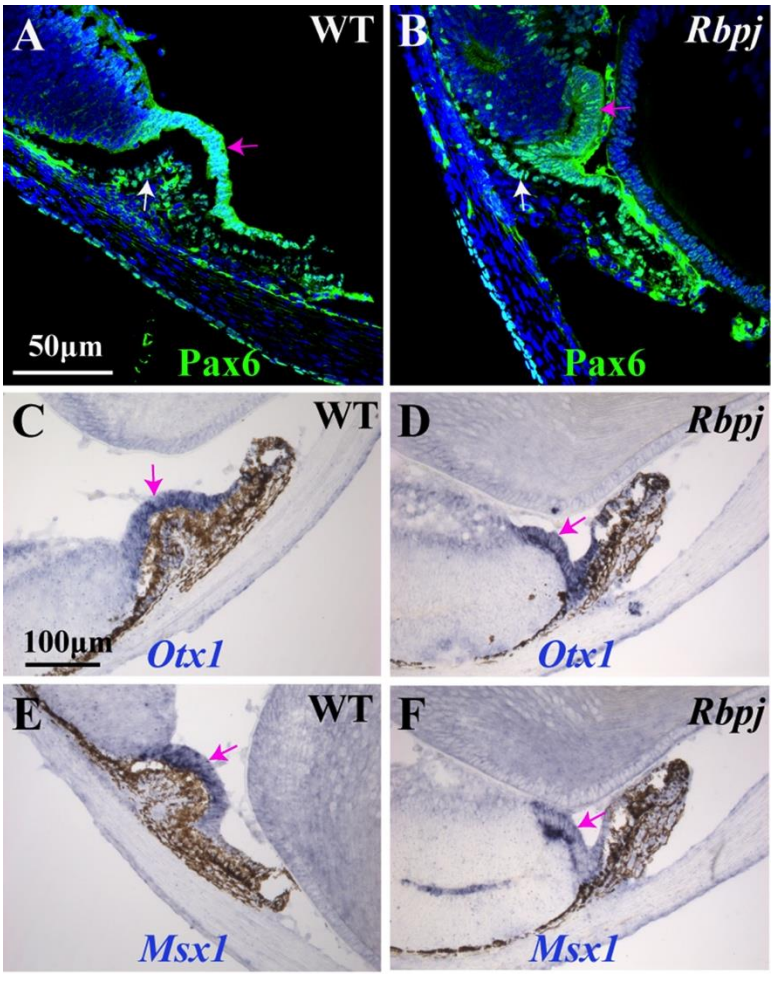

A
WT
B
Rbpj
50μm
Pax6
Pax6
C
WT
D
Rbpj
100μm
Otx1
Otx1
E
WT
F
Rbpj
Msx1
Msx1

Figure 4.3 RBPJ is required to maintain N-cadherin expression in the OCE of the CB.

(**A-A'**) N-cadherin is expressed in the apical junctions between the ICE and the OCE, as well as basolateral sides of the ICE and the OCE in the WT CB. Expression of N-cadherin remains unchanged in the *Notch2* mutant CB (**C-C'**), but its levels are drastically reduced in the ICE-OCE junction and the OCE of the *Rbpj* mutant CB (**B-B'**). Although the ICE is separated from the OCE, N-cadherin is normally expressed in the ICE in *Rbpj* mutant CBs. White arrow indicates the separated ICE. **A'**, **B'** and **C'** are higher magnification of **A**, **B** and **C**, respectively. White arrowheads denote the apical junctions, whereas red arrowheads show the basal side of the OCE cells. Scale bar: 50 μm.

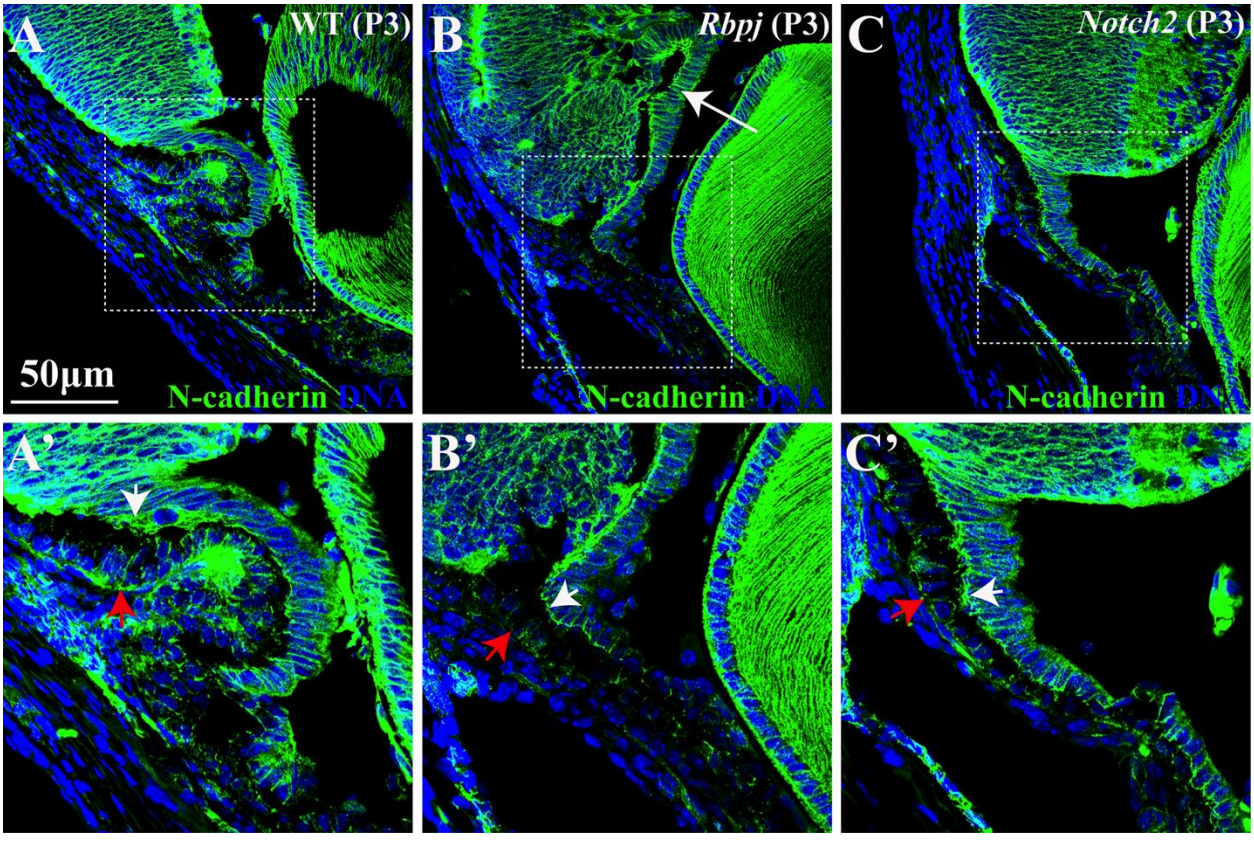

A
WT (P3)
50µm
N-cadherin DNA
B
Rbpj (P3)
N-cadherin DNA
C
Notch2 (P3)
N-cadherin DNA
A'
B'
C'

Figure 4.4 N-cadherin is important for CB morphogenesis.

(**A-F**) The CB undergoes morphogenesis in WT at P0 (**A**), P3 (**C**) and P7 (**E**). (**B-B'**, **D-D'**, **F-F'**) The CB shows deficits in morphogenesis in *Ncad CKO* mutants at P0 (**B-B'**), P3 (**D-D'**) and P7 (**F-F'**). Asterisks indicate stem-like folding of ICE. (**G-G'**) N-cadherin has been efficiently removed from both CB layers. (**H**) Quantification of percentage of CBs with morphogenesis defects in *Ncad CKO* mutants (n=23 for P3, n=10 for P7, and n=12 for P30).

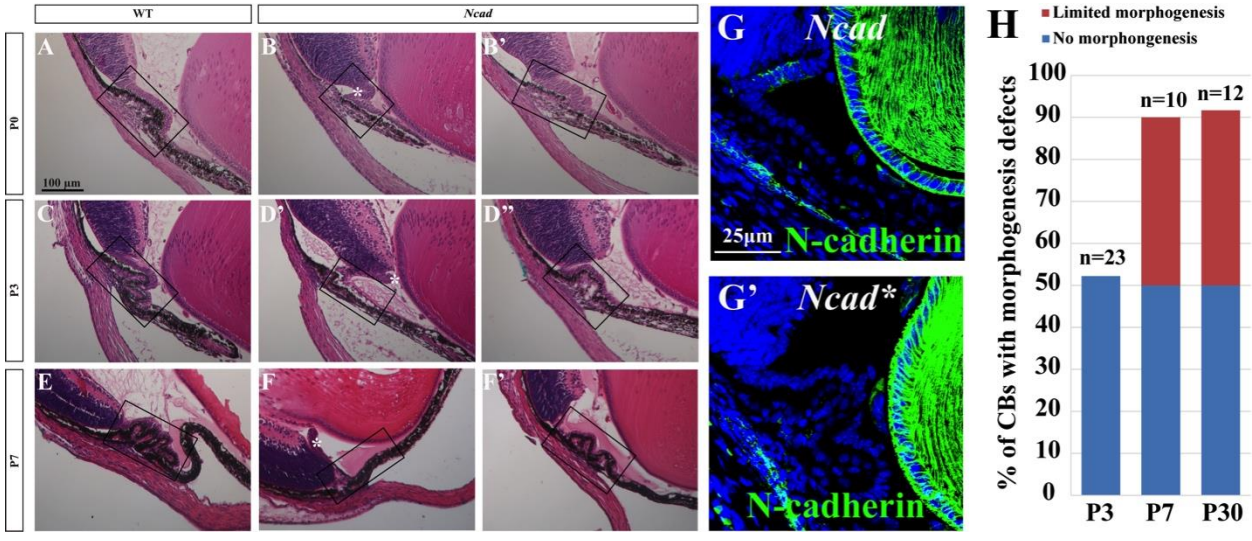

WT
Ncad
P0
P3
P7
A
B
B'
C
D'
D''
E
F
F'
100 µm
G
Ncad
25µm
N-cadherin
G'
Ncad*
N-cadherin
H
Limited morphogenesis
No morphongenesis
% of CBs with morphogenesis defects
100
90
80
70
60
50
40
30
20
10
0
n=23
n=10
n=12
P3
P7
P30

Figure 4.5 RBPJ is essential for cell proliferation in the OCE.

(**A-C**) BrdU labels proliferative S-phase cells in both ICE and OCE of WT CBs (**A-A''**) and *Rbpj* mutant CBs (**B-B''**). Broken lines highlight the OCE regions. (**C**) Quantitative analysis shows a significant reduction in BrdU-positive cells in the OCE of the *Rbpj* mutant CBs compared to the control CBs. N = 13 for WT controls, and N = 8 for *Rbpj* mutant CBs. Scale bar: 50 µm.

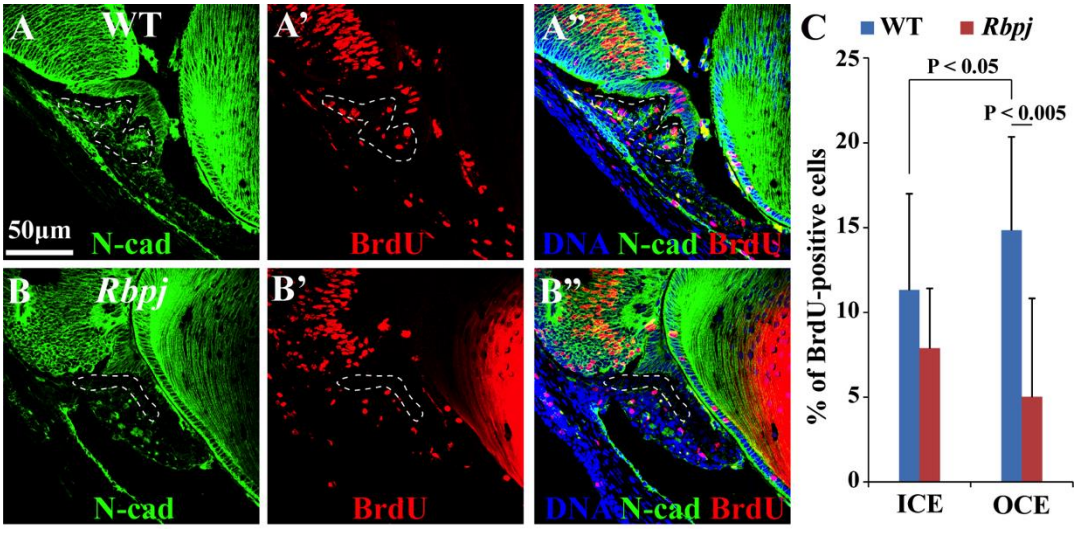

A
WT
A'
A''
50μm
N-cad
BrdU
DNA N-cad BrdU
B
Rbpj
B'
B''
N-cad
BrdU
DNA N-cad BrdU
C
WT
Rbpj
P < 0.05
P < 0.005
% of BrdU-positive cells
25
20
15
10
5
0
ICE
OCE

Figure 4.6 RBPJ is required for maintaining BMP signaling in the OCE of the CB.

(**A**, **B**) pSmad1/5/8 is strongly expressed in the OCE and stromal cells of the WT CB at P0 (**A**) and P3 (**B**). (**C**, **D**) Both the OCE and stromal cells show a dramatic reduction of pSmad1/5/8 in the *Notch2* mutant P0 (**C**) and P3 (**D**) CB. Broken lines outline the OCE, and arrowheads indicate the stroma cells. (**E**, **F**) pSmad1/5/8 expression is diminished in the *Rbpj* mutant P0 (**E**) and P3 (**F**) OCE, but its expression is largely normal in the underlying stromal cells. Scale bar: 50 μm.

(G) Quantitative PCR results show that *Chrdl1* and *Nbl1expression* does not change in *Rbpj* mutant CB, whereas *Notch2* expression is decreased by half.

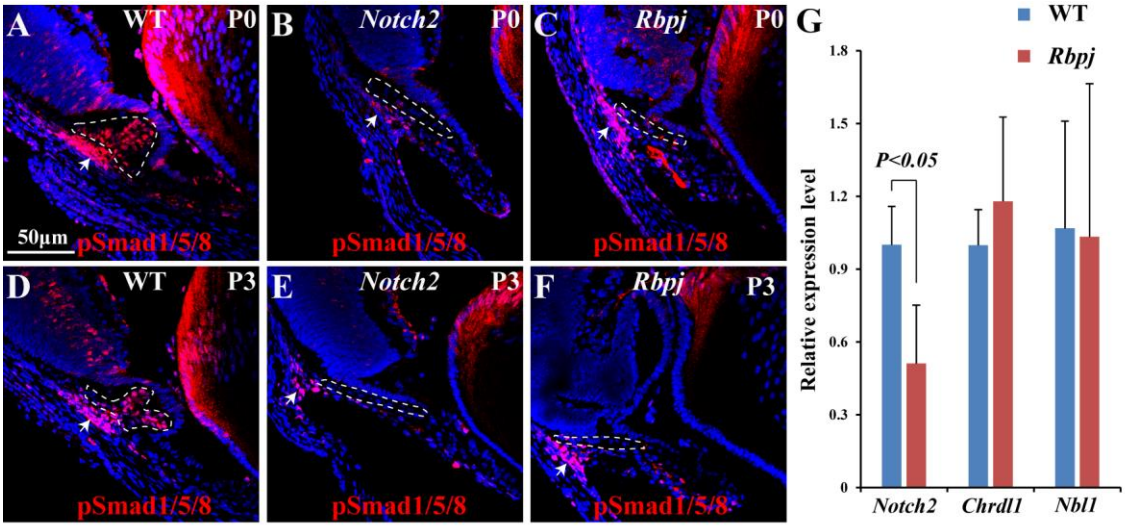

A
WT
P0
50μm
pSmad1/5/8
B
Notch2
P0
pSmad1/5/8
C
Rbpj
P0
pSmad1/5/8
D
WT
P3
pSmad1/5/8
E
Notch2
P3
pSmad1/5/8
F
Rbpj
P3
pSmad1/5/8
G
WT
Rbpj
P<0.05
Relative expression level
1.8
1.5
1.2
0.9
0.6
0.3
0
Notch2
Chrdl1
Nbl1

Figure 4.7 RBPJ regulates CB secretion.

(**A** and **B**) Col IX accumulates at the surface of the CB and the retina in WT control P0 (**A**) and P3 (**B**) eyes. Expression levels of Col IX are not affected in *Rbpj* mutant eyes (**E** and **F**) and *Notch2* mutant eyes (**C** and **D**). (**H** and **I**) Optc is expressed in the ICE of the CB and deposited at the basal side of the CB at P0 (**H**) and P3 (**I**). (**L-M**) Optc expression in the ICE is drastically reduced in the *Rbpj* mutant CBs (**J**, **K**), whereas its level in *Notch2* mutant CBs remains unaffected (**J**, **K**). Red arrows indicate the surface of the lens and white arrows denote the surface of the retina. Arrowheads show ICE cells.

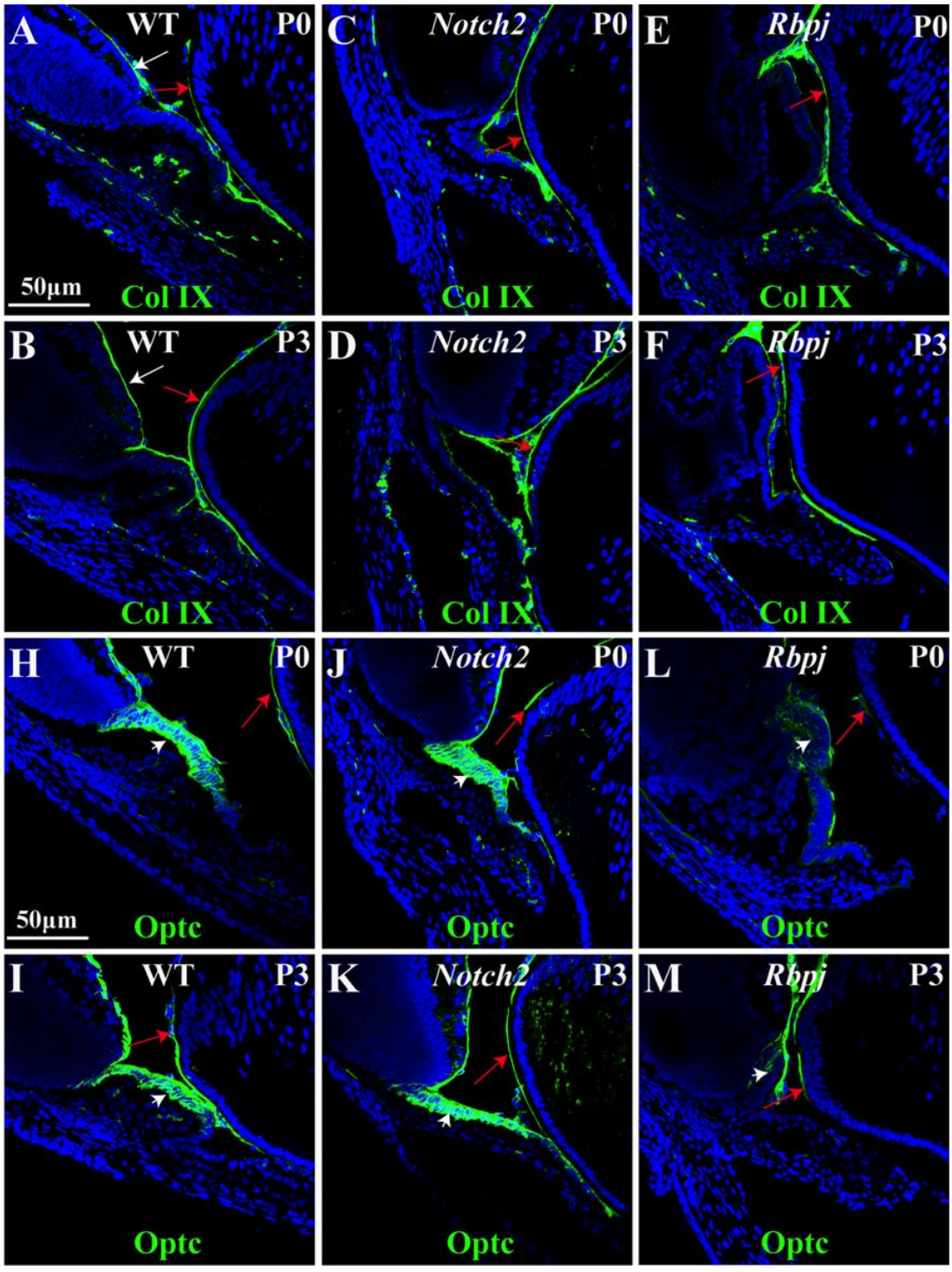
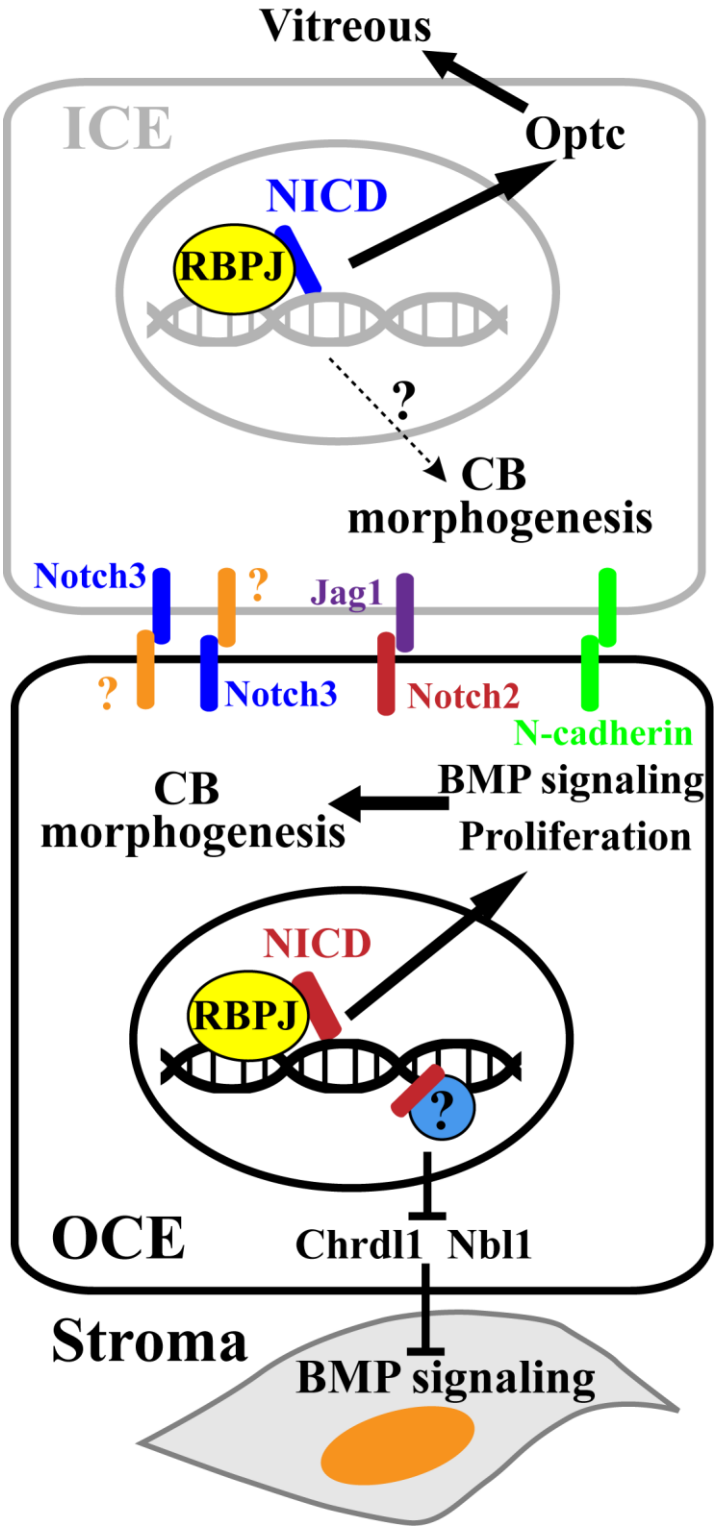

A
WT
P0
50µm
Col IX
C
Notch2
P0
Col IX
E
Rbpj
P0
Col IX
B
WT
P3
Col IX
D
Notch2
P3
Col IX
F
Rbpj
P3
Col IX
H
WT
P0
50µm
Optc
J
Notch2
P0
Optc
L
Rbpj
P0
Optc
I
WT
P3
Optc
K
Notch2
P3
Optc
M
Rbpj
P3
Optc

Figure 4.8 Working model on Notch signaling-mediated regulation of CB morphogenesis.

Notch3 is expressed in the ICE and OCE of the CB, whereas Notch2 is restricted to the OCE. RBPJ-dependent Notch3 signaling in the ICE may regulate the expression of Optc and controls secretion of the CB. Notch3 signaling in the ICE may also modulate CB morphogenesis. The ligand Jag1 from the ICE activates Notch2 signaling in the OCE. The activated Notch2 signaling in the OCE regulates cell proliferation and BMP signaling through RBPJ to facilitate CB morphogenesis. In addition, RBPJ-independent Notch2 signaling can repress the expression of secreted BMP inhibitors Chrdl1 and Nbl1 to maintain BMP signaling in the stroma cells. RBPJ modulates the expression of N-cadherin in the OCE possibly through Notch3-mediated signaling.


Vitreous
ICE
Optc
NICD
RBPJ
?
CB
morphogenesis
Notch3
?
Jag1
?
Notch3
Notch2
N-cadherin
CB
morphogenesis
BMP signaling
Proliferation
NICD
RBPJ
?
OCE
Chrdl1
Nbl1
Stroma
BMP signaling

CHAPTER FIVE: CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

Based on experimental results, I have the following conclusions:

1. **RBPJ-dependent Notch2 signaling is required for BMP signaling and epithelial morphogenesis in the developing CB.**

   Canonical Notch signaling requires the activity of both Notch receptors and transcription effector RBPJ. Here I show that absence of either Notch2 or RBPJ abrogates the morphogenesis of the CB. I further characterized that RBPJ-dependent Notch2 signaling regulates cell proliferation and maintains active BMP signaling in the OCE, which are important for CB morphogenesis.

2. **RBPJ-independent Notch2 signaling regulates BMP signaling non-autonomously in the ciliary stroma cells.**

   Notch signaling can crosstalk with BMP signaling via interaction of downstream components. Here I identified a novel link between Notch2 and BMP that RBPJ-independent Notch2 signaling maintains BMP signaling non-autonomously in the stroma cells via repressing the expression of secreted BMP inhibitors Chrdl1 and Nbl1.

3. **RBPJ holds two layers together possibly by maintaining N-cadherin expression in the OCE during CB morphogenesis.**

Cadherin-mediated adherens junction is essential for epithelial morphogenesis. Here I show that Notch2-independent RBPJ maintains N-cadherin expression in the OCE of the developing CB. The depletion of RBPJ in the CB leads to decreased homophilic accumulation of N-cadherin at the apical junctions, and the subsequent separation of the ICE from the OCE. I further characterized the phenotype of *Ncad* CKO mutant CBs and concluded that N-cadherin is required for the morphogenesis of the CB.

4. RBPJ modulates Optc expression and secretion in the ICE of the CB.

Aqueous humor secretion is one of the major functions of the CB. RBPJ but not Notch2 controls Optc expression in the ICE, whereas Col IX expression is not regulated by Notch2/RBPJ-mediated signaling. Reduced Optc expression in the *RBPJ* mutant eyes correlates with loss of the vitreous body and degeneration of major ocular structures, suggesting an important role of RBPJ in modulating CB secretion function.

FUTURE DIRECTIONS

The ciliary body has been acknowledged as an important structure for maintaining normal eye functions. However, the regulation of its development and function remains poorly understood. In this study, I have characterized important roles of Notch2 and RBPJ in the eye, and this provides novel insights into the development of the ciliary body and potential glaucoma targets. However, there are still many interesting and exciting questions awaiting exploration.

1. To investigate RBPJ-dependent and –independent Notch2 signaling in CB development

Canonical Notch signaling relies on the cooperation of Notch receptors and effector RBPJ. To investigate the roles of Notch2 and RBPJ, I conditionally removed Notch2 and RBPJ from the developing CB, respectively. No obvious defects were observed in the RPE, suggesting Notch2 and RBPJ are dispensable for the development of RPE. Results from others also support this idea (Forrester 2004, Sarode et al 2014). I observed a severe disruption of formation of the CB in both *Notch2* and *RBPJ* mutant eyes, suggesting the importance of Notch2 and RBPJ in the developing CB. However, I also noticed substantial phenotypical differences between *Notch2* and *RBPJ* mutant CBs, indicating they might employ different mechanisms to regulate CB morphogenesis and function.

A comprehensive comparison of the transcriptome profile will reveal genome-wide differences between RBPJ and Notch2 signaling. To determine differential gene expression between *RBPJ* and *Notch2* mutant OCE cells, I can use a laser-capture microdissection approach to precisely harvest OCE cells and perform RNA-seq subsequently (Figure 5.1). This method has been

proved to be highly sensitive and effective to analyze gene expression in specific cell types within a tissue (Morrison et al 2012).

2. To investigate the role of cell proliferation during CB morphogenesis

Differential cell proliferation rates in the CB provide cellular basis for the fold formation (Napier & Kidson 2005, Stroeva 1967). As I observed, deletion of Notch2 or RBPJ leads to drastic and significant decreases of cell proliferation in the OCE, demonstrating that Notch2 and RBPJ are required for high level of cell proliferation of the OCE. Notch signaling has been shown to be able to promote cell proliferation if ectopically activated in the RPE and the CB, and its effect on cell proliferation depends on the presence of RBPJ (Forrester 2004, Sarode et al 2014). Consistent with this idea, the proliferation rate in *Notch2* mutant OCE cells is comparable to that of *RBPJ* mutant OCE cells (Figure 4.5) (Zhou et al 2013), suggesting Notch2 regulates cell proliferation through RBPJ dependent mechanisms.

However, it remains unclear whether decreased cell proliferation in *Notch2* and *RBPJ* mutants is the cause for morphogenesis defects or the consequence of the reduction of cell surface in unfolded epithelium. A direct genetic evidence could be generated by ectopically overexpressing p21 cell cycle inhibitor during the time of fold formation (Abbas & Dutta 2009).

3. To investigate the role of cell adhesion in the CB morphogenesis

Dynamic formation of adherens junctions during epithelium morphogenesis generates adhesive forces and interacts with cytoskeleton to regulate tissue morphology (Harris & Tepass 2010). I have identified that N-cadherin instead of E-cadherin is highly expressed in the developing CB, especially at the apical junctions between the ICE and the OCE. I further confirmed the role of N-cadherin in the CB development by conditionally removing N-cadherin. The fold formation is abolished in half of N-cadherin-depleted CBs. Redundant roles of other cadherin family proteins may likely explain phenotypical variations of *Ncad* mutant CBs (Honjo et al 2000).

Surprisingly, expression of N-cadherin is severely reduced in the OCE of *Rbpj* mutants, but not in *Notch2* mutant CBs, suggesting Notch2-independent RBPJ regulates the stability of N-cadherin in the OCE. RBPJ has been shown to be able to interact with other transcriptional coactivators other than NICD to activate expression of downstream targets (Beres et al 2006, Lelievre et al 2011, Masui et al 2007, Obata et al 2001). It is important to identify interacting partners of RBPJ in OCE cells for understanding the mechanism of this novel Notch2-independent RBPJ function.

4. To investigate how Notch2/RBPJ signaling regulates BMP signaling

BMP signaling plays an important role during the development of anterior segments including the ciliary body. Disruption of BMP signaling in the developing eye can cause severe dysgenesis of the ciliary body (Chang et al 2001, Zhao et al 2002, Zhou et al 2013). BMP signaling is active in both OCE cells and underlying stroma cells (Figure 3.4 and 4.6). Deletion of Notch2 or RBPJ diminishes BMP signaling in the OCE cells, whereas BMP signaling in the stroma cells is only

affected in *Notch2* mutant CBs but not *Rbpj* counterparts. These results suggest canonical RBPJ-dependent Notch2 regulates BMP signaling in the OCE cells cell-autonomously, in addition to a mechanism of RBPJ-independent non-autonomous regulation of BMP signaling in the underlying stroma cells.

Microarray and qPCR experiments identified two secreted BMP inhibitors, *Chrdl1* and *Nbl1*, up-regulated about two folds in *Notch2* mutant OCE cells (Figure 3.5). This might be a possible mechanism of the non-autonomous regulation of BMP signaling in the stroma cells by Notch2. However, these two genes are not regulated by RBPJ as their expression remain normal in *RBPJ* mutant CBs (Figure 4.6 G). More evidence of RBPJ-independent Notch functions has emerged recently (Andersen et al 2012). For example, NICD can associate with β-catenin and negatively regulates Wnt/β-catenin signaling in stem and colon cancer cells (Kwon et al 2011). It will be even more complicated if the regulation is indirect. *In vitro* analysis with cell lines might provide more insights into the molecular mechanisms.

5. To investigate how RBPJ regulates ciliary body secretion in the ICE

Secretion from the CB provides components that constitute the aqueous humor and the vitreous body (Beebe 1986). I observed a severe decrease of the expression of vitreous protein Optc and the absence of vitreous body in *RBPJ* but not *Notch2* CKO mutant eyes, demonstrating that RBPJ regulates ciliary body secretion to maintain normal ocular structures. As I and others (Bao & Cepko 1997, Lindsell et al 1996, Zhou et al 2013) have shown, expression of Notch2 is highly restricted in the OCE of the CB (Figure 3.2). This might explain why Notch2 is dispensable for

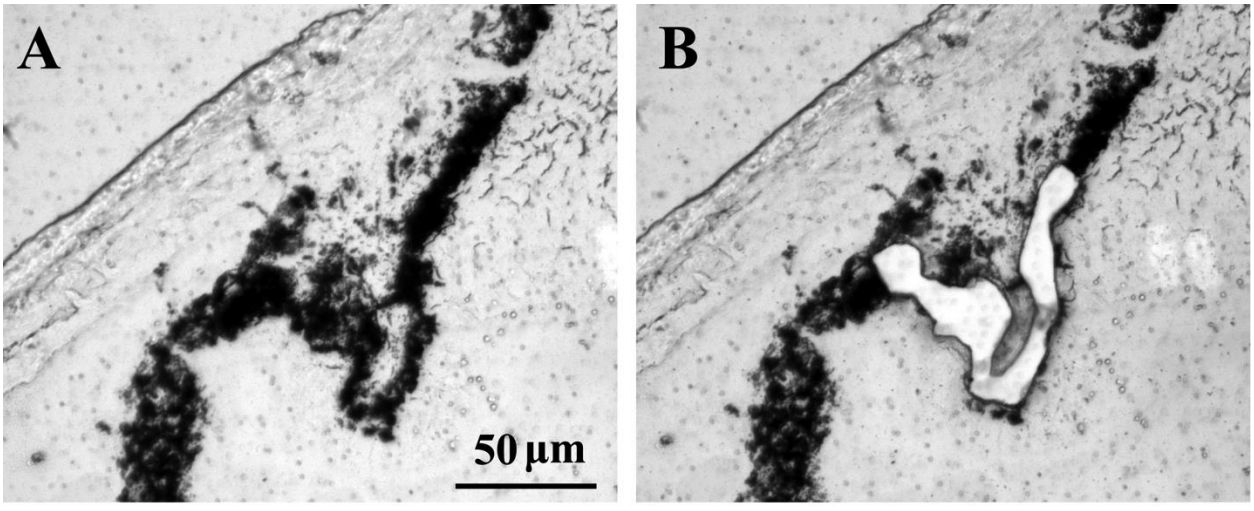
the regulation of CB secretion. It has been suggested that Notch3 is expressed in the ICE (Kitamoto et al 2005, Lindsell et al 1996), however no obvious eye defects were reported in *Notch3* null mice (Kitamoto et al 2005, Krebs et al 2003), suggesting additional Notch receptor might work together with Notch3 to control CB secretion in the ICE. Careful immunohistochemistry and mRNA *in situ* hybridization experiments may reveal the Notch receptors expressed in the ICE. Again, it is still possible that RBPJ regulates CB secretion independent of Notch receptors. Optc could serve as an entry point to uncover this mechanism.

In summary, I have established a great model to study signaling pathway functions in the regulation of the development and function of the ciliary body. This study reveals not only important biological roles of Notch2 signaling in the ciliary body development, but also provide novel insight into the regulation of ciliary body secretion by modulating Notch/RBPJ signaling pathways, which might lead to the development of a novel glaucoma therapy.

Figure 5.1 Laser-capture microdissection isolates CB tissues for RNA sequencing.

(A) Bright field image of a section of the CB before laser-capture microdissection.

(B) Bright field image of the same section shows precise isolation of OCE cells with laser-capture microdissection.



Before After

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