NOTCH2 REGULATES BMP4 AND MORPHOGENESIS

IN THE DEVELOPING MOUSE CILIARY BODY

by

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Abstract

Notch is an evolutionarily conserved cell-signaling pathway that is ubiquitous throughout development from *Drosophila* to humans. Defects in the Notch pathway result in several human genetic diseases and are associated with acquired disease states including cancer. Using the mouse eye as a model, we have discovered a new role for Notch2 in development and a novel interaction between the Notch2 and BMP4 pathways.

The ciliary body is an important part of the anterior eye which secretes aqueous humor and controls lens accommodation. The ciliary body also modulates the trabecular meshwork which maintains proper intraocular pressure by regulating its outflow. Using conditional Cre-loxP knockout technology, we selectively removed the Notch2 receptor from the pigmented outer ciliary epithelium in mouse. The loss of Notch signaling resulted in severe dysmorphogenesis of the ciliary body while sparing the iris. Contributing to this lack of morphogenesis is a significant reduction in proliferation across multiple time points. Cell-adhesion both dorsally and ventrally involving N-cadherin, α-catenin, and β-catenin is also disrupted. Thirdly, a ventral specific reduction of pSMAD1/5/8 was detected. Using a GFP reporter line which identified Cre expression, we determined that Notch2 affects N-cadherin and cell adhesion in a cell autonomous manner, while pSMAD1/5/8 is regulated both cell autonomously and non-cell autonomously only on the ventral side of the eye. This is a novel interaction between the Notch and BMP pathways.

To further define the roles of Notch2 versus BMP4, we crossed the same Cre-line with Alk2/Alk3 mice to conditionally remove the BMPR1 receptors. While previous work showed that BMP4 heterozygotes displayed ciliary body dysmorphogenesis, our new cross demonstrates that BMP4 signaling in the outer ciliary epithelium is necessary for ciliary body morphogenesis. In the Alk2/Alk3 mice, N-cadherin, α-catenin, and β-catenin were all present, indicating that they are downstream of Notch2 and not BMP4. This is consistent with our Notch2 knockout studies. Notch2 staining is also retained in the DM61/Alk2$^{fx/fx}$/Alk3$^{fx/fx}$ mice, further confirming that Notch2 is upstream of BMP4.

Finally, to confirm the role of adhesion in the ciliary body, we crossed our Cre-line with an N-cadherin floxed line. The DM61/N-cadherin$^{fx/fx}$ mice recapitulated the Notch2 phenotype and confirmed the necessity of N-cadherin in ciliary body morphogenesis. Histologic analysis suggest a positive feedback loop between N-cadherin and Notch2, as Notch2 was reciprocally downregulated in the DM61/N-cadherin$^{fx/fx}$ mice. BMP4 signaling was also downregulated.

Taken together, we have discovered a new role for Notch2 in eye development. Notch2 is a necessary signal in regulating the morphogenesis of ciliary body development. It does this, at least in part, through a concerted program of proper proliferation and adhesion. In parallel with regulating adhesion and possibly upstream of proliferation, Notch2 regulates BMP4 signaling through a novel mechanism. This study is the first to provide any evidence of signaling pathway integration in anterior eye development. Proper

development of the anterior eye is significant in several human diseases, including primary congenital glaucoma. These findings may have far-reaching implications across both development and disease, especially in Notch biology.

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We have been fortunate in this project to have the assistance of both internal and external collaborators. Dr. Shuyi Chen discovered that conditionally knocking out Notch2 in the RPE causes morphologic defects in the ciliary body, which forms a foundation of my

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ABBREVIATIONS

Genes:
BMP4/7 = Bone morphogenetic protein 4/7
Bmpr = Bone morphogenetic protein receptor
Cdc = Cell division cycle related
Chx10 = Ceh-10 homeo domain containing homolog
Col IX = Collagen IX
Conn43 = Connexin 43
Dll = Delta (Notch ligand)
EGF = Epidermal growth factor
FGF = Fibroblast growth factor
Hes = Hairy and enhancer of split
Hey = Hairy/E(spl)-related with YRPW motif
Jag = Jagged (Notch ligand)
LacZ = β-galactosidase
Lef1 = Lymphoid enhancer-binding protein factor1
Lmx1b = LIM homeobox transcription factor 1 beta
Mitf = Microphthalmia-associated transcription factor
Msx1 = Homeobox, msh-like 1
N-cadherin = Neuronal cadherin
Otx1 = Orthodenticle homolog 1
Pax6 = Paired box gene 6
P-cadherin = Placental cadherin
Pttg1 = Pituitary tumor-transforming 1
rSMAD = Receptor - mothers against decapentaplegic homolog
SMAD = Mothers against decapentaplegic homolog
TGF-β = Transforming growth factor-beta
Tryp = Tyrosinase-related protein
Wnt2b = Wingless-type MMTV integration site family member 2b

Terms:
AGS = Alagille Syndrome
ASD = Anterior segment dysgenesis
CADASIL = Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CB = Ciliary body
CMZ = Ciliary marginal zone
CSL family = CBF1, Su(H), Lag-1 family
DN = Dominant negative
IACUC = Institutional Animal Care and Use Committee

ICE = Inner ciliary epithelium
IOP = Intraocular pressure
KO = Knockout
miRNA = MicroRNA
NICD = Notch intracellular domain
NR = Neural retina
OCE = Outer ciliary epithelium
PCG = Primary congenital glaucoma
PEST = Proline (P), glutamic acid (E), serine (S), and threonine (T) rich peptide sequence
piRNA = Piwi-interacting RNA
POAG = Primary open angle glaucoma
RGC = Retinal ganglion cell
RNA = Ribonucleic acid
RPE = Retinal pigmented epithelium
snoRNA = Small nucleolar RNA
shRNA = Short hairpin RNA
TM = Trabecular meshwork
UTR = Untranslated region

Reagents:
BCIP = 5-bromo-4-chloro-3'-indolyphosphate
BSA = Bovine serum albumin
DAPI = 4',6-diamidino-2-phenylindole
DEPC = Diethylpyrocarbonate
HRP = Horseradish peroxidase
NBT = Nitro-blue tetrazolium
PBS = Phosphate buffered saline
SDS = Sodium dodecyl sulfate
TBST = Tris-buffered saline plus Tween-20
TdT = Terminal deoxynucleotide transferase

CHAPTER ONE: BACKGROUND

NOTCH

Notch Pathway Overview

The Notch signaling pathway is an evolutionarily conserved pathway which participates in diverse developmental roles. Since its discovery in *Drosophila* in 1917 by Thomas Hunt Morgan, four mammalian homologue receptors have been identified. These receptors are membrane bound with an intracellular domain and an EGF-repeat rich extracelluar domain. Upon binding of the extracellular EGF-repeats by a Delta or Jagged (Serrate) ligand, proteolytic cleavage releases the Notch Intracellular Domain (NICD) which then translocates to the nucleus, binds a CSL family transcription factor, and co-activates or represses its target genes (Fortini, 2009). Three Delta homologue ligands and two Jagged (Serrate homologue) ligands have also been characterized in mammals (Figure 1.1).

During its history, Notch has been shown to be important in cell fate, proliferation, differentiation, tissue boundary formation, apoptosis, cellular migration, and other processes (Artavanis-Tsakonas et al., 1999). Such a range of functions underscores its evolutionary importance in development. Within the eye alone, multiple receptors and ligands are expressed. Notch1 and Delta are expressed in the retina, whereas Jagged has been shown in the tip of the optic cup and lens. Notch2 is expressed during lens development and throughout the RPE early in development followed by a restriction of

expression to only the tips of the optic cup (Bao and Cepko, 1997; Saravanamuthu et al., 2009).

Genetic Diseases Associated With Notch

Defects in the Notch pathway have been shown to be the cause of four human genetic diseases: Alagille Syndrome (AGS), spondylocostal dysostosis, Hajdu-Cheney Syndrome, and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). Dysregulation of Notch signaling also has a role in various leukemias and lymphomas, breast cancer, colorectal cancer, and other malignancies (Florena et al., 2007; Aster et al., 2008; Joshi et al., 2009; Chu et al., 2011). The genetic diseases associated with Notch signaling and their associated defective component(s) are summarized in Table 1.1. As the focus of this project is Notch2, only AGS and Hajdu-Cheney Syndrome will be discussed further.

As described by Dr. Alagille, AGS is characterized by liver, heart, eye, skeletal, craniofacial, and kidney abnormalities (Alagille et al., 1987). One of the unique features of AGS is the variability of penetrance of the respective symptoms. The disease is characterized into two subtypes: AGS1 and AGS2. In AGS1, the disease is caused by a mutation in the Jag-1 ligand (Li et al., 1997; Oda et al., 1997). This mutation is found in 94% of patients (Warthen et al., 2006). AGS2 results from mutations in the Notch2 receptor gene. AGS2 also has less cardiac and renal involvement (McDaniell et al., 2006). In terms of ocular involvement, Hingorani et al. characterized a variety of AGS

dysmorphologies including posterior embryotoxon, iris abnormalities, diffuse fundus hypopigmentation, speckling of the RPE, and optic disc anomalies (Hingorani et al., 1999).

Shortly after discovery of the role of Notch in AGS, an effort was made to develop a mouse model of the disease. Gridley and coworkers soon discovered that despite the work done on discovering the role of Jag1 in AGS, making an accurate mouse model proved difficult. Homozygous Jag1 null alleles turned out to be embryonic lethal, and heterozygotes displayed eye defects but none of the other physical signs of AGS (Xue et al., 1999). A few years later McCright and Gridley developed a double-heterozygote of Jag1 and Notch2 which accurately displayed the AGS phenotype (McCright et al., 2002). This was followed by the discovery of Notch2 mutations as a second cause of human AGS (McDaniell et al., 2006). These studies have contributed to improved detection of AGS (Samejima et al., 2007) and a better understanding of AGS genetics, but more work still needs to be done. The actual mechanism and downstream signals of Alagille Syndrome are not known. In eye development, the role of Notch2 outside the lens has yet to be discovered. Just as importantly, the integration of Notch2 with other signaling pathways in anterior eye development needs to be investigated.

Hajdu-Cheney Syndrome is an autosomal dominant disorder of the connective tissue. Severe osteoporosis due to extreme osteolysis is the cardinal phenotype. Patients with this rare disease may also present with characteristic features including micrognathia,

hypertelorism, elongated philtrum, wormian skull bones, and acro-osteolysis of the distal phalanges. It was recently shown that the causative defect in the Notch2 gene is frame-shift inducing mutations in the last exon (Isidor et al., 2011; Simpson et al., 2011). The last exon codes for a PEST domain which helps to regulate degradation of the protein. Loss of this domain, then, results in a putative gain-of-function mutation. It is hypothesized that this increased Notch2 activity alters the osteoblast/osteoclast balance in favor of increased bone resorption. How this amplification in Notch2 activity may result in the other features of Hajdu-Cheney Syndrome is not yet known.

Notch biology remains an important field of research. Numerous genetic and acquired disorders have been traced back to defects in this pathway. Additionally, numerous tissues rely on Notch and its interactions with other conserved signaling mechanisms for proper development and tissue maintenance. This project seeks to add valuable insight into the Notch pathway and how it interacts with other pathways which may be directly beneficial for patients who suffer from Notch related illnesses.

ANTERIOR EYE DISEASES AND DEVELOPMENT

Why Study the Anterior Eye?

The anterior eye is subject to numerous acquired and congenital disease states making the study of its development relevant to human medicine. Most famous amongst anterior eye

disorders is glaucoma. Glaucoma is the second leading cause of blindness in the United States, and the leading cause worldwide (Resnikoff et al., 2004).

Glaucoma is a form of optic neuropathy resulting from the death of retinal ganglion cells (RGCs). The pathologic loss of RGCs damages the optic nerve which is composed of the axons of the RGCs and leads first to peripheral visual field loss and then to overall visual field defects potentially including blindness. A significant risk factor for developing glaucoma is increased intraocular pressure (IOP) (Kwon et al., 2009). The pressure in the eye may be increased in a number of ways. In normal physiology, aqueous humor produced by the ciliary body enters the posterior chamber of the anterior eye, exits through the pupil of the iris into the anterior chamber, and escapes through the outflow tracks of the trabecular meshwork (TM) and Schlemm's canal (Figure 1.2). These structures funnel excess fluid into either the nasal cavity or the venous circulation. Increased IOP may result from the dysgenesis or obstruction of these outflow tracks. The primary regulator of IOP is the TM, not the ciliary body. However, TM contractility is modulated by the neighboring stromal musculature of the ciliary body (Stumpff and Wiederholt, 2000; Tamm, 2009). Taken together, the ciliary body plays a unique role as (1) the secretory organ which secretes aqueous humor and (2) a muscular tissue which cooperates with the TM in regulating outflow and IOP.

Glaucoma has several classifications and etiologies including both primary and secondary forms. Primary glaucoma may be further divided into primary open-angle glaucoma

(POAG), primary closed-angle glaucoma, and primary congenital glaucoma. Secondary forms may derive from pathologic and/or iatrogenic causes which will not be discussed here. In POAG, neuropathy of the optic nerve results despite the normal, gross appearance of the distance between the cornea and iris (irido-corneal angle). There are both normotensive and increased IOP variants. While POAG may be either juvenile-onset or adult-onset in nature, both have genetic components such as links to the myocillin (MYOC) gene (Kwon et al., 2009; Rao et al., 2011). Around 4% of adult-onset cases and over 10% of juvenile onset cases have been linked to mutations in this gene (Kwon et al., 2009).

In primary closed-angle glaucoma, the irido-corneal angle is either sufficiently reduced or eliminated resulting in obstruction of the outflow tract and increased IOP. This physical occlusion of the outflow tracts may quickly lead to a medical emergency and must be addressed promptly.

Anterior Segment Dysgenesis (ASD)

Anterior segment dysgenesis (ASD) is an umbrella term for several different genetic diseases which result in defects in the anatomical structure of the anterior eye. These patients usually have an increased risk of developing glaucoma early in life. Mutations in a number of genes have been identified in association with human disorders which cause PCG. Examples of ASD include Axenfeld-Rieger's Syndrome, Nail-Patella Syndrome, and defects in CYP1b1.

Patients with Axenfeld-Riegers Syndrome have a 50% increased likelihood of developing glaucoma. This disorder is associated with corneal and iris defects resulting in thinning of the iris and closure of the irido-corneal angle (Idrees et al., 2006). Both mouse and human studies have identified causal mutations for these defects in the Pitx2 and Foxc1 genes (Gould et al., 2004; Idrees et al., 2006). Loss of the transcription factor Lmx1b results in Nail-Patella Syndrome. This syndrome is best known for defects in the skeletal system including the nails and kneecaps. An increased rate of fractures and scoliosis is also associated with this disorder (Towers et al., 2005). These patients also have a very high predisposition towards developing glaucoma (Dreyer et al., 1998; McIntosh et al., 1998; Vollrath et al., 1998; Pressman et al., 2000). Lastly, mutations in the CYP1b1 gene also cause PGC. CYP1b1 catalyzes the rate limiting step of retinoic acid biogenesis by converting all-*trans*-retinol to all-*trans*-retinal (Chen et al., 2000). Mutations in CYP1b1 have been isolated in human patients, especially those with a high rate of inbreeding, and confirmed genetically in mouse (Bejjani et al., 1998; Bejjani et al., 2000; Libby et al., 2003).

In addition to ASD defects which lead to glaucoma, a number of other defects are present in human populations. One of the best examples involves the Pax6 gene. Pax6 is a master regulator of early eye development from *Drosophila* to humans (Chow and Lang, 2001; Graw, 2010). In *Drosophila*, the homologue for the Pax6 gene is named *eyeless* due to the extreme lack-of-eye phenotype when it is knocked out. Later in development, it has been shown to also affect the iris. Mutations in Pax6 result in aniridia and have been well

described in mouse and human (Ashery-Padan et al., 2000; Davis et al., 2009; Graw, 2010).

As the tools for both human and mouse genetics continue to evolve and improve, our understanding of the pathways which regulate ASD will expand. This expanded knowledge is necessary to develop new therapies to treat patients suffering from ASD. An example is provided by the work on CYP1b1. While confirming the human phenotype in mouse, Libby et al. observed differences in severity of the phenotype between albino and pigmented mouse strains (Libby et al., 2003). This keen observation led to the discovery of the tyrosinase gene as a phenotype enhancer for both $CYP1b1^{-/-}$ and $FOXC1^{-/-}$ mice. Treatment of subsequent $CYP1b1^{-/-}$ albino mice with L-dopa *in utero* led to a remarkable rescuing of the phenotype. This provides an outstanding example of the necessity of development biology in studying the anterior eye. Further studies may reveal similar targets for drug therapy that can be used in the clinic.

CILIARY BODY OVERVIEW AND DEVELOPMENT

Introduction

The development of the vertebrate eye has been well studied and reviewed (Chow and Lang, 2001). The process begins early in development with evagination of the optic stalk. The stalk develops into the optic vesicle which releases inductive signals to cause lens placode formation from surface ectoderm. Upon invagination of the lens placode,

reciprocally inductive signals direct optic cup and lens vesicle formation. The inner layer of the optic cup is the future neural retina, and the outer layer the future retinal pigmented epithelium (RPE) layer. The tip of the optic cup develops into the ciliary body and iris regions. Several studies have now elicited key pathways and transcription factors which delineate ciliary body from retinal development.

The ciliary body has essential roles in eye physiology as both a secretory and muscular tissue (Figure 1.2). Aqueous humor is produced by the neural derived inner ciliary epithelium (ICE) and secreted into the anterior chamber. Aqueous humor bathes the avascular lens and cornea providing necessary nutrients (Hyer, 2004). Proper IOP also maintains the shape of the eye including the cornea which is the major refractive structure of the eye (Beebe, 1986). Besides secreting aqueous humor into the anterior chamber, the posterior ICE also secretes components of the vitreous humor and perhaps of the inner limiting lamina of the retina as well (Bishop et al., 2002; Dong et al., 2002; Halfter et al., 2005). These posterior secretions may play a role not only in later maintenance of the vitreous body but also in development of the retinal architecture.

Another essential physiologic role performed by the ciliary body is lens accommodation. While the main refractor of the eye is the cornea, fine tuning and focusing of an image is performed by the lens. Zonule fibers produced by the ciliary body bridge the distance between the ciliary body and lens and make this possible. Upon contraction of the ciliary muscles, the ciliary body enlarges in size and the fibers relax, allowing the lens to fatten

and increase its refractive capacity for near objects. Conversely, upon relaxation of the ciliary muscles, the zonule fibers become taut, stretching the lens to focus on more distant objects. The condition of presbyopia in older individuals occurs when the lens becomes brittle, reducing its ability to change shape and requiring the use of reading glasses for near vision.

The final aspect of ciliary body physiology discussed here is its role as a drug target. When an optometrist or medical doctor wishes to dilate the eye, both the adrenergic receptors of the iris and muscarinic receptors of the ciliary body must be targeted for full dilation. This is why multiple drops are often used. The ciliary body is also targeted in first-line therapy for open-angle glaucoma. Drugs such as beta-blockers target the ciliary body to reduce aqueous humor production. This helps to reduce the pressure of the eye and the progression of the disease.

Cell Determination of the Optic Cup

Understanding the forces which regulate cell determination is a fundamental question throughout biology. The eye has proven to be an excellent model for studying neural cell determination due to its accessibility, small number of cell types, and manageable architecture (Cepko et al., 1996; Livesey and Cepko, 2001). Midway through eye development, the anterior eye is home to a pool of progenitor cells in a region referred to as the ciliary marginal zone (CMZ). These progenitor cells will either become retinal cells or ciliary body/iris progenitors. In some species, a pool of adult stem cells is

maintained in the CMZ. It was thought that such was the case in humans as well (Coles et al., 2004; Tropepe et al., 2000), but later studies have been controversial and some feel that RPE cells with the capacity to proliferate were isolated rather than true stem cells (Cicero et al., 2009). Regardless, the CMZ remains a productive model to study cell fate determinants with clinical application to human diseases such as aniridia and glaucoma.

To date, the majority of the studies have focused on signaling pathways which either provide cues for maintenance of a progenitor state or cues for further differentiation. FGF 4/8, Wnt2b, and BMP 4/7 pathways have all been shown to play roles in the specification of the ciliary marginal zone and differentiation of the anterior eye structures. Table 1.2 provides a summary of the following recent studies relating to the CMZ and ciliary body development.

In an interesting set of experiments, Dias da Silva et al. showed strong evidence for an FGF-mediated factor in anterior eye development (Dias da Silva et al., 2007). Using a retroviral vector, a LacZ tagged FGF construct was injected into embryonic chick eyes at the optic vesicle stage. Surprisingly, the RPE transdifferentiated around the site of injection into neural retina. This was supported by both histologic appearance and immunohistochemical markers. Further away from the site of injection, the newly formed neural layer transitioned back to the unaffected RPE. In the transition zone between the nascent neural layer and RPE, ciliary margin specific markers such as collagen IX (Col IX) and thymosinβ4 were detected. However, no morphogenesis was reported. A

mechanism was proposed whereby the FGF gradient overlapping with endogenous BMP signaling known to be present in the anterior eye synergistically induced an environment favorable for ciliary margin specification. The authors suggested that FGF and BMP could be acting directly to cause this change or indirectly through a secondary mechanism such as Wnt2b, another signaling mechanism known to be important in ciliary margin specification.

The role of Wnt2b in anterior eye development has been debated in recent years. While undeniably important, its function is in dispute. In a pair of papers, Kubo et al. found an increase in retinal progenitor cell proliferation using *in vitro* explant cultures and suggests that Wnt2b helps to inhibit differentiation of the retinal progenitor cells by repressing Notch signaling (Kubo et al., 2003; Kubo et al., 2005). However, Cho and Cepko reported no increase in retinal progenitor cell proliferation rates either *in vitro* or *in vivo* using constitutively active β-catenin and Wnt2b constructs in chick (Cho and Cepko, 2006). In their dominant negative studies, Cho and Cepko also found that peripheral fate markers were decreased and accompanied by iris hypoplasia. The authors suggest that Wnt2b does not play a role in retinal progenitor proliferation; rather, that Wnt2b promotes conversion to the peripheral cell fates of ciliary body and iris. This is in agreement with the role of the Wnt homologue *wingless* in *Drosophila* eye development (Acampora et al., 1996; Baonza and Freeman, 2002; Tomlinson, 2003). Liu et al. agrees with Cepko's group that Wnt signaling induces transdifferentiation from neural retina to CM (Liu et al., 2007). However, Liu did not find Lef-LacZ, a canonical Wnt reporter, activation in their explants suggesting a non-canonical role for Wnt2b. Citing their own

unpublished work and other reports, Liu et al. suggest that Otx1 and Msx1, known to be present in ciliary body development, may be downstream of Wnt2b and not BMP4/7 in this situation, or alternatively they may be effectors of a convergence of these two pathways. The caveat to Liu's work which they did acknowledge is that they were using explant cultures which were not verified *in vivo*.

Established microarray protocols and emerging techniques such as single-cell microarrays have also found a home in studying anterior eye cell fates. Kubota et al. used a subtractive cDNA library approach in the chick (Kubota et al., 2004). cDNA was reverse-transcribed from mRNA extracted from both the central retina and the optic cup. The central retina genes were then subtracted from the ciliary epithelial genes to identity specific anterior eye markers. Known ciliary body genes such as Col IX and Nidogen 1 were used as positive controls to verify the approach. New genes such as Thrombospondin 4 and Chondromodulin were added to the growing library of anterior eye/ciliary body specific markers. More recently, single-cell microarray technology was used in mouse to identify progenitor cell characteristics (Trimarchi et al., 2009). The power of this approach comes from recognizing that neighboring progenitor cells may have different fates. Subtle differences between neighbor cells would not show up on traditional microarrays or even subtractive libraries. New techniques such as these are essential to better understand the molecular regulation of cell-fate determination in the eye and other tissues.

Induction of the Ciliary Body by the Lens

The lens has long been a classic model to study tissue induction and reciprocal signals in development. Early in development, the optic vesicle induces pre-lens surface ectoderm to invaginate (Chow and Lang, 2001). Reciprocal signals cause a folding of the optic vesicle into its future bi-layer structure. Later in development, the lens also plays an important role in the organization of the anterior eye.

Early studies provided strong evidence that the lens plays a key role in the development of the ciliary body and iris. In mice it was observed that regions of the optic cup where the lens did not contact due to congenital malformations failed to produce the ciliary body and iris (Beebe, 1986). Stroeva removed the lens in chick embryos and neither the ciliary body nor iris formed (Stroeva, 1967). Remarkably, neural retina replaced these structures. Also, transplanted supernumerary lenses induced ectopic ciliary body and iris structures in neonatal rats (Stroeva, 1967). In another study, replacing the lens with cellulose beads affected the neural crest migration into the stroma of the iris and cornea (Beebe and Coats, 2000). Presumably, the chemotactic and/or inductive signals lost due to replacing the lens with inert cellulose were responsible for the loss of migration. These and other experiments led the field to believe with some certainty that the lens was essential and perhaps sufficient to induce the ciliary body and iris to develop.

The advent of mouse genetics, however, brought the role and necessity of the lens into question. Ablation of the lens using diphtheria toxin was reported to result in multiple phenotypes including a loss of the ciliary body and iris (Harrington et al., 1991).

However, a more recent study also using diphtheria toxin under a lens-specific α-crystallin promoter reported the proper specification of the optic cup tip in the mutant mice with identifying genes such as BMP4/7, Otx1, and Opticin still expressed (Zhang et al., 2007). And while the ciliary body still developed folds, the iris failed to form. This later study brought back into question the necessity of the lens in ciliary body and iris development. These seemingly contradictory results may be explained, however, by the timing of the onset of the transgene and its expression level. Many signaling mechanisms are dosage sensitive, and perhaps all are temporally and spatially sensitive. This can make interpreting knockout and knock-in studies very difficult at times. One interpretation of the seemingly contradictory lens specification results would be that lens is not essential for the early specification of the ciliary body and lens, but it may play a role later in the differentiation and morphogenesis of these structures.

Several papers support the hypothesis that the lens may play a greater role in the differentiation and morphogenesis of the ciliary body and iris than in the specification of these structures. BMP4 is one of the signaling genes which has been identified as specific to the ciliary body and iris. A BMP4 heterozygous mouse has extension of the ciliary body and iris from the retina, but lacks the folding of the ciliary body (Chang et al., 2001). Overexpression of Noggin, a BMP-antagonist by the lens mimics this phenotype, and co-expression of Noggin with BMP7 in the lens beautifully rescues the defect (Zhao et al., 2002). These studies suggest a role for BMP4 and the lens in the morphogenesis of the ciliary body and lens after proper cell fate decisions have already occurred. Additional studies utilizing the manipulative capacity of the chick model further suggest

that the lens plays a later role in anterior eye development. In chick, expression of the CMZ specific gene Col IX is unaffected by removal of the lens, suggesting that cell specification remained intact (Hyer, 2004). Recently, injected lentivirus was used to create artificial RPE/neural retina boundaries to mimic the tip of optic cup (Kitamoto and Hyer, 2010). Wnt2b was found to be expressed at these artificial interfaces even distal and separate from the lens. Additionally, neural retina transdifferentiated from RPE proximal to the lens did not express Wnt2b. This group completed the study by introducing ectopic lenses which failed to induce Wnt2b expression. Taken together, these results strongly support the role of the lens in the development and differentiation of the ciliary body and iris after specification of the ciliary marginal zone by other factors.

Cell Adhesion in the Ciliary Body

Tissue morphogenesis is a complex process which requires the coordination of several events including cell adhesion. The eye presents a unique situation in which a single epithelium is folded back on itself during development to form an epithelial bi-layer connected by an apical-apical junction. The ciliary body and iris develop from the tip of this fold. This creates an environment enriched in numerous zonula adherens, zonula occludens, gap junctions, nectins, and other cell adhesion components. The unique and consistent morphogenesis of the ciliary body likely requires precise coordination of these proteins in parallel with other cellular changes such as rates of proliferation and cell determination cues.

A productive avenue in early anterior eye studies focused on the ultra-structural components of the ciliary body. Tight-junctions were described at the apical-apical junction of the neural derived inner ciliary epithelium (ICE) and RPE derived outer ciliary epithelium (OCE) (Raviola, 1971; Raviola and Raviola, 1978). Raviola also described the structure of the zonule fibers (Raviola, 1971). Prior to this study, it was commonly believed that the zonule fibers originated from the ICE. Electron microscopy, however, showed that they actually originate from the pigmented OCE, pierce the inner limiting membrane between the two layers, travel between the cells of the ICE, and then insert into the lens. Gap junctions were also described between cells laterally but more abundantly at the apical-apical junction of the two layers. Later studies have shown these gap junctions to primarily be composed of Connexin43 (Conn43) (Coffey et al., 2002). Conn43 is also necessary for the production of aqueous humor. Conditional deletion of Conn43 in mouse results in decreased intraocular pressure, but morphogenesis is unchanged (Calera et al., 2006; Calera et al., 2009).

In addition to gap junctions, nectins and cadherins have also been studied in the ciliary body. Unlike the gap junctions, the conditional knockout of either Nectin1 or Nectin3 results in complete abrogation of ciliary body morphogenesis and the lack of a vitreous body (Inagaki et al., 2005). Normally, nectins 1 and 3 localize to the apical-apical junction of the OCE and ICE and the apicolateral junction of the OCE. Nectin 1 also localizes to the apicolateral junctions of the non-pigmented ICE. P-cadherin's profile matches that of nectin 3 (Inagaki et al., 2005), but knocking out P-cadherin produces no phenotype suggesting it is not essential to morphogenesis (Radice et al., 1997). However,

it should be noted that anterior eye structures such as the ciliary body are often overlooked when reporting phenotypes. Despite the clear enrichment of cell adhesion factors at the tip of the optic cup, their role in morphogenesis is as of yet unknown. This is likely a key and necessary component of the development of the anterior eye.

Ciliary Body Morphogenesis

As an important part of eye physiology and considering the attention the eye receives in developmental biology, it is surprising how little is known concerning the mechanisms of development of the ciliary body. In the now classic study, Coulombre and Coulombre (Coulombre and Coulombre, 1957) showed the role of IOP in chick ciliary body morphogenesis. Upon insertion of a catheter and subsequent depressurization of the intraocular space, the ciliary body failed to develop properly. They proposed that mechanistic constraints of the developing eye combine with the increased IOP to cause the folding. Napier points out, though, that this model fails to explain why folding only occurs in the ciliary body region (Napier and Kidson, 2007). Also, the invasiveness of the technique makes it difficult to accurately assess the true requirements of IOP in ciliary body development. A third cause for pause in accepting these results is that the loss of pressure was likely accompanied by a loss of secreted factors as well which may confound the results. Another potential mechanism proposed but untested is the role of the underlying capillary bed. Each ciliary fold has a mesodermally derived capillary at its core (Gage et al., 2005). Combined with the knowledge that the ICE loses some of its lateral cellular adhesions during morphogenesis, possibly to increase flexibility, the capillary network could act as a scaffold on which to develop (Bard and Ross, 1982).

While the mechanisms of morphogenesis are still undiscovered, the stepwise progression of ciliary body morphogenesis has been carefully studied on both the histological and ultrastructural levels. In addition to systematically describing the histologic progression of ciliary body folding, Napier and Kidson carefully measured proliferation rates of the ICE and OCE and cellular height changes during morphogenesis (Napier and Kidson, 2005). The OCE had significantly higher rates of proliferation than the ICE. This would create a mass action force pushing against the neural derived layer and possibly contributing to morphogenesis. They concluded that differential proliferation rates between the two layers combined with cell height changes contributed to proper folding. These findings need further study, however, as they are observational only. Specific testing of altered proliferation rates and/or cell heights may help determine the necessity of these changes in development.

Cell Signaling in Ciliary Body Development

Despite the lack of knowledge regarding the mechanisms of ciliary body development, several genes and pathways have been reported to affect ciliary body development. In mouse, BMP4 heterozygotes have been shown to be haploinsufficent and display anterior eye defects including ciliary body dysmorphogenesis and increased IOP (Chang et al., 2001). Using transgenic mice overexpressing the BMP inhibitor Noggin in a lens-specific manner, Zhao et al. showed significant ciliary body developmental defects ranging from agenesis to dysmorphogenesis and hypoplasia (Zhao et al., 2002). Msx1 and Otx1, noted ciliary body specific markers, were both down-regulated. While this only shows that they

may be downstream of BMP in this situation, a similar phenotype had already been shown in an Otx1 knockout, further solidifying its important role in ciliary body development (Acampora et al., 1996). The study concluded by elegantly rescuing the phenotype through co-expression of BMP7 under a lens-specific promoter (Zhao et al., 2002).

Two transcription factors require our attention: Lmx1b and Pax6. Lmx1b knockout studies in mouse display a similar phenotype to Otx1 and BMP4 with a lack of ciliary body folding and iris hypoplasia (Pressman et al., 2000). These defects are passed on in an autosomal dominant manner. Lmx1b was also the first transcription factor specifically linked to trabecular meshwork development and maintenance. Lmx1b floxed mice crossed with a neural crest specific Cre line showed defects in ciliary body development (Liu and Johnson, 2010). This makes Lmx1b unique in the literature so far in that it provides definitive evidence of the importance of the periocular mesenchyme and neural crest in ciliary body and iris development.

Pax6 is a master regulator of the eye and was first discovered genetically in *Drosophila*. The phenotype was a complete lack of eyes. The gene was given the appropriate name of *eyeless*. In evolutionarily higher systems, the crucial role of Pax6 in eye development is conserved. In addition to its role in early eye development, Pax6 is strongly expressed in the ICE and OCE and has multiple roles later in eye development including ciliary body and iris formation. This is true in human and mouse as Pax6 haploinsufficiency causes

aniridia in humans (Hsieh et al., 2002; Davis et al., 2009). In mouse, using a Tryp2-Cre line specific to the pigmented epithelium, heterozygous $Pax6^{fx/+}$ mice show iris hypoplasia with normal ciliary bodies and lens. Homozygous conditional knockouts display a complete lack of ciliary body folding and a very short iris (Davis-Silberman et al., 2005; Davis et al., 2009). While these results reflect and support the human phenotype, a molecular explanation has yet to be discovered. Since Pax6 has a very important cell fate role in early eye development, a similar mechanism could be in place here. However, other cellular activities such as cell proliferation, adhesion, cell maintenance, or some combination of these could also account for this lack of development. Additionally, as previously discussed, cell fate decisions in the optic cup appear to occur prior to ciliary body and iris morphogenesis. Pax6, then, may be a useful marker for proper cell fate in both the ICE and OCE.

One of the greatest surges in biology during the last several years has been the interest in new classes of RNAs such as piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), and microRNAs (miRNAs). Of these small RNAs, miRNAs and optic cup development recently intersected. MiRNAs were recently shown to have a role in the development of the anterior eye. MiRNAs are small, non-coding RNAs that modulate the expression of other mRNAs in the cells. These RNAs are usually approximately 22 base pairs in length and complement regions of target mRNAs to form duplexes that mark the mRNA for degradation (Bartel, 2009). 3' UTRs are common target sites. Using a variety of Cre-lines with differing spatial and temporal expression patterns, Davis et al. reported multiple roles for the miRNA pathway in anterior eye development (Davis et al., 2011).

Dicer1 floxed alleles (Dicer1$^{fx/fx}$) crossed with Tyrp2-Cre mice (specific to the pigmented epithelium) produced a very severe phenotype with no ciliary body folding and a very short, almost non-existent iris. Dicer1$^{fx/fx}$ crossed with an α-Cre specific to the neuronal inner layer displays a hypoplastic iris and no ciliary body folding. In both cases, the structures are still positive for Pax6 and Conn43, suggesting that proper cell fate decisions have occurred. However, at E15.5, the α-Cre mice show reduced Mitf and increased Chx10 expression. Alterations in dosage of these mutually repressive transcription factors may play a role in the future development of this region. BMP4 was also down-regulated in the α-Cre mutant which may indicate that BMP4 is either downstream of the miRNA pathway or is responding to a feedback loop in this situation.

In summary, the ciliary body is a unique structure which provides an excellent model for future developmental studies. From a developmental perspective, the ciliary body is composed of tissue from neural and non-neural ectoderm, mesodermally derived vasculature, and neural crest derived musculature. Additionally, it interacts with periocular mesenchyme and the surface ectoderm derived lens during development. These numerous tissue types and interactions are fertile ground for studying the integration of reciprocally inductive signaling mechanisms in a manageable and accessible structure. From a clinical perspective, developmental defects of the anterior segment result in aniridia and/or glaucoma for a significant patient population. An increased understanding of the developmental processes of the anterior eye may lead to improved therapies and superior outcomes for these patients.

Figure 1.1. Overview of the Notch Signaling Pathway, adapted from Gridley (Gridley, 2003)

(1) Delta-like ligand (DLL) or Jagged (JAG) ligand binding to NOTCH receptor.

(2) Cleavage of Notch Intracellular Domain (NICD) by γ-secretase.

(3) Translocation of NICD to the nucleus

(4) NICD displaces histone deacetylase (HDAc) and other co-repressors to activate RPBJ and gene transcription

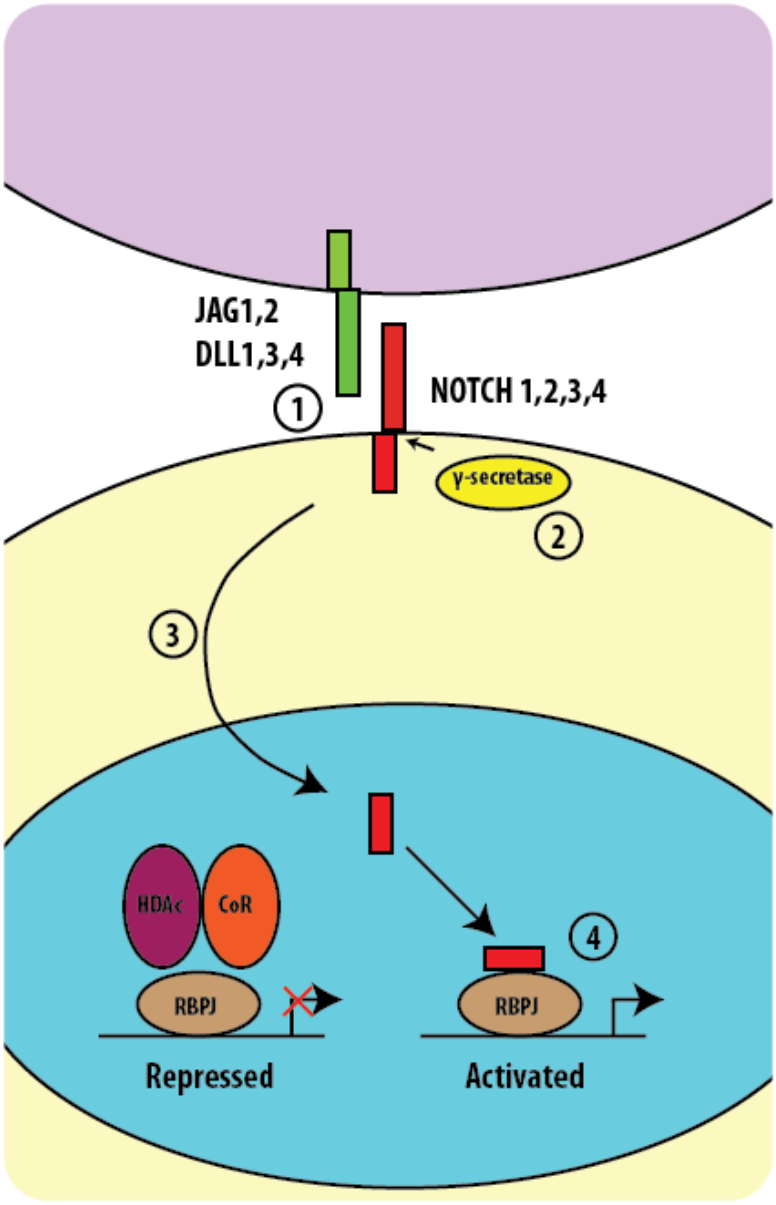

JAG1,2
DLL1,3,4
NOTCH 1,2,3,4
1
γ-secretase
2
3
HDAc
CoR
RBPJ
4
RBPJ
Repressed
Activated

Table 1.1. Heritable Diseases of the Notch Pathway

This table provides a summary of known monogenic diseases caused be defects in the Notch signaling pathway. Alagille Syndrome is unique in that it may be caused by mutations in either Jag1 and/or Notch2, although mutations in Jag1 constitute nearly 90% of all cases. Notch2 also holds the distinction as being the only Notch family member, mutations in which are known to cause two distinct diseases: Alagille Syndrome and Hajdu-Cheney Syndrome. Alagille Syndrome is due to a loss-of-function mutation, while Hajdu-Cheney is believed to be caused by a gain-of-function mutation.

Disease	**Notch Component(s)**
Alagille Syndrome	Jag1, Notch2
Spondylocostal dysostosis	Dll-3
Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL)	Notch3
Hajdu-Cheney Syndrome	Notch2

Figure 1.2. Schematic of Human Eye

This figure presents an oversimplified schematic of the human eye. The eye can be divided into anterior and posterior chambers. The lens provides the boundary between the two chambers. The anterior chamber may be further divided into anterior and posterior zones with the iris separating the two. The vitreous humor occupies posterior chamber, while the aqueous humor supports the anterior structures by filling the space between the lens and cornea. The ciliary body is an extension of the retina and RPE layers and lies posterior to the iris (yellow), with which is it also continuous. The ciliary body is also mechanically connected to the lens by virtue of the zonule fibers which enable the ciliary body to control lens accommodation.

The ciliary body secretes aqueous humor into the posterior zone of the anterior chamber behind the iris. The fluid travels through the pupil into the anterior zone and out through the trabecular meshwork and Schlemm's canal (not pictured).

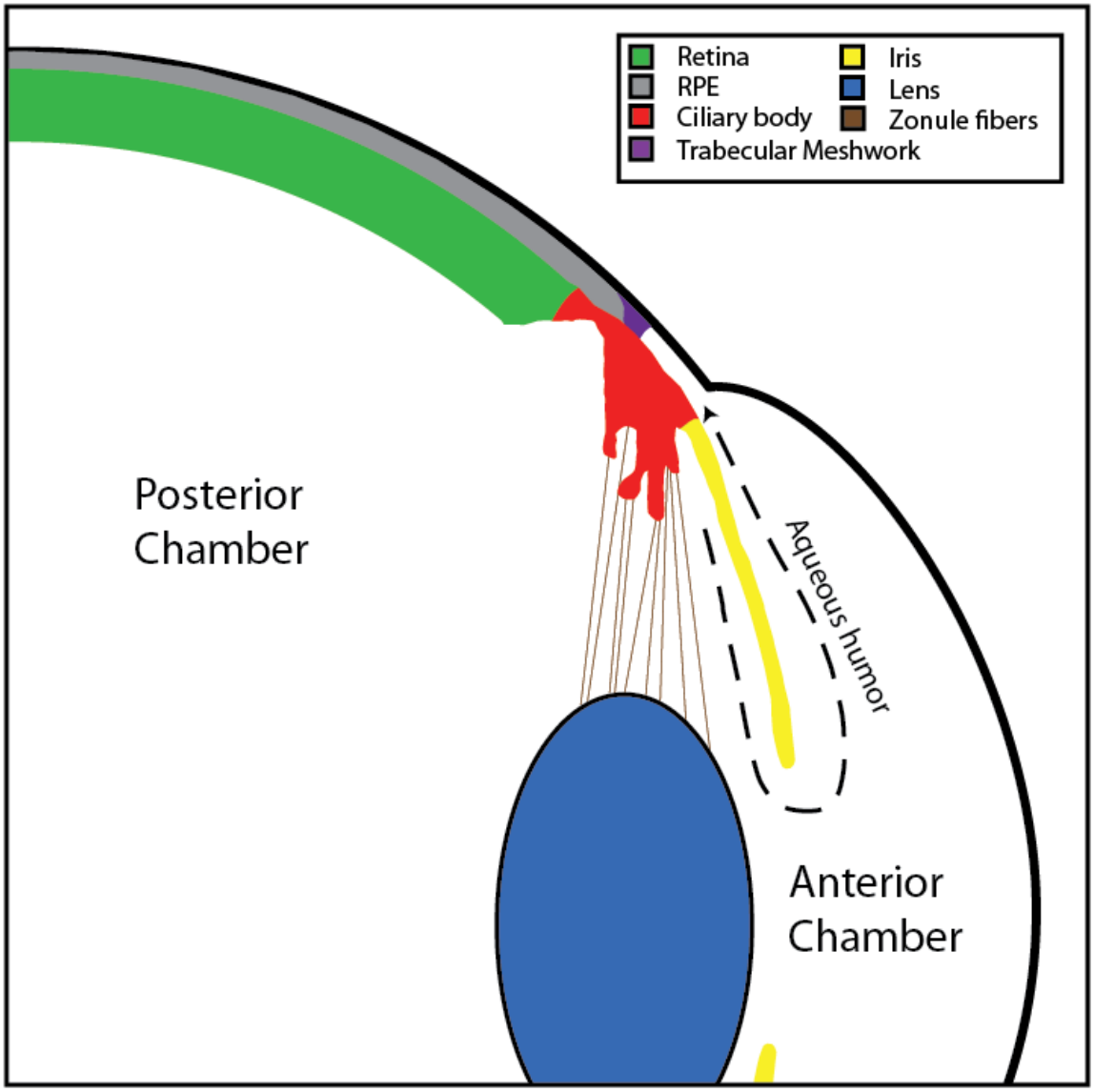

Retina
RPE
Ciliary body
Trabecular Meshwork
Iris
Lens
Zonule fibers
Posterior Chamber
Aqueous humor
Anterior Chamber

Table 1.2. Summary of Current Literature Regarding Development of the Ciliary Body

Summary of the last ten years of ciliary body related research. While excellent signaling pathway and cell biology research has been done regarding the cell fate of the ciliary marginal zone and anterior eye structures, most studies involving the ciliary body directly have been observational only in reporting phenotypes.

Author	Date	Pathway	Findings
Acampora et al.	1996	Otx1	Anterior eye (including CB) defects in $Otx^{+/-}$.
Pressman et al.	2000	Lmx1b	Mouse knockout shows lack of ciliary body folding and iris hypoplasia.
Chang et al.	2001	BMP4	Anterior eye (including CB) dysmorphogenesis and increased IOP in $BMP4^{+/-}$.
Kubo et al.	2003/ 2005	Wnt2b	Increase in retinal progenitor cell proliferation rate upon Wnt2b activation using explant cultures.
Zhao et al.	2004	BMP4/7	Overexpressed Noggin and showed similar phenotype to Chang et al.; Msx1/Otx1 downregulated; rescued by overexpressing BMP7.
Davis-Silberman et al., Davis et al.	2005, 2009	Pax6	Ciliary body and iris are sensitize to pax6 dosage. Conditional knockouts show complete lack of ciliary body folding and very short iris.
Cho and Cepko	2006	Wnt2b	Disagrees with Kubo; finds loss of retinal peripheral fate markers in Wnt2b DN studies.
Liu et al.	2007	Wnt2b	Similar to Cepko; but no Lef activation suggesting non-canonical Wnt signaling.
Dias da Silva et al.	2007	FGF4/8	FGF4/8 can induce transdifferentiation of RPE to NR; transition zone back to RPE shows ciliary body specific markers.
Liu and Johnson	2010	Lmx1b	Lmx1b regulates trabecular meshwork development and maintenance.
Davis et al.	2011	Dicer1	No ciliary body folding and dysplastic iris. Normal Pax6 and Conn43 suggesting that proper cell fate specification has occurred. BMP4 downregulated.

CHAPTER TWO: MATERIALS AND METHODS

MAINTENANCE OF MOUSE STRAINS

All mice were housed and maintained in the Stowers Institute Laboratory Animals Support Facility under the supervision of a veterinarian. All experiments and procedures involving animals were first approved by the Institutional Animal Care and Use Committee (IACUC) of the Stowers Institute and performed in accordance with The Association for Research in Vision and Ophthalmology's (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Genotyping for all strains was performed with primers previously published.

HISTOLOGY

Tissue for all experiments was fixed using either 4% PFA or a modified Davidson's fixative overnight (Latendresse et al., 2002). For pSMAD staining, tissue was fixed in 4% PFA plus Cocktail 1 and 2 phosphatase inhibitors (1:500, Sigma or Calbiochem). For paraffin sectioning, samples were dehydrated using increasing percentages of alcohol solution up to 70% alcohol. Samples were then sent to the Stowers Histology Core for infiltration, processing, and sectioning (5µm). Tissue intended for cryosections were infiltrated with 15% and then 30% sucrose in PBS. Following sufficient infiltration, embedding in Tissue-Tek O.C.T. compound and flash-freezing in isopentane was carried out. Sections between 10 and 12µm were processed on a Leica 3050S cryostat. Hematoxylin and eosin staining was performed on a Leica Autostainer XL.

Standard immunofluorescent staining procedures were used. Paraffin slides were deparaffinized and cryosections were dried for 20 minutes prior to antigen retrieval. Antigen retrieval was performed at 95°C for 10 minutes for paraffin slides and at 60°C for 15 minutes for cryosections in citrate buffer, followed by 3 TBST washes for 5 minutes each. Slides were blocked with Powerblock (Biogenex) solution for at least 10 minutes, and then the primary antibody was applied overnight at 4°C. Primary antibodies came from the following sources: pSMAD1/5/8 antibody (1:200, Cell Signaling); pSMAD1/5 (1:100, Cell Signaling); α-catenin (1:100, Invitrogen); β-catenin (1:100, Invitrogen); Notch2 (1:250, Abcam); Jag1 (1:100, Santa Cruz Biotechnology); BrdU (1:500, Megabase Research Products); anti-GFP (1:300, Invitrogen); Connexin43 (1:500, Cell Signaling); N-Cadherin (1:4, Developmental Studies Hybridoma Bank); Ncam 5B8 (1:2 Developmental Studies Hybridoma Bank). Day two of staining involved 3 TBST washes for 5 minutes, secondary antibody (1:300, Invitrogen) or Phalloidin stain (1:300, Invitrogen) for 1 hour at room temperature, and TBST 3x5 minutes. Counterstaining and mounting was performed using either DAPI (1:1000, Invitrogen) and Vectashield Mounting Media (H-1000, Vector Laboratories) or Vectashield Mounting Media plus DAPI (H-1200). During the course of this project, new lots of pSMAD1/5/8 antibody lost their manufacturers rating for immunofluorescence. Therefore, this antibody was replaced in the middle of the project with pSMAD1/5 (1:100, Cell Signaling). To improve the signal of this antibody, a biotinylated secondary antibody (1:300, Invitrogen) followed by a strepavidin-conjugated fluorophore (1:500, Invitrogen) were used.

Fluorescent images were taken using a Leica SP5 confocal microscope. Counting of BrdU positive cells was done on a Leica DM5500. H&E pictures were taken with either a Zeiss AxioPlan or Leica DM5500.

TUNEL ASSAY

The ApopTag Fluorescein In-Situ Apoptosis Detection Kit (Chemicon) was used for the TUNEL assay. The first step involves treating the slides to a citrate buffer heated to 95˚C for 10 minutes for antigen retrieval. Next, equilibration buffer is applied to the slide, tapped off, and working-strength TdT enzyme is applied. The slides are incubated with the enzyme in a dark, humidified chamber for 1 hour at 37˚C. The slides are then washed in a Coplin jar in the stop/wash buffer. Counter staining will be performed with DAPI. After mounting, the slides will be examined under a confocal microscope.

PLASMID PRODUCTION

cDNA for RT-PCR was made using the SuperScript III reverse transcriptase (Invitrogen) on RNA extracted using TRIzol (Invitrogen) from E10.5-E14.5 mouse brain tissue. Genes for transcribing *in-situ* hybridization probes were cloned using cDNA reverse transcribed using the SuperScript III enzyme (Invitrogen) into the Promega P-GEM Easy vector using the following primers: BMP4-ACTTCGAGGCGACACTTCTACA, ACGTCACTGAAGTCCACGTATA; Ptmb14-CACCTCATTTGCATAGAAGC, TACATCCTGCAGGACATC; and Tgfb1li4-GACTTCAGCAGCTAGATTCC,

GAACAGACCGAAGAGATGTGCT. The RNA probe for Jag1 was transcribed off of a clone, which was a kind gift from Dr. Nian Zhang. The probe for WFDC1 was transcribed off of a commercially available clone obtained from ATCC.

IN-SITU HYBRIDIZATION

Primers for the ISH probes were ordered through Integrated DNA Technologies and cloned into Promega P-GEM Easy Vectors. Digoxigenin is added to the transcription reaction to label the RNA probes. Transcription is checked on a gel. After the reaction, the nascent RNA probes are purified using either alcohol precipitation or a column. After centrifugation and removal of the supernatant, the pellet is resuspended in TE or water.

Day 1 of the hybridization process starts with deparaffinization of the slides in RNAse free solutions (or drying for 20 minutes if cryosections). Slides are washed in DEPC-treated PBS for 20 minutes. The slides are then prehybridized with hybridization solution for 3-4 hours without probe at 65˚C in a sealed humid chamber (In-Slide Out, Boeckman). Plastic coverslips are placed on the slides to prevent excess evaporation. Probe diluted in hybridization solution is then applied to the slides at individually determined concentrations, coverslips are reapplied, and the slides are reinserted into the 65˚C humid chamber overnight. Day 2 the slides are washed and blocked in 10% normal sheep serum (NSS). Anti-digoxygenin antibody (1:2000, Roche) is diluted in the NSS, applied to the slides, and incubated overnight at 4˚C. On day three the slides are washed in TBST and alkaline phosphatase buffer. Color is developed by applying a NBT (4.5

µl/ml, Promega) and BCIP (3.5 µl/ml, Promega) solution and keeping the slides in a dark, humidified chamber at room temperature. Slides are generally left overnight, washed in PBS, and mounted using 80% glycerol in PBS.

WESTERN BLOTS

OCE tissue from the ciliary body and iris were dissected from mutant and littermate control mice age P3. Tissue was placed in PBS and either used immediately or stored at -80ºC. After removal of PBS, tissue was resuspended in 10 µl of an SDS lysis solution + 2% β-mercaptoethanol. Tissue was homogenized using a hand-held, motorized, rotating pestle. Buffer and tissue were then heated at 99°C for 15 minutes and spun down in a table-top centrifuge at top speed for one minutes. Samples were then loaded onto a 10% SDS gel (gels made by Stowers Media Prep) and electrophoresed in a cold room for 1 hour. After running the gel, samples were transferred to a nitrocellulose membrane and blocked with 5% BSA. Primary antibodies (Bmpr1a, Bmpr1b, Bmpr2, Smad1, Smad5, Smad8; Santa Cruz) (pSMAD1/5/8; Cell Signaling) (N-Cadherin; gift from Dr. Nian Zhang) (anti-GFP; Invitrogen) (anti-FLAG; Sigma) (β-actin; Abcam) were diluted in 1% BSA and incubated with membrane overnight at 4ºC. Membranes were washed in TBST for one hour. The next day, secondary HRP-conjugated antibodies (Promega) were diluted 1:10,000 in 1% BSA and applied to membranes for one hour followed by TBST washes. Western Lightning Plus-ECL reagent was used to develop signal. All blots were performed in triplicate experiments with biologic replicates.

Analyses of western blot intensities were performed using Adobe Photoshop CS4. Average intensities of bands were multiplied by pixels counted with background signal then subtracted. Bands were then divided by intensities of corresponding β-actin controls to normalize for protein levels. Averages of three separate bands were used for statistical analysis (Student's t-test) in Microsoft Excel.

BRDU INJECTIONS

Two hours prior to culling, the pups (or pregnant female for embryonic ages) were injected intraperitoneally (IP) with BrdU at a dose of 5 µl/g body weight (concentration 20 µg/µl). After tissue fixation in 4% PFA and processing, either cryosections (10-12µm) or paraffin sections (5µm) were taken. Immunostaining was performed using an anti-BrdU antibody (1:500, Megabase Research Products). A Leica DM 5500 B fluorescent microscope was used for the primary analysis, while pictures for publication were taken with a Leica SP5 confocal microscope. BrdU positive cells were normalized to the total number of cells for a percentage of BrdU positive cells. At least 10 ciliary bodies from at least three mice were used at all ages except for E17.5.

MICROARRAY ANALYSIS

RPE derived tissue from the ciliary body and iris were dissected under a dissecting microscope at age P0. RNA extraction was carried out using the TRIzol reagent (Invitrogen). The RNA was purified and underwent one round of amplification. A mouse

Affymetrix chip was used to run the microarray. With the help of Stowers Microarray Core Facility and Bioinformatics Group several angles of analysis were performed. In addition to determining fold change, log fold change, and p-values, GO term (GOrilla plots) and Kegg pathway analysis were performed. Both semi-quantitative and quantitative real-time PCR techniques were used to verify the microarray findings.

INTRAOCULAR PRESSURE MEASUREMENTS

All intraocular pressure (IOP) measurements were taken using a TonoPen XL (Tonolab). Measurements were taken in triplicate and averaged to ensure consistency. Measurements were taken precisely in the middle of the cornea, and the TonoPen was always held in a horizontal position.

ELECTRORETINOGRAMS

The HMS-ERG device by Ocuscience, LLC, was used to take all electrophysiologic measurements. Both thread and needle electrodes were also obtained from Ocuscience, LLC. Animals were placed under anesthesia using a cocktail of ketamine, xylazine, and acepromazine. During anesthesia animals were placed on a hot pad equipped with a rectal thermometer to maintain appropriate body temperature (TC-1000, American Medical). A single needle electrode was placed subcutaneously on the dorsal side of the mouse just anterior to the tail to serve as a ground. Two additional needle electrodes were placed subcutaneously in the cheeks for reference electrodes. Thread electrodes were placed

over the eyes and covered with an aclar contact (Ocuscience, LLC). 1% methylcellulose (TheraTears) was used as a lubricant. Electrophysiologic protocols used were preprogrammed into the HMS-ERG machine. All animals were supervised until recovered from anesthesia. All processing of recordings were performed using ERG-View software (Ocuscience, LLC).

CHAPTER THREE: NOTCH2 REGULATES BMP4 SIGNALLING IN THE DEVELOPING MOUSE CILIARY BODY

SUMMARY

Notch is a conserved and prominent signaling pathway both in development and disease. Notch receptors are activated and gene transcription is subsequently altered for varied reasons including tissue boundary formation, proliferation, apoptosis, and cell fate decisions. In this study, we show a novel role for Notch2 in the development of the vertebrate eye as an essential signaling mechanism for ciliary body morphogenesis. The ciliary body adjusts the lens for visual accommodation, secretes aqueous humor into the anterior eye, and modulates the outflow tracts of the aqueous humor. Using conditional Cre-loxP technology, we selectively delete the Notch2 receptor from the outer, pigmented layer of the ciliary body. This knockout results in a lack of morphogenesis of the ciliary body. Careful analysis of the phenotype reveals a significant loss in cell proliferation and adhesion which likely directly contribute to the loss of morphogenesis. Using Cre-reporter mice, we discovered that Notch2 regulates the activation of BMP4 pathway receptor-SMADs through a non-cell autonomous mechanism, and Notch2 regulates adhesion in a cell-autonomous manner. As far as we know, this is a novel interaction between the pathways. Further genetic knockouts of BMPR1, N-cadherin, and α-catenin mice enabled us to more clearly determine the epitasis of these different cellular processes.

INTRODUCTION

Notch is an evolutionarily conserved pathway that plays significant roles in both developmental and pathologic situations. In mammals, four Notch receptors and five ligands (Delta-like 1,3,4 and Jagged 1,2) have been identified. Upon binding of the Notch receptor by its ligand, a series of proteolytic cleavages ensues resulting in release of the Notch Intracellular Domain (NICD). The NICD translocates to the nucleus where it binds a CSL-family DNA binding protein such as RBPJK and classically targets basic helix-loop-helix transcription factors such as the Hes and Hey proteins (Lai, 2004). Notch signaling's varied roles in cell fate determination, tissue boundary formation, proliferation, and other processes have been well-studied and reviewed (Artavanis-Tsakonas et al., 1999; Lai, 2004; Louvi and Artavanis-Tsakonas, 2006; Fortini, 2009). In addition to its role in development, defects in the Notch pathway have also been associated with several heritable diseases (Li et al., 1997; Louvi et al., 2006; Isidor et al., 2011; Simpson et al., 2011) , and its misregulation has been associated with a variety of cancers including leukemias and lymphomas, breast cancer, and colon cancer (Florena et al., 2007; Koch and Radtke, 2007; Aster et al., 2008).

Discovering novel interactions between Notch and other signaling pathways continues to be a driving force in the field. Notch and TGF-β, for example, have been linked on several levels including TGF-β induction of Jagged-1 and Hey1, and synergistic targeting of effector genes (Guo and Wang, 2009). Notch signaling may also directly affect rSMAD transcription levels (Fu et al., 2009). Recently it was shown in human and mouse

that RBPJK and SMAD4 physically interact and co-activate the N-cadherin promoter (Li et al., 2011). Further discovery of interactions between pathways is critical to integrating what is currently known in signal transduction biology in development. Integration of pathways is also important for improving our panels of biomarkers for diseases such as cancer and understanding the role that these signals play in their growth and maintenance.

The eye has long been a favorite model in developmental neurobiology. However, oft-overlooked is the ciliary body which secretes the aqueous humor and regulates lens accommodation. Previous studies have primarily focused on the mechanisms behind the cell fate switch which differentiates the future ciliary body and iris from the retina early in development. Multiple reports have discovered roles for Wnt2b and FGF4/8 in this critical step (Kubo et al., 2003; Kubo et al., 2005; Cho and Cepko, 2006; Dias da Silva et al., 2007). The step-wise progression of wild-type ciliary body morphogenesis has been carefully described (Napier and Kidson, 2005; Napier and Kidson, 2007). Also, pathways including BMP4 and the Otx1 transcription factor have been reported to be necessary in ciliary body morphogenesis (Acampora et al., 1996; Chang et al., 2001; Zhao et al., 2002; Davis et al., 2011). More recently, a conditional knockout of Dicer and the microRNA pathway was shown to also have a role in ciliary body morphogenesis (Davis et al., 2011). However, a careful exploration of the cellular mechanisms of ciliary body morphogenesis has not been reported. Neither has any level of integration of signaling pathways nor effector genes been demonstrated.

Here we demonstrate a novel role for Notch2 in eye development. Using conditional knockout technology in mouse, we show that Notch2 regulates the proliferation and morphogenesis of the ciliary body. Using Z/EG reporter mice, we determine that while Notch2 regulates adhesion in the ciliary body in a cell-autonomous manner, it regulates the phosphorylation of rSMADs non-cell autonomously. We also demonstrate for the first time both histologically and genetically the essential role of the adhesion components N-cadherin and α-catenin in the development of the ciliary body.

RESULTS

Notch2 Expression in the Eye

The presence of Notch1 and Notch2 in the developing vertebrate eye has been known for some time (Bao and Cepko, 1997). But while Notch1 and its role in retinal development has been increasingly studied (Jadhav et al., 2006a; Jadhav et al., 2006b; Yaron et al., 2006), little attention has been paid to Notch2 which is localized to the retinal pigmented epithelium (RPE). In order to assess the role of Notch2, we crossed conditional Notch2 floxed mice ($Notch2^{fx/fx}$) (McCright et al., 2006) with mice carrying a RPE specific Cre-recombinase under the Trp1 promoter (Mori et al., 2002). Hereafter we shall refer to these as DM61-Cre mice. The resulting progeny showed a complete lack of ciliary body morphogenesis (Figure 3.1). Surprising to us was the degree of specificity in the mutant mice as no other structures appear to be affected. The retina, iris, and lens all develop normally, although the iris appears slightly delayed in its development. We carefully characterized the phenotype by examining time points ranging from E17.5 to P7.

Additional sections from adult mice confirmed that the phenomenon was not simply a delay in development (Supplemental Figure 5.1A, 5.1C). In normal ciliary body development, the prospective ciliary body and iris are morphologically distinct from the retina by age E17.5 (Figure 3.1A-B). This delineation was no longer present in our DM61/Notch2$^{fx/fx}$ mice (Figure 3.1C). By P0, a single fold in both the neural derived inner ciliary epithelium (ICE) and the retinal pigmented epithelium (RPE) derived outer ciliary epithelium (OCE) is clearly visible in the wild-type, but again absent in the knockout mice (Figure 3.1D-F). Through the first week of life, continued folding and proliferation normally occurs in the wild-type ciliary body. The DM61/Notch2$^{fx/fx}$ mice showed no apparent folding or morphogenetic development during this time-frame. Some variation of the phenotype was observed, however, and it was determined that our DM61-Cre line does not express Cre very well on the dorsal side of the eye. Crossing the DM61-Cre with a Z/EG EGFP reporter line confirmed that the Cre protein was uniformly expressed ventrally but not dorsally (Supplemental Figure 5.1B, 5.1D) (Novak et al., 2000). Histologic sections oriented dorsal/ventral and stained with hematoxylin and eosin correlated these genetic data with the morphologic variation of the DM61/Notch2$^{fx/fx}$ mice (Supplemental Figure 5.1A, 5.1C). These results indicate that Notch2 plays a necessary and novel role in the development of the ciliary body.

Notch Pathway Components in the Ciliary Body

To further characterize the phenotype, it was important to determine the components of the Notch pathway relevant ciliary body development. Immunofluorescent staining for

activated (cleaved) Notch2 protein confirmed the efficiency of the DM61/Notch2$^{fx/fx}$ cross (Figure 3.2A-B). Notch2 is retained in the neural derived inner ciliary epithelium (ICE) of the DM61/Notch2$^{fx/fx}$ mice but lost in the RPE derived outer ciliary epithelium (OCE) of the knockouts. Further immunofluorescence studies revealed the presence of the Jag1 in the ICE. In the wild-type, Jag1 staining is enriched at the apical-apical junction between the ICE and OCE (Figure 3.2C). In the DM61/Notch2$^{fx/fx}$ mice, however, the staining is more diffuse across the cells (Figure 3.2D). This finding regarding Jag1 was validated by our collaborators who conditionally knocked out Jag1 using a Six3-Cre line (personal communication with Drs. Richard Libby, Amy Kiernan, and Zhipeng Yanl). Knocking out the ligand phenocopied our knockout of the Notch2 receptor remarkably well. As with the DM61/Notch2$^{fx/fx}$ mice, morphogenesis of the ciliary body is suppressed while the iris extends normally from the retina. These results conclusively show that Jag1 is the ligand for Notch2. We may also conclude that Notch2 signaling is occurring across the apical-apical junction from the ICE with the ligand to the OCE with the receptor.

Ciliary Body Cell Determination is Independent of Notch2

A common role for the Notch family is to regulate cell fate and tissue boundary formation. One mechanism by which is does this is through lateral inhibition (Fortini, 2009; Sprinzak et al., 2010). Differences in expression levels of ligands versus receptors between cells are amplified through lateral inhibition and may promote different cell fates. To determine whether or not the lack of ciliary development is due to a change in

cell determination, we performed immunofluorescence and *in-situ* hybridization on numerous markers for both the Notch2 and Jag1 mutants.

In-situ hybridization was performed on several previously described markers for the ciliary body including WFDC1, Ptmb4, and Tgfb1i4 (Figure 3.2G-L) (Thut et al., 2001; Rowan et al., 2004). In each case, there was no apparent change in expression levels (see arrows). These markers, however, are only specific to the ICE. To assess cell fate in the OCE, we stained for Pax6 and Connexin 43 (Conn43). Conn 43 is essential for aqueous humor production and is located at the apical-apical junction of the ICE and OCE (Calera et al., 2006; Calera et al., 2009). Pax6 is strongly expressed in both the ICE and OCE at the period in development (Davis et al., 2009). Both mutant and wild-type samples showed robust expression of Pax6 (3.2E-F) and Conn43 (3.4G-H). From these findings we conclude that Notch2 does not affect the cell fate determination of the ciliary body. This is in agreement with previous studies showing that the cell fate of the ciliary body and iris occur earlier in development in the ciliary marginal zone (Kubo et al., 2003; Kubo et al., 2005; Cho and Cepko, 2006).

Notch2 Regulates Cell Proliferation in the Ciliary Body

We hypothesized that the reduction in size of the ciliary body may be due to a decrease in cell proliferation. To assess rates of proliferation we performed a comprehensive BrdU labeling pulse-chase experiment. BrdU positive cells in both the ICE and OCE were counted and normalized against the total number of cells in each layer. Mice were

analyzed at E17.5, P0, P3, P5, and P7 time points (Figure 3.3A-K). While ICE rates of proliferation were unchanged in the DM61/Notch2$^{fx/fx}$ mice, OCE rates were significantly reduced at P3, P5, and P7 (Figure 3.3L). This time period includes the peak proliferative period from P3-P5 and the most dramatic changes in folding and morphogenesis (Napier and Kidson, 2005). To ensure that the reduction in cell number was due solely to decreased proliferation, we also performed TUNEL staining on several time-points to assess changes in apoptosis (Supplemental Figure 5.2A-C). No TUNEL positive cells were observed in either the wild-type or DM61/Notch2$^{fx/fx}$ mice. We also compared the proliferative rates between the ICE and OCE layers. In normal development, the rate of proliferation of the OCE is statistically higher than the ICE (Supplemental Figure 5.3A) (Napier and Kidson, 2005). This higher level of cell division in the pigmented layer may contribute to the buckling and folding of tissue layers during morphogenesis. In the DM61/Notch2$^{fx/fx}$ mice, not only is there a significant difference between wild-type and mutant OCE's, but the significant difference between the ICE and OCE proliferation rates is lost (Supplemental Figure 5.3B). Taken together, we can conclude that Notch2 regulates proliferation during ciliary body development. Also, the reduction in ciliary body cell number in the Notch2$^{fx/fx}$ conditional knockout is due solely to reduced proliferation and not increased apoptosis or some combination of the two.

Notch2 Regulates Cell Adhesion in the Ciliary Body

Correct morphogenesis is dependent on proper cell adhesion. Our analysis of the wild-type developing ciliary body revealed strong expression of N-Cadherin in the OCE. As

expected, this staining is localized to both the lateral and basal surfaces of the cellular membrane (Figure 3.4C). During development, the OCE begins as a monolayered extension of the RPE. It then buckles and folds back on itself to form a bi-layer sharing a basal substrate. For this to occur, proper lateral and basal adhesion is likely essential. Staining of the DM61/Notch2$^{fx/fx}$ mice showed a near complete absence of the N-cadherin staining in comparison with the wild-type controls (Figure 3.4D). Similar results for α-catenin and β-catenin were observed (Figure 3.4A-B). To further confirm this finding we used a phalloidin stain to observe changes in F-actin during morphogenesis. In wild-type mice, F-actin is enriched at both basal and lateral cell junctions (Figure 3.4E). In DM61/Notch2$^{fx/fx}$ mice, the lateral enrichment is lost while some basal staining remains (Figure 3.4F, see arrows). Despite these changes in cell adhesion, other aspects of adhesion remained unchanged. And, as previously mentioned, Conn43, which helps maintain the apical-apical junction between the ICE and OCE is still present (Figure 3.4G-H. This would suggest that certain cell adhesion proteins may be more important than others in ciliary body morphogenesis and that not all cell adhesion is under the influence of Notch2 signaling. Combined with our BrdU studies, these data suggest that a concerted program of cell-adhesion and differential rate of proliferation is necessary to drive ciliary body morphogenesis.

Notch2 Regulates BMP4 Phosphorylation

Previously, the role of BMP4 has been reported in relation to ciliary body development. A BMP4 heterozygote displayed a similar phenotype to the DM61/Notch2$^{fx/fx}$ mice

(Chang et al., 2001; Zhao et al., 2002). To assess BMP4 activity in our mice, we began by staining for phosphorylated-SMAD1/5/8 (pSMAD1/5/8) using P3 sections as a representative midpoint in development. These sections showed distinct nuclear staining in the OCE (Figure 3.5A). However, when sections from DM61/Notch2$^{fx/fx}$ mice were similarly stained, almost no nuclear staining was visible (Figure 3.5B). To further investigate the level at which Notch2 might regulate BMP4, western blots were performed on P3 tissue from ciliary body and iris OCE (Figure 3.5C). Antibodies against BMP receptors (Bmpr1a, Bmpr1b, and Bmpr2), SMAD1, SMAD5, SMAD8, and pSMAD1/5/8 were applied and developed. All blots were performed in triplicate with biologic replicates. BMP receptors in the conditional knockouts all showed equal bands with the controls. The non-phosphorylated SMADs also showed similar expression levels between the mutant and controls. Only pSMAD1/5/8 showed any change. Quantification of this change revealed a nearly 60% decrease in the amount of pSMAD present in the Notch2$^{fx/fx}$ mice (Figure 3.5D, p-value < 0.001). These data not only confirm our immunofluorescence results, but also suggest that the regulation of BMP4 is at or upstream the level of SMAD phosphorylation.

Notch2 Regulates BMP4 Cell Autonomously and Non-Cell Autonomously

A caveat to genetic knockout studies is distinguishing cause from effect in the observed phenotype. To enhance our understanding of the adhesion and BMP4 phenotypes, we crossed our DM61/Notch2$^{fx/fx}$ mice onto the Z/EG reporter background. Sections from these mice stained with anti-GFP antibody show us every cell where genetic

recombination has occurred and where Notch2 has been removed. As previously mentioned, the DM61-Cre line is poorly expressed on the dorsal side of the eye. This provided us with the chance to test our phenotypic observations on genetic mosaics.

Although BMP4 is restricted to the ventral side of the eye early in development, it is strongly expressed throughout the ciliary body along all axes (Chang et al., 2001). Using eyes oriented both dorsal/ventral and nasal temporal, we show that BMP4 is not only expressed but the pathway is also activated across all axes (Supplemental Figure 5.1E-5.1H). Again using our DM61/Notch2$^{fx/fx}$/Z/EG mice, we examined the presence of pSMAD1/5 in the GFP mosaics. Ventrally, both GFP(+) and GFP(-) cells were negative for pSMAD1/5 expression (Figure 3.6B). GFP- cells (see plus sign) immediately adjacent to GFP(+) cells (see asterisk) are clearly negative for pSMAD1/5. These data indicate that BMP4 signaling is affected in both Notch2 deficient cells and in their neighboring cells. Dorsally, both GFP(+) and GFP(-) cells were positive for pSMAD1/5 expression (Figure3.6A). Taken together, these results provide additional insight into the manner in which Notch2 regulates BMP4 signaling in the ciliary body. First, Notch2 regulates BMP4 signaling in a ventral specific manner. Second, it regulates BMP4 both cell autonomously and non-cell autonomously. These results help to explain the high penetrance of the phenotype on the ventral side despite the mosaicism in the DM61-Cre. They are also in conformity with our previous conclusions that Notch2 is regulating BMP4 at or above the level of SMAD phosphorylation.

Notch2 Regulates Cell-Adhesion in a Cell Autonomous Manner

We hypothesized that GFP positive cells where Notch2 was knocked out on the dorsal side would show the same changes in cell adhesion that were observed ventrally. Indeed such was the case. Patches of GFP(+)/Notch2(-) cells (Figure 3.7A, see asterisk) were shown to be N-cadherin negative right next to GFP(-)/Notch2(+) cells which were positive for the same marker on the dorsal side (Figure 3.7A, see arrow). Similar results were found for α-catenin and β-catenin (Figure 3.7C, 3.7E). This provides strong evidence that Notch2 affects cell adhesion across the entire ciliary body, regardless of axial position. These data also suggest that the changes in cell adhesion are in fact due to the loss of Notch2 and not due to a secondary effect in the knockout or reduction in BMP4 signaling.

This approach also enabled us to determine that Notch2 is regulating cell-adhesion in a cell-autonomous manner. We examined cells which were in between a GFP(+)/β-catenin(-) cell (see asterisk) and a GFP(-)/β-catenin(+) cell (see plus sign). These middle cells showed adhesion deficits on the cellular border shared with the GFP(+)/β-catenin(-) cell but intact adhesion on the borders with the GFP(-)/β-catenin(+) cell (Figure 3.6F). Taken together, Notch2 regulates the cell-adhesion proteins N-cadherin, α-catenin, and β-catenin independent of both eye axis and BMP4 signaling and in a cell-autonomous manner.

Notch2 Microarray

To gain a more global appreciation of how Notch2 maybe be affecting these changes in proliferation and cell adhesion, we performed a microarray to examine the transcriptional differences between the DM61/Notch2$^{fx/fx}$ mice and their littermate controls. Careful dissections of OCE tissue from the ciliary body and iris of P0 pups were carried out. After RNA extraction, purification of the RNA and one round of amplification were performed prior to hybridization on an Affymetrix chip.

As a positive control for the microarray results, we looked at canonical targets in the Notch and BMP pathways. Taken as an aggregate, the pathways showed little change (Supplemental Figure 5.4). However, two genes stood out as canonical targets: hairy/enhancer-of-split related with YRPW motif 1(Hey1) and inhibitor of DNA binding 2 (ID2) (Figure 3.8). Hey1 is a basic-helix-loop-helix (bHLH) transcription factor which normally acts as a transcriptional repressor under the direction of Notch signaling. ID2 is a helix-loop-helix (HLH) transcription factor targeted by BMP signaling which also usually acts as a transcriptional repressor. The reduced levels of transcription of both of these genes not only identified a potential target for Notch2 in the ciliary body but also confirmed our findings of pSMAD1/5/8 being downregulated.

In addition to mining the microarray results by applying standard statistical filters (Supplemental Figure 5.5), we also organized the statistically significant genes by gene ontology (GO) terms to look at significant changes in these terms as a whole

(Supplemental Table 5.1). Organizing these terms in to GOrilla plots provides an easy way to visual which cellular compartments and processes had the greatest transcriptional changes (Supplemental Figure 5.7) (Eden et al., 2009). Processes including cell cycle and DNA replication came back as statistically significant (Supplemental Figure 5.6A) along with chromosomal and DNA structure components (Supplemental Figure 5.6B). Not surprisingly, the cell proliferation plot showed some of the most significant changes. Some of the key cell cycle regulators which were altered include Cdc20, Cdc14a, and Pttg1.

A third method of analysis which we applied to our microarray results is Kegg Pathway analysis. Similar to GO term analysis, genes are analyzed as an aggregate against expected normal variation to see what processes are changed more as a whole than expected. Kegg Pathway analysis looks at known cell signaling pathways, however, instead of GO terms. Our analysis resulted in three pathways which showed statistically significant variation beyond what was expected: Cell Cycle, p53, and TGFβ (Supplemental Figures 5.7-5.9). The Cell Cycle and p53 pathways made intuitive sense due to the proliferative defects in our DM61/Notch2$^{fx/fx}$ mice. The TGFβ superfamily was initially a little surprising due to the lack of transcriptional change within BMP signaling by itself. But the Kegg pathway better reflects not only downstream targets of BMP signaling but also probably compensatory mechanisms by the broader superfamily.

Our microarray results provide a basis for many future projects both in our lab and others. These results add additional cell cycle genes to those previously known to be possible Notch and/or BMP targets in both development and disease. These results also revealed Hey1 and ID2 as likely downstream targets of Notch2 and BMP4 in ciliary body development.

Bmpr1 Conditional Knockout Mice Further Define Notch2-BMP4 Relationship

To better understand the epistatic relationship of Notch2, BMP4, and the characteristics of our ciliary body phenotype, we crossed the DM61-Cre mice with double-homozygous Alk2/Alk3 BMP-receptor mice (Mishina et al., 2002; Dudas et al., 2004). This cross results in mice which lack BMPR1 receptors and are thus incompetent to transduce the BMP4 signal.

Consistent with the previously mentioned BMP4 heterozygous mice, these mice display a lack of ciliary body morphogenesis (Figure 3.9A-B). Next, BrdU labeling at age P3 was carried out to evaluate proliferation. Overall proliferation in the wild-type was reduced compared to the Notch2 wild-type and increased variability was observed in both wild-type and mutant ciliary bodies (Figure 3.9C). For these reasons, no statistical significance was observed. Staining with the pSMAD1/5 antibody confirmed the efficiency of our knockout (Figure 3.9D). As expected, no pSMAD1/5 positive nuclei were observed in the knockout layer. Notch2 staining is retained in both the ICE and OCE, providing genetic evidence that BMP4 is downstream of Notch2 (Figure 3.9E). In our

DM61/Notch2$^{fx/fx}$/Z/EG mice, we observed that Notch2(-) cells in the dorsal ciliary body were negative for adhesion but positive for pSMAD1/5. This finding suggested that BMP4 does not regulate adhesion within the OCE. The DM61/A2$^{fx/fx}$/A3$^{fx/fx}$ mice confirm this hypothesis. N-cadherin, α-catenin, and β-catenin were all clearly expressed in the ICE and OCE (Figure 3.9E-F).

N-cadherin Is Necessary for Ciliary Body Morphogenesis

In order to determine whether or not N-cadherin is necessary for ciliary body development, we crossed N-cadherin$^{fx/fx}$ mice (Kostetskii et al., 2005) with our DM61-Cre. Removing N-cadherin phenocopied the DM61/Notch2$^{fx/fx}$ ciliary bodies (Figure 3.10A-B), confirming the necessity of N-cadherin and proper adhesion in ciliary body morphogenesis. Further interrogation of the phenotype showed significant proliferation defects (p-value < .00001) at age P3, the peak of ciliary body morphogenesis (Figure 3.10C). While the loss of N-cadherin staining confirmed the phenotype, the DM61/N-cadherin$^{fx/fx}$ ciliary bodies unexpectedly lost staining for Notch2 activation (Figure 3.10D). This suggests that a positive feedback look exists between Notch2 and N-cadherin in the ciliary body. Stainings for α-catenin and β-catenin were disrupted, as expected, further confirming the loss of adhesion (Figure 3.10E). Lastly, we stained for and observed a loss of pSMAD1/5 signal (Figure 3.10F). The loss of pSMAD1/5 staining in our DM61/N-cadherin$^{fx/fx}$ mice is consistent with the Notch2-N-cadherin positive feedback loop data. Taken together, the DM61/N-cadherin$^{fx/fx}$ mice show a very similar phenotype to the DM61/Notch2$^{fx/fx}$ mice in morphologic, proliferative, and adhesion

defects. These results confirm the necessity of N-cadherin and proper adhesion in proper ciliary body morphogenesis.

Discussion

The ciliary body is an important structure in the anterior eye. First, it secretes the aqueous humor which bathes the avascular lens and cornea and maintains proper intraocular pressure. Second, its stromal musculature assists the trabecular meshwork in regulating aqueous humor outflow (Tamm, 2009). Thirdly, contraction of the ciliary body muscle controls lens accommodation for near versus far vision. These functions are critical to the health and performance of the eye throughout an individual's lifetime.

Developmentally, the ciliary body is an interesting model. The inner ciliary epithelium is derived from the neural retina, while the outer ciliary epithelium extends from the non-neural RPE. Each fold of the ciliary body has a mesenchymally derived capillary at its core, and the ciliary body muscle is contributed by the neural crest. Additionally, ciliary body morphogenesis occurs postnatally, opening up the possibility of combining the power of mouse genetics with external manipulation and/or gene transfer. These unique characteristics make the ciliary body an excellent model to study tissue-tissue interactions and signal transduction pathways and their integrations.

While the eye has long been a favorite model for developmental biologist, most studies focus on either the lens or cornea anteriorly, or the retina posteriorly. Phenotypic reports of ciliary body dysgenesis have been published. These reports have knocked out BMP4, Dicer, Lmx1b, Pax6, and Otx1 (Acampora et al., 1996; Pressman et al., 2000; Chang et al., 2001; Zhang and Yang, 2001; Davis-Silberman et al., 2005; Davis et al., 2009; Liu and Johnson, 2010; Davis et al., 2011). However, no integration between pathways has been reported. Nor have any mechanisms of morphogenesis been proposed based on any experimental data. In our current study, we report for the first time a role for Notch2 in the development of the ciliary body. We have not only characterized the Notch2 phenotype but have also defined roles for Notch2 as a key regulator of several aspects of ciliary body development including cell proliferation, adhesion, and the BMP4 pathway.

The Notch pathway has been shown to play proliferative roles in varied contexts including both development and disease (Artavanis-Tsakonas et al., 1999; Koch and Radtke, 2007). In the retina, for example, Notch1 promotes proliferation and maintenance of a progenitor state in retinal progenitor cells (Jadhav et al., 2006a; Jadhav et al., 2006b; Yaron et al., 2006). Maintenance of this pool is critical to achieve the proper types of retinal cells and total numbers of cells. Notch2 appears to play a more specific role in the ciliary body. Cell fate decisions occur early on in ciliary body development, prior to morphogenesis, and are unchanged in the DM61/Notch2$^{fx/fx}$ mutant mice. What is changed is the rate of proliferation of the OCE. Previously, the rates of proliferation of both the ICE and OCE were published (Napier and Kidson, 2005). In wild-type mice, the authors observed that the rate of proliferation of the OCE was statistically greater than

that of the ICE and concluded that these differential rates between the layers may provide a driving force behind the morphogenesis of this structure. In our DM61/Notch2$^{fx/fx}$ mice, we are able to experimentally support this hypothesis. The rate of proliferation in the ICE was unchanged while the rate of proliferation in the OCE was significantly reduced to the point that there was no longer any significant difference between the two layers. While our data are experimental, they are not conclusive that differential proliferative rates between the ICE and OCE are needed for proper morphogenesis of the ciliary body. It is possible that the proliferative defects are secondary to other factors and not necessary for proper morphogenesis. However, a basic need for proper morphogenesis is to have sufficient tissue for folding, suggesting that proper proliferation is most likely necessary.

In addition to identifying proliferative defects through BrdU labeling, we were also able to identify 3 different cell cycle genes (Pttg1, Cdc14a, Cdc20) which were significantly reduced (p-value < .05) on our microarray. In a recent genome-wide shRNA screen, all three genes were individually shown to be sufficient to halt the cell cycle when knocked down (Neumann et al., 2010). These genes may be direct or indirect targets of Notch signaling. An indirect possibility would be through the BMP4 pathway. Although our DM61/Alk2$^{fx/fx}$/Alk3$^{fx/fx}$ mice did not show a significant reduction in proliferation, the variability may have been simply due to poor sections or injections. The similar phenotype to the DM61/Notch2$^{fx/fx}$ and DM61/Ncad$^{fx/fx}$ mice suggest that proliferation may indeed be affected. Both Notch and BMP have been linked to the proliferative activities of numerous types of cancers (Bolós et al., 2009; Masuda et al., 2011). These new markers may be useful in the clinical setting.

Proper cell adhesion can play an essential role in the morphogenesis of a tissue. In *Xenopus*, morpholino knock down of N-cadherin and Ncam each result in delamination and disorganization of the retina (Rungger-Brandle et al., 2010). In Drosophila, cone cell morphogenesis is dependent on a Nephrin-homologue (Grillo-Hill and Wolff, 2009). Not all adhesion, however, is essential to morphogenesis, even in the ciliary body. Conn43, the gap junction necessary for aqueous humor production, is unchanged in our mutant. Conn43 knockouts do not show any morphogenetic defects (Calera et al., 2006; Calera et al., 2009). Additionally, P-cadherin knockouts don't report an eye phenotype (Radice et al., 1997). However, it should be noted that anterior eye structures such as the ciliary body are often overlooked when reporting phenotypes.

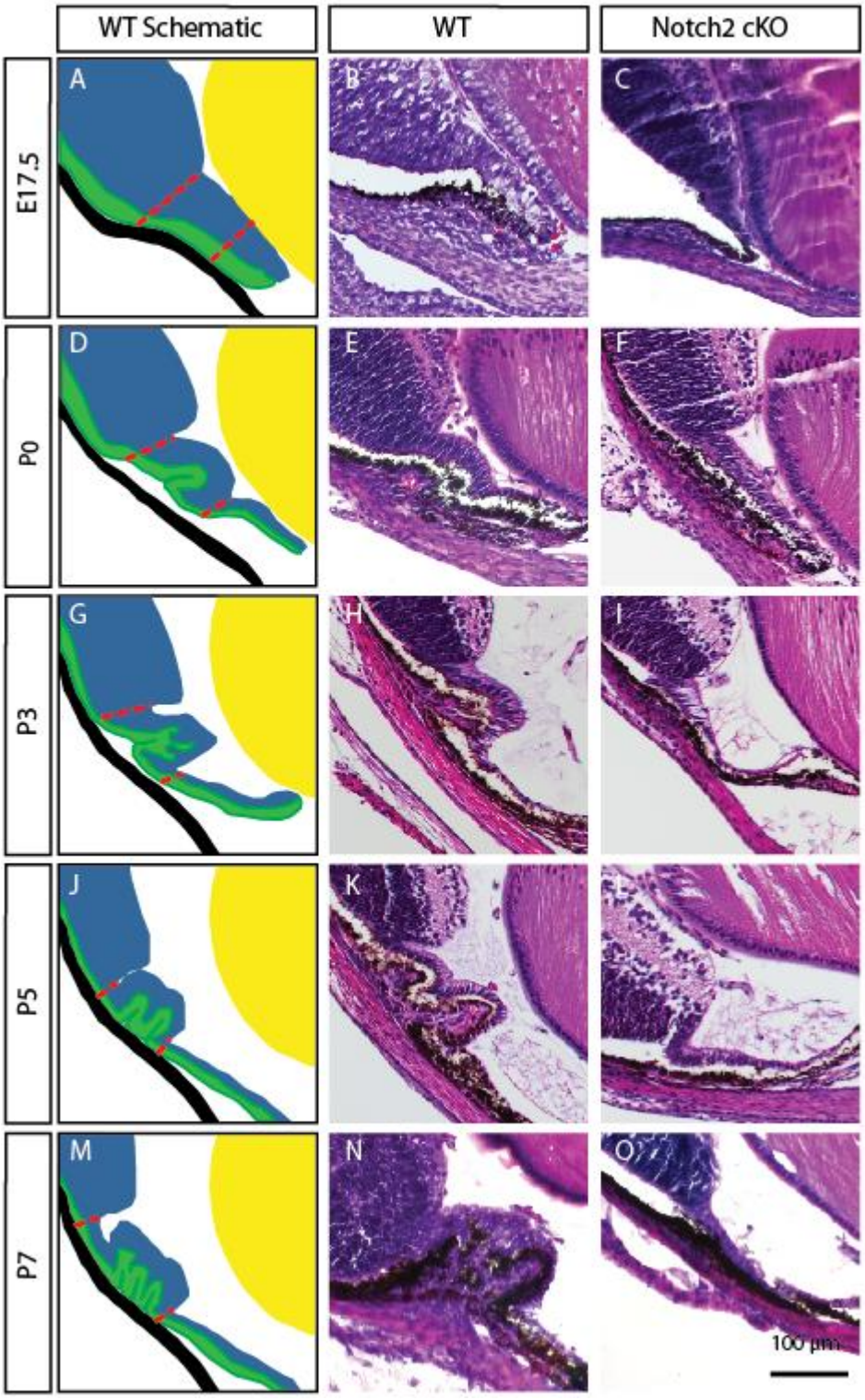
We have shown that Notch2 regulates cell adhesion in the developing mouse ciliary body. DM61/Notch2$^{fx/fx}$ mice lost staining for the N-cadherin/catenin complex in the OCE. Through our GFP mosaics, we have shown that Notch2 regulates N-cadherin, α-catenin, and β-catenin in a cell autonomous manner on both dorsal and ventral sides. Our DM61/Alk2$^{fx/fx}$/Alk3$^{fx/fx}$ cross confirmed that Notch2 interacts with these adhesion proteins independent of BMP4 signaling. Furthermore, our DM61/N-cadherin$^{fx/fx}$ cross revealed not only that N-cadherin is necessary for proper morphogenesis of the ciliary body, but that a positive feedback loop between Notch2 and N-cadherin exists in the developing ciliary body. The proliferative defects seen in the N-cadherin knockouts also support the reduction in Notch2 signaling.

This project has provided valuable, new integration data between Notch2, BMP4, and N-cadherin. We have identified a novel interaction whereby Notch2 is regulating BMP4 signaling at or above the level of SMAD phosphorylation. Our GFP mosaics provide strong evidence that Notch2 is regulating BMP4 both cell autonomously and non-cell autonomously. A likely possibility is that Notch2 is affecting a secreted BMP4 inhibitor. Numerous secreted molecules have the ability to either bind the BMP4 or its ligand extracellularly. Further studies are necessary to identify the biochemical mechanism of this novel interaction.

In conclusion, we have utilized the power of mouse genetics to determine the epistatic relationship of several necessary components of ciliary body morphogenesis. We have established Notch2 as a new regulator of anterior eye development. Notch2 regulates N-cadherin and cell adhesion in a cell autonomous manner through a positive feedback loop. Conversely, Notch2 controls BMP4 signaling at or above the level of SMAD1/5/8 phosphorylation in a non-cell autonomous manner. We have also identified 3 genes as potential Notch targets of the cell cycle through our microarray study. This study is the first to provide any evidence of signaling pathway integration in anterior eye development. Proper development of the anterior eye is important for several human diseases, including glaucoma. These findings may have far-reaching implications across both development and disease, especially in Notch biology.

Figure 3.1. Normal Ciliary Body Development and DM61/Notch2$^{fx/fx}$ phenotype.

(A, D, G, J, M) Oversimplified schematic of ciliary body development. The sclera/cornea is in black, lens in yellow, retina and retina derived layers of the ciliary body and iris are in blue, and the RPE and RPE derived layers of the ciliary body and iris are in green. The ciliary body (prospective ciliary body at E17.5) is outlined with a red dotted line. **(A-C)** At age E17.5 the prospective ciliary body and iris extend from the retina and are demarcated from the retina by a single notch. **(D-F)** At P0 a single fold is present in both the OCE (RPE derived) and ICE (neural derived) layers of the ciliary body. The ciliary body is outlined with red, dotted lines. Morphogenesis continues through ages P3 **(G-I)**, P5 **(J-L)**, and P7 **(M-O)**. Normal development is complete by age P8.


WT Schematic
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Notch2 cKO
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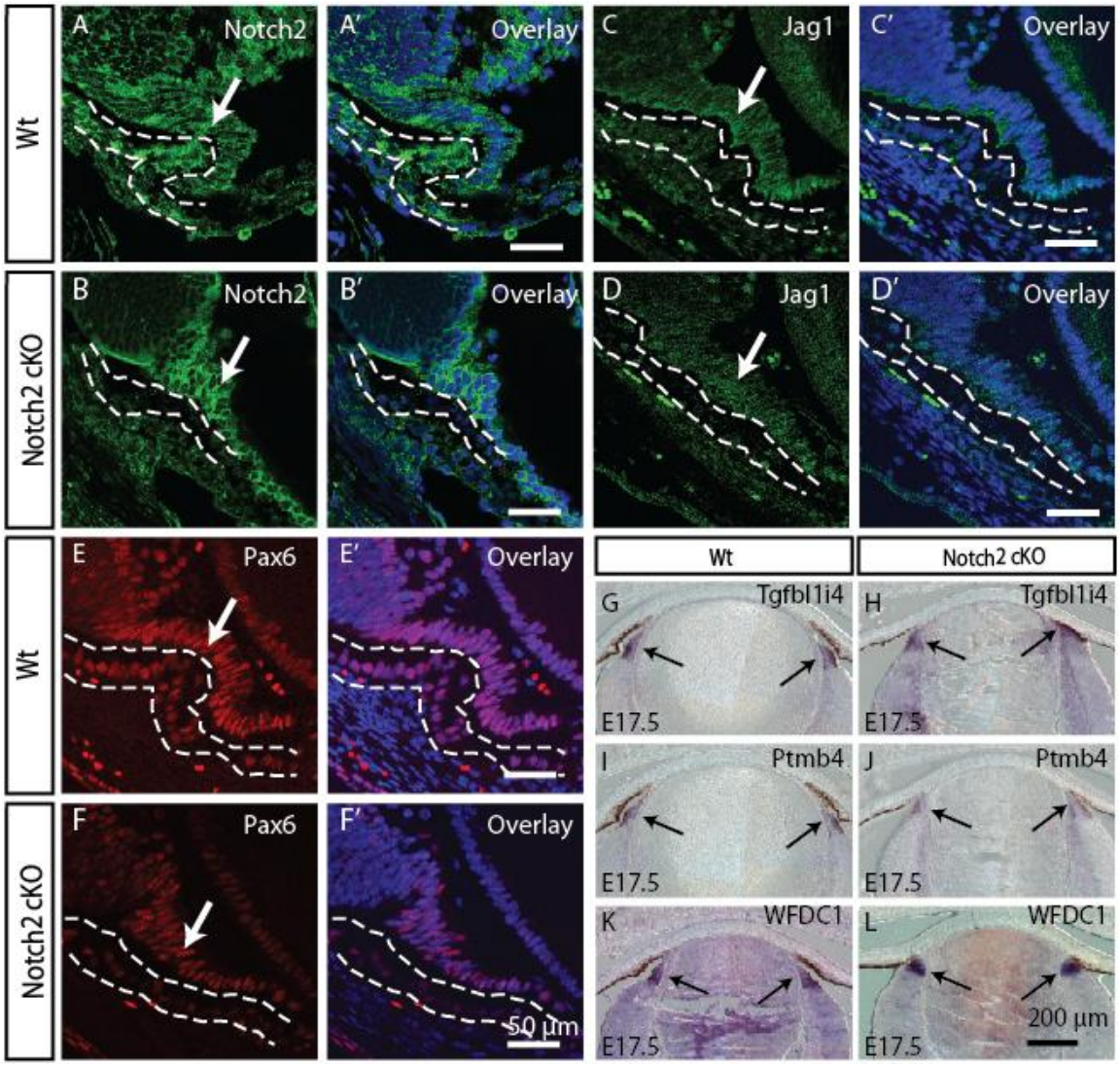
100 µm

Figure 3.2. Notch2, Jag1 Expression and Cell Fate Indicators

In all confocal pictures, the OCE (knockout layer) is outlined with a white, dotted line. All confocal pics are age P3. **(A-B)** Confocal images confirm the lack of Notch2 protein in the OCE of the DM61/Notch2$^{fx/fx}$ mice. **(C-D)** Jag1 staining shows localization of the Jag1 ligand to the apical-apical junction between the ICE and OCE in the wild-type but diffuse staining in the Notch2 cKO. **(E-F)** Pax6 staining in both the ICE and OCE is conserved between wild-type and mutant. In-situ hybridization at age E17.5 of ciliary body specific markers including Tgfbl1i4 **(G-H)**, Ptmb4 **(I-J)**, and WFDC1 **(K-L)**. Arrows point to prospective ciliary body and iris region. These in-situ hybridizations together with the Pax6 staining suggest that cell fate is unchanged in the ciliary body of the DM61/Notch2$^{fx/fx}$ mice.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G
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WFDC1
200 µm
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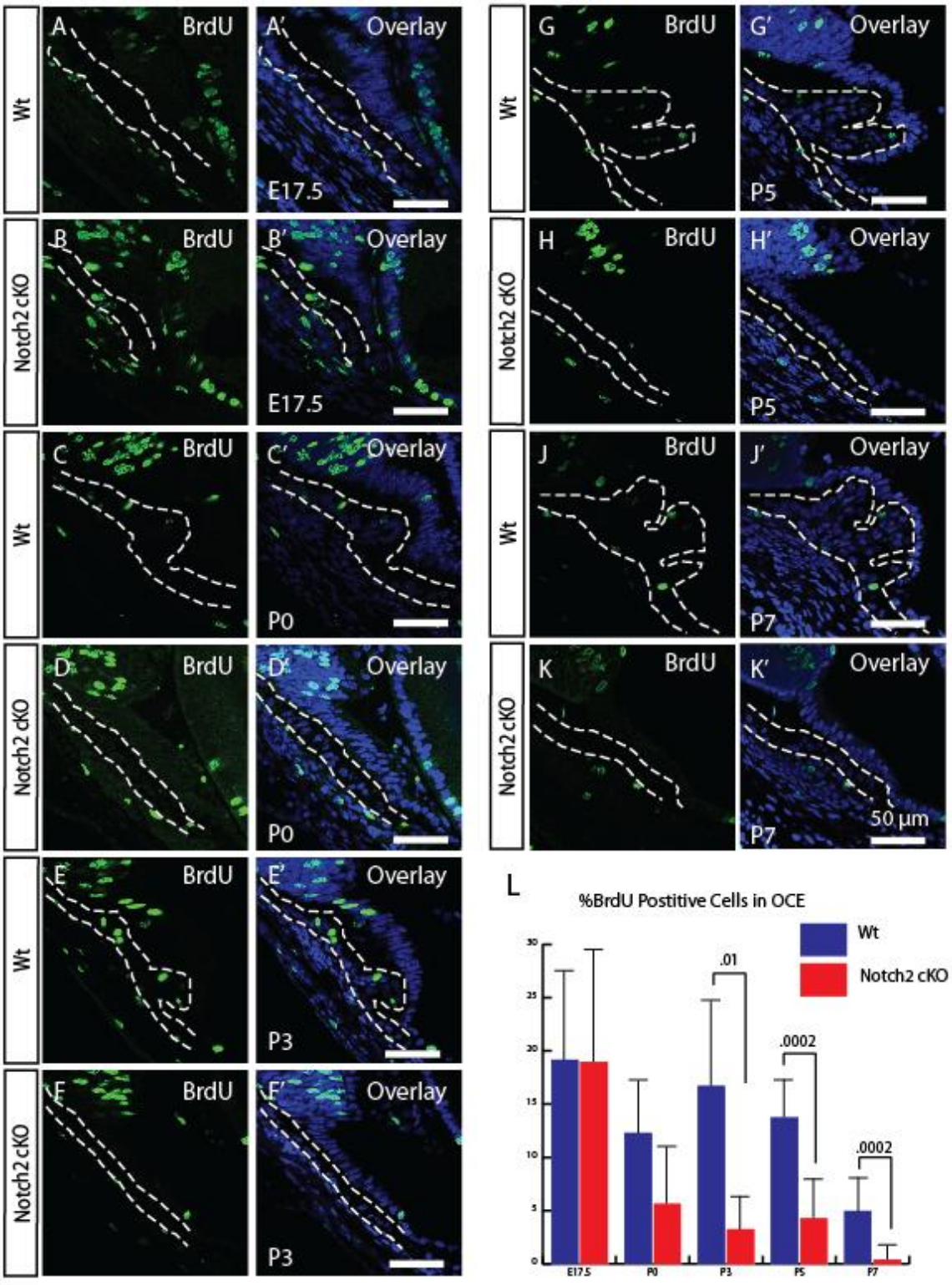
Figure 3.3. Cell Proliferation Defects in DM61/Notch2$^{fx/fx}$ Mice

(A-K) Confocal images of staining for BrdU positive cells from age E17.5 to P7. **(L)** Numbers of BrdU positive cells in the OCE (outlined with white, dotted line) were normalized to the total number of cells to view the rate in proliferation across the development of the ciliary body. There is a significant reduction in the percentage of dividing cells at ages P3, P5, and P7. Asterisk (*) = p-value < .01. Double-cross (‡) = p-value < .0002.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G
G'
P5
H
H'
J
J'
P7
K
K'
50 µm
L
%BrdU Postitive Cells in OCE
Wt
Notch2 cKO
.01
.0002
.0002
E17.5
P0
P3
P5
P7

Figure 3.4. Adhesion Defects in DM61/Notch2$^{fx/fx}$ mice.

Age P3 confocal images of adhesion components of the developing ciliary body. OCE outlined in white, dotted line. **(A)** α-catenin and β-catenin co-localize in the OCE to the basal and lateral sides of the cells in the OCE in the wild-type mice. **(B)** DM61/Notch2$^{fx/fx}$ mice retain α-catenin and β-catenin staining in the ICE but lose this adhesion in the OCE. **(C-D)** Staining for N-cadherin shows similar results. **(E-F)** Phalloidin staining for f-actin further confirms the loss of adherens junctions in the DM61/Notch2$^{fx/fx}$ mice. **(G-H)** Conn43, a gap junction essential for aqueous humor production, maintains its expression in both wild-type and DM61/Notch2$^{fx/fx}$ mice.

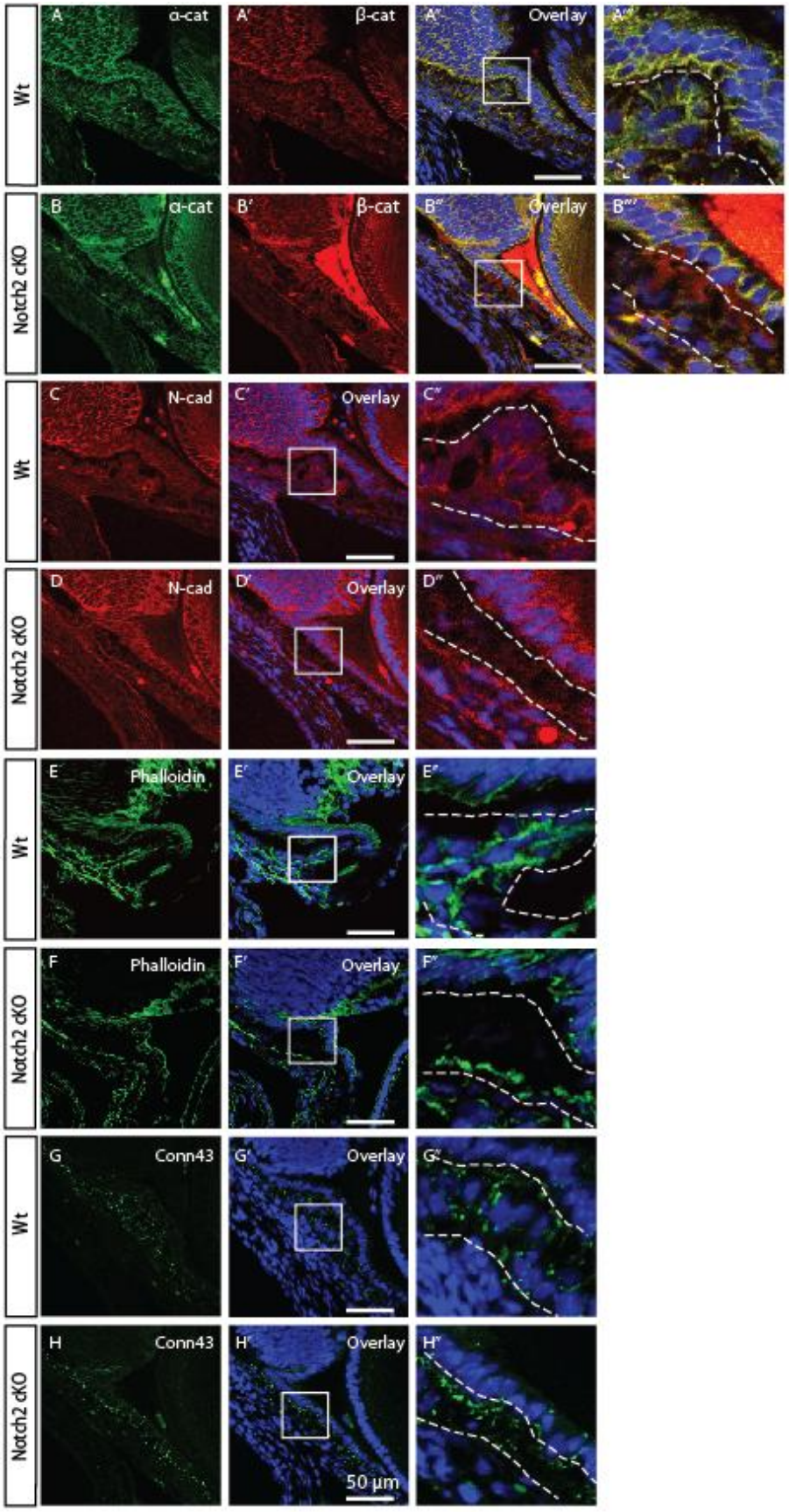
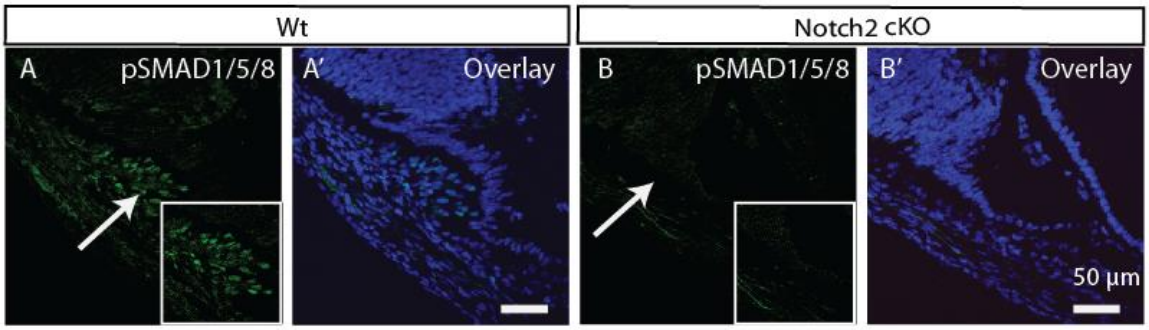
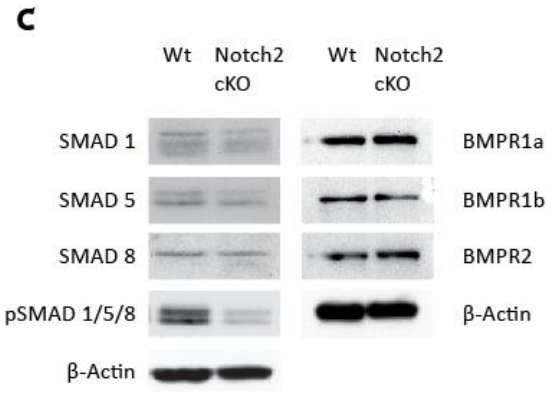

Wt
A
α-cat
A′
β-cat
A″
Overlay
A‴
Notch2 cKO
B
α-cat
B′
β-cat
B″
Overlay
B‴
Wt
C
N-cad
C′
Overlay
C″
Notch2 cKO
D
N-cad
D′
Overlay
D″
Wt
E
Phalloidin
E′
Overlay
E″
Notch2 cKO
F
Phalloidin
F′
Overlay
F″
Wt
G
Conn43
G′
Overlay
G″
Notch2 cKO
H
Conn43
H′
Overlay
H″
50 µm

Figure 3.5. Analysis of BMP4 Signaling

BMP4 signaling is down-regulated in DM61/Notch2$^{fx/fx}$ mice. **(A)** Wild-type mice at age P3 show strong, nuclear expression of pSMAD1/5/8 (see arrow and insert). **(B)** pSMAD1/5/8 staining is lost in the DM61/Notch2$^{fx/fx}$ mice. **(C)** Western blots of OCE tissue from the ciliary body and iris (age P3) show stable expression of SMAD1, SMAD5, SMAD8, BMPR1a, BMPR1b, and BMPR2 in both wild-type and Notch2 mutant mice. **(D)** 60% decrease in pSMAD1,5,8 expression between wild-type and Notch2 cKO bands (p-value < .001). All intensities normalized to β-actin control.


Wt
Notch2 cKO
A
pSMAD1/5/8
A'
Overlay
B
pSMAD1/5/8
B'
Overlay
50 µm


C
Wt
Notch2 cKO
Wt
Notch2 cKO
SMAD 1
SMAD 5
SMAD 8
pSMAD 1/5/8
β-Actin
BMPR1a
BMPR1b
BMPR2
β-Actin

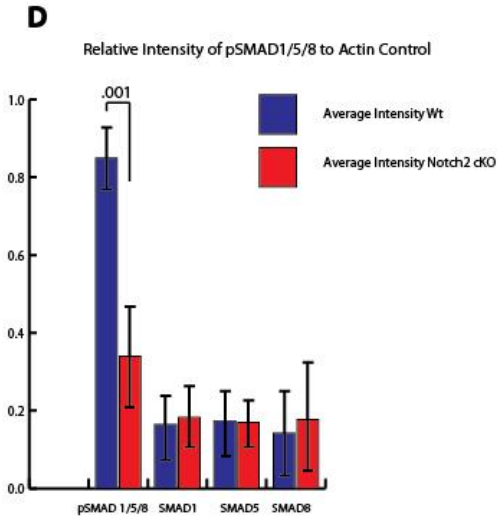

D
Relative Intensity of pSMAD1/5/8 to Actin Control
.001
Average Intensity Wt
Average Intensity Notch2 cKO
1.0
0.8
0.6
0.4
0.2
0.0
pSMAD 1/5/8
SMAD1
SMAD5
SMAD8

Figure 3.6. Notch2 Regulates BMP4 Signaling in a Ventral, Cell Autonomous, and Non-cell Autonomous Manner

(A) pSMAD1/5 staining is present in the dorsal ciliary body regardless of the cells GFP (+) (see asterisk) or GFP (-) (see plus sign) status. **(B)** GFP (+) cells (see asterisk) in the ventral ciliary body are negative for pSMAD1/5 staining. Adjacent GFP (-) cells are also negative for pSMAD1/5 staining. Therefore, Notch2 is affecting BMP4 signaling in a non-cell autonomous manner. All images are age P3.

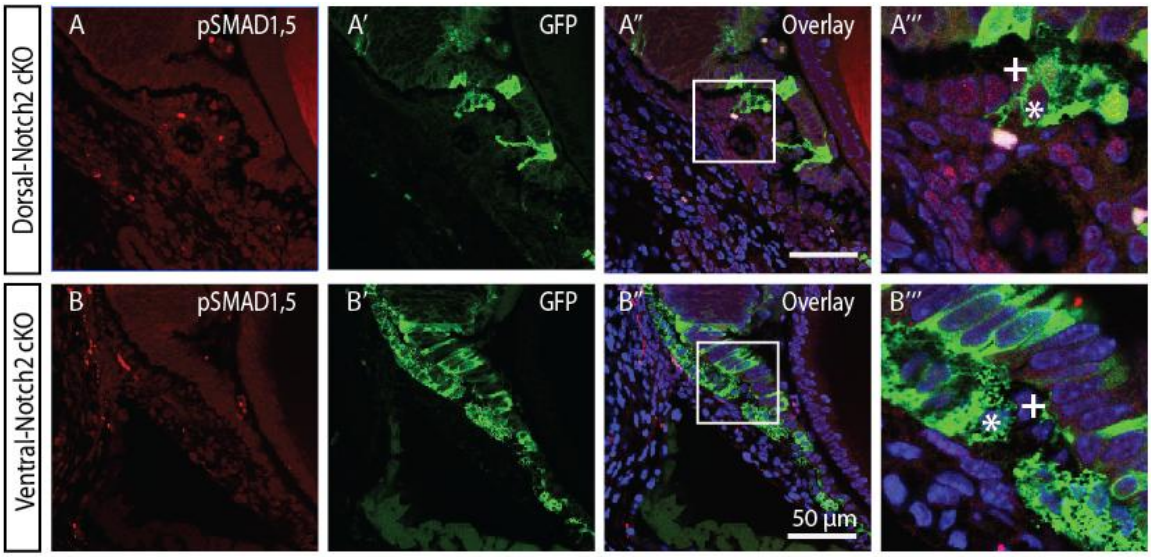

Dorsal-Notch2 cKO
Ventral-Notch2 cKO
A
pSMAD1,5
A′
GFP
A″
Overlay
A‴
B
pSMAD1,5
B′
GFP
B″
Overlay
B‴
50 µm

Figure 3.7. Notch2 Cell Autonomously Regulates Adhesion in the Developing Ciliary Body

(A-B) Dorsal and ventral N-cadherin and GFP staining. Notch2 regulates adhesion both dorsally and ventrally as shown by GFP (+) cells lacking N-cadherin (see asterisk). Some GFP (-) cells are also negative for N-cadherin staining (see plus sign). Arrow shows N-cadherin positive cell for reference. **(C-F)** Similar results found for both α- and β-catenin. **(F''')** Arrow shows GFP (-)/ β -catenin (+) cell one cell-diameter away from a GFP(+) cell. This indicates that the GFP (-) cell (see plus sign) is competent to make adhesions despite being immediately adjacent to a GFP (+) cell (see asterisk). Taken together, Notch2 is regulating adhesion in a cell autonomous manner. All images are age P3.

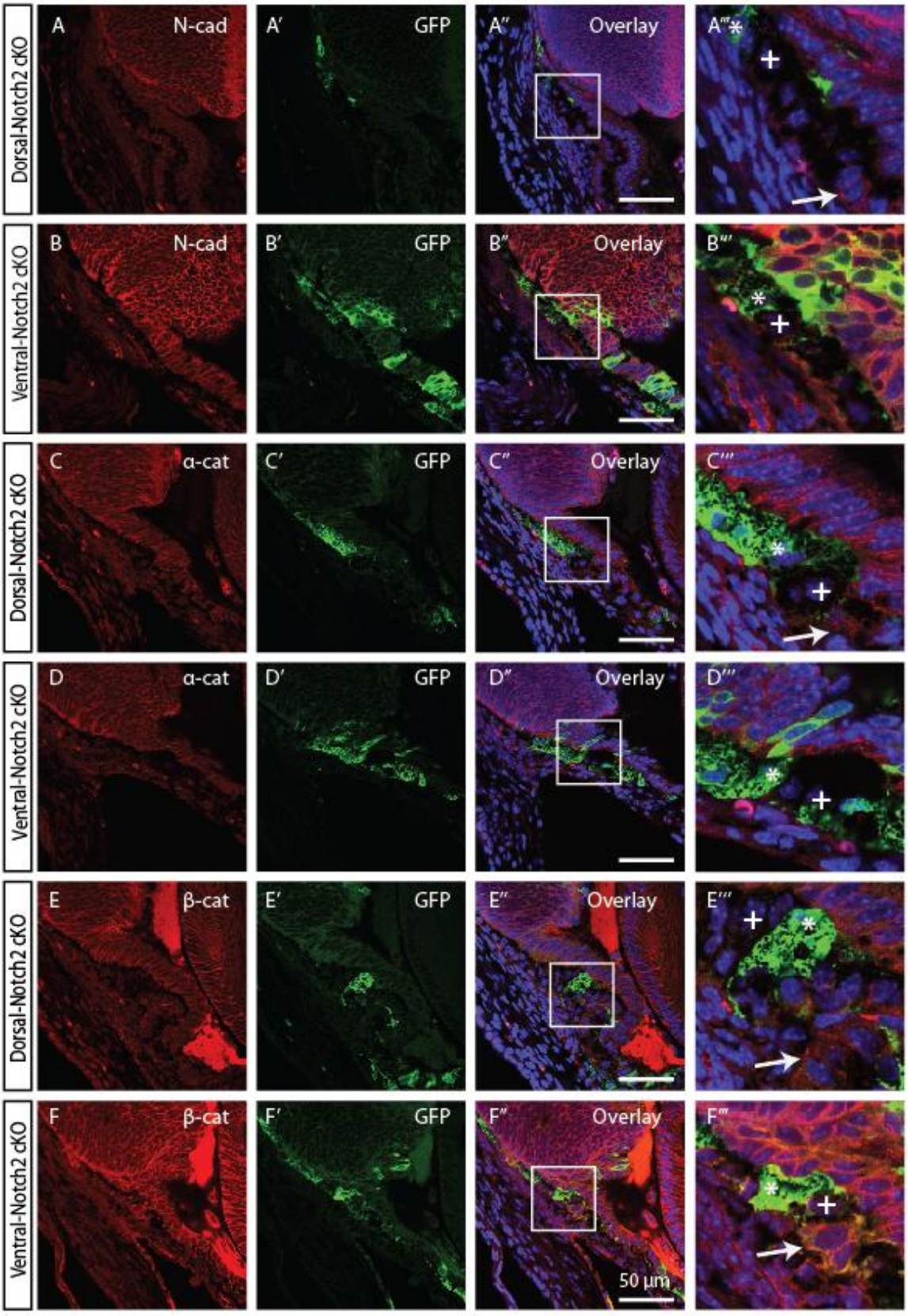

Dorsal-Notch2 cKO
A
N-cad
A′
GFP
A″
Overlay
A‴
Ventral-Notch2 cKO
B
N-cad
B′
GFP
B″
Overlay
B‴
Dorsal-Notch2 cKO
C
α-cat
C′
GFP
C″
Overlay
C‴
Ventral-Notch2 cKO
D
α-cat
D′
GFP
D″
Overlay
D‴
Dorsal-Notch2 cKO
E
β-cat
E′
GFP
E″
Overlay
E‴
Ventral-Notch2 cKO
F
β-cat
F′
GFP
F″
Overlay
F‴
50 µm

Figure 3.8. qRT-PCR Confirmation of Key Microarray Genes

Key genes from our DM61/Notch2$^{fx/fx}$ microarray. Three cell cycle genes and one downstream target each from Notch2 and BMP4 are shown. Black bars show fold changes based on microarray results. Gray bars show confirmation of microarray results by qRT-PCR.

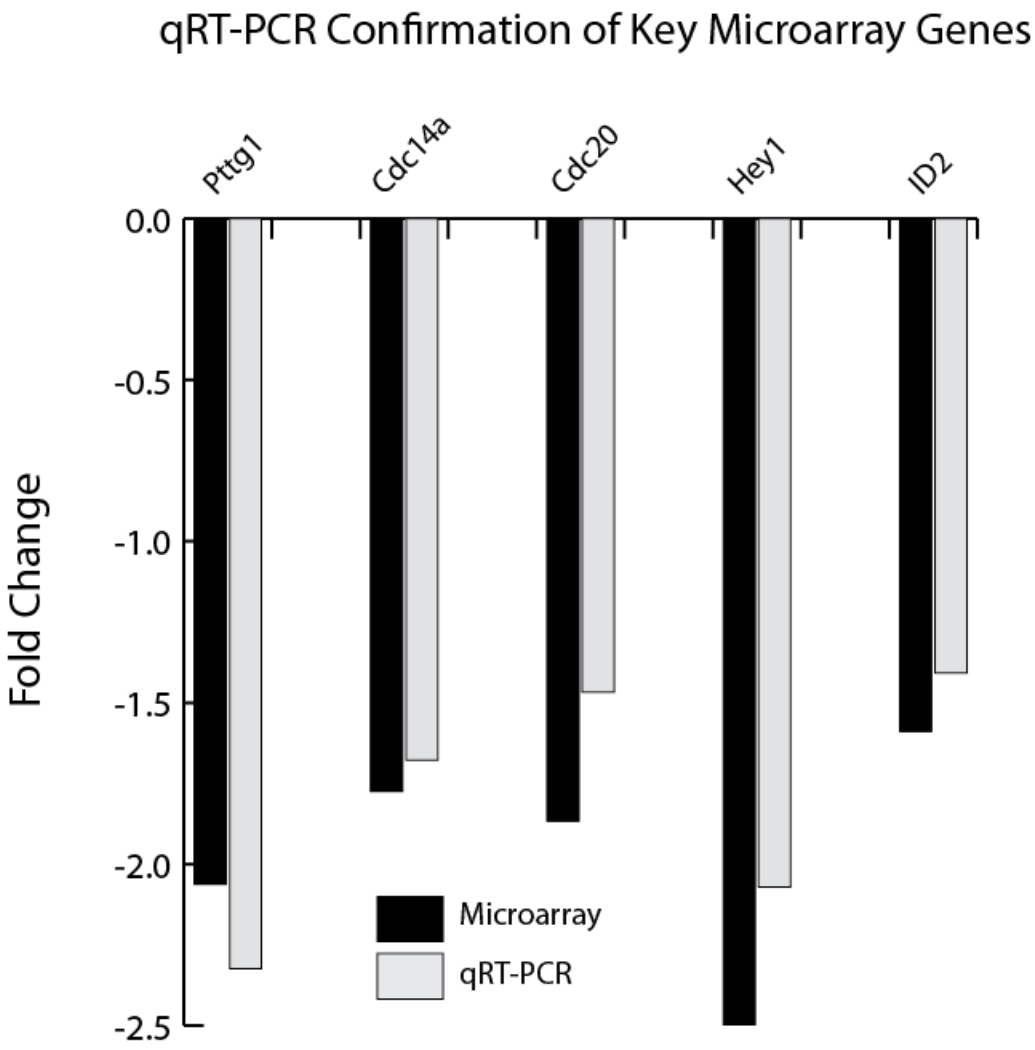

qRT-PCR Confirmation of Key Microarray Genes
Pttg1
Cdc14a
Cdc20
Hey1
ID2
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Fold Change
Microarray
qRT-PCR

Figure 3.9. BMP4 Signaling In the OCE Is Necessary for Ciliary Body Development

(A) Control DM61/A2$^{fx/fx}$/A3$^{fx/fx}$ ciliary body. **(B)** Mutant DM61/A2$^{fx/fx}$/A3$^{fx/fx}$ ciliary body. **(C)** BrdU studies in the DM61/A2$^{fx/fx}$/A3$^{fx/fx}$ mice showed wide variability and no statistical significance. **(D)** pSMAD1/5 staining is lost in the DM61/A2$^{fx/fx}$/A3$^{fx/fx}$ mice, confirming the lack of BMP4 signaling. **(E)** Notch2 and N-cadherin expression are retained in A2/A3 cKO. **(F)** α-catenin and β-catenin expression are also maintained in the cKO. All images are age P3.

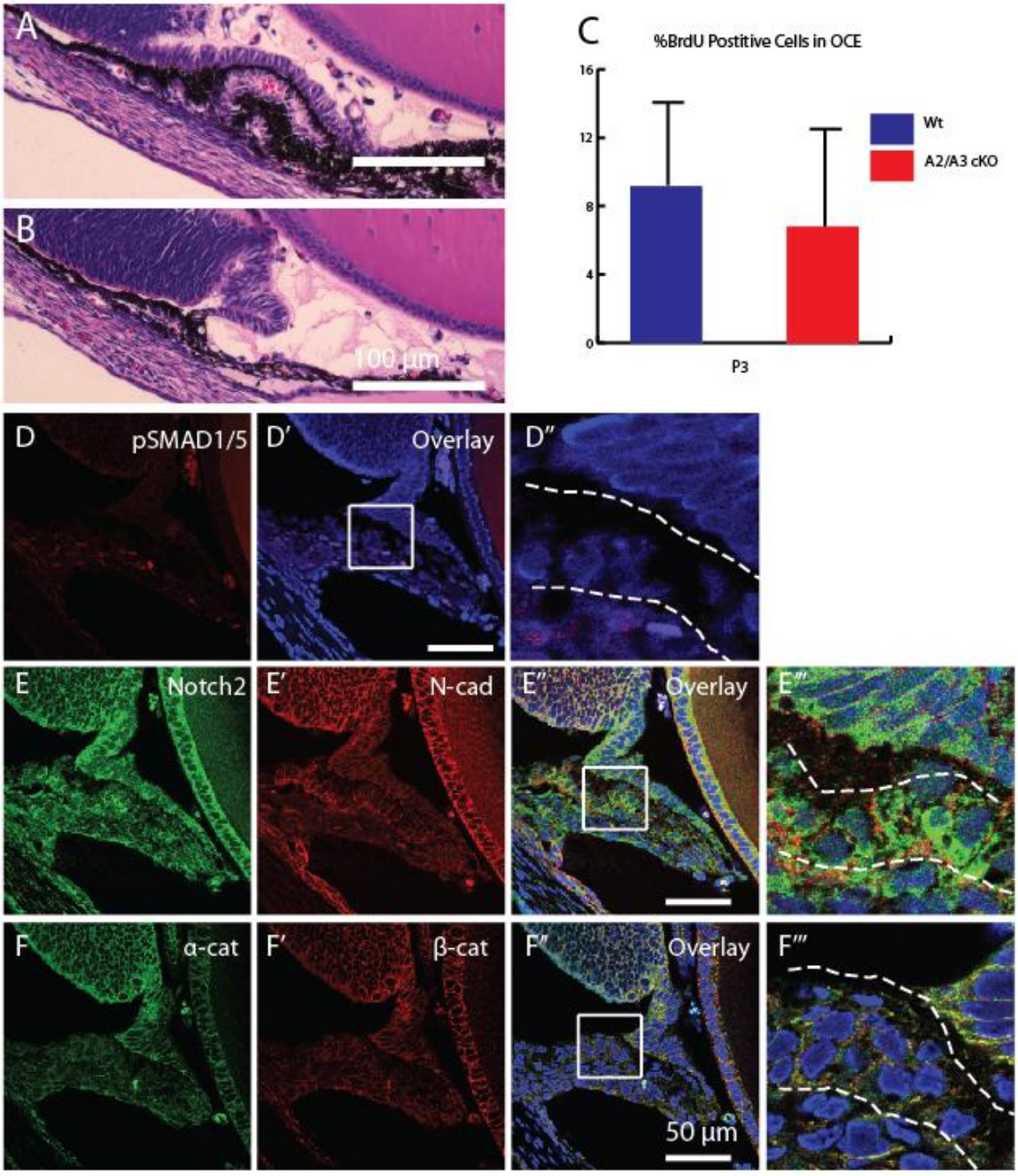

A
B
100 µm
C
%BrdU Postitive Cells in OCE
16
12
8
4
0
P3
Wt
A2/A3 cKO
D
pSMAD1/5
D′
Overlay
D″
E
Notch2
E′
N-cad
E″
Overlay
E‴
F
α-cat
F′
β-cat
F″
Overlay
F‴
50 µm

Figure 3.10. N-Cadherin Is Necessary For Ciliary Body Morphogenesis

(A) Control DM61/Ncad$^{fx/fx}$ ciliary body. **(B)** Mutant DM61/Ncad$^{fx/fx}$ ciliary body. **(C)** DM61/Ncad$^{fx/fx}$ mice show proliferation defects in the OCE similar to those of the DM61/Notch2$^{fx/fx}$ mice. Asterisk (*) = p-value < .00001. **(D)** Notch2 and N-cadherin stainings are lost in the N-cadherin cKO. This confirms the knockout and suggests a positive feedback loop between Notch2 and N-cadherin. **(E)** α-catenin and β-catenin expression is disrupted in the cKO. **(F)** pSMAD1/5 staining is abolished in the DM61/Ncad$^{fx/fx}$ mice. These results provide conclusive evidence that N-cadherin is necessary for ciliary body development. All images are age P3.

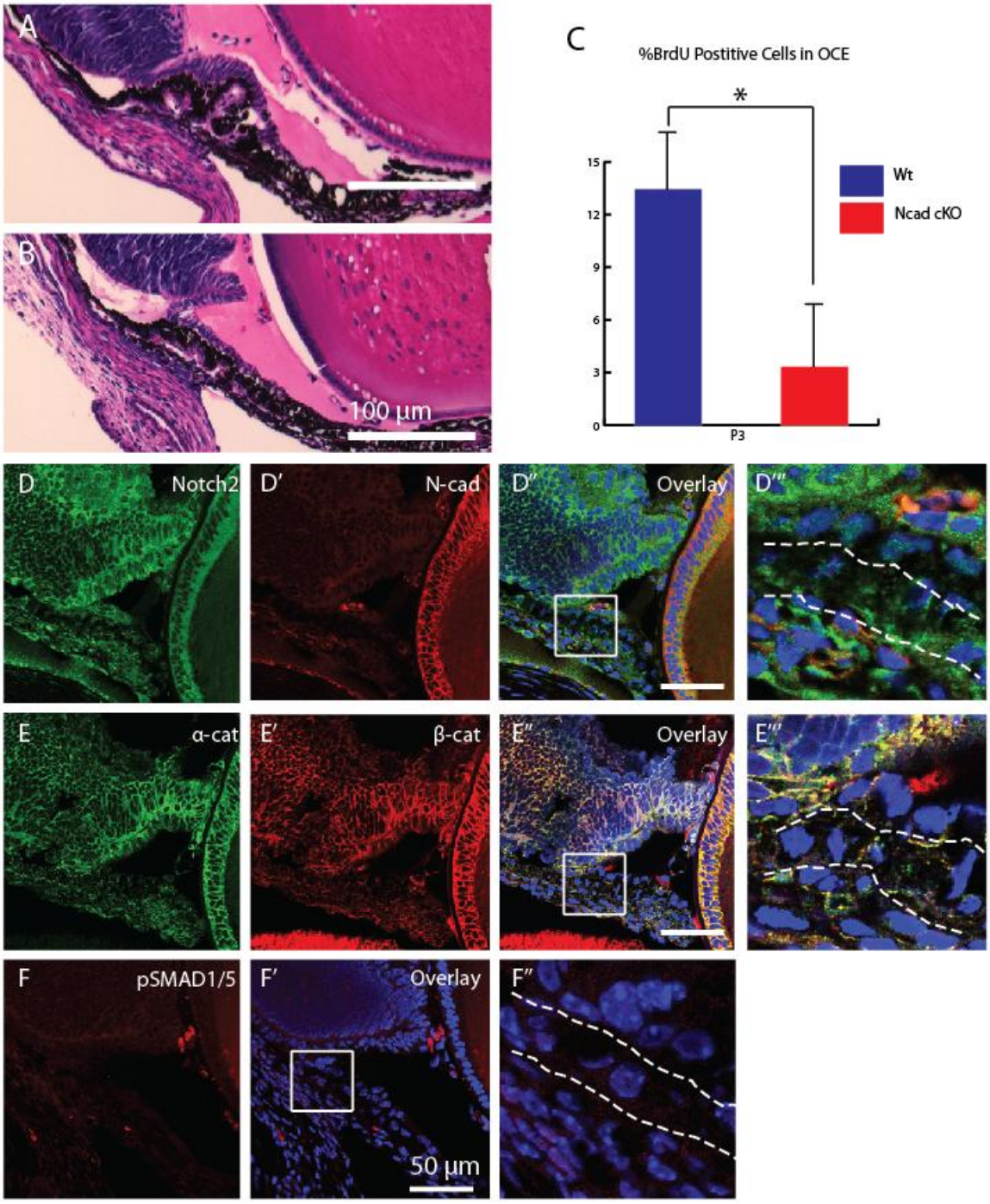
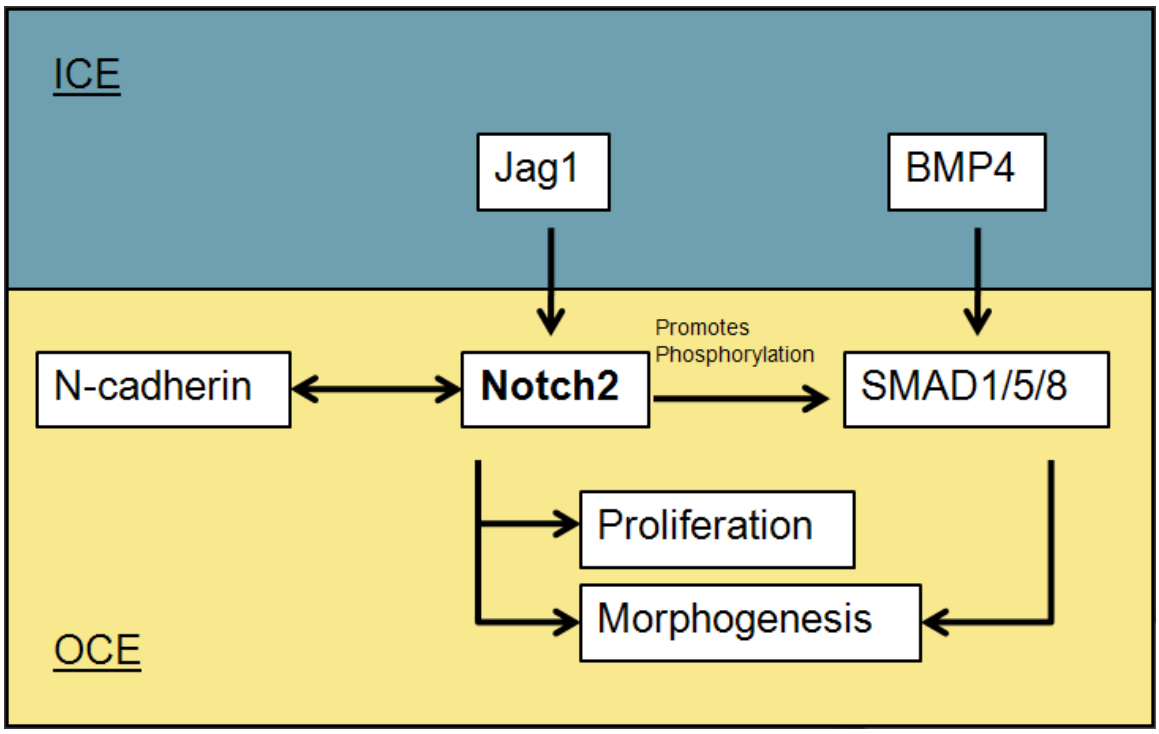

A
B
100 µm
C
%BrdU Postitive Cells in OCE
*
15
12
9
6
3
0
P3
Wt
Ncad cKO
D
Notch2
D′
N-cad
D″
Overlay
D‴
E
α-cat
E′
β-cat
E″
Overlay
E‴
F
pSMAD1/5
F′
Overlay
50 µm
F″

Figure 3.11. Model of Notch2 Regulation of Ciliary Body Morphogenesis

Model of the role of Notch2 in ciliary body development. Jag1 signals from the non-pigmented ICE to the Notch2 receptor on the OCE to activate the Notch2 pathway. Notch2 affects cell adhesion independently of BMP4 signaling and in a cell autonomous manner. A positive feedback loop between Notch2 and N-cadherin exists. Notch2 also regulates cell proliferation and morphogenesis. Finally, Notch2 promotes the phosphorylation of SMAD1/5/8 and thus BMP4 signaling in a cell autonomous and non-cell autonomous manner. BMP4 signaling also regulates morphogenesis of the ciliary body


ICE
Jag1
BMP4
Promotes
Phosphorylation
N-cadherin
Notch2
SMAD1/5/8
Proliferation
Morphogenesis
OCE

CHAPTER FOUR: CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

The main conclusions of this study may be summed up in the following:

1. **Notch2 is required for morphogenesis and proliferation in the developing mouse ciliary body.**

   We have identified Notch2 as a novel regulator of ciliary body development. Conditional removal of Notch2 from the pigmented outer ciliary epithelium abrogates folding and development of the ciliary body while leaving the iris intact. One mechanism by which it does this is through regulating cell proliferation which is significantly reduced in our DM61/Notch2$^{fx/fx}$ mice. Apoptosis was not increased in our mutant.

2. **Notch2 regulates N-cadherin and cell adhesion in ciliary body development.**

   Appropriate cell adhesion is essential for correct morphogenesis. Here we show a role Notch in regulating the adhesion proteins N-cadherin, α-catenin, and β-catenin. DM61/Notch2$^{fx/fx}$ mice show a loss of N-cadherin and the supporting α- and β-catenin proteins in a cell autonomous manner according to GFP labeled mosaics. Our further genetic studies with DM61/Ncad$^{fx/fx}$ mice confirm the essential role of N-cadherin in ciliary body development and suggest a positive feedback loop between Notch2 and N-cadherin.

3. Notch2 cell autonomously and non-cell autonomously regulates BMP4 signaling in ciliary body development.

Notch and BMP are two of the most important and conserved pathways in cell signaling. DM61/Notch2$^{fx/fx}$ mice display a loss of BMP4 signaling which has previously been shown to be essential for ciliary body development. Using genetic mosaics, we determined that Notch2 is regulating BMP4 both in a cell autonomous and non-cell autonomous manner. This would be a novel interaction between the two pathways.

FUTURE DIRECTIONS

Science, by nature, generally takes a reductionist approach to problems. Such was the case with this project. Using mouse genetics as our base, we have isolated a new role for Notch2 in the eye. New insights into developmental biology, Notch biology, and the genetics of glaucoma have been revealed. However, a number of questions remain unanswered and provide exciting opportunities for future projects.

Additional Roles for Notch2

Early in eye development, Notch2 is restricted to the RPE layer. It is only in later embryonic and early postnatal stages that its expression shifts anteriorly to the ciliary body. Our initial cross of the Notch2$^{fx/fx}$ mice with the DM61-Cre line was intended to assess the role of Notch2 in RPE development. We performed functional studies including IOP measurements and ERGs without finding any phenotype (Supplemental Figure 5.9). Nor were any obvious defects at the light microscopy level observed in the RPE. However, a closer look may in fact reveal some phenotype. One method of testing would be to look at the ultrastructural level using electron microscopy. RPE cells have delicate protrusions which support photoreceptors and phagocytose aged segments.

Another possible line of testing would include utilizing the immortalized ARPE-19 cell line. Although these cells are an immortalized adult cell line, they provide a convenient model to test various genetic constructs and drugs on RPE cells. Both Notch1 and Notch2

are activated in these cells (Notch1 more than Notch2) according to our own unpublished observations. Knocking down Notch2 or overexpressing Notch2 and then scoring for proliferation and/or adhesion defects may be useful. This may also be a productive strategy to more directly identify the links between Notch2 and the cell cycle genes we identified through our microarray. Established cell lines greatly facilitate more in-depth biochemistry and molecular biology. However, there are inherent limitations to cell lines, especially in development where tissue-tissue interactions and their reciprocally inductive signals are so essential.

One of the challenges of studying anterior eye development, and eye development in general, is the lack of an *in vitro* model. Some biologists have taken advantage of chick models to manipulate the lens and introduce various vectors. While the avian approach has its positives, there are fundamental disadvantages to using the chick to study anterior eye development. Foremost is the lack of genetic tools. More and more genes are being linked to anterior segment dysgenesis and other human diseases. Mouse geneticists have been able to reproduce many of these deformities. Another clear advantage of mammalian models is the basic anatomy of the eye. While chicks have a ciliary marginal zone and iris, the ciliary body region never undergoes folding. This lack of morphogenesis not only makes it impossible to study ciliary body development after the point of cell fate specification, it also calls into question the validity of studying other anterior components such as the trabecular meshwork in a non-mammalian system.

Recently, however, a break-through occurred which may facilitate future developmental studies in the eye. Sasai and colleagues in Japan successfully grew eyes in a 3-D *in vitro* culture from mouse ES cells (Eiraku et al., 2011). It is unknown as of yet whether or not this cultured model will have application to anterior eye development since the authors did not take the culture that far, but it certainly offers the potential of a manipulatable mammalian model. Introducing gene therapy vectors, morpholinos, and electroporation techniques to quickly test specific questions in eye development may now be a reality.

Molecular Links Between Notch2 Ciliary Body Developmental Processes

This project has extensively utilized mouse genetics to determine the epistatic relationship of Notch2 with BMP4, cell proliferation, and cell adhesion in the developing ciliary body. The molecular links, however, between Notch2 and these processes is still unclear. One challenge with mammalian CNS development is developing appropriate *in vitro* models to test candidate genes and protein-protein interactions. Cell lines provide convenient models for basic cell biology, but often fall short in understanding tissue-tissue interactions and more complex developmental problems.

One possibility in the anterior eye may be to directly inject viruses containing shRNA or overexpression vectors into the developing tissue. Since the anterior eye undergoes much of its development postnatally, it opens up the possibility of external manipulate postpartum. Preliminary studies in our lab have demonstrated the feasibility of this approach. Injections of lentivirus directly into the ciliary body at age P0 has resulted in

vector being delivered to the proper cell layers. Continued refinement of the procedure is needed, however, to achieve the necessary efficiency and reproducibility required to make this a viable technique.

In terms of proliferation, we have successfully identified multiple candidate cell cycle genes. All of which are capable of halting the cell cycle if affected. *In vitro* studies using cell lines which express Notch may be useful in testing for direct links between Notch signaling and these genes. Luciferase reporters containing the promoter elements of these genes and ChIP assays may be effective techniques to assess the interactions between Notch2 and the specific genes. In our experience, the current Notch2 and RBPJk antibodies are not of sufficient quality for ChIP assays. So recombinant Notch2 tagged with FLAG or HIS would be required for good results.

Cell adhesion can be more difficult to study *in vitro*. However, both immortalized and primary RPE cell lines form adherens and tight junctions *in vitro*. Adhesion complex formation may be a useful screening tool for an *in vitro* shRNA screen to identify novel regulators of adhesion. Our microarray results would be able to provide likely gene candidates for such an endeavor.

Lastly, determining the exact interaction between Notch2 and BMP4 is an essential next step in this project. We have been able to determine several characteristics of the

interaction in the current study; namely that the BMP4 ligand is transcriptionally unchanged (according to the microarray), the BMP receptors are unaffected at the transcriptional and protein level, and that Notch2 regulates BMP4 both cell autonomously and non-cell autonomously. These results suggest an extracellular interaction that can affect neighboring cells.

Our microarray data suggest two candidate genes that meet this profile: Neuroblastoma-like 1 (Nbl1) and Chordin-like 1 (Chrdl1). Both proteins are secreted inhibitors of the BMP pathway, and both are upregulated approximately two-fold on the microarray. While a two-fold change is not very impressive as far as transcriptional changes go, it very likely still has biologic relevance for a couple of reasons. First, signaling cascades are amplified at each step. A two-fold change at the beginning of a signaling pathway will end up being many times that further down the pathway. Second, the BMP4 pathway has already been shown in several instances to be susceptible to haploinsufficiency. Even in the anterior eye, the original BMP4 report was of a BMP4 heterozygote mouse showing ciliary body defects. So decreasing the amount of free BMP4 through increasing a secreted inhibitor would likely be sufficient to induce ciliary body defects. An example of this lies in renal development. In an elegant set of experiments, knocking out a single copy of Gremlin, a BMP4 secreted inhibitor, resulted in a halt in nephric bud development (Walsh et al., 2010). Despite little change in BMP4 levels, downstream targets of BMP4 were markedly increased due to the reduction in the Gremlin inhibitor. Impressively, full bud development was then restored by concomitantly decreasing BMP4 in the Gremlin heterozygotes. Taken together, these experiments demonstrate the

extremely tight regulation of signaling pathways and that even slight changes in signaling can have significant developmental results.

Chrdl1 appears to be the most likely candidate. It has, in fact, previously been described in eye development (Sakuta et al., 2001). It was even noted to be ventrally localized in the developing retina. While retinal expression does not necessarily indicate anterior eye development, it is certainly suggestive and would correlate with our observation that Notch2 only affects BMP4 signaling in the ventral ciliary body. A quick way to test for expression would be through *in-situ* hybridization. While *in-situ* hybridization may not be sensitive enough to show a two-fold change between wild-type and mutant, it should be able to show expression in the normal ciliary body and whether or not there are significant differences between the dorsal and ventral sides of the eye. *In vitro* studies may appear convenient for testing a BMP inhibitor. However, secreted proteins can be difficult to assess *in vitro*. Also, Chrdl1 has been shown to have opposite effects on BMP4 signaling depending on the presence or absence of twisted gastrulation (Kane et al., 2008; Larman et al., 2009). These results highlight the tissue specificity of developmental interactions. They also make clear that to be certain, one must directly test Chrdl1 *in vivo* in the ciliary body. To mimic the Chrdl1 overexpression, the previously mentioned virus injections may be an attractive possibility. Another option would be to create a knock-in mouse to overexpress the inhibitor. While making knock-in mice takes time, it would nicely complement our previous genetic studies and provide strong *in vivo* evidence of the role of Chrdl1.

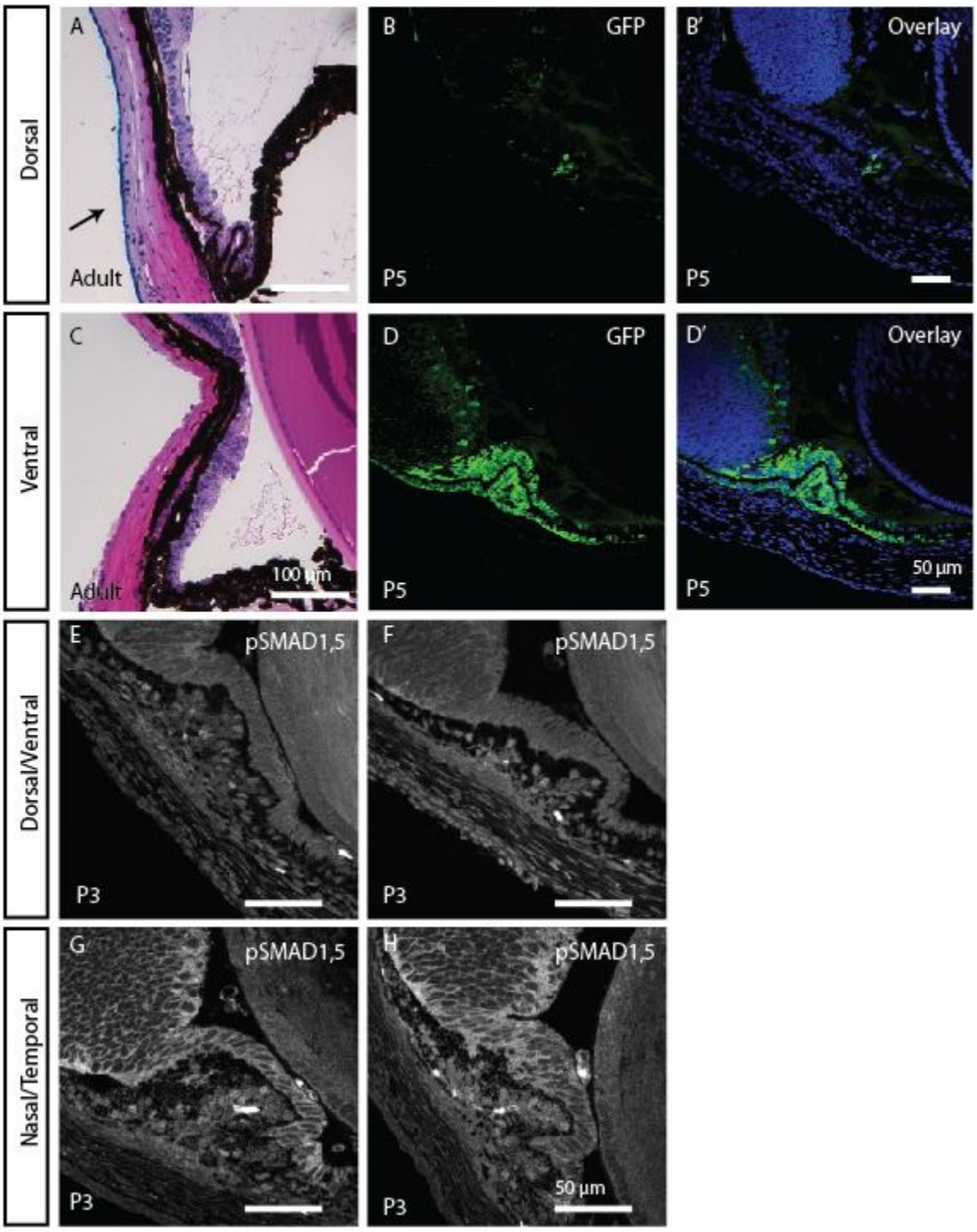
Tissue-Tissue Interactions

The ciliary body is a small, yet developmentally complex organ. Previous studies, including the present one, have focused on interactions between the layers of the ciliary body itself, or with the lens which is in contact with the ciliary body early in development. Future areas of study should include the neural crest cells which make up the ciliary body musculature and the endothelial cells of the developing vasculature. At the core of each ciliary body fold in a capillary (Gage 2005). Notch has already been shown to play an important role in the angiogenesis of the mammalian eye (Ahmad et al., 2011). Whether or not Notch is affecting the capillaries of the ciliary body, it is likely that the mesenchymal endothelial cells are both receiving signals and sending signals with the surrounding tissue to organize proper development.

CHAPTER FIVE: SUPPLEMENTAL FIGURES

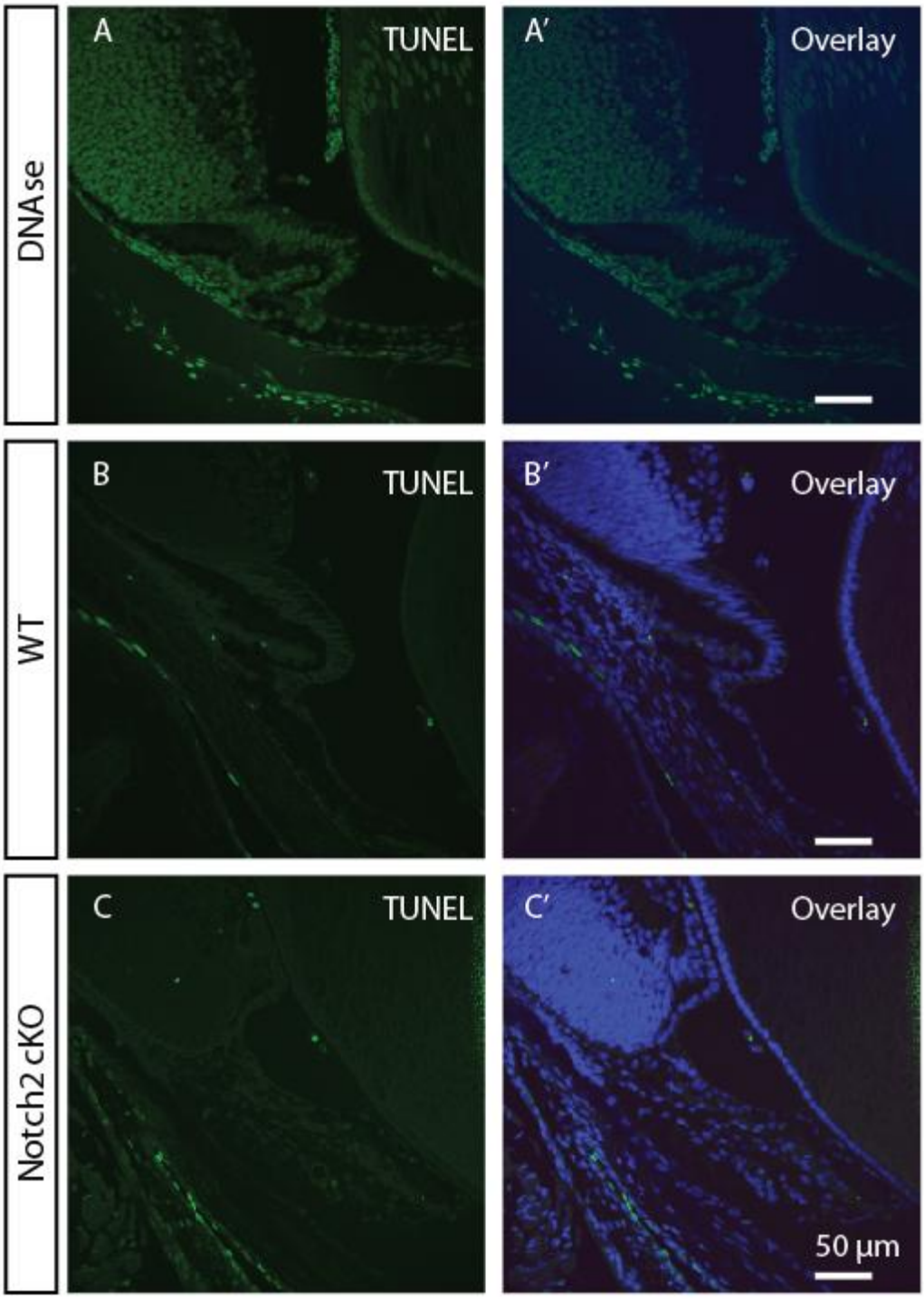
Supplemental Figure 5.1. Axes Variations in DM61/Notch2$^{fx/fx}$ Mice

(A,C) Adult DM61/Notch2$^{fx/fx}$ mice. **(A)** Dorsal side of eye of adult DM61/Notch2$^{fx/fx}$ mouse showing no phenotype. Immediately after dissection, eyes were marked dorsally with green histology dye to mark dorsal/ventral axis (see arrow). **(C)** Ventral side of adult DM61/Notch2$^{fx/fx}$ showing lack of ciliary body phenotype. **(B,D)** DM61/Z/EG mice showing high expression of Cre on ventral side **(D)** and low Cre expression dorsally **(B)**. **(E-H)** pSMAD1/5 expression is uniform across all axes. **(E)** Dorsal, **(F)** ventral, **(G)** nasal, **(H)** temporal.


Dorsal
A
Adult
B
GFP
P5
B'
Overlay
P5
Ventral
C
Adult
100 µm
D
GFP
P5
D'
Overlay
50 µm
P5
Dorsal/Ventral
E
pSMAD1,5
P3
F
pSMAD1,5
P3
Nasal/Temporal
G
pSMAD1,5
P3
H
pSMAD1,5
50 µm
P3

Supplemental Figure 5.2. TUNEL Staining of DM61/Notch2$^{fx/fx}$ Mice

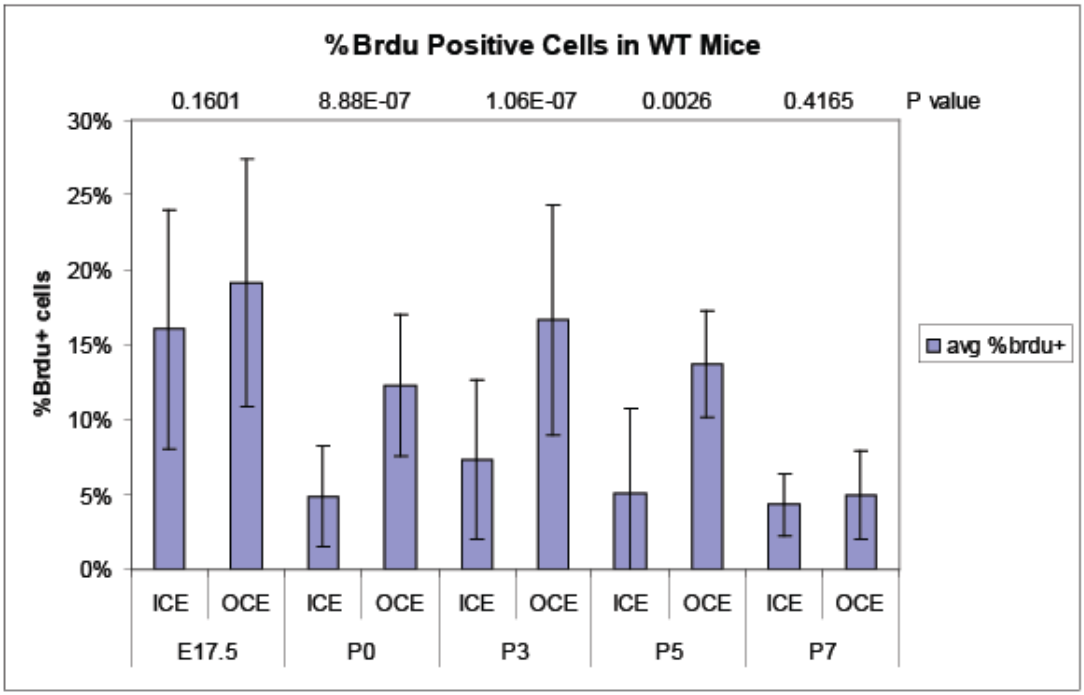
DM61/Notch2$^{fx/fx}$ mice do not show an increase in TUNEL positive cells. **(A)** DNAse treated positive control to ensure the staining worked. **(B)** Wild-type section shows no TUNEL positive nuclei. **(C)** DM61/Notch2$^{fx/fx}$ mice show no positive TUNEL staining. All sections are age P3.


DNAse
A
TUNEL
A′
Overlay
WT
B
TUNEL
B′
Overlay
Notch2 cKO
C
TUNEL
C′
Overlay
50 µm

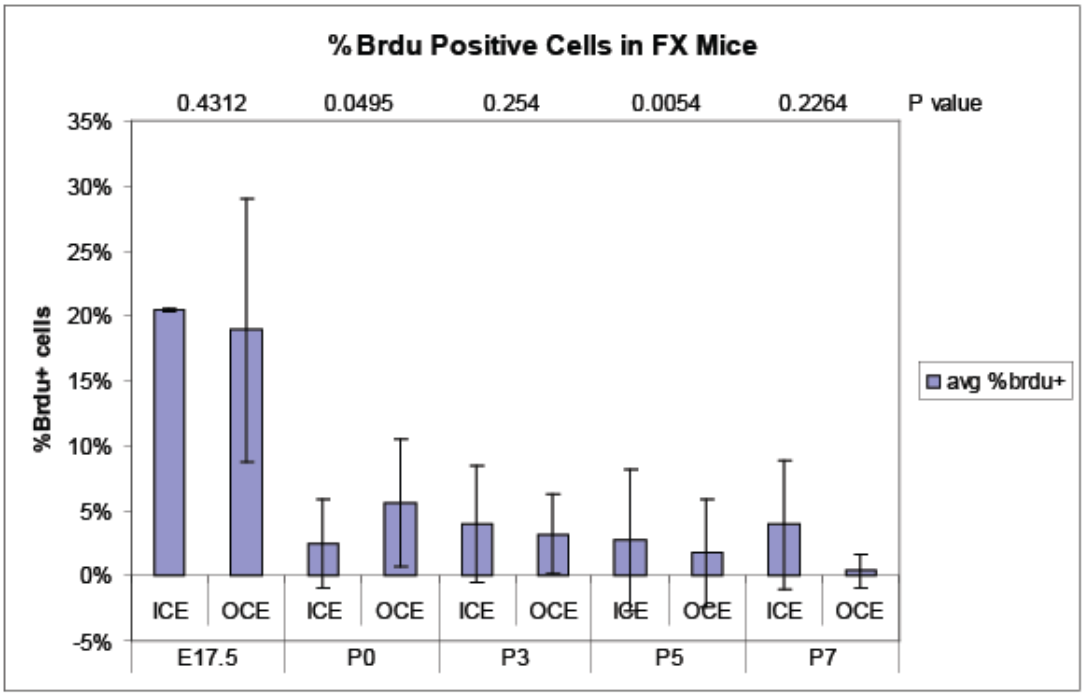
Supplemental Figure 5.3. ICE versus OCE Proliferation in Wild-type and DM61/Notch2$^{fx/fx}$ Mice

(A) In the wild-type ciliary body, the average percentage of BrdU + cells is higher in the OCE than the ICE during development. This differential rate in proliferation may contribute to the folding process. **(B)** In DM61/Notch2$^{fx/fx}$ mice, the significant decrease in the OCE's rate of proliferation is no longer any different than the ICE's rate.

A


%Brdu Positive Cells in WT Mice
0.1601
8.88E-07
1.06E-07
0.0026
0.4165
P value
30%
25%
20%
15%
10%
5%
0%
%Brdu+ cells
ICE
OCE
E17.5
P0
P3
P5
P7
avg %brdu+

B


%Brdu Positive Cells in FX Mice
0.4312
0.0495
0.254
0.0054
0.2264
P value
35%
30%
25%
20%
15%
10%
5%
0%
-5%
%Brdu+ cells
ICE
OCE
E17.5
P0
P3
P5
P7
avg %brdu+

Supplemental Figure 5.4. Heat-Map for Notch and BMP Signaling Pathways

Heat map projection of genes in the Notch and BMP signaling pathways showing little transcriptional change within the pathways. Notably, Hey1 is downregulated 2.5-fold and ID2 is downregulated 1.6-fold. Hey1 and ID2 genes are canonical Notch and BMP targets, respectively, and act as positive controls for this data set. Done in conjunction with Madeleine Gogol of Stowers Bioinformatics.

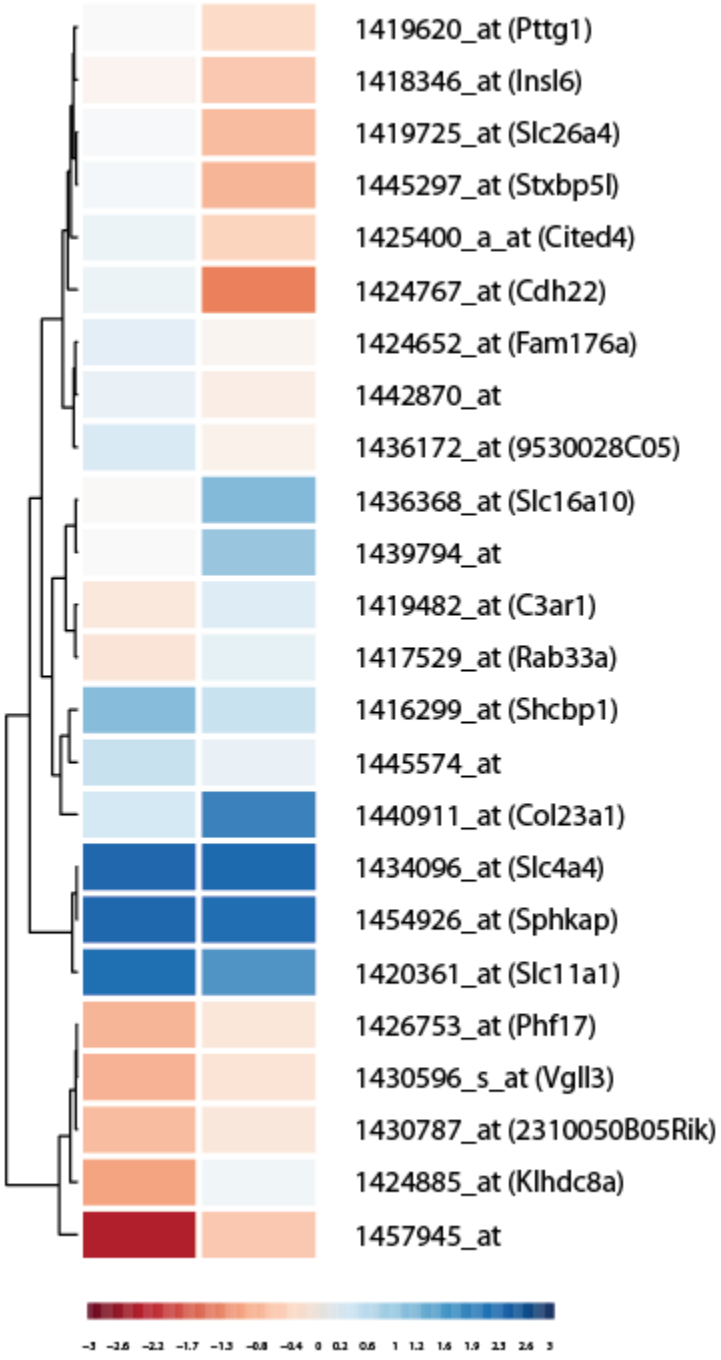
Supplemental Table 5.1. GO Enrichment Terms

GO Enrichment analysis with FDR 0.10 as the threshold. GO Terms listed below are those which came back with a higher count of significant genes than one would expect. Done in conjunction with Hua Li of Stowers Bioinformatics.

GOBPID	ExpCount	Count	Term
GO:0000279	7	40	M phase
GO:0022403	8	42	cell cycle phase
GO:0007049	17	61	cell cycle
GO:0000280	5	32	nuclear division
GO:0007067	5	32	mitosis
GO:0022402	9	44	cell cycle process
GO:0000087	5	32	M phase of mitotic cell cycle
GO:0048285	5	32	organelle fission
GO:0051301	6	36	cell division
GO:0000278	6	36	mitotic cell cycle
GO:0007059	1	12	chromosome segregation
GO:0000070	0	7	mitotic sister chromatid segregation
GO:0000819	0	7	sister chromatid segregation
GO:0006996	26	54	organelle organization
GO:0016043	47	79	cellular component organization
GO:0006260	4	15	DNA replication
GO:0006259	9	24	DNA metabolic process
GO:0030261	0	5	chromosome condensation
GO:0000226	2	10	microtubule cytoskeleton organization
GO:0007091	0	4	mitotic metaphase/anaphase transition
GO:0007076	0	4	mitotic chromosome condensation
GO:0007017	4	13	microtubule-based process
GO:0001570	1	6	vasculogenesis
GO:0007051	0	4	spindle organization
GO:0009186	0	2	deoxyribonucleoside diphosphate metabolic proce

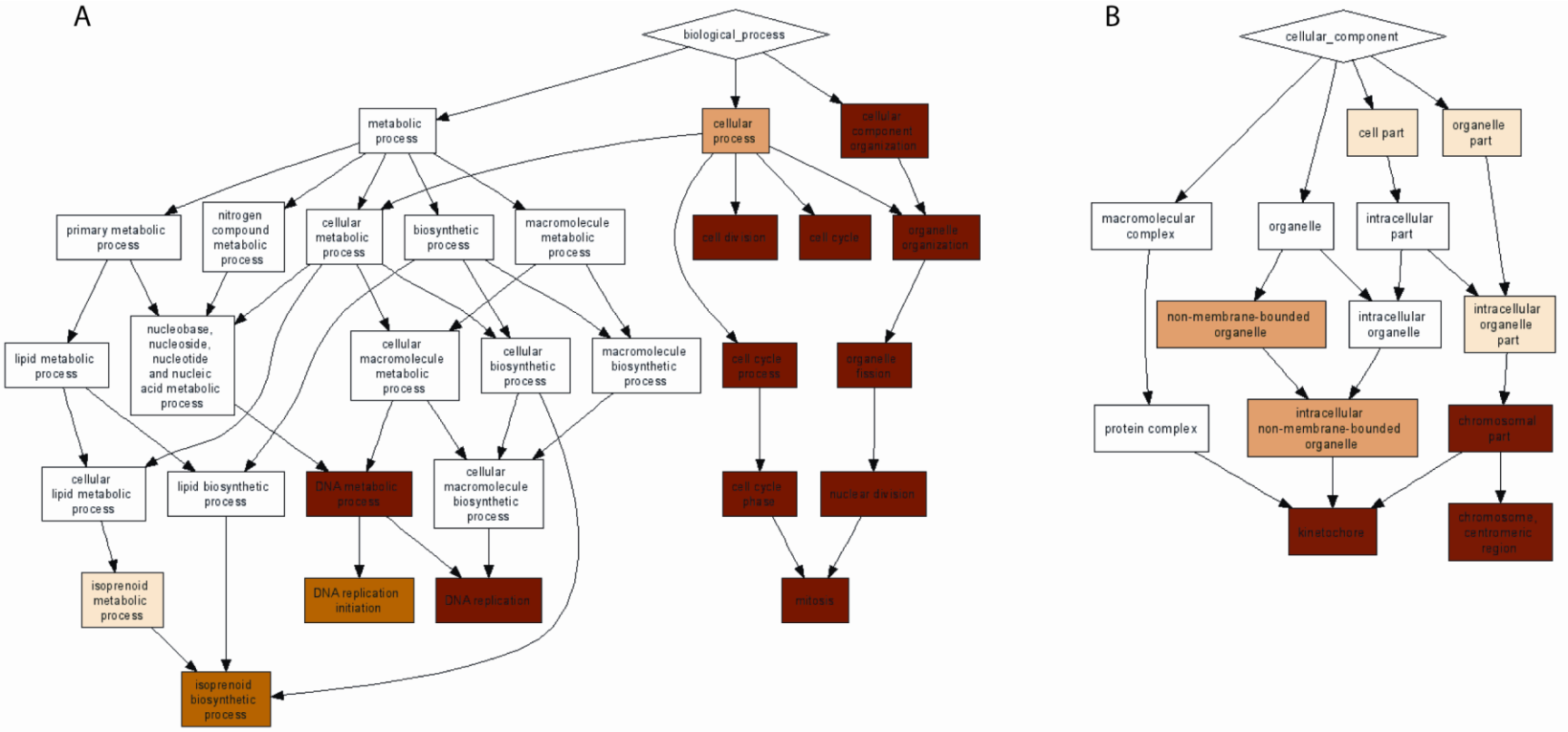
Supplemental Figure 5.5. Clustered Heat-Map

Heat map representation of microarray genes filtered with FDR .05 and a 2-fold change criteria. Brackets connect genes with similar functions. Done in conjunction with Madeleine Gogol of Stowers Bioinformatics.


1419620_at (Pttg1)
1418346_at (Insl6)
1419725_at (Slc26a4)
1445297_at (Stxbp5l)
1425400_a_at (Cited4)
1424767_at (Cdh22)
1424652_at (Fam176a)
1442870_at
1436172_at (9530028C05)
1436368_at (Slc16a10)
1439794_at
1419482_at (C3ar1)
1417529_at (Rab33a)
1416299_at (Shcbp1)
1445574_at
1440911_at (Col23a1)
1434096_at (Slc4a4)
1454926_at (Sphkap)
1420361_at (Slc11a1)
1426753_at (Phf17)
1430596_s_at (Vgll3)
1430787_at (2310050B05Rik)
1424885_at (Klhdc8a)
1457945_at
−3 −2.6 −2.2 −1.7 −1.3 −0.8 −0.4 0 0.2 0.6 1 1.2 1.6 1.9 2.3 2.6 3

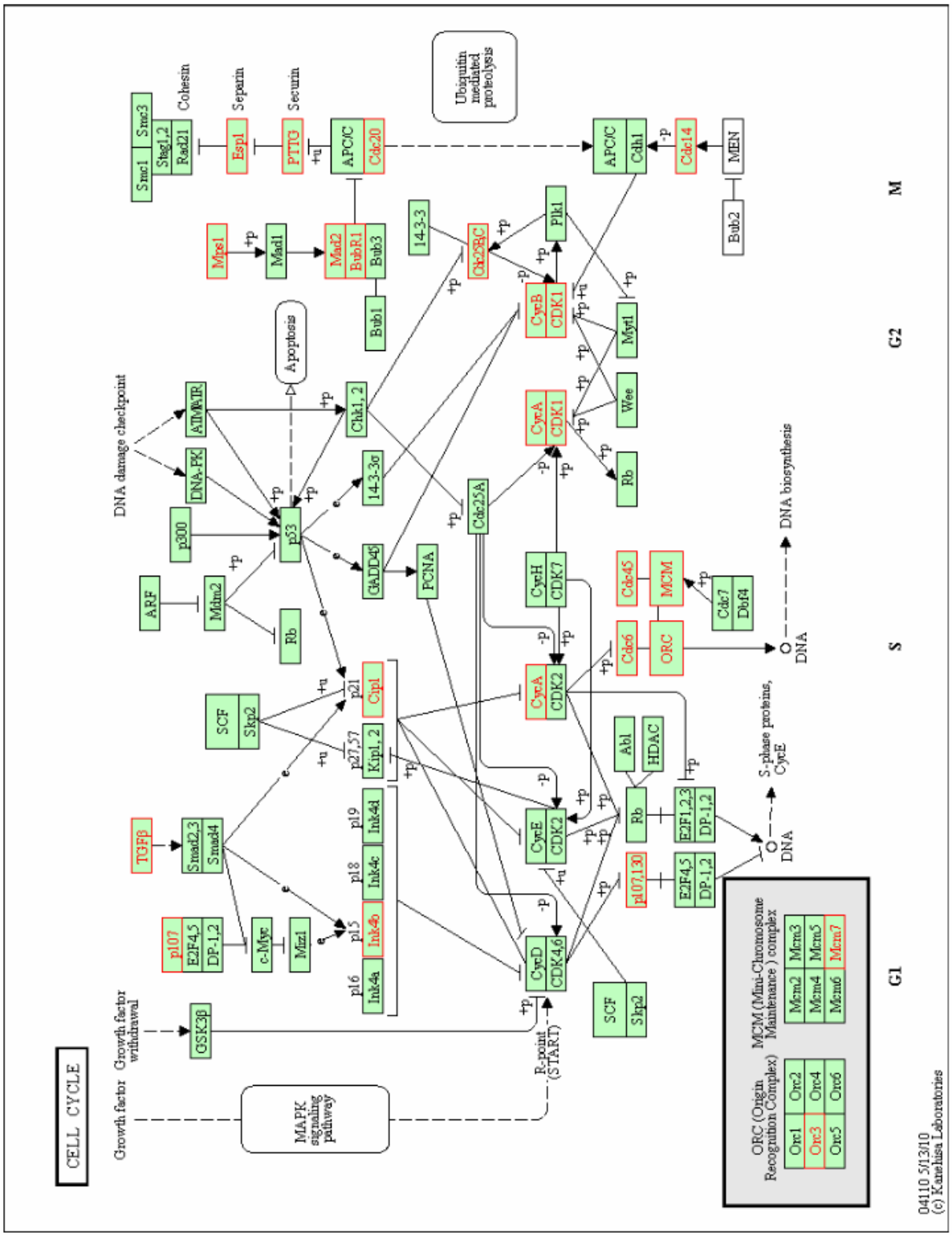
Supplemental Figure 5.6. GOrilla Plots

(A) GOrilla Plot showing cellular processes which as an aggregate of related genes came back statistically significant. More significant processes including cell cycle and DNA replication are a darker shade. **(B)** GOrilla Plot showing cellular components which as an aggregate of related genes came back statistically significant. More significant processes including chromosome and DNA structure components are a darker shade. Done in conjunction with Madeleine Gogol of Stowers Bioinformatics.


A
biological_process
metabolic process
cellular process
cellular component organization
primary metabolic process
nitrogen compound metabolic process
cellular metabolic process
biosynthetic process
macromolecule metabolic process
cell division
cell cycle
organelle organization
lipid metabolic process
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
cellular macromolecule metabolic process
cellular biosynthetic process
macromolecule biosynthetic process
cell cycle process
organelle fission
cellular lipid metabolic process
lipid biosynthetic process
DNA metabolic process
cellular macromolecule biosynthetic process
cell cycle phase
nuclear division
isoprenoid metabolic process
DNA replication initiation
DNA replication
mitosis
isoprenoid biosynthetic process
B
cellular_component
cell part
organelle part
macromolecular complex
organelle
intracellular part
non-membrane-bounded organelle
intracellular organelle
intracellular organelle part
protein complex
intracellular non-membrane-bounded organelle
chromosomal part
kinetochore
chromosome, centromeric region

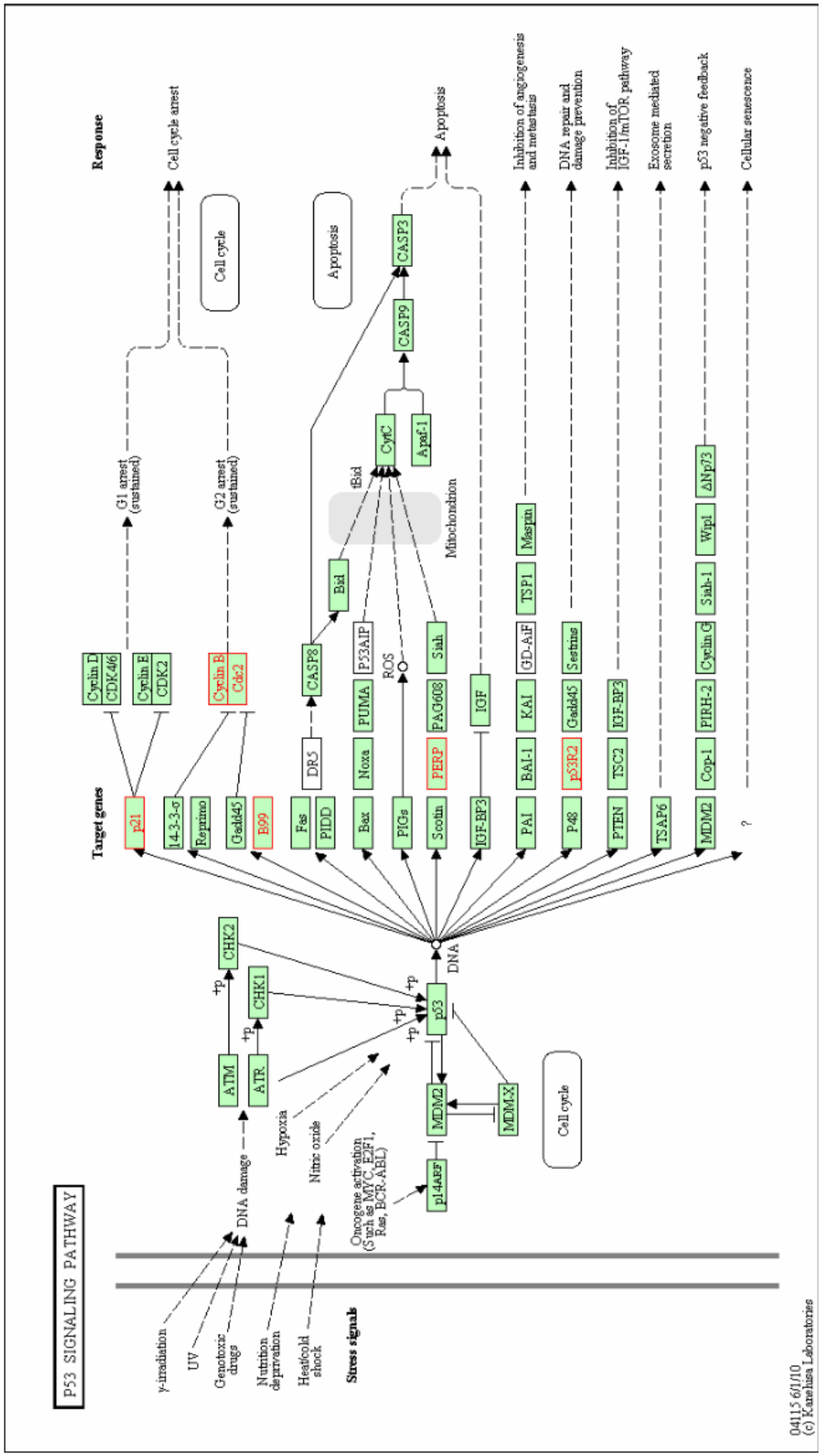
Supplemental Figure 5.7. Kegg Pathway—Cell Cycle

Diagram showing the Kegg Pathway related to the Cell Cycle extracted from our microarray. A higher number of genes (in red) came back statistically significant related to this pathway than expected. Done in conjunction with Hua Li of Stowers Bioinformatics.


CELL CYCLE
Growth factor
Growth factor withdrawal
MAPK signaling pathway
GSK3β
R-point (START)
G1
S
G2
M
DNA damage checkpoint
ATM/ATR
DNA-PK
p300
p53
Chk1, 2
14-3-3σ
GADD45
PCNA
Cdc25A
Apoptosis
ARF
Mdm2
Rb
SCF
Skp2
p21
Cip1
p27,57
Kip1, 2
TGFβ
Smad2,3
Smad4
p107
E2F4,5
DP-1,2
c-Myc
Mxi1
p15
Ink4b
p16
Ink4a
p18
Ink4c
p19
Ink4d
CycD
CDK4,6
CycE
CDK2
CycA
CDK2
CycH
CDK7
CycA
CDK1
CycB
CDK1
Wee
Myt1
Plk1
Chk2/Rad
14-3-3
Mps1
Mad1
Mad2
BubR1
Bub3
Bub1
Bub2
MEN
Cdc14
APC/C
Cdh1
APC/C
Cdc20
PTTG
Securin
Esp1
Separin
Smc1
Smc3
Stag1,2
Rad21
Cohesin
Ubiquitin mediated proteolysis
Abl
HDAC
E2F1,2,3
DP-1,2
E2F4,5
DP-1,2
p107,130
Cdc6
ORC
Cdc45
MCM
Cdc7
Dbf4
S-phase proteins, CycE
DNA
DNA
DNA biosynthesis
ORC (Origin Recognition Complex)
Orc1
Orc2
Orc3
Orc4
Orc5
Orc6
MCM (Mini-Chromosome Maintenance) complex
Mcm2
Mcm3
Mcm4
Mcm5
Mcm6
Mcm7
04110 5/13/10
(c) Kanehisa Laboratories

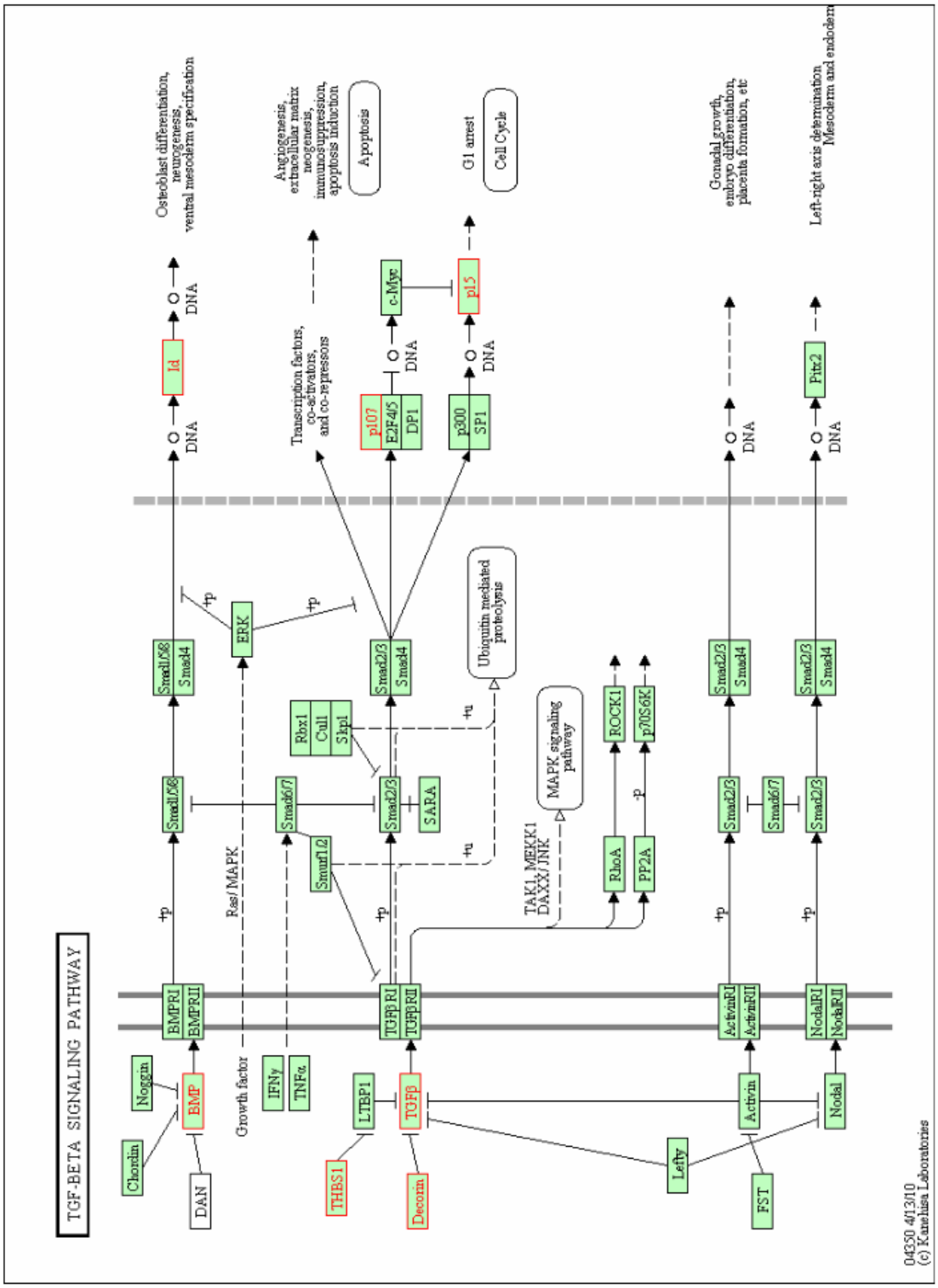
Supplemental Figure 5.8. Kegg Pathway—p53 Signaling Pathway

Diagram showing the Kegg Pathway related to the p53 signaling pathway extracted from our microarray. A higher number of genes (in red) came back statistically significant related to this pathway than expected. Done in conjunction with Hua Li of Stowers Bioinformatics.


P53 SIGNALING PATHWAY
Response
Cell cycle arrest
G1 arrest (sustained)
G2 arrest (sustained)
Cell cycle
Apoptosis
Target genes
p21
14-3-3-σ
Reprimo
Gadd45
B99
Fas
PIDD
Bax
PIGs
Scotin
IGF-BP3
DR5
Noxa
PUMA
PERP
PAG608
IGF
CASP8
P53AIP
ROS
Siah
Bid
tBid
Mitochondrion
CytC
Apaf-1
CASP9
CASP3
Apoptosis
Cyclin D
CDK4/6
Cyclin E
CDK2
Cyclin B
Cdc2
PAI
BAI-1
KAI
GD-AIF
TSP1
Maspin
Inhibition of angiogenesis and metastasis
p48
p53R2
Gadd45
Sestrins
DNA repair and damage prevention
PTEN
TSC2
IGF-BP3
Inhibition of IGF-1/mTOR pathway
TSAP6
Exosome mediated secretion
MDM2
Cop-1
PIRH-2
Cyclin G
Siah-1
Wip1
ΔNp73
p53 negative feedback
?
Cellular senescence
DNA
p53
MDM2
MDM-X
p14ARF
ATM
ATR
CHK2
CHK1
+p
DNA damage
Hypoxia
Nitric oxide
Oncogene activation (Such as MYC, E2F1, Ras, BCR-ABL)
Cell cycle
Stress signals
γ-irradiation
UV
Genotoxic drugs
Nutrition deprivation
Heat/cold shock
04115 6/1/10
(c) Kanehisa Laboratories

Supplemental Figure 5.9. Kegg Pathway—TGFβ Signaling Pathway

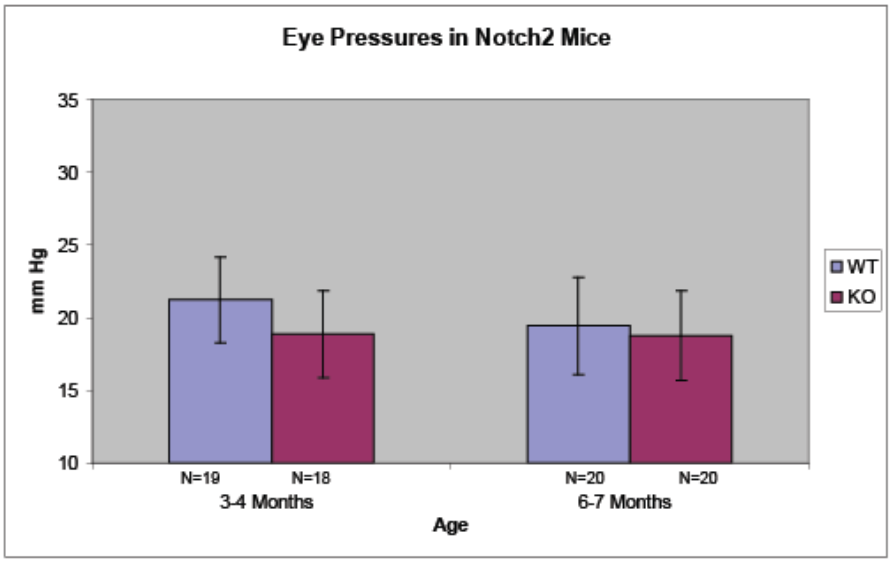
Diagram showing the Kegg Pathway related to the TGFβ signaling pathway extracted from our microarray. A higher number of genes (in red) came back statistically significant related to this pathway than expected. Done in conjunction with Hua Li of Stowers Bioinformatics.


TGF-BETA SIGNALING PATHWAY
Noggin
Chordin
BMP
DAN
BMPRI
BMPRII
Smad1/5/8
Smad4
Id
DNA
Osteoblast differentiation, neurogenesis, ventral mesoderm specification
Growth factor
Ras/MAPK
ERK
IFNγ
TNFα
Smad6/7
Smurf1/2
LTBP1
THBS1
TGFβ
Decorin
TGFβRI
TGFβRII
Smad2/3
Smad4
SARA
Rbx1
Cul1
Skp1
Ubiquitin mediated proteolysis
TAK1, MEKK1
DAXX/ JNK
MAPK signaling pathway
RhoA
PP2A
ROCK1
p70S6K
Transcription factors, co-activators, and co-repressors
Angiogenesis, extracellular matrix neogenesis, immunosuppression, apoptosis induction
Apoptosis
p107
E2F4/5
DP1
c-Myc
p300
SP1
p15
G1 arrest
Cell Cycle
Lefty
Activin
FST
ActivinRI
ActivinRII
Nodal
NodalRI
NodalRII
Gonadal growth, embryo differentiation, placenta formation, etc
Left-right axis determination, Mesoderm and endoderm
Pitx2
04350 4/13/10
(c) Kanehisa Laboratories

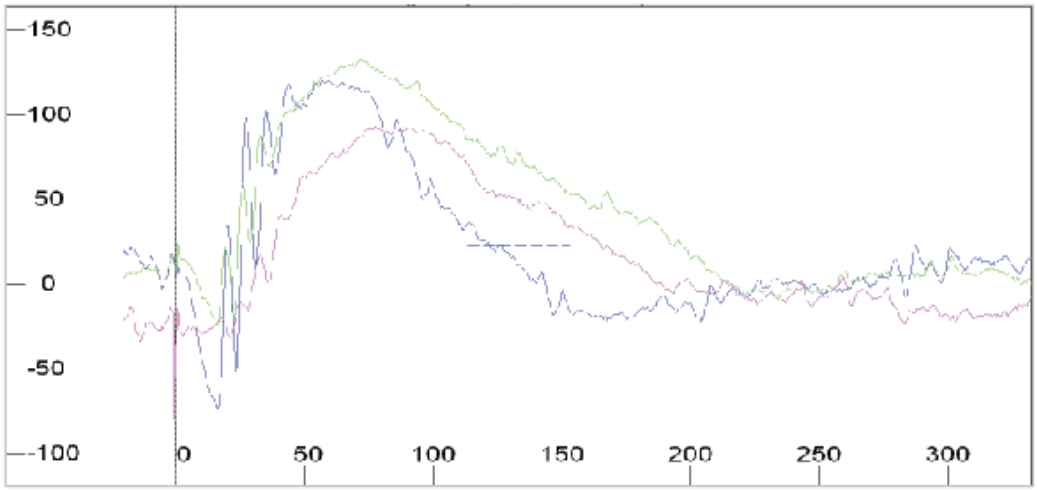
Supplemental Figure 5.9. Functional Analysis of DM61/Notch2$^{fx/fx}$ Mice

(A) Intraocular pressure measurements in DM61/Notch2$^{fx/fx}$ mice. We found no decrease in intraocular pressure in DM61/Notch2$^{fx/fx}$ either at 3-4 months or 6-7 months of age. **(B)** ERG measurements of adult DM61/Notch2$^{fx/fx}$ mice showed no alterations in visual response to light. Black and pink lines are representative wild type measurements. Green line is a representative mutant. Measurements shown were averages taken of 4 flashes 10 seconds apart at 1000 mcd.s/m^2 as part of the Scotopic 0 Series of the HMS-Erg.

A


Eye Pressures in Notch2 Mice
35
30
25
20
15
10
mm Hg
N=19
N=18
N=20
N=20
3-4 Months
6-7 Months
Age
WT
KO

B


150
100
50
0
-50
-100
0
50
100
150
200
250
300

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