# THE ROLE OF AP-2α AND AP-2β IN RETINAL DEVELOPMENT

# THE ROLE OF AP-2α AND AP-2β IN HORIZONTAL CELL DEVELOPMENT AND AMACRINE CELL PATTERNING

By

MIZNA ZAVERI, B.SC. (HONOURS)

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AUTHOR: Mizna Zaveri, B.Sc. (Honours) (McMaster University)

SUPERVISOR: Dr. Judith A. West-Mays

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## ABSTRACT

Previous studies from our lab have shown that the Activating Protein- 2 (AP-2) transcription factors, *AP-2α* and *AP-2β,* are important in retinal development. It was discovered that these are co-expressed in developing horizontal cells and postmitotic amacrine cells. To understand their role in retinogenesis, and the impact of their deletion on the adult retina, a double mutant mouse model was created, AP-2$\alpha^{KI/flox}$/AP-2$\beta^{-/flox}$. The neural retina of the AP-2$\alpha^{KI/flox}$/AP-2$\beta^{-/flox}$ mice was examined in the current study using histological, immunofluorescent and electron microscopy (EM) techniques at embryonic, post-natal and adult stages. These double mutants displayed a variety of abnormalities in the inner retina. Loss of AP-2α and AP-2β at E10.5 led to a complete absence of developing and mature horizontal cells. This loss was associated with changes in the outer plexiform layer, which diminished from two to four months of age. There were also defects with photoreceptor ribbons in which triad synapses failed to form, and instead led to rudimentary, spherical-shaped ribbons. There was also significant retraction of photoreceptor axons. Furthermore, this study was able to infer a role of AP-2α and AP-2β as acting upstream of the Onecut-1 protein, which targets Lim1 and Prox1 to direct horizontal cell genesis. Examining amacrine cells of the double mutants shows evidence that AP-2α and AP-2β are involved in the mosaic arrangement pattern of amacrine cell bodies and axons. Previous work on embryonic double mutants displayed clustering of amacrine cells. This study observed abnormalities in the dendrites of the inner plexiform layer, which consists of amacrine cell processes. Taken together, the work presented in

this thesis implicates the redundant requirement of both AP-2α and AP-2β in development of horizontal cells and patterning of amacrine cells in the neural retina.

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| | |
|---|---|
| AC | Amacrine Cell |
| AP-2 | Activating Protein - 2 |
| BC | Bipolar Cell |
| bHLH | basic Helix-loop-Helix |
| bp | base pair |
| BOFS | Branchio-Oculo-Facial Syndrome |
| CNS | Central Nervous System |
| CNTF | Ciliary Neurotrophic Factor |
| DAPI | Diamino-2-phenylindole |
| DNA | Deoxyribonucleic Acid |
| Dscam | Down syndrome cell adhesion molecule |
| E | Embryonic Day |
| EGF | Epidermal Growth Factor |
| ERG | Electroretinogram |
| GC | Ganglion Cell |
| GCL | Ganglion Cell Layer |
| H&E | Hematoxylin & Eosin |
| HC | Horizontal Cell |
| INL | Inner Nuclear Layer |
| IPL | Inner Plexiform Layer |
| KO | Knockout |
| NGF | Nerve Growth Factor |
| NR | Neural Retina |
| Oc-1 | Onecut-1 |
| ONL | Outer Nuclear Layer |
| OPL | Outer Plexiform Layer |
| OV | Optic Vesicle |
| P | Post-natal Day |
| PCR | Polymerase Chain Reaction |
| PGK | Phosphoglycerokinase |
| PR | Photoreceptor |
| RPC | Retinal Progenitor Cell |
| RPE | Retinal Pigmented Epithelium |
| Shh | Sonic hedgehog |
| TEM | Transmission Electron Microscopy |
| TF | Transcription Factor |
| TGFα | Transforming Growth Factor α |

# CHAPTER ONE

General Introduction

### 1. 1 Eye Development in Vertebrates

The eye is a highly specialized organ in vertebrates and develops from various interactions that occur in the neural tube. These interactions allow cranial ectodermal placodes to arise, and eventually form into neurons and sensory epithelia (Gilbert, 2006). Beginning at gastrulation, the neuroepithelium comes into contact with the surface ectoderm which causes the induction of the optic field (Fig. 1). The neuroepithelium then thickens and protrudes outward to form the optic vesicles, which then invaginate inward to form the optic cup (Fig. 1) (Chow and Lang, 2001). The surface ectoderm overlying the developing optic vesicle forms the lens placode. This lens placode then invaginates inward to form the lens pit (Chow and Lang, 2001; Gilbert, 2006). The optic cup differentiates into the inner and outer retina, which give rise to the neural retina (NR) and the retinal pigmented epithelium (RPE) respectively while the lens placode pinches off to form a lens vesicle (Chow and Lang, 2001). This lens vesicle forms a transparent lens that allows light to enter the retina. The cornea begins to form from the overlying surface ectoderm (Hilfer and Yang, 1980; Chow and Lang, 2001; Gilbert, 2006).

### 1.2 The Vertebrate Retina

Although the NR and RPE arise from cells of the anterior neuroepithelium, they are structurally and functionally different. The RPE is a multifunctional monolayer of non-neuronal, cuboidal melanosome-containing cells. It is required for maintenance of the blood-retinal barrier, the water and ion flow between the NR and the choroid, and absorption of light, protection against free radicals, and control of retinoid metabolism (Bok, 1993). On the other hand, the NR consists of neurons and photoreceptors required

for vision. In mice, neural retinogenesis commences at embryonic day (E)10.5 and ends at post-natal day (P)11. During these three weeks, multipotent retinal progenitor cells (RPCs) give rise to five classes of neurons (Levine *et al*., 2004). These cells emerge in a timely and ordered mode, beginning with the genesis of ganglion cells (GC), followed by horizontal cells (HC), cone photoreceptors (PR), amacrine cells (AC), rod photoreceptors, bipolar cells (BC) and finally the non-neuronal Muller glia cells (Fig. 2) (Marquardt, 2003; Zhang *et al*., 2011). It is interesting to note that there is some overlap in the genesis of the retinal cell types. Even though all these cells initially emerge from the outer surface of the retina, they migrate to occupy various fixed positions within the retina. Cells that are born at the same time, such as horizontal and amacrine cells, can possess the ability to reside in different layers (Wong and Godinho, 2004). Cell migration has been found to occur radially for rods, bipolar and Muller glia cells, while cones, horizontal and amacrine cells migrate tangentially to reach their final positions (Wong and Godinho, 2004).

Retinal cell fate and differentiation from the multipotent RPCs depends on various inhibitory and excitatory factors including TGFα (transforming growth factor), EGF (epidermal growth factor), NGF (nerve growth factor), CNTF (ciliary neurotrophic factor), as well as various basic-Helix-Loop-Helix (bHLH) transcription factors (TFs), which are co-expressed in the multipotent RPCs. These TFs include *Foxn4*, *Pax6*, *Six3*, *Rx1*, *Chx10*, *Hes1* and Helix-span-Helix TFs such as *AP-2α* (West-Mays *et al*., 1999; Marquardt, 2003; Li and Dashwood, 2004; Li *et al*., 2004, Bassett *et al*., 2007). Throughout retinal development, some cell lineages continue the expression of these

factors while others are down-regulated. Various combinations of TFs are then responsible for determining the identity of each retinal cell type (Marquardt, 2003).

### 1.2.1 Horizontal Cell Development

Retinal horizontal cells are interneurons that lie within the inner nuclear layer (INL). They are responsible for processing visual information laterally by modulating visual signals between photoreceptors and bipolar cells (Cervetto and MacNichol, 1972; Wu, 1992). Like the other retinal neuronal population, HCs arise from a combination of transcription factors that regulate the expression of surface receptors or signal transduction cascades that allow retinal progenitor cells to respond to extrinsic cues that determine cell fate and differentiation. Extensive studies have been conducted to identify the transcriptional controllers of HC development. Foxn4 is responsible for initiating HC and AC differentiation and activating downstream genes required for the birth of these cell types (Xiang and Li, 2013). Loss of Foxn4 leads to an elimination of both AC and HCs, while causing an excess number of PRs to develop (Li *et al*., 2004). Foxn4 specifies both AC and HC cell fate by directly regulating the expression of downstream genes such as *Ptf1a, Neurod1, Neurod4* and *Lhx1*, which are essential for amacrine and horizontal cell genesis (Xiang and Li, 2013). A recent study has identified another essential transcriptional regulator of horizontal cell development known as Onecut-1 (Oc-1) (Wu *et al*., 2013). The expression of Oc-1 is downregulated in *Foxn4*$^{-/-}$ retinas but remains the same in *Ptf1a*$^{-/-}$ mutants (Wu *et al*., 2013). Loss of Oc-1 in the murine retina results in a complete loss of horizontal cells, which indicates that Foxn4 functions upstream of Oc-1,

and that Ptf1a and Oc-1 work in parallel to specify horizontal cell genesis (Wu *et al*., 2013). Ptf1a expressing precursor cells that also express Prox1 allow these cells to be directed towards a HC fate, while those Ptf1a+ cells expressing Math3 and NeuroD are directed towards an AC fate (Poche and Reese, 2013). This is supported by the loss of amacrine cells in a *Math3/NeuroD* double knockout (KO) and horizontal cells remain intact (Inoue *et al*., 2002). A *Ptf1*a null model on the other hand shows a complete loss of HCs and most ACs (Fujitani *et al*., 2006), while a *Prox1* deletion results in a complete loss of HCs without affecting AC genesis (Dyer *et al*., 2003). Therefore, Foxn4 allows RPCs to differentiate into either HCs or ACs, while the expression of Prox1 directs these cells to commit to a HC fate. Nevertheless, the pathway to HC fate from the retinal progenitor pool has not been completely characterized (Fig. 3).

### 1.2.2 Amacrine Cell Development

Amacrine cell genesis relies on many of the similar transcription factors as those required for horizontal cell differentiation. Foxn4 expression in a retinal progenitor cell, followed by Ptf1a, NeuroD and Math3 are required for the path towards AC birth. As previously mentioned, loss of Foxn4 results in ablation of both ACs and HCs, and causes a downregulation in the expression of Math3 and NeuroD (Fig. 3) (Li *et al*.,2004). The latter two are necessary for amacrine cell differentiation, however they are not sufficient since misexpression of either gene does not induce the birth of amacrine cells (Inoue *et al*., 2002). Aside from Foxn4, a key player in amacrine cell fate determination is the homebox gene, *Sox2*. It has been shown to promote the activation of another homebox

transcription factor, Pax6 and the bHLH gene *NeuroD* (Lin Ouchi Sato 2009). Pax6 has been shown to be important in determining amacrine cell fate since Pax6-deficient RPCs become restricted to AC cell fate alone (Marquardt*et al*., 2001). However misexpression of the gene in an RPC does not contribute to the birth of these neurons until the misexpression is combined with a bHLH gene such as *Math3* or *NeuroD* (Inoue, 2002). Interestingly, misexpression of Pax6 with Math3 leads to the production of amacrine and horizontal cells, while Pax6 with NeuroD leads solely to the production of amacrine cells (Inoue *et al*., 2002). Therefore, it is suggested that NeuroD contributes more specifically to AC differentiation than Math3.

Upon differentiation, these neurons migrate to their final position, and respond to environmental cues for organizing their cell bodies in a non-random pattern in the retina. Regular patterning of retinal nerve cells is essential for visual processing. These retinal mosaics allow visual images to be processed uniformly across the various neurons. Amacrine cell mosaic is particularly important for extracting visual data related to direction of motion of the ON and OFF visual pathways (Reese 2007; Whitney *et al*. 2008).

Retinal mosaic patterning is not well understood, particularly the arrangement of amacrine cells in both the inner nuclear layer (INL) and ganglion cell layer (GCL). All retinal neurons establish a specific pattern of distribution of their soma to minimize distance to homotypic cells, while their respective dendrites are distributed to evenly distribute over the retinal surface. Studies have shown that the absence of the Down Syndrome cell adhesion molecules (Dscam) causes clustering of somas of melanopsin-

positive RGCs and dopaminergic ACs, and fasciculations of their retinal processes (Fuerst *et al*., 2008, 2009). Not only is there a decrease in intercellular spacing amongst the cells (Keeley *et al*., 2012), the mutation also causes an increase in the number of these two cell types (Fuerst *et al*., 2008, 2009). It is believed that the presence of Dscam prevents cell-cell interactions that mediate adhesion, which subsequently allows dendrites to freely extend amongst other homotypic dendrites (Keeley *et al*., 2012).

Cell adhesion molecules, such as Dscam, are regulated by a variety of transcription factors. For example, the TF Pax6 was shown to control the expression of members of the cadherins family such as the neural adhesion molecule L1 (Chalepakis *et al*., 1994) while the TF AP-2α has been shown to control the expression of E-cadherin (Behrens *et al*., 1991; Pontoriero *et al*., 2008). However, the regulators of cell adhesion molecules remain largely unknown, and their discovery would lead to a greater insight in the development controls that are involved in retinal mosaic patterning. Discovery of these regulators will not only allow us to understand the pathways required for neuronal commitment to their specific fate, but also identify the genes required for dictating synaptic interactions to understand the complex network of retinal connectivity further.

### 1. 3 Activating Protein-2 and Eye Development

The Activating Protein-2 (AP-2) family consists of a class of five different transcription factors: AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε, which form homo- or hetero- dimers. AP-2 proteins are expressed in humans and mice, while orthologs have

been found in frogs and fish. They bind to target sequences and regulate the transcription of target genes (Eckert *et al*., 2005). These proteins have a basic DNA-binding domain, coded by exons 4-7 that binds to the palindromic consensus sequence 5'-GCCNNNGGC-3', followed by a characteristic helix-span-helix motif that facilitates dimerization and DNA binding (Eckert *et al*., 2005). AP-2 proteins are initially expressed in the primitive ectoderm of invertebrates and vertebrates, and their expression is also seen in the emerging neural crest in vertebrates. Their general function has been elucidated to be cell-type-specific stimulation and proliferation during embryonic development. Studies have shown that these proteins are involved in regulating embryonic expression in tissues such as: neural crest derivatives, neural, epidermal and urogenital tissues (Hilger-Eversheim *et al*., 2000).

The AP-2α family member is expressed in numerous tissues such as the lens and retina throughout embryonic development (Pontoriero *et al*., 2000; Bassett *et al*., 2007, 2010, 2012). *AP-2α* germ-line KO mice have severe developmental defects, including craniofacial and ocular abnormalities. Due to the death of migrating neural crest cells, the cranial neural tube fails to close, leading to a craniofacial cleft. The abdomen also fails to close, resulting in exposed organs. Anomalies have also been observed in facial structures such as the jaw and chin (Hilger-Eversheim *et al*., 2000). Our lab has shown that the *AP-2α* germ-line KO mice exhibit a variety of ocular mutant phenotypes, including absent cornea and eyelids, failed or defective lens induction, and abnormalities in the optic cup (presumptive retina) (West-Mays *et al*., 1999). In these mice, the RPE was absent on the dorsal aspect of the optic cup, and replaced by a cell layer similar to the inner NR. Also,

distinct ganglion cell and inner plexiform layers were not present. Similar optic cup defects were also observed in *AP-2α* chimeric mice generated from a combination of *AP-2α* $^{-/-}$ and *AP-2α* $^{+/+}$ cells (West-Mays *et al*., 1999). However, the *AP-2α* germ-line KO mice die prenatally from severe developmental defects (Hilger-Eversheim *et al*., 2000) and thus ocular development could not be examined beyond birth. Therefore, conditional KOs, in which *AP- 2α* is deleted from specific tissues were required to further investigate its developmental role. For example, the *Le-AP-2α* mice were created and utilized in our lab by employing Cre-loxP technology, in which the *AP-2α* gene was deleted from the developing lens placode (Pontoriero *et al*., 2008). These mice displayed defects in tissues derived from the placode, including the cornea, lens and eyelid; abnormalities similarly observed in the *AP-2α* germ-line KOs; demonstrating a cell autonomous role for *AP-2α* in the lens-placode derived tissues (Pontoriero *et al*., 2008).

Interestingly, recent clinical data has shown that mutations in the human *AP-2α* gene, *TFAP2A* result in craniofacial and eye defects (Milunsky *et al*., 2008), further demonstrating the importance of studying this protein as a model for human ocular diseases. The condition is known as Branchio-Oculo-Facial Syndrome (BOFS), which is a dominantly inherited birth defect in which there are a range of craniofacial malformations including skull deformity, cleft lip and a malformed nasal tip (Fujimoto *et al*., 1987; Milunsky *et al*., 2008). BOFS patients have eye defects including those observed in *AP-2α* KO mice such as anophthalmia, microphthalmia, primary aphakia (absence of the lens), coloboma of the iris, retina or optic nerve and eyelid (Milunsky *et al*., 2008; Stoetzl *et al*., 2009; Tekin *et al*., 2009; Gestri *et al*., 2009). BOFS is caused by

missense mutations in the AP-2α protein that alter amino acids in the DNA binding domain.

To examine whether *AP-2α* also has a cell autonomous role in the developing retina, our lab once again employed Cre-loxP technology to conditionally delete AP-2α from the presumptive retina; named the Ret-AP-2 mice (Bassett *et al*., 2007). Detailed examination of these conditional KO mice revealed no detectable abnormalities in the neural retina (Bassett *et al*., 2007). A possible explanation proposed for this finding was that a compensatory role was being played by another member of the AP-2 family, AP-2β (Bassett *et al*,, 2012). In order to analyze a potential compensatory role, expression of AP-2β and other members was carried out (Bassett *et al*., 2007). Embryonic expression of AP-2β was found to be in a similar manner of AP-2α, with AP-2β being localized in the INL, while expression of the protein was observed in the INL and a subset of cells in the GCL in the developing and adult retina (Fig. 4). AP-2γ was observed in both the INL and the GCL, while AP-2δ transcripts were found in the GCL only (Bassett *et al*., 2007*). AP-2β* germ-line KO mice have normal neural crest cells, cranial structures and neural tissues, even though these tissues normally show high expression of AP-2β (Moser *et al*., 1997). Thus, a compensatory mechanism may be in place due to the presence of normal AP-2α. Anomalies in the kidneys of these mice lead to polycystic kidney disease, resulting in lethality at E16.5 (Moser *et al*., 1997). In humans, *AP-2β* mutations have been found in Char syndrome, an autosomal dominant disease characterized by facial and hand disfiguration and in some cases teeth, feet and eyes (Moser *et al*., 1995; Satoda *et al*., 2000; Slavotinek *et al*., 1997; Zhao *et al*., 2001).

It is interesting to note that there is a high degree of conservation in the intron-exon structure, and the protein sequences between AP-2α and AP-2β, implying a common ancestor (Moser *et al*., 1995). At the amino acid level, the two have a 92% similarity in the DNA binding and dimerization regions. AP-2α and AP-2β bind to each other *in vitro,* and to identical sites on a given promoter, and are able to regulate transcription of the same genes (Zhu *et al*., 2001). Also, both AP-2α and AP-2β are able to bind to one another creating a heterodimer that binds to the promoter for the proto-oncogene c-erbB-2 (Bosher *et al*., 1996). These factors together, with the observation that AP-2β expression was higher in *AP-2α* deleted regions, led to the hypothesis that AP-2β may be compensating for the loss of AP-2α (Bassett *et al*., 2010). Thus, the study of double *AP-2α/AP-2β* germ-line KO mice became important for our lab. These mice have much earlier and more severe eye defects (defective lens placode and optic cup defects) than single mutants (Bassett *et al*., 2007). This provides support for redundancy in the roles these two genes play in development. Unfortunately, these mutants die by E12.5, *before* retinal development can be studied. Thus, a mutant model in which Ret-AP-2α mutants were crossed on to an *AP-2β* germ-line KO background was obtained (Bassett *et al*., 2012). The resultant mice have *AP-2*α conditionally deleted from the developing retina, and have both copies of *AP-2β* deleted from all tissues ($AP2\alpha^{lacZki/flox}$/ $AP\text{-}2\beta^{-/-}$). The Ret-AP-2α/AP-2$\beta^{-}$ deficient retinas showed a striking loss of horizontal cells and misplacement of amacrine cells, which reside in the INL, that were not detected upon deletion of either family member alone. This demonstrated a critical overlapping role for *AP-2α* and *AP-2β* in horizontal and amacrine cell development (Bassett *et al*., 2012).

These mutants, however, could not be examined past birth since the *AP-2β* germ-line deletion results in neonatal death. Thus, one of the main goals of this thesis is to examine retinogenesis in post-natal mice.

# CHAPTER TWO

Rationale, Main Hypothesis & Research Aims

## 2.1 Rationale for Study

This study aims to gain a better understanding of retinal development by elucidating and characterizing the defects that arise by genetic deletions. Since the retina is an extension of the brain, it can also be used as a model to learn about the central nervous system (CNS), and in particular, neurodevelopment. Retinal progenitor cells develop into intricate and specialized neurons with specific numbers of cells, arrangement and migration patterns, which are an excellent asset to understand neural development. As a non-essential organ, the eye can be manipulated to facilitate our understanding of the genetic codes involved in neurodevelopment. Consequently, our lab has acquired the *AP-2β* "floxed" mouse line, which allows the selective deletion of *AP-2β* exon 6 from the retina. These mice are to be bred with the *AP-2α* "floxed" line to generate a homozygous floxed line for *AP-2α* and *AP-2β*. These mice were incorporated into the breeding scheme outlined in Figure 5 to examine mutants post-natally. The purpose of this project was to create and analyze retinal defects in a mouse line, *AP-2α* $^{KI/flox}$*/AP2-β*$^{KO/flox}$, in which one copy of *AP-2α* and *AP-2β* was germ-line deleted, while the other was conditionally deleted from the retina at E10.5. These double conditional KOs were expected to only display defects in the developing retina. Thus, this approach enabled the mutants to survive well into adulthood, and thereby facilitated our investigation of these two genes in retinogenesis. Studying *AP-2α* and *AP-2β* allows us to elucidate their required role(s) in cell fate determination of horizontal cells, and in amacrine cell mosaic patterning.

## 2.2 Main Hypothesis

*AP-2α* and *AP-2β* are expressed in horizontal cells and amacrine cells and the two genes play redundant roles in retinogenesis. The deletion of both these genes from the developing and postnatal retina will result in the loss of horizontal cells and further potential defects in the mosaic patterning of the amacrine cell population, which is not observed in single KO models.

## 2.3 Specific Aims

*2.3.1. Generate a mouse model for a double conditional deletion of AP-2α and AP-2β in the developing retina.*

The breeding scheme described in Figure 5 was employed to obtain double conditional knockouts, $AP\text{-}2\alpha^{KI/flox}/AP\text{-}2\beta^{KO/flox}$. The genotype of each resultant mouse was confirmed through PCR using the primer sequences described in Table 1. Immunohistochemistry was utilized to confirm the conditional deletion of both proteins, using AP-2α and AP-2β specific antibodies. Subsequently, histological examination were carried out to determine potential structural defects in the neonatal and adult KOs in the regions of deletion.

*2.3.2 Examine and analyze the horizontal cell population of the double KO mice.*

Postnatal mice were examined for defects in the inner nuclear layer, with a specific emphasis on detecting the loss of horizontal cells in postnatal mice. These studies

were conducted through the use of polyclonal antibodies for AP-2α and AP-2β to confirm regions of deletion and cell-specific markers of horizontal cells. Since the loss of HCs contributes to further defects in photoreceptors (Haeseleer *et al*., 2004; Chang *et al*., 2006; Specht *et al*., 2007; Sonntag *et al*., 2012; Wu *et al*., 2013), these changes were also evaluated in adult mice aged 2 months and older through electron microscopy.

*2.3.2 Examine and analyze the amacrine cell population of the double KO mice.*

Due to the subtle defects in amacrine cell clustering observed in embryonic double KOs by Bassett *et al*. (2012), postnatal mice were examined for amacrine cell defects beginning at P14 as retinogenesis is complete by P11. Although the focus of these studies were based on the observational defects of cholinergic amacrine cells (Bassett *et al*., 2012), a variety of amacrine cell markers were employed to examine potential defects in other amacrine cell subtype populations.

# CHAPTER THREE

Experimental Design

### 3.1 Generation of Mouse Lines

All animal procedures were performed in accordance with the Association for Research in Vision & Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. For genotyping, DNA was extracted from neonatal tail samples using the Direct PCR (tail) Lysis Reagent (Viagen), or adult ear clips using the EZNA Tissue DNA Kit (Omega Bio-Tek). Mouse lines were generated according to Figure 5, and genotypes were determined by well-established PCR protocols (Table 1).

The series of crosses used to generate *AP-2α* $^{KI/flox}$/*AP-2β*$^{KO/flox}$ are depicted in Figure 5. Two different AP-2 alleles were used: An *AP-2α*$^{ki7lacZ/+}$ null allele [due to a germ-line IRES-lacZ knock-in insertion disrupting exon 7] (Brewer *et al*., 2002); and a *AP-2β* null allele [due to a germ-line insertion of a PGK-neo cassette disrupting exon 4] (Moser *et al*., 1997).

*AP-2α* $^{ki7lacZ/+}$ mice were crossed with *α-Cre*$^{+/-}$ transgenic mice (Marquardt *et al*., 2001) expressing Cre recombinase under control of the retina-specific ‘‘Pax6 *α* enhancer,’’ from the murine Pax6 gene. *AP-2α* $^{ki7lacZ/+}$ / *α-Cre*$^{+/-}$ mice were crossed with *AP-2β* $^{+/-}$ mice to generate mice that retained the α-Cre transgene, and had only one functional copy of the *AP-2α* and *AP-2β* genes (*AP-2α*$^{ki7lacZ/+}$ / *AP-2β* $^{+/-}$ /*α-Cre*$^{+/-}$).

In a separate cross, homozygous *AP-2α* $^{lox}$ mice (Brewer *et al*., 2004) were bred with mice homozygous for the *AP-2β*$^{lox}$ allele (Green *et al*., unpublished), in which exons 5 and 6 of *AP-2α*, and exon 6 of *AP-2β*, respectively are flanked by single loxP sites to obtain *AP-2α/β*$^{lox}$ mice that were homozygous floxed for both alleles.

In the final cross, the *AP-2α*$^{ki7lacZ/+}$ / *AP-2β* $^{+/-}$ /*α-Cre*$^{+/-}$ mice were bred with *AP-2α* $^{lox/lox}$ and *AP-2β*$^{lox/lox}$ mice, resulting in double conditional mutants that are heterozygous for *AP-2α* and *AP-2β* null alleles, with the second copies of *AP-2α* and *AP-2β* deleted by Cre-mediated excision. This final cross in the breeding scheme is expected (based on Mendelian genetics) to result in 12.5% (1/8th) of the offspring being double mutants, therefore numerous breeding pairs were required. Littermates used as controls either contained two functional copies of *AP-2α* and two functional copies of *AP-2β* in the retina, or were missing one functional copy of either *AP-2α* or *AP-2β* if the former was not available (Table 1).

**3.2 Histology**

Neonates or dissected whole eyes were collected from mice euthanized by $CO_2$ overdose. Tissue was either fixed in 10% neutral buffered formalin (Sigma-Aldrich,Oakville, ON) overnight, processed and embedded in paraffin (Paraplast tissue embedding media, Fisher Scientific, Waltham, MA), or double embedded in paraffin-celloidin (1% CedarLane). Embryonic Onecut studies were performed on tissue that was fixed in 4% PFA overnight at 4◦C, cryoprotected overnight in 30% sucrose/PBS, and embedded in Tissue-Tek OCT (Sakura-Finetek, Torrance, CA). Serial sections were cut 4 μm (paraffin) or 10 μm (frozen) in thickness and used for immunofluorescent analysis or haematoxylin and eosin (H&E) staining.

### 3.3 Immunofluorescence

Paraffin-embedded sections were deparaffinized in xylene, hydrated (through 100%, 95% and 70% ethanol, followed by water), treated with 10 mM sodium citrate buffer (pH 6.0; boiling for 20 minutes) for antigen retrieval, blocked with normal serum and incubated with primary antibodies overnight at 4°C. For all co-localizations, both primaries and both secondaries were mixed and incubated simultaneously. Indirect immunofluoresence was performed using the primary antibodies found in Table 2. All stains were mounted with Pro Long Gold antifade reagent containing 4,6-diamino-2-phenylindole (DAPI) (Invitrogen – Molecular Probes, Burlington, ON). All staining was visualized with a microscope (Leica, Deerfield, IL) equipped with an immunofluorescence attachment, and images were captured with a high-resolution camera and associated software (Open-Lab; Improvision, Lexington, MA). Images were reproduced with image-management software (Photoshop 7.0; Adobe Systems Inc., Mountain View, CA). To quantify immunolabeled cells, a 600 μm length of retinal section equidistant from the periphery was scored on a minimum of 3 different sections from 3 animals and expressed as the mean ±SEM. To quantify retinal thickness, sections were measured from the peripheral retina on a minimum of 3 different sections from 3 different animals, with 5 measurements per section. Data were tested for significance by one-way ANOVA or Student's t-test where appropriate. (GraphPad Prism 6 Software).

Least significant difference post hoc comparisons were made when statistical significance ($P < 0.05$) was found between observations.

### 3.4 Electron Microscopy

Prior to enucleation, eyes were marked with a Davidson® dye (Bradley Products, Inc., Cedarane, Burlington ON) to indicate dorsal, nasal, temporal and ventral regions. The anterior parts of the eyes were then removed, and the neural retinas were separated from the pigment epithelium. The retinas were then quartered in to their respective regions: dorsal, nasal, temporal and ventral. These regions were then fixed by immersion in a primary fixative, 2% PFA/2.5% Glutaraldehyde, buffered in 0.15M Cacodylate Buffer, overnight at 4 C. Retinas were then washed in 0.15 M Cacodylate Buffer and post fixed in a 1:1 solution of 2% aqueous OsO4/0.2 M Cacodylate Buffer, 2 hours at 4°C, and then washed in 0.15 M Cacodylate Buffer. Retinas were then serially dehydrated in alcohol, and embedded in Spurr's resin in preparation for transmission electron microscopy (TEM) analysis. Thin sections (60-80 nm) were obtained with an ultramicrotome (Reichert–Jung Ultracut E Microtome; American Instruments) using a diamond knife, collected onto copper 75/300 mesh grids (Electron Microscopy Sciences), and stained with 2% (w/v) uranyl acetate and Reynolds' lead citrate. Sections were viewed using a JEOL 100CX electron microscope at an accelerating voltage of 60 keV, and digital images were collected and stored on a computer for subsequent viewing and analysis.

**Table 1**. PCR Protocols for Genotyping.

| Alleles | Primers | Conditions | Products |
|---|---|---|---|
| AP-2α $^{KI/LacZ}$ vs AP-2α $^{+/+}$ | **Alpha 6/7** 5′-GAA AGG TGT AGG CAG AAG TTT GTC AGG GC -3′ **Alpha 3′KO** 5′-CGT GTG GCT GTT GGG GTT GTT GCT GAG GTA C -3′ **IRESUP** 5′-GCT AGA CTA GTC TAG CTA GAG CGG CCC GGG -3′ | 45 s at 95°C, 1min at 67°C, 1min 10 sec at 72°C for 33cycles | *Tcfap2a*ki7lacZ 300 bp *Tcfap2a*+ 500 bp |
| AP-2α $^{lox}$ vs AP-2α $^{+}$ | **Alflox4** 5′-CCC AAA GTG CCT GGG CTG AAT TGA C-3′ **Alfscsq** 5′-GAA TCT AGC TTG GAG GCT TAT GTC-3′ | 45 s at 95°C, 45 s at 65°C, 1 min at 72°C for 39 cycles | *Tcfap2a*lox 560 bp *Tcfap2a*+ 490 bp |
| AP-2β $^{KO/+}$ vs AP-2β $^{+/+}$ | **4 Exon DW** 5′-CCT CCC AAA TCT GTG ACT TCT-3′ **PGK-PolyA DW** 5′-CTG CTC TTT ACT GAA GGC TCT TT-3' **4 Exon Rev** 5′-TTC TGA GGA CGC CGC CCA GG-3′ | 45 s at 95°C, 45 s at 58°C, 1 min at 72°C for 37 cycles | *Tcfap2b*– 380 bp *Tcfap2b*+ 221 bp |
| AP-2β $^{lox}$ vs AP-2β $^{+/+}$ | **BFL1** 5'-GTC TGT TTA GAA CCT GGC TCA GCC AG-3' **BLF2** 5'-TCT GGC AAG GCT CTT TCG GGG CAC TC-3' **SD5P33** 5'-CGC AGC GCA TCG CCT TCT ATC GCC TT-3' | 2 min at 95°C, 45 s at 95°C, 3 min at 70°C for 34 cycles | *Tcfap2β*lox 550 bp *Tcfap2β*+ 450 bp |
| α-Cre | **Cre1** 5′-GCT GGT TAG CAC CGC AGG TGT AGA G-3′ **Cre3** 5′-CGC CAT CTT CCA GCA GGC GCA CC-3′ | 45 s at 95°C, 1 min at 67°C, 1 min 10 sec at 72°C for 33 cycles | Presence of *α-Cre* transgene 420 bp |

**Table 2**. List of Antibodies for Immunofluorescence

| ANTIBODY | SUPPLIER | WORKING CONCENTRATION |
|---|---|---|
| Mouse anti-AP-2α | Developmental Studies Hybridoma Bank (3B5), University of Iowa, Iowa City, IA | 1:1 |
| Rabbit anti-AP-2β | Cell Signaling Technology #2509, Danvers, MA | 1:50 |
| Mouse anti-AP-2γ | Clone 6E4/4, Sigma Aldrich, Oakville, ON | 1:800 |
| Mouse anti-β-Catenin | BD Transductions, Cat # 610153 | 1:100 |
| Goat anti-β3 (BHLHB5) | E-17 #sc-6045, Santa Cruz Biotechnology, Santa Cruz, CA | 1:400 |
| Mouse anti-Calbindin | Clone CL-300, Product no. C8666, Sigma Aldrich, Oakville, ON | 1:250 |
| Goat anti-Calretinin | N-18; Santa Cruz Biotechnology #sc-11644, Santa Cruz, CA | 1:800 |
| Goat anti-Glyt1 | Millipore-Chemicon, Billerica, MA | 1:5000 |
| Rabbit anti-GAT1 | Abcam, Cambridge, MA | 1:250 |
| Rabbit anti-Islet1/2 | K5; from T. Jessell Lab, Columbia University, New York, NY | 1:500 |
| Mouse anti-NF160 | Clone NN18, Sigma Aldrich #N5264, Oakville, ON | 1:300 |
| Rabbit anti-Onecut-1 | HNF6, Santa Cruz Biotechnology, Santa Cruz, CA | 1:100 |
| Rabbit anti-Otx2 | Abcam, Cambridge, MA | 1:100 |
| Rabbit anti-Pax6 | Covance #PRB-278P | 1:50 |
| Rabbit anti-Sox2 | Millipore-Chemicon #AB5603 | 1:1000 |
| Mouse anti-Syntaxin1 | HPC-1, Sigma Aldrich (S0664), Oakville, ON | 1:2000 |
| **Secondary:** | | |
| Alexa Flour 568 | Invitrogen – Molecular Probes, Burlington, ON | 1:200 |
| Alexa Flour 488 | Invitrogen – Molecular Probes, Burlington, ON | 1:200 |

# CHAPTER FOUR

Results

### 4.1 Generation of *AP-2α* and *AP-2β* Retinal Conditional Mutants

Multiple double mutants were successfully generated by the breeding scheme described in Figure 5. The breeding scheme employed *AP-2α* and *AP-2β* heterozygotes so that Cre-loxP-mediated excision was only required for one allele, while the function of the second allele was removed by disrupting the DNA binding exons 7 and 4, respectively. Mice from the final cross were genotyped for each AP-2 allele: αKI lacZ, αflox, βhet, βflox, and the presence of Cre recombinase was also detected through PCR. Once a sample was positive for all five genetic fragments, the eyes were then processed and the expression of AP-2α and AP-2β was examined. Since the Pax6 α-Cre is only active in the dorsal, nasal and temporal regions, it was imperative to detect the regions of deletion of these genes in each sample to conduct further analysis. In order to examine retinogenesis through to completion, WT and KO eyes were obtained at various stages from P14 to two months of age. To ensure *AP-2* deletion from the peripheral neural retina, we examined AP-2α and AP-2β expression via immunofluorescence in each of the mutants obtained (Fig. 4). AP-2α/β expression was readily detected in the central retina, with a clear deletion in the periphery. Nearby sections from these results were used for all future analyses, as described below.

### 4.2 A Complete Loss of Horizontal Cells

Embryonic data on double KOs revealed a striking loss of horizontal cells (HC) at embryonic stages (Bassett *et al*., 2012). To assess HCs in the post-natal double KO mice, immunofluorescence labeling was performed using horizontal cell-type specific markers

at P14. Calbindin, a calcium-binding protein which aids in calcium transport, is expressed by HCs located in the INL, and is expressed in a subset of ACs in the INL and GCL. Deletion of AP-2α and AP-2β affected these HCs, but not the ACs (Fig. 6D-F). To further assess HC deficiency, other markers were utilized including Neurofilament 160 which is found in the cystoskeleton of particular neurons (Fig. 6A-C), β-catenin which is found in all neuronal processes and is essential for neuronal lamination and, (Fig. 6G-I) and Onecut-1 which is a precursor of HC development (Fig. 7). A complete loss of these interneurons is found only in the Cre-positive regions of the peripheral retina of the double mutants (Fig. 7B) as shown by immunolabeling with the above described markers; this loss is not seen in littermates used as controls.

### 4.3 The Genetic Cascade for Development of Horizontal Cells

Previous work by Bassett et. al (2012), demonstrated the early loss of horizontal cell markers Lim-1 and Prox-1 in embryonic *AP-2α/β* mutants, determining that HCs did not form during early development in the double mutants. Thus, it was concluded that *AP-2α* and *AP-2β* are required for horizontal cell genesis. Wu et el. (2013) have determined that the transcription factor Onecut-1 (Oc1) lies upstream of Lim-1 and Prox-1, but downstream of FoxN4. Therefore, in order, to decipher the genetic cascade involved in horizontal cell development, it was imperative to examine Oc1 expression in *AP-2α/β* double KOs, and conversely examine the expression of these AP-2s in $Oc1^{-/-}$ KOs. At E16.5, Oc1 expression remained absent in our double KOs (Fig. 7B, D). This finding suggested that AP2α and AP-2β work upstream of Lim1, Prox1 and Oc1 in

horizontal cell development. We also examined the expression of both AP-2α and AP-2β in *Oc1*$^{-/-}$ single KOs and *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ double KOs (obtained from the Mu lab in Buffalo, NY). Interestingly, both AP-2α and AP-2β are expressed in WT and *Oc1*$^{-/-}$ KOs (Fig. 8E, F), as well as *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ double KOs (Fig. 8H, I). These findings suggest that these AP-2s may work upstream of Onecut proteins to regulate horizontal cell development..

**4.4 Abnormalities in the Outer Plexiform Layer of AP-2α/β Double KOs**

Another structural defect in the double KOs was observed in older animals. By two months of age, there is a thinning of the outer plexiform layer (OPL) over time in the peripheral regions of the mutants, which is not observed in control littermates (Fig. 9A, B). By 4 months, the OPL has completely disappeared, leading to an indistinguishable difference between the ONL and INL (Fig. 9D). Measurements of the OPL were recorded over the two and four month period. Post-hoc analysis showed there was significant decrease between the control and mutant retinas at two months, as well as at four months $P < 0.001$(Fig. 11). There was also a significant reduction in OPL thickness between the two and four month stage, $P<0.05$ (Fig. 11), thereby demonstrating an overall reduction in this synaptic layer over time. There were no differences in OPL thickness between the control retinas and the central retina of mutants (not shown). The OPL is composed of photoreceptor terminals, and HC and BC processes. We propose that one possible cause of this absence of the OPL in the double KO retinas is due to the loss of HCs in these regions, as demonstrated earlier.

HCs have shown to be required for photoreceptor structure and viability. Loss of HCs has shown to cause defects in synaptic connections between photoreceptors and bipolar cells. These specialized synaptic contacts, triadic ribbon synapses, are formed by a presynaptic photoreceptor ribbon, horizontal cell dendrites and ON bipolar cell dendrites. The triad is responsible for signal transmission between PRs, HCs and BCs, in order to regulate photoreceptor output. Structural changes in the classic triadic ribbon synapse have been observed in HC-ablated retinas (Haeseleer *et al*., 2004; Chang *et al*., 2006; Specht *et al*., 2007; Sonntag *et al*., 2012; Wu *et al*., 2013).

We wanted to examine whether the loss of HCs in our double mutants would also result in changes within the OPL and the triad. Examination of these synaptic connections in the OPL by transmission electron microscopy (TEM) allowed for the assessment of structural changes in the double KOs. We were able to observe similar structural defects in the synaptic connections in the OPL as those reported in the literature regarding HC-ablated retinas. We observed a complete absence of triad ribbon synapses of rod photoreceptors consistent with a loss of horizontal cell processes (Fig. 10B, D**)**. Although the triad was absent, there were remnants of spherical ribbon-like structures present in these retinas (Fig. 10B, arrows), which are normally thin, long elongated structures (Fig. 10A). Furthermore, there were observable defects in the photoreceptors, which exhibited retracted axons (Fig. 10D, arrows). Due to the lack of Cre recombinase activity in the central retina, it was confirmed that these abnormalities were due to a direct cause of the deletion, as the central retina mimicked WT conditions with an intact thick OPL and triad ribbon synapses. These abnormalities were not found in single AP-2α and AP-2β mutants.

### 4.5 Double *AP-2α* and *AP-2β* Conditional Mutants Display Retinal Dysplasia

Retinas from double KO mice displayed some abnormal cellular lamination. For example, at P17, the mutant retinas exhibited irregular folding and arrangement of the nuclear and synaptic layers. The irregularities vary across mutants, including a folded and upside down retina (Fig. 12B), photoreceptor whorls embedded within the outer segments (Fig. 12C), and excessive thickening of multiple nuclear and/or synaptic layers (Fig. 12B, C). Recently, we discovered that our mice lines contain a spontaneous mutation, a single nucleotide deletion that causes early onset retinal degeneration. The mutation occurs in the *Crb1* gene and causes various forms of retinal degeneration including light fundus spots, whorls, rosettes, dysplasia and degeneration (Mattapalil *et al*., 2012). Interestingly however, none of the reported rd8 histological abnormalities are similar in appearances as those present in the double KOs. The documented cases of rd8 have not shown inverted or upside down retinas, or embedded whorls, which are all present in these KOs. Early work by West-Mays *et al*. (1999) reported an absence of an RPE in AP-2α germ line KOs, in which the dorsal aspect of the RPE was replaced by a neural retina-like structure, similar to Fig. 12B. On the other hand, some of the WT littermates do exhibit a milder version of dysplasia. We wondered whether abnormal cellular proliferation was a factor that would explain the excess retina, however examination of the proliferation marker PCNA, did not show any changes (data not shown). The synaptic processes, as labeled by Syntaxin-1 (Fig. 13) are also intact, even in the abnormal dysplastic regions.

### 4.6 Amacrine Cell Development

Given the overlapping expression of AP-2α and AP-2β in postmitotic amacrine cells, it was imperative to examine their development in postnatal stages in the double KOs. The adult retina expresses the two proteins in both the glycinergic and GABA-ergic amacrine population., with a 77% overlap in their expression pattern (Bassett *et al*., 2012). The GABA-ergic expressing amacrine cells are found as early as E14.5 to E16.5, while the glycinergic population peaks at P0, and is not detectable until postnatal stages. Therefore, the expression pattern of only the former cell type could be examined by Bassett *et al*. (2012). It was discovered that there was an absence of a band of BHLHB5-positive GABAergic amacrine cells, which was present in control littermates. Examination of this marker in post-natal double mutant retinas has not revealed any abnormalities in the number of BHLHB5 expressing amacrine cells (Fig. 14). There are also no observed alterations in the mosaic patterning of these ACs. Immunofluorescence initially suggested an overall decrease in the population of these cells (Fig. 14A-B). Thus, quantification of this subpopulation of ACs in the peripheral retina was conducted, and no statistical difference was observed in the total number of BHLHB5+ cells in the regions of *AP-2α/β* deletion. This observation does differ from the absent band of these ACs observed in embryonic *AP-2α/β* KOs by Bassett *et al*. (2012). Therefore, BHLHB5 expression should be analyzed in wholemount retinas to accurately determine a genuine reduction in the number of these cells, as histological sections do not represent the entire view of the retina.

Further examination of ACs revealed some other abnormalities that have not previously been reported. Calretinin-positive cells depicted abnormal sublaminar stratification in the inner plexiform layer (IPL). There are five sublaminae within the IPL; the anti-Calretinin antibody marks ACs and GCs as well as the three IPL strata at the borders of S1 and S2, S2 and S3, S3 and S4. These distinct bands of dendrites were completely absent in the double KOs (Fig. 15). It is interesting to note that due to the nature of the Pax6 α-Cre, the central regions of the retina mimic WT conditions, while the periphery consists of the region of deletion. We employed this phenomenon as an internal control during analysis of the double KOs. This allowed us to note that Calretinin staining is normal in the central region (data not shown), where the AP-2s continue to be expressed but expression is abnormal in the areas of deletion. This suggests a bonafide correlation of the phenotype, as opposed to an artefact.

Cholinergic amacrine cells are the earliest subset of GABA-ergic ACs that are found in the developing retina. Previous studies examining embryonic stages of double AP-2α /β KOs revealed altered immunostaining of SOX2 and ISL1/2 positive ACs, in which these cell types formed irregular cellular arrangements in the mutants as compared to controls (Bassett *et al*., 2012). Examination of these markers in postnatal stages did not reveal striking abnormalities in the positioning or patterning of these cells (Fig.16). Due to the limitation in obtaining double mutants, the amacrine cell studies are currently incomplete. The data from BHLHB5 and Calretinin expression does indicate exciting preliminary results, which could potentially infer a novel redundant role of AP-2α and AP-2β in amacrine cell mosaic patterning.

# CHAPTER FIVE

Discussion

Retinogenesis is a complex process that involves numerous transcription factors that give rise to the varying retinal cell types throughout development. Interestingly, many of these factors have varying functions, as well as redundant and compensatory roles in the genesis of retinal neurons. Early in embryogenesis, these factors impact cell fate of varying retinal neurons, their spatial arrangement, as well as both homotypic and heterotypic neural connections that require precise organization within the retinal framework. In particular, AP-2α and AP-2β are expressed throughout eye development, in the optic cup (West-Mays *et al*., 1999), the developing lens (Pontoriero *et al*., 2008; Kerr *et al*., 2014) and the neural retina (Basset *et al*., 2007; Bassett *et al*., 2012). Lens-placode specific and retinal conditional deletion of *AP-2α* does not impact retinogenesis (Dwivedi *et al*., 2005, Pontoriero *et al*., 2008 and Bassett *et al*., 2007, respectively); however, conditional deletion of both *AP-2α* and *AP-2β* from the developing retina leads to impaired retinal development (Bassett *et al*., 2012).

### 5.1 The redundant role of AP-2α and AP-2β in the development of retinal horizontal cells

The current study served to establish key members of horizontal cell development by examining the roles of AP-2α and AP-2β. We have previously shown the potential redundancy of these genes in HC development (Bassett *et al*., 2012). However, since the study could not utilize *AP-2β* floxed mice, the progression of loss could not be examined in post-natal animals, as *AP-2β* null mice die at birth. Together these findings demonstrate that complete horizontal cell depletion occurs due to a deletion of *AP-2α* and *AP-2β* in post-natal and adult stages confirmed the idea that these two members are

involved in HC genesis. The development of these neurons has been suggested to occur in a step-wise manner, such that Foxn4 acts most upstream in retinal progenitor cells to initiate the process of making these cells competent for a horizontal cell (Wu *et al*., 2013) as well as for an amacrine cell fate (Li *et al*., 2004). From this population emerge two others, one that exclusively expresses Ptf1a, while a second population emerges which expresses both Ptf1a and Oc1 (Fig. 3). Interestingly, both factors are essential but not individually sufficient for HC development. This study has introduced two new players in this genetic cascade. Due to the absence of Oc1 in *AP-2α/AP-2β* double KOs and the intact expression of these AP-2s in *Oc1* and *Oc1/2* KOs, we propose that cell populations that first express AP-2α/AP-2β and subsequently Oc1 are then able to adopt HC fate. It has been previously shown that AP-2s act upstream of Lim1 and Prox1 (Bassett *et al*., 2012), which are also downstream of Oc1 (Wu *et al*., 2013). Therefore it would be interesting to examine whether AP-2α and AP-2β are up- or down- regulated in Onecut KO models. Preliminary data from the Mu Lab (SUNY at Buffalo, Buffalo NY) suggests that AP-2α and AP-2β are upregulated by two-fold in the *Oc1* KOs (Sapkota *et al*., unpublished). This further supports the model that AP-2α and AP-2β regulate Oc1 expression, which could then act as a negative feedback mechanism to control AP-2 expression. Further work on examining AP-2s in Foxn4 and Ptf1a mutants will illustrate this complex hierarchy of factors involved in HC development. It is also interesting to note that Ptf1a is required for amacrine cell formation (Fujitani *et al*., 2006; Nakhai *et al*., 2007). Since AP-2α and AP-2β are expressed in a subset of amacrine cell populations, it

would be interesting to ascertain how Ptf1a and AP-2s collaborate to regulate cholinergic and/or GABAergic amacrine cells in development.

### 5.2 Horizontal cells are vital for the maintenance of rod photoreceptors and retinal integrity

The present study examined downstream effects of horizontal cell loss by employing the *AP-2β* floxed mouse line, which allowed the double KOs to survive well into adulthood. During retinal development, horizontal cell processes recruit synaptic ribbons to photoreceptors by creating sites of contact at the synapse. This contact and recruitment then promotes a second HC process to arrive at the synapse, followed by the addition of an ON-bipolar cell dendrite. Together, this complex forms a synaptic ribbon triad. We examined whether the loss of HCs affected this structure and discovered disruptions in these contacts. A complete absence of the ribbon triad was observed in *AP-2α/AP-2β* double KO, and only remnant-like spherically shaped ribbons were seen in these mutants. This absence was not noted in single mutants, littermate controls or in mutants with areas of intact AP-2 expression. The absence of the triad has been extensively recorded in studies examining HC-ablated retinas, thus confirming our findings. These changes resembled effects caused by genetic mutations, such as the loss of Onecut-1 (Wu *et al*., 2013), mutations induced by toxins such as diphtheria (Sonntag *et al*., 2012), and effects caused by the loss of photoreceptor synaptic proteins, such as CaBP4 (Haeseleer *et al*., 2004) and bassoon (Specht *et al*., 2007). The development of photoreceptor synapse formation is integral to transmission of signals from the rods to bipolar cells and subsequently to the inner retina. Although untested, physiological tests

would depict a severely compromised electroretinogram (ERG). The presence of spherical ribbon-like structures leads to further questions about synaptic contacts between photoreceptors and bipolar cells, and if the processes of other classes of bipolar cells are affected.

We have also observed the dependence of horizontal cells on retinal lamination and integrity. Over time, starting from as early as two months of age, mice lacking both AP-2α and AP-2β begin to lose the entirety of the OPL. Eventually by four months of age, the inner and outer nuclear appear as one, with a complete absence of the OPL. This loss is indicative of retinal degeneration, a phenomenon that has also been observed in multiple studies involving depletion or deletion of the horizontal cell population (Sonntag *et al*., 2012; Wu *et al*., 2013). The mechanisms and survival signals, whether trophic or electrical, through which HCs promote photoreceptor survival and retinal lamination is currently unknown. Their discovery could potentially impact clinical outcomes that involve photoreceptor survivability and vision loss. At present, however, horizontal cell loss has not been reported in the human population.

### 5.3 Amacrine cell patterning may be dependent on AP-2α and AP-2β expression

Due to the overlap in expression of AP-2α and AP-2β in the amacrine cell population, it was imperative to examine this cell type in this double KO study. Embryonic studies revealed abnormal cellular arrangement of cholinergic ACs, as shown by immunostaining with ISL1/2 and SOX2, and an absence of a band of BHLHB5 expressing ACs in the *AP-2α/AP-2β* double mutants. This study was not able to confirm clustering of cholinergic ACs, or a decrease in BHLHB+ cells, but we observed an

alteration in the sublamination of neuronal processes in the IPL of the double mutants by examining the expression of calretinin (Fig. 15). Embryonic studies proposed that AP-2α and AP-2β may be involved in determining cell fate or survival of AP-2-expressing amacrine cells, as well as their possible roles in the positioning of these cell types in the inner retina. The current study further leads to the possibility that these AP-2 proteins are involved in of cellular patterning of these cells, in particular their processes. It is possible that subsequent to cell fate determination, AP-2α and AP-2β are involved in the organization of a distinct pattern of amacrine cell bodies, and in directing neural growth, branching and axon fasciculation. Surprisingly, there were no changes detected in the BHLHB5-expressing population of GABAergic cells. It is certainly possible that another AP-2 member, such as AP-2γ, may be compensating the fate of this particular cell type as AP-2γ is expressed in these cells. The absence of distinct bands of dendritic processes in the IPL of double mutants suggested that these proteins could direct final position of neuronal processes, which can no longer be attained in their absence.

Much of the work on amacrine cells in this study was performed on histological sections. Due to difficulties in obtaining double mutants and antibody incompatibilities, confirming these results in wholemount retinas was unsuccessful. It is certainly imperative to examine the expression of these markers in wholemount retinas, as opposed to histological sections, to gain an accurate view of the field of amacrine cell spacing in the mutants. Regular patterning of retinal nerve cells is essential for visual processing. These retinal mosaics allow visual images to be processed uniformly across the various neurons. Amacrine cell mosaic is particularly important for extracting visual data related

to direction of motion of the ON and OFF visual pathways (Reese 2007; Whitney *et al*. 2008). Nearest-neighbor and Voronoi-based analyses have become the favorable means used to examine patterning of retinal mosaics (Reese 2007; Whitney *et al*., 2008). These indices examine the degree of randomness in spacing between neighboring homotypic cells. It would be beneficial to examine the spacing between cholinergic amacrine cells and to further evaluate whether axonal processes are altered in their arrangement or morphology in the double mutants. These studies could elucidate the mechanism by which neuronal migrations occur by potentially revealing any downstream targets of AP-2s involved in these developmental processes. Any abnormalities from littermates may indicate a role of AP-2s in amacrine cell patterning. Examining the true arrangement of amacrine cell bodies, as well as the interaction of their respective processes with homotypic neighbors can only be performed in whole-tissue samples. The current study, however, does provide strong evidence for the hypothesis of AP-2s playing a crucial role in amacrine mosaic patterning. Currently, few studies have examined the key regulators of amacrine cell spacing and arrangement. For example, the adhesion protein podocalyxin has been implicated to play a role in the growth and migration of neuronal processes, while Dscam has been shown to prevent cell-cell adhesions that allow dendrites to migrate to their appropriate destination within the retina (Keeley *et al*., 2012). Interestingly, AP-2α has been shown to directly regulate E-cadherin expression in the developing lens (Pontoriero *et al*., 2008) and that N-cadherin expression is upregulated in corneal cells in which AP-2α has been overexpressed (West-Mays *et al*., 2003). Since multiple other cadherins are involved in the retina, such as R-cadherin and

cadherin-6, -7 and -11 (Honjo *et al*., 2000), it is possible that these adhesion molecules may be under the control of AP-2 proteins. Another gene known as *ERBB2* has also been demonstrated to be a target of AP-2s (Bosher *et al*., 1996) and is expressed in the IPL during embryonic and postnatal retinogenesis. It is responsible for mediating neuregulin-1 signal, which is a promoter of neuronal survival and neurite growth (Bermingham-McDonogh *et al*., 1996).

This study has shown that AP-2α/β proteins are required for horizontal cell genesis, but not amacrine cell genesis. However, these AP-2 proteins may play a role in the regulation of cell adhesion molecules that are involved in homotypic interactions to determine amacrine cell spacing and dendrites. AP-2s are not involved in cell fate determination, commitment or migration of ACs, but they may be involved in the arrangement and spacing of nuclei or dendrites. Therefore, this study illustrates the different roles played by the same set of genes but in different cell types of the retina. Studying the role of developmental genes provides important information about the progression of genetic processes that regulate eye formation, thus increasing our understanding of molecular interactions that could be involved in congenital birth defects involving AP-2s in the eye. Since the retina is an extension of the CNS, the work from this study and those involving the retina are crucial for understanding neuronal development and interactions within the overall neuronal circuitry of the mammalian system.

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# FIGURES

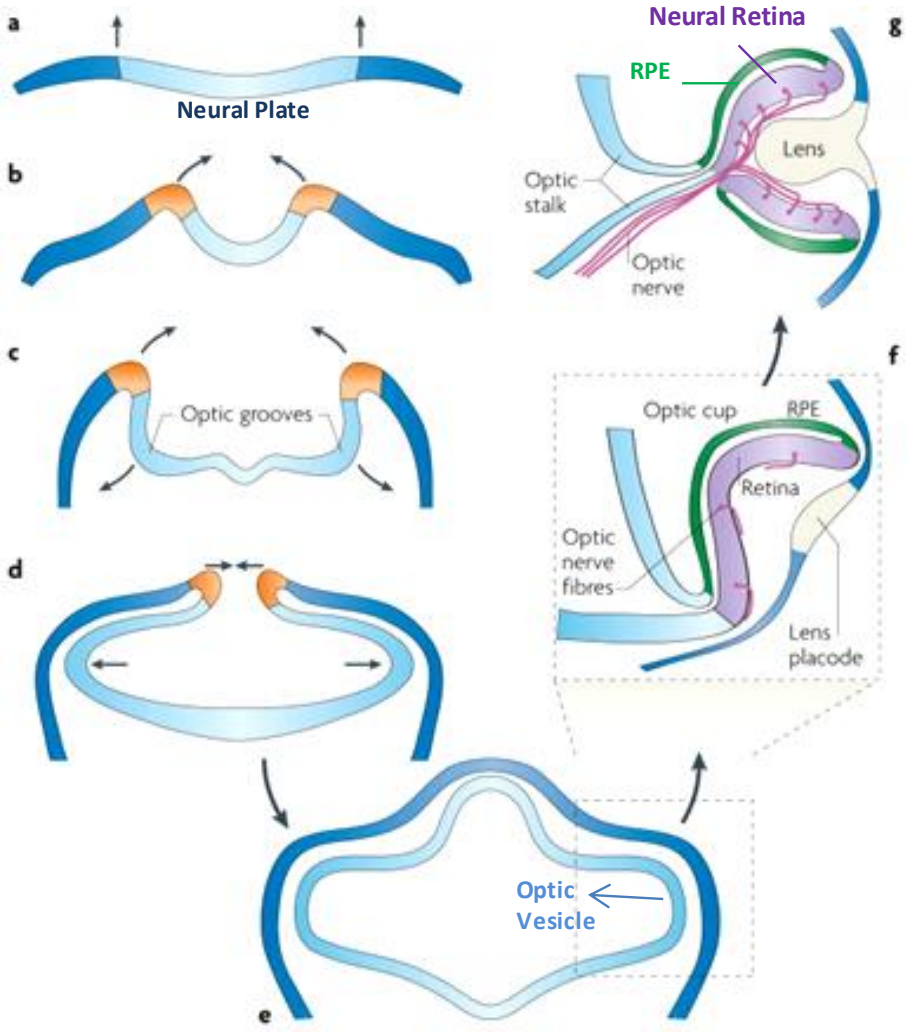


**Figure 1. Eye Development**. Development of the vertebrate eye cup begins with the neural tube, as it folds upwards (a) and inwards (b) causing the optic grooves to evaginate (c). The neural folds then begin to approach one other (d), while the optic vesicles (OV) bulge outwards. Once the folds come in contact, the neural tube pinches off (e). At this point, the forebrain grows upwards and the OVs continue to protrude outwards. They eventually contact the surface ectoderm and cause induction of the lens placode. The lens placode pinches off (f) to form into a lens vesicle and the OV invaginate inward to form the optic cup, which will give rise to the RPE and the neural retina (g). Adapted from Lamb *et al.*, 2007.

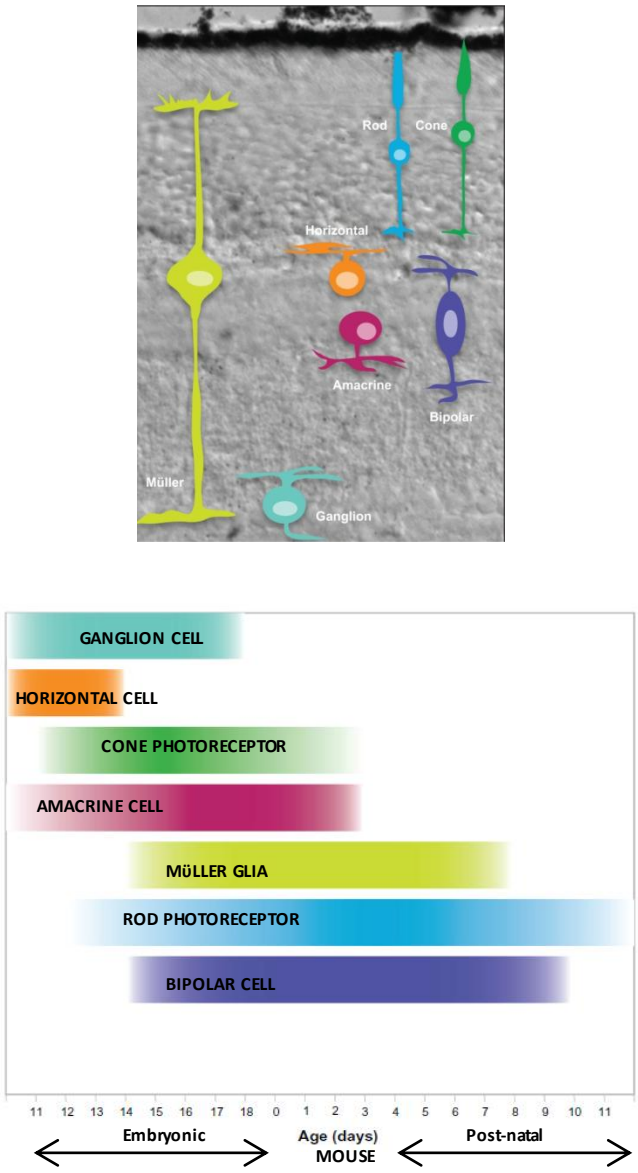


**Figure 2. The Mammalian Retina.** (Top Panel): There are six classes of neurons in the mammalian retina: rods, cones, horizontal cells, bipolar cells, amacrine cells and retinal ganglion cells (RGCs), as well as the Muller glia cells. (Bottom Panel): All seven retinal cell types derive from a pool of proliferating pluripotent precursors. Experiments have revealed that these cells are not generated at the same time, but instead undergo a defined order of commitment: first, RGCs, followed by cone, amacrine and horizontal cells, and ending with bipolar and rod photoreceptor neurons, as well as Muller glia. Retinogenesis is completed by P11. Adapted from Zhang *et al*., 2011.

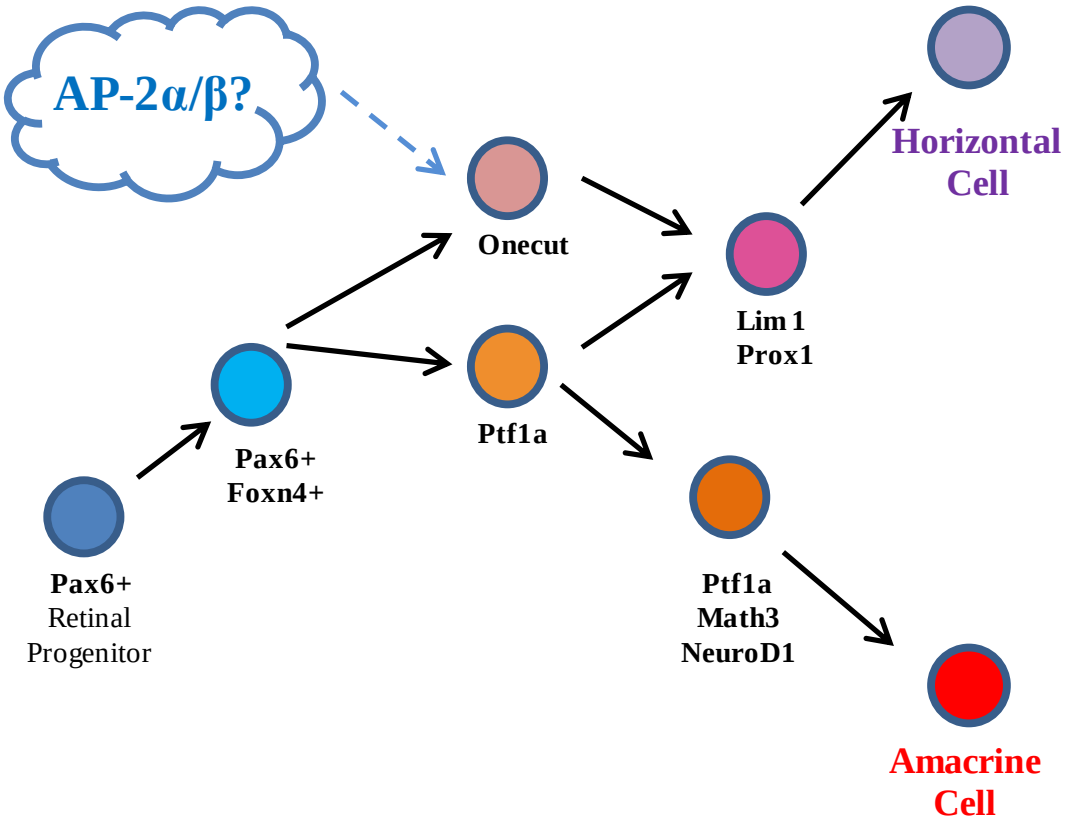


**Figure 3. Horizontal Cell and Amacrine Cell Development Pathway**. The expression of early transcription factors such as Pax6 and Foxn4 allow the retinal progenitor cells to begin a pathway to develop into both horizontal and amacrine cells. Parallel pathways have been shown to exist for HC development involving Onecut and Ptf1a. We propose that AP-2α and AP-2β may also play a redundant role in the pathway to HC development.

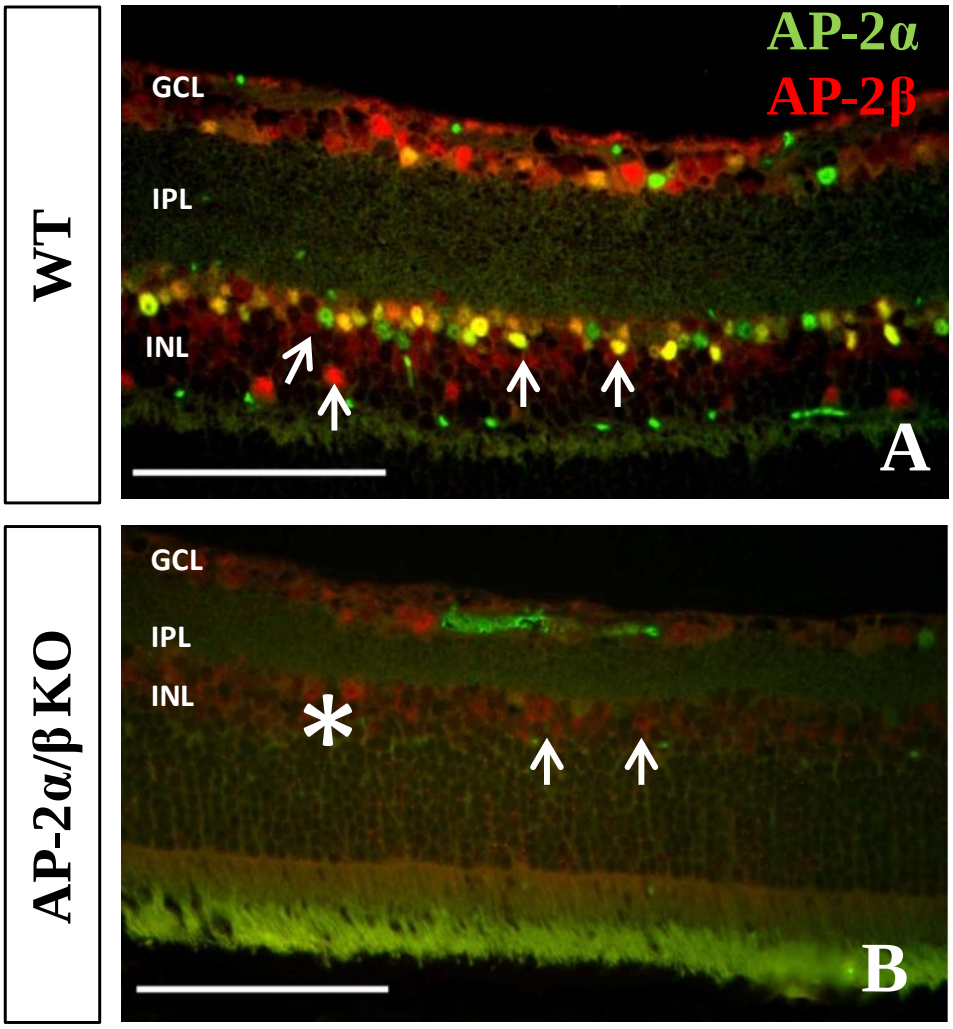


**Figure 4. AP-2α/β Expression in the Retina.** WT (A), AP-2α/β KO (B). AP-2α expression parallels AP-2β in the developing retina. Both proteins are expressed in the INL, where developing horizontal and amacrine cells reside and they are expressed in displaced amacrine cells in the GCL. This expression is completely lost in the AP-2α/β KO (B, *). GCL, gangion cell layer; IPL, inner plexiform layer, INL, inner nuclear layer. Scalebars: 100μm.

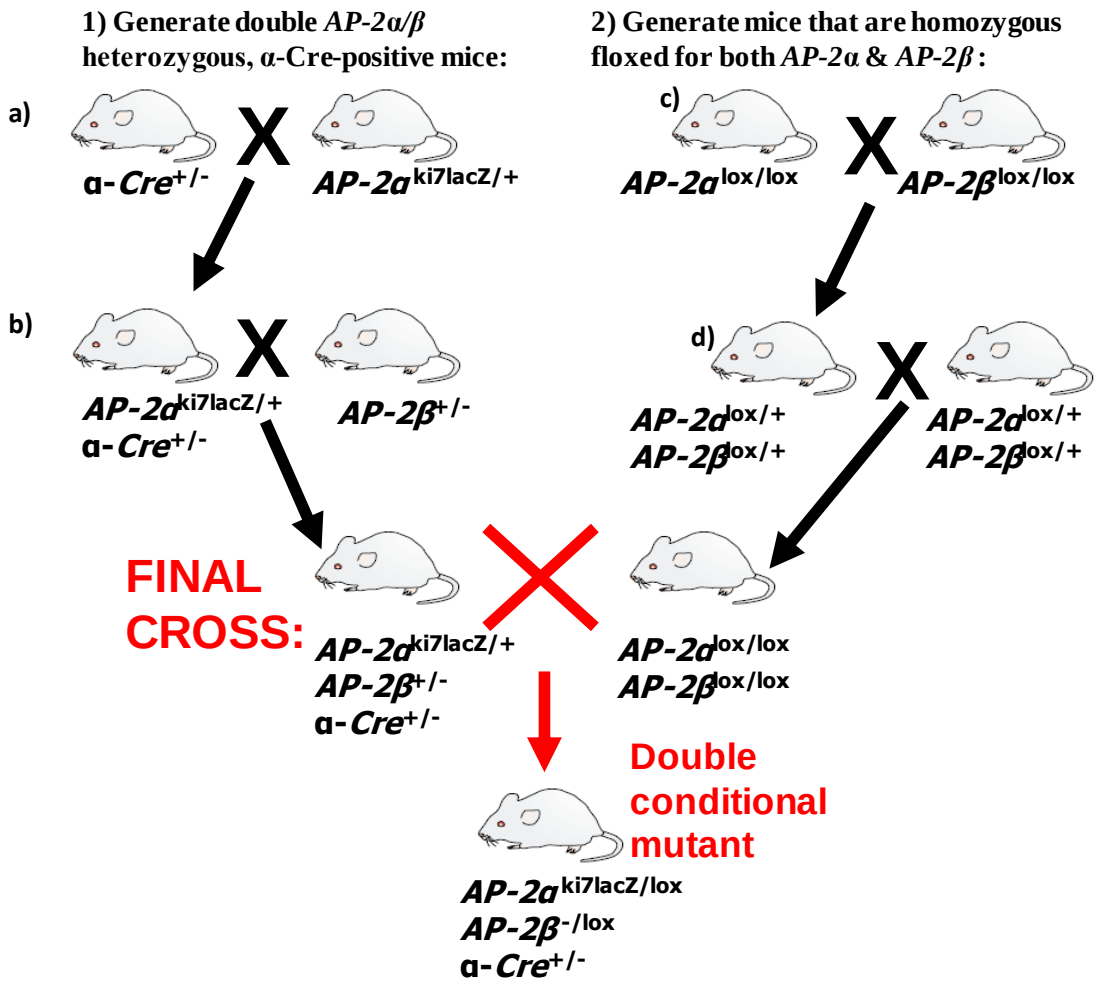
1) Generate double AP-2α/β heterozygous, α-Cre-positive mice:
2) Generate mice that are homozygous floxed for both AP-2α & AP-2β:
a)
α-Cre+/-
AP-2αki7lacZ/+
c)
AP-2αlox/lox
AP-2βlox/lox
b)
AP-2αki7lacZ/+
α-Cre+/-
AP-2β+/-
d)
AP-2αlox/+
AP-2βlox/+
AP-2αlox/+
AP-2βlox/+
FINAL CROSS:
AP-2αki7lacZ/+
AP-2β+/-
α-Cre+/-
AP-2αlox/lox
AP-2βlox/lox
Double conditional mutant
AP-2αki7lacZ/lox
AP-2β-/lox
α-Cre+/-

**Figure 5. Generation of Double Conditional AP-2α/β Retinal Mutants.** (1, 2) The breeding scheme uses *AP-2α* and *AP-2β* heterozygotes so that Cre-loxP-mediated excision is only required for one allele. (a) Mice heterozygous for the *AP-2α* gene are crossed with heterozygous *α-Cre*$^{+/-}$ transgenic mice expressing Cre recombinase. b) The resulting *AP-2α*$^{ki7lacZ/+}$ */ α-Cre*$^{+/-}$ mice are crossed with *AP-2β*$^{+/-}$ heterozygous mice to generate *AP-2α*$^{ki7lacZ/+}$ */ AP-2β* $^{+/-}$ */ α-Cre*$^{+/-}$ (2). (c) In a separate series of crosses, mice homozygous for the *AP-2α*$^{lox}$ allele are crossed with mice homozygous for the *AP-2β*$^{lox}$ allele. The resulting offspring (d) are interbred to regain the floxed alleles to homozygosity, generating *AP-2α*$^{lox/lox}$ */ AP-2β*$^{lox/lox}$ mice. In the final cross, the *AP-2α*$^{ki7lacZ/+}$ */ AP-2β* $^{+/-}$ */ α-Cre*$^{+/-}$ mice were bred with *AP-2α*$^{lox/lox}$ */ AP-2β*$^{lox/lox}$ mice, resulting in double conditional mutants.

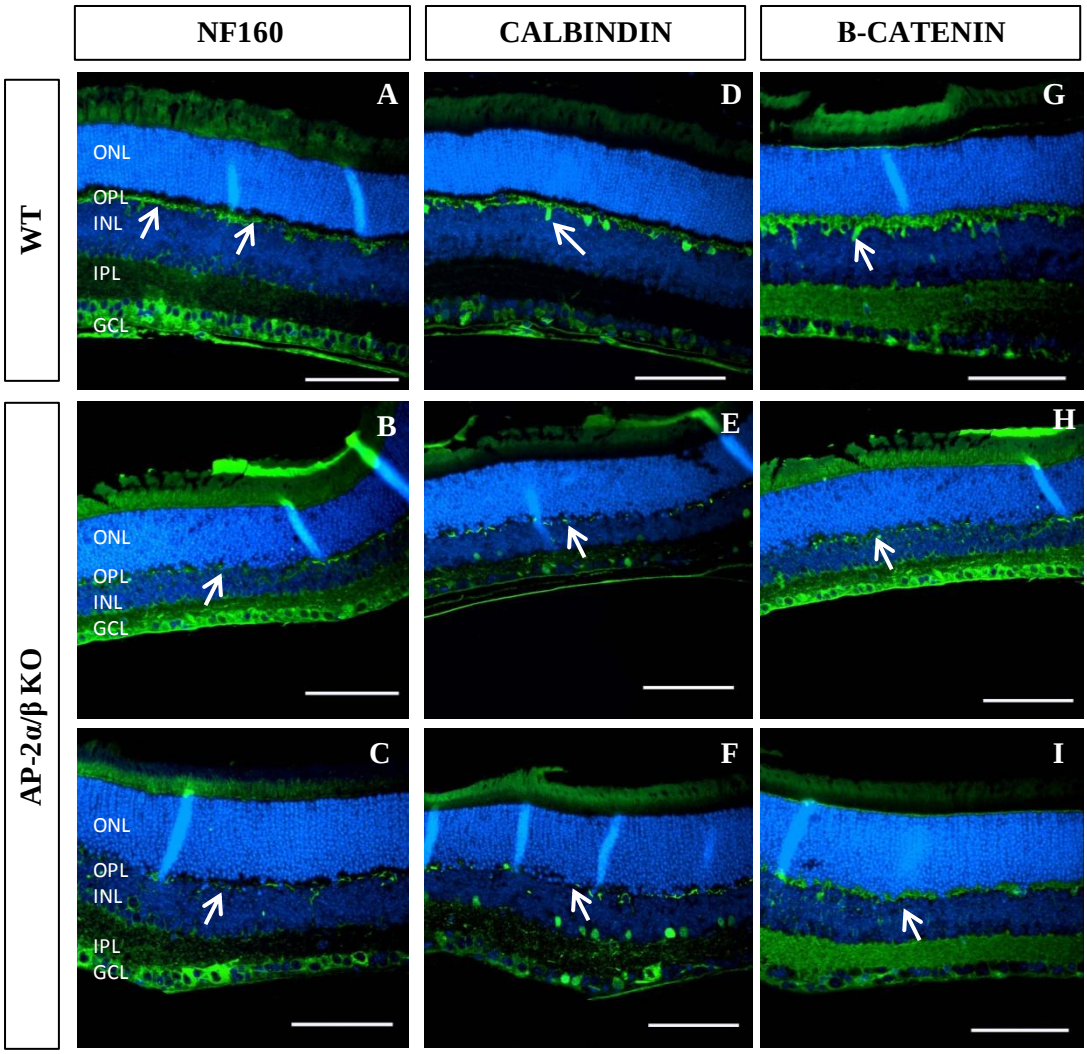


**Figure 6. Horizontal Cell Loss**. Photomicrographs showing expression of HC markers in WT and AP-2α/β KO mice. Wild-type (A, D, G) depict expression of horizontal cell bodies and axons (green, arrows). Neurofilament 160 (NF160) (A) And β-catenin (G) label neuronal processes in the outer plexiform layer (arrows) - their expression is absent in the periphery of the mutant retinas (arrows) (B, C) and (H, I), respectively. Calbindin is a horizontal cell and axon specific marker (D, arrows), expression of this marker was lost in the mutants (E, F). Scalebars: 100µm.

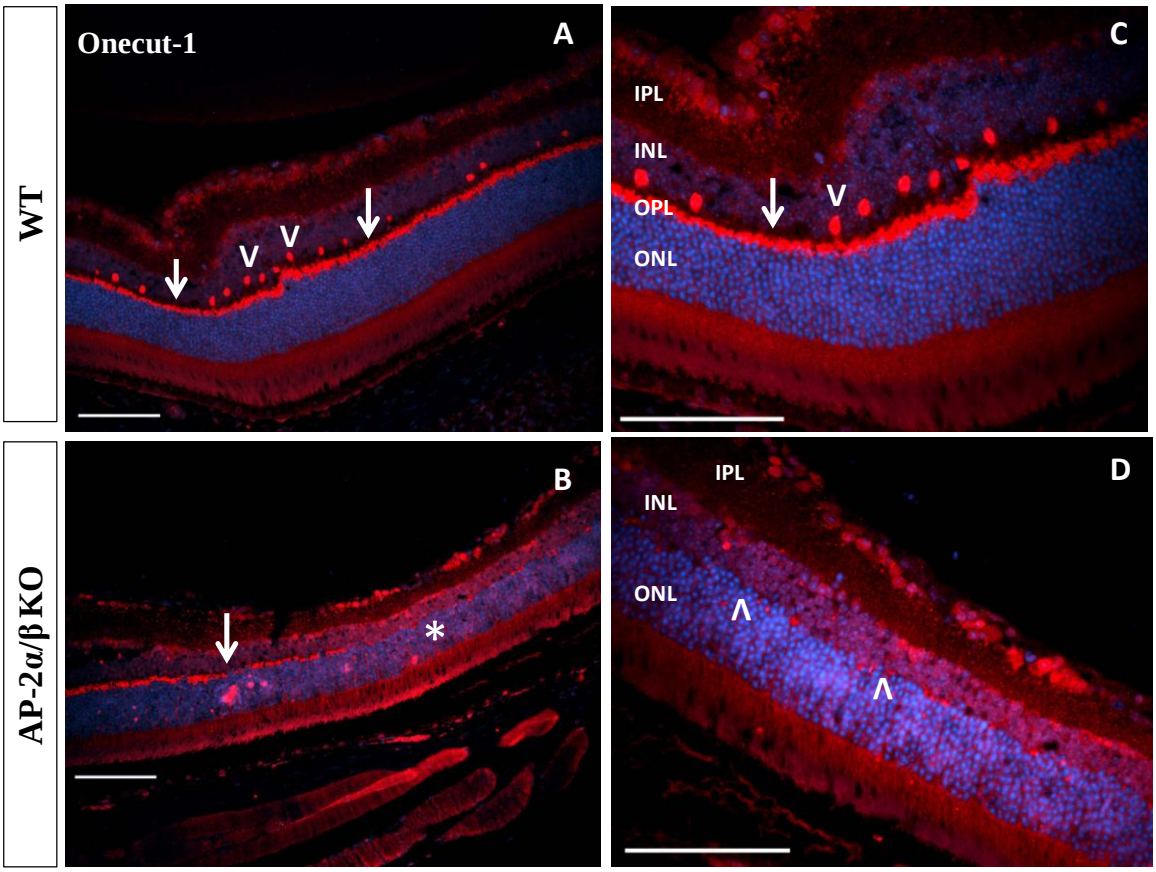


**Figure 7. Onecut-1 Expression**. WT (A, C). Onecut-1 is a marker of horizontal cell bodies (arrow heads) and their respective axons (arrows). The loss of horizontal cells is evident in the mutant retinas (B, D), however this loss is only present in the peripheral regions of the retina, where AP-2α and AP-2β are deleted. (B *). Examining the deleted region of the periphery (D) shows a full loss of horizontal cell soma and axonal processes (arrowheads). Scalebars: 100μm.

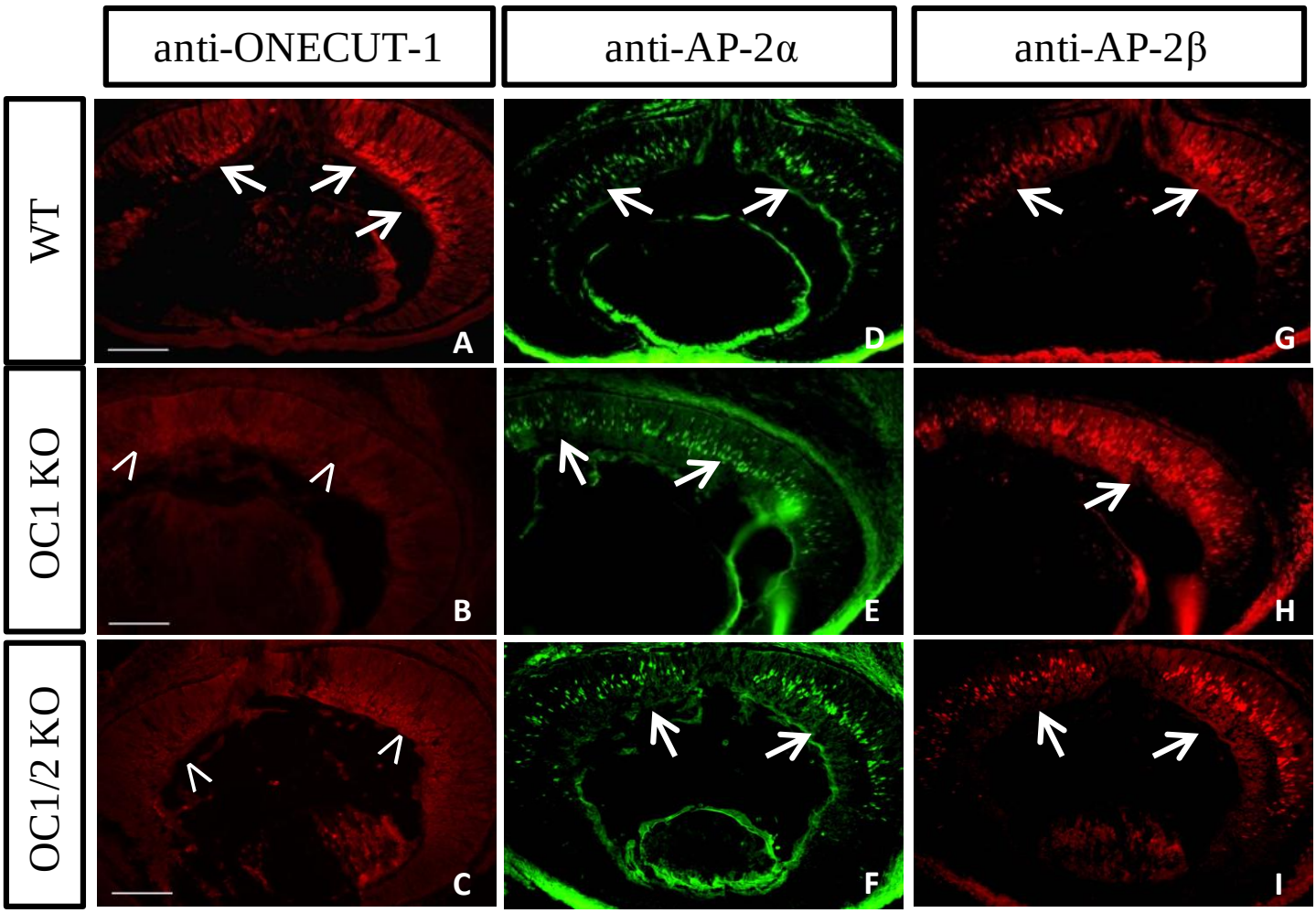
anti-ONECUT-1
anti-AP-2α
anti-AP-2β
WT
OC1 KO
OC1/2 KO
A
B
C
D
E
F
G
H
I

**Figure 8. AP-2 and Onecut-1 Expression**. WT (A, D, G) Oc1, AP-2α and AP-2β are expressed in the developing retina, in the presumptive inner nuclear layer (arrows). Oc1 expression is clearly lost in both Oc1 KO and Oc1/2 KOs at E14.5 (B, C, arrowheads), and interestingly both AP-2α and AP-2β expression is intact in these single and double Onecut KOs (E, F, H, I, arrows). Scalebars: 100μm.

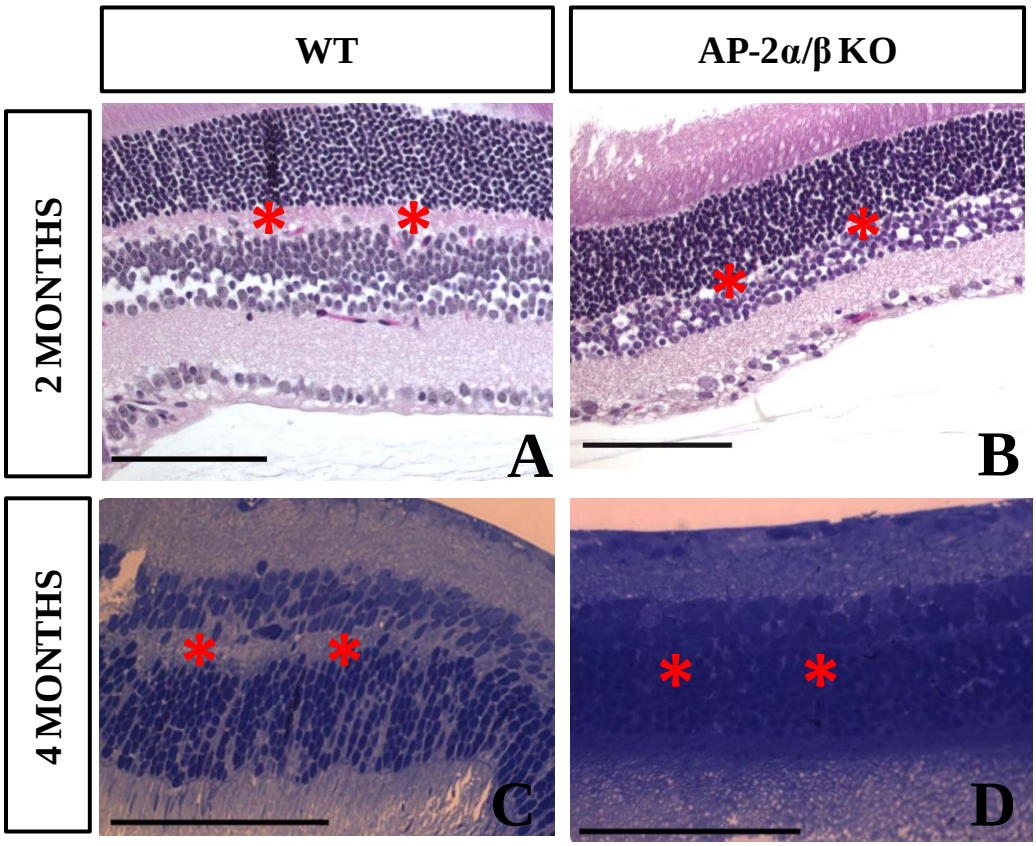


**Figure 9. Absent Outer Plexiform Layer**. (A, C) Wild type retina, 2 months and 4 months, respectively. All the nuclear and synaptic layers are intact, particularly the OPL (*). (B) Mutant retina, 2 months, the OPL has diminished significantly. (D) Mutant retina, 4 months. The OPL is practically absent, so that the border between the ONL and the INL is no longer distinguishable. Scalebars: 100μm.

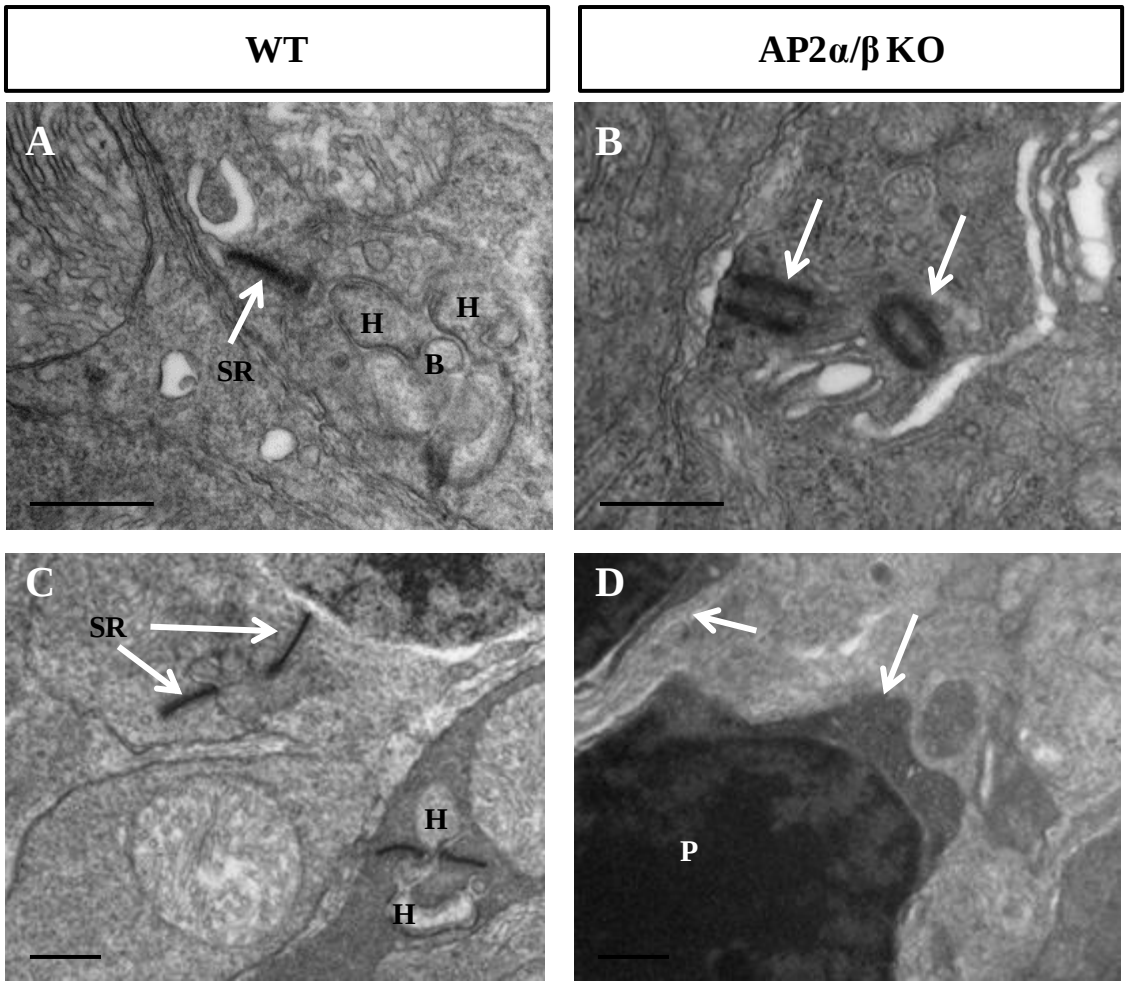


**Figure 10. Changes in the Outer Plexiform Layer.** (A, C) WT retina, 4 months, exhibit typical ribbon synapses involving horizontal and bipolar cells. There are no observable defects in photoreceptor axon terminals. (B, D) At the same age, double mutants lack rod ribbon synapses, and instead sporadically display spherical-like ribbons in the outer plexiform of the retina (B, arrows), and exhibit axon retraction (D, arrows). SR, synaptic ribbon; H, horizontal cell; B, bipolar cell; P, photoreceptor. Scalebars: 500 nm.

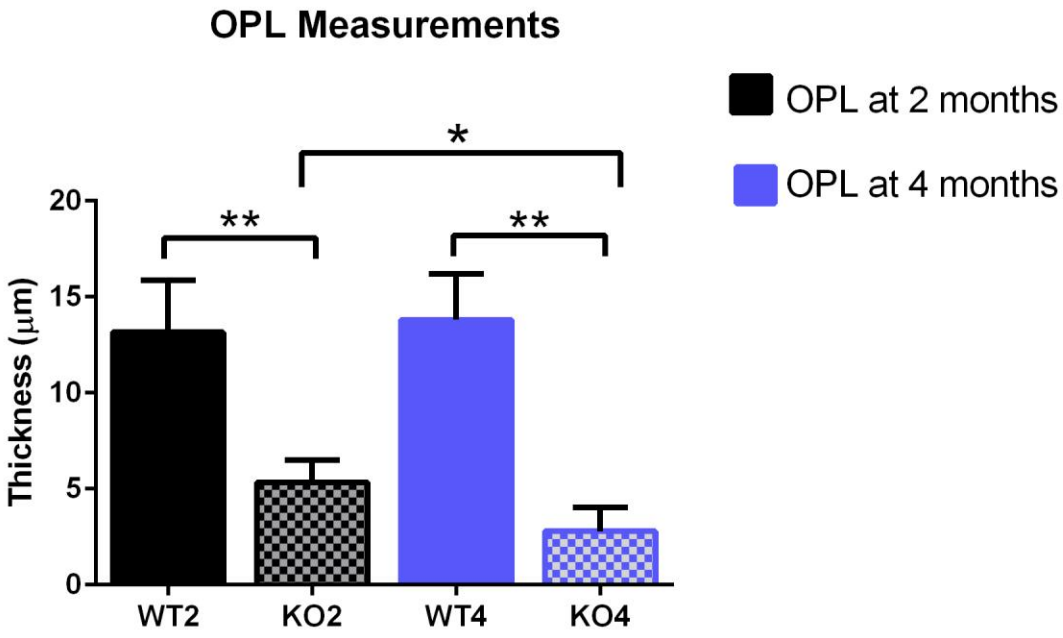


**Figure 11. Outer Plexiform Layer Diminishes Over Time.** Examination of the thickness of the OPL revealed striking changes at two and four months, black and blue bars respectively. The OPL of double KOs was significantly reduced at both the two and four month stage when compared to age-matched controls (**P<0.001). The OPL was also significantly reduced between the two and four month mark (*P<0.05). Bars represent the mean± SEM of counts from 3 animals.

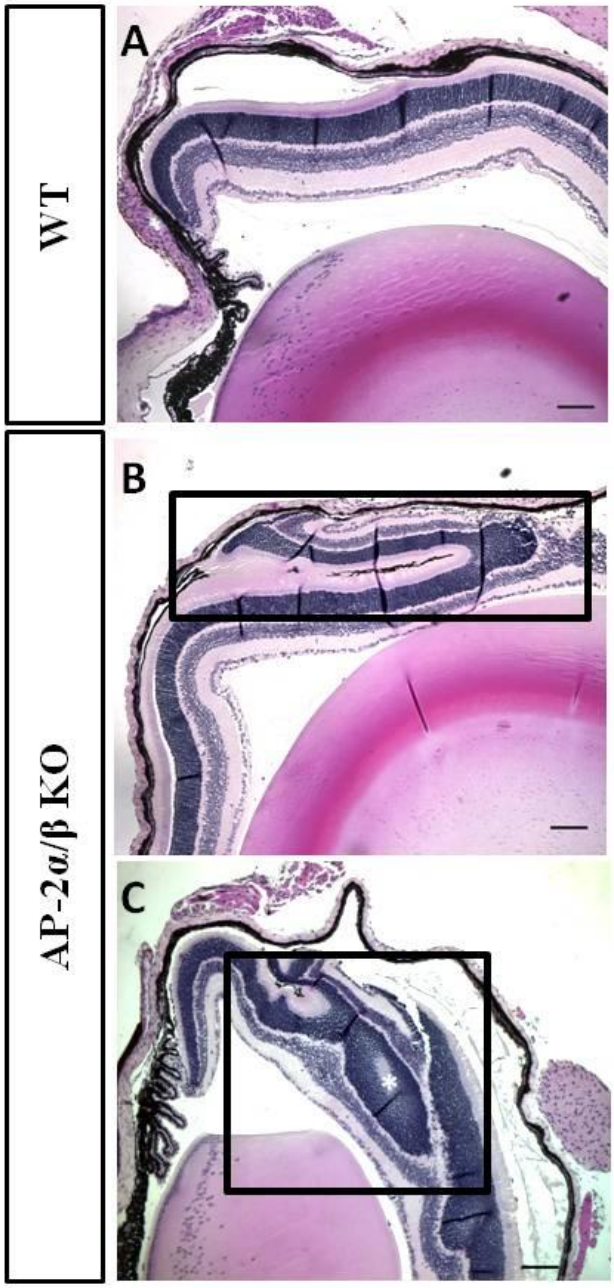


**Figure 12. Retinal Dysplasia.** Variable phenotypes were observed in the mutant retinas. (A) Wild-type. (C-D) AP-2α/β knockouts. The mutant retinas display significant abnormal folding (B, C boxes), including an inverted, double retina (B), multi-layering of both nuclear and synaptic retinal layers (C), and whorls of photoreceptors embedded within the outer segments (C*). Scalebars: 100µm.

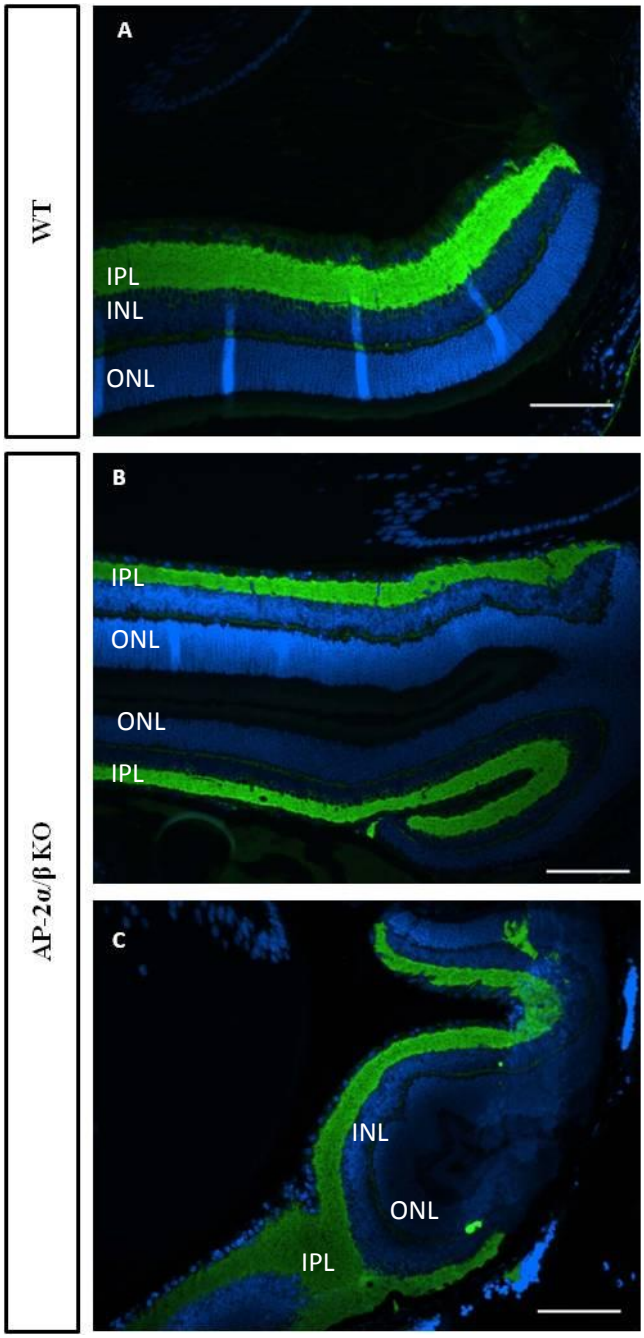


**Figure 13. Syntaxin-1 Expression Remains Unchanged.** (A) Syntaxin-1 (green) in the wild-type retina is predominantly expressed in the IPL. The disrupted layers of the dysplasic retina continue to possess intact synaptic processes of the IPL (B, C). IPL, inner plexiform layer, INL, inner nuclear layer, ONL, outer nuclear layer. Scalebars: 100μm.

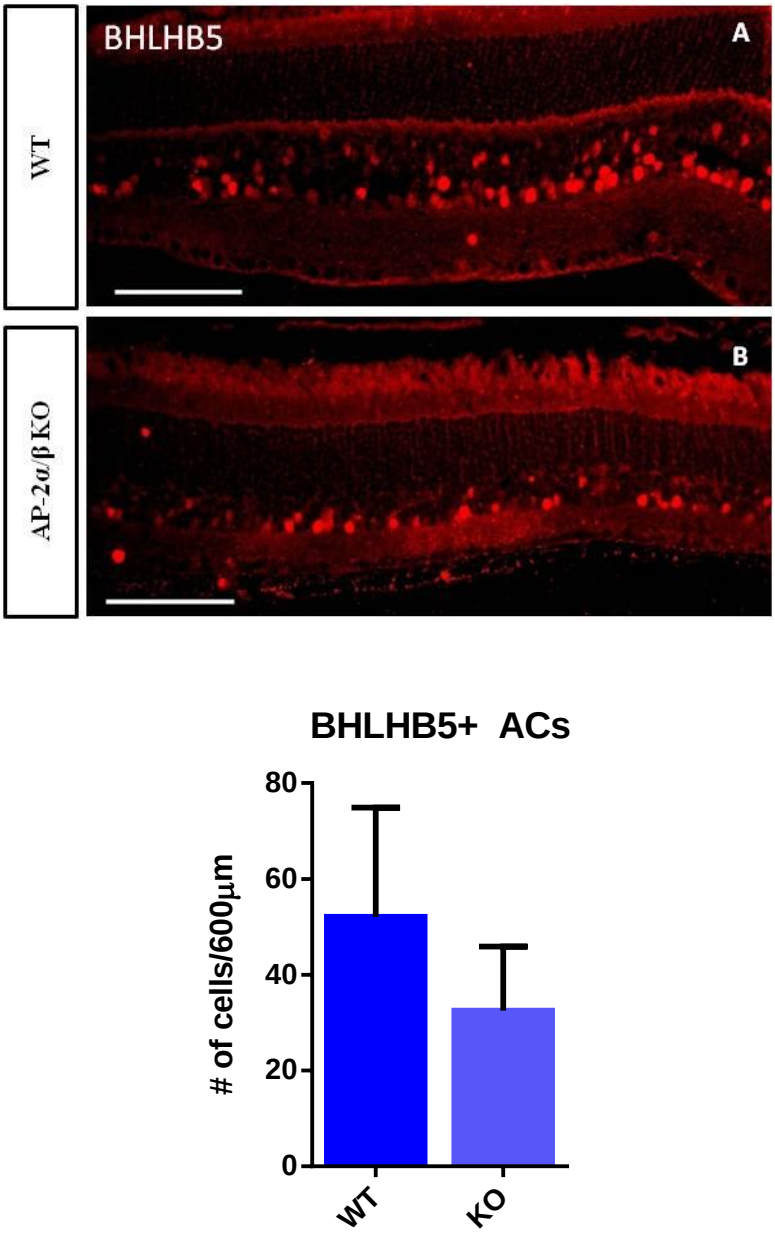


**Fig 14. BHLHB5 Expression as a Marker of GABAergic Amacrine Cells**. (A) WT. (B) AP-2α/β KO. Examination of the GABAergic marker, BHLHB5 did not reveal any abnormalities in the expression of this subpopulation of ACs (A,B). Quantification of BHLHB5+ ACs was conducted and did not reveal a significant change in total cell count, $P \geq 0.05$ Scalebars: 100µm.

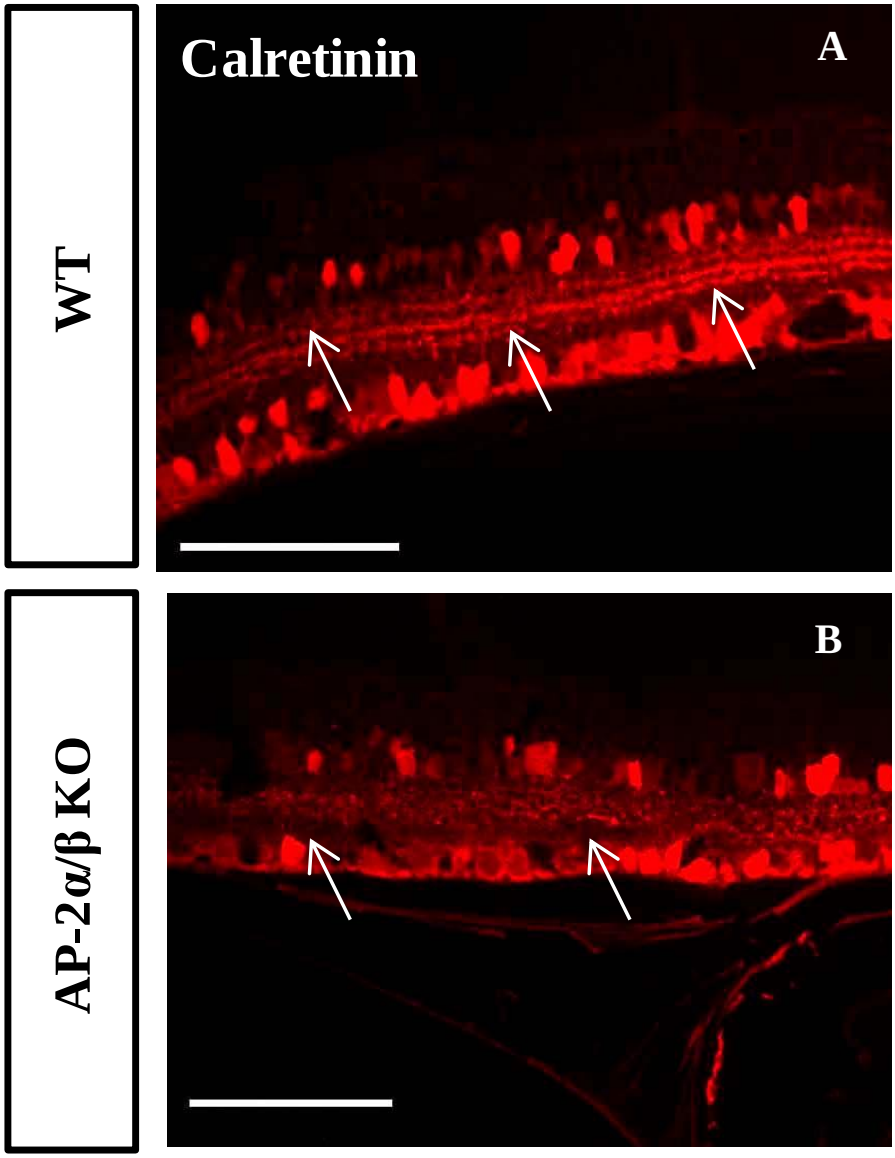


**Figure 15. Calretinin Expression as a Marker of GABAergic Amacrine Cells**. (A) WT. The distinct pattern of three dendritic bands (A, arrows) is completely absent from the IPL of the mutant retinas (B, arrows). Scalebars: 100μm.

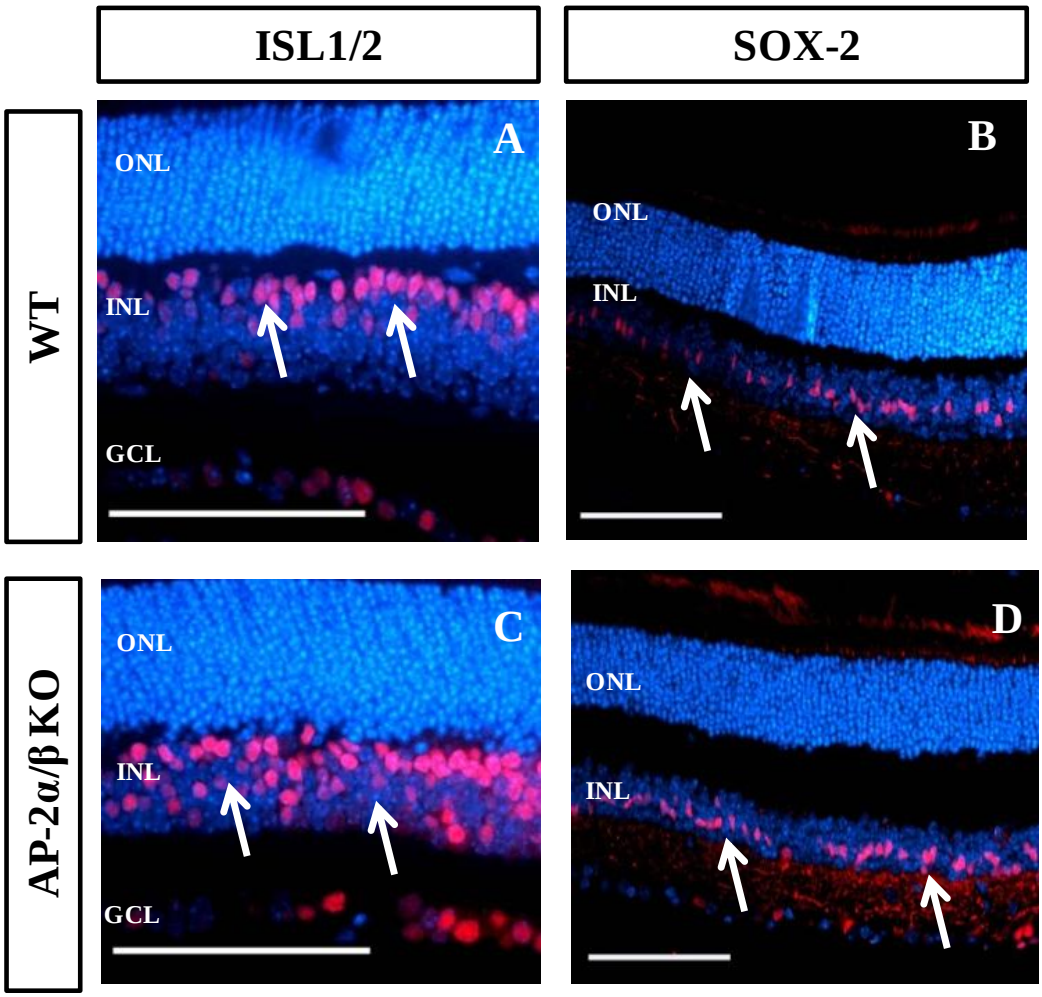


**Figure 16. Expression of Cholinergic Amacrine Cells**. Examination of Isl1/2 positive ACs (A,C) and Sox-2 positive ACs (B, D) did not reveal alteration in the staining pattern of these populations. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scalebars: 100µm.