THE REGULATORY ROLE OF AP-2β IN CORNEAL DEVELOPMENT

CORNEAL EPITHELIAL CELL FATE AND STRATIFICATION ABNORMALITIES OBSERVED FOLLOWING CONDITIONAL DELETION OF AP-2β IN THE NEURAL CREST CELLS OF THE MOUSE

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ABSTRACT

The cornea is an anterior eye structure that is specialized to be transparent. It is comprised of an endothelial monolayer, avascular stroma and stratified epithelium. Anterior ocular tissues, including the corneal endothelium and stroma, develop from the periocular mesenchyme (POM), which derives from neural crest cells (NCCs). Activating Protein-2β (AP-2β) is highly expressed in the POM. Our lab has used a conditional knock-out model in which AP-2β is deleted in the NCCs, known as the AP-2β NCC KO mouse, to investigate the role of this protein in anterior segment development. In these mutants we previously observed a closed iridocorneal angle, as well as corneal abnormalities including an absent endothelium, stromal neovascularization, and failed epithelial stratification. The present thesis sought to investigate corneal defects in the AP-2β NCC KO mouse, and particularly the corneal epithelium given that it arises from the surface ectoderm rather than POM. PAS and IHC staining showed changes to epithelial cell fate and stratification. We observed that Keratin-12, a marker of differentiated corneal epithelium, was absent, and Keratin-15, a limbal and conjunctival marker, was expanded across the epithelium. Changes to the basement membrane and integrin expression were also evident. Given the non-NCC origin of the epithelium we hypothesize that abnormalities in the corneal epithelium of the AP-2β NCC KO mouse result from changes to regulatory signaling from the POM-derived stroma. Our investigation of Wnt/β-Catenin signaling suggested an important role for this pathway through its interactions with growth factor Bmp4, which is supressed in the mutant. Ultimately these results indicate that AP-2β expression in the POM is crucial for normal corneal epithelial cell fate and stratification.

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LIST OF ABBREVIATIONS

AP-2	Activating Protein-2
ASD	Anterior Segment Dysgenesis
BM	Basement Membrane
Bmp4	Bone Morphogenic Protein-4
E	Embryonic Day
ECM	Extracellular Matrix
Fzd7	Frizzled-7
K12	Keratin-12
K15	Keratin-15
KO	Knock-out
HDs	Hemidesmosomes
IHC	Immunohistochemistry
Itga6	Integrin Alpha 6
Itga9	Integrin Alpha 9
LESCs	Limbal Epithelial Stem Cells
LSCD	Limbal Stem Cell Deficiency
MET	Mesenchyme-to-Epithelial Transition
NCCs	Neural Crest Cells
P	Post-Natal day
PAS	Periodic Acid Schiff
Pax6	Paired Box Protein-6
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
POM	Periocular Mesenchyme
RA	Retinoic Acid
SE	Surface Ectoderm
TAC	Transient Amplifying Cell
WT	Wild-Type

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Anatomy of the Anterior Segment of the Eye

The anterior segment of the vertebrate eye is composed of highly specialized structures that optimize retinal function, allowing for light to be transformed into the bio-electric signals necessary for vision. Comprised of a posterior and anterior chamber, the anterior segment of the eye contains clear aqueous humour, produced by the ciliary body, while the posterior segment of the eye contains vitreous humour (Figure 1) (Prada et al., 2019). The aqueous humour is secreted into the posterior chamber, between the lens and iris, and then travels through the pupil (the space within the iris) into the anterior chamber, between the iris and cornea. It serves to provide nutrients to specialized anterior eye structures that are devoid of blood vessels: the lens and cornea (Chen et al., 2016). These structures need to be transparent in order to refract incoming light. The aqueous humour finally flows through the trabecular meshwork, and then exits the eye into the vasculature via Schlemm's canal (Figure 1). It should be noted that in addition to the trabecular drainage pathway, aqueous humour can also enter the extracellular spaces of the iris root at the iridocorneal angle region and travel towards the sclera, entering lymphatic drainage.

The cornea is a specialized tissue in the anterior segment of eye that is essential for vision. It is completely transparent and refracts light prior to the lens, while also acting as a protective barrier for all the interior eye structures (Nowell and Radtke, 2017). The outermost, anterior cellular layer of the cornea is a stratified squamous, 6-7 cell epithelium (Chen et al., 2016). The innermost, posterior layer is an endothelial monolayer. Between the epithelium and endothelium is a highly organized stroma composed of keratocytes and collagen bundles (Chen et al., 2016). The cornea is completely devoid of blood vessels,

known as angiogenic privilege, allowing for the unobstructed passage of light and resulting clarity of vision (Gage et al., 2014). Essential nutrients are instead supplied via the aqueous humour. Lymphatic privilege is also characteristic of the cornea (Chen et al., 2016).

1.2 Embryonic Ocular Development

Various structures in the anterior segment of the eye are derived from the periocular mesenchyme (POM) tissue, including the ciliary body muscle, iris stroma, trabecular meshwork, Schlemm's canal and both the corneal endothelium and stroma (Figure 2) (Johnston et al., 1979; Williams and Bohnsack, 2015). Crucially, the POM arises from the cranial neural crest cells and the mesoderm (Gage et al., 2005; Gould et al., 2004). Neural crest cells (NCCs) are multipotent embryonic stem cells that originate at the neural plate border during gastrulation and migrate from folds of the neural ectoderm as the neuroepithelium closes to form the neural tube (Cvekl and Tamm, 2004). There are three different subpopulations of NCCs: (1) the cardiac NCCs, (2) the trunk NCCs, and (3) the cranial NCCs (Brewer et al., 2004). Migrating cranial NCCs give rise to a multitude of different tissue types including the POM in the region posterior to the optic cup during ocular development. The ocular surface ectoderm (SE), another embryonic lineage, gives rise to the lens, eyelid epidermis, conjunctiva and corneal epithelium (Figure 2) (Cvekl and Tamm, 2004; Gould et al., 2004). Components of the ciliary body and iris derive from the neural ectoderm (Williams and Bohnsack, 2015).

Vertebral eye development begins with the emergence of the optic pit, a bilateral evagination of the neuroepithelium of the diencephalon, derived from the embryonic neural

tube (Chow and Lang, 2001). Further evagination sees the pit expanding into the surrounding POM cells towards the overlying SE, forming the optic vesicle (Figure 3) (Chow and Lang, 2001; Swamynathan, 2013). The POM cells are derived from the NCCs and the mesoderm. At this point, embryonic day 9.5 (E9.5), a local thickening of the SE occurs, forming the lens placode (Chow and Lang, 2001). Through E10.5, the optic vesicle and lens placode both invaginate, the resulting space being the lens pit (Figure 3) (Beebe and Coats, 2000; Chow and Lang, 2001). The tissue surrounding the lens pit is the optic cup, and the inner layer will go on to become the neural retina, while the outer gives rise to the retinal pigmented epithelium (Cvekl and Tamm, 2004). At this point, the lens placode begins a process of separation from the SE, remaining connected by the lens stalk to the SE (Cvekl and Tamm, 2004). The lens vesicle detaches from the SE at E11.5, after which POM cells migrate into the region between the lens and SE between E12.5-13.5 (Figure 3) (Cvekl and Tamm, 2004; Gage et al., 2008; Swamynathan, 2013). The SE gives rise to the presumptive corneal epithelium, which is initially only 2 layers, but develops into a 6-7-layer stratified epithelium. In mice this migration occurs in a single wave, and by E13.5, 4-7 layers of mesenchymal cells are present with the numbers continuously increasing, resulting in flat layers of mesenchymal cells separated by loose extracellular matrix – early makings of the stroma (Figure 3) (Cvekl and Tamm, 2004; Gage et al., 2008). Through E14.5-15.5, the POM cells flatten and connect to those adjacent, forming an endothelial monolayer via mesenchyme-to-epithelial transition (MET) (Figure 3) (Cintron et al., 1983; Cvekl and Tamm, 2004). As embryonic development proceeds, the POM cells between the endothelium and epithelium continue to secrete loose extracellular matrix (ECM) and

differentiate into mature Keratocytes, which in turn secrete ECM components including collagen type I, V and VI, as well as keratan sulfate (Fini, 1999; Funderburgh et al., 2003; Linsenmayer et al., 1983). In humans, POM migration occurs in two waves, with the first forming the endothelium between the lens epithelium and overlying corneal epithelium through MET, and the second giving rise to the stromal keratocytes (Hay, 1980).

Concurrent with corneal development, the anterior periphery of the optic cup expands into the space between the forming cornea and lens (Cvekl and Tamm, 2004). Additionally, the ciliary body and iris are formed from another influx of POM cells. At E16.5, the iris stroma becomes separates from the posterior cornea, the space between is the anterior chamber (Cvekl and Tamm, 2004; Smith et al., 2001). Continuing through till E19.5, the iris elongates and the iridocorneal angle becomes populated by precursor cells for the aqueous outflow structures, the trabecular meshwork and Schlemm's canal (Cvekl and Tamm, 2004). Development of these structures will largely occur post-natally (Smith et al., 2001).

1.3 Corneal Epithelial Structure

In mice, the primitive 1-2 cell layer corneal epithelium arises from the overlying anterior SE though its further development does not progress until after birth (Figure 3). Upon eyelid opening at P12, this structure beginning to stratify, reaching 5-6 layers by P21, and becoming the fully mature 6-7 layer stratified squamous non-keratinized epithelium by 8-10 weeks of age (Lwigale, 2015; Rodrigues et al., 1987; Zieske, 2004). For humans, the epithelium similarly arises from the head SE, however stratification is complete upon birth

(Swamynathan, 2013). The corneal epithelium has a distinct basal layer of columnar cells which are adhered to a posterior basement membrane (BM) (McKay et al., 2020). The BM is composed of ECM which is secreted by the basal epithelial cells, and, to a lesser extent, the anterior stromal keratocytes. While the cornea is specialized to allow for the passage of light, the adjacent conjunctival tissue is not transparent. The conjunctiva is a vascular structure and the epithelium does not undergo the same organized stratification as the cornea (Gipson, 2007). The conjunctival epithelium is also defined by the presence of goblet cells, which secrete mucins into the tear film (Gipson, 2007).

Stem cells located in the limbus between the corneal and conjunctival epithelia are responsible for maintenance and repair of the corneal epithelium. These cells, known as the limbal epithelial stem cells (LESCs), are slow-dividing and have a high proliferative capacity (Lavker et al., 2020; Lehrer et al., 1998; Nowell and Radtke, 2017; Schermer et al., 1986). They give rise to transient amplifying cells (TACs) which migrate centripetally, moving towards the central cornea, while also proliferating, giving rise to terminally differentiated cells (Lehrer et al., 1998; Yazdanpanah et al., 2017). In mice, the LESCs are located in the basal limbal epithelium, however the human limbus is more complex. Structures known as the Palisades of Vogt are located at the human limbus, these have limbal epithelial crypts where the LESCs are found (Mort et al., 2012; Townsend, 1991). The LESCs have long been a focal point for research into corneal development, and wound healing. While some studies contend that stem cells are not solely confined to the limbal region, suggesting their presence in the basal corneal epithelium (Li et al., 2017; Tanifuji-Terai et al., 2006), the majority of research agrees on the limbal localization.

1.4 Anterior Segment Dysgenesis and Glaucoma

Anterior Segment Dysgenesis (ASD) describes a range of conditions in which abnormalities occur in the anterior segment of the eye. Issues with POM patterning and migration are thought to be the underlying cause of most ASD. Mutations in a number of different genes cause ASD including, *Pax6*, *Pitx2*, *Pitx3*, *Focc1*, *Foxe3*, *Eya1*, *Cyp1b1*, *Lmx1b*, and *Maf* (Cvekl and Tamm, 2004; Gould and John, 2002; Ito and Walter, 2014). While each of the specific pathologies caused by these mutations vary, ASD is a common characteristic. In 50% of cases ASD is accompanied by closed angle glaucoma (Ito and Walter, 2014). Glaucoma is characterized by a loss of retinal ganglion cells (RGCs) and excavation of the optic nerve head (ONH), resulting in accompanying defects to visual fields. Elevated intraocular pressure (IOP) is a major risk factor. Often in ASD the structural abnormalities of the anterior segment can result in anatomical and functional defects to the aqueous humour drainage systems, leading to a buildup of fluid and subsequently a higher IOP, favourable for glaucoma development (Kupfer and Kaiser-Kupfer, 1978, 1979). As such, congenital ASD conditions affecting the iridocorneal angle are a common cause of close angle glaucoma.

1.5 Key Genes Expressed in the POM for Anterior Segment Development

As explained in the previous sections, the POM gives rise to a number of anterior segment structures, including the corneal stroma and endothelium. Various transcription factors expressed in the POM are crucial for the regulation of proper development into the subsequent mature tissues. Severe pathologies including ASD and glaucoma can result in

response to changes to expression of these transcription factors, notably Foxc1, Pitx2 and Lmx1b. Mice which are homozygous null for *Focx1*, which encodes Foxc1, are characterized by adhesion between the cornea and lens, and a thickened corneal epithelium (Kidson et al., 1999). These mice do not survive past birth; however, mice that are heterozygous for *Foxc1* do survive and exhibit less severe ASD with cloudy corneas, close angle phenotypes, and irregular drainage structures (Smith et al., 2000). Pitx2 is expressed in the POM as early as E9.5 and continues to be expressed in the POM-derivates that become the angle structures, iris and corneal stroma. Mice which are homozygous null for *Pitx2* fail to form a corneal endothelium and anterior chamber, while the corneal epithelium is thickened and undifferentiated (Gage et al., 1999; Lu et al., 1999); however, these mice do not survive past E15.5. Transcription factor Lmx1b is observed in the POM and early developing cornea at E10.5. Mice which are homozygous null for *Lmx1b* are characterized by disorganization of the stromal keratocytes, hypoplasia of the iris and ciliary body, as well as generally smaller eyes (Pressman et al., 2000).

Axenfeld-Rieger syndrome, is an autosomal dominant syndrome in humans recognized by various congenital anterior segment defects (Tumer and Bach-Holm, 2009). Both Foxc1 and Pitx2 in the POM have been implicated. Nail-patella syndrome in humans has been found to be result from mutations in Lmx1b in the POM (Knoers et al., 2000; Lichter et al., 1997).

1.6 Activating Protein-2 Transcription Factors in the POM

The Activating Protein-2 (AP-2) family of transcription factors, including AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε, are retinoic acid responsive proteins involved in ocular development (Barzago et al., 2017; Bassett et al., 2012; Bassett et al., 2007; Bassett et al., 2010; Kerr et al., 2014; Martino et al., 2016; Pontoriero et al., 2008; West-Mays et al., 1999). Expression of AP-2 transcription factors is first evident in the pre-migratory NCC population, and therefore they are present in various NCC derivatives including pigmented cells, facial structures, and cranial and dorsal root ganglia among others (Brewer et al., 2004). The AP-2 family has been shown to regulate cell cycle, differentiation and apoptosis through a variety of mechanisms (Hilger-Eversheim et al., 2000). Of the five members of the AP-2 family, AP-2α and AP-2β are of particular interest in the developing eye (Bassett et al., 2012). Overlapping expression of both AP-2α and AP-2β is observed in the NCC-derived POM around the lens placode (West-Mays et al., 1999). By E15.5 however, AP-2β expression becomes predominant in the POM, and highly expressed in POM-derived tissues, such as the corneal endothelium and stroma (Martino et al., 2016). AP-2β is also strongly expressed in other POM derived tissues such as the trabecular meshwork, ciliary body muscle and iris stroma (Bassett et al., 2007; Martino et al., 2016; West-Mays et al., 1999). Of importance, AP-2β and AP-2α are also expressed in the embryonic SE that gives rise to the corneal epithelium (Martino et al., 2016). Our lab sought to develop a model in which we could observe the effects of AP-2β deletion on the developing anterior segment and ultimately achieve an understanding of the regulatory role this transcription factor plays.

1.7 AP-2β Neural Crest Cell Knock-Out Mouse Model

AP-2β germ-line null mice die pre-natally (Martino et al., 2016). Therefore, in order to study the role of AP-2β in anterior segment development, our lab used Wnt1Cre transgenic mice to conditionally delete AP-2β in the NCCs which contribute to the POM. This mutant is created with Cre-LoxP technology and uses a Wnt1-Cre transgene to target a floxed *Tfap2b* allele, which encodes AP-2β (Chen et al., 2016; Martino et al., 2016). Cre-recombinase is expressed under direction of Wnt1 regulatory sequences, to limit expression to the neural crest cell population. In our experimental mutants, known as AP-2β neural crest knockout (AP-2β NCC KO) mice, AP-2β is deleted in the NCCs when Wnt1Cre is first expressed in these cells at E8.5. In our breeding scheme (Figure 4), male mice hemizygous for the *Wnt1Cre* transgene are crossed with female mice heterozygous for the *Tfap2b* allele ($Tfap2b^{+/-}$). Male offspring that possess the *Wnt1Cre* transgene and are heterozygous for the *Tfap2b* allele ($Wnt1Cre^{Tg/0}$, $Tfap2b^{+/-}$) are bred with female mice possessing a floxed *Tfap2b* allele ($Tfap2b^{lox/lox}$). The resulting mutants express the Cre transgene and have one null allele for *Tfap2b*, and one floxed *Tfap2b* allele that is disrupted by Cre-recombinase excision ($Wnt1Cre^{Tg/0}$, $Tfap2b^{-/lox}$). Therefore, the resulting mice have one active copy of *Tfap2b* in all tissues, except the NCC and its derivatives, where it has been knocked out by Cre-recombinase.

Previous investigation of the AP-2β NCC KO model by our laboratory has confirmed that AP-2β expression is conditionally deleted in the POM and its derivatives (Martino et al., 2016). We have shown that at E15.5, AP-2β expression in the POM is significantly reduced relative to wild-type (WT) mice and at E18.5, AP-2β is suppressed in

the POM-derived structures including the corneal stroma and endothelium (Figure 5). We continue to see AP-2β expression in the SE-derived corneal epithelium. The AP-2β NCC KO mice present with a phenotype characteristic of ASD. An abnormal mutant phenotype has been observed as early as E15.5, including a disorganized corneal stroma and adherence between the lens and cornea (Martino et al., 2016). At 2-3 months of age, we have observed various anterior segment defects including a closed iridocorneal angle, corneolenticular adhesions, and significant corneal abnormalities (Figure 6). Prior investigation centred around the glaucomatous changes, raised intraocular pressure (3-fold greater relative to the controls) and loss of retinal ganglion cells, that accompany the closed iridocorneal angle. We have also investigated the effects of AP-2β deletion on POM-derived ocular drainage structures such as the trabecular meshwork and Schlemm's canal. Presently, we have directed our focus towards the corneal abnormalities that we observe in the AP-2β NCC KO mouse. In the mutant we have found the corneal endothelium to be absent, and the stroma to be hypercellular and vascular. Particularly drawing our focus is the corneal epithelium, which fails to stratify despite being of a non-POM embryonic origin.

1.8 Clinical Implications

Corneal blindness describes a range of pathologies in which the function of the cornea is compromised, and vision becomes severely impaired. Limbal stem cell deficiency (LSCD) is a congenital condition that leads to corneal blindness (Richardson et al., 2018). This condition results when the LESCs become depleted or lose function, presenting in the form of corneal ulceration, inflammation, neovascularization and conjunctivalization

(Richardson et al., 2018). The limbal region, and the role of the stem cells, is a highly researched area, in large part due to the role that these cells may play in corneal healing and repair. Additionally, epithelial and limbal transplants are among the main treatment measures for corneal blindness, especially in the case of congenital conditions. Given the severe corneal epithelial pathologies observed in the AP-2β NCC KO mice, AP-2β expression in the POM appears to play an important role in the regulation of corneal epithelial development. The corneal epithelial and stromal phenotypes we have observed do resemble the LSCD congenital condition. Further investigation of corneal epithelial development and differentiation in our model could provide insight into this blinding condition, and treatment of these pathologies.

1.9 Bi-Directional Stromal-Epithelial Signaling

As outlined above, AP-2β NCC deletion exhibits significant abnormalities with regard to corneal structure. The corneal epithelial defects are of interest, given that this tissue does not arise from the POM, and as such epithelial AP-2β expression should not be directly affected. We suspect that the defects observed in the epithelium are non-autonomous, and likely occur as a result of interaction with tissues derived from the POM in which AP-2β expression has been affected. Regulation of corneal epithelial development, including both cell fate and stratification, has been shown extensively to be dependent on bi-directional signaling which occurs between the POM-derived stroma and the SE-derived epithelium. Major pathways are involved in the interactions between these tissues, including Wnt/β-Catenin, Retinoic Acid (RA), and TGF-β signaling (Gage et al.,

2008; Ma and Lwigale, 2019; Matt et al., 2005; Molotkov et al., 2006). Genetic manipulation of these key regulatory pathways between the POM and SE have been shown to result in abnormalities in one or both of the developing and mature corneal stroma and epithelium (Gage et al., 2008; Ma and Lwigale, 2019; Matt et al., 2005; Molotkov et al., 2006).

Retinoic acid (RA) signaling is involved in various facets of ocular development. In epithelial tissues including the neuroretina, lens and prospective corneal epithelium, RA is synthesized by Raldh1 and Raldh3. RA impacts POM development by binding to the RA receptors (RAR) of these cells (Molotkov et al., 2006). Abnormalities with the RA signaling pathway can cause a range of ocular pathologies, often including corneal developmental defects. For example, the knock-down of POM RARa/b receptors with a Wnt1Cre driver results in the POM layer between the lens and SE becoming thickened, ultimately leading to issues with stromal organization (Matt et al., 2005).

TGF-β signaling, while often associated with wound healing, has also suggested to play a role in corneal development, cell migration and differentiation. The TGF-β receptor 2 (TGF-βR2) has been found to be an important part of corneal TGF-β signaling (Webber et al., 2016). In the mouse POM, transcriptome profiling has shown that TGF-βR2 is highly upregulated at E14.5 and E16.5, which suggests a potential role in regulating POM migration (Ma and Lwigale, 2019). Mutants that are deficient for TGF-βR2 are observed to be unable to phosphorylate Smad2, not express transcription factors Foxc1 and Pitx2, and display abnormal differentiation of keratocytes and collagen production (Ittner et al., 2005). Also, based on the finding that TGF-β expression results in upregulation of genes

that activate Wnt signaling, such as Hmga2, it is suspected that cross-talk occurs between these pathways (Ma and Lwigale, 2019).

Wnt/β-Catenin signaling has been studied extensively in the context of corneal development and has been suggested to play a role in epithelial stratification and fate determination. Concurrent with epithelial stratification, between P1-21 Wnt/β-Catenin signaling in the stroma gradually decreases (Zhang et al., 2015). It has been observed that in the case of β-Catenin gain-of-function in the stroma, epithelial stratification is impaired (1-2 layers at P21), while β-Catenin loss-of-function in the stroma leads to increased, early stratification (4-5 layers at P10) (Zhang et al., 2019; Zhang et al., 2015). These effects result from the Wnt/β-Catenin pathway regulating the expression of growth factors, like Bone morphogenic protein-4 (Bmp4), and transcription factors within both the stroma and epithelium. Stromal Wnt/β-Catenin signaling has also been found to play a role in determining epithelial phenotype through interacting the aforementioned Pitx2, and its downstream effector Dkk2, an inhibitor of Wnt signaling. Models in which Pitx2 or Dkk2 were conditionally knocked-down in the POM resulted in the corneal epithelium adopting a conjunctival fate due to elevation of Wnt/β-Catenin in the stroma (Gage et al., 2014; Gage et al., 2008). Furthermore, upregulation of components of the Wnt/β-Catenin signaling pathway, including crucial receptor Frizzled-7 (Fzd7), specifically at the basal limbal epithelium suggests an important role in maintaining the LESC niche (Sartaj et al., 2018). Ultimately, dysregulated Wnt/β-Catenin signaling between the stroma and epithelium could be central to causing the observed AP-2β NCC KO phenotype.

CHAPTER 2

RATIONALE, MAIN HYPOTHESIS, RESEARCH AIMS

2.1 Rationale for the Study

Proper development and maintenance of corneal structure is essential for vision to occur. The cornea is highly specialized to be transparent and allow for the refraction of light, however defects of the cornea, resulting from injury or congenital conditions, can impair vision and lead to corneal blindness. The process by which the POM gives rise to structures that form the anterior segment of the eye, including the corneal endothelium and stroma, is highly controlled and involves many different regulators. The transcription factor AP-2β is highly expressed in the developing POM and its derivatives. Our lab has used the AP-2β NCC KO mouse model to investigate how deletion of this protein impacts anterior segment development. In the cornea specifically, deletion of AP-2β in the NCCs resulted in a failure of the endothelium to develop and the stroma was disorganized, hypercellular and vascular. Of particular interest, the corneal epithelium fails to stratify despite this structure being derived from the surface ectoderm. This observation highlights the complex bi-directional signaling between the POM-derived stroma and SE-derived epithelium. Given the non-NCC origin of the epithelium, we suspect that the epithelial abnormalities in the AP-2β NCC KO mouse result due to changes in regulatory signaling from the POM-derived stroma.

In-depth investigation of the corneal epithelial abnormalities observed in the AP-2β NCC KO mouse will improve our understanding of the role that AP-2β plays as a regulator of POM development. Characterizing the changes to epithelial phenotype in greater detail will illustrate how AP-2β deletion affects key cell signaling pathways that promote stratification and determine cell fate. With the epithelium being of SE rather than

POM origin, regulatory pathways between the stroma and epithelium are focal points for this research. Ultimately by improving our understand of how AP-2β deletion elicits the observed changes on epithelial phenotype, crucial regulators of corneal epithelial development can be identified. The findings from this research may be applied to clinical work on congenital corneal epithelial pathologies, such as Limbal Stem Cell Deficiency, and healing in response to injury.

2.2 Main Hypothesis

Activating Protein-2 beta (AP-2β) expression in the periocular mesenchyme (POM) is essential for regulation of normal corneal epithelial development.

2.3 Research Aims

2.3.1 To use the AP-2β NCC KO mouse model to determine the effects of AP-2β deletion in the POM on stratification and phenotypic patterning of corneal epithelial cells.

In our previous research into the effects of AP-2β NCC KO on the anterior segment, we have observed that the corneal epithelium fails to stratify and that the epithelial basement membrane is absent. The present research seeks to elucidate in greater depth, how the epithelium changes due to deletion of AP-2β in the POM. High magnification imaging of the cornea will be conducted with ocular sections stained for Periodic Acid Schiff (PAS). This will allow us to compare structural differences between the WT and mutant across the surface epithelia, including the cornea, limbus and conjunctiva. Immunohistochemistry

(IHC) with immunofluorescent imaging will be employed to compare the expression of major corneal epithelial markers between WT and mutant mice. Keratin filaments are commonly used to differentiate between epithelial tissues – in the cornea we will check for the expression of Keratin-12, a marker of differentiated corneal epithelial cells, and Keratin-15, a marker of limbal and conjunctival epithelium. Transcription factors closely associated with cell fate, such as Pax6, will also be investigated. These results will indicate whether the cell fate of the corneal epithelium has been altered. To follow up on the observed failure to stratify, we will conduct IHC for Proliferating Cell Nuclear Antigen (PCNA), which identifies those cells undergoing proliferation. Additionally, to gain insight into the absence of the BM in the mutant, staining will be conducted for specific integrins. Changes to integrin expression could impact adhesion between the basal epithelium and BM. These experiments will ultimately indicate phenotypic differences between the WT and AP-2β NCC KO mouse. Beyond the differentiated central epithelial cells, we will also be able to determine if changes occur to the LESC population. Furthermore, conducting IHC for these phenotypic markers at different developmental timepoints (P7, P14, and 2-3-months) can allow us to observe how expression changes over time and if there is variance between the WT and mutant. Better understanding the mutant epithelial abnormalities on a cellular level will help focus our investigation into how deletion of AP-2β in the POM affects regulatory signaling and development of the epithelium.

2.3.2 To identify AP-2β dependent signaling pathways between the stroma and epithelium that ultimately regulate epithelial development.

The corneal epithelium is derived from the surface ectoderm, and our previous research has shown that while our mutant does not express AP-2β in the POM derived structures, including the corneal stroma and endothelium, this transcription factor is not eliminated from the SE and subsequent epithelium. Extensive research has shown that complex bi-direction regulatory signaling pathways occur between the stroma and epithelium which are essential for development and homeostasis. We hypothesize that in the AP-2β NCC KO mouse, deletion of AP-2β in the POM has disrupted regulatory signaling between the stroma and epithelium, leading to the changes in phenotype and stratification we observe. Strong evidence in the literature suggests that the Wnt/β-Catenin pathway is among those which occurs between the stroma and epithelium, and that it has a regulatory role in determining epithelial phenotype and stratification. As a result, we will conduct IHC for components of this pathway, including β-Catenin, Axin-2 and Fzd7, to determine if it is affected by AP-2β NCC KO. We will also investigate important growth factors and transcription factors, such as Bmp4 and P63 respectively, which have been shown to influence epithelial cell fate, while also being involved with stromal-epithelial signaling, and Wnt/β-catenin signaling specifically. The results from these experiments could potentially indicate how deletion of AP-2β in the NCC and subsequent POM, results in significant abnormalities to the SE-derived epithelium.

CHAPTER 3

EXPERIMENTAL DESIGN

3.1 Animal Husbandry

All procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were performed at McMaster University and mice were housed in the Central Animal Facility. Adult ear clippings were used for genotyping, with DNA being extracted using the EZNA Tissue DNA kit (Omega Bio-Tek). The AP-2β NCC KO mouse model was investigated with regard to differences in corneal structure and cell phenotype.

3.2 Generation of AP-2β NCC KO Mice

The breeding scheme described below that was used to generate the AP-2β NCC KO mice is also shown in Figure 4. This mutant mouse model has been used extensively by our lab for previous research studies. The AP-2β NCC KO mice was generated using Cre-LoxP technology. *Tfap2b*, the gene encoding AP-2β, has loxP sites flanking exon 4, and this floxed region was conditionally deleted from the NCC tissue through the use of a Cre transgene that is under the control of the Wnt1 promoter. The mutant was produced through a series of breeding crosses, in which mice hemizygous for the Wnt1Cre transgene, $Wnt1Cre^{Tg/0}$ ($H2az2^{Tg(Wnt1\text{-}cre)11Rth}$ Tg (Wnt1-GAL2)11Rth/J, Jackson Lab, Bar Harbor, ME), were bred with mice heterozygous for *Tfap2b*, $Tfap2b^{+/-}$ (Chen et al., 2016; Martino et al., 2016). From this first cross, males found to be $Wnt1Cre^{Tg/0}$; $Tfap2b^{+/-}$ were bred with female mice possessing the loxP flanked *Tfap2b* allele, $Tfap2b^{lox/lox}$. Offspring of this cross include the AP-2β NCC KO mice, $Wnt1Cre^{Tg/0}$; $Tfap2b^{-/lox}$, as well as littermates possessing

a minimum of one active copy of *Tfap2b* which were used as age-matched controls. The desired mutant will therefore have one active copy of *Tfap2b* in all tissues with the exception of the NCCs, where Wnt1 directs Cre recombinase to excise the loxP flanked exon, thus deleting AP-2β expression in the NCCs and NCC-derived tissues, including the POM. Necessary genotyping was conducted following standard PCR protocols (Table 1). Inbreeding between mouse lines for the final cross was avoided. The background strain used for all breeding crosses was C57BL/6J (Charles River, Wilmington, MA).

3.3 Histology

Eyeballs were enucleated from mice euthanized by CO_2 and fixed in 4% PFA for 2 hours prior to washes with 1xPBS and incubation overnight in 70% ethanol. Eyes were then processed (McMaster Immunology Research Centre Histology Core Facility, McMaster University) and embedded in paraffin wax. Sectioning was conducted at a thickness of 4 μm for both Periodic Acid Schiff (PAS) staining and immunohistochemistry (IHC) (Bassett et al., 2007).

3.4 Immunohistochemistry and Image Analysis

Paraffin embedded sections were deparaffinized in xylene and then rehydrated in decreasing ethanol concentrations (100%, 95%, and 70% ethanol), before a final wash in water. Heat mediated antigen retrieval was performed, with sections being placed in 10mM sodium citrate at pH 6.0 heated to 80-90^{o}C and maintained at this temperature for 20 minutes. Tris EDTA buffer at pH 9.0 was used according to the same procedure for Itag6.

Having unmasked the protein epitope, an incubation was commenced for 1 hour with the normal serum from the host-animal of the secondary antibody, at 5% in 1xPBS to block non-specific staining. A subsequent overnight incubation was conducted at 4^{o}C with the primary antibody at an appropriate dilution in 1xPBS (Bassett et al., 2007). The following primary antibodies were used: anti-K12 (Abcam, ab185627; 1:100), anti-K15 (Biolegend, 833904; 1:100), anti-PCNA (DAKO, M0879; 1:250), anti-Pax6 (Biolegend, 901301; 1:100), anti-Itga6 (Abcam, ab181551; 1:500), anti-Itag9 (Abcam, ab140599; 1:50), anti-Axin-2 (Abcam, ab32197; 1:50) anti-β-Catenin (BD Biosciences, 610154; 1:200), anti-Fzd7 (Abcam, ab64636; 1:250), anti-Bmp4 (Abcam, ab155033; 1:100), anti-pSmad1/5 (Cell Signaling Technologies, 9511; 1:50) and anti-P63 (Abcam, ab124762; 1:250). After washes with PBS, the appropriate Alexa Fluor secondary antibody, Alexa Fluor 568 (Invitrogen, Molecular Probes, Burlington, CA; 1:200), was added to the sections at a concentration of 1:200 diluted in 1xPBS with 1.5% normal serum from the host. A 1-hour incubation time was followed by three 5-minute washes with 0.1% Tween-20 solution. Mounting was conducted with ProLong Gold containing DAPI (Invitrogen, Molecular Probes, Burlington, CA) (Bassett et al., 2007).

Imaging was performed using a Leica microscope, with a bright-field attachment for imaging of PAS staining, and fluorescent attachment for immunofluorescence. A high-resolution camera and LasX software were used to acquire images. Quantification of nuclei/cells was performed using ImageJ software with the "Cell Counter" plug-in. Statistical analysis comparing cell counts for sections of WT and AP-2β NCC KO mutants was conducted using unpaired two-tailed t-tests ($p<0.05$) (Prism9, GraphPad Software, La

Jolla, CA, USA). When possible, two central corneal images of the same magnification were counted for each epithelial sample section. These values from the same section were averaged and considered to be the value for that one sample in order to ensure representative quantification and limit error. In all experiments, sample size (n) refers to individual eyeballs. When possible eyeballs from different mice were used in experiments.

Table 1. PCR protocols for genotyping

Alleles	Primers	Conditions	Products
Wnt1Cre	**Cre1** 5'-GCT GGT TAG CAG CGC AGG TGT AGA G-3' **Cre 3** 5'-CGC CAT CTT CCA GCA GGC GCA CC-3'	33 cycles of: 45 sec at 95°C 1 min at 67°C 1 min 10 sec at 72 °C	Cre transgene at 420bp
Tfap2b	**4 Exon DW** 5'-CCT CCC AAA TCT GTG ACT TCT-3' **PGK-PolyA** 5'-CTG CTC TTT ACT GAA GGC TCT TT-3' **4 Exon Rev** 5'-TTC TGA GGA CGC CGC CCA GG-3'	37 cycles of: 45 sec at 95 °C 45 sec at 58 °C 1 min at 72°C	$Tfap2b^-$ at 380bp $Tfap2b^+$ at 221bp

CHAPTER 4

RESULTS

4.1 Aim 1

4.1.1 Corneal Periodic Acid Schiff Staining

While only the corneal stroma and endothelium are derived from the POM, all three corneal tissues in the AP-2β NCC KO mice, including the SE-derived epithelium, displayed abnormalities. As shown with PAS staining in Figure 7, at 2-3 months of age, WT littermate mice exhibited all three specialized and distinct corneal tissues with a monolayer endothelium, avascular stroma and stratified epithelium. In contrast, the endothelium was absent in mature AP-2β NCC KO mice and the iris adhered to the corneal tissue. In the central WT epithelium, 6-7 layers of stratified squamous cells with a clear basal columnar layer were seen, posterior to which is the darkly PAS-stained epithelial basement membrane (Figure 7 A). The peripheral corneal epithelium, approaching the limbus between the cornea and conjunctiva, exhibited fewer stratified layers (Figure 7 C). In comparison, the corneal epithelium of the 2-3-month AP-2β NCC KO mice was not stratified, being composed of only 1-2 layers of flatter, smaller cells, with no distinct columnar basal cell layer (Figure 7 B, D). Both the epithelial and endothelial (Descemet's membrane) basement membranes were also absent in the AP-2β NCC KO mice compared to WT, as revealed by PAS staining (Figure 7).

4.1.2 Epithelial Expression of Keratin Filaments

Epithelial tissues are often differentiated based on the expression of different keratin filaments. In the cornea, mature differentiated epithelial cells can be identified based on expression of the Keratin-12 (K12) protein (Gage et al., 2014; Moll et al., 1982; Tseng et

al., 1982; Yoshida et al., 2006). The corneal limbal epithelial cells and conjunctival epithelium do not express K12, and instead can be identified by expression of Keratin-15 (K15), a putative marker of LESCs (Di Girolamo et al., 2015; Yoshida et al., 2006). These characteristic markers were used herein to determine the identity of the epithelial cells in the AP-2β NCC KO mice. K12 staining in the 2-3-month WT mouse revealed the expected expression pattern, with consistent high levels of K12 across the central cornea, tapering off at the periphery of the epithelium as it approaches the limbus and conjunctiva where no K12 was observed (Figure 8 A, C). In the AP-2β NCC KO model K12 expression was completely absent from the entire corneal epithelium (Figure 8 B, D). The expression pattern for K12 observed in the mature WT and mutant mice was consistent with the distribution of K12 at earlier developmental timepoints. As early as P7, K12 was present in the non-stratified WT epithelium, while being absent from the P7 mutant (Figure 9 A, B). Following eyelid opening, and the initiation of stratification, K12 expression was observed to be further upregulated in the P14 WT compared to the P7 timepoint (Figure 9).

Immunostaining for K15 in the 2-3-month WT demonstrated that the central cornea is devoid of expression, instead K15 was upregulated in the epithelium of the conjunctiva and corneal limbal epithelial cells (Figure 10 A, C). In comparison, the 2-3-month AP-2β NCC KO mice exhibited a shift in expression, with K15 being found throughout the central and peripheral corneal epithelium, continuous with the expression in the limbus and conjunctiva (Figure 10 B, D). Unlike K12 expression, which remained consistent at earlier timepoints, we observed that the K15 pattern in the cornea varied over time. In the unstratified WT epithelium at P7, K15 staining was observed across the entire extent of the

cornea rather than being confined to limbal and conjunctival regions as in the mature cornea (Figure 11 A). This expression pattern was the same as that observed for the P7 AP-2β NCC KO mice (Figure 11 B). Following eyelid opening, at P14 we saw that the WT phenotype does match that seen in the mature cornea, with K15 being confined to the limbal region and absent from the rest of the cornea (Figure 11 C). Similarly, for the mutant at P14, the observed phenotype matched that seen for the 2-3-month timepoint, with K15 staining continuing across the full extent of the corneal epithelium (Figure 11 D).

4.1.3 Epithelial Expression of PCNA

To determine the localization of epithelial cells actively undergoing proliferation, IHC was conducted using PCNA, a marker of proliferation that tags cells in the early G1 and S cell cycle phases (Gan et al., 2001). In the 2-3-month WT, specific nuclear PCNA expression was seen continuously across the basal corneal epithelium and throughout the limbus and conjunctiva (Figure 12 A, C). Staining for PCNA in the AP-2β NCC KO was observed to be comparable to the WT, with consistent nuclear expression (Figure 12 B, D). Cell counts conducted to determine the percentage of PCNA-positive basal cells revealed no significant difference between the WT and mutant (Figure 12 E).

4.1.4 Epithelial Expression of Pax6

Based on the observed phenotypic changes to the corneal epithelium, we investigated the expression of transcription factor Paired box protein-6 (Pax6), a known regulator of corneal cell fate that has been found to bind directly to the promoter of K12

(Kitazawa et al., 2017; Shiraishi et al., 1998). The Wnt/β-Catenin regulatory pathway has also been shown to negatively regulate Pax6, and as such inhibit the generation of a differentiated epithelial phenotype (Mukhopadhyay et al., 2006). Immunostaining for Pax6 in 2-3-month WT mice revealed high nuclear expression in all stratified layers, across the central and peripheral cornea, continuing throughout the limbus and conjunctiva (Figure 13 A, C). We observed no difference in the AP-2β NCC KO model, with a similar proportion of epithelial cells exhibiting nuclear Pax6 expression across both the central and peripheral cornea, extending through the limbus and conjunctiva (Figure 13 B, D). Cell counts confirmed this observation, with no significant difference between the percentage of Pax6-positive cells in the WT and mutant (Figure 13 E).

4.1.5 Epithelial Expression of Integrins

In the AP-2β NCC KO model, a key morphological difference is that the epithelial basement membrane (BM) is absent. Integrins are transmembrane receptors which function as alpha-beta heterodimers. Crucially, they are an essential component of intermediate filament based hemidesmosomes (HDs), which ensure adhesion between basal corneal epithelial cells and BM (McKay et al., 2020). Integrin alpha 6 beta 4 (Itga6b4) is an integral membrane component of HDs and forms a connection between the keratin filaments present in the cytoplasm of basal epithelial cells and laminin-332 in the BM (Litjens et al., 2006; Stepp et al., 1990). Decreased integrin expression results in poor adhesion to the BM and subsequently can impact epithelial stratification and phenotype (McKay et al., 2020).

Adhesion between these structures allows for transmission of cell signals between stromal keratocytes and basal epithelial cells.

Given the important role of integrins in facilitating BM production and adhesion, as well as the observed absence of a BM in the AP-2β KO model, IHC for Itga6 was conducted. Itga6 was selected as an indicator for the Itga6b4 heterodimer that is essential for HDs. In the 2-3-month WT mouse, staining for Itga6 was observed as expected at the basal membrane of the basal epithelial cells (Figure 14 A, C). Continuous expression was seen across the central and peripheral corneal epithelium, extending through the limbal region, but not into the conjunctiva. In the 2-3-month mutant, staining for Itga6 was present along the basal membrane of the basal epithelial cells of the central and peripheral cornea, however contrasting sharply with the WT, the mutant expression was not limited to the basal membrane (Figure 14 B, D). Itga6 staining in the mutant was observed at the lateral and apical membranes of basal cells, as well as in the membranes of non-basal cells.

Integrin alpha 9 (Itga9), which forms a heterodimer with Integrin beta 1 (Itgb1), has been found to be upregulated in the limbal region (Pajoohesh-Ganji and Stepp, 2005; Singh et al., 2009; Stepp et al., 1995). Unlike Itga6 which participates in cell-matrix adhesion, Itga9 is predominantly located in the apical and lateral membranes of basal epithelial cells (Pajoohesh-Ganji et al., 2004). Itga9 has been suggested to be necessary for TAC migration from the limbus to the central cornea (Singh et al., 2009). Given evidence for its role as a limbal marker, we conducted IHC for Itga9. In the 2-3-month WT mouse, Itga9 was observed consistently across the central and peripheral cornea in the apical and lateral membranes of basal epithelial cells (Figure 15 A, C). No upregulation of this Itga9 was

seen at the limbus. The mutant at 2-3 months displayed the same pattern of staining as the WT, with consistent expression of Itga9 at the lateral and apical membranes of basal cells throughout the central, peripheral and limbal epithelia (Figure 15 B, D).

4.2 Aim 2

4.2.1 Wnt/β-Catenin Signaling Pathway Components

We hypothesize that the epithelial abnormalities observed in the AP-2β NCC KO mouse, as discussed in Aim1, result due to dysregulation of signaling pathways between the stroma and epithelium, caused by deletion of AP-2β in the POM from which the stroma arises. Wnt/β-Catenin regulatory signaling has been researched extensively as a bi-directional pathway that is involved with epithelial stratification and differentiation. Canonical Wnt/β-Catenin signaling is initiated when Wnt ligands bind to a heterodimeric cell surface receptor, which consists of a Frizzled (Fzd) and a LRP5/6 subunit (Logan and Nusse, 2004). This binding initiates intracellular signaling which stabilizes β-Catenin and promotes its translocation to the nucleus for the regulation of target genes. Wnt/β-Catenin signaling has also been found to be upregulated in the limbal region, suggested to play a role in maintaining LESCs.

We conducted IHC for β-Catenin, as a key component of the signaling pathway, and Axin-2, a downstream effector. Ultimately the staining for both Axin-2 and β-Catenin displayed a high degree of variability between samples (data not shown). A consistent pattern of epithelial staining was not recognizable for samples within either the WT or mutant groups. Further, β-Catenin staining in the stroma was inconsistent. The high degree

of variability and ambiguity of these IHC results prevents us from drawing any conclusions about Wnt/β-Catenin signaling from these IHC experiments.

We also attempted IHC for Fzd7 as a major receptor in the Wnt/β-Catenin pathway, and a suggested marker of LESCs. In the 2-3-month WT, Fzd7 was observed to be upregulated in the limbus and conjunctiva, with lower expression in the central corneal epithelium (Figure 16 A, C). The 2-3-month mutant was observed to have consistent Fzd7 expression across the limbal and corneal epithelia at staining levels comparable to those seen in the limbal region of the WT (Figure 16 B, D). However, in both WT and mutant, the Fzd7 stain was unspecific and variability was evident between samples. As such, despite these results suggesting an upregulation of Fzd7 in the WT limbus and in the mutant central cornea, they should be treated as preliminary findings and follow-up is needed to draw a conclusion.

4.2.2 Growth Factor Bmp4

Another protein implicated in corneal cell fate determination is Bmp4, which is suggested to be necessary for stratification and the promotion of cell differentiation (Mohan et al., 1998; Shen et al., 2020). It has also been shown that Bmp4 is negatively regulated by Wnt/β-Catenin signaling (Gouveia et al., 2019; Zhang et al., 2015). Immunostaining for Bmp4 in the 2-3-month WT mouse revealed that the central corneal epithelium exhibited nuclear expression, which was most consistent in the basal layer (Figure 17 A). This expression was continuous throughout the peripheral cornea, but tapered off at the limbal region, and no Bmp4 expression was observed in the limbal or conjunctival epithelial cells

(Figure 17 C). A significantly different expression pattern was observed for the mutant, with a complete absence of Bmp4 staining across the central and peripheral corneal regions, as well as the limbus and conjunctiva (Figure 17 B, D). Together with the observed staining for K12, these results support the notion that Bmp4 plays a role in promoting stratification and differentiation of corneal epithelial cells. At earlier timepoints we observed a different expression pattern relative to the definitive phenotype seen at 2-3 months. At P7 consistent low levels of Bmp4 were seen throughout the conjunctival, limbal and corneal epithelia; however, specific nuclear staining was not observed (Figure 18 A). Additionally, high levels of stromal keratocyte Bmp4 expression was evident through the central cornea and at the angle region. The mutant at P7 showed very similar expression to the WT, including the high stromal Bmp4 levels (Figure 18 B). At the P14 stage, post-eyelid opening, Bmp4 staining in the WT showed specific nuclear expression within the central and peripheral cornea, with much supressed, non-nuclear staining at the limbus (Figure 18 C). Stromal staining was also observed to be significantly reduced at this stage relative to the P7. Contrasting with this, Bmp4 expression in the mutant at P14 was similar to P7, and predominantly non-nuclear through the conjunctival, limbal and corneal epithelia (Figure 18 D). In the stroma Bmp4 was observed at similar levels to those of the P14 WT.

Bmp4 binding to its receptor complexes initiates a Smad-dependent pathway by which Smad1 and Smad5 are phosphorylated, and form a complex enters the nucleus and regulates transcription factors to control gene expression (Tiwari et al., 2020; von Bubnoff and Cho, 2001). IHC was conducted for phosphorylated Smad1/5 (pSmad1/5). In the WT at 2-3 months, we observed consistent nuclear expression of pSmad1/5 across the corneal

epithelium, continuing through the limbus and conjunctiva (Figure 19 A, C). The same expression pattern was seen in the AP-2β NCC KO mouse (Figure 19 B, D). Pre-eyelid opening, prior to stratification, P7 IHC for pSmad1/5 revealed consistent nuclear expression in the epithelium and stromal keratocytes for both the WT and mutant (data not shown). Bmp4 also initiates Smad-independent downstream signaling to control important regulatory pathways. Our observation of unchanged pSmad1/5 expression in the mutant despite suppression of Bmp4 suggests that if Bmp4 is involved in generating the abnormal epithelial phenotype, this likely occurs through dysregulation of Bmp4 Smad-independent pathways. However, the IHC experiments conducted for pSmad1/5 were preliminary and additional follow up is needed to confirm these results.

4.2.3 Transcription Factor P63

As a putative LESC marker, P63 was also investigated in the AP-2β NCC KO model to provide insight into the epithelial phenotypic changes and the potential role played by the LESCs (Lavker et al., 2020). Immunostaining for 2-3-month WT mice demonstrated consistent nuclear expression of P63 across the basal epithelial layer of the central and peripheral cornea (Figure 20 A). Similar levels of basal epithelial expression were evident in both the limbus and conjunctiva (Figure 20 C). Stromal keratocyte expression of P63 was also consistently observed across the central and peripheral regions of the cornea (Figure 20 A, C). The same pattern of P63 expression was seen for the mutant. Staining for P63 in the mutant corneal epithelium showed consistent expression across the central and peripheral cornea, with a similar proportion of cells displaying nuclear staining (Figure 20

B, D). Also like the WT, no difference in P63 expression was observed between the limbal and conjunctival epithelia relative to the corneal epithelium. The levels of P63 seen in the stroma of the central and peripheral cornea were also comparable to those seen in the WT (Figure 20 B, D). Cell counting confirmed these observations, with no significant difference between the percentage of basal cells stained for P63 between the WT and mutant (Figure 20 E). Looking at the P7 and P14 timepoints, consistent nuclear localization of P63 across the corneal and limbal epithelia was observed in both the WT and mutant (Figure 21). At P7, P63 was highly expressed by the stromal keratocytes in both WT and mutant, with stromal expression still being evident, though decreased at P14.

CHAPTER 5

DISCUSSION, FURTHER DIRECTIONS AND CONCLUSION

Earlier observations of the corneal abnormalities in the AP-2β NCC KO mutant, prompted the present research to determine, in greater depth, how the cornea is altered and potential underlying genetic causes. In response to AP-2β deletion in the NCCs, it is not surprising that POM-derived tissues including the corneal endothelium and stroma would be impacted. This has been observed in the form of an absent endothelium and a hypercellular, vascular stroma (Martino et al., 2016). However, it was also found that the SE-derived corneal epithelium was adversely affected, with smaller, flatter cells as well as absent stratification. Given the non-NCC origin of the epithelium, the defects that we observed suggest that proper epithelial development is contingent on intact expression of AP-2β in the POM-derived stroma. We hypothesize that AP-2β expression in the POM is essential for regulation of normal corneal epithelial development. The results of our research revealed that, beyond the reduced stratification we had observed in previous studies, the epithelial cell fate is significantly altered, highlighted by the loss of K12, a marker of differentiated epithelial cells. Our investigation of regulatory signaling between the stroma and epithelium, and specifically the Wnt/β-Catenin pathway, has suggested that Bmp4 plays an important role in influencing both epithelial stratification and phenotype in the mutant. Considering our findings in relation to other studies in the field, promising future directions are clear for researching the cornea in the AP-2β NCC KO model. Ultimately by investigating the effects of AP-2β deletion in the POM on the cornea we are improving our understanding of normal corneal development and the complex regulatory signaling that is necessary.

5.1 Epithelial Cell Fate in the AP-2β NCC KO Mouse

By examining keratin filament expression, we were able to determine the epithelial identity of the corneal cells and demonstrate that phenotypic changes occurred in the AP-2β NCC KO corneal epithelium. For example, the loss of K12 expression within the mutant corneal epithelium indicates that these cells no longer have the differentiated corneal epithelial cell phenotype (Figure 8) (Gage et al., 2008; Moll et al., 1982; Tseng et al., 1982). This combined with the expansion of K15 expression, a limbal and conjunctival marker, across the extent of the corneal epithelium (Figure 10) provided further evidence of an altered cell fate (Di Girolamo et al., 2015; Yoshida et al., 2006). One possible conclusion to extrapolate from these findings is that a conjunctival-like phenotype has expanded throughout the central corneal epithelial region. In addition to K15 expression, other features of conjunctivalization observed in the mutant included neovascularization of the stroma, loss of the epithelial BM, and absent epithelial stratification (Chen et al., 2016; Martino et al., 2016). However, one prominent feature of conjunctivalization missing in the AP-2β NCC KO corneal epithelial region is the presence of goblet cells (Gage et al., 2008). This suggests that the epithelium has not completely shifted to a conjunctival phenotype. As observed in the 2-3-month WT littermate mice, K15 is expressed in the conjunctiva, but also in the corneal limbal epithelium (Figure 10). Thus, K15 expression in the central cornea of the mutant may be indicative of an expansion of the basal limbal epithelial cells across the entire cornea. Fitting with this hypothesis is the observation of decreased stratification and an absent PAS-stained BM, neither of which are present in mature WT limbal epithelium (Figure 7). Importantly, goblet cells are not a feature of the limbal

epithelium, and as such we would not expect to see them in the central cornea of the AP-2β NCC model if indeed these cells are now of limbal cell fate. We plan to investigate conjunctival specific markers and conduct limbal cell fate mapping experiments in our mutant to achieve a greater understanding of phenotypic differences observed as a result of AP-2β deletion in the POM.

Pax6 as a regulator of corneal epithelial cell fate has been shown to bind directly to the promoter of K12 (Kitazawa et al., 2017; Li et al., 2015; Shiraishi et al., 1998). Further, Pax6 deficient LESCs in culture have been shown to revert to a conjunctival phenotype, with specific loss of K3/K12 markers (Li et al., 2015), and in cases of severe ocular disease, deletion of Pax6 results in loss of K12 and increased K10, the latter being a marker for conjunctival tissue (Li et al., 2008). Together this suggests an important role for Pax6 in controlling the differentiated corneal epithelial cell phenotype. As such we expected that in the mature WT, Pax6 would be expressed in the regions where we observed K12, and indeed we found consistent nuclear epithelial Pax6 expression across the cornea (Figure 13) in the WT. Interestingly, despite the deficiency of K12 in the AP-2β NCC KO, we did not observe a significant difference in Pax6 expression as compared to the WT. While Pax6 may be involved in regulation of K12 expression in corneal epithelial cells, the lack of K12 expression observed in the AP-2β NCC KO mutant suggests that other regulators are required. Additional known transcriptions factors that positively regulate K12 include ESE-1 and KLF6 (Chiambaretta et al., 2002; Yoshida et al., 2000). The Kruppel-like factors (KLF) protein family, specifically KLF5 and KLF6, have also been shown to play a role in corneal epithelial development and maintenance including regulating expression of various

keratins (Kenchegowda et al., 2012). Thus, further investigation is required to determine the mechanism by which K12 expression is lost in the AP-2β NCC KO mutant epithelium.

If the basal limbal phenotype is indeed expanded in the AP-2β NCC KO mouse, this raises questions about the important stem cell population that is normally localized to this region. In 1986, Schermer et al. found evidence of stem cells located in the limbal region of the cornea (Schermer et al., 1986). The basal limbus is comprised of stem cells and their TAC progeny. The LESCs are recognised by their quiescence, but also proliferative capacity, and the expression of putative stem cell markers: N-cadherin, ABCB5, ABCG2, p63, Lrig1 and K15 (Lavker et al., 2020). Early TAC cells are present in the limbal basal layer among the LESCs, and at this point, are phenotypically indistinguishable; while, mature TAC cells migrate towards the basal central epithelium (Lehrer et al., 1998). Determining the impact of AP-2β KO on the LESCs may be crucial in understanding observed epithelial phenotypic changes. Given the reduced stratification in the mutant, in combination with K15 expansion potentially indicating that the LESCs have been impacted in our mutant, we sought to determine the location of proliferating cells. This was accomplished through IHC for PCNA, a marker of proliferation that tags cells in the early G1 and S cell cycle phases (Gan et al., 2001). We did not however observe any difference in PCNA expression between the WT and mutant at 2-3-months (Figure 12).

A key structural difference between the WT and AP-2β NCC KO corneas was the absence of a PAS-positive corneal epithelial BM in the mutant (Figure 7). In the WT, the BM was observed to be continuous across the central and peripheral cornea, only tapering off at the limbal region. The BM is composed of ECM secreted by the epithelial cells, and,

to a lesser extent, the anterior stromal keratocytes. The localization of this structure ensures its involvement with stromal-epithelial bi-directional signaling, and it has been suggested to play an essential role in initiating epithelial stratification (Chung et al., 1992; Singh et al., 2009). Without the BM, the normal regulatory pathways which occur between the stroma and epithelium, including the aforementioned Wnt/β-Catenin pathway, may be impacted and result in changes to cell phenotype or stratification. Adherence between the BM and basal epithelial cells is also essential for bi-directional signaling and involves integrins, a component of hemidesmosomes, binding to their ligands in the BM (Litjens et al., 2006; McKay et al., 2020; Stepp et al., 1990). Changes to the composition of the BM or basal cell integrin expression can also impact normal pathways (Litjens et al., 2006; McKay et al., 2020; Stepp et al., 1990). While PAS staining of the BM was absent in the AP-2β NCC KO cornea, which could indicate that the BM has not developed, another possibility is that a BM forms which has a different composition that is not revealed by PAS staining. Our lab plans to conduct additional investigation into the BM and determine if it is absent, as indicated by PAS staining, through the use of high magnification Transmission Electron Microscopy (TEM). This technique would reveal if a BM ultrastructure was still present in the mutant. Such a finding would align with the theory that a BM with a differential architecture forms, rather than the BM not developing entirely. An abnormal BM composition could be the result of AP-2β deletion impacting the capacity of the POM-derived stromal keratocytes to produce sufficient and proper ECM components for the BM. In turn, this could lead to issues with adhesion to the basal epithelium or impact the ability of regulatory proteins to travel between the stroma and epithelium. Further

investigation into how the BM is altered in our mutant could provide insight into changes in the regulatory signalling pathways at large.

The Integrin alpha 6 beta 4 heterodimer is essential for hemidesmosome based adhesion between the basal epithelial cells and BM. As expected, we observed consistent expression of Itga6 in the 2-3-month WT along the basal membrane of basal epithelial cells (Figure 14). In the mutant, this highly specific Itga6 staining was not observed, and while there was still basal membrane expression, high levels of Itga6 were also observed at the apical and lateral cell membranes of both basal and apical epithelial cells. This change could potentially indicate that the ligands which Itga6 binds to in the BM, such as Laminin-332, are no longer present, leading to its abnormal localization in the epithelial cell membranes, and ultimately suggesting that BM composition changes in the mutant. Expression of Itag6 in apical and lateral membranes of the mutant epithelial cells could also impact cell adhesion, and lead to changes in cell signaling or even stratification.

Previous studies have shown that Integrin alpha 9 (Itga9) is upregulated in the limbal region (Pajoohesh-Ganji and Stepp, 2005; Singh et al., 2009; Stepp et al., 1995). While Itga9 is expressed throughout all epithelial basal cells at post-natal day 10, it is limited to the limbus by day 21 (Pajoohesh-Ganji et al., 2004; Singh et al., 2009). In a conditional Itga9 KO model, using K14-Itga9 null mice, it was observed that basal cells were smaller and flatter, with overall decreased epithelial thickness and 33% less cell proliferation based on BrdU staining (Singh et al., 2009). Itga9 is largely associated with cell-cell adhesion and cell migration, with primary ligands including Tenascin-C, and the EIIIA segment of Fibronectin (Singh et al., 2009; Yokosaki et al., 1994). Unlike Itga6

which participates in cell-matrix adhesion, Itga9 is predominantly located in the apical and lateral membranes of basal epithelial cells (Pajoohesh-Ganji et al., 2004). Based on the phenotype in the Itga9 null mouse and studies looking at corneal epithelial repair, Itga9 has been suggested to be necessary for TAC migration from the limbus to the central cornea (Singh et al., 2009). Despite the findings of previous studies, our own IHC for Itga9 did not confirm this limbal localization. In the WT, we observed Itag9 expression to be confined to the apical and lateral cell membranes of basal epithelial cells; however, this pattern was consistent across the cornea, with no evidence of upregulation at the limbus (Figure 15). For the AP-2β NCC KO, we did not observe any difference relative to the WT, though due to the smaller and flatter epithelial cells, the staining appeared less specific.

5.2 Stromal-Epithelial Signaling in the AP-2β NCC KO Mouse

Since the corneal epithelium is of SE rather than POM origin, we hypothesized that the abnormalities observed are non-autonomous, and occur due to interaction with the POM-derived stroma in which AP-2β expression has been affected. The Wnt/β-Catenin signaling pathway between the stroma and epithelium has been shown in the literature to be important for determining stratification and cell fate.

Previous studies have shown that dysregulation of the Wnt/β-Catenin signaling pathway can lead to features of a conjunctivalization (Figure 22). Crucially, this includes an absence of K12, which we observe in our mutant model as discussed above. Dkk2 is an antagonist of Wnt/β-Catenin signaling, and a downstream effector of transcription factor Pitx2, that is normally expressed in the epithelium and POM (Gage et al., 2008). When

Pitx2 or Dkk2 are deleted, corneal stromal and epithelial tissue adopt a phenotype similar to the conjunctiva (Gage et al., 2014; Gage et al., 2008). This conjunctivalization results in blood vessels in the stroma, while in the corneal epithelium, goblet cells are present, K12 is absent, and conjunctival epithelial marker K4 is expressed (Gage et al., 2014) (Figure 18). Gage et al., also found that Axin-2, a downstream effector of Wnt/β-Catenin signaling, was upregulated in the epithelium of the Dkk2 and Pitx2 KO mice (Gage et al., 2008). Furthermore, Mukhopadhyay et al. saw that Pax6 expression is absent in the basal epithelial cells following Dkk2 KO as early as P10 (Mukhopadhyay et al., 2006). This led them to suggest that in the absence of Dkk2, Wnt signaling suppresses Pax6, resulting in the loss of corneal specific gene expression (Mukhopadhyay et al., 2006) (Figure 22). We conducted IHC for β-Catenin and Axin-2 in order to investigate the signaling pathway described here, as features such as a loss of K12 expression align with our mutant phenotype. Both of these experiments however produced inconclusive results, with a high degree of variation between samples of the same groups and generally unspecific staining (data not shown).

The Wnt/β-Catenin pathway has ligands and receptors all across the corneal epithelium, however this pathway is suggested to be most active in the limbus (Gage et al., 2008; Liu et al., 2003). Fzd7 is an essential receptor in Wnt/β-Catenin signaling and has been shown to be absent from the central cornea, and upregulated in the limbal region, specifically the LESCs based on co-localization with GFP in label retaining experiments (Sartaj et al., 2018). Ligands from the mesenchyme or epithelium may initiate the Wnt pathway in LESCs by binding to Fzd7. β-Catenin and Axin-2 have also been found to be

relatively upregulated in Fzd7-expressing cells (Sartaj et al., 2018). Knockdown of Fzd7 results in a significant decrease to the expression of putative LESC markers, suggesting that Wnt signaling helps maintain the LESC phenotype (Mei et al., 2014). Our finding of K15 expansion across the epithelium in the AP-2β NCC KO suggests that the LESCs may be implicated in establishing the mutant phenotype. As a result, we conducted IHC for Fzd7 as a suggested marker of the stem cells, as well as an indicator of Wnt/β-Catenin signaling. In the mature WT, we observed an upregulation of Fzd7 at the limbus and conjunctiva, relative to reduced, inconsistent expression in the central corneal epithelium (Figure 16). In the mutant, staining was observed consistently at similar levels in the limbus and central corneal epithelium. The finding of limbal localization in the WT supports the notion that Fzd7 is a LESC marker. Additionally, the upregulation of Fzd7 in the central cornea of the mutant implies that Wnt/β-Catenin signaling along the central cornea has increased, aligning with previous studies which suggest upregulation of this pathway leads to conjunctival features such as a loss of K12. Despite these results seeming promising, the IHC staining for Fzd7 was unspecific and variation existed between samples. These findings should be viewed as preliminary and more specific localization of this receptor in the WT and mutant cornea is needed for a definitive conclusion to be made.

Previous research has shown that Bmp4 is an important regulator of corneal stratification as well as involved in determining epithelial cell phenotype (Gouveia et al., 2019; Zhang et al., 2015). We investigated Bmp4 to determine if its expression was altered by AP-2β NCC KO. In the 2-3-month WT, we observed high levels of nuclear Bmp4 throughout the central corneal epithelium, though expression was absent from the

epithelium of the limbus and conjunctiva (Figure 17). This contrasted sharply with the complete absence of Bmp4 staining from the epithelium of the mature mutant cornea. The similar expression patterns for K12 and Bmp4 support the notion that Bmp4 has a function in determining cell fate. From immunostaining at earlier timepoints (P7) we also observed that prior to eyelid opening, the WT and mutant resemble each other closely, with low levels of epithelial and stromal expression (Figure 18). However, at P14, after eyelid opening and the initiation of stratification, the WT shows specific nuclear expression in the central cornea. This upregulation of Bmp4 in the central epithelial nuclei following eyelid opening, as well as its absence in the non-stratified mutant, strongly suggests its role in stratification.

Bmp4 and its receptors are expressed in human and mouse corneal epithelial cells and cell nuclei, however they are also expressed by the stromal keratocytes (Maruyama-Koide et al., 2017; Mohan et al., 1998; Zhang et al., 2015). The process by which Bmp4 regulates corneal epithelial development likely involves cross-talk between the mesenchyme and epithelium to regulate necessary gene expression. Various studies have investigated Bmp4 in the in the context of its relationship with the Wnt/β-Catenin pathway. In mice, Zhang et al. found evidence that when β-Catenin expression is suppressed in stromal keratocytes, upregulation of Bmp4 is observed in the stroma and epithelium, as well as increased stratification with 4-5 cell layers as early as P10 (Zhang et al., 2015). Another study from Zhang et al. found that β-Catenin overexpression leads to Bmp4 downregulation along with decreased stratification, with only 1-3 cell layers at P21 (Zhang et al., 2019). Further experimentation showed that between P10-21, Bmp4 expression in

the stroma increases, and for mice treated with recombinant Bmp4 daily from P0-8, early stratification was evident, with 3-4 cell layers at P10 relative to 1-2 in the control (Zhang et al., 2015). They suggested that β-Catenin inhibits Bmp4 expression in the stroma prior to eyelid opening; however, after eyelid opening, Wnt/β-Catenin signaling is reduced, allowing for upregulation of Bmp4 in the stroma, which promotes epithelial stratification through Smad-dependent activation of transcription factor P63 (Figure 23) (Zhang et al., 2019; Zhang et al., 2015). Although these findings focused on paracrine signaling from Bmp4 produced in the stroma, the general expression pattern concurs with our observation of reduced stratification in the absence of Bmp4 for the mutant corneal epithelium (Figure 17) and paralleled our findings of nuclear Bmp4 expression beginning in the P14 samples (Figure 18). Despite stromal Bmp4 not being observed in our 2-3-month mice, as mentioned previously, the mechanisms of Bmp4 signaling are complex and uncertain, and likely involve extensive bi-directional signaling between the stroma and epithelium (Shen et al., 2020).

Our results showed a distinct expression pattern for Bmp4 in the WT, with nuclear expression in the central and peripheral epithelial regions, but not in the limbal epithelium (Figure 17). A study from Gouveia et al. using human LESCs found evidence that nuclear β-Catenin suppresses Bmp4-mediated cell differentiation as a means of maintaining the stem cell phenotype (Gouveia et al., 2019). In mice, various studies have identified an upregulation of Wnt/β-Catenin signaling at the limbal region, including high levels of the receptor Fzd7, providing further support for the notion of Wnt/β-Catenin signaling suppressing Bmp4 in the LESCs (Gage et al., 2008; Liu et al., 2003; Nakatsu et al., 2013;

Sartaj et al., 2018). Thus, while Bmp4 is supressed in the LESCs by Wnt/β-Catenin signaling, the central cornea exhibits high Bmp4 expression, contributing to stratification and differentiation. Based on these studies, and the reduced levels of Bmp4 found in the AP-2β NCC KO mice, further investigation of Wnt/β-Catenin signaling in the mutants may provide valuable insight into how the corneal epithelial phenotype and stratification are regulated by AP-2β.

Transcription factor P63 is frequently investigated in corneal epithelial research and is hypothesized to play an important role in maintaining proliferative potential and initiating stratification (Lavker et al., 2020; Lehrer et al., 1998). While some studies, often with human tissue, have found evidence that P63 is upregulated in the limbal region as a marker of LESCs (Chen et al., 2004; Kawasaki et al., 2006; Nakatsu et al., 2013), others have observed expression of P63 throughout the basal epithelial cells of the entire cornea in mice (Li et al., 2017; Zhang et al., 2019). In the mature WT mouse, we found that P63 was expressed consistently in basal epithelial cells across the cornea, including both central and limbal regions (Figure 20). In addition, preferential limbal expression of P63 was not observed at earlier timepoints P7 and P14 (Figure 21). As mentioned above, P63 has been proposed as a downstream effector of Bmp4 that promotes stratification (Zhang et al., 2015) after upregulation by pSmad1/5. However, despite the absence of Bmp4 from our mutant, we did not observe any significant change in P63 expression relative to the WT. Across the central and limbal epithelium, a similar proportion of basal epithelial cells displayed nuclear expression of P63 for both the WT and the AP-2β NCC KO model (Figure 20). These findings suggest that the deficient stratification observed as a consequence of AP-2β

deletion occurs via a mechanism independent of P63. Despite conflicting evidence in mice, P63 has been shown extensively to be a LESC marker in humans (Chen et al., 2004; Kawasaki et al., 2006; Nakatsu et al., 2013). Proteins such as YAP and SOX2 have been suggested to regulate P63 in human LESCs in order to maintain proliferative capacity and an undifferentiated state (Bhattacharya et al., 2019; Gouveia et al., 2019). Additional investigation of P63 and its associated regulators, may give further insight into the impact of AP-2β deletion on the LESC population.

While previous studies did find evidence for Bmp4 Smad-dependent regulation of P63, we did not find any change to P63 despite Bmp4 being absent in the mutant (Zhang et al., 2019; Zhang et al., 2015). Further, the IHC we conducted for pSmad1/5 revealed consistent nuclear expression across all regions of the corneal and limbal epithelia in both the WT and AP-2β NCC KO mice (Figure 19). The differential expression of Bmp4 between WT and mutant suggests a role for this protein, however unchanged expression of pSmad1/5 and P63 suggest that this signaling axis is not involved in causing stratification or cell fate changes. Bmp4 also initiates downstream Smad-independent signaling, influencing important pathways such as P38 MAPK and ERK1/2 signaling (Shen et al., 2020). In our model, Bmp4 suppression caused by AP-2β deletion may impact stratification and differentiation through altering Smad-independent effectors of Bmp4 signaling rather than P63 through Smad-dependent mechanisms.

5.3 Future Directions and Conclusions

AP-2β deletion in the NCC-derived POM, as carried out in the AP-2β NCC KO model was shown to indirectly elicit profound effects on cell phenotype and stratification of the SE-derived corneal epithelium. Perhaps the most significant finding from this research is the change in epithelial cell fate that we identified based on expression of keratin filaments. The absence of K12 in the mutant indicates that these cells are not differentiated epithelial cells, however the expression of K15 across the entire corneal epithelium could be indicative of a shift in cell fate towards a conjunctival or a basal limbal cell phenotype. Given the absence of goblet cells, it is more likely that the limbal epithelial phenotype is expanded, but additional experiments are needed to confirm this theory. Conducting IHC with a conjunctival specific marker, such as Keratin-7 or Keratin-4 (Gage et al., 2008), could provide the necessary evidence. The absence of staining for these conjunctival markers in the mutant central cornea would confirm that these epithelial cells have not taken on a conjunctival phenotype.

The LESCs are extensively researched in the context of corneal epithelial development and repair. They are also involved in medical treatments for corneal pathologies and their damage can lead to corneal defects that cause blindness, as is the case in Limbal Stem Cell Deficiency. Given the changes to cell fate and stratification we observed in the mutant mouse, it is important that we understand how AP-2β NCC KO influences this critical stem cell population. While K15 did successfully stain the basal limbal region, this staining was also present in the conjunctiva. This was similarly the case with the Fzd7 receptor, which preliminary IHC showed was upregulated in the limbus and

conjunctiva, but increased staining specificity is required to draw a conclusion. In order to accurately investigate the LESC population in our AP-2β NCC KO model, as well as visualize the changes to this cell population over time, we plan to conduct lineage tracing experiments using a pulse-chase model. The results of these experiments in combination with the findings of this thesis may ultimately improve our understanding exactly how cell fate is changed in our mutant and highlight avenues for further exploration.

Compelling evidence in the literature directed our focus to Wnt/β-Catenin signaling as a regulatory pathway between the stroma and epithelium. IHC that we conducted for components of this pathway, including β-Catenin and Axin-2, produced results that were inconclusive due to high intra-group variation and unspecific staining. In working with these same antibodies, anti-Fzd7, anti-β-Catenin and anti-Axin-2, other studies appeared to have more success producing specific stains using cryo-sectioning for IHC rather than paraffin sections. Going forward, re-testing these antibodies with cryo-sections may allow us to draw conclusions regarding the localization of these proteins and determine if there is differential expression in the AP-2β NCC KO mouse. Previous studies have found evidence that Bmp4 is supressed by Wnt/β-Catenin signaling, and that its expression is necessary for stratification and differentiation (Gouveia et al., 2019; Zhang et al., 2019; Zhang et al., 2015). Our IHC for Bmp4 conclusively showed that it is not expressed in the limbal region of the WT, and that it is absent from the mutant epithelium. These significant findings warrant follow-up. The IHC we conducted for P63 and pSmad1/5 showing no change between WT and mutant suggests that Smad-dependent activation of P63 is not affected. However, the effects of altered Bmp4 expression in the mutant may be elicited via Smad-

independent pathways, such as P38 MAPK signaling, which studies have implicated in epithelial development and repair (Shen et al., 2020). Such alternative effectors of Bmp4 signaling warrant investigation in the context of the AP-2β NCC KO model.

The AP-2β NCC KO cornea exhibited an absence of stratification, an expansion of K15 and loss of K12 expression. Although this keratin profile suggests conjunctival-like features, the absence of goblet cells is notable, and thus we support the alternative hypothesis, that the limbal epithelial phenotype is expanded across the entire corneal surface in the mutant. The absence of Bmp4 expression in the KO epithelium may have contributed to the reduction in stratification and altered cell fate since Bmp4 is a known regulator of these processes. Cross-talk between the stroma and epithelium has been shown to be critical in the development and maintenance of these tissues (Pearton et al., 2005; Walker et al., 2020). We hypothesize that the defects we have observed in the epithelium of AP-2β NCC KO mutant mice are the result of changes to regulatory signaling from the POM-derived stroma in which AP-2β has been deleted. Our current findings, in combination with those of previous studies, suggest that Wnt/β-Catenin signaling in the POM-derived corneal stroma may regulate epithelial Bmp4 expression. Further investigation of epithelial abnormalities in the AP-2β NCC KO model has the potential to improve our understanding of the underlying genetic causes that account for disorders involving the corneal epithelium.

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FIGURES

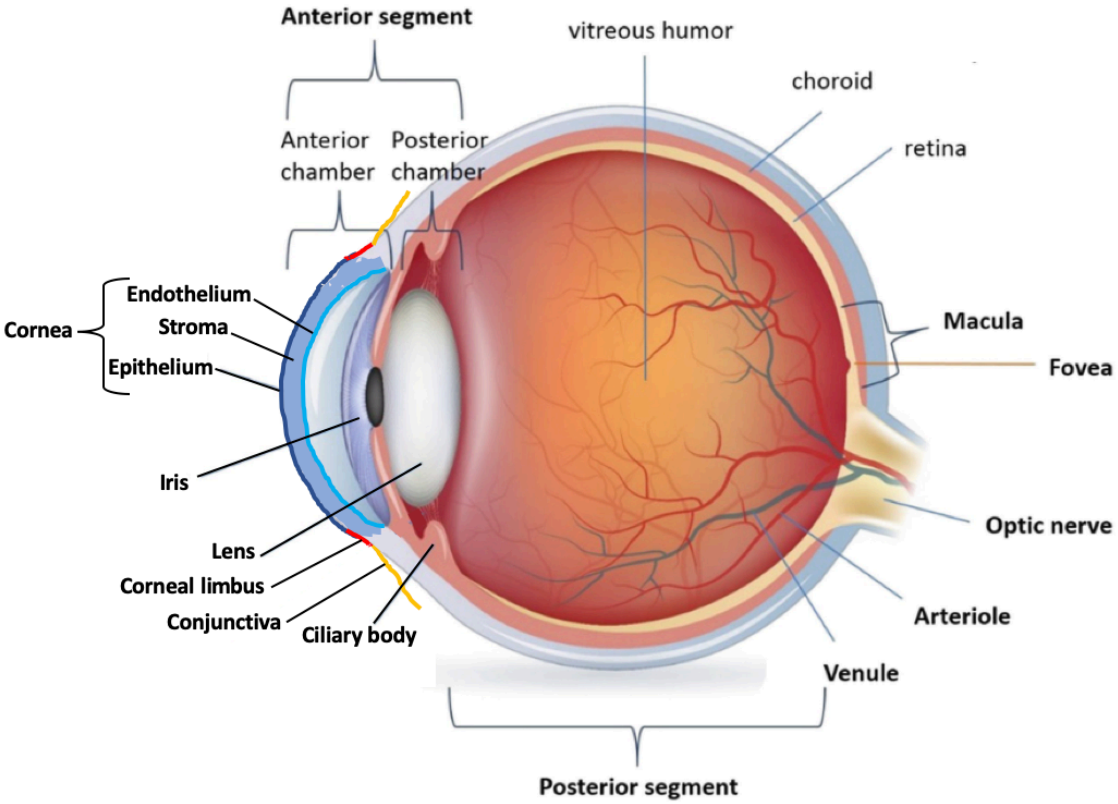

Figure 1. Anterior segment anatomy. The anterior segment of the eye consists of the lens and those structures anterior, including the iris, ciliary body, cornea, trabecular meshwork and Schlemm's canal. The anterior chamber of the anterior segment of the eye is the space between the cornea and iris, while the posterior chamber is between the iris and lens. Aqueous humour secreted by the ciliary body moves from the posterior chamber, through the pupil (gap in the iris) to the anterior chamber. The anterior surface of the eye consists of a transparent cornea, which is continuous with the conjunctiva with a distinct limbus between. The cornea itself has three layers, the epithelium, stroma and endothelium. Adapted from: (Cioffi, 2020)

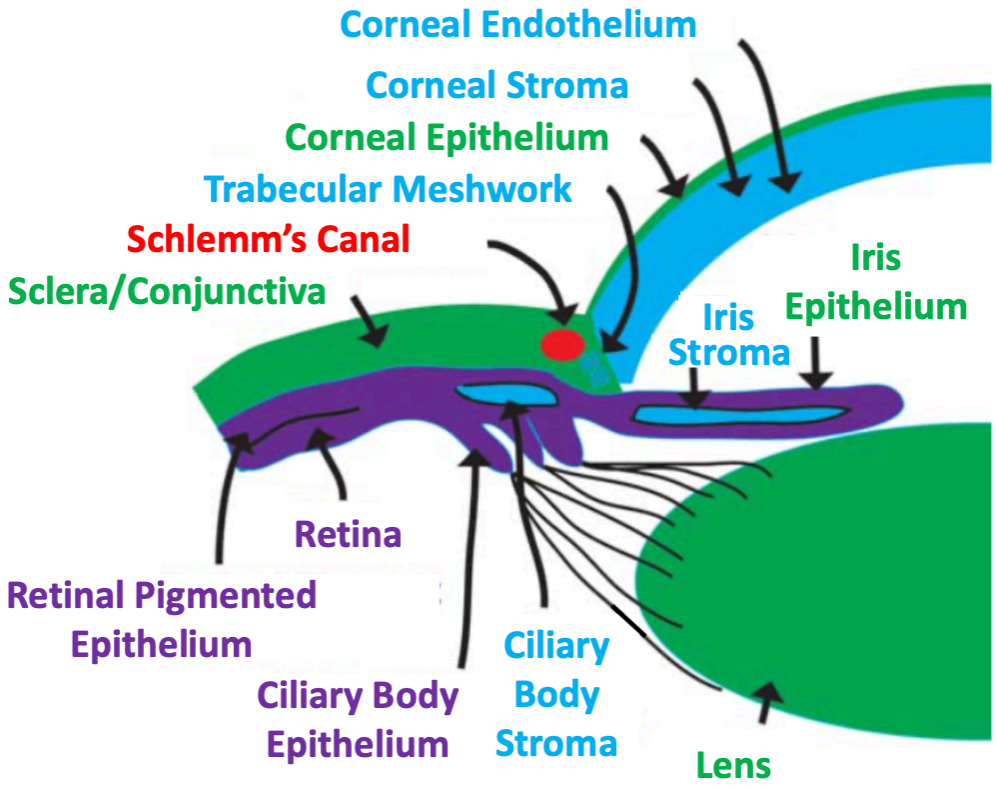

Figure 2. Embryonic origins of anterior segment tissues. Tissues derived from the neural crest cells, and subsequently the periocular mesenchyme (POM), are in blue: the iris stroma, the corneal stroma and endothelium, the trabecular meshwork, and the ciliary body stroma. Tissues in green are derived from the surface ectoderm: the lens, corneal epithelium, and sclera. Purple denotes structures originating from the neural ectoderm: the retina, retinal pigment epithelium, ciliary body epithelium and iris epithelium. Schlemm's canal, derived from the mesoderm component of the POM, is in red. Adapted from: (Williams and Bohnsack, 2015)

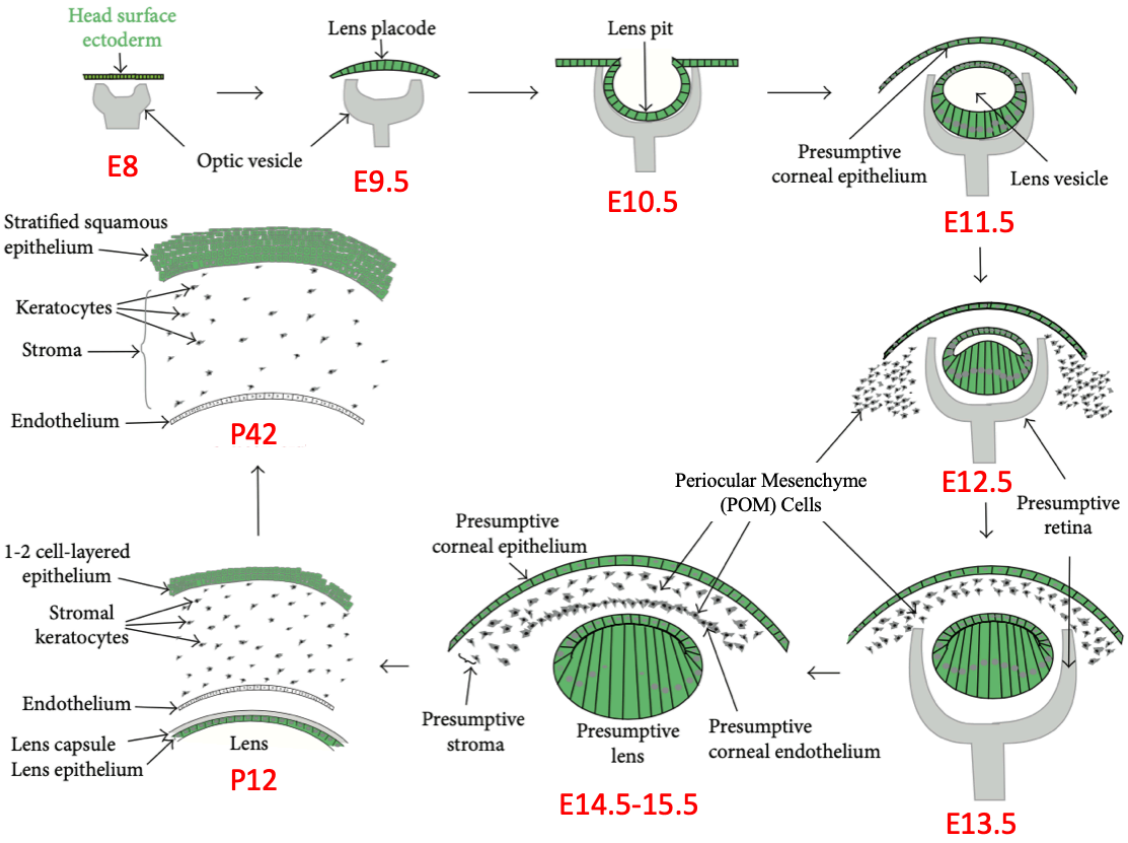

Figure 3. Murine development of the anterior segment and cornea, E8-P42. By E8, evagination of the optic pit towards the surface ectoderm forms the optic vesicle. At E9.5 the lens placode develops from a thickening of the surface ectoderm and by E10.5 invagination of both the optic vesicle and lens place creates the lens pit. The lens placode begins to separate and develops into the lens vesicle, which detaches from the surface ectoderm at E11.5. The overlying surface ectoderm gives rise to the presumptive corneal epithelium. Between E12.5-13.5, POM cells migrate into the region between the lens and presumptive epithelium. By E13.5 layers of POM cells have migrated into this region, which develop into the presumptive corneal endothelium and stroma through E14.5-15.5. Further differentiation of these structures results in a 1-2 layered epithelium, ECM secreting stromal keratocytes and an endothelial monolayer. The epithelium remains unstratified up until P12, at which point eyelid opening initiates stratification and the epithelium becomes a stratified squamous structure. Adapted from: (Swamynathan, 2013)

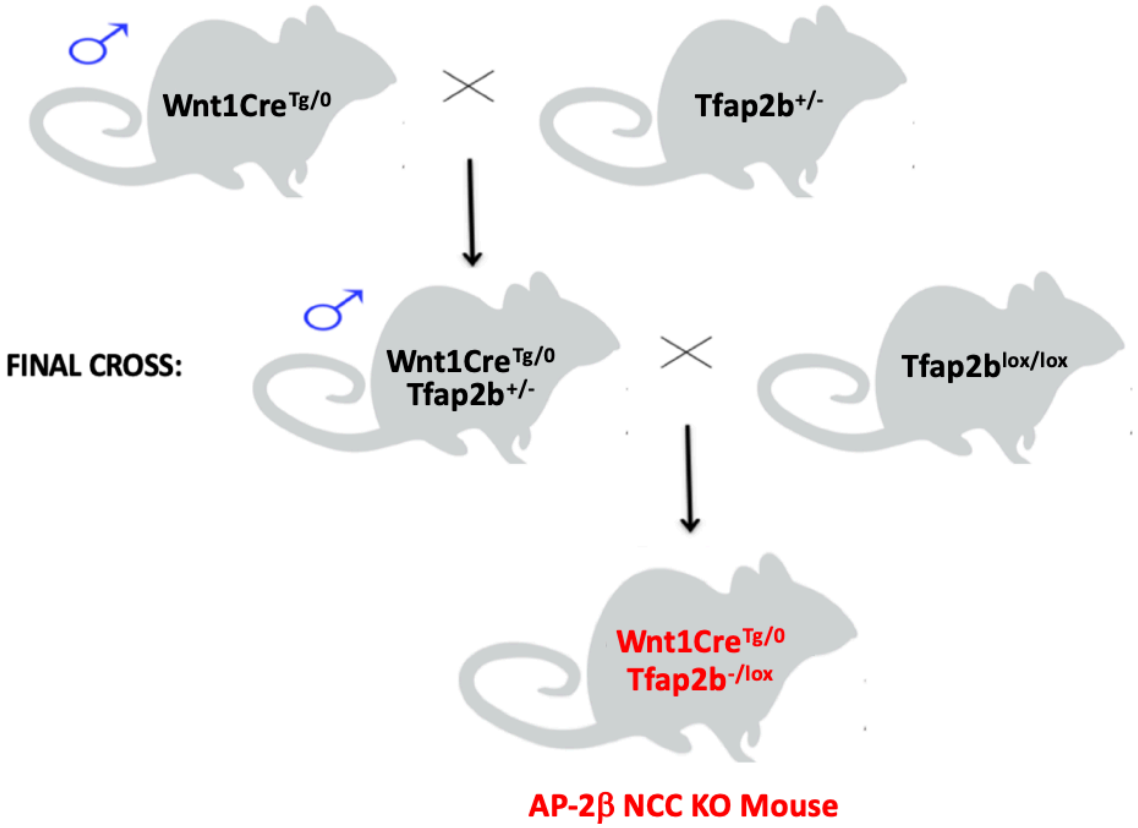

Figure 4. Breeding of AP-2β NCC KO mutant mice. Transgenic male mice hemizygous for *Wnt1Cre*$^{Tg/0}$ (Cre recombinase is expressed under direction of Wnt1 regulatory sequences, to limit expression to the neural crest cell population) are crossed with female mice heterozygous for the *Tfap2b* allele (*Tfap2b*$^{+/-}$), which encodes AP-2β. The male offspring that possess the Wnt1Cre transgene and are heterozygous for the *Tfap2b* allele (*Wnt1Cre*$^{Tg/0}$, *Tfap2b*$^{+/-}$) are bred with female mice possessing a floxed *Tfap2b* allele (*Tfap2b*$^{lox/lox}$). The resulting mutants are hemizygous for the Cre transgene, and have one null allele for *Tfap2b* and one floxed *Tfap2b* allele that is disrupted by Cre-recombinase excision (*Wnt1Cre*$^{Tg/0}$, *Tfap2b*$^{-/lox}$). Therefore, the resulting mice have one active copy of *Tfap2b* in all tissues, except the NCC (and its derivatives), where it has been knocked out by Cre-recombinase.

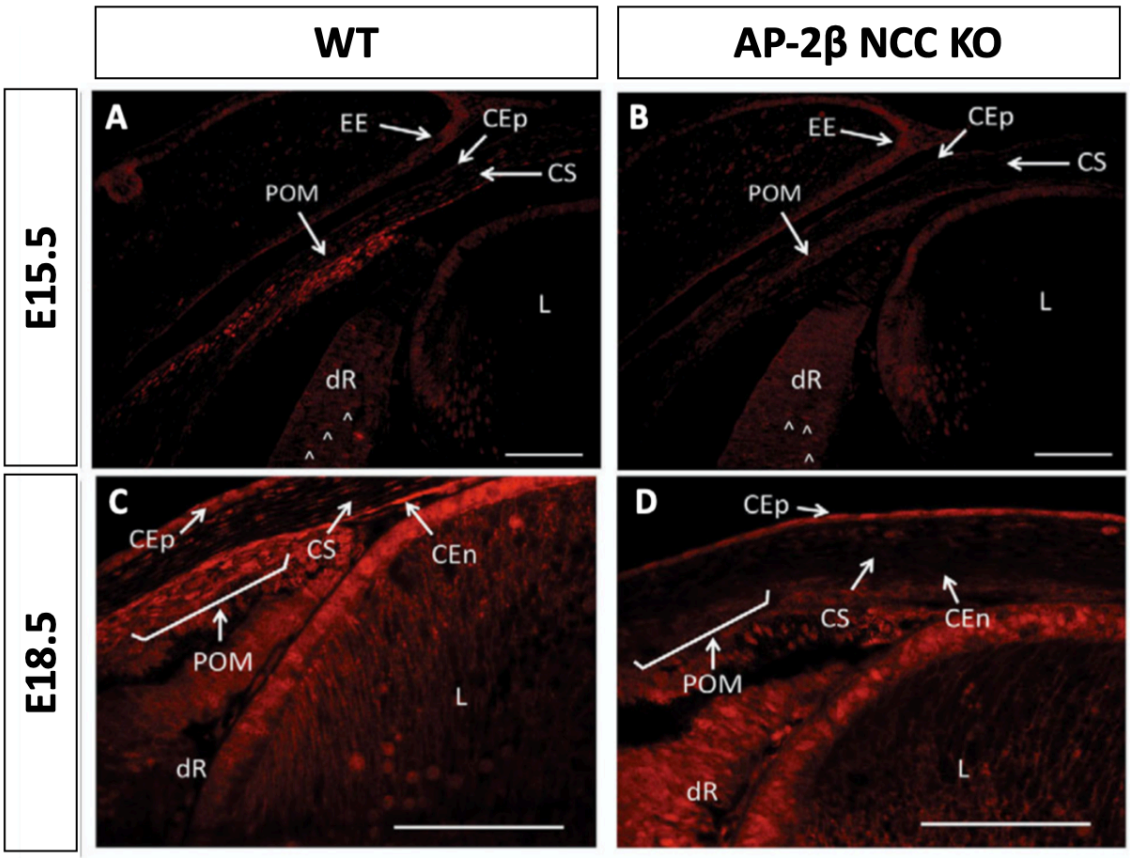

Figure 5. Embryonic AP-2β expression in WT and AP-2β NCC KO mice. WT mice exhibit high AP-2β expression in the POM at E15.5 (A), and in POM-derived tissues such as the stroma and endothelium by E18.5 (C). In the AP-2β NCC KO mutant, considerably less AP-2β expression is visible in the migrating POM at E15.5 (B). At E18.5, POM derived structures including the corneal stroma and endothelium display significantly less expression of AP-2β relative to the WT (D). AP-2β expression by non-NCC derived tissues like the corneal epithelium was unaffected. EE, eyelid epidermis; POM, periocular mesenchyme; CEp, corneal epithelium; CS, corneal stroma; CEn, corneal endothelium; L, lens; dR, developing retina. Adapted from: (Martino et al., 2016)

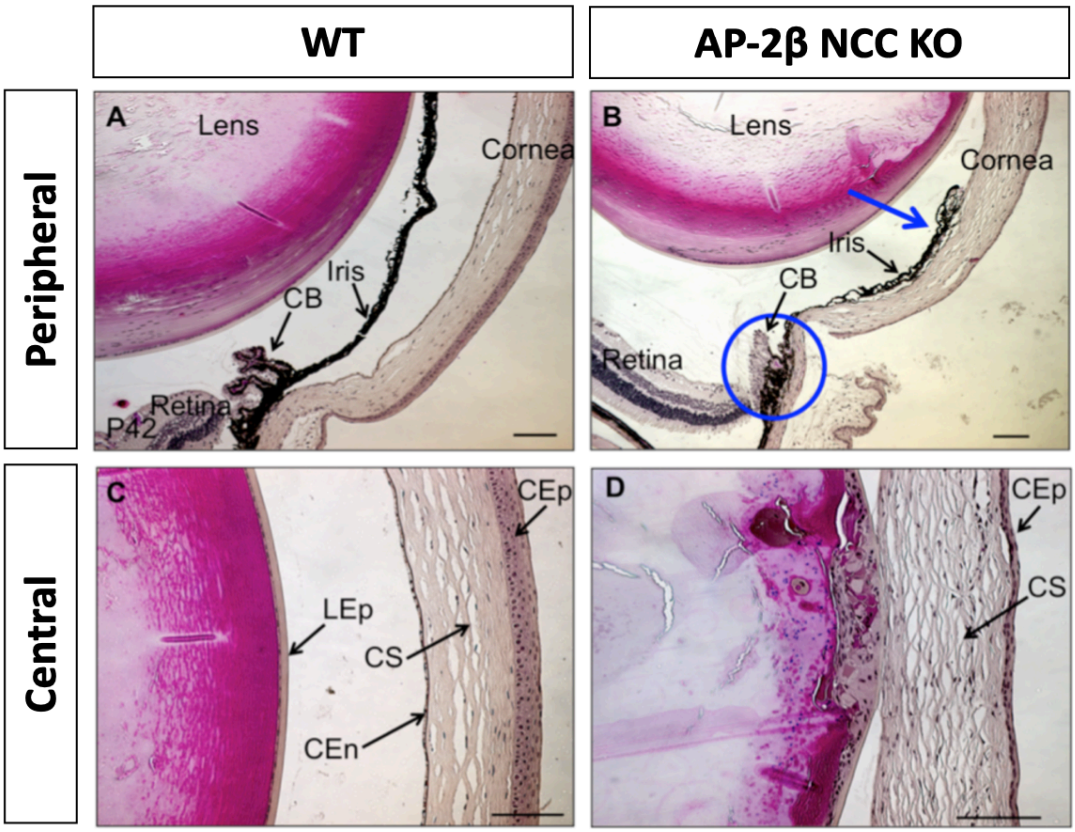

Figure 6. 2-3-month H&E-stained sections of the anterior segment of the eye in WT and AP-2β NCC KO mice. In the P42 WT, the angle region has properly formed, with no adherence observed between lens, iris and cornea (A). In the P42 mutant, the iris is adhered to the posterior cornea (blue arrow) and the ciliary body is atrophied (blue circle) (B). The three distinct layers of the cornea are visible in the P42 WT, and a distinct anterior chamber present between the lens and cornea (C). In the mutant, corneolenticular adhesions are observed, in addition to an absent corneal endothelium, disorganized, hypercellular stroma, and significantly reduced stratification (D). CB, ciliary body; CEn, corneal endothelium; CEp, corneal epithelium; CS, corneal stroma; LEp, lens epithelium. Scale bars 100μm. Adapted from: (Martino et al., 2016)

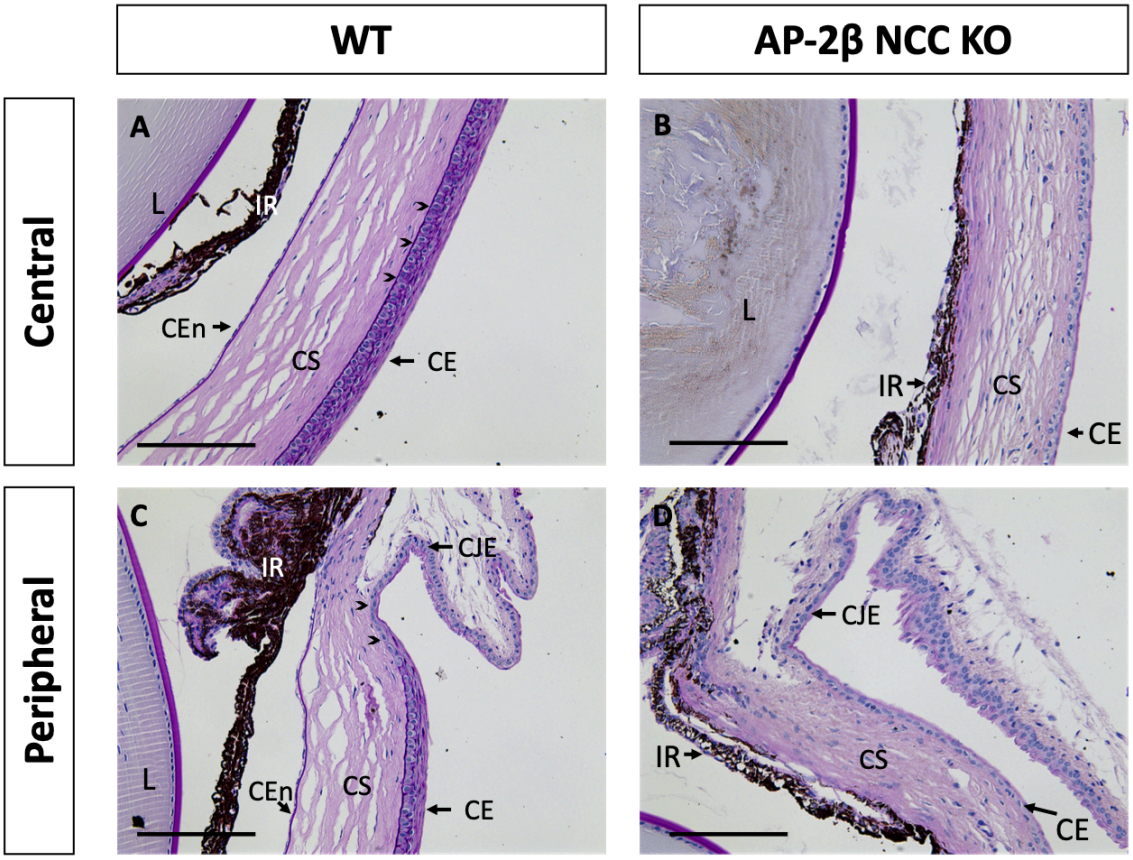

Figure 7. PAS staining of the anterior segment from 2-3-month-old WT and AP-2β NCC KO mice. In the WT mice (A, C), the corneal endothelium, stroma and epithelium are properly formed and distinct. A 6-7-layer stratified squamous epithelium is observed, with a dark purple stained epithelial basement membrane (A, arrowheads) posterior to the basal epithelial columnar cell layer. At the corneal periphery, the limbal region (C, arrowheads) is evident prior to conjunctiva. PAS staining of the mutant mice (B, D) displays the anticipated abnormal phenotype seen in our previous studies. The corneal endothelium is absent, with the iris adhering to the posterior cornea, and the stroma is hypercellular relative to the WT. Epithelial stratification is absent, and cells are smaller and flatter, with staining for the basement membrane also being absent. CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; L, lens. Scale bars represent 150 μm.

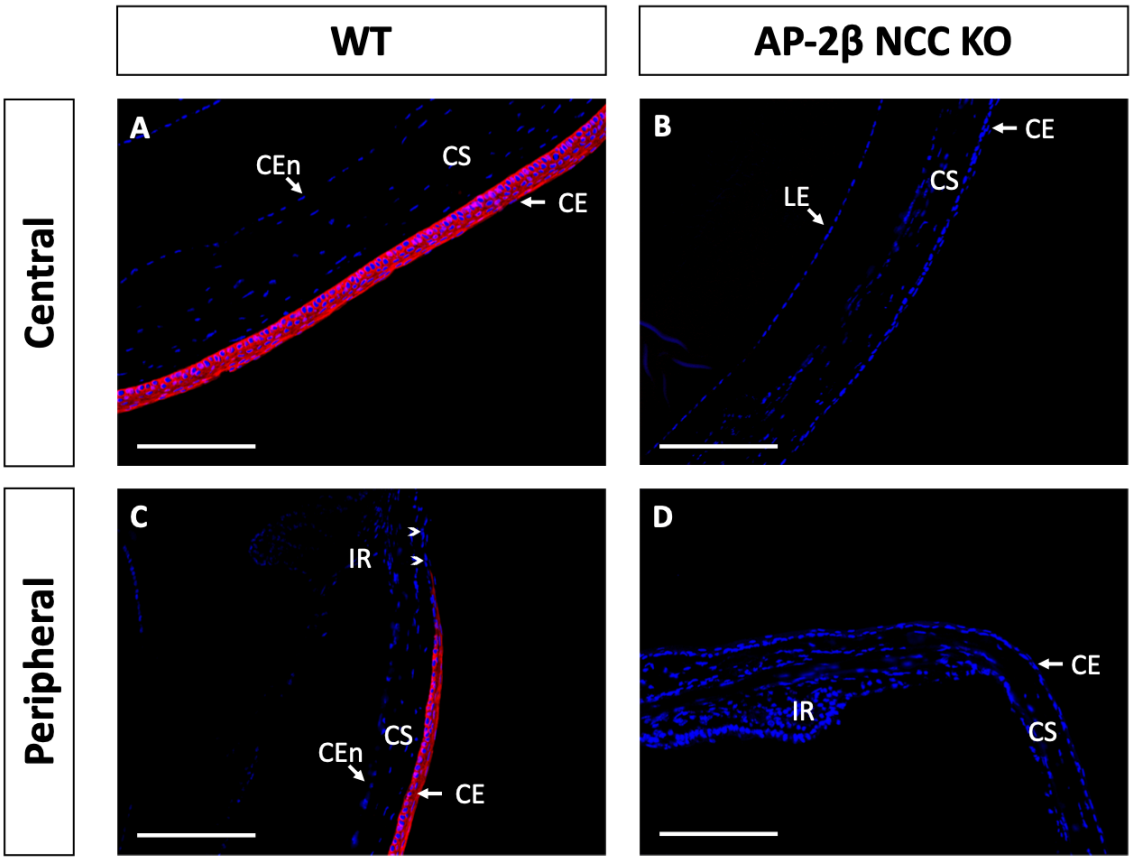

Figure 8. K12 IHC staining of the cornea from 2-3-month-old WT and AP-2β NCC KO mice. WT mice (A, C; n=8) exhibit high expression of K12 across the central (A) and peripheral corneal epithelium (C). Peripheral K12 staining tapers off at the limbal region (arrowheads). Mature KO mice (B, D; n=8) do not express K12 in the central corneal epithelium (B) or at the periphery (D). CE, corneal epithelium; CEn, corneal endothelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 150 μm.

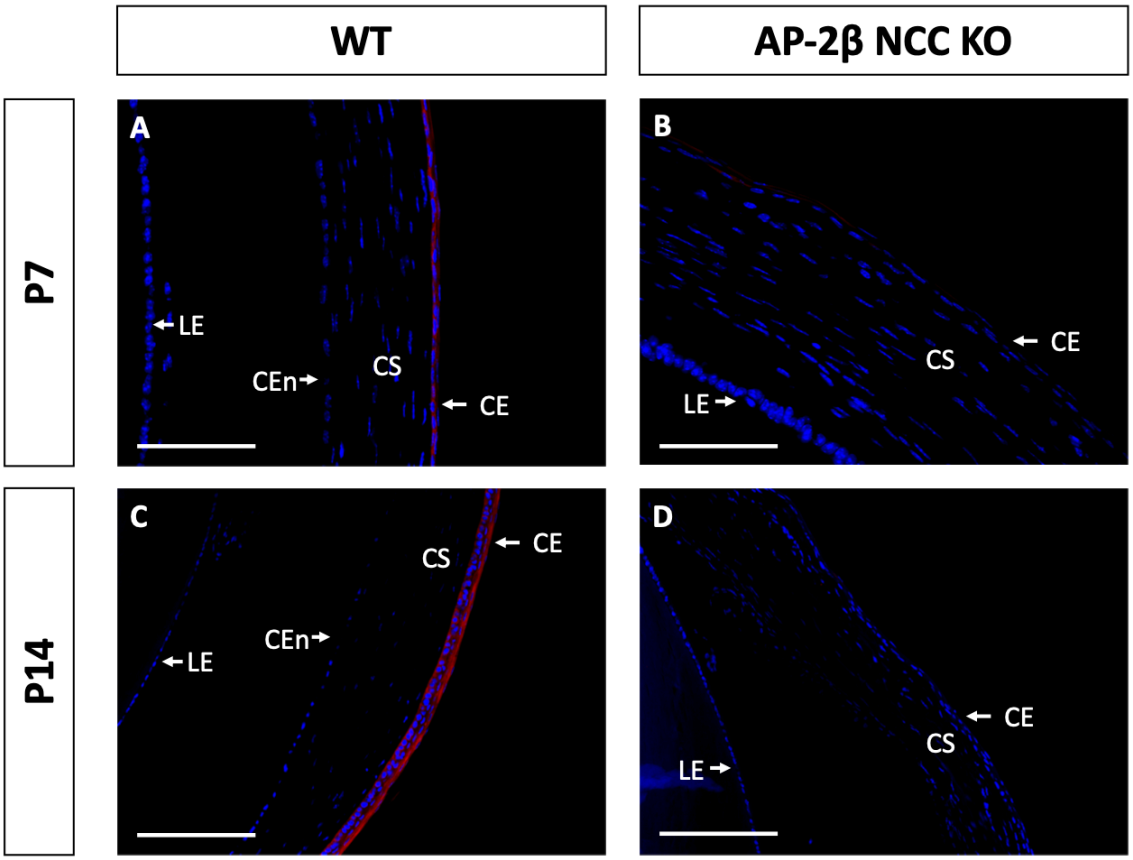

Figure 9. K12 IHC staining of the cornea from WT and AP-2β NCC KO mice at P7 and P14. K12 is expressed consistently in the unstratified corneal epithelium of the P7 WT mouse (A; n=3), however K12 is absent in the epithelium of the P7 mutant (B; n=4). Post-eyelid opening, at P14, K12 is highly expressed in the WT central stratified corneal epithelium (C; n=3). No expression of K12 is observed in the mutant epithelium at P14 (D; n=3). CE, corneal epithelium; CEn, corneal endothelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 75 μm (A, B) and 150 μm (C, D).

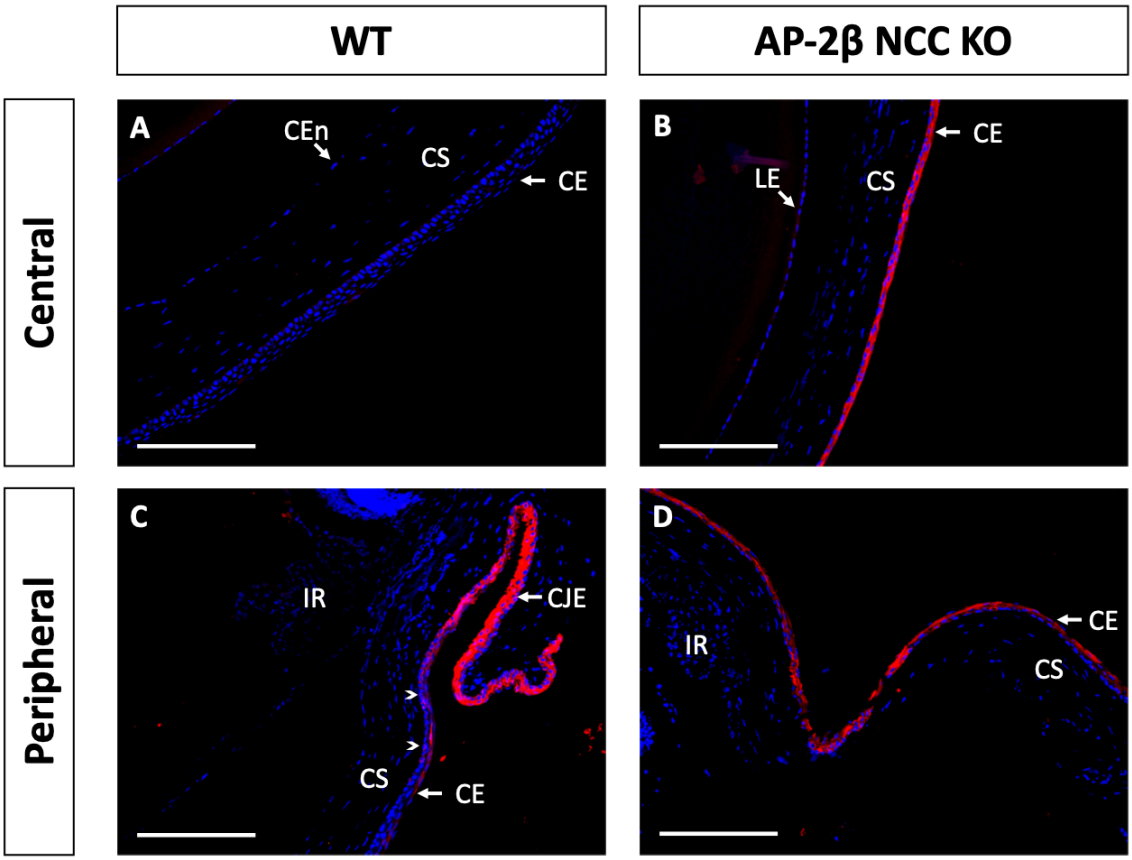

Figure 10. K15 IHC staining of the cornea from 2-3-month-old WT and AP-2β NCC KO mice. In the WT (A, C; n=8) expression of K15 is limited to the conjunctival and limbal epithelia (C). K15 staining tapers off at the transition to the peripheral corneal region (arrowheads) with the differentiated corneal epithelium not staining for K15 (A). In contrast, for the mutant (B, D; n=7), K15 is observed at high levels across the central (B) and peripheral cornea (D), continuous with the staining observed at the limbus and conjunctival epithelia. CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 150 μm.

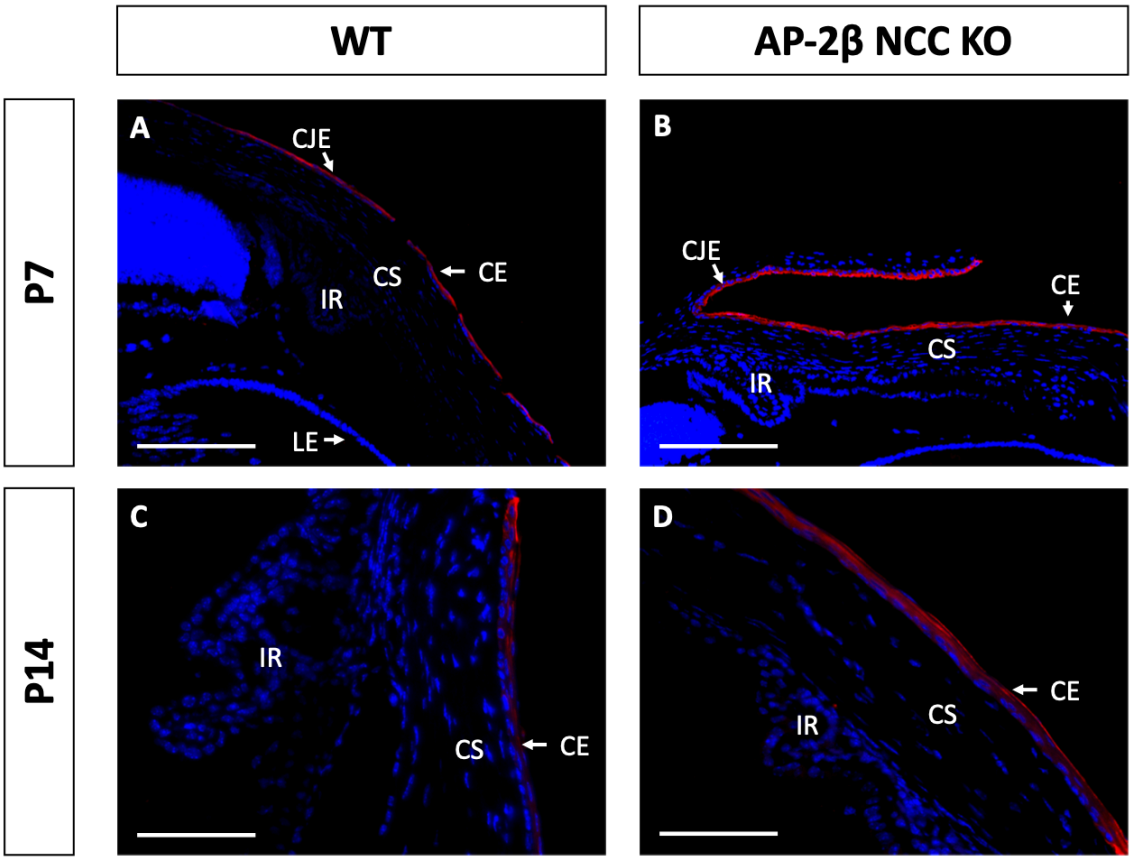

Figure 11. K15 IHC staining of the cornea from WT and AP-2β NCC KO mice at P7 and P14. K15 is observed to be expressed continuously across epithelium of the conjunctiva, limbus and cornea of P7 WT mice (A; n=3). The same expression pattern is seen for the mutant at the P7 timepoint (B; n=4). At P14, WT K15 expression is present in the limbus, however it tapers off significantly approaching the peripheral corneal epithelium (C; n=4). In contrast, the mutant exhibits continuous expression of K15 across all of the conjunctival, limbal and corneal epithelia (D; n=3). CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 150 μm (A, B) and 75 μm (C, D).

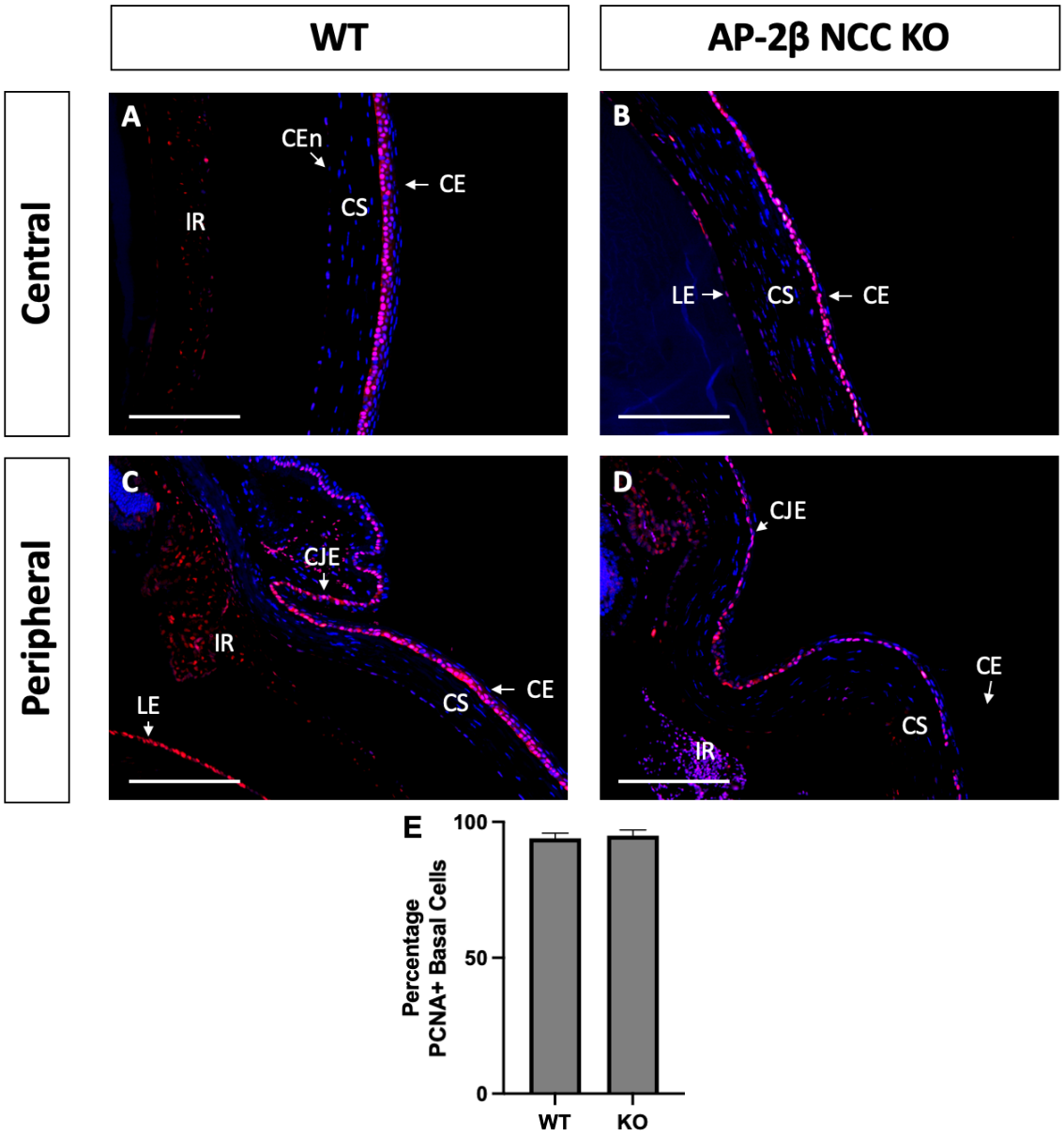

Figure 12. PCNA IHC staining of the cornea from 2-3-month WT and AP-2β NCC KO mice. Nuclear expression of PCNA is observed for basal epithelial cells across the central and peripheral cornea, continuous with staining for the limbus and conjunctiva (A, C; n=3). In the 2-3-month mutant, PCNA expression appears in the same pattern as the WT, with consistent basal nuclear staining across the cornea, limbus and conjunctiva (B, D; n=4). Cell counts indicated no significant difference ($p>0.05$) in percentage of PCNA-positive basal cells between the WT (93.94% ± 1.92) and mutant (94.94% ± 2.08) (E). Multiple counts were conducted to achieve mean value for individual sample; sample means were averaged to produce group mean. Error bars indicate standard deviation. CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 150 μm.

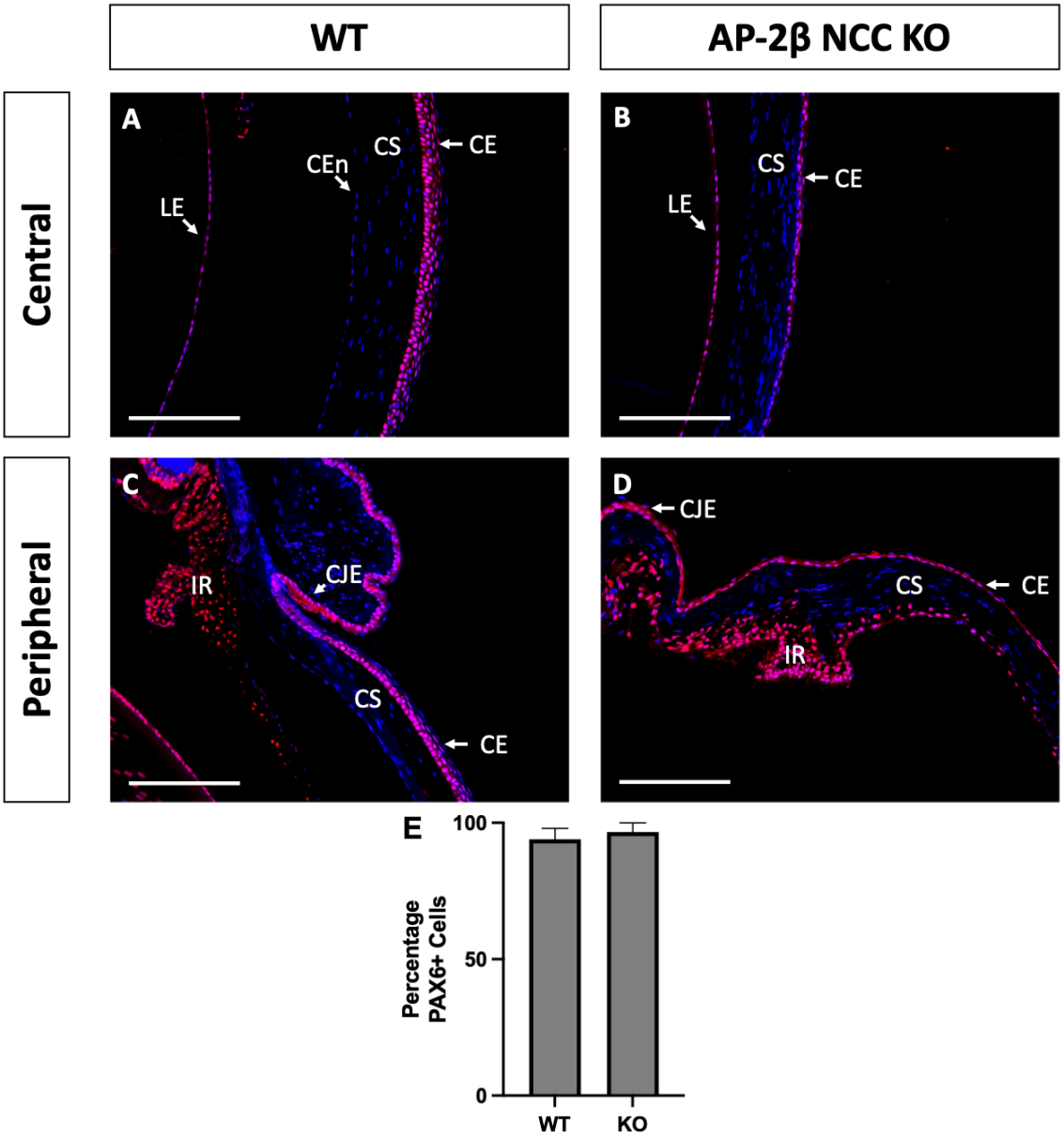

Figure 13. Pax6 IHC staining of the cornea from 2-3-month-old WT and AP-2β NCC KO mice. In WT mice (A, C; n=4), consistent Pax6 nuclear epithelial staining is observed in the central region (A) and the periphery (C), continuous with expression in the limbus and conjunctiva. The expression pattern of Pax6 in the mutant (B, D; n=3) was observed to be the same as that which was seen in the WT. Cell counts indicated no significant difference ($p>0.05$) in percentage of Pax6-positive cells between the WT (93.92% ± 3.99) and mutant (96.59% ± 3.29) (E). Multiple counts were conducted to achieve mean value for individual sample; sample means were averaged to produce group mean. Error bars indicate standard deviation. CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 150 μm.

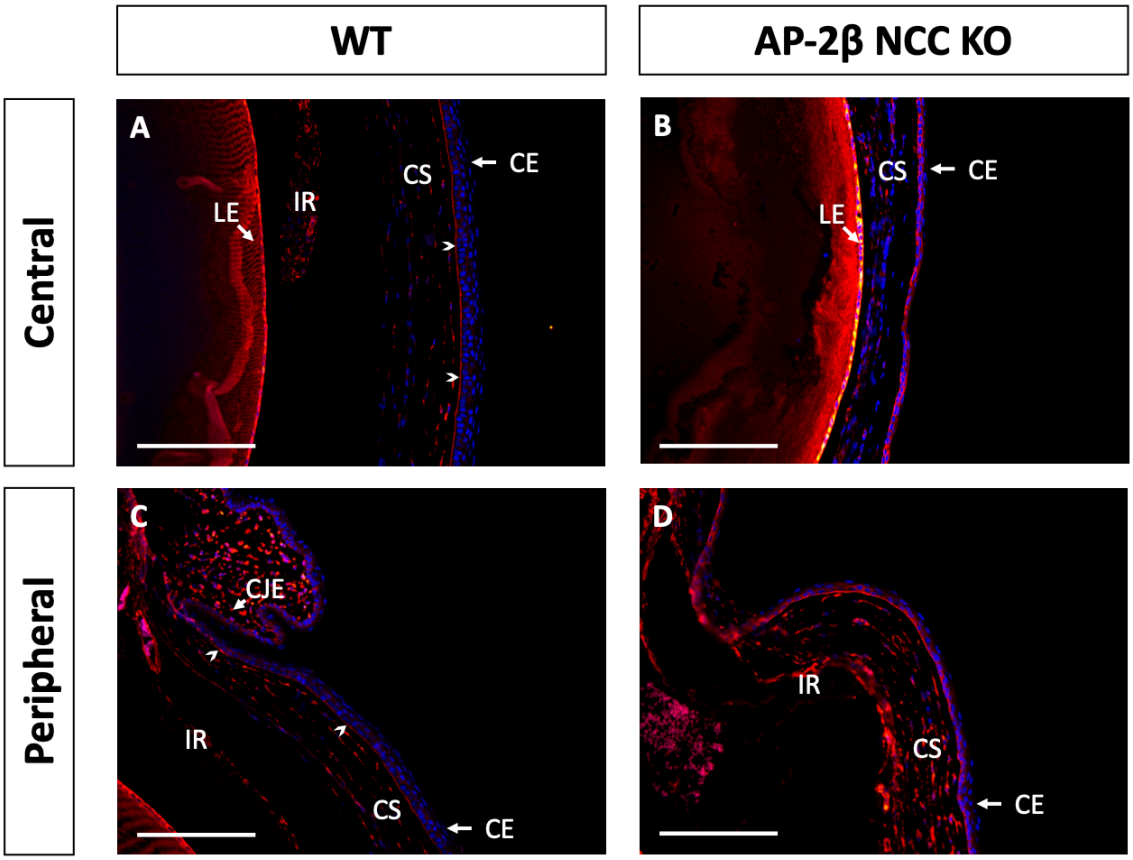

Figure 14. Itga6 IHC staining of the cornea from 2-3-month-old WT and AP-2β NCC KO mice. In the 2-3-month WT, expression of Itga6 is seen (arrows) consistently across the basal membranes of basal epithelial cells within the central and limbal cornea (A, C; n=4). Staining in the mutant for Itga6 was present at the apical and lateral aspect of cells, as opposed to the exclusively basal membrane localization in the WT (B, D; n=4). CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 150 μm.

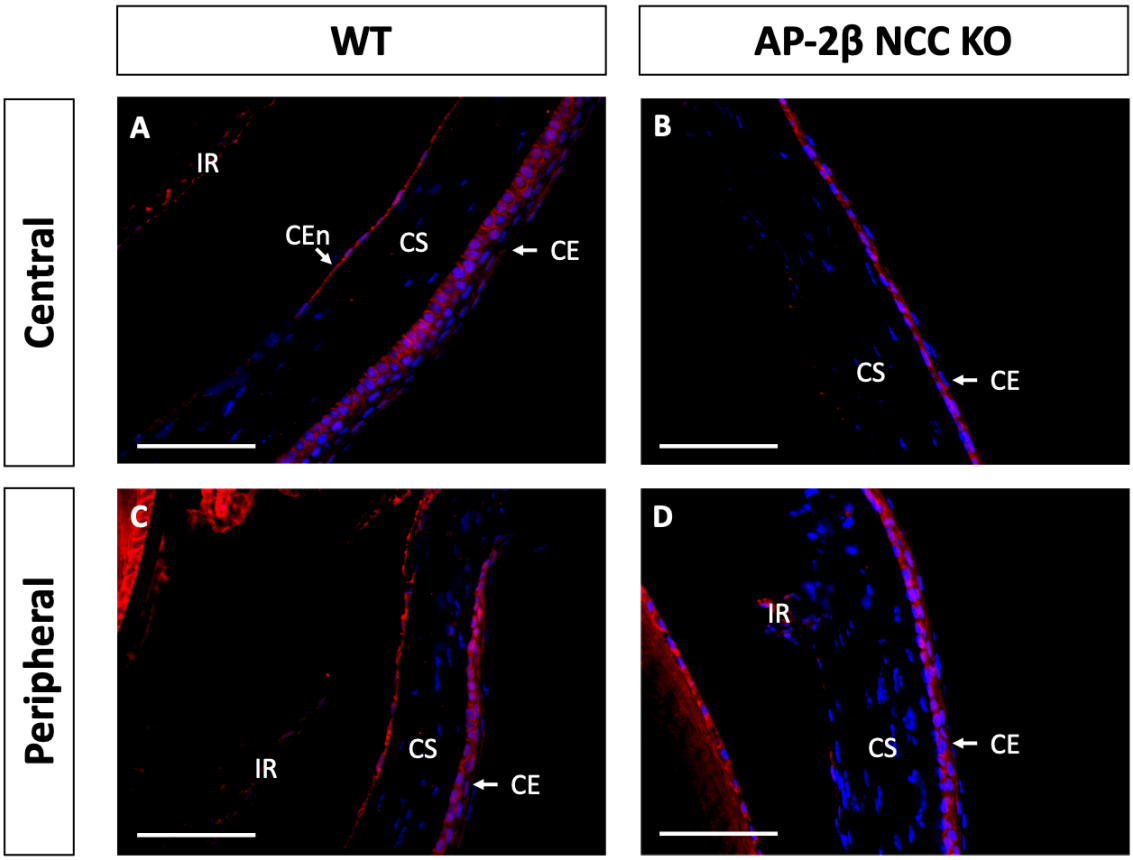

Figure 15. Itga9 IHC staining of the cornea from 2-3-month-old WT and AP-2β NCC KO mice. In the 2-3-month WT mouse, Itga9 expression is observed consistently across the central and limbal cornea, with staining predominantly localized to the lateral and apical membranes of basal epithelial cells (A, C; n=5). Itga9 expression in the KO is observed at similar levels to the WT across the extent of the cornea (B, D; n=5). CE, corneal epithelium; CEn, corneal endothelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 75 μm.

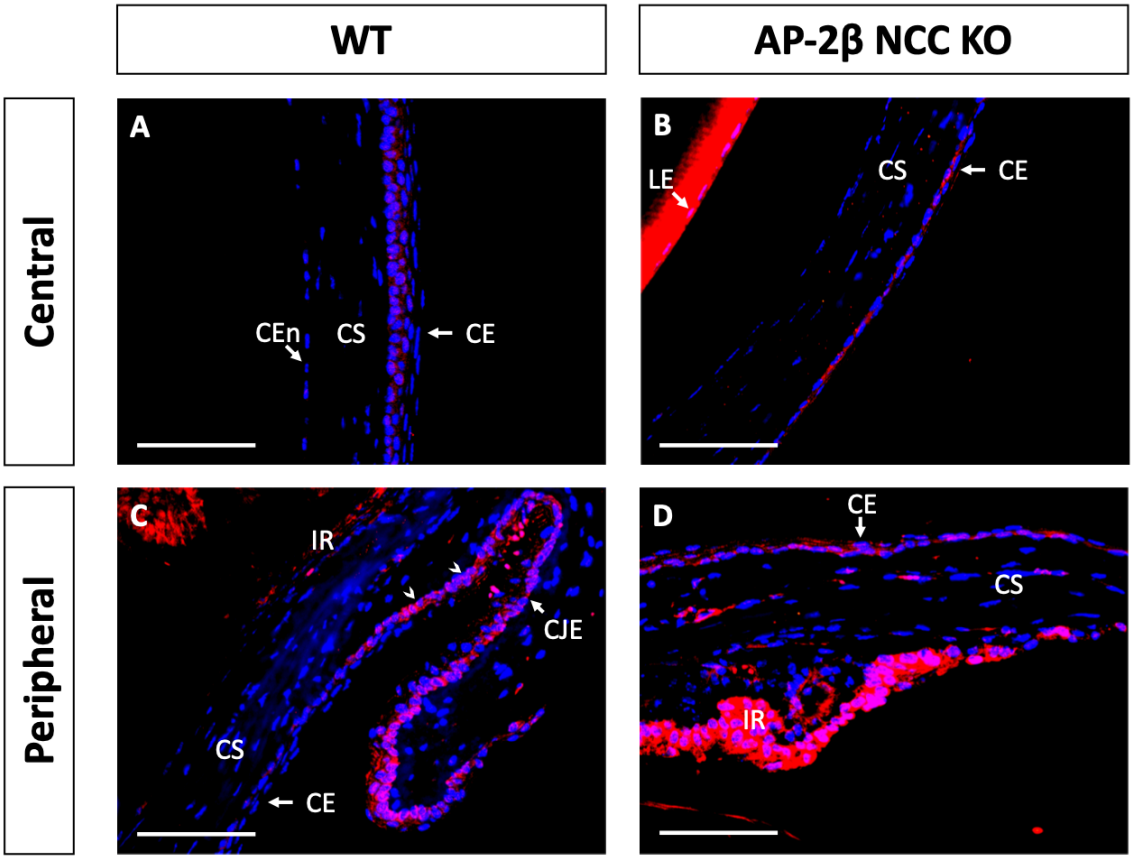

Figure 16. Fzd7 IHC staining of the cornea from 2-3-month-old WT and AP-2β NCC KO mice. In the 2-3-month WT mouse Fzd7 is upregulated in the limbus (arrows) and conjunctiva relative to the central epithelium (A, C; n=5). In the mutant, expression of Fzd7 is consistent across the corneal and limbal epithelia (B, D; n=5). CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 75 μm.

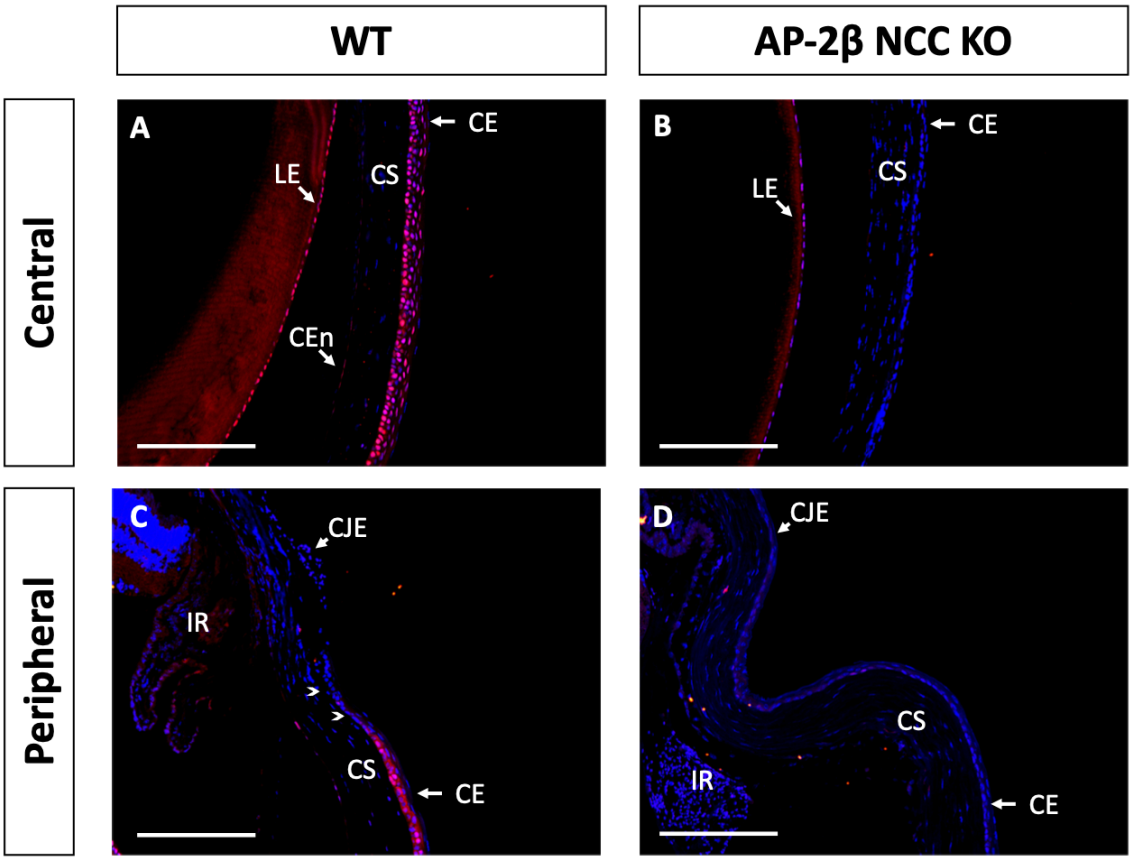

Figure 17. Bmp4 IHC staining of the cornea from 2-3-month-old WT and AP-2β NCC KO mice. WT mice (A, C; n=5) display high nuclear expression of Bmp4 across the central corneal epithelium (A), with the most consistent expression observed for the basal epithelial layer. Nuclear staining continues throughout the peripheral (C) corneal epithelium, however Bmp4 is expression is absent from the limbal (arrowheads) and conjunctival epithelial regions. Bmp4 expression is not conserved in the mutant (B, D; n=5) with staining being absent from the central (B) and peripheral regions (D). CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 150 μm.

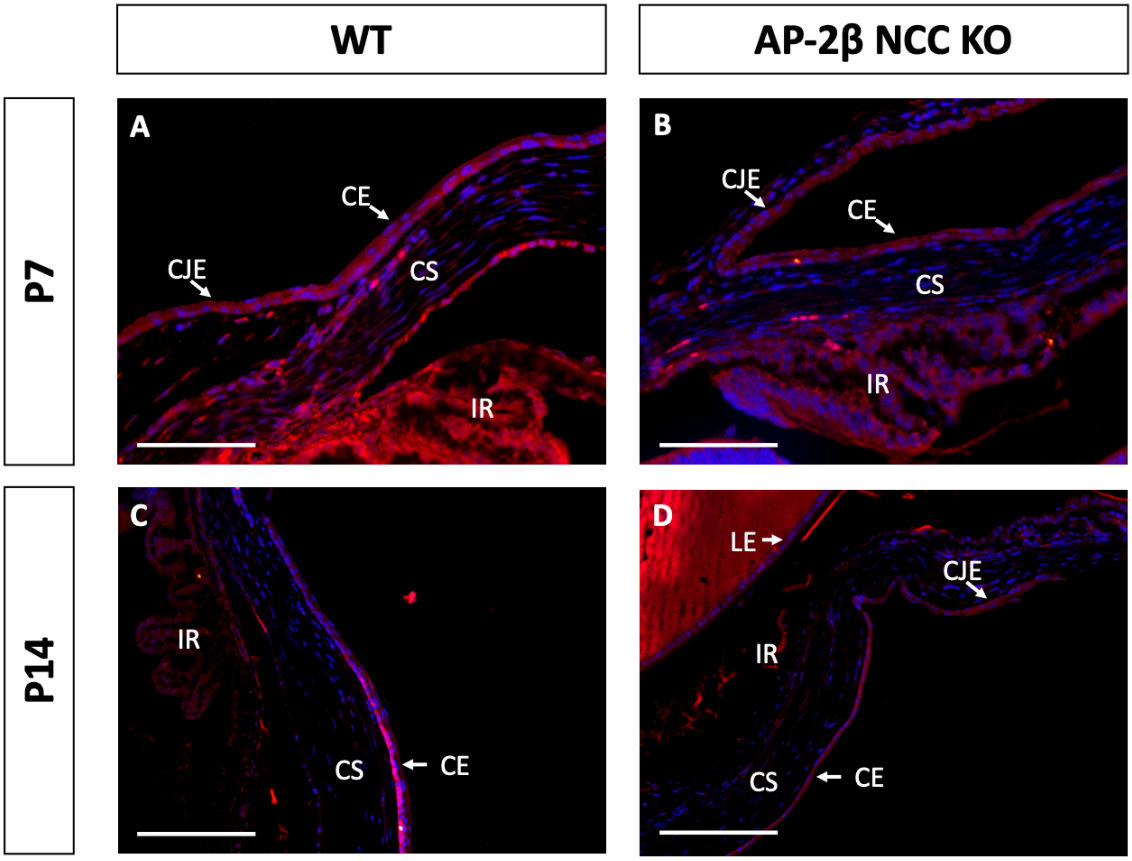

Figure 18. **Bmp4 IHC staining of the cornea from WT and AP-2β NCC KO mice at P7 and P14.** At P7, the pattern of Bmp4 expression is similar between the WT (A; n=4) and mutant (B; n=4), with consistent cytoplasmic staining across the corneal epithelium, limbus and conjunctiva, in addition to expression in the stroma. At P14 in the WT, we observe reduced stromal expression, as well as specific expression of Bmp4 in the basal corneal epithelium, which is not seen in the limbus or conjunctiva of the WT (C; n=4). The mutant at P14 (D; n=4) has similarly decreased stromal expression of Bmp4, but specific upregulated basal epithelial expression of Bmp4 is not observed. CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 75 μm (A, B) and 150 μm (C, D).

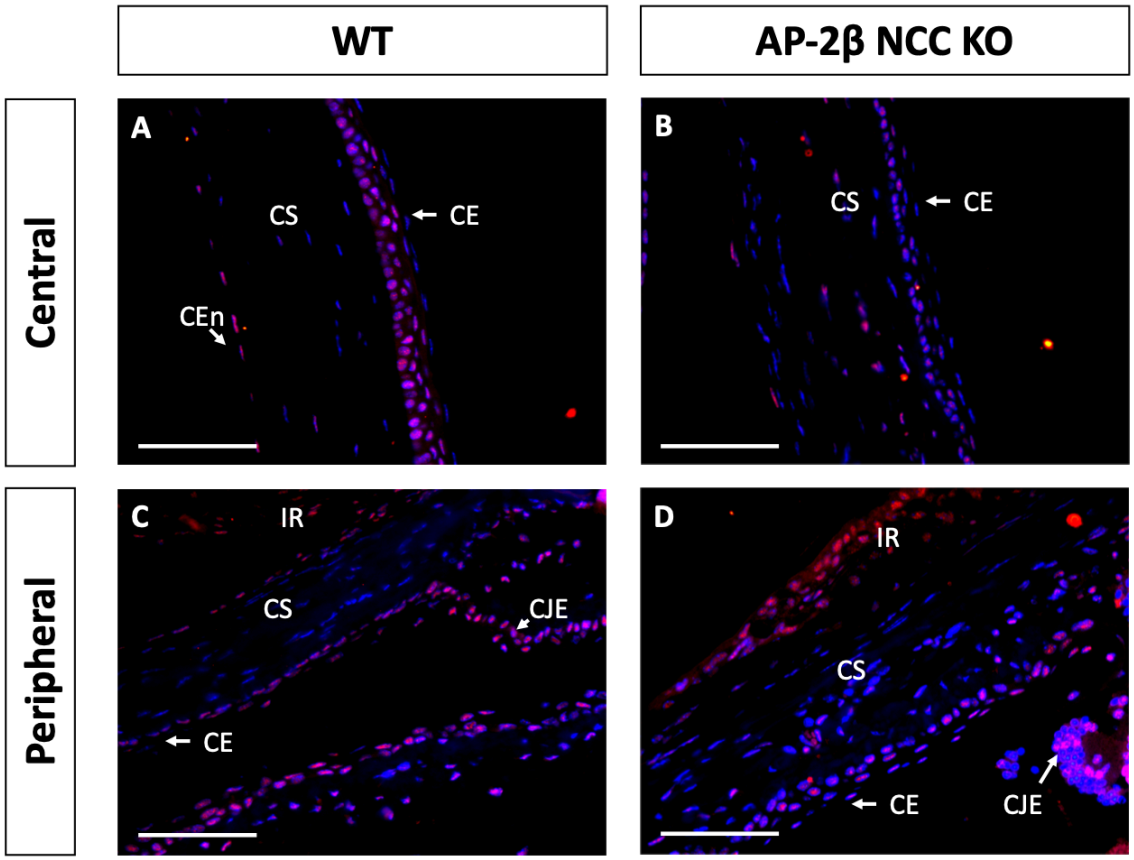

Figure 19. pSmad1/5 IHC staining of the cornea from 2-3-month-old WT and AP-2β NCC KO mice. In the 2-3-month WT, consistent nuclear expression of pSmad1/5 is observed across the corneal epithelium, continuous with the limbal and conjunctiva epithelia (A, C; n=3). In the mutant, the same expression pattern is observed as in the WT (B, D; n=4). CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 75 μm.

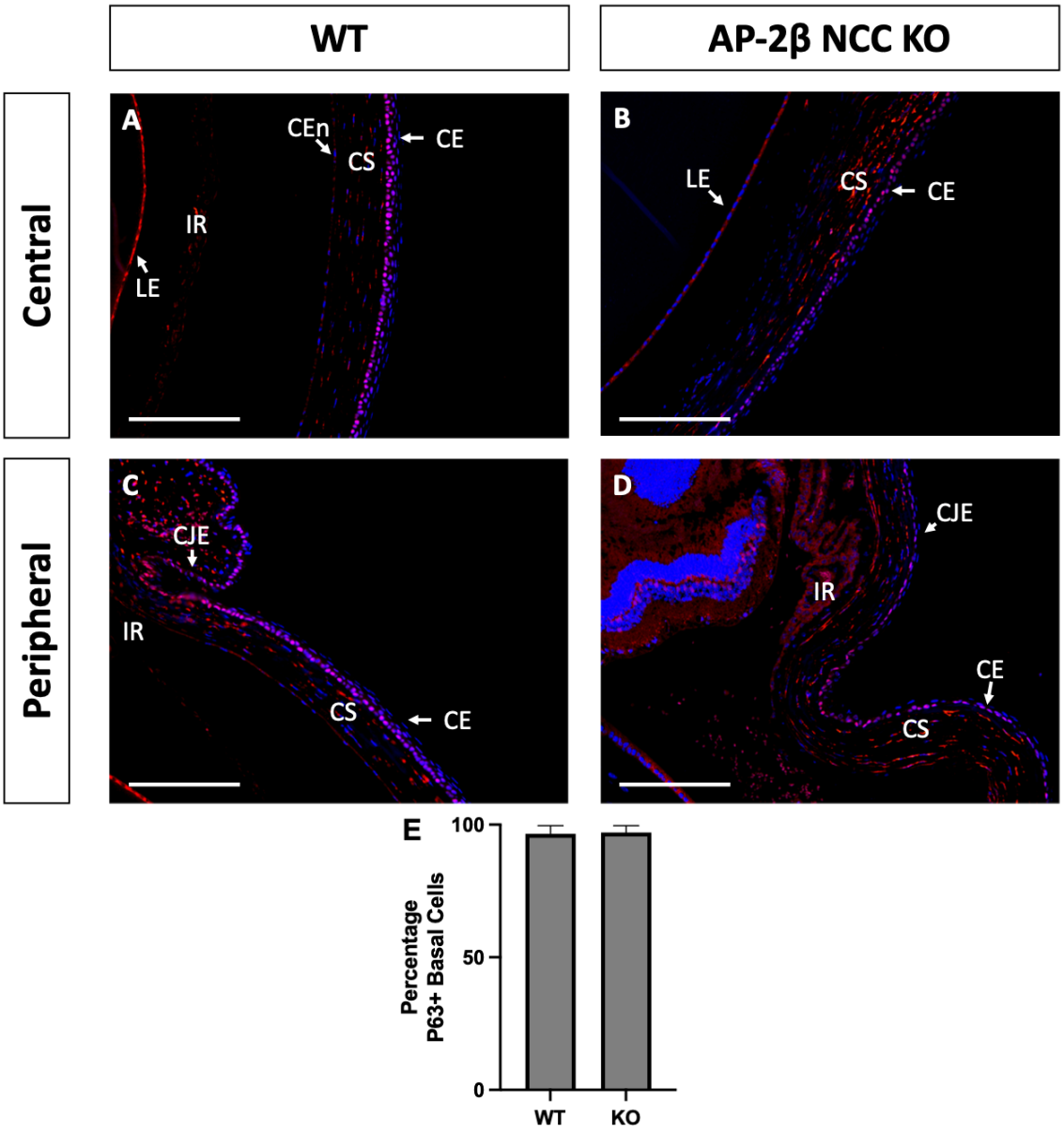

Figure 20. P63 IHC staining of the cornea from 2-3-month-old WT and AP-2β NCC KO mice. P63 in WT mice (A, C; n=4) is expressed consistently throughout the basal cells of the corneal, limbal and conjunctival epithelia. Stromal expression of P63 is observed across the entire cornea. The mutant cornea (B, D; n=4) has the same pattern of P63 expression as the WT, nuclear staining being consistent across the cornea, limbus and conjunctiva. Cell counts indicated no significant difference ($p>0.05$) in the percentage of basal cells expressing P63 between the WT (96.53%±3.12) and mutant (97.06%±2.56) (E). Multiple counts were conducted to achieve mean value for individual sample; sample means were averaged to produce group mean. Error bars indicate standard deviation. CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 150 μm.

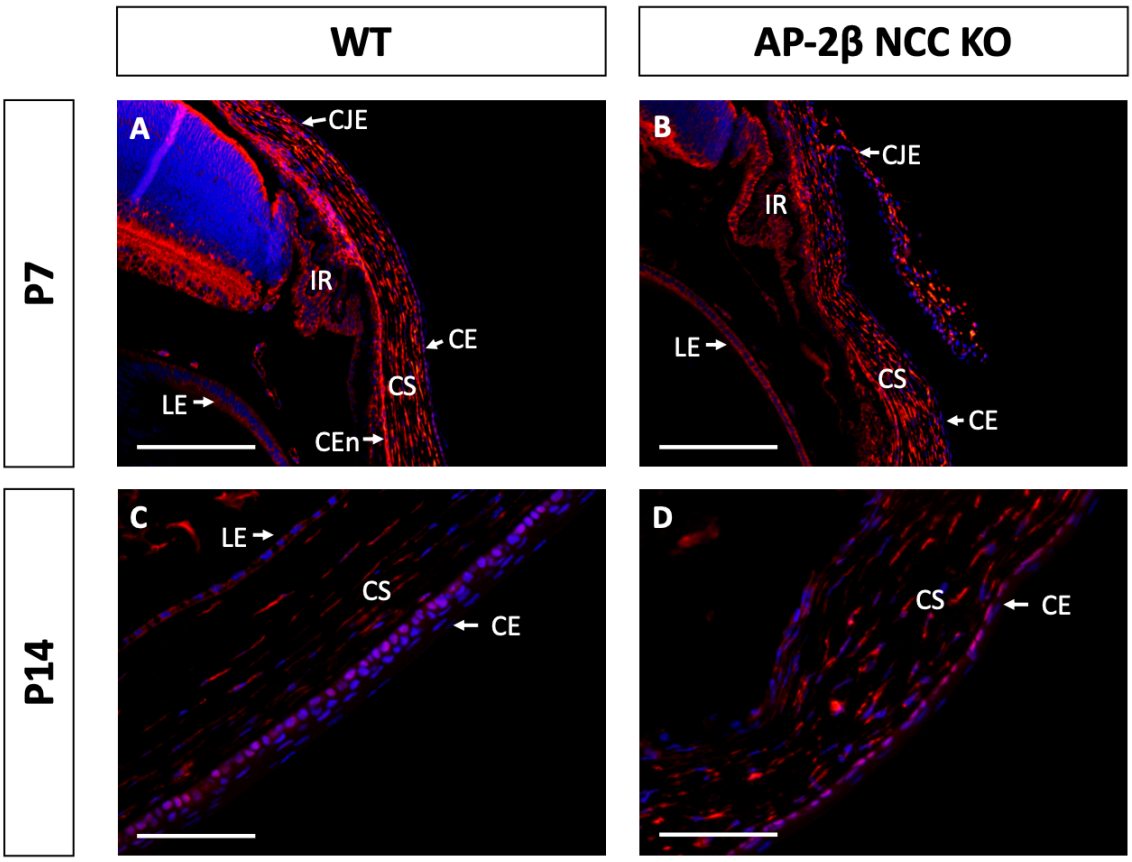

Figure 21. P63 IHC staining of the cornea from WT and AP-2β NCC KO mice at P7 and P14. The pattern of P63 expression is similar between the WT (A; n=4) and mutant (B; n=4) at P7, with high stromal keratocyte expression as well as consistent nuclear staining of the corneal, limbal and conjunctival epithelia. In the WT at P14, initial stratification is observed and P63 expression remains confined to the basal cell nuclei of the corneal epithelium (C; n=3). In the KO, though stratification is not seen, nuclear basal epithelial cell expression of P63 is consistent across the epithelium (D; n=3). Stromal expression of P63 is similar for the WT and mutant at P14. CE, corneal epithelium; CEn, corneal endothelium; CJE, conjunctival epithelium; CS, corneal stroma; IR, iris; LE, lens epithelium. Scale bars represent 150 μm (A, B) and 75 μm (C, D).

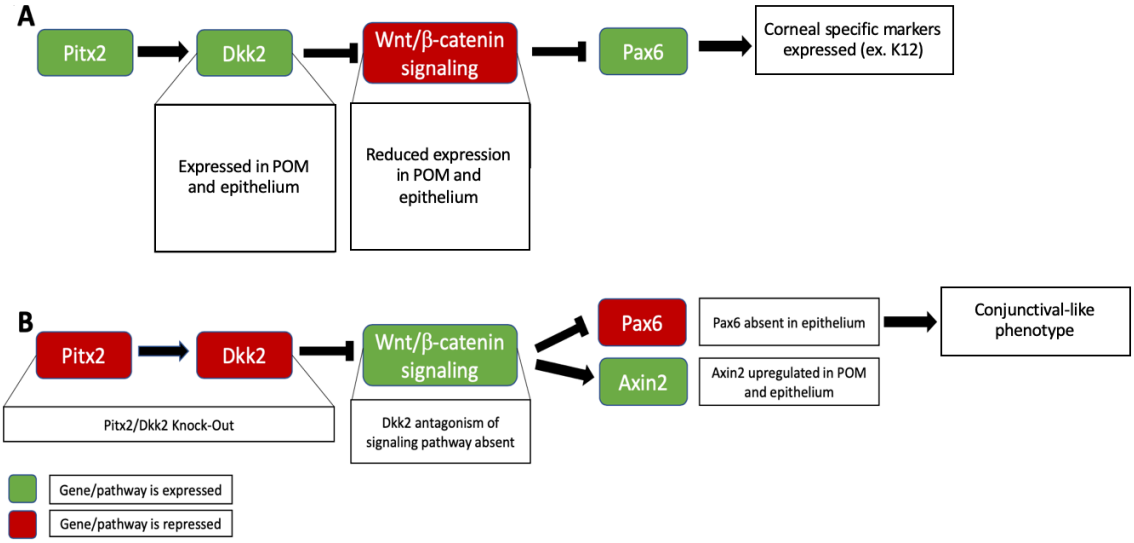

Figure 22. Canonical Wnt/β-Catenin pathway in development of the differentiated corneal epithelial phenotype. (A) In the WT mouse, Dkk2 is expressed in the epithelium and POM downstream of transcription factor Pitx2 and inhibits the Wnt/β-Catenin pathway. Uninhibited by Wnt signaling, transcription factor Pax6 promotes the expression of corneal epithelium specific markers include K12. (B) When Pitx2 or Dkk2 are knocked down, Wnt signaling is no longer antagonised by Dkk2. Pax6 expression is subsequently inhibited, preventing corneal-specific differentiation in favour of a conjunctival phenotype. Downstream effectors of Wnt signaling such as Axin-2 are upregulated in the POM and epithelium. Adapted from: (Walker et al., 2020)

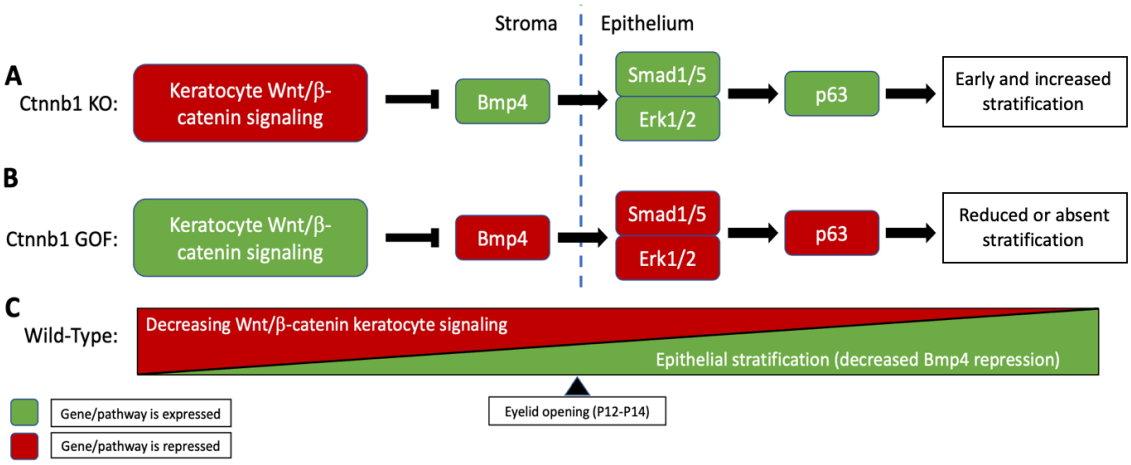

Figure 23. Canonical Wnt/β-Catenin pathway in epithelial stratification. (A) Repression of the Wnt/β-Catenin pathway through β-Catenin (ctnnb1) KO in the stroma results in upregulation of Bmp4, which activates P63 in the epithelium through phosphorylation of Smad1/5 and Erk1/2. Increased stratification is evident. (B) Increased Wnt/β-Catenin signaling as a result of β-Catenin (ctnnb1) gain-of-function inhibits Bmp4 production in the stroma, preventing Smad-dependent upregulation of P63 in the epithelium. Stratification is reduced or absent. (C) In the WT mouse, as eyelid opening approaches (P12-14), Wnt/β-Catenin signaling in stromal keratocytes decreases, removing inhibition of Bmp4 production, which becomes increasingly expressed and promotes stratification upon eyelid opening. Adapted from: (Walker et al., 2020)