THE ROLES OF HH AND AP-2 IN LENS DEVELOPMENT

THE ROLES OF HEDGEHOG AND AP-2 SIGNALING IN THE REGULATION OF LENS DEVELOPMENT

By

CHRISTINE KERR, Honours B.Sc., B. Ed

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AUTHOR: Christine L. Kerr, Honours B.Sc., B.Ed

SUPERVISOR: Dr. Judith West-Mays

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ABSTRACT

Lens development is an intricate process governed by growth factor signaling and a hierarchy of transcription factors that regulate important processes required for normal lens development.

Midline hedgehog (Hh) signaling has been implicated in lens defects including cyclopia and lens degeneration in rodents and fish. A lens specific model of hedgehog signaling has not been examined, and it was unknown whether the lens is able to respond to Hh signals. To investigate this question, and to determine any consequences of abnormal Hh signaling on lens development, a mouse model of constitutively active smoothened in the surface ectoderm and derivatives, (including the lens), was created. These mutants exhibited ectopic expression of FoxE3 by E12.5, and ectopic Pax6 expression by E15.5, along with deregulation of the lens cell cycle and lens degeneration.

Similar to the Hh signaling pathway, normal expression of the transcription factor Activating Protein-2 (AP-2, *tcfap2*), in the lens, was shown to be essential for the maintenance of an epithelial cell phenotype, and the regulation of the lens cell cycle. AP-2α has been shown to be important at the placode stage of development for correct separation of the lens vesicle away from the overlying surface ectoderm. Defects resulting from the loss of AP-2α at this stage do not manifest until E12.5, at time at which AP-2β expression is lost in the lens, suggesting possible redundancy between the two AP-2 family members in early lens development.

To investigate this possible redundancy, *Tcfap2a* and *Tcfap2b* were conditionally deleted from the lens at E9.5 (AP-2α/β DKOs). These family members were shown to play redundant roles during early lens development, with the double mutants exhibiting more severe defects than those seen in the AP-2α single knockout model A more nasally positioned lens stalk and a rotated lens were observed. Severe corneal defects and deregulation of the lens cell cycle were also evident.

Roles for AP-2α in later lens development were unknown. To examine whether or not this transcription factor continues to play a role in lens epithelial cell maintenance subsequent to lens vesicle separation, a mouse model with *Tcfap2a* conditionally deleted from the lens during these later stages of development was created (MRL10-AP-2α). These mutants displayed a disorganized and multilayered lens epithelial cell layer with elongated epithelial cells that abnormally expressed fiber cell specific β/γ crystallins. These mutants also exhibited defects in cell adhesion between the epithelium and fiber cells, as well as between the epithelium and capsule, and exhibited fiber cell defects including vacuoles.

Together, the work presented in this thesis outline previously unknown roles for Hh and AP-2 signaling in lens development. Both Hh and AP-2 are required for the maintenance of a normal lens epithelial cell phenotype and regulation of the cell cycle. This thesis also illustrates the requirement (and redundant roles) for AP-2α and AP-2β at the lens placode stage of development.

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LIST OF ABREVIATIONS

AP-2	Activating Protein-2
ARVO	Association for Research in Vision and Ophthalmology
BCNS	Basal cell nevous syndrome
BMP	Bone morphogenic protein
BOFS	Branchio-Oculo-Facial Syndrome
Cdk	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CEZ	Central epithelial zone
ChIP	Chromatin immunoprecipitation
CKI	Casein kinase I
CNS	Central nervous system
Cos2	Costal2
Crect	Surface ectoderm specific Cre
Cryaa	αA-Crystallin
Cyclin-Cdk	Cyclin-Cyclin dependent kinase
Dhh	Desert hedgehog
DKO	Double knockout
dyl	Dysgenic lens
E	Embryonic day
EMT	Epithelial to mesenchymal transition
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
G1	Gap1 phase

G2	Gap2 phase
GABA	γ-aminobutyric acid
GCL	Ganglion cell layer
GSK-3β	Glycogen synthase kinase-3β
GZ	Germative zone
H&E	Haematoxylin and eosin
Hh	Hedgehog
Ihh	Indian hedgehog
INL	Inner nuclear layer
IR	Inner retina
IRES	Internal ribosomal entry sequence
ISL1	Islet1
ISL2	Islet2
KO	Knockout
L	Lens
LE	Lens epithelium
LoxP	locus of crossing over
LPi	Lens pit
LPl	Lens placode
M	Mitosis
MIP26	Major intrinsic protein 26
nbl	neuroblast layer
Npc1	Niemann-Pick C1
NR	Neural retina

OCT	optimal cutting temperature
onbl	outer neuroblast layer
P	Postnatal day
Pax6	Paired box 6
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PH3	Phospho Histone-3, Phosphorylated Histone H3
PLE	Presumptive lens ectoderm
PKA	Protein Kinase A
PNS	Peripheral nervous system
POM	Periocular mesenchyme
Ptch	Patched
R	Retina
RPE	Retinal pigmented epithelium
S	Synthesis phase
SE	Surface ectoderm
Shh	Sonic hedgehog
Smo	Smoothened
SuFu	Suppressor of fused
Tcfap2	Gene name for AP-2 in rodents
Tfap2	Gene name for AP-2 in humans
TGF-β	Transforming growth factor-β
TUNEL	Terminal uridine Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling
TZ	Transition zone

WT	Wild type
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside
YFP	Yellow fluorescence protein
ZO-1	Zona Occludens-1
α-SMA	α-Smooth muscle actin
β-TrCP	β-transducin repeats-containing proteins

1 CHAPTER 1 - General Introduction

1.1 History of Lens Development

The lens provides an excellent model to study development. Since it is comprised of only two cell types (epithelial and fiber cells), this system allows scientists the opportunity to learn the cellular and molecular mechanisms regulating embryonic induction and morphogenesis. Lens induction has been studied for over 100 years, and the use of classic experiments, transplantation studies and cloning and knockout models has advanced our understanding of lens induction from the classical "single inducer" model, to the five phased model of competence, bias, inhibition, specification and differentiation that we have come to appreciate today (Grainger et al., 1988)

Hans Spemann, a German embryologist was awarded the Nobel Prize in Physiology or Medicine in 1935 for the discovery of embryonic induction. He conducted the first experiments in lens induction in 1901, where he showed that presumptive retinal tissue is required for lens induction. When he ablated the presumptive retina at the neural plate stage, lens induction failed (Spemann, 1901). In 1904, W.H. Lewis provided evidence that the optic vesicle is sufficient for lens induction, and saw the development of lens-like structures after transplanting optic vesicle beneath non lens ectodermal regions (Lewis, 1904).

This simple model illustrating the optic vesicle being the sole tissue necessary for lens induction was questioned after Mencle and Spemann each performed experiments showing lens could form even in the absence of optic vesicle (Mencl, 1903; Spemann, 1907). More recent studies have illustrated that the optic vesicle is not the only tissue

required for lens induction. Early studies failed to determine whether the source of induced lens was from the ectoderm of the donor, or from lens cells remaining after surgery removing the presumptive lens ectoderm (PLE). It was determined that when PLE was removed, lenses formed due to the fact that host lens cells were left adhering to the optic vesicle after the removal of the PLE (Stone, 1940).

Years of research has led us to now accept the five phase model of lens induction mentioned above. Each phase is distinguished by the expression of multiple gene products, including transcription factors in a specific spatio-temporal manner at different stages of embryonic ocular development. Lens competence begins when animal cap ectoderm responds to mesoderm-inducing signals and neural-inducing signals (Nieuwkoop, 1956). During this stage, Sox-3 genes are expressed along with bone morphogenetic proteins (BMPs) and work to make animal cap ectoderm lens competent (Nishimatsu and Thomsen, 1998; Zygar et al., 1998). Lens bias occurs when inductive signals from tissues such as the neural plate create a lens forming bias on a specified region of the head ectoderm (Henry and Grainger, 1990). BMP's, retinoic acid, Pax6 and Otx2, Six3 and Optx2 are all expressed during the lens biasing phase of lens induction (Cheyette et al., 1994; Pannese et al., 1995; Wawersik et al., 1999; Zygar et al., 1998). The third phase, inhibition, occurs to ensure that the lens biased region of ectoderm surrounding the eye forms a lens. Genes expressed during lens bias are expressed in a region of anterior ectoderm much larger than the eye, and thus, a lateral inhibitory mechanism is required and it is believed that signals from migrating neural crest cells

around the optic vesicle work to inhibit ectoderm not in the region of the eye from becoming lens (Von Woellwarth, 1961). Lens specification occurs to ensure that presumptive lens ectoderm becomes committed to a lens fate and to ensure that this tissue will act as a lens without any further inductive instructions (Henry and Grainger, 1990). Pax6 is expressed during this phase and regulates the expression of other genes important in lens development including crystallins (Cvekl et al., 1995a; Cvekl et al., 1995b), which indicate the onset of the final phase, differentiation. During this final phase, several genes including Sox3, L-Maf, Pitx3, Prox1 and Pax6 are expressed to activate crystallin gene expression activating the differentiation program (Cvekl et al., 1995a; Cvekl et al., 1995b; Del Rio-Tsonis et al., 1999; Ogino and Yasuda, 1998; Zygar et al., 1998).

1.2 Ocular Development

Ocular development begins during the early neurula stage, when the diencephalon evaginates bilaterally, forming the optic pits. As the optic pits continue to evaginate, the optic vesicles form and eventually come into close proximity with non-neural surface ectoderm (SE). This close tissue contact allows for inductive signaling, leading to the formation of the lens and cornea (Chow and Lang, 2001). With the close association of the SE and optic vesicles, the SE thickens to form a lens placode at E9.5. By E10.5, the lens placode and outer layer of the optic vesicle invaginates to form the lens pit and optic cup respectively. The optic cup eventually forms the mature retina. The lens pit continues to invaginate, forming a lens vesicle, which by E12.5, has completely separated

away from the overlying SE. By E13.5, primary lens fiber cells have elongated towards the anterior pole of the lens and fill in the lumen of the lens vesicle, while a monolayer of epithelial cells occupy the anterior pole of the lens. Anterior epithelial cells proliferate throughout life, with those cells that migrate below the lens equator differentiating into secondary lens fiber cells. These secondary lens fiber cells eventually lose their nuclei and become transcriptionally silent throughout the lifetime of the mature lens (Chow and Lang, 2001; Lang, 2004; Lovicu and McAvoy, 2005; Lovicu. F.J., 2004; Medina-Martinez and Jamrich, 2007). (Fig 1.1). In order for lens development to proceed correctly, critical growth factors and transcription factors are expressed at specific times in specific cells of the lens, ensuring that correct signaling leads to a normally developed mature lens. When abnormalities in the production and/or expression of these factors goes awry, defects in lens development arise.

1.3 Regulators of Lens Placode Development and Lens Differentiation

Throughout the development of the ocular lens, several growth factors and transcription factors function in a complex genetic hierarchy to regulate proper lens development. Within the ocular media, growth factors exist to influence lens cell polarity as illustrated through lens inversion and re-implantation experiments (Yamamoto, 1976), as well as the growth, and differentiation of specific lens cell types (Lovicu and McAvoy, 2005). Fibroblast Growth Factors (FGFs) exist within the aqueous and vitreous humors of the eye in a gradient and determine anterior-posterior patterns of lens cell behaviour

(Lovicu and Overbeek, 1998; Robinson et al., 1995). High concentrations of FGFs exist in the vitreous humor and prompt cells below the lens equator to migrate and differentiate into fiber cells. Lower concentrations of FGFs exist in the aqueous humor triggering cells above the lens equator to proliferate (McAvoy and Chamberlain, 1989; Schulz et al., 1993). FGFs play a critical role in the initiation of fiber cell elongation and are important in early lens development (Faber et al., 2001; Lovicu and McAvoy, 1989, 1992). BMPs are also required for correct lens induction and fiber cell differentiation. In order for the optic vesicle to develop lens inducing properties, BMP4 expression is necessary (Furuta and Hogan, 1998). Transforming Growth Factor β (TGFβ) is an additional growth factor expressed in the lens and plays a developmental role in secondary fiber cell differentiation (de Iongh et al., 2001).

In addition to growth factors, transcription factors are expressed in the lens to regulate lens development. Figure 1.2 illustrates the cascade of transcription factors that are critical in ensuring lens development proceeds normally (Fig 1.2). At the top of the hierarchy is Pax6, a paired-like homeobox gene that is necessary and sufficient for proper lens development (Pichaud and Desplan, 2002). The Pax6 gene is essential for eye development, and in humans, mice and flies carrying a loss of function mutation in Pax6, eyes fail to develop (Glaser et al., 1992; Hill et al., 1992; Jordan et al., 1992; Quiring et al., 1994). Pax6 expression in the lens is controlled by two enhancers; the SIMO enhancer (Kleinjan et al., 2001) and an ectodermal enhancer. The second of these two enhancers is active in the lens placode at E8.75, in the lens vesicle at E10.5, and in the

lens epithelium beginning at E11.5 (Kammandel et al., 1999; Williams et al., 1998; Xu et al., 1999). Pax6 is expressed in two waves, with its early phase of expression shown to be critical for correct placode development (van Raamsdonk and Tilghman, 2000). Pre-placodal expression of Pax6 regulates the expression of Sox2 and Six3, both important in lens placode formation (Ashery-Padan et al., 2000; Chow and Lang, 2001; Huang et al., 2011; Smith et al., 2009). The second wave of Pax6 expression, following early placode development, is important for regulating genes involved in lens vesicle formation (Plageman et al., 2010).

Multiple transcription factors work downstream of Pax6 including FoxE3, a member of the forkhead family of transcription factors important in lens epithelial cell proliferation and lens vesicle separation. In the lens, FoxE3 is coexpressed with Pax6 at the lens placode and lens pit stages of development. Its expression becomes confined to the lens epithelium at E12.5 (Brownell et al., 2000). Dysgenic lens (dyl) mice with mutations in the DNA binding domain of the *FoxE3* gene exhibit a range of lens phenotypes. These mice have smaller lens placodes, leading to smaller lenses. They also exhibit anterior segment abnormalities including failed lens vesicle closure and separation, and reduced lens epithelial cell proliferation and failed fiber cell denucleation. These mice also get cataracts in later development (Blixt et al., 2000; Brownell et al., 2000). FoxE3 is implicated in two human diseases known as Peter's Anomaly, and Aphakia. Peter's Anomaly is a congenital ocular disorder characterized by a central corneal opacity, a keratolenticular adhesion and sometimes anterior polar cataracts

(Semina et al., 2001). Congenital Primary Aphakia is a rare congenital ocular abnormality characterized by failed lens formation. As a result, the anterior segment of the eye fails to develop. In Secondary Aphakia, lens formation does occur, however the lens degenerates and is reabsorbed perinatally (Valleix et al., 2006)

Six3 belongs to the six-homeodomain family and has been shown to be important in lens cell proliferation and maintenance. In mice, Six3 is first expressed in the lens placode and is activated along with Pax6 and Sox2 at this stage to induce lens placode formation (Cvekl and Duncan, 2007; Liu et al., 2006). Six3 is later expressed in the lens epithelium (Oliver et al., 1995). This transcription factor is important in lens cell proliferation and survival, and when Six3 is inactivated in the lens placode, extensive apoptosis of lens progenitor cells occurs (Cvekl and Duncan, 2007; Liu et al., 2006)

While many of the factors discussed thus far function in lens placode formation, lens epithelial cell proliferation and maintenance, an additional set of genes is required in lens fiber cell development. Primary fiber cell differentiation begins at E11.5 when cells at the posterior of the lens migrate towards the anterior pole of the lens where they form adhesions as differentiation progresses. *β-crystallin* and *γ-crystallin* are genes expressed during primary fiber cell development, and they continue to be expressed as secondary lens fiber cells develop throughout life(Charlton-Perkins et al., 2011). Mutations in these important fiber cell specific genes have been shown to lead to the formation of cataracts in mice and humans (Chambers and Russell, 1991; Chen et al., 2007; Graw, 1999, 2009, 2010). The *Maf* genes are also an important family of genes expressed in the lens. c-Maf

is expressed in the lens placode at E10.5, but its expression in primary fiber cells at E11.5 is far more abundant than in the presumptive lens epithelium. c-Maf remains expressed in secondary lens fiber cells at adult stages of development (Kawauchi et al., 1999). In homozygous *c-Maf* knockout (KO) mice, failed fiber cell elongation and denucleation occurs after E11.5, and since C-maf regulates β/γ-crystallins, the expression of these genes in fiber cells was also disrupted in the KOs (Kawauchi et al., 1999). An additional gene crucial in correct fiber cell elongation is *Prox1*and it is expressed in lens fiber cells at E12.5 (Wigle et al., 1999). *Prox1* is required for the removal of proliferating epithelial cells from the cell cycle, so that these cells may begin their fiber cell terminal differentiation process. In *Prox1* KO mice, cell cycle inhibitors $p27^{kip1}$ and $p57^{kip2}$ are down regulated resulting in failed fiber cell elongation and a hollow lens (Wigle et al., 1999). Lens development is a complex process, and it is clear that a myriad of growth factors, transcription factors and genes are required for normal lens development. Their expression and their influence on the expression of downstream targets are critical for the progression of vertebrate lens development.

1.4 Cell Adhesion in the Developing Lens

Cellular adhesion is a critical process in embryonic development, cell differentiation and growth and the formation of different tissues. The development and maintenance of lens shape is highly dependent on correct migration and adhesion of cells in this tissue. Epithelial cells at the lens equator migrate posteriorly into the transition

zone where differentiation is initiated. Concomitant with the elongation of fiber cells, fiber cells migrate posteriorly along the capsule, and anteriorly along the epithelial cell-fiber cell junction to form a symmetric fiber cell mass (Zelenka, 2004). In order for these cellular migrations to progress, cells must create and break cell-cell and cell-matrix adhesions (Zelenka, 2004). Any disruption in the migration and adhesion of cells in the lens will result in an aberrant lens shape. A number of adhesions at different locations in the lens must be present for correct lens growth and development. For example, lens cells adhere to each other at epithelial-capsule interfaces, epithelial-fiber cell interfaces, epithelium-epithelium interfaces and fiber-fiber interfaces. As different types of adhesions are created and broken, different cellular adhesion molecules, including integrins, vinculin, plakoglobins, cadherins, catenins and Zona-Occludens protein 1 (ZO-1) are expressed. (Beebe et al., 2001; Zelenka, 2004).

Three main types of cellular junctions exist to mediate normal cellular processes and are critical in maintaining homeostasis in the lens. Gap junctions allow passive diffusion of metabolites, ions and cell signaling molecules; adherens junctions hold adjacent cells together though physical interactions, and tight junctions function as a paracellular barrier (Arora et al.; Boswell et al., 2009; Denker et al., 1996; Meng and Takeichi, 2009). ZO-1, a tight junction protein is responsible for mediating the interactions between the cytoskeleton and tight junction proteins. It is expressed in the apical region of the lens epithelium, and is also a marker of apical-basal lens cell polarity (Arora et al.; Fanning et al., 1998). During the formation of lens gap junctions, ZO-1

transports the gap junction protein Connexon-43 to the plasma membrane (Arora et al.; Rhett et al.). Cadherins, including E-cadherin and N-cadherin are adherens junctions proteins. Cadherins on adjacent cells interact with each other through their extracellular domain while they interact with the cytoskeleton through their cytoplasmic domain, and this is mediated through cadherin-catenin binding. This binding is facilitated through the binding of E-cadherin to p120- catenin, which in turn associates with β-catenin. α-catenin interacts with β-catenin and binds this entire adherens junction complex to the actin cytoskeleton. (Bajpai et al., 2008).

The growth of the lens is dependent on the continual addition of fiber cells at the periphery. Lens fiber cell differentiation can be broken down into four stages which can be defined by the type of cell adhesions present. These stages can further be discerned based on the cell adhesion molecules expressed during each stage of fiber cell differentiation (Beebe et al., 2001). During the elongation phase of differentiation, epithelial cells that have exited the cell cycle at the transition zone begin to elongate. Adherens junctions formation occurs at the apical and basal ends of the fiber cells as well as along their lateral surfaces, and the cells form adhesions with the capsule and epithelium (Beebe et al., 2001). During this first stage, N-cadherin, a Ca^{2+}-dependent cellular adhesion molecule involved in controlling cell polarity and tissue morphology is expressed (Vleminckx and Kemler, 1999; Xu et al., 2002). The apical/basal adherens junctions dissolve with the completion of the elongation stage, and fiber cells lose their contact with the epithelium and capsule. At this time, the lateral membrane complexes of

fiber cells are re-structured and become more highly folded and eventually fiber cells become locked to their neighbours (Beebe et al., 2001). At this time, vinculin, (a cytoskeletal protein expressed in cell-cell and cell-matrix adhesions) is upregulated (Beebe et al., 2001; Critchley, 2004). The expression of paxillin (an adaptor protein present in focal adhesions) marks the third stage of differentiation at which time the lateral membranes of fiber cells fuse, creating pores between adjacent cells. This stage is accompanied by a loss of fiber cell nuclei (Beebe et al., 2001; Shestopalov and Bassnett, 2000; Turner et al., 1990). Finally the loss of membrane-bound organelles marks the final stage of fiber cell differentiation (Beebe et al., 2001). Cellular adhesion is key in maintaining proper lens shape throughout life, and the creation and dissolution of adhesions within the lens, along with proper expression of adhesion molecules is important in facilitating the maintenance of a proper lens structure.

1.5 The Cell Cycle in the Developing Lens

During key stages of development, tight regulation of both proliferation and the cell cycle is critical. The lens has emerged as an ideal tissue to study cell cycle regulation due to the fact that over 40 years of research has allowed for precise characterization of proliferative activity in the lens. This research has determined the zones in the lens with high and low proliferation and correlated this data with the developmental stage of the animal in many models including chick, rat and mouse (Griep, 2006). During the lens pit and lens vesicle stages, proliferation is observed throughout the entire lens. Subsequent to

the lens vesicle stage, proliferation is confined to the lens epithelium, and as previously mentioned in this introduction; fiber cells will have exited the cell cycle and lost their nuclei. During postnatal lens development, the lens epithelium can be divided into three zones with differing proliferative capacities. The central epithelial zone (CEZ) displays very low amounts of proliferation; in fact, most of the cells in this region are in a quiescent stage, but retain proliferative capacity. Posterior to the central epithelial zone and anterior to the lens equator sits the germinative zone (GZ). It is in this region where high levels of proliferation are observed. The transition zone lies posterior to the lens equator and no cellular proliferation is observed in this region, as these cells will have exited the cell cycle and begun their differentiation process (Griep, 2006). (Fig 1.3).

The cell cycle consists of 4 stages; G1 (gap 1), S (DNA synthesis), G2 (gap 2) and M (mitosis). Specific checkpoints ensure the cell completes one stage of the cell cycle before progressing to the next, and the restriction point determines if a cell will progress past the G1 stage,, or enter into the G0 quiescent stage (Draetta, 1994; Ohtsubo and Roberts, 1993; Pardee, 1989; Quelle et al., 1993). Progression through the cell cycle is promoted by cyclin/cyclin-dependent kinase (Cyclin-Cdk) complexes (Harper and Elledge, 1996; Sherr and Roberts, 1995). Cyclin D forms complexes with Cdk4 and Cdk6 and functions in the G1 phase of the cell cycle, and is also required for the G1-S phase transition. Cyclin E partners with Cdk2 and also functions in the G1 phase of the cell cycle. Cyclin A associates with Cdk2 and Cdc2 and functions both within S phase and at the G2-M phase transition. Finally, cyclin B complexes with Cdc2 and is required for a

cells entry into M phase (Baldin et al., 1993; Draetta, 1994; Dulic et al., 1992; Hunter and Pines, 1994; Koff et al., 1992; Meyerson et al., 1992; Zindy et al., 1992). While these cyclin/Cdk complexes promote the movement of a cell through the cell cycle, they are negatively regulated by Cyclin Dependent Kinase Inhibitors (CDKI's) including $p27^{kip1}$ and $p57^{kip2}$ (Sherr and Roberts, 1995; Zindy et al., 1992).

In the lens, these cyclin/Cdk complexes along with specific CDKI's are expressed in different regions. Cyclin D1 and cyclin D2, along with cyclin A, cyclin B and cyclin E are expressed in the lens epithelium (Fromm and Overbeek, 1996; Gao and Zelenka, 1997; Gomez Lahoz et al., 1999). Cdk2 and Cdc2 are all found in the lens epithelium, while Cdk4 is found in the lens epithelium at the lens equator where epithelial cells begin to differentiate into fiber cells (Fromm and Overbeek, 1996). The CDKI $p57^{kip2}$ is expressed in high levels at the transition zone, while $p27^{kip1}$ is expressed at the transition zone, and at lower levels throughout the epithelium (Griep, 2006; Zhang et al., 1998). E2F family members (involved in activation and repression of the cell cycle) are also expressed in the lens epithelium along with their target gene *c-myc* (Griep, 2006; Harris et al., 1992; Rampalli et al., 1998). Fewer cell cycle regulators are expressed within the fiber cells than in the lens epithelium. Cyclin D1/D2-Cdk4 and cyclin B-Cdc2 are expressed at the transition zone suggesting that they play critical roles in both cellular proliferation and differentiation (Gao et al., 1999; He et al., 1998). Tight cell cycle regulation is important in maintaining a normally developing lens. It is required for proper proliferation of lens epithelial cells, and the correct transition between

proliferation and differentiation for the progression of fiber cell development. Many factors involved in lens development work to maintain highly regulated cell cycle activity, and mutations involving these factors can cause cell cycle defects and deregulation.

1.6 Hedgehog Signaling in Lens Development

One signaling pathway known to regulate the cell cycle is the hedgehog (Hh) signaling pathway. The Hh pathway interacts with cell cycle regulators at different stages of the cell cycle, and when abnormalities in Hh signaling arise, the cell cycle becomes de-regulated, leading to abnormal proliferation (Roy and Ingham, 2002). The Hh gene family, first discovered in *Drosophila,* encodes several proteins that play a role in mediating inductive interactions important in vertebrate development (Ingham, 2001). The Hh genes encode a family of secreted signaling proteins that are essential in the myriad of developmental processes during embryogenesis, including growth, patterning, morphogenesis and tissue formation (Ingham and McMahon, 2001). Lipid modifications play an important role in the maturation of Hh proteins. Mature Hh proteins are covalently coupled to cholesterol at their carboxy-terminal end, and are palmitoylated at a highly conserved cysteine residue at the proteins' amino-terminal end (Ingham, 2001).

In vertebrates, the Hh family includes three homologues [Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh)] (Huangfu and Anderson, 2006). Shh and Ihh have been shown to play important roles in embryogenesis, specifically in

the patterning of the limb and neural tube, while Dhh has been implicated in embryonic development as well as other processes such as the regulation of spermatogenesis (Bitgood et al., 1996; Echelard et al., 1993).

Hh signals through an intricate cascade of events that ultimately results in the activation or repressions of Hh target genes. In the presence of the Hh ligand, Hh binds to its receptor, Patched (Ptch), a multiple membrane spanning protein. Smoothened (Smo), a seven-transmembrane spanning effector protein usually repressed by Ptch and internalized inside the cell, is then localized to the primary cilium where it activates downstream signaling events (Huangfu and Anderson, 2006). The mechanism resulting in the activation of smo is still unknown, as neither Ptch nor Hh interact directly with this protein. It is hypothesized that smo is regulated by intermediate components that signal between Ptch and smo. Ptch is observed to share structural homology to the Niemann-Pick C1 (Npc1) protein, which is important in cholesterol trafficking (Scott and Ioannou, 2004). Similar to Npc1, Ptch exhibits a sterol sensing domain, which has led to evidence that Ptch regulates smo activity through the transportation of small endogenous hydrophobic molecules that bind to smo and act both synergistically and antagonistically to regulate smo activity (Carstea et al., 1997; Chen et al., 2002; Huangfu and Anderson, 2006).

Three crucial zinc finger transcription factors, Gli1, Gli2 and Gli3, work to either activate or repress the transcription and translation of Hh target genes (Fuccillo et al., 2006; Jia et al., 2009) In the absence of Hh, Gli1 and Gli2 (activator forms of Gli) are

phosphorylated by Protein Kinase A (PKA), Casein Kinase I (CKI) and Glycogen Synthase Kinase-3β (GSK-3β) and targeted for degradation by the proteasome in a β-TrCP mediated pathway. These kinases are also required to modify Gli3 into its repressor state in preparation for its translocation to the nucleus to repress the transcription of Hh target genes (Varjosalo and Taipale, 2007; Wang and Li, 2006). With the activation of the Hh pathway, activator forms of Gli (Gli1 and Gli2), normally sequestered in the primary cilium by the kinesin-related protein, Costal-2 (Cos2), and suppressor of fused (SuFu), are translocated to the nucleus where they activate transcription and translation of hedgehog related target genes including cell cycle genes *cyclin D*, *cyclin E*, and *myc* (Huangfu and Anderson, 2006; Varjosalo and Taipale, 2007). (Fig 1.4).

Hh signaling has been shown to be critical for proper forebrain and eye development. In early embryogenesis, precise ventral midline Shh signaling is crucial in mediating proper bilateral division of the forebrain and eye fields (Menuet et al., 2007; Retaux et al., 2008). The detrimental effects of improper Shh signaling on forebrain and eye development is well illustrated by the condition Cyclopia. When a lack of Shh signaling is present in the animal, the forebrain fails to develop as it should, and a single central eye forms in the middle of the face (Retaux et al., 2008).

Perhaps the most well studied model of Hh signaling is that seen the *Astyanax* Cavefish. This fish, which has evolved from surface dwelling fish, expresses excessive Shh signaling leading to blindness due to extreme eye degeneration throughout

development (Yamamoto et al., 2004). In this model, midline Shh expression is expanded in anterior domains of the brain, including the floor plate, the hypothalamus, the zona limitans intrathalamica and the subpallium (Menuet et al., 2007). Eye degeneration in cavefish is observed starting in the early stages of embryonic development where the optic vesicle is reduced in size, and the ventral portion of the optic cup is lost. Transcription factors Pax2a and Vax1, expressed normally in the optic stalk, show expression domains that have expanded into the optic cup, thus modifying customary expression of these factors in cavefish optic primordia (Yamamoto et al., 2004). Cavefish also display reduced expression of the transcription factor Pax6 at the neural plate stage, reduced expression of the anti-apoptotic factor αA-crystallin in the developing lens, and display smaller lenses that undergo extensive apoptosis (Retaux et al., 2008). Interestingly, the cavefish retina initially develops normally, however, subsequent to lens degeneration, retinal neurons undergo cell death leading to the complete degeneration of the retina and disappearance of the eye (Retaux et al., 2008).

Each of the three vertebrate homologues of Hh has been found to play important roles in eye development. While Dhh expression has been identified in the central retinal pigmented epithelium (RPE), Ihh has been shown to demonstrate important roles in the development of both the RPE and scleral mesenchyme (Dakubo et al., 2008; Perron et al., 2003). Dakubo et al., have illustrated that the RPE and scleral mesenchyme are direct targets of Ihh signaling, and require correct Ihh signaling for proper pigmentation patterning of the RPE and condensation of mesenchymal cells for accurate development

of the sclera (Dakubo et al., 2008). In humans, mutations in the Ptch receptor PTCH1, which cause an activation of the Hh pathway even in the absence of Hh ligands, result in Gorlin Syndrome. This disorder results in various ocular defects, including defects in the retina, iris and lens (including cataract formation) (Hahn et al., 1996; Maity et al., 2005; Taylor et al., 2006). **In examining factors important in lens development, one goal of this dissertation is to determine how Hh signaling impacts lens development. This thesis examines the hypothesis that the lens will respond to abnormal Hh signaling resulting in deregulated lens development. Increased knowledge of this process in lens development will help to further our understanding of human ocular disorders.**

1.7 Activating Protein-2 Transcription Factors

The Activating Protein-2 (AP-2) transcription factors are essential for proper development and morphogenesis of a myriad of tissues and organs during embryogenesis. Like Hh signaling, AP-2 genes have also demonstrated roles in cell cycle regulation. Abnormal AP-2 expression has been shown to both promote and repress cellular proliferation and differentiation and aberrant AP-2 expression has been observed in multiple cancers, including skin, breast, thyroid and cervical cancer (Beger et al., 2001; Chiefari et al., 2002; Wang et al., 2006; Williams et al., 2009).

AP-2 transcription factors belong to the helix-span-helix class of transcription factors, and is made up of 5 family members in rodents and humans (AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε) that are each encoded by a separate gene (*Tcfap2a-e* in rodents and

TCFAP2A-E in humans) (Eckert et al., 2005; Feng et al., 2008). The AP-2 transcription factors bind to the palindromic consensus sequence, 5'-GCCN$_3$-GGC-3', and have also been shown to bind to additional GC binding motifs differing from the consensus sequence (Eckert et al., 2005; Mohibullah et al., 1999; Williams and Tjian, 1991a). The C-terminal region of AP-2 contains a DNA binding and dimerization domain that spans approximately 200 amino acids, while the N-terminus of the protein contains a short proline-rich sequence for transactivation (Fig 1.5). (Williams and Tjian, 1991a). This characteristic domain structure is highly conserved between species and has been shown to be between 60% and 99% homologous at the amino-acid level (Eckert et al., 2005). The AP-2 proteins bind DNA as a homodimer or heterodimer, and it has been shown that when AP-2 loses its ability to dimerize, it is no longer able to bind to DNA. AP-2 has also been shown to form dimers in solution without the presence of DNA (Williams and Tjian, 1991a).

The AP-2 family members have both overlapping and unique expression patterns throughout development. During early development, AP-2α, AP-2β and AP-2γ are primarily expressed in neural crest cells and the SE. With the progression of development, AP-2α, AP-2β and AP-2γ display overlapping and unique patterns of expression within the facial ectoderm, limbs, and regions of the Central Nervous System (CNS) as well as the Peripheral Nervous System (PNS), urogenital tissues, cornea and lens (Chazaud et al., 1996; Eckert et al., 2005; Moser et al., 1995; Moser et al., 1997b; Williamson et al., 1996). AP-2δ has been shown to be expressed in the developing heart,

CNS and retina while the fifth AP-2 family member, AP-2ε has shown expression restricted to the CNS, specifically the olfactory system (Feng and Williams, 2003; Zhao et al., 2003).

1.8 Ocular Expression of AP-2

AP-2 family members are expressed in a myriad of tissues contributing to ocular development including the SE, forebrain, neural crest cells and their derivatives as well as the retina (Bassett et al., 2012; West-Mays et al., 1999). AP-2α and AP-2β expression is first detected in the SE in the mouse at E8.75. These proteins continue to be co-expressed at E9.5 within the lens placode and the neural crest-derived mesenchymal cells between the evaginating optic cup and forebrain and within those cells adjacent to the lens placode. At E10.5, AP-2α and AP-2β remain coexpressed within the SE and also are expressed in epithelial cells lining the lens pit. The separation of the lens vesicle at E12.5 marks the period of time when AP-2α has become uniquely expressed in the anterior lens epithelium, and the expression of AP-2β from this region of the lens is lost (West-Mays et al., 1999). The expression pattern of AP-2α and AP-2β remains overlapping in the SE ectoderm at this stage, within cells fated to differentiate into the corneal epithelium. By E15, AP-2α is also expressed within the eyelids and corneal epithelium (Pontoriero et al., 2008; West-Mays et al., 1999). (Fig 1.6). Our lab has also reported AP-2α expression in the developing retina. AP-2α is first detected in the retina at E13.5 in the presumptive inner nuclear layer within the central retina. The number of AP-2 expressing cells within

this layer increases between E13.5 and E15.5 with expression extending to the peripheral retina. At postnatal (P) day 0, AP-2α is expressed in the ganglion cell layer and continues to be expressed within the inner nuclear layer (Bassett et al., 2007). Both amacrine and horizontal cells have been shown to co-express AP-2α and AP-2β (Bassett et al., 2012)

1.9 The Role of AP-2 in the Context of Eye Development

Extensive work has been done examining the role of AP-2α in eye development in our lab. Our work has illustrated important roles of AP-2α in the development of ocular tissues including the cornea, lens and retina (Bassett et al., 2007; Bassett et al., 2010; Pontoriero et al., 2008; West-Mays et al., 2002; West-Mays et al., 1999). Early work by West-Mays et al., illustrated a role for AP-2α in early lens vesicle development (West-Mays et al., 1999). In this work, AP-2α germ-line KO and AP-2α chimeric mutant mice were generated, and displayed aberrant ocular development. Although eyes of the AP-2α null mutants appeared similar to wild type (WT) littermates at E9.5-E10.5, defects were observed beginning at E12.5. By this stage, 38% of these mutants lacked one or both eyes, while 62% of the KO mutants had eye or eye rudiments embedded inside the head, surrounded by an excess of mesenchymal and neural tissue (West-Mays et al., 1999). Lens development was perturbed beginning at E11.5 with failed lens induction in some mutants. The SE, which normally forms the lens vesicle, was also seen to be induced to invaginate into the optic cup. Those lenses that did form were abnormal in shape and

reduced in size, and remained connected to the overlying SE by a lens stalk, which as a result, caused failed lens epithelial cell development (West-Mays et al., 1999). This lens stalk phenotype is reminiscent of the human congenital condition Peter's Anomaly (Guercio and Martyn, 2007). Fiber cell specific Major Intrinsic Protein 26 (MIP26) was misexpressed in the AP-2α null lens, while the expression of the lens epithelial marker, Pax6, was absent from the lens. Optic cup and corneal defects were also observed in the AP-2α null and chimeric mice (West-Mays et al., 1999).

After the examination of the AP-2α null mice, questions regarding the direct cause of the ocular phenotypes remained unanswered. It was not clear whether ocular developmental defects were resulting from an intrinsic loss of AP-2α, and whether or not defects were occurring due to a loss of AP-2α in specific eye tissues (Pontoriero et al., 2008). To address these questions, a conditional deletion of Tcfap2a from the lens placode and its derivatives of mice (Le-AP-2α) was created in our lab, by employing Cre-loxP technology, allowing for the examination of a possible cell autonomous role of AP-2α in lens development (Pontoriero et al., 2008). Our findings illustrated that the loss of AP-2α from the lens placode leads to defects confined to the lens placode and its derivatives. These defects closely resembled the germline KO phenotype. The conditional Le-AP-2α mutants displayed a lens vesicle that had failed to separate away from the overlying SE, illustrated by a lens stalk, similar to that seen in the AP-2α germline KO mutants. Pigmented cells were observed in some of the mutant cells within the lens stalk, with some of these cells extending into the lining the cornea (Pontoriero et

al., 2008). Unlike the AP-2α germline KO mutants, optic cup defects were not evident in the Le-AP-2α conditional mutants, further solidifying the hypothesis that AP-2α has a cell autonomous role in early lens morphogenesis at the lens vesicle stage of development (Pontoriero et al., 2008).

Interestingly, in both the AP-2α germline KOs and Le-AP-2α conditional mutants, although AP-2α was absent from lens at the lens placode stage of development (E9.5), initial defects were not observed until the lens vesicle stage, around E12-E12.5. This time point also corresponds with the time at which AP-2β expression is lost from the lens. This suggests that AP-2β may exhibit redundancy with AP-2α at the lens placode and lens pit stages of development.

1.10 Possible redundancy and cooperation of AP-2 family members during embryogenesis and ocular development

Germline deletions of AP-2α and AP-2β in mouse models have all illustrated specific roles for each gene in development. Due to their unique expression in certain tissues, their absence causes developmental abnormalities that could not be compensated for by another AP-2 family member in those tissues. AP-2$\alpha^{-/-}$ germline KO mice show extensive non ocular abnormalities, including defective development of the face, skull, sensory organs and cranial glia. These mice also display cranio-abdominoschisis and failed cranial closure and die prenatally (Schorle et al., 1996). AP-2$\beta^{-/-}$ germline KO mice die between P1 and P2 as a result of renal epithelial cell apoptosis, and also exhibit

low levels of noradrenaline and noradrenaline synthesizing dopamine β-hydroxylase in the PNS as well as abnormal noradrenaline neuron development in the locus coeruleus (Hong et al., 2008; Moser et al., 1997a).

Although the AP-2 family members appear to have specific developmental functions in those tissues in which they are uniquely expressed, animal models examining those tissues which coexpressed AP-2 family members provide support for the idea that these family members may exhibit redundancy or cooperation within these tissues. These two family members have been shown to play compensatory roles in retinal development (Bassett et al., 2012). Bassett et al., showed that the loss of AP-2α and AP-2β resulted in a loss of horizontal cells and an altered pattern of expression of amacrine cell markers in the *Tcfap2a/b* double mutants. BHLHB5, normally expressed in postmitotic GABAergic amacrine cells, was absent from the inner nuclear layer of the double mutants at birth (Bassett et al., 2012). An altered pattern of staining of Islet1 (ISL1) and Islet2 (ISL2) (expressed in retinal ganglion cells and cholinergic amacrine cells), and Sox2 (expressed in cholinergic amacrine cells and Muller glia cells), was also observed in the *Tcfap2a/b* mutants (Bassett et al., 2012).

As mentioned previously, AP-2α and AP-2β are coexpressed in the developing SE and lens until the lens vesicle separates away from the SE at E12.5, at which time AP-2β expression is lost from the lens. It is hypothesized that AP-2β compensates for the loss of AP-2α in these tissues in early lens development, since lens defects do not occur until between E12 and E12.5 when AP-2β expression has been lost from the lens. Preliminary

studies carried out in the West-Mays lab examining AP-2α/AP-2β germline KO mutant mice display earlier lens defects beginning at the lens placode stage of development and failed lens formation by E10.5. Due to embryonic lethality of these mice at E12.5, lens development cannot be studied further. **In continuing to investigate important factors involved in lens development, the goal of this dissertation is to both further investigate the role of AP-2α in later stages of lens development, subsequent to lens vesicle separation, and to discern any redundant roles that AP-2α and AP-2β may have during early lens development.**

1.11 AP-2 in human ocular disorders

TFAP2A and *TFAP2B* have both been implicated in human disorders. *TFAP2B* has been implicated in Char Syndrome. This autosomal dominant disorder results from missense mutations in the *TFAP2B* gene and results in patient ductus arteriosus, facial abnormalities and hand anomalies (Satoda et al., 2000; Zhao et al., 2011).

Within the last few years, mutations in *TFAP2A* have been studied and have been shown to result in the condition called Branchio-Oculo Facial Syndrome (BOFS). This condition is a rare autosomal dominant disorder characterized by a range of phenotypes, including ocular phenotypes (Milunsky et al., 2008). BOFS can occur as a result of a 3.2 Mb deletion on chromosome 6 that includes *TFAP2A* (Milunsky et al., 2008). Further investigation into the genetic cause of this disorder illustrated that a missense mutation within the DNA binding domain of *TFAP2A* (exon 4 and exon 5), also resulted in BOFS

in some patients (Milunsky et al., 2008). The loss of function mutation of *TFAP2A* in BOFS patients is shown to result in many phenotypes, including craniofacial abnormalities such as a malformed pinnae, thick nasal tip, up-slanted eyes, and cleft lip which may or may not be accompanied by cleft palate. This disorder also presents with hearing loss, small teeth, dysplastic nails and scalp cysts, and in rare occasions, renal abnormalities, stunted growth, developmental delays and cancer have been noted (Lin et al., 1995; Milunsky et al., 2008). Interestingly, this disorder also presents with a range of ocular phenotypes including microphthalmia, anophthalmia, cataract, coloboma, ptosis and strabismus (Lin et al., 1995; Milunsky et al., 2008).

1.12 Rationale for the study

Hh signaling has been studied in several models and has been shown to play roles in lens development. Hh overexpression in zebra fish results in failed lens formation (Barth and Wilson, 1995; Cornesse et al., 2005; Dutta et al., 2005)**,** while excessive Shh signaling in cavefish leads to eye degeneration (Yamamoto and Jeffery, 2000; Yamamoto et al., 2004). In addition, a loss of midline Shh signaling in Shh KO mice results in cyclopia (Chiang et al., 1996). Although these studies illustrate the importance of correct Hh signaling in regulating lens-specific gene expression and lens development, the effects of Hh signaling, especially an upregulation of Hh signaling, specifically in the lens has not been examined. A detailed examination of a lens specific model mimicking excessive Hh signaling will determine the ability of the lens to respond to Hh signals, as

well as outline the genes important in lens development that are regulated by Hh signaling in the lens.

Research has also illustrated roles for AP-2α in early lens development at the placode stage of development (Pontoriero et al., 2008; West-Mays et al., 1999), Defects in the Le-AP-2α placode specific KO model did not arise until E12. As mentioned previously, studies have shown a possible redundant role for AP-2α and AP-2β in development (Bassett et al., 2012). Since defects in the Le-AP-2α conditional KO model did not arise until after AP-2β expression was lost from the lens, possible redundancy of AP-2α and AP-2β in the early stages of lens development, beginning at E9.5, will be examined through the generation of a placode specific *Tcfap2a/Tcfap2b* double KO (AP-2α/AP-2β DKO) model.

Although the requirement for AP-2α in early lens development has previously been examined (Pontoriero et al., 2008; West-Mays et al., 1999), its role in later stages of lens development, subsequent to lens vesicle separation remains unknown. AP-2α is expressed in the lens epithelium throughout life, and to determine its role at later developmental stages, a lens specific conditional KO model with *Tcfap2a* deleted from the epithelium at the lens vesicle stage (E12.5) of development will help elucidate its roles at these later developmental stages.

1.13 Hypotheses and implications of my thesis research

My Ph.D research focused on furthering an understanding of genes involved in lens development. This thesis focused on gaining a better understanding of how two gene families, including the *Hh* and *Tcfap2* families are involved in lens development. Little is known about the role that Hh plays in lens development, and although much research on AP-2 in eye development has been examined during early stages of lens development, its roles in later development, as well as its potential compensatory roles with other AP-2 members during lens development was unknown, and required further study. My research resulted in three projects which further resulted in one published paper. My research hypotheses were:

1. The lens will respond to abnormal Hh signaling resulting in deregulated lens development.
2. *Tcfap2a* and *Tcfap2b* act redundantly in early stages of lens development.
3. *Tcfap2a* is required after lens vesicle separation to maintain a normal lens epithelial phenotype.

These three hypotheses were studied and resulted in the following publication (from hypothesis 1)

Kerr, C.L., et al. *Activation of the hedgehog signaling pathway in the developing lens stimulates ectopic FoxE3 expression and disruption of fiber cell differentiation.* Investigative Ophthalmology and Vision Science. 2012. **53**(7): p. 3316-30

My Ph.D studies have demonstrated the ability of the developing lens to respond to Hh signals. We have also learned the importance of normal Hh signaling in maintaining a normal lens epithelial phenotype, and correct cell cycle regulation in the developing lens. This thesis also demonstrates that *Tcfap2a* acts redundantly with *Tcfap2b* in early lens development to regulate lens vesicle separation and the lens cell cycle. Finally, my Ph.D studies have illustrated the importance of correct AP-2α expression subsequent to lens vesicle separation in the maintenance of a correct lens epithelial phenotype.

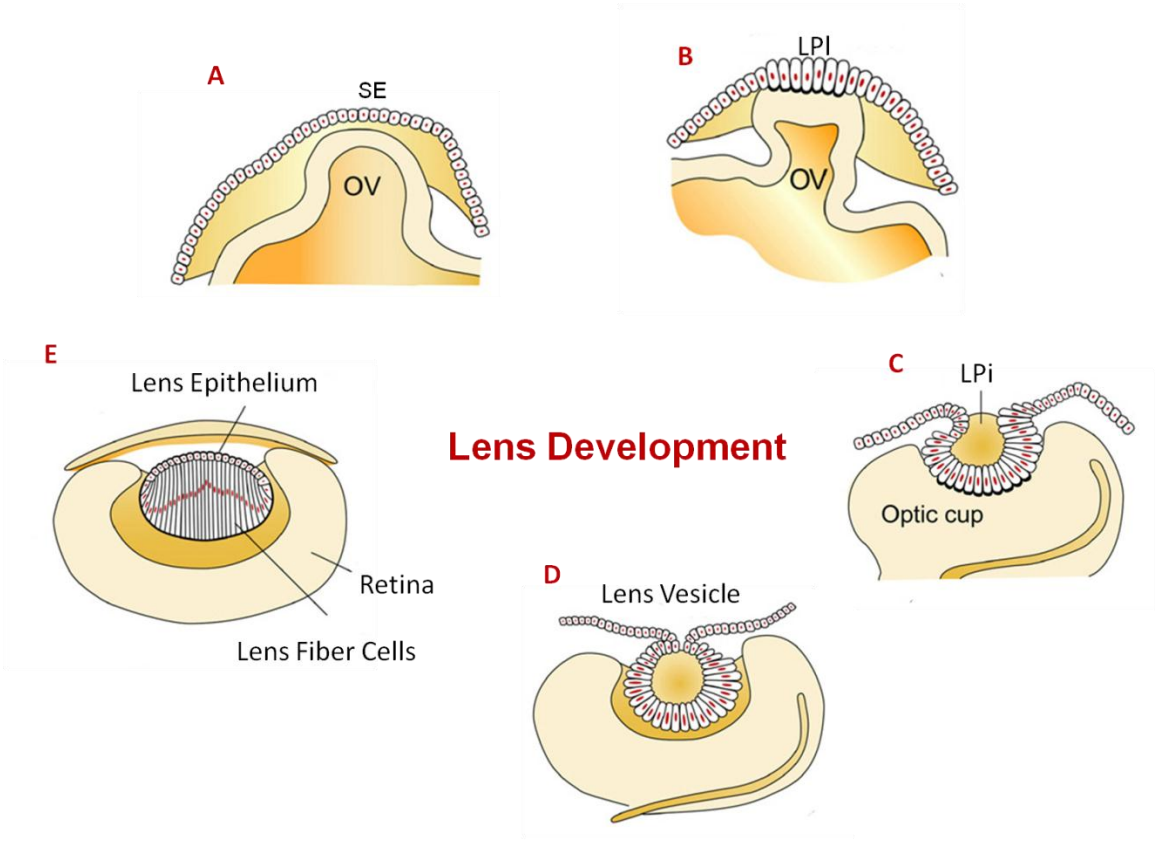

Figure 1.1 Vertebrate eye development.
Vertebrate eye development progression requires the interaction of several embryonic tissues. Subsequent to the formation of the optic vesicle (OV), this tissue along with the neuroepithelium, contacts the surface ectoderm (SE) to induce the formation of the lens placode (LPl) (A and B). The optic vesicle collapses inward giving rise to the optic cup (C) which differentiates into the mature retina (E). The LPl invaginates inward to form the lens pit (LPi) (C). The lens pit forms a lens vesicle (LV) (D), which continues to invaginate and eventually separates away from the overlying SE. Cells of the lens vesicle differentiate into two lens cell types. Primary lens fiber cells elongate at the posterior end of the lens, losing their nuclei to become transcriptionally silent throughout the life of the animal. Cells at the anterior end of the lens differentiate into anterior lens epithelial cells which continue to proliferate throughout life (E). adapted from (Andley, 2008).

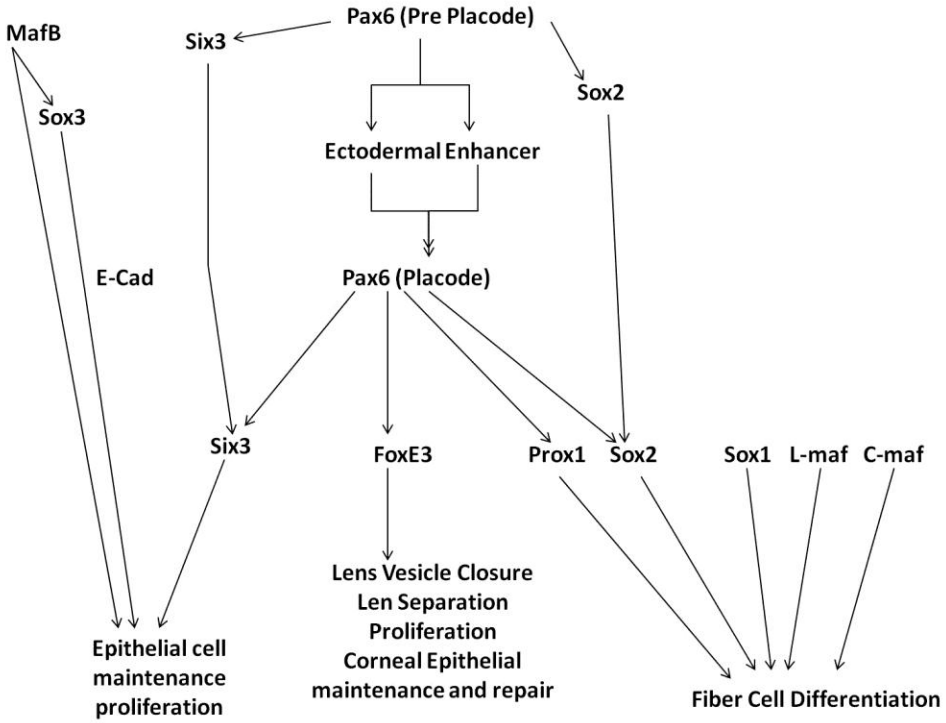

Figure 1.2 Cascade of transcription factors regulating lens development.
This cascade illustrates the numerous factors that act in the complex process of vertebrate eye development. Pax6 acts early in the development of presumptive eye primordia, and is again crucial in the formation of the lens placode. Downstream of Pax6, transcription factors such as Six3 and FoxE3 function in epithelial maintenance, vesicle closure and proliferation, while Prox1 and the Maf genes, along with the crystallins (not shown) work to achieve proper fiber cell elongation and differentiation. Factors involved in lens development are still being discovered, and the complex cascade will continue to grow adapted from (Chow and Lang, 2001).

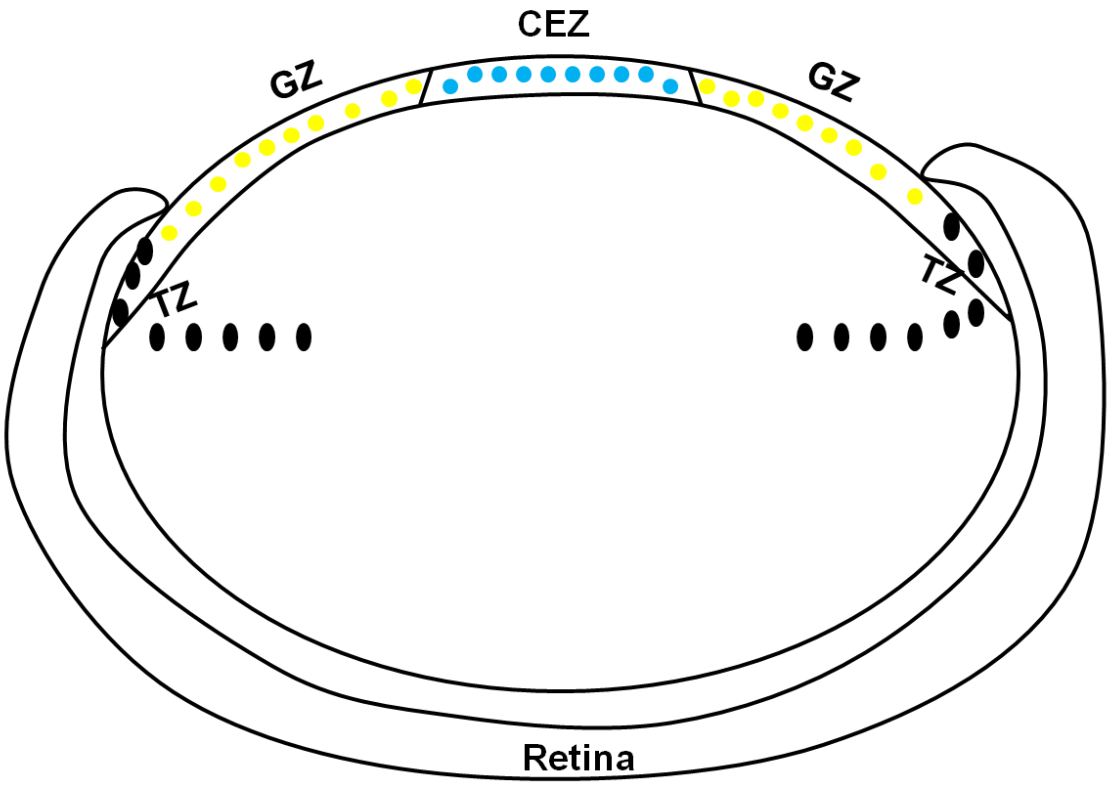

Figure 1.3 Proliferation zones of the developing lens.
During postnatal lens development, the lens epithelium can be divided into three zones with differing proliferative capacities. The central epithelial zone (CEZ) displays very low amounts of proliferation (blue); in fact, most of the cells in this region are in a quiescent stage, but retain proliferative capacity. Posterior to the CEZ, and anterior to the lens equator sits the germinative zone (GZ). It is in this region where high levels of proliferation are observed (yellow). The transition zone (TZ) lies posterior to the lens equator and no cellular proliferation is observed in this region (black), as these cells will have exited the cell cycle and begun their differentiation process. adapted from (Griep, 2006).

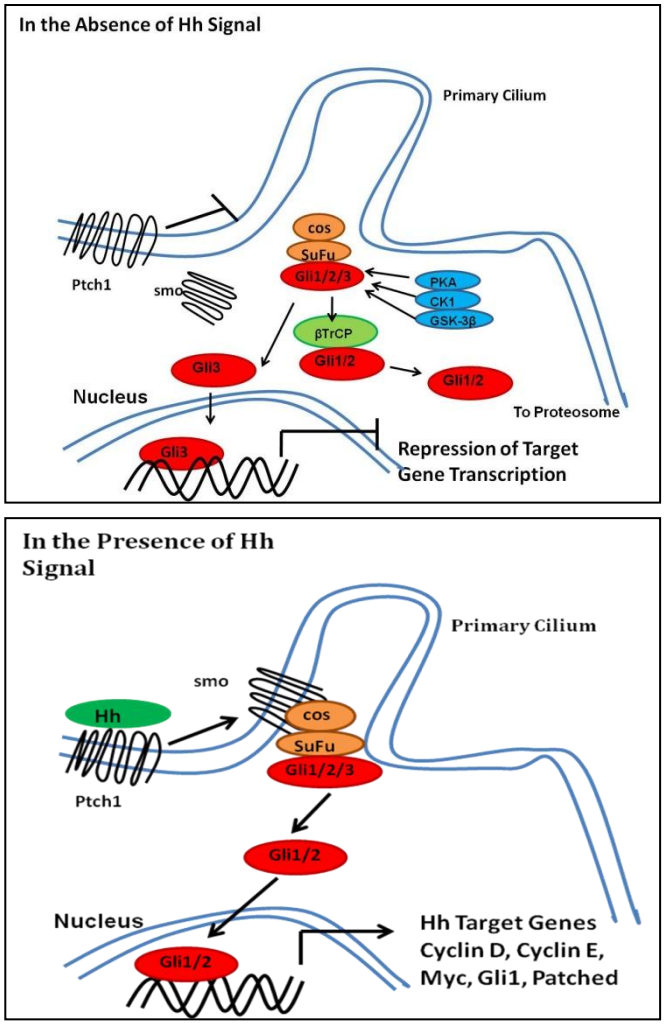

Figure 1.4 Hedgehog signaling pathway.
In the absence of Hh, activator forms of Gli are phosphorylated by PKA, CK1 and GSK-3β and sent to the proteasome for degradation. Gli3, the repressor form of the transcription factor is translocated to the nucleus where repression of Hh target genes occurs. With the binding of Hh to its receptor patched (Ptch1), the repression of Ptch1 on smo is released, and smo activates downstream signaling events. Gli1/2, activator forms of the Gli transcription factors are translocated to the nucleus to activate expression of Hh target genes. adapted from (Huangfu and Anderson, 2006).

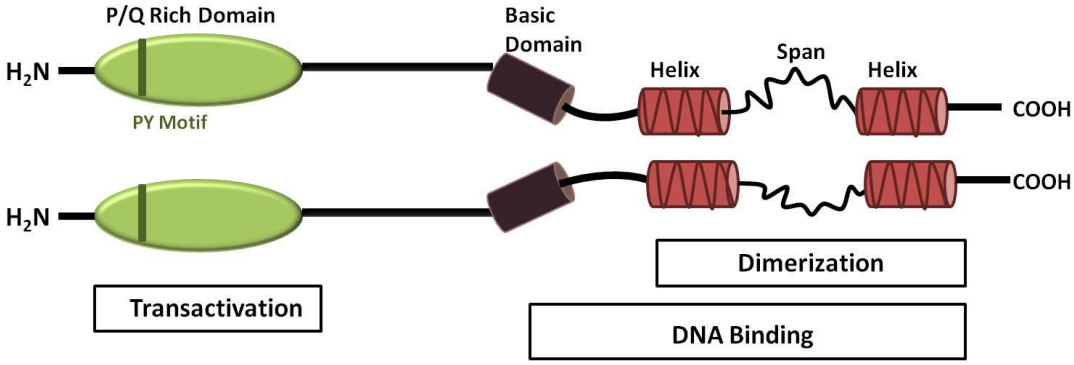

Figure 1.5 Protein structure of an AP-2 homodimer.
The AP-2α protein consists of a C-terminal helix-span-helix motif for DNA dimerization, followed by a basic region. The basic domain, along with the helix-span-helix motif, facilitates DNA binding at the consensus sequence 5'-GCCN_3GG-3'. The N-terminal region of the protein includes a proline/glutamine rich domain involved in transactivation. adapted from (Eckert et al., 2005).

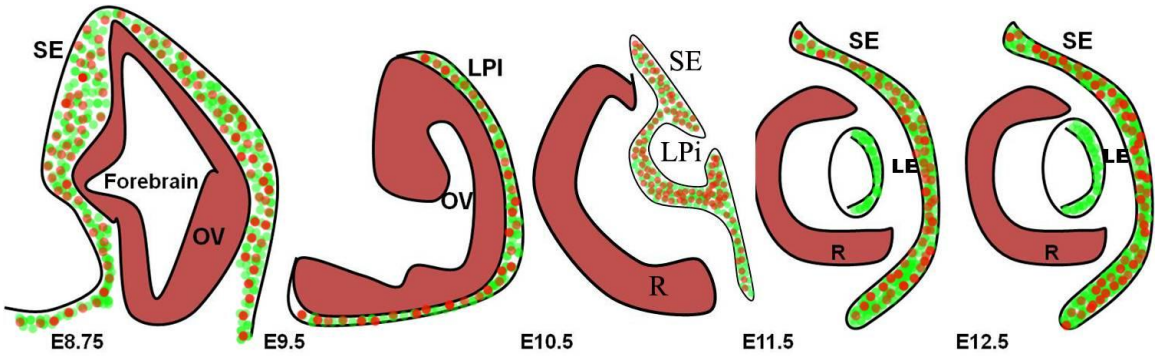

Figure 1.6 Expression of AP-2α and AP-2β in the developing lens.
AP-2α staining is denoted with green dots. AP-2β expression is denoted with red dots. AP-2α and AP-2β are seen to be expressed in the cells of the surface ectoderm at E8.75 and the developing lens placode at E9.5. At E10.5, AP-2α and AP-2β are expressed in the epithelial cells lining the lens pit. By E12.5, AP-2α is expressed in anterior epithelial cells of the lens vesicle and the SE, while AP-2β expression has disappeared from the lens, but persists in the overlying SE**.** AP-2α and AP-2β are expressed in cells of the retina at E12.5. SE-Surface Ectoderm; OV-Optic Vesicle; LPl-Lens Placode; L-lens, LE-Lens Epithelium; R-Retina.

2 CHAPTER 2 - Activation of the hedgehog signaling pathway in the developing lens stimulates ectopic Foxe3 expression and disruption in fiber cell differentiation

The work described in this chapter has been published in Investigative Ophthalmology and Visual Science (IOVS)

I have carried out all of the studies in this manuscript, with the exception of the breeding and collection of different stages of the WT and activated smoothened mouse embryos, which was performed in the laboratory of Dr. Trevor Williams at the University of Colorado Denver, by Jian Huang.

INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE (IOVS), June 2012, p. 3316-3330
Vol. 53, No. 7
doi: 10.1167/iovs.12-9595

Abstract

The hedgehog genes encode extracellular signaling proteins responsible for patterning and tissue formation during embryogenesis. Signal transduction of this pathway is mediated through activation of the transmembrane proteins smoothened and patched, stimulating downstream signaling resulting in the activation or repression of hedgehog target genes. Hedgehog signaling is implicated in eye development, and defects in hedgehog signaling components have been shown to result in defects of the retina, iris and lens. In this study we assess the consequences of constitutive hedgehog signaling in the developing mouse lens using Cre-LoxP technology to express the conditional M2 smoothened allele in the embryonic head and lens ectoderm. Although initial lens development appeared normal, morphological defects were apparent by E12.5 and became more significant at later stages of embryogenesis. Altered lens morphology correlated with ectopic expression of FoxE3, which encodes a critical gene required for human and mouse lens development. Later, inappropriate expression of the epithelial marker Pax6 as well as fiber cell markers c-maf and Prox1 also occurred indicating a failure of appropriate lens fiber cell differentiation accompanied by altered lens cell proliferation and cell death. Our findings demonstrate that the ectopic activation of downstream effectors of the hedgehog signaling pathway in the mouse lens disrupts normal fiber cell differentiation by a mechanism consistent with a sustained epithelial cellular developmental program driven by FoxE3.

2.1 Introduction

In vertebrates, the Hedgehog (Hh) family comprises three developmentally important homologues, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh) (Huangfu and Anderson, 2006; Jia and Jiang, 2006). In a receiving cell, signal transduction driven by the Hh ligand relies on the Patched (Ptch) and Smoothened (Smo) transmembrane proteins. In the absence of Hh, Smo is repressed by Ptch and internalized leading to an inhibitory transcriptional output. When Hh is present, it binds to Ptch and relieves the repression of Smo generating a transcriptional cascade via Gli proteins resulting in the activation or repression of Hh target genes (Huangfu and Anderson, 2006). Hh signaling is critical for many developmental processes in multiple vertebrate and invertebrate species. With respect to human eye formation, disruptions in Hh signaling components including *SHH* and *PTCH1* can result in cyclopia, due to an underlying defect in brain and facial development. Furthermore, *PTCH1* mutations, which lead to activation of the Hh pathway even in the absence of Hh (Williams et al., 2003) results in Gorlin Syndrome (BCNS), which can often present with defects in the retina, iris or lens, including cataracts (Hahn et al., 1996; Maity et al., 2005; Taylor et al., 2006).

In a broader evolutionary context, an expanded domain of Shh expression in the developing central nervous system (CNS) is responsible for the inhibition of eye development in the blind cavefish via its effects on the optic cup (Yamamoto et al., 2004). A number of studies in fish and amphibians have further provided further evidence

that Hh activity is important in regulating lens formation (Buxton et al., 2004; Davey et al., 2006; Kondoh et al., 2000). For example, over-expression of Hh in zebrafish results in suppression of lens formation (Barth and Wilson, 1995; Dutta et al., 2005). Similarly, exaggerated Hh activity through suppression of the Hh inhibitor, Xhip, in the prospective lens ectoderm in Xenopus also leads to loss of lens placode formation (Cornesse et al., 2005). Conversely, loss of Hedgehog signaling in non-neural ectoderm has been shown to result in a conversion of the pituitary to lens (Karlstrom et al., 1999; Kondoh et al., 2000; Varga et al., 2001) The expression and function of an intact Hh pathway also is associated with the process of lens regeneration in the newt (Tsonis et al., 2004). These data strongly suggest that the appropriate control of Hh signaling in lower vertebrates is an important mechanism for regulating lens specific gene expression and lens development.

In the mouse, Shh and to a lesser extent, Ihh, have been shown to have specific roles in eye formation, including development of the retina and the scleral mesenchyme (Dakubo et al., 2008; Levine et al., 1997; Perron et al., 2003; Wallace and Raff, 1999; Zhang and Yang, 2001a, b). For example, studies in *Ihh* knockout (KO) mice have shown that there is a loss of Hh target gene expression in the periocular mesenchyme and this results in defects in the posterior sclera, including a deformed ocular shape and fragile ocular globe. The *Ihh* KO mice also exhibit abnormalities of the RPE, including abnormal pigment distribution, as well as disruption in photoreceptor specification in the neural retina. Shh has been found to be expressed in the retinal ganglion cells of the

mouse retina and when overexpressed in these cells there is reduced retinal ganglion cell population, whereas mice with inhibited Shh activity results in increased retinal ganglion cell number (Zhang and Yang, 2001a). Thus, not surprisingly, *Shh* KO mice exhibit, in addition to cyclopia, a disrupted optic stalk and failed neural retina formation (Chiang et al., 1996). Correspondingly, Ptch has been shown to be expressed in the neural retina, RPE, iris and at low levels in the cornea of the mouse eye. The expression of Ptch has also been shown to overlap with Shh expression in the developing embryonic mouse eyelids (Motoyama et al., 1998; Takabatake et al., 1997). In contrast, Ptch expression has yet to be observed in the normal mouse lens (Takabatake et al., 1997).

Despite the findings for Hh in lens development in lower vertebrates and effects of Hh mutations on lens defects in humans, the role of Hh signaling, particularly expanded Hh levels, in the development and differentiation of the murine lens has not been investigated. The lens placode is derived from a region of SE overlying the optic vesicle. It is detected first, at the morphological level, as a thickening of the SE. The invagination of the lens placode gives rise to a lens pit which subsequently separates away from the overlying SE giving rise to a hollow lens vesicle. This vesicle ultimately matures into a specialized polar lens structure, with an anterior lens epithelium and central lens fiber cell region (Chow and Lang, 2001; Lovicu. F.J., 2004; McAvoy, 1980). To determine how aberrant Hh signaling may influence lens development and differentiation, we created mouse mutants that exhibit constitutive Hh signaling in the SE of the head and lens. Previous studies have identified a mutation in Smo, termed M2,

that prevents its interaction with, and repression by, Ptch proteins (Xie et al., 1998). Subsequently, this mutation has been engineered into the Rosa26 locus of the mouse genome downstream of a LoxP-Stop-LoxP cassette (Jeong et al., 2004). We combined this allele with an early ectodermal specific Cre recombinase transgene to activate SmoM2 expression from E9.5 onwards. We demonstrate that, while the lens placode does form in these mutants, there are major alterations in the expression of key regulatory molecules associated with aberrant differentiation and disorganized lens cell morphology. Our findings showed further that the developing mouse lens is capable of responding to altered Smo activity, suggesting that aspects of the Hh pathways that influence lens formation are conserved in vertebrate development.

2.2 Materials and Methods

2.2.1 Generation of activated smo mutant mice

All animal 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. Mice containing the activating mutation in Smo (Jeong et al., 2004; Srinivas et al., 2001; Xie et al., 1998) were obtained from the Jackson Laboratories, Bar Harbor, ME (STOCK Gt(ROSA)26Sor$^{tm1(Smo/EYFP)Amc}$/J, stock # 005130), and were crossed with transgenic mice expressing a new Cre-recombinase specific to the SE and its derivatives (Crect), to generate the activated smo mutant mice. Crect mice use an ectodermal enhancer from *Tcfap2a* to drive Cre expression specifically in the ectoderm, and will be described elsewhere (Yang, Melvin and Williams T, manuscript in preparation). Noon on the day of vaginal plug detection was considered day 0.5 (E0.5) of embryogenesis. Mice were genotyped using DNA extracted from tail or yolk sac samples using the DNeasy tissue kit (Qiagen, Valencia, CA). Genotyping was performed using the primers oIMR0316, oIMR0872, oIMR1416 and oIMR3621, and the following conditions: 1 cycle of 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute, and 72°C for 2 to generate a 425 bp product for the WT Rosa26 allele and 173 bp product for the EYFP component of the targeted locus. To detect the Cre allele, PCR was performed using the primers Cre1: 5'-GCT CCT TAG CAC CGC AGG TGT AGA G-3', Cre3: 5'-CGC CAT CTT CCA GCA

GGC GCA CC-3', and the following conditions: 120 seconds at 95ºC, 35 cycles of 95ºC for 45 seconds, 67ºC for 45 seconds, 72ºC for 60 seconds, followed by 72ºC for 10 minutes to generate a 421 bp product for the cre allele.

2.2.2 Histology

Whole embryos were collected corresponding to Gt(ROSA)26Sor$^{tm1(Smo/EYFP)Amc}$/J;Crect mutants and wild type (WT) litter mates. Embryonic tissue was fixed in 10% neutral buffered formalin overnight at room temperature and then transferred into 70% ethanol until processing. Whole embryos (E12.5) or embryo heads (E15.5, E18.5 and P0) were processed and embedded in paraffin. Serial sections were cut at a thickness of 4μm and used for Hematoxylin and Eosin (H&E) staining as well as immunofluorescent analysis. For all stages examined in the activated smo study, sample sizes of 3 lenses from 3 animals were stained.

2.2.3 Immunofluorescence and TUNEL assay

Indirect immunofluorescence was performed using the following primary antibodies: goat polyclonal green fluorescent protein (GFP), Bioshop Canada, Burlington, ON (1:250); rabbit polyclonal Pax6, Covance, Princeton, NJ (1:50); mouse monoclonal Pax6, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA (1:5); rabbit polyclonal FoxE3, developed by Dr. Peter Carlsson, University of Goteborg, Goteborg Sweden, (1:1000); rabbit polyclonal β-Crystallin, rabbit polyclonal γ-Crystallin, provided by Dr. Samuel Zigler Jr., Chief of lens and cataract biology section of the National Eye Institute, National Institute of Health, Bethesda, MD (1:200); The

mitosis marker anti-phospho-Histone H3 (rabbit polyclonal, Upstate Biotechnology, Lake Placid, NY) was used to detect mitotic cells (1:30); mouse monoclonal PCNA was used to detect cells in S phase of the cell cycle, Dako, Burlington, ON (1:750). We also used mouse monoclonal Cyclin D1, Santa Cruz, Santa Cruz, CA (1:100); mouse monoclonal $p27^{kip1}$, BD Transduction (1:350); goat polyclonal $p57^{kip2}$, Santa Cruz (1:100); rabbit polyclonal prox1, Covance (1:100); goat polyclonal c-maf, Santa Cruz (1:200); goat polyclonal Calretinin, Santa Cruz (1:25). Fluorescent secondary antibodies were either Alexa Fluor 488 (Goat anti-mouse and goat anti-rabbit), or Alexa Flour Fluor 568 (goat anti-mouse and donkey anti-goat), Invitrogen-Molecular Probes, Burlington, ON, used 1:200 for 1 hour at room temperature. Paraffin-embedded sections were deparaffinized in xylene, hydrated (through 100%, 95%, 70% ethanol, followed by water), treated with 10mM sodium citrate buffer (pH 6.0; boiling for 20 minutes) for antigen retrieval, blocked with normal serum and incubated with primary antibodies overnight at 4°C. For colocalization studies, both primaries were mixed and incubated simultaneously, followed by both secondaries. Each stain included a negative control with no primary antibody. Terminal uridine Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) was performed using the ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit (Millipore-Chemicon, Billerica, MA), according to the manufacturer's instructions for fluorescent staining of paraffin-embedded tissue. Following immunofluorescence or the TUNEL assay, stained slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlington, ON) or Prolong

Gold® antifade reagent with DAPI (Invitrogen). All H&E and fluorescent stains were visualized with a microscope equipped with a fluorescence attachment, and images were captured with a high-resolution camera and associated software (Open-Lab; Improvision, Lexington, MA). Images were reproduced for publication with image-management software (Photoshop 7.0; Adobe Systems Inc., Mountain View, CA).

2.2.4 LacZ assay

To examine Hh signaling pathway activity, activated smo mutants were bred with Ptch1-LacZ reporter mice (Ptch/^{tm1Mps}) (Goodrich et al., 1997). LacZ staining was performed to examine Ptch expression as a readout of Hh signaling. Embryonic tissue was fixed overnight in 4% paraformaldehyde (PFA) followed by cryoprotection in 30% sucrose. Tissue was embedded in ornithine carbamoyltransferase (OCT) for cryosectioning. Slides were washed in PBS and double distilled water (ddH2O). An X-gal dilution buffer containing 5mM pottassium ferricyanide crystalline, 5mM potassium ferricyanide trihdrate, 1mM magnesium chloride, and PBS was warmed to 37ºC, and 40mg/ml X-gal stock solution was added to the dilution buffer and applied to the slides containing 8μm cryosections. Slides were incubated overnight at 37ºC and viewed the following day.

2.3 Results

2.3.1 Verification of constitutive expression of smo in SE and SE derivatives

Constitutively active smo was fused to a Yellow Fluorescent Protein (YFP) in the smo construct. This fusion protein allowed for the verification of properly expressed constitutively active smo in the SE and SE derivatives (including the skin, eyelids, cornea, and lens). GFP and YFP differ by only a single amino acid (due to a mutation at T203Y) (Zimmer, 2002), and, therefore, antibodies raised against full length GFP can be used to detect YFP expression. Immunostaining for GFP revealed expression of the constitutively active smo allele in the SE and its derivatives, including the lens (Fig 2.1). GFP staining was seen within the corneal epithelium and lens at E12.5, E15.5 and E18.5 in the Gt(ROSA)26Sor$^{tm1(Smo/EYFP)Amc}$/J mice containing the Crect transgene. These mutants will be referred to as "activated smo mutants" throughout this study**.**

As an independent method to measure the activity of the Hh signaling pathway in the activated smo mutant eye, we bred them with the Ptch1-LacZ reporter line and assayed for beta-galactosidase activity. Ptch1 is a transcriptional target of the Hh pathway and can be used as a readout for pathway activity (Goodrich et al., 1997). At E12.5 in otherwise wild-type mice, the mutant Ptch1 allele drove LacZ expression in the neuroepithelium of the brain and within the spinal cord (Fig 2.1G). LacZ expression was not detected in the eyes of these mice (Fig. 2.1G). In contrast, in activated smo mutants, expression was expanded. Not only was LacZ expression detected within the neuroepithelium and spinal cord of the mutants, but its expression also was detected

within the entire lens and SE (Fig. 2.1H). Similar results were obtained at P0 with ß-gal expression in the lens observed only in the presence of the activated smo allele (compare Fig. 2.1I and J). Further analysis indicated that Ptch1-LacZ activity in the developing lens was observed only in a small number of cells (<5%) at E10.5, but was more widespread by E11.5, indicating the response to smo activation occurred by E11.5, consistent with the timing of Cre transgene activity (Table 1 and data not shown). Note that the cre transgene used in our study is not expressed in the retina, as revealed by the lack of GFP and Ptch1-LacZ expression in this tissue (Fig 2.1). Taken together, these findings demonstrate Ptch1-LacZ expression occurred in the same regions of the eye where we observed GFP expression in the activated smo mutants. The data indicate further that the cells of the lens have the capacity to respond to Hh signal transduction.

2.3.2 Activated smo mutants exhibit aberrant and disorganized lens morphology

Since lens cell morphology resulting from excessive Hh signaling had yet to be explored in a mouse model we first performed a gross morphological examination of eyes of the Crect activated smo mutants (Fig. 2.2). Analysis of E10.5-E12.5 embryos revealed that gross morphological defects were apparent most readily by E12.5, corresponding to the day after the major up-regulation of Ptch1-LacZ expression (Fig 2.2D and data not shown). At E12.5 the lens was misshapen and displayed a much thicker lens epithelial region than that observed in the wild type (WT) lens at the same stage (Fig. 2.2D, black arrow). An aberrant group of cells also was seen to be developing within the lumen of

the lens vesicle of the smo mutants (Fig 2.2D, pink arrow). As development progressed to E15.5, the WT lens displayed a distinct lens epithelial and fiber cell region. However, in the smo mutants, the lens epithelial and fiber cell regions were disorganized, with the mutant lens protruding further away from the optic cup towards the cornea (Fig. 2.2E). The anterior portion of the mutant lens appeared to adhere to the overlying SE (Fig. 2.2E, black star), whereas the WT had completed its separation. This adhesion also was evident at E12.5, but was not as pronounced as in the E15.5 embryonic lens. By E18.5, extensive lens epithelial and fiber cell disorganization persisted in the mutant lens. The completely misshapen lens was smaller than a WT E18.5 lens, and continued to protrude even further away from the optic cup than was observed at E15.5 (Fig. 2.2C, F). Throughout these stages of embryonic development, vacuoles and cavities also were evident throughout the entire mutant lens region. These data indicate that a constitutively active mutation in smo expressed in the SE and derivatives causes defective and disorganized embryonic lens cell morphology.

2.3.3 Activated smo mutants display abnormal patterns of proliferation and apoptosis within the developing lens

Hh signaling has been shown to regulate cell proliferation (Huangfu and Anderson, 2006). Thus, we assayed for the phosphorylated form of Histone 3 (PH3) as a readout for cells in the mitotic stage of the cell cycle (Fig 2.3) (Hans and Dimitrov, 2001). Proliferating cells were detected within the lens epithelial and fiber cell regions of

the activated smo mutant and WT lens at E12.5. When examining lenses of the mutants and WT at this early stage, it was evident that many more cells stain positively for PH3 in the mutant compared to the WT (Fig 2.3A, D). WT lenses at E15.5 and E18.5 exhibited PH3 staining that was confined to the lens epithelial cell layer (Fig. 2.3B, C), while activated smo mutants at equivalent stages displayed numerous PH3 positively stained cells within the lens epithelial and fiber cell regions (Fig 2.3E, F). Similar results were obtained by staining for Proliferating Cell Nuclear Antigen (PCNA), which labels cells in the S phase of the cell cycle (data not shown) (Al-Hussaini et al., 2008).

Evolutionary models of eye degeneration, including the Iberian Mole and the blind cave fish, share some lens specific phenotypes with our mouse model (Carmona et al., 2008; Menuet et al., 2007; Retaux et al., 2008). These models can both be caused by excess Shh signaling and exhibit lens degeneration. Therefore, TUNEL reactions were carried out to examine cell death in WT and mutant lenses (Fig 2.3). Lens vesicle closure is occurring around E12.5 during normal embryonic development, and, thus, some TUNEL-positive staining can be expected at this stage in the region where this separation is occurring (Lovicu. F.J., 2004). Normal amounts of apoptosis were seen in the lens epithelial regions of the WT and activated smo mice examined at E12.5 (Fig. 2.3G, J). However, abnormal TUNEL staining was observed in the posterior region of the lens vesicle of the activated smo mutants at this stage (Fig 2.3J). As expected, no apoptosis was observed in E15.5 and E18.5 WT lenses (Fig 2.3H, I). However, abnormal amounts of apoptosis were observed in the lens epithelial and posterior fiber cell region of the

mutant lens at both of these stages (Fig 2.3K, L). (The table provides a summary of the observed changes in the mutant, including those pertaining to the cell proliferation and cell death data).

2.3.4 Abnormal patterns of expression of cell cycle proteins and abnormal cell cycle behaviour occur during lens development in smo mutants

To understand better the aberrant proliferative and apoptosis patterns observed in the activated smo mutants, we performed an investigation into the expression patterns of the cell cycle promoting factor Cyclin D1, and also examined the expression of $p27^{kip1}$ and $p57^{kip2}$, which are negative regulators of the cell cycle (Griep, 2006). Cyclin D1 is required for the G1 to S phase transition in the cell cycle, and is expressed normally in proliferating lens epithelial cells and equatorial lens fiber cells, with expression absent from those fiber cells that have completed their differentiation process (Gomez Lahoz et al., 1999; Yamamoto et al., 2008). Importantly, Cyclin D1 also is a known target of the Hh pathway (Kenney and Rowitch, 2000), and, thus, we reasoned that its expression pattern may be altered due to the constitutive activation of smo in our mutants. Although Cyclin D1 expression appeared normal at E12.5 in the smo mutants (Fig. 2.4C), its expression became aberrant with the progression of lens development. In the WT lens at E16.5, Cyclin D1 was expressed in the proliferating lens epithelial cells and at the lens equator (Fig. 2.4B). At equivalent stages in the activated smo mutants, however, Cyclin D1 expression was scattered throughout the lens epithelial region, and, interestingly, was expressed in the posterior fiber cell compartment (Fig. 2.4D), unlike WT lenses at this

stage (Fig. 2.4B). A similar trend was seen at E18.5, with Cyclin D1 expressed throughout the anterior and posterior lens compartments in the mutant lens (data not shown).

During development, the terminal differentiation of specific cell types requires that cells exit the cell cycle correctly. Cell cycle exit and terminal differentiation is of particular importance during lens development, as cells fated to differentiate into fiber cells must complete these pathways successfully (Zhang et al., 1998). $p27^{kip1}$ and $p57^{kip2}$ are cyclin-dependent kinase inhibitors (CDKIs) belonging to the $p21^{cip1}$ family, and are required for inhibition of kinases involved in the G1/S transition of the cell cycle (Griep, 2006; Pateras et al., 2009; Zhang et al., 1998). $p27^{kip1}$ is expressed normally in lens fiber cells, specifically at the transition zone of the lens, while $p57^{kip2}$ is expressed in the lens epithelium and fiber cells, though its expression in the epithelium pales in comparison to its levels of expression at the transition zone of the lens (Griep, 2006). $p27^{kip1}$ expression appeared normal in the earlier stages of lens development at E12.5, displaying expression throughout the lens vesicle of the WT and mutant lenses (Fig 2.4E, G). However, with the progression of development to E15.5, $p27^{kip1}$ was expressed aberrantly throughout the entire anterior lens epithelial regions and posterior fiber cell regions of the activated smo mutant lens (Fig 2.4F, H). The expression patterns of $p57^{kip2}$ were similar to those seen for $p27^{kip1}$. Although $p57^{kip2}$ expression appeared normal at E12.5 (Fig 2.4I, K), its expression became quite irregular later in development at E15.5. $p57^{kip2}$ expression was concentrated at the transition zone of the WT lens, but was expressed throughout the

majority of the anterior and posterior regions of the mutant lens (Fig. 2.4J, L). The expression patterns of $p27^{kip1}$ and $p57^{kip2}$ at E18.5 were similar to those seen at E15.5, with expression of both CDKIs seen throughout the lens epithelial and fiber cell regions of the mutant lens (the Table and data not shown).

2.3.5 An activated smo mutation results in abnormal expression patterns of lens epithelial cell markers

Pax6 and FoxE3 are proteins that have important roles in lens development, and their expression becomes confined to the epithelial compartment of the differentiating lens. Immunofluorescent analysis of these two lens epithelial cell markers in the activated smo mutants revealed defective patterns of expression. Pax6 expression was examined at E12.5, E15.5, and E18.5 in the WT and mutant lens (Fig. 2.5). At E12.5, the expression pattern of Pax6 in the WT and mutant lenses appeared similar, with Pax6 expressed specifically in the lens epithelium (Fig 2.5A, D). However, by E15.5, Pax6 expression in the activated smo mutants became disrupted. While the WT lens displayed a pattern of Pax6 expression that was confined to the lens epithelial cell layer (Fig 2.5B), the mutant lens at this stage showed Pax6 expression throughout the entire lens region (Fig 2.5E), a trend that persisted at E18.5 (compare Fig 2.5C, F).

FoxE3 also was investigated in the developing lens at E12.5 and E15.5 (Fig 2.5). By E12.5, the pattern of FoxE3 expression already was disrupted in the activated smo mutant lens. While its expression was confined to the lens epithelial cells in the WT lens

(Fig 2.5G), FoxE3 expression not only was seen within the lens epithelial region of the mutant lens, but its expression also had expanded into the posterior fiber cell region (Fig 2.5I). A similar pattern of FoxE3 expression was observed by E15.5 in the activated smo lens, with expression evident throughout the lens epithelial and fiber cell regions (Fig. 2.5J). Thus, the ectopic expression of FoxE3 in the smo mutants preceded the ectopic expression of Pax6. (see the Table).

2.3.6 Final patterns of fiber cell differentiation are perturbed in activated smo mutant lenses

In the developing and adult vertebrate lens, the crystallins, including αA-Crystallin, αB-Crystallin, β-Crystallin and γ-Crystallin are expressed in specific patterns within the differentiating fiber cells. Thus, to examine fiber cell development in the activated smo mutants, expression of β-Crystallin and γ-Crystallin was explored (Fig 2.6). γ-Crystallin expression appeared normal in the activated smo mutants at E12.5, E15.5, and E18.5. Expression was observed in the posterior region of the lens vesicle at E12.5, and within the fiber cell region of the lens during the later stages of development (Fig 2.6A-F). β-Crystallin, another excellent marker of fiber cell differentiation, is detected first within elongating primary fiber cells. In subsequent stages of embryonic development, its expression is initiated at the time when secondary fibers begin to differentiate away from the transitional zone of the lens (Lovicu. F.J., 2004). Like that of γ-Crystallin, β-Crystallin expression appeared normal in the activated smo mutants, with

expression seen throughout the fiber cell region of the lens vesicle at E12.5 and throughout the fiber cell region of the lens during subsequent stages of embryonic development (Fig. 2.6G-L). Interestingly, although fiber cells in the activated smo mutants expressed γ-crystallin and β-crystallin in a relatively normal manner, cells in this region showed clear cell morphological defects when viewed with DAPI. Specifically, in contrast to the wild-type situation at 15.5 and E18.5, the cells in the posterior region of the mutant lenses failed to lose their nuclei (Fig. 2.6E, F, K, L).

In the mouse, Pax6 has been found to repress the transcription of genes encoding β-Crystallin (Duncan et al., 1998; Duncan et al., 2004). Normally, β-Crystallin is expressed in fiber cells, where Pax6 expression has been down regulated, while the expression of Pax6 remains confined to the lens epithelium (Lovicu. F.J., 2004). Expression patterns of β-Crystallin and Pax6 were examined simultaneously by double immunostaining for each lens cell marker (Fig 2.6). At E12.5, staining for Pax6 and β-Crystallin expression within the developing lens of the activated smo mutants and WT littermates appeared normal, with Pax6 expressed in the developing lens epithelium and fiber cells and β-Crystallin expressed within the fiber cell region (Fig. 2.6M, P). By E15.5, however, it is evident from examination of the WT lens, that Pax6 expression had become confined to the lens epithelial region, while its expression within the β-Crystallin-expressing fiber cell region had been down regulated (Fig. 2.6N). In the activated smo mutants at this stage, Pax6 expression was seen not only throughout the lens epithelial cell region, but also in the β-Crystallin-positive fiber cell region (Fig

2.6Q). Similarly, while these lens cell markers were expressed correctly in the E16.5 WT lens, Pax6-positive cells also stained positive for β-Crystallin in the activated smo mutants at this stage (Fig. 2.6O, R). This trend continued at E18.5 (data not shown).

The aberrant fiber cell phenotype of the activated smo mutants was analyzed further by examining the expression patterns of Prox1 and c-maf, both important in regulating fiber cell elongation and differentiation (Lang, 2004; Ring et al., 2000). By E13, c-maf becomes highly expressed at the equatorial region of the lens where proliferatively active anterior epithelial cells begin their differentiation process into secondary fiber cells (Kawauchi et al., 1999; Lovicu. F.J., 2004; Ring et al., 2000). Expression of c-maf appeared normal in the activated smo mutants at E12.5, with expression observed throughout the entire lens, appearing most pronounced throughout the fiber cell region (Fig. 2.7D). By E15.5 however, c-maf expression had become disrupted in the smo mutants. While WT lenses displayed strong c-maf expression at the lens equator, and to a lesser extent throughout the anterior epithelium (Fig. 2.7B) c-maf expression was abnormal and expanded throughout the entire lens region of the activated smo mutants (Fig. 2.7E). By E18.5 in the WT lens c-maf expression appeared highly concentrated at the lens equator, where secondary fiber cell differentiation is occurring, whereas c-maf expression again was abnormal and expanded throughout the entire lens region of the activated smo mutants (Fig. 2.7C, F).

Similar to c-maf, Prox1 also is expressed in the head ectoderm and lens placode early in development. With the progression of development to E12.5, Prox1 is expressed

throughout the entire lens region, and its expression is maintained in the lens epithelium and lens transition zone throughout embryonic development (Duncan et al., 2002; Lovicu. F.J., 2004). Expression of Prox1 in the activated smo mutants at E12.5 was normal and observed throughout epithelial and fiber cell compartments of the developing lens (Fig. 2.7J). In WT lenses at E15.5 and E18.5, Prox1 was expressed correctly throughout the anterior lens epithelium and lens transition zone (Fig. 2.7H, I); however, its pattern of expression in the mutant lenses at these stages was expanded, encompassing the entire anterior epithelial and posterior fiber cell regions (Fig. 2.7K, L; and Table 2.1).

2.3.7 An activating mutation in smo results in retinal disorganization and degeneration at P0

Often, following the degeneration of the lens, retinal morphology and retinal cell survival can be impacted negatively. Also as shown in blind cavefish, which exhibit an expanded domain of Shh expression in the CNS, retinal degeneration follows extensive lens degeneration (Alunni et al., 2007; Strickler et al., 2007). Thus, due to the lens degeneration observed in the activated smo mutants we examined the embryonic retina in this model. Gross retinal morphology, cell proliferation, cell death, and the expression of retinal specific markers were normal through E18.5 (Fig 2.8). However, at P0, the latest stage the mice survive, retinal defects became apparent. For example, at P0 retinal lamination was disrupted in the activated smo mutant retina and, unlike WT retinas, the mutant retina exhibited only a neuroblast layer (nbl) and no distinct ganglion cell layer

(GCL) (Fig 2.8H). Retinal markers Pax6 and Calretinin were also examined (Fig. 2.9A-H). By P0, Pax6 is expressed strongly in the GCL and inner nuclear layer (INL) of WT mice, but expression is not observed within the outer neuroblast layer (onbl). In contrast, P0 smo mutants lacked retinal lamination and a distinct GCL, along with the strong GCL Pax6 expression domain. Instead, Pax6 expression was maintained aberrantly in the nbl as well as in a small population of cells staining weakly positive for Pax6 in the disorganized inner retina (IR) of these mutants (Fig. 2.9F). Calretinin, a calcium binding protein that labels amacrine and ganglion cells, also was examined. By P0, Calretinin expressing cells appeared to have lost their normal architecture in the activated smo retinas, and expression was dispersed within the IR (Fig 9H). Proliferation and apoptosis were also examined through staining with PCNA and TUNEL, respectively (Fig. 2.9I-P). While cellular proliferation in the activated smo retinas appeared normal at E18.5 (Fig. 2.9M), at P0 an increase in proliferation was seen within the IR of the mutant (Fig. 2.9N). Patterns of apoptosis in the activated smo retinas also appeared similar to those observed in WT littermates at E18.5 (Fig. 2.9K, O). However at P0, while a small number of cells label TUNEL-positive in the INL and outer nuclear layer in WT and mutant retinas, TUNEL-positive cells also were apparent in the disorganized IR of the activated smo mutants. (Fig. 2.9L, P; and the Table).

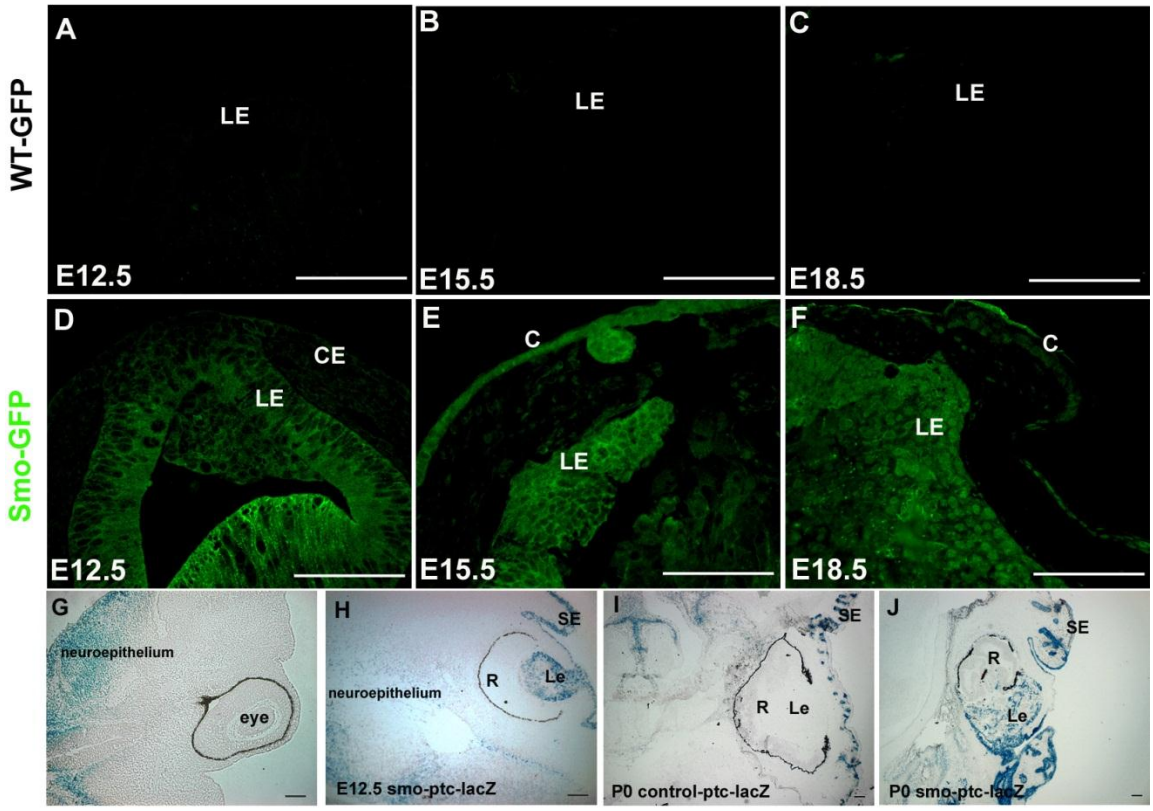

Figure 2.1 Verification of constitutively active smoothened allele expression in SE and SE derivatives.

Sections of WT (A, B and C) and Crect activated Smo mutant (D, E, and F) mouse eyes at E12.5 (A, D), E15.5 (B, E) and E18.5 (C, F) immunostained for GFP expression. Ptch1-LacZ (G, I) and Crect Smo-Ptch1-LacZ (H, J) at E12.5 (G-H) and P0 (I-J) were stained with X-gal to examine for Ptch1 expression as indicated by LacZ staining. The blue staining seen in the wild-type P0 samples in the surface ectoderm overlying the eye represents expression in the hair follicles associated with the eyelids. CE, corneal epithelium; LE, lens epithelium; C, cornea, SE, surface ectoderm; Le, lens; R, retina. All scale bars represent 100μm.

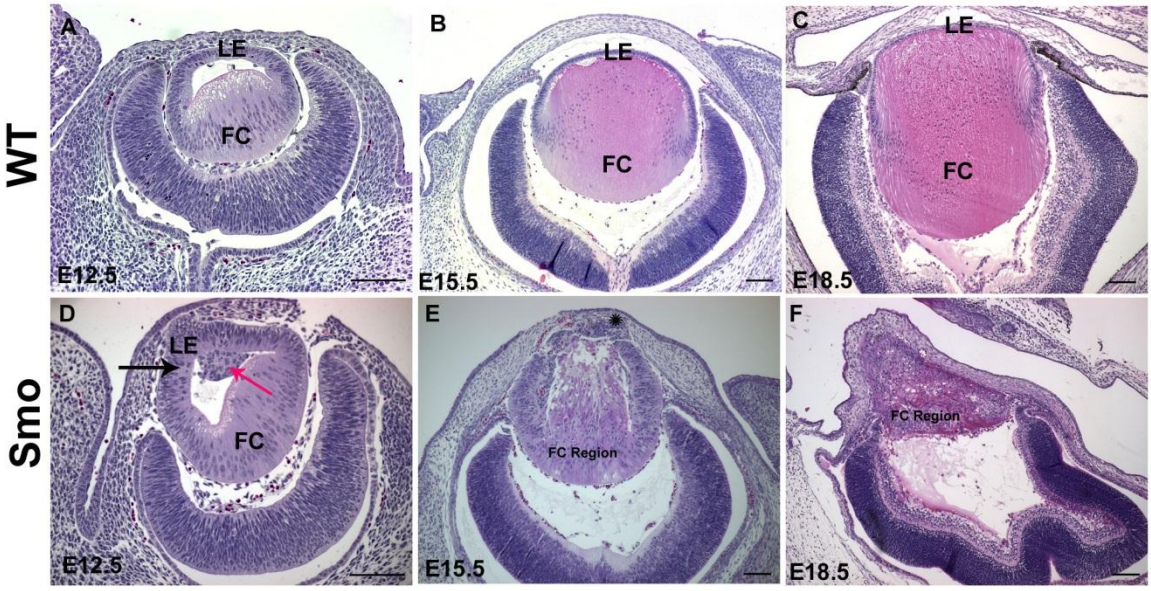

Figure 2.2 Hematoxylin and eosin stains of WT and activated smo mutant lenses.
At E12.5 (A, D) defective lens morphology in the mutant (D) was characterized by a thicker than normal lens epithelial region (black arrow) and a misshapen lens. An aberrant group of cells developed in the mutant lens vesicle lumen (pink arrow; D). By E15.5 (B, E), the WT lens displayed distinct lens epithelial and fiber cell layers while the mutant lens was disorganized, with the lens protruding away from the optic cup. An adhesion of the lens to the overlying SE was also observed (black star) (E). By E18.5 (C, F), the mutant lens was smaller than that of the WT, remained disorganized, and continued to protrude even further away from the optic cup (C and F). LE, lens epithelium; FC, fiber cells. All scale bars represent 100μm.

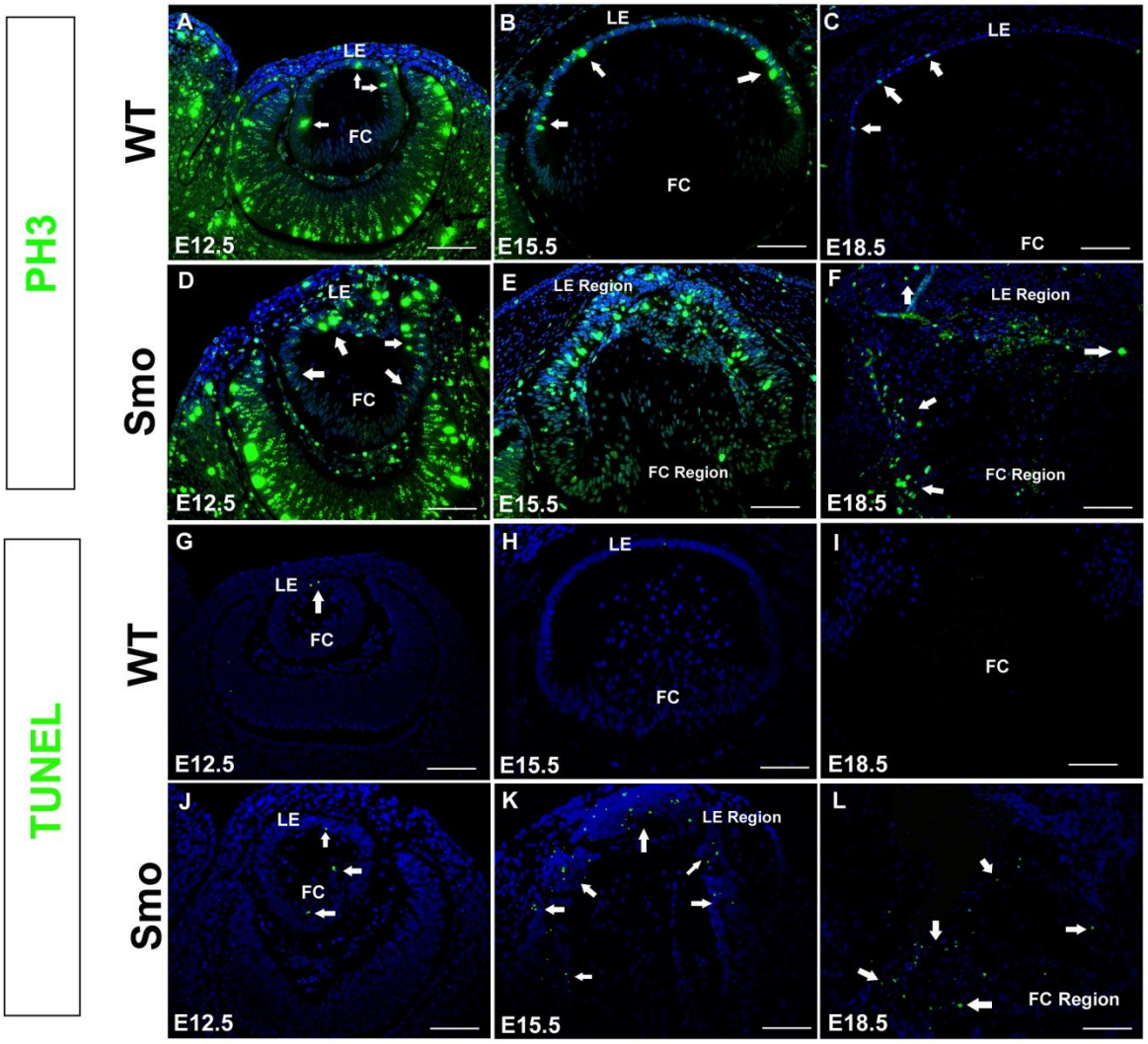

Figure 2.3 Patterns of proliferation and cell death are abnormal in developing activated smo lens.

Sections of WT and Smo mutant mouse (Smo) eyes at the indicated time points immunostained for expression of phosphohistone H3 or detection of TUNEL as shown. White arrows indicate proliferating cells (A-F) or TUNEL positive cells (G-L) respectively. Slides were also stained with DAPI to highlight nuclei (blue). LE, lens epithelium; FC, fiber cells. All scale bars represent 100μm.

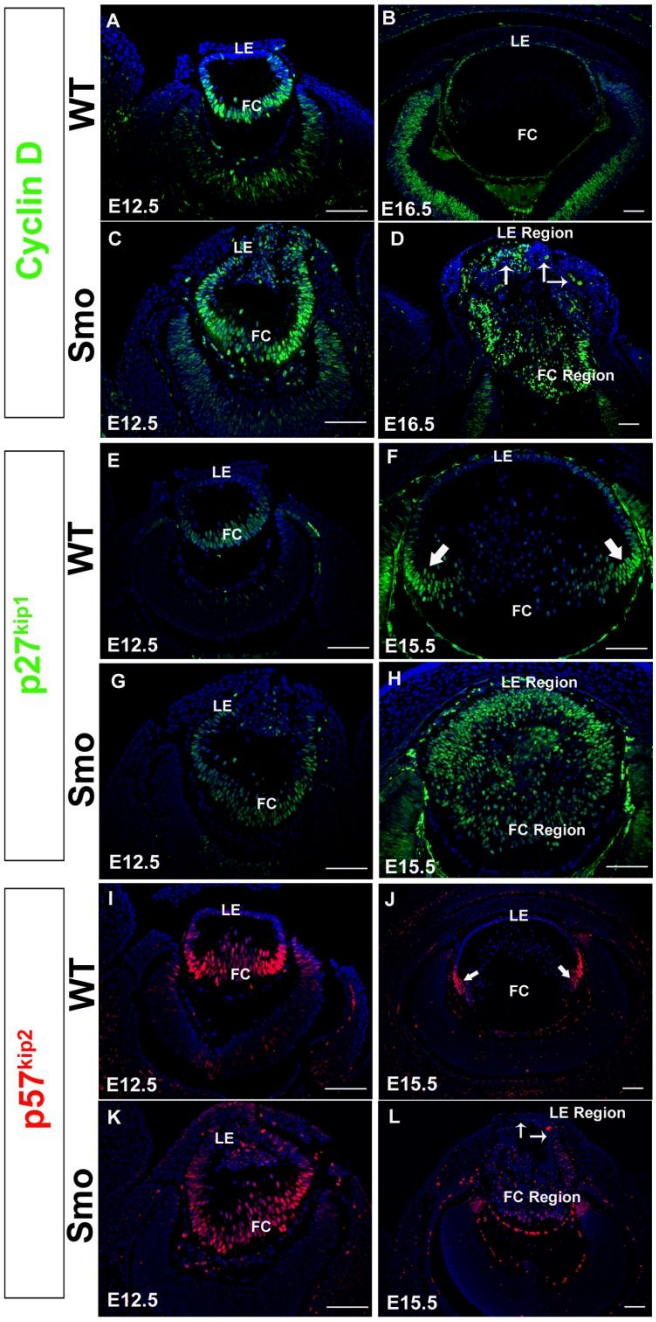
Cyclin D
WT
Smo
A
LE
FC
E12.5
B
LE
FC
E16.5
C
LE
FC
E12.5
D
LE Region
FC Region
E16.5
p27kip1
WT
Smo
E
LE
FC
E12.5
F
LE
FC
E15.5
G
LE
FC
E12.5
H
LE Region
FC Region
E15.5
p57kip2
WT
Smo
I
LE
FC
E12.5
J
LE
FC
E15.5
K
LE
FC
E12.5
L
LE Region
FC Region
E15.5

Figure 2.4 Cell cycle promoting and inhibiting factors Cyclin D1, p27kip1, and p57kip2 are ectopically expressed in the activated smo lens.

Sections of WT (A, B, E, F, I, J) and activated Smo mutant (C, D, G, H, K, L) mouse eyes at E12.5 (A, C, E, G, I, K), E15.5 (F, H, J L) and E16.5 (B, D) immunostained for expression of cyclin D (A-D, green), $p27^{kip1}$ (E-H, green) or $p57^{kip2}$ (I-L, red). Slides were also stained with DAPI to highlight nuclei (blue). White arrows (F, J) show localized expression of $p27^{kip1}$ and $p57^{kip2}$ within the transitional zone of the lens. White arrows (D, L) show expression of cyclin D and $p57^{kip2}$ in cells within the lens epithelial region of the mutant. LE, lens epithelium; FC, fiber cells. All scale bars represent 100μm.

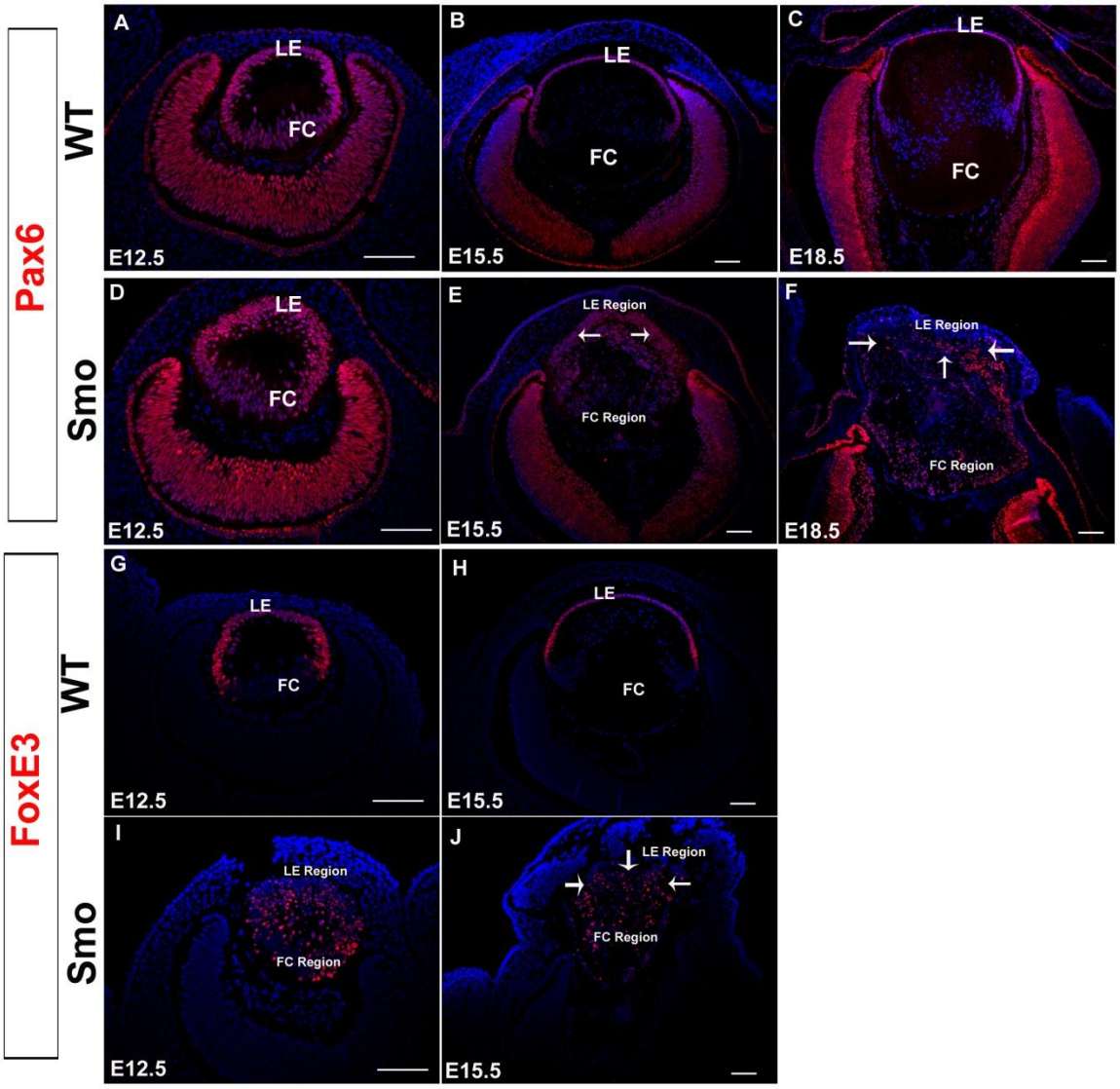
Pax6
WT
Smo
FoxE3
WT
Smo
A
LE
FC
E12.5
B
LE
FC
E15.5
C
LE
FC
E18.5
D
LE
FC
E12.5
E
LE Region
FC Region
E15.5
F
LE Region
FC Region
E18.5
G
LE
FC
E12.5
H
LE
FC
E15.5
I
LE Region
FC Region
E12.5
J
LE Region
FC Region
E15.5

Figure 2.5 Pax6 and FoxE3 are ectopically expressed in developing activated smo lens.

Normal sections of WT (A, B, C, G, H) and activated Smo mutant (D, E, F, I, J) mouse eyes at E12.5 (A, D, G, I), E15.5 (B, E, H, J) and E18.5 (C, F) immunostained for expression of Pax6 (A-F, red) or Foxe3 (G-J, red). White arrows (E, F, J) indicate Pax6 (E, F) and FoxE3 (J) staining within the lens epithelial cell region. Slides were also stained with DAPI to highlight nuclei (blue). LE, lens epithelium; FC, Fiber cells. All scale bars represent 100μm.

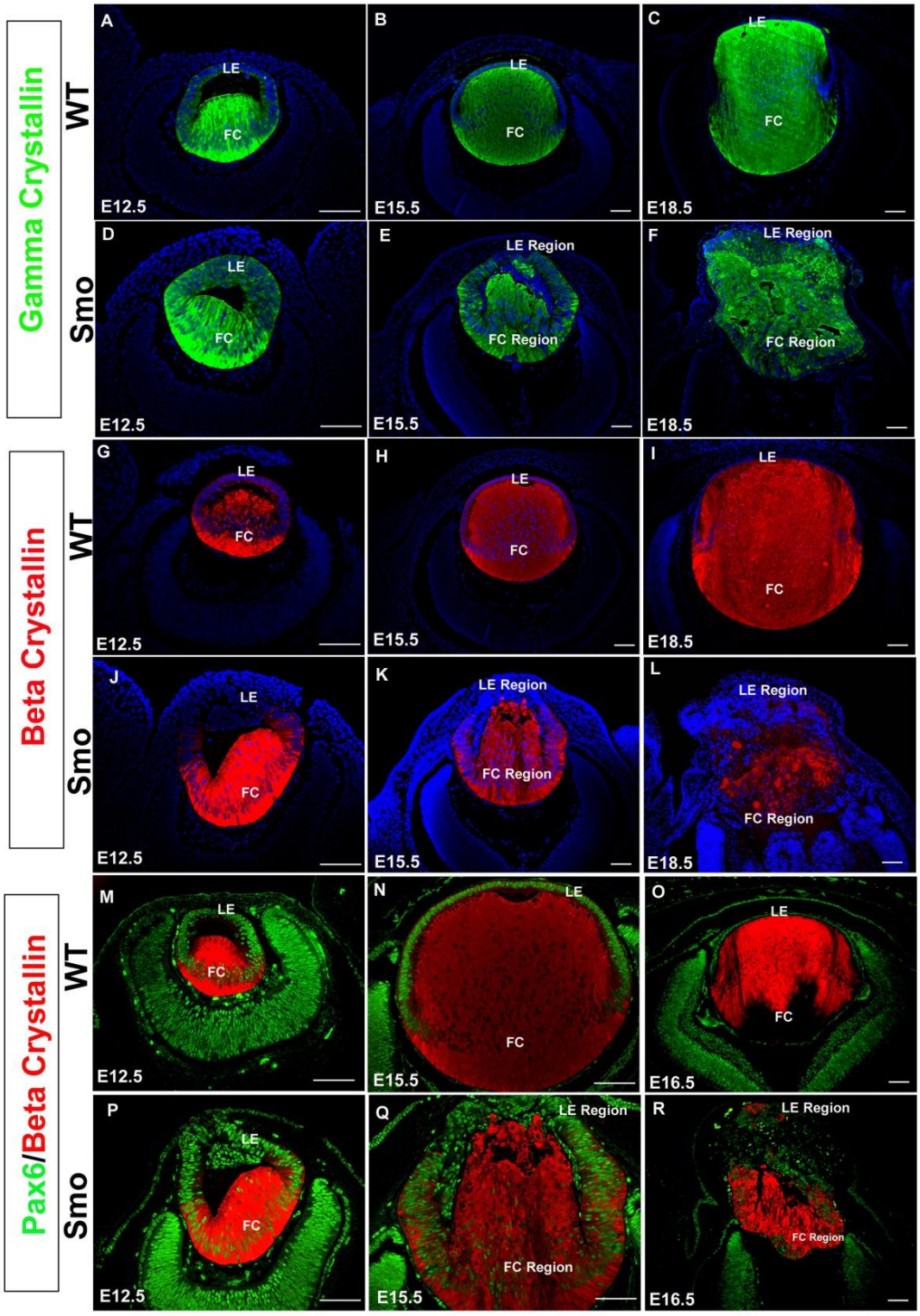
Gamma Crystallin
WT
Smo
A
B
C
D
E
F
LE
FC
LE Region
FC Region
E12.5
E15.5
E18.5
Beta Crystallin
WT
Smo
G
H
I
J
K
L
LE
FC
LE Region
FC Region
E12.5
E15.5
E18.5
Pax6/Beta Crystallin
WT
Smo
M
N
O
P
Q
R
LE
FC
LE Region
FC Region
E12.5
E15.5
E16.5

Figure 2.6 γ-Crystallin and β-Crystallin are expressed in an appropriate spatial pattern, though crystallin expressing FCs fail to de-nucleate and maintain Pax6 expression.

Sections of WT (A- C, G- I, M-O) and activated Smo mutant (D- F, J-L, P-R) mouse eyes at E12.5 (A, D, G, J, M, P), E15.5 (B, E, H, K, N, Q) and E18.5 (C, F, I, L ,O, R) immunostained for expression of the γ-crystallin lens fiber cell marker (A-F, green), β-Crystallin (G-L, red) or both Pax6 and β-Crystallin (M-R, green and red respectively). Slides were also stained with DAPI to highlight nuclei (blue). LE, lens epithelium; FC, fiber cells. All scale bars represent 100μm.

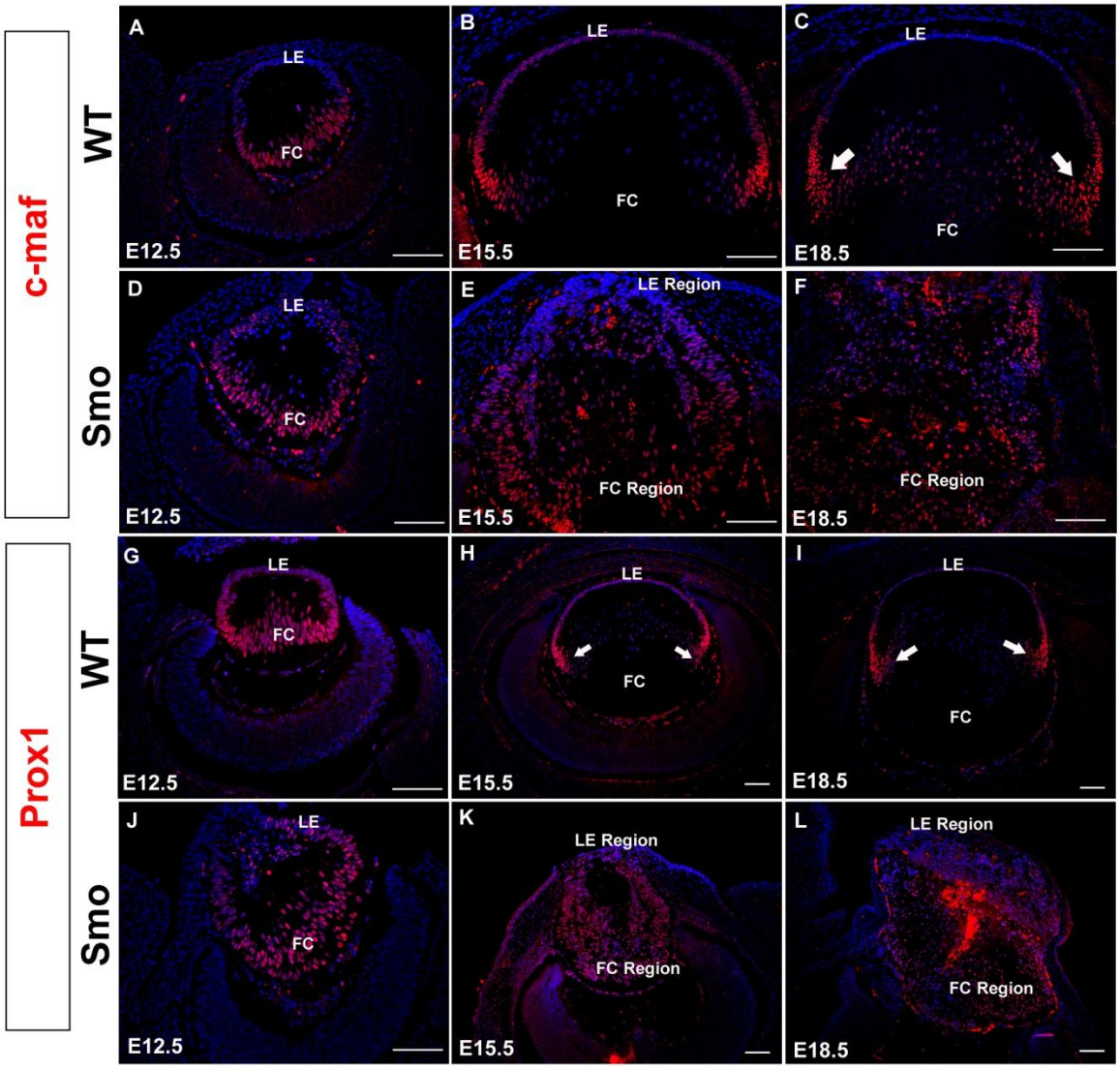

Figure 2.7 c-maf and Prox1 expression is expanded throughout the anterior and posterior lens of the activated smo mutants.

Sections of WT (A- C, G- I) and activated Smo mutant (D- F, J-L) mouse eyes at E12.5 (A, D, G, J), E15.5 (B, E, H, K) and E18.5 (C, F, I, L) immunostained for expression of c-maf (A-F, red), or Prox1 (G-L, red). Slides were also stained with DAPI to highlight nuclei (blue). White arrows show c-maf and Prox1 expression at the equatorial transitional zone. LE, lens epithelium; FC, fiber cells. All scale bars represent 100μm.

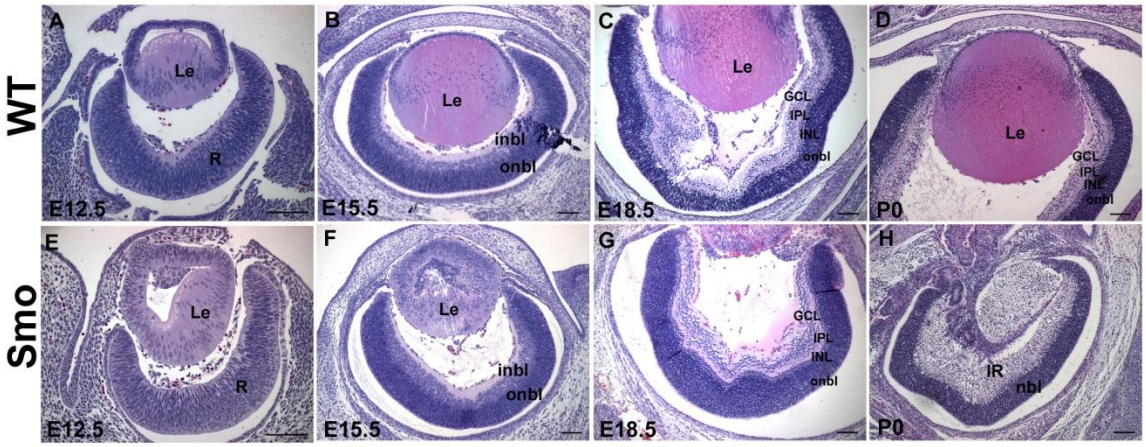

Figure 2.8 Retinal lamination is lost between E18.5 and P0.
Sections of WT (A- D) and activated Smo mutant (E-H) mouse eyes at E12.5 (A, E), E15.5 (B, F), E18.5 (C, G) and P0 (D, H) stained with Hematoxylin and Eosin. Le, lens; R, retina; inbl, inner neuroblast layer; onbl, outer neuroblast layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; IR, inner retina; nbl, neuroblast layer. All scale bars represent 100μm.

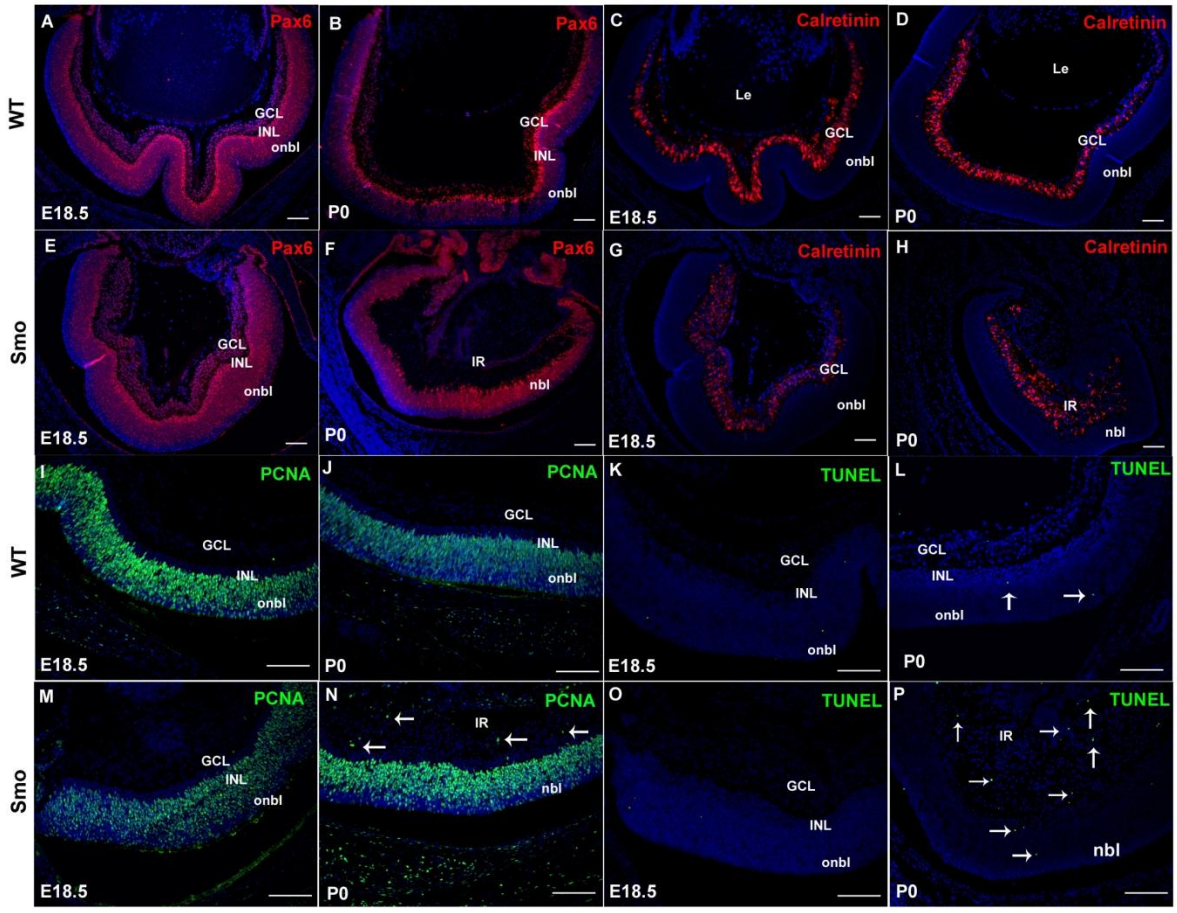
WT
Smo
WT
Smo
A
Pax6
GCL
INL
onbl
E18.5
B
Pax6
GCL
INL
onbl
P0
C
Calretinin
Le
GCL
onbl
E18.5
D
Calretinin
Le
GCL
onbl
P0
E
Pax6
GCL
INL
onbl
E18.5
F
Pax6
IR
nbl
P0
G
Calretinin
GCL
onbl
E18.5
H
Calretinin
IR
nbl
P0
I
PCNA
GCL
INL
onbl
E18.5
J
PCNA
GCL
INL
onbl
P0
K
TUNEL
GCL
INL
onbl
E18.5
L
TUNEL
GCL
INL
onbl
P0
M
PCNA
GCL
INL
onbl
E18.5
N
PCNA
IR
nbl
P0
O
TUNEL
GCL
INL
onbl
E18.5
P
TUNEL
IR
nbl
P0

Figure 2.9 Activated smo mutants show disorganized retinal morphology and expression of Pax6 and Calretinin while exhibiting abnormal retinal cell proliferation and death by P0.

Sections of WT (A-D, I-L) and activated Smo mutant (E-H, M-P) mouse eyes at E18.5 and P0. Slides were immunostained for expression of Pax6 (A-B, E-F) and Calretinin (C-D, G-H), as well as PCNA (I-J, M-N) and TUNEL (K-L, O-P). White arrows indicate irregular proliferation and cell dealth in the IR of the mutants. Le, lens; R, retina; inbl, inner neuroblast layer; onbl, outer neuroblast layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; IR, inner retina; nbl, neuroblast layer. All scale bars represent 100μm.

Marker	**Wild-type spatial localization**	**Stage at which difference from wt was first observed**	**Ectopic expression in Fiber Cell compartment of mutant lens**	**Additional comments**
Ptc1-LacZ	-Not present in lens or retina at PO -lacZ staining associated with hair follicles in eyelid; within neuroepithelium and spinal cord	E10.5-limited number of lacZ positive cells in the mutant lens pit and surrounding ectoderm E11.5 – widespread lacZ expression in mutant lens and surrounding ectoderm	+	-
FoxE3	LE	E12.5	+	-
Pax6 (lens)	LE	by E15.5 (lens)	+	-
β and γ-crystallin	FCs	by E15.5	NA	Failed FC denucleation
c-maf	Lens TZ	by E15.5	+	-
Prox-1	LE and TZ	by E15.5	+	-
Cyclin D	LE and TZ	by E15.5	+	-
$p27^{kip1}$	LE (low levels) and TZ	by E15.5	+	-
$p57^{kip2}$	TZ	by E15.5	+	-
Phosphohistone H3 (lens)	LE and TZ	by E15.5	+	-
Pax6 (retina)	GCL and nbl	P0	NA	Expression persists in mutant nbl at PO and also observed in IR
Calretinin (retina)	Ganglion and amacrine cells	P0	NA	Disorganized expression in the IR
PCNA (retina)	Ganglion cell layer, INL, onbl	P0	NA	Abnormal proliferation in IR
TUNEL	cell death in LV at E12.5, Small amount of cell death in INL and onbl	E15.5 (lens) P0 (Retina)	NA	-excessive cell death in LE and FCs -abnormal cell death in IR

Table 2.1 Summary of differences in marker expression in activated smoothened mutant versus wild-type eyes.

Legend: LE- Lens Epithelium, FC- Fiber Cells, TZ – Transition Zone, GCL – Ganglion Cell Layer, nbl- neuroblast layer, IR- Inner Retina, onbl- outer neuroblast layer, PCNA- Proliferating Cell Nuclear Antigen, LV- Lens Vesicle, NA- Not Applicable

2.4 Discussion

Previous studies in vertebrates have shown that during early development Hh signals can regulate specification and fate of the head ectoderm into lens, and that appropriate levels of Hh signaling impact lens-specific gene expression and lens development. For example, in zebrafish, disrupted Hh signals can cause the formation of ectopic lenses at the expense of the adenohypophysis. Furthermore, when Hh signaling is exaggerated in the ectoderm, lens placode formation is suppressed completely (Barth and Wilson, 1995; Cornesse et al., 2005; Dutta et al., 2005). However, the role of Hh signaling in mammalian lens development is less understood, even though it has been demonstrated that mutations in human Hh signaling components that result in activation of the Hh pathway can cause cataracts (Taylor et al., 2006). In our study, we explored the consequence of excessive Hh signaling through constitutive activation of smo in the SE of the head, at a time by which the lens placode already is specified (E8.5). Our findings show that constitutive activation of smo did not appear to impact negatively early development of the lens, including the lens pit and vesicle stages, but did disrupt later stages of lens differentiation, with the first defect apparent at E12.5. By E15.5 the fiber cells of the mutant lens were shown to have remained in the cell cycle and failed to differentiate terminally. Accompanying this was the persistent epithelialization of cells in the fiber cell compartment.

The ectopic expression of FoxE3 in the fiber cell compartment was the earliest expression defect observed in the activated smo mutants at E12.5. FoxE3, a forkhead

transcription factor gene, is a critical regulator of lens vesicle separation, and importantly also has been shown to regulate the normal proliferation patterns in the vertebrate lens (Blixt et al., 2000). Interestingly, ectopic and sustained FoxE3 expression in the posterior region of the lens has been shown to cause a vacuolated lens with fiber cells that maintain a partial epithelial phenotype (Landgren et al., 2008), similar to the phenotype observed in the activated smo lens. In this previous model, the α-A-crystallin (*Cryaa*) promoter was used to drive ectopic, transgenic expression of *Foxe3* in differentiating mouse lens fibers. This resulted in an increase of mRNAs normally enriched in lens epithelial cells, as determined my microarray profiling, and was consistent with an epithelialization of the transgenic fibers. Similar to our activated smo mutants, some aspects of fiber differentiation were unaffected, such as the expression of the crystallins. However, loss of fiber cell organelles and antimitotic signaling were affected. These data suggest that the early ectopic expression of FoxE3 in the activated smo mutants may be responsible for the subsequent defects observed in the fiber cells.

Like FoxE3, Pax6 expression becomes confined to the lens epithelium after E12.5 (Walther and Gruss, 1991). Although Pax6 was found to be expressed in a normal manner in the smo mutants at E12.5, its expression pattern became ectopic by E15.5, with staining evident throughout the epithelial and posterior fiber cell regions. The ectopic expression of Pax6 may also have contributed to the altered fiber cell phenotype. For example, it has been demonstrated in Pax6 transgenic mice, which over express Pax6 in lens fiber cells, that there is a disorganized and vacuolated fiber cell region. These fiber

cells failed to lose their nuclei, leading to disrupted fiber cell elongation and differentiation (Duncan et al., 2004), features reminiscent of the phenotype observed in our activated smo mutants. Ectopic expression of Pax6 also was shown to reduce drastically the expression of βB1-crystallin in the fiber cells of the transgenic lenses (Duncan et al., 2004). This outcome is not surprising, since Pax6 has been shown to repress directly the expression of βB1-crystallin (Collinson et al., 2001; Cui et al., 2004; Duncan et al., 2004). However, in our activated smo mutant model, βB1-crystallin and γ-crystallin, two markers of fiber cell development, elongation and differentiation, (Andley, 2007; Lovicu. F.J., 2004; Robinson and Overbeek, 1996) were expressed in a spatially correct pattern throughout the fiber cell region. Since the fiber cell region of the activated smo lenses showed co-expression of Pax6 and βB1-crystallin, it is possible that the levels of Pax6 in our mutants are below the threshold necessary to repress crystallin gene expression.

Another possibility for the sustained expression of βB1-crystallin in the fiber cells of the activated smo lens is that an additional, positive regulator of crystallin gene expression, c-maf, also was found to be expressed ectopically in the mutant lens, away from its normal localization in the equatorial region of the lens. C-maf has been shown to be critical for proper lens fiber cell differentiation and elongation, and importantly, in the regulation of β and γ-crystallin expression (Kawauchi et al., 1999; Ring et al., 2000). C-maf homozygous null mutants also were shown to exhibit cessation in primary fiber cell differentiation and elongation by E12.5, as well as defects in secondary fiber cell

development, including reduced expression of β-crystallin and absent γ-crystallin (Kawauchi et al., 1999; Ring et al., 2000). Kawauchi et al, also reported that c-maf regulates directly the expression of γ-crystallin (Kawauchi et al., 1999). Thus, the ectopic expression of c-maf in the posterior cells of the activated smo lens may be responsible for maintaining the βB1-crystallin and γ-crystallin expression observed, despite the persistent expression of the repressor, Pax6.

The constitutive activation of smo also resulted in defective patterns of proliferation and cell death. Staining with the proliferation-specific markers PH3 and PCNA revealed proliferating cells in the fiber cell region of the mutant lens, at embryonic stages in which no proliferation should be observed. Hh signaling has been shown to regulate cell proliferation and differentiation in other systems. For example, activation of the Hh pathway in the regenerating newt lens was shown to cause an increase in lens vesicle cell proliferation (Tsonis et al., 2004). Aberrant expression of cell cycle regulators also was observed in our smo mutant lens model. In particular, cyclin D, which is known to be downstream of Hh (Kenney and Rowitch, 2000) and functions in the G1 phase of the cell cycle to promote proliferation, normally is localized within the lens epithelium and fiber cells at the lens equator (Baldin et al., 1993; Draetta, 1994; Gomez Lahoz et al., 1999). However, in the activated smo mutants the domain of Cyclin D, expression was expanded such that it was observed ectopically throughout the posterior fiber cell compartment. Interestingly, a study by Rowitch et al. illustrated that ectopic Hh pathway activation in central nervous system precursor cells during

embryogenesis resulted in increased proliferation, and maintenance of these cells in an undifferentiated state (Rowitch et al., 1999). We postulate that a similar mechanism is operating in the activated smo mutants, resulting in alterations in both cell cycle regulation and fiber cell differentiation.

The $p27^{kip1}$ and $p57^{kip2}$, both belonging to the family of $p21^{cip1}$ CDKIs, cooperate with each other to promote proliferating lens epithelial cells at the lens equator to withdraw from the cell cycle, and begin their differentiation process into lens fiber cells. These CDKIs work by inhibiting kinases required for the G1 – S phase transition (Harper and Elledge, 1996; Matsuoka et al., 1995; Zhang et al., 1998). At E15.5 of development, both CDKIs are highly expressed at the transition zone of the lens, where they work to inhibit G1 phase cyclins, allowing these cells to exit the cell cycle and differentiate into secondary fiber cells (Lovicu and McAvoy, 1999; Zhang et al., 1998; Zhao et al., 2008). In the mutant lens at E15.5 and onward, however, $p27^{kip1}$ and $p57^{kip2}$ were expressed ectopically into the fiber cell region, mirroring the ectopic cyclin D1 expression discussed previously. Not surprisingly, Prox1, which promotes the expression of $p27^{kip1}$ and $p57^{kip2}$, also was shown to have escaped its normal spatial localization within the transition zone of the lens (Duncan et al., 2002; Wigle et al., 1999), and was expressed ectopically throughout the anterior and posterior of the mutant lenses, similar to the expression pattern observed for each CDKI examined in our study. Together, these data suggest that ectopic Prox1-driven expression of $p27^{kip1}$ and $p57^{kip2}$ is functioning to counteract the cyclin D proliferation caused by altered smo activity.

The activated smo lenses also exhibited lens degeneration, illustrated by positive TUNEL staining observed throughout the lens epithelial and fiber cell regions onwards of E15.5. The inappropriate expression of cell cycle promoting and inhibiting factors in the lens fiber cell compartment may have been a determinant in the apoptotic response. Additionally, as outlined previously, Hh signaling has been shown to suppress lens cell fate during lens placode formation (Barth and Wilson, 1995; Cornesse et al., 2005; Dutta et al., 2005), and a consequence of the prolonged and exaggerated Hh signaling may be lens cell death, as an attempt to suppress further development of the lens. Nonetheless, lens degeneration did appear to have an impact on the retina in the activated smo mutants. While the mutant retina exhibited normal morphology throughout the majority of embryonic development, in late embryonic and early postnatal stages, following lens degeneration, aberrant retinal morphology became evident. For example, by P0, the retina displayed abnormal amounts of apoptosis and a loss in laminar organization. Despite this, retinal cell markers, including Pax6, and calretinin were expressed. Interestingly, retinal degeneration in the blind cavefish does not occur due to a defect in proliferation, or as a result of incorrect patterning of critical retinal development markers, including Pax6, but rather, as a result of programmed cell death in the retina subsequent to lens apoptosis and a loss of protective signals offered from the lens to the retina during development (Alunni et al., 2007; Strickler et al., 2007). It is important to reiterate that the Cre used in our studies (Crect) is active only in the surface SE and its derivatives, and not in the retina. Thus, the fact that these retinal defects occurred following the peak of

intense lens cell death and degeneration suggests that the degenerating lens in our smo mutants had a role. The lens may have lost its protective effect on the retina or, alternatively, provided a new signal that induced retina degeneration.

In conclusion, our studies have illustrated that active Hh signaling in the mammalian lens can lead to changes in cell cycle regulation, and expression of critical molecules responsible for lens development and differentiation. On one hand, sustained Hh signaling in fish and amphibians can lead to the suppression of lens formation or lens degeneration during development. Therefore, our findings raise the possibility that the mammalian eye anlagen has retained components of the regulatory network that might suppress embryonic lens formation if the lens is positioned inappropriately within the head. Conversely, the Hh signaling pathway has been shown to be critical for normal lens regeneration in the newt, even though the pathway has not been associated with normal lens development in this species. Therefore, it is possible that the Hh pathway could function in mammalian wound healing following lens injury or be used in lens regeneration. Further studies will be required to examine if the Hh pathway is associated with mammalian lens repair and regeneration. Finally, the Crect Smo M2 mouse also may serve as a model to study aspects of BCNS (Gorlin Syndrome), known to affect the lens and cornea. In this context, it may be valuable to determine if Foxe3 activation also is associated with BCNS in ocular pathology.

3 CHAPTER 3 – Cooperative role for AP-2α and AP-2β in Normal Lens Development

Abstract

AP-2α and AP-2β are co-expressed in the developing lens placode in early development. Following the separation of the lens vesicle, AP-2β expression is lost from the lens epithelium by E12.5. The conditional deletion of AP-2α from the lens in the Le-AP-2α single conditional KOs (KO) resulted in defects in lens development that were not seen until E12, the time at which AP-2β expression is lost from the epithelium. Potential cooperative roles of AP-2α and AP-2β in early stages of lens development were examined in the current study using a double conditional deletion of Tcfap2a/b in the lens. AP-2α and AP-2β flox'ed mouse lines were mated with the LeCre transgenic mouse line, to conditionally delete *Tcfap2a* and *Tcfap2b* from the lens placode and its derivatives at E 9.5. DKO lenses were small and mispositioned, with a lens stalk located toward the nasal aspect of the face. P4 mutants displayed microphthalmia, with variation in eye size between mutants and between eyes of the same mutant. The DKO mutants displayed increased proliferative markers in the lens epithelium and fiber cells, as well as additional cell cycle abnormalities. The DKO lenses also exhibited a disorganized cornea with abnormal N-cadherin expression within the corneal epithelium and a loss of N-cadherin expression in the corneal endothelium. Thus, our studies have revealed a cooperative role for AP-2α and AP-2β during lens development. A loss of both of these genes in early lens development results in a more severe phenotype than that seen in the single Le-AP-2α KOs, illustrating that both genes are necessary for proper lens development and have redundant roles in early stages of lens development.

3.1 Introduction

Vertebrate eye development occurs due to the interactions of multiple tissues and a complex network of inductive signals all working to form a properly oriented mature eye. In early eye development an area in the centre of the forebrain is specified to become the visual field. *Shh* and *Six3* genes are expressed in this single eye field and are responsible for further separation of this central eye field into bilateral ocular fields (Graw, 2010). Ultimately, a lens and cornea form from the surface ectoderm (SE), while the retina forms from the anterior neural plate (Graw, 2010). Mouse lens development begins with the formation of a lens placode, characterized by a thickening of the SE at embryonic day (E) 9.5. This lens placode invaginates inward forming a lens pit at E10.5. The anterior region of the developing lens adheres to the overlying SE, however, subsequent to the formation of the lens pit, this adhesion is abolished, and a lens vesicle is formed at E12.5 (Chow and Lang, 2001; Graw, 2010; Gunhaga). Cells at the posterior of the lens vesicle elongate towards the anterior of the lens forming primary fiber cells, while the anterior region is composed of a single layer of epithelial cells that proliferate throughout life. Epithelial cells at the lens equator exit the cell cycle and differentiate into secondary fiber cells which lose their organelles and nuclei, becoming transcriptionally silent throughout life (Chow and Lang, 2001; Graw, 2010; Gunhaga).

The Activating Protein-2 (AP-2) transcription factors are a family of five highly homologous proteins (AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε) that bind DNA as homo or heterodimers on specific GC rich consensus sequences (Mohibullah et al., 1999). The

AP-2 proteins contain a C-terminal helix-span helix domain and basic region to facilitate DNA binding and dimerization, and an N-terminal proline rich transactivation domain (Williams and Tjian, 1991a, b). During development, AP-2 is expressed in several embryonic tissues including migratory neural crest cells and its derivatives (pigment cells, dorsal root ganglia and facial structures including the eye), the central and peripheral nervous system, limbs, renal and urogenital tissues (Bauer et al., 1998; Chazaud et al., 1996; Mitchell et al., 1991; Monge and Mitchell, 1998; Moser et al., 1995; Moser et al., 1997b). AP-2α null mice die before birth, and display a myriad of defects including failed cranial closure, craniofacial abnormalities as well as abnormal morphology of the sensory organs and cranial ganglia. These AP-2α null mutants also display optic cup defects and evidence of aborted lens development (Schorle et al., 1996). An examination of the ocular phenotypes in the AP-2α null and chimeric mice illustrated that a germline loss of AP-2α caused phenotypes ranging from anophthalmia (lack of eyes) to lens defects including a lens stalk (an adhesion of the lens epithelium to the overlying SE) and the misexpression of lens proteins including Pax6 and MIP26 during embryonic development (West-Mays et al., 1999). To determine a cell autonomous role for AP-2α in lens development, mice with a conditional deletion of AP-2α (Le-AP-2α) from the lens placode and its derivatives using the LeCre line of mice were examined (Ashery-Padan et al., 2000; Pontoriero et al., 2008). When AP-2α was deleted from the lens placode in the Le-AP-2α mutants beginning at E9.5, morphological defects similar to those seen in the AP-2α null mice were observed. Le-AP-2α mutants displayed a lens

stalk (Pontoriero et al., 2008). Microarray analysis of Le-AP-2α lenses showed the differential expression of 415 genes compared to WT littermates. E-cadherin, responsible for maintaining a lens epithelial cell phenotype was among these differentially expressed genes. Consistent with findings that AP-2α directly regulates E-cadherin expression (Batsche et al., 1998; Decary et al., 2002), E-cadherin expression in the Le-AP-2α mutants was reduced in embryonic and postnatal lenses (Pontoriero et al., 2008).

Previous studies from our lab have shown that multiple AP-2 proteins are expressed in the developing eye such as AP-2α, AP-2β, and AP-2γ. AP-2α and AP-2β demonstrate overlapping expression in the SE and lens placode at E9.5, and their expression continues to overlap in the cornea and lens epithelium at E10.5. Following lens vesicle separation at E12.5, AP-2α remains expressed in the lens epithelium, while AP-2β expression is lost from this anterior lens structure (West-Mays et al., 1999). Interestingly, defects in the Le-AP-2α conditional mutants were not seen until E12, after the loss of AP-2β from the lens epithelium (Pontoriero et al., 2008), suggesting possible cooperative roles for AP-2α and AP-2β in lens placode development.

Using Cre-LoxP technology to address the possible redundancy between AP-2α and AP-2β in early lens development, we created a mutant mouse model with a conditional deletion of both *Tcfap2a* and *Tcfap2b* from the lens placode and its derivatives beginning at E9.5. These double mutants (AP-2α/β DKO) displayed abnormal lens, corneal and retinal morphology at embryonic and postnatal stages. AP-2α/β DKOs displayed a turned lens with a lens stalk located toward the nasal aspect of the face. The

DKO lens was disorganized with no clear transition zone and failed fiber cell denucleation at E15.5 and P4. AP-2α/β DKO lenses displayed increased levels of proliferation and lens cell cycle abnormalities as demonstrated through expression studies using PH3, Cyclin D1, $p27^{kip1}$ and $p57^{kip2}$. Corneal morphology was more severely disrupted in the AP-2α/β DKOs than in the Le-AP-2α single KO mutants. Incorrect corneal lamination was evident beginning in early embryonic development. N-cadherin was also incorrectly expressed within the corneal epithelium, suggesting an altered corneal epithelial phenotype. Together, the current study demonstrates the importance, and necessity for both AP-2α and AP-2β expression at the lens placode stage to ensure normal lens and corneal development.

3.2 Materials and Methods

3.2.1 Generation of AP-2α/β Double Mutant Mice

All animal 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. To generate mice with a double conditional deletion of *Tcfap2a* and *Tcfap2b*, several mouse crosses were performed. Heterozygous *Tcfap2*$^{ki7lacZ/+}$ mice (with a *Tcfap2a:LacZ* KI null allele) (Brewer et al., 2002) were first crossed with heterozygous *LeCre* mice that expressed Cre-recombinase in cells of the early lens placode (Ashery-Padan et al., 2000). Mice heterozygous for both the *Tcfap2a:LacZ* allele and the cre transgene were then mated with heterozygous *Tcfap2b*$^{+/-}$ mice (possess a *Tcfap2b*$^{-}$ allele as a result of a germline insertion of a PGK-neo cassette disturbing exon 4 of the AP-2 gene) (Moser et al., 1997a) to generate mice heterozygous for the cre transgene that also had one functional allele of the *Tcfap2a* and *Tcfap2b* genes. In a set of parallel crosses, homozygous *Tcfap2a*$^{lox/lox}$ *flox'ed* mice (which include loxP sites flanking the DNA binding and dimerization domains of exon 5 and 6 of the AP-2α gene) (Brewer et al., 2004) were crossed with homozygous *Tcfap2b*$^{lox/lox}$ *flox'ed* mice (which include loxP sites flanking exon 6 of the AP-2α gene) (Williams, unpublished). In a final cross to generate the double conditional AP-2α/β double mutants, homozygous *Tcfap2a*$^{lox/lox}$*/Tcfap2b*$^{lox/lox}$ mice were crossed with *Tcfap2a*$^{ki7lacZ/+}$*/Tcfap2b*$^{+/-}$*/Le-Cre*$^{+/-}$ mice to generate cre positive mice with the successful deletion of both alleles of AP-2α and both alleles of AP-2β (of genotype *Tcfap2a*$^{ki7lacZ/lox}$*/Tcfap2b*$^{-lo/x}$*/Le-Cre*$^{+/-}$) (See Fig

3.11 for breeding scheme)**.** Noon on the day of vaginal plug detection was considered day 0.5 (E0.5) of embryogenesis. Mice were genotyped using DNA extracted from tail or ear clipping samples using the DNeasy tissue kit (Qiagen). For *Cre* allele detection, PCR was performed using the primers Cre1: 5'-GCT CCT TAG CAC CGC AGG TGT AGA G-3', Cre3: 5'-CGC CAT CTT CCA GCA GGC GCA CC-3', and the following conditions: 120 seconds at 95ºC, 35 cycles of 95ºC for 45 seconds, 67ºC for 45 seconds, 72ºC for 60 seconds, followed by 72ºC for 10 minutes in order to generate a 421 bp product for the cre allele. To detect the *Tcfap2a*^{ki7lacZ} allele, PCR was performed using the primers Alpha 6/7: 5'-GAA AGG TGT AGG CAG AAG TTT GTC AGG GC -3', Alpha 3'KO: 5'-CGT CTG GCT GTT GGG GTT GTT GCT GAG GTA C-3', IRESUP: 5'–GCT AGA CTA GTC TAG CTA GAG CGG CCC GGG-3', and the following conditions: 45 seconds at 95ºC, 1min at 67ºC, 1 minute and 10 seconds at 72ºC for 33 cycles to generate a 300bp *Tcfap2a*^{ki7lacZ} allele and a 500bp WT allele. To detect the *Tcfap2b*$^{-}$ allele, PCR was performed using the primers 4 Exon DW: 5'–CCT CCC AAA TCT GTG ACT TCT-3', 4 Exon Rev: 5'–TTC TGA GGA CGC CGC CCA GG-3', and PGK-PolyA DW: 5'-CTG CTC TTT ACT GAA GGC TCT TT-3', and the following conditions: 45 seconds at 95ºC, 45 seconds at 58ºC, 1 minute at 72ºC for 37 cycles to generate a 380bp *Tcfap2b*$^{-}$ allele and a 221bp WT allele. To detect the *Tcfap2a*^{lox} allele, PCR was performed using the primers Alflox4: 5'-CCC AAA GTG CCT GGG CTG AAT TGA C-3', and Alfscsq: 5'-GAA TCT AGC TTG GAG GCT TAT GTC-3', and the following conditions: 45 seconds at 95ºC, 45 seconds at 65ºC, 1 minute at 72ºC for 39

cycles, to generate 560bp *Tcfap2a*^{lox} allele and a 490bp WT allele. To detect the *Tcfap2b*^{lox} allele, PCR was performed using the following primers BFL1: 5'-GTC TGT TTA GAA CCT GGC TCA GCC AGA GGC TGG-3', and BFL2: 5'-TCT GGC AAG GCT CTT TCG GGG CAC TCA CAG CAG-3', and SD5P33: 5'-CGC AGC GCA TCG CCT TCT ATC GCC TT-3', and the following conditions: 2 minutes at 95ºC, 45 seconds at 95ºC, 3 minutes at 70ºC for 34 cycles to generate a 550bp *Tcfap2b*^{lox} allele and a 450bp WT allele.

3.2.2 Histology

Whole embryos and postnatal eyes were collected at different stages corresponding to AP-2α/β DKOs, Le-AP-2α single KOs and WT littermates. Embryonic tissue was fixed in 10% neutral buffered formalin overnight at room temperature and then transferred into 70% ethanol until processing. Whole embryo's (E13.5), embryo heads (E15.5) or eyes (P4) were processed and embedded in paraffin. Serial sections were cut at a thickness of 4μm and used for Hematoxylin and Eosin staining as well as immunofluorescent analysis. For the E13.5 embryos examined, a sample size of 6 lenses from 3 animals was stained (for each genotype). For the E15.5 embryos examined, a sample size of 2 DKO lenses from 1 animal were stained, and 3 lenses from 3 single KO and 3 WT animals were stained. For the P4 stage examined, a sample size of 4 lenses from 2 DKO animals were stained and 3 lenses from 3 single KO and 3 WT animals were stained.

3.2.3 Immunofluorescence

Indirect immunofluorescence was performed using the following primary antibodies: mouse monoclonal AP-2α, Developmental Studies Hybridoma Bank (1:1); Rabbit polyclonal AP-2β, Cell Signaling (1:50); rabbit polyclonal Pax6, Covance (1:50); rabbit polyclonal β-Crystallin provided by Dr. Samuel Zigler Jr., Chief of lens and cataract biology section of the National Eye Institute (1:200); The mitosis marker anti-Phospho-Histone H3 (rabbit polyclonal, Upstate) was used to detect mitotic cells (1:30); mouse monoclonal Cyclin D1, Santa Cruz (1:100); mouse monoclonal $p27^{kip1}$, BD Transduction (1:350); goat polyclonal $p57^{kip2}$, Santa Cruz (1:100); Fluorescent secondary antibodies were either Alexa Fluor 488 (Goat anti-mouse and goat anti-rabbit), or Alexa Flour Fluor 568 (goat anti-mouse and donkey anti-goat), Invitrogen-Molecular Probes, used 1:200 for 1 hour at room temperature. Paraffin-embedded sections were deparaffinized in xylene, hydrated (through 100%, 95%, 70% ethanol, followed by water), treated with 10mM sodium citrate buffer (pH 6.0; boiling for 20 minutes) for antigen retrieval, blocked with normal serum and incubated with primary antibodies overnight at 4°C. Each stain included a negative control with no primary antibody. All H&E and fluorescent stains were visualized with a microscope equipped with a fluorescence attachment, and images were captured with a high-resolution camera and associated software (Open-Lab; Improvision, Lexington, MA). Images were reproduced for publication with image-management software (Photoshop 7.0; Adobe Systems Inc., Mountain View, CA).

3.3 Results

3.3.1 Successful deletion of AP-2α and AP-2β protein expression in AP-2α/β DKO lenses

AP-2α is normally expressed in the lens placode, SE and within a region of periocular mesenchyme (POM) fated to become RPE at E9.5 (Bassett et al., 2010; West-Mays et al., 1999). Throughout ocular development, AP-2α remains expressed within the lens epithelium and cornea, and also within amacrine cells of the neural retina (NR) (Bassett et al., 2012; Bassett et al., 2007; West-Mays et al., 1999). In agreement with these findings, WT lenses at E13.5, E15.5 and P4 showed clear nuclear AP-2α protein expression within the lens epithelium and cornea (Fig 3.1A-C). In the Le-AP-2α single KOs, and AP-2α/β DKOs, AP-2α expression was successfully deleted from the lens epithelium and cornea at equivalent stages (Fig 3.1D-I) while its expression was retained within the retina.

AP-2β shares overlapping expression patterns with AP-2α in embryonic and postnatal stages of development. During embryogenesis, both AP-2 proteins are co-expressed in the SE and lens placode at E9.5 and remain co-expressed in the lens until E11.5 (West-Mays et al., 1999). AP-2α and AP-2β are also co-expressed within cells of the developing and postnatal cornea and retina (Bassett et al., 2007; Bassett et al., 2010; West-Mays et al., 1999). With the separation of the lens vesicle at E11.5, AP-2β expression is lost from the lens, but remains expressed in the cornea and retina (Bassett et al., 2007; West-Mays et al., 1999). As shown in figure 3.2, AP-2β was successfully

deleted in the AP-2α/β DKOs. WT and Le-AP-2α single mutant corneas and retinas stained positively for AP-2β expression at E13.5, E15.5 and P4 (Fig 3.2A-F) while AP-2β expression was deleted from the cornea in the AP-2α/β DKOs at these developmental stages (Fig 3.2G-I).

3.3.2 AP-2α/β DKOs exhibit abnormal lens morphology

The conditional deletion of AP-2α and AP-2β from the lens beginning at the lens placode stage of development resulted in lens defects that were more severe in the AP-2α/β DKO vs. the single Le-AP-2α KOs. For example, DKO lenses (Fig 3.3G-I) appeared smaller than both WT (Fig 4 A-C) and Le-AP-2α (Fig 4 D-F) lenses at all stages examined (Fig 3.3). As previously reported, the Le-AP-2α KO lens exhibited a lens stalk (an adhesion of the anterior region of the lens to the overlying SE) (Pontoriero et al., 2008), and this trend was again seen in the DKO model. However, at E13.5, the lens stalk appeared to be situated more nasally in the DKO model, causing the lens to rotate resulting in an epithelium that was closer to the corneal stroma compared to that of the Le-AP-2α single KO (Fig 3.3D,G). At E15.5, the DKO lens stalk appeared to have rotated laterally, adhering to tissue located on the lateral sides of the lens. Interestingly, at this stage, the lens had failed to separate not only from the anterior region where cornea normally develops, but also adhered to tissue on the lateral sides of the lens not normally present in WT or Le-AP-2α eyes (Fig 3.3H). At E15.5 and P4, the DKO lens occupied much of the space where the vitreous humor is normally situated (Fig 3.3H-I). The DKO

lens also appeared to be rotated laterally at E15.5 and P4. Beginning at E15.5 (Fig 3.3H) and persisting in one mutants at P4 (not shown), it appeared that the anterior lens epithelium had rotated almost 90º laterally. Denucleated cells remained in the fiber cell regions of all mutant lenses examined at E15.5 and P4 (Fig 3.3H-I). One AP-2α/β DKO mutant lens at P4 exhibited a pronounced lens stalk with what appeared to consist of epithelial cells protruding into the corneal region (Fig 3.3I). This lens also exhibited a disorganized transition zone, lacked a true lens bow region and again occupied the vitreal space that normally separates the lens and retina (Fig 3.3H). Variation in DKO lens size (between lenses of the same animal) was seen throughout postnatal development, and some DKO mutants exhibited bilateral anophthalmia at P29 (data not shown).

3.3.3 AP-2α/β DKOs exhibit corneal defects

During development, the cornea is derived from cells of the SE as well as migratory neural crest cells (Hay, 1980). The cornea is made up of three layers. An outer epithelium is derived from differentiated SE; an inner endothelium is derived from mesenchymal cells of neural crest origin, and a stroma which is composed of keratocytes separated by tightly packed and arranged collagen lamellae (Chow and Lang, 2001; Fini et al., 1997; Hay, 1980; Saika et al., 2001; Trainor and Tam, 1995). AP-2α has been shown to be important in corneal development, and its deletion in the Le-AP-2α single KO mice resulted in defects in the corneal epithelium and corneal stroma (Dwivedi et al., 2005). These mutant corneas exhibited corneal epitheliums with variations in the number

of stratified layers (2-10), a decrease in E-cadherin expression and increased cellular proliferation. A thin Bowman's layer and abnormalities in the corneal stroma were also evident. The abnormal corneal morphology of the AP-2α/β DKOs was severely exacerbated compared to the defective corneal morphology in the Le-AP-2α mutants. At E13.5, the DKO cornea appeared thinner than both WT and Le-AP-2α corneas (see Fig 3.3A, D, F). At this stage it had become difficult to discern where the different layers of the cornea begin and end. At E15.5, the double mutant cornea was extremely thin and adhered to the entire anterior portion of the lens (see Fig 3.3H). Corneal morphology remained disrupted and disorganized in the AP-2α/β DKO lens at P4 (Fig 3.4). Cells within the corneal stroma region of the DKO (Fig 3.4C) were not as tightly packed as they were within WT (Fig 3.4A) and Le-AP-2α single KO corneas (Fig 3.4B). No real corneal layers could be discerned in the DKO corneal region (Fig 3.4C and refer to Fig 3.3I).

To begin to better understand the corneal defect occurring due to the loss of AP-2α and AP-2β, immunofluorescence staining with N-cadherin, normally found in the corneal endothelium (Reneker et al., 2000) was performed. N-cadherin was normally expressed in the corneal endothelium of WT mice at P4 (Fig 3.4D, gold arrowhead). N-cadherin expression appeared to be reduced and patchy in the Le-AP-2α mutants (Fig 3.4E, gold arrowhead) and appeared to be lost in the AP-2α/β DKO corneal endothelium (Fig 3.4F). Interestingly, cells within the corneal epithelium of the P4 AP-2α/β DKO

abnormally expressed N-cadherin, a marker not traditionally expressed in this region (Fig 3.4F, white star).

3.3.4 Pattern of Pax6 expression in DKO lens suggests a mis-positioned lens

Due to the morphological defects observed in the AP-2α/β DKO lens and cornea, Pax6 and β-Crystallin expression were examined as markers of epithelial and fiber cell development respectively. Pax6 is expressed in the eye in two waves, pre-placode and post-placode formation (Chow and Lang, 2001). Normally Pax6 expression is confined to the lens epithelium of the developing and postnatal lens and is also expressed in the developing corneal epithelium (Chow and Lang, 2001; Davis et al., 2003; Davis and Reed, 1996). Pax6 appeared to be expressed normally in the DKO lens epithelium at E13.5, though it was expressed in cells of the lens stalk (Fig 3.5G). However, its expression had become abnormal by E15.5. While Pax6 was expressed in the lens epithelium of both WT (Fig 3.5B) and Le-AP-2α single KOs (Fig 3.5E), its expression appeared to be expanded throughout the entire lens region in the DKO at E15.5 (Fig 3.5F). Interestingly, a tight zone of Pax6 expression around the anterior/lateral/posterior aspects of the DKO lens was evident in one mutant at this stage, suggesting that the lens may be rotated or mispositioned approximately 90 degrees (Fig 3.5F). At E15.5 and P4, Pax6 expression was expanded in the lens stalk regions (Fig 3.5G-J). Pax6 expression in the corneal epithelium appeared to be patchy in the Le-AP-2α single KOs, however, this phenotype was not attenuated in the AP-2α/β DKO corneal epithelium at P4 (not shown).

As a result of the fiber cell defects revealed through hematoxylin and eosin staining (including a small lens with a lack of denucleation in fiber cells at E15.5 and P4, vacuoles and a disorganized transition zone), β-crystallin expression was examined. β-crystallin is a fiber cell specific marker and its expression begins early in development, and persists throughout life (Bloemendal et al., 2004; Wang et al., 2004). No difference in β-crystallin expression was seen in DKO lenses (Fig 3.6C-D) compared to WT lenses (Fig 3.6A-B) at E13.5 or P4, however, the lack of fiber cell denucleation is evident through DAPI staining at P4 in the DKO fiber cell region (Fig 3.6D, and refer to H&E Fig 3.3H-I).

3.3.5 AP-2α/β DKO lenses exhibit abnormal amounts of cellular proliferation

AP-2 has been shown to regulate cellular proliferation (Dwivedi et al., 2005; Eckert et al., 2005; Moser et al., 1997b). A deletion of AP-2α in the cornea has been shown to lead to an increase in proliferation in cells of the corneal epithelium (Dwivedi et al., 2005). Although the DKO lens is morphologically smaller than that of WT and Le-AP-2α single KO lenses, it takes up the majority of space between the posterior region of the lens and the retina, usually occupied by the vitreous humor (See Fig 3.3H-I). To assess DKO lens cell proliferation, we examined PH3 expression, which labels cells undergoing mitosis (Hans and Dimitrov, 2001). A few cells in the lens epithelium of WT (Fig 3.7A-C) and Le-AP-2α single KOs (Fig 3.7D-F) expressed PH3, with an abnormally large amount of cells in the AP-2α/β DKO lens staining PH3 positive at all stages

examined (Fig 3.7G-I). Proliferating cells were observed in both the lens epithelial and fiber cells regions, and were also observed in the lens stalk region of the DKO lens (Fig 3.7G-I). An increase in proliferating cells were also evident in the retinas of the DKO eyes at all stages (Fig 3.7G-I).

3.3.6 AP-2α and AP-2β are required to maintain normal cell cycle activity in the lens

AP-2 has been shown to play a role in cell cycle activity (Hilger-Eversheim et al., 2000; Moser et al., 1997b; Zeng et al., 1997), thus we examined cell cycle promoting and inhibiting factors in the lenses of the AP-2α/β DKOs. The cell cycle promoting factor Cyclin D1, which binds to specific cyclin dependent kinases (Cdk) to promote G1 to S phase transition of cells (Ewen et al., 1993; Hinds et al., 1992) was examined at E13.5, E15.5 and P4 in WT, Le-AP-2α single KOs and AP-2α/β DKO lenses. WT and Le-AP-2α single KO lenses at all stages showed normal Cyclin D1 expression in the lens epithelium and developing fiber cells at the transition zone of the lens (Fig 3.8A-F, white arrows). Interestingly, the expression of Cyclin D1 in the lens epithelium and at the transition zone of the AP-2α/β DKO lens appeared to be down regulated at E13.5 (Fig 3.8G, dashed circles). By E15.5, Cyclin D1 expression appeared ectopic within the DKO lens, with expression evident in both the anterior and posterior compartments of the lens, a trend that continued at P4 (Fig 3.8H-I, white arrows). Where Cyclin D1 expression was mostly confined to the transition zone in WT and Le-AP-2α single KO lenses at P4 (Fig 3.8C, F,

white arrows), it was expressed throughout the entire lens epithelium in the AP-2α/β DKO lens, with a large cluster of cells within the lens stalk region expressing this cell cycle promoting factor (Fig 3.8I, white star).

$p27^{kip1}$ and $p57^{kip2}$ are CDKIs in the $p21^{cip1}$ family responsible for inhibiting Cdks involved in the G1-S phase transition, including Cyclin D1/Cdk4/6 (King et al., 1994). During lens development, $p27^{kip1}$ and $p57^{kip2}$ are of particular importance in the differentiation of epithelial cells to fiber cells. These cell cycle inhibitors are responsible for causing lens epithelial cells to exit the cell cycle, allowing them to begin their differentiation process (Griep, 2006; Pateras et al., 2009; Zhang et al., 1998). As a result of the abnormal Cyclin D1 expression seen in the DKO lenses, $p27^{kip1}$ and $p57^{kip2}$ expression was examined. $p27^{kip1}$ is most predominantly expressed in the fiber cells during early lens development, and at the lens transition zone with the progression of lens development (Griep, 2006).

$p27^{kip1}$ was normally expressed in the fiber cells at E13.5 in WT and single KO lenses (Fig 3.9A, D), and its expression appeared confined to the lens transition zone of WT and single KO lenses at E15.5 (Fig 3.9B, E, white arrows) and P4 (Fig 3.9C, F, white arrows). Although $p27^{kip1}$ expression appeared relatively normal in the AP-2α/β DKO lens at E13.5, with expression observed in the fiber cells of the lens (Fig 3.9G), its expression was ectopic at both E15.5 (Fig 3.9H, dashed circle) and P4 (Fig 3.9I, white arrows). Its expression was no longer tightly confined to the transition zone in the DKO lens, but expressed throughout the entire lens compartment.

$p57^{kip2}$ is normally expressed at low levels in the lens epithelium and highly expressed at the transition zone (Griep, 2006). $p57^{kip2}$ was expressed normally in WT and Le-AP-2α KOs at E13.5 (Fig 3.10A, D). Although $p57^{kip2}$ was expressed in a spatially normal manner in the AP-2α/β DKOs, it appeared to be upregulated in the transition zone of the DKO lens at this stage (Fig 3.10G, gold arrows). While $p57^{kip2}$ was expressed at the transition zone of WT and single KO lenses at E15.5 and P4 (Fig 3.10B, E, white arrows), its expression in the DKO lens mirrored that of $p27^{kip1}$, being expressed ectopically throughout the entire lens compartment (Fig 3.10H-dashed circle, I-white arrows).

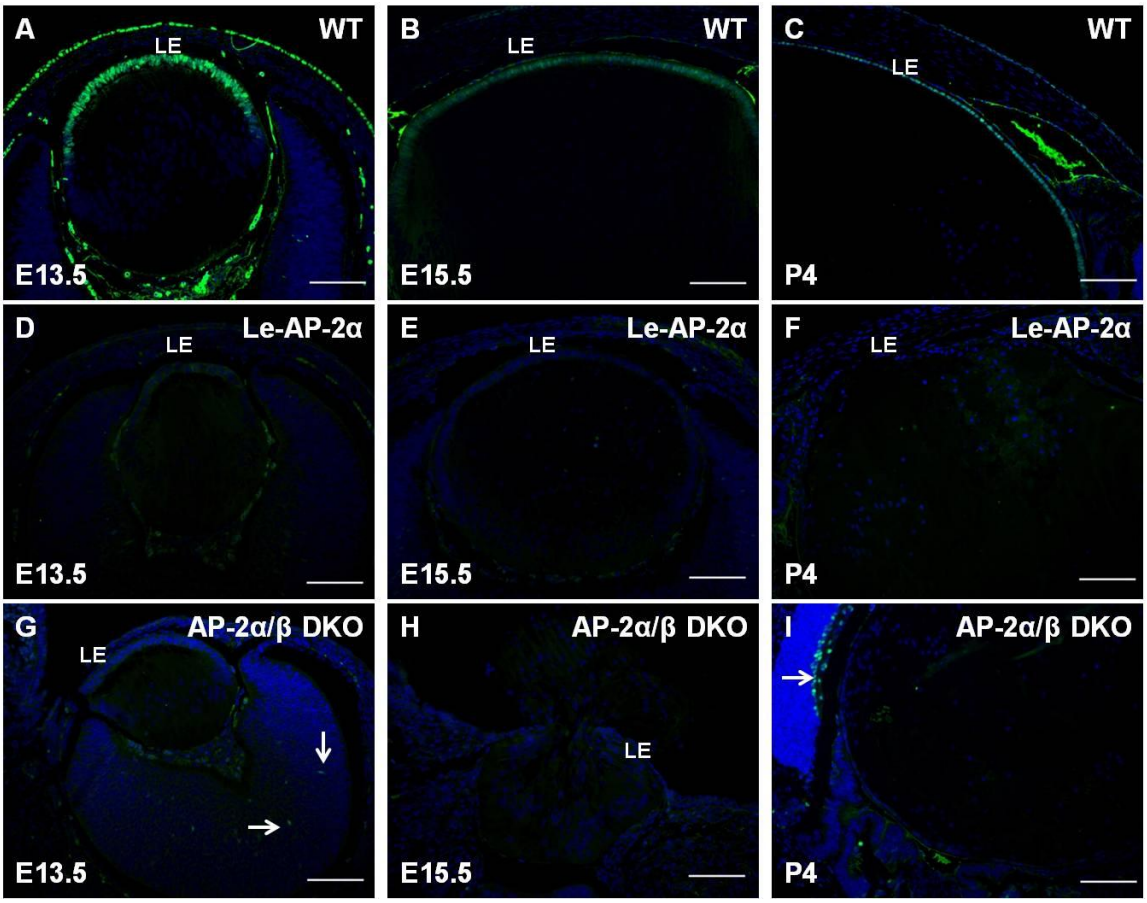

Figure 3.1 Successful deletion of AP-2α from AP-2α/β DKO lens.

AP-2α stains nuclei of the WT lens epithelium at E13.5, E15.5 and P4 (A-C). AP-2α is successfully deleted from epithelial cell nuclei in Le-AP-2α (D-F) and AP-2α/β DKOs (G-I) while it remains expressed in single and DKO retina (eg G,I, white arrows). LE-Lens Epithelium. All scalebars represent 100μm

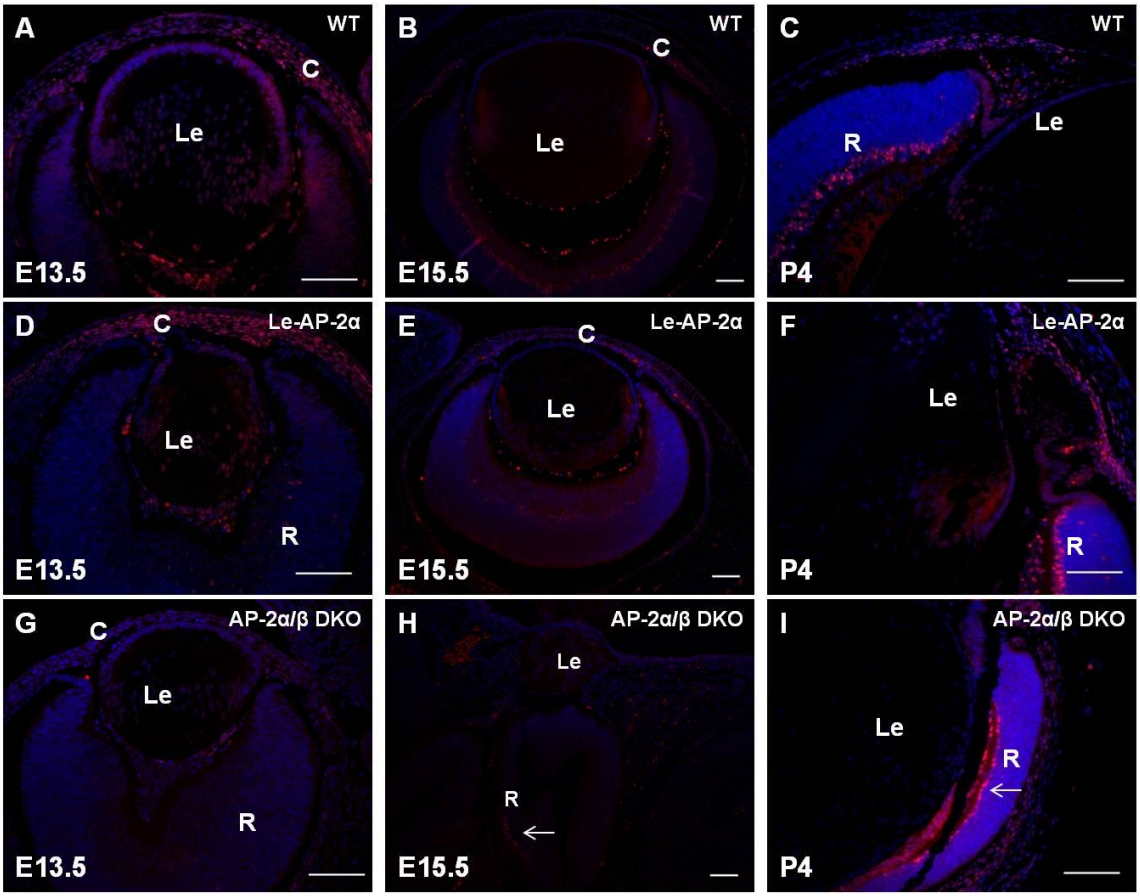

Figure 3.2 Successful deletion of AP-2β from AP-2α/β DKO lens

AP-2β is expressed in the WT and Le-AP-2α cornea and retina at E13.5, E15.5 and P4 (A-F). AP-2β is successfully deleted from the DKO cornea (G-I) while still expressed in the retina (H-I, white arrows). C-Cornea. Le-Lens. R-Retina. All scalebars represent 100μm.

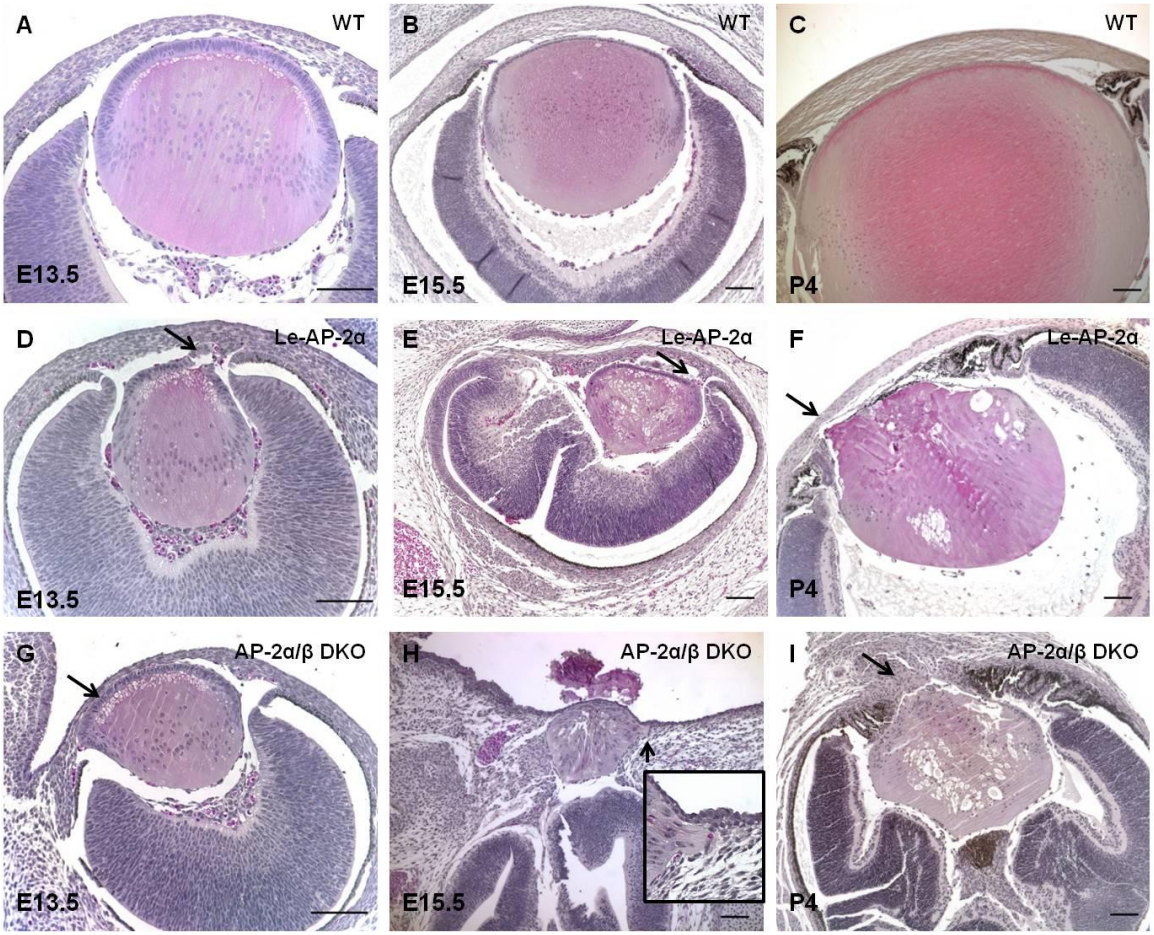

Figure 3.3 Morphology of AP-2αβ DKO lenses.

Normal WT lenses at E13.5, E15.5 and P4 show a single layered epithelium that has separated away from the surface ectoderm (A-C). Le-AP-2α single KOs display a lens stalk (D-F illustrated with black arrows). A more nasally located lens stalk is seen in the DKO at E13.5, resulting in an epithelium that is closer to the corneal stroma (G black arrow). The lens appears to be rotated laterally at E15.5 with a laterally placed lens stalk (H, stalk outlined with black arrow and shown in inset). A large lens stalk is present at P4 in the DKO (I). A lack of fiber cell denucleation and a disorganized bow region is evident at E15.5 and P4 (H,I). All scalebars represent 100μm.

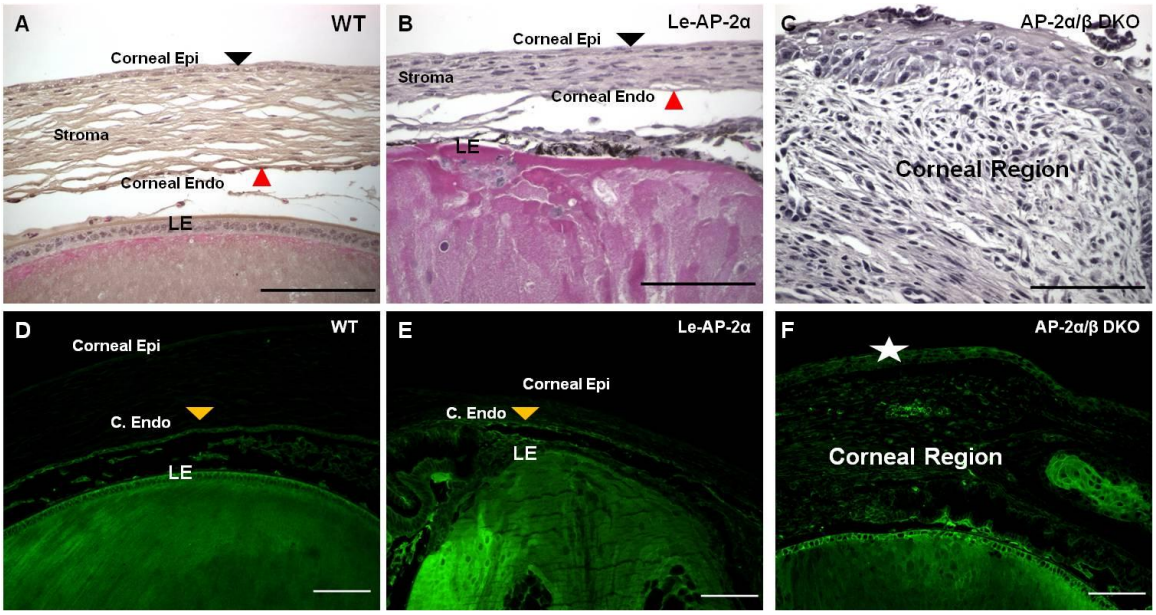

Figure 3.4 AP-2α/β DKO exhibits abnormal corneal morphology, loss of true corneal endothelial and corneal epithelial phenotypes.
The DKO cornea appears to have a thicker stroma (C) than seen in the LE-AP-2α (B) and WT (A) at P4. No true corneal epithelium or corneal endothelium can be seen in the DKO eye (C). N-cadherin staining illustrates a loss of a corneal endothelium in the DKO (F), while N-cadherin is abnormally expressed in the DKO corneal epithelium (F, white star). No distinct corneal endothelium is evident in the DKO (F). Corneal Epi-Corneal Epithelium (Illustrated by black arrowheads). Corneal.Endo-Corneal Endothelium (Illustrated by red and gold arrowheads). LE-Lens Epithelium. All scalebars represent 100μm.

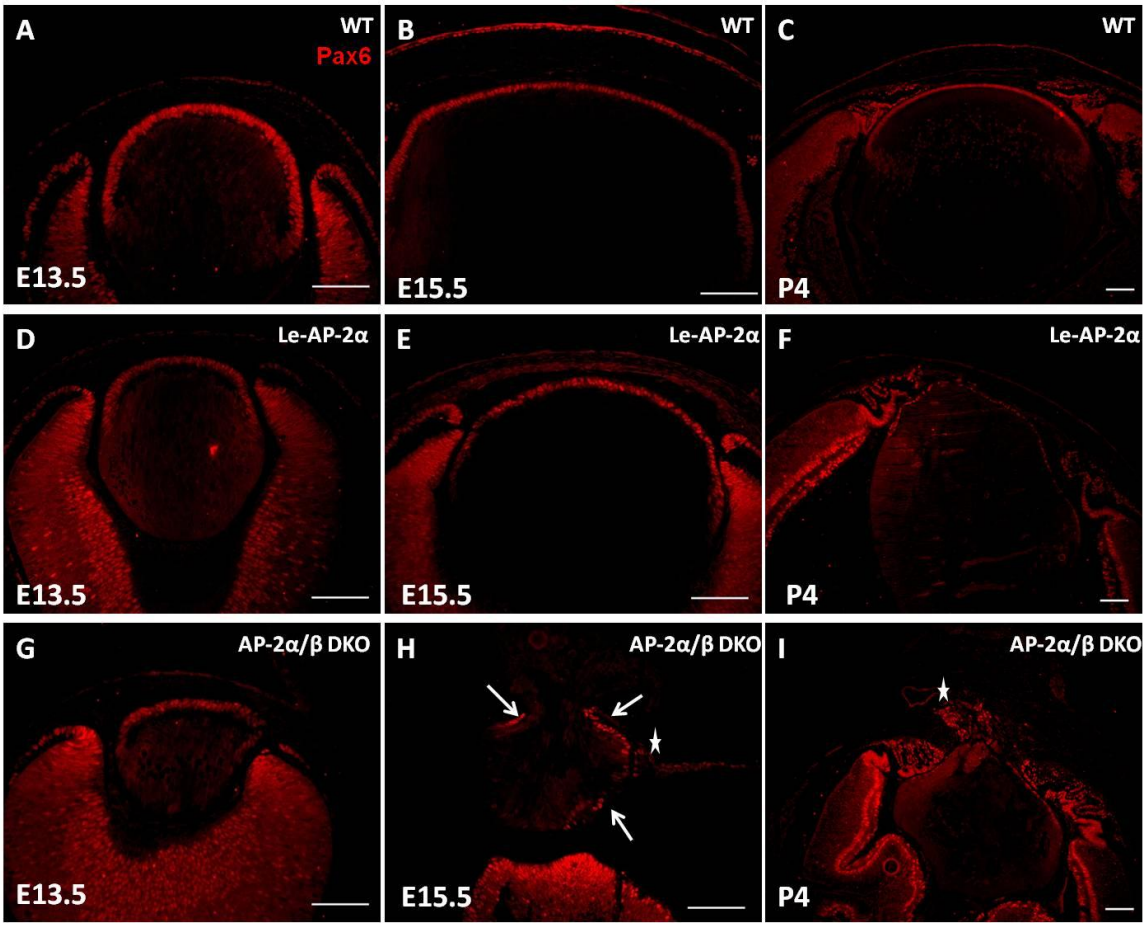

Figure 3.5 Pax6 expression in AP-2α/β DKO illustrates possible mis-positioning of lens.

Pax6 is expressed in the lens epithelium of WT and Le-AP-2α KOs at E13.5, E15.5 and P4 (A-F). Pax6 is expressed in the DKO lens stalks at all stages (G, H, I, white star illustrates Pax6 expression in lens stalk at E15.5 and P4). A possible rotated zone of Pax6 expression is seen at E15.5 and P4 (H, J, white arrows) while Pax6 is expressed in the fiber cell region in the DKO at E15.5 and P4. All scalebars represent 100μm.

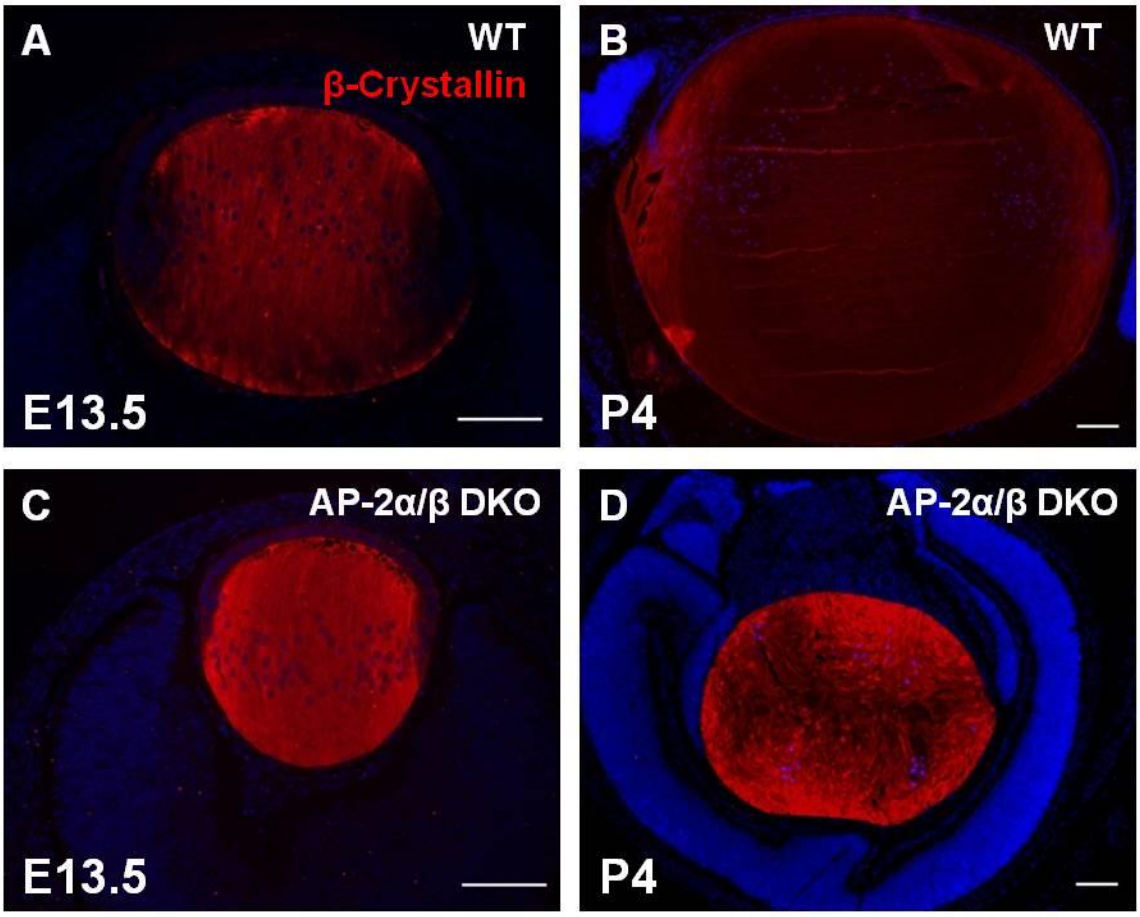
A
WT
β-Crystallin
E13.5
B
WT
P4
C
AP-2α/β DKO
E13.5
D
AP-2α/β DKO
P4

Figure 3.6 β-Crystallin Expression in the DKO Lens
β-Crystallin expression is normal in DKO lenses at E13.5 and P4 (C-D) compared to WT at equivalent stages (A-B). DAPI staining in the fiber cell region of the P4 DKO lens illustrates failed fiber cell denucleation. All scalebars represent 100μm.

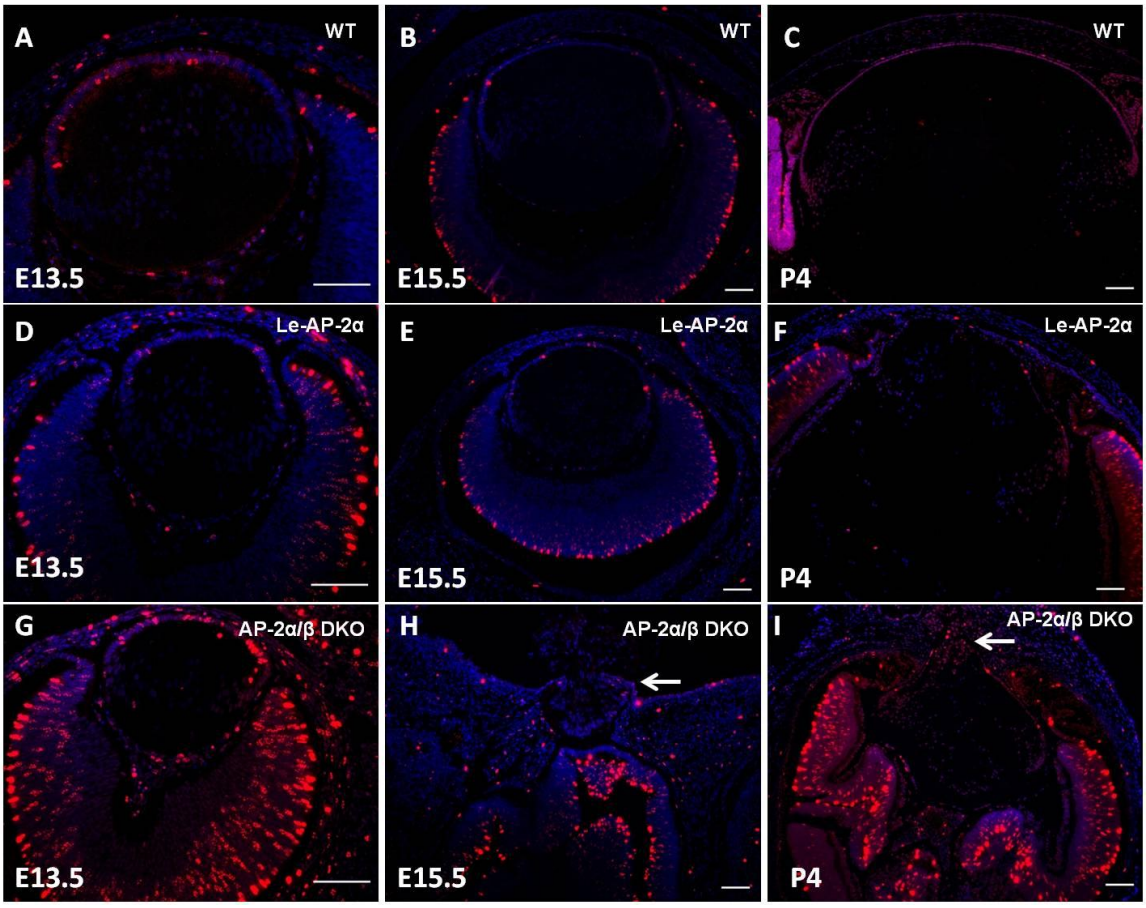

Figure 3.7 Phosphohistone 3 expression is increased in DKO lenses.

While a few cells stain positive for PH3 (red) in the lens epithelium of WT (A-C) and Le-AP-2α KOs (D-F), PH3 staining is increased in DKO lenses at E13.5, E15.5 and P4 (G-I). PH3 positive cells are also observed in the lens stalks at E15.5 and P4 (H, I white arrows) and in the fiber cell region of these lenses (H,I). All scalebars represent 100μm.

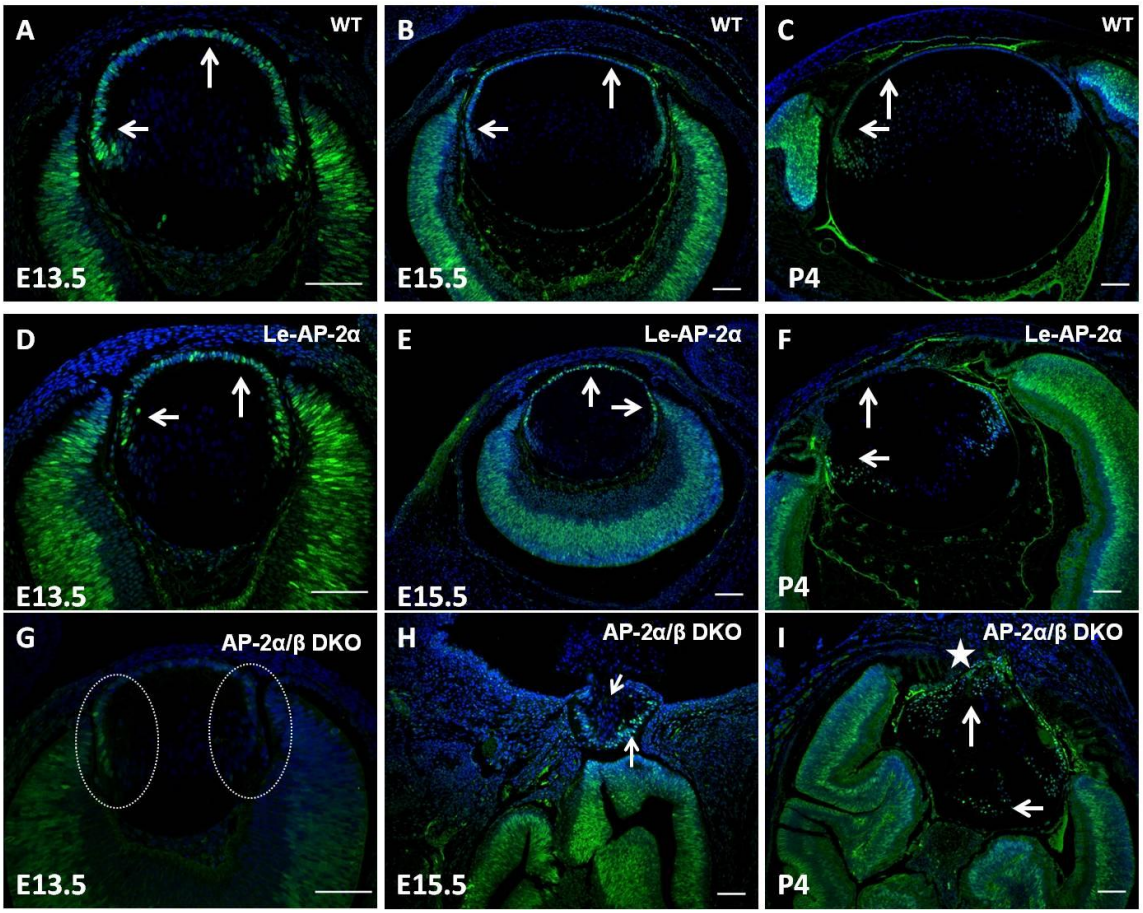

Figure 3.8 Cyclin D1 is expressed in the fiber cell regions of the DKO lens.
Cyclin D1 is normally expressed in the lens epithelium and transition zone of WT (A-C, white arrows) and Le-AP-2α KOs (D-F, white arrows). Cyclin D1 expression appears down regulated in the DKO lens at E13.5 (G, dashed circles), and is expressed abnormally in the fiber cell regions of the DKO lens at E15.5 and P4 (H, I, white arrows). Cyclin D1 is expressed in the lens stalk region at P4 (I, white star). All scalebars represent 100μm.

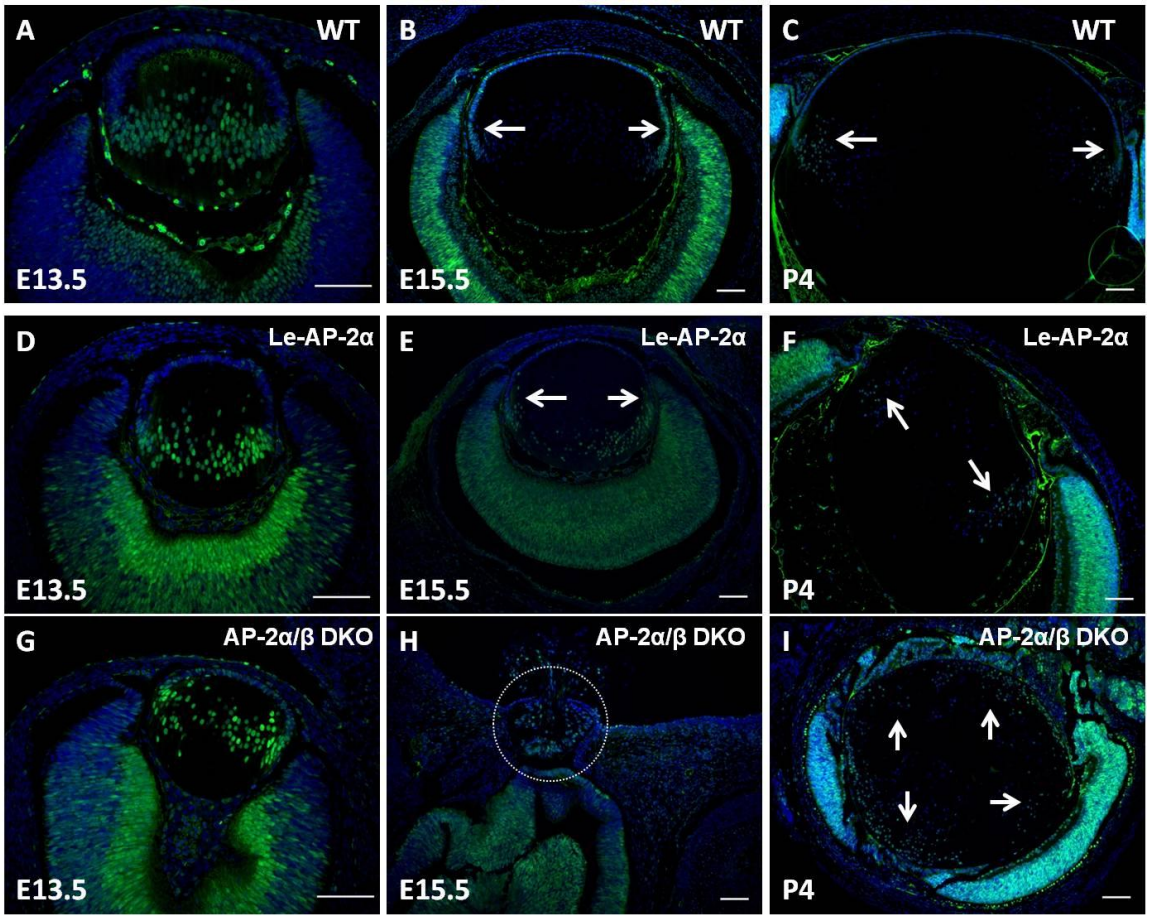

Figure 3.9 p27^{kip1} is expressed ectopically in DKO lens fiber cells.
p27^{kip1} is expressed normally predominately at the transition zone of WT (A-C, white arrows) and Le-AP-2α KOs (D-F, white arrows). p27^{kip1} is abnormally expressed in fiber cells at E15.5 (H, dashed circle) and P4 (I, white arrows) in DKO lenses. All scalebars represent 100μm.

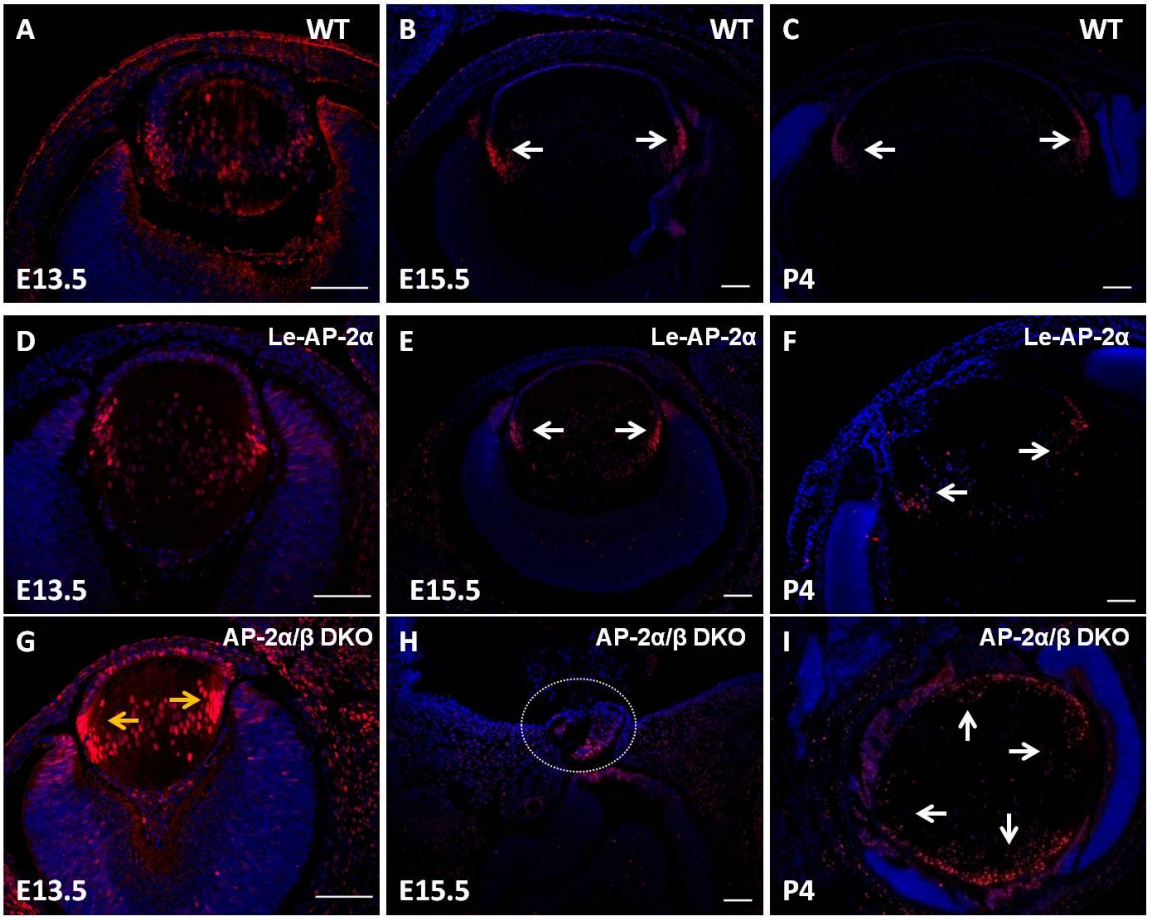

Figure 3.10 p57^{kip2} is expressed ectopically in DKO lens fiber cells.
p57^{kip2} is expressed normally within the epithelium (E13.5) and transition zone (all stages) of WT (A-C, white arrows) and Le-AP-2α KOs (D-F, white arrows). p57^{kip2} appears upregulated in the transition zone at E13.5 (G, yellow arrows), and abnormally expressed in fiber cells at E15.5 (H, dashed circle) and P4 (I, white arrows) in DKO lenses. All scalebars represent 100μm.

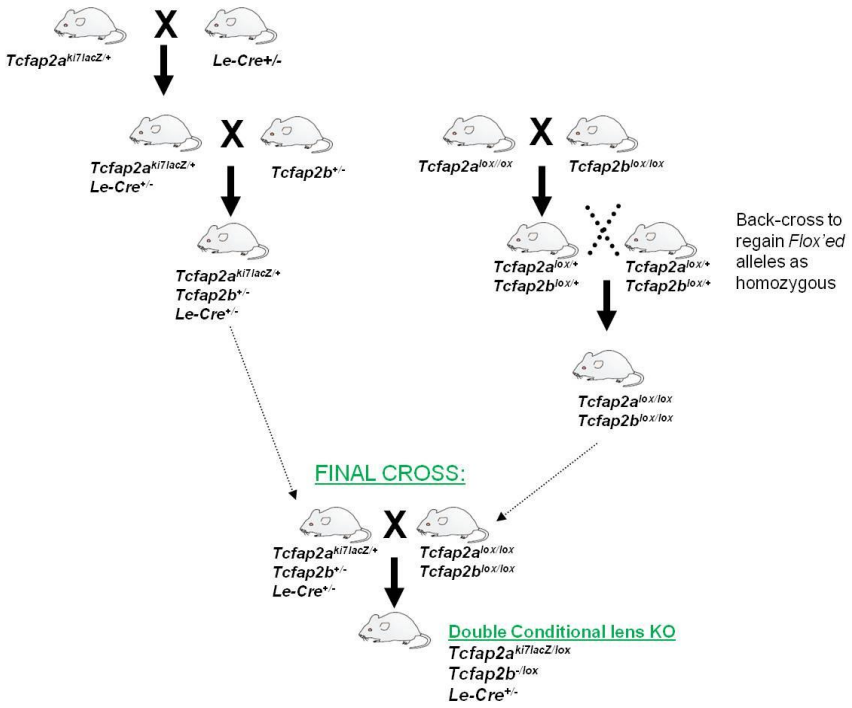

Figure 3.11 Generation of the conditional lens placode specific AP-2α/AP-2β DKO model.

Mice heterozygous for *Tcfap2a* and *Tcfap2b* will be used so that cre-mediated excisions of only one allele is needed. *Tcfap2a*$^{ki7lacZ/+}$ mice will be crossed with Le-Cre$^{+/-}$ transgenic mice to generate cre positive mice that are heterozygous for *Tcfap2a* (*Tcfap2a*$^{ki7lacZ/+}$/Cre$^{+/-}$). These mice will be crossed with heterozygous *Tcfap2b*$^{+/-}$ mice to generate mice heterozygous for *Tcfap2a* and *Tcfap2b* that are also cre positive (*Tcfap2a*$^{ki7lacZ/+}$/*Tcfap2b*$^{+/-}$/Cre$^{+/-}$). In a separate series of crosses, *Tcfap2a flox'ed* mice (*Tcfap2a*$^{flox/flox}$), will be crossed with *Tcfap2b flox'ed* mice (*Tcfap2b*$^{flox/flox}$). The resulting offspring will be interbred to generate homozygous *flox'ed* alleles, generating homozygous *Tcfap2a/b* mice (*Tcfap2a*$^{flox/flox}$/*Tcfap2b*$^{flox/flox}$). In the final cross, these mice will be crossed with the *Tcfap2a*$^{ki7lacZ/+}$/*Tcfap2b*$^{+/-}$/Cre$^{+/-}$ mice to generate a double conditional KO mouse line heterozygous for the *Tcfap2a* and *Tcfap2b* null alleles, with the second copy of *Tcfap2a* and *Tcfap2b* deleted by cre-mediated excision (*Tcfap2a*$^{ki7lacZ/lox}$/*Tcfap2b*$^{-/flox}$/Cre$^{+/-}$). (Private communication from Dr. Erin Bassett, Vision Program, Ottawa Hospital Research Institute).

3.5 Discussion

Previous studies involving AP-2 have demonstrated important roles for this transcription factor in eye development. Numerous studies have demonstrated the requirement for AP-2α in eye development (including the development of the cornea, lens and retina), and its role in ocular disease has also been examined in the literature (Milunsky et al., 2008). AP-2α germline KOs exhibit several ocular defects. Some of these mutants displayed a complete lack of one or both eyes, while others displayed evidence of failed lens induction. In this case, mesenchymal cells occupied the inner region of the optic cup where SE should normally invaginate to form the lens vesicle. The germline KOs that did develop a lens displayed a lens stalk phenotype where the anterior region of the lens remained attached to the overlying SE (West-Mays et al., 1999). The epithelial marker Pax6 and the fiber cell marker MIP26 were also misexpressed in these KOs. AP-2α has been shown to have binding sites on the promoters of both of these genes (Ohtaka-Maruyama et al., 1998; Plaza et al., 1995). AP-2α's role in lens epithelial cell maintenance was also demonstrated when it was ectopically expressed in lens fiber cells causing the formation of small eyes with pockets of multilayered epithelium and lens transition zone defects (West-Mays et al., 2002). Finally the conditional deletion of AP-2α from the lens placode also resulted in corneal defects (Dwivedi et al., 2005) and lens defects (Pontoriero et al., 2008), including a lens stalk, and a reduction in E-cadherin expression; another gene which AP-2α has been shown to interact with (Batsche et al., 1998; Decary et al., 2002; Pontoriero et al., 2008). The lens stalk phenotype observed in

the germline and Le-AP-2α mutants resembles the Pax6 null mouse phenotype , and the human ocular disorder Peter's Anomaly, which is characterized by defects in several genes, including Pax6 (Hanson et al., 1994). Interestingly, AP-2α and Pax6 share similar expression patterns in the lens and their interactions may possibly contribute to these developmental and disease phenotypes.

Although the role that AP-2α plays in eye and lens development is becoming better understood, lens phenotypes in the Le-AP-2α mutants did not present until E12 suggesting another factor may compensate for the loss of AP-2α in earlier stages of lens development (Pontoriero et al., 2008). AP-2α shares a common expression pattern with its family member AP-2β in early lens development. They have been shown to play compensatory roles in retinal development (Bassett et al., 2012). For example, the loss of AP-2α and AP-2β in the retina resulted in a loss of horizontal cells and an altered pattern of expression of amacrine cell markers in the *Tcfap2a/b* double mutants. BHLHB5, normally expressed in postmitotic GABAergic amacrine cells, was absent from the inner nuclear layer of the double mutants at birth (Bassett et al., 2012). An altered pattern of staining of ISL1 and ISL2 (expressed in retinal ganglion cells and cholinergic amacrine cells), and Sox2 (expressed in cholinergic amacrine cells and Muller glia cells), was also observed in the *Tcfap2a/b* mutants (Bassett et al., 2012). Although AP-2α and AP-2β have been shown to play this redundant role in retinal development, possible redundant roles of these transcription factors in the lens were not known. In the current study, we explored the possible compensatory roles of AP-2α and AP-2β in early lens development

through the conditional deletion of both genes in the lens placode beginning at E9.5. Our findings showed that the deletion of AP-2α and AP-2β resulted in a more nasally located lens stalk and more severe lens and corneal defects than those seen in the Le-AP-2α single KOs.

The conditional deletion of both AP-2α and AP-2β in the AP-2α/β DKOs increased the severity of morphological defects seen in the lens compared to those observed in the Le-AP-2α single KO model. Initially, it was thought that a loss of AP-2α and AP-2β at the lens placode stage of development might impede lens development so much that lens induction may fail altogether. After carrying out our studies, we have seen that lens induction does in fact occur, however, for lens development to progress normally, both AP-2α and AP-2β must be expressed. It is hypothesized that AP-2α and AP-2β function downstream of the initial lens specifying signals and therefore do not need to be present for the lens placode to form. However, AP-2α and AP-2β are both likely required to interact with other lens development factors including Pax6 and E-cadherin in order for normal lens development to occur.

Our findings show that AP-2α and AP-2β are both required for proper positioning of the lens within the head. This is evidenced by the locations of the lens stalks observed in the AP-2α/β DKOs. At E13.5, the stalk seen in the DKO lens is located more towards the nasal aspect of the face compared to that observed in the Le-AP-2α KO. The stalk appeared to initiate closer to the lens equator and this resulted in a rotated lens with the epithelium located closer to the corneal stroma. By E15.5, the deletion of both genes

caused a unique lens stalk phenotype. The stalk was located at the lateral aspect of the lens, and was intermeshed with surrounding tissue and continuous with an overlying thin layer of tissue in the region where cornea normally forms. It appears that the deletion of both AP-2 genes resulted in a mispositioned lens at E15.5, and this phenotype was also seen in one DKO lens at P4. This possible mispositioning of the lens can be seen clearly by looking at Pax6 staining at E15.5 and P4. Pax6 normally stains the lens epithelium (Chow and Lang, 2001) and in these lenses, the pattern of epithelial Pax6 staining normally observed at the anterior region of the lens appeared to be rotated laterally 90 degrees. Perhaps the laterally located lens stalk is determining the "anterior" axis in the DKO lens, resulting in the rotated lenses seen at E15.5 and P4.

Pax6 is also expressed in the lens stalk regions at all stages examined. Grimm et al., suggests that the persistent expression of Pax6 in the lens stalk inhibits the lens from developing beyond a rudimentary lens vesicle (Graw, 1999; Grimm et al., 1998). The deletion of both AP-2α and AP-2β may be sequestering lens development at the lens vesicle stage. AP-2 binds to promoters as both homo (AP-2α/AP-2α) and heterodimers (AP-2α/AP-2β) (Williams and Tjian, 1991a). As mentioned previously AP-2 interacts with Pax6 as evidenced by AP-2 binding sites on the Pax6 promoter (Plaza et al., 1995; Sivak et al., 2004). Perhaps AP-2 interacts with Pax6 as an AP-2α/β heterodimer at the lens vesicle stage of development, allowing for complete and proper separation of the lens vesicle from the overlying SE, and the progression of development past the lens vesicle stage toward a fully mature lens. The lack of fiber cell denucleation at E15.5 and

P4, and the expression of Pax6 in the fiber cells also suggest that the DKO lens is remaining in an immature state.

The AP-2α/β DKOs also exhibited severely abnormal corneal morphology. Both AP-2α and AP-2β are expressed within the developing cornea (West-Mays et al., 1999). Previous studies have illustrated the requirement for AP-2α in corneal development. The loss of AP-2α in the Le-AP-2α single KO mutants exhibited corneal epithelial and corneal stroma defects (Dwivedi et al., 2005). These mutants displayed a decrease in stratified layers of the corneal epithelium with disrupted organization and packing of corneal epithelial cells. The corneal epithelium had a thin Bowman's layer that was absent in some regions, leading to defects in the corneal stroma. A decrease in E-cadherin staining within the corneal epithelium was also observed (Dwivedi et al., 2005). The loss of both AP-2α and AP-2β caused even more sever morphological defects in the AP-2α/β DKO cornea. The loss of both genes at P4 gave rise to an intensely disorganized cornea with a stroma more disorganized than that seen in the Le-AP-2α mutants. No clear corneal endothelium was visible in the DKO cornea, and in the most severely disrupted eyes, no clear corneal epithelium was visible.

In an attempt to understand the exacerbated corneal phenotype in the AP-2α/β DKOs, N-cadherin expression was examined. N-cadherin is a cellular adhesion molecule normally expressed in the corneal endothelium (Reneker et al., 2000). The corneal endothelium is a monolayer of hexagonally shaped cells that have a dual function to restrict the flow of fluids into the stroma, and to supply nutrients from the aqueous humor

to the stroma. This is accomplished through the apical junction complex, composed of specialized tight junctions and adherens junctions (Vassilev et al., 2012). The loss of N-cadherin in the corneal endothelium has been shown to lead to stromal edema (due to an unbalanced influx of fluids into the stroma from the anterior chamber of the eye) and epithelial dysgenesis (Vassilev et al., 2012). N-cadherin was expressed in the corneal endothelium of both WT and Le-AP-2α KOs. A loss of N-cadherin expression was observed in the AP-2α/β DKO corneal endothelium. In Vassilev, et al.'s study, in mutants where N-cadherin was deleted from the corneal endothelium, the thickness of the stroma was increased (Vassilev et al., 2012). Similarly, the AP-2α/β DKO stroma appeared thicker than that of WT and Le-AP-2α corneal stroma, and this may be a result of the loss of N-cadherin expression in the DKO corneal endothelium. AP-2α and AP-2β at the placode stage of development appear to be important in maintaining a normal corneal phenotype. The loss of these genes in the DKO resulted in a corneal epithelial region that was thicker than that observed in WT and Le-AP-2α KOs, and also abnormally expressed N-cadherin. N-cadherin was not expressed in the Le-AP-2α KO corneal epithelium, so it is unlikely that N-cadherin is compensating for the loss of E-cadherin (Dwivedi et al., 2005) in this single KO model. It is possible that the cells in this layer of the DKO model are not of true corneal epithelial phenotype, and that AP-2α and AP-2β are important in maintaining N-cadherin expression in the corneal endothelium, maintaining a corneal epithelial cell phenotype, and maintaining overall corneal integrity.

AP-2 has been shown to play roles in cellular proliferation and cell cycle regulation. The proliferative capabilities of AP-2 have been studied in several types of epithelial cells, and results of these studies have indicated that AP-2 can have both promoting and inhibitory roles in cellular proliferation (Braganca et al., 2003; Johnson, 1996; Maytin et al., 1999). In mammary carcinoma cell lines, AP-2α is shown to positively regulate the growth promoting factors TGFα and the ErbB (Wang et al., 1997). However, in breast cancer tissue, AP-2α expression inhibits cellular proliferation even in the presence of an overexpression of ErbB2 (Friedrichs et al., 2005; Pellikainen et al., 2004). In skin development, a loss of AP-2α in the epidermis leads to an increase in proliferation (Wang et al., 2006). This same trend is seen in Le-AP-2α single KOs, where the loss of AP-2α leads to increased proliferation in the corneal epithelium (Dwivedi et al., 2005).

Proliferation was explored in our AP-2α/β DKO model. Although the overall size of the DKO eye is smaller than that of WT and Le-AP-2α eyes, the lens appeared to occupy most of the space where the vitreous humor usually resides. To determine if the loss of AP-2α and AP-2β were causing increased proliferation in the lens, PH3 expression was explored. The loss of AP-2α in the Le-AP-2α single KOs did not appear to increase the amount of proliferation occurring in the lens compared to that of the WT. However, proliferation in the AP-2α/β DKO lens appeared increased over that of the Le-AP-2α mutant lens. Cells expressed PH3 within the DKO lens epithelium at all stages examined, and PH3 expressing cells were also observed in the lens stalks of the double

mutants at all stages. This interesting finding may shed light on the role that AP-2 plays in lens cell proliferation. AP-2α and AP-2β may act redundantly, and AP-2β expression at the placode stage of development is able to maintain normal levels of lens proliferation in the absence of AP-2α in the Le-AP-2α mutants. The loss of both genes in the DKO model resulted in the abnormal patterns of proliferation.

Abnormal cell cycle regulation was also evident in the AP-2α/β DKO model. As with cellular proliferation, AP-2 has also been implicated in regulation of the cell cycle (Hilger-Eversheim et al., 2000; Moser et al., 1997a; Zeng et al., 1997). Cyclin D1 expression in the Le-AP-2α KO and AP-2α/β DKO model was explored. Cyclin D1 is a cell cycle promoting factor necessary for the G1-S phase transition of the cell cycle (Ewen et al., 1993; Hinds et al., 1992). Cyclin D1 is normally expressed in the lens epithelium and at the transition zone of the developing and postnatal lens (Griep, 2006). Cyclin D1 expression in the Le-AP-2α mutants appeared normal; however, Cyclin D1 was expressed aberrantly within the fiber cell regions of the E15.5 and P4 DKO lens indicating that fiber cells in this region that should have exited the cell cycle are transitioning from G1 to S phase. Not surprisingly, $p27^{kip1}$ and $p57^{kip2}$ which were expressed normally in the Le-AP-2α KO, showed an expression pattern in the DKO model that mirrored that of Cyclin D1. These CDKI's are responsible for forming complexes with Cyclin D/cdk4/cdk6 to remove cells expressing these Cyclin/cdk complexes from the cell cycle. It appears that the loss of AP-2α and AP-2β is preventing fiber cells in the DKO model from exiting the cell cycle, and that $p27^{kip1}$ and $p57^{kip2}$

expression is turned on in these cells to attempt to remove them from the cell cycle. These results suggest that AP-2β may have compensated for the loss of AP-2α at the placode stage in the Le-AP-2α KOs, and was able to correctly remove fiber cells from the cell cycle. The loss of both transcription factors does not cause cell cycle arrest at the G1 phase, and cells in the fiber cell region of the DKO lens incorrectly remain in the cell cycle.

Further studies are ongoing to continue to elucidate redundant functions of AP-2α and AP-2β in the early stages of lens development. The current study has illustrated the importance of the presence of both AP-2α and AP-2β at the lens placode stage of development. It appears that these transcription factors both play an important role in allowing the lens to separate away from the SE, and that their absence may be sequestering the lens in a lens vesicle state. Our findings also illustrate the importance of these transcription factors in normal corneal development. Interestingly, the loss of both transcription factors seems to shed light on their cooperative roles in the lens with regard to cellular proliferation and cell cycle regulation.

4 CHAPTER 4 - AP-2α is required after lens vesicle formation to maintain lens integrity

Abstract

AP-2α has been shown to be important in early lens development, and a conditional deletion of AP-2α at the lens placode stage of development results in failed lens vesicle separation from the overlying SE. Although the role of AP-2α in early stages of lens development has been explored, its role during later developmental stages is unknown. To determine the requirement for AP-2α in later stages of lens development, subsequent to lens vesicle separation, this study utilizes the MLR10-cre transgenic line of mice, to conditionally delete AP-2α from the lens epithelium beginning at E10.5. The loss of AP-2α after lens vesicle separation resulted in morphological defects beginning at E18.5. By P4, a small highly vacuolated lens with a multilayered lens epithelium was evident in the MLR10-AP-2α mutants. Epithelial cells were elongated and abnormally expressed fiber cell specific proteins β and γ-crystallins. Cell-cell adhesion between the epithelium and fiber cells, and epithelium and basement membrane of the capsule was disrupted and mutant lenses misexpressed the cell adhesion molecules ZO-1, N-cadherin and β-catenin. Cell death was also observed in the fiber cells of the mutant lens beginning at P4, and in the lens epithelium between P14 and P30, the latter of which led to regions that were devoid of cells. This chapter demonstrates that AP-2α is not only required during early stages of lens vesicle development, but is also required during subsequent stages of lens development in maintaining a normal lens epithelial cell phenotype.

4.1 Introduction

Transcription factors are critical regulators of key developmental processes including cell growth and differentiation and are required for the regulation of other developmentally important genes. Numerous transcription factors are required for the development of different tissues, and each exhibits a characteristic spatio-temporal pattern of expression. In lens development, a hierarchy of transcription factors exists, with Pax6 (Paired box 6) functioning at the top (Chow and Lang, 2001; Lovicu. F.J., 2004). Pax6 is expressed in two waves, with its early phase of expression shown to be critical for correct placode development (van Raamsdonk and Tilghman, 2000). Pre-placodal expression of Pax6 regulates the expression of Sox2 and Six3, which are both important in formation of the lens placode (Ashery-Padan et al., 2000; Chow and Lang, 2001; Huang et al., 2011; Smith et al., 2009). The second wave of Pax6 expression, following early placode development, is important for regulating genes involved in lens vesicle formation (Plageman et al., 2010), including FoxE3 (Brownell et al., 2000). Additional regulators have been identified and shown to play important roles in earlier stages of lens induction, such as the Activating Protein-2 alpha (AP-2α) transcription factor, yet its function(s) in later stages of lens differentiation is less understood.

The AP-2 family of transcription factors includes five highly homologous members (AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε) known to be important in differentiation, cell growth and apoptosis (Eckert et al., 2005; Feng and Williams, 2003; Hilger-Eversheim et al., 2000; West-Mays et al., 2003). AP-2α and AP-2β have been

shown to be co-expressed in a number of ocular tissues during development, including the lens placode (Bassett et al., 2012; Bassett et al., 2007; West-Mays et al., 1999). Previous investigations of *Tcfap2a* germ-line (null) and chimeric mice have shown that AP-2α is an important regulator of early lens development, with germ-line mutant mice exhibiting lens defects reminiscent of mutants in the Pax6 regulatory pathway (Pontoriero et al., 2008; West-Mays et al., 2002). For example, *Tcfap2a* null mice exhibited a range of abnormal ocular phenotypes, including anophthalmia (lack of eyes) and a persistent adhesion of the lens to the overlying SE, forming a lens stalk. Because AP-2α is expressed in both the developing lens placode and the developing neural retina, tissues that provide critical signals to each other during development, it remained unclear whether or not the ocular defects observed in the *Tcfap2a* null mouse were due to the intrinsic loss of AP-2α expression. Furthermore, because of its broader developmental expression pattern, deletion of AP-2α in mice also led to additional developmental defects in the head, including excencephaly as well as a dysmorphogenesis of the developing face (West-Mays et al., 1999; Zhang et al., 1996). Thus, these defects may have contributed, in a non–cell-autonomous manner, to the ocular defects observed in the *Tcfap2a* mutants. To address this, a conditional KO with AP-2α (Le-AP-2α) deleted from the lens placode and its derivatives was created using the lens placode specific LeCre (Ashery-Padan et al., 2000; Pontoriero et al., 2008). Similar to the germline KOs, the conditional Le-AP-2α mutants exhibited lens defects, including a persistent lens stalk, as well as decreased E-cadherin staining in the lens epithelium beginning at E12.5

(Pontoriero et al., 2008). These findings demonstrated an autonomous role for AP-2α in early lens vesicle development.

Following lens vesicle development, AP-2α continues to be expressed in the lens epithelium and its expression ceases at the lens transitional zone where epithelial cells differentiate into fiber cells (West-Mays et al., 1999). Thus, while it is known that AP-2α is required for proper lens vesicle formation, it remained unknown what its requirement in later stages of development may be in maintaining a normal lens epithelial phenotype. The current study examines the role of AP-2α at stages of lens development subsequent to lens vesicle separation. Cre-LoxP technology was employed in conjunction with the MLR10 line of mice (Zhao et al., 2004), which results in the conditional deletion of AP-2α from the lens epithelium after lens vesicle separation. The MLR10-cre transgenic mouse line incorporates a Pax6 consensus binding sequence in the αA-crystallin promoter that drives cre expression to not only the fiber cells, but also the lens epithelium beginning at E10.5 (Zhao et al., 2004). Results demonstrated that the MLR10-AP-2α mutant lenses were smaller than normal, with a vacuolated fiber cell region and a multilayered epithelium. Cells in the mutant lens epithelium were elongated and expressed the fiber cell specific proteins β and γ crystallins. A fragile adhesion of cells was evident between the epithelial/fiber cell and epithelial/capsule borders with an expanded and disorganized zone of ZO-1 staining. Misexpression of cell adhesion molecules β-Catenin and N-cadherin in the fiber cell region of the mutant lens was also observed. Together these findings indicate that AP-2α expression in the lens epithelium is

required subsequent to lens vesicle separation in maintenance of the lens epithelial cell phenotype.

4.2 Materials and Methods

4.2.1 Generation of the MLR10-AP-2α Mutants

To generate a line of MLR10-cre/AP-2$\alpha^{flox/flox}$ mice (further referred to as MLR10-AP-2α mutants), MRL10-cre mice (Zhao et al., 2004) were crossed with AP-2α^{lacZKI} (Brewer et al., 2002) The progeny of this cross (AP-2α^{lacZKI}/MLR10-cre) were crossed with mice homozygous for the *Alflox* allele, which contain LoxP sits flanking the DNA binding and dimerization domain of AP-2α (Brewer et al., 2004), to generate a conditional mutant with AP-2α deleted from the lens epithelium beginning at E10.5 (See supplemental Fig 4.12). PCR for the *MLR10cre* transgene was performed with the primers Cre1 and Cre3 that corresponds to nucleotides 1090-1114 and 1489-1511 of the *Cre-recombinase gene*, respectively. PCR analysis was performed for 35 cycles of 95°C for 45 seconds, 67°C for 45 seconds, and 72°C for 1.5 minutes, to generate a 420 bp fragment. To detect the AP-2α:*LacZKI* allele, PCR genotyping was performed using the forward primer Alpha6/7 and reverse primers Alpha3'KO and IRESUP under the following conditions:35 cycles of 95°C for 45 seconds, 70°C for 45 seconds and 72°C for 1 minute. This generated a 500 bp WT product and a 300 bp *LacZKI* product as outlined in (Pontoriero et al., 2008).

4.2.2 Histology

In the collection of embryonic stages, noon on the day of vaginal plug detection is considered E0.5 of embryogenesis. Whole embryos and postnatal eyes were collected

from MLR10-AP-2α mutants and WT littermates. Embryonic tissue was fixed in 10% neutral buffered formalin overnight at room temperature and then transferred into 70% ethanol until processing. Whole embryo's (E18.5) or postnatal eyes (P4, P14, P30, P48) were processed and embedded in paraffin. Serial sections were cut at a thickness of 4μm and used for Hematoxylin and Eosin staining as well as immunofluorescent analysis as outlined in (Bassett et al., 2012; Kerr et al., 2012) For all stages examined in this study, sample sizes of 3 lenses were stained.

4.2.3 Immunofluorescence

Immunofluorescence was performed on 4μm paraffin sections, using the following primary antibodies: rabbit polyclonal Pax6, Covance (1:50); mouse monoclonal ZO-1, Zymed (1:40); rabbit polyclonal β-Crystallin, rabbit polyclonal γ-Crystallin, all provided by Dr. Samuel Zigler Jr., Chief of lens and cataract biology section of the National Eye Institute (1:200); mouse monoclonal α-sma, Sigma (1:100); mouse monoclonal AP-2α (3B5), Developmental Studies Hybridoma Bank (1:1); mouse monoclonal β-Catenin, BD Transduction (1:100); rabbit polyclonal Collagen IV, Abcam (1:200); mouse monoclonal N-cadherin, BD Transduction (1:100). Fluorescent secondary antibodies were either Alexa Flour 488 (Goat anti-mouse and goat anti-rabbit), or Alexa Flour 568 (goat anti-mouse and donkey anti-goat), Invitrogen-Molecular Probes, used 1:200 for 1 hour at room temperature). Paraffin-embedded sections were deparaffinized in xylene, hydrated (through 100%, 95%, 70% ethanol, followed by

water), treated with 10mM sodium citrate buffer (pH 6.0; boiling for 20 minutes) for antigen retrieval, blocked with normal serum and incubated with primary antibodies overnight at 4°C. Each stain included a negative control with no primary antibody. All stains were visualized with a microscope (Leica, Deerfield, IL) equipped with an immunofluorescence attachment, and all images were captured with a high-resolution camera and associated software (Open-Lab; Improvisation, Lexington, MA).

4.2.4 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

To examine cells undergoing apoptosis within lenses of mutant and WT littermates, TUNEL staining was carried out using the ApopTag® Plus Fluorescein In Situ Apoptosis Detection Kit, Chemicon International. 4μm paraffin-embedded sections were deparaffinized in xylene, hydrated (through 100%, 95%, 70% ethanol, followed by PBS) and treated with 10mM sodium citrate buffer (pH 6.0; boiling for 20 minutes) for antigen retrieval. Proteinase K (20ug/mL) was applied to slides for 15 min at room temperature followed by two washes with PBS. After application of equilibration buffer, slides were treated with working strength TdT enzyme and incubated in a humidified chamber at 37°C for 1 hour. Working strength stop wash buffer was applied to all slides followed by washing in PBS. Slides were treated with Anti-Digoxigenin Conjugate and incubated at room temperature for 30 minutes. Slides were washed in PBS and mounted with Vectashiled mounting medium containing DAPI (Vector Laboratories, Burligame,

CA). TUNEL experiment included a positive control slide treated with DNAse after antigen retrieval, followed by processing with other slides as previously described. All slides were visualized with a microscope (Leica, Deerfield, IL) equipped with an immunofluorescence attachment, and all images were captured with a high-resolution camera and associated software (Open-Lab; Improvisation, Lexington, MA).

4.3 Results

4.3.1 Targeted Deletion of AP-2α in the Lens Epithelium Results in Altered Epithelial and Fiber Cell Morphology

To determine the requirement for AP-2α in later stages of lens development, subsequent to lens vesicle separation, a line of mice possessing a conditional knockout of AP-2α in the lens epithelium beginning at E10.5 (MLR10-AP-2α) was created using the Cre-loxP recombination approach (Gu et al., 1994). Immunofluorescent staining for AP-2α protein expression was carried out in WT and MLR10-AP-2α mutant embryonic lenses to ensure correct deletion of AP-2α from the lens epithelium and the timing of deletion. The majority of lens epithelial cells in the MLR10-AP-2α mutants exhibited an absence in AP-2α expression beginning at E11.5 (Fig. 4.1A, D). At P4, P14 and P30 AP-2α protein expression was seen in the WT lens epithelium (Fig. 4.1B-D), but was absent in the mutant epithelium at equivalent stages (Fig. 4.1E-H). However, a small number of lens epithelial cells in the mutant lens retained AP-2α protein expression due to the mosaic nature of the MLR10 Cre mouse model (Ashery-Padan et al., 2000) (Fig. 4.1F-G, dashed circles).

Histological analyses of the MLR10-AP-2α mutant mice were carried out at multiple developmental stages. Lens development in the MLR10-AP-2α mutants proceeded normally prior to E18.5. However, defects became apparent at E18.5 in the mutant lens and persisted throughout postnatal development. At E18.5, small vacuoles

were present in the anterior portion of the fiber cell compartment (Fig. 4.2E) and at later stages of postnatal development the vacuoles were large and occupied more of the fiber cell compartment (Fig. 4.2F-H). The size of the mutant lens also appeared smaller than that of WT mice at all postnatal stages examined (Fig 4.2B-D and F-H).

In comparison to the WT lens epithelium, which consisted of a monolayer of cuboidal shaped epithelial cells (Fig. 4.2I), the epithelial cells in the MLR10-AP-2α mutant lens appeared elongated in shape beginning at P4 (Fig. 4.2L). At this stage, small pockets of multilayered epithelial cells were also observed in the mutant epithelium, a phenotype which was exacerbated as postnatal development progressed (Fig. 4.2L, circles). Nuclei were observed in fiber cell regions of the mutant lens at P14 and P30 in an area that is normally devoid of nuclei suggesting a problem with fiber cell denucleation in the MLR10-AP-2α mutant lens (Fig. 4.2M, N, black arrows). By P30, this uniform spacing of nuclei was disrupted in the mutant lens epithelium, with large gaps observed between nuclei in this region (Fig. 4.2N, inset and Fig 4.5F, dashed lines).

Beginning at P4 and persisting throughout postnatal development, adhesion at the epithelial-fiber cell interface and at the epithelial-capsule interface was disrupted in the MLR10-AP-2α lens in areas where detachment was evident (Fig. 4.2M, black arrows). Immunostaining for Col IV, a component of the basement membrane of the lens capsule (Kelley et al., 2002), further illustrated disrupted adhesion between the epithelium and the capsule at P4, P14 and P30 (Fig. 4.3D-F, insets within figure), with some instances of the capsule breaking away from the mutant epithelium as seen in the lens at P14 (Fig.

4.3E, inset) These findings suggest that a loss of AP-2α in the lens epithelium subsequent to lens vesicle separation causes weakened adhesion between cells of the lens epithelium and lens capsule.

Since we had observed areas that lacked cells in the epithelial region of the MLR10-AP-2α mutant lens, TUNEL staining was carried out to determine any aberrant pattern of programmed cell death. The mutant lens epithelium did not exhibit any abnormal levels of cell death at P4, however, TUNEL staining was seen in the fiber cell region of the mutant lens and this was not observed in the WT lens (Fig. 4.4C, white arrows). By P14, cells in the central lens epithelium (Fig. 4.4D, white arrows), as well as at the transition zone (not shown) were also found to stain positive for TUNEL in the MLR10-AP-2α mutant lens, as compared to no labeled cells in the WT lens (Fig. 4.4B). Some cells between the lens epithelium and lens capsule also labeled positive for TUNEL staining (Fig. 4.4D, yellow arrow). Cell death was also again observed in the fiber cell region of the mutant lens at P14 (Fig 4.4D, inset).

4.3.2 Altered Expression Pattern of Lens Epithelial and Fiber Cell Markers in the MLR10-AP-2α Mutant Lens

Pax6 is a developmentally important protein that is necessary for proper lens development. At E12.5, Pax6 expression is known to be confined to the lens epithelium and maintained here throughout the remainder of embryonic and postnatal development. In the MLR10-AP-2α mutants during embryonic development Pax6 was detected in the

lens epithelium, similar to that observed in WT lenses. At P4, Pax6 was also observed to have a normal expression pattern in the epithelium of the mutant lens (Fig. 4.5D). However, at P30 Pax6 expression was observed in the fiber cell compartment, an area normally devoid of Pax6 expression (Fig. 4.5E, F). Gaps between Pax6 expressing cells were also evident in the mutant lens at P30, further illustrating a loss of epithelial cells in this region (Fig. 4.5F, dashed lines).

E-cadherin is a Ca^{2+}-dependent cell-cell adhesion molecule that is expressed within the developing and postnatal lens epithelium (Braga, 2000; Larue et al., 1996; Nose and Takeichi, 1986; Xu et al., 2002). AP-2α has been shown to bind to the E-cadherin promoter and our laboratory has shown that a loss of AP-2α during early lens placode development resulted in a decrease in E-cadherin mRNA and protein expression in the Le-AP-2α mutant lens (Pontoriero et al., 2008). We therefore examined E-cadherin protein expression in the MLR10-AP-2α mutants using immunofluorescence. No difference in the expression of E-cadherin was observed in the mutant lens compared to their wild-type littermates during embryonic and postnatal development (Fig. 4.6C-D). N-cadherin, is an additional Ca^{2+} dependent cell adhesion molecule expressed in the developing and postnatal mouse lens. At postnatal stages, N-cadherin is normally expressed in the epithelial cells and newly differentiated fiber cells at the transition zone of the lens. Mature fiber cells in the centre of the lens do not normally express this cell adhesion molecule (Xu et al., 2002). We had noted that the morphological phenotype of the MLR10-AP-2α mutant lens greatly resembled that of N-cadherin KO mouse lenses

(Pontoriero et al., 2009). We therefore examined N-cadherin expression in the MLR10-AP-2α mutant lens. N-cadherin expression was observed in the lens epithelium and fiber cells of the transitional zone in WT lenses at P4, P14 and P30 (Fig. 4.7A-C), whereas N-cadherin expression was absent in the central mature fiber cell region of the WT lens (Fig. 4.7B-C). Like WT mice, N-cadherin expression in MLR10-AP-2α mutant lens was detected in the epithelium and transitional zone at P4, P14 and P30 (Fig. 4.7D-F). However, unlike WT littermates, N-cadherin continued to be expressed in the central fiber cell region of the mutant lens at P14 and P30 (Fig. 4.7E, F).

β-catenin is normally expressed in the lens epithelium and required for epithelial cells to maintain their proliferative ability and initiate fiber cell differentiation and polarity. However, β-catenin is no longer required in fiber cells that have begun to elongate and differentiate in the cortex of the lens (Cain et al., 2008). We also examined β-Catenin expression and although it was detected in its normal pattern in the lens epithelium and at the transition zone of both the WT and MLR10-AP-2α mutant lens (Fig. 4.8A-C) its expression had aberrantly expanded into the posterior fiber cell compartment of the mutant lens at all postnatal stages examined (Fig. 4.8D-F).

In the developing vertebrate lens, αA-crystallin, β-crystallin, and γ-crystallin are all expressed in differentiating fiber cells. βB1-crystallin and γ-crystallin are markers of fiber cell development, elongation and differentiation and are normally expressed in the fiber cell region of the lens, and not within the lens epithelium (Andley, 2007; Lovicu. F.J., 2004; Robinson and Overbeek, 1996). Since the shape of the lens epithelial cells in

the MLR10-AP-2α mutants appeared elongated beginning at P4, βB1-cyrstallin expression was analyzed. During all stages of embryonic development, βB1-crystallin was expressed normally in the MLR10-AP-2α mutants (not shown). However, at P4 βB1-crystallin was not only expressed within the fiber cell region of the mutant lens, but was also detected in the lens epithelium (Fig. 4.9D), unlike WT lenses in which βB1-crystallin was absent from the lens epithelium (Fig. 4.9A). By P14, the expression of βB1-cyrstallin in the mutant lens epithelium appeared even stronger than at P4, and this expression persisted in the mutant lens epithelium at P30 (Fig. 4.9E-F). No βB1-crystallin expression was observed in the WT lens epithelium at equivalent stages (Fig. 4.9B-C). To determine if other fiber cell specific crystallins were also expressed in the mutant lens epithelium, γ-crystallin localization was explored at P4 and P14. Similar to βB1-crystallin expression, γ-crystallin was also detected in the mutant lens epithelium at these stages (Fig. 4.9H, J), while the lens epithelium of WT littermates at equivalent stages was devoid of γ-crystallin expression (Fig. 4.9G, I).

4.3.3 ZO-1 Misexpression in the MLR10-AP-2α Mutant Lens Indicates Altered Polarity

Tight junctions mediate adhesion between lens epithelial cells and indicate the cell polarity of lens epithelial and fiber cells (Cain et al., 2008; Shin et al., 2006). Due to the altered morphological shape and multilayered nature of the epithelial cells in the MLR10-AP-2α mutant lens, ZO-1 expression was examined during early and later stages

of postnatal lens development. ZO-1 is a tight junction protein that is normally expressed in the apical region of lens epithelial cells at the epithelial-fiber cell interface and its expression is confined to a tight domain spanning from the proliferative zone of the lens to the equatorial region (Nielsen et al., 2003). In WT lenses ZO-1 was expressed normally at the apical aspect of the lens epithelium and was properly localized between the proliferative zone and the lens equator at P4, P14 and P30 (Fig. 4.10A-C). In comparison in the mutant lens although ZO-1 expression was observed at the epithelial-fiber cell interface, it appeared weaker than in the WT lens, and its distribution more diffuse (Fig. 4.10D-F). ZO-1 was also observed to have strong punctate expression in the central fiber cell region around the vacuoles within the fiber cell region of the mutant lens and this was not seen in the WT lens (Fig. 4.10E, inset).

α-smooth muscle actin (α-SMA) expression is indicative of cells transitioning from an epithelial to a mesenchymal (EMT) phenotype and it is not normally expressed in the lens. Abnormal expression of α-SMA in the lens is observed during anterior subcapsular cataract formation, and this is correlated with a multilayered epithelium (Lovicu et al., 2004). Due to the multilayering seen in our mutant epithelium we examined α-SMA in the MLR10-AP-2α mutants to determine if the cells had undergone EMT. At P48, a large region of multilayered epithelium was examined for α-SMA expression (Fig. 4.11D, white star), and revealed no α-SMA expression. These findings demonstrate that EMT is not occurring in the lens epithelium of the MLR10-AP-2α mutant mice.

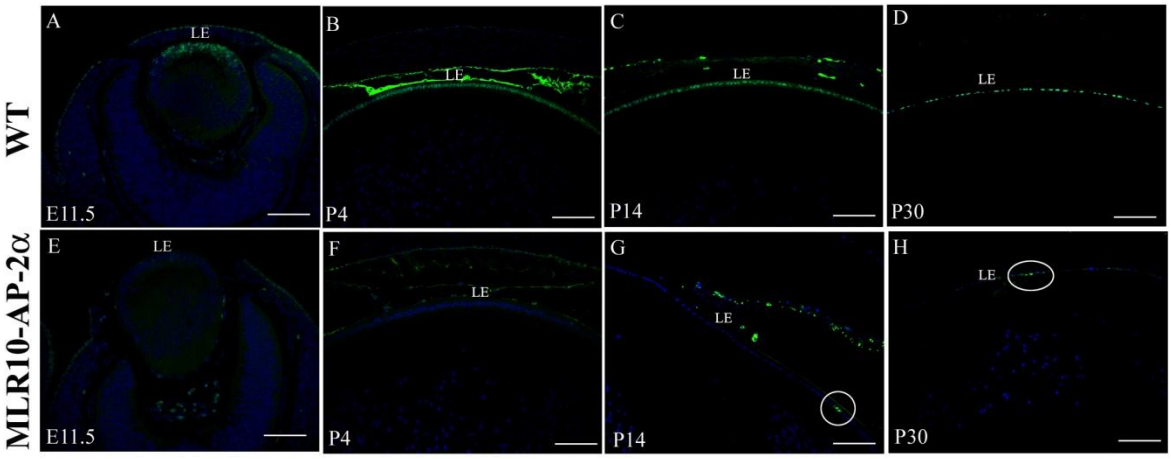

Figure 4.1 Deletion of AP-2α from Lens Epithelium.
WT lenses (A-D) show nuclear staining of AP-2α in the lens epithelium, while MLR10-AP-2α mutants (E-H) show lack of AP-2α protein expression. The MLR10cre transgenic line of mice works in a mosaic nature, and thus dashed circles display some cells that retain AP-2α expression. Scalebars represent 100μm.

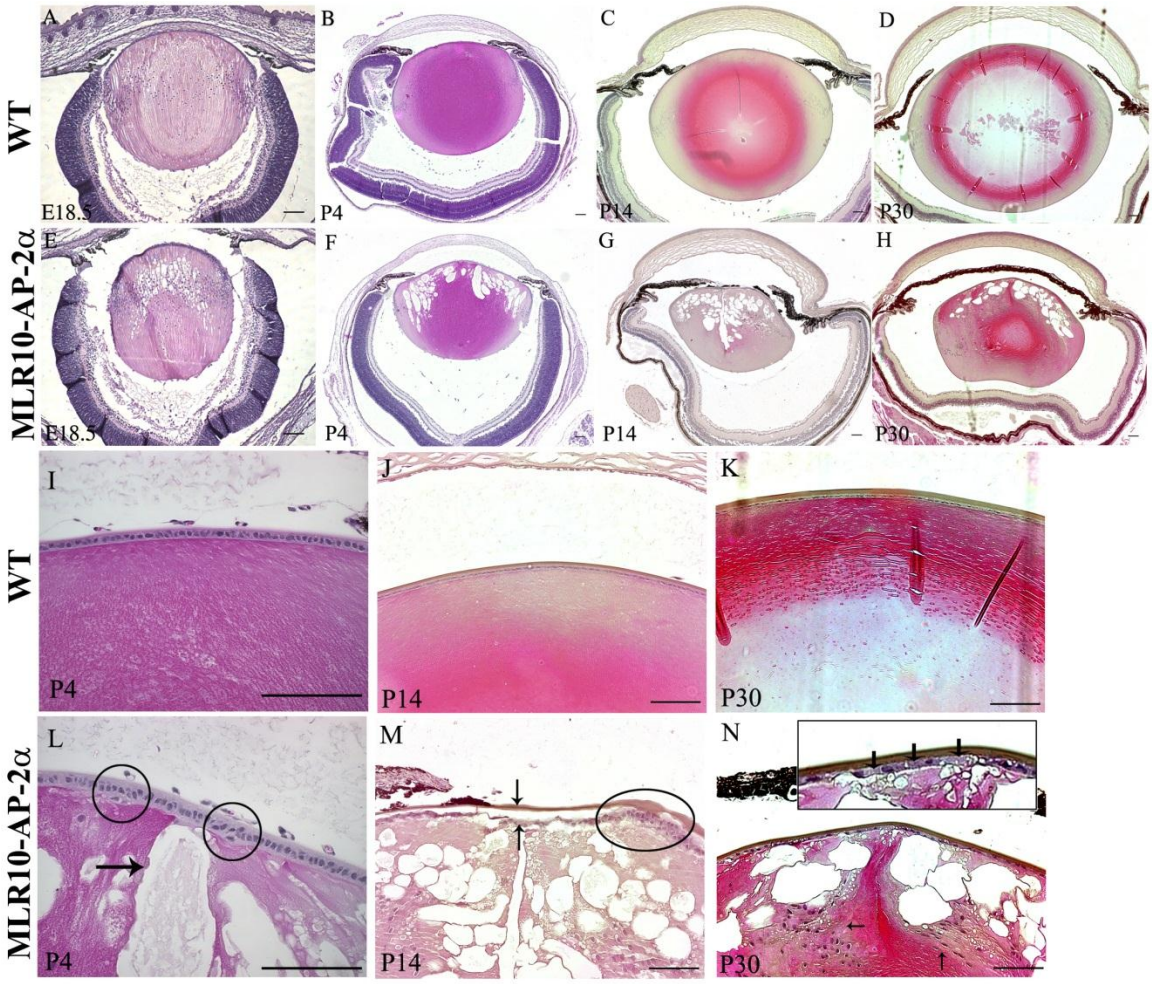

Figure 4.2 Deletion of AP-2α from lens epithelium leads to morphological abnormalities and defects in lens integrity.

Defective lens morphology in the mutant lens (E-H) begins at E18.5 with small vacuoles that become larger and persist throughout postnatal development (E-H. black arrow in L). Mutant lenses (E-H) are smaller in size than WT lenses (A-D) after E18.5. Epithelial cells are elongated at P4 (L) than in WT littermates (I). Multilayering is evident in the mutant lens epithelium at all stages (L, M, dashed circle). Adhesion of cells at epithelial-fiber cell and epithelial-capsule interfaces is fragile (M, double arrow). By P30, the uniform spacing between epithelial cells is lost, with large gaps between epithelial cells present (N, inset, black arrows). All scalebars represent 100μm.

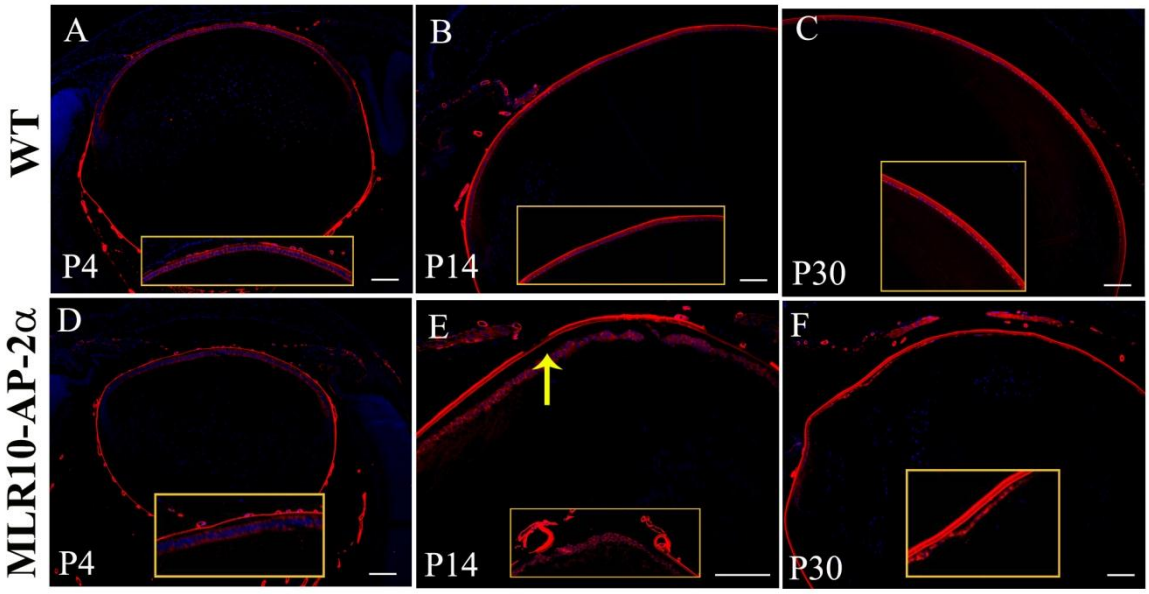

Figure 4.3 Normal Collagen IV expression in mutant lens capsule.
Collagen IV is expressed normally in the capsule of WT (A-C) and mutant (D-F) at P4, P14 and P30. Collagen IV staining clearly illustrates loss of adhesion between epithelium and capsule (D-inset, E-yellow arrow, F-inset). Staining also illustrates breakages of the capsule with the epithelium protruding outwards (E-inset). All scalebars represent 100μm.

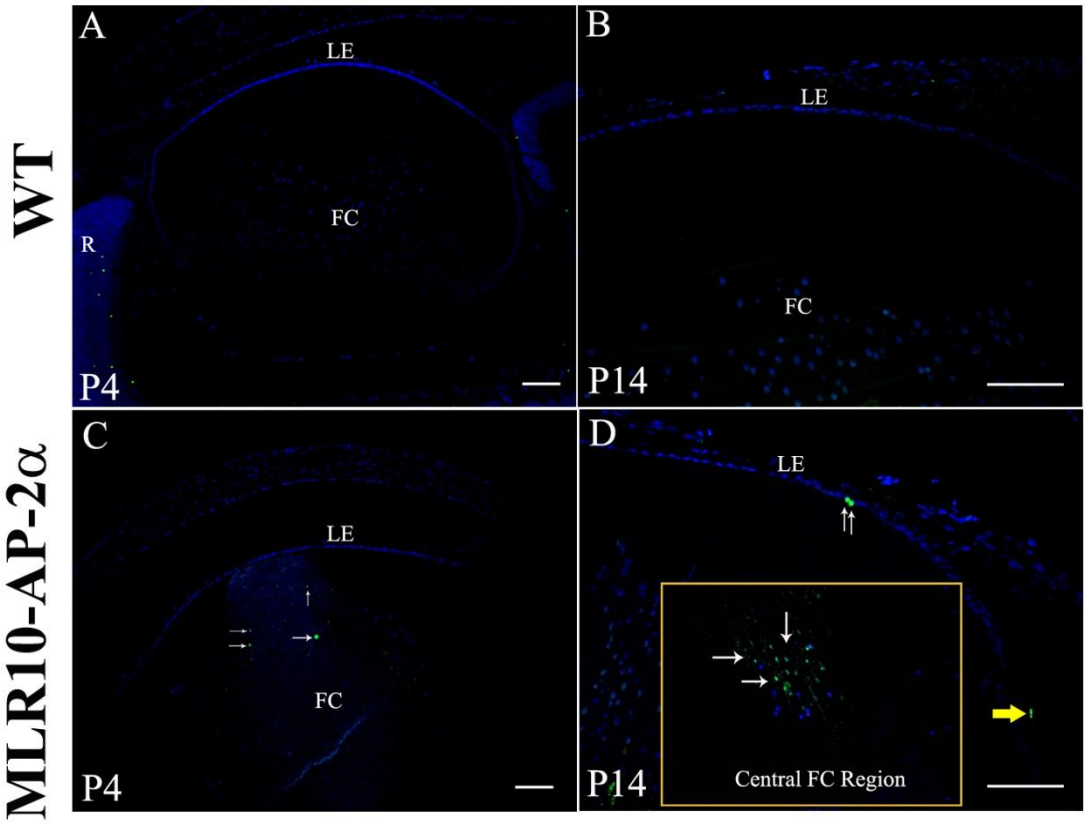

Figure 4.4 MLR10-AP-2*α* mutants display abnormal TUNEL staining in Fiber Cells and Lens Epithelium at P4 and P14.

Cells in the fiber cell region of the mutant lens display abnormal amounts of apoptosis at P4 and P14 (C, D white arrows) compared to WTs at equivalent stages (A,B). Aberrant lens epithelial apoptosis is also observed in the central epithelium and transition zone/capsule area at P14 (D, white/yellow arrows). All scalebars represent 100μm.

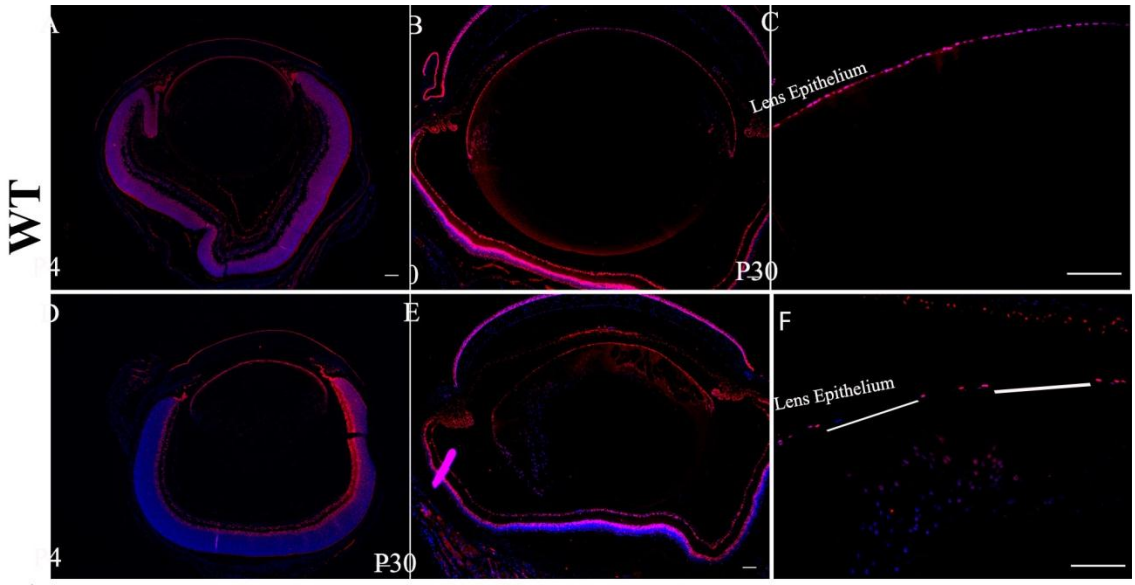

Figure 4.5 Pax6 expressed in lens epithelium of mutant lenses.
WT (A-C), and mutant (D-F) lenses express Pax6 in its normal spatial location in the lens epithelium at P4 (A,D) and P30 (B-C, E-F). Gaps between epithelial cells in the mutant lens are visible here (F, dashed line). All scalebars represent 100µm.

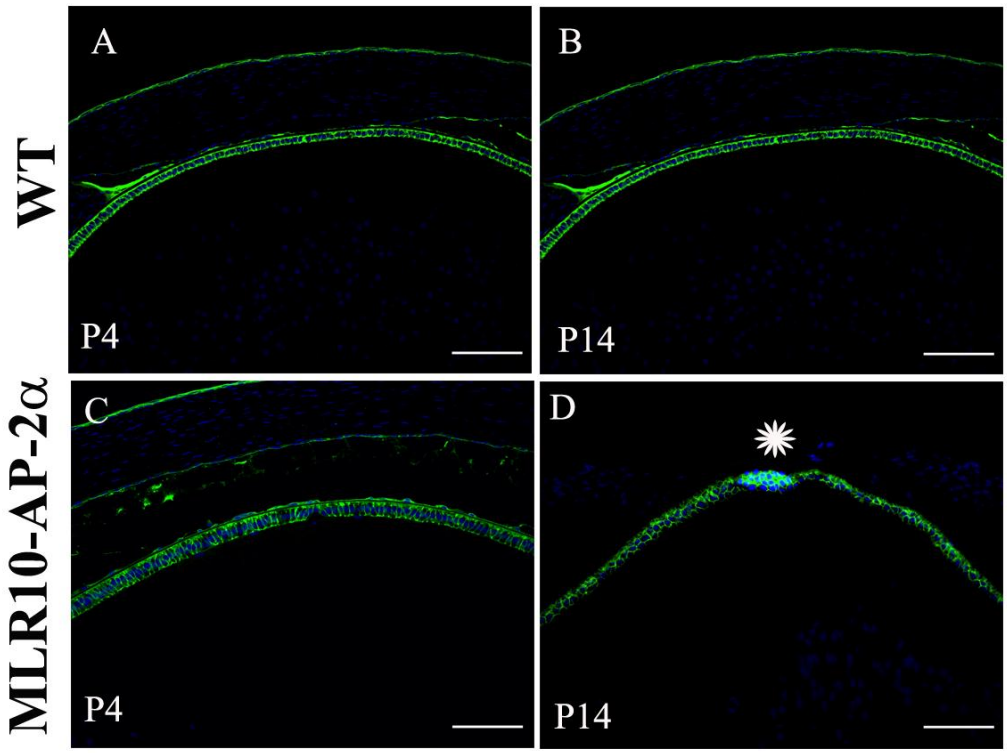

Figure 4.6 Deletion of AP-2α in lens epithelium subsequent to lens vesicle separation does not affect E-cadherin expression.
WT (A,B) and mutant (C,D) lenses express E-cadherin normally in the lens epithelium at P4 (A,C) and P14 (B,D). E-cadherin expression is maintained even in regions of multilayering in mutant lenses at P4 and P14 (C, D, white star indicates pocket of multilayered epithelium in P14 lens). All scalebars represent 100μm.

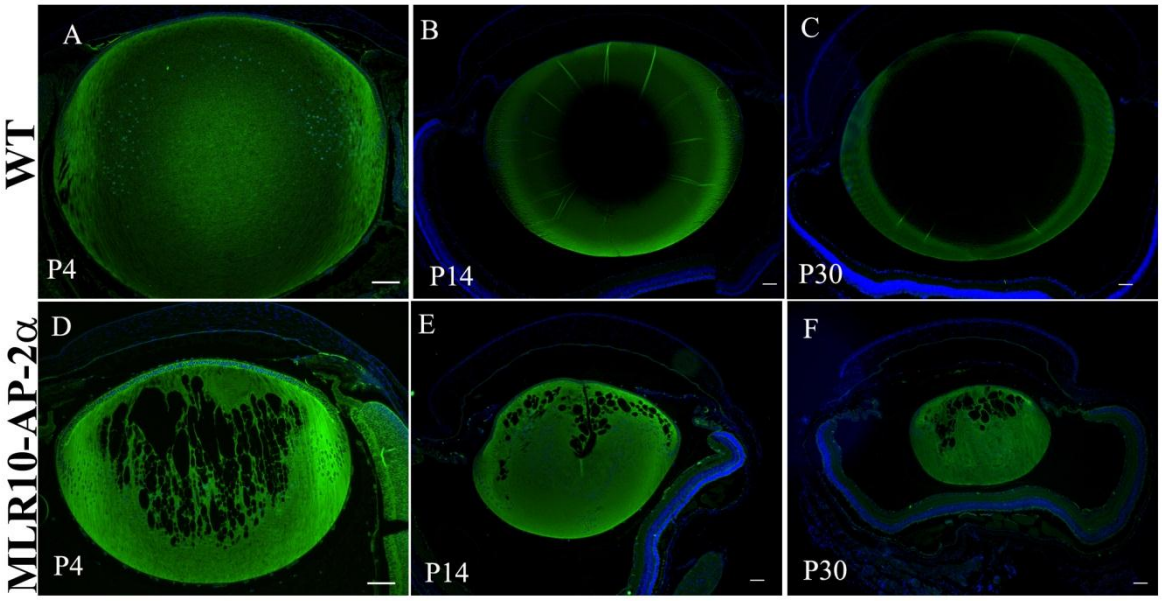

Figure 4.7 MLR10-AP-2α mutants maintain N-cadherin expression in central cortex of fiber cells.

N-cadherin is expressed in WT lenses (A-C) in the lens epithelium and at the lens transition zone at P4, P14 and P30. No N-cadherin expression occurs in the mature fiber cell region of WT lenses at P14 and P30 (B,C). Mutant lenses (D-F) express N-cadherin in lens epithelium and transition zone (D-F), but maintain expression in central fiber cell region (E-F). All scalebars represent 100μm.

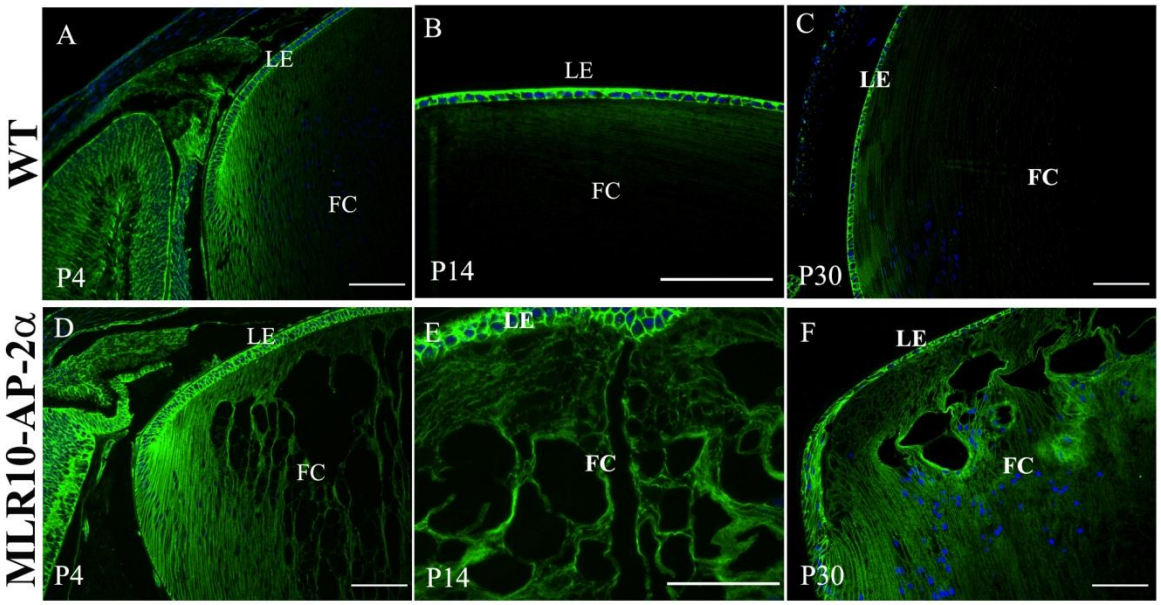

Figure 4.8 β-Catenin expression maintained in posterior fiber cells of MLR10-AP-2α lens.

WT lenses express β-Catenin in the lens epithelium at lens transition zone (A-C). Expression is maintained here in mutant lenses, though expression also occurs in posterior fiber cell compartment (D-F). All scalebars represent 100μm.

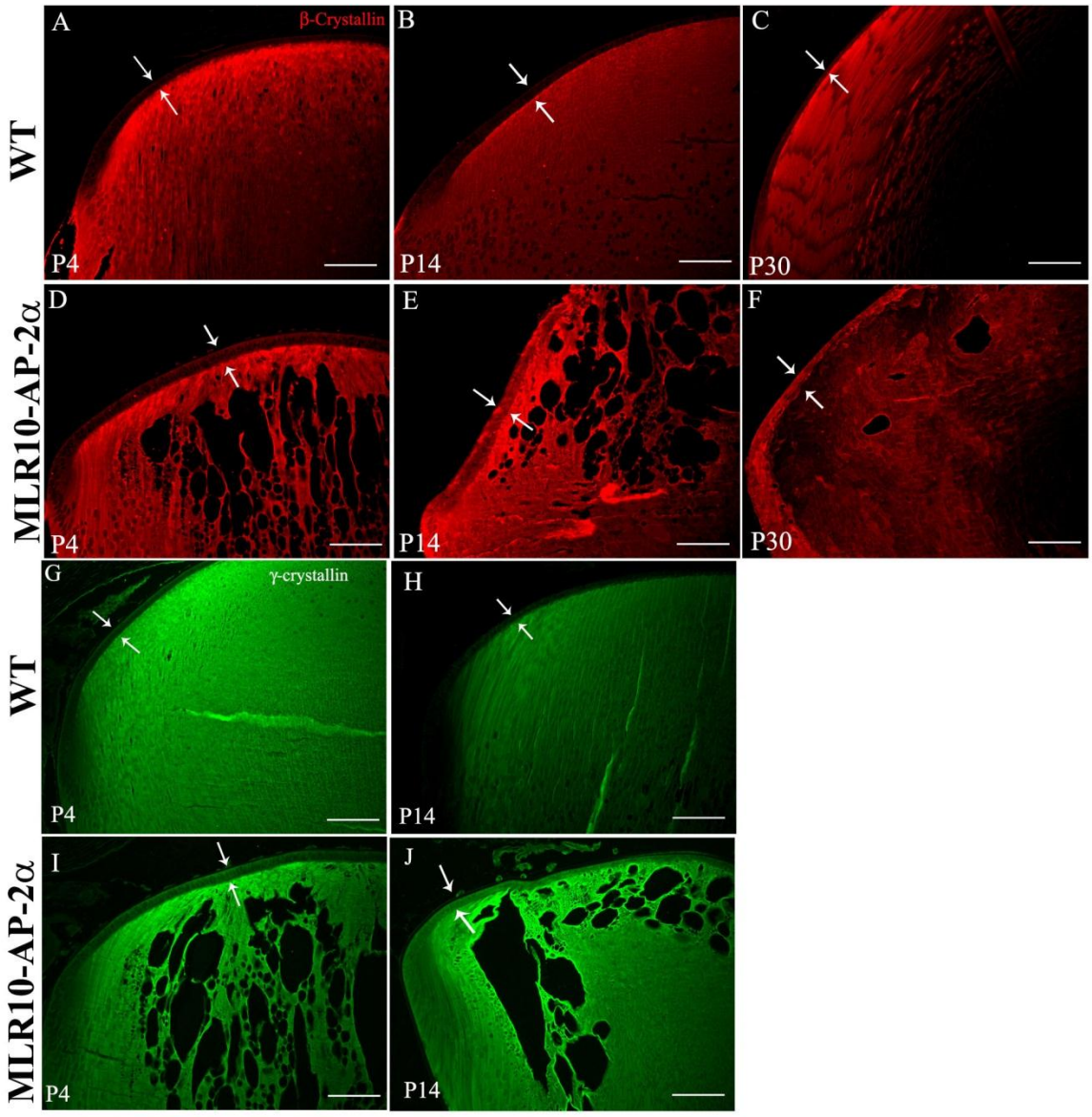

Figure 4.9 βB1-Crystallin and γ-Crystallin is expressed in mutant lens epithelium. WT lenses (A-C) illustrate βB1-Crystallin expression in fiber cells, but lack expression in the epithelium at P4, P14, and P30 (A-C, white arrows indicate position of lens epithelium). βB1-Crystallin expression is present in mutant lens epitheliums (D-F). γ-Crystallin expression lacks in WT lens epithelium at P4 and P14 (G, I), while some γ-Crystallin is expressed in the mutant. All scale bars represent 100μm.

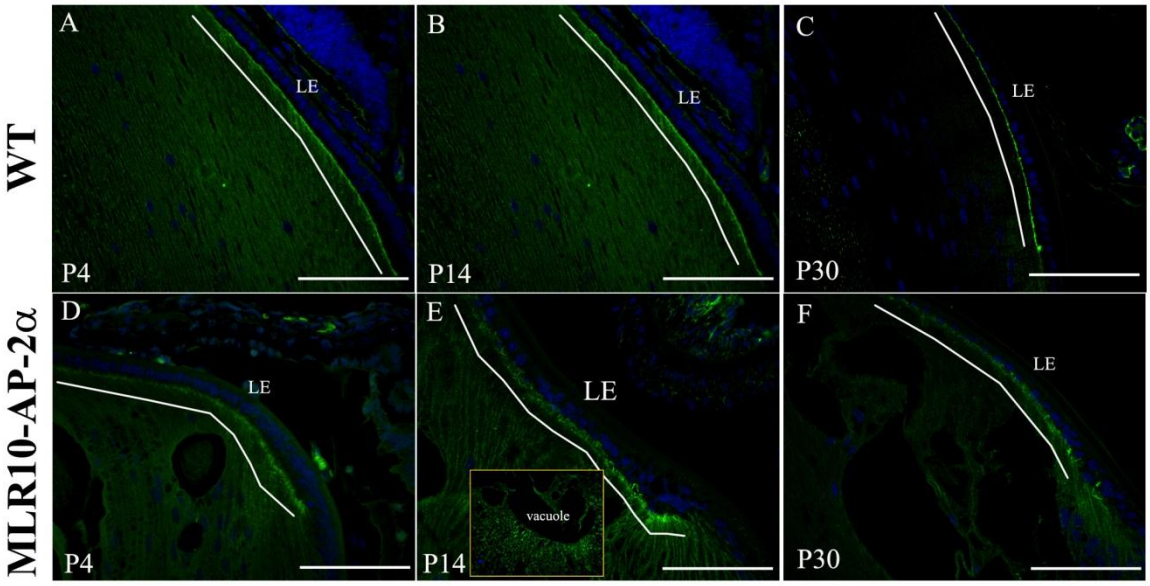

Figure 4.10 ZO-1 expression is irregular in MLR10-AP-2α mutant lens.
ZO-1 expression in WT (A-C) lenses is confined to a tight band at the apical region of the lens epithelium (A-C, white dashed line) at P4, P14 and P30. ZO-1 is expressed at the apical region of the mutant lens epithelium, however, expression appears weaker and more diffuse (D-F, dashed white line). ZO-1 stains strongly around vacuoles at P14 (E, inset). All scalebars represent 100μm.

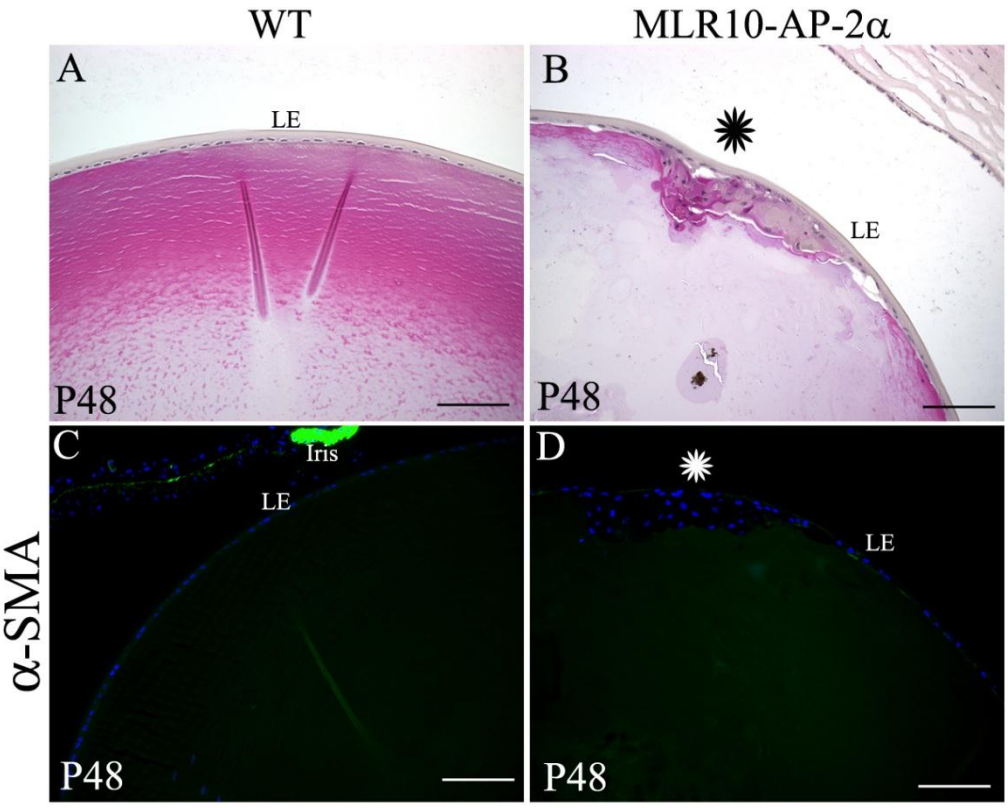

Figure 4.11 Mutant lenses do not form anterior subcapsular cataracts.
H&E staining of P48 mutant lens illustrates large plaque like multilayered region of epithelium (B, black star). Staining with α-SMA in WT (C) and mutant lenses (D) reveals no positive expression of α-SMA in the multilayered mutant epithelium (D, white star). Positive control α-SMA staining is seen in the iris in WT (C) eye. All scalebars represent 100μm.

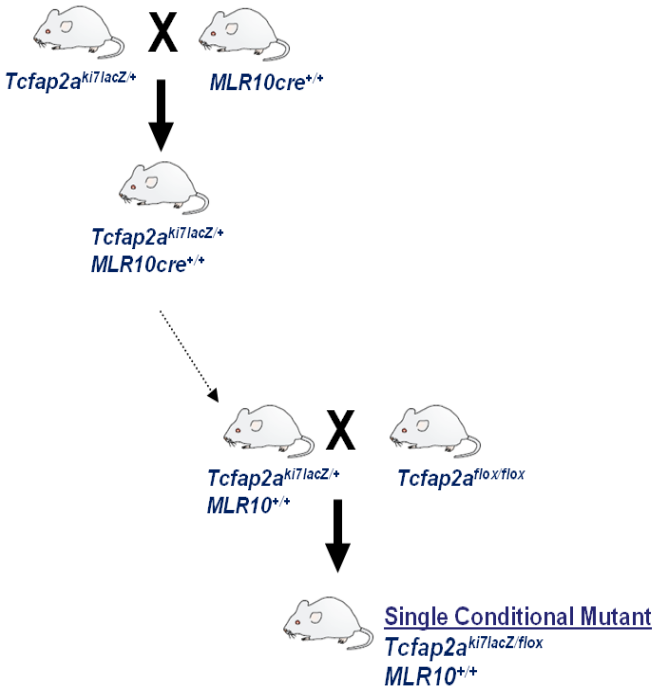

Figure 4.12 Generation of the AP-2α/MLR10 mutants.
A series of mouse breedings were carried out to generate mice of genotype *Tcfap2a*$^{KI7lacZ/flox}$/MLR10Cre$^{+/+}$. *Tcfap2a*$^{KI7lacZ/+}$ were first crossed with homozygous MLR10-cre mice to generate cre positive offspring with one allele of AP-2α deleted. The *Tcfap2a*$^{KI7lacZ/+}$/MLR10Cre$^{+/+}$ offspring were then crossed with our *Tcfap2a*$^{flox/flox}$ mice to generate mice with both alleles of AP-2α deleted from the lens beginning at E10.5 (*Tcfap2a*$^{KI7lacZ/flox}$/MLR10Cre$^{+/+}$). (Private communication from Dr. Erin Bassett, Vision Program, Ottawa Hospital Research Institute).

4.4 Discussion

An earlier role for AP-2α in lens development, and specifically in separation of the lens vesicle from the overlying placodal tissue had been revealed previously using a conditional deletion of AP-2α in the lens placode (Le-AP-2α) (Pontoriero et al., 2008). Since the role of AP-2α in later stages of lens development and differentiation are not well understood, we utilized the MLR10-Cre transgenic line of mice to target AP-2α expression in the lens at stages subsequent to lens vesicle separation. The MLR10-Cre transgenic line uses the αA-crystallin promoter with the incorporation of a Pax6 consensus sequence to drive cre expression into both the epithelium and fiber cell compartments of the lens beginning at E10.5 (Zhao et al., 2004). Using this system AP-2α was successfully deleted from the lens epithelium at E10.5, allowing for the elucidation of its roles in lens epithelial cell development and maintenance. Deletion of AP-2α in the lens epithelium, subsequent to vesicle separation, resulted in altered epithelial cell architecture accompanied by morphological defects in the fiber cell compartment of the lens. Importantly, fiber cell specific crystallins were also aberrantly expressed in the mutant lens epithelium. Together these data suggest that AP-2α is required to maintain an epithelial cell phenotype in the lens.

By day 4 of postnatal development lens epithelial cells in the MLR10-AP-2α mutant lens had taken on a more elongated cellular shape than those of wild-type littermates. Previous studies have shown that changes in epithelial cell shape can be the result of defects in both cell polarity and cell-cell adhesion (Rivera et al., 2009). In

agreement with this, in the MLR10-AP-2α mutant lens the expression of ZO-1, a tight junction protein involved in cell polarity, appeared to have a lower level of expression and was organized more diffusely at the apical region of epithelial cells. Morphological assessment also revealed disrupted adhesion at the epithelial-fiber cell interface. Interestingly, no changes in the expression of E-cadherin were observed in the MLR10-AP-2α mutant lenses from this study despite the fact that previous studies in which AP-2α was conditionally deleted from the lens at the placode stage of development (E9.5) (Le-AP-2α), did revealed a reduction in expression of E-cadherin (Pontoriero et al., 2008). Microarray data further showed that E-cadherin was differentially expressed in the Le-AP-2α mutants, and quantitative chromatin Immunoprecipitation (ChIP) experiments showed that AP-2α directly interacts with the E-cadherin promoter (Pontoriero et al., 2008). The difference in the finding between the mutant models is not surprising given that transcription factors are known to be required to regulate a set of genes at one particular stage of development, but no longer needed at another. For example, Pax6 has been shown to be required for the regulation of crystallin genes during early stages of lens development, but no longer required to regulate their expression during later stages of embryogenesis (Shaham et al., 2009). Thus, the change in shape of the lens epithelial cells in the MLR10-AP-2α mutant lens may be more attributed to the altered ZO-1 localization.

The epithelium of the MLR10-AP-2α mutant lens also exhibited regions of multilayering. Multilayering in the lens epithelium can be indicative of an EMT of lens

epithelial cells, which is observed in the formation of anterior subcapsular cataracts. This multilayering is typically accompanied by the expression of α-SMA along with a loss of epithelial specific proteins such as Pax6 and E-cadherin (Hales et al., 1994; Lovicu et al., 2004; Rungger-Brandle et al., 2005; Schmitt-Graff et al., 1990) However, no α-SMA expression was observed in the multilayered regions of the MLR10-AP-2α mutant lens and E-cadherin and Pax6 expression were normally expressed. Previous studies in flies have shown that a loss of normal cell adhesion contributes to a multilayered epithelium (Bilder and Perrimon, 2000). Thus, the multilayering of the epithelium in our mutant lens is likely the result of altered polarity or adhesion rather than a transformation of the cells.

It was also observed that lens epithelial cells in the postnatal MLR10-AP-2α mutant lens aberrantly expressed the fiber cell specific proteins βB1-crystallin and γ-crystallin suggesting that these cells had taken on characteristics of fiber cells. The change in expression of these crystallins may have been directly related to the loss of AP-2α in these cells. Although the direct, negative regulation of the crystallin promoters by AP-2 has not been previously reported, earlier work suggests that AP-2 interacts with other proteins known to repress crystallin expression, such as Pax6. Duncan et, al., have shown a requirement for Pax6 in the repression of the βB1-Crystallin gene (Duncan et al., 1998) and further demonstrated that β-crystallin expression in the lens epithelium is inhibited when Pax6 is upregulated in this region (Duncan et al., 1996). Interestingly, AP-2α has been show to physically interact with Pax6 and cooperate in the regulation of gene expression in ocular tissues (Sivak et al., 2004). Thus, it is possible that AP-2α may

inhibit the expression of crystallins in the lens epithelium through the interaction with other regulators. It has however been shown that the deletion of Pax6 alone in the lens epithelium is not sufficient to cause increased expression of β-crystallin in the lens epithelium as we have observed in our mutant (Shaham et al., 2009). Thus, unlike Pax6, the expression of AP-2α in the lens epithelium may be necessary to suppress β-crystallin expression.

The requirement for correct AP-2α expression in the lens has also been demonstrated in a study in which AP-2α was ectopically expressed in fiber cells (West-Mays et al., 2002). Under control of the αA-crystallin promoter, the AP-2α gene, whose expression is normally confined to the lens epithelium, was ectopically expressed in the differentiated lens fiber cell compartment. Ectopic AP-2α expression resulted in defects that included the inability of newly formed fiber cells to migrate normally in the transitional zone, along with inhibition of fiber cell denucleation and reduced expression of the fiber cell-specific protein MIP26 (West-Mays et al., 2002). These data are in line with findings of the current study in which loss of AP-2α in the lens epithelium of the MLR10-AP-2α mutants results in an altered epithelial phenotype with some features of fiber cells. Thus, together these two separate models support the role for AP-2α in maintenance of a lens epithelial cell phenotype and as a negative regulator of fiber cell differentiation.

The conditional loss of AP-2α subsequent to lens vesicle separation, specifically in lens epithelial cells, also resulted in fiber cell defects. Epithelial markers including

Pax6 and N-cadherin are not normally expressed in mature fiber cells (Cain et al., 2008; Xu et al., 2002), but were expressed at P14 and P30 in the fiber cells of the MLR10-AP-2α mutants. β-catenin is not required in fiber cells that have begun their differentiation and elongation processes (Cain et al., 2008) but was also observed in the mutant lens fiber cells. Together, this evidence suggests that the fiber cells in MLR10-AP-2α mutants had remained in an immature state. AP-2α is not normally expressed in fiber cells, with expression ceasing at the transitional zone of the lens where epithelial cells differentiate in to fiber cells. Thus, the MLR10 cre used in this study only targeted AP-2α expression in the lens epithelium. As a result, the fiber cell defects observed in MLR10-AP-2α mutant lens must have occurred as a consequence of the epithelial cell defects. We had observed that adhesion at the epithelial cell-fiber cell interface was disrupted in the mutants. Adhesion between fiber cells and epithelial cells has been shown to be important in regulating fiber cell elongation and maturation as illustrated in chick studies (Beebe et al., 2001). Thus, it is possible that the abnormal fiber cell-epithelial cell contact in the MLR10-AP-2α mutant lens may have altered the proper maturation of fiber cells and ultimately caused the aberrant fiber cell death observed.

In summary, the current study has shown that AP-2α is not only required during early stages of lens placode development but is also important for regulating later stages of morphogenesis of the lens. In particular we have shown that targeted deletion of AP-2α in the lens epithelium at stages subsequent to lens vesicle separation resulted in a loss of epithelial cell architecture and aberrant expression of fiber cell proteins. These

findings demonstrate that AP-2α is important in maintaining a normal lens epithelial cell phenotype and that in its absence features of fiber cell differentiation appear.

5 CHAPTER 5 - General Discussion

Hh and AP-2 signaling in the regulation of lens development

In striving to continually further the field of vision research, scientists are studying signaling pathways, expression patterns and the interactions of genes and transcription factors to elucidate their roles in eye development and disease. The studies carried out within this thesis have examined some of the lesser understood pathways and transcription factors involved in lens development. Although the importance of midline Shh signaling in the development of the ocular fields has been well documented, Hh signaling in the lens, including the ability of the lens to respond to these signals were much less understood. The AP-2 family of transcription factors has been shown to play key regulatory roles in eye and lens development; however where they act within the hierarchy of lens development regulators were not well understood. AP-2α has been shown to be important in early lens development, but its role in later stages of lens development was not clear. Compensation for an early loss of AP-2α by other AP-2 family members expressed in the lens was an additional facet that needed to be addressed. In carrying out the experiments within this thesis, key roles of both the Hh pathway and AP-2 signaling in lens development were addressed. Interestingly, this thesis illustrates that Hh and AP-2 signaling share a primary critical developmental role; that being the maintenance of a normal epithelial phenotype in the development of the ocular lens. Additionally, this thesis has shown that defects in both Hh signaling and AP-2 signaling results in deregulation of the lens cell cycle.

Excessive Hh signaling results in sustained epithelial cell development and cell cycle deregulation

Shh, Ihh and Dhh all play important developmental roles in embryonic development (Bitgood et al., 1996; Echelard et al., 1993). Hh signaling occurs when the Hh ligand binds to its receptor Ptch. This in turn causes Smo to become localized to the primary cilium in preparation to activate downstream signaling events (Huangfu and Anderson, 2006). The zinc finger transcription factors Gli1, Gli2 and Gli3 work to activate (Gli1/2) and repress (Gli3) transcription and translation of Hh target genes including the cell cycle promoting factors Cyclin D and Cyclin E (Huangfu and Anderson, 2006; Varjosalo and Taipale, 2007). Hh signaling has been studied in several models including cavefish, zebrafish, newt and mice. Overexpression of Hh signaling in zebra fish results in suppression of lens formation (Barth and Wilson, 1995; Cornesse et al., 2005; Dutta et al., 2005). Normal Hh signaling has been shown to be important in lens regeneration in the newt (Tsonis et al., 2004). Excessive midline Shh signaling in cavefish has been demonstrated to lead to lens and subsequent eye degeneration (Yamamoto and Jeffery, 2000; Yamamoto et al., 2004). In addition, a loss of midline Shh signaling in *Shh* KO mice results in cyclopia (Chiang et al., 1996).

Although these studies illustrated the importance of correct Hh signaling in regulating lens-specific gene expression and lens development, the effects of Hh signaling, especially an upregulation of Hh signaling, specifically in the lens had not been examined. The first study in this thesis (Chapter 2) utilized the SmoM2 mutation in conjunction with the early Crect transgene, to create the constitutive activation of smo in

the head SE and lens beginning at E8.75. This study illustrated the ability of the lens to respond to altered smo activity and resulted in lens defects including sustained epithelialization of cells in the fiber cell region and abnormal activation of the lens cell cycle.

The constitutive activation of smo in the head SE and lens, beginning at E8.75, in the activated smo mutants resulted in the adhesion of the anterior portion of the lens to the SE between E12.5 and P0. The first defect in lens protein expression to be observed was the ectopic expression of FoxE3 at E12.5. The expression of FoxE3 at this stage is normally confined to the lens epithelium, but was instead expressed in both the anterior and posterior regions of the lens in the activated smo mutants. Previous studies have shown that the ectopic expression of FoxE3 in lens fiber cells results in fiber cells that retain an epithelial phenotype (Landgren et al., 2008). Subsequent to the ectopic FoxE3 expression, Pax6 was also ectopically expressed with an expanded domain of expression in the lens epithelium as well as abnormal expression in the fiber cell region beginning at E15.5. These results illustrate that normal Hh signals in the lens are required to maintain a normal lens epithelium.

Interestingly, the results of chapter 2 of this thesis were corroborated by studies presented recently at the 2013 ARVO international conference. The De Iongh lab presented data illustrating a role for correct Hh signaling in early lens development for normal lens epithelial cell maintenance (De Iongh, 2013). These studies showed for the first time, the expression of Hh signaling pathway components Ptch1, Gli2 and Gli3 in

the WT lens epithelium at E12.5. This study examined the effects of a loss of smo from the lens at the lens placode stage of development and demonstrated the necessity for normal Hh signaling in the lens between E9.5 and E13 (De Iongh, 2013). The loss of smo expression at E9.5 resulted in a massive reduction of FoxE3 expression. These mutants also displayed decreased PH3 staining in the lens epithelium between E14.5 and E16.5 and epithelial cell death, demonstrating that the early loss of smo in the lens resulted in a loss of epithelial cells (De Iongh, 2013).

Therefore, chapter 2 of this thesis, in conjunction with new studies presented at ARVO 2013, illustrated for the first time, the importance of normal Hh signaling in the lens and in the maintenance of a normal epithelial phenotype. Lens cell cycle defects were also observed in both models described. The activated smo mutants presented in this thesis demonstrated abnormal G2-M phase transitions, as an increase in PH3 positive cells in both the epithelium and fiber cell region was observed. The loss of smo resulted in decreased PH3 expression in cells of the lens epithelium (De Iongh, 2013). Therefore, normal levels of Hh signaling are critical in early lens development for the maintenance of the lens epithelium by regulating FoxE3 expression, and are also required to maintain tight regulation of the lens cell cycle to ensure lens development progresses as it should.

The human disorder Gorlin Syndrome (also known as BCNS) is caused by a Ptch1 receptor mutation that results in uninhibited cell growth and proliferation (Hahn et al., 1996; Maity et al., 2005; Taylor et al., 2006). This disease results in cancer, but also presents with congenital cataracts and clouding of the cornea (Lueder and Steiner, 1995;

Ortega Garcia de Amezaga et al., 2008). Future studies can utilize the SmoM2-Crect model to study aspects of Gorlin Syndrome in a lens specific animal model and can be used to examine FoxE3 expression to help determine if an activation of FoxE3 is responsible for the ocular defects observed with this disease.

Roles for AP-2 in Lens Development

With ongoing research, particularly in our laboratory, the role that AP-2 plays in eye and lens development is becoming better understood. Germline AP-2α KO studies have illustrated a role for AP-2α in the development of several tissues and organs, including the eye (Schorle et al., 1996; West-Mays et al., 1999). AP-2α germline KO mice died perinatally and exhibited extreme morphological defects including cranio-abdominoschisis, abnormal structure of the face, skull, brain and cranial ganglia, increased apoptosis in the brain and ocular defects (Schorle et al., 1996; West-Mays et al., 1999). These findings provided evidence that AP-2α is required for early development of the lens vesicle. The AP-2α KO mice presented with a range of phenotypes from a complete lack of eyes (anophthalmia), to a persistent adhesion of the lens vesicle to the overlying SE (lens stalk) and the misexpression of Pax6 and in lens epithelial cells and fiber cells respectively. These mutants also exhibited optic cup defects (West-Mays et al., 1999). As a result of the extreme craniofacial abnormalities in the AP-2α germline KOs, it was unknown whether the eye and lens defects in these mutants resulted due to secondary defects or if AP-2α played a cell-autonomous role in

the development of the lens. To answer this question, a lens placode specific conditional KO (Le-AP-2α) was created with *Tcfap2a* deleted from the lens placode beginning at E9.5 (Pontoriero et al., 2008). These conditional KO mice displayed abnormalities beginning at E12, including a lens stalk and reduced expression of E-cadherin, a cell adhesion molecule important in the maintenance of a lens epithelial cell phenotype. These results provided insights into the cell autonomous requirement for AP-2α during lens development. Interestingly, unlike the AP-2α germline KOs, the Le-AP-2α KOs did not have optic cup defects, further demonstrating tissue specific roles for AP-2α in early lens development (Pontoriero et al., 2008). Ectopic expression of AP-2α in lens fiber cells has provided further evidence that a normal pattern of expression of AP-2α is required for lens epithelial cell maintenance. These mutants (αA-AP-2α) displayed a lens epithelium with pockets of multilayered cells illustrating the importance of correct AP-2α expression in lens epithelial cell development (West-Mays et al., 2002).

Realizing that lens defects did not begin until E12 in the Le-AP-2α placode conditional KOs, it remained unknown whether or not another AP-2 family member may compensate for the loss of AP-2α in early lens placode development (Pontoriero et al., 2008). AP-2α and AP-2β are coexpressed in cells of the lens placode (E9.5), and lens pit (E10.5). AP-2β expression is lost from the lens at E11.5 while AP-2α remains expressed in this region throughout development (Bassett et al., 2012; West-Mays et al., 1999). Therefore, the second study in this thesis (Chapter 3) examines the possible redundant roles of AP-2α and AP-2β at the placode stage of development through the creation of a

conditional AP-2α/β DKO mouse model. These mutants exhibited more severe defects than seen in the Le-AP-2α single KO model, including increased cell proliferation and lens cell cycle defects, a turned lens, and extreme corneal defects which illustrated the necessity for correct expression of both AP-2α and AP-2β at the lens placode stage of development.

AP-2α and AP-2β are both required at the placode stage of development

A placode specific conditional KO model with a deletion of both *Tcfap2a* and *Tcfap2b* was created using Cre-loxP technology to assess the possible redundant roles of these AP-2 family members in early lens development. The Le-AP-2α single KO model revealed lens defects including a lens stalk and decreased expression of E-cadherin (Pontoriero et al., 2008). However, these defects were not seen until E12, around the time at which AP-2β expression is lost from the lens, while AP-2α remains expressed (Bassett et al., 2012; West-Mays et al., 1999). In the Le-AP-2α single KO model, the lens placode stage of development was not disrupted, indicating that AP-2α alone did not have an intrinsic role in the specification of the lens placode (Pontoriero et al., 2008). Chapter 3 of this thesis attempted to understand the importance of AP-2β in early lens development, including any role it may have in lens placode specification. Would the loss of AP-2α and AP-2β cause failed lens placode development?

Although the AP-2α/β DKO mutants had phenotypes that were more severe than those seen in the Le-AP-2α model, as well as some new phenotypes specific to this DKO

model, lens formation did progress beyond the placode stage, indicating that the loss of AP-2α and AP-2β at E9 was not sufficient to inhibit placode formation. The LeCre transgene is not activated until E9 and future studies could be geared toward examining both AP-2α single and AP-2α/β DKO models using a cre that is expressed in the placode earlier. One possibility is to use the Crect cre from chapter 2 of this thesis to drive early (E8.75) cre expression to the SE and its derivatives (including the lens). Future studies should also examine earlier stages of AP-2α/β DKO's to examine for any subtle abnormalities in lens placode development, which may have contributed to the defects seen beginning at E13.5.

The AP-2α/β DKOs exhibited a lens stalk that appeared to be located more toward the nasal aspect of the face than seen in the Le-AP-2α KO model. Interestingly, Pax6 was found to be expressed within the region of the lens stalk. The persistent expression of Pax6 in the lens stalk has been shown to inhibit the lens from developing beyond the lens vesicle stage (Grimm et al., 1998). AP-2α has already been shown to be required for lens vesicle separation (Pontoriero et al., 2008). In the single mutants however, Pax6 expression in the lens stalk region is retained, but still appears to follow the tight zone of expression within the lens epithelium (Pontoriero et al., 2008). In the DKO model however, the zone of Pax6 expression within the lens stalk region is expanded. AP-2 has binding sites on the Pax6 promoter (Sivak et al., 2004). Pax6 and AP-2α have also been shown to interact in corneal maintenance and repair (Sivak et al., 2004). Sivak, et al., demonstrated that Pax6 regulates the activity of the transcriptional promoter for

Gelatinase B. This promoter has two Pax6 binding sites, and is regulated by the direct binding of one Pax6 to a Pax6 response element on the promoter, and the indirect binding of a second Pax6 through an interaction with AP-2α to regulate corneal repair and corneal maintenance (Sivak et al., 2004). AP-2α and Pax6 have also been shown to cooperate during lens development, as mice heterozygous for both AP-2α and Pax6 exhibit more severe defects than mice heterozygous for either gene alone (Makhani et al., 2007). Although we know that AP-2 and Pax6 cooperate during lens development, we do not understand how they cooperate. AP-2 binds DNA as either a homo or heterodimer, and it is perhaps the heterodimer form (AP-2α/AP-2β) that is required to interact with Pax6, to downregulate its expression in the lens stalk, allowing lens vesicle separation to take place. Co-immunoprecipitation assays could be carried out in the future to determine the AP-2 dimer type functioning at the lens vesicle stage of development to determine if the loss of both AP-2α and AP-2β is preventing the inhibition of Pax6 in the lens stalk, and thus inhibiting lens vesicle separation from occurring.

Examination of the AP-2α/β DKO model also illustrated new phenotypes not seen in the Le-AP-2α single KO model. Cellular proliferation and cell cycle regulation were altered in the DKO model but normal in the single KO model compared to WT littermates. More cells in the epithelial region of the DKO lenses were found to express PH3 and Cyclin D1. Additionally cells in the fiber cell region that are normally transcriptionally silent were also seen to proliferate and express Cyclin D1. CDKI's $p27^{kip1}$ and $p57^{kip2}$ were also ectopically expressed throughout the lens, most likely

attempting to remove those cells aberrantly expressing Cyclin D1 from the cell cycle. These new phenotypes seen in the DKO model illustrate that AP-2β may compensate for a loss of AP-2α at the lens placode stage of development in the single mutants, and that the expression of these transcription factors are crucial in the regulation of the early lens cell cycle. Future studies could examine if AP-2 acts as an α/β heterodimer to regulate cellular proliferation.

Additionally, the variability exhibited between mutants in the AP-2α/β DKO model requires further examination. Some DKO lenses exhibited severe misplacement within the optic cup, while other mutant mice displayed bilateral anophthalmia. A possible explanation for this variability may be due to the multiple genetic backgrounds of mice used to generate our DKO model. Further breedings are required to place this model on the same genetic background to determine if the variability observed is due to these mixed genetic backgrounds, or a result of the loss of AP-2α and AP-2β from the lens at the placode stage of development. Microphthalmia and anophthalmia does occur a small percentage of the time when using C57 black mice for breeding (used in this project). In the future, to definitively determine the cause of the bilateral anophthalmia and microphthalmia seen in the DKO model, another mouse strain, such as FVB could be used for breeding. Alternatively, similar defects are seen in BOFS, and further investigation of the BOFS mutant mice already being examined in our lab may reveal whether or not mutations in AP-2α that affect their ability to dimerize with other AP-2s including AP-2β may result in both microphthalmia and anophthalmia. Additionally, in

humans, mutations in the *Sox2* gene leading to the production of non-functional Sox2 protein results in SOX2 Anophthalmia Syndrome and also results in microphthalmia (Fantes et al., 2003). An examination of Sox2 expression in the DKO model should also be examined in the future to determine if a loss of AP-2α and AP-2β at the placode stage is effecting Sox2 expression contributing to the observed anophthalmia and microphthalmia seen in our mutants. To date, anophthalmia has only been observed at later postnatal stages of development suggesting that the loss of AP-2α and AP-2β may be affecting downstream gene activity leading to the eventual degeneration of the eye over time.

AP-2α is required for normal lens epithelial cell maintenance subsequent to lens vesicle separation

With a better understanding of the role of AP-2α, and its close family member AP-2β in early stages of lens development, the role of AP-2α during later stages of lens development, subsequent to lens vesicle separation, remained unknown. AP-2α has been shown to play important roles in lens epithelial cell maintenance (Pontoriero et al., 2008; West-Mays et al., 2002; West-Mays et al., 1999) and therefore, the third study (Chapter 4) of this thesis set out to determine if AP-2α is also required in the lens at later stages subsequent to lens vesicle separation, and if so, what is its role in the maintenance of a correct lens epithelial cell phenotype?

Through an examination of the MLR10-AP-2α line of mice, with a conditional deletion of *Tcfap2a* from the lens epithelium after lens vesicle separation, I have shown

that AP-2α is in fact required not only in the early stages of lens development, but is also required later, to maintain a normal lens epithelial cell phenotype.

The MLR10-AP-2α mutants exhibited a multilayered epithelium with elongated epithelial cells, altered epithelial cell polarity and defects in cell adhesion at the epithelial-fiber cell and epithelial-capsule interfaces. Defects in both cell polarity and cell-cell adhesion have been reported to result in elongated lens epithelial cell shape (Rivera et al., 2009), and it was seen that a loss of AP-2α after lens vesicle separation resulted in defects in both cell polarity and cell-cell adhesion. Expression of the cell adhesion and cell polarity marker ZO-1 at the apical region of the mutant lens was diffuse and sparse suggesting that defects in epithelial cell polarity and adhesion lead to the elongated epithelial cells in the MLR10-AP-2α mutants. AP-2α appears to play a role in lens cell adhesion and has already been shown to have binding sites on the E-cadherin promoter (Pontoriero et al., 2008). It appears that AP-2α in some way regulates the expression ZO-1 after lens vesicle separation and its absence ultimately results in abnormal epithelial cell architecture due to breakdowns in the pathways that regulate lens cell polarity and adhesion. The type of interaction that AP-2α has (if any exists) with adhesion molecules important in lens development including ZO-1 or integrins is not known. To determine a possible direct interaction with these proteins at stages subsequent to lens vesicle separation, microarray and ChIP analysis on the MLR10-AP-2α mutants could be carried out. In striving to learn more about the exact placement of AP-2 in the genetic cascade of regulators important in lens development, the results

yielded from these experiments would increase our understanding of the interaction of AP-2α with other adhesion molecules after lens vesicle separation, and help us understand in greater depth how AP-2α works in the lens to maintain a normal epithelial phenotype.

The most striking phenotype in our model was the expression of the fiber cell specific proteins βB1-crystallin and γ-crystallin in the mutant lens epithelium. To date, AP-2α has not been shown to directly regulate β/γ-crystallin gene expression. AP-2α has been shown to genetically interact with the Pax6, and their association has been shown to regulate gene expression in ocular tissues (Sivak et al., 2004). Interestingly, Pax6 is also known to inhibit β-crystallin expression in the lens epithelium (Duncan et al., 1996). However, when Pax6 is absent from the lens epithelium after lens vesicle separation, β-crystallin expression is not induced in this region (Shaham et al., 2009). Therefore, it may be possible that prior to lens vesicle separation, when Pax6 is expressed in the entire lens, it is directly inhibiting β-crystallin expression as outlined by the Duncan lab (Duncan et al., 1996). However, after lens vesicle separation, AP-2α may be the key factor interacting with the β-crystallin promoter to downregulate its expression in the lens epithelium. AP-2α has been shown to play different roles in different tissues at different stages of development, and this finding helps further elucidate the specific roles that AP-2α plays in the lens during early and later stages of its development. Future studies should be aimed at determining this possible direct interaction between AP-2α and β-

crystallin in the lens epithelium to continue to understand its roles in the transcriptional activation and/or repression of important genes in lens development.

Fiber cell defects including a lack of fiber cell denucleation, as well as the expression of epithelial markers Pax6, N-cadherin and β-catenin were also seen in the MLR10-AP-2α mutants. AP-2α has been implicated previously in fiber cell development. When AP-2α was ectopically expressed in lens fiber cells, abnormal migration of newly formed fiber cells at the transition zone along with decreased expression of the fiber cell marker MIP26 and a lack of fiber cell denucleation were observed (West-Mays et al., 2002). Chapter 4 of this thesis illustrated that a loss of AP-2α in the epithelium resulted in an altered epithelial phenotype with these cell exhibiting fiber cell characteristics, including the expression of fiber cell specific crystallins in the elongated epithelial cells. Together, this data show that AP-2α is necessary to maintain the lens epithelial phenotype as well as acts as a negative regulator of fiber cell differentiation.

The examination of the MLR10-AP-2α mutants have allowed us to gain a better understanding of the differing roles that AP-2α plays during early vs. later stages of lens development, and is shedding light on how it works to regulate different genes at these different stages of development to allow proper lens development to progress. E-cadherin expression in the Le-AP-2α conditional placode KO mice was down regulated in the lens epithelium. However, when E-cadherin expression was examined in the MLR10-AP-2α mutants, it was found to be expressed normally. As mentioned above, it

was illustrated that although Pax6 has been shown to inhibit β-crystallin expression in the early lens epithelium, it is not required after lens vesicle separation for the regulation of this fiber cell specific gene (Duncan et al., 1996; Shaham et al., 2009). Similarly, it is possible that although AP-2α regulates E-cadherin expression at the lens placode stage of development, other regulators may play a role in regulating its expression after lens vesicle separation and this has been illustrated in this thesis.

Closing Statement

This thesis has demonstrated roles for normal Hh and AP-2 signaling in lens development, and has shed some light on the placement of both Hh and AP-2 within the genetic hierarchy of transcription factors and regulators involved in lens development (See Fig. 5.1). Hh signaling has been shown to be important in lens development between E9.5 and E13 (De Iongh, 2013). Abnormal Hh signaling (through the constitutive activation of smo) just prior to the lens placode stage of development led to abnormal lens development and deregulated expression of important lens development regulators including FoxE3 and Pax6. This thesis illustrated that abnormal Hh signaling in the early stages of lens development first results in abnormal FoxE3 expression, and later results in aberrant Pax6 expression. This suggests that Pax6 may not work alone to regulate FoxE3 expression in lens development. The work of this thesis has demonstrated that AP-2 may work in a parallel pathway with Pax6 at the placode stage of development and that an AP-2α/β heterodimer may be required to interact with Pax6 in

these early stages to allow the lens to progress through the lens vesicle stage and separate away from the SE. AP-2α has already been shown to interact with E-cadherin at the placode stage of development (Pontoriero et al., 2008). Perhaps at this early stage, AP-2α is required to interact with both Pax6 and E-cadherin simultaneously to ensure lens vesicle separation occurs normally. This thesis has also demonstrated a potential interaction of AP-2α with the β-crystallin promoter at the lens vesicle stage of development to inhibit its expression in the lens epithelium (Figure 5.1).

Mutations in genes required for eye development often result in ocular defects associated with congenital birth defects. Continual examination of the genes associated with these defects provides insights into their normal developmental roles, and how mutations in these genes lead to disease phenotypes. Hh and AP-2 signaling are both required for normal lens development. As mentioned previously, both mutations in Hh and AP-2 have been implicated in the human diseases Gorlin Syndrome and BOFS respectively (Hahn et al., 1996; Maity et al., 2005; Milunsky et al., 2008; Taylor et al., 2006). Studying lens specific mouse models of these disorders will greatly expand our understanding of the specific roles of Hh and AP-2α in the ophthalmic pathology observed in their respective diseases. Although a lens specific mouse model of Gorlin Syndrome has not yet been created, a lens specific mouse model of BOFS has been, and the ocular phenotypes associated with the disease are currently being studied in the West-Mays lab. As mentioned previously, BOFS can results from a deletion of one copy of the *TFAP2A* gene, or from a missense mutation in the DNA binding domain of the *TFAP2A*

gene (Milunsky et al., 2008). The latter of the mutations results in more severe phenotypes indicating a dominant negative phenomenon, where mutant AP-2α protein may dimerize with other WT AP-2α proteins, and possibly other AP-2s such as AP-2β, resulting in defects seen in BOFS patients. Preliminary data from the examination of the BOFS dominant negative mice in our lab illustrate similarities to lens defects seen in both AP-2α KO mice, and more severe defects seen in the AP-2α/β DKO mice presented in this dissertation including microphthalmia, Primary Aphakia (missing lens) and mispositioning of the lens. The BOFS and AP-2α/β DKO models should be further studied and compared to determine the non-cell autonomous roles of the dominant negative AP-2α allele in BOFS, and the possibility that these severe defects may be occurring in these patients due to the interactions of dominant negative AP-2α with WT AP-2α and AP-2β in lens development.

In this thesis, the requirement for normal Hh signaling and AP-2α/β expression in early lens development has been demonstrated. The role of AP-2α in later stages of lens development, subsequent to lens vesicle separation has also been illustrated, and future studies examining these signaling events will continue to determine their specific roles in lens and eye development and disease.

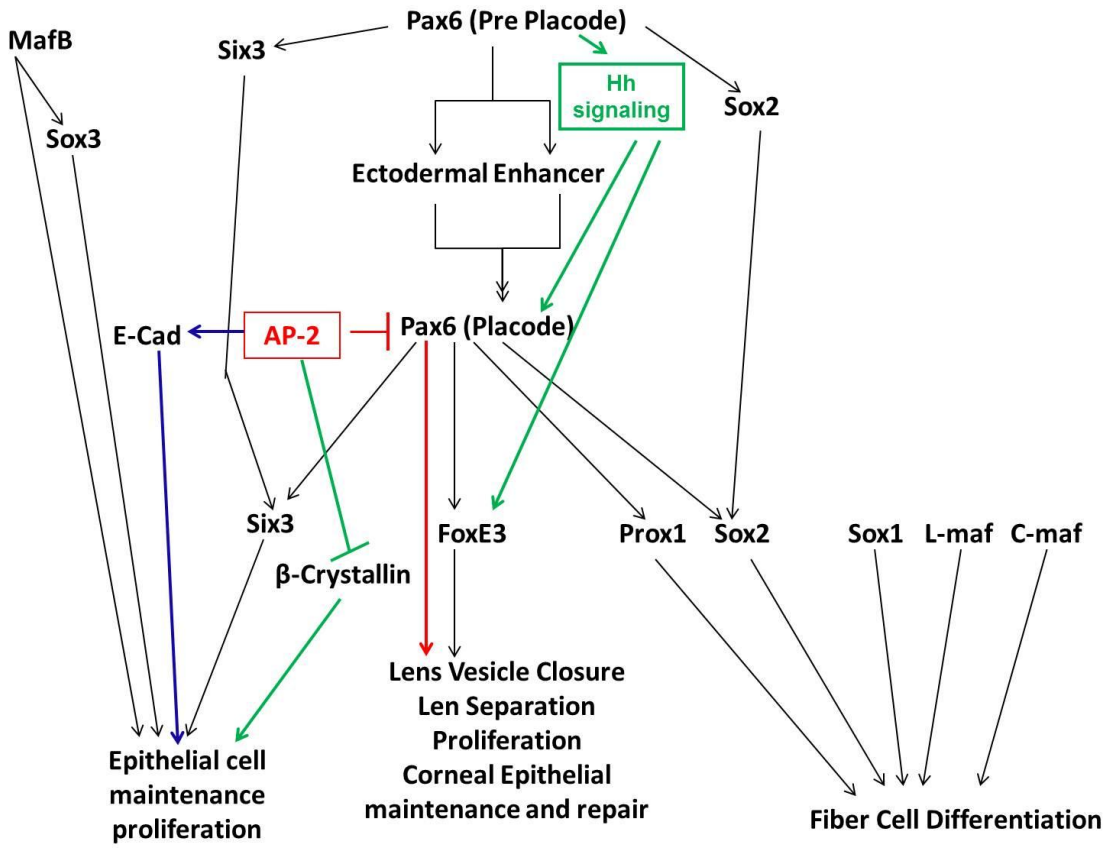

Figure 5.1 Proposed Position of Hh and AP-2 within the hierarchy of lens development regulators.

Abnormalities in Hh signaling beginning prior to lens placode formation result in abnormal lens development and deregulated expression of FoxE3 and later, Pax6. Based on the knowledge gained in this thesis, it is believed that AP-2 is sufficient for lens placode development, but interacts with Pax6 at the lens vesicle stage, to ensure proper separation of the lens away from the surface ectoderm. The interaction of AP-2α with E-cadherin is important in early lens development for lens vesicle separation to occur (Pontoriero et al., 2008). However, results from the DKO studies suggest that AP-2α and AP-2β may be required in heterodimer form to interact with Pax6 and E-cadherin to ensure proper lens vesicle separation. This thesis has also demonstrated the possible interaction of AP-2α with β-crystallin to inhibit its expression in the lens epithelium. adapted from (Chow and Lang, 2001).

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