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# AP-2α is Required After Lens Vesicle Formation to Maintain Lens Integrity

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## Abstract

**Background:** Transcription factors are critical in regulating lens development. The AP-2 family of transcription factors functions in differentiation, cell growth and apoptosis, and in lens and eye development. AP-2α, in particular, is important in early lens development, and when conditionally deleted at the placode stage defective separation of the lens vesicle from the surface ectoderm results. AP-2α's role during later stages of lens development is unknown. To address this, the MLR10-Cre transgene was used to delete AP-2α from the lens epithelium beginning at embryonic day (E) 10.5.

**Results:** 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 epithelium was evident in the MLR10-AP-2α mutants. Epithelial cells appeared elongated and expressed fiber cell specific βB1 and γ-crystallins. Epithelial cell polarity and lens cell adhesion was disrupted and accompanied by the misexpression of ZO-1, N-Cadherin, and β-catenin. Cell death was observed in the mutant lens epithelium between postnatal day (P) 14 and P30, and correlated with altered arrangements of cells within the epithelium.

**Conclusions:** Our findings demonstrate that AP-2α continues to be required after lens vesicle separation to maintain a normal lens epithelial cell phenotype and overall lens integrity and to ensure correct fiber cell differentiation.

### Keywords

transcription factors; AP-2; lens development; lens epithelium; differentiation

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## Introduction

Transcription factors are critical regulators of key developmental processes including cell growth and differentiation. In lens development, a hierarchy of transcription factors functions to drive lens formation and patterning. Pax6 (Paired box 6) is positioned at the top of this hierarchy and controls multiple aspects of eye development characterized by two waves of expression (Chow and Lang, 2001; Lovicu and Robinson, 2004). The early phase of expression is critical for correct placode development (van Raamsdonk and Tilghman, 2000). During this preplacodal stage, Pax6 regulates the expression of the transcription factors Sox2 and Six3, which are both important in the formation of the lens placode (Ashery-Padan et al., 2000; Chow and Lang, 2001; Smith et al., 2009; Huang et al., 2011). The second wave of Pax6 expression, following early placode development, is important for regulating genes involved in lens vesicle formation, including FoxE3 (Brownell et al., 2000; Plageman et al., 2010). Additional studies have shown that AP-2α can also influence the earlier stages of lens induction (Pontoriero et al., 2008; West-Mays et al., 1999), yet whether this transcription factor can also function in later stages of lens differentiation, akin to Pax6, 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 (Hilger-Eversheim et al., 2000; Feng and Williams, 2003; West-Mays et al., 2003; Eckert et al., 2005). Mice lacking *Tfap2a*, the gene encoding AP-2α, have multiple developmental defects affecting the head and face including exencephaly and facial clefting (Zhang et al., 1996; West-Mays et al., 1999). Furthermore, *Tfap2a* null mice, as well as chimeras containing AP-2α null cells, have severe defects in early lens development, with a range of abnormal ocular phenotypes including anophthalmia (lack of eyes) and persistent adhesion of the lens to the overlying surface ectoderm, forming a lens stalk (West-Mays et al., 2002; Pontoriero et al., 2008). In this respect, mutations in AP-2α generate lens phenotypes reminiscent of those caused by early loss of Pax6.

AP-2 proteins are co-expressed in several ocular tissues during development, including the lens placode (West-Mays et al., 1999; Bassett et al., 2007, 2012). However, AP-2α is also expressed in the developing neural retina, and in multiple tissues in the embryonic head, including the neural crest and surface ectoderm. Therefore, further studies were required to determine if the normal formation of the lens required the cell autonomous activity of AP-2α, or whether this morphogenetic process relied on AP-2α function in adjacent tissues. To address this question, the lens placode specific LeCre transgene (Ashery-Padan et al., 2000) was used for conditional deletion of *Tfap2a* (Le-AP-2α) from the lens placode and its derivatives (Ashery-Padan et al., 2000; Pontoriero et al., 2008). Similar to the germline knockouts, 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; Ashery-Padan et al., 2000; Pontoriero et al., 2008). Thus, while it is known that AP-2α is required for proper lens vesicle formation, the requirement for this transcription factor in regulating further development and maintenance of the lens remains to be determined. Here, we have used the MLR10-Cre transgene, which drives Cre recombinase expression in the lens fiber and epithelial cells from E10.5 (Zhao et al., 2004), to ablate AP-2α function after lens vesicle separation. The MLR10-cre line was created as a means to conditionally delete a gene from the lens epithelium and/or fiber cell region at the lens vesicle stage of development. The insertion of a Pax6 consensus sequence into the αA-crystallin promoter allowed for gene deletions in the epithelium in addition to the fiber cells at the lens vesicle stage (Zhao et al., 2004). AP-2α expression is unique to the lens epithelium in the developing and postnatal lens, and therefore, the MLR10-cre acts to delete its expression from this specific region of the lens in our mutants. Our findings indicate that a loss of AP-2α leads to molecular, cellular, and phenotypic changes in lens development that demonstrate a continuing requirement for this transcription factor for the integrity of the lens epithelium and associated fiber cells.

## Results

### Targeted Deletion of AP-2α in the Lens Epithelium Results in the Loss of an Epithelial Monolayer and Cortical Cataracts

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 embryonic day (E) 10.5 (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 wild-type (WT) and MLR10-AP-2α mutant embryonic lenses to assess the extent and timing of AP-2α deletion from the lens epithelium. The majority of lens epithelial cells in the MLR10-AP-2α mutants exhibited an absence of AP-2α expression beginning at E11.5 (compare Fig. 1A with E). At postnatal day (P) 4, P14 and P30 AP-2α protein expression was seen in the WT lens epithelium (Fig. 1B–D), but was absent in the mutant lens epithelium at equivalent stages (Fig. 1F–H). As illustrated, a small number of lens epithelial cells in the mutant lens escaped Cre-mediated deletion of AP-2α, which has also been seen in other studies using this Cre (Shaham et al., 2009). Thus, a small proportion of cells in the lens epithelium were found to retain AP-2α protein expression at all stages examined. Representations of this occurrence can be seen in mutant lens sections at E11.5, P14, and P30 (Fig. 1E,G,H, white 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 before E18.5. 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 of MLR10-AP-2α mice (Fig. 2E) and at later stages of postnatal development the vacuoles were large and occupied more of the fiber cell compartment (Fig. 2F–H). Failed fiber cell denucleation was also evident in the MLR10-AP-2α mutants at P14 and P30 (compare Fig. 2J,K with M,N). Vacuoles and failed fiber cell denucleation contribute to opacities within the fiber cell region of the lens causing a loss of critical lens

clarity (Song et al., 1997; Wride, 2011). Posterior lens vacuoles and failed fiber cell denucleation have been indicated as characteristics of cortical cataracts in both mice and humans (Song et al., 1997; Hsu et al., 2006; Rivera et al., 2009; Wride, 2011). Cortical cataract formation was indeed evident at P14 in the mutant lens (Fig. 2P, black arrow).

In comparison to the WT lens epithelium, which consisted of a monolayer of cuboidal shaped epithelial cells (Fig. 2I), the epithelial cells in the MLR10-AP-2α mutant lens appeared elongated in the anterior–posterior direction beginning at P4 (Fig. 2L). In addition, in the MLR10-AP-2α lens, cell adhesion at the epithelial-fiber cell interface and at the epithelial-capsule interface appeared disrupted as evidenced by gaps between the layers (Fig. 2J,M, black arrows). These adhesion defects, as well as abnormal adhesions between neighboring fiber cells has been shown to result in disruptions in suture formation (Maddala et al., 2011), and this is also evident in fiber cells of our mutants. An example of this is shown at P14 (Fig. 2G, purple arrow). The spacing between nuclei within the lens epithelium was also disrupted in the mutant lens at P30. Nuclei of lens epithelial cells are uniformly spaced as seen in WT lenses at P30 (Fig. 2K); however, large gaps between nuclei are evident in the mutant at this stage (Fig. 2N, inset, and see Fig. 5F, white lines).

Small pockets of multilayered epithelial cells were also observed in the MLR10-AP-2α mutant epithelium, a phenotype which was exacerbated as postnatal development progressed (Fig. 2L,M, circles). Multilayering in the lens epithelium can be indicative of an epithelial to mesenchymal transition (EMT) of lens epithelial cells, which is observed in the formation of anterior subcapsular cataracts (ASC). This multilayering is typically accompanied by the expression of α-SMA along with a loss of epithelial specific proteins such as E-cadherin (Schmitt-Graff et al., 1990; Hales et al., 1994; Lovicu et al., 2004; Rungger-Brandle et al., 2005). No evidence of EMT or ASC was seen in our mutants, as α-SMA was not detected within multilayered regions of the lens epithelium in our MLR10-AP-2α mutants (data not shown). In addition, E-cadherin expression was expressed in all mutant lens epithelial cells at P4 and P14, including cells within the multilayered epithelial regions (Fig. 3C,D, white star). The E-cadherin staining intensity appeared brighter in the central region of mutant epithelium (denoted by the white star) (Fig. 3D), and we attribute this increased staining to the clustering of the cells.

### MLR10-AP-2α Mutant Lenses Exhibit Apoptosis in the Lens Epithelium

Because 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 if programmed cell death correlated with the pathology. In WT lenses, no cell death was detected in the lens epithelium at P4 (data not shown) or P14 (Fig. 4A). No TUNEL positive cells were detected in the epithelium of the MLR10-AP-2α lens at P4 (data not shown), but by P14 several such cells were found in the central lens epithelium (Fig. 4B, white arrows), as well as the transition zone near the lens capsule (Fig. 4B, yellow arrow), and this is hypothesized to contribute to the large gaps between epithelial cells observed at P30.

### Loss of AP-2α Results in Altered Lens Cell Fates and Differentiation

Pax6 is an important protein that is necessary for proper lens development. At E12.5, Pax6 has been shown to be expressed in the lens epithelium and at lower levels in the fiber cells in the transition zone near the lens equator. Pax6, however, is not normally expressed in fiber cells within the central region of the lens that have undergone terminal differentiation (Shaham et al., 2009). In embryonic stages, Pax6 expression in the MLR10-AP-2α mutants was found to be similar to that of WT littermates, with no apparent abnormalities (data not shown). At P4, Pax6 was also observed to have a normal expression pattern in the epithelium of the mutant lens as compared to wild-type (Fig. 5C). However, at both P4 and P30, Pax6 expression in the MLR10-AP-2α mutants was observed in fiber cells in the central fiber cell compartment, an area that is normally devoid of fiber cell nuclei and Pax6 expression (Fig. 5C,D).

N-cadherin is a $Ca^{2+}$ 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 center of the lens do not show immunoreactivity for this cell adhesion molecule (Xu et al., 2002). This is thought to be due to the fact that once fiber cells have completed elongation, and have formed new adhesions with neighboring fiber cells, the epitopes for N-cadherin are masked due to the rearrangement of fiber cell adhesion complexes in these mature cells (Beebe et al., 2001). N-cadherin expression was examined in the lens epithelium and fiber cells of the transitional zone in WT lenses at P4, P14, and P30 (Fig. 6A–C), where it was absent in the central mature fiber cell region of the WT lens, as previously described (Fig. 6B,C). Like WT mice, N-cadherin expression in MLR10-AP-2α mutant lenses was detected in the epithelium and transitional zone at P4, P14, and P30 (Fig. 6D–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. 6E,F). This finding suggests that the mutant cells in the central fiber cell region may not be fully differentiated. These data, in conjunction with the expression of Pax6 in the mutant fiber cell region of the lens, suggest that these fiber cells have not undergone normal fiber cell differentiation.

In addition, we examined β-catenin expression. 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). Although β-catenin 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. 7A–C), its expression had aberrantly expanded into the posterior fiber cell compartment of the mutant lens at all postnatal stages examined (Fig. 7D–F), further illustrating abnormal fiber cell differentiation.

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 expressed in the fiber cell region of the lens, and not usually observed within the lens epithelium (Lovicu and Robinson, 2004; Andley, 2007); however, there have been some reports of low level expression in the

postnatal lens epithelium. Because 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 (data 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. 8D), unlike WT lenses in which βB1-crystallin was absent from the lens epithelium (Fig. 8A). 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. 8E,F). No βB1-crystallin expression was observed in the WT lens epithelium at equivalent stages (Fig. 8B,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. 8H,J), while the lens epithelium of WT littermates at equivalent stages was devoid of γ-crystallin expression (Fig. 8G,I).

### 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 (Shin et al., 2006; Cain et al., 2008). 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 throughout the lens, with its expression levels decreasing immensely three weeks after birth (Nielsen et al., 2003). In WT lenses, ZO-1 is expressed in the apical region of lens epithelial cells at the epithelial-fiber cell interface and along the lateral membranes of outer cortical fiber cells in this region (Nielsen et al., 2003). At the epithelial-fiber cell interface, 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 seen to be normally expressed at the apical aspect of the lens epithelium and on the lateral membranes of outer cortical fiber cells. Its expression was properly localized between the proliferative zone and the lens equator at P4, P14, and P30 (Fig. 9A–C). Consistent with the study by Neilson et al., ZO-1 expression is observed to have decreased in the WT lens by P30 (Fig. 9C). In comparison in the mutant lens although ZO-1 expression was observed along the apical border of the epithelial-fiber cell interface, it appeared weaker than in the WT lens, and its distribution more diffuse (Fig. 9D–F). The expanded domain of ZO-1 expression appeared to illustrate a larger amount of ZO-1 staining localized on the fiber cells where they contact the lens epithelium (Fig. 9D–F). ZO-1 was also observed to have strong punctate expression in the central fiber cell area in the region surrounding the vacuoles of the mutant lens. This increased ZO-1 expression in the mutant fiber cells was not seen in the WT lens (Fig. 9E, inset).

## Discussion

An earlier study, in which AP-2α was conditionally deleted from the lens placode and its derivatives (Le-AP-2α), illustrated a crucial role for AP-2α in early lens development (Pontoriero et al., 2008). A loss of AP-2α at the lens placode stage resulted in failed separation of the lens epithelium from the overlying surface ectoderm (Pontoriero et al.,

2008). Because the role of AP-2α in later stages of lens development and differentiation were not well understood, we used 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 organization and lens cell differentiation and fate determination. Deletion of AP-2α in the lens epithelium, subsequent to vesicle separation, resulted in altered epithelial cell architecture, aberrant differentiation of epithelial and fiber cells, accompanied by morphological defects in the fiber cell compartment of the lens.

## Loss of AP-2α Results in Altered Lens Morphology and Cataracts

The loss of AP-2α from the lens vesicle did not result in overt lens defects during the embryonic-fetal lens developmental stages (E10.5–E16). However, during the fetal to postnatal developmental stages the morphology of the lens was severely disrupted in the MLR10-AP-2α mutants. Interestingly, it has been shown that during this period of development the expression profile of transcription factors, cellular receptors and structural proteins changes dramatically (Carper et al., 1986; Srinivasan et al., 1998; Sinha et al., 2001; Kelley et al., 2002; Min et al., 2004). Defects in the lens epithelium were evident at E18.5 in the MLR10-AP-2α mutants and included regions of multilayering that was unlike wild-type littermates, which exhibited a layer of simple cuboidal epithelium. Multilayering of the lens epithelium can be indicative of EMT of lens epithelial cells, a feature observed in the formation of ASC. 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 (Schmitt-Graff et al., 1990; Hales et al., 1994; Lovicu et al., 2004; Rungger-Brandle et al., 2005). However, no α-SMA expression was observed in the multilayered regions of the MLR10-AP-2α mutant lens epithelium, and E-cadherin and Pax6 expression were normally expressed indicating that ASCs are not present in this model. Previous studies in flies have shown that a loss of normal cell adhesion and polarity 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 (discussed in next section).

The postnatal MLR10-AP-2α mutants also exhibited failed fiber cell denucleation and large vacuoles in the posterior fiber cell region of the lens. These phenotypes have been reported in mice and humans, as factors contributing to cortical cataracts (Song et al., 1997; Hsu et al., 2006; Rivera et al., 2009; Wride, 2011) and cortical cataracts were in fact present in our postnatal mutants (See Fig. 2). AP-2α has been implicated in the human disorder branchio-oculo-facial syndrome (BOFS). This disorder results from a deletion of one copy of *TFAP2A* (gene that encodes AP-2α), or from a missense mutation in the DNA binding domain of the *TFAP2A* gene (Milunsky et al., 2008). Of interest, this disorder presents with a myriad of clinical facial and ocular abnormalities including cortical cataracts (Milunsky et al., 2008; Dumitrescu et al., 2012). Together, these data suggest that normal AP-2α expression in the lens epithelium is critical in maintaining an organized epithelial cell layer,

as well as in promoting normal fiber cell differentiation and fiber cell clarity. In addition, the data highlight the importance of our mouse model in understanding the phenotypes (such as cataracts) observed in human ocular diseases such as BOFS.

## AP-2α is Required to Maintain a Normal Lens Epithelial Cell Phenotype

This study illustrates the importance of AP-2α expression in the lens epithelium subsequent to lens vesicle separation to ensure normal development and differentiation of the epithelium and subsequent fiber cell differentiation. Growth factors, including FGFs, and BMPs, cell adhesion molecules such as E-cadherin and N-cadherin as well as numerous transcription factors including Pax6, FoxE3, Prox1, and AP-2α have been shown to be critical for normal lens development and differentiation (West-Mays et al., 1999; Ferreira-Cornwell et al., 2000; Lovicu and Robinson, 2004; Lovicu and McAvoy, 2005; Pontoriero et al., 2009; Shaham et al., 2009). Although it is becoming better understood, it remains unclear how these factors precisely interact to ensure the development of a normal lens; however, a loss of one or more of these factors from the lens results in abnormal lens cell differentiation. For example, studies have illustrated that the conditional loss of Pax6 from the lens epithelium resulted in the inability of lens epithelial cells to complete fiber cell differentiation. These Pax6 mutant epithelial cells accumulated at the lens equator and in the posterior of the lens, and failed to differentiate into lens fiber cells (Shaham et al., 2009). Similarly, a loss of FoxE3 also resulted in lens epithelial defects and subsequent, abnormal fiber cell differentiation (Medina-Martinez et al., 2005).

Synonymous to these studies, the findings of this study illustrate that AP-2α is required subsequent to lens vesicle separation to ensure normal development and differentiation of lens epithelial and fiber cells. Strikingly, we 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. Low but detectable levels of βB1-crystallin and γ-crystallin have been reported in postnatal rat epithelial lens explants and in mouse lens epithelium in vivo (Wang et al., 2004). However, the level of staining we observed in the MLR10-AP-2α mutant lens epithelium was much greater than that observed in wild-type littermates, and more similar to that typically seen in fiber cells. The increased expression of βB1-crystallin and γ-crystallin in the MLR10-AP-2α mutant lens epithelium 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 suggest that β-crystallin expression in the lens epithelium is inhibited when Pax6 is up-regulated in this region (Duncan et al., 1998). Of interest, AP-2α has been shown 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 been shown however 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 βB1-crystallin expression, thus allowing normal progression of development of lens epithelial cells.

Further evidence corroborating the role for AP-2α as a negative regulator of fiber cell differentiation comes from a study by West-Mays et al, where AP-2α was ectopically expressed in newly differentiated lens fiber cells (West-Mays et al., 2002). 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 MIP (major intrinsic protein) (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 targeted deletion of AP-2α from the lens epithelium of the MLR10-AP-2α mutant lenses also caused secondary defects in cells within the fiber cell region. For example, cells in this region failed to lose their nuclei, indicating that they had not terminally differentiated. The expression of Pax6 was also observed in the mutant postnatal central fiber cell region. Pax6 is normally expressed in the epithelium and in lower levels at the lens equator, but should be absent in the central terminally differentiated fiber cell region (Shaham et al., 2009). This finding is consistent with studies illustrating that ectopic Pax6 expression in cells in the fiber cell region results in disruptions in fiber cell differentiation and the maintenance of a partial epithelial phenotype (Duncan et al., 2004; Carmona et al., 2008; Kerr et al., 2012). β-catenin expression was also detected in the posterior regions of the mutant lens, further suggesting aberrant fiber cell differentiation in this region. In agreement with our study, the overexpression of β-catenin in the lens has previously been shown to result in disruptions in fiber cell differentiation (Shaham et al., 2009). Finally, the postnatal MLR10-AP-2α mutants also displayed N-cadherin expression throughout the entire fiber cell region, whereas wild-type littermates show the central region devoid of N-cadherin staining. A previous study has shown that the detection of N-cadherin by immunolocalization, in the central mature fiber cell region is complicated by epitope masking (Beebe et al., 2001). The authors suggest that once fiber cells have completed elongation, and have formed new adhesions with neighboring fiber cells, the N-cadherin epitopes in this mature region are masked due to the rearrangement of fiber cell adhesion complexes in these specific mature cells. We observed a similar lack of N-cadherin expression was in the wild-type lenses examined and this was most likely a reflection of this phenomenon. However, the fact that the mutant lens did not demonstrate this masking further suggests that the fiber cells are not fully differentiating.

The secondary fiber cell defects observed in our model are not surprising given the disorganization of the epithelium observed in the MLR10-AP-2α mutant lens epithelium. Models illustrating a disorganized lens epithelium, with phenotypic characteristics similar to those seen in our mutant model (e.g., multilayering, disruptions in the uniform spacing of epithelial cells) also exhibit a lack of complete fiber cell differentiation. For example, a study examining the loss of *Dlg-1* (the mouse homologue of the tumor suppressor

Drosophila, Discs large [*dlg*]), illustrated how a disorganized and multilayered epithelium was accompanied by characteristic fiber cell defects including a disorganized bow region, failed fiber cell denucleation, vacuoles and abnormal fiber cell differentiation (Rivera et al., 2009). Thus, it appears that AP-2α expression is required to maintain a normal lens epithelial cell fate; furthermore, this ensures that fiber cells differentiate properly.

## Targeted Deletion of AP-2α From the Lens Epithelium Results in Abnormal Epithelial Cell Polarity and Adhesion and Subsequent Secondary Fiber Cell Defects

Normal polarity of cells, along with normal cell adhesion, is critical in proper cell development and differentiation. ZO-1, a protein important in both cell polarity and cell adhesion (Nielsen et al., 2003), was examined in the MLR10-AP-2α mutant model. ZO-1 is a tight junction protein, required to mediate adhesions between lens epithelial cells, and plays a role in determining normal cell polarity of epithelial and fiber cells (Shin et al., 2006; Cain et al., 2008). In comparison to WT littermates, ZO-1 expression was disrupted in the MLR10-AP-2α mutants, appearing weak and very diffuse. Additionally, ZO-1 expression was also strong and punctate around the vacuoles within the fiber cell region of the mutant lens. Punctate staining of ZO-1 in the posterior region of the lens has been considered indicative of abnormal tight junction formation in lens fiber cells (Cain et al., 2008) and may contribute to the adhesion defects seen in the fiber cell region of our mutant model. Because ZO-1 was found to be aberrantly expressed in both the epithelial and fiber cell regions it may have contributed to the adhesion defects observed in the postnatal MLR10-AP-2α lens between the fiber/epithelial cell interface.

A correctly polarized lens is important for normal epithelial and fiber cell development. Epithelial cells must be correctly polarized to drive normal fiber cell differentiation, and fiber cells must be correctly polarized to elongate both anteriorly and posteriorly to develop transient adhesions with the anterior epithelium and posterior capsule (Lovicu and Robinson, 2004). A recent study has shown, for the first time, that the lens epithelium possesses polarizing cues (including wnt5) to properly orient and polarize elongating fiber cells and that the resulting epithelial-fiber cell interactions are critical in maintaining the highly polarized lens structure (Dawes et al., 2014). This study demonstrated that rat lens epithelial explants treated with FGF showed normal polarized localization of ZO-1 consistent with normal ZO-1 expression in the epithelium and underlying fibers of WT in vivo lenses (Dawes et al., 2014). However, when these explants were treated with the Wnt secretion inhibitor IWP2, the polarizing wnt5 signal appeared to be blocked, and ZO-1 expression was observed to be abnormal (Dawes et al., 2014). Thus, one possible consequence of our targeted deletion of AP-2α in the MLR10-AP-2α mutants may have been to negatively impact polarizing cues from the epithelium. Thus, AP-2α, as a transcription factor, may be important in regulating genes involved in the communication system between the epithelium and developing fiber cells and disruptions in critical polarizing cues (which may manifest themselves as disruptions in ZO-1 expression in our mutants) may have caused the lack of normal epithelial-fiber cell adhesions and the abnormal fiber cell differentiation evident in our model. Future studies identifying the downstream targets of AP-2α that are affected in our mutant may give further clues regarding the signals responsible for lens cell polarization.

In summary, the current study has shown that AP-2α is not only required during early stages of lens placode development but it 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. The loss of AP-2α also resulted in defects in epithelial cell fate determination and abnormal differentiation and development of both epithelial and fiber cells. These findings demonstrate that AP-2α is important in maintaining a normal lens epithelial cell phenotype and that in its absence these cells exhibit features of fiber cell differentiation. In addition, the expression of AP-2α is required to preserve lens epithelial cell organization to ensure normal fiber cell differentiation, and when lost, it appears that the intrinsic self-assembly mechanisms occurring between epithelial and fiber cells are negatively affected.

## Experimental Procedures

### Generation of the MLR10-AP-2α Mutants

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 a line of MLR10-Cre/AP-2α$^{flox/lacZKI}$ mice (hereafter referred to as MLR10-AP-2α mutants), MLR10-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 (Brewer et al., 2004), which contains LoxP sites flanking the DNA binding and dimerization domain of AP-2α, to generate a conditional mutant with AP-2α deleted from the lens epithelium beginning at E10.5. As AP-2α's expression in the lens is unique to the lens epithelium, and its expression ceases at the transition zone, the MLR10-cre transgenic lines deletes AP-2α only where it is expressed in the lens epithelium. PCR for the *MLR10-Cre* transgene was performed with the primers Cre1 and Cre3 corresponding 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 sec, 67°C for 45 sec, and 72°C for 1.5 min, with a 420nt product indicating the presence of Cre recombinase allele. 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 sec, 70°C for 45 sec and 72°C for 1 min. This generated a 500 bp WT product and a 300 bp *LacZKI* product as outlined in (Pontoriero et al., 2008).

### Histology

Noon on the day of vaginal plug detection was considered embryonic day (E) 0.5. Whole embryos and postnatal eyes were collected from MLR10-AP-2α mutants and wild-type (WT) littermates. Embryonic tissue was fixed in 10% neutral buffered formalin overnight at room temperature and then transferred into 70% ethanol until processing. Whole embryos (E18.5) or postnatal eyes (P4, P14, P30) were processed and embedded in paraffin. Serial sections were cut at a thickness of 4 μm 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.

### Immunofluorescence

Immunofluorescence was performed on 4μm paraffin sections, using the following primary antibodies: rabbit polyclonal Pax6, Covance, Princeton, NJ (1:50); mouse monoclonal ZO-1, Invitrogen, Burlington, ON, CA (1:40); rabbit polyclonal βB1-Crystallin, rabbit polyclonal γ-Crystallin, all provided by Dr. Samuel Zigler Jr., Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD (1:200); mouse monoclonal α-Sma, Sigma, Oakville, ON, CA (1:100); mouse monoclonal AP-2α (3B5), Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA (1:1); mouse monoclonal β-Catenin, BD Transduction, Burlington, ON, CA (1:100); rabbit polyclonal Collagen IV, Abcam, Toronto, ON, CA (1:200); mouse monoclonal N-Cadherin, BD Transduction, Burlington, ON, CA (1:100). Fluorescent secondary antibodies were either Alexa Fluor 488 or 568 (Goat anti-mouse and goat anti-rabbit), Invitrogen-Molecular Probes, Burlington, ON, CA, used 1:200 for 1 hr at room temperature. Paraffin-embedded sections were deparaffinized in xylene, hydrated (through 100%, 95%, 70% ethanol, followed by water), treated with 10 mM sodium citrate buffer (pH 6.0; boiling for 20 min) for antigen retrieval, and 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).

### 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 (Bellirica, MA). Four micrometer paraffin-embedded sections were deparaffinized in xylene, hydrated (through 100%, 95%, 70% ethanol, followed by phosphate buffered saline [PBS]) and treated with 10 mM sodium citrate buffer (pH 6.0; boiling for 20 min) for antigen retrieval. Proteinase K (20 μg/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 hr. 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 min. Slides were washed in PBS and mounted with ProLong Gold Antifade Reagent containing DAPI (Life Technologies, CA). TUNEL experiments included a positive control slide treated with DNAse after antigen retrieval, followed by processing with other slides as previously described (Loo, 2011).

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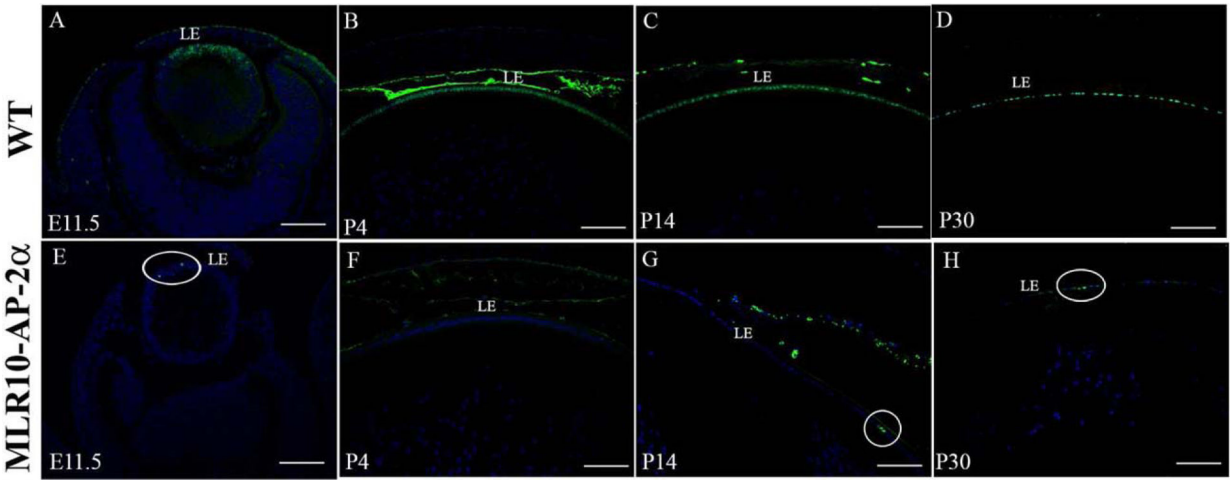


**Fig. 1.**

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 MLR10-cre transgenic line of mice works in a mosaic nature, and thus white circles display some cells that retain AP-2α expression. LE-Lens Epithelium. Green staining, AP-2α; Blue staining, DAPI. Scale bars = 100 μm.

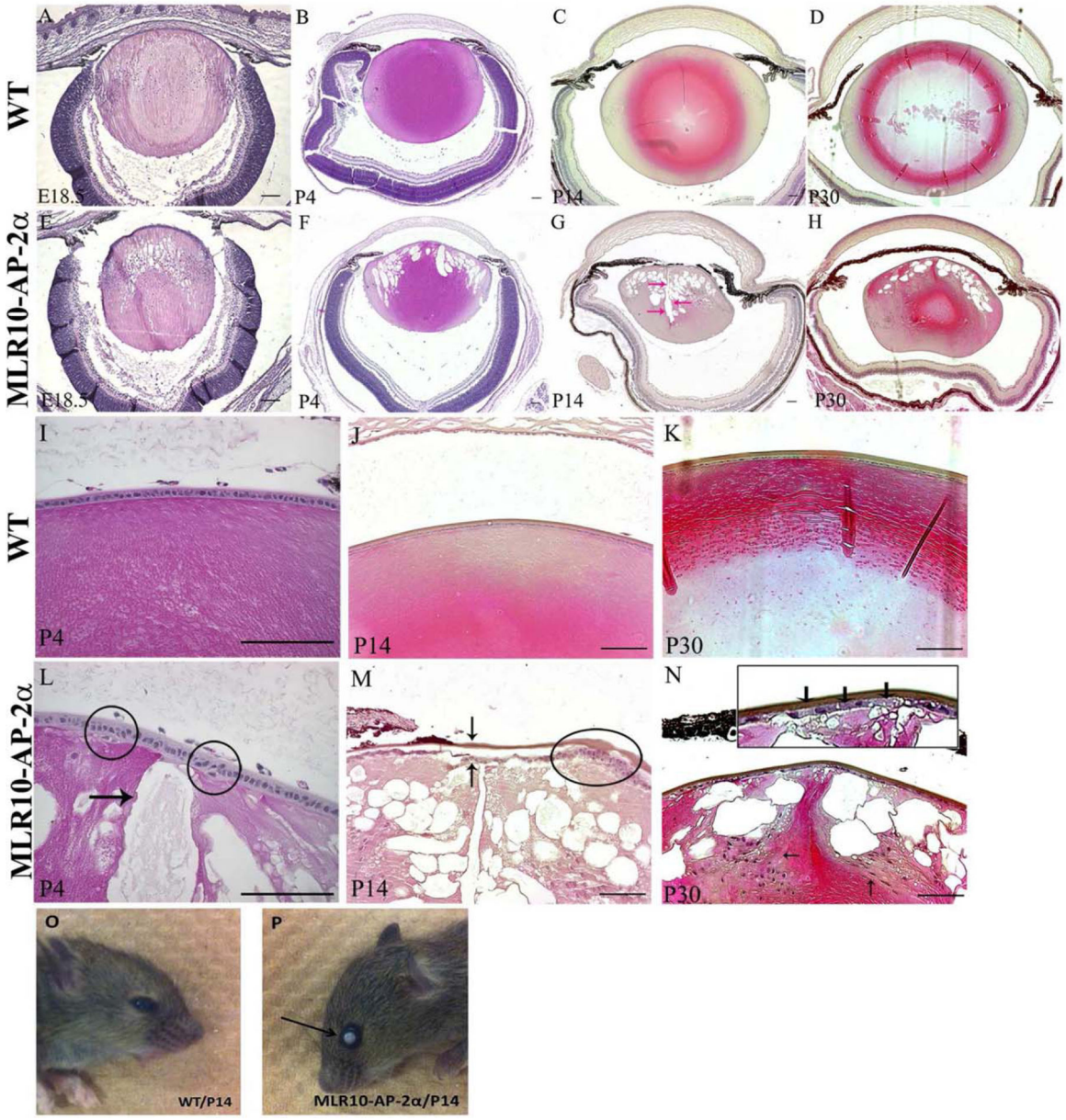


**Fig. 2.**

Deletion of AP-2α from lens epithelium leads to morphological abnormalities, defects in lens integrity and cortical cataracts. 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 more elongated in the MLR10-AP-2α mutant lens at P4 (L) than in lenses of WT littermates (I). Multilayering is evident in the mutant lens epithelium at all stages (L, M, circles). Adhesion of cells at epithelial-fiber cell and epithelial-capsule interfaces is fragile (M, double arrow). Suture formation is defective in mutant fiber cells as seen at P14 (G, purple arrow). Nuclei are observed in the anterior region of the fiber cell compartment at P30 (N, black arrows). At P30, the uniform spacing

between epithelial cells is lost, with large gaps between epithelial cells present (N, inset, black arrows). Cataracts are evident through the white opacity at P14 in the mutant lens (P, black arrow). Scale bars = 100 μm.

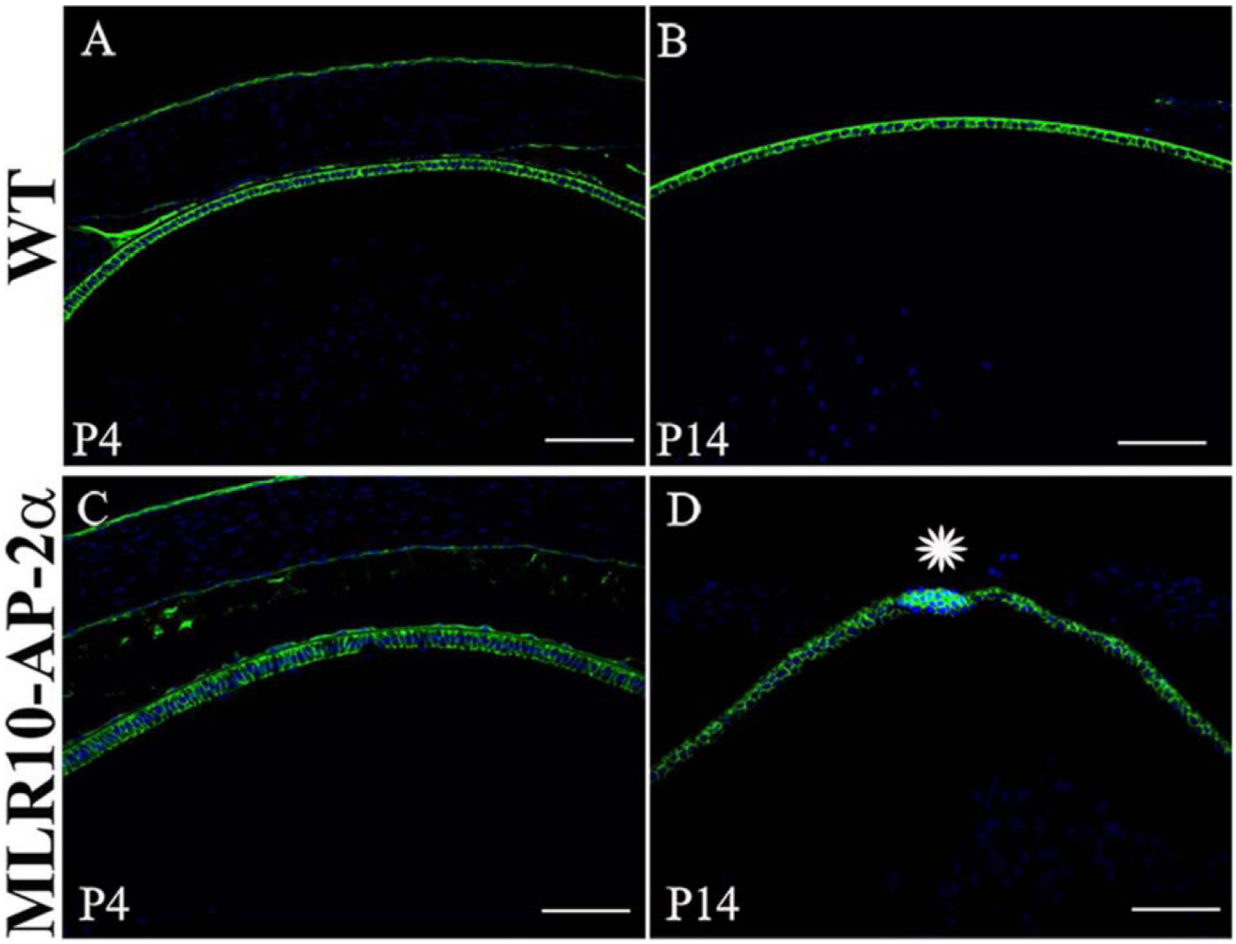


**Fig. 3.**

E-cadherin remains expressed in regions of multilayered epithelium in the MLR10-AP-2α mutants. E-cadherin is expressed within the WT epithelium (**A,B**). E-cadherin remains expressed in regions of multilayering in the mutant epithelium (**C,D**) and this is seen very clearly at P14 (D, white star). Green staining, E-cadherin; Blue staining, DAPI. Scale bars = 100 μm.

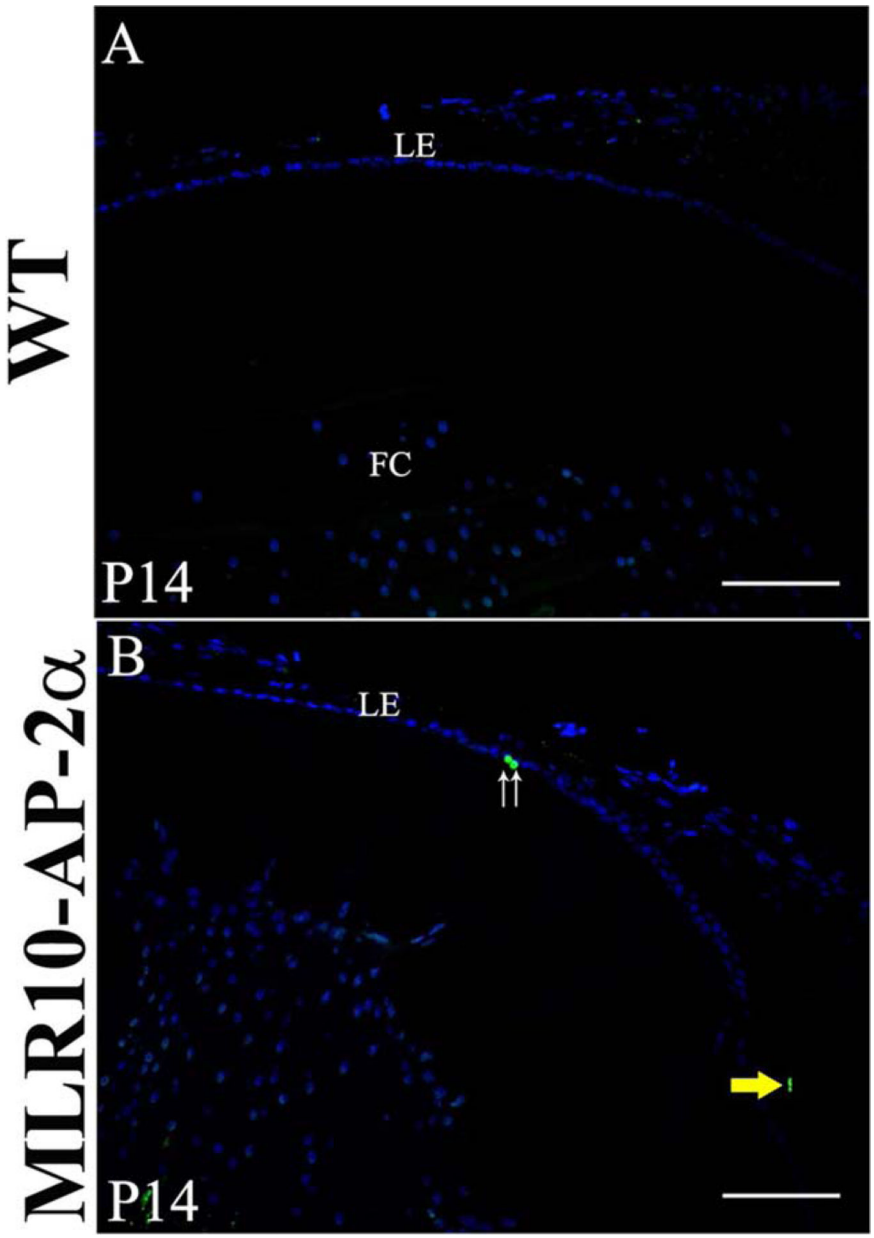


**Fig. 4.**

MLR10-AP-2α mutants display abnormal TUNEL staining in fiber cells and lens epithelium at P4 and P14. Aberrant lens epithelial apoptosis is observed in the central epithelium and transition zone/capsule area at P14 (**B**, white/yellow arrows), but absent from the lens epithelium and transition zone/capsule area of the WT (**A**) Green staining, TUNEL positive nuclei. Blue Staining, DAPI. Scale bars = 100 μm.

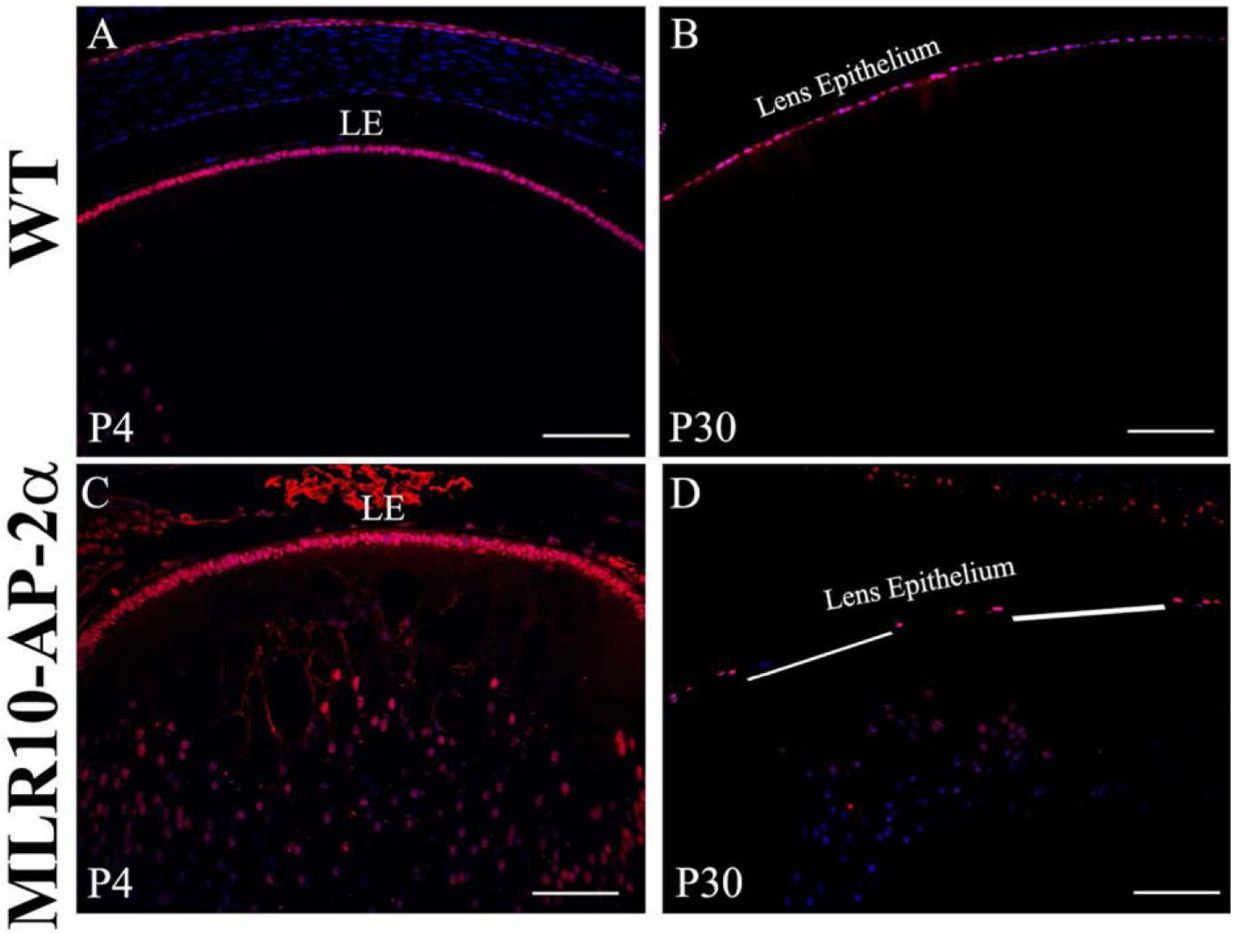


**Fig. 5.**

Pax6 expressed in lens epithelium of mutant lenses. WT (A,B), and mutant (C,D) lenses express Pax6 in its normal spatial location in the lens epithelium at P4 (**A,C**) and P30 (**B,D**). Gaps between epithelial cells in the mutant lens are visible at P30 (D, white line). Pax6 staining is also evident in the fiber cell region of the lens at P30 (D). LE-Lens Epithelium. Red staining, Pax6. Blue Staining, DAPI. Scale bars = 100 μm.

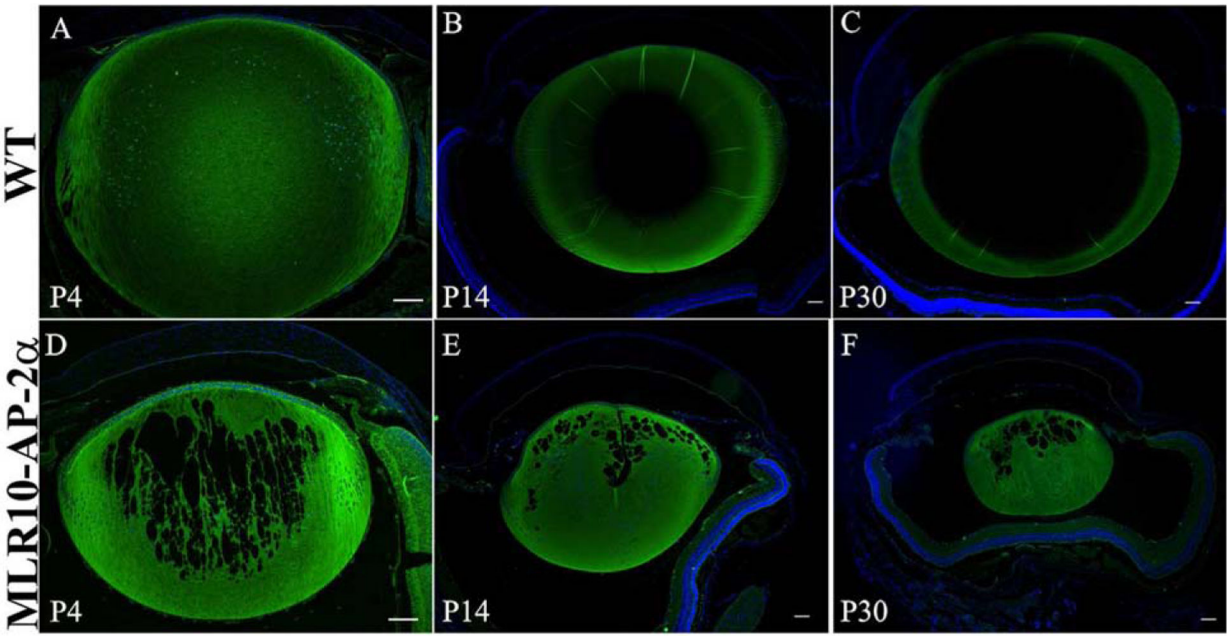


**Fig. 6.**

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). Green staining, N-cadherin; Blue staining, DAPI. Scale bars = 100 μm.

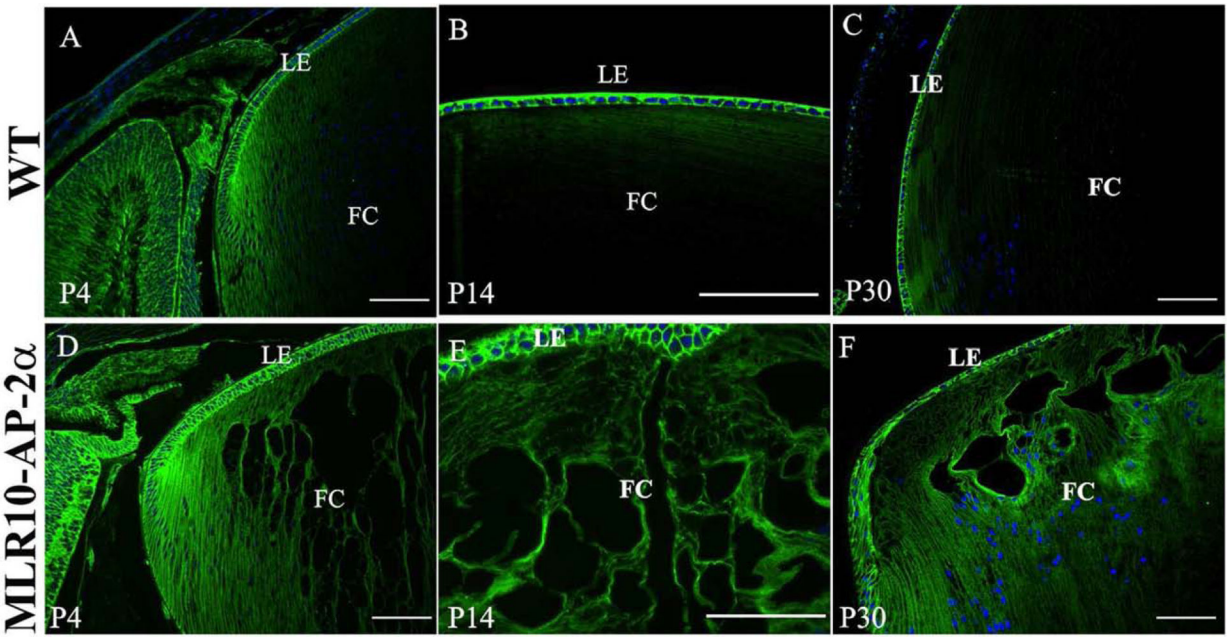


**Fig. 7.**

β-Catenin expression is 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**). Green staining, β-catenin; Blue staining, DAPI. Scale bars = 100 μm.

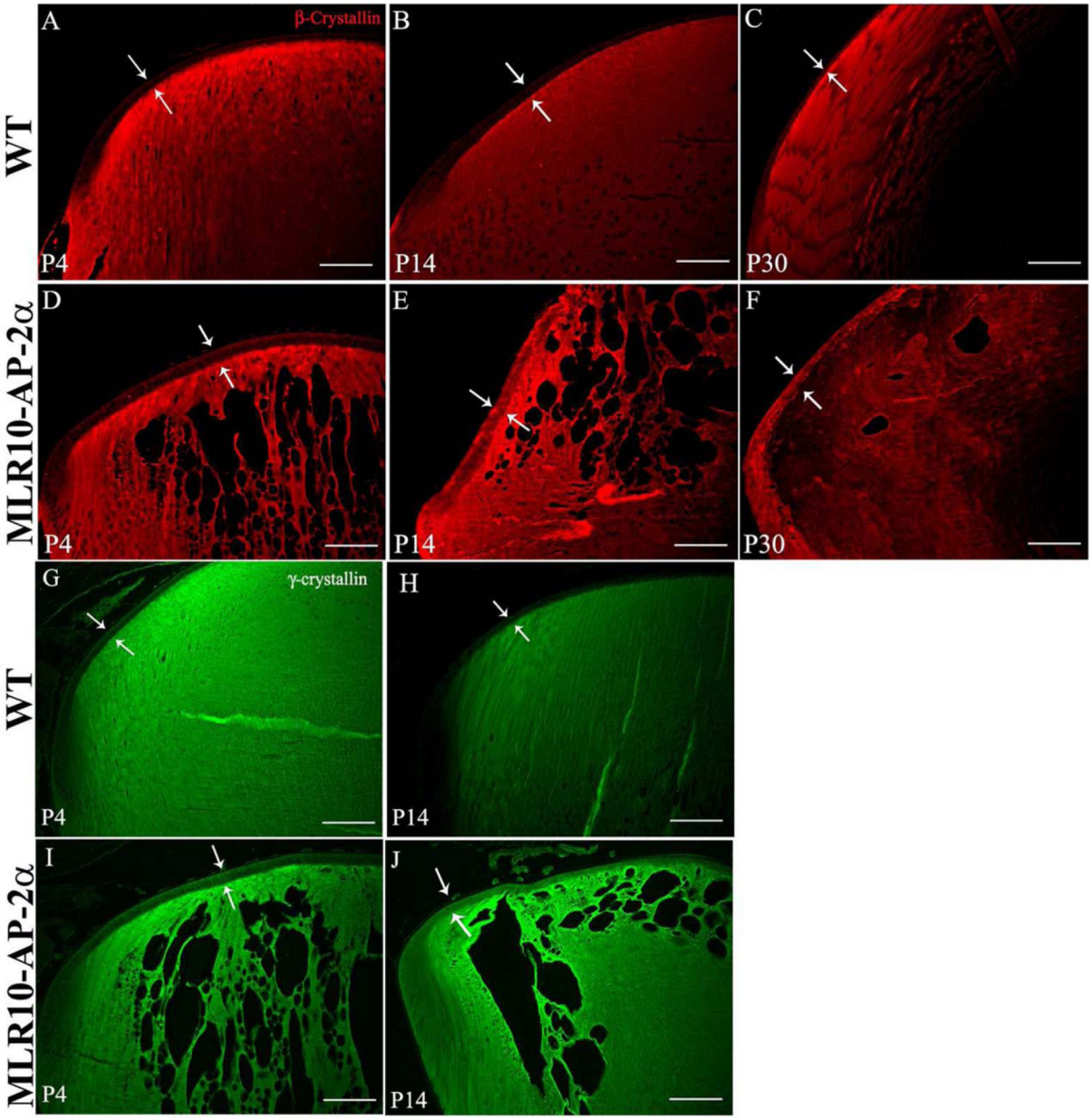


**Fig. 8.**

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,H**), while some γ-Crystallin is expressed in the mutant (**I,J**). Red staining, βB1-crystallin; Green staining, γ-crystallin. Scale bars = 100 μm.

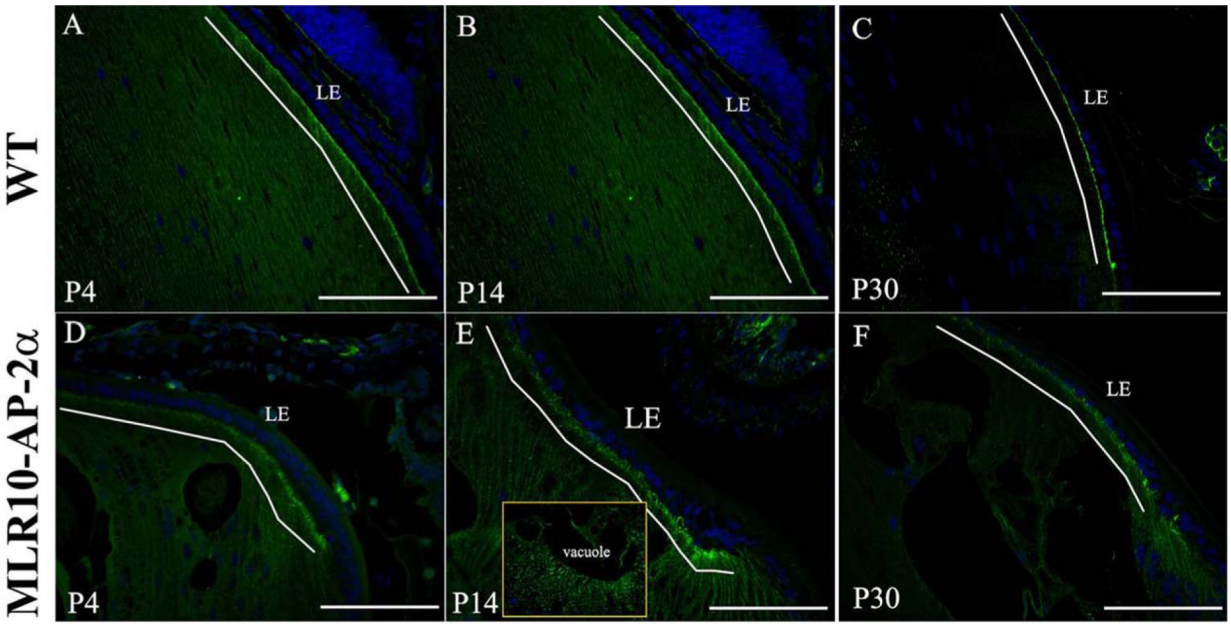


**Fig. 9.**

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). LE-Lens Epithelium. Green Staining, ZO-1; Blue staining, DAPI. Scale bars = 100 μm.