Loss of Axin2 Causes Ocular Defects During Mouse Eye Development

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Purpose. The scaffold protein Axin2 is an antagonist and universal target of the Wnt/β-catenin pathway. Disruption of *Axin2* may lead to developmental eye defects; however, this has not been examined. The purpose of this study was to investigate the role of Axin2 during ocular and extraocular development in mouse.

Methods. Animals heterozygous and homozygous for a $Axin2^{lacZ}$ knock-in allele were analyzed at different developmental stages for reporter expression, morphology as well as for the presence of ocular and extraocular markers using histologic and immunohistochemical techniques.

Results. During early eye development, the $Axin2^{lacZ}$ reporter was expressed in the periocular mesenchyme, RPE, and optic stalk. In the developing retina, $Axin2^{lacZ}$ reporter expression was initiated in ganglion cells at late embryonic stages and robustly expressed in subpopulations of amacrine and horizontal cells postnatally. Activation of the $Axin2^{lacZ}$ reporter overlapped with labeling of POU4F1, PAX6, and Calbindin. Germline deletion of *Axin2* led to variable ocular phenotypes ranging from normal to severely defective eyes exhibiting microphthalmia, coloboma, lens defects, and expanded ciliary margin. These defects were correlated with abnormal tissue patterning in individual affected tissues, such as the optic fissure margins in the ventral optic cup and in the expanded ciliary margin.

Conclusions. Our results reveal a critical role for Axin2 during ocular development, likely by restricting the activity of the Wnt/β-catenin pathway.

Keywords: axin2, Wnt, coloboma, reporter, RPE

Signaling by Wnt/β-catenin controls several critical processes in the developing and adult vertebrate eye; it regulates proliferation, differentiation, dorsoventral patterning, differentiation of the RPE, ciliary body, and iris, and modulates lens development and retinal angiogenesis.[1–21] In addition, the pathway is fine-tuned by antagonists that are critical for normal eye development,[22–31] and can be activated by Wnt-unrelated agonists (e.g., Norrin).[32] In mice and humans, 19 Wnt ligands are identified that bind to several surface receptors, including the Frizzled family of transmembrane proteins that activate different pathways.[33] The canonical Wnt/β-catenin pathway is the best characterized and functions through stabilization of β-catenin, its translocation into the nucleus, and activation of T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors. Axis inhibition proteins (AXIN1, AXIN2) act as scaffold proteins and associate with the Wnt signaling components Dishevelled, the serine/threonine kinase GSK3b, APC, and CKI to control β-catenin degradation[34–39] (for reviews, see Refs. 40–42). In the absence of a Wnt signal, this "destruction" complex phosphorylates β-catenin, thereby targeting it for subsequent degradation through the ubiquitin-proteasome pathway. Activation of Frizzled receptors and LRP5/6 coreceptors perturbs formation of the destruction complex, allowing stable β-catenin to accumulate in the cytoplasm. Axis inhibition 1 (*Axin1*) is ubiquitously expressed and loss of function in mouse results in early embryonic lethality; *Axin2* (conductin, axil) expression is more restrictive and is transcriptionally activated by Wnt/β-catenin signaling.[43,44] Thereby, AXIN2 acts as a negative feedback regulator, and its activity is modulated by tankyrase and CDC20.[43,45–47] Consistent with being a Wnt target, lineage tracing in mouse revealed that *Axin2* is expressed in cell populations responsive to Wnt/β-catenin signaling, often in cells with stem cell capacity.[48–51] In humans, *Axin2* mutations are associated with colorectal cancer and oligodontia.[52,53] Mice with an inactivated *Axin2* gene survive, with defects in skull formation (premature fusion of the posterior-frontal suture, reminiscent of craniosynostosis in humans) and bone remodeling.[54–57]

Transgenic mouse reporter lines have been used to determine Wnt/β-catenin pathway activity during mouse eye development. Possibly due to variegation effects, expression in the developing and adult retina varies among the different reporter lines.[58–61] Importantly, we demonstrated in the TOPgal line that this TCF/LEF reporter is activated in embryonic retinal progenitor cells in the absence of β-catenin expression.[62] Thus, TCF/LEF reporter lines can be expressed independently of Wnt/β-catenin signaling, which confounds a faithful analysis of Wnt/β-catenin activation.

To obtain a more accurate and comprehensive picture of Wnt/β-catenin activation during embryonic and postnatal eye development, we analyzed expression of the $Axin2^{lacZ}$ reporter, which drives expression of *LacZ* from the endogenous *Axin2* locus.[63] It was generated by inserting *lacZ* into the endogenous start codon, thereby replacing most of exon 2 and

inactivating the *Axin2* gene.[63] Our results show that *Axin2^{lacZ}* activation starts during late embryogenesis in ganglion cells, and is postnatally upregulated in horizontal cells and amacrine cells, and occasionally in photoreceptors. Furthermore, it was recently noted that *Axin2$^{lacZ/lacZ}$* mice display ocular abnormalities, but a detailed investigation is lacking.[55,57] Here, we demonstrate that disruption of *Axin2* results in severe ocular defects during optic cup morphogenesis, such as abnormal development of the anterior segment and a defect in closure of the optic fissure.

Materials and Methods

Mice

Axin2^{lacZ} mice were obtained from Jackson Laboratory (City, State, Country) and maintained on a C57BL/6 genetic background (Charles River, Hollister, CA, USA).[63] Animals heterozygous and homozygous for the *Axin2^{lacZ}* allele are here referred to as *Axin2^{lacZ}* and *Axin2$^{lacZ/lacZ}$* mice, respectively. Noon on the day of detection of the vaginal plug is counted embryonic day 0.5 (E0.5). Animals were genotyped by PCR using the following primer combinations: Cs: 5′-AAG CTG CGT CGG ATA CTT GAG A-3′, Cwt: 5′-AGT CCA TCT TCA TTC CGC CTA GC-3′, and ClacZ: 5′-TGG TAA TGC TGC AGT GGC TTG-3′. These primers produce the *Axin* wt (493 bp) and *Axin lacZ* amplicons (400 bp).[57] In the course of this study, we found that the *Axin2^{lacZ}* mouse line contained the Rd8 mutation, which is caused by a mutation in *Crb1*.[64,65] Rd8 can lead to postnatal retinal disorganization, obvious at postnatal day 14 (P14), and subsequent photoreceptor degeneration[66]; however, we did not observe abnormal lamination of the adult retina. We also did not detect differences in *Axin2^{lacZ}* reporter expression in adult eyes between Rd8 heterozygous and Rd8 homozygous mutants. The reporter expression did vary in the photoreceptor layer of adult animals that did not correlate with Rd8 heterozygosity or homozygosity. Animal experiments were performed according to the guidelines of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Utah Institutional Animal Care and Use Committee.

X-gal Labeling

Embryos were fixed with 4% paraformaldehyde for 10 to 15 minutes (E11 embryos) or 20 minutes (postnatal eyes) at room temperature. Standard X-gal labeling was performed on whole embryos (E11) or directly on cryostat sections (adult eyes) for 12 to 24 hours at 30°C and postfixed with paraformaldehyde.

Immunohistochemistry

Heads and eyes were fixed in 4% paraformaldehyde, cryoembedded, and sectioned, usually at 12 µm. If necessary, cryostat sections were treated for antigen retrieval with hot citrate buffer (pH 6) or with 1% Triton X-100. The following primary antibodies or markers were used: BRN3A/POU4F1 (1:50, #AB1585; EMD Millipore, Billerica, MA, USA), BRN3 (1:50, sc-6026; Santa Cruz Biotechnology, Dallas, TX, USA), CALB1 (1:500, #AB1778; EMD Millipore), F-actin/Phalloidin (1:500, #A12379; Life Technologies, Carlsbad, CA, USA), β-galactosidase (1:5000, #55976; Cappel/MP Biomedicals, Aurora, OH, USA), β-galactosidase (1:750, generous gift from Nadean Brown, University of California, Davis, CA, USA), Laminin (1:2000, #ab30320; Abcam, Cambridge, MA, USA), LEF1 (1:100, #C12A5; Cell Signaling, Danvers, MA, USA), MITF (1:400, #X1405M; Exalpha, Exalpha Biologicals, Shirley, MA, USA), OTX1/2 (1:1500, #NG1734326; EMD Millipore), PAX2 (1:100, #PRB-276P; Covance/BioLegend, Dedham, MA, USA), PAX6 (1:300, #AB2237; EMD Millipore), PITX2 (1:1000, #PA1020-100; Capra Science, Angelholm, Sweden), PROX1 (1:1000, #11-002; AngioBio Co., San Diego, CA, USA), SOX9 (1:50, #AB5535; EMD Millipore), TCF4 (1:100, #2569; Cell Signaling Technology), and VSX2 (1:300, #X1180P; Exalpha Biologicals). These antibodies were used in combination with the following secondary antibodies: Alexa 488/568/647–conjugated secondary antibodies (1:1000; Life Technologies), donkey-anti-goat TRITC (1:500, #705-025-147; Jackson ImmunoResearch, West Grove, PA, USA), and donkey-anti-sheep TRITC (1:500, #713-165-003; Jackson ImmmunoResearch). We analyzed 5 and 4 *Axin2$^{lacZ/lacZ}$* embryos with uni- or bilateral ocular defects at E12.5 and E15.5, respectively (see also Results for further details). Each marker was analyzed in two to four tissue sections in central regions of each eye, and several markers were analyzed repeatedly.

Imaging

Epifluorescent images were taken with an Olympus XM10 camera (Olympus, Tokyo, Japan) on an upright Olympus BX51 microscope and were processed in Adobe Photoshop (CS3) (Adobe Systems, Inc., San Jose, CA, USA). Confocal images were taken with an Olympus FV1000 and processed using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and Adobe Photoshop (CS3). All other images were taken with an Olympus U-CMAD3/Microfire camera mounted on the aforementioned microscope or on an Olympus SZX12 stereomicroscope.

Results

Reporter activation of *Axin2^{lacZ}* was detectable in the dorsal and ventral RPE of the optic cup at E11 (Figs. 1A, 1B), consistent with previous results in transgenic TCF/LEF reporters.[2,62,67–69] However, in contrast to the TOPgal and TCF/LEF reporter lines, *Axin2^{lacZ}* was not activated in the embryonic retina up to E14.5 (Fig. 1C).[62,68,69] At this age, few individual cells in the ganglion cell layer (GCL) started to show expression, and the number of cells in the GCL increased subsequently (Fig. 1D). At early postnatal ages, cells in the inner nuclear layer (INL) began to express *Axin2^{lacZ}* (Fig. 1E). At approximately P15, reporter activation was detectable in the GCL, and in diverse populations in the INL (Fig. 1F), and this pattern did not change significantly in the GCL and INL (Figs. 1G, 1H); however, occasionally robust labeling of the photoreceptor layer was observed in adult eyes (Fig. 1H). This variability in reporter activation did not correlate with the Rd8 mutation; we observed either presence or absence of this *LacZ* expression pattern in the photoreceptor layer in animals heterozygous as well as homozygous for Rd8. Thus, the reason for the variable *Axin2^{lacZ}* reporter activation in adult photoreceptors is unclear. Interestingly, Liu et al.[50] observed an increase of X-gal labeling in the outer nuclear layer (ONL) when retinal explants of *Axin2^{lacZ}* reporter mice are stimulated with exogenous R-spondin that can activate Wnt/β-catenin signaling in the presence of Wnts. This suggests that the *Axin2^{lacZ}* reporter expression pattern in photoreceptors in our study may be activated in response to endogenous levels of Wnt signaling. Thus, endogenous activation of Wnt signaling may vary between individual animals, causing variable labeling of the ONL as observed in Figures 1G and 1H.

To identify retinal cell types expressing the *Axin2^{lacZ}* reporter, we performed colabeling with several antibodies recognizing differentiated retinal cell populations in P15 to P21

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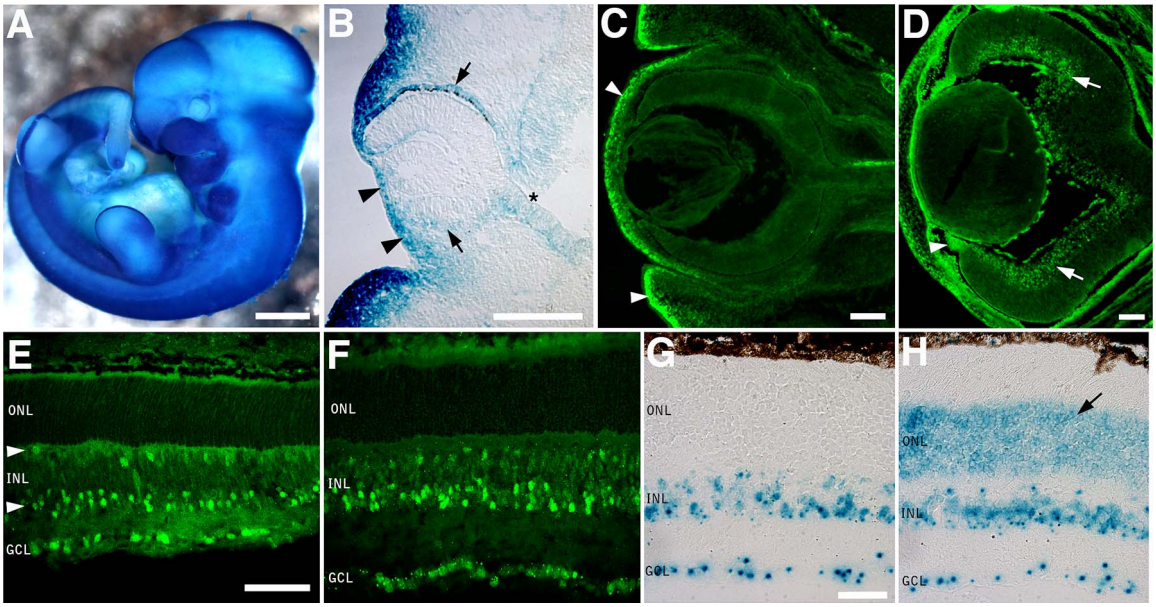

Figure 1. Dynamic activation of *Axin2*^{lacZ} in the developing and mature mouse eye. Whole embryos, and coronal and transversal cryostat ocular sections were stained with X-gal substrate to detect β-galactosidase activity or labeled with a β-galactosidase antibody. (**A**) At E11.5, the *Axin2*^{lacZ} reporter is activated in many regions of the mouse embryo, including the eye, limbs, facial primordia, and the dorsal brain. (**B**) In the E11.5 optic cup, *Axin2*^{lacZ} is expressed in the dorsal and ventral RPE (*arrows*), extraocular and corneal mesenchyme (*arrowheads*), and in the optic stalk (*asterisks*). (**C**) At E14.5, *Axin2*^{lacZ} expression is robust in the extraocular and eyelid mesenchyme (*arrowheads*). (**D**) At E17.5, expression is detectable cells in the differentiated/ presumptive GCL (*arrows*) and in the ciliary margin (*arrowhead*). (**E**) Shortly after birth (postnatal day 5: P5), cells in the GCL and several cells in the presumptive INL show reporter activation (*arrowheads*). (**F**) At P15, the *Axin2*^{lacZ} reporter is robustly expressed in several cell populations in the INL and in cells in the GCL. (**G**, **H**) *Axin2*^{lacZ} reporter activation in the adult retina of Rd8 homozygous mutant mice. (**G**) The *Axin2*^{lacZ} expression pattern is largely maintained in the adult retina. (**H**) In addition, we observed several animals exhibiting variable *Axin2*^{lacZ} reporter activation in the photoreceptor layer, shown here in most if not all photoreceptor cells (*arrow*). We found the presence or absence of this *LacZ* expression pattern in the photoreceptor layer in animals heterozygous and homozygous for Rd8; thus, we conclude that it did not correlate with Rd8. *Scale bars*: (**A**) 1 mm, in (**B**, **C**, **D**) 100 μm, in (**E**) 50 μm, in (**G**) 20 μm.

eyes. The calcium-binding protein Calbindin (CALB1) is present in horizontal cells and amacrine cells in the INL and in the GCL (Fig. 2A).[70] Activation of *Axin2*^{lacZ} overlapped with CALB1-positive horizontal cells in the INL and few CALB1-positive amacrine cells in the GCL (Figs. 2B, 2C); *Axin2*^{lacZ} was also expressed in PAX6-positive amacrine cells in the INL and GCL (Figs. 2D–F). In addition, *Axin2*^{lacZ} reporter expression was observed in many ganglion cells that are labeled for the transcription factor POU4F1 (Figs. 2G–I). Furthermore, we investigated whether Müller glia labeled for SOX9 show *Axin2*^{lacZ} expression. As previously reported,[50] we observed little overlap (Figs. 2K, 2L). Finally, transcriptional activation downstream of Wnt/β-catenin signaling is mediated by TCF/LEF transcription factors. With the exception of a few cells, *Axin2*^{lacZ} reporter-expressing cells did not show TCF4 colabeling (Figs. 2N–P). Because LEF1 is not detectable in the adult central retina (Supplementary Fig. S1), this suggests that other downstream effectors may be used in *Axin2*^{lacZ}-expressing cells, such as TCF1; however, we were not able to find a satisfying TCF1 antibody. Overall, our results showed that the *Axin2*^{lacZ} reporter is activated in ganglion and horizontal cells, as well as in amacrine cell populations. Subsequently, we examined the role of Axin2 in development of these cell populations by analyzing mice homozygous for the *Axin2*^{lacZ} reporter (*Axin2*$^{lacZ/lacZ}$), which results in global inactivation of the *Axin2* gene. Interestingly, although we detected severe defects in early ocular development in some *Axin2*$^{lacZ/lacZ}$ mice (see below), no changes in differentiation of retinal cell populations labeled for PAX6, CALB1, POU4F1, TCF4, and SOX9 protein were observed in mutant eyes without obvious abnormalities (Supplementary Fig. S2).

Homozygous Axin2 inactivation resulted in variable ocular defects in 36% of the *Axin2*$^{lacZ/lacZ}$ embryos and 25% of *Axin2*$^{lacZ/lacZ}$ eyes between E12.5 and E15.5 (Fig. 3; $n = 44$ embryos, 17 litters). We analyzed five *Axin2*$^{lacZ/lacZ}$ embryos with ocular defects in eight eyes at E12.5 and four *Axin2*$^{lacZ/lacZ}$ embryos with four abnormal eyes at E15.5. We observed coloboma, anterior segment with variable lens abnormalities and ventral optic cup defects in *Axin2*$^{lacZ/lacZ}$ embryos at E12.5 (Figs. 3A–F; see below). At later stages, eyelid closure defects were occasionally detectable and affected eyes can be severely microphthalmic (Figs. 3G–K). *Axin2*$^{lacZ/lacZ}$ mice showed cranial defects as previously described (craniosynostosis; Figs. 3I, 3K, arrowheads).

Histologic and immunohistochemical analysis of the ocular defects in *Axin2*$^{lacZ/lacZ}$ embryos revealed that colobomatous eyes in *Axin2*$^{lacZ/lacZ}$ embryos exhibited a defect in closure of the optic fissure, confirmed by persistent presence of the basement membrane marker Laminin (Figs. 4A–D; six of six colobomatous eyes; $n = 4$ embryos). The transcription factor PAX2 is required for optic fissure closure during optic cup morphogenesis and *Pax2* mutations in humans cause coloboma, among other developmental abnormalities. (for review, see Ref. 71) We observed that PAX2 labeling showed a largely normal pattern in both margins of the optic fissure of *Axin2*$^{lacZ/lacZ}$ optic cups (Fig. 4F; four of five colobomatous eyes; $n = 3$ embryos). In addition, apicobasal polarity appeared undisturbed, because F-actin showed normal apical distribution in the fissure margins (Fig. 4H; four of five colobomatous eyes; $n = 3$ embryos). The transcription factor *Pitx2* is expressed in the periocular mesenchyme and positively regulated by Wnt/β-catenin signaling.[72] Mutations in *Pitx2* cause Axenfeld-Rieger syndrome resulting from abnormal

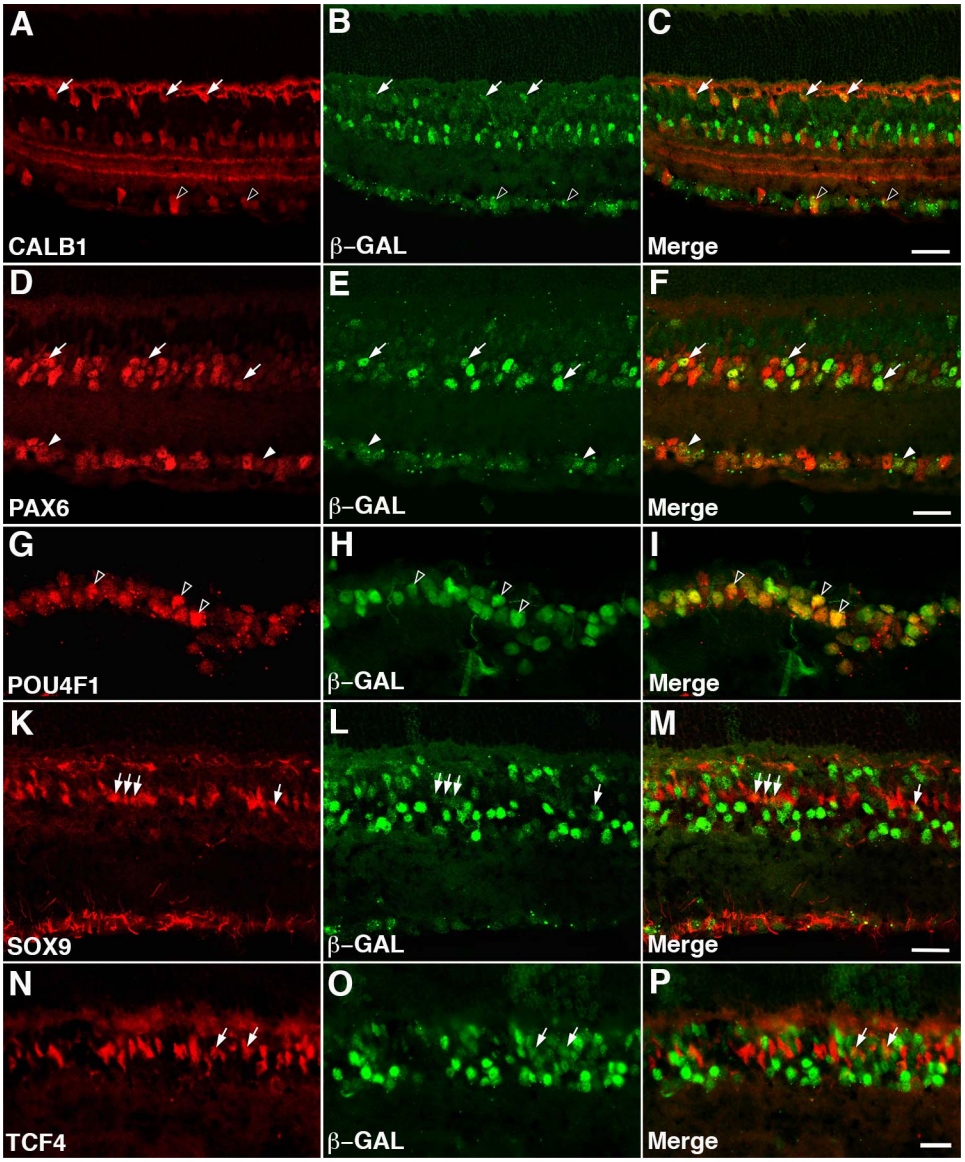

Figure 2. The *Axin2*^{lacZ} reporter is present in specific cell populations in the postnatal retina. Transversal sections were double-labeled with antibodies detecting cell type–specific markers (*red*; **A**, **D**, **G**, **K**, **N**) and β-gal protein (*green*; **B**, **E**, **H**, **L**, **O**), merge shown in (**C**, **F**, **I**, **M**, **P**). (**A**–**C**) At P15, *Axin2*^{lacZ} reporter-positive horizontal cells and cells in the GCL colabel for CALB1 (*arrows* and *open arrowheads*, respectively). (**D**–**F**) PAX6-positive amacrine cells (*arrows*) and cells in the GCL (*arrowheads*) show *Axin2*^{lacZ} reporter activation at P15. (**G**–**I**) Reporter activation in P15 ganglion cells is confirmed by POU4F1 colabeling (*arrowheads*). (**K**–**M**) Very rarely, labeling of the *Axin2*^{lacZ} reporter overlapped with the Müller Glia marker SOX9, shown here at P21 (*arrows*). (**N**–**P**) Few *Axin2*^{lacZ} reporter-expressing cells colabel for TCF4 protein (**P**, *arrows*). *Scale bars*: (**C**) 30 μm, in (**F**, **M**) 20 μm, in (**P**) 10 μm.

anterior segment development, which can lead to coloboma. However, we observed no obvious changes in mesenchymal PITX2 labeling in *Axin2*$^{lacZ/lacZ}$ embryos (Fig. 4J; five of five colobomatous eyes; $n = 3$ embryos).

Although general patterning of the optic cup neuroepithelium into retina and RPE appeared normal in *Axin2*$^{lacZ/lacZ}$ optic cups (see Fig. 6), we observed changes of gene expression particularly in cells lining the margins of the optic fissure (Fig. 5). Visual system homeobox 2 (VSX2) protein is normally found in retinal progenitors throughout the optic cup at E12.5 (Fig. 5A). Several affected *Axin2*$^{lacZ/lacZ}$ optic cups showed absent VSX2 labeling in cells comprising the margins of the open optic fissure (Fig. 5B; arrows; three of five colobomatous eyes, $n = 4$ embryos). Retinal pigment epithelium markers such as OTX and MITF are normally not present in the region of the optic fissure after closure is completed, except in the RPE in the ventral optic cup (Figs. 5C, 5E, 5G). Interestingly, in *Axin2*$^{lacZ/lacZ}$ optic cups, OTX1/2 and MITF were extended into the optic fissure margins that exhibited a defect in closure, suggesting that RPE tissue persists in the optic fissure (Figs. 5D–H) (MITF: eight of eight colobomatous eyes; $n = 5$ embryos; OTX2: five of eight colobomatous eyes; $n = 5$ embryos). Immunolabeling for *LacZ* did not reveal a consistent, robust increase in activity of the *Axin2*^{lacZ} reporter, occasionally the reporter appeared weakly upregulated in the margins of *Axin2*$^{lacZ/lacZ}$ eyes (Fig. 5J, arrow; four of eight colobomatous eyes, $n = 5$ embryos). Normally LEF1 labeling is not robustly detectable in the ventral RPE in the optic fissure (Supplementary Fig. S3A), and we did not observe an increase of LEF1 in *Axin2*$^{lacZ/lacZ}$ eyes

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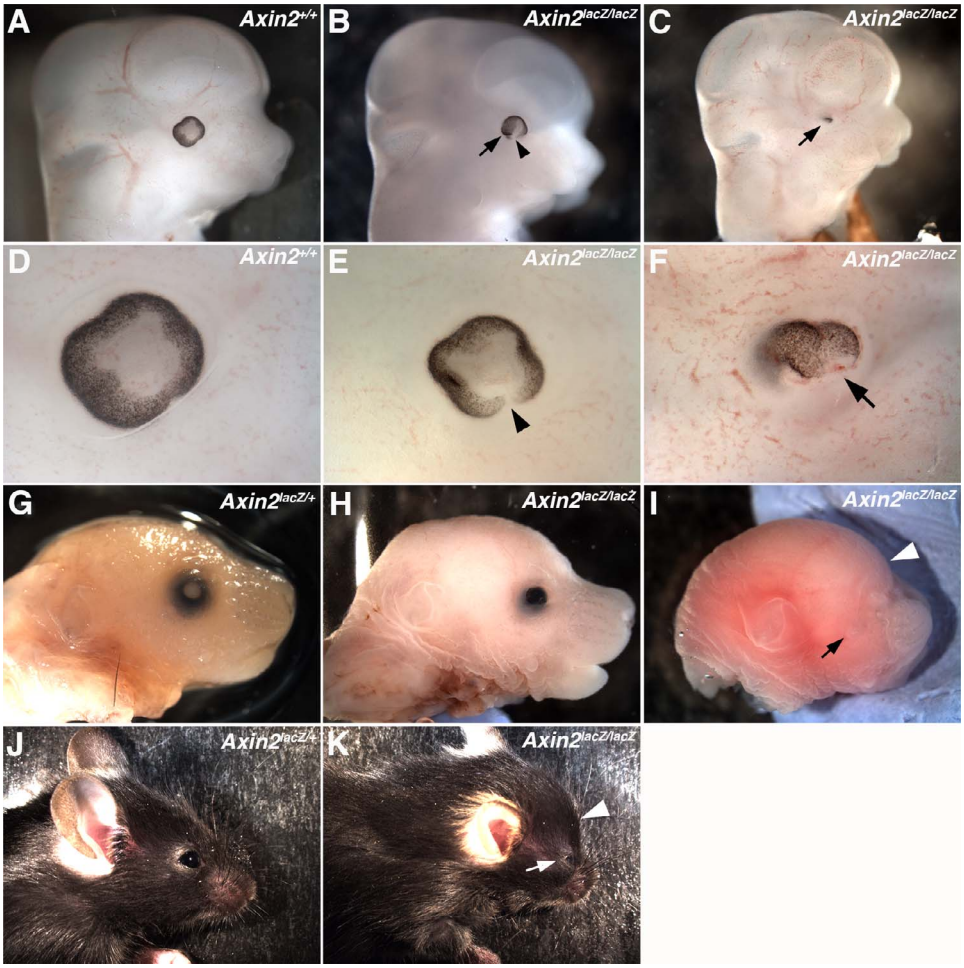

Figure 3. Eye development defects in *Axin2*$^{lacZ/lacZ}$ embryos, in particular abnormal pigment and lens formation, coloboma and microphthalmia. (**A**) Lateral view of control eye at E12.5. (**B–F**) Range of severities of ocular phenotypes found in E12.5 *Axin2*$^{lacZ/lacZ}$ embryos, for example, hyperpigmentation (**B**, *arrow*) and coloboma (**B**, *arrowhead*) or, in rare cases, largely absent pigment and severe microphthalmia (**C**). (**D**) Control eye at higher magnification. (**E**) Mild ocular defect with ventral coloboma (*arrowhead*). (**F**) Severe hyperpigmentation dorsally, possibly ventral rotation of the eye resulting in severely reduced ventral optic cup (*arrow*). (**G**) Lateral view of control embryo at E17.5 showing closed eyelid. (**H**) *Axin2*$^{lacZ/lacZ}$ embryos at E17.5 with eyelid closure defect and hyperpigmentation. (**I**) Extreme microphthalmia in *Axin2*$^{lacZ/lacZ}$ embryos at E17.5. (**J**) Adult control animal at P23. (**K**) Adult *Axin2*$^{lacZ/lacZ}$ embryos with microphthalmia (*arrow*). *White arrowheads* in **I**, **K** point to cranial defects (craniosynostosis).

(Supplementary Fig. S3B). Thus, Wnt/β-catenin activation in the domain of persistent RPE in the optic fissure margins was not clearly detectable.

At later embryonic ages, ectopic expansion of the optic cup periphery became more evident (Figs. 3H, 6). At E15.5, the ectopic tissue in the *Axin2*$^{lacZ/lacZ}$ optic cup margin did not show labeling for the retinal marker VSX2 (Fig. 6B; arrow; three of four affected eyes; $n = 4$ embryos). Paired box 6 (Pax6) protein was present in the ciliary margin of controls (Fig. 6C) and in the ectopic tissue in the margin of *Axin2*$^{lacZ/lacZ}$ optic cups (Fig. 6D; three of four affected eyes; $n = 4$ embryos). In control eyes, OTX1/2 protein is normally found in the RPE, retinal progenitors, and lower levels are present diffusely in the presumptive ciliary body and iris (Fig. 6E; arrow). In *Axin2*$^{lacZ/lacZ}$ eyes, the ectopic tissue in the dorsal optic cup showed a widespread OTX1/2 labeling pattern, reminiscent of the ciliary margin in controls (Fig. 6F; arrow; four of four affected eyes; $n = 4$ embryos). Compared with control eyes, lens vesicles appeared smaller in three of four affected *Axin2*$^{lacZ/lacZ}$ eyes and labeled for Pax6 and Prox1 protein (Figs. 6D, 6H), suggesting that some aspects of lens differentiation can be maintained. Elevated expression of the Wnt target LEF1 confirmed that the ectopic tissue in *Axin2*$^{lacZ/lacZ}$ eyes acquired a peripheral retina fate (Fig. 6J; arrow; three of four affected eyes; $n = 4$ embryos). Similar to E12.5, PITX2 showed largely normal expression in *Axin2*$^{lacZ/lacZ}$ mesenchyme (Fig. 6L; three of three affected eyes; $n = 3$ embryos). Collectively, our data suggest that the hyperplastic tissue in *Axin2*$^{lacZ/lacZ}$ eyes shows features of presumptive ciliary body and iris.

Discussion

The purpose of our study was to characterize activation of the universal target and negative regulator of the Wnt/β-catenin pathway Axin2 in the developing mouse eye using the knock-in *LacZ* reporter *Axin2*^{lacZ}.[63] In addition, we investigated the ocular phenotype in *Axin2*$^{lacZ/lacZ}$ eyes. Our results demonstrate that *Axin2*^{lacZ} is dynamically expressed; it becomes activated in subpopulations of diverse retinal cell types mostly during postnatal differentiation. During optic cup morphogenesis, *Axin2*^{lacZ} is activated in the presumptive RPE, ciliary

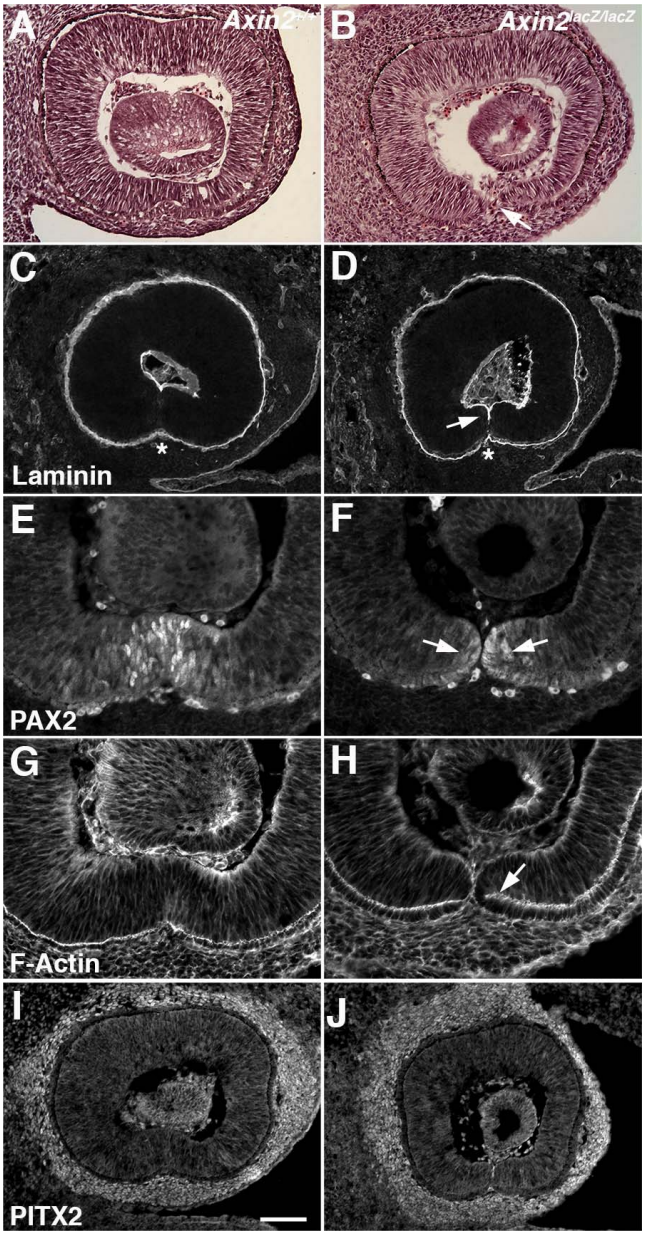

Figure 4. Defects in closure of the optic fissure in *Axin2*$^{lacZ/lacZ}$ embryos. Sagittal views of E12.5 optic cups. (**A**, **B**) Hematoxylin and eosin–stained control (**A**) and *Axin2*$^{lacZ/lacZ}$ optic cup (**B**). *Arrow* points to the abnormally open optic fissure in the ventral optic cup of mutant eyes. (**C**) Laminin labeling in the basement membrane surrounds ocular tissues in control eyes and is not detectable in the optic fissure (*asterisk*). (**D**) Persistent Laminin labeling in the *Axin2*$^{lacZ/lacZ}$ ventral optic cup (*arrow*). *Asterisk* marks the optic fissure. (**E**, **F**) Pax2 is present in the ventral optic cup of controls (**E**) and *Axin2*$^{lacZ/lacZ}$ eyes (**F**, *arrows*). (**G**) F-Actin localization marks the apical border of retina and RPE in controls. (**H**) Apical distribution of F-Actin is maintained in *Axin2*$^{lacZ/lacZ}$ optic cups (*arrow*). (**I**) The transcription factor PITX2 is present in the extraocular mesenchyme surrounding the optic cup. (**J**) In *Axin2*$^{lacZ/lacZ}$ embryos, extraocular mesenchyme labeling of PITX2 appears normal. *Scale bar*: 100 μm.

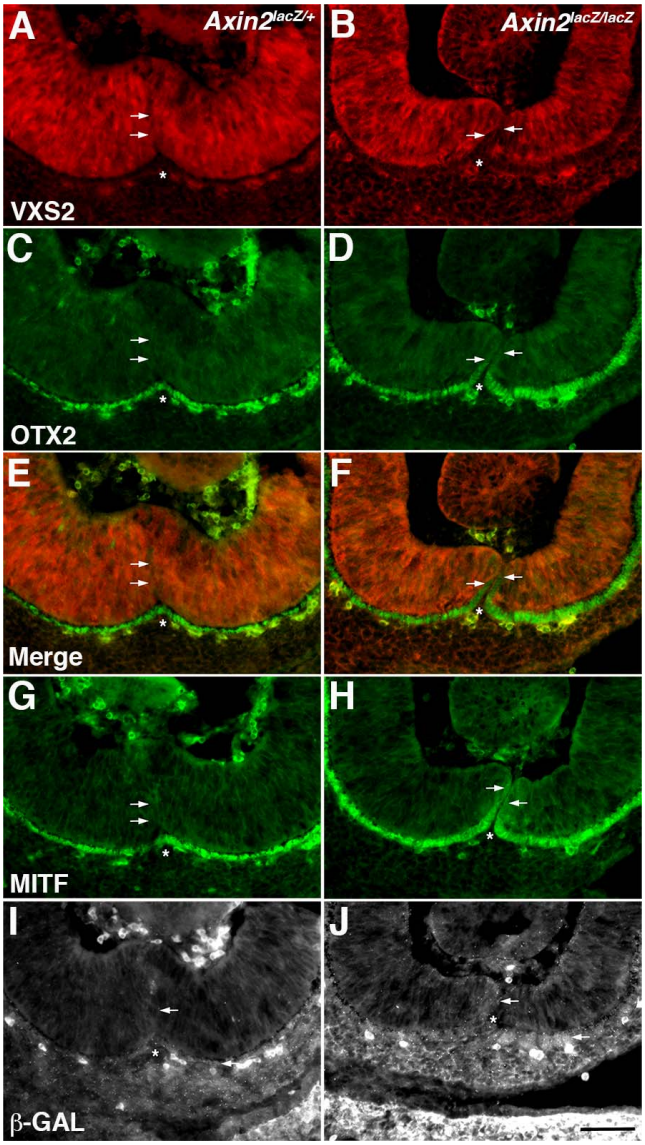

Figure 5. Persistence of RPE tissue in the optic fissure margins in *Axin2*$^{lacZ/lacZ}$ ventral optic cups. Sagittal views of E12.5–E13.0 ventral optic cups. (**A**) Control embryo showing continuous VSX2 labeling in the ventral retina (*arrows*), also in the region of the fused optic fissure (*asterisks*). (**B**) In some of the affected *Axin2*$^{lacZ/lacZ}$ ventral optic cups, VSX2 labeling is discontinuous in the region of the optic fissure (*arrows*). (**C**) In controls, OTX1/2 labeling in the ventral optic cup is confined to the RPE and absent in the optic fissure (*arrows*). (**D**) The *Axin2*$^{lacZ/lacZ}$ ventral optic cup shows persistent OTX1/2 labeling in the margins of the optic fissure that is continuous with the RPE in the ventral optic cup (*arrows*). (**E**, **F**) Merge of VSX2 and OTX1/2 staining in control (**E**) and *Axin2*$^{lacZ/lacZ}$ optic cups (**F**). (**G**) Similar to OTX1/2, MITF is restricted to the ventral RPE and excluded from the optic fissure region in control embryos (*arrows*). (**H**) In the margins of the defective optic fissure of *Axin2*$^{lacZ/lacZ}$ embryos, MITF is persistent (*arrows*). (**I**, **J**) In *Axin2*$^{lacZ/lacZ}$ optic cups, *Axin2*^{lacZ} reporter expression does not show robust upregulation in the optic fissure margins and in the RPE, as shown by β-GAL immunolabeling (**J**, *arrows*). *Scale bar*: 30 μm.

body, and iris, and in the extraocular mesenchyme. Furthermore, we show that Axin2 exerts distinct roles during ocular development; on disruption of the *Axin2* gene, microphthalmia, coloboma, and an expanded ciliary margin are observed. These phenotypes occur with varying severity and incomplete penetrance, suggesting that other factors may interact with Axin2 to regulate ocular development.[73] In humans, ocular defects have not been associated with *Axin2* mutations, thus, it is possible that Axin2 has distinct, species-specific functions in the developing eye of mouse and humans.

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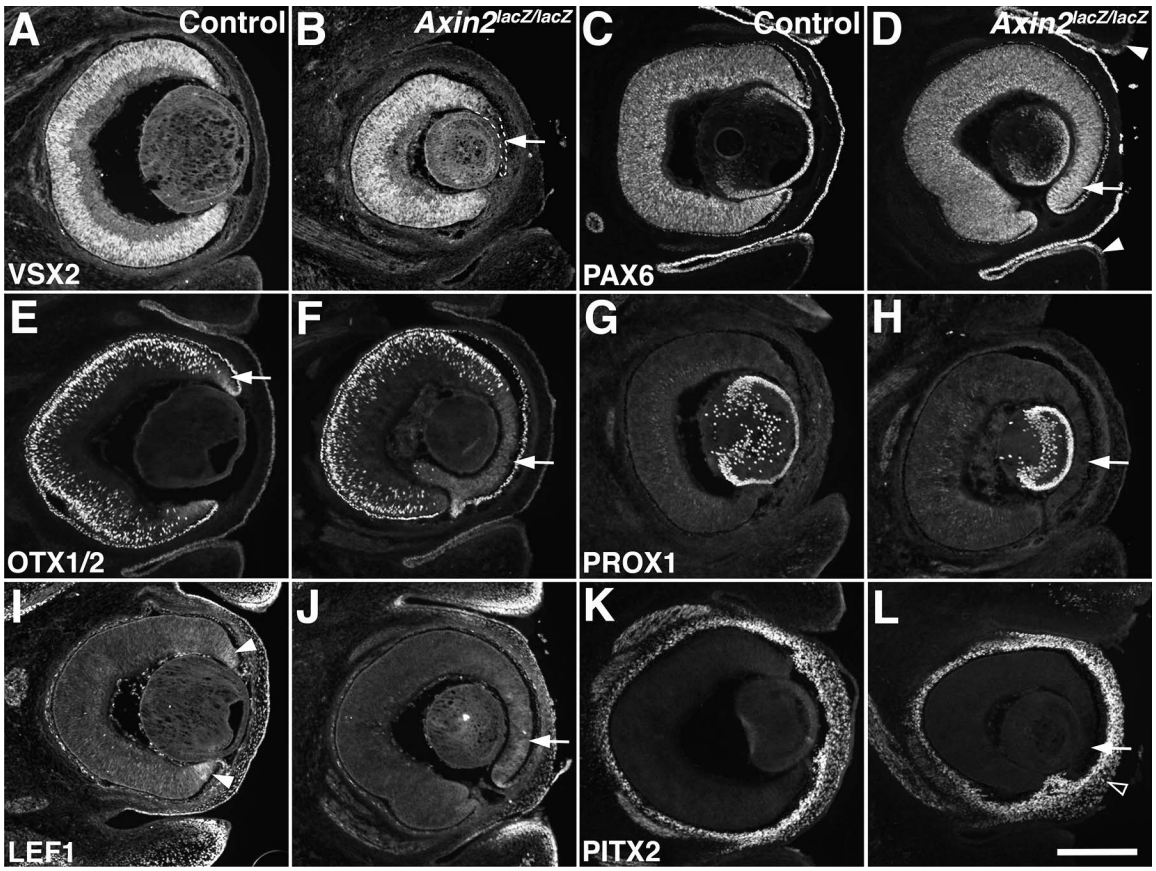

Figure 6. *Axin2*$^{lacZ/lacZ}$ embryonic eyes show ectopic expansion of the peripheral optic cup. Coronal views of E15.5 optic cups. (**A**) Protein labeling for the homeobox transcription factor VSX2 in retinal progenitor cells in controls. (**B**) VSX2 labeling pattern in the central retina of *Axin2*$^{lacZ/lacZ}$ optic cups appears normal. *Arrow* points to the expanded area in the dorsal ciliary margin that exhibits reduced VSX2 labeling (*dotted lines*). (**C**, **D**) The paired homeobox transcription factor Pax6 is present in many ocular and extraocular tissues. (**D**) Pax6 labeling is found in the ectopic tissue in *Axin2*$^{lacZ/lacZ}$ dorsal optic cups (*arrow*). *Arrowheads* label the eyelid primordia. (**E**, **F**) Otx1/2 protein is normally found in the RPE, surface ectoderm, retinal progenitors, and ciliary margin. (**F**) In *Axin2*$^{lacZ/lacZ}$ embryos, the abnormally expanded region of the dorsal optic cup shows a OTX1/2 labeling pattern typical for the ciliary margin. (**G**, **H**) PROX1 labeling in the embryonic lens is not significantly altered in *Axin2*$^{lacZ/lacZ}$ embryos. (**I**, **J**) The HMG transcription factor LEF1 is present in the eyelid and corneal mesenchyme, and in the ciliary margin (*arrowheads*). (**J**) The abnormal expansion of the dorsal optic cup in *Axin2*$^{lacZ/lacZ}$ mutants shows increased LEF1 labeling (*arrow*). (**K**, **L**) PITX2 labeling in the extraocular mesenchyme is not altered in *Axin2*$^{lacZ/lacZ}$ embryos (**L**). *Arrows* in (**B**, **D**, **F**, **H**, **J**, **L**) point to abnormal, expanded area in the dorsal optic cup of *Axin2*$^{lacZ/lacZ}$ embryos. *Scale bar*: 300 μm.

Optic Cup Morphogenesis Requires Proper Levels of Wnt Signaling

Axin2 acts as an inhibitor of the Wnt/β-catenin pathway; therefore, homozygous disruption in *Axin2*$^{lacZ/lacZ}$ mice is likely to result in increased nuclear localization of β-catenin and possibly in ectopic Wnt pathway activation.[56,57,69] However, Axin2 is considered a relatively weak inhibitor,[46] which could explain the variable penetrance and range of early ocular defects in *Axin2*$^{lacZ/lacZ}$ mutants. In *Axin2*$^{lacZ/lacZ}$ optic cups that display a closure defect of the optic fissure, we observed that labeling for the RPE markers OTX1/2 and MITF are persistent in the fissure margins. The Wnt/β-catenin pathway transcriptionally regulates expression of MITF and OTX2.[1,2,4] Thus, we expected that loss of *Axin2* leads directly to elevated Wnt activity resulting in abnormal maintenance or upregulation of RPE gene expression in the optic fissure margins; however, our results do not indicate a robust increase of *Axin2* activation in the optic fissure. It is possible that the reporter is not sensitive enough to detect potentially small changes of pathway activation in the optic fissure margins. Alternatively, the ubiquitous Axin1 may compensate for loss of *Axin2* and prevent significant upregulation of the pathway. Finally, we cannot exclude that Axin2 is regulated independent of Wnt/β-catenin signaling.

Interestingly, disruption of FGF signaling, which is required for retinal patterning in the optic cup, shows a very similar effect of persistent RPE labeling in the optic fissure margins, associated with colobomata.[74,75] Collectively, these and our present study show that proper acquisition of cell organization in the marginal cells of the optic fissure is crucial for the progression to closure and fusion. It will be interesting to further investigate the nature of the downstream molecular events that ensure subsequent attachment and fusion of the fissure margins.

In addition, our results demonstrate that the expanded ciliary margin shows elevated LEF1 labeling, consistent with an enlarged domain of Wnt/β-catenin pathway activation in this area (Fig. 6J). The observed defects in the anterior segment in *Axin2*$^{lacZ/lacZ}$ eyes are in agreement with previous studies demonstrating that balanced levels of Wnt/β-catenin pathway activation are required for proper eye development. A secreted antagonist, Dkk1, binds to the coreceptor LRP5/6, resulting normally in downregulation of the pathway.[22] Haploinsufficiency of *Dkk1* leads to defects in optic cup morphogenesis similar to *Axin2*$^{lacZ/lacZ}$ mutants, such as microphthalmia,

coloboma, lens abnormalities, and anterior segment defects.[26] Similar abnormalities are observed on ablation of the forkhead transcription factor *Foxg1*.[76–78] The abnormal expansion of ciliary margin tissue in *Foxg1* mutants could be explained by ectopic Wnt pathway activation. However, shh expression is decreased early in *Foxg1* mutants, which may contribute to the ocular defects.[76,77] In *apc* mutant zebrafish, an expanded ciliary margin zone and coloboma were observed.[27,29] Consistent with a role in cell fate determination, ectopic Wnt/β-catenin pathway activation in the retina by constitutively active β-catenin leads to transdifferentiation into peripheral fate.[5,79] Finally, several of the above-mentioned mouse models exhibit microphthalmia and coloboma to some degree, which may be due to ectopic Wnt/β-catenin activity, as discussed previously. Together with mouse models displaying reduced or absent Wnt/β-catenin pathway activity that are associated with coloboma and peripheral eye defects, our data are in agreement with the notion that Wnt/β-catenin activity needs to be tightly regulated during normal optic cup morphogenesis.[12,21]

Comparison Between *Axin2^{lacZ}* and Transgenic Wnt/β-Catenin Reporter Lines

Our study shows *Axin2^{lacZ}* activation in the dorsal optic cup, extraocular mesenchyme, differentiating ciliary body and iris, and optic stalk in the embryonic eye, which is consistent with other transgenic Wnt/β-catenin reporter lines. In addition, we observed *Axin2^{lacZ}* reporter expression in the differentiating cells of the GCL and inner INL in a pattern similar to the transgenic TCF/LEF line.[69] Our results demonstrate that *Axin2^{lacZ}* is activated in the adult retina in horizontal and occasionally in photoreceptor cells (Fig. 1), both of which are novel findings. However, a TCF/LEF-independent regulation of the *Axin2* gene cannot be excluded, which could be responsible for some of the reporter activation in retinal cell populations.[80,81]

In agreement with others, we observed very little overlap of *Axin2^{lacZ}* with markers for Müller glia (this study).[50] Under certain conditions, however, Müller glia show stem cell potential (for review, see Ref. 82) and exhibit *Axin2^{lacZ}* reporter expression in retinal explants after exogenous Wnt pathway activation.[50] Interestingly, ectopic Wnt pathway activation, due to complete inactivation of *Axin2*, causes Müller glia to proliferate and produce retinal progenitors.[50]

Recently, it was shown that retinal amacrine cells expressing the G-protein–coupled receptor Lgr5 display regenerative potential.[83] Lgr5 is a Wnt pathway target and bound by secreted R-spondin proteins to modulate Wnt pathway activation. It is possible that *Axin2^{lacZ}* overlaps with expression of Lgr5 in amacrine cells, because both populations express Pax6 in a similar pattern (this study: Fig. 2F; Ref. 83: Supplementary Fig. S2G). Both Axin2 and Lgr5 are stem cell markers in other systems; thus, some of the adult expression pattern of *Axin2^{lacZ}* may reflect a regenerative potential of retinal cells. Further studies are required to address the precise role of Axin2 in the diverse cell types in the adult retina.

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Investigative Ophthalmology & Visual Science

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