Loss of Axin2 Causes Ocular Defects During Mouse Eye Development

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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 degradation34–39 (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 oligodendroglioma.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 Axin2lacZ reporter, which drives expression of LacZ from the endogenous Axin2 locus.55 It was generated by inserting lacZ into the endogenous start codon, thereby replacing most of exon 2 and
inactivating the \textit{Axin2} gene.\textsuperscript{63} Our results show that \textit{Axin2}\textsubscript{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 \textit{Axin2}\textsubscript{lacZ} mice display ocular abnormalities, but a detailed investigation is lacking.\textsuperscript{55,57} Here, we demonstrate that disruption of \textit{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**

\textit{Axin2}\textsubscript{lacZ} mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a C57BL/6 genetic background (Charles River, Hollister, CA, USA).\textsuperscript{63} Animals heterozygous and homozygous for the \textit{Axin2}\textsubscript{lacZ} allele are here referred to as \textit{Axin2}\textsubscript{lacZ} and \textit{Axin2}\textsubscript{lacZ}\textsubscript{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 CCG CTA GGA ATG CAA TCT TCA TCA TTC CGC GTA CTG CC-3′, and ClacZ: 5′-TGG TAA TGC AAG GCC TTG-3′. These primers produce the \textit{Axin2}\textsubscript{wt} (493 bp) and \textit{Axin2}\textsubscript{lacZ} amplicons (400 bp).\textsuperscript{57} In the course of this study, we found that the \textit{Axin2}\textsubscript{lacZ} mouse line contained the Rdh8 mutation, which is caused by a mutation in \textit{Crb1}.\textsuperscript{64} A heterogeneous Rd8 homozygous mutants. The \textit{Axin2}\textsubscript{lacZ} 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, cryosectioned, 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), Factin/Phalloidin (1:500, #A12379; Life Technologies, Carlsbad, CA, USA), β-galactosidase (1:5000, #5579; 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; Exalpa, Exalpa Biologicals, Shirley, MA, USA), OTX1/2 (1:1500, #NG1753426; EMD Millipore), PAX2 (1:100, #PRB-276P; Covance/BioLegend, Dedham, MA, USA), PAX6 (1:300, #AB2237; EMD Millipore), TFI1X (1:100, #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 (1:500, #X1180P; Exalpa 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 Immunoresearch). We analyzed 5 and 4 \textit{Axin2}\textsubscript{lacZ}\textsubscript{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-CMADS/Microfire camera mounted on the aforementioned microscope or on an Olympus SZX12 stereo-microscope.

**Results**

Reporter activation of \textit{Axin2}\textsubscript{ 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.\textsuperscript{2,62,67–69} However, in contrast to the TOPgal and TCF/LEF reporter lines, \textit{Axin2}\textsubscript{lacZ} was not activated in the embryonic retina up to E14.5 (Fig. 1C).\textsuperscript{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 \textit{Axin2}\textsubscript{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. 1I). 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 \textit{Axin2}\textsubscript{lacZ} reporter activation in adult photoreceptors is unclear. Recently, Liu et al.\textsuperscript{50} observed an increased X-gal labeling in the outer nuclear layer (ONL) when retinal explants of \textit{Axin2}\textsubscript{lacZ} reporter mice are stimulated with exogenous R-spondin that can activate Wnt/β-catenin signaling. Thus, endogenous activation of \textit{Axin2}\textsubscript{lacZ} reporter expression pattern in photoreceptor cells in our study may be induced 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 \textit{Axin2}\textsubscript{lacZ} reporter, we performed colabeling with several antibodies recognizing differentiated retinal cell populations in P15 to P21...
eyes. The calcium-binding protein Calbindin (CALB1) is present in horizontal cells and amacrine cells in the INL and in the GCL (Fig. 2A).⁷° Activation of Axin2lacZ overlapped with CALB1-positive horizontal cells in the INL and few CALB1-positive amacrine cells in the GCL (Figs. 2B, 2C). Axin2lacZ was also expressed in PAX6-positive amacrine cells in the INL and GCL (Figs. 2D–F). In addition, Axin2lacZ 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 expression. As previously reported,⁹⁰ we observed little overlap (Figs. 2K, 2L). Finally, transcriptional activation downstream of Wnt/b-catenin signaling is mediated by TCF/LEF transcription factors. With the exception of a few cells, Axin2lacZ 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 Axin2lacZ expressing cells, such as TCF1; however, we were not able to find a satisfying TCF1 antibody. Overall, our results showed that the Axin2lacZ 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 Axin2lacZ reporter (Axin2lacZ/lacZ), which results in global inactivation of the Axin2 gene. Interestingly, although we detected severe defects in early ocular development in some Axin2lacZ/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 Axin2lacZ/lacZ embryos and 25% of Axin2lacZ/lacZ eyes between E12.5 and E15.5 (Fig. 3; n = 44 embryos, 17 litters). We analyzed five Axin2lacZ/lacZ embryos with ocular defects in eight eyes at E12.5 and four Axin2lacZ/lacZ embryos with four abnormal eyes at E15.5. We observed coloboma, anterior segment with variable lens abnormalities and ventral optic cup defects in Axin2lacZ/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). Axin2lacZ/lacZ mice showed cranial defects as previously described (craniosynostosis; Figs. 3L, 3K, arrowheads).

Histologic and immunohistochemical analysis of the ocular defects in Axin2lacZ/lacZ embryos revealed that colobomatous eyes in Axin2lacZ/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 Axin2lacZ/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 periciliary mesenchyme and positively regulated by Wnt/b-catenin signaling.² Mutations in Pitx2 cause Axenfeld-Rieger syndrome resulting from abnormal
anterior segment development, which can lead to coloboma. However, we observed no obvious changes in mesenchymal PITX2 labeling in Axin2lacZ/lacZ embryos (Fig. 4J; five of five colobomatous eyes; n = 3 embryos).

Although general patterning of the optic cup neuroepitheliulm into retina and RPE appeared normal in Axin2lacZ/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 Axin2lacZ/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 Axin2lacZ/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 Axin2lacZ reporter, occasionally the reporter appeared weakly upregulated in the margins of Axin2lacZ/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 Axin2lacZ/lacZ eyes.
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 Axin2lacZ/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 Axin2lacZ/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 Axin2lacZ/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 Axin2lacZ/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 Axin2lacZ/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 Axin2lacZ/lacZ mesenchyme (Fig. 6L; three of three affected eyes; n = 3 embryos). Collectively, our data suggest that the hyperplastic tissue in Axin2lacZ/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 Axin2lacZ. In addition, we investigated the ocular phenotype in Axin2lacZ/lacZ eyes. Our results demonstrate that Axin2lacZ is dynamically expressed; it becomes activated in subpopulations of diverse retinal cell types mostly during postnatal differentiation. During optic cup morphogenesis, Axin2lacZ is activated in the presumptive RPE, ciliary...
Axin2 Function During Eye Development

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. 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.
Optic Cup Morphogenesis Requires Proper Levels of Wnt Signaling

Axin2 acts as an inhibitor of the Wnt/β-catenin pathway; therefore, homozygous disruption in Axin2lacZ/lacZ mice is likely to result in increased nuclear localization of β-catenin and possibly in ectopic Wnt pathway activation. However, Axin2 is considered a relatively weak inhibitor, which could explain the variable penetrance and range of early ocular defects in Axin2lacZ/lacZ mutants. In Axin2lacZ/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. 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. 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. 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 Axin2lacZ/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. Haploinsufficiency of Dkk1 leads to defects in optic cup morphogenesis similar to Axin2lacZ/lacZ mutants, such as microphthalmia,
coloboma, lens abnormalities, and anterior segment defects. Similar abnormalities are observed on albino for 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 aptc mutant zebrafish, an expanded ciliary margin zone and coloboma were observed. 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. 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.

**Comparison Between Axin2lacZ and Transgenic Wnt/β-Catenin Reporter Lines**

Our study shows Axin2lacZ activation in the dorsal optic cup, extracocular 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 Axin2lacZ reporter expression in the differentiating cells of the GCL and inner INL in a pattern similar to the transgenic TCF/LEF line. Our results demonstrate that Axin2lacZ 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.

In agreement with others, we observed very little overlap of Axin2lacZ with markers for Müller glia (this study). Under certain conditions, however, Müller glia show stem cell potential (for review, see Ref. 82) and exhibit Axin2lacZ reporter expression in retinal explants after exogenous Wnt pathway activation. Interestingly, ectopic Wnt pathway activation, due to complete inactivation of Axin2, causes Müller glia to proliferate and produce retinal progenitors.

Recently, it was shown that retinal amacrine cells expressing the G-protein-coupled receptor Lgr5 display regenerative potential. Lgr5 is a Wnt pathway target and bound by secreted R-spondin proteins to modulate Wnt pathway activity. It is possible that Axin2lacZ 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 Axin2lacZ 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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