Cyp1b1 Regulates Ocular Fissure Closure Through a Retinoic Acid–Independent Pathway

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PURPOSE. Mutations in the CYP1B1 gene are the most commonly identified genetic causes of primary infantile-onset glaucoma. Despite this disease association, the role of CYP1B1 in eye development and its in vivo substrate remain unknown. In the present study, we used zebrafish to elucidate the mechanism by which cyp1b1 regulates eye development.

METHODS. Zebrafish eye and neural crest development were analyzed using live imaging of transgenic zebrafish embryos, in situ hybridization, immunostaining, TUNEL assay, and methacrylate sections. Cyp1b1 and retinoic acid (RA) levels were genetically (morpholino oligonucleotide antisense and mRNA) and pharmacologically manipulated to examine gene function.

RESULTS. Using zebrafish, we observed that cyp1b1 was expressed in a specific spatiotemporal pattern in the ocular fissures of the developing zebrafish retina and regulated fissure patency. Decreased Cyp1b1 resulted in the premature breakdown of laminin in the ventral fissure and altered subsequent neural crest migration into the anterior segment. In contrast, cyp1b1 overexpression inhibited cell survival in the ventral ocular fissure and prevented fissure closure via an RA-independent pathway. Cyp1b1 overexpression also inhibited the ocular expression of vsx2, pax6a, and pax6b and increased the extraocular expression of sbbα. Importantly, embryos injected with human wild-type but not mutant CYP1B1 mRNA also showed colobomas, demonstrating the evolutionary and functional conservation of gene function between species.

CONCLUSIONS. Cyp1b1 regulation of ocular fissure closure indirectly affects neural crest migration and development through an RA-independent pathway. These studies provide insight into the role of Cyp1b1 in eye development and further elucidate the pathogenesis of primary infantile-onset glaucoma.

Keywords: congenital glaucoma, neural crest, CYP1B1, retinoic acid, coloboma

In primary infantile-onset glaucoma (Online Mendelian Inheritance in Man [OMIM] 231300), elevated intraocular pressures resulting from the malformation of the trabecular meshwork lead to irreversible damage to the optic nerve, cornea, and sclera. Children with primary infantile-onset glaucoma often require multiple eye surgeries; however, eye pressures can be difficult to control, and many affected individuals are blind.1,2 Autosomal recessive mutations in the CYP1B1 gene are the most commonly identified genetic causes of primary infantile-onset glaucoma.3 Despite its association with human disease, the role of CYP1B1 in eye development remains unknown. Cyp1b1 knockout mice showed angle abnormalities involving Schlemm’s canal, trabecular meshwork, cornea, and iris; yet these defects did not lead to elevations in intraocular pressure.4 Additional animal studies are required to understand the function of CYP1B1 during embryogenesis.

The CYP1B1 gene encodes a cytochrome p450 enzyme that regulates the in vitro synthesis of the essential morphogen retinoic acid (RA) through the conversion of retinol (vitamin A) into retinaldehyde and subsequently retinaldehyde into RA in a pathway independent of aldehyde dehydrogenases.5 Similar to other RA synthesis enzymes (e.g., aldh2 [aldh1a2] and aldh3 [aldh1a3]), cyp1b1 is expressed in the developing dorsal and ventral retina. In contrast, RA degradation enzymes, including cyp26a1, cyp26b1, and cyp26c1, are localized to the cranial and caudal retina.6–9 Together, these enzymes create RA gradients in the periocular mesenchyme centered on the dorsal and ventral axis of the eye.

We previously demonstrated that in zebrafish, RA produced by the developing retina regulates the formation of neural crest–derived structures in the eye and craniofacial region.10,11 The neural crest is a transient population of stem cells that contribute to many structures in the anterior segment, including the corneal stroma and endothelium, sclera, iris stroma, ciliary body stroma, and trabecular meshwork.12,13 The genetic or toxic disruption of ocular neural crest migration, proliferation, survival, and differentiation is responsible for many congenital eye diseases, including primary infantile-onset glaucoma, Axenfeld-Rieger syndrome (OMIM 180500), and Peters anomaly (OMIM 604229).14–16 Thus, investigating the molecular regulation of neural crest development is important for understanding the basis of congenital eye diseases.

In the present study, we demonstrated that cyp1b1 is expressed in the dorsal and ventral retina and retinal pigment epithelium, specifically in the ocular fissures, and maintains fissure patency, such that decreased expression caused premature closure, while overexpression resulted in colobomas. Although cyp1b1 regulated local RA levels, the effect on
fissure closure was independent of RA. Cyp1b1 regulated the expression of vsx2, Pax6, and sbb, genes clinically associated with colobomas. Importantly, we demonstrated evolutionary and functional conservation between the human and zebrafish forms of cyp1b1. These studies better define the role of Cyp1b1 in eye development and, importantly, provide insight into the pathogenesis of primary infantile-onset glaucoma.

**Materials and Methods**

**Animal Care and Animal Strains**

Zebrafish (Danio rerio) were raised in a breeding colony under a 14-hour light/10-hour dark cycle as previously described.10,11,17,18 Embryos were maintained at 28.5°C and staged as previously described.19 The transgenic strains Tg(sox10:EGFP) and Tg(fox3:GFP) were gifts from Thomas Schilling and Mary Halloran. The strains were crossed into the casper (rofl/nacef, nacre/) background as indicated.20–22 The Tg(RARE:mCherry) transgenic strain was generated using Gateway cloning techniques as previously described.23 Animal protocols were performed in accordance with the guidelines for the humane treatment of laboratory animals established by the University of Michigan Committee on the Use and Care of Animals (IACUC, protocol 10205) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Morpholino Oligonucleotides and mRNA Injections**

Translation blocking, 5-base pair mismatch, and standard control antisense morpholino oligonucleotides (MO; Gene Tools, LLC, Corvallis, OR, USA) directed against zebrafish Cyp1b1 (ATG and 5’ UTR targeted) and Raldh2 (ATG and 5’ UTR targeted) (Supplementary Table S1) were reconstituted in deionized water. Concentrations yielding consistent and reproducible phenotypes were determined for each MO. One-cell-stage embryos were injected with 1 to 2 nL MO at a concentration of 0.1 to 0.25 mM (2.1–2.4 ng/nL).

Two nonoverlapping translation blocking MO against Cyp1b1 were used to confirm the observed phenotype. The ATG-targeted MO against Cyp1b1 was previously verified for efficacy by confirming decreased protein expression, but this antibody is no longer available.24 No commercially available antibodies against human CYP1B1 with predicted cross-reactivity with zebrafish cyp1b1 showed consistent detection of the zebrafish protein by Western blot. The same ATG-targeted MO sequence and same range of concentrations as were used by Timme-Laragy et al.24 were used in these experiments. While the ATG-targeted Cyp1b1 MO is insensitive to the human mRNA coding sequence, there is overlap with the initial part of the zebrafish coding sequence. In contrast, the 5’ UTR-targeted Cyp1b1 MO is insensitive to both the zebrafish and human mRNA sequences (Supplementary Table S1). The 5’ UTR Cyp1b1 MO was used in cyp1b1 mRNA rescue experiments. In the current studies, both the ATG- and 5’ UTR-targeted Cyp1b1 MO showed similarity of phenotype.

Similarly, 2 nonoverlapping translation blocking Raldh2 MO were used to confirm the knockdown phenotype. The ATG-targeted Raldh2 MO, but not the 5’ UTR-targeted Raldh2 MO, overlapped with the zebrafish coding sequence (Supplementary Table S1). The 5’ UTR Raldh2 MO was used in raldh2 mRNA rescue experiments. Morpholino oligonucleotide knockdown (ATG or 5’ UTR) decreased Raldh2 protein while raldh2 mRNA overexpression increased Raldh2 protein detected by whole-mount immunostaining (Supplementary Figs. S1A-E).

The coding regions of zebrafish cyp1b1 and raldh2 genes and GFP were cloned into the pCS2þ expression vector, and the sequences were verified. The human CYP1B1 coding region was cloned into the pCRII-TOPO expression vector. Site-directed mutagenesis using Pfu Turbo (QuikChange Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA) and custom-designed primers were used to introduce point mutations (Supplementary Table S2). Capped mRNA was synthesized (mMessage mMACHINE kit; Ambion Biosystems, Foster City, CA, USA) and resuspended at 50 to 150 ng/µL in nuclease-free water. Approximately 1 to 2 nL (50–300 pg total RNA) of zebrafish cyp1b1 or raldh2 mRNA or human CYP1B1 (wild type or mutant) mRNA was coinjected with phenol red and GFP mRNA (50 ng/µL; 50–100 pg RNA) into one-cell-stage embryos.

For each experiment, ~100 embryos were injected with MO and/or mRNA, and all experiments were replicated a minimum of three times. Embryos were counted, and phenotypes for developmental delay, abnormal craniofacial development, and coloboma were assessed at 72 and 96 hours post fertilization (hpf). The percentage of embryos with these phenotypes in each group was calculated. Morpholino- or mRNA-injected embryos were compared with control-injected embryos. Each experiment (of ~100 embryos/group) was used as an experimental unit. The mean with standard error is shown in the text and tables. The data were statistically analyzed using ANOVA with post hoc Tukey pairwise tests, and P < 0.05 was considered statistically significant. Images shown are representative of all experiments.

**Pharmacologic Treatments**

All-trans retinoic acid (RA; Sigma-Aldrich Corp., St. Louis, MO, USA), diethylaminobenzaldehyde (DEAB; Sigma-Aldrich Corp.), and phenylthiourea (PTU; Sigma-Aldrich Corp.) were diluted in dimethylsulfoxide (DMSO; Sigma-Aldrich Corp.) at a 1000× final concentration. The pharmacologic treatments were administered in the embryo media at final concentrations of 25 to 50 nM RA, 10 µM DEAB, and 0.003% PTU. Treatments were initiated between 3 and 24 hpf as indicated. The medium was changed every 24 hours as indicated. Approximately 50 embryos were included for each condition, and all experiments were replicated a minimum of three times. Embryos were counted and phenotypes were assessed as described above. Images shown are representative of all experiments.

**Imaging**

Embryos were analyzed under a M205FA combi-scope (Leica Microsystems CMS GmbH, Wetzlar, Germany). Representative images of at least four embryos in each group per experiment were obtained using brightfield DFC290 (Leica Microsystems CMS GmbH) and/or fluorescent ORCA-ER (Hamamatsu, Hamamatsu City, Japan) cameras. Sections were imaged under a DM6000B upright microscope (Leica Microsystems CMS GmbH) equipped with a DFC500 camera (Leica Microsystems CMS GmbH). The images were processed and analyzed using Adobe Photoshop (San Jose, CA, USA), LAS software (Leica Microsystems CMS GmbH), and/or AF6000 software (Leica Microsystems CMS GmbH).

Time-lapse imaging of Tg(fox3:GFP) embryos was performed as previously described.23 Briefly, the embryos were imaged using a TCS SP5 MP multiphoton microscope (Leica Microsystems CMS GmbH) fitted with a Mai Tai DeepSee Ti-Sapphire laser (SpectraPhysics; Newport Corp., Irvine, CA, USA). The images were obtained every 20 minutes during the described time frame. Each time-lapse experiment was...
Repeated three or four times. Images and movies included are representative of all experiments.

Immunostaining and TUNEL Assay

Staged zebrafish embryos were fixed in 4% paraformaldehyde overnight at 4°C. For sectioning, the embryos were cryoprotected in successive sucrose solutions, embedded in optical cutting temperature (OCT) compound, and subsequently sectioned at 10 μm.

Whole-mount immunostaining was performed as previously described using primary rabbit anti-laminin (1:200; Sigma-Aldrich Corp.), primary rabbit anti-Raldh2 (1:100; GeneTex, Irvine, CA, USA), and secondary goat anti-rabbit IgG conjugated to Cy3 or 488 (1:1000; Abcam, Cambridge, MA, USA). Section immunostaining for GFP was performed using mouse anti-GFP directly conjugated to FITC (1:100; EMD Millipore, Billerica, MA, USA).

The percentage of embryos with continuous laminin staining in the distal ocular fissure was calculated. Morpholino- or mRNA-injected embryos were compared with control-injected embryos. The experiments were repeated three times, and each experiment (of ~20 embryos/group) was used as an experimental unit. The mean with standard error is shown in the text. The data were statistically analyzed using ANOVA with post hoc Tukey comparison with those of control-injected embryos.

Immunostaining and TUNEL labeling (TUNEL) assay was performed as previously described using standard protocols. Briefly, apoptotic cells were detected through the TdT-mediated incorporation of digoxigenin-labeled deoxyuridine triphosphate (Roche Life Sciences, Indianapolis, IN, USA). Sheep anti-digoxigenin conjugated to rhodamine (Roche Life Sciences) was used to detect the TUNEL signal. The sections were costained with 4',6-diamidino-2-phenylindole (DAPI). For quantification, three to five consecutive sections through the equator of the lens of at least four embryos per group were counted. The total number of cell nuclei and TUNEL-positive cells in the eyes of each section were manually counted. The percentage of TUNEL-positive cells in the eyes of MO- or mRNA-injected embryos was compared with those of control-injected embryos. The data were statistically analyzed using ANOVA with post hoc Tukey pairwise tests, and P < 0.05 was considered statistically significant. Images shown are representative of all experiments.

In Situ Hybridization

In situ hybridization was performed through standard protocols using digoxigenin- or fluorescein-labeled RNA antisense probes. The cyp1b1 RNA probe was 1047 base pairs, the vsx1 RNA probe was 797 base pairs, the rpx2 RNA probe was 503 base pairs, the sbba RNA probe was 911 base pairs, the sbbb RNA probe was 876 base pairs, the pax6a RNA probe was 458 base pairs, and the pax6b RNA probe was 316 base pairs. All probes were hybridized at 56°C overnight. For colorimetric reactions and signal comparisons, the embryos were developed for equal amounts of time. Sense controls were also developed in parallel to ensure specific staining. The embryos were cryoprotected, embedded, and sectioned at 10 μm. The sections were washed in phosphate-buffered saline, coverslipped, and imaged as described above.

Western Blotting

Embryos were injected as described above, and total protein was isolated at 24 hpf. Twenty micrograms of protein was combined with sample buffer (10% glycerol, 62.5 mM Tris base, pH 6.8, 2.5% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 0.002% bromophenol blue), boiled for 5 minutes, and loaded onto a 12% acrylamide gel (BioRad, Hercules, CA, USA). Protein was transferred to a nitrocellulose membrane (Life Technologies, Waltham, MA, USA), blocked in PBS/0.1% Tween-20/3% nonfat dry milk, and subsequently incubated with rabbit polyclonal anti-CYP1B1 (1:500, Ab157578, Ab78044; Abcam), mouse monoclonal anti-β-actin (1:1000, Ab8226; Abcam) overnight at 4°C. The membrane was washed in PBS/0.1% Tween-20 and subsequently incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA) or anti-mouse-HRP conjugated secondary antibody (1:1000, Cell Signaling Technology). Pierce ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA) was used for chemiluminescent detection. Commercially available antibodies (Abcam Ab157578 and Ab78044) against human CYP1B1 (~61 kDa) did not show cross-reactivity with the zebrafish form (~60 kDa) by Western blotting (see Fig. 7).

Methylacrylate Sections

Zebrafish embryos at 96 hpf were fixed in 2% paraformaldehyde/1.5% glutaraldehyde overnight at 4°C, followed by embedding in methylacrylate. The blocks were sectioned at 5 μm. The sections were stained with Lee’s stain, coverslipped, and subsequently imaged as described above.

Results

Cyp1b1 Overexpression Inhibited Ocular Fissure Closure

Previously published studies have established an effective MO that blocks Cyp1b1 translation; however, these studies did not assess eye or craniofacial development. Because mutations in CYP1B1 are associated with primary infantile-onset glaucoma, reflecting abnormalities in the neural crest–derived iridocorneal angle, the Tg(sax1:EGFP) and Tg(foxa3;GFP) transgenic zebrafish strains in which GFP is expressed in different subpopulations of neural crest cells were used to analyze neural crest and eye development.

Two different translation blocking Cyp1b1 MOs (ATG or 5′ UTR) were used to determine the effects of knockdown on neural crest and eye development and verify consistency of phenotype. At 72 hpf, the most common phenotypes in Cyp1b1 ATG and 5′ UTR MO knockdown embryos included developmental delays of 12 to 24 hours and craniofacial abnormalities in 20.5 ± 12.1% and 26.8 ± 11.1% of embryos, respectively (Fig. 1A; Supplementary Table S3). This finding was not significantly different compared with the percentage of developmentally delayed and abnormal Cyp1b1 ATG mismatch MO injected (12.1 ± 9.8%, P = 0.56) or Cyp1b1 5′ UTR mismatch MO injected (21.0 ± 7.1%, P = 0.97). Further, there were no significant differences between mismatch control-injected embryos and uninjected (8.3% ± 6.5%, P = 0.98, ATG mismatch, and P = 0.10, 5′ UTR mismatch) embryos.

At 96 hpf, MO knockdown of Cyp1b1 had a minimal effect on craniofacial neural crest development, including the pharyngeal arches (PA), ceratohyal cartilage (Ch), and Meckel’s (Mk) cartilage (Fig. 1D; Supplementary Fig. S2B) compared with controls (Fig. 1C; Supplementary Fig. S2A) and uninjected (Fig. 1B) embryos. Although we previously showed that Cyp1b1 knockdown delayed eye development at 48 hpf,26 by 96 hpf, anterior segment and retinal development (Fig. 1D; Supplementary Fig. S2B) had caught up to control-injected
FIGURE 1. Overexpression of cyp1b1 inhibited ocular fissure closure via an RA-independent pathway. The percentage of 72 hpf embryos with developmental delay and/or craniofacial abnormalities (A) due to Cyp1b1 MO (ATG or 5' UTR) knockdown, cyp1b1 (mRNA) overexpression, Raldh2 MO knockdown, raldh2 (mRNA) overexpression compared to uninjected, mismatch (MM) MO-injected or gfp mRNA-injected controls. ([a] P < 0.0001; [b] P < 0.001; [c] P < 0.01; [d] P < 0.05). Live images of uninjected (B) and mismatch control-injected (C, G) Tg(sox10:EGFP) embryos showed that at 96 hpf, neural crest contributed to the pharyngeal arches (PA) and the ceratohyal (Ch) and Meckel’s (Mk) cartilage of the jaw.
Sections of 96 hpf Tg(fosd3::GFP) embryos showed that fosd3-positive neural crest cells contributed to the iris stroma in the dorsal (B, C, G, arrowhead) and ventral (double arrowhead) indocorneal angle. In addition, differentiated retinal photoreceptors expressed fosd3, which was unrelated to neural crest cells. Injection of 5' UTR Cyp1b1 MO had minimal effect on craniofacial neural crest (D) and overall eye (D') development. Overexpression of cyp1b1 through mRNA injection disrupted neural crest-derived Meckel's cartilage formation (E, K) and inhibited ocular fissure closure, resulting in prominent colobomas (closed arrows, E, E'). The injection of Cyp1b1 5' UTR MO significantly decreased the percentage of injected embryos (Fig. 1A; Supplementary Table S3). Furthermore, at 72 hpf, Cyp1b1 knockdown decreased the percentage of injected embryos, which were not observed in gfp-injected or uninjected embryos. At 96 hpf, cyp1b1 overexpression inhibited Mk cartilage development (Fig. 1E) and caused eye wall defects and colobomas (Figs. 1E, 1E', arrows). Although loss of the retinal pigment epitheliad layer was observed in the area of the coloboma, the retinal layers were properly differentiated. In addition, fosd3-positive cells were observed in the neural crest-derived iris stroma (arrowheads and double arrowheads). The injection of Cyp1b1 ATG or 5' UTR MO significantly decreased the percentage of embryos (20.9 ± 6.0%, P = 0.002 and 27.9 ± 4.8%, P = 0.03) with ocular and craniofacial defects due to cyp1b1 mRNA injection (Supplementary Table S3; Figs. 1A, 1E, 1F'). Although decreased cyp1b1 caused only mild developmental delays and abnormalities, the overexpression of cyp1b1 demonstrated that this gene plays a role in ocular fissure closure and cranial neural crest development.

**Cyp1b1 Expression in the Ocular Fissure Was Regulated Through RA**

The role of cyp1b1 in eye development has been hypothesized to involve RA, as cyp1b1 regulates RA synthesis in vitro. We determined whether cyp1b1 expression was regulated through alterations in RA levels. In situ hybridization revealed that cyp1b1 was expressed in the dorsal (arrowhead) and ventral (closed arrow) retina and retinal pigment epithelium at 24 hpf (Figs. 2A, 2B, 2C) and 48 hpf (Figs. 2A', 2B', 2C') in untreated and DMSO-control treated embryos. Cyp1b1 expression was spatially and temporally correlated with the superior and inferior ocular fissures. Diethylnitrobenzaldehyde is a pan-aldehyde dehydrogenase inhibitor that decreases endogenous RA activity (raldh2 and raldh3). Treatment at 12 hpf with 10 μM DEAB did not alter cyp1b1 expression in the developing eye at 24 hpf (Fig. 2D), but did increase cyp1b1 expression in the ventral migrating neural crest (open arrowhead) compared with untreated embryos (Fig. 2C). In contrast, treatment with exogenous RA (25 nM) at 12 hpf inhibited cyp1b1 expression in the eye at 24 hpf (Fig. 2E) and 48 hpf (Fig. 2F). Thus, cyp1b1 expression was affected by pharmacologic alterations in RA levels.

To determine whether the converse was also true, a transgenic line (Tg[RAL:emCHERRY]), in which mCherry expression is driven by canonical RA response elements (RARE), was utilized. At 24 hpf, RA expression in the craniofacial region was greatest in the eye and the olfactory placode in uninjected and control-injected embryos (Figs. 2F, 2G; Supplementary Figs. S3A, S3B). At 48 hpf, within the eye, diffuse RA activity was observed throughout the retina, lens, and cornea (Fig. 3F'). Cyp1b1 MO knockdown did not show alterations in RA activity in the developing head at the doses tested (Fig. 2H; Supplementary Fig. S3C). In the eye, decreased RA activity was observed in the retinal pigment epithelium (Fig. 3H') in the area of cyp1b1 expression (Fig. 3B') compared with control-injected (Fig. 3G') and uninjected (Fig. 3F') embryos. In contrast, the overexpression of cyp1b1 by injecting mRNA at the one-cell stage caused a diffuse increase in mCherry expression throughout the craniofacial region (Fig. 3I; Supplementary Fig. S3D). Further, in the eye, increased RA activity was observed in the retinal pigment epithelium and retina (Fig. 3I', arrows) and in the region of the ocular fissure (arrowhead) compared with the eyes of control-injected (Fig. 3G') and uninjected (Fig. 3F') embryos. These studies suggest that cyp1b1 expression was regulated by RA, and the overexpression of cyp1b1 may alter local RA levels in and around the developing eye.

**Cyp1b1 Regulation of Ocular Fissure Closure Was Independent of RA**

Since cyp1b1 expression was regulated through RA, we assessed whether cyp1b1 and raldh2 worked together to control eye and neural crest development. As previously demonstrated, raldh2 overexpression (Figs. 1H, 1H') and Raldh2 MO knockdown (Figs. 1I, 1I'; Supplementary Figs. S2C, S2C) significantly decreased eye size and inhibited neural crest-derived jaw and PA formation in 56.2 ± 17.7% (P < 0.002) and 80.4 ± 5.9% (P = 0.0001) of injected embryos, respectively (Fig. 1A; Supplementary Table S3). The coinjection of Raldh2 MO and raldh2 mRNA significantly decreased the percentage of embryos with craniofacial and ocular defects (20.5 ± 6.8%, P = 0.01), demonstrating the specificity of these reagents (Supplementary Table S3; Figs. 1A, 1H, 1H'). To determine whether the Cyp1b1-mediated regulation of neural crest and eye development reflects the regulation of RA synthesis, Cyp1b1 MO was coinjected with raldh2 mRNA. At 72 hpf, Cyp1b1 knockdown decreased the percentage of embryos with small eyes and craniofacial defects (31.4 ± 9.0%, P = 0.04, Fig. 1A; Supplementary Table S3) resulting from the overexpression of raldh2. At 96 hpf, Cyp1b1 knockdown with raldh2 overexpression improved eye size and craniofacial cartilage formation (Figs. 1K, 1K'; Supplementary Figs. S2D, S2D). Cyp1b1 overexpression decreased the percentage of 72
hpf Raldh2 MO knockdown embryos with developmental delay, small, malformed eyes and craniofacial abnormalities (53.5 ± 7.7%, P = 0.0001, Fig. 1A; Supplementary Table S3). At 96 hpf, the overexpression of cyp1b1 improved eye size and jaw and PA development in Raldh2 MO knockdown embryos (Figs. 1L, 1L'; Supplementary Figs. S2E, S2E'). However, decreased RA through Raldh2 MO knockdown did not decrease the percentage of cyp1b1 mRNA-injected embryos with ocular fissure defects at 72 hpf (30.0 ± 8.7%, P = 0.17, Supplementary Table S3) and 96 hpf (Fig. 1L', Supplementary Fig. S2E'). However, decreased RA through Raldh2 MO knockdown did not decrease the percentage of cyp1b1 mRNA-injected embryos with ocular fissure defects at 72 hpf (30.0 ± 8.7%, P = 0.17, Supplementary Table S3) and 96 hpf (Fig. 1L', Supplementary Fig. S2E'). However, decreased RA through Raldh2 MO knockdown did not decrease the percentage of cyp1b1 mRNA-injected embryos with ocular fissure defects at 72 hpf (30.0 ± 8.7%, P = 0.17, Supplementary Table S3) and 96 hpf (Fig. 1L', Supplementary Fig. S2E').

This finding was further confirmed using a DEAB dose-response curve (1–5 μM) in combination with cyp1b1 overexpression (Supplementary Fig. S4; Supplementary Table S4). Treatment of GFP mRNA-injected embryos with 1 μM DEAB (at 3 hpf) did not significantly affect ocular fissure closure (28.0 ± 7.0%, P = 0.10) compared with untreated GFP-injected controls (3.3 ± 1.0%). However, treatment with 2 or 5 μM DEAB significantly increased the percentage of embryos (38.0 ± 9.4% [P = 0.008] and 55.2 ± 4.6% [P < 0.0001], respectively) with ocular closure defects at 72 hpf. At 96 hpf in GFP-injected control embryos, increasing concentrations of DEAB (Supplementary Figs. S4E, S4G, S4I) altered PA and Ch and Mk cartilage formation, decreased eye size, and delayed ocular fissure closure (arrowhead) compared with untreated (Supplementary Fig. S4A) and DMSO-control treated (Supplementary Fig. S4C) embryos. Treatment with DEAB did not improve or rescue the ocular fissure defects in cyp1b1 mRNA-injected embryos, but rather significantly increased the percentage of embryos with defects at each concentration (82.2 ± 13.4% at 1 μM DEAB [P = 0.0007], 84.5 ± 14.4% at 2 μM DEAB [P = 0.0003], and 100% at 5 μM DEAB [P < 0.0001]). Cyp1b1 mRNA-injected embryos treated with 1, 2, or 5 μM DEAB showed open ocular fissures (arrowheads), ocular wall defects (arrows), small eyes, and jaw and PA defects (Supplementary Figs. S4F, S4H, S4J). Concentrations of DEAB higher than 5 μM were lethal in both GFP- and cyp1b1 mRNA-injected embryos (data not shown). Thus, the effect of cyp1b1 overexpression on ocular fissure closure was not mediated through RA.

Next, the effect of cyp1b1 on cell survival in the developing eye, particularly in the ocular fissure, was assessed. At 24 hpf,
TUNEL assay of the eyes of embryos injected with cyp1b1 mRNA (5.9 ± 2.8%, Figs. 3E, 3F) showed a statistically significant (P = 0.005) 2-fold increase in apoptosis compared with uninjected (2.6 ± 1.1%, Fig. 2A) and control-injected embryos (1.8 ± 0.8% Fig. 3B). Many of the apoptotic cells were specifically localized to the inferior ocular fissure (arrows). Raldh2 5' UTR (D) but not ATG (C) MO knockdown significantly decreased cell survival throughout the developing eye compared to uninjected (A) and control injected (B). Similarly, overexpression of raldh2 (E') increased apoptosis in the eye. The percentage of apoptotic cells in 24 hpf developing eyes (F) of embryos injected with Cyp1b1 MO (ATG or 5' UTR), cyp1b1 mRNA, Raldh2 (ATG or 5' UTR) MO, raldh2 mRNA compared to uninjected and control injected (*P < 0.01).

Cyp1b1 Maintained the Patency of the Inferior Ocular Fissure

Ocular fissure closure is a complex process, requiring optic vesicle evagination, spatial specification along the proximal-distal and ventral-dorsal axes, breakdown of the basement membrane that demarcates the fissure, and fusion of the two sides (reviewed in Refs. 27, 28). The temporal and spatial expression of cyp1b1 in the ocular fissure and the colobomatous defects resulting from cyp1b1 overexpression suggested that cyp1b1 plays a role in fissure closure. We used whole-mount laminin staining to examine basement membrane integrity in the ocular fissure as a measure for the timing of ocular fissure closure. In uninjected (Figs. 4A–A”) and control-injected (Figs. 4B–B”) embryos, laminin staining was found in the distal ocular fissure (arrows) between 36 and 54 hpf. In uninjected embryos, laminin staining was found in a continuous line indicating presence of the basement membrane between the two sides of the ocular fissure in 100% of 36 and 42 hpf embryos, 93.4 ± 2.9% of 48 hpf embryos, and 79.1 ±
11.7% of 54 hpf embryos (Fig. 4F). Similarly, control-injected embryos showed laminin staining in 100% of 36 hpf, 99.3 ± 6.1% of 42 hpf, 92.1 ± 8.8% of 48 hpf, and 79.8 ± 8.8% of 54 hpf embryos, (Fig. 4G). Injection of Cyp1b1 ATG (Fig. 4C–C’’’) or 5’ UTR (Fig. 4D–D’’’) MO knockdown showed discontinuous staining of laminin at 48 hpf (C’’, D’’’) and 54 hpf (C’’’, D’’’), suggesting premature basement membrane breakdown. In cyp1b1 mRNA-injected embryos, laminin staining remained prominent within the fissure at all time points (E–E’’’). The percentage of embryos with continuous laminin staining in the distal ocular fissure at 36, 42, 48, and 54 hpf (G), (*P < 0.001).

Cyp1b1 Regulates Ocular Fissure Closure

The neural crest migrates into the anterior segment of the eye, contributing to the cornea, iris, and iridocorneal angle. Because cyp1b1 was expressed in the retina and retinal pigment epithelium and alterations in cyp1b1 affected fissure closure, we used time-lapse imaging to investigate whether cyp1b1 affected neural crest migration into the developing eye. Between 30 and 60 hpf, foxd3-positive neural crest cells [Tg(foxd3:GFP)] entered the anterior segment of the eye (Fig. 5A–J). Foxd3-positive cells migrated between the surface ectoderm and optic cup, with the majority of cells localized to the dorsal–posterior quadrant (Figs. 5E–H, dorsal denoted with “d”, ventral denoted with “v”, anterior denoted with “a” and posterior denoted with “p”). Additional cells travelled...
Cyp1b1 Regulates Ocular Fissure Closure

Tyrosinase Did Not Modify the Effect of Cyp1b1 in Zebrafish Eye Development

Previously published studies in mice have demonstrated that tyrosinase modifies the effect of Cyp1b1 knockout; however, the specific interactions between these two enzymes in neural crest and anterior segment development remain unknown. This relationship was further investigated in zebrafish embryos. In 24 hpf wild-type zebrafish embryos, the tyrosinase gene (tyr) was expressed in the migrating neural crest and in the retinal pigment epithelium in uninjected and control-injected embryos (Figs. 6A, 6B). At 48 hpf, tyr expression (Figs. 6A', 6A'', 6B') followed the skin pigmentation pattern and was also observed in the cranial neural crest (arrowheads), retinal pigment epithelium, migrating neural crest cells in the ocular fissure (arrowheads and open arrows), and iris stroma (open arrows). At 48 hpf, the Cyp1b1 knockdown decreased tyr expression in the cranial neural crest (arrowheads) and ocular fissure (Figs. 6C, 6C'). Overexpression of cyp1b1 did not alter tyr expression (Figs. 6D, 6D', 6D'') at 24 and 48 hpf. Mutant mice deficient for both tyr and Cyp1b1 genes showed a worse anterior segment phenotype. We utilized pharmacologic and genetic strategies to decrease tyrosinase activity. Phenylthiourea is often used in zebrafish embryos to inhibit tyrosinase and subsequent ocular phenotypes. We compared the effect on the anterior segment of uninjected embryos treated with 0.003% PTU and embryos treated with 0.003% PTU before administration of cyp1b1 MO. In contrast to uninjected embryos, PTU did not cause a significant percentage of embryos with developmental delays and craniofacial abnormalities (17.6 ± 7.0%, P = 0.99) compared with untreated/uninjected embryos. Compared with PTU-treated uninjected embryos, PTU did not cause a significant increase in the percentage of embryos with developmental delays and craniofacial abnormalities when administered prior to 22 hpf or at concentrations higher than 0.003%. To inhibit tyrosinase activity prior to tyr and cyp1b1 expression, embryos were treated with 0.003% PTU between 10 and 12 hpf. Treatment of uninjected embryos with PTU did not significantly increase the percentage of embryos with developmental delays and craniofacial abnormalities (17.6 ± 7.0%, P = 0.99) compared with untreated/uninjected embryos. Compared with PTU-treated uninjected embryos, PTU did not cause a significant increase in the percentage of embryos with developmental delays and craniofacial abnormalities when administered prior to 22 hpf or at concentrations higher than 0.003%.
increase in developmental delays or abnormalities in control-injected (26.3 ± 3.5%, P = 0.28) or Cyp1b1 MO-injected embryos (29.0 ± 7.5%, P = 0.85). In addition, we utilized the casper mutant strain, which has decreased tyr expression in neural crest-derived cells. Injection of Cyp1b1 MO in casper embryos did not significantly increase the percentage of embryos with craniofacial abnormalities or developmental delays (25.0 ± 11.0%) compared to control-injected (19.4 ± 12.8%, P = 0.71) and uninjected casper embryos (8.4 ± 10.4%). Hence, in zebrafish, alterations in tyrosinase expression and activity did not alter the Cyp1b1 effect on eye development.

**Cyp1b1 Differentially Regulated Genes Associated With Colobomas**

Because Cyp1b1 modulation of ocular fissure closure was not mediated through RA, genes clinically associated with colobomas were investigated as downstream targets. In situ hybridization at 24 hpf demonstrated that cyp1b1 overexpression inhibited the expression of the homeobox transcription factor vsx2 in the developing eye (Figs. 7D, 7D') compared with Cyp1b1 MO knockdown (Figs. 7C, 7C'), control-injected (Figs. 7B, 7B'), and uninjected (Figs. 7A, 7A') embryos. Further, in situ hybridization revealed the presence of vsx2-expressing tissue bridging the area between the optic cups (Fig. 7D', arrow) in response to cyp1b1 overexpression. In contrast, cyp1b1 overexpression increased sbbd expression in the floor plate between the developing eyes (Figs. 7H, 7H', arrowheads) compared with Cyp1b1 MO knockdown (Figs. 7G, 7G), control-injected (Figs. 7F, 7F'), and uninjected (Figs. 7E, 7E') embryos. Alterations in cyp1b1 levels did not affect the expression of vsx1 and sbbd (data not shown) at 24 hpf. Although the expression of pax6a and pax6b was not affected at 24 hpf (data not shown), the expression of these two pax6 genes was decreased in the retina in response to cyp1b1 overexpression at 48 hpf (Figs. 7I, 7I') compared with Cyp1b1 MO knockdown (Figs. 7K, 7K'), control-injected (Figs. 7J, 7J'), and uninjected (Figs. 7L, 7L') embryos. Thus, cyp1b1 overexpression altered the expression of key signaling molecules involved in eye development and ocular fissure closure.

**Human CYP1B1 and Zebrafish cyp1b1 Genes Are Evolutionarily and Functionally Conserved**

Mutations in CYP1B1 disrupt the development of the neural crest-derived trabecular meshwork and outflow channels and are the most commonly identified genetic causes of primary infantile-onset glaucoma. To investigate whether Cyp1b1...
function is conserved between humans and zebrafish, human CYP1B1 mRNA was injected into zebrafish embryos at the one-cell stage. Western blotting verified human CYP1B1 protein expression in 24 hpf mRNA-injected embryos (Fig. 8G). Similar to the effects observed with the zebrafish gene, the overexpression of human CYP1B1 caused large colobomas in 71.8 ± 17.9% of embryos (P = 0.0003, Supplementary Table S5), reflecting the defective closure of the ocular fissure (Figs. 8B, 8B') compared to controls (Figs. 8A, 8A'). Further, similar to the overexpression of zebrafish cyp1b1, human CYP1B1 overexpression disrupted neural crest-derived jaw (Mk and Ch cartilage) and PA formation. The ATG MO-mediated knockdown of endogenous zebrafish Cyp1b1 showed improvement of craniofacial defects, but did not statistically significantly decrease the percentage of embryos with colobomas and craniofacial defects (36.4 ± 17.0, P = 0.06) due to human CYP1B1 overexpression (Figs. 8C, 8C').

The genetic analysis of patients affected with primary infantile-onset glaucoma showed that disease-causing mutations typically occur in the second or third exon of the CYP1B1 gene. Using site-directed mutagenesis, three mutations (genomic DNA nomenclature based on GenBank U56438) were introduced into the coding region of human CYP1B1, and the mRNAs for each of these mutants were transcribed and injected into one-cell embryos (Supplementary Table S2). The g.3976G>C mutation results in a tryptophan to cysteine (W57C) amino acid substitution that disrupts the hinge region connecting the N-terminal membrane-bound domain to the cytosolic portion of CYP1B1. The g.4490G>A mutation results in a glutamic acid to lysine (E229K) amino acid substitution that alters the ionic bonds necessary for substrate binding. Unlike wild-type human CYP1B1 mRNA, injecting g.3976G>C, g.4490G>A, or g.8168G>A mutant CYP1B1 mRNA into one-cell-stage zebrafish embryos did not significantly affect eye size, neural crest development, or ocular fissure closure (Supplementary Table S5; Figs. 8D, 8D', 8E, 8E', 8F, 8F') compared with control embryos. Thus, these studies demonstrated the evolutionary conservation of CYP1B1 gene function between humans and zebrafish.

**DISCUSSION**

In primary infantile-onset glaucoma, mutations in the CYP1B1 gene lead to increased intraocular pressure and subsequent vision loss resulting from damage to the optic nerve, cornea, and sclera. However, the mechanism by which CYP1B1 regulates eye development has not been defined, and the in vivo substrate for this cytochrome p450 enzyme during embryogenesis has not previously been identified. In the present study, we used a zebrafish model to improve our understanding of the pathophysiology of primary infantile-onset glaucoma.

CYP1B1 mutations associated with primary infantile-onset glaucoma have primarily been detected in the coding region, comprising the second and third exons of this gene. The CYP1B1 protein comprises an N-terminal membrane-binding domain, a short “hinge” region, and a large C-terminal cytosolic domain. Disease-causing point mutations frequently affect highly conserved regions in the cytosolic portion, including the hinge-binding region, substrate-binding region, and the substrate access channel. For example, the g.4490G>A (E229K) mutation, one of the most commonly identified point mutations associated with primary infantile-onset glaucoma, destabilizes ionic interactions between helices in the substrate-binding domain, resulting in decreased protein activity. In the present study, the overexpression of either the zebrafish cyp1b1 or human CYP1B1 yielded a similar phenotype: colobomatous defects and abnormal jaw and PA formation. However, the injection of human mRNA containing clinically significant point mutations had less effect on eye and
proximal–distal axes, breakdown of the basement membrane spatial patterning of the optic cup along the dorsal–ventral and Ocular fissure closure is a complex process that requires a role for less well understood (Stach T, et al. inferrior ocular fissure is well defined, the closure of the and immediately surrounding the ocular fissures. Although the
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overexpression of cyp1b1 expression in the developing eye is regulated through developmental pathways, and this enzyme has an endogenous substrate. In vitro studies have shown that Cyp1b1 catalyzes both enzymatic steps in the conversion of retinol into RA. This mechanism represents an alternative pathway from the well-characterized canonical pathway, which uses alcohol dehydrogenases to convert retinol into retinaldehyde dehydrogenases to convert retinaldehyde into RA. In mice, chickens, and zebrafish, the developing eye expresses retinaldehyde dehydrogenases in the dorsal and ventral retina. The local synthesis of RA is counteracted by degradation enzymes (Cyp26a1, Cyp26b1, and Cyp26c1) expressed in the cranial and caudal retina. The specific expression of synthesis and degradation enzymes generates RA gradients, where the highest concentrations of RA are localized along the dorsal-ventral axis of the developing eye. Retinoic acid is critical for both craniofacial and ocular neural crest development. We previously demonstrated that decreased or increased RA levels disrupt neural crest contributions to the cornea, iris, jaw, and PA in zebrafish. The present study demonstrated that cyp1b1 expression was regulated by alterations in RA levels and the overexpression of cyp1b1 increased RA levels throughout developing zebrafish embryos. Further, the increase in RA as a result of cyp1b1 overexpression decreased the microphthalmia induced through RA deficiency (Raldh2 MO). Cyp1b1 knockdown had minimal effect on RA expression and eye development. However, decreased Cyp1b1 expression improved the effects of increased RA as a result of the overexpression of raldh2. Taken together, cyp1b1 may regulate RA synthesis in vivo through a secondary pathway that can be either up- or downregulated when the primary aldehyde dehydrogenase pathway is genetically or pharmacologically altered; however, the primary endogenous role of cyp1b1 in eye development seems to be independent of RA.

A key function of cyp1b1 may be the regulation of ocular fissure closure. Cyp1b1 expression in the developing eye was localized to the retina and retinal pigment epithelium within and immediately surrounding the ocular fissures. Although the inferior ocular fissure is well defined, the closure of the superior fissure prior to its ventral counterpart in zebrafish is less well understood (Stach T, et al. IOVS 2013;54:ARVO E-Abstract 3362). Cyp1b1 expression was correlated with the patency of ocular fissures, as cyp1b1 expression is highest between 24 and 36 hpf, after which expression becomes attenuated and is undetectable at 60 hpf. This finding suggests a role for cyp1b1 in maintaining the patency of ocular fissures. Ocular fissure closure is a complex process that requires proper optic vesicle evagination from the forebrain, specific spatial patterning of the optic cup along the dorsal-ventral and proximal-distal axes, breakdown of the basement membrane enveloping the anterior and posterior edges, and tissue fusion. In zebrafish embryos, the ventral fissure closure is initiated in the middle of the optic cup and proceeds anteriorly and posteriorly between 36 and 72 hpf. In the present study, the premature breakdown of laminin in the basement membrane was observed in Cyp1b1 MO knockdown embryos, suggesting premature ocular fissure closure compared with control MO-injected and uninjected embryos. Time-lapse imaging further revealed that the premature closure of the fissure disrupted neural crest migration adjacent to and through the ocular fissure into the anterior segment. In mouse and chicken embryos, increased and decreased RA result to affect neural crest contributions to the cornea and iris at 96 hpf; thus, we propose that this mechanism may account for the specific disruption of the neural crest-derived trabecular meshwork in primary infantile-onset glaucoma. In humans, neural crest cells travel in three separate waves, with the first wave generating the components of the cornea, the second wave contributing to the iris, and the third wave forming the trabecular meshwork and angle structures. While neural crest cells destined for the cornea and iris enter the anterior segment, the early closure of the ocular fissure may prevent neural crest specifically destined for the trabecular meshwork from migrating into the anterior segment. Differences in the effects on the neural crest in cyp1b1 mutant zebrafish, mice, and chickens may reflect species differences in the number of waves of neural crest migration (e.g., two in chickens and one in mice) and anatomic differences in aqueous outflow pathways. Taken together, the regulation of ocular fissure patency may explain the mechanism by which cyp1b1 affects neural crest contributions to the anterior segment of the eye.

Interestingly, overexpression of cyp1b1 resulted in large colobomas. CYP1B1 mutations have not been reported in cases of inferior colobomas, but this gene is not included in the typical genetic screens used in clinical testing. Notably, a CYP1B1 mutation was identified in a patient with a rare superior coloboma (Stach T, et al. IOVS 2013;54:ARVO E-Abstract 3362). However, whether this variant is a gain-of-function mutation remains unknown, indicating that additional studies are required to further identify and assess mutations in human CYP1B1 in cases of colobomas.

Given the timing of the changes in the expression of sbb, pax6, and vsx2 and the increased apoptosis in the inferior ocular fissure, we hypothesized that cyp1b1 overexpression had an early effect on the spatial patterning of the optic cup. In humans, heterogeneous mutations in SHH are associated with holoprosencephaly and a spectrum of eye malformations, including anophthalmia, microphthalmia, and colobomas. Sbb is expressed in the floor plate of the diencephalon and is required for patterning the optic cup through the specific spatial expression of transcription factors. Sbb regulates Vax2 and Pax2 in the optic stalk and ventral neural retina to maintain the expression of these transcription factors within the more proximal structures of the eye. Mice with Vax2 or Pax2 mutations show the inhibition of basement membrane breakdown in the fissure, resulting in colobomas. In contrast, Sbb inhibits Pax6, restricting its expression to the distal optic cup, which is critical for lens induction from the surface ectoderm. Human mutations in Pax6 are most commonly associated with aniridia (OMIM 706108), an autosomal dominant congenital eye disease characterized by cataract, limbal stem cell deficiency, glaucoma, and hypoplasia of the iris, optic nerve, and fovea. While large disruptions in gene function resulting from frameshift or nonsense mutations lead to these widespread ocular defects, rare missense PAX6 mutations have been associated with colobomas. In mice, Pax6 regulates other genes associated with colobomas, such as Vsx2 (Dox10), Maf1, and Six3. The current studies showed that in zebrafish, cyp1b1 overexpression increased sbb gene expression in the midline floor plate, while decreasing pax6a, pax6b, and vsx2 gene expression in the developing retina. Notably, RA is also a key regulator of ocular fissure closure, as both increased and decreased RA result in colobomas in mice and zebrafish. Exposure to exogenous
RA inhibits Sbb expression in the midline floor plate.\textsuperscript{62} We observed that the overexpression of cyp1b1 not only increased RA levels throughout the developing head and eye, but also increased sbb expression. Furthermore, genetically or pharmacologically decreasing endogenous RA synthesis to compensate for cyp1b1 overexpression did not rescue the observed colobomatous defects. It is unclear whether these signaling pathways are affected only in the overexpression state or represent normal endogenous targets of cyp1b1. We did not observe alterations in the levels of sbb, pax6, or vsx2 with decreased Cyp1b1, suggesting that there are additional targets of Cyp1b1 that regulate fissure patency. Taken together, with decreased Cyp1b1, suggesting that there are additional did not observe alterations in the levels of state or represent normal endogenous targets of cyp1b1 pensate for increased RA levels throughout the developing head and eye, but also observed that the overexpression of zebrafish and human forms of the CYP1B1 Cyp1b1 evolutionarily conserved.\textsuperscript{68} These results improve the current understanding of the complex signals that regulate eye development and, importantly, provide insight into the molecular pathogenesis of primary infantile-onset glaucoma.

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