

# Retinoic acid receptor signaling regulates choroid fissure closure through independent mechanisms in the ventral optic cup and periorcular mesenchyme

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**Retinoic acid receptor (RAR) signaling is required for morphogenesis of the ventral optic cup and closure of the choroid fissure, but the mechanisms by which this pathway regulates ventral eye development remain controversial and poorly understood. Although previous studies have implicated neural crest–derived periorcular mesenchyme (POM) as the critical target of RA action in the eye, we show here that RAR signaling regulates choroid fissure closure in zebrafish by acting on both the ventral optic cup and the POM. We describe RAR-dependent regulation of eight genes in the neuroepithelial cells of the ventral retina and optic stalk and of six genes in the POM and show that these ventral retina/optic stalk and POM genes function independently of each other. Consequently, RAR signaling regulates ventral eye development through two independent, nonredundant mechanisms in different ocular tissues. Furthermore, the identification of two cohorts of genes implicated in ventral eye morphogenesis may help to elucidate the genetic basis of ocular coloboma in humans.**

Coloboma is a common visual system disorder in humans characterized by the failure of the choroid fissure in the ventral optic cup to close (1). Gestational vitamin A deficiency causes disruption of the anterior eye segment, microphthalmia, and coloboma (2–4). These defects are phenocopied in mice lacking various combinations of the *Aldh1A1–3* retinoic acid (RA)-producing enzymes, or retinoic acid receptors (RARs), indicating that vitamin A is required for RA synthesis and RAR activation during choroid fissure closure (2, 3, 5).

*Aldh1A1/3* are expressed in restricted domains along the retinal dorsoventral (DV) axis (2, 5). However, DV polarity is normally established in *Aldh1A* or RAR mutant eyes (2, 3, 5). Furthermore, retinal development proceeds normally after abrogation of RAR $\alpha$ , the only RAR expressed in the mouse retina at embryonic day 10.5 (E10.5) (3), suggesting that RAR signaling within the retina does not have a major role in eye morphogenesis (3).

A major RA target is the neural crest–derived periorcular mesenchyme (POM) surrounding the developing eye (3, 5), where RAR signaling controls expression of the transcriptional regulators *FoxC1a*, *Eya2*, and *Pitx2* (2, 5). RAR-dependent regulation of POM development is instrumental in anterior segment formation (2, 3, 5) and, in addition, neural crest–specific knockout of all RAR genes in mice causes coloboma and eversion of the ventral optic cup (3), suggesting that RA acts via the POM to guide the morphogenetic movements of the optic cup. It remains unclear how POM influences ventral eye morphogenesis and what role, if any, RA exerts directly on optic cup cells.

In this study, we elucidate the actions of RA on choroid fissure closure through analysis of 14 genes that we show depend on RAR signaling for their expression in and around the developing zebrafish eye. Eight of these genes are expressed in the ventral retina (VR)/optic stalk (OS), demonstrating a robust transcriptional response to RAR signaling within the optic cup, and six genes are expressed in POM. Both of these genetic programs are

required for choroid fissure closure, but they function independently of each other. Thus, RAR signaling controls ventral eye morphogenesis through two distinct, nonredundant mechanisms operating, respectively, in the ventral optic cup and in the surrounding POM.

## Results

**Inhibition of RAR Signaling During Eye Morphogenesis Causes Coloboma Without Affecting Eye Polarity.** In zebrafish, *aldh1A3* expression is initiated in the evaginating optic vesicles at the 3- to 6-somite stage (3–6s), at 11–12 h postfertilization (hpf) (Fig. S1), followed by transcription of *aldh1A2* at around 20s (19 hpf) (6). RA synthesis is thus likely to be initiated during optic vesicle evagination and maintained during optic cup formation. Given this timing, to block RAR signaling in and around the forming eye, we treated zebrafish embryos with AGN194310 (AGN), a small-molecule inhibitor of all RARs (7), from 3 to 6 s up to 60 hpf.

At least 75% of AGN-treated embryos displayed coloboma at 60 hpf ( $n = 87$ ). The most affected eyes showed a marked gap in pigmentation in the ventral optic cup (Fig. 1A), and histological sections highlighted both the lack of retinal pigmented epithelium and a shortening of the retina ventral to the optic nerve head (Fig. 1A). Defective closure of the choroid fissure was already detectable by differential interference contrast microscopy at 48 hpf (Fig. 1A). At 60 hpf, embryos treated with AGN until 25–26 hpf or treated from 18s (18 hpf) showed coloboma (at least 60%,  $n > 40$  in each case). Temporally restricted AGN treatments between 17–18s and 25–26 hpf also caused coloboma at 64 hpf (at least 37%,  $n = 106$ ). Thus, the developmental period during which AGN treatments interfere with choroid fissure closure includes the time window between 17–18s and 25–26 hpf, although stages flanking this window contribute to the penetrance of the phenotype.

The choroid fissure serves as a route for retinal axons and blood vessels out of and into the eye, respectively. In control eyes, retinal axons form a compact bundle at the optic nerve head and project contralaterally at the midline (Fig. 1B). After

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The authors declare no conflict of interest.

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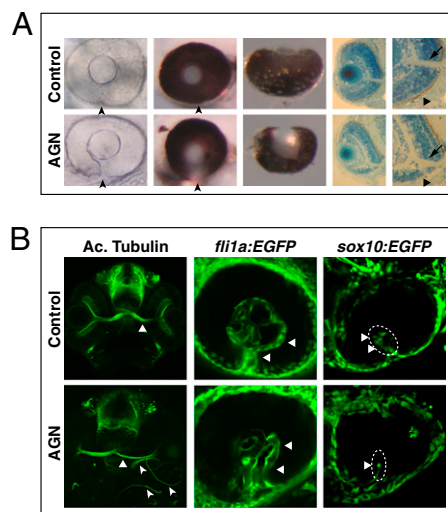
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**Fig. 1.** Inhibition of RAR signaling causes coloboma in zebrafish. (A) Coloboma (arrowheads) in embryos treated with 2.5–5  $\mu$ M AGN from 3 to 6 s. (Left) Lateral view of 48-hpf phenylthiourea-treated embryos. (Center) Lateral (Center Left) and ventral (Center Right) views of 60-hpf eyes. (Right) Sections of 60-hpf eyes showing the lack of retinal pigmented epithelium (triangles) and a shortening of the retina ventral to the optic nerve head (arrows) in the AGN-treated embryo. (B, Left) Immunostaining with an anti-acetylated (Ac.) tubulin antibody at 48–50 hpf, showing abnormal retinal axon trajectories in the AGN-treated embryo (arrowheads), besides the normal contralateral projection (triangles). (Center) *fli1a:EGFP* transgenic embryos at 48 hpf. The AGN-treated embryo exhibits disrupted vascularization (triangles). (Right) *sox10:EGFP* transgenic embryos at 32–36 hpf. The AGN-treated embryo shows less POM cells within the choroid fissure (triangles).

AGN treatments, we observed axons projecting ipsilaterally and aberrantly around the eye or leaving the retina from ectopic locations (Fig. 1B). Vascularization of the retina through the choroid fissure, as detected by using the endothelial cell reporter *fli1a:EGFP* (8), was also disorganized, with both the patterning and diameter of the hyaloid vessels affected (Fig. 1B). The eye vascular system also contains pericytes originating from neural crest–derived POM cells migrating inside the choroid fissure (9). Using a *sox10:EGFP* reporter labeling neural crest–derived POM (10), we found that AGN treatments caused a significant decrease in the number of EGFP-positive cells within the choroid fissure at 26–28 hpf (DMSO:  $n = 6.3 \pm 1.7$ , 12 eyes; AGN:  $n = 2.8 \pm 1.9$ , 12 eyes;  $P < 0.001$ ) (Fig. 1B).

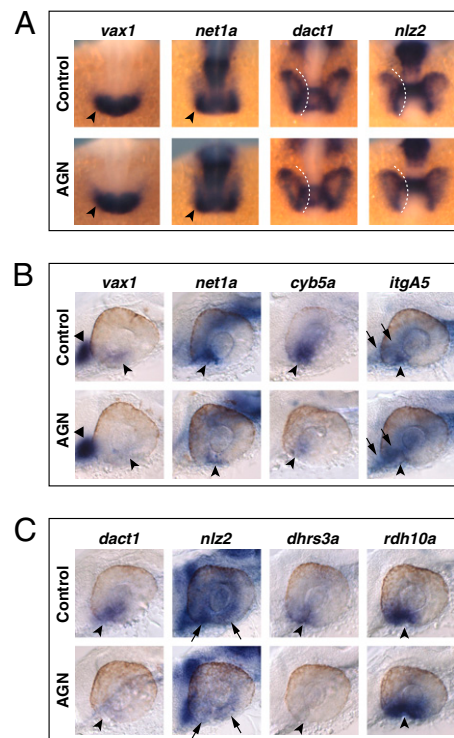
Since coloboma phenotypes can arise because of impaired DV patterning of the eye (1), we examined the effects of AGN treatments on the expression of markers of eye polarity but found no changes at 24–25 hpf and 31 hpf (Fig. S2A–C). Ventral patterning of the eye depends on Shh and Fgf8 signals from the ventral forebrain and/or OS (11, 12), and AGN-treated embryos showed no appreciable effects on the expression of Shh and Fgf8 pathway genes or ventral forebrain markers (Fig. S2D).

**RAR Signaling Regulates Gene Expression in Optic Cup Cells Flanking the Choroid Fissure and in the POM.** To identify genes misregulated in the developing eye region upon RAR inhibition, we selected ~40 genes that were candidates for being regulated by RAR signaling based on restricted expression near sites of ocular RA production and/or previous studies implicating them in ventral optic cup morphogenesis. Expression analyses of these genes in DMSO- and AGN-treated embryos yielded 14 genes showing robust transcriptional changes in or around the developing eye upon AGN treatments (see below and Table S1).

These 14 genes could be grouped into 8 primarily expressed in VR/OS cells flanking the choroid fissure (*vax1*, *net1a*, *cyb5a*,

*itgA5*, *dact1*, *nlz2*, *dhrs3a*, and *rdh10a*) and 6 primarily expressed in POM within the fissure and/or around the optic cup (*foxC1a*, *nlz1*, *twist1a*, *eya2*, *pix2*, and *lmx1b1*) (Fig. S3). The VR/OS gene *itgA5* was also expressed in extraocular tissue ventral and medial to the eye, corresponding to neural crest and/or endodermal derivatives (Fig. S3) (13). The POM gene *pix2* is expressed as two different isoforms (*pix2a* and *pix2c*) generated by the use of two promoters and alternative splicing (14), and we found that *pix2a* is the predominant isoform in the POM (Fig. S3B).

We determined the effects of RAR signaling on the VR/OS and POM genes by treating embryos with AGN from 3s and analyzing their expression at different time points (18s, 24–25 hpf and 31 hpf). AGN treatments from 3s did not cause major changes in the early expression patterns of the VR/OS genes *vax1*, *net1a*, *dact1*, and *nlz2* in the optic vesicle at 18s (Fig. 2A). *cyb5a* and *dhrs3a* were barely detectable in the eye at this stage. By contrast, 24- to 25-hpf embryos showed down-regulation of *vax1*, *net1a*, *cyb5a*, *itgA5*, *dact1*, and *nlz2* in the VR/OS, whereas other expression sites of these genes were unaffected (Fig. 2B and C, Fig. S4A and B, and Table S1). *Dhrs3a* and *Rdh10a* are enzymes involved in RA metabolism and, in other contexts, feedback regulation by RA activates *dhrs3a* and represses *rdh10a* expression (15). As expected, RAR inhibition eliminated *dhrs3a* and increased *rdh10a* expression in the VR/OS (Fig. 2C, Fig. S4B,



**Fig. 2.** RAR signaling regulates gene expression in the VR/OS. (A–C) Embryos treated with DMSO or 10  $\mu$ M AGN from 3s and hybridized at 18s (A) or 24–25 hpf (B and C) with the indicated probes. (A) Dorsal/anterior views of heads. AGN treatment has no major effects on the expression of *vax1* and *net1a* at the optic vesicle/forebrain junction (arrowheads) and of *dact1* and *nlz2* in the optic vesicle. Dashed lines highlight the proximal edge of the optic vesicle. (B and C) Lateral views of eyes. AGN treatment causes down-regulation of *vax1*, *net1a*, *cyb5a*, *itgA5*, *dact1*, *nlz2*, and *dhrs3a* and up-regulation of *rdh10a* in the VR/OS (arrowheads). Triangles point to *vax1* expression in the ventral forebrain (B). Arrows point to *itgA5* expression in extraocular tissue at the back of the eye (B) and *nlz2* expression on each side of the choroid fissure (C). Panels are representative images of the following numbers ( $n$ ) of independent experiments: *vax1*, *net1a*,  $n = 4$ ; *itgA5*,  $n = 3$ ; and *cyb5a*, *dact1*, *nlz2*, *dhrs3a*, *rdh10a*,  $n = 2$ . Approximately 15–30 embryos were assayed for each condition and probe in each experiment.

and Table S1). Similar changes for most of these genes were also observed at 31 hpf (Figs. S5 A and B and S6 A and B). Abrogation of RAR signaling did not generally compromise ventral eye development, as other VR/OS-expressed genes were not obviously affected (Fig. S2).

POM genes also showed severely reduced expression upon AGN treatments. *foxC1a* and *nlz1*, but not *twist1a*, were already down-regulated at 18s (Fig. 3A). At 24–25 hpf and 31 hpf, both *foxC1a* and *nlz1* were down-regulated in POM surrounding the dorsal, anterior, and ventral eye, whereas *twist1a* expression was decreased in the dorsal POM (Fig. 3C, Figs. S4C, S5B, and S6B, and Table S1). *eya2*, *pitx2*, and *lmx1b1* expression in POM within the choroid fissure and/or in anterior POM became clearly detectable only by 31 hpf and was reduced in AGN-treated embryos (Fig. 3C, Fig. S6C, and Table S1).

The results described above suggest that RAR signaling acts early to initiate POM gene expression and later to maintain expression of the VR/OS genes. AGN treatments started at 23 hpf lasting until 31 hpf caused down-regulation of the VR/OS expression of *vax1* and *dhrs3a*, but not *net1a* or *cyb5a* (Fig. S5 C and D). AGN treatments from 18s did, however, lead to reduced *net1a* and *cyb5a* expression (Fig. S5D). AGN treatments from

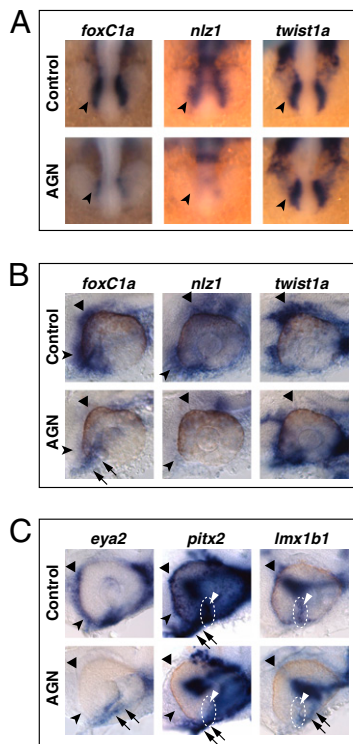
23 hpf also caused down-regulation of *foxC1a* and *nlz1* (Fig. S5C). Thus, RAR signaling is necessary beyond 18s for the maintenance of both POM and VR/OS gene expression.

The relative transcriptional levels of some of the VR/OS and POM genes were quantified by real-time PCR on RNA purified from 31-hpf dissected heads of DMSO- or AGN-treated embryos. Supporting in situ hybridization (ISH) data, *vax1*, *nlz2*, and *cyb5a* were significantly down-regulated and *dhrs3a* expression was nearly abolished in AGN-treated heads, whereas *rdh10a* was up-regulated (Fig. 4A). Expression levels of the POM genes *foxC1a*, *nlz1*, and *lmx1b1* were also significantly lower in AGN-treated heads (Fig. 4A). AGN treatments significantly down-regulated *pitx2a*, but not *pitx2c*, consistent with the differential expression of these isoforms in the POM (Fig. 4A). The relative levels of *vax2* and *nkx2.1b* were not significantly changed, confirming that AGN treatments did not cause a global down-regulation of ventral eye and forebrain gene expression (Fig. 4A).

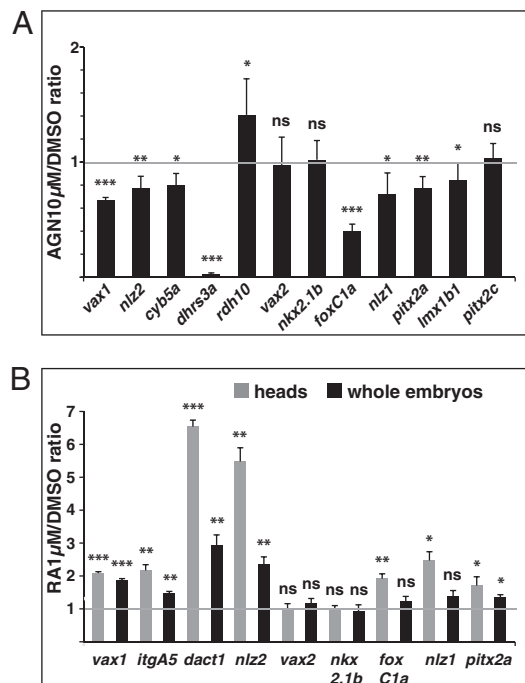
#### Increased RA Signaling Leads to Up-Regulation of Most VR/OS and POM Genes.

To assess whether exogenous RA could promote expression of the VR/OS and POM genes, embryos were treated from 3s with DMSO or 0.125–0.5  $\mu$ M RA and analyzed by ISH at 25–29 hpf. Confirming previous observations (16), exogenous RA expanded *pax2* expression in the eye (Fig. S7A), but other markers of DV eye polarity were not evidently affected (Fig. S7B). RA treatments led to expanded expression of the VR/OS genes *nlz2*, *dhrs3a*, *dact1*, and *itgA5*, but not *vax1*, *net1a*, and *cyb5a*, in the dorsal retina (Figs. S7A and S8A). Expression of the POM genes *foxC1a*, *nlz1*, *pitx2*, and *lmx1b1* also showed dose-dependent increase after RA treatments (Fig. S8B).

RA treatments from early somitogenesis disrupted eye morphogenesis (Fig. S8). To address the role of RA in conditions where optic cup formation was preserved, we started RA treat-



**Fig. 3.** RAR signaling regulates gene expression in POM. (A–C) Embryos treated with DMSO or 10  $\mu$ M AGN from 3s and hybridized at 18s (A), 24–25 hpf (B), or 31 hpf (C) with the indicated probes. (A) Dorsal/anterior views. In the AGN-treated embryos, *foxC1a* and *nlz1*, but not *twist1a*, are down-regulated in migrating POM (arrowheads). (B and C) Lateral views of eyes. AGN treatment decreases *foxC1a*, *nlz1*, *eya2*, *pitx2*, and *lmx1b1* expression in both anterior-ventral (arrowheads) and anterior-dorsal (black triangles) POM. AGN treatment decreases *twist1a* expression in dorsal POM (black triangles). Expression of *pitx2* and *lmx1b1* within the choroid fissure is also diminished (white dashed circles and triangles). Residual *foxC1a*, *eya2*, and *lmx1b1* staining is detectable in a ventro-medial location behind the eye (arrows). *pitx2* is expressed in extraocular mesodermal tissue in both the control and the AGN-treated embryo (arrows). Panels are representative images of the following numbers of independent experiments: *pitx2*,  $n = 4$ ; *foxC1a*,  $n = 3$ ; and *nlz1*, *twist1a*, *eya2*, *lmx1b1*,  $n = 2$ . Approximately 10–30 embryos were assayed for each condition and probe in each experiment.



**Fig. 4.** Quantification of gene expression changes after manipulations of RAR signaling. (A and B) Real-time PCR quantification of gene expression in 31- to 33-hpf dissected heads (A) or 25- to 28-hpf dissected heads and whole embryos (B) after treatments with the indicated reagents from 3s, shown as the mean ratio between AGN or RA and DMSO conditions in six (A) or three (B) biological replicates. Error bars show SDs. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns, nonsignificant ( $P \geq 0.05$ ) according to two-tailed Student's  $t$  test.



ments at 18s. RA (1  $\mu$ M) caused ectopic activation of *dact1*, *nlz2*, and *itgA5* in the dorsal retina (Fig. 5A) and expanded the domains of *foxC1a*, *nlz1*, *lmx1b1*, and *pitx2* in the anterior POM (Fig. 5B). These higher RA doses slightly reduced *vax1* expression in the VR/OS, while activating it ectopically in the dorsal telencephalon and diencephalon (Fig. 5A).

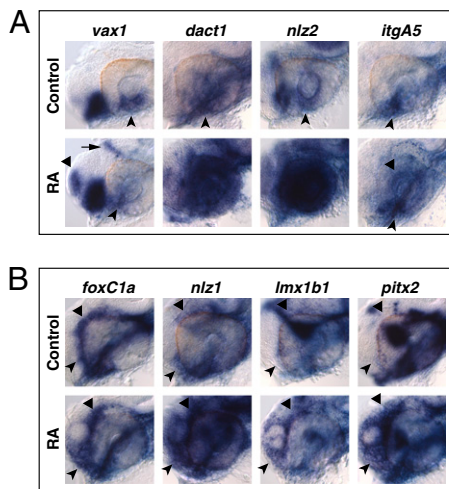
Real-time PCR analysis confirmed ISH results. *vax1*, *dact1*, and *nlz2* showed dose-dependent up-regulation in embryos treated with 0.125–1  $\mu$ M RA (Fig. 4B and Fig. S9A). Similar or stronger effects were detected in heads dissected from treated embryos than in whole embryos, confirming that RA regulates these genes in the eye and forebrain region (Fig. 4B). Dissected heads also showed significant up-regulation of the POM genes *foxC1a*, *nlz1*, and *pitx2a* (Fig. 4B). No significant changes in the expression levels of *vax2* and *nkx2.1b* were observed (Fig. 4B and Fig. S9A).

To determine the time course of the response of the VR/OS and POM genes to RA, we treated embryos with 0.5  $\mu$ M RA from 18s and harvested them between 0.5 and 6 h later. The shortest analyzed incubation time in RA (30–50 min) was sufficient to detect up-regulation of both VR/OS and POM genes by ISH and/or real-time PCR (Fig. S9 B and C), indicating rapid transcriptional responses to RA.

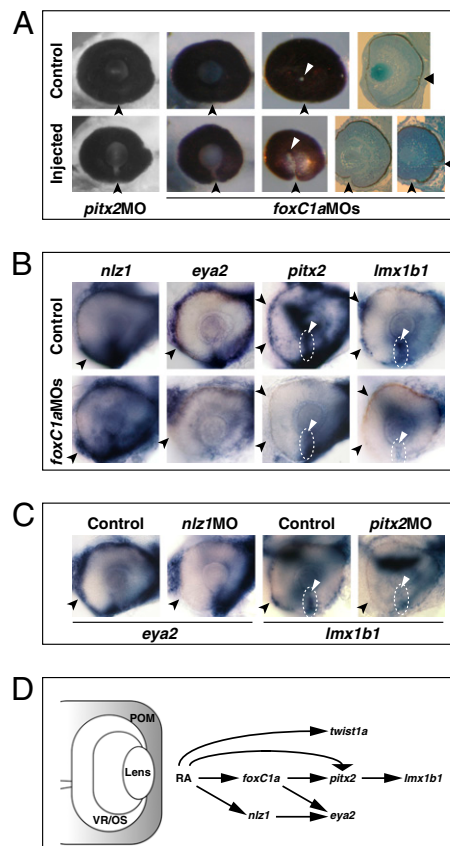
**Abrogation of FoxC1a, Nlz1, or Pitx2 Causes Coloboma Without Affecting Expression of VR/OS Genes.** Roles in choroid fissure closure are well documented for genes expressed in VR/OS cells (11, 17, 18), but the requirements for POM genes are less well studied. Consequently, we abrogated the activity of selected RAR-regulated POM genes with morpholino antisense oligonucleotides (MOs) and assessed ventral eye morphogenesis and the expression of VR/OS and other POM genes. We focused on *foxC1a* and *nlz1*, the earliest RAR-regulated genes in POM, and *pitx2* because it is a direct RAR target in mouse POM (19).

At 72 hpf, embryos injected with either *foxC1a* MOs or *pitx2* MO showed a narrow coloboma (*foxC1a* MO: 48%,  $n = 63$ ; *pitx2* MO: 40%,  $n = 90$ ) in which the choroid fissure had failed to fuse

ventral to the optic disk (Fig. 6A). Abrogation of Nlz1 is also reported to cause coloboma (18). Despite defective choroid fissure morphogenesis, there were no evident effects on the expression of the VR/OS genes *vax1*, *net1a*, *cyb5a*, and *itgA5* in *foxC1a*, *nlz1*, or *pitx2* morphants (Fig. S10 A–C and Table S1), indicating that abrogation of POM gene activity probably leads to coloboma independently of the activity of VR/OS genes. FoxC1a was also not required for the expression of *nlz1* and *twist1a* in POM (Fig. 6B and Fig. S11A). By contrast, expression of *eya2*, *pitx2*, and *lmx1b1* was reduced in the choroid fissure POM and/or in the anterior POM of *foxC1a* morphants (Fig. 6B and Table S1). In *nlz1* morphants, *eya2* expression in the anterior POM was decreased (Fig. 6C and Table S1), whereas *foxC1a*, *twist1a*, *pitx2*, and *lmx1b1* expression patterns were not obviously affected (Fig. S11 B and C). In *pitx2* morphants, *lmx1b1* expression was reduced in the anterior POM and within the choroid fissure (Fig. 6D and Table S1), whereas *foxC1a*, *nlz1*, and *eya2* were largely unaffected (Fig. S11D).



**Fig. 5.** Exogenous RA activates genes expressed in the VR/OS and in POM. (A and B) Lateral views of eyes from 31-hpf hybridized embryos treated with DMSO or 1  $\mu$ M RA from 18s. (A) Arrowheads point at the expression of *vax1*, *dact1*, *nlz2*, and *itgA5* in the VR/OS. RA treatment reduces *vax1* expression in the VR/OS while inducing it ectopically in the dorsal telencephalon (triangle) and diencephalon (arrow). The RA-treated embryos show *dact1* and *nlz2* expression throughout the retina and ectopic *itgA5* expression in the dorsal retina (triangle). (B) RA treatment increases *foxC1a*, *nlz1*, *lmx1b1*, and *pitx2* expression in anterior-ventral (arrowheads) and/or anterior-dorsal (triangles) POM. Approximately 20 embryos for each condition and probe were used for these analyses.



**Fig. 6.** Abrogation of FoxC1a, Nlz1, and Pitx2 function causes coloboma and affects gene expression in POM. (A) Eyes of embryos injected with *pitx2* MO or *foxC1a* MOs showing coloboma at 72 hpf, as detected in lateral view (Left and Center Left), posterior view (Center Right), and histological sections (Right). The injected eyes exhibit an open choroid fissure (arrowheads) ventral to the optic disk (triangles). (B and C) Lateral views of 32-hpf eyes. Panels are representative images of two to three independent experiments. Approximately 10–20 embryos were used for each condition and probe in each experiment. (B) Decreased expression of *eya2*, *pitx2*, and *lmx1b1*, but not *nlz1*, in choroid fissure POM (dashed circles and triangles) and/or anterior POM (arrowheads) in the *foxC1a* MO-injected embryos. (C) Decreased expression of *eya2* in anterior POM (arrowheads) of an *nlz1* MO-injected embryo and of *lmx1b1* in anterior POM (arrowheads) and within the choroid fissure (dashed circles and triangles) of a *pitx2* MO-injected embryo. (D) Proposed model of the molecular mechanisms of RAR-dependent RA signaling during development of the zebrafish POM (highlighted in gray). See text for details.

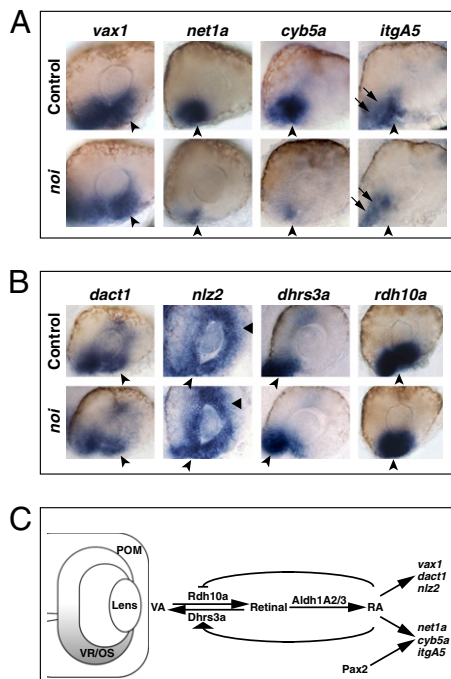
### Some VR/OS Genes Are Regulated in Parallel by RAR Signaling and Pax2

Pax2 is expressed in the VR/OS, and abrogation of Pax2 function leads to coloboma and reduced expression of *net1a* (17), prompting us to further explore the events downstream of RAR signaling and Pax2 during choroid fissure morphogenesis. RAR signaling can promote, but is not required for, *pax2* expression in the ventral eye (Figs. S24 and S7A). Vice versa, *pax2a* (*noi*) mutants showed normal VR/OS expression of *dhrs3a* and *rdh10a*, indicating that ocular RA signaling is intact in this mutant (Fig. 7B). *dact1* and *nlz2* were also unaffected in *noi* mutant eyes (Fig. 7B), whereas expression of *net1a*, *cyb5a*, and *itgA5* in the VR/OS was reduced (Fig. 7A). *foxC1a*, *nlz1*, *pitx2*, and *lmx1b1* were not affected in *noi* mutants (Fig. S11E), indicating that any alterations to the VR/OS in *noi* mutants have no obvious effects on POM genes.

Together with the data described above, these results show that RAR signaling and Pax2 can regulate the expression of some VR/OS genes in parallel and that, in both cases, this regulation is likely to be independent of the POM (Fig. 7C).

### Discussion

In this study, we show that RAR signaling is required for choroid fissure closure through regulation of distinct genetic programs in the VR/OS and the POM. In both fish and mammals, RAR signaling is required for ventral eye morphogenesis and gene regulation in POM, but not for DV patterning of the retina. We show, however, that RAR signaling controls genes expressed in the ventral optic cup, and implicated in its morphogenesis, independently of POM. This finding indicates that not all RA actions in ventral eye development are mediated through POM cells.



**Fig. 7.** Pax2 regulates some VR/OS genes in parallel to RAR signaling. (A and B) Lateral views of 25- to 26-hpf eyes. Approximately 15–20 control or mutant embryos for each probe were used for these analyses. (A) The *noi* embryos show down-regulation of *net1a*, *cyb5a*, and *itgA5*, but not *vax1*, in the VR/OS (arrowheads). Arrows point to *itgA5* expression in extraocular tissue at the back of the eye. (B) Expression of *dact1*, *nlz2*, *dhrs3a*, and *rdh10a* in ventral eye cells is not affected in the *noi* mutants (arrowheads). Triangles point to *nlz2* dorsal retinal expression. (C) Proposed model of the molecular mechanisms of RAR signaling in the developing zebrafish VR/OS (highlighted in gray). See text for details.

We describe RAR-dependent expression of six transcription factor-encoding genes in POM. *foxC1a*, *eya2*, and *pitx2* are known RA targets from mouse studies (2, 5), to which we add *nlz1*, *twist1a*, and *lmx1b1*. Neural crest-specific abrogation of either RARs or Pitx2 disrupts formation of the VR and/or OS in mice (3, 20), as does abrogation of Lmx1b in fish (21). In these cases, however, the morphogenetic movements driving the eyes away from the brain are compromised, and the developing VR/OS may be abnormally exposed to midline signals that influence eye development (11, 12).

Our functional analyses of FoxC1a and Pitx2 in zebrafish reveal more restricted roles for POM in the fusion of the lips of the ventral optic cup on each side of the choroid fissure. Thus, *foxC1a* and *pitx2* morphants (in this study) or *nlz1* morphants (18) show subtle colobomata, without overt effects on earlier eye morphogenesis or on the expression of VR/OS genes. Fewer POM cells enter the choroid fissure after inhibition of RAR function, and we speculate that POM cells invading the fissure facilitate the fusion of the ventral lips of the optic cup. Decreasing the supply of vitamin A in mouse embryos at the time of choroid fissure closure causes coloboma and persistence of the basal lamina within the fissure (4), suggesting that POM cells migrating inside the choroid fissure might have a role in basal lamina degradation before fissure closure.

The epistasis analyses of POM genes in this study and reported elsewhere (19) allow us to construct a tentative network of genetic interactions downstream of RAR signaling required for POM development (Fig. 6D). In this model, RAR signaling activates *foxC1a* and *nlz1* expression in early neural crest cells migrating in the eye region. In turn, FoxC1a collaborates with active RARs and with Nlz1 to promote *pitx2* and *eya2* expression, respectively, whereas Pitx2 promotes *lmx1b1* expression. Although there are caveats to making conclusions based on gene expression changes, this model provides a useful framework for future studies.

Our study challenges the hypothesis that RA signaling mediates eye morphogenesis solely through the POM (3). Neural crest-specific RAR knockout mice show similar eye phenotypes to ubiquitous knockout embryos, whereas abrogation of RAR function in the mouse retina at E10.5 causes no obvious retinal phenotype. However, neural crest-specific RAR mutant mice and *Aldh1A1/A3* mutant mice show normal optic cup formation up to E10.5, but not later (2, 3, 5). Instead, *Aldh1A1/A2/A3* mutants show clear defects in the ventral invagination of the optic cup by E10.5, possibly because of the lack of Aldh1A2-dependent RAR signaling in the optic vesicle before E10.5 (2). Altogether, current studies do not rule out the possibility of RAR signaling acting directly upon optic cup cells before E10.5 to regulate eye morphogenesis in mice.

We report on a cohort of genes regulated by RAR signaling in the developing VR/OS—*vax1*, *net1a*, *cyb5a*, *itgA5*, *dact1*, and *nlz2*—besides the RA pathway genes *dhrs3a* and *rdh10a* (Fig. 7C). RAR-dependent regulation of their expression is centered around a developmental window (between 18s and 23 hpf) coinciding with the invagination of the optic cup. Abrogation of Vax1 or Nlz2 function causes coloboma (11, 18). Dact1 functions in morphogenetic processes by regulating the planar cell polarity pathway (22). Netrins and integrins are implicated in cell migration in various contexts (e.g., refs. 23 and 24) and consequently may also be involved in the morphogenetic movements leading to choroid fissure closure. They could also be implicated in the defective retinal vascularization and optic nerve formation associated with RAR-dependent coloboma given their known roles in axon guidance and blood vessel formation (e.g., ref. 25).

It is unlikely that changes in expression of VR/OS genes upon RAR inhibition are an indirect consequence of abnormal development of POM or other nearby tissues because expression of VR/OS genes is not decreased in *foxC1a*, *nlz1*, or *pitx2* mor-

phants (in this study) or *lmx1b1* morphants (21), and we see no obvious changes in midline tissues that could secondarily compromise ventral eye development (11, 12). Analysis of *noi* mutants reinforces the idea that regulation of *net1a*, *cyb5a*, and *itgA5* expression is independent of POM. Finally, some VR/OS and POM genes show rapid transcriptional activation in response to RA, suggesting that their expression may be directly regulated by RAR signaling.

In conclusion, we show that functional RAR signaling is required not only for proper POM development but also for the correct regulation of a genetic program within the VR/OS that is important for choroid fissure closure.

## Materials and Methods

**Zebrafish.** Embryos were obtained and staged as previously described (26). Melanization was prevented with phenylthiourea when necessary. Embryos were treated in the dark with 2.5–10  $\mu$ M AGN (7) or with 0.125–1  $\mu$ M *all-*

*trans* RA (Sigma), diluted from 25 mM stocks in DMSO. Controls received identical amounts of DMSO.

**MOs.** MOs were obtained from Gene Tools or Open Biosystems and dissolved in water at a stock concentration of 1 mM. MO aliquots were heated for 5–10 min at 65 °C before use and injected at the 1- to 4-cell stage.

**Real-Time PCR.** cDNA was prepared from total RNA extracted from 20 to 40 embryos or dissected embryonic heads and amplified on a Rotor-Gene 3000 (Corbett Life Science) using Qiagen kits.

Further details on transgenic and mutant lines, treatment protocols, MOs, and analyses can be found in *SI Materials and Methods*.

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